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1 **MALDI-TOF MS as a promising tool to assess potential virulence of *Vibrio tapetis* isolates**

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2 Alexandra Rahmani<sup>1†</sup>, Maaïke Vercauteren<sup>2†</sup>, Katleen Vranckx<sup>3</sup>, Filip Boyen<sup>2</sup>, Adeline Bidault<sup>1</sup>, Vianney  
3 Pichereau<sup>1</sup>, Annemie Decostere<sup>2</sup>, Christine Paillard<sup>1,\*\*</sup> and Koen Chiers<sup>2\*\*</sup>

4 <sup>1</sup> Univ Brest, CNRS, IRD, Ifremer, UMR 6539 LEMAR, F-29280 Plouzane, France

5 <sup>2</sup> Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent  
6 University, Salisburylaan 133, 9820 Merelbeke, Belgium

7 <sup>3</sup> Applied Maths NV, Sint-Martens-Latem, Belgium

8 <sup>†</sup> Shared first authors

9 <sup>\*\*</sup> Shared last authors

10 Alexandra Rahmani: Univ Brest, CNRS, IRD, Ifremer, UMR 6539 LEMAR, F-29280 Plouzane, France;  
11 [Alexandra.rahmani@univ-brest.fr](mailto:Alexandra.rahmani@univ-brest.fr); <https://orcid.org/0000-0001-8888-1145> ; +33298498743

12 Maaïke Vercauteren: Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary  
13 Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium; [Maaïke.vercauteren@ugent.be](mailto:Maaïke.vercauteren@ugent.be);  
14 <https://orcid.org/0000-0002-7618-143X>

15 Katleen Vranckx: Applied Maths NV (bioMérieux SA), Sint-Martens-Latem, Belgium;  
16 [Katleen.vranckx@biomerieux.com](mailto:Katleen.vranckx@biomerieux.com); <https://orcid.org/0000-0002-1980-9809>

17 Filip Boyen: Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine,  
18 Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium; [filip.boyen@ugent.be](mailto:filip.boyen@ugent.be) ;  
19 <https://orcid.org/0000-0002-4777-6880>

20 Adeline Bidault: Univ Brest, CNRS, IRD, Ifremer, UMR 6539 LEMAR, F-29280 Plouzane, France;  
21 [adeline.bidault@univ-brest.fr](mailto:adeline.bidault@univ-brest.fr)

22 Vianney Pichereau: Univ Brest, CNRS, IRD, Ifremer, UMR 6539 LEMAR, F-29280 Plouzane, France;  
23 [Vianney.pichereau@univ-brest.fr](mailto:Vianney.pichereau@univ-brest.fr) , <https://orcid.org/0000-0003-1078-9407>

24 Annemie Decostere: Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary  
25 Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium; [annemie.decostere@ugent.be](mailto:annemie.decostere@ugent.be);  
26 <https://orcid.org/0000-0001-5150-555X>

27 Christine Paillard: Univ Brest, CNRS, IRD, Ifremer, UMR 6539 LEMAR, F-29280 Plouzane, France;  
28 [Christine.paillard@univ-brest.fr](mailto:Christine.paillard@univ-brest.fr); <https://orcid.org/0000-0002-8429-918X>

29 Koen Chiers: Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine,  
30 Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium; [koen.chiers@ugent.be](mailto:koen.chiers@ugent.be);  
31 <https://orcid.org/0000-0002-7449-1047>

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43 **ABSTRACT**

44 *Vibrio tapetis*, the etiological agent of Brown Ring Disease, mainly affects the Manila clam *Ruditapes*  
45 *philippinarum*. Although this bacterium is mainly known as a clam pathogen, it has been isolated from  
46 several fish species. The main aim of the present study was to further explore the variability of 27 *V. tapetis*  
47 isolates from bivalves and fish, considering three different aspects; *in vitro* virulence based on the loss of  
48 clam hemocyte adhesion properties, detection of the gene *virB4* encoding for an essential component of  
49 the Type IV Secretion System, and MALDI-TOF MS characterization based on whole cell extracts. Finally,  
50 these approaches were compared and evaluated for their ability to differentiate the potential  
51 pathogenicity of the 27 isolates against the Manila clams. Among the 11 *V. tapetis* isolates from the  
52 common dab isolated in 2018 in Belgium, only one (2BB) showed intermediate *in vitro* virulence against  
53 the Manila clam, and seven carried the *virB4* gene while none of the *V. tapetis* previously isolated from  
54 fish in 2003 showed the presence of this particular gene. Finally, the peak protein profiles generated with  
55 MALDI-TOF MS analysis from all 27 *V. tapetis* strains showed a clear clustering of clam pathogenic and  
56 nonpathogenic isolates suggesting that a new isolate of *V. tapetis* that would cluster within the clam  
57 pathogenic isolates could be potentially pathogenic to the Manila clam. Thereupon, MALDI-TOF MS typing  
58 allows rapid and cost-efficient identification of *V. tapetis* isolates and can be defined as a complementary  
59 method of the traditional qPCR that opens new perspectives to study the virulence of *V. tapetis* isolates  
60 but also to perform environmental monitoring in order to prevent outbreaks.

61 **KEYWORDS:** *Vibrio tapetis*, MALDI-TOF MS, *virB4*, hemocyte cytotoxicity assay, Manila clam, fish

62 **ABBREVIATIONS**

63 BRD: Brown ring Disease

64 FSSW: Filtered Sterilized Sea Water

65 HPLC: High performance liquid chromatography

- 66 MALDI-TOF MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
- 67 MLSA: Multilocus sequence analysis
- 68 RAPD-PCR: Randomly Amplified Polymorphic DNA PCR
- 69 T4SS: Type IV secretion system
- 70 TSA: Tryptic Soy Agar
- 71 TSB: Tryptic Soy Broth
- 72
- 73

