

# **Towards an understanding of genetic resistance to *Flavobacterium psychrophilum* in Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792))**

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Thesis submitted for the degree of Doctor of Philosophy



By

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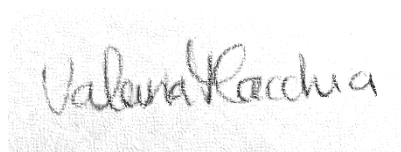
## Declaration

I hereby declare that the work and results presented in this thesis was conducted by me at the Institute of Aquaculture, University of Stirling, Scotland. The work presented in this thesis has not been previously submitted for any other degree or qualification.

The GWAS on *F. psychrophilum* resistance traits in commercial rainbow trout and Atlantic salmon breeding populations from the AquaGen nucleus analysis presented in Chapter 2 was conducted by Dr Fabian Grammes (AquaGen Norway).

The literature consulted has been cited and where appropriate, collaborative assistance has been acknowledged.

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A handwritten signature in dark ink, reading 'Valeria Macchia', is positioned above a horizontal line.

Valeria Macchia

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## Peer-reviewed Publications

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Mathissen, H., Duan, Y., Marana, M.H., Zuo, S., Karami, A., Jafaar, R.M., Jørgensen, L.G., Kania, P.W., Dalsgaard, I., Madsen, L., Nielsen, T., Grammes, F., Moen, T., Ødegaard, J., Macchia, V., Buchmann, K. (2022). Validation of a QTL for *Flavobacterium psychrophilum* resistance in rainbow trout *Oncorhynchus mykiss*. *Aquaculture Reports*, 30, 101573. <https://doi.org/10.1016/j.aqrep.2023.101573>.

## Scientific Conferences and Meeting

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- PhD symposium- October 2019
- 4th International Conference on Integrative Salmonid Biology – November 2019
- European Aquaculture Society, Online conference- May 2021
- European Association of Fish Pathologists, Online conference 20-23 September 2021.
- European Association of Fish Pathologists, Conference 13-16 September 2022.

## Training and Workshops

- RNA-seq Data Analysis, Edinburgh Genomics- June 2020
- Essential Bioinformatics, Edinburgh Genomics- May 2020
- SAIC- Digital Skills Workshop – March 2021
- ARCH-UK Proteomic Workshop – November 2019
- The Search for Selection by Bruce Walsh (Roslin Institute)- August 2018
- ScotPIL course (E1/L), Glasgow University – October 2018
- ScotPIL course (Fish Module), Stirling University – March 2019

## Abstract

Flavobacteriosis is a widespread challenge for the aquaculture sector, with infections reported in salmonids farmed in Scotland, Chile, Norway, and Canada, causing major welfare, environmental and economic concerns. A better understanding of the interactions between the host immune system and the pathogens are required for the development of new methods to control or prevent disease outbreaks. The current treatment and prevention programmes are limited to antimicrobial therapy and vaccines (ALPHA JECT® IPNV-Flavo) delivered by injection and therefore not suitable for fry, which is when the fish are most vulnerable to this pathogen. As the sector continues to move away from antibiotic treatments, a genetic breakthrough could be the key to preventing *Flavobacterium psychrophilum* infections in Atlantic salmon and rainbow trout. The selection of families of fish with resistance or decreased susceptibility to this pathogen offers another possibility for addressing the problems posed by *F. psychrophilum*. Therefore, the aim of this thesis was to study *F. psychrophilum* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), and Atlantic salmon (*Salmo salar* L.) resistant and susceptible families, and to uncover the genetic underpinnings of resistance to *F. psychrophilum*. To achieve this, a successful immersion challenge model for *F. psychrophilum* in Atlantic salmon and rainbow trout, which mimics the natural infection route, was developed. Rainbow trout and Atlantic salmon were challenged using this newly developed challenge model, and a genome wide association study (GWAS) was conducted in both species, which identified genetic markers for disease resistance. In rainbow trout resistance to *F. psychrophilum* is a polygenic trait, while in Atlantic salmon the trait is oligogenic, which suggests that the trait is influenced by few genes with large effects (Chapter 2). A SYBR Green quantitative polymerase chain reaction (qPCR) was developed, validated, and used to quantify *F. psychrophilum* load in Atlantic salmon and rainbow trout head kidney tissues at different time points post challenge. However, there was not a clear relationship between fish genotype and bacterial load in head kidney tissues (Chapter 3). Since *F. psychrophilum* infection was not solely localised to the head kidney, and low bacterial load was determined in this tissue, the mucosal immune response of Atlantic salmon and rainbow trout was investigated by immunohistochemistry (IHC) by detecting and quantifying *F. psychrophilum* in different tissues (gills, head kidney, and gut), and the IHC supported the qPCR results of low

bacterial load in the head kidney. Similarly, immunoglobulin T (IgT) positive cells were detected in all mucosal tissues, with the highest detection in the secondary lamellae of the gills, suggesting the importance of this immunoglobulin (Ig) in teleosts (Chapter 4). Finally, for the first time the transcriptomic response to *F. psychrophilum* infection in Atlantic salmon head kidney was investigated to unravel genes and pathways that take part in disease resistance. Genes and pathways up regulated in infected Atlantic salmon head kidney included those related to the immune response, cell chemotaxis and iron homeostasis; pattern recognition receptors (PRRs), complement molecules, inflammatory cytokines, chemokines, and iron-related genes, which indicated these genes participate in the response of Atlantic salmon to *F. psychrophilum* challenge (Chapter 5). This present study is the first to describe a *F. psychrophilum* immersion challenge model that can be used to study host-pathogen interactions, and molecular and immunological techniques that allow for the rapid detection of this bacterium in infected fish tissues. Moreover, the transcriptomic study provided an insight into the immune system and the defence mechanisms of Atlantic salmon against *F. psychrophilum*, which is an important step forward towards preventing infections by this bacterium in farmed Atlantic salmon.

## Table of Contents

|                                                                                                                                                                                                             |           |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Declaration .....                                                                                                                                                                                           | i         |
| Acknowledgements .....                                                                                                                                                                                      | ii        |
| Peer-reviewed Publications .....                                                                                                                                                                            | iii       |
| Scientific Conferences and Meeting .....                                                                                                                                                                    | iii       |
| Training and Workshops .....                                                                                                                                                                                | iii       |
| Abstract .....                                                                                                                                                                                              | iv        |
| Table of Contents .....                                                                                                                                                                                     | vi        |
| List of Figures .....                                                                                                                                                                                       | ix        |
| List of Tables .....                                                                                                                                                                                        | xvi       |
| List of Abbreviations and Definitions .....                                                                                                                                                                 | xviii     |
| Chapter 1: General Introduction .....                                                                                                                                                                       | 1         |
| <b>1.1 BACKGROUND .....</b>                                                                                                                                                                                 | <b>1</b>  |
| <b>1.2 FLAVOBACTERIOSIS .....</b>                                                                                                                                                                           | <b>3</b>  |
| <b>1.3 TAXONOMY OF THE AETIOLOGICAL AGENT.....</b>                                                                                                                                                          | <b>5</b>  |
| <b>1.4 PATHOGENESIS .....</b>                                                                                                                                                                               | <b>7</b>  |
| <b>1.5 SALMONID FISH IMMUNE SYSTEM.....</b>                                                                                                                                                                 | <b>8</b>  |
| 1.5.1 MUCOSAL RESPONSE TO <i>FLAVOBACTERIUM PSYCHROPHILUM</i> .....                                                                                                                                         | 9         |
| <b>1.6 SALMONID RESPONSE TO <i>F. PSYCHROPHILUM</i>-INFECTION.....</b>                                                                                                                                      | <b>14</b> |
| <b>1.7 DIAGNOSIS .....</b>                                                                                                                                                                                  | <b>16</b> |
| 1.7.1. ENZYME-LINKED IMMUNOSORBENT ASSAY .....                                                                                                                                                              | 16        |
| 1.7.2 IMMUNOHISTOCHEMISTRY .....                                                                                                                                                                            | 17        |
| 1.7.3 MOLECULAR DIAGNOSIS .....                                                                                                                                                                             | 17        |
| <b>1.8 CURRENT TREATMENTS AND PREVENTION.....</b>                                                                                                                                                           | <b>18</b> |
| <b>1.9 SALMONID GENOME BIOLOGY.....</b>                                                                                                                                                                     | <b>20</b> |
| <b>1.10 RNA-SEQUENCING .....</b>                                                                                                                                                                            | <b>21</b> |
| <b>1.11 SELECTIVE BREEDING .....</b>                                                                                                                                                                        | <b>27</b> |
| <b>1.12 AIMS AND OBJECTIVES.....</b>                                                                                                                                                                        | <b>33</b> |
| Chapter 2: Development of an Immersion Challenge Model for <i>Flavobacterium psychrophilum</i> with the aim to test (or show) resistance to flavobacteriosis in Atlantic salmon and rainbow trout fry ..... | 34        |
| <b>2.1 INTRODUCTION .....</b>                                                                                                                                                                               | <b>34</b> |
| <b>2.2 MATERIALS AND METHODS.....</b>                                                                                                                                                                       | <b>38</b> |
| 2.2.1 BACTERIAL ISOLATES, CULTURE MEDIA AND GROWTH CONDITIONS.....                                                                                                                                          | 38        |
| 2.2.2 EXPERIMENTAL ANIMALS .....                                                                                                                                                                            | 40        |
| 2.2.3 IMMERSION CHALLENGES .....                                                                                                                                                                            | 40        |
| 2.2.4 TWO-ROUND NESTED PCR TO CONFIRM MORTALITIES DUE TO <i>F. PSYCHROPHILUM</i> INFECTION .....                                                                                                            | 44        |
| 2.2.5 GENOTYPING .....                                                                                                                                                                                      | 47        |
| 2.2.6 GENOME-WIDE ASSOCIATION STUDY AND HERITABILITY.....                                                                                                                                                   | 47        |
| 2.2.7 STATISTICAL ANALYSES .....                                                                                                                                                                            | 47        |
| 2.2.8 ETHICAL APPROVAL .....                                                                                                                                                                                | 48        |
| <b>2.4 RESULTS.....</b>                                                                                                                                                                                     | <b>49</b> |
| 2.4.1 BACTERIAL ISOLATES.....                                                                                                                                                                               | 49        |

|                                                                                                                                                                                   |            |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| 2.4.2 EXPERIMENTAL ANIMALS .....                                                                                                                                                  | 55         |
| 2.4.3 PRELIMINARY CHALLENGES .....                                                                                                                                                | 55         |
| 2.4.4 MAIN CHALLENGE .....                                                                                                                                                        | 55         |
| <b>2.4.3 HERITABILITY AND GWAS.....</b>                                                                                                                                           | <b>62</b>  |
| <b>2.5 DISCUSSION .....</b>                                                                                                                                                       | <b>65</b>  |
| <b>2.6 CONCLUSION.....</b>                                                                                                                                                        | <b>69</b>  |
| Chapter 3: SYBR Green qPCR assay for <i>F. psychrophilum</i> detection in Atlantic salmon and rainbow trout tissues .....                                                         | 71         |
| <b>3.1 INTRODUCTION .....</b>                                                                                                                                                     | <b>71</b>  |
| <b>3.2 MATERIALS AND METHODS .....</b>                                                                                                                                            | <b>77</b>  |
| 3.2.1 BACTERIAL ISOLATES.....                                                                                                                                                     | 77         |
| 3.2.2 EXPERIMENTAL ANIMALS .....                                                                                                                                                  | 77         |
| 3.2.3 SAMPLING TANK CHALLENGE .....                                                                                                                                               | 78         |
| 3.2.4 SAMPLING .....                                                                                                                                                              | 78         |
| 3.2.5 BACTERIAL ISOLATES AND DNA EXTRACTION .....                                                                                                                                 | 80         |
| 3.2.6 TISSUE DNA EXTRACTION .....                                                                                                                                                 | 81         |
| 3.2.7 TISSUE DNA EXTRACTION FOR GENOTYPING .....                                                                                                                                  | 82         |
| 3.2.8 <i>FLAVOBACTERIUM PSYCHROPHILUM</i> PCR .....                                                                                                                               | 84         |
| 3.2.9 SYBR GREEN QUANTITATIVE PCR (qPCR).....                                                                                                                                     | 85         |
| 3.2.10 PREPARATION OF STANDARD CURVE.....                                                                                                                                         | 88         |
| 3.2.11 GENOTYPING .....                                                                                                                                                           | 89         |
| <b>3.3 STATISTICAL ANALYSIS .....</b>                                                                                                                                             | <b>89</b>  |
| <b>3.4 RESULTS.....</b>                                                                                                                                                           | <b>91</b>  |
| 3.4.1 qPCR PRIMERS SENSITIVITY, EFFICIENCY, AND SPECIFICITY .....                                                                                                                 | 91         |
| 3.4.2 TISSUE DNA EXTRACTION .....                                                                                                                                                 | 99         |
| 3.4.2 qPCR STANDARD CURVE.....                                                                                                                                                    | 100        |
| 3.4.3 SYBR GREEN qPCR ASSAY FOR <i>F. PSYCHROPHILUM</i> DETECTION IN ATLANTIC SALMON AND RAINBOW TROUT TISSUES.....                                                               | 108        |
| <b>3.5 DISCUSSION .....</b>                                                                                                                                                       | <b>113</b> |
| <b>3.6 CONCLUSIONS.....</b>                                                                                                                                                       | <b>116</b> |
| Chapter 4: Characterisation of antibodies for the detection of <i>Flavobacterium psychrophilum</i> and IgT positive cells in infected Atlantic salmon and rainbow trout fry ..... | 117        |
| <b>4.1 INTRODUCTION .....</b>                                                                                                                                                     | <b>117</b> |
| <b>4.2 MATERIALS AND METHODS.....</b>                                                                                                                                             | <b>121</b> |
| 4.2.2 CLONING OF MABS .....                                                                                                                                                       | 121        |
| <b>4.2.1 BACTERIAL CULTIVATION.....</b>                                                                                                                                           | <b>121</b> |
| <b>4.2.3 CHARACTERISATION OF ANTI-<i>F. PSYCHROPHILUM</i> MABS BY IFAT .....</b>                                                                                                  | <b>124</b> |
| <b>4.2.4 SPECIFICITY OF MABS TESTED BY DIRECT ELISA.....</b>                                                                                                                      | <b>125</b> |
| <b>4.2.5 1D SDS PAGE (1-DE) AND SILVER STAINING OF <i>F. PSYCHROPHILUM</i> ISOLATES ..</b>                                                                                        | <b>128</b> |
| <b>4.2.6 WESTERN BLOT .....</b>                                                                                                                                                   | <b>128</b> |
| <b>4.2.7 2D SDS-PAGE .....</b>                                                                                                                                                    | <b>131</b> |
| 4.2.7.1 PREPARATION OF BACTERIAL PELLETS FOR 2D GEL ELECTROPHORESIS .....                                                                                                         | 131        |
| 4.2.7.2 PROTEIN QUANTIFICATION AND CLEAN UP .....                                                                                                                                 | 131        |
| 4.2.7.3 2D SDS PAGE (2-DE).....                                                                                                                                                   | 132        |
| <b>4.2.8 HISTOPATHOLOGICAL PROCESSING .....</b>                                                                                                                                   | <b>135</b> |
| <b>4.2.9 DETECTION OF <i>F. PSYCHROPHILUM</i> IN ATLANTIC SALMON AND RAINBOW TROUT TISSUES BY IMMUNOHISTOCHEMISTRY USING ANTI-<i>F. PSYCHROPHILUM</i> MABS.....</b>               | <b>137</b> |

