

1 **Whole cell inactivated autogenous vaccine effectively protects red**
2 **Nile**
3 **tilapia (*Oreochromis niloticus*) against francisellosis via**
4 **intraperitoneal**
5 **injection**

6 **Short running title:** *Francisella* vaccine for Nile tilapia

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26 **Acknowledgements**

27 JGRP was a Ph.D. candidate in Aquatic Veterinary Studies at the Institute of Aquaculture
28 University of Stirling partially sponsored by the National Council for Science and Technology
29 in Mexico (CONACyT) under the call “Convocatoria de Becas al Extranjero 2010 segundo
30 periodo” Register number: 214096.

31 MAMR was an MSc student in Aquatic Veterinary Studies at the Institute of Aquaculture
32 University of Stirling class 2014 partially sponsored by Fundación para el futuro de Colombia
33 COLFUTURO.

34 Rearing, breeding, and maintenance of naïve, immunised and challenged Nile tilapia was
35 carried out by Mr. Keith Ranson at the Tropical Aquarium and Mr. Niall Auchinachie at the
36 Aquatic Research Facility, Institute of Aquaculture, University of Stirling.

37 The hypodermic needles to vaccinate the fish were kindly provided by Robert Wittmann and
38 Phil Brown at Aqualife Services Ltd. www.aqualifeservices.com

39 The adjuvant used in this study was kindly provided by SEPPIC, Puteaux Cedex, France
40 <https://www.seppic.com>

41 **Abstract**

42 *Francisella noatunesis* subsp. *orientalis* is a pathogen of tilapia and other warm water fish for
43 which no vaccines are commercially available. In this study a whole cell formalin inactivated

44 vaccine was developed using the highly virulent isolate STIR-GUS-F2f7 and the oil-based
45 adjuvant Montanide™ ISA 763A VG. The efficacy of the vaccine was assessed in red Nile
46 tilapia via intraperitoneal (i.p.) injection using homologous experimental infection and
47 correlates of protection such as seral antibody production and bacterial loads in the spleen. For
48 immunisation, fish were i.p. injected with 0.1mL of the vaccine, the adjuvant alone or PBS. At
49 840 degree days post vaccination all fish were i.p. injected with 4.0×10^3 CFU/fish of pathogenic
50 bacteria. The RPS at the end of the trial was 100% in the vaccinated group with significantly
51 higher survival than in the adjuvant and control groups. The RPS in the adjuvant group was
52 42%, and no significant difference was seen in survival between this and the PBS group.
53 Moreover, significantly higher antibody titres in the serum and significantly lower bacterial
54 loads in the spleen were detected in the vaccinated fish by ELISA and qPCR, respectively.
55 These findings highlight the potential of autogenous vaccines for controlling francisellosis in
56 tilapia.

57 **Keywords:** tilapia vaccines; *Francisella* vaccines; *Francisella noatunensis* subsp. *orientalis*;
58 francisellosis in tilapia; autogenous vaccines; tilapia diseases.

59 **1 Introduction**

60 *Francisella noatunensis* subsp. *orientalis* is a facultative intracellular pathogen responsible of
61 francisellosis in several warm water fish species (Colquhoun & Duodu, 2011). In farmed Nile
62 tilapia (*Oreochromis niloticus* L.), outbreaks caused by *Francisella noatunensis* subsp.
63 *orientalis* usually occur in intensive culture systems with high stocking densities when water
64 temperature drops below 25°C (Ramirez-Paredes et al., 2017b; Soto et al., 2009; Soto et al.,
65 2012; Soto et al., 2018). Acute episodes of francisellosis in tilapia are commonly triggered by
66 husbandry handling procedures and result in high levels of morbidity (80-90%) and mortality
67 (50-90%) that can affect fish at all production stages (Birkbeck et al., 2011; Colquhoun and
68 Duodu, 2011).

69 *Francisella noatunensis* subsp. *orientalis* is one of the most pathogenic bacteria for tilapia
70 (Klinger-Bowen et al., 2016; Pulpipat et al., 2019; Ramirez-Paredes 2017b; Soto et al., 2009)
71 and one of the main challenges that this industry currently faces globally. Nevertheless, despite
72 the relevance of this pathogen, there are still no commercial vaccines available for its
73 prevention and control.

74 Soto et al. (2011) developed the first experimental vaccine for tilapia against *Francisella*
75 *noatunensis* subsp. *orientalis*. This was a live attenuated strain, generated with an insertional
76 mutation in the intracellular growth *locus C* gene (*iglC*) of strain recovered in Costa Rica which
77 conferred a relative percentage survival (RPS) of 87.5% after experimental infection by
78 immersion. However, due to the inherent risks of using live genetically modified
79 microorganism (virulence reversion, dissemination into the environment and potential
80 transmission to other species), most of the countries where tilapia is farmed do not grant
81 licences to live genetically modified vaccines. Therefore although such vaccine was shown to

82 be reasonably efficacious in that and further trails (Soto et al., 2014), it has not being licensed
83 and is not commercially available in any country.

84 In the present study, a whole cell inactivated autogenous vaccine against *Francisella*
85 *noatunensis* subsp. *orientalis* was developed in 2014 for the first time. The vaccine was
86 formulated using the commercial oil adjuvant Montanide™ ISA 763A VG and a highly virulent
87 isolate (STIR-GUS-F2f7) recovered in 2012 from diseased red tilapia fingerlings in the United
88 Kingdom (Ramirez-Paredes et al., 2017b). The efficacy of the vaccine was assessed by
89 measuring survival rates after experimentally infecting vaccinated fish by intraperitoneal
90 injection and its potency expressed in terms of RPS. Additionally, relevant correlates of
91 protection including specific antibody (IgM) kinetics and quantification of bacterial loads in
92 the spleen by qPCR were assessed.

93 **2 Materials and Methods**

94 **2.1 Bacterial strain and experimental fish**

95 *Francisella noatunensis* subsp. *orientalis* (STIR-GUS-F2f7) was isolated in November 2012
96 from a moribund red Nile tilapia, farmed in UK (Ramirez-Paredes et al., 2017b). The strain
97 had been stored at -80 °C in Modified Mueller-Hinton II cation-adjusted broth supplemented
98 with 2% IsoVitaleX (MMHB) and 20% sterile glycerol. The strain was cultured in cystine heart
99 agar with 2% bovine haemoglobin (CHAH) and MMHB following culture conditions indicated
100 by Ramirez-Paredes et al. (2017b).

101 Healthy naïve red Nile tilapia, 6-7 months/~11g (7-13 g), were obtained from the Tropical
102 Aquarium (TA) at the Institute of Aquaculture, University of Stirling, Scotland UK. Fish in
103 these facilities are maintained in recirculation systems with water at 28 °C +/- 2 °C, and

104 confirmed to be free of francisellosis by bacteriological and molecular methods prior to the
105 study as outlined by Ramirez-Paredes et al. (2017b).