74 **1. INTRODUCTION**

75 *Vibrio tapetis* is the etiological agent of Brown Ring Disease (BRD), mainly affecting Manila clam *Ruditapes*  
76 *philippinarum*, and responsible for mass mortalities in cultured clams (Maes and Paillard, 1992; Paillard et  
77 al., 1994). Clams are amongst the most heavily traded species in the global aquaculture market with Manila  
78 clam being the second major cultured bivalve in the world with a yearly production of over 4.4 million ton  
79 (FAO, 2018; Smits et al., 2020). BRD is characterized by a brown organic, conchiolin deposit, between the  
80 pallial line and the edge of the shell (Paillard and Maes, 1995). The reference strain *V. tapetis* CECT4600,  
81 first isolated from a cultured Manila clam exhibiting BRD in France, has been well characterized (Borrego  
82 et al., 1996; Paillard and Maes, 1990). Since 1990, *V. tapetis* was identified in BRD of various bivalve hosts  
83 such as common cockle *Cerastoderma edule* (Novoa et al., 1998; Paillard and Maes, 1990), rayed artemis  
84 *Dosinia exoleta* (Paillard, 2004a), pink clam *Polititapes rhomboïdes* (Paillard, 2004a), grooved carpet shell  
85 *R. decussatus* (Maes and Paillard, 1992; Novoa et al., 1998) and *Venerupis aurea* (Maes and Paillard, 1992),  
86 This suggests that *V. tapetis* is able to cross species barriers (Paillard, 2016).

87 Since 2003, some studies described the isolation of *V. tapetis* from cultivated or captive held aquatic  
88 vertebrates such as corkwing wrasse *Symphodus melops* ((Jensen et al., 2003), Atlantic halibut  
89 *Hippoglossus hippoglossus* (Reid et al., 2003), Dover sole *Solea solea* (Declercq et al., 2015), fine flounder  
90 *Paralichthys adspersus* and red conger eel *Genypterus chilensis* (Levican et al., 2017). *V. tapetis* was also  
91 pinpointed as causative agent of ulcerative skin lesions in the wild-caught common dab *Limanda limanda*  
92 (Vercauteren et al., 2019).

93 In the past, *V. tapetis* has been described as a homogenous taxon based on traditional methods such as  
94 bio- or serotyping (such as indole production, growth in NaCl and production and utilization of different  
95 nutrients) (Allam et al., 1996; Castro et al., 1996; Figueras et al., 1996; Paillard, 2004b). Later studies, using  
96 advanced genetic or experimental techniques revealed that this species is more heterogeneous. To date,  
97 three distinct subspecies of *V. tapetis* are described, i.e. subsp. *tapetis* (Balboa and Romalde, 2013), subsp.  
98 *britannicus* (Balboa and Romalde, 2013) and subsp. *quintayensis* (Levican et al., 2017). However, using

99 genotyping methods, such as Randomly Amplified Polymorphic DNA (RAPD) PCR (Romalde et al., 2002)  
100 and multilocus sequence analysis (MLSA) (Gulla et al., 2017) different clusters were defined between *V.*  
101 *tapetis* subspecies. This indicates that the phylogeny of *V. tapetis* is not yet completely unraveled.

102 From an epidemical point of view, differences between *V. tapetis* isolates might provide valuable  
103 information regarding the diagnosis of BRD outbreaks and detection of virulent isolates. Furthermore,  
104 typing of isolates can also help examining the geographical and host distributions supporting a more  
105 ecological approach for studying host-pathogen-environment interactions (Paillard, 2016; Rodríguez et al.,  
106 2006).

107 Several studies have indeed pointed towards differences between clam pathogenic and non-pathogenic  
108 *V. tapetis* strains. *In vivo* infection into the pallial cavity of the clams revealed that none of the tested  
109 strains derived from fish are able to induce BRD in manila clam (Bidault et al., 2015; Choquet, 2004; Dias  
110 et al., 2018). In addition, these last authors have pointed out that *virB4*, an essential gene coding for  
111 nucleoside triphosphatase of the Type IV Secretion System (T4SS), was only present in strains pathogenic  
112 to the Manila clam and therefore suggested to be a discriminative tool to differentiate between  
113 pathogenic and non-pathogenic strains (Dias et al., 2018). Furthermore, the *virB4* real-time PCR assay  
114 offers a method for quantification of *V. tapetis* in the extrapallial fluids of the Manila clam (Bidault et al.,  
115 2015).

116 The main aim of the present study was to further explore the variability between the 27 *V. tapetis* isolates  
117 from bivalves and fish, considering three different aspects; *in vitro* virulence based on the loss of hemocyte  
118 adhesion properties after contact with *V. tapetis* (Choquet et al., 2003), detection of the *virB4* gene using  
119 the TaqMan qPCR (Bidault et al., 2015), and protein-based matrix-assisted laser desorption/ionization  
120 time-of-flight mass spectrometry (MALDI-TOF MS) characterization. Finally, these approaches were  
121 compared and evaluated for their ability to discriminate the potential virulence of the 27 isolates against  
122 the Manila clams.

## 123 2. MATERIALS AND METHODS

124 **2.1. Bacterial isolates and cultivation**

125 The *V. tapetis* isolates used in this study were isolated from nine different host species (five bivalves and  
126 four fish species) in various countries between 1988 and 2017. Table 1 summarizes the information of all  
127 isolates with the year, place and host of first isolation and a synthesis of the existing knowledge about  
128 their virulence. After isolation, all isolates were frozen at -80 °C for further analysis

129 For the cytotoxicity bioassay, only the isolates derived from common dab and the CECT4600 reference  
130 strain were used. All isolates were cultivated on Tryptic Soy Agar (TSA; Difco™) supplemented with 1.5 %  
131 NaCl and were allowed to grow for two days at  $16 \pm 1$  °C (fish isolates) or  $18 \pm 1$  °C (reference clam isolates).

132 For the *virB4* gene detection only common dab isolates were used. All isolates were cultured in TSA  
133 supplemented with 1.6 % NaCl for PCR and Tryptic Soy Broth (TSB; Difco™) supplemented with 1.6 % NaCl  
134 for qPCR. For MALDI-TOF analysis, all 27 isolates were cultivated in triplicate (biological replicates,  
135 Supplementary file 1) on TSA supplemented with 1.5 % NaCl. Isolates were grown for minimally two days  
136 at  $16 \pm 1$  °C (fish isolates) or  $18 \pm 1$  °C (clam isolates). We chose to use the optimal or common  
137 temperatures for the strains to ensure optimal growth of the isolates.