|                                                                                                                                                                                          |            |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <b>4.2.10 DETECTION OF <i>F. PSYCHROPHILUM</i> IN TISSUES BY IMMUNOHISTOCHEMISTRY WITH PAB 151</b>                                                                                       | <b>138</b> |
| <b>4.2.11 IMMUNOHISTOCHEMISTRY FOR DETECTION OF IgT POSITIVE CELLS IN TISSUE</b>                                                                                                         | <b>138</b> |
| <b>4.2.12 ETHICAL APPROVAL</b>                                                                                                                                                           | <b>139</b> |
| <b>4.3 RESULTS</b>                                                                                                                                                                       | <b>140</b> |
| 4.3.1 CLONING OF MABS                                                                                                                                                                    | 140        |
| 4.3.2 CHARACTERISATION OF ANTI- <i>F. PSYCHROPHILUM</i> MABS BY IFAT                                                                                                                     | 140        |
| 4.3.3 SPECIFICITY OF MABS TESTED BY DIRECT ELISA                                                                                                                                         | 142        |
| 4.3.4 1D SDS PAGE (1-DE), SILVER STAINING AND WESTERN BLOTTING OF <i>F. PSYCHROPHILUM</i> ISOLATES                                                                                       | 145        |
| 4.3.5 2D SDS PAGE, SILVER STAINING AND WESTERN BLOTTING OF <i>F. PSYCHROPHILUM</i> ISOLATES                                                                                              | 148        |
| 4.3.6 DETECTION OF <i>F. PSYCHROPHILUM</i> IN ATLANTIC SALMON AND RAINBOW TROUT TISSUES BY IFAT                                                                                          | 151        |
| 4.3.7 IMMUNOHISTOCHEMISTRY FOR DETECTION OF <i>F. PSYCHROPHILUM</i> IN ATLANTIC SALMON AND RAINBOW TROUT TISSUES                                                                         | 152        |
| 4.2.9 IMMUNOHISTOCHEMISTRY FOR DETECTION OF IgT POSITIVE CELLS IN ATLANTIC SALMON AND RAINBOW TROUT TISSUES                                                                              | 159        |
| <b>4.4 DISCUSSION</b>                                                                                                                                                                    | <b>164</b> |
| <b>4.5 CONCLUSION</b>                                                                                                                                                                    | <b>167</b> |
| Chapter 5: Transcriptomic Analysis of Atlantic salmon head kidney post- <i>Flavobacterium psychrophilum</i> infection using RNA sequencing to identify global changes in gene expression | 169        |
| <b>5.1 INTRODUCTION</b>                                                                                                                                                                  | <b>169</b> |
| <b>5.2 METHODS</b>                                                                                                                                                                       | <b>174</b> |
| 5.2.1 EXPERIMENTAL CHALLENGE                                                                                                                                                             | 174        |
| 5.2.2 RNA EXTRACTION AND SEQUENCING                                                                                                                                                      | 174        |
| 5.2.3 ATLANTIC SALMON RNA-SEQ STUDY                                                                                                                                                      | 176        |
| 5.2.4 CROSS-SPECIES COMPARISON                                                                                                                                                           | 177        |
| <b>5.3 RESULTS</b>                                                                                                                                                                       | <b>179</b> |
| 5.3.1 SAMPLING AND SEQUENCING                                                                                                                                                            | 179        |
| 5.3.2 DIFFERENTIAL EXPRESSION AND GO ENRICHMENT ANALYSIS                                                                                                                                 | 188        |
| 5.3.3 CROSS-SPECIES COMPARISON                                                                                                                                                           | 195        |
| <b>5.4 DISCUSSION</b>                                                                                                                                                                    | <b>198</b> |
| 5.4.1 DIFFERENTIALLY EXPRESSED GENES RELATED TO IMMUNE RESPONSE                                                                                                                          | 199        |
| 5.4.2 DIFFERENTIALLY EXPRESSED GENES RELATED TO CELL CHEMOTAXIS                                                                                                                          | 202        |
| 5.4.3 DIFFERENTIALLY EXPRESSED GENES RELATED TO IRON HOMEOSTASIS                                                                                                                         | 204        |
| <b>5.5 Conclusion</b>                                                                                                                                                                    | <b>207</b> |
| Chapter 6: General Discussion                                                                                                                                                            | 208        |
| Appendix                                                                                                                                                                                 | 216        |
| References                                                                                                                                                                               | 220        |

## List of Figures

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |           |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| <b>Figure 1.1</b> Gross signs of disease in Atlantic salmon challenged with <i>F. psychrophilum</i> . Haemorrhaging of the dorsal musculature (a) and bleeding orbitals (b) in infected Atlantic salmon (Johansen et al., 2015). In rainbow trout the bacterium has been linked with ulceration (c) and fin rot (d) (Bernardet & Bowman, 2006). Figure 1.1 I naturally <i>F. psychrophilum</i> infected Atlantic salmon, showing extensive petechiation and large haemorrhagic areas in skeletal muscle (Nielsen et al., 2011). Images adapted from Bernardet & Bowman (2006) and Nielsen et al. (2011)..... | <b>4</b>  |
| <b>Figure 1.2</b> Morphology of <i>F. psychrophilum</i> colonies exhibiting a smooth margin (a) and exhibiting spread margins (b). Image adapted from Cain & LaFrentz (2007).....                                                                                                                                                                                                                                                                                                                                                                                                                            | <b>6</b>  |
| <b>Figure 1.3</b> Schematic representation of MALT and their anatomical localisation in teleost. Image adapted from Salinas et al. (2022).....                                                                                                                                                                                                                                                                                                                                                                                                                                                               | <b>12</b> |
| <b>Figure 1.4</b> Illustration of the main steps involved in a transcriptome analysis using RNA-Seq are: experimental design, library construction, sequencing of the library on a NGS platform, and analysis using bioinformatic tools for interpretation. Image adapted from Sudhagar et al. (2018).....                                                                                                                                                                                                                                                                                                   | <b>23</b> |
| <b>Figure 2.1</b> Schematic representation of the Atlantic salmon preliminary and main immersion challenges tanks, parameters (tank size, oxygen levels, photoperiod regime, water temperature), number of animals challenged, bacterial isolate and dosage used, trial length and material sampled.....                                                                                                                                                                                                                                                                                                     | <b>42</b> |
| <b>Figure 2.2</b> Schematic representation of the rainbow trout preliminary and main immersion challenges tanks, parameters (tank size, oxygen levels, photoperiod regime, water temperature), number of animals challenged, bacterial isolate and dosage used, trial length and material sampled.....                                                                                                                                                                                                                                                                                                       | <b>43</b> |
| <b>Figure 2.3</b> Rainbow trout (n=1024) main immersion challenge with isolate <i>F. psychrophilum</i> 19_5. A) Cultivation of bacteria in MVB (ca. 6 L), B) Rainbow trout fry treated with hydrogen peroxide (200 mg/L) in a 90 L tank for 1 h. C) Schematic diagram of what is happening in the tank in Figure 3B, in which the animals are treated with hydrogen peroxide to strip off the mucous to expose the fish to the bacteria D) Head kidney swab specimen from moribund rainbow trout, MVA plate showing clear growth of <i>F. psychrophilum</i> -like colonies.....                              | <b>46</b> |
| <b>Figure 2.4</b> Agarose gel (1%) used to confirm the presence of <i>F. psychrophilum</i> from representative colonies recovered from moribund animals sampled from natural outbreaks of flavobacteriosis in Atlantic salmon and rainbow trout using a nested PCR that targets the 16S rRNA gene. From left to right the wells were filled with 100 bp gene ruler (position 1), Atlantic salmon isolate 6_S and 18_S (position 2 and 3), rainbow trout isolate 19_5 and 365a (position 4 and 5), positive control (position 6), and a non-template control (NCT) (position 7).....                          | <b>50</b> |
| <b>Figure 2.5</b> Kaplan-Meier survival curves of Atlantic salmon in the preliminary challenge with two isolates derived from Atlantic salmon ( <i>F. psychrophilum</i> 6_S and <i>F. psychrophilum</i> 18_S) at two concentrations, specifically $2 \times 10^6$ and $2 \times 10^7$ CFU/mL. Fish with a mean mass of $2.4 \pm 0.237$ g (n=30 per treatment group) were exposed to hydrogen peroxide (200 mg/L) for 1 h prior to immersion challenge with the bacteria for 4 h.....                                                                                                                         | <b>58</b> |
| <b>Figure 2.6</b> Kaplan-Meier survival curves of rainbow trout in the preliminary challenge with one isolate derived from rainbow trout ( <i>F. psychrophilum</i> 19_5) at $2 \times 10^7$ CFU/mL. Fish (n=15) with a mean mass of $1.8 \pm 0.247$ g; were exposed to hydrogen peroxide (200 mg/L) for 1 h prior to immersion challenge with the bacteria for 4 h.....                                                                                                                                                                                                                                      | <b>59</b> |

**Figure 2.7** Kaplan-Meier survival curves of Atlantic salmon in the main challenge with *F. psychrophilum* 6\_S (for Atlantic salmon) and *F. psychrophilum* 19\_5 (for rainbow trout) at  $2 \times 10^7$  CFU/mL. Atlantic salmon (n=1,187;  $1.8 \pm 0.237$ g) and rainbow trout (n=1,024;  $1.8 \pm 0.247$  g;) were exposed to hydrogen peroxide (200 mg/L) for 1 h prior to immersion challenge with the bacteria for 4 h (Atlantic salmon) or 1.5 h (rainbow trout).....**60**

**Figure 2.8** *F. psychrophilum* nested PCR used to confirm representative colonies recovered from moribund or deceased fish during the 2019 Atlantic salmon main immersion challenge trial. Top lane: 100-bp gene ruler (position 1 and 12), positive control (position 2), samples 2-11 (position 3-11); bottom lane: 100bp gene ruler (position 1 and 8), samples 12-15 (position 2-7) and NTC (position 8).....**61**

**Figure 2.9** Manhattan plots for genome-wide association analysis of *F. psychrophilum* resistance in Atlantic salmon. The figure shows the  $-\log_{10}$  (p-value) of the test statistics for each SNP plotted against the physical positions of the markers on the chromosomes. The two lines in the panel indicate significance threshold after accounting for multiple testing using either: Bonferroni correction (dotted line) or False Discovery Rate correction (dashed line). Fabian Grammes (Senior Scientist, AquaGen Norway) .....**63**

**Figure 2.10** Manhattan plots for genome-wide association analysis of *F. psychrophilum* resistance in rainbow trout. The figure shows the  $-\log_{10}$  (p-value) of the test statistics for each SNP plotted against the physical positions of the markers on the chromosomes. The two lines in the panel indicate significance threshold after accounting for multiple testing using either: Bonferroni correction (dotted line) or False Discovery Rate correction (dashed line). Figure provided by Fabian Grammes (Senior Scientist, AquaGen Norway).....**69**

**Figure 3.1** Schematic representation of the challenge trials, time at which the challenges started, sampling time points and challenge termination point (Atlantic salmon: 21 dpc and rainbow trout: 31 dpc).....**79**

**Figure 3.2** The red box illustrates the location of skin tissue dissected (from the bottom of the dorsal fin until the bottom of the anal fin) from rainbow trout fry for DNA extraction for genotyping.....**83**

**Figure 3.3** *F. psychrophilum* PCR to test the primers, targeting the *rpoC* gene, specificity on different *F. psychrophilum* isolates at 50ng/  $\mu$ L. Samples were run agarose gel (1%). From left to right: 100bp GeneRuler Ladder (position 1). *F. psychrophilum* 6\_S (position 2), *F. psychrophilum* 19\_5 (position 3), *F. psychrophilum* 469 (position 4), *F. psychrophilum* 468 (position 5), *F. psychrophilum* JIP02/86 (position 6) and NTC (position 7). PCR product is expected at  $\sim 164$ bp.....**96**

**Figure 3.4** *F. psychrophilum* PCR to test the primers, targeting the *rpoC* gene, specificity on different non- *F. psychrophilum* bacteria genera isolates at 50ng/  $\mu$ L. Samples were run agarose gel (1%). From left to right: 100bp GeneRuler Ladder (position 1), *Aeromonas salmonicida* sub. *salmonicida* (position 2), *Francisella orientalis* (position 3), *Vibrio anguillarum* (position 4), *Escherichia coli* (5), *Yersinia ruckeri* (6), *Edwardsiella piscicida* (7) NTC position (8 and 9) and *F. psychrophilum* JIP02/86 (position 10). PCR product is expected at  $\sim 164$ bp.....**97**

**Figure 3.5** PCR analysis run to test *EF1-a* primers specificity on DNA samples extracted from two Atlantic salmon head kidney randomly selected from 0 dpc (position 2 and 3), and one randomly selected rainbow trout head kidney from 0 dpc at 50ng/  $\mu$ L. Samples were run agarose gel (1%). From left to right: 100-bp GeneRuler Ladder (position 1), Atlantic salmon head kidney (position 2), Atlantic salmon head kidney

(position 3), rainbow trout head kidney (position 4), Atlantic salmon liver extracted by Vera et al. (2017) (position 5). PCR product is expected at ~ 78 bp.....98

**Figure 3.6** Agarose gel (1%) used to check DNA integrity and quality. Lambda HindIII ladder (Position 1 and 6), Atlantic salmon head kidney day 0 post-challenge (position 2), Atlantic salmon head kidney day 3 post-challenge (position 3), rainbow trout head kidney day 6 post-challenge (position 4), and rainbow trout head kidney day 10 post-challenge (position 5).....99

**Figure 3.7** qPCR (*rpoC*) standard curve developed using seven serial dilutions of isolate *F. psychrophilum* 6\_S (black line) and *F. psychrophilum* 19\_5 (grey dotted line). Standards range in concentration from  $10^1$  to  $10^7$  theoretical number of isolate *F. psychrophilum* 6\_S (x-axis). The Ct was within threshold when values were between 15 and 35 (y-axis). For Atlantic salmon (n=3, p>0.05) the slope was of -3.30 ( $R^2 = 0.998$ ), indicating an efficiency of 100 % and for rainbow trout (n=3, p>0.05) the slope was -3.25 ( $R^2 = 0.994$ ), indicating an efficiency of 101%.....101

**Figure 3.8** Representative image of melt curve for qPCR, targeting the *rpoC* gene in *F. psychrophilum* 6\_S, standard curve developed using seven serial dilutions of isolate *F. psychrophilum* 6\_S. Standards range in concentration from  $10^1$  to  $10^7$  with all demonstrating a melting point of ca. 72 °C showing a single product being amplified.....102