106 **2.2 Vaccine preparation and stability**

107 For vaccine preparation, an overnight culture (~18 h) was prepared in 15 mL of broth using 5x
108 50 mL centrifuge tubes. The next day, the culture was centrifuged at 3500 x g for 20 min, the
109 bacterial pellet washed 3 times with sterile 1x phosphate buffered saline (PBS) and adjusted,
110 with PBS, to an OD₆₀₀ of 1.0 (2.6×10^9 CFU/mL).

111 For bacterial inactivation, the suspension was left mixing overnight at 4 °C in a sterile glass
112 vessel, slow stirring at 100 rpm with 0.5% formalin (Formaldehyde 40% w/v) (Sigma-Aldrich,
113 Dorset, UK). The formaldehyde was neutralised using 1/100 dilution of 15% sodium
114 metabisulphite. This solution was then centrifuged, washed 3 times with PBS and readjusted
115 to an OD₆₀₀ of 1.0. Inactivation of the bacteria and sterility was confirmed by inoculating a
116 subsample of the suspension onto CHAH and incubating at 28 °C for 7 days.

117 For emulsification, the formalin killed bacterial suspension was mixed with a commercial oil
118 adjuvant Montanide™ ISA 763A VG (SEPPIC, Puteaux Cedex, France), following the
119 manufacturer's guidelines for water-in-oil (W/O) emulsion in a 30/70 distribution of the
120 continuous and dispersed phases (30% antigen to 70% adjuvant). Briefly, the emulsion was
121 homogenised for 5 min with a hand blender as follows: 4000 rpm for the first 3 min, 4500 rpm
122 for 30 s, 4000 rpm for 1 min and 4500 rpm for 30 s. The initial concentration of the inactivated
123 bacteria (2.6×10^9 CFU/mL) was reduced with the emulsification to obtain a final concentration
124 in the vaccine of 1.3×10^9 CFU/mL, to provide a dose of 1.3×10^8 CFU/fish when 0.1 mL of
125 the vaccine was administered a normal dose.

126 The final volumes to produce the vaccine were 100 mL of bacteria in PBS, 500 μ L of 40%
127 formalin and 1 mL of 15% sodium metabisulphite. The total volume of formalin-killed bacteria
128 obtained was 60 mL of which 33 mL were added to 77 mL of adjuvant to obtain a final vaccine
129 volume of 110 mL (Supplementary File 1).

130 As indicated by the adjuvant manufacturer and Aucouturier et al. (2001), the vaccine emulsion
131 was stored at 4 °C and its stability checked after 1, 15 and 60 days post preparation by
132 performing a visual inspection of the emulsion. A droplet test was also performed to confirm
133 that a water-in-oil emulsification had been successfully prepared by dropping 20 μ L of the
134 emulsion into a beaker containing bi-distilled water and confirming the droplet retained its
135 shape. The conductivity of the emulsion was tested using an electrical conductivity meter
136 (Cole-Parmer, England, UK) in a 50 mL tube at 4 °C. The droplet size was observed under
137 light microscope at 400X by placing a drop of vaccine on a slide glass with a cover slip without
138 crushing the preparation. Finally, the syringeability in and out of fish was measured using a 1
139 mL disposable insulin syringe with an integrated 30G x 8 mm needle and with stainless steel
140 removable needles of different gauges i.e. 22G x 7 mm and 21G x 8 mm (Aqualife Services
141 Ltd., Stirling, UK).

142 **2.3 Vaccine safety test**

143 To rule out possible side effects during the trial, thirty fish were intraperitoneally (i.p.) injected
144 with twice the normal dose, i.e. 0.2 mL of the vaccine and kept at 28 °C in the TA for 30 days.
145 Fish were anaesthetised with a dose of 100 mg/L of Tricaine Pharmaq 1000 mg/g (TPQ)
146 (Pharmaq, Hampshire, UK) prior to the injection. After injection, the fish were checked twice
147 a day during the first week examining them for acute side effects, such as changes in behaviour,
148 lesions around the injection zone, sudden mortalities due to toxicity or other signs that could
149 be related to the formulation of the vaccine. Fish were euthanized after ~840 degree days (DD)

150 (30 days at 28 °C) and necropsied to examine them for signs of chronic side effects such as
151 internal lesions or adhesions.

152 **2.4 Vaccination**

153 A total of 468 red Nile tilapia were used in this experiment. The fish were equally distributed
154 into 9 experimental tanks i.e. 3 treatment groups with 3 replicate tanks per group and 52 fish
155 per tank. The treatments groups included (1) vaccinated fish, (2) fish injected with 1x sterile
156 PBS and (3) fish injected with adjuvant only i.e. emulsified with 1x PBS (Table 1). The
157 vaccinated and control groups were used to investigate the efficacy of the vaccine, while the
158 adjuvant-only group was included to investigate possible immunostimulatory effects of the
159 Montanide™ ISA 763A VG in the tilapia and if such stimuli could result in protection.

160 All the fish were i.p. injected with 0.1 mL of their respective treatment i.e. vaccine, adjuvant
161 or PBS using stainless hypodermic needles 22G x 7 mm and 21G x 8 mm (Aqualife Services
162 Ltd., Stirling, UK). Prior to injection, fish were anaesthetised with a dose of 100 mg/L of TPQ.
163 All fish were maintained in the TA at 28 °C for 30 days (~840 DD) for development of
164 immunity. During this period, fish were fed twice at a daily rate of 2% biomass and sampled
165 at four time points during the trial for serology. Twenty fish per replicate tank (60 per
166 treatment) were used for the experimental challenge to evaluate vaccine efficacy.

167 **2.5 Experimental infections**

168 A pre-challenge was performed to confirm the dose that resulted in 60% mortality (LD₆₀) in a
169 group of naïve fish, housed in similar conditions to those in the vaccine study. This was carried
170 out by injecting 20 fish with 2.4×10^4 CFU/fish in a 21 days trial. The dose was selected based
171 on previous studies (Ramirez-Paredes 2015; Soto et al., 2009). For the main challenge
172 experiment 20 fish in replicate tanks (n=60 fish per treatment) were infected with a single dose

173 of 4.0×10^3 CFU/fish live bacteria at 31 days post vaccination (d.p.v.) (Table 1 and
174 Supplementary File 2).

175 The pre-challenge and main challenge were performed following the methodology previously
176 described (Ramirez-Paredes et al., 2017b). Briefly, fish were moved from the TA into a flow-
177 through system at the Aquatic Research Facility (ARF), where the water was maintained at 23
178 ± 2 °C to replicate the natural environmental conditions at which the disease occurs.