138 **2.2. In vitro hemocyte cytotoxicity bioassay**

139 Virulence of *V. tapetis* isolates derived from common dab was tested using the standardized *in vitro*  
140 hemocyte cytotoxicity bioassay (Choquet et al., 2003). This test is based on the ability of *V. tapetis* virulent  
141 strains to induce a rounding phenotype on infected hemocytes, increasing the number of non-adherent as  
142 compared to the negative control. *V. tapetis* induces during its phagocytosis, a process of cytoskeletal  
143 inhibition and cell rounding. Thus, this adhesion test provides not only morphological but also functional  
144 characterization. The type strain CECT4600 was used as the positive control, and filter-sterilized seawater  
145 (FSSW) as negative control. Animals used in this study were Manila clams (4 cm Manila clam, February  
146 2018) from the SATMAR shellfish aquaculture site in Landeda (Finistère, France). Clams were allowed to  
147 acclimate in oxygenated seawater at 14°C for 14 days. Hemolymph was harvested from the adductor  
148 muscle, pooled after quality check and the hemocytes were enumerated using a Malassez counting grid.



149 100 µL of hemolymph ( $5 \cdot 10^5$  hemocyte/mL) was added in 24-well plates and kept for a few minutes to let  
150 hemocytes adhere to the bottom of the plate. Then, 100 µl of bacterial suspension prepared with FSSW,  
151 was added at a bacteria/hemocyte ratio of 25/1. Hemocyte exposure to each of the bacterial isolates was  
152 performed in triplicate. In negative control samples, 100 µl of FSSW was added to the hemolymph. After  
153 3 h of incubation, the number of non-adherent hemocytes was measured (used as a proxy of *V. tapetis*  
154 cytotoxicity) using flow cytometry after addition of 4 µl of SYBR–Green solution in DMSO (nucleic acid gel  
155 stain, dilution 1 : 10 000, Life Technology, USA) as already described (Choquet et al., 2003). A ratio was  
156 calculated by dividing the number of non-adherent hemocytes in samples exposed to bacteria by the  
157 number of non-adherent cells in negative controls. Statistical analyses were performed using a pairwise  
158 Student test to determine significant differences in non-adherent cell ratios between bacterial suspensions  
159 and both positive and negative control samples as already performed by Choquet et al. (2003).

### 160 **2.3. *VirB4* gene detection**

161 A PCR assay was performed with common dab isolates, in which a fragment of 173 bp of the *virB4* gene  
162 was amplified using primers 170513 (5'-TTAAAAGTGGCGGAGGAATG-3') and 170514 (5'-  
163 AAGCTCTGCATCGGTTAGGA-3') and GoTAQ polymerase. Subsequently, a Taqman real-time qPCR  
164 quantification was performed in triplicate according to the standardized method previously described  
165 (Bidault et al., 2015). The positive control used for both of these experiments was the strain *V. tapetis*  
166 CECT4600.

### 167 **2.4. MALDI-TOF MS characterization**

168 An ethanol formic acid extraction was performed, based on MALDI Biotyper protocol (Bruker Daltonics,  
169 Bremen Germany). Briefly, bacterial cultures were suspended in nuclease-free water aliquoted in 1.5 mL  
170 Eppendorf tubes. Ethanol (70 %) dissolved in high performance liquid chromatography (HPLC) grade water  
171 was added to the suspension and tubes were centrifuged twice ( $20\,000 \times g$ , 2 min) upon which the ethanol  
172 was removed. Thereafter, 20 µL of 70% formic acid (in HPLC grade water) was added to the pellet. To finish  
173 the extraction, 20 µL of acetonitrile was added. One µL of each extract was spotted eight-fold (technical

174 replicates) on a MALDI target plate (Bruker Daltonics, Bremen, Germany), air dried and covered with 1  $\mu$ L  
175 of alpha-cyano-4-hydroxycinnamic acid matrix (Bruker Daltonics, Bremen, Germany). All spots were  
176 processed in triplicate (technical replicates, Supplementary file 1) with an Autoflex III Smartbeam MALDI-  
177 TOF MS, recording masses ranging from 2 000 to 20 000 Da using standard settings (flexControl 1.4, version  
178 3.4, Bruker Daltonic, Bremen, Germany). The obtained raw spectra were imported in BioNumerics 7.6.3  
179 (Applied Maths NV, Sint-Martens-Latem, Belgium) for data analysis. Preprocessing of the data was  
180 performed according to (Giacometti et al., 2018). After preprocessing, peaks were detected using the  
181 continuous wavelet transform method with a signal-to-noise threshold of two. The spectra were  
182 summarized in an average spectrum per biological replicate (Supplementary file 1) and all replicates with  
183 less than 95% similarity to this summary spectrum were removed from the analysis. Of *V. tapetis* 2BB,  
184 RD0705 and RP2.3, only two biological replicates were implemented in the analyses due to inconsistencies  
185 in the data or low similarity (< 50%) with other replicates of the same isolate. The resulting summary  
186 spectra were used to construct an UPGMA dendrogram using a Pearson similarity coefficient.

### 187 **3. RESULTS AND DISCUSSION**

188 Infectious diseases remain one of the main limiting factors in the aquaculture of clams, therefore  
189 increasing the value of research on the variability of those infectious agents (Smits et al., 2020). The wide  
190 host (at least fourteen different host species) and geographic distribution (at least seven different  
191 countries) of *V. tapetis* and linked questions on possible variability of isolates have been discussed in  
192 previous research with a deep interest in finding a discriminative test for assessing pathogenicity of *V.*  
193 *tapetis* isolates against clams (Bidault et al., 2015). Furthermore, the discovery of new isolates from skin  
194 ulcerations in the common dab (Vercauteren et al., 2018) might urge for further exploration of pathogenic  
195 markers using available techniques.

