EFFECT OF ALGAL-DERIVED COMPOUNDS ON GROWTH AND SURVIVAL OF THE FISH PATHOGEN *Francisella noatunensis* subsp. *orientalis*

Thesis submitted to the University of Stirling for the degree of Doctor of Philosophy in Aquatic Veterinary Studies

by

Winarti Achmad Sarmin Djainal

MSc. Aquaculture Science, BSc. (Hons) Aquaculture

INSTITUTE OF AQUACULTURE

UNIVERSITY OF STIRLING, STIRLING, SCOTLAND

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To my beloved family, especially to my mum (Zuraida) and my dad (Achmad Sarmin). Love you. x

Supervisors and external advisers

Professor Alexandra Adams
Institute of Aquculture, University of Stirling
Stirling, Scotland, United Kingdom

Dr. Andrew Desbois
Institute of Aquculture, University of Stirling
Stirling, Scotland, United Kingdom

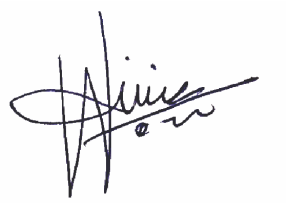
Dr. David Penman
Institute of Aquculture, University of Stirling
Stirling, Scotland, United Kingdom

Professor Dave Hoole
School of Life Sciences
Keele University, Keele, United Kingdom

Declaration

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted by any other degree.

Winarti Achmad Sarmin Djainal



Candidate's signature : _____

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List of Abbreviations

+w	Weak reactivity
3Rs	Re-placement, reduction and refinement
AMPs	Antimicrobial peptides
ARF	Aquatic research facility
AU	Absorbance units
AUC	Area under the curve
AUS	Austria
B	Brackish-water;
cDNA	Complementary DNA
CF	Culture filtrate
CFU	Colony forming unit
CHAH	Cysteine heart agar supplemented with bovine haemoglobin
CR	Costa Rica
d	day
DHA	docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpf	day post algae feeding
dpi	day post infection
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
F	Freshwater
FAO	Food and Agriculture Organization
Fm	Farm
Fnn	*Francisella noatunensis* subsp. *noatunensis*
Fno	*Francisella noatunensis* subsp. *orientalis*
GALT	Gut-associated lymphoid tissue
gDNA	Genomic DNA
GLA/DGLA	Gamma-linolenic acid / Dihomo-γ-linolenic acid
h	hour/hours
H & E	Haematoxylin and eosin staining

HCL	Hydrochloric acid
HK	Head kidney
hk	heat-killed
HSWB	High salt wash buffer
Id tests	Primary identification tests
IgG-HRP	Immunoglobulin G-horseradish peroxidase
iglC	Intracellular growth locus C gene
IHC	Immunohistochemistry
IM	Intramuscular
IP	Intraperitoneally
JPN	Japan
Kb	Kilo base-pair
bp	base-pair
KOH	Potassium hydroxide
LD_{40}	Lethal Dose causing 40% mortality
LD_{50}	Lethal Dose causing 50% mortality
LPS	Lipopolysaccharides
LSWB	Low salt wash buffer
LVS	live vaccine strain
MARCO	macrophage receptor with collagenous structure
MAS	Motile aeromonads septicaemia
MBC	Minimum bactericidal concentration
MEX	Mexico
MHB	Mueller-Hinton II broth supplemented with glucose and IsoVitaleX
MIC	Minimum inhibitory concentration
mins	Minutes
MMCs	Melanomacrophages centres
MOI	Multiplicity of infection
MRSA	methicillin-resistant *S. aureus*
MT	Million tones
NaCl	Natrium Chloride
NBF	Neutral buffer formalin
NBT	Nitroblue tetrazolium

NCIMB	National collection of industrial, food and marine bacteria
NE	Northern Europe
NO	Nitric Oxide
nr	Not reported
NVI	Norwegian Veterinary Institute
O/F	Oxidation/Fermentation
OD	Optical density
OMVs	Outer membrane vesicles
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PLOs	Piscirickettsia-like organisms
PMA	Phorbol 12-myristate 13-acetate
Pop	Populations
PUFAs	Long Chain-Polyunsaturated fatty acids
RLOs	Rickettsia-like organisms
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
RT-PCR	Real-time PCR
S	Sea/salt-water
secs	Seconds
T	Temperature
TAE	Tris–Acetic–EDTA
TET	Tetracycline
TSA	Trypticase soy agar
TSB	Tryptic soy broth
v/v or w/v	Volume per volume or Weight per volume
Wi	Wild

Abstract

Piscine francisellosis, caused by *Francisella noatuenensis* subsp *orientalis* (*Fno*), is an emerging infectious disease in the tilapia industry, but no effective commercial treatments or vaccines are available. The use of immunostimulants is a promising method to control diseases in aquaculture, and various algae and algal-derived compounds are potent immunostimulants for improving immune status. Algae produce a great variety of secondary metabolites that exert a broad spectrum of biological activities. The aim of this thesis was to evaluate the effectiveness of algal compounds against *Fno in vitro* and *in vivo* and determine their potential to control francisellosis infection in Nile tilapia *Oreochromis niloticus* L. under experimental conditions, and in an alternative host, namely the greater wax moth *Galeria mellonella*. Some of the algae and their compounds (*Chlorella* sp., alginic acid, and ß-glucan) exerted antimicrobial activity *in vitro* against *Fno, Aeromonas hydrophila* and *Streptococcus agalactiae* and stimulated responses of Nile tilapia macrophages (Chapter 2). An immersion challenge model for *Fno* STIR-GUS-F2f7 was developed in two genetic groups of Nile tilapia, and the homo gold strain was more susceptible to infection than wild type (Chapter 3). *In vivo* trials were conducted in Nile tilapia homo gold where fish were fed diets supplemented with 10% *Scenedesmus quaricauda*, 10% *Haematococcus pluvialis*, and 0.1% or 0.2% alginic acid or ß-glucan, and then challenged with *Fno* and co-infected with *S. agalactiae* (Chapter 4). The *Fno* challenge failed to produce mortality; however, co-infection resulted in high mortalities in all groups. As the *in vivo* trial in tilapia could not be to repeated, a *G. mellonella* model for *Fno* was validated. *Fno* doses between 0.7–1.7 x 10^8 CFU mL^{-1} killed *G. mellonella*, while tetracycline, alginic acid and ß-glucan rescued the wax moth from lethal doses of bacteria (Chapter 5).

Key words: Nile tilapia; francisellosis; immunostimulants; algae.

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CHAPTER 1
GENERAL INTRODUCTION

1.1 World tilapia aquaculture industry

The farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants, in freshwater (warm or cold-water), brackish-water and seawater environments is known as aquaculture. The aquaculture industry has become an important source of food for human consumption, as the capture fishing industry has declined, and wild stocks diminished. According to FAO (2016) global coverage of aquaculture production statistics has continued to improve, with a record 200 countries and territories. As a result, production of farmed food fish worldwide is growing more rapidly than all other food animal producing sectors; its contribution to global supplies increased from 55.7 million tonnes (MT) in 2009 to 73.8 MT in 2014, which comprises 49.8 MT of finfish, 16.1 MT of mollusks, 6.9 MT of crustaceans, and 7.3 MT of other aquatic animals including frogs, freshwater turtles, sea cucumbers, sea urchins, sea squirts and jellyfish (Josupeit, 2007; FAO, 2014, 2016; Fitzsimmons, 2016).

Half of total global aquaculture production in 2016 was finfish, amounting to 49.8 MT with an estimated first-sale value of US$99.2 billion (FAO, 2016). Furthermore, the number of finfish species included 362 species including hybrids, and of the various cultured species among this group, tilapia is one of the most popular farmed fish (FAO, 2016). Tilapia *Oreochromis* spp. is one of the most-exploited inland water fish species in tropical to sub-tropical ecosystems and most-produced fish species in aquaculture since 2004 (FAO, 2016). Tilapia has been one of the major food-fish aquaculture species since 1994 in Taiwan (Chen *et al.*, 1994), and in sub-Saharan Africa, even though tilapia were recently replaced with the carnivorous North African catfish *Clarias gariepinus*. Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) are the main cultivated fish species in the Brazilian aquaculture (Assis *et al.*, 2017; Sebastião *et al.*, 2017).

Tilapia have many attributes that are considered to be ideal for aquaculture such as their hardy nature, fast growth, and tolerance to a wide range of environmental conditions (such as temperature, salinity, low dissolved oxygen, etc.) (El-Sayed, 2006). Tilapia are currently farmed in fresh-, brackish- and even seawater environments, since some tilapia are euryhaline and can

tolerate ranges of water salinity even up to 10 ppt (Josupeit, 2005; El-Sayed, 2006). In addition, tilapia has high fecundity and the ability to reproduce in captivity with a short generation time, while also feeding on low trophic levels and accepting artificial feeds immediately after yolk-sac absorption (El-Sayed, 2006). Tilapia have a relatively lower unit value compared with other types of farmed fish and are typically relative easy-to-raise in simple aquaculture systems with low levels of technology, and antibiotics and chemicals are often not needed (Josupeit, 2005; El-Sayed, 2006; Little *et al.*, 2008; Jeffery *et al.*, 2010). Furthermore, tilapia can be reared in water of relatively low oxygen content and over a broad range of water temperatures, from 15 to 40°C (Josupeit, 2005; Soto and Revan, 2012; Joshi and Ravindranath, 2017). Tilapia culture is also not necessarily dependent on fishmeal diets as tilapia species can be either omnivorous or herbivorous, which provides greater food security and is therefore a good candidate for food production from aquaculture (Josupeit, 2005).

Tilapia culture has been established in many tropical and subtropical regions, including areas beyond their native ranges, where they have been introduced for various purposes, *e.g.* farming as food fish, insect and aquatic weed control, recreational fishing, tuna bait and research purposes (El-Sayed, 2006; Fitzsimmons, 2015). Tilapia farming is the most widespread type of aquaculture and it occurs in more than 140 countries and territories on all continents, except Antarctica (El-Sayed, 2006; Josupeit, 2007; FAO, 2014, 2016; Fitzsimmons, 2016). However, because commercially farmed tilapia are yet to be reflected separately in national statistics in Canada and some European countries, the true number of producer countries is higher (FAO, 2014). The annual production of farmed tilapia has increased from over 2.5 MT in 2005 (Josupeit, 2007), to 4.5 MT in 2012 (FAO, 2014), then to 5.6 MT in 2015 (Figure 1.1), and it is expected to reach 8.5 MT in 2026 (Fitzsimmons, 2016). China is the world's largest producer of tilapia, but production continues to spread and Indonesia, Egypt, the Philippines, Thailand, Bangladesh, Burma, Vietnam, Latin America, Ghana are significant producers, while commercial culture also occurs in France, Germany, the Netherlands, Great Britain, Belgium, Spain, Canada, Korea, Japan and most states in the US (Fitzsimmons, 2016). Tilapia are

known as a ‘democratic fish’ for people living in Sub-Saharan Africa, since the fish is consumed as an affordable source of protein for the poorest farmers while at the same time being a premium product for sale into international and upscale markets (Mapfumo, 2015). Also called aquatic chicken because of wide range of production systems (El-Sayed, 2006; Fitzsimmons *et al.*, 2011; Mapfumo, 2015; Joshi and Ravindranath, 2017).

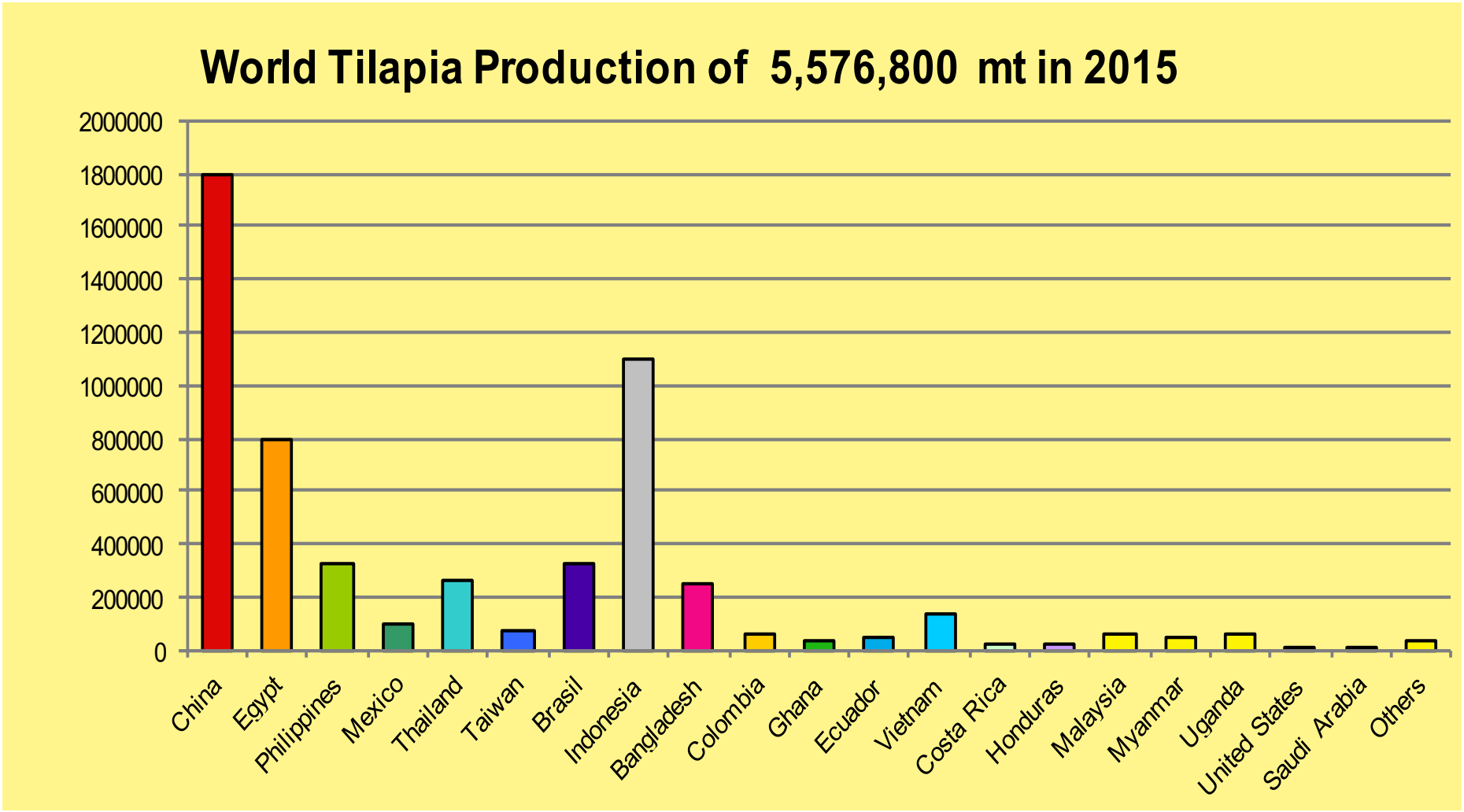

Figure 1.1 World tilapia production of 5,576,800 million tonnes in 2015 from the regions of tilapia rapid production growth (Fitzsimmons, 2016).

Based on their geographical distribution, tilapia is one of the most widely introduced fishes in the world, especially the Mozambique tilapia *Oreochromis mossambicus* (Linnaeus, 1758) which was introduced from 1940-1950 (Figure 1.2). Later they were known as one of the most invasive pest fishes because of their good adaptability to various environmental conditions and rapid breeding, competing with and even displacing endemic species (Joshi and Ravindranath, 2017). As a consequence, tilapia was included in the list of 100 of the 'World's Worst Alien Invasive Species' by the International Union for Conservation of Nature (Fitzsimmons, 2015; Fitzsimmons and Zengeya, 2015). However, tilapia continue to spread worldwide and for farming purpose the more desirable Nile tilapia species was introduced and disseminated worldwide from 1960–1980 (Figure 1.3) (Joshi and Ravindranath, 2017).

Among other tilapia species, Nile tilapia *O. niloticus* is one of the most commercially important farmed species in the aquaculture industry (Josupeit, 2005; El-Sayed, 2006). Nile tilapia can grow to a length of 60 cm and 3.6–4.3 kg and it is the most popular species in terms of both capture fisheries and aquaculture. As an aquaculture species, Nile tilapia grows very fast and its production accounts for ~80% of the total tilapia production worldwide (FAO, 2016). Other tilapia species are also gaining some popularity in certain parts of the world, *e.g.* Blue tilapia *O. aureus*, redbreast tilapia *Tilapia rendalli*, redbelly tilapia *Tilapia zilii*, three-spotted tilapia *O. andersonii* and longfin tilapia *O. macrochir* (El-Sayed, 2006; Joshi and Ravindranath, 2017).

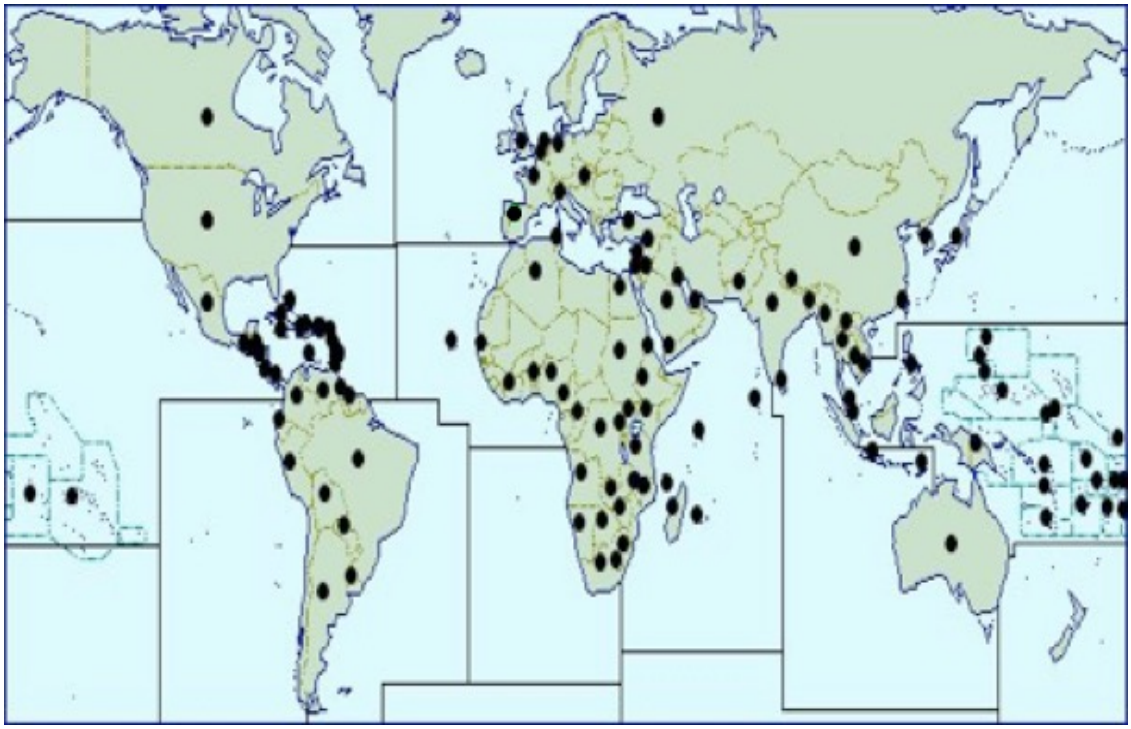

Figure 1.2 Geographical distribution of Mozambique tilapia *O. mossambicus* worldwide (black dot: present) [Source: Centre for Agriculture and Bioscience International–Invasive Species Compendium (Fitzsimmons, 2015)].

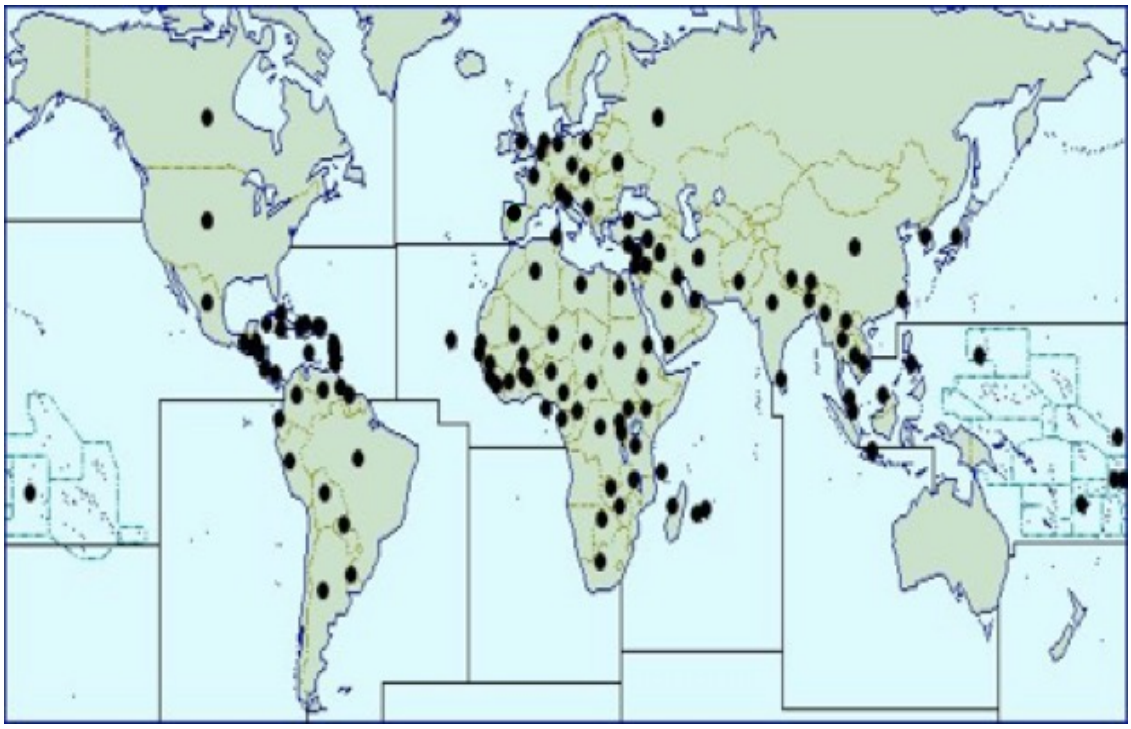

Figure 1.3 Geographical distribution of Nile tilapia *O. niloticus* worldwide (black dot: present) [Source: Centre for Agriculture and Bioscience International–Invasive Species Compendium (Fitzsimmons and Zengeya, 2015)].

Some tilapia species are easy to hybridize and are highly fecund which has allowed crossing of several species to develop strains with the best traits from each of the parent species. The aim was to produce tilapia species with greater consumer acceptance, including a more edible fillet product, salt tolerance and living in seawater increases tastiness, and fast growth with low animal protein intake (Josupeit, 2005; Fitzsimmons *et al.*, 2011; FAO, 2016). The world production of tilapia hybrids was 333,300 tonnes in 2010, however, production could be greater than this estimate as there are many tilapia hybrids worldwide that have not been reported (FAO, 2016). In addition, some strains of red tilapia have arisen from selective breeding of a hybrid of *Oreochromis* spp., including populations from Florida, Hawaii, Taiwan, South African and Israel (Table 1.1) (El-Sayed, 2006; Fitzsimmons *et al.*, 2011; Joshi and Ravindranath, 2017). The main rationale to produce red Nile tilapia hybrids is to develop marketable strains and to gain a premium price due to its attractive color, and in certain Asian communities it is believed that red color is the color of “good luck” (Figure 1.4).

Table 1.1 Hybrids of red tilapia produced from genetic mutants (all-males produced by hormonal treatment)

No.	Name of hybrid	Cross between	Chief traits
1	Taiwanese Red Tilapia Hybrid (first reported Red Tilapia - 1960)	Mutant Reddish-Orange Female *O. mossambicus* X Normal Male *O. niloticus*	Tolerates higher salinities
2	Florida Red Tilapia Hybrid (1970) (Mozambique Tilapia X Zanzibar Tilapia)	Red-Gold Male *O. mossambicus* X Normal Female *O. hornorum*	Tolerates higher salinities
3	Israeli Red Tilapia Hybrid (Nile Tilapia X Blue Tilapia)	Red Female Nile Tilapia from Egypt *O. niloticus* X Wild-type Male Blue Tilapia from Israel *O. aureus*	Tolerates higher salinities
4	South African 'Red-5' Strain of Mozambique Tilapia	Red *O. mossambicus* X Best-performing Normal *O. mossambicus* and Backcrossed with Red	The most salt-tolerant Red Tilapia
5	Israeli ND-56 Red Tilapia (Tetra-hybrid Strain) **(Figure 1.4)**	Female *O. niloticus* ND-5 Family-line X Male *O. niloticus* ND-6 Family-line	Uniform orange colour skin, salt tolerant

[Source: Fitzsimmons et al., (2011); Joshi and Ravindranath, (2017)]

Figure 1.4 Red Nile tilapia, "ND-56 or commonly known as Saint Peter or San Pietro" is an orange colour tetra-hybrid tilapia developed in Israel and obtained by crossing two F1 generation (hybrid males *O. niloticus* X *O. aureus* and females *O. mossambicus* X *O. uroleps hornorum*) (Hilsdorf *et al.*, 2002; Marengoni *et al.*, 2015) [Photo credit: Fitzsimmons et al., 2011].

Tilapia comprise about 70 to 100 species of mainly warm-freshwater fish belonging to the family Cichlidae (Steffens and Hilge, 1999). The word 'tilapia' originally came from A. Smith (its initiator), to sound like a Bushman word meaning 'fish' (Trewavas, 1982a). Tilapia are originally from the lakes of tropical Africa and the Levant (Eastern Mediterranean countries). The first trials of tilapia culture were recorded in Kenya in the 1920s and were successful internationally introduced during the second half of the 20th century and used for mass production in aquaculture (Josupeit, 2005; El-Sayed, 2006; Joshi and Ravindranath, 2017).

Based on breeding behaviour and feeding habits, tilapia were divided into two subgenera *e.g. Tilapia* (substrate spawners) and *Sarotherodon* ('brush-toothed') (mouth brooders) first described by Smith (1840) and Ruppell (1852), respectively (Trewavas, 1982a) (Table 1.2). The substrate spawners build nests on the bottom, while mouth brooders incubate the fertilized eggs and hatched fry in the mouth (Trewavas, 1982a). Based on the parent that performs the mouth brooding behavior, *Sarotherodon* were divided into two genera comprising the genus *Sarotherodon* male mouth brooders (paternal) or both parental sexes mouth-brooders (bi-parental), and the genus *Oreochromis* (mountain cichlids) are female parents (maternal mouth-brooders) (Trewavas, 1982a; b; Steffens and Hilge, 1999; El-Sayed, 2006).

Table 1.2 Genera of tilapia (Trewavas, 1982a; b)

Genus	Sub-genus	Species	Other species
Tilapia A. Smith	(three to six)	*T. sparmanii* A. Smith	*T. rendalli* Boulenger *T. zilli* Gervais
Sarotherodon Ruppell		*S. melanotheron* Ruppell	*S. galilaeus* (Linn.) *S. linnellii* (Lonnberg)
Oreochromis Gunther	*Oreochromis* Gunther	*O. hunteri* Gunther	*O. (O.) niloticus* (Linn.) *O. (O.) mossambicus* (Peters) *O. (O.) aureus* (Steindachner) *O. (O.) spilurus* (Gunther)
	Nyasalapia Thys	*O. (N.) squamipinnis* (Gunther)	O*. (N.) macrochir* (Boulenger) *O. (N.) variabilis* (Boulenger) *O. (N.) angolensis* (Treawavas)
	Alcolapia Thys	*O. (A.) grahami* (Boulenger) *= O. alcalicus grahami*	*O. (A.) alcalicus alcalicus* (Hilgendorf)
	Neotilapia Regan	*O. (N.) tanganicae* (Boulenger)	None
Danakilia Thys		*D. franchetti* (Vinciguerra)	None

1.2 Bacteria diseases of tilapia

Disease outbreaks are a major problem for the tilapia farming industry and caused significant economic losses (Birkbeck *et al.*, 2011). This is not just due to mortality but also associated decreases in the quality of the surviving fish such as reduced growth rates and/or product quality (Ramirez-Paredes, 2015). As with all species of farmed fish, tilapia are affected by disease, and susceptibility of tilapia to infection can easily be increased when farming conditions are poor (Dong *et al.*, 2017). This commonly occurs in intensive culture systems with high stocking densities, excessive handling, poor water quality, and when biosecurity is inadequate, and such conditions lead to increased stress and may trigger an infectious disease to spread (Dong *et al.*, 2017). Opportunistic bacteria are one of the most important groups of pathogens associated with disease outbreaks and cause massive economic losses in farm-raised cultured tilapia (Colquhoun and Duodu, 2011). Several bacterial diseases can affect tilapia such as *Enterococcus* sp., mycobacteriosis caused by *Mycobacterium* spp., vibriosis caused by *Vibrio* spp., columnaris disease caused by *Flavobacterium columnare*, motile aeromonas septicemia (MAS) caused by *Aeromonas hydrophila*, streptococcosis caused by *Streptococcus* spp., and the emerging disease “francisellosis” caused by *Francisella* spp. Among other bacterial pathogens in tilapia, *A. hydrophila* and *Streptococcus* spp*.,* are commonly found and caused disease outbreaks in natural and cultured tilapia, while, *Francisella* spp., particularly *Francisella noatunensis* subsp. *orientalis* is an emerging pathogen in tilapia worldwide.

Motile aeromonads are rod-shaped in appearance with a diameter and length of 1.0–3.5 and 0.3–1.0 µm, respectively. They are Gram-negative, catalase and oxidase positive, facultative aerobic, non-sporulating and motile (Cipriano, 2001). *Aeromonas* spp., generally live in freshwater containing high organic matter and they are capable of living at 15–37°C with an optimum temperature of 20–30°C and pH between 5.5–9.0 (Cipriano, 2001). *A. hydrophila* causes red spot disease, haemorrhagic septicaemia or bacterial haemorrhagic septicaemia or MAS that affects nearly all species of freshwater fish including tilapia (Austin and Austin, 2007).

Streptococcus spp., causing streptococcosis, are colonies of 1 mm in diameter, spherical, Gram-positive, fermentative, catalase-negative, oxidase-positive, non-motile and do not grow at temperatures of 10, 37 or 45°C but can grow at pH 9.6 (Austin and Austin, 2007). The clinical symptoms of these diseases are serpentine swimming, skin lesions and necrotizing myositis, lethargy, meningoencephalitis and panophthalmitis, abdominal swelling, stomach and intestines filled with gelatinous fluid or yellowish in some fish, a turgid gallbladder, swollen liver, kidneys and spleen congestion, brain and liver haemorrhages, small haemorrhagic lesions in the eye, exophthalmos and corneal opacity (Amal *et al.*, 2015). There are six species that are as pathogenic to fish, namely *Streptococcus iniae*, *S. agalactiae*, *S. dysgalactiae*, *S. phocae*, *S. parauberis* and *S. ictaluri*, *S. agalactiae* and *S. iniae* are the most common species causing disease outbreaks in cultured tilapia and these species are associated with high economic losses worldwide (Perera *et al.*, 1994; Evans *et al.*, 2003; Mian *et al.*, 2009; Abuseliana *et al.*, 2010; Figueiredo *et al.*, 2012; Huang *et al.*, 2013; Kayansamruaj *et al.*, 2014; Amal *et al.*, 2015; Assis *et al.*, 2017).

1.3 *Francisella* spp.

Bacteria belonging to the genus *Francisella* (Francisellaceae) have “emerged” as serious pathogens in both wild and farmed fish of various species from numerous geographical territories worldwide for over the last two decades. *Francisella* spp. cause piscine francisellosis and are the causative agents of chronic granulomatous and pyogranulomatous lesions in fish, and these are divided into certain subspecies, depending mainly on whether the disease occurs *e.g.* in warm- and/or temperate water fish, namely *Francisella noatunensis* subsp. *orientalis* (*Fno*; syn. *F. asiatica*) or cold-water environments, specifically *F. noatunensis* subsp. *noatunensis* (*Fnn*; syn. *F. piscida*). Recently, Kamaishi *et al.*, (2010) and Brevik *et al.*, (2011) proposed a novel aquatic bacterial species called *F. halioticida* subsp. *novicida* (=LMG26062T, =DSM23729T) that infected abalone in marine cold-water.

Commonly, the gross signs of francisellosis infection of fish is their appearance at the water's surface or near aeration, in addition to anorexia, dyspenea, lethargic behaviour, lack of appetite, abdominal distension, gasping, some presence of numerous focal to multifocal haemorrhagic skin lesions scattered over the abdomen (petechial on the sides) and also apparent in the dorsal and ventral fins, and excess of deficit of skin mucus (Chern and Chao, 1994; Hsieh *et al.*, 2006; Jeffery *et al.*, 2010; Ramirez-Paredes, 2015). Fish become emaciated, anaemic, have pale or darkened body colourations, loss of scales, show ulcerations in severe cases, erosions, fins appear pale and light fraying, gills are pale and mildly thickened, exhibit mild to moderate lateral or bilateral exophthalmia and display a marked to severe diffuse branchial pallor, corneal opacity and perforation, enlargement of the abdominal cavity due to varying degrees of bloody ascites with or without swollen organs, and fish may be listless in a moribund stage (Chen *et al.*, 1994; Mauel and Miller, 2002; Mauel *et al.*, 2003, 2005, 2007, Soto *et al.*, 2009b, 2011b; Colquhoun and Duodu, 2011).

Internally the infected fish have empty intestines and very large gall bladders (Soto *et al.*, 2009a; Jeffery *et al.*, 2010). Both subspecies of *Francisella* can cause multiple chronic granulomatous infections (white nodules) in several internal organs such as the spleen (Colquhoun and Duodu, 2011); anterior and posterior kidney, liver, heart (Ottem *et al.*, 2009); intestine, gastrointestinal walls (Birkbeck *et al.*, 2011); testes, ovary, choroid gland (Mauel *et al.*, 2003); eye, swim-bladder (Hsieh *et al.*, 2006); stomach, mesenteric fat (Lin *et al.*, 2016); and the gills (Ortega *et al.*, 2016). Head kidneys are markedly enlarged with up to 90% parenchymal replacement by coalescing granulomatous inflammation (Soto *et al.*, 2011b). The spleen can be enlarged by ~5 to 50-fold in size or up to 10-fold heavier than normal fish, with variable-sized white nodules (Chern and Chao, 1994; Mauel *et al.*, 2003, 2005, 2007; Soto *et al.*, 2011b). Infected spleens comprise numerous, up to 2-microns wide, cystic, fluid-filled cavities randomly distributed throughout the tissue, while the fluid within the cystic cavities is clear, tan or serous (Soto *et al.*, 2011b). Basophilic granules may be observed within enlarged vacuolated cells of the kidney, spleen, ascites and ulcerated skin (Chen *et al.*, 1994).

Outbreaks of diseases caused by members of the genus *Francisella* have been reported from a variety of fish and shellfish species in different geographical locations and aquatic environments worldwide. These constitute an important group of fish pathogens, with the potential for significant effects on the health of cold- and warm-water species from fresh-, brackish-, and sea-water. Cod and tilapia are well-known to be highly susceptible hosts of *Fnn* and *Fno,* respectively, resulting in high morbidity and mortality rate (Colquhoun and Duodu, 2011; Birkbeck *et al.*, 2011). The number of outbreak reports resembling francisellosis have increased over the last two decades and have been reported in various warm-water fish species especially tilapia, and *Fno* is considered to be an emergent disease worldwide.

1.4 Piscine francisellosis, an emerging pathogen in tilapia

1.4.1 Aetiological agent

Diagnosis of francisellosis is challenging due to the highly fastidious properties of the micro-organisms, low numbers of bacteria needed to cause significant mortality, the presence of multiple infectious agents, and the emergent nature of the disease (Soto *et al.*, 2009a, 2011b). Rickettsia-like organisms (RLOs), piscirickettsia-like organisms (PLOs) or *Mycobacterium* spp., have been identified as the cause of disease in wild and farmed fish for the past seven decades, and early efforts to characterize these diseases were hindered by difficulties in recovering and culturing the responsible agent(s). However, recently the use of molecular diagnostic methods has confirmed that the bacteria most often causing these diseases belong to the families Piscirickettsiaceae and Francisellaceae (Birkbeck *et al.*, 2011). The first report of disease outbreaks that confirmed the involvement of a *Francisella* sp. in piscine francisellosis was in farmed three-line grunt *Parapristipoma trilineatum* from Japan in 2005 (Kamaishi *et al.*, 2005). The bacteria were found mainly in the kidney and spleen from which DNA was extracted. Then the small subunit rRNA was amplified by PCR and sequenced, this showed 97.3-98.5% homology to *Francisella* spp., with *F. philomiragia* determined to be the closest

neighbor. The causative agent was identified as a highly virulent bacterium from the genus Francisella, and the named *Francisella noatunensis* subsp. *orientalis* was proposed by Ottem *et al.*, (2009).

1.4.2 Histopathology

Histological examination of fish with francisellosis reveals the presence of multifocal granulomatous formation and numerous small, pleomorphic cocco-bacilli that stain basophilic with hematoxylin and eosin or magenta-colored bodies stained with Giemsa (Chern and Chao, 1994; Mauel *et al.*, 2003, 2007; Soto *et al.*, 2009a; Nguyen *et al.*, 2016). The most dramatic histopathological changes include vascular lesions with thrombosis, necrosis and oedema with a granulomatous response, and these are found in the haemopoietic tissues such as anterior kidney and spleen (Chern and Chao, 1994; Chen *et al.*, 1994). These are indications that these tissues have been targeted by the organism (Jantrakajorn and Wongtavatchai, 2016).

Granulomatous inflammation in the meninges with focal and mild inflammatory cellular infiltration, and necrosis and thrombotic lesions have also been reported in the hepatocytes of the liver, brain, heart, mesentery, testis, pancreas, ovaries, muscle, pseudo-branch, nasal capsule, adipose tissue (Chen *et al.*, 1994), testes, choroid gland (Mauel *et al.*, 2003, 2005, 2007), and in the eye (Jeffery *et al.* 2010). According to Mauel *et al.*, (2003), based on histopathological examination of 28 affected Nile tilapia by francisellosis in Latin American fish farms, granulomas were in the muscle, gills, spleen, kidney, liver, heart, and testis, at levels of 10.7, 35.7, 82.1, 92.8, 32.1, 28.5 and 14.2%, respectively. A 100% presence of granulomas was oberved in the organs of 120 tilapia in Taiwan in the spleen and kidneys, 85.8% in the liver, 72.5% in gills, 63.3% in gonads, 56.7% in gastrointestinal, 47.5% in heart, 40% in swim bladder and 30.8% on eyes (Hsieh *et al.,* 2006). Granulomas progress according to the age of the fish, as demostrated using 187 spleens of infected Nile tilapia of various ages and showed 15% in fingerling fish (1–10g; n=80), 75% in market-size fish (500–750 g; n=83) and 100% in brood-stock (≥1,000 g; n=24) (Mauel *et al.*, 2007). The initial lesions were multi-focal aggregations of

large foamy cells (mixed macrophages, lymphocytes and neutrophils) containing coco-bacilli in their cytoplasm which were frequently observed in the necrotizing or granulomatous lesions, with necrosis gradually developed in the central area, and finally, fibrous encapsulation formed in the outermost zone (Hsieh *et al.*, 2006; Ortega *et al.*, 2016).

1.4.3 Treatment

There is no commercially available effective treatments or vaccine to control *Fno* infection at present, although it is considered to be one of the most pathogenic bacteria in tilapia culture. Antibiotics have been used extensively to attempt to treat the disease and are considered as one of the most important tools for a rapid first treatment option for *Francisella* infections in mammals (including humans) as well as fish, but they are costly and difficult to deliver to the intracellular environment where these pathogens localize (Smith *et al.*, 1997). Numerous antibiotics have been used to control piscine francisellosis in tilapia and the efficacy of these therapies have been assessed in *vitro* and/or *in vivo* efficacies (Table 1.3). However, there are currently few approved antibiotics to control aquatic *Francisella* infections of teleosts, and wide-spread use of antibiotics to control these infections, in the face of continued emergence of antibiotic-resistant human and animal pathogens, is unlikely to be sanctioned by regulators (Navas, 2002).

Table 1.3 Francisellosis treatment using various antibiotics by *in vitro* and/or *in vivo* efficacies

No	Antibiotic	Concentration (μg mL^{-1})	*Fno* Isolate Used	Method	Reference
		In Vitro			
1	Oxytetracycline, Tetracycline, Erythromycin, and Chloramphenicol	0.01, 0.04, 0.1, 4.0, 1.0, 4.0, 10, 40, and 100	RLO suspected *Fno*	MIC and MBC (the MBC was ten times higher that of the MIC)	(Chern and Chao, 1994)
2	Romet, Terramycin, and Florfenicol			antimicrobial disc diffusion (Kiby–Bauer) (NCCLS 2003)	(Mauel *et al.*, 2007)
3	Florfenicol	2		MIC broth dilution method	(Soto *et al.*, 2010a)
4	Florfenicol	10		Tilapia head kidney-derived macrophages cells culture	(Soto *et al.*, 2010a)
5	Florfenicol	10 and 100		Lactate dehydrogenase cytotoxicity assays	(Soto *et al.*, 2010a)
6	Enrofloxacin, gentamycin, neomycin, oxytetracycline, tetracycline, florfenicol, streptomycin, novobiocin, amikacin, ciprofloxacin, imipenem, atifloxacin, meropenem, tobramycin, nitrofurantoin, oxolinic acid, and levofloxacin; and resistant to penicillin, amoxicillin, ampicillin, piperacillin, oxacillin, erythromycin, novobiocin, tylosin tartrate, clyndamycin, sulphathiazole, sulphadimethoxine, trimethropin / sulfamethoxazole, piperacillin / tazobactam constant 4, ticarcillin / clavulanic acid constant 2, ampicillin / sulbactam 2:1 ratio, aztreonam, ceftiofur, cefazolin, cefepime, cefotetan Na, ceftriaxone, ceftazidime, cefoxitin, cefuroxime and vancomycin	0.25, 0.5, 1.0			(Soto *et al.*, 2012a)
7	Gentamicin, enrofloxacin, ampicillin, oxytetracycline, erythromycin, florfenicol, flumequine, and oxolinic acid	0.002 – 16.00	*Fno* isolates by 3 fish i.e. three-line grunt, hybrid stripe bass, and		(Soto *et al.*, 2016)

No	Antibiotic	Concentration (μg mL^{-1})	*Fno* Isolate Used	Method	Reference
			tilapia		
8	Enrofloxacin, kanamycin, gentamicin, tetracycline, oxytetracycline, florfenicol, oxolinic acid, and streptomycin	2 – 30	Nothern Europe and Ehime-1	Disc diffusion assay (Kirby–Bauer)	(Ramirez-Paredes, 2015)
9	Enrofloxacin, Gentamycin, Ceftiofur, Neomycin, Erythromycin, Oxytetracycline, Tetracycline, Amoxicillin, Spectinomycin, Sulphadimethoxine, Trimethoprim / sulfamethoxazole, Florfenicol, Sulphathiazole, Penicillin, Streptomycin, Novobiocin, Tylosin tartrate, Clyndamycin, Amikacin, Ampicillin, Ampicillin/sulbactam, Aztreonam, Cefazolin, Cefepime, Cefotetan Na, Ceftriaxone, Ceftazidime, Cefuroxime, Ciprofloxacin, Gatifloxacin, Meropenem, Imipenem, Nitrofurantoin, Cefoxitin, Piperacillin, Piperacillin/tazobactam constant 4, Ticarcillin/clavulanic acid constant 2, Tobramycin, and Cefpodoxime	0.12–1024 or 2/38–0.5/9.5 or 2:1 ratio (4/2–32/16 mμ mL^{-1}) or (128/4–16/4 mμ mL^{-1}) >128 or (64/2–16/2 mμ mL^{-1})	Nothern Europe and Ehime-1	MIC methods (*i.e.* GN2F and AVIAN1F Sensi-titre Plates)	(Ramirez-Paredes, 2015)
		In Vivo			
10	Oxytetracycline	30–50mg kg^{-1} body weight per day		Oral administration in 75 g Mozambique tilapia at low temperature 18°C treated for 14 d	(Chen *et al.*, 1994)
11	Oxytetracycline	4g/2.2kg body weight per day		in 10 g Blackchin tilapia for 10 d	(Mauel *et al.*, 2003)
12	Oxytetracycline	2.5g kg^{-1} of food per day		in post-larvae of red tilapia (<1–2 cm) for 10 d	(Iregui *et al.*, 2011)
13	Florfenicol	15 mg kg^{-1} of fish body weight		Medicated-feed trials in tilapia for 10 d	(Soto *et al.*, 2010a)
14	Florfenicol	0, 15 and 20 mg kg^{-1} body weight per day		Nile tilapia for 10d medicated-feed treatment and 21 d *Fno* challenge	(Soto *et al.*, 2013a)

1.4.4 Prevention and control

As with other infectious diseases, good husbandry and bio-security practices are key to prevention of piscine francisellosis in tilapia culture (McDermott and Palmeiro, 2013). In addition, the disease can be reduced by improved management practices which are essential to prevent many infectious agents including *Fno*, and this typically comprises using low stocking densities with a decreased biomass per site; a high standard of water conditions in the cage environment; separating year classes at each site to prevent transmission of the agent between year classes; elimination of dead and clinically diseased fish; appropriate disposal of blood from harvested fish; removal of infected brood stock and their eggs; and the routine disinfection of eggs (Branson and Diaz-Munoz, 1991; Turnbull, 1993; Rodger and Drinan, 1993; Almendras and Fuentealba, 1997).

There is no effective and efficient method of treatment to prevent and control francisellosis, but vaccination approaches hold promise as they have been used in the control of many other fish diseases (Adams, 2016), and vaccines have playe an important role in sustainable aquaculture production (Lagos *et al.*, 2017). Several researchers have reported successful vaccine development to control piscine francisellosis. For example, an autologous live attenuated vaccine in tilapia was developed, with a deletion mutation at the intracellular growth locus C gene (*iglC*) (Soto *et al.*, 2009b), and this vaccine conferred a relative percentage survival of 87.5% in Nile tilapia after experimental infection by immersion (Soto *et al.*, 2011a).

In addition, a current patented vaccine has been developed based on a genetically attenuated *Fno* strain in tilapia (Hawke and Soto, 2012). An autologous *Fno* inactivated (formalin killed) vaccine proved to be 100% effective and protected red Nile tilapia after IP challenged under laboratory conditions (Ramirez-Paredes, 2015). An outer membrane vesicles (OMV) based vaccine against *Fno* has also been developed and assessed using mass spectrometry both *in vivo* and *in vitro* (Lagos *et al.*, 2017). According to the

same authors, the OMV *Fno* vaccine protected zebrafish from subsequent challenge with a lethal dose of *Fno* (Brudal *et al.*, 2015).

1.5 *Francisella noatunensis* subsp. *orientalis*

1.5.1 Historical background and taxonomy

The RLO and PLO groups include both the Piscкirickettsiaceae and the Francisellaceae families, which are relatively closely related and similar in morphology and pathogenesis (Mikalsen *et al.*, 2008; McDermott and Palmeiro, 2013). In spite of morphologic similarities, the genera *Piscirickettsia* and *Francisella* belong to the y-proteobacteria and are, consequently, only vaguely linked to the true Rickettsia (α–proteobacteria) (McDermott and Palmeiro, 2013).

The first report of the disease outbreaks caused by RLOs in tilapia was observed in Kaoshiung area (southern Taiwan) and soon thereafter spread to at least 37 farms around the island from October 1992 to April 1993, where the species infected were Mozambique tilapia *O. mossambicus*, Nile tilapia *O. niloticus*, Blue tilapia *O. aureus*, redbelly tilapia *Tilapia zillii* and Wami tilapia *T. hornorum* (Chern and Chao, 1994; Chen *et al.*, 1994; Hsieh *et al.*, 2006). Outbreaks of francisellosis have also been reported in Hawaii, Jamaica, Indonesia and continental US (Southern California, Florida and South Carolina) during the 1990s to 2003 (Mauel and Miller, 2002; Mauel *et al.*, 2003). *Fno* was characterized in Hawaiian tilapia *O. mossambicus* and *S. melanotheron* in 1994–2003 (Mauel *et al.*, 2003). Occurrences of other PLOs have also been reported in farmed tilapia from the continental US (Florida, California and South Carolina) during 2001-2003 (Mauel *et al.*, 2005). Importantly, molecular techniques have retrospectively confirmed that some of the outbreaks previously reported as cases of RLOs, PLOs or mycobacteriosis-like were in fact piscine francisellosis caused by *Francisella* spp., (Hsieh *et al.*, 2006; Birkbeck *et al.*, 2007; Mauel *et al.*, 2007).

Francisella-like organisms have also been detected in hybrid striped bass (*Morone chrysops* x *M. saxatilis*) and ornamental cichlids (Ostland *et al.*, 2006; Hsieh *et al.*, 2007). The name *F. noatunensis subsp. orientalis* was proposed by Ottem *et al.* (2009) for *Francisella* sp. from *Parapristipoma trilineatum* and on the basis of a few phenotypic traits, was described as the type strain of the subspecies *Fno*. Subsequently, most *Francisella* isolates from tilapia worldwide have been confirmed to belong to this subspecies (Ottem, 2011).

Based on phylogenetic analysis, the *F. philomiragia* representatives (despite their various sources or origins) grouped together in all the phylogenetic trees in the same major branch with the *F. noatunensis* clades (Sjödin *et al.*, 2012). However, the *F. noatunensis* species do have several genetic, phenotypic, and physiological characteristics that discriminate them from *F. philomiragia* (Birkbeck *et al.*, 2011; Sjödin *et al.*, 2012; Challacombe *et al.*, 2017). The two subspecies of *F. noatunensis* (subsp. *noatunensis*) and subsp. *orientalis* (Kamaishi *et al.*, 2005; Ottem *et al.*, 2009) were separated in all the trees, reflecting fish host specificity (Challacombe *et al.*, 2017). Furthermore, PCR and sequence comparison of the 16S ribosomal RNA of the majority of the francisellosis cases have made it possible to place *Fno* at 96.1–97.72% identity to the human pathogen *F. tularensis*, 97.4% identity to *F. novicida*, 98–99.07% identity to *F. philomiragia*, and 99–99.22% identity to the *Fnn*, and 99.5–100% identity to other strains isolated from warm water fish species (Kamaishi *et al.*, 2005; Ostland *et al.*, 2006; Hsieh *et al.*, 2006; Birkbeck *et al.*, 2007; Ottem *et al.*, 2007, 2009; Mauel *et al.*, 2007; Mikalsen *et al.*, 2007; Lin *et al.*, 2016).

1.5.2 Epizootiology

Endemic bacteria in an environment usually only cause sporadic disease in fish but can induce an epizootic episode in the confines present in aquaculture facilities (Mauel and Miller, 2002). Epizootics caused by *Fno* are often severe and result in high mortality and economic losses. Several outbreaks resembling francisellosis have been observed in wild and cultured tilapia and almost all species of tilapia are particularly susceptible to this disease at any size of fish, either in the same group of cichlids (see details in Chapter 3 Table 3.1) or non-

chiclids, and *Fno* has been reported as a causative agent to cause disease worldwide. Moreover, piscine francisellosis in warm-water fish species does not only affect the tilapia industry worldwide, but also affects other important aquaculture sectors such as the ornamental fish industry (Table 1.4). In summary, the potential impact of *Fno* on fish health is extensive since the geographic distribution of francisellosis is expansive (Figure 1.5), and their host range is broad (Mauel *et al.*, 2003).

Table 1.4 Reports of natural outbreaks of piscines francisellosis caused by *Fno* in warm water fish species both fresh- and sea-water worldwide

Fish Species		Year	Location	Mortality	Pop.	Size	Water	T (°C)	References
Three-line grunt or Isaki fish	*Parapristipoma trilineatum*	1999–2000	Bungo Chanel (Oita, Kochi and Ehime) Japan	5–60%	Fm	6–510 g	F	17–30	(Fukuda *et al.*, 2002; Kamaishi *et al.*, 2005)
Hybrid striped bass / sunshine bass	*Morone chrysops* X *M. saxatilis*	2006	Southern California, USA	87.5–100%	Fm	7–11 g	F	26±1	(Ostland *et al.*, 2006)
Ornamental cichlid fish:		1998–2002	Southern Taiwan		Fm		F		(Hsieh *et al.*, 2007)
Firebird	*Aulonocara rubescens*								
Elegans	*Pseudotropheus elegans*								
Zebra	*Pseudotropheus zebra*								
Rhodes's chilo	*Chilotilapia rhoadesii*								
Malawi eyebiter	*Dimidiochromis compressiceps*								
Brown discus	*Symphysodon aequifasciatus*								
Deep-water hap	*Haplochromis electra*								
Electric blue hap	*Sciaeno- chromis fryeri*								
Blue-white libido	*Labidochromis caeruleus* and *Placidochromis milomo*								
Frontosa cichlid	*Cyphotilapia frontosa*								
Indo-Pacific reef fish species:		2011–2012	Atlanta, Georgia, USA	63%	Fm	5–6 cm	S (34 ppt)	25.5	(Camus *et al.*, 2013)
fairy wrasses including:	*Cirrhilabrus* spp. (Temmnick & Schlegel),								
Orangeback	*C. aurantidorsalis* (Allen & Kuiter)								
Solor	*C. solorensis* (Bleeker)								
Exquisite	*C. exquisitus* (Smith)								
Lubbock's	*C. lubbocki* (Randall & Carpenter)								

Fish Species		Year	Location	Mortality	Pop.	Size	Water	T (°C)	References
Blue-green damselfish	*Chromis viridis* (Cuvier)	2012	South Carolina, USA	100%	Fm	1.5–2.5 cm	S (32 ppt)	21	
Ornamental marine grunts:		2012–2013	Florida Keys, North America	8.2–100%	Fm		B/S (23–33 g/L)	22.9–25.4	(Soto *et al.*, 2014a)
French grunt	*Haemulon flavolineatum*								
Caesar grunt	*H. carbonarium*								
Tomtate	*H. aurolineatum*								
Porkfish	*A. virginicus*								
Bluestriped grunt	*H. sciurus*								
Smallmouth grunt	*H. chrysargyreum*								
Sailor's Choice	*H. parra*								
Spanish Grunt	*H. macrostomum*								
Ornamental Malawi cichlids:	*Nimbochromis venustus* Boulenger 1908, *Nimbochromis linni* Burgess and Axelrod 1975, *Aulonocara stuartgranti* "maleri" Meyer and Riehl 1985, *Placidochromis* sp. "blue hongi", *Protomelas* sp. "Steveni Taiwan", *Naevochromis chryosogaster* Trewavas 1935, *Copadichromis mloto* and *Otopharynx tetrastigma*	2014	Austria		Fm		F	24	(Lewisch *et al.*, 2014)
Guapote tigre	*Parachromis managuensis*		Costa Rica and others part of Latin America						

Pop., Populations; T, Temperature; +Wi, wild; Fm, farm; F, freshwater; B, brackish-water; S, Sea/salt-water

Figure 1.5 Geographical distribution of piscine francisellosis in warm water fish species caused by *Fno* worldwide (red dots: infected tilapia, green dots: other fish, black dots: both tilapia and other fish).

1.5.3 Morphology and biochemical characteristics

1.5.3.1 Cell morphology

On agar media the colonies of bacteria are smooth and grey-greyish (Hsieh *et al.*, 2006), convex (Soto *et al.*, 2009a, 2011a, 2013a; Jantrakajorn and Wongtavatchai, 2016; Lin *et al.*, 2016), or grey-greenish (Ramirez-Paredes, 2015), and mucoid with pale yellow pigment (Nguyen *et al.*, 2016).

Fno is a facultative intracellular bacterium that can be observed within the cytoplasmic vacuoles of phagocytes and can survive and replicate in the host cells such as macrophages (an attribute that contributes to its virulence), freely in cytosol or within the epithelial cell cytoplasm revealing a high proliferative activity (Hsieh *et al.*, 2006; Ottem *et al.*, 2009; Colquhoun and Duodu, 2011; Birkbeck *et al.*, 2011; Jantrakajorn and Wongtavatchai, 2016). Ultra-structurally, the bacteria contain an abundance of electron dense ribosome-like particles and electron-lucent areas, and a double cell wall with occasional rippled appearance and undulating projections at some points in the outer wall (Chen *et al.*, 1994; Mauel *et al.*, 2003, 2005; Iregui *et al.*, 2011). A large amounts of myelin figures surrounded the bacteria within host cell cytoplasm (Iregui *et al.*, 2011). These characteristics signposted that the baceria can cause massive host epithelial cell damage associated with concomitant alteration of the electrolyte balance (Iregui *et al.*, 2011).

1.5.3.2 *In vitro* cultivation (diagnosis and culture)

Members of the *Francisella* genus are difficult to culture (Petersen *et al.*, 2004), and *Fno* is a fastidious bacterial pathogen that grows slowly on agar and crucially, can be out-competed by other more rapidly growing bacteria during primary isolation attempts, which thereby increase false-negative results (Hsieh *et al.*, 2006; Mikalsen *et al.*, 2009; Soto *et al.*, 2010b). Several researchers have successfully used several selective solid and liquid media to isolate *Fno* from infected tilapia, and these are listed in Table 1.5. Typically, the agar media used to isolate *F. tularensis* and environmental or *Francisella* spp. were used modified to select for *Fno* from infected fish (Baker *et al.*, 1985; Petersen *et al.*, 2004).

Table 1.5 Media used for primary isolation of *Fno* from infected tilapia *Oreochromis* spp.

Type of media	T (°C)	Reference
Solid agar media		
Thayer-Martin II agar		(Hsieh *et al.*, 2006)
	27	(Soto and Revan, 2012)
Modified Thayer-Martin II agar	22–25	(Soto *et al.*, 2009a)
	27	(Soto *et al.*, 2013c; Klinger-Bowen *et al.*, 2016)
Cysteine heart agar + 2% bovine hemoglobin	25	(Soto *et al.*, 2009a; b; Jantrakajorn and Wongtavatchai, 2016)
	28	(Soto, 2010; Soto *et al.*, 2011b, 2013b; Leal *et al.*, 2014; Ramirez-Paredes, 2015; Assis *et al.*, 2016, 2017)
	26	(Ortega *et al.*, 2016)
Cysteine heart agar with 5% sheep blood	20	(Lin *et al.*, 2016)
Cysteine heart agar + 5–9% chocolatized sheep blood	22–25	(Soto *et al.*, 2009a; b)
Modified Thayer-Martin (McLeod)	22–25	(Mikalsen and Colquhoun, 2010; Soto *et al.*, 2011b)
Tryptic soy agar + 1% D-glucose + 0.1% Cysteine + 10% sheep blood	28	(Sjödin *et al.*, 2012; Challacombe *et al.*, 2017)
	28	(Nguyen *et al.*, 2016)
Cysteine heart agar with 5% tilapia blood	28	(Ramirez-Paredes, 2015)
Tryptic soy agar + 5% sheep blood	25	(Jantrakajorn and Wongtavatchai, 2016)
Liquid media		
Mueller Hinton + 3% foetal bovine serum + 1% glucose + 0.1% cysteine	25	(Soto *et al.*, 2009a)
Modified Mueller-Hinton II cation-adjusted broth supplemented + 2% IsoVitaleX™ + 0.1% glucose	22–25	(Soto *et al.*, 2009a; b, 2013b)
	28	(Soto *et al.*, 2011b; Leal *et al.*, 2014; Ramirez-Paredes, 2015)
	25	(Klinger-Bowen *et al.*, 2016)
	20	(Lin *et al.*, 2016)
Modified Mueller-Hinton II cation-adjusted broth supplemented + 2% IsoVitaleX™ + 0.1% glucose + 30 ppt NaCl	25/30	(Soto and Revan, 2012)
Tryptic soy broth supplemented + 1% D-glucose + 0.2% cysteine	28	(Nguyen *et al.*, 2016)

The growth of *Fno* isolates from diseased tilapia can also be supported in cell culture. For example, cell lines used successfully to isolate *Fno* from infected tilapia tissues (spleen and/or kidney) include Chinook salmon embryo (CHSE-214) cell lines (Chen *et al.*, 1994; Hsieh *et al.*, 2006). Moreover, RLOs suspected to be *Fno* replicated in tilapia ovary cells (TO-2), and epithelial papilloma of carp/epithelioma papulosum cyprinid (EPC) cell lines at 25°C (Chern and Chao, 1994). Inoculation of infected tissue into the cell lines cultures gave maximum cell cytotoxicity between 5 and 7 days post-inoculation (Birkbeck *et al.*, 2011).

1.5.3.3 Biochemical characteristics

Fno had negative reaction for reduction of nitrate, resazurin, and glycine, while they were strongly positive for ß–lactamase, proline and sucrose (Hsieh *et al.*, 2006). The *Fno* Ehime-1 isolate tested positive for In-Doxyl phosphate and phenylalanine arylamidase (Ottem *et al.*, 2009) (Table 1.6). The detection ranges of these techniques between studies sometimes inconsistence indicate that commercial kits (such as API Kits) are not suitable techniques to biochemically and phenotypically analyse these bacteria or any other member of *Francisella* genus (Chapter 3.4).

1.5.3.4 Serological characterization

Rabbit polyclonal antisera raised to whole cells of *F. tularensis*, *F. novicida* or with a monoclonal antibody to LPS from *F. tularensis* or *F. novicida* confirmed that the LPS of whole cells of *Fno* to be immunochemically distinct from these other *Francisella* spp. (Kay *et al.*, 2006). However, *Fnn* and *Fno* (Ehime-1=LMG24544T isolate) appeared serologically to be immunologically related, with both salmon and tilapia isolates agglutinated by a polyclonal antiserum raised against *Fnn* (strains GM2212, NVI 5330, and *F. piscidida*) (Birkbeck *et al.*, 2007; Ottem *et al.*, 2009, 2011).

Table 1.6 Physiological and biochemical characteristics of *Fno* Japan (Ehime-1 isolate)

Characteristics		Characteristics	
Optimal temperature °C	28–28.5	Alkaline phospatase	+
Cysteine required for growth	+	Esterase (C 4)	+
Gram-stained	-	Esterase lipase (C 8)	+
Cytochrome oxidase activity	-	Lipase (C 14)	+
Motility	-	Leucine arylamidase	-
Catalase	+w	Valine arylamidase	-
Indole	+w	Tyrosine arylamidase	nr
Arginine dehydrolase	-	Alanine arylamidase	nr
Lysine decarboxylase	-	Histidine arylamidase	nr
Ornithine decarboxylase	-	Arginine arylamidase	nr
CIT, Sodium Citrate	+	Trypsin	-
H_2S production	-	α-chymotrypsin	+
Urease	-	Acid phosphatase	+
Tryptophanase	-	Napthol-AS-BI-phosphohydrolase	+
IND, Tryptophane	-	α-galactosidase	-
VP, Sodium Pyruvate	+	ß-galactosidase	+
Gelatine hydrolysis	+	ß-glucuronidase	-
D-Glucose	-	α- glucuronidase	-
D-Mannitol	-	α-glucosidase	nr
INO, Inositol	-	ß-glucosidase	-
D-Sorbitol	-	B-N-acetyl-ß-glucosaminidase	-
Rhamnose	-	α-mannosidase	-
Sucrose	-	α-fucosidase	-
D-Melibiose	-	O-Nitrophenyl N-Acetyl-ß-D-Glucosaminide (ONAG)	nr
AMY, Amygdalin	-	N-Acetyl-ß-Glucosaminidase	nr
α-Arabinose	-	InDoxyl phosphate (IDP)	nr
D-saccharose		ß-lactamase	nr

+w, weak reactivity reported by Mikalsen & Colquhoun (2010); nr, not reported
Data presented are based on combined results published by Mikalsen & Colquoun (2010), (Hsieh *et al.*, 2006; Birkbeck *et al.*, 2007, 2011; Ottem *et al.*, 2009; Ramirez-Paredes, 2015)

1.5.4 Pathogenesis of *Francisella noatunensis* subsp. *orientalis*

Fno replicates in the skin mucus and enters the fish via skin lesions, the gills and gastrointestinal tract to elicit a local inflammatory response with formation of papules and ulcers *in situ* (Hsieh *et al.*, 2006; Soto *et al.*, 2011b, 2013c; Dong *et al.*, 2016a; Pradeep *et al.*, 2017). Granuloma lesions of the gills were reported to appear at an early stage of the disease (Chern and Chao, 1994). The infective agent might enter from any of the proposed routes and then it spreads to the endothelial cells of capillary beds via the blood where acute vasculitis occurs (Chern and Chao, 1994). Then the pathogen replicates in various organs, followed by perivascular leucocytic inflammatory infiltration, and these lesions cause degeneration, lead to focal necrosis, chronic infiltration of inflammatory cells, and multifocal and diffuse necrosis of which the last stage is typically granulomatous formation (Chern and Chao, 1994; Chen *et al.*, 1994). *Fno* can invade parenchymal tissue resulting in the formation of granulomas and abscesses following lympho-hematogenous dispersion (Hsieh *et al.*, 2006). The digestive system may be another possible site for the bacteria entry as lesions have been found in the intestinal tract, and it is possible that the transfer of the disease between fish can take place via faeces or intestine-content fluid (Chern and Chao, 1994). The bacteria can be extremely infectious and <10 colony forming units (CFU) of *Fno* injected intra-coelomically can lead to a colonization and significant pathology to the anterior kidney and spleen (Soto *et al.*, 2009a). A dose as low as 23 bacteria can result in mortality in tilapia fingerlings (Soto *et al.*, 2009a).

1.6 Algae and their role in aquaculture

Recently, research on the application of algae for aquaculture has been performed in a variety of areas, such as for wastewater treatment, used in gnotobiotics and synbiotics, as a growth hormone, in vaccines delivery, and as potential immunostimulants and as a general feed additive.

Algae are a polyphyletic and paraphyletic group of organisms which are included in the class of low-level plants (Patterson, 2000; Hallmann, 2007). They are generally photosynthetic but as protists they have no true stems, roots, leaves or vascular system (Hallmann, 2007), and so they obtain nutrients typically via osmosis (Gupta and Abu-Ghannam, 2011). Most algae are eukaryotes, including the Rhodophyta (red algae), Chlorophyta (green algae), Phaeophyta (brown algae) (O'Sullivan *et al.*, 2010; Gupta and Abu-Ghannam, 2011; Panayotova *et al.*, 2013), Bacillariophyta (diatoms) and Dinoflagellates, while only prokaryotic algae are the Cyanophyta (also known as the Cyanobacteria or blue-green algae (Hallmann, 2007). Algae size varies from giant multicellular organisms known as macro-algae ('seaweeds') to tiny single-celled species which are termed microalgae. Algae live in the water columns or on the surface and are found in freshwater and ocean waters. The macro-algae are typically found in the littoral zones as well as sub-tidal areas up to a depth of around 100 m where very little light is available for photosynthesis, while the microalgae occupy in both benthic and littoral habitats and also as the water column where they are called phytoplankton (Gupta and Abu-Ghannam, 2011).

Annual global aquaculture production of algae has expanded at 8% per year in the past decade, up from 13.5 tonnes in 2005, to 27.3 tonnes in 2014, and algae farming is practised in about 50 countries now (FAO, 2016). Algae are potentially useful as human food and for the production of bioactive compounds (Chojnacka *et al.*, 2012). Amid different valuable alga-derived compounds there are polysaccharides (*e.g.* sulphated galactan and polysaccharides), photosynthetic pigments (*e.g.* carotenoids, xanthophyll and phycobiliproteins), phycosterols, bioactive peptides, polyunsaturated fatty acids (PUFA), betaines, taurine, polyphenols (especially phlorotannins), minerals, vitamins and others (Chojnacka *et al.*, 2012). There have been many studies on the application of algae for humans use *e.g.* for human food (Batista *et al.*, 2013), chemicals and pharmaceuticals (Table 1.7), biofuel or biodiesel (Talebi *et al.*, 2013), bio-plastid (Balaji *et al.*, 2013), wastewater treatment (Posadas *et al.*, 2013), animal feed (Leonard *et al.*, 2010a; b; Sweeney *et al.*, 2011) and for aquaculture.

Table 1.7 Algal species and their bioactive compounds with possible effect on human health

Class / Species	Bioactive compounds	Possible health effects	References
Antibacterial activity			
Chlorococcum sp, Chlorella sp., and Synechococcus sp. (blue-green alga)	Linolenic acids (C18:3), and Crude extracts	Gram-positive bacteria	(Ohta *et al.*, 1994)
Corallina officinalis, *Cystoseira barbata*, *Dictyota dichotoma*, *Halopteris filicina, Cladostephus spongiosus, f. verticillatus* and *Ulva rigida*	Crude extracts	*Staphylococcus aureus, Micrococcus luteus, Enterococcus faecalis*, *Escherichia coli,* and *Enterobacter aerogenes*	Taskin et al. 2007
Phaeodactylum tricornutum	(9Z)-hexadecenoic acid (palmitoleic acid; C16:1 n-7) and (6Z, 9Z, 12Z)-hexadecatrienoic acid (HTA; C16:3 n-4)	*Bacillus cereus, B. weihenstephanensis, Listonella anguillarum, Planococcus citreus, S. epidermidis, S. aureus,*	Desbois et al. 2008
Kappaphycus sp and *Padina* sp	Crude extracts (powder)	*Pseudomonas flouresences, P. aeruginosa, S. aureus, Vibrio cholera, Proteus mirabilis, P. vulgaris, E. coli, Citrobacter* sp, *B. megaterium, Enterobacter* sp, *Klebsiella pneumoniae, Salmonella typhi,* and *Shigella flexneri*	Press et al. 2009
Phaeodactylum tricornutum	eicosapentaenoic acid (EPA; C20:5 n-3)	*L. anguillarum, M. luteus, Photobacterium* sp.*, P. citreus, B. cereus, B. weihenstephanensis, S. epidermidis, S. aureus,*	Desbois et al. 2009
26 marine *Rhodophyceae* (8 Ceramiales, 7 Gelidiales, 9 Gigartinales, 1 Bonnemaisoniales and 1 Rhodymeniales)	Crude extracts (powder)	*Enterococcus faecalis*, *S. aureus, E. coli,* and *K. pneumoniae*	Rhimou et al. 2010
Phaeophyceae (*Cystoesira myrica, C. trinodes, Padina gymnospora, Sargassum dentifolium, S. hystrix*), Rhodophyceae (*Actinotrichia fragilis*), and Bryopsidophyaceae (*Caulerpa racemosa, and Codium fragile*)	Crude extracts (powder)	*E. coli*, *S. aureus*, *E. feacalis*, *Salmonella* sp., *B. cereus* and *P. aeruginosa*	(Salem, 2011)
Chlorophyta (*Enteromorpha compressa, E. intestinalis, C. scalpelliformis, C. racemosa, Chaetomorpha linum*), and Rhodophyta (*Gracilaria folifera, Hypnea valentiae*)	Crude extracts (powder)	*B. subtilis, S. aureus, E. aerogenes, E. coli, K. pneumonia,* and *P. aeruginosa*	Jebasingh et al. 2011
Phaeophyceae (*Petalonia fascia, Stypocaulon scoparium*) and Chlorophyceae (*Cladophora prolifera, Codium fragile*)	Crude extracts (powder)	*S. aureus, S. epidermidis, Salmonella typhimurium, Enterobacter aerogenes, Enterococcus faecalis* and *Escherichia coli*	Taşkin 2011
Haliptilon attenuatum, Spyridia filamentosa, Cystoesira foeniculacea, Dictyota dichotoma var. intricate, Caulerpa racemosa, C. frolifera, Dasya rigidula, and *Ulva linza*	Crude extracts (powder)	*E. coli, Aeromonas hydrohilla, Yersinia enterocolitica,* and *S. aureus*	(Taskin *et al.*, 2012)
Gracilaria edulis, Sargassum wightii, Ulva lactuca	Crude extracts (lycophilized)	*V. cholerae, Salmonella typhii, E. coli* and *K. pneumonia*	(D.Radhika *et al.*, 2012)
Sargassum wightii	Crude extracts	*S. aureus*, *E. coli*, *Klebsiella* sp., *K. pneumoniae*, *P.*	(Devi *et al.*, 2012)

Class / Species	Bioactive compounds	Possible health effects	References
		aeruginosa, *P. proteus*, *V. cholerae*, *Salmonella paratyphi*, *S. typhi,* and *Shigella sonnie*	
Phaeophyceae (*S. wightii* Greville), Cladophoracea (*C. Linum*), and Dictyotaceae (*Padina Gymnospora*)	Crude extracts	*P. aeruginosa, S. typhi, S. aureus, Erwinia amylopora, Enterobacter aerogenes, P. vulgaris K. pneumonia, E. coli,* and *Enterococcus faecalis*	Rosaline et al. 2012
S. illicifolium	Crude extracts (powder)	*S. epidermidis, B. subtilis,* and *E. coli*	Selvarani et al. 2013
Phaeodactylum tricornutum	six LC-PUFAs (DGLA, DHA, EPA, GLA, 15-hydroxyeicosatrienoic acid [HETrE], and 15-hydroxyeicosapentaenoic acid [15-OHEPA])	*Propionibacterium acnes* and *Staphylococcus aureus*	(Desbois and Lawlor, 2013)
Enteromorpha linza	Stearidonic acid (SA, C18:4 n-3) and gamma-linolenic acid (GLA, C18:3 n-6)	*Prevotella intermedia, Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Candida albicans, Fusobacterium nucleatum subsp. vincenti*, and *Streptococcus mutans*	(Park *et al.*, 2013)
Chlorella ellipsoidea	Chloroplast	produced defensin, are small cationic peptides that act in the first line of defence against pathogens (*Escherichia coli)*	(Bai *et al.*, 2013)
Antibacterial and antifungal activities			
Chlorophyta (*Bryopsis pennata*, *Caulerpa racemosa*, *C. scalpelliformis, C. taxifolia, Codium iyengarii, C. shameelii, Ulva fasciata, U. intestinalis, U. lactuca*), Phaeophyta (*Colpomenia sinuosa, Cystoseira indica, Dictyota dichotoma, D. hauckiana, Iyengaria stellate, Padina antillarum, P. pavonica, Sargassum boveanum, S. ilicifolium, S. swartzii, S. tenerrimum, S. vulgare, Stoechospermum polypodioides, Stypopodium shameelii*), and Rhodophyta (*Botryocladia leptopoda, Champia compressa, G. corticata, Hypnea musciformis, H. valentia, Osmundea pinnatifida, Sarconema filiforme, Scinaia saifullahii, Solieria robusta*)	Crude extracts	Bacteria *i.e*. *B. subtilis, Corynebacterium diptheriae, S. aureus, Streptoccus pyogenes, P. aeruginosa, Proteus mirabilis, K. pneumonia, Shigella dysenteriae, Salmonella typhi,* Fungi *i.e. Asperigillus flavus, A. niger, Candida albican, C. glabrata, Pseudallescheria boydii, Trichophyton longifusus, T. schoenleninii* Animal pathogens *i.e. Microspermum canis*, *Trichophyton mentagrophytes, T. simii* Plant pathogens *i.e. Fusarium moliforme, F. solani*, *Mucor* sp.	(Rizvi and Shameel, 2005)
Chlorophyta (*Bryopsis plumosa, U. fasciata, Acrosiphonia rientalis, Chaetomorpha antennina, Grateloupia filicina*), Rhodophyta (*H. pannosa, G. corticata, Centroceras clavulatum, Portieria ornemannii, Cheilosporum spectabile*), and Phaeophyta (*P. tetrastromatica, S. wightii, Stocheospermum marginatum, Chnoospora bicanaliculata*)	Crude extracts	*Candida albican, Streptococcus* sp., *Enterococcus faecalis, S. epidermidis, S. aureus, E. coli, Micrococcus luteus B. subtilis*, *P. aeruginosa, Klebsiella pneumoniae*, *Proteus mirabilis,*	(Shanmughapriya *et al.*, 2008)
Sargassum sp.	Crude extracts	inhibition of lipid peroxidation, and glutathione-S-	Patra et al. 2008

Class / Species	Bioactive compounds	Possible health effects	References
		transferase and antimicrobial properties (*S. aureus, E. coli, and B. subtilis*)	
Sargassum wightii and *Kappaphycus alwarezii*	Organic extracts	*Streptococcus faecalis, S. pneumonia, B. subtilis, B. aureus, V. cholera, V. parahaemolyticus, E. coli, S. aureus, K. pneumonia, P. aeruginosa, Proteus, Citrobacter, Microsporum gypsum, Aspergeillus niger, A. flavus, A. fumigatus Trichophyton rubrum,*	(Bibiana *et al.*, 2012)
Gracilaria corticata	Crude extracts (polysaccharide like compound)	*Streptococcus pneumonia, S. aureus, Proteus mirabilis, E. coli, Pseudomonas aeruginosa, Candida albicans* and *Candida* spp.	Govindasamy et al. 2012
Cyanobacteria (*Lyngbya aestuarii* and *Aphanothece bullosa*)	Crude extracts (lycophilized)	*Candida albicans* and *Leishmania donovain*	(Kumar *et al.*, 2013)
Antiviral activity			
Gymnogongrus griffithsiae and *Cryptonemia crenulata*	sulphated polysaccharides (kappa/iota/nu carrageenan *G3d* and the dl-galactan hybrid *C2S-3*)	against dengue virus was dependent on virus serotype and host cell	(Talarico *et al.*, 2005)
Sphaerococcus coronopifolius (*Rhodophytha*, *Gigartinales*) and *Boergeseniella thuyoides* (*Rhodophyta*, *Ceramiales*)	Sulfated polysaccharides (galactose, 3,6-anhydrogalactose, uronics acids, sulfate)	inhibited replication of the Human Immunodeficiency Virus (HIV) and *Herpes simplex* virus type 1 (HSV-1)	(Bouhlal *et al.*, 2011)
Toxicity of algae			
Ulva rigida	Ethanolic extracts	anti-hyperglycemic, anti-oxidative and genotoxic/antigenotoxic in diabetes mellitus	(Celikler *et al.*, 2009)
Undaria pinnatifida	fucoidan	Genotoxicity	(Kim *et al.*, 2010; Chung *et al.*, 2010)
Spatoglossum schröederi (Dictyotaceae)	sulfated polysaccharides: heterofucan (rich L-fucose)	Toxicity	Almeida-Lima et al. 2011,
Caulerpa cupressoides var. *lycopodium*	sulphated polysaccharides	Antinociceptive activity and acute toxicological study	(Rodrigues *et al.*, 2013)
Antitumor, anticancer, antiobesity, antioxidant			
Haematococcus pluvialis	Carotenoid astaxanthin	inhibitory effect on *H. pylori* infection which is in humans associated with chronic type B gastritis, peptic ulcer disease, and gastric carcinoma	(Wang *et al.*, 2000)
Porphyridium cruentum, Chaetoceros sp.*, Chlorella* sp., *C. capsulate, C. autotrophica, Dunalliela tertiolecta, Isochrysis* sp., *Isochrysis galbana* var. tahitiana, *Nannochloropsis oculata, Phaeodactylum tricornutum, Rodhosorus marinus, Tetraselmis*	sulphate exopolysaccharides,	can block adherence to human gastrointestinal pathogens *Helicobacter pylori*	(Guzman-Murillo and Ascencio, 2000)

Class / Species	Bioactive compounds	Possible health effects	References
sp., *T. suecica, Botrycoccus sudeticus, B. braunii, Chlamydomonas Mexicana, Chlorococcum oleofaciens, Dysmorphococcus globosus, Hormotilopsis gelatinosa, Neochloris oleoabundans,* and *Ochromonas danica*			
Haematococcus pluvialis	Astaxanthin	Exert beneficial effects in diabetes, with preservation of -cell function (reducing glucose toxicity)	(Uchiyama *et al.*, 2002)
Undaria pinnatifida	Fucoxanthin	attenuates white adipose tissue (WAT) weight gain and hyperglycemia in diabetic/obese	(Maeda *et al.*, 2005; Hosokawa *et al.*, 2010)
Sargassum fulvellum and *Sargassum thunbergii*	Crude extracts	Antipyretic, analgesic, and anti-inflammatory activities:	Kang et al. 2008
Cladosiphon okamuranus	Fucoidan	induced apoptotic cell death through a caspase-8-dependent pathway in human breast cancer MCF-7 cells	Yamasaki-Miyamoto et al. 2009
Cladosiphon okamuranus	sulfated polysaccharides: fucoidans	Cardioprotective activity: induced myocardial infarction	Thomes et al. 2010
Undaria pinnatifida	Fucoidan (O-acetylated sulphated galactofucan)	PC-3 (prostate cancer), HeLa (cervical cancer), A549 (alveolar carcinoma), and HepG2 (hepatocellular carcinoma) cells,	Synytsya et al. 2010
Undaria pinnatifida	Fucoxanthin	improves plasma and hepatic lipid metabolism and blood glucose concentration	(Woo *et al.*, 2010)
Undaria pinnatifida	Fucoidan	induced apoptosis of A549 human lung cancer cells through down-regulation of p38, PI3K/Akt, and the activation of the ERK1/2 MAPK pathway	(Boo *et al.*, 2011)
Undaria pinnatifida	Fucoxanthin	prevent insulin resistance and hepatic fat accumulation that is partly mediated by modulating the hepatic glucose and lipid homeostasis in the high fat induced obese	(Park *et al.*, 2011)
Sargassum myriocystum	Crude extracts	Antioxidant: for reactive oxygen, radical scavenging capacities and antioxidant activities	(Badrinathan *et al.*, 2011)
Cladosiphon okamuranus	Fucoidan	suppresses tumor growth and prolongs survival times in a colon tumor-bearing mouse model	(Azuma *et al.*, 2012)
Codium dimorphum, *Ulva lactuca, Phyllospora comosa*, *Sargassum* sp., *Prionitis linearis* and *Corallina vancouveriensis*	Protein kinase A (PKA) and fatty acids	involved in the regulation of an increasing number of physiological processes including immune function, cardiovascular disease, memory disorders and cancer	(Zivanovic and Skropeta, 2012)
Haematococcus pluvialis	Astaxanthin	a. Improves cognitive function in the healthy aged individuals b. Anticancer of skin activity	(Katagiri *et al.*, 2012)
Porphyra columbina	Water soluble proteins	Bioactive properties of peptides: immunomodulatory	(Cian *et al.*, 2012)

Class / Species	Bioactive compounds	Possible health effects	References
	(phycobiliproteins)	properties, antihypertensive activity and antioxidant capacity	
Ecklonia cava Kjellman	phlorotannins	induce sleep via positive allosteric modulation of gamma-aminobutyric acid type A-benzodiazepine receptor	(Cho *et al.*, 2012)
Haematococcus pluvialis	Total carotenoids, astaxanthin, astaxanthin monoester, and astaxanthin diester.	inhibited of UV-DMBA-induced skin cancer through inhibition of accumulation of ROS and tyrosinase enzyme activity	(Rao *et al.*, 2013)
Undaria pinnatifida	Fucoidan	induced the apoptosis of PC-3 cells human prostate cancer cells	(Boo *et al.*, 2013)
Eisenia bicyclis	Water-soluble polysaccharides i.e. laminaran (a glucan with ß-(1→6) side chains linked to a ß-(1→3) backbone) and fucoidan (1,3-linked fucose residues, some 1,6-, 1,2,6-, 1,4,6-linked galactose residues and traces of mannose and xylose)	Inhibited the colony formation of human melanoma SK-MEL-28 and colon cancer DLD-1 cells	Ermakova et al. 2013

1.6.1 Aquatic animal nutrition

In aquaculture, the major application for microalgae is with respect to nutrition, and these species are used fresh (as the sole part of the diet or as an additive to supplement basic nutrients), as live feed, for colouring salmonid flesh and for inducing other beneficial biological activities (Spolaore *et al.*, 2006). The applications of microalgae as a source of live fish food is because they have key qualities such as suitable nutrition composition, appropriate size for ingestion, easy to digest, fast growth rates up to high densities, and easy with which to vary cultivation conditions (Spolaore *et al.*, 2006). Microalgae are commonly used commercially as live feeds for all growth phases of bivalve molluscs (*e.g.* oysters, scallops, abalone, crustaceans, and for clams and mussels) and shrimp juveniles, and are essential in both marine and freshwater fish rearing systems especially during larval stages (Spolaore *et al.*, 2006). The most frequently cultured species for these purposes are *Anabaena cylindrica* and *Microcystis aeruginosa* for Nile tilapia larvae (Northcott *et al.*, 1991).

In aquaculture food chains, microalgae such as *Scenedesmus abundans*, *Monoraphidium minitum* and *Chlorella vulgaris*, are also utilized as feed for the larval and early juvenile phases of zooplankton such as rotifers *Brachionus calyciflorus* which ultimately are fed to Redbelly tilapia larvae (Isik *et al.*, 1999). Furthermore, levels of dietary PUFA including docosahexaenoic acid (22:6n–3, DHA) and arachidonic acid (20:4n–6, AA) are important during the larval stages when the need for these PUFA can exceed the dietary input from the live feeds used in commercial aquaculture during rapid growth and development (Patil *et al.*, 2007). For example, Gilthead seabream *Sparus aurata* L. larvae were fed to rotifers (*B. rotundiformis*) and *Artemia* nauplii enriched with docosahexaenoic acid-rich dinoflagellate *Crypthecodinium* sp., and varying arachidonic acid was tested on growth, survival and resistance to handling stress of the fish, especially during the more sensitive stages of metamorphosis (Koven *et al.*, 2001). Enriched *Artemia* nauplii with a low or high-level AA strongly reduced the cortisol response after air exposure of both 28 and 50 d post-hatch larvae (Van Anholt *et al.*, 2004).

Several micro- and macro-algae are widely used as fish feed additives. For example, fresh and dried samples of *Anabaena* sp. were fed to Silver carp *Hypophthalmichthys molitrix* and Nile tilapia (Ekpo and Bender, 1989). *Porphyra yezoensis* was evaluated as a partial replacement for fish meal in intensively-farmed Nile tilapia feed (Stadtlander *et al.*, 2013). Meanwhile, laminaran ß(1,3)-D-Glucan isolated from *L. hyperborean* was used as a feed additive of Atlantic salmon, *Salmo salar* L. (Dalmo *et al.*, 1998); *L. japonica, G. lemaneiformis* and *S. pallidum* were fed to the Pacific abalone *Haliotis discus hannai* (Qi *et al.*, 2010); and *U. clathrata* of juvenile white shrimp *Litopenaeus vannamei* (Cruz-Suárez *et al.*, 2010). Finally, dietary glycoprotein extracted from *Hizikia fusiformis* was found to promote the growth rate of juvenile olive flounder *Paralichthys olivaceus* (Choi *et al.*, 2014). Most of the algae on those studies were prepared by mixing the fresh or dry algae powder directly into the diets (Ekpo and Bender, 1989; Dalmo *et al.*, 1998; Cruz-Suárez *et al.*, 2010; Qi *et al.*, 2010; Stadtlander *et al.*, 2013) or extracting the algal-derived compounds before adding to the diet (Choi *et al.*, 2014).

1.6.2 Gnotobiotic and Synbiotic

The gnotobiotic and synbiotic concept as applied to aquaculture feed formulation is still in very early development. Gnotobiotic (originally from Greek words *i.e.* roots *gnostos* 'known' and *bios* 'life') relating to or denoting an environment for rearing or culturing organisms in which all the microorganisms are either known or excluded (Marques *et al.*, 2006). Synbiotics is nutritional supplementation with products which combine both probiotics and prebiotics to create a form of synergism (Schrezenmeir and de Vrese, 2001). For instance, the use of microalgae *Dunaliela tertiolecta* in gnotobiotic *Artemia franciscana* cysts was able to protect *Artemia* against two opportunistic and virulence bacterial strains *e.g. Vibrio campbellii* and *V. proteolyticus* (Marques *et al.*, 2006). Meanwhile, a globulated feed combining dried *Spirulina platenis* and mass cultivated sporulated lactic acid bacteria *Lactobacillus* sp. cultures was formulated and showed beneficial health implications on the catfish *Arius seemani* (Chandra *et al.*, 2010). Further, encapsulating the diet together with bacteria in an aqueous phase provided an optimum environment for the

bacteria to grow, secrete the enzyme and digest the protein available from the diet and in the capsule walls (Chandra *et al.*, 2010).

1.6.3 Immunostimulants

Natural or synthetic substances which are able to activate non-specific immune responses or utilized as adjuvants to boost the specific immune response are known as immunostimulants (Anderson, 2004). Algae are a potent source of biologically active substances that can produce different important secondary metabolites with potential pharmacological activities (Tables 1.7 and 1.8). They can be applied for prophylaxis and therapy of fish diseases and may be used as immunostimulants in aquaculture or as health-promoting feed additives for aquaculture (Boo *et al.*, 2013). In humans they have been shown to have various functions (Table 1.7), as well as in aquaculture species, thus much research about the application of algae to improve fish immune responses and resistance against various pathogens has been performed and is described in Table 1.8.

Table 1.8 Algal species and their bioactive compounds with possible effect as immunostimulants in aquaculture

Class / Species	Bioactive compounds	Possible health effects	Fish species	Against	References
Laminaria hyperborean	Laminaran [ß(1,3)-D-Glucan]	Chemiluminescent response	blue gourami, *Trichogaster trichopterus*	*A. hydrophila, in vivo*	(Samuel *et al.*, 1996)
L. hyperborean	Laminaran [ß(1,3)-D-Glucan]	Antibacterial activity	Atlantic salmon, *Salmo salar* L.,	*A. salmonicida* and *V. salmonicida in vivo*	(Dalmo *et al.*, 1998)
Diatom *Skeletonema costatum*	Organic extracts	Antibacterial activity, *in vitro* (disk diffusion assay)		*A. hydrophila, A. salmonicida, Listonella anguillarum, Serratia liquefasciens, V. alginolyticus, V. mytili, Vibrio* spp. and *Yersinia ruckeri*	(Naviner *et al.*, 1999)
(21) of marine and freshwater microalgal strains	Sulphate exopolysaccharides	Antibacterial activity	Spotted sand bass *Paralabrax maculatofasciatus*	*A. veronii* and the strains associated with red-sore diseases *i.e.*, *V. campbellii, V. ordalii* and *S. saprophyticu*	(Guzman-Murillo and Ascencio, 2000)
Ulva rigida, Enteromorpha sp., *Codium tomentosum, Fucus vesiculosus, Pelvetia canaliculata, Dictyota dichotoma, Chondrus crispus* and *Porphyra umbilicalis*	Protein-free water-soluble extracts and polysaccharide fractions	Increased respiratory burst activity and IL-1 ß expression	Turbot, *Psetta maxima* (*in vitro* cell cultures)		(Castro *et al.*, 2004) (Castro *et al.*, 2006)
Spirulina platensis	Crude extracts	Antiviral and antibacterial activities	*Penaeus monodon*	White Spot Syndrome Virus and *V. harveyi*	(Hemtanon *et al.*, 2005)
Gracilaria corticata, *U. fasciata* and *Enteromorpha compressa*	Methanolic extracts	Antibacterial activity, *in vitro* (i.e., disk diffusion and MIC methods)		*Edwardsiella tarda*, *V. alginolyticus*, *P. fluorescens*, *P. aeruginosa* and *A. hydrophila*	(Choudhury *et al.*, 2005)
Asparagopsis armata, Ceramium rubrum, Drachiella minuta, Falkenbergia rufolanosa, G. cornea and *Halopitys incurvus*	Dichloromethane, and methanole extracts	Antibacterial activity, *in vitro* (disk diffusion assay)		*A. salmonicida* subsp. *salmonicida, A. hydrophila* subsp. *hydrophila, P. anguilliseptica, V. anguillarum, Yersinia ruckeri*	(Bansemir *et al.*, 2006)
Hydropuntia cornea (formerly *Gracilaria cornea*)	Ethanolic extract	Immunostimulatory and antibacterial activities	Sole, *Solea sinegalensis*	*Photobacterium damselae* ssp. *piscicida*	(Díaz-Rosales *et al.*, 2007)
Porphyridium cruentum	Crude extracts	Induced respiratory burst activity	Sole	*Photobacterium damselae* subsp. *piscicida*	(Díaz-Rosales *et al.*, 2008).
L. nigrescens	Sodium alginate	Antiviral and antibacterial activities	Fingerling orange spotted grouper, *Epinephelus coioides*	*Streptococcus* sp. and iridovirus	(Yeh *et al.*, 2008)
Chondrus crispus, Eucheuma cottonii, E. spinose, Gigartina aciculaire and *G. pistillata*	Carrageenan	Antibacterial activity	*White shrimp L. vannamei*	*V. alginolyticus*	(Yeh and Chen, 2008)
Gracilaria edulis, *Calorpha peltada* and	Ethanolic extracts	Antibacterial activity, *in vitro* (disk		*Escherichia coli, Enterobacter*	(Kolanjinathan *et al.*,

Class / Species	Bioactive compounds	Possible health effects	Fish species	Against	References
Hydroclothres sp.,		diffusion assay)		*aerogenes*, *Staphylococcus aureus*, *P. aeruginosa*, *S. faecalis* and *Bacillus cereus*	2009)
Bovine lactoferricin containing transgenic microalgae which transferred to protoplast of *Nannochloropsis oculata*		Antibacterial activity	Medaka fish *Oryzias latipes*	*V. parahaemolyticus*	(Li and Tsai, 2009)
Brown algae (*e.g.* phaeophyceae) *Laminariae* sp.	*Laminaran,* ß*-1,3-D-glucan and short branched* ß*-1,6-D-glucan,*	Increased the immune response and disease resistance	Nile tilapia *Oreochromis niloticus*	*A. hydrophila*	(El-Boshy *et al.*, 2010)
Gracilaria fisheri	Ethanol, methanol, chloroform and extracts	Antibacterial activity (disk diffusion assay and *in vivo*)	Post-larvae and juvenile of *Penaeus monodon*	*V. harveyi* both *in vitro* and *in vivo*	(Kanjana *et al.*, 2011)
Macrocystis pyrifera and *Fucus vesiculosus*	Alginic acid and fucoidan	Immunostimulatory activity	Atlantic cod, *Gadus morhua*	*In vitro* cell cultures	(Caipang *et al.*, 2011)
Cyanoprokaryota Anabaena wisconsinense		Increased immune response and disease resistance	Nile tilapia	*A. sabriia, Actinobacter anitratus* and *A. veronii*	(Dawah *et al.*, 2011)
Spirulina (Arthrospir platensis)		Increased immune response and disease resistance	Nile tilapia	*A. hydrophila*	(Abdel-Tawwab and Ahmad, 2009; Ragap *et al.*, 2012)
Chetomorpha linum	Acetone, methanol, diethyl ether, ethyl acetate, and hexane extracts	Antibacterial activity, *in vitro* (disk diffusion assay)		*P. aeruginosa, V. alginolyticus, A. hydrophila, V. parahaemolyticus, E. aerogenes*	(Vijayakumar *et al.*, 2012)
Gracilaria edulis, G. verrcosa, Acanthospora spicifera, U. facita, U. lacta, Kappaphycus spicifera, Sargassum ilicifolium, S. wightii, Padina tetramatica and *P. gymonospora*	Hot water extracts	Enhanced the immune response and disease resistance	Mullet *Mugil cephlus*	*V. alginolyticus*	(Rajasekar *et al.*, 2012)
M. pyrifera	Alginic acid	heighten the transcription of the selected immune-related genes in the spleen cells	Atlantic cod	*In vitro* cell cultures	(Caipang *et al.*, 2012)
Commercial immunostimulant and probiotic *e.g.,* Algae® *(*artemia and rotifer enrichments) and *Aquabacteriae*® (AquaGrow DHA, extract of microalgae*, Crypthecodinium,* high in Omega-3 DHA, and AquaGrow ARA)		Antibacterial activity	Sea bass and sea bream	*P. Fluorescence*	(Saad *et al.*, 2013)
Spirulina platensis		Increased immune response and disease resistance	Nile tilapia	*Pseudomonas fluorescens*	(Ibrahem *et al.*, 2013)
Hizikia fusiformis	Glycoprotein	Enhanced significantly IL-2 and -6 levels	Juvenile olive flounder *Paralichthys olivaceus*	*In vitro* cell cultures	(Choi *et al.*, 2014)
Chondrococcus hornemanni (Kuetzing, 1847)	Methanol extracts	Antibacterial activity and increased	Clownfish *Amphiprion*	*V. parahaemolyticus* and *A.*	(Ganeshamurthy *et al.*,

Class / Species	Bioactive compounds	Possible health effects	Fish species	Against	References
		white blood cell count and respiratory burst activity	*sebae*	*hydrophila Providencia rettgeri* and *V. alginoticus*	2013, 2014)
Sargassum sp	Alginate	Enhanced non- specific immune system	Walking catfish *Clarias* sp.		(Isnansetyo *et al.*, 2014)
Sargassum cristaefolium and *Fucus vesiculosus*	Fucoidan	Increased the immune response	Nile tilapia		(Isnansetyo *et al.*, 2016)
Green alga *Ulva clathrata*		Enhanced immune response	Nile tilapia		(del Rocío Quezada-Rodríguez and Fajer-Ávila, 2017)

1.7 The potential of greater wax moth larvae "*Galleria mellonella*" as an alternative *in vivo* model of bacterial pathogenesis

An insect is an attractive alternative host that offers many benefits over the native host, and these systems can provide valuable insight due to the structural and functional similarities between immune systems of insects and other animals (Kavanagh and Reeves, 2004; Fuchs and Mylonakis, 2006; Browne *et al.*, 2013b).

Regarding the advantages of using insects instead of vertebrates in infection studies, insects can be used in large numbers, their short life span makes them ideal for high-throughput studies, they are inexpensive, easily cultivable and manipulated, they do not require special lab equipment, there are few legal constraints to using them in experiments and they are more ethically acceptable compared to using vertebrates in similar studies (Scully and Bidochka, 2006; Wojda, 2016; Tsai *et al.*, 2016). In addition, results acquired using insects as model hosts can be confirmed using mammalian or vertebrate models, and their replacement for the hosts in suitable conditions should reduce mammalian or other vertebrate suffering (Scully and Bidochka, 2006).