**Figure 3.9** Representative image of performance of *F. psychrophilum* specific SYBER Green qPCR amplifying the *rpoC* gene using seven serial dilutions of isolate *F. psychrophilum* 6\_S STC. The amplification curves (in duplicates) show the fluorescence development over time using a dilution range of  $10^1$  to  $10^7$  copies/  $\mu$ L of the *F. psychrophilum* DNA standard.....103

**Figure 3.10** qPCR (*EF1- $\alpha$*  gene) standard curve developed using seven serial dilutions of Atlantic salmon head kidney DNA (black line) and rainbow trout head kidney DNA (grey dotted line). Standards range in concentration from  $10^2$  to  $10^7$  theoretical number of isolate *F. psychrophilum* 6\_S (x-axis). The Ct was within threshold when values were between 15 and 35 (y-axis). For Atlantic salmon (n=3, p>0.05) the slope was of -3.20 ( $R^2 = 0.997$ ), indicating an efficiency of 103 % and for rainbow trout (n=3, p>0.05) the slope was -3.33 ( $R^2 = 0.997$ ), indicating an efficiency of 100%.....105

**Figure 3.11** Representative image of melt curve for qPCR, targeting the *EF1- $\alpha$*  gene in Atlantic salmon head kidney, standard curve developed using seven serial dilutions of isolate *F. psychrophilum* 6\_S. Standards range in concentration from  $10^2$  to  $10^7$  with all demonstrating a melting point of ca. 78 °C showing a single product being amplified.....106

**Figure 3.12** Representative image of performance of SYBER Green qPCR amplifying the *EF1- $\alpha$*  gene using seven serial dilutions of Atlantic salmon head kidney. The amplification curves (in duplicates) show the fluorescence development over time using a dilution range of  $10^2$  to  $10^7$  copies/  $\mu$ L of Atlantic salmon head kidney DNA.....107

**Figure 3.13** *F. psychrophilum* DNA copy number ( $\log_{10}$ ) in Atlantic salmon head kidney challenged by immersion with *F. psychrophilum* 6\_S. Fish was sampled at different time points post-challenge (i.e., 0 [n=10], 3 [n=18], 6 [n=18] and 10 [n=24] dpc) and qPCR was used to detect and quantify bacterial abundance in the head kidney tissues of Atlantic salmon with different genotypes on chromosome 9 at the different time points. Day 0 is not represented in the figure as no detection was observed. N/N represents the 'susceptible' genotype, Y/Y the 'resistant' genotype and Y/N the heterozygous genotype. There is not a significant difference in bacterial load between

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |     |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| fish genotypes on day 3, 6 and 10 ( $p > 0.05$ ), while there is a significant difference between 0 vs 3,6,10 dpc regardless of the fish genotype ( $p < 0.05$ ).....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 109 |
| <b>Figure 3.14</b> <i>F. psychrophilum</i> DNA copy number ( $\log_{10}$ ) in Atlantic salmon head kidney challenged by immersion with <i>F. psychrophilum</i> 6_S. Fish was sampled at different time points post-challenge (i.e., 0 [n=10], 3 [n=18], 6 [n=18] and 10 [n=24] dpc) and qPCR was used to detect and quantify bacterial abundance in the head kidney tissues of Atlantic salmon with different genotypes on chromosome 5 at the different time points. Day 0 is not represented in the figure as no detection was observed. N/N represents the 'susceptible' genotype, Y/Y the 'resistant' genotype and Y/N the heterozygous genotype. There is not a significant difference in bacterial load between fish genotypes on day 3, 6 and 10 ( $p > 0.05$ ), while there is a significant difference between 0 vs 3, 6, 10 dpc regardless of the fish genotype ( $p < 0.05$ ).....        | 110 |
| <b>Figure 3.15</b> <i>F. psychrophilum</i> DNA copy number ( $\log_{10}$ ) in Atlantic salmon head kidney challenged by immersion with <i>F. psychrophilum</i> 19_5. Fish was sampled at different time points post-challenge (i.e., 0 dpc [n=7], 3 dpc [n=15], 6 dpc [n=15], 10 dpc [n=16]) and qPCR was used to detect and quantify bacterial abundance in the head kidney tissues of Atlantic salmon with different genotypes on chromosome 3 at the different time points. Day 0 is not represented in the figure as no detection was observed. N/N represents the 'susceptible' genotype, Y/Y the 'resistant' genotype and Y/N the heterozygous genotype. There is not a significant difference in bacterial load between fish genotypes on day 3, 6 and 10 ( $p > 0.05$ ), while there is a significant difference between 0 vs 3,6,10 dpc regardless of the fish genotype ( $p < 0.05$ )..... | 111 |
| <b>Figure 3.16</b> <i>F. psychrophilum</i> DNA copy number ( $\log_{10}$ ) in Atlantic salmon head kidney challenged by immersion with <i>F. psychrophilum</i> 19_5. Fish was sampled at different time points post-challenge (i.e. 0 dpc [n=7], 3 dpc [n=15], 6 dpc [n=15], 10 dpc [n=16]) and qPCR was used to detect and quantify bacterial abundance in the head kidney tissues of Atlantic salmon with different genotypes on chromosome 8 at the different time points. Day 0 is not represented in the figure as no detection was observed. N/N represents the 'susceptible' genotype, Y/Y the 'resistant' genotype and Y/N the heterozygous genotype. There is not a significant difference in bacterial load between fish genotypes on day 3, 6 and 10 ( $p > 0.05$ ), while there is a significant difference between 0 vs 3,6,10 dpc regardless of the fish genotype ( $p < 0.05$ ).....  | 112 |
| <b>Figure 4.1</b> Diagram used to show a representative ELISA assay plate layout. In this representative plate, the 96-well plate was coated with IoA_240, <i>F. psychrophilum</i> JIP 02/96 and <i>Y. ruckeri</i> NCIMB 2194. The plate was then incubated with 100 $\mu$ L/well of the mAb 9G7, 32, 5A9, 5, 8 and PBS (negative control) for 1 h at RT.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | 127 |
| <b>Figure 4.2</b> Diagram illustrating the western blotting apparatus used for this study and how to assemble the protein gel in the cast. For the purpose of the illustration the blotting paper, protein gel and nitrocellulose membrane are not to size, however blotting paper and nitrocellulose membrane need to be cut to the size of the protein gel during before being assembled in the blotting apparatus.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | 130 |
| <b>Figure 4.3</b> Image illustrating the 2D SDS PAGE set up. The IPG strips were inserted on top of the gel, and a precision protein dual colour molecular weight standard (marker) was added to the anodic end of the strip and finally sealed with warm agarose sealing solution. The gels were run at 100 V for 90 min.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 134 |
| <b>Figure 4.4</b> Histopathology processing. Figure 4.4 (a) tissue fixed using paraffin wax; (b) microtome used for sectioning the paraffin blocks (c); heated slides dryer used to dry the sectioned tissues (i.e., representative image of head tissues) on positive charged slides for antigen retrieval analysis.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | 136 |

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |            |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <b>Figure 4.5</b> Representative image of characterisation of anti- <i>F. psychrophilum</i> mAbs and pAbs on isolate <i>F. psychrophilum</i> 240 by IFAT. The bacterial smear was incubated with (A) anti- <i>F. psychrophilum</i> pAb 151 (B) mAb 9G7, (C) mAb 32 and (D) anti-KHV mAb (negative control).....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | <b>141</b> |
| <b>Figure 4.6</b> Characterisation of anti- <i>F. psychrophilum</i> mAbs (9G7, 32, 5A9, 8 and 5) by ELISA. mAbs specificity was tested in duplicates on different <i>F. psychrophilum</i> isolates (n=7), <i>F. hydatidis</i> (n=1), <i>A. salmonicida</i> isolates (n=2), <i>Y. ruckeri</i> (n=1) and <i>V. anguillarum</i> (n=1).....                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | <b>143</b> |
| <b>Figure 4.7</b> 1D-SDS PAGE and Western Blotting was performed on different strains of <i>F. psychrophilum</i> isolates. (A) Whole cell lysate treated with proteinase K of the following bacterial strains IoA_240 (lane 1), IoA_350 (lane 2), Fp. <sup>T</sup> 1947 (lane 3) and <i>F. hydatidis</i> (lane 4), <i>F. aquatile</i> (lane 5), <i>F. psychrophilum</i> 18_S (lane 6), <i>V. anguillarum</i> (lane 7), <i>A. salmonicida</i> (NCIMB 1002) (lane 8) and <i>A. salmonicida</i> (MT 423)(lane 9) were separated by SDS-PAGE and stained with silver stain. Western blot was performed following SDS-PAGE, the gels were blotted onto nitrocellulose membranes and incubated with anti- <i>F. psychrophilum</i> monoclonal antibodies (B) mAb 9G7, (C) mAb 32 and (D) TBS (negative control blot)..... | <b>146</b> |
| <b>Figure 4.8</b> Representative 2D gels electrophoresis of the protein profile of the whole cell lysate of two <i>F. psychrophilum</i> isolates (A) IoA_240 and (B) Fp. <sup>T</sup> 1947 (60 µg protein). Duplicate gels were run for each treatment group as technical replicates. The gels were stained with Bio-Safe™ Coomassie Stain and the marker used was Precision Plus Protein Dual Colour Molecular Weight Standard Marker (10-250 kDa).....                                                                                                                                                                                                                                                                                                                                                           | <b>149</b> |
| <b>Figure 4.9</b> Western blot analysis of <i>Flavobacterium psychrophilum</i> strains. Representative 2D Western blot gels of <i>F. psychrophilum</i> isolates 240 separated by 2D SDS-PAGE and then blotted onto nitrocellulose and reacted with mAb 9G7 (A) and mAb 32 (B) and TBS- negative control (C). These western blots were performed in duplicates. Precision Plus Protein Dual Color Molecular Weight Standard Marker (10-250 kDa) was used.....                                                                                                                                                                                                                                                                                                                                                       | <b>150</b> |
| <b>Figure 4.10</b> Representative visual scoring of <i>F. psychrophilum</i> infection in Atlantic salmon gills following IHC with anti- <i>F. psychrophilum</i> pAb 151. Brown staining indicates presence of <i>F. psychrophilum</i> colonies.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | <b>153</b> |
| <b>Figure 4.11</b> Immunohistochemical detection of antigen uptake following <i>F. psychrophilum</i> immersion challenge in Atlantic salmon in the skin epithelium at 3 dpc around the mouth. Detection of <i>F. psychrophilum</i> cells using anti- <i>F. psychrophilum</i> pAb 151 (size of specimens: 200 µm and magnification to 20 µm). Detection and quantification of cells were carried out by an individual observer(Valeria Macchia)..                                                                                                                                                                                                                                                                                                                                                                   | <b>154</b> |
| <b>Figure 4.12</b> Immunohistochemical detection of antigen uptake following <i>F. psychrophilum</i> immersion challenge in mucosal tissues of Atlantic salmon. Detection of <i>F. psychrophilum</i> cells using anti- <i>F. psychrophilum</i> pAb 151. The black arrows point to the brown staining ( <i>F. psychrophilum</i> colonies) in the different mucosal tissues (A) the gills, (B) the head kidney o and (C) the gut (mid-intestine) of an Atlantic salmon sampled 3 dpc (size of specimens: 20 µm). Detection and quantification of cells were carried out by an individual observer. Detection and quantification of cells were carried out by an individual observer. Detection and quantification of cells were carried out by an individual observer (Valeria Macchia) .....                        | <b>155</b> |
| <b>Figure 4.13</b> Immunohistochemical detection of antigen uptake following <i>F. psychrophilum</i> immersion challenge in different tissues of Atlantic salmon. Detection of <i>F. psychrophilum</i> cells using anti- <i>F. psychrophilum</i> pAb 151. The black arrows point to the brown staining ( <i>F. psychrophilum</i> colonies) in the different tissues (A) the skin,                                                                                                                                                                                                                                                                                                                                                                                                                                  |            |

(B) the gut (mid-intestine) and (C) the gills of a rainbow trout sampled 3 dpc (size of specimens: 10  $\mu$ m and 20  $\mu$ m). Negative control slide of gill tissues(D). Detection and quantification of cells were carried out by an individual observer(Valeria Macchia)..156

**Figure 4.14** Immunohistochemical detection of IgT<sup>+</sup> cells in mucosal organs following immersion challenge with *F. psychrophilum*. Representative image of a magnified Atlantic salmon skin (A), gill (B) and (C) gut tissues (specimen size: 20  $\mu$ m) at 3 dpc. In the mucosal organs, IgT positive cells were identified as oval dark red spots. Detection and quantification of cells were carried out by an individual observer (Valeria Macchia).....160

**Figure 4.15** Immunohistochemical detection of IgT<sup>+</sup> cells in mucosal organs and spleen following immersion challenge with *F. psychrophilum*. Representative image of a magnified rainbow trout skin (A), gill (B) and (C) gut tissues, (D) negative control- gill tissue (specimen size: 20  $\mu$ m) at 3 dpc. In the organs, IgT positive cells were identified as oval dark red spots. Detection and quantification of cells were carried out by an individual observer (Valeria Macchia).....161

**Figure 4.16** Box plot representing the mean number of IgT positive cells (n= 3 fish per time point) detected in 3 fields of view by an individual observed in *F. psychrophilum* infected Atlantic salmon mucosal tissues (gills, gut and spleen) sampled at 0 (n=3), 3 (n=3), 6 (n=3) and 10 (n=3) dpc.....162

**Figure 4.17** Box plot representing the mean number of IgT positive cells (n= 3 fish per time point) detected in 3 fields of view by an individual observed in *F. psychrophilum* infected rainbow trout mucosal tissues (gills, gut, and spleen) sampled at 0 (n=3), 3 (n=3), 6 (n=3) and 10 (n=3) dpc.....163

**Figure 5.1** Boxplots of log-count per million (log-CPM) values showing expression distributions of filtered unnormalised data for each sample. The line dividing the box represents the median of the data and top and bottom of the box shows the upper and lower quartiles respectively. The samples are organised by their median, and the whiskers show the highest and lowest values, and it is possible to see that one sample (VS-10-3) is an outlier.....180

**Figure 5.2** Boxplots of log-CPM values showing expression distributions of filtered and Trimmed Mean of M-values (TMM) normalised data for each sample. The line dividing the box represents the median of the data and top and bottom of the box shows the upper and lower quartiles respectively. The samples are organised by their median, and the whiskers show the highest and lowest values.....181

**Figure 5.3** Scatterplot of the biological coefficient of variation (BCV) against the average abundance of each gene (n= 26,700). The vertical axis of the BCV plot shows square-root estimates of the common, also known as biological coefficient of variation (BCV), trended and tagwise negative binomial (NB) dispersions. The NB dispersions tend to be higher for genes with very low counts.....183

**Figure 5.4a** Principal component analysis (PCA) plot of the top 13,000 genes of Atlantic salmon head kidney samples infected with *F. psychrophilum*. The samples were collected at day 0 (control fish), day 3,6 and 10 post challenge. RNA-seq samples (n=56) clustered according to their gene expression. The labels on the points indicate the fish genotypes (i.e. Y/Y, Y/N and N/N) on chromosome 5.....184