#### 196 **3.1. Most of the *V. tapetis* isolates from the common dab are unable to induce hemocyte toxicity.**

197 To gain insight into the pathogenicity of *V. tapetis* to the Manila clam, an *in vitro* cytotoxicity assay was  
198 developed based on the cell-rounding and subsequent loss of adherence of hemocytes following exposure

199 to *V. tapetis* (Choquet et al., 2003). This bioassay was previously performed for many *V. tapetis* strains  
200 pathogenic to bivalves (Table 1), whereby strains IS9 and CECT4600 induced the highest loss of adhesion  
201 of the hemocytes (i.e. *in vitro* cytotoxicity). Most of the other strains from clams displayed intermediate  
202 cytotoxicity and only a few showed no cytotoxicity (Table 1). Remarkably, the GDE and GTR-I strains, both  
203 isolated from bivalves but not from the Manila clam, showed no cytotoxicity *in vitro*. Complete genome  
204 analyses of these two strains revealed strong differences with clam isolates (Dias et al., 2018) and these  
205 two strains were genetically closer to the LP2 strain derived from corkwing wrasse. This results further  
206 substantiates the observed difference in cytotoxicity towards Manila clam hemocytes (Dias et al., 2018).

207 The common dab isolates, 2BG, 2AE, 2AC and 2BW tested in this study, showed non-adherent cells ratios  
208 after bacterial exposure fluctuating around 1. The 2BU isolate displayed a ratio of  $0.85 \pm 0.14$  and remained  
209 clearly below 1 in all replicates. The ratios found with isolates 2BC, 2BT, 2AU, and 2BA ranged between 1  
210 and 1.5. All isolates showed similar ratios with the FSSW control, therefore indicating negative cytotoxicity  
211 (i.e. causing no additional loss of adherence of hemocytes) ( $pvalue > 0.05$ ; Fig 1). The isolate 2BB showed  
212 the highest cytotoxic activity with an average ratio of  $2.27 \pm 0.26$  (Fig 1). The cytotoxicity of 2BB was  
213 evaluated to be intermediate since the amount of non-adherent hemocytes was significantly higher as  
214 compared to the FSSW control ( $pvalue: 0.0005$ ) but was intermediate considering the positive control  
215 strain ( $pvalue: 0.31$ ) These results are consistent with previous ones obtained with strains isolated from  
216 fish (e.g. LP2 and HH6087) which mostly showed negative cytotoxicity towards clam hemocytes (Dias et  
217 al., 2018).

218 Comparison between different *in vitro* cytotoxicity bioassays is complicated since the non-adherent  
219 hemocyte ratio might vary depending on the susceptibility of hemolymph to *V. tapetis*. This is illustrated  
220 with the strain LP2, which showed intermediate cytotoxicity in Choquet et al. (2003) and negative  
221 cytotoxicity in Dias et al. (2018). In the present study, this bias risk was reduced, by using one pool of  
222 hemocytes and using a standardized analysis of the data comparing the results to both a positive  
223 (CECT4600) and negative control (FSSW), as already described in Choquet et al. (2003).

224 **3.2. First detection of the *virB4* gene in non-cytotoxic fish isolates**

225 Recently, a rapid and accurate Taqman real-time PCR assay for detection and quantification of *V. tapetis*  
226 in extrapallial fluids of the Manila clam has been developed, based on the presence of the *virB4* gene,  
227 which encodes a component of the T4SS (Bidault et al., 2015). Since the T4SS has been described to be  
228 essential for virulence in other pathogenic species such as *Helicobacter pylori* and *Legionella pneumophila*  
229 (Voth et al., 2012) ,this PCR analysis was suggested as a possible screening tool to differentiate between  
230 clam pathogenic and non-pathogenic isolates (Dias et al., 2018). Among 17 fully sequenced *V. tapetis*  
231 genomes, in a previous study, the T4SS genes cluster was identified only in the genomes of isolates virulent  
232 to the Manila clam (based on *in vivo* assays), thus proving that these strains do not only carry the *virB4*  
233 gene but the entire T4SS gene cluster and also suggesting a role for this T4SS system in *V. tapetis*  
234 pathogenicity towards clams (Dias et al., 2018). It should be noted that FPC1121, an isolate from Manila  
235 clam in Japan, does show *in vivo* virulence towards Manila clam, even though the *virB4* gene is not present.  
236 Surprisingly, in the isolates from common dab, the *virB4* gene was found to be present in seven out of the  
237 11 isolates (2BM, 2AU, 2BA, 2BT, 2BU, 2AC and 2BG). This study is therefore the first to provide evidence  
238 that *V. tapetis* isolated from fish can carry the *virB4* gene, in contrast with previously reported results (Dias  
239 et al., 2018). It needs however to be elucidated if these *virB4* positive isolates carry the entire cluster  
240 coding for T4SS and/or are able to induce BRD during *in vivo* experiments. Therefore, it seems that it was  
241 not the only gene that determines virulence, as expected.

242

243 **3.3. MALDI-TOF MS analysis reveals 3 clusters of *V. tapetis* isolates**

244 All 27 *V. tapetis* isolates included in this study were analyzed using the MALDI-TOF MS method. This  
245 method allows sensitive and rapid identification of microorganisms and is now widely used in different  
246 fields such as clinical microbiology, epidemiological studies and water or food borne pathogens (Maier et  
247 al., 2006; Singhal et al., 2015). The technique is broadly used to identify microorganisms at the species  
248 level, but has recently been shown valuable for strain typing (Sandrin et al., 2013).

