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1 **Amoebae can promote the survival of *Francisella* species in the aquatic**
2 **environment**

3

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23 Running title: Amoebae favour *Francisella* environmental survival

24

25 **ABSTRACT**

26 *Francisella tularensis*, a tier 1 select agent, is the causative bacterium of tularemia, a zoonosis
27 with a large animal reservoir. However, *F. tularensis*, like many other *Francisella* species
28 (including *F. novicida* and *F. philomiragia*), is assumed to have an aquatic reservoir. The
29 mechanisms of *Francisella* species persistence in surface water remain poorly characterized.
30 In this study, we deeply investigated the long-term interactions of the tularemia agent *F.*
31 *tularensis* subsp. *holarctica*, *F. novicida* or *F. philomiragia* with amoebae of the
32 *Acanthamoeba* species. In amoeba plate screening tests, all the *Francisella* species tested
33 resisted the attack by amoebae. In *in vitro* infection models, intra-amoebic growth of
34 *Francisella* varied according to the involved bacterial species and strains, but also the amoeba
35 culture medium used. In co-culture models, the amoebae favoured *Francisella* survival over
36 16 days. Such enhanced survival was likely dependent on direct contact between bacteria and
37 amoebae for *F. novicida* and on amoeba-excreted compounds for *F. novicida* and for *F.*
38 *tularensis*. In a spring water co-culture model, amoebae again enhanced *F. novicida* survival
39 and preserved bacterial morphology. Overall, our results demonstrate that amoebae likely
40 promote *Francisella* survival in aquatic environments, including the tularemia agent *F.*
41 *tularensis*. However, bacteria-amoebae interactions are complex and depend on the
42 *Francisella* species considered.

43

44 **KEYWORDS:** *Francisella*, *Francisella tularensis*, amoebae, tularemia, microbial ecology

45

46 INTRODUCTION

47 *Francisella tularensis* is a Gram-negative, facultative intracellular bacterium, causing the
48 potentially life-threatening zoonosis tularemia. This microorganism is classified as a category
49 A potential agent of biological threat by the US Centers for Disease Control and Prevention
50 (CDC) ¹. Taxonomically, the *F. tularensis* species is divided into four subspecies: *F.*
51 *tularensis* subsp. *tularensis* (Type A) and *F. tularensis* subsp. *holarctica* (Type B) are the
52 etiological agents of tularemia. *F. tularensis* subsp. *mediasiatica* is restricted to central Asia
53 and Russia, and has never been isolated from humans. *F. tularensis* subsp. *novicida* is an
54 aquatic bacterium of low virulence in humans, also considered a different species, *F. novicida*
55 ^{2,3}. For clarity, in our study we will differentiate *F. tularensis* (type A and type B tularemia
56 agents) from the aquatic bacterium *F. novicida*. *F. tularensis* can infect a wide range of
57 animals, which constitute the primary reservoir of this species ⁴. Ticks and other arthropods
58 (especially mosquitoes) are vectors of this pathogen ⁴. More recently, the hydro-telluric
59 environment has been highlighted as a probable major reservoir of this zoonotic agent and as
60 a source of human infections ⁵. Although *F. tularensis* has been detected in natural water
61 sources ⁵, the role of the aquatic environment as a reservoir of this species remains to be
62 firmly established. Furthermore, the mechanisms and conditions of *F. tularensis* long-term
63 survival in this environment have to be characterized. Some intracellular bacteria such as
64 *Legionella pneumophila* and *Mycobacterium avium* persist in natural ecosystem by using free
65 living amoebae as a multiplication niche and reservoir ⁶. Amoebae such as *Acanthamoeba* and
66 *Vermamoeba* species are ubiquitous in water and soil ⁷. A few experimental studies have
67 investigated the relationships between *Francisella* sp. and amoebae, with contradictory results
68 ⁸⁻¹⁴. Most of these studies have involved the live vaccine strain (LVS) of *F. tularensis*, *F.*
69 *novicida*, *F. philomiragia*, or *F. noatunensis* ⁸⁻¹⁴. Only two studies have evaluated the

70 interactions with amoebae of virulent *F. tularensis* type A strains ^{8,14} and only one with a
71 virulent *F. tularensis* type B strain ¹⁴.

72 In mammalian cells, it is well established that *Francisella* species multiplies intracellularly
73 thanks to an atypical Type VI secretion system (T6SS) encoded in the *Francisella*
74 Pathogenicity Island (FPI) ¹⁵⁻¹⁷. *F. tularensis* possesses two almost identical copies of the FPI
75 in its genome, while *F. novicida* and *F. philomiragia* only possess a single copy ^{17,18}. *F.*
76 *novicida* harbors another genetic locus, that we termed FNI, likely encoding a different T6SS
77 of unknown function ^{17,19}. The role of *Francisella* T6SSs in persistence/replication within
78 amoebae remains unclear ^{9,11,12,20}.

79 In the present study, we conducted a detailed characterization of the interactions of three
80 *Francisella* species, including a virulent type B strain, with two amoebic species in order to
81 further characterize the potential role of amoebae in the long-term survival of these species in
82 the aquatic environment.

83

84 **MATERIALS AND METHODS**

85 **BACTERIAL STRAINS**

86 The following strains of *Francisella* sp. were used (Table S1): the reference strains *F.*
87 *philomiragia* ATCC 25015, *F. novicida* U112 (CIP 56.12), and *F. tularensis* subsp. *holarctica*
88 LVS NCTC 10857; one clinical strain of *F. philomiragia* (Ft47); six clinical strains of *F.*
89 *tularensis* subsp. *holarctica* (Ft5, Ft6, Ft7, Ft46, Ft62, Ft74), *F. novicida* Δ FPI mutant ²¹, *F.*
90 *novicida* Δ FNI mutant ¹⁹, and the *F. novicida* Δ FPI Δ FNI double mutant. Clinical strains of *F.*
91 *tularensis* subsp. *holarctica* were isolated from French patients suffering from the different
92 tularemia forms. It was previously shown that all French clinical strains belong to clade B.44
93 and display low genetic diversity (genomes sequences of these clinical strains are available at
94 DDBJ/EMBL/GenBank under the BioProject number PRJNA551589) ²². Therefore, for most
95 experiments, the Ft6 strain was used as representative of clinical strains. Clinical strain of *F.*