**Figure 5.4b** Principal component analysis (PCA) plot of the top 13,000 genes of Atlantic salmon head kidney samples infected with *F. psychrophilum*. The samples were collected at day 0 (control fish), day 3,6 and 10 post challenge. RNA-seq samples (n=56) clustered according to their gene expression. The labels on the points indicate

the fish genotypes (i.e. Y/Y, Y/N and N/N) on chromosome 9.....185

**Figure 5.6** Volcano plot for differential gene expression (n=26,700). Scattered points represent genes: the x-axis is the log fold change (logFC) which represents the difference between the levels of expression for each gene between the control vs infected animals, whereas the -log<sub>10</sub> of the p-value for the contrast: Infected (sampling points after D0) vs control. Dashed lines indicate cut-offs for significance Volcano plot. Genes with an FDR < 0.05 and logFC > 1 were considered to be differentially expressed (pink dots). The most statistically significant genes are towards the top of plot, the most up regulated genes are towards the right, the most down regulated genes are towards the left.....187

**Figure 5.7** Barcode plot of up regulated gene sets (red bars, top of the plot) and down regulated gene set (blue bar, bottom of plot) of the Langevin et al. (2012) data against the DE genes identified in the Atlantic salmon study (n=717). The curve (or worm) above the barcode shows the relative local enrichment of the bars in each part of the plot. The dotted horizontal line indicates neutral enrichment. All genes are ranked from left to right by decreasing log-fold change for the contrast and the genes within the gene set are represented by vertical bars, forming the barcode-like pattern.....189

**Figure 5.8** Barcode plot of up regulated gene sets (red bars, top of the plot) and down regulated gene set (blue bar, bottom of plot) of the Marancik et al. (2015) data against the DE genes identified in the Atlantic salmon study (n=250). The curve (or worm) above the barcode shows the relative local enrichment of the bars in each part of the plot. The dotted horizontal line indicates neutral enrichment. The dotted horizontal line indicates neutral enrichment. All genes are ranked from left to right by decreasing log-fold change for the contrast and the genes within the gene set are represented by vertical bars, forming the barcode-like pattern.....190

**Figure 5.9** Dot plot visualising significant GO enrichment analysis results (FDR < 0.05). The y-axis displays the different (biological process) GO terms, while the x-axis shows the -log<sub>10</sub> of the Elim p-value (testing for enrichment of the corresponding GO term within the DE genes). The size of the dots corresponds to the number of DE genes associated with each GO term.....192

**Figure 5.10** Heatmaps, showing the scaled gene expression for the DE genes annotated to the GO terms: **A:** Immune response GO:0006955; **B:** cell chemotaxis GO:0060326; **C:** defence response GO:0006952; **D:** innate immune response GO:0045087; **E:** cellular iron GO:0006879. In the diagram each column (n=56) represents an individual animal (i.e. head kidney) divided according to the day post challenge. The scale represents normalised gene expression (expression mean cpm / standard deviation), the lighter the colour in the scale (i.e. yellow), the higher the expression of the gene). The lightest colour (i.e. yellow) in the scale represent the highly expressed genes, and the darkest colour (i.e. dark blue) represent the lowly expressed genes.....193

**Figure 5.11** Heatmap showing the scaled gene expression for the commonly DE genes expressed in rainbow trout following *F. psychrophilum* infection in Langevin et al. (2012) and Marancik et al. (2015) studies and in *F. psychrophilum* infected (sampling points after D0) vs control Atlantic salmon head kidney. The scale represents normalised gene expression (scaled for each gene within each study), with the lightest colour (i.e. yellow) representing the highly expressed genes, and the darkest colour (i.e. dark blue) representing the lowly expressed genes. In Figure 5.10 each column represents a study (i.e. rainbow trout\_ Langevin et al. (2012) [omy\_lang];

|                                                                                                                                             |     |
|---------------------------------------------------------------------------------------------------------------------------------------------|-----|
| rainbow trout_Marancik et al. (2015) [omy_maran]; Atlantic salmon [ssa]) and the experimental group (i.e. control vs infected animals)..... | 197 |
|---------------------------------------------------------------------------------------------------------------------------------------------|-----|

## List of Tables

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| <b>Table 1.1</b> Comparison of RNA-seq with other methods for surveying transcriptome. Table extrapolated from Qian et al., (2014).....                                                                                                                                                                                                                                                                                                                                                                                                                            | 24  |
| <b>Table 1.2</b> Representative publications on RNA-seq analysis used to analyse fish transcriptomes in response to pathogens.....                                                                                                                                                                                                                                                                                                                                                                                                                                 | 25  |
| <b>Table 1.3</b> High-density SNP chips developed for aquaculture species.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | 29  |
| <b>Table 2.1</b> Characteristics of the <i>F. psychrophilum</i> isolates used for the Atlantic salmon and rainbow trout immersion challenge model.....                                                                                                                                                                                                                                                                                                                                                                                                             | 39  |
| <b>Table 2.2</b> Top 20 sequences producing significant alignments when blasting nucleotide sequence of 16S rRNA from each bacterial isolate in NCBI.....                                                                                                                                                                                                                                                                                                                                                                                                          | 51  |
| <b>Table 2.3</b> Table showing the different statistical comparisons made between in the preliminary and main challenge trials.....                                                                                                                                                                                                                                                                                                                                                                                                                                | 56  |
| <b>Table 2.4</b> Outline of the preliminary and main challenges with <i>F. psychrophilum</i> in Atlantic salmon and rainbow trout, including the bacterial isolates used and their origins, the challenge concentrations, and percentage of sampled mortalities where <i>F. psychrophilum</i> was recovered by bacteriological culture and confirmed by PCR.....                                                                                                                                                                                                   | 57  |
| <b>Table 3.1</b> Techniques used to identify bacteria.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 72  |
| <b>Table 3.2</b> Primers used for qPCR of Atlantic salmon and rainbow trout <i>EF1-α</i> and <i>rpoC</i> genes.....                                                                                                                                                                                                                                                                                                                                                                                                                                                | 87  |
| <b>Table 3.3</b> Blasting results obtained from the NCBI website of the top 15 sequences producing significant alignments when blasting the PCR product produced following the <i>F. psychrophilum</i> PCR assay targeting the <i>rpoC</i> gene of <i>F. psychrophilum</i> .....                                                                                                                                                                                                                                                                                   | 92  |
| <b>Table 4.1</b> List of bacterial species cultured for the characterisation of the anti- <i>F. psychrophilum</i> mAbs .....                                                                                                                                                                                                                                                                                                                                                                                                                                       | 123 |
| <b>Table 4.2</b> Characterisation of anti- <i>F. psychrophilum</i> mAbs (9G7, 32, 5A9, 8 and 5) by ELISA. mAbs specificity was tested in duplicates on different <i>F. psychrophilum</i> isolates (n=7), <i>F. hydati</i> (n=1), <i>A. salmonicida</i> isolates (n=2), <i>Y. ruckeri</i> (n=1), <i>V. anguillarum</i> (n=1) and PBS. The ELISA plates were read with a spectrophotometer and the cut-off point was determined to be 3 × average negative control (PBS, OD: 0.364). OD >0.7-1.0 (++) . OD > 0.4- 0.6 (+). No reactivity=negative reactions (-)..... | 144 |
| <b>Table 4.3</b> Western blotting of the different bacterial species tested by 1D SDS PAGE to determine reactivity of mAb 9G7 and mAb 32 with different bacterial species and isolates to investigate cross reactivity with non- <i>F. psychrophilum</i> species. The staining intensity to the band at the 250 kDa was determined by a single observer: (+++) very strong band, (++) strong band, (+) weak band, (-) no reaction.....                                                                                                                             | 147 |
| <b>Table 4.4</b> Detection of <i>F. psychrophilum</i> cells by IHC using anti- <i>F. psychrophilum</i> pAb. Brown staining intensity (indicating the presence of <i>F. psychrophilum</i> cells) in Atlantic salmon mucosal tissues (gills, skin, gut and head kidney) sampled from 3 fish per time point was assessed by a single researcher and blind checked by another researcher. Scoring scale 0= clear, 1= light, 2= moderate and 3= advanced.....                                                                                                           | 157 |
| <b>Table 4.5</b> Detection of <i>F. psychrophilum</i> cells by IHC using anti- <i>F. psychrophilum</i> pAb. Brown staining intensity (indicating the presence of <i>F. psychrophilum</i> cells) in rainbow trout mucosal tissues (gills, skin, gut and head kidney) sampled from 3 fish                                                                                                                                                                                                                                                                            |     |

|                                                                                                                                                                |            |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| per time point was assessed by a single researcher and blind checked by another researcher. Scoring scale 0= clear, 1= light, 2= moderate and 3= advanced..... | <b>158</b> |
| <b>Table 5.1</b> Quantitative trait loci discovered for resistance to <i>Flavobacterium psychrophilum</i> in rainbow trout.....                                | <b>170</b> |
| <b>Table 5.3</b> Common DE genes identified across the Langevin et al. (2012), Marancik et al. (2015) and the Atlantic salmon transcriptomic studies.....      | <b>198</b> |

## List of Abbreviations and Definitions

|                    |                                                   |
|--------------------|---------------------------------------------------|
| °C                 | Degree Celsius                                    |
| omy                | Oncorhynchus mykiss                               |
| QTL                | Quantitative trait loci                           |
| EF1 $\alpha$       | Elongation factor 1 $\alpha$                      |
| AWERB              | Animal Welfare and Ethical Review Body            |
| BPI                | Bactericidal permeability-increasing protein-like |
| BCWD               | Bacterial cold-water disease                      |
| BKD                | Bacterial kidney disease                          |
| BSA                | Bovine serum albumin                              |
| BLAST              | Basic Local Alignment Search Tool                 |
| bp                 | base pair                                         |
| CATH               | Cathelicidins                                     |
| Buffer A1          | Guanidinium chloride                              |
| Buffer ATL         | Sodium dodecyl sulfate                            |
| C3                 | Complement component 3                            |
| CCL19              | C-C motif chemokine 19-like                       |
| CCL4               | C-C motif chemokine 4 like                        |
| CCL8               | C-C motif chemokine 8                             |
| CFU                | Colony forming unit                               |
| CH25H              | Cholesterol 25-Hydroxylase                        |
| CLRs               | C-type lectin receptor                            |
| cm                 | Centimetre                                        |
| CPM                | Centrifugal force                                 |
| Ct                 | Cycle threshold                                   |
| CXL11              | C-X-C motif chemokine 11-like                     |
| d                  | Days                                              |
| DAMPs              | Damage-associated molecules patterns              |
| DAPI               | 4',6-diamidino-2-phenylindole                     |
| ddH <sub>2</sub> O | Double distilled water                            |
| DE                 | Differential expression                           |

|                                |                                                         |
|--------------------------------|---------------------------------------------------------|
| DEGs                           | Differentially expressed genes                          |
| dH <sub>2</sub> O              | Distilled water                                         |
| DMSO                           | Dimethyl sulfoxide                                      |
| DNA                            | Deoxyribonucleic acid                                   |
| dpc                            | Days post-challenge                                     |
| dpi                            | Days post-infection                                     |
| e.g.                           | <i>exempli gratia</i>                                   |
| ER                             | Endoplasmic reticulum                                   |
| EDTA                           | Ethylenediaminetetraacetic acid                         |
| EDTA                           | Exempli gratia (for example)                            |
| EF1 $\alpha$                   | Ethylenediaminetetraacetic acid                         |
| <i>EF1-<math>\alpha</math></i> | Elongation factor alpha                                 |
| ELISA                          | Enzyme-linked immunosorbent assay                       |
| ERD                            | Enteric red mouth disease                               |
| EtBr                           | Ethidium bromide                                        |
| <i>F. psychrophilum</i>        | <i>Flavobacterium psychrophilum</i>                     |
| FAO                            | Food and Agriculture Organization of the United Nations |
| FCS                            | Foetal calf/bovine serum                                |
| FDR                            | False discovery rate                                    |
| GD                             | Bacterial gill Disease                                  |
| GO                             | Geno ontology                                           |
| GWAS                           | Genome-wide association study                           |
| GS                             | Genomic selection                                       |
| h                              | Hours                                                   |
| H <sub>2</sub> O <sub>2</sub>  | Hydrogen peroxide                                       |
| HCK                            | Hemopoietic cell kinase                                 |
| HSMI                           | Heart and Skeletal Muscle Inflammation                  |
| IEF                            | Isoelectric focusing                                    |
| IFAT                           | Immunofluorescent antibody technique                    |
| IFNs                           | Interferons                                             |
| IgT                            | immunoglobulin T                                        |
| IHC                            | Immunohistochemistry                                    |

|              |                                                               |
|--------------|---------------------------------------------------------------|
| IL-10        | Interleukin 10                                                |
| IL-1 $\beta$ | Interleukin 1 beta                                            |
| IL-22        | Interleukin 22                                                |
| IL-6         | Interleukin 6                                                 |
| IL-8         | Interleukin 8                                                 |
| IIs          | Interleukins                                                  |
| IoA          | Institute of Aquaculture                                      |
| IPN          | Infectious Pancreatic Necrosis                                |
| ISG          | Interferon stimulated genes                                   |
| JAK-STAT     | Janus kinase-signal transducer and activator of transcription |
| kg           | Kilogram                                                      |
| L            | Litre                                                         |
| LECT2        | Leukocyte cell-derived chemotaxin-2-like                      |
| Log2         | Log base 2                                                    |
| logFC        | log fold change                                               |
| LPS          | Lipopolysaccharide                                            |
| M            | Molar                                                         |
| m IgM        | Mucosal immunoglobulin M                                      |
| mAb          | Monoclonal antibody                                           |
| MAC          | Membrane attack complex                                       |
| MALT         | Mucosa-associated lymphoid tissue                             |
| MAOA         | Anacker and Ordal medium                                      |
| MAPSs        | MBL-associated serine proteases                               |
| MAS          | Marker-assisted selection                                     |
| mg           | Milligrams                                                    |
| min          | Minutes                                                       |
| mL           | Millilitres                                                   |
| mM           | Millimolar                                                    |
| mPCR         | Molecular typing using multiplex PCR                          |
| MVA          | Modified Veggietone Agar                                      |
| MVB          | Modified Veggietone Broth                                     |
| NaCl         | Sodium chloride                                               |

|             |                                                           |
|-------------|-----------------------------------------------------------|
| NB          | Negative binomial                                         |
| NCBI        | National Centre for Biotechnology Information             |
| NGS         | next generation sequencing technologies                   |
| NO          | Nitric oxide                                              |
| NRTKs       | Non-receptor tyrosine kinases                             |
| OD          | Optical density                                           |
| p           | p-value                                                   |
| pAb         | Polyclonal antibody                                       |
| PAMPs       | Pathogen-associated molecular patterns                    |
| PCA         | Principal component analysis                              |
| PD          | Pancreas Disease                                          |
| pIgR        | Polymeric immunoglobulin receptor                         |
| ppm         | Sodium chloride                                           |
| ppt         | Parts per thousand                                        |
| PRRs        | Pattern recognition receptor                              |
| PUFAs       | N-polyunsaturated fatty acids                             |
| QL          | Quasi-likelihood                                          |
| QN          | Quantile normalisation                                    |
| qPCR        | Quantitative polymerase chain reaction                    |
| CPM         | Counts-per-million                                        |
| RAS         | Recirculating aquaculture systems                         |
| RGNNV       | Red-spotted grouper nervous necrosis virus                |
| RNA-seq     | RNA sequencing                                            |
| ROS         | Reactive oxygen species                                   |
| <i>rpoC</i> | $\beta'$ DNA-dependent RNA polymerase                     |
| RT          | Room temperature                                          |
| RTFS        | Rainbow trout fry syndrome                                |
| s.d.        | Standard deviation                                        |
| SC          | secretory component                                       |
| SDS-PAGE    | Sodium dodecyl-sulfate polyacrylamide gel electrophoresis |
| Sec         | Seconds                                                   |
| SGIV        | Singapore grouper iridovirus                              |

|               |                                    |
|---------------|------------------------------------|
| SNP           | Single nucleotide polymorphism     |
| SOCS          | Suppressor of cytokine signalling  |
| SRS           | Salmonid Rickettsial Septicaemia   |
| SRs           | Scavenger receptors                |
| TBS           | Tris-buffered saline               |
| TBST          | Tris-buffered saline with Tween-20 |
| TLR22         | Toll-like receptor 22              |
| TLR3          | Toll-like receptor 3               |
| TLR8          | Toll-like receptor 8               |
| TMB           | 3,3' 5,5' tetramethylbenzidine     |
| TNF           | Tumor necrosis factors             |
| TNF- $\alpha$ | Tumor necrosis factor alpha        |
| TSA           | Tryptone soya agar                 |
| UDG           | Uracil-DNA glycosylase             |
| V             | Volt                               |
| v/v           | Volume/volume                      |
| w/v           | Weight/volume                      |
| CH25H         | Cholesterol 25-Hydroxylase         |
| $\mu\text{g}$ | Micrograms                         |
| $\mu\text{l}$ | Microliters                        |
| $\mu\text{m}$ | Micrometres                        |