Recently, many researchers have begun to use a variety of insects as alternative infection models to study various aspects of opportunistic pathogens (especially of mammals), including strain virulence testing of different strains, elucidation of putative virulence factors, analysis of the immune-related gene expression and to test efficacy of antimicrobial agent therapy. Such insect alternative hosts used successfully include the lesser wax moth *Achoria grisella* Fabric (Pierpoint, 1957), tobacco horn worm or sphinx moth *Manduca sexta* (Bravo *et al.*, 1992; Sahoo *et al.*, 2002), migratory locust *Locusta migratoria* (Mullen and Goldsworthy, 2003; Weers and Ryan, 2003), forest tent caterpillar moth *Malacosoma disstria* (Giannoulis *et al.*, 2007), the greater wax moth *Galleria mellonella* (Bidla *et al.*, 2009), fruit fly *Drosophila melanogaster* (Stenbak *et al.*, 2004; Vonkavaara *et al.*, 2008, 2013), Egyptian cotton leaf

worm *Spodoptera littoralis* (Boisduval) (Guz *et al.*, 2013) and Silk worm *Bombyx mori* (Goncu and Parlak, 2009; Suzuki *et al.*, 2016).

1.7.1 *G. mellonella* as experimental infection models

Recently, successful studies have been conducted with the fish pathogen *V. anguillarum* using the *G. mellonella* model (McMillan *et al.*, 2015). *G. mellonella* (order Lepidoptera, and family Pyralidae) lives in beehives, and feeds with on wax and pollen, and this species is considered to be a pest causing bee, bumblebee or wasp-galleriosis (Wojda, 2016). Their life cycle is approximately 7–8 weeks, and from the egg the larvae undergo 6 larval phases (which takes 5–6 weeks at 25–28°C) before reaching the last instar stage (Wojda, 2016). Then the pre-pupae and pupae form and after an additional 2 weeks, the adult moths hatch (Wojda, 2016). The last instar wax worm larvae are available commercially as bait for fishermen and for keepers of reptilian pets, but they have also been commonly used as an alternative host for studying bacterial pathogenesis. Indeed, various opportunistic human pathogens have also been studied in the *G. mellonella* (Table 1.9).

Table 1.9 Reports of experimental pathogenesis of various opportunistic human bacterial pathogens in the *G. mellonella* patho-system

Pathogens	Reference
Gram-negative bacteria	
P. aeruginosa	(Jander *et al.*, 2000; Andrejko *et al.*, 2008; Hill *et al.*, 2014; Mizerska-Dudka and Andrejko, 2014)
Klebsiella pneumonia	(McLaughlin *et al.*, 2014)
Legionella pneumophila	(Harding *et al.*, 2012, 2013; Aurass *et al.*, 2013)
Escherichia coli	(Mizerska-Dudka and Andrejko, 2014)
Cryptococcus neoformans	(Mylonakis *et al.*, 2005; Trevijano-Contador *et al.*, 2015)
Burkholderia cepacia complex	(Seed and Dennis, 2008)
Yersinia pseudotuberculosis	(Champion *et al.*, 2009)
Acinetobacter baumanii	(Peleg *et al.*, 2009; Jacobs *et al.*, 2014)
Type A, *F. tularensis* and type B *F. tularensis* subsp. *holarctica* LVS	(Aperis *et al.*, 2007; Ahmad *et al.*, 2010)
F. novicida	(McKenney *et al.*, 2012; Dean and van Hoek, 2015)
F. philomiragia	(Propst *et al.*, 2016)
Campylobacter jejuni	(Senior *et al.*, 2011)
Gram-positive bacteria	
S. pneumoniae (pneumococcus)	(Evans and Rozen, 2012)
Group A Streptococcus (*S. pyogenes*)	(Olsen *et al.*, 2011)
S. mutans	(Abranches *et al.*, 2011)
E. faecalis	(Park *et al.*, 2007; Lebreton *et al.*, 2009; Gaspar *et al.*, 2009; Hanin *et al.*, 2010; Zhao *et al.*, 2010; Michaux *et al.*, 2011; Gaca *et al.*, 2012; La Rosa *et al.*, 2012, 2013; Benachour *et al.*, 2012; Martini *et al.*, 2015)
E. faecium	(Lebreton *et al.*, 2011, 2012; Chibebe Junior *et al.*, 2013)
S. aureus	(Desbois and Coote, 2011; Browne *et al.*, 2015)
L. monocytogenes	(Mukherjee *et al.*, 2010; Joyce and Gahan, 2010)

1.7.2 The *G. mellonella* immune system

Insect immune systems are comprised of two main components, the cellular and humoral immune responses, and protection is provided by anatomical and physiological barriers (Figure 1.6). The first barriers of defense in insects against pathogens is the cuticle, which is a structurally and chemically complex barrier designed to prevent or retard the entry of pathogens into the haemoceol (the body cavity) (Clarkson and Charnley, 1996). The cuticle serves a function analogous to the skin in mammals (Kavanagh and Reeves, 2007). Cellular immunity is provided through rapid synthesis and mobilization of immune cells mediated by phagocytic cells, named haemocytes, that are found within the haemolymph (Kavanagh and Reeves, 2007; Tsai *et al.*, 2016). Up to 30% of haemocytes in some insect species are associated with internal organs such as the fat body, trachea or digestive system but the majority circulate freely within the haemolymph (Ratcliffe, 1985). These cells play a critical role analogous to mammalian blood immune cells or similar cells in other vertebrates, such as roles in non-self-recognition (Kavanagh and Reeves, 2007; Tsai *et al.*, 2016).

In addition, humoral immunity is coordinated by soluble effector molecules that immobilize or kill the microbial pathogens (Tsai *et al.*, 2016), and this consists of the processes of melanisation, haemolymph clotting and wound healing in response to injury (Vilmos and Kurucz, 1998). The humoral immune response includes the release of anti-microbial peptides (AMPs), complement-like proteins, opsonins (*e.g.* apolipophorin-III, peptidoglycan recognition proteins, cationic protein 8 and hemolin), and melanin (by phenoloxidase pathway and melanisation) (Kavanagh and Reeves, 2007; Tsai *et al.*, 2016). Several distinct types of AMPs are produced in the immune response to infection including an inducible metalloproteinase inhibitor, lysozyme, five moricin-like peptides, two cecropins, defensin, transferrin, Gm proline-rich peptides 1 and 2, Gm anionic peptide 1 and 2, galiomycin, gloverin, gallerimycin, inducible serine protease inhibitor 2, x-tox and heliocin-like peptide (Kavanagh and Reeves, 2007; Tsai *et al.*, 2016). All defense components in the *G. mellonela* immune system are interrelated and mutually cross-regulated which can trigger activation of humoral and cellular mechanisms and *vice versa*, and these responses are

involved in wound healing (Figure 1.6) (Wojda, 2016). Further, haemocytes can be triggered by humoral components but they also conceal particles affecting humoral responses.

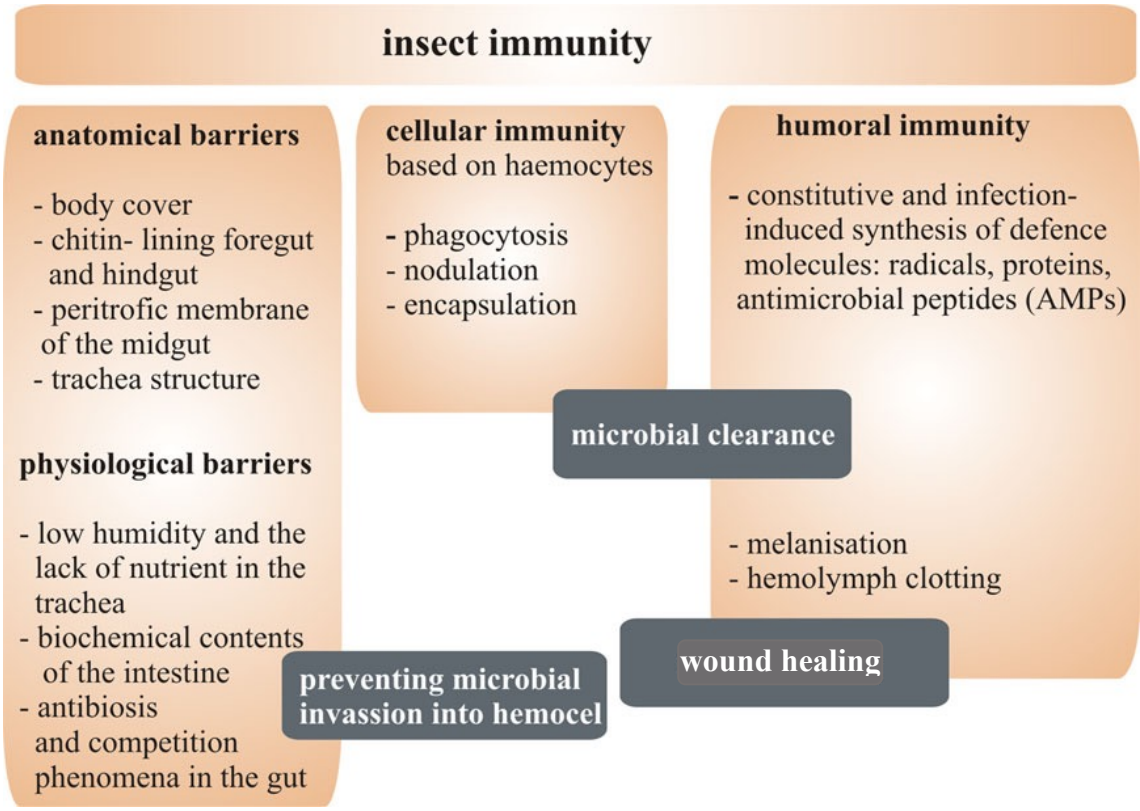

Figure 1.6 Streamlined system exhibiting insect immunity which the Insects are normally protected by anatomical and physiological barriers and by cellular and humoral immune responses (Wojda, 2016).

1.7.3 Use of *G. mellonella* to evaluate efficacy of antimicrobial agents

The *G. mellonella* model is a good alternative for the evaluation of antimicrobial agent effectiveness *in vivo* and decreases the probability of an antimicrobial agent that acts well in *in vitro* studies from progressing to ineffective performance in a mammalian or vertebrate model (Desbois and Coote, 2012; Tsai *et al.*, 2016). In order to evaluate novel antimicrobial compounds effectiveness and potential toxicity, normally, they are screened *in vitro* first (Tsai *et al.*, 2016). Then successful candidate compounds are assessed in a mammalian or vertebrate model, such as in a murine or rodent model, prior to their potential application in humans or other intended target mammal species (Desbois and Coote, 2012; Tsai *et al.*, 2016). *In vivo* examinations are essential to identify potential activity losses owing to host factors, *e.g.* most critically by metabolic processes, anti-infective defence mechanisms, mortification by host enzymes and the physiological conditions effect such as pH (Zak and O'Reilly, 1991). Several studies have administered antimicrobial agents such antibiotics and other bioactive compounds to infected *G. mellonella* larvae (Table 1.10).

Table 1.10 Experimental *in vivo* antimicrobial agents in the *G mellonella* against human pathogens (combined data with Desbois and Coote, 2011)

Antimicrobial agent	Pathogens	Effective dose	Time administration	Reference
		Gram-negative		
Ciprofloxacin	*F. tularensis* LVS	20 mg kg^{-1}	A single dose at 1 hbi or 2 hai after inoculation, two doses 6 and 24, or two doses 24 and 48 h after inoculation	(Aperis *et al.*, 2007)
Streptomycin		15 mg kg^{-1}	A single dose 1 h before inoculation, a single dose 2 h after inoculation, or two doses 6 and 24 h after inoculation	
Levofloxacin		15 mg kg^{-1}	A single dose 1 h before inoculation, a single dose 2 h after inoculation, two doses 6 and 24 h after inoculation, or two doses 24 and 48 h after inoculation	
Azithromycin,	*Acinetobacter baumannii*	0.77 mg kg^{-1}	A single dose 2 h after inoculation[b]	(Ahmad *et al.*, 2010)
Ciprofloxacin		0.62 mg kg^{-1}	A single dose 2 h after inoculation[b]	
Colistin		2.5 mg kg^{-1}	A single dose within 30 mins of inoculation; ineffective against a multidrug- resistant strain	(Hornsey and Wareham, 2011)
Colistin + teicoplanin		2.5 + 10 mg kg^{-1}	Two doses within 30 mins of inoculation	
Colistin + vancomycin		2.5 + 10 mg kg^{-1}	Two doses within 30 mins of inoculation	
Gentamicin		5 mg kg^{-1}	A single dose within 30 mins of inoculation; ineffective against a gentamicin- resistant strain	
Vancomycin		10 mg kg^{-1}	A single dose given within 30 mins of inoculation	
Gentamicin		6 mg kg^{-1}	A single dose within 30 mins of inoculation	(Peleg *et al.*, 2009)
Meropenem		60 mg kg^{-1}	A single dose within 30 mins of inoculation; ineffective against a carbapenem- resistant strain	
Bacteriophages: KS4, KS4-M, KS12, and KS14	*Burkholderia cenocepacia*	various multiplicity of infection values of 0.001– 5000	A single dose immediately after inoculation, a single dose 6 h after inoculation, or a single dose 12 h after inoculation; ineffective when given as a single dose 24 h after inoculation in all cases	(Seed and Dennis, 2009)
Baicalin hydrate (antibiofilm compound)		1.79 mg kg^{-1}	A single dose within 1 h after inoculation[a]	(Brackman *et al.*, 2011a)
Cinnamaldehyde		1.32 mg kg^{-1}	A single dose within 1 h after inoculation[a]	

Antimicrobial agent	Pathogens	Effective dose	Time administration	Reference
Cinnamaldehyde + tobramycin	*Burkholderia multivorans*	1.32 + 0.32 mg kg^{-1}	Two doses within 1 h after inoculation[a]; significantly more effective than either compound given alone at these doses	(Brackman *et al.*, 2011a)
Tobramycin		0.32 mg kg^{-1}	A single dose within 1 h after inoculation[a]	
Baicalin hydrate (antibiofilm compound)		1.79 mg kg^{-1}	A single dose within 1 h after inoculation[a]	
Cinnamaldehyde		1.32 mg kg^{-1}	A single dose within 1h after inoculation[a]	
Cinnamaldehyde + tobramycin		1.32 + 0.32 mg kg^{-1}	Two doses within 1 h after inoculationa; significantly more effective than either compound given alone at these doses	
Tobramycin		0.32 mg kg^{-1}	A single dose within 1 h after inoculation[a]	
Cefotaxime + piperacillin	*P. aeruginosa*	100 + 70 mg kg^{-1}	Two doses within 2 h after inoculation; significantly more effective than either compound given alone at these doses [b]	(Krezdorn *et al.*, 2014)
Cefotaxime + amikacin		100 + 50 mg kg^{-1}	Two doses within 2 h after inoculation; significantly more effective than either compound given alone at these doses [b]	
Piperacillin + amikacin		70 + 50 mg kg^{-1}	Two doses within 2 h after inoculation; significantly more effective than either compound given alone at these doses [b]	
Piperacillin + levofloxacin		70 + 100 mg kg^{-1}	Two doses within 2 h after inoculation; significantly more effective than either compound given alone at these doses [b]	
Cefotaxime + meropenem + piperacillin		100 + 2 + 70 mg kg^{-1}	Three doses within 2 h after inoculation; significantly more effective than either compound given alone or dual at these doses [b]	
Cefotaxime + meropenem + amikacin		100 + 2 +50 mg kg^{-1}	Three doses within 2 h after inoculation; significantly more effective than either compound given alone or dual at these doses [b]	
Cefotaxime + meropenem + levofloxacin		100 + 2 +100 mg kg^{-1}	Three doses within 2 h after inoculation; significantly more effective than either compound given alone or dual at these doses [b]	
Cefotaxime + piperacillin + amikacin		50 +70 + 100 mg kg^{-1}	Three doses within 2 h after inoculation; significantly more effective than either compound given alone or dual at these doses [b]	
Cefotaxime + piperacillin + levofloxacin		50 +70 + 100 mg kg^{-1}	Three doses within 2 h after inoculation; significantly more effective than either compound given alone or dual at these doses	
Cefotaxime + amikacin +levofloxacin		50 + 50 + 100 mg kg^{-1} levofloxacin	Three doses within 2 h after inoculation; significantly more effective than either compound given alone or dual at these doses [b]	

Antimicrobial agent	Pathogens	Effective dose	Time administration	Reference
Meropenem + piperacillin + colistin		2 + 70 + 10 mg kg^{-1}	Three doses within 2 h after inoculation; significantly more effective than either compound given alone or dual at these doses [b]	
Meropenem + piperacillin + amikacin		2 + 70 + 100 mg kg^{-1}	Three doses within 2 h after inoculation; significantly more effective than either compound given alone or dual at these doses [b]	
Meropenem + piperacillin + levofloxacin		2 +70 + 100 mg kg^{-1}	Three doses within 2 h after inoculation; significantly more effective than either compound given alone or dual at these doses [b]	
Meropenem + colistin + amikacin		2 +10 + 100 mg kg^{-1}	Three doses within 2 h after inoculation; significantly more effective than either compound given alone or dual at these doses [b]	
Meropenem + colistin + levofloxacin		2 + 10 + 100 mg kg^{-1}	Three doses within 2 h after inoculation; significantly more effective than either compound given alone or dual at these doses [b]	
Meropenem + amikacin + levofloxacin		2 + 50 + 100 mg kg^{-1}	Three doses within 2 h after inoculation; significantly more effective than either compound given alone or dual at these doses [b]	
Piperacillin + colistin + amikacin		70 + 10 + 50 mg kg^{-1}	Three doses within 2 h after inoculation; significantly more effective than either compound given alone or dual at these doses [b]	
Piperacillin + colistin + levofloxacin		70 + 10 +100 mg kg^{-1}	Three doses within 2 h after inoculation; significantly more effective than either compound given alone or dual at these doses [b]	
Piperacillin + amikacin + levofloxacin		70 + 50 + 100 mg kg^{-1}	Three doses within 2 h after inoculation; significantly more effective than either compound given alone or dual at these doses [b]	
Gram-positive				
Daptomycin	*S. aureus*	10 mg kg^{-1}	Three doses 2, 26, and 50 h after inoculation	(Desbois and Coote, 2011)
Penicillin		0.5 mg kg^{-1}	Three doses 2, 26, and 50 h after inoculation; ineffective against a penicillin- resistant strain	
Vancomycin		10 mg kg^{-1}	Three doses 2, 26, and 50 h after inoculation	
Hamamelitannin (antibiofilm compound)		4.84 mg kg^{-1}	A single dose within 1 h after inoculation[a]	(Brackman *et al.*, 2011a)
Hamamelitannin (antibiofilm compound) + vancomycin		4.84 + 0.8 mg kg^{-1}	A single dose within 1 h after inoculationa; significantly more effective than either compound given alone at these doses[a]	
Vancomycin		0.8 mg kg^{-1}	A single dose within 1 h after inoculation[a]	
Silver and zinc ions		ca. 1.5 ppm zinc ions or 0.2 ppm	A single 20 mL dose 20 mins after inoculation	(Coughlan *et al.*, 2010)

Antimicrobial agent	Pathogens	Effective dose	Time administration	Reference
Echinocandins (*e.g.* caspofungin)		0.19 µg mL^{-1}	A single dose within 24 after inoculation [b]	(Kelly and Kavanagh, 2011)
Daptomycin		6 or 10 mg kg^{-1}	A single dose within 1 h after inoculation	(Luther *et al.*, 2014)
Linezolid	*E. faecalis* or *E. faecium*	600 mg kg^{-1}	A single dose within 1 h after inoculation	
Gentamicin		1.3 mg kg^{-1}	A single dose within 1 h after inoculation	
Rifampin		300 mg kg^{-1}	A single dose within 1 h after inoculation	
Gentamicin + daptomycin	*E. faecalis*	1.3 + 6 mg kg^{-1}	A single dose within 1 h after inoculation; significantly more effective than either compound given alone at these doses[b]	
Gentamicin + linezolid	*E. faecium*	1.3 + 600 mg kg^{-1}	A single dose within 1 h after inoculation; significantly more effective than either compound given alone at these doses[b]	
Gentamicin + daptomycin		1.3 + 6 mg kg^{-1}	A single dose within 1 h after inoculation; significantly more effective than either compound given alone at these doses[b]	
Fungal				
Laminarin, a polymer of b-1,3 glucan from *L. digitata*		60 µg 20 $µL^{-1}$	A single dose within 24 h before inoculation	(Bergin *et al.*, 2006)
Mannan, from *S. cerevisiae*		60 µg 20 $µL^{-1}$	A single dose within 24 h before inoculation	
Yeast extract ß-glucan		7.5, 15, 30 or 60 mg $larva^{-1}$	A single dose within 24 h before inoculation [b]	(Mowlds *et al.*, 2010)
Echinocandins (*e.g.* caspofungin)	*C. albican*	0.0475 or 0.19 µg mL^{-1}	A single dose within 24 before inoculation [b]	(Kelly and Kavanagh, 2011)
Probiotic *Lactobacillus acidophilus*		10^5 cells $larva^{-1}$	A single dose within 1 h before or after inoculation (slight increased survival of the larvae)	(Vilela *et al.*, 2015)
Acetylcholine		50 µg $larva^{-1}$	A single dose within 1 h after inoculation [a]	(Rajendran *et al.*, 2015)

In each of these studies, efficacy is considered to be a significant increase in wax moth larva survival (either after a specific incubation time or the total lifespan) compared to the control group. In all cases, the antimicrobial agents were delivered by direct injection into the hemocoel and effective doses have been converted to mg kg^{-1} to allow simple comparisons between studies and to doses used in humans.

[a] Assumes mean mass of 250 mg $larva^{-1}$, [b] Assumes mean mass of 325 mg $larva^{-1}$,

1.8 Aim of the study

A thorough understanding of pathogens associated with tilapia commercial production and how to prevent disease, especially with the increased worldwide production of cultured tilapia, will be of great significance to fish health professionals and commercial producers. Therefore, the overall goal of this research was to evaluate the effectiveness of bioactive algal compounds on the growth and survival of francisellosis *in vitro* and *in vivo* and determine their potential to control francisellosis infection under experimental conditions in Nile tilapia *O. niloticus*, *in vivo*. *In vivo* tests were also conducted in an alternative insect *in vivo* model, the wax moth *G. mellonella*.

The specific objectives of this thesis were to:

1. Screen algal-derived compounds *in vitro* for immunostimulatory and antibacterial activities (using tilapia primary head kidney macrophages and disc diffusion assays)

2. Develop an immersion challenge for *Francisella noatunensis* subsp. *orientalis* in Nile tilapia *O. niloticus* L. and evaluate response to infection.

3. Examine the effect of feeding algal-derived compounds (in feed) on the innate immune response of Nile tilapia homo gold *in vivo*, challenged with single and co-infections of economically significant bacterial pathogens.

4. Development an alternative *in vivo* infection model for *Francisella noatunensis* subsp. *orientalis* in wax moth larva *Galleria mellonella* and assess the effects of antibacterial compounds, including algal-derived compounds.

CHAPTER 2

IN VITRO SREENING OF ALGAL-DERIVED COMPOUNDS FOR IMMUNOSTIMULATORY AND ANTIBACTERIAL ACTIVITIES

2.1 Introduction

Intensification of aquaculture practices leads to the emergence of several pathogenic organisms. The control of disease outbreaks is one of the great challenges for the aquaculture industry worldwide. Numerous compounds have been used to control fish diseases, including antibiotics, probiotics, prebiotics and immunostimulants. Immunostimulants is a naturally occurring compound that activate the innate humoral and cellular defense mechanisms by increasing the host's resistance against to infectious disease by viruses, bacteria, fungi and parasites (Galindo-Vilegas and Hosokawa, 2004; Bricknell and Dalmo, 2005). Immunostimulants can be divided into several groups depending on their sources: bacterial products (*e.g.* biostim, peptidoglycan or lipopolysaccharides/LPS), nutritional factors as immunostimulants, hormones/cytokines, phytochemicals [*e.g.* Glycyrrhizin (Licorice), Ete (Tunicate), *Quillaja saponica* (Soap tree), etc]; ß-1,3-glucans of yeast, mycelial fungi and mushrooms; a number of synthetic products (*e.g.* besatin, muramyl peptides, lipopeptides, levamisole, etc); animal-derived products like chitin; and algae (*e.g.* Spirulina) or algal-derived compounds (*e.g.* fucoidan, Laminaran, alginates, etc).

Algae are able to produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities, therefore they are considered as a source of bioactive compounds (Chojnacka *et al.*, 2012). Recently, a variety of biological activities for algal compounds have been reported including antibacterial (Park *et al.*, 2013; Desbois and Lawlor, 2013), antifungal (Kumar *et al.*, 2013), antitumor (Azuma *et al.*, 2012), anticancer (Ermakova *et al.*, 2013), anti-obesity (Park *et al.*, 2011), antiviral (Bouhlal *et al.*, 2011) and antioxidant (Badrinathan *et al.*, 2011) (as described in Chapter 1, Section 1.6). In addition, with regards to aquaculture, extracts from macro and micro algae have led to the discovery of a diversity of secondary metabolites with antimicrobial activities against fish pathogens (as described in Chapter 1, Section 1.6.3). Therefore, interest in macro and micro algae as a potential and promising source of pharmaceutical agents has increased (Choudhury *et al.*, 2005; Bansemir *et al.*, 2006; Caipang *et al.*, 2011; Vijayakumar *et al.*, 2012;

Choi *et al.*, 2014).

In order to determine which antimicrobial agents should be considered for treating a bacterial pathogen, *in vitro* antimicrobial susceptibility testing is recommended as the first stage. The various bacterial pathogens that cause disease in aquatic animals often require specific growth conditions and these vary considerably from routine terrestrial bacterial pathogens (Hawke *et al.*, 2006). Thus, it is become desirable to develop antimicrobial testing standards for bacteria that require particular conditions, such as lower temperature, semisolid media or supplemented media (*e.g.* NaCl, serum, blood, etc.) (Hawke *et al.*, 2006). A variety of antimicrobial susceptibility testing protocols have been used for screening antimicrobial agents against bacterial pathogens *in vitro*. For example, the disc diffusion assay (Ganeshamurthy *et al.*, 2013), various microplate methods *e.g.* minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (Desbois *et al.*, 2009), cell lines (Fierro-Castro *et al.*, 2013) and primary cell cultures (Caipang *et al.*, 2011). The disc diffusion assay is commonly used to screen antimicrobial agents, owing to its simplicity, rapidity and low cost (Desbois and Smith, 2010). In addition, this assay can be applied to screen many samples simultaneously, irrespective of the solvent used during preparation and the output data is easy to interpret (Desbois and Smith, 2010). Another alternative method for this purpose is the use of primary cell cultures and such *in vitro* assays have been used to select new immunostimulant candidates for aquaculture. These may constitute a cost-effective alternative to *in vivo e*xperimentation, as they may give more reproducible results under a highly controlled experimental environment (Iliev *et al.*, 2005a; b).

The most important immunocompetent organs and tissue of fish include the kidney (anterior/or head and posterior/or caudal), thymus, spleen, liver and mucosa-associated lymphoid tissues (Galindo-Villegas and Hosokawa, 2004; Zapata *et al.*, 2006). In fish, myelopoiesis generally occurs in the head kidney and/or spleen, whereas thymus, kidney and spleen are the major lymphoid organs (Zapata *et al.*, 2006). The thymus is considered as the primary T cell organ, while the head kidney is considered the primary B cell organ. In addition,

head kidney and spleen present macrophage aggregates, also known as melano-macrophage centers (Alvarez-Pellitero, 2008). Macrophages play a role in both the innate and the adaptive immune system. In the innate immune system, they act as phagocytic cells, phagocytosing pathogens and producing oxygen and nitrogen radicals (Alvarez-Pellitero, 2008). In the adaptive immune system, macrophages act as professional antigen presenting cells. As such, macrophages can provide a bridge between the innate and adaptive immune response. Altogether, macrophage differentiation exhibits a wide array of functional and phenotypic heterogenecity. In the literature, a further distinction has been made between classical and innate activation of macrophages (Gordon, 2013). Classical activation is induced by stimulation with both IFN-α/ß and a microbial trigger such as LPS, whereas innate activation is induced by stimulation with LPS (or other microbial triggers) alone (Gordon, 2013). Although classically and innate activated macrophages are induced by different stimuli, their functions overlap. Innate activated macrophages have microbicidal activity and produce pro-inflammatory cytokines, reactive oxygen species (ROS) and NO (Gordon and Taylor, 2005).

Immunostimulants, which primarily activate non-specific cellular mechanisms mediating innate immunity, are considered to be safe and cost-effective drugs for the health protection of aquaculture fish, as they enhance their innate immunity and provide sufficient protection against a range of fish pathogens (Galindo-Vilegas and Hosokawa, 2004). Most immunostimulants are recognized as specific pathogen-associated molecular patterns (PAMPs) (Akira and Takeda, 2004), which are detected by pattern-recognition receptors (PRRs) in the host cells, such as Toll-like (Akira *et al.*, 2006) and RIG-1 receptors (Zou *et al.*, 2010). The binding of these receptors with their respective PAMPs can trigger intracellular signalling pathways leading to the production and release of pro-inflammatory cytokines, which mediate the onset of the innate immune responses (Rebl *et al.*, 2010). Microbicidal/cytotoxic activities are of major importance among the innate effector responses such as phagocytosis, increased respiratory burst and release of bactericidal molecules in macrophages, granulocytes and other cells of the innate immune system (Galindo-Villegas and Hosokawa, 2004). Therefore, the immune effects of

immunostimulant candidates are mostly studied by assessing and focusing on the activation of macrophages and granulocytes (Galindo-Villegas and Hosokawa, 2004).

In fish, immunostimulants that are PAMPs or PAMP-like molecules, like LPS, ß-glucans and double-stranded RNAs, also increase non-specific protection by activating pro-inflammatory responses and microbicidal activities through PRRs (Iliev *et al.*, 2005a). However, for other immunostimulants, like algal-derived compounds the mechanisms by which they elicit pro-inflammatory and microbicidal responses are unclear, especially in tilapia. Preliminary selection, screening and *in vivo* testing of drugs as new immunostimulant-candidates is quite difficult and laborious (Bricknell and Dalmo, 2005). This is in part because not all aquaculture fish species respond in the same way to a given immunostimulant, as there are important adaptive and genetic differences between them affecting the fish immune system (Bricknell and Dalmo, 2005), as well as in the environmental conditions they are grown in (Bowden *et al.*, 2007; Bowden, 2008). Moreover, modifications in the molecular form and purification processing of PAMPs used as immunostimulants may result in notable differences in immune responses, as has been reported for LPS (Iliev *et al.*, 2005b).

The aim of this Chapter was to screen algal-bioactive compounds as candidate immunostimulants by *in vitro* testing. A disc diffusion assay and a primary cell culture system of tilapia head kidney-derived macrophages were used. The ability of the head kidney-derived macrophages was evaluated after stimulation with different algal bioactive compounds to produce ROS, as well as bactericidal killing capacity.

2.2 Materials and methods

2.2.1 Bacteria

2.2.1.1 Bacterial strains and culture conditions

Three bacterial species were used: *Fno* STIR-GUS-F2f7, *A. hydrophila* T4 strain, and two isolates of *S. agalactiae* (type strain NCIMB 701348 and *S. agalactiae* fish isolate). The *Fno* isolate was kindly provided by Jose Gustavo Ramirez-Paredes, University of Stirling, while *A. hydrophila* and *S. agalactiae* were kindly provided by the Bacteriology Laboratory, Institute of Aquaculture, University of Stirling, Scotland, UK). *Fno* STIR-GUS-F2f7 was recovered from infected red Nile tilapia *Oreochromis niloticus* L cultured on a Northern Europe fish farm with a re-circulating system in 2012 (Ramirez-Paredes, 2015; Ramírez-Paredes *et al.*, 2017). The *A. hydrophila* T4 strain was originally isolated from *Labeo rohita* during an outbreak of epizootic ulcerative syndrome in Bangladesh, while, the *S. agalactiae* fish isolate originated from infected tilapia in Columbia (recovered in 2008).

Culture of the bacteria was performed on agar and in broth media at 28°C, the optimal temperature for *in vitro* growth (Baker *et al.*, 1985; Kamaishi *et al.*, 2005; Ramirez-Paredes, 2015). *Fno* was cultured on Cysteine Heart Agar (Melford Laboratories, UK) supplemented with 10% bovine haemoglobin solution (also known as CHAH media) (Becton Dickenson BBL, Sparks, MD, USA) for 48 h at 28°C. The liquid medium was cation-adjusted Mueller-Hinton II broth supplemented with both 0.1% glucose and 2% IsoVitaleX (Becton Dickenson BBL, Sparks, MD, USA) (MHB). Broth cultures were grown overnight at 28°C in an orbital shaker at 150 rpm and bacteria were frozen at -70°C in broth medium containing 20% glycerol (Soto *et al.*, 2009a; Colquhoun and Duodu, 2011; Ramirez-Paredes, 2015) for later use.

A. hydrophila and *S. agalactiae* were inoculated onto trypticase soy agar (TSA) (Oxoid Ltd., Basingstoke, UK) and liquid media was tryptic soy broth (TSB) (Oxoid Ltd., Basingstoke, UK). The agar plate was sealed with parafilm (Bemis Company, Inc., Neenah, USA), and incubated at 28˚C for 24 h for *A. hydrophila*

and at least 48 h for *S. agalactiae*. Frozen stocks of *A. hydrophila* and *S. agalactiae* were prepared using protect cyopreservative beads by picking up some colonies (4–5 large or medium colonies) of *A. hydrophila* and *S. agalactiae* from agar media at 24 or at least 48 h culture, respectively, with a 10 µL sterile disposable plastic inoculating loop (Nunc^{TM}, ThermoFisher Scientific, Denmark) and transferring these into the bead vial (TS80-MX, Technical Service Consultants, UK) for each bacterium. The vial lid was replaced, the tube inverted 10 times to ensure agitation, left for 2 mins, then the cryofluid was removed using a sterile Pasteur pipette (201CS10, Copan Diagnostic LTD, USA), and stored at -70°C.

The bacterial isolate was confirmed as *Fno*, *A. hydrophila*, and *S. agalactiae* according to the methods described by Frerichs and Millar (1993), including primary identification (Id) tests and biochemical profiles. The primary Id tests included: Gram staining, catalase, oxidase, Oxidation/Fermentation (O/F) of glucose and motility tests were performed as described by Frerichs and Millar (1993). Biochemical tests were performed using the commercially available API 20E for *A. hydrophila* and *Fno*, while ZYM kits (BioMerieux®; Marcy l'Etoile, France) only for *Fno*, and these were conducted as described in the manufacturer's guidelines except the inoculated strips were incubated at 28°C and read at 24 h.

2.2.1.2 Bacterial growth curves

Growth curves were prepared for *Fno* STIR-GUS-F2f7, *A. hydrophila* T4 strain, and *S. agalactiae* type strain. This was done in order to determine the phase of bacterial growth (*e.g.* lag phase, the starting phase, exponential phase, stationary phase and a die-off phase) over time. Measurements of turbidity were made using a spectrophotometer, expressed as absorbance values (A^o) or optical density with interval measurements until the bacteria reach either the exponential, stationary, or die-off phase.

Briefly, frozen glycerol or protect beads of the bacterial cultures were sub-cultured by inoculating onto CHAH or TSA and incubated at 28˚C for 24 h for *A.*

hydrophila and at least 48 h for *Fno* and *S. agalactiae*. Once growth had been achieved, the purity of the cultures was confirmed, and then some colonies of each bacteria (*i.e.* a loop full of colonies of *Fno*, five colonies of *S. agalactiae*, and two colonies of *A. hydrophila*) were transferred into 10 mL of of MHB or TSB, incubated overnight at 150 rpm and 28˚C in an orbital shaker and prepared as a starter bacterial culture. Forty microliters of overnight cultures were spread on CHAH, sealed with parafilm, and incubated at 28˚C for at least 48 h to confirm the purity. The overnight cultures were diluted by transferring 1 mL into 99 mL of sterile MHB or TSB in 500 mL Duran glass bottles (Sigma-Aldrich, Merk, UK), and gently inverting to distribute the cells for the growth curve observation. The duplicate samples were prepared for experimental comparison. Forty microliters of each bacterial broth cultures were plated out onto CHAH or TSA to confirm purity as mentioned previously. One millilitre of the bacterial culture was aseptically added into a polystyrene semi-micro cuvette (Fisherbrand^{TM}, Fisher Scientific, UK), and the turbidity of the culture was checked at absorbance of 600 nm for *Fno* (Vojtech *et al.*, 2009a) and 610 nm for *A. hydrophila* and *S. agalactiae* (Mian *et al.*, 2009) using a spectrophotometer (Cecil CE-2014; Buck Scientific, Inc., USA) and compared to either a sterile MHB or TSB standard.

Drop counts of bacteria (bacteria in broth culture) were performed to enumerate the actual number of viable bacteria per mL (colony forming units, CFU mL^{-1}), and this was done by serially diluting the bacterial suspension (10-fold dilutions) in 1X PBS (Miles and Misra, 1938). A 1X PBS (0.02M Phosphate and 0.15M NaCl) was contained of 0.876 g L^{-1} $NaH_2PO_4.2H_2O$ (BDH, Laboratories Supplies, England), 2.56 g L^{-1} $Na_2HPO_4.2H_2O$ (BDH), and 8.77 g L^{-1} NaCl (Sigma Aldrich, UK), and pH 7.2. Briefly, five hundred microliters of the culture were added into 4.5-mL of a sterile PBS in a bijuox, and serial dilutions (10-fold dilutions) was prepared from 10^{-1} to 10^{-7}, and plating 6x 20 µL drops of each tenfold dilution onto CHAH or TSA. These were then left to dry flat at room temperature for at least 1 h, sealed using parafilm, and viable colonies were counted after 24 or at least 48 h of incubation at 28°C (Figure 3.1B, C, D, and E). The absorbance measurements and drop counts of the bacteria cultures

were made at 3, 6, 9, 12, 15 h and every 24 h until the exponential phase was reached.

2.2.1.3 Preparation of bacteria inocula for antibacterial activity

Three bacteria (*Fno* STIR-GUS-F2f7, *A. hydrophila* T4 strain, and *S. agalactiae* fish isolate) were selected to test antibacterial activity. Twenty microliters of *Fno* from frozen stock was spreaded onto CHAH using a sterile disposable plastic loop, the agar plate was sealed and incubated for 24 and 48 h at 28˚C, while for *A. hydrophila* and *S. agalactiae*, cryo-ball from frozen stock was directly streaked onto TSA. These were used in the antibiotic disc diffusion assay. Bacterial cultures in 15-mL of MHB or TSB in a polypropylene 30 mL universal tube (Sterilin™; Thermo Fisher Scientific, UK) were obtained from the previous cultures of bacteria on agar medium by picking up a loop full of *Fno*, or two colonies of *A. hydrophila,* or five colonies of *S. agalactiae* using a sterilie metal bacteriological loop, and grown 28˚C in an orbital shaker (150 rpm) to middle log-phase growth (Ottem *et al.*, 2007). Based on the growth curve results for each bacterium, the age of bacteria culture inocula used for this present study and for the next experiments in other Chapters will be an inocula at middle log phase at 19.5 (*Fno*), 13.5 (*S. agalactiae*), and 4.5 h (*A. hydrophila*) (Section 2.3.3). The same volume of sterile MHB or TSB in a universal tube without bacteria was added and used as the negative control. After incubation time, the bacterial cells were collected by centrifugation (Sigma 4K15; Sigma Laboratory Centrifuges, Shropshire, UK) at 3,000 x g for 15 mins at 4°C. Then the supernatant was discarded, and the cells was re-suspended in 10 mL of sterile PBS by vortexing. Centrifugation and resuspension in PBS process was repeated twice to complete the washing of the cells. The cell suspension was adjusted to the desired absorbance units (AU) using PBS for *in vitro* assays. A bacterial drop count was then performed, as described in Section 2.2.1.2.

2.2.2 Experimental animals

Nile tilapia homo gold were obtained from the Tropical Aquarium at the Institute of Aquaculture (University of Stirling, UK). The fish were stocked into 20 L tanks

with recirculating, aerated freshwater with individual air stones at 27±2°C under constant aeration and filtration. The tilapia were fed twice daily with commercial pellets (Skretting Trout Nutra 25, UK) and kept on a 12 h light: 12 h dark cycle. Head kidney macrophages were isolated from tilapia specimens with body masses ranging 170–200 g.

2.2.3 *In vitro* screening algal-derived compounds for immunostimulatory and antibacterial activities

2.2.3.1 Algae

Three freeze-dried samples of algal species were used: green algae, *Chlorella* sp. provided by Algamax^{TM} from Florida USA; a diatom, *Chaetoceros calcitrans* (Paulsen) Takano 1968, and a green algae *Nannochloropsis* sp. provided by Professor Fatimah Md. Yusoff from University Putra Malaysia, Malaysia. In addition, two commercially available algal compounds were also used, alginic acid and ß-1,3-glucan. The alginic acid extracted from *Macrocystis pyrifera* (giant kelp) was obtained from Sigma-Adrich, UK. A single source of ß-1,3-glucan from *Euglena gracilis* was used and supplied as product Algamune AM by Algal Scientific Corporation, Plymouth Michigan USA. Algamune AM contained 50% ß-1,3-glucan, beneficial omega-3 fatty acids, vitamins, and trace elements, and the product had been approved as a feed material for sale in the EU (EU Reg. No 68/2013 “7.1.2 dried algae *E. gracilis)* (Algal Scientific, USA).

2.2.3.2 Preparation of algal extracts

The three freeze-dried algal samples were extracted using the method described by Foo *et al.,* (2015a; b). Fifty millilitres of ethanol were added to 0.1 g of lyophilized microalgae biomass and homogenized (Ultra-Turrax T25 digital, rpm x 1000 IKA®-WERKE GmbH & Co. KG, Staufen, Germany) at 9500 rpm/min for 15 mins. Extracts were then sonicated (Kerry ultrasonic water bath; model pulsatron 125) for 30 mins at room temperature (RT) then filtered through Whatman No. 1 filter paper. Pellets were collected, and another 50 mL of ethanol added for the second extraction. This procedure was repeated three

times. The pooled filtrates containing solvent and extracts were separated from each other under reduced pressure (Rotavapor R-220, Buchi, Postfach, Flawil, Switzerland and continued with Nitrogen Evaporator N-EVAP^{TM} 112, OA-HEAT^{TM} model 8125, Organomation, USA) (Figure 2.1). Extrcats were kept in -70˚C for at least 1 h prior to completely removed the solvent from extracts with lyophilisation (Christ Alpha 1-4 LSC freeze dryer, SciQuip, The Scientific Equipment Specialists, UK), and then subsequently stored at -20˚C until further analysis. Then extraction procedure was repeated with other solvent systems: 100% methanol, 100% acetone and distilled water. The dry crude extract from each algal was re-dissolved using the same solvents to achieve stock solution at 20 mg mL^{-1} and then diluted to get working solution 10 mg mL^{-1}.

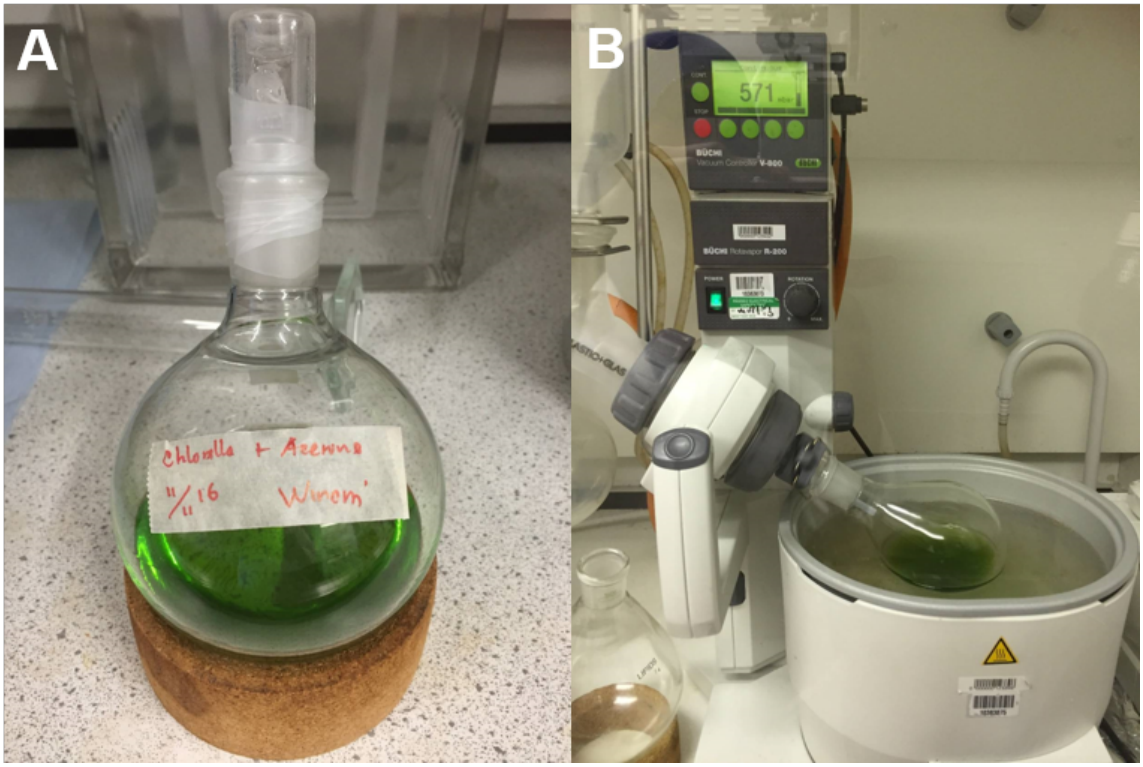

Figure 2.1 Algal extractions (Foo *et al.*, 2015a; b): (A) extracts of *Chlorella* sp. in acetone; and (B) collected filtrates containing solvent and extracts were separated from each other under reduced pressure (Rotavapor R-220, Buchi, Postfach, Flawil, Switzerland).

2.2.3.3 Isolation of head kidney macrophages

Phagocytes were isolated from the head kidney (HK) of Nile tilapia homo gold using the method of Sayeed (2003) with some modifications. The fish were sacrificed by a Schedule 1 method, specifically by anaesthetizing with at least 5 mL of 10% benzocaine in 1 L of water (from the same tank where the fish was reared) and a lethal blow to the head. The fish were bled to remove potential contaminating red blood cells from the macrophage cell cultures. Briefly, the HK was removed aseptically and placed on a piece of sterile cell strainer 100 mm, 100 µm mesh size (Fisherbrand®, Thermo Scientific, UK) placed over a small petridish containing 5 mL of ice-cold Leibovitz medium (L-15) with phenol red or RPMI-1640 without phenol red (GIBCO™, Thermo Fisher Scientific, USA) and 20 µL heparin (Sigma-Aldrich, UK). Heparin, clinically has been used as an anticoagulant, but also has various anti-inflammatory and immunomodulatory properties (Young, 2008; Ludwig, 2009; Sarah *et al.*, 2015). Therefore, just small amount of heparin used in this study. HK cell suspensions were obtained by gently macerating the tissue through the mesh with the blunt end of 1 mL syringe plunger to form a cell suspension. The diluted cell suspension was then carefully layered over 7 mL of lymphocyte separation medium, Histopaque®-10771 steril-filtered, density: 1.077 g mL^{-1} (Sigma-Aldrich, UK) in a universal tube and centrifuged at 1100 x g for 45 mins at 4˚C acceleration 8 and decrease 2. The buffy coat layer was carefully collected at the L-15 medium-Histopaque interface using a sterile Pasteur pipette (210CS10, Copan Diagnostics Inc, USA) (this fraction is enriched with macrophages). The cells suspension was placed in a plastic universal tube and washed with 20 mL of cold PBS by centrifugation at 600 x g for 7 mins. The pellet was re-suspended with 10 mL RPMI medium. The viable cell concentration was determined using an improved Neubauer haemocytometer (0630030, Superior, Marienfield, Germany) and microscope counting. The concentration of cells was adjusted to 1 x 10^7 cells mL^{-1} with RPMI and kept on ice until further use.

2.2.3.4 Respiratory burst assay (as an immunostimulatory activity)

Reactive Oxygen Species (ROS) production by macrophages was assessed using the reduction of NBT to formazan as a measure of intracellular

superoxide anion production as described previously (Rook *et al.*, 1985; Fierro-Castro *et al.*, 2013). Briefly, three Nile tilapia homo gold (170–200 g) were killed, bled, and HK phagocytes isolated. Cell concentration was adjusted to 1 x 10^7 cells mL^{-1} with RPMI, and 100 µL of cells were placed into each well of a sterile 96 well tissue culture plate, with three replicate wells for each treatment per fish. Three different controls were also included in each plate: negative control (cells with RPMI medium and 1 mg mL^{-1} of NBT), positive control (cells with 1 µg mL^{-1} Phorbol 12-myristate 13-acetate [Sigma-Aldrich, UK] and NBT) and a control for absorbance (blank wells) (Figure 2.2). The plate was wrapped with aluminium foil and incubated for 2 h at RT (22˚C). This temperature was selected based on the preliminary temperature testing of respiratory burst for the best growth of primary (tilapia macrophage) cell culture (data not shown).

NBT solution was prepared at 1 mg mL^{-1} in RPMI medium. In addition, PMA solution was made by removing 5 mL of NBT solution above and added 5 µL of PMA stock solution (1 mg mL^{-1} in ethanol) to give a final concentration of 1 µg mL^{-1}. Algal-derived compounds were also prepared using NBT solution above (at 1 mg mL^{-1}) at 50 µg mL^{-1} for each algal and solvent of extractions. A 100 µl of corresponding algae solution in 1 mg mL^{-1} of NBT for each solvent was added into the replicate wells for each treatment after the monolayers were washed gently three times with PBS (at the same temperature with cells incubation) to remove non-adherent cells. In addition, controls and blank wells were included in each plate (Figure 2.2). The macrophage monolayers plate was wrapped again with foil and incubated for 1, 3, 6 or 24 h, at RT. Then at the end of incubation the respiratory burst reaction was stopped by fixing the cells with 100% methanol (Merck, UK) for 30 secs (added directly into each well), washed three times with 70% (v/v) methanol, and allowed to dry (max. overnight). The formazan was solubilized by adding into each well 120 µL 2M KOH (potassium hydroxide) (Panreac) and 140 µL DMSO (dimethyl sulfoxide) (Sigma-Aldrich, UK). The content of each well was mixed carefully, and air bubbles removed with a tip. The absorbance of the wells was measured at 490 nm using a Synergy™ HTX Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). The results were expressed as the stimulation index and calculated as { ($\ddot{X}$ sample - $\ddot{X}$ blank) / ($\ddot{X}$ control - $\ddot{X}$ blank) }–1.

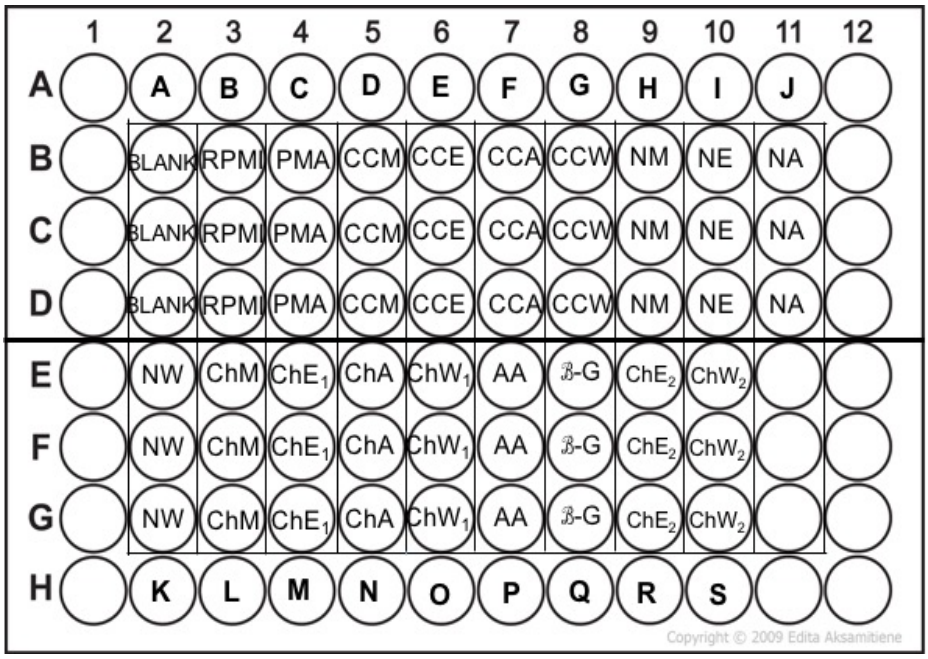

Figure 2.2 Schematic representation of respiratory burst assay 96 well tissue culture plate layout where tilapia head kidney primary cells culture were stimulated with algal-derived compounds in a 96 wells tissue culture plate: (A) blank wells; (B) cells+RPMI+NBT; (C) cells+PMA+NBT; cells+*C. calcitrans*+methanol (CCM) (D), +ethanol (E), +acetone (CCA) (F), and +water (CCW) (G); cells+*Nannochloropsis* sp.+methanol (NM) (H), +ethanol (NE) (I), +acetone (NA) (J), and +water (NW) (K); cells+Chlorella sp.+methanol (ChM) (L), +ethanol 1 (ChE1) (M), +acetone (ChA) (N) and +water 1 (ChW1) (O); cells+alginic acid (AA) (P); cells+ß-glucan (ß-G) (Q); cells+*Chlorella* sp.+ethanol 2 (ChW_2) (R); and +water 2 (ChW_2) (S).

2.2.3.5 Antibacterial activity

The antimicrobial activity of three crude algal extracts which were extracted with four different solvents (Section 2.2.2.2), and two other algal compounds that were dissolved in sterile distilled water were tested with three different bacteria (Section 2.2.1.1), by using disc-diffusion and primary cell (macrophages) culture assays.

A. Antibiotic disc diffusion assay

Prior screening of the different algal extracts and compounds using the disc diffusion assay, the antibiotics sensitivities of the three bacterial pathogens were determined. The results were used as a positive control for the algae screening in the disc diffusion assay. The CHAH medium was utilized for *Fno* in the disc-diffusion method (mainly, due to the fastidious nature of *Fno* and its inability to grow in Mueller–Hinton based agar), and TSA was used for *S. agalactiae* and *A. hydrophila*. The bacteria were tested for susceptibility to the following antibiotics, all purchased from Sigma-Aldrich Ltd (Poole, UK): ampicillin (A9393-5g); penicillin G sodium (1502701-150g); erythromycin (E5389-5g); gentamicin (G1397-10 mL); kanamycin (K1377-1g); and tetratcycline (T3383-25g). Streptomycin, kanamycin, gentamycin, penicillin and ampicillin were dissolved in a sterile water, while tetracycline was dissolved in water+100% ethanol (v/v) (1:1) and erythromycin in 1M HCL.

Briefly, bacteria were grown on the agar plate for each bacterium and incubated at 28˚C, as described at Section 2.2.1.1. A visible quantity of microbial growth from the inocula agar plate (more than one colony *e.g.* 3–5 colonies) was swabbed onto new agar using a sterile cotton swab wooden stick in peel packs (Fisherbrand™, FisherScientific, UK). The bacterial swab was initiated at the centre of the petridish, and then streaked across the plate in multiple rapid strokes until reaching the top of the plate (Figure 2.3a). The plate was then turned 180° and the streaking process repeated exactly as before (Figure 2.3b). When finished the plate was turned 90° and starting from the centre of the petridish, again streaked across the plate until reaching the top (Figure 2.3c), and finally, the plate was turned 180° and for the final time repeat the streaking process exactly as before (Figure 2.3d). It was importantly to make sure that the agar surface was evenly covered with the microbial inocula.

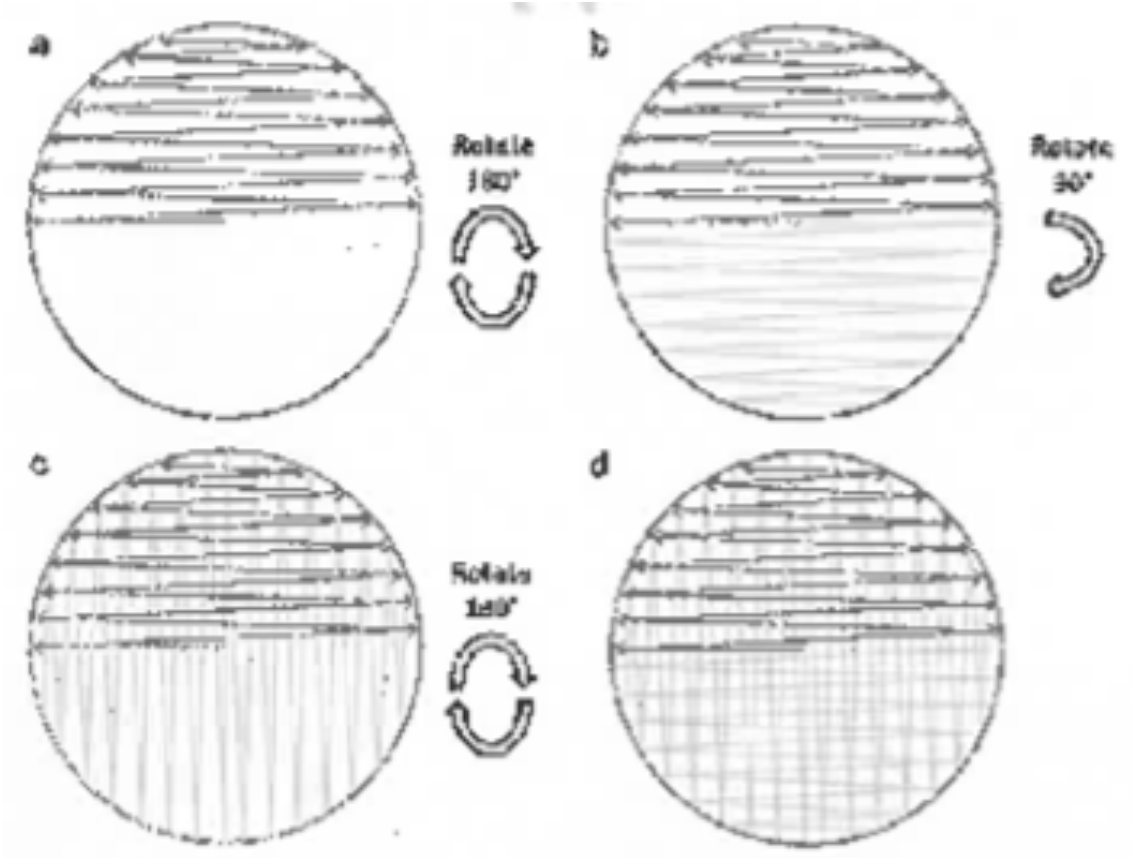

Figure 2.3 Graphic illustration of how to evenly spread the bacterial inocula using a sterile cotton wool swab across the agar plate (steps A to D) (Desbois and Smith, 2010).

Prior to loading antibiotics onto the paper discs, a piece of rigid cardboard (approximately 20 x 30 cm) in aluminium foil was prepared to rest the paper disc on during the loading and drying steps. The foil-covered board was sprayed with absolute ethanol, wiped clean with blue tissue roll and this step repeated with sterile water. Once the board surface had dried completely, sterile paper discs (6 mm diameter Whatman^{TM}, GE Healthcare Life Science, UK) were placed on the board, and arranged in rows using sterile plastic forceps. Twenty microliters of antibiotic were pipetted onto the centre of the first sterile paper disc using a sterile tip and pipette, and this process was repeated using a separate disc for each antibiotic and concentration (5 and 50 µg 20 μL^{-1} for each antibiotic). The concentrations were selected after pre-eliminary test of antibiotics to get the low and medium inhibitons on agar for each bacterium (data not shown). Then the paper discs were dried completely at RT. Negative control paper discs were prepared by loading these with volumes equal to the maximum quantities of solvents used to prepare the antibiotic solutions (*e.g.* water, water and ethanol or 1M HCL) loaded onto any of the test discs. After all the antibiotics and negative controls loaded paper discs were dried completely at RT, the discs were then placed onto the agar surface using sterile plastic forceps and pressed down gently on each disc to ensure that it was attached to the surface. The agar plates were inverted and incubated at 28˚C for 24 and 48 h, as previously mentioned in Section 2.2.1.1. The antibacterial activity was determined by measuring diameters of the clean inhibitory zone of bacterial growth around each agar paper disc and calculated as a total area of clear zone minus the area of the disc in mm^2.

B. Screening algal-bioactive compounds using the agar disc diffusion method

The anti-bacterial activity of algal extracts, and purified compounds were tested using the disc-diffusion technique according to Abd El-Aty *et al.* (2014). Initially, bacterial strains were grown to mid log phase (Section 2.2.1.3). The bacterial suspensions were then adjusted to 0.5 and 0.1 AU A_{600} or A_{610} with a sterile PBS. A 100 µL bacterial inocula from each bacterium either at AU 0.5 or 0.1 was seeded onto agar media (CHAH or TSA) and spread on the agar surface

using a sterile glass rod to get a uniform bacterial growth on the test plates, and then allowed to dry at RT. Dried algae samples were extracted and dissolved using four different solvent *viz.* 100% ethanol, 100% methanol, 100% acetone, and sterile distilled water, as described in Section 2.2.2.2. The sterile filter paper discs were loaded with 20 µL of respective algal extracts, and the total quantity of each extract was increased by reloading the disc with further sample (20 µL each time) until impregnated by 800 µg mL^{-1}, and then allowed to dry at RT. Discs impregnated with an equivalent volume of carrier solvent (ethanol, methanol, acetone or sterile water) or 5 µg mL^{-1} of Tetracycline (which was tested to give a particular zone size against all tested bacteria) were used as positive and negative controls, respectively. For each assay, all tested, and control discs were dried, and then laid onto the agar surface of a petri plate that had been suitably seeded with test bacteria using sterile forceps. The plates were incubated at 4°C for 5 h. After incubation, the plates were normalized at RT for 10 mins. The plates were inverted and incubated at 28°C for 24 (*A. hydrophila*) and at least 48 h (*Fno* and *S. agalactiae*) and then inspected for the appearance of clearing around each disc. The antibacterial activity was determined and calculated as described in Section 2.2.2.6A.

C. Tilapia head kidney primary cells (macrophages) cultures and respiratory burst assay

The respiratory burst activity of Nile tilapia homo gold head kidney primary cell cultures was determined to investigate the response of macrophages to bacterial infection. This was performed using two different methods of macrophage isolation (viz. collecting the cells with and without cells separation medium to compare both methods). The cells were infected with the three different bacteria pathogens (Section 2.2.1.3), at two different doses, 1 x 10^6 and 1 x 10^8 CFU mL^{-1} and control (no bacteria), as well as with fresh and heat-inactivated bacteria. The results were applied in the next phase of work to select the best bacteria concentration for cell infections.

Macrophage respiratory burst activity was evaluated after incubation of cells with nitroblue tetrazolium (NBT) (Sigma-Aldrich, UK) and Phorbol 12-myristate

13-acetate (PMA) (Sigma-Aldrich, UK) (Chung and Secombes, 1988; Secombes, 1990). Four Nile tilapia homo gold (100–15 g) were killed, bled and the HK phagocytes were isolated aseptically following the method described in Section 2.2.2.4. Primary cell cultures were prepared with and without cells separation steps. When cells were separated, the macrophages were harvested as described in Section 2.2.2.4. Both cells cultures were adjusted to 1 x 10^7 cells mL^{-1} with L-15 medium with phenol red. A 100 µl volume of cells from each cell culture was placed into each well of a 96 well tissue culture plate (167008, Nunclon™ Delta Surface, ThermoFisher Scientific, Denmark) and at least two or three wells were prepared for each treatment per fish. The plate containing macrophage monolayers was then wrapped with aluminium foil and incubated for 2 h at RT.

Two different controls were included in each plate: a positive control of treatment *i.e.* cells with 1 µg mL^{-1} PMA in NBT solution (1 mg mL^{-1}) and a control of cells with RPMI medium and lysis buffer to determine the number of attached cells. The lysis buffer contained 0.1M citric acid (100813M, BDH, UK), 1.0% Tween 20 (P-1379, Sigma-Aldrich, UK), 0.05% (w/v) crystal violet and distilled water. NBT solution at 1 mg mL^{-1} was prepared and PMA was then added to the NBT solution to get a final concentration of 1 µg mL^{-1}, as described in Section 2.2.2.5. The bacteria inocula was cultured, collected and washed in PBS (as Section 2.2.1.3), and then the concentration was adjusted with RPMI medium supplemented with 2% foetal calf serum (F2442, Sigma Life Science, UK) at absorbance (600 or 610 nm) equal to 1 x 10^8 or 1 x 10^6 cells mL^{-1}. In addition, duplicate inocula was also prepared for treatment with heat killed bacteria (at 60°C for 1 h). Bacteria drop counts were performed on either CHAH or TSA for each bacteria concentration including heat killed bacteria (before and after), as Section 2.2.1.2.

After incubation, the monolayers were washed gently three times with sterile L-15 medium with phenol red, then a 100 µl of bacteria inocula at each concentration was added into 3 replicate wells for each treatment. Positive and negative controls were also included. A 100 µl RPMI medium was added into each well instead of bacteria for groups with no bacterial infection. The plates

were wrapped with foil and incubated for 1 h at RT. Cells supernatant was gently taken off and replaced with 100 µl NBT solution (1 mg mL^{-1}) into three replicate wells for all treatments that were infected with different concentrations of bacteria or cells with no bacteria. 100 µl of NBT solution containing PMA (1 µg mL^{-1}) was added to another three replicate wells for each treatment group, and 100 µl lysis buffer was added to the two remaining wells (Figure 2.4). Again, the monolayer plates were wrapped with foil and incubated for another 1 h at RT. The number of adherent cells in the wells containing lysis buffer was determined by counting the number of released nuclei with a hemocytometer. The respiratory burst reaction was stopped, fixed, washed, dried overnight, the formazan was solubilized, and the absorbance measured at 610 nm (Section 2.2.2.5). The results were adjusted and expressed as an absorbance at 610 nm for 10^5 cells.

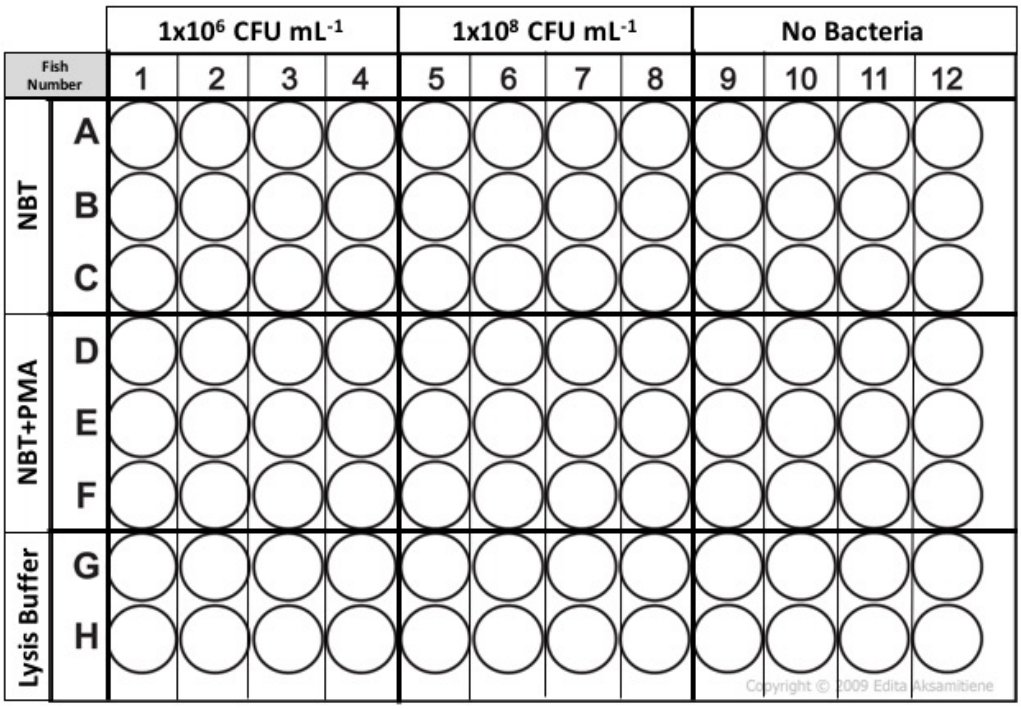

Figure 2.4 Schematic representation of respiratory burst assay 96 well tissue culture plate layout where tilapia head kidney primary cells cultures were infected with different concentrations of fresh (live) and heat killed bacteria. One plate was used for each method of cells isolation, each bacterial species and at each concentration including negative and positive controls. For example, the plate above was the layout for primary (macrophages) with the cells separations steps infected with two concentrations of live *Fno*).

D. Bactericidal activity using MTS assay

The bactericidal activity of the tilapia head kidney primary cell cultures against three different bacteria *i.e*. *A. hydrophila* T4 strain, *S. agalactiae* fish isolate, and *Fno* STIR-GUS F2f7 were studied using a CellTitre 96® Aqueous non-radioactive cell proliferation assay, as described by Fierro-Castro et al. (2013), originally described by Secombes (1990), and as described in the manufacturer's guidelines (Promega, Madison, USA). The CellTitre 96® Aqueous assay was comprised of a solution of novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-2(4-sulfophenyl)-2H-tetrazolium, inner salt (a salt formed by reaction within the molecule of a compound having both acid and basic properties); MTS] and an electron coupling reagent (phenazine methosulfate; PMS). MTS is bio-reduced by cells into a formazan product that is soluble in tissue culture medium (Barltrop *et al.*, 1991).

One Nile tilapia homo gold (155 g) was killed, bled, HK collected, and macrophages harvested. These were adjusted to 1 x 10^7 cells mL^{-1}, as in Section 2.2.2.4. One hundred microliters of cells were seeded onto five replicate wells for each treatment, incubated for 2 h at RT, and then washed gently three times with PBS when the incubations were finished, as in Section 2.2.2.5. The HK macrophages were then stimulated with 60 µL of ß-glucan, alginic acid or water extracts of *Chlorella* sp. at 50 µg mL^{-1}, as described in Section 2.2.2.5, for 3 h at RT. Positive and negative controls were also included using cells simulated with PMA in RPMI medium (1 µg mL^{-1}) and infected with bacteria, unstimulated cells with RPMI medium with and without bacterial infections, and blank wells with RPMI medium (Figure 2.5).

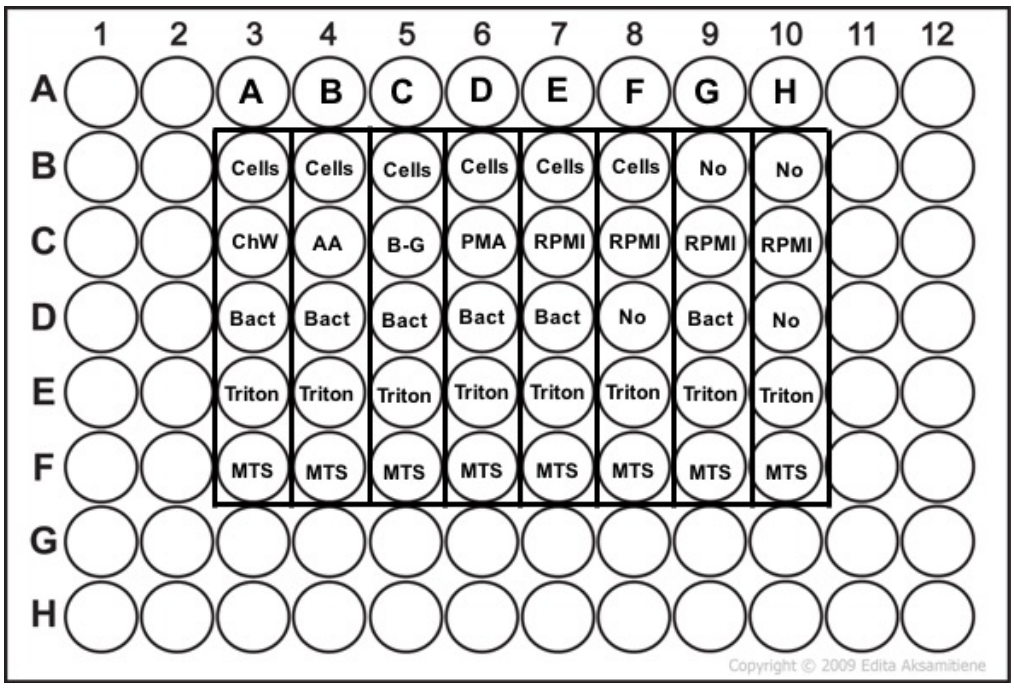

Figure 2.5 Schematic representation of the layout of 96 wells tissue culture plate for determining bactericidal activity using the MTS assay where tilapia head kidney primary macrophages cultures were infected with live bacteria (one plate was used for each bacteria): (A) cells stimulated with water extract of *Chlorella* sp. (ChW); (B) with alginic acid (AA); (C) ß-glucan (B-G); (D) PMA; and (E, F, G and H) controls negative RPMI medium without stimulant, and with and without either cells or bacteria.

Bacteria were grown, harvested, washed and adjusted at A_{600} or A_{610} equal to 5 x 10^8 and 5 x 10^9 cells mL^{-1} (Section 2.2.1.3) using the standard curves for *Fno* (Chapter 2, Section 2.3.3), *S. agalactiae* (Wongsathein, 2012) and *A. hydrophila* (Poobalane *et al.*, 2010). Following incubation of cells with algal compounds, 20 mL $well^{-1}$ of a bacterial suspension in PBS was adjusted to a ratio multiplicity of infection (MOI) of 100 and 1000 bacteria $cells^{-1}$ was added. The plates were centrifuged at 150 x g for 5 mins to bring the bacteria into contact with the cells and incubated at RT for 1 h. After this, the cells were lysed by addition of 40 µL $well^{-1}$ 0.5% Triton X-100 (Scharlau, Spain) in PBS. The number of viable bacteria present in the wells was determined using a commercial CellTiter 96® Aqueous kit by adding 24 µL $well^{-1}$ of the kit. The plates were incubated for 4 h at 28˚C and then the absorbance was read at 490 nm in a Microplate Reader (Section 2.2.2.5). The bacterial killing was expressed as an absorbance of 490 nm minus control of blank wells.

2.2.4 Statistical analysis

Data were examined using a one-way analysis of variance (ANOVA), general linear model, and Tukey's test for pairwise comparison of means. All statistical tests were performed using Minitab software (Version 16) under licence to the University of Stirling. A p-value of less than 0.05 was considered to indicate a significant difference between groups. Statistical differences were examined between groups at each time point.

2.3 Results

The results in this chapter include bacterial growth curves and *in vitro* testing of algal-derived compounds (screening for immunostimulatory and anti-bactericidal activities) using tilapia head kidney primary cell cultures, disc diffusion and anti-bactericidal assays.

2.3.1 Bacterial culture and identification

The *Fno* isolate STIR-GUS-F2f7 gave a single colony type when cultured on CHAH media. Cysteine and haemoglobin were required for growth and the

optimal growth occurred at the temperature 28°C. Standard biochemical assays were performed to determine the phenotypic and biochemical characteristics of the bacterium and the observations obtained in this present study were in line with previous descriptions for *Francisella* bacteria and especially for *Fno* species (Birkbeck *et al.*, 2007, 2011; Ottem *et al.*, 2009; Ottem, 2011; Ramirez-Paredes, 2015) as shown in Table 2.1. Phenotypically, the colonies were small, round, mucoid, convex, smooth, and greenish on CHAH after incubation at least 48 h at 28°C (Figure 2.6). The bacterium was Gram-negative, coccoid to short rods that were oxidase negative, non-motile, catalase positive, and negative for hydrolysis of glucose, oxidase and fermentation. Characteristics of the isolate are shown in Table 2.1 and Figure 2.7.

Biochemical profiles and phenotypic characterization of the isolate were determined with regard to utilization of specific substrates such as carbohydrate fermentation and enzymatic activity, using API 20 E and ZYM (Figure 2.8). The API 20 E kit demonstrated three positive reactions: sodium citrate and pyrupate (VP/Voges–Proskauer), and gelatine (Table 2.2). The ability of the isolate to utilise citrate as a carbon source, produce acetoin from sodium pyruvate and hydrolyze gelatine were confirmed in this present study as well as in Ramirez-Paredes (2015).

Furthermore, with API ZYM kits the test results were positive for alkaline phosphatase, esterase, esterase lipase, acid phosphatase, and napthol-AS-BI-phosphohydrolase (Table 2.2).

Table 2.1 Results of primary identification tests at 28°C for the *Fno* STIR-GUS-F2f7 isolated from red Nile tilapia farmed in Northern Europe

Bacteria	Gram stain	Oxidase	O/F	Catalase	Motility
Fno STIR-GUS-F2f7	-	-	-/-	+	-

Identification: + stands for positive, – stands for negative

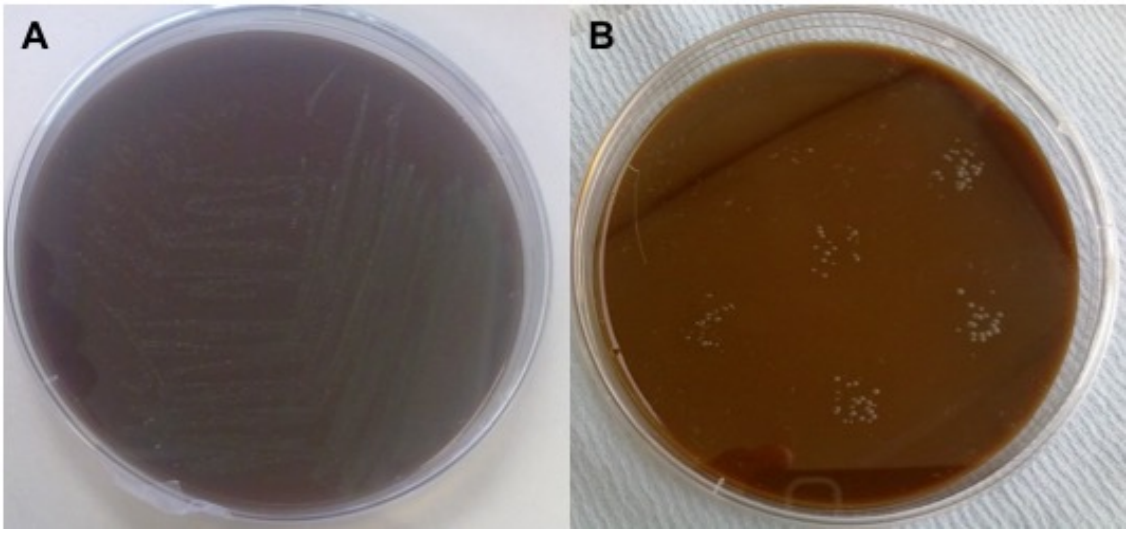

Figure 2.6 *Fno* colonies on the CHAH plates after incubation at 28°C for at least 48 h: (A) streaked and (B) dropped counts of *Fno*.

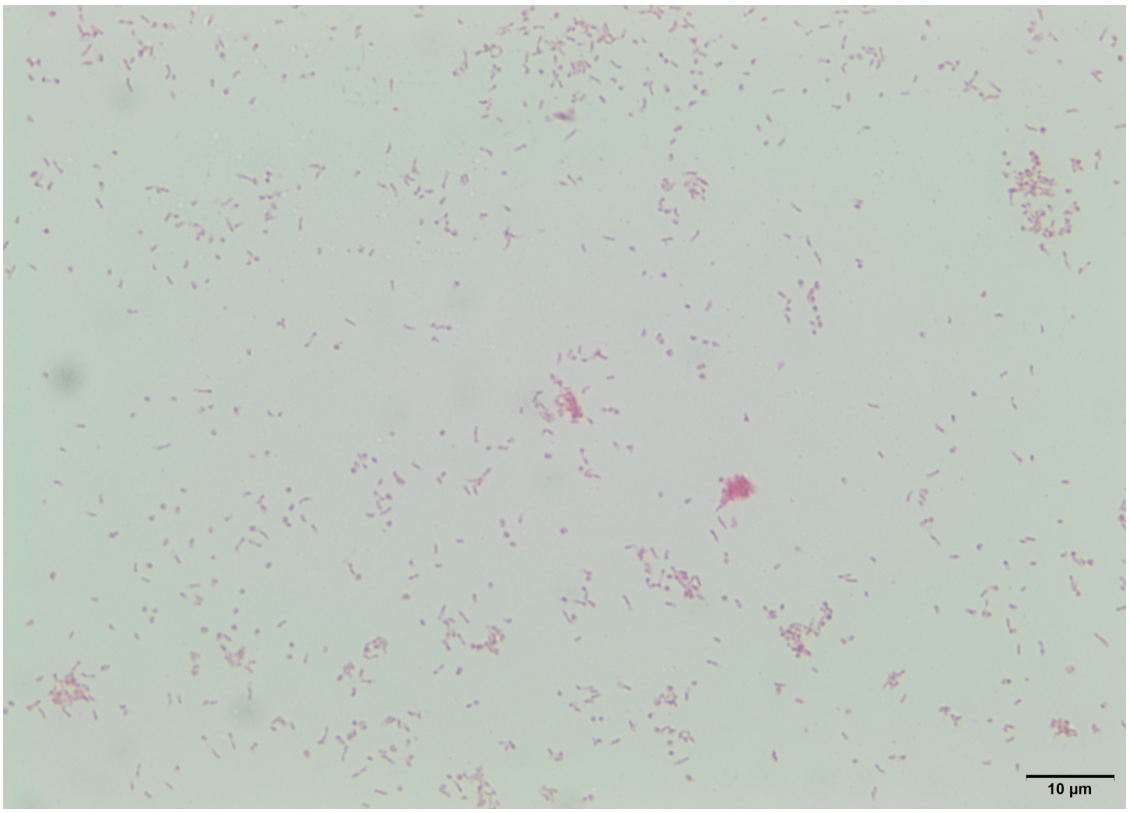

Figure 2.7 Gram staining of *Fno* STIR-GUS-F2f7 strain cultured on CHAH for 72 h at 28°C.

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Table 2.2 Biochemical characteristics and phenotypic characterization at 28°C of the *Fno* STIR-GUS-F2f7 isolated from red Nile tilapia farmed in Northern Europe as determined using API 20E and ZYM kits

API 20E	Result	API ZYM	Result
ONPG, Ortho-nitrophenyl galactosidase	-	Control	-
ADH, Arginine	-	Alkaline phospatase	+
LDC, Lysine	-	Esterase (C 4)	+
ODC, Ornithine	-	Esterase lipase (C 8)	+
CIT, Sodium Citrate	+	Lipase (C 14)	-
H2S, Sodium Thiosulphate	-	Leucine arylamidase	-
URE, Urea	-	Valine arylamidase	-
TDA, Tryptophane	-	Cystine arylamidase	-
IND, Tryptophane	-	Trypsin	-
VP, Sodium Pyruvate	+	α-chymotrypsin	-
GEL, Gelatine	+	Acid phosphatase	+
GLU, Glucose	-	Napthol-AS-BI-phosphohydrolase	+
MAN, Mannitol	-	α-galactosidase	-
INO, Inositol	-	ß-galactosidase	-
SOR, Sorbitol	-	ß-glucuronidase	-
RHA, Rhamnose	-	α- glucuronidase	-
SAC, Sucrose	-	ß-glucosidase	-
MEL, Melibiose	-	N-acetyl-ß-glucosaminidase	-
AMY, Amygdalin	-	α-mannosidase	-
ARA, Arabinose	-	α-fucosidase	-

Key: + stands for positive, – stands for negative

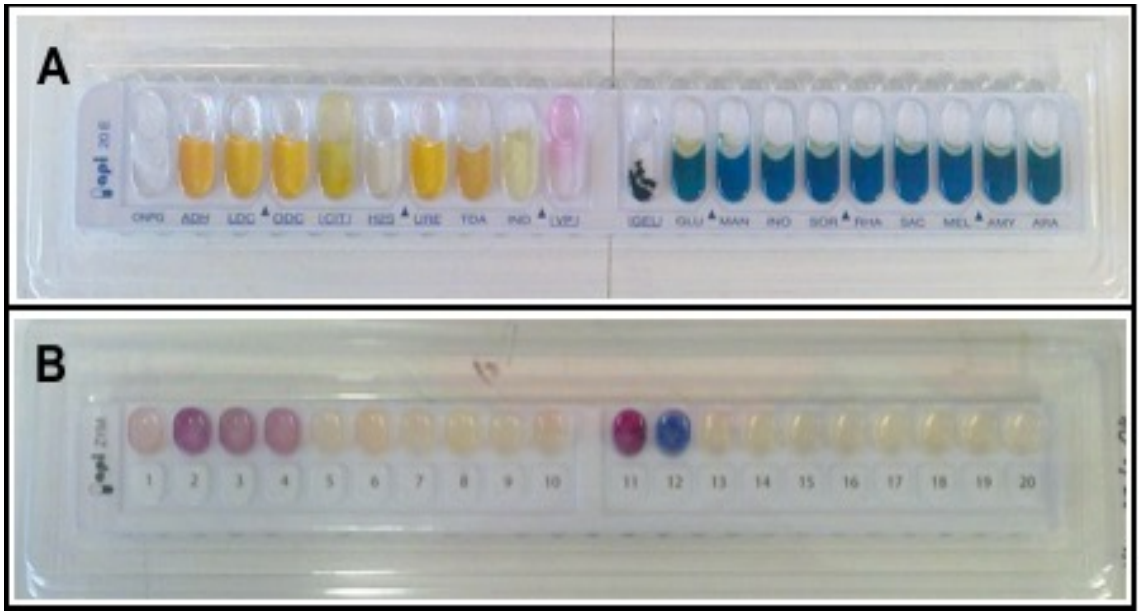

Figure 2.8 Biochemical characteristics of the *Fno* STIR-GUS-F2f7 isolated from red Nile tilapia farmed in Europe after 24 h incubation at 28°C as determined using: (A) API 20 E, and (B) ZYM kits.

A. hydrophila and *S. agalactiae* grew as pure cultures on TSA media with optimal growth at 28˚C. Standard biochemical assays were performed to determine the phenotypic and biochemical characteristics of the bacterium and the observations obtained in this present study were in line with previous descriptions for *A. hydrophila* (Poobalane *et al.*, 2010) and *S. agalactiae* (Wongsathein, 2012) species as shown in Table 2.3. Phenotypically, *A. hyrophila* isolates appeared cream in colour, and as quite large colonies on TSA after 24 h, while *S. agalactiae* presented as smooth, light cream in colour and as small colonies on TSA at least after 48 h, after incubation of both bacteria at 28˚C (Figure 2.9). *A. hydrophila* was Gram-negative with rod-shaped that were oxidase and catalase positive, motile, and fermentative (Table 2.3). In addition, *S. agalactiae* was Gram-positive with coccoid, spherical colonies that were oxidase positive, catalase negative, non-motile, and fermentative (Table 2.3). Characteristics of the isolates are shown in Figure 2.10 and 2.11, as Gram-negative and positive bacteria, respectively. Biochemical profiles and phenotypic characterization of *A. hydrophila* isolates were determined using API 20 E, which demonstrated 11 positive reactions as describe in Table 2.4 and Figure 2.12; API 20 E original data for *S. agalactiae* are not shown.

Table 2.3 Primary identification test at 28°C of bacteria used in these studies

Bacteria	Gram stain	Oxidase	O/F	Catalase	Motility
A. hydrophila (T4)	-	+	Fermentative	+	+
S. agalactiae type strain (NCIMB 701348)	+	+	Fermentative	-	-
S. agalactiae	+	+	Fermentative	-	-

Identification: + stands for positive, – stands for negative

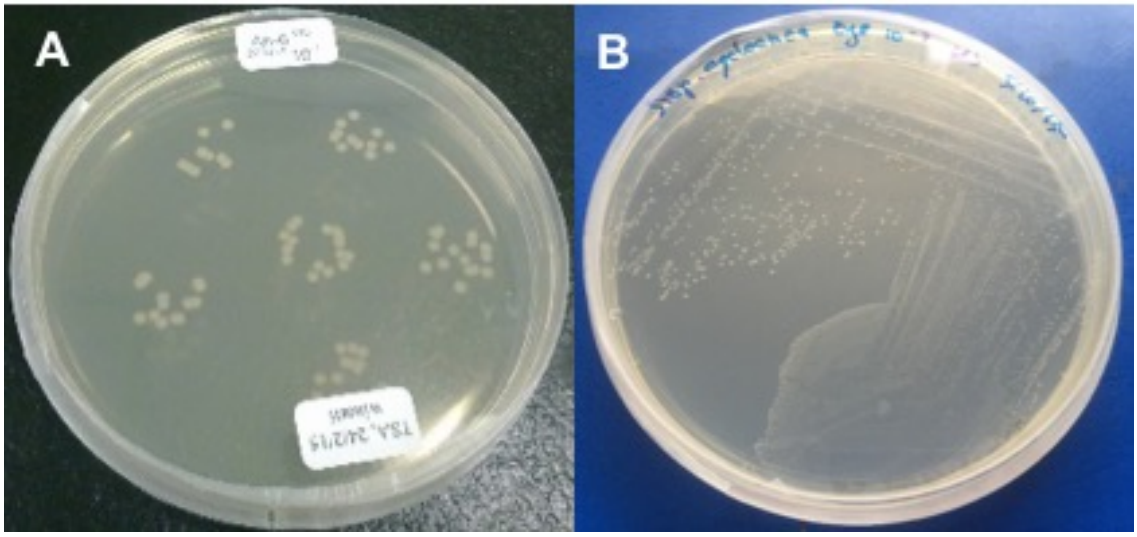

Figure 2.9 Bacteria colonies on the TSA plate after incubation at 28°C: (A) *A. hydrophila* T4 strain after 24 h; and (B) *S. agalactaie* after 48 h.

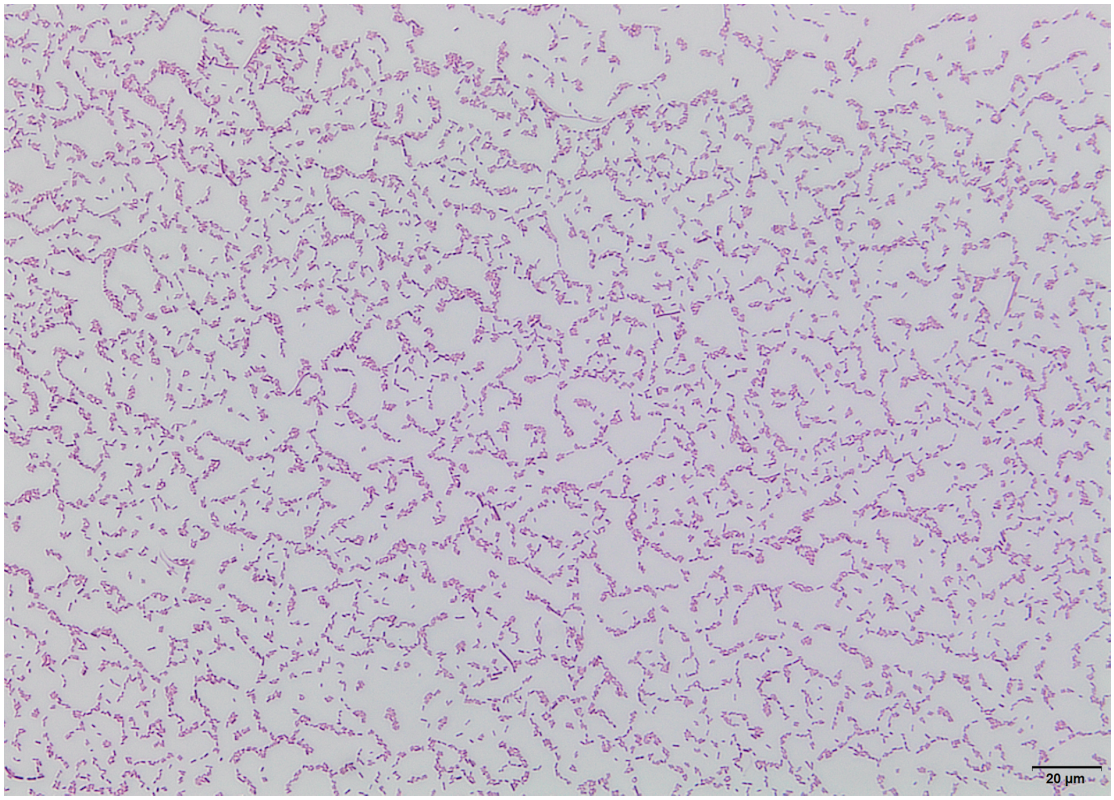

Figure 2.10 Gram stain of *A. hydrophila* T4 strain grown on TSA after 24 h of incubation at 28˚C, short-rod cell with 0.3-1.0 µm in diameter, and 1.0-3.5 µm in length.

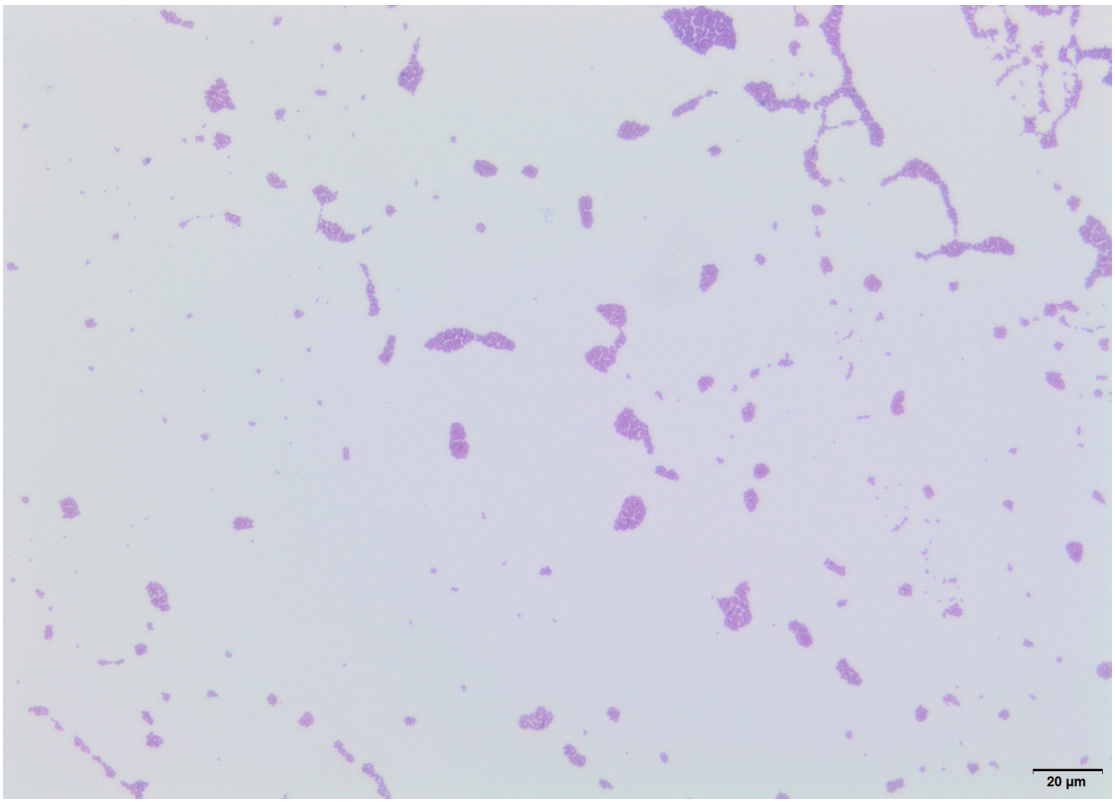

Figure 2.11 Gram stain of *S. agalactiae* strain grown on TSA after 48 h of incubation at 28˚C, coccus cell with 0.5–2.0 µm in diameter.

Table 2.4 Biochemical characteristics and phenotypic characterization of *A. hydrophila* T4 strain (at 28°C) as determined using API 20 E kit

API 20 E	Results
ONPG, Ortho-nitrophenyl galactosidase	+
ADH, Arginine	+
LDC, Lysine	+
ODC, Ornithine	-
CIT, Sodium Citrate	+
H2S, Sodium Thiosulphate	-
URE, Urea	-
TDA, Tryptophane	-
IND, Tryptophane	-
VP, Sodium Pyruvate	+
GEL, Gelatine	+
GLU, Glucose	+
MAN, Mannitol	+
INO, Inositol	-
SOR, Sorbitol	-
RHA, Rhamnose	-
SAC, Sucrose	+
MEL, Melibiose	-
AMY, Amygdalin	+
ARA, Arabinose	+

Identification: + stands for positive, – stands for negative

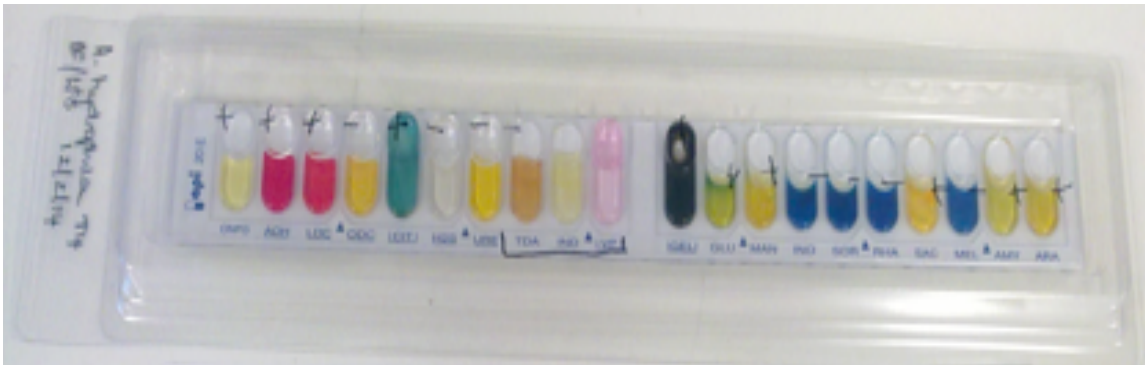

Figure 2.12 Biochemical characteristics of the *A. hydrophila* T4 strain after 24 h incubation at 28°C as determined using API 20E kit.