96 *philomiragia* (Ft47) was isolated from a French patient suffering from a systemic infection
97 with bacteraemia. All *Francisella* strains used in this study are owned by the French National
98 Reference Center for *Francisella* (Grenoble University Hospital, Grenoble, France). Specific
99 authorizations were obtained from the Agence Nationale de Sécurité du Médicament et des
100 produits de santé (ANSM, authorization number ADE-103892019-7) for *F. tularensis* strains.
101 For experiments, *Francisella* sp. strains were grown on chocolate agar media supplemented
102 with PolyViteX® (bioMérieux, Marcy-l'Etoile, France), incubated at 35°C in a 5% CO₂-
103 enriched atmosphere for one day for *F. philomiragia* and *F. novicida* strains, and two days for
104 *F. tularensis* strains. Experiments were all conducted in a biosafety level 3 (BSL3) laboratory.
105 The *F. novicida* ΔFPIΔFNI double mutant (U112 ΔFTN_1309-1325::*aphA*, ΔFTN_0037-
106 0054::*FRTsc*) was generated in the wild-type U112 strain by transforming the ΔFNI mutant
107 with genomic DNA from the ΔFPI mutant using chemical transformation. Briefly, the ΔFNI
108 mutant grown in Tryptic Soy Broth (TSB), 0.1% L-cys and 0.4% glucose until an O.D.600nm
109 of 0.9 was washed once and 10-fold concentrated in chemical transformation buffer (Tris
110 50mM, NaCl 270mM, MgSO₄, 25 mM, CaCl₂, 20 mM, L-Arg 2mM, L-His 1mM, L-Met
111 2mM, L-Asp 3mM, Spermine 0,2mM; MnCl₂ 35mM, pH6.8). 500 μl of the obtained bacterial
112 solution was incubated for 20 min at 37°C with shaking 100rpm with 1μg gDNA from the
113 ΔFPI strain (ΔFTN_1309-1325::*aphA*). 1ml TSB, 0.1% L-cys and 0.4% glucose was then
114 added to the bacteria which were further incubated for 2 h at 37°C before plating on selective
115 plates (Tryptic Soy Agar (TSA), 0.1% L-cys, 10 μg/ml Kanamycin). Following clonal
116 isolation, gene deletions were verified by PCR on gDNA using FPI and FNI flanking primers.
117 Other bacterial strains used as controls included *Staphylococcus epidermidis* ATCC 1228 and
118 *Legionella pneumophila* CIP107629T. *S. epidermidis* ATCC 1228 was grown on sheep blood
119 agar medium (bioMérieux, Marcy-l'Etoile, France) and *L. pneumophila* on Buffered Charcoal
120 Yeast Extract (BCYE) medium (Oxoid, Cambridge, UK).

121

122 AMOEBAE AND AMOEBAE CULTURE MEDIA

123 We used *Acanthamoeba castellanii* (Neff) and *A. polyphaga* (Linc-AP1) strains. Amoebae
124 were grown axenically in peptone-yeast extract-glucose medium (PYG, Eurobio, France) at
125 27°C, in 75-cm² cell culture flasks (Falcon, Corning Incorporated, Life Sciences, Durham,
126 USA). Peptone-yeast extract-glucose medium (PYG = ATCC medium 712) is a rich culture
127 medium (2% proteose peptone, 0.1% yeast extract, 0.1 M glucose, 4 mM MgSO₄ · 7H₂O, 0.4
128 mM CaCl₂, 0.1% sodium citrate dihydrate, 0.05 mM Fe(NH₄)₂(SO₄)₂ · 6H₂O, 2.5 mM
129 Na₂HPO₄ · 7H₂O, 2.5 mM KH₂PO₄) supporting *Acanthamoeba* sp. living in trophozoite form
130 and multiplication.

131 For experiments, several amoebae culture media were used. PYG without glucose medium
132 (PYG/wg) presents the same composition as PYG medium except that it does not contain
133 glucose. This medium supports *Acanthamoeba* sp. survival in trophozoite form for 16 days
134 but does not allow amoebae multiplication (data not shown). Starvation medium (SM) is a
135 less nutritive medium (1 mM Na₂HPO₄, 1 mM KH₂PO₄, 0.016 mM MgSO₄ · 7H₂O, 0.027
136 mM CaCl₂ · 2H₂O, 2 mM NaCl, 0.005 mM (NH₄)₂Fe(SO₄)₂ · 6H₂O, 0.2% yeast extract, 1.8%
137 glucose)²³ allowing *Acanthamoeba* sp. survival in trophozoite form for 16 days without
138 amoebae multiplication (data not shown). Page's amoeba saline (PAS = ATCC medium 1323)
139 is a poor medium (1 mM Na₂HPO₄, 1 mM KH₂PO₄, 0.016 mM MgSO₄ · 7H₂O, 0.027 mM
140 CaCl₂ · 2H₂O, 2 mM NaCl) allowing *Acanthamoeba* sp. survival in cystic form without
141 multiplication.

142

143 AMOEBEA PLATE TESTS (APT)

144 This test allows evaluation of interactions of bacteria with an amoebae monolayer established
145 on the surface of an agar plate^{10,24}. APT were performed using *A. castellanii* or *A. polyphaga*,

146 for *F. tularensis* (LVS, and clinical Ft5, Ft6, Ft7, Ft46, Ft62, and Ft74 type B strains), *F.*
147 *philomiragia* (ATCC 25015, or Ft47 clinical strain), and *F. novicida* U112.
148 Amoebae grown in PYG medium in 75-cm² cell culture flask were harvested at 90%
149 confluence by rapping the flask to bring amoebae into suspension. Amoebae were counted,
150 centrifuged 10 min at 1000 g, and resuspended in fresh PYG medium at a concentration of
151 2.67×10^6 cells/ml. The amoebae suspension (1.5 ml) was spread on chocolate agar plates and
152 allowed to dry 1-2h to form an amoebae monolayer on the surface of the agar medium. For
153 each bacterial strain tested, a suspension of 10^9 CFU/ml was prepared in Phosphate-Buffered
154 Saline (PBS, Thermo Fisher Scientific). Tenfold serial dilutions (from 10^9 to 10^6 CFU/ml) of
155 this suspension were prepared, and 10 μ l of each dilution was inoculated on the amoebae
156 monolayer. The agar plates were then incubated at 30°C for 10 days, and examined daily for
157 bacterial growth. As a bacterial growth control, chocolate agar plates without amoeba were
158 inoculated with the same bacterial suspensions. For negative and positive APT controls, *S.*
159 *epidermidis* ATCC 1228 and *L. pneumophila* CIP107629T respectively were inoculated to
160 amoebae monolayers using the same protocol except that BCYE agar plates were used for *L.*
161 *pneumophila*. For *Francisella* strains, the results of APTs were evaluated visually and semi-
162 quantitatively according to the lowest bacterial inoculum allowing colony formation and the
163 intensity of the bacterial growth (equal or inferior) in comparison to the amoeba-free growth
164 control. *Francisella* growth on the amoeba monolayer was also compared to that of *L.*
165 *pneumophila* on the same protozoa. Experiments were performed twice.

166

167 GROWTH OF FRANCISELLA STRAINS IN AMOEBAE CULTURE MEDIA

168 We tested the capacity of *F. philomiragia* ATCC 25015, *F. novicida* U112, and the LVS and
169 Ft6 strains of *F. tularensis* to grow in the amoebae culture media PYG, PYG/wg, SM, and
170 PAS, in the absence of amoeba. Each medium was inoculated with 10^5 CFU/ml of the tested

171 bacterial strain, spread in 24-well plates and then incubated at 27°C. At different time
172 intervals over a 16-day period, an aliquot of each culture medium was sampled, serially
173 diluted and spread onto chocolate agar plates to determine CFU counts. Experiments were
174 performed twice, each time in triplicate.

