

**Efficacy of an inactivated whole-cell injection vaccine for Nile tilapia,  
*Oreochromis niloticus* (L), against multiple isolates of *Francisella noatunensis*  
*subsp. orientalis* from diverse geographical regions**

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## Abstract

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

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

## 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

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

## 2. Materials and Methods

### 2.1. Fish and rearing conditions

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

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

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

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

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

## 2.2. Bacteria and culture conditions

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

for 18 h (mid log phase) at 160 rpm at 28°C followed by harvesting of the cells by centrifugation at 3000  $\times$ g for 5 min and pellets were resuspended in sterile PBS to the appropriate optical density at OD<sub>600</sub>. The colony-forming units (CFU) per mL was estimated using a 6 x 6 drop plate method, following the published protocol [26] in conjugation with CHAH plates. Plates were incubated for 72 h at 28°C to obtain colony count.

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

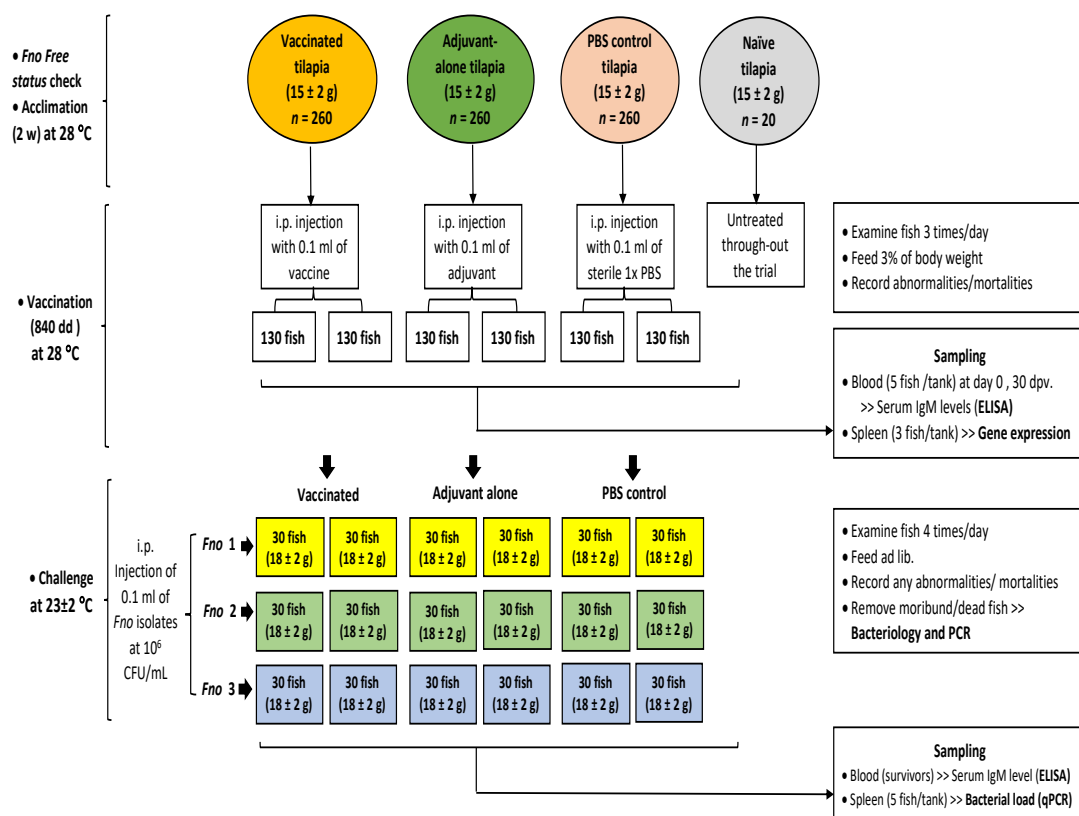
Isolate ID	Designation	Source	Isolation year	Reference
<i>Fno</i> 1	AVU-STIR-GUS-F2f7	Red Nile tilapia (Europe)	2012	[19]
<i>Fno</i> 2	NVI-5409 *	Nile tilapia (Central America)	2006	Unpublished
<i>Fno</i> 3	AVU-Fran-Cos1	Blue tilapia (North America)	2013	Unpublished