# Chapter 1: General Introduction

## 1.1 Background

Population growth has led to an increase in global seafood production and global food fish consumption increased at an average annual rate of 3.1% from 1961 to 2017, with a total global production of 214 million tonnes in 2020, worth ca. USD 424 billion (FAO, 2022). Fish is a major source of animal protein, essential amino acids, n-polyunsaturated fatty acids (PUFAs), minerals (calcium, phosphorous, iodine, zinc, iron and selenium), trace elements and vitamins (D and B) (Hosomi et al., 2012; Rice & Garcia, 2011) especially in low income and food deficient countries (Kawarazuka & Béné, 2011). In 2020, aquaculture accounted for 49.2% of the total fish production (FAO, 2022). The main 'staple' salmonid species that dominate aquaculture production are Atlantic salmon (*Salmo salar*) and followed by coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*). Atlantic salmon has been one of the main contributors to the growth of fisheries global trade and aquaculture production. Atlantic salmon aquaculture is the most profitable and advanced salmonid production industry. In 2018, 2.44 million tonnes of farmed Atlantic salmon were produced globally, mainly in Norway (53% global share), Chile (27% global share), Scotland (7% global share), and Canada (5% global share) (FAO, 2021). In 2020, Atlantic salmon and rainbow trout exports accounted for 18.4% of the value of all aquatic products, and despite COVID-19 pandemic, their trade has remained resilient (FAO, 2022).

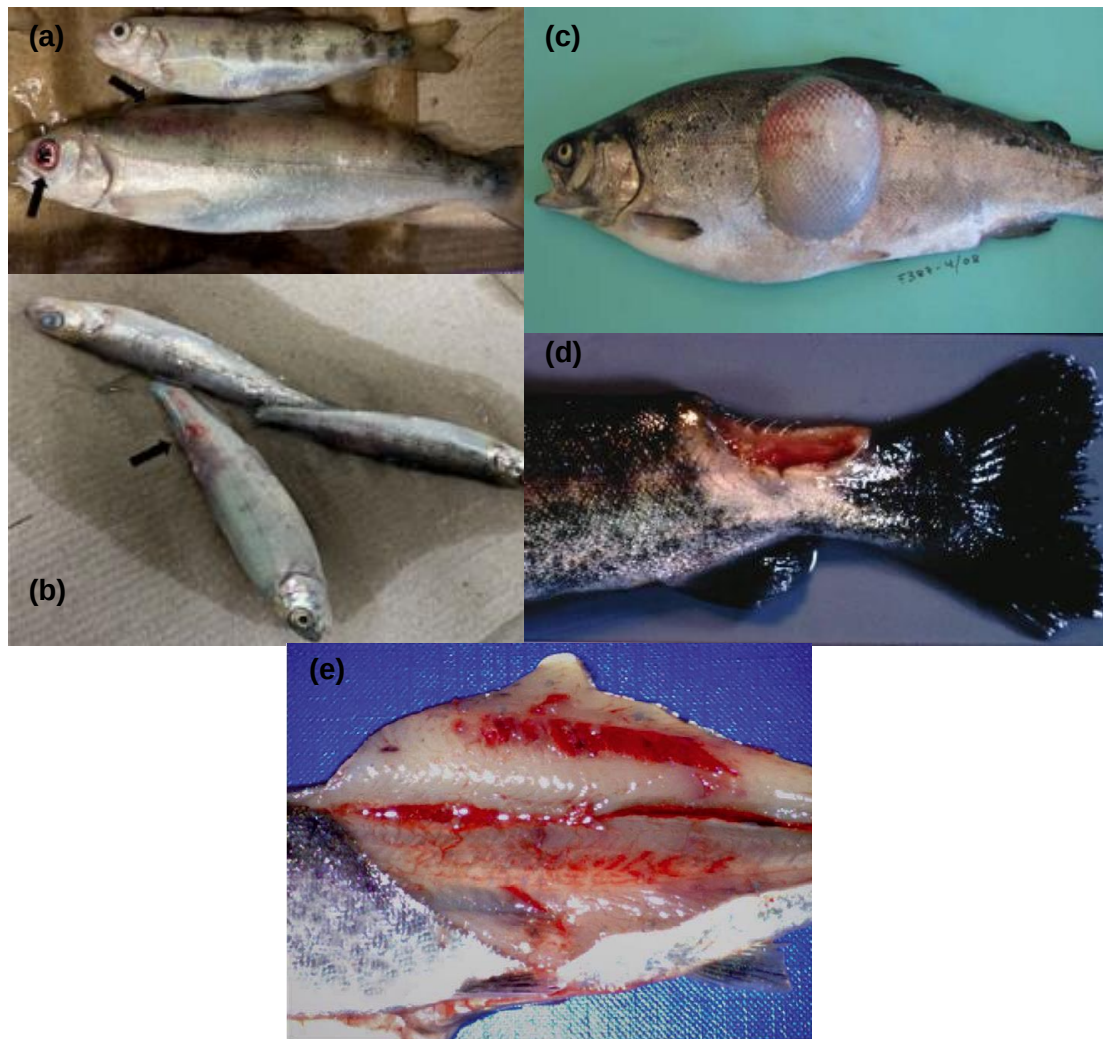
In Europe, salmonids are the major aquaculture food producing species, and in 2019 rainbow trout was one of the most valuable species farmed accounting for about 16% of all aquaculture production (Gyalog, 2022; EuroStat, 2021). In the UK, trout farming was introduced by a Danish entrepreneur in 1970 and it has grown widely since, especially in central and southern Scotland, South England and North Yorkshire (Petr & Swar, 2002). Trout production in the UK has seen a 105-fold increase, from 1,927 tonnes in 1980 to 20,000 tonnes in 2020 (FAO, 2022). Scottish salmon production started in the 1970s and in 2018 contributed £878 million to the Scottish economy. Scotland has been the largest producer during the last decade in the EU and is the third largest globally, producing 203,881 tonnes in 2019 (Scottish Government, 2020).

The intensification of aquaculture and the globalisation of aquatic product trading have led to the emergence and re-emergence of numerous infectious diseases, including bacterial, viral and parasitic diseases. Therefore, disease control represents a major challenge to the aquaculture sector (FAO, 2022). Aquaculture species differ in their defences, with invertebrates lacking adaptive immunity, and relying on their innate immune system, which is complex and not fully understood (Elaswad & Dunham, 2018; Pernet et al., 2016). The aquaculture industry has responded to infectious diseases using a variety of approaches, including best management practice (Little & Bunting, 2016), the use of therapeutants (Cabello et al., 2013) and large investments have been made in selective breeding for disease resistance (Kumar & Engle, 2016). In aquaculture, bacteria, viruses, parasites and fungi have caused serious economic losses to the global finfish aquaculture, and diseases potentially cost the sector \$6 billion in loss each year (Stentiford et al., 2017). The most prevalent infectious diseases in farmed Atlantic salmon are viral diseases: Pancreas Disease (PD), Salmonid Rickettsial Septicaemia (SRS), Infectious Pancreatic Necrosis (IPN), Heart and Skeletal Muscle Inflammation (HSMI), Infectious Salmon Anaemia (ISA) and Piscine myocarditis virus (PMCV); parasitic diseases: amoebic gill disease (AGD), *Lepeophtheirus salmonis* (sea-lice) infection (MOWI, 2020). In farmed rainbow trout some of the most important infectious diseases are Enteric redmouth disease (ERM), Rainbow trout fry syndrome (RTFS), Furunculosis, Vibriosis, Bacterial kidney disease (BKD), Bacterial gill disease (GD), and Infectious Pancreatic Necrosis (IPN) (FAO 2022). Bacterial diseases constitute one of the most significant threats to future expansion and sustainability of salmonid farming, and at present, RTFS caused by the Gram-negative bacterium *Flavobacterium psychrophilum* has been affecting freshwater salmonids worldwide (Loch & Faisal, 2018) and it is considered the most important rainbow trout disease hindering European production of rainbow trout (Fraslin et al., 2018).

## 1.2 Flavobacteriosis

Flavobacteriosis is a bacterial disease caused by the Gram-negative bacterium *Flavobacterium psychrophilum* (*F. psychrophilum*) (Loch & Faisal, 2018). Rainbow trout fry and fingerlings with weights between 0.2 g and 10 g at water temperatures below 10°C are particularly susceptible to *F. psychrophilum* infection (Rangdale et al., 1996), and infection can result in high mortalities (up to 70%), with survivors having the potential to develop detrimental deformities (Nematollahi et al., 2003).

*F. psychrophilum* was firstly isolated from the kidney and external lesions of a juvenile coho salmon (Borg, 1960). However, the disease was first recognised in the 1980s as its occurrence had been reported in Western European rainbow trout hatcheries. Clinical signs of RTFS include erosion of tissues, particularly the caudal fin, lower jaw skin ulcerations, pale or necrotic gills, excess mucus production, pale liver and kidney, enlarged spleen, and spinal abnormalities (Figure 1c and 1d) (Barnes & Brown, 2011). Infection with *F. psychrophilum* is also referred to as bacterial cold-water disease (BCWD) and the bacterium has been associated with occasional cases of fin rot, ulceration, necrotic myositis and haemorrhaging of the dorsal musculature, bleeding orbitals (Figure 1a and 1b), extensive petechiation and large haemorrhagic areas in skeletal muscle in Atlantic salmon in Norway (Figure 1e) (Nilsen et al., 2011). More recently, *F. psychrophilum* has also been isolated from Atlantic salmon fry (<1 g) following several disease outbreaks in Scotland and this has prompted some unease in the industry (e.g., Anonymous, 2021).



**Figure 1.1** Gross signs of disease in Atlantic salmon challenged with *F. psychrophilum*. Haemorrhaging of the dorsal musculature (a) and bleeding orbitals (b) (arrowed) in infected Atlantic salmon (Johansen et al., 2015). In rainbow trout the bacterium has been linked with ulceration (c) and fin rot (d) (Bernardet & Bowman, 2006). Figure 1.1 (e) naturally *F. psychrophilum* infected Atlantic salmon, showing extensive petechiation and large haemorrhagic areas in skeletal muscle (Nielsen et al., 2011). Images adapted from Bernardet & Bowman (2006) and Nielsen et al. (2011).

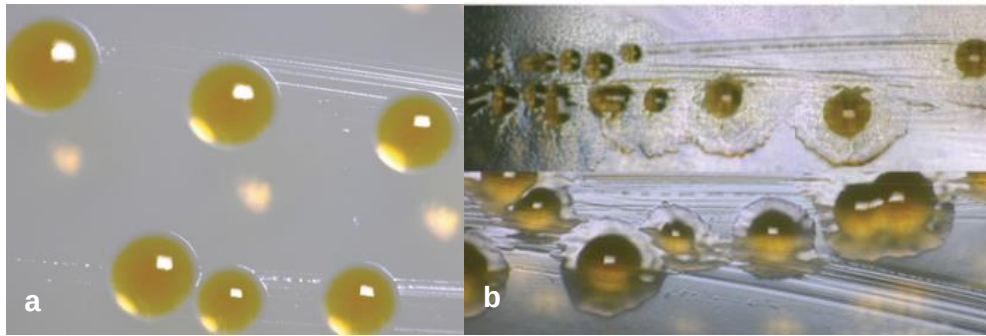
### 1.3 Taxonomy of the aetiological agent

*F. psychrophilum*, previously designated as *Cytophaga psychrophila* and *Flexibacter psychrophilium* is a Gram-negative slender rod bacterium with rounded ends ranging from 0.3-0.75 µm in diameter and 2-7 µm in length depending on the conditions of the culture media. It is a fastidious, slow-growing aquatic organism that requires a specialised media for optimal growth in the laboratory (Cain & LaFrentz, 2007).

Its isolation using classical agar cultivation is very difficult because of the slow growth of the bacterial cells and the overgrowth or inhibition of other bacteria (Michel et al., 1999). *F. psychrophilum* does not grow on rich media such as blood agar or brain heart infusion agar, but it requires a specific media, low in nutrients to grow (Nematollahi et al., 2003). It weakly grows on tryptone soya agar (TSA) (Madetoja et al., 2002; Wiklund & Dalsgaard, 2002). The bacterium prefers to grow in a reduced nutrient concentration media such as *Cytophaga* agar (Starliper & Schill, 2011), modified cytophaga with the addition of new born calf serum (Brown et al., 1997), foetal calf serum (Michel et al., 1999), horse serum (Wagner et al., 2012), calf/fish blood cells (Ellen Lorenzen & Olesen, 1997), sugars and skimmed milk (Wagner et al., 2012), antibiotics (Kumagai & Nawata, 2010) or activated charcoal (Álvarez & Guijarro, 2007). The bacterium is strictly aerobic, and the optimum incubation temperature is between 4°C and 10°C, but the fastest doubling time of the bacterium occurs at 15°C, on Anacker and Ordal medium (MAOA) containing 3% fish or horse blood, 0.2-0.5% NaCl (Crump et al., 2001). Tryptone yeast extract agar has been the most commonly used medium for isolation of *F. psychrophilum* from diseased fish (Holt et al., 1987), however more recently Hoare et al. (2017) has demonstrated good growth of different *F. psychrophilum* isolates on Modified Veggietone (MV) medium.