2.3.2 Bacterial growth curves

The relationship between the absorbance value and the number of cells mL^{-1} through the growth curve of *Fno* STIR-GUS-F2f7 was obtained by simple linear regression analysis *e.g*. y = 0.2355x + 2.8582 with and R^2 value of =0.8237 (Figure 2.13) and by using this equation the growth of the bacteria was known and the curve was plotted (Figure 2.14). The typical bacterial growth phases for the *Fno* isolate included lag phase at 0–3 h, log phase at 3–27 h with the mid log phase at 19.5 to 20 h, and stationary phase was reached after 30 h (Figure 2.14). These findings are similar tp the previous study with the same *Fno* isolate (Ramirez-Paredes, 2015). No bacterial cultures were recovered from the negative control MHB sample only. The primary id test results confirmed that the isolates were *Fno* in at each time sampling point on the growth curve (Gram-negative, cocco-bacilli, non-motile, catalase positive and oxidase negative).

In addition, the growth curve of *A. hydrophila* and *S. agalactiae* were obtained through the relationship between the absorbance value with the number of cells mL^{-1} by simple linear regression analysis of y=0.8837x+7.7462 and y=1.1547x+7.3718, and with R^2 value of =0.99651 and =0.90489, respectively (Figures 2.15 and 2.17). The typical bacterial growth phases for the *A. hydrophila* and *S. agalactiae* isolates used in this study had similar pattern for lag phase at 0–3 h, log phase at 3–15 h and stationary phase was reached after 24 h, while the differences found in the mid log phase that occurred at 4.5 and 13.5 h, respectively (Figures 2.16 and 2.18). The primary id test results confirmed of *A. hydrophila* and *S. agalactiae* isolates from all samples at each time sampling point on the growth curves.

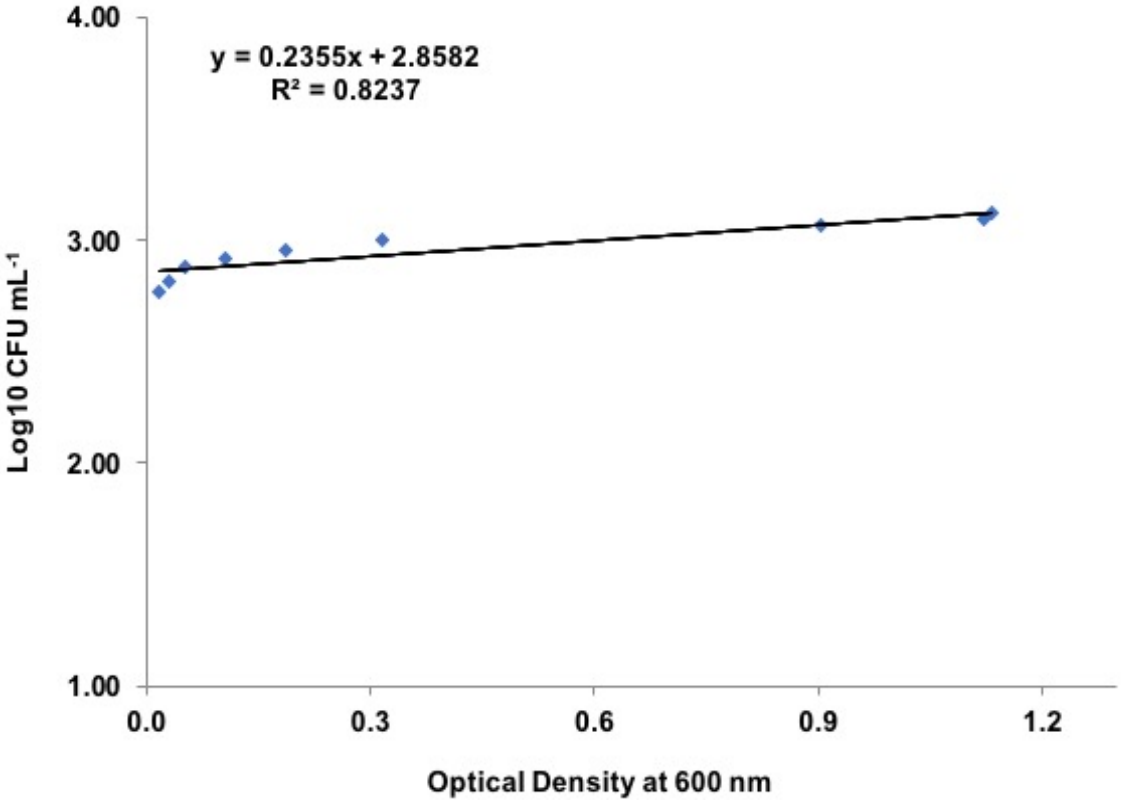

Figure 2.13 Scatter graph plot at A_{600} for *Fno* STIR-GUS-F2f7 cell growth suspensions against time and viable CFU mL^{-1} showing a significant correlation between these parameters.

Figure 2.14 Growth curve of *Fno* STIR-GUS-F2f7 at A_{600} nm.

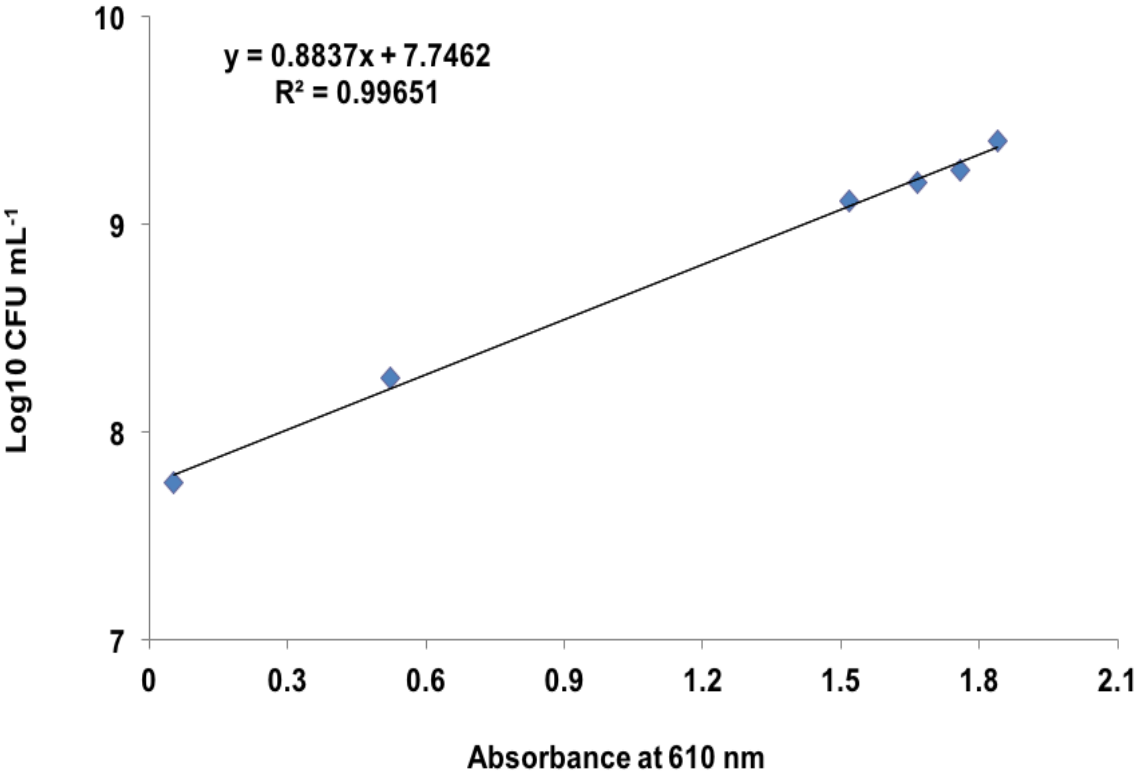

Figure 2.15 Scatter graph plotting at A_{610} for *A. hydrophila* (T4) cell growth suspensions against time and viable CFU mL^{-1} showing a significant correlation between these parameters.

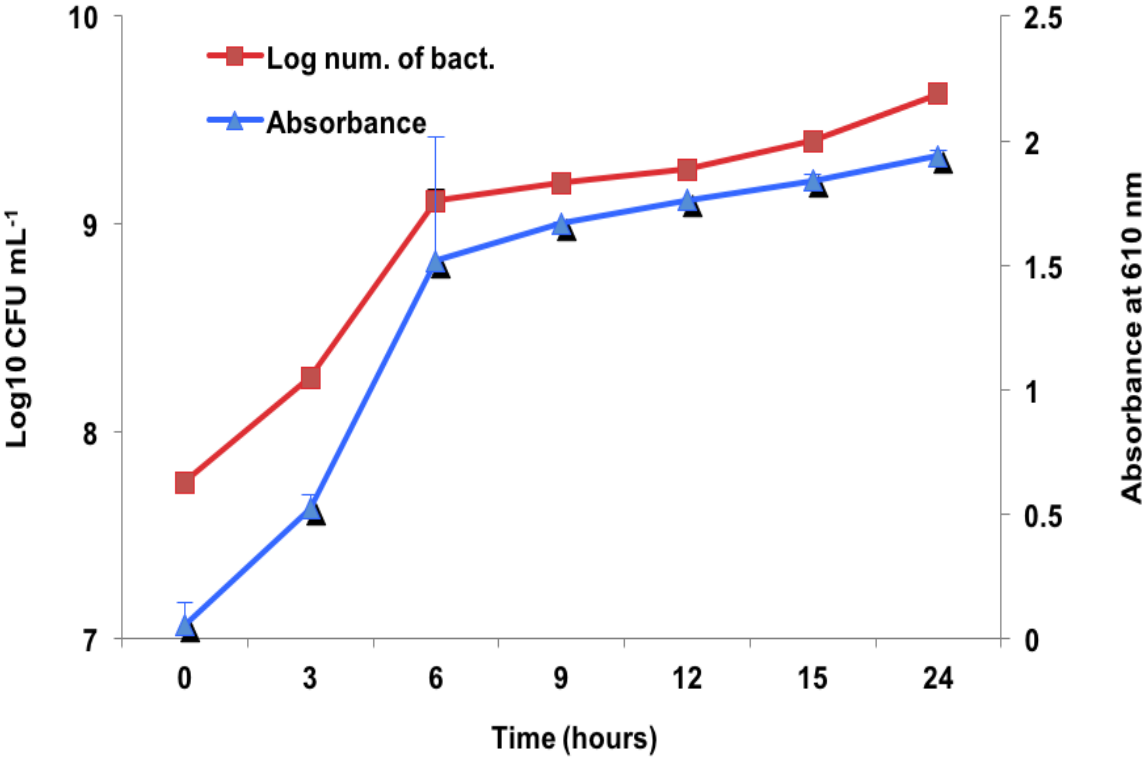

Figure 2.16 Growth curve of *A. hydrophila* (T4) at A_{610} nm.

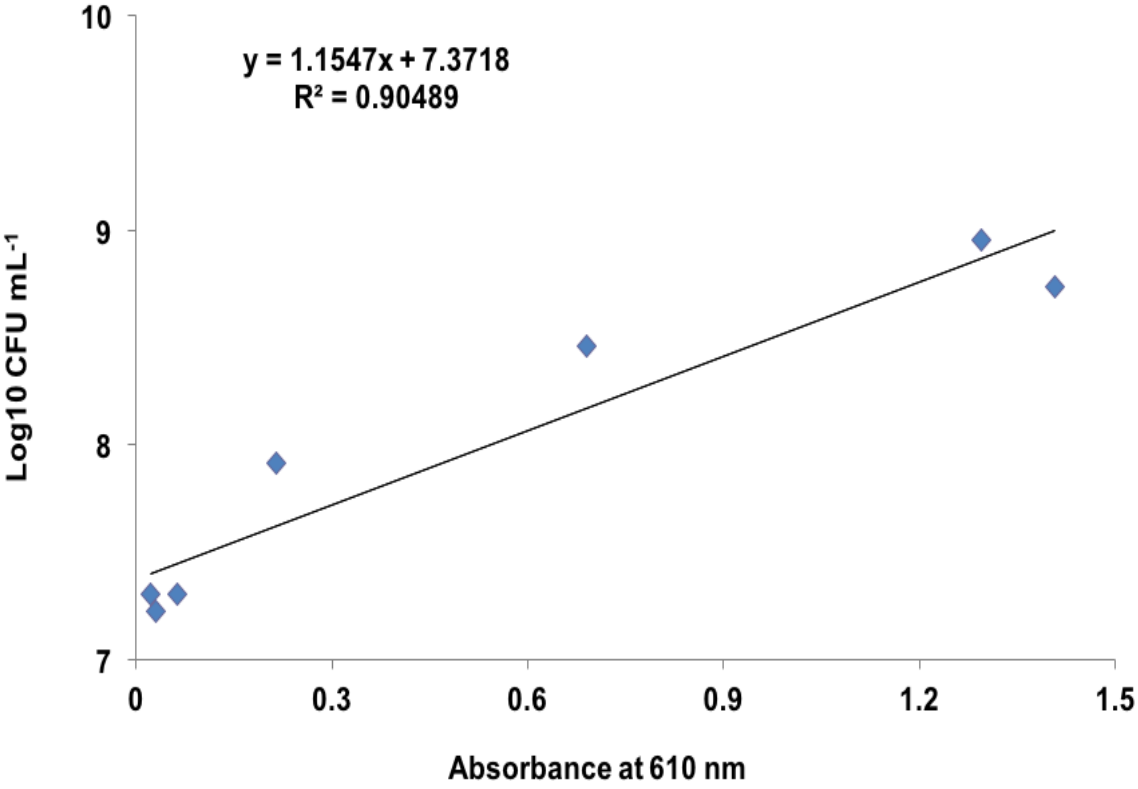

Figure 2.17 Scatter graph plotting at A_{610} for *S. agalactiae* type strain (NCIMB 701348) cell growth suspensions against time and viable CFU mL^{-1} showing a significant correlation between these parameters.

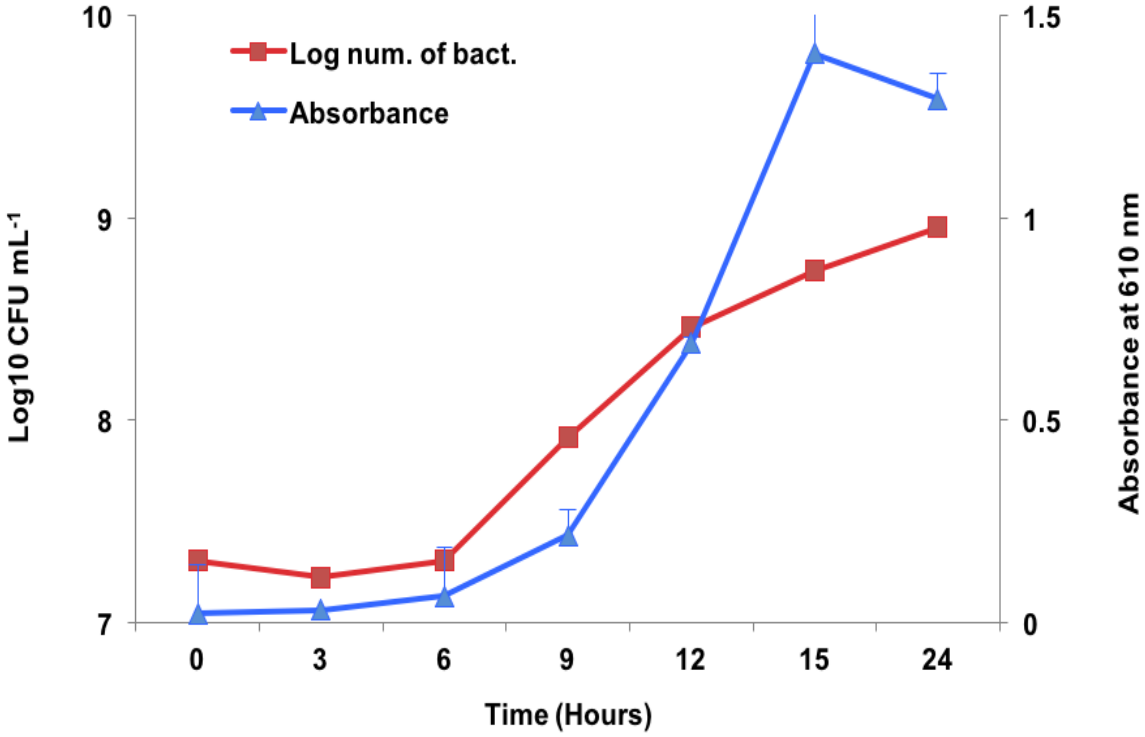

Figure 2.18 Growth curve of *S. agalactiae* type strain (NCIMB 701348) at A_{610} nm.

2.3.3 *In vitro* screening of algal bioactive compounds

Data obtained during *in vitro* work using Nile tilapia homo gold head kidney primary cells culture included: 1) immunostimulatory activity of algal-derived compounds using respiratory burst assay and 2) anti-bacterial activity (including bacterial sensitivity to antibiotics and to algal-derived compounds, tilapia head kidney primary cell (macrophage) culture, bacterial infections by respiratory burst assay and bactericidal activity using an MTS kit).

2.3.3.1 Immunostimulatory activity of algal bioactive compounds using respiratory burst assay

Primary cell cultures from Nile tilapia homo gold HK were separated using lymphocyte separation medium to obtain macrophages that were used *in vitro* assays to screen the algal bioactive compounds (Figure 2.19). In order to determine whether algal-derived compounds were able to modulate the respiratory burst activity of tilapia HK macrophages, the possible stimulation of primary macrophage cultures by algal extracts using different solvents of extractions (aqueous, methanolic, acetonolic and ethanolic) was tested. The macrophages were cultured for 1, 3, 6 and 24 h in the presence of 50 µg mL^{-1} of either each algal extract or compound.

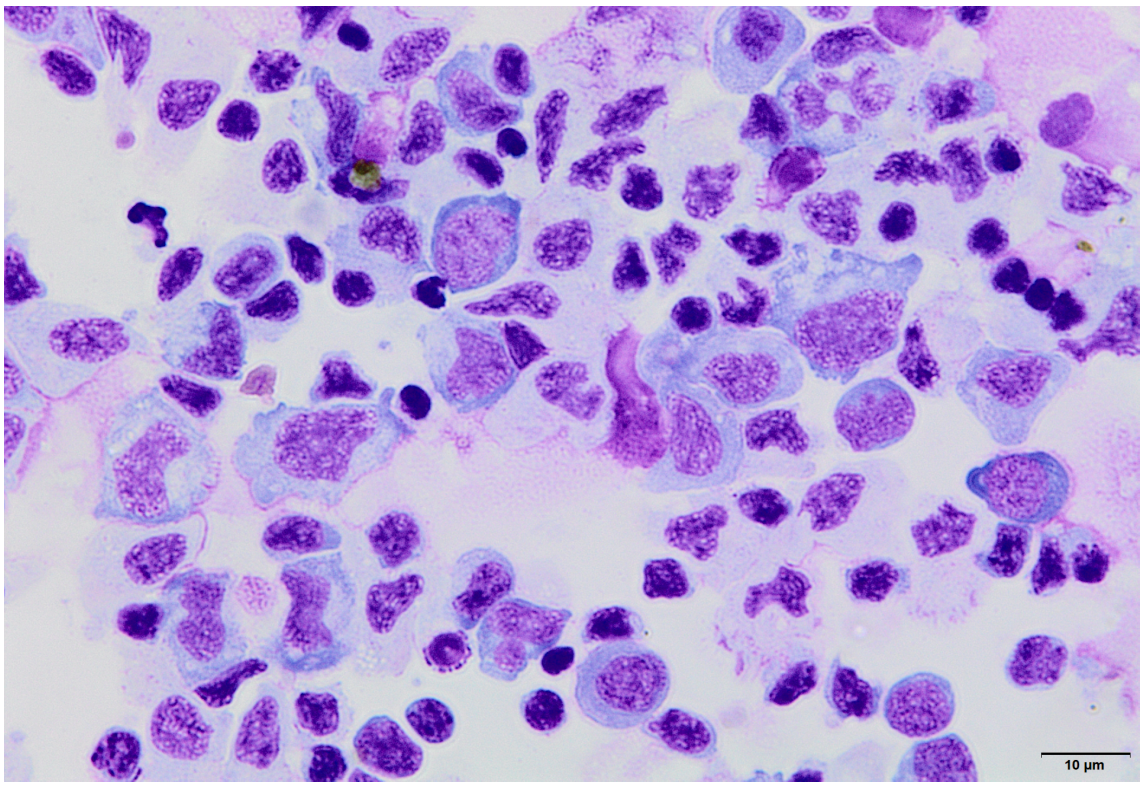

Figure 2.19 Macrophages collected using lymphocyte separation medium followed by cytospin.

The results showed that after 1, 3, 6 and 24 h of exposure with 50 µg mL^{-1} of different extracts of algal-derived compounds, respiratory burst activity of the HK macrophages had increased stimulation indices for some of the compounds, while others inhibited the respiratory burst activity (Figures 2.20, 2.21, 2.22 and 2.23). In general ROS production started to increase 1 h after the exposure and increased further until 6 h and then sharply decreased by 24 h. The HK macrophages treated with PMA as a positive control showed a similar trend with an increase in all time point compared to the negative control.

In more detail, there was significant differences were found at 1 h post incubation especially between PMA (the highest) and *Nannochloropsis* sp ethanol extract (the lowest). Meanwhile, the algal-derived compounds that gave the high stimulation at 1 h were water extracts of *Chlorella* sp. (0.33–0.34), followed by ß-glucan (0.22), *C. calcitrans* water extract (0.20) and alginic acid (0.13), with some compounds actually inhibiting the ROS activity (*e.g.* methanol, ethanol and acetone extracts of *C. calcitrans*, methanol and ethanol extracts of *Chlorella* sp., while *Nannochloropsis* sp. showed inhibition in all extracts) (Figure 2.20).

No significant differences were observed at 3 h post incubation, eventhough the high stimulation index was again recorded in water extracts of *Chlorella* sp. (0.91–0.95), then the *Chlorella* sp. ethanol-1 extracts (0.62), alginic acid (0.50), water extracts of *C. calcitrans* and *Nanonocloropsis* sp. (0.37), and ß-glucan (0.34), while the ethanol-2 extracts of *Chlorella* sp. inhibited the activity (Figure 2.21).

After 6 h of exposure, two treated macrophage groups showed inhibition of ROS activities (*Nannocloropsis* sp. in acetone and ethanol extracts). In contrast, the acetone extracts of *Chlorella* sp. produced the highest activity (0.85) followed by ethanolic-2 extracts of the same algal (0.77), ß-glucan (0.75), alginic acid (0.55), and then water (0.43–0.54) and ethanol-1 (0.44) extracts of *Chlorella* sp. (Figure 2.22). At this time point, there was significant differences in ROS activities were observed between groups.

Finally, the ROS production sharply declined at 24 h post exposure in all groups of treated macrophages with algal stimulants including in the PMA positive control, while three groups inhibited the activity (acetone and ethanol extracts of *C. calcitrans* with the high inhibition observed with acetone extract of *Nannochloropsis* sp. (-0.23) (Figure 2.23). There was no significant difference in ROS production were found among groups at this time point.

Overall, a 3 h exposure appeared to be the optimal time to stimulate the macrophages with algal-derived compounds, and this resulted in ROS production in almost all treated groups, with the high index found in water followed by ethanol-1 extracts of *Chlorella* sp., alginic acid, water extracts of *C. calcitrants* and *Nanonocloropsis* sp., and ß-glucan. Since, water extracts of *Chlorella* sp., alginic acid and ß-glucan resulted in increased ROS activity at all the time points, these extracts/compounds were used for the next phase of the *in vitro* work (bactericidal activity with MTS kit).

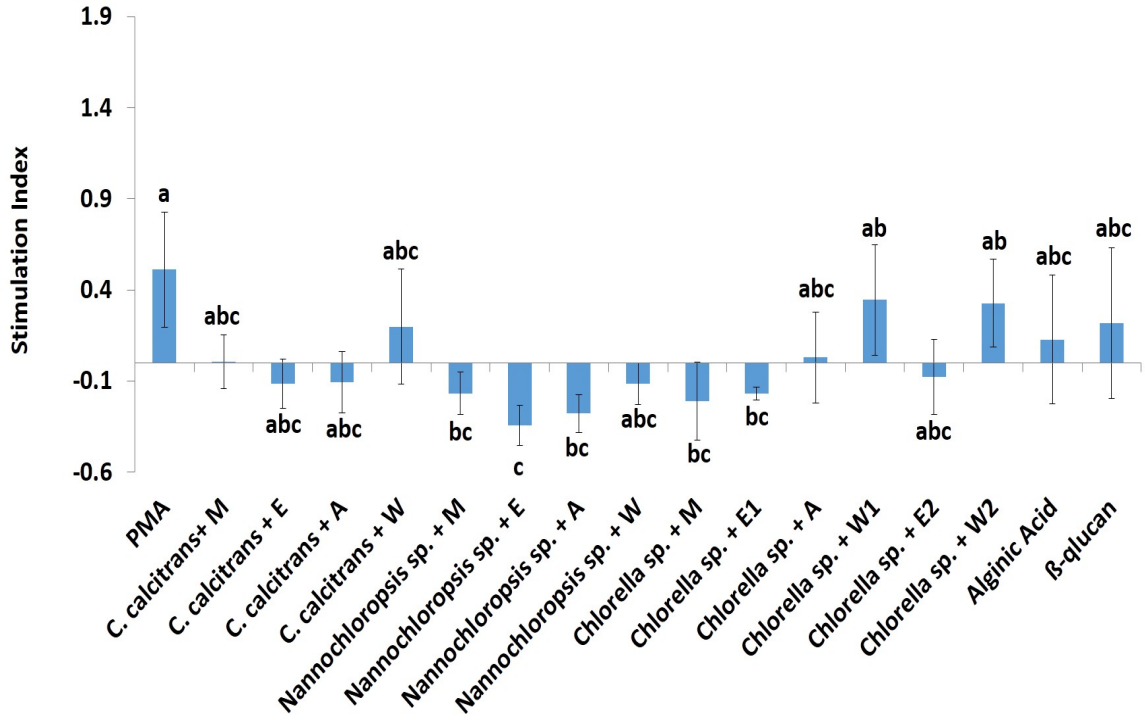

Figure 2.20 Intracellular ROS production in Nile tilapia homo gold head kidney macrophages after exposure to 50 μg mL^{-1} of different algal compounds and solvent extractions, and PMA for 1 h. The graph shows results from a representative assay as stimulation indices against controls (control values are zero). Bars represent the mean of 3 fish and wells+SD. Groups that do not share a letter are significantly different.

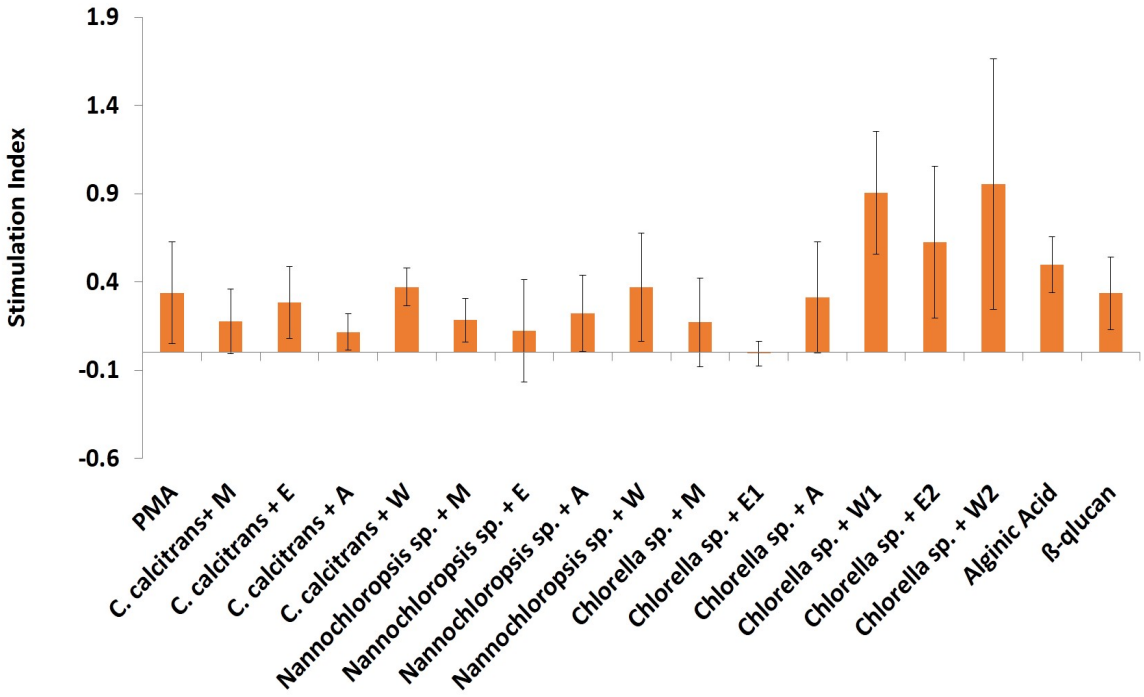

Figure 2.21 Intracellular ROS production in Nile tilapia homo gold head kidney macrophages after exposure to 50 μg mL^{-1} of different algal compounds and solvent extractions, and PMA for 3 h. The graph shows results from a representative assay as stimulation indices against controls (control values are zero). Bars represent the mean of 3 fish and wells+SD. No significant different between all treatments groups.

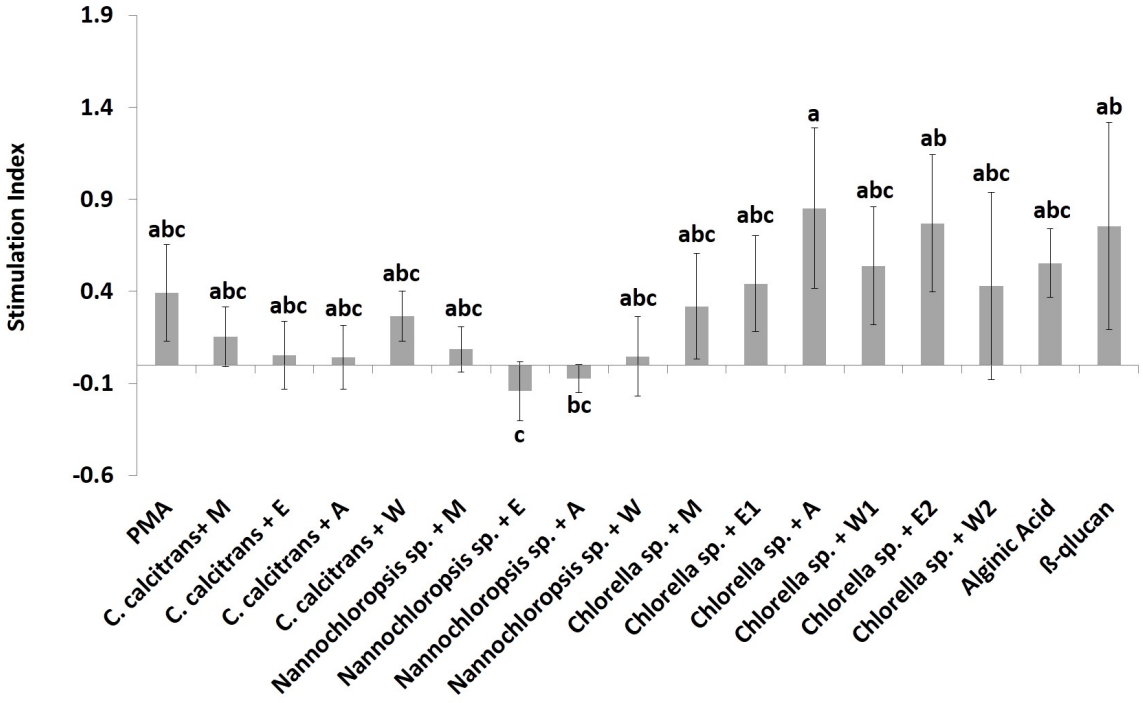

Figure 2.22 Intracellular ROS production in Nile tilapia homo gold head kidney macrophages after exposure to 50 μg mL^{-1} of different algal compounds and solvent extractions, and PMA for 6 h. The graph shows results from a representative assay as stimulation indices against controls (control values are zero). Bars represent the mean of 3 fish and wells+SD. Groups that do not share a letter are significantly different.

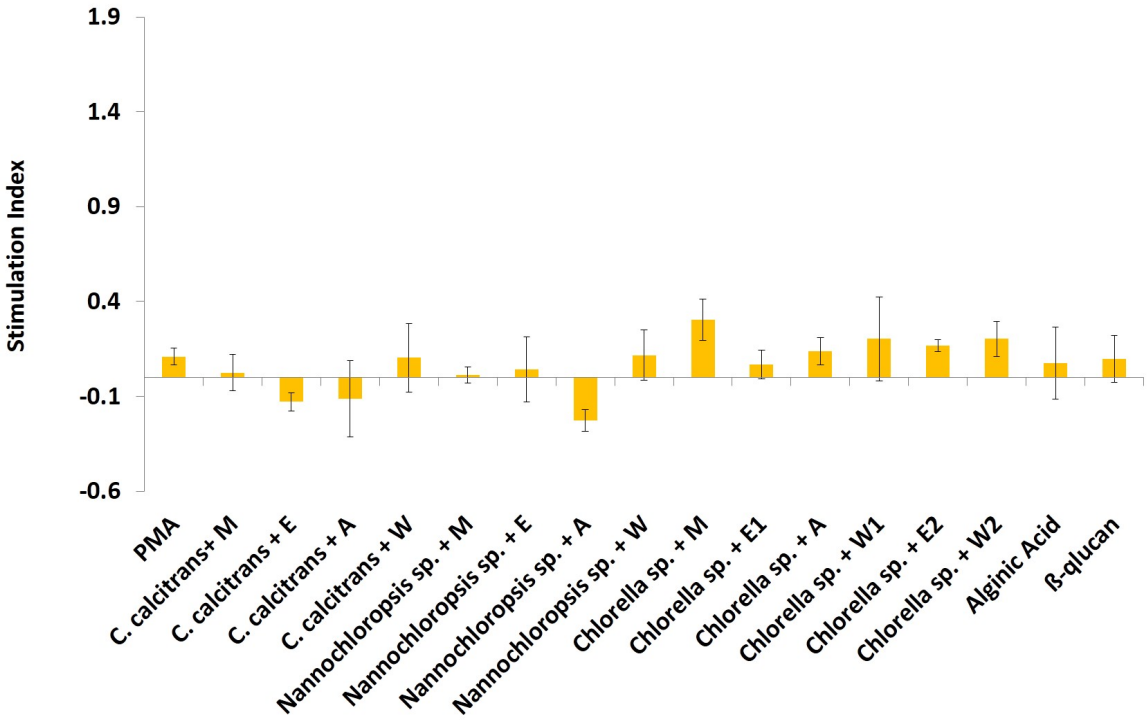

Figure 2.23 Intracellular ROS production in Nile tilapia homo gold head kidney macrophages after exposure to 50 μg mL^{-1} of different algal compounds and solvent extractions, and PMA for 24 h. The graph shows results from a representative assay as stimulation indices against controls (control values are zero). Bars represent the mean of 3 fish and wells+SD. No significant different between all treatments groups.

2.3.3.2 Antibacterial activity

A. Bacterial sensitivity to antibiotics

Seven antibiotics were used to study antibacterial activity against three fish bacteria pathogens, *A. hydrophila, S. agalactiae* and *Fno* STIR-GUS-F2f7, using the agar disc diffusion method. *A. hydrophila* was shown to be susceptible to streptomycin and tetracycline at 5 and 50 µg 20 μL^{-1}, and kanamycin, and gentamicin at 50 µg 20 μL^{-1}, while it was resistant to lower dose of kanamycin and gentamicin (5 µg 20 μL^{-1}), and both doses with penicillin, erythromycin and ampicillin (Table 2.5). In contrast, *S. agalactiae* was found to be resistant to the five lower doses of antibiotics (5 µg 20 μL^{-1}) and susceptible to the higher dose (50 µg 20 μL^{-1}) of streptomycin, kanamycin, penicillin, ampicillin and tetracycline (Table 2.24). In addition, *Fno* appeared to be susceptible to streptomycin, kanamycin, gentamicin, and tetracycline, while resistant to penicillin, erythromycin, and ampicillin, all at both doses (Table 2.5). The highest inhibition zone recorded was with tetracycline and *Fno* for both doses 5 and 50 µg 20 μL^{-1} at 17 and 30 mm, followed by *S. agalactiae* at 16 and 28 mm and *A. hydrophila* at 13 and 21 mm, respectively (Figure 2.24). No inhibition was observed in negative controls of water or water with 50% ethanol. In contrast, with 1M HCL solvent alone or with erythromycin, a clear zone was observed normally at 2 mm in all control agar or tested plates against all bacteria (data not shown).

Table 2.5 The mean zone diameters (mm) of antimicrobial activity of different concentrations of antibiotics, performed by the disc-diffusion method against *Fno*, *A. hydrophila,* and *S. agalactiae*

Antibiotic	Concentration	*Fno* (mm)	*A. hydrophila* (mm)	*S. agalactiae* (mm)
Streptomycin	5µg 20µL^{-1}	14	9	-
	50µg 20µ L^{-1}	22	11	9
Kanamycin	5µg 20µL^{-1}	20	-	-
	50µg 20µ L^{-1}	27	15	11
Gentamycin	5µg 20µL^{-1}	17	-	-
	50µg 20µ L^{-1}	27	14	-
Penicillin	5µg 20µL^{-1}	-	-	-
	50µg 20µ L^{-1}	-	-	19
Erythromycin	5µg 20µL^{-1}	-*	-*	-*
	50µg 20µ L^{-1}	-*	-*	-*
Ampicillin	5µg 20µL^{-1}	-	-	-
	50µg 20µ L^{-1}	-	-	26
Tetracycline	5µg 20µL^{-1}	**17**	**13**	16
	50µg 20µ L^{-1}	**30**	**21**	**28**
Water	100%	-	-	-
Water+50% ethanol	100+50%	-	-	-
HCL	1M	-*	-*	-*

-* there was inhibition zone, but it was cause by solvent (1M HCL) alone instead of erythromycin (data not shown).

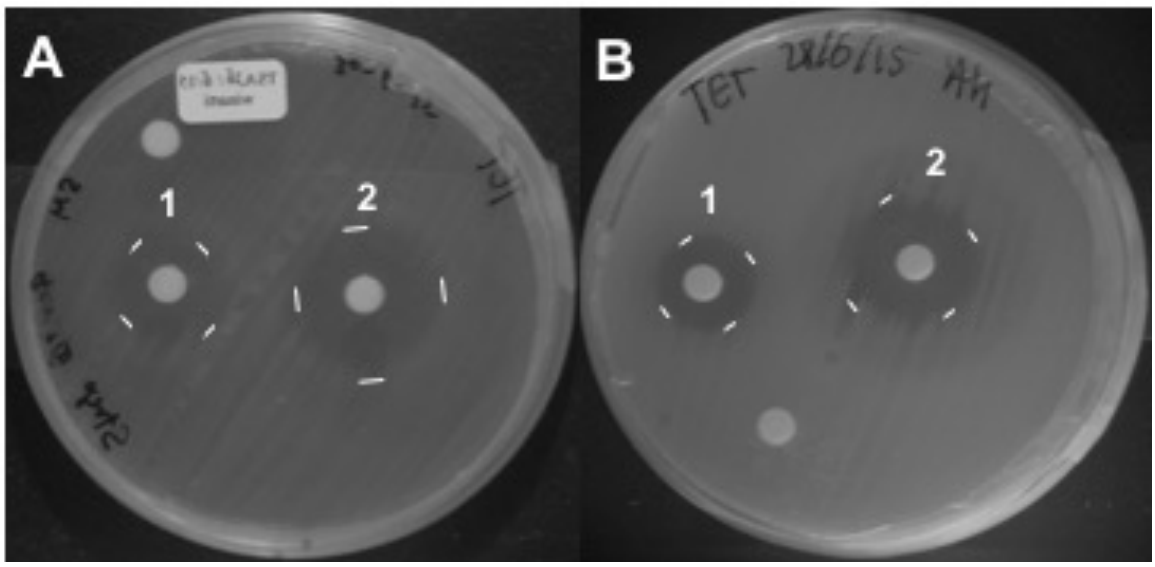

Figure 2.24 Inhibitory activity of tetracycline against *A. hydrophila* and *S. agalactiae* (data not shown for *Fno*) on TSA: (A) the second highest inhibition zone recorded in *S. agalactiae* for both doses 5 and 50 µg 20 µL^{-1} at 16 (1) and 28 (2) mm; (B) followed by and *A. hydrophila* at 13 (1) mm and 21 (2) mm, respectively.

B. Sensitivity of bacteria to algal compounds

The antibacterial activity of three species of algae extracts and dissolved in four different solvents (methanol, ethanol, acetone, and water) and two compounds (ß-glucan and alginic acid) were tested against 3 tilapia pathogens *viz. Fno*, *S. agalactiae* and *A. hydrophila* (Table 2.6). No zones of inhibition were observed for any of the algae extracts or compounds against *A. hydrophila* at either of the doses used (5.64 x 10^8 and 1.2 x 10^8 CFU mL^{-1}). The *C. calcitrans* and *Chlorella* sp. extracts in methanol, ethanol and acetone, the *Nannochloropsis* sp. ethanol extracts did, however, produce a zone of inhibition with *S. agalactiae* at a lower dose (1 x 10^8 CFU mL^{-1}), while at the higher dose (3.42 x 10^8 CFU mL^{-1}) only the methanol and acetone extracts of *C. calcitrans* and *Chlorella* sp. showed clear zones of inhibition. The highest inhibitory effect on *S. agalactiae* was found with the *C. calcitrans* methanol extract (5 mm) at the lower dose of bacteria, followed by *Chlorella* sp. methanol and acetone extracts at 4 mm; while at the higher dose the *C. calcitrans* methanol extract and in *Chlorella* sp. methanol and acetone extracts gave zones of inhibition of 3 mm. Interestingly, even though the zones of inhibition were small, ß-glucan and alginic acid dissolved in water gave 1 mm zones of inhibition with *S. agalactiae* at the lowest bacterial dose. Moreover, with *Fno,* zones of inhibition were observed for all algae species in all solvents for both high and low doses of *Fno* (8.42 x 10^8 CFU mL^{-1} and 1 x 10^8 CFU mL^{-1}). A clear zone of inhibition was also found in water extracts of *C. calcitrans, Nannochloropsis* sp., *Chlorella* sp. and ß-glucan against *Fno* at the lowest dose, although it was small (1–1.5 mm). In contrast, no inhibition was observed with alginic acid. The highest inhibition zone was recorded for the *C. calcitrans* methanol extracts (4 and 3.5 mm) followed by the *C. calcitrans* acetone extracts and *Chlorella* sp. methanol extracts, with zones of inhibition of 4 and 3 mm against *Fno* at low and high doses, respectively. No activity was recorded for the negative controls for any of solvents against all tested bacteria. A large zone of inhibition was observed for the positive control with tetracyline at 5 µg 20 $µL^{-1}$ in both low and high doses; *at* 18 and 16 mm for *Fno*, while *S. agalactiae* and *A. hydrophila* both had zones of inhibition of 17 and 16 mm, respectively.

Table 2.6 The mean zone diameters (mm) of antimicrobial activity of different algal extracts (at different solvent of extractions and compounds using disc-diffusion method against *Fno*, *A. hydrophila* and *S. agalactiae*

Species of Algae	Type of Solvent	Cont. (µg)	*Fno* (mm)		*A hydrophila* (mm)		*S. agalactiae* (mm)	
			AU 0.1 ($1x10^8$ CFU mL^{-1})	AU 0.5 ($8.42x10^8$ CFU mL^{-1})	OD 0.1 ($1.2x10^8$ CFU mL^{-1})	AU 0.5 ($5.64x10^8$ CFU mL^{-1})	AU 0.1 ($1x10^8$ CFU mL^{-1})	AU 0.5 ($3.42x10^8$ CFU mL^{-1})
C. calcitrans	Methanol	800	**4***	**3.5***	-	-	**5***	**3***
	Ethanol	800	3	2.5	-	-	2	-
	Acetone	800	**4***	3*	-	-	3	2
	Water	800	1	-	-	-	-	-
Nannochloropsis sp.	Methanol	800	2	1.5	-	-	-	-
	Ethanol	800	2	2	-	-	2	-
	Acetone	800	2	1.5	-	-	-	-
	Water	800	1.5	-	-	-	-	-
Chlorella sp.	Methanol	800	**4***	3*	-	-	4*	**3***
	Ethanol	800	2.5	2	-	-	1	-
	Acetone	800	2.5	1.6	-	-	4*	**3***
	Water	800	1	-	-	-	-	-
ß-qlucan	Water	800	1	-	-	-	1	-
Alginic Acid	Water	800	-	-	-	-	1	-
Tetracycline	50% Ethanol	5	18	16	17	16	17	16
Negative control	Water		-	-	-	-	-	-
	Methanol	100%	-	-	-	-	-	-
	Ethanol	100%	-	-	-	-	-	-
	Acetone	100%	-	-	-	-	-	-

*** The highest and second highest of inhibition zones**

C. Respiratory burst

Statistically, there was no significant difference in respiratory burst activity of macrophages isolated using different methods or when fresh or heat-inactivated bacteria (*Fno, A. hydrophila* and *S. agalactiae*,) were used at any of the doses tested (Figure 2.25). The bactericidal assays using the MTS kit were performed using separated macrophages and used the highest dose of live bacteria.

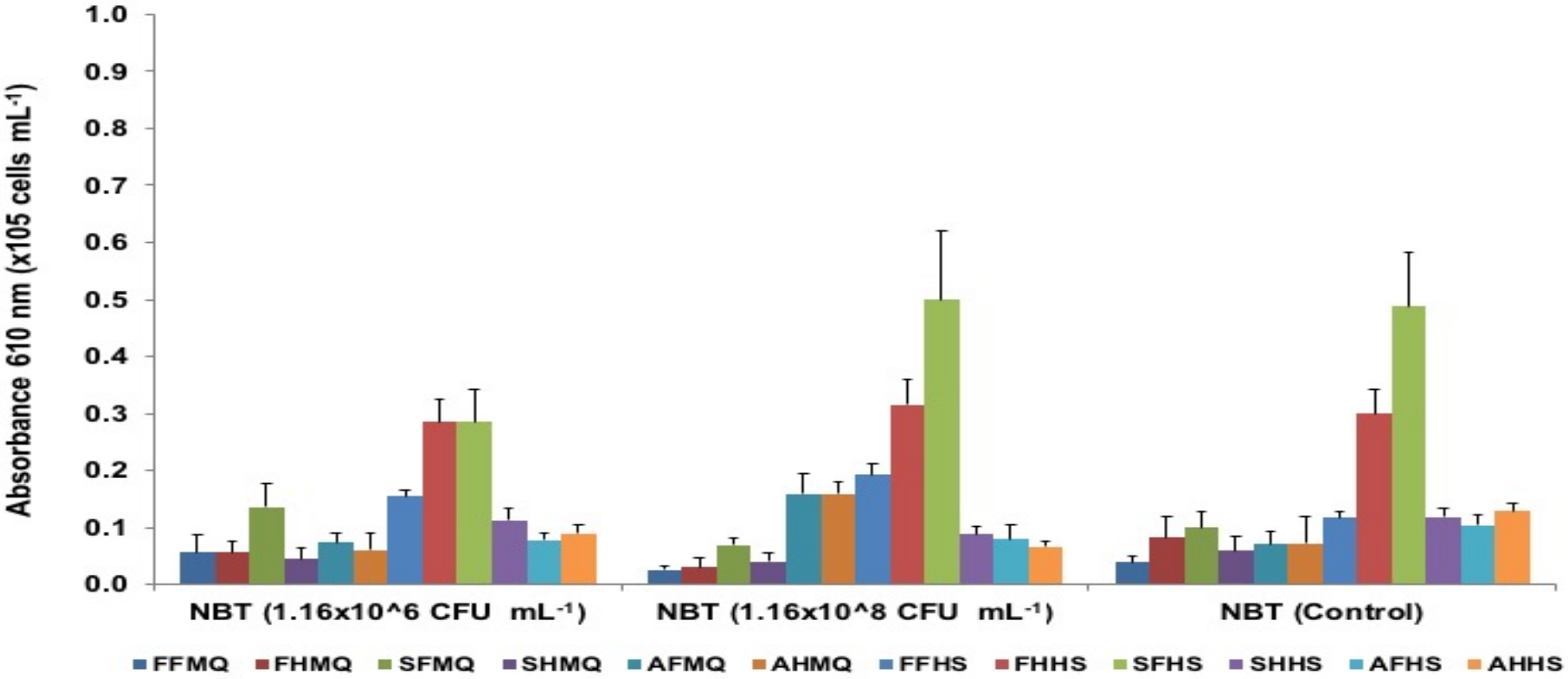

Figure 2.25 Respiratory burst activity of tilapia macrophages isolated by different methods with and without cells separation medium in the response to lived and heat-inactivated bacterial infections *e.g. Fno* (FFMQ & FFHS*,* live *Fno* vs macrophages or vs HK suspension; FHMQ & FHHS, heat killed *Fno* vs macrophages or vs HK suspension); *A. hydrophila* (AFMQ & AFHS*,* live *A. hydrophila* vs macrophages or vs HK suspension; AHMQ & AHHS, heat killed *A. hydrophila* vs macrophages or vs HK suspension) and *S. agalactiae* (SFMQ & SFHS)*,* live *S. agalactiae* vs macrophages or vs HK suspension; SHMQ & SHHS*,* heat killed *S. agalactiae* vs macrophages or vs HK suspension), at two different doses (viz. $1x10^6$ and $1x10^8$ CFU mL^{-1}). No significant different between all treatments groups.

D. Bactericidal activity using MTS kit

Since treatment of tilapia macrophages *in vitro* with ß-glucan, alginic acid, and water extracts of *Chlorella* sp. enhanced their ability to generate a respiratory burst, this study was undertaken to see if these extracts/compounds may also have bactericidal activity. At concentrations of 50 ug mL^{-1} ß-glucan, alginic acid, or water extracts of *Chlorella* sp., each algal extract/compound, give optimal stimulation of respiratory burst activity (Section 2.3.3.1), and so this concentration was used in all the killing assays (bactericidal activity). Bactericidal activity was shown by an increasing in brown colour on each well, with the darker the colour meaning higher bacteria killing (Figure 2.26).

All extracts/compounds tested exhibited bactericidal activity against *Fno* either at MOI 1:100 or 1:1000. The positive control of PMA showed a significant difference of bacterial killing on both MOI compared to other algae stimulants (Figures 2.27 and 2.28). Bacterial killing at MOI 1:1000 (Figure 2.28) showed much higher killing compared to 1:100 (Figure 2.27). A little higher killing of the bacteria observed in the three groups of algae with the highest killing in macrophages treated of *Chlorella* sp. and ß-glucan (MOI 1:100), and ß-glucan (MOI 1:1000). There were significant differences in the killing capacity of the macrophages treated algae groups compared to group of macrophages infected with *Fno* alone on MOI 1:100, while no significant differences on MOI 1:1000 (Figures 2.27 and 2.28, respectively).

The bacterial killing activity for *A. hydrophila* and *S. agalactiae* gave unreliable results (data not shown) and no further assay optimisation was conducted.

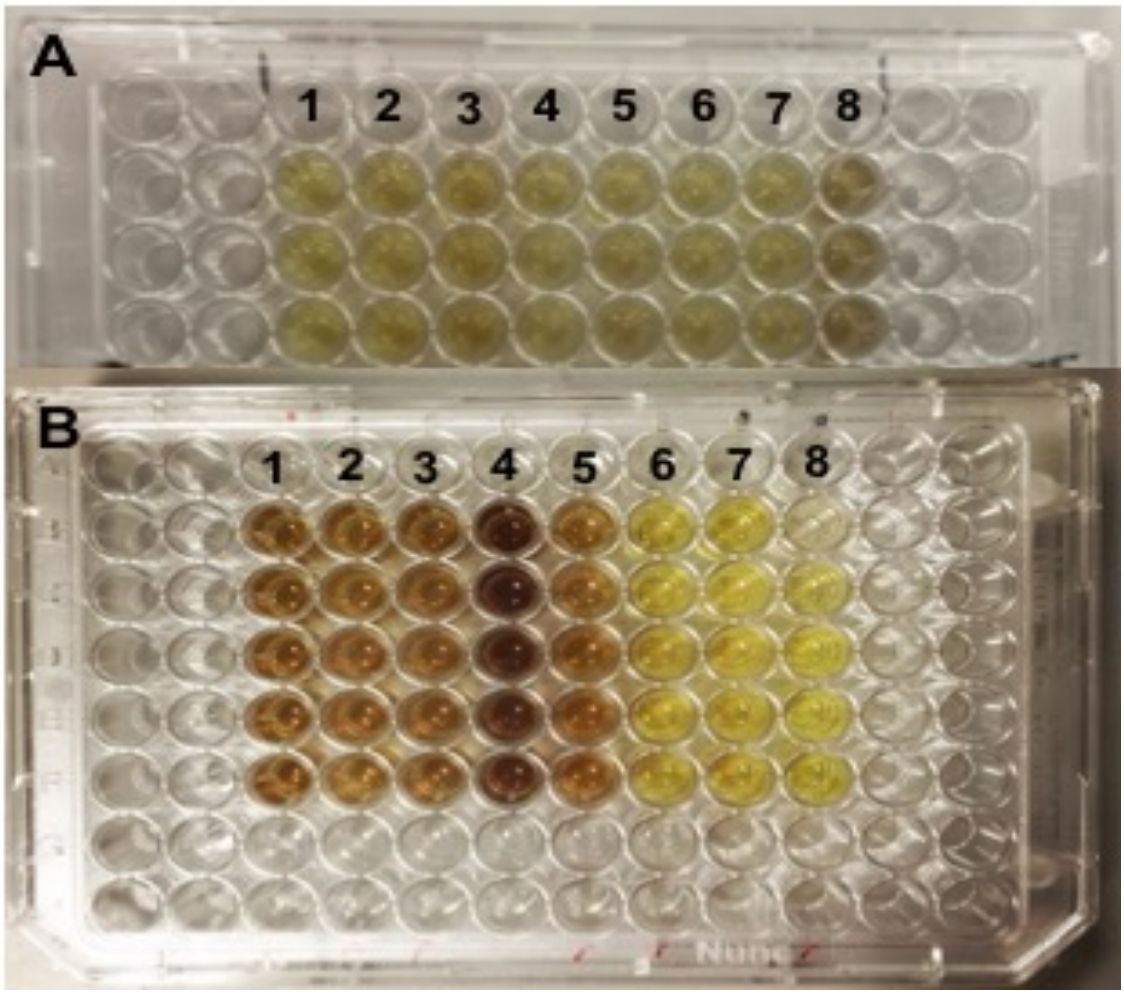

Figure 2.26 Bactericidal activity using MTS/PMS kit on 96 wells plates, positive reaction shown by the increasing of brown colour as darker brown colour meaning higher bacteria killing. (A) MOI 1:100: 1=blank (RPM)I, 2=Fno, 3=*Chlorella* sp., 4=alginic acid, 5=ß-glucan, 6=laminarin and 7=zymosan (data not shown), 8=PMA. (B) MOI 1:1000: 1= *Chlorella* sp., 2=alginic acid, 3=ß-glucan, 4=PMA, 5=*Fno*, 6=RPMI+*Fno* (no cells), 7=RPMI+cells and 8=blank (RPMI).

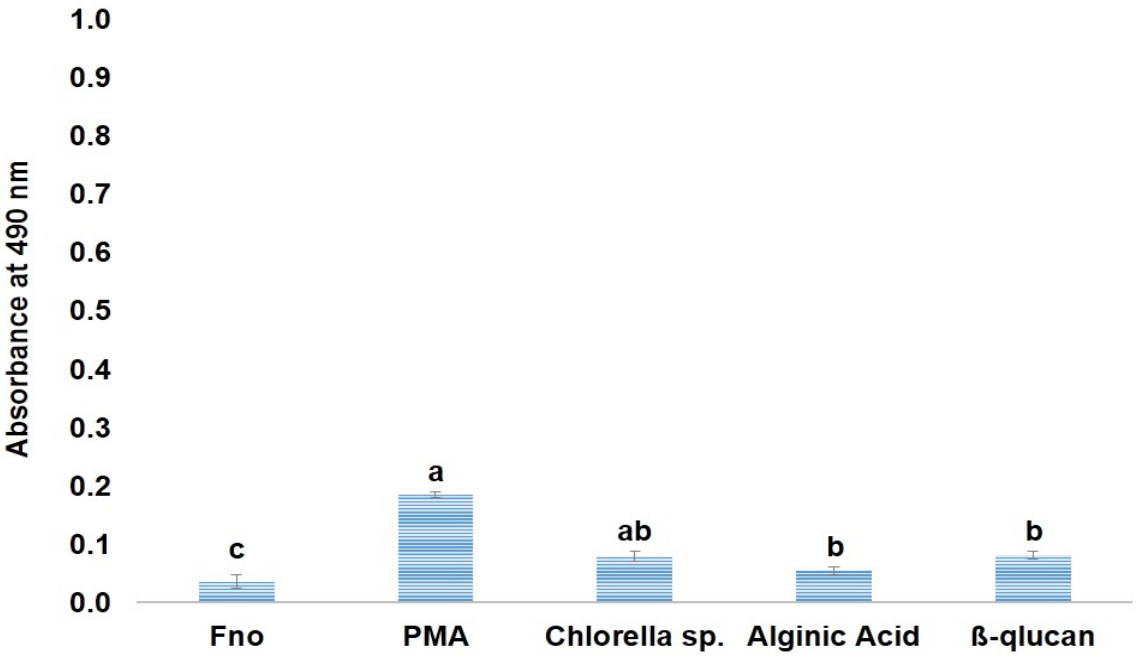

Figure 2.27 Stimulation of the bactericidal activity against *Fno* (at MOI 100) in Nile tilapia homo gold head kidney macrophages after exposure to 50 μg mL^{-1} of *Chlorella* sp., alginic acid and ß-glucan, and 1 μg mL^{-1} PMA. The graph shows results from a representative assay as A_{490} minus blank (blank values are zero). Bars represent the mean of 3 wells+SD. Groups that do not share a letter are significantly different.

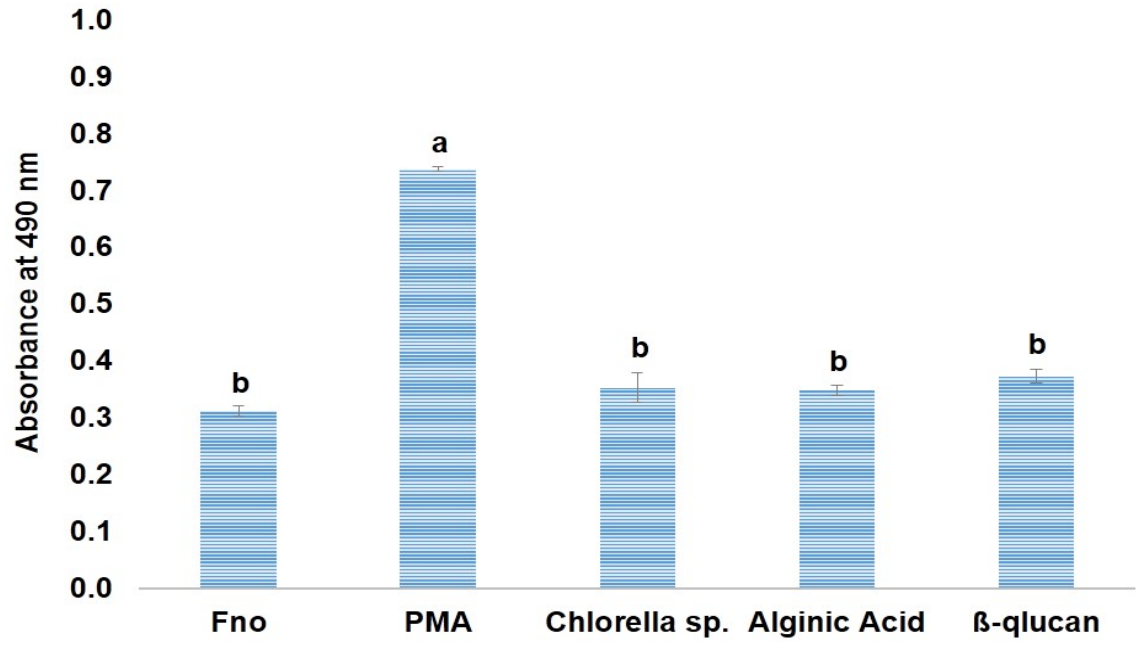

Figure 2.28 Stimulation of the bactericidal activity against *Fno* (at MOI 1000) in Nile tilapia homo gold head kidney macrophages after exposure to 50 μg mL^{-1} of *Chlorella* sp., alginic acid and ß-glucan, and 1 μg mL^{-1} PMA. The graph shows results from a representative assay as A_{490} minus blank (blank values are zero). Bars represent the mean of 5 wells+SD. Groups that do not share a letter are significantly different.

2.4 Discussion

Antimicrobial activity of algae has been documented with respect to the presence of compounds belonging to various chemical classes comprising fatty acids, indoles, terpenes and phenols (Sameeh *et al.*, 2016). Numerous types of antibiotics are produced by algae and each of them kill microbes in a unique way. For example, several disrupt the structure of the bacterial cell wall; others interrupt with the production of essential proteins; and others interfere with the transformation (metabolism) of nucleic acids (Flodin and Whitfield, 2000; Jin *et al.*, 2006). In this chapter, algal-derived compounds were screened, and their potential was assessed as immunostimulatory compounds by respiratory burst assay, while antibacterial activities of the compounds were determined using a disc diffusion assay and MTS assay.

Based on API 20 E, this results with the *Fno* STIR-GUS-F2f7 isolate used in the study are similar to those previously reported for *Fno* isolated from tilapia and three-line grunt (Ehime-1, Japan) (Birkbeck *et al.*, 2007, 2011; Ottem *et al.*, 2009; Mikalsen and Colquhoun, 2010; Ottem, 2011; Ramirez-Paredes, 2015). In contrast, *Francisella* like organisms isolated from tilapia showed a positive reaction for carbohydrate utilization (sucrose) (Hsieh *et al.*, 2006); while Brevik *et al.* (2011) and Colquhoun *et al.* (2013) showed a negative reaction for the gelatin hydrolase with the same isolate from Japan (*Fno* DSM21254T). In addition, in this present work, enzymatic profiles identical to the one found in the present study, have been previously demonstrated when comparing biochemical and phenotypic characteristics of *Francisella* sp., *Fno* from tilapia and three-line grunt (Ehime-1) (Birkbeck *et al.*, 2007, 2011; Ottem *et al.*, 2009; Mikalsen and Colquhoun, 2010; Ottem, 2011). In contrast, the results of Ramirez-Paredes (2015), using the same *Fno* isolate (STIR-GUS-F2f7) and Ehime-1, found a different enzymatic profile to the one in this study *e.g.* 3 different positive enzymes: lipase (C14), α-chymotrypsin and ß-galactosidase. In addition, Brevik *et al.* (2011), using *Fno* DSM21254T, found a positive result for lipase, which was not found in this study.

Algae, including micro and macro-algae, are able to produce a great variety of secondary metabolites characterized by a broad spectrum of antimicrobial activities (Chojnacka *et al.*, 2012). Thus, they are broadly screened to isolate drugs or bioactive substances all over the world (Chapter 1, Section 1.6.6). Accordingly, the present study was focused to screen extracts of *C. calcitrans, Nannochloropsis* sp., and *Chlorella* sp. as well as two commercially available compounds namely alginic acid and ß-glucan for the potential of antimicrobial activity against three tilapia bacterial pathogens using disc diffusion assay as presented in Table 2.6. No antimicrobial activity was observed for any of the algal extracts/compounds tested against *A. hydrophila* at both A_{610}. *C. calcitrans* and *Chlorella* sp. were however, observed to be active and inhibited the growth of *S. agalactiae* at both high and low bacterial doses, while *Nannochloropsis* sp, alginic acid and ß-glucan were also active at the lower dose. In addition, almost all algae extracts/compounds showed inhibition zones against *Fno* at both doses, except for ß-glucan (only at AU 0.1), while alginic acid had no inhibition at all. A major role in displaying the antibacterial activity using algae as a source is affected by the solvent system used for the extraction. In the present study, among four different solvents extracts tested, methanol exhibited the highest inhibitory effect followed by acetone, ethanol then water. For example, the highest inhibitory of *S. agalactiae* was found in *C. calcitrans* methanol extracts, followed by *Chlorella* sp. methanol and acetone extracts, while against *Fno* the highest inhibition zone recorded in *C. calcitrans* methanol extracts followed by the same algal but with acetone extracts and *Chlorella* sp. methanol extracts at both AU. Therefore, the results clearly showed that the methanol solvent systems were more efficient in extracting active compounds from the algae.

Antimicrobial activity in algae methanolic extracts has been reported against Gram-negative and Gram-positive bacteria and fungi *in vitro* using the agar disc diffusion method. For example, methanol extracts of *Stoechospermum marginatum* (brown alga) and *Cladophora prolifera* (green alga) at 500 µg mL^{-1} had moderate bacterial activity against *S. aureus, Klebsiella* sp. and *V. cholera* at 1.2 x 10^8 CFU mL^{-1} per bacterium (Ely *et al.*, 2004). All the test methanolic

extracts of six algae species (*e.g. Corallina officinalis, Cystoseira barbata, Dictyota dichotoma, Halopteris filicina, Cladostephus spongiosus f. verticillatus* and *U. rigida*), except *C. officinalis*, were shown to inhibit growth *S. aureus*. On the other hand, the highest inhibition activity among all the extracts was shown to be with *E. aerogenes* by *C. officinalis*, while, an extract of *C. barbata* showed broader activity against all three Gram-positive test organisms (*S. aureus, M. luteus* and *E. faecalis*) as well as three Gram-negative organisms (*E. aerogenes* and *E. coli*) at 10^7–10^8 CFU mL^{-1} (Taskin *et al.*, 2007). A high antimicrobial activity of the methanolic extracts of the seaweeds (*Himanthalia elongata, L. sachharina* and *L. digitate*) at 60 mg mL^{-1}, has also been reported, where these had the capacity to inhibit Gram-negative food pathogens and food spoilage bacteria at 1 x 10^6 CFU mL^{-1}. The results showed almost 100% inhibition against *L. monocytogenes*; 98% and 93% inhibition were achieved against *P. aeruginosa* by *H. elongata* and *L. saccharina* extracts, respectively (Gupta *et al.*, 2010). In recent investigation by Sameeh *et al.*, (2016), the acetone extract of *P. boryana* Thivy had strong antibacterial inhibition against the Gram-negative bacteria *Neisseria gonorrhoeae* and *Moraxella catarrhalis* followed by Gram-positive methicillin-resistant *S. aureus*, while, ethanol extracts of *P. boryana* Thivy and *Enteromorpha* sp. showed a broad spectrum of antimicrobial activity against Gram (+) and Gram (-) bacteria in addition to Candida.

Furthermore, with regards to fish pathogenic bacteria, several studies have reported an antimicrobial activity of methanolic extracts of algae. Inhibition activity was observed at 500 µg 50 μL^{-1} methanolic extracts of *G. corticata, U. fasciata*, and *E. compressa* against *P. fluorescens*, *V. alginolyticus* and *E. tarda* (10^6 bacteria mL^{-1}) (Choudhury *et al.*, 2005). *Ceramium rubrum, Falkenbergia rufolanosa* and *Halopitys incurvus* (2 mg $disc^{-1}$) were demonstrated to have weak inhibiting activities against *V. anguillarum, P. anguilliseptica* and *A. salmonicida* (Bansemir *et al.*, 2006). *Anabaena wisconsinense* and *Oscillatoria curviceps* methanol extracts of (1.5 mg 30 μL^{-1} each algal) were observed have antibacterial effects against *Lactobacillus* sp, *P. anguilliseptica*, *A. hydrophila, Bacillus firmus, P. fluorescens* (1.2 x 10^7 CFU mL^{-1} each bacterium) and the

fungi *Apergillus niger* and *Saprolegnia parasitica* (El-sheekh *et al.*, 2008). In addition, the same study found that the ethanolic extracts of *O. curviceps* and had maximum inhibition activity against *Lactobacillus* sp. and *A. hydrophila*, whereas *A. wisconsinense* had high inhibition activity against *A. hydrophila*. The methanol extract of *Chetomorpha linum* at 5 mg mL^{-1} showed a high inhibition zone against *V. alginolytics* and *P. aeruginosa* (Vijayakumar *et al.*, 2012), while with the same solvent, *S. wightii, U. lacta* and *P. tetramatica* at 50 mg mL produced maximum zone of inhibition against *V. harveyi* (1.2×10^8 CFU mL^{-1}) (Rajasekar *et al.*, 2012). *Chondrococcus hornemanni* methanol extracts (100 mg mL^{-1}) had a broad and high antibacterial activity against four fish pathogens including *Providencia rettgeri, A. hydrophila, V. alginoticus* and *V. parahaemolyticus* (Ganeshamurthy *et al.*, 2013). It is well-defined, that the use of organic solvents always provides a higher efficiency in extracting compounds for antimicrobial activities compared to water-based methods (Lima-Filho *et al.*, 2002).

In the present study, *C. calcitrans, Chlorella* sp. and *Nannochloropsis* sp. extracts had the capacity to inhibit Gram-negative and Gram-positive bacteria, with the highest inhibition activities observed against *Fno*. Gram-negative bacteria are more conspicuous pathogens compared to the Gram-positive bacteria (Gupta *et al.*, 2010). To prevent certain drugs and antibiotics from penetrating the cell thus accounting for the high resistance of these bacteria to antibiotics, they have an additional lipopolysaccharide layer on the outer surface (Dowling, 2004). In contrast, none of algae extracts used showed activity against *A. hydrophila*. It could be due to the fact that this bacterium has a thicker cell wall, thus needs a higher concentration of algal extract to penetrate the cell, as reported in some studies. For example, *A. wisconsinense* and *O. curviceps* methanol extracts at 1.5 mg 30 μL^{-1} each algal (Vijayakumar *et al.*, 2012), while with *Chondrococcus hornemanni* need a higher concentration of 100 mg mL^{-1} (Ganeshamurthy *et al.*, 2013) to inhibit the growth of *A. hydrophila*.

The kidney often referred to as the head kidney tissue is important in hematopoiesis and immunity in fish and it is predominantly a lympho-myeloid

compartment (Press and Evensen, 1999). Early in development, the entire kidney is involved in production of immune cells and the early immune response, as the fish mature, blood flow through the kidney is slow, and exposure to antigens occurs (Press and Evensen, 1999). There appears to be a concentration of melanomacrophage centers; these are aggregates of reticular cells, macrophages, lymphocytes and plasma cells; they may be involved in antigen trapping and may play a role in immunologic memory (Secombes *et al.*, 1982; Galindo-Villegas and Hosokawa, 2004). The head or anterior kidney (pronephros), the active immune part, is formed with two Y-arms, which penetrate underneath the gills. In addition, this structure of the kidney has a unique feature, and it is a well innervated organ, and the kidney is also an important endocrine organ, homologous to mammalian adrenal glands, releasing corticosteroids and other hormones. Thus, the kidney is a valuable organ with key regulatory functions and the central organ for immune-endocrine interactions and even neuroimmuno-endocrine connections (Press and Evensen, 1999).

Phagocytes (neutrophils and macrophages) are cellular components of natural immunity and the activation in mammals is usually correlated with a sudden increase in oxygen utilization, leading to the production of reactive oxygen species which have potent microbicidal activities and play an important role in the host defense against microorganisms (Castro *et al.*, 2004). In addition, ROS and formation processes are activated by the opsonization process and cause respiratory burst. In this process, NADPH-oxidase complex produces superoxide anion (O2-) and the anion is subsequently converted to hydrogen peroxide (H2O2) with antibacterial activity (Castro *et al.*, 2004). Fish neutrophils and macrophages also generate ROS (Secombes, 1996) which have been associated with the ability of phagocytes to kill bacterial pathogens (Ellis, 2001). There are two mechanisms of phagocytosis, oxygen-dependent and oxygen-independent mechanisms, of which the former can be detected by NBT, a substrate that forms blue formazan as a result of O2 reduction in the phagocytosis process (Sarlin and Philip, 2011). The respiratory burst activity of fish phagocytes can be modulated *in vitro* and *in vivo* by many substances and

some are known as immunostimulants; these include algae and their products such as either glucans or alginic acid.

Three algal species, a green algal, *Chlorella* sp. a diatom, *C. calcitrans* and a green algal, *Nannochloropsis* sp., which were extracted with 4 different solvents (*i.e.* methanol, acetone, ethanol and water), and two substances viz. alginic acid and ß-glucan which are derived from *M. pyrifera* and *E. gracilis*, respectively, were evaluated for their ability to stimulate the cellular immune responses of Nile tilapia homo gold HK leukocytes. Although there were no significant differences between the groups, different algae extracts (at 50 µg mL^{-1}) were able to stimulate cellular respiratory burst activity of Nile tilapia, especially at 3 and 6 h after treatment. A clearer pattern and high activity was observed in cells incubated with water extracts of *Chlorella* sp., beta-1,3-glucan and alginic acid compared to other algae stimulants. However, the ROS activity sharply declined in all groups at 24 h after treatment. Probably at 24 h of exposure the macrophages are already finished. The results from present study are in agreement as well as in contrast with the one reported by Caipang *et al.* (2011), that after incubation of cod (*Gadus morhua*) HK leukocytes with alginic acid from the same source as in present study, resulted in significantly elevated respiratory burst activity at 3 and 24 h post-incubation in a dose-dependent manner (either 10 or 100 µg mL^{-1}). Not many studies have been conducted with respect to the immune-related cells *in vitro* by primary cultures of HK leukocytes after administration of alginate or alginic acid, especially produced by algae, with most of the studies showing the fish immune responses to this algal derivative at the organismal level. ß-glucan also modulated respiratory burst in Nile tilapia homo gold HK leukocytes in the present study. This is in agreement with a previous report that ß-glucan from the alga *E. gracilis* was led to a significant increase of ROS production at a higher concentration of 10 mg mL^{-1} (Díaz-Rosales *et al.*, 2007). Laminaran, a ß-glucan obtained from the brown alga *L. hyperborea*, was also found increase the intracellular production of superoxide anion by salmon *Salmo salar* L macrophages (Dalmo and Seljelid, 1995).

Moreover, there are some studies on the use of ß-glucan as immunostimulant in cellular immune responses of different fish species *in vitro* especially for MacroGard, but not many from algae. For example, 72 h incubation of ß-glucan from yeast (a microparticulate 13-1,3- and 13-1,6-1inked glucan from cell walls of *S. cerevisiae*) at 1 µg mL^{-1} produced an increase in respiratory burst activity Rainbow trout, *Oncorhynchus mykiss* (Novoa et al., 1996). Three particulated ß-glucans from yeast (Macrogard, Fibosel, and VitaStim) and a particulate glucan from the fungus *Schizophyllum commune* (Sigma) induced a respiratory burst in phagocytes of turbot (*Psetta maxima*) and gilthead seabream (*Sparus aurata*) after 1 h incubation which increased with the concentration of glucan (at 0.5, 1, 2.5, 5 7.5, 10, 25, 50 and 100 µg mL^{-1}) and reached a maximum when the glucan concentrations were in the range 250–500 µg mL^{-1} (Castro et al., 1999). In addition, when the incubation period used was 2 h, the maximum responses induced by Macrogard, Fibosel and the Sigma glucan were at concentrations of 50–100 µg mL^{-1}, and with higher glucan concentrations the response tended to decrease, while VitaStim increased the respiratory burst with the glucan concentration, reaching a maximum at concentrations of 250–500 µg mL^{-1} (Castro et al., 1999). Atlantic salmon macrophages treated with yeast ß-glucan of 0.1–1 µg mL^{-1} gave a maximum respiratory burst response at 4 to 7 d after the addition, whereas 10 µg mL^{-1} had no effect and 50 µg mL^{-1} was inhibitory; in addition, the glucan also triggered respiratory burst activity directly, but this occurred only at relative high concentrations with a maximal effect at ≥200 µg mL^{-1} (Jørgensen and Robertsen, 1995). In contrast, in the present study ß-glucan from *E. gracilis* showed stimulation activity on tilapia HK macrophages at a concentration of 50 µg mL^{-1} and shorter times of incubation (3 and 6 h). Meanwhile, EcoActiva paste (at 0.001–0.1% v/v) was shown to stimulate Snapper macrophages after both 1 and 3 h incubation followed by PMA stimulation (Cook *et al.*, 2001). Three-commercial ß-glucans were able to modulate the intracellular O^{-}_{2} production of Atlantic salmon head kidney macrophages *in vitro*, after cultured 3 and 7 d in the presence of 0.1–10 µg mL^{-1} of the ß-glucan products (Bridle *et al.*, 2005).

In addition, in the present study, the low ROS activity observed in macrophages stimulated with algae extracted using organic solvents (methanol, acetone, and ethanol) could be due to inhibition of the ROS activity by the solvents. These results are in contrast with a previous study where an ethanolic extracts from *Hydropuntia cornea,* at high as 10 mg mL^{-1}, increased superoxide anion production in sole phagocytes compared to the aqueous extracts (Díaz-Rosales *et al.*, 2007). However, several authors have reported the immunostimulant capacity of aqueous extracts from several algal species. For example, the presence of aqueous extracts from *U. rigida* and *Chondrus crispus* which is associated with the polysaccharidic fraction were stimulated respiratory burst in turbot *Psetta maxima*, L. (1758) phagocytes (Castro *et al.*, 2004, 2006). The capacity of aqueous extracts from *Fucus distichus* and mycosporine from *Porphyra leucosticta* were showed increased respiratory burst activity of sole phagocytes after infection with *P. damselae* subsp. *piscicida* (Díaz-Rosales *et al.*, 2005).

Despite the fact that the water extracts of *Chlorella* sp., alginic acid and ß-glucan (50 µg mL^{-1}) in the present study increased the capacity of macrophages to produce superoxide, they showed capacity to kill the virulent strains of *Fno* with significant difference between the groups observed especially on MOI 1:100. In agreement with previous studies, especially for ß-glucan, 1 µg mL^{-1} of yeast ß-glucan was gave maximum O^{-}_{2} production but showed no killing activity on Atlantic salmon HK macrophages bactericidal activity using MTT assay for an avirulent and virulent strain of *A. salmonicida* at MOI 10:1 and 1:1, respectively (Jørgensen and Robertsen, 1995). In addition, the growth of *A. salmonicida* and *V. anguilarum* in both treated cod cells (with 100 µg mL^{-1} alginic acid from *M. pyrifera,* which had significant increase in respiratory burst activity at 3 and 24 h), and control supernatants of the compounds was assessed using MTT assay and found significantly lower level than those observed in the bacterial culture medium (Caipang *et al.*, 2011). The supernatants, however, from the treated cells had significantly higher bacterial growth compared with supernatants of the control cells (Caipang *et al.*, 2011). These are in contrast to the correlation between increased respiratory burst and

bactericidal activity with respect to the rainbow trout head kidney macrophages activated *in vitro* which capable of secreting a soluble macrophage-activating factor after stimulation with concanavalin A (Graham and Secombes, 1988).

An activation of cellular response by immunostimulant is associated with specific interactions of one or more cell surface receptors (Meena *et al.,* 2013). For example, glucans are believed to activate innate immunity by binding to specific receptors on monocyte/macrophages, neutrophils and natural killer cells (Muller *et al.,* 2000). Different ß-glucans are associated with different or same type of receptors, which different types of ß-glucan receptors are including: Scavenger Receptor, complement receptor, lactosylceramide, dectin-1 and toll-like ß-glucan receptors (Meena *et al.,* 2013). A distinct class A member of the Scavenger Receptor family, MARCO (macrophage receptor with collagenous structure), is present in several fish species, and knockdown experiments in zebrafish confirmed a role for MARCO in rapid phagocytosis of *Mycobacterium marinum* (Benard *et al.,* 2014). Teleost fish do express genes encoding for the alpha- (CD11(b)-like) and beta-unit (CD18) of complement receptor 3 (Mikrou *et al.,* 2009; Nakao *et al.,* 2003). Toll-like receptors like TLR2 could possibly sense ß-glucans and are expressed in several fish species (Pietretti and Wiegertjes, 2014), but the presence of Dectin-1 (the prototypical C-type lectin receptor for ß-glucan), is limited to mammalian genomes (Sattler *et al.,* 2012).

Furthermore, several earlier reports are in line and correlate with the results from the present study above. For instance, *in vitro* macrophages isolated from ß-glucan-injected rainbow trout and Atlantic salmon had increased capacity to kill *A. salmonicida* (Jorgensen *et al.*, 1993; Jørgensen *et al.*, 1993) and the enhanced killing ability could be associated with increased respiratory burst activity for trout (Jørgensen *et al.*, 1993). In addition, ethanolic extracts from *H. cornea* and ß-glucan at 10 mg mL^{-1} each stimulated enhanced superoxide anion production in sole phagocytes as well as bactericidal activity against *P. damselae* subsp. *piscicida* MOI 1:10 (cells:bacteria) (Díaz-Rosales *et al.*, 2007). A wide range of biologically active mechanisms to generate ROS which

are involved in bactericidal activity exist in mammalian macrophages such as production of nitrogen oxide (Green *et al.*, 1991), limitation of intracellular iron availability, phagosome acidification, release of lysosomal enzymes in the phagosome, cationic peptides and production of defensins (Adams and Hamilton, 1984). Therefore, a more multifaceted picture of such mechanisms will most possibly appear, as studies of antimicrobial mechanisms of fish macrophages are extended (Jørgensen and Robertsen, 1995). Whether treatment of macrophages with algal-derived compounds will cause more efficient killing of a less specialized pathogens is not yet known and needs further studies.

The bactericidal activity can be attributed due to the presence of halogenated secondary metabolites, such as bromophenols and phlorotannins that are produced by algae (Nagayama *et al.*, 2002). In addition, halogen-containing terpenoids, acetylens and phenols have been identified in several algae species as biologically active compounds (Konig and Wright, 1997; Vairappan *et al.*, 2001). According to Sameeh *et al.*, (2016) the results from disc diffusion assay were found to parallel the highest total phenolic and flavonoid content in acetone and ethanol extract, thus the antimicrobial activity of *P. boryana* Thivy and *Enteromorpha* sp. might be attributed to their phenolic and flavonoid content. Mohamed *et al.* (2015) also reported a positive relationship between antimicrobial activity potential and concentration of phenolic compounds of crude extracts. Phenolic compounds were found to be present in the hot-water extracts of seaweed *S. wightii* which were tested using GC-MS chromatogram (Thirunavukkarasu *et al.*, 2015). One of the most important classes of natural products are phenolic compounds. Phenolics act as antioxidants by impeding enzymes involved in radical generation and demonstrated anticancer, antibacterial, anti-allergic, anti-diabetes, anti-aging, and anti-HIV activities, while flavonoids are a widely distributed group of natural compounds and the most important natural phenolics (Fresco *et al.*, 2006; Li *et al.*, 2011). In addition, phenols are important antioxidants in most algae, due to their ability to scavenge free radicals such as singlet oxygen, superoxide and hydroxyl radicals (Shanab *et al.*, 2011). According to Bricknell and Dalmo (2005), algal

polyphenolic constituents might directly activate innate defense mechanisms by acting on receptors and triggering gene activation, which might result in production of antimicrobial molecules (Lin *et al.*, 2011).

In spite of positive effects of algal-derived compounds on fish, there are also some studies on the potential negative effects of the compounds especially produce by cyanobacteria (Rymuszka, *et al.,* 2008). For instance, after exposures for 24 h, microcystin-RR inhibited glutathione S-transferase in liver, gills, intestine and brain of *Corydoras paleatus,* enhanced activities of glutathione reductase, guaiacol peroxidase, and glutathione peroxidase, in liver but inhibited in gills, enhanced catalase activity in liver, activated antioxidant response in liver at low toxin concentrations, followed by a drop at the highest microcystin-RR levels, inhibited detoxification activity in liver and brain in a dose-dependent way, and induced peroxidation of lipids in brain of exposed fish (Cazenave, *et al.,* 2006). An administration of microcystin-RR (10 nmol L^{-1}) caused a massive calcium influx resulting in elevation of reactive oxygen species in *Carassius auratus*, rapid disruption of mitochondrial membrane potential, and depletion of adenosine triphosphate (Zhang, *et al.,* 2007). The lymphocyte proliferation of rainbow trout inhibited after application of microcystin-LR at a concentration of 40 mg mL-1 but significantly increased at a concentration of 1 mg mL^{-1} (Rymuszka, *et al.,* 2007). Cyanobacterial hepatotoxins, microcystins-LR induced apoptosis in lymphocytes of carp 2 h after incubation, whereas high toxin concentrations induced necrosis in lymphocytes in a time and concentration-dependent manner, inhibited phagocytosis without affecting apoptosis or glutathione levels and induced a significant re-organization of the actin cytoskeleton in phagocytes, which subsequently collapsed around the nucleus leading to cell shrinkage and the disappearance of filopodi (Rymuszka, 2013).

2.5 Conclusions

These studies indicate that algae and its derivatives have potential as immunostimulants for Nile tilapia in order to combat bacterial infections *in vitro*.

The results clearly signify, based on disc diffusion assay that among the four different solvents used for extraction that methanol extracts exhibited the highest inhibitory effect, especially for *Fno* and *S. agalactiae*, followed by acetone, ethanol then water extracts. Eventhough no significant differences were observed between the groups, different algal extracts (50 µg mL^{-1}) were able to stimulate cellular respiratory burst activity of Nile tilapia homo gold HK macrophages cells cultures especially at 3 and 6 h. The cells incubated with water extracts of *Chlorella* sp., ß-glucan and alginic acid showed higher ROS activity in the HK macrophages compared to other algae extracts/compounds. These water extracts of *Chlorella* sp., alginic acid and ß-glucan (50 µg mL^{-1}), however, gave little capacity to kill the virulent strains of *Fno* (MTS kit assay). This study has provided evidence that algal-derived compounds have potential use as immunostimulants in tilapia *in vitro*. However, further work is required to investigate the potential of algal-derived compounds to modulate the fish immune response *in vivo*. In Chapter 4, ß-glucan, alginic acid and two additional algae species were evaluated to determine their effects in Nile tilapia homo gold against bacterial pathogens, especially *Fno*. An immersion challenge model for *Fno* in Nile tilapia was developed in Chapter 3 prior to the *in vivo* srcreening trial (Chapter 4).

CHAPTER 3

DEVELOPMENT OF AN IMMERSION CHALLENGE FOR *Francisella noatunensis* subsp. *orientalis* IN TWO SPECIES OF NILE TILAPIA *Oreochromis niloticus* L AND EVALUATION OF RESPONSE TO INFECTION

3.1 Introduction

Tilapia aquaculture produces a potentially sustainable source of high-quality protein and its delicate white meat is desired by consumers throughout the world. All species of tilapia are native to Africa and the Near East, and these are now one of the most important cultured group of fish. Indeed, global production of tilapia is second only to carp, and they are one of the main traded fish-food commodities worldwide (Josupeit, 2005; Mapfumo, 2015; FAO, 2016).

Increase in production and intensification of tilapia aquaculture has led to an increase in outbreaks of infectious diseases, in particular bacterial diseases. This has been characterized as one of the main obstructions to the full growth of tilapia culture. Piscine francisellosis caused by the highly virulent bacteria *Francisella noatunensis* subsp. *orientalis* is an emerging problem for the tilapia industry. *Fno* is a small pleomorphic, Gram-negative coccobacillus recently described as an emergent bacterial pathogen that causes of systemic granulomatous disease in a variety of fish species especially tilapia. This bacterium has been implicated as the cause of mortalities in tilapia that are reared in a variety of different culture systems, including fresh- brackish- and sea-water. The number of reports of outbreaks resembling francisellosis has increased over for the last two decades and have been reported worldwide (Chern and Chao, 1994; Chen *et al.*, 1994; Mauel *et al.*, 2007, 2003, 2005; Hsieh *et al.*, 2006; Soto *et al.*, 2009a, 2013c; Jeffery *et al.*, 2010; Iregui *et al.*, 2011; Leal *et al.*, 2014; Ramirez-Paredes, 2015; Jantrakajorn and Wongtavatchai, 2016; Lin *et al.*, 2016; Ortega *et al.*, 2016; Nguyen *et al.*, 2016; Sebastião *et al.*, 2017). All species of tilapia, wild and cultured, throughout the world have been reported to be susceptible to *Fno*, as listed in Table 3.1.

Nile tilapia and red Nile tilapia hybrids (*Oreochromis* spp.) are the species most affected by piscine francisellosis, from larvae to broodstock (Table 3.1). Several predisposing factors for francisellosis are associated with a great variance in mortality rate (from 5 to 95%) in tilapia. These includes water temperatures, water quality, co-infection, and handling stress (Soto *et al.*, 2009b, 2012b; Assis *et al.*, 2017). This disease poses a substantial constraint to the potential of

tilapia farming and has in several cases led to significant associated economic losses owing to high mortality, reduced weight gain, antibiotic and medication costs (Bondad-Reantaso *et al.*, 2005; Soto *et al.*, 2009b). Therefore, a better understanding of the epidemiology and epizootiology of the infectious agent is critical to healthy food production and commercial farming (Klinger-Bowen *et al.*, 2016).

Tilapia piscine francisellosis can be present as an acute disease with high mortality rates and few clinical signs or as a sub-acute to chronic infection with varying clinical signs (Soto *et al.*, 2009a; b, 2011b; Iregui *et al.*, 2011). Mostly infected fish present with external clinical signs, such as the fish swimming lethargically, lack of appetite, anorexia, becoming anaemic and emaciated, some fish showing darker or pale body colourations, skin with excess mucus or lower than normal, focal areas of hemorrhage or petechiae on the skin, loss of scales, ulcerations in severe cases, pale and light fraying of fins, corneal opacity, mild to moderate lateral or bilateral exophthalmia, varying degrees of bloody ascites, and listless at the moribund stage (Chern and Chao, 1994; Chen *et al.*, 1994; Mauel *et al.*, 2003, 2005, 2007). The gills exhibit epithelial hyperplasia with mild to severe fusion of secondary lamellae and necrosis with a patchy white appearance caused by loss of intralamellar spaces (Mauel *et al.*, 2003, 2005, 2007). Upon macroscopic and microscopic examination, several internal organs (mainly spleen and kidney) are enlarged varying to 5–50x greater than the normal organ size and contain widespread multifocal white nodules (as ring-shaped foci) varying in size and focal areas of necrosis, and in some cases fish were found with hepatic lipidosis (Chern and Chao, 1994; Mauel *et al.*, 2003, 2007; Soto *et al.*, 2011b; Ramirez-Paredes, 2015). In several cases, white nodules were observed in gills, liver, choroid gland, and sporadically in the gastrointestinal walls and mesenteric fat (Soto *et al.*, 2009a). Histological examination reveals the presence of multifocal granulomatous lesions containing numerous small, pleomorphic cocco-bacilli (Chern and Chao, 1994; Chen *et al.*, 1994; Mauel *et al.*, 2003; Soto *et al.*, 2009a, 2011b).

Cold stress, poor water conditions and water circulation, and high fish density have major effect on the development of piscine francisellosis in tilapia on fish

farms, as well as in controlled laboratory studies (Chern and Chao, 1994; Chen *et al.*, 1994; Mauel *et al.*, 2003, 2005; Duodu and Colquhoun, 2010). Outbreaks of piscine francisellosis began in freshwater cultured ponds in the Kaoshiung area of southern Taiwan (Chen *et al.*, 1994) between October 1992 to February 1993, and soon thereafter spread around the island (northern, central and southern Taiwan) with at least 37 tilapia farms infected, including several stocks, hatcheries, brackish- and seawater-ponds cultured (Chern and Chao, 1994; Hsieh *et al.*, 2006). In contrast, in several regions of Thailand, francisellosis in tilapia was limited to freshwater culture, and not observed in southern Thailand, where tilapia production is based on brackish- and sea-culture; however, it could be related to the geographical location, because the water temperature in that region is in the range (27–30°C) year-round (Jantrakajorn and Wongtavatchai, 2016).

Table 3.1 Reports of natural outbreaks of piscines francisellosis caused by *Francisella noatuenensis* subsp., *orientalis* in tilapia species worldwide

Fish Species		Year	Location	Mortality	Pop.	Size	Water	T (°C)	Reference
Mozambique tilapia	*O. mossambicus*	1992-1993	Taiwan	20–95%	Fm	30–639 g	F, B, S	15	(Chern and Chao, 1994)
		1994	Oahu, Hawaii	60%	Wi, Fm	nr	F, B	21.5–29.2	(Mauel *et al.*, 2003)
		2001-2004	Northern, Central & Southern Taiwan	nr	Fm	nr	F	nr	(Hsieh *et al.*, 2006)
		2000-2004	Indonesia	nr	Fm	nr	F	nr	(Mauel and Miller, 2002; Mauel *et al.*, 2003; Ottem *et al.*, 2009)
		2006	Southern California, USA Island of Oahu, Hawaii	nr	Fm	nr	F	nr	(V. E. Ostland unpubl. obs.)
		2010-2012		nr	Fm	nr	F	nr	(Soto *et al.*, 2013c)
Nile tilapia	*O. niloticus/ Tilapia nilotica*	1992	Southern Taiwan	75%	Fm	40–200 g	F	nr	(Chen *et al.*, 1994)
		1992-1993	Taiwan	20–95%	Fm	30–639 g	F, B, S	15	(Chern and Chao, 1994)
		1995	Japan	nr	Fm	nr	F	nr	(Wada *et al.*, 1995)
		2001-2004	Northern, central & Southern Taiwan	nr	Fm	nr	F, B	nr	(Hsieh *et al.*, 2006)
		2007	Alajuela, Costa Rica	50–60%	Fm	13–20 g	F	28–30	(Soto *et al.*, 2009a)
		2010	England, UK	20%	Fm	0.5–5 g	F	24–27	(Jeffery *et al.*, 2010)
		2004-2007	Central/Latin America	nr	Fm	10–1000 g	F	21–28	(Mauel *et al.*, 2007)
		2010	Midwest, US	nr	Fm	10–100 g	F	nr	(Soto *et al.*, 2011b)
		2012	Central Mexico	40%	Fm	200–350 g	F	26	(Ortega *et al.*, 2016)
		2012-2013	Minas Gerais, Brazil	30–60%	Fm	2.04–109.3 g	F	≤22	(Leal *et al.*, 2014)
		2012-2014	Central, Northern, North-eastern, & Western, Thailand	10–60%	Fm	10–800 g	F	24–27	(Jantrakajorn and Wongtavatchai, 2016)
		2015	Minas Gerais, Brazil	nr	Fm	35.5–508.9	F	winter	(Assis *et al.*, 2016)
		2015	Santa Catarina, Southern Brazil	55%	Fm	61–407 g	F F	25	(Jatoba *et al.*, 2016)
		2015	West Parana & Sao Paolo, Brazil	5–50%	Fm	3–500 g		20–22	(Sebastião *et al.*, 2017)
		2015	Brazil	1.48–37.5%	Fm	36.2–117.2 g	F	22.2–24	(Assis *et al.*, 2017)

Fish Species		Year	Location	Mortality	Pop.	Size	Water	T (°C)	Reference
Red Nile tilapia hybrids	*Oreochromis* spp.,	2008	Central Thailand (Nam Sai Farms, Prachinburi) Southern Colombia	nr	Fm	150–200 g	F	nr	(Pradeep *et al.*, 2017)
		2009	Island of Oahu, Hawaii	50%	Fm	1–2 cm	F	26–27	(Iregui *et al.*, 2011)
		2010-2012	Kaneohe & Waimanalo	nr	Fm	nr	F	nr	(Soto *et al.*, 2013c) (Soto *et al.*, 2013c)
		2011	Oahu, Hawaii Oahu, Hawaii	nr	Fm	nr	F	nr	(Tamaru *et al.*, 2011)
		2012	Northern Europe	nr	Fm	8–10 cm	F	28	(Soto *et al.*, 2013c)
		2011-2012	Kanchanaburi, Thailand	60%	Fm	7–13 g	F	23±2	(Ramirez-Paredes, 2015)
		2013	Central, Northern, Northeastern, & Western,	50–60%	Fm	10 cm	F	26–27	(Nguyen *et al.*, 2016) (Dong *et al.*, 2016a)
		2012-2014	Thailand	10–60%	Fm	55–850 g	F	24–27	(Jantrakajorn and Wongtavatchai, 2016)
	O. mossambicus X *O. niloticus*	1992-1993	Taiwan	20–95%	Fm	30–639 g	F, B, S	15	(Chern and Chao, 1994)
	O. niloticus X *O. aureus*	1992-1993	Taiwan	20–95%	Fm	30–639 g	F, B, S	15	(Chern and Chao, 1994)
		2002	Florida	nr	Fm	5–20 cm	F	22–<25	(Mauel *et al.*, 2005)
		2002-2003	South Carolina	nr	Fm	nr	F	22–<25	(Mauel *et al.*, 2005)
		2013	Guangzhou, South China	40–50%	Fm	11 cm	F	20–25	(Lin *et al.*, 2016)
	O. mossambicus X *O. hornorum*	2001	Southern California	nr	Fm	20–90 g.	F	22–<25	(Mauel *et al.*, 2005)
Blackchin tilapia	*Sarotherodon melanotheron*	1994	Oahu, Hawaii	60%	Wi, Fm	nr		21.5–29.2	(Mauel *et al.*, 2003; Soto *et al.*, 2012b)
Blue tilapia	*O. aureus*	1992-1993	Taiwan	20–95%	Fm	30–639 g	F, B, S	15	(Chern and Chao, 1994)
		2010-2011	Island of Oahu, Hawaii	nr	Fm	nr	F	nr	(Soto *et al.*, 2013c)
Redbelly tilapia	*Tilapia zillii*	1992-1993	Taiwan	20–95%	Fm	30–639 g	F, B, S	15	(Chern and Chao, 1994)
Koilapia/Wami tilapia	*O. or T. hornorum*	1992-1993	Taiwan	20–95%	Fm	30–639 g	F, B, S	15	(Chern and Chao, 1994)
		2011	Kaneohe & Waimanalo Oahu, Hawaii	nr	Fm	nr	F	nr	(Tamaru *et al.*, 2011)
		2010-2011	Island of Oahu, Hawaii	nr	Fm	nr	F	nr	(Soto *et al.*, 2013c)
Unisexual broods	All-male *O. aureus*	1992-1993	Taiwan	20–95%	Fm	30–639 g	F, B, S	15	(Chern and Chao, 1994)
	all-male *O. mossambicus*	1992-1993	Taiwan	20–95%	Fm	30–639 g	F, B, S	15	(Chern and Chao, 1994)

Pop., populations; nr, not reported; Wi, wild; Fm, farm; F, freshwater; B, brackish-water; S, Sea/salt-water

According to Soto and Revan (2012), both temperature and salinity are important factors in the culturability and persistence of *Fno* in both sea- and fresh-water microcosms, as *Fno* is able to survive under starvation conditions in both sea- and freshwater microcosm for a period of 8 d, and culturable *Fno* persists for longer periods of time and at higher numbers in seawater. In additon, the pathogenic properties of the bacteria suspended in water microcosms appear to decrease after only 24 h and become non-infective after 2 d in the absence of the fish host (Soto and Revan, 2012). Furthermore, environmental temperature and fish size has been shown to have a significant effect on the development of piscine francisellosis in tilapia cultured in fresh-, brackish-, and sea-water and were found to play a substantial role in its epidemiology (in the outcome of *Fno* infection) in field studies, as well as in controlled laboratory studies (Soto *et al.*, 2012c, 2013c, 2014b; Klinger-Bowen *et al.*, 2016). Water temperature is important in the development of both specific and non-specific immunity in cold-blooded animals, such as fish. Tilapia will be growth optimally in intensive culture with the water temperature between 25 and 32°C; however, they can survive in a wider temperature range (15 to 40°C) (Josupeit, 2005; Soto and Revan, 2012).