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

### 2.3. Fish vaccination and sampling

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

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



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

#### 2.4. Fish challenge and sampling

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

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

Isolates **	CFU/mL	CFU/fish
<i>Fno</i> 1	$1.12 \times 10^6$ CFU/mL	$1.12 \times 10^5$ CFU/mL
<i>Fno</i> 2	$1.23 \times 10^6$ CFU/mL	$1.23 \times 10^5$ CFU/mL
<i>Fno</i> 3	$1.28 \times 10^6$ CFU/mL	$1.28 \times 10^5$ CFU/mL

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

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



## 2.5. Enzyme-linked immunosorbent assay (ELISA)

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

## 2.6. Immunoblotting

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

## 2.7. RNA isolation and cDNA synthesis

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

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

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

Expression (REST®) Software [30]. All the primers used in this study were analysed for self-annealing using NCBI Blast sequence analyser [31].

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

Ten spleen samples were randomly collected from surviving fish in the different treatments 15 dpc and preserved in 95% ethanol (Sinopharm, China) for quantification of *Fno* load using real-time qPCR. DNA from 20 mg sub-samples of the spleen tissue were extracted using a DNeasy Blood and Tissue kit (Qiagen, UK), following the manufacturer's protocol. Real-time qPCR was performed to quantify the *Fno* genomic load in copy numbers using a dilution range of  $10^7$  to  $10^1$  copies/  $\mu$ L of DNA plasmid standard containing the unique gene (*Fno* FSC 771-hypothetical protein gene) previously described [32], using primers listed in Table 1, following the published qPCR protocol [22]. All samples were run in triplicate. After the run, analysis was performed using the default calculation of the threshold fluorescence (Ct value).

### 2.9. Statistical analysis

Data processing was performed using Microsoft Excel 2013, while GraphPad prism version 7 (GraphPad software Inc., San Diego, CA, USA) was used to conduct pairwise Kaplan-Meier survival analyses [33] with subsequent Mantel-Cox log-rank tests applied to the mortality data to calculate the survival probabilities and to compare the survival distributions of fish in each experimental group. One-way ANOVA with a Tukey *post hoc* test was performed to analyse the differences in optical density (OD<sub>450</sub>) values representing antibody responses in serum samples and *Fno* load between the different treatments (vaccinated, adjuvant-alone and PBS control groups). In all cases a *p*-value of < 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).