On MV medium, the colonies have a diameter of 1-5 mm and they appear bright yellow, with a slightly-raised centre, rhizoid and they adhere tightly to the medium surface. Högfors-Rönholm & Wiklund (2010) identified two different *F. psychrophilium* colony phenotypes isolated from diseased fish, described as yellow with regular edges (smooth colonies) and yellow with spread margins (rough colonies) (Figure 1.2). Evensen & Lorenzen (1996) performed an enzyme-linked immunosorbent assay (ELISA) using the double antibody sandwich method and their study revealed that among 45 *F. psychrophilum* isolates (25 from Danish rainbow trout and 20 from other European countries), three distinct serotypes were captured.

Among the Danish isolates a major serotype was found (serotype Th) as well as two minor serotypes (serotype Fd and FpT), which were identified and confirmed by slide agglutination.



**Figure 1.2** Morphology of *F. psychrophilum* colonies exhibiting a smooth margin (a) and exhibiting spread margins (b). Image adapted from Cain & LaFrentz (2007).

## 1.4 Pathogenesis

Studies on *F. psychrophilum* pathogenesis are scarce, however various experimental infection studies have revealed that this bacterium causes heavy mortalities (Borg, 1960; Lorenzen & Olesen, 1997; Rangdale et al., 1996; Nematollahi et al., 2003). Disease transmission mode and portal of entry are still unclear and need to be elucidated. It was reported that in sub-optimal environmental conditions, the bacterium gains access to the host across the skin and gills of immunosuppressed salmonids (Roberts, 1976). Rochat et al. (2017) identified *F. psychrophilum* in the lumen and the mucosa of the stomach of naturally infected wild rainbow trout fry and ayu, *Plecoglossus altivelis*, suggesting that the gastro-intestinal tract acts as a portal of entry. Rangdale et al. (1996) demonstrated that this agent can be transferred horizontally as well as vertically from the broodfish to the offspring, and the bacterium can survive in stream water for months by adapting a different morphology to cope with starvation (Vatsos et al., 2003), and the presence of sediments in water increases the survival of *F. psychrophilum* (Madetoja et al., 2003).

Successful colonisation of the bacterium on the host tissue depends on its ability to adhere. Adhesion and mobility are facilitated by the presence of a thin and partially compacted slime layer that surrounds *F. psychrophilum* cells (Cipriano & Holt, 2005). Rainbow trout fry infected with *F. psychrophilum* cells develop anaemia, which is caused by the ability of the bacterium to partially lyse and agglutinate erythrocytes (Ellen Lorenzen & Olesen, 1997). This can explain why *F. psychrophilum* cells grows better in a medium supplemented by blood cells. Proteolytic enzymes are responsible for massive tissue damage and contribute to the invasion of the pathogen (Rochat et al., 2019). Secades et al. (2001) purified and characterised a 55-kDa psychrophilic protease produced by *F. psychrophilum*. Extracellular protease production depends on environmental conditions and they have been showing to be sensitive to repression by different carbohydrates and nitrogen sources (Secades et al., 2001) and pathogenesis of *F. psychrophilum* infections are facilitated by lytic activity.

Rangdale et al. (1999) demonstrated that lysosomal activity occurs within the phagocytes of naturally infected rainbow trout spleen tissues and *F. psychrophilum* bacteria and their metabolites induce the production of reaction oxygen species (ROS) by kidney head phagocytes (Decostere et al., 2000). The liposaccharide fraction of *F. psychrophilum* is strongly immunogenic (Dumetz et al., 2008) and the O-chain is

composed by repeating trisaccharides, containing a unique sugar which can be used as a specific target for diagnostic purposes. A plasmid of 2.9 Kb has been identified and linked with virulence (Nematollahi et al., 2003). The serum of infected rainbow trout contain high levels of antibodies to *F. psychrophilum* cells, however when the bacterial cells are incubated in the serum, there is only a slight decrease in cell count suggesting that antibodies alone cannot protect the fish against virulent *F. psychrophilum* (Wiklund & Dalsgaard, 2002).

## **1.5 Salmonid fish immune system**

The vertebrate immune system is a dynamic and complex network that plays a key role in maintaining the integrity of the body by detecting and neutralising pathogens, to maintain homeostasis (Pulendran & Davis, 2020.) The immune system is involved in fighting against infections, causing or contributing to disease pathophysiology. The immune system is divided into innate and adaptive arms which are both composed of different cell types and humoral components, that together maintain homeostasis (Albert-Vega et al., 2018). The innate response contributes to the instant protection of tissues by tackling pathogens entering the host, and these response acts through three defence mechanisms: 1) physical barriers 2) cellular components and 3) humoral responses (Gomez et al., 2013). The mucosal barriers are the first line of defence between the fish and its immediate niches. The main role of the mucus is to entrap and slough microbes, and it contains many factors such as antimicrobial peptides (AMPs), lysozymes, lectins, proteases, immunoglobulins (Igs; IgT and IgM), that provide innate immunity (Dash et al., 2018). When pathogenic microorganisms enter the host, cellular and humoral innate defence mechanisms are activated (Magnadóttir, 2006). Phagocytes (neutrophils and macrophages) are the main cells involved in engulfing and destroy the pathogen. During phagocytosis there is production of reactive oxygen species (ROS) and consequent production of free radicals (FR), myeloperoxidases, lysozymes and nitric oxide (NO), and these compounds lead to the formation of potent bactericides to combat microorganisms (Biller & Takahashi, 2018). Bridging the innate and adaptive immunity, macrophages secrete a broad selection of inflammatory mediators to orchestrate the type and magnitude of an inflammatory response. Phagocytic cells produce pro-inflammatory cytokine (including interleukin 1 beta [IL-1 $\beta$ ] and tumour necrosis factor alpha [TNF- $\alpha$ ]), as well as chemotactic cytokines known as chemokines (including interleukin-8

[IL-8]) to alert and recruit cells involved in the adaptive immune response (Fu & Harrison, 2021). Phagocytes contain, kill and process the internalised pathogens for antigen presentation by leading antigenic ligands to initiate activation and clonal expansion of T and B cells and ultimately developing the adaptive response.

### **1.5.1 Mucosal response to *Flavobacterium psychrophilum***

Studies have shown disruption of the fish skin mucus to be a critical element in the establishment of *F. psychrophilum* infection in rainbow trout (Henriksen et al., 2013; Madetoja et al., 2000; Martínez et al., 2004). The mucosal barriers are the first line of defence between the fish and its immediate niches, playing an important role in the protection against pathogens, and containing various antibacterial and inflammatory factors such as lysozyme, immunoglobulin, complement, lectins, acute-phase proteins, proteases and extracellular traps (Koshio, 2016; Subramanian et al., 2007; Van et al., 2020). These barriers are colonised with microbial communities that have a unique relationship with the mucosal immune system. Despite the high exposure of the mucosal immune system to an antigen-rich environment, fish can avoid excessive immune responses and maintain tissue homeostasis (Salinas et al., 2021).

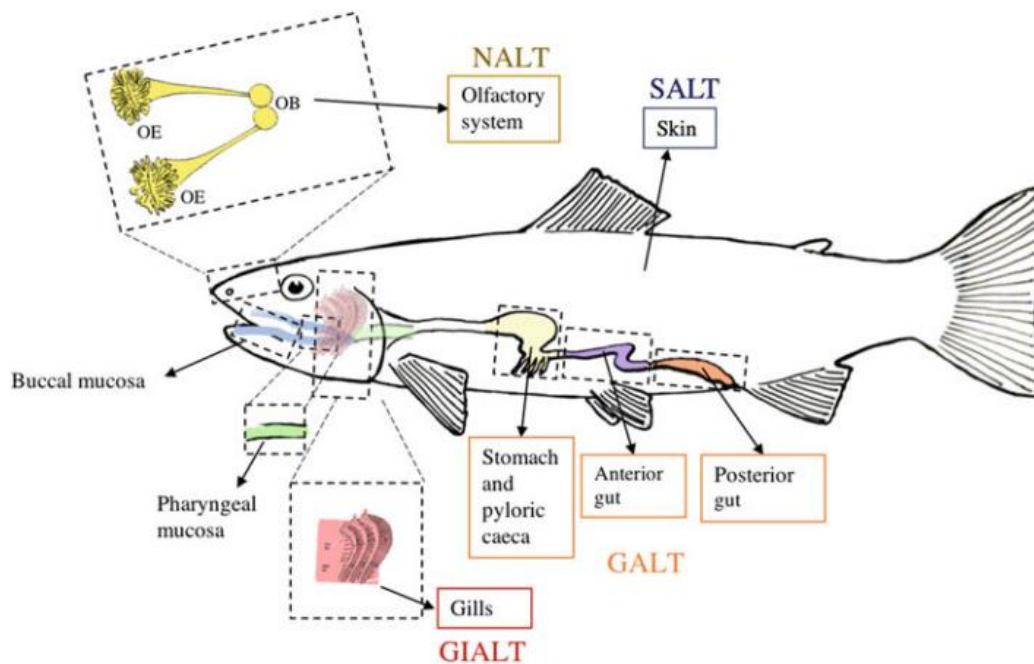
Hoare et al. (2022) highlighted the importance of rainbow trout mucus during immersion challenge studies with *F. psychrophilum*. Greater levels of actins were observed in the skin mucus of rainbow trout fry challenged by immersion compared with injection delivery, and the authors suggest these may have a role in mucosal immunity by potentiating the macrophage response to pathogens (Hoare et al., 2022). Actins are highly conserved proteins of many types of eukaryotic cells and are involved in a variety of process including cell motility, cytoplasmic streaming and transport, cytokinesis, and phagocytosis (Weeds, 1982). Beta-actin ( $\beta$ -actin) has been shown to activate endothelial NOS (eNOS), also known as nitric oxide synthase 3, thereby increasing nitric oxide (NO) production (Kondrikov et al., 2010). Migration of macrophages is a key process for a variety of physiological functions, such as pathogen clearance by invading the extracellular matrix based on their adaptable morphology, and the local degradation of matrix components. Hoare et al., (2022) discovered higher levels of actin in the mucus of immersion infected fish compared to fish infected by injection, suggesting that the presence of actin may not simply be residuals from damaged cells, but this protein could have a role in mucosal immunity by potentiating the macrophage response to pathogens. Similarly,  $\beta$ -actin was a

significant component of Atlantic salmon mucus following sea lice, *Lepeophtheirus salmonis*, infection and it has been localised extracellularly (Easy & Ross, 2009). Easy & Ross, (2009) observed cleavage products of both transferrin and actin and these appeared more pronounced in mucus from infected fish suggesting an increase in protease activity. Proteases have an important role in both the innate and adaptive immune systems that include activities such as complement activation, initiation of proinflammatory responses and the generation of peptides from foreign antigens that are then presented to the major histocompatibility complex in the adaptive immune response (Carroll, 2006; Zavašnik-Bergant & Turk, 2006). Additionally, cleaved transferrin in salmon mucus following sea lice infection suggest that the action of proteases and may be an early step in the immune response that would ultimately include activation of macrophage-type cells in the epidermis to produce NO (Stafford & Belosevic, 2003).

Mucosal barriers are the interface between the environment and the fish host, making it the target for vaccine development and immune interventions in aquaculture (Miquel-Clopés et al., 2019). The route of infection in challenge studies with *F. psychrophilum* is important, as it is suspected to have a mucosal route of entry (Hoare et al., 2019). Most experiments in fish are conducted using intraperitoneal or intramuscular injection as these methods allow a more standardized infection model to be used. However, they completely bypass the primary natural barriers of mucus and epithelium, thereby promoting an 'unnatural' infection.

Zebrafish, *Danio rerio*, has been used as a model to visualise and compare the development of *Vibrio anguillarum* infection on and in the fish following injection or immersion. Schmidt et al., (2017) revealed that the tissues and organs where bacteria were detected differed significantly as a result of time as well as treatment (injection vs. immersion). In the immersion challenged group, bacteria were initially found on external surfaces including gut. After 24 h *V. anguillarum* still persisted in gut but had now also spread to the blood. In contrast, in the injection group, bacteria were found in the blood throughout all sampling times, as well as in the hypodermis and body cavity at most sampling times. During an immersion challenge with *F. psychrophilum* in rainbow trout, Hoare et al. (2017) determined that significant higher numbers of bacteria were needed to cause infection and morbidity/mortality by the mucosal route than by injection, revealing how effective the mucosal barriers of fish are in preventing infection.

Recent research has highlighted the importance of mucosal immunity in fish (Salinas, 2015). As shown in Figure 1.3, the main mucosa-associated lymphoid tissues (MALT) of teleosts are the gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), the gill-associated lymphoid tissue (GIALT) and the recently discovered nasopharynx-associated lymphoid tissue (NALT) (Salinas et al., 2022). In teleosts MALT, diffuse B and T lymphocytes form a dynamic network for the induction and regulation of secretory antibodies and cytotoxic T lymphocyte (CTL) responses (Kunisawa & Kiyono, 2005). B cells, plasma cells and Igs defend the complex environment that defines mucosal barriers. In rainbow trout, secretory immunoglobulins (slgs) can be detected in the skin, gut, gills, nasal, buccal, and pharyngeal mucus (Kole et al., 2019; Yu et al., 2019). In Atlantic salmon, Løken et al. (2020) identified a bursa of Fabricius-like structure in cloaca of Atlantic salmon which contains IgM<sup>+</sup> and IgT<sup>+</sup> B-cells and, therefore, the mucus surrounding this structure should also contain slgs, and thus it might represent an additional teleost MALT.



**Figure 1.3** Schematic representation of MALT and their anatomical localisation in teleosts. Teleost mucosal immune responses have been mostly investigated in the skin, gut and gills. As a result, the skin associated lymphoid tissue (SALT), gut-associated lymphoid tissue (GALT) and gill-associated lymphoid tissue (GIALT) are the best described MALTs in teleosts. The list of fish MALTs has been considerably expanded, with new studies reporting the presence of a nasopharynx-associated lymphoid tissue (NALT) as well as a buccal- and pharyngeal-associated lymphoid tissues in teleosts. Image taken from Salinas et al. (2022).