Francisellosis outbreaks in cultured and wild tilapia commonly occur only during the cooler seasons of the year, from October to February (Chern and Chao, 1994; Jantrakajorn and Wongtavatchai, 2016) or October to April (Mauel *et al.*, 2005) and were not recorded during the warmer months (Mauel *et al.*, 2003; Leal *et al.*, 2014; Assis *et al.*, 2016; Lin *et al.*, 2016; Nguyen *et al.*, 2016). Similarly, natural outbreaks of francisellosis in intensively cultured tilapia seems to be restricted to certain water temperatures *e.g.* in Brazil the disease occurred when the water temperature was 22°C or lower (Leal *et al.*, 2014), ranging from 20 to 25°C in China (Lin *et al.*, 2016), and 24 to 27°C in the UK and Thailand where Nile tilapia fry were reared in an indoors recirculation system (Jeffery *et al.*, 2010; Jantrakajorn and Wongtavatchai, 2016). The clinical disease essentially disappeared when the water temperature rose to ≥24°C (Leal *et al.*, 2014), or decreased significantly from 24 to 29°C and disappeared when water temperature exceeded 30°C (Lin *et al.*, 2016) to 31°C (Jantrakajorn and Wongtavatchai, 2016).

Experimental infections have been reported in Nile tilapia cultured at 15 or 25°C and significantly higher mortality was observed at these temperatures compared to fish cultured at 30°C (Chern and Chao, 1994; Soto *et al.*, 2012c). Additionally, in Hawaiian tilapia the mortalities were shown to be much greater between 21.5 and 26.3°C with the first mortalities occurring on day 15 and these doubled almost daily thereafter; no mortalities were observed between 26.5 and 29.2°C (Mauel *et al.*, 2003). The importance of water temperature in *Fno* infection was demonstrated by measuring *Fno* level by PCR and real-time PCR (RT-PCR) following infection at different temperatures, and the results showed that the infection was more liable to occur in colder water with higher levels of *Fno* detected that at higher temperatures (Soto *et al.*, 2013c). This show that francisellosis is a seasonal disease (Lin *et al.*, 2016). Furthermore, smaller fish (fry, fingerlings and young adults, live weight ≤100 g) appeared to be more susceptible to *Fno* infection than larger fish (Soto *et al.*, 2009b, 2011a, 2012c, 2013c; Jeffery *et al.*, 2010; Iregui *et al.*, 2011; Leal *et al.*, 2014).

Koch's postulates have been fulfilled in controlled experiments with different routes of exposure. For instance, healthy Nile tilapia of 40—50 g were injected intraperitoneally with 0.1-mL of the homogenized spleen tissue supernatant (1:10) from infected tilapia at concentrations 10^{-1} to 10^{-4} dilution of supernatant and the mortalities were recorded up to 14 d (Chen *et al.*, 1994). Two intramuscular (IM) challenge tests were also performed with differently sized Nile tilapia. The first experiment was performed with 30 g fish receiving an injection of 0.05-mL of the splenic supernatant from naturally infected tilapia at 10^{-5} to 10^{-6} $TCID_{50}$ mL^{-1}. These fish were maintained at 15°C and the mortalities were recorded for 21 d. A second IM challenge test subsequently performed on 50–60 g tilapia using 0.2-mL of the diluted crude supernatant (10-fold) from the spleen of dead fish from initially IM challenge, and the clinical signs of francisellosis were observed (Chern and Chao, 1994). A co-habitation challenge method has also been demonstrated for *Fno* in Nile tilapia (30 g) by keeping uninfected fish with naturally infected fish in the same tank, and this was performed at 15 and 30°C, and the appearance of disease signs and mortalities were examined for up to 58 d, as well as in 30 to 60 g Blackchin

tilapia *Sarotherodon melanotheron* that were observed for 23 d at 22°C (Chern and Chao, 1994; Mauel *et al.*, 2003).

Experimental infection with different administration routes have also been demonstrated to be effective in inducing disease using various bacterial isolates of *Fno* (Table 3.2). Meanwhile, differences in the susceptibility of various tilapia species to infection with *Fno* have been observed during both natural outbreaks and controlled experimental infections. In a previous study, infectivity of various species of tilapia with spleen supernatant from naturally infected tilapia was observed by both IM and cohabitation routes, including Blue tilapia, Mozambique tilapia, Redbelly tilapia, Red tilapia hybrids *i.e. O. mossambicus* X *O. niloticus* and *O. niloticus* X *O. aureus*, and all male *O. aureus* and *O. mossambicus* (Chern and Chao, 1994). The molecular and isolation methods confirmed that *Fno* was prevalent in different cultured species of tilapia and their hybrids, which included Mozambique tilapia *O. mossambicus* (Peters), Koilapia *O. urolepis hornorum* (Trewavas), Blue tilapia *O. aureus*, and Nile tilapia *O. niloticus* hybrids obtained from 20 different geographical locations throughout the island of Oahu Hawaii (Soto *et al.*, 2013c). Ramirez-Paredes (2015) described different susceptibility to *Fno* STIR-GUS F2f7 by IP in red and wild-type Nile tilapia *O. niloticus* L fingerlings (6–7 months, 11±4 g) that had been recovered from infected red Nile tilapia in Europe. Recently, an immersion challenge was performed in Mozambique tilapia *O. mossambicus*, Blue tilapia *O. aureus* and Koilapia/Wami tilapia *O. urolepis hornorum* (10 g in weight for each species) to *Fno* at 10^3 CFU mL^{-1} for 1 h under static conditions and comparing two populations of each tilapia species *i.e.*, 'resident fish' defined as fish maintained in tanks since the initial challenge and maintained for 25 d, and 'naıve fish' defined as fish added to tanks once temperature in water reached <26°C at 21 weeks post-challenge and maintained for 4 weeks (Klinger-Bowen *et al.*, 2016). The results showed that Mozambique tilapia appears more susceptible to piscine francisellosis than Koilapia and Blue tilapia (Klinger-Bowen *et al.*, 2016).

Table 3.2 Confirmed of different experimental challenge methods to inducing francisellosis using various strains of *Fno* isolates

Tilapia species	*Fno* isolate	Origin of isolate	Route of exposure	Purpose	T (°C)	For	Reference
Nile tilapia (length 9 cm & weight 18.9 g)	LADL 07-285A	from cultured Nile tilapia suffering a natural outbreak of francisellosis in freshwater ponds in Costa Rica	0.1-mL (~10^7 CFU $fish^{-1}$) by IP 0.1–0.2 mL (~10^7 CFU $fish^{-1}$) by gill spraying (GS)	To inducing francisellosis and analysed by a complete clinical, bacteriological & histopathological examinations, and PCR	23–25	10 d	(Soto *et al.*, 2009a)
Nile tilapia (length 9 cm & weight 18.9 g)	LADL 07-285A		IP & immersion challenges (treatment for 3 h) with serial dilutions of 2.3 to 10^{-9} and 10^{-1} to 10^{-8}, respectively		23–25	40 d	(Soto *et al.*, 2009b)
	LADL 07-285A and attenuated *Fno* Δ*iglC** Strains		3 x 10^8 or 1.5 x 10^8 CFU $fish^{-1}$ by IP and 3.73107 or 1.8 x 10^7 CFU^{-1} mL by bath challenge for 3 h		23–25	30 d	(Soto *et al.*, 2009b)
Nile tilapia (mean weight 72 g)	wild type *Fno* LADL 07-285A and an attenuated *Fno* strain containing a mutation in the intracellular growth locus C (*iglC*) gene (essential to intracellular survival)		By bath challenge at concentration 10^3 CFU mL^{-1} for both bacteria	To comparing the distribution of bacteria to multiple organs, associated with lesion development after 3, 24, 48, 96, and 192 h post infection by RT-PCR & histopathology	25	8 d	(Soto *et al.*, 2013b)
Nile tilapia (average weight of 45 g)	*Fno*-12	From infected Nile tilapia cage farmed in Brazil	IP injection with a 0.1-mL, corresponding to a final dose of 10^6 CFU $fish^{-1}$	To inducing francisellosis and analysed by a complete clinical, bacteriological and qPCR	26	15 d	(Leal *et al.*, 2014)
red Nile tilapia (average length 11 cm)	*Fno* gz201301	Isolated from infected red tilapia spleen tissues in China.	IP injected with 0.2-mL of *Fno* and supernatant filtered from infected liver homogenate at 1.0 x 10^8 CFU mL^{-1}	To inducing francisellosis and analysed by a complete clinical, bacteriological and histopathological tests, & PCR	20–25	30 d	(Lin *et al.*, 2016)
red tilapia (average 10 cm in length)	*Fno* VMCU-FNO131	From infected red tilapia cultured in floating cage systems in Thailand	IP injected with 0.1-mL of different doses of 1.08 x 10^6 and 1.08 x 10^4 CFU mL^{-1}	To inducing francisellosis and analysed by complete clinical, bacteriological and histopathological examinations	26–27	21 d	(Nguyen *et al.*, 2016)
red tilapia *Oreochromis* sp	*Fno* VMCU-FNO131		IP injected with 0.1 mL, corresponding to 1.5 x 10^6 CFU $fish^{-1}$	To inducing francisellosis and & analyzed by duplex PCR, histological assessment and *in situ* hybridization	nr	21 d	(Dong *et al.*, 2016b)
Nile Tilapia (body weight 90±12 g)	*Fno*	From infected Nile tilapia cultured in ponds and net cages in Thailand	IP challenge with 0.1-mL *Fno* suspension at a dose 0.8 x 10^5 CFU $fish^{-1}$	Splenic tissue was collected at 6, 24, 48, 72, and 96 hpi to look at the pro- and anti-implamatory immune gene expressions	25–28	4 d	(Jantrakajorn and Wongtavatchai, 2016)

In order to screen bioactive algal compounds *in vivo*, and to determine their potential to control francisellosis, an immersion challenge model needed to be first developed. This route of challenge is preferred to injection (either by IP or IM), as immersion (and co-habitation challenge) better resemble the natural infection process. At present, no information exists on the susceptibility of Nile tilapia *O. niloticus* L. to *Fno* STIR-GUS-F2f7 by immersion challenge. In addition, although some research has been performed on immune response to infection following injection challenge, there is little information so far following immersion challenge. Thus, the aim of this Chapter was to develop an immersion challenge model for *Fno* STIR-GUS-F2f7 to induce francisellosis so that this could then be used to screen bioactive algal compounds *in vivo* (Chapter 4). External and internal gross pathology and pathological changes in the tissues were evaluated by histopathological and Gram Twort examination, and immunohistochemistry. Meanwhile, the relative susceptibilities of two commonly farmed Nile tilapia strains, homo gold and wild-type to *Fno* were determined after immersion challenge.

3.2 Materials and methods

3.2.1 Bacterial strain

Fno STIR-GUS-F2f7 was recovered from frozen stock, cultured and prepared for the challenge as described in Chapter 2, Sections 2.2.1.1 to 2.2.1.3. To fulfill Koch's postulates the bacterium was passaged through a susceptible host, reisolated and then used to infect a new host and a dose response to infection demonstrated.

3.2.2 Bacteria standard curve

The number of bacteria present at different absorbance units of diluted *Fno* were determined to produce a standard curve. The A_{600} of cell suspensions ranging from approximately 0.1 to 1.0 AU was determined and the numbers of viable cells were expressed as CFU mL^{-1}. The bacteria were subcultured on CHAH, MHB cultured was prepared, incubated to the middle logarithmic phase growth, collected and washed in PBS (Chapter 2, Sections 2.2.1.1 and 2.2.1.3).

The crude cell suspension was subsequently adjusted spectrophotometrically to an initial A_{600} of 1.0 AU with PBS. The process was repeated to prepare cell suspensions with A_{600} of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9 AU. Bacterial CFU per mL values were verified for each cell suspension by drop count method on CHAH according to Chapter 2, Section 2.2.1.2. Colonies were counted for each dilution of each cell suspension. Mean CFU mL^{-1} were calculated for each bacterial suspension and a standard curve produced by plotting the actual A_{600} against CFU mL^{-1}. The standard curve was used to determine bacterial concentrations in cell suspensions.

3.2.3 Experimental animals

Nile tilapia homo gold and wild type fingerlings were sourced and cultured as described in Chapter 2, Section 2.2.2. Fish were cultured from larvae for 7 months to get the size required for the experimental challenge. Prior to use for experimental challenges, a sub-sample of the population (10 fish) were confirmed as negative for *Francisella* spp., by complete clinical, bacteriological (culture of head-kidney and spleen on CHAH) and molecular PCR analysis (Soto *et al.*, 2009a). Head kidney and spleen tissues were sampled from another 5 fish for histological analysis (haematoxilyn and eosin/H & E, and Gram Twort staining, and immunohistochemistry/IHC) as negative control (naïve fish). Tilapia weighing 10 ± 4 g were used for the subsequent challenge studies. Fish were transferred into a flow-through system in the Aquatic Research Facility (ARF) (Institute of Aquaculture, University of Stirling, UK) where they were subjected to bacterial passage or challenge by immersion.

3.2.4 Bacterial passage

Prior to performing any challenge studies, bacteria were passaged in Nile tilapia homo gold to enhance and verify their virulence properties for subsequent challenge trials, as the isolate had been frozen in storage for a long period of time. The bacterial inocula was prepared as previously mentioned in Section 3.2.1. Passage was performed using one fish, and the passage was conducted twice. Nile tilapia homo gold was used for the passage and

anesthetized with 2 or 3 mL of 10% benzocaine (Sigma Aldrich, UK) in 1 L of water (from the same tank where the fish was reared) in a bucket, prior to the challenge. Then, fish was injected IP with a high concentration of live bacteria (0.1-mL of a cell suspension in a sterile PBS at an A_{600} of 2.460 for the first passage and 0.607 for the second passage) and returned to the initial tanks. After injection, fish were monitored at least four times per day and after 3 d, or as soon as first clinical signs appeared or abnormal / "nonresponsive" behaviour (no reaction when pushing the fish with a small net) was apparent, the fish were sacrificed as describe in Chapter 2, Section 2.2.3.3. During necropsy, the sacrificed fish were sampled for bacterial recovery, as described in Section 3.2.6. The isolates recovered from the head kidney and spleen from the first and second passages were purified by sub-culturing uniform colonies consistent with *Fno* morphology onto fresh CHAH agar plates using the culture conditions as described previously in Chapter 2, Section 2.2.1.1. Primary identification tests were applied to determine the identity of the colonies and MHB was used to propagate isolate for the experimental challenge study, and the glycerol frozen stocks were prepared for later use (as Chapter 2, Section 2.2.1.1).

3.2.5 Experimental design of the susceptibility Nile tilapia homo gold and wild type to *Fno* infection

A dose-dependent experimental challenge was performed using *Fno* to compare survival rates of different strains of Nile tilapia when infected with different bacterial doses and to provide a description of the clinical signs and the pathogenesis of the infection. Sixty fish of each Nile tilapia homo gold and wild type were selected, divided into three experimental groups of 20 fish and placed into 3.5-L aquarium tanks (2.8 L water) each with an individual filter and air stone for aeration per tank, in a flow-through system with UV-treated water (Figure 3.1). This species naturally shows hierarchical aggression, and therefore strong aeration and a high stocking density were used to prevent this behaviour. The fish were acclimated for 10 d prior to the immersion challenge. Water temperature was maintained at 23±2°C to replicate the natural conditions at which the disease occurs (Colquhoun and Duodu, 2011), and a 12 h light: 12

h dark cycle was operated. Fish were fed with a commercial fish feed at 3% of body weight twice daily during acclimation and only once daily during infection as the fish lost appetite.

Immersion challenge was carried out in the same tank (3.5 L) as above but with 2.2 L of static water by turning off the water inflow on each tank and supplying only aeration (one air stone per tank). The *Fno* isolate from infected head kidney from the second passage after the Id tests (Section 3.2.4) was cultured, collected and washed in PBS, and drop counts performed on CHAH as described Chapter 2, Section 2.2.1.2. Three bacteria concentrations were used for the challenge using a *Fno* suspension with an A_{600} of 0.4 (~1 x 10^9 CFU mL^{-1}) such that 2.2, 22 or 220 mL was added to the tank water to give final concentration at 1 x 10^5, 1 x 10^6, and 1 x 10^7 CFU mL^{-1}, respectively. Each group of Nile tilapia received its respective treatment for 3 h, as described by Soto *et al*. (2013). After 3 h the water inflow was turned on and the fish were maintained for 40 d. Fish were fasted for 24 h prior to the challenge and 12 h following bacteria addition and then once daily feeding resumed. During the experiment, fish were monitored at least four times per day by observing clinical signs, any mortalities were recorded. Dead and moribund fish were removed daily, and moribund fish were euthanized, then examined for gross signs and aseptically sampled for recovery of bacteria as described in Section 3.2.4. At the end of the challenge (40 d), all surviving fish were sacrificed, and complete necropsies were performed on all fish. Head kidney and spleen from all freshly dead, moribund and surviving fish were sampled for bacterial recovery, histopathological examination, Gram Twort staining and immunohistochemistry, and stored in 95% ethanol (Fisher chemicals, Thermo Fisher Scientific, UK) for subsequent PCR. The Lethal Dose causing 50% mortality (LD_{50}) at 40 d post infection (dpi) was calculated according to the following formula (Reed and Muench, 1938; Ramakrishnan, 2016):

Log^{10} 50% end point dilution = Log^{10} of dilution showing mortality next above 50% – (difference of logarithms × logarithm of dilution factor)

$$\text{Difference of logarithms} = \frac{[(\text{mortality at dilution next above 50\%}) - 50\%]}{[(\text{mortality next above 50\%}) - (\text{mortality next below 50\%})]}$$

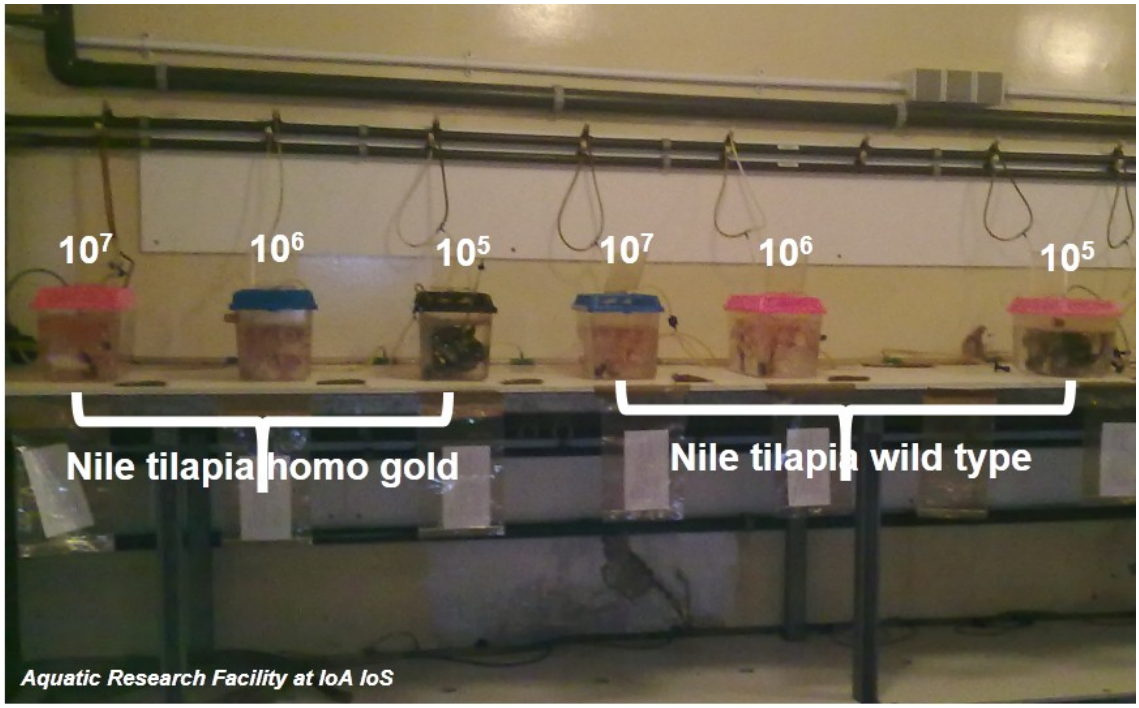

Figure 3.1 Experimental design of the susceptibility of two different strains of Nile tilapia, homo gold and wild type. Three tanks were used (3.5 L each tank and 2.8 L water) for three different concentration of *Fno* STIR-GUS-F2f7 (10^5, 10^6 and 10^7 CFU mL^{-1}) each tilapia strain and maintained for 35 d in Aquatic Research Facility Institute of aquaculture, University of Stirling.

The experiments were approved by the Animal Welfare and Ethical Review Body, University of Stirling. All animal experiments were conducted at the Institute of Aquaculture (University of Stirling, UK) in accordance with the Animals (Scientific Procedures) Act 1986 and complied with local institutional regulations. These experiments were performed without replicate groups of fish or control tanks, as stipulated in the Project Licence, owing to the nature of the experiment.

3.2.6 Bacterial recovery

Bacteria were recovered from head kidney and spleen tissues as described previously (Petersen *et al.*, 2004; Ramirez-Paredes, 2015). Briefly, head kidney and spleen samples were collected aseptically by dissection of tilapia exhibiting signs of disease and a small part of each tissue was homogenised in a 1.5-mL Eppendorf tube (APEX Tough Microtube; Alphalaboratories, Hampshire, UK) containing 100 µL of PBS. The cells were lysed by grinding 10 to 20 times with a 1.5-mL microtube pestle plastic tissue homogenizer (Sigma Aldrich, UK) (Petersen *et al.*, 2009; Ahmad *et al.*, 2010). Twenty microliters of each homogenate were put onto a CHAH plate and streaked using a 10 µL sterile plastic inoculating loop (Nunc™; Thermo Scientific™, UK) to permit colony isolation. Inoculated plates were sealed using parafilm and incubated at 28˚C. The plates were checked daily for up to 10 d especially after 72h of inoculation and then examined for the presence of bacterial colonies (*Fno* colonies were expected to appear at 3 d). Once colonies of *Fno* were observed, the isolate was purified, identified and stored as glycerol frozen stock (as described at Chapter 2, Sections 2.2.1.1).

3.2.7 Histopathological analyses

At the end of the experiment all surviving fish were killed and tissue samples, specifically head kidney and spleen, were removed and fixed in 10% neutral buffered formalin (NBF) (v:v) for a minimum of 24 h: 10% NBF contained sodium dihydrogen phosphate (monohydrate) and disodium hydrogen phosphate (anhydrous). Naïve fish were used as controls. Tissues were

prepared for sectioning, and H & E staining was performed according to the standard protocols of the Histology Laboratory at the Institute of Aquaculture, University of Stirling. Briefly, tissue samples were trimmed to a suitable size using a scalpel and placed into standard tissue cassettes for routine overnight processing with a tissue processor (tissue processor2000; Thermo Scientific, Shandon Citadel, USA), before the tissues were embedded in paraffin wax (EG1160 Leica Histoembedder; Leica Biosystems, Nussloch, Germany). The surface layer of the wax block was trimmed with a microtome (Leica RM 2255; Leica Biosystems, Nussloch, Germany) to expose the complete surface of the tissue and then soaked in water for at least 30 mins prior to cutting. Four micrometer-thick sagittal sections were cut, floated on a waterbath (Raymond A Lamb, London, UK), mounted onto clean glass slides (Solmedia® Supplying Science, Shrewsbury, UK) and transferred into an oven (Windsor Incubator; Sandrest, England, UK) at 60˚C overnight or for at least 1 h before staining.

Sections were deparaffinised through a series of 100% xylene (Fisher chemicals, Thermo Fisher Scientific, UK) for 3 and 2 mins (twice), rehydrated in absolute ethanol for 2 mins, placed into methylated spirit (Fisher chemicals, Thermo Fisher Scientific, UK) for 1 min, washed in running water for 30–60 secs, stained with Mayer's haematoxylin "Z" (Cellpath, Newton, UK) for 5 mins, then washed in water again. Each section was dipped quickly three times in 1% acid alcohol (contained 1 L methylated spirit and 10 mL hydrochloric acid), washed in running water, counterstained with eosin and washed again in water. Eosin was contained 8 parts of 1% eosin (in distilled water) and 1 part of Putt's eosin (in a mixture of distilled water, potassium dichromate, ethanol and picric acid) (Cellpath, Newton, UK) (8:1). Dehydration continued through a series of absolute ethanol for 2 and 1 mins (twice), then the slides were cleared with xylene for 5 mins before mounting with Pertex® (HistoLab® Products Ab, Gothenburg, Sweden), and drying in a fume hood for at least 10-15 mins. Then slides were examined using a light microscopy (upright metallurgical microscope BX53M) and images were collected with a digital camera (SC100; Media Cybernetics) and cellSens 1.17 software (Olympus, Japan). Duplicate sections were subjected to immunohistochemistry and Gram Twort staining to aid the localisation of the bacteria in the tissues.

3.2.8 Immunohistochemistry

The presence and distribution of *Fno* in infected and naïve head kidney and spleen tissues of Nile tilapia was determined by IHC. Formalin-fixed paraffin-embedded tissues were sectioned at a thickness of 4 µm and heat fixed in the oven at 60˚C for at least 1 h as Sections 3.2.7, and then subjected to IHC identification according to standard protocols in the Aquatic Vaccine Laboratory at the Institute of Aquaculture, University of Stirling. Sections were deparaffinised and rehydrated through a series of xylene (5 mins twice), a series of ethanol (*i.e.*100% for 5 mins and followed by 70% for 5 mins) and then rinsed with distilled water. The tissue section was marked with a ring around it using a PAP pen to allow small volumes of liquid to be added onto the section. Sections were pre-treated with 3% hydrogen peroxide (H_2O_2) (Fisher Scientific, Thermo Fisher Scientific, UK) in methanol (Fisher chemicals, Thermo Fisher Scientific, UK) (v:v) for 10 mins to block endogenous peroxidase activity and then washed three times in PBS. All incubations were performed in a humidified chamber at room temperature (RT) (22˚C) to keep the sections moist. Non-specific binding of the secondary antibody was blocked by incubation with normal goat serum (Sigma Aldrich, UK) diluted 1:10 in PBS for 15 mins. The serum was discarded, slides tapped dry and then rabbit antisera against *Francisella noatunensis* subsp. *noatunensis* (NCIMB 14265 serum (diluted 1:1200 in PBS) was added to the sections and incubated for 1 h. Dilutions of 1:100, 1:500, 1:1000, 1:1400, 1:2000, 1:5000, 1: 10,000, 1: 20,000, and 1: 30,000 were also evaluated to optimise the IHC method. The primary antibody was kindly provided by Dr. Duncan Colquhoun (Norwegian Veterinary Institute [NVI], Oslo Norway). Slides were then washed three times with PBS.

The samples were then incubated for 30 mins with goat anti-rabbit antibody (immunoglobulin G [IgG]) conjugated to horseradish peroxidase (HRP) (Sigma Aldrich Inc., USA) diluted 1:200. Slides were washed washed again, as described before, and after incubation for 10 mins with permanent chromogen (Immpact™ DAB peroxidase substrate; Vector Laboratories, Inc, USA), the reaction was stopped by rinsing the sections in running tap water. Negative controls consisted of infected tissues and naïve fish which were incubated with

PBS and/or primary antibody and PBS, respectively. The slides were counterstained with Mayer's haematoxylin for 4 mins, followed by rinsing in tap water until the water ran clear. The slides were dehydrated in a graded series of ethanols (70% for 5 mins followed by 100% for 5 mins) and cleared in xylene (5 mins twice) prior to adding a coverslip with Pertex mounting media. Slides were examined using light microscopy, as described in Section 3.2.7. Positive staining in tissues appeared brown in colour.

3.2.9 Gram Twort

Gram Twort staining was performed according to the methods of Twort (1924) and Ollet (1947) as described by Bancroft and Marilyn (2008) with some modification**.** Formalin-fixed paraffin-embedded tissues for Gram Twort were prepared, trimmed, cut, heat fixed, deparaffinised, rehydrated and washed as described in Sections 3.2.7. Slides were stained with 2% Lilie's crystal violet that comprised 10 g crystal violet (Merck Chemical, Germany) in 100 mL 95% ethanol and 4 g ammonium oxalate (Sigma Aldrich, UK) in 400 mL distilled water, for 1 min and rinsed in running tap water. The slides were treated with 0.4% lugol's iodine, which contained 1 g iodine (Fisher Scientific, UK) and 2 g potassium iodide (BDH; Laboratories Supplies, England) in 100 mL water, for 1 min, rinsed in water, and drained before flooded with acetone (Fisher chemicals, UK) for 2–5 secs. After each of these steps the slides were rinsed in running water and counterstained with Twort's stain in a closed Wheaton coplin staining jar (S5516-6EA; Sigma Aldrich, UK) for 5 mins, then rinsed in water again. Twort's stain was divided into two stain solutions: a stock solution contained 1 part of 0.2% neutral red (Sigma Aldrich, USA) and 9 parts 0.2% fast green (Acros Organic; Thermo Fisher Scientific, UK) in 95% ethanol (1:9) and a working solution prepared by diluting the stock solution in distilled water (1:3). Each section was dehydrated, cleared and mounted, as described in Section 3.2.7. Slides were examined using an upright metallurgical microscopy, as in Section 3.2.7. Gram-positive and negative organisms stained blue or black and red or pink, respectively, though nuclei presented in red and varying shades of green for collagen, red blood cells and cytoplasm.

3.2.10 PCR analysis

3.2.10.1 DNA extraction and 16S rRNA gene amplification

PCR was conducted on spleen samples fixed in ethanol. *Francisella* genus DNA can be amplified by PCR (Forsman *et al.*, 1994). Genomic DNA (gDNA) was extracted from spleen tissues from each strain of naïve and infected Nile tilapia, an *Fno* culture and sterile PBS was used as a negative control. Healthy spleen tissue samples were collected prior to experimental infection (Section 3.2.3), while infected tissues were collected at 40 d after *Fno* infection.

A. Ethanol fixation of tissues

Naïve and infected tilapia spleen samples with or without gross signs of francisellosis were collected specifically in areas where white multifocal nodules were visible. A small piece of tissue (approximately 300 mg) was immediately fixed and stored in a 7-mL polystyrene bijou containers (Sterilin™; Thermo Fisher Scientific, UK) with 5 mL of 95% ethanol in a tissue:fixative proportion of 1:5 (weight per volume, w:v) and kept at RT prior to DNA extraction.

B. Bacteria preparation

Fno was recovered from frozen glycerol stock and cultured on CHAH and then MHB as described in Chapter 2, Sections 2.2.1.1 and 2.2.1.2. After 19.5 h of incubation on an orbital shaker, a bacteria suspension was prepared and mixed by vortexing and then 1.5 mL of the suspension was transferred into sterile Eppendorf tubes. The bacterial pellet was collected by centrifugation at 13,000 x g for 2 mins at RT (Sigma 1-14 microcentrifuge; SIGMA Laborzentrifugen GmbH; Osterode am Harz, Germany). The supernatant was discarded and a further 1.5 mL of broth culture was added and centrifuged exactly as before. This step was repeated if necessary to obtain a sufficient bacterial pellet. Before continuing with DNA extraction, the bacteria pellet was washed by adding 1 mL of NaCl-Tris-EDTA (STE) buffer (0.1 M NaCl (Sigma-Aldrich, UK), 10 mM Tris hydrochloride (Fisher chemicals, Thermo Fisher Scientific, UK), and

1 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, UK], pH 8.0), pipetting up and down, and centifuging as before.

C. Genomic DNA extraction

Extraction of gDNA from the spleens of dead, moribund and surviving fish was performed using a combine the Realpure Genomic DNA Extraction Kit (Durviz S.L.; Spain) and SSTNE DNA extraction protocol (unpublished: pers. comm. Dr Kerry Bartie).

Briefly, each spleen sample (approximately 100 mg) or a large *Fno* pellet was resuspended in 300 µL lysis solution (SSTNE buffer) and 3 µL of 10 mg mL^{-1} Proteinase K (Bioline, UK). Samples were mixed by flicking several times and centrifugation at 17,000 x g for 1 min at RT (IEC Microlite Centrifuge; Thermo Electron Corporation, Massachusetts, USA**)** to ensure they were covered completely by the solution, and then incubated overnight at 55°C on a heatblock to allow lysis to occur (Eppendorf Thermomixer R; QBDI Grant Instrument Cambridge Ltd., UK).

Samples were incubated at 70°C for 15 mins to inactivate the Proteinase K, and then cooled to RT. Five microliters of 2 mg mL^{-1} RNase (Sigma-Aldrich, UK) was added to each sample and the tube contents were mixed by invertion and centrifugation at 17,000 x g for 1 min at RT, and then incubated at 37°C for 1 h, before cooling to RT. In the protein precipitation step, a further 180 µl of protein precipitation solution was added to each sample, mixed by vortexing at high speed for 30 secs and incubated at 4°C for 15 mins. Precipitated protein formed a white pellet at the bottom of the tube after centrifugation at 17,000 x g for 10 mins at RT. The supernatant containing the DNA was pipetted into a fresh sterile 1.5 mL eppendorf, and this step was repeated, and the supernatant retained and transferred to the same tube as the first supernatant. An equal volume of isopropanol (300 µL; Fisher chemicals, Thermo Fisher Scientific, UK) was added to the supernatant, mixed by inversion (4–6 times), and centrifuged at 17,000 x g for 10 mins at RT. The supernatant was removed by decanting and pipetting.

The resulting DNA was washed in 1 mL of 70% ethanol, inverted several times, centrifuged for 2 secs at RT, and then incubated at RT for 30 mins to gently rehydrate the DNA. Tubes were placed onto a paddle mixer (VWR Tube Rotator Plus, USA) for 1 h, and then centrifuged at 17,000 xg for 10 mins at RT. The ethanol was poured off and replaced with a further 900 µL of 70% ethanol, and then incubated at RT for 1 h on the paddle mixer. After this incubation, tubes were centrifuged at 17,000 x g for 10 mins at RT. The supernatant was carefully removed by pouring off, followed by centrifugation for 2 secs at RT, and then residual 70% ethanol was removed by pipette. The DNA pellet was air-dried for at least 1 h at RT. To dissolve the DNA, 50–100 µL of the kit hydration buffer (depending on pellet size) was added to each sample. The sample was centrifuged briefly and incubated for up to 1 h at RT to slowly reconstitute the DNA pellet (or overnight at 4°C or on ice). Samples were kept at 2–8°C or at -20°C to -80°C for long-term storage.

Extracted DNA was quantified by NanoDrop according to manufacturer's instructions (ND-1000 spectrophotometer, NanoDrop Technologies Inc., Montchanin, DE, UK, and resuspended in Milli-Qure Water (Millipore Milli-Q Biocel "ZMQP60F01" Ultra-Pure Water Purifier, UK) to 50 ng μL^{-1}. DNA samples were mixed by brief vortexing and short centrifugation and measured for concentration and purity on the NanoDrop. The purity of DNA was assessed by the ratios of absorbance A_{260}/A_{280} and A_{260}/A_{230}. The value of wavelength reading 260 nm allows calculation of DNA concentration in the templates, meanwhile the value of 280 nm showed the amount of protein in the samples. Absorbance at 230 nm is the result of other contamination. A A_{260}/A_{280} ratio of approximately 1.8–2.0 represents good quality DNA, while A_{260}/A_{230} expected values are commonly in the range of 2.0–2.2.

D. Gel electrophoresis

All DNA samples were run on a 1% (w/v) agarose gel (Bioline, UK) containing 0.1 µg mL^{-1} ethidium bromide (Sigma-Aldrich, UK) in 0.5% Tris–Acetic–EDTA (TAE) buffer (0.2 mM Tris, 0.1 mM acetic acid [Fisher chemicals, UK], and 0.005 mM EDTA) against a known amount of lambda *Hin* dIII marker (New

England Biolabs, UK) to confirm the quantity and integrity of the DNA. Each well was loaded with 1 µL of DNA sample, and 0.4 µL 6X loading dye (Thermo Scientific, Loughborough, UK); 0.4 µL 1 Kb (kilo base pair) DNA ladder (BioLabs Inc., UK) was added to the first well. Electrophoresis was performed at 80 V and 140 mVa for 45 mins in wide Mini-Sub® cell GT electrophoresis cell (Bio-Rad, UK) with energy supply EPS 301 (GE healthcare Biosciences AB, Sweden). The DNA was visualized under UV illumination (Bio Imaging INGENIUS, Syngene, UK).

3.2.10.2 Polymerase chain reaction

PCR was conducted using the *Francisella* genus specific-primers targeting the 16S rRNA sequence (F11, 5´-TAC CAG TTG GAA ACG ACTGT-3´ and F5, 5´-CCT TTC TGA GTT TCGCTC C-3´) (Forsman *et al.*, 1994). Tissue sampled from infected Nile tilapia homo gold and wild-type spleens yielded the appropriately amplified PCR products of 1150 bp.

Firstly, to amplify the 16S rRNA gene, 1 µL of the DNA template at 50 ng μL^{-1} was added to 25 µL of reaction mixture, which consisted of 12.5 µL of 2X ReddyMix PCR master mix (1.5 mM $MgCl_2$) (Thermo Fisher Scientific, Inc., UK), 0.2 µL of each primer (forward and reverse primers) at 10 ng μL^{-1}, and 11.1 µL Milli-Qure water. PCR amplification was run on a Biometra TGradient Professional thermocycler (Goettingen, Germany) using the following protocol: denaturing for 3 mins at 95°C; 35 cycles of 94°C for 30 secs, 60°C for 60 secs, and 72°C for 60 secs; and an extension period at 72°C for 5 mins. DNA extracted from the spleen of naïve Nile tilapia for each strain and a no template control (PBS), and a reaction containing DNA extracted from *Fno* STIR-GUS-F2f7, were also performed and these acted as negative and positive controls, respectively. Amplifiied of PCR products were visualized by gel electrophoresis as described in Section 3.2.10.1.D.

3.3 Results

Data obtained during the experimental immersion challenge in Nile tilapia homo gold and wild type with *Fno* STIR-GUS-F2f7 were: 1) *Fno* standard curve, *Fno* recovery from tilapia passages and immersion challenge; 2) susceptibility of Nile tilapia to *Fno* infection; 3) histopathology, IHC and Gram Twort staining; and 4) PCR analysis.

3.3.1 *Fno* standard curve

A bacterial standard curve of viable CFU against absorbance at 600 nm was produced for *Fno* STIR-GUS-F2f7 with an R^2 value of 0.988 (Figure 3.2). No bacterial growth was detected in the MHB negative control sample. Based on the standard curve results, an A_{600} value of 0.4 in PBS yielded an *Fno* concentration of ~1.03 x 10^9 CFU mL^{-1}. All the samples of the *Fno* isolate at each AU point on the standard curve were confirmed by primary id tests: results indicated the *Fno* was Gram-negative, cocco-bacilli, non-motile, catalase positive and oxidase negative (as Chapter 2, Section 2.3.1).

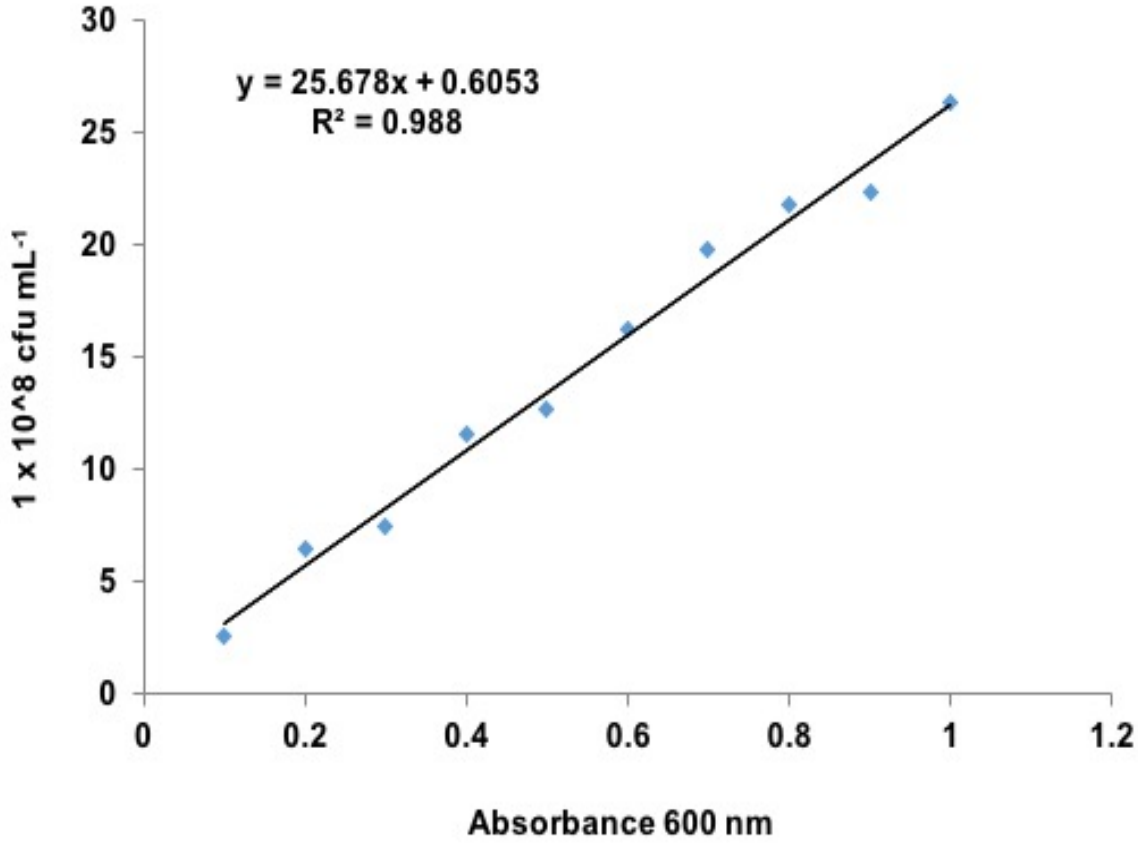

Figure 3.2 Scatter graph plotting A_{600} of *Fno* STIR-GUS-F2f7 cell suspensions against viable CFU mL^{-1} showing a significant correlation existed between these parameters.

3.3.2 *Fno* recovery from passage prior to experimental immersion challenge

Fish died at 24 h during the first passage of *Fno* STIR-GUS-F2f7 in both Nile tilapia strains, probably due to the high dose, while during the second passage with a lower dose the fish died at 48 h post-inoculation. Samples were taken onto CHAH plates from the first and second bacterial passages from these fish and were overgrown with contaminants after 24 h cultured at 28°C, with *Fno* colonies observed after 72 h. The contaminants were mostly white milk or brownish in colours, while *Fno* were greyish and/or greenish, and with bigger colonies compared to *Fno* (Figure 3.3). All purified bacteria samples from head kidney and spleen of each tilapia strain after the first and second passages were confirmed to be *Fno,* according to Chapter 2, Section 2.2.1.1.

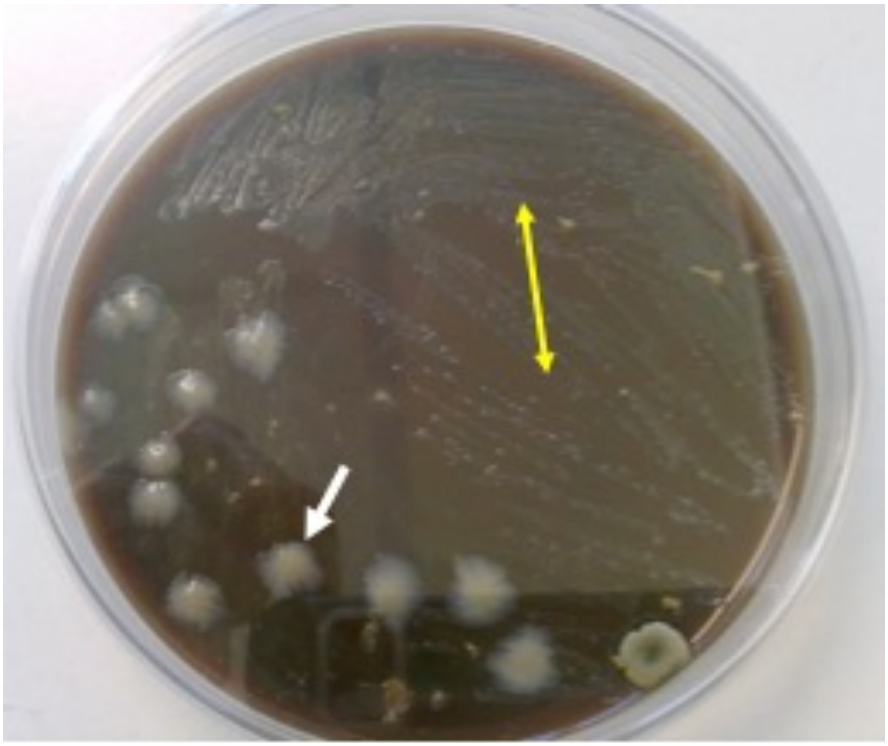

Figure 3.3 *Fno* recovered at 2nd passages from infected anterior kidney homogenate Nile tilapia homo gold 48 hpi and after 72 h incubation at 28°C on CHAH (10^{-2} dilution on PBS). *Fno* was greenish in colour (double yellow arrow), and some bacterial contaminants were also present (white arrow).

3.3.3 Susceptibility of Nile tilapia to *Fno*

3.3.4.1 *Fno* dose response

An immersion challenge was performed successfully in both Nile tilapia strains, with an *Fno* dose-dependent mortality response observed. Cumulative percentage mortality levels of 10, 50, and 65, and 35, 30, and 75 were obtained at *Fno* concentrations of 1.03 x 10^5, 1.03 x 10^6 and 1.03 x 10^7 CFU mL^{-1} for homo gold and wild-type tilapia, respectively after 40 dpi (Figure 3.4 and 3.5). Immersion challenge with 1.03 x 10^7 CFU mL^{-1} in Nile tilapia homo gold and wild type caused rapid mortality, with a cumulative mortality of 55% within 10 and 14 dpi, respectively. Most deaths occurred between day's 5 to 21 and 7 to 31 dpi for homo gold and wild-type, respectively. Based on the percentage cumulative mortalities, the medium lethal dose of 50% mortality (LD_{50}) observed from the immersion challenged for both Nile tilapia strains infected with *Fno* STIR-GUS-F2f7 at 40 dpi was 1.03 x $10^{6.7}$ and 1.03 x $10^{6.4}$ CFU mL^{-1} for the homo gold and wild-type, respectively.

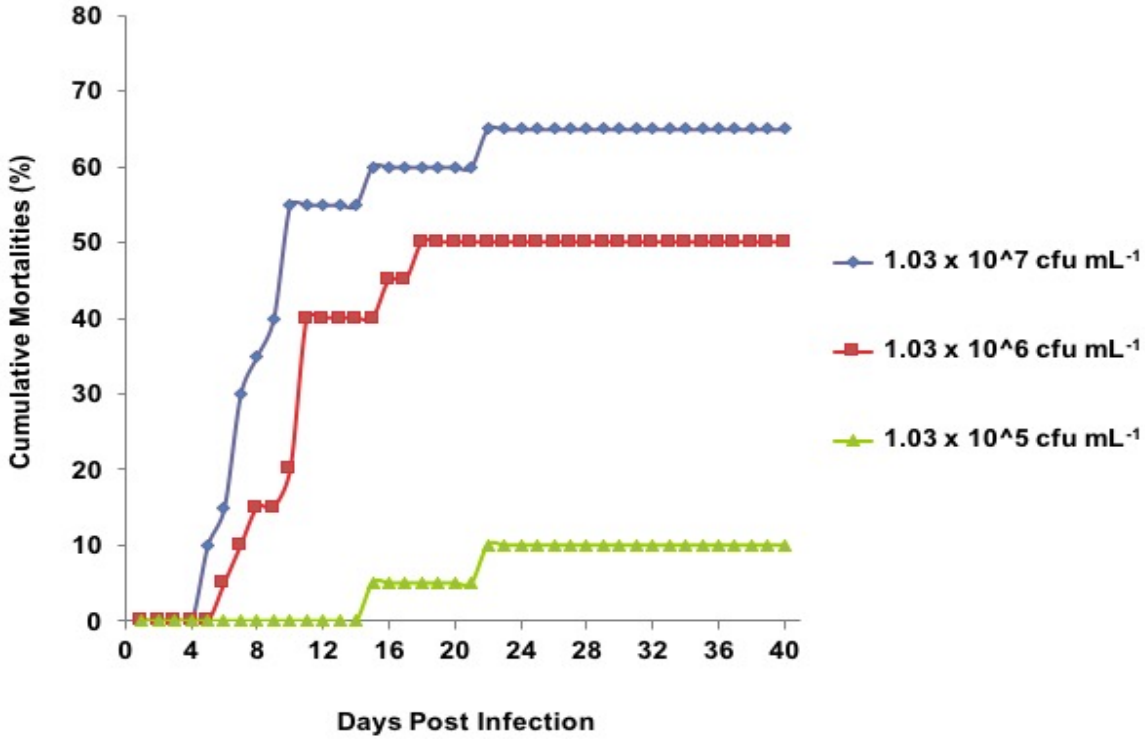

Figure 3.4 Mortality of Nile tilapia homo-gold over 40 d following immersion challenge with different concentrations of *Fno* STIR-GUS-F2f7 CFU mL^{-1}; 20 fish were infected per treatment.

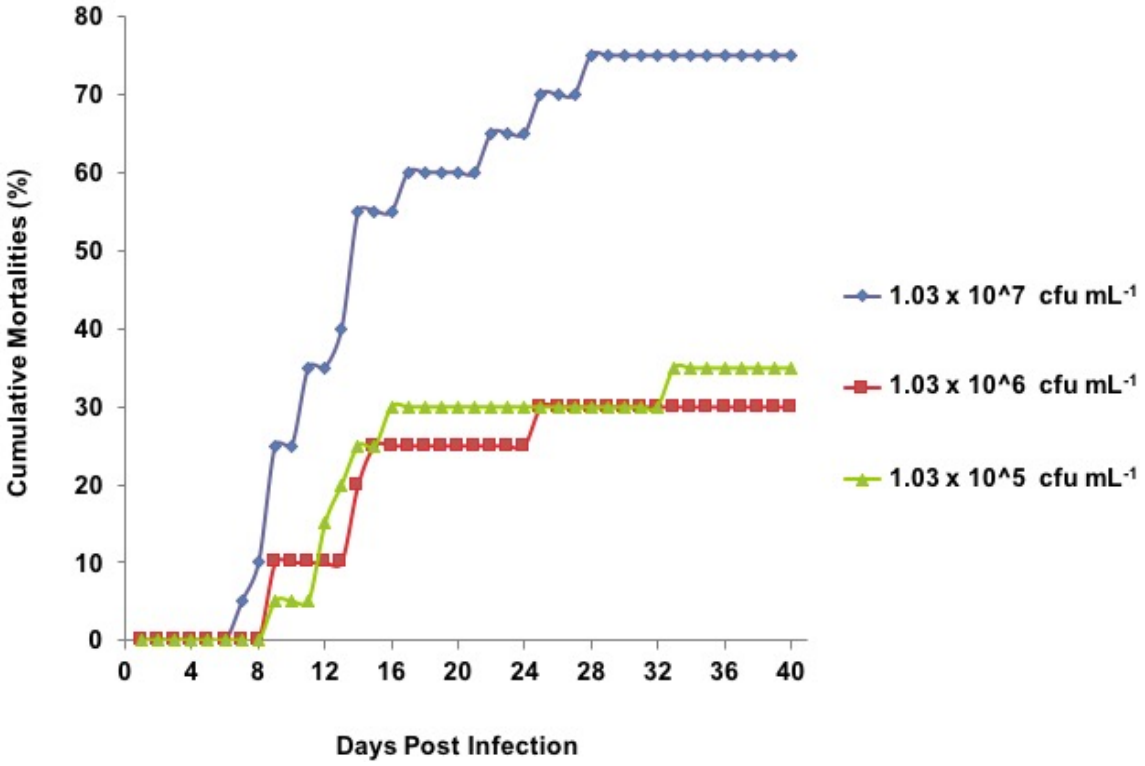

Figure 3.5 Mortality of Nile tilapia wild type over 40 d following immersion challenged with different concentrations of *Fno* STIR-GUS-F2f7 CFU mL^{-1}; 20 fish were infected per treatment.

3.3.4.2 Gross pathology and clinical signs

The external clinical signs and gross pathology expected of francisellosis were observed in dead and moribund fish in both Nile tilapia homo gold and wild-type after the *Fno* challenge (Figures 3.6 and 3.7). The first obvious clinical signs appeared 4–6 d post-challenge in all affected fish depending on the dose administered and consisted of lethargy, anorexia, inability to maintain normal buoyancy and displayed abnormal swimming behaviour, which always preceeded fish becoming stationary at the bottom of the tank 24 to 48 h prior to death. External gross lesions observed on all the examined fish included dark pigmentation and/or the fish exhibited a pale body colour and showed red markings (petechialy haemorrhage) on the skin, operculum, fins and tail. Common signs included haemorrhages or abrasions on the mouth, signs of acites or emaciation and lost scales. In addition, some fish were found with rare symptoms, such as over-production of mucus on the skin, frayed fins, exophthalmia or opacity of eye, and pale and/or presence of numerous white granulomas on gills with a lot of mucus production.

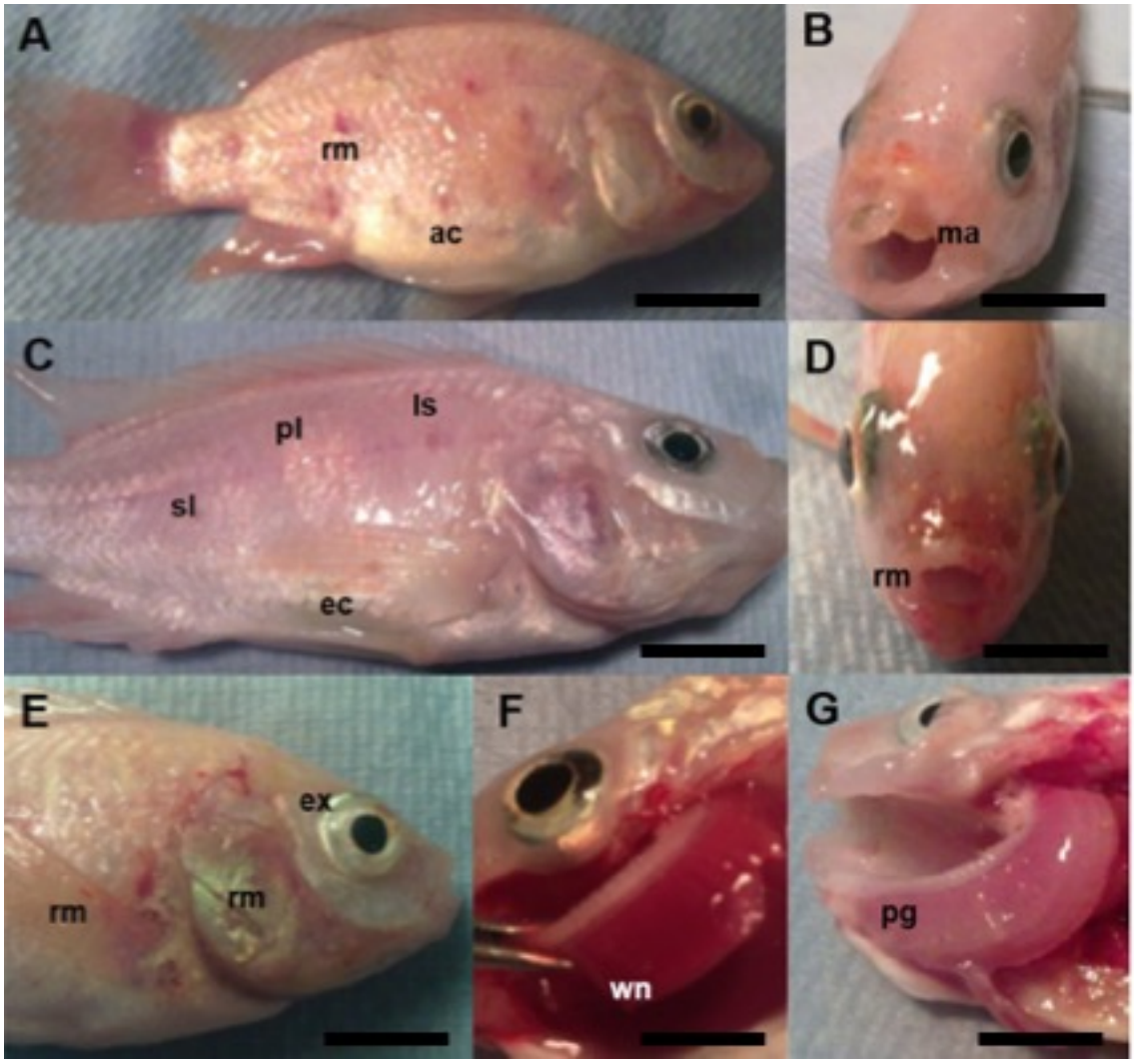

Figure 3.6 External gross pathological findings in Nile tilapia homo gold after 40 d immersion challenge with *Fno* STIR-GUS-F2f7. (A) Red marks on the skin and tail (rm), ascites (ac) and excess mucus production (at 1.03 x 10^6 CFU mL^{-1}); (B) mouth abrasion (ma) (at 1.03 x 10^7 CFU mL^{-1}); (C) pale body colour with scales loss (sl), emaciation (ec), and lesions on skin (ls) (at 1.03 x 10^5 CFU mL^{-1}); (D) red marks on mouth (at 1.03 x 10^6 CFU mL^{-1}); (E) red marks on surrounding operculum with exophthalmia (ex) (at 1.03 x 10^5 CFU mL^{-1}); (F) white nodules on gills (wn) (at 1.03 x 10^5 CFU mL^{-1}); and (G) pale gills (pg) with overproduction of mucus (at 1.03 x 10^7 CFU mL^{-1}). Scale bar = 1.0 cm.

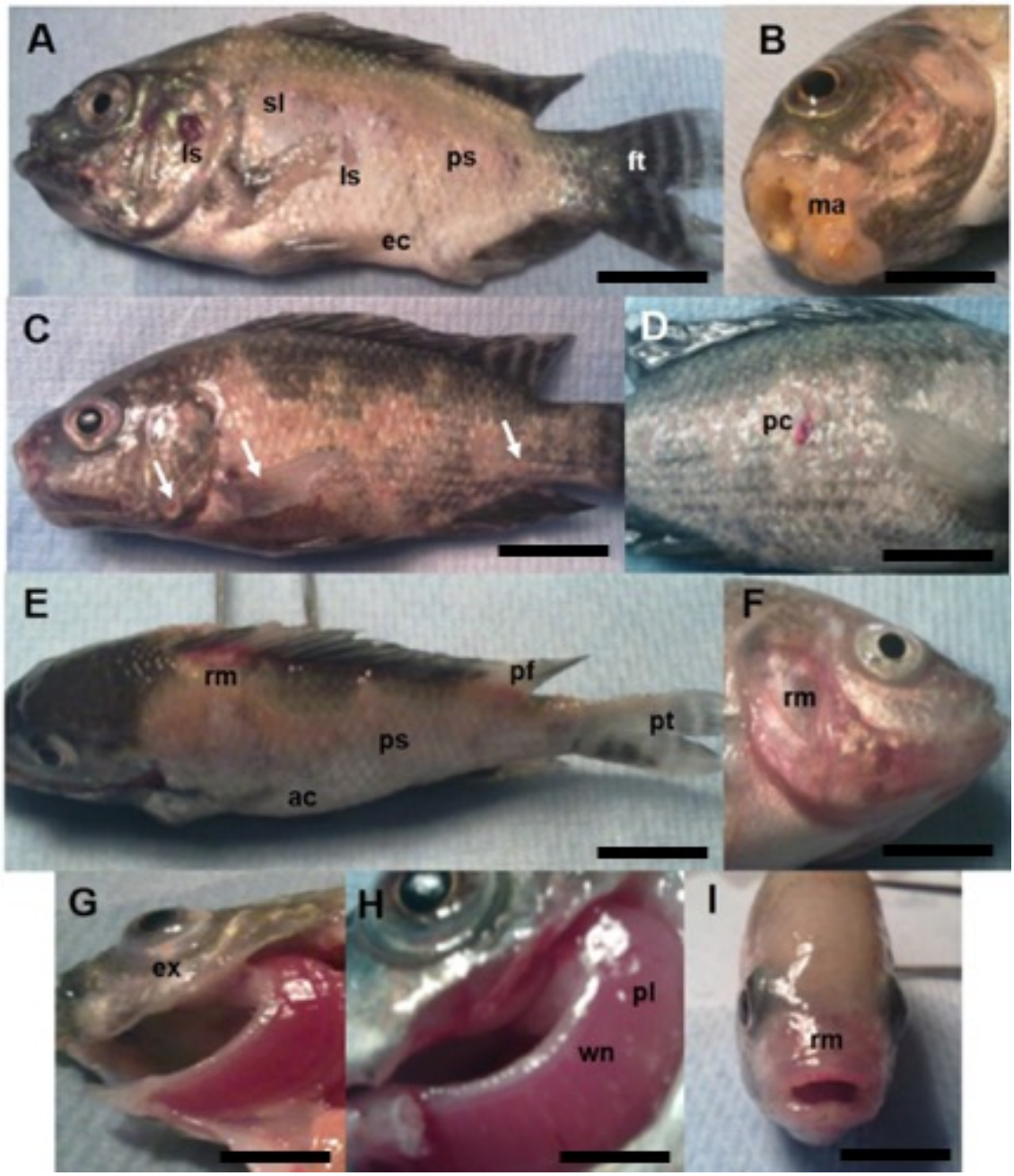

Figure 3.7 External gross lesions in tilapia wild type after 40 d immersion challenge with *Fno* STIR-GUS-F2f7: (A) Pale (pl) skin and emaciation (ec) with loss of scales (sl), frayed tail (ft), and lesion (ls) on skin and operculum (infected with *Fno* at concentration 1.03 x 10^7 CFU mL^{-1}); (B) mouth abrasion (ma) (at 1.03 x 10^6 CFU mL^{-1}); (C, D) red marked/petechiae (pc) on skin, operculum and fin (arrows); (E) pale skin (ps), fins (pf) and tail (pt), and red marks (rm) on pectoral fin (at 1.03 x 10^7 CFU mL^{-1}); (F) red marks on operculum (at 1.03 x 10^6 CFU mL^{-1}); (G) exopthalmia; (H) pale and white nodules on gills (wn) (at 1.03 x 10^5 CFU mL^{-1}); and (I) red marks on mouth (at 1.03 x 10^5 CFU mL^{-1}). Scale bar = 1.0 cm.

At necropsy, the internal organs were examined and splenomegaly, renomegaly or hepatomegaly observed, with either haemorrhagic or pale tissues. These symptoms were observed in fish that started to die on days 5 and 7 at the two highest concentrations of 1.03×10^6 and 1.03×10^7 CFU mL^{-1} following *Fno* exposure in both tilapia strains. Most of the fish presented with widespread multifocal white nodules in the spleen and anterior kidney. The gross pathological changes of the organs, including the spleen and kidney varied from being just slightly swollen to most of the viscera being covered with white granulomas. At the terminal stages of the infection, in some severe cases fish presented enlarged internal organs especially the spleen, as described in Chen *et al.*, (1994), Chern and Chao (1994) and Ramirez-Paredes (2015). The spleen was enlarged 5–50 times and was covered completely with and penetrated by granulomas over 60 to 90% of the tissue surface and contained air bubbles (Figures 3.8 and 3.9). Among the tissues, posterior kidney and liver were also most strongly affected by degeneration and presence of granulomas in posterior kidney, while in some fish, the livers were hyperaemic or yellowish in colour with hepatic lipidosis.

During the challenge experiment, more sub-acute disease occurred at early post *Fno* infection (5–14 d) with the highest concentration (1.03×10^6 and 1.03×10^7 CFU mL^{-1} in homo gold, and 1.03×10^7 CFU mL^{-1} in wild-type). In this sub-acute disease, almost all infected fish showed typical petechial haemorrhagies on skins, fins and tail, haemorrhages in liver, while white nodules slightly presented on the spleen and anterior kidney. In contrast, fish subjected to a low dose of *Fno* (1.03×10^5 or 1.03×10^6 CFU mL^{-1}) or after 15 d post infection displayed a chronic disease showing pale body colour, skin and operculum lesions. Signs of ascites or emaciation were also observed with frayed fins, abrasions on the mouth, lost scales, and corneal opacity with/without later or bilateral exophthalmia. In this chronic disease phase, common internal gross phatological changes were found in examined fish, including hepatic lipidosis in the liver that were pale and yellowish in colour, enlarged intestine and stomach with empty food or atrophy, and multifocal white nodules on gills, anterior and posterior kidney, spleen and mesenteric fat.

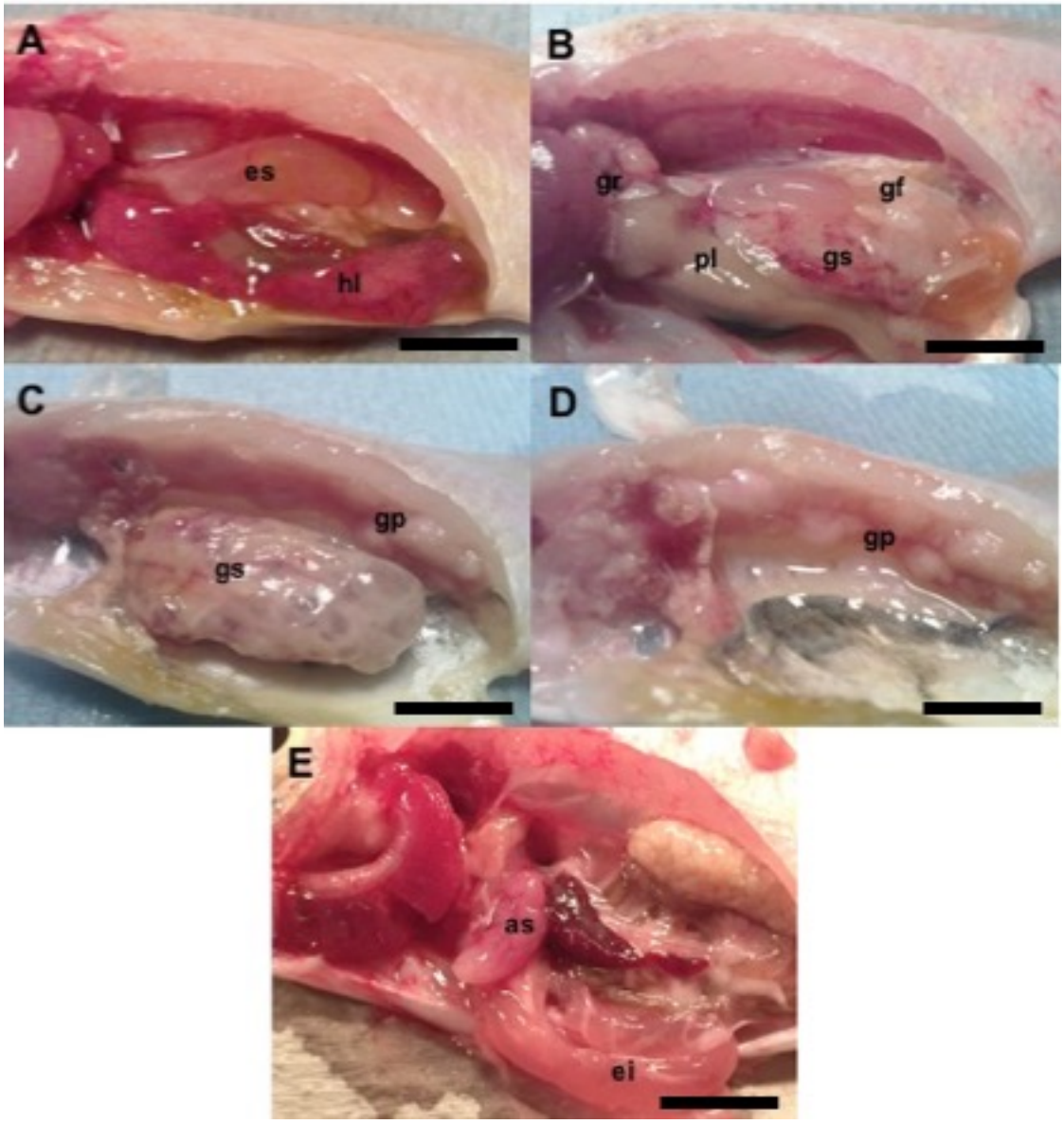

Figure 3.8 Internal gross pathological findings in Nile tilapia homo gold 40 d after immersion challenge with *Fno* STIR-GUS-F2f7: (A) at an early stage of francisellosis (at 1.03 x 10^7 CFU mL^{-1}) haemorrhages on liver (hl), slight granulomas on spleen, and enlarged stomach (es) empty of food; (B) granulomas on anterior kidney and spleen with a marked splenomegaly (gs) and renomegaly (gr); and pale liver with yellowish in colour (at 1.03 x 10^6 CFU mL^{-1}); and (C) at terminal stages of disease spleen enlarged and protruded up to 50X and completely covered by granuloma with air bubbles inside (at 10^6 CFU mL^{-1}); (D) granulomas on almost whole of posterior kidney (at 1.03 x 10^5 CFU mL^{-1}); and (E) atropy stomach (as) and enlarged intestine (ei). Scale bar = 1.0 cm.

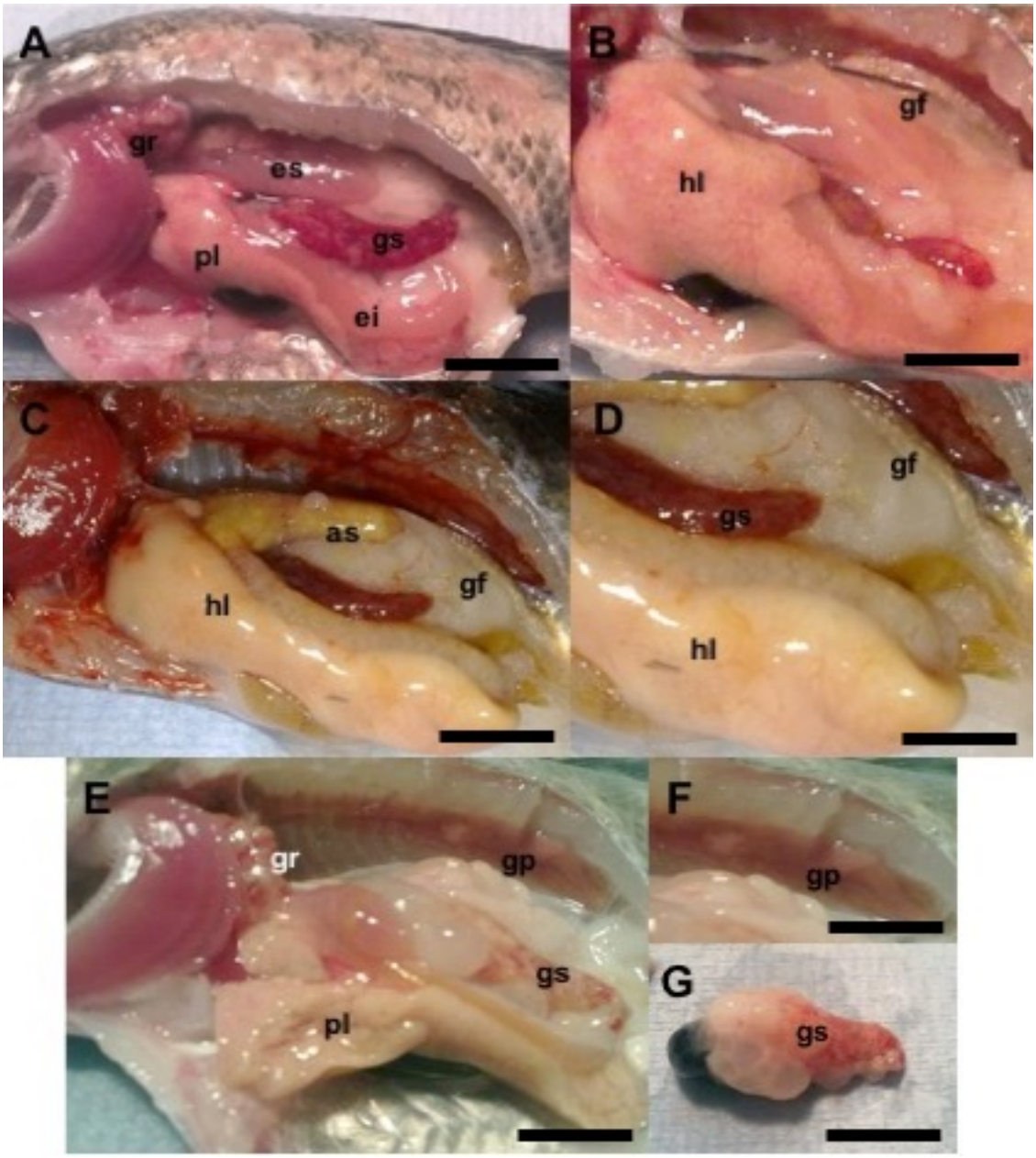

Figure 3.9 Internal gross pathological findings in Nile tilapia wild type 40 d after immersion challenge with *Fno* STIR-GUS-F2f7: (A) pale liver (pl), granulomas on anterior kidney (gr) and spleen (gs) with splenomegaly and renomegaly, and enlarged stomach (es) and intestine (ei) empty of food (at 1.03 x 10^7 CFU mL^{-1}); (B) pale liver with hepatic lipidos (hl) and white spot on mesenteric fat (gf); (C & D) hepatic lipidosis liver with yellowish in colour and white nodules in mesenteric fat and atropy stomach (as); (E & G) spleen enlarged up to 10X and covered by granulomas containing air bubbles, and (F) the same fish showed granulomas on posterior kidney (at 1.03 x 10^6 CFU mL^{-1}). Scale bar = 1.0 cm.

3.3.4 *Fno* recovery from challenged tilapia

Pure cultures of *Fno* Stir-GUS-F2f7 were recovered on CHAH plates from homogenates of spleen and anterior kidney from each infected fish (freshly dead and moribund fish) after immersion challenge of homo gold and wild-type Nile tilapia. Initially, the bacterial contaminants overgrew on all CHAH plates (24 h after the inoculation), while *Fno* colonies had appeared mostly after 192 h of incubation at 28°C. The morphology of the colonies was as expected (Figure 3.10), and the identities of purified bacterial isolated from head kidney and spleen samples from each tilapia strain after 192 h incubation on CHAH were confirmed by Gram stain (Figure 3.11) and PCR. Moreover, no *Fno* was detected from any surviving fish, in either tilapia strains at the completion of the experimental challenge at 40 dpi.

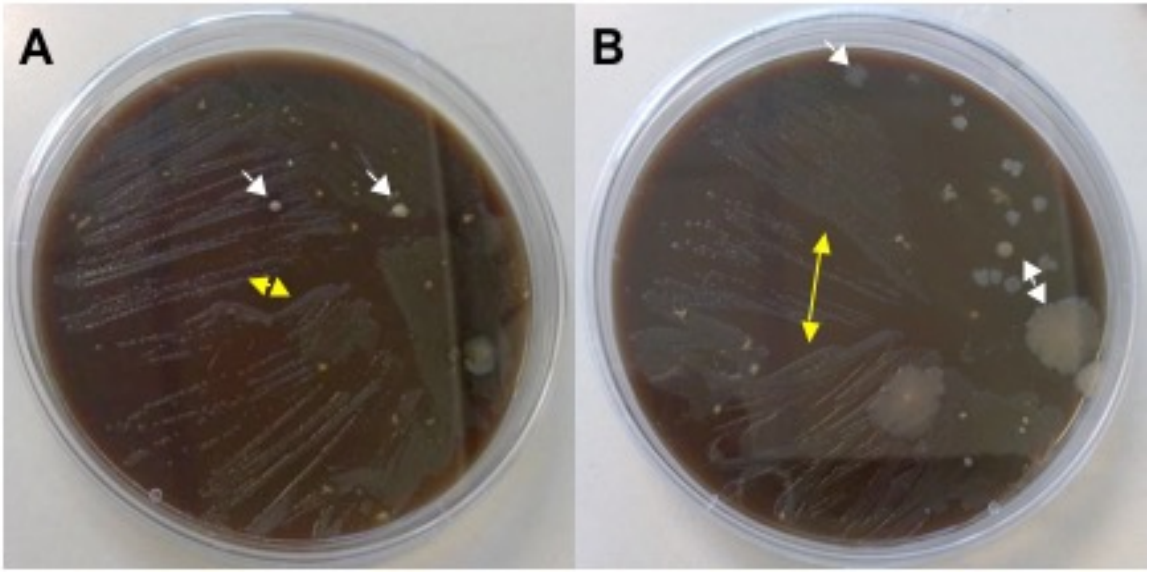

Figure 3.10 *Fno* recovered from infected Nile tilapia homo gold (A) and wild-type (B) anterior kidney homogenate 10 days' post infection and at concentration 1.03 x 10^7 CFU mL^{-1} after 8 d incubation at 28ºC on CHAH (10^{-2} dilution on PBS). *Fno* was greenish in colour (double yellow arrow) while some bacterial contaminants were also present (white arrows).

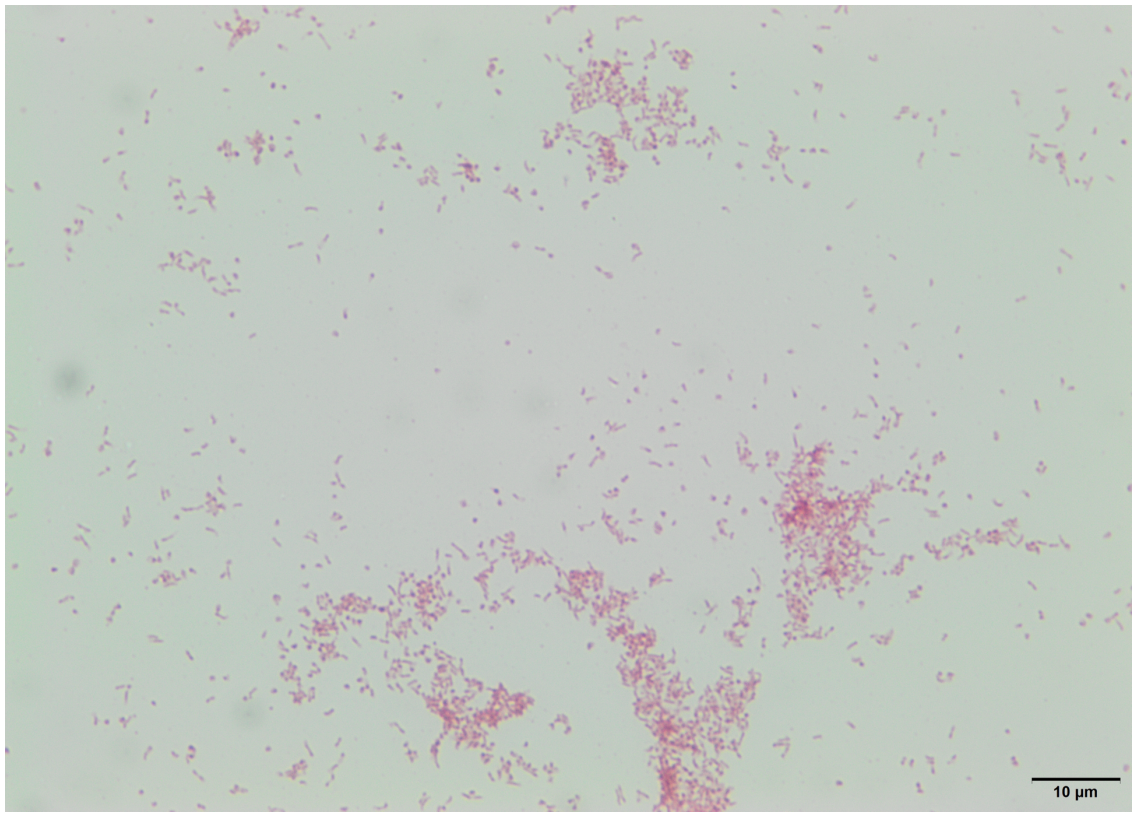

Figure 3.11 Gram staining of *Fno* STIR-GUS-F2f7: isolate recovered and grown on CHAH from infected Nile tilapia homo gold at day 10 d post infection with 1.03 x 10^7 CFU mL^{-1} and cultured for 8 d at 28ºC on CHAH.

3.3.5 Histopathology

The histological observations were consistent with previous descriptions of francisellosis in tilapia (Chern and Chao, 1994; Hsieh *et al.*, 2006; Soto *et al.*, 2009a, b; Soto *et al.*, 2013c; Ramirez-Paredes, 2015). Microscopic lesions appeared similar in both of Nile tilapia homo gold and wild-type and were consistent with francisellosis infection as were other signs, including widespread granulomatous inflammation in multiple organs (*e.g.* spleen and head kidney). The granulomas in the spleen and head kidney dominated by large numbers of hypertrophied macrophages (the morphology varied between granulomas), lymphocytes, and neutrophils (Figures 3.12 and 3.13). In addition, small pleomorphic cocco-bacilli bacteria were observed mostly inside cytoplasmic vacuoles of hypertrophied macrophages inside the granulomas in the spleen, and head kidney. The centers of the granulomas in both organs often had progressively established marked coagulative necrotic small areas or complete necrosis, and fibrous encapsulation finally formed in the outermost zone. The granulomas in infected spleen and head kidney increased noticeably in size and were commonly found in multiple formations. In the most severely affected samples the granulomatous inflamation covered around 80% of the splenic and renal tissues of all infected fish, causing extensive parenchyma loss (Figures 3.12B and E, and 3.13B and E).

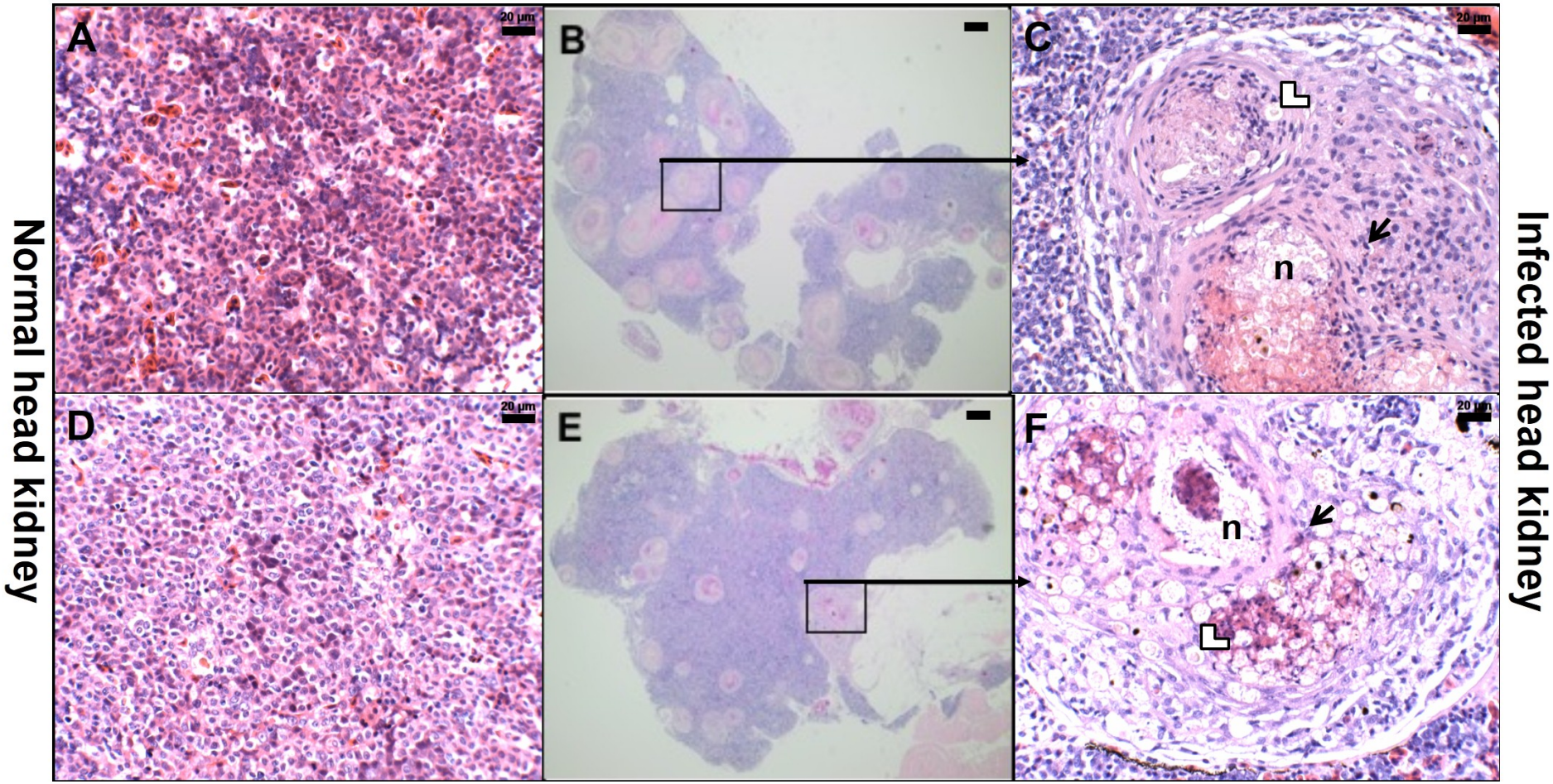

Figure 3.12 Histopathology findings of Nile tilapia homo gold (at top) and wild-type (below) head kidney (stained with haematoxylin and Eosin staining): (A and D) non-infected head kidney showed normal renal parenchyma and stroma. On the other hand, (B, C, E and F) severely infected head kidney with *Fno* at (1.03 x 10^6 CFU mL^{-1}) 40 d post infection presented widespread granulomas comprising many lymphocytes (arrow) and macrophages (arrow head) with large vacuoles containing small coccoid bacteria and marked coagulative necrosis (n). Scale bar: A, C, D and F = 20 µm; B and E = 100 µm.

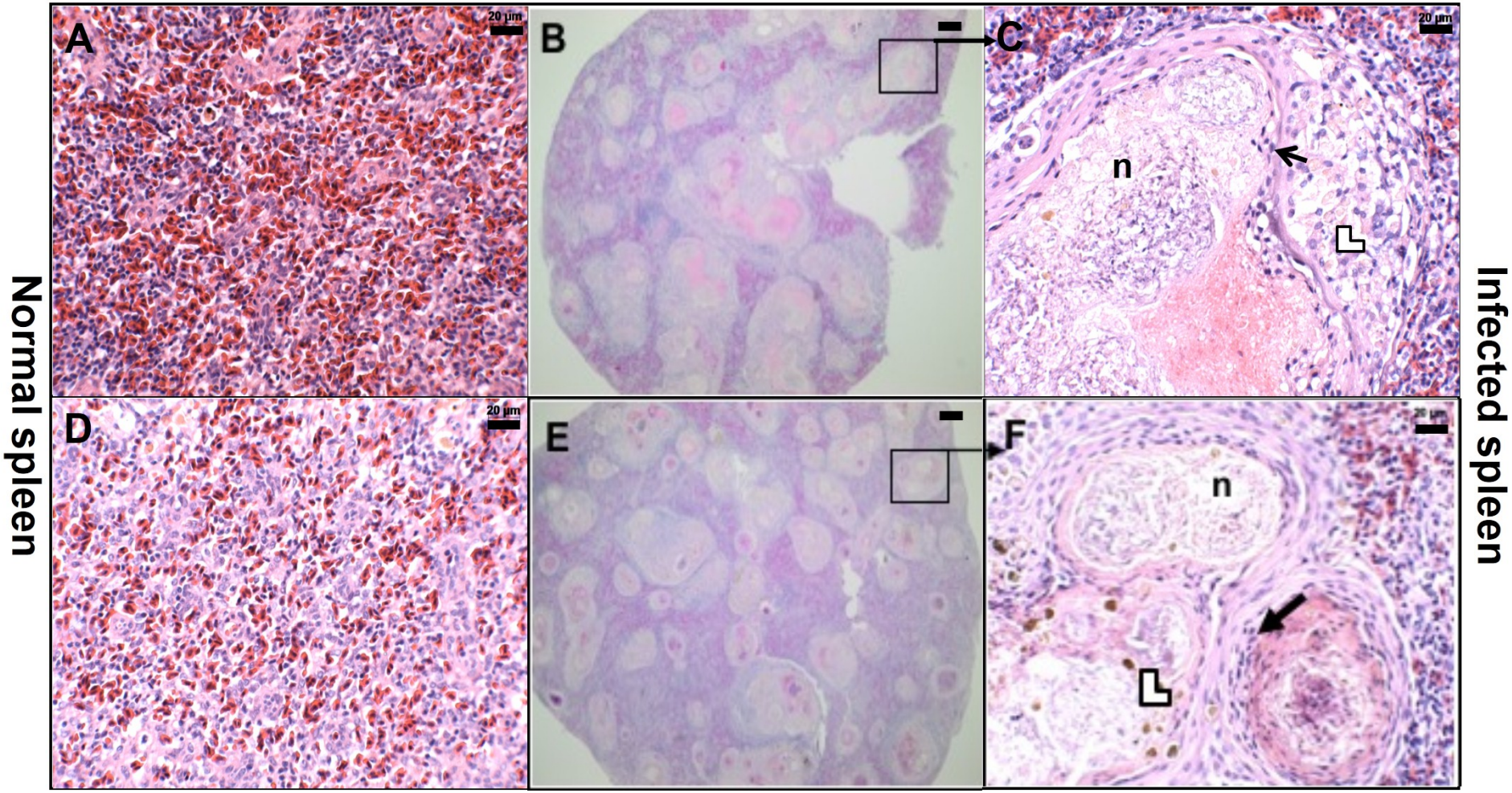

Figure 3.13 Histopathology findings of Nile tilapia homo gold (at top) and wild-type (below) spleen (stained with haematoxylin and Eosin staining): (A and D) normal spleen showed normal splenic parenchyma and stroma. On the other hand, (B, C, E and F) severely infected spleen with *Fno* (1 x 10^6 CFU mL^{-1}) at 40 d post infection presented widespread granulomas comprising many lymphocytes (arrow) and macrophages (arrow head) with large vacuoles containing small coccoid bacteria and marked coagulative necrosis (n). Scale bar: A, C, D and F = 20 µm; B and E = 100 µm.

3.3.6 Immunohistochemistry

Immunohistochemistry was perfomed to investigate the relationship between granulomas and *Fno* in the infected tissues for both Nile tilapia strains. The assay was optimised using different concentrations of rabbit anti-*Fnn* antibodies. The results showed good detection and low background when using the anti-*Fnn* antibody at 1:1200. A positive reaction was observed in different tissues, including the spleen, and anterior kidney, as confirmed by a light brown colour localised mainly in the large foamy cells of the splenic and renal granulomas (Figures 3.14 and 3.15). *Fno* was not detected in any of the negative control tissues.

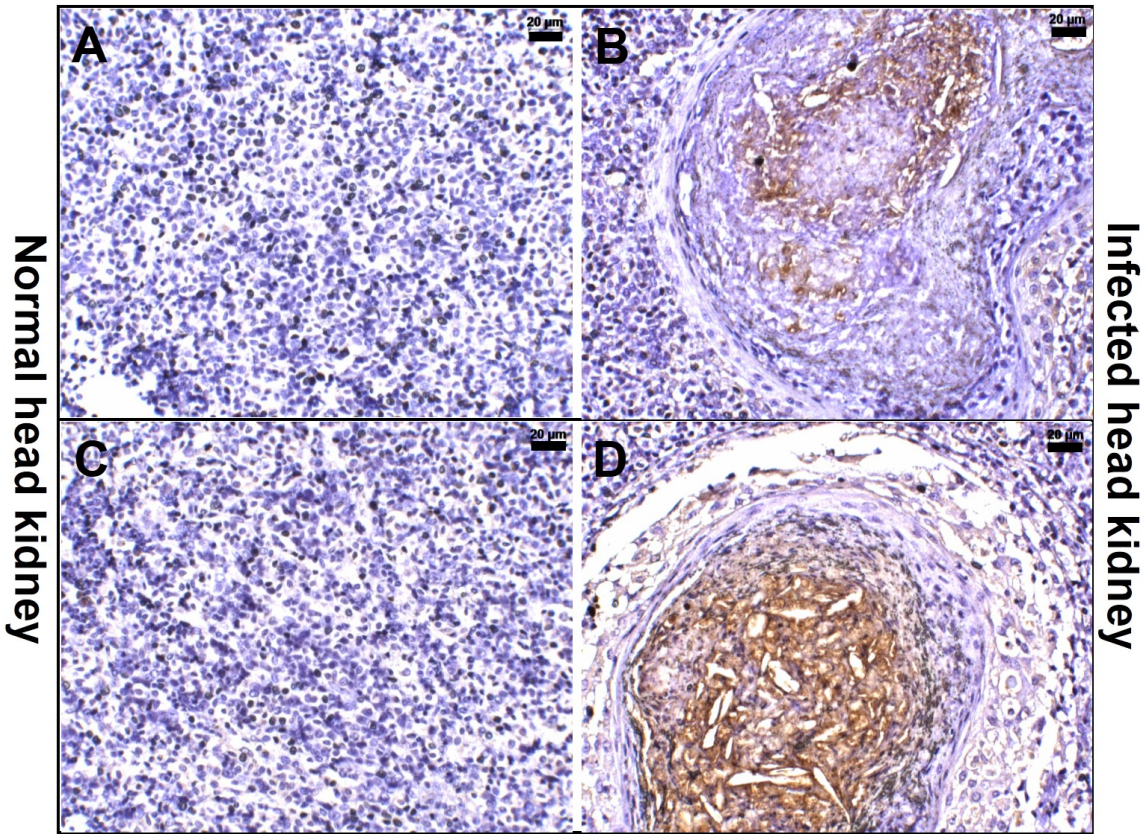

Figure 3.14 Immunohistochemical detection of *Fno* in sections of head kidney from homo gold (top) and wild-type (below) Nile tilapia: (A & C) sections of normal head kidney, and (B & D) positive and severely infected head kidney (challenged with *Fno* at 1.03 x 10^6 CFU mL^{-1}) 40 d after immersion challenge. A positive reaction appears as a light brown colour. Scale bar: A, B, C and D = 20 µm.