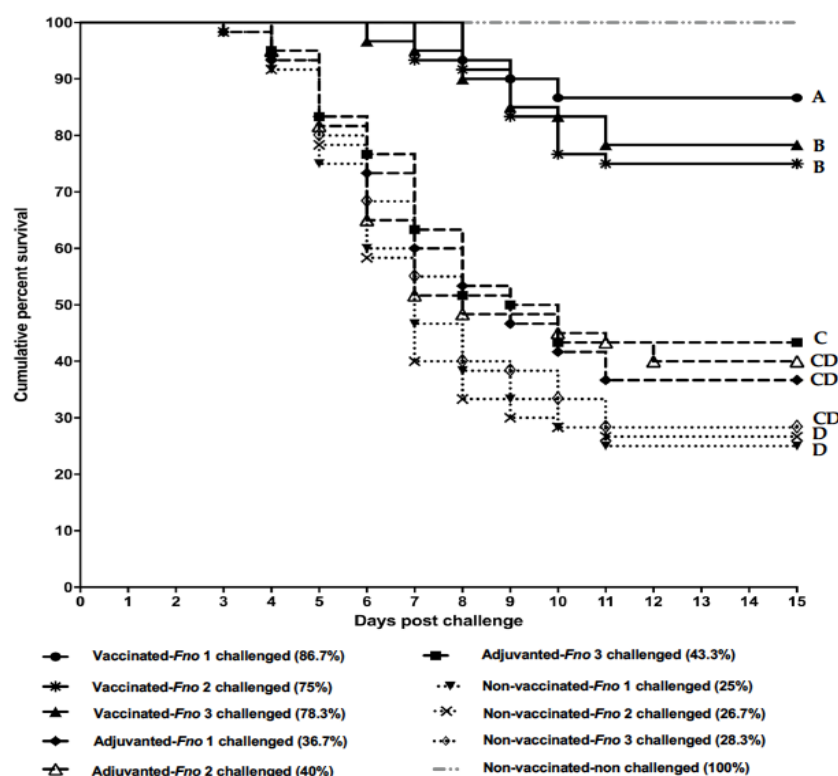
#### 3.2. Vaccine efficacy

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 %  $\pm$  SD of 2 parallel tanks holding 30 fish/tank/challenge group)

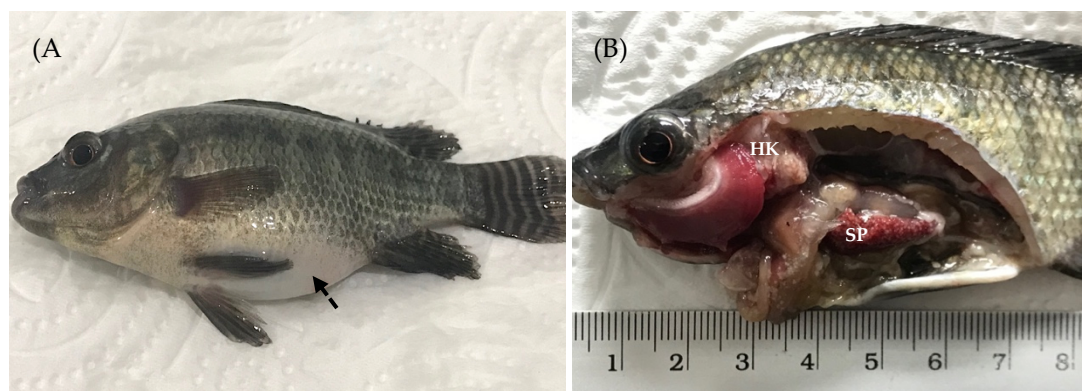
<i>Fno</i> isolate	Cumulative mortality in vaccinated fish ( <i>n</i> = 60)	Cumulative mortality in adjuvant-alone fish ( <i>n</i> = 60)	Cumulative mortality in PBS control fish ( <i>n</i> = 60)
<i>Fno</i> 1	13.3% ( $\pm$ 0.49)	63.3% ( $\pm$ 1.33)	75% ( $\pm$ 1.8)
<i>Fno</i> 2	25 % ( $\pm$ 0.82)	60% ( $\pm$ 1.68)	73.3% ( $\pm$ 1.26)
<i>Fno</i> 3	21.7 % ( $\pm$ 0.56)	56.7% ( $\pm$ 1.5)	71.7 % ( $\pm$ 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 ( $p < 0.05$ ).

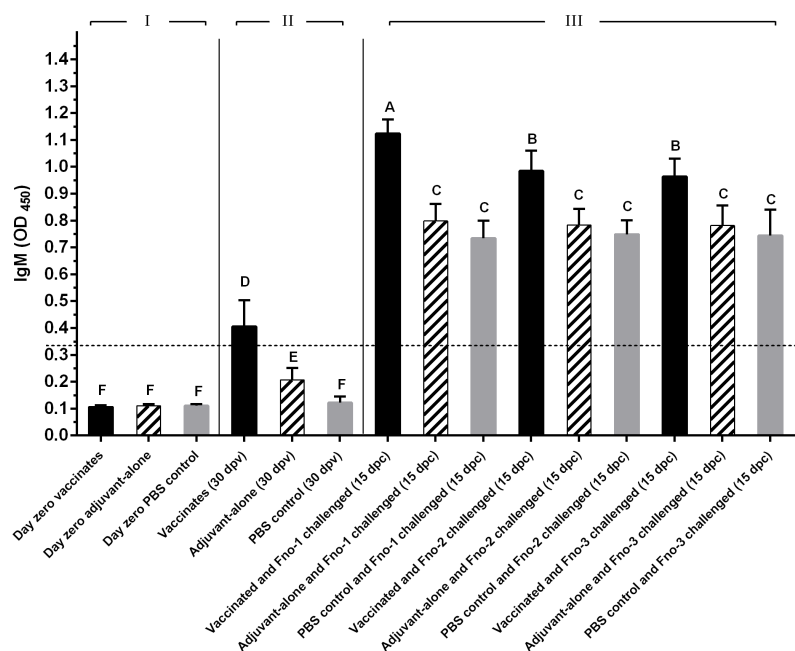
Signs of *Fno* infection were clearly evident upon necropsy of recent mortalities and moribund fish, including ascites, enlargement of the spleen and head kidney with the presence of creamy nodules on these tissues (Figure 3). Detection of *Fno* in spleen of moribund fish from the different challenge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



