1 # Efficacy of an inactivated whole-cell injection vaccine for Nile tilapia,

2 # *Oreochromis niloticus* (L), against multiple isolates of *Francisella noatunensis*

3 # *subsp. orientalis* from diverse geographical regions

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29 **Abstract**

30 Francisellosis, induced by *Francisella noatunensis* subsp. *orientalis* (*Fno*), is an emerging bacterial
31 disease representing a major threat to the global tilapia industry. There are no commercialised
32 vaccines presently available against francisellosis for use in farmed tilapia, and the only available
33 therapeutic practices used in the field are either the prolonged use of antibiotics or increasing water
34 temperature. Recently, an autogenous whole cell-adjuvanted injectable vaccine was developed that
35 gave 100% relative percent survival (RPS) in tilapia challenged with a homologous isolate of *Fno*. In
36 this study, we evaluated the efficacy of this vaccine against challenge with heterologous *Fno* isolates.
37 Healthy Nile tilapia, *Oreochromis niloticus* (~15g) were injected intraperitoneally (i.p.) with the
38 vaccine, adjuvant-alone or phosphate buffer saline (PBS) followed by an i.p. challenge with three *Fno*
39 isolates from geographically distinct locations. The vaccine provided significant protection in all
40 groups of vaccinated tilapia, with a significantly higher RPS of 82.3% obtained against homologous
41 challenge, compared to 69.8% and 65.9% with the heterologous challenges. Protection correlated with
42 significantly higher specific antibody responses, and western blot analysis demonstrated cross-isolate
43 antigenicity with fish sera post-vaccination and post-challenge. Moreover, a significantly lower
44 bacterial burden was detected by qPCR in conjunction with significantly greater expression of IgM,
45 IL-1 β, TNF-α and MHCII, 72 hours post-vaccination (hpv) in spleen samples from vaccinated tilapia
46 compared to fish injected with adjuvant-alone and PBS. The *Fno* vaccine described in this study may
47 provide a starting point for development a broad-spectrum highly protective vaccine against
48 francisellosis in tilapia.

49

50 **Keywords:** Inactivated vaccine, cross protection, immune response, *Francisella noatunensis* subsp.
51 *orientalis*.

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1. Introduction

Francisella noatunensis subsp. *orientalis* (*Fno*) is a serious emerging bacterial pathogen affecting a wide range of ornamental and farm-raised cichlids globally [1-7]. Due to its fastidious nature, high infectivity (~ 23 CFU can induce clinical disease), wide host range, various routes of transmission, capacity to survive in multiple environments and co-existence with other pathogens, it has been highlighted as one of the major threats to the tilapia aquaculture industry, with mortalities of > 90% reported [8]. In tilapia farms, several strategies have been adopted to control francisellosis. The conventional practice of increasing the water temperature from 25 to 30°C was previously reported to inhibit the development of francisellosis in infected tilapia and other susceptible ornamental fish [2,9]. Treating with approved antibiotics like oxytetracycline (Terramycin®) and florfenicol (Aquaflor®) for up to 10 days has also been reported to be effective [9-11]. Although the latter can potentially reduce fish mortality due to francisellosis [9], the use of antibiotics is not ideal as infected fish usually suffer from anorexia and there is a risk of the bacteria developing antibiotic resistance [12,13]. Currently no commercial prophylactic treatments are available for use against *Fno* in farmed fish. The broad emergence of *Fno* outbreaks globally has raised concerns of a potential francisellosis pandemic, thus efforts to develop protective vaccines against *Fno* have increased. Such vaccines should be safe, have a high level of efficacy, provide cross-protection, be cost effective and be easy to administer [14].

In a previous study, the highest Relative Percent Survival (RPS) obtained in a vaccination trial in tilapia was 87.5 % using a live attenuated immersion vaccine [15]. However, live attenuated vaccines are not easily registered in all countries due to concerns relating to safety. Recently, an autogenous injectable whole cell adjuvanted bacterin developed using a virulent *Fno* isolate obtained from diseased tilapia farmed in Europe [16]. Following intraperitoneal (i.p.) vaccination and challenge with the homologous vaccine isolate, this vaccine stimulated protective antibodies and resulted in a high level of protection (RPS of 100%) [16]; however, cross protection of this vaccine

79 against heterologous isolates is unknown. Previous studies examining *Fno* genetic diversity using
80 PCR-based typing or sequencing methods did not discriminate between *Fno* isolates from different
81 countries, thus demonstrating a clonal behavioral pattern among these isolates [4, 17-19]. Minor
82 antigenic differences between *Fno* isolates from distinct geographical regions have been highlighted
83 in a previous proteomic study [20]. The capacity of the vaccine for cross protection, therefore, should
84 be addressed. The aims of the current study were to investigate the efficacy of the recently developed
85 injectable vaccine [16] by i.p. challenge with multiple *Fno* isolates from diverse geographical origins
86 and evaluate the immune response to vaccination in Nile tilapia, *O. niloticus* (L).

87 **2. Materials and Methods**

88 ***2.1. Fish and rearing conditions***

89 Nile tilapia, *O. niloticus*, of mean weight 13 ± 0.8 g and an average length 10 ± 0.13 cm were obtained
90 from a commercial tilapia farm in central Thailand, and transported to the research aquarium of Fish
91 Vet Group Asia Ltd. (FVGAL), in Chonburi, Thailand. Upon arrival, the fish were transferred to 100
92 L tanks in a recirculation system within a temperature-controlled room supplied with dechlorinated
93 water, aerated with air stones and acclimated to their new conditions prior to the experiment. Water
94 quality was maintained as follows: temperature 28°C ± 1, dissolved oxygen (DO) 6.5-7 mg/L, pH 7-
95 7.5, free ammonia ≤0.1 mg/L, nitrite ≤0.25 mg/L, nitrate ≤0.2 mg/L. The photoperiod in the room was
96 maintained at 12 h light: 12 h dark with a 30 min transition. Fish were acclimated for 2 weeks and fed
97 at a rate of 3% body weight per day with a commercial tilapia feed (Charoen Pokphand Foods Public
98 Company Limited (CPF-PCL), Thailand). The *Fno*-free status of the tilapia was determined prior to
99 commencing the vaccination study using samples of spleen and head kidney from six fish.
100 Bacteriology analysis and a *Francisella* genus specific PCR targeting a partial sequence of the 16S

101 rRNA gene using primers listed in Table 1 were performed as previously described [2, 21]. Extraction
102 of DNA from head kidney and spleen samples was performed using DNeasy Blood and Tissues kit
103 (QIAGEN, Germany) following the manufacturer's instructions.

