



**HAL**  
open science

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

Aurélie Hennebique, Julien Peyroux, Camille Brunet, Amandine Martin,  
Thomas Henry, Masa Knezevic, Marina Santic, Sandrine Boisset, Max Maurin

### ► **To cite this version:**

Aurélie Hennebique, Julien Peyroux, Camille Brunet, Amandine Martin, Thomas Henry, et al.. Amoebae can promote the survival of Francisella species in the aquatic environment. *Emerging microbes & infections*, 2021, 10.1080/22221751.2021.1885999 . hal-03132662

**HAL Id: hal-03132662**

**<https://hal.science/hal-03132662>**

Submitted on 5 Feb 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

3

4 Aurélie Hennebique<sup>1,2</sup>, Julien Peyroux<sup>2</sup>, Camille Brunet<sup>2</sup>, Amandine Martin<sup>3</sup>,  
5 Thomas Henry<sup>3</sup>, Masa Knezevic<sup>4</sup>, Marina Santic<sup>4</sup>, Sandrine Boisset<sup>1,2</sup>, Max  
6 Maurin<sup>1,2\*</sup>

7

8 <sup>1</sup> *Centre National de Référence des Francisella, Institut de Biologie et de Pathologie, Centre*  
9 *Hospitalier Universitaire Grenoble Alpes, Grenoble, France.*

10 <sup>2</sup> *Université Grenoble Alpes, Centre National de la Recherche Scientifique, TIMC-IMAG,*  
11 *UMR5525, Grenoble, France.*

12 <sup>3</sup> *CIRI, Centre International de Recherche en Infectiologie, Univ Lyon, Inserm U1111,*  
13 *Université Claude Bernard Lyon 1, CNRS, UMR5308, ENS de Lyon, Labex Ecofect, Univ*  
14 *Lyon, F-69007, LYON, France*

15 <sup>4</sup> *Faculty of Medicine, University of Rijeka, Department of Microbiology and Parasitology,*  
16 *Rijeka, Croatia*

17

18 \* Corresponding author: Prof. M. Maurin, Service de Bactériologie-Hygiène Hospitalière,  
19 Institut de Biologie et de Pathologie, Centre Hospitalier Universitaire Grenoble Alpes,  
20 CS10217, 38043 Grenoble Cedex 9, France. Tel: +33476765479

21 Email: [mmaurin@chu-grenoble.fr](mailto:mmaurin@chu-grenoble.fr)

22

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) <sup>1</sup>. 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 <sup>2,3</sup>. 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 <sup>4</sup>. Ticks and other arthropods  
58 (especially mosquitoes) are vectors of this pathogen <sup>4</sup>. 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 <sup>5</sup>. Although *F. tularensis* has been detected in natural water  
61 sources <sup>5</sup>, 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 <sup>6</sup>. Amoebae such as *Acanthamoeba* and  
66 *Vermamoeba* species are ubiquitous in water and soil <sup>7</sup>. A few experimental studies have  
67 investigated the relationships between *Francisella* sp. and amoebae, with contradictory results  
68 <sup>8-14</sup>. Most of these studies have involved the live vaccine strain (LVS) of *F. tularensis*, *F.*  
69 *novicida*, *F. philomiragia*, or *F. noatunensis* <sup>8-14</sup>. Only two studies have evaluated the

70 interactions with amoebae of virulent *F. tularensis* type A strains <sup>8,14</sup> and only one with a  
71 virulent *F. tularensis* type B strain <sup>14</sup>.

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) <sup>15-17</sup>. *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 <sup>17,18</sup>. *F.*  
76 *novicida* harbors another genetic locus, that we termed FNI, likely encoding a different T6SS  
77 of unknown function <sup>17,19</sup>. The role of *Francisella* T6SSs in persistence/replication within  
78 amoebae remains unclear <sup>9,11,12,20</sup>.