179 Prior to performing the challenges, fish were acclimated for 10 days and fed twice daily at a
180 rate of 2% biomass. For the infection process, fish were anaesthetised with a dose of 100 mg/L
181 of TPQ and i.p. injected with the dose of bacteria as stated above. During the course of the
182 infection the numbers of mortalities, as opposed to moribund and near-moribund fish, were
183 kept to a minimum with continuous observations. The following criteria were considered as
184 humane endpoints of infection, based on whether the fish were moribund or near moribund and
185 the clinical signs they presented with one of the following severe signs: poor or no response to
186 stimuli (slow or unable to swim off when touched with net), bilateral exophthalmia, total loss
187 of equilibrium or total loss of buoyancy, or three of the following signs: unilateral
188 exophthalmia, emaciation, hypo/hyperventilation, oedema, irregular swimming or tank
189 placement, partial loss of balance, marked darkening of skin, lesions, haemorrhaging or natural
190 concurrent infection.

191 Diseased fish classed as moribund or near moribund (humane endpoint) were euthanised by
192 Schedule 1 method (S1-M) i.e. with an overdose of TPQ followed by confirmation of death by
193 brain destruction immediately after detection. All the euthanised fish were recorded throughout
194 the experiments and accounted for posterior mortality and survival statistical analyses.

195 To confirm specific mortalities, all moribund fish were necropsied, checked for gross
196 pathology and sampled for bacteriology and histopathology as previously described (Ramirez-

197 Paredes et al., 2017b). The experiment was concluded when there was a period of at least five
198 days with no mortalities. At the end of the trial all surviving fish were euthanized by S1-M and
199 blood sampled to measure specific antibody levels in the serum by ELISA. Additionally the
200 spleen of all survivors was collected and preserved in 95% ethanol for later bacterial
201 quantification in the spleen by qPCR.

202 **2.6 ELISA development for specific IgM detection in tilapia serum**

203 An indirect enzyme linked immunosorbent assay (ELISA) was developed to measure the level
204 of specific anti *Francisella noatunensis* subsp. *orientalis* IgM in the serum of fish. This was
205 used to monitor the antibody kinetics throughout the immunisation period and at the end of the
206 challenge in the survivors. For this, eight fish per replicate tank were euthanised by S1-M and
207 bled from the caudal vein at 4, 9, 15 and 30 d.p.v. Additionally, all surviving fish were
208 euthanised and sampled at 40 days post challenge (d.p.c.) (Table 1). Blood samples were stored
209 overnight at 4 °C and centrifuged at 3000 ×g for 5 min for serum collection which was
210 subsequently stored at -20 °C until analysis.

211 The ELISA was performed according to Adams *et al.*, (1995) with modifications. The 96-well
212 ELISA plates (Immulone®4 HBX-USA) were coated with 100 µL of 1% w/v poly-L-lysine in
213 carbonate–bicarbonate buffer and incubated for 60 min at room temperature (~21 °C). Plates
214 were then washed three times with a low salt wash buffer (LSWB) (0.02 mol/L Trizma base,
215 0.38 mol/L NaCl, 0.05% (v/v) Tween 20, pH 7.2). Plates were coated with a bacterial
216 suspension of isolate STIR-GUS-F2f7 re-suspended to an OD₆₀₀ of 0.4 (~1.0 x 10⁹ CFU/mL)
217 with PBS. This was then added to each well (100 µL/well) and the plates were incubated
218 overnight at 4 °C. The following day 50 µL of a 0.05% v/v solution of glutaraldehyde in LSBW
219 was added to the plates and these were incubated at 21 °C for 20 min to fix the bacteria to the
220 plate before washing the ELISA plates three times with LSBW.

221 Non-specific binding was prevented by first adding 100 μ L/well of 1/10 dilution of a 30% stock
222 solution of hydrogen peroxide (Sigma-Aldrich, Dorset, UK) and incubating for 1h at room
223 temperature. The plates were washed three times as before and further blocking was performed
224 by incubating them after adding 250 μ L/well of 5% w/v marvel (Premier Foods Group Ltd,
225 England UK) in distilled water for 3 h at room temperature. Thereafter the plates were washed
226 three times with 1x LSBW, and 100 μ L/well of serum from 5 fish from the 3 different
227 vaccinated groups (4, 9 and 30 dpv) and challenge survivors were added to the plates at a 1/500
228 dilution in LSBW containing 1 % bovine serum albumin (BSA, Fisher scientific). Both positive
229 (vaccinated and challenged fish serum) and negative (naïve fish serum) were used in each plate
230 using the same dilution as tested sera. The plates were then incubated overnight at 4 °C and
231 after the incubation, plates were washed five times with high salt wash buffer (HSWB) (0.02
232 mol/L Trizma base, 0.5 mol/L NaCl, 0.01% (v/v) Tween 20, pH 7.4) and left to soak for 5 min
233 on the last wash. An anti-tilapia IgM monoclonal antibody (Aquatic Diagnostics Ltd, Stirling,
234 UK) was added (100 μ L/well) and the plates were incubated at room temperature for 60 min.
235 The plates were again washed using 1x HSWB before adding 100 μ L/well of goat anti-mouse
236 immunoglobulin-G, conjugated with horseradish peroxidase (Sigma-Aldrich, Dorset, UK)
237 diluted 1/3000 in LSBW with 1% BSA. Plates were incubated for 60 min at room temperature
238 and then washed again with HSWB as previously described. Substrate/chromogen (15 mL of
239 substrate buffer [5.25 g citric acid, 2.05 g of sodium acetate, distilled water up to 15 mL, pH
240 5.4] containing 5 μ L of hydrogen peroxide (Fisher) and 150 μ L of trimethyl-benzidine (TMB)
241 di-hydrochloride (Sigma-Aldrich, Dorset, UK) was added to the plates, which were incubated
242 for 5 min at room temperature. The reaction was terminated with the addition of 5 μ L/well of
243 2M H₂SO₄ and the absorbance measured at OD₄₅₀ using a 96-well plate spectrophotometer
244 (Biotek Instruments, Friedrichshall, Germany).

