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1 # First isolation of virulent *Tenacibaculum maritimum*
2 # strains from diseased orbicular batfish (*Platax orbicularis*)
3 # farmed in Tahiti Island

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Abstract

The orbicular batfish (*Platax orbicularis*), also called 'Paraha peue' in Tahitian, is the most important marine fish species reared in French Polynesia. Sudden and widespread outbreaks of severe 'white-patch disease' have occurred since 2011 in batfish farms one to three weeks after the transfer of juveniles from bio-secured hatcheries to lagoon cages. With cumulative mortality ranging from 20 to 90%, the sustainability of aquaculture of this species is severely threatened.

In this study, we describe for the first time the isolation from diseased batfish of several strains belonging to the species *Tenacibaculum maritimum,* a major pathogen of many marine fish species. Histopathological analysis, an experimental bath challenge and a field monitoring study showed that *T. maritimum* is associated with white-patch disease. Moreover, molecular and serological analyses performed on representative strains revealed some degree of genetic diversity among the isolates, a finding of primary importance for epidemiological studies and for the development of management and control strategies such as vaccination.

1 Introduction

The orbicular batfish (*Platax orbicularis,* family *Ephippidae*) is a fish species inhabiting the top 30 metres depth over tropical reefs of the western Pacific [1]. It is widely distributed from the Indo-Pacific region to the Red Sea in East Africa, with a northern limit in south Japan and a southern limit in Australia and New Caledonia [2]. In French Polynesia, the batfish is highly appreciated as a food by the local population, but the wild stock is rapidly decreasing, probably as a result of high fishing pressure and climate change that severely disturb the coral communities [3]. To promote local sustainable aquaculture, the governmental department of marine resources (DRM) decided to focus effort on captive breeding and rearing of orbicular batfish. Since 2011, the governmental hatchery VAIA (Vairao, Tahiti, French Polynesia) has produced eighty thousand fry annually, reared inland in bio-secured conditions (i.e. in filtered and UV treated seawater). Four production cycles are completed

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42 per year, allowing an annual production of 50–80 metric tons. One to two-month-old juvenile fish
43 (mean weight, 1 and 10 g) are then transferred to different fish farms in lagoons. However, recurrent
44 mortalities occur almost systematically during the first two months of growth in the lagoon cages,
45 causing losses of 20–90% of production and threatening the sustainability of aquaculture based on this
46 species. One to three weeks following their transfer to net cages in the lagoon, the fish show
47 symptoms of disease, with loss of appetite, frayed fins, whitish patches on the tegument, followed by
48 ulcers, necrosis and death. Little was known about the status of pathogens and diseases associated with
49 this tropical fish species under local farming conditions and, consequently, this disease was simply
50 named 'white-patch disease', based on the clinical symptoms. Light microscopy examination of
51 fragments of skin lesions revealed abundant rod-shaped and gliding bacteria potentially belonging to
52 the genus *Tenacibaculum*.

53 The genus *Tenacibaculum* (family *Flavobacteriaceae,* phylum *Bacteroidetes*) currently comprises 31
54 validly named species (http://www.bacterio.net/tenacibaculum.html), all retrieved from marine
55 environments [4]. Among these, *T. dicentrarchi*, *T. discolor*, *T. finnmarkense*, *T. gallaicum*, *T.*
56 *maritimum, T. piscium* and *T. soleae* are responsible for ulcerative conditions that affect a large variety
57 of cultured and wild marine fish species and are collectively known as tenacibaculosis [5].
58 Tenacibaculosis is generally associated with gross external lesions such as ulcerative and necrotic skin
59 lesions, haemorrhagic mouth, frayed fins and tail rot [5]. The disease was originally described in 1977
60 in cultured red (*Pagrus major*) and black (*Acanthopagrus schlegeli*) seabream in Japan [6]. The
61 causative agent was subsequently identified as *Tenacibaculum maritimum* (formerly *Flexibacter*
62 *maritimus*) [7], a filamentous, Gram negative, gliding bacterium. Since then, *T. maritimum* has been
63 shown to be responsible for considerable losses in marine aquaculture worldwide, affecting a large
64 variety of wild and cultured marine fish species. For example, *T. maritimum* has been found to be
65 associated with mortality events occurring in Atlantic salmon (*Salmo salar*) in Australia [8], Chinook
66 salmon (*Oncorhynchus tshawytscha*) in Canada [9], rainbow trout (*Oncorhynchus mykiss*) in Australia
67 [8], sole (*Solea senegalensis*) and turbot (*Scophthalmus maximus*) in Spain [10], sea bass
68 (*Dicentrarchus labrax*) in Europe [11], Japanese flounder (*Paralichthys olivaceus*) in Japan [12], and

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69 black damselfish (*Neoglyphieodon melas*) and Picasso triggerfish (*Rhinecanthus assasi*) in Egypt [13].
70 Although other *T. maritimum* isolates have also been retrieved from outbreaks in other countries and
71 host fish species, pathogenicity has not been confirmed using experimental challenges in any of these
72 examples [14,15].

73 The aims of the present study were to investigate the recurrent and acute outbreaks occurring in *Platax*
74 *orbicularis* farms in French Polynesia, to characterize the causative agent using bacteriological,
75 histological and molecular analyses and then to conduct experimental challenges to confirm this
76 causality, thus fulfilling Koch's postulates.

77 2 Materials and methods

78 2.1 Ethic statement

79 In the absence of *adhoc* ethical committees in French Polynesia, *in vivo* experiments reported in the
80 present study fulfill all the sections of deliberation no 2001-16 APF from the Assembly of French
81 Polynesia issued in the Journal Officiel de Polynésie française on the 1st February 2001, dealing on
82 domestic or wild animal welfare. Nevertheless, we used several guidelines in the present study and
83 followed animal care and ethic guidelines (16,17). In particular, fish were euthanized using an
84 overdose of Benzocaine (150 mg L^{-1} stock solution prepared in ethanol). This method of euthanasia,
85 reproducible and safe to the operator, induces a depression of the central nervous system activity, rapid
86 unconsciousness and death of *P. orbicularis*, without compromising further microbiological and
87 histological analyses. The criterion used to exercise humane endpoint was that moribund fish
88 displaying the typical sign of white-patch disease had lost their ability to maintain an upright position
89 in the tanks and were not evasive to netting. During all experiments of this study, fish were monitored
90 by trained fish health specialists to make sure that the ethical aspects were secured.

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2.2 Sampling of diseased fish

Ten symptomatic orbicular batfish (mean weight 5.1 +/- 2.3 g) were recovered from two different farms located in Tahiti island. They were sampled during two severe outbreaks in 2013 and 2016 that had caused >50% cumulative mortality just 2 weeks after the fish had been transferred from the VAIA hatchery to net cages in the Tahiti lagoon. All fish showed erosion and ulceration of the skin surface. After being euthanized with an overdose of Benzocaine (150 mg L^{-1}), they were examined by microscopy and microbiological and histological techniques.

2.3 Direct microscopic examination and isolation of bacteria

Skin lesion scrapings from moribund batfish were collected using sterile surgical scalpels. Wet mount preparations were then examined under a light microscope (Leica DM 1000 LED). For each bacterial isolation, a sterile swab cotton-tipped applicator (COPAN) was used. Smears of skin samples were deposited onto plates of *Flavobacteriaceae*-selective marine agar (FSMA) developed by an accredited veterinary diagnostic laboratory (Labofarm, Loudéac, France). A total of ten dominant bacterial strains were isolated after sub-culture (Table 1).

Table 1. List of bacterial strains retrieved from farmed *Platax orbicularis* affected by white-patch disease, with their sources and characteristics. Virulence was evaluated by experimental bath challenge on *T. maritimum*-free batfish. Significant differences ($p<0.05$) in mortality rate (see paragraph 3-3) between non-infected and infected fish are indicated by 'yes' or 'no'. Isolate identification was performed using EzBioCloud software [18], based on >99% identity of their 16S rRNA sequences with the closest type strain. ST refers to the MLST sequence type. n/a: not analysed.

Strains	Source and date of isolation	GPS location	16S rDNA GenBank accession number	Bacterial species	Virulence	ST
TFA4	Skin lesions, Tautira lagoon, Tahiti, 2013	17°47’50’’ S, 149°07’14” W	MW690171	*T. maritimum*	yes	ST168
Aq 9-66	Skin lesions, Tautira lagoon, Tahiti, 2013	17°47’50’’ S, 149°07’14” W	MW690177	*T. mesophilum*	no	
Aq 9-67	Skin lesions, Tautira lagoon, Tahiti, 2013	17°47’50’’ S, 149°07’14” W	MW690178	*T. mesophilum*	no	
Aq 16-83	Skin lesions, Vairao lagoon, Tahiti, 2016	17°48’22’’ S, 149°17’36’’ W	MW690172	*T. maritimum*	n/a	n/a
Aq 16-84	Skin lesions, Vairao lagoon, Tahiti, 2016	17°48’22’’ S, 149°17’36’’ W	MW690173	*T. maritimum*	yes	n/a

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Aq 16-85	Skin lesions, Vairao lagoon, Tahiti, 2016	17°48'22'' S, 149°17'36'' W	MW690174	*T. maritimum*	n/a	ST167
Aq 16-87	Skin lesions, Vairao lagoon, Tahiti, 2016	17°48'22'' S, 149°17'36'' W	MW690175	*T. maritimum*	yes	n/a
Aq 16-88	Skin lesions, Vairao lagoon, Tahiti, 2016	17°48'22'' S, 149°17'36'' W	MW690176	*T. maritimum*	n/a	ST167
Aq 16-89	Skin lesions, Vairao lagoon, Tahiti, 2016	17°48'22'' S, 149°17'36'' W	MW690180	*T. maritimum*	n/a	ST167
Aq 16-91	Skin lesions, Vairao lagoon, Tahiti, 2016	17°48'22'' S, 149°17'36'' W	MW690179	*T. mesophilum*	n/a	

112

2.4 Histopathological examination

114 Skin fragments of approximately 1 cm^2 were collected from four fish showing typical skin lesions of
115 the white-patch disease using a sterile surgical scalpel. Samples were fixed in Davidson's solution
116 (25% formaldehyde, 37.5% ethanol, 12.5% acetic acid and 25% water) for 48 hours at room
117 temperature, then washed and kept in 70% ethanol. Skin samples were progressively dehydrated in an
118 ascending series of alcohol (70 to 100% ethanol) followed by a xylene bath, using a dehydration
119 automate (Leica, ASP 300S), then embedded in paraffin, cut into 3-µm sections using a rotary
120 microtome (Microm HM 340E, Thermo Fisher Scientific) and stained with haematoxylin–eosin (H-E)
121 using a fully-automated integrated stainer (Leica, CV5030 autostainer XL). Several sections were
122 analysed to ensure reproducibility, using a Leica DM 1000 LED microscope equipped with a Dino-
123 Lite camera (AnMo Electronics).

2.5 Molecular and serological studies

125 16S rDNA sequences were PCR-amplified using the universal 27F and 1492R primers (Table S1) and
126 the purity and length of the amplicons were verified by agarose gel electrophoresis. Amplicons were
127 Sanger sequenced by GATC-biotech (https://www.gatc-biotech.com) using the six universal
128 sequencing primers listed in Table S1. For each strain, the six sequences were visualized and aligned
129 to create a consensus sequence (with > 2X coverage over 80% of the sequences) using Benchling
130 software (2020). For primary taxonomic assignation, the 16S rRNA consensus sequences were
131 searched against the EzBioCloud database [18] (accession numbers are given in Table S2). In addition,

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132 a tentative phylogenetic tree was constructed using the MAFFT online service [19]. The evolutionary
133 distance was calculated using 1000 bootstrap replicates (Fig S1).

134 To characterize the genetic diversity of presumptive *T. maritimum* strains in greater depth, multi-locus
135 sequence analysis (MLSA) was performed on four selected isolates (TFA4, Aq 16-85, Aq 16-88, Aq
136 16-89) using sequences retrieved from their draft genomes [20]. These isolates were selected
137 according to their background information: Aq 16-85, Aq 16-88 and Aq 16-89 were sampled from
138 three different infected fish during an outbreak at the Vairao fish farm in 2016, while strain TFA4 was
139 isolated from a symptomatic fish at the Tautira fish farm in 2013 (Table 1). The MLSA profile defined
140 by Habib et al. (2014) [21] consists of the sequences of seven housekeeping genes (*atpA*, *gyrB*, *dnaK*,
141 *glyA*, *infB*, *rlmN* and *tgt*). The profiles of the new allele and sequence types (ATs and STs,
142 respectively), were generated and analysed using the *Tenacibaculum* pubMLST database
143 (https://pubmlst.org/tenacibaculum/) [22]. Results were visualized using the incremented Interactive
144 Tree of Life (iTOL) v3 tool [23].

145 To examine the isolates identified as *T. maritimum* more closely, their serotype was determined as
146 described by Avendaño-Herrera et al. (2004b) [24]. This method uses a slide agglutination test and a dot
147 blot assay on both whole-cell preparations and heat stable O antigens of each strain. Antisera against
148 serovars O1 (PC503.1), O2 (PC424.1) and O3 (ACC13.1) were used in all assays.

149 2.6 Experimental infection by immersion and quantification of 150 *Tenacibaculum maritimum* in mucus samples by real-time qPCR

151 A batch of *Platax orbicularis* fingerlings (mean weight 9.7 +/- 2.6 g) reared at the VAIA bio-secured
152 hatchery was transferred for acclimatization to a 1-m^3 fibreglass tank containing seawater (salinity, 32
153 PPT; water temperature of 26–27°C) for 10 days. Prior to infection, 494 fish were randomly selected
154 and transferred to nine 150-L tanks (50 fish per tank) filled with 5-µm filtered seawater. Three groups
155 were tested in triplicate: (i) non-infected fish (NIF), (ii) fish infected with *T. maritimum* strain TFA4
156 (IF) and (iii) fish with impaired mucus (IM) infected with *T. maritimum* strain TFA4 (IM-IF). The

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157 mucus of the latter fish was partially removed by gently wiping one side of the fish with a sponge
158 soaked in filtered seawater. Fish were challenged with a pure culture of strain TFA4 obtained by
159 incubation at 27°C for 48 h (stationary phase) in autoclaved nutrient broth composed of 4 g L^{-1}
160 peptone and 1 g L^{-1} yeast extract (Becton, Dickinson and Co., Sparks, MD, USA) in 5µm-filtered
161 seawater under orbital shaking at 200 rpm. Bath challenges were performed for two hours with strain
162 TFA4 at a final concentration of 5.3 10^4 CFU mL^{-1} for groups IF and IM-IF or with nutrient broth in
163 the case of the mock-treated NIF control group. The infected fish were then rinsed twice with filtered
164 seawater to remove all non-adherent bacteria, and fish from the NIF control group were manipulated
165 in the same way. Mortality was monitored twice daily from day 0 (D0) until day 5 (D5) at which point
166 the fish were euthanized using 150 mg L^{-1} Benzocaine. Any fish that died or were found moribund
167 over the experimental period (D0–D5) were promptly removed from the tanks during the monitoring.
168 The non-parametric Kaplan–Meier method (R package *survival*) was used to test for differential
169 survival performances among groups at the same date or within groups throughout the sampling
170 period. Differences were considered significant at $P < 0.05$.

171 At 24 h post-infection, before the onset of mortality, four fish displaying skin lesions were randomly
172 sampled from the two infected groups (IF and IM-IF) in addition to four fish from the NIF group.
173 These were used to quantify *T. maritimum* cells in fish mucus using TAQMAN real time PCR (see
174 primers and probe in suppl. Table 1) following the protocol developed by Fringuelli et al. (2012) [23]
175 with minor modifications. Briefly, skin mucus samples were obtained from lesions on symptomatic
176 fish using cotton swabs (COPAN), directly diluted in 1.5-mL micro-centrifuge tubes containing 0.5
177 mL of lysis solution (0.1 M EDTA pH 8; 1 % SDS and 200 µg ml^{-1} proteinase K) and incubated
178 overnight at 55°C. DNA was extracted using the conventional phenol/chloroform/isoamyl alcohol
179 (25/24/1) method. DNA quantity and purity were assessed using a NanoDrop ND 1000
180 spectrophotometer (Thermo Fisher Scientific). In order to obtain a standard curve, bacterial cells of
181 strain TFA4 from a stationary phase culture in nutrient broth were enumerated using a Malassez
182 counting chamber (2.35 10^8 bacteria ml^{-1}) and DNA from 1 ml of the bacterial suspension was
183 extracted. The DNA was then spiked at a final concentration of 1.33 ng μl^{-1} in salmon sperm gDNA

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184 (SSD, Thermo Fisher) at 10 ng μl^{-1} in artificial seawater (ASW, Sigma), then serially diluted 10-fold
185 in SSD at 10 ng μl^{-1} in ASW. A linear range of values was obtained for PCR amplification on a
186 Mx3000 Thermocycler (Agilent) using Brilliant III Ultra-Fast QPCR Master Mix (Agilent) following
187 the supplier's recommendations (5 µl DNA at 10 ng μl^{-1} in a total reaction volume of 20 µl), with six
188 successive sample 10-fold dilutions tested in triplicate. Cycle threshold (Ct) values ranged from 16.05
189 to 33.14, corresponding to 1.44 10^5 to 2.06 10^1 cells of strain TFA4 per PCR well, while correlation
190 (linear regression with r^2 coefficient) and qPCR reaction efficacy were 0.995 and 99.9%, respectively.

191 2.7 Detection and quantification of *Tenacibaculum maritimum* 192 during a field episode of tenacibaculosis

193 Juvenile batfish (mean weight 10 +/- 3.3 g) reared in the bio-secured facilities of the VAIA hatchery
194 were carefully transferred (D0) to the Tahiti Fish Aquaculture farm in Tautira lagoon and kept in a
195 single net cage of 1 m^3 (167 fish/m^3 density). Ten fish were collected at five sampling times: day 6
196 before transfer (D-6) (i.e. in the VAIA hatchery) and D1, D9, D17 and D36 post-transfer to Tautira
197 lagoon. When gross signs of the white-patch disease were observed, five moribund fish (symptomatic)
198 and five apparently healthy ones (asymptomatic) were sampled. These fish were euthanized as detailed
199 above and the liver, posterior intestine and some skin mucus (collected with a cotton swab in the
200 lesion area in the case of symptomatic fish) were individually and aseptically sampled and preserved
201 in 500 µl RNAlater (Ambion) at -80°C. Approximatively 100 mg of tissue were used to quantify *T.*
202 *maritimum* by qPCR [23]. Throughout this survey, no curative treatments were given, mortality was
203 monitored daily and moribund animals (euthanatized with an overdose of benzocaine) or dead fish
204 were removed and discarded.

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205 # 3 Results

206 ## 3.1 Microscopic examination and isolation of bacteria

207 Two severe white-patch disease outbreaks, occurring within the first 2 months following transfer to
208 net cages in a lagoon, were recorded in 2013 and 2016. These outbreaks occurred in two
209 geographically distinct fish farming areas of Tahiti island (Tautira lagoon: 17°47'50'' S, 149°07'14"
210 W, and Vairao lagoon: 17°48'22'' S, 149°17'36'' W) with cumulative mortality reaching 80% and
211 62%, respectively. The main clinical signs were loss of appetite, erratic swimming and ulcerative skin
212 lesions (Fig 1A). Wet mount examination of the skin mucus of diseased batfish revealed a significant
213 amount of long (6.3 +/- 0.6 µm) and rod-shaped bacteria (Fig 1B).

214 **Fig 1. Examination of fish lesions.** A) Gross clinical signs of the white-patch disease of batfish
215 characterized by: (i) circular discoloration areas of various sizes, apparently randomly distributed on
216 the skin surface; (ii) skin lesions, ulcers, scale loss (white arrow) and areas of haemorrhagic necrosis
217 (red arrow); and (iii) frayed (usually caudal) fins (black arrow). B) Microscopic examination of skin
218 lesions reveals abundant, long, slender, rod-shaped bacteria. Numerous bacteria remain adhered to the
219 fish scales (white arrows) while others detached after the fragment of lesion was crushed. C) View of
220 the skin surface at the interface between apparently healthy and damaged zones. D) An apparently
221 healthy zone observed under a surgical Q-Scope microscope (AnMo Electronics).

222

223 Histopathological examination of skin lesions from moribund batfish revealed that the epidermis and
224 dermis were severely damaged, with clusters of filamentous, *Tenacibaculum*-like bacteria and
225 scattered inflammatory cells (Fig 2). In contrast, no evidence of histopathological changes was noticed
226 in the internal organs.

227 **Fig 2. Two representative cross sections (H-E staining) of *Platax orbicularis* fingerlings affected**
228 **by white-patch disease.** A1 and B1: Severe necrosis affecting the hypodermis and dermis layers with
229 invasion of *Tenacibaculum* cells (arrows) visible at a higher magnification (A2 and B2) and detection
230 of inflammatory cells in damaged areas (asterisks).

231

232 Ten strains were isolated from samples of 10 moribund batfish exhibiting the typical signs of white-
233 patch disease using *Flavobacteriaceae*-selective marine agar (Table 1). Two different colony

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234 morphotypes were observed after 48 h of incubation at 27°C: the first morphotype consisted of pale,
235 translucent colonies with uneven edges, extremely adherent to the agar (Fig 3A), while the second
236 morphotype consisted of orange, opaque, diffuse and strongly iridescent colonies (Fig 3B, C).

237 **Fig 3. Representative strains isolated from symptomatic *Platax orbicularis*.** A) colonies of
238 *Tenacibaculum maritimum*, strain TFA4. B) and C) colonies of *Tenacibaculum mesophilum*, strain Aq
239 16-91, with different camera shooting angles revealing the iridescent phenotype.

240 3.2 Genomic and serological characterization

241 Analysis of nearly complete 16S rRNA sequences revealed that all isolates belonged to the genus
242 *Tenacibaculum*. Seven strains (TFA4, Aq 16-83, Aq 16-84, Aq 16-85, Aq 16-87, Aq 16-88 and Aq 16-
243 89) shared 99.65% to 99.79% sequence identity with the *T. maritimum* type strain NBRC 15946^{T}, with
244 at least 98,9% coverage. The three remaining strains (Aq 9-66, Aq 9-67 and Aq 16-91) displayed
245 99.37 to 99.93% sequence identity with the *T. mesophilum* type strain DSM 13764^{T}, with at least
246 98,9% coverage. A tentative phylogenetic tree was drawn using MAFFT (Fig S1). The seven strains
247 displaying the first morphotype clustered with the *T. maritimum* type strain, while the three strains
248 belonging to the second morphotype clustered with the *T. mesophilum* type strain; bootstrap values
249 were 100% and 91%, respectively.

250 The results of the MLSA analysis (Fig 4) performed on *T. maritimum* strains TFA4, Aq 16-85, Aq 16-
251 88 and Aq 16-89 showed that none matched exactly with any of the sequence types (ST) already
252 described in the pubMLST database. They were therefore treated as belonging to new STs: ST168,
253 which was attributed to strain TFA4; and ST167, which was attributed to strains Aq 16-85, Aq 16-88
254 and Aq 16-89. Analysis of the number of locus variants revealed that these novel STs only share three
255 allele types (AT), corresponding to loci *gyrB*, *infB* and *rlmN*, which reveal genetic heterogeneity
256 among these two groups of isolates. The single and double locus variant analyses (SLV and DLV)
257 were fairly congruent with the phylogenetic tree based on the concatenated nucleotide sequences of
258 the seven housekeeping genes (Fig 4). SLV analysis showed that ST168 (TFA4) shares 6/7 loci with
259 ST2, which up to now only included strain ACC13.1 (referenced as 002 in the pubMLST database),

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260 isolated from a diseased Senegalese sole (*Solea senegalensis*) in Portugal. In the DLV, TFA4 shared
261 5/7 loci with ST3, 4, 10, 35, 36, and 130, which essentially comprise isolates from the south of Europe
262 (except for strain 4646, isolated in Australia). Interestingly, strains Aq 16-85, Aq 16-88 and Aq 16-89
263 (ST167) formed a singleton, meaning that they displayed at least three different ATs compared with
264 all the strains included in the pubMLST database. These results revealed the existence of at least two
265 genetically distinct groups of *T. maritimum* isolates in Tahitian fish farms.

266 **Fig 4. Genomic and background information on all *T. maritimum* isolates in the pubMLST**
267 **database.** Strains isolated in this study are shown in red type. Neighbor-joining tree based on the
268 concatenated nucleotide sequences of the 7 housekeeping genes (3894 bp). Other information: strain
269 number, strain name, country of isolation, fish host species, year of isolation, sequence type, and its
270 allelic combination.
271

272 Serological analysis revealed two different serogroups among the *T. maritimum* strains. Slide
273 agglutination tests showed that strain TFA4 specifically reacted with the anti-O3 antiserum, while
274 strains Aq 16-85, Aq 16-88 and Aq 16-89 specifically reacted with the anti-O1 antiserum.

275 3.3 Pathogenicity assays using an immersion challenge

276 All batfish that were experimentally infected with *T. maritimum* TFA4 using the immersion challenge
277 (IF group) exhibited typical clinical signs of white-patch disease starting from 24 h post-infection (PI),
278 although no mortality was recorded at this time point (Fig 5). At 30 h PI, fish in this group underwent
279 significant (chi-squared-test with simulated p-value correction, $P = 0.017$) mortality, with a survival
280 probability of 94.9% compared with 100% (no death events) in the non-infected fish group (NIF). A
281 sudden mortality event was observed in the IF group between 30 and 48 h PI, with 94.9% and 25%
282 survival probabilities, respectively. From 82 h until the end of the monitoring period (120 h PI), no
283 increase in mortality was observed (7.1% survival probability for the IF group at 72 h PI to 120 h PI)
284 even though all batfish displayed typical clinical signs of the disease. Nevertheless, the intensity of
285 ulcerative skin lesions (i.e. the number and area of whitish patches) in the IF group from 72 h to 120 h
286 PI was lower than that observed before 72 h PI (data not shown). In the IM-IF group, not only did
287 batfish experience the highest mortality rate because all fish died, giving a survival probability of 0%

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288 from 30h PI, but they also died significantly earlier than those of the IF group with intact skin mucus
289 (log-rank test comparing the survival curves from 0 h PI to 30 h PI, P = 0.02). No mortality was
290 recorded in the NIF control group during the entire trial.

291 **Fig 5 Survival curves of bath-challenged batfish *Platax orbicularis*.** NIF, non-infected fish; IF, fish
292 infected with 5.3 10^4 CFU mL^{-1} of strain TFA4 for 2 hours; IM-IF, infected fish from which mucus
293 had been partially removed before the bath challenge.

294

295 All skin samples that were collected from diseased fish in the IF group before the onset of mortality
296 were found positive by real time PCR assay, with an average load of 7.8 10^8 +/- 1.4 10^8 *T. maritimum*
297 bacteria per µg of total extracted DNA. In contrast, no *T. maritimum* was detected in any of the four
298 analysed skin samples in the NIF group. The virulence potential of two (Aq 16-84 and Aq 16-87) and
299 three (Aq 9-65, Aq 9-66 and Aq 9-67) strains belonging to the species *T. maritimum* and *T.*
300 *mesophilum,* respectively, was also evaluated using the same immersion challenge protocol. Results of
301 this separate trial showed that the two *T. maritimum* strains exhibited levels of virulence similar to that
302 of strain TFA4, whereas the three *T. mesophilum* strains were avirulent at a similar infection dose
303 (6.1 10^4 CFU mL^{-1}), with no mortality recorded in the groups IF and NIF during a seven-day post-
304 infection survey (data not shown).

305 3.4 Kinetics of *Tenacibaculum maritimum* infection during a field 306 episode of tenacibaculosis

307 To gain insight into *T. maritimum* pathogenesis under natural field conditions, a batch of batfish was
308 monitored from its production under bio-secured conditions at the VAIA hatchery to its rearing in a
309 net cage on a private farm in Tautira lagoon. Soon after the transfer to the net cage in the lagoon, a
310 severe outbreak of white-patch disease was observed, with the first typical signs appearing from D1
311 post-transfer and mortality from D3 (Fig 6).

312 **Fig 6. Cumulative mortalities of a batch of batfish during a natural outbreak following their**
313 **transfer to a net cage in Tautira lagoon.** Fish were 10 g (mean weight) and reared at an initial
314 density (D0) of 167 fish/m^3.

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315

316 Two peaks of mortality occurred, at D3–D6 and D13–D31. The second peak was higher, with
317 cumulative mortalities increasing significantly from 11.2% at D13 to 74.5% at D31. No subsequent
318 mortality then occurred among the surviving batfish until the end of the study period (D36).

319 Six days before the transfer to the lagoon cage (D-6) all sampled fish were qPCR negative for *T.*
320 *maritimum* (Fig 7).

321

322 **Fig 7. Kinetics of *Tenacibaculum maritimum* bacterial cells quantified by qPCR in the mucus of**
323 **asymptomatic (white boxes) and symptomatic (grey boxes) batfish from D-6 (at VAIA hatchery)**
324 **to D36 post transfer to net cage in the lagoon.** Quantification results are expressed in numbers of *T.*
325 *maritimum* cells per µg of total extracted gDNA. Because zero values (no detection) cannot be
326 represented on a logarithmic scale, an arbitrary value of 1.1 was assigned to these negative results.
327 Each box-plot shows mean (white circle), the 25th to 75th percentile (rectangular box), the minimum
328 and maximum values (dots at the extremities), as well as individual quantification (black dots) from 5
329 to 10 batfish per sampling time and group of batfish.

330

331 In contrast, the mucus of all batfish sampled just one day (D1) after the transfer to the lagoon net cages
332 was found positive for *T. maritimum* by qPCR, although very different bacterial loads (mean values,
333 $9.98\ 10^6 \pm 1.57\ 10^7$ cells per µg DNA) were observed. At D9, during the first stationary phase of
334 mortality (D6–D13), asymptomatic batfish showed significantly lower bacterial loads in their mucus
335 compared with D1 (Kruskal Wallis test; $p = 0.020$). Nevertheless, the amount of *T. maritimum* at D9
336 was significantly higher ($p = 0.047$) in batfish exhibiting clinical signs of tenacibaculosis compared
337 with asymptomatic fish. During the second peak of mortality, the discrepancy between asymptomatic
338 and symptomatic batfish was even more pronounced ($p = 0.016$). At the end of the mortality events
339 (D36), *T. maritimum* DNA was absent from most sampled surviving fish (8/10). Indeed, only two
340 asymptomatic batfish among the 10 tested were found positive by qPCR but at very low levels (8.36
341 $10^2 \pm 6.02\ 10^2$ cells per µg DNA), signalling the end of the outbreak.

342 qPCR results on the liver of the same batfish sampled for their mucus revealed that only one fish
343 among the 50 tested, sampled at D1, was positive for *T. maritimum* at a low level ($1.19\ 10^4$ cells per

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344 µg gDNA). Similar results were obtained with the posterior intestine: only seven batfish were found
345 qPCR positive, with the low value of 1.11 $10^4 \pm$ 1.37 10^4 cells per µg gDNA (data not shown).

346 4 Discussion

347 Orbicular batfish aquaculture in French Polynesia started in 2004 and the first symptoms of white-
348 patch disease were observed in 2006. In this study, we showed that the white-patch disease decimating
349 farmed *Platax orbicularis* is associated with *T. maritimum* infection. To our knowledge, this is the first
350 time that this bacterium has been isolated in French Polynesia, and also the first time it has been
351 retrieved from batfish. *T. maritimum* has been associated with a large variety of marine fish species:
352 38 according to Nowlan et al. (2020) [5], including the orbicular batfish (this study). Surprisingly,
353 *Platax orbicularis* is the only tropical fish in which this pathogen has been reported so far.
354 Nevertheless, the range of susceptible hosts for this bacterium is probably underestimated. In French
355 Polynesia, tenacibaculosis has dramatic consequences for batfish farms, which often suffer mortality
356 levels over 50%. In addition, the disease may also be of serious concern regarding the diversification
357 of aquaculture programs launched by local authorities.

358 In this study, we described a reproducible bath challenge protocol that demonstrated that *T. maritimum*
359 is able to infect orbicular batfish by immersion, thus fulfilling Koch's postulates. Experimental
360 infection procedures using immersion challenges have been broadly used with fish pathogens in recent
361 years because they are likely to mimic the natural infection process more accurately than injection
362 challenges. In particular, immersion does not bypass the first line of fish defence (i.e. the skin mucus
363 barrier), unlike the more common subcutaneous, intraperitoneal and intramuscular injection routes. It
364 has also been reported that, compared with immersion, some injection challenges fail to induce
365 tenacibaculosis [11,26–28] or can lead to high mortality rates in negative controls due to stress and
366 local lesions caused by the injection [29]. However, comparative analyses of challenge protocols are
367 rather difficult to perform due to the many factors reported to influence pathogenicity, such as the
368 bacterial strain [9,27,28], culture conditions (i.e. growth medium and temperature), infection dose

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369 [12,26], duration of immersion [30], physical [27] and chemical characteristics of seawater,
370 zootechnical practices (e.g. fish density and animal feed), and the host fish species [8], physiological
371 status (e.g. age [27]) and genetic background (e.g. susceptible, resistant). In this study, no significant
372 difference in mortality rates was observed when batfish were infected with strains TFA4, Aq 16-87, or
373 Aq 16-84, although these strains differed in some genetic traits. However, due to the high virulence of
374 these strains in our immersion challenge model, further studies using a lower dose or shorter
375 immersion time might reveal virulence differences between these strains. Although physical alteration
376 of the fish skin was not necessarily seen before morbidity and mortality, batfish with impaired mucus
377 developed clinical signs more rapidly and experienced higher mortality rates (100% mortality at 23 h
378 post infection) than those which mucus was intact. These results are in agreement with similar studies
379 performed on other fish species [11,30]. Indeed, mucus has been largely documented as an important
380 component of the fish innate immune system and a physical and chemical barrier against pathogens
381 [31].

382 Some of the isolates from diseased batfish were shown to belong to another *Tenacibaculum* species, *T.*
383 *mesophilum*, a bacterium initially reported in a marine sponge. In addition, *T. mesophilum* strain
384 HMG1 was shown to degrade malachite green, an antimicrobial that has long been used in aquaculture
385 but now banned in many countries [32]. Immersion challenges performed with strains Aq 9-66 and Aq
386 9-67 showed that both strains were totally avirulent. Further studies would be needed, however, to
387 determine whether *T. mesophilum* strains can play a role in the pathogenesis of tenacibaculosis,
388 primarily caused by *T. maritimum*, by acting as secondary colonizers of the lesions.

389 Although this study was conducted with only 10 isolates, an unexpected diversity of *T. maritimum*
390 isolates was found. Our results demonstrate the presence of two distinct groups: strains Aq 16-85, Aq
391 16-88 and Aq 16-89, belonging to serotype O1 and to sequence type ST167; and strain TFA4,
392 belonging to serotype O3 and to sequence type ST168. Such a diversity among *T. maritimum* isolates
393 was also noticed among the Australian isolates (Fig 4). In agreement with Van Gelderen et al. (2010)
394 [33], no correlation between serotype and geographic distribution was observed in the present study.

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395 Additional work is needed to make a deeper exploration of the genetic diversity of *Tenacibaculum*
396 strains associated with batfish in French Polynesia in order to evaluate their virulence potential and
397 develop management and disease control strategies. Because the natural ecology of *T. maritimum* is
398 still unknown, more in-depth epidemiological studies will also be necessary to decipher the mode of
399 transmission and the natural route of infection of this pathogen.

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508
Supporting information

509 **S1 Fig. Phylogenetic relationships of 16S rDNA nucleotide sequences of the strains recovered in**
510 **this study and the type strains of all *Tenacibaculum* species (See Table S2 for accession number)**.
511 The phylogenetic tree was constructed with the MAFFT online tool using the neighbor-joining (NJ)
512 method with Jukes Cantor substitution model on all gap-free sites (1316pb) based on an alignment of
513 32 members of the genus *Tenacibaculum* performed with the L-INS-i method. Numbers at each
514 branch indicated the percentage bootstrap values on 1,000 replicates. The 16S rDNA sequence of
515 *Pseudotenacibaculum haliotis* (strain FDZSB0410) was used as an outgroup.
516
517 **S1 Table. List of PCR primers and probe used in this study.**
518

Name	Target gene	Sequence (5'-3')	Reference
27F	*16S rDNA*	AGAGTTTGATCMTGGCTCAG	Universal sequencing primer
1492R	*16S rDNA*	TACGGYTACCTTGTTACGACTT	Universal sequencing primer
785F	*16S rDNA*	GGATTAGATACCCTGGTA	Universal sequencing primer

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805R	*16S rDNA*	GACTACCAGGGTATCTAATC	Universal sequencing primer
518R	*16S rDNA*	GTATTACCGCGGCTGCTGG	Universal sequencing primer
1100F	*16S rDNA*	YAACGAGCGCAACCC	Universal sequencing primer
MAR 4 fwd	*16S rDNA*	TGCCTTCTACAGAGGGATAGCC	Fringuelli et al. (2012) [25]
MAR rev	*16S rDNA*	CTATCGTTGCCATGGTAAGCCG	Fringuelli et al. (2012) [25]
MAR probe	*16S rDNA*	Texas Red -CACTTTGGAATGGCATCG- BHQ2	Fringuelli et al. (2012) [25]

519

520 **S2 Table. Accession number of the type strains used in S1 Fig.**

Type strains	**Genome accession number**
Tenacibaculum adriaticum B390	AM412314
Tenacibaculum aestuarii SMK-4	DQ314760
Tenacibaculum aestuariivivum JDTF-79	MF193601
Tenacibaculum agarivorans HZ1	MSMP01000073
Tenacibaculum aiptasiae a4	EF416572
Tenacibaculum amylolyticum MBIC4355	AB032505
Tenacibaculum ascidiaceicola RSS1-6	KT231981
Tenacibaculum caenipelagi HJ-26M	KC832834
Tenacibaculum crassostreae JO-1	EU428783
Tenacibaculum dicentrarchi 35/09	FN545354
Tenacibaculum discolor DSM 18842	RCCS01000002
Tenacibaculum finnmarkense DSM 28541	KT270385
Tenacibaculum gallaicum A37.1	AM746477
Tenacibaculum geojense YCS-6	HQ401023
Tenacibaculum halocynthiae P-R2A1-2	JX912707
Tenacibaculum haliotis RA3-2	KX450476
Tenacibaculum holothuriorum S2-2	LAPZ01000023
Tenacibaculum insulae JDTF-31	MF765760.1
Tenacibaculum jejuense KCTC 22618	LT899436
Tenacibaculum litopenaei B-I	DQ822567
Tenacibaculum litoreum CL-TF13	AY962294
Tenacibaculum lutimaris DSM 16505	RAQM01000002
Tenacibaculum maritimum NCIMB 2154	KT270382.1
Tenacibaculum mesophilum DSM 13764	jgi.1107970
Tenacibaculum ovolyticum IFO 15947	AB078058
Tenacibaculum piscium TNO020	GU124766
Tenacibaculum sediminilitoris YKTF-3	KU696540
Tenacibaculum singaporense TLL-A2	MG641897
Tenacibaculum skagerrakense D30	AF469612
Tenacibaculum soleae LL04 12.1.7	AM746476
Tenacibaculum todarodis LPB0136	CP018155

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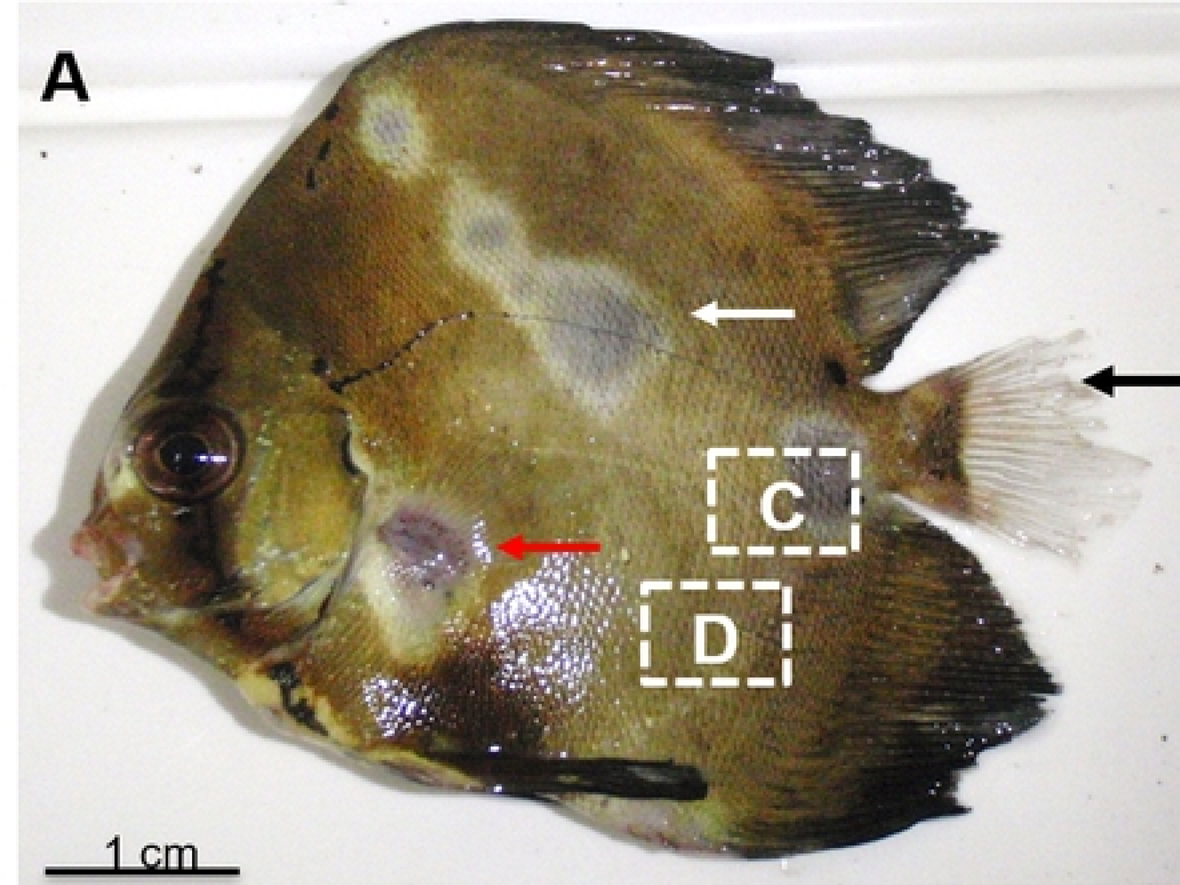
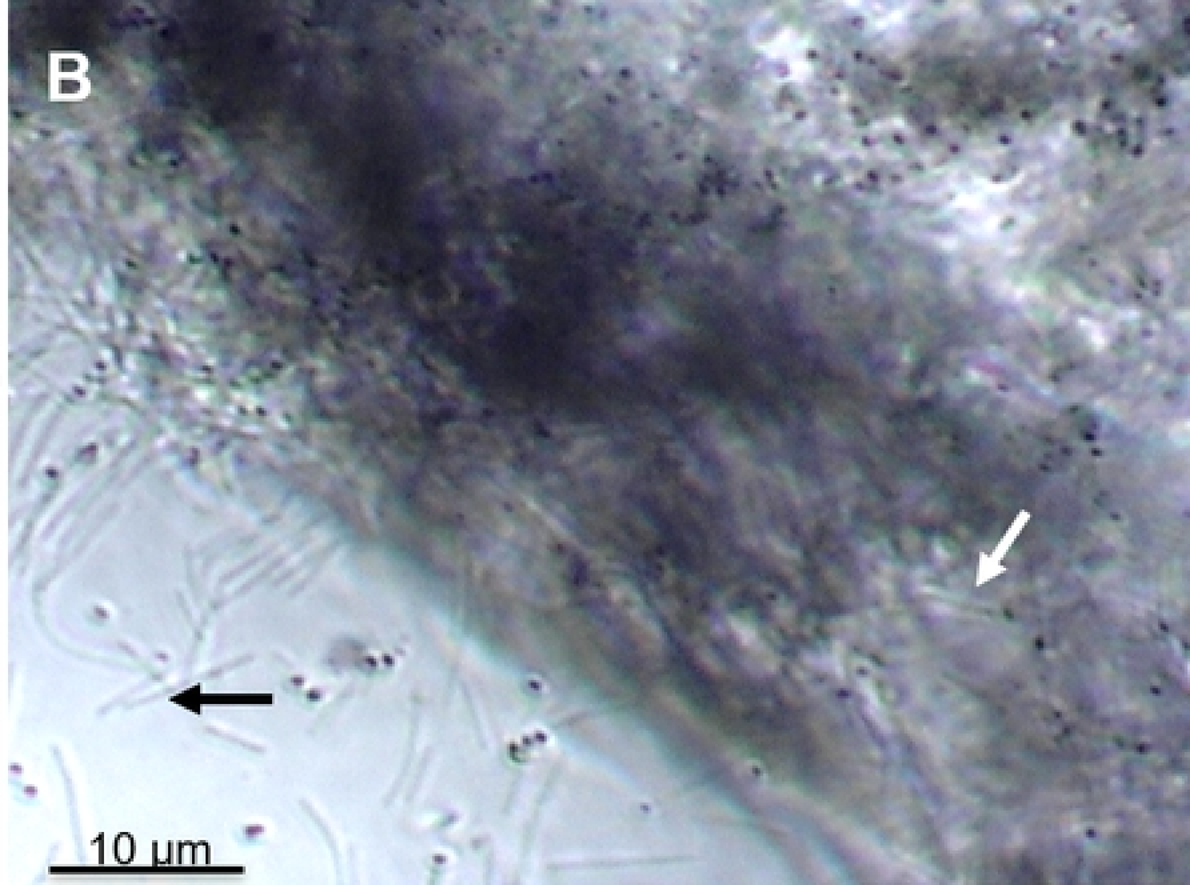
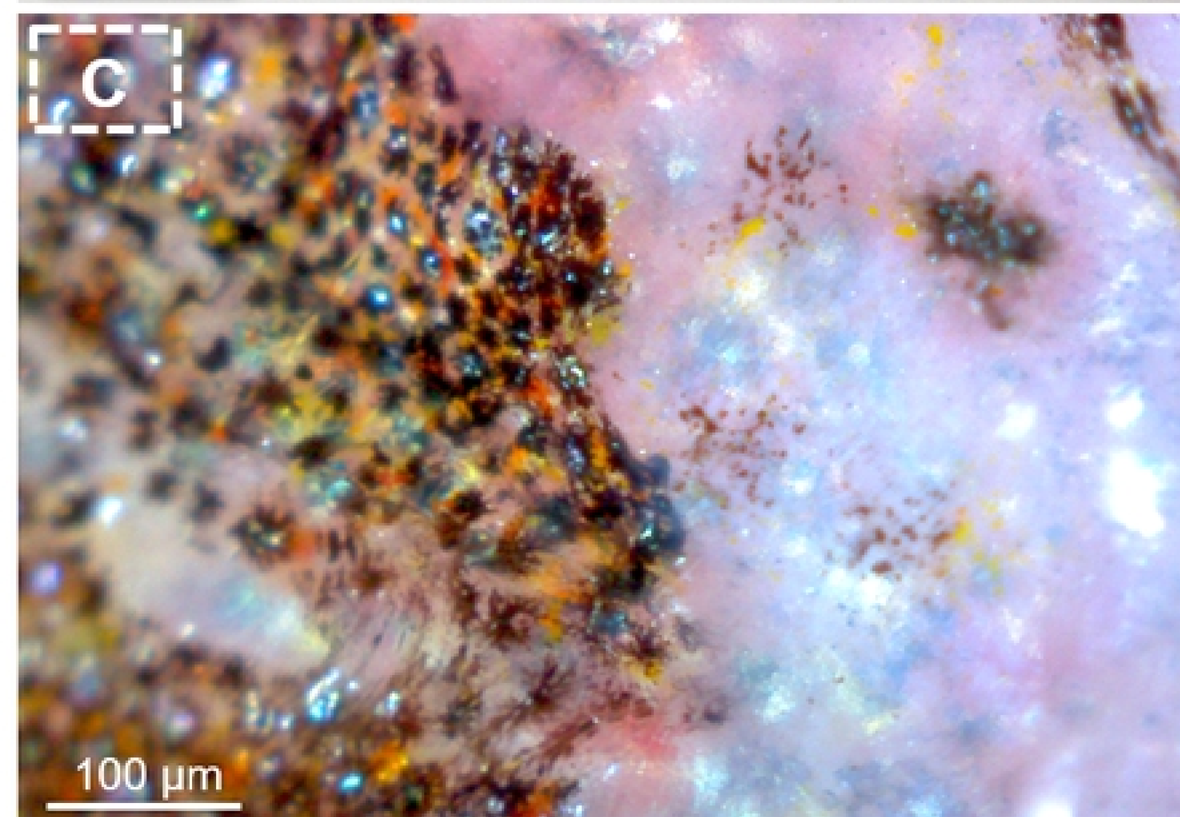
Tenacibaculum xiamenense WJ-1	JX984443

521







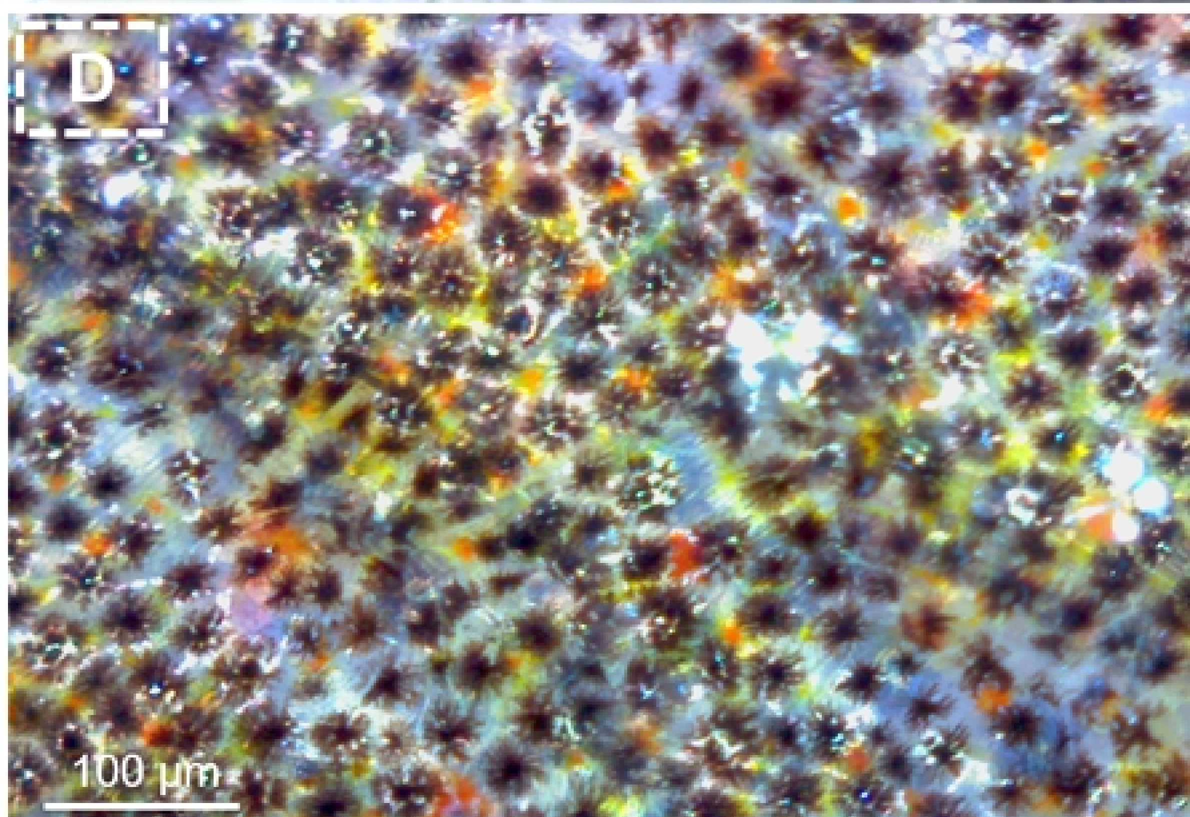


Figure 1

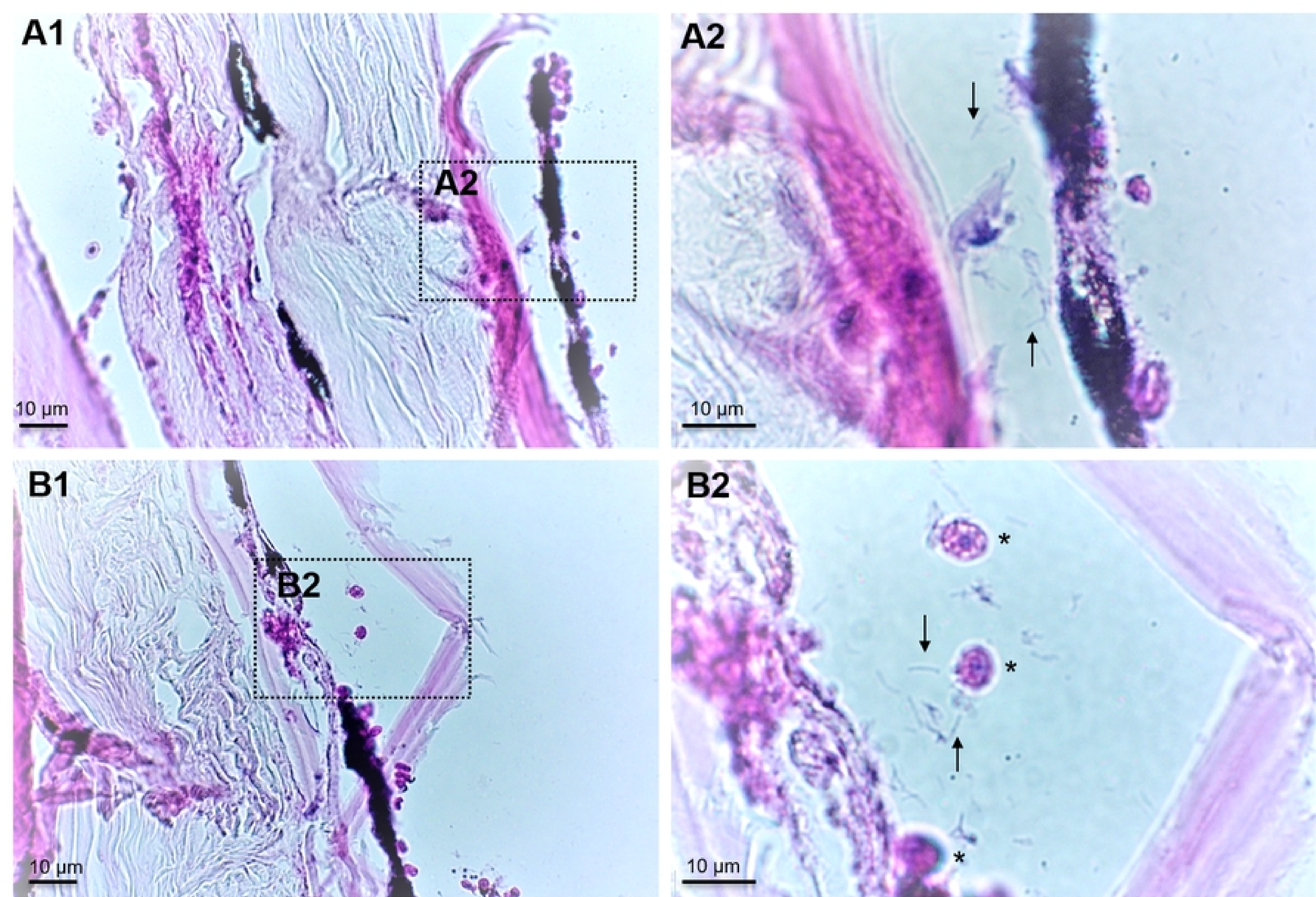


Figure 2

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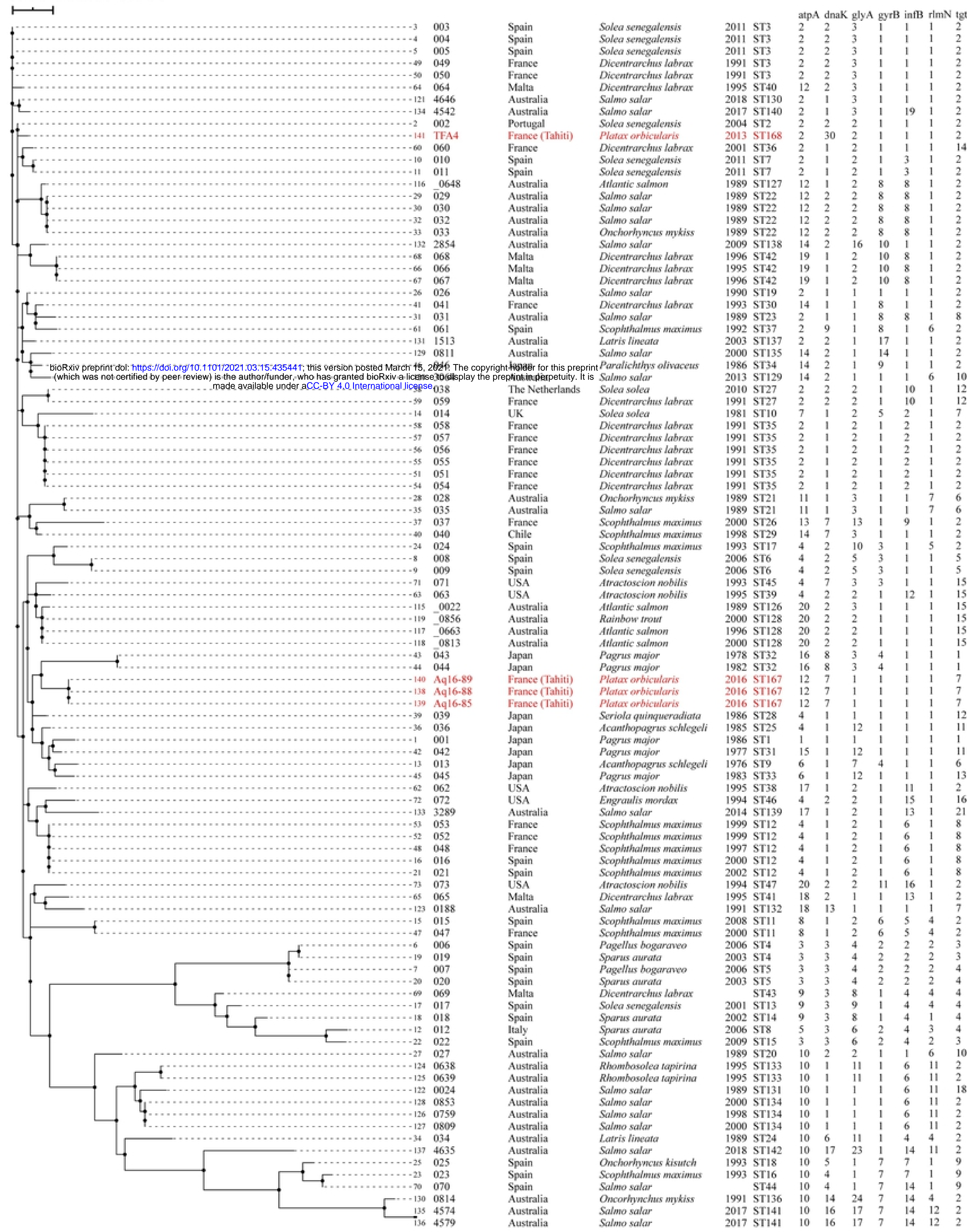


Figure 4

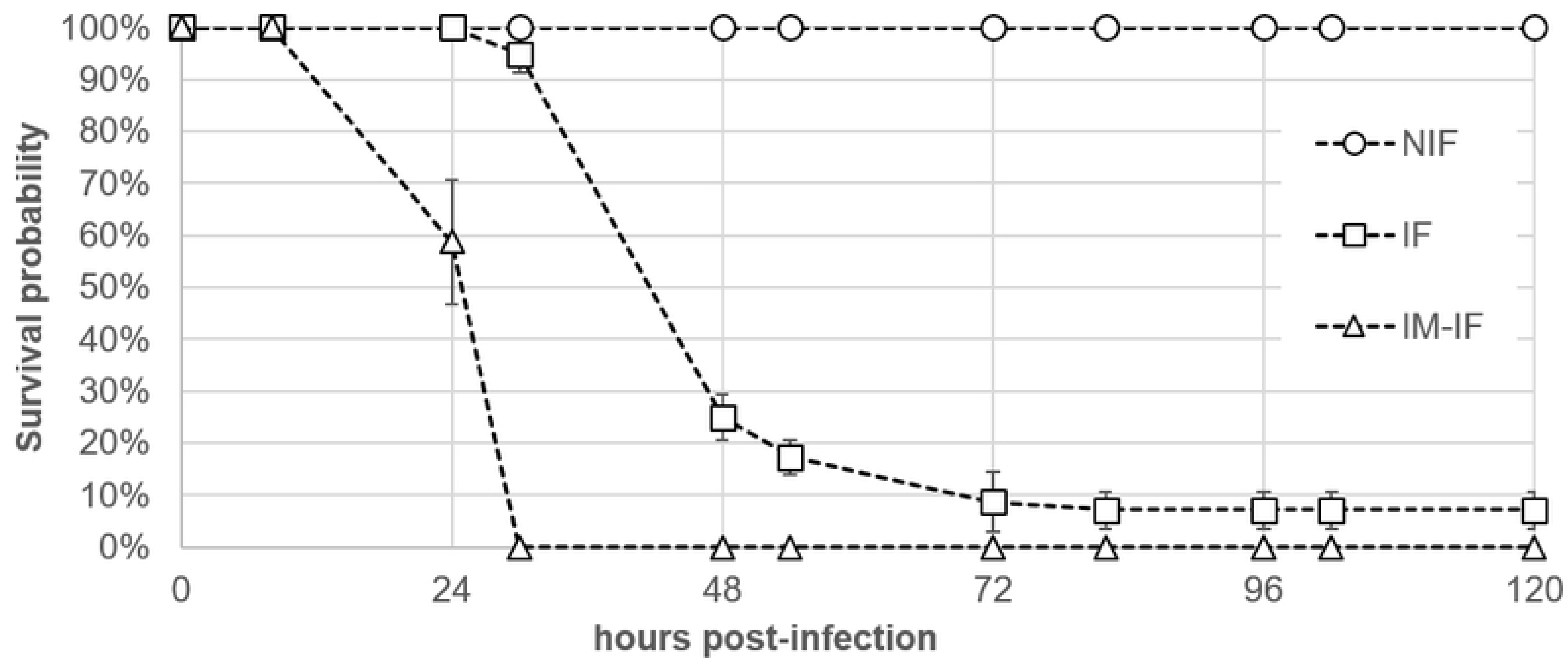


Figure 5

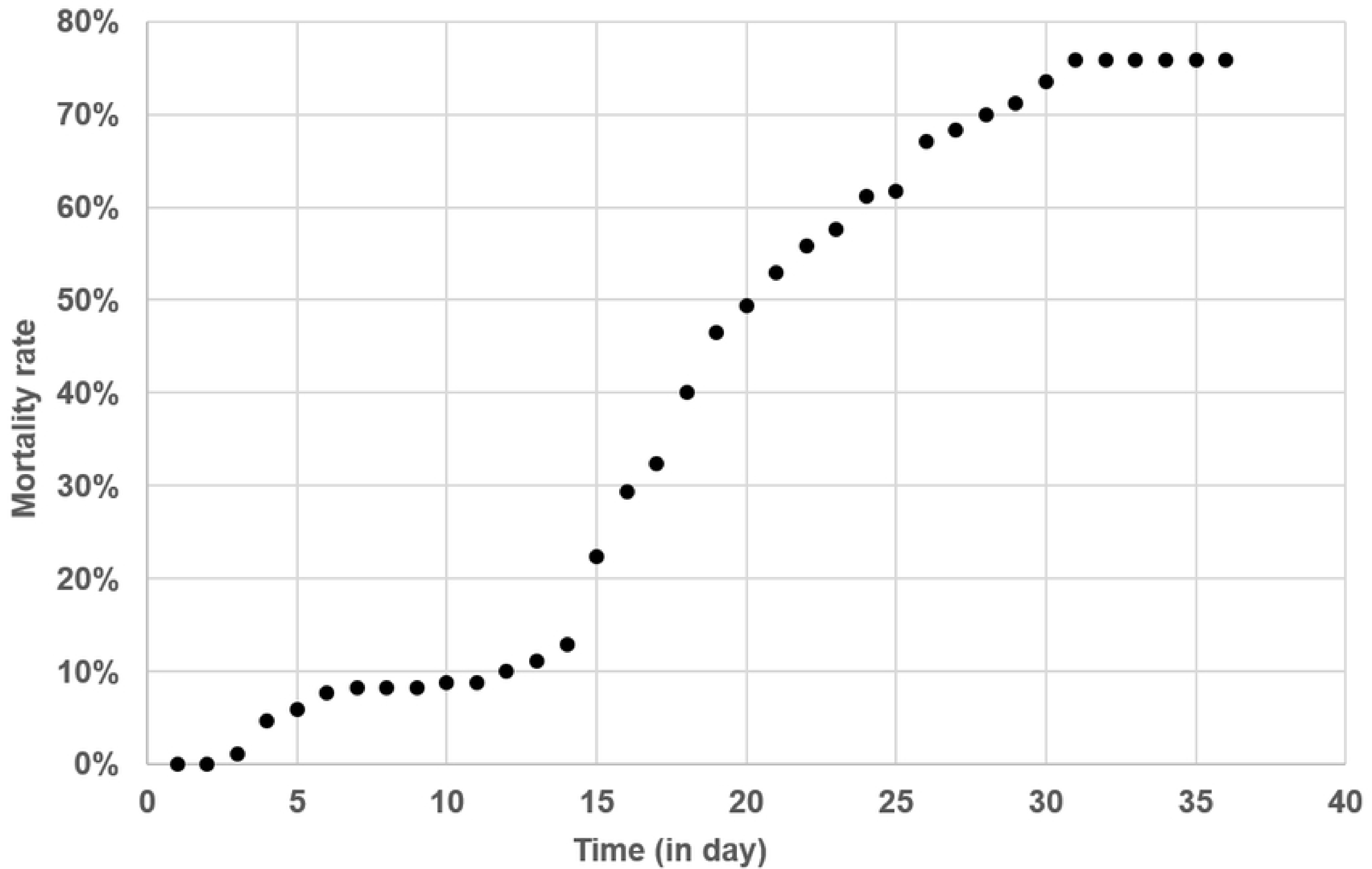


Figure 6

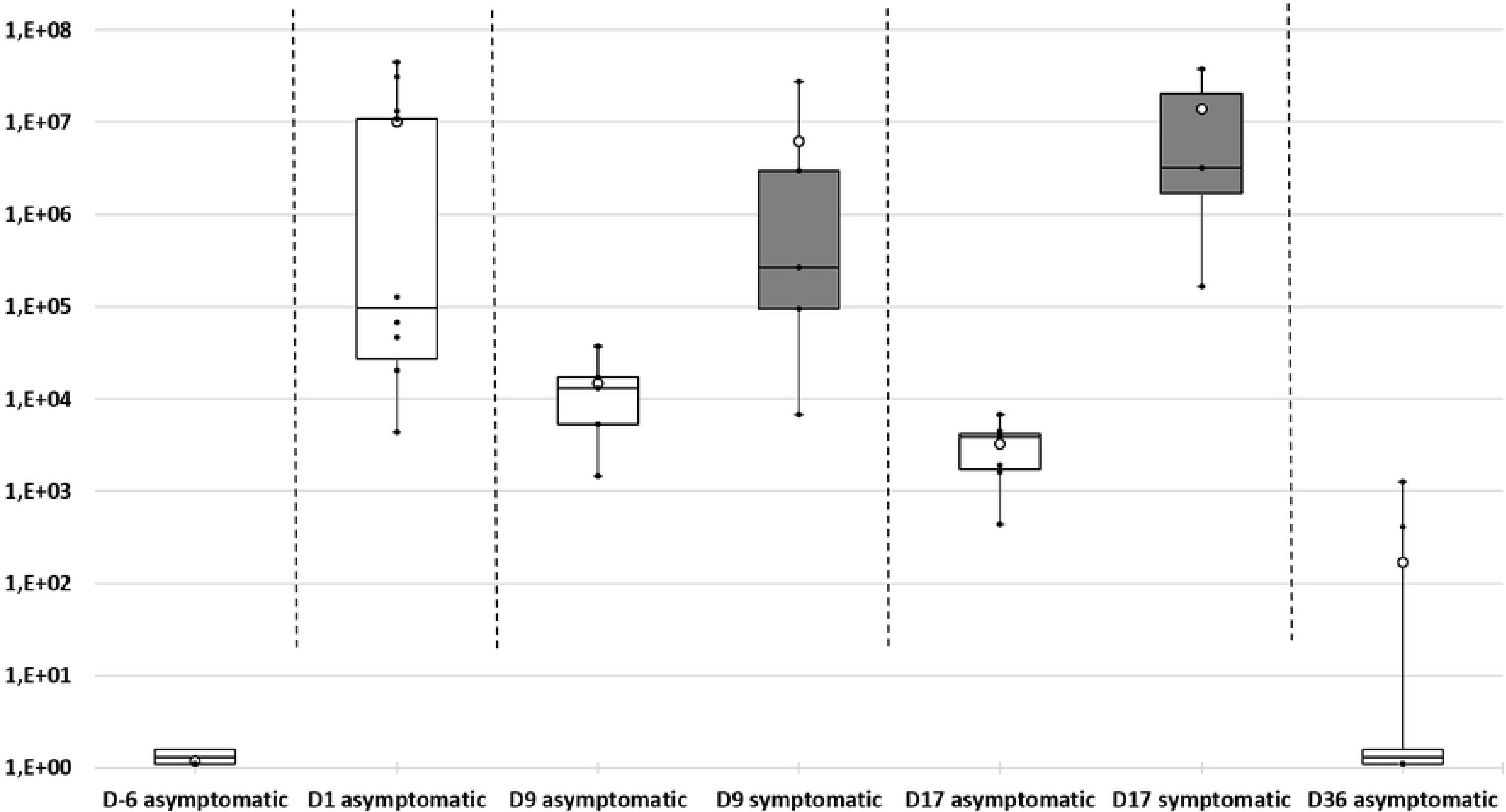