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*  $\Delta$ FPI mutant <sup>21</sup>, *F.*  
90 *novicida*  $\Delta$ FNI mutant <sup>19</sup>, and the *F. novicida*  $\Delta$ FPI $\Delta$ 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) <sup>22</sup>. 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% CO<sub>2</sub>-  
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, MgSO<sub>4</sub>, 25 mM, CaCl<sub>2</sub>, 20 mM, L-Arg 2mM, L-His 1mM, L-Met  
111 2mM, L-Asp 3mM, Spermine 0,2mM; MnCl<sub>2</sub> 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-cm<sup>2</sup> 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 MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.4  
128 mM CaCl<sub>2</sub>, 0.1% sodium citrate dihydrate, 0.05 mM Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> · 6H<sub>2</sub>O, 2.5 mM  
129 Na<sub>2</sub>HPO<sub>4</sub> · 7H<sub>2</sub>O, 2.5 mM KH<sub>2</sub>PO<sub>4</sub>) 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 Na<sub>2</sub>HPO<sub>4</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 0.016 mM MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.027  
136 mM CaCl<sub>2</sub> · 2H<sub>2</sub>O, 2 mM NaCl, 0.005 mM (NH<sub>4</sub>)<sub>2</sub>Fe(SO<sub>4</sub>)<sub>2</sub> · 6H<sub>2</sub>O, 0.2% yeast extract, 1.8%  
137 glucose)<sup>23</sup> 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 Na<sub>2</sub>HPO<sub>4</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 0.016 mM MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.027 mM  
140 CaCl<sub>2</sub> · 2H<sub>2</sub>O, 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<sup>10,24</sup>. 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-cm<sup>2</sup> 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  $2.67 \times 10^6$  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  $\mu$ 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  $5.3 \times 10^5$  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*  $\Delta$ FPI, *F. novicida*  $\Delta$ FNI, or *F. novicida*  $\Delta$ FPI $\Delta$ FNI. Infections in  
266 PYG/wg and SM were performed for *A. polyphaga* and either WT *F. novicida* U112 or *F.*  
267 *novicida*  $\Delta$ FPI $\Delta$ FNI. Co-culture without inserts were performed for *A. polyphaga* and WT *F.*  
268 *novicida* U112 or *F. novicida*  $\Delta$ FPI $\Delta$ 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 AMOEBIA 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 10<sup>6</sup> 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 10<sup>6</sup>  
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 10<sup>9</sup> CFU/ml dilution (3 log inferior to the  
313 amoeba-free control and the *L. pneumophila* control).

314

### 315 GROWTH OF FRANCISELLA STRAINS IN AMOEBIAE 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<sup>9,11</sup> 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  $3 \times 10^5$  to  $8 \times 10^5$  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*  $\Delta$ FPI, *F. novicida*  $\Delta$ FNI, and *F. novicida*  $\Delta$ FPI $\Delta$ 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  $\Delta$ FPI $\Delta$ 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  $\Delta$ FPI $\Delta$ FNI decreased progressively and similarly over time despite bacterial counts  
465 slightly higher for *F. novicida*  $\Delta$ FPI $\Delta$ FNI (Figure S3).

466 Finally, during co-culture without inserts, *A. polyphaga* enhanced the survival of either *F.*  
467 *novicida* WT or *F. novicida*  $\Delta$ FPI $\Delta$ 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.* <sup>24</sup> to study the  
504 interactions between *L. pneumophila* and amoebae <sup>24</sup>. 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 <sup>6</sup>. 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.* <sup>10</sup> showing higher resistance of *F. philomiragia* compared to *F. novicida* in an *A.*  
514 *castellanii* APT model and those of Thelaus *et al.* <sup>25</sup> 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 <sup>22</sup>. 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.* <sup>25</sup>.