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

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

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



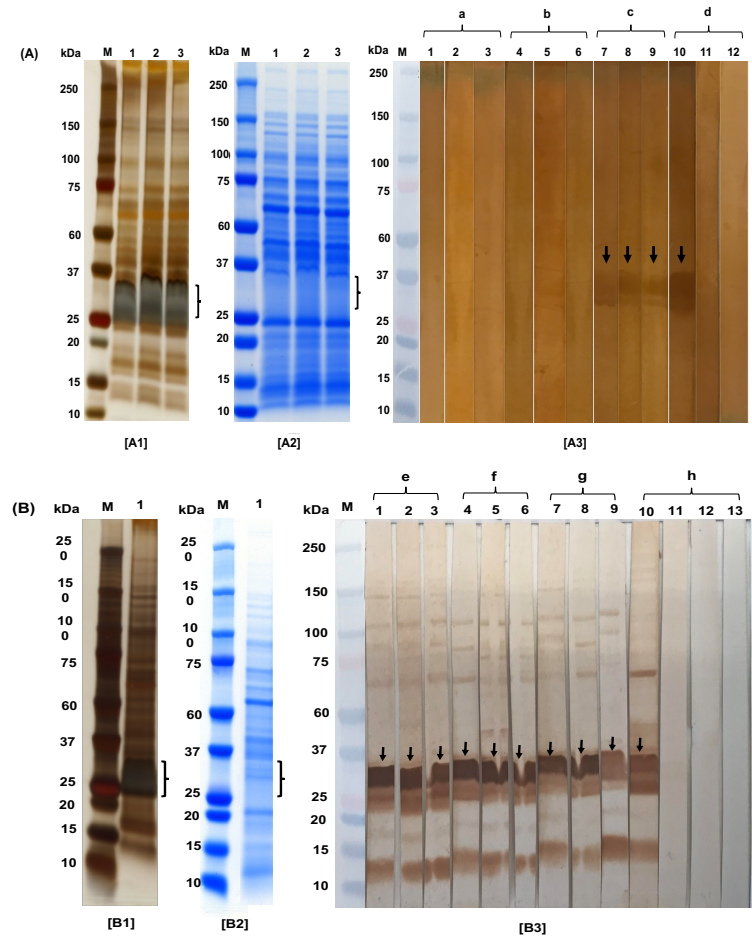
**Figure 4.** Specific antibody response of tilapia following i.p. injection of vaccine, adjuvant-alone or PBS at 30 dpv (840 dd) and 15 dpc with multiple *Fno* isolates. Each bar represents the average serum IgM at OD<sub>450</sub> of 10 fish/ treatment. [I] sera from fish before vaccination, [II] sera 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<sub>0</sub>) 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 immunoreactive 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