245 **2.7 Determination of bacterial load by quantitative PCR (qPCR)**

246 The bacterial load in the spleen of surviving fish was determined at the end of the challenge
247 experiment using a qPCR protocol previously described by Duodu et al. (2012). For this, 10 of
248 the spleens previously preserved in 95% ethanol were randomly selected from each treatment
249 for genomic DNA extraction. The DNA was extracted from 20 mg of the fixed spleen samples
250 using the DNeasy blood and tissue kit (Qiagen, UK). The concentration of the extracted DNA
251 was measured using a nanodrop ND-1000 Spectrophotometer (Thermo Scientific, UK) and
252 standardised to 100 ng/ μ L with Milli-Q water (Thermo Scientific, UK). One μ L of each DNA
253 sample was visualised in a UV illuminator (Bio Imaging, Syngene) after electrophoresis on a
254 1% (w/v) agarose gel (Sigma-Aldrich, Dorset, UK) containing 0.1 μ g/mL ethidium bromide
255 (Sigma-Aldrich, Dorset, UK) in Tris-EDTA (TAE) buffer. The assay was performed in a
256 LightCycler[®] 2.0 (ROCHE) using a 20 μ L reaction volume consisting of 0.3 μ M from each
257 primer (Eurofins Genomics, UK), 1x Luminaris color HiGreen[™] qPCR master mix
258 (ThermoScientific), 1 μ L DNA template and nuclease free water (ThermoScientific) up to 20
259 μ L. The PCR cycling conditions were 50 °C for 2 min for uracil-DNA glycosylase enzyme
260 activity, 95 °C for 10 min to start denaturing the UNG enzyme and activate the DNA
261 polymerase enzyme then 45 cycles at 95 °C for 15 s and 60 °C for 1 min. Melting curve analysis
262 was performed with 1 cycle at 95 °C for 30 s, 55 °C for 30 s and 95 °C for 30 s. All samples
263 were run in triplicate. After the run, analysis was performed using the default calculation of the
264 quantification cycles (Cq values).

265 **2.8 Statistical analysis**

266 The vaccine efficacy was estimated by calculating the relative percent survival (RPS). This
267 value indicates the proportional relationship between mortality in the vaccinated group and the
268 unvaccinated group according to Amend (1981) using the following equation:

269
$$RPS = \left[1 - \left(\frac{\% \text{ mortality in vaccinated fish}}{\% \text{ mortality in non-vaccinated fish}} \right) \right] \times 100\%$$

270 The RPS of the adjuvant-only group was also calculated as a comparison. Differences in
271 survival were determined using the product limit method of Kaplan and Meier and the Log-
272 rank (Mantel–Cox) test was used to compare survival curves. The specific antibody levels in
273 the 3 different treatments, at the different time points were analysed by one-way ANOVA
274 followed by Welch's test. The bacterial loads quantified by qPCR were also analysed with a
275 one way ANOVA, paired comparisons between vaccinated and unvaccinated fish were
276 analysed using a Tukey's test. In all cases, a p-value of < 0.05 was considered significant. All
277 statistical analyses were carried out using the GraphPad Prism 8.02 software package
278 (GraphPad Software Inc., San Diego, CA, USA).

279 **2.9 Ethics**

280 The vaccination, infection and associated procedures were performed in accordance with the
281 UK Animal (Scientific Procedures) Act 1986 and the University of Stirling Animal Welfare
282 and Ethical Review Body (AWERB) regulations. All the protocols were approved by the
283 University of Stirling AWERB.

284 **3 Results**

285 **3.1 Vaccine stability**

286 An appropriate water-in-oil emulsion was observed in the drop test, with a conductivity of
287 30 $\mu\text{s}/\text{cm}$. The microscopic particles observed were 1 μm in size and were homogenously
288 distributed in the continuous and dispersed phase of the mixture, showing a dense liquid
289 compatible with the 70/30 emulsion. No separation of the liquid phases was observed when
290 examined 24 h after emulsification, indicating that the vaccine was suitably stable for injection
291 into the fish. The results of these tests when performed at 15, 30 and 60 days post-preparation

292 confirmed that the emulsion remained stable during this time. During the syringeability test,
293 the needles in the insulin syringes were not successful administering the vaccine as due to the
294 viscosity of the final product the process was slow, complicated and some of the syringes broke
295 while injecting the fish. For this reason hypodermic needles 22G x 7 mm and 21G x 8mm were
296 used to vaccinate the fish (Supplementary File 3).

297 **3.2 Vaccine safety**

298 The 30 fish injected with the double dose of vaccine remained healthy, with no acute side
299 effects evident, such as changes in behaviour i.e. lethargy, lack of feeding, aggression, gaping,
300 flashing etc. No adhesions between mesentery and the internal wall of the peritoneal cavity or
301 between sections of the intestines or the intestines and other organs were seen and therefore
302 the fish were classified as “0” according to the Speilberg scoring system (Midtlyng et al., 1996).
303 All fish had a droplet of the vaccine in the peritoneal cavity, always located dorsal, caudal and
304 lateral to the right (below the swim-bladder, posterior section of the peritoneal cavity). Smaller
305 vaccines droplets could also be observed distributed randomly within the peritoneal cavity in
306 about 30% of the fish. The droplets were contained in a smooth soft transparent sac with
307 vascularisation. Although 12 fish (40%) had dark pigments within the peritoneal cavity and
308 mesenteric fat, these were not linked to the vaccine as peritoneal pigmentation is a common
309 feature of tilapia. As expected, during the immunisation period (~840 DD) all fish vaccinated
310 with the normal dose remained healthy and no mortalities or signs of acute toxicity or chronic
311 side effects were seen.

312 **3.3 Bacterial infection**

313 Administration of 2.4×10^4 CFU/fish produced a cumulative mortality of 90% by 21 d.p.c. in
314 the pre-challenge test. On the basis of this result, it was decided to challenge the vaccinated

315 fish with a log₁₀ lower dose to test the efficacy of the vaccine, with the actual dose used
316 determined to be 4.0 x 10³ CFU/fish based on colony counts.

317 When moribund fish were dissected, granulomas were observed in their spleens and kidneys,
318 with varying degrees of severity (Figure 1). Some spleens were larger than normal, with dark
319 red coloration and white nodules in 80-90% of the parenchyma, others were bright red with 90-
320 100% granulomas and some others showed splenomegaly with large nodules and a white
321 membranous lining of the capsule of the spleen, which extended over the majority of the
322 peritoneal cavity. Head kidneys appeared enlarged, haemorrhagic and full of granulomas, and
323 in some cases the organ protruding ventrally towards the anterior section of the peritoneal
324 cavity, in contact with the spleen, liver, and gut sections. Other organs such as the gonads, gut
325 and posterior kidney were also affected. Bacterial recovery was achieved from 100% of the
326 moribund fish sampled.

327 Histopathological sections of the affected organs showed typical granuloma lesions. The
328 bacteria were seen frequently contained within enlarged macrophages or found covered with
329 fibrin and enclosed by accumulation of phagocytic cells, that outlined by inflammatory cells
330 and fibroblasts. Concomitant mononuclear infiltration and increased vascularisation were also
331 noted around the in the affected areas (Figure 2).

332 **3.4 Vaccine efficacy**

333 A significant level of protection against the bacterial challenge was obtained for the vaccinated
334 group compared to the control and adjuvant groups ($p < 0.0001$). (Figure 3). However, no
335 significant difference was seen between adjuvant and control treatment (Table 2). The
336 mortalities in the control group started by 8 d.p.c. and reached an average of $63.3 \pm 2.9\%$ by
337 34 d.p.c. The mortalities in the adjuvant group started at 13 d.p.c. and reached an average of
338 $36.6 \pm 18.9\%$ by 32 d.p.c. All the fish in the vaccinated group survived until the end of the

339 experiment by 40 d.p.c. The RPS value at the end of the experiment was 100% for the
340 vaccinated group and 42% for the adjuvant group. The mortality data is presented in
341 Supplementary File 4.