104 **Table 1.** Primers sequences used in the study

Target Gene	Oligo sequence (5′ - 3′)	Genbank accession no.	Product Size	Annealing temperature	Ref.
Francisella spp. *16S rRNA*	F11: TACCAGTTGGAAACGACTGT R5: CCTTTTTGAGTTTCGCTCC	NR_074666.1	1140 pb	50 °C	[21]
Hypothetical protein gene	F: CATGGGAAACAAATTCAAAAGGA R: GGAGAGATTTCTTTTTAGAGGAGCT	JQ780323.1	85 pb	60 °C	[22]
β-actin	F: CCACACAGTGCCCATACTACGA R: CCACGCTCTGTCAGGATCTTCA	XM_003443127	144 bp	60 °C	[23]
EF-1α	F: GCACGCTCTGCTGGCCTTT R: GCGCTCAATCTTCCATCCC	NM_001279647	250 bp	57 °C	[24]
IgM	F: GGGAAGATGAGGAAGGAAATGA R: GTTTTACCCCCCTGGTCCAT	KC708223	120 bp	57 °C	[24]
TNF-α	F: CTTCCCATAGACTCTGAGTAGCG R: GAGGCCAACAAAATCATCATCCC	NM_001279533	161 bp	60 °C	[23]
IL-1β	F: TGCACTGTCACTGACAGCCAA R: ATGTTCAGGTGCACTTTGCGG	XM_019365844	113 bp	57 °C	[23]
MHC-II	F: ACTGACTGGGACCCGTCCAT R: ACAGGAAGCAGCCGCTTTTA	XM_003459253	204 bp	57 °C	[25]

105 F: Forward primer, R: Reverse primer, bp: Base pair, Ref: Reference.

106 ***2.2. Bacteria and culture conditions***

107 Bacterial isolates used in this study included three virulent isolates of *Fno* collected from separate
108 francisellosis outbreaks in tilapia from three different geographical locations (Table 2). For challenge
109 experiment, bacteria were cultured in cysteine heart agar supplemented with 1% bovine hemoglobin
110 (CHAH) (Becton Dickenson BBL, USA), following previously published protocol [19]. A single
111 colony from the agar plate was inoculated into Modified Muller Hinton broth (MMHB) (Difco, USA)
112 containing 2% IsoVitaleX and 0.1% glucose as described by [2]. The *Fno* broth culture was incubated

113 for 18 h (mid log phase) at 160 rpm at 28°C followed by harvesting of the cells by centrifugation at
114 3000 *xg* for 5 min and pellets were resuspended in sterile PBS to the appropriate optical density at
115 OD_{600}. The colony-forming units (CFU) per mL was estimated using a 6 x 6 drop plate method,
116 following the published protocol [26] in conjugation with CHAH plates. Plates were incubated for 72
117 h at 28°C to obtain colony count.

118 **Table 2.** *Fno* isolates used in the challenge trial

Isolate ID	Designation	Source	Isolation year	Reference
Fno 1	AVU-STIR-GUS-F2f7	Red Nile tilapia (Europe)	2012	[19]
Fno 2	NVI-5409 *	Nile tilapia (Central America)	2006	**Unpublished**
Fno 3	AVU-Fran-Cos1	Blue tilapia (North America)	2013	**Unpublished**

119 AVU: Aquatic Vaccine Unit Bacterial Culture Collection, * Isolate supplied by Dr Duncan Colquhoun,
120 Norwegian Veterinary Institute (NVI), Oslo, Norway.

121 ***2.3. Fish vaccination and sampling***

122 Preparation of the inactivated-adjuvanted *Fno* vaccine was performed using the virulent *Fno* isolate
123 (*Fno* 1), (**Table 2**) as described in [16]. Following a two-week acclimation period, fish (15 ± 0.2 g) were
124 divided into four groups: a vaccination group (*n* = 260), an adjuvant-alone group (*n* = 260), a PBS
125 control group (*n* = 260) and a naïve group (*n* = 20). The fish were stocked in 100 L recirculation tanks
126 filled with chlorine-free water. The vaccination, adjuvant-alone and PBS control groups consisted of
127 duplicate tanks with 130 fish each, whilst the naïve group consisted of a single tank of 20 fish (**Figure**
128 **1**). Fish were starved for 24 h, anaesthetised with 10 % benzocaine in 100 % ethanol (Sigma, UK) and
129 i.p. injected with 0.1 mL of vaccine, adjuvant-alone (Montanide, Seppic, France) or sterile PBS. The
130 naïve group did not receive any treatments during the experiment. Fish were fed 3% of their body
131 weight /day 24 h after injection and water quality was monitored throughout the trial. Fish were
132 maintained at 28°C ± 1 for 30 days (840 degree days (dd)) and checked regularly for any

133 abnormalities. Prior to sampling, the fish were starved for 24 h and then euthanised with an overdose
134 of benzocaine at 6, 24 and 72 h post-vaccination (hpv) and the spleen sampled from three fish/tank (*n*
135 = 6) from each group. Tissue samples were stored in 1 mL of RNA later (Sigma, UK) at 4°C overnight,
136 then the RNA Later was removed, and tissues were stored at -80°C until use. Blood samples were
137 collected by lethal caudal vein puncture from five fish per tank at day zero (D_0) and 30 days post-
138 vaccination (30 dpv) to measure IgM levels post-vaccination by enzyme-linked immunosorbent assay
139 (ELISA). Blood was transferred to micro-centrifuge tubes, kept at 4°C overnight then centrifuged at
140 3000 × *g* for 10 min. Serum was collected and kept at -20°C until used.

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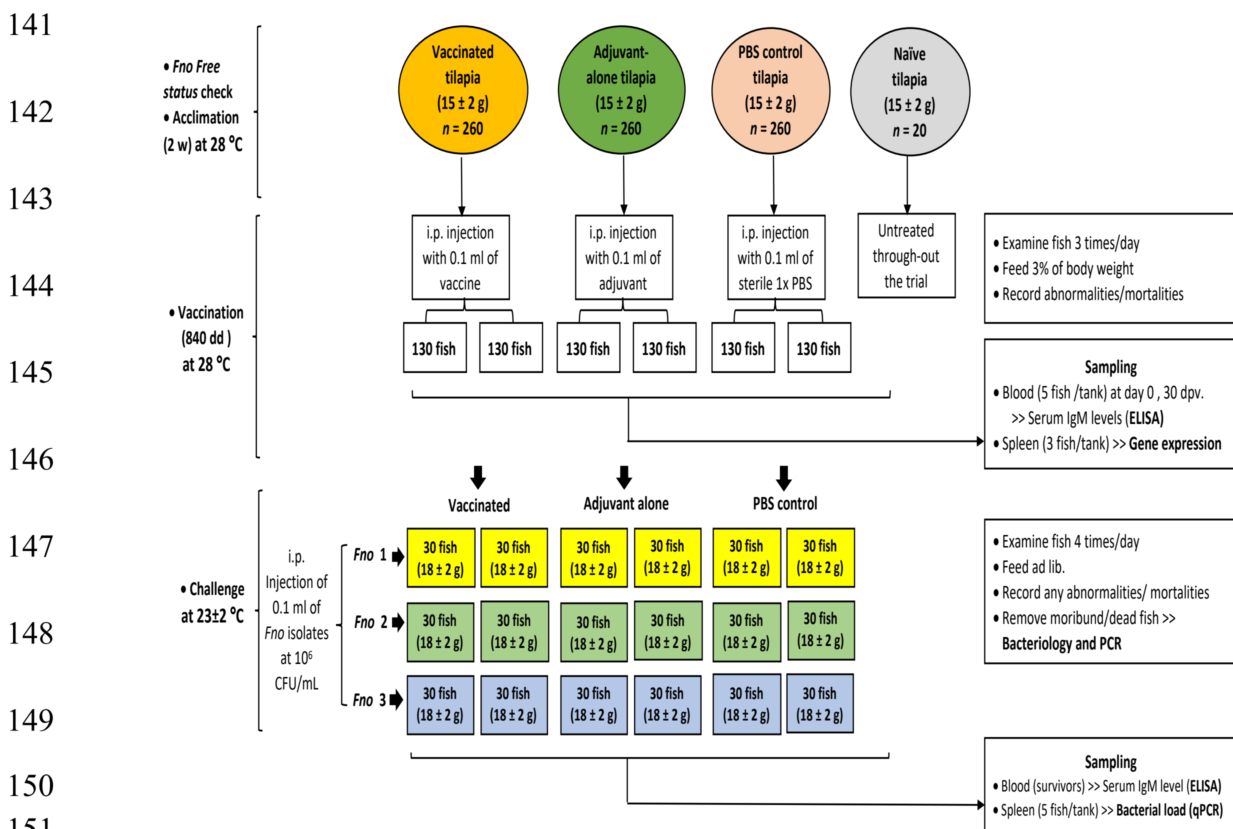

152 **Figure 1.** Experimental design of the *Francisella noatunensis* subsp. *orientalis* (*Fno*) vaccination trial
153 involving heterologous isolate challenge. dd: degree days, w: weeks, *n:* number of fish/group.