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

### 3.5. Analysis of immune gene expression by RT-qPCR

The relative expression of *IgM*, *IL-1 $\beta$* , *TNF- $\alpha$*  and *MHCII* in tilapia RNA samples was first normalised against  *$\beta$ -actin* and *EF-1 $\alpha$* . The relative fold change in expression of these genes in RNA samples extracted from spleen of vaccinated and adjuvant-alone tilapia compared to the PBS injected fish is summarised in [Table 5](#). At 6 hpv, there was a significant up-regulation of *IL-1 $\beta$*  in both vaccinated and adjuvant-alone groups with significantly higher expression in the vaccinated group ( $p < 0.001$ ) than in the adjuvant-alone group ( $p < 0.01$ ). A significant up-regulation of *TNF- $\alpha$*  ( $p < 0.001$ ) and *MHCII* ( $p < 0.01$ ) was observed in the vaccinated group only at 6 hpv. At 24 hpv, a significantly higher expression of *TNF- $\alpha$*  and *MHCII* was observed in vaccinated fish ( $p < 0.001$ ) compared to the adjuvant-alone and PBS-control fish ( $p < 0.01$  and  $p < 0.05$ , respectively). Furthermore, *MHCII* was significantly down-regulated in the adjuvant-alone group ( $p < 0.01$ ). At 72 hpv, a significant up-regulation of *IgM*, *IL-1 $\beta$* , *TNF- $\alpha$*  and *MHCII* ( $p < 0.01$ ) was observed in vaccinated tilapia spleen samples accompanied with significant down-regulation of *MHCII* ( $p < 0.01$ ) in the adjuvant-alone group.

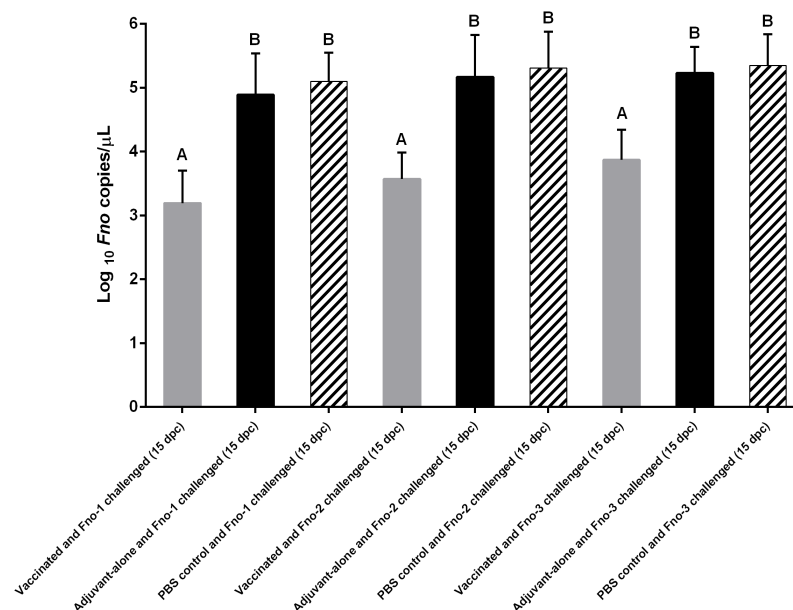
387 **Table 5.** Relative expression of pro-inflammatory and immune related genes in spleen samples of vaccinated and adjuvant injected tilapia at 6 h, 24 h and 72 h  
388 post vaccination (hpv) compared to the non-vaccinated control group.

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

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

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



**Figure 6.** *Fno* load (Log<sub>10</sub> of mean copies/ $\mu$ L  $\pm$ SD) quantified by qPCR in spleen of survivors after i.p. challenge with *Fno* 1, 2 and 3 isolates in the different treatment groups. Each bar represents average of *Fno* load of 10 spleen samples/treatment. Groups that do not share letters are significantly different ( $p < 0.05$ ).

## 4. Discussion

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

with either homologous, or heterologous *Fno* isolates, compared to fish injected with adjuvant-alone or mock vaccinated with PBS. The results obtained are in agreement with a previous study using the same vaccine [16], which induced significant protection in tilapia, demonstrated by a RPS value of 100% compared to 46.6% in the adjuvant-alone group post-challenge with the homologous *Fno* isolate. Furthermore, the RPS values obtained for vaccinated tilapia in this study were similar to those reported in tilapia vaccinated with a live-attenuated *Fno* vaccine after immersion challenge using a self-genotype *Fno* isolate that resulted in RPS of 68.8 % - 87.5 % [15]. Interestingly, the survival rates in the current study were higher than those obtained in zebrafish, *Danio rerio*, i.p. immunised with an *Fno*-outer membrane vesicle (OMVs)-derived vaccine and i.p. challenged with the same *Fno* vaccine isolate [34]. This suggests a weaker stimulation of the zebrafish immune system by OMVs compared to the adjuvanted whole cell vaccine used in the current study, although differences in susceptibility between tilapia and zebrafish to *Fno* may account for this variation.