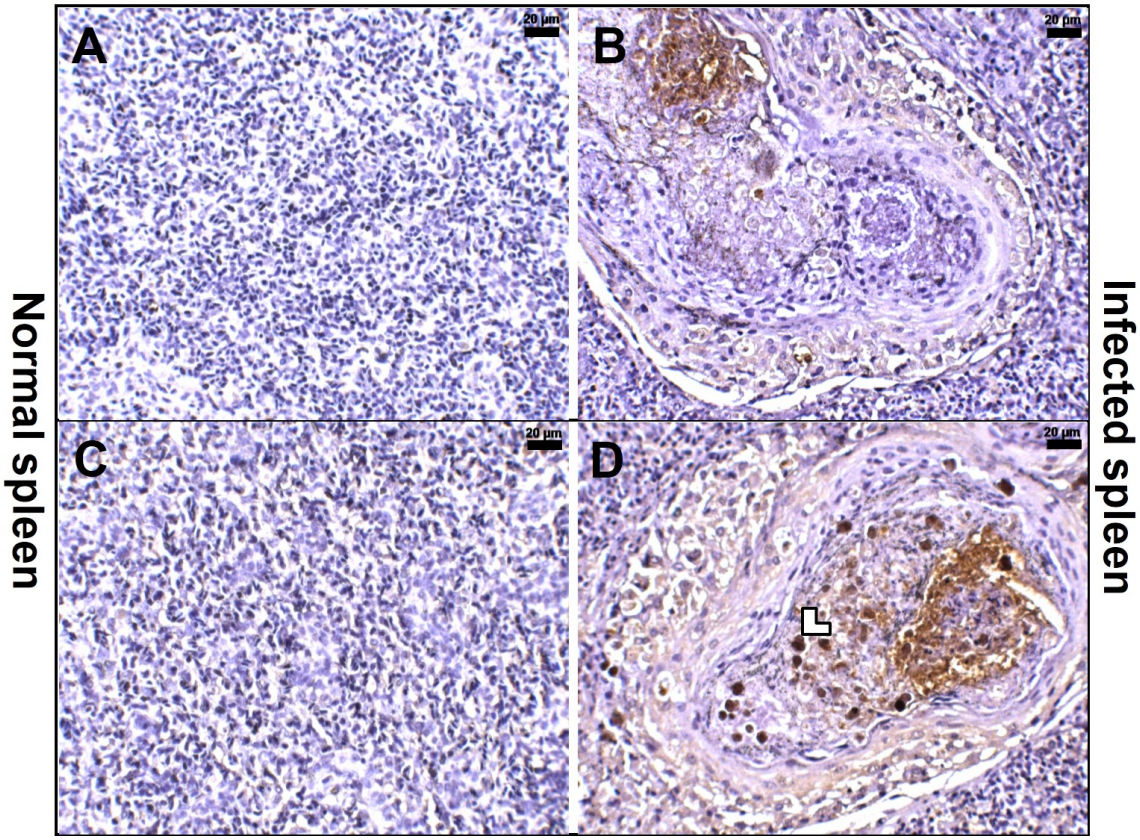

Figure 3.15 Immunohistochemical detection of *Fno* in sections of spleen from homo gold (top) and wild-type (below Nile tilapia): (A & C) sections of normal spleen, and (B & D) positive and severely infected spleen (challenged with *Fno* at 1.03 x 10^6 CFU mL^{-1}) 40 d after immersion challenge. A positive reaction appears as a light brown colour. Dark brown staining are the centres of melanomacrophages (arrow head). Scale bar: A, B, C and D = 20 µm.

3.3.7 Gram Twort

The Gram-Twort stain is a variation of the standard Gram stain to highlight bacteria localization and host interaction, which is used in histopathology for the demonstration of Gram-positive and Gram-negative bacteria in formalin-fixed sections (Perdoni *et al.*, 2014). Gram Twort observations revealed the existence of spaciously distributed granulomatous inflammation and a greater number of melanomacrophages in the spleen, anterior and posterior kidneys from both infected Nile tilapia strains. In both tilapia strains, large numbers of small pleomorphic cocco-bacilli bacteria were also presented inside the cytoplasmic vacuoles and/or inside the nucleus of the macrophages, lymphocytes, and even in erythrocytes and melanomacrophages (Figures 3.16, 3.17, 3.18, 3.19, 3.20 and 3.21).

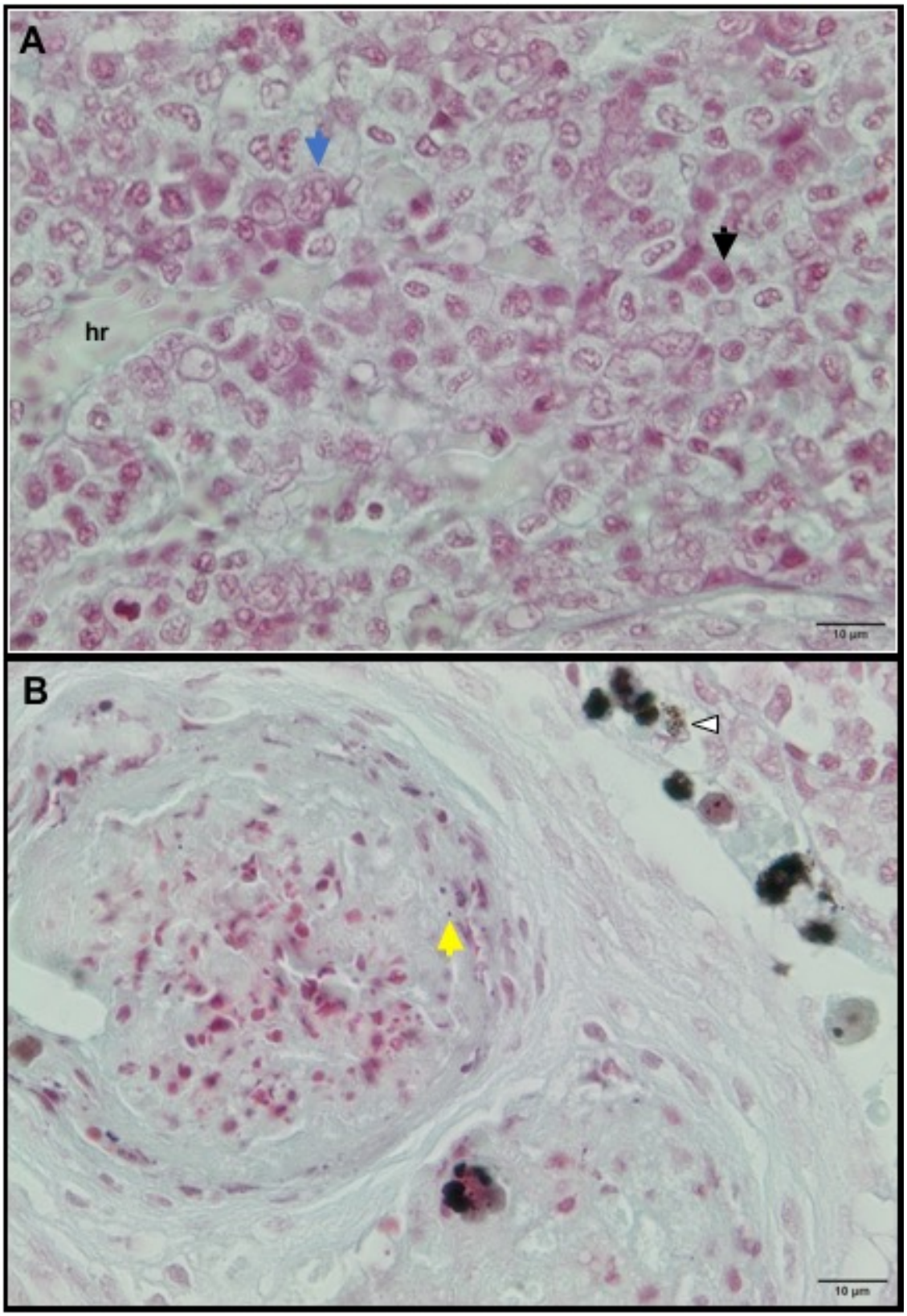

Figure 3.16 Gram Twort photomicrographs Nile tilapia homo gold spleen severely infected with *Fno* (1.03 x 10^6 CFU mL^{-1}) 40 d post infection: (A) cocco-bacilli bacteria inside macrophages (blue arrow), lymphocytes (black arrow) and hemorrhage (hr) in splenic arterioles; and (B) many small pleomorphic cocco-bacilli bacteria inside melanomacrophages (white arrowhead) (presented *Fno* escaping from the phagocytes) within and surrounding granulomas (yellow arrow).

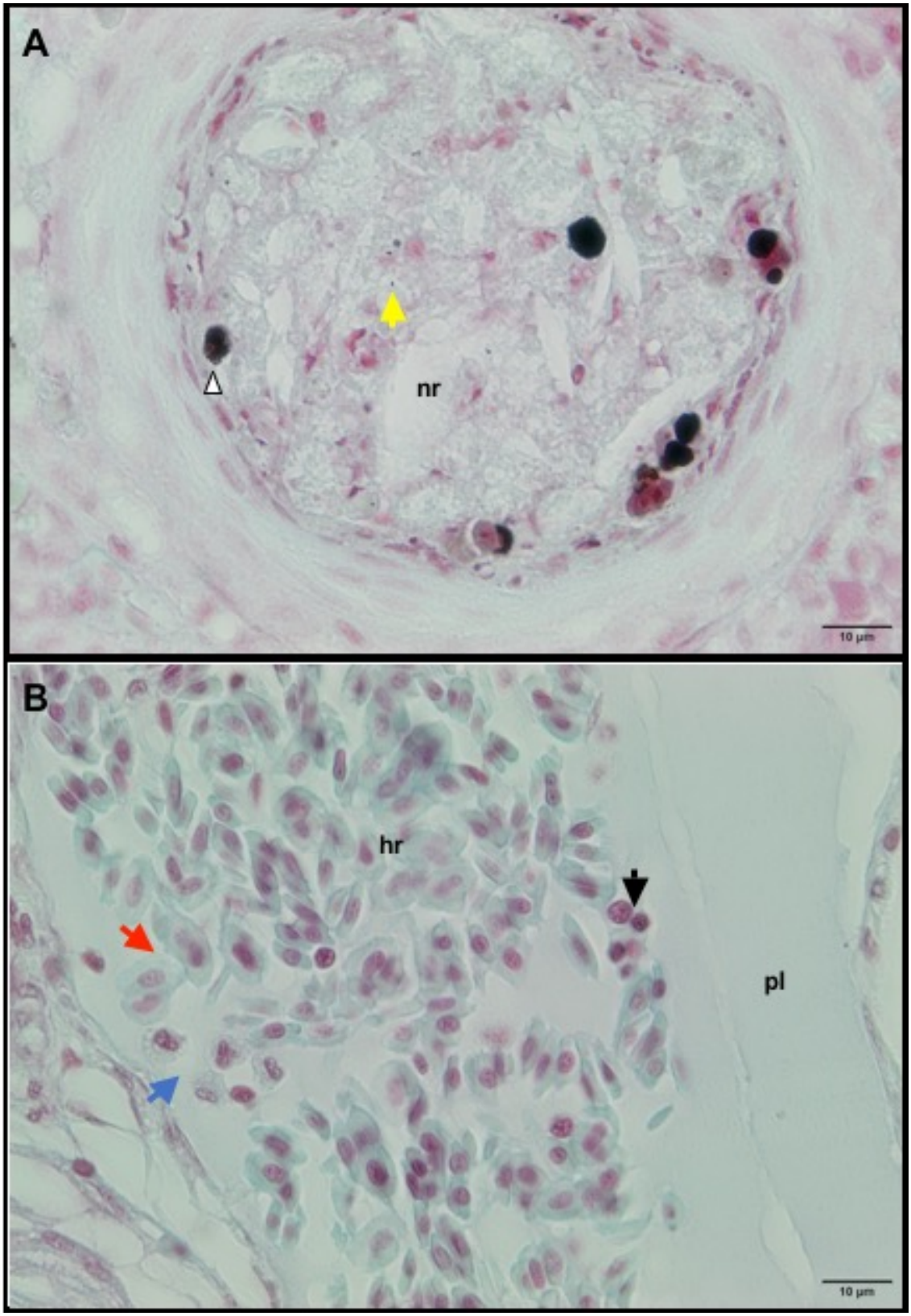

Figure 3.17 Gram Twort photomicrographs Nile tilapia homo gold head kidney severely infected with *Fno* (1.03 x 10^6 CFU mL^{-1}) 40 dpi: (A) typical granuloma containing small coccoid bacteria (yellow arrow), inside melanomacrophages (white arrowhead) and necrosis (nr); and (B) hemorrhage (hr) and large formation of pale eosinophilic protein lakes (pl) of renal arterioles composed with macrophages (blue arrow) and lymphocytes (black arrow) containing coccoid bacteria and inside erythrocytes (red arrow).

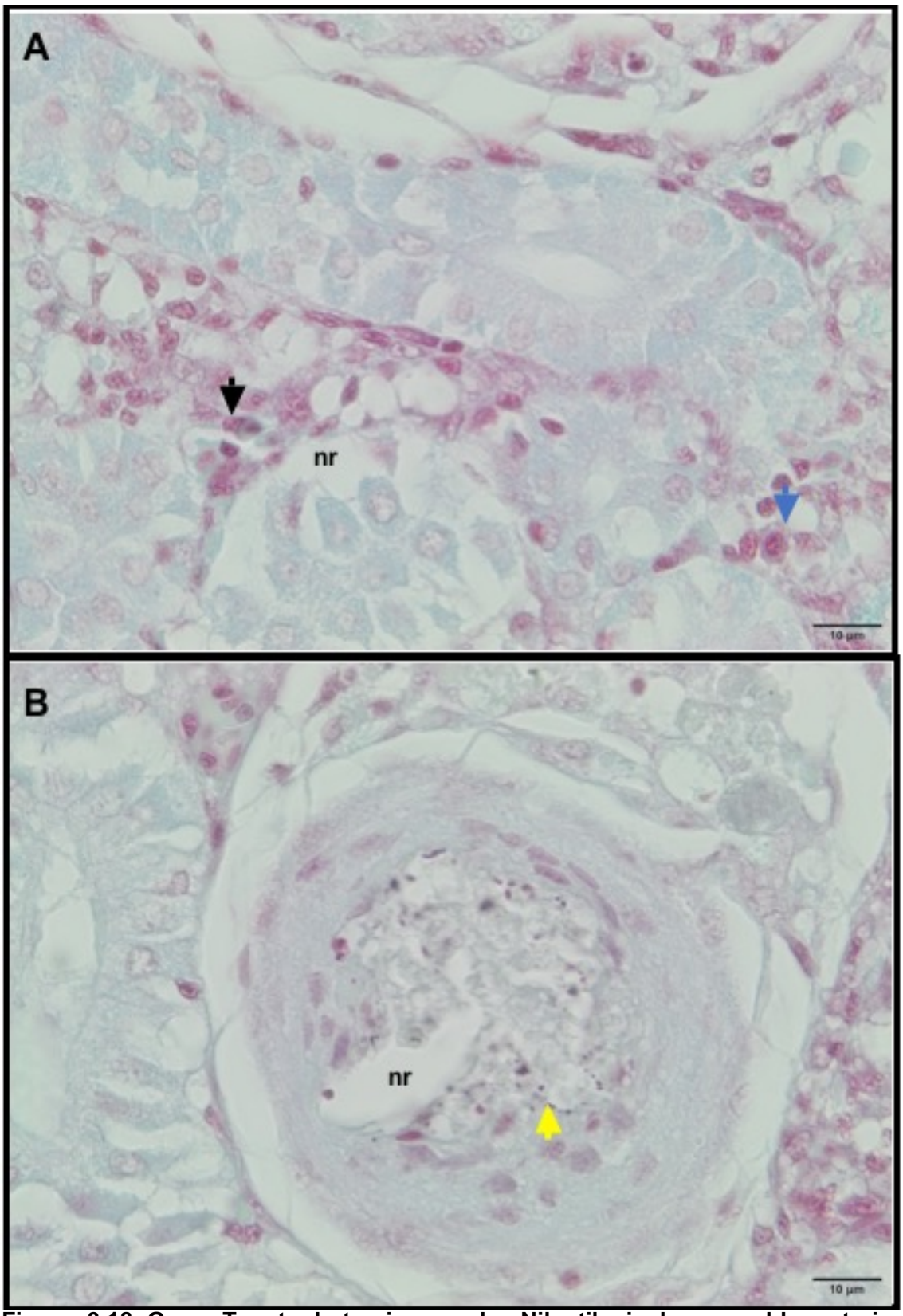

Figure 3.18 Gram Twort photomicrographs Nile tilapia homo gold posterior kidney severely infected with *Fno* (1.03 x 10^6 CFU mL^{-1}) 40 d post infection: (A) necrosis (nr) of renal tubules containing macrophages (blue arrow) and surrounded with lymphocytes (black arrow) containing coccoid bacteria; and (B) many small pleomorphic cocco-bacilli bacteria inside the granuloma (yellow arrow) and necrosis (nr).

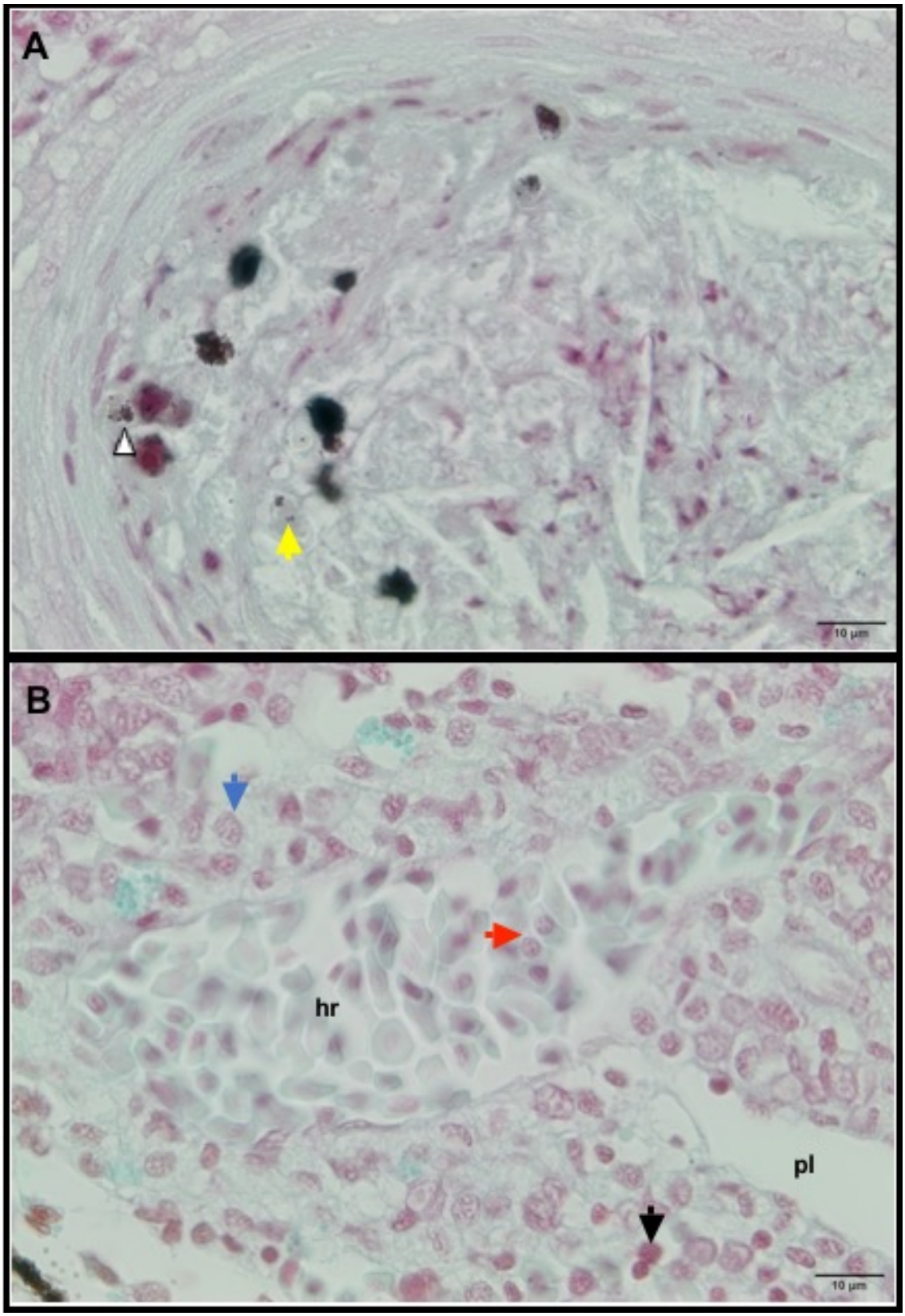

Figure 3.19 Gram Twort staining Nile tilapia wild-type spleen severely infected with *Fno* (1.03×10^6 CFU mL^{-1}) 40 d post infection: (A) many small pleomorphic cocco-bacilli bacteria inside melanomacrophages (white arrowhead) (presented *Fno* escaping from the phagocytes) and within granulomas (yellow arrow); and (B) hemorrhage (hr) of splenic arterioles followed by necrosis and formation of pale eosinophilic protein lakes (pl) surrounded with macrophages (blue arrow), lymphocytes (black arrow) containing coccoid bacteria inside the nucleus and in erythrocytes (red arrow).

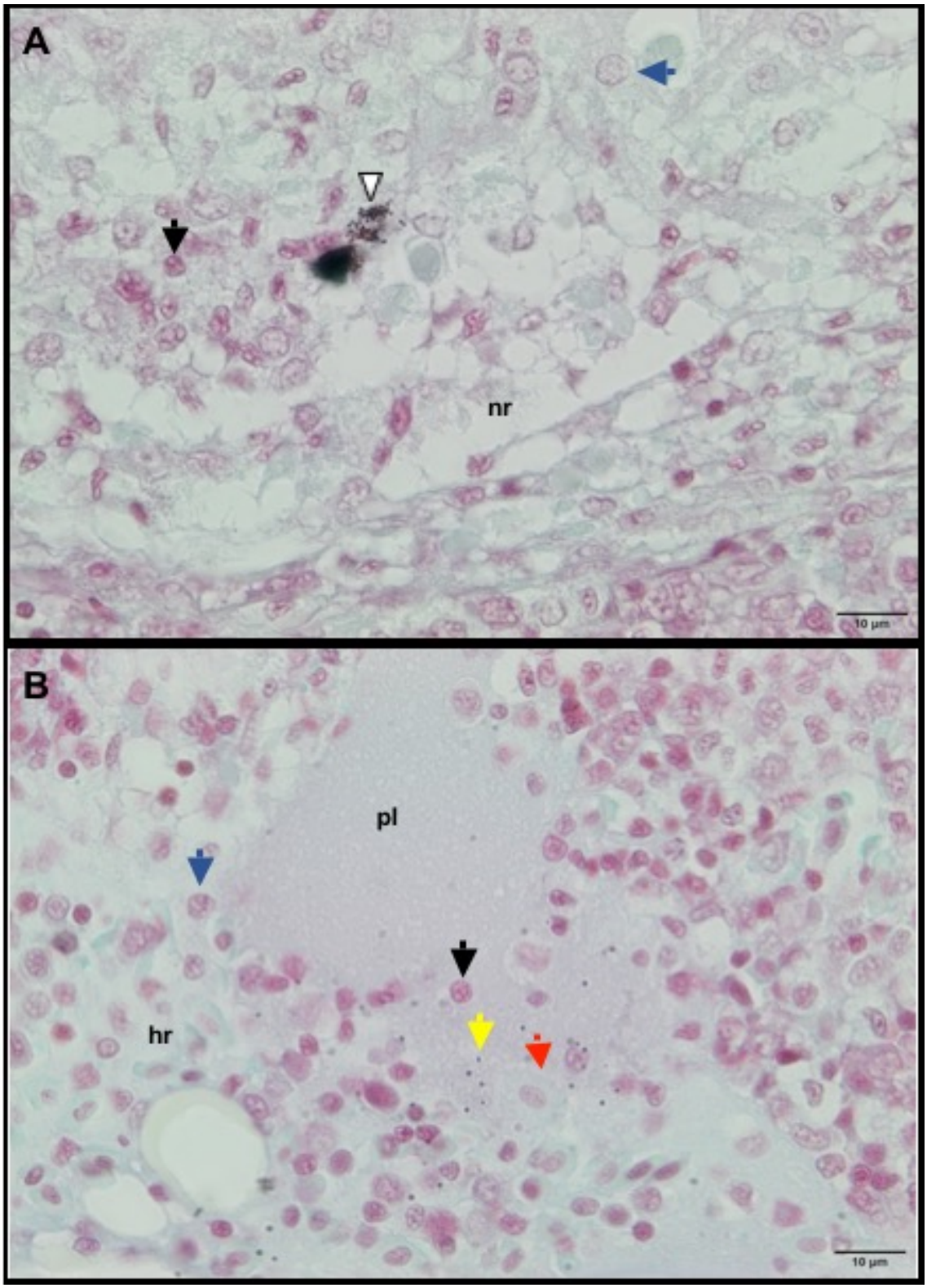

Figure 3.20 Gram Twort photomicrographs Nile tilapia wild-typed head kidney severely infected with *Fno* (1.03 x 10^6 CFU mL^{-1}) 40 dpi: (A) necrosis (nr) of renal arterioles containing macrophages (blue arrow), lymphocytes (black arrow) containing coccoid bacteria inside the nucleus, as well as melanomacrophages (presented bacteria escaping from melanomacrophages phagocytes) (white arrowhead); and (B) hemorrhage (hr) and the formation of pale eosinophilic protein lakes (pl) in renal arterioles surrounding with circulation of the bacteria that escaping from melanomacrophages as the colour are dark brown (yellow arrow), macrophages (blue arrow) and lymphocytes (black arrow) containing the bacteria inside the nucleus and in erythrocytes (red arrow).

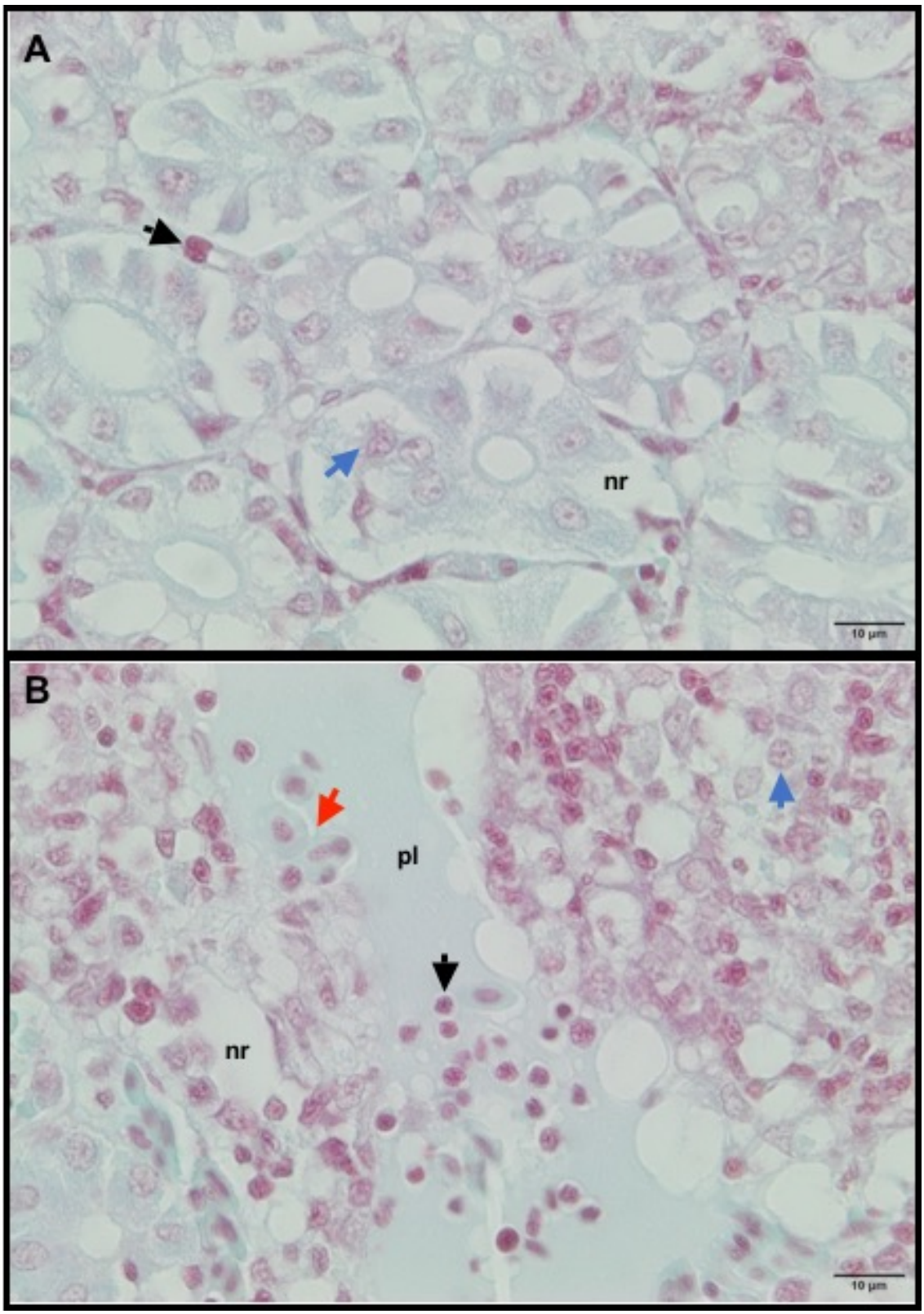

Figure 3.21 Gram Twort photomicrographs Nile tilapia wild-typed posterior kidney severely infected with *Fno* (1.03 x 10^6 CFU mL^{-1}) 40 dpi: (A) necrosis (nr) of renal tubules containing macrophages (blue arrow) and surrounded with lymphocytes (black arrow) containing coccoid bacteria; and (B) necrosis (nr) of renal arterioles often resulted in hemorrhages and the formation of pale eosinophilic protein lakes (pl) surrounded with macrophages (blue arrow) and lymphocytes (black arrow) containing coccoid bacteria inside the nucleus and in erythrocytes (red arrow).

3.3.8 PCR analysis

Tissue samples from the infected tilapia spleens yielded bands on agarose gels of the expected size (1,150 kb) after PCR amplification with the *Francisella* genus specific primers, while no band was observed in non-infected control fish (Figures 3.22 and 3.23). All surviving fish (at 40 dpi) from both genetic backgrounds showed very weak bands.

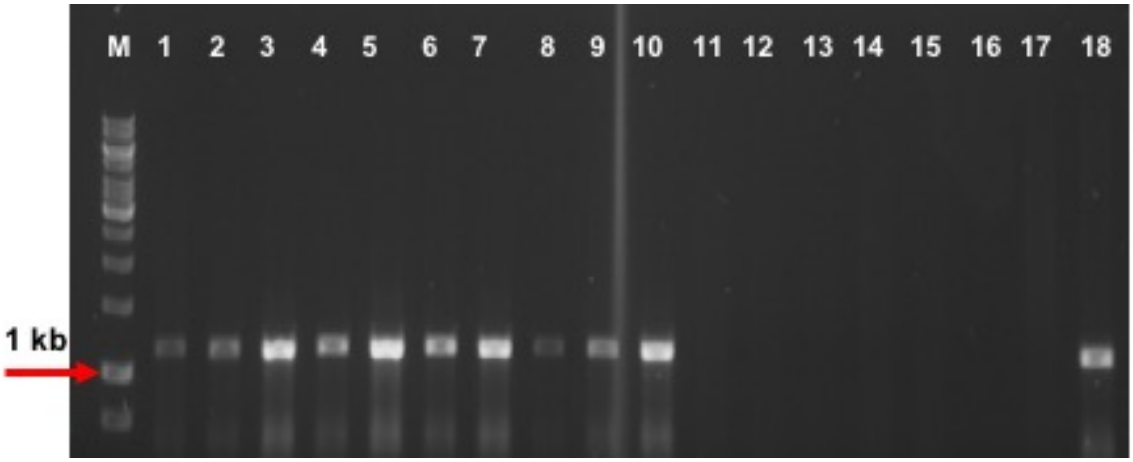

Figure 3.22 PCR amplification of tissue sampled from *Fno* infected Nile tilapia homo gold: lane M, 1 kilo base-pair ladder; lane 1–10, moribund and freshly dead fish; lane 11–15, fish surviving to 40 d post infection; lane 16, negative control (PBS); lane 17, naïve Nile tilapia homo gold; lane 18, *Fno.*

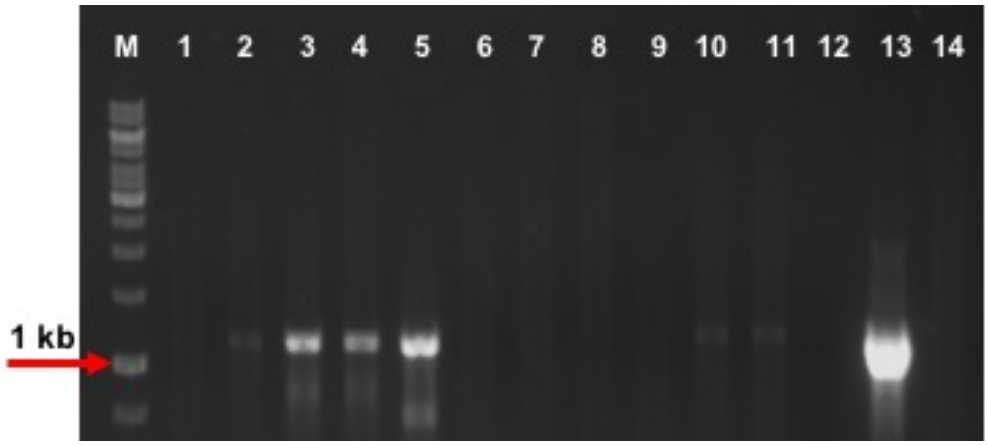

Figure 3.23 PCR amplification of tissue sampled from *Fno* infected Nile tilapia wild type: lane M, 1 kilo base-pair ladder; lane 1–5, moribund and freshly dead fish; lane 6–11, fish surviving to 40 d post infection h; lane 12, negative control (PBS); lane 13, *Fno*; lane 14, naïve Nile tilapia wild-type.

3.4 Discussion

Francisellosis, caused by *Fno*, is a major emerging bacterial disease affecting intensive tilapia farming systems and is known to cause significant economic losses globally. It is therefore important to have methods to control this disease. The results of Chapter 2 provided evidence that algal-derived compounds have potential use as immunostimulants in tilapia *in vitro* and the next logical step would be to test these *in vivo* in tilapia. In order to do this an *Fno* challenge model for *Fno* STIR-GUS-F2f7 to induce francisellosis was required and this was performed in the two most common farmed tilapia globally, homo gold (red) and wild-type Nile tilapia. In addition, gross pathology, histopathology, Gram Tworf, and immunohistochemistry findings, as well as PCR, were utilised to gain insight into the pathophysiology of francisellosis in both species following immersion challenged with *Fno* STIR-GUS-F2f7 isolated originally from infected red Nile tilapia in Northern Europe (Ramirez-Paredes, 2015; Ramírez-Paredes *et al.*, 2017).

An experimental immersion challenge was successfully developed with both strains of Nile tilapia. The results suggested that both Nile tilapia strains were susceptible to *Fno* infection. The immersion challenge route was chosen because this method more closely mimics the conditions presented during natural infection. This will therefore be related to the normal transmission of the bacterium *i.e.* horizontal transmission through water or direct contact between fish and exploits the natural portal of bacteria entry into the fish (Soto *et al.*, 2009b). Moreover, it more closely mimics natural disease progression due to the pathogen contacting directly with the innate immune system present in the skins, gills, gastrointestinal mucosa and other organs.

Immersion challenge with *Fno* STIR-GUS F2f7 in this present study resulted in rapid mortality in both tilapia strains tested (homo gold and wild-type), with a cumulative mortality of 55% at a concentration of 1.03×10^7 CFU mL^{-1} within 10 and 14 dpi, respectively for the two strains (Figures 3.4 and 3.5). These cumulative mortality rates are higher when compared with a previous study, even when a lower dose was used (Soto *et al.*, 2009b). The differences could

be due to the variability of bacterial isolates, differences in the age of bacteria when used for the challenge (*e.g.* how long they have been kept frozen, or how successful the bacterial passage was), geographical differences where the bacteria were isolated (how severe the disease outbreaks were), and/or difference in virulence of the *Fno* strain with STIR-GUS-F2f7, or differences in the fish, stocking densities, tank conditions, or water parameters where the challenge was performed.

The surviving fish became carriers of the pathogen as PCR analysis showed low levels of *Fno* 40 d after exposure to the pathogen, as seen in natural infections. Almost all surviving challenged fish showed lesions (granulomas) in the spleen, head and posterior kidneys which caused extensive and direct damage of hematopoietic and osmoregulatory organs (Soto *et al.*, 2009b, 2011b). *Fno* infection may not kill the fish quickly but might weaken the fish's ability to osmoregulate and trigger the fish be immunosuppressed, possibly making the fish immunocompromised and more susceptible to re-infection or co-infection with opportunistic bacteria found in the water system at farm facilities (*e.g. Streptococcus* sp). Cases of co-infection by infectious agents in fish farms (*i.e.* multiple) have been reported in Nile tilapia, including *S. iniae* and *Gyrodactylus niloticus* (Xu *et al.*, 2007)*, F. columnare* and *A. veronii*; *F, columnare*, *A. veronii* and *Iridovirus*; *F, columnare*, *A. veronii* and *V. cholera; F, columnare*, *A. veronii* and *S. agalactiae; F, columnare*, *A. veronii* and *Plesiomonas shigeloides;* and *F. columnare*, *A. veronii, S. agalactiae* and iridovirus (Dong *et al.*, 2015b), *A. jandaei* and *A. veronii* (Dong *et al.*, 2015a, 2017), *F, columnare* and *Fno* (Dong *et al.*, 2016a), and *S. agalactiae* and *Fno* (Assis *et al.*, 2016, 2017). Assis *et al.* (2017) found that the co-infection of Nile tilapia by *S. agalactiae* and *Fno* was caused by the same genotypes previously associated with outbreaks in Brazil and these authors suggested that co-infection was not associated with an adaptation of the pathogens, but with the conditions of chronic hypoxia experienced by the Nile tilapia resulting in stress, immunosuppression and a predisposition to infection.

The findings in the present study are consistent with the clinical signs observed in previous studies, which include lethargy, anorexia and abnormal swimming

during early infection (Soto *et al.*, 2009a; b; Ramirez-Paredes, 2015). In addition, the disease progression was consistent with sub-acute to chronic infection, and thus there was alteration in body pigmentation, petechial haemorrhages, loss of scales, lesions on the skin, frayed fins, pale gills, opacity of the eye and exopthalmia. Interestingly, the most obvious clinical signs found were abrasion on the mouth for both Nile tilapia strains: this had not been observed previously (Chern and Chao, 1994; Chen *et al.*, 1994; Mauel *et al.*, 2007, 2003, 2005; Hsieh *et al.*, 2006; Soto *et al.*, 2009a, 2013c; Jeffery *et al.*, 2010; Iregui *et al.*, 2011; Leal *et al.*, 2014; Ramirez-Paredes, 2015; Jantrakajorn and Wongtavatchai, 2016; Lin *et al.*, 2016; Ortega *et al.*, 2016; Nguyen *et al.*, 2016; Sebastião *et al.*, 2017). In contrast to other reports, the external gross pathology started with the appearance of petechial haemorrhages at 5 dpi in all infected fish of both homo gold and wild-type strains (Figures 3.6 and 3.7) (Hsieh *et al.*, 2006; Soto *et al.*, 2009b, 2013b; Ramirez-Paredes, 2015). This is thought to be due to immersion exposure of the fish to the bacteria used in the present study, and bacteria will be expected to enter through the skin, the first barrier of fish immune system, prior to causing an infection and the appearance of gross signs as a response of the fish to the pathogen, even though this is contrary to previous work (Soto *et al.*, 2009b). However, the skin, gills and gastrointestinal tract of Nile tilapia are the likely to be portals used by *Fno* to enter fish following immersion challenge according to real-time PCR results (Soto *et al.*, 2013b). The gills from both infected Nile tilapia strains were commonly found to be pale in colour, with or without excess mucus production, as well as the presence of white nodules (Mauel *et al.*, 2005; Hsieh *et al.*, 2006; Soto *et al.*, 2009a, 2011b; Tamaru *et al.*, 2011; Ramirez-Paredes, 2015; Ortega *et al.*, 2016; Nguyen *et al.*, 2016).

Gross lesions in the infected Nile tilapia strains in the present study are in line with previous studies that have also observed marked renomegaly and splenomegaly (typically 5–50 times enlarged than normal size) (Figures 3.8 and 3.9) (Chern and Chao, 1994; Mauel *et al.*, 2007). The increases in bacterial numbers in the body tissues also paralleled this to enhance the presence, size and number of granulomas typical of francisellosis, which were found predominantly in the spleen, anterior and posterior kidney segments (Soto *et*

al., 2013b). In the present study, granulomas or white nodules were found in the spleen, anterior and posterior kidney, gills, and mesenteric fat (Figures 3.8 and 3.9). These results are similar to those described previously, especially the mesenteries that were fibrous with multiple fine nodules (granulomas) and adhesions to the viscera (Chen *et al.*, 1994; Soto *et al.*, 2011b; Jantrakajorn and Wongtavatchai, 2016). In the present study enlarged livers were shown to be haemorrhagic or pale and yellowish in colour (Chern and Chao, 1994) with hepatic lipidosis, but no white nodules were present as reported elsewhere for *Fno* infection (Mauel *et al.*, 2003, 2007; Jantrakajorn and Wongtavatchai, 2016). Additionally, some of the infected fish in the present study had ascites with serosanguinous fluid within the abdominal cavity or massive expansion of internal organs such as spleen, anterior kidney and liver, and this is in line to previous research (Jantrakajorn and Wongtavatchai, 2016).

Francisella spp. are capable of penetrating intact skin or penetrating via skin injury to elicit a local inflammatory response with formation of papules and ulcers *in situ*. As a facultative intracellular organism this bacterium can invade parenchymal tissue resulting in the formation of granulomas and abscesses following lymphohematogenous dispersion (Hsieh *et al.*, 2006). In the current study, a chronic general granulomatous infection of both Nile tilapia strains was diagnosed as an infection caused by *Fno* organisms from the results of histopathology, and strongly supported by immohistochemistry and Gram twort investigations. According to Soto *et al.* (2013c), the presence of granulomas in the spleen samples was related to species, for instance Mozambique tilapia *O. mossambicus* were more likely to show granulomas than Wami tilapia *O. hornorum*. Based on histophatology results, in the present study both infected Nile tilapia strains showed similar number of granulomas in the spleen, however, in the head kidney, homo gold displayed more granulomas than wild-type (Figures 3.12 and 3.13).

Moreover, infected tissues showed large numbers of bacteria to be present in macrophages and lymphocytes, which is perhaps not surprising as the genus *Francisella* spp. are facultative intracellular pathogens that require living inside host cells and usually survive in membrane-bound vacuoles (Chern and Chao,

1994; Chen *et al.*, 1994; Soto *et al.*, 2009b). All infected fish of both tilapia strains in the present study showed individual hypertrophied cells and the presence of numerous melanomacrophage centres (MMCs) in the spleen, head and posterior kidneys. MMCs are macrophage aggregates or a group of pigments containing cells which are usually located in lymphoid tissue such as the kidney and spleen, and are also found in the liver, and play an important role in the response to infectious agents (Dong *et al.*, 2016a). In addition, the huge numbers of small pleomorphic cocco-bacilli bacteria mostly presented inside the nucleus of leucocytes including the erythrocytes, and MMCs, confirmed that the organisms were likely escaping from the phagolysosome of the phagocytes and spreading into the cytoplasm (Figure 3.16, 3.19 and 3.20). *Fno* could replicate in the cytoplasmic vacuoles of the MMCs and cause them to turn brown due to the melanisation process. This is the first report that *Fno* can infect tilapia erythrocytes. Furthermore, *F. tularensis* can parasitise erythrocytes *in vivo* during experimental murine infection and persist in human erythrocytes *in vitro* for ≥72 h (Horzempa *et al.*, 2011). Thus, given the long lifespan of erythrocytes *in vivo*, *F. tularensis* could potentially persist in this environment for several months (Horzempa *et al.*, 2011). Still, further research is required to assess whether *Fno* STIR-GUS-F2f7 can invade the nucleus of leucocytes, especially macrophages and/or erythrocytes.

The virulence of *Francisella* spp., has principally been associated with the ability to enter, survive and replicate in phagocytic cells of the innate immune system, such as polymorphonuclear leukocytes and macrophages (Allen, 2003; Golovliov *et al.*, 2003; Lindgren *et al.*, 2004; Clemens *et al.*, 2005; Barker and Klose, 2007; Dai *et al.*, 2011; Steiner *et al.*, 2014). Previous studies have described the intracellular localization, survival and replication of *F. noatunensis* in fish *e.g.* the interaction of *Fnn*, NCIMB 14265^{T} with phagocytic cells of Atlantic cod ncluding the monocytes, macrophages, neutrophils and phagocytic B-cells (Furevik *et al.*, 2011). *F. noatunensis* NCIMB 14265^{T} was found in membrane-enclosed vacuoles of macrophages during the initial phase of infection, and at later stages, apparently surrounded by partially intact membranes (Bakkemo *et al.*, 2011). In addition, the release of bacterial-derived membrane vesicles from intracellular *F. noatunensis* demonstrated an event

suggestive of promoting phagosomal membrane degradation allowing escape of the bacteria to cytoplasm (Bakkemo *et al.*, 2011). *Fnn* was found to replicate within cod leucocytes and inhibit respiratory burst activity, a potent pathogen killing mechanism, in cod leucocytes (Vestvik *et al.*, 2013). Both neutrophils and macrophages uptake *Fno, Fnn* and *F. tularensis* subsp. *novicida* in zebrafish *Danio rerio* embryos, where macrophages are the main site of replication (Brudal *et al.*, 2014). The internalization and replication of *Fnn* were demonstrated in primary monocyte/macrophage cultures and an epithelial-like cell line derived from Atlantic cod larvae cells (Bakkemo *et al.*, 2016). Moreover, *Fno* LADL 07-285A demonstrated efficient intracellular survival and replication within both 4 h-head kidney-derived macrophages (HKDM) and 5 d-HKDM from tilapia, and eventually killed the host cell by inducing necrosis (Soto *et al.*, 2010c). Meanwhile, *Fno* LADL 07-285A and two *Fno* knockout intracellular growth loci C (*iglC*) strains were demonstrated with replication in the endothelial *O. mossambicus* bulbus arteriosus cell line (Soto *et al.*, 2017). The beginning of a degenerative phase in granulomas that progressed to more widespread coagulative necrosis by *Francisella* spp., signified by the appearance of necrotic macrophages or circulating leucocytes, allows the replicating bacteria to escape target cells when nutrients become limited (Soto *et al.*, 2013b).

The study of the biochemical and the phenotypic properties of bacteria has mainly been used for bacterial classification and is commonly been based on their growth characteristics and utilization of specific substrates (Cohan, 2002). Biochemical tests applied mainly in the characterization of the *Fno* include the fermentation of carbohydrates and enzymatic activities using standardised biochemical tests notably API 20E and API ZYM kits (Birkbeck *et al.*, 2007, 2011; Ottem *et al.*, 2009; Mikalsen and Colquhoun, 2010; Ottem, 2011; Brevik *et al.*, 2011b; Ramirez-Paredes, 2015). However, other researchers have described difficulty in interpretation using the API 20E or ZYM systems and the results are sometimes not accurate or reliable when using commercial kits (Mikalsen *et al.*, 2007, 2009). Several factors could influence the differences in the results when using the kits, especially for Gram positive bacteria *e.g.,* the variability of bacterial isolates / strains, there may be a lack of useful

information or no information available in the existing databases, differences in the age of bacteria and concentration of the bacterial inocula used, and variable incubation temperature for enzyme reactions (Ravelo *et al.*, 2001; Evans *et al.*, 2002; Vandamme *et al.*, 2016). Moreover, according to O'Hara, (2005) misidentification using such kits has occurred and these kits are not optimized for *Francisella* bacteria. These results also highlight the need for reliable methodologies to characterise these bacteria and eventually establish minimum standards for the delineation of species and subspecies within this genus (Wongsathein, 2012).

Fno isolate STIR-GUS-F2f7 grew well on CHAH which is consistent with previous studies, even though it grew slowly on the agar (*e.g.* 3 d for *Fno* recovery from passages and 8 d from the challenged trial) (Soto *et al.*, 2009a; b, 2013b; Jeffery *et al.*, 2010; Leal *et al.*, 2014; Ramirez-Paredes, 2015; Jantrakajorn and Wongtavatchai, 2016; Lin *et al.*, 2016; Ortega *et al.*, 2016). Therefore, CHAH was deemed to be a suitable agar for primary bacterial recovery in natural outbreaks as well as for use in laboratory testing. The *Fno* STIR-GUS-F2f7 was re-isolated in pure culture from the experimentally immersion challenged fish from infected head kidney and spleen tissues in both Nile tilapia strains and tests confirmed that this bacterium was the causative agent of the disease outbreaks. Although the intracellular cocco-bacillus in tilapia was successfully recovered on CHAH, its isolation was difficult due to the presence of a wide range of bacterial contaminations in the inocula at primary isolation (Figures 3.3 and 3.10).

The common growth of mixed infections/infiltration of environmental or host bacteria is a particularly relevant problem in diagnostic work. Currently, there is no selective agar medium for the re-isolation of *Francisella* species, especially for *Fno* from various samples. Therefore, a CHAH (cysteine heart agar suplemented with bovine haemoglobin) with antibiotic supplementation could be useful for isolating *Fno*. Several researchers have published selective agars for reisolation of *Francisella* species, including a CHAH containing colistin, amphotericin, lincomycin, trimethoprim and ampicillin for selection of *F. tularensis* (Petersen *et al.*, 2004, 2009); *F. philomiragia* from environmental

material (Berrada and Telford, 2010); including fungizone® with ampicillin were used successfully for isolation of *Fnn* (Brevik *et al.*, 2011a). Moreover, some antibiotics were added into CHA but with different supplementation of blood, to recover *Fno* from infected fish*, e.g.* CHA supplemented with rabbit blood, and polymixin B with and without ampicilin) (Soto *et al.*, 2009a); CHAH, with polymixin B (100 units mL^{-1}) and ampicillin (50 µg mL^{-1}) were added when needed to make the primary isolation media selective, which aided in recovery of the bacteria from infected fish tissues; and kanamycin (15 µg mL^{-1}) (Soto *et al.*, 2009a; b) and/or tetracycline (10 µg mL^{-1}) (Soto *et al.*, 2010c) were used for recovery of transformed *Fno* following electroporation; CHAH with ampicillin alone (Lewisch *et al.*, 2014), and CHA supplemented with 10% sheep blood and polymixin B (100 units mL^{-1}) (Nguyen *et al.*, 2016). However, selective agar medium could be used to differentiate and identify *Fno*, nevertheless further identification and characterisations are required, and such a test should not be used alone. At the completion of the experimental challenge, no *Fno* was re-isolated on CHAH while very weak bands were detected by PCR from surviving fish.

Molecular techniques, such as a PCR, are increasingly used to identify many different bacterial pathogens including *Francisella* sp., especially *Fno* (Mauel *et al.*, 2003; Hsieh *et al.*, 2007; Soto *et al.*, 2009a; Jeffery *et al.*, 2010; Duodu *et al.*, 2012; Hansen *et al.*, 2013; Leal *et al.*, 2014; Ramirez-Paredes, 2015; Dong *et al.*, 2016a; Jantrakajorn and Wongtavatchai, 2016; Lin *et al.*, 2016; Ortega *et al.*, 2016; Nguyen *et al.*, 2016). This method could be used as an alternative method in routine diagnosis for specific detection and identification of the pathogen from various sources, due to its accuracy, sensitivity, and low cost. In addition, PCR provides results more quickly than conventional bacterial identification methods. The presence of pathogens may be able to be detected at earlier stages of infection, and in carrier fish tissue, when the number of bacteria in tissues may be very low. In the present study, almost all freshly dead and moribund fish produced the same product size expected for *Fno* STIR-GUS-F2f7. PCR is regarded as useful in the confirmation of *Fno* within piscine francisellosis infections (Mauel *et al.*, 2003; Hsieh *et al.*, 2007; Soto *et al.*, 2009a; Ortega *et al.*, 2016).

However, in the current study although 10 of 10 and 4 of 5 freshly dead and moribund fish yielded positive results, the intensity of bands in 3 and 1 cases was weak for homo gold and wild type, respectively; while one band from a wild-type was almost undetectable. Interestingly, the surviving fish from both Nile tilapia strains at 40 dpi showed very weak bands, indicating that the fish might still be a carrier but with insufficient DNA to detect by PCR. Like other diagnostic techniques, PCR also has some limitations such as limits on specificity and sensitivity, results being based on band-size discrimination and measured at end-point (plateau) only, and lack of quantification when compared to RT-PCR (Duodu *et al.*, 2012; Soto *et al.*, 2013c; Ramirez-Paredes, 2015). Therefore, inclusion of further molecular technologies *e.g.* RT-PCR might have added value to the present study to quatify levels of *Fno*.

Closely related fish species of the same genus can demonstrate varying degrees of susceptibility to bacterial diseases. For example, based on PCR and RT-PCR analysis, Mozambique tilapia co-cultured with Koilapia is more susceptible to *Fno* infection (and develop granulomas in the spleen) than the Koilapia (Soto *et al.*, 2013c). Similarly, Mozambique tilapia were found more susceptible to piscine francisellosis than Blue tilapia and Koilapia, as only Mozambique tilapia resulted in viable *Fno* in the spleen of surviving fish (based on RT-PCR), displayed consistent mortalities, and had granulomas (Klinger-Bowen et al., 2016). Based on observation during the challenge and cumulative mortalities, in the present study, Nile tilapia homo gold was more susceptible than wild-type tilapia to *Fno* STIR-GUS-F2f7, since homo gold exhibited signs of infection and mortalities earlier (from day 5) than wild-type (day 7) (Figures 3.4 and 3.5). Nile tilapia homo gold reached cumulative mortality of 55% within 10 dpi and wild type at 14 dpi. In addition, Nile tilapia homo gold had more granulomas in both spleen and anterior kidney than wild-type. The identification of resistant and susceptible populations of tilapia could be useful to identify genes related to the resistance against this disease (Klinger-Bowen *et al.*, 2016).

3.5 Conclusion

In the present study, an experimental immersion challenge model for *Fno* was established and this provided data regarding the susceptibility of different genetic groups of Nile tilapia homo gold and wild-type (the most important tilapia farmed species worldwide) to *Fno* STIR-GUS-F2f7. Both Nile tilapia strains were susceptible to francisellosis, with homo gold showing higher susceptibility which resulted in faster disease progression and more granulomas in both spleen and anterior kidney than wild-type. The *in vivo* challenge model developed will be useful in testing the bioactive algal compounds, including those previously assessed *in vitro* (Chapter 2).

CHAPTER 4

DIETARY ADMINISTRATION OF ALGAL-DERIVED COMPOUNDS TO NILE TILAPIA HOMO GOLD *Oreochromis niloticus* L **CHALLENGED WITH BACTERIAL PATHOGENS**

4.1 Introduction

Tilapia aquaculture is a very fast-developing industry worldwide (Chapter 1, Section 1.1). Unmanaged fish culture practices, the intensification of culture and adverse environmental conditions do, however, affect fish health leading to high numbers of infectious and non-infectious disease outbreaks and production losses. Recently, outbreaks of piscine francisellosis, caused by *Fno,* have been reported in a wide variety of warm fish species, and especially in tilapia aquaculture worldwide (see Chapters 1 and 2). The use of antibiotics has been recommended to control francisellosis in tilapia, including oxytetracycline (Chen *et al.*, 1994; Mauel *et al.*, 2003; Iregui *et al.*, 2011), and oral administration of florfenicol in Nile tilapia (Soto *et al.*, 2010a, 2013a) was shown to significantly reduced mortalities (see Chapter 1, Section 1.4.3). The emergence of antibiotic-resistant microorganisms, however, is an important obstacle to their extensive use (Miranda and Zemelman, 2002). The utilization of chemotherapeutants, chemical disinfectants and antibiotics for controlling disease has been widely criticized as it leads to a negative impact, including residue accumulation in the tissue, development of the drug resistance and immunosuppression, thus resulting in reduced consumer preference for food fish treated with antibiotics (Miranda and Zemelman, 2002), as well as concerns over the environment and wildlife protection.

Prevention of disease is much more desirable than intervention to stop and reverse the disease process once it begins. The use of vaccines, dietary supplement of probiotics, prebiotics and immunostimulants may help to reduce the susceptibility of fish to diseases (Meena *et al.*, 2013). There are currently no commercial probiotics, prebiotics or vaccines to prevent piscine francisellosis. However, vaccines are under development as described in Chapter 1, Section 1.4.4 (Soto *et al.*, 2009b; Lagos *et al.*, 2017). Although vaccination has been shown to be an effective prophylactic method for disease control in fish (Midtlyng *et al.*, 1996), there are some methodological problems related to high costs, labour intensive process, stress and the often-undesirable side-effects of the adjuvants incorporated in injectable vaccines (Midtlyng *et al.*, 1996; Ellis, 1999). Hence, instead of chemotherapeutic agents, probiotics, prebiotics and

vaccines, one of the most promising methods for controlling diseases in aquaculture is strengthening the defence mechanisms of fish through prophylactic administration of immunostimulants (Robertsen, 1999; Meena *et al.*, 2013).

Immunostimulants are naturally occurring compounds that modulate the immune system by promoting phagocytic cell function, increasing their bactericidal activity and/or non-specific cytotoxic cells and antibody production, increasing host resistance to infectious pathogens, and they have been widely used in aquaculture (Sakai, 1999; Ai *et al.*, 2007; Selvaraj *et al.*, 2009; Ringø *et al.*, 2010). Already, remarkable success has been achieved using immunostimulants as an environmentally friendly approach to disease management, as they have few unwanted effects on the host and can be given as a feed additive (Sakai, 1999; Peddie *et al.*, 2002; Raa, 2013). Several compounds, including plant extracts, bacterial polysaccharides, chitin, ß-glucans, and variety of alga-derived compounds have been used to enhance immunity and disease resistance in a number of fish species. ß-glucan administration has been reported to augment antibody production, complement activity, lysozyme activity, phagocytic activity and respiratory burst in channel catfish *Ictalurus punctatus* (Chen and Ainsworth, 1992), Atlantic salmon *Salmo salar* (Solem *et al.*, 1995), carp (Vera-Jimenez *et al.,* 2013) and striped catfish *Pangasianodon hypophthalmus* (Sirimanapong *et al.*, 2015). Recently, various studies on the utilization of algae to treat various diseases both in mammals and fish have proven algae as a potent, valuable and promising immunostimulant for improving immune status and controlling disease, especially in fish culture, as described in Chapter 1, Section 1.6.

In the present study (Chapter 2), *in vitro* screening of bioactive algae compounds was performed, and the results showed that ß-glucan, alginic acid and *Chlorella* sp. had positive immunostimulatory and antibacterial activities against three fish bacterial pathogens (*Fno* and *S. agalactiae*). The aim of this Chapter was to determine the potential of two of these algal-derived compounds (ß-glucan and alginic acid) in disease management, in particular against *Fno, in vivo* in Nile tilapia. Two other additional algae (*Scenedesmus*

quadricauda and *Haematococcus pluyvialis*) were also tested. The *in vivo* trials included evaluation of the growth and survival of tilapia when were fed such products, as well as their effect on non-specific immune parameters (tested using macrophages isolated from fish treated fed algae supplemented diets) and their resistance against Francisellosis (using the immersion challenge model developed in Chapter 3). In addition, the effect of co-infection with Streptococcocis caused by *S. agalactiae* in the fish surviving *Fno* challenge was also determined.

4.2 Materials and methods

4.2.1 Bacterial strains

Fno STIR-GUS-F2f7 and *S. agalactiae* fish isolates were used in this study; they were cultured and prepared for the challenges as described in Chapter 2, Sections 2.2.1.1 and 2.2.1.3.

4.2.2 Experimental animals

Nile tilapia homo gold fingerlings were obtained from the same source and parents as fish used in Chapter 2, Section 2.2.2. The tilapia were fed and maintained, as described in Chapter 2, Section 2.2.2. Fish weighing 15±2 g were used. All animal experiments were approved by the Animal Welfare and Ethical Review Body (University of Stirling, UK) in accordance with the Animals (Scientific Procedures) Act 1986 and complied with local institutional regulations (Chapter 3, Section 3.2.5).

4.2.3 *In vivo* trials in Nile tilapia homo gold

Experimental trials were performed where fish were fed algal-derived products and then challenged with *Fno*. The survivors were then challenged with a co-infection with *S. agalactiae.*

4.2.3.1 Experimental fish feed

A. Algae

Two freeze-dried samples of whole dried microalgae as well as two algal-derived compounds were used: *H. pluvialis* (red algae) and *S. quadricauda* (green algae) kindly provided by Algamax™; and two algal compounds (alginic acid from *M. pyrifera* and ß-glucan from *E. gracillis*, as described in Chapter 2, Section 2.2.3.1). The whole freeze-dried algae were made into a fine powder using a blender (Blender 8011EG, Waring®, USA), while the algal compounds underwent no pre-treatment prior to addition to the fish feed.

B. Preparation of experimental fish diets

Seven different diets were made up according to Levine and Villamar (2015), with algal powder or compounds combined with 37.1% protein commercial trout premix (Skretting standard expanded 60, UK) which is routinely used on fish farms, 40% water and the binder carboxymethyl cellulose (Sigma-Aldrich, UK) at 3% inclusion rate. Briefly, the commercial diet was crushed using a Waring blender and then mixed together with each of the algal powders or compounds in a Hobart mixer at the desired concentration (10% for *H. pluvialis* and *S. quadricauda*, and two different concentrations, 0.1 and 0.2%, for ß-glucan and alginic acid for each compound) for 15 mins. Then tap water was slowly added and the feed mixed for a further 15 mins to form a soft dough. The diets were then cold extruded (re-pelleting) through a pellet machine (California Pellet Mill Co; San Francisco, Calif, Crawfordsville, IND) into an appropriately sized pellet (3 mm diameter) (Figure 4.1). The resulting feed was then allowed to air dry at RT with a portable heater, dried and stored at 4˚C until further use.

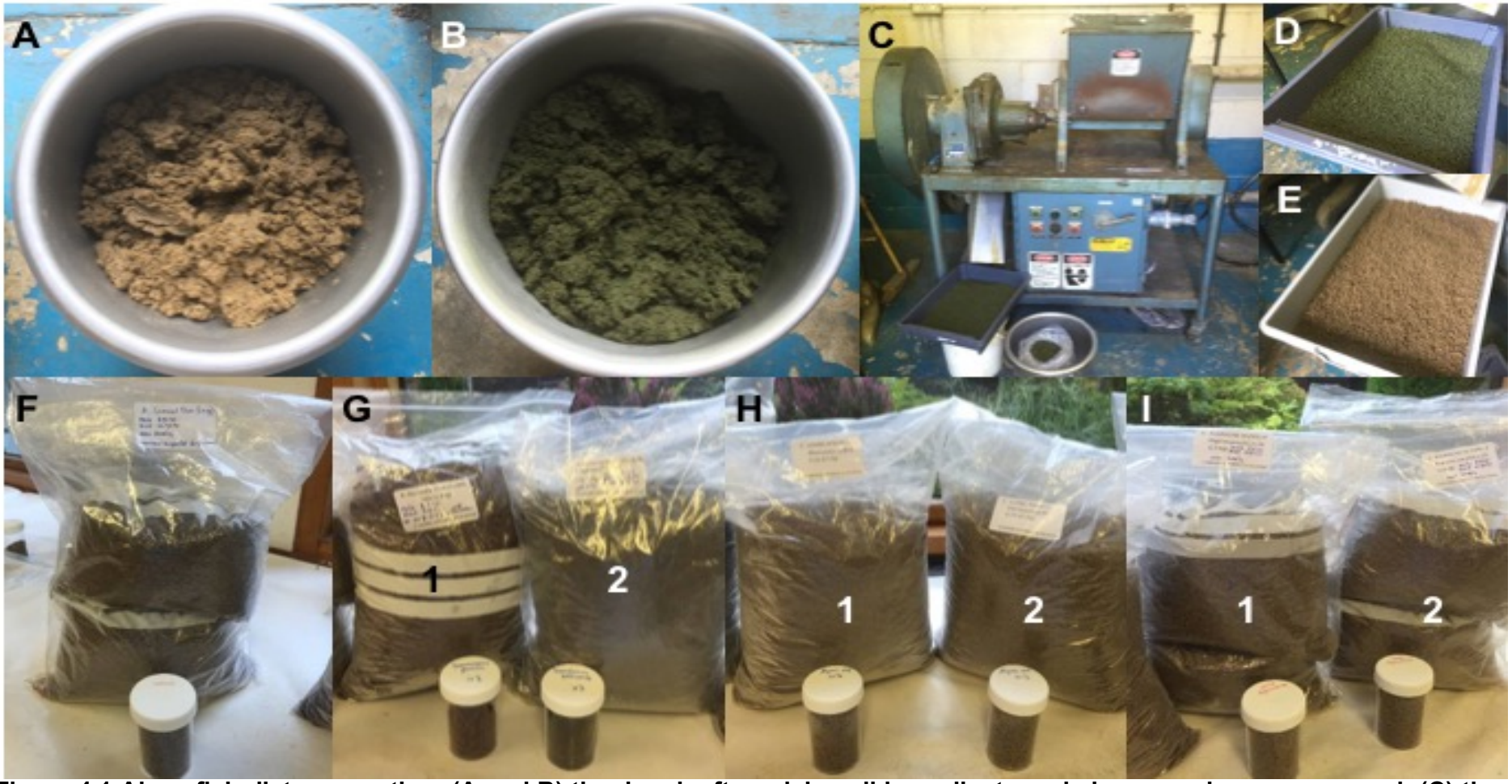

Figure 4.1 Algae fish diet preparation: (A and B) the dough after mixing all ingredients and algae powder or compound; (C) the dough then cold extruded (re-pelleted) through a pellet machine into proper sized of pellets; (D) *S. quadricauda* and (E) control pellets ready for drying; (E–H) the diets ready to use (at least 2.5 kg for each diet), (E and F) control diet without algae, (G) *H. pluvialis* diet (1) and *S. quadricauda* (2), (H) alginic acid at 0.1 (1) and 0.2% (2), and (I) ß-glucan at 0.1 (1) and 0.2% (2).

4.2.3.2 Experimental design

A. Fish husbandry

Naïve Nile tilapia homo gold fingerlings were fed the different diets and used in the challenge trials. Fish were cultured for 7 months to reach a suitable size (15±2 g), as described in Chapter 3, Section 3.2.4. Prior to the trials, a total of 21 tanks 120 L round plastic aquaria were each stocked with 45 fish per tank, and each tank was provided with individual filters and air stones for aeration in a recirculation water system (Figure 4.2). The fish were confirmed to be free from bacteria before use in this study, particularly from *Francisella* spp., and *S. agalactiae* infection, as described in Chapter 3, Section 3.2.4. Water temperature was maintained at 27±2°C.

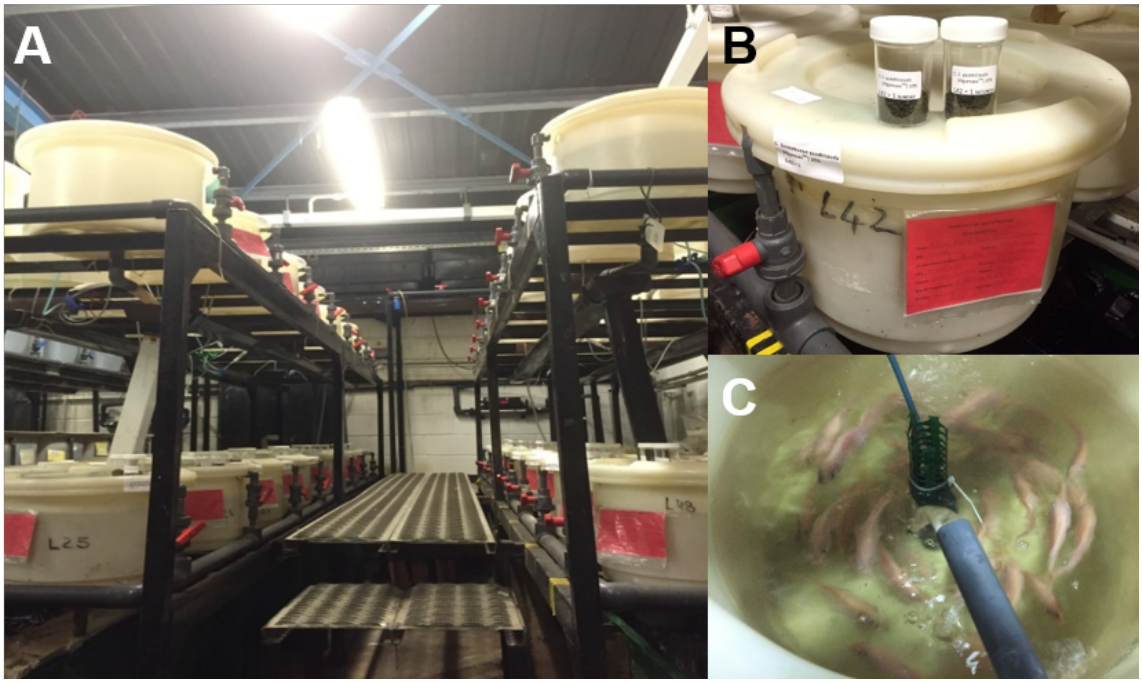

Figure 4.2 Installations of 21 tanks 120 L round plastic aquaria were used for algae feeding trials in Nile tilapia homo gold.

B. Feeding regimes and sampling

Seven treatment groups (triplicate group of 45 tilapia per groups) were used. These comprised non-supplemented commercial diet (negative control group), commercial diet containing *H. pluvialis* or *S. guadricauda* at 10% and diet containing 0.1 or 0.2% of alginic acid or ß-glucan. The fish were fed two times per day (at 10.00 am and 16.00 pm) at 3% body weight for 28 d (plus 7 d of feed acclimation prior to the trial).

Blood and head kidney from 3 fish per group were sampled for functional assays, lysozyme and respiratory burst activities, at 0, 7, 14, 21 and 28 d post algae feeding (dpf).

C. Respiratory burst activity

Macrophages were isolated and respiratory burst activity was measured (as described in Chapter 2, Sections 2.2.3.3, and 2.2.3.5C, respectively. The macrophage concentrations were adjusted to 1 x 10^7 cells mL^{-1} with L-15 medium (with phenol red), 100 µl of cells were placed into a well of sterile 96 wells tissue culture plate and at least three wells were prepared for each algae diet group.

D. Lysozyme activity

Plasma lysozyme activity was measured by the turbidimetric method (Parry *et al.*, 1965; Ellis, 1990). Briefly, *Micrococcus lysodeikticus* (M3770, Sigma-Aldrich, UK) was used as substrate at a concentration of 0.2 mg mL^{-1} in 0.04 M sodium phosphate buffer, which contained 0.04 M solution of $NaH_2PO_42H_2O$ (monosodium phosphate) (71500, Sigma-Aldrich, UK) and 0.04 M solution of $Na_2HPO_42H_2O$ (sodium phosphate) (30412, Sigma-Aldrich, UK) in distilled water, at pH 5.8. One hundred and ninety microliters of substrate were added to 10 µL of plasma in triplicate wells in a 96 wells microtiter plate (Sterilin™ Clear Microtiter™ Plates, ThermoFisher Scientific, UK). Optical density was measured at 540 nm after 1 and 5 mins using a Microplate Reader (Chapter 2, Section 2.2.3.4). The average of all replicates for each sample at the 0 and 5

mins reading were then calculated using the equation of [(0 average – 5 averages) *5)/100] then multiplying by 1000 (*1000). A unit of lysozyme activity was defined as the amount of sample causing a decrease in OD of 0.001 min^{-1}.

4.2.3.3 Experimental challenge with *Fno*

At 28 dpf, fish were transferred into 10 L tanks with individual filters and air stones for aeration in a flow-through system with UV-treated water in the Aquatic Research Facility (ARF) (Institute of Aquaculture, University of Stirling, UK) where they were subjected to challenge by immersion with *Fno* (Figure 4.3). A virulent strain of *Fno* STIR-GUS-F2f7 isolated from infected Nile tilapia homo gold head kidney from the second passage (Chapter 3, Section 3.3.2) was cultured, collected, washed, adjusted to the desired AU value A_{600} in PBS, and drop counts were performed on CHAH, as in Chapter 2, Section 2.2.1.2. Each group of tilapia, 24 fish $tank^{-1}$ (25±2 g) were immersed in a dose of *Fno* (1 x 10^5 CFU mL^{-1}) in 3 L of water in static tank of 10 L capacity with aeration for 3 h. The fish were maintained for 35 d at 23±2˚C. The fish were fed with the diets two times per day (at 10.00 am and 16.00 pm) at 1% body weight during the challenge trial. Clinical signs of disease and mortalities were monitored and recorded at least 4 times per day during the experiment. Dead and moribund fish were examined for external and internal clinical signs and sampled by head kidney swabs for bacterial recovery (Chapter 3, Section 3.2.6). At the end of experiment (35 d), blood samples were collected from three surviving fish per tank to determine specific antibody titre. The rest of surviving fish were continued in the infection with *S. agalactiae* (Section 4.2.3.4C).

Figure 4.3 Installations of 21 tanks 10 L plastic aquaria used for *Fno* challenge trial and continued co-infection with *S. agalactiae* for surviving Nile tilapia homo gold for 21 d in ARF Institute of aquaculture (University of Stirling, UK). From top left to rigt then below left to right: (A) fish fed with control diet, (B) 10% *H. pluvialis,* (C) 10% *S. quadricauda*, (D and E) 0.1% and 0.2% alginic acid, respectively, and (F and G) fish fed with 0.1 and 0.2% ß-glucan, respectively.

ELISA was used to quantify the specific antibody development against *Fno* following challenge. Ninety-six-well microtiter plates were coated with 100 µL of 1% w/v poly-L lysine (P8920; Sigma-Aldrich, UK) in carbonate-bicarbonate buffer (C3041; Sigma Life Science, UK) and incubated for 60 mins at RT. The plate was then washed three times with low salt wash buffer (LSWB): 100 µL well^{-1} of *Fno* re-suspended in PBS (at 0.4 AU A_{600} = 1 x 10^8 bacteria mL^{-1}) were then added into each well and incubated overnight at 4˚C. The LSWB contained trisma base (T-1503; Sigma-Aldrich, UK), NaCl (S-9625; Sigma-Aldrich, UK), and Tween 20 at pH 7.3. The bacteria were fixed with 50 µl well^{-1} of 0.05% v/v of glutaraldehyde (G-6403; Sigma-Aldrich, UK) in LSWB and incubated for a further 20 mins at RT, followed by discarding the contents of the plates, which were then washed three times with LSWB. A 100 µl well^{-1} of hydrogen peroxide (H_2O_2) (1:10 of 30% stock solution) was added to prevent non-specific reactions, incubated for 1 h, followed by discarding the contents and the plate was washed 3 times with LSWB. Non-specific binding sites were then blocked with 300 µL well^{-1} of 4% (w/v) casein (dried milk "Marvel", UK) in distilled water and incubated for 3 h, at RT or overnight at 4˚C, followed by washing 3 times with LSWB.

Doubling-dilutions of fish serum in PBS were prepared starting at 1/32 dilution, and 100 µl well^{-1} added to triplicate wells, and incubated for 3 h, at RT or overnight at 4˚C. Controls were included on each plate (pre-immune serum or serum from healthy fish and PBS as negative controls, while sera from infected fish were used as positive control). The plates were washed 5 times with High Salt Wash Buffer (HSWB); this was composed of the same ingredients as LSWB but had a higher salt concentration and Merthiolate was added for long term storage (T-5125; Sigma-Aldrich, UK) at pH 7.7. Before removing the contents, the plate was incubated with the last wash with HSWB for 5 mins. A 100 µL well^{-1} of anti-fish IgM monoclonal antibody (anti-tilapia 1/75 in PBS) (Aquatic Diagnostic Ltd, UK) was then added and incubated for 1 h, at RT, followed by further washing. Next, a 100 µl well^{-1} of goat anti-mouse IgG-HRP (A4416; Sigma Life Science, UK) diluted 1:1000 in conjugate buffer, 1% w/v bovine serum albumin (A9647, Sigma Life Science, UK) in LSWB, was added and the plate incubated for 1 h, at RT. The plates were then washed 5 times

with HSWB as before and incubating for 5 mins on last wash, then incubated with a 100 µL well^{-1} chromogen in substrate buffer (containing H_2O_2 substrate), for 2–3 mins at RT. The chromogen comprised 42 mM 3'3'5'5'tetramethylbenidine dihydrochloride (T-0440; Sigma Life Science, UK) and acetic acid (Fisherbrand®, Thermo Scientific, UK). Substrate buffer contained citric acid and sodium acetate (30104, BDH, UK). The enzymatic reaction was stopped by adding 50 µL well^{-1} of 2M H_2SO_4 (sulphuric acid) (10276, BDH, UK). The absorbance was measured using a micro-plate reader (ELISA reader) at OD 450 nm (Chapter 2, Section 2.2.3.4). Blank ELISA reader was used against wells filled with chromogen and stop solution.

4.2.3.4 Experimental infection with *S. agalactiae*

Prior to the co-infection trial, *S. agalactiae* was passaged to enhance the virulence of bacteria and pre-challenge experiments were performed to determine the LD_{40}.

A. Bacterial passage

The passage was performed by IP injecting fish with 0.1 mL of a high concentration (1.5 A_{610}) of *S. agalactiae* using one fish, and the passage was conducted twice (as described in Chapter 3). The *S. agalactiae* isolate was cultured, collected and washed in PBS, and drop counts performed on TSA as described in Chapter 2, Section 2.2.1.2 and 2.2.1.3. Sacrificed fish were sampled for bacterial recovery by streaking the infected tissues (*e.g.* brain, eye, head kidney and spleen) using a sterile plastic loop onto TSA, and incubated at 28°C for at least 48 h. The isolates recovered from infected tissues from the first and second passages were purified as required by picking and sub-culturing uniform colonies consistent with *S. agalactiae* morphology onto a new TSA using the culture conditions, as described previously (Chapter 2, Section 2.2.1.1). Primary identification tests were applied to determine the identity of the colonies and TSB was used to propagate isolate for the experimental challenge studies, and the frozen stock in protect beads were prepared for later use (as Chapter 2, Section 2.2.1.1).

B. Pre-challenge

A group of tilapia, fifteen tilapia (30±2 g) were placed into each of a series of 10 L tank in a flow-through system. The fish then challenged by IP injection with three different doses of *S. agalactiae i.e.* 1 x 10^6, 1 x 10^7 and 1 x 10^8 CFU $fish^{-1}$ without replication. Each fish received 0.1 mL of bacteria were maintained for 30 d at 25±2°C. Clinical signs and mortalities were monitored and recorded at least 4 times per day during the experiment. Fish showing clinical signs and/or moribund fish were sacrificed. Dead and moribund fish were sampled for bacterial recovery and identification (as described in Section 4.3.3.2C). At the end of the experiment survivors were sacrificed.

C. Co-infection challenge with *S. agalactiae*

Fish with francisellosis infections often die from secondary co-infections with Streptococcocis. Thus, fish fed the different diets that had been challenged and survived a *Fno* challenge were challenged with *S. agalactiae* (at day 51). Surviving tilapia from all treatment group replicates were adjusted to 19 fish per group and maintained in the co-infection trial for 21 d at 25±2˚C. The fish were subjected to co-infection with *S. agalactiae* by IP at 5 x 10^5 CFU $fish^{-1}$. The bacterial challenge dose was selected based on the dose response of *S. agalactiae* (Sections 4.2.3.4B and 4.3.3.2A). Clinical signs and mortalities were monitored and recorded at least 4 times per day during the experiment. Dead and moribund fish were sampled by brain, eye, spleen and head kidney swabs for detection of *S. agalactiae*. At the end of the experiment (21 d) survivors were killed, sampled and disposed of in accordance with the animal protection Schedule 1.

4.2.4 Statistical analysis

Data were examined using a one-way ANOVA, general linear model, and Tukey's test for pairwise comparison of means. All statistical tests were performed using Minitab software (Version 16) under licence to the University of Stirling, except survival analysis. The percentage of survival was calculated for each group. Survival differences between treatments groups of the algae diets, were compared for significance with the logrank test using the statistical package R where desired. A p-value of less than 0.05 was considered to indicate a significant difference between groups. Statistical differences were examined between groups at each time sampling point and within groups over time.

4.3 Results

Experimental trials were performed *in vivo* to determine the potential of algal-derived compounds in disease management. Nile tilapia were fed different algal-derived products (ß-glucan and alginic acid pre-tested *in vitro* in Chapter 2 and two other algae, *S. quadricauda* and *H pluvialis*) and then fish were experimentally challenged with *Fno* 28 dpf for a period of 35 d using an immersion challenge (developed in Chapter 3). As fish with francisellosis infection often die from secondary co-infections with Streptococcosis the survivors of the *Fno* challenge were co-infected with *S. agalactiae* for another 21 d.

Respiratory burst and lysozyme activities were measured following feeding, while cumulative mortality and specific antibody titres were measure following the *Fno* and *S. agalatiae* challenges.

4.3.1 Response following algal feeding

4.3.1.1 Respiratory burst activity

No significant differences were observed in the respiratory burst activity of Nile tilapia homo gold HK macrophages fed different experimental diets compared to control groups, at any of the time points measured, with or without PMA, with high variation in the results observed at 14 dpf (Figure 4.4). The group fed with 0.2% Ag50 (ß-glucan) did show a significantly lower respiratory burst activity than fish fed with the diet supplemented with 0.2% Aa (alginic acid).

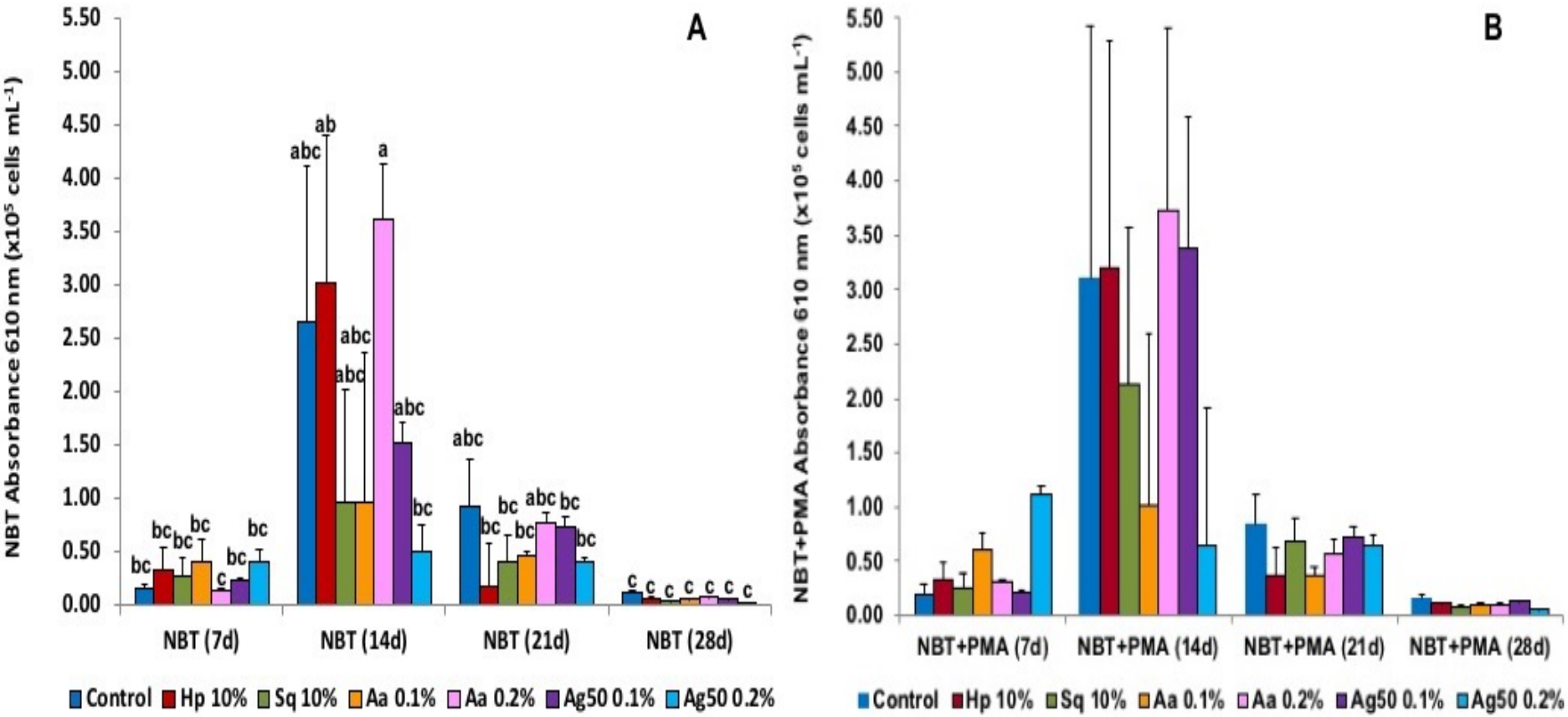

Figure 4.4 Comparison of respiratory burst activity by head kidney macrophages from Nile tilapia homo gold fed with different algae diets over 28 d: (A) NBT, and (B) NBT+PMA. The algae diets included a control diet without algae (Control/dark blue bar) *H. pluvialis* 10% (Hp 10%/red bar), *S. quadricauda* (Sq 10%/green bar), alginic acid 0.1% (Aa 0.1%/orange bar), alginic acid 0.2% (Aa 0.2%/pink bar), ß-glucan 0.1% (Ag 0.1%/purple bar), and ß-glucan 0.2% (Ag 0.2%/light blue bar). Values are presented as mean of triplicates per group±SD. Groups that do not share a letter are significantly different.

4.3.1.2 Lysozyme activity in plasma

Lysozyme activity in the Nile tilapia homo gold showed high variability during the algae feeding trial (Figure 4.5). There was no significant difference in plasma lysozyme activity were found among groups at any time point or within any experimental group over time.

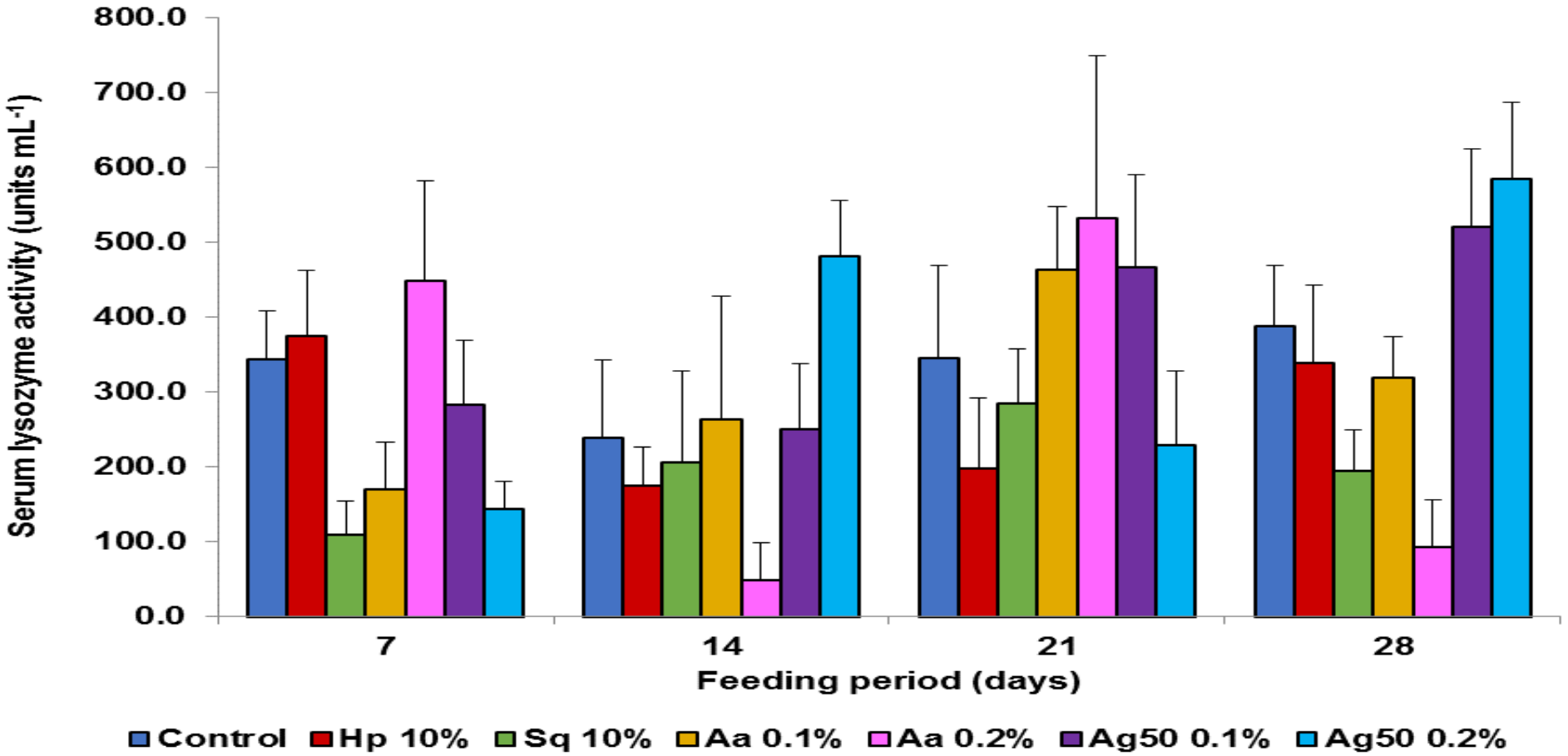

Figure 4.5 Lysozyme activity in serum from Nile tilapia homo gold fed with different algae diets (control/dark blue bar; Hp 10%, *H. pluvialis*/red bar; Sq 10%, *S. quadricauda*/green bar; Aa, alginic acid at 0.1 and 0.2%/orange and pink bars, respectively; and Ag50, ß-glucan at 0.1 and 0.2%/purple and light blue bars, respectively) over 28 d. Values are presented as mean of triplicates per group±SD. No significant different between the algae dietary treatment groups was seen compared to normal control diet.

4.3.2 Survival of fish fed different diets following challenge with *Fno*

4.3.2.1 Cumulative survival

Percentage cumulative survival of Nile tilapia homo gold after immersion challenge with *Fno* is shown in Figure 4.6. Based on the drop count results, the actual concentration of *Fno* used was ~1.05 x 10^5 CFU mL^{-1}. Five days post infection, typical external gross pathology caused by *Fno* infection was observed (as seen previously, Chapter 3, Section 3.3.4.2) in almost all fish, with the appearance of petechial haemorrhages on the skin and surrounding the mouth of infected fish. Fish, however, then recovered with few mortalities. At the end of the experiment, no significant difference in cumulative survival between dietary groups was observed.

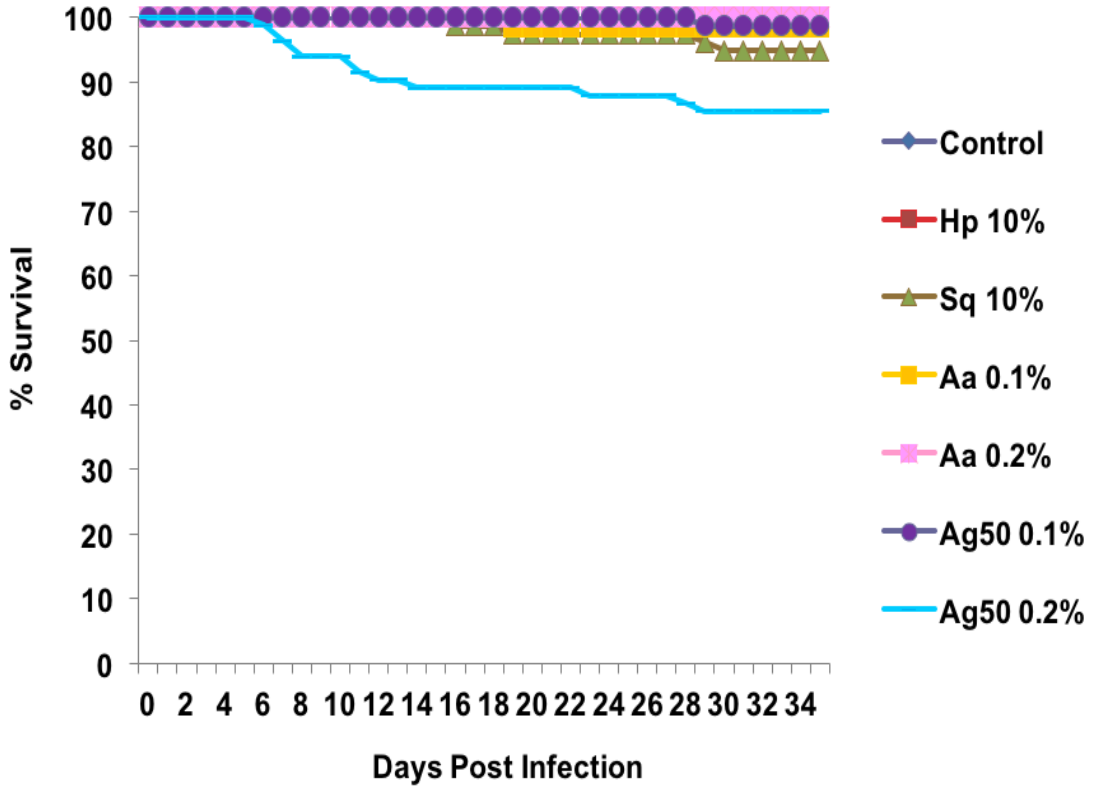

Figure 4.6 Cumulative percentage survival of Nile tilapia homo gold, which fed with different algae diets (*i.e.* Hp/*H.pluvialis* 10%, Sq/*S. quadricauda* 10%, Aa/alginic acid at 0.1 and 0.2%, Ag50/ß-glucan at 0.1 and 0.2%) for 28 d then challenged by immersion with *Fno* at 1.05 x 10^5 CFU mL^{-1} for 3 h and maintained for 35 d. No significant difference observed between algae dietary groups compared to control diet.

4.3.2.2 *Fno*-specific antibodies in plasma

The *Fno*-specific antibody (IgM) titres in plasma of *Fno* infected Nile tilapia homo gold had increased significantly by 35 d in the control dietary (*Fno* infected) group ($p>0.001$) compared to the control dietary uninfected group as expected (Figure 4.7). These significant differences were also observed in the group fed with alginic acid 0.1% ($p>0.0028$), 10% *H. pluvialis* ($p>0.0131$), 10% *S. quadricauda* ($p>0.0148$), and ß-glucan 0.1% ($p>0.0408$) diets and then challenged with *Fno*.

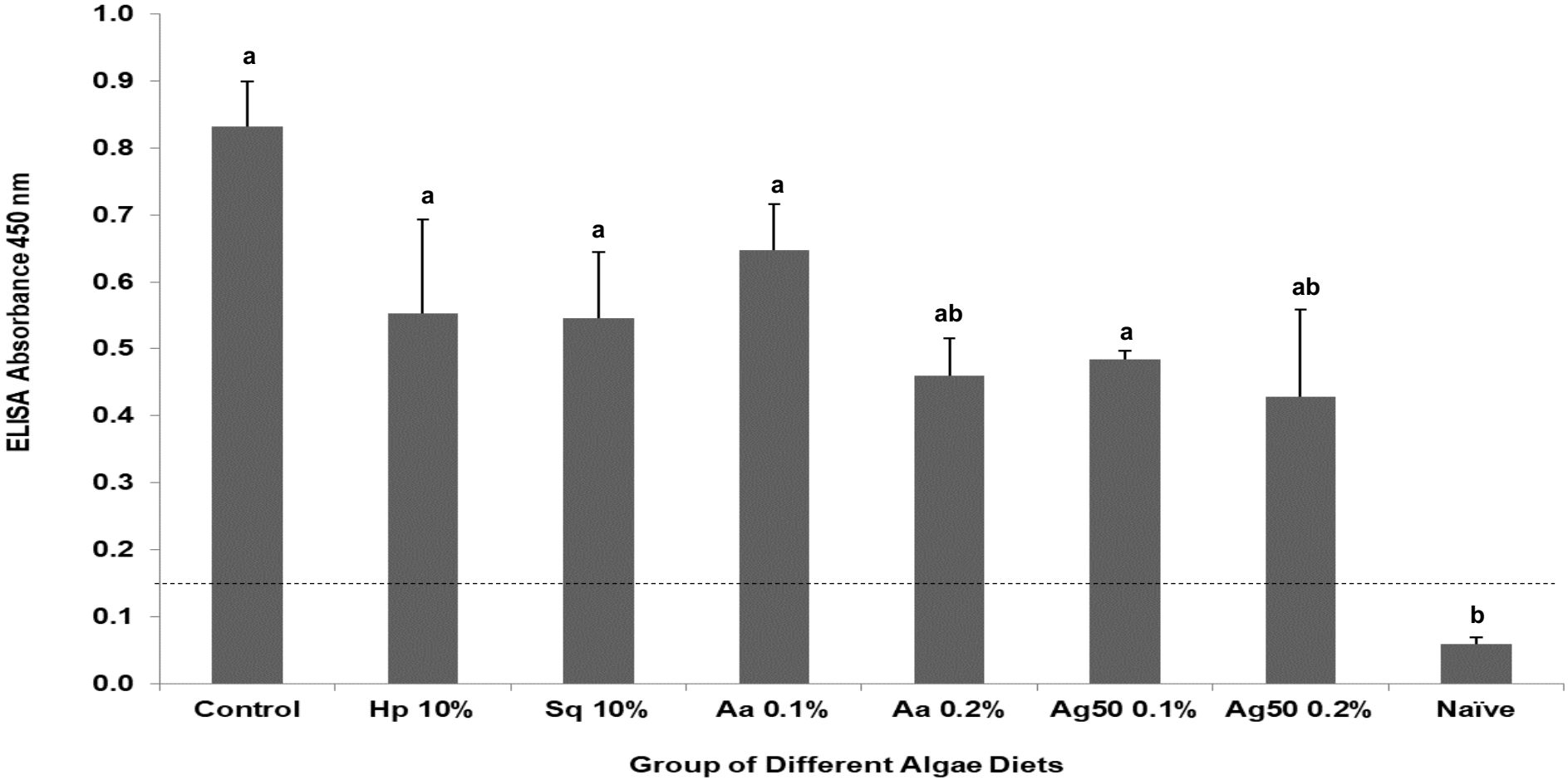

Figure 4.7 Plasma *Fno* specific antibody titres of Nile tilapia homo gold fed with different algae diets for 28 d and challenged by *Fno* immersion for 3 h (1.05 x 10^5 CFU mL^{-1}) for 35 d. Values are presented as mean of triplicates per group±SD at A_{450} nm. Groups that do not share a letter are significantly different. (Dash line is expressed a cut off at 0.142)

4.3.3 *S. agalactiae* challenge trials

4.3.3.1 *S. agalactiae* passage

Fish were sampled 3 d after IP challenge with a high dose of *S. agalactiae* by IP for the first and second passages. Four tissue samples (*e.g.* brain, eye, spleen and head kidney) from each fish per passage were taken and streaked onto TSA plates. Pure *S. agalactiae* colonies were observed without any contaminants after at least 48 h culture at 28˚C. The bacteria observed were light cream in colour, and were small colonies as expected.