249 The main peaks generated by the MALDI-TOF MS were found between 2000 and 7500 Da. Based on the  
250 peak profiles, the constructed dendrogram (Fig 2) of the investigated spectra revealed some clearly  
251 delineated clusters. In total three distinct clusters (named A, B and C) were defined, based on differences  
252 in protein profiles (Fig 2). All strains that were derived from BRD outbreaks in cultured Manila clam  
253 between 1988 - 1996 clustered together in cluster B with only a 29.6 % similarity with other isolates. The  
254 strains derived from the common cockle (IS9) and from the carpet shell clam, *R. decussatus* (RD0705) were  
255 also included in this cluster. All these strains were collected during the BRD emergence in Europe, which  
256 might explain the limited variability between the isolates. The two strains that were isolated from the  
257 rayed artemis (GDE) and pink clam (GTR-I) were clustered together with isolates from fish, in cluster A.  
258 The latter cluster could be further divided in four sub-clusters (Fig 2). Interestingly, one isolate (2BW)  
259 demonstrated different protein profiles and was included in a separate cluster C. This bacterium was  
260 isolated as a co-culture with *Pseudoalteromonas* sp. and *Psychrobacter submarinus* from a skin ulcer in dab.  
261 *In vitro* assays demonstrated the absence of the *virB4* gene and hemocyte cytotoxicity. Therefore, it is  
262 possible that this isolate represents a non-pathogenic strain for clams and/or dab. The virulence of this  
263 isolate should be characterized towards clams and fish to elucidate this clustering. Increasing the number  
264 of isolates (from different hosts and geographic origin) would corroborate our results and fortify the  
265 importance of MALDI-TOF MS analysis for differentiate between clam pathogenic and non-pathogenic *V.*  
266 *tapetis* isolates.

267 The MALDI-TOF MS clustering in the present study showed some similarities with previously reported data  
268 based on genome analyses (Dias et al., 2018). The latter have demonstrated that the two strains isolated  
269 from the rayed artemis (GDE) and pink clam (GTR-I) clustered together with LP2 isolated from corkwing  
270 wrasse, and were genetically distant from *V. tapetis* strains isolated from clams.

271 FPC1121 is the only clam pathogenic strain that was clustered together with fish isolates, based on the  
272 MALDI-TOF MS profiling. FPC1121 was isolated from cultivated Manila clam in Japan (Table 1, Matsuyama  
273 et al., 2010). The original report clearly described the brown deposit and mass mortalities of Manila clam

274 due to this strain, which was confirmed by *in vivo* virulence assays (Matsuyama et al., 2010). This clustering  
275 of FPC1121 is an interesting result; detailed genetic analysis would be interesting to explore the  
276 phylogenetic position of this strain and the linked genetic differences.

### 277 **3.4. Value of the assays to differentiate isolates according to their host-species (Fig 3)**

278 *In vivo* virulence tests are necessary to determine the ability for an isolate to induce BRD. However, several  
279 tests have been developed to characterize the *in vitro* virulence regarding Manila clam.

280 The *in vitro* cytotoxicity assay has been used as a tool to evaluate the pathogenicity of *V. tapetis* strains  
281 for clams (Dias et al., 2018; Bidault et al., 2015). This assay is based on the correlation between the  
282 cytotoxic activity of bacteria to clam hemocytes and the *in vivo* pathogenicity. As demonstrated in Table  
283 1, such a correlation was not found for strain RD0705. Indeed, this strain causes no hemocytes cytotoxicity  
284 *in vitro* although it can cause BRD *in vivo* in the Manila clam (Table 1) (Dias et al., 2018; Novoa et al., 1998).  
285 Since RD0705 was isolated from another clam species (i.e. the grooved carpet shell), it is tempting to  
286 speculate that this inconsistency might be related to host specificity. However, it could also suggest that  
287 cytotoxicity towards hemocytes is not the only virulence factor involved in the development of BRD. Beside  
288 this exception, it should be recalled that the analysis of the virulence profiles of many *V. tapetis* strains  
289 isolated from clam revealed a good correlation between the cytotoxic activity to clam hemocytes and the  
290 *in vivo* pathogenicity.

291 Another assay commonly used to detect *V. tapetis* isolates pathogenic to clams is the search for the *virB4*  
292 gene encoding part of the T4SS (Dias et al., 2018). In our study, we have demonstrated for the first time  
293 that the *virB4* gene can also be present in fish isolates. Since these isolates did not display *in vitro* virulence  
294 to clam hemocytes and they clustered separately based on their protein profile, it could be possible that  
295 they are not pathogenic for clams. *In vivo* studies should be performed to confirm the pathogenicity  
296 towards clams and if so, this might question the use of *virB4* detection as a pertinent marker for  
297 pathogenicity to clams.

298 The MALDI-TOF dendrogram showed a good clustering of *V. tapetis* from different origins. However, this  
299 clustering was not correlated with the presence of the *virB4* gene. In fact, the clustering was more  
300 correlated with the host species from which they were isolated. Based on the results of the different assays  
301 in the present study, it could be hypothesized that MALDI-TOF clustering could differentiate between clam  
302 pathogenic and nonpathogenic isolates.

### 303 ***3.5. Implications in the context of virulent strain detection***

304 Based on these results, MALDI-TOF MS analysis seems to be a promising tool to indirectly evaluate the  
305 pathogenicity of a *V. tapetis* isolate towards the Manila clam. Nevertheless, although the presented  
306 MALDI-TOF MS isolate typing might be a rapid, cost-effective and powerful tool in identifying *V. tapetis*  
307 isolates, the Taqman real-time qPCR remains necessary to quantify the load of *V. tapetis* during infection.  
308 Although some inconsistencies exist, the *in vitro* assay is also believed to give a good indication of virulence  
309 towards Manila clam in some cases.

310 Regarding the value of each test described in this study to determine *V. tapetis* isolates pathogenicity to  
311 the Manila clam, we can consider the MALDI-TOF MS as a new promising screening tool which can be  
312 complementary to the tools already used, increasing reliability of the screening.

## 313 **4. CONCLUSION**

314 In conclusion, the discovery of new *V. tapetis* isolates derived from common dab was linked with the need  
315 for a further exploration of variability of *V. tapetis* isolates. The currently used assays (toxicity to  
316 hemocytes and search of the *virB4* gene), have previously been shown to be interesting to differentiate  
317 between *V. tapetis* pathogenic and non-pathogenic clam isolates. Nevertheless, in this study, *V. tapetis*  
318 isolates from dab showed inconsistencies with results of previously pinpointed techniques questioning  
319 their discriminative power. In contrast, the MALDI-TOF MS analysis proved to be a promising tool with the  
320 possible ability to differentiate between pathogenic and non-pathogenic clam isolates. This can be used  
321 as a complementary discriminative test for virulence of clam isolates.