The difference in the level of protection against the homologous isolate (i.e. *Fno* 1 isolate) obtained in the current study (RPS 82.3%) and the previous study [16] (RPS 100%) may be attributed to differences in the genetic make-up and susceptibility of the fish used. Wild type Nile tilapia obtained from a commercial farm were used in the present study and therefore would have been exposed to stressors commonly associated with the farm environment, while hybrid red tilapia raised in an in-house aquatic research facility were used for the previous vaccine study [16]. Differences in bacterial inoculum were also used in the experimental challenge between the two experiments. A bacterial dose of  $10^5$  CFU/fish induced ~ 70 % mortality in control fish in the current study, while  $10^3$  CFU/fish and  $10^5$  CFU/fish resulted in the same level of mortality in Nile tilapia and zebrafish, respectively [16, 34], which may have influenced the RPS values obtained in the different studies. A

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

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

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

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

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

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

A significant up-regulation of *IgM* transcription was noted in the spleen of immunised tilapia at 72 h<sub>pv</sub>. This indicates that activation of B cells in response to vaccination is correlated with the increased serum *IgM* detected 30 dpv. Our results are consistent with the findings of an earlier study [34], where the authors reported an up-regulation of *IgM* at 7 dpv that was maintained to 21 dpv following i.p. immunisation of zebrafish with *Fno*-derived OMVs. There is lack of information regarding the role of cellular immunity against piscine francisellosis and most of our understanding is based on results from vaccine experiments with *Francisella tularensis*. It was reported that *F. tularensis* has the ability to trigger T-cell mediated immune responses, mainly antigen-specific IFN- $\gamma$  responses [41, 42] and a strong cell-mediated immune response has also been suggested to prevent *Francisella* spp. infection in other vertebrates [43, 44]. In a recent study [34], a significant up-regulation of IFN- $\gamma$ -1 transcription in zebrafish 24 h post-immunisation with *Fno*-derived OMVs was reported that remained up-regulated until 21 dpv. These authors suggested that IFN- $\gamma$  prevents *Fno* from escaping from the zebrafish phagosomes containing *Fno* cells post-infection. Also, *IL-12* and *IL-17* appears to drive a strong T-cell proliferation in Atlantic cod challenged with *F. noatunensis* subsp. *noatunensis* (*Fnn*) [45]. In the current study, the transcription of *MHCII* was significantly up-regulated in the spleen of vaccinated fish 6 h<sub>pv</sub> and at 24-72 h<sub>pv</sub>, where a 4.5-fold change was detected, while a significant down-regulation was observed in the adjuvant alone group. This suggests successful recognition of *Fno* cells in the vaccinated fish and presentation of the antigen by antigen presenting 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-1 $\beta$*  and *TNF- $\alpha$* , which are  
503 produced primarily by activated macrophages in the spleen of the fish [46]. *IL-1 $\beta$*  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-1 $\beta$*  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-1 $\beta$*  expression was up-regulated in the splenic cells of Nile tilapia 24-  
508 96 hpc with *Fno* [48]. *TNF- $\alpha$*  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- $\alpha$*  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- $\alpha$*  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- $\alpha$*   
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- $\alpha$*  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



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

Analysis of the bacterial burden in the vaccinated fish showed they did become infected, albeit with significantly lower bacterial loads than the adjuvant-alone and PBS control fish 15 dpc. Thus, the protection provided by the developed vaccine may be associated with the ability to enhance clearance and limit dissemination of the infection. This supports the application of using bacterial load quantification as a measure of vaccine efficacy against *Fno*. Future histological studies investigating the inflammatory and tissue-associated damage post-challenge between vaccinated and control tilapia would allow greater insights into the protection mechanisms of the developed vaccine at the tissue level.