342 **3.5 Kinetics of specific IgM response of vaccinated fish**

343 The results of the ELISA confirmed that there was no significant difference in the levels of
344 specific anti *Francisella noatunensis* subsp. *orientalis* IgM between vaccinated, adjuvant and
345 control groups in the first 15 d.p.v. However, the vaccinated fish started to produce
346 significantly higher levels of specific IgM by 30 d.p.v. ($p < 0.001$) compared to the other two
347 groups (Figure 4). No specific antibody response was seen in serum sampled from the adjuvant
348 or control groups prior to challenge. All the survivors showed increased antibody production
349 after the infection with the vaccinated fish displaying the highest titres (Figure 4).

350 **3.6 Quantitative PCR (qPCR) for estimation of bacteria load in the spleens of infected** 351 **fish**

352 Molecular quantification of bacterial loads in the spleen of survivors revealed significantly
353 lower *Francisella noatunensis* subsp. *orientalis* loads i.e. pathogen genome copies, in the
354 vaccinated than in the adjuvant-only ($p < 0.05$) and PBS-injected ($p < 0.001$) fish. The fish
355 immunised only with the adjuvant displayed significantly lower loads than the control fish
356 ($p < 0.05$) (Figure 5).

357 **4 Discussion**

358 Francisellosis remains one of the most important infectious diseases in aquaculture for which
359 no licenced vaccines are available. Autogenous vaccines are custom made formulations that
360 have the potential to be rapidly developed and deployed when no off-the-shelf fully licensed
361 vaccines exist. In principle autogenous vaccines must be inactivated (killed) and derived from

362 pathogens isolated directly from the sites where they will be delivered i.e. have the capacity to
363 confer immunity against homologous challenges (Haskell et al, 2004).

364 Under these circumstances, the formulation of autogenous vaccines is a logical scenario but as
365 yet, remains as an unexplored option to control francisellosis in tilapia. The present study was
366 therefore carried out using a whole cell inactivated vaccine against *Francisella noatunensis*
367 subsp. *orientalis*, which had an excellent efficacy in red Nile tilapia using a homologous isolate
368 to assess its efficacy.

369 The vaccine here developed was found to be safe with adequate bacterial inactivation achieved,
370 residual formaldehyde neutralisation and absence of acute or chronic side effects to the fish
371 during the safety test. Although the safety test was run for 30 days, the first 7 days are
372 considered the most critical for this assessment.

373 The vaccine was also considered stable as the use of a high speed mixer resulted in an efficient
374 water-in-oil 70/30 (70% adjuvant / 30% antigen) emulsion that was easy to administer using
375 stainless steel 21-22 gauge needles. In the emulsion, the antigenic component was the primary
376 inducer of the specific adaptive immune response while the adjuvant enhanced and extended
377 such response.

378 According to Tafalla et al. (2014) such role of the adjuvants is achieved in part by acting as a
379 delivery vehicles for the antigens and also by stimulating immune system through inherent
380 immunostimulatory properties. Although the exact mechanisms of how adjuvants work are
381 mostly unknown, according to Cox & Coulter (1997) five modes of action have been
382 recognised: (1) Immunomodulation: the ability to modify the cytokine network. (2)
383 Presentation: the ability to preserve the conformational integrity of an antigen and to present
384 the antigen to appropriate immune effector cells. (3) Cytotoxic T-lymphocyte (CTL) induction:
385 induction of CD8+ CTL responses. (4) Targeting: the ability to deliver an immunogen to

386 immune effector cells, generally via antigen presentation cells. (5) Depot generation:
387 generation of a short-term or long-term depot to give a continuous or pulsed release.

388 In the present study, fish in the adjuvant-only group had a better survival rate (RPS 42%) than
389 the control fish, making clearly evident that the commercial adjuvant Montanide™ ISA 763 A
390 VG acted as an immunostimulant for Nile tilapia. It is thought that this could have been caused
391 by immunomodulation, CTL induction and/or depot generation mode of actions as no specific
392 antibody production was seen in this group at any point after vaccination (Cox & Coulter, 1997;
393 Tafalla et al., 2014). The immunostimulation properties of the adjuvant allowed the fish to
394 control bacterial replication to some degree and this was reflected in the qPCR results of the
395 only adjuvant group where significantly lower bacterial loads than in the control fish were seen.

396 If the immunostimulation properties were seen in the only adjuvant group it can be assumed
397 that these properties were also present in the vaccine group. However further research
398 comparing the expression of cell mediated immune genes in adjuvant, vaccinated and control
399 groups is needed to clarify if these mechanisms of immunity are stimulated by the adjuvant
400 ISA 763A VG and to better understand the interactions between this adjuvant and the tilapia
401 immune system.

402 As discussed by Munang'andu & Evensen (2015) the type of adaptive immune response is
403 highly influenced by the site of antigen uptake i.e. antigens deposited extracellularly primarily
404 evoke humoral immune responses, while antigens deposited intracellularly evoke both humoral
405 and cellular mediated immune responses. It is believed that in this study the external aqueous
406 phase of the water-in-oil emulsion secured a correct preservation and incorporation of the killed
407 antigen (antigen delivery role) into the intracellular space of the immune effector cells (local
408 antigen presenting cells, CD8+, CD4+, etc.) and this resulted in a gradually increasing and

409 continuous release of specific IgM (Aucouturier et al., 2001; Cox & Coulter, 1997; Tafalla et
410 al. 2014).

411 It is possible that such a strong humoral immune response controlled the extracellular stage of
412 the bacterial infection especially during the early stages of the infection presumably preventing
413 entry and replication of live pathogenic bacteria into the immune cells of the fish (Soto et al.,
414 2010). Most importantly although some individual variation was seen amongst the vaccinated
415 fish, the average humoral response reached a signature of protective immunity as defined by
416 Pulendran et al. (2010) by day 25 p.v. that fell well within the concept of herd immunity
417 (Anderson and May, 1985; Gudding, 2014). Further research is needed to understand the exact
418 mechanisms that induced the potent and rapid humoral response seen in the vaccinated fish and
419 if the vaccine is also able to stimulate cellular immune response.

420 Previous reports of *Francisella* infections in humans have reported development of immunity
421 in naturally infected individuals (Koskela & Salminen, 1986). Interestingly the antibody titres
422 in the survivors of the control group was above the threshold of protection confirming that this
423 group of fish had already developed humoral immunity to *Francisella noatunensis* subsp.
424 *orientalis* infection. It is possible that this immunity had potentiated the immunity conferred
425 by the vaccine and adjuvant. These observations correlated with the bacterial loads seen in
426 spleen determined from the qPCR in all the treatments. Whether the survivors from these
427 groups would have eventually eliminated the infection or became carriers or vertical/horizontal
428 transmitters stills remains unknown.