322 This approach allows rapid and cost-efficient identification of *V. tapetis* species and opens new  
323 perspectives to study the virulence of *V. tapetis* isolates but also to perform environmental monitoring in  
324 order to prevent outbreaks.



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425

426

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430 **AUTHORS CONTRIBUTIONS**

431 The collaboration has been initiated by AD, KC, CP and VP. *In vitro* virulence assays were performed by MV  
432 and AR. *virB4* detection was performed by MV, AR and AB. MALDI TOF MS experiments and analyses were  
433 performed by MV, FB and KV. The article was written by MV, AR, CP, VP, KC and AD.

434 **CONFLICTS OF INTEREST**

435 Alexandra Rahmani declares that she has no conflict of interest.

436 Maaïke Vercauteren declares that she has no conflict of interest.

437 Katleen Vranckx is an employee of Applied Maths NV (bioMérieux SA).

438 Filip Boyen declares that he has no conflict of interest.

439 Adeline Bidault declares that she has no conflict of interest.

440 Vianney Pichereau declares that he has no conflict of interest.

441 Annemie Decostere declares that she has no conflict of interest.

442 Christine Paillard declares that she has no conflict of interest.

443 Koen Chiers declares that he has no conflict of interest.

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453 in the study design, data collection, analysis or the writing process of the manuscript.

454 **Compliance with Ethical Standards**

455 All applicable international, national, and/or institutional guidelines for the care and use of animals were  
456 followed.

457 This article does not contain any studies with human participants performed by any of the authors.

458 **TABLES**

459 **Table 1:** Information on the *V. tapetis* isolates of clams, other bivalves and fish with host species, common name of the host species, description of the health  
460 of the host species, year and location of isolation and a reference for each isolate. Furthermore, a synthesis is provided of the existing knowledge on (1) *in*  
461 *vivo* virulence, estimating the possibility of the isolate to cause Brown Ring Disease in Manila clam following experimental infection. The isolate indicated with  
462 an asterisk is able to cause Brown Ring Disease in pink clam but not in Manila clam; (2) *in vitro* cytotoxicity against hemocytes of the Manila clam ((-): no  
463 cytotoxicity, (±) intermediate cytotoxicity, (+):cytotoxicity. a: negative in Dias et al, 2018 and intermediate in Choquet, 2004. b: negative in Dias et al, 2018  
464 and intermediate in Choquet et al, 2003 And (3) presence of the *virB4* gene from Bidault et al., 2015; (+) : presence, (-): absence. References for the virulence  
465 analyses are: Bidault et al., 2015; Dias et al., 2018; Novoa et al, 1998; Choquet et al, 2004; Matsuyama et al., 2010. Nd: Not determined; \*: Determined in this  
466 study.

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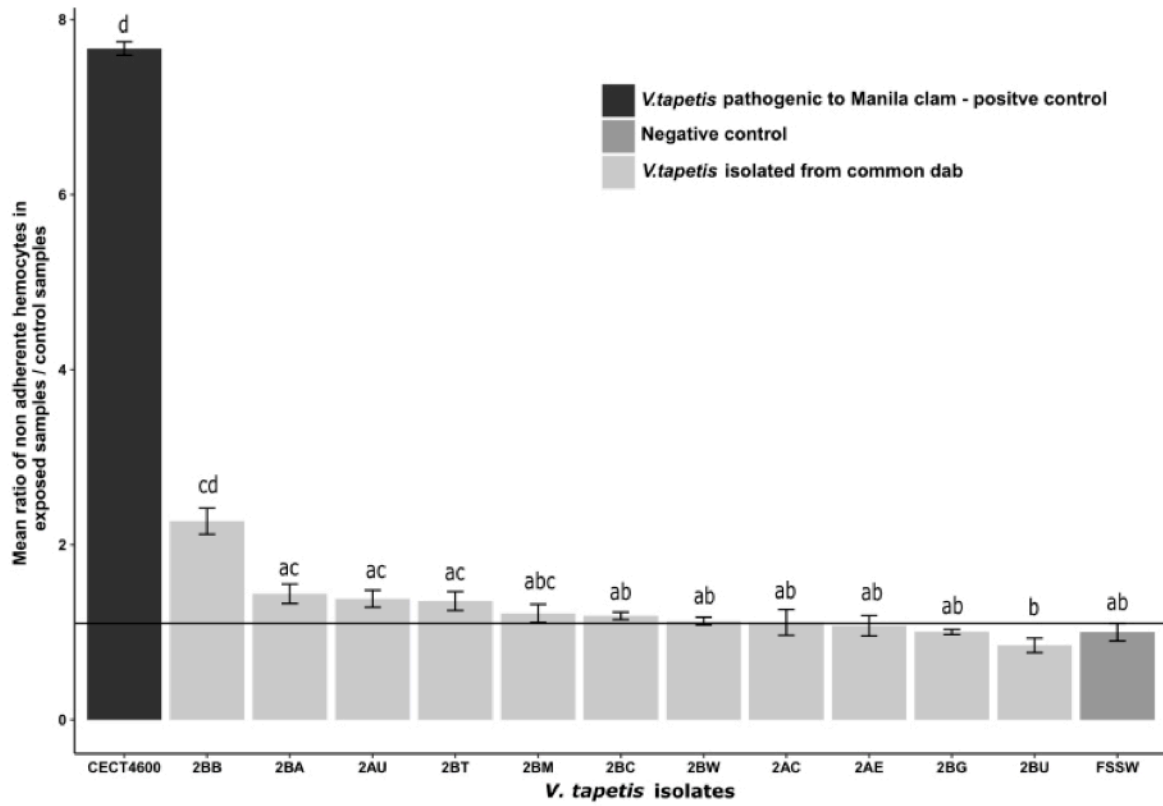
Isolates	Host species	Common name	Isolated from	Year	Location	Isolate reference	<i>In vivo</i> virulence (1)	<i>In vitro</i> cytotoxicity (2)	<i>virB4</i> gene detection (3)
<b>CLAMS</b>									
RD0705	<i>Ruditapes decussatus</i>	Grooved carpet shell	Animals with BRD signs	1992	Spain, Galice	(Novoa et al., 1998)	+	-	+
CECT4600	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1990	France, Landeda	(Borrego et al., 1996; Paillard and Maes, 1995)	+	+	+
FPC1121	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	2008	Japan	(Matsuyama et al., 2010)	+	Nd	-
IS1 (VP1)	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1988	France, Landeda	(Paillard and Maes, 1990)	+	±	+
IS5	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1991	France, Landeda	(Borrego et al., 1996)	+	±	+
P16B	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1995	France, Golfe du Morbihan	(Allam et al., 2002)	+	±	+
RP11.2	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1990	France, Landeda	(Borrego et al., 1996)	+	±	+
RP2.3.	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1990	France, Landeda	(Borrego et al., 1996)	+	±	+
RP8.17	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1990	France, Landeda	(Borrego et al., 1996)	+	± <sup>-a</sup>	+
RP9.7	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1990	France, Landeda	(Borrego et al., 1996)	+	±	+
UK6	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1996	Great Britain	(Allam et al., 2000)	+	±	+
<b>OTHER BIVALVES</b>									
IS9	<i>Cerastoderma edule</i>	Common cockle	Healthy animals	1990	France, Quiberon	(Borrego et al., 1996)	+	+	+
GTR-I	<i>Polititapes rhomboïdes</i>	Pink clam	Animals with BRD signs	2008	France, Glénan	(Dias et al., 2018)	-*	-	-
GDE	<i>Dosinia exoleta</i>	Rayed artemis	Animals with BRD signs	2003	France, Glénan	(Dias et al., 2018)	-	-	-
<b>FISH</b>									

