UNDERSTANDING THE FISH PATHOGEN *Flavobacterium psychrophilum* DIVERSITY FOR THE CONTROL OF RAINBOW TROUT FRY SYNDROME IN THE UNITED KINGDOM

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Declaration

This thesis has been composed in its entirety by me, 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.

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Abbreviations

AMOX	Amoxicillin
$AMOX_{10}$	10 µg Amoxicillin,
AMP	Ampicillin
AS	Atlantic salmon
BCWD	Bacterial cold water disease
bp	Base pairs
BSA	Bovine serum albumin
CAMH	Cation-adjusted Mueller-Hinton
cDNA	Complementary DNA
CFU	Colony-forming units
CLSI	Clinical and Laboratory Standards Institute
CO_{WT}	Epidemiological cut-off values
CS	Coho salmon
C3	Complement factor 3
DNA	Deoxyribonucleic acid
DPI	Day post immunisation
DW	Distilled water
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENRO	Enrofloxacin
$ENRO_5$	5 µg Enrofloxacin
ERY	Erythromycin
FCA	Freund's complete adjuvant
FCS	Foetal calf serum
FFN	Florfenicol
FFN_{30}	30 µg Florfenicol
FKC	Formalin-killed bacterial cells
FLUQ	Flumequine
HSW	High salt wash
H_2O_2	Hydrogen peroxide
IFN	Interferon

IgM	Immunoglobulin M
IgT	Immunoglobulin T
IL-1β	Interleukin 1β
i.m.	Intramuscular
i.p.	Intraperitoneal
kb	Kilobase pairs
LPS	Lipopolysaccharide
LSW	Low salt wash buffer
MH	Mueller Hinton
MIC	Minimal inhibitory concentration
MLST	Multilocus sequence typing
MV	Modified Veggietone medium
NaCl	Sodium chloride
NRI	Normalised resistance interpretation
NWT	Non-wild type
OD	Optical density
OTC	Oxytertracycline
OTC_{30}	30 µg Oxytetracycline
OXO	Oxolinic acid
OXO_2	2 µg Oxolinic acid
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PFGE	Pulsed-field gel electrophoresis
PRI	Ormetoprim/sulphadimethoxine (1/19)
rep-PCR	Repetitive polymerase chain reaction
REST®	Relative Expression Software Tool
RNA	Ribonucleic acid
RPS	Relative percentage survival
RSD	Relative standard deviation
RSMT	Red spotted masu trout
RTFS	Rainbow trout fry syndrome
RT	Rainbow trout
RT-qPCR	Real-time quantitative PCR
SAA	Acute serum amyloid A

s.c.	Subcutaneous
SE	Standard error
STE	Sodium chloride-Tris-EDTA buffer
SXT	Trimethoprim/sulphamethoxazole (1/19)
SXT_{25}	25 µg Trimethoprim/sulphamethoxazole (1/19)
TAE	Tris–Acetic–EDTA
TBE	Tris–Borate–EDTA
TE	Tris-EDTA
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TLR2	Toll-like receptor 2
TSA	Tryptone soy agar
TSI	Target specific ingestion of single phagocyte
TYES	Tryptone yeast extract salts
UK	United Kingdom
USA	United States of America
WT	Wild type

Abstract

Rainbow trout represents the most prominent species in freshwater farming in UK aquaculture. One of the common diseases constraining rainbow trout production and increasingly causing problems in Atlantic salmon (*Salmo salar* L.) hatcheries worldwide is rainbow trout fry syndrome (RTFS) or bacterial cold water disease (BCWD). During the last 20 years, the development of a commercial vaccine against RTFS has been hindered by the prevalence of a wide range of the fish pathogen *F. psychrophilum*, thus the current treatment of choice is the use of antibiotics. Studies involved in understanding the innate and adaptive immune response of vaccinated rainbow trout fry using inactivated whole cell are still lacking. Therefore, the aim of this thesis is to characterise the strain diversity and antibiotic susceptibility of UK *F. psychrophilum* isolates, evaluate the efficacy of a whole-cell formalin-killed polyvalent vaccine, which was developed based on the characterisation results of this study, and investigate the immune response in trout fry following the immersion vaccination via the changes in expression of relevant immune genes.

A total of 315 *F. psychrophilum* isolates, 293 of which were collected within the UK, were characterised using four genotyping methods and a serotyping scheme. A high strain diversity was identified among the isolates with 54 pulsotypes, ten $(GTG)_5$-PCR types, two 16S rRNA allele lineages, seven plasmid profiles and three serotypes. The predominant profile observed within the *F. psychrophilum* isolates examined was PFGE cluster II – $(GTG)_5$-PCR type r1 – 16S rRNA lineage II – serotype Th (n= 70/156, 45%). The characterisation results not only revealed the wide distribution within the UK and the persistence within a site of predominant pulsotypes, but also the presence of unique genotypes in certain sites or countries. Co-existence of genetically and serologically heterogeneous isolates within each farm was detected, highlighting the reasons this disease is so difficult to control, especially by vaccination. The occurrence over time of *F. psychrophilum* pulsotypes within a site could provide important epidemiological data for farm management and the development of site-specific vaccines.

The antimicrobial susceptibilities of 140 *F. psychrophilum* strains, 125 of which were from the UK, were evaluated by the broth microdilution (MIC) and disc diffusion methods. There was evidence of reduced susceptibilities to three of the main antimicrobials used in UK aquaculture. Broth microdilution testing showed that only 12%

of 118 UK isolates tested were WT to oxolinic acid (MIC CO_{WT} ≤0.25 mg L^{-1}), 42% were WT for oxytetracycline (MIC CO_{WT} ≤0.25 mg L^{-1}), and 66% were WT for amoxicillin. In contrast, all the isolates tested were WT (MIC CO_{WT} ≤2 mg L^{-1}) for florfenicol, the antimicrobial of choice for RTFS control in the UK. Despite the imprecision of disc diffusion-based CO_{WT} values due to high standard deviations, there was a high categorical agreement between the classification of the strains (into WT or NWT) by MIC and disc diffusion methods for florfenicol (100%), oxolinic acid (99%), amoxicillin (97%) and oxytetracycline (94%). In general, this study showed that the UK *F. psychrophilum* isolates examined remain susceptible to florfenicol and also stresses the importance of performing susceptibility testing using standardised methods and CO_{WT} values. Several statistically significant associations between genotypes and the reduced susceptibilities of *F. psychrophilum* strains were revealed.

A whole-cell formalin killed polyvalent vaccine against RTFS/BCWD was developed by combining three genetically and serologically divergent strains, recently collected from UK farms. The efficacy of this polyvalent vaccine was evaluated after immersion vaccination in 5 g trout and bath challenge using hydrogen peroxide as a pre-stressor with a virulent heterologous isolate of *F. psychrophilum* strain. Significant protection was achieved with an RPS of 84%. The combination of exposure to hydrogen peroxide prior to bath challenge may be an alternative to an injection challenge with 12 g trout, although further standardisation and optimisation of the challenge model is required. Changes in the innate immune response of trout fry following the initial vaccination included the up-regulation of the *interleukin 1 β* (*IL-1β*) gene in head kidney at 4 h and the up-regulation of *toll-like receptor-2* (*TLR-2*) in skin at day 2. While the expression levels of *C3* was unchanged, the down regulation of *CD8-α* in head kidney and spleen and *CD4-1* in spleen were documented. *IgM* and *IgT* transcripts were found to be up-regulated in hind-gut two days post-vaccination.

Understanding the strain diversity and the antibiotic susceptibility of UK *F. psychrophilum* isolates could help improve the control strategies, such as preventing the spreading of pathogenic *F. psychrophilum* clones between fish farms, reducing the use of antibiotics in RTFS/BCWD treatment and monitoring the development of acquired antibiotic resistance mechanisms. Moreover, strain characterisation data of UK *F. psychrophilum* species has assisted in selecting suitable candidates for developing an effective RTFS vaccine.

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Chapter 1

General Introduction

1.1.Overview of rainbow trout production worldwide and within the UK

1.1.1. Rainbow trout production

Aquaculture is one of the fastest-growing food-producing sectors in the world and one of the most important sources of high quality proteins for human consumption. Aquaculture production for the intended use as human food has risen from 32.4 million tonnes in 2000 to 73.8 million tonnes in 2014, amounting to a value of US $ 160 billion (Tidwell and Allan, 2001; FAO, 2016a).

Salmonidae are one of the most important groups of fish farmed in aquaculture (FAO, 2016a) and are classed as the major animal food producing species in Europe, with rainbow trout representing the most prominent species in freshwater farming (Rodgers and Furones, 2009; FAO, 2016a). In Europe, production of farmed rainbow trout contributed the largest portion (294,615 tonnes, 36%) of world production in 2014 (Figure 1.1), amounting to a value of US $ 1.34 billion (FAO, 2016a).

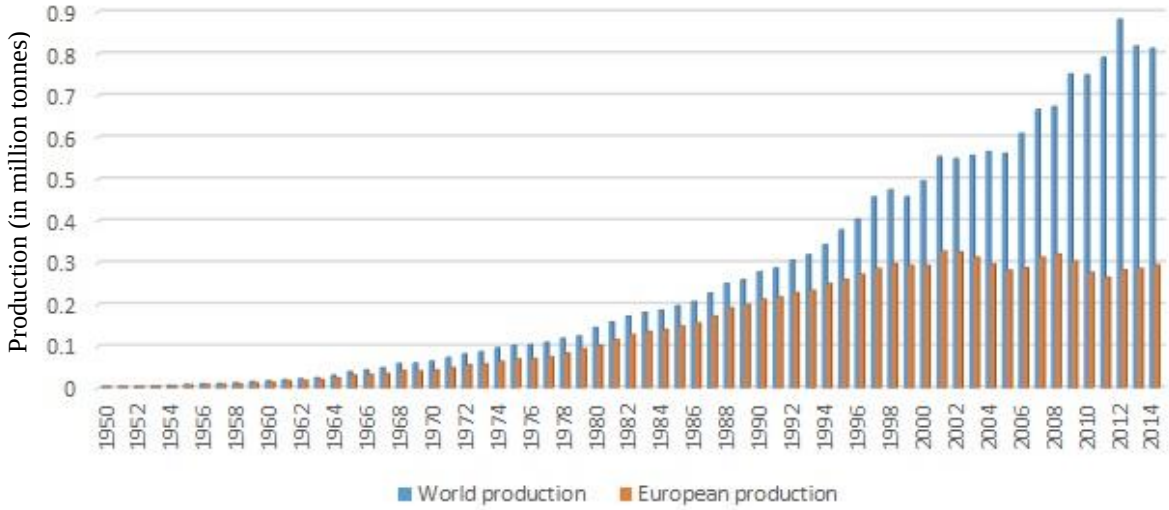


Figure 1.1. Aquaculture production for rainbow trout (*Oncorhychus mykiss*) from 1950 to 2014 in the world and Europe (FAO, 2016a).

Trout has been farmed widely in the UK, but particularly in central and southern Scotland, the South of England and North Yorkshire, since it was first introduced in the early 1970s by a Danish entrepreneur (Nepal *et al.*, 2002; British Trout Association, 2016). In 2012, UK rainbow trout production was 14,590 tonnes, with 8,109 tonnes in England, 5,670 tonnes in Scotland, 563 tonnes in Northern Ireland and 248 tonnes in Wales (Ellis *et al.*, 2015). The production of farmed rainbow trout in the UK has increased

7.7-fold since 1980 (from 1,927 tonnes to 14,800 tonnes in 2014) and has been around 14,000 tonnes annually over the last three decades (FAO, 2016a).

1.1.2. Rainbow trout

Rainbow trout (*Oncorhychus mykiss*) is a highly commercial fish species for both market and sport trade. In the wild, adult rainbow trout have normal weights of 2 – 3 kg and can reach a maximum size, weight and age of 120 cm total length, 25.4 kg and 11 years respectively (Woynarovich *et al.*, 2011). Farmed rainbow trout are hatched from eggs and raised in inland freshwater facilities with various production options (Figure 1.2) until they reach the desired size for the table trade, which usually varies between 250 g to >900 g (Munro and Wallace, 2015).

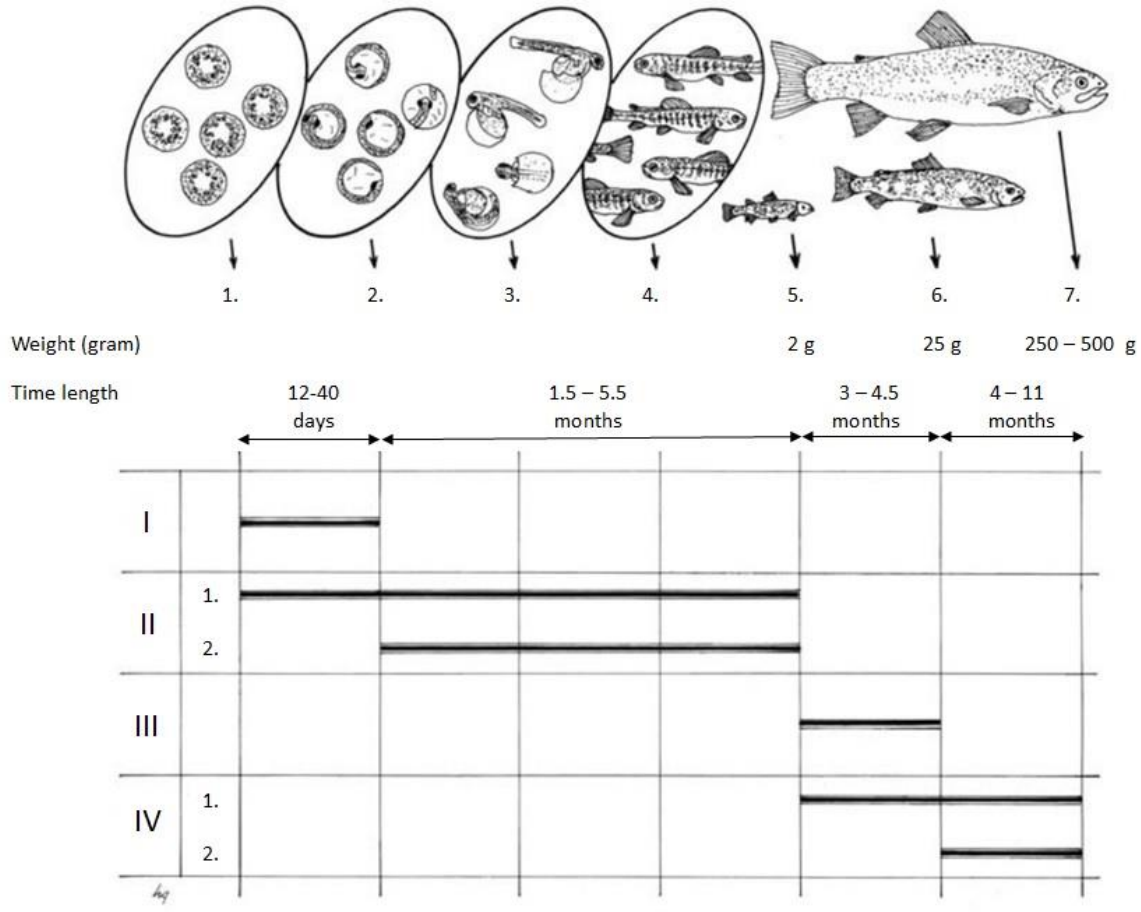


Figure 1.2. Four different production options for rainbow trout farming (Woynarovich *et al.*, 2011).

Development stages: **1.** Fertilised eggs; **2.** Eyed eggs; **3.** Hatched sac fry; **4.** Swim-up fry; **5.** Fry; **6.** Fingerlings; **7.** Table fish

Phase of production: **I.** Eyed egg production; **II.** Fry production; **III.** Fingerling production; **IV.** Table fish production

Rainbow trout can be affected by a variety of diseases caused by bacteria (e.g. furunculousis, enteric red-mouth disease, columnaris disease, Flavobacterium septicaemia), viruses (e.g. viral haemorrhagic septicaemia, infectious pancreatic necrosis, infectious haematopoietic necrosis), fungi (e.g. saprolegniasis) and parasites (e.g. whirling disease caused by protozoa) (Woynarovich *et al.*, 2011; FAO, 2016b). A recent survey with the participation of producers, vets and health professionals in the UK confirmed that rainbow trout fry syndrome (RTFS) caused by the bacterium *Flavobacterium psychrophilum* is one of the key diseases that are constraining current rainbow trout production (Verner-Jeffreys and Taylor, 2015).

1.2. Rainbow trout fry syndrome

Rainbow trout fry syndrome (RTFS) or bacterial cold water disease (BCWD) is a serious septicaemic condition of rainbow trout (*Oncorhynchus mykiss*) fry and fingerlings over a weight range of 0.2 to 10.0 g at water temperatures below 10^{o}C (Faruk, 2002; Nematollahi *et al.*, 2003a). Many different names have been given to this disease, such as peduncle disease, saddleback disease and BCWD in the USA; a visceral form of BCWD in France; fry mortality syndrome in Denmark; visceral myxobacteriosis in Italy and rainbow trout fry anaemia or RTFS in the UK (Holt., 1993; Rangdale, 1995; Nematollahi *et al.*, 2003a; Cipriano and Holt, 2005).

This disease is now considered as one of the main threats to the rainbow trout culture industry worldwide and particularly in Europe. In fry and fingerlings, a mortality rate up to 90% is typical of an acute systemic bacterial infection, while in larger and older fish, the infection causes a more chronic form of the disease and causes lower mortality (Barnes and Brown, 2011; Nilsen *et al.*, 2011a). There are a number of reasons for the differences in fish mortality rates and water temperature is considered as the key factor. Normally disease occurs from 4°C to 10°C, but at 15°C the disease can become a severe epizootic. However, water temperatures from 16^{o}C to 21^{o}C are typical in BCWD outbreaks in Japanese ayu *Plecoglossus altivelis altivelis* (Barnes and Brown, 2011).

1.2.1. Typical pathologies and clinical disease signs

After first being described in 1946 in USA, disease associated with *F. psychrophilum* infection has been reported worldwide, with diseased fish showing characteristic behaviour and clinical signs, including lethargy, cessation of feeding, and eroded fin tip

in the early stage of RTFS; and bilateral exophthalmia, pale gills and swollen abdomen in the later stages of the disease. In late-stage infection, lesions can be noted on the lateral sides, snout-jaw region, musculature between dorsal fin and back of the head; necrosis at the site of bacterial colonisation may progress until the vertebral column is exposed (Figure 1.3) (Starliper and Schill, 2011). Additional clinical signs have also been reported, such as anaemia, hyperpigmentation of the skin, enlarged spleen and haemorrhage on the gills, liver and kidney during acute RTFS; while spiral or erratic swimming behavior, blackened caudal region and spinal deformation are evident during chronic disease episodes (Kent *et al.*, 1989; Faruk, 2002; Nematollahi *et al.*, 2003a; Buller, 2004; Noga, 2010; Starliper and Schill, 2011).

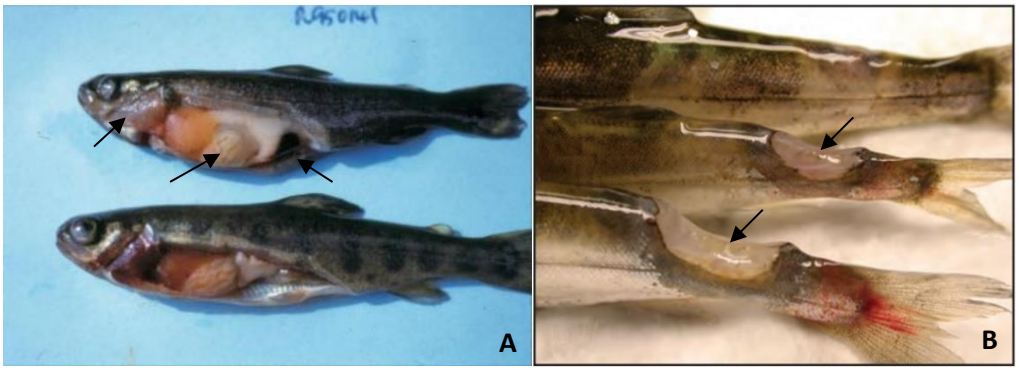


Figure 1.3. Rainbow trout fry syndrome caused by *Flavobacterium psychrophilum*. (A) In European hatcheries, the upper fish (infected) shows pale gills and liver, and enlarged spleen, compared to lower fish (control) (Robert, 2012). (B) Deep and scooping ulcers characteristic of severe bacterial colonisation in BCWD on coho salmon fingerlings (Meyers *et al.*, 2008).

1.2.2. Typical histopathological changes

Histological examination in naturally and experimentally infected fry reveals focal necrosis in spleen, liver, and kidney; cellular degeneration; splenic hypertrophy; increased eosinophilia and haemosiderin in the kidney; necrosis, pyknosis and lymphocytic infiltration of the dermis and underlying muscle block (Faruk, 2002; Nematollahi *et al.*, 2003a; Starliper and Schill, 2011). Chronic forms of BCWD may manifest as necrotic scleritis and cephalic osteochondritis; periostitis, osteitis, meningitis, and periosteal proliferation of vertebrae at the junction of the vertebral column and cranium (Kent *et al.*, 1989; Starliper and Schill, 2011).

1.2.3. Aetiology

Buller (2004) noted that a number of bacteria including *F. columnare*, *Janthinobacterium* spp., *Micrococcus luteus* and *Planococcus* spp. have been associated with RTFS presentation. This led to speculation that the establishment and mortality level of RTFS/BCWD might be influenced by the presence of other non-*F. psychrophilum* pathogens. However, based on many epizootiological analyses, *F. psychrophilum* is likely to be the causative agent of RTFS as the prevalence of this bacterium is considerably higher than that of any other organisms detected in RTFS outbreaks (Noga, 2010; Starliper and Schill, 2011). The bacterium may also be cultured from internal organs like brain, kidney, liver, spleen and heart of diseased fish (Starliper and Schill, 2011).

1.2.4. Treatment

To control *F. psychrophilum* infection, the use of antibiotics is currently the treatment of choice (Smith *et al.*, 2016) as no commercial vaccines are available. The combination of antibiotics with other therapeutic chemicals has also been tried, such as florfenicol-medicated diets and hydrogen peroxide baths (Gultepe and Tanrikul, 2006). However, the use of antibiotics for prophylactic purposes or indiscriminate antimicrobial therapy can lead to the emergence and selection of *F. psychrophilum* strains with antibiotic resistance mechanisms. After every treatment, antibiotics kill sensitive bacteria, but resistant germs may be left to grow and increase in the number (Starliper and Schill, 2011; Sundell *et al.*, 2013).

Instead of using antibiotics, warm water treatment has been demonstrated as a possible cure for BCWD in ayu (*Plecoglossus altivelis altivelis*). Fish were treated with water warmed at 28ºC for 3 days, starting 24 h after immersion in a live bacterial suspension of *F. psychrophilum* (10^7 CFU mL^{-1}) for 30 minutes. This was shown to lead to a lower cumulative mortality (18%), compared to the control fish maintained at 18ºC (90%) (Sugahara and Eguchi, 2012).

With the successful identification of several *F. psychrophilum* bacteriophages, phage therapy against *F. psychrophilum* has shown promise *in vitro* (Stenholm *et al.*, 2008; Kim *et al.*, 2010a; Castillo *et al.*, 2012). The use of probiotic bacteria has also been suggested to be a promising alternative to the use of antibiotics in aquaculture (Ström-Besto and Wiklund, 2011; Korkea-aho *et al.*, 2011; Boutin *et al.*, 2012; Burbank *et al.*, 2012).

1.2.5. Prevention and control

In the absence of approved, cost-effective vaccination methods, egg disinfection is one of the main preventive measures against RTFS/BCWD. Povidone–iodine is routinely used in surface disinfection of fertilised and eyed eggs (Cipriano and Holt, 2005; Starliper and Schill, 2011). However, povidone–iodine was found not to be effective to completely eliminate *F. psychrophilum* from eggs (Brown *et al.*, 1997; Kumagai *et al.*, 1998). Disinfection of the egg surface by hydrogen peroxide or glutaraldehyde has also been reported (Cipriano and Holt, 2005).

Maintenance of optimum water quality is considered to be an important RTFS/BCWD preventive measure (Taylor, 2004; Barnes and Brown, 2011). Water used for maintaining eggs and emergent fry should be filtered and ultraviolet-irradiated or ozonated in order to eliminate any potential pathogens (Hedrick *et al.*, 2000; Cipriano and Holt, 2005). Controlling organic loads and the nitrite concentration in farm water may also help reduce *F. psychrophilum* infectivity (Nematollahi *et al.*, 2003b).

Fish experiencing stress can be prone to health problems (Barnes and Brown, 2011). Stress has been shown to be associated with fish culture practices (e.g. high rearing densities, physical handling during grading, vaccination and transport). Physical handling not only can cause immunosuppression, but also may lead to cutaneous lesions, providing the ideal point-of-entry for a pathogen (Cipriano and Holt, 2005). Fish food should be used and stored properly to maintain its high quality, as low quality diets (e.g. high oxidised lipid concentrations) can elevate rainbow trout mortality after *F. psychrophilum* challenge (Daskalov *et al.*, 2000). In addition, good fish husbandry can minimise the introduction and transmission of pathogens, such as by routinely disinfecting the equipment and tools used in fish production, regularly examining fish health, quarantining infected fish and applying appropriate treatments (Cipriano and Holt, 2005; Starliper and Schill, 2011).

The use of *F. psychrophilum*-resistant fish lines may also help reduce RTFS/BCWD related mortalities (Henryon *et al.*, 2005; Vallejo *et al.*, 2010; Langevin *et al.*, 2012; Weber *et al.*, 2013). Rainbow trout is able to express additive genetic variation for resistance to RTFS, allowing selective breeding to establish RTFS-resistance fish lines (Henryon *et al.*, 2005).

1.3. *Flavobacterium psychrophilum*

1.3.1. Historical background

F. psychrophilum was first isolated in 1948 from diseased juvenile coho salmon, *Oncorhynchus kisutch* in Washington, USA (Borg, 1948; Nematollahi *et al.*, 2003a; Cipriano and Holt, 2005). This bacterium was successfully cultivated in a nutrient-dilute medium, reproduced the disease and fulfilled Koch's postulates (Cipriano and Holt, 2005). Since then, the causal relationship between *F. psychrophilum* and BCWD/RTFS has been established.

1.3.2. Epizootiology

Although juvenile rainbow trout and coho salmon are particularly susceptible to RTFS/BCWD, *F. psychrophilum* infection can affect all species of salmonid fish (Holt, 1993). This bacterium has been reported to cause disease in sockeye salmon (*Oncorhynchus nerka*), chinook salmon (*Oncorhynchus tshawytscha*), chum salmon (*Oncorhynchus keta*), masou salmon (*Oncorhynchus masou*), lake trout (*Salvelinus namaycush*), brook trout (*Salvelinus fontinalis*), and cutthroat trout (*Oncorhynchus clarki*) (Holt, 1993; Amita *et al.*, 2000; Nematollahi *et al.*, 2003a).

F. psychrophilum can also present in non-salmonid fish, such as Japanese eel (*Anguilla japonica*), European eel (*Anguilla anguilla*), common carp (*Cyrpinus carpio*), crucian carp (*Carassius carassius*), tench (*Tinca tinca*), ayu (*Plecoglossus altivelis altivelis*), pale chub (*Zaco platypus*), perch (*Perca fluviatilis*), and roach (*Rutilis rutilis*) (Lehmann *et al.*, 1991; Wakabayashi *et al.*, 1994; Liu *et al*., 2001; Nematollahi *et al.*, 2003a). These findings suggest that various fish species may be asymptomatic carriers and become reservoirs of *F. psychrophilum* infection for other more susceptible salmonid species (Nematollahi *et al.*, 2003a; Cipriano and Holt, 2005, Barnes and Brown, 2011).

Originally, the geographical distribution of *F. psychrophilum* was thought to be limited to North America, but currently *F. psychrophilum* is considered as a worldwide pathogen occurring in freshwater aquaculture throughout North America, Chile, Peru, Turkey, Japan, Korea, Australia and many countries in Europe, such as Germany from 1987 (reviewed in Nematollahi *et al.*, 2003a), France from 1988 (Bernardet *et al.*, 1988), Denmark from 1991 (Lorenzen *et al*., 1991), the UK from 1992 (Santos *et al.*, 1992), Italy from 1992 (reviewed in Nematollahi *et al.*, 2003a), Spain from 1993 (Toranzo and Baria, 1993) and Finland from 1994.

1.3.3. Taxonomy

The nomenclature of the pathogen causing RTFS/BCWD has been changed several times due to the confusion and controversy associated with the taxonomy of the yellow-pigmented gliding bacteria since the pioneering work of Borg in 1948. Based on biochemical characteristics, this bacterial pathogen was initially thought to be a myxobacterium and named *Cytophaga psychrophila* (Borg, 1948; Pacha, 1968; Nematollahi *et al.*, 2003a; Cipriano and Holt, 2005). Leadbetter (1974) realised that such classification was inappropriate as this pathogen did not produce fruiting bodies or degrade complex polysaccharides and suggested that the species belonged within the genus *Flexibacter* of the family Cytophagaceae (Cipriano and Holt, 2005). In 1989, based on phenotypic characterisations and the reorganisation of the entire Cytophaga-Flexibacter-Flavobacterium phylogenetic branch, Bernardet and Grimont (1989) provisionally reclassified this bacterium as *Flexibacter psychrophilus* (Nematollahi *et al.*, 2003a; Cipriano and Holt, 2005). However, some inconsistencies of this reclassification were observed, such as DNA composition (32 – 33 mol % G + C) and the poor gliding ability of BCWD isolates in comparison with other fish pathogenic members of the Order Cytophagales noted by Bernardet and Keronuault (1989). Finally, the description of the Flavobacterium genus was later amended by Bernardet *et al.* (1996) and included all the BCWD isolates, taxonomically identified as *Flavobacterium psychrophilum* (Cipriano and Holt, 2005).

1.3.4. Morphology and biochemical characteristics

1.3.4.1. Cell morphology

F. psychrophilum cells are Gram-negative, strictly aerobic, weakly refractile, flexible and slender rods with rounded ends (Faruk, 2002; Nematollahi *et al.*, 2003a). Actively growing cells of *F. psychrophilum* are 0.3 – 0.75 µm in diameter and 2 – 7 µm in length depending on the conditions of the culture media. A few cells may be attached end-to-end and consequently appear in filamentous forms, 10 – 40 µm long. In older culture, cells have pleomorphic forms, and can appear involuted, branched or thickened at the ends. No microcysts or fruiting bodies are formed. This bacterium does not have pili and has smaller cell dimensions (0.3 x 2.0-2.5 µm) in the lesion of naturally infected fish compared to growth in culture media (Holt, 1993).

On specialised agar, *F. psychrophilum* produces bright yellow, smooth and glossy colonies with the diameter of 1 – 5 mm, a slightly raised center and thin spreading

margins (Barnes and Brown, 2011). Like *F. columnare*, these colonies do not adhere to the medium surface (Faruk, 2002; Starliper and Schill, 2011). Two different colony phenotypes of *F. psychrophilum* can be collected from diseased fish, described as yellow with regular edges (smooth colonies) and yellow with spreading margins (rough colonies) (Figure 1.4) (Pacha, 1968, Bernardet and Kerouault, 1989; Högfors-Rönnholm and Wiklund, 2010). The irreversible phase variation from the smooth to rough phenotypes has been reported (Högfors-Rönnholm and Wiklund, 2010).

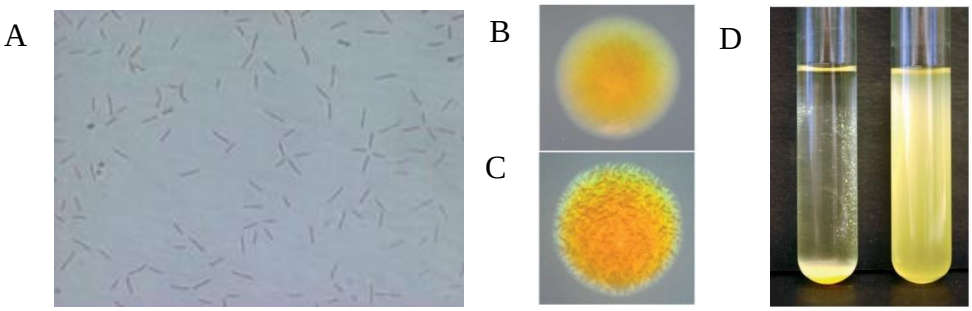


Figure 1.4. *Flavobacterium psychrophilum*. (A) Gram-stained *F. psychrophilum* cells using a 100X objective len. (B) Smooth colony and (C) Rough colony on tryptone yeast extract salts (TYES) agar. (D) Autoagglutinated smooth phenotype cells at the bottom of the tube (left), and a non-agglutinating and uniform growth mode of the rough colony type cells (right) in TYES broth (Högfors-Rönnholm and Wiklund, 2010).

1.3.4.2. *In vitro* cultivation

An optimised medium formulation, incubation temperature and gentle handling, which avoids strong stirring, vortexing and centrifugation, are critical to improve the overall viability and growth of *F. psychrophilum* cells *in vitro* (Michel *et al.*, 1999; Faruk, 2002).

The species *F. psychrophilum* is not able to grow or grows poorly on high nutrient concentration media, such as brain heart infusion agar, tryptone soy agar (TSA), triple sugar iron agar and blood agar (Nematollahi *et al.*, 2003a). Some authors have observed weak growth of *F. psychrophilum* isolates on TSA (Bustos *et al*., 1995; Lumsden *et al*., 1996), while others were unable to demonstrate any growth (Santos *et al*., 1992; Brown *et al.*, 1997; Ekman *et al.*, 1999). The bacterium prefers reduced nutrient concentration media, such as Cytophaga (CA) medium, also called Anacker and Ordal medium (Starliper and Schill, 2011) and Tryptone Yeast Extract Salts - TYES (Antaya, 2008). Oplinger and Wagner (2012) concluded that CA medium provided better growth of *F. psychrophilum* than TYES.

Some authors have attempted to modify the medium formulation in order to improve the recovery and the growth of *F. psychrophilum* from infected fish. There have been many modifications in the CA medium formation, such as the addition of new born calf serum (Dalsgaard, 1993; Brown *et al.*, 1997), foetal calf serum (Obach and Baudin Laurencin, 1991; Michel *et al.*, 1999), horse serum (Oplinger and Wagner, 2012), calf/fish blood cells (Lorenzen *et al.*, 1997), sugars and skimmed milk (Daskalov *et al.*, 1999; Cepeda *et al.*, 2004; Oplinger and Wagner, 2012), antibiotics (Kumagai *et al.*, 2004), and activated charcoal (Álvarez and Guijarro, 2007); or alteration of the medium components by increasing concentrations of tryptone and the beef extract (Lorenzen, 1993; Rangdale *et al.*, 1997).

Possibly due to the strain variation, a discrepancy in the sodium chloride (NaCl) concentration range suitable for *F. psychrophilum* growth has been reported. Pacha (1968) observed growth in 0.8% NaCl but not at concentrations greater than 2%, while Bernardet and Kerouault (1989) noted no growth at concentrations greater than 0.5%. In another study, at a salinity of 3%, the number of culturable *F. psychrophilum* cells reduced below the detection limit after one day, while at a salinity of 0.6%, the number of culturable bacterial cells reduced but then recovered (Madetoja *et al.*, 2003).

Flavobacterium psychrophilum can grow in temperatures ranging from 4 to 23^{o}C. However, the optimum incubation requirements for the growth of *F. psychrophilum* are 15 – 20^{o}C and 48 – 96 h (Faruk, 2002; Starliper and Schill, 2011). This bacterium can achieve an optimum generation time of 2 h at 15^{o}C (Holt, 1993).

1.3.4.3. Gliding ability

The gliding ability of *F. psychrophilum* is the active movement over surfaces without the aid of flagella. Compared with other fish pathogens such as *F. columnare* and *F. maritimus*, the gliding movement of *F. psychrophilum* is so slow and weak that it is difficult to observe, and is only apparent after prolonged observation (Bernadet and Kerouault, 1989; Schmidtke and Carson, 1995; Lorenzen *et al.*, 1997). Because this bacterium has no pili, the mechanism of this gliding movement is unknown (Faruk, 2002; Nematollahi *et al.*, 2003a). Thiol oxidoreductase-like protein (tlpB) is associated with the gliding ability of *F. psychrophilum* as *tlpB*-interrupted *F. psychrophilum* mutant was deficient in this motility and then restored this ability after the complementation with the *tlpB* loci (Álvarez *et al.*, 2006).

This gliding motion is strongly influenced by nutrient concentrations in the culture media. As the nutrient depletion increases, spreading is favoured and the diameter of the colonies is broader (Pérez-Pascual *et al.*, 2009). The gliding motility of *F. psychrophilum* is also suggested to be involved in the erosion pattern, which has the shape of tubular boreholes, in the fins of Atlantic salmon (*Salmo salar*) (Martínez *et al.*, 2004).

1.3.4.4. Biochemical characteristics

Flavobacterium psychrophilum is weakly reactive in biochemical tests (Barnes and Brown, 2011). The bacterium is unable to use simple and complex carbohydrates, such as cellulose, starch, aesculin, chitin, xanthine, lysine and ornithine (Bernardet and Kerouault, 1989; Nematollahi *et al.*, 2003a). However, this bacterium is actively proteolytic with a capacity to degrade collagen, fibrinogen, gelatin, casein, tyrosine, albumin, fish muscle extract and litmus milk (Ostland *et al.*, 2000; Nematollahi *et al.*, 2003a). *F. psychrophilum* has also been shown to have weak catalase and oxidase activity, but does not produce hydrogen sulphide, indole and lysine and ornithine decarboxylase (Nematollahi *et al.*, 2003a). Most *F. psychrophilum* isolates produce flexirubin-like pigments. There are conflicting reports on elastin hydrolysis, nitrate reduction, tributyrin degradation and chondroitin sulphate lysis by the bacterium (Nematollahi *et al.*, 2003a; Starliper and Schill, 2011). The variability seen in the results of these tests could be attributed to differences in isolate origin, or the methods employed such as the variability of medium formulation or medium supplement concentration (Pacha, 1968; Starliper and Schill, 2011).

1.3.5. Serological characterisation

There have been several reports on the serotypes of *F. psychrophilum*; however, it is difficult to compare the results between the studies due to the various techniques involved and data interpretation (Faruk *et al.*, 2002). Pacha (1968) developed rabbit antisera against three *C. psychrophila* strains isolated during different outbreaks from eight hatcheries and observed a strong serological relatedness of 10 isolates examined and no cross-reaction of these antisera with 24 non-pathogenic cytophagas associated with fish. Lorenzen and Olesen (1997) used an enzyme-linked immunosorbent assay (ELISA) and slide agglutination to analyse the serological differentiation among 45 *F. psychrophilum* isolates from Denmark during 1990 – 1993 and other European countries during 1986 –

1994. Three main serotypes were found: serotype "Th", including a major subtype Th-1 and a minor subtype Th-2, was dominant and represented most of isolates (27/38 isolates); serotype "Fd" contained a few isolates (8/38); and serotype "Fp^T", defined by the type strain *F. psychrophilum* NCIMB 1947^T, comprised of isolates (3/38) not obtained from clinical BCWD or RTFS situations. Subsequently, Izumi and Wakabayashi (1999) reported three host-dependent serotypes among 112 *F. psychrophilum* isolates from Japan, USA and Europe. Serotype O-1 contained 5 isolates from infected coho salmon from Japan and the USA, serotype O-2 consisted of 42 isolates from ayu and serotype O-3 included 32 isolates from rainbow trout (Izumi and Wakabayashi, 1999). In addition, Mata *et al.* (2002) identified seven host-specific serovars among 34 *F. psychrophilum* isolates from six fish species and seven countries by both slide agglutination and biotin-avidin ELISA. Serovar 1 was found only in isolates originating from salmon (four isolates); serovar 2 and 3 in 24 rainbow trout isolates; serovar 4, 5 and 6 in isolates from eel, carp and tench respectively (one isolate per serovar); and serovar 7 probably unique for ayu isolates (three isolates) (Mata *et al.*, 2002). There have also been attempts to harmonise the three serotyping schemes and identify additional serotypes (Mata *et al* 2002, Izumi *et al* 2003a) (Table 1.1). To date, the serotyping schemes established by Lorenzen and Olesen (1997) and by Izumi and Wakabayashi (1999) are most commonly used to serotype *F. psychrophilum* isolates (Mata *et al.* 2002).

Table 1.1. Serotypes of *F. psychrophilum* based upon thermostabile antigens

Fish host	Serotyping scheme		
	Lorenzen and Olesen (1997)	Izumi and Wakabayashi (1999)	Mata *et al.* (2002)
Salmon	Fp^T	O-1	1
Trout	Fd	-	2a
Trout	Th-2	-	2b
Trout	Th-1	O-3	3
Eel	-	-	4
Carp	-	-	5
Tench	-	-	6
Ayu	-	O-2	7

Madetoja *et al.* (2001) reported five of 37 Finnish isolates from rainbow trout, sea trout and brook trout possessed a serotype different from those previously described by Lorenzen and Olesen (2007). Moreover, a novel serotype (O-4) of *F. psychrophilum* was recorded in isolates from amago, *Oncorhynchus masou rhodurus* Jordan & Gilbert, in Japan (Izumi *et al.*, 2003a). Therefore, the occurrence of untypeable *F. psychrophilum* isolates still remains (Lorenzen and Olesen, 1997; Izumi and Wakabayashi, 1999; Izumi *et al*., 2003a).

The correlation of serotypes with any specific strain characteristic or virulence level of *F. psychrophilum* has been investigated in several studies. The predominant serotypes Th and Fd were found in the majority of isolates collected from rainbow trout having clinical signs of RTFS/BCWD (Lorenzen and Olesen, 1997; Madsen and Dalsgaard, 1999; Dalsgaard and Madsen, 2000), while isolates belonging to serotype Fp^T were speculated to be more pathogenic for coho salmon than for rainbow trout (Toranzo and Barja, 1993; Lorenzen and Olesen, 1997). Madetoja *et al.* (2000, 2001) reported the simultaneous presence of serologically heterogeneous *F. psychrophilum* isolates from the same farm, and even from the same individual fish, suggesting the involvement of different isolates in the same RTFS/BCWD outbreak.

Högfors-Rönnholm and Wiklund (2010) documented that smooth *F. psychrophilum* cells could not be serotyped using the slide agglutination method due to the cross-reaction with all three anti-sera proposed by Lorenzen and Olesen (1997) and with the negative controls (pre-immune serum and 0.9% NaCl), while rough cells represented three different reaction patterns including Th, Th/Fp^T or Fp^T/Th, indicating the rough phenotype cells shared multiple antigens. Meanwhile, Valdebenito and Avendaño-Herrera (2009) suggested that the majority of the Chilean *F. psychrophilum* belong to the Th-Fp^T serotype.

Based on the different reaction patterns with two rabbit antisera against virulent and non-virulent strains of *F. psychrophilum*, Faruk *et al.* (2002) distinguished five groups among 33 isolates obtained from diverse geographical locations. However, no apparent correlation between the serotype and geographical origin, the host fish species, or the virulence of the isolate was observed. This study found that the antiserum against the virulent strain (B97026P1) cross-reacted with a number of non-*F. psychrophilum* bacteria (10/26 strains) at the highest level of 61.3%, while the other antiserum against a non-virulent strain (32/97) showed cross-reactivity of less than 2.9% (2/26 strains), in comparison with the 100% reactivity of two strains used to prepare the antisera.

1.3.6. Genetic characterisation

The complete genome sequence of the virulent *F. psychrophilum* JIP02/86 (ATCC 49511) strain from France has been reported (Duchaud *et al.*, 2007). The genome is a circular chromosome consisting of 2,861,988 base pair (bp) with the G+C content of 32.54% and 2,432 predicted open reading frames (ORFs) (Duchaud *et al.*, 2007). Protein-coding genes involved in toxin production, stress response systems, the gliding motility, adhesion mechanisms and biofilm formation were identified and need to be further assessed in large-scale functional analysis (Duchaud *et al.*, 2007). In 2014, the genome of the *F. psychrophilum* CSF259-93 from USA was sequenced and used to select a line of rainbow trout with increased genetic resistance against BCWD (Wiens *et al.*, 2014). The genome of CSF259-93 consists of 2,900,735 bp and 2,701 predicted ORFs (Wiens *et al.*, 2014). Comparative sequence analysis between strains JIP 02/86 and CSF259-93 revealed 1,471 single nucleotide polymorphisms present within predicted protein-coding genes (Wiens *et al.*, 2014). Recently, a draft genome sequence of *F. psychrophilum* strain isolated from ayu in Japan has been described with a total of 2,705,210 bp, a G+C content of 32.5% and 2,608 predicted protein-coding sequences (Shimizu *et al.*, 2016). Whole genome analysis of *F. psychrophilum* strains will assist in the understanding of the pathogenic mechanisms of this bacterium.

Many DNA-based genotyping methods have been used to differentiate *F. psychrophilum* strains for epidemiological investigation. By using DNA-DNA hybridisation, Lorenzen *et al.* (1997) found that *F. columnare* NCIMB 2248^T was only 3% related to *F. psychrophilum* NCIMB 1947^T and all 32 Danish *F. psychrophilum* isolates from rainbow trout were highly related to the type strain NCIMB 1947^T. Random amplified polymorphic DNA (RAPD) PCR with primers OPH 06 and OPH 08 was used successfully by Chakroun *et al.* (1997) in the differentiation of *F. psychrophilum* from nine related *Flavobacterium* species and in intra-specific typing of 177 *F. psychrophilum* strains isolated from 14 different countries. Some RAPD patterns showed a strong correlation with the fish host, but no clear association between the patterns and the geographical origin of the isolates was detected. In contrast, genetic homogeneity was observed among 20 Chilean isolates from 10 Atlantic salmon and rainbow trout farms, and no correlation between the RAPD profile and the fish host was found (Valdebenito and Avendaño-Herrera, 2009). On the other hand, based on a ribotyping technique, Chakroun *et al.* (1998) found several RNA gene restriction patterns (ribotypes) possessing a clear relationship with the host, but not with the isolate location among 85

strains originating from 13 countries and 10 fish species. These results were in agreement with the findings of Madetoja *et al.* (2001) that no significant differences between 37 Finnish *F. psychrophilum* isolates during 1993 – 1996 and 12 isolates from Estonia, Sweden and USA were noted.

PCR – restriction fragment length polymorphism (PCR-RFLP) targeting an anonymous 290 bp DNA marker and *gyrB* gene has been employed to investigate the genetic variability of 242 Japanese, USA and European *F. psychrophilum* isolates, which were subsequently classified into four genotypes (A-R, A-S, B-R, B-S) (Izumi *et al.*, 2003b). The relationship between these four PCR-RFLP genotypes and the host species was found to be statistically significant (Izumi *et al*., 2003b; Nagai and Nakai, 2011). Valdebenito and Avendaño-Herrera (2009) also used the same PCR-RFLP assay to identify two predominant genotypes (B-S and B-R) among 20 Chilean isolates, but there seemed to be no relationship between these genotypes and host fish species. Polymorphism analysis using the *ppiC* gene has been widely used in Japan to divide *F. psychrophilum* isolates strains into two strain types (A and B) possibly related to the fish host and the virulence to ayu (Fujiwara-Nagata *et al.*, 2012). In addition, Fujiwara-Nagata *et al.* (2012) also analysed two single nucleotide polymorphisms in the *gyrA* gene of 232 Japanese *F. psychrophilum* isolates and were able to differentiate isolates potentially harmful to ayu (G-C type) from potentially not harmful isolates (A-C, A-T and G-T type). Repetitive element PCR (rep-PCR) using ERIC and REP primers was employed to investigate the genetic diversity of 20 Chilean *F. psychrophilum* isolates (Valdebenito and Avendaño-Herrera, 2009). The results revealed genetic homogeneity among these isolates based on the REP patterns, while ERIC patterns were suggested to be unsuitable for genetic studies on *F. psychrophilum* due to variations in band intensity, variability and problems with reproducibility (Valdebenito and Avendaño-Herrera, 2009).

To map the intra-species phylogeny of *F. psychrophilum*, Soule *et al.* (2005a) prepared reciprocal subtractive libraries for virulent and non-virulent strains by combining suppression subtractive hybridisation and microarrays, and then assessed 32 USA and European strains from seven different fish hosts. Two genetic lineages were identified reflecting host specificity (Soule *et al.*, 2005a). The majority of strains (11/13) from 'Lineage I' were from salmon and almost all of the strains (18/19) from 'Lineage II' were sourced from rainbow trout (Soule *et al.*, 2005a). This hypothesis was further confirmed by detecting the 16S rRNA sequence variants between these two genetic

lineages using 16S rDNA microarray and PCR-RFLP (Soule *et al.*, 2005b). The authors found that most strains (11 of 14) defined as 'Lineage I' possessed two alleles (ATCC and CSF) and all 15 strains from 'Lineage II' contained only the CSF allele. Ramsrud *et al.* (2007) developed a simpler PCR assay to rapidly differentiate *F. psychrophilum* strains based on the presence of one or both of these 16S rRNA alleles. Applying this 16S allele-specific PCR, Nilsen *et al.* (2011b) was able to assign Norwegian isolates from rainbow trout (nine isolates) and brown trout (four isolates) to lineage II, while those from Atlantic salmon belonged to both lineages (five isolates in lineage I and five in lineage II). The same PCR assay failed to classify 59 Canadian isolates originating from 52 rainbow trout, 5 Arctic charr, one Atlantic salmon and one brook trout (Hesami *et al.*, 2008) and 20 Chilean isolates from 10 Atlantic salmon, nine rainbow trout and one unknown source (Valdebenito and Avendaño-Herrera, 2009) according to their host species, as evidenced by the majority of isolates (43/59 and 18/20, respectively) belonging to 'Lineage II'.

Macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) has been used for the genetic analysis of various pathogenic bacteria. PFGE was applied for the first time in the study of Arai *et al.* (2007) using 64 Japanese *F. psychrophilum* isolates from 19 prefectures. A total of 42 genotypes grouped into 20 clusters were observed and a clear difference between PFGE genotypes of isolates from ayu and those from other fish hosts was noted (Arai *et al.*, 2007). Chen *et al.* (2008) found a greater average similarity of PFGE patterns of 85 *F. psychrophilum* isolates in commercially raised rainbow trout (*O. mykiss* Walbaum) compared to 54 isolates from spawning coho salmon (*O. kisutch* Walbaum) (88% vs. 70% respectively). Meanwhile, Del Cerro *et al.* (2010) found 17 PFGE patterns among 25 Spanish isolates from 12 fish farms during 2005 – 2008 and suggested that the population of potentially pathogenic *F. psychrophilum* isolates in northern Spain was quite heterogeneous. In contrast, PFGE analysis of 14 Chilean *F. psychrophilum* field strains revealed two PFGE clusters correlated with host of origin (rainbow trout and Atlantic salmon) and indicated that the Chilean isolates were closely related, but clearly different from type strain ATCC 49418^{T} (Avendaño-Herrera *et al.*, 2009). The low diversity of 12 Chilean *F. psychrophilum* isolates from nine different salmonid production sites was also demonstrated by direct genome restriction fragment analysis using conventional polyacrylamide gel electrophoresis (Castillo *et al.*, 2012). Similarly, the majority (38/42) of Finnish isolates from 21 fish individuals at three different farms during 2009 – 2010 showed a high level of genetic homogeneity with the

band similarity exceeding 94% in PFGE analysis (Sundell *et al.*, 2013). The concurrence of more than one PFGE profile at the same outbreaks was also recorded (Del Cerro *et al.*, 2010; Sundell *et al.*, 2013).

To better understand the population structure and evolution mode of *F. psychrophilum*, multilocus sequence typing approach (MLST) has been applied based on variability in eleven housekeeping genes (Nicolas *et al.*, 2008). A total of 33 sequence types (STs) and three clonal complexes (CCs) were resolved among 50 isolates from six different geographical areas (North America, Europe, Israel, Chile, Tasmania and Japan) and 10 fish species over a period of more than 20 years (1981 – 2003) (Nicolas *et al.*, 2008). The pattern of polymorphism was characterised by a particularly low level of diversity (fewer than four differences per kilobase on average) and a high recombination rate, contributing to a high level of species cohesiveness. In addition, the analysis showed the worldwide geographic distribution and the host specificity of several CCs, perhaps reflecting the transmission routes and/or adaptive niche specialisation (Nicolas *et al.*, 2008). A MLST scheme based on seven loci as proposed by Nicolas *et al.* (2008) has been employed by various studies on 66 French *F. psychrophilum* isolates (Siekoula-Nguedia *et al.*, 2012), 44 Norwegian and Chilean isolates (Apablaza *et al.*, 2013), 94 Chilean isolates (Avendaño-Herrera *et al.*, 2014) and 560 isolates from four Nordic countries (Nilsen *et al.*, 2014). In general, these data supported the hypothesis of an epidemic population structure of *F. psychrophilum* due to the expansion of genetically closely related isolates. A large globally spread clonal complex of the species *F. psychrophilum* almost exclusive to the rainbow trout host has been identified and shown to have sequence type ST2 or ST10 as the predicted common ancestor (Siekoula-Nguedia *et al.*, 2012; Apablaza *et al.*, 2013; Avendaño-Herrera *et al.*, 2014; Nilsen *et al.*, 2014). However, the observed wide geographical distribution of *F. psychrophilum* isolates also indicates a large potential of this bacterium for spreading over long distances and between different salmonid host species (Apablaza *et al.*, 2013; Avendaño-Herrera *et al.*, 2014). The MLST database is available online (http://pubmlst.org/fpsychrophilum) for further epidemiological surveys and future management of RTFS/BCWD outbreaks.

1.4. Antibiotic susceptibility of *Flavobacterium psychrophilum*

1.4.1. Antibiotics in aquaculture – mechanisms of action

Since the formal discovery of the antibiotic penicillin by Flemming in 1929, hundreds of antibiotics derived from natural sources or having synthetic origins have been developed

and used widely to treat infectious diseases (Giguère, 2006a; Romero *et al.*, 2012). Antibacterial drugs can be distinguished by their capacity to kill (i.e. a bactericidal effect) or inhibit (i.e. a bacteriostatic effect) the growth of microorganisms (Romero *et al.*, 2012). The mechanisms of action of antibacterial drugs fall into five categories (Figure 1.5): inhibition of cell wall synthesis, damage to cell membrane function, inhibition of nucleic acid synthesis or function, inhibition of protein synthesis, and inhibition of folic acid synthesis (Giguère, 2006a).

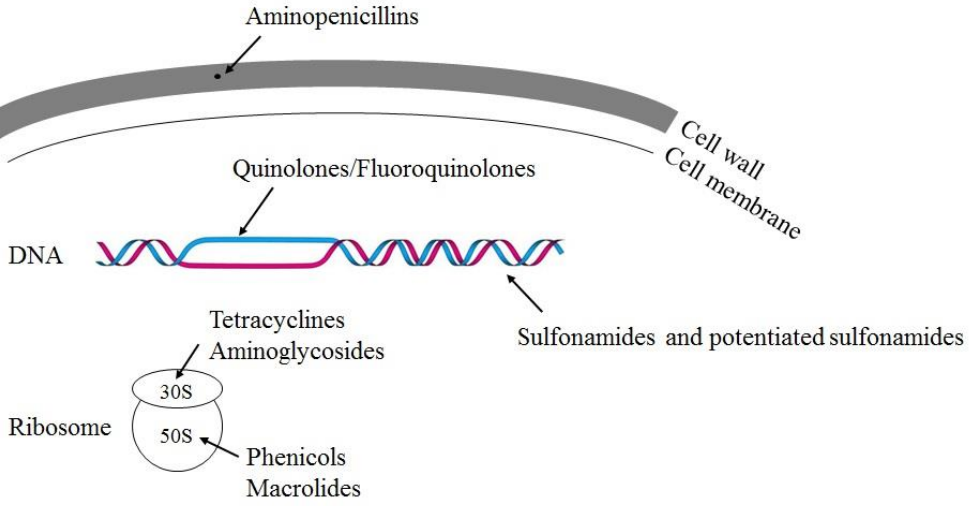


Figure 1.5. Action sites of commonly used antibiotics in aquaculture (Giguère, 2006a).

Like other animal food production industries, aquaculture is also reliant on the use of antimicrobial agents for anti-infective therapy. Therefore, knowledge on the susceptibility of fish pathogens, such as *F. psychrophilum*, to antimicrobial drugs are essential for monitoring and ensuring the efficacy of any treatment (Smith *et al.*, 2016). Several classes of antibacterial drugs known to be used at varying frequencies in global aquaculture and the *in vitro* susceptibilities of *F. psychrophilum* to several antibiotics within these classes are described below (CLSI, 2006). However, comparisons of antimicrobial susceptibility data of *F. psychrophililum* between studies are difficult due to differences in the testing protocols and the interpretive criteria used.

1.4.1.1. Aminoglycosides

Members of aminoglycosides class are bactericidal antibiotics, which inhibit bacterial protein synthesis at the ribosomal level. Aminoglycosides (e.g., streptomycin, gentamicin, kanamycin) are used primarily to treat serious infection caused by aerobic Gram-negative bacteria or combined with cell-wall active compounds to produce

synergistic effects (CLSI, 2006; Dowling, 2006a). The activity of aminoglycosides is varied due to the different levels of susceptibility to aminoglycoside-inactivating enzymes between agents (CLSI, 2006). The most common resistance mechanism to aminoglycoside is bacterial enzyme production to modify the antibiotics, from which more than 50 enzymes have been detected (Sykes and Papich, 2014). In addition, reduced drug uptake may also be one of aminoglycoside resistance mechanisms caused by bacteria (Sykes and Papich, 2014). The use of these antibacterial drugs has been monitored carefully due to their extended residue time in some animals (CLSI, 2006).

Pacha (1968) reported that ten *F. psychrophilum* isolates from the USA were susceptible to 10 µg dihydrostreptomycin. Forty five years later, streptomycin, closely related in structure to dihydrostreptomycin, was found to be effective at controlling the infection of three representative USA *F. psychrophilum* isolates at the recommended concentration of ≥5,000 mg L^{-1} (Oplinger and Wagner, 2013). However, in one study from Turkey, the majority of *F. psychrophilum* isolates showed reduced susceptibility to 10 µg gentamicin based on the inhibition zone data (≤12 mm, 14/20 isolates) and minimum inhibitory concentration (MIC) values (≥8 µg mL^{-1}, 19/20 isolates) (Kum *et al.*, 2008). Similarly, using broth microdilution methods to determine MIC values, Hesami *et al.* (2010) identified 92% of 75 Canadian *F. psychrophilum* isolates exhibiting high MICs (≥1 µg mL^{-1}) for gentamicin.

1.4.1.2. Aminopenicillins

Aminopenicillins are a group of antibiotics belonging to the penicillin family that contain a beta-lactam ring crucial for the antibacterial activity (Cunha, 1992). Beta-lactam antibiotics interfere with peptidoglycan synthesis in the bacterial cell wall by inhibiting the transpeptidase and other peptidoglycan-active enzymes (Prescott, 2006a). These antibacterial drugs (e.g. ampicillin, amoxicillin, bacampicillin, talampicillin) have an extended-spectrum activity and are able to penetrate penicillin-susceptible Gram-positive bacteria, as well as Gram-negative bacteria (CLSI, 2006). Aminopenicillins are susceptible to destruction by beta-lactamases produced by bacteria (CLSI, 2006). Amoxicillin resistance genes were observed on plasmids, non-conjugative plasmids, and also in chromosome of bacterial isolates in a study of Akortha *et al*. (2011) using *Streptococcus viridians*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Moraxella catarrhalis* and *Staphylococcus* species.

Penicillin (10 units) was described as effective to kill ten USA *F. psychrophilum* isolates, although penicillin has not been recorded in any literature for controlling RTFS/BCWD outbreaks (Pacha, 1968). The combination of penicillin (2.5 x 10^6 IU) and streptomycin (2,500 mg L^{-1}) was demonstrated to work synergistically and found to be more effective against *F. psychrophilum* infection than streptomycin alone (Oplinger and Wagner, 2013).

In the UK, the occurrence of several *F. psychrophilum* isolates (4/48) showing high MIC values to amoxicillin (AMOX, ≥2 µg mL^{-1}) was documented on the farms that had an apparent treatment failure with this drug, suggesting comparative resistance to AMOX (Rangdale *et al.*, 1997). Recently, using the standardised broth microdilution method (CLSI, 2014a), Smith *et al.* (2016) reported 13% of 22 UK *F. psychrophilum* isolates collected during 2006 – 2013 showing reduced susceptibility to AMOX.

Amoxicillin was introduced into Denmark for use in aquaculture in 1993 (Schmidt *et al.*, 2000). From 1994 to 1995, no *F. psychrophilum* isolates manifesting reduced susceptibility to this antimicrobial drug were recorded in Denmark (Bruun *et al.* 2000; Dalsgaard and Madsen, 2000). An increase of resistance proportion to 36% (34/97 Danish isolates) for AMOX was reported in 1998 (Bruun *et al.* 2000). However, none of the 15 Danish isolates retrieved during 1988 – 1998 had reduced susceptibility to this antibiotic in the study of Smith *et al.* (2016). From 1997 to 1999, the frequency of Danish isolates exhibiting decreased susceptibility to AMOX with MICs between 1 and 2 µg mL^{-1} increased to 50% (44/89 isolates) (Schmidt *et al.*, 2000). Recently, 36% of Danish isolates (5/14 isolates collected during 1999 – 2012) manifesting reduced susceptibility to AMOX was recorded (Smith *et al.* 2016). In addition, the prevalence of isolates less susceptible to AMOX from the environment (52%) has been reported as being higher than isolates recovered from fish (31%) (Bruun *et al.*, 2000).

The frequency of *F. psychrophilum* isolates from Turkey with decreased susceptibility to amoxicillin-clavulanic acid has been shown to be inconsistent depending on the testing methods adopted (Kum *et al.*, 2008). The disc diffusion test revealed 19/20 isolates with an inhibition zone diameter of ≤14 mm with 30 µg amoxicillin-clavulanic acid), while in the MIC assay, only 3/20 isolates had elevated MIC values ≥128 µg mL^{-1} (Kum *et al.*, 2008). In another study involving *F. psychrophilum* isolates from Canada, 50 of 75 isolates (67%) showed high MIC values (≥4 µg mL^{-1}) against ampicillin, a beta-lactam antibiotic closely related to AMOX (Hesami *et al.*, 2010).

1.4.1.3. Quinolones/Fluoroquinolones

Quinolones or fluoroquinolones are a large and expanding class of synthetic antimicrobial agents (Walker and Dowling, 2006). This class includes quinolones (e.g., oxolinic acid, flumequine, nalidixic acid, sarafloxacin) and fluoroquinolones (e.g., enrofloxacin, piromidic acid). The main mechanism of action is based on the inhibition of DNA-gyrase activity of Gram-positive and Gram-negative bacteria (CLSI, 2006). Mechanisms of fluoroquinolones resistance are DNA gyrase mutations, reduced bacterial permeability, and active drug efflux (Sykes and Papich, 2014).

Rangdale *et al.* (1997) reported MIC values for enrofloxacin (0.00098 – 0.25 µg mL^{-1}), sarafloxacin (0.000195 – 8 µg mL^{-1}) and oxolinic acid (OXA, 0.03125 – 16 µg mL^{-1}) from 47 UK *F. psychrophilum* isolates, and considered these isolates to be susceptible to the three quinolone antibiotics examined. However, these authors noted that OXA may be inactivated by exposure to Ca^{2+} and Mg^{2+} in the water and in the intestine of the fish at the farms; thus the *in vitro* susceptibility testing of OXA may be underestimated. Recently, the frequency of UK isolates manifesting reduced susceptibility to OXA was reported at 70% (15/22) based on a MIC epidemiological cut-off (CO_{WT}) value for OXA (≤0.25 µg mL^{-1}) calculated by the normalised resistance interpretation (NRI) method (Smith *et al.*, 2016).