4.3.3.2 Susceptibility of Nile tilapia homo gold to *S. agalactiae*

A. *S. agalactiae* dose response

Challenge of Nile tilapia homo gold with *S. agalactiae* at all doses resulted in rapid and acute mortality, with a cumulative mortality of 100% within 12 dpi at the highest dose of 1.12 x 10^8 CFU mL^{-1}, while other doses reached 100% mortality by 21 dpi (Figure 4.8). Most deaths occurred between 1 to 11 d and 1 to 21 d, for the highest dose and two other doses groups, respectively.

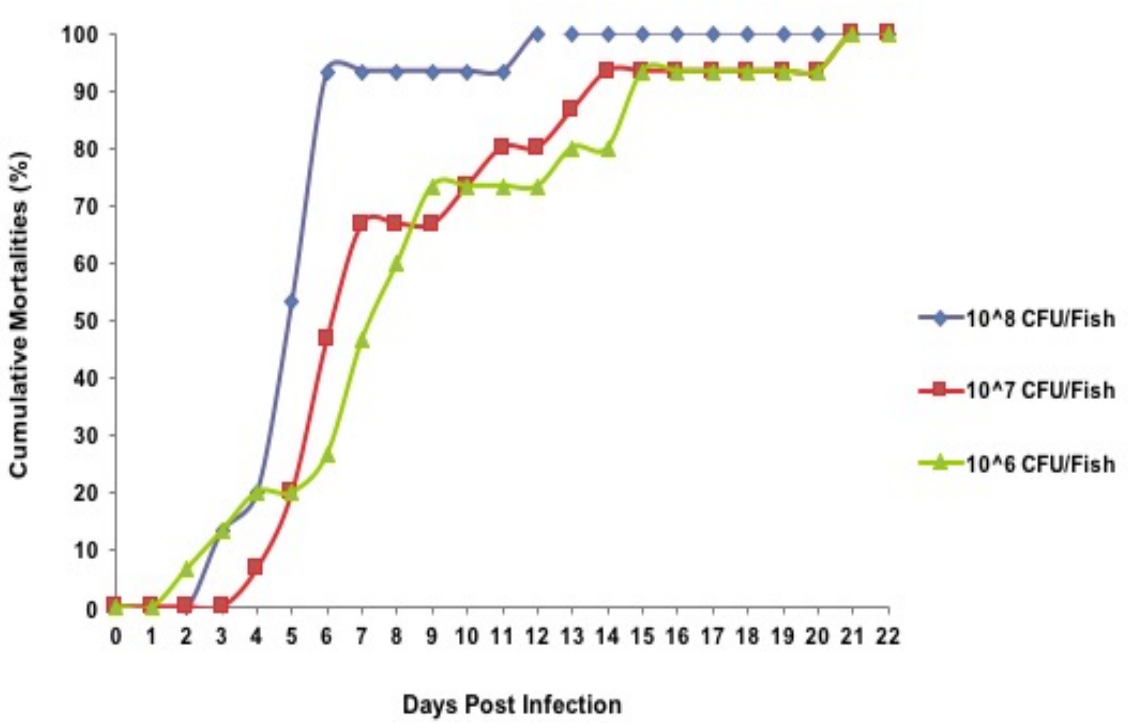

Figure 4.8 Percentage cumulative mortality of Nile tilapia homo gold following IP challenge with different doses of *S. agalactiae*; 15 fish were infected per dose.

B. Gross pathology and clinical signs

The external clinical signs and gross pathology expected from streptococcocis were observed in dead and moribund fish after challenged, as shown in Figures 4.10 and 4.11. The first clinical signs appeared 24 h post-challenge in the highest dose of 1.12 x 10^8 CFU $fish^{-1}$ followed by the lower doses of 1.12 x 10^7 and 1.12 x 10^6 CFU $fish^{-1}$ at 5 dpi. The clinical signs consisted of lethargy, anorexia, failure to maintain normal buoyancy and display of abnormal swimming behaviour where fish were swimming erratically close to the water surface, formed letter C body shape, and followed by signs of jumping on the top of the tank a few hours prior to death (Figure 4.9). Obvious external gross lesions were observed on the examined fish, mostly related to the eye, including graded of severe exophthalmia either lateral or bilateral, turbid and bulging eyes, corneal opacity of eye, small hemorrhagic occurs in the eye, and some fish with over-produced of mucus on skin (Figure 4.10). In addition, internal pathology was showed abdominal swelling, the presence of fluid in the peritoneal cavity, stomach and intestines filled with gelatinous fluid or yellowish in some fish, enlarged liver, spleen and kidneys, and a turgid gallbladder, liver haemorrhages, and kidneys and spleen congestion (Figure 4.11).

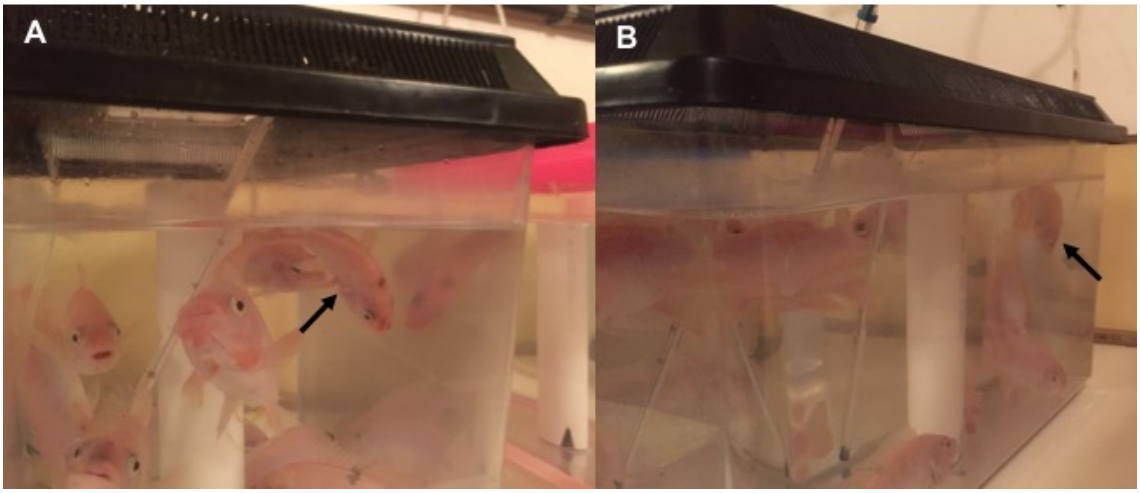

Figure 4.9 Infected Nile tilapia homo gold after IP dose response challenge with *S. agalactiae* showed abnormal swimming behaviour which were swim erratically close to water surface and some of them formed letter C body shape (black arrows): (A and B) the same tank (K2) infected with 1.12 x 10^7 CFU fish $^{-1}$, (A) from the front and (B) from side (10 L plastic tank).

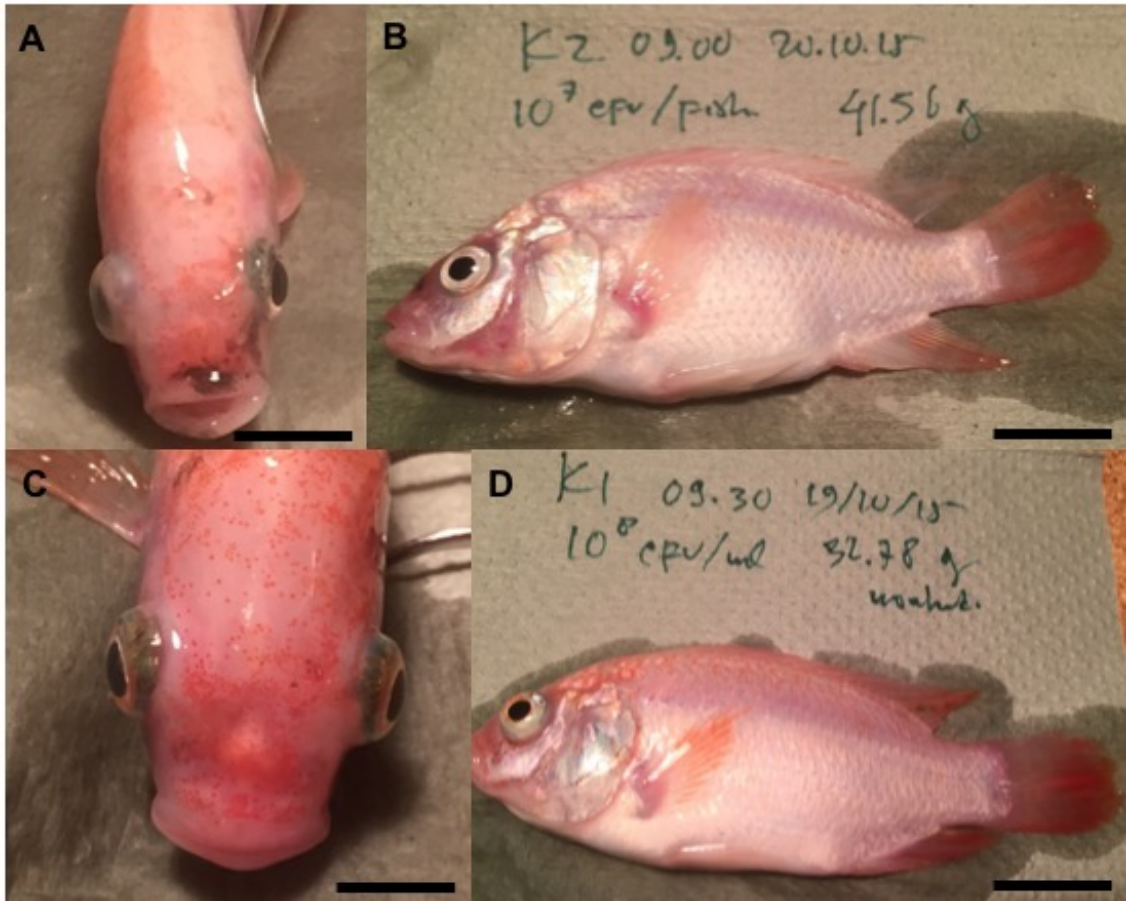

Figure 4.10 External gross pathological findings in Nile tilapia homo gold after IP challenged with *S. agalactiae*: (A) lateral exophthalmos with corneal opacity and protruding eye at 10^6 CFU fish^{-1}; (B) ascites with body cavity filled with fluid or swollen tissues at 10^7 CFU fish^{-1}; (C) bilateral exophthalmos at 10^7 CFU fish^{-1}; and (D) severe exophthalmia with turbid and bulging eyes at 10^8 CFU fish^{-1}. Scale bar = 1.0 cm.

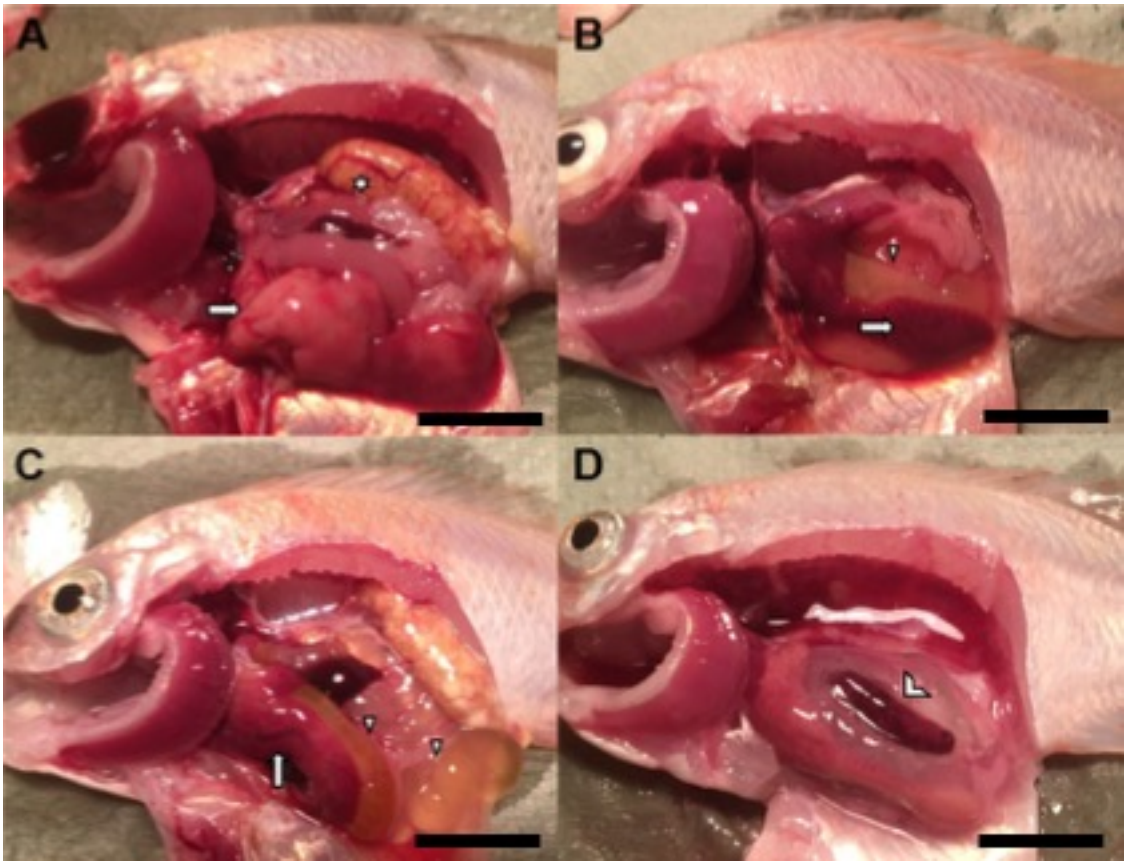

Figure 4.11 Internal gross pathological findings in Nile tilapia homo gold after IP challenged with *S. agalactiae*: (A, B, and C) liver haemorrhages (arrows) at 10^7 to 10^8 CFU fish^{-1}; (A) haemorrhages on ovary at 10^8 CFU fish^{-1}; (B and C) stomach and intestines filled with gelatinous or yellowish fluid (arrows head) at 10^7 to 10^8 CFU fish^{-1}; and (D) spleen congestion (open arrow head) at 10^6 CFU fish^{-1}. Scale bar = 1.0 cm.

C. Bacterial recovery

Pure cultures of *S. agalactiae* isolates were successfully recovered on TSA plates from all the infected Nile tilapia homo gold (freshly dead and moribund fish) after IP challenge by inoculating homogenates of brain, eye, spleen and head kidney. No contaminants grew on the plates and *S. agalactiae* colonies observed mostly after at least 48 h of incubation at 28°C. The morphology of colonies was light cream with small colonies as expected (Figure 4.14). After 3 d post inoculation on TSA, all purified bacterial samples from brain, eye, spleen and head kidney from each tilapia strain were confirmed as *S. agalactiae* (Chapter 2, Section 2.2.1.1).

D. Co-infection challenge with *S. agalactiae*

An *S. agalactiae* dose that ideally caused 40% mortality was to be used for the co-infection trial. However, as doses used in the pre-challenge all resulted in 100% mortality an estimate was made for the dose to use. Therefore, a dose 10-times lower than the lowest previous dose was used and 5 x 10^5 CFU $fish^{-1}$ was selected to co-infect the survivors. The IP challenge dose was retrospectively determined by drop count as 2 x 10^5 CFU $fish^{-1}$. No significant difference in cumulative survival was observed between the groups with high mortality in all groups (Figure 4.12).

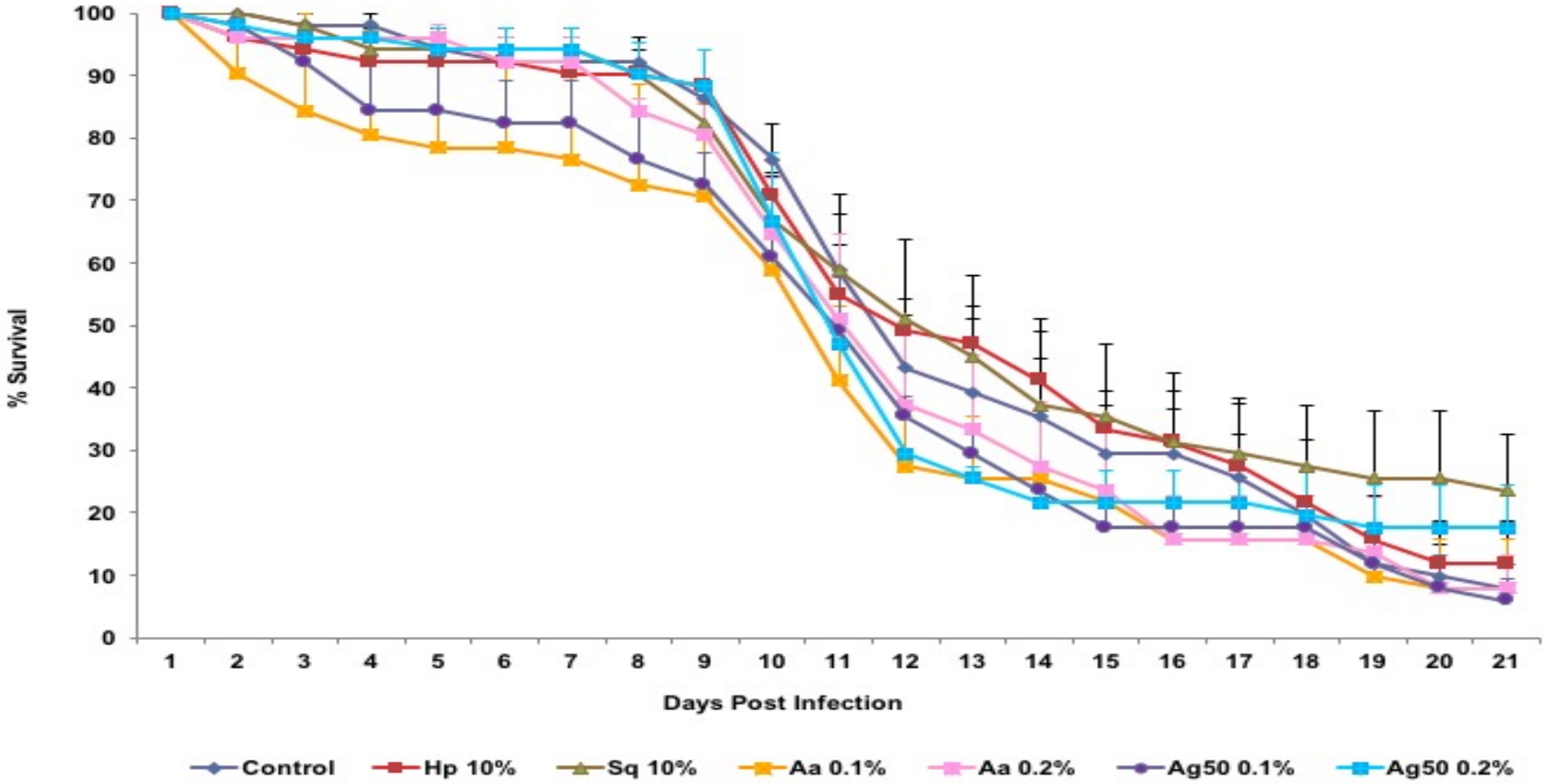

Figure 4.12 Percentage cumulative survival of surviving Nile tilapia homo gold (that had survived from *Fno* challenge) after co-infected by IP injection with *S. agalactiae* (2 x 10^5 CFU $fish^{-1}$) and maintained for 21 d. No significant different between algae diet treatment groups (Hp/*H.pluvialis* 10%, Sq/*S. quadricauda* 10%, Aa/alginic acid at 0.1 and 0.2%, Ag50/ß-glucan at 0.1 and 0.2%) compared to the control diet group.

4.4 Discussion

Intensive tilapia production systems with high stocking densities are a driving force in the establishment and distribution of emergent infectious diseases, including piscine francisellosis. This disease is characterized by a systemic granulomatous infection and high mortality rate particularly in temperate regions, where temperatures drop below 23°C, caused by the highly virulent bacteria *Francisella noatunensis* subsp. *orientalis*, is considered as one of the most important diseases of cultured tilapia worldwide (Sebastião *et al.*, 2017). The use of immunostimulants can be an effective means of enhancing disease resistance in fish (Petit and Wiegertjes, 2016). The mechanism of protection appears not to be by stimulating specific immune responses, but by increasing non-specific (or innate) defense mechanisms (Raa, 2013). The main components of the innate immune system are monocytes, macrophages, granulocytes and humoral elements such as lysozyme and the complement system (Thirunavukkarasu *et al.*, 2015). Stimulation of the innate immune system both in mammals and in fish by phagocytes is triggered by the enhanced production of both nitrogen and oxygen intermediates (Bridle *et al.*, 2005).

In this chapter, the immunostimulatory potential of orally administered algal-derived compounds in Nile tilapia homo gold against *Fno* and co-infection with *S. agalactiae* was investigated. Immunostimulatory effects were measured *in vitro* by analysis of respiratory burst (in macrophages isolated from fish fed different diets containing algal-derived compounds) and lysozyme activity (in the plasma from fish). In addition, *Fno*-specific antibodies (IgM) in plasma, and resistance to experimental challenge with *Fno* and *S. agalactiae*, respectively, were measured. Based on the *in vitro* results (Chapter 2), *Chlorella* sp. was supposed to be used for the *in vivo* trial, however, insufficient amount of this was available to prepare feed pellets.

The respiratory burst activity of macrophages started to increase during the first week (at 7 dpf) and the peak occurred at 14 dpf compared to other time points (Figure 4.4). The response then sharply decreased at 21 dpf until the end of

experiment (28 dpf). This is in line with some previous studies, especially for ß-glucan. For example, the peak chemiluminescent response of blue gourami phagocytes after IP injection with 10 mg kg^{-1} laminaran [ß(1,3)-D-Glucan] from *L. hyperborean,* was observed at 14 d and then reduced significantly at 22 dpi (Samuel *et al.*, 1996). On the other hand, respiratory burst activity in the striped catfish *Pangasianodon hypophthalmus* fed with 0.2% fungal β-glucan was found to increase within one week of being fed the diets with the highest respiratory burst activity seen at day 7 (Sirimanapong, 2013; Sirimanapong *et al.*, 2015).

Increased respiratory burst activity following administration of alginate or alginic acid has been well documented in fish and shrimps by other groups. For example, sodium alginate extracted from *M. pyrifera* enhanced migration of carp head kidney phagocytes to the peritoneal cavity, and increased phagocytic activity (Fujiki and Yano, 1997). An IP injection of Ergosan containing 1% of alginic acid extract from the brown alga *L. digitata* resulted in enhanced respiratory burst activity in rainbow trout, *Oncorhynchus mykiss* (Peddie *et al.*, 2002), and seabass, *Dicentrarchus labrax* (Bagni *et al.*, 2005). The white shrimp *Litopenaeus vannamei* injected with sodium alginate at 20 μg g^{-1} increased its respiratory burst after 2 d, while injection with higher doses at 50 μg g^{-1} maintained a higher phagocytic activity and clearance efficiency to *V. alginolyticus* after 4 d (Cheng *et al.*, 2005). In contrast, tiger shrimp *Penaeus monodon* L fed a diet containing 1.0 and 2.0 g kg^{-1} sodium alginate had significantly increased cytosolic SOD activity but decreased respiratory burst (Liu *et al.*, 2006). Grouper, *Ephinephelus coioides* exhibited significantly increased in respiratory burst of HK phagocytes following IP injection of *V. parahaemolyticus* (Cheng *et al.*, 2007) or oral administration of sodium alginate after challenge with *Streptococcus* sp. and iridovirus (Yeh *et al.*, 2008).

ß-glucan is also widely recognized for its immunostimulatory capacities as reported in invertebrates, fish and mammals and is symbolize a diverse group of linear and branched polysaccharides functioning as structural or storage components in plants (*e.g.* wheat), yeast (*e.g*. *Saccharomyces* genus) (Tokunaka *et al.*, 2000), fungi (*e.g. C. neoformans*) (Reese *et al.*, 2007),

mushrooms (*e.g.* shiitake) (Wasser and Weis, 1999) some bacteria (*e.g.* Rhizobiaceae family) (Breedveld and Milleri, 1994), and algae (*e.g. Laminaria* sp., *E. gracilis*). Several authors have reported the use of algae or algae-derived substances as immunostimulants, for instance laminaran [ß(1,3)-D-Glucan] from *L. hyperborean* were produced significantly enhanced production more superoxide anion after IP (15 mg kg^{-1}), or peroral (150 mg kg^{-1}) administration in Atlantic salmon, *Salmo salar* L (Dalmo *et al.*, 1996). Hot-water extracts from *G. tenuistipitata* are able to increase the production of oxygen radicals in phagocytes of extract-injected white shrimp *Litopenaeus vannamei* and its resistance against *V. alginolyticus* (Hou and Chen, 2005). Previous studies have been reported immunostimulation of ß-glucan from yeast (known as MacroGard) or fungal on the fish macrophages such as dietary administration of commercial ß-glucans increases macrophage respiratory burst activity in African catfish, *Clarias gariepinus* (Burchell) (Yoshida *et al.*, 1995), and gilthead seabream *Sparus auratus* (Castro *et al.*, 1999).

On the other hand, in the present work, although respiratory burst activity appeared to increase in a number of the algal-fed groups at day 14, the level was not significantly different to the control group at this time point and the variation was so high that no firm conclusion could be reached. High variation in lysozyme was also observed with no significant difference between dietary groups.

Lysozyme was measured as it is an important protein found in the plasma of fish. This enzyme attacks the ß-1,4-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan of bacterial cell walls causing lysis either directly or in conjunction with complement system and phagocytosis by head kidney macrophages (Ellis, 2001; Paulsen *et al.*, 2001). Previous studies have demonstrated the increase of lysozyme activity during dietary of immunostimulants. For example, seabass that were fed a diet containing sodium alginate from brown algae *L. digitata* and *Ascophyllum nodosum* were shown have increased lysozyme activity at 15 dpf (Sakai, 1999). Similarly, hot-water extract of *Sargassum wightii* incorporated in fed diet significantly enhanced the lysozyme activity in mullet *Mugil cephalus* at the first

week of the experiments (Thirunavukkarasu *et al.*, 2015). In addition, lysozyme activity was found significantly higher in the striped catfish fed with 0.2% yeast β-glucan at 7 dpf, after which the trend in enzyme activity decreased (Sirimanapong, 2013; Sirimanapong *et al.*, 2015). This was possibly not observed in the present study due to the high variation within sample groups

The *Fno* (~1.05 x 10^5 CFU mL^{-1}) immersion challenge of tilapia, following the feeding with different diets, did not perform as expected and infected fish recovered quickly, with only few mortalities observed in the fish fed Ag (0.1%) *i.e.* β -glucan (Figure 4.7). Based on the previous *Fno* dose responses experiment (Chapter 3, Section 3.3.4.1), the *Fno* concentration used in this study should have induced 40% mortality of the fish. Future experiments, using a higher dose of *Fno* could be used and qPCR analysis of samples to determine levels of *Fno* following infection should be included as fish did appear to become infected but then recovered. In addition, the original immersion pre-challenge of *Fno* was performed with single tanks (*i.e.* without any replicate tanks), due to Home Office Licence constraints, and this could be improved.

Due to the *Fno* challenge failure in the present study the potential of different products in mitigating disease could not be assessed. Survival rates of infected fish are usually increased after administration of various immunostimulants such as algae or its derivatives. For instance, feeding with ß-glucan reduced the mortality of large yellow croaker after challenging with *V. harveyi* (Khotimchenko and Vaskovsky, 1990), and IP injection of 20 mg kg^{-1} laminarin was sufficient to protected blue gourami *Trichogaster trichopterus* against *A. hydrophila* (Samuel *et al.*, 1996). While a single IP injection of 5 mg mL^{-1} of yeast ß-glucan 6 d before challenge enhanced significantly the survival against an infection of *A. hydrophila* in zebrafish *Danio rerio* (Rodríguez *et al.*, 2009). Dietary High-M alginate (isolated from *Durvillaea antarctica*) or sodium alginiate extracted from *M. pyrifera* was shown to increase the survival of juvenile turbot challenged with *V. anguillarum* (Skjermo *et al.*, 1995), better resistance was shown against atypical *A. salmonicida* in cod (Vollstad *et al.*, 2006), increased resistance of larvae or juvenile of turbot *Scophthalmus maximus* L., and halibut

Hippoglossus hippoglossus L. was shown against *V. anguillarum* (Skjermo *et al.*, 1995; Skjermo and Bergh, 2004). In addition, a dietary supplement of High-M alginate, ß-glucan, a polysaccharide (chrysolaminaran) from the marine diatom *Chaetoceros mülleri*, and yeast-glucan enhanced larvae of Atlantic cod *Gadus morhua* L. resistance against colonization and possible infection by the opportunistic bacteria and pathogens such as *Pseudomonas*- and *Vibrio*-like bacteria (Skjermo *et al.*, 2006). Tiger shrimp juveniles fed with a medicated diet containing 10 g dry weight of *Spirulina platensis* per kg for 7 d and challenged by injection with White Spot Syndrome Virus suffered no mortality versus 60% in controls (Hemtanon *et al.*, 2005), and oral administarion with diet containing polysaccharide extracted from marine green algae, *Acrosiphonia orientalis* (4 g kg^{-1}) led to better survival rate against the same virus (Manilal *et al.*, 2009).

In natural disease outbreaks in the aquatic environment, fish are commonly exposed to heterogeneous infectious macro or micro-organisms such as multiple bacterial pathogens which are probably responsible for disease outbreaks and considerably outweigh single infections (Kotob *et al.*, 2016; Dong *et al.*, 2017). In this present study, secondary infection of the surviving Nile tilapia homo gold following being fed with different algae diets and immersion challenge with *Fno* was performed with *S. agalactiae* by IP injection for 21 d and resulted with very low percentage cumulative survival in all dietary groups (Figure 4.15). No significant difference was observed in any of the dietary groups. This could be due to the high dose of *S. agalactiae* used in this study (2 x 10^5 CFU $fish^{-1}$), as the dose was only 10-times lower than the dose that caused 100% mortality in single *S. agalactiae* infection (Section 4.3.3.2A). However, even with this high challenge dose of bacteria, after 21 d post co-infection, higher survival levels were observed in some algae dietary groups *e.g. S. quadricauda* (23.5%) or ß-glucan 0.2% (17.6%) compared to the infected control dietary group (7.84%) or ß-glucan 0.1% (5.88%) (Figure 4.15). As the differences were not significant no conclusion can be made about stimulation from the algae diet to the immune system of treated Nile tilapia homo gold and protection against secondary infection. This present study is, however, the first report of the secondary infection in Nile tilapia following dietary administration of immunostimulants and main challenge with *Fno*.

With respect to future challenges, the use of a lower dose (aimed at reaching 40% mortality, for example) may allow more subtle effects of the immunostimulants to be seen. In addition, how the presence of one pathogen can affect the load of other pathogens and how the host mortality rate will be changed during co-infection in comparison with single infection are little known (Bradley and Jackson, 2008). It is important to understand how defensive immunity to a specific pathogen can occur in the host infected with multiple pathogens, how frequent co-infections can be and the potentially important impact that co-infections can have on the development of a disease (Lello *et al.*, 2004). Thus, further investigation of the innate and adaptive immune responses, genes expression, bacterial load in the infected fish tissue *e.g.* for the main and secondary bacteria, and bacterial clearance mechanism of the resistant host will be useful for the development of new effective vaccine-based disease prevention and disease control strategies.

4.5 Conclusion

The non-specific immune responses of Nile tilapia, measured in this study (*i.e.* respiratory burst and lysozyme activities) were differentially stimulated by different algal-derived compounds even though there were no significant differences between dietary groups and the control group and the variation was so high that no firm conclusion could be reached. *Fno* immersion challenge failed to produce mortality in any of the dietary groups despite being successful previously. Fish did appear to become infected but then recovered. Future experiments, using a higher dose of *Fno* could be used and qPCR analysis of samples to determine levels of *Fno* following infection should be included. The immersion pre-challenge of *Fno* should be improved and standardized prior repeating the *in vivo* trial. The secondary infection with *S. agalactiae* resulted in very low cumulative survival in all algae dietary groups as the bacterial dose used was too high. Thus, further experiments are required, and it is recommended that the dose of bacteria used for the *in vivo* immunostimulants trial should be lower than that normally used to test vaccine efficacy. The lower challenge regime aimed at 40% mortality level might have enabled any the differences between algae dietary groups to be detected. Unfortunately, the

trials in this study could not be repeated as the aquarium had been closed for refurbishment. Thus, an alternative *in vivo* challenge model (wax moth larvae *G. mellonella*) was developed and used to test algal-derived compounds against *Fno* infection (Chapter 5).

CHAPTER 5

DEVELOPMENT OF A WAX MOTH LARVA *Galleria mellonella* INFECTION MODEL OF *Francisella noatunensis* subsp. *orientalis* TO TEST THE *IN VIVO* EFFECTS OF ANTIBACTERIAL COMPOUNDS

5.1 Introduction

Francisella species are intracellular pathogens, and *F. tularensis*, a related mammalian pathogen, can survive and replicate in many different cell types such as dendritic cells, neutrophils, hepatocytes, and lung epithelial cells, but macrophages appear to be the primary target of this bacterium (Pechous *et al.*, 2009; Santic *et al.*, 2010). Similar to *F. tularensis, Fnn* is also found to localise, survive, and replicate inside phagocytic cells of fish including the monocytes and macrophages (Bakkemo *et al.*, 2011, 2016; Vestvik *et al.*, 2013), neutrophils and phagocytic B-cells (Furevik *et al.*, 2011). In addition, *Fno, Fnn* and *F. tularensis* subsp. *novicida* proliferate in neutrophils and macrophages (Brudal *et al.*, 2014). Meanwhile, *Fno* can enter, survive, and multiply within tilapia macrophages and in the endothelial *O. mossambicus* bulbus arteriosus cell line (Soto *et al.*, 2009b, 2010c, 2017). Currently, there are no commercially available treatments or vaccines to control *Fno* infection, although it is considered to be one of the most pathogenic bacteria in tilapia.

In order to address francisellosis problems, a deeper understanding of the infection process is required, as this may lead to new and improved treatments, vaccines and management practices. Several studies have been performed in vertebrate aquatic hosts to study bacterial pathogens, especially for the *Francisella* genus. These include zebrafish for *Fnn* (Vojtech *et al.*, 2009b) and their embryos for *Fno, Fnn* and *F. tularensis subsp. novicida* (Brudal *et al.*, 2014). However, performing experiments with fish are costly, and can pose biohazard risks due to large volumes of contaminated water (McMillan *et al.*, 2015). Such experimental models require full ethical review, make high-throughput studies a challenge, necessitate specialist infrastructure (*e.g.* aquaria), and require biosecurity procedures to assure the safety of aquatic animal stocks and personnel, especifically for zoonotic pathogens (Zak and O'Reilly, 1991; Chamilos *et al.*, 2007; Froquet *et al.*, 2007; McMillan *et al.*, 2015). Furthermore, good practice prescribed by European guiding principles (Hartung, 2010) recommends that researchers should adhere to the principles of the 3Rs, *i.e.* the replacement, reduction and refinement of the use of animals in experiments, particularly vertebrates (Jander *et al.*, 2000). Therefore, there is

strong justification for pursuing new more ethically acceptable alternative infection models when studying bacterial pathogens of fish, in order to reduce vertebrate experimentation and adhere to the principles of the 3Rs.

Currently, few studies have identified suitable alternative infection models for investigating bacterial pathogens of fish, but those available include the crustacean *Artemia franciscana* for *A. hydrophila, V. anguillarum* and *V. harveyi* (Defoirdt *et al.*, 2005); the nematode *Caenorhabditis elegans* for *Vibrio* spp. (Brackman *et al.*, 2011b); the freshwater ciliate *Tetrahymena thermophile* for *Aeromonas* spp. (Pang *et al.*, 2012); and the amoeba *Dictyostelium discoideum* for *Aeromonas* spp. and *Fnn* (Froquet *et al.*, 2007; Lampe *et al.*, 2016). However, each of these models is correlated with inadequacies such as a requirement for specialist facilities, the need for time-consuming and specific training to achieve competence, the lack of important aspects of immune complexity, or being unsuited to the study of certain pathogen virulence traits (McMillan *et al.*, 2015). However, one alternative host associated with various desirable benefits not befitting the aforementioned examples is the larva of the insect *G. mellonella* (Lepidoptera: Pyralidae; the greater wax moth) (Desbois and Coote, 2012). The insect innate immune system is divided into humoral and cellular defense responses, and there is functional similarity in the immune responses to invasive infection between insects and fish with respect to pathogen recognition, the inducible production of lysozyme, and antimicrobial peptides, the production of reactive intermediates of oxygen and nitrogen species, phagocytosis of invading microbes, and the cascades that regulate coagulation and melanization of hemolymph (Neumann *et al.*, 2001; Lavine and Strand, 2002; Agius and Roberts, 2003; Magnadóttir, 2006; Cytryńska *et al.*, 2007; Jiang *et al.*, 2010). In addition, the cellular defences of the insect are facilitated by haemocytes which are found in the hemolymph system, and haemocyte cells are capable of phagocytosing, encapsulating, or engulfing foreign cells or bacterial invaders that breach the cuticle in a way that is analogous to the cellular responses observed in fish (Mullett *et al.*, 1993; Lavine and Strand, 2002). In addition, several basic components of the bacterial infection process, such as toxin production, cell adhesion and cell

invasion, are also likely to be important in both insects and fish (Jander *et al.*, 2000).

There are many of advantages for the utility of *G. mellonella* as an experimental model in these applications including the fact that perfoming experiments using this kind of larvae is simple to perform, the larvae do not need feeding, and results may often be obtained within a relatively short space of time. There is also lack of legal constraints as the larvae are not covered by legislation covering experimental vertebrate animals and experiments on insects are arguably more ethically acceptable in comparison to vertebrates (Desbois and Coote, 2012). Large numbers of the larvae can be kept inexpensively without any special husbandry procedures during the period prior to the trial, although it is advisable to use them within 14 days of receipt as prolonged pre-incubation can affect susceptibility to infection (Mowlds and Kavanagh, 2008; Browne *et al.*, 2013a). Furthermore, during experiments the larvae are kept in Petri dishes with infected material made safe by autoclaving and the techniques needed to work with this model can be achieved after only limited instruction, while it permits high-throughput experiments of pathogenicity and virulence factors (Desbois and Coote, 2012; McMillan *et al.*, 2015). This model may be a particularly useful for laboratories that study microbial pathogenesis and do not want to allocate the resources and commitment necessary to study a pathogen in the native hosts (Cytryńska *et al.*, 2007; Aperis *et al.*, 2007; Jiang *et al.*, 2010; Desbois and Coote, 2012; McMillan *et al.*, 2015). Other attributes that make *G. mellonella* larvae an attractive model system includes their relatively large size (250 mg in weight and 1.5–3.5 cm in length), thus enabling inoculation of individual larvae with precise doses of the pathogen by injection, and subsequent examination of the pathology (*e.g.* to obtain haemolymph and haemocytes, and to isolate organs for further analysis) (Jander *et al.*, 2000; Scully and Bidochka, 2006). Most importantly, larvae are commercially available worldwide and inexpensive to purchase, as they are used as fish bait and as live feed for some animals in captivity (Mylonakis *et al.*, 2005; Kavanagh and Fallon, 2010a).

G. mellonella has been used widely to assess virulence of microbial pathogens, host innate immune responses, and as an *in vivo* model for evaluating the potency of antimicrobial agents, with results comparable to those obtained using mammalian models (Jander *et al.*, 2000; Brennan *et al.*, 2002; Mylonakis *et al.*, 2005; Propst *et al.*, 2016). Furthermore, the *G. mellonella* model could support many bacterial infections under a variety of different environmental and experimental conditions, including human pathogens (see Chapter 1 Section 1.7) and the genus *Francisella* (Aperis *et al.*, 2007; Ahmad *et al.*, 2010; McKenney *et al.*, 2012; Dean and van Hoek, 2015; Propst *et al.*, 2016).

In the present study (Chapter 4), due to lack of time (due to facility refurbishment) to repeat the *in vivo* challenge in fish (Nile tilapia), *G. mellonella* larvae were proposed as a possible alternative host model for administration of algal-derived compounds to treat *Fno* infections. However, this alternative infection model must be validated for each bacterial pathogen to make sure that an infection occurs, virulence associates with that observed in the native fish host, and conserved virulence mechanisms are involved during infection (McMillan *et al.*, 2015). Importantly, several studies have been conducted with fish pathogens using the *G. mellonella* model and these include *V. anguillarum* (McMillan *et al.*, 2015), *Flavobacterium psychrophilum* (Guest *et al.*, unpublished) and *A. salmonicida* (Lawlor and Desbois, unpublished), through this system has never been investigated for its uselfulness for *Fno* studies. Thus, the aim of the work presented in this Chapter was to assess the suitability of *G. mellonella* as an alternative model for *Fno* and to investigate and improve our understanding of the virulence and pathogenicity of different strains of *Fno*. In addition, the efficacy of antibacterial agents such as antibiotics and algal-derived compounds (immunostimulants) were also examined in this insect *in vivo* model.

5.2 Materials and methods

5.2.1 Bacteria isolates and culture conditions

Five isolates of *Fno* used in this study were isolated from separate outbreaks of francisellosis from different countries worldwide, and were kindly provided by Dr Jose Gustavo Ramirez-Paredes, University of Stirling. Isolates were collected from infected Nile tilapia, with the exception of the Japanese and Austrian strains that were isolated from outbreaks in three-lined grunt and ornamental African cichlids, respectively (Table 5.1). Bacterial cultures of *Fno* were grown on CHAH or MHB (Chapter 2, Section 2.2.1.1). Culture media, PBS and water were sterilised by autoclaving at 121°C for at least 1.5 h.

Table 5.1 Origin of the strains of *Fno* isolates used in these stud

Isolate	Location	Year	Infected Fish	Reference
NE STIR-GUS -F2f7	Northern Europe	2012	Red Nile tilapia *O. niloticus* L	(Ramírez-Paredes *et al.*, 2017)
AUS	Austria	2014	Ornamental African cichlids	(Lewisch *et al.*, 2014)
CR#	Costa Rica	2007	Nile tilapia *O. niloticus* L	-
MEX	Mexico	2012	Nile tilapia *O. niloticus* L	(Ortega *et al.*, 2016)
JPN*	Japan (Ehime-1)	1999–2000	Three-lined grunt/isaki fish *Parapristipoma trilineatum*	(Fukuda *et al.*, 2002; Kamaishi *et al.*, 2005)

#*Fno* Costa Rica was provided by Dr Duncan Colqouhun, NVI, Oslo. * *Fno* type strain

5.2.2 Preparation of bacterial inocula

The five isolates of *Fno* were recovered from frozen glycerol stocks (20 µL each strain) onto CHAH and incubated at 28˚C for at least 48 h. One loopful of each bacterium was inoculated into 15 mL of MHB and incubated in an orbital shaker at 28°C, 150 rpm for 19.5 h to the middle of logarithmic phase (as described in Chapter 2, Section 2.3.3). Bacterial cells were harvested by centrifugation at 3000 x g, 4°C for 15 mins. The cells were washed by resuspension in 10 mL of PBS, vortexed, re-centrifuged as above and the process was repeated once more. After the final wash, the pellet was re-suspended in 15 mL PBS, and then cell density was determined by measuring at absorbance 600 nm and adjusted to AU 0.6. The bacterial concentration was then calculated and adjusted with PBS to the desired concentration using the *Fno* NE STIR-GUS-F2f7 standard curve (as described in Chapter 3, Section 3.3.1). Bacterial suspensions were diluted with PBS to the desired CFU mL^{-1}, and then serially diluted in PBS and plated on CHAH to enumerate CFU per mL (as described in Chapter 2, Section 2.2.1.2).

5.2.3 Experimental animals

Final instar stage *G. mellonella* larvae were purchased from UK Waxworms Ltd (Sheffield, UK). Larvae were sorted to remove moribund, discoloured and dead larvae, and those used for experiments were between 250 and 350 mg and had uniform cream colouration. The larvae were kept in the dark at 4°C and used within one week from receipt. The larvae were used for injection experiments and as a source of haemolymph samples.

5.2.4 *G. mellonella* infection trials

Each experimental group contained 12 larvae for all experiments and most experiments were repeated using larvae from different batches to give n = 24. There were no any overall differences in the larvae used between batches in all experiments. *G. mellonella* larvae were injected with 10 µL of bacterial inocula, antibiotic solution, algal-bioactive compounds or PBS by using a 50-µL Hamilton syringe (Sigma-Aldrich, Ltd) into the haemocoel of each larva via the

last left pro-leg, once the larvae have been cooled on ice. Consecutive washes of 1% (w/v) sodium hypochlorite, 70% ethanol and sterile water were used to clean the syringe in between experimental groups following the protocol of McMillan *et al.* (2015). After injection, larvae were kept in a disposable plastic Petri dishes (8.5 cm in diameter), incubated in the dark at 28°C for up to 288 h, and inspected every 24 h for survival. The incubation temperature of 28°C was selected based on the killing of *G. mellonella* at different temperatures after a preliminary *Fno* inoculation trial (Section 5.3.1). The larvae were considered dead when they displayed no response or movement to a tactile stimulus given by gently nudging the larvae with a sterile inoculation loop.

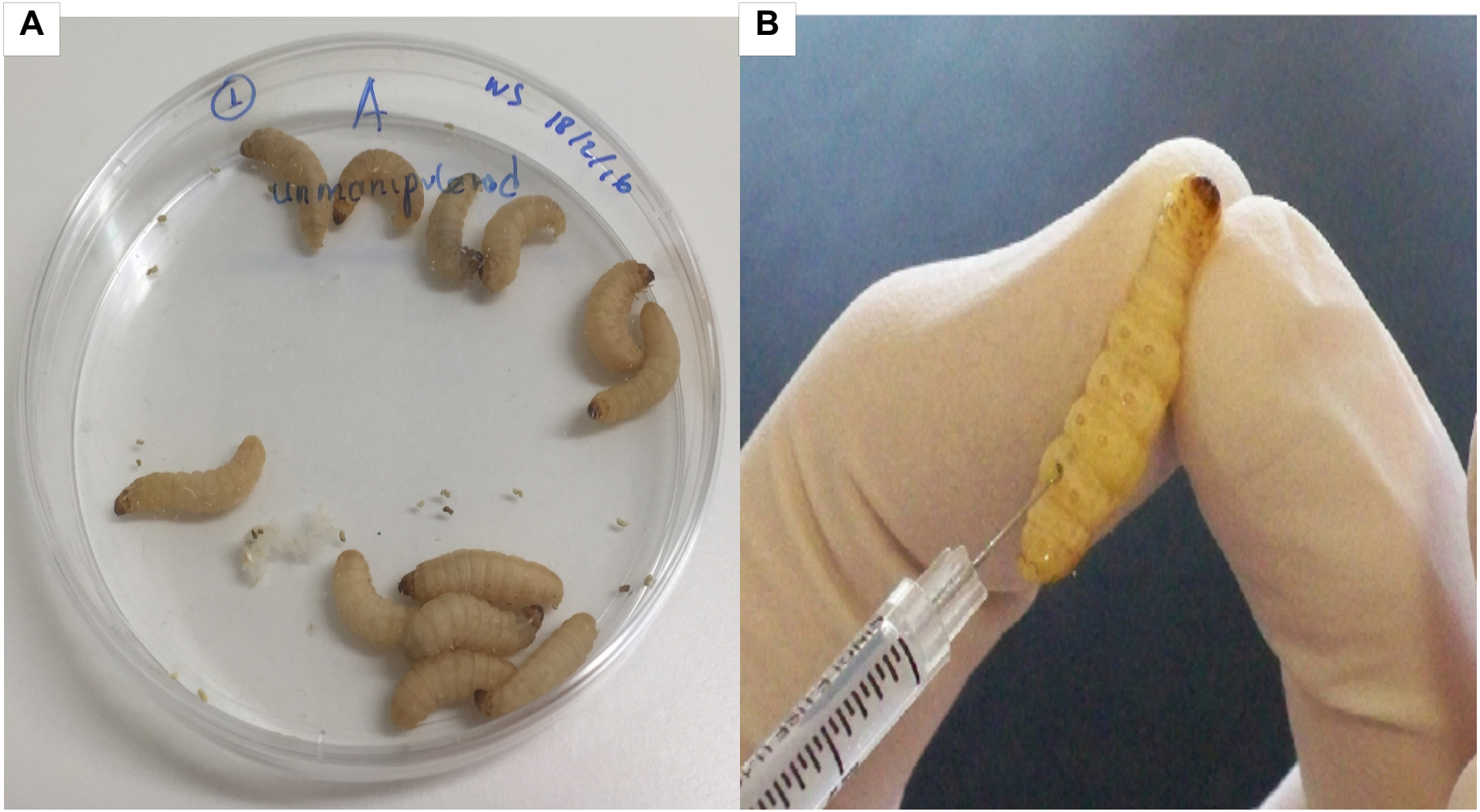

Figure 5.1 The greater wax moth larvae *G. mellonella*: (A) the last instar stage of larvae ready to use in experiments and (B) each larva is injected into the haemocoel via the last left pro-leg (Kavanagh and Fallon, 2010). Photo source (B): http://aqualandpetsplus.com/Live%20Food,%20Wax%20Worms.htm).

5.2.5 Virulence of different isolates of *Fno* in *G. mellonella*

Two different trials were run. The first trial evaluated the effect of temperature of incubation after inoculation of bacterial inocula on mortality of *G. mellonella*. The isolate used for this trial was the one with greatest virulence in fish, *Fno* STIR-GUS-F2f7 (Section 5.3.1 and 5.3.2), and this was injected at 1 x 10^9 CFU mL^{-1} and the larvae were incubated at 15, 22, 25, 28 or 37°C for 288 h. The second trial investigated the virulence of five different *Fno* isolates from different geographical regions (Table 5.1). For this trial, larvae were injected with culture filtrate and different concentrations of live bacteria (1 x 10^8, 5 x 10^8, 1 x 10^9 or 5 x 10^9 CFU mL^{-1}). Heat-killed bacteria (5 x 10^9 CFU mL^{-1}) were also administered for each bacteria strain.

Each *Fno* isolate from each strain was cultured, collected, washed in PBS, diluted to the desired concentration and then drop counts performed on CHAH, as described in Chapter 2, Section 2.2.1.2. The relative virulence of five different *Fno* isolates was assessed by inoculating larvae with 10 µL of suspensions containing 1 x 10^8, 5 x 10^8, 1 x 10^9 and 5 x 10^9 CFU mL^{-1}. To examine the effect of sterile culture filtrates on larval survival, culture supernatant from each culture was passed through a sterile polyethersulfone 0.22-µm filter (Millipore, Watford, Herts, UK) and then injected into the larvae as above. Meanwhile, the effects of heat-killed bacteria on larval survival were assessed with washed bacterial suspensions that had been adjusted with PBS to approximately 1 x 10^9 CFU mL^{-1} (for *Fno* STIR-GUS-F2f7 only) and 5 x 10^9 CFU mL^{-1} (for all 5 isolates). Bacteria were heat-killed at 60°C for 60 mins, or 90°C for 30 mins, and killing was confirmed by the absence of colonies forming when 100 µL of bacterial suspension was plated across CHAH plates and incubated at 28°C for at least 48 h. Ten microlitres of heat-killed bacterial suspension was injected into the larvae as above.

The following negative control groups were always included in experiments: the first group included larvae injected with PBS only to assess the mortality related to the impact of physical trauma by the injection and handling, a second group ('unmanipulated') was used to control for background larval mortality, as this

group underwent no injections. Larvae were incubated and inspected as above (Section 5.2.4) and percentage survival was calculated for each group.

5.2.6 Correlation of virulence of *Fno* isolates in *O. niloticus* and *G. mellonella* infection models

The virulence of 5 *Fno* isolates was calculated as total AUC of the cumulative larval survival which was in the *G. mellonella* larvae in this present study using total concentrations of *Fno* at 10^8 and 10^9 (injected at 10 µL $larva^{-1}$, n=24), while in the red Nile tilapia *O niloticus* infection model virulence was determined after IP injection at total concentrations of *Fno* 10^6 and 10^7 (injected at 1 mL $fish^{-1}$, n=6).

5.2.7 Treatment of infected larvae with antimicrobials

In order to corroborate that larvae could be rescued from *Fno* infection by antibiotic therapy, *in vivo* antibacterial activity testing was performed according to Desbois and Coote (2011). There were five groups of treatments (including control groups) for each *Fno* isolate *i.e.* a group of larvae injected with bacteria and antibiotic (treatment group), and four control groups: the unmanipulated group; a group injected with PBS instead of bacteria and treated with antibiotic; a group injected with the bacterial inocula and PBS; and a group injected with PBS only. The second control group was used to assess the toxicity of the tetracycline treatment. Tetracycline was selected as the antibiotic due to its efficacy against *Fno* STIR-GUS-F2f7 by agar disc diffusion assay (as described in Chapter 2, Section 2.3.3.2A). The tetracycline was diluted in sterile PBS and administered once by a separate injection. Larvae were inoculated with 10 µL of *Fno* at ~1 x 10^9 CFU mL^{-1} for each of the five *Fno* isolates (Section 5.2.1). Ten microlitres of tetracycline at 10 µg kg^{-1} (equal to 10 mg g^{-1} body weight) was injected into the larvae at 2, 24, and 48 h post infection (hpi), as described by Desbois and Coote (2011). All treated larvae except unmanipulated group were injected with the same number of injections, including the PBS inoculated group, but PBS was injected into the larvae instead of bacteria and tetracycline. As multiple injections were necessary in this experiment, a different pro-leg was used for each injection, starting from the left last pro-leg and rotating left to right

and moving proximally up the body (*i.e.* injecting through the left last proleg, right last proleg, penultimate left proleg and penultimate right proleg, as needed).

5.2.8 Visualization of *Fno* in the larvae cells

Fno STIR-GUS-F2f7 was selected for visualization in the *G. mellonella* cells. The bacterial inocula was prepared as described previously in Section 5.2.2 and adjusted to ~1 x 10^9 CFU mL^{-1} in PBS. The larvae were injected with 10 µL of this suspension and incubated at 28°C as above. In this case, each experimental group contained 20 larvae to ensure there would be sufficient surviving larvae to sample at each time point. Three larvae were sampled before and after injections (at 24, 48, 72 and 96 hpi) for histopathology (H & E staining), Gram Twort staining and IHC. Larvae were anesthetized on ice for at least 30 mins and then placed into 10% buffered formalin. To fix the internal organs and block melanisation, the larvae were stored in this solution at 4°C for 24 h (Perdoni *et al.*, 2014).

5.2.8.1 Histopathology

Tissue section preparation (*e.g.* cassetting, processing, blocking out and microtomy) and H & E staining were performed according to standard protocols used in the Histology Laboratory at the Institute of Aquaculture, University of Stirling and Perdoni et al. (2014), with some modifications. Briefly, whole larvae were dissected transversally into six equal transverse sections using a scalpel, and each half of the tissue was wrapped in biopsy tissue paper prior to placing into standard tissue cassettes for processing overnight using a tissue processor and subsequent embedding of the samples in paraffin wax. The procedure was performed carefully to avoid squeezing the larval tissues. Six rings of larval tissues were positioned in each paraffin block (*e.g.* one in the distal part, four in the middle, and one in the proximal part). The paraffin blocks were trimmed, sectioned, and then 4 µm thick tissue sections were stained with haematoxylin and eosin, as described in Chapter 3, Section 3.2.7. Slides were examined using a light microscope and images were collected (Chapter 3, Section 3.2.7).

Duplicate sections (4 μm thick) were subjected to Gram Twort staining and IHC to aid the localisation of the bacteria in the tissues.

5.2.8.2 Gram-Twort

Gram Twort staining was performed according to the standard method using a protocol modified from Twort (1924) and Ollet (1947) in Bancroft and Marilyn (2008), as decribed in Chapter 3, Section 3.2.9.

5.2.8.3 Immunohistochemistry

IHC was performed using the method described (in Chapter 3, Section 3.2.8) and Ramos-Vara (2005) with slight modifications. To optimise the IHC method for wax moth larvae sections, serum dilutions of 1:400, 1:500, 1:1000, 1:1200, 1:1300, and 1:1400 were assessed. After incubation with goat anti-rabbit IgG-HRP conjugate (diluted to 1:200) for 30 mins, the samples were incubated with chromogen and substrate (DAB peroxidase) for 1 min, before the reaction was stopped by immersing the sections in tap water (as Chapter 3, Section 3.2.8). Unmanipulated (*i.e.* naïve) larvae were used as negative controls and these were incubated either with PBS or with primary antibody in PBS, respectively.

5.2.9 Recovery of *Fno* from infected wax moth larvae

5.2.9.1 CHAH plate antibiotic testing

Fno STIR-GUS-F2f7 and Japan isolates were used to assess growth of *Fno* in the larval haemolymph over 288 h. First, it was necessary to determine an antibiotic regimen that could be added to the agar to prevent the growth of non-*Fno* contaminants (introduced from the larval surface or gut contents) while having no effect to the recovery of *Fno*. Antibiotics selected for evaluation were ampicillin and penicillin (as earlier antibiotic agar disc diffusion assays indicated that *Fno* was not susceptible to either of these antibiotics; see Chapter 2, Section 2.3.3.2A, as well as antibiotics that have been used elsewhere to recovered *Francisella* spp. from various samples *i.e.* colistin (C4461-100mg; Sigma Aldrich Ltd, UK); and ampothericin B (A2942-20 mL; Sigma Aldrich Ltd,

UK) (Petersen *et al.*, 2004, 2009, Soto *et al.*, 2009a; b, 2010c). Various concentrations were evaluated for each antibiotic in agar [penicillin: 1, 2.5 and 5 mg L^{-1}; ampicillin: 1, 2.5, 7.5 and 10 mg L^{-1}; colistin: 1, 2.5 and 7.5 mg L^{-1}; and ampothericin: 1 and 2.5 mg L^{-1}]. Bacteria inoculations were prepared, washed and collected as described previously (Section 5.2.2). The absorbance of cell suspensions was adjusted to 0.6 AU at A_{600} nm, and then diluted with PBS to ~1 x 10^9 CFU mL^{-1} (as described in Section 5.2.2). Each *Fno* strain was serially diluted in PBS and 100 µL of suspension spread onto CHAH with or without antibiotics at the different concentrations to enable CFU comparison after incubation at 28°C for at least 48 h. Experimental CHAH plates were performed in triplicate for each bacterium and each antibiotic concentration.

Moreover, the experiment was repeated by mixing two antibiotics in the agar plate to investigate whether this would be even better at preventing growth of contaminants. For this, penicillin and ampothericin B were selected and tested at two different concentrations *i.e.* 1 and 2.5 mg L^{-1} of each agent against both *Fno* STIR-GUS-F2f7 and Japan isolates.

5.2.9.2 *Fno* burden from infected *G. mellonella* haemolymph

Fno STIR-GUS-F2f7 and Japan strains were selected to assess bacterial burden in the larvae during infection, as these strains represent the *Fno* isolates with highest and lowest virulence in fish in the present study, respectively. Groups of 175 *G. mellonella* larvae were infected with 1 x 10^9 CFU mL^{-1} of one of these isolates. The *Fno* inocula were prepared and adjusted to ~1 x 10^9 CFU mL^{-1} in PBS, as described previously in Section 5.2.2. After injection, the larvae were incubated at 28°C, and 3 surviving larvae in each group were selected at random for determination of bacterial load at 2, 4, 8, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 288 hpi. Haemolymph was selected for *Fno* recovery based on a preliminary investigation of *Fno* burden from infected larva haemolymph and body tissue that revealed that bacterial burden was greater in the haemolymph rather than body tissue (data not shown). In addition, according to McMillan et al. (2015), approximately 95% of *V. anguillarum* Vib79 were retrieved from the haemolymph rather than body

tissues. Haemolymph was also collected from the unmanipulated group larvae and larvae inoculated with PBS only.

Prior to sampling surviving larvae for haemolymph, larvae were placed on aluminium foil and cooled on ice for at least 30 mins (Figure 5.2). Then the body surface was sterilized by spraying with 70% ethanol and wiping with tissue paper. Then the last abdominal segment (final 2 mm of the body) was removed aseptically by cutting off the posterior abdominal segment using sterile scissors and harvesting the haemolymph according to McMillan *et al.* (2015) and Senior *et al.* (2011). The haemolymph (approximately 5–10 µL) was drained from each larva into a sterile 0.5 mL micro-centrifuge tube (APEX Though Microtube, Alpha Laboratories, Hampshire, UK), pipetted up and down thirty times to lyse the cells (Senior *et al.*, 2011), and then briefly disrupted on a vortexer. Ten-fold serial dilutions were performed by adding 10 µL of larva haemolymph into 90 µL sterile PBS in a sterile 96-well tissue culture plate from 10^{-1} to 10^{-3} or 10^{-4}, and then 10 µL of each dilution and the crude sample was plated on CHAH containing the antibiotics best suited to prevent growth of contaminants (*i.e.* penicillin and amphotericin B both at 1 mg L^{-1}) to allow for enumeration of bacteria. Nevertheless, Gram staining and primary identification tests were perfomed to confirm that recovered colonies from infected larvae were indeed *Fno* (Chapter 2, Section 2.2.1.1).

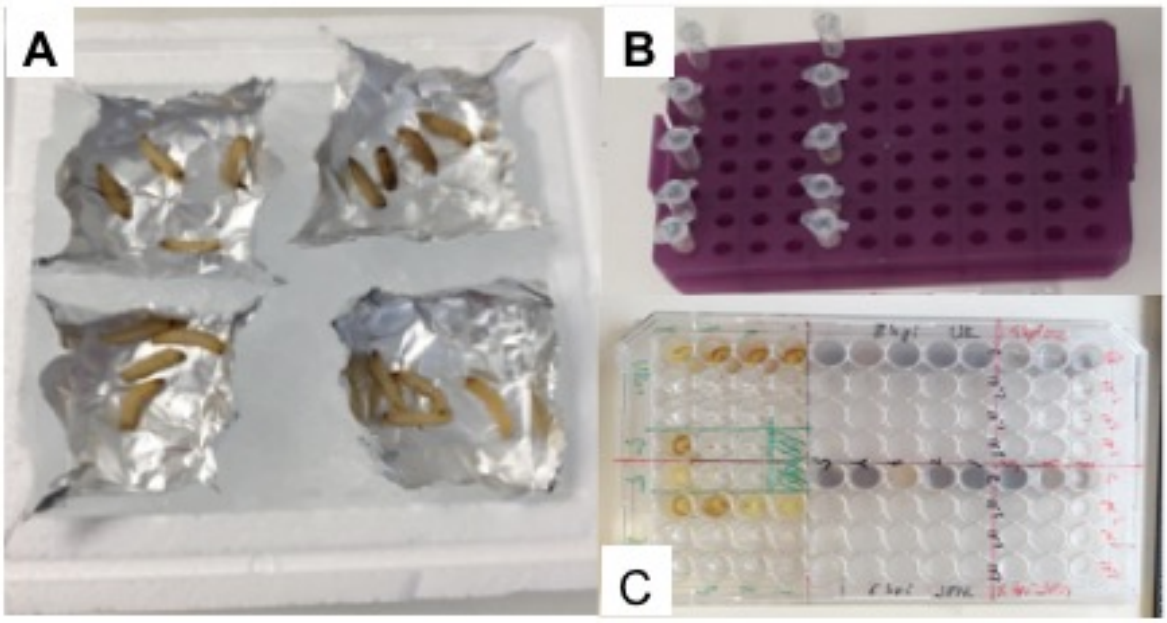

Figure 5.2 Sampling of surviving larvae and collection of haemolymph: (A) larvae were placed on foil and anesthetized on ice for at least 30 mins; (B) the haemocoel was drained and collected into sterile 0.5 mL of micro-centrifuge tubes; and (C) serial dilution of 5 or 10 µL of larva haemolymph was performed in 95 or 90 µL PBS in a sterile 96 well tissue culture plate.

5.2.10 In vivo antibacterial activity of algal-derived compounds in *G. mellonella*

In vivo antibacterial activity of different algal-derived compounds was performed using the *G. mellonella* assay to assess virulence of *Fno* STIR-GUS-F2f7 in the presence of ß-glucan and alginic acid. In these trials, 12 larvae were used per group.

5.2.10.1 Dose response of different algal-derived compounds

First, a dose response for algal-derived compounds was performed to assess the toxicity of these compounds according to Mowlds *et al.* (2010). The larvae of *G. mellonella* were inoculated (10 µL) with different concentrations of ß-glucan or alginic acid dissolved in PBS (0.5, 1.0, 2.5, 5.0, 7.5, 10, 15, 20, 25, 50 or 100 µg larva^{-1}). After inoculation, the larvae were incubated and inspected as described in Section 5.2.4. Negative control groups were included (*i.e.* unmanipulated group and larvae inoculated with PBS only). ß-glucan and alginic acid are commercialy available algal-derived compounds as described in Chapter 2, Section 2.2.3.1.

5.2.10.2 In vivo antibacterial activity of algal-derived compounds

In vivo antibacterial activity was performed in order to investigate survival of *G. mellonella* larvae after injection with algal-derived compounds (as Section 5.2.9.1), followed by challenge with *Fno* STIR-GUS-F2f7. The experiment was carried out with two different doses of algal-derived compounds, and these were administered at two times before the larvae were challenged with *Fno*, *e.g.* 1 and 24 h before challenge. The larvae were injected (10 µL) with different concentrations of ß-glucan or alginic acid at 7.5 and 10 or at 20 and 25 µg larva^{-1}, respectively. The concentrations of algal-derived compounds used were selected based on the results from the toxicity dose response trials as described in Section 5.3.6.1. One or 24 h later the larvae were challenged with two or three different doses of *Fno e.g.* at 1 x 10^8 or 5 x 10^8 CFU mL^{-1} or with 1 x 10^8 or 5 x 10^8 or 1 x 10^9 CFU mL^{-1}, respectively. The bacterial inocula were prepared and adjusted to the desired concentrations as described previously in

Section 5.2.2. Controls were included, and these consisted of an unmanipulated group, larvae that received PBS and bacteria (each dose) inoculations only, larvae that were injected with algal-derived compounds (each compound and concentration) and PBS. Each larval received the same number of injections, except the unmanipulated group. Twelve larvae were employed per group, and these were incubated and inspected as described in Section 5.2.4. Larvae group survival rates were determined daily and up to 288 h post-injections (Section 5.2.4). Results were expressed as percentage survival of larvae in each group.

5.2.11 Statistical analyses

Survival rates of the larval groups were plotted using the Kaplan-Meier method in Microsoft Excel. Survival differences between groups were compared with the logrank test in R. A p-value of less than 0.05 was considered to indicate a significant difference between groups. Virulence of the 5 *Fno* isolates in red Nile tilapia and *G. mellonella* models of infection were calculated using area under the curve (AUC) of cumulative survival in each group of larvae and fish, and the correlation was plotted and assessed using Spearman's Rank Correlation Coefficient.

5.3 Results

5.3.1 Virulence of different strains of *Fno* in *G. mellonella*

First, in order to evaluate whether wax moth larvae would be an appropriate model for assessing virulence of *Fno,* it was necessary to confirm that *Fno* was capable of establishing an infection in the insect. This validation also comprised ensuring that the bacteria replicated inside the larvae and that antibiotic treatment, *i.e.* tetracycline therapy could rescue infected larvae. Initial experiments were performed to evaluate the killing of *G. mellonella* at different incubation temperatures (at 15, 22, 25, 28 and 37°C) after inoculation of *Fno* STIR-GUS-F2f7 at 1.05 x 10^9 CFU mL^{-1}. Mortalities in larvae groups were greater as post-inoculation temperatures increased; except that larvae incubated at 37°C showed lower mortality compared to 25 and 28°C groups

(Figure 5.3 and Appendix 4.1). Larval pupations occurred faster in general at the higher temperatures, with quickest pupation observed at 28°C compared to the other groups, and pupation also occurred especially in the unmanipulated and PBS only groups (Table 5.2). Killing of larvae by *Fno* appeared to be more effective at 28˚C, and this is known to be a more effective growth temperature for *Fno*. Thus, for future trials 28°C for 288 h was elected as the larva incubation temperature.

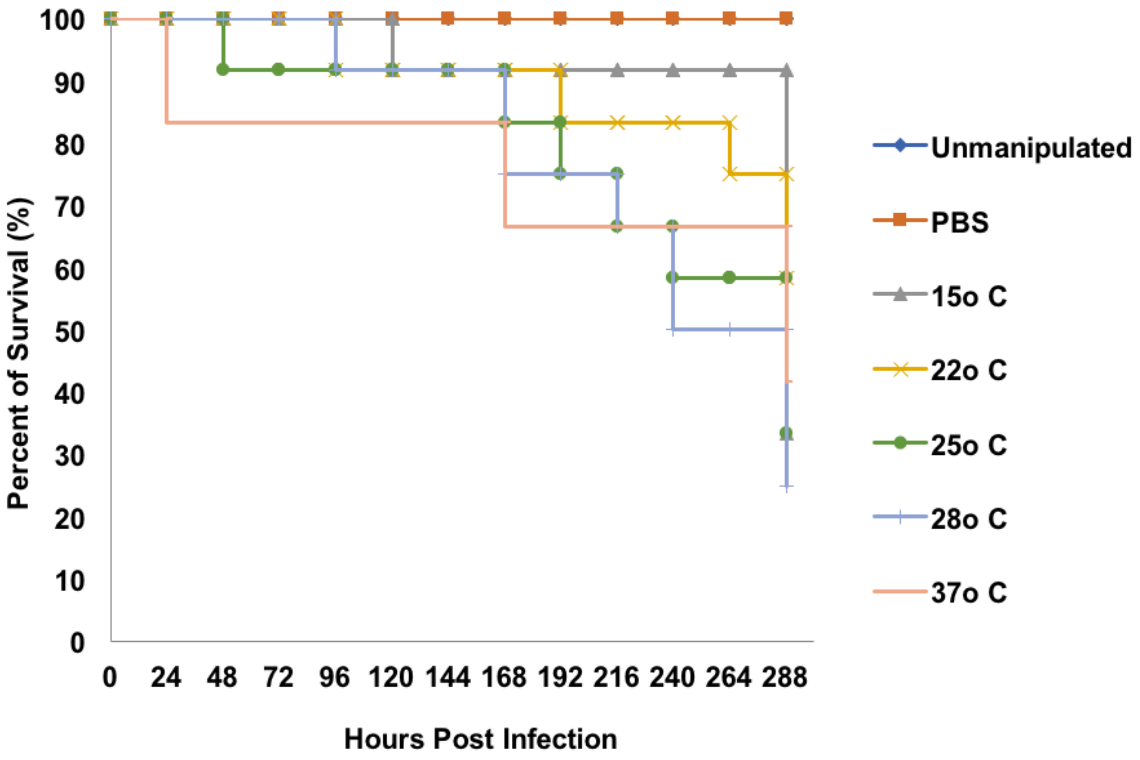

Figure 5.3 Survival of larvae infected with *Fno* STIR-GUS-F2f7 at 1.05 x 10^9 CFU mL^{-1} and incubated at 15, 22, 25, 28 and 37°C. There was 100% survival in all uninfected and PBS control groups at all temperatures (n=12).

Table 5.2 Larva pupation after challenge with *Fno* STIR-GUS-F2f7 at 1.05 x 10^9 CFU mL^{-1} and incubated at different temperatures

No.	Incubation Temperature (°C)	Number of pupae		
		Unmanipulated	PBS Given	*Fno* STIR-GUS-F2f7
1.	15	0	0	1
2.	22	1	0	0
3.	25	3	0	0
4.	28	7	5	0
5.	37	5	3	0

Five *Fno* isolates were then tested for virulence in the insect host. The results were showed a significant difference between all treatmeants (Figures 5.3, 5.4, 5.5, 5.6 and 5.7). Larval mortalities in the groups occurred in a dose-dependent manner for each isolate, indicating that greater inocula caused faster and larger mortality (Figures 5.3, 5.4, 5.5, 5.6 and 5.7). However, importantly, mortality also depended on the isolate used, for example, after injection with 2.4–6.7 x 10^9 CFU mL^{-1}, the final percentage mortality was 100% when *Fno* Austria was used; 96% with *Fno* STIR-GUS-F2f7, *Fno* Costa Rica, and *Fno* Mexico isolates; while only 50% mortality was observed when the *Fno* Japan was used. After injection at a lower dose of 0.8–1.3 x 10^9 CFU mL^{-1}, the time to death started between 24 and 96 h (thus slightly later than the 24–72 h for the higher dose) and percentage mortality was lower in all groups. Mortality was again lowest (29%) for *Fno* Japan followed by 83% in the *Fno* STIR-GUS-F2f7 group, 92% for *Fno* Austria, and *Fno* Costa Rica groups, and 96% for *Fno* Mexico. Injection of 3.8–6.6 x 10^8 CFU mL^{-1} resulted in greater percentage mortality (21–79%) compared to the groups of larvae that received 0.7–1.8 x 10^8 CFU mL^{-1} (8–58%). There were no deaths in the groups of larvae injected with PBS only or the unmanipulated group.

5.3.1.1 Heat-killed bacteria

When larvae were injected with heat killed *Fno* of the five isolate groups (heat killed at 90˚C for 30 mins) at a dose of 2.4–6.7x10^9 CFU mL^{-1}, the final percentage larval mortality ranged between 12–54%. Interestingly, greater percentage mortalities were observed in larvae that were injected with the same dose of bacteria that had been heat-killed at lower temperature for longer (*i.e.* 60˚C for 60 mins compared to 90˚C for 30 mins), with larvae mortality ranging from 67–83%. There was a significant difference between all treatments (Figures 5.3, 5.4, 5.5, 5.6 and 5.7).

5.3.1.2 Bacterial filtrate

Sterile culture filtrate (bacteria supernatant) from each isolate had a slight effect on larval survival, with final survival rates of 71% for *Fno* Austria, 79% for *Fno* Costa Rica, and 92% for *Fno* STIR-GUS-F2f7, *Fno* Mexico and *Fno* Japan.

There was a significant difference especially for *Fno* Austria and Costa Rica (Figures 5.4 and 5.5).

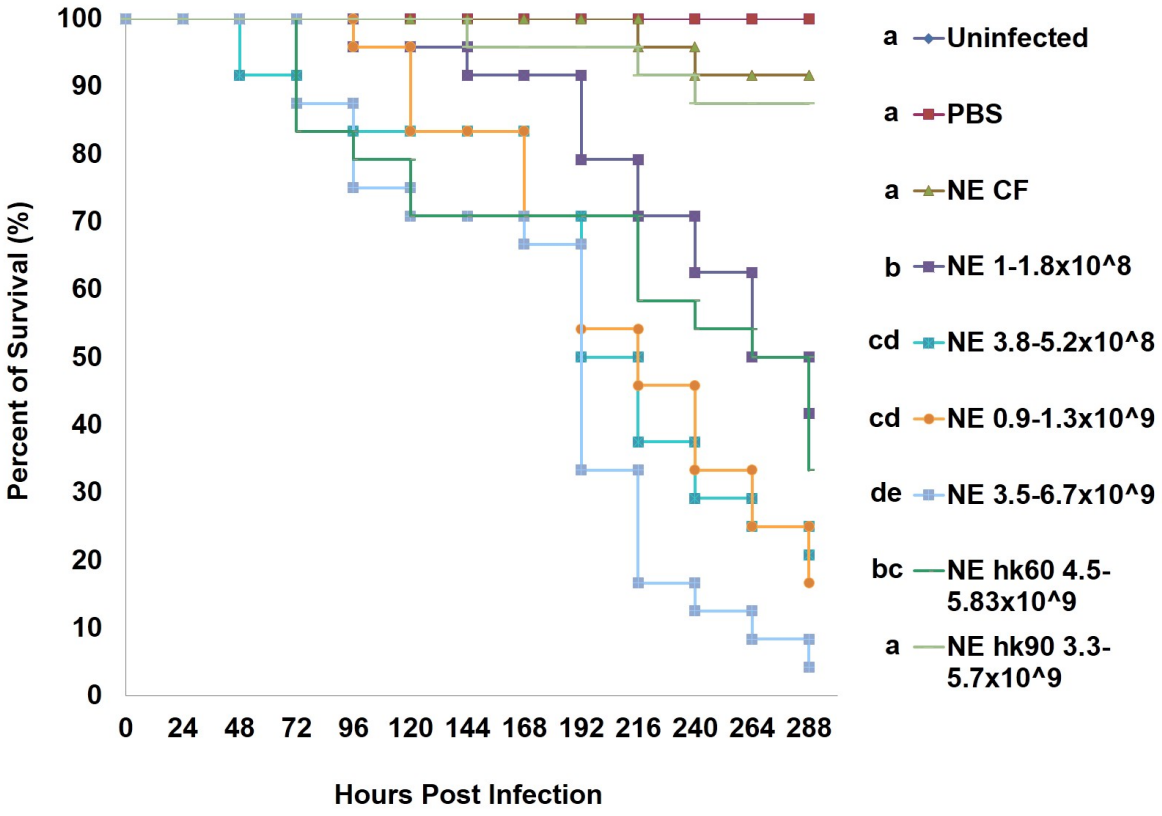

Figure 5.4 Killing of *G. mellonella* larvae by *Fno* STIR-GUS-F2f7 depended on the dose of bacteria inoculated. Kaplan Meier plots of *G. mellonella* survival after injection of different inocula of the bacteria showed that injection with 3.6–6.7 x 10^9 CFU mL^{-1} resulted in higher death rate, compared to injection with 0.9–1.3 x 10^9 CFU mL^{-1}, or 3.8–5.2 x 10^8 mL^{-1} or 1–1.8 x 10^8 CFU mL^{-1}. Injection with 3.6–6.7 x 10^9 CFU mL^{-1} bacteria heat killed at 60˚C for 60 mins resulted in higher death rate compared to 90˚C for 30 mins. There was higher death rate with the group of larvae received injection of culture filtrate compared to PBS only or unmanipulated groups. The larvae were maintained at 28˚C for 288 h (n=24). Groups that do not share a letter are significantly different.

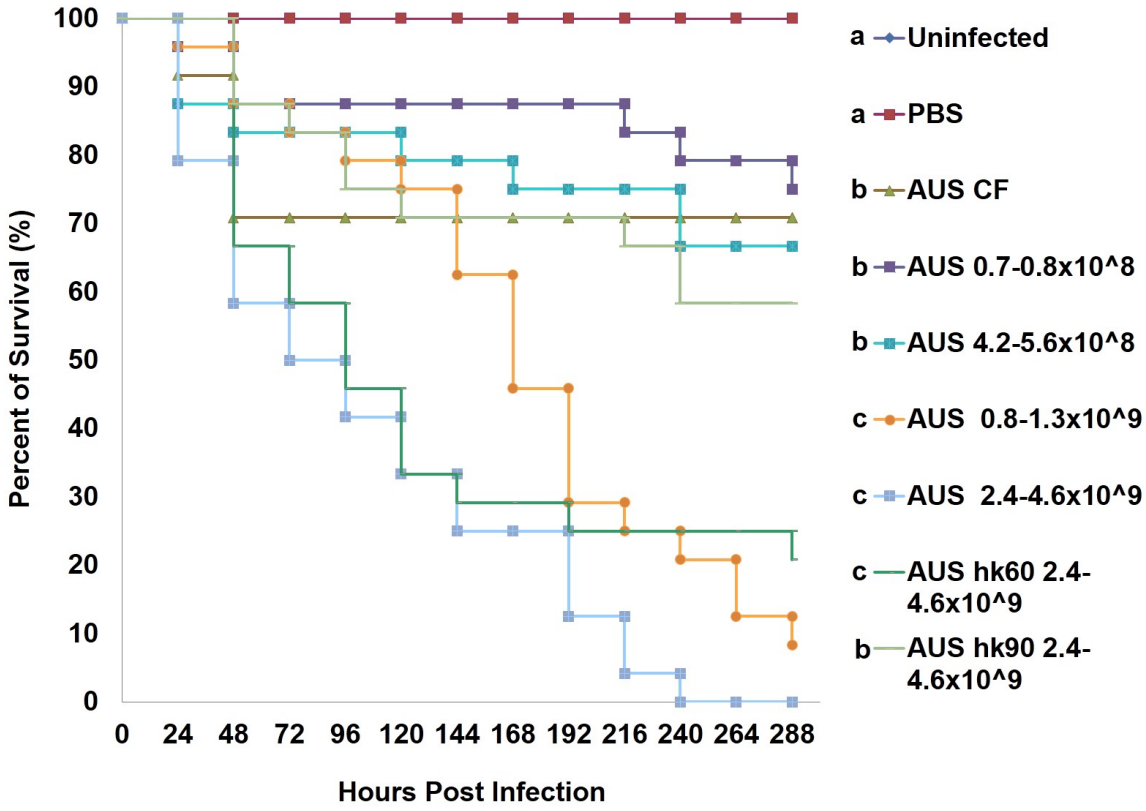

Figure 5.5 Killing of *G. mellonella* larvae by *Fno* Austria isolate depended on the dose of bacteria inoculated. Kaplan Meier plots of *G. mellonella* survival after injection of different inocula of the bacteria showed that injection with 2.4–4.6 x 10^9 CFU mL^{-1} resulted in higher death rate, compared to injection with 0.8–1.3 x 10^9 CFU mL^{-1}, or 4.2–5.6 x 10^8 or 0.7–0.8 x 10^8 CFU mL^{-1}. Injection with 2.4–4.6 x 10^9 CFU mL^{-1} bacteria heat killed at 60˚C for 60 mins resulted in higher death rate compared to 90˚C for 30 mins. There was higher death rate with the group of larvae received injection of culture filtrate compared to PBS only or unmanipulated groups. The larvae were maintained at 28˚C for 288 h (n=24). Groups that do not share a letter are significantly different.

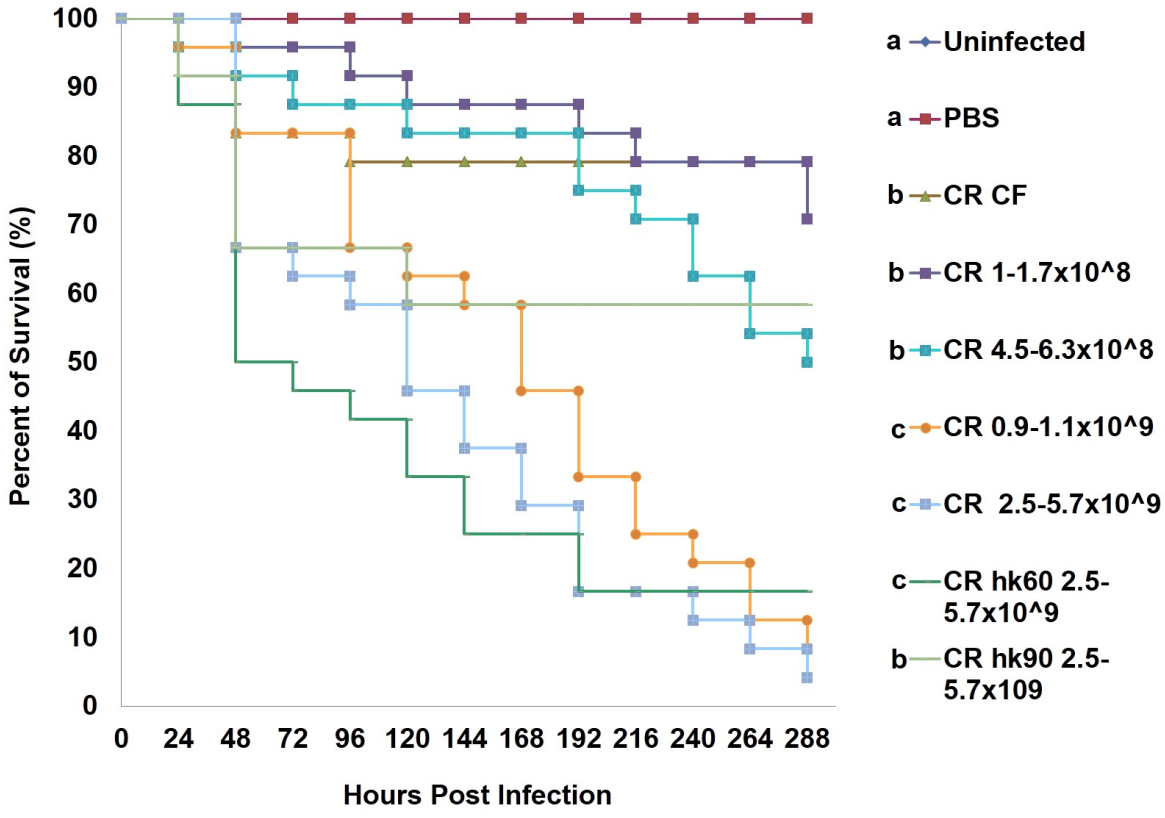

Figure 5.6 Killing of *G. mellonella* larvae by *Fno* Costa Rica isolate depended on the dose of bacteria inoculated. Kaplan Meier plots of *G. mellonella* survival after injection of different inocula of the bacteria showed that injection with 2.5–5.7 x 10^9 CFU mL^{-1} resulted in higher death rate, compared to injection with 0.9–1.1x10^9 CFU mL^{-1}, or 4.5–6.3 x 10^8 CFU mL^{-1} or 1–1.7 x 10^8 CFU mL^{-1}. Injection with 2.5–5.7x10^9 CFU mL^{-1} bacteria heat killed at 60˚C for 60 mins resulted in higher death rate compared to 90˚C for 30 mins. There was higher death rate with the group of larvae received injection of culture filtrate compared to PBS only or unmanipulated groups. The larvae were maintained at 28˚C for 288 h (n=24). Groups that do not share a letter are significantly different.

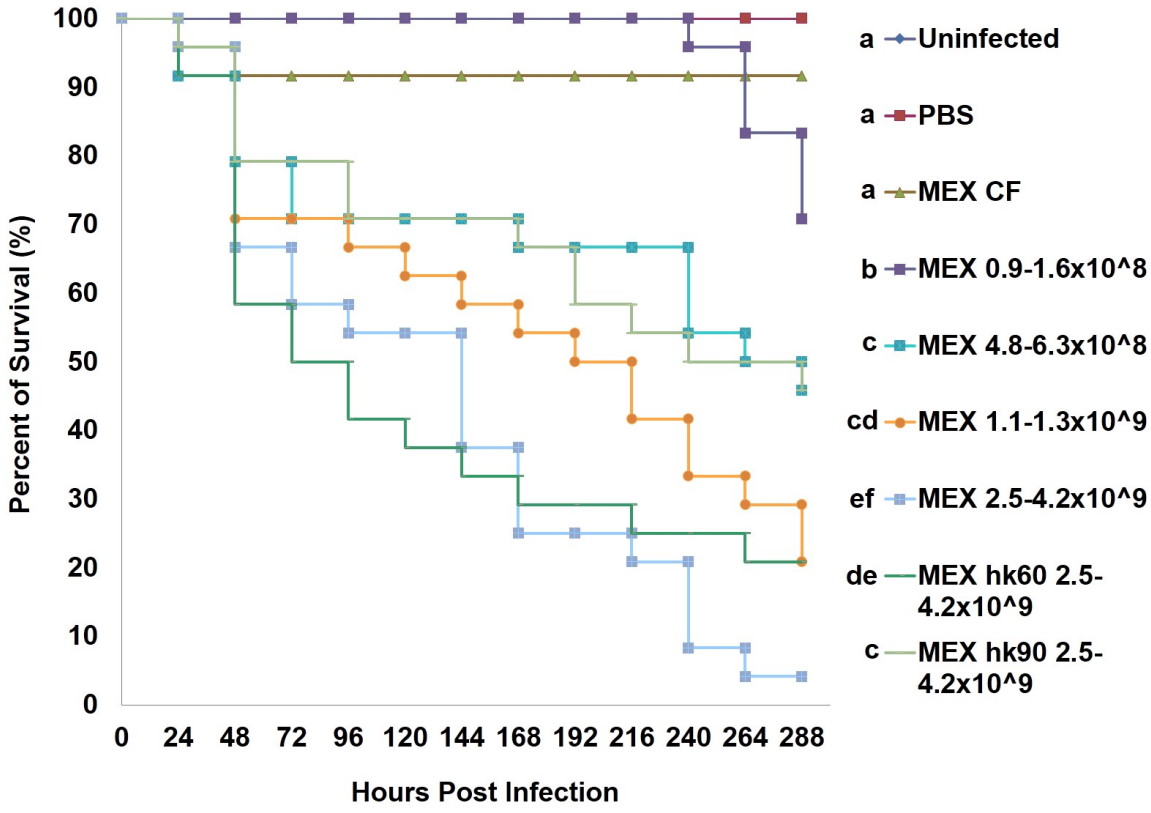

Figure 5.7 Killing of *G. mellonella* larvae by *Fno* Mexico isolate depended on the dose of bacteria inoculated. Kaplan Meier plots of *G. mellonella* survival after injection of different inocula of the bacteria showed that injection with 2.5–4.2 x 10^9 CFU mL^{-1} resulted in higher death rate, compared to injection with 1.1–1.3 x 10^9 CFU mL^{-1}, or 4.8–6.3 x 10^8 CFU mL^{-1}or 0.9–1.6 x 10^8 CFU mL^{-1}. Injection with 2.5–4.2 x 10^9 CFU mL^{-1} bacteria heat killed at 60˚C for 60 mins resulted in higher death rate compared to 90˚C for 30 mins. There was higher death rate with the group of larvae received injection of culture filtrate compared to PBS only or unmanipulated groups. The larvae were maintained at 28˚C for 288 h (n=24). Groups that do not share a letter are significantly different.

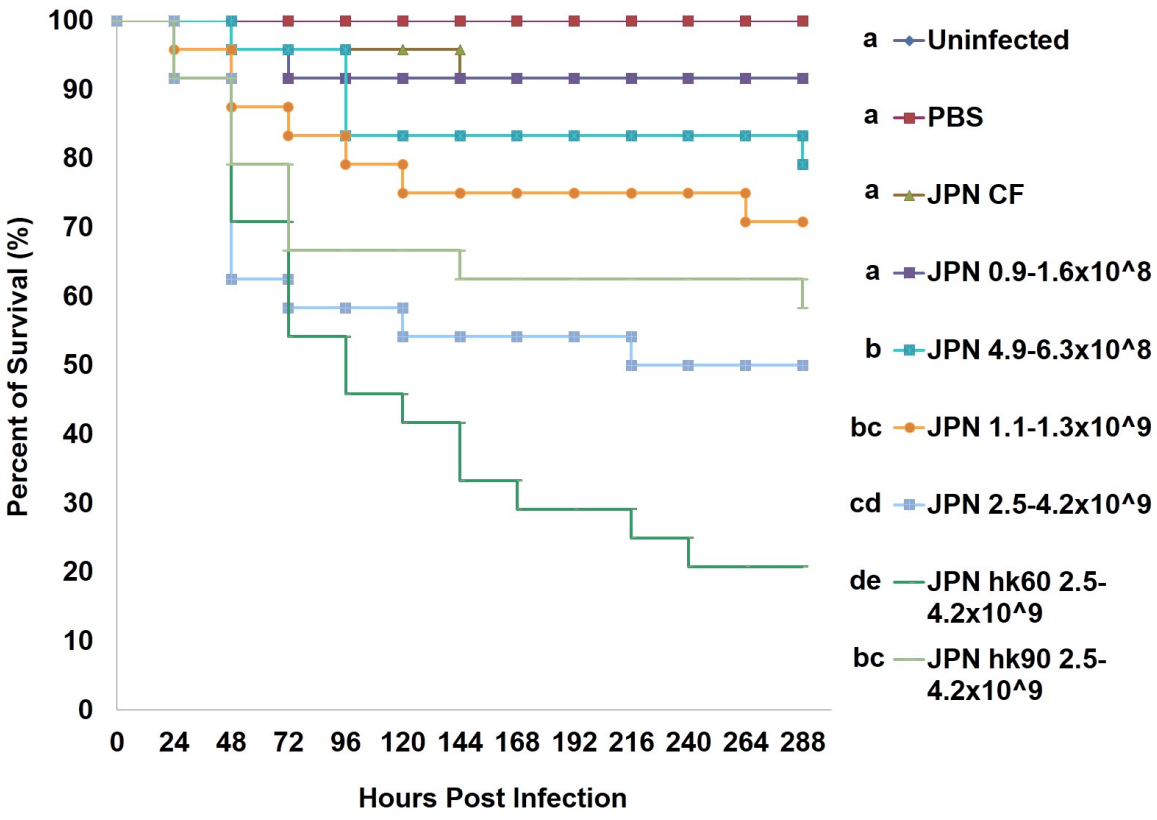

Figure 5.8 Killing of *G. mellonella* larvae by *Fno* Japan isolate depended on the dose of bacteria inoculated. Kaplan Meier plots of *G. mellonella* survival after injection of different inocula of the bacteria showed that injection with 2.5–4.2 x 10^9 CFU mL^{-1} resulted in higher death rate, compared to injection with 1.1–1.3 x 10^9 CFU mL^{-1}, or 4.9–6.3 x1 0^8 CFU mL^{-1} or 0.9–1.6 x 10^8 CFU mL^{-1}. Injection with 2.5–4.2 x 10^9 CFU mL^{-1} bacteria heat killed at 60˚C for 60 mins resulted in higher death rate compared to 90˚C for 30 mins. There was higher death rate with the group of larvae received injection of culture filtrate compared to PBS only or unmanipulated groups. The larvae were maintained at 28˚C for 288 h (n=24). Groups that do not share a letter are significantly different.

5.3.1.3 Change in larvae body colouration

After injection with PBS, generally the *G. mellonella* showed normal cream body colorations until the end of the experiments (288 h), similar to the unmanipulated group larvae (Figure 59A). In addition, groups of larvae injected with live *Fno* in (for the five isolates), *i.e.* 0.7–1.8 x 10^8, 3.8–6.3 x 10^8, 0.8–1.3 x 10^9 and 2.5–6.7 x 10^9 CFU mL^{-1}, showed no change in body colour within a few minutes after injection (Figure 5.9C, D, E and F). In contrast, when the larvae were injected with *Fno* culture filtrate (supernatant), some larvae started to melanise within just few minutes after injection (Figure 5.9B). Moreover, larvae injected with heat killed *Fno* at 2.4–6.7 x 10^9 CFU mL^{-1}, where *Fno* had been heat killed at 60˚C for 60 mins (Figure 5.10G) showed greater degrees of melanisation than larvae injected with *Fno* that had been heat killed for 90˚C for 30 mins (Figure 5.9H), and this was consistent for each of the five isolates tested. During 288 hpi, the groups of larvae injected with different concentrations of live bacteria developed melanisation progressively and this occurred more quickly at the greater *Fno* concentrations (Figure 5.10D, E, F, G and H). Moreover, larvae started pupated sometimes within 96 hpi, and this mostly occurred in the unmanipulated group, followed by groups injected groups with PBS only, while few larvae pupated in groups injected with *Fno* culture filtrate, while the rest of the larvae were observed to show progressive melanisations (Figures 11 and 12) or remained with cream body colour (Figure 5.10A, B and C). So, on those three groups the larvae did not stress too much and the larvae endocrine system were still working very well. No pupation occurred in the groups injected with live *Fno* or with the heat-killed bacteria (Figure 5.10D to H), as the bacteria caused stress in the larvae and inhibited the larvae pupation.