175

176 AMOEBAE INFECTION WITH FRANCISELLA MODELS

177 We evaluated intra-amoebic growth of *Francisella* strains (*F. philomiragia* ATCC 25015, *F.*
178 *novicida* U112, and *F. tularensis* LVS and Ft6 strains) in *A. castellanii* or *A. polyphaga*. Two
179 models were evaluated either using PYG/wg or SM media as the amoebae culture
180 supernatant.

181 For the first model, a suspension of 5.3×10^5 cells/ml in PYG/wg medium was prepared for
182 each amoeba strain tested, using the same procedure described for APT experiments. Then,
183 the amoebae suspensions were dispensed in five 24-well plates (950µL per well, each plate
184 containing five wells for each amoeba strain), and amoebae were allowed to adhere for 1 h at
185 27°C. For each *Francisella* sp. strain tested, a bacterial suspension (50 µl of 10^8 CFU/ml in
186 PYG/wg) was added to wells containing either *A. castellanii* or *A. polyphaga* (in triplicate) to
187 obtain a multiplicity of infection (MOI) of 10. The plates were centrifuged at 1000 g for 20
188 min to optimize contact between bacteria and amoebae, and incubated 40 min at 27°C. Then,
189 the culture supernatant was removed, the amoebae layer was washed three times with PAS,
190 and new PYG/wg medium containing 100 µg/ml of gentamicin was added to remove non-
191 phagocytized bacteria. The plates were further incubated 1h at 27°C. The amoebae
192 monolayers were then washed three times with PAS and finally incubated in new PYG/wg
193 medium at 27°C. This time of the experiment corresponded to T0. Plates were incubated
194 during seven days at 27°C. CFU counts were determined at T0, day one (D1), D2, D5 and D7
195 of incubation of cell cultures (one plate per each time). Therefore, the culture supernatant of

196 each well was removed and amoebae were lysed with 1% saponin for 10 min at 27°C
197 followed by vigorous pipetting. The amoebic lysate was serially diluted and spread onto
198 chocolate agar plates to determine intra-amoebic CFU counts. In parallel, the removed
199 supernatant was also serially diluted and spread onto chocolate agar plates to determine extra-
200 amoebic CFU counts. The bacterial detection limit was 10 CFU/ml.

201 For the second model, the same procedure as above was performed, but SM was used instead
202 of PYG/wg and extra-amoebic CFU counts were not determined.

203 Experiments were performed twice, each time in triplicate.

204

205 AMOEBAE AND FRANCISELLA CO-CULTURE MODELS

206 We developed two co-culture models to evaluate growth of *Francisella* strains in the presence
207 of amoebae, with or without direct contact of bacteria with these protozoa.

208 In the first model, *A. castellanii* or *A. polyphaga* were infected with *F. philomiragia* ATCC
209 25015, *F. novicida* U112, or *F. tularensis* Ft6 strains, using the same procedure as above and
210 SM as the amoeba culture medium. However, killing of non-phagocytized bacteria using
211 gentamicin was not performed. The plates were incubated at 27°C for 16 days. At T0, D2, D7,
212 D12 and D16, amoebae were lysed by addition of 1 ml of 2% saponin during 10 min at 27°C
213 and vigorous pipetting without removing the amoebae supernatant. The lysates were serially
214 diluted and spread onto chocolate agar plates in order to determine the total (i.e. intra- and
215 extra-amoebic) CFU counts. Control wells without amoeba were prepared using the same
216 protocol. The bacterial detection limit was 20 CFU/ml.

217 In the second model, co-culture conditions were the same as above, but bacteria and amoebae
218 were separated by a cell culture insert with a pore size of 0.4 µm (Millicell Merck Millipore,
219 Darmstadt, Germany). Therefore, there was no direct contact between these microorganisms,
220 but possible interactions due to a shared culture medium. In co-culture with inserts model, at

221 each time point listed previously, supernatant above the insert was sampled, serially diluted
222 and spread onto chocolate agar plates in order to determine CFU counts. After this step, insert
223 was removed and 1 ml of 2% saponin was added on the amoebae supernatant for 10 min at
224 27°C followed by vigorous pipetting. The lysate was spread onto chocolate agar plate in order
225 to check for the absence of bacteria.

226 Experiments were performed twice, each time in triplicate.

227

228 AMOEBAE SURVIVAL

229 During infection and co-culture experiments, wells with infected or uninfected amoebae were
230 incubated in similar conditions. At each time point, amoebic morphology and adherence of
231 infected and uninfected amoebae were checked under a microscope. After amoebae
232 detachment through vigorous pipetting, determination of the percentage of mortality were
233 performed using trypan blue dye in a counting chamber. In addition, for co-culture
234 experiments that lasted for 16 days, total amoebae counting was also performed throughout
235 the experiment.

236

237 IMMUNOFLUORESCENCE DETECTION OF FRANCISELLA IN INFECTED AMOEBAE

238 At different time points of the infection experiments in PYG/wg or SM media, infection of
239 amoebae with *Francisella* strains was evaluated by immunofluorescence and confocal
240 microscopy. Infection experiments were performed as described above in 24-well plates,
241 except that a 12 mm microscopy slide (SPL Life Sciences, Korea) was added in each well at
242 the beginning of the experiments. At the different incubation times previously indicated,
243 microscopy slides were removed from wells, fixed with 3.7 % paraformaldehyde for 20 min
244 at room temperature and then washed with PBS. Amoebae were permeabilized with PBS-
245 Triton X-100 0.2% for 10 min at room temperature followed by two PBS washes. Blocking

246 was performed with 3% bovine serum albumin for 30 min at room temperature. Rabbit anti-
247 *Francisella tularensis* primary antibody (# TC-7005, Tetracore, Rockville, USA) diluted
248 1:100 in PBS-BSA 0.3% was added for 1 h in humid chamber at room temperature followed
249 by three washes with PBS-BSA 0.3%-Tween 0.01%. This anti-*Francisella tularensis*
250 antibody was shown to also labelled *F. philomiragia* (data not shown). Then, AlexaFluor 594
251 goat-anti-rabbit secondary antibody (# A-11012, Invitrogen, Thermo Fisher Scientific,
252 Rockford, USA) diluted 1:1000 in PBS-BSA 0.3%, together with AlexaFluor 488 phalloidin
253 (Invitrogen, Thermo Fisher Scientific, Rockford, USA) were added for 1 h at obscurity in a
254 humid chamber at room temperature, respectively for *Francisella* and amoebae actin staining.
255 Slides were then washed with PBS-BSA 0.3%-Tween 0.01% twice, then with PBS twice.
256 They were mounted with ProLong Glass (Invitrogen, Thermo Fisher Scientific, Rockford,
257 USA) and examined by immunofluorescent microscopy (on a Nikon Eclipse TS100) and
258 confocal microscopy. Confocal microscopy was performed using a confocal laser scanning
259 microscope (CLSM, LSM710, Zeiss, Jena, Germany) equipped with a Plan-Apochromat
260 63×/1.40 Oil DIC M27 objective.