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156 *2.4. Fish challenge and sampling*

157 At 30 dpv (840 dd), fish in vaccinated, adjuvant-alone and PBS control groups (18 ± 0.5 g) were
158 anaesthetised with benzocaine as described previously and each group was divided into three sub-
159 groups. Each group consisted of duplicate 100 L recirculation tanks with an integral UV system
160 (TMC, UK) and 30 fish/tank (**Figure 1**). Each sub-group was i.p. injected with 0.1 mL of one of the
161 three *Fno* isolates. The isolates included one homologous isolate (the vaccine isolate; i.e. *Fno* 1) and
162 two heterologous isolates (*Fno* 2 and 3). The bacterial isolates were grown as described above. Doses
163 of *Fno* isolates used in the challenge experiment are shown in **Table 3**. These doses were determined
164 from a pre-challenge experiment (**data not shown**) and represent the bacterial concentration that
165 induced 70% mortality (LD_{70}) in the control fish. A sample of each bacterial inoculum was removed
166 at the time of challenge and the dose (CFU) for each isolate was determined using the drop plate
167 method **[25]**.

168 **Table 3.** Calculated dose of *Fno* isolates used in the challenge trial post-vaccination

Isolates **	CFU/mL	CFU/fish
Fno 1	1.12 × 10^6 CFU/mL	1.12 × 10^5 CFU/mL
Fno 2	1.23 × 10^6 CFU/mL	1.23 × 10^5 CFU/mL
Fno 3	1.28 × 10^6 CFU/mL	1.28 × 10^5 CFU/mL

169 ** Optical density (OD_{600}) set at 0.1 for all isolates.

170 Fish were maintained for 15 days at 23 ± 2°C, examined four times per day and water quality was
171 monitored. Fish received feed *ad libitum*, mortalities were removed, moribund fish were sampled,
172 and occurrence of the disease was confirmed by bacteriology and PCR. The surviving fish at 15 days
173 post challenge (15 dpc) were euthanised and blood was sampled for serum, and the relative percent
174 survival (RPS) was calculated according to **[27]**.

175 ***2.5. Enzyme-linked immunosorbent assay (ELISA)***

176 An indirect ELISA was performed to assess the specific IgM levels in serum of vaccinated, adjuvant-
177 alone and PBS control tilapia sera at day zero (D_0), 30 dpv and 15 dpc with the three different *Fno*
178 isolates according to [28].

179

180 ***2.6. Immunoblotting***

181 The whole cell proteins of the three *Fno* isolates were resolved on 1D SDS-PAGE and a 1D western
182 blot was performed as described [28]. Serum samples collected from fish at 30 dpv and 15 dpc from
183 the different treatments were used to perform the immunoblotting to analyse cross-reactivity of the
184 serum from vaccinated, adjuvant-alone and control fish 30 dpv (prior to challenge) with the
185 homologous (*Fno* 1) and heterologous *Fno* isolates (*Fno* 2 and 3) and analyse cross reaction of the
186 vaccine isolate (*Fno* 1) antigen with serum of fish from the different challenge groups at 15 dpc.

187

188 ***2.7. RNA isolation and cDNA synthesis***

189 RNA was extracted from ~ 40-50 mg of the spleen samples collected from the vaccinated, adjuvant-
190 alone and PBS control groups at 6, 24, 72 hpv using TRI Reagent (Sigma, UK) following the
191 manufacturer's protocol. RNA samples were stored at -70 °C until further use. RNA quantity and
192 quality were determined using the Nanodrop ND-1000 Spectrophotometer (ThermoFisher Scientific,
193 UK). RNA integrity was checked by gel electrophoresis on 1.0% agarose gel. Potential DNA residues
194 in RNA samples were removed using a DNA-free kit (Ambion, ThermoFisher Scientific, UK)
195 according to the manufacturer's instructions. The cDNA was synthesised using a High Capacity
196 cDNA Reverse Transcription Kit (Applied Biosystem, USA) following the manufacturer's protocol.

197 ***2.8. Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)***

198 Spleen samples taken at 6 h, 24 h and 72 h post-vaccination were analysed by qRT-PCR to quantify
199 the expression of immune related genes: *IgM, TNF-α, IL-1β and MHC-III*. All RT-qPCR assays were
200 performed in white 96-well plates using an Eppendorf® Realplex2 Mastercycler gradient S instrument
201 (Eppendorf, UK) with SYBR® Green I master mix (Thermo Scientific, UK) and primers (MWG
202 Eurofins genomics, UK) as listed in **Table 1**. The RT-qPCR was performed in a 20 µL reaction mix
203 consisting of 1x SYBR® Green I buffer, 0.3 µM from forward and reverse primers and 5 µL of the ten-
204 fold diluted cDNA. The cycling conditions were 94 °C for 15 s, 40 cycles of denaturation at 95 °C for
205 30 s, annealing at the optimal temperature of each primer as indicated in **Table 1** for 30 s and a final
206 extension at 72°C for 1 min. Melting curve analysis included amplification at 60°C to 90°C with 0.1°C
207 increments per second to evaluate the qPCR products specificity. Samples were run in duplicates and
208 each qPCR run included RT negative (RT^-) and non-template controls (NTC) (Milli-Q water only).
209 Serial dilutions of a pool of all cDNA samples were prepared in nuclease free water including seven
210 dilutions at 1:10, 1:20, 1:50, 1:100, 1:500, 1:1000 and 1:10000. The threshold cycle (Ct) values of these
211 dilutions were plotted versus log concentration to generate a standard curve in the *Realplex* software
212 V2.2 (Eppendorf, UK). The quality of the generated standard curve was evaluated using the slope
213 curve and the correlation co-efficient (R^2). The efficiency of the amplification of the qPCR targets was
214 judged by the line slope following the equation, $E= (10^{-1/slope}) -1$. The expression of the target genes in
215 this study was normalised to the expression of *β-actin* and *EF-1α*. The fold change in the expression
216 of the target genes in spleen samples of vaccinated and adjuvant-alone injected fish compared to the
217 unvaccinated-control samples was calculated following the $2^{-\Delta\Delta C_T}$ method **[29]** using the Relative

218 Expression (REST©) Software [30]. All the primers used in this study were analysed for self-annealing

219 using NCBI Blast sequence analyser [31].

220 ***2.9. Quantification of bacterial load in survivor fish by quantitative real-time PCR (qPCR)***

221 Ten spleen samples were randomly collected from surviving fish in the different treatments 15 dpc

222 and preserved in 95% ethanol (Sinopharm, China) for quantification of *Fno* load using real-time

223 qPCR. DNA from 20 mg sub-samples of the spleen tissue were extracted using a DNeasy Blood and

224 Tissue kit (Qiagen, UK), following the manufacturer`s protocol. Real-time qPCR was performed to

225 quantify the *Fno* genomic load in copy numbers using a dilution range of 10^7 to 10^1 copies/ µL of DNA

226 plasmid standard containing the unique gene (*Fno* FSC 771-hypothetical protein gene) previously

227 described [32], using primers listed in **Table 1**, following the published qPCR protocol [22]. All

228 samples were run in triplicate. After the run, analysis was performed using the default calculation of

229 the threshold fluorescence (Ct value).