429 The correlation between the RPS values, differences in the survival rate, antibody titres and
430 bacterial loads observed in this study confirmed that the vaccine developed here was 100%
431 effective in protecting red Nile tilapia from the bacterial challenge administered by i.p.
432 injection. These results provide a strong support that this approach could be further explored

433 at a commercial level to develop autogenous vaccines as a solution to control francisellosis in
434 farms or areas where the problem is endemic.

435 Further research is needed to investigate the efficacy of this vaccine in scenarios such as higher
436 challenge doses and other infection routes i.e. immersion and cohabitation. Moreover, this
437 approach could also be used to explore the possibility of cross protection between isolates from
438 different origins, as previous studies have revealed high genetic homogeneity at genome level
439 between isolates obtained from different countries, fish species, and over time (Gonçalves et
440 al., 2016; Ramirez-Paredes 2015; Ramirez-Paredes et al., 2017a; Sjödin et al., 2012).

441 **5 Conclusion**

442 The whole cell inactivated vaccine developed in this study provided excellent results,
443 protecting red Nile tilapia against experimentally induced francisellosis (RPS = 100%) using
444 homologous isolate. The protection correlated with systemic IgM responses and bacterial loads
445 as confirmed by ELISA and qPCR, respectively.

446 **6 Conflict of interest**

447 The authors declare that the research was conducted in the absence of any commercial or
448 financial relationships that could be construed as a potential conflict of interest

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Table 1. Experimental design of vaccination trial and sampling point for correlates of protection.

Feature	Vaccine	Adjuvant	Control
Immunisation (No. fish/ replicate)	52 x 3	52 x 3	52 x 3
Inoculum (100 µl via i.p. injection)	1.3x10 ⁹ CFU/ml + Adjuvant	70% Adjuvant + 30% PBS	PBS
Sampling points for serology (d.p.v.)	4, 9, 15 and 30	4, 9, 15 and 30	4, 9, 15 and 30
No. fish/ No. replicates	20 x 3	20 x 3	20 x 3
Challenge dose	10 ³ CFU/fish	10 ³ CFU/fish	10 ³ CFU/fish
Sampling for pathology, serology and qPCR	40 d.p.c.	40 d.p.c.	40 d.p.c.

d.p.c. = days post challenge.

Table 2. Survival analysis of different treatment groups showing results between treatments and the overall comparisons results.

Comparisons between treatments						Overall Comparisons			
Treatment 1	vs	Treatment 2	Log-rank Mantel (and Cox) test	df	significance	Are the survival curves significantly different?	Log-rank Mantel (and Cox) test	df	significance
vaccinated		control	38.6	5	<0.0001	yes	41.39	8	<0.0001
vaccinated		adjuvant	30.12	5	<0.0001	yes			
control		adjuvant	7.357	5	0.1954	no			