261

262 STUDY OF THE T6SS IMPACT ON FRANCISELLA AND AMOEBAE INTERACTIONS

263 The role of the *F. novicida* T6SS in amoebae interactions was evaluated using the previously
264 described models. APT were performed for *A. castellanii* and either wild-type (WT) *F.*
265 *novicida* U112, *F. novicida* Δ FPI, *F. novicida* Δ FNI, or *F. novicida* Δ FPI Δ FNI. Infections in
266 PYG/wg and SM were performed for *A. polyphaga* and either WT *F. novicida* U112 or *F.*
267 *novicida* Δ FPI Δ FNI. Co-culture without inserts were performed for *A. polyphaga* and WT *F.*
268 *novicida* U112 or *F. novicida* Δ FPI Δ FNI. Experiments were performed twice, each time in
269 triplicate.

270

271 AMOEBAE AND FRANCISELLA CO-CULTURES IN SPRING WATER

272 Spring water (pH 7.5) was collected from a large permanent spring in Croatia and then
273 autoclaved at 121 °C for 15 min. *F. novicida* U112 and *A. castellanii* were inoculated in 1 L
274 of spring water in glass bottles, with shaking, and capped with cap half-loosened, at room
275 temperature. Amoebae and bacteria concentrations were of: 10^6 *F. novicida*/ml alone, 10^7 *F.*
276 *novicida*/ml alone, 10^6 *F. novicida*/ml together with 10^7 *A. castellanii*/ml, 10^7 *F. novicida*/ml
277 together with 10^6 *A. castellanii*/ml, 10^6 *A. castellanii*/ml alone and 10^7 *A. castellanii*/ml alone.
278 Two hours after inoculation, most of the amoebae were adhered to the bottle, the rest were
279 floating in the water. Growth kinetics of bacteria and amoeba were followed every five days
280 in a period of 30 days. The suspensions were homogenized by shaking before each sampling.
281 The number of *F. novicida* at each time point was determined by plating serial dilutions on
282 BCYE agar plates. In order to determine the number of amoeba cells, at each time point 1 ml
283 of water sample was transferred to 24 well plate, and left for 2 h at room temperature to allow
284 amoeba to adhere. It was followed by analysing the samples using light microscopy.
285 Experiments were performed twice, each time in triplicate.
286 During these experiments in spring water, morphology and structure of *F. novicida*, after 15
287 days incubation alone in spring water or in co-incubation with *A. castellanii* in spring water
288 was studied by TEM in comparison to control bacteria grown on BCYE agar. Bacteria were
289 prepared for TEM by negative staining. Bacterial suspension was applied to the Carbon
290 Coated 200 mesh Cooper Grid for 2 min, and drawn off from the edge of the grid with filter
291 paper. After that, the grid was stained using 10 µl of 2% phosphotungstic acid for 1 min and
292 again drained with the filter paper. The grid was placed directly into the grid box and allowed
293 to air dry before observation. By transmission electron microscopy on a Zeiss 902A, we
294 observed morphology of bacteria, including their size, shape and density. Ten fields for each
295 sample were randomly photographed.

296

297 STATISTICAL ANALYSIS

298 A two-tailed Student t-test was used to compare bacterial loads in the different experimental
299 models, using a significance level of 0.05.

300

301 RESULTS

302 AMOEBA PLATE TESTS

303 The APT was first performed as a screening assay of interactions between a monolayer of
304 amoebae and *Francisella* sp strains. Figure S1 shows some visual results of the APT tests. All
305 *Francisella* strains tested were able to pass through the amoebae monolayer forming bacterial
306 colonies, demonstrating resistance of these bacteria to the predatory properties of amoebae. *F.*
307 *philomiragia* (ATCC 25015 and Ft47) growth was observed at the 10⁶ CFU/ml dilution with
308 the same intensity with or without amoebae and similar to the *L. pneumophila* control. For the
309 clinical strains of *F. tularensis*, and *F. novicida* U112, growth was observed at the 10⁶
310 CFU/ml dilution with or without amoebae but with inferior intensity in the presence of
311 amoebae and compared to the *L. pneumophila* control. Finally, for the LVS strain, growth on
312 the amoebae monolayer was observed at the 10⁹ CFU/ml dilution (3 log inferior to the
313 amoeba-free control and the *L. pneumophila* control).

314

315 GROWTH OF FRANCISELLA STRAINS IN AMOEBAE CULTURE MEDIA

316 In order to define the most suitable supernatant medium for further evaluations of *Francisella*
317 and amoebae interactions, we evaluated the growth of *F. tularensis* LVS and Ft6 strains, *F.*
318 *philomiragia* ATCC 25015, and *F. novicida* U112 in the amoebae culture media PYG, PYG
319 without glucose (PYG/wg), starvation medium (SM), and PAS. In the absence of amoeba, the
320 four bacterial strains showed strong growth in PYG and PYG/wg media, with an increase of

321 more than 4 log of CFU counts after 16 days of incubation of cultures (Figure S2). In contrast,
322 these strains were unable to grow in SM or PAS media, with a progressive decrease in CFU
323 counts up to undetectable level within 5 to 7 days for PAS and 5 to 16 days for SM (Figure
324 S2).

325

326 AMOEBAE INFECTION WITH FRANCISELLA IN PYG/wg

327 Since APT revealed interactions between *Francisella* sp. and amoebae, we needed to better
328 characterise these interactions. In this aim, we first used an infection model in a rich culture
329 medium (i.e. PYG/wg) previously described in the literature^{9,11} but with longer incubation
330 period. Infections of *A. castellanii* or *A. polyphaga* with *F. philomiragia* ATCC 25015, *F.*
331 *novicida* U112, or *F. tularensis* (LVS or Ft6 strains) were performed.

332 During the first two days of incubation of cultures, we observed a significant increase in
333 intracellular CFU counts for *F. philomiragia* in *A. polyphaga* (3.04 log CFU/ml, $p < 0.01$) and
334 *A. castellanii* (3.46 log CFU/ml, $p < 0.001$) and for *F. novicida* in *A. polyphaga* (1.50 log
335 CFU/ml, $p < 0.001$) and *A. castellanii* (1.73 log CFU/ml, $p < 0.05$). Then, a progressive
336 decrease (for *F. philomiragia*) or stagnation (for *F. novicida*) of intra-amoebic CFU counts
337 was observed the following five days. CFU counts of both *Francisella* strains in the amoebae
338 culture supernatant evolved similarly to the intra-amoebic CFU counts with difference
339 between them at each time point of one log or less (Figure 1).

340 As for *F. tularensis* Ft6, we observed a weaker and slower increase in CFU counts within the
341 amoebic compartment, with a peak at five days of incubation, also more marked for *A.*
342 *polyphaga* (2.22 log CFU/ml, $p < 0.01$) than for *A. castellanii* (1.07 log CFU/ml, not
343 significant (NS)). Here again, CFU counts in culture supernatant evolved similarly to the
344 intra-amoebic CFU counts with less of one log difference between them at each time point
345 (Figure 1).

346 In contrast, the LVS strain did not display any intra- or extra-amoebic growth, but a
347 progressive decline in intra-amoebic CFU counts during the seven days of the experiments, of
348 2.91 log CFU/ml ($p < 0.01$) for *A. polyphaga* and to undetectable level for *A. castellanii* ($p <$
349 0.001) (Figure 1).