230

231 ***2.9. Statistical analysis***

232 Data processing was performed using Microsoft Excel 2013, while GraphPad prism version 7

233 (GraphPad software Inc., San Diego, CA, USA) was used to conduct pairwise Kaplan-Meier survival

234 analyses [33] with subsequent Mantel-Cox log-rank tests applied to the mortality data to calculate the

235 survival probabilities and to compare the survival distributions of fish in each experimental group.

236 One-way ANOVA with a Tukey *post hoc* test was performed to analyse the differences in optical

237 density (OD_{450}) values representing antibody responses in serum samples and *Fno* load between the

238 different treatments (vaccinated, adjuvant-alone and PBS control groups). In all cases a p-value of <

239 0.05 was considered significant. The expression of the target genes in both vaccinated and adjuvant-

alone injected fish samples was considered significantly different from that of the control samples when a p-value of <0.05 was obtained.

3. Results

3.1. Screening of tilapia for vaccination

Tilapia tissues sampled prior to the experiment were negative for *Fno* when screened by bacteriology and PCR (**Figure S1**).

3.2. Vaccine efficacy

Mortalities started between 6- 8 days post-challenge (dpc) in the vaccinated groups and between 3-4 dpc in the adjuvant-alone and PBS groups **(Figure 2)**. The non-vaccinated fish had the lowest level of survival at 15 dpc with the three different *Fno* isolates. Fish injected with the adjuvant-alone had higher survival rates compared to fish injected with PBS, however these differences were not significant ($p>0.05$). The vaccinated fish demonstrated significantly higher levels of survival ($p<0.001$) than both the adjuvant-alone and the PBS injected groups post-challenge with the different *Fno* isolates **(Table 4)**. The RPS values obtained in the vaccinated fish were 82.3%, 69.8% and 65.9%, while the adjuvant-alone group had RPS values of 15.6%, 20.9% and 18.2% post-challenge with *Fno* 1, *Fno* 2 and *Fno 3* isolates, respectively. No significant differences ($p<0.05$) were observed in level of survival obtained with the homologous isolate (*Fno* 1) compared to the heterologous isolates (*Fno* 2 and 3 isolates) in the adjuvant-alone or PBS-injected groups. In the vaccinated group, however, fish challenged with the homologous isolate displayed significantly higher survival ($p<0.05$) than fish challenged with the heterologous isolates, which were not significantly different to each other ($p>0.05$) **(Figure 3)**.

Table 4. Accumulated mortality in the different groups of fish after challenge (Average mortality % ± SD of 2 parallel tanks holding 30 fish/tank/challenge group)

Fno isolate	Cumulative mortality in vaccinated fish (*n*= 60)	Cumulative mortality in adjuvant-alone fish (*n*= 60)	Cumulative mortality in PBS control fish (*n*= 60)
Fno 1	13.3% (± 0.49)	63.3% (± 1.33)	75% (± 1.8)
Fno 2	25 % (± 0.82)	60% (± 1.68)	73.3% (± 1.26)
Fno 3	21.7 % (± 0.56)	56.7% (± 1.5)	71.7 % (± 1.74)

n: number of fish per challenge group

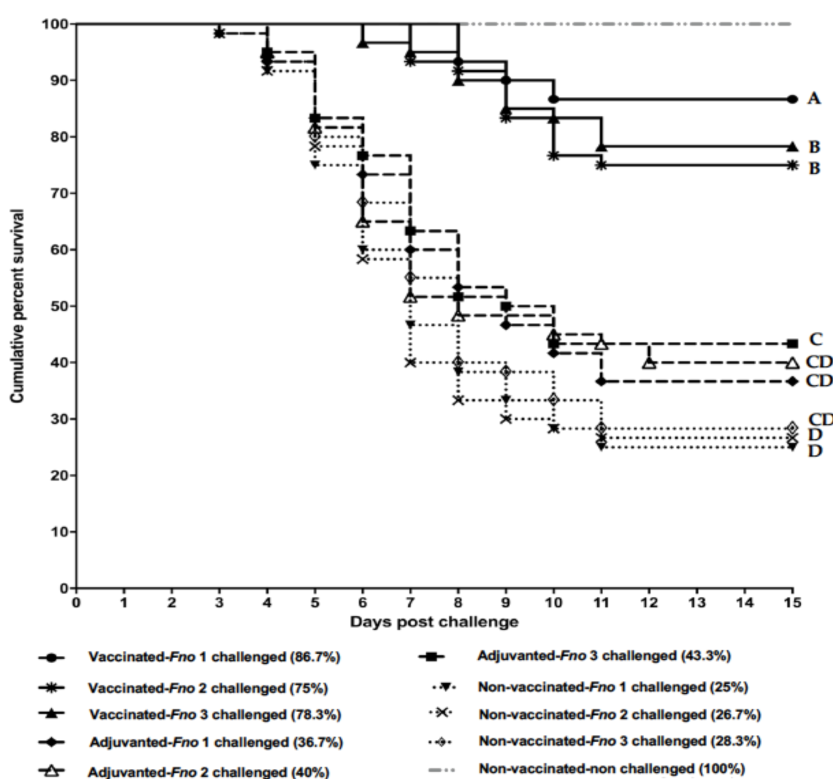

Figure 2. Kaplan-Meier (Log-rank Mantel Cox) representation of cumulative survival of tilapia fingerlings 15 dpc with 10^6 CFU/mL of *Fno* 1, *Fno* 2 and *Fno* 3. Each curve represents the average results of two parallel tanks holding 30 fish/tank/challenge group. The non-vaccinated, non-challenged curve represents data from 1 tank with 20 fish. Groups that do not letters are significantly different (p<0.05).

289 Signs of *Fno* infection were clearly evident upon necropsy of recent mortalities and moribund fish,
290 including ascites, enlargement of the spleen and head kidney with the presence of creamy nodules
291 on these tissues (**Figure 3**). Detection of *Fno* in spleen of moribund fish from the different challenge
292 groups was confirmed by bacteriology (**Figure S2**) and conventional PCR (**Figure S3**).

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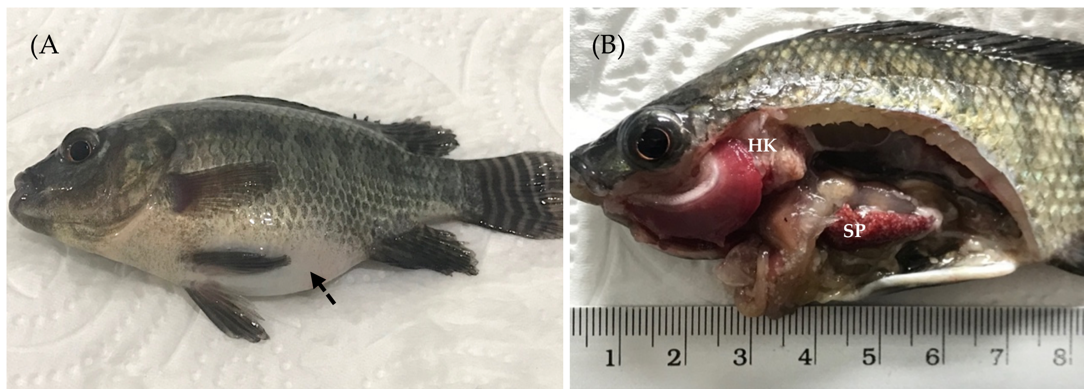

300 **Figure 3.** Clinical signs of francisellosis in moribund (A) and recently dead (B) tilapia after
301 heterologous i.p. challenge with three *Fno* isolates. (A) Ascites (dashed arrow); (B) enlargement of
302 spleen (SP) and head kidney (HK) with appearance of white nodules on their surfaces.