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 <sup>9,11</sup> 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.* <sup>8</sup>. 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.* <sup>9</sup> 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)<sup>8</sup>.  
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 strains<sup>8</sup> 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 strains<sup>14</sup>. 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 onset<sup>14</sup>. 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.*<sup>14</sup> 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 <sup>10</sup>. Gustafsson *et al.* also demonstrated the same positive effect of culture media  
601 preconditioned by *A. palestinensis* on the growth of *F. tularensis* LVS <sup>26</sup>. 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 <sup>17</sup>. 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*<sup>8-13</sup>. Conversely, other publications reported a lack of multiplication of *F.*  
623 *novicida*, LVS or type A and type B clinical strains<sup>8,14</sup>. 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)<sup>10,11</sup>. 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 widespread<sup>7</sup>. 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 pathogens<sup>5</sup>. 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 <sup>5</sup>. 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 <sup>5</sup> and have to be further investigated.

651

## 652 **ACKNOWLEDGEMENTS**

653 The authors thank Yves Usson for providing access to confocal microscope of the TIMC-  
654 IMAG laboratory of the Grenoble-Alpes University.

655

## 656 **CONFLICT OF INTEREST**

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

658

## 659 **FUNDING STATEMENT**

660 This work was supported by the Direction Generale de l'Armement (DGA, ANR-17-ASTR-  
661 0024), by the LABEX ECOFECT (ANR-11-LABX-0048) of Université de Lyon, within the  
662 program "Investissements d'Avenir" (ANR-11-IDEX-0007) operated by the French National  
663 Research Agency (ANR) and by Croatian Science Foundation (HRZZ- IP-2016-9003) and  
664 University of Rijeka (UNIRI-BIOMED-128).

665

666 **REFERENCES**

- 667 1 Dennis DT, Inglesby TV, Henderson DA *et al.* Tularemia as a biological weapon: medical  
668 and public health management. *JAMA* 2001; **285**: 2763–2773.
- 669 2 Johansson A, Celli J, Conlan W *et al.* Objections to the transfer of *Francisella novicida* to  
670 the subspecies rank of *Francisella tularensis*. *Int J Syst Evol Microbiol* 2010; **60**: 1717–  
671 1718; author reply 1718-1720.
- 672 3 Busse H-J, Huber B, Anda P *et al.* Objections to the transfer of *Francisella novicida* to the  
673 subspecies rank of *Francisella tularensis* - response to Johansson *et al.* *Int J Syst Evol*  
674 *Microbiol* 2010; **60**: 1718–1720.
- 675 4 Sjöstedt A. Tularemia: history, epidemiology, pathogen physiology, and clinical  
676 manifestations. *Ann N Y Acad Sci* 2007; **1105**: 1–29.
- 677 5 Hennebique A, Boisset S, Maurin M. Tularemia as a waterborne disease: a review. *Emerg*  
678 *Microbes Infect* 2019; **8**: 1027–1042.
- 679 6 Greub G, Raoult D. Microorganisms resistant to free-living amoebae. *Clin Microbiol Rev*  
680 2004; **17**: 413–433.
- 681 7 Rodríguez-Zaragoza S. Ecology of free-living amoebae. *Crit Rev Microbiol* 1994; **20**: 225–  
682 241.
- 683 8 El-Etr SH, Margolis JJ, Monack D *et al.* *Francisella tularensis* type A strains cause the rapid  
684 encystment of *Acanthamoeba castellanii* and survive in amoebal cysts for three weeks  
685 postinfection. *Appl Environ Microbiol* 2009; **75**: 7488–7500.
- 686 9 Santic M, Ozanic M, Semic V, Pavokovic G, Mrcic V, Kwaik YA. Intra-vacuolar  
687 proliferation of *F. novicida* within *H. vermiformis*. *Front Microbiol* 2011; **2**: 78.
- 688 10 Verhoeven AB, Durham-Colleran MW, Pierson T, Boswell WT, Van Hoek ML. *Francisella*  
689 *philomiragia* biofilm formation and interaction with the aquatic protist *Acanthamoeba*  
690 *castellanii*. *Biol Bull* 2010; **219**: 178–188.
- 691 11 Lauriano CM, Barker JR, Yoon S-S *et al.* MglA regulates transcription of virulence factors  
692 necessary for *Francisella tularensis* intraamoebae and intramacrophage survival. *Proc*  
693 *Natl Acad Sci USA* 2004; **101**: 4246–4249.
- 694 12 Lampe EO, Brenz Y, Herrmann L *et al.* Dissection of *Francisella*-host cell interactions in  
695 *Dictyostelium discoideum*. *Appl Environ Microbiol* 2015; **82**: 1586–1598.
- 696 13 Abd H, Johansson T, Golovliov I, Sandström G, Forsman M. Survival and growth of  
697 *Francisella tularensis* in *Acanthamoeba castellanii*. *Appl Environ Microbiol* 2003; **69**: 600–  
698 606.