Oxolinic acid was introduced into Denmark for use in aquaculture in 1986 (Schmidt *et al.,* 2000). Since 1996, quinolone agents have not been used for RTFS treatment in Denmark as florfenicol has become the agent of choice, but quinolone antibiotics are still used for treatment of other bacterial diseases in Danish farms (Smith *et al.*, 2016). From 1994 to 1995, around 55% of 250 Danish *F. psychrophilum* isolates manifested reduced susceptibility (with inhibition zone diameters <30 mm) to 10 µg oxolinic acid (Dalsgaard and Madsen, 2000). Smith *et al.* (2016) observed little of change in the prevalence of isolates manifesting reduced susceptibility to OXA (73% and 79%) in Danish isolates during 1988-1998 (15 isolates) and 1999-2012 (14 isolates) respectively, while Bruun *et al.* (2000) reported an increase of this frequency in 387 Danish isolates during 1994-1998 ranging from 50% to 100%. Meanwhile, another collection of 89 Danish isolates collected during 1997-1999 exhibited decreased susceptibility to OXA with high MIC values ranging between 4 and 16 µg mL^{-1} (Schmidt *et al.*, 2000).

Although OXA has been banned for use in aquaculture in Finland since 2001, the frequency of Finnish *F. psychrophilum* isolates collected during 2009 – 2010 manifesting reduced susceptibility to 2 µg OXA was noted to be high (74% of 41 isolates, with inhibition zone diameters ≤13 mm) (Sundell *et al.*, 2013). Similarly, the proportion of isolates with acquired resistance phenotypes to quinolone antimicrobial drugs in Canada was also found to be high: 83% of 75 isolates with MICs ≥0.5 µg mL^{-1} for OXA, 58% with MICs ≥0.25 µg mL^{-1} for ENRO and 50% with MICs ≥1 µg mL^{-1} for flumequine (Hesami *et al.* 2010). In Chile, the majority of *F. psychrophilum* isolates (34/40, 85%) showed very high MIC values (≥16 µg mL^{-1}) for OXA (Henríquez-Núñez *et al.* 2012). Conversely, Turkish *F. psychrophilum* isolates were found to be susceptible to quinolone agents, with only 10 or 15% of 20 isolates showing reduced susceptibility to enrofloxacin (inhibition zone diameters ≤15 mm with 5 µg enrofloxacin and MIC values ≥8 µg mL^{-1}) in studies of Kum *et al.* (2008) or Boyacioğlu and Akar (2012) respectively.

1.4.1.4. Macrolides

Macrolides (e.g. erythromycin) are a class of natural products produced by streptomycetes. They are characterised and classified based on the number of atoms (12 – 16) comprising the lactone ring (Giguère, 2006b). Macrolides reversibly bind to the 50S subunit of the ribosome leading to inhibition of protein synthesis (CLSI, 2006). Although macrolides exhibit many advantages, such as high intracellular concentration, broad distribution in tissue, prolonged half-life and strong antibacterial activity, antibiotics belonging to this class are mostly under-used in veterinary medicine (Giguère, 2006b). Resistance to macrolides results from reduced bacterial permeability (for gram-negative bacteria), target modifications (i.e. adding a methyl group to the 50S subunit RNA using a ribosomal methylase to prevent the macrolide from binding to the ribosome), active drug efflux, and enzymatic inactivation by bacterial esterases (Sykes and Papich, 2014).

Erythromycin is primarily used to treat the infection caused by Gram-positive bacteria, but this antibiotic was demonstrated to be effective against 13 USA *F. psychrophilum* isolates at the recommended concentrations of >2,000 mg L^{-1} and a 15-min exposure (Pacha, 1968; Oplinger and Wagner, 2013). Similarly, 12 of 75 Canadian isolates showed high MIC values (≥32 µg mL^{-1}) for erythromycin (Hesami *et al.*, 2010), while all of 62 UK and Danish isolates used in the study of Smith *et al.* (2016) were

considered to be fully susceptible to this antimicrobial drug with MICs below 8 µg mL^{-1}. However, the majority of Turkish *F. psychrophilum* isolates (65 or 100% of 20 isolates) were described as being less susceptible to 15 µg erythromycin with inhibition zone diameters ≤13 mm or MIC values ≥8 µg mL^{-1} respectively (Kum *et al.*, 2008).

1.4.1.5. Phenicols

Phenicols are a group of substances with broad-spectrum activity and can be divided into non-fluorinated compounds (e.g., chloramphenicol, thiamphenicol) and fluorinated compounds (e.g., florfenicol) (CLSI, 2006). Antibacterial drugs of this class inhibit microbial protein synthesis by irreversibly binding to a receptor site on the 50S subunit of ribosome. Resistance to chloramphenicols results from porin mutations, drug efflux, or enzymatic inactivation by chloramphenicol acetyltransferase enzymes (Sykes and Papich, 2014). The fluorinated compounds are less susceptible to chloramphenicol acetyltransferase-mediated resistance from bacteria. The presence of a region containing resistance genes to florfenicol (*floR*), tetracycline (*tet*X), streptothricin and chloramphenicol acetyltransferase gene was detected in *Chryseobacterium* spp. from rainbow trout (Verner-Jeffreys *et al.*, 2017). However, this resistance gene cassette was not widely distributed in Flavobacteriaceae isolates (Verner-Jeffreys *et al.*, 2017).

Chloramphenicol has been prohibited for use in food-producing animals or aquaculture in many countries because of its toxic effects, such as bone marrow depression and aplastic anemia (CLSI, 2006; Dowling, 2006b). The use of this antibiotic prior to any antigenic stimulus or vaccination trial may affect immune responses as chloramphenicol may suppress antibody production (Dowling, 2006b).

Florfenicol (FFN) is currently considered as the main treatment of choice for controlling RTFS/BCWD outbreaks. *F. psychrophilum* isolates manifesting reduced susceptibility to FFN seems to be very exceptional in Europe and only a few strains (10/48 strains) with unusually high MIC values (≥8 µg mL^{-1}) have been documented (Rangdale *et al.*, 1997). All the *F. psychrophilum* isolates collected from the UK, Denmark, France and Spain were demonstrated to be fully susceptible to FFN in various earlier studies (Rangdale *et al.*, 1997; Bruun *et al.*, 2000; Michel *et al.*, 2003; Del Cerro *et al.*, 2010; Smith *et al.*, 2016). Using NRI methods, Smith *et al.* (2016) suggested the CO_{WT} value for FFN to be at ≤2 µg mL^{-1}. Nevertheless, moderate and high frequencies of *F. psychrophilum* isolates having reduced susceptibility to FFN has been reported in

Turkey (16 and 25% of 20 isolates with MICs ≥8 μg mL^{-1} in studies of Kum *et al.* (2008) and Boyacioğlu and Akar (2012) respectively), Chile (92.5% of 40 isolates with MICs ≥2.35 μg mL^{-1}, Henríquez-Núñez *et al.*, 2012) and Canada (52.8% of 75 isolates with MICs ≥4 μg mL^{-1}, Hesami *et al.*, 2010).

1.4.1.6. Sulfonamides and Potentiated Sulfonamides

The use of sulfonamides as single antibacterial agents has diminished due to widespread acquired resistance. However, in combination with diaminopyrimidines (e.g. ormetoprim–sulfadimethoxine, ormetoprim–sulfamonomethoxine, sulfamerazine, sulfamethazine, sulfisoxazole, trimethoprim–sulfadiazine, trimethoprim–sulfamethoxazole), the antibacterial activity can be enhanced (Prescott, 2006b). This combination interferes with DNA synthesis by inhibiting sequential steps in the synthesis of folic acid and subsequently purines (Prescott, 2006b). Although ormetoprim–sulfadimethoxine and trimethoprim–sulfamethoxazole are mostly used in aquaculture, it is recommended to test each component of the combination to obtain the most reliable susceptibility testing result (CLSI, 2006; Minogue *et al*., 2012). Resistance to potentiated sulfonamides results mainly from plasmid-mediated production of altered dihydrofolate reductase or dihydropteroate synthetase, from overproduction of bacterial dihydrofolate reductase, and from decreased permeability (Sykes and Papich, 2014).

Historically, for the effective control of BCWD in salmonids, sulphonamides were suggested to be orally administered at between 220 and 400 mg kg^{-1} day^{-1} (reviewed in Rangdale *et al.* 1997). However, consistently high MICs against the combinations of sulfonamides and diaminopyrimidines (e.g. trimethoprim/sulfadiazine, ormetoprim/sulphadimethoxine, trimethoprim/sulphamethoxazole) have been reported among *F. psychrophilum* isolates in various countries, such as the UK (Rangdale *et al.*, 1997; Smith *et al.*, 2016), Denmark (Bruun *et al.*, 2000; Schmidt *et al.*, 2000), Turkey (Kum *et al.*, 2008) and Canada (Hesami *et al.*, 2010). The calculated MIC CO_{WT} values for ormetoprim/sulphadimethoxine and trimethoprim/sulphamethoxazole according to the NRI method were ≤320 (16/304) μg mL^{-1} and ≤20 (1/19) μg mL^{-1} respectively (Smith *et al.*, 2016). Although Smith *et al.* (2016) concluded that all the *F. psychrophilum* isolates examined in their study were fully susceptible to potentiated sulphonamides, other studies have suggested that the *F. psychrophilum* species may have an intrinsic resistance to this class of compounds due to the omission of the biochemical pathways

on which the drug acts (Rangdale *et al.*, 1997; Bruun *et al.*, 2000; Schmidt *et al.*, 2000; Kum *et al.*, 2008; Hesami *et al.*, 2010).

1.4.1.7. Tetracyclines

These compounds are bacteriostatic antibiotics that inhibit bacterial protein synthesis by reversibly binding to receptors on the 30S subunit of the ribosome (CLSI, 2006). Tetracyclines (e.g. tetracycline, oxytetracycline, doxycycline, minocycline) exhibit a broad spectrum activity against Gram-positive and Gram-negative bacteria. However, their usefulness has been reduced with the occurence of antibiotic resistance (Giguère, 2006c). Resistance to tetracyclines primarily results from active drug efflux, mediated by the *tetK* gene, while resistance mediated by *tetM* gene confers resistance to all tetracyclines (Sykes and Papich, 2014).

In 1968, ten *F. psychrophilum* isolates from the USA were demonstrated to be sensitive to 30 µg tetracycline and 30 µg chlortetracycline (Pacha, 1968). Similarly, no resistance to doxycycline among 47 UK *F. psychrophilum* isolates was recorded in 1997 (Rangdale *et al.*, 1997). However, high MIC values (MIC_{90} of ≥32 µg mL^{-1}) for oxytetracycline (OTC) among these 47 UK isolates were observed, indicating that the emergence of bacterial resistance to OTC could be associated with the long term high dosage use of OTC in the UK (300 mg kg^{-1} fish/day for 10-14 days) since this antibiotic was introduced in 1994 for use in aquaculture (Rangdale *et al.*, 1997). Recently, Smith *et al.* (2016) reported that 15 of 22 UK *F. psychrophilum* isolates (70%) showed reduced susceptibility to OTC with a CO_{WT} value of ≤0.125 mg L^{-1}.

In Denmark, although the use of OTC in freshwater aquaculture ceased almost completely in the early nineties due to the inefficacy of the treatment, little change in the susceptibility to OTC in Danish isolates has been observed (Bruun *et al.*, 2000; Smith *et al.*, 2016). Dalsgaard and Madsen (2000) documented the frequencies of Danish isolates manifesting the reduced susceptibility to 80 µg OTC in 1994 and 1995 at 53% and 76% of the isolates examined respectively. Meanwhile, Bruun *et al.* (2000) recorded consistently around 70% of 387 isolates having decreased susceptibility to OTC in Denmark during 1994 – 1998. From 1997 to 1999, 63 of 89 Danish isolates (71%) were found to be less susceptible to OTC with MICs between 1 and 8 µg mL^{-1} (Schmidt *et al.*, 2000). Using a standardised testing method (CLSI, 2014a) and the NRI method to interpret the susceptibility data, the prevalence of Danish *F. psychrophilum* isolates

showing acquired resistance phenotype to OTC during 1988 – 1998 and 1999 – 2012 was 27% and 21% respectively (Smith *et al.*, 2016).

High MIC values for OTC were also found in the majority of *F. psychrophilum* isolates from other countries, such as 61% of Canadian isolates (46/75) having MICs ≥4 μg mL^{-1} (Hesami *et al.* 2010), 80% of Spanish isolates (20/25) showing MICs between 2.5 and 9.7 μg mL^{-1} (Del Cerro *et al.* 2010) and 90% of Chilean isolates (36/40) exhibiting MIC of ≥0.125 μg mL^{-1} (Henríquez-Núñez *et al.* 2012). Meanwhile, the frequencies of *F. psychrophilum* isolates from Turkey manifesting reduced susceptibility to OTC were inconsistent between two testing methods used, according to the disc diffusion test (20% with 30 μg OTC, Kum *et al.*, 2008) and MIC assay (62-75%, Kum *et al.*, 2008; Boyacioğlu and Akar, 2012).

1.4.2. Antibiotics licensed in the UK, Europe and worldwide for use in aquaculture

With the intensification and diversification of aquaculture, the availability of effective treatments for infectious disease control is a critical requirement for a successful aquaculture industry (Verner-Jeffreys and Taylor, 2015). In comparison with agricultural and medicinal use, the market for aquaculture antibiotics is small and the approval is expensive. The use of antibacterial drugs in aquaculture is controlled by regulations, particularly in Europe and the USA in order to ensure the safety and efficacy of the drugs in the treatment of fish disease and also the safety for human and other animals consuming the treated fish product (Rodgers and Furones, 2009).

Table 1.2. List of commonly used antibiotics in UK aquaculture (Verner-Jeffreys and Taylor, 2015).

Antibiotics	Regulatory status	Diseases used to control	Dosing, duration and number of treatments
Florfenicol (Florocol™)	UK Licensed veterinary medicine for use in salmon for control of furunculosis	RTFS Enteric redmouth disease (ERM) and furunculosis (limited use)	10 mg kg^{-1} body weight (bw) daily for 10 d
Oxolinic acid (Branzil Vet™)	Prescription only medicines (POM)	ERM, furunculosis	10 mg kg^{-1} bw

	Not licensed in UK. Licensed veterinary medicine for control of trout diseases in other European MS (Denmark; listed in EU Regulation 37/2010 Table 1)		daily for 10 d
Oxytetracycline (Aquatet™)	POM UK Licensed veterinary medicine for use in furunculosis due to *Aeromonas salmonicida* and columnaris disease in Atlantic salmon, and furunculosis and ERM in rainbow trout	ERM, furunculosis	75 mg kg^{-1} bw daily for 10 d
Amoxicillin (Vetremox™)	POM UK Licensed veterinary medicine for use in salmon for control of furunculosis	Furunculosis/ other Gram negative septicaemias. Occasionally for RTFS	80 mg kg^{-1} bw daily for 10 d

In the UK, three antibiotics (florfenicol, oxytetracycline and amoxicillin) are licensed for use in aquaculture by the UK Veterinary Medicines Directorate (VMD) (http://www.vmd.defra.gov.uk/) (Table 1.2). Although oxolinic acid is licensed for control of trout diseases in European Member States but not in the UK, veterinarians and health professionals expressed a preference for using this antibiotic where possible to treat fish diseases (Verner-Jeffreys and Taylor, 2015). A survey of eight different UK rainbow trout producers revealed that florfenicol was the treatment of choice in the industry and that most batches of rainbow trout were treated with this agent at least once during every production cycle (Verner-Jeffreys and Taylor, 2015). These florfenicol treatments were generally considered to be very effective (Verner-Jeffreys and Taylor, 2015). Where other antimicrobials (oxytetracycline, oxolinic acid or amoxicillin) were used, the therapeutic response was reported as either mixed or poor (Verner-Jeffreys and Taylor, 2015).

In Europe, the Salmonidae are classed as a major animal food producing species and seven antibiotics are approved for use in aquaculture: amoxicillin, florfenicol, flumequine, oxolinic acid, oxytetracycline, sarafloxacin and sulfadiazinetrimethoprim (Rodgers and Furones, 2009). In the USA, florfenicol and oxytetracycline are approved

for aquaculture use by the U.S. Food and Drug Administration (www.fda.gov/cvm) (Oplinger and Wagner, 2013; Van Vliet *et al.*, 2017). Conversely, Japan, where aquaculture is considered as an important industry, 27 antimicrobial agents have been approved for 13 different fish species (Treves-Brown, 2000; Rodgers and Furones, 2009).

1.4.3. Resistance mechanisms and transfer

Antimicrobial resistance is defined as the ability of a microorganism to render a normally active concentration of an antimicrobial agent ineffective (Boerlin and White, 2006). There are three fundamental phenotypes of antimicrobial resistance (susceptibility, intrinsic resistance and acquired resistance) in bacteria (Boerlin and White, 2006). Four main antimicrobial resistance mechanisms of bacteria have been identified: changes in membrane permeability to reduce the penetration of drugs into the bacterial cell; extrusion of the drugs from the cell by efflux pumps; enzyme degradation of the antimicrobial drugs; and modifications of the antimicrobial target in the bacteria (Figure 1.6) (Boerlin and White, 2006).

The failure of antibiotic treatment and the recurrence of bacterial infections are also associated with a specialised survivor – a persister (Lewis, 2013). Persisters are non-growing dormant cells exhibiting their tolerance to antibiotics, and their mechanism of the formation is still unknown (Lewis, 2013).

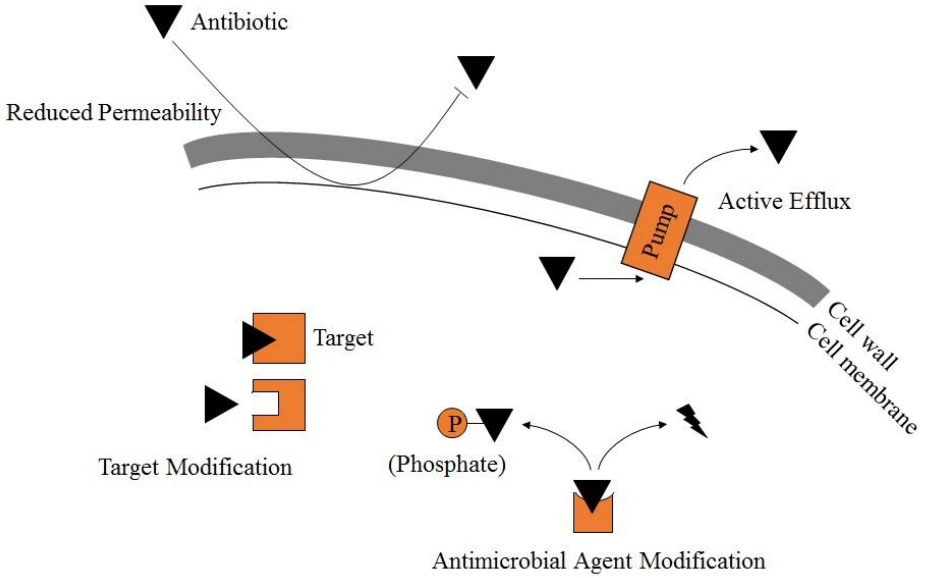


Figure 1.6. Four main antimicrobial resistance mechanisms of bacteria. ***Reduced permeability*** can occur in either the outer membrane or the cell membrane. ***Active efflux*** can pump the antimicrobial drugs back into the periplasmic space or in the outer milieu. ***Antimicrobial agent modification*** is performed by bacterial enzymes inside the cell, in the periplasmic space or even outside the cells. ***Target modifications*** happen in both surface-exposed and intracellular targets (Boerlin and White, 2006).

1.4.3.1. Intrinsic resistance

Intrinsic resistance is defined as the unusual susceptibility of a bacterial species to a particular drug (Romero *et al.*, 2012). This may be a result of structural or biochemical characteristics inherent in the wild-type microorganism (Boerlin and White, 2006). The *F. psychrophilum* species is suggested to be intrinsically resistant to potentiated sulfonamides as no evidence of field efficacy with this group of antimicrobial drugs has been documented and very few susceptible isolates have been found in *in vitro* antimicrobial susceptibility testing (Rangdale *et al.*, 1997; Bruun *et al.*, 2000; Schmidt *et al.*, 2000; Kum *et al.*, 2008; Hesami *et al.*, 2010).

1.4.3.2. Acquired resistance

Antimicrobial resistance can be acquired often through genetic changes in a normally susceptible microorganism, representing a major concern due to the often transmissible nature of resistance mechanisms (Boerlin and White, 2006; Romero *et al.*, 2012). This type of resistance can originate from a mutation in the genes involved in normal physiological processes and cellular structures (Boerlin and White, 2006).

The resistance to quinolones (nalidixic acid and oxolinic acid) in the *F. psychrophilum* species has been demonstrated to be associated with chromosomal mutations in the *gyrA* gene and was transferred vertically in a bacterial population (Bruun *et al.*, 2003a; Izumi and Aranishi, 2004). Therefore, this gene has been suggested as a target for detecting quinolone-resistant *F. psychrophilium* strains. However, Henríquez-Núñez *et al.* (2012) observed no correlation between quinolone resistance and the *gyrA* mutation in 40 Chilean *F. psychrophilum* isolates. Reduced susceptibility to tetracycline has been suggested to be associated with polymorphisms of the 16S rRNA gene (Soule *et al.*, 2005b). Isolates belonging to the genetic lineage I were found to be more susceptible to tetracycline (inhibition zone diameter of 36.4 ± 5.3 mm) compared with isolates from lineage II (27.1 ± 5.3 mm) (Soule *et al.*, 2005b). These authors also found a statistically significant association between this reduced susceptibility and a tetracycline resistance gene (*tet*(A)-like gene) (Soule *et al.*, 2005b).

Foreign genes can be responsible for the acquisition and transferring of the antimicrobial resistance between bacteria by three processes (Figure 1.7): transformation (the uptake of naked DNA from the external environment), transduction (the transfer of DNA through infection by bacteriophage) and conjugation (the transfer of plasmids by cell-to-cell mating) (Boerlin and White, 2006).

Oxytetracycline resistance determinants in *F. psychrophilum* strains have been speculated to be located in mobile elements (transposons or plasmids) or due to non-specific changes in membrane permeability (Bruun *et al.*, 2003a). The possibility of transferring OTC-resistance gene located in large plasmids (>25 kb) between *Aeromonas salmonicada* strains into *F. psychrophilum* has been investigated *in vitro* (Bruun and Schmidt, 2003b). However, no transfer was detected, suggesting that the bacterial species of the recipient (incompatibility) and conditions under which plasmid transferring occurs are important for a successful conjugation.

An association between plasmid content and acquired antimicrobial resistance has been studied in 25 Spanish *F. psychrophilum* strains, but no such relationship could be established as some isolates resistant to OTC did not possess a plasmid, whereas isolates susceptible to OTC harboured a 3.5 kb plasmid (Del Cerro *et al.*, 2010). These authors speculated that antimicrobial resistance determinants in *F. psychrophilum* are possibly carried in mobile genetic elements (such as transposons) located in the bacterial chromosome (Del Cerro *et al.*, 2010).

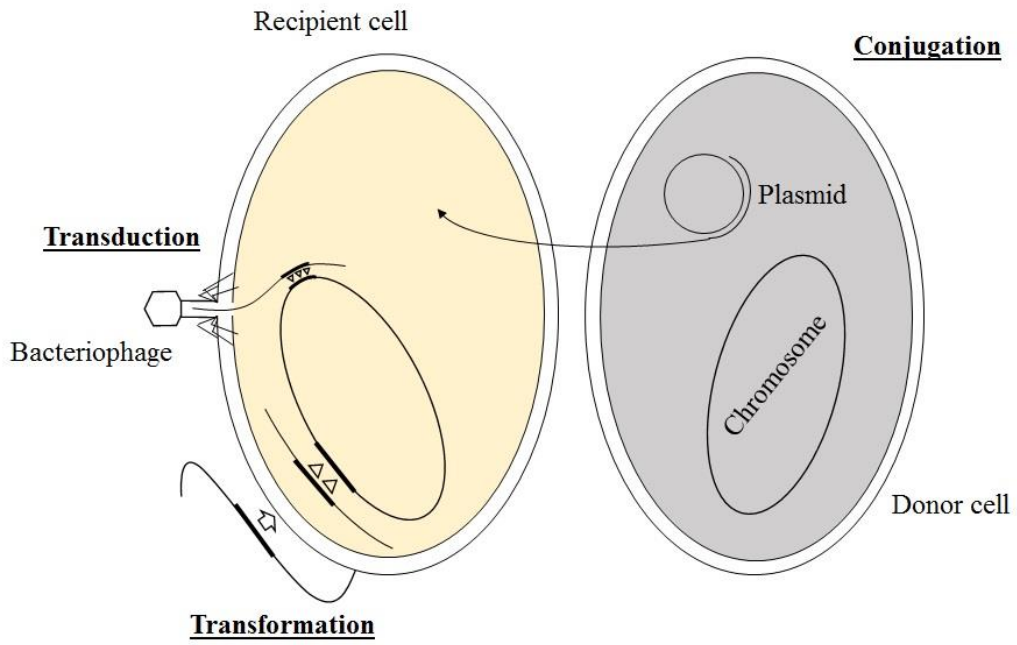


Figure 1.7. Three mechanisms of horizontal transfer of genetic material between bacteria. White arrows represent the movement of genetic material and recombinant events; bold black lines indicate antimicrobial resistance genes or gene clusters. In ***transduction***, DNA of a bacteriophage is injected into the recipient cell and then integrates into the chromosome via a lysogenic phase. In ***transformation***, naked DNA is taken up by a competent cell and may recombine with homologous sequences present in the recipient's genome. In ***conjugation***, a replicated plasmid is transferred from a donor bacterium to a recipient cell. The plasmid may contain a transposon carrying antimicrobial resistance genes (Boerlin and White, 2006).

In addition, acquired resistance can be to a single agent or to some agents within an antimicrobial class or from several different classes (Boerlin and White, 2006). Three or more resistance phenotypes (sulfadiazine-trimethoprim, OXA and OTC) have been found in 64 of 89 Danish *F. psychrophilum* isolates (72%) (Schmidt *et al.*, 2000).

1.4.4. Occurrence of plasmids in *Flavobacterium psychrophilum*

There have been several studies examining the plasmid content of the *F. psychrophilum* isolates. Although there is a discrepancy in the estimation of plasmid sizes, possibly due to method differences, most of the *F. psychrophilum* isolates studied possess a single small plasmid (Dalsgaard, 1993; Nematollahi *et al.*, 2003a). Madsen and Dalsgaard (2000) observed the majority of Danish *F. psychrophilum* isolates (284/299) habouring a

3.3 kb plasmid. Meanwhile, other studies reported plasmids of 3.2 kb (Lorenzen *et al.*, 1997), 3.4 kb (Izumi and Aranishi, 2004) and 3.5 kb (Chakroun *et al.*, 1998; Del Cerro *et al.*, 2010; Kim *et al.*, 2010b; Henríquez-Núñez *et al.*, 2012). This small plasmid is suggested to be correlated with the virulence of *F. psychrophilum* strains (Madsen and Dalsgaard, 2000). Lorenzen *et al.* (1997) found only one plasmid of 3.2 kb among 35 European isolates originating from clinical outbreaks of either BCWD or RTFS, while other plasmid profiles were harboured by isolates (n= 10) originating from fish with chronic or no RTFS/BCWD clinical signs.

A large plasmid (50 kb) was detected in a Danish *F. psychrophilum* strain manifesting reduced susceptibility to oxytetracycline, but plasmid sequencing and curing or transfer experiments need to be performed to confirm the role this large plasmid as a resistance plasmid (Bruun *et al.*, 2003b).

Other plasmids (100 kb, 23 kb, 3.5 kb, 2.7 kb, 2.4 kb and 1.8 kb) were characterised among 128 Japanese *F. psychrophilum* isolates from ayu, pale chub *Zacco platypus* and crucian carp *Carassius cuvieri* (Kim *et al.*, 2010b). Meanwhile, *F. psychrophilum* type strain NCIMB 1947^{T} was shown to have only one plasmid of approximately 2.6 kb (Lorenzen *et al.*, 1997; Madsen and Dalsgaard, 2000; Chakroun *et al.*, 1998; Izumi and Aranishi, 2004; Del Cerro *et al.*, 2010; Henríquez-Núñez *et al.*, 2012).

The role of plasmids in the pathogenesis of *F. psychrophilum* infection is still unknown. A relationship between serological differences and plasmids has been suggested but this requires further investigation (Lorenzen and Olesen, 1997). These authors observed that the majority of isolates of serotype Th and Fd (32/35) had a plasmid of 3.2 kb, while five different plasmid profiles were found among nine isolates from serotype Fp^{T}. On the other hand, Sundell *et al.* (2013) found no connection between the *in vitro* phase conversion from the smooth cell phenotype to the rough cell phenotype and the acquisition or loss of a plasmid.

1.4.5. Antimicrobial susceptibility testing and interpretive criteria

Smith *et al.* (2013) stressed the need for standardised and internationally recognised protocols for laboratory *in vitro* antimicrobial susceptibility testing in monitoring and surveillance programmes and the use of standardised methods to calculate epidemiological cut-off values (CO_{WT}) for data interpretation.

Due to the lack of standardised procedures for antimicrobial susceptibility testing of specific fish pathogens needing lower incubation temperatures, susceptibility data of *F. psychrophilum* generated from different methods or growth media differ from laboratory to laboratory, making any comparison difficult (Alderman and Smith, 2001). In addition, clear differences in the results between the disc diffusion and MIC agar dilution methods, when testing the antimicrobial susceptibility of *F. psychrophilum*, have been reported (Kum *et al.*, 2008; Henríquez-Núñez *et al.*, 2012). Recently, a standardised method for microdilution MIC testing of *F. psychrophilum* has been published in the VET04-A2 guideline of the Clinical and Laboratory Standards Institute (CLSI, 2014a).

Among various interpretative methods used in antimicrobial susceptibility data analysis, normalised resistance interpretation (NRI) is an objective method to define the wild-type population, which is not affected by resistance development, on the susceptible side of the inhibition zone diameter histogram (Kronvall, 2003) and MIC distribution (Kronvall, 2010). This method offers a new statistically based tool in comparative susceptibility studies such as global surveillance of the reduced susceptibility and in intra-laboratory quality control (Kronvall, 2010). To date, there have been two studies using NRI to calculate the CO_{WT} values for *F. psychrophilum* MIC data and inhibition zone diameters in 40 Chilean isolates (Henríquez-Núñez *et al.*, 2012), and in 61 UK and Danish isolates (Smith *et al.*, 2016).

1.5. Vaccine development against *Flavobacterium psychrophilum*

Disease prevention by vaccination is one of the most important preventive measures in aquaculture, contributing to a sustainable aquaculture industry with low use of antimicrobials (Gudding, 2014). Despite the severe impact of BCWD or RTFS on the salmonids and rainbow trout industry, no commercial vaccines against this disease are currently available, though there are many reports of attempts to develop vaccines for BCWD or RTFS (Cipriano and Holt, 2005; Gómez *et al.*, 2014). However, it is difficult to compare the results of the different studies due to differences in fish species, vaccine preparations and methods of immunisation and challenge (Appendix 1).

1.5.1. Potential inactivated vaccines

Inactivated bacterial vaccines, depending upon the inactivation of whole pathogen, account for the majority of commercial vaccines available for use in aquaculture because of their safety and effectiveness (Munang'andu *et al.*, 2014). The main purpose of

inactivation is to obtain non-viable cells whilst retaining the conformational structure, which is essential to stimulate a protective immune response (Gómez *et al.*, 2014; Munang'andu *et al.*, 2014). There are two main methods to inactivate the pathogen, namely physical (ultraviolet, heat, sonication) and chemical (formalin) (Munang'andu *et al.*, 2014).

Heat inactivated whole cell bacterins provided a relative percent survival (RPS, the ratio of the proportion of observed survivors in a group of diseased fish to the proportion of expected survivors in a comparable set of disease-free fish; Amend, 1981) of 80% against the visceral form of BCWD in rainbow trout fry of 2.2 g (90 days post-hatch) previously immunised by intraperitoneal (i.p.) injection (Obach and Baudin-Laurencin, 1991). These authors also observed no protection in groups of fish <0.5 g (<50 days post-hatch), indicating that the fish should be at least 1 g in size prior to vaccination to ensure good protection. A formalin-killed *F. psychrophilum* cell (FKC) preparation elicited no significant protection and considerably low antibody titres in i.p. immunised rainbow trout fry of 3 g (LaFrentz *et al.*, 2002). In contrast, FKC and sonicated FKC of *F. psychrophilum* provided complete protection and induced significantly increased antibody titres in immunised rainbow trout (mean weight, 104 g) when i.p. administered without Freund's complete adjuvant (FCA) (Högfors *et al.*, 2008).

Several adjuvants have been used to improve vaccine efficacy in aquaculture worldwide. For fish vaccines, oil adjuvants and water-soluble adjuvants are the commonly used emulsion formulations (Taffala *et al.*, 2014). Rahman *et al.* (2000) documented a higher RPS (60%) and humoral antibody production in ayu than the vaccine alone (40%) following the intramuscular (i.m.) injection of *F. psychrophilum* FKC in an oil-based adjuvant with minimal water solubility (either Montanidae ISA 763A or a squalene emulsion). However, residues of vaccine with a minimal water soluble adjuvant were observed in ayu for at least 4 weeks after vaccination (Rahman *et al.*, 2003). Therefore, Rahman *et al.* (2003) investigated the efficacy, retention period and toxicity of highly water-soluble adjuvants (either Montanidae IMS 1311 or 1312VG) in ayu. These authors reported that 10 µL of vaccine containing 5 µL of highly water soluble adjuvant and 5 µL of FKC produced promising protection against RTFS/BCWD at 4 weeks post-vaccination, conferring high levels of protection (RPS up to 67%), a short retention period and no toxicity.

RPS values of 100% and 83% following i.p. injection were obtained in coho salmon of 5 – 27 g and rainbow trout fry of 3 g respectively, previously i.p. immunised

with FKC emulsified in FCA (Holt, 1987; LaFrentz *et al.*, 2002). The association between antibody titres and levels of protection from *F. psychrophilum* challenge was observed, suggesting the important role of antibody in conferring significant protection (LaFrentz *et al.*, 2002). However, the contribution of non-specific stimulation to the protection may also be important, as evidenced by the fact that coho salmon or rainbow trout immunised with FCA alone exhibited high RPS values (78% and 51% respectively) (Holt, 1987; LaFrentz *et al.*, 2002). In contrast, Högfors *et al.* (2008) documented weak non-specific antibody titres in plasma and no significant protection in rainbow trout of 104 g i.p. injected with FCA alone. Similarly, water-in-oil vaccine formulations containing four non-*F. psychrophilum* fish pathogens or adjuvant only produced mortality following challenge of 96% or 98% respectively, strongly supporting that non-specific immune responses induced by mineral oil or other bacterial components cannot protect the fish from *F. psychrophilum* infection (Fredriksen *et al.*, 2013a).

Intraperitoneal injection of mineral oil-adjuvanted vaccine consisting of formalin- or heat-inactivated *F. psychrophilum* of Fd and Th serotypes effectively triggered higher plasma antibody levels and provided higher RPS values (77% or 89% respectively) in rainbow trout (50 g), compared to non-vaccinated fish (Madetoja *et al.*, 2006). In addition, under the field conditions, the vaccine containing both of these formalin-inactivated *F. psychrophilum* strains produced high protection against *F. psychrophilum* with a cumulative mortality rate of 9% in a challenge 7 months post-vaccination.

An i.p. injection vaccine comprising FKC of *F. psychrophilum* strain of serotypes Fd and Th and four other fish pathogens was developed in a water-in-oil formulation and elicited protection levels of 44 – 67% in rainbow trout (37 g) after an i.m. injection challenge (Fredriksen *et al.*, 2013a). Similarly, divalent and multivalent water-in-oil formulated vaccines were prepared, then administered by i.p. injection route in rainbow trout of 33 g and provided high protection (78 – 80%) following an i.m. challenge (Fredriksen *et al.*, 2013b). However, bacterial antigens in polyvalent vaccines should be carefully selected in order to avoid the inhibitory effect of many immunobiological processes in the specific response of fish (Nikoskelainen *et al.*, 2007).

Other administration routes of vaccines are employed due to the time and cost involved in mass injection immunisation of fish, especially fry (<2 g) held in hatchery systems. The delivery methods via immersion, oral, cohabitation are not only more feasible for the farmers, but also minimise handling stress (Cipriano and Holt, 2005; Starliper and Schill, 2011). Immersion vaccination trials of salmonids against RTFS have

resulted in varying success. Immersion vaccination of coho salmon using FKC evoked protection (RPS of 68%) but not as high as the injection of the FKC in the adjuvant provided (RPS of 100%) (Holt, 1987). Obach and Baudin-Laurencin (1991) also obtained similar results, which indicated that the administration of heat inactivated vaccine via immersion provided significant protection in fish at 50 days post-hatch or later, but RPS values (14 – 47%) were considerably varied and lower than those via the injection route (80%). Additionally, following immersion immunisation with FKC, a RPS of 13% was reported in fry and no significant antibody responses were generated in immunised juvenile rainbow trout (LaFrentz *et al.*, 2002).

Numerous experiments on oral vaccines in aquaculture have been reported, but the protective effect of these vaccines is uncertain due to poor protection and high consumption of antigen (Lillehaug, 2014). Oral vaccination has been suggested as a booster after primary immersion vaccination (Thinh *et al.*, 2009). The efficacy of oral administration of *F. psychrophilum* FKC was evaluated in ayu of 0.5 g fed either every day for 2 weeks or 5 days over 2 weeks (Kondo *et al.*, 2003). After immersion challenge at 7 weeks post-vaccination, a significant increase in RPS values (up to 88%) in orally administered fish was obtained, as compared to the control fish. Although an increase in the agglutination titres in the serum of orally administered fish was not detected, skin mucus of these fish agglutinated *F. psychrophilum* and inhibited bacterial attachment, suggesting that oral vaccination could prevent surface colonisation and penetration of the pathogen in the fish host (Kondo *et al.*, 2003). Aoki *et al.* (2007) observed that oral vaccination by logarithmic phase *F. psychrophilum* FKC produced higher survival rates (RPS of 85 – 89%) in rainbow trout of 1.6 g than the stationary phase FKC (RPS of 22 – 42%). The differences in the protein patterns of logarithmic and stationary phase culture supernatants in SDS-PAGE analysis suggested that membrane vesicles on the cell surface are released into the medium during the stationary phase (Aoki *et al.*, 2007). The vesicles did not confer any protection when used as an oral vaccine, but oral administration of the combination of FKC and the membrane vesicles provided nearly complete protection (94 – 100%) in juvenile rainbow trout after the bath challenge (Aoki *et al.*, 2007). Therefore, membrane vesicles, probably consisting of outer membrane lipids, outer membrane proteins and soluble periplasmic components, were considered as an adjuvant necessary to obtain efficient protection (Aoki *et al.*, 2007).

1.5.2. Potential subunit vaccines

Surface characterisation of *F. psychrophilum* species has revealed several candidate antigens for developing subunit vaccines. Some of these antigens have been tested as potential vaccines in fish against the bacterial infection.

A vaccine formulation composed of the outer membrane fraction (OMF, 10 µg) of *F. psychrophilum*, applied i.p. in 2 g trout and 1.7 g ayu, conferred higher RPS values (up to 95%) than those in fish immunised with FKC (up to 46%) (Rahman *et al.*, 2002). The ability of the OMF vaccine to increase levels of protection and antibody titres was probably due to high protective antigens (outer membrane proteins and LPS) present in this preparation. These findings were supported by the study of LaFrentz *et al.* (2004), in which near complete protection (RPS of 94%) following a subcutaneous (s.c.) injection challenge was elicited in rainbow trout of 2.8 g i.p immunised with a high molecular mass protein fraction (70 – 100 kDa) emulsified with FCA. In addition, this protein fraction, which is presumed to be components of the glycocalyx of *F. psychrophilum*, exhibited significantly lower mean cumulative mortality (6%) when compared to saline controls (80 – 96%) and other treatments with 18 – 28 kDa protein fraction/FCA (66%), FKC/FCA (41%) and 41 – 49 kDa protein fraction/FCA (40%) (LaFrentz *et al.*, 2004). Recently, a crude LPS extract from *F. psychrophium*, which is mainly represented as a 16 kDa proteinase K-resistant band, was used for immunisation of rainbow trout (3 g) at doses of 10 or 25 µg with or without FCA by i.p. injection (LaFrentz *et al.*, 2014). Significant antibody responses against *F. psychrophilum* were observed in the crude LPS/FCA treatment groups and only specific for low-molecular mass proteins (14 – 22 kDa), while no specific antibodies were exhibited for *F. psychrophilum* LPS (LaFrentz *et al.*, 2014). Minimal levels of protection were conferred (RPS of 13% and 25%) for fish immunised with 10 or 25 µg of crude LPS/FCA respectively (LaFrentz *et al.*, 2014).

Another candidate for the development of subunit vaccines is the immunogenic 25 – 33 kDa protein fraction identified by Högfors *et al.* (2008). This fraction (5 µg) in combination with FCA induced a significantly elevated humoral immune response and protected i.p. immunised rainbow trout of 15 g from *F. psychrophilum* infection with cumulative mortality of 10 – 15% (Högfors *et al.*, 2008). Moreover, the use of an oil adjuvant in an i.p. injection administration with the subunit vaccine appears necessary (Högfors *et al.*, 2008).

Vaccination trials by i.p. injection with a fraction highly enriched with a 18 kDa outer membrane-associated OmpH-like protein with or without FCA (7 µg or 14 µg of

protein respectively) in rainbow trout of 2.8 g were demonstrated to induce high titres of protective antibodies with the RPS of 89% and 51% respectively (Dumetz *et al.*, 2006). Similarly, Dumetz *et al.* (2007) evaluated the potential of a membrane glycoprotein OmpA (i.e., P60; Merle *et al.*, 2003) as a vaccine candidate. The results revealed that 50 µg of OmpA emulsified with FCA developed a high specific anti-OmpA antibody titre in rainbow trout of 2.8 g following i.p. injection immunisation, suggesting that OmpA could be a promising candidate for vaccine development against *F. psychrophilum* infection (Dumetz *et al.*, 2007).

Recombinant heat shock proteins, HSP60 and HSP70 of *F. psychrophilum*, expressed and purified from *E. coli* were emulsified with FCA and used as i.p. injection vaccines containing a total of 8 µg of protein for each 2 g rainbow trout (Plant *et al.*, 2009). Although antibody titres were elevated against these two proteins, especially against HSP70, no protective responses against a s.c. injection challenge with *F. psychrophilum* strain were observed. Similarly, three immunogenic proteins of *F. psychrophilum*, elongation factor-Tu (EFTU), SufB Fe-S assembly protein (SufB) and ATP synthase β, were cloned into *E. coli*, purified, emulsified in FCA and subsequently used as vaccines in 2-g rainbow trout (Plant *et al.*, 2011). However, the results indicated that these recombinant proteins were not suitable vaccine candidates for *F. psychrophilum* when administered via i.p. injection (Plant *et al.*, 2011).

A putative antigen (FL1493) was distinguished by an ability to form high molecular mass multimers and immunoreactivity with convalescent rainbow trout sera and a monoclonal antibody (FL-43) (Gliniewicz *et al.*, 2012). This antigen was expressed in *E. coli* as a recombinant protein, emulsified in FCA and tested as an i.p. injection vaccine in rainbow trout (2 g) (Gliniewicz *et al.*, 2012). However, this vaccine did not demonstrate a protective effect against the s.c. injection challenge with the parental strain (Gliniewicz *et al.*, 2012).

Using immunoproteomic analyses of ayu antiserum against *F. psychrophilum*, three proteins, 3-hydroxyacyl-CoA dehydrogenase (HCD), ATP synthase beta subunit (atpD), and glutamate dehydrogenase (gdhA) located on the cell surface of *F. psychrophilum*, were used for vaccine trials (Kato *et al.*, 2014). Recombinant *E. coli* cells expressing each of these proteins were inactivated with formalin and then i.p. injected into ayu of 2.2 g (Kato *et al.*, 2014). The vaccination produced significantly high antibody titers against the three recombinant proteins in vaccinated fish serum and

significantly longer survival times compared to the PBS-injected group, despite low RPS values (32% – 37%) (Kato *et al.*, 2014).

1.5.3. Potential attenuated vaccines

A first attenuated *F. psychrophilum* strain was developed by using transposon insertion mutagenesis to disrupt a gene coding for the ExbD2 protein, a member of the TonB complex (Álvarez *et al.*, 2008). The mutant was approximately 450-fold attenuated compared to the parent, suggesting that a functional ExbD2, involved in iron uptake, is essential for full virulence in *F. psychrophilum* (Álvarez *et al.*, 2008). The i.m. injection of this attenuated strain into rainbow trout (5 g) provided a high RPS value of 82% in vaccinated rainbow trout fry against i.m. injection challenge with the parental strain (Álvarez *et al.*, 2008). Skin lesions were observed in vaccinated fish after the challenge but healed in a few days (Álvarez *et al.*, 2008).

Two attenuated *F. psychrophilum* strains were developed by passaging the virulent strain on TYES plates containing increasing concentrations of rifampicin (LaFrentz *et al.*, 2008). These rifampicin resistant strains were generated and one of them (CSF259-93B.17) was described as highly attenuated (LaFrentz *et al.*, 2008). Compared to the wild-type strain, the 259-93B.17 strain showed five differentially expressed proteins, the roles of which are still unknown (LaFrentz *et al.*, 2008). An i.p. immunisation of rainbow trout (2.4 g) using the CSF259-93B.17 strain elevated specific antibody titres and exhibited a significantly protective immune response (RPS of 45% at 8 weeks post-vaccination) after the s.c. challenge with the virulent parent strain (LaFrentz *et al.*, 2008). Immersion exposure of the CSF259-93B.17 strain in rainbow trout (3.4 g) developed protective immune responses at 10 weeks post-vaccination (LaFrentz *et al.*, 2008). Gliniewicz and colleagues (2012) identified a mutation in the *rpoB* gene of the CSF259-93B.17 strain, which encodes the β-subunit of the RNA polymerase, and an altered protein profile with six specific proteins of this rifampicin resistant strain.

Another attenuated *F. psychrophilum* strain was developed by culturing the CSF259-93B.17 strain under iron-limited conditions and termed CSF259-93B.17 ILM (Long *et al.*, 2013). Immunisation of coho salmon (3.6 g) using CSF259-93B.17 and CSF259-93B.17 ILM strains by i.p injection or 1-h immersion (with adipose fin removal) resulted in significantly higher antibody titres against *F. psychrophilum*, compared to the mock immunised fish (Long *et al.*, 2013). After a s.c. injection challenge with *F. psychrophilum*, RPS values of CSF259-93B.17 ILM immunisation (73% and 98%)

were higher than those of CSF259-93B.17 immunisation (46% and 90%) in both immersion- and injection-delivery methods respectively (Long *et al.*, 2013).

The efficacy of the live attenuated vaccine CSF259-93B.17 ILM was further evaluated in rainbow trout (2.5 g) and was improved by altering the diet prior to vaccination with a commercially available health enhancing aquaculture feed (Sudheesh *et al.*, 2016). Using a shorter immersion time (3 min) and without removing the adipose fin of the vaccinated fish, the immunisation not only produced significantly increased antibody titres but also provided significant protection in rainbow trout with RPS values of 61% for the normal diet and 69% for the alternative diet (Sudheesh *et al.*, 2016).

1.5.4. Potential live wild-type based vaccines

A potential immersion vaccine containing live wild-type *F. psychrophilum* strains has been examined. Rainbow trout fry (1 g) were immersed in live *F. psychrophilum* cells (serotype Th) for 30 min and challenged by i.p. injection 26 days or 47 days later with a more pathogenic strain (Lorenzen *et al.*, 2010). Although specific antibody titres were detected only in 25% of the immunised fish at the time of challenge, high levels of protective response (RPS of 88% at 26 days and 60% at 47 days) were obtained against the unnatural challenge route, bypassing the intrinsic defence barriers of the fish (Lorenzen *et al.*, 2010). However, the use of wild-type based vaccines under field conditions is probably a risk due to the release of potentially virulent pathogens into the aquaculture environment.

1.5.5. Potential DNA vaccines

DNA vaccination is a form of genetic immunisation that uses a gene or genes encoding protective antigens and can produce protection against intracellular pathogens by mimicking the natural route of infection (Plant *et al.*, 2009; Biering and Salonius, 2014). Plant *et al.* (2009) investigated the efficacy of DNA vaccines against *F. psychophilum* infection using the pVAX1 vector and genes encoding heat shock proteins HSP60 and HSP70 in rainbow trout (2 g) immunised by i.m. injection. Although low antibody responses and no protection were observed following the s.c. injection challenge at 6 or 10 weeks post-vaccination, HSP genes were confirmed to be translated in the host cells and subsequently stimulated the immune response against HSP60 at low levels (Plant *et al.*, 2009).

1.5.6. Passive immunisation

The role of antibody in conferring protection to rainbow trout fry (mean weight of 1 – 1.3 g) was determined by passive immunisation with anti-*F. psychrophilum* sera collected from convalescent or immunised adult rainbow trout or goat (LaFrentz *et al.*, 2003). The antisera were i.p. injected into fry, which were then challenged by s.c. injection with a virulent *F. psychrophilum* strain (LaFrentz *et al.*, 2003). Higher RPS values (57%) were obtained in fry administered with anti-*F. psychrophilum* serum from immunised adult fish than those from convalescent fish (9 – 42%). Goat antiserum did not confer any protection to fry, suggesting that trout antibodies are important but not the only factors in providing complete protection to *F. psychrophilum* (LaFrentz *et al.*, 2003). Similarly, Kato *et al.* (2015) performed passive immunisation against *F. psychrophilum* infection in 2 g ayu by i.p. injection. Although the RPS value was low (21%), survival times of antiserum-immunised group were significantly different from those of the control serum-immunised and PBS-immunised groups (Kato *et al.*, 2015). Despite the protective effect observed in several studies, passive immunisation is not a practical solution for mass vaccination.

In general, despite many attempts to develop vaccines for BCWD or RTFS caused by *F. psychrophilum* (Appendix 1), no commercial vaccines are currently available for the prevention of this disease. New strategies are needed to develop vaccines against *F. psychrophilum* infection, which are cross-protective, cost-effective and applicable to small fish.

1.6. Interaction of *Flavobacterium psychrophilum* with the fish immune system

1.6.1. Overview of fish immunity

Fish are a wide and diverse group of vertebrates comprising about 40,000 species. In the history of evolution, fish have undergone at least two rounds of whole-genome duplication, which supposedly has led to the expansion of some immune molecular families in several fish species, resulting in the functional effects of this molecular diversification compared to mammals (reviewed in Castro and Taffala, 2015). In addition, living in pathogen-rich aquatic environment, fish are suggested to have a well-functioning mucosal immune system (Rombout *et al.*, 2011).

A pathogen penetrating via fish mucosal tissues, such as gills, skin or gut is primarily blocked by epithelial or mucosal barriers. The mucosal layers not only have the

capability of trapping the invader but also contain various antimicrobial factors (lectins, lysozymes, pentraxins, complement proteins, antibacterial peptides and natural antibodies) to eliminate the infectious agent (Castro and Taffala, 2015). Passing through these physical barriers successfully, the pathogen then confronts the humoral and cellular components of the innate immune system, including different cell types bearing invariable receptors (pattern recognition receptors) with the ability to recognise conserved molecular characteristics of many microorganisms. At the same time, antigen-specific lymphocytes bearing variable receptors with the ability to specifically recognise exclusive molecules of a pathogen will also be activated, setting the basis for further secondary responses (Figure 1.8) (Magnadóttir, 2006; Castro and Taffala, 2015).

1.6.2. Lymphoid organs

1.6.2.1. Thymus

The thymus is considered as the primary organ for functional T lymphocyte development and is present in all jawed vertebrates (Secombes and Wang, 2012). The teleost thymus is located on the dorsolateral region of the gill chamber and possesses a highly conserved cortex/medulla architecture (Castro and Taffala, 2015).

1.6.2.2. Kidney

Functions of the kidney in fish includes the hematopoietic, reticulo-endothelial, endocrine, and secretory systems (Secombes and Wang, 2012; Castro and Taffala, 2015). The anterior portion of the kidney (defined as anterior kidney, pronephros, or head kidney) is the main site for hematopoiesis and consists primarily of hematopoietic and lymphoid tissue with some adrenal-like endocrine tissues (Castro and Taffala, 2015). Meanwhile, the posterior kidney works as a renal system (Secombes and Wang, 2012; Castro and Taffala, 2015).

The fish head kidney appears to be the primary organ for antibody production with the presence of proliferating B cell precursors and plasma cells (Secombes and Wang, 2012; Castro and Taffala, 2015). The development of B cells occurs in the anterior kidney, which are then distributed via the blood to the spleen and posterior kidney for activation, leading to the plasmablast stage and mature plasma cells, which are then relocated in the anterior kidney to become long-lived (Zwollo *et al.*, 2008; Ye *et al.*, 2011).

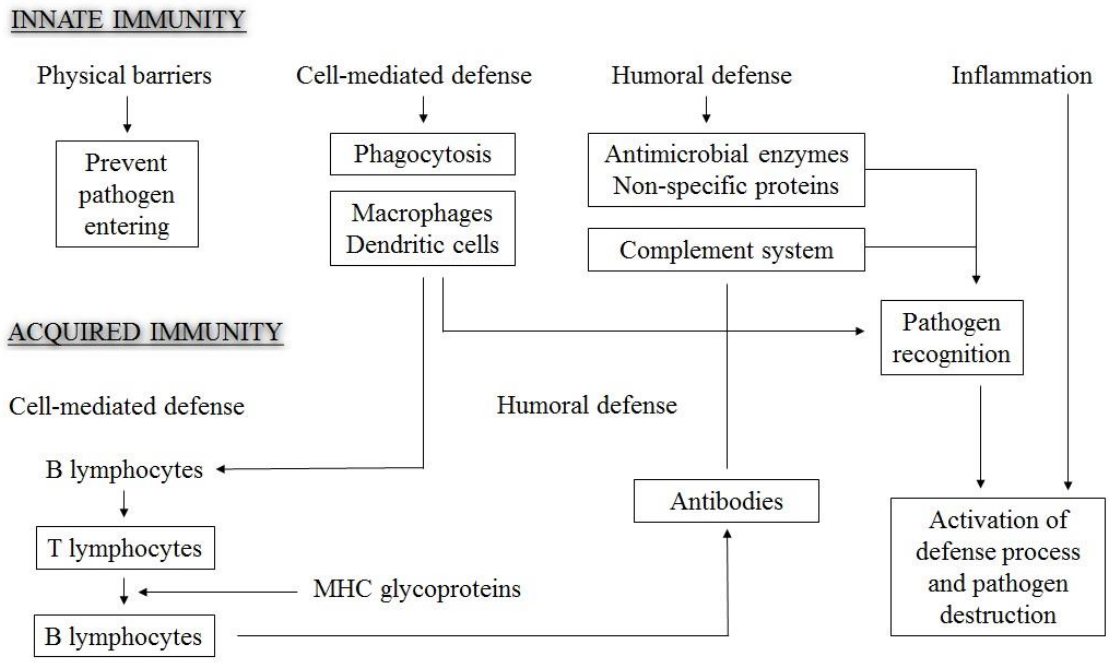


Figure 1.8. The concept of fish immune system (Biller-Takahashi and Urbinati, 2014). MHC, major histocompatibility complex.

1.6.2.3. Spleen

A spleen is found in virtually all vertebrates and serves several functions, such as blood filtration, erythrocytic destruction, antigen presentation and antibody production (Zapata, 1982; Castro and Taffala, 2015). Spleen structures of teleosts and mammals are similar, with blood vessels and differentiated red pulp and white pulp (Secombes and Wang, 2012; Castro and Taffala, 2015).

The spleen can be a major reservoir of disease. In comparison with other organs (e.g. skin, muscle, gills and liver), spleen and kidney contain the highest levels of *F. psychrophilum*, possibly leading to the suppression of the acquired immune system triggered by this pathogen (Orieux *et al.*, 2013). A positive correlation between spleen size and resistance to *F. psychrophilum* has been documented but whether this is due to greater filtering capacity or improved immune functions is unknown (Hadidi *et al.*, 2008).

1.6.3. Mucosal-associated lymphoid tissues

Teleosts are among the first vertebrate animals to exhibit mucosal immune defenses (Peterson, 2015). The aquatic environment presents continuous and intimate surface contact of potential pathogens unique to the mucosal tissues of fish. The amplitude and

efficacy of fish mucosal responses can be remarkably impacted by environmental factors, such as photoperiod, ambient water temperature and water quality (Tort *et al.*, 2004; Niklasson *et al.*, 2011; Peterson, 2015). Constitutive mucosal tissues in teleost fish include the gills, integument and intestine (Castro and Taffala, 2015).

1.6.3.1. Gills

The gills are considered to be an important portal of entry for several bacterial fish pathogens (Henriksen, 2013). The gill filament and lamellar epithelium are covered by a surface mucous layer, which is an initial physical barrier to pathogens and environmental insults (Peterson, 2015). This mucous layer contains various bioactive substances, including enzymes, antimicrobial peptides and preformed antibodies of IgM isotype released from the systemic circulation (Olsen *et al.*, 2011). IgT positive cells were found in the epithelial lining of gills and CD8 positive cells at the base of the primary gill filaments, indicating the potential for a rapid response against invading pathogens (Olsen *et al.*, 2011). In younger life stages, where immunity is not yet fully developed, the dense layer of IgT may act as a shield against pathogens (Chettri *et al.*, 2012).

1.6.3.2. Skin

The skin of fish is the largest mucosal tissue. The epidermal mucous layer of fish contains various preformed peptides, enzymes, agglutinating lectins, analogous resident dendritic cells, lymphocytes (B cells and plasma cells) and secreted antibodies (IgM and IgT), thus contributing significantly to the immune defense against pathogens (Davidson *et al.*, 1997; Hatten *et al.*, 2001; Nakamura *et al.*, 2004; Zhao *et al.*, 2008). Detectable antibodies in the skin mucus display a lag phenomenon in contrast to serum antibodies during parenteral systemic vaccinations, regardless of the population of plasma cells in the epidermis (Rombout *et al.*, 2011). That may be explained by transport of the antibodies through the epidermis to the mucous layer or possibly due to the timing of antibody production in a given systemic response (Rombout *et al.*, 2011).

1.6.3.3. Gut

Gut-associated lymphoid tissue (GALT) is widely assumed to be a constant feature among vertebrates (Peterson, 2015), although in fish there is lack of the well-defined Peyer's patches (Lin *et al.*, 2005) or the localised lymphocytic aggregates compared to mammals, birds and reptiles (Peterson, 2015). GALT of fish includes frequent intermixed

populations of immune cells as well as loosely aggregated and scattered lymphocytes, eosinophilic granule cells, rodlet cells, neutrophils, and macrophages within the intestinal lamina propria (Reite and Evensen, 2006). Although serum immunoglobulin IgM is transported into the intestinal mucosa when pathogens are encountered, locally produced soluble antibodies (IgM, IgT/IgZ) can be positively secreted by GALT-analog plasma cells (Flajnik, 2010). The hindgut, or posterior intestinal segment, was initially discovered as the preferential site of bacterial antigen uptake and processing, eliciting both systemic and mucosal immune responses to intra-intestinal pathogens (Rombout *et al.*, 1989).

1.6.4. Innate immunity

The most important cells in the first line of defense in fish are the phagocytes, including neutrophils and monocytes/macrophages, and the non-specific cytotoxic cells, which are thought to be the pre-cursor for the natural killer cells in mammals (Magnadóttir, 2006). Furthermore, epithelial and dendritic cells also contribute to the innate immune response. The pathogens are engulfed by phagocytes and processed in the phagolysosomes. Parts of the destroyed microbe are then transported to the surface of antigen-presenting cells and are presented to T cells, thereby initiating the adaptive immune response (Magnadóttir, 2006).

1.6.4.1. Pathogen recognition

Once a pathogen succeeds in breaching the physical barriers and penetrating into the epithelium, pattern recognition receptors (PRRs) on immune cells, mainly dendritic cells, and macrophages, are activated in response to a conserved motif in pathogens, such as bacterial lipopolysaccharides, fungal β-glucan, viral double stranded RNA and unmethylated CpG motifs in bacterial DNA (Magnadóttir, 2006; Castro and Taffala, 2015). These receptors are designated as pathogen-associated molecular patterns (PAMP), or damage-associated molecular patterns (DAMP) in response to cell components released during cell damage (Castro and Taffala, 2015). There are two sub-groups of PRRs categorised based on function: endocytic PRRs and secreted signaling PRRs (Magnadóttir, 2006).

The endocytic PRRs, including mannose receptors, scavenger receptors and opsonin receptors, are located on the surface of phagocytes and promote attachment,

engulfment and destruction of microorganisms but do not transmit an intracellular signal (Magnadóttir, 2006).

Signaling PRRs are also known as toll-like receptors (TLRs), located on the cell surface or intracellularly in the membrane of endosomes. In mammals, TLR3, 7, 8, and 9 are primarily involved in viral detection, while TLR1, 2, 4, 5, 6, 7, 8, and 9 recognise highly conserved bacterial structures (Pietretti and Wiegertjes, 2014). At least 16 TLRs have been identified in teleost fish, but only a few of them (TLR2, TLR3, TLR5M, TLR5S and TLR22) have been characterised in regards to ligand specificity (Pietretti and Wiegertjes, 2014). Although many similarities are seen with mammalian TLRs, remarkable distinct features of teleostean TLR cascades have been discovered (Rebl *et al.*, 2010; Pietretti and Wiegertjes, 2014).

Similar to human TLR5, fish TLR5 recognises flagellin, a protein monomer making up the bacterial filament found on nearly all motile bacteria (Pietretti and Wiegertjes, 2014). Rainbow trout presents a membrane-bound and soluble TLR5, while cyprinids seem to express membrane-form TLR5 only (Pietretti and Wiegertjes, 2014). Evenhuis and Cleverland (2012) observed no significant differences in the expression of *TLR5* in rainbow trout following an injection challenge with *F. psychrophilum* compared to the control group, possibly due to the lack of flagella in *F. psychrophilum*.

1.6.4.2. Inflammation

The activation of PRRs leads to the release of inflammatory mediators, which increase the movement of plasma cells and leucocytes into infected tissue. Acute inflammation is a localised protective response to tissue injury or infection, aiming not only to eliminate pathogens, confine the area, stimulate the immune response and incite wound healing, but also to contribute to antigen presentation by immune cells (Murphy, 2012a). The activation of cell-adhesion molecules expressed by endothelial cell walls and slower blood flow facilitate the migration of leucocytes into the tissue (Murphy, 2012a).

Neutrophils are the first leucocytes to migrate to the site of inflammation by chemotaxis. Neutrophils are very efficient at sweeping up surface-associated, but not fluid-borne, bacteria (Colucci-Guyon *et al.*, 2011). The phagocytic ability of neutrophils is essential for the elimination of microbes and cellular remains before apoptosis, removal in inflammatory exudates (e.g. pus), or reverse migration of neutrophil occurs (Henry *et al.*, 2013).

Secondly, the phagocytotic monocytes arrive and differentiate into macrophages or dendritic cells depending on cytokines in the environment (Henriksen, 2013). In contrast to neutrophils, macrophages effectively phagocytise bacteria from blood or fluid-filled body cavities (Colucci-Guyon *et al.*, 2011) and present antigens to the B and T cells, associated with the adaptive immune response. Furthermore, eosinophils and basophils are important contributors in combating invading microbes (Murphy, 2012b).

The activation of the complement system also mediates inflammation through the cleavage of C3, C4 and C5 to recruit leucocytes to the site of tissue injury (Boshra *et al.*, 2006). This cleavage activity enhances phagocytic activity of head kidney leucocytes three- to four fold, which is not seen in mammals (Boshra *et al.*, 2006). The level of inflammation induced is a balance between the pro-inflammatory and the anti-inflammatory cytokines (Henriksen, 2013).

In teleosts, both IgM^+ and IgT^+ B cells were demonstrated to actively internalise beads or bacteria and then lead to phagolysosome fusion (Li *et al.*, 2006; Zhang *et al.*, 2010). Although their contribution to pathogen clearance is lower than other phagocytes, this phagocytic activity facilitates the development of a higher capacity of antigen-presenting B cells in fish (Catro and Taffala, 2015).

1.6.5. Adaptive immune response

1.6.5.1. Antigen presentation

In fish, antigen presentation appears to take place locally where the antigen is present (Castro *et al.*, 2014a). Major histocompatibility complex (MHC) class I molecules are resident in nearly every nucleated cell of the body and bind to intracellular peptides produced during an intracellular infection or through a cell cycle alteration (tumor cells) (Castro and Taffala, 2015). MHC I presents the peptides on the cell surface and displays them to Tc lymphocytes, leading to the killing of target cells (Castro and Taffala, 2015). An up-regulation of MHC I was observed in head kidney of *F. psychrophilum*-infected rainbow trout shortly after an immersion challenge (Henriksen *et al.*, 2015a), while no expression changes of this transcript was found in gills of challenged fish (Henriksen *et al.* 2015b). A negative correlation between MHC I transcript abundance and bacterial load in gills was found at 192 h post-challenge (Henriksen *et al.*, 2015b).