303

304 ***3.3. Specific antibody (IgM) response post-vaccination and challenge***

305 Vaccinated fish had significantly higher levels of specific antibody (IgM) (OD_{450} at 1:500 dilution) in
306 their serum at 840 dd than the adjuvant or PBS injected fish as measured by ELISA. No specific
307 antibody response was detected in fish prior to vaccination. Analysis of serum IgM levels post-
308 challenge with the three different *Fno* isolates (15 dpc) indicate that the vaccinated fish had
309 significantly higher levels of antibody against *Fno* ($p<0.05$) compared to the adjuvant and PBS injected
310 fish (**Figure 4**). In addition, the serum IgM level in the *Fno* 1 challenged fish was significantly higher
311 ($p<0.05$) than that of fish challenged with *Fno* 2 or *Fno* 3 isolates.

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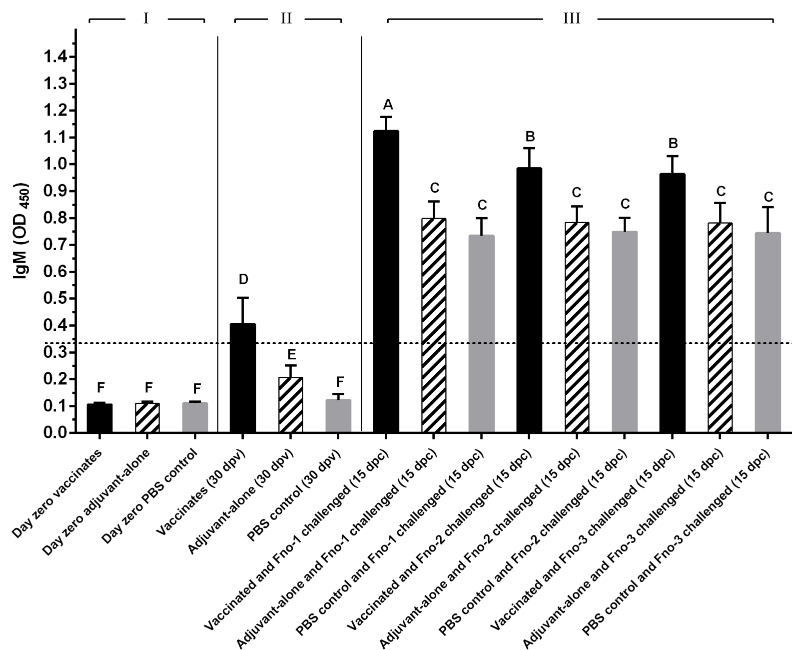

Figure 4. Specific antibody response of tilapia following i.p. injection of vaccine, adjuvant-alone or PBS at 30 dpv (840 dd) and 15 dpc with multiple *Fno* isolates. Each bar represents the average serum IgM at OD_{450} of 10 fish/ treatment. [I] sera from fish before vaccination, [II] sera from fish 30 dpv, [III] sera from fish 15 dpc. The dashed line represents the cut-off (3X the average absorbance of the negative control (PBS)). Groups that do not share letters are significantly different ($P < 0.05$). dpv: days-post vaccination, dpc: days-post challenge.

3.4. Immunoblotting

The *Fno* isolates from the three geographical regions showed a similar profile when subjected to 1D SDS-PAGE **(Figure 5A)**. Coomassie Blue and Silver staining revealed a conserved abundant protein band between 20-37 kDa. This band was strongly antigenic in different *Fno* isolates when serum sampled from the vaccinated fish 30 dpv was used **(Figure 5A)**. The intensity of the immunoreactive region varied between the different antigen used, where the UK antigen (homologous or vaccine isolate) showed higher intensity than the other heterologous antigens. No immunoreactivity was observed with serum sampled at day zero (D_0) or with serum from the adjuvant or PBS injected fish sampled at 30 dpv. The same immunoreactive band (i.e. 20-37 kDa) was also observed with the

vaccine isolate antigen (i.e. *Fno* 1) when blotted with serum from fish surviving the challenge with the different *Fno* isolates (**Figure 5B**).

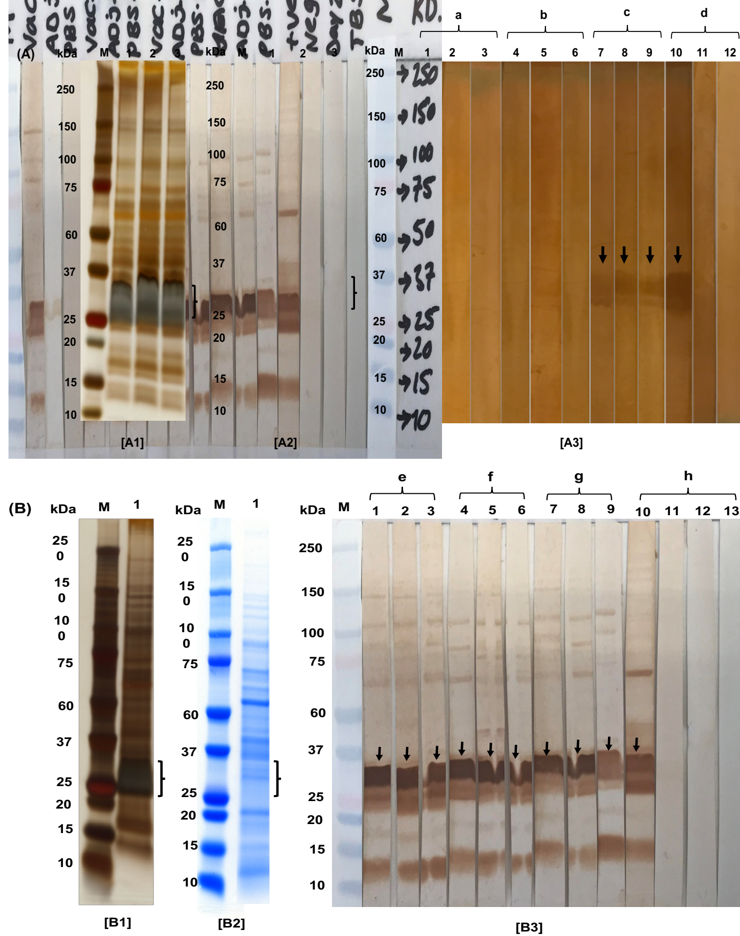

Figure 5. SDS-PAGE and Western blot analysis of whole cell lysates of *Fno*. Immunoreactivity of serum of vaccinated, adjuvant-alone and control tilapia 30 dpv against homologous and heterologous *Fno* isolates (A) and immunoreactivity of serum of survivor tilapia 15 dpc with the different *Fno* isolates in vaccinated, adjuvant-alone and control groups against *Fno* 1 (vaccine isolate) (B). Immunoreactive band on the blots is marked by black arrows and its corresponding protein band on the reference gels is marked by brackets. A1: 1D reference SDS PAGE gel stained with silver stain; M: marker; 1: *Fno* 1 isolate; 2: *Fno* 2 isolate; 3: *Fno* 3 isolate. A2: 1D reference SDS PAGE stained with Coomassie blue stain; 1: *Fno* 1 isolate; 2: *Fno* 2 isolate; 3: *Fno* 3 isolate. A3: 1D western blot showing the antigenic band observed using sera from PBS control (a); adjuvant-alone (b) and vaccinated tilapia (c) against whole cells lysate of *Fno* 1 (Lanes 1,4,7); *Fno* 2 (Lanes 2,5,8); *Fno* 3 (Lanes 3,6,9). d: western blot control sera; 10: positive control serum; 11: negative control serum; 12: TBS (Tris-buffer saline) (internal control). B1: 1D reference SDS PAGE stained with silver stain. M: marker; 1: *Fno* 1 isolate. B2: 1D reference SDS PAGE stained with Coomassie blue stain; 1: *Fno* 1 isolate. B3: 1D western blot showing the immunreactive band of the vaccine isolate (*Fno* 1) following blotting with sera from different challenge groups 15 dpc. e: sera from fish challenged with *Fno* 1 isolate in vaccinated group (Lane 1); adjuvant-only group (Lane 2); PBS control group (Lane 3); f: sera from fish challenged with *Fno* 2 isolate in vaccinated group (Lane 4); adjuvant-alone group (Lane 5); PBS control group (Lane

367 6); g: sera from fish challenged with *Fno* 3 isolate in vaccinated group (Lane 7); adjuvant-alone group
368 (Lane 8); PBS control group (Lane 9). d: western blot control sera. 10: positive control serum; 11:
369 negative control serum; 12: day zero serum; 13: TBS.