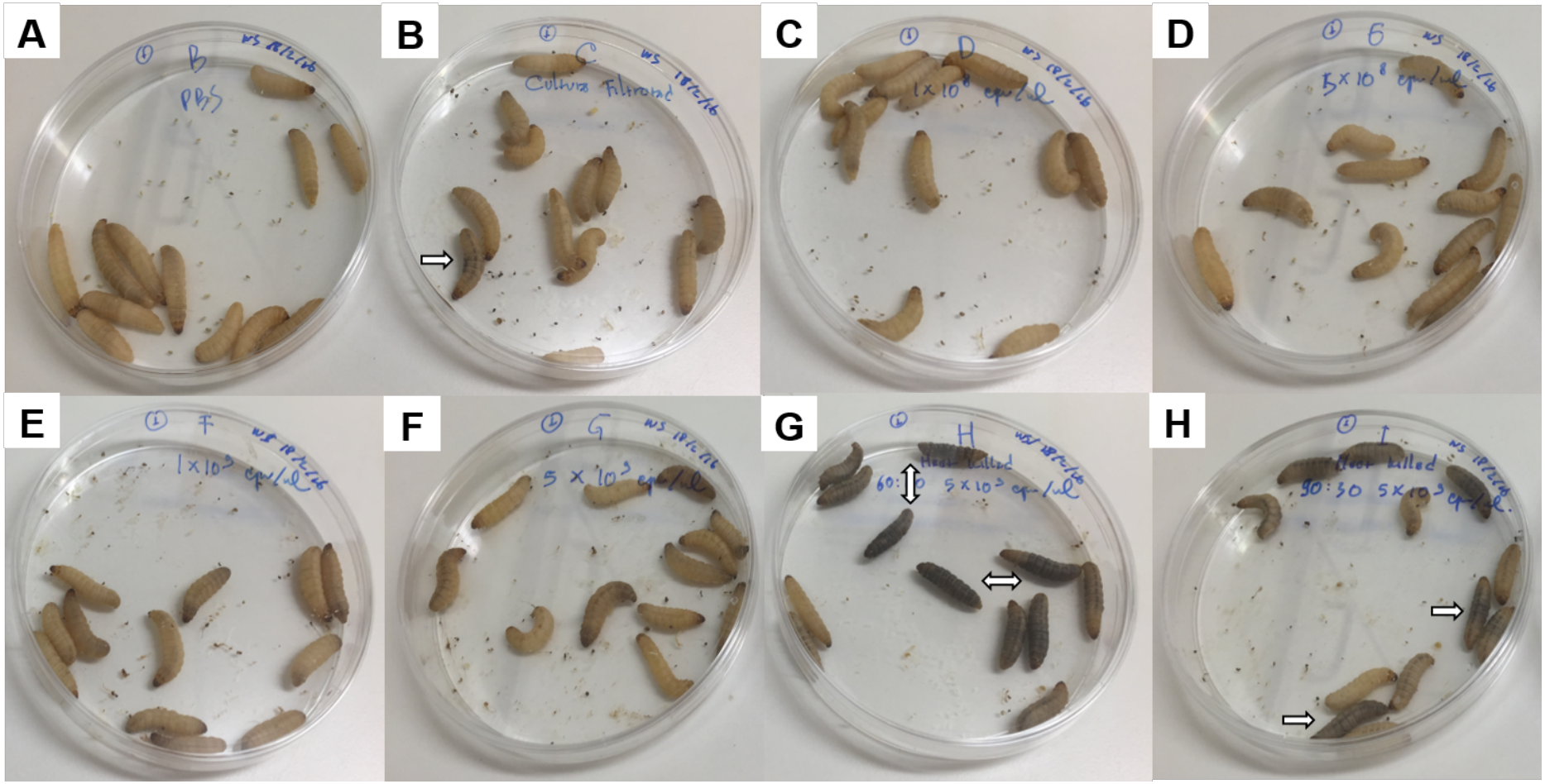

Figure 5.9 Appearance of groups of *G. mellonella* within a few minutes after injection with different concentrations of *Fno* STIR-GUS-F2f7: (A) larvae injected with PBS showed normal cream body colorations; (B) larvae injected with *Fno* culture filtrate (supernatant) where only a few larvae found started to melanise (white arrow); (C–F) groups of larvae injected with live bacteria with different concentrations of live bacteria from low to high *e.g.* 0.7–1.8 x 10^8, 3.8–6.3 x 10^8, 0.8–1.3 x 10^9, and 2.5–6.7 x 10^9 CFU mL^{-1}, showed no melanisation produced after the injection and this developed later after infection; and (G–H) larvae injected with heat killed *Fno* at 2.4–6.7 x 10^9 CFU mL^{-1} produced melanisation within just few minutes after injection with *Fno* heat killed at 60˚C for 60 mins (H) showing more melanised larvae compare to *Fno* heat killed at 90˚C for 30 mins (I) (white arrows). Petridish diameter is 8.5 cm (n=12).

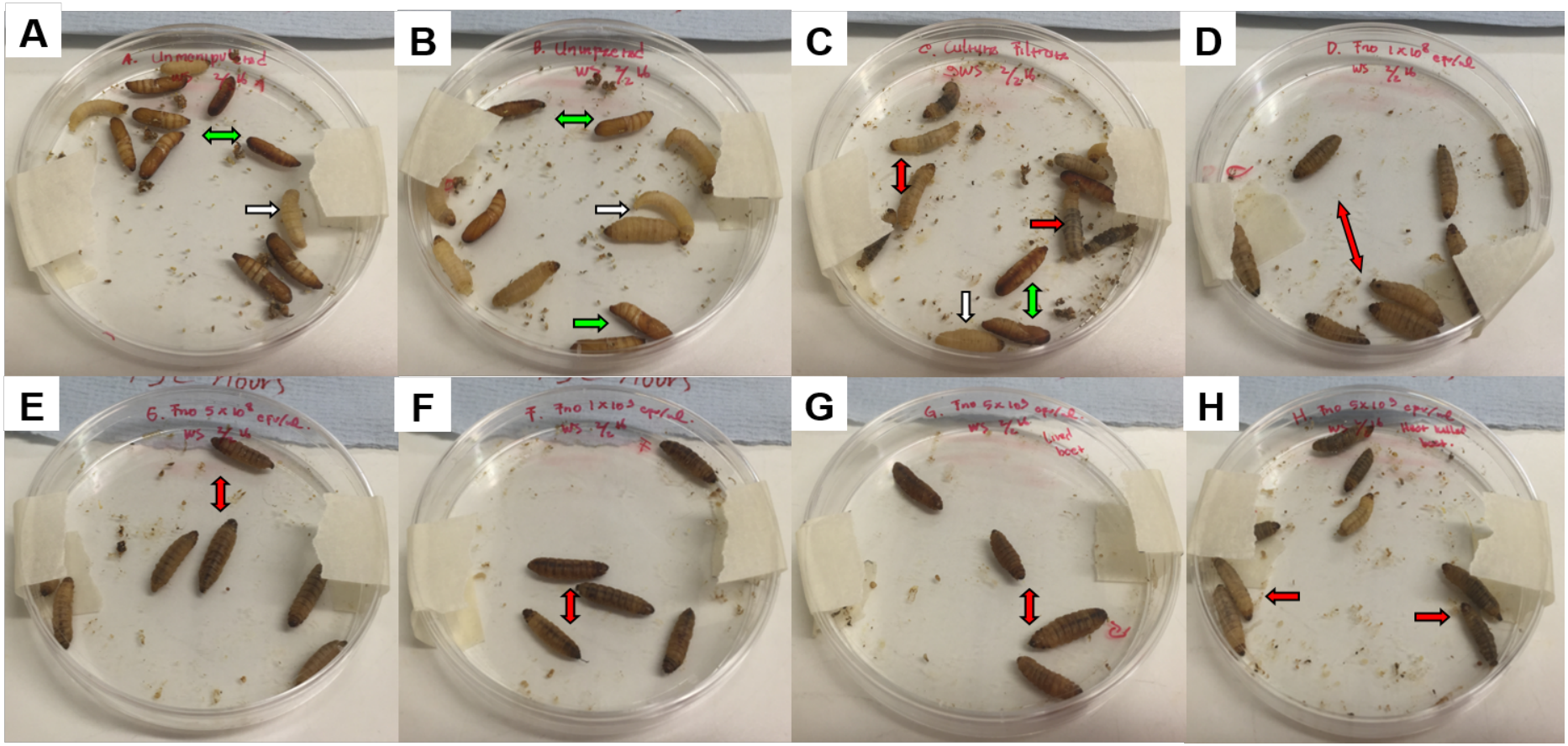

Figure 5.10 Appearance of *G. mellonella* from second batches of larvae, at 192 hpi with or without injection with different concentrations of *Fno* STIR-GUS-F2f7: (A) unmanipulated group of larvae, almost all larvae pupated (white arrow), while the rest have normal with cream body colour (green arrow); (B) in the group injected with PBS several larvae pupated (white arrows) but some still showed the normal cream body colorations (green arrow); (C) larvae injected with *Fno* culture filtrate, few larvae pupated (white arrow), while others showed progressive melanisations (red arrows) or remained normal with cream colour (yellow arrow); (D–G) groups of larvae injected with different concentration of live bacteria from low to high doses *e.g.* (D) 0.7–1.8 x 10^8, (E) 3.8–6.3 x 10^8, (F) 0.8–1.3 x 10^9, and (G) 2.5–6.7 x 10^9 CFU mL^{-1}, produced progressive melanisation over the time after the injection; and (H) larvae injected with *Fno* heat killed at 90˚C for 30 mins at 2.4–6.7 x 10^9 CFU mL^{-1} developed little melanisation since it started at just a few minutes after injection. Petridish diameter is 8.5 cm (n=12).

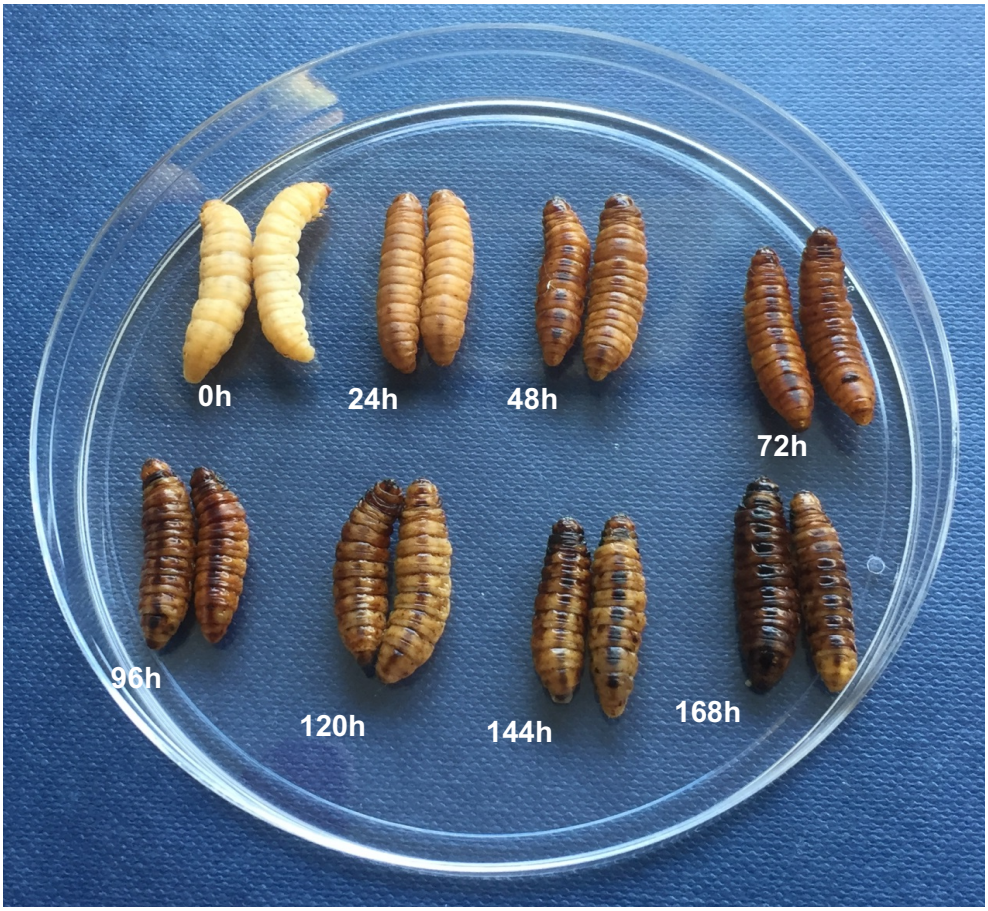

Figure 5.11 Representative images to show wax moth larvae demonstrated progressive melanisation over time after injection with *Fno.* From left to right: group of two of *G. mellonella* after challenge with an inocula of *Fno* STIR-GUS-F2f7 (0.92 x 10^9 CFU mL^{-1}) at different time points (*i.e.* first rows at 0, 24, 48 and 72 hpi, and second rows at 96, 120, 144 and 168 hpi). Infected larvae showed progressively increasing melanisation. Petridish diameter is 8.5 cm.

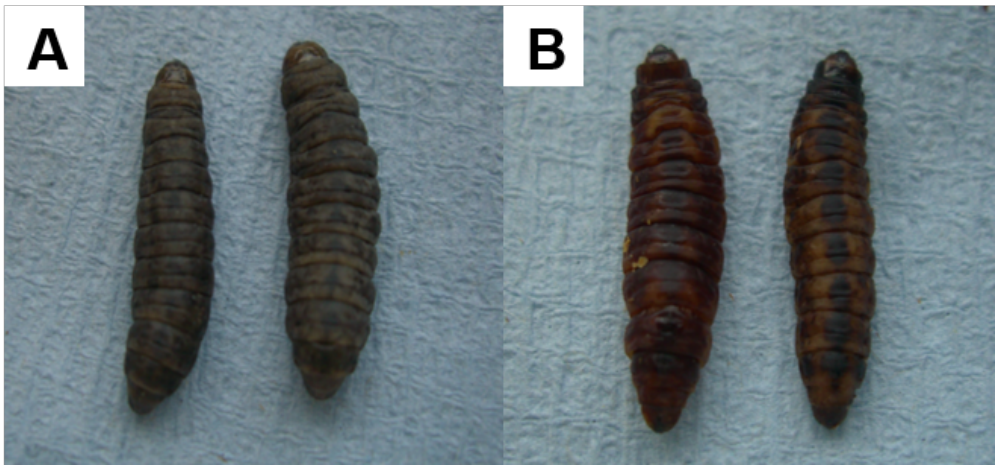

Figure 5.12 Typical final melanisation when the injected *G. mellonella* died after challenge with *Fno*: (A) the larvae are totally black and this occured mostly after acute mortality; and (B) final progressive melanisation after infection, with the larvae dying more slowly having turned a dark brown colour and with the patternation was time observed often with a curved body shape and happened during chronic mortality.

5.3.2 Correlation of virulence of *Fno* isolates in *O. niloticus* and *G. mellonella* infection models

The relative virulence of each *Fno* isolate in the larva was compared with virulence determined previously for each isolate in a red Nile tilapia *O. niloticus* infection model (Shahin, unpublished). Interestingly, there was a highly significant positive correlation between relative virulence of the 5 isolates of *Fno* in *G. mellonella* larvae and tilapia models of infection, and the Spearman rho for rank (larvae) and rank (fish) was 0.900 ($p=0.037$) (Figure 5.13). This result indicates that more virulent *Fno* strains in *O. niloticus* were also more virulent in the insect host when injected. Overall, from the present results *Fno* STIR-GUS-F2f7, which was isolated from an outbreak in red Nile tilapia in Northern Europe, was the most virulent isolate compared to other 4 *Fno* strains, while *Fno* Japan showed the weakest virulence in *G. mellonella* infection model and was not tested completely in the fish model as it was determined to be almost avirulent in preliminary trials.

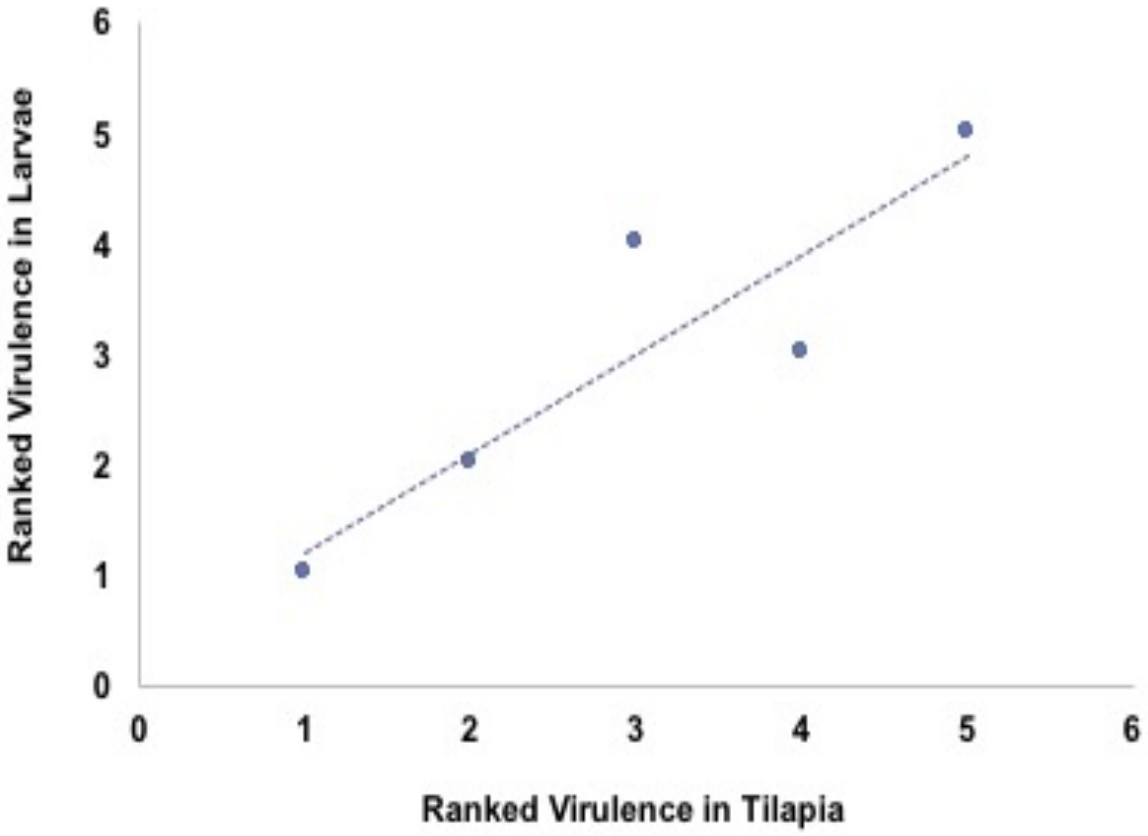

Figure 5.13 Spearman's Rank Correlation Coefficient of the virulence 5 *Fno* isolates in the *G. mellonella* larvae (calculated as total AUC of the cumulative larval survival with *Fno* concentrations at 10^8 and 10^9 CFU mL^{-1}; injected at 10 µL $larva^{-1}$, n=24), and in the *O. niloticus* infection model (total AUC of the cumulative tilapia survival determined after IP injection at *Fno* concentrations of 10^6 and 10^7 CFU mL^{-1}; injected at 1 mL $fish^{-1}$, n=6; Shahin, unpublished). *Fno* STIR-GUS-F2f7 and *Fno* Japan showed as the most and weakest virulence in *G. mellonella* and naïve *O. niloticus* hosts, respectively. *Fno* Japan was not tested completely in the fish model as it was determined to be almost avirulent in preliminary trials (data not shown).

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5.3.3 Antibiotic therapy in the *G. mellonella–Fno* system

The role of tetracycline as an antibacterial therapy was evaluated as part of the validation of the model to demonstrate that the infection is real and infected individuals could be rescued from an otherwise lethal infection. In addition, the antibiotic therapy was also performed with regard to investigating whether the *G. mellonella–Fno* system could be used to study the effect of antibacterial agents. Three doses of tetracycline (10 mg g^{-1}) were administered at 2, 24 and 48 h after the inoculation of larvae for all strains of *Fno*, and tetracycline had been chosen based on the results from the antibiotics disc diffusion assay testing on CHAH where *Fno* was deemed to be highly susceptible to the action of tetracycline (Chapter 2, Section 2.3.3.2A).

Tetracycline was effective in prolonging the life span of larvae infected with each *Fno* isolate as percentage larval survival was significantly improved when the larvae were treated with three doses of tetracycline at 10 mg g^{-1}, compared to groups of larvae infected with same dose of bacteria and PBS only (Figures 5.14, 5.15, 5.16, 5.17 and 5.18). In order to rule out any killing of larvae by tetracycline, this antibiotic was administered at the same doses to larvae that had not been injected with bacteria and there were no deaths in these groups (Figures 5.14, 5.15, 5.16, 5.17 and 5.18).

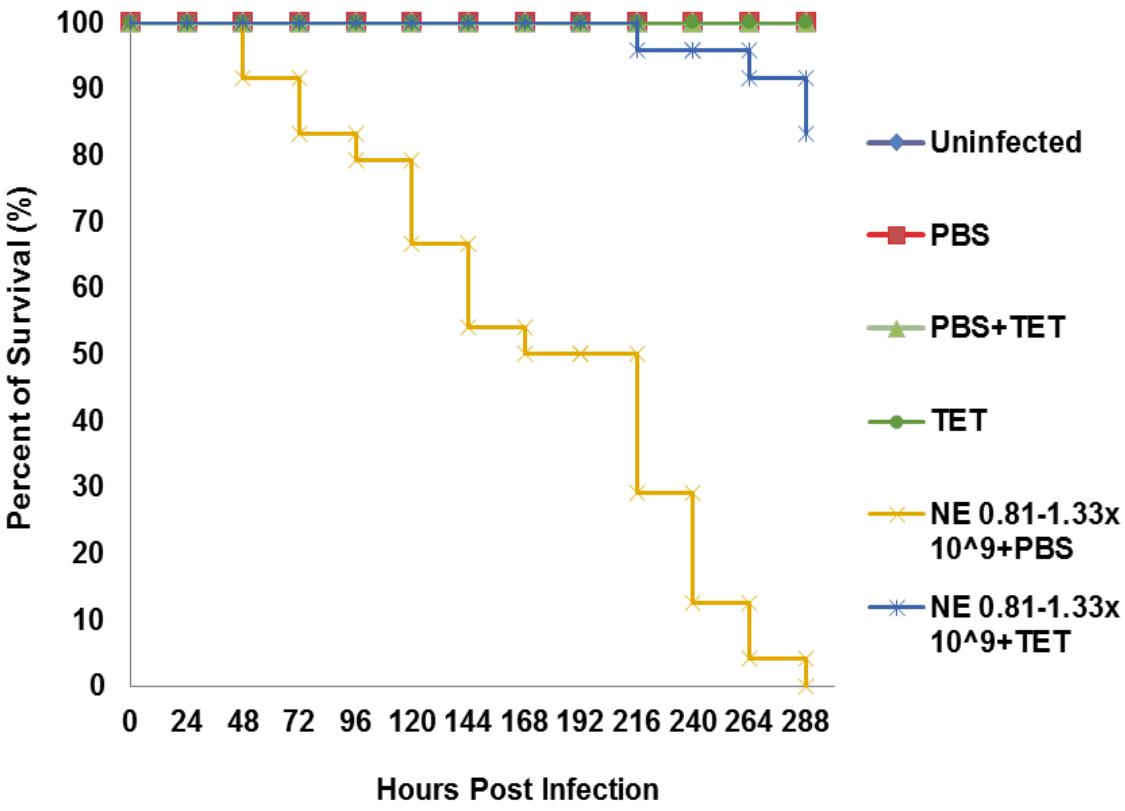

Figure 5.14 Tetracycline increased percentage survival of *G. mellonella* after challenge with *Fno* STIR-GUS-F2f7 at 0.81–1.33 x 10^9 CFU mL^{-1} and treated with 10 mg g^{-1} tetracycline in 10 µL PBS at 2, 24, and 48 h post injection (infected+TET). A control group received the *Fno* inocula and PBS instead of TET (Infected–TET), while the TET only group controlled for the toxicity of the antibiotic. The larvae were kept at 28°C for 288 h (n=24).

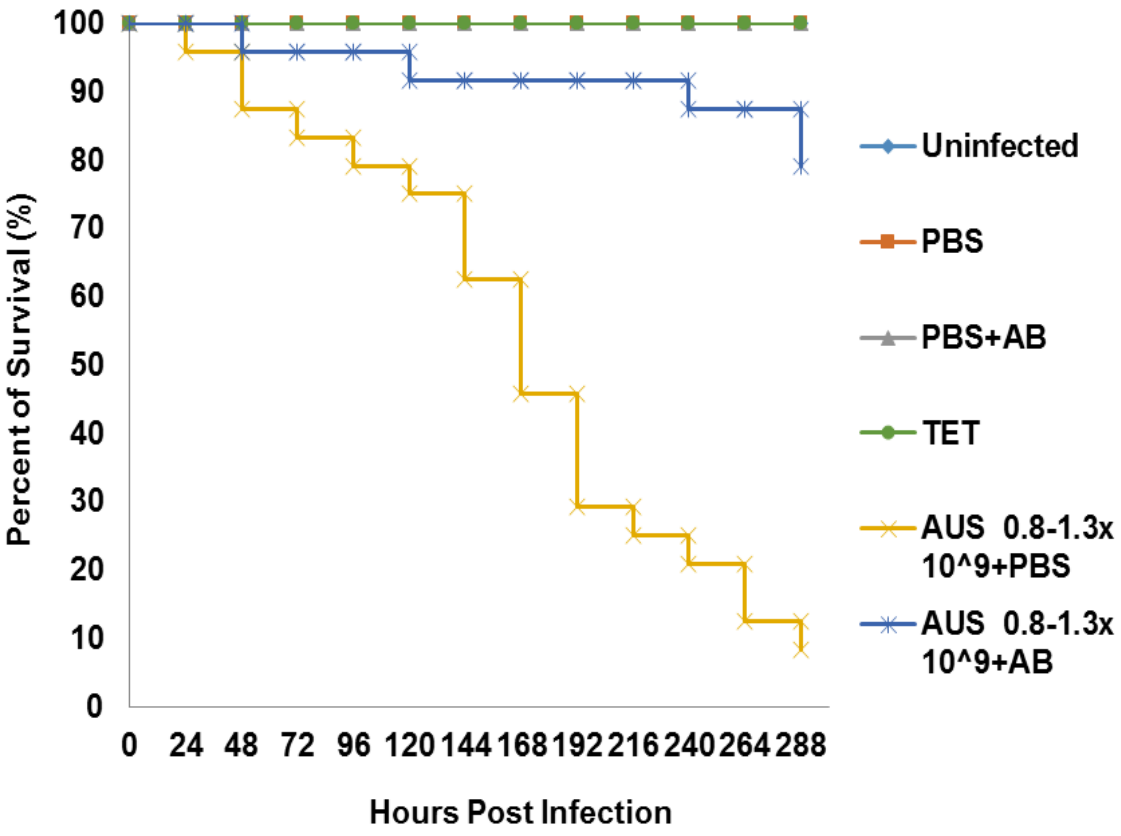

Figure 5.15 Tetracycline increased percentage survival of *G. mellonella* after challenge with *Fno* Austria at 0.8–1.3 x 10^9 CFU mL^{-1} and treated with 10 mg g^{-1} tetracycline in 10 µL PBS at 2, 24, and 48 h post injection (infected+TET). A control group received the *Fno* inocula and PBS instead of TET (Infected–TET), while the TET only group controlled for the toxicity of the antibiotic. The larvae were kept at 28°C for 288 h (n=24).

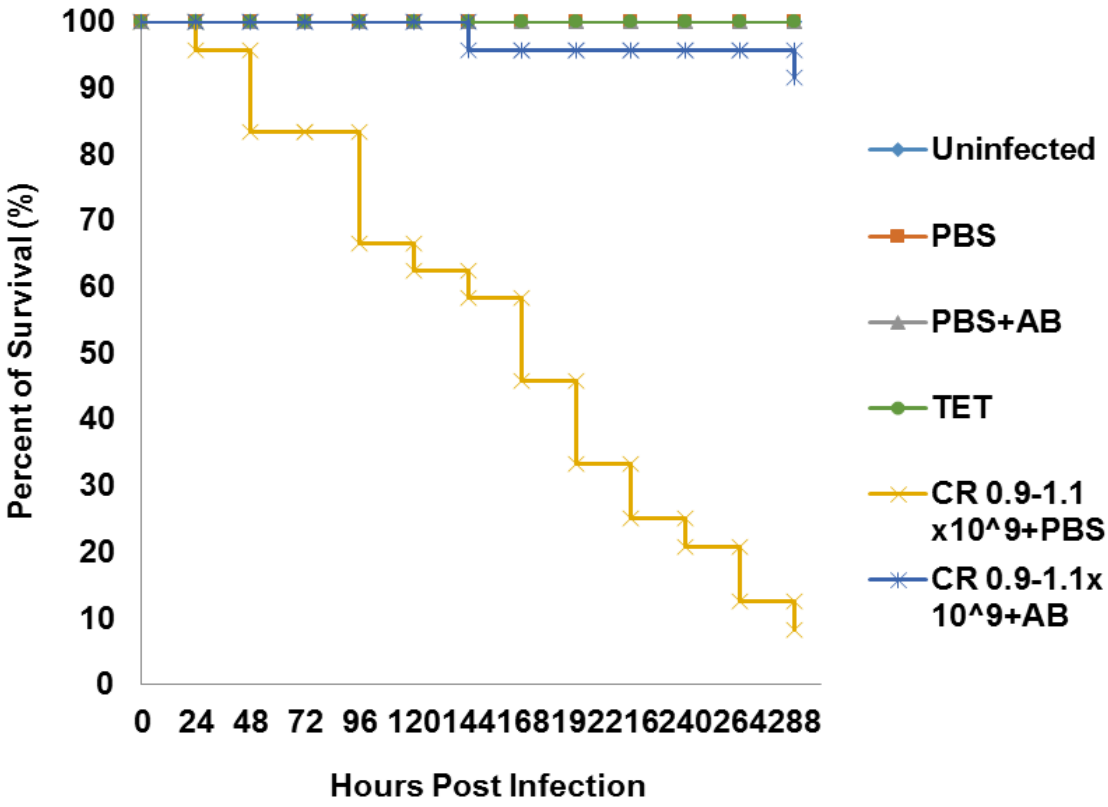

Figure 5.16 Tetracycline increased percentage survival of *G. mellonella* after challenge with *Fno* Costa Rica at 0.9–1.1x10^{9} CFU larva^{-1} and treated with 10 mg g^{-1} tetracycline in 10 µL PBS at 2, 24, and 48 h post injection (infected+TET). A control group received the *Fno* inocula and PBS instead of TET (Infected–TET), while the TET only group controlled for the toxicity of the antibiotic. The larvae were kept at 28°C for 288 h (n=24).

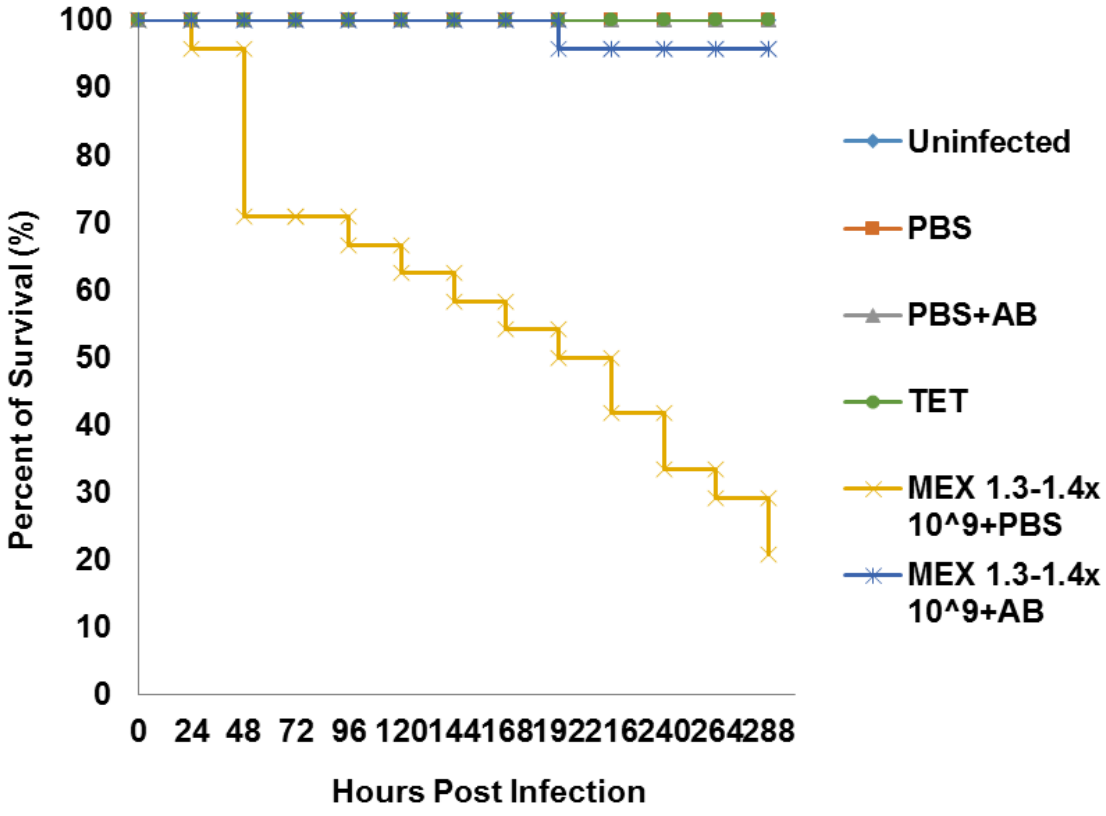

Figure 5.17 Tetracycline increased percentage survival of *G. mellonella* after challenge with *Fno* Mexico at 1.3–1.4 x 10^9 CFU mL^{-1} and treated with 10 mg g^{-1} tetracycline in 10 µL PBS at 2, 24, and 48 h post injection (infected+TET). A control group received the *Fno* inocula and PBS instead of TET (Infected–TET), while the TET only group controlled for the toxicity of the antibiotic. The larvae were kept at 28°C for 288 h (n=24).

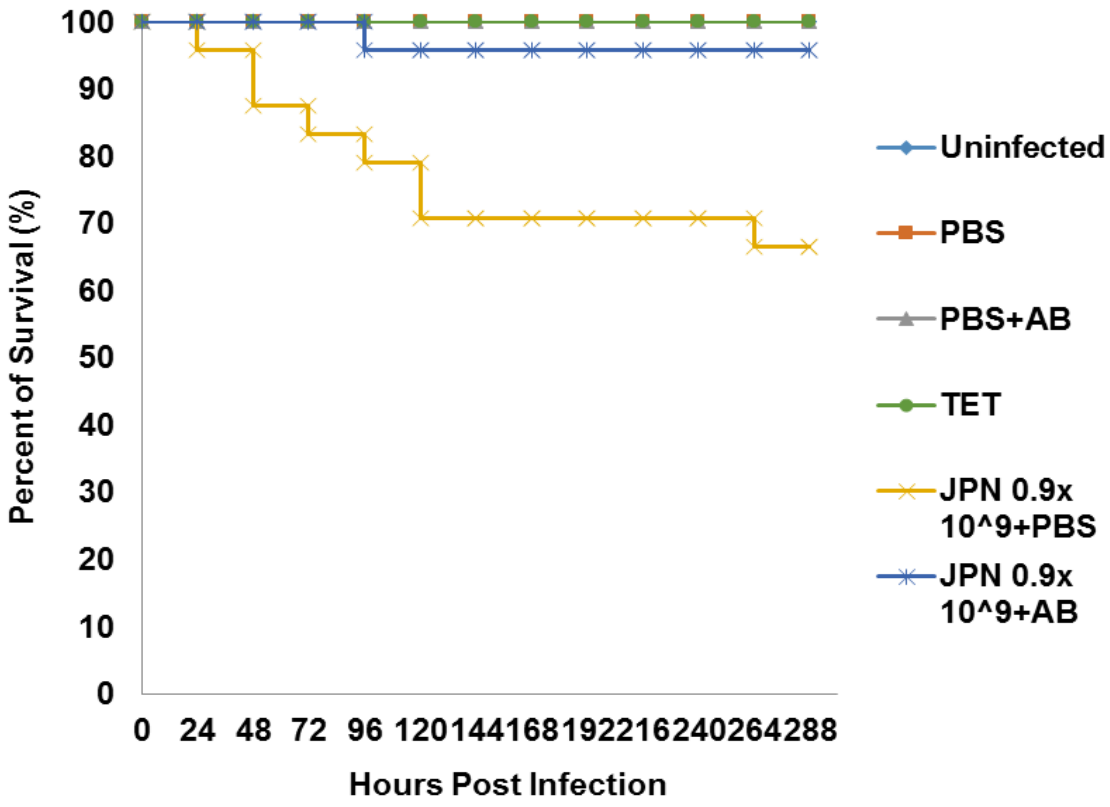

Figure 5.18 Tetracycline increased percentage survival of *G. mellonella* after challenge with *Fno* Japan at 0.9 x 10^9 CFU mL^{-1} and treated with 10 mg g^{-1} tetracycline in 10 µL PBS at 2, 24, and 48 h post injection (infected+TET). A control group received the *Fno* inocula and PBS instead of TET (Infected–TET), while the TET only group controlled for the toxicity of the antibiotic. The larvae were kept at 28°C for 288 h (n=24).

5.3.4 Visualization of *Fno* in the larvae cells

Larvae injected with 9.2 x 10^8 CFU mL^{-1} of *Fno* STIR-GUS-F2f7 were studied to determine the fate of the bacteria in the host, and larval tissues were stained with H & E, Gram Tworf and IHC. The arrangement of six different rings in a paraffin block facilitated the study of the infection throughout the larvae and allowed for tridimensional reconstruction of the larval anatomy (Figure 5.19).

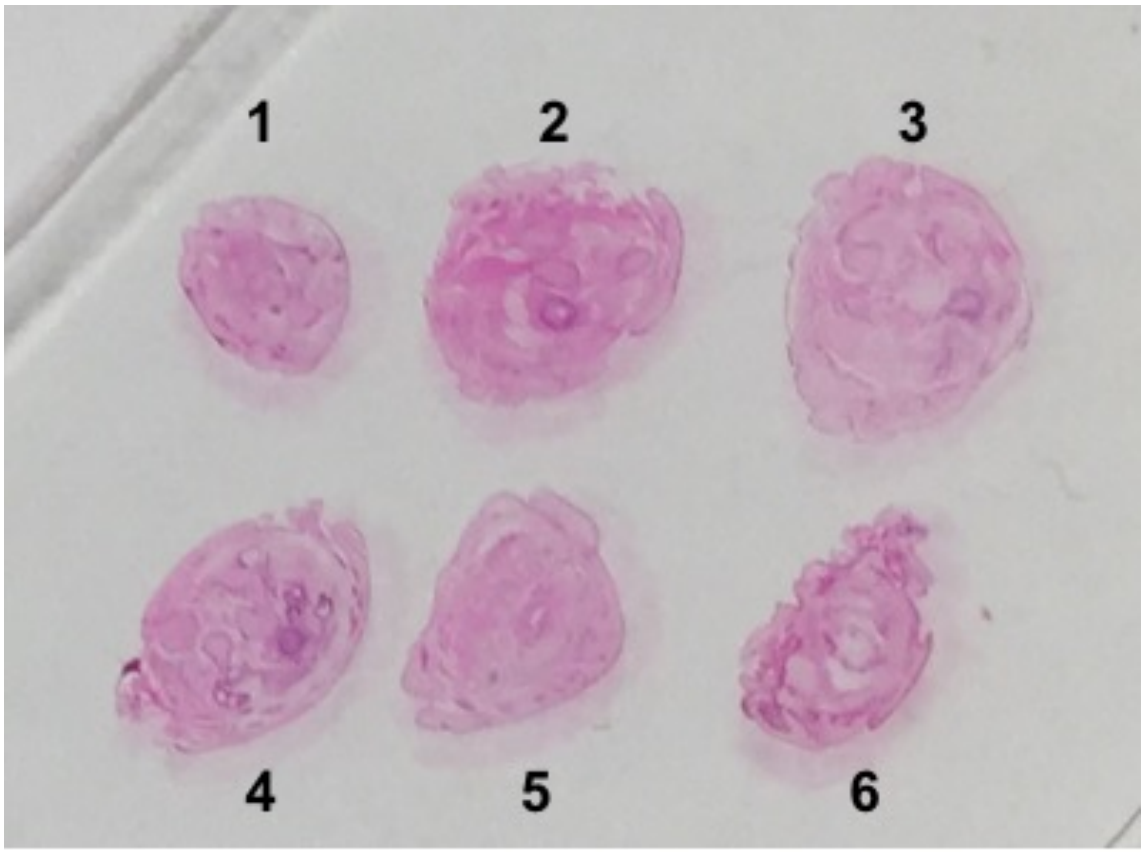

Figure 5.19 Larval tissues were processed and stained with H & E, with controls processed in parallel, proximal (1), middle (2–5) and distal (6) transversal sections are visible on a slide.

5.3.4.1 Haematoxylin and eosin staining

Histological analysis of the pathologichal changes in *G. mellonella* infected with *Fno* STIR-GUS-F2f7 at 9.2 x10^8 CFU mL^{-1} was performed using H & E staining at 24, 48, and 72 hpi. The larval immune cells were observed morphologically in different tissues, and in ‘‘nodulations’’ (encapsulations of large invading pathogens) which are produced from formation of huge numbers of hemocytes in layers as a part of the *G. mellonella* response to infection by pathogens, such as fungi or parasites (Mylonakis *et al.*, 2005) (Figures 5.22, 5.23 and 5.24). Haemocytes are involved in clotting and formation of nodules with encapsulation at different infectious sites. Haemocytes appear as oval or round cells of various sizes, and sometimes look like small immature cells, with a variable number and type of cytoplasmic granules and vacuoles in areas far away from where the infectious agent could be seen, while at infection sites, haemocytes formed multicellular aggregates, *i.e.* nodules, to entrap the invading *Fno*.

Few haemocytes were observed in the control unmanipulated larvae. These were mostly sub-cuticular and found around the muscle fibres (Figure 5.21A), in the fat body, around tracheal walls (Figure 5.21B, C, D and E), and in discrete aggregates surrounding the intestinal outer layer (Figure 5.21E, and F). At 48 hpi, compared to control larvae, infected larvae showed an increase of haemocytes in the fat body, subcuticular areas and surrounding tracheal walls, while early stages of nodulations were observed, and these small nodules were usually found around the fat body (Figure 5.22B, C and F). Meanwhile, early stages of necrosis were observed in the fat body by the presence of hypertrophied fat body cells and/or circulation haemocytes inside the fat body which were expanding more widely into the whole fat body (Figure 5.22A and B). Moreover, after infection, haemorrhages and the formation of pale eosinophilic protein lakes were observed in the fat body, surrounding the subcuticular area, in the first proximal gastrointestinal tract, and around the tracheal area, and this led to tissues necrosis (Figure 5.22A, B, C, D, and E). This result is in line with previously reported findings when larvae were inoculated with *C. albicans* (Sherry *et al.*, 2014; Rajendran *et al.*, 2015).

At 72 h, an enormous number of haemocytes were observed surrounding the tracheal walls, fat body, close to the subcuticular area and around the muscle fibres, and nodulations had also formed (Figure 5.23B and D). At this stage, nodules of various sizes were observed (small to large and confluent), and contained melanised haemocytes (Figure 5.23A, B, C and D), while intermediate stages of necrosis had spread to the subcuticular area and this was apparent in the tracheal walls and around the gastrointestinal tract (Figure 5.23C, E and F). Furthermore, at 96 h, giant hypertrophied haemocytes were observed surrounding the fat body and muscle fibres (Figure 5.24A), while numerous enlarged circulating haemocytes were found around the subcuticular area (Figure 5.24B). At this stage, not many nodules were observed and those that were seen were small in size (Figure 5.24C, D and E). Meanwhile, tissue necrosis was greater than at 72 h, especially around the tracheal walls, and surrounding and inside the gastrointestinal tract, and this had resulted in haemorrhages and the formation of pale eosinophilic protein lakes (Figure 5.24C, D and F). In mature nodules, melanin deposition was observed, and spindle cells had formed clots (Figure 5.24E).

Highlighting the role of encapsulation as a response to infection in the larvae, haemocyte recruitment was observed in almost all the larval organs, and spindle cells were observed to form clots in the nodules to entrap coccobacilli bacteria. Specifically, round haemocytes had acquired a spindle shape to stratify around the infectious agent in the external parts of melanised nodules (Figures 5.22B and F; 5.23A, B, C, D and F; 5.24C, D and E).

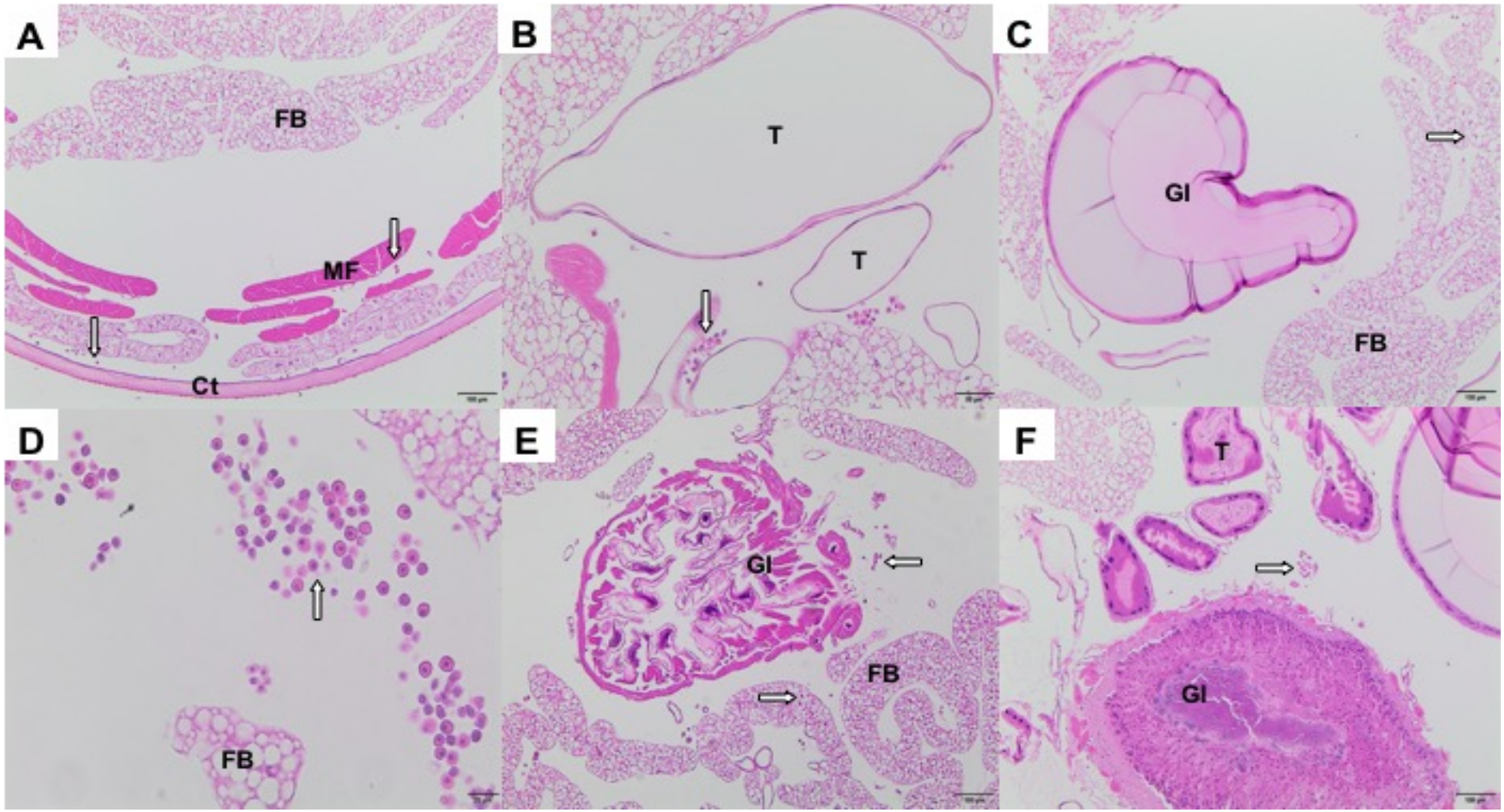

Figure 5.20 Unmanipulated control larvae (A-F) stained by H & E: (A) haemocytes (arrows) monolayer localised sub-cuticularly (Ct) and around the muscle fibres (MF) and fat body (FB); (B, C, D & E) scattered haemocytes surrounding the tracheal walls (T) in normal conditions, and around and in the normal fat body (FB); and (E & F) small aggregates of haemocytes surrounding the gastro intestinal tract (GI) in normal conditions. Scale bar: B and D = 50 µm, and A, C, E and F = 100 µm.

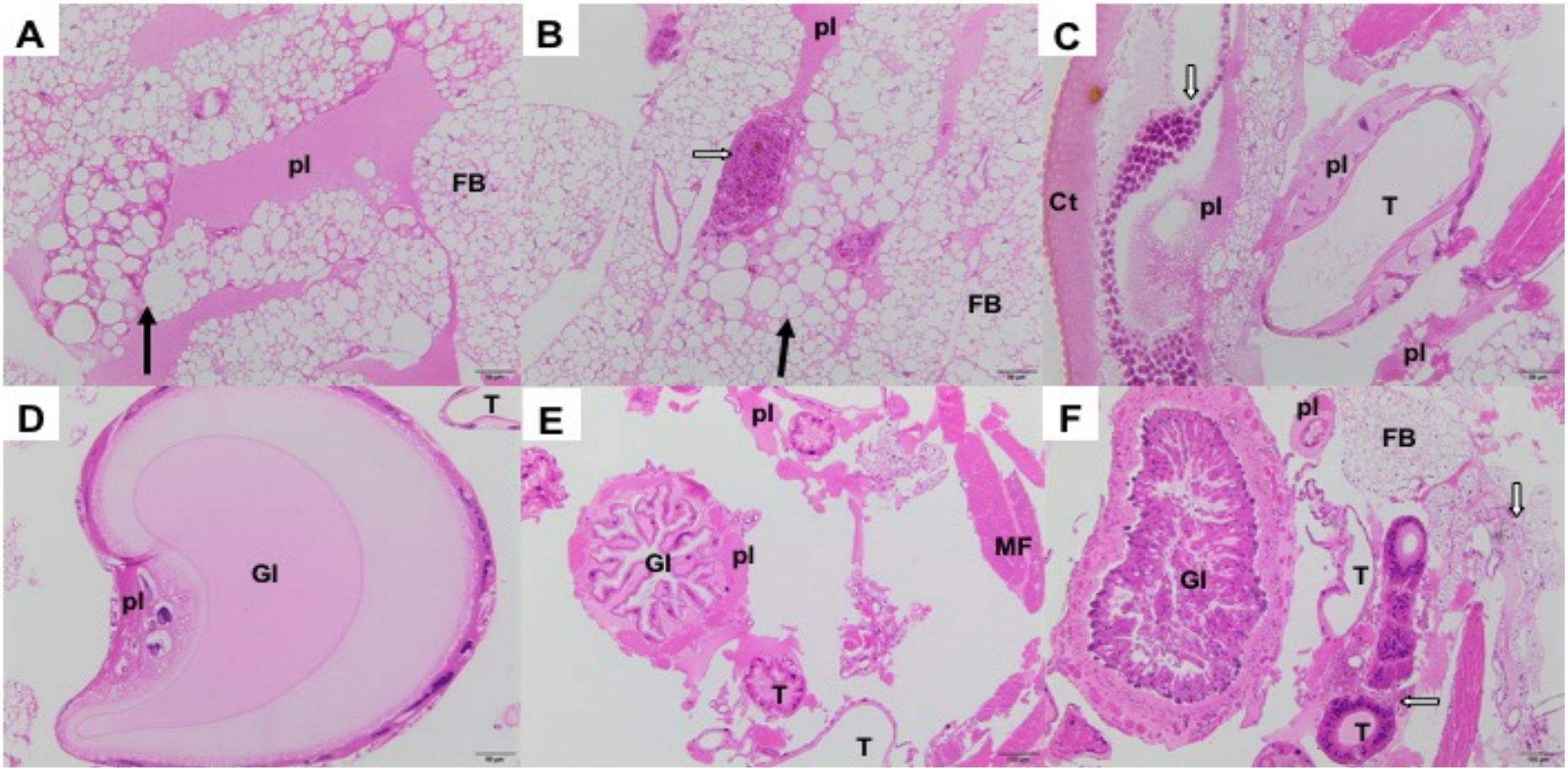

Figure 5.21 Cellular immune response in larvae infected with *Fno* STIR-GUS-F2f7 ($9.2x10^8$ CFU mL^{-1}) (A–F) at 48 hpi by H & E staining: (A) *Fno* infection process started with vacuolated fat body cells extending and expanding into the whole fat body (FB) (black arrow), and necrosis of the fat body often resulted in haemorrhages of haemolymph and the formation of pale eosinophilic protein lakes (pl): (B) white arrow highlights pronounced early stages of small aggregation, melanisation, and merger of haemocytes into nodules which is representative of haemocyte recruitment and activation in the fat body with vacuolated cells; (C) huge haemocyte recruitment in the subcuticular (Ct) area surrounded by pale eosinophilic protein lakes (pl) and this lakes were found even around the tracheal walls (T); (D & E) pale eosinophilic protein lakes (pl) around the tracheal walls and surrounding the first proximal gastrointestinal tract (Gl); and (F) haemocytes increased in number in the fat body and surrounded the tracheal walls to then form early stage of nodulations associated with pale eosinophilic protein lakes (pl). Scale bar: A, B, C and D = 50 µm, and E and F = 100 µm.

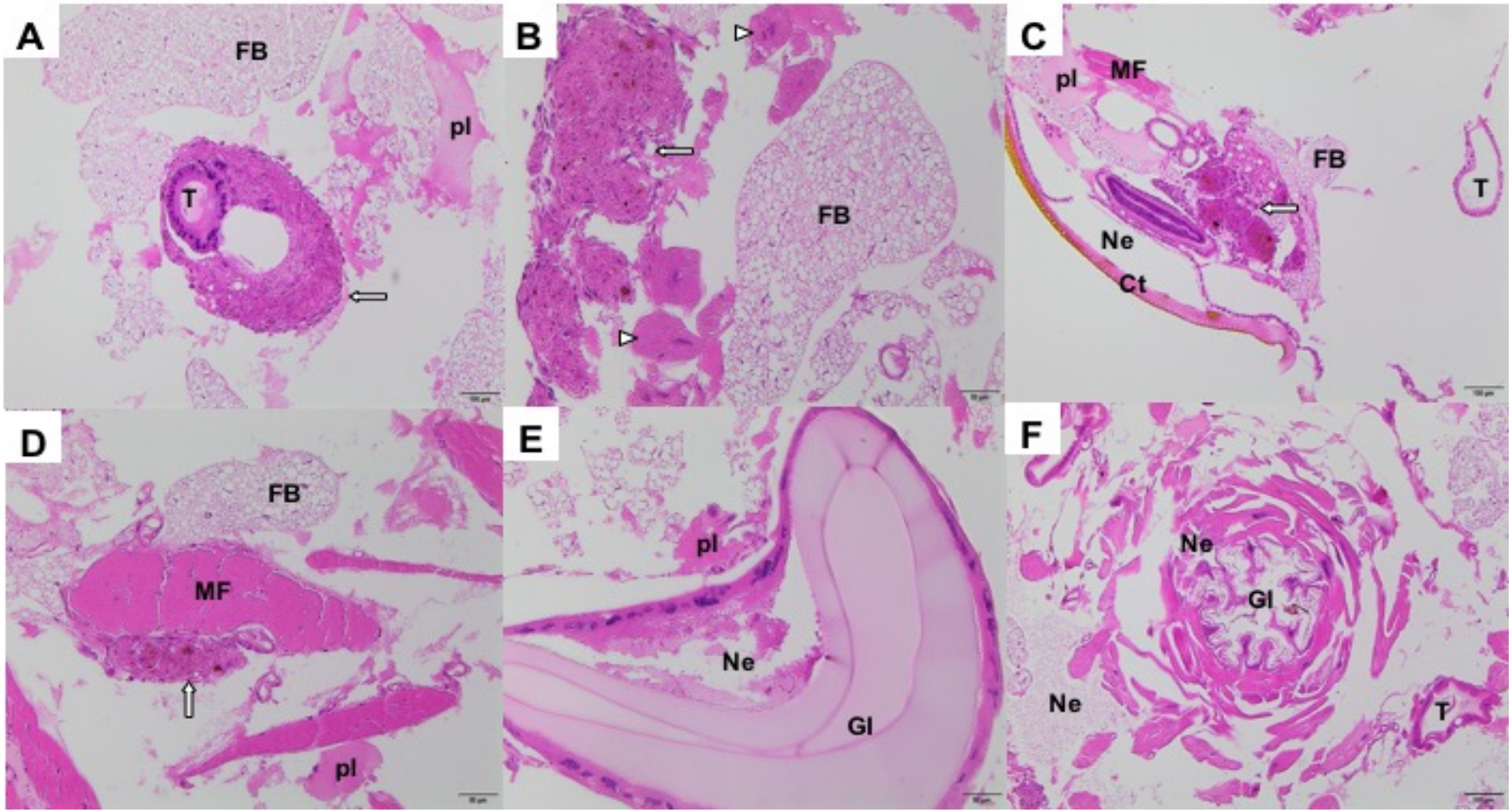

Figure 5.22 Cellular immune response in infected larvae with *Fno* STIR-GUS-F2f7 (9.2 x 10^8 CFU mL^{-1}) (A–F) at 72 hpi by H & E staining: (A, B, C & D) huge melanised nodules, and evidence of haemocytes recruitment surrounding the nodules (white arrows) in the tracheal wall (T), the fat body (FB) and close to the subcuticular (Ct) area; (B) few hugely enlarged hemocytes during the infection process (white arrows head); (C & E) early necrosis (Ne) of subcuticular area, in the tracheal walls, and in and surrounding the gastrointestinal tract (Gl). Scale bar: B, D and F = 50 µm, and A, C and F = 100 µm.

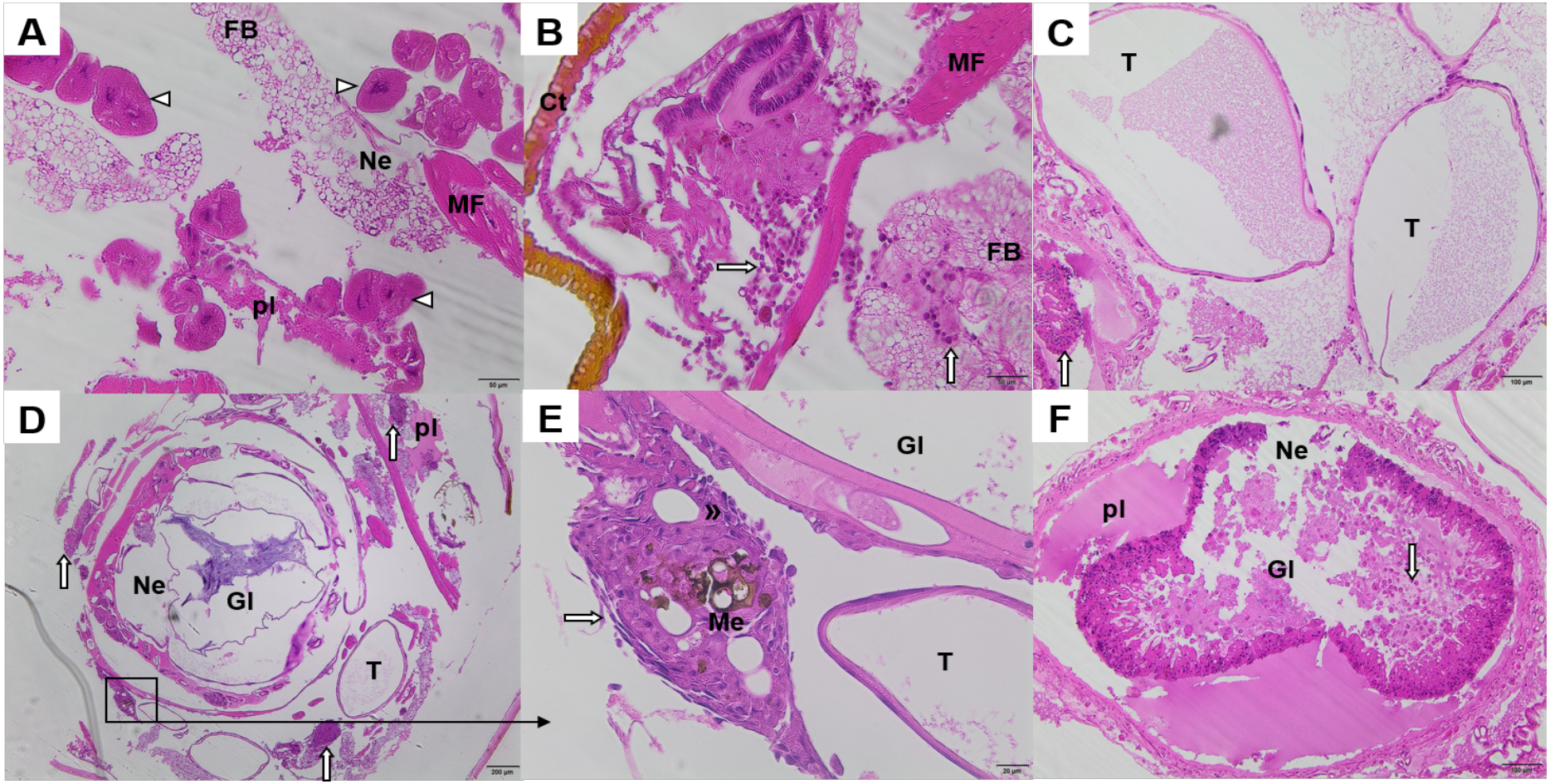

Figure 5.23 Cellular immune response in infected larvae with *Fno* STIR-GUS-F2f7 ($9.2x10^8$ CFU mL^{-1}) (A–F) at 96 hpi by H & E staining; (A) giant hypertrophication of circulation hemocytes (white arrows head) surrounding the fat body (FB) and muscle fibres (MF) during the latter stages of infection process; (B) many circulating haemocytes surrounding the subcuticular area (Ct); (C) necrosis (Ne) inside the tracheal walls; (C, D & E) some nodules of small size observed (white arrows); (E) melanine (Me) deposition in mature nodules with spindle cells forming clots, and newer round haemocytes (») recruited from the haemolymph surrounding the gastrointestinal tract (Gl); and (E and F) large necrosis in gastrointestinal tract (Gl) resulted in haemorrhage of haemocytes and the formation of pale eosinophilic protein lakes (pl). Scale bar: E = 20 µm, A and B = 50 µm, and C, D and F = 100 µm.

5.3.4.2 Gram-Twort staining

A *G. mellonella-Fno* interaction study was performed by Gram Twort staining where the cells in unmanipulated control larvae nuclei appeared as red, with varying shades of green for collagen, red blood cells (haemocytes), and cytoplasm (Figure 5.24). In infected larvae, bacteria appeared as red or pink in the cells. At 48 hpi, the bacteria had reached and progressively replicated inside enlarged un-vacuolated and vacuolated haemocytes to entrap the bacteria and the haemocyte cells had mostly lost their nuclei or had enlarged nuclei (Figure 5.25A, C and D). Melanised haemocytes were observed to surround and be inside large necrotic areas in the fat body (Figure 5.25E). In that area, the haemocytes had retained or lost their nuclei, containing bacteria and presented *Fno* escaping from the phagocytes or because of cells necrosis (Figure 5.25E). Enormous numbers of haemocytes had been recruited and activated to form the early stage of nodulation in the fat body while the bacteria contained inside melanised haemocytes (Figure 5.25B). At 72 hpi, larger nodules had formed in the fat body with the haemocytes and melanised haemocytes found to contain cocco-bacilli bacteria (Figure 5.26A and F). Haemocyte lakes were observed in the fat body and led to a large area of necrosis with haemocyte cells fully occupied by the bacteria (Figure 5.26B and C). At 96 hpi, huge numbers of enlarged and multiply vacuolated haemocyte were present and multiple nodules were observed surrounding the fat body and the tracheal walls and the subcuticular area with the haemocyte cells containing bacteria (Figure 5.27A, E and F). The early stages of necrosis on the haemocyte cells was found leading to extensive necrosis of the organs (Figure 5.27B, C and D).

Great numbers of haemocytes containing the bacteria were also observed inside the muscle fibres (Figures 5.25B and 5.26D) and surrounding the sub-cuticular area (Figure 5.26E). Necrosis of the vascular tissue often ensued upon the obliteration of the arteriolar walls, with the consequent formation of proteinaceous fluid lakes and hemorrhages of haemolymph composed of enomorous haemocytes containing huge numbers of small pleomorphic coccobacilli bacteria present inside cytoplasmic vacuoles and inside the

nucleus of haemocytes, as well as in melanized haemocytes cells (Figures 5.25, 5.26 and 5.27). The invasion of coccobacilli bacteria inside and surrounding the tracheal walls and gastrointestinal track also led to necrosis of the organs (Figures 5.26F and 5.27F).

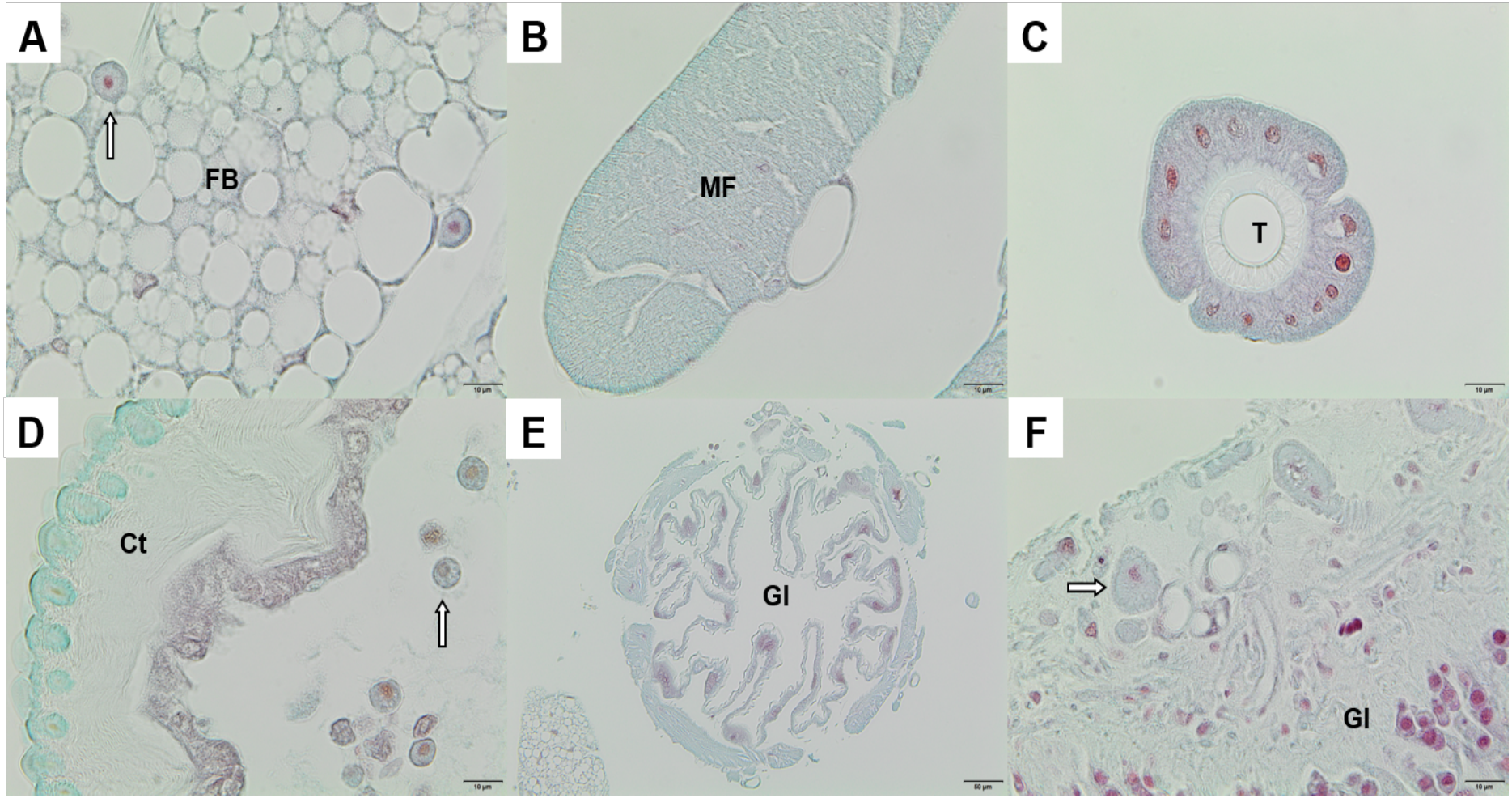

Figure 5.24 Gram Twort staining of unmanipulated control larvae in healthy condition, with normal immune cells, where the nuclei are red, and varying shades of green for collagen, red blood cells (haemocytes), and cytoplasm: (A, D, & F) the presence of a small number of scattered haemocytes (arrows) form monolayer localised around and in the normal fat body, circulating in the haemolymph, around the sub-cuticular area, and surrounding the gastrointestinal tract in normal larvae conditions. (A) fat body (FB); (B) muscle fibre (MF); (C) tracheal wall (T); (D) cuticle (Ct); (E & F) proximal and distal gastrointestinal tract (GI). Scale bar: A to F = 10 µm.

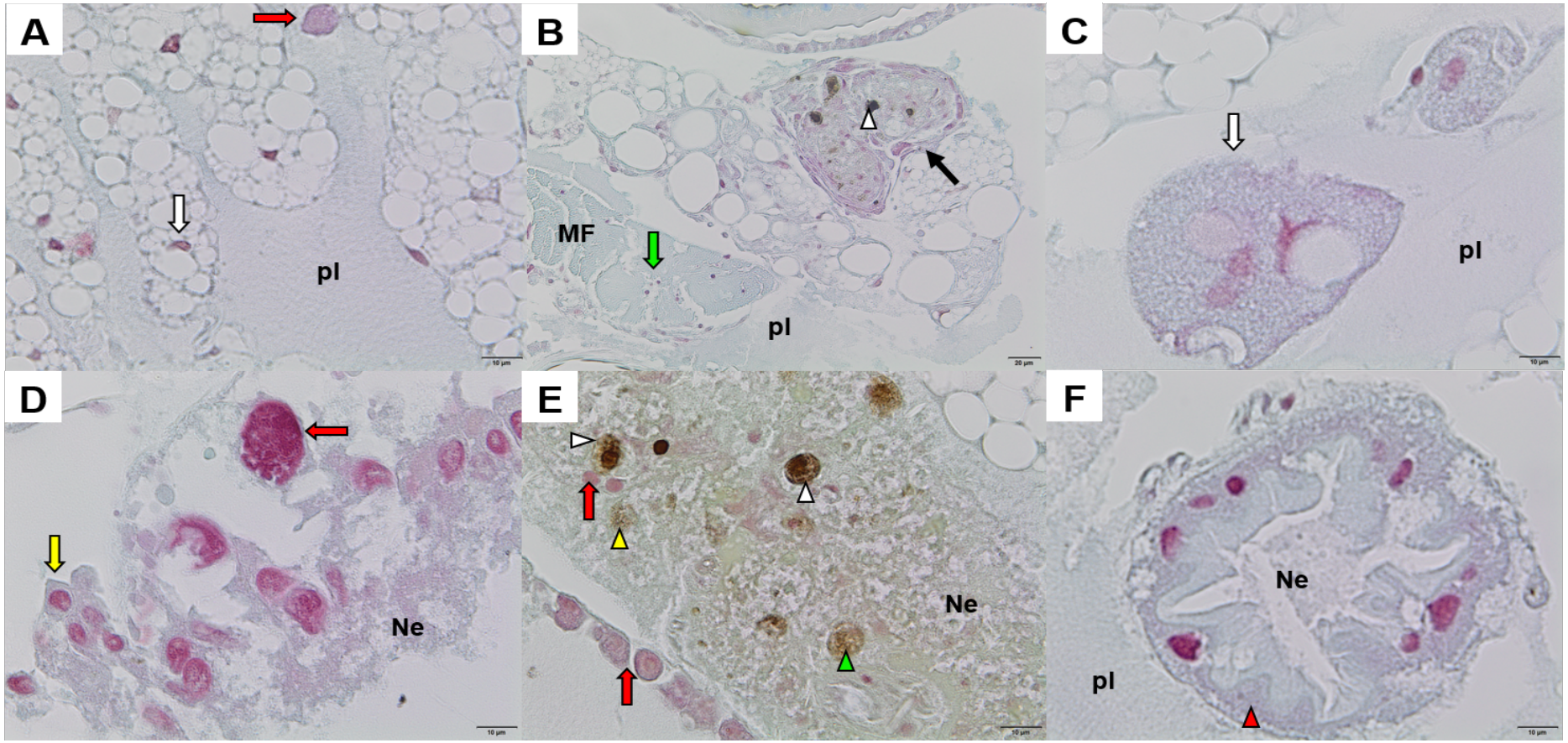

Figure 5.25 Gram Twort staining of larvae infected with *Fno* STIR-GUS-F2f7 ($9.2x10^8$ CFU mL^{-1}) (A–F) at 48 hpi, *Fno* appear red or pink in colour: (A) cocco-bacilli bacteria inside vacuolated (white arrow) and unvacuolated haemocytes (red arrow) where the cell has lost its nuclei and this is totally occupied by the bacteria; (B) nodule formation as a result of enormous haemocyte recruitment and activation with the appearance of multiple melanised haemocytes in the fat body, as well as haemocytes containing bacteria inside the muscle fibre (green arrow); (C) enlarged haemocytes containing the bacteria and several vacuoles inside to entrap the bacteria (white arrow); (D) many enlarged haemocytes have mostly lost their nuclei and these have been replaced by a huge number of bacteria (red arrow), and several haemocytes observed to have enlarged nuclei containing bacteria (yellow arrow); (E) enlarged haemocytes that have lost their nuclei and now contain bacteria (red arrows) are observed to surround and are inside large necrotic areas. And several haemocytes with melanised cells which had (white arrows head) or lost (green arrow head) their nuclei, containing bacteria and present *Fno* escaping from the phagocytes or because of cells necrosis (yellow arrow head); and (F) early stages of cocco-bacilli bacteria invading the inside and surrounding the tracheal walls (red arrow head). Scale bar: A, C to F = 10 µm, and B = 20 µm.

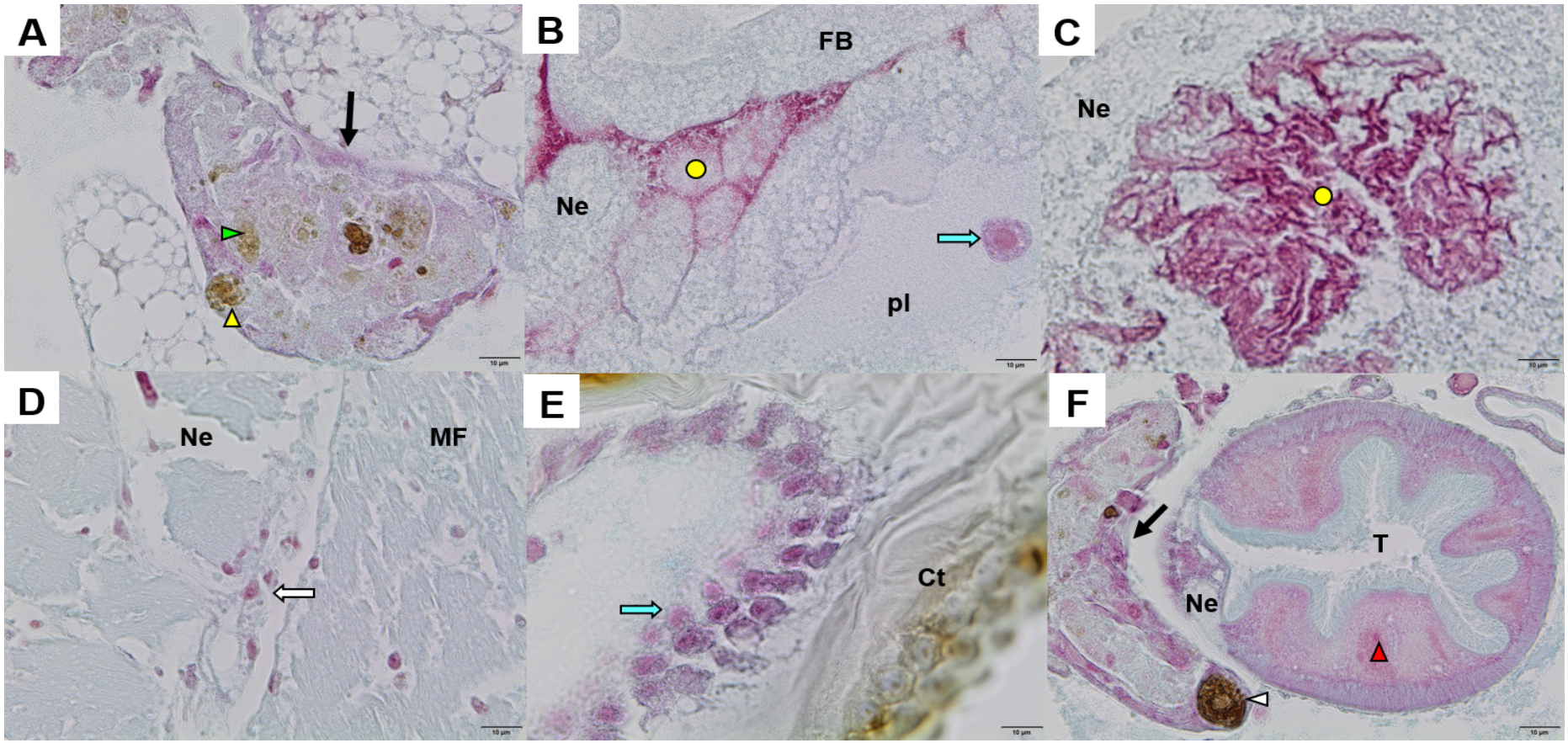

Figure 5.26 Gram Twort staining of larvae infected with *Fno* STIR-GUS-F2f7 (9.2 x 10^8 CFU mL^{-1}) (A–F) at 72h post infection, *Fno* appear red or pink in colour: (A) large nodules formed in the fat body containing the bacteria and the presence of melanized haemocytes (black arrow) containing bacteria inside the cells (yellow arrow head) or escaping from phagocytes (green arrow head); (B) invasion and expansion of a small pleomorphic coccobacilli bacteria inside a huge number of haemocytes (haemocytes lakes) in the fat body (yellow round shape), and haemocytes fully occupied by the bacteria including inside the enlarged nuclei (blue arrow); (C) fully expansion of the coccobacilli bacteria in a larger area of necrosis which formed from haemocytes lakes (yellow round shape); (D) haemocytes containing bacteria spreading inside muscle fibres (green arrow); (E) haemocytes fully occupied by the bacteria including inside the enlarged nuclei (blue arrow) and surrounding the sub-cuticular area; and (F) mature stage of coccobacilli bacteria invasion into the cells surrounding the tracheal walls led to necrosis of the organ (red arrow head), and nodule formation (black arrow) around the tracheal walls containing the bacteria inside the hamocytes and melanised haemocyte cells (white arrow head). Scale bar: A to F = 10 µm.

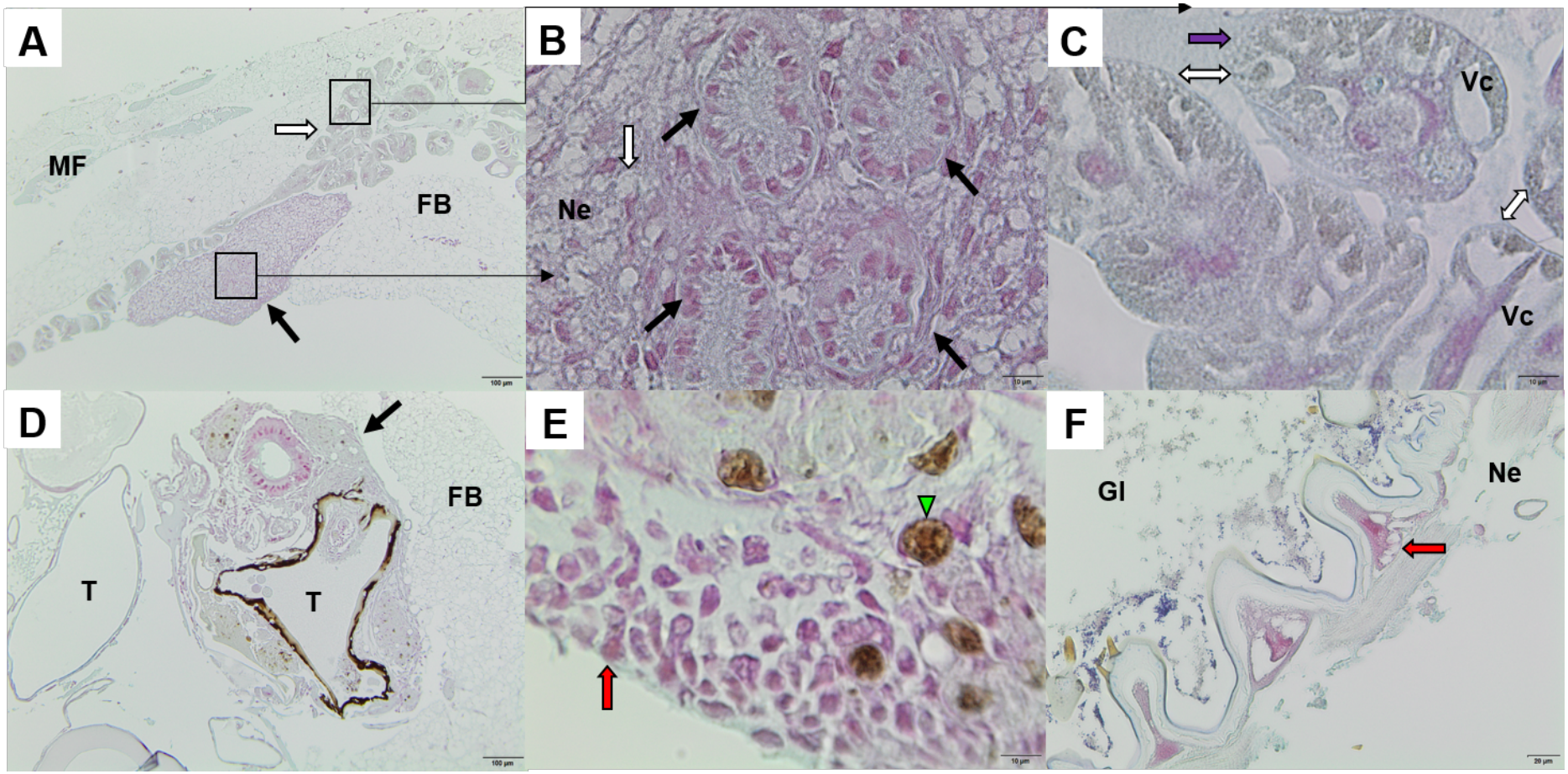

Figure 5.27 Gram Twort staining of larvae infected with *Fno* STIR-GUS-F2f7 (9.2 x 10^8 CFU mL^{-1}) (A–F) at 96 hpi, *Fno* appear red or pink in colour: (A) expansion of enlarged and vacuolated haemocytes containing bacteria (white arrow) surrounding the fat body; (B) formation of multiple nodules with many haemocytes containing the bacteria in the fat body; (C) huge enlarged haemocytes containing the bacteria and several vacuoles to entrap the bacteria (white arrows) and the appearance of early stages of necrosis (purple arrow) leading to extensive necrosis; (D) large nodule formation (black arrow) containing huge haemocytes and the cells fully occupied by the bacteria, surrounding the tracheal walls and causing extensive necrosis; (E) appearance of melanised cells (green head arrow) inside nodules containing enormous haemocytes lacking nuclei but fully occupied by a huge number of bacteria (red arrow); and (F) the bacteria invaded the gastrointestinal tract which led to necrosis (red arrow head). Scale bar: A and D = 100 µm, F = 20 µm, and B, C, and E = 10 µm.

5.3.4.3 Immunohistochemistry

IHC, used to localise *Fno* in infected larvae, was carried out using anti-*Fnn* primary antibodies that were shown previously to cross react with *Fno* (Chapter 3, Section 3.3.6). During method optimisation, anti-*Fnn* antibodies diluted to 1:1300 resulted in good detection and low background in the larvae tissue sections compared to the other dilutions tested (Section 5.2.8.3). A positive reaction was observed as a light brown colour localised mostly in the haemocyte nodulations in different larvae organs. No detection of *Fno* was obtained in any of the negative control fixed tissues (Figure 5.28).

Injection of larvae with *Fno* were resulted in the rapid progression of an infection. The bacteria invasion were commenced at 48 hpi and characterized by the presence of simultaneous pleomorphic coccobacilli. The bacteria were observed to be mainly encapsulated in small nodules preferentially located in the fat body, the surrounding sub-cuticular area, and the tracheal walls, but observed especially in the pale eosinophilic protein lakes that had resulted from haemorrhage of enormous numbers of haemocytes that then ultimately led to necrosis of the organs (Figure 5.29A, B and D). The bacteria were detected on the outer layer of the gastrointestinal tract and had entered inside the gastrointestinal tract, even during the early initial phase of the infection (48 h) (Figure 5.29E and F). Replicating bacteria had invaded muscle fibres by 72 hpi (Figure 5.30B) and were present in the sub-cuticular area (Figure 5.30C). In the infected larvae, *Fno* infection had induced nodule formation to entrap the bacteria and there was strong melanisation and a tendency to converge into large aggregates, while nodules had mostly formed within the fat body (Figure 5.30A and D) and surrounding the tracheal wall system (Figure 5.30E), which led to necrosis of it (Figure 5.30F). At later phases of infection (96 h), multiple nodules were observed to surround the sub-cuticular area where the bacteria were found inside haemocytes, as well as inside muscle fibres, while very large nodulations contained large numbers of bacteria which led to extensive necrosis of the organs (Figure 5.31A, B, C and D). *Fno*-positive reactions were also present inside and surrounding the proximal and distal area of gastrointestinal tract, inside the tracheal walls and inside the vacuolated cells of the fat body (Figure 5.31E and F).

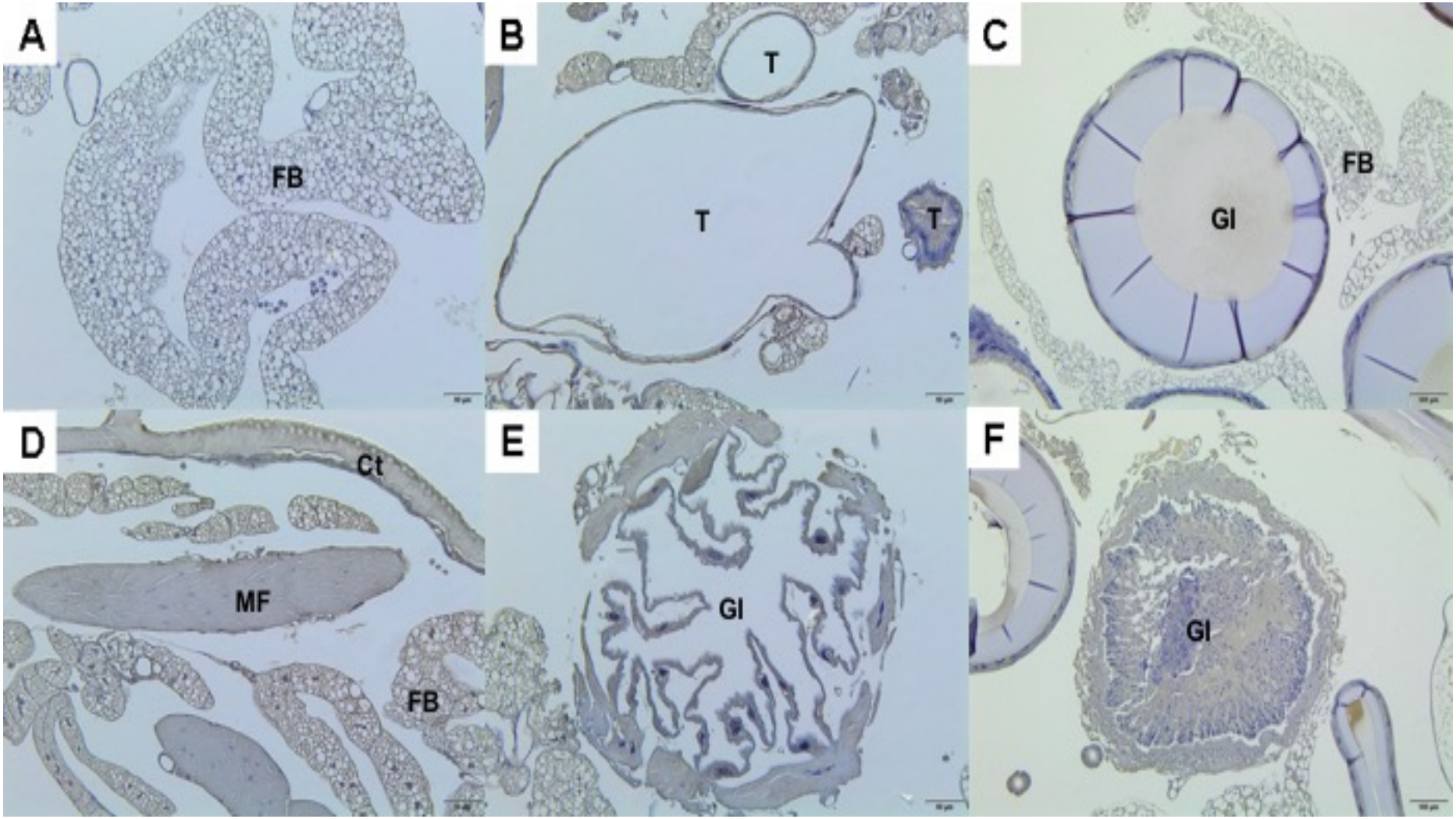

Figure 5.28 Unmanipulated control larvae (A-F) at 0 h which had been incubated with primary polyvalent rabbit antisera against *Francisella noatunensis* subsp. *noatunensis* NCIMB 14265 serum and stained with DAB peroxidase: (A) fat body (FB); (B & C) tracheal walls (T); (D) muscle fibres (MF) surrounding sub-cuticular area (Ct); and (E & F) proximal and distal gastrointestinal tract (GI) in normal conditions. Scale bar A to F = 50 µm.

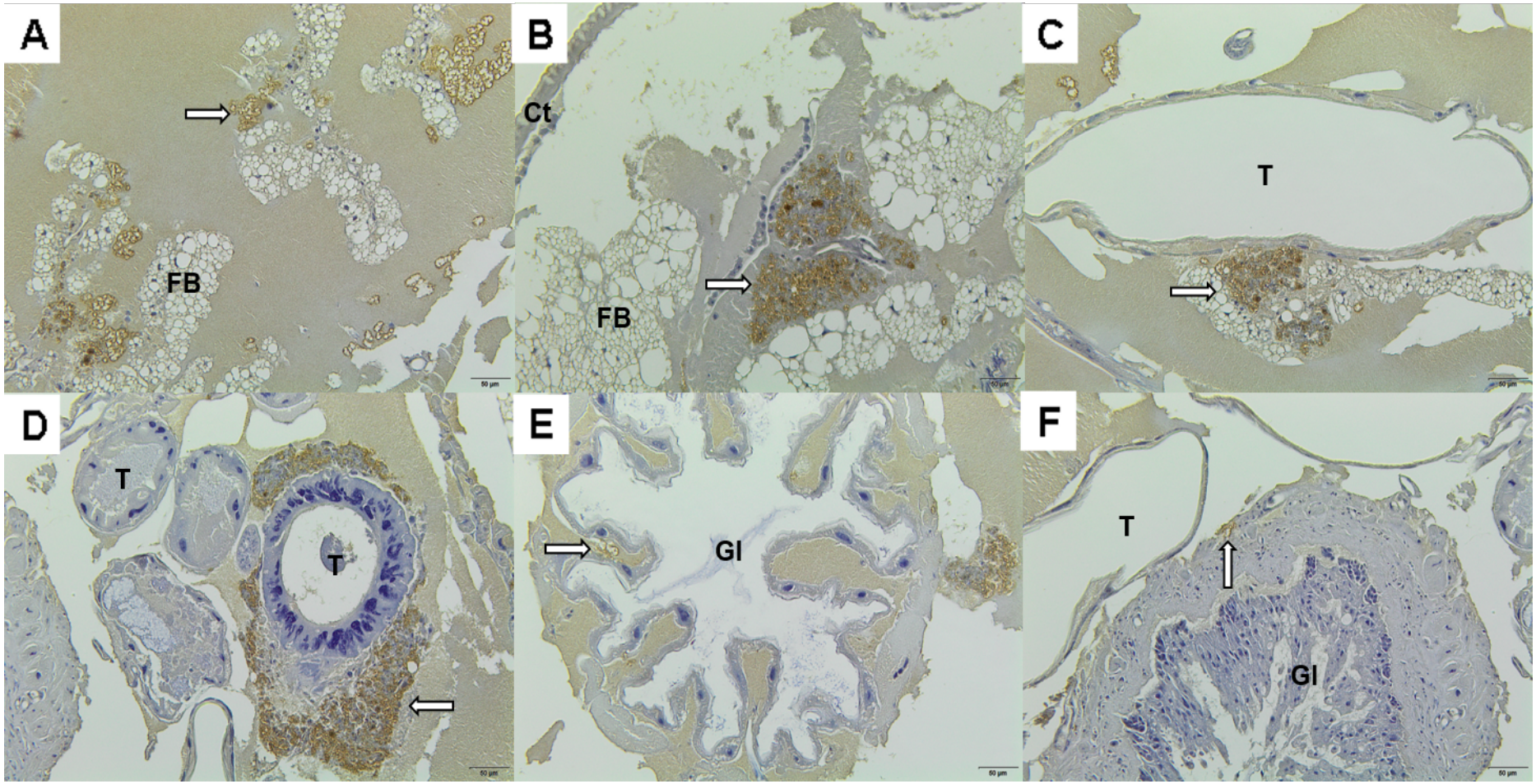

Figure 5.29 Immunohistochemical staining of paraffin sections of *G. mellonella* infected with *Fno* at 9.2 x 10^8 CFU mL^{-1} at 48 h post injection and after incubation with primary polyvalent rabbit antisera against *Francisella noatunensis* subsp. *noatunensis* NCIMB 14265 serum and stained with DAB peroxidase; positive IHC staining is apparent by light brown colouration (arrows): (A, B, C, & D) the bacteria were observed in the fat body (FB), around the sub-cuticular area (Ct), and the tracheal walls (T), and mostly in the pale eosinophilic protein lakes that had resulted from haemorrhage of enormous numbers of haemocytes caused by an early stage of necrosis of the organ; and (E & F) *Fno* found inside and surrounding the proximal and distal area of the gastrointestinal tract (GI). Scale bar: A, B, D and E = 50 µm, and C and F = 100 µm.

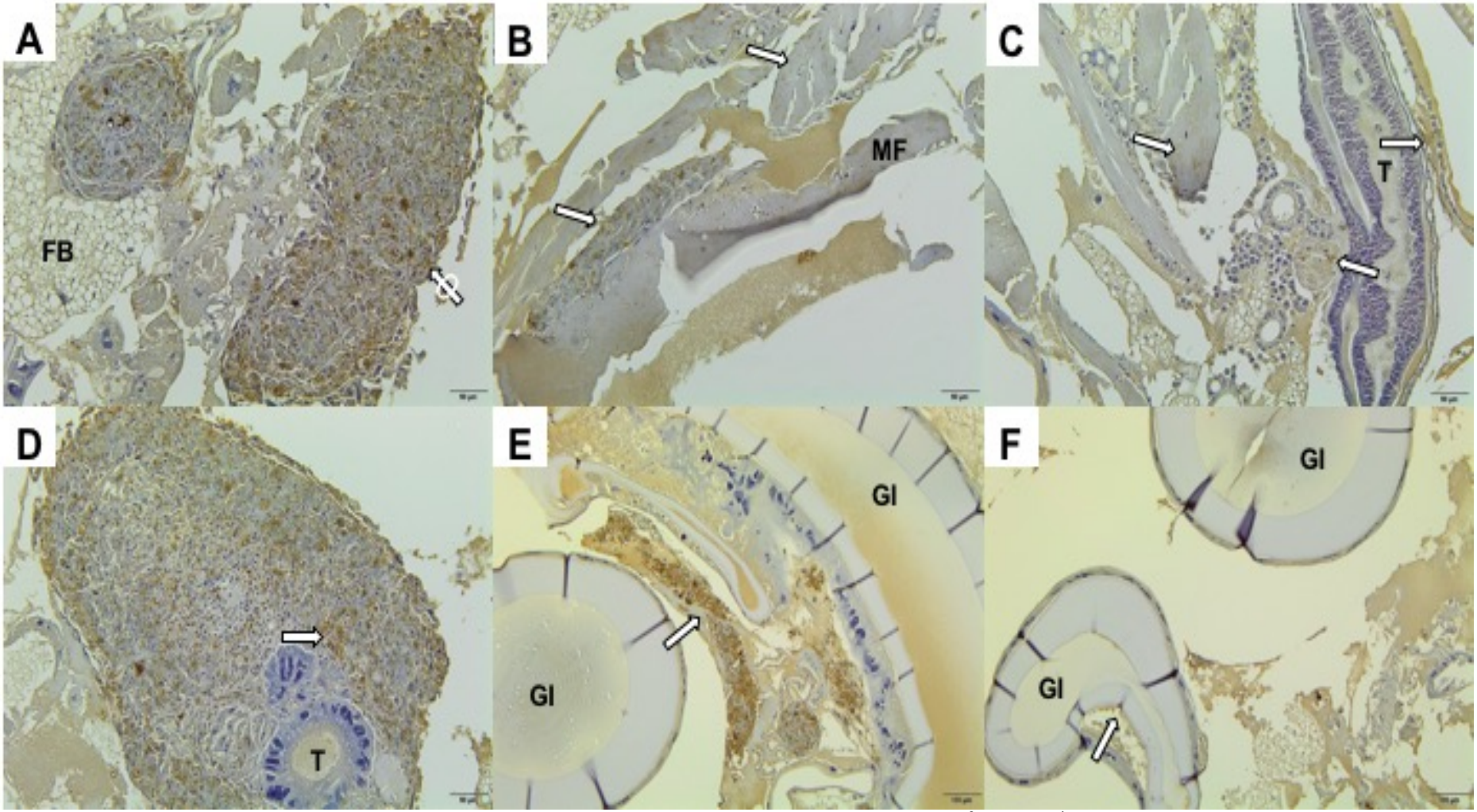

Figure 5.30 Immunohistochemical staining of paraffin sections of *G. mellonella* infected with *Fno* at 9.2 x 10^8 CFU mL^{-1} at 72 h post infection and stained with DAB peroxidase; positive IHC staining visualized by light brown colour (arrows): (A & D) *Fno* entrapped in huge nodules composed of enormous haemocytes in the fat body (FB) and surrounding the tracheal walls (T); (B) and inside muscle fibres (MF); (C) (E) surrounding the gastroinstestinal tract (GI): and (F) the bacteria invasion caused necrosis of the gastrointestinal tract (GI). Scale bar: A, B, C and D = 50 µm, and E and F = 100 µm.