- 699 14 Buse HY, Schaefer lii FW, Rice EW. Enhanced survival but not amplification of *Francisella*  
700 spp. in the presence of free-living amoebae. *Acta Microbiol Immunol Hung* 2017; **64**: 17–  
701 36.
- 702 15 Gray CG, Cowley SC, Cheung KKM, Nano FE. The identification of five genetic loci of  
703 *Francisella novicida* associated with intracellular growth. *FEMS Microbiol Lett* 2002; **215**:  
704 53–56.
- 705 16 Nano FE, Zhang N, Cowley SC *et al.* A *Francisella tularensis* pathogenicity island required  
706 for intramacrophage growth. *J Bacteriol* 2004; **186**: 6430–6.
- 707 17 Broms JE, Sjostedt A, Lavander M. The role of the *Francisella tularensis* Pathogenicity  
708 Island in type VI secretion, intracellular survival, and modulation of host cell signaling.  
709 *Front Microbiol* 2010; **1**. doi:10.3389/fmicb.2010.00136.
- 710 18 Eshraghi A, Kim J, Walls AC *et al.* Secreted effectors encoded within and outside of the  
711 *Francisella* Pathogenicity Island promote intramacrophage growth. *Cell Host Microbe*  
712 2016; **20**: 573–583.
- 713 19 Rigard M, Broms JE, Mosnier A *et al.* *Francisella tularensis* IgG belongs to a novel family  
714 of PAAR-Like T6SS proteins and harbors a unique N-terminal extension required for  
715 virulence. *PLoS Pathog* 2016; **12**: e1005821.
- 716 20 Köppen K, Chen F, Rydzewski K *et al.* Screen for fitness and virulence factors of  
717 *Francisella* sp. strain W12-1067 using amoebae. *International Journal of Medical*  
718 *Microbiology* 2019; **309**: 151341.
- 719 21 Weiss DS, Brotcke A, Henry T, Margolis JJ, Chan K, Monack DM. *In vivo* negative selection  
720 screen identifies genes required for *Francisella* virulence. *Proc Natl Acad Sci USA* 2007;  
721 **104**: 6037–6042.
- 722 22 Kevin M, Girault G, Caspar Y *et al.* Phylogeography and genetic diversity of *Francisella*  
723 *tularensis* subsp. *holarctica* in France (1947-2018). *Front Microbiol* 2020; **11**: 287.
- 724 23 Reteno DG, Benamar S, Khalil JB *et al.* Faustovirus, an asfarvirus-related new lineage of  
725 giant viruses infecting amoebae. *J Virol* 2015; **89**: 6585–6594.
- 726 24 Albers U, Reus K, Shuman HA, Hilbi H. The amoebae plate test implicates a paralogue of  
727 lpxB in the interaction of *Legionella pneumophila* with *Acanthamoeba castellanii*.  
728 *Microbiology (Reading, Engl)* 2005; **151**: 167–182.
- 729 25 Thelaus J, Andersson A, Mathisen P, Forslund A-L, Noppa L, Forsman M. Influence of  
730 nutrient status and grazing pressure on the fate of *Francisella tularensis* in lake water.  
731 *FEMS Microbiology Ecology* 2009; **67**: 69–80.
- 732 26 Gustafsson K. Growth and survival of four strains of *Francisella tularensis* in a rich  
733 medium preconditioned with *Acanthamoeba palestinensis*. *Can J Microbiol* 1989; **35**:  
734 1100–1104.

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