Meanwhile, MHC class II are found only on specific cell types of fish, such as macrophages, dendritic cells, B cells, thrombocytes, and acidophilic granulocytes, which have been assumed to have antigen-presenting properties (Castro and Taffala, 2015).

MHC class II exposes parts of extracellular proteins that have been taken up and processed through phagocytosis on the surface by these cells (Castro and Taffala, 2015). T cells, usually $CD4^+$ Th cells, are activated and secrete cytokines to modulate the adaptive immune response after recognising this MHC II:antigen complex (Castro and Taffala, 2015).

The expression of genes encoding for the MHC class II was noted to be down-regulated in the spleen of naturally diseased fish (Orieux *et al.*, 2013) and head kidney of immersion-challenged rainbow trout with *F. psychrophilum* (Henriksen *et al.*, 2015a). The repression of the immune response in spleen and head kidney triggered by the pathogen may lead to a disease outbreak occurring (Orieux *et al.*, 2013). Meanwhile, no changes in the expression of *MHC II* transcript was observed in rainbow trout gills following an immersion challenge with *F. psychrophilum,* and transcript abundance of *MHC II* was found to be negatively correlated with bacterial load in gills at 192 h post-challenge (Henriksen *et al.*, 2015b).

1.6.5.2. Cytotoxic responses

Natural killer-like cells and T cytotoxic (Tc) cells are responsible for identifying and killing antigen-presenting cells in the context of MHC class I or II. Natural killer cells sense the modified expression of MHC-I to identify altered cells, whereas Tc cells, also called $CD8^+$ T cells, bind to a certain antigenic peptide exposed in a MHC-I complex via the co-receptor protein CD8 (Randelli *et al.*, 2008).

CD8 is a glycoprotein expressed on T cells (Ellmeier *et al.* 1998). Regulation of CD8 is repressed in rainbow trout naturally infected with *F. psychrophilum* (Orieux *et al.*, 2013), while it is up-regulated in the spleen and kidney of s.c. injection-challenged fish (Overturf and LaPatra, 2006). In an immersion challenge with *F. psychrophilum*, no significant change in the regulation of *CD8* expression was found in the head kidney of rainbow trout (Henriksen *et al.*, 2015a), while a late over-expression (after 192 h) of this transcript was reported in gills of challenged fish (Henriksen *et al.*, 2015b).

On the other hand, T cells carrying the co-receptor protein CD4 recognise the MHC II:peptide complex. $CD4^+$ T cells differentiate into T_h1 and T_h2 effector cells (also known as T helper cells), which respectively activate macrophages and stimulate antibody production through B cell proliferation (Randelli *et al.*, 2008). The expression of *CD4* did not present any change in the head kidney and gills of rainbow trout after the

fish were immersion-challenged with *F. psychrophilum*, leaving no clear picture of either a T_h1 or T_h2 response in protection against this bacterium (Henriksen *et al.*, 2015a,b).

The development and functionality of regulatory T cells, which generally turn the immune response down to maintain self-tolerance and immune homeostasis, are regulated by FoxP3, a member of the FOX protein family (Sakaguchi *et al*., 2009). Two paralogue genes, *FoxP3a* and *FoxP3b*, have been identified in rainbow trout but their functions are still largely unknown (Wang *et al.*, 2010a). In an immersion-based *F. psychrophilum* challenge with or without H_2O_2 pretreatment in rainbow trout, down-regulation of *FoxP3a* in the *F. psychrophilum* group and down-regulation of *FoxP3b* in both treatment groups was observed (Henriksen *et al.*, 2015a). A positive correlation with pathogen load and transcripts of these two genes was found early after exposure to only *F. psychrophilum*, suggesting that *F. psychrophilum* does not induce any tolerance-associated reactions (Henriksen *et al.*, 2015a).

Transcript abundance for the gene encoding the T-cell receptor beta (*TCR-β*), considered as a relative measure for T cells, did not show any significant increase in rainbow trout following injection challenge with *F. psychrophilum* (Evenhuis and Cleverland, 2012). Meanwhile, this gene was down-regulated in the spleen of rainbow trout following an immersion-based challenge with *F. psychrophilum* (Henriksen *et al.*, 2015a). Therefore, the importance of the cell-mediated immune response in protection against *F. psychrophilum* infection needs further investigation.

1.6.5.3. Antibody production

Compared to mammals, the overall production of antibodies in jawed fish is slower and weaker, and the antibodies are of a lower affinity and diversity (Randelli *et al.*, 2008). In salmonids, the secondary antibody response is of a lesser degree, compared to mammals, and the response time for antibody production (at least 4 – 6 weeks) is temperature-dependent (Ellis, 2001).

B cells express immunoglobulins (Igs) either on the cell surface as antigen receptors, defining different B cell types, or secrete them as soluble Igs or antibodies. The secreted antibody acts as neutralizing antibodies by binding to a pathogen and in some cases directly interfering with the replication capacity of the pathogen (Stafford *et al.*, 2006). Furthermore, the formation of antibody-pathogen complexes facilitates not only phagocytosis by the host leucocytes, but also bactericidal activity of the host complement system (Stafford *et al.*, 2006). In addition to the predominant antibody IgM, specific IgD

and IgT/IgZ have been characterised in teleosts, suggesting that all three Igs cooperate in pathogen clearance although their specific functions are still unclear (Zhang *et al.*, 2010; Xu *et al.*, 2013; Castro and Taffala, 2015). Among the three Igs, constitutive expression of *IgM* transcripts were found to be greatest in gill, skin, gut and lymphocytes of rainbow trout, followed by *IgT* (Makesh *et al.*, 2015).

1.6.6. Cellular components

The interaction of *F. psychrophilum* and the innate host immune system has been investigated in several studies. Rangdale *et al.* (1999) reported extensive lysosomal activity within granulocytes, which were present in apparently increased numbers compared to lymphocytes, plasma cells and thrombocytes in the spleen of rainbow trout fry with a natural RTFS infection. However, little evidence of an effective phagocytic response, and numerous filamentous bacteria within phagocytic vehicles together with extensive cellular degeneration were observed, indicating the phagocytic response to the pathogen was not effective (Rangdale *et al.*, 1999). An increasing number of *F. psychrophilum* viable cells over the course of time were found in the spleen phagocytic cells of i.p. *F. psychrophilum*-injected rainbow trout fry, aged 10 weeks (Decostere *et al.*, 2001). This suggested that surviving within phagocytes protects bacteria from complement and lysozyme activity and other humoral defence mechanisms of the host, constituting an important virulence trait (Decostere *et al.*, 2001). However, in the experimental models with fish aged 5 months and older, phagocytosis was not induced and no viable intracellular *F. psychrophilum* bacteria were isolated (Decostere *et al.*, 2001). These findings clearly demonstrated the age related antibacterial activity of spleen phagocytes to *F. psychrophilum* infections (Decostere *et al.*, 2001). In comparison with head kidney macrophages, spleen macrophages might be a 'safe site' for *F. psychrophilum* to reside due to significantly weaker bactericidal activity and lower production of reactive oxygen residue (Nematollahi *et al.*, 2005).

Using a chemiluminescence technique, *F. psychrophilum* and their metabolites were shown to stimulate head kidney phagocytes from mature rainbow trout *in vitro* by a heat labile carbohydrate component (Lammens *et al.*, 2000). Similarly, Wiklund and Dalsgaard (2003) also demonstrated that the *in vitro* association of *F. psychrophilum* with rainbow trout kidney phagocytes was mediated by opsonin independent cell-receptor adhesion, which involved carbohydrates on the bacterial surface. Furthermore, this type of binding was elucidated to be non-cytotoxic for phagocytes, suggesting the cytotoxic

effect for phagocytes is not necessary for the virulence of *F. psychrophilum* (Wiklund and Dalsgaard, 2003). In constrast, Nematollahi *et al.* (2005) observed that the cytotoxic effects of *F. psychrophilum* for trout macrophages were recorded and positively correlated with bacterial virulence. In addition, both virulent and avirulent *F. psychrophilum* strains adhered equally to macrophages, suggesting that the difference in bacterial survival is attributed to a difference in resistance to killing by macrophages (Wiklund and Dalsgaard, 2003). Although non-virulent strains of serotype Fp^T exhibited a stronger association with phagocytes, resulting in a high rate of bacterial killing after 2 h incubation, a relationship between the virulence or serotype of *F. psychrophilum* isolates and their capacity to stimulate or associate with the phagocytes was not observed (Lammens *et al.*, 2000; Wiklund and Dalsgaard, 2003).

1.6.7. Humoral components

1.6.7.1. Complement system

The complement system is an ancient innate immune mechanism present in both vertebrate and invertebrate species. This enzyme cascade system consists of several proteins and glycoproteins, either plasma- or membrane-associated. Functions of the complement system include pathogen clearance, promotion of inflammatory responses, elimination of cell debris, and modulation of the adaptive immune response (Taylor *et al.*, 1998; Walport, 2001; Castro and Taffala, 2015).

Complement proteins are normally present as inactive precursors until the system is activated by one of three pathways: the classical pathway, the alternative pathway, and the lectin pathway, all resulting in the formation of the protease C3 convertase (Castro and Taffala, 2015). The classical pathway requires the antigen:antibody complexes, part of the specific immune response, for the activation of the C1 component that, together with C2 and C4 components, lead to the formation of the protease C3 convertase (Castro and Taffala, 2015). Meanwhile, the alternative and lectin pathways are parts of the innate response and can be activated by C3 hydrolysis or antigens (Castro and Taffala, 2015). The alternative pathway is initiated by the spontaneous activation and binding of the C3 component to pathogen surfaces, leading to the interaction with plasmatic factors B and D and, finally, to the formation of the protease C3 convertase (Castro and Taffala, 2015). The lectin pathway is activated by the interaction of a C-type lectin, such as mannose-binding lectin and ficolins, with sugar moieties found on the surface of microbes,

activating a system of proteases that cleave the C2 and C4 components in a similar way as the classical pathway (Taylor *et al.*, 1998; Walport, 2001; Castro and Taffala, 2015).

In rainbow trout serum, complement consumption by *F. psychrophilum* strains was observed, but the bacterial cells were found to be resistant to the bactericidal effect of the activated complement, even in the presence of specific antibodies (Wiklund and Dalsgaard, 2002). These results indicated that *F. psychrophilum* isolated from rainbow trout are unaffected by both classical and alternative complement pathways in rainbow trout serum. Furthermore, *F. psychrophilum* could survive in immune sera of rainbow trout for up to 72 h without a reduction in viability, suggesting that an elevated antibody level against this bacterium cannot provide complete protection against invading virulent *F. psychrophilum* (Wiklund and Dalsgaard, 2002).

On the other hand, Kato *et al.* (2015) reported that bacterial opsonisation stimulated the phagocytic activity of leucocytes and the bactericidal activity of the humoral component in ayu, suggesting that both alternative and classical complement pathways are associated with the elimination of *F. psychrophilum* by the ayu immune system. In addition, high values for cumulative percent survival of ayu (up to 60%) were obtained when *F. psychrophilum* was opsonised in a high concentration of antiserum, implying that specific antibody production may be important for ayu immunity against *F. psychrophilum* (Kato *et al.*, 2015). Conversely, Nagai and Nakai (2011) reported that *F. psychrophilum* isolates from ayu not only resisted complement-killing by normal ayu serum, but also grew well up to 116-fold of CFU in this serum at 18^{o}C after 24-h incubation. The survival of ayu *F. psychrophilum* isolates in immune ayu serum was observed but no significant change in their growth was recorded, suggesting the static effect of the complement system activated via the classical pathway (Nagai and Nakai, 2011).

The correlation between host specificity and bacterial virulence was determined by inoculating *F. psychrophilum* isolates in sera prepared from different fish species (Nagai and Nakai, 2011). *F. psychrophilum* isolates from ayu were pathogenic to ayu but not to red spotted masu trout (RSMT). Meanwhile, other salmonid and cyprinid isolates were not pathogenic to ayu but some of them were pathogenic to RSMT, indicating different host-pathogen interactions in *F. psychrophilum* infection (Nagai and Nakai, 2011).

Complement factor C3 is the central component of the complement system and is cleaved by the protease C3 convertase into a small (C3a) and a large (C3b) fragment

(Ellis, 1999). The small soluble C3a fragment increases vascular permeability and then recruits phagocytes to the infection site (Ellis, 1999). C3b is deposited on the membrane, tags bacteria and thus enhances the recognition and phagocytosis of polymorphonuclear cells and macrophages (Ellis, 1999). The expression level of *C3* was found to be increased significantly in the spleen and kidney of rainbow trout 5 days post-infection by s.c. injection with *F. psychrophilum* (Overturf and LaPatra, 2006). When a greater dose of *F. psychrophilum* was used, *C3* transcripts in the liver were also significantly elevated. Moreover, *C3* was strongly induced in the *F. psychrophilum*-resistant fish line, suggesting that complement factor C3 may play an important role in the variation of the susceptibility to *F. psychrophilum* infection in fish (Langevin *et al.*, 2012).

1.6.7.2. Cytokines

a. Chemokines

Chemokines are involved in the regulation of the migration, maturation, and functionality of the recruited immune cells in response to inflammation (Warnock *et al.*, 2000) and also act as homeostatic mediators under normal physiological conditions (Laing and Secombes, 2004; Zlotnik, 2006).

CXCL8 (also known as IL-8) acts as an inflammatory mediator for attracting and activating neutrophils and T-lymphocytes to the area of infection. It is released by macrophages and other cell types, such as epithelial cells and endothelial cells (Hedges *et al.*, 2000). The expression of *IL-8* was elevated significantly in spleen, kidney and lower intestine of rainbow trout following an injection challenge with *F. psychrophilum* (Overturf and LaPatra, 2006; Evenhuis and Cleverland, 2012), while there was a relative decrease in expression of *IL-8* with an increasing injection dose of *F. psychrophilum* (Overturf and LaPatra, 2006). Meanwhile, a significant correlation between *IL-8* transcript abundance in the lower intestine and bacterial loads in spleen by day 10 post challenge was observed (Evenhuis and Cleverland, 2012).

b. Interleukins

Interleukins (ILs) are mainly produced by helper $CD4^+$ T cells, monocytes, macrophages and endothelial cells and promote the development and differentiation of T and B cells, and hematopoietic cells (Brocker *et al.*, 2010).

IL-1β has been reported in many different teleost species (Secombes *et al.*, 2011). IL-1β is a pro-inflammatory cytokine in mammals and is associated with adhesion,

colonisation and invasion of bacterial pathogens in rainbow trout (Komatsu *et al.*, 2009). *IL-1β* was up-regulated in gills, spleen, liver and head kidney of naturally infected rainbow trout with *F. psychrophilum* and could act as a marker of bacterial infection in rainbow trout (Orieux *et al.*, 2013). An up-regulation of *IL-1β* was also observed in the head kidney and gills of 2.2 g rainbow trout immediately after experimental exposure to *F. psychrophilum*, indicating a pro-inflammatory response (Henriksen *et al.*, 2015a,b).

The IL-6 family is a group of both pro- and anti-inflammatory cytokines (IL-6, IL-11, IL-31), which regulate hematopoiesis, inflammation, immune responses and bone homeostasis (Costa *et al.*, 2011). No significant changes in the expression of *IL-6* were recorded in the kidney of 2.2 g rainbow trout following an immersion-based challenge with *F. psychrophilum* (Henriksen, 2015a).

IL-10 is an anti-inflammatory cytokine and primarily associated with macrophages, although many other cell types can secrete this cytokine (Lutfalla *et al.*, 2003). IL-10 plays a central role in regulation of the inflammatory response, in which the innate response is inhibited by an up-regulation of IL-10, and also has multiple effects involved in an antibody-based Th2-type response (Hummelshoj *et al.*, 2006; Raida and Buchmann, 2008). The expression of other cytokines, such as pro-inflammatory cytokines IL-1β and IL-6, are regulated by IL-10 at a transcriptional level (Seppola *et al.*, 2008). *IL-10* transcripts were down-regulated in head kidney of rainbow trout (2.2 g) following an immersion-based challenge with *F. psychrophilum*, regardless of H_2O_2 pre-treatment (Henriksen *et al.*, 2015a), and in gills of fish from the H_2O_2 + *F. psychrophilum* group (Henriksen *et al.*, 2015b). A positive correlation between *IL-10* transcript abundance and bacterial load in head kidney of H_2O_2 + *F. psychrophilum* challenged fish is suggested to be a part of a repressive effect on the immune response triggered by *F. psychrophilum* (Henriksen *et al.*, 2015a).

In mammals, IL-17 members act as stimulators for pro-inflammatory gene expression, and promote cellular infiltration through pro-inflammatory cytokines and chemokines (Kono *et al.*, 2011). Teleost homologues to IL-17 genes have been found in many teleost species, including rainbow trout (IL-17c1 mainly expressed in gill and skin; and IL-17c2 in spleen, head kidney and brain) (Wang *et al.*, 2010b). Although teleost IL-17C genes are considered as major responders to lipopolysaccharides (LPS), the effects of IL-17c1 and IL-17c2 in fish are unclear (Kono *et al.*, 2011; Wang *et al.*, 2010b). In an immersion-based *F. psychrophilum* challenge with or without H_2O_2 pretreatment using rainbow trout, no significant change in *IL-17c2* transcripts was observed in the head

kidney and gills of fish from both treatment groups (Henriksen *et al.*, 2015a,b). Down regulation of *IL-17c1* was noted in the head kidney of fish from both groups but faster in the H_2O_2 + *F. psychrophilum* group, suggesting a suppressive effect on the immune system triggered by *F. psychrophilum* (Henriksen *et al.*, 2015a). Meanwhile, an early up-regulation of *IL-17c1* was noted in gills of H_2O_2 + *F. psychrophilum* challenged fish (Henriksen *et al.*, 2015b).

IL-4 decreases the production of T_h1 cells, macrophages and dendritic cells, while it activates CD4+ cells for the differentiation into T_h2 cells (Secombes *et al.*, 2011). Similar to IL-10, IL-4 has an anti-inflammatory effect by inhibiting production of pro-inflammatory cytokines (e.g. IL-1β and IL-6) (Murphy, 2012d). IL-4 and IL-13 are functionally closely related cytokines and localised in tandem on the genome (Ohtani *et al.*, 2008). *IL-4/13A* transcript was down-regulated in the head kidney of rainbow trout, previously immersed in *F. psychrophilum* broth culture (Henriksen *et al.*, 2015a), while this transcript did not present any changes in gills of challenged fish (Henriksen *et al.*, 2015b). A positive correlation with bacterial load was seen for IL-4/13A in gills of fish following the *F. psychrophilum*-immersion challenge with H_2O_2 pre-treatment (Henriksen *et al.*, 2015b).

c. Interferons

Interferons (IFN) are involved in anti-viral defenses and are classified into three subfamilies (I, II and III). In fish, type I and II IFNs have been reported, and at least two type II IFN genes are found in some species. Type I IFNs are produced by any cell type in response to a viral infection (Boehm *et al.*, 1997). Type II IFN (IFN γ) is produced by activated T cells and natural killer cells in response to intracellular pathogens, such as viruses and bacteria (Boehm *et al.*, 1997). *IFN-γ* was elevated moderately on day 3 and significantly on day 10 post injection challenge with *F. psychrophilum* in rainbow trout, compared with the mock challenged fish (Evenhuis and Cleverland, 2012).

d. Tumor necrosis factor

Tumor necrosis factor (TNF) is a superfamily of cytokines with a wide range of functions involved in inflammation, host defense, autoimmunity, organogenesis, cellular apoptosis and differentiation (Ware, 2003). TNF-α is the best known member of TNF family and has been described in several fish species as a pro-inflammatory mediator (Roca *et al.*,

2008). In an injection challenge with *F. psychrophilum*, transcript abundance of *TNF-α* was significantly increased and conclusively induced the inflammation directly in the intestine of rainbow trout (Evenhuis and Cleverland, 2012).

e. Transforming growth factor

Transforming growth factor (TGF) superfamily includes multifunctional cytokines involved in cell growth, migration, differentiation and apoptosis (ten Dijke and Hill, 2004). TGF-β is the best known family member but the information on its biological functions in the fish immune system is still limited. Evenhuis and Cleverland (2012) observed the increase of *TGF-β* transcript in the intestine of rainbow trout following an injection challenge with *F. psychrophilum*. Conversely, *TGF-β* was down-regulated in spleen of naturally RTFS-affected fish compared to the normally appearing fish (Orieux *et al.*, 2013). Both of these studies also found no correlation between the bacterial load and the expression of *TGF-β* transcript, suggesting that the expression of this transcript is not correlated with systemic infection of *F. psychrophilum* in rainbow trout.

1.6.7.3. Antibody

a. IgM

Teleost IgM typically exists in tetrameric form but can also be secreted as both a monomer and dimer structure, enhancing the epitope binding ability of IgM (Watts *et al.*, 2001). Transcript abundance of *IgM* significantly increased in the intestine of rainbow trout following a *F. psychrophilum*-injection challenge (Evenhuis and Cleveland, 2012). Correspondingly, IgM levels in serum, skin mucus and gill mucus were significantly elevated in fish i.p. immunised with a live attenuated *F. psychrophilum* but not by anal intubation- and bath- immunisation (Makesh *et al.*, 2015). These results suggest that IgM production is important for both systemic and mucosal immune response to an injection challenge or vaccination with *F. psychrophilum*. In addition to local production of IgM, a portion of mucus IgM may be passively transported from blood or through the polymeric Ig receptors (pIgR) found in all mucosal surfaces (Makesh *et al.*, 2015).

On the other hand, naturally *F. psychrophilum* infected fish did not present an increased plasma IgM titre and had an even lower titre in comparison with healthy appearing fish (Orieux *et al.*, 2013). This low IgM titre in plasma might be attributed to the repression of the immune response in the diseased fish. Henriksen *et al.* (2015a,b) also reported no significant regulation of *IgM* transcript in the head kidney and gills of

immersion-challenged rainbow trout (2.2 g) with *F. psychrophilum*. However, a positive correlation between bacterial load and *IgM* transcript abundance was observed in naturally and experimentally *F. psychrophilum*-infected fish (Evenhuis and Cleveland, 2012; Henriksen *et al.*, 2015a,b).

The highest *IgM* transcript abundance was observed in tissues that were directly exposed to the bacterin depending upon the immunisation route (e.g. blood for i.p. injection, skin and gill for bath and intestine for anal intubation) but this transcript abundance did not result in any significantly increased IgM antibody in the mucus of these organs (Makesh *et al.*, 2015). This study also reported a time lag in antibody secretion (peaking at 28 days post immunisation, DPI) compared to transcript production (peaking at 7 DPI) in blood of rainbow trout i.p. immunised with a live attenuated *F. psychrophilum*.

b. IgT/IgZ

Recently, IgT and IgZ have been described in rainbow trout and zebrafish respectively (Hansen *et al.*, 2005; Danilova *et al.*, 2005; Zhang *et al.*, 2010). IgT is considered to be a specialised Ig in the mucosal immunity of rainbow trout, equivalent to IgA in mammals. IgT positive B cell populations have been identified prevalently in the gut-associated lymphoid tissue in rainbow trout (Zhang *et al.*, 2010) and in gill epithelia (Olsen *et al.*, 2011). *IgT* gene was down-regulated in the spleen of diseased farm fish due to *F. psychrophilum* infection and even more repressed in antibiotic-treated diseased fish, suggesting that antibiotic treatment leads to a repression of antibody production (Orieux *et al.*, 2013). Similarly, H_2O_2 is suggested to delay the production of antibodies in the early phase of infection, as evidenced by the earlier down-regulation of *IgT* gene in the head kidney of rainbow trout following the *F. psychrophilum*-immersion challenge with H_2O_2 pre-treatment, compared to the challenge without H_2O_2 pre-treatment (Henriksen *et al.*, 2015a). *IgT* was down-regulated in both head kidney and gills of challenged fish, regardless of the pre-treatment with H_2O_2 (Henriksen *et al.*, 2015a,b). In contrast, immunised rainbow trout (35 g) with a live attenuated *F. psychrophilum* strain by bath and anal intubation routes presented up-regulation of *IgT* transcript in gills (53-fold) and gut (8-fold) respectively, suggesting the local presence of IgT-producing B lymphocytes (Makesh *et al.* 2015). *IgT* was up-regulated very late in the skin (42 DPI), indicating the lack of IgT secreting B lymphocytes in this organ or could be due to the movement of these cells to the skin (Makesh *et al.* 2015).

c. IgD

With the identification of an IgD homolog in catfish, this Ig has been found in all vertebrates except birds, indicating that IgD represents an ancient Ig class (Bengten *et al.*, 2002). The IgD antibody titres and *IgD* transcript did not change significantly in blood of rainbow trout (35 g) immunised by i.p. injection, bath and anal intubation routes with a live attenuated *F. psychrophilum* vaccine (Makesh *et al.*, 2015). However, *IgD* transcript was up-regulated in gills (347-fold) and intestine (20-fold) at 3 DPI in bath immunised and anal intubation-immunised groups respectively, suggesting that IgD producing B lymphocytes are present locally and express IgD transcripts in response to the mucosal vaccination (Makesh *et al.* 2015). Similar to *IgT*, the late expression of *IgD* in skin (42 DPI) could indicate the lack of IgD-secreting B lymphocytes in the skin or be due to the migration of these cells to the skin. These results reveal that in addition to IgT, IgD is also potentially involved in mucosal immunity in teleosts.

d. Natural antibodies

Natural antibodies have been described in the serum of teleosts (Sinyakov *et al.*, 2002; Castro and Taffala, 2015) at any time without apparent antigenic stimulation. They play an important role in the innate immune response and are linked to the adaptive immune response, in order to give broad protection against invading pathogens (Whyte, 2007).

Unexpectedly high titres of natural antibodies (1:16384) against whole *F. psychrophilum* cells were found in rainbow trout, which were never immunised against this bacterium (Wiklund and Dalsgaard, 2002). These high antibody levels could be developed through the natural infection by *F. psychrophilum* or represent the cross-reacting antibodies induced by common epitopes in the bacterial peptidoglycan layer from the normal bacterial flora in the water.

1.6.7.4. Other humoral factors

The expression of *SAA* gene encoding acute serum amyloid A (SAA), an acute phase protein synthesised in the liver under pro-inflammatory conditions, and the presence of SAA protein were assessed in immune-relevant tissues and plasma of healthy, naturally *F. psychrophilum*-infected and PAMP-injected fish (Villarroel *et al.*, 2008). The *SAA*

gene was clearly up-regulated in epithelial barriers (liver, skin, intestine, gills) and lymphoid organs of naturally and experimentally infected rainbow trout, suggesting an important role of *SAA* gene in local response to injury and inflammation (Villarroel *et al.*, 2008, 2009; Evenhuis and Cleverland, 2012). *SAA* transcript abundance and the bacterial loads were positively correlated in liver, skin, intestine, gills (Evenhuis and Cleverland, 2012) and head kidney (Henriksen *et al.*, 2015a). However, no significant regulations of *SAA* were recorded in head kidney of 2.2-g rainbow trout following an immersion-based challenge with *F. psychrophilum* (Henriksen *et al.*, 2015a), while down-regulation of *SAA* was observed in gills of the fish from H_2O_2 + *F. psychrophilum* group (Henriksen *et al.*, 2015b).

However, SAA protein was not detected in the plasma or any other biological fluid of infected or challenged fish, possibly due to the extremely fast clearance for this protein (Villarroel *et al.*, 2008). Furthermore, amyloid A-derived aggregates were detected for the first time in the skeletal muscle of infected rainbow trout, a lower vertebrate, and are suggested as relevant markers for controlling fish meat quality (Villarroel *et al.*, 2009).

Metallothionein is a family of cysteine-rich, low molecular weight (0.5 – 14 kDa) proteins, localised to the membrane of the Golgi apparatus. The synthesis of metallothionein is involved in the inflammatory process and the oxidative stress caused by the inflammatory acute phase response (Sigel, 2009; Inoue *et al.*, 2009). Metallothionein A (*mt-a*) was overexpressed in gills, liver and spleen of diseased rainbow trout due to *F. psychrophilum* infection and appears to be a marker of bacterial infection in fish (Orieux *et al.*, 2013).

In general, understanding of the innate and adaptive immune response of rainbow trout to *F. psychrophilum*, especially at the mucosal surfaces, is essential for the development of an efficacious RTFS/BCWD vaccine (Makesh *et al.*, 2015). There have been several studies involved in the immune response of naturally infected (Villarroel *et al.*, 2008; Orieux *et al.*, 2013), injection-challenged fish (Overturf and LaPatra, 2006; Evenhuis and Cleveland, 2012) and bath-challenged fry (Henriksen *et al.*, 2015a,b). Recently, the antibody response (IgM, IgD and IgT) of immunised rainbow trout weighing 35 g to live attenuated *F. psychrophilum* was investigated (Makesh *et al.*, 2015). However similar studies on vaccinated fry, especially using inactivated whole cell vaccines, are needed.

1.7. Aims of the study

In the UK, *F. psychrophilum* was first reported in diseased rainbow trout in 1992 (Santos *et al.* 1992). However, studies on the epidemiology, antibiotic susceptibility and vaccine development based on *F. psychrophilum* strains recovered in the UK are limited. Therefore, the overall aim of the present study was to investigate the diversity of the UK *F. psychrophilum* isolates as a basis for setting up improved RTFS control strategies, especially with regard to vaccination programs. The three main objectives were:

- To characterise the strain diversity of *F. psychrophilum* isolates present in the UK based on their genetic and serological characteristics, and to determine the predominant profiles of *F. psychrophilum* strains circulating within the country.
- To investigate the antimicrobial susceptibility of representative UK *F. psychrophilum* isolates from different genotypes, serotypes, locations and dates of sampling; to establish the epidemiological cut-off values for the antibiotics tested; to examine the correlation of the susceptibility data obtained from the disc diffusion test and standardised broth microdilution assay; and to identify any possible association between the genotype and antimicrobial susceptibility of *F. psychrophilum* strains.
- To evaluate the efficacy of a whole-cell formalin killed polyvalent vaccine, which was developed based on the genotyping and serotyping characterisation results, in protecting rainbow trout fry against *F. psychrophilum* infection; and to investigate the expression of immune-relevant genes in rainbow trout fry following the immersion administration of this vaccine.

Chapter 2

Genotypic and serological characterisation of *Flavobacterium psychrophilum* isolates from salmonids within the United Kingdom

2.1. Introduction

Rainbow trout fry syndrome (RTFS) or bacterial cold water disease (BCWD) is a severe septicemic condition of rainbow trout (*Oncorhynchus mykiss*) fry and fingerlings at water temperatures below 10^{o}C. It is considered to be one of the main threats to the rainbow trout culture industry worldwide (Faruk, 2002; Starliper and Chill, 2011). The aetiological agent of RTFS and BCWD is *Flavobacterium psychrophilum*, a Gram-negative, filamentous, psychrotrophic bacterium (Bernardet *et al*., 1996). It was first isolated in 1948 from diseased juvenile coho salmon, *Oncorhynchus kisutch* in Washington, USA (Borg, 1948). Although many attempts have been made to develop a commercial vaccine against RTFS during the last 20 years, this has been hindered by the prevalence of a wide range of *F. psychrophilum* strains (Gómez *et al*., 2014).

Macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) can be used to investigate the genetic variation of a bacterial population. With its high discriminatory ability and reproducibility, PFGE has been used successfully for molecular epidemiological characterisation of *F. psychrophilum* isolates in Japan (Arai *et al*., 2007), USA (Chen *et al*., 2008), Chile (Avendaño-Herrera *et al*., 2009), Spain (Del Cerro *et al*., 2010) and Finland (Sundell *et al*., 2013). These studies have shown that the genetic diversity of *F. psychrophilum* in these areas is associated with both geographical origin and the fish host from which the isolates were collected. Furthermore, PFGE analysis has been used to determine the source of BCWD infection (Arai *et al*., 2007) and to investigate the horizontal and vertical transmission of *F. psychrophilum* within and between rearing facilities (Chen *et al*., 2008).

Molecular typing methods have also been used to differentiate *F. psychrophilum* strains. Repetitive PCR (rep-PCR), a fast and simple typing method, has been deployed to characterise *F. psychrophilum* isolates from several outbreaks in Chile (Valdebenito and Avendaño-Herrera, 2009), showing that a major genetic group of isolates is possibly dominant on farms. A six-base polymorphism in the variable stem-loop region of the 16S rRNA gene, distinguishing the CSF 259-93 and ATCC 49418 alleles (Soule *et al*., 2005a,b), has been analysed to investigate the host-specific association (trout vs. salmon) of *F. psychrophilum* genetic 16S rRNA lineages (Ramsrud *et al*., 2007; Valdebenito and Avendaño-Herrera, 2009; Nilsen *et al*., 2011). Plasmid profiling has also been used as a characterisation method and could differentiate *F. psychrophilum* isolates with regards to pathogenicity (Chakroun *et al*., 1998; Lorenzen *et al*., 1997; Madsen and Dalsgaard,

2000), serotype (Lorenzen and Olesen, 1997) and antimicrobial susceptibility (Izumi and Aranishi, 2004; Del Cerro *et al*., 2010; Henríquez-Núñez *et al*., 2012).

Serotyping is useful for both diagnosis and sero-epidemiological studies. Three serotypes (Th, Fd, Fp^T) identified by Lorenzen and Olesen (1997) and three O-antigen groups (O-1, O-2 and O-3) established by Izumi and Wakabayashi (1999) are the most commonly used serotyping schemes (Madsen and Dalsgaard, 2000). In addition, Mata *et al*. (2002) found seven distinguishable serovars (1-7), allowing further epidemiological assays and routine identification of *F. psychrophilum*. There has also been an attempt to harmonise the three serotyping schemes above and identify additional serotypes (Mata *et al*., 2002, Izumi *et al*., 2003). Some serotypes were clearly associated with the fish species from which the isolates were collected. Serotypes Th and O-3 are proposed to be broadly similar and predominant among *F. psychrophilum* strains isolated from rainbow trout in RTFS/BCWD outbreaks (Lorenzen and Olesen, 1997; Izumi and Wakabayashi, 1999).

In the UK, *F. psychrophilum* was first reported in diseased rainbow trout in 1992 (Santos *et al*., 1992). However, to date, no studies on the epidemiology of *F. psychrophilum* strains recovered in the UK have been published. Understanding the population structure of this fish pathogen is important for predicting outbreaks and setting up effective RTFS/BCWD control strategies, such as vaccination programs. The aim of this chapter was to describe the diversity of *F. psychrophilum* isolates from RTFS-affected fish on farms within the UK using a combination of molecular and serotyping methods.

2.2. Materials and methods

2.2.1. Bacterial isolates and growth conditions

A total of 303 *F. psychrophilum* isolates and 12 reference *F. psychrophilum* strains were recovered from kidney, spleen, skin, gill, lesions, eye and/or internal organs of 293 rainbow trout (*O. mykiss*), 21 Atlantic salmon (*S. salar* L.) and one coho salmon (*O. kisutch*). This collection comprised 293 *F. psychrophilum* isolates collected from 27 sites within the UK, two of which were unknown, between 2005 – 2015 (20 sites in Scotland, six in England and one in Northern Ireland), and nine isolates from single farm outbreaks within France and Ireland (Table 2.1). Thirteen reference strains were also included in this study for comparative purposes (Table 2.2). Among the 315 isolates studied, 214 isolates were retrieved from diseased fish, five isolates from apparently healthy fish and the remaining 96 isolates from fish with unknown health conditions.

Presumptive *F. psychrophilum* colonies were selected after preliminary biochemical and phenotypic characterisation using Gram staining, motility and oxidase testing. For all the experiments, the *F. psychrophilum* isolates were routinely grown in modified Veggietone (MV) medium (veggitones GMO-free soya peptone (Oxoid, UK), 5 g L^{-1}; yeast extract [Oxoid, UK], 0.5 g L^{-1}; magnesium sulphate heptahydrate [Fisher chemicals, UK], 0.5 g L^{-1}; anhydrous calcium chloride [BHD], 0.2 g L^{-1}; dextrose [Oxoid, UK], 2 g L^{-1}; agar [solid medium; Oxoid, UK], 15 g L^{-1}; pH 7.3) at 15^{o}C for 72 – 96 h. Broth cultures were shaken at 140 rpm. Stock cultures were maintained at -70^{o}C in tryptone–yeast extract–salts medium supplemented with glucose [FLP – tryptone (Oxoid, UK), 4.0 g L^{-1}; yeast extract, 0.4 g L^{-1}; anhydrous calcium chloride, 0.2 g L^{-1}; magnesium sulphate heptahydrate, 0.5 g L^{-1}; D(+)-glucose (Sigma, UK), 0.5 g L^{-1}; Cepeda *et al.*, 2004] with 10% glycerol and on Protect-Multi-purpose cryobeads (Technical Service Consultants Ltd, UK).

Table 2.1. Details of 303 *F. psychrophilum* isolates collected from 27 fish farm sites within the UK and two farms in Europe during 2005 – 2015.

Site	Year of isolation	No. of sampling times	Host source	No. of individual fish sampled	No. of isolates
Scot I	2005 - 2015	16	RT	35	87
Scot II	2013	2	RT	2	2
Scot III	2011 - 2015	5	RT	13	44
Scot IV	2013	1	RT	3	5
Scot V	2013 - 2015	4	RT	25	55
Scot VI	2009	1	RT	1	1
Scot VII	2007	1	RT	1	1
Scot VIII	2005	1	RT	1	1
Scot IX	2006	1	AS	1	1
Scot X	2011 - 2013	3	AS	3	3
Scot XI	2015	1	AS	4	4
Scot XII	2010	1	AS	1	1
Scot XIII	2005	1	AS	1	1
Scot XIV	2013	2	AS	2	2
Scot XV	2013	2	AS	8	8
Scot XVI	2014 - 2015	4	RT	8	14
Scot XVII	2007	1	RT	1	1
Scot XVIII	2009	1	RT	1	1
Unknown	2009-2012	2	RT	2	2
Sub-total Scotland	*2005 - 2015*	*50*	*RT/AS*	*113*	*234*
Eng I	2013	3	RT	8	24
Eng II	2015	1	RT	4	13
Eng III	2015	1	RT	1	2
Eng IV	2015	1	RT	1	1
Eng V	2007	1	RT	1	1
Eng VI	2007	1	RT	1	1
N Ire I	2013	2	RT	9	17
Sub-total UK	*2005 - 2015*	*10*	*RT/AS*	*138*	*293*
Ireland	2006	1	AS	1	1
France	unknown-2013	2	RT	5	9
	Total	Total	63		144

RT, rainbow trout; AS, Atlantic salmon.

Table 2.2. Origins and typing summary of the twelve reference strains of *F. psychrophilum* used in this study.

Strains	Isolation source	Year	Host source	Pulsotype	Serotype	rep-PCR profile	16S rRNA allele	Plasmid profile	MLST based sequence type[5]
046-04 Idaho [1]	USA	2004	RT	K	Th	r1b	CSF	p1	
NCIMB 1947^T	USA	unknown	CS	Y	Fp^T	r1b	Both	p2	ST13
CSF 259-93 [1]	USA	1993	RT	N	Fd	r1a	CSF	p1	ST10
302-95, Idaho [1]	USA	1995	RT	L1	Fd	r1a	CSF	p1	
1/3 Th 1994 [2]	Denmark	1994	RT	T3	Th	r1a	CSF	p1	
NCIMB 13384 [2]	Denmark	1990	RT	U1	Fd	r1a	CSF	p1	
NCIMB 13383 [2]	Denmark	1990	RT	T3	Th	r1a	CSF	p1	ST2
32/97 chile [3]	Chile	1997	RT	H	Fd	r2	CSF	p1	
59/95 chile [3]	Chile	1995	RT	T6	Fd	r1a	CSF	p8	
P13 3/96 Th [4]	Finland	1996	RT	T3	Th	r1a	ND	p1	
P5 10/96 Th [4]	Finland	1996	RT	T3	Th	r1a	CSF	p1	
P8 3/96 Fd [4]	Finland	1996	RT	T3	Fd	r1a	ND	p1	

RT, rainbow trout; CS, coho salmon; ND, not determined
Reference: [1] Wiens *et al*., 2014; [2] Lorenzen and Olesen, 1997; [3] Faruk *et al*., 2002; [4] Madetoja *et al*., 2001; [5] Online multilocus sequence typing (MLST) database for *F. psychrophilum*: http://pubmlst.org/fpsychrophilum

2.2.2. DNA extraction

Bacterial DNA was extracted using the REALPURE Genomic DNA Extraction kit (Durviz, Spain). A cell pellet of 3-mL MV broth culture was harvested by centrifugation (Heraeus Pico17, Thermo Scientific, UK) at 13,000 × *g* for 1 min, washed in 1.0 mL of NaCl-Tris-EDTA (STE) buffer (0.1 M NaCl [Sigma, UK], 10 mM Tris hydrochloride [Fisher chemicals, UK], 1 mM ethylenediaminetetraacetic acid [EDTA; Sigma, UK], pH 8.0) and then resuspended in 300 µL of the Lysis buffer, containing 3 µL of 10 mg mL^{-1} Proteinase K (Bioline, UK). After an overnight incubation at 55^{o}C, 5 µL of 2 mg mL^{-1} RNase A (Sigma, UK) was added to the cell lysate and incubated at 37^{o}C for 1 h. A further 180 µL of the Protein precipitation solution was added to the lysis mixture, vigorously shaken for 30 s and incubated on ice for 15 min. The protein precipitate was removed by centrifugation at 17,000 × *g* for 10 min at 22^{o}C and the supernatant was transferred into a new tube containing an equal volume of isopropanol (Fisher chemicals, UK). Each tube was inverted 4 – 6 times and centrifuged at 17,000 × *g* for 10 min. The resulting DNA pellet was washed in 1.0 mL of 70% ethanol (Fisher chemicals, UK) for 2 h. Finally, the DNA pellet was collected by centrifugation at 17,000 × *g* for 5 min, dried at 50^{o}C for 10 min and resuspended in 50 - 100 µL of the kit Hydration buffer depending on the pellet size. DNA samples were kept at 4^{o}C for short term storage or at -20^{o}C for long term storage.

The concentration of DNA samples was measured using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, UK). Each DNA sample was standardised to 50 ng μL^{-1} with Milli-Q distilled water (DW). An aliquot (1 µL) of each DNA sample was visualised under UV illumination (Bio Imaging INGENIUS, Syngene) following electrophoresis on a 1.0% (weight per volume, w/v) agarose gel (Bioline, UK) containing 0.1 µg mL^{-1} ethidium bromide (Sigma, UK) in Tris–Acetic–EDTA (TAE) buffer (0.2 mM Tris, 0.1 mM acetic acid [Fisher chemicals, UK], and 0.005 mM EDTA). A lambda DNA/HindIII marker (New England Biolabs, UK) was used to confirm the size, quantity and quality of the standardised DNA samples.

2.2.3. 16S rRNA Nested PCR

All 315 study isolates were confirmed as *F. psychrophilum* species using the nested PCR method targeting the 16S ribosomal RNA gene (Toyama *et al*., 1994) with modifications (K.L. Bartie, unpublished protocol). PCR constituents (25 µL total volume) included two first round universal primers, 20F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1500R

(5′-GGTTACCTTGTTACGACTT-3′) amplifying an amplicon size of 1500 base pair (bp; PCR1), and two second round species-specific primers, PSY1 (5′-CGATCCTACTTGCGTAG-3′) and PSY2 (5′-GTTGGCATCAACACACT-3′) amplifying an expected amplicon size of 1080 bp (PCR2). Reaction mixtures consisted of ReddyMix PCR Master Mix (ABgene, Thermo Scientific, UK), 0.2 µM of each primer pair (Eurofins MWG Operon) and 50 ng of DNA template (PCR1). PCR2 used 5 µL of the 1:100 diluted PCR1 amplicon as the DNA template in a 25-µL reaction volume. PCR amplifications were carried out in a TProfessional Basic Thermocycler gradient (Biometra, Germany). The following PCR thermal cycle conditions were used during both PCR1 and PCR2: 94°C for 5 min, followed by 35 amplification cycles of denaturation of DNA at 94°C for 30 s, annealing of primers at 45°C for 90 s and extension at 72°C for 120 s. After the last cycle, a final extension was carried out at 72°C for 5 min. A 5 µL aliquot of the PCR2 product was electrophoresed in a 1.0% (w/v) agarose gel (Bioline, UK) containing 0.1 µg mL^{-1} ethidium bromide in TAE buffer. A 100-bp DNA ladder (GeneRuler Express DNA Ladder; Fermentas, Fisher Scientific, UK) was used as molecular size marker to confirm the presence of the *F. psychrophilum* species specific amplicon of 1080 bp. The gel was visualised under UV illumination (Bio Imaging INGENIUS, Syngene).

2.2.4. Macrorestriction analysis by pulsed-field gel electrophoresis (PFGE)

The PFGE protocol was performed as described previously (Bartie *et al*., 2012) on the 315 *F. psychrophilum* isolates using restriction enzyme *Sac*I (New England BioLabs, UK) (Chen *et al*., 2008). A cell pellet of 1.5-ml MV broth culture was collected by centrifugation at 13,000 × *g* for 1 min, washed once in 1.0 mL of STE buffer and resuspended in the same buffer to an optical density (OD) at 525 nm of 1.0 - 1.3 (CE 2041, Celcil, UK). Standardised bacterial suspensions were mixed with an equal volume of 2% low melting point agarose (Flowgen Bioscience, UK) at 55ºC, then immediately loaded into a disposable plug mould (Bio-Rad Laboratories, UK) and allowed to solidify at 22ºC. Bacterial cells in each plug were then lysed in 3 mL of lysis buffer (10 mM Tris, 1 mM EDTA, 1% sarcosine [Sigma, UK], pH 8.0) containing 0.1 mg mL^{-1} proteinase K overnight at 55ºC with gentle shaking at 100 rpm. Prior to digestion with the restriction enzyme, the plugs were washed three times for at least 15 min in 5 mL of dilute Tris-EDTA (TE) buffer (10 mM Tris; 0.1 mM EDTA pH 8.0). The first wash was carried out at 55ºC and the remaining washes at 22ºC. The plugs were then washed twice in 3 mL of

dilute TE buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma, UK), and twice in 5 mL of dilute TE buffer at 22ºC. The plugs were stored in 5 mL of dilute TE buffer at 4ºC for long term storage.

The chromosomal DNA in the plugs was digested overnight at 37ºC with 5 U of restriction enzyme *Sac*I (New England Biolabs, UK) in a 200-µL reaction volume. The digested DNA fragments were separated on a 1.0% (w/v) UltraPure and trade Agarose-1000 (Invitrogen, Fisher Scientific, UK) in 0.25 X Tris–Borate–EDTA (TBE) buffer (89 mM Tris base [Fisher chemicals, UK], 89 mM boric acid [Fisher chemicals, UK], 2 mM EDTA, pH 8.0) using a Rotaphor® Type V system (Biometra, Germany). The electrophoresis conditions comprised switch times of 2 - 6 s at 200 V at 15ºC for 37 h. Following electrophoresis, the gels were stained for 30 min in 1 µg mL^{-1} ethidium bromide solution, destained in DW for at least 1 h and visualised under UV illumination (Bio Imaging INGENIUS, Syngene). A Low Range I PFG marker (New England BioLabs, UK) was used on the gel as a molecular size marker. Two isolates were considered different when the DNA profiles showed at least one band difference (Del Cerro *et al*., 2010).

2.2.5. Repetitive PCR (rep-PCR) genomic fingerprinting

All 315 *F. psychrophilum* isolates were typed using a rep-PCR method based on the single $(GTG)_5$ repetitive primer as previously described by Bartie *et al.* (2012). The 25-µL rep-PCR reaction mixture consisted of 1X MyTaq™ HS Mix (Bioline, UK), 0.5 µM of $(GTG)_5$ primer (MWG, Eurofins), 2.5 mM of magnesium chloride (Sigma, UK) and 50 ng of DNA template. The PCR amplification conditions included an initial denaturation at 95ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 30 s, annealing at 40ºC for 30 s and extension at 65ºC for 3 min; and a final extension cycle at 65ºC for 10 min. Ten microlitres of each PCR product was separated on a 1.5% UltraPure and trade Agarose-1000 (Invitrogen, Fisher Scientific, UK) in chilled TAE buffer. A GeneRuler Express DNA Ladder (Fermentas, Fisher Scientific, UK) was used as a molecular size marker. Following electrophoresis, the gel was stained for 30 min in 1 µg mL^{-1} ethidium bromide and destained in DW for at least 1 h. Gel image was captured as described in section 2.2.3.

2.2.6. 16S rRNA allele PCR

To investigate the presence of one or both 16S rRNA alleles, a PCR assay was performed as described by Ramsrud *et al.* (2007) on 169 selected *F. psychrophilum* isolates from different genotypes and origins. Two individual PCR tests using primers specific for the ATCC 49418 coho salmon allele (A49418: 5′-CGTCAAGCTACCTCACGAGGT-3′ and AF49418: 5′-ATAGTGAGTTGGCATCAACACAC T-3′) and the CSF 259-93 rainbow trout allele (A259-93: 5′-GAAACACTCGGTCGTGACCG-3′ and AR259-93: 5′-GACAACCATGCAGCACCTTG-3′) resulted in amplification products of 298 bp and 600 bp, respectively. The 25-µL reaction mixtures included 1X MyTaq™ HS Mix (Bioline, UK), 0.2 µM of each primer (MWG, Eurofins) and 50 ng of DNA template. The PCR program included one denaturation cycle at 95^{o}C for 3 min, followed by 30 cycles at 95^{o}C for 15 s, 61^{o}C for 15 s and 72^{o}C for 20 s, and concluded with a final 72^{o}C step for 10 min. Five microlitres of each PCR product was electrophoresed on 1.0 % (w/v) agarose gel containing 0.1 µg mL^{-1} ethidium bromide in TAE buffer. A GeneRuler Express DNA Ladder (Fermentas, Fisher Scientific, UK) was used as molecular size marker to confirm the presence or absence of the ATCC or CSF allele. The gel was visualised under UV illumination (Bio Imaging INGENIUS, Syngene).

2.2.7. Plasmid profiling

Plasmid profiles of 185 isolates from representative genotypes and origins were investigated. The extraction of plasmid DNA from *F. psychrophilum* isolates was performed as previously described (Bartie *et al.*, 2012). A cell pellet of 3-mL MV broth culture was harvested by centrifugation (Heraeus Pico17, Thermo Scientific, UK) at 13,000 × *g* for 1 min, washed in 1.0 mL of STE buffer and resuspended in 250 µL of ice cold Solution I (50 mM glucose, 25 mM Tris, 10 mM EDTA pH 8.0) containing 10 µg mL^{-1} RNase A. The bacterial cells were lysed by adding 250 µL of freshly prepared Solution II (0.2 N sodium hydroxide [Fisher chemicals, UK], 1% [w/v] sodium dodecyl sulfate [Fisher chemicals, UK]) and immediately mixed by gentle inversion. The lysate was neutralised by adding 300 µL of ice cold Solution III (3 M potassium acetate [BHD, UK] pH 4.8), mixed well by gentle inversion and incubated on ice for 5 min. Denatured genomic DNA and cellular proteins in the mixture were pelleted by centrifugation at 17,000 × *g* for 10 min. The supernatant containing the plasmid DNA was transferred into a new tube and precipitated by adding an equal volume of isopropanol (Fluka, UK) and centrifuging at 17,000 × *g* for 10 min. The DNA pellet was washed in 0.5 mL of 70%

ethanol (Fisher chemicals, UK), centrifuged at 17,000 × *g* for 2 min, air-dried, dissolved overnight in 50 µL of TE buffer at 4ºC and then stored at 4ºC for a short term usage or at -20 ºC for long term preservation. The plasmids were separated on a 0.7% UltraPure and trade Agarose-1000 (Invitrogen, Fisher Scientific, UK) gel in chilled TAE buffer. Following electrophoresis, the gel was stained for 30 min in 1 µg mL^{-1} ethidium bromide solution, destained in DW for at least 1 h and visualised under UV illumination (Bio Imaging INGENIUS, Syngene). The approximate molecular size of the plasmids was estimated using a Supercoiled DNA ladder (New England BioLabs, UK) and the known plasmid contents of two reference strains *Escherichia coli* V517 and 39R861 (Macrina *et al*., 1978). Plasmid profiles were identified by differences in size and band intensity.

2.2.8. Serological characterisation

Absorbed polyclonal antisera against three *F. psychrophilum* strains (NCIMB 1947^T, NCIMB 13384 and NCIMB 13383) were kindly supplied by Dr Tom Wiklund from Laboratory of Aquatic Pathobiology, Åbo Akademi University, Finland. Titres of the antisera were determined using an enzyme-linked immunosorbent assay (ELISA) as previously described (Lorenzen and Olsen, 1997; Faruk *et al*., 2002). Two replicates at 1:20,000, 1:50,000, 1:100,000, 1:200,000, 1:500,000, 1:1,000,000 and 1:2,000,000 dilutions of each antiserum were prepared in phosphate buffered saline (PBS) (0.02 M sodium dihydrogen phosphate (Fisher chemicals, UK), 0.02 M disodium hydrogen phosphate (Fisher chemicals, UK), 0.15 M sodium chloride, pH 7.2) supplemented with 1% bovine serum albumin (BSA; Fisher chemicals, UK). The titre was defined as the reciprocal value of the highest dilution of a serum sample showing three times the mean absorbance value of the negative control (PBS).

Serotypes of 181 *F. psychrophilum* strains having different genotypes and origins were examined. A bacterial pellet from 1.0 mL MV broth culture was harvested by centrifugation (Heraeus Pico17, Thermo Scientific, UK) at 13,000 × *g* for 1 min and washed twice in PBS. The bacterial suspension was adjusted to an OD at 525 nm of 0.4 (CE 2041, Celcil, UK) and heat treated at 55ºC for 15 min (Lorenzen and Olesen, 1997). This suspension was equivalent to a concentration of 1 x 10^8 colony forming units (CFU) per millilitre (Faruk, 2000).

Flat-bottomed 96-well plates (Nunc, Fisher Scientific, UK) were coated with 50 µl $well^{-1}$ of 0.05 % (w/v) poly L-Lysine (Sigma, UK) in 0.5 M carbonate bicarbonate buffer (Sigma, UK), pH 9.6, for 1 h and then washed twice with low salt wash (LSW)

buffer (0.02 M Tris, 0.38 M sodium chloride, 0.05 % Tween-20, pH 7.3). The plates were coated with 100 µl well^{-1} of heat-treated bacterial suspensions. Plates were incubated overnight at 4^{o}C and then washed three times with LSW buffer before post coating with 250 µl well^{-1} of 1 % bovine serum albumin (BSA) in PBS for 2 h at 22^{o}C. Each plate was again washed three times in LSW buffer before the addition of the diluted polyclonal antisera (100 µl well^{-1}) and PBS (negative control). After 1 h incubation at 22^{o}C, the plates were washed five times with high salt wash (HSW) buffer (0.02 M Tris, 0.5 M sodium chloride, 0.1 % Tween 20, pH 7.7), with a 5 min soak on the last wash, to remove unbound antibodies. Goat anti-rabbit immunoglobulin-G (IgG) labelled with horseradish peroxidase (HRP; SAPU: Diagnostics Scotland, UK), diluted 1:1000 in PBS, was then added (100 µl well^{-1}) and plates incubated at 22^{o}C for 1 h. Plates were then washed five times with HSW buffer to remove excess HRP conjugate. Chromogen (42 mM tetramethyl-benzidine dihydrochloride [Sigma, UK] in acetic acid: water, 1:2) at 1% concentration was prepared in substrate buffer (0.1 M citric acid [Sigma, UK] and 0.1 M sodium acetate [Sigma, UK], pH 5.4) containing 0.03 % H_2O_2 [Sigma, UK]). The freshly prepared substrate solution was added at 100 µl well^{-1}. The reaction was stopped after 4 min by adding 50 µl well^{-1} of 2 M sulphuric acid (Fisher chemicals, UK). The reaction was read using a MR 5000 ELISA reader (Synergy HT, Biotek) at 450 nm.

2.2.9. Data analysis

DNA profiles resulting from the PFGE and rep-PCR methods were visualised and the gel images were captured using Bioimaging INGENIUS system (Syngene). Numerical analysis of the DNA fingerprints was performed using Gel Compar II software (Applied Maths, Belgium). Dendrograms were constructed using the unweighted average pair group method of arithmetic averages (UPGMA), with the Jaccard similarity coefficient and a 0.8% tolerance and a 0.3% optimisation (PFGE) or a 0.4% tolerance (rep-PCR). A cut-off at 95% similarity of the Jaccard's coefficient was used to indicate $(GTG)_5$-PCR types, equivalent to three band difference.

According to the guidelines for interpreting chromosomal DNA restriction patterns produced by PFGE (Tenover *et al.*, 1995), a PFGE group was defined as a group of possibly related isolates with a banding pattern similarity of ≥80 % (fewer than seven bands different). A cut-off at 95% similarity of the Jaccard's coefficient (fewer than three bands different) was used to indicate PFGE pulsotypes containing closely related isolates. DNA fingerprinting profiles showing ≥95% similarity were identified as PFGE subtypes.

Diversity values (D) were calculated based on Simpson's original index (Σ pi^2), using formulation described by Hunter and Gaston (1988), $D = 1- \Sigma pi^2$. A diversity index is defined as a quantitative measure that reflects the probability that two entities represent different types.

2.3. Results

2.3.1. Phenotypic characterisation and molecular identification of *F. psychrophilum*

All suspected yellow colonies were confirmed as *F. psychrophilum* using 16S rRNA nested PCR. The PCR1 products of the colonies that were negative in this test (e.g. lane 4-5 in Figure 2.1) were sequenced and none of them were identified as *F. psychrophilum*. On the other hand, the identification of *F. psychrophilum* using the 16S rRNA nested PCR was consistent with the PCR1 sequencing results.

The 16S rRNA nested PCR yielded PCR products with expected sizes: 1500 bp (PCR1) and 1080 bp (PCR2) (Figure 2.1), confirming the identification of 315 isolates as *F. psychrophilum*. Amplicons were not observed in the negative control (no DNA template added). These 315 *F. psychrophilum* isolates were biochemically homologous regardless of the source of isolation. The phenotypic and biochemical tests showed that all the isolates were Gram-negative, long, slender rods and they were slowly gliding according to the motility test and weakly positive for the oxidase test.

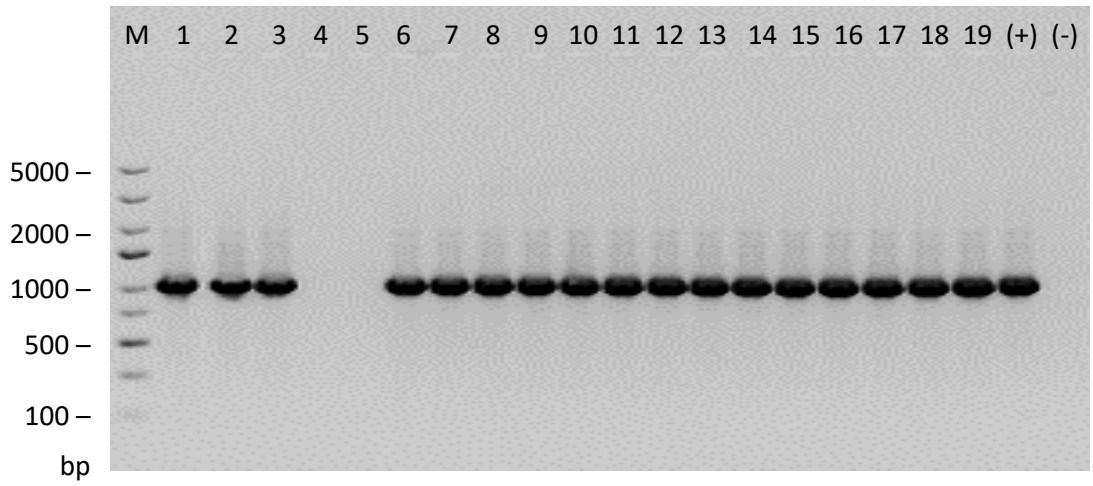


Figure 2.1. Agarose gel of the second round PCR product (1080 bp) using the specific primer pair (PSY1 and PSY2) of the diagnostic 16S rRNA nested PCR for *F. psychrophilum*. Lane 1-19: representative DNA samples of suspected *F. psychrophilum* colonies; lane (+): positive control (*F. psychrophilum* type strain NCIMB 1947^{T}); lane (-): negative control (DW); lane M: GeneRuler Express DNA Ladder (Fermentas, Fisher Scientific, UK).

2.3.2. Macrorestriction analysis by PFGE

Three hundred and fourteen *F. psychrophilum* isolates were successfully typed using *Sac*I PFGE. A total of 54 pulsotypes, each containing approximately 22 fragments ranging in size from 20 - 200 kb were identified (Figure 2.2). Seven pulsotypes (E1, E4, P6, Q1, T3, T5 and U1) contained 2 – 3 subtypes. Seven PFGE groups (≥80% similarity) and 27 singletons were identified, based on the criteria of Tenover (Tenover *et al*., 1995) (Figure 2.3). The diversity index of the whole strain collection based on the cut-off at 80% similarity using the Jaccard's coefficient was calculated at 0.87.

Two major PFGE clusters at a similarity of 40% and their outliers were revealed in the dendrogram. Cluster I was composed of two PFGE groups and seven singletons (A – I) and 75 *F. psychrophilum* isolates retrieved from 11 sites in Scotland, Northern Ireland, Chile and France. Cluster I was found to be associated with the large predominant cluster II formed at the 47% similarity level. Cluster II with five PFGE groups and ten singletons (J – X) contained the majority of the study isolates (226/314, 72%), 216 of which were collected from 20 sites within the UK and the remaining ten were reference strains from the USA, Denmark, Chile and Finland. The CSF 259-93 reference strain from USA was found to belong to cluster II. A collection of outliers to clusters I and II

were represented by ten remaining singletons Y – HH. The *F. psychrophilum* type strain NCIMB 1947^{T} was located in this collection of outliers together with 12 other isolates obtained within the UK and Ireland.