Teleosts, like mammals, have IgH isotypes specialised in mucosal immunity: IgT, IgD and IgZ, and their abundance varies between species (Danilova et al., 2005). IgT is detectable at the protein level in several mucosal secretions using immunoblotting or ELISA (Salinas et al., 2011) and has been identified as a specialised mucosal Ig. The structural and functional analysis of IgT protein has only been conducted in rainbow trout (Parra et al., 2016). IgT is expressed as a monomer (~180 kDa) in serum, while it is found mostly as a polymer associated by non-covalent interactions in mucus (ca. 4–5 monomers), but small amounts of monomeric IgT are also found in body fluid. Compared to IgM, IgT concentration is much lower in mucus (Kong et al., 2019). When the fish is infected, pathogen-specific titres of IgT are highly prevalent in the mucus from different mucosal sites, while IgM titres are overwhelmingly dominant in the serum (Kong et al., 2019; Tacchi et al., 2014). In rainbow trout vaccinated by immersion against *F. psychrophilum*, high relative percent survival was reported, which could be caused by the contribution of non-specific immune mechanisms, indicated by a significant up-regulation of interleukin 1 beta (*IL1-β*) in head-kidney and toll-like receptor 2 (*TLR-2*) in skin of vaccinated fish, and the induction of the mucosal antibody IgT, as evidenced by proliferation of IgT positive cells in systemic organs, up-regulation of IgT in the hind-gut, and an increase of total IgT in the serum of vaccinated fish (Hoare et al., 2017). IgT-depleted rainbow trout were highly susceptible to the mucosal parasite Ich, (*Ichthyophthirius multifiliis*) while no IgM or IgD responses were observed. Additionally, IgT has shown to be the prevalent teleost Ig coating the MALTs microbiota (Kong et al., 2020; Tacchi et al., 2014; Xu et al., 2020). Fish depleted of IgT in the microbiota exhibited dysbiosis (imbalances of the microbial community), and the microbiota was able to translocate across the epithelium and reach systemic circulation, demonstrated by high levels of LPS in the plasma ( Xu et al., 2020).

These results suggest that IgT might contribute to the immediate protection of the host at mucosal surfaces against the natural infection route of pathogens, supporting the hypothesis that IgT-producing B-lymphocytes are present locally, thus resulting in increased expression of this immunoglobulin upon stimulation by vaccination by immersion (Makesh et al., 2015).

To emphasise the role of IgT in mucosal surfaces, Xu et al., (2020) demonstrated that polymeric Ig receptor (pIgR) facilitates the transport of sIgs across the epithelia and into the mucus of luminal areas through transcytosis (Gurevich et al., 2003). pIgR has

been detected by immunohistochemistry (IHC) in rainbow trout gut, gills, skin, buccal and pharyngeal cavity, and olfactory epithelium, and trout secretory component (SC) has been found associated with IgT and IgM in the mucus of all these mucosal sites (Yu et al., 2019). SC has not been detected in the serum of several tested teleost, but in the mucus, it binds to both microbiota and bacterial pathogens, possibly contributing to innate immunity and/or homeostasis of microbial communities in the fish mucosal surfaces (Kelly & Salinas, 2017). plgR is also highly expressed in the teleost liver, where it could be responsible for transporting systemic Igs into the fish gut, as occurs in mammals (Brandl et al., 2017). In teleost, plgR expression is regulated in response to infection. For example, in grass carp, *Ctenopharyngodon Idella*, infected with *F. columnare*, plgR was up regulated in mucosal tissues after 4 h post-infection (Pei et al., 2019). Similar results were observed when olive flounder, *Paralichthys olivaceus*, was infected with *V. anguillarum* (Sheng et al. 2019)

## **1.6 Salmonid response to *F. psychrophilum*-infection**

In recent years, advances in next-generation sequencing technologies (NGS) have allowed a better understanding and characterisation of molecular responses in salmonids with regards to physiological and immune responses (Aballai et al., 2017; Núñez-Acuña et al., 2017; Valenzuela-Muñoz et al., 2017). Microarray analysis and, more recently, RNA-sequencing has been used to analyse transcriptomic responses to *F. psychrophilum* in resistant, susceptible and control rainbow trout lines (Langevin et al., 2012; Marancik et al., 2015; Paneru et al., 2016). Rivas-Aravena et al., (2019) used RNA-seq analysis to understand molecular mechanisms underlying *F. psychrophilum* *in vivo* pathogenesis in skeletal tissues, given that the clinical signs of RTFS in juvenile fish include ulcerative lesions of the skeletal muscle, especially near the peduncle, followed by progressive necrosis (Nematollahi et al., 2003). All these studies have discovered important aspects of infection and immune response in rainbow trout to *F. psychrophilum*. Marancik et al. (2015) identified that the transcriptional response included induction of complement factors, acute-phase proteins (APP), cytokines, chemokines and other genes associated with innate immunity. Complement factors including complement c3, complement c9, complement c4-b-like and complement c1q-like proteins have been up regulated after infection.

During the acute-phase response (APR), the generation of complement has been previously reported in rainbow trout and has been associated with bactericidal activity (Whyte, 2007). Several APP encoding genes, including *serum amyloid A* and *TLR5* were also highly expressed after infection, and similar trends have been previously observed (Langevin et al., 2012). Immune factors have shown to be expressed during the salmonid APR after bacterial infection, these include *IL-1*, *IL-11*, and *IL-17*, which exert diverse pro-inflammatory and defensive actions including recruitment of inflammatory cells (Carrington et al., 2004; Jørgensen et al., 2001; Wang et al., 2010).

Other enzymes with regulatory anti-inflammatory functions, have been found up-regulated by *F. psychrophilum* infection in rainbow trout (Langevin et al., 2012). NADH dehydrogenase 1 alpha subcomplex subunit 5 is part of the mitochondrial respiratory complex and has a regulatory role on inflammation. The metalloredutase *STEAP-4* regulates degradation of the NF- $\kappa$ B inhibitory molecule I $\kappa$ B $\alpha$  and pho-STAT3, appearing as a negative regulator of inflammation, which is known to down-regulate the production of inflammatory mediators such as *IL-8* and *IL-6* in humans (Tanaka et al., 2012).

Paneru et al. (2016) identified several long non-coding DNA (lncRNAs) associated with genetic resistance against *F. psychrophilum* and identified immune-relevant protein-coding genes including complement components, cytokines, chemokines, and several signalling molecules involved in immunity. In mammals, lncRNAs regulate various immune responses including the interferon response, inflammatory processes, and other aspects of innate and adaptive immune responses (Peng et al., 2010; Wang et al., 2014).

Despite the fact that knowledge on *F. psychrophilum* is expanding, limited studies have looked at *F. psychrophilum* infection in Atlantic salmon mainly due to the lack of a reliable and reproducible challenge model for *F. psychrophilum* (Bruce et al., 2021; Fredriksen et al., 2016). Therefore, to improve the welfare of Atlantic salmon culture, further research is needed looking at the development of an immersion challenge model that mimics the more 'natural' route of infection, which will allow scientists to develop tools to easily diagnose this bacterium and allow studies to look at the immune response of Atlantic salmon to *F. psychrophilum*.

## 1.7 Diagnosis

Diagnosis of *F. psychrophilum* infections relies mainly on macroscopic symptoms, microscopic examination of fresh samples of fish spleens, and cultures of samples from tissues on non-selective agar medium (Strepparva et al., 2014). Diagnosis of RTFS can be successfully achieved through histology. This powerful tool can be used to describe pathological features of the disease in formalin fixed paraffin embedded tissues, particularly in the spleen. The most common and distinctive pathological features observed in diseased fish are loss of splenic border definition, fibrinous inflammation, and intracellular oedema within the spleen. The spleen cells are colonised by numerous filamentous bacteria (Barnes & Brown, 2011). However, this method has some disadvantages, including the fact that preparation of paraffin embedded tissue can be time-consuming, and that the method is subject to human-error while processing the tissue and preparing the slides, and it can also be difficult sometimes to identify specific cell types (Hillman, 20001).

### 7.1. Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) is a labelled immunoassay used to measure antibodies, antigens, proteins, glycoproteins, and hormones in biological samples. The detection of these products is achieved when an antibody-antigen complex is formed, producing a measurable result. An antibody is a type of protein produced by an individual's immune system, and it has specific regions that bind to antigens. The antigen is a substance (molecule) produced by a foreign source, and once it is bound to the antibody, it triggers a cascade of events through immune response (Alhajj & Farhana, 2022).

Long et al. (2012) developed a sensitive and specific *F. psychrophilum* ELISA using mAb FL43, which Lindstrom et al. (2009) generated against the outer membrane of *F. psychrophilum*. This study demonstrated that the ELISA could detect moderate to high levels of the bacteria in healthy looking fish. The advantage of using an ELISA is that it has the capacity for high throughput sample processing and requires minimal instrumentation and technical expertise (Lindstrom et al., 2009).

### 1.7.2 Immunohistochemistry

Immunohistochemistry (IHC) is a technique that exploits the principal that antibodies bind specifically to a given antigen in biological tissues. The antigen-antibody interactions can be visualised using fluorescent dyes or enzyme-substrate colour reactions (Zhang et al., 2017). Despite the progress in biotechnology, IHC tests can give rise to false positive and false negative results due to different factors, including fixation, cross-reactivity, antigen retrieval, sensitivity, and specificity of antibodies (Hillman, 2000). IHC protocols can be optimised accordingly to the experimental requirement, including antigen retrieval to unmask epitopes which are lost during chemical cross-linking over fixation. Antisera raised against *F. psychrophilum* (anti-*F. psychrophilum* polyclonal antibody) has been applied in IHC (Hoare et al., 2017). A specific IHC analysis was performed by using formalin-fixed and paraffin-wax-embedded infected naturally and experimentally infected fry rainbow trout. Skin, gill, and hind-gut samples were examined for the presence of *F. psychrophilum* at 4 h, day 2 and day 7 post vaccination by IHC. Staining by IHC for *F. psychrophilum* was observed in the skin epithelium from 4 h post challenge, while positive staining for *F. psychrophilum* was observed after day 2 post challenge on the gills and in the hindgut (enterocytes) (Hoare et al., 2017).

### 1.7.3 Molecular Diagnosis

Molecular techniques have been extensively used to diagnose RTFS. Polymerase chain reaction (PCR) has been developed to amplify *F. psychrophilum* DNA in infected tissue (Toyama et al., 2011). Rapid and sensitive detection of *F. psychrophilum* was also achieved through the development of a quantitative polymerase chain reaction (qPCR) (Orieux et al., 2011). Orieux et al. (2011), developed an assay that exhibited high specificity for the 16S *rRNA* gene of *F. psychrophilum* and low amounts of DNA were detected in the environment or infected animals, highlighting the fact that the technique can be used to monitor disease spread as well as to study healthy carriers or reservoirs of the disease. For a sensitive and specific diagnostic PCR, a species-specific marker gene is necessary.  $\beta'$  *DNA-dependent RNA polymerase* (*rpoC*), a single copy gene present in *Flavobacterium* spp. has been used to determine phylogenetic relationships and mutation rates in different genera and species and has

been shown to be more variable at the interspecific level than the 16S *rRNA* gene (Strepparava et al., 2014). Strepparava et al., (2014) developed a highly sensitive and a specific qPCR to detect *F. psychrophilum* in different sample types (water and fish samples) without the need for culturing. qPCR allowed reliable detection and quantification of *F. psychrophilum* in the samples with low pathogen densities ( $\geq 20$  copies, i.e., 66 *F. psychrophilum* cells/ml sampled).

## 1.8 Current treatments and prevention

The use of antibiotics is currently the treatment of choice for controlling RTFS outbreaks (Gómez et al. 2014) and, 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/>). However there is concern about the development of antibiotic resistance (Ngo et al., 2018). Ngo et al. (2018), broth microdilution testing showed that only 12% of UK isolates (rainbow trout and Atlantic salmon isolates) were susceptible to oxolinic acid ( $\text{MIC CO}_{\text{WT}} \leq 0.25$  mg/L) and 42% were susceptible to oxytetracycline ( $\text{MIC CO}_{\text{WT}} \leq 0.25$  mg/L). While all the isolates tested were susceptible ( $\text{MIC CO}_{\text{WT}} \leq 2$  mg/L) to florfenicol, which is the major antimicrobial used for flavobacteriosis control in the UK, there is still concern that resistance to this antimicrobial will be developed.

The provision of safe and effective vaccination programmes is one of the key tools to ensure the sustainable development of the aquaculture sector and therefore help meet growing global protein demands, whilst minimising the use of drugs and chemicals as they enhance the species immunity against pathogens (Mondal & Thomas, 2022).

Advances in biotechnology have had a great impact on the development of new vaccines against several infectious pathogens for the prevention and control of fish diseases and are used routinely in aquaculture, especially for Atlantic salmon (*Salmo salar*), while in a limited capacity (or not at all) in other fish species due to lack of vaccines, poor performance or cost (Adams, 2019). The most important step in developing an effective vaccine is identification of 'potentially' protective antigens and confirming their protective response in the host species against the pathogen of interest. Vaccine development depends on pathogen type, fish species, administration method, availability of reagents, and whether a challenge model has been developed to efficacy test the vaccine candidates. Identifying protective antigens is not easy and

requires a variety of approaches. Successful fish vaccines have been developed and are commercially available against Vibriosis, Enteric Red Mouth (ERM) and Furunculosis. Today, 24 fish vaccines are available for a wide range of species, most of which are formalin killed whole cell vaccines (Adams & Subasinghe, 2019). When using a commercial vaccine in fish it is important to take into consideration the fish species, the immune system status, the fish production cycle, the question of when the fish is vulnerable to a pathogen, farming technology including handling and mechanisation, environmental and stress factors, nutrition and cost benefits. Bacterial diseases cause major challenges for rainbow trout, carp, tilapia and catfish aquaculture. Moreover, there are only a few successful vaccines against viral diseases and these cause significant problems in salmonid and marine finfish (Rodger, 2016). A commercial vaccine against *F. psychrophilum* is available to Atlantic salmon farmers in Chile (ALPHA JECT® IPNV-Flavo), however this vaccine, like the majority of commercial vaccines, is administered by intraperitoneal injection, but this is not suitable for fry when the fish are most vulnerable to this pathogen (Wahli & Madsen, 2018). In contrast, Hoare et al. (2017) assessed the efficacy of a polyvalent, whole cell vaccine immersion vaccine containing formalin-inactivated *F. psychrophilum* to induce protective immunity in rainbow trout. The immersion vaccine provided significant protection to the rainbow trout fry with a relative percent survival of 84% ( $p < 0.0001$ ), however this vaccine efficacy still requires evaluation as the relative standard deviation (RSD) in mortality between replicate tanks was high in both unvaccinated (61.5%) and vaccinated fish (47.25%).

The selection of families of fish with resistance or decreased susceptibility to this pathogen, or genomic selection that exploits genetic markers to calculate the genomic estimated breeding values of selection candidates, offer other possibilities for addressing the problems posed by *F. psychrophilum*, and several studies have demonstrated the potential use of such approaches (Fraslin et al., 2020; Liu et al., 2018; Vallejo et al., 2017; Wiens et al., 2013). Genetic improvement programs via selective breeding are focused on increasing the welfare of farmed animals and on increasing the productivity yield as well as improving their product quality.