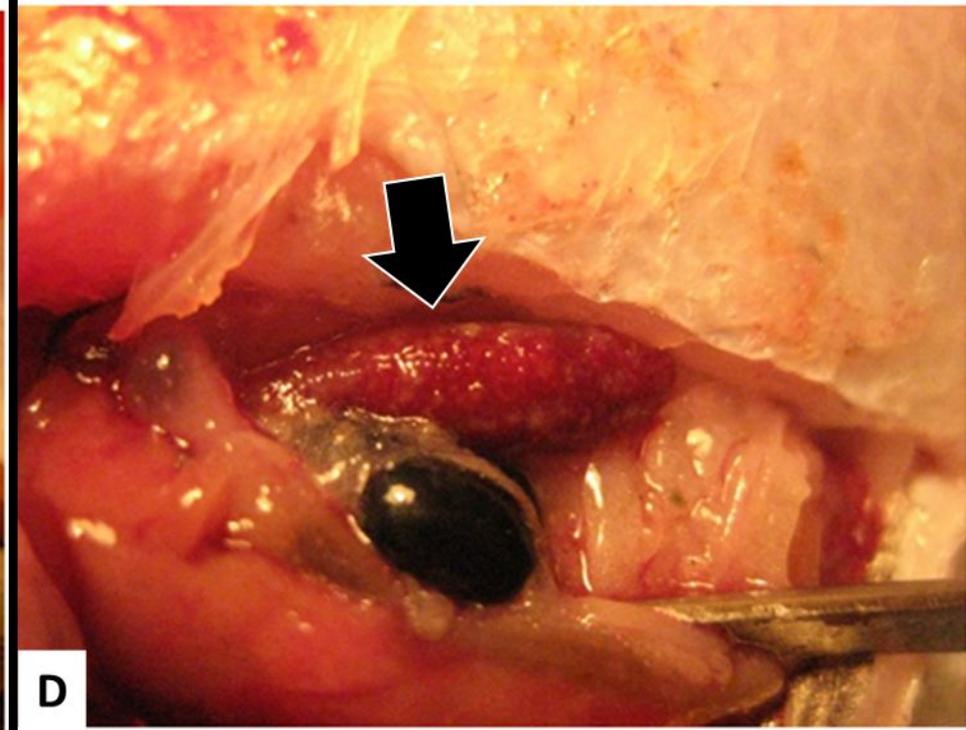
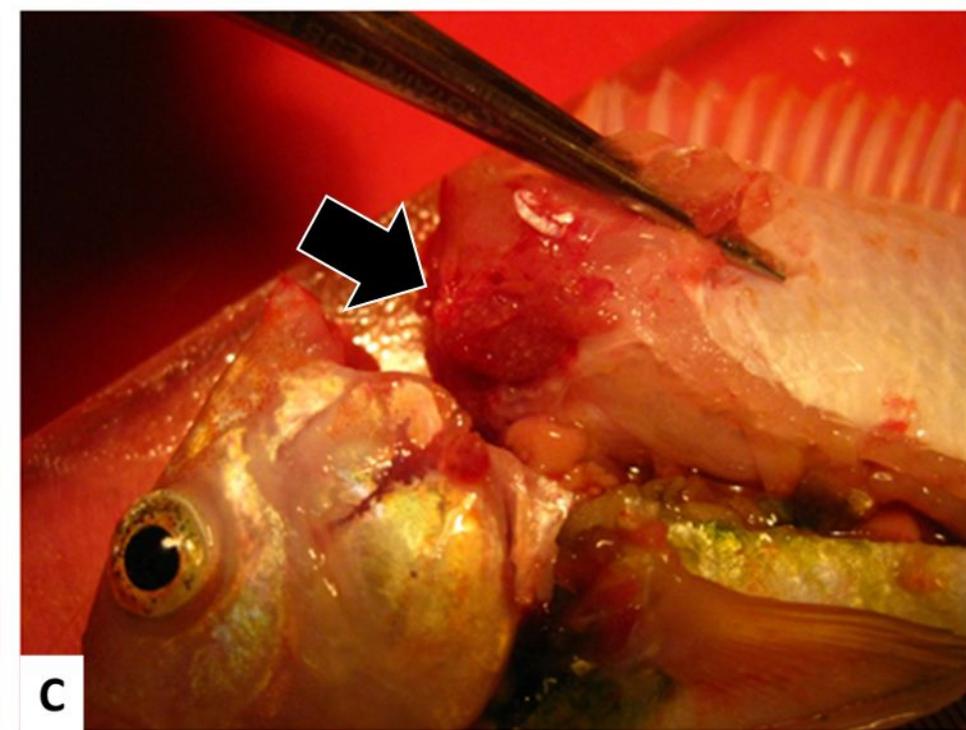
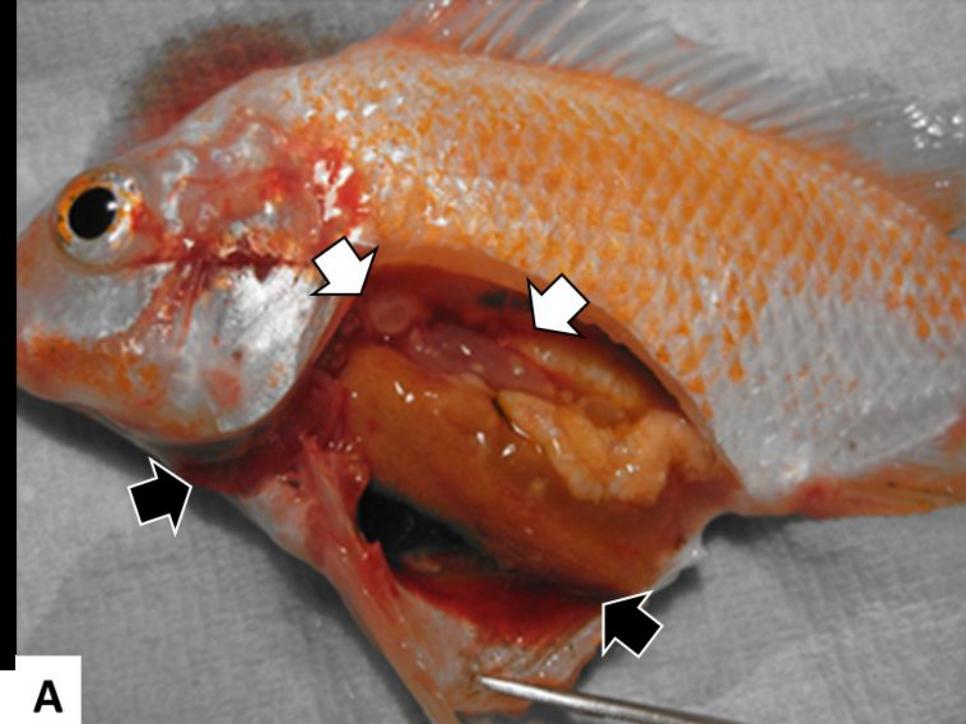
539 **Figure 1. Gross pathology of experimentally infected naïve red Nile tilapia showing varying degrees of affection. A.**
540 Necropsy of moribund fish displaying erratic swimming and ascites, the black arrows point at enlarged and haemorrhagic organs
541 including gills, wall of the peritoneal cavity and liver. The white arrows point at formation of white nodules in head kidney and
542 mesentery. **B.** Spleen from fish shown in A, the organ presents severe affection with white nodules covering 100% of the
543 parenchyma. **C.** Head kidney with moderate white nodule formation. **D.** Spleen from fish shown in C, the organs displays
544 moderate affection with white nodules in the parenchyma.