370 ***3.5. Analysis of immune gene expression by RT-qPCR***

371 The relative expression of *IgM, IL-1β, TNF-α* and *MHCII* in tilapia RNA samples was first normalised
372 against *β-actin* and *EF-1α*. The relative fold change in expression of these genes in RNA samples
373 extracted from spleen of vaccinated and adjuvant-alone tilapia compared to the PBS injected fish is
374 summarised in **Table 5**. At 6 hpv, there was a significant up-regulation of *IL-1β* in both vaccinated
375 and adjuvant-alone groups with significantly higher expression in the vaccinated group ($p < 0.001$)
376 than in the adjuvant-alone group ($p <0.01$). A significant up-regulation of *TNF-α* ($p <0.001$) and *MHCII*
377 ($p <0.01$) was observed in the vaccinated group only at 6 hpv. At 24 hpv, a significantly higher
378 expression of *TNF-α* and *MHCII* was observed in vaccinated fish ($p <0.001$) compared to the adjuvant-
379 alone and PBS-control fish ($p < 0.01$ and $p < 0.05$, respectively). Furthermore, *MHCII* was significantly
380 down-regulated in the adjuvant-alone group ($p < 0.01$). At 72 hpv, a significant up-regulation of *IgM,*
381 *IL-1β, TNF-α* and *MHCII* ($p <0.01$) was observed in vaccinated tilapia spleen samples accompanied
382 with significant down-regulation of *MHCII* ($p < 0.01$) in the adjuvant-alone group.

383

384

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386

387 **Table 5.** Relative expression of pro-inflammatory and immune related genes in spleen samples of vaccinated and adjuvant injected tilapia at 6 h, 24 h and 72 h
388 post vaccination (hpv) compared to the non-vaccinated control group.

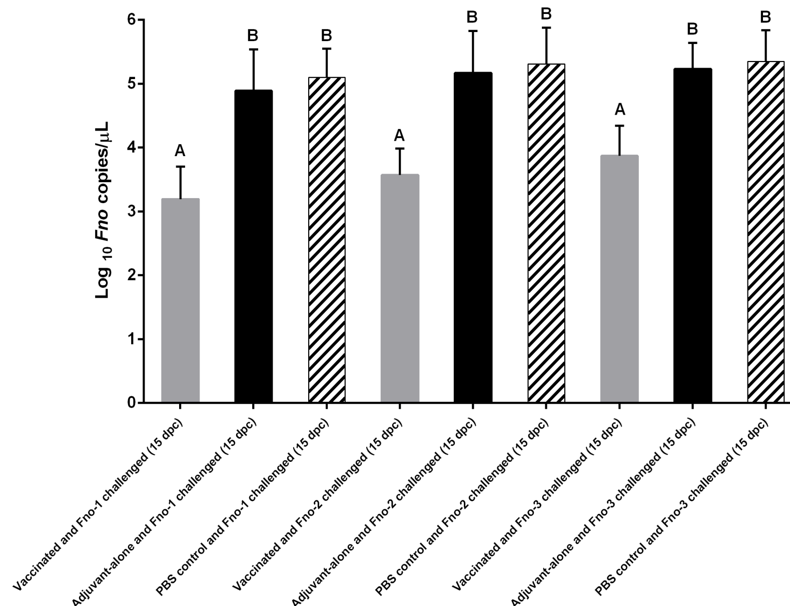
Gene	Treatment	**6 hpv**		**24 hpv**		**72 hpv**	
		Expression	SE	Expression	SE	Expression	SE
IgM	**Vaccinated**	2.306	0.261-22.013	3.077	1.248-7.406	**4.956** ↑↑	2.384 - 11.362
	Adjuvant-alone	1.443	0.403-4.624	2.800	0.694 - 14.70	0.777	0.274 - 3.188
IL-1β	**Vaccinated**	**7.884** ↑↑↑	3.685 - 12.028	**5.811** ↑↑↑	1.911 - 11.435	**4.977** ↑↑	3.319 - 9.852
	Adjuvant-alone	**5.761** ↑↑	1.728 - 37.970	**4.404** ↑↑	1.713 - 11.199	**4.269** ↑	0.951 - 28.387
TNF-α	**Vaccinated**	**2.467** ↑↑↑	1.949 - 3.108	**2.991** ↑↑↑	2.164 - 3.998	**4.539** ↑↑	2.543 - 12.118
	Adjuvant-alone	1.188	0.876 - 1.659	**1.473** ↑	1.112 - 2.199	1.692	0.895 - 4.483
MHCII	**Vaccinated**	**3.409** ↑↑	1.854 - 4.927	**4.190** ↑↑	2.048 - 7.672	**4.506** ↑↑	2.815 - 6.063
	Adjuvant-alone	0.770	0.414 - 1.428	**0.627** ↓↓	0.435 - 0.861	**0.395** ↓↓	0.267 - 0.587

389 (↑ or ↓), (↑↑ or ↓↓) and (↑↑↑ or ↓↓↓) indicates significant up or down regulation relative to controls at ($p < 0.05$), ($p < 0.01$) and ($p < 0.001$), respectively.

390 ### 3.6. *Fno* load in surviving fish post-challenge

391 Quantification of the bacterial burden (copies/ μL) in the spleen samples from the different fish 392 treatments showed significantly higher *Fno* loads ($p<0.05$) in the PBS control and adjuvant-alone 393 groups compared to the vaccinated group after challenge with the different *Fno* isolates, respectively 394 (**Figure 6**).

395 396 397 398 399 400 401 402 403



404 **Figure 6**. *Fno* load (Log10 of mean copies/μL ±SD) quantified by qPCR in spleen of survivors after i.p. 405 challenge with *Fno* 1, 2 and 3 isolates in the different treatment groups. Each bar represents average 406 of *Fno* load of 10 spleen samples/treatment. Groups that do not share letters are significantly different 407 ($p < 0.05$).