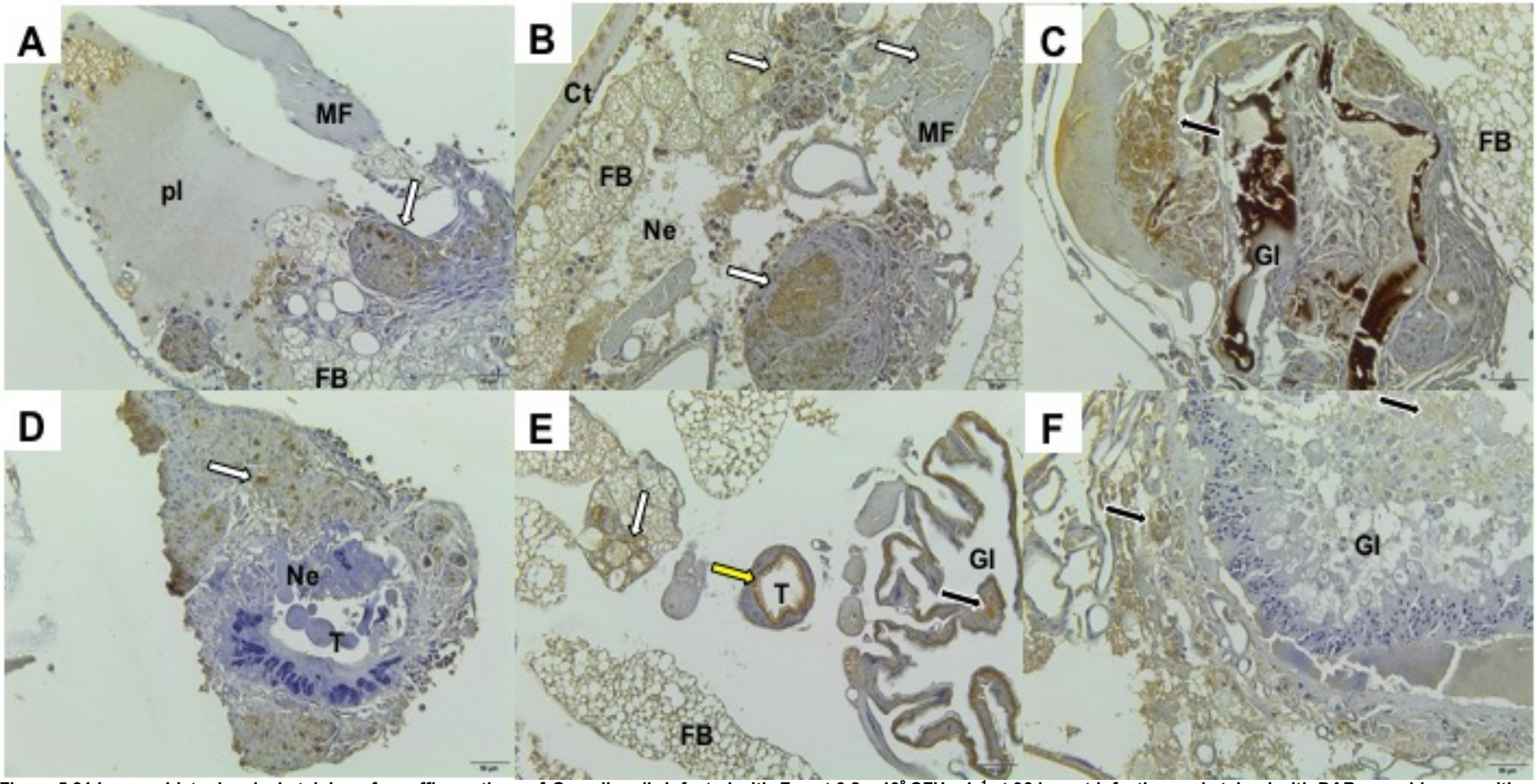

Figure 5.31 Immunohistochemical staining of paraffin sections of *G. mellonella* infected with *Fno* at 9.2 x 10^8 CFU mL^{-1} at 96 h post infection and stained with DAB peroxidase; positive IHC staining visualized by light brown colour (arrows): (A) some nodules (white arrow) and large of protein eosinophilic lake (pl) around the fat body (FB) and muscle fibre (MF): (B) multiple nodulations surrounding the sub-cuticular area (Ct) where the bacteria are found inside the haemocytes, inside muscle fibres (MF), and necrosis (Ne) around the fat body (FB); (C & D) gigantic nodulations led to extensive necrosis (Ne) of the gastrointestinal tract (Gl) and the tracheal walls (T), while an enormous number of bacteria are observed inside the haemocytes in the nodules (black and white arrows); and (E & F) positive *Fno* reaction detected inside and surrounding the proximal and distal regions of the gastrointestinal tract (Gl) (black arrows), and inside the tracheal walls (T) (yellow arrow) and vacuolated cells in the fat body (FB) (white arrows). Scale bar: A to F = 50 µm.

5.3.5 *Fno* recovery from infected *G. mellonella*

5.3.5.1 Antibiotic agar plates testing

Fno STIR-GUS-F2f7 and *Fno* Japan grew well on CHAH agar supplemented with penicillin and ampothericin B at 1 and 2.5 mg L^{-1} (Appendix 4.2). Therefore, these two antibiotics in combination at these concentrations were selected for further experiments in combination. *Fno* STIR-GUS-F2f7 grew on CHAH containing the two antibiotics and at two different concentrations as above for each antibiotic, while *Fno* Japan did not grow well even at concentration of 1 mg L^{-1} (Figure 5.32). Thus, CHAH supplemented with mixed penicillin and ampothericin B at 1 mg L^{-1} of each antibiotic was selected for *Fno* STIR-GUS-F2f7 and *Fno* Japan burden experiments.

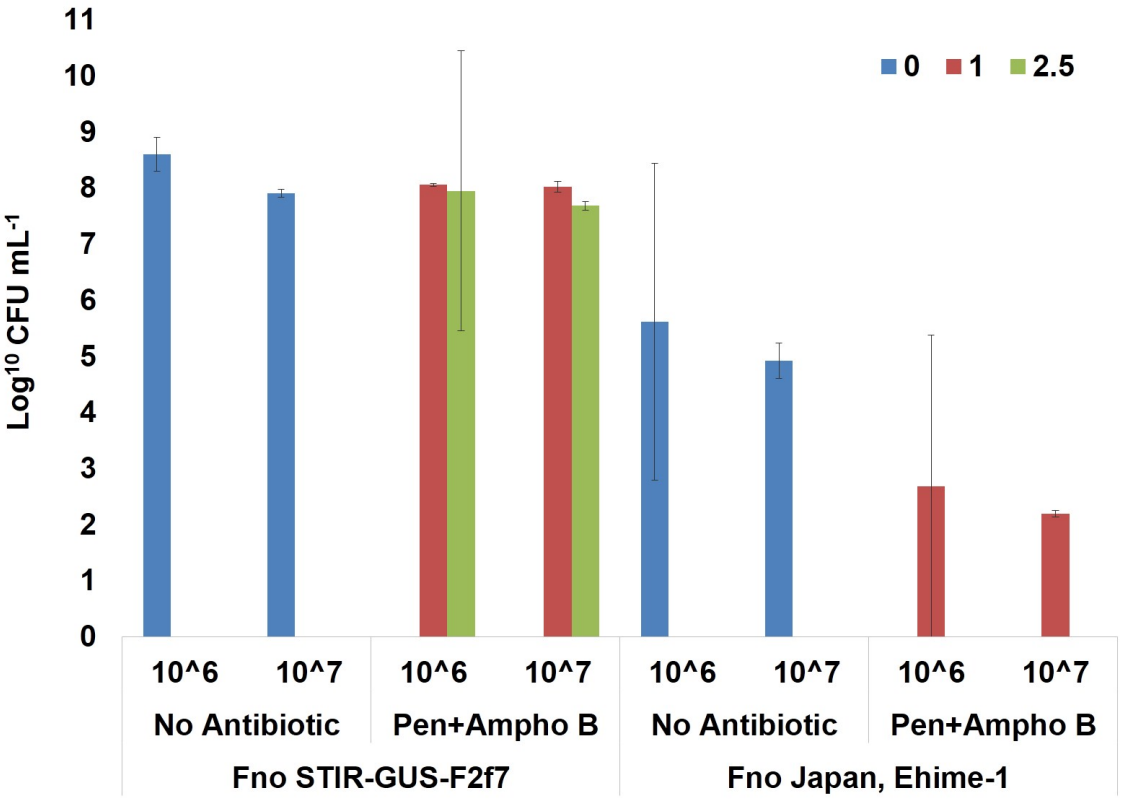

Figure 5.32 Log number CFU mL^{-1} of *Fno* STIR-GUS-F2f7 and *Fno* Japan. A 100 µL of bacterial inocula per dilution spreaded onto CHAH containing mixed of two antibiotics (penicilin and ampothericin B) and at two different concentrations (*i.e.* 1 and 2.5 mg L^{-1}) of each antibiotic and compare with normal CHAH without antibiotic (0) for each bacterium. Bars represent the mean of 4 plates+SD.

5.3.5.2 *Fno* burden from larvae haemolymph

In order to evaluate tissue burden of *Fno* in the larval haemolymph over time, groups of larvae were infected with virulent *Fno* STIR-GUS-F2f7 and the weakly virulent *Fno* Japan. CHAH supplemented with the penicillin and amphotericin B (at 1 mg L^{-1} of each antibiotic) was used. Closer investigation of the growth of each isolate in the larva revealed that *Fno* STIR-GUS-F2f7 replicated faster than *Fno* Japan, remained detectable for longer in the host (192 h vs 288 h) and reached a greater final burden at 288 h in the haemolymph than less virulent isolates (Figure 5.33).

Based on the *Fno* burden graph, there was an initial decrease in bacteria burden in the haemolymph for both *Fno* STIR-GUS-F2f7 and *Fno* Japan up to 96 h and 72 h, respectively. Following this, the burden of both isolates slightly increased. Subsequently, *Fno* STIR-GUS-F2f7 continued to persist within the larvae until 264 h before a final decrease in burden at 288h, while the burden of *Fno* Japan decreased sharply at 120 h with no bacteria recovered after 192 h.

Importantly, *Fno* STIR-GUS-F2f7 and *Fno* Japan isolates recovered from infected larval haemolymph showed grayish or greenish colonies. In addition, when the isolates were tested using primary identification tests they were similar with the *Fno* isolates used as inocula. The recovered isolates were Gram negative (Figures 5.34 and 5.35), cocco-bacilli, non-motile and oxidase negative.

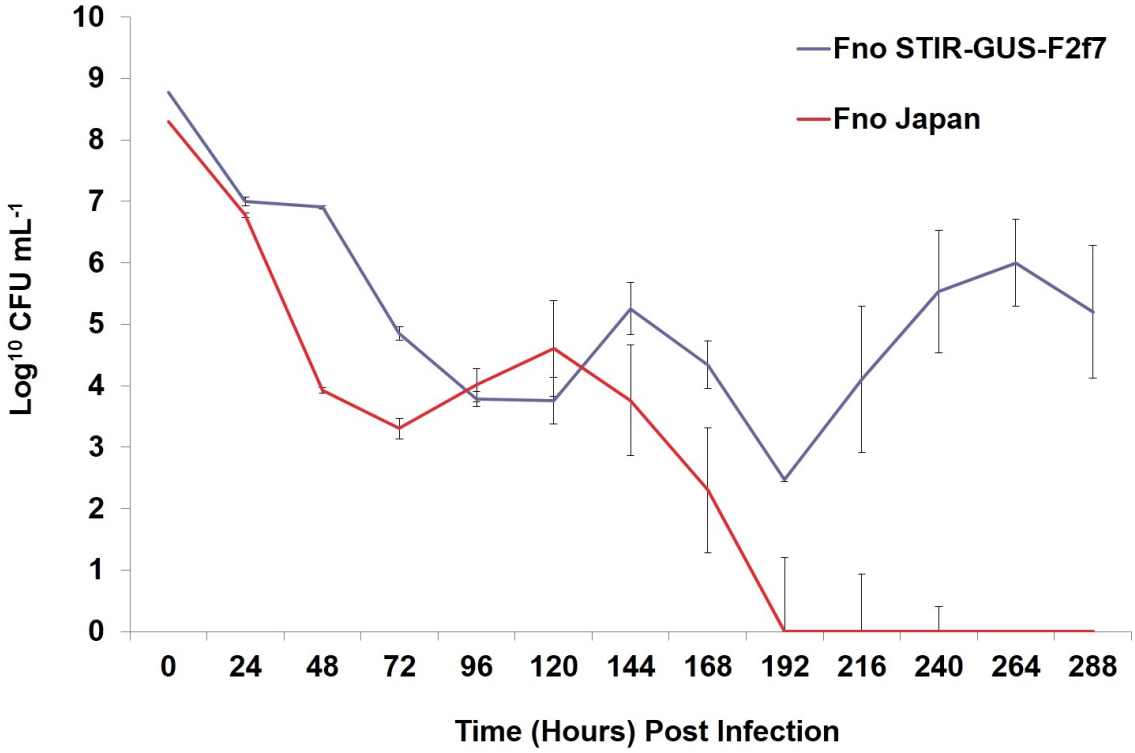

Figure 5.33 Replication of *Fno* STIR-GUS-F2f7 and *Fno* Japan in the haemolymph of *G. mellonella* larvae during 288 h post infection. The CFU mL^{-1} value at 0 h refers to the PBS inocula of 5.7 x 10^{8} and 1.6 x 10^{8} CFU mL^{-1} *Fno* STIR-GUS-F2f7 and *Fno* Japan, respectively. Data from the unmanipulated and PBS control groups are not shown. Bars represent the mean of 5 larvae+SD.

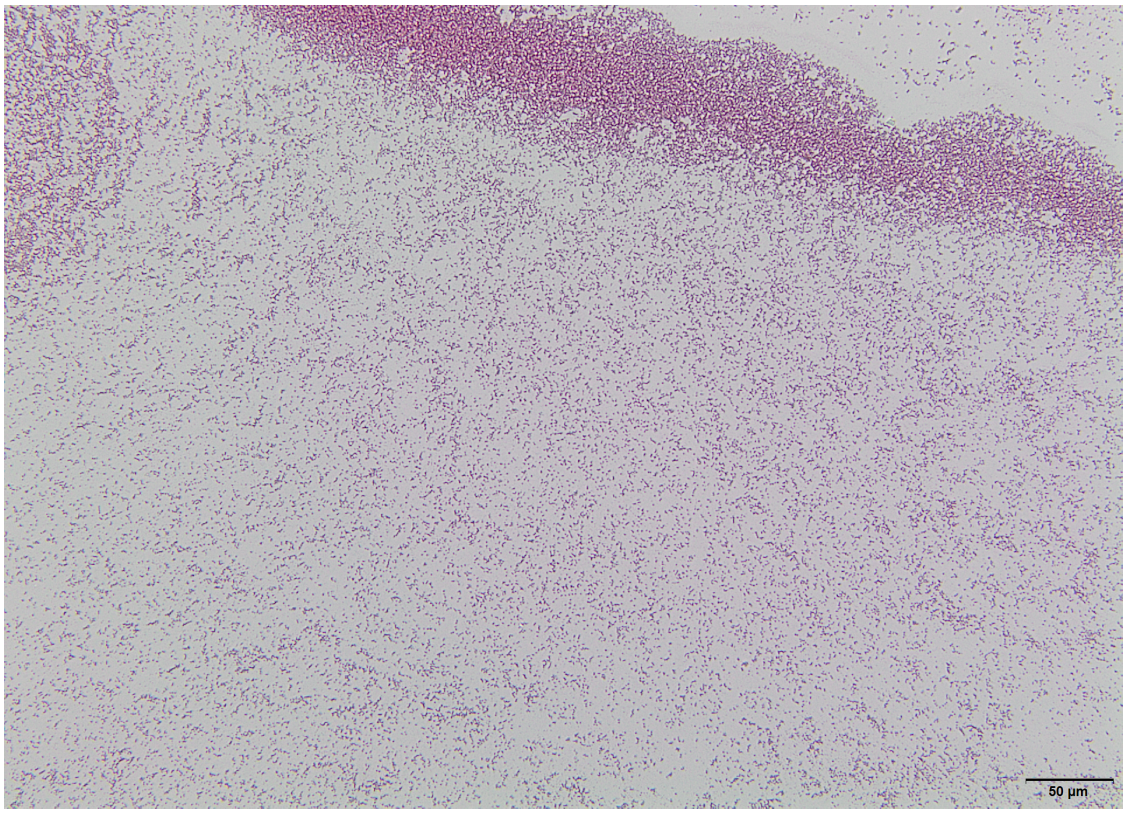

Figure 5.34 Gram stain of *Fno* STIR-GUS-F2f7 isolate recovered from infected larvae and grown on CHAH supplemented with penicillin and amphotericin B at 1 mg L^{-1} after 10 d of incubation at 28˚C.

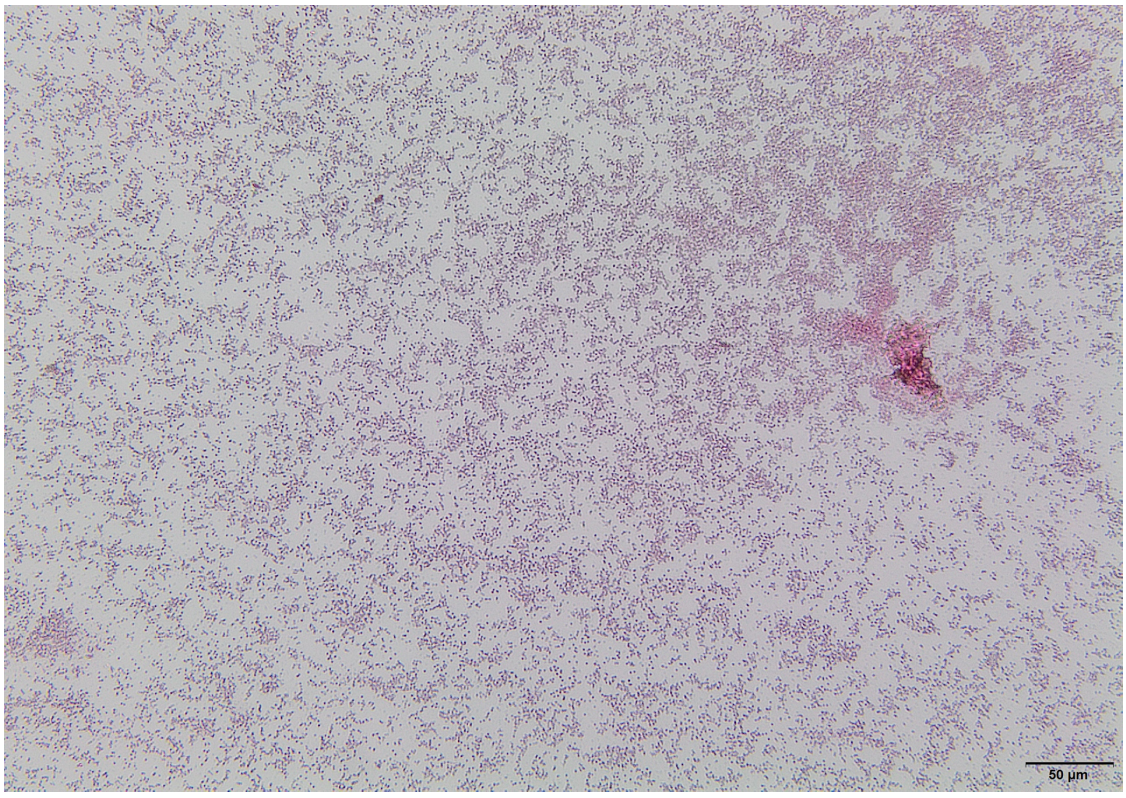

Figure 5.35 Gram stain of *Fno* Japan isolate recovered from infected larvae and grown on CHAH supplemented with penicillin and amphotericin B at 1 mg L^{-1} after 10 d of incubation at 28˚C.

5.3.6 *In vivo* antibacterial activity of algal-derived compounds in *Fno*-infected *G. mellonella*

5.3.6.1 Dose response of algal-bioactive compounds and *Fno* vaccines

In order to establish whether different concentrations of algal-bioactive compounds could be toxic to the larvae, *G. mellonella* were first injected with various concentrations of ß-glucan and alginic acid, and incubated for 288 h at 28˚C. The results demonstrated that larvae receiving ß-glucan showed 100% survival at 0.5 to 10 (Figures 5.36), while alginic acid showed 100% survival at all concentrations (at 0.5 to 100). ß-glucan treatment groups showed higher larvae survival at the lower concentrations at 15, 20, 25 and 100 µg larva^{-1} with 92, 83, 75 and 58% survival, respectively (Figure 5.36). The larvae showed 100% survival in the control unmanipulated and PBS only groups (Figures 5.36). Collectively, these data suggest that ß-glucan and alginic acid are not toxic at certain concentrations.

G. mellonella larvae showed melanisation within minutes after inoculation especially at higher doses of ß-glucan (15, 20, 25, 50 and 100 µg larva^{-1}) (Figure 5.37). In contrast, no melanisation was observed at any of the larvae injected with alginic acid as well as with lower concentrations of ß-glucan (at 0.5, 1.0, 2.5, 5, 7.5 and 10 µg larva^{-1}) (Figure 5.37). Larvae that died had soft body tissue with total or slight melanisation in groups that treated with ß-glucan.

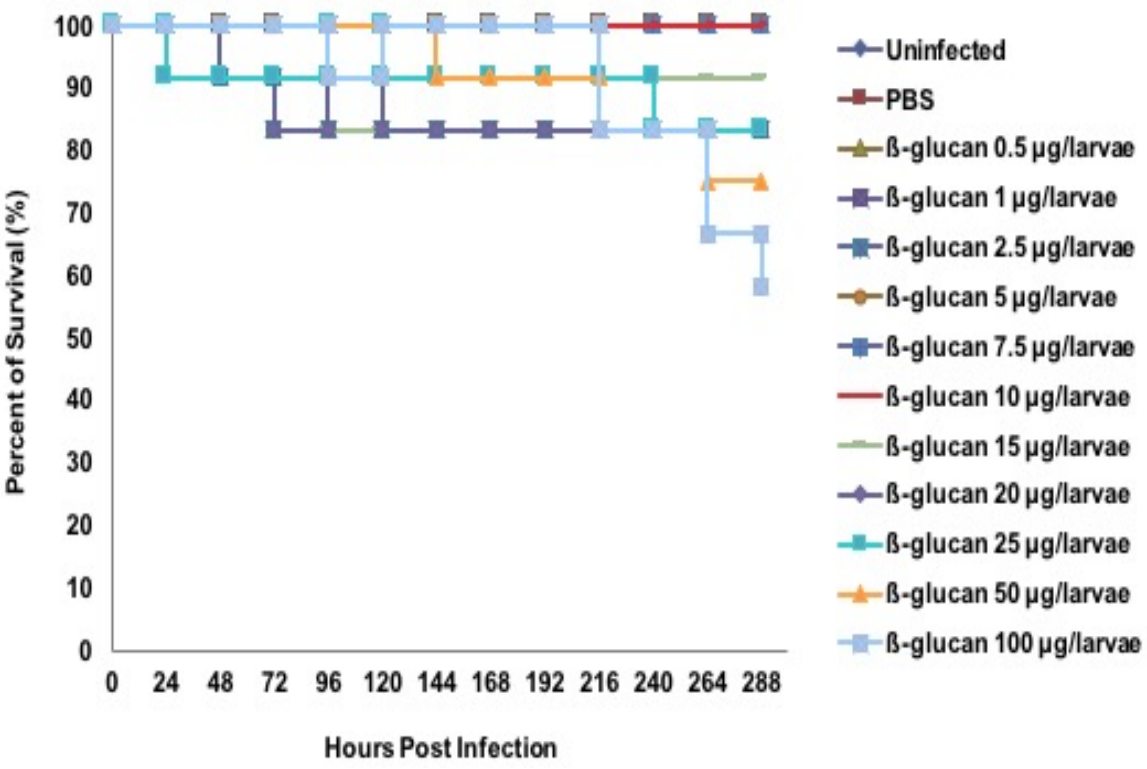

Figure 5.36 Survival of *G. mellonella* larvae after injection with different concentrations of ß-glucan at 10 µL larva^{-1} during 288 h (n=12).

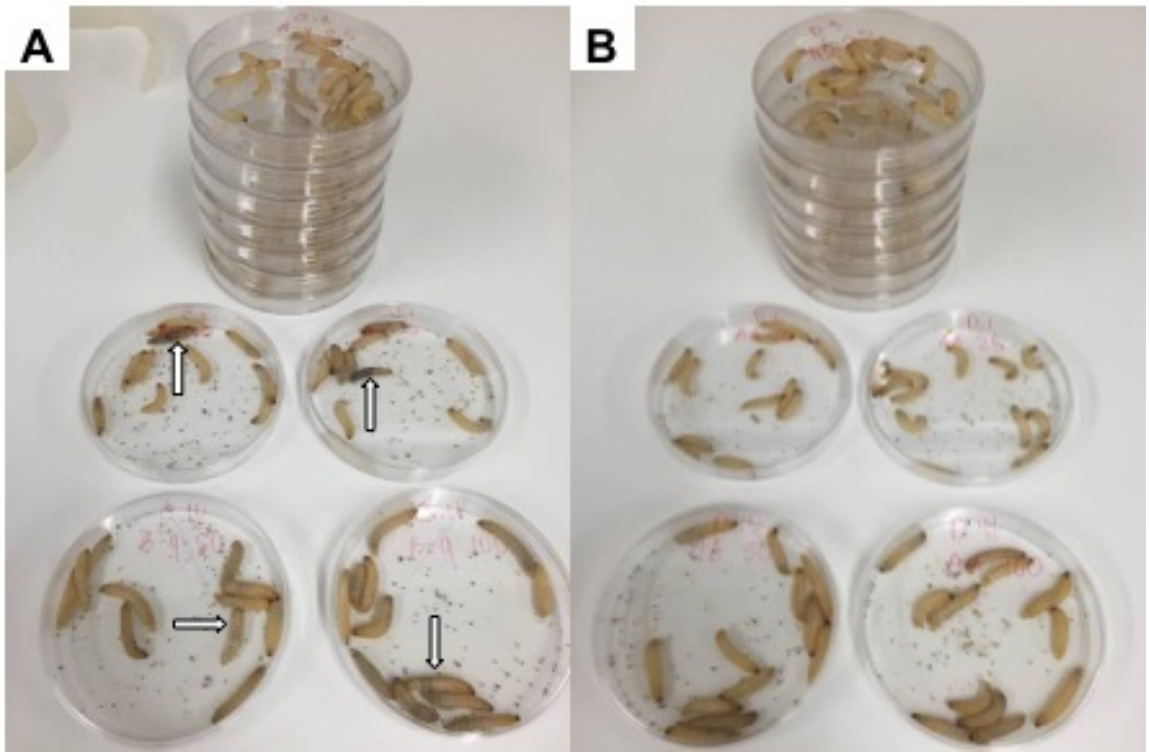

Figure 5.37 Dose responses of *G. mellonella* to different algal-derived compounds at different concencentrations of inoculations and incubated for 288 h at 28˚C: (A) ß-glucan and (B) alginic acid, both compounds at concentrations of 0.5, 1.0, 2.5, 5.0, 7.5, 10, 15, 20, 25, 50 and 100 µg larva^{-1}. Larvae showed progressive melanisation at higher concentrations of ß-glucan (20, 25, 50 and 100 µg larva^{-1}) (arrows), while none was observed in inoculated alginic acid groups (n=12).

5.3.6.2 Antibacterial activity of algal-derived compounds in *Fno*-challenged *G. mellonella*

The effect of ß-glucan and alginic acid on *Fno* pathogenicity *in vivo* was investigated to see if either offered a protective effect to *G. mellonella* against *Fno* infection. Larvae of *G. mellonella* were injected with two different concentrations of ß-glucan (at 7.5 and 10 µL larva^{-1}), and alginic acid (20 and 25 µg larva^{-1}). All groups were incubated for 1 or 24 h at 28°C. Afterwards, larvae from both incubation groups were injected with live *Fno* STIR-GUS-F2f7 inocula doses of 1 x 10^8 and 5 x 10^8 CFU mL^{-1}, with larvae from the 24 h incubation group also injected with a dose 1 x1 0^9 CFU mL^{-1}. The results establish that groups of larvae inoculated with PBS and challenged 1 h later with live *Fno* at 1 x 10^8 and 5 x 10^8 CFU mL^{-1} showed survival rates of 33 and 17%, respectively (Figures 5.39 and 5.40). Meanwhile, larvae inoculated with PBS and challenged after 24 h with different doses of *Fno* (1 x 10^8, 5 x 10^8 and 1 x 10^9 CFU mL^{-1}) showed survival rates of 58, 42, and 0%, respectively (Figures 5.38, and 5.39). In addition, 100% larval survival was observed in the control unmanipulated and double PBS injection groups at 1 and 24 h after second PBS inoculation, as well as control ß-glucan+PBS (7.5 and 10 µg larva^{-1}) and alginic acid+PBS (20 and 25 µg larva^{-1}) groups (Figures 5.38, and 5.39).

Larvae inoculated with ß-glucan at 7.5 or 10 µg larva^{-1} at 1 or 24 h prior to different doses of *Fno* challenged showed little increase in survival compared to control *Fno* only groups at the same dose and time of administrations (Figure 5.38). The percentage larval survival at 1 h was higher of 42 and 52% (7.5 µg larva^{-1}), and 58 and 42% (10 µg larva^{-1}) after challenge with *Fno* at 1 x 10^8 and 5 x 10^8 CFU mL^{-1}, respectively (Figure 5.38). Meanwhile, the percentage larval survival at 24 h was higher of 83, 42 and 33% (7.5 µg larva^{-1}), and 42, 50 and 0% (10 µg larva^{-1}) after challenge with *Fno* at 1 x 10^8, 5 x 10^8 and 1 x 10^9 CFU mL^{-1}, respectively (Figure 5.38). No significant differences were observed between all the groups, with the exception of larvae treated with ß-glucan inoculations of 7.5 µg larva^{-1} at 24 h before challenge with live *Fno* at 1 x 10^9 CFU mL^{-1} which showed significantly higher survival compared to the *Fno* control group ($p=0.0396$) (Figure 5.38).

In addition, larvae injected with alginic acid at 20 or 25 µg larva^{-1} at 1 or 24 h before challenge with different concentrations of *Fno* found had higher survival compared to *Fno* alone control groups at the same dose and time of administrations (Figure 5.39). The percentage larval survival at 1 h was 50 and 58% (20 µg larva^{-1}), and 67 and 50% (25 µg larva^{-1}) post-challenge with *Fno* at 1 x 10^8 and 5 x 10^8 CFU mL^{-1}, respectively (Figure 5.39). Meanwhile, the larva survival at concentration of 20 µg larva^{-1} after 24 h post challenge with *Fno* at 1 x 10^8 and 5 x 10^8 CFU mL^{-1} was 100, 83 and 25%, respectively (Figure 5.39). The larva survival at 25 µg larva^{-1} after 24 h post challenge with *Fno* at 1 x 10^8, 5 x 10^8 and 1 x 10^9 CFU mL^{-1} was 83, 67 and 33%, respectively (Figure 5.39). There were highly significant differences in the survival between groups of larvae that were treated with alginic acid of 20 ($p= 0.0183$) and 25 ($p=0.0308$) µg larva^{-1} at 1 h prior the challenge with 5 x 10^8 CFU mL^{-1} compared to control *Fno* groups. Groups treated with alginic acid of 20 µg larva^{-1} at 24 h prior to infection with 1 x 10^8 CFU ($p= 0.0318$) and 5 x 10^8 CFU ($p= 0.0386$) mL^{-1} showed significant differences when compared to *Fno* control groups.

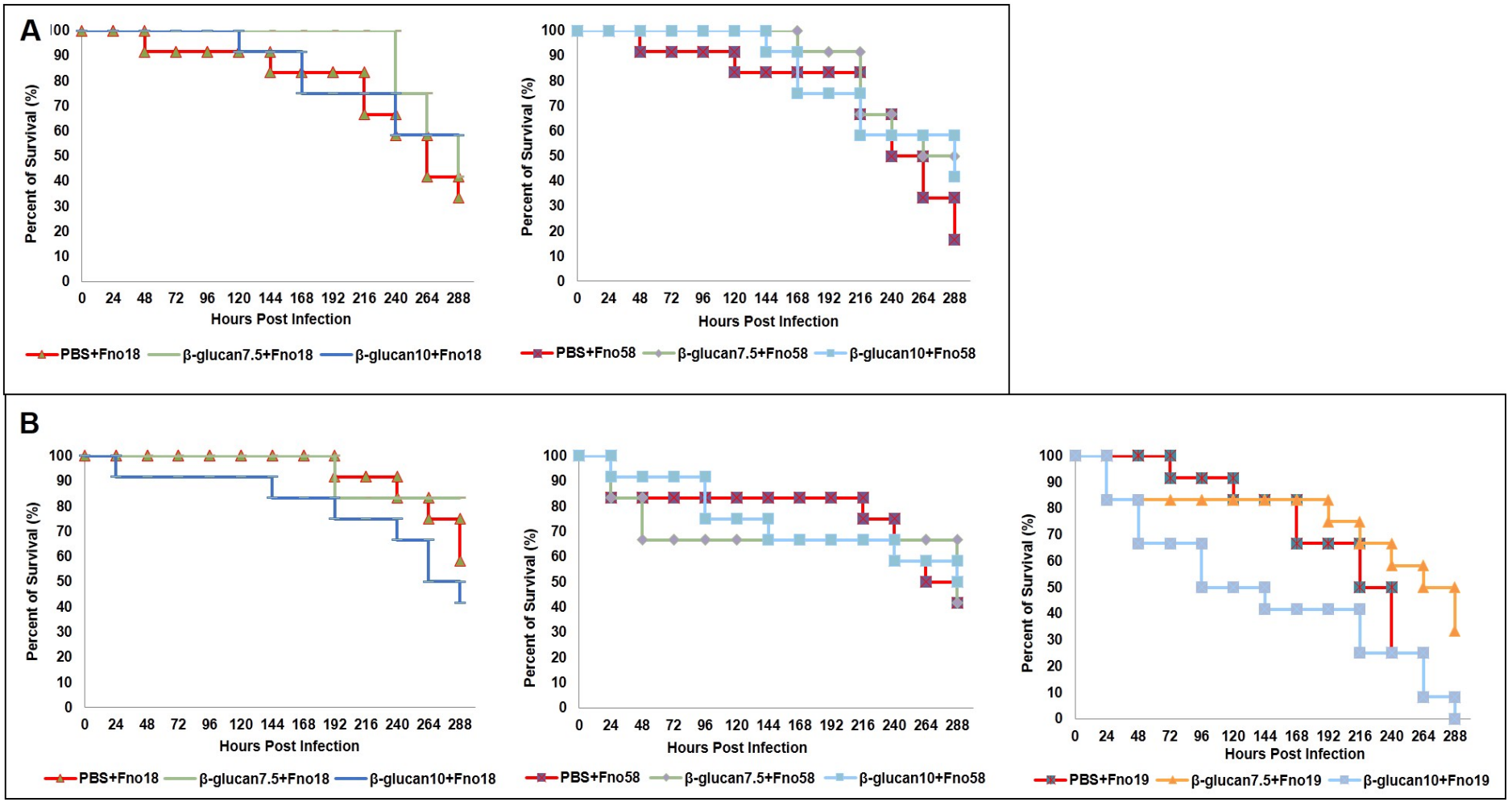

Figure 5.38 Larvae that received ß-glucan inoculations of 7.5 (ß-glucan7.5) or 10 (ß-glucan10) µg larva^{-1}: (A) at 1 h prior challenged with live *Fno* at 1 x 10^{8} CFU mL^{-1} (*Fno*18) and 5 x 10^{8} CFU mL^{-1} (*Fno*58) showed little increase of survival 42 and 52%, and 58 and 42%, respectively; while, (B) at 24 h prior challenged with live *Fno* at 1 x 10^{8} CFU mL^{-1} (*Fno*18), 5 x 10^{8} CFU mL^{-1} (*Fno*58) and 1 x 10^{9} CFU mL^{-1} (*Fno*19) showed 83, 42 and 33% (p=0.0396), and 42, 50 and 0%, respectively, compared to control *Fno* at the same doses and times (n=12).

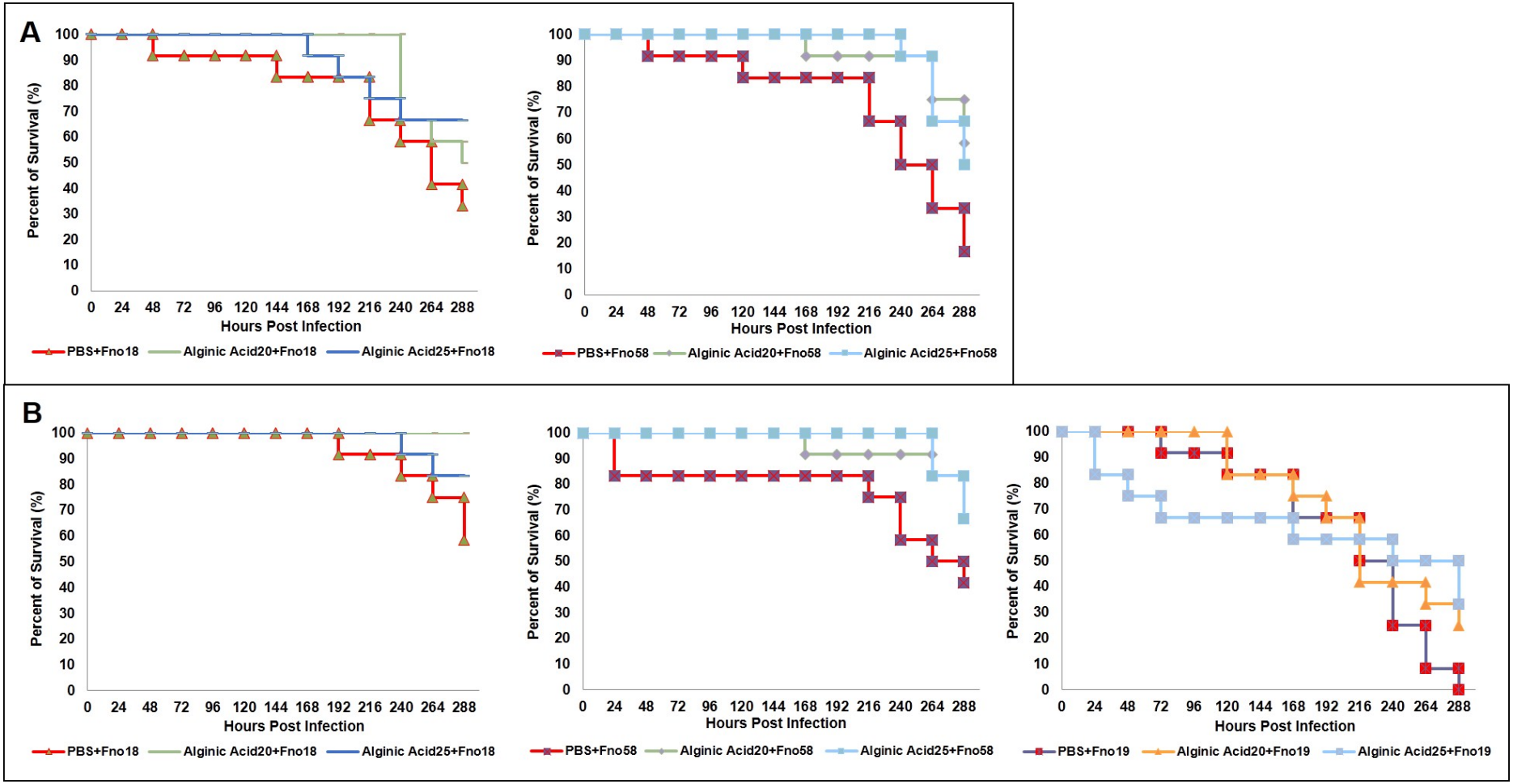

Figure 5.39 Larvae that received alginic acid inoculations of 20 (Alginic Acid20) or 25 (Alginic Acid25) µg larva^{-1}: (A) at 1 h prior challenged with live *Fno* at 1 x 10^{8} CFU mL^{-1} (*Fno*18) and 5 x 10^{8} CFU mL^{-1} (*Fno*58) showed higher survival of 50 and 58% (p= 0.0183), and 67 and 50% (p=0.0308), respectively; in addition, (B) at 24 h prior challenged with live *Fno* at 1 x 10^{8} CFU mL^{-1} (*Fno*18), 5 x 10^{8} CFU mL^{-1} (*Fno*58) and 1 x 10^{9} CFU mL^{-1} (*Fno*19) showed 100 (p= 0.0318), 83 and 25%, and 83 (p= 0.0386), 67 and 33%, respectively, compared to control *Fno* at the same dose and time of administrations (n=12).

5.4 Discussion

In vivo studies of pathogen virulence are often complicated by experimental costs, ethical concerns, adherence to the principles of 3Rs and the use of vertebrates or mammals as model hosts. There are not many alternative infection models available to study pathogens especially those produced in aquaculture. This chapter aimed to validate the use of the *G. mellonella* larvae as an alternative model to investigate microbial pathogenic mechanisms and to develop an invertebrate host system for the study of francisellosis in fish. This disease is caused by *Fno*, an emerging pathogen that infects many warm water fish species, especially tilapia: it reduces farm productivity and causes significant economic losses globally. The current experiment with the new insect model included examination of pathogen virulence, the efficacy of antibacterial agents, as well as immunostimulants presently used in fish for other diseases.

In the present study, *Fno* was confirmed to establish systemic infection of *G. mellonella* larvae as this bacterium killed the insect in a dose-dependent manner, depending on the incubation temperature following inoculation, and the bacteria replicated *in vivo* in the insect, while antibiotics and algal-derived compounds (immunostimulants) could rescue the insect from lethal bacterial inocula. In addition, melanisation and phagocytosis were involved *G. mellonella* in the host response to the *Fno* infection.

In this work, the results showed that larvae injected with *Fno* were killed faster at intermediate temperatures (25 or 28°C) (Figure 5.3). In fish, change in water temperature can induce stress, predisposing fish to pathogens which are more virulent at certain temperature ranges. Environmental temperature can have a significant effect on the development of piscine francisellosis and can play a substantial role in its epidemiology (in the outcome of *Fno* infection) as observed in field and controlled laboratory studies (Soto *et al.*, 2012c, 2013c, 2014b; Klinger-Bowen *et al.*, 2016). The mortalities caused by *Fno* mainly occur during the cold months of the year in cultured and wild tilapia and are not recorded typically during the warmer months (Mauel *et al.*, 2003; Leal *et al.*, 2014; Assis *et al.*, 2016; Lin *et al.*, 2016). During experimental infections, Nile

tilapia reared at 15 or 25˚C developed francisellosis and have significantly higher mortality and splenic *Fno* concentrations compared to fish reared at 30˚C (Chern and Chao, 1994; Soto *et al.*, 2012c). Additionally, in Hawaiian tilapia, mortalities were shown to be much greater between 21.5 and 26.3˚C with the first mortalities occurring on day 15 and these doubled almost daily thereafter; otherwise no mortality was observed between 26.5 and 29.2˚C (Mauel *et al.*, 2003). Meanwhile, in the present study, the optimal temperature of *Fno* STIR-GUS-F2f7 to kill both strains of Nile tilapia (homo gold and wild type) was 23±2˚C. With regards to the *G. mellonella* larvae, in the present study, the killing appeared to be more effective at 28˚C, since it is a more effective growth temperature for *Fno* in laboratory studies (on CHAH and MHB). Therefore, higher mortality rates of *G. mellonella* larvae infected by *Fno* were found at 28˚C than at higher or lower temperatures; in addition, this could be attributed to an increase in bacterial virulence and increased susceptibility of the insect to infection. Moreover, in this present work, it was also observed that the insect larvae developed faster, and more larvae attained pupation at higher temperatures except 37˚C (Table 5.2). These results are in line with a similar previous study (Cymborowski, 2000), as at 18˚C, the last instar larvae did not pupate but when transferred to 30˚C they initiated development and pupation in a circadian manner, as the larvae endocrine system work very well on that temperature. Thus, the time of larvae incubation used in this study is quite optimal for the growth of the insects, and larvae pupation is an indication that the larvae are not stressed.

In Nile tilapia, a dose of *Fno* equivalent to 23 CFU can cause mortality (Soto *et al.*, 2009b), while a dose of 3.45 x 10^5 CFU mL^{-1} caused very low mortality in zebrafish (Vojtech *et al.*, 2009b). In this present study (Chapter 3, Section 3.3.4.1), a dose of 1.03 x 10^5 CFU mL^{-1} caused 10 and 30% mortalities in Nile tilapia homo gold and wild type, respectively, and the lowest *Fno* inocula that killed the larvae was 0.7–0.8 x 10^8, 0.9–1.6 x 10^8, 1–1.1 x 10^8, 1–1.7 x 10^8 and 0.9–1.6 x 10^8 CFU $larva^{-1}$ for *Fno* Austria, Mexico, Northern Europe, Costa Rica and Japan isolates, respectively. However, greater inocula (*e.g.* 0.7–1.7 x 10^8 CFU mL^{-1} and above) were necessary to cause mortalities in the larvae similar to the native tilapia host. That a higher dose of bacteria is needed to cause

mortality in the greater wax moth larvae could be attributed to species-specific host-pathogen interactions. Considering its highly virulent nature, infection with as few as 10 cells of *F. tularensis* can cause severe disease in humans (Saslaw *et al.*, 1961). According to previous studies in *G. mellonella* larvae, injection of *F. tularensis* LVS at the lowest concentration of 3 x 10^3 CFU $larva^{-1}$ resulted in 75% survival after 12 d of infection (Aperis *et al.*, 2007). Therefore, it can be suggested that the ability of haemocytes to clear F. tularensis is dependent on the subspecies and its virulence (Aperis *et al.*, 2007).

In this present work, heat-killed *Fno* Japan (at 60°C for 60 mins) resulted in greater larval mortality than live bacteria (both at doses of 2.5–4.2 x 10^9 CFU mL^{-1}), eventhough there was no significant difference between these treatments (Figure 5.7). Live *Fno* Japan had the lowest virulence compared to other *Fno* isolates so the larval mortality is normally low, while the greater mortality in the heat-killed bacteria could have been due to toxicity of bacterial cells (same concentration for live and heat-killed groups). Injection with *Fno* in the five isolates (2.4–6.7 x 10^9 CFU $larva^{-1}$) heat-killed at 60˚C for 60 mins caused 67–83% mortality while injection with *Fno* (2.4–6.7 x 10^9 CFU $larva^{-1}$) heat-killed at 90˚C for 30 mins resulted in 12–54% mortality (Figures 5.4, 5.5, 5.6, 5.7 and 5.8). As heat-killing at 60˚C for 60 mins showed higher mortality compared to 90˚C for 30 mins, it seems likely that bacterial toxins are released at 60˚C but not killed, so cause greater mortality in larvae than the 90˚C treatment or the larval deaths occured due to high concentration of the bacteria, and viable bacteria are not required for death. In addition, in the present study, few mortalities were observed when the larvae were injected with culture filtrate from each *Fno* isolate (Figures 5.4, 5.5, 5.6, 5.7 and 5.8). This could be indicated the presence or lack of extracellular toxins.

Crucially, there was significant positive correlation between the pathogenicity of different *Fno* isolates evaluated in native host (tilapia) and an alternative host *G. mellonella* infection models (Figure 13). This is a key evidence when validating an alternative host for a particular pathogen, even though it is rarely performed or achieved possibly due to the undesirable number of animals required (Jander *et al.*, 2000; Olsen *et al.*, 2011; McMillan *et al.*, 2015).

Importantly, in this present study, additional fish were not needed with respect to the correlation in virulence of *Fno* isolates in tilapia and *G. mellonella* as previous data on tilapia were used (Shahin, unpublished). The correlation between virulence in *G. mellonella* and tilapia models suggests that the *G. mellonella-Fno* system might be used for determining virulence of isolates or for studying known virulence factors, and for the identification of new genes in *Fno* involved in virulence as well as studies on antibacterial compounds.

Studies of microbial pathogenesis, including phagocytosis, can be perform in *G. mellonella* larvae where the analysis of insect responses to pathogens can provide an indication of the mammalian (Aperis *et al.*, 2007; Ahmad *et al.*, 2010), and fish (McMillan *et al.*, 2015) responses to the pathogen. The host response of *G. mellonella* to resist microbial infections comprises structural and passive barriers, as well as cellular and humoral defenses that are mediated by haemocytes within the haemolymph; larvae do not have an adaptive immune system (Aperis *et al.*, 2007; Seed and Dennis, 2008). The insect response includes phagocytosis (encapsulation of large invading pathogens by layers of haemocytes) and melanisation where different types of haemocytes have been identified in *G. mellonella* (Kavanagh and Reeves, 2004). Physical effects such as body colour change can be observed when the bacteria replicate and increase in the larvae (Aperis *et al.*, 2007). Melanisation is a part of the insect response to infection and plays an essential role in the larvae cellular defense mechanisms by recognition of antigens and/or pathogens. The melanisation response to the infection is a synthesis and deposition of melanin to encapsulate pathogen at the wound site followed by haemolymph coagulation and opsonisation, typically starts with distinctive balck spots on the cream colored larvae (Tang, 2016; Tsai *et al.*, 2016). Melanin development is catalysed by prophenoloxidase cascade in haemocytes that leads to melanization (Aperis et al., 2007; Olsen et al., 2011; Harding *et al.*, 2012). In the present study, the larvae demonstrated obvious signs of progressive melanisation during the progression of the infection by *Fno* (Figures 5.10, 5.11 and 5.12). This result is in agreement with others bacteria infection in the *G. mellonella* studies *e.g., P. aeruginosa* (Jander *et al.*, 2000), *F. tularensis* (Aperis *et al.*, 2007), *Acinetobacter baumannii* (Peleg *et al.*, 2009), *Listeria*

monocytogenes (Joyce and Gahan, 2010), Group A *Streptococcus* (Olsen *et al.*, 2011), *Campylobacter jejuni* (Senior *et al.*, 20111), and *Legionella pneumophila* (Harding *et al.*, 2012).

In this present study, various histochemical stainings were used evaluate different morphological aspects of *G. mellonella-Fno* interactions. More information regarding host-immune response in relation to the pathogen virulence was provided and chronological aspects of host-pathogen interaction were also documented including immune cell maturation, and the progression of organ damage. Based on the slides obtained by processing the larvae at different time points of infection, *Fno* was easily monitored and showed aggressive invasion of host gastrointestinal walls within a short time (Figure 5.29). By 48 h, *Fno* cells were observed inside haemocytes (Figures 5.25A and 5.29A), followed by a progressively increasing number of bacteria cells within hemocytes, indicating successful phagocytosis (Figures 5.25B and 5.29B). At 48 h, *Fno* cells in the larvae haemolymph was found greater than at the later time points (72 to 288 h) (Figure 5.33), showing that the bacteria replicated rapidly in the larva haemolymph upon the bacteria gaining acces into the host. At 72 h after injection, a significant number of bacteria were found surrounded by layers of haemocytes which continued to aggregate in nodules and there was a tendency to confluence (Figures 5.26A and F, and 5.30A, D and E), in a process known as nodulation. These observations are in line with previous studies, and this process has been described previously with *G. mellonella* in association with pathogens (Kavanagh and Reeves, 2004). Moreover, there may be some similarities with the granulomas detected in the tissues associated with *Fno* infection in warm water fish species especially tilapia (Chern and Chao, 1994; Mauel *et al.*, 2007; Ramirez-Paredes, 2015; Sebastião *et al.*, 2017). By 96 h, the invading of *Fno* resulted in large necrotic areas in the larval tissues caused by haemorrhages and the formation of pale eosinophilic protein lakes (haemocytes lakes) or the necrosis of enormous numbers and enlarged haemocytes as well as other larvae cells (Figures 5.23C, D and F, 5.27A, B, C, D and E, and 5.31A, B, C, D and F). This result shares similarity with the development of necrosis in infected tilapia tissues (especially in spleen and kidneys) caused by *Fno* infection (Soto *et al.*, 2013b).

The presence of a small number of haemocytes was detected in healthy larvae found in the sub-cuticular area, in the hemolymph, and in close association with aero-digestive tracts (Figures 5.20 and 5.24). After the initial phases of infection, the number of haemocytes increased, including circulating haemocytes, such cells are the immune cells attracted to the site of *Fno* replication, which is in agreement with a previous report (Kavanagh and Reeves, 2004). The morphological switch of haemocytes from round into spindle cells was observed in the encapsulation development where the nodules increased in number and in dimension during the progress of the infection (Figures 5.25A to E; 5.26A, D and E; 5.27A, B, C and E).

Encapsulation, a primary defence response in insects, typically takes place in cases where the invading organism is too large to be phagocytized (Perdoni *et al.*, 2014). During the process of encapsulation, haemocytes attach to the invading pathogen and one another in multiple layers to form a nodule, in which the pathogen becomes trapped (Perdoni *et al.*, 2014). In larvae infected with *Fno*, this feature was highly noticeable, and bacteria replication induced phagocytosis by huge numbers of haemocyte and melanised haemocytes prior to formation of nodulations (Figures 5.21–5.23, 5.25–5.27 and 5.29–5.31). While encapsulation is generally considered to be employed against much larger pathogens (> 10 μm) (Ratner and Vinson, 1983; Pech and Strand, 1996), previous literature suggests that insects also utilise encapsulation against large numbers of pathogens (Dubovskiy *et al.*, 2016). As such, it can be suggested that the high doses of *Fno* utilised in this study resulted in the use of encapsulation and nodule formation by the larvae host, as well as phagocytosis.

In this present study, the burden of *Fno* in *G. mellonella* larvae was assessed post-inoculation to clarify if the bacteria replicated in the host during infection up to a threshold where death occured. It was confirmed that *Fno* can survive in *G. mellonella* larvae and proliferate inside the hemocoel and kill the larvae, even though the bacteria underwent phagocytosis by haemocytes. The bacteria not only survived and replicated within the larvae but were also recovered from inoculated larval haemolymph. As *Fno* is an intracellular bacterium, it was

recovered from the haemolymph at all time points by lysing the haemocoel with PBS and breaking the cells by pipetting and vortexing. *Fno* STIR-GUS-F2f7, the isolate showing greatest virulence replicated to a greater extent inside the insect haemolymph to bring about faster larval mortality than the less virulent *Fno* Japan isolate, presumably by more effectively combating the innate immune defences to exploit the host (Figure 33). This is in agreement with a previous study where 11 wild-type isolates of *V. anguilarum* were compared using *G. mellonella* and fish *Salmo salar* models of infection and more virulent strains replicated faster and reached greater burden in the haemolymph than less virulent isolates (McMillan *et al.*, 2015).

Moreover, in the present study, the pattern of bacterial replication in the infected larvae showed a decrease followed by an increase, and these results are in contrast with previous study (McMillan *et al.*, 2015). This pattern occurred twice with *Fno* STIR-GUS-F2f7, while it occurred once with *Fno* Japan (Figure 5.33). In terms of *Fno* STIR-GUS-F2f7, as the experiment lasted 288 h, it is unclear whether the observed pattern of increases and decreases would have continued to occur or whether a plateau would have been reached at a later time. It could be suggested that tissue invasion in the later stages of infection lead to CFU decreases in the haemolymph but may have caused CFU increases in other tissues which were not sampled. However, this suggestion would require additional studies and could involve the sampling of fat body or gastrointestinal track. In addition, although there were several increases of bacteria replication in infected *G. mellonella* larvae for both *Fno* during 288 h of infection, CFUs were not equal to or greater than the CFU mL^{-1} at 0 h (the initial *Fno* injection inocula in PBS). However, even though the number of the bacteria was low in infected larvae, it still caused mortality in the larvae, especially with the most virulent *Fno* STIR-GUS-F2f7 isolate. This evidence suggests that viable and replicating *Fno* cells are needed to establish a systemic infection of *G. mellonella*. Gram staining of isolates confirmed that the bacteria recovered after the infection challenge were indeed *Fno* (Figure 5.34 and 5.35).

In addition to *in vivo* immune-defence responses, the efficacy of antibacterial agents or algal-derived compounds to treat *Fno* infections can be evaluated in

the *G. mellonella* model. The treatment with tetracycline prolonged survival of *Fno* infected larvae for each bacteria strain when the drugs were administered at 2, 24 and 48 h post infection. This result is in agreement with previous observations (Chern and Chao, 1994; Mauel *et al.*, 2007; Soto *et al.*, 2012a, 2016; Ramirez-Paredes, 2015). However, with regards to algal-derived compounds, a limitation of the *G. mellonella* model, is that the compounds need to be injected into the larvae. This does not directly compare to the fish model, as antibiotics/antibacterials would be applied in either a bath or oral treatments.

The results presented in this study are the first to report that administration of algal-derived compounds (ß-glucan from *E. gracillis* and alginic acid from *M. pyrifera*) primes the immune response of *G. mellonella* larvae and protects the insect from challenge at 1 and 24 h later with otherwise lethal doses of *Fno* at 1 x 10^8, 5 x 10^8, and 1 x 10^9 CFU larva^{-1} (Figures 4.38 and 5.39). Previous work has demonstrated that the larvae of *G. mellonella* can withstand a lethal inocula of *C. albicans* (10^6 cells) for 72 h if pre-exposed to a non-lethal dose of yeast *S. cerevisiae* yeast or polysaccharide (*e.g.* mannan, laminarin) 24 h previously, which is mediated by increased expression of a number of antimicrobial peptides and the appearance of other peptides in the challenged larvae (Bergin *et al.*, 2006). Similarly, a correlation between the amount of ß-glucan administered and the degree of immune priming was evident and ß-glucan administered at 15, 30 or 60 mg larva^{-1} was sufficient to render the larvae resistant to *C. albicans* (1x10^6 cells) infection for 72 h, while the larvae that received a lower ß-glucan dose of 0.93 mg larva^{-1} showed little increase in survival relative to that seen in PBS inoculated larvae (Mowlds *et al.*, 2010).

This present study indicates that prior an exposure to sub-lethal dose of algal-derived compounds primes the *G. mellonella* immune system and allows the larvae to withstand a subsequent lethal *Fno* infection. Indeed, provided they are applied as a prophylactic measure, it has become evident that algal-derived compounds can be a potential immunostimulants, while the range of potential ways in which the compounds may affect bacteria, especially in fish could be via immunostimulation, more direct antibacterial activity, via affects on intestinal microbiome or immunity, systemic immunity and increased protection from a

subsequent pathogenic challenge (Dalmo and Bogwald, 2008). However, even though *in vivo* antibacterial agents in this work can protect *G. mellonella* larvae from *Fno* infection, how the agents are modulating host innate immune responses and protecting the larvae during *Fno* infection as well as the gene expression of proteins involved in the immune system is unknown; therefore, further investigation is still needed. While this research continues to demonstrate the efficacy and suitability of the *G. mellonella* model for algal-derived compounds and infection testing, there continue to be limitations in the administration of antibacterial agents/compounds. In the larvae, algal compounds (alginic acid and glucan) are injected for testing while these types of compounds would be administered by orally in fish subjects. Therefore, as such, traditional research it still necessary to link the *G. mellonella* model more closely to practices in fish.

5.5 Conclusion

In this present study, the *G. mellonella* model for *Fno* was validated, indicating that the greater wax moth larvae can be applied as an alternative model for *Fno* infections. The killing appeared to be more effective at 28˚C with the lowest *Fno* concentrations that killed the larvae being 0.7–1.7 x 10^8 CFU $larva^{-1}$, while melanisation and phagocytosis were involved in the *G. mellonella* host response to the infection. The greatest and least virulent isolates of *Fno* successfully replicated *in vivo* in *G. mellonella*, while tetracycline and algal-derived compounds (alginic acid and ß-glucan) at certain concentrations could rescue the insect from lethal bacterial inocula. This model is likely to be highly suited to the study of virulence in other fish and aquaculture pathogens; however, still the *G. mellonella* model requires full validation for each particular pathogen before its suitability for studying that microbe can be ensured and before a direct comparison with infections in fish *in vivo* can be completed. The model may also prove particularly useful in investigating the *in vivo* intracellular survival of *Fno* within macrophages, an area of some dispute. Finally, the model allows screening for natural variations in the virulence of *Fno* field isolates, which would prove invaluable for tracking specifically virulent strains in the aquaculture industry.

CHAPTER 6
GENERAL DISCUSSION AND FINAL CONCLUSION

6.1 Final discussion

6.1.1 Framework of this study

The rapid growth of tilapia farming is a consequence of the adaptability and tolerance of tilapia to a wide range of environments and intensive cultivation systems (El-Sayed, 2006). As a result, tilapia culture has expanded, and occurs in more than 140 countries with global production expected to reach the 8.5 MT in 2026 (Fitzsimmons, 2016). Intensification of tilapia culture practices has resulted in an increase of disease outbreaks on farms, and bacterial agents are responsible for a large number of the major epizootics. Piscine francisellosis caused by the bacterium *Fno* is an infectious emerging disease in tilapia but no effective commercial treatments or vaccines are currently available.

The main aim of this thesis was to evaluate the effectiveness of bioactive algal compounds on the growth and survival of francisellosis *in vitro* and *in vivo* and determine their potential to control francisellosis infection in Nile tilapia *O. niloticus* L. and in the wax moth *G. mellonella*, under experimental *in vivo* conditions. To meet this aim, four objectives were defined, specifically: 1) to screen algal-derived compounds *in vitro* for immunostimulatory and antibacterial activities (using tilapia primary head kidney macrophages and disc diffusion assays); 2) to develop an immersion challenge for *Fno* in Nile tilapia *O. niloticus* L. and evaluate response to infection; 3) to examine the effect of feeding algal-derived compounds (in feed) on the innate immune response of Nile tilapia homo gold *in vivo*, challenged with single and co-infections of economically significant bacterial pathogens; and 4) to develop an alternative *in vivo* infection model for *Fno* in wax moth larva *G. mellonella* and assess the effects of antibacterial compounds, including algal derived compounds.

Information on *Fno* in aquaculture is mainly focused on reporting cases of disease outbreaks (Chen *et al.*, 1994; Hsieh *et al.*, 2006; Sebastião *et al.*, 2017) and/or experimental challenges by various methods, including gill spraying (Soto *et al.*, 2009a), IP (Chen *et al.*, 1994; Soto *et al.*, 2009a; Leal *et al.*, 2014; Jantrakajorn and Wongtavatchai, 2016; Lin *et al.*, 2016; Dong *et al.*, 2016b;

Nguyen *et al.*, 2016), IM injection (Chern and Chao, 1994) or cohabitation (Chern and Chao, 1994; Mauel *et al.*, 2003). Limited information is available on pathogenesis and virulence determinants, especially by immersion challenge (Soto *et al.*, 2009b, 2013b). Moreover, at the start of this project, no information was available regarding the possible application of algal-derived compounds to treat francisellosis in Nile tilapia, and no validated alternative hosts were available to study pathogenicity or virulence. Thus, the present study was designed to afford a sequential and multidisciplinary approach to expand the available knowledge of *Fno* and possible approaches to its control.

First, an *in vitro* study was performed to screen algal-derived compounds for immunostimulatory and anti-bacterial activities using the disc diffusion assay and primary Nile tilapia homo gold head kidney cell cultures (Chapter 2). The products giving the most promising results from the *in vitro* studies were taken forward for an *in vivo* trial in Nile tilapia to evaluate the effectiveness of the algal-derived compounds on the growth and survival of tilapia in a francisellosis immersion challenge. For this, an immersion challenge for *Fno* STIR-GUS-F2f7 had to be developed in Nile tilapia (homo gold/red and wild type) in order to investigate the LD_{40} and evaluate response to the infection (Chapter 3). The *in vivo* trial sought to investigated whether concurrent bacterial pathogens were involved in naturally diseased Nile tilapia, thus *Fno*-challenged fish were exposed to a subsequent streptococcocis challenge, using an IP challenge with *S. agalactiae* (Chapter 4). The *in vivo* dietary administration of algal immunostimulants and challenge trials failed to demonstrate a beneficial effect against *Fno* and *S. agalactiae* challenges. Due to ethical considerations and time contraints, it was decided to investigate whether an alternative host model, specifically *G. mellonella*, could be developed and applied to generate *in vivo* activity data on algal-derived extracts, with promising products then evaluated in subsequent fish trials (Chapter 5). The *G. mellonella* alternative host was validated through various means including showing a correlation between virulence different *Fno* strains in tilapia and insect models (Chapter 5).

6.1.2 Immunostimulatory and anti-bacterial activities of algal-derived compounds against bacterial pathogens

Disease outbreaks are mainly caused by stress-induced factors that make the fish more susceptible to infectious pathogens, such as poor water quality and husbandry practices, high stocking densities, low seed quality and poor control of the microbial condition in intensive rearing facilities (Dong *et al.*, 2017). The use of chemotherapeutants, chemical disinfectants and antibiotics is commonplace due to disease outbreaks in culture tilapia (Soto *et al.*, 2016). Antibitoic use has been criticized for negative impacts such as residual accumulation in the tissue, development of drug resistance and immunosuppression, and so there is reduced consumer preference for food fish treated with antibiotics, as well as concerns over the environment and wildlife protection (Anderson, 1992). Therefore, other methods of prevent and control bacteria disease in fish are required. Immunostimulants such as dietary supplements are currently being adopted by the aquaculture industry as an alternative to antibiotics, prebiotics, probiotics or vaccines. The mechanism of immunostimulants to prevent and control diseases, is by enhancing the fish immune defences and thereby increasing immunocompetency and disease resistance (Bricknell and Dalmo, 2005). Algae are able to produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities, therefore they are considered as a source of bioactive compounds for immunostimulant and antibacterial activity against fish pathogen (Chojnacka *et al.*, 2012; Vijayakumar, *et al.,* 2012).

In the Chapter 2, *in vitro* studies were performed to screen algal-derived compounds for immunostimulatory and antibacterial activities as candidate immunostimulants, using Nile tilapia homo gold primary head kidney cell cultures and disc diffusion assay. Extracts (using methanol, acetone, ethanol or water) of three algal species, *Chlorella* sp., *C. calcitrans*, and *Nannochloropsis* sp., and two algal-derived substances, alginic acid and ß-glucan, were tested for their abilities to inhibit the growth of three bacterial pathogens using disc diffusion assay (Chapter 2). The results showed that methanol was the most efficient solvent in extracting antibacterial compounds from algae. Methanol

extracts of *C. calcitrans* had the highest inhibitory activity against *Fno* and *S. agalactiae,* followed by methanol extracts of *Chlorella* sp. Algae produce a great variety of secondary metabolites that exhibit broad spectrum of antimicrobial activities (Chojnacka *et al.*, 2012). Several studies have reported the antimicrobial activity of algae extracts against fish pathogenic bacteria (Section 1.6.6).

The algal extracts were also screened for immunostimulatory activity in primary Nile tilapia homo gold head kidney cells cultures (Chapter 2). The results demonstrated that even though no significant differences were observed between the groups, different algal extracts at 50 µg mL^{-1} stimulated cellular respiratory burst activity especially at 3 and 6 h after inoculations, and greatest activity was observed in the cells incubated with water extracts of *Chlorella* sp., ß-glucan and alginic acid. There are few reports of effects of algae and algal-derived substances on immune-related cells, such as primary HK leukocytes. Nevertheless, alginic acid from the same source as in the present study induced significantly increased ROS production by cod HK leukocytes (Caipang *et al.*, 2011), while ß-glucan enhanced ROS activity in sole phagocytes (Díaz-Rosales *et al.*, 2007) and laminaran, a ß-glucan obtained from *L. hyperborea*, increased the intracellular production of the superoxide anion by salmon macrophages (Dalmo and Seljelid, 1995). In addition, aqueous extracts of *U. rigida* and *C crispus* stimulated respiratory burst activity in turbot phagocytes (Castro *et al.*, 2004, 2006), and an ethanolic extract of *H. cornea* increased superoxide anion production in sole phagocytes (Díaz-Rosales *et al.*, 2007).

Previous studies have reported a great deal of variation with regards to the concentrations and incubation times of various algae-derived compounds which elicit increased macrophage respiratory burst activities *in vitro* and *in vivo,* and this is further underlined by species-specific responses (Bridle *et al.*, 2005). For example, respiratory burst activity of Atlantic salmon macrophages after incubation with MacroGard at 1 µg mL^{-1} for 4 d, or 0.1 or 1 µg mL^{-1} for 7 d, then stimulated with PMA, was significantly increased compared to the control (0 µg mL^{-1}). In contrast, when incubated with higher concentrations at 10 µg mL^{-1} there was no significant difference in ROS production compared to the control,

while at 50 µg mL^{-1} the activity was inhibited (Jørgensen and Robertsen, 1995). According to Castro *et al.* (1999), high concentrations of ß-glucan (25–500 µg mL^{-1}) can modulate ROS production in head kidney macrophages of turbot and gilthead sea bream head kidney macrophages.

With regard to anti-bactericidal assays using Nile tilapia homo gold HK macrophages and the MTS kit, the water extracts of *Chlorella* sp., alginic acid and ß-glucan (50 µg mL^{-1}) showed little capacity to kill the virulent strains of *Fno*. Still further *in vitro* studies are needed to investigate whether *in vitro* algae-derived compounds can cause more efficient killing of other less specialized pathogens by fish marophages. Several *in vitro* cell cultures have been developed to stimulate the cellular immune response and investigate the bactericidal activity. For example, macrophages isolated from ß-glucan-injected rainbow trout and Atlantic salmon show increased capacity to kill *A. salmonicida* (Jorgensen *et al.*, 1993; Solem *et al.*, 1995). An alginic acid from *M. pyrifera* was demonstrated to inhibit the growth of *A. salmonicida* and *V. anguilarum* in both the cod treated cells and control supernatants of the compounds using MTT assay (Caipang *et al.*, 2011). Aqueous extracts of *F. distichus* and mycosporine from *P. leucosticta* increased respiratory burst activity of sole phagocytes after infection with *P. damselae* subsp. *piscicida* (Díaz-Rosales *et al.*, 2005). Finally, an ethanolic extract of *H. cornea* and ß-glucan enhanced bactericidal activity against *P. damselae* subsp. *piscicida* (Díaz-Rosales *et al.*, 2007). This is the first report of the *in vitro* screening of bioactive algal compounds using different assays and bacterial fish pathogens.

6.1.3 Pathogenicity of *Fno* STIR-GUS-F2f7 in native (tilapia) and alternative (*G. mellonella*) hosts

In Chapter 3, it was preferable to use the natural infection route of *Fno*, and thus immersion challenge was used as this closely replicates *Fno* transmission in Nile tilapia culture systems. In the experimental immersion challenge study, *Fno* STIR-GUS-F2f7 was pathogenic to fish and successfuly infected both Nile tilapia strains. In this model, the bacteria were highly virulent, and mortality was consistently 55% at concentration 1.03 x 10^7 CFU mL^{-1} of tank water within 10

and 14 dpi in both Nile tilapia homo gold and wild type (Chapter 3). These cumulative mortality rates are higher when compared with a previous study, even though a lower dose was used (Soto *et al.*, 2009b), but comparison of virulence of the strains using a standardised challenge models is needed to corroborate this. The susceptibility of various tilapia species to francisellosis has been reported during both natural outbreaks and controlled experimental infections (Chern and Chao, 1994; Soto *et al.*, 2013c; Ramirez-Paredes, 2015; Klinger-Bowen *et al.*, 2016). In the present study, both Nile tilapia strains were susceptible to francisellosis, with homo gold showing higher susceptibility which resulted in faster disease progression and more granulomas in both spleen and anterior kidney than wild-type. This observation is in contrast with a previous study in which the latter was found to be more resistant to the infection (Ramirez-Paredes, 2015). Whether the differences between these studies is due to the virulence of the bacterial strains, the current families tested or is indeed characteristic of the fish population and/or environmental conditions are unknown; therefore, further study is still required.

Typical clinical signs caused by *Fno* after immersion challenge included the appearance of petechial haemorrhages on the skin and abrasion on the mouth for both Nile tilapia strains. These signs have not been observed previously (Chern and Chao, 1994; Chen *et al.*, 1994; Mauel *et al.*, 2007, 2003, 2005; Hsieh *et al.*, 2006; Soto *et al.*, 2009a; b, 2013b; c; Jeffery *et al.*, 2010; Iregui *et al.*, 2011; Leal *et al.*, 2014; Ramirez-Paredes, 2015; Jantrakajorn and Wongtavatchai, 2016; Lin *et al.*, 2016; Ortega *et al.*, 2016; Nguyen *et al.*, 2016; Sebastião *et al.*, 2017). The internal gross and histopathological lesions that developed during the *Fno* infection were also investigated (Chapter 3). After 5 and 7 dpi, in both homo gold and wild type, the formation of granulomas containing of bacteria were observed mostly in the spleen, anterior and posterior kidney, which is in agreement with natural outbreaks and previous experimental studies (Chern and Chao, 1994; Mauel *et al.*, 2007; Soto *et al.*, 2013b; Ramirez-Paredes, 2015). All infected fish of both tilapia strains in the present study showed individual hypertrophied cells (mostly macrophages) surrounding and within the granulomas, and huge numbers of small pleomorphic cocco-bacilli bacteria were observed predominantly presented

inside the nucleus of leucocytes, in the melanomacrophages centres, and inside the erythrocytes and their nuclei. These latter two observations have not been reported previously.

In Chapter 5, *G. mellonella* was validated as an alternative host to study *Fno* virulence. Five *Fno* isolates established systemic infection of *G. mellonella* larvae; the bacteria killed them in a dose-dependent manner and the optimal temperature for killing the larvae was 28°C.

Importantly, there was significant positive correlation between the pathogenicity of different *Fno* isolates in native host (tilapia) and the alternative host *G. mellonella* host (Chapter 5). These results indicate that more virulent *Fno* strains in *O. niloticus* were also more virulent in the insect. *Fno* STIR-GUS-F2f7 with the greater virulence replicated to a greater extent inside the insect haemolymph to bring about faster larval mortality than the least virulent isolate (*Fno* Japan). These results are in agreement with previous study that showed correlation in virulence between *G. mellonella* and Atlantic salmon models for 11 wild-type isolates of *V. anguilarum*, and these differences were due to ability to replicate in the insect host (McMillan *et al.*, 2015). Based on the gross and histopathological observations it was found that phagocytosis*,* nodulations and melanisation were involved in the *G. mellonella* host response to the *Fno* infection. This process has been described previously with *G. mellonella* in association with pathogens (Kavanagh and Reeves, 2004). The larvae immune responses may have some similarities with the fish responses in respect to the process to phagocytose the bacteria by formation of granuloma inflammation and in the process to develop necrosis in the tissues associated with *Fno* infection in warm water fish species, especially tilapia (Soto *et al.*, 2013b; Sebastião *et al.*, 2017).

In the present studies (Chapters 3 and 5), after immersion challenge, *Fno* is thought to enter through the skin, the first barrier of fish immune system, gain access to the bloodstream and spread to the internal organs (*e.g.* spleen, kidneys, etc). In *G. mellonela* larvae, after IP injection, the bacterium gained access directly to the haemolymph, infected the haemocytes and spread rapidly

to the internal organs (*e.g.* cuticle, fat body, muscle fibres and gastrointestinal track). The fastidious nature of *Fno* (slow growth and nutritional requirements) and its ability to enter into a viable but not cultivable state has complicated the primary isolation of the pathogen (Hsieh *et al.*, 2006; Mikalsen *et al.*, 2009; Soto *et al.*, 2010b). However, in this present study, the viable bacteria were recovered mostly from hematopoietic organs under investigation with proper agar media and method of isolation in both Nile tilapia and *G. mellonella*. Gram staining of isolates confirmed that bacteria can be recovered after an infection challenges both Nile tilapia and *G. mellonella.* Further study is required to identify the bacteria isolates by molecular identifications such as PCR and RT-PCR assays. The insect stress-regulated enzyme phenoloxidase, is one of the first immune molecules and normally activated following infections (Bidla *et al.*, 2009). Thus, further study is needed to measure levels of enzyme phenoloxidase in larvae after infection with *Fno* and to compare the enzyme levels in uninfected or infected larvae. This is the first report of the pathogenicity of *Fno* STIR-GUS-F2f7 in native (Nile tilapia) and alternative (*G. mellonella*) hosts.

6.1.4 Innate immune response and disease resistance of Nile tilapia to *Fno* and *S. agalactiae* after feeding dietary algal-derived compounds

Several studies have been conducted on the application of immunostimulants, particularly algal-derived compounds, to inducing fish and shrimp non-specific immune responses such as respiratory burst and lysozyme activities. For instance, laminaran from *L. hyperborean* significantly enhanced production of superoxide anion of Atlantic salmon (Dalmo *et al.*, 1996). Ergosan containing 1% of alginic acid extract from *L. digitata* enhanced respiratory burst activity in rainbow trout (Peddie *et al.*, 2002) and seabass (Bagni *et al.*, 2005). Hot-water extracts of *G. tenuistipitata* increased the production of oxygen radicals in phagocytes of white shrimp (Hou and Chen, 2005). A sodium alginate from *M. pyrifera* increased respiratory burst activity of white shrimp (Cheng *et al.*, 2005) and grouper (Cheng *et al.*, 2007; Yeh *et al.*, 2008). Similarly, a sodium alginate from *L. digitata* and *A. nodosum* enhanced lysozyme activity in seabass (Sakai,

1999), while a hot-water extract of *S. wightii* significantly enhanced the lysozyme activity of mullet (Thirunavukkarasu *et al.*, 2015).

In Chapter 4, algae and algal derivative were used in Nile tilapia homo gold diets (10% *H. pluvialis*, 10% *S. quadricauda*, 0.1 and 0.2% β-glucan, and 0.1 and 0.2% alginic acid) for 28 d to assess any immunostimulant activity. The non-specific immune responses of Nile tilapia, measured in this study (*i.e.* respiratory burst and lysozyme activities) were differentially stimulated by different algal-derived compounds, even though there were no significant differences between dietary groups and the control group and the variation was so high that no firm conclusion could be reached.

In the present study, 28 d after being fed with different algae diets, Nile tilapia homo gold were experimentally infected by immersion with *Fno* STIR-GUS-F2f7 (~$1.05x10^5$ CFU mL^{-1}) (Chapter 4). However, *Fno* immersion challenge failed to produce mortality in any of the dietary groups despite being successful previously. Fish did appear to become infected but then recovered. Future experiments, using a higher dose of *Fno,* could use qPCR analysis of samples to determine levels of *Fno* following infection. The immersion pre-challenge of *Fno* should be improved and standardized prior repeating the *in vivo* trial. Many challenge models have been developed to investigate the health status of the fish and the survival rate of infected fish can be enhanced after oral administrations of various algae immunostimulants. For example, dietary alginate from *A. nodosum* reduced mortality of turbot juvenile (Skjermo *et al.*, 1995), while High-M alginate from *D. antarctica* increased the survival of halibut larvae against *V. anguillarum* (Skjermo and Bergh, 2004), and the same alginate increased cod resistance to atypical *A. salmonicida* (Vollstad *et al.*, 2006). Dietary supplement of High-M alginate and ß-glucan from *C. mülleri* enhanced cod larval resistance against *Pseudomonas*- and *Vibrio*-like bacteria (Skjermo *et al.*, 2006). The secondary infection of the surviving Nile tilapia homo gold with *S. agalactiae* after algal treatment and main challenge with *Fno* resulted in very low cumulative survival in all dietary groups. The low survival could have been due to the huge dose of *S. agalactiae* used in this study (2 x 10^5 CFU $fish^{-1}$). The dose used previously caused 100% mortalities of fish in

single infection. Therefore, further study is needed, and the use of a lower bacteria dose (aiming for LD_{40}) for the *in vivo* immunostimulants trial is recommended. This could enable any the differences between algae dietary groups to be detected. This is the first report of co-infection in Nile tilapia after dietary administration of different algae-derived compounds. Moreover, whether the presence of one pathogen can affect the load of other pathogens and how defensive immunity to a specific pathogen can occur in the host infected with multiple pathogens are unknown (Lello *et al.*, 2004; Bradley and Jackson, 2008). Therefore, further studies are required to investigate the innate and adaptive immune responses, genes expressions, bacterial load in the infected fish tissue (*e.g.* for the main and secondary pathogens), and bacterial clearance mechanism of the resistant host will be useful for the development of new effective vaccine-based disease prevention and disease control strategies.

This present study is the first report of dietary administration of algal-derived compounds in Nile tilapia in order to control francisellosis. The differences in immunostimulatory abilities reported in *in vivo* studies and the present study are most likely explained by differences in experimental factors such as species-specific immune system responses, water temperatures, diet and compound formulations, and feed intakes, which can cause great variations in the results. For example, respiratory burst activity of snapper *Pagrus auratus* HK macrophages was enhanced both *in vitro* (Cook *et al.*, 2001), and after dietary administration of 0.1% v/v EcoActiva paste containing ß-glucan (Cook *et al.*, 2003). Three commercial ß-glucan products were able to stimulate the respiratory burst activity of Atlantic salmon HK macrophages *in vitro*, however, dietary incorporation of the three ß-glucans was unable to stimulate the *in vivo* respiratory burst activity of HK macrophages, or serum lysozyme production, and did not increase resistance against amoebic gills disease (Bridle *et al.*, 2005).

The immunostimulatory potential of ß-glucan seems to be influenced by different physico-chemical parameters and this may account for some of the contradictory conclusions present in the literature such as solubility, molecular weight, degree of branching and tertiary structure (Tabata and Ikada, 1988;

Hunter *et al.*, 2002). ß-glucan containing ß-1,3 and ß-1,6 glycosidic linkages are known to have a potent stimulatory effect on innate immune responses, which is linked to the degree of branching of these ß-1,3 and ß-1,6 polymer chains (Bridle *et al.*, 2005). In mammals, uptake via the digestive tract and presentation to the gut-associated lymphoid tissue (GALT) is closely associated with particle size (Tabata and Ikada, 1988). ß-glucan particles of approximately 1 µm diameter that do not form aggregates en route through the digestive system are absorbed by the GALT more efficiently than poorly processed β-glucan particles that readily form aggregates when exposed to water (Hunter *et al.*, 2002). Thus, different manufacturing processes may explain differences in the dose–response and timing of respiratory burst activation between different commercial ß-glucan formulations (Bridle *et al.*, 2005). In contrast, the response induced by *Ulva rigida* polysaccharides extracts appears to be longer lasting and the stimulatory capacity is not directly related to the size of the molecules, as both large and small polysaccharides induced stimulation of turbot phagocyte respiratory burst; however, their activity *in vivo* still needs to be determined (Castro *et al.*, 2006). In the present study, a linear ß-1,3-glucan (syn. paramylon), a storage carbohydrate of euglenid algae, that is produced in high amounts (up to 90% of dry cell mass) by *E. gracilis* was used. The particles of this ß-glucan were large in size, which made it difficult to dissolve in PBS, and caused the formation of aggregates in the tilapia HK macrophage cultures (data not shown). However, whether this led to great variations in the non-specific immune response of Nile tilapia *in vitro* and *vivo* remains unknown, and further study is needed. These conditions could be applied for alginic acid as well, but again further study is required.

6.1.5 Efficacy of antimicrobial agents in the *G. mellonella* to *Fno* infection

Due to the failure of the *Fno* immersion challenge following feeding with diets containing different algae species and compounds (Chapter 4), and the lack of time to repeat *in vivo* in fish, *in vivo* trials were conducted in *G. mellonella*.

The *G. mellonella* model has been shown to be a good alternative for the rapid evaluation of antimicrobial agent effectiveness *in vivo*, prior to *in vivo* testing in mammalian or vertebrate models (Desbois and Coote, 2012; Tsai *et al.*, 2016). In the present study (Chapter 5), *in vivo* immune-defence responses, with regard to the use of antibiotics as treatment of *Fno* infection, were first evaluated in the *G. mellonella* model system. Prior to *in vivo* treatment of *G. mellonella* with antibiotics, *in vitro* antibiotic sensitivity to three different bacterial pathogens (*Fno*, *A. hydrophila* and *S. agalactiae*) was performed using disc diffusion assay. Tetracycline rescued *G. mellonella* from *Fno* infection. In addition, algal-derived compound immunostimulants (alginic acid and ß-glucan) primed the immune response of *G. mellonella* larvae and protected the larvae to withstand subsequent lethal doses of *Fno* infection (Chapter 5). In the present study, even though *in vivo* antibacterial agents prolonged *G. mellonella* larvae survival from *Fno* infection, the mechanism which agents modulate the host innate immune responses during *Fno* infection and gene expression of proteins involved in the immune system are unknown. Thus, further investigation is still required. This is the first to report that administration of algal-derived compounds primes the immune response of *G. mellonella* larvae and protects the insect from lethal doses of *Fno*. However, according to Dalmo and Bogwald (2008), the variety of possible ways in which the compounds might affect bacteria, especially in fish, include via immunostimulation, antibacterial activity, affects on intestinal microbiome or immunity, systemic immunity and increased protection from infectious pathogens. Therefore, how the agents are protecting the *G. mellonella* larvae during *Fno* infection as well as the gene expression of proteins involved in the immune system is unknown; therefore, further investigation is still needed.

6.2 Final conclusions

This study has provided evidence from *in vitro* and *in vivo* methods that algal-derived compounds have potential use as immunostimulants to control francisellosis infection in Nile tilapia as a safer and eco-friendly approaches in health management strategy. Sreening of such products is, however, difficult with both *in vitro* and *in vivo* testing results showing high variation and low

reproduciblility of disease challenges in fish. Alternative *in vivo* models, such as *G. mellonella*, may therefore be useful in future studies.

Chlorella sp. was found to be a good candidate as immunostimulant based on *in vitro* screening. Thus, it would be interesting to investigate their activity further *in vivo* and to find out if they can modulate the fish immune response, increase immunocompetency and disease resistance. However, proper therapeutic doses of these algal-derived compound immunostimulants, diet formulations, application methods and administration regimes for different fish species and age groups of fish, as well as effective bacterial challenge doses have to be investigated and confirmed to prevent possible side-effects and to achieve optimal immunostimulatory and antimicrobial response on the host before application of such products in aquaculture.

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APPENDICES

Appendix 4.1: Survival of larvae infected with *Fno* STIR-GUS-F2f7 at 1.05 x 10^9 CFU mL^{-1} and incubated at 15, 22, 25, 28 and 37°C

Time (h)	Unmanipulated	PBS	15 °C	22 °C	25 °C	28 °C	37 °C
0	100	100	100	100	100	100	100
24	100	100	100	100	100	100	83
48	100	100	100	100	92	100	83
72	100	100	100	100	92	100	83
96	100	100	100	92	92	92	83
120	100	100	92	92	92	92	83
144	100	100	92	92	92	92	83
168	100	100	92	92	83	75	67
192	100	100	92	83	75	75	67
216	100	100	92	83	67	67	67
240	100	100	92	83	58	50	67
264	100	100	92	75	58	50	67
288	100	100	33	58	33	25	42

Appendix 4.2: Antibiotic agar plates testing

Fno STIR-GUS-F2f7 grew well on various antibiotic-containing agar plates and showed a similar number of colonies were detected on CHAH supplemented with each concentration of colistin (1, 2.5, and 7.5 mg L^{-1}) and amphotericin B (1 and 2.5 mg L^{-1}) compared to CHAH without antibiotics (Figure 5.33). CHAH supplemented with penicillin gave similar results at each concentration (1, 2.5, and 5 mg L^{-1}) although low number of colonies were recovered at 1 mg L^{-1} at the 10^7 dilutions, and for ampicillin colonies recovered were low at each concentration (1, 2.5, and 7.5 mg L^{-1}) and dilutions, while no *Fno* grew at 10 mg L^{-1} ampicillin (Figure 5.32a).

Fno Japan, CHAH supplemented with ampicillin consistently gave low numbers of colonies in all concentrations, compared to CHAH without antibiotics. However, CHAH supplemented with penicillin, colistin and amphotericin B showed a similar number of *Fno* colonies only at 1, 2,5 and 5 mg L^{-1} in dilution of 10^6 and 2.5 in 10^7 (penicillin), 1 and 7.5 mg L^{-1} in 10^6 (colistin), 1 and 2.5 mg L^{-1} in 10^6 and 1 mg L^{-1} in 10^7 (ampothericin B) (Figure 5.32b).

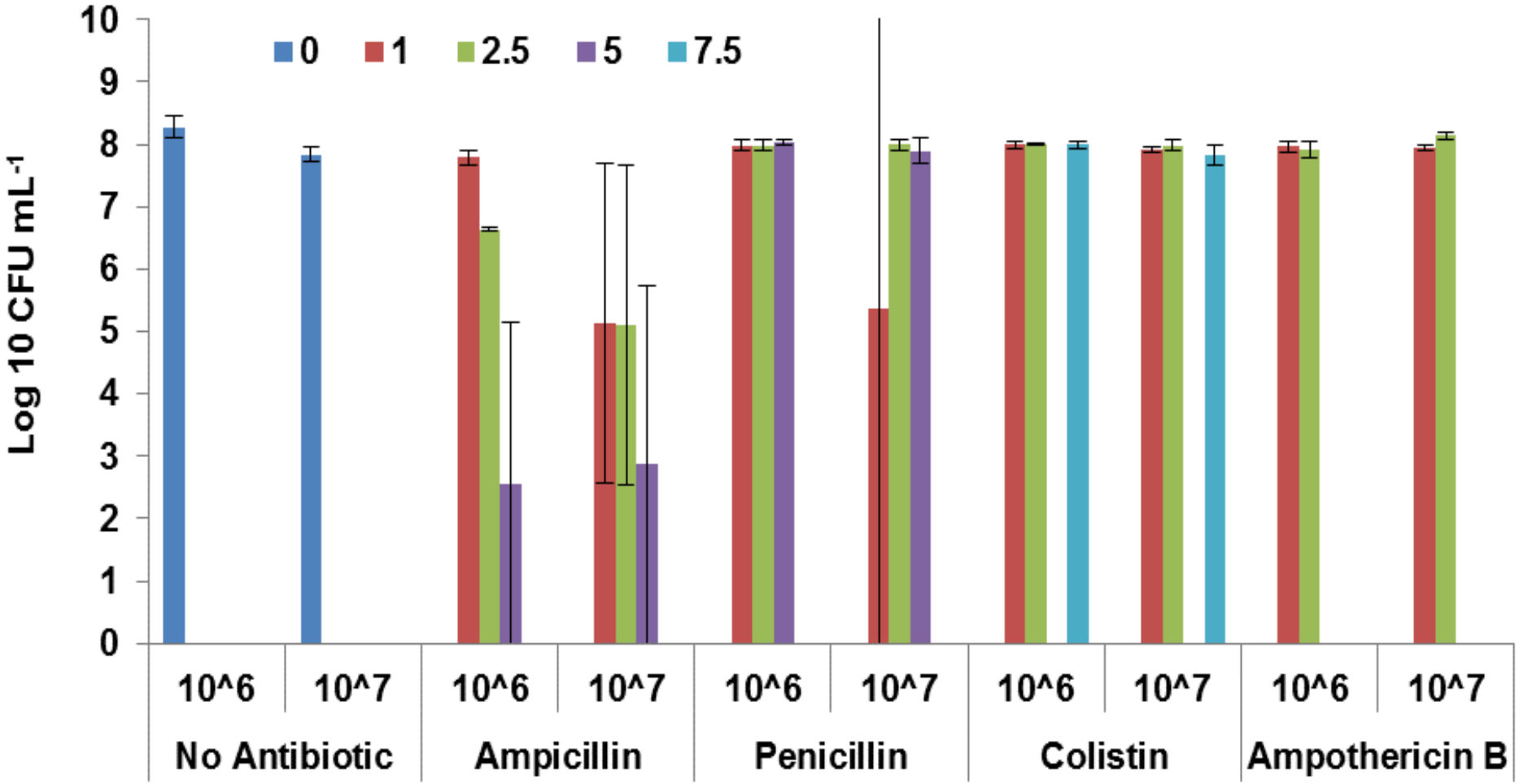

Figure 5.32a Log number CFU mL^{-1} of *Fno* STIR-GUS-F2f7 in 100 µL spreaded per dilution on CHAH containing different antibiotics (*e.g.* ampicillin, penicillin, colistin and amphotericin B), concentrations and dilutions, and compared with normal CHAH without antibiotic. Bars represent the mean of 4 plates+SD) (n=4).

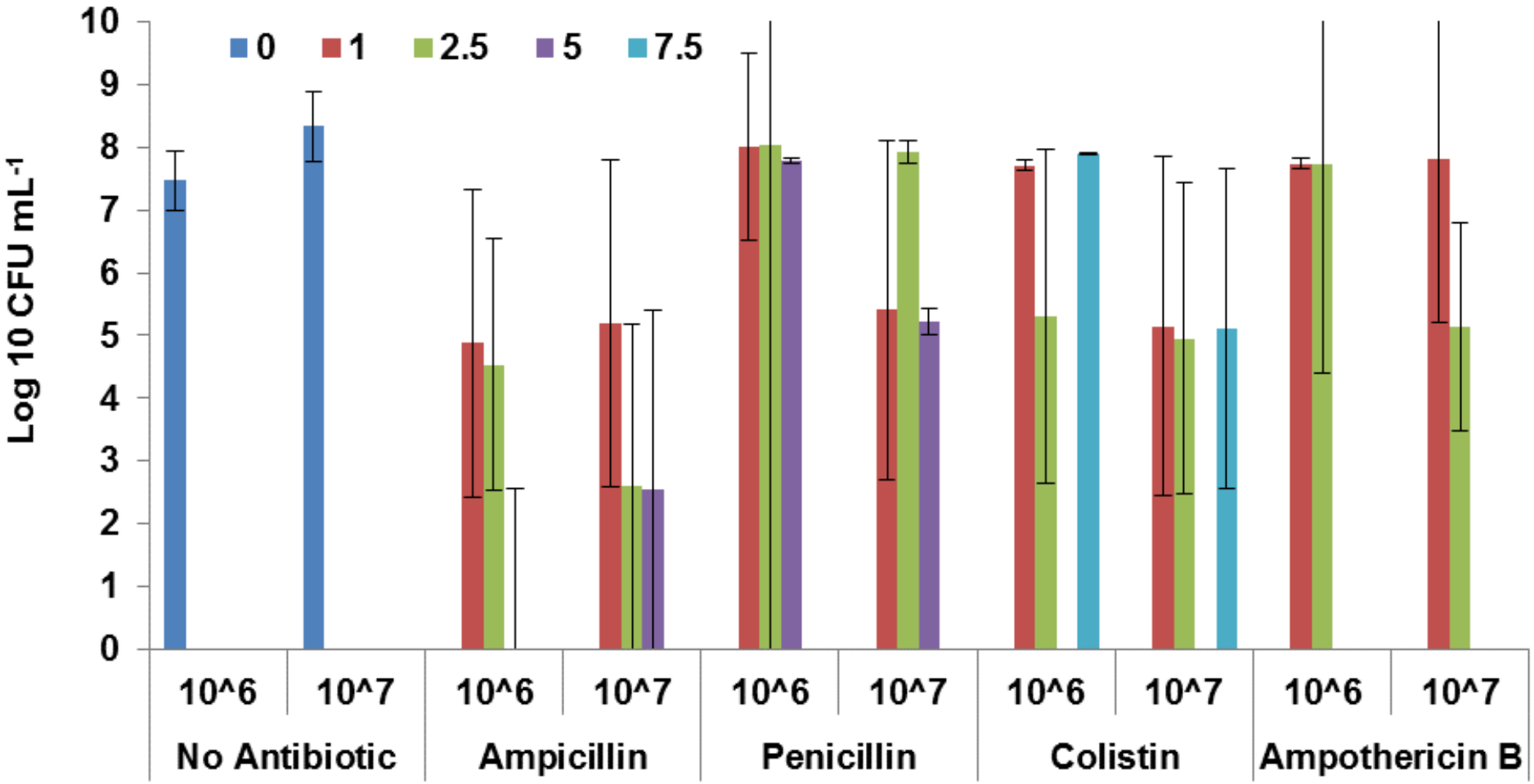

Figure 5.32b Log number CFU mL^{-1} of *Fno* Japan in 100 µL spreaded per dilution CHAH containing different antibiotics (*e.g.* ampicillin, penicillin, colistin and amphotericin B), concentrations and dilutions, and compare with normal CHAH without antibiotics. Bars represent the mean of 4 plates+SD) (n=4).