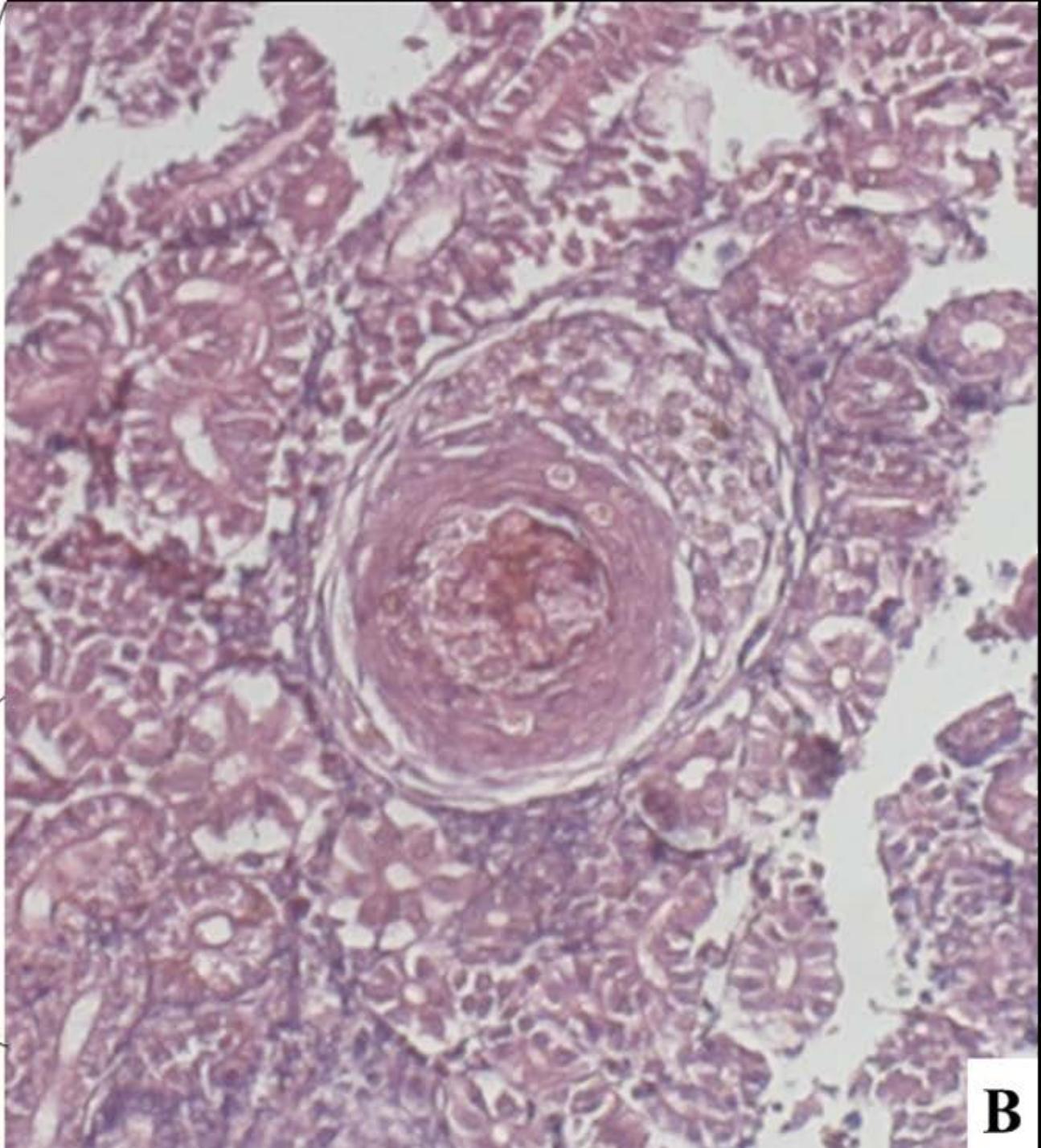
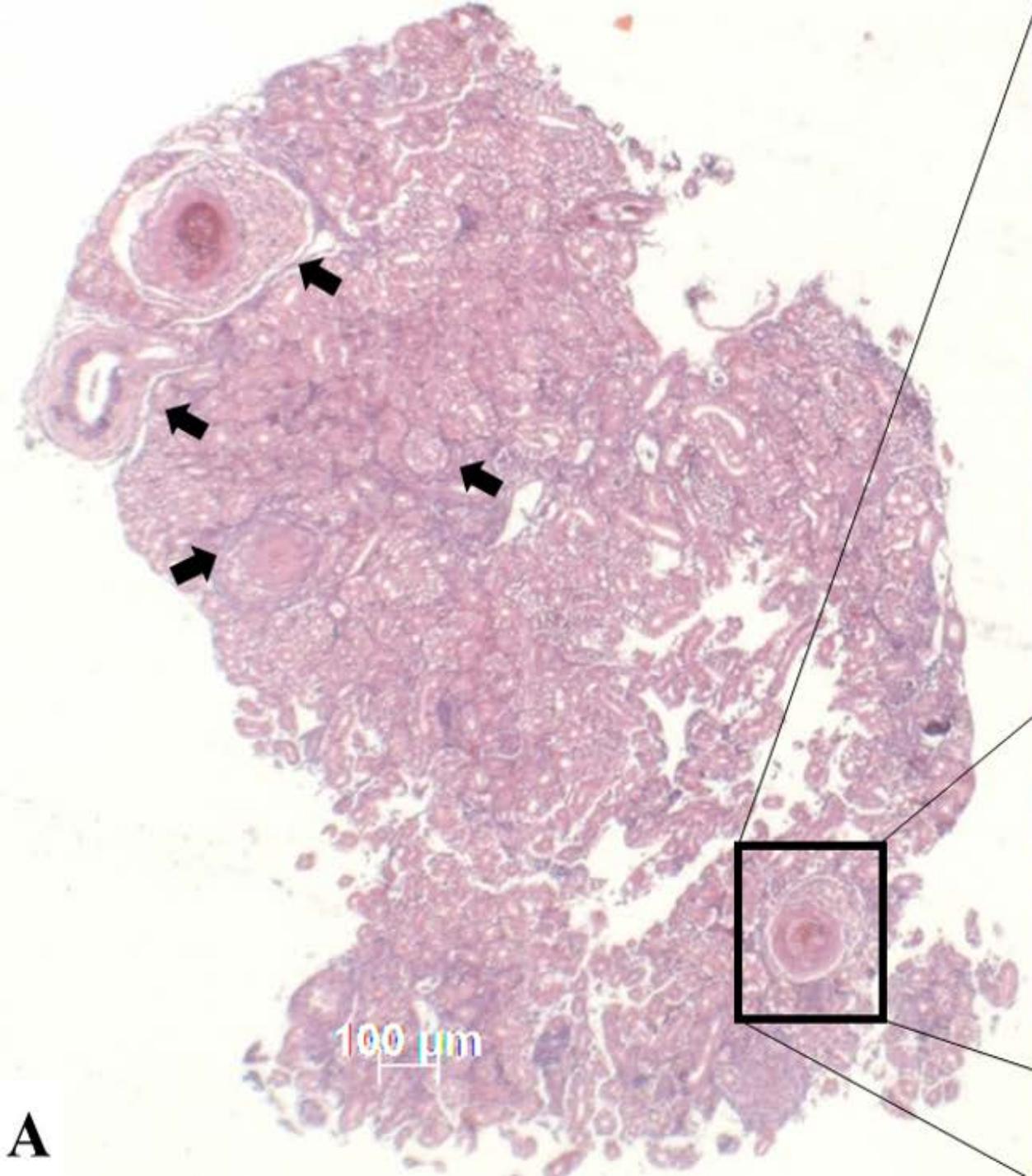
545 **Figure 2. Histological sections of the kidney of tilapia experimentally infected with francisellosis stained with H&E. A.**
546 The arrows indicate fully formed granulomas. Scale bar = 100 µm. **B.** Higher magnification 100x of one the granulomas with
547 necrotic centre, encased by macrophages, mononuclear leukocytes and fibroblasts.

548 **Figure 3. Cumulative percentage survival of red Nile tilapia during the main experimental challenge.** The three replicate
549 tanks of the three treatment groups (vaccinated, adjuvant alone and control) were experimentally infected with the homologous
550 strain STIR-GUS-F2f7 at 840 degree days post vaccination. Survival for each replicate tank is shown. Relative percent survival
551 (RPS) of the vaccinated group was 100% and RPS of the adjuvant only group was 42%.

552 **Figure 4. Serum antibody levels of vaccinated, adjuvant-injected and PBS control tilapia post vaccination and post**
553 **challenge.** Each bar represents average OD₄₅₀ values of 5 fish per treatment \pm SD. Horizontal dashed line represents cut-off
554 calculated from average of the background multiplied by 3. Vertical dashed line divides vaccinated fish at days 4, 9, 15 and 30
555 post vaccination from challenge survivors at day 40 post challenge. Asterisks indicates significant difference, ns= not significant
556 different ($p \geq 0.05$), * ($p \leq 0.05$), *** ($p \leq 0.0001$) d.p.v. = days post vaccination, d.p.c. = days post challenge.

557 **Figure 5. *Francisella noatunensis* subsp. *orientalis* loads on the spleen of vaccinated, adjuvant-injected and control group**
558 **survivors at day 40 post challenge.** Each bar represents average copy number of 10 spleen samples per treatment \pm SD.
559 Asterisks indicate significance difference * ($p \leq 0.05$), *** ($p \leq 0.0001$).





Vaccine efficacy assessment of *Francisella* autogenous vaccine in Nile tilapia