408

409 ## 4. Discussion

410 Following the emergence of piscine francisellosis outbreaks worldwide, high mortality and serious 411 economic losses have been reported in farm-raised tilapia due *Fno* infections. Efforts to develop 412 potent, safe, cost-effective vaccines against *Fno* have therefore become a priority for the tilapia 413 industry. The vaccine tested in this study conferred significant protection to tilapia (~15 g) challenged

414 with either homologous, or heterologous *Fno* isolates, compared to fish injected with adjuvant-alone
415 or mock vaccinated with PBS. The results obtained are in agreement with a previous study using the
416 same vaccine [16], which induced significant protection in tilapia, demonstrated by a RPS value of
417 100% compared to 46.6% in the adjuvant-alone group post-challenge with the homologous *Fno*
418 isolate. Furthermore, the RPS values obtained for vaccinated tilapia in this study were similar to those
419 reported in tilapia vaccinated with a live-attenuated *Fno* vaccine after immersion challenge using a
420 self-genotype *Fno* isolate that resulted in RPS of 68.8 % - 87.5 % [15]. Interestingly, the survival rates
421 in the current study were higher than those obtained in zebrafish, *Danio rerio*, i.p. immunised with
422 an *Fno*-outer membrane vesicle (OMVs)-derived vaccine and i.p. challenged with the same *Fno*
423 vaccine isolate [34]. This suggests a weaker stimulation of the zebrafish immune system by OMVs
424 compared to the adjuvanted whole cell vaccine used in the current study, although differences in
425 susceptibility between tilapia and zebrafish to *Fno* may account for this variation.
426 The difference in the level of protection against the homologous isolate (i.e. *Fno* 1 isolate)
427 obtained in the current study (RPS 82.3%) and the previous study [16] (RPS 100%) may be attributed
428 to differences in the genetic make-up and susceptibility of the fish used. Wild type Nile tilapia
429 obtained from a commercial farm were used in the present study and therefore would have been
430 exposed to stressors commonly associated with the farm environment, while hybrid red tilapia raised
431 in an in-house aquatic research facility were used for the previous vaccine study [16]. Differences in
432 bacterial inoculum were also used in the experimental challenge between the two experiments. A
433 bacterial dose of 10^5 CFU/fish induced ~ 70 % mortality in control fish in the current study, while 10^3
434 CFU/fish and 10^5 CFU/fish resulted in the same level of mortality in Nile tilapia and zebrafish,
435 respectively [16, 34], which may have influenced the RPS values obtained in the different studies. A

436 higher level of protection is frequently obtained with live attenuated vaccines due to the induction of
437 both a strong cell mediated immunity and humoral immunity [14]. The inactivated *Fno* bacterin used
438 in our study not only appears to confer comparable protection to a live attenuated vaccine [15], but
439 also it removes concerns relating to potential reversion to virulence and release of the live genetically
440 modified organisms into the environment, a potential complication associated with live attenuated
441 vaccines [35].

442 It is of note that, the vaccine studied here stimulated a strong humoral immune response in the
443 vaccinated fish, however, more studies are required to investigate the effect of this vaccine on
444 cellular-mediated immunity. Generally, bacterial vaccines in fish aim to trigger a specific antibody
445 response that provide protection against subsequent infections [36]. The antibody response post-
446 vaccination is a widely used parameter to examine vaccine efficacy in fish and other higher
447 vertebrates when correlating with protection [35, 37, 38]. In the current study, a strong correlation
448 between specific antibody production and the level of protection was observed. At 30 dpv, a
449 relatively weak antibody response was observed in vaccinates, although it was significantly higher
450 ($p< 0.05$) than in both adjuvant-alone and PBS control group fish. This corroborates previous results
451 with this vaccine [16], this was, however, in contrast to weak mucosal or serum antibody responses
452 obtained with the live attenuated *Fno* vaccine administered to tilapia by immersion or the OMVs-
453 derived *Fno* injectable vaccine trialed in zebrafish [15, 34]. In the present study, elevated levels of
454 specific serum IgM were measured in all treatment groups at 15 dpc, when fish were challenged with
455 the different *Fno* isolates, with significantly higher IgM values in the vaccinated fish. Furthermore,
456 significantly higher *Fno* specific IgM levels were detected in the fish challenged with a homologous
457 isolate (*Fno* 1), which correlated with significantly higher survival in this group compared to the fish

458 challenged with the heterologous *Fno* isolates (*Fno* 2 and 3). It is not surprising to get a higher level
459 of protection in fish challenged with a homologous isolate as they are likely to elicit a stronger
460 immune response than fish challenged with heterologous isolates.

461 The protective mechanism of immunity against piscine francisellosis is yet to be determined. The
462 correlation found between the survival of vaccinated fish and antibody levels in serum or mucus
463 post-challenge in tilapia **[15]**, zebrafish **[34]** and Atlantic cod **[39]** highlights the importance of
464 antibody-mediated immunity in protection against *Francisella* infection in fish. This was also
465 observed here in response to the current vaccine. Moreover, the specific antibody produced in
466 response to vaccination/or infection with the majority of Gram-negative bacteria act synergistically
467 with the complement system leading to a direct bactericidal effect on the invading bacteria or can
468 assist phagocytic cells activity, mainly facilitated via Fc receptor bearing macrophage-like cells and
469 NK cells to destroy the engulfed bacterial cells including intracellular bacteria **[15]**. This was
470 previously demonstrated by the ability of antibodies in the serum of tilapia immunised with a live
471 attenuated *Fno* vaccine to co-stimulate phagocytosis of *Fno* by head kidney derived macrophages
472 (HKDM), which was hampered by either heat inactivated or naïve serum **[15]**.

473 The antigenicity of the vaccine master seed may be a major factor in the efficacy of the vaccine
474 against heterologous bacterial isolates **[40]**. Immunoblotting in the present study showed cross
475 reaction between *Fno* isolates with sera obtained from vaccinated tilapia and also between the vaccine
476 isolate (i.e. *Fno* 1) and tilapia serum post-challenge with either the homologous or heterologous *Fno*
477 isolates. Taken together with the induced high survival rates (i.e. RPS), the cross-protection ability of
478 the developed vaccine against challenge with multiple *Fno* isolates was highlighted. Further studies
479 using other geographically distinct *Fno* isolates will give us more insights into the efficacy, and in

480 particular, establishment of the cross-protective nature of the developed vaccine. Future work may
481 also include development and efficacy testing of bivalent or polyvalent *Fno* vaccines.

482 A significant up-regulation of *IgM* transcription was noted in the spleen of immunised tilapia at
483 72 hpv. This indicates that activation of B cells in response to vaccination is correlated with the
484 increased serum IgM detected 30 dpv. Our results are consistent with the findings of an earlier study
485 [34], where the authors reported an up-regulation of *IgM* at 7 dpv that was maintained to 21 dpv
486 following i.p. immunisation of zebrafish with *Fno*-derived OMVs. There is lack of information
487 regarding the role of cellular immunity against piscine francisellosis and most of our understanding
488 is based on results from vaccine experiments with *Francisella tularensis*. It was reported that *F.*
489 *tularensis* has the ability to trigger T-cell mediated immune responses, mainly antigen-specific IFN-γ
490 responses [41, 42] and a strong cell-mediated immune response has also been suggested to prevent
491 *Francisella* spp. infection in other vertebrates [43, 44]. In a recent study [34], a significant up-regulation
492 of *IFN-γ-1* transcription in zebrafish 24 h post-immunisation with *Fno*-derived OMVs was reported
493 that remained up-regulated until 21 dpv. These authors suggested that *IFN-γ* prevents *Fno* from
494 escaping from the zebrafish phagosomes containing *Fno* cells post-infection. Also, *IL-12* and *IL-17*
495 appears to drive a strong T-cell proliferation in Atlantic cod challenged with *F. noatunensis* subsp.
496 *noatunensis* (*Fnn*) [45]. In the current study, the transcription of *MHCII* was significantly up-regulated
497 in the spleen of vaccinated fish 6 hpv and at 24-72 hpv, where a 4.5-fold change was detected, while
498 a significant down-regulation was observed in the adjuvant alone group. This suggests successful
499 recognition of *Fno* cells in the vaccinated fish and presentation of the antigen by antigen presenting
500 cells (APCs), which is a key event in triggering of a subsequent adaptive immune response [14].