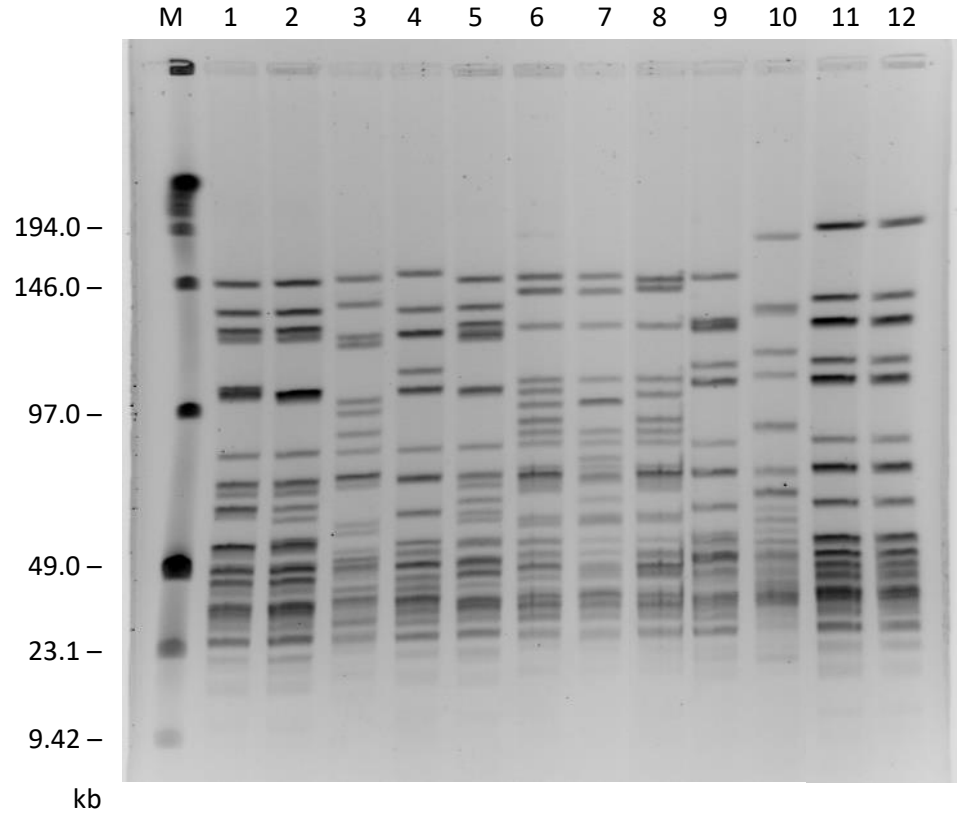
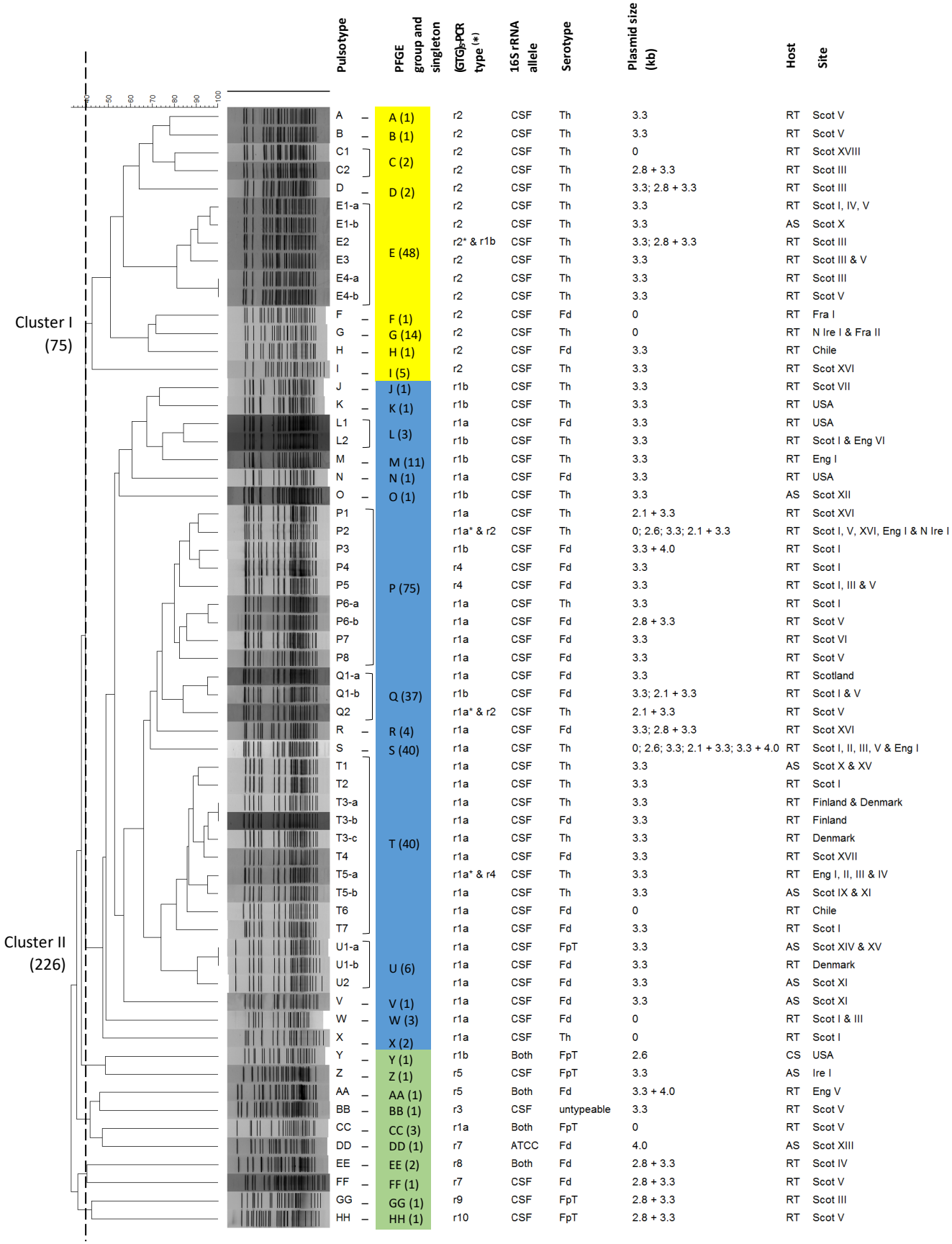


Figure 2.2. Pulsed-field gel electrophoresis (PFGE) banding pattern of 12 representative *F. psychrophilum* isolates using the *Sac*I restriction enzyme. Lane 1, 2, 9: USA reference strains from rainbow trout (046-04 Idaho (pulsotype K), 302-95 Idaho (pulsotype L1) and CSF 259-93 (pulsotype N) respectively); lane 3: *F. psychrophilum* type strain NCIMB 1947T (USA) from coho salmon (pulsotype Y); lane 4, 8: Chilean reference strains from rainbow trout [59/95 (pulsotype T6) and 32/97 (pulsotype H) respectively]; lane 5: an English isolate from rainbow trout (site Eng I, pulsotype M); lane 6: a French strain from rainbow trout (pulsotype F); lane 7: a Northern Irish isolate from rainbow trout (site N Ire I, pulsotype G); lane 10: a Scottish isolate from rainbow trout (site Scot V, pulsotype CC); lane 11: a Scottish isolate from Atlantic salmon (site Scot XV, pulsotype U1); lane 12: a Scottish isolate from Atlantic salmon (site Scot XIV, pulsotype U1). Lane M: Low Range I PFG marker (New England BioLabs, UK).



Pulsotype	PFGE group and singleton	$(GTG)_5$-PCR type (*)	16S rRNA allele	Serotype	Plasmid size (kb)	Host	Site
A	A (1)	r2	CSF	Th	3.3	RT	Scot V
B	B (1)	r2	CSF	Th	3.3	RT	Scot V
C1	C (2)	r2	CSF	Th	0	RT	Scot XVIII
C2		r2	CSF	Th	2.8 + 3.3	RT	Scot III
D	D (2)	r2	CSF	Th	3.3; 2.8 + 3.3	RT	Scot III
E1-a	E (48)	r2	CSF	Th	3.3	RT	Scot I, IV, V
E1-b		r2	CSF	Th	3.3	AS	Scot X
E2		r2* & r1b	CSF	Th	3.3; 2.8 + 3.3	RT	Scot III
E3		r2	CSF	Th	3.3	RT	Scot III & V
E4-a		r2	CSF	Th	3.3	RT	Scot III
E4-b		r2	CSF	Th	3.3	RT	Scot V
F	F (1)	r2	CSF	Fd	0	RT	Fra I
G	G (14)	r2	CSF	Th	0	RT	N Ire I & Fra II
H	H (1)	r2	CSF	Fd	3.3	RT	Chile
I	I (5)	r2	CSF	Th	3.3	RT	Scot XVI
J	J (1)	r1b	CSF	Th	3.3	RT	Scot VII
K	K (1)	r1b	CSF	Th	3.3	RT	USA
L1	L (3)	r1a	CSF	Fd	3.3	RT	USA
L2		r1b	CSF	Th	3.3	RT	Scot I & Eng VI
M	M (11)	r1b	CSF	Th	3.3	RT	Eng I
N	N (1)	r1a	CSF	Fd	3.3	RT	USA
O	O (1)	r1b	CSF	Th	3.3	AS	Scot XII
P1	P (75)	r1a	CSF	Th	2.1 + 3.3	RT	Scot XVI
P2		r1a* & r2	CSF	Th	0; 2.6; 3.3; 2.1 + 3.3	RT	Scot I, V, XVI, Eng I & N Ire I
P3		r1b	CSF	Fd	3.3 + 4.0	RT	Scot I
P4		r4	CSF	Fd	3.3	RT	Scot I
P5		r4	CSF	Fd	3.3	RT	Scot I, III & V
P6-a		r1a	CSF	Th	3.3	RT	Scot I
P6-b		r1a	CSF	Fd	2.8 + 3.3	RT	Scot V
P7		r1a	CSF	Fd	3.3	RT	Scot VI
P8		r1a	CSF	Fd	3.3	RT	Scot V
Q1-a	Q (37)	r1a	CSF	Fd	3.3	RT	Scotland
Q1-b		r1b	CSF	Fd	3.3; 2.1 + 3.3	RT	Scot I & V
Q2		r1a* & r2	CSF	Th	2.1 + 3.3	RT	Scot V
R	R (4)	r1a	CSF	Fd	3.3; 2.8 + 3.3	RT	Scot XVI
S	S (40)	r1a	CSF	Th	0; 2.6; 3.3; 2.1 + 3.3; 3.3 + 4.0	RT	Scot I, II, III, V & Eng I
T1	T (40)	r1a	CSF	Th	3.3	AS	Scot X & XV
T2		r1a	CSF	Th	3.3	RT	Scot I
T3-a		r1a	CSF	Th	3.3	RT	Finland & Denmark
T3-b		r1a	CSF	Fd	3.3	RT	Finland
T3-c		r1a	CSF	Th	3.3	RT	Denmark
T4		r1a	CSF	Fd	3.3	RT	Scot XVII
T5-a		r1a* & r4	CSF	Th	3.3	RT	Eng I, II, III & IV
T5-b		r1a	CSF	Th	3.3	AS	Scot IX & XI
T6		r1a	CSF	Fd	0	RT	Chile
T7		r1a	CSF	Fd	3.3	RT	Scot I
U1-a	U (6)	r1a	CSF	FpT	3.3	AS	Scot XIV & XV
U1-b		r1a	CSF	Fd	3.3	RT	Denmark
U2		r1a	CSF	Fd	3.3	AS	Scot XI
V	V (1)	r1a	CSF	Fd	3.3	AS	Scot XI
W	W (3)	r1a	CSF	Fd	0	RT	Scot I & III
X	X (2)	r1a	CSF	Th	0	RT	Scot I
Y	Y (1)	r1b	Both	FpT	2.6	CS	USA
Z	Z (1)	r5	CSF	FpT	3.3	AS	Ire I
AA	AA (1)	r5	Both	Fd	3.3 + 4.0	RT	Eng V
BB	BB (1)	r3	CSF	untypeable	3.3	RT	Scot V
CC	CC (3)	r1a	Both	FpT	0	RT	Scot V
DD	DD (1)	r7	ATCC	Fd	4.0	AS	Scot XIII
EE	EE (2)	r8	Both	Fd	2.8 + 3.3	RT	Scot IV
FF	FF (1)	r7	CSF	Fd	2.8 + 3.3	RT	Scot V
GG	GG (1)	r9	CSF	FpT	2.8 + 3.3	RT	Scot III
HH	HH (1)	r10	CSF	FpT	2.8 + 3.3	RT	Scot V

Figure 2.3. Dendrogram of 314 *F. psychrophilum* isolates constructed using the UPGMA method, based on 62 PFGE banding patterns obtained using *Sac*I. For each banding pattern; *Sac*I-PFGE pulsotype, pulsotype group and singleton (number of isolates), $(GTG)_5$-PCR type, 16S rRNA allele, serotype, plasmid size, fish host and site code are shown. A vertical dashed line placed at 40% similarity classified the isolates into two *Sac*I-PFGE clusters I (in yellow) and II (in blue) (number of isolates in brackets). An asterisk (*) highlights the dominant $(GTG)_5$-PCR type present in each *Sac*I-PFGE subtype. RT, rainbow trout; AS, Atlantic salmon; CS, coho salmon. One isolate untypeable using PFGE-Sac*I* method is not included in the dendrogram.

2.3.3. Rep-PCR using the $(GTG)_5$ primer

Analysis of the 315 *F. psychrophilum* isolates by rep-PCR using the $(GTG)_5$ primer revealed 12-14 fragments ranging in size from 600 to 5000 bp, predominated by two intense bands of *ca.* 1800 bp and *ca.* 2600 bp (Figure 2.4A). Eleven distinct rep-PCR banding patterns were classified into ten $(GTG)_5$-PCR types, r1 to r10, based on subtle banding differences. The majority of the isolates (306/315, 97%) fell into a related cluster at a similarity level of 56%, consisting of groups r1 – r4, characterised by the presence of intense bands at 900 bp, 1800 bp and *ca.* 2600 bp (Figure 2.4B).

The $(GTG)_5$-PCR type r1 included 63% (198/315) study isolates and possessed two subtypes (r1a and r1b) defined by a band intensity difference at 2300 bp. Isolates belonging to this major $(GTG)_5$-DNA type r1 were widely distributed in Chile, Denmark, Finland, the USA and 20 sites within the UK including England, Scotland and Northern Ireland, and from three different host species: rainbow trout, Atlantic salmon and coho salmon. In contrast, $(GTG)_5$-DNA subtype r1b was found mainly distributed within the UK (England, 12/48 isolates; and Scotland, 34/48 isolates).

The other two numerically dominant $(GTG)_5$-DNA types, r2 and r4, represented 78/315 (25%) and 29/315 (9%) isolates, respectively. The $(GTG)_5$-DNA type r2 was detected in Chile, France and the UK from rainbow trout and Atlantic salmon, while the type r4 was from rainbow trout sites within the UK only. The remaining $(GTG)_5$-DNA types contained one or two *F. psychrophilum* isolates from the UK. Multiple $(GTG)_5$-PCR types co-existing at the same site were found in 7/27 sites within the UK. Site Scot V possessed four distinct $(GTG)_5$-PCR types (r1, r2, r3 and r7) when sampled in June 2015.

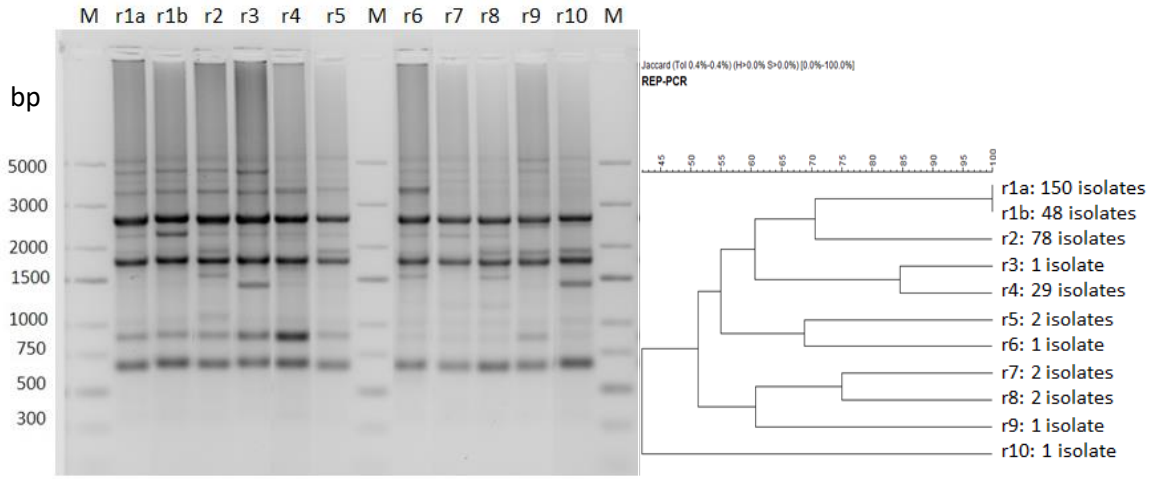


Figure 2.4. Repetitive PCR (rep-PCR) of *F. psychrophilum* isolates using $(GTG)_5$ primer. (A) Ten representative $(GTG)_5$-PCR types obtained from the 315 *F. psychrophilum* isolates tested. Lane M contains the GeneRuler Express DNA Ladder (Fermentas, Fisher Scientific, UK). Numbers on the left indicate the size of the bands of the molecular size marker in bp. (B) Dendrogram created using the GelCompar II software package (Applied Maths, Belgium) based on the $(GTG)_5$-PCR profiles and UPGMA algorithm with the Jaccard's similarity coefficient (0.4% tolerance setting).

2.3.4. 16S rRNA allele distribution

Two independent PCR assays for the detection of CSF 259-93 and ATCC 49418 alleles produced amplicons that were clearly distinguishable with expected sizes of 600 bp and 298 bp respectively (Figure 2.5).

The majority of the study isolates (162/169, 96%) contained the CSF 259-93 allele only, corresponding to 16S rRNA lineage II (Table 2.3). Only six of the 169 tested *F. psychrophilum* isolates, including the type strain NCIMB 1947^T were positive for both the ATCC 49418 and the CSF 259-93 allele (five from rainbow trout and one from coho salmon) and one isolate obtained from rainbow trout possessed the ATCC 49418 allele only. These seven isolates were classed as 16S rRNA lineage I.

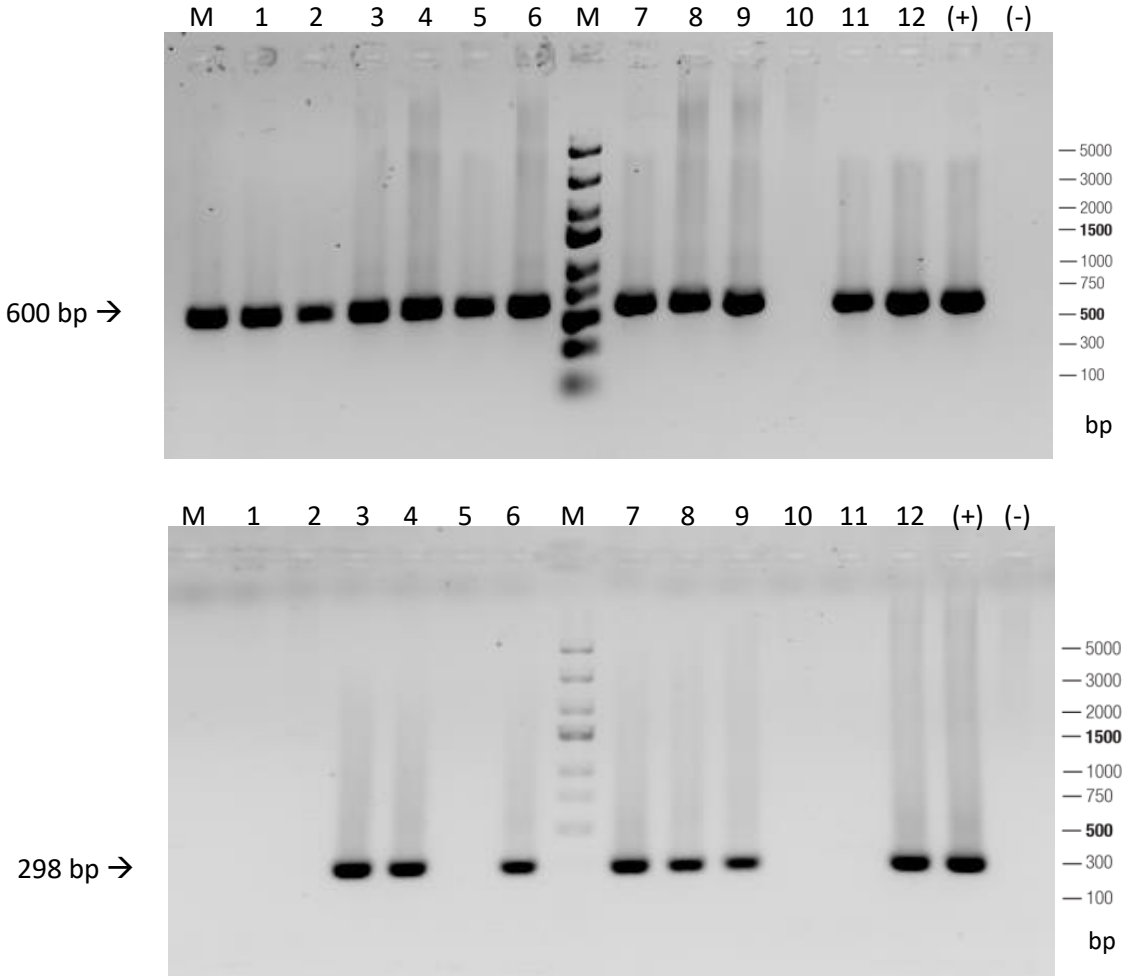


Figure 2.5. PCR products generated from *F. psychrophilum* DNA samples using two primer pairs for detecting the CSF 259-93 allele (600 bp, above) and the ATCC 49418 allele (298 bp, below). Lane 1-12: representative *F. psychrophilum* DNA sample extracts; lane (+): positive control (reference strain CSF 259-93 upper panel and type strain NCIMB 1947^{T} lower panel); lane (-): negative control (DW); lane M: GeneRuler Express DNA Ladder (Fermentas, Fisher Scientific, UK).

Table 2.3. Distribution of 16S rRNA alleles among 169 *F. psychrophilum* isolates tested.

Host	Lineage I		Lineage II	Total number of isolates
	ATCC allele	ATCC and CSF alleles	CSF allele	
Rainbow trout	1	5	142	148
Salmon	0	1	20	21
Total number of isolates	1	6	162	169

2.3.5. Plasmid profiling

Plasmid profiling revealed seven distinct profiles among 185 *F. psychrophilum* isolates (p1 – p7; Figure 2.6). These profiles were composed of one 3.3 kb plasmid (profile p1, n= 118), one 2.6 kb plasmid (p2, n= 3), one 4.0 kb plasmid (p3, n= 1), two plasmids of 3.3 and 2.1 kb (p4, n= 32), two plasmids of 4.0 and 3.3 kb (p5, n= 3), two plasmids of 3.3 and 2.8 kb (p6, n= 10) or no plasmid bands observed (p7, n= 18).

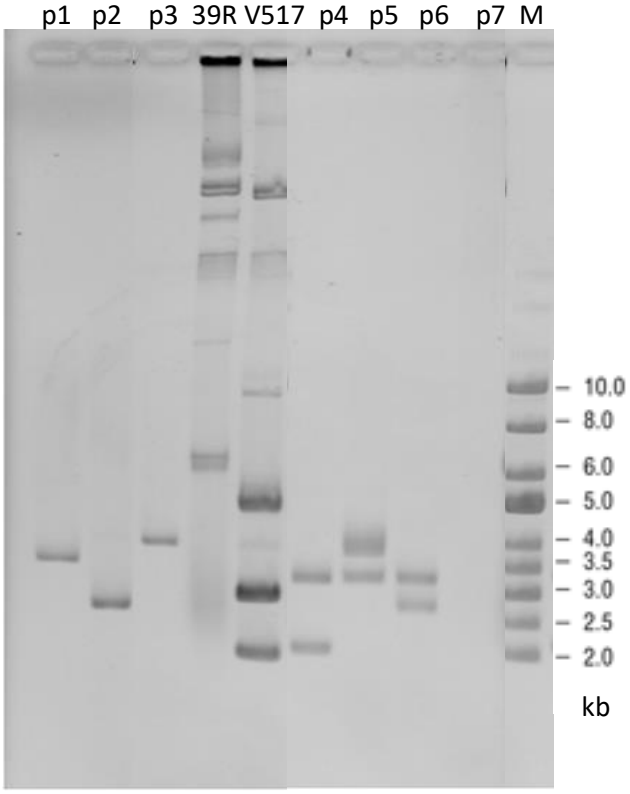


Figure 2.6. Seven plasmid profiles (p1 – p7) identified among 185 *F. psychrophilum* isolates separated in a 0.7 % agarose gel in TAE buffer. Lane 39R: *E. coli* 39R816 (7.0, 36-63-147 kb); Lane V517: *E. coli* V517 (2.1, 2.7-3.0, 3.9, 5.1-5.5, 7.2, 54 kb); Lane M: Supercoiled DNA ladder (New England BioLabs, UK). Profile p1: one 3.3 kb plasmid; p2: one 2.6 kb plasmid; p3: one 4.0 kb plasmid; p4: two plasmids of 3.3 and 2.1 kb; p5: two plasmids of 4.0 and 3.3 kb; p6: two plasmids of 3.3 and 2.8 kb; and p7: or no plasmid detected.

One hundred and sixty seven isolates (90%) possessed plasmids, of which 73% (122/167) had a single plasmid and the remainder harboured a combination of two plasmids. The *F. psychrophilum* type strain NCIMB 1947^{T} possessed one plasmid of 2.6 kb. The plasmid profile (p1) was found to be most widespread and detected in *F. psychrophilum* isolates retrieved from the UK (five sites in England and 14 sites in

Scotland), Denmark, Finland, Ireland, Chile and the USA. The simultaneous infection of more than one isolate harboring different plasmid profiles was observed at seven sites within the UK.

2.3.6. Serotyping

Antisera titres were determined by ELISA with homologous antigens (Table 2.4). One hundred and eighty of the 181 isolates examined could be serotyped, with 120, 49 and 11 isolates belonging to serotype Th, Fd and Fp^T, respectively. One Scottish isolate from rainbow trout (site Scot V) did not react with any of the antisera used. No isolates cross-reacting with more than one antisera were observed. The majority of isolates from rainbow trout (109/161, 68%) and Atlantic salmon (11/19, 58%) possessed serotype Th. The co-existence of two different serotypes, Th and Fd or Th and Fp^T, was recorded in six and one UK site respectively.

Table 2.4. Antigens and rabbit antisera titres included in this study

Antigen	Antiserum	Titre
F. psychrophilum NCIMB 13383	anti – Th	1,000,000
F. psychrophilum NCIMB 13384	anti – Fd	100,000
F. psychrophilum NCIMB 1947^T	anti – Fp^T	200,000

2.3.7. Population structure of the UK *F. psychrophilum* isolates

The PFGE groups E (cluster I), P, Q and the singleton S (cluster II) were numerically dominant and accounted for 200 of the 292 study isolates (68%). The *F. pychrophilum* isolates belonging to PFGE group P (n= 75) including eight pulsotypes were found to predominate within the UK originating from eight sites in Scotland, England and Northern Ireland. Singleton (pulsotype) S contained 40 isolates originating from four Scottish sites and one English site. In contrast, PFGE group E with four pulsotypes (n= 48) and PFGE group Q with two pulsotypes (n= 37) contained isolates exclusive to Scottish sites. In addition to the shared pulsotypes, most of the PFGE singletons (24/27) were unique to certain sites, e.g. pulsotypes A, B, BB, CC, FF and HH for site Scot V. There were two pulsotypes (HH and T1) found in healthy appearing fish at site Scot V and Scot XV respectively.

The majority of UK *F. psychrophilum* isolates (186/292, 64%) were collected from three Scottish sites (Scot I, Scot III and Scot V) over 3 – 11 years. The genetic heterogeneity over 3 – 11 years of *F. psychrophilum* isolates within each site is summarised in Figure 2.7. Site Scot I included 87 isolates that were collected from 35 fish samples over 16 sampling points during 11 years (2005 – 2015), and were categorised into five PFGE groups and 3 singletons belonging mainly to cluster II (D= 0.67, Figure 2.7A). Four sampling points revealed heterogeneous isolates including at least one of the three numerically dominant PFGE groups and singleton. The co-infection of isolates of distinct PFGE groups in an individual fish was noted in two fish.

In site Scot III, three PFGE groups and four singletons were identified among 44 isolates retrieved from 13 fish and five sampling points during 2011 – 2015 (D= 0.40, Figure 2.7B). The mixed infection with heterogeneous isolates, including isolates of the numerically dominant PFGE group E and singleton S, was recorded in June 2015. The *F. psychrophilum* isolates recovered from site Scot III in this study belonged to the two major PFGE clusters and their outliers.

Three PFGE groups and seven singletons were recognised among 55 *F. psychrophilum* isolates from site Scot V from June 2013 to June 2015 over four sampling points (D= 0.82, Figure 2.7C). Four numerically dominant PFGE groups and a singleton were detected simultaneously at this site in June 2015. Six individual fish showed co-infection of *F. psychrophilum* isolates belonging to two distinct pulsotypes. Similar to site Scot III, *F. psychrophilum* isolates from site Scot V were also distributed in the two major PFGE clusters and their outliers.

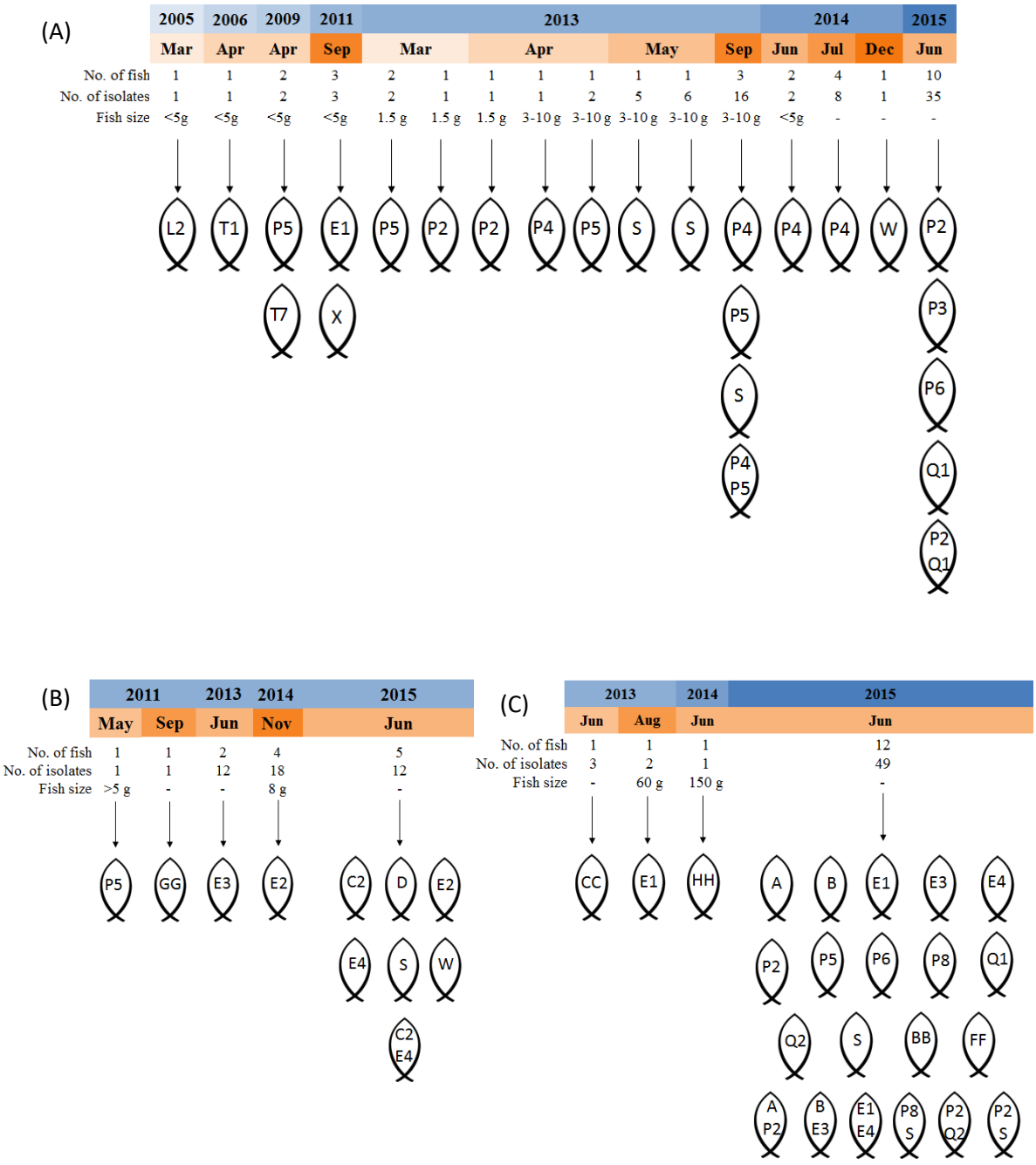


Figure 2.7. The occurrence over 3 – 11 years of *F. psychrophilum* pulsotypes at three Scottish sites; (A) site Scot I, (B) site Scot III, (C) site Scot V. Fish sizes from several sampling points are known. The co-infection of two distict pulsotypes within the same fish are indicated at each site.

By combining genotypic and serotypic methods, the predominant profile within the UK *F. psychrophilum* population, PFGE cluster II – $(GTG)_5$-PCR type r1 – 16S rRNA lineage II – serotype Th (70/156 isolates, 45%) was noted. The simultaneous infection of isolates harboring distinct pulsotypes, $(GTG)_5$-PCR types, serotypes or plasmids profiles

was observed in eight, seven, seven and seven of the 27 UK sites sampled respectively. The combination of these methods assisted in understanding the population diversity of UK *F. psychrophilum* isolates. In PFGE analysis, there was one non-typeable isolate by this technique due to the DNA degradation but this isolate was amenable to the $(GTG)_5$-PCR method. The exclusive $(GTG)_5$-PCR type r6 of this isolate possibly indicated that its distinctly degradation-sensitive DNA hindered PFGE procedure. Similarly, one non-serotypeable isolate possessed unique pulsotype BB and $(GTG)_5$-PCR type r3. The relationship between the genotype, defined by PFGE and $(GTG)_5$-PCR, 16S rRNA allele, plasmid profile and serotype, is represented in the PFGE dendrogram (Figure 2.3) and Table 2.5.

Table 2.5. The distribution of *F. psychrophilum* isolates according to the serotype in comparison to the size of plasmid(s)

Size of plasmid (kb)	Serotype Th	Fd	Fp^T	unknown
3.3	70	36	3	1
2.6	2	0	1	0
4.0	0	1	0	0
0	12	4	1	0
2.8 + 3.3	3	5	2	0
2.1 + 3.3	29	1	0	0
3.3 + 4.0	1	2	0	0

2.3.8. Genetic relationships inferred between the UK *F. psychrophilum* isolates and the reference isolates sourced from abroad

Within the major PFGE cluster I, a subpopulation containing three pulsotypes (F – H, clustered at 68% similarity) suggested the genetic relatedness of the UK, French and Chilean isolates. Notably, six Northern Irish isolates recovered in 2013 possessed the same pulsotype as eight French isolates also collected in 2013 and appeared to be genetically related to another French isolate, sampling date of is unknown. A Chilean isolate obtained in 1997 was distantly related to these Northern Irish and French isolates.

Within the major PFGE cluster II, 15 UK *F. psychrophilum* isolates of a subpopulation consisting of one PFGE group and five singletons (J – O) showed greater homology to USA isolates than to each other (e.g. a Scottish isolate of pulsotype J or O

and an USA isolate of pulsotype K or N respectively; Scottish and English isolates [pulsotype L2 and M] and an USA isolate of pulsotype L1). Another genetically related subpopulation composed of two PFGE groups (T – U) inferred a close relationship between the UK isolates and isolates of European origin. Particularly, PFGE group T (n= 40) comprised of seven pulsotypes and appeared to be the predominant genotype of European *F. psychrophilum* isolates used in this study. The UK *F. psychrophilum* isolates belonging to PFGE group T were identified from 2006, earlier than the isolates from the numerically dominant PFGE groups (P, E, Q) and singleton S that were detected from 2009.

2.4. Discussion

Flavobacterium psychrophilum is an important bacterial pathogen in the salmonid industry worldwide. The presence of this pathogen on fish farms requires close surveillance and the use of antibiotics for treatment and disease control. Hence, the analysis of the species genetic diversity and population structure using molecular typing methods is essential to improve our understanding of this pathogen and, in turn, endemic disease control at the farm level. PFGE is able to give an indication of fine-scale genetic differences between isolates, making the technique suitable for outbreak tracing, and, as in this study for examining the heterogeneity of isolates circulating in a particular region or epidemiological unit (farm or related group of rearing facilities) (Arai *et al.*, 2007; Chen *et al.*, 2008). A total of 45 different pulsotypes from the 292 UK *F. psychrophilum* isolates examined were identified. PFGE-based analyses have also been successfully deployed to investigate the genetic diversity of *F. psychrophilum* in other geographical regions. Arai *et al.* (2007) described 20 PFGE groups for 81 Japanese isolates from 19 prefectures; Chen *et al.* (2008), 32 pulsotypes for 139 USA isolates from four sites; and Del Cerro *et al.* (2010), 10 PFGE groups for 25 Spanish isolates from 12 fish farms. A high genetic relatedness was found in Chilean isolates (12 isolates from six sites in the study of Avendaňo-Herrera *et al.*, 2009) and Finnish isolates (42 isolates from three sites, [Sundell *et al.*, 2013]) where the majority of them (12/12 and 38/42 respectively) had the band similarity exceeding 87.5% and 94% respectively. Comparison of results in the present study with those of the two other studies in which the *Sac*I enzyme was used in PFGE (Chen *et al.*, 2008; Avendaňo-Herrera *et al.*, 2009) reveals a considerable difference in the minimum degrees of homology, i.e. 72 % and 54 % in the studies by Avendaňo-Herrera *et al.* and Chen *et al.*, respectively, and 32% in this work. This

indicates a higher overall genetic diversity of the UK *F. psychrophilum* isolates, observed by PFGE using *Sac*I enzyme (D= 0.86).

Using rep-PCR, the majority of UK *F. psychrophilum* isolates (284/293, 97%) were distributed into three predominant $(GTG)_5$-PCR types (r1, r2 and r4) showing minor differences in the banding patterns with a computed similarity level of 60%. In contrast, genetic homogeneity was detected between 20 Chilean *F. psychrophilum* isolates that belonged to a unique group with the computed similarity level at 89.7% based on a rep-PCR method using the REP primer (Valdebenito and Avendaño-Herrera, 2009). The results in the present study, where the $(GTG)_5$- PCR types r1 and r2 were found to be associated with PFGE cluster II and PFGE cluster I respectively, suggested rep-PCR could be utilised as a rapid diagnostic marker to assess genetic variation within the *F. psychrophilum* species in the laboratories of fish farms lacking sophisticated gene analysis equipment, although the reproducibility and performance of the method needs to be controlled by standardisation (Valdebenito and Avendaño-Herrera, 2009; Bartie *et al.,* 2012).

Although a large number of pulsotypes were recovered overall, most of the 292 UK isolates (68%) could be resolved into three genetically distinct PFGE groups P, E, Q and the singleton S, that were consistently recovered from farms across the UK during 2009 – 2015. This suggests that these particular pulsotypes may possess certain selective advantages, over the wide range of other pulsotypes recovered that favored the transmission and colonisation of salmonids in UK farming conditions. The recovery of similar PFGE groups from a wide variety of rainbow trout sites over an extended period likely reflects the structure of the UK rainbow trout industry, where there is widespread movement of live fish between sites (e.g. from hatcheries to ongrowing facilities), clearly favoring the spread of associated pathogens, such as *F. psychrophilum* (Brynildsrud *et al.*, 2014). Moreover, the persistence of isolates belonging to a certain predominant pulsotype within a site could increase the risk of epizootic episodes in consecutive years, as suggested by Madetoja *et al.* (2002). These results broadly support the contention of Nilsen *et al.* (2014) that *F. psychrophilum* displays an epidemic population structure in the regions where the pathogen is circulating.

The relatively clonal population structure of the species *F. psychrophilum* has been proposed previously and represents the species emergence within the developing rainbow trout industry and subsequent global dissemination of pathogenic clones (Nilsen *et al.*, 2014), via trade in eggs and live fish over the last 40 years, particularly into the

UK and other Northern European countries (Brynildsrud *et al.*, 2014). In the present study, three *F. psychrophilum* subpopulations (F – H, J – O and T – U) included isolates from the UK, European countries, Chile and USA. Furthermore, a visual comparison of the PFGE profiles from previous studies and in the present study suggests the following matches: PFGE genotype 2 (Avendaňo-Herrera *et al.*, 2009) = PFGE group T (this work); PFGE profiles ID05 and ID06 (Chen *et al.*, 2008) = PFGE group L (this work), indicating the genetic relatedness of the UK *F. psychrophilum* isolates and those found from abroad.

The different values of diversity index calculated from the PFGE analysis data of three sites (Scot I, Scot III and Scot V) pointed to an elevated population heterogeneity for isolates from site Scot V. The different D values and the similar number of isolates for these three sites suggest an influence of geographical origin and fish sizes on *F. psychrophilum* strain dissemination and evolution. The occurrence of unique PFGE singletons (24/27) and $(GTG)_5$-PCR types (r5 – r10) in certain sites or countries possibly represents local adaption of particular strains to sites or environmental niches (Arai *et al.*, 2007; Del Cerro *et al.*, 2010). These genotypes could be representatives of a wider group of environmental isolates that may represent less virulent endemic isolates and act more like opportunistic pathogens, and so are infrequently recovered. The high genetic heterogeneity of these isolates may result from continuous recombination of circulating isolates, with only some of these variants possessing fitness advantages that favour strain persistence (Nilsen *et al.*, 2014).

A concurrent mixture of isolates belonging to distinct pulsotypes, $(GTG)_5$-PCR types, serotypes or plasmid profiles observed in this study is in keeping with the observations of Madetoja *et al.* (2002), Chen *et al.* (2008), Del Cerro *et al.* (2010) and Sundell *et al.* (2013). Madetoja *et al.* (2002) found the simultaneous presence of serologically and genetically heterogeneous isolates at the same farm, from the same fish and in ovarian fluid of farmed female brood fish. Chen *et al.* (2008) also observed co-infection of *F. psychrophilum* in eight coho salmon with up to four distinct PFGE profiles, while Del Cerro *et al.* (2010) detected up to three different PFGE clusters co-existing in the same fish farm. These results reveal the importance of examining more than a single colony from an infected fish in monitoring the genetic population structure of *F. psychrophilum*. Although how the characteristics of these heterogeneous isolates, i.e. virulence and host specificity, contribute to the success and severity of such mixed infection remains unknown, the concurrent existence of isolates possessing genotypic and

phenotypic diversity creates an opportunity for genetic acquisition and recombination among *F. psychrophilum* isolates (Nilsen *et al.*, 2014).

There have been limited studies on linking specific pulsotypes of *F. psychrophilum* with severe RTFS/BCWD outbreaks in fish farms, especially those involving the coinfection of genetically heterogeneous isolates (Nilsen *et al.*, 2014). In the current study, pulsotypes G and P2 were identified simultaneously during two RTFS outbreaks in May and July 2013 in the same batch of fish from site N Ire I. The majority (5/6) of the *F. psychrophilum* isolates obtained from the first outbreak were assigned to pulsotype G, while ten out of 11 isolates in the second outbreak belonged to pulsotype P2. Meanwhile, Atlantic salmon site Scot XV had a BCWD outbreak with the simultaneous presence of isolates of two pulsotypes T1 and U1 but no disease outbreaks were reported when only isolates of pulsotype T1 were found in apparently healthy fish. However, isolates belonging to pulsotype T1 were associated with a raised mortality of Atlantic salmon from site Scot X, indicating the potential of these isolates to be pathogenic on another site. These findings suggested that more than one virulent isolate could coexist in the fish stock but only one strain tends to be associated with a disease outbreak. The selection of virulent strains may depend on some underlying factors related to fish husbandry of the farm (Madetoja *et al.*, 2002; Sundell *et al.*, 2013). Although the contribution of multiple strains within an outbreak, as well as the variation in virulence, is still unclear, Sundell *et al.* (2013) suggested that together with the majority of isolates within a farm belonging to a homogeneous group, a minority of isolates showing different genetic and serological characteristics is always present and might be required in the pathogenic process, resulting in a systemic infection. In addition, apparently healthy fish carrying *F. psychrophilum* isolates might act as reservoirs for shedding pathogens into surrounding water and be responsible for the wide dissemination of diverse isolates within and between sites (Chen *et al.*, 2008).

Due to the limited numbers of *F. psychrophilum* isolates derived from salmon hosts in comparison with rainbow trout (22 vs 293 isolates), a host-specific association between pulsotypes, as reported previously (Arai *et al.*, 2007; Chen *et al.*, 2008; Avendaño-Herrera *et al.*, 2009), was not able to be observed, although five singletons (O, V, Y, Z and DD) exclusive for Atlantic salmon and coho salmon were noted. Three PFGE groups (E, T and U) contained *F. psychrophilum* isolates from both rainbow trout and Atlantic salmon. Although these isolates were classified into the same PFGE group and could possibly be considered to be genetically related, the PFGE banding patterns

between isolates from different fish hosts showed subtle differences. The impact of these subtle differences on the characteristics (i.e. host specificity) of the isolates needs further investigation.

The correlation between $(GTG)_5$-PCR types and the fish host was not evident, which is consistent with the findings of Valdebenito and Avendaño-Herrera (2009). The *F. psychrophilum* type strain NCIMB 1947^T was assigned to the major $(GTG)_5$-PCR type r1, together with other rainbow trout and Atlantic salmon isolates from five different countries. In addition, the distribution of the CSF 259-93 and ATCC 49418 16S rRNA gene polymorphisms among *F. psychrophilum* isolates also did not reflect host-specificity. Similar studies on 59 Canadian isolates originating from 52 rainbow trout, five Arctic charr, one Atlantic salmon and one brook trout (Hesami *et al.*, 2008) and 20 Chilean isolates from ten Atlantic salmon, nine rainbow trout and one unknown source (Valdebenito and Avendaño-Herrera, 2009) also observed that the majority of isolates (43/59 and 18/20, respectively) were classed as lineage II, regardless of the fish host. In contrast, Nilsen *et al.*, (2011) found that Norwegian isolates from rainbow trout (nine isolates) and brown trout (four isolates) were assigned to lineage II, while those from Atlantic salmon were from both lineages (five isolates in lineage I and five in lineage II).

Low molecular weight plasmids of 3.3 kb, found either alone or in a combination with other plasmid, were detected in 152 out of the 169 UK isolates (90%) examined in this study. This plasmid size (3.3 kb) was similar to the findings reported by Madsen and Dalsgaard (2000), who also observed that the majority of Danish *F. psychrophilum* isolates (284/299) haboured a 3.3 kb plasmid. Meanwhile, other studies reported plasmids of 3.2 kb (Lorenzen *et al.*, 1997), 3.4 kb (Izumi and Aranishi *et al.*, 2004) and 3.5 kb (Chakroun *et al.*, 1998; Del Cerro *et al.*, 2009; Kim *et al.*, 2010; Henríquez-Núñez *et al.*, 2012). This discrepancy in the estimation of plasmid sizes could be due to methodological differences, as evidenced by the slight difference in the plasmid size of the type strain NCIMB 1947^T, which was determined to be 2.6 kb in the current study and by Lorenzen *et al.* (1997) and Madsen and Dalsgaard (2000), but was estimated to be 2.7 kb in the studies of Chakroun *et al.* (1998), Del Cerro *et al.* (2009) and Henríquez-Núñez *et al.* (2012), and 2.8 kb by Izumi and Aranishi (2004).

Lorenzen *et al.* (1997) observed that the Danish *F. psychrophilum* isolates containing one 3.2 kb plasmid were from RTFS/BCWD clinical outbreaks, while isolates with an additional 2.6 kb plasmid were from chronically infected fish and isolates without plasmids or with two plasmids of different size were from fish without classical signs of

RTFS/BCWD. However, no such association between plasmid profiles and virulence was revealed in the present study, where isolates containing one 3.3 kb plasmid was retrieved not only from diseased fish in acute or chronic RTFS outbreaks but also from non-symptomatic fish. That might appear contradictory with the observations of the cited literature but specific environmental conditions and the current overuse of antimicrobial agents at fish farms could exert selective pressure on the bacterial population, favouring the genetic acquisition and recombination among pathogenic *F. psychrophilum* isolates and isolates in the environment. Another explanation is that in spite of the similar size, the characteristics and roles of the 3.3 kb plasmids from different isolates may be diverse (E. Duchaud, personal communication). Further studies are required to confirm the heterogeneity of the similar sized plasmids occurring within *F. psychrophilum* isolates from various host species and clarify their roles in disease outbreaks. To date, only one plasmid pCP1 (3.4 kb) has been completely sequenced but the function of *F. psychrophilum* plasmids still remains unclear (Duchaud *et al.*, 2007).

Serotype Th was predominantly observed in 113 out of 165 isolates typed from 20 sites in the UK, while serotypes Fd and Fp^T were detected in 42 and 9 isolates and from 12 and 4 UK sites, respectively. This indicates that isolates obtained from RTFS/BCWD outbreaks in the UK mainly belonged to serotype Th. These findings are consistent with those of Lorenzen and Olesen (1997), in which serotype Th was the major serotype amongst the isolates examined (27/38 isolates, including two isolates from the UK), while the remaining isolates belonged to two minor serotypes, Fd (8/38 isolates) and Fp^T (3/38 isolates). Isolates belonging to serotype Fp^T were more commonly recovered from salmon hosts (Lorenzen and Olesen, 1997) and were less pathogenic to rainbow trout during challenge experiments, in comparison with Th and Fd serotypes (Madsen and Dalsgaard 1999, 2000; Madetoja *et al.*, 2002). In the present study, serotype Fp^T was recovered from four diseased Atlantic salmon, one apparently healthy rainbow trout and one diseased rainbow trout collected from four UK sites. However, the lower pathogenicity of this serotype in rainbow trout needs to be confirmed by further fish challenge trials. All of the *F. psychrophilum* isolates in the present study reacted with only one antisera and no cross-reaction was observed, indicating that the ELISA is a reliable method for defining serotypes (Lorenzen and Olesen, 1997). Several *F. psychrophilum* isolates (9 out of 45 and 18 out of 64) were shown to cross-react with two absorbed antisera using slide agglutination in the studies of Lorenzen and Olesen (1997) and Madetoja *et al.* (2002) respectively. One untypeable isolate retrieved from

rainbow trout in this study could represent another serotype in other serotyping schemes of Izumi and Wakabayashi (1999) and Mata *et al.* (2002) or a new serotype of *F. psychrophilum* as suggested previously by Lorenzen and Olesen (1997), Izumi and Wakabayashi (1999), and Madetoja *et al.* (2002).

The combination of genetic and phenotypic typing methods could aid in evaluating *F. psychrophilum* population diversity. A relationship between the genotype and antigenicity of *F. psychrophilum* isolates was observed in this work and in previous studies (Madsen and Dalsgaards, 2000; Madetoja *et al.*, 2001, 2002), although the contribution of such correlation to RTFS outbreaks is unknown. On the other hand, no correlation was found between the plasmid profiles and genotypes determined by PFGE, $(GTG)_5$-PCR and 16S rRNA allele PCR methods in the present study, as isolates of each genotype had different plasmid contents, consistent with the findings of Del Cerro *et al.* (2010) and Sundell *et al.* (2013) on 25 Spanish and 42 Finnish isolates respectively, who also applied PFGE as a genotyping method. A connection between plasmids and serological differences has been suggested by Lorenzen and Olesen (1997), who found that most isolates of serotype Th and Fd had a 3.2-kb plasmid, while isolates of serotype Fp^T exhibited more diverse plasmid patterns. A similar situation was also detected in the present study. However, eight isolates assigned to serotype Fp^T were not sufficient to reveal any plasmid variation within this group, compared with the two more prevalent serotypes Th (118 isolates) and Fd (50 isolates). Despite the limited value for epidemiological analysis (Del Cerro *et al.*, 2010; Sundell *et al.*, 2013), plasmid profiles could be used to differentiate isolates exhibiting the same genotypes.

Further work to confirm the population structure of UK *F. psychrophilum* isolates could be accomplished by applying multilocus sequence typing (MLST) and whole genome sequencing in order to enable inter-laboratory comparison and global *F. psychrophilum* surveillance. A large globally spread clonal complex (CC) of the species *F. psychrophilum* almost exclusive to the rainbow trout has been identified by MLST and is shown to have the sequence type ST2 or ST10 as the predicted common ancestor (Nicolas *et al.*, 2008; Siekoula-Nguedia *et al.*, 2012; Avendaño-Herrera *et al.*, 2014; Nilsen *et al.*, 2014). The fact that the *Sac*I-PFGE cluster II from this study included two reference strains CSF 259-93 (ST10) and NCIMB 13383 (ST2) suggested that the isolates belonging to the PFGE cluster II could be related to those of CC-ST2 or CC-ST10 in MLST analysis. The reference strain NCIMB 1947^T (ST13) was shown to be a singleton by both methods. These results indicate a likely overlap between the genetic

relationships inferred by PFGE and MLST in investigating the population structure of *F. psychrophilum*.

2.5. Conclusions

PFGE data on the UK *F. psychrophilum* diversity broadly supports the hypothesis of an epidemic population structure of this bacterium. The country-wide distribution of several PFGE groups or singletons may reflect local fish farming practices, where there can be widespread movement of live fish between sites. The simultaneous presence of genetically and serologically divergent clones additionally confound RTFS prevention and control. Understanding the genetic diversity present at particular sites is crucial in preventing the spread of epidemic clones and allowing in-depth studies to assess the differences between environmental isolates and clinical isolates. Further work is required to determine the *in vivo* biological importance of genetic and serological heterogeneity observed *in vitro* in the UK *F. pychrophilum* population and in vaccine development against RTFS/BCWD.

Chapter 3

Antimicrobial susceptibility of *Flavobacterium psychrophilum* isolates from the United Kingdom

3.1. Introduction

To date, *F. psychrophilum* infection of cultured salmonids has been found throughout North America, Europe and elsewhere in Turkey, Australia, Peru, Japan and Korea (Barnes and Brown, 2011), causing considerable losses worldwide. In the absence of a commercial vaccine against RTFS/BCWD, the use of antibiotics is currently the treatment of choice for controlling disease outbreaks, resulting in concerns about the development of antimicrobial resistance in *F. psychrophilum* (Gómez *et al.*, 2014).

Several studies have examined the antimicrobial susceptibility of *F. psychrophilum* isolated from the USA (Pacha, 1968, Soule *et al.* 2005), the UK (Rangdale *et al.*, 1997; Verner-Jeffreys and Taylor, 2015; Smith *et al.*, 2016), Denmark (Lorenzen *et al.*, 1997; Bruun *et al.*, 2000, 2003; Dalsgaard and Madsen, 2000; Schmidt *et al.*, 2000), France (Michel *et al.*, 2003), Japan (Izumi and Aranishi, 2004), Turkey (Kum *et al.*, 2008; Durmaz *et al.*, 2012; Boyacioğlu and Akar, 2012; Boyacioğlu *et al.*, 2015), Canada (Hesami *et al.*, 2010), Spain (Del Cerro *et al.*, 2010), Norway (Nilsen *et al.*, 2011), Chile (Henríquez-Núñez *et al.*, 2012) and Finland (Sundell *et al.*, 2013). However, differences in the medium and growth conditions used in these studies and variations in the interpretive criteria applied make comparisons between the studies difficult.

Smith *et al.* (2013) stressed the need for standardised and internationally recognised protocols for laboratory *in vitro* susceptibility testing for monitoring and surveillance programmes and the use of standardised methods to calculate epidemiological cut-off values (CO_{WT}) for interpretation of the data collected in such surveys. Recently, a standard method for microdilution minimal inhibitory concentration (MIC) testing of *F. psychrophilum* was published in the VET04-A2 guidelines of Clinical and Laboratory Standards Institute (CLSI, 2014a).

Normalised resistance interpretation (NRI) is an objective method to define the wild-type population, which is not affected by resistance development, on the sensitive side of the inhibition zone diameter histograms (Kronvall, 2003) and MIC distributions (Kronvall, 2010). This method offers a new statistically based tool in comparative susceptibility studies such as global surveillance of reduced susceptibility and performance of intra-laboratory quality controls. To date, there have been two studies using NRI to calculate the CO_{WT} values for *F. psychrophilum* MIC data and inhibition zone diameters in Chilean isolates (Henríquez-Núñez *et al.*, 2012), and in UK and Danish isolates (Smith *et al.*, 2016).

In the UK, oxytetracycline (OTC), amoxicillin (AMOX) and florfenicol (FFN) are the three antimicrobial agents licensed for use in aquaculture by the UK Veterinary Medicines Directorate (Verner-Jeffreys and Taylor, 2015). Florfenicol appears to be the only effective treatment for RTFS, while reduced susceptibilities to the other two antimicrobials have been observed among the UK *F. psychrophilum* isolates (Rangdale *et al.*, 1997; Verner-Jeffreys and Taylor, 2015; Smith *et al.*, 2016). However, data on the antimicrobial susceptibility of *F. psychrophilum* isolates circulating within the UK are still limited.

The aim of this chapter was to evaluate the antimicrobial susceptibility of 140 *F. psychrophilum* strains, 125 of which were obtained within the UK during 2005 – 2015, by the disc diffusion and standardised broth microdilution methods following M42-A (CLSI 2006) and VET04-A2 (CLSI, 2014a) guidelines respectively, recommended by CLSI for aquatic bacteria with optimal growth temperature below 35^{o}C. Several antibiotics commonly used in rainbow trout production (e.g. florfenicol, oxytetracycline, amoxicillin, oxolinic acid, enrofloxacin, trimethoprim/sulphamethoxazole) were used for susceptibility testing. Normalised resistance interpretation (NRI) (Kronvall, 2003; 2010) was applied to calculate CO_{WT} values for the interpretation of these data.

3.2. Materials and methods

3.2.1. Bacterial strains and growth conditions

Based on the strain characterisation data in Chapter 2, a total of 140 *F. psychrophilum* isolates with genetically and serologically different characteristics and from different sampling time and sites were selected for antimicrobial susceptibility testing. This collection comprised 125 isolates obtained within the UK during 2005-2015 and 15 strains from other countries (France, Denmark, Finland, Ireland, Chile and USA) included for purposes of comparison (Table 3.1). In relation to host distribution, the 123, 16 and one *F. psychrophilum* isolates were obtained from rainbow trout, Atlantic salmon and coho salmon respectively (Table 3.1).

F. psychrophilum isolates were grown in diluted Mueller-Hinton (MH) broth (Sigma-Aldrich, UK; 3 g L^{-1}) at 18^{o}C for 72 – 96 h. Broth cultures were shaken at 140 rpm (ISF-1-W, Kuhner, Switzerland). Stock cultures were maintained at -70^{o}C in tryptone–yeast extract–salts medium supplemented with glucose (FLP – tryptone (Oxoid, UK), 4.0 g L^{-1}; yeast extract (Oxoid, UK), 0.4 g L^{-1}; anhydrous calcium chloride (BHD, UK), 0.2 g L^{-1}; magnesium sulphate heptahydrate (Fisher Chemicals, UK), 0.5 g L^{-1};

D(+)-glucose (Sigma, UK), 0.5 g L^{-1}; Cepeda *et al.*, 2004) with 10% glycerol (Fisher Chemicals, UK) and on Protect-Multi-purpose cryobeads (Technical Service Consultants Ltd, UK).

The quality control strain *Escherichia coli* NCIMB 12210 (ATCC® 25922) was grown on trypticase soy broth (TSB; Oxoid, UK) for 16 – 20 h at 37ºC, shaken at 140 rpm.

Table 3.1. Summary of 125 UK *F. psychrophilum* isolates and 15 reference strains analysed for antimicrobial susceptibility in this study.

Location	**Host source**	**Site**	**Year of isolation**	**No. of sampling times**	**No. of strains**	**No. of unique genotypes[(a)]**	**No. of plasmid profiles [(a)]**
Scotland	*RT(93)/AS(14)*	*20 sites in total*	*2005-2015*	*46*	*107*	*47*	*7*
		Scot I	2005-2014	16	26	11	4
		Scot II	2013	1	1	1	1
		Scot III	2011-2015	4	15	9	4
		Scot IV	2013	1	5	2	2
		Scot V	2013-2015	4	29	16	5
		Scot VI	2009	1	1	1	1
		Scot VII	2007	1	1	1	1
		Scot VIII	2005	1	1	1	1
		Scot IX	2006	1	1	1	1
		Scot X	2011-2013	2	3	2	1
		Scot XI	2015	1	4	3	1
		Scot XII	2010	1	1	1	1
		Scot XIII	2005	1	1	1	1
		Scot XIV	2013	1	2	1	1
		Scot XV	2013	2	3	2	1
		Scot XVI	2014-2015	4	9	4	3
		Scot XVII	2007	1	1	1	1
		Scot XVIII	2009	1	1	1	1
		Unknown (2)	2009-2012	2	2	2	2

Location	Host source	Site	Year of isolation	No. of sampling times	No. of strains	No. of genotypes(*)	No. of plasmid profiles (*)
England	*RT*	*6 sites in total*	*2007-2015*	*8*	*15*	*5*	*2*
		Eng I	2013	3	8	4	2
		Eng II	2015	1	3	1	1
		Eng III	2015	1	1	1	1
		Eng IV	2015	1	1	1	1
		Eng V	2007	1	1	1	1
		Eng VI	2007	1	1	1	1
Northern Ireland	*RT*	*N Ire I*	*2013*	*2*	*3*	*2*	*2*
Total UK isolates	**RT(111)/AS(14)**	**27 sites**	**2005-2015**	**56**	**125**	**51**	**7**
Chile	RT		1995-1997		2	2	2
Denmark	RT		1990-1994		3	3	1
Finland	RT		1996		2	2	1
France	RT		1986-2013		3	2	1
Ireland	AS		2006		1	1	1
USA(b)	RT(3)/CS(1)		unknown - 2004		4	4	2
Total reference strains	**RT(13)/AS(1)/CS(1)**		**unknown - 2013**		**15**	**13**	**3**

RT, rainbow trout; AS, Atlantic salmon; CS, coho salmon; [a] Genotypes and plasmid profiles of *F. psychrophilum* isolates were determined by PFGE, rep-PCR and plasmid profiling in Chapter 2; [b] including the *F. psychrophilum* type strain NCIMB 1947^{T}.

3.2.2. Minimum inhibitory concentration (MIC) testing

The MIC testing for *F. psychrophilum* isolates was performed once for each isolate using Sensititre CMP1MSP plates (Trek Diagnostic Systems; ThermoScientific.com/microbiology). These test plates were 96-well, dry-form plates that contained two-fold serial dilution of the following antimicrobial agents: ampicillin (AMP) 0.03–16 mg L^{-1}, amoxicillin (AMOX) 0.25–16 mg L^{-1}, erythromycin (ERY) 0.25–128 mg L^{-1}, enrofloxacin (ENRO) 0.002–1 mg L^{-1}, florfenicol (FFN) 0.03–16 mg L^{-1}, flumequine (FLUQ) 0.008–4 mg L^{-1}, ormetoprim/sulphadimethoxine (1/19, PRI) 0.008/0.15–4/76 mg L^{-1}, oxolinic acid (OXO) 0.004–2 mg L^{-1}, oxytetracycline (OTC) 0.015–8 mg L^{-1} and trimethoprim/sulphamethoxazole (1/19, SXT) 0.015/0.3–1/19 mg L^{-1}.

The MIC assays were performed using the broth microdilution protocol recommended in the CLSI guideline VET04-A2 (CLSI, 2014a). A 3-mL aliquot of a 72 h broth culture of *F. psychrophilum* incubated at 18ºC was adjusted to an OD at 525 nm of 0.14 to 0.20 (Spectrophotometer CE 2041, Celcil, UK), equivalent to 5 x 10^7 CFU mL^{-1}, using diluted cation-adjusted Mueller-Hinton (CAMH) broth (BD Diagnostic Systems, UK and Ireland; 4 g L^{-1}). Similarly, an overnight TSB-broth culture of *E. coli* NCIMB 12210 shaken at 37ºC was adjusted to an OD at 525 nm of 0.25 to 0.30, equivalent to 2 x 10^8 CFU mL^{-1}. The inoculum was prepared by adding separately 30 µL and 110 µL of the OD-adjusted suspension of *E. coli* and *F. psychrophilum* respectively in 11 mL of diluted CAMH broth (4 g L^{-1}). All the wells of the microtitre plate were inoculated with 100 µL of the inoculum and covered with an adhesive seal. The plates were incubated at 18ºC for 72 – 96 h. The MIC values were recorded as the lowest concentration of antimicrobial agent that inhibited the visible growth of bacteria.

Colony counts on the inoculum suspension were performed to ensure that the final inoculum density was close to 5.0 x 10^5 colony-forming units (CFU) per millilitre. A 10-µL aliquot of the inoculum was diluted in 10 mL of sterile saline (0.9% NaCl, Sigma, UK). After mixing, a 100-µL aliquot was spread over the surface of diluted CAMH plate containing 1.5% agar (Oxoid, UK) and then incubated at 18ºC for 72 – 96 h. After incubation, the presence of approximately 50 colonies indicated a standardised inoculum concentration of 5.0 x 10^5 CFU mL^{-1}.

3.2.3. Disc diffusion testing

Disc diffusion susceptibility of the *F. psychrophilum* strains was determined by the protocol suggested in guideline M42-A (CLSI, 2006). The test was performed once for each isolate on diluted MH plates (3 g L^{-1}) containing 1.5% agar and 5% foetal calf serum (FCS; Gibco, Fisher chemicals, UK) and incubated at 15ºC for 68 – 72 h. Antimicrobial agent discs (Oxoid, UK) containing 10 µg amoxicillin ($AMOX_{10}$), 5 µg enrofloxacin ($ENRO_5$), 30 µg florfenicol (FFN_{30}), 2 µg oxolinic acid (OXO_2), 30 µg oxytetracycline (OTC_{30}) and 25 µg trimethoprim/sulphamethoxazole (1/19, SXT_{25},) were employed.