## **1.9 Salmonid Genome Biology**

The salmonids, a group of relatively primitive teleosts, includes over 70 species, and are naturally distributed in freshwater, brackish water and marine habitats in the Northern hemisphere and they have been introduced to South America, Australia, Africa, and the Middle East. Salmonids have an economic, societal and scientific significance; however populations are declining (Hendry, 2004). Salmonids have a vast range of adaptations, displaying great phenotypic diversity, which has allowed researchers to study the adaptive divergence and ecological speciation which has been potentially facilitated by a whole genome duplication (WGD) in their common ancestor ~95 Mya (Macqueen & Johnston, 2014; Bernatchez et al. 2010). The 'Functional Annotation of All Salmonid Genomes' (FAASG) is an initiative that has emerged to develop an integrative understanding of the functional genomic basis of phenotypic variation. FAASG has several applications, including an improved understanding of aquaculture production, and improving the efficiency and sustainability of aquaculture production (Macqueen et al. 2017). The FAASG initiative follows the latest publications of the Atlantic salmon (Lien et al. 2016) and rainbow trout (Berthelot et al. 2014) genomes. Genome-wide analysis has offered a valuable tool to investigate remodelling and divergence of duplicated genome content and functions during the post-WGD rediploidization process. Genotyping by sequencing, whole genome-resequencing and high-density SNP array have transformed population genomics (Houston et al. 2014; Palti et al. 2015; Yanez et al. 2016). Population genomics can be applied in salmonids without a genome sequence, using conserved regions with rainbow trout or Atlantic salmon (e.g., Nichols et al. 2016). Genome-wide approaches have been also applied in breeding programs to improve accuracy of selection of key traits, including disease resistance, through genomic selection (e.g., Vallejo et al. 2016) or by identifying major effect loci (e.g., Moen et al. 2015). More recently, genome editing in salmonids for gene knockout has been successful (Yano et al. 2020; Dehler et al. 2016), which allows validation of candidate functional genomic elements and causative polymorphisms. The FAASG initiative will explore further salmonid research and its applications.

## 1.10 RNA-sequencing

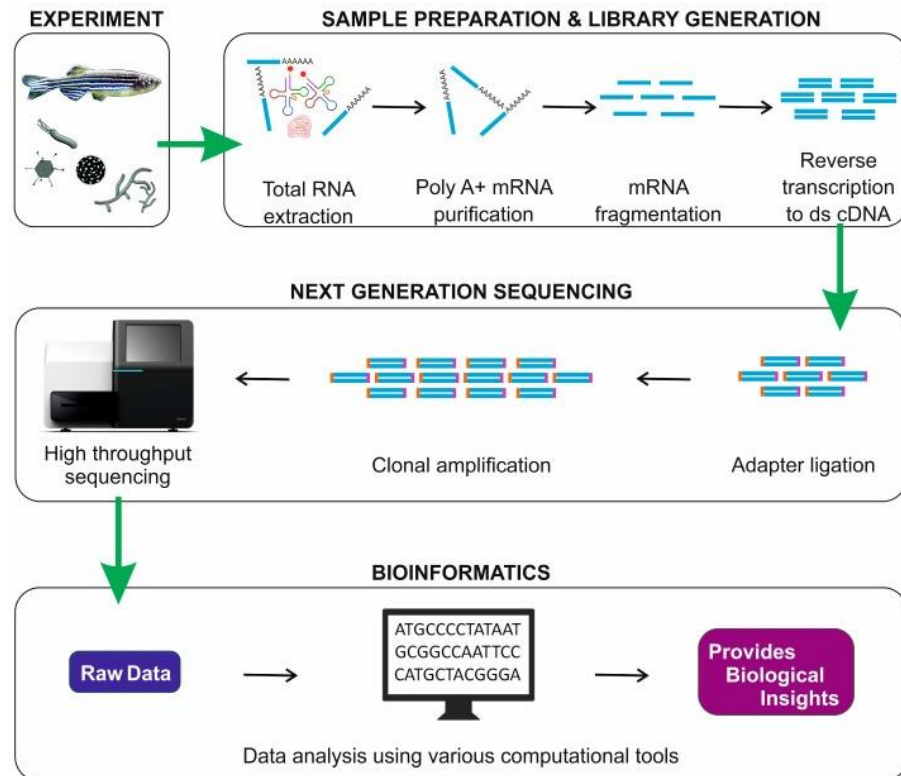
Genomic advancements are becoming increasingly essential for the genetic enhancement of farmed salmon as well as for the conservation of wild salmon stocks. The landmark publications of the salmon genome (Lien et al. 2016) and rainbow trout genome (Berthelot et al. 2014; Pearse et al. 2020) provided reference assemblies that advanced possibilities for high-resolution genomic analyses. These resources are available in databases including NCBI and Ensembl with associated annotations in support of transcriptomics.

Transcriptomics refers to the study of the transcriptome, the complete set of RNA transcripts (protein-coding messenger RNA and non-coding RNA) in a specific cell, tissue, or organ, under specific circumstances using methods such as microarray, tag-based sequencing methods and RNA-sequencing (Table 1.1) (Qian et al., 2014). Genetic improvement for enhanced disease resistance in fish is an increasingly utilized approach to mitigate endemic infectious disease in aquaculture, and the use of high-throughput sequencing has transformed salmonid genetics, in particular through the ease of generating genome-wide genetic marker datasets (Manzoni et al., 2018). The comparison of transcriptomes allows the identification of differentially expressed genes in a sample, or in response to different treatments. It is a powerful tool to determine the relationship between the genotype and the phenotype, to give a better understanding of the underlying pathways and mechanisms that control cell fate, development, and disease progression. Transcriptome analyses do not only quantify gene expression of different samples but also support the annotation of the transcriptome and help to determine the functional structure of each gene in the genome (Costa et al., 2010). RNA-seq has been developed to overcome the limitations of other methods used to survey transcriptomes (Table 1.1) and has been applied to a substantial range of fish biology studies (Table 1.2). RNA-seq employs NGS technologies to sequence complementary DNA (cDNA) directly from an RNA sample of interest (Sudhagar et al., 2018).

Transcriptomic analysis by RNA-seq is a three-step method, which includes library construction, sequencing on a specific NGS platform (for example various Illumina platform), and bioinformatic analysis (Figure 1.4). After the signal processing, the NGS platform generates millions of short sequences, known as reads (Sudhagar et al., 2018). Given the large amount of RNA-seq data, various bioinformatic tools, such as

those consolidated within Bioconductor have been developed for RNA-seq analysis. This software is free, open source and mainly based on the R programming language (Amezquita et al., 2020). Different Bioconductor components are organised as R packages and can be combined to form the best analysis pipeline according to the research of interest. Quality assessment is the first step for the RNA-seq data analysis. Once all reads have been appropriately filtered and mapped, they can then be counted, and gene expression levels can thus be inferred from the total counts of reads belonging to the exons of a particular gene. For differential expression (DE) analysis of the RNA-seq data, normalisation is required to adjust for between-sample differences and a commonly used tool in Bioconductor includes edgeR and DeSeq2 (Robinson et al., 2010). RNA-Seq can be performed against a reference genome, and this is routinely used for functional genomic investigations focussed on evolution (e.g. He et al. 2017; Robertson et al. 2017), aquaculture (e.g. Robledo et al. 2017) and physiology (e.g. Gillard et al. 2018). Mapping against a reference genome, compared to a transcriptome assembly, also has the advantage that highly similar duplicated regions can be distinguished in the analysis, assuming such regions have been correctly assembled. A wide range of RNA-seq approaches are available as well as mapping-based genomic analysis. To standardise pipelines and to allow comparability between studies, the Functional Annotation of All Salmonid Genomes (FAASG) initiative (Macqueen et al. 2017) has been established, and as the genetic and functional data are generated, the genome browser Salmonbase.org (Samy et al. 2017) has been used to analyse and visualise these data.

Comparison of the fish transcriptome before and after an immune challenge can be used to identify and characterise immune-related genes involved in specific immune responses pathways to better understand fish immune system and fish-host interactions. RNA-seq studies regarding fish immune responses to pathogens have been performed on economically important species (Sudhagar et al., 2018).



**Figure 1.4** Illustration of the main steps involved in a transcriptome analysis using RNA-Seq are: experimental design, library construction, sequencing of the library on a NGS platform, and analysis using bioinformatic tools for interpretation. Image taken from Sudhagar et al. (2018).

**Table 1.1** Comparison of RNA-seq with other methods for surveying transcriptome. Table extrapolated from Qian et al., (2014).

|                                                               | <b>Tilling microarray</b> | <b>Tag-based sequencing</b> | <b>RNA-Seq</b>                          |
|---------------------------------------------------------------|---------------------------|-----------------------------|-----------------------------------------|
| Principle                                                     | Hybridization             | Sanger sequencing           | Parallelized high-throughput sequencing |
| Resolution of data                                            | Several to 100 bp         | Single base                 | Single base                             |
| Sensitivity                                                   | Low                       | Moderate                    | High                                    |
| Throughput                                                    | High                      | Low                         | High                                    |
| Turnaround time                                               | Long                      | Long                        | Short                                   |
| Required amount of RNA samples                                | High                      | High                        | Low                                     |
| Cost per sample (excluding equipments)                        | High                      | High                        | Relatively low                          |
| Reliance on existing genomic sequence                         | Yes                       | No                          | No                                      |
| Linear dynamic range of expression levels                     | <2 orders of magnitude    | Not practical               | Limited only by sequencing depth        |
| Discovery of unknown transcribed regions                      | Limited                   | Yes                         | Yes                                     |
| Detection of differences in isoformic and allelic expressions | Limited                   | Limited                     | Yes                                     |
| Detection of mutations                                        | Limited                   | Yes                         | Yes                                     |
| Determination of splicing sites                               | Limited                   | Yes                         | Yes                                     |
| Identification of UTRs                                        | Limited                   | Limited                     | Yes                                     |

**Table 1.2** Representative publications on RNA-seq analysis used to analyse fish transcriptomes in response to pathogens

| <b>Fish</b>                              | <b>Bacterial Species</b>               | <b>Sample</b>                                  | <b>Sequencing Platform</b>    | <b>Reference</b>                  |
|------------------------------------------|----------------------------------------|------------------------------------------------|-------------------------------|-----------------------------------|
| Atlantic salmon                          | <i>Infectious salmon anaemia virus</i> | Head kidney, liver & gills                     | Illumina MiSeq                | (Valenzuela-Miranda et al., 2015) |
| Atlantic salmon                          | <i>Infectious salmon anaemia virus</i> | Spleen                                         | Illumina MiSeq                | (Dettleff et al., 2017)           |
| Atlantic salmon                          | <i>Caligus rogercresseyi</i>           | Skin                                           | Illumina Hiseq 4000           | (Robledo, et al., 2018)           |
| Atlantic salmon                          | <i>Piscirickettsia salmonis</i>        | Head kidney and liver                          | Illumina Hiseq 4000           | (Moraleta et al., 2021)           |
| Blunt snout bream                        | <i>Aeromonas hydrophila</i>            | Blood, liver, gill, intestine, spleen & kidney | Illumina HiSeq 2000           | (Tran et al., 2015)               |
| Channel catfish                          | <i>Edwardsiella ictaluri</i>           | Intestine                                      | Illumina HiSeq 2000           | (Li et al., 2012)                 |
| Channel catfish                          | <i>Flavobacterium columnare</i>        | Gill                                           | Illumina HiSeq 2000           | (Peatman et al., 2013)            |
| Common carp                              | <i>Aeromonas hydrophila</i>            | Spleen                                         | Illumina HiSeq 2000           | (Jiang et al., 2016)              |
| European sea bass                        | <i>Vibrio anguillarum</i>              | Head kidney & hind gut                         | Genome Sequencer FLX Titanium | (Chaves-Pozo et al., 2017)        |
| Grouper                                  | <i>Betanodavirus</i>                   | Kidney cell line                               | Illumina HiSeq 2000           | (Lu et al., 2012)                 |
| Hybrid of channel catfish & blue catfish | <i>Edwardsiella ictaluri</i>           | Liver                                          | Illumina HiSeq 2500           | (Wang et al., 2013)               |

|                        |                                                                  |                             |                             |                         |
|------------------------|------------------------------------------------------------------|-----------------------------|-----------------------------|-------------------------|
| Japanese sea bass      | <i>Vibrio anguillarum</i>                                        | Head kidney, liver & spleen | Illumina HiSeq 2000         | (Zhao et al., 2016)     |
| Koi carp               | <i>Cyprinid Herpesvirus 3</i>                                    | Spleen                      | Illumina HiSeq 2500         | (Lee et al., 2016)      |
| Large yellow croaker   | <i>Poly I:C (viral mimic)</i>                                    | Spleen                      | Illumina Genome Analyzer II | (Mu et al., 2014)       |
| Nile tilapia           | <i>Streptococcus agalactiae</i>                                  | Spleen                      | Illumina Genome Analyzer    | (Wang et al., 2016)     |
| Olive flounder         | <i>Heat inactivated viral hemorrhagic septicemia virus</i>       | Kidney                      | Illumina HiSeq 2500         | (Hwang et al., 2017)    |
| Orange spotted grouper | <i>Vibrio alginolyticus</i>                                      | Whole animal                | Illumina HiSeq 2000         | (Wang et al., 2014)     |
| Rainbow trout          | <i>Flavobacterium psychrophilum</i>                              | Whole animal                | Illumina HiSeq 2000         | (Marancik et al., 2015) |
| Sockeye salmon         | <i>Piscine reovirus/ infectious hematopoietic necrosis virus</i> | Kidney                      | Illumina HiSeq 2000         | (Polinski et al., 2016) |
| Tilapia                | <i>Streptococcus iniae</i>                                       | Spleen                      | Illumina HiSeq 2000         | (Zhu et al., 2015)      |

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### **1.11 Selective breeding**

Selective breeding is a key tool for the development of aquaculture because precise phenotypic records and quantitative genetics analysis allows the selection of desirable individuals based on the estimated breeding values (Gjedrem & Baranski, 2010). Atlantic salmon genetic improvement through selective breeding started in Norway in the early 1970s (Gjøen & Bentsen, 1997), and resulted in the improvement of important economic traits such as growth, sexual maturation and disease resistance (Gjedrem et al., 2012). Today, selective breeding targets not only growth but product quality, including feed efficiency and adaptation to novel feeds (Palaikostas, 2021). There are several selection strategies available for genetically improving the performance of aquaculture species. Individual (mass) Selection is the most commonly used and most successful method of selection in aquaculture and it is a relatively simple and cost-effective program (Rye et al., 2009). Initially, in mass selection programs full-sib groups from individual pairs of parents are reared separately and then a number from each group are pooled and reared together, to keep low levels of inbreeding. Subsequently, individuals with the desirable phenotypic traits are selected. When heritability is high for a specific trait, mass selection is effective and at this point the phenotypic values are approximately the same as the breeding values (Tave, 1993). In contrary, when spawning can be controlled, heritability is low and the trait cannot be measured on the live individual, then mass selection will not be effective and family-based selection should be considered.

Family selection is a more intricate program which uses sib-information. These programs are used in most aquaculture production sites as they allow successful selection of traits that can only be recorded on dead fish and challenge tests (Rye et al., 2009). Furthermore, family selection is more effective than individual selection when the heritability of a trait is low. However, family selection requires that each family produced is kept separate and that all fish are tagged before testing in common test