LP2	<i>Symphodus melops</i>	Corkwing wrasse	Kidney of fish suffering vibriosis	1999	Norway, Bergen	(Jensen et al., 2003)	-	±/-. <sup>b</sup>	-
HH6087 (CECT8161)	<i>Hipoglossus hipoglossus</i>	Atlantic halibut	Kidney of moribund fish	2002	Great Britain, Glasgow	(Reid et al., 2003)	-	-	-
2AC	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	+*
2AE	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	-*
2AU	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	+*
2BA	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	+*
2BB	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	±*	-*
2BC	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	-*
2BG	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	+*
2BM	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	+*
2BT	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	+*
2BU	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	+*
2BW	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	-*

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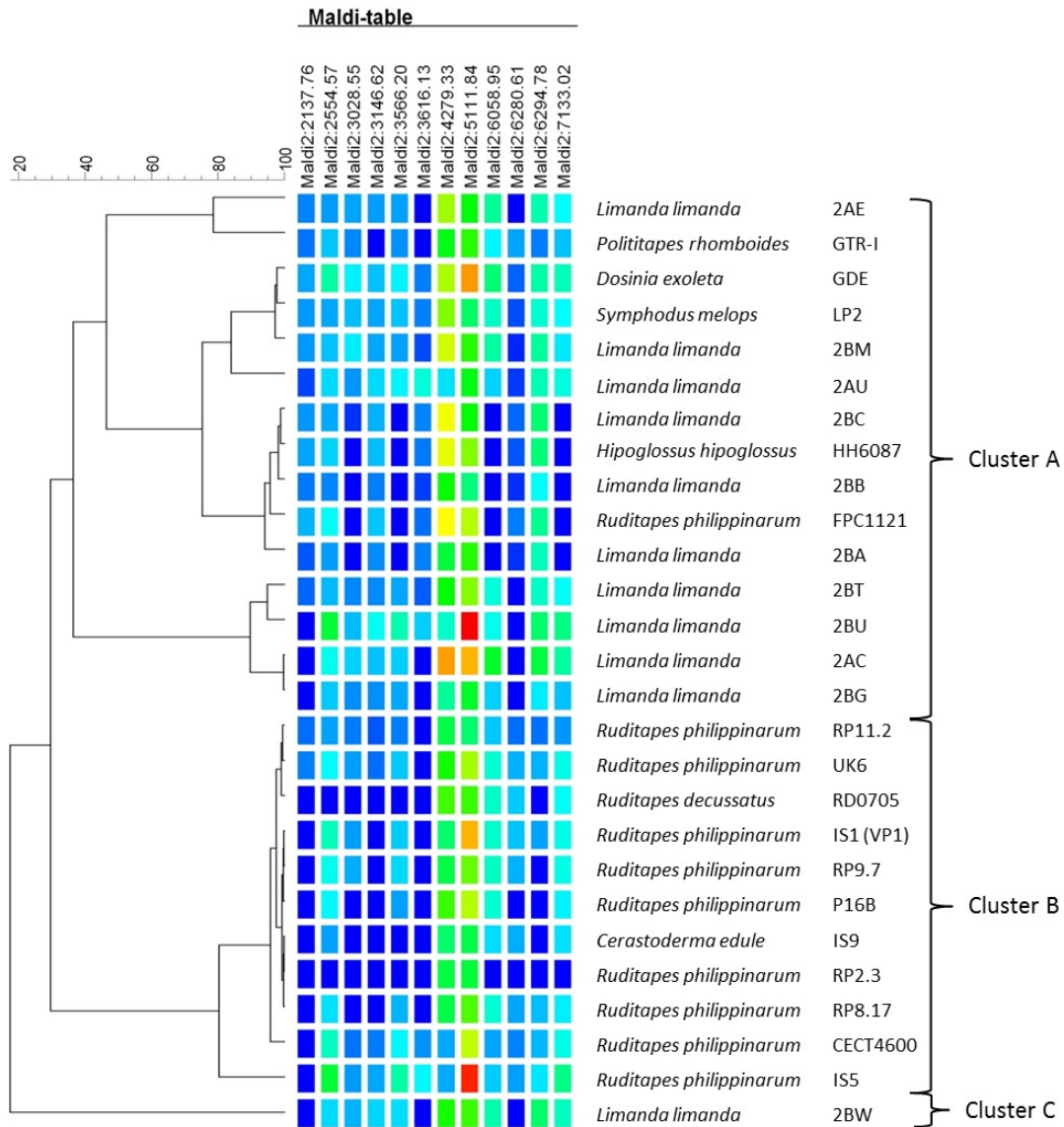
475

476 **Fig 1** Effect of isolates of *V. tapetis* from common dab on non-adherent cell ratio. Incubation time: 3h.