The inoculums were prepared by adjusting 3-mL aliquots of a 72 h broth culture of *F. psychrophilum* (shaken at 15ºC) to an OD of 0.14 to 0.20 at 525 nm, using diluted MH broth. A sterile cotton swab (VWR, UK) was dipped into the standardised inoculum suspension within 15 min of preparation. Surplus moisture was removed from the swab by rotating several times while pressing firmly against the inside wall of the tube above the liquid. The swab containing the inoculum was streaked over the entire surface of the diluted MH plate. Two antibiotic discs ($AMOX_{10}$ and OXO_2 or OTC_{30} and SXT_{25}) were applied on the same plate using a commercial dispenser (Thermo Scientific, UK). FFN_{30} and $ENRO_5$ discs were applied on separate plates manually using sterile forceps. The plates were incubated in an inverted position at 15ºC for 68 – 72 h. After incubation, the diameter of each inhibition zone was measured in millimetres by a ruler.

3.2.4. Quality control

As specified in VET04-A2 (CLSI, 2014b) the quality control strain *Escherichia coli* NCIMB 12210 (ATCC® 25922) was included in every MIC test run and was assayed on diluted CAMHB at 18ºC as described above. However, for the disc diffusion method, no quality control ranges have been established for any organisms requiring diluted MH plate at low temperature (CLSI, 2006). Therefore, the *F. psychrophilum* type strain NCIMB 1947^T was used every test run in order to monitor the performance of the method.

3.2.5. Statistical analysis

The antimicrobial susceptibility patterns of *F. psychrophilum* strains used in this study were determined by application of CO_{WT} values. These values were calculated for both the MIC and disc diffusion data using the NRI method described by Kronvall (2003; 2010). This method allowed isolates to be categorised as fully susceptible (wild type, WT) or manifesting reduced susceptibility (non-wild type, NWT) by CO_{WT}.

MIC distributions were analysed by NRI (Kronvall, 2010). A fully automatic Excel spreadsheet for performing these NRI analyses is available on-line (http://www.bioscand.se/nri/). The NRI method was used with permission from the patent holder, Bioscand AB, TÄY, Sweden (European patent No 1383913, US patent No 7,465,559). Peak values were established using 3-point rolling means, while the mean and standard deviation of the calculated normalised distributions of WT strains were calculated using a plot of probit values against the MIC values. For MIC data, the CO_{WT} was set at two standard deviations above the mean, giving a 97.7% inclusion of WT observations. To generate MIC CO_{WT} that could be applied to observational data sets, CO_{NRI} was rounded up to the next highest dilution used in the test protocol. In data sets where a small percentage (<5%) of the WT observations were "below-scale", these observations were treated as having the MIC value immediately below the limit of the plate quantitation (Smith *et al.*, 2016).

Normalised resistance interpretation for zone histograms was performed according to the method of Kronvall (2003) with the modification that peak values were established using 8-point rather than 4-point rolling means. The mean and standard deviation of the calculated standard distribution of WT strains were calculated using a plot of probit values of the cumulative frequencies against the zone diameters. Disc diffusion-based CO_{WT} was set at two and a half standard deviations below the mean, giving a 99.4% inclusion of WT observations (Smith *et al.*, 2007).

Chi-square test was performed in order to examine the statistical association between the antimicrobial susceptibility results and the genotypes (e.g. PFGE groups/singletons, $(GTG)_5$-PCR types and plasmid profiles determined in Chapter 2, with more than five strains) of *F. psychrophilum* strains using GraphPad Prism version 6 (GraphPad Software Inc., San Diego, CA, USA). The null hypothesis for this test was that antibiotic susceptibility pattern and the genotype of *F. psychrophilum* strains are independent. When the null hypothesis was rejected ($p < 0.05$), post-hoc cellwise test by calculating residuals was applied to investigate further the statistically significant chi-square test results (Beasley and Schumacker, 1995; García-Pérez and Núñez-Antón, 2003) using the SPSS 21.0 Software® (IBM, Armonk, New York, USA) and the groups considered to be significantly different if $p < 0.05$.

3.3. Results

3.3.1. Quality control

The MIC values obtained with the quality control reference strain *E. coli* NCIMB 12210, grown at 18°C for 72 – 96 h in diluted CAMH broth, were within the acceptable range published by CLSI in VET04-A2 guideline (CLSI, 2014b).

F. psychrophilum type strain NCIMB 1947^T was included in all disc diffusion tests and the inhibition zone data of this strain were 56 – 72 mm for $AMOX_{10}$, 60 – 75 mm for $ENRO_5$, 57 – 64 mm for FFN_{30}, 64 – 86 mm for OTC_{30}, 45 – 56 mm for OXO_2 and 16 – 44 mm for SXT_{25}. The mean of the ranges of these zone sizes for these six agents against the *F. psychrophilum* type strain was 16.5 ± 7.6 mm.

3.3.2. NRI analysis of susceptibility data

The distribution of MIC values of 140 *F. psychrophilum* strains for ten antimicrobial agents is shown in Tables 3.2 and 3.3. On the basis of the analysis of MIC data, the CO_{WT} values of ten antimicrobial agents is presented in Table 3.4. The distribution of disc diffusion zones of the isolates for six antimicrobials is displayed in Figure 3.1 and the inhibition zone-based CO_{WT} values of four antimicrobial agents is shown in Table 3.5.

3.3.2.1. Oxytetracycline

MIC data for OTC showed a clear bimodal distribution (Table 3.2). The modal group with lower MICs was assumed to represent the WT group. NRI analysis calculated the standard deviation of the log_2 normalised WT distribution as 0.67 and a CO_{WT} value of ≤0.25 mg L^{-1} (Table 3.4). Applying this cut-off, seventy-seven (55%) of the 140 strains analysed were categorised as NWT.

The disc diffusion zone sizes for OTC_{30} were also bimodal (Figure 3.1A). NRI analysis of these data calculated a standard deviation of the normalised WT distribution of 5.05 mm and a CO_{WT} value of ≥61 mm (Table 3.5). Applying this cut-off, seventy-nine (56%) of the 140 strains analysed were categorised as NWT.

The categorisation of isolates resulting from applying the cut-off of ≤0.25 mg L^{-1} to the MIC data agreed with the disc zone cut-off of ≥61 mm to the zone data for 94% of the 140 isolates studied (Figure 3.2A).

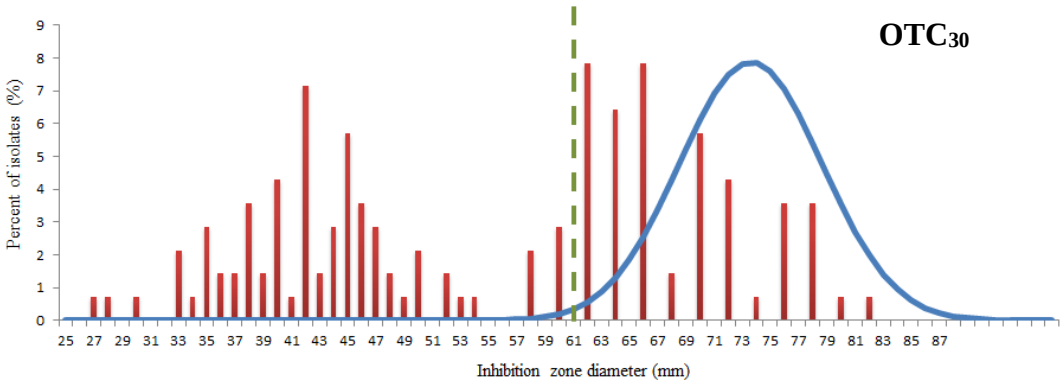
3.3.2.2. Amoxicillin and ampicillin

The distribution of MIC values for both AMP and AMOX appeared to be bimodal (Table 3.2). However, for AMOX, 98 observations (100% of the lower MIC modal observations) and for AMP, 24 observations (24% of the lower MIC modal observations), were recorded as "below-scale". On this basis, neither of these data sets were considered suitable for NRI analysis.

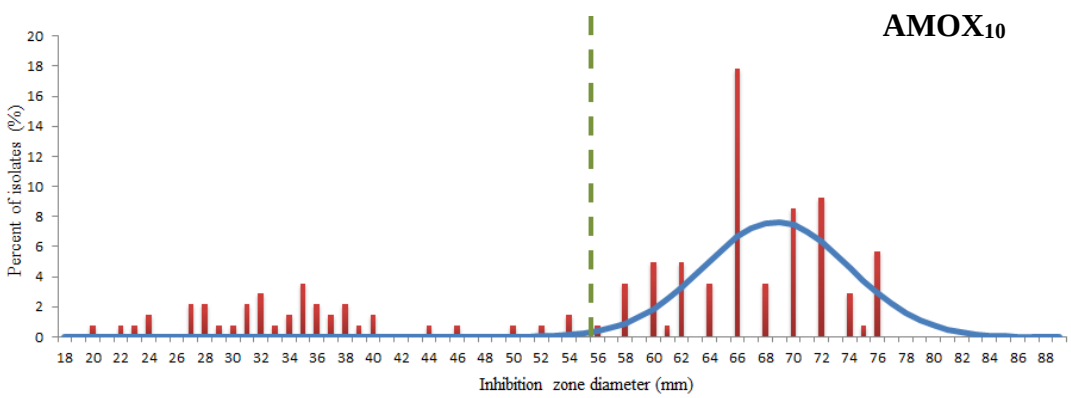
As in both MIC data sets, there was a separation of the low MIC and high MIC modal groups, thus the results were considered suitable for estimating CO_{WT} by visual examination. The provisional values generated by this subjective method were ≤0.25 mg L^{-1} for AMOX and ≤0.125 mg L^{-1} for AMP. A scatterplot of the paired MIC data for these two beta-lactam agents (Figure 3.3A) demonstrated a high correlation between them and also demonstrated that there was 100% agreement between the categorisation of isolates resulting from the application of these provisional cut-off values.

The disc diffusion zone sizes for $AMOX_{10}$ were also bimodal (Figure 3.1B). NRI analysis of these data calculated a standard deviation of the normalised WT distribution of 5.2 mm and a CO_{WT} value of ≥56 mm (Table 3.5). The categorisation of isolates resulting from applying the estimated cut-off of ≤0.25 mg L^{-1} to the AMOX MIC data agreed with the disc zone cut-off of ≥56 mm to the AMOX zone data for 97% of the 140 isolates studied (Figure 3.2B).

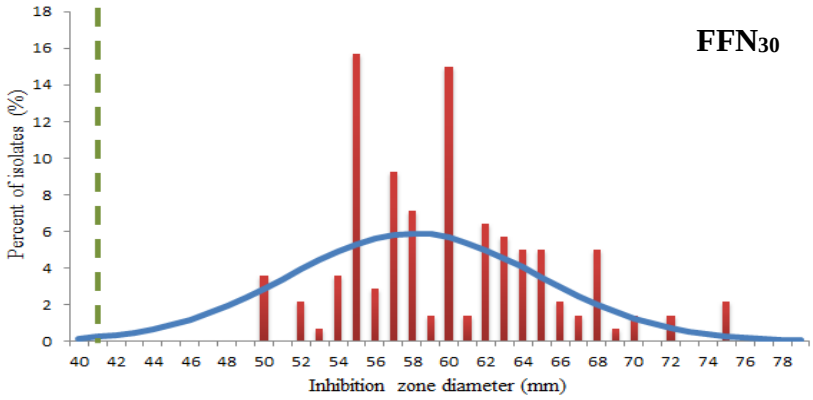
A


OTC30
Percent of isolates (%)
Inhibition zone diameter (mm)

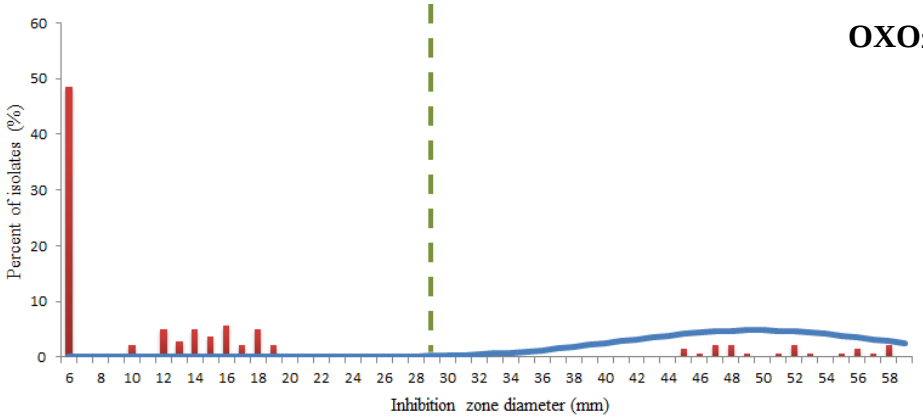
B


AMOX10
Percent of isolates (%)
Inhibition zone diameter (mm)

C


FFN30
Percent of isolates (%)
Inhibition zone diameter (mm)

D


OXO2
Percent of isolates (%)
Inhibition zone diameter (mm)

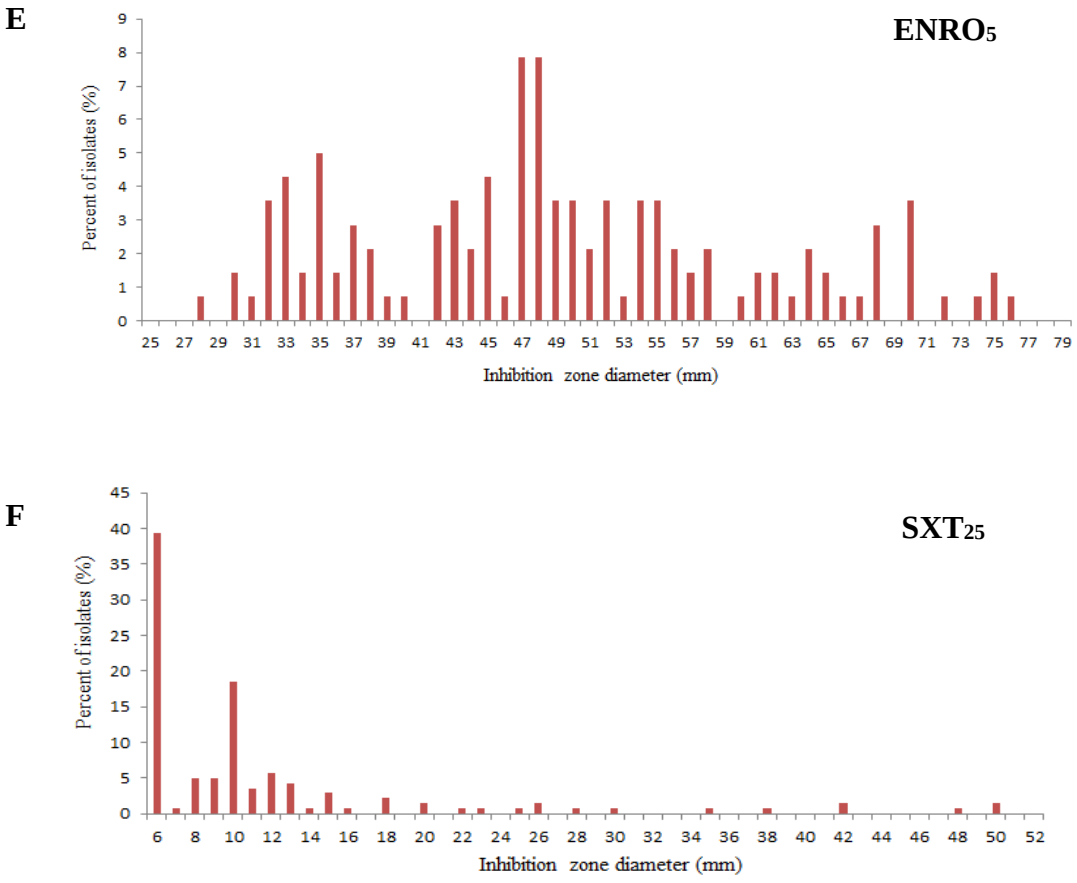


Figure 3.1. Distribution of 140 *F. psychrophilum* strains according to inhibition zone diameters generated by the disc diffusion test for 30 µg oxytetracycline (A), 10 µg amoxicillin (B), 30 µg florfenicol (C), 2 µg oxolinic acid (D), 5 µg enrofloxacin (E) and 25 µg trimethoprim/sulphamethoxazole (F). The continuous line represents 8 point rolling means, the vertical dashed line represents the calculated inhibition zone-based CO_{WT} value.

Table 3.2. MIC values (mg L^{-1}) determined for 140 *F. psychrophilum* isolates with eight different antibiotics. Shaded areas indicate the MIC values that could not be determined using the Sensititre CMP1MSP plates. Off scale indicates the number of strains where MIC values lay outside of the test range that could be determined using these plates.

	Off scale	0.002	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	Off scale
β lactams																
AMOX	98										19	14	9			
AMP	24						71	3			15	17	10			
Macrolides																
ERY	4									6	24	98	8			
Phenicols																
FFN								3	9	72	56					
Quinolones																
ENRO				8	14	3	3	53	17	14	28					
FLUQ						3	19	1	1	3	44	14	13			42
OXO							8	15	1	1	6	51				58
Tetracyclines																
OTC						13	45	5		2		7	33	32		3
	Off scale	0.002	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	Off scale

AMP: ampicillin; AMOX: amoxicillin; ERY: erythromycin; ENRO: enrofloxacin; FFN: florfenicol; FLUQ: flumequine; OXO: oxolinic acid; OTC: oxytetracycline

Table 3.3. MIC values (mg L^{-1}) determined for potentiated sulphonamide drugs (ormetoprim/sulphadimethoxine (PRI) and trimethoprim/sulphamethoxazole (SXT)) against 140 *F. psychrophilum* isolates. Shaded areas indicate MIC values that could not be determined using the Sensititre CMP1MSP plates. Off scale indicates the number of strains where MIC values lay outside of the range that could be determined using these plates.

	Off scale	0.008/0.15	0.015/0.30	0.03/0.59	0.06/1.19	0.12/2.38	0.25/4.75	0.5/9.5	1/19	2/38	4/76	Off scale
PRI						1	2	16	31	44	29	17
SXT					1	11	40	32	50			6

PRI: ormetoprim/sulphadimethoxine (1/19); SXT: trimethoprim/sulphamethoxazole (1/19).

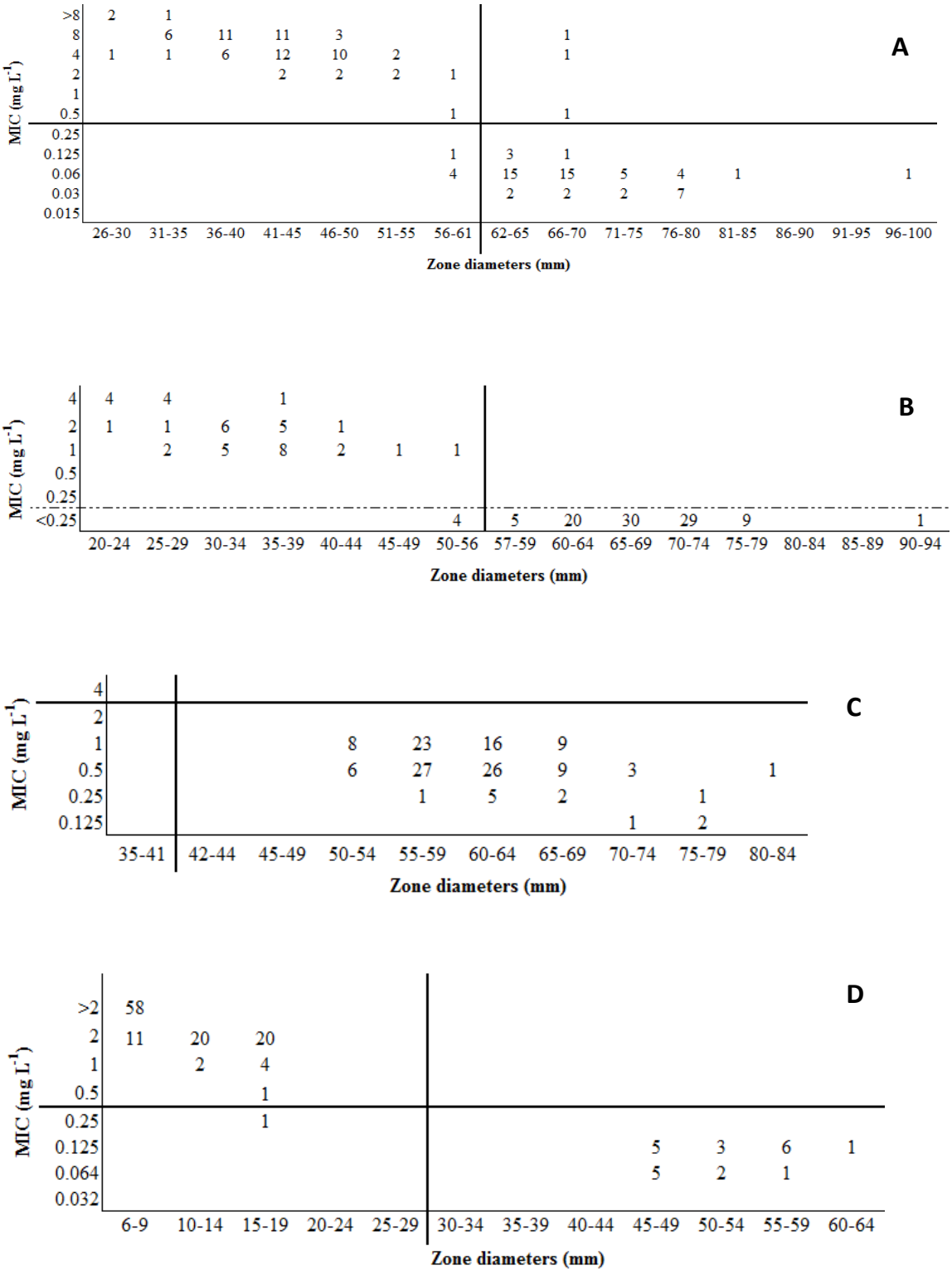
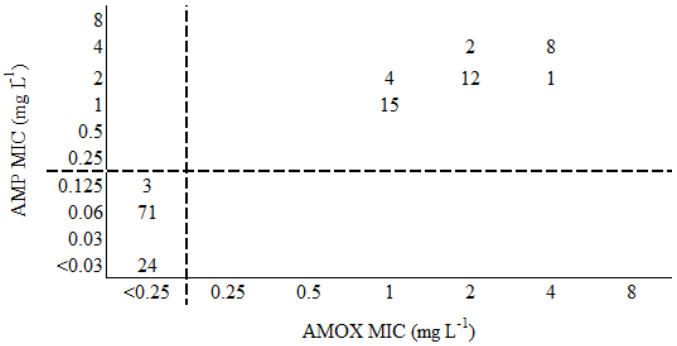
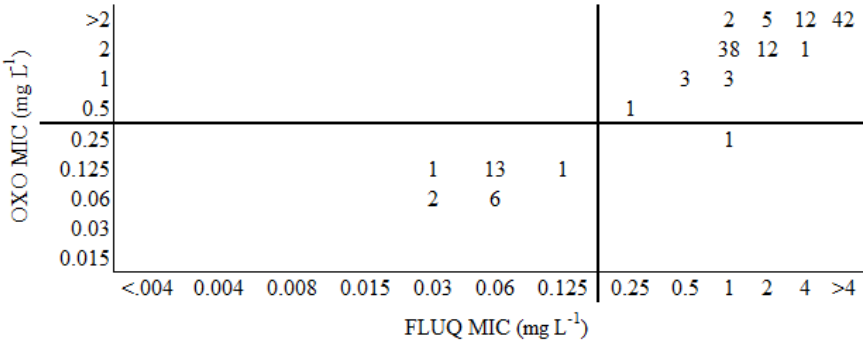


Figure 3.2. Plot of 140 paired MIC values versus disc diffusion zone diameters for oxytetracycline (A), amoxicillin (B), florfenicol (C) and oxolinic acid (D). A continuous thick line presents the calculated CO_{WT} value of the microbial agent. A dashed line represents an estimated CO_{WT} value by visual examination.

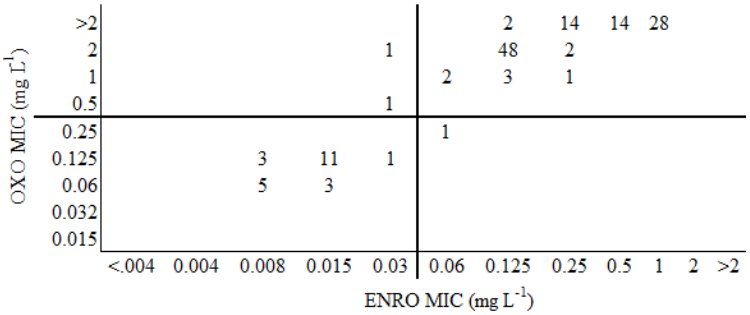
A


AMP MIC (mg L-1)
8
4
2
1
0.5
0.25
0.125
0.06
0.03
<0.03
2
8
4
12
1
15
3
71
24
<0.25
0.25
0.5
1
2
4
8
AMOX MIC (mg L-1)

B


OXO MIC (mg L-1)
>2
2
1
0.5
0.25
0.125
0.06
0.03
0.015
2 5 12 42
38 12 1
3 3
1
1
1 13 1
2 6
<.004
0.004
0.008
0.015
0.03
0.06
0.125
0.25
0.5
1
2
4
>4
FLUQ MIC (mg L-1)

C


OXO MIC (mg L-1)
>2
2
1
0.5
0.25
0.125
0.06
0.032
0.015
2 14 14 28
1
48 2
2 3 1
1
1
3 11 1
5 3
<.004
0.004
0.008
0.015
0.03
0.06
0.125
0.25
0.5
1
2
>2
ENRO MIC (mg L-1)

D

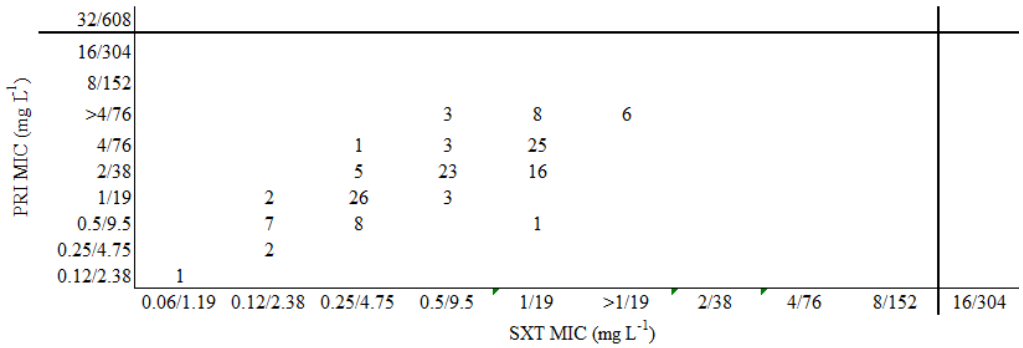

PRI MIC (mg L-1)
32/608
16/304
8/152
>4/76
4/76
2/38
1/19
0.5/9.5
0.25/4.75
0.12/2.38
3 8 6
1 3 25
5 23 16
2 26 3
7 8 1
2
1
0.06/1.19
0.12/2.38
0.25/4.75
0.5/9.5
1/19
>1/19
2/38
4/76
8/152
16/304
SXT MIC (mg L-1)

Figure 3.3. Plot of 140 paired MIC values between antimicrobial agents within the beta-lactams (A: ampicillin (AMP) and amoxicillin (AMOX)), quinolones (B: oxolinic acid (OXO) and flumequine (FLUQ); C: oxolinic acid (OXO) and enrofloxacin (ENRO)) and potentiated sulphonamide group (D: ormetoprim/sulphadimethoxine (PRI) and trimethoprim/sulphamethoxazole (SXT)). A continuous thick line represents the calculated cut-off value of the microbial agent. A dashed line indicates the estimated cut-off value by visual examination.

Table 3.4. Cut-off values (CO_{WT}) calculated using normalised resistance interpretation from the MIC data of ten antimicrobial agents.

[a] Standard deviation of the normalised distribution of MIC values for WT strains.

[b] CO_{WT} values of AMP and AMOX were estimated by visual examination.

Agent	No. of WT observations (%)	Standard deviation [a] (log_2 mg L^{-1})	CO_{WT} (mg L^{-1})
AMP[b]	98 (70%)	-	≤0.125
AMOX[b]	98 (70%)	-	≤0.25
ENRO	25 (18%)	0.69	≤0.032
ERY	140 (100%)	1.01	≤8
FFN	140 (100%)	0.71	≤2
FLUQ	23 (16%)	0.54	≤0.125
OTC	63 (45%)	0.67	≤0.25
OXO	24 (17%)	0.67	≤0.25
PRI	140 (100%)	1.39	≤16/304
SXT	140 (100%)	1.71	≤8/152

Table 3.5. Cut-off values (CO_{WT}) calculated using normalised resistance interpretation from the inhibition zone data of four antimicrobial agents.

* Standard deviation of the normalised distribution of MIC values for WT strains.

Agent	No. of WT observations (%)	Standard deviation* (mm)	CO_{WT} (mm)
$AMOX_{10}$	94 (67%)	5.20	≥56
FFN_{30}	140 (100%)	6.70	≥41
OTC_{30}	61 (44%)	5.05	≥61
OXO_2	23 (16%)	8.23	≥29

3.3.2.3. Florfenicol

MIC data for FFN showed a clear unimodal distribution (Table 3.2). NRI analysis calculated a standard deviation of the log_2 normalised WT distribution of 0.71 and a CO_{WT} value of ≤2 mg L^{-1} (Table 3.4).

The disc diffusion zone sizes for FFN_{30} were also unimodal (Figure 3.1C). NRI analysis of these data calculated a standard deviation of the normalised WT distribution of 6.7 mm and a CO_{WT} value of ≥41 mm (Table 3.5).

Applying the cut-off of ≤2 mg L^{-1} to the MIC data and the disc zone cut-off of ≥41 mm to the zone data both categorised 100% of the 140 isolates studied as WT (Figure 3.2C).

3.3.2.4. Oxolinic acid, Flumequine and Enrofloxacin

The MIC values of OXO, FLUQ and ENRO were bimodally distributed (Table 3.2). NRI analysis calculated a standard deviation of the log_2 normalised WT distribution of 0.67, 0.54 and 0.69 for OXO, FLUQ and ENRO respectively. The MIC CO_{WT} values calculated from these data were ≤0.25 mg L^{-1} for OXO, ≤0.125 mg L^{-1} for FLUQ and ≤0.032 mg L^{-1} for ENRO (Table 3.4). When these CO_{WT} values were applied, 116 (83%), 117 (84%) and 115 (82%) of the 140 strains were categorised as NWT with respect to OXO, FLUQ and ENRO respectively.

Scatterplots of the MIC data for OXO against FLUQ and ENRO (Figure 3.3B and 3.3C) demonstrated a very high (>98%) categorical agreement in both cases.

The disc diffusion zone sizes for OXO_2 were bimodal (Figure 3.1D). NRI analysis of these data calculated a standard deviation of the normalised WT distribution of 8.23 mm. The disc CO_{WT} value was calculated as ≥29 mm (Table 3.5). Applying the cut-off of ≤0.25 mg L^{-1} to the MIC data for OXO and the disc zone cut-off of ≥29 mm to the zone data resulted in 99% agreement in the categorisation of the 140 isolates studied (Figure 3.2D).

The disc diffusion zone sizes for ENRO did not show any visually obvious high zone modal group and were not subject to NRI analysis (Figure 3.1E).

3.3.2.5. Erythromycin

MIC values of ERY resembled a unimodal distribution. NRI analysis calculated a standard deviation of the log_2 normalised WT distribution of 1.01 and a CO_{WT} value of ≤8 mg L^{-1} (Table 3.2 and 3.4). This value determined that all 140 *F. psychrophilum* strains analysed were WT strains for ERY.

3.3.2.6. Ormetoprim/Sulphadimethoxine and Trimethoprim/Sulphamethoxazole

The distributions of the MIC values for these two potentiated sulpha agents were diverse but appeared to be unimodal (Table 3.2). NRI analysis generated provisional CO_{WT} values for PRI and SXT of ≤320 mg L^{-1} and ≤160 mg L^{-1}, respectively. However, the standard deviations calculated for the log_2 normalised distribution of these putative WT observations, 1.39 and 1.71 for PRI and SXT respectively, were higher than those recorded for all other agents in this work (Table 3.4). Therefore, the validity of these CO_{WT} values was questionable.

The disc diffusion zone sizes for SXT_{25} did not show any visually obvious high zone modal group and were not subject to further NRI analysis (Figure 3.1F).

3.3.3. Frequencies of UK *F. psychrophilum* strains with reduced susceptibility

The 125 isolates from 27 sites within the UK in this study had been isolated between 2005 and 2015 with the majority (110 isolates, 88%) being retrieved between 2011 and 2013. These isolates were selected for antimicrobial susceptibility testing on the basis of representative genotypes observed among 315 *F. psychrophilum* isolates examined in Chapter 2. Among 125 UK strains analysed, 51 different genetic profiles defined by PFGE and rep-PCR, and 7 plasmid profiles were found (Table 3.1). However, within this

strain set, there were four groups including two to three isolates that possessed the same site origin, sampling time point, genetic characteristics and antimicrobial susceptibilities. In order to prevent the overrepresentation of a certain strain in the strain set, these seven replicate isolates were eliminated from the analysis and the frequencies of NWT phenotypes circulating the UK during 2005 – 2015 were estimated from the MIC data and inhibition zone data for 118 isolates.

Applying the MIC-based CO_{WT} values calculated or estimated in this work (Table 3.3) to the MIC data from the remaining 118 UK isolates, the frequencies of *F. psychrophilum* isolates with reduced susceptibility were 92% for FLUQ, 90% for ENRO, 88% for OXO, 58% for OTC, 34% for AMOX, 32% for AMP and 0% for FFN, ERY, SXT and PRI. While the frequencies of the UK isolates manifesting reduced susceptibility determined by the inhibition zone-based CO_{WT} values (Table 3.4) were 92% for OXO_2, 59% for OTC_{30}, 36% for $AMOX_{10}$ and 0% for FFN_{30}.

The reduced susceptibility to three main antibiotics (OTC, AMOX and OXO) over time (2005 – 2015) of the UK *F. psychrophilum* isolates is summarised in Table 3.6. These data revealed that evidence of isolates with the reduced susceptibility to OTC and OXO was detected at two UK sites in 2005, while the reduced susceptibility to AMOX was not reported until 2007 or 2008 at one site depending on the disc diffusion test or MIC method applied respectively. Although OXO was not licensed for use in the UK, the occurrence of *F. pychrophilum* isolates with reduced susceptibility to this drug increased considerably from one of three sites in 2005 to all the UK sites examined since 2013, with a frequency of NWT isolates up to 96 – 98%. The prevalence of isolates manifesting the NWT phenotype for OTC within the UK was significantly unchanged over time (2005 – 2015) with the highest frequency of NWT isolates (22 of 39 isolates from eight of ten sites) recorded in 2013. The presence of isolates with reduced susceptibility to AMOX increased during 2005 - 2015 from no isolates found in 2005 to 20 out of 53 isolates observed at seven of eight sites in 2015.

Based on MIC analysis, 24 of 118 isolates presented a reduced susceptibility to all three antimicrobials (OTC, AMOX and OXO), 37 isolates manifested a decreased susceptibility to both OTC and OXO, 16 isolates were NWT to both AMOX and OXO, and four isolates were WT to these three antibiotics. Based on the disc diffusion results, 27 of 118 isolates exhibited the reduced susceptibility to OTC, AMOX and OXO, 40 isolates were NWT to both OTC and OXO, 18 isolates were NWT to both AMOX and OXO, and five isolates were WT to all three antibiotics. No isolates with reduced

susceptibility to both OTC and AMOX were identified, regardless of the method of analysis used.

The co-infection of isolates having different antimicrobial susceptibility levels to OTC, AMOX and OXO was reported in seven UK sites and from eight sampling time points (Table 3.7).

Table 3.6. The occurrence of *F. psychrophilum* isolates with reduced susceptibility determined by MIC data-based and inhibition zone-based CO_{WT} values in UK sites during the sampling years (2005 – 2015) for three commonly used antimicrobial agents (oxytetracycline, amoxicillin and oxolinic acid) against 118 UK *F. psychrophilum* isolates. The number of isolates is shown in brackets.

Year	Total no. of sites (Total no. of study isolates)	No. of sites containing NWT isolates determined by MIC data-based CO_{WT} values			No. of sites containing NWT isolates determined by inhibition zone-based CO_{WT} value		
		OTC	AMOX	OXO	OTC_{30}	$AMOX_{10}$	OXO_2
2005	3 (3)	2	0	1	2	0	1
2006	2 (2)	2	0	2	2	0	2
2007	4 (4)	3	0	3	3	1	3
2009	4 (4)	1	1	3	1	1	3
2010	1 (1)	1	0	0	1	0	0
2011	4 (4)	2	1	3	2	2	3
2013	10 (39)	8 (28)	4 (15)	10 (36)	8 (27)	4 (14)	10 (36)
2014	4 (8)	3 (4)	2 (4)	4 (7)	3 (4)	2 (4)	4 (8)
2015	8 (53)	5 (25)	7 (20)	8 (51)	5 (28)	7 (20)	8 (52)

Table 3.7. The co-infection of *F. psychrophilum* isolates having different antimicrobial susceptibility levels determined by MIC data-based inhibition zone-based and CO_{WT} values to three commonly used antimicrobial agents (oxytetracycline, amoxicillin and oxolinic acid) in seven UK sites.

Site	Sampling date	Total no. of study isolates	No. of NWT isolates determined by MIC data-based CO_{WT} values			No. of NWT isolates determined by inhibition zone-based CO_{WT} value		
			OTC	AMOX	OXO	OTC_{30}	$AMOX_{10}$	OXO_2
Scot I	12/09/2013	3	1	3	3	2	3	3
	09/06/2015	7	3	4	6	4	4	6
Scot III	12/06/2015	9	2	1	9	3	1	9
Scot IV	31/07/2015	5	3	0	5	2	0	5
Scot V	11/06/2015	24	14	10	22	15	10	23
Scot XVI	12/06/2015	5	1	1	5	1	1	5
Eng I	11/06/2013	2	1	1	2	1	1	2
N Ire I	03/07/2013	2	1	1	1	1	1	1

3.3.4. The correlation between antimicrobial susceptibility and genotypes of the *F. psychrophilum* strains

In order to investigate a possible association between genotype and antimicrobial susceptibility of *F. psychrophilum* strains, Pearson Chi-square test was applied between PFGE groups/singletons, $(GTG)_5$-PCR types or plasmid profiles containing more than five isolates and with the elimination of seven replicate isolates mentioned in Section 3.4.3. Tables 3.8 and 3.9 show the susceptibility patterns for each of the PFGE group/singleton based on MIC data and inhibition zone data respectively. Based on MIC data, statistically significant dependence of antibiotic susceptibilities (e.g. full susceptibility (WT) and reduced susceptibility (NWT)) on the *F. psychrophilum* PFGE groups/singletons were recorded for beta-lactams (AMP and AMOX, $p< 0.0001$) and OTC (p= 0.0014). Inhibition zone data also found such significant dependence for $AMOX_{10}$ ($p< 0.0001$) and OTC_{30} (p= 0.001). Regardless of the susceptibility testing method employed, post-hoc cellwise comparison revealed the over-representation ($p< 0.05$) of the WT phenotype to beta-lactams and OTC in PFGE group E, the NWT phenotype to beta-lactams in PFGE group P and the NWT phenotype to OTC in PFGE singleton S.

A high variability of susceptibility profiles was found in the set of 30 PFGE groups/singletons represented by less than five strains (Table 3.8 and 3.9). These profiles ranged from singletons that were fully susceptible to six antibiotics tested, such as singleton Y and one strain untypeable by *Sac* I-PFGE method, to PFGE groups that manifested reduced susceptibility to most of the tested antibiotics, such as PFGE group A (i.e. showing NWT phenotype to all the tested antibiotics).

Table 3.8. Number of strains from each PFGE group/singleton showing full susceptibility and reduced susceptibility to six antibiotics according to MIC testing by broth microdilution method.

PFGE group/singleton	Total no. of strains	No. of strains											
		AMP		AMOX		OTC		OXO[(b)]		ENRO[(b)]		FLUQ[(b)]	
		WT	NWT	WT	NWT	WT	NWT	WT	NWT	WT	NWT	WT	NWT
E [(a)]	15	**15**	0	**15**	0	**13**	2	0	15	0	15	0	15
P [(a)]	30	5	**25**	5	**25**	13	17	0	30	0	30	0	30
Q [(a)]	8	7	1	7	1	4	4	0	8	0	8	0	8
S [(a)]	13	4	9	4	9	1	**12**	0	13	0	13	0	13
T [(a)]	19	15	4	15	4	9	10	4	15	4	15	4	15
A	1	0	1	0	1	0	1	0	1	0	1	0	1
B	1	1	0	1	0	1	0	0	1	0	1	0	1
C	2	2	0	2	0	2	0	0	2	0	2	0	2
D	2	2	0	2	0	2	0	0	2	0	2	0	2
F	1	1	0	1	0	1	0	1	0	1	0	0	1
G	4	4	0	4	0	0	4	2	2	2	2	2	2
H	1	1	0	1	0	0	1	1	0	1	0	1	0
I	1	1	0	1	0	1	0	0	1	0	1	0	1
J	1	1	0	1	0	0	1	0	1	0	1	0	1
K	1	1	0	1	0	0	1	1	0	1	0	1	0
L	3	3	0	3	0	0	3	2	1	2	1	2	1
M	3	3	0	3	0	0	3	0	3	0	3	0	3
N	1	1	0	1	0	0	1	1	0	1	0	1	0
O	1	1	0	1	0	0	1	1	0	1	0	1	0
R	4	4	0	4	0	4	0	0	4	0	4	0	4
U	5	5	0	5	0	1	4	0	5	0	5	0	5

PFGE group/singleton	Total no. of strains	No. of strains											
		AMP		AMOX		OTC		OXO[b]		ENRO[b]		FLUQ[b]	
		WT	NWT	WT	NWT	WT	NWT	WT	NWT	WT	NWT	WT	NWT
V	1	1	0	1	0	0	1	0	1	1	0	0	1
W	2	2	0	2	0	0	2	0	2	0	2	0	2
X	1	1	0	1	0	0	1	0	1	0	1	0	1
Y	1	1	0	1	0	1	0	1	0	1	0	1	0
Z	1	1	0	1	0	0	1	1	0	1	0	1	0
AA	1	1	0	1	0	0	1	1	0	1	0	1	0
BB	1	1	0	1	0	0	1	1	0	0	1	0	1
CC	1	1	0	1	0	1	0	1	0	1	0	1	0
DD	1	1	0	1	0	0	1	0	1	1	0	0	1
EE	2	2	0	2	0	0	2	0	2	0	2	0	2
FF	1	1	0	1	0	1	0	1	0	1	0	0	1
GG	1	1	0	1	0	0	1	1	0	1	0	1	0
HH	1	1	0	1	0	0	1	0	1	1	0	0	1
untypeable	1	1	0	1	0	1	0	1	0	1	0	1	0

WT: wild type, fully susceptible; NWT: non wild-type, manifesting reduced susceptibility; AMP: ampicillin; AMOX: amoxicillin; OTC: oxytetracycline; OXO: oxolinic acid; ENRO: enrofloxacin; FLUQ: flumequine. Statistically significant results ($p< 0.05$)), indicating the overexpression of a antimicrobial susceptibility pattern in a certain genotype, by post-hoc cellwise comparison are shown in red. [a] These PFGE groups/singletons were included in the statistical tests due to the presence of more than five strains; [b] The chi-square tests for MIC data of quinolones were not valid because of the total small sample sizes showing WT phenotype to these drugs.

Table 3.9. Number of strains from each PFGE group/singleton showing full susceptibility and reduced susceptibility to three antibiotics according to disc diffusion testing.

PFGE group/singleton	Total no. of strains	No. of strains					
		$AMOX_{10}$		OTC_{30}		OXO_2[(b)]	
		WT	NWT	WT	NWT	WT	NWT
E [(a)]	15	**15**	0	**12**	3	0	15
P [(a)]	30	5	**25**	12	18	0	30
Q [(a)]	8	6	2	4	4	0	8
S [(a)]	13	4	9	0	**13**	0	13
T [(a)]	19	15	4	9	10	4	15
A	1	0	1	0	1	0	1
B	1	1	0	1	0	0	1
C	2	2	0	2	0	0	2
D	2	2	0	2	0	0	2
F	1	1	0	1	0	1	0
G	4	4	0	1	3	2	2
H	1	1	0	0	1	1	0
I	1	1	0	1	0	0	1
J	1	0	1	0	1	0	1
K	1	1	0	0	1	1	0
L	3	3	0	0	3	2	1
M	3	3	0	0	3	0	3
N	1	1	0	0	1	1	0
O	1	1	0	0	1	1	0
R	4	4	0	4	0	0	4
U	5	5	0	1	4	0	5
V	1	1	0	0	1	0	1
W	2	2	0	0	2	0	2
X	1	1	0	0	1	0	1
Y	1	0	1	1	0	1	0
Z	1	1	0	0	1	1	0
AA	1	1	0	0	1	1	0
BB	1	1	0	0	1	0	1
CC	1	1	0	1	0	1	0
DD	1	1	0	0	1	0	1
EE	2	2	0	0	2	0	2
FF	1	1	0	1	0	1	0
GG	1	1	0	0	1	1	0
HH	1	1	0	0	1	0	1
untypeable	1	1	0	1	0	1	0

WT: wild type, fully susceptible; NWT: non wild-type, manifesting reduced susceptibility; $AMOX_{10}$: amoxicillin 10 µg; OTC_{30}: oxytetracycline 30 µg; OXO_2:

oxolinic acid 2 µg. Statistically significant results ($p< 0.05$)), indicating the overexpression of a antimicrobial susceptibility pattern in a certain genotype, by post-hoc cellwise comparison are shown in red; [(a)] These PFGE groups/singletons were included in the statistical tests due to the presence of more than five strains; [(b)] The chi-square test for OXO_2 of quinolones was not valid because of the total small sample size showing WT phenotype to this drug.

Similarly, Tables 3.10 and 3.11 show the susceptibility patterns for each of the $(GTG)_5$-PCR types/subtypes tested by broth microdilution assay and disc diffusion test respectively. Based on the MIC data, the chi-square test revealed statistically significant dependence of antibiotic susceptibility on the *F. psychrophilum* $(GTG)_5$-PCR type/subtype for AMP, AMOX ($p< 0.0001$) and OTC ($p< 0.0001$). Inhibition diameter data also identified such significant dependence for $AMOX_{10}$ ($p< 0.0001$) and OTC_{30} ($p< 0.0001$). Regardless of the susceptibility testing method applied, statistically significant associations ($p< 0.05$) were observed by post-hoc cellwise comparison between the full susceptibility to beta-lactams and $(GTG)_5$-PCR type r2; between $(GTG)_5$-PCR type r4 and both NWT phenotype to beta-lactams and WT phenotype to OTC; and between the reduced susceptibility to OTC and $(GTG)_5$-PCR subtype r1a.

An over-representation of WT phenotype to beta-lactams in $(GTG)_5$-PCR subtypes r1a and r1b was only recorded in the broth microdilution MIC data analysis ($p< 0.05$). Likewise, a correlation between the full susceptibility to OTC and $(GTG)_5$-PCR type r2 was only found in the data analysis of the disc diffusion method ($p< 0.05$).

Variable patterns of antimicrobial susceptibility obtained in $(GTG)_5$-PCR types represented by less than five strains were observed in both broth microdilution and disc diffusion methods (Table 3.10 and 3.11). The strain belonging to $(GTG)_5$-PCR type r6 and one of two strains of r7 were fully susceptible up to six antibiotics tested, while strains of $(GTG)_5$-PCR type r8 exhibited reduced susceptibility up to four antibiotics tested.

Table 3.10. Number of strains from each $(GTG)_5$-PCR type/subtype showing full susceptibility and reduced susceptibility to six antibiotics according to MIC testing by the broth microdilution method.

$(GTG)_5$-PCR type/subtype	Total no. of strains	No. of strains											
		AMP		AMOX		OTC		OXO		ENRO		FLUQ	
		WT	NWT	WT	NWT	WT	NWT	WT	NWT	WT	NWT	WT	NWT
r1a (*)	71	**41**	30	**41**	30	21	**50**	7	64	7	64	7	64
r1b (*)	15	**15**	0	**15**	0	7	8	5	10	5	10	5	10
r2 (*)	28	**27**	1	**27**	1	19	9	5	23	4	24	5	23
r4 (*)	9	0	**9**	0	**9**	**9**	0	0	9	0	9	0	9
r3	1	1	0	1	0	0	1	1	0	0	1	0	1
r5	2	2	0	2	0	0	2	2	0	2	0	2	0
r6	1	1	0	1	0	1	0	1	0	1	0	1	0
r7	2	2	0	2	0	1	1	1	1	2	0	1	1
r8	2	2	0	2	0	0	2	0	2	0	2	0	2
r9	1	1	0	1	0	0	1	1	0	1	0	1	0
r10	1	1	0	1	0	0	1	0	1	1	0	0	1

WT: wild type, fully susceptible; NWT: non wild-type, manifesting reduced susceptibility; AMP: ampicillin; AMOX: amoxicillin; OTC: oxytetracycline; OXO: oxolinic acid; ENRO: enrofloxacin; FLUQ: flumequine. Statistically significant results ($p < 0.05$), indicating the overexpression of a antimicrobial susceptibility pattern in a certain genotype, by post-hoc cellwise comparison are shown in red; (*) indicates the $(GTG)_5$-PCR types/subtypes included in the statistical tests due to the presence of more than five strains.

Table 3.11. Number of strains from each $(GTG)_5$-PCR type/subtype showing full susceptibility and reduced susceptibility to three antibiotics according to disc diffusion testing.

$(GTG)_5$-PCR type/subtype	Total no. of strains	No. of strains					
		$AMOX_{10}$		OTC_{30}		OXO_2	
		WT	NWT	WT	NWT	WT	NWT
r1a (*)	71	40	31	20	**51**	7	64
r1b (*)	15	13	2	5	10	4	11
r2 (*)	28	**27**	1	**19**	9	4	24
r4 (*)	9	0	**9**	**8**	1	0	9
r3	1	1	0	0	1	0	1
r5	2	2	0	0	2	2	0
r6	1	1	0	1	0	1	0
r7	2	2	0	1	1	1	1
r8	2	2	0	0	2	0	2
r9	1	1	0	0	1	1	0
r10	1	1	0	0	1	0	1

WT: wild type, fully susceptible; NWT: non wild-type, manifesting reduced susceptibility; $AMOX_{10}$: amoxicillin 10 µg; OTC_{30}: oxytetracycline 30 µg; OXO_2: oxolinic acid 2 µg. Statistically significant results ($p < 0.05$)), indicating the overexpression of a antimicrobial susceptibility pattern in a certain genotype, by post-hoc cellwise comparison are shown in red; (*) indicates the $(GTG)_5$-PCR types/subtypes included in the statistical tests due to the presence of more than five strains.

Tables 3.12 and 3.13 show the susceptibility patterns for each plasmid profile based on MIC data and inhibition zone data respectively. The chi-square test when applied on the MIC data revealed significant dependence of antibiotic susceptibility on *F. psychrophilum* strain's plasmid profile for OTC (p= 0.0009). Susceptibility data generated by the disc diffusion method also found such significant association for OTC_{30} (p= 0.0332). When post-hoc cellwise comparison was applied on these significant chi-square results, the over-representation of WT or NWT phenotype to OTC in the plasmid profile containing one plasmid of 3.3 kb or a combination of 2.1 kb and 3.3 kb plasmids respectively were only observed in MIC data ($p < 0.05$).

Table 3.12. Number of strains from each plasmid profile showing full susceptibility and reduced susceptibility to six antibiotics according to MIC testing by the broth microdilution method.

Plasmid sizes (kb)	Total no. of strains	No. of strains											
		AMP		AMOX		OTC		OXO		ENRO		FLUQ	
		WT	NWT	WT	NWT	WT	NWT	WT	NWT	WT	NWT	WT	NWT
0 (*)	12	10	2	10	2	3	9	4	8	4	8	4	8
3.3 (*)	77	53	24	53	24	**43**	34	12	65	11	66	11	66
2.1 + 3.3 (*)	21	10	11	10	11	2	**19**	0	21	0	21	0	21
2.8 + 3.3 (*)	10	9	1	9	1	5	5	2	8	3	7	2	8
2.6	3	3	0	3	0	3	0	1	2	1	2	1	2
4.0	1	1	0	1	0	0	1	0	1	1	0	0	1
3.3 + 4.0	3	2	1	2	1	0	3	1	2	1	2	1	2

WT: wild type, fully susceptible; NWT: non wild-type, manifesting reduced susceptibility; AMP: ampicillin; AMOX: amoxicillin; OTC: oxytetracycline; OXO: oxolinic acid; ENRO: enrofloxacin; FLUQ: flumequine. Statistically significant results ($p < 0.05$)), indicating the overexpression of a antimicrobial susceptibility pattern in a certain genotype, by post-hoc cellwise comparison are shown in red; (*) indicates the plasmid profiles included in the statistical tests due to the presence of more than five strains.

Variable patterns of antimicrobial susceptibility obtained in plasmid profiles represented by less than five strains were observed in both broth microdilution and disc diffusion methods. Strains belonging to profile containing two plasmids of 3.3 kb and 4.0 kb manifested reduced susceptibility to six tested antibiotics, while one strain belonging to plasmid profile of 2.6 kb was fully susceptible to six antibiotics tested.

Table 3.13. Number of strains from each plasmid profile showing full susceptibility and reduced susceptibility to three antibiotics according to disc diffusion testing.

Plasmid sizes (kb)	Total no. of strains	No. of strains					
		$AMOX_{10}$		OTC_{30}		OXO_2	
		WT	NWT	WT	WT	WT	NWT
0 (*)	12	10	2	4	8	4	8
3.3 (*)	77	51	26	38	39	11	66
2.1 + 3.3 (*)	21	10	11	3	18	0	21
2.8 + 3.3 (*)	10	9	1	4	6	2	8
2.6	3	2	1	2	1	1	2
4.0	1	1	0	0	1	0	1
3.3 + 4.0	3	2	1	0	3	1	2

WT: wild type, fully susceptible; NWT: non wild-type, manifesting reduced susceptibility; $AMOX_{10}$: amoxicillin 10 µg; OTC_{30}: oxytetracycline 30 µg; OXO_2: oxolinic acid 2 µg. (*) indicates the plasmid profiles included in the statistical tests due to the presence of more than five strains.

3.4. Discussion

3.4.1. Data precision

3.4.1.1. Precision of MIC data sets

The precision of any CO_{WT} value is a function of the precision of the observational data used to calculate it. Smith *et al.* (2012) demonstrated that the standard deviations of the normalised distributions of the $\log_2$ WT observation calculated by the NRI analysis could provide a proxy measurement of precision. In this work, the median value of standard deviations calculated for ENRO, ERY, FFN, FLUQ, OTC and OXO (Table 3.4) was 0.68 $\log_2$ mg L^{-1}. This is very similar to the median of 0.67 $\log_2$ mg L^{-1} reported by Smith *et al.* (2016) for data generated in two laboratories for the same six antimicrobial agents. It is also similar to the median of 0.80 $\log_2$ mg L^{-1} reported for 25 data sets obtained by single laboratories at 35°C and published by EUCAST

(http://www.eucast.org/mic_distributions/; Smith *et al.*, 2016). These comparisons suggest that the MIC data sets obtained in this work for ENRO, ERY, FFN, FLUQ, OTC and OXO were of an acceptable level of precision and were of sufficient quality that they could be used to calculate CO_{WT} values.

The standard deviations calculated for potentiated sulphonamide MIC data**,** 1.39 $\log_2$ mg L^{-1} and 1.71 $\log_2$ mg L^{-1} for PRI and SXT respectively in this work, were considerably larger and were taken to indicate significant imprecision. Smith *et al.* (2016) also reported very low precision in the MIC data they obtained for these agents. NRI is based on the assumption that the $\log_2$ MIC values for WT isolates show a normal distribution and are randomly distributed about the mean. Therefore, in data obtained from a study of isolates that manifested fully susceptible phenotypes for two related agents such as PRI and SXT, one would not predict any correlation between their MIC values. However, analysis of the paired MIC values for PRI and SXT (Figure 3.3D) obtained in this work demonstrated a significant correlation (Spearman's r = 0.808). A similar analysis of the PRI and SXT data performed by Smith *et al.* (2016) on 61 *F. psychrophilum* isolates also revealed a significant correlation (Spearman's r = 0.873). These analyses suggest that the assumption that the MIC data for PRI and SXT obtained in this work had been obtained from a set of WT isolates is possibly incorrect. The possibility that all the isolates were more correctly categorised as NWT is consistent with the conclusion made by a number of authors (Rangdale *et al.*, 1997; Soltani *et al.*, 1995; Bruun *et al.*, 2000; Schmidt *et al.*, 2000; Valdebenito and Avendaño-Herrera, 2009) that members of the *F. psychrophilum* species could be intrinsically resistant to these agents.

Another possible explanation for the low precision levels of PRI and SXT has been offered by Minogue *et al.* (2013) who investigated the small reduction in susceptibility resulting from the possession of the *sul1* gene in *Aeromonas salmonicida* in a study with 17 isolates. These authors found that a group of *A. salmonicida* strains with an apparent WT phenotype to SXT included both WT and NWT strains with respect to the sulphamethozaxole component of SXT. If this is a general phenomenon, the large standard deviations for normalised distributions of the putative WT strains of *F. psychrophilum* may result from the fact that the assumed low MIC modal group represented WT strains is incorrect. To resolve this issue it would be necessary to investigate susceptibility to SXT and PRI by determining the MIC value for each of the two components of these mixtures separately.

3.4.1.2. Precision of disc diffusion data sets

The mean of the ranges of zone sizes (16.5 ± 7.6 mm) for six agents against the *F. psychrophilum* type strain NCIMB 1947^T can be compared to the mean ranges of acceptable zone sizes for *E. coli* NCIMB 12210 provided in the guideline M42-A (CLSI, 2006) for tests performed at higher temperatures. These were 7.7 ± 0.8 mm at 35°C, 8.0 ± 1.5 mm at 28°C and 11.8 ± 2.0 mm at 22°C. Likewise, the standard deviation of the normalised distributions of the four inhibition zone sets that could be analysed were 5.2 mm, 6.7 mm, 5.05 mm and 8.23 mm for $AMOX_{10}$, FFN_{30}, OTC_{30} and OXO_2 respectively (Table 3.5). The mean of these measures of precision (6.3 mm) can be compared with the mean values of 3.9 mm for 13 data sets of *Aeromonas salmonicida* obtained at 22°C for 44 – 48 h (Miller and Reimschuessel, 2006; Smith *et al.*, 2007, 2012; Smith and Kronvall, 2015) and 2.5 mm for 19 data sets of 52 *Edwardsiella tarda* isolates and 54 *Vibrio harveyi* isolates obtained at 28°C for 24 h (Lim *et al.*, 2016). The low level of precision of the zone data sets suggests that any CO_{WT} calculated from them should be treated with some caution. Smith and Kronvall (2015) have presented evidence that a decrease in precision as incubation temperature decreased and time increased is an inherent property of data generated by disc diffusion assays.

3.4.2. Categorical agreements

For four agents (FFN, OXO, AMOX and OTC), it was possible to calculate CO_{WT} values from both the MIC data and inhibition zone data and determine the percentage agreement between the categorisation of the 140 strains for the two methods. The values of the categorical agreements were 100% for FFN, 99% for OXO, 97% for AMOX and 94% for OTC. The high level of categorical agreement found suggests that although the disc diffusion protocol used in this work generated data of low precision, these inhibition zone data and the CO_{WT} values calculated from them may have some value in detecting strains of reduced susceptibility. It should, however, be noted that Smith and Kronvall (2015) demonstrated that reduced temperatures and prolonged incubation time necessary for *F. psychrophilum* growth increased not only the level of intra-laboratory variation but also the level of inter-laboratory variation in the data generated. This suggests that any inhibition zone CO_{WT} values calculated in one laboratory may be of value in interpreting zone data produced in that laboratory but may be misleading if applied to zone data obtained in another laboratory. In other words, the CO_{WT} values for MIC data calculated in this work are probably laboratory-independent and of general or ‘universal’

applicability. However, it is probably safer to treat the any CO_{WT} values for inhibition zone data as only of local applicability. Each laboratory using disc diffusion would have to generate their own CO_{WT} values.

3.4.3. Frequencies of UK *F. psychrophilum* strains with reduced susceptibility

Florfenicol, licensed for use in the UK since 1999, is the first treatment of choice for controlling RTFS outbreaks (Verner-Jeffreys and Taylor, 2015). It is a fluorinated structural analog of thiamphenicol and chloramphenicol and has a bacteriostatic effect by binding to the 50S ribosomal subunit and inhibiting bacterial protein synthesis (Hesami *et al.*, 2010; Romero *et al.*, 2012). This work has supported the effectiveness of FFN against UK *F. psychrophilum* isolates, in accordance with the observations of Rangdale *et al.* (1997) and Smith *et al.* (2016) in 36 and 22 UK isolates collected during 1990-1994 and 2006-2013 respectively. The failure to detect any *F. psychrophilum* strains manifesting reduced susceptibility to FFN has been reported in previous studies of strains of European origin (Bruun *et al.*, 2000; Schmidt *et al.*, 2000; Michel *et al.*, 2003). In contrast, a high frequency (52.8%) of isolates inhibited by high concentration of FFN (≥2 μg mL^{-1}) was observed by Hesami *et al.* (2010) in 75 Canadian *F. psychrophilum* isolates. These authors found a unimodal distribution of FFN MIC data and no difference in the MICs for FFN among isolates collected before and after 1996, the date when FFN was approved in Canada. Henríquez-Núñez *et al.* (2012), on the basis of MIC data, also reported a very high (90.5%) frequency of isolates showing MIC values for FFN ranging from 4 to 32 μg mL^{-1}. However, the results of their disc diffusion assays on the same 40 Chilean isolates suggested that the vast majority were fully susceptible to FFN (with zone sizes from 60 to 70 mm). Applying the MIC-based CO_{WT} value for FFN calculated in this study, high frequencies of Canadian and Chilean isolates could be categorised as NWT isolates assumed to have an acquired resistance determinant to FFN. These findings would be a concern in the development and transmission of the resistance mechanism to FFN within the global *F. psychrophilum* population, despite the insufficient information on strain sets and differences in the methods and the interpretive criteria of susceptibility data used in these studies.

Oxytetracycline, an analog of tetracycline, has been licensed mainly for the treatment of furunculosis and enteric redmouth disease since 1994 in the UK (Verner-Jeffreys and Taylor, 2015). Tetracyclines inhibit protein synthesis by binding to the 30S ribosomal subunit (Romero *et al.*, 2012). The frequency of UK isolates (58%) with

reduced susceptibility to OTC obtained in this study is consistent with the observation (65%) of Smith *et al.* (2016). Bruun *et al.* (2000) recommended the MIC breakpoint at 0.25 mg L^{-1} for OTC based on the consideration of pharmacokinetic data and MIC values of 387 Danish strains collected in 1994-1998 and reported a 68% frequency of isolates manifesting reduced susceptibility. Higher frequencies of isolates (82% and 90%) showing MIC values for OTC above 0.25 µg mL^{-1} were observed in 75 Canadian (Hesami *et al.*, 2010) and 40 Chilean (Henríquez-Núñez *et al.*, 2012) *F. psychrophilum* isolates respectively. Although OTC has not been used mainly for RTFS treatment in the UK at present since the introduction of FFN in 1999, the OTC data from this work did not indicate any decline in the occurrence and spreading of isolates manifesting reduced susceptibility to this drug in the UK sites over 2005 – 2015. However, this recent frequency (58%) is considerably lower than that in the study of Rangdale *et al.* (1997), which was around 88% of isolates had MIC values for OTC ranging from 0.5 to 64 µg mL^{-1} in a total of 47 isolates examined, 36 of which was collected from UK during 1990 – 1994.