501 The rapid activation of pro-inflammatory cytokines in response to the vaccine in the current
502 study was evidenced by an early (6 hpv) significant up-regulation of *IL-1β* and *TNF-α*, which are
503 produced primarily by activated macrophages in the spleen of the fish [46]. *IL-1β* is mainly involved
504 in lymphocyte activation, leukocyte migration, phagocytosis and diverse bactericidal activities [47].
505 These findings agree with a previous study, where a significantly higher *IL-1β* expression was
506 detected in kidney cells of adult zebra fish vaccinated with *Fno*-derived OMVs at 1 dpv compared to
507 control fish [34]. Moreover, *IL-1β* expression was up-regulated in the splenic cells of Nile tilapia 24-
508 96 hpc with *Fno* [48]. *TNF-α* is a well-known pro-inflammatory cytokine, associated with the killing
509 of infected cells, inhibiting intracellular pathogen replication, apoptosis, up-regulating the
510 transcription of various immune-related genes and recruiting leukocytes to the site of inflammation
511 [48]. In contrast to the significant up-regulation of *TNF-α* transcription 6 hpv observed in the current
512 study, down-regulation of this cytokine was noted in the head kidney of zebrafish vaccinated with
513 *Fno*-derived OMVs 1-21 dpv [34]. While challenge with *Fno* successfully induced up-regulation of
514 *TNF-α* 6-96 hpc in tilapia and 24 hpc and 48 hpc in adults and larval zebrafish, respectively [34, 48,
515 49]. This suggests that whole cell *Fno* antigens, not found in the OMVs, may induced *TNF-α*
516 stimulation by splenic leukocytes. Despite up-regulation of these cytokines in the adjuvant injected
517 group, the fold change of their transcription was lower and of shorter duration than that obtained
518 with the vaccine at 6, 24 and 72 hpv. Notably, up-regulation of *TNF-α* started earlier at 6 hpv in the
519 spleen of vaccinated fish rather than in those receiving the adjuvant alone (24 hpv), implying that the
520 response was induced by antigen and not a non-specific induction by the adjuvant. This result is
521 supported by antibody responses detected by ELISA and western blot analyses in this study, and
522 other studies [39], where the anti-*Fno* IgM in the sera of adjuvant-alone treated fish pre-challenge was

523 significantly lower in the ELISA and showed no recognition of specific protein bands in western

524 blotting.

525 Analysis of the bacterial burden in the vaccinated fish showed they did become infected, albeit

526 with significantly lower bacterial loads than the adjuvant-alone and PBS control fish 15 dpc. Thus,

527 the protection provided by the developed vaccine may be associated with the ability to enhance

528 clearance and limit dissemination of the infection. This supports the application of using bacterial

529 load quantification as a measure of vaccine efficacy against *Fno*. Future histological studies

530 investigating the inflammatory and tissue-associated damage post-challenge between vaccinated and

531 control tilapia would allow greater insights into the protection mechanisms of the developed vaccine

532 at the tissue level.

533

534 **5. Conclusions**

535 The current study represents the first report of a protective oil-based adjuvanted inactivated

536 injectable vaccine against multiple isolates of *Fno* from diverse geographical origins for Nile tilapia

537 following injection immunisation and injection challenge. The significantly higher RPS in the

538 vaccinated fish was correlated with significantly higher specific antibody responses, lower bacterial

539 burden and greater expression of *IgM, IL-1β, TNF-α and MHCII* transcripts 72 hpv in comparison to

540 the adjuvant alone or PBS control fish. This highlights the importance of antibody-mediated immune

541 responses in the control of *Fno* infection in tilapia. In addition, the potential of the current vaccine to

542 cross protect against different isolates of *Fno* was highlighted by immunoblotting. Taking into

543 account the relatively short production cycle of tilapia in most of the tropical countries (6-9 months),

544 a desirable vaccine must induce a significant long-term protection against *Fno*. Thus, future work will

545 investigate the duration of protection induced by the developed vaccine, and efficacy testing against
546 more *Fno* isolates under field conditions. In conclusion, the whole-cell inactivated vaccine described
547 in the present study may provide a starting point for developing a broad-spectrum highly protective
548 vaccine against *Fno* outbreaks in tilapia.

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775 **Supplementary Materials**

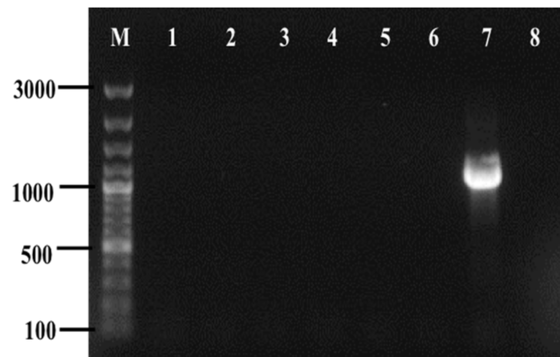

Figure S1: *Francisella* genus specific 16S rRNA PCR for screening tilapia for the presence of *Fno* prior to vaccination. 1% agarose gel showing negative results for *Fno* in tested fish. M: 100bp Molecular marker; lane 1-6: head kidney and spleen pool of 6 naïve tilapia; lane 7: Positive control; lane 8: negative control (Milli-Q water only).

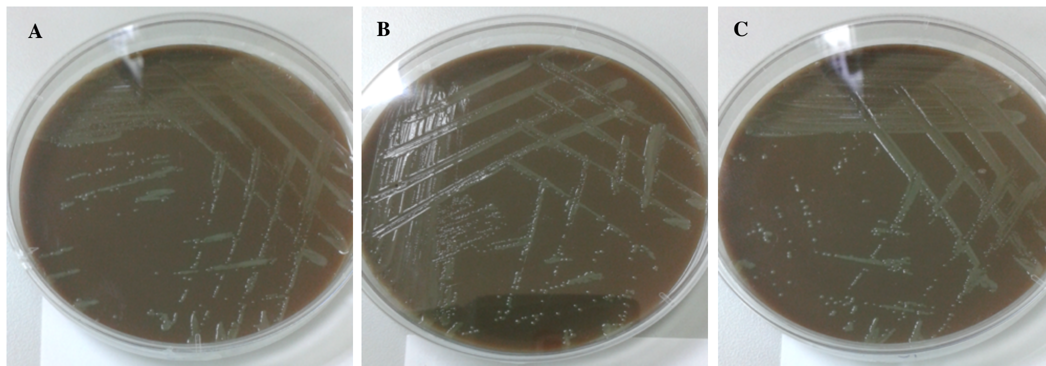

Figure S2: Grey, semi translucent and mucoid *Fno* colonies retrieved from spleen homogenate of moribund tilapia after i.p. challenge with *Fno* 1 (A), 2 (B) and 3 (C) isolates on CHAH.

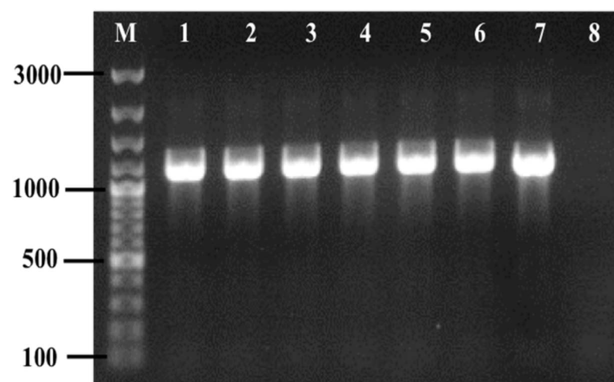

Figure S3: *Francisella* genus specific 16S rRNA PCR for detection of *Fno* in moribund fish and mortalities post-challenge with three different *Fno* isolates. 1% agarose gel showing amplicon of ~ 1140 bp. M: DNA ladder. Lanes 1 – 6: spleen of representative moribund fish (Lanes 1-3) and recently dead (Lanes 4-6) post the heterologous challenge with *Fno* 1 (Lanes 1,4), 2 (Lanes 2,5) and 3 (Lanes 3,6); lane 7: positive control; lane 8: negative control (MQ- water only).