477 Results are presented by a mean ratio of non-adherent hemocytes (i.e. round hemocytes) in presence of

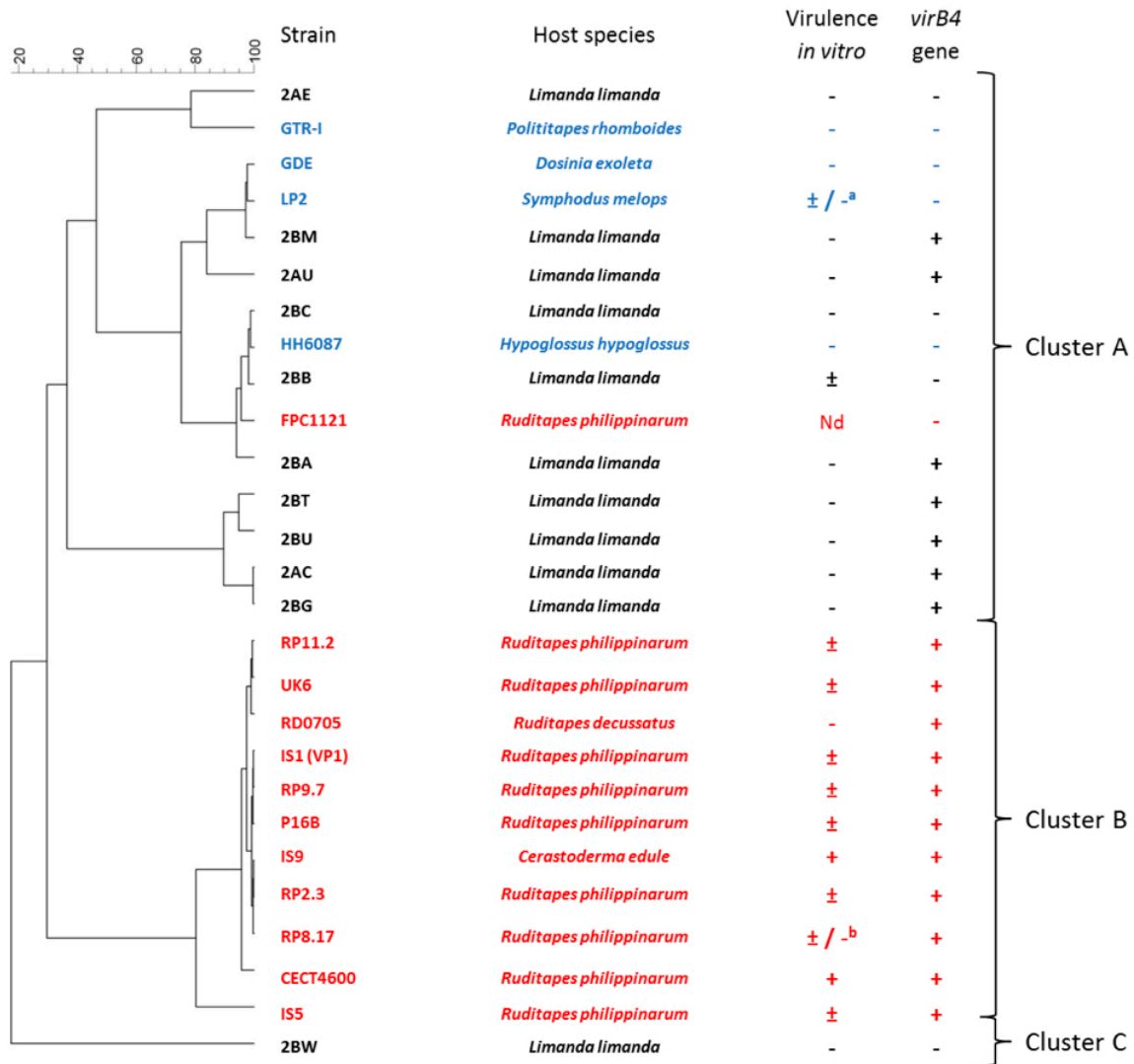
478 bacteria to the number of non-adherent cells after incubation with filtered sterile seawater (FSSW). Letters

479 depict significant differences (Pairwise Student test). Error bar = Standard Deviation/  $\sqrt{n}$  (n = 3 replicates)



480

481 **Fig 2** Dendrogram, based on the MALDI-TOF peak lists, of all *V. tapetis* isolates based on the complete  
 482 spectra. Peak intensity is represented in the heat map using different colors ranging from blue (low  
 483 intensity), over green (low intermediate) to yellow (intermediate), orange (high intermediate) and red  
 484 (high intensity).



485

486 **Fig 3** Dendrogram, based on the MALDI-TOF peak lists, of *V. tapetis* isolates derived from bivalves and fish

487 combined with *in vitro* virulence (hemocyte cytotoxicity assay) and presence of the *virB4* gene. In red:

488 isolates that can induce Brown Ring Disease *in vivo* after injection in the pallial cavity. In blue: isolates that

489 cannot induce Brown Ring Disease after injection in the pallial cavity. In black: isolates where no *in vivo*

490 assay has been performed against the Manila clam. References of the virulence profiles can be found in

491 Table 1. Cytotoxicity *in vitro* is depicted as follows: (-): no cytotoxicity, (±) intermediate cytotoxicity, (+):

492 comparable cytotoxicity as the reference strain (CECT4600). <sup>a</sup>: negative in Dias et al, 2018 but intermediate

493 in Choquet et al, 2003, <sup>b</sup>: negative in Dias et al, 2018 but intermediate in Choquet et al, 2004. Presence (+)

494 or absence (-) of the *virB4* gene as assessed using the TaqMan PCR method is also included. Nd: Not

495 determined