Amoxicillin has been licensed in the UK in the control of furunculosis and other Gram-negative septicaemias in salmon, and occasionally for RTFS outbreaks, since 1999 (Verner-Jeffreys and Taylor, 2015). Beta lactam agents are bactericidal by interfering bacterial cell wall synthesis (Romero *et al.*, 2012). The frequency of UK isolates (34%) with reduced susceptibility to AMOX obtained in this study is higher than those (16%, 14% and 12%) obtained by Rangdale *et al.* (1997), Smith *et al.* (2016) and Bruun *et al.* (2000) respectively. This higher frequency could be due to a widespread use of AMOX in UK aquaculture for the treatment of other bacterial fish diseases since 1999, which may increase a possibility of developing and transmitting a resistance mechanism to AMOX between fish pathogens. Ampicillin is a beta-lactam antibiotic that is closely related to AMOX. A frequency (32%) of the UK isolates with NWT phenotype to AMP observed in this study was considerably lower than the observation of Hesami *et al.* (2010), who reported 82 % of the Canadian isolates having MICs values ranging from 0.25 to ≥16 µg mL^{-1}.

Quinolones and fluoroquinolones function primarily by inhibiting the DNA-gyrase enzyme, which catalyses the topological changes necessary for bacteria DNA replication (CLSI, 2006). The very high frequencies of the UK isolates with the NWT phenotype to OXO (88%), FLUQ (92%) and ENRO (90%) from this work were consistent with those observed by Hesami *et al.* (2010) in 75 Canadian isolates (83%,

86% and 79% respectively), Bruun *et al.* (2000) in 387 Danish isolates (100% for OXO) and Henríquez-Núñez *et al.* (2012) in 40 Chilean isolates (100% for OXO). The fact that OXO is not licensed in the UK but in other European Member States for controlling trout diseases (Verner-Jeffreys and Taylor, 2015) could possibly indicate the spread of the isolates exhibiting reduced susceptibility to quinolones into the UK via trade in eggs and live fish. A trend of increased frequency of UK *F. psychrophilum* isolates manifesting reduced susceptibility to OXO during 2005 – 2015 was also observed in this study. Similarly Bruun *et al.* (2000) reported the increased prevalence of resistance to OXO in Danish *F. psychrophilum* strains from around 50% to 100% during 1994 – 1998. The high percentage agreement in the categorisation of the *F. psychrophilum* strains between MIC data for OXO and FLUQ or ENRO observed in this work and Smith *et al.* (2016) suggests that the MIC data for OXO could be used as a predictor of reduced susceptibility to the FLUQ and ENRO. Adoption of this would reduce the cost of routine susceptibility testing.

Using sulphonamides individually for antibacterial effect has been diminished by widespread acquired resistance; but, when combined with diaminopyrimidines, this mixture displays a synergistic effect by inhibiting sequential steps in the synthesis of folic acid and purines required for bacterial DNA synthesis (Prescott, 2007). PRI and SXT are examples of this combination used in aquaculture. Despite the low precision of SXT and PRI MIC data observed this work, high MIC values for these two drugs have been reported in previous studies (Rangdale *et al.*, 1997; Bruun *et al.*, 2000; Lorenzen *et al.*, 1997; Kum *et al.*, 2008; Hesami *et al.*, 2010; Smith *et al.*, 2016). The modal MIC values for the UK strains obtained in this work were 20 mg L^{-1} for SXT (1/19) and 40 mg L^{-1} for PRI (1/19), considerably higher than those observed by Smith *et al* (2016), which were 2.5 mg L^{-1} and 10 mg L^{-1} respectively. In earlier studies, *F. psychrophilum* strains were found to have MIC data for trimethoprim/sulphadiazine (1:5) with modal values at 16 mg L^{-1} (Rangdale *et al.*, 1997) and 128 mg L^{-1} (Bruun *et al.*, 2000); for trimethoprim/sulphamethoxazole (1:20) at 50 mg L^{-1} (Lorenzen *et al.*, 1997); for SXT (1/19) at 4 mg L^{-1} (Kum *et al.*, 2008) and ≥20 mg L^{-1} (Hesami *et al.*, 2010).

Erythromycin is not used therapeutically against *F. psychrophilum* infection. Therefore, the susceptibility testing for ERY has limited value. All the UK isolates with full susceptibility to ERY observed in this work is not surprising, and is consistent with the finding of Hesami *et al.* (2010) with the majority (83%) of 75 Canadian isolates being fully susceptible to ERY.

The Kirby-Bauer disc diffusion test (Bauer *et al.*, 1966), the antimicrobial susceptibility test most widely used in diagnostic laboratories, was conducted in parallel with the MIC method for investigating the antimicrobial susceptibility of the 118 UK *F. psychrophilum* isolates examined. There have been many studies using the disc diffusion test to evaluate the antimicrobial susceptibility of *F. psychrophilum* strains (Pacha, 1968; Dalsgaard and Madsen, 2000; Soule *et al.*, 2005; Kum *et al.*, 2008; Nilsen *et al.*, 2011b; Boyacioğlu and Akar, 2012; Durmaz *et al.*, 2012; Henríquez-Núñez *et al.*, 2012; Michel *et al.*, 2013; Sundell *et al.*, 2013). However, to date, there is no standardised disc diffusion test developed for use with organisms whose optimal growth temperature is below 22^{o}C, such as *F. psychrophilum*. The disc diffusion testing protocol from the guideline M42-A (CLSI, 2006) applied in the present study has not been formally accepted as a standard by CLSI. In addition, no quality control ranges have been established for any organisms grown on diluted MH plate at low temperature (CLSI, 2006) to monitor the performance of the method. Therefore, these studies used different methods and importantly a variety of the interpretive criteria in categorising their isolates as manifesting reduced susceptibility. As a consequence, the meaningful comparison of the frequencies of strains with reduced susceptibility both within and between countries is difficult. Similar to this work, Henríquez-Núñez *et al.* (2012) applied the NRI method in analysing the disc diffusion data of 40 Chilean *F. psychrophilum* strains obtained according to the guideline M42-A (CLSI, 2006). These authors found the majority of Chilean isolates with inhibition zones for OTC_{30} and FFN_{30} in the region of 30 – 42 mm and 60 – 70 mm respectively and no inhibition zones for OXA_2. With the exception of FFN_{30}, these inhibition zone ranges were smaller than those obtained in the present study (27 – 82 mm for OTC_{30} and 6 – 60 mm for OXA_2).

In general, among the four common agents (FFN, OTC, AMOX and OXO) used in attempts to control RTFS in the UK, the fact that FFN is the most effective treatment was reflected by the full *in vitro* susceptibility to FFN of all the 118 UK *F. psychrophilum* isolates analysed in this work regardless of the testing methods, in accordance with the observations of Rangdale *et al.* (1997) and Smith *et al.* (2016). Previously, the survey of Verner-Jeffreys and Taylor (2015) revealed that florfenicol was the treatment of choice in the industry and that most batches of rainbow trout were treated with this agent at least once during every production cycle. These florfenicol treatments were generally considered very effective. Where other antimicrobials (oxytetracycline, oxolinic acid or amoxicillin) were used, the therapeutic response was reported as either mixed or poor.

That the UK *F. psychrophilum* strains were determined to be *in vitro* fully susceptible to FFN during two recent decades of sampling (1997 – 2016) is encouraging. However, data on the antimicrobial susceptibility of *F. psychrophilum* strains circulating in UK hatcheries and fish farms are limited. Thus, the evaluation of FFN effectiveness within the UK may not be complete. In addition, the current heavy reliance on FFN for controlling RTFS in the UK may be facilitating the development of resistance to FFN by *F. psychrophilum*, which could affect the viability of the UK rainbow trout industry. Both oxytetracycline and amoxicillin were shown to be moderately effective against the UK *F. psychrophilum* strains and so could be considered as alternative antimicrobials to FFN in RTFS management, contributing to a reduction in use of FFN.

The co-infection of *F. psychrophilum* isolates manifesting different antimicrobial susceptibility patterns was observed in this study, as previously reported by Bruun *et al.* (2000) and Henríquez-Núñez *et al.* (2012). The succession of the RTFS/BCWD outbreaks could occur if strains with different antibiograms co-exist in the farm, where the strains with reduced susceptibility take over when one antimicrobial agent is used for the treatment.

The improper and repetitive use of an antimicrobial agent in aquaculture can result in bacteria manifesting reduced susceptibility to the drug and an increase in frequency of these strains within the population. Resistance mechanisms in bacteria can be intrinsic or involve a number of genetic mechanisms, such as chromosomal mutation, expressing a latent resistant gene, or acquiring new genetic material (chromosomal or extrachromosomal DNA) (Guardabassi and Courvalin, 2006). In the present study, statistical tests were applied and revealed some significant associations between PFGE groups/singletons, $(GTG)_5$-PCR types/subtypes or plasmid profiles and the antimicrobial susceptibilities of the *F. psychrophilum* strains. The reduced susceptibility to AMP and AMOX was over-represented in PFGE group P and $(GTG)_5$-PCR type r4, while NWT phenotype to OTC was found to be associated to PFGE singleton S, $(GTG)_5$-PCR subtype r1a and a combination of two plasmids (2.1 kb and 3.3 kb). The over-representation of WT phenotype to OTC in plasmid profile containing only 3.3 kb plasmid suggests that the 2.1-kb plasmid in this combination might be responsible for the reduced susceptibility to OTC. Previously, Bruun and colleagues (2003a) speculated that OTC resistance determinants in *F. psychrophilum* strains could be located in mobile elements (transposons or plasmids) or a result of unspecific changes in membrane permeability. In contrast, Del Cerro *et al.* (2010) did not observe any relationship between plasmid content

and acquired antimicrobial resistance in 25 Spanish *F. psychrophilum* strains as some isolates resistant to OTC did not possess any plasmid, whereas isolates susceptible to OTC harboured a 3.5 kb plasmid. Therefore, these authors suggested that antimicrobial resistance determinants in *F. psychrophilum* were possibly carried in mobile genetic elements (such as transposons) located in the bacterial chromosome. Meanwhile, the most common resistance mechanism to aminopenicillins (e.g. ampicillin and amoxicillin) has been demonstrated to be mediated by bacterial beta-lactamases (CLSI, 2006), suggesting the investigation of the presence of beta-lactamase-coding genes in plasmids or chromosome of *F. psychrophilum* isolates. To date, there have been no reports on the correlation between genotypes and antibiotic susceptibilities of the *F. psychrophilum* species. Del Cerro *et al.* (2010) and Sundell *et al.* (2013) genotyped *F. psychrophilum* strains using PFGE and investigated the antimicrobial susceptibility of these strains but these authors did not perform any statistical analysis to investigate the association between genotypes and susceptibilities to antibiotics. Antimicrobial susceptibility testing with a larger set of isolates from each genotype is needed in order to confirm the associations between genotypes and antibiotic susceptibility patterns observed in the present study. Consequently, further comparisons on the genetic differences using whole genome sequencing between genotypes having different antimicrobial susceptibilities could improve the understanding of antibiotic resistance mechanisms, such as PFGE group E or $(GTG)_5$-PCR type r2 (WT phenotype to beta-lactams) and PFGE group P or $(GTG)_5$-PCR subtype r4 (NWT phenotype to beta-lactams); PFGE group E or $(GTG)_5$-PCR type r4 (WT phenotype to OTC) and PFGE group S or $(GTG)_5$-PCR subtype r1a (NWT phenotype to OTC).

No association between genotypes and the susceptibility patterns for quinolones was observed in this work. Larger sets of isolates should be examined to maximise the statistical power of the analysis on the susceptibility to quinolones, especially between PFGE groups/singletons. Previously, the reduced susceptibility to quinolones (nalidixic acid and oxolinic acid) in *F. psychrophilum* was demonstrated to be associated with chromosomal mutations in *gyrA* gene and transferred vertically in the bacterial population (Bruun *et al.*, 2003a; Izumi and Aranishi, 2004). However, Henríquez-Núñez *et al.* (2012) observed no correlation between quinolone resistance and *gyrA* mutation in Chilean *F. psychrophilum* isolates. Further work on the *gyrA* gene of the UK *F. psychrophilum* strains is needed to identify whether there is any association between this gene and genotypes established in the present study.

3.5. Conclusions

The present study applied a standard broth microdilution MIC protocol (CLSI 2014a) and NRI analysis to interpret the *in vitro* susceptibility data of *F. psychrophilum* strains from mainly UK sources. Based on the estimated frequencies of NWT phenotypes in UK *F. psychrophilum* strains, florfenicol is still the most effective treatment for controlling RTFS. A standardised susceptibility testing method, associated with appropriate interpretation of the data obtained, would represent a cost-effective and an essential element in monitoring the prudent use of antibiotics in aquaculture. The MIC data generated from this work together with data from other studies using the standard CLSI protocol could contribute to setting up internationally agreed MIC epidemiological cut-off values for investigating the antimicrobial susceptibilities of *F. psychrophilum*. The correlation between MIC CO_{WT} and disc-diffusion CO_{WT} in the classifications of the WT and NWT strains suggests that inhibition zone sizes have some value within an individual laboratory but imprecision concerns may limit intra-laboratory comparison. Because of the importance of antimicrobial susceptibility surveillance to RTFS/BCWD management, it is crucial to constantly monitor and understand the associations between a particular genotype and reduced susceptibility to the commonly used antibiotics, particularly with regard to FFN, for RTFS/BCWD treatment within the UK.

Chapter 4

Efficacy of a polyvalent RTFS vaccine in rainbow trout (*Oncorhynchus mykiss*) following an immersion immunisation and bath challenge with *Flavobacterium psychrophilum*

4.1. Introduction

Flavobacterium psychrophilum is a well-known fish pathogen which causes significant mortalities in cultured salmonids worldwide (Faruk, 2002; Starliper and Chill, 2011). Despite several attempts to develop vaccines for BCWD or RTFS caused by *F. psychrophilum*, no commercial vaccines are currently available for the prevention of this disease. This is due to the limited information regarding *F. psychrophilum* strain diversity and the immune system of fry, which are highly susceptible to the disease. Current RTFS or BCWD management includes chemical and antibiotic treatments, resulting in a concern about the potential development of antimicrobial resistance by *F. psychrophilum* (Cipriano and Holt, 2005).

Various vaccine formulations against RTFS and BCWD have been developed to date with varying degrees of success (Gómez *et al*., 2014). Heat or formalin inactivated bacterial whole-cell vaccines have been the most commonly tested vaccines, resulting in relative percent survival (RPS) values ranging from 60 – 83% (Obach and Baudin Laurencin, 1991; Rahman *et al*., 2000; LaFrentz *et al*., 2002; Kondo *et al*., 2003; Aoki *et al*., 2005; Madetoja *et al*., 2006; Fredriksen *et al*., 2013a,b). Several immunogenic antigens have been identified as potential candidates for developing subunit vaccines (Crump *et al*., 2001; Rahman *et al*., 2002; Merle *et al*., 2003; LaFrentz *et al*., 2004, 2007, 2011; Crump *et al*., 2005; Dumetz *et al*., 2006, 2007, 2008; Sudheesh *et al*., 2007). Although most of these potential candidates have not been tested as vaccines in fish, the efficacy of those that have been tested have resulted in high RPS values of 88 – 95% (Rahman *et al*., 2002; LaFrentz *et al*., 2004; Dumetz *et al*., 2006). In comparison with subunit and whole-cell inactivated vaccines, live attenuated *F. psychrophilum* strains created by mutating the *exbD2* gene (Álvarez *et al*., 2008) or culturing the bacterium in a rifampicin containing medium (LaFrentz *et al*., 2008; Gliniewicz *et al*., 2012; Long *et al*., 2013; Makesh *et al*., 2015; Sudheesh *et al*., 2016) may offer longer-lasting protection. However, safety issues are of concern when releasing live vaccine into the aquaculture environment (Gómez *et al*., 2014). Therefore, at present, live vaccines are not licensed for use in the UK and European countries. Additionally, the use of wild-type strains (Lorenzen *et al*., 2010) or anti-*F. psychrophilum* sera from convalescent or immunised adult trout (LaFrentz *et al*., 2003) for developing potential vaccines has been evaluated, conferring protection with RPS values up to 88%, 42% and 57% respectively.

Furthermore, the ability to produce cross protection in 32 – 36 g trout using a combination of two *F. psychrophilum* serotypes (Fd and Th) has been investigated in

previous studies of Nikoskelainen *et al.*, (2007), Fredriksen *et al.* (2013a,b), in which intraperitoneal vaccines comprising formalin-killed cells of two *F. psychrophilum* strains of serotypes Fd and Th with/without other fish pathogens were developed. Nikoskelainen *et al.* (2007) suggested that serologically different *F. psychrophilum* shared protective antigens when a polyvalent vaccine containing *Aeromonas salmonicida*, *Listonella anguillarum* and *F. psychrophilum* serotype Fd showed the target specific ingestion of single phagocyte (TSI) against *A. salmonicida*, *L. anguillarum*, both *F. psychrophilum* Fd and Th serotypes, but not against *F. psychrophilum* Fp^T serotype. The Th serotype was suggested to be a suppressive serotype of *F. psychrophilum* in trout when a polyvalent vaccine with similar formulation containing both *F. psychrophilum* serotypes Fd and Th showed TSI against only *A. salmonicida* and *F. psychrophilum* serotype Fd (Nikoskelainen *et al.*, 2007). These authors recommended that bacterial antigens in polyvalent vaccines should be carefully selected in order to avoid the inhibitory effect of many immunobiological processes on the specific response of fish. On the other hand, Fredriksen *et al.* (2013a,b) reported high protection levels (44 – 67% and 78 – 80% respectively) induced by a polyvalent vaccine containing (Th + Fd serotype) *F. psychrophilum* isolate and four additional fish pathogens in rainbow trout following i.m. challenge with *F. psychrophilum* serotype Th.

Bearing in mind that RTFS and BCWD is a serious septicemic condition of salmonid fish ranging from 0.2 to 10 g (Faruk, 2002; Nematollahi *et al*., 2003a), vaccination against this disease should be carried out as early as possible. A new injectable vaccine against *F. psychrophilum* and Infectious Pancreatic Necrosis Virus administered as a nano dose (0.025 ml) has recently been developed by Pharmaq and approved by the Chilean authorities for use in 28 g fish. As the disease affects small fish from 0.2 g, vaccination by immersion or in-feed administration is more feasible and preferable to administration by injection (Gómez *et al*., 2014). So far, inactivated immersion vaccines with RPS values of less than 50% have not given sufficient and durable protection against RTFS/BCWD in salmonid fry (Lorenzen *et al*., 2010). This could be explained by the application of an unsuitable fish challenge method via injection against which immersion/oral vaccines may not protect (Lorenzen *et al*., 2010). In 2013, Henriksen and colleagues (2013) established a reproducible challenge method by immersion, including pre-treatment with hydrogen peroxide (H_2O_2) for rainbow trout fry.

An inadequate understanding of the innate and adaptive immune response of rainbow trout to *F. psychrophilum*, especially at the mucosal surfaces, is a constraint to

the development of an efficacious RTFS/BCWD vaccine (Makesh *et al*., 2015). Several studies have been published on the immune response of naturally infected (Villarroel *et al*., 2008; Orieux *et al*., 2013), injection-challenged fish weighing 10-50 g (Overturf and LaPatra, 2006; Evenhuis and Cleveland, 2012) and bath-challenged fry (Henriksen *et al*., 2015a,b). Recently, the immune response of immunised rainbow trout weighing 35 g to live attenuated *F. psychrophilum* was investigated (Makesh *et al*., 2015), however similar studies on vaccinated fry, especially using inactivated whole cell vaccines, are lacking.

Based on the results of the genotyping and serotyping characterisation performed in Chapter 2, a whole-cell formalin killed polyvalent vaccine against RTFS/BCWD was developed by combining three genetically and serologically divergent strains, recently collected from UK farms. This combination was chosen to confer broad-spectrum protection against *F. psychrophilum* infection. The present chapter aimed to investigate the efficacy of this polyvalent vaccine to induce protection in rainbow trout fry after bath challenge with a heterogeneous *F. psychrophilum* strain possessing a different genotype compared to the three strains used in the vaccine. The specific IgM response in serum and the expression levels of seven immune genes (*TLR2*, *IL-1β*, *C3*, *CD4-1*, *CD8-α*, *IgM* and *IgT*) in response to the immersion vaccination from different mucosal and lymphoid organs of fry were also examined.

4.2. Materials and methods

4.2.1. Experimental animals

Rainbow trout fry (*O. mykiss*) (mean weight of 3 g) were supplied by a fish farm in the East of Scotland and transported to the aquarium at the Institute of Aquaculture, University of Stirling. After arrival, the fry were acclimatised in flow-through tap water (5 L min^{-1}) at 15 ± 1^{o}C for at least two weeks in a 300 L tank with continuous aeration. The fry were fed to satiation daily using Inicio feed with pellet size of 1.1 mm (BioMar). All experimental procedures with live fish were carried out in accordance with the U.K. Animals (Scientific Procedures) Act 1986 and associated guidelines (EU Directive 2010/63/EU for animal experiments).

4.2.2. Culture of *F. psychrophilum*

Flavobacterium psychrophilum strains were routinely grown in modified veggietone (MV) medium at 15^{o}C for 72 h, 140 rpm. Stock cultures were maintained at -70^{o}C in tryptone–yeast extract–salts medium supplemented with glucose (Cepeda *et al*., 2004)

with 10% glycerol (Fisher chemicals, UK) and on Protect-Multi-purpose cryobeads (Technical Service Consultants Ltd, UK).

4.2.3. Preparation of the polyvalent inactivated vaccine

On the basis of genotyping and serotyping data of 315 *F. psychrophilum* isolates, three representative strains (AVU-1T/13, serotype Fd; AVU-2T/13, serotype Th; and AVU-3S/13, serotype Fp^T) recovered from diseased rainbow trout and Atlantic salmon in UK farms were selected to make a polyvalent whole cell vaccine.

A cell pellet of 72-h MV broth culture of each strain was collected by centrifugation at 3,000 × *g* for 15 min, washed twice in sterile phosphate buffered saline (PBS) (0.02 M sodium dihydrogen phosphate [Fisher chemicals, UK], 0.02 M disodium hydrogen phosphate [Fisher chemicals, UK], 0.15 M NaCl [Sigma, UK], pH 7.2), and resuspended in PBS to an optical density (OD) of 1.0 at 525 nm, equivalent to a concentration of 2 x 10^8 colony forming units (CFU) per mL^{-1}. Formalin was added to the adjusted bacterial suspension to a final concentration of 0.5%. The mixture was stirred slowly at 4°C for 48 h, then neutralised by adding 0.15% sodium metabisulphite (Sigma, UK) and incubated at 4°C for a further 72 h with slow stirring and gentle inversion. The bacterial pellet was harvested by centrifugation at 3,000 × *g* for 15 min, washed twice in sterile PBS buffer and adjusted to OD_{525} of 1.0 in PBS. The purity and inactivity of the bacteria was monitored by streaking out the bacterial suspension during the procedure on MV agar plate and then incubating at 15°C for 72 – 96 h.

Inactivated *F. psychrophilum* strains were concentrated and mixed in equal parts to form a polyvalent vaccine at a final concentration of 1 x 10^9 CFU mL^{-1}. The polyvalent whole cell vaccine was kept at 4°C until use.

Table 4.1. The diversity of *F. psychrophilum* strains used in the polyvalent inactivated vaccine and challenge trials. (*): different genotypes are coded by different letters.

Strain	PFGE pulsotype(*)	$(GTG)_5$-PCR type(*)	Plasmid profiles(*)	16S rRNA lineage(*)	Serotype
AVU-1T/13	a	e	h	i	Fd
AVU-2T/13	b	e	h	i	Th
AVU-3T/13	c	f	h	i	Fp^T
AVU-1T/07	d	g	h	i	Th

4.2.4. Fish vaccination

Duplicate groups of 30 fish (mean weight of 4.72 ± 1.01 g) were immersed in a 1:10 dilution of the polyvalent whole cell vaccine for 30 s and then transferred to 25-L flow through tanks (500 mL min^{-1}) with aeration at 15^{o}C. Duplicate control groups were immersed in tank water for 30 s. Three hundred and fifteen degree days following the primary vaccination, both groups of fish were booster vaccinated with the same dose and treatment as the primary vaccination.

4.2.5. Sampling

At each sampling time point, three fish from each tank (six fish per group in total) were euthanised using an overdose of benzocaine (500 mg L^{-1}). For gene expression analysis by qPCR, tissues (second gill arch, hind-gut, head kidney, skin and spleen) were sampled at 4 h, day 2 and day 7 post – initial vaccination. Tissues were placed in RNA-later (Sigma, UK) and stored at 4^{o}C overnight. RNA-later was then removed and tissues were stored at -70^{o}C. Blood was collected six weeks post-initial vaccination (630 degree days) by caudal puncture using a 25G x 16 mm needle (Terumo, Scientific Laboratory Supplies, UK) and 1 mL syringe (Terumo, Scientific Laboratory Supplies, UK). Blood was placed in a 1.5 mL Eppendorf tube and allowed to clot overnight at 4^{o}C. Serum was collected following centrifugation at 3,000 × *g* for 5 min and stored at -20^{o}C.

4.2.6. Pre-challenge of unvaccinated fish using a heterogeneous *F. psychrophilum* strain

F. psychrophilum AVU-1T/07 (serotype Th) was retrieved from the spleen of a diseased rainbow trout fry in Scotland in 2007. The susceptibility of the rainbow trout to bath challenge with *F. psychrophilum* was determined by performing a pre-challenge test on a small group of unvaccinated fish with three bacterial concentrations for LD_{50}. One group of 18 fish (mean weight of 7.54 ± 2.03 g) was immersed in 3 L of hydrogen peroxide solution (H_2O_2, Sigma, UK) at 200 mg L^{-1} for 1 h with aeration (Henriksen *et al.*, 2013). Six of these fish were then immediately immersed in 1 L of bacterial solution containing *F. psychrophilum* AVU-1T/07 at 1.5 x 10^8 CFU mL^{-1}, 1.5 x 10^7 CFU mL^{-1} or 1.5 x 10^6 CFU mL^{-1} in static conditions with aeration for 5 h. The viable numbers of the *F. psychrophilum* strain used for the pre-challenge test was determined using the drop count (Miles/Misra) method in Section 4.2.7.1. The pre-challenge test was carried out in 10 L flow-through tanks with aeration for 21 days. Moribund fish and mortalities were

sampled by streaking out spleen and kidney on MV plates and incubating at 15°C for 72-96 h. Identification of *F. psychrophilum* was performed using a nested PCR described in Section 2.2.3.

4.2.7. Challenge with a heterogeneous *F. psychrophilum* strain

4.2.7.1. Preparation of F. psychrophilum strain

A single colony of *F. psychrophilum* strain AVU-1T/07, grown on a MV agar plate as described in Section 4.2.1, was inoculated into 100 mL of MV broth at 15°C for 72 h with shaking at 140 rpm. This pre-inoculum was transferred to 10 L of MV broth in conical flasks with a medium volume to container volume ratio of 1:5. The inoculum was incubated at 15°C for 24 h and shaken at 140 rpm. A cell pellet of a 24-h MV broth culture was collected by centrifugation at 3000 × *g* for 15 min, washed once in sterile PBS, resuspended in the same buffer and adjusted to an OD_{525} of 1.0.

The viable numbers of *F. psychrophilum* cells from the adjusted bacterial suspension used for the challenge was determined using the drop count (Miles/Misra) method. Briefly, the adjusted bacterial suspension was diluted 1:2 and then further diluted ten-fold serially seven times. A 20-µL amount of each ten-fold dilution sample was dropped onto the six marked sections of an MV agar plate. The plates were incubated at 15°C for 72 – 96 h. The number of bacteria in the original 1:2 diluted bacterial suspension was calculated by multiplying the average number of colonies per drop by 50 and the dilution factor.

4.2.7.2. Challenge

At six weeks (630 degree days) post-initial vaccination, rainbow trout fry (mean max 12.43 ± 2.33 g) were challenged with a heterogeneous *F. psychrophilum* strain AVU-1T/07 by immersion. The fish were starved for 24 h prior to the challenge. Fry were first pre-immersed in 3 L of H_2O_2 at 200 mg L^{-1} for 1 h in static aerated 5-L tanks and then subsequently transferred into static 25-L tanks containing 3 L of bacterial suspension at 1.9 x 10^8 CFU mL^{-1}, and immersed for 5 h with aeration. The water temperature was maintained at 15°C throughout the challenge.

Mortalities were recorded daily for 32 days. Moribund and dead fish were sampled for *F. psychrophilum* recovery by streaking out the spleen and kidney on MV plates and incubating the plates at 15°C for 72 – 96 h. The presence of *F. psychrophilum* in these fish was confirmed by 16S rRNA nested PCR as described in Section 2.2.3.

4.2.8. Enzyme-linked immunosorbent assay (ELISA) for detecting specific IgM in serum

Due to the limited amount of blood obtained from fry, specific IgM in serum was examined using ELISA with only two of three *F. psychrophilum* strains used in the vaccine. In addition, blood sample from one vaccinated fish was lost during storage, so blood samples of five fish from the vaccinated group and six fish from control group were analysed in ELISA.

A flat-bottom 96 well plate (Immulon-HBX, Nunc) was sensitised with 50 µl well^{-1} of 0.05% (w/v) poly L-Lysine (Sigma, UK) in 0.5 M carbonate bicarbonate buffer (Sigma, UK), pH 9.6, for 1 h and then washed twice with low salt wash (LSW) buffer (0.02 M Tris [Fisher chemicals, UK], 0.38 M NaCl [Fisher chemicals, UK], 0.05 % Tween-20 [Sigma, UK], pH 7.3). The plate was coated with 100 µl well^{-1} of *F. psychrophilum* solution (AVU- 1T/ 13 or AVU-2T/ 13) at the concentration of 1 x 10^8 CFU mL^{-1}, and incubated overnight at 4^{o}C. All the wells were fixed with 50 µl well^{-1} of 0.05% (v/v) glutaraldehyde (Sigma, UK) for 20 min, washed three times with LSW buffer and then blocked by adding 250 µl well^{-1} of 3% casein in distilled water (DW) and incubating the plates for 2 h at 22^{o}C. The plate was again washed three times in LSW buffer before adding 100 µl well^{-1} of fish serum samples (diluted 1:32 in PBS buffer containing 1% Tween 20) or PBS (negative control) in duplicate. Following an overnight incubation at 4^{o}C, the plate was washed five times with high salt wash (HSW) buffer (0.02 M Tris, 0.5 M NaCl, 0.1 % Tween 20, pH 7.7), with a 5 min soak on the last wash to remove unbound antibodies. Anti-trout monoclonal antibody (mAb, Aquatic Diagnostics Ltd.) diluted 1:33 in PBS buffer containing 1% of bovine serum albumin (BSA; Fisher chemicals, UK) was added (100 µl well^{-1}) to the plate and incubated at 22^{o}C for 1 h. Then the plate was washed five times in HSW buffer and incubated with 100 µl well^{-1} of horse anti-mouse immunoglobulin-G (IgG) labelled with horseradish peroxidase (Sigma, UK) diluted 1:4000 in conjugate buffer (LSW buffer containing 1% BSA) for 1 h at 22^{o}C. Following the final five washes in HSW buffer to remove the excess HRP conjugate, chromogen (42 mM tetramethyl-benzidine dihydrochloride [Sigma, UK] in solution of acetic acid [Fisher chemicals, UK] diluted 1:2 in water) was prepared in substrate buffer [0.1 M citric acid (Sigma, UK), 0.1 M sodium acetate (Sigma, UK), pH 5.4)] containing 0.03 % hydrogen peroxide and added (100 µl well^{-1}) into the plate. After a 10-min incubation at 22^{o}C, the reaction was stopped by adding 50 µl well^{-1} of 2 M sulphuric acid (Fisher chemicals, UK). The absorbance was read on a Synergy HT

Microplate reader (Biotek) at 450 nm. Reactions were considered positive when the mean absorbance readouts in wells with fry serum was at least three times higher than in the parallel wells with the negative control (PBS).

4.2.9. Reverse transcription quantitative real-time PCR (RT-qPCR)

4.2.9.1. RNA extraction

RNA was extracted from 30 to 40 mg of each tissue sample (three fish per tank, six fish per group in total) using TRI Reagent (Sigma, UK) following the manufacturer's instructions. Tissue was transferred into a 1.5-mL screw cap tube containing 0.5 mL of TRI Reagent, incubated on ice for 15 min and homogenised for 30 – 60 s using Mini-Beadbeater (BioSpec Products) until the tissue was significantly disrupted. Following a 5-min incubation at 22^{o}C, 50 µL of 1-bromo-3-chloropropane (BCP; Sigma, UK) was added into the homogenised samples, shaken vigorously for 15 s and incubated at 22^{o}C for 15 min. The aqueous phase was separated by centrifugation at 20,000 × *g* for 15 min at 4^{o}C and transferred (150 µL) into a new tube. The resulting RNA was precipitated by adding 75 µL of RNA precipitation solution (1.2M sodium chloride; 0.8M sodium citrate sesquihydrate [Sigma, UK]) and 75 µL of 100% isopropanol (Fluka, UK), inverting gently 4 – 6 times, incubating at 22^{o}C for 10 min and centrifuging at 20,000 × *g* for 10 min at 4^{o}C. After the supernatant was removed, the RNA pellet was washed in 1 mL of 75% ethanol (Fisher chemicals, UK) for 15 min at 22^{o}C and harvested by centrifugation at 20,000 × *g* for 5 min. The RNA pellet was air dried at 22^{o}C for 3 – 5 min until all visible traces of ethanol were gone, resuspended in 50 µL of RNase free water (Thermo Fisher Scientific, UK) and incubated at 22^{o}C for 30 min to aid resuspension. All steps involving RNA were carried out in an RNase-free environment using filtered tips and gloves. RNA samples were stored at -70^{o}C until further use.

RNA quantity and quality were determined using the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, UK). RNA integrity was checked using agarose gel electrophoresis (1.0 % agarose gel [Bioline, UK] containing 0.1 µg mL^{-1} ethidium bromide [Sigma, UK]) in Tris–Acetic–EDTA (TAE) buffer (0.2 mM Tris [Fisher chemicals, UK], 0.1 mM acetic acid [Fisher chemicals, UK], and 0.005 mM EDTA [Sigma, UK]).

4.2.9.2. DNase treatment

Contaminating DNA in the RNA samples was removed using the DNA-*free*™ kit (Ambion). The RNA concentration was adjusted to the same level (2 µg) in each sample by adding nuclease-free water (Thermo Fisher Scientific, UK). The DNase digestion mix consisted of DNase I buffer, 2 units (U) of rDNase I and 2 µg of the RNA sample. Following a 30 min incubation at 37ºC, the DNase was inactivated by adding DNase inactivation reagent and incubating at 22ºC for 2 min. The treated RNA was collected by centrifugation at 10,000 × *g* for 90 s and transferred into a new tube. The quantity and quality of the DNase-treated RNA was examined using the Nanodrop ND-1000 Spectrophotometer.

4.2.9.3. cDNA synthesis

Synthesis of cDNA was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) according to the manufacturer's instructions. A 20-µL reverse transcription (RT) master mix consisted of 1X RT buffer, 4 mM dNTP, 1X RT random primers and 0.5 µM oligo dT (MWG), 50 U of MultiScribe™ Reverse Transcriptase, 20 U of RNase inhibitor (Applied Biosystem) and 0.5 µg of the DNase-treated RNA sample. RT-PCR amplification was carried out in a TProfessional Basic Thermocycler gradient (Biometra, Germany) using thermal cycle conditions including 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. The cDNA was aliquoted and kept at -20ºC until further use. A minus-RT control (RT^-) was prepared by excluding MultiScribe™ Reverse Transcriptase in the RT master mix in order to examine the contamination of genomic DNA in RNA samples. Nuclease-free water was used to dilute cDNA 10 times before storage at -20ºC.

4.2.9.4. RT-qPCR

All real-time quantification PCR assays were conducted in colourless 96-well plates using the Eppendorf® RealPlex^2 Mastercycler gradient S instrument with the SYBR® Green I master mix (Thermo Scientific, UK) and primers (MWG) as listed in Table 4.1. A 20-µL reaction mix consisted of 1X SYBR® Green I buffer, 0.3 µM each of the forward and reverse primers and 5 µL of the ten-fold diluted cDNA. The thermal profile consisted of 94°C for 15 s, followed by 40 amplification cycles of denaturation at 95°C for 30 s, annealing at the optimal temperature (Table 4.1) for each primer pair for 30 s and extension at 72°C for 120 s. Melting curve analysis was carried out from 60ºC to

95^{o}C in 0.1^{o}C per second increments to assess the specificity of the RT-qPCR products. Non-template and RT^{-} controls were included on every plate. A pool of all available cDNAs was diluted at 1:5; 1:10; 1:20; 1:50; 1:100; 1: 1,000 and 1: 10,000 in nuclease-free water. The threshold cycle (Ct) values of these diluted samples were used to generate a standard curve plot of cycle number versus log concentration in the *realplex* software V2.2 (Eppendorf). For each sample, two replicate wells were used. The plates were covered by adhesive seals (Starlab) before running the assay.

All the primers used in this study (Table 4.1) were analysed using the NCBI Blast sequence analyser (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). The quality of the standard curve was judged by the slope of the curve and the correlation coefficient (r). The slope of the line was used to estimate the efficiency of the target amplification using the equation E= ($10^{-1/slope}$)-1.

4.2.10. Statistical analysis

The GraphPad Prism version 6 program (GraphPad Software Inc., San Diego, CA, USA) was used to generate graphs and analyse for statistical differences. The normality of the raw data from serum antibody responses was checked using the Kolmogorov-Smirnov test. Then the significant difference between vaccinated group and control group was analysed by the two-tailed unpaired t-test.

The relative standard deviation (RSD = (standard deviation/mean) x 100) was calculated for mortality rates of vaccinated group and control group following the bath challenge. Kaplan-Meier survival curves were generated and a log-rank test was used to compare the survival curves of the two groups (Kaplan and Meier, 1958; Peto *et al*., 1977). The RPS value of this trial was calculated using the following equation (Amend, 1981):

$$\text{RPS} = \left[1 - \frac{\text{average \% mortality of vaccinated fish}}{\text{average \% mortality of unvaccinated fish}}\right] \times 100$$

Although PCR efficiencies of two reference genes EF-1α and β-actin are low (87.2% and 88.6% respectively), their correlation coefficients (R^2) are greater than 99% (Table 4.1) and the raw Ct values for these two genes range from 12.43-17.33, which is acceptable for reliable RT-qPCR quantification.

The expression of target genes was normalised to the expression of two reference genes (*EFL-1α* and *β-actin*). The fold change in expression of the target genes in vaccinated samples compared to the unvaccinated sample was calculated according to the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) using the Relative Expression Software Tool (REST©) software (Pfaffl *et al*., 2002). The error range (SE) is asymmetrically distributed relative to the mean value and estimated using $\Delta\Delta C_T$ plus the standard deviation and $\Delta\Delta C_T$ minus the standard deviation (Table 4.3) (Livak and Schmittgen, 2001). Expression significantly different from the control group was calculated by the Pair Wise Fixed Reallocation Randomisation Test© (2000 randomisation). A *p*-value of <0.05 was considered statistically significant.

Table 4.2. Details of primers used in the RT- qPCR

Gene	Oligo sequence	Product size (bp)	Annealing temperature (ºC)	Efficiency	R^2	Genbank accession no.	Reference
EF-1α	F: 5'-GATCCAGAAGGAGGTCACCA-3' R: 5'-TTACGTTCGACCTTCCATCC-3'	150	58ºC	87.2%	0.992	NM_001124339.1	Dixon *et al*., 2013
β-actin	F: 5'-CAGCCCTCCTTCCTCGGTAT-3' R: 5'-AGCACCGTGTTGGCGTACA-3'	110	54ºC	88.6%	0.990	NM_001124235.1	Palti *et al*., 2007
TLR-2	F: 5'-GATCCAGAGCAACACTCTCAACAT-3' R: 5'-CTCCAGACCATGAAGTTGACAAAC-3'	282	58ºC	85.3%	0.970	LK933545.1	Abós *et al*., 2013
C3	F: 5'-GAGATGGCCTCCAAGAAGATAGAA-3' R: 5'-ACCGCATGTACGCATCATCA-3'	91	58ºC	89.2%	0.980	L24433.1	Cortés *et al*., 2013
IL-1β	F: 5'-GACATGGTGCGTTTCCTTTT-3' R: 5'-ACCGGTTTGGTGTAGTCCTG-3'	122	54ºC	85.1%	0.977	AJ278242.2	Castro *et al*., 2014b
IgT	F: 5'-AACATCACCTGGCACATCAA-3' R: 5'-TTCAGGTTGCCCTTTGATTC-3'	80	54ºC	76.3%	0.972	AY870266.1	Castro *et al*., 2014c
IgM	F: 5'-TGCGTGTTTGAGAACAAAGC-3' R: 5'-GACGGCTCGATGATCGTAAT-3'	107	54ºC	81.7%	0.997	AH014877.2	Castro *et al*., 2014c
CD4-1	F: 5'-GAGTACACCTGCGCTGTGGAAT-3' R: 5'-GGTTGACCTCCTGACCTACAAAGG-3'	121	58ºC	88.9%	0.996	NM_001124539.1	Bernard *et al*., 2006
CD8-α	F: 5'-ACGACTACACCAATGACCACAACC-3' R: 5'-CAGTGATGATGAGGAGGAGGAAGA-3'	160	58ºC	91.2%	0.970	AF178055.1	Castro *et al*., 2014b

4.3. Results

4.3.1. Vaccine efficacy

No mortality was recorded immediately following the immersion vaccination. Pre-challenge experiment revealed the bacterial concentration of *F. psychrophilum* strain AVU-1T/07 for LD_{50} was 1.5 x 10^8 CFU mL^{-1}. Therefore, this bacterial concentration was used to challenge the vaccinated fish six weeks post-vaccination. Mortalities started 8 days post-challenge in the control groups and continued until the 23rd day of the challenge. In contrast, mortalities in the vaccinated group occurred 17 and 20 days post-challenge. All the moribund and dead fish showed at least one of the following clinical signs: haemorrhage, splenomegaly, lesion in the trunk, and eroded tail and mouth (Figure 4.1). The presence of *F. psychrophilum* was found in 20 moribund and dead fish sampled during the challenge, but not in any of 16 representative survivors (four fish per replicate tank) following the termination of the challenge (Figure 4.2 and 4.3).

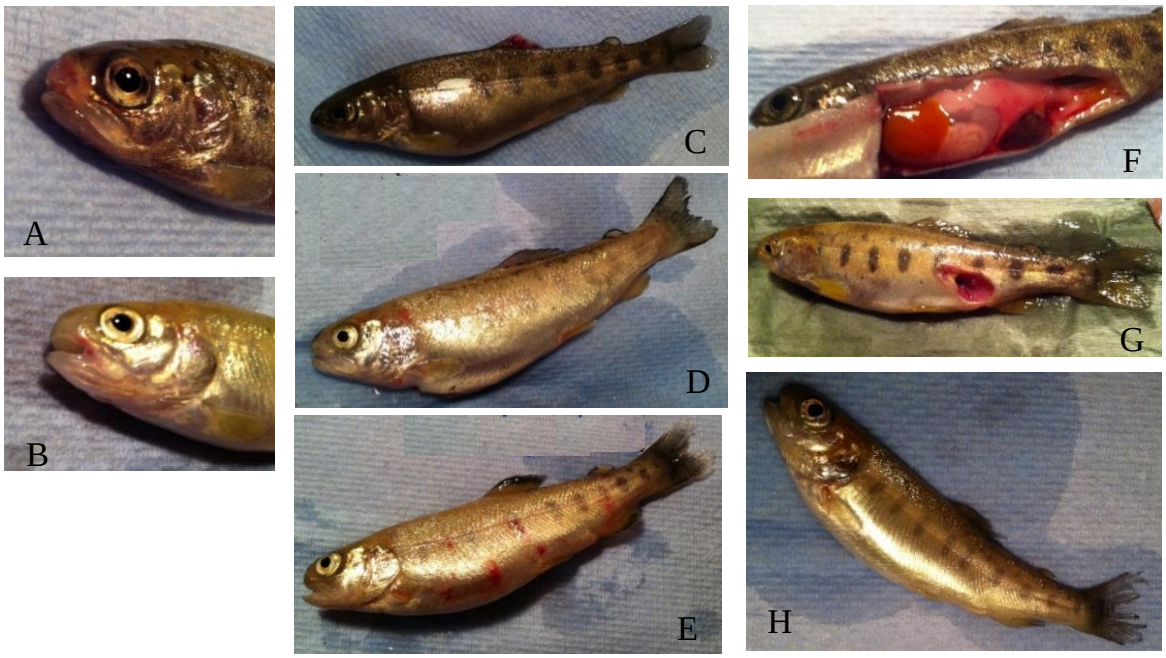


Figure 4.1. Clinical signs of RTFS in rainbow trout (moribund and mortalities) following bath challenge with a heterogeneous *F. psychrophilum* strain AVU-1T/07. Eroded mouth (A); haemorrhage on mouth (B), dorsal fin (C), operculum, pelvic fin (D) and the trunk (E); splenomegaly and blurred spleen margins (F); lesion on the trunk (G); eroded tail (H).

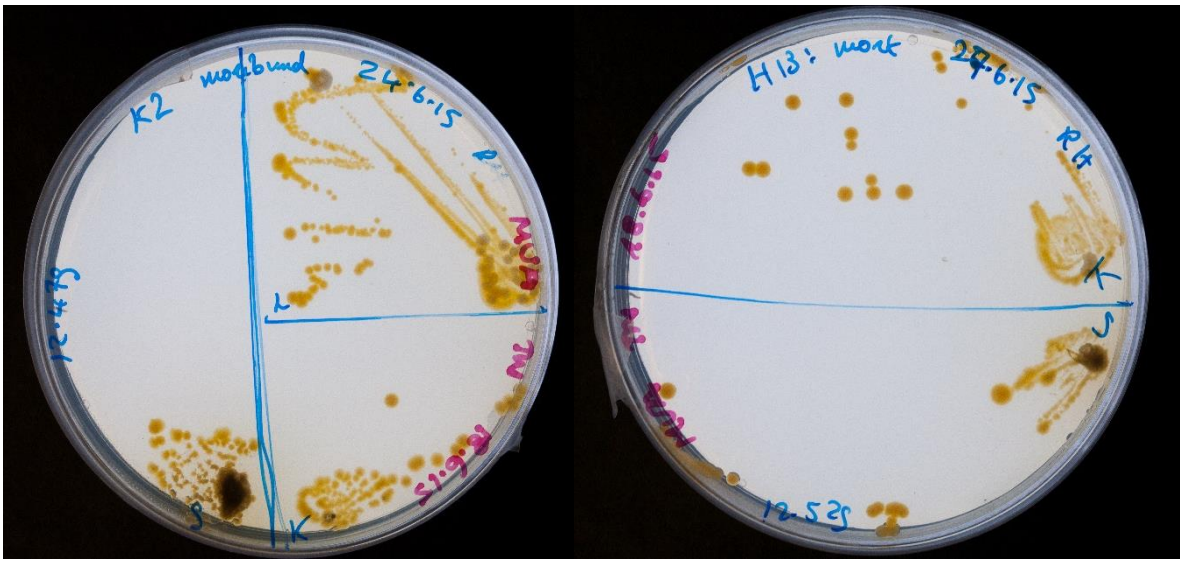


Figure 4.2. Yellow pigmented bacterial colonies retrieved from spleen, kidney and lesion of moribund and dead fish after the bath challenge with *F. psychrophilum* strain AVU-1T/07.

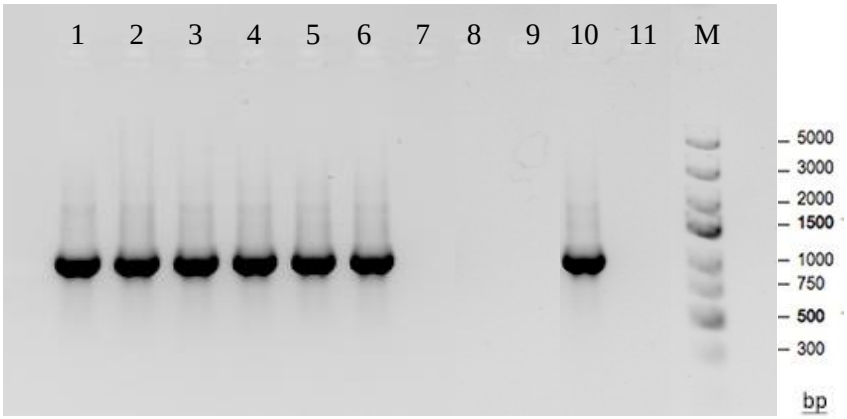


Figure 4.3. 16S rRNA nested PCR for detecting the presence of *F. psychrophilum* in moribund fish or mortalities during the challenge. The gel shows the second round PCR amplicons (1080 bp). Lanes 1-6: moribund/mortalities; lanes 7-9: survivor fish; lane 10: positive control; lane 11: negative control (DW) and lane M: GeneRuler Express DNA Ladder (Fermentas, Fisher Scientific, UK).

The average cumulative mortality was 49.1% in the unvaccinated group and 7.9% in the vaccinated group (Table 4.2). The survival rates from the challenge study are presented as a Kaplan-Meier plot (Figure 4.4). The calculated RPS value induced by the polyvalent inactivated vaccine was 84%.

Table 4.3. Average mortality percent of rainbow trout fry after the bath challenge with a heterogeneous *F. psychrophilum* strain AVU-1T/07. Average values for weight and mortality rate are stated along with standard deviation. "n" represents the number of fish; SD, standard deviaton; RSD, the relative standard deviation value.

Group	Replicate	Weight (± SD, g)	n	% mortality (n)	Average % mortality	RSD
Control	1	12.46 (± 3.25)	18	27.78 (5)	49.19 (± 30.27)	61.54%
	2	12.75 (± 2.43)	17	70.59 (12)		
Vaccinated	1	12.08 (± 2.02)	19	5.26 (1)	7.90 (± 3.73)	47.25%
	2	12.47 (± 1.49)	19	10.53 (2)		

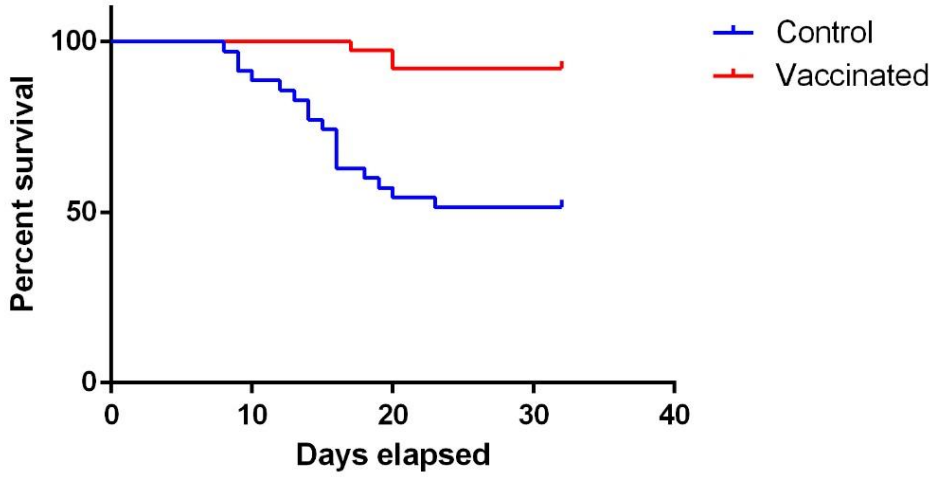


Figure 4.4. Survival rates after the bath challenge of vaccinated and control groups with *F. psychrophilum* strain AVU-1T/07. Each survival curve represents the average results for two parallel tanks ($p < 0.0001$, n= 35 fish for control group, n= 38 fish for vaccinated group).

4.3.2. Specific antibody response

Levels of specific IgM (OD_{450}; 1:32) in sera of immersion vaccinated fry were above the cut-off in two out of five fry when tested against the *F. psychrophilum* vaccine strain AVU-1T/13 (serotype Fd) and negative titres were recorded in all fry samples against the vaccine strain AVU-2T/13 (serotype Th). The naïve control fry showed positive titres

against AVU-1T/13 (4/6 samples) and AVU-2T/13 (1/6 samples) (Figure 4.5). IgM levels six weeks post-vaccination to both homologous *F. psychrophilum* strains were not significantly different in the vaccinated group when compared to the unvaccinated group.

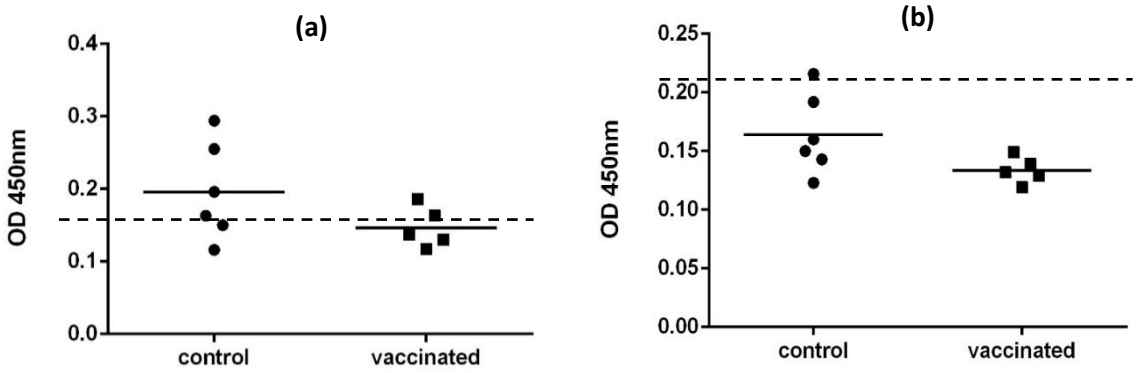


Figure 4.5. Serum antibody responses of fry six weeks post-vaccination by immersion with a polyvalent whole cell inactivated vaccine (■, n= 5) and of naïve control fry (●, n= 6). (a): against the vaccine strain AVU- 1T/13; (b) against the vaccine strain AVU-2T/13. The serum dilution was at 1:32. The dashed line represents the cut-off as determined by three times the absorbance of the negative control (PBS).

4.3.3. Gene expression (RT-qPCR)

The integrity of extracted RNA was checked by electrophoresis on a 1.0% agarose gel (Figure 4.6) prior to treatment using DNase. Fold change in gene expression of immune relevant genes in gill, hind-gut, skin, head kidney and spleen is summarised in Table 4.3.

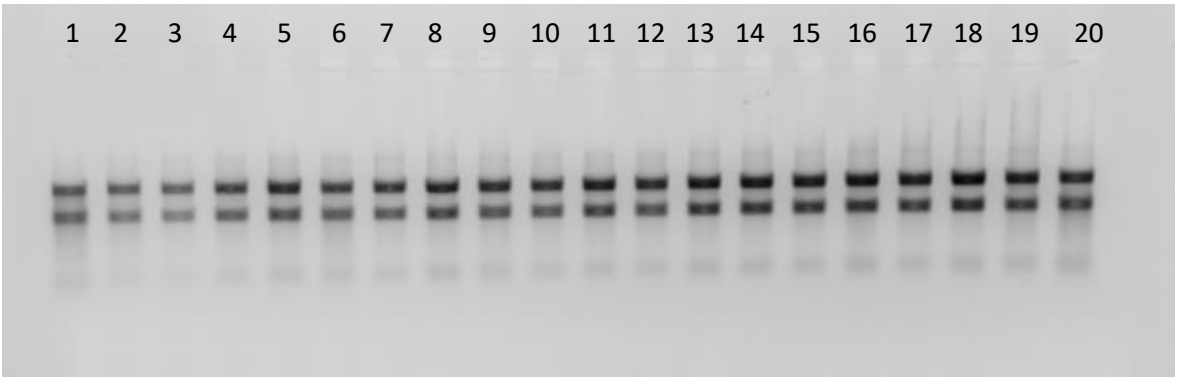


Figure 4.6. Agarose gel electrophoresis revealing RNA integrity in 1.0% agarose gel of the total extracted RNA from skin (lanes 1-4), kidney (lanes 5-8), gill (lanes 9-12), hind-gut (lanes 13-16) and spleen (lanes 17-20).

The relative expression of *TLR-2*, *C3*, *IL-1β*, *IgT*, *IgM*, *CD4-1* and *CD8-α* was normalised against *EF-1α* and *β-actin*. After 4 h post-vaccination, *IL-1β* was significantly up-regulated in the head kidney ($p < 0.05$). At day 2 post-vaccination, a significant up-regulation of *TLR-2* was observed in the skin and up-regulation of *IgT* and *IgM* was found in the hind-gut, while *IL-1β* was down-regulated in the gill and *CD8-α* was down-regulated in both head kidney and spleen ($p < 0.05$). At day 7 post-vaccination, significant down-regulation of both *CD4-1* ($p < 0.01$) and *CD8-α* ($p < 0.05$) was found in spleen.

Table 4.4. Expression in fold change and standard error (SE) of seven relevant immune genes in five organs of rainbow trout fry at three time points post-initial vaccination (n= 6 fish per group). Fold change of genes in vaccinated groups compared to controls ± SE. ↑ and ↓ indicate significant up-regulation and down-regulation relative to the control respectively ($p< 0.05$). ↑↑ and ↓↓ indicate significant up-regulation and down-regulation relative to the control respectively ($p< 0.01$).

	4 h		Day 2		Day7	
	Expression	SE	Expression	SE	Expression	SE
Gill						
TLR-2	1.989	0.528 - 6.877	2.129	0.680 - 7.022	1.776	0.729 - 4.139
C3	1.016	0.626 - 1.431	1.025	0.762 - 1.317	1.215	0.664 - 2.342
IL-1β	0.679	0.445 - 1.040	**0.552** ↓	0.358 - 0.837	0.651	0.431 - 1.009
IgT	3.963	0.669 - 65.100	2.722	0.791 - 66.208	0.713	0.504 - 1.197
IgM	0.808	0.378 - 1.699	1.309	0.854 - 2.217	1.088	0.656 - 1.960
CD4-1	0.924	0.674 - 1.366	0.933	0.799 - 1.119	0.922	0.704 - 1.235
CD8-α	0.726	0.505 - 1.029	0.709	0.378 - 1.364	1.071	0.587 - 2.075
Hind-gut						
TLR-2	1.808	0.549 - 18.535	1.340	0.818 - 2.121	0.562	0.300 - 1.125
C3	0.444	0.096 - 1.279	0.429	0.148 - 1.236	0.611	0.149 - 2.025
IL-1β	1.000	0.514 - 2.051	0.955	0.532 - 1.936	0.678	0.372 - 1.337
IgT	0.742	0.319 - 2.565	**2.127** ↑	1.166 - 4.228	0.230	0.017 - 1.083
IgM	1.381	0.721 - 2.533	**2.035** ↑	0.466 - 3.485	0.510	0.149 - 2.111
CD4-1	1.126	0.928 - 1.473	1.269	0.788 - 1.986	0.853	0.461 - 1.691
CD8-α	0.873	0.537 - 1.595	0.666	0.290 - 1.726	0.538	0.246 - 1.268

	4 h		Day 2		Day7	
	Expression	SE	Expression	SE	Expression	SE
Skin						
TLR-2	2.080	0.895 - 5.022	**4.348** ↑	1.819 - 7.875	1.611	0.525 - 3.783
C3	1.038	0.732 - 1.397	0.958	0.829 - 1.274	1.093	0.846 - 1.409
IL-1β	1.019	0.586 - 1.728	0.475	0.232 - 1.099	0.971	0.540 - 1.745
IgT	1.183	0.750 - 1.904	1.322	0.542 - 3.646	0.491	0.204 - 1.535
IgM	0.730	0.530 - 1.133	1.729	0.767 - 6.848	1.100	0.342 - 6.770
CD4-1	0.772	0.465 - 1.363	1.272	0.884 - 1.639	0.974	0.705 - 1.375
CD8-α	0.679	0.341 - 1.256	0.711	0.309 - 1.560	0.713	0.421 - 0.955
Head kidney						
TLR-2	1.031	0.451 - 2.220	0.918	0.564 - 1.238	0.791	0.503 - 1.265
C3	0.677	0.098 - 4.091	1.444	0.427 - 5.269	5.260	0.667 - 36.548
IL-1β	**1.819** ↑	0.901 - 4.013	1.001	0.417 - 2.254	1.347	0.639 - 3.058
IgT	0.995	0.505 - 1.947	1.199	0.845 - 1.638	2.189	0.202 – 48.134
IgM	0.988	0.713 - 1.368	0.784	0.531 - 1.261	0.654	0.415 - 1.095
CD4-1	0.944	0.656 - 1.315	1.128	0.834 - 1.546	1.191	0.827 - 1.616
CD8-α	0.788	0.562 - 1.133	**0.426** ↓	0.212 - 0.773	0.947	0.475 - 1.614
Spleen						
TLR-2	1.823	0.536 - 6.035	1.995	0.683 - 5.727	1.536	0.406 - 4.075
C3	0.793	0.390 - 1.689	1.129	0.661 - 1.864	0.810	0.362 - 1.566
IL-1β	2.494	0.976 - 8.678	0.813	0.457 - 1.579	1.002	0.719 - 1.617
IgT	0.898	0.411 – 2.454	1.572	0.737 – 3.106	1.230	0.461 - 3.188
IgM	1.184	0.595 - 2.287	0.863	0.475 - 1.364	0.628	0.348 - 1.507
CD4-1	0.869	0.626 - 1.216	0.903	0.581 - 1.357	**0.769** ↓↓	0.625 - 0.891
CD8-α	0.701	0.459 - 1.045	**0.485** ↓	0.244 - 1.027	**0.627** ↓	0.394 - 0.946

4.4. Discussion

RTFS and BCWD outbreaks usually occur when fish are 0.2 g to 10 g, thus an immersion or oral vaccine against *F. psychrophilum* needs to be developed. A new polyvalent whole cell vaccine was developed by combining three genetically and serologically diverse *F. psychrophilum* strains to confer broad-spectrum protection against *F. psychrophilum* infections. The efficacy of this vaccine, delivered by immersion, was examined in the current study as an RPS value, in addition to examining the immune response of fry by serology and relative expression of several immune genes. The polyvalent vaccine provided significant protection against a heterogeneous *F. psychrophilum* strain when administered to rainbow trout fry (mean weight of 4.72 ± 1.01 g) by immersion (Figure 4.4, $p < 0.0001$). A RPS value of 84% obtained in the present study was greater than those of previous studies using immersion administration route (14 – 47%, Obach and Laurencin, 1991; 13%, LaFrentz *et al*., 2002; 28 – 45%, LaFrentz *et al*., 2008). Lorenzen *et al.* (2010) observed a high RPS value (88%) following immersion exposure of fry to a combination of two live *F. psychrophilum* isolates, while no protection was seen in the group immersed in the inactivated vaccine. The low RPS values obtained in studies where immersion vaccination was used may be due to the incompatible challenge procedures, such as i.p. or subcutaneous injection used (Lorenzen *et al*., 2010). Inactivated or attenuated bacteria in immersion vaccines may not be able to reach the key immune tissues such as the kidney and spleen for stimulating the systemic immune response (Lorenzen *et al*., 2010). Therefore, in order to evaluate the efficacy of an immersion vaccine, a reproducible experimental bath infection for *F. psychrophilum* is essential.

Bath challenge in combination with a stress factor as H_2O_2 treatment), as described by Henriksen *et al.* (2013), produced a clinical RTFS infection in trout fry with an average mortality rate of 49% in the current work. Generally H_2O_2 causes injury to the gills with pathological changes, including extensive epithelial lifting and necrosis (Rach *et al.*, 1997). Although the mortality was lower than 60%, the desired mortality level for testing the potency of fish vaccines (Amend, 1981), the RPS was high. The fact that the challenged fry averaged approximately 12 g possibly reduced the effectiveness of the bath challenge as other studies used much smaller fish (1 g to 6 g) for bath challenge (Madsen and Dalsgaard, 1999; Garcia *et al*., 2000; Madetoja *et al*., 2000; Kondo *et al*., 2003; Aoki *et al*., 2005; Henriksen *et al*., 2013). Previous researchers found a correlation between the level of immunocompetence and size differences in rainbow trout, suggesting that larger fish possessed better protection against *F. psychrophilum*

(Madetoja *et al*., 2000; Aoki *et al*., 2005). Therefore, the average mortality of nearly 50% obtained in the current study with trout of 12 g indicated that the combination of stress and bath challenge may be an alternative to the injection challenge. However, there was a considerable difference in mortality rates between two replicate tanks (20.78% and 70.59%) in the control group (Table 4.2). This variation (RSD of 60%) was higher than that (40%) of Henriksen *et al.* (2013), who challenged the fry of 1.1 g. These results indicated that the reproducibility of the stress and bath challenge model might be influenced by the fish size (average weight of 12 g). Thus, one way to reduce this variation could be to use larger experimental groups or more replicates (Henriksen *et al*., 2013).