## 5. Conclusions

The current study represents the first report of a protective oil-based adjuvanted inactivated injectable vaccine against multiple isolates of *Fno* from diverse geographical origins for Nile tilapia following injection immunisation and injection challenge. The significantly higher RPS in the vaccinated fish was correlated with significantly higher specific antibody responses, lower bacterial burden and greater expression of *IgM*, *IL-1 $\beta$* , *TNF- $\alpha$*  and *MHCII* transcripts 72 hpc in comparison to the adjuvant alone or PBS control fish. This highlights the importance of antibody-mediated immune responses in the control of *Fno* infection in tilapia. In addition, the potential of the current vaccine to cross protect against different isolates of *Fno* was highlighted by immunoblotting. Taking into account the relatively short production cycle of tilapia in most of the tropical countries (6-9 months), a desirable vaccine must induce a significant long-term protection against *Fno*. Thus, future work will

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

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**Research Ethics:** All experimental procedures with live fish were carried out in accordance with the UK animals (Scientific Procedures) Act 1986 and associated guidelines (EU Directive 2010/63/EU for animal experiments) and were approved by the Animal Welfare and Ethical Review Body (AWERB) of the Institute of Aquaculture, University of Stirling, UK and the NRCT regulations in Thailand.

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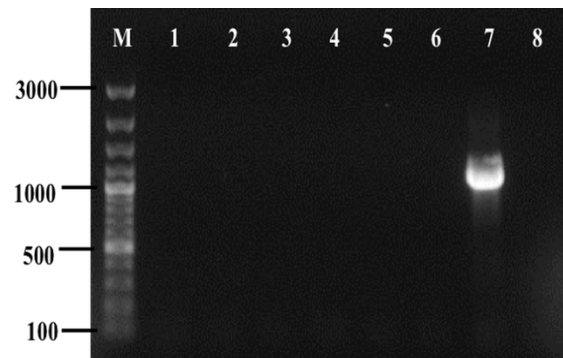
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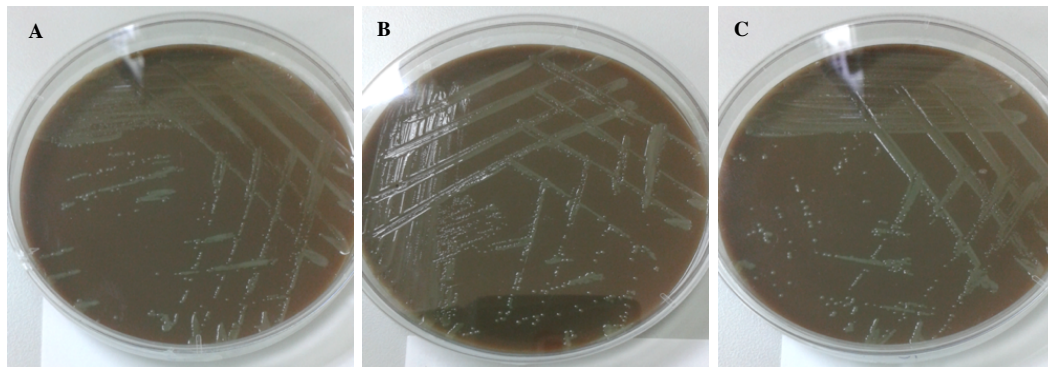
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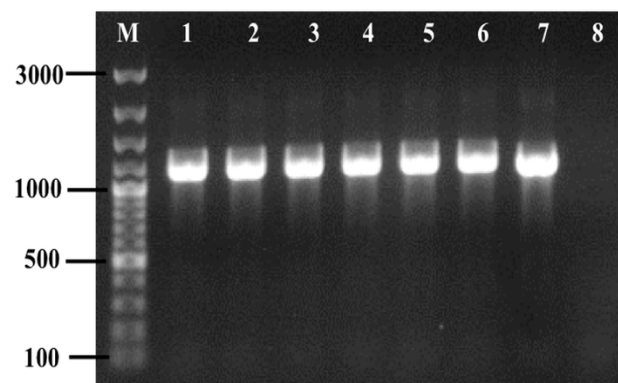
## Supplementary Materials



**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).