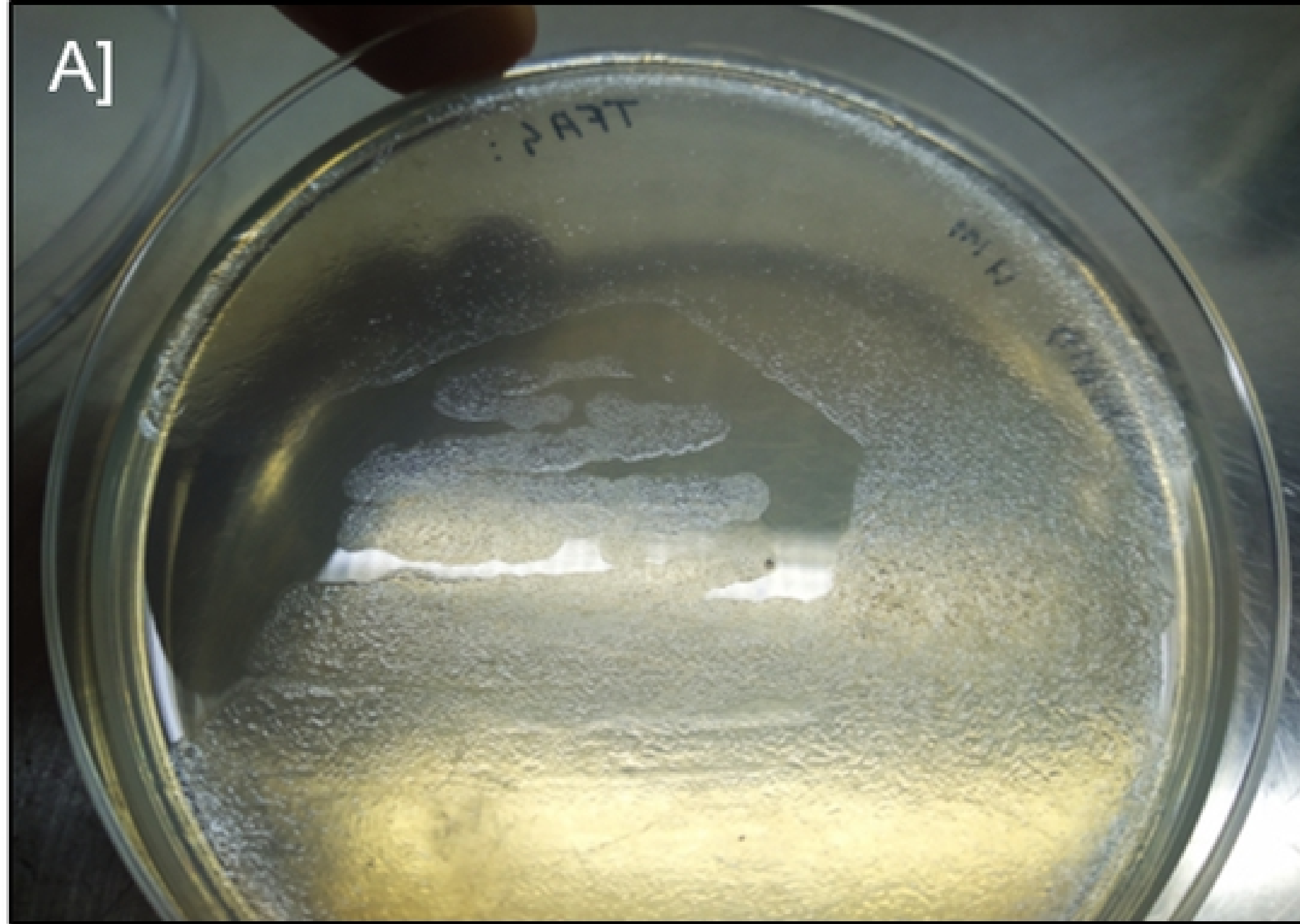
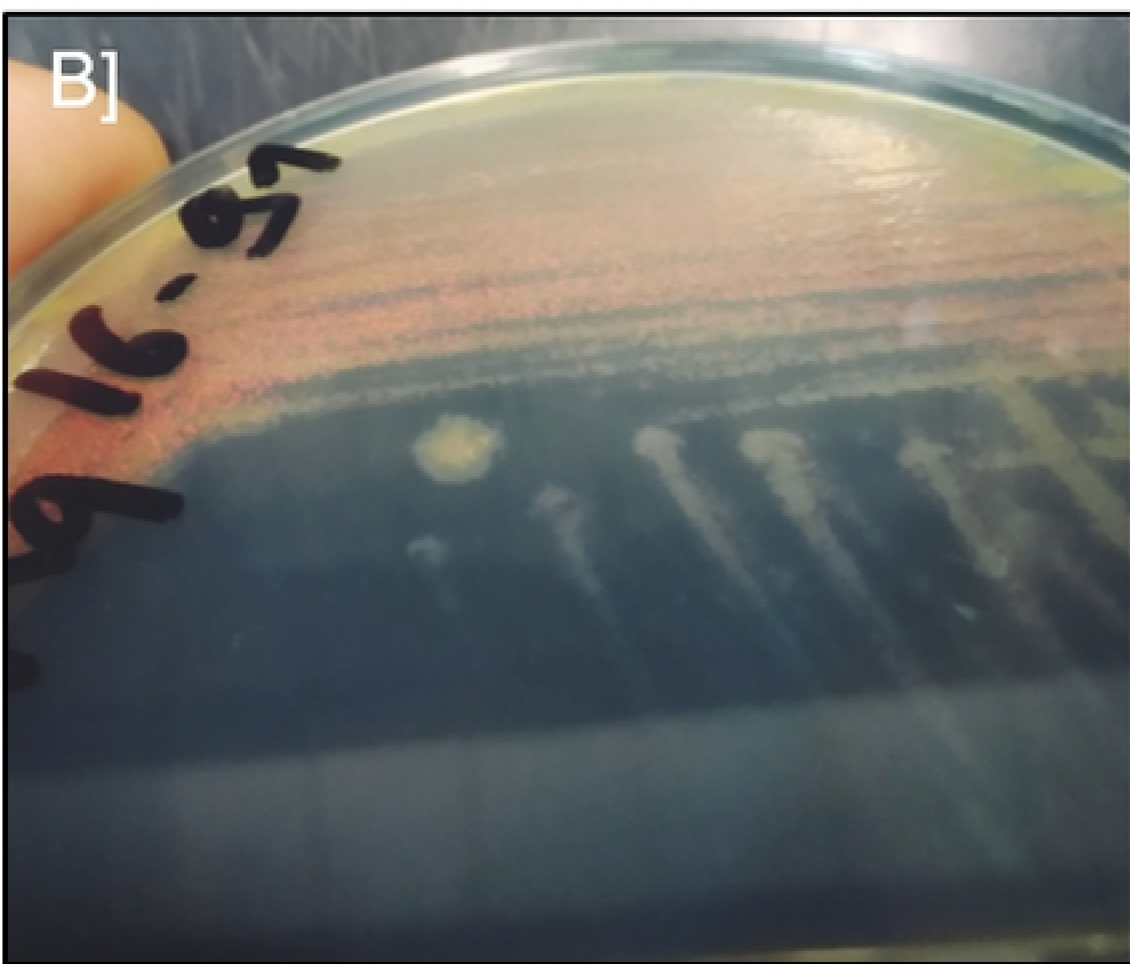


Figure 7





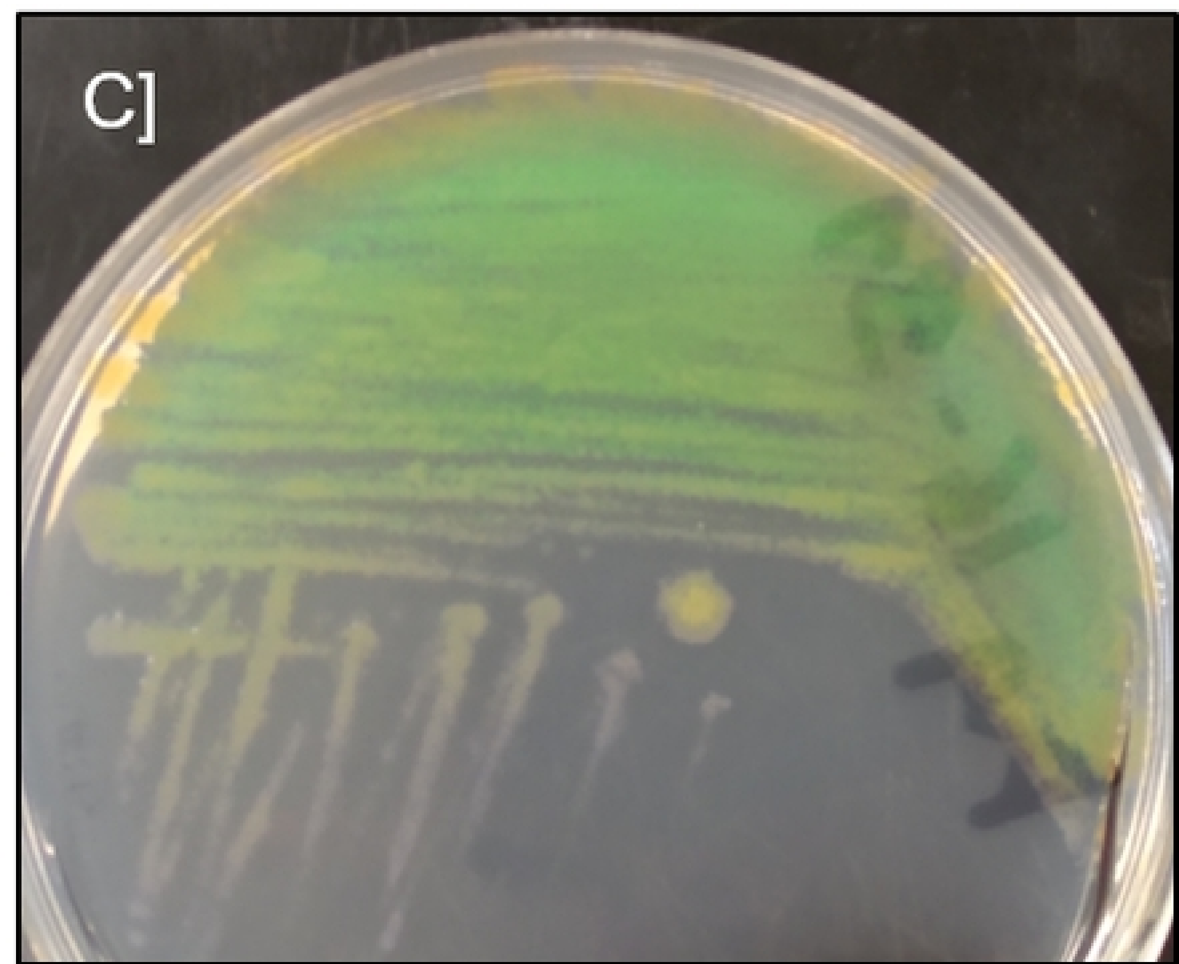


Figure 3