Serum IgM levels six weeks post-initial vaccination by immersion were not significantly different between control and vaccinated groups, in agreement with previous reports (LaFrentz *et al*., 2002; Valdenegro-Vega *et al*., 2013; Makesh *et al*., 2015). Makesh *et al.* (2015) observed no change in serum IgM level in 35 g rainbow trout vaccinated by the bath route up to 56 days post-vaccination using a live attenuated *F. psychrophilum* strain. No change in specific IgM level in fish serum following immersion is unclear but could involve the dose delivered and the inactivation of vaccine *F. psychrophilum* strains used in this thesis. On the other hand, the levels of specific IgM in fish serum were considered "positive" only in two out of five vaccinated fry samples against the vaccine strain of serotype Fd, and in none of serum samples against the other strain of serotype Th. Previously, Nikoskelainen *et al.* (2007) has suggested that Th serotype was a suppressive serotype of *F. psychrophilum* in trout when a polyvalent vaccine with similar formulation containing *Aeromonas salmonicida, Listonella anguillarum* and both *F. psychrophilum* serotypes Fd and Th showed TSI against only *A. salmonicida* and *F. psychrophilum* serotype Fd. These authors speculated that there could be an inhibitory effect of many immunobiological processes caused by bacterial antigens in polyvalent vaccines on the specific response of fish. Further studies to compare the efficacies of the polyvalent vaccine used in this thesis and monovalent vaccines on the immune response of trout are necessary. Although the polyvalent vaccine did not stimulate the specific antibody responses in sera of immunised fry, a high level of protection (RPS of 84%) was still observed against the immersion challenge of a heterogeneous isolate of serotype Th, which was performed three weeks post-booster vaccination. Lorenzen *et al.* (2010) also observed a high level of protection to i.p. injection challenge (RPS of 88 %) when only 25% (2/8) of the fish showed positive IgM

titres at the time of challenge following a bath immunisation using two live wild-type *F. psychrophilum* strains. It is suggested that the high RPS levels in the study of Lorenzen *et al.* (2010) and the present study could be partially due to the contribution of non-specific immune mechanisms.

In order to investigate the effect of the polyvalent whole cell vaccine on the immune system of rainbow trout fry at the molecular level, the expression levels of several genes related to innate and adaptive immunity (*TLR-2*, *IL-1β*, *C3*, *CD4-1*, *CD8-α*, *IgM* and *IgT*) were examined. Toll-like receptors (TLR) are a well-characterised family of receptors, responsible for sensing and triggering the innate immune response (Abós *et al*., 2013). TLR-2 receptor is involved in detecting highly conserved structures of bacterial origin (Castro and Tafalla, 2015). In the present work, the amount of *TLR-2* transcripts in the skin increased two-fold 4 h post-initial vaccination and was significantly up-regulated two days post-initial vaccination. These results indicated that the inactivated *F. psychrophilum* cells present in the immersion vaccine come into contact with skin-associated lymphoid tissues of the fish and are recognised by the TLR-2 receptors on the innate immune cells. The activation of pattern recognition receptors on immune cells, like TLR-2, is the first key factor in the triggering of a subsequent immune response (Castro and Tafalla, 2015).

Interleukin 1β (IL-1β) is a pro-inflammatory cytokine in mammals and was first described in rainbow trout in 1999 (Zou *et al*., 1999). The expression of *IL-1β* has been linked to the adhesion of live *A. salmonicida* to intestinal epithelial cells (Komatsu *et al*., 2009) and considered as a marker for bacterial infection in diseased fish (Orieux *et al*., 2013). In the present study, initially *IL-1β* was significantly up-regulated in head kidney 4 h post-vaccination and then down-regulated in gill at day 2. These results indicated that a pro-inflammatory response was observed at an early stage (4 h) after the immersion immunisation using formalin-killed *F. psychrophilum* cells. The reason for the down-regulation of *IL-1β* observed two days post-vaccination in gill is unclear but could be related to the expression of anti-inflammatory molecules (e.g. IL-10) because inflammation is a balance between the expression levels of pro and anti-inflammatory cytokines. Henriksen *et al.* (2015 a,b) also found the initial up-regulation (4 h post challenge) and later down-regulation (192 h post-challenge) of *IL-1β* in head kidney and gill of trout fry of 1.2 g following bath challenge in *F. psychrophilum* solution with or without a preceding H_2O_2 immersion. The reduced inflammation or suppressed immune response elicited by *F. psychrophilum* might be due to a release of cortisol as a response

of stress or due to virulence factors expressed by *F. psychrophilum* (Henriksen *et al.*, 2015 a,b).

Complement factor C3 is the central component of the complement system, an ancient innate immune mechanism present in both vertebrate and invertebrate species (Castro and Tafalla, 2015). The complement system of rainbow trout is analogous to mammals and plays an important role in defence against infection (Tomlinson *et al*., 1993). The importance of C3 is illustrated in C3-deficient humans and mice, which have a greater susceptibility to bacterial infection (Daha, 2010; Wessels *et al*., 1995; Langevin *et al*., 2012). A similar mechanism was observed in trout infected with *F. psychrophilum* by the intramuscular injection route where *C3* showed a stronger induction following infection in a resistant rainbow trout line, compared with a susceptible line (Langevin *et al*., 2012). The *C3* gene was also demonstrated to be significantly up-regulated in kidney, spleen and liver of fish five days post-subcutaneous injection with virulent strains of *F. psychrophilum*, *Aeromonas salmonicida* and infectious haematopoietic necrosis virus (Overturf and LaPatra, 2006). In the current study, the expression levels of *C3* in five organs were not significantly different in vaccinated group and control group. The lack of a differential response of *C3* observed in the present study could be due to the immersion immunisation route and the inactivation of *F. psychrophilum* cells in the vaccine.

The down regulation of *CD8-α* was found in head kidney and spleen two days post-vaccination and in the spleen seven days post-vaccination. CD8 is primarily known as a marker for cytotoxic T-cells and is able to recognise intracellularly derived antigens in the context of major histocompatibility complex (MHC) class I molecules (Castro and Taffala, 2015). Orieux *et al*. (2013) speculated that the suppression of some immune genes, such as *mhc-2*, *tgh-β*, and *CD8-α* contributed to the low serum IgM titre in a previous study, in which diseased fish from natural RTFS outbreaks presented lower plasma IgM titres (87 immunoglobulin monomer (UIg)) than apparently healthy fish (1092 UIg). In addition, no change in the expression of *CD8* was demonstrated in head kidney following a bath challenge using *F. psychrophilum* (Henriksen *et al*., 2015a), while in contrast injection challenge significantly elevated the expression levels of *CD8* in kidney and spleen (Overturf and LaPatra, 2006). On the other hand, the down-regulation of *CD4-1* in spleen seven days post-vaccination was observed in the current study. CD4-positive T cells are able to recognise extracellularly derived antigens presented by MHC II molecules and differentiate into either Th1 effector cells or Th2

cells to regulate the action of other immune cells, mainly B cells (Henriksen *et al*., 2015a, b). Previously, no change in *CD4* level was observed when fish were bath challenged with *F. psychrophilum* with or without preceding H_2O_2 immersion (Henriksen *et al*., 2015a, b). Indeed, the effect of the repression of *CD8-α* and *CD4-1* following an immersion vaccination using formalin-killed *F. psychrophilum* cells is unclear, but could be related to the lower titre of IgM measured in serum of vaccinated fish compared to control fish, as suggested by Orieux *et al.* (2013). Further work is required to examine the expression of *CD8-α* and *CD4-1* pre-challenge and post challenge in immersion vaccinated fry in order to investigate the importance of the cell-mediated immunity response in protection against *F. psychrophilum* infection.

Vaccination strategies are generally targeted to stimulate antibody responses in fish to provide protection against subsequent infection (LaFrentz *et al*., 2002). IgM is thought to play a role in both systemic and mucosal immunity, and seems to be directly related to protection (Solem and Stenvik, 2006; Zhang *et al*., 2010; Castro and Taffala, 2015; Makesh *et al*., 2015). However, the recent discovery of IgT in mucosal-associated lymphoid tissues has changed that paradigm (Zhang *et al*., 2010). IgT is considered as a specialised Ig in mucosal immunity in rainbow trout, equivalent to IgA in mammals (Zhang *et al*., 2010). In the present study, an up-regulation of *IgM* was noted in hind-gut two days post-initial vaccination. Such early up-regulation of *IgM* in mucosal tissues (e.g. hind-gut) suggests that IgM plays a role not only in systemic immune response but also in mucosal immunity (Makesh *et al.*, 2015). Although the immersion vaccination produced such significant over-expression of *IgM* transcripts, the serum IgM levels in immersion vaccinated fish showed no significant changes six weeks post-vaccination, compared to control group. Such a time lag in antibody production compared to transcript production was also reported by Makesh *et al.* (2015). These authors observed peak *IgM* expression at 7 days post-immunisation in gills and skin of fish (35 g), which were bath-immunised using a live attenuated vaccine, but reported no change in serum IgM levels in immunised fish at 56 days post immunisation. On the other hand, *IgT* was significantly up-regulated in hind-gut two days post-initial vaccination in the present work. These results are consistent with the findings that IgT and IgT^+ B cells were prevalent in the gut of rainbow trout and represented 54.3% of all B cells (Zhang *et al*., 2010), while Makesh *et al.* (2015) reported significantly greater and earlier *IgT* expression in gills of bath-immunised fish at 3 days post immunisation, compared with i.p.-injected or anal-intubated fish. The up-regulated expression of *IgT* in hind-gut in immersion immunised

fish in the current study suggests that IgT might contribute to immediate protection to the host at mucosal surfaces against the natural infection route of pathogens. Additionally the significant up-regulation of *IgT* as early as two days post-vaccination supports the hypothesis that IgT producing B lymphocytes are present locally, thus resulting in increased expression of this immunoglobulin upon stimulation by bath vaccination (Makesh *et al*., 2015), and stresses that IgT is a major antibody isotype inducing mucosal immunity. Further sampling points pre- and post-challenge to examine the expression levels of *IgT* in both mucosal and systemic organs against *F. psychrophilum* infection would improve the understanding of the induction of mucosal immunity by immersion vaccination. Moreover, investigation of non-specific immune factors such as lysozyme, alternative complement and anti-microbial peptides in serum and mucus of vaccinated fish would allow greater insight into the protective mechanism induced by immersion vaccination against *F. psychrophilum*.

4.5. Conclusions

The present study is one of the first to demonstrate significant protection (RPS of 84%) of rainbow trout induced by a polyvalent inactivated whole cell vaccine against RTFS/BCWD following immersion immunisation route and heterogeneous immersion challenge. This vaccine stimulated the up-regulation of genes related to both innate and adaptive immunity that may contribute to protect rainbow trout against *F. psychrophilum*. Immersion vaccination induced a significant increase in *IgT* and *IgM* transcripts in the hind-gut two days post-vaccination as measured by gene expression, suggesting that IgT is a major antibody isotype inducing mucosal immunity, while IgM seems to have some role in mucosal immunity. Although bath challenge with a pre-treatment of H_2O_2 appreared to be a promising alternative to injection for challenging rainbow trout up to 12 g, futher work is still needed to standardise and optimise the method. Further investigation is needed to elucidate the long-term protection of this polyvalent vaccine, demonstrate the broad-spectrum protection of the vaccine against other genetically and serologically different *F. psychrophilum* strains, evaluate the expression level of specific antibodies secreted into mucus of immersion vaccinated fish, and improve the reproducibility of the bath challenge model for *F. psychrophilum*.

Chapter 5
General discussion and final conclusions

5.1. General discussion

The UK aquaculture (finfish and shellfish) industry retains a leading position within Europe (1st by value, 3rd by production tonnage) (Ellis *et al.*, 2015). While Atlantic salmon continues to dominate UK aquaculture production in harvest tonnage and value, rainbow trout represents the most prominent species in freshwater farming (Rodgers and Furones, 2009; Ellis *et al.*, 2015). Rainbow trout fry syndrome (RTFS) caused by the bacterium *Flavobacterium psychrophilum* is one of the major diseases which is currently constraining rainbow trout production worldwide (Verner-Jeffreys and Taylor, 2015). A survey by these authors reported that clinical signs of this disease were observed by all eight different UK rainbow trout producers surveyed, and the main treatment used for the disease was florfenicol.

Despite many attempts to develop a commercial vaccine against RTFS during the last 20 years, this has been hindered by the prevalence of a wide range of *F. psychrophilum* strains (Gómez *et al.*, 2014). In the UK, *F. psychrophilum* was first reported in internal organs and skin lesions of diseased rainbow trout in 1992 (Santos *et al.*, 1992). However, to date, no studies on the epidemiology of the UK *F. psychrophilum* strains have been published. Therefore, in Chapter 2, the strain diversity of *F. psychrophilum* isolates from RTFS-affected fish on farms within the UK was described using a combination of genotypic and serotyping methods. Although the 292 UK *F. psychrophilum* isolates showed an overall high genetic diversity, the majority of isolates (200/292) could be placed into four main PFGE groups/singleton (P, E, Q and S), which may have selective advantages over other circulating pulsotypes. The wide distribution within the UK and the persistence within a site of these predominant pulsotypes could reflect the structure of the UK rainbow trout industry, where there is widespread movement of live fish between sites, which increases the risk of epizootic episodes occurring in consecutive years (Madetoja *et al.*, 2002). These results broadly support the contention of Nilsen *et al.* (2014) that *F. psychrophilum* displays an epidemic population structure, in which highly successful clones have expanded within a generally recombinant bacterial population, in the regions where it is circulating. In addition, the close genetic similarity observed between some of the UK *F. psychrophilum* study isolates and those found from abroad (Chen *et al.*, 2008; Avendaño-Herrera *et al.*, 2009) was also observed in this study and may represent the emergence of a clonal *F. psychrophilum* population within the developing rainbow trout industry and subsequent global dissemination of pathogenic clones (Nilsen *et al.*, 2014).

Besides the predominant pulsotypes recovered, the occurrence of unique PFGE singletons (24/27 singletons) and $(GTG)_5$-PCR types (r5 – r10) in certain sites or countries possibly represents local adaption of particular strains to sites or environmental niches (Arai *et al.*, 2007; Del Cerro *et al.*, 2010). A concurrent mixture of isolates belonging to distinct pulsotypes, $(GTG)_5$-PCR types, serotypes or plasmid profiles was recorded in this study, in keeping with the observations of Madetoja *et al.* (2002), Chen *et al.* (2008), Del Cerro *et al.* (2010) and Sundell *et al.* (2013). There have been limited studies on linking specific pulsotypes of *F. psychrophilum* with severe RTFS/BCWD outbreaks in fish farms, especially those involving the co-infection of genetically heterogeneous isolates, the contribution of which to the success and severity of such mixed infection remains unknown (Nilsen *et al.*, 2014). However, the findings of this study suggest that more than one virulent *F. psychrophilum* strain, such as pulsotype T1 and U1 or pulsotypes G and P2, could coexist in the fish stock but only one strain tends to be associated with a disease outbreak depending on some underlying factors related to fish husbandry of the farm, such as environmental conditions (e.g. water, temperature) and fish health status (Madetoja *et al.*, 2002; Sundell *et al.*, 2013). Moreover, apparently healthy fish carrying *F. psychrophilum* might act as a reservoir for shedding the pathogen into surrounding water (Chen *et al.*, 2008).

Due to the limited numbers of *F. psychrophilum* isolates derived from salmon hosts in comparison with rainbow trout, a host-specific association between pulsotypes, $(GTG)_5$-PCR types or 16S rRNA lineages, as reported previously (Arai *et al.*, 2007; Ramsrud *et al.*, 2007; Chen *et al.*, 2008; Avendaño-Herrera *et al.*, 2009; Nilsen *et al.*, 2011), was not clearly observed. The strain set comprising representative and equivalent numbers of isolates recovered from rainbow trout and salmon should be established in order to investigate the relationship between fish host and *F. psychrophilum* genotypes, thus improving the understanding of the transmission and colonisation of this pathogen between these different fish species.

Plasmids of 3.3 kb, found either alone or in combination with other plasmids were detected in 90% of the UK isolates examined in this study. The presence of this small plasmid in isolates collected from diseased fish in acute or chronic RTFS outbreaks and also from apparently healthy fish indicates either genetic acquisition between clinical *F. psychrophilum* isolates and other isolates in the aquatic environment, or the carriage of similar sized (~ 3.3 kb) but heterogeneous plasmids occurring within *F. psychrophilum*

isolates. Further studies on characterising these plasmids are required to determine their function within *F. psychrophilum*.

The UK *F. psychrophilum* isolates obtained from RTFS/BCWD outbreaks mainly belonged to serotype Th (113/165 isolates, with 11 isolates from Atlantic salmon), while serotypes Fd and Fp^T were detected in 42 (including three isolates from Atlantic salmon) and 9 isolates (including four Atlantic salmon isolates) respectively, consistent with the findings of Lorenzen and Olesen (1997) on European isolates. Previous authors have noted that isolates belonging to serotype Fp^T were more commonly recovered from salmon hosts (Lorenzen and Olesen, 1997) and less pathogenic to rainbow trout during the challenge experiments, in comparison with Th and Fd serotypes (Madsen and Dalsgaard, 1999, 2000; Madetoja *et al.*, 2002). The association between the virulence of the UK isolates and the serotype Fp^T should be investigated in a strain set containing more isolates recovered from salmon and then confirmed by fish challenge experiments. One untypeable isolate (pulsotype BB, $(GTG)_5$-PCR type r3, 16S rRNA allele CSF and 3.3 kb plasmid) collected from rainbow trout could represent another serotype in other serotyping schemes of Izumi and Wakabayashi (1999) and Mata *et al.* (2002) or a new serotype of *F. psychrophilum*, which perhaps should be considered along with the three currently used serotypes (Th, Fd and Fp^T), when serotyping the UK *F. psychrophilum* isolates. The ELISA technique employed in this study could be further optimised to resolve the classification of Th subtypes (Th-1 and Th-2), as mentioned by Lorenzen and Olesen (1997).

The combination of genetic and phenotypic typing methods employed in the present study has been shown to be useful when evaluating population diversity. The predominant profile within the UK *F. psychrophilum* population, PFGE cluster II – $(GTG)_5$-PCR type r1 – 16S rRNA lineage II – serotype Th (70/156 isolates, 45%) was noted. The $(GTG)_5$- PCR types r1 and r2 were found to be associated with PFGE cluster II and PFGE cluster I respectively, suggesting rep-PCR could be used as a rapid genotyping method for *F. psychrophilum* species. A relationship between the genotype and antigenicity of *F. psychrophilum* isolates was observed in this work, in which isolates belonging to each pulsotype showed a single serotype, in agreement with previous studies (Madsen and Dalsgaards, 2000; Madetoja *et al.*, 2001, 2002). However, no correlation was found between the plasmid profiles and serotypes or genotypes determined by PFGE, $(GTG)_5$-PCR and 16S rRNA allele PCR methods, as isolates of each genotype had different plasmid contents, in agreement with the findings of Del Cerro *et al.* (2010) and

Sundell *et al.* (2013). A likely overlap between the genetic relationships inferred by PFGE and multilocus sequence typing (MLST, Nicolas *et al.*, 2008; Siekoula-Nguedia *et al.*, 2012; Avendaño-Herrera *et al.*, 2014; Nilsen *et al.*, 2014) in investigating the population structure of *F. psychrophilum* was suggested when analysing the PFGE data of several reference strains used in this study. The *F. psychrophilum* isolates belonging to *Sac*I-PFGE cluster II could be related to those of clonal complexes CC-ST2 or CC-ST10 in MLST analysis. Further work applying MLST and whole genome sequencing in such investigations on the UK *F. psychrophilum* population could enable inter-laboratory comparison and global *F. psychrophilum* surveillance.

In the absence of a commercial vaccine against RTFS/BCWD, the use of antibiotics is currently the treatment of choice for controlling disease outbreaks, resulting in concern regarding the development of antimicrobial resistance by *F. psychrophilum* (Gómez *et al.*, 2014). The threat posed by antibiotic resistance also stresses the importance of performing routine susceptibility testing using reliable standardised methods (e.g. VET04-A2 guideline of CLSI) and interpretive criteria (e.g. epidemiological cut-off values, CO_{WT}) (Smith *et al.*, 2013). Therefore, in Chapter 3, the antimicrobial susceptibility of 118 UK *F. psychrophilum* strains selected on the basis of genotyping, serotyping and plasmid profiling data from Chapter 2 was evaluated. The results showed that among the three agents (florfenicol, oxytetracycline and amoxicillin) used to control RTFS in the UK, florfenicol (FFN) is still the most effective treatment, as evidenced by the full *in vitro* susceptibility to FFN of all the strains examined with MIC values ranging from 0.12 mg L^{-1} to 1 mg L^{-1} and inhibition zone ranging from 50 mm to 75 mm, in accordance with the observations of Rangdale *et al.* (1997) and Smith *et al.* (2016), where MIC values were documented at 0.00098 – 16 mg L^{-1} and 0.12 – 1 mg L^{-1} respectively. The fact that the UK *F. psychrophilum* strains were determined to be *in vitro* fully susceptible to FFN over the last two decades (1997 – 2016) is encouraging. However, due to the limited data on the antimicrobial susceptibility of *F. psychrophilum* strains circulating in UK hatcheries and fish farms, the evaluation of FFN effectiveness within the UK may not be fully complete. In addition, the current heavy reliance on FFN for controlling RTFS in the UK may be facilitating the development of resistance to FFN by *F. psychrophilum*, which could affect the viability of the UK rainbow trout industry. Both oxytetracycline and amoxicillin were moderately effective against the UK *F. psychrophilum* strains. They could be considered as

alternative antimicrobials to FFN in RTFS management, contributing to a reduction in use of FFN.

There have been many studies determining the antimicrobial susceptibility of *F. psychrophilum* strains (Pacha, 1968; Rangdale *et al.*, 1997; Lorenzen *et al.*, 1997; Dalsgaard and Madsen, 2000; Schmidt *et al.*, 2000; Bruun *et al.*, 2000, 2003; Michel *et al.*, 2003; Izumi and Aranishi, 2004; Soule *et al.* 2005; Kum *et al.*, 2008; Del Cerro *et al.*, 2010; Hesami *et al.*, 2010; Nilsen *et al.*, 2011; Henríquez-Núñez *et al.*, 2012; Durmaz *et al.*, 2012; Boyacioğlu and Akar, 2012; Sundell *et al.*, 2013; Verner-Jeffreys and Taylor, 2015; Boyacioğlu *et al.*, 2015; Smith *et al.*, 2016). However, these studies used different testing methods and importantly a variety of the interpretive criteria in categorising their isolates as manifesting reduced susceptibility. As a consequence, the meaningful comparison of the frequencies of strains with reduced susceptibility both within and between countries is difficult. The existence of these variables has stressed the need for standardised procedures adapted specifically for testing the antibiotic susceptibility of the *F. psychrophilum* species, such as the VET04-A2 guideline of CLSI and normalised resistance interpretation method. The CO_{WT} values for MIC data calculated in this work are probably laboratory-independent and of general or 'universal' applicability, while the low levels of precision of the zone data sets have previously been attributed to the low incubation temperature (15^{o}C) and the long incubation time (72-96 h) (Smith and Kronvall, 2015). However, the high percentage agreement between the categorisation (full susceptibility, WT; or reduced susceptibility, NWT) of the 140 *F. psychrophilum* strains obtained by analysing the observed MIC values and those of the disc diffusion data suggests that, although the disc diffusion protocol used in this work generated data of low precision, these inhibition zone data and the CO_{WT} calculated from them may have some value in detecting strains of reduced susceptibility. In spite of this, any CO_{WT} calculated from inhibition zone data should be treated with some caution and each laboratory using the disc diffusion test should generate their own set of CO_{WT} values.

Because of the importance of antimicrobial susceptibility surveillance to RTFS/BCWD management, it may be helpful to understand the associations between a particular genotype and reduced susceptibility to the main antibiotics, which are commonly used in RTFS/BCWD treatment within the UK. Based on the genotyping results in Chapter 2 and the antimicrobial susceptibility data in Chapter 3, several significant associations between PFGE groups/singletons, $(GTG)_5$-PCR types/subtypes or plasmid profiles and the antimicrobial susceptibilities of *F. psychrophilum* strains were

revealed using the Chi-square test and post-hoc cellwise comparison (Beasley and Schumacker, 1995; García-Pérez and Núñez-Antón, 2003). The reduced susceptibility to AMP and AMOX was statistically over-represented in PFGE group P and $(GTG)_5$-PCR type r4, while the NWT phenotype to OTC was associated with PFGE singleton S, $(GTG)_5$-PCR subtype r1a and the combination of two plasmids (2.1 kb and 3.3 kb), of which the 2.1 kb plasmid may be responsible for the reduced susceptibility to OTC. Previously, Bruun *et al.* (2003a) suggested that OTC resistance determinants in *F. psychrophilum* strains could be located in mobile elements, such as plasmids. Further studies on the 2.1 kb plasmid are needed to prove the correlation of this plasmid and NWT phenotype to OTC. In addition, a larger set of *F. psychrophilum* isolates from each genotype is needed in order to confirm the associations between genotypes and antibiotic susceptibility patterns observed in this study; and to further compare the genetic differences (e.g. whole-genome sequencing) between genotypes exhibiting different antimicrobial susceptibilities, such as PFGE group E or $(GTG)_5$-PCR type r2 (WT phenotype to beta-lactams) and PFGE group P or $(GTG)_5$-PCR subtype r4 (NWT phenotype to beta-lactams); PFGE group E or $(GTG)_5$-PCR type r4 (WT phenotype to OTC) and PFGE group S or $(GTG)_5$-PCR subtype r1a (NWT phenotype to OTC).

Disease prevention by vaccination is one of the most important preventive measures in aquaculture, contributing to a sustainable aquaculture practice with low use of antimicrobials (Gudding, 2014). Based on the genotyping and serotyping characterisation results obtained in Chapter 2, a whole-cell formalin killed polyvalent vaccine against RTFS/BCWD was developed by combining three genetically and serologically divergent strains, recently collected from UK farms. This combination would be expected to confer a broad-spectrum protection against *F. psychrophilum* infection. The protective efficacy of this polyvalent vaccine in rainbow trout fry (mean weight of 4.72 ± 1.01 g) after bath challenge with a *F. psychrophilum* strain possessing a heterogeneous pulsotype compared to the vaccine strains, was investigated in Chapter 4. The significant protection achieved (RPS of 84%) in this study was higher than those of previous studies using a similar vaccination route (14 – 47%, Obach and Laurencin, 1991; 13%, LaFrentz *et al.*, 2002; 28 – 45%, LaFrentz *et al.*, 2008; and 0%, Lorenzen *et al.*. 2010), which could be due to the incompatible challenge procedures used, such as intraperitoneal or subcutaneous injection used in these earlier studies.

Bath challenge was possible as this was performed in combination with a stress factor (H_2O_2 treatment), as described by Henriksen *et al.* (2013). This resulted in a

promising *F. psychrophilum* experimental infection challenge in rainbow trout fry with an average weight of 12 g, which resulted in a mean cumulative mortality of 49% in the present study. Earlier researchers found a correlation between the level of immunocompetence and size differences in rainbow trout, suggesting that larger fish were less susceptible to infection with *F. psychrophilum* (Madetoja *et al.*, 2000; Aoki *et al.*, 2005). Although the bath challenge model results obtained here indicated that the combination of a pre-treatment using H_2O_2 and bath challenge may be a promising alternative to injection challenge, further standardisation and optimisation of the method is required. Larger experimental groups or more replicates are needed to reduce the variation between the replicates of the experimental model (Henriksen *et al.*, 2013).

The expression levels of several immune genes (*TLR-2*, *IL-1β*, *C3*, *CD4-1*, *CD8-α*, *IgM* and *IgT*) in response to the immersion vaccination from different mucosal and lymphoid organs of fry were examined in order to investigate the effect of the polyvalent whole cell vaccine to immune system of rainbow trout fry at the molecular level. The significant up-regulation of *toll-like receptor-2* (*TLR-2*) in skin of fry two days post-vaccination indicated that the inactivated *F. psychrophilum* cells present in the vaccine stimulated these important innate receptors of the fry, which trigger all subsequent immune responses (Castro and Carolina, 2015). A pro-inflammatory response was observed at an early stage (4 h) following immersion immunisation using formalin-killed *F. psychrophilum* cells via significantly up-regulated *interleukin 1 β* (*IL-1β*) transcripts in head kidney. However, the reason for the down-regulation of *IL-1β* at day 2 post-vaccination in gills was unclear and contrasted with earlier reports (Henriksen *et al.*, 2015a,b). No change in the expression of *complement factor 3* (*C3*), the central component of the complement system, was recorded in the present study and could be due to the immersion immunisation route used and the inactivation of *F. psychrophilum* cells in the vaccine. *CD8-α* was down-regulated in head kidney and spleen two days post-vaccination and in spleen seven days post-vaccination, while the down-regulation of *CD4-1* was observed in spleen seven days post-vaccination. Previously Orieux *et al.* (2013) reported that naturally diseased fish showed down-regulation of *CD8-α*, which may contribute to the lower serum IgM, compared to apparently healthy fish, while Henriksen *et al.* (2015a) demonstrated no change in the expression levels of *CD8-α* and *CD4* in bath-challenged fry with *F. psychrophilum*. Therefore, further work is required to examine the expression of *CD8-α* and *CD4-1* pre-challenge and post challenge in

immersion vaccinated fry in order to investigate the importance of the cell-mediated immunity response in protection to *F. psychrophilum* infection.

Vaccination strategies are generally targeted to stimulate antibody responses in fish to provide protection from fish pathogen challenge (LaFrentz *et al.*, 2002). In the present study, serum IgM levels six weeks post-initial vaccination by immersion were not significantly different in control and vaccinated groups, as reported previously (LaFrentz *et al.*, 2002; Valdenegro-Vega *et al*., 2013; Makesh *et al*., 2015). No change in specific IgM level in fish serum following immersion is unclear but could involve the dose delivered and the inactivation of vaccine *F. psychrophilum* strains used in this thesis. The positive levels of serum IgM in vaccinated fish were only found against the vaccine strain of serotype Fd, but not against the strain of serotype Th. Previously Nikoskelainen *et al.* (2007) suggested that Th serotype was a suppressive serotype of *F. psychrophilum* in trout when using a polyvalent vaccine, possibly due to an inhibitory effect caused by bacterial antigens in the polyvalent vaccine. Therefore, further studies to compare the efficacies of the polyvalent vaccine used in this thesis and relevant monovalent vaccines on the immune response of trout are essential. Although the polyvalent vaccine did not stimulate the specific antibody responses in sera of immunised fry, a high level of protection (RPS of 84%) was still observed against the immersion challenge of a heterogeneous isolate of serotype Th. It is suggested that this high RPS level could be partially due to the contribution of non-specific immune mechanisms. On the other hand, at molecular level, *IgM* transcripts were found to be up-regulated in hind-gut two days post-initial vaccination, suggesting that IgM plays a role not only in systemic immune response but also in mucosal immunity. Although the up-regulation of *IgM* was induced by immersion vaccination, serum IgM levels in vaccinated fish showed no significant changes six weeks post-immersion vaccination, compared to control group. Such time lag in antibody production compared to transcript production has been also reported in a previous study of Makesh *et al.* (2015). The up-regulated expression of *IgT* in the hind-gut of fish at day 2 following immersion immunisation in the current study suggests that IgT might contribute to the immediate protection to the host at mucosal surfaces against the natural infection route of pathogens (Zhang *et al.* 2010; Makesh *et al.* 2015). Moreover, the significant up-regulation of *IgT* in hind-gut as early as two days post-vaccination supported the hypothesis that IgT producing B lymphocytes are present locally, thus resulting in increased expression of this immunoglobulin upon stimulation by bath vaccination (Makesh *et al.* 2015), and stressed that IgT is a major antibody

isotype inducing mucosal immunity. Futher analysis on the expression levels of IgT in both mucosal and systemic organs at different time points pre- and post-challenge could help improve the understanding of the induction of mucosal immunity by immersion vaccination against *F. psychrophilum* infection.

5.2. Final conclusions

The UK *F. psychrophilum* diversity broadly supports the hypothesis of an epidemic population structure of this bacterium. Understanding the genetic diversity of *F. psychrophilum* present in particular countries or sites where RTFS is a problem is crucial for monitoring the spread of epidemic clones, setting up preventative RTFS/BCWD control strategies (e.g. vaccination programmes) and managing the egg and fish trade between farms. Further work is required to determine the *in vivo* biological importance of genetically and serologically divergent *F. psychrophilum* clones simultaneously present in the UK sites, and to assess the functional differences between environmental and clinical isolates.

PFGE was able to give an indication of the genetic differences between the *F. psychrophilum* isolates studied, making the method suitable for tracing outbreaks and as performed in the present study showing the heterogeneity of isolates circulating in a particular region or epidemiological unit (farm or related group of rearing facilities). Rep-PCR could be utilised as a rapid diagnostic marker to assess genetic variation within the *F. psychrophilum* species in laboratories lacking expensive gene analysis equipment.

Florfenicol is still the most effective treatment for controlling RTFS outbreaks within the UK. However, there is a reasonable chance that oxytetracycline and amoxicillin would be effective and could be considered as alternative antimicrobials for RTFS management, contributing to a reduction in use of florfenicol. Furthermore, this study also stresses the importance of performing susceptibility testing using standardised methods and CO_{WT} values, in association with every therapeutic treatment and in monitoring the development of antibiotic resistance mechanisms.

The MIC data generated from this work together with data from other studies using the standard CLSI protocol could contribute to setting up internationally agreed MIC epidemiological cut-off values for investigating the antimicrobial susceptibilities of *F. psychrophilum*. The correlation between MIC CO_{WT} and disc-diffusion CO_{WT} in the classifications of the WT and NWT strains suggested the inhibition zone data and their

calculated CO_{WT} values may have some value in detecting strains of reduced susceptibility, although disc-diffusion CO_{WT} values should be treated with some caution.

A dependence of the antimicrobial susceptibility patterns on particular *F. psychrophilum* genotypes was identified in the current study. A larger set of isolates from each genotype is needed in order to confirm such significant associations and to further improve the understanding of antibiotic resistance mechanisms by characterising the genetic differences between genotypes exhibiting different antimicrobial susceptibilities.

A polyvalent inactivated whole cell vaccine against RTFS/BCWD has been developed on the basis of the genotyping and serotyping data generated in this study. This vaccine stimulated the expression of genes related to innate and adaptive immunity post-immersion vaccination and provided protection (RPS of 84%) in rainbow trout fry against a bath challenge using a heterogeneous *F. psychrophium* strain. Although IgM appears to play a role not only in the systemic immune response but also in mucosal immunity, IgT is suggested to be a major antibody isotype inducing mucosal immunity. Moreover, bath challenge with H_2O_2 pre-treatment could be a promising alternative to injection challenge of rainbow trout at the weight up to 12 g, although further work is required to improve and standardise the method. Further investigation is also needed to elucidate the long-term protection of this polyvalent vaccine, evaluate the expression level of specific antibodies secreted into mucus of immersion vaccinated fish, and improve the reproducibility of the bath challenge model for *F. psychrophilum*.

In general, understanding *F. psychrophilum* strain diversity on the genetics, serology and antibiotic susceptibility would help improve the control measures and reduce inappropriate antibiotic therapies against RTFS/BCWD outbreaks, thus preventing the spread of pathogenic isolates between farms and the development of antibiotic resistance mechanisms. Moreover, strain characterisation data of the *F. psychrophilum* species will also assist in selecting suitable candidates for developing effective vaccines against *F. psychrophilum* infection.

References

Abós, B., Castro, R., Pignatelli, J., Luque, A., Gonzalez, L. and Tafalla, C. (2013) Transcriptional heterogeneity of IgM^+ cells in rainbow trout (*Oncorhynchus mykiss*) tissues. *PloS One*, 8 (12), pp. e82737.

Akortha, E.E., Aluyi, H.S.A. and Enerrijioki, K.E. (2011) Transfer of amoxicillin resistance gene among bacterial isolates from sputum of pneumonia patients attending the university of Benin teaching hospital, Benin city, Nigeria. *Shiraz E-Medical Journal* **12**, pp. 179-188.

Alderman, D. and Smith, P. (2001) Development of draft protocols of standard reference methods for antimicrobial agent susceptibility testing of bacteria associated with fish diseases. *Aquaculture,* 196 (3), pp. 211-243.

Álvarez, B. and Guijarro, J. (2007) Recovery of *Flavobacterium psychrophilum* viable cells using a charcoal-based solid medium. *Letters in Applied Microbiology,* 44 (5), pp. 569-572.

Álvarez, B., Secades, P., Prieto, M., McBride, M. and Guijarro, J. (2006) A mutation in *Flavobacterium psychrophilum* tlpB inhibits gliding motility and induces biofilm formation. *Applied and Environmental Microbiology,* 72 (6), pp. 4044-4053.

Álvarez, B., Álvarez, J., Menéndez, A. and Guijarro, J.A. (2008) A mutant in one of two exbD loci of a TonB system in *Flavobacterium psychrophilum* shows attenuated virulence and confers protection against cold water disease. *Microbiology,* 154 (4), pp. 1144-1151.

Amita, K., Hoshino, M., Honma, T. and Wakabayashi, H. (2000) An investigation on the distribution of *Flavobacterium psychrophilum* in the Umikawa River. *Fish Pathology,* 35 (4), pp. 193-197.

Amend D. F. (1981) Potency testing of fish vaccines. *Developments in Biological Standardization,* 49, pp. 447-454.

Antaya, C.L. (2008) Current eco-economical impacts of *Flavobacterium psychrophilum*. *MMG 445 Basic Biotechnology eJournal,* 4 (1), pp. 16-21.

Aoki, M., Kondo, M., Kawai, K. and Oshima, S. (2005) Experimental bath infection with *Flavobacterium psychrophilum*, inducing typical signs of rainbow trout *Oncorhynchus mykiss* fry syndrome. *Diseases of Aquatic Organisms,* 67 (1-2), pp. 73-79.

Aoki, M., Kondo, M., Nakatsuka, Y., Kawai, K. and Oshima, S. (2007) Stationary phase culture supernatant containing membrane vesicles induced immunity to rainbow trout *Oncorhynchus mykiss* fry syndrome. *Vaccine,* 25 (3), pp. 561-569.

References

Apablaza, P., Løland, A., Brevik, Ø., Ilardi, P., Battaglia, J. and Nylund, A. (2013) Genetic variation among *Flavobacterium psychrophilum* isolates from wild and farmed salmonids in Norway and Chile. *Journal of Applied Microbiology*, 114, pp. 934-946.

Arai, H., Morita, Y., Izumi, S., Katagiri, T. and Kimura, H. (2007) Molecular typing by pulsed-field gel electrophoresis of *Flavobacterium psychrophilum* isolates derived from Japanese fish. *Journal of Fish Diseases*, 30 (6), pp. 345-355.

Avendaño-Herrera, R., Araya, P. and Fernández, J. (2009) Molecular analysis of *Flavobacterium psychrophilum* isolates from salmonid farms in Chile. *Bulletin of The European Association of Fish Pathologists*, 29 (6), pp. 184-192.

Avendaño-Herrera, R., Houel, A., Irgang, R., Bernardet, J., Godoy, M., Nicolas, P. and Duchaud, E. (2014) Introduction, expansion and coexistence of epidemic *Flavobacterium psychrophilum* lineages in Chilean fish farms. *Veterinary Microbiology*, 170 (3), pp. 298-306.

Barnes, M.E. and Brown, M.L. (2011) A review of *Flavobacterium psychrophilum* biology, clinical signs, and bacterial cold water disease prevention and treatment. *Open Fish Science Journal*, 4, pp. 40-48.

Bartie K.L., Austin F.W., Diab A., Dickson C., Dung T.T., Giacomini M. and Crumlish M. (2012) Intraspecific diversity of *Edwardsiella ictaluri* isolates from diseased freshwater catfish, *Pangasianodon hypophthalmus* (Sauvage), cultured in the Mekong Delta, Vietnam. *Journal of Fish Diseases* 35, pp. 671–682.

Bauer, A.U., Kirby W.M., Sherris J.C. and Track M (1966) Antibiotic susceptibility testing by a standardized single disc method. *Journal of Clinical Pathology* 45, pp. 493–494.

Beasley, T.M. and Schumacker, R.E. (1995) Multiple regression approach to analysing contingency tables: post-hoc and planned comparison procedures. *The Journal of Experimental Education*, 64 (1), pp. 79-93.

Bengten, E., Quiniou, S.M., Stuge, T.B., Katagiri, T., Miller, N.W., Clem, L.W., Warr, G.W. and Wilson, M. (2002) The IgH locus of the channel catfish, *Ictalurus punctatus*, contains multiple constant region gene sequences: different genes encode heavy chains of membrane and secreted IgD. *Journal of Immunology*, 169 (5), pp. 2488-2497.

Bernard, D., Six, A., Rigottier-Gois, L., Messiaen, S., Chilmonczyk, S., Quillet, E., Boudinot, P. and Benmansour, A. (2006) Phenotypic and functional similarity of gut intraepithelial and systemic T cells in a teleost fish. *Journal of Immunology (Baltimore, Md.: 1950)*, 176 (7), pp. 3942-3949.

Bernardet, J., Baudin-Laurencin, F. and Tixerant, G. (1988) First identification of *Cytophaga psychrophila* in France. *Bull Eur Assoc Fish Pathol,* 8, pp. 104-105.

Bernardet, J. and Kerouault, B. (1989) Phenotypic and genomic studies of "*Cytophaga psychrophila*" isolated from diseased rainbow trout (*Oncorhynchus mykiss*) in France. *Applied and Environmental Microbiology,* 55 (7), pp. 1796-1800.

Bernardet J-F., Segers P., Vancanneyt M., Berthe F., Kersters K. and Vandamme P. (1996) Cutting a Gordian Knot: Emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (Basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *International Journal of Systematic Bacteriology* 46, pp. 128-148.

Biering, E. and Salonius, K. (2014) DNA vaccines. *Fish Vaccination*, Wiley-Blackwell, pp. 47-55.

Biller-Takahashi, J. D. and Urbinati, E. C. (2014) Fish Immunology. The modification and manipulation of the innate immune system: Brazilian studies. *Anais da Academia Brasileira de Ciências* 86 (3), pp. 1484-1506.

Birnholm H.C. and Doly J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* 7, pp. 1513-1524.

Boehm, U., Klamp, T., Groot, M. and Howard, J. (1997) Cellular responses to interferon-γ. *Annual Review of Immunology,* 15 (1), pp. 749-795.

Boerlin, P. and White, D.G. (2006) Antimicrobial resistance and its epidemiology. *Antimicrobial Therapy in Veterinary Medicine.* Wiley-Blackwell, pp. 22-44.

Borg, A. F. (1948). Studies on myxobacteria associated with diseases in salmonid fishes. *Ph.D. Thesis*. University of Washington. Seattle.

Boshra, H., Li, J. and Sunyer, J. (2006) Recent advances on the complement system of teleost fish. *Fish & Shellfish Immunology,* 20 (2), pp. 239-262.

Boutin, S., Bernatchez, L., Audet, C. and Derôme, N. (2012) Antagonistic effect of indigenous skin bacteria of brook charr (*Salvelinus fontinalis*) against *Flavobacterium columnare* and *F. psychrophilum*. *Veterinary Microbiology*, 155 (2), pp. 355-361.

Boyacioğlu, M. and Akar, F. (2012) Isolation of *Flavobacterium psychrophilum* causing rainbow trout fry syndrome and determination of an effective antibacterial treatment in rainbow trout (*Oncorhynchus mykiss*) fry. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi,* 18 (2), pp. 197-203.

Boyacioğlu, M., Kum, C., Kirkan, S., Sekkin, S., Parin, U., Karademir, U. and Akar, F. (2015) Comparison of *in vitro* and *in vivo* antibacterial efficacy for the control of *Flavobacterium*

psychrophilum in rainbow trout (*Oncorhynchus mykiss*) fry: the first genotypical evidence in West Aegean region of Turkey. *Turkish Journal of Veterinary and Animal Sciences*, 39, pp. 314-321.

British Trout Association (2016) Trout farming. Online at http://britishtrout.co.uk/trout/farming-trout/ (assessed 21st August 2016).

Brocker, C., Thompson, D., Matsumoto, A., Nebert, D.W. and Vasiliou, V. (2010) Evolutionary divergence and functions of the human interleukin (IL) gene family. *Human Genomics*, 5 (1), pp. 1.

Brown, L., Cox, W. and Levine, R. (1997) Evidence that the causal agent of bacterial cold-water disease Flavobacterium psychrophilum is transmitted within salmonid eggs. *Diseases of Aquatic Organisms*, 29 (3), pp. 213-218.

Bruun, M.S., Schmidt, A.S., Madsen, L. and Dalsgaard, I. (2000) Antimicrobial resistance patterns in Danish isolates of *Flavobacterium psychrophilum*. *Aquaculture*, 187 (3), pp. 201-212.

Bruun, M.S., Schmidt, A.S., Dalsgaard, I. and Larsen, J.L. (2003a) Conjugal transfer of large plasmids conferring oxytetracycline (OTC) resistance: transfer between environmental aeromonads, fish-pathogenic bacteria, and *Escherichia coli*. *Journal of Aquatic Animal Health*, 15 (1), pp. 69-79.

Bruun, M.S., Madsen, L. and Dalsgaard, I. (2003b) Efficiency of oxytetracycline treatment in rainbow trout experimentally infected with *Flavobacterium psychrophilum* strains having different *in vitro* antibiotic susceptibilities. *Aquaculture*, 215 (1), pp. 11-20.

Brynildsrud O., Feil E.J., Bohlin J., Castillo-Ramirez S., Colquhoun D., McCarthy U., Matejusova I.M., Rhodes L.D., Wiens G.D. and Verner-Jeffreys D.W. (2013) Microevolution of *Renibacterium salmoninarum*: evidence for intercontinental dissemination associated with fish movements. *International Society for Microbial Ecology*, 1-11.

Buller, N.B. (2004) Aquatic animal species and organism relationship. *Bacteria from fish and other aquatic animals: a practical identification manual.* Wallingford: CABI Publishing, pp. 38-78.

Burbank, D., LaPatra, S., Fornshell, G. and Cain, K. (2012) Isolation of bacterial probiotic candidates from the gastrointestinal tract of rainbow trout, *Oncorhynchus mykiss* (Walbaum), and screening for inhibitory activity against *Flavobacterium psychrophilum*. *Journal of Fish Diseases*, 35 (11), pp. 809-816.

Bustos, P., Calbuyahue, J., Montaña, J., Opazo, B., Entrala, P. and Solervicens, R. (1995) First isolation of *Flexibacter psychrophilus*, as causative agent of rainbow trout fry syndrome (RTFS),

producing rainbow trout mortality in Chile. *Bulletin of the European Association of Fish Pathologists*, 15 (5), pp. 162-164.

Castillo, D., Higuera, G., Villa, M., Middelboe, M., Dalsgaard, I., Madsen, L. and Espejo, R. (2012) Diversity of *Flavobacterium psychrophilum* and the potential use of its phages for protection against bacterial cold water disease in salmonids. *Journal of Fish Diseases*, 35 (3), pp. 193-201.

Castro, R., Martínez-Alonso, S., Fischer, U., de Haro, N.Á., Soto-Lampe, V., Wang, T., Secombes, C.J., Lorenzen, N., Lorenzen, E. and Tafalla, C. (2014a) DNA vaccination against a fish rhabdovirus promotes an early chemokine-related recruitment of B cells to the muscle. *Vaccine*, 32 (10), pp. 1160-1168.

Castro, R., Abós, B., Pignatelli, J., von Gersdorff Jorgensen, L., Gonzalez Granja, A., Buchmann, K. and Tafalla, C. (2014b) Early immune responses in rainbow trout liver upon viral hemorrhagic septicemia virus (VHSV) infection. *PloS One*, 9 (10), pp. e111084.

Castro, R., Bromage, E., Abós, B., Pignatelli, J., Gonzalez Granja, A., Luque, A. and Tafalla, C. (2014c) CCR7 is mainly expressed in teleost gills, where it defines an $IgD^{+}IgM^{-}$ B lymphocyte subset. *Journal of Immunology (Baltimore, Md.: 1950)*, 192 (3), pp. 1257-1266.

Castro, R. and Tafalla, C. (2015) Overview of fish immunity. *Mucosal Health in Aquaculture*, Academic Press USA, pp. 3-55.

Cepeda, C., García-Márquez, S. and Santos, Y. (2004) Improved growth of *Flavobacterium psychrophilum* using a new culture medium. *Aquaculture*, 238 (1), pp. 75-82.

Chakroun, C., Grimont, F., Urdaci, M.C. and Bernardet, J. (1998) Fingerprinting of *Flavobacterium psychrophilum* isolates by ribotyping and plasmid profiling. *Diseases of Aquatic Organisms*, 33 (3), pp. 167-177.

Chakroun, C., Urdaci, M.C., Faure, D., Grimont, F. and Bernardet, J. (1997) Random amplified polymorphic DNA analysis provides rapid differentiation among isolates of the fish pathogen *Flavobacterium psychrophilum* and among *Flavobacterium* species. *Diseases of Aquatic Organisms*, 31 (3), pp. 187-196.

Chen, Y., Davis, M., LaPatra, S., Cain, K., Snekvik, K. and Call, D. (2008) Genetic diversity of *Flavobacterium psychrophilum* recovered from commercially raised rainbow trout, *Oncorhynchus mykiss* (Walbaum), and spawning coho salmon, *O. kisutch* (Walbaum). *Journal of Fish Diseases*, 31 (10), pp. 765-773.

Chettri, J.K., Raida, M.K., Kania, P.W. and Buchmann, K. (2012) Differential immune response of rainbow trout (*Oncorhynchus mykiss*) at early developmental stages (larvae and fry) against

the bacterial pathogen *Yersinia ruckeri*. *Developmental & Comparative Immunology,* 36 (2), pp. 463-474.

Cipriano, R.C. and Holt, R.A. (2005) *Flavobacterium psychrophilum,* cause of bacterial cold-water disease and rainbow trout fry syndrome. *Fish Disease Leaflet No. 86*. United States Department of the Interior, U.S. Geological Survey, National Fish Health Research Laboratory, Kearneysville, WV.

CLSI (2006) *Methods for Antimicrobial Disk Susceptibility Testing of Bacteria Isolated from Aquatic Animals,* Approved Guideline M42-A. Clinical and Laboratory Standards Institute, Wayne, PA.

CLSI (2014a) *Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated from Aquatic Animals, Approved Guideline VET04-A2*. Clinical and Laboratory Standards Institute, Wayne, PA.

CLSI (2014b) *Performance Standards for Antimicrobial Susceptibility Testing of Bacteria Isolated from Aquatic Animals; Second Informational Supplement*. CLSI document VET03/04-S2. Clinical and Laboratory Standards Institute, Wayne, PA.

Colucci-Guyon, E., Tinevez, J.Y., Renshaw, S.A. and Herbomel, P. (2011) Strategies of professional phagocytes in vivo: unlike macrophages, neutrophils engulf only surface-associated microbes. *Journal of Cell Science,* 124 (Pt 18), pp. 3053-3059.

Cortés, R., Teles, M., Tridico, R., Acerete, L. and Tort, L. (2013) Effects of Cortisol Administered through Slow-Release Implants on Innate Immune Responses in Rainbow Trout (*Oncorhynchus mykiss*). *International Journal of Genomics,* 2013, pp. 619714.

Costa, M.M., Maehr, T., Diaz-Rosales, P., Secombes, C.J. and Wang, T. (2011) Bioactivity studies of rainbow trout (*Oncorhynchus mykiss*) interleukin-6: effects on macrophage growth and antimicrobial peptide gene expression. *Molecular Immunology,* 48 (15), pp. 1903-1916.

Crump, E.M., Burian, J., Allen, P.D. and Kay, W.W. (2005) Identification and expression of a host-recognized antigen, FspA, from *Flavobacterium psychrophilum*. *Microbiology,* 151 (9), pp. 3127-3135.

Crump, E.M., Perry, M.B., Clouthier, S.C. and Kay, W.W. (2001) Antigenic characterization of the fish pathogen *Flavobacterium psychrophilum*. *Applied and Environmental Microbiology,* 67 (2), pp. 750-759.

Cunha, B.A. (1992) Aminopenicillins in urology. *Urology,* 40 (2), pp. 186-190.

Dalsgaard, I. (1993) Virulence mechanisms in *Cytophaga psychrophila* and other *Cytophaga*-like bacteria pathogenic for fish. *Annual Review of Fish Diseases,* 3, pp. 127-144.

Dalsgaard, I. and Madsen, L. (2000) Bacterial pathogens in rainbow trout, *Oncorhynchus mykiss* (Walbaum), reared at Danish freshwater farms. *Journal of Fish Diseases,* 23 (3), pp. 199-209.

Danilova, N., Bussmann, J., Jekosch, K. and Steiner, L.A. (2005) The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. *Nature Immunology,* 6 (3), pp. 295-302.

Daskalov, H., Austin, D. and Austin, B. (1999) An improved growth medium for *Flavobacterium psychrophilum*. *Letters in Applied Microbiology,* 28 (4), pp. 297-299.

Daskalov, H., Robertson, P. and Austin, B. (2000) Influence of oxidized lipids in diets on the development of rainbow trout fry syndrome. *Journal of Fish Diseases,* 23 (1), pp. 7-14.

Davidson, G.A., Lin, S., Secombes, C.J. and Ellis, A.E. (1997) Detection of specific and 'constitutive'antibody secreting cells in the gills, head kidney and peripheral blood leucocytes of dab (*Limanda limanda*). *Veterinary Immunology and Immunopathology,* 58 (3), pp. 363-374.

Decostere, A., D'Haese, E., Lammens, M., Nelis, H. and Haesebrouck, F. (2001) In vivo study of phagocytosis, intracellular survival and multiplication of *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum), spleen phagocytes. *Journal of Fish Diseases,* 24 (8), pp. 481-487.

Del Cerro, A., Márquez I. & Prieto J.M. (2010) Genetic diversity and antimicrobial resistance of *Flavobacterium psychrophilum* isolated from cultured rainbow trout, *Onchorynchus mykiss* (Walbaum), in Spain. *Journal of Fish Diseases*, 33, pp. 285-291.

Dixon, B., Luque, A., Abós, B., Castro, R., Gonzalez-Torres, L. and Tafalla, C. (2013) Molecular characterization of three novel chemokine receptors in rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology,* 34 (2), pp. 641-651.

Dowling, P.M. (2006a) Aminoglycosides. *Antimicrobial Therapy in Veterinary Medicine.* Wiley-Blackwell, pp. 207-230.

Dowling, P.M. (2006b) Chloramphenicol, thiamphenicol and florfenicol. *Antimicrobial Therapy in Veterinary Medicine.* Wiley-Blackwell Publishing, pp. 241-248.

Duchaud, E., Boussaha, M., Loux, V., Bernardet, J., Michel, C., Kerouault, B., Mondot, S., Nicolas, P., Bossy, R. and Caron, C. (2007) Complete genome sequence of the fish pathogen *Flavobacterium psychrophilum*. *Nature Biotechnology,* 25 (7), pp. 763-769.

Durmaz, Y., Onuk, E.E. and Çiftci, A. (2012) Investigation of the presence and antibiotic susceptibilities of *Flavobacterium psychrophilum* in rainbow trout farms (*Oncorhynchus mykiss* Walbaum, 1792) in The Middle and Eastern Black Sea Regions of Turkey. *Ankara Üniversitesi Veteriner Fakültesi Dergisi* 59, pp. 141-146.

References

Dumetz, F., Duchaud, E., LaPatra, S.E., Le Marrec, C., Claverol, S., Urdaci, M. and Le Hénaff, M. (2006) A protective immune response is generated in rainbow trout by an OmpH-like surface antigen (P18) of *Flavobacterium psychrophilum*. *Applied and Environmental Microbiology*, 72 (7), pp. 4845-4852.

Dumetz, F., LaPatra, S., Duchaud, E., Claverol, S. and Le Henaff, M. (2007) The *Flavobacterium psychrophilum* OmpA, an outer membrane glycoprotein, induces a humoral response in rainbow trout. *Journal of Applied Microbiology*, 103 (5), pp. 1461-1470.

Dumetz, F., Duchaud, E., Claverol, S., Orieux, N., Papillon, S., Lapaillerie, D. and Le Hénaff, M. (2008) Analysis of the *Flavobacterium psychrophilum* outer-membrane subproteome and identification of new antigenic targets for vaccine by immunomics. *Microbiology*, 154 (6), pp. 1793-1801.

Ekman, E., Börjeson, H. and Johansson, N. (1999) *Flavobacterium psychrophilum* in Baltic salmon Salmo salar brood fish and their offspring. *Diseases of Aquatic Organisms*, 37 (3), pp. 159-163.

Ellis, A. (1999) Immunity to bacteria in fish. *Fish & Shellfish Immunology*, 9 (4), pp. 291-308.

Ellis, A. (2001) Innate host defense mechanisms of fish against viruses and bacteria. *Developmental & Comparative Immunology*, 25 (8), pp. 827-839.

Ellis, T., Gardiner, R., Gubbins, M., Reese, A. and Smith, D. (2015) Aquaculture statistics for the UK, with a focus on England and Wales 2012. *Centre for Environment Fisheries & Aquaculture Science (Cefas) Weymouth*.

Ellmeier, W., Sunshine, M.J., Losos, K. and Littman, D.R. (1998) Multiple developmental stage–specific enhancers regulate CD8 expression in developing thymocytes and in thymus-independent T cells. *Immunity*, 9 (4), pp. 485-496.

Evenhuis, J.P. and Cleveland, B.M. (2012) Modulation of rainbow trout (*Oncorhynchus mykiss*) intestinal immune gene expression following bacterial challenge. *Veterinary Immunology and Immunopathology*, 146 (1), pp. 8-17.

FAO (2016a) Fisheries and Aquaculture Department. Online at http://www.fao.org/figis/servlet/SQServlet?file=/work/FIGIS/prod/webapps/figis/temp/hqp_1799287356044186708.xml&outtype=html (accesed 21st August 2016).

FAO (2016b) Cultured Aquatic Species Information Programme *Oncorhynchus mykiss* (Walbaum, 1792) at http://www.fao.org/fishery/culturedspecies/Oncorhynchus_mykiss/en (accessed 21st August 2016).

Faruk M.A.R. (2000) Characterisation of *Flavobacterium psychrophilum*, the causative agent of rainbow trout fry syndrome. Ph.D. Thesis. University of Stirling, Scotland.

Faruk, M. (2002) A review on rainbow trout fry syndrome (RTFS). *Pakistan Journal of Biological Sciences*, 5 (2), pp. 230-233.

Faruk, M.A., Campbell, R., Thompson, K., Rangdale, R. and Richards, R. (2002) Characterisation of *Flavobacterium psychrophilum*, the causative agent of rainbow trout fry syndrome (RTFS), using rabbit serum. *Bulletin of the European Association of Fish Pathologists*, 22 (6), pp. 354-365.

Flajnik, M.F. (2010) All GOD's creatures got dedicated mucosal immunity. *Nature Immunology*, 11 (9), pp. 777-779.

Fredriksen, B.N., Furevik, A., Gauthier, D., Egenberg, M., Paulsen, E.D. and Brudeseth, B. (2013a) Intramuscular challenge of rainbow trout (*Oncorhynchus mykiss*) with two Norwegian field strains of *Flavobacterium psychrophilum*. *Fish & Shellfish Immunology*, 35 (2), pp. 595-598.

Fredriksen, B.N., Olsen, R.H., Furevik, A., Souhoka, R.A., Gauthier, D. and Brudeseth, B. (2013b) Efficacy of a divalent and a multivalent water-in-oil formulated vaccine against a highly virulent strain of *Flavobacterium psychrophilum* after intramuscular challenge of rainbow trout (*Oncorhynchus mykiss*). *Vaccine*, 31 (15), pp. 1994-1998.

Fujiwara-Nagata, E., Ikeda, J., Sugahara, K. and Eguchi, M. (2012) A novel genotyping technique for distinguishing between *Flavobacterium psychrophilum* isolates virulent and avirulent to ayu, *Plecoglossus altivelis altivelis* (Temminck & Schlegel). *Journal of Fish Diseases*, 35 (7), pp. 471-480.

Gao, P, Mao, D., Luo, Y., Wang, L., Xu, B. and Xu, L. (2012) Occurrence of sulphonamide and tetracycline-resistant bacteria and resistance genes in aquaculture environment. *Water Research* 46, pp. 2355-2364.

Garcia, C., Pozet, F. and Michel, C. (2000) Standardization of experimental infection with *Flavobacterium psychrophilum*, the agent of rainbow trout *Oncorhynchus mykiss* fry syndrome. *Diseases of Aquatic Organisms*, 42 (3), pp. 191-197.

García-Pérez, M.A. and Núñez-Antón, V. (2003) Cellwise residual analysis in two-way contingency tables. *Educational and Psychological Measurement*, 63 (5), pp. 825-839.

Giguère, S. (2006a) Antimicrobial drug action and interaction: an introduction. *Antimicrobial Therapy in Veterinary Medicine.* Wiley-Blackwell, pp. 3-10.

Giguère, S. (2006b) Macrolides, azalides, and ketolides. *Antimicrobial Therapy in Veterinary Medicine.* Wiley-Blackwell, pp. 191-206.

Giguère, S. (2006c) Tetracyclines and Glycylcyclines. *Antimicrobial Therapy in Veterinary Medicine.* Wiley-Blackwell, pp. 231-240.

Gliniewicz, K., Plant, K., LaPatra, S., LaFrentz, B., Cain, K., Snekvik, K. and Call, D. (2012) Comparative proteomic analysis of virulent and rifampicin-attenuated *Flavobacterium psychrophilum*. *Journal of Fish Diseases*, 35 (7), pp. 529-539.

Gómez, E., Mendez, J., Cascales, D. and Guijarro, J.A. (2014) *Flavobacterium psychrophilum* vaccine development: a difficult task. *Microbial Biotechnology*, 7 (5), pp. 414-423.

Grove, S., Johansen, R., Reitan, L.J. and Press, C.M. (2006) Immune-and enzyme histochemical characterisation of leukocyte populations within lymphoid and mucosal tissues of Atlantic halibut (*Hippoglossus hippoglossus*). *Fish & Shellfish Immunology*, 20 (5), pp. 693-708.

Guardabassi, L. and Courvalin, P. (2006) Modes of antimicrobial action and mechanisms of bacteria resistance. In: Aarestrup, F.M. (Ed.), Antimicrobial resistance in bacteria of animal origin. American Society for Microbiology Press, Washington, D.C., pp. 1–18.

Guardiola, F.A., Cuesta, A., Arizcun, M., Meseguer, J. and Esteban, M.A. (2014) Comparative skin mucus and serum humoral defence mechanisms in the teleost gilthead seabream (*Sparus aurata*). *Fish & Shellfish Immunology*, 36 (2), pp. 545-551.

Gudding, R. (2014) Vaccination as a preventive measure. *Fish Vaccination*, Wiley-Blackwell, pp. 12-21.

Gultepe, N. and Tanrikul, T.T. (2006) Treatment methods of *Flavobacterium psychrophilum*: cause of rainbow trout fry syndrome (RFTS) and bacterial coldwater disease (BCWD) in Turkey. *Journal of Fisheries International*, 1 (2-4), pp. 102-105.

Hadidi, S., Glenney, G.W., Welch, T.J., Silverstein, J.T. and Wiens, G.D. (2008) Spleen size predicts resistance of rainbow trout to *Flavobacterium psychrophilum* challenge. *The Journal of Immunology*, 180 (6), pp. 4156-4165.

Hansen, J.D., Landis, E.D. and Phillips, R.B. (2005) Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. *Proceedings of the National Academy of Sciences of the United States of America*, 102 (19), pp. 6919-6924.

Hatten, F., Fredriksen, Å., Hordvik, I. and Endresen, C. (2001) Presence of IgM in cutaneous mucus, but not in gut mucus of Atlantic salmon, *Salmo salar*. Serum IgM is rapidly degraded when added to gut mucus. *Fish & Shellfish Immunology*, 11 (3), pp. 257-268.