350 For the four bacterial strains tested, microscopic observations of infected and uninfected
351 amoebae showed conservation of adherence and maintenance in trophozoite form of these
352 protozoa at each time point of the experiments and amoebae mortality rates were inferior to
353 10% (data not shown).

354

355 AMOEBAE INFECTION WITH FRANCISELLA IN SM

356 In the absence of amoebae, we observed that PYG/wg supported a progressive growth of
357 *Francisella* strains over time, whereas SM did not. Indeed, to prevent any extracellular
358 growth of *Francisella* that may interfere with intra-amoebic CFU counts monitoring, we then
359 developed an infection model in poor culture medium (i.e. SM), precluding extracellular
360 growth of these bacteria. *A. castellanii* or *A. polyphaga* were infected with *F. philomiragia*
361 ATCC 25015, *F. novicida* U112, or *F. tularensis* (LVS or Ft6 strains) using the same
362 procedure as above but SM as the culture supernatant.

363 In this model, a rapid decrease of intra-amoebic CFU counts was observed up to undetectable
364 level within one to seven days of incubation of cultures depending on the *Francisella* sp.
365 strain considered (Figure 2).

366 For the four bacterial strains tested, microscopic observations of infected and uninfected
367 amoebae showed conservation of adherence and maintenance in trophozoite form of these
368 protozoa at each time point of the experiments and amoebae mortality rates were inferior to
369 10% (data not shown).

370

371 AMOEBAE AND FRANCISELLA CO-CULTURES IN SM

372 Since APT revealed that *Francisella* sp. resisted to amoebae and infection in SM did not
373 suggested intra-amoebic survival of *Francisella* sp., we developed co-culture models in order
374 to evaluate if amoebae could promote *Francisella* sp. survival whether intracellular or
375 extracellular. In co-culture model, after the infection step, extra-cellular bacteria were not
376 killed. This model was supposed to be closer to the interactions between amoebae and
377 bacteria that may occur in nature. Co-cultures of *A. castellanii* or *A. polyphaga* with *F.*
378 *tularensis* Ft6, *F. philomiragia* ATCC 25015 or *F. novicida* U112 were established using SM
379 as the amoebae culture medium.

380 We observed an enhanced survival of both *F. philomiragia* and *F. novicida* in the presence of
381 *A. castellanii* or *A. polyphaga*, compared to amoeba-free SM medium (Figure 3). The *F.*
382 *philomiragia* and *F. novicida* CFU counts declined during the first seven to 12 days of
383 experiments, both in the presence of *A. castellanii* (3.99 log CFU/ml, $p < 0.001$; and 3.73 log
384 CFU/ml, $p < 0.001$, respectively) or *A. polyphaga* (1.19 log CFU/ml, $p < 0.001$; and 1.08 log
385 CFU/ml, $p < 0.01$, respectively) (Figure 3). However, we then observed an increase in *F.*
386 *philomiragia* CFU counts between days 12 and 16 for *A. castellanii* (1.97 log CFU/ml, NS),
387 and days 7 and 16 for *A. polyphaga* (2.28 log CFU/ml, $p < 0.05$). Between days 7 and 16, *F.*
388 *novicida* CFU counts significantly increased in *A. castellanii* (2.93 log CFU/ml, $p < 0.001$),
389 and remained relatively stable in *A. polyphaga* (Figure 3). In contrast, CFU counts steadily
390 and significantly decreased in the absence of amoebae, to undetectable level for *F.*
391 *philomiragia* ($p < 0.01$), and by 5.19 log CFU/ml ($p < 0.001$) after 16 days for *F. novicida*
392 (Figure 3). Therefore, there was a clear persistence of both *F. philomiragia* and *F. novicida* in
393 the presence of either amoeba species, compared to amoeba-free culture conditions. For *F.*
394 *novicida*, the difference in CFU counts at D16 was 4.32 log CFU/ml ($p < 0.001$) between
395 amoeba-free and *A. castellanii* cultures, and 3.93 log CFU/ml ($p < 0.001$) between amoeba-

396 free and *A. polyphaga* cultures. For *F. philomiragia*, the difference in CFU counts between
397 amoeba-free and either *A. castellanii* or *A. polyphaga* cultures was more than four log (p
398 could not be evaluated).

399 For *F. tularensis* Ft6, CFU counts progressively and similarly decreased between T0 and D16
400 in the presence or absence of amoebae: 3.53 log CFU/ml reduction (p < 0.001) in the presence
401 of *A. castellanii*; 1.88 log CFU/ml (p < 0.001) with *A. polyphaga*; and 3.27 CFU/ml (p <
402 0.001) without amoebae (Figure 3). At day 16 of incubation, CFU counts were not statistically
403 different between Ft6 alone and Ft6 with *A. castellanii* and was slightly superior for Ft6 with
404 *A. polyphaga* than for Ft6 alone (p < 0.05) (Figure 3).

405 For the three bacterial strains tested, microscopic observations of infected and uninfected
406 amoebae showed conservation of adherence and maintenance in trophozoite form of these
407 protozoa. Total amoebae counts varied from 3 x 10⁵ to 8 x 10⁵ amoebae/ml and amoebae
408 mortality rates were inferior to 10% (data not shown).

409

410 AMOEBAE AND FRANCISELLA CO-CULTURES IN SM IN THE PRESENCE OF 411 INSERTS

412 We tried to further investigate the mechanisms implicated in the enhanced survival of *F.*
413 *novicida* U112 and *F. philomiragia* ATCC 25015 in co-culture with amoebae. In this aim, we
414 established new co-cultures of these species in the presence of *A. castellanii* or *A. polyphaga*.
415 However, direct contact between bacteria and amoebae was prevented by using cell culture
416 inserts, while these microorganisms still shared the same culture medium.

417 Enhanced survival of *F. novicida* in the presence of either of the two amoebae species was
418 partially lost when bacteria and amoebae were separated by an insert. *F. novicida* CFU counts
419 progressively decreased between T0 and D16 in wells with inserts (3.56 log CFU/ml
420 reduction (p < 0.001) for *A. castellanii* and 2.91 CFU/ml reduction (p < 0.01) for *A.*

421 *polyphaga*) (Figure 4). For each amoeba species, CFU counts in wells with versus without
422 inserts were significantly different at D16 ($p < 0.01$ and $p < 0.001$ for *A. castellanii* and *A.*
423 *polyphaga*, respectively) (Figure 4). However, bacterial counts at D16 in wells with amoebae
424 and inserts were significantly higher than in wells with bacteria alone ($p < 0.05$ for *A.*
425 *castellanii* and inserts and $p < 0.001$ *A. polyphaga* and inserts).

426 *F. philomiragia* ATCC 25015 co-culture experiments with inserts gave non reproducible
427 results within and between experiments and thus were not included in this study.

428 Although Ft6 survival was not favoured by the presence of amoebae, we performed co-
429 cultures with insert for this bacterial strain. Surprisingly, *F. tularensis* Ft6 better survived
430 when it was separated from the amoebae compared to co-cultures without inserts. When
431 bacteria were separated from amoebae by an insert, CFU counts decreased during the first
432 seven days (0.61 log CFU/ml ($p < 0.01$) reduction for *A. castellanii* and 1.54 log CFU/ml
433 reduction ($p < 0.001$) for *A. polyphaga*) and then increased up to 16 days (1.66 log CFU/ml
434 increase ($p < 0.05$) for *A. castellanii* and 2.74 log CFU/ml increase ($p < 0.05$) for *A.*
435 *polyphaga*), for both amoebae species (Figure 4). CFU counts were significantly higher in
436 wells with insert than in wells without inserts at D16 ($p < 0.05$ both for *A. castellanii* and *A.*
437 *polyphaga*) (Figure 4).

438 For the two bacterial strains, no bacteria were obtained from the amoebae supernatants
439 lysates demonstrating that bacteria did not pass through the insert during the 16 days of the
440 co-culture experiment.

441

442 IMMUNOFLUORESCENCE DETECTION OF FRANCISELLA IN INFECTED AMOEBAE

443 Immunofluorescence staining and confocal microscopy were performed for the two amoebae
444 species and the four *Francisella* strains at T0, D2 and D7 of infection in PYG/wg, and at T0
445 and D7 of infection in SM. Confocal microscopy confirmed that the four *Francisella* strains

446 tested were located inside *A. polyphaga* or *A. castellanii* amoebae. No major variation was
447 observed over time in PYG/wg, whereas bacteria disappeared at D7 in SM. Figure 5 shows
448 confocal microscopy of *A. polyphaga* infected with *F. tularensis* Ft6, either in PYG/wg at T0,
449 D2 and D7, or in SM at T0.

450

451 STUDY OF THE T6SS IMPACT ON FRANCISELLA AND AMOEBAE INTERACTIONS

452 In order to evaluate the role of the T6SS on *F. novicida* interactions with amoebae, we tested
453 T6SS-deleted mutants compared to the wild-type (WT) U112 strain of *F. novicida* in the
454 previously described models.

455 In APT model, *F. novicida* Δ FPI, *F. novicida* Δ FNI, and *F. novicida* Δ FPI Δ FNI gave the
456 same results with *A. castellanii* compared to the WT strain (Figure S1).

457 During infection in PYG/wg of *A. polyphaga*, the intra-amoebic counts of *F. novicida* WT
458 and Δ FPI Δ FNI strains evolved similarly although subtle but significant differences were
459 observed at D2 and D7. There was a raise in CFU counts during two or five days, followed by
460 a relative stabilization (Figure S3). Here again, CFU counts evolved similarly inside and
461 outside amoebae, and were not significantly different between the mutant and WT strains over
462 time (Figure S3).

463 During infection in SM of *A. polyphaga*, the intra-amoebic CFU counts of *F. novicida* WT
464 and Δ FPI Δ FNI decreased progressively and similarly over time despite bacterial counts
465 slightly higher for *F. novicida* Δ FPI Δ FNI (Figure S3).

466 Finally, during co-culture without inserts, *A. polyphaga* enhanced the survival of either *F.*
467 *novicida* WT or *F. novicida* Δ FPI Δ FNI in a similar manner. In contrast, the bacterial count of
468 these two strains progressively and similarly decreased over time in amoeba-free medium
469 (Figure S3).

470

471 AMOEBAE AND FRANCISELLA CO-CULTURE IN SPRING WATER

472 To better mimic the natural conditions of *Francisella* sp. survival in aquatic environments, *F.*
473 *novicida* U112 and *A. castellanii* co-cultures were performed in spring water and at room
474 temperature, during 30 days.

475 In co-culture with amoebae, *F. novicida* showed enhanced survival compared to amoeba-free
476 water samples, regardless of doses. During the observed period, bacterial counts in co-culture
477 were higher in comparison to samples with no amoebae ($p < 0.05$ at D5, D10, D15, and D20,
478 NS at D25 and D30 for *F. novicida* at 10^6 CFU/ml and $p < 0.01$ at D5, D10, and D15, $p <$
479 0.001 at D20, $p < 0.01$ at D25 and NS at D30 for *F. novicida* at 10^7 CFU/ml) (Figure 6, A).
480 Moreover, *F. novicida* survived for a longer period of time in the presence of amoebae.

481 Unlike previous models, in the spring water co-culture model, amoebic counts were not stable
482 during the experiments and decreased progressively. Besides, in co-culture models, amoebic
483 counts decreased slightly faster in comparison to control water samples with no bacteria (NS
484 at D5, $p < 0.01$ at D10, $p < 0.01$ at D15, $p < 0.001$ at D20, NS at D25 and D30 for *A.*
485 *castellanii* at 10^6 cells/ml and NS at D5, $p < 0.05$ at D10, $p < 0.001$ at D15, $p < 0.01$ at D20, p
486 < 0.05 at D25 and NS at D30 for *A. castellanii* at 10^7 cells/ml) (Figure 6, B).

487 Morphology of *F. novicida*, after 15 days incubation alone in spring water or in co-incubation
488 with *A. castellanii* in spring water was also studied by transmission electron microscopy
489 (TEM). Multiple morphological changes of *F. novicida* cells were observed after 15 days
490 incubation in amoeba-free spring water. Bacteria showed disorganized cytoplasm separated
491 from the cell wall, with multiple clumping and highly undefined cell wall. In contrast,
492 *F. novicida* co-cultured with amoebae in spring water were roundly shaped and showed well
493 preserved cell structures with smooth and intact cell wall, similar to the control bacteria
494 grown on BCYE agar (Figure 7).

495

496 **DISCUSSION**

497 The aim of our study was to evaluate *in vitro* survival or even proliferation of
498 *Francisella* species in the presence of amoebae over an extended period. This could help to
499 understand their long-term survival in natural aquatic environments. Therefore, we set-up
500 different models of interactions between *F. tularensis*, *F. philomiragia*, or *F. novicida* with
501 two amoeba species, *A. castellanii* and *A. polyphaga*.

502 We first evaluated the interactions between *Francisella* and amoebae using the
503 Amoeba Plate Test (APT). This screening test was developed by Albers *et al.* ²⁴ to study the
504 interactions between *L. pneumophila* and amoebae ²⁴. The APT evaluates the ability of a
505 specific bacterial strain to pass through a layer of amoebae to form colonies on an agar plate.
506 A negative APT usually indicates that the tested bacterium is unable to kill amoebae either
507 directly or through intra-amoebic multiplication. Conversely, a positive test usually indicates
508 that the bacterium is able to resist the attack by amoebae and possibly multiply within these
509 protozoa ⁶. Interestingly, we observed a positive APT for all tested *Francisella* strains but
510 with varying results. We observed a strong growth with *F. philomiragia*, a medium growth
511 with *F. novicida* and the clinical strains of *F. tularensis*, and the weakest growth with the LVS
512 strain. As for *F. philomiragia*, our results are in agreement with those reported by Verhoeven
513 *et al.* ¹⁰ showing higher resistance of *F. philomiragia* compared to *F. novicida* in an *A.*
514 *castellanii* APT model and those of Thelaus *et al.* ²⁵ showing that *F. philomiragia* was the
515 least edible *Francisella* species for the ciliate *Tetrahymena pyriformis*. Regarding *F.*
516 *tularensis* subsp. *holarctica* strains, LVS was less efficient to resist to amoebae than the
517 clinical strains. This might be due to the attenuated virulence of this vaccine strain.
518 Interestingly, the six clinical strains of *F. tularensis* subsp. *holarctica* displayed similar
519 results, suggesting a homogenous amoeba resistance between strains of this subspecies,
520 correlating with the low genetic diversity of French Type B strains ²². Overall, the APT

521 demonstrated some interactions between *Francisella* sp. and amoebae, with variations
522 between *Francisella* species that could suggest different degree of adaptation to protozoan
523 predation, as previously suggested by Thelaus *et al.* ²⁵.

524 To better characterise interactions between *Francisella* and amoebae, we then used
525 different cell models: 1/ an infection model in rich culture medium previously described in the
526 literature ^{9,11} but with longer incubation period; 2/ an infection model in poor culture medium
527 developed to prevent any extracellular growth of *Francisella*; and 3/ co-culture models in
528 which amoebae and *Francisella* were allowed to interact through direct contact or not.

529 We first evaluated the ability of *Francisella* to multiply in amoeba culture media
530 (PYG, PYG/wg, SM and PAS) in the absence of these protozoa. In models evaluating the
531 ability of bacteria to grow inside eukaryotic cells, the culture supernatant should not support
532 their extracellular growth. *F. philomiragia*, *F. novicida*, and *F. tularensis* Ft6 and LVS strains
533 displayed a strong growth in PYG and PYG/wg. This observation was previously reported by
534 El-Etr *et al.* ⁸. Because we incubated our cultures for up to 16 days, we considered that an
535 extracellular growth of bacteria may lead to erroneous results. Thus, in our experiments, we
536 did not use the PYG and PYG/wg media but rather the SM medium for which no bacterial
537 growth was observed.

538 In the infection in PYG/wg model, use as control, we observed a two- to four-log
539 increase in CFU counts of *F. novicida*, *F. philomiragia* and Ft6 strains within amoebae during
540 the first two or five days of infection (Figure 1). Confocal microscopy confirmed that bacteria
541 were present inside the amoebae (Figure 5). These findings were similar to those previously
542 reported by Santic *et al.* ⁹ for *F. novicida* and *A. castellanii* or *V. vermiformis*. Yet, the next
543 days of the experiments we observed a stagnation or reduction in intra-amoebic CFU counts.
544 However, extracellular CFU counts overlaid intracellular CFU counts. The LVS strain did not
545 exhibit any CFU increase in the intra- or extra-amoebic compartments, while this bacterium

546 was able to grow in amoeba-free PYG/wg. This result suggests that the LVS strain was
547 phagocytosed and digested by the amoebae, which may be correlated to the low virulence of
548 this vaccine strain. Because of extra-amoebic growth of most *Francisella* strains tested, we
549 considered this model not suitable for long-term evaluation of the intra-amoebic growth of
550 these bacteria.

551 The infection in SM model was designed to allow only intra-amoebic growth of
552 *Francisella* strains, by using SM as the culture medium. The SM supported the survival of *A.*
553 *castellanii* and *A. polyphaga* for 16 days in trophozoite forms (data not shown), but did not
554 allow growth of the tested *Francisella* sp. strains (Figure S2). In this model, no intra-amoebic
555 replication of *F. novicida*, *F. philomiragia*, LVS and Ft6 was observed. A rapid decrease of
556 intra-amoebic CFU counts was observed (Figure 2). Confocal microscopy confirmed that
557 *Francisella* sp. were localised inside the amoebae at T0, but then disappeared at D7 post
558 infection (Figure 5), a result correlating with reduction in CFU counts. El-Etr *et al.* also
559 reported a decrease in intracellular counts of LVS and some clinical strains of *F. tularensis*
560 subsp. *tularensis*, in an infection model using a poor culture medium (High-salt buffer)⁸.
561 However, in the same study, an increase in intra-amoebic CFU counts was observed for *F.*
562 *novicida* U112 and others *F. tularensis* subsp. *tularensis* clinical strains⁸ suggesting that
563 intra-amoebic growth could depend on the *Francisella* species and strain studied and also on
564 experimental protocol used.

565 Since APT revealed that *Francisella* sp. resisted to amoebae and infection in SM did
566 not suggested intra-amoebic survival of *Francisella* sp., we developed co-culture models. The
567 aim of the co-culture model was to evaluate if amoebae could promote *Francisella* sp.
568 survival whether intracellular or extracellular. Enhanced survival of *F. philomiragia* and *F.*
569 *novicida* was observed in the presence of amoebae, while we found a rapid loss of
570 cultivability of these bacteria alone in amoeba-free medium (Figure 3). Buse *et al.* also

571 showed that several amoebae species enhanced bacterial survival but did not enable
572 multiplication of *F. novicida*, LVS and type-A and type-B clinical strains¹⁴. Unlike this
573 study, we observed a real multiplication of both *F. novicida* and *F. philomiragia* after seven
574 to 12 days of incubation (Figure 3). However, Buse *et al.* stopped their experimentations after
575 10 days of co-incubation, maybe just before bacterial multiplication onset¹⁴. This suggested
576 that amoebae could promote the survival of these two *Francisella* species. Two mechanisms
577 should be considered to explain this enhanced survival in the presence of amoebae: 1/ a direct
578 “physical” interaction between bacteria and amoebae; 2/ the production of nutritive elements
579 by amoeba in the culture supernatant that could be useful for *Francisella* survival and/or a
580 degradation of toxic compounds by amoebae. In order to investigate these two hypotheses, we
581 repeated the same co-culture experiments with the addition of inserts to physically separate
582 bacteria from amoebae while allowing them to share the same medium. Enhanced survival of
583 *F. novicida* was partially lost when an insert separated amoebae and bacteria (Figure 4). This
584 result suggested that enhanced survival of *F. novicida* in co-culture with amoebae could be
585 partly dependent on direct contact between bacteria and protozoa but also on amoeba-excreted
586 compounds. Experiments with *F. philomiragia* gave too heterogeneous results within and
587 between experiments to draw any conclusion.

588 Although the Ft6 strain did not survive in amoeba co-cultures without insert, we observed an
589 enhanced survival of this strain when separated from the amoebae (Figure 4). We can
590 hypothesize that, because *F. tularensis* strains (including Ft6) have a lower growth rate than
591 *F. philomiragia* and *F. novicida*, phagocytosis by amoebae may outcompete bacterial growth
592 in co-culture models without insert leading to a reduction in bacterial CFU counts. In contrast,
593 when amoebae cannot phagocytize Ft6 because of the insert, this bacterium may benefit from
594 amoeba-excreted compounds. This differs slightly from the observations of Buse *et al.*¹⁴ who
595 reported an enhanced survival of a *F. tularensis* type B strain in contact with amoebae, which

596 may indicate that results are dependent on the bacterial strain used or on the experimental
597 protocol. Verhoeven *et al.* reported that the inoculation of *F. philomiragia* ATCC 25015 in
598 PYG medium preconditioned by *A. castellanii* (i.e. growth supernatant of *A. castellanii* from
599 which the amoebae were removed) reduced biofilm production and increased bacterial growth
600 ¹⁰. Gustafsson *et al.* also demonstrated the same positive effect of culture media
601 preconditioned by *A. palestinensis* on the growth of *F. tularensis* LVS ²⁶. These two studies
602 suggested that several *Francisella* species could benefit from elements excreted by amoebae
603 during co-culture with these protozoans.

604 Co-culture models of *F. novicida* with *A. castellanii* were then performed in a spring water
605 environment and at room temperature to be as close as possible from natural ecosystem. In
606 this model, amoebae again favored *F. novicida* survival. Indeed, bacterial counts were higher
607 in the presence of amoebae compared to amoeba-free spring water and survival time of *F.*
608 *novicida* was longer in the presence of amoebae (Figure 6, A). In addition, bacterial
609 morphology was preserved in presence of amoebae compared to amoeba-free spring water
610 (Figure 7).

611 In all the models tested, we did not observe any difference between WT *F. novicida*
612 and a *F. novicida* strain deleted of the two loci encoding T6SS, strongly suggesting that *F.*
613 *novicida* does not rely on its T6SSs to interact with amoeba. This result is in sharp contrast
614 with the key role of the FPI-encoded T6SS in promoting bacterial replication in mammalian
615 cells ¹⁷. However, this fits well with our infection and co-culture experiments results that
616 suggest an absence of *Francisella* sp. replication inside amoebae but a *Francisella* sp.
617 survival and multiplication in presence of amoebae, probably extracellularly.

618 Overall, data available in the literature on the interactions between *Francisella* sp. and
619 amoebae are conflicting. Some studies described the multiplication of *F. noatunensis*, *F.*
620 *philomiragia*, *F. novicida*, LVS, and clinical strains of *F. tularensis* subsp. *tularensis* inside or

621 in association with amoebae such as *A. castellanii*, *V. vermiformis* and *Dictyostelium*
622 *discoideum*⁸⁻¹³. Conversely, other publications reported a lack of multiplication of *F.*
623 *novicida*, LVS or type A and type B clinical strains^{8,14}. However, these studies used very
624 different experimental protocols, especially in terms of amoebae culture media. Previous
625 works reporting multiplication of *Francisella* strains inside or in the presence of amoebae for
626 12 or 20 days have used rich media such as PYG medium (ATCC medium 712)^{10,11}. For such
627 models, it may be difficult to distinguish intra- from extra-amoebic growth of bacteria, and
628 increase in CFU counts should be interpreted cautiously. To overcome this potential bias, we
629 decided to work in SM medium, which did not support growth of *Francisella* strains used in
630 this study. We evaluated for the first time the interactions of amoebae with several virulent
631 clinical strains of *F. tularensis* subsp. *holarctica*. Although more tedious, these models can be
632 considered more relevant to study ecological aspects of this major human pathogen compared
633 to the attenuated LVS strain or strains belonging to other *Francisella* species. Hence, very
634 different results were obtained in this study between the studied *Francisella* species, but also
635 between LVS and clinical strains of *F. tularensis* subsp. *holarctica*.

636 In our models, *F. philomiragia*, *F. novicida* and virulent type B strain of *F. tularensis*
637 did not multiply inside amoebae, but could survive and multiply in an environment containing
638 amoebae suggesting a potential commensal relationship between these two microorganisms.
639 The interaction conditions established in our co-culture models likely occur in the aquatic
640 environment where amoebae are widespread⁷. We found that *F. novicida* and *F. philomiragia*
641 survive in the presence of amoebae. These two species are considered to have a primary
642 aquatic reservoir, which fits well with the water-borne nature of the rare human infections
643 caused by these two pathogens⁵. We also demonstrated that fully virulent *F. tularensis* strains
644 may benefit from the presence of amoeba to survive in the environment. This may partly
645 explain why human tularemia cases are occasionally associated with water-born transmission

646 ⁵. However, the variable ability of *Francisella* species to interact with amoebae, as described
647 in the literature, also suggests that amoebae should not be considered the only reservoir of
648 these bacteria in the aquatic environment. Other mechanisms potentially involved may
649 include survival of *Francisella* sp. in biofilms, in mosquitoes larvae, and in water as viable
650 but non-culturable (VBNC) forms ⁵ and have to be further investigated.

651

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655

656 **CONFLICT OF INTEREST**

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

658

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665

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735

736 **FIGURE LEGENDS**

737 **Fig 1. Infection of *A. castellanii* and *A. polyphaga* by *Francisella* sp. in PYG/wg.**

738 The figure shows one experiment made in triplicate. Similar results were obtained in a second
739 experiment. The error bars represent standard deviations. Comparison of T0 versus D2 (or D5
740 for Ft6), and D2 (or D5 for Ft6) versus D7. NS: not significant; *: $p < 0.05$; **: $p < 0.01$; ***:
741 $p < 0.001$

742

743 **Fig 2. Infection of *A. castellanii* and *A. polyphaga* by *Francisella* sp. in SM.**

744 The figure shows one experiment made in triplicate. Similar results were obtained in a second
745 experiment. The error bars represent standard deviations. Comparison of T0 versus D7. NS:
746 not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

747

748 **Fig 3. Co-culture of *A. castellanii* and *A. polyphaga* with *Francisella* sp. in SM.**

749 The figure shows one experiment made in triplicate. Similar results were obtained in a second
750 experiment. The error bars represent standard deviations. Comparison of T0 versus D7, D12
751 or D16; or D16 versus D7 or D12. Comparison of bacteria with amoebae and bacteria alone at
752 D16. NS: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

753

754 **Fig 4. Co-culture with inserts of *A. castellanii* and *A. polyphaga* with *Francisella* sp. in
755 SM.**

756 The figure shows one experiment made in triplicate. Similar results were obtained in a second
757 experiment. The error bars represent standard deviations. Comparison of T0 versus D7 or
758 D16; or D7 vs D16. Comparison of bacteria with amoebae separated or not by an insert at
759 D16. NS: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

760

761 **Fig 5. Confocal microscopy of *A. polyphaga* infected by *F. tularensis* Ft6.**

762 A) T0 in PYG/wg; B) D2 in PYG/wg; C) D7 in PYG/wg; D) T0 in SM; No more bacteria
763 were observed inside amoeba at D7 in SM (data not shown). A') orthogonal view of T0 in
764 PYG/wg; D') orthogonal view of T0 in SM. Green: actin (phalloidin Alexa Fluor 488). Red:
765 *Francisella* sp. (rabbit primary antibody against *Francisella*, goat secondary antibody against
766 rabbit Alexa Fluor 594).

767

768 **Fig 6. Growth kinetics of *F. novicida* (A) and *A. castellanii* (B) in spring water.**

769 The figure shows one experiment made in triplicate. Similar results were obtained in a second
770 experiment. The error bars represent standard deviations. Comparison at each time point of
771 (A) *F. novicida* with or without amoebae (B) *A. castellanii* with or without bacteria. NS: not
772 significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Fn: *F. novicida*; Ac: *A. castellanii*

773

774 **Fig 7. Morphology and structure of *F. novicida*, after 15 days incubation in spring water**
775 **(A), co-incubation with *A. castellanii* in spring water (B), and as a control after growth**
776 **on BCYE agar (C) by TEM.**

777