Hedges, J.C., Singer, C.A. and Gerthoffer, W.T. (2000) Mitogen-activated protein kinases regulate cytokine gene expression in human airway myocytes. *American Journal of Respiratory Cell and Molecular Biology,* 23 (1), pp. 86-94.

Hedrick, R., McDowell, T., Marty, G., Mukkatira, K., Antonio, D., Andree, K., Bukhari, Z. and Clancy, T. (2000) Ultraviolet irradiation inactivates the waterborne infective stages of *Myxobolus cerebralis*: a treatment for hatchery water supplies. *Diseases of Aquatic Organisms,* 42 (1), pp. 53-59.

Henriksen, M. M. M. (2013). *Flavobacterium psychrophilum* experimental challenge and immune response. *Ph.D. Thesis*. Technical University of Denmark, Copenhagen.

Henriksen, M.M.M., Kania, P.W., Buchmann, K. and Dalsgaard, I. (2015a) Evaluation of the immune response in rainbow trout fry, *Oncorhynchus mykiss* (Walbaum), after waterborne exposure to *Flavobacterium psychrophilum* and/or hydrogen peroxide. *Journal of Fish Diseases,* 38 (1), pp. 55-66.

Henriksen, M.M.M., Kania, P.W., Buchmann, K. and Dalsgaard, I. (2015b) Effect of hydrogen peroxide and/or *Flavobacterium psychrophilum* on the gills of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases,* 38 (3), pp. 259-270.

Henríquez-Núñez, H., Evrard, O., Kronvall, G. and Avendaño-Herrera, R. (2012) Antimicrobial susceptibility and plasmid profiles of *Flavobacterium psychrophilum* strains isolated in Chile. *Aquaculture,* 354-355, pp. 38-44.

Henry, K.M., Loynes, C.A., Whyte, M.K. and Renshaw, S.A. (2013) Zebrafish as a model for the study of neutrophil biology. *Journal of Leukocyte Biology,* 94 (4), pp. 633-642.

Henryon, M., Berg, P., Olesen, N.J., Kjær, T.E., Slierendrecht, W.J., Jokumsen, A. and Lund, I. (2005) Selective breeding provides an approach to increase resistance of rainbow trout (*Onchorhynchus mykiss*) to the diseases, enteric redmouth disease, rainbow trout fry syndrome, and viral haemorrhagic septicaemia. *Aquaculture,* 250 (3), pp. 621-636.

Hesami, S., Allen, K.J., Metcalf, D., Ostland, V.E., MacInnes, J.I. and Lumsden, J.S. (2008) Phenotypic and genotypic analysis of *Flavobacterium psychrophilum* isolates from Ontario salmonids with bacterial coldwater disease. *Canadian Journal of Microbiology,* 54 (8), pp. 619-629.

Hesami, S., Parkman, J., MacInnes, J.I., Gray, J.T., Gyles, C.L. and Lumsden, J.S. (2010) Antimicrobial susceptibility of *Flavobacterium psychrophilum* isolates from Ontario. *Journal of Aquatic Animal Health,* 22 (1), pp. 39-49.

References

Högfors, E., Pullinen, K., Madetoja, J. and Wiklund, T. (2008) Immunization of rainbow trout, *Oncorhynchus mykiss* (Walbaum), with a low molecular mass fraction isolated from *Flavobacterium psychrophilum*. *Journal of Fish Diseases,* 31 (12), pp. 899-911.

Högfors-Rönnholm, E. and Wiklund, T. (2010) Hemolytic activity in *Flavobacterium psychrophilum* is a contact-dependent, two-step mechanism and differently expressed in smooth and rough phenotypes. *Microbial Pathogenesis,* 49 (6), pp. 369-375.

Holt, R. A. (1987). *Cytophaga psychrophila*, the causative agent of bacterial cold-water disease in salmonid fish. *Ph.D. Thesis*. Oregon State University.

Holt, R. A. (1993) Bacterial cold-water disease. *Bacterial Disease of Fish,* Wiley-Blackwell, Oxford, pp. 3-22.

Hu, Y., Xiang, L. and Shao, J. (2010) Identification and characterization of a novel immunoglobulin Z isotype in zebrafish: implications for a distinct B cell receptor in lower vertebrates. *Molecular Immunology,* 47 (4), pp. 738-746.

Hummelshoj, L., Ryder, L. and Poulsen, L.K. (2006) The role of the interleukin-10 subfamily members in immunoglobulin production by human B cells. *Scandinavian Journal of Immunology,* 64 (1), pp. 40-47.

Hunter P.R. and Gaston M.A. (1988) Numerical Index of the Discriminatory ability of typing systems: an application of Simpson's index of diversity. *Journal of Clinical Microbiology* 26, pp. 2465-2466.

Inoue, K., Takano, H., Shimada, A. and Satoh, M. (2009) Metallothionein as an anti-inflammatory mediator. *Mediators of Inflammation*, 2009, pp. 101659.

Izumi, S. and Wakabayashi, H. (1999) Further study on serotyping of *Flavobacterium psychrophilum*. *Fish Pathology*, 34, pp. 89-90.

Izumi, S. and Aranishi, F. (2004) Relationship between *gyrA* mutations and quinolone resistance in *Flavobacterium psychrophilum* isolates. *Applied and Environmental Microbiology*, 70 (7), pp. 3968-3972.

Izumi, S., Liu, H., Aranishi, F. and Wakabayashi, H. (2003a) A novel serotype of *Flavobacterium psychrophilum* detected using antiserum against an isolate from amago, *Oncorhynchus masou rhodurus* Jordan & Gilbert, in Japan. *Journal of Fish Diseases,* 26 (11-12), pp. 677-680.

Izumi, S., Aranishi, F. and Wakabayashi, H. (2003b) Genotyping of *Flavobacterium psychrophilum* using PCR-RFLP analysis. *Diseases of Aquatic Organisms,* 56 (3), pp. 207.

Kaplan, E.L., Meier P. (1958) Nonparametric-estimation from incomplete observations. *Journal of the American Statistical Association,* 53, pp. 457- 81.

Kato, G., Suzuki, K., Sakai, T., Kawakami, M., Takano, T., Matsuyama, T. and Nakayasu, C. (2015) The role of a specific antibody against *Flavobacterium psychrophilum* infection in ayu sweetfish, *Plecoglossus altivelis altivelis* (Temminck & Schlegel, 1846). *Journal of Fish Diseases,* 38 (1), pp. 107-112.

Kato, G., Sakai, T., Suzuki, K., Yamaguchi, K., Takano, T., Matsuyama, T. and Nakayasu, C. (2014) Antigenic proteins of *Flavobacterium psychrophilum* recognized by ayu *Plecoglossus altivelis antisera*. *Diseases of Aquatic Organisms,* 108 (2), pp. 103-112.

Kent, M. (1989) Spiral swimming behavior due to cranial and vertebral lesions associated with *Cytophaga psychrophila* infections in salmonid fishes. *Diseases of Aquatic Organisms,* 6, pp. 11-16.

Kim, J.H., Gomez, D.K., Nakai, T. and Park, S.C. (2010a) Isolation and identification of bacteriophages infecting ayu *Plecoglossus altivelis altivelis* specific *Flavobacterium psychrophilum*. *Veterinary Microbiology,* 140 (1), pp. 109-115.

Kim, J.H., Gomez, D.K., Nakai, T. and Park, S.C. (2010b) Plasmid profiling of *Flavobacterium psychrophilum* isolates from ayu (*Plecoglossus altivelis altivelis*) and other fish species in Japan. *Journal of Veterinary Science,* 11 (1), pp. 85-87.

Komatsu, K., Tsutsui, S., Hino, K., Araki, K., Yoshiura, Y., Yamamoto, A., Nakamura, O. and Watanabe, T. (2009) Expression profiles of cytokines released in intestinal epithelial cells of the rainbow trout, *Oncorhynchus mykiss*, in response to bacterial infection. *Developmental & Comparative Immunology,* 33 (4), pp. 499-506.

Kondo, M., Kawai, K., Okabe, M., Nakano, N. and Oshima, S. (2003) Efficacy of oral vaccine against bacterial coldwater disease in ayu *Plecoglossus altivelis*. *Diseases of Aquatic Organisms,* 55 (3), pp. 261-264.

Kono, T., Korenaga, H. and Sakai, M. (2011) Genomics of fish IL-17 ligand and receptors: a review. *Fish & Shellfish Immunology,* 31 (5), pp. 635-643.

Korkea-aho, T., Heikkinen, J., Thompson, K., von Wright, A. and Austin, B. (2011) *Pseudomonas* sp. M174 inhibits the fish pathogen *Flavobacterium psychrophilum*. *Journal of Applied Microbiology,* 111 (2), pp. 266-277.

Kronvall, G. (2003) Determination of the real standard distribution of susceptible strains in zone histograms. *International Journal of Antimicrobial Agents*, 22 (1), pp. 7-13.

Kronvall, G., Kahlmeter, G., Myhre, E. and Galas, M.F. (2003) A new method for normalized interpretation of antimicrobial resistance from disk test results for comparative purposes. *Clinical Microbiology and Infection*, 9, pp. 120-132.

References

Kronvall, G. (2010) Normalized resistance interpretation as a tool for establishing epidemiological MIC susceptibility breakpoints. *Journal of Clinical Microbiology,* 48 (12), pp. 4445-4452.

Kum, C., Kirkan, S., Sekkin, S., Akar, F. and Boyacioğlu, M. (2008) Comparison of *in vitro* antimicrobial susceptibility in *Flavobacterium psychrophilum* isolated from rainbow trout fry. *Journal of Aquatic Animal Health,* 20 (4), pp. 245-251.

Kumagai, A., Takahashi, K., Yamaoka, S. and Wakabayashi, H. (1998) Ineffectiveness of iodophore treatment in disinfecting salmonid eggs carrying *Cytophaga psychrophila. Fish Pathology*, 33 (3), pp. 123-128.

Kumagai, A., Nakayasu, C. and Oseko, N. (2004) Effect of tobramycin supplementation to medium on isolation of *Flavobacterium psychrophilum* from ayu *Plecoglossus altivelis*. *Fish Pathology*, 39 (2), pp. 75-78.

LaFrentz, B., LaPatra, S., Jones, G., Congleton, J., Sun, B. and Cain, K. (2002) Characterization of serum and mucosal antibody responses and relative per cent survival in rainbow trout, *Oncorhynchus mykiss* (Walbaum), following immunization and challenge with *Flavobacterium psychrophilum*. *Journal of Fish Diseases,* 25 (12), pp. 703-713.

LaFrentz, B., LaPatra, S., Jones, G. and Cain, K. (2003) Passive immunization of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Flavobacterium psychrophilum*, the causative agent of bacterial coldwater disease and rainbow trout fry syndrome. *Journal of Fish Diseases,* 26 (7), pp. 377-384.

LaFrentz, B.R., LaPatra, S.E., Jones, G.R. and Cain, K.D. (2004) Protective immunity in rainbow trout *Oncorhynchus mykiss* following immunization with distinct molecular mass fractions isolated from *Flavobacterium psychrophilum*. *Diseases of Aquatic Organisms,* 59 (1), pp. 17.

LaFrentz, B.R., Lindstrom, N.M., LaPatra, S.E., Call, D.R. and Cain, K.D. (2007) Electrophoretic and Western blot analyses of the lipopolysaccharide and glycocalyx of *Flavobacterium psychrophilum*. *Fish & Shellfish Immunology,* 23 (4), pp. 770-780.

LaFrentz, B.R., LaPatra, S.E., Call, D.R. and Cain, K.D. (2008) Isolation of rifampicin resistant *Flavobacterium psychrophilum* strains and their potential as live attenuated vaccine candidates. *Vaccine,* 26 (44), pp. 5582-5589.

LaFrentz, B., LaPatra, S., Call, D., Wiens, G. and Cain, K. (2011) Identification of immunogenic proteins within distinct molecular mass fractions of *Flavobacterium psychrophilum*. *Journal of Fish Diseases,* 34 (11), pp. 823-830.

LaFrentz, B.R., LaPatra, S.E., Call, D.R. and Cain, K.D. (2014) Immunization of rainbow trout *Oncorhynchus mykiss* (Walbaum) with a crude lipopolysaccharide extract from *Flavobacterium psychrophilum*. *Aquaculture Research,* 45 (3), pp. 476-483.

Laing, K.J. and Secombes, C.J. (2004) Chemokines. *Developmental & Comparative Immunology,* 28 (5), pp. 443-460.

Lammens, M., Decostere, A. and Haesebrouck, F. (2000) Effect of *Flavobacterium psychrophilum* strains and their metabolites on the oxidative activity of rainbow trout *Oncorhynchus mykiss* phagocytes. *Diseases of Aquatic Organisms,* 41 (3), pp. 173-179.

Langevin, C., Blanco, M., Martin, S.A., Jouneau, L., Bernardet, J., Houel, A., Lunazzi, A., Duchaud, E., Michel, C. and Quillet, E. (2012) Transcriptional responses of resistant and susceptible fish clones to the bacterial pathogen *Flavobacterium psychrophilum*. *PloS One,* 7 (6), pp. e39126.

Lehmann, J., Mock, D., Stürenberg, F. and Bernardet, J. (1991) First isolation of *Cytophaga psychrophila* from a systemic disease in eel and cyprinids. *Diseases of Aquatic Organisms,* 10 (3), pp. 217-220.

Lewis, K. (2013) Platforms for antibiotic discovery. *Nature Reviews Drug Discovery*, **12**, pp. 371-387.

Li, J., Barreda, D.R., Zhang, Y., Boshra, H., Gelman, A.E., LaPatra, S., Tort, L. and Sunyer, J.O. (2006) B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. *Nature Immunology,* 7 (10), pp. 1116-1124.

Lillehaug, A. (2014) Vaccination strategies and procedures. *Fish Vaccination*, Wiley-Blackwell, pp. 140-152.

Lim, Y-J., Kim, D-H., Roh, H.J., Park, M-A., Park, C-I. and Smith, P. (2016) Epidemiological cut-off values for disc diffusion data generated by standard test protocols from *Edwardsiella tarda* and *Vibrio harveyi*. *Aquaculture International*. DOI 10.1007/s10499-016-9977-0

Lin, S., Davidson, G., Secombes, C. and Ellis, A. (1998) A morphological study of cells isolated from the perfused gill of dab and Atlantic salmon. *Journal of Fish Biology,* 53 (3), pp. 560-568.

Liu, H., Izumi, S. and Wakabayashi, H. (2001) Detection of *Flavobacterium psychrophilum* in various organs of ayu *Plecoglossus altivelis* by in situ hybridization. *Fish Pathology,* 36 (1), pp. 7-12.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods,* 25 (4), pp. 402-408.

Long, A., Fehringer, T.R., Swain, M.A., LaFrentz, B.R., Call, D.R. and Cain, K.D. (2013) Enhanced efficacy of an attenuated *Flavobacterium psychrophilum* strain cultured under iron-limited conditions. *Fish & Shellfish Immunology,* 35 (5), pp. 1477-1482.

Lorenzen, E., Dalsgaard, I., From, J., Hansen, E., Horlyck, V., Korsholm, H., Mellergaard, S. and Olesen, N. (1991) Preliminary investigations of fry mortality syndrome in rainbow trout. *Bulletin of the European Association of Fish Pathologists,* 11 (2), pp. 77-79.

Lorenzen, E. (1993) The importance of the brand of the beef extract in relation to the growth of *Flexibacter psychrophilus* in Anacker and Ordals medium. *Bulletin of the European Association of Fish Pathologists (United Kingdom),* 13 (2), pp. 64-65.

Lorenzen E. and Olesen N. J. (1997) Characterization of isolates of *Flavobacterium psychrophilum* associated with coldwater disease or rainbow trout fry syndrome II: serological studies. *Diseases of Aquatic Organisms*, 31, pp. 209-220.

Lorenzen, E., Dalsgaard, I. and Bernardet, J. (1997) Characterization of isolates of *Flavobacterium psychrophilum* associated with coldwater disease or rainbow trout fry syndrome I: phenotypic and genomic studies. *Diseases of Aquatic Organisms,* 31, pp. 197-208.

Lorenzen, E., Brudeseth, B., Wiklund, T. and Lorenzen, N. (2010) Immersion exposure of rainbow trout (*Oncorhynchus mykiss*) fry to wildtype *Flavobacterium psychrophilum* induces no mortality, but protects against later intraperitoneal challenge. *Fish & Shellfish Immunology,* 28 (3), pp. 440-444.

Lovy, J., Wright, G.M. and Speare, D.J. (2008) Comparative cellular morphology suggesting the existence of resident dendritic cells within immune organs of salmonids. *The Anatomical Record,* 291 (4), pp. 456-462.

Lumsden, J., Ostland, V. and Ferguson, H. (1996) Necrotic myositis in cage cultured rainbow trout, *Oncorhynchus mykiss* (Walbaum), caused by *Flexibacter psychrophilus*. *Journal of Fish Diseases,* 19 (2), pp. 113-119.

Lutfalla, G., Crollius, H.R., Stange-thomann, N., Jaillon, O., Mogensen, K. and Monneron, D. (2003) Comparative genomic analysis reveals independent expansion of a lineage-specific gene family in vertebrates: the class II cytokine receptors and their ligands in mammals and fish. *BMC Genomics,* 4 (1), pp. 1.

Macrina F.L., Kopecko D.J., Jones K.R., Ayers D.J. and McCowen S.M. (1978). A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. *Plasmid* 1, pp. 417–420.

Madetoja, J., Nyman, P. and Wiklund, T. (2000) *Flavobacterium psychrophilum*, invasion into and shedding by rainbow trout *Oncorhynchus mykiss*. *Diseases of Aquatic Organisms,* 43 (1), pp. 27-38.

Madetoja, J., Hänninen, M., Hirvelä-Koski, V., Dalsgaard, I. and Wiklund, T. (2001) Phenotypic and genotypic characterization of *Flavobacterium psychrophilum* from Finnish fish farms. *Journal of Fish Diseases,* 24 (8), pp. 469-479.

Madetoja J., Dalsgaard I. and Wiklund T. (2002) Occurrence of *Flavobacterium psychrophilum* in fish-farming environments. *Diseases of Aquatic Organisms*, 52, pp. 109-118.

Madetoja, J., Nystedt, S. and Wiklund, T. (2003) Survival and virulence of *Flavobacterium psychrophilum* in water microcosms. *FEMS Microbiology Ecology,* 43 (2), pp. 217-223.

Madetoja, J., Lönnström, L., Björkblom, C., Uluköy, G., Bylund, G., Syvertsen, C., Gravningen, K., Norderhus, E. and Wiklund, T. (2006) Efficacy of injection vaccines against *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases,* 29 (1), pp. 9-20.

Madsen, L. and Dalsgaard, I. (1999) Reproducible methods for experimental infection with *Flavobacterium psychrophilum* in rainbow trout *Oncorhynchus mykiss*.*Diseases of Aquatic Organisms*, 36 (3), pp. 169-176.

Madsen, L. and Dalsgaard, I. (2000) Comparative studies of Danish *Flavobacterium psychrophilum* isolates: ribotypes, plasmid profiles, serotypes and virulence.*Journal of Fish Diseases,* 23 (3), pp. 211-218.

Magnadóttir, B. (2006) Innate immunity of fish (overview). *Fish & Shellfish Immunology,* 20 (2), pp. 137-151.

Makesh, M., Sudheesh, P.S. and Cain, K.D. (2015) Systemic and mucosal immune response of rainbow trout to immunization with an attenuated *Flavobacterium psychrophilum* vaccine strain by different routes. *Fish & Shellfish Immunology,* 44 (1), pp. 156-163.

Martínez, J.L., Casado, A. and Enríquez, R. (2004) Experimental infection of *Flavobacterium psychrophilum* in fins of Atlantic salmon *Salmo salar* revealed by scanning electron microscopy. *Diseases of Aquatic Organisms,* 59 (1), pp. 79-84.

Mata, M., Skarmeta, A. and Santos, Y. (2002) A proposed serotyping system for *Flavobacterium psychrophilum*. *Letters in Applied Microbiology,* 35 (2), pp. 166-170.

Merle, C., Faure, D., Urdaci, M. and Le Henaff, M. (2003) Purification and characterization of a membrane glycoprotein from the fish pathogen *Flavobacterium psychrophilum*. *Journal of Applied Microbiology,* 94 (6), pp. 1120-1127.

Meyers, T., Burton, T., Bentz, C. and Starkey, N. (2008) Bacterial coldwater disease. *Common diseases of wild and cultured fishes in Alaska*, Fish Pathology Laboratories, Alaska Department of Fish and Game.Anchorage, Alaska, pp. 14-16.

Michel, C., Antonio, D. and Hedrick, R.P. (1999) Production of viable cultures of *Flavobacterium psychrophilum*: approach and control. *Research in Microbiology,* 150 (5), pp. 351-358.

Michel, C., Kerouault, B. and Martin, C. (2003) Chloramphenicol and florfenicol susceptibility of fish-pathogenic bacteria isolated in France: comparison of minimum inhibitory concentration, using recommended provisory standards for fish bacteria. *Journal of Applied Microbiology* 95, pp. 1008-1015.

Minogue, E., Barry, T., Carroll, C. and Smith, P. (2012) Setting epidemiological cut-off values for *Aeromonas salmonicida* disc diffusion data capable of discriminating between strains on the basis of their possession of *sul1* genes. *Aquaculture,* 364, pp. 329-332.

Munang'andu, H.M., Mutoloki, S. and Evensen, Ø. (2014) Non-replicating vaccines. *Fish Vaccination*, Wiley-Blackwell, pp. 22-32.

Munro, L. and Wallace, I. (2015) *Scottish Shellfish Farm Production Survey 2014.* Marine Scotland Science, The Scottish Government, Edinburgh.

Murphy, K. (2012a) Induced innate responses to infection. *Janeway's immunobiology*. Eighth edition. Garland Science, New York pp. 99-121.

Murphy, K. (2012b) The first lines of defence. *Janeway's immunobiology*. Eighth edition. Garland Science, New York, pp. 38-47.

Murphy, K. (2012c) Immunological memory. *Janeway's immunobiology.* Eighth edition. Garland Science, New York, pp. 448-460.

Murphy, K. (2012d) Priming of naïve T cells by pathogen-activated dendritic cells. *Janeway's immunobiology*. Eighth edition. Garland Science, New York, pp. 353-365.

Nagai, T. and Nakai, T. (2011) Growth of *Flavobacterium psychrophilum* in fish serum correlates with pathogenicity. *Journal of Fish Diseases,* 34 (4), pp. 303-310.

Nakamura, O., Tazumi, Y., Muro, T., Yasuhara, Y. and Watanabe, T. (2004) Active uptake and transport of protein by the intestinal epithelial cells in embryo of viviparous fish, *Neoditrema ransonneti* (Perciformes: Embiotocidae). *Journal of Experimental Zoology Part A: Comparative Experimental Biology,* 301 (1), pp. 38-48.

Nematollahi, A., Decostere, A., Pasmans, F. and Haesebrouck, F. (2003a) *Flavobacterium psychrophilum* infections in salmonid fish. *Journal of Fish Diseases,* 26 (10), pp. 563-574.

Nematollahi, A., Decostere, A., Pasmans, F., Ducatelle, R. and Haesebrouck, F. (2003b) Adhesion of high and low virulence *Flavobacterium psychrophilum* strains to isolated gill arches of rainbow trout *Oncorhynchus mykiss*. *Diseases of Aquatic Organisms,* 55 (2), pp. 101-107.

Nematollahi, A., Pasmans, F., Haesebrouck, F. and Decostere, A. (2005) Early interactions of *Flavobacterium psychrophilum* with macrophages of rainbow trout *Oncorhynchus mykiss*. *Diseases of Aquatic Organisms,* 64 (1), pp. 23-28.

Nepal, A., Basnyat, S., Lamsal, G., Joshi, P. and Mulmi, R. (2002) Economics of rainbow trout farming system in Nepal. *Cold Water Fisheries in the Trans-Himalayan Countries,* 431, pp. 179.

Nicolas, P., Mondot, S., Achaz, G., Bouchenot, C., Bernardet, J. and Duchaud, E. (2008) Population structure of the fish-pathogenic bacterium *Flavobacterium psychrophilum*. *Applied and Environmental Microbiology,* 74 (12), pp. 3702-3709.

Niklasson, L., Sundh, H., Fridell, F., Taranger, G. and Sundell, K. (2011) Disturbance of the intestinal mucosal immune system of farmed Atlantic salmon (*Salmo salar*), in response to long-term hypoxic conditions. *Fish & Shellfish Immunology,* 31 (6), pp. 1072-1080.

Nikoskelainen, S., Verho, S., Järvinen, S., Madetoja, J., Wiklund, T. and Lilius, E. (2007) Multiple whole bacterial antigens in polyvalent vaccine may result in inhibition of specific responses in rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology,* 22 (3), pp. 206-217.

Nilsen, H., Olsen, A., Vaagnes, Ø., Hellberg, H., Bottolfsen, K., Skjelstad, H. and Colquhoun, D. (2011a) Systemic *Flavobacterium psychrophilum* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum), farmed in fresh and brackish water in Norway. *Journal of Fish Diseases,* 34 (5), pp. 403-408.

Nilsen, H., Johansen, R., Colquhoun, D.J., Kaada, I., Bottolfsen, K., Vågnes, Ø. and Olsen, A.B. (2011b) *Flavobacterium psychrophilum* associated with septicaemia and necrotic myositis in Atlantic salmon *Salmo salar*: a case report. *Diseases of Aquatic Organisms,* 97 (1), pp. 37-46.

Nilsen, H., Sundell, K., Duchaud, E., Nicolas, P., Dalsgaard, I., Madsen, L., Aspan, A., Jansson, E., Colquhoun, D.J. and Wiklund, T. (2014) Multilocus sequence typing identifies epidemic clones of *Flavobacterium psychrophilum* in Nordic countries. *Applied and Environmental Microbiology,* 80 (9), pp. 2728-2736.

Noga, E.J. (2010) Problem 38: Bacterial cold water disease. *Fish disease: diagnosis and treatment.* Second edition. Ames, IA: Wiley-Blackwell, pp. 169-170.

Obach, A. and Laurencin, F.B. (1991) Vaccination of rainbow trout *Oncorhynchus mykiss* against the visceral form of coldwater disease. *Diseases of Aquatic Organisms,* 12 (1), pp. 13-15.

References

Ohtani, M., Hayashi, N., Hashimoto, K., Nakanishi, T. and Dijkstra, J.M. (2008) Comprehensive clarification of two paralogous interleukin 4/13 loci in teleost fish. *Immunogenetics,* 60 (7), pp. 383-397.

Oplinger, R.W. and Wagner, E.J. (2012) Effects of media ingredient substitution and comparison of growth of *Flavobacterium psychrophilum* among four Media. *Journal of Aquatic Animal Health,* 24 (1), pp. 49-57.

Oplinger, R.W. and Wagner, E.J. (2013) Control of *Flavobacterium psychrophilum*: tests of erythromycin, streptomycin, osmotic and thermal shocks, and rapid pH change. *Journal of Aquatic Animal Health,* 25 (1), pp. 1-8.

Orieux, N., Douet, D., Le Hénaff, M. and Bourdineaud, J. (2013) Prevalence of *Flavobacterium psychrophilum* bacterial cells in farmed rainbow trout: characterization of metallothionein A and interleukin1-β genes as markers overexpressed in spleen and kidney of diseased fish. *Veterinary Microbiology,* 162, pp. 127-135.

Olsen, M.M., Kania, P.W., Heinecke, R.D., Skjoedt, K., Rasmussen, K.J. and Buchmann, K. (2011) Cellular and humoral factors involved in the response of rainbow trout gills to *Ichthyophthirius multifiliis* infections: molecular and immunohistochemical studies. *Fish & Shellfish Immunology,* 30 (3), pp. 859-869.

Ostland, V.E., Byrne, P.J., Hoover, G. and Ferguson, H.W. (2000) Necrotic myositis of rainbow trout, *Oncorhynchus mykiss* (Walbaum): proteolytic characteristics of a crude extracellular preparation of *Flavobacterium psychrophilum*. *Journal of Fish Diseases,* 23, pp. 329-336.

Overturf, K. and LaPatra, S. (2006) Quantitative expression (Walbaum) of immunological factors in rainbow trout, *Oncorhynchus mykiss* (Walbaum), after infection with either *Flavobacterium psychrophilum, Aeromonas salmonicida*, or infectious haematopoietic necrosis virus. *Journal of Fish Diseases,* 29 (4), pp. 215-224.

Pacha, R.E. (1968) Characteristics of *Cytophaga psychrophila* (Borg) isolated during outbreaks of bacterial cold-water disease. *Applied Microbiology,* 16 (1), pp. 97-101.

Pérez-Pascual, D., Menéndez, A., Fernández, L., Méndez, J., Reimundo, P., Navais, R. and Guijarro, J.A. (2009) Spreading versus biomass production by colonies of the fish pathogen *Flavobacterium psychrophilum*: role of the nutrient concentration. *International Microbiology,* 12 (4), pp. 207-214.

Peterson, T.S. (2015) Overview of mucosal structure and function in teleost fishes. *Mucosal Health in Aquaculture,* Academic Press USA, pp. 55-65.

Peto, R., Pike, M.C., Armitage, P., Breslow, N.E., Cox, D.R., Howard, S.V., Mantel, N., McPherson, K., Peto, J. and Smith, P.G. (1997) Design and analysis of randomized clinical trials requiring prolonged observation of each patient. II. Analysis and examples. *British Journal of Cancer*, 35(1), pp. 1-39.

Pietretti, D. and Wiegertjes, G.F. (2014) Ligand specificities of Toll-like receptors in fish: indications from infection studies. *Developmental & Comparative Immunology,* 43 (2), pp. 205-222.

Plant, K., LaPatra, S. and Cain, K. (2009) Vaccination of rainbow trout, *Oncorhynchus mykiss* (Walbaum), with recombinant and DNA vaccines produced to *Flavobacterium psychrophilum* heat shock proteins 60 and 70. *Journal of Fish Diseases,* 32 (6), pp. 521-534.

Plant, K., LaPatra, S., Call, D. and Cain, K. (2011) Immunization of rainbow trout, *Oncorhynchus mykiss* (Walbaum), with *Flavobacterium psychrophilum* proteins elongation factor-Tu, SufB Fe-S assembly protein and ATP synthaseβ. *Journal of Fish Diseases,* 34 (3), pp. 247-250.

Pfaffl, M.W., Horgan, G.W. and Dempfle, L. (2002) Relative expression software tool (REST© for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research,* 30 (9), pp. e36.

Prescott, J.F. (2006a) Beta-lactam antibiotics: Penam penicillins. *Antimicrobial Therapy in Veterinary Medicine.* Wiley-Blackwell, pp. 121-138.

Prescott, J.F. (2006b) Sulfonamides, diaminopyrimidines, and their combinations. *Antimicrobial Therapy in Veterinary Medicine*. Wiley-Blackwell, pp. 249-262.

Prescott J. F. (2007) Sulfonamides, diaminopyrimidines, and their combinations. In: Antimicrobial Therapy in Veterinary Medicine 4th edition, Wiley-Blackwell, pp. 249-262.

Rach, J.J., Schreier, T.M., Howe, G.E. and Redman, S.D. (1997) Effect of species, life stage, and water temperature on the toxicity of hydrogen peroxide to fish. *The Progressive Fish-Culturist* 59, pp. 41-46.

Rahman, M., Ototake, M., Iida, Y., Yokomizo, Y. and Nakanishi, T. (2000) Efficacy of oil-adjuvanted vaccine for coldwater disease in ayu *Plecoglossus altivelis*. *Fish Pathology,* 35 (4), pp. 199-203.

Rahman, M.H., Kuroda, A., Dijkstra, J.M., Kiryu, I., Nakanishi, T. and Ototake, M. (2002) The outer membrane fraction of *Flavobacterium psychrophilum* induces protective immunity in rainbow trout and ayu. *Fish & Shellfish Immunology,* 12 (2), pp. 169-179.

Rahman, M. H., Ototake, M. and Nakanishi, T. (2003) Water-soluble adjuvants enhance the protective effect of *Flavobacterium psychrophilum* vaccines in ayu *Plecoglossus altivelis*. *Fish Pathology*, 38 (4), pp. 171-176.

Raida, M.K. and Buchmann, K. (2008) Development of adaptive immunity in rainbow trout, *Oncorhynchus mykiss* (Walbaum) surviving an infection with *Yersinia ruckeri*. *Fish & Shellfish Immunology*, 25 (5), pp. 533-541.

Ramsrud, A., LaFrentz, S., LaFrentz, B., Cain, K. and Call, D. (2007) Differentiating 16S rRNA alleles of *Flavobacterium psychrophilum* using a simple PCR assay. *Journal of Fish Diseases*, 30 (3), pp. 175-180.

Randelli, E., Buonocore, F. and Scapigliati, G. (2008) Cell markers and determinants in fish immunology. *Fish & Shellfish Immunology*, 25 (4), pp. 326-340.

Rangdale, R., Richards, R. and Alderman, D. (1997) Minimum inhibitory concentrations of selected antimicrobial compounds against *Flavobacterium psychrophilum* the causal agent of rainbow trout fry syndrome (RTFS). *Aquaculture*, 158 (3), pp. 193-201.

Rangdale, R. E. (1995) Studies on rainbow trout fry syndrome (RTFS). *Ph.D. Thesis*. Stirling University, Stirling, Scotland.

Rangdale R.E., Richards R.H. and Alderman D.J. (1997) Minimum inhibitory concentrations of selected antimicrobial compounds against *Flavobacterium psychrophilum* the causal agent of rainbow trout fry syndrome (RTFS). *Aquaculture* 158, pp. 193-201.

Rangdale, R., Richards, R. and Alderman, D. (1999) Histopathological and electron microscopical. *The Veterinary Record*, 144, pp. 251-254.

Rebl, A., Goldammer, T. and Seyfert, H. (2010) Toll-like receptor signaling in bony fish. *Veterinary Immunology and Immunopathology*, 134 (3), pp. 139-150.

Reite, O.B. and Evensen, Ø. (2006) Inflammatory cells of teleostean fish: a review focusing on mast cells/eosinophilic granule cells and rodlet cells. *Fish & Shellfish Immunology*, 20 (2), pp. 192-208.

Roberts, R. (2012) The bacteriology of teleosts. *Fish Pathology*. Fourth edition, Wiley-Blackwell, pp. 339-382.

Roca, F.J., Mulero, I., Lopez-Munoz, A., Sepulcre, M.P., Renshaw, S.A., Meseguer, J. and Mulero, V. (2008) Evolution of the inflammatory response in vertebrates: fish TNF-alpha is a powerful activator of endothelial cells but hardly activates phagocytes. *Journal of Immunology (Baltimore, Md.: 1950)*, 181 (7), pp. 5071-5081.

Rodgers, C. and Furones, M. (2009) Antimicrobial agents in aquaculture: practice, needs and issues. *Options Méditerranéennes,* 86, pp. 41-59.

Rombout, J., Bot, H. and Taverne-Thiele, J. (1989) Immunological importance of the second gut segment of carp. *Journal of Fish Biology,* 35 (2), pp. 167-178.

Rombout, J.H., Abelli, L., Picchietti, S., Scapigliati, G. and Kiron, V. (2011) Teleost intestinal immunology. *Fish & Shellfish Immunology,* 31 (5), pp. 616-626.

Romero, J., Feijoó, C.G. and Navarrete, P. (2012) *Antibiotics in aquaculture-use, abuse and alternatives.* INTECH Open Access Publisher.

Sakaguchi, S., Wing, K., Onishi, Y., Prieto-Martin, P. and Yamaguchi, T. (2009) Regulatory T cells: how do they suppress immune responses? *International Immunology,* 21 (10), pp. 1105-1111.

Santos, Y., Huntly, P., Turnbull, A. and Hastings, T. (1992) Isolation of *Cytophaga psychrophila* (*Flexibacter psychrophilus*) in association with rainbow trout mortality in the United Kingdom. *Bulletin of the European Association of Fish Pathologists,* 12 (6), pp. 209-210.

Schmidt, A.S., Bruun, M.S., Dalsgaard, I., Pedersen, K. and Larsen, J.L. (2000) Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. *Applied and Environmental Microbiology,* 66 (11), pp. 4908-4915.

Schmidtke, L. and Carson, J. (1995) Characteristics of *Flexibacter psychrophilus* isolated from Atlantic salmon in Australia. *Diseases of Aquatic Organisms,* 21 (2), pp. 157-161.

Schwarz, S., Kehrenberg, C., Doublet, B. and Cloeckaert, A. (2004) Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiology Reviews* **28**, pp. 519-542.

Secombes, C.J., Wang, T. and Bird, S. (2011) The interleukins of fish. *Developmental & Comparative Immunology,* 35 (12), pp. 1336-1345.

Secombes, C. and Wang, T. (2012) The innate and adaptive immune system of fish. *Infectious Disease in Aquaculture: Prevention and Control,* 231, pp. 3-68.

Seppola, M., Larsen, A.N., Steiro, K., Robertsen, B. and Jensen, I. (2008) Characterisation and expression analysis of the interleukin genes, IL-1β, IL-8 and IL-10, in Atlantic cod (*Gadus morhua* L.). *Molecular Immunology,* 45 (4), pp. 887-897.

Shimizu, M., Goda, H., Yamasaki, K., Oshima, S-I., Ohnishi, K., Osaki, Y., Kataoka, S. and Imajoh, M. (2016). Draft genome sequence of *Flavobacterium psychrophilum* strain KTEN-1510 with genotype A/G-C, isolated from an ayu (*Plecoglossus altivelis altivelis*) in the Kagami River, Kochi, Japan. *American Society for Microbiology,* 4 (1), pp. e01762-15.

Siekoula-Nguedia, C., Blanc, G., Duchaud, E. and Calvez, S. (2012) Genetic diversity of *Flavobacterium psychrophilum* isolated from rainbow trout in France: predominance of a clonal complex. *Veterinary Microbiology*, 161 (1), pp. 169-178.

Sigel, A., Sigel, H. and Sigel, R.K. (2009) *Metallothioneins and related chelators.* Royal Society of Chemistry.

Sinyakov, M.S., Dror, M., Zhevelev, H.M., Margel, S. and Avtalion, R.R. (2002) Natural antibodies and their significance in active immunization and protection against a defined pathogen in fish. *Vaccine*, 20 (31), pp. 3668-3674.

Smith, P, Ruane, N.M., Douglas, I., Carroll, C., Kronvall, G. & Flemming G.T.A. (2007) Impact of inter-lab variation on the estimation of epidemiological cut-off values for disc diffusion susceptibility test data for *Aeromonas salmonicida*. *Aquaculture*, 272, pp. 168-179.

Smith, P., Schwarz, T. and Verner-Jeffreys, DW. (2012). Use of normalised resistance analyses to set interpretive criteria for antibiotic disc diffusion data produce by *Aeromonas* spp. *Aquaculture*, 326–329, pp. 27-35.

Smith,P., Alday-Sanz, V., Matysczak, J., Moulin, G., Lavilla-Pitogo, C.R. and Prater, D. (2013) Monitoring and surveillance of antimicrobial resistance in mircoorganisms associated with aquatic animals. *Scientific and Technical Review*, 32, pp. 583-593.

Smith, P. and Kronvall, G. (2015) Effect of incubation temperature and time on the precision of data generated by antibiotic disc diffusion assays. *Journal of Fish Diseases*, 38, pp. 629-536.

Smith, P., Endris, R., Kronvall, G., Thomas, V., Verner-Jeffreys, D., Wilhelm, C. and Dalsgaard, I. (2016) Epidemiological cut-off values for *Flavobacterium psychrophilum* MIC data generated by a standard test protocol. *Journal of Fish Diseases*, 39, pp. 143-154.

Solem, S.T. and Stenvik J (2006) Antibody repertoire development in teleosts—a review with emphasis on salmonids and *Gadus morhua* L. *Dev. Comp. Immunol*, 30, pp. 57–76.

Soltani, M., Shanker, S. and Munday, B.L. (1995) Chemitherapy of *Cytophaga/Flecibacter*-like bacteria (CFLB) infections in fish: studies validating clinical efficacies of selected antimicrobials. *Journal of Fish Diseases*, 18, pp. 555-565.

Soule, M., Cain, K., LaFrentz, S. and Call, D.R. (2005a) Combining suppression subtractive hybridization and microarrays to map the intraspecies phylogeny of *Flavobacterium psychrophilum*. *Infection and Immunity*, 73 (6), pp. 3799-3802.

Soule, M., LaFrentz, S., Cain, K., LaPatra, S. and Call, D.R. (2005b) Polymorphisms in 16S rRNA genes of *Flavobacterium psychrophilum* correlate with elastin hydrolysis and tetracycline resistance. *Diseases of Aquatic Organisms*, 65 (3), pp. 209-216.

Stafford, J.L., Wilson, M., Nayak, D., Quiniou, S.M., Clem, L.W., Miller, N.W. and Bengten, E. (2006) Identification and characterization of a FcR homolog in an ectothermic vertebrate, the channel catfish (*Ictalurus punctatus*).*Journal of Immunology (Baltimore, Md.: 1950),* 177 (4), pp. 2505-2517.

Starliper, C.E. and Schill W. B. (2011) Bacterial coldwater disease of fishes caused by *Flavobacterium psychrophilum*. *Journal of Advanced Research,* 2 (2), pp. 97-108.

Stenholm, A.R., Dalsgaard, I. and Middelboe, M. (2008) Isolation and characterization of bacteriophages infecting the fish pathogen *Flavobacterium psychrophilum*. *Applied and Environmental Microbiology,* 74 (13), pp. 4070-4078.

Ström-Bestor, M. and Wiklund, T. (2011) Inhibitory activity of *Pseudomonas* sp. on *Flavobacterium psychrophilum, in vitro*. *Journal of Fish Diseases,* 34 (4), pp. 255-264.

Sudheesh, P.S., LaFrentz, B.R., Call, D.R., Siems, W.F., LaPatra, S.E., Wiens, G.D. and Cain, K.D. (2007) Identification of potential vaccine target antigens by immunoproteomic analysis of a virulent and a non-virulent strain of the fish pathogen *Flavobacterium psychrophilum*. *Diseases of Aquatic Organisms,* 74 (1), pp. 37-47.

Sudheesh, P., Zimmerman, J. and Cain, K. (2016) Dietary effects on immunity, stress, and efficacy of two live attenuated *Flavobacterium psychrophilum* vaccine formulations. *Aquaculture,* 454, pp. 35-43.

Sugahara, K. and Eguchi, M. (2012) The use of warmed water treatment to induce protective immunity against the bacterial cold-water disease pathogen *Flavobacterium psychrophilum* in ayu (*Plecoglossus altivelis*). *Fish & Shellfish Immunology,* 32 (3), pp. 489-493.

Sundell, K., Heinikainen, S. and Wiklund, T. (2013) Structure of *Flavobacterium psychrophilum* populations infecting farmed rainbow trout *Oncorhynchus mykiss*. *Diseases of Aquatic Organisms,* 103 (2), pp. 111-119.

Sykes, J.E. and Papich, M.G. (2014) Chapter 8: Antibacterial drugs. *Canine and feline infectious diseases*, Saunders, an imprint of Elsevier Inc, pp. 66-86.

Tafalla, C., Bøgwald, J., Dalmo, R.A., Munang'andu, H.M. and Evensen, Ø. (2014) Adjuvants in fish vaccines. *Fish Vaccination,* Wiley-Blackwell, pp. 68-84.

Taylor, P.W. (2004) Detection of *Flavobacterium psychrophilum* in eggs and sexual fluids of Pacific salmonids by a polymerase chain reaction assay: implications for vertical transmission of bacterial coldwater disease. *Journal of Aquatic Animal Health,* 16 (2), pp. 104-108.

Taylor, P., Botto, M. and Walport, M. (1998) The complement system. *Current Biology,* 8 (8), pp. R259-R261.

ten Dijke, P. and Hill, C.S. (2004) New insights into TGF-β–Smad signalling. *Trends in Biochemical Sciences,* 29 (5), pp. 265-273.

Tenover F.C., Arbeit R.D., Goering R.V., Mickelsen, P.A., Murray B.E., Persing D.H. and Swaminathan B. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterias strain typing. *Journal of Clinical Microbiology*, 33, pp. 2233-2239.

Thinh, N.H., Kuo, T.Y., Hung, L.T., Loc, T.H., Chen, S.C., Evensen, O. and Schuuman, H.J. (2009). Combined immersion and oral vaccination of Vietnamese catfsh (*Pangasianodon hypophthalmus*) confers protection against mortality caused by *Edwardsiella ictaluri*. *Fish Shellfsh Immunol*, 27, pp. 773–776.

Tidwell, J.H. and Allan, G.L. (2001) Fish as food: aquaculture's contribution. Ecological and economic impacts and contributions of fish farming and capture fisheries.*EMBO Reports,* 2 (11), pp. 958-963.

Tomlinson, S., Stanley, K.K. and Esser, A.F. (1993) Domain structure, functional activity, and polymerization of trout complement protein C9. *Developmental & Comparative Immunology,* 17 (1), pp. 67-76.

Toranzo, A. and Baria, J. (1993) Fry mortality syndrome (FMS) in Spain, isolation of the causative bacterium *Flexibacter psychrophilus*. *Bulletin of the European Association of Fish Pathologists,* 13 (1), pp. 30-32.

Tort, L., Rotllant, J., Liarte, C., Acerete, L., Hernandez, A., Ceulemans, S., Coutteau, P. and Padros, F. (2004) Effects of temperature decrease on feeding rates, immune indicators and histopathological changes of gilthead sea bream *Sparus aurata* fed with an experimental diet. *Aquaculture,* 229 (1), pp. 55-65.

Toyama T., Kita-Tsukamoto K. and Wakabayashi H. (1994) Identification of *Cytophaga psychrophila* by PCR targeted 16S ribosomal RNA. *Fish Pathology,* 29, pp. 271-275.

Treves-Brown, K. (2000) The law. *Applied Fish Pharmacology.* Springer, pp. 26-54.

Valdebenito, S. and Avendaño-Herrera, R. (2009) Phenotypic, serological and genetic characterization of *Flavobacterium psychrophilum* strains isolated from salmonids in Chile. *Journal of Fish Diseases,* 32 (4), pp. 321-333.

Valdenegro-Vega, V.A., Crosbie, P., Vincent, B., Cain, K.D. and Nowak, B.F. (2013) Effect of immunization route on mucosal and systemic immune response in Atlantic salmon (*Salmo salar*). *Veterinary Immunology and Immunopathology,* 151 (1), pp. 113-123.

Vallejo, R.L., Wiens, G.D., Rexroad, C., Welch, T.J., Evenhuis, J.P., Leeds, T.D., Janss, L.L. and Palti, Y. (2010) Evidence of major genes affecting resistance to bacterial cold water disease in rainbow trout using Bayesian methods of segregation analysis. *Journal of Animal Science,* 88 (12), pp. 3814-3832.

Van Vliet D., Loch T.P., Smith P. & Faisal M. (2017) Antimicrobial susceptibilities of *Flavobacterium psychrophilum* isolates from the Great Lakes Basin, Michigan. *Microbial Drug Resistance*. Doi:10.1089/mdr.2016.0103.

Verner-Jeffreys, D.W. and Taylor, N.J. (2015) Review of freshwater treatments used in the Scottish freshwater rainbow trout aquaculture industry. *Scottish Aquaculture Research Forum Report SARF10.* Centre for Environment Fisheries & Aquaculture Science (Cefas) Weymouth.

Verner-Jeffreys, D.W., Brazier, T., Perez, R.Y., Ryder, D., Card, R.M., Welch, T.J., Hoare, R., Ngo, T., MacLaren, N., Ellis, R., Bartie, K.L., Feist, S.W., Rowe, W.M.P., Adams, A. and Thompson, K.D. (2017) Detection of the florfenicol resistance gene *flo*R in *Chryseobacterium* isolates from rainbow trout. Exception to the general rule? *FEMS Microbiol Ecol*. Doi: 10.1093/femsec/fix015.

Villarroel, F., Casado, A., Vásquez, J., Matamala, E., Araneda, B., Amthauer, R., Enriquez, R. and Concha, M.I. (2008) Serum amyloid A: a typical acute-phase reactant in rainbow trout? *Developmental & Comparative Immunology,* 32 (10), pp. 1160-1169.

Villarroel, F., Zambrano, Á., Amthauer, R. and Concha, M.I. (2009) Detection of up-regulated serum amyloid A transcript and of amyloid AA aggregates in skeletal muscle lesions of rainbow trout infected with *Flavobacterium psychrophilum*. *Veterinary Immunology and Immunopathology,* 130 (1), pp. 120-124.

Wakabayashi, H., Toyama, T. and Iida, T. (1994) A study in serotyping of *Cytophaga psychrophila* isolated from fishes in Japan. *Fish Pathology*, 29 (2), pp. 101-104.

Walker, R.D. and Dowling, P.M. (2006) Flouroquinolones. *Antimicrobial Therapy in Veterinary Medicine.* Wiley-Blackwell, pp. 263-284.

Walport, M.J. (2001) Complement. First of two parts. *New England Journal of Medicine,* 344 (14), pp. 1058-1066.

Wang, T., Monte, M.M., Huang, W., Boudinot, P., Martin, S.A. and Secombes, C.J. (2010a) Identification of two FoxP3 genes in rainbow trout (*Oncorhynchus mykiss*) with differential induction patterns. *Molecular Immunology,* 47 (16), pp. 2563-2574.

Wang, T., Martin, S.A. and Secombes, C.J. (2010b) Two interleukin-17C-like genes exist in rainbow trout *Oncorhynchus mykiss* that are differentially expressed and modulated. *Developmental & Comparative Immunology,* 34 (5), pp. 491-500.

Ware, C.F. (2003) The TNF superfamily. *Cytokine & Growth Factor Reviews,* 14 (3-4), pp. 181-184.

Warnock, R.A., Campbell, J.J., Dorf, M.E., Matsusawa, A., McEvoy, L.M. and Butcher, E.C. (2000) The role of chemokines in theh microenvironmental control of T versus B cell arrest in Peyer's Patch high endothelial venules. *The Journal of Experimental Medicine*, 191 (1), pp. 77-88.

Watts, M., Munday, B. and Burke, C. (2001) Immune responses of teleost fish. *Australian Veterinary Journal,* 79 (8), pp. 570-574.

Weber, G.M., Wiens, G.D., Welch, T.J., Hostuttler, M.A. and Leeds, T.D. (2013) Comparison of disease resistance between diploid, induced-triploid, and intercross-triploid rainbow trout including trout selected for resistance to *Flavobacterium psychrophilum*. *Aquaculture,* 410, pp. 66-71.

Wessels, M.R., Butko P., Ma M., Warren H.B., Lage A.L. and Carroll, M.C. (1995) Studies of group B streptococcal infection in mice deficient in complement component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity. *Proc Natl Acad Sci USA*, 92, pp. 11490–11494.

Whyte, S.K. (2007) The innate immune response of finfish–a review of current knowledge. *Fish & Shellfish Immunology,* 23 (6), pp. 1127-1151.

Wiens, G.D., LaPatra, S.E., Welch, T.J., Rexroad, C.,3rd, Call, D.R., Cain, K.D., LaFrentz, B.R., Vaisvil, B., Schmitt, D.P. and Kapatral, V. (2014) Complete genome sequence of *Flavobacterium psychrophilum* strain CSF259-93, used to select rainbow trout for increased genetic resistance against bacterial cold water disease.*Genome Announcements,* 2 (5), pp. 10.1128/genomeA.00889-14.

Wiklund, T. and Dalsgaard, I. (2002) Survival of *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*) serum *in vitro*. *Fish & Shellfish Immunology,* 12 (2), pp. 141-153.

Wiklund, T. and Dalsgaard, I. (2003) Association of *Flavobacterium psychrophilum* with rainbow trout (*Oncorhynchus mykiss*) kidney phagocytes *in vitro*. *Fish & Shellfish Immunology,* 15 (5), pp. 387-395.

Woynarovich, A., Hoitsy, G. and Moth-Poulsen, T. (2011) The rainbow trout. *Small-scale rainbow trout farming.* Food and Agriculture Organization of the United Nations, pp. 5-13.

Xu, Z., Parra, D., Gómez, D., Salinas, I., Zhang, Y-A, Jørgensen, L.V.G., Heinecke, R.D., Buchmann, K., LaPatra, S. and Sunyer, O. (2013) Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. *Proceedings of the National Academy of Sciences*, 110 (32), pp. 13097-13102.

Ye, J., Kaattari, I.M., Ma, C. and Kaattari, S. (2013) The teleost humoral immune response. *Fish & Shellfish Immunology*, 35 (6), pp. 1719-1728.

Ye, J., Kaattari, I. and Kaattari, S. (2011) Plasmablasts and plasma cells: reconsidering teleost immune system organization. *Developmental & Comparative Immunology*, 35 (12), pp. 1273-1281.

Zapata, A. (1982) Lymphoid organs of teleost fish. III. Splenic lymphoid tissue of Rutilus rutilus and Gobio gobio. *Developmental & Comparative Immunology*, 6 (1), pp. 87-94.

Zhang, Y., Salinas, I., Li, J., Parra, D., Bjork, S., Xu, Z., LaPatra, S.E., Bartholomew, J. and Sunyer, J.O. (2010) IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nature Immunology*, 11 (9), pp. 827-835.

Zhao, X., Findly, R.C. and Dickerson, H.W. (2008) Cutaneous antibody-secreting cells and B cells in a teleost fish. *Developmental & Comparative Immunology*, 32 (5), pp. 500-508.

Zlotnik, A. (2006) Chemokines and cancer. *International Journal of Cancer*, 119 (9), pp. 2026-2029.

Zou, J., Grabowski, P.S., Cunningham, C. and Secombes, C.J. (1999) Molecular cloning of interleukin 1β from rainbow trout *Oncorhynchus mykiss* reveals no evidence of an ICE cut site. *Cytokine*, 11 (8), pp. 552-560.

Zwollo, P., Haines, A., Rosato, P. and Gumulak-Smith, J. (2008) Molecular and cellular analysis of B-cell populations in the rainbow trout using Pax5 and immunoglobulin markers. *Developmental & Comparative Immunology*, 32 (12), pp. 1482-1496.

Appendix 1. *Flavobacterium psychrophilum* vaccine development studies. RPS: Relative percent survival; RT: rainbow trout; i.p.: intraperitoneal; i.m.: intramuscular; s.c.: subcutaneous; FKC: Formalin-killed *F. psychrophilum* cells; FCA: Freund's complete adjuvant; CPM: cumulative percent mortality.

Study	Vaccine type	Immunisation route	Challenge route	RPS	Fish species	Fish weight
Potential inactivated vaccines						
Obach and Baudin Laurencin, 1991	Heat inactivated whole cell bacterins	i.p. injection	i.p. injection	80%	RT	2.2 g (90 days post-hatch)
		2-min immersion	i.p. injection	14 - 47%	RT	>50 days post-hatch
				No protection	RT	<0.5 g (<50 days post-hatch)
LaFrentz *et al.*, 2002	FKC	i.p. injection	s.c. injection	15%	RT	3 g
	FKC emulsified in FCA	i.p. injection	s.c. injection	83%	RT	3 g
	FKC	2-min immersion	s.c. injection	13%	RT	3 g
LaFrentz *et al.*, 2004	FKC emulsified in FCA	i.p. injection	s.c. injection	29 - 57%	RT	2.8 g
Högfors *et al.*, 2008	FKC and sonicated FKC of *F. psychrophilum*	i.p. injection	i.m. injection	100%	RT	104 g
Rahman *et al.*, (2000)	FKC	i.p. injection	i.m. injection	40%	ayu	1.5 g
	FKC in the adjuvant Montanidae	i.p. injection	i.m. injection	60%	ayu	1.5 g
	FKC in the adjuvant Squalene	i.p. injection	i.m. injection	68%	ayu	1.5 g

Study	Vaccine type	Immunisation route	Challenge route	RPS	Fish species	Fish weight
Rahman *et al.*, 2002	FKC	i.p. injection	i.m. injection	46% at challenge dose of 1 x 10^6 45% at challenge dose of 3 x 10^5	RT	2.1 g
	FKC	i.p. injection	i.m. injection	35% at challenge dose of 5 x 10^5 41% at challenge dose of 5 x 10^4	ayu	1.7 g
Rahman *et al.*, (2003)	FKC	i.p. injection	i.m. injection	33%	ayu	1.7 g
	FKC in the adjuvant IMS-1311	i.p. injection	i.m. injection	67%	ayu	1.7 g
	FKC in the adjuvant IMS-1312	i.p. injection	i.m. injection	73%	ayu	1.7 g
	FKC in the adjuvant IMS-2212	i.p. injection	i.m. injection	46%	ayu	1.7 g
	FKC in the adjuvant ISA-763A	i.p. injection	i.m. injection	61%	ayu	1.7 g
Madetoja *et al.*, 2006	FKC of Fd and Th serotypes in oil-based adjuvant	i.p. injection	i.m. injection	77%	RT	50 g
	Heat-inactivated *F. psychrophilum* of Fd and Th serotypes in oil-based adjuvant	i.p. injection	i.m. injection	89%	RT	50 g
	FKC of Fd and Th serotypes in oil-based adjuvant (under field condition)	i.p. injection	i.m. injection	CPM of vaccinated and non-vaccinated fish was 9% and 100% respectively	RT	14 g
Fredriksen *et al.*, 2013a	FKC of serotype Fd and Th in water-in-oil adjuvant	i.p. injection	i.m. injection	44 - 67%	RT	37 g

Study	Vaccine type	Immunisation route	Challenge route	RPS	Fish species	Fish weight
Fredriksen *et al*., 2013b	FKC of serotype Fd and Th and infectious pancreatic necrosis virus (in water-in-oil adjuvant)	i.p. injection	i.m. challenge	80%	RT	33 g
	FKC of serotype Fd and Th, and other fish pathogens (in water-in-oil adjuvant)	i.p. injection	i.m. challenge	78%	RT	33 g
Kondo *et al.*, 2003	FKC of *F. psychrophilum*	Oral (every days for 2 weeks, 15 times)	immersion (at 7 weeks post vaccination)	43% at challenge dose of 2.1×10^7 59% at challenge dose of 1.4×10^8	ayu	0.5 g (75 days post-hatching)
		Oral (5 days over 2 weeks)	immersion	50% at challenge dose of 2.1×10^7 63% at challenge dose of 1.4×10^8	ayu	0.5 g (75 days post-hatching)
Aoki *et al.*, 2007	FKC of *F. psychrophilum* at logarithmic phase	Oral	Bath	85 - 89%	RT	1.6 g
	FKC of *F. psychrophilum* at stationary phase	Oral	Bath	22 - 42%	RT	1.6 g
	Stationary phase culture supernatant of *F. psychrophilum*	Oral	Bath	5%	RT	1.6 g
	FKC of *F. psychrophilum* at stationary phase and membrane vesicles in the medium	Oral	Bath	94 - 100%	RT	1.6 g

Study	Vaccine type	Immunisation route	Challenge route	RPS	Fish species	Fish weight
Potential subunit vaccines						
Rahman *et al.*, 2002	Outer membrane fraction (10 µg)	i.p. injection	i.m. injection	95% at challenge dose of 1×10^6 93% at challenge dose of 3×10^5	RT	2.1 g
	Outer membrane fraction (10 µg)	i.p. injection	i.m. injection	64% at challenge dose of 5×10^5 71% at challenge dose of 5×10^4	ayu	1.7 g
LaFrentz *et al.*, 2004	protein fraction (70-100 kDa) emulsified in FCA	i.p. injection	s.c. injection	66 - 94%	RT	2.8 g
	protein fraction (41-49 kDa) emulsified in FCA	i.p. injection	s.c. injection	26 - 58%	RT	2.8 g
	protein fraction (18-28 kDa) emulsified in FCA	i.p. injection	s.c. injection	6 - 31%	RT	2.8 g
LaFrentz *et al.*, 2014	Crude LPS extract from *F. psychrophilum* 10 µg	i.p. injection	s.c. injection	No protection	RT	3 g
	Crude LPS extract from *F. psychrophilum* 25 µg	i.p. injection	s.c. injection	No protection	RT	3 g
	Crude LPS extract from *F. psychrophilum* 10 µg in FCA	i.p. injection	s.c. injection	13%	RT	3 g
	Crude LPS extract from *F. psychrophilum* 25 µg in FCA	i.p. injection	s.c. injection	15%	RT	3 g
Högfors *et al.*, 2008	25 - 33 kDa protein fraction (5 µg) in FCA	i.p. injection	i.m. injection	Cumulative percent mortality (CPM) of 10%	RT	15 g
	25 - 33 kDa protein fraction (10 µg) in FCA	i.p. injection	i.m. injection	CPM of 15%	RT	15 g

Study	Vaccine type	Immunisation route	Challenge route	RPS	Fish species	Fish weight
Dumetz *et al*., 2006	18 kDa outer membrane protein	i.p. injection	s.c. injection	54%	RT	2.8 g
	18 kDa outer membrane protein (in FCA)	i.p. injection	s.c. injection	89%	RT	2.8 g
Plant *et al*., 2009	Recombinant heat shock proteins of *F. psychrophilum* HSP90 and HSP70 (8 µg)	i.p. injection	s.c. injection	No protection	RT	2 g
Plant *et al*., 2011	Recombinant elongation factor-Tu (EFTU), SufB Fe-S assembly protein (SufB) and ATP synthase β (in FCA)	i.p. injection	s.c. injection	No protection	RT	2 g
Gliniewicz *et al*., 2012	Recombinant FL1493 of *F. psychrophilum* (in FCA)	i.p. injection	s.c. injection	No protection	RT	2 g
Kato *et al*., 2014	Formalin-killed recombinant *E. coli* cells expressing 3-hydroxyacyl-CoA dehydrogenase (HCD), ATP synthase beta subunit (atpD) or glutamate dehydrogenase (gdhA) of *F. psychrophilum*	i.p. injection	i.p. injection	32 - 37%	ayu	2.2 g
Potential attenuated vaccines						
Alvarez *et al*., 2008	An attenuated *F. psychrophilum* strain having a disrupted gene coding for ExbD2 protein	i.m. injection	i.m. injection	82%	RT	5 g

Study	Vaccine type	Immunisation route	Challenge route	RPS	Fish species	Fish weight
LaFrentz *et al*., 2008	An attenuated *F. psychrophilum* strain (CSF259-93B.17) developed by passaging in increasing concentrations of rifampicin	i.p. injection	s.c. injection	29 - 45%	RT	2.4 g
		immersion (with adipose fin removal)	s.c. injection	0 - 45%	RT	3.4 g
Long *et al.*, 2013	CSF259-93B.17	i.p. injection	s.c. injection	90%	coho salmon	3.6 g
	CSF259-93B.17 ILM (developed by culturing CSF259-93B.17 under iron-limited conditions	i.p. injection	s.c. injection	98%	coho salmon	3.6 g
	CSF259-93B.17	1-h immersion (with adipose fin removal)	s.c. injection	46%	coho salmon	3.6 g
	CSF259-93B.17 ILM	1-h immersion (with adipose fin removal)	s.c. injection	73%	coho salmon	3.6 g
Sudheesh *et al.*, 2016	CSF259-93B.17 ILM and normal diet	3-min immersion	s.c. injection	61%	RT	2.5 g
	CSF259-93B.17 ILM and altered diet	3-min immersion	s.c. injection	69%	RT	2.5 g
Potential live wild-type vaccines						
Lorenzen *et al.*, 2010	Live wild-type *F. psychrophilum* cells	30-min immersion	i.p. injection	88% at 26 days 60% at 47 days	RT	1 g
Potential DNA vaccines						
Plant *et al.*, 2009	DNA vaccine using pVAX1 vector containing HSP60 or HSP70 genes	i.m. injection	s.c. injection	No protection	RT	2 g

Study	Vaccine type	Immunisation route	Challenge route	RPS	Fish species	Fish weight
Passive immunisation						
LaFrentz *et al*., 2003	Anti-*F. psychrophilum* serum from convalescent adult RT	i.p. injection	s.c. injection	9 - 42%	RT	1.0 - 1.4 g
	Anti-*F. psychrophilum* serum from immunised adult RT	i.p. injection	s.c. injection	57%	RT	1.0 - 1.4 g
	Anti-*F. psychrophilum* serum from goat	i.p. injection	s.c. injection	No protection	RT	1.0 - 1.4 g
Kato *et al*., 2015	Anti-*F. psychrophilum* serum	i.p. injection	i.p. injection	21%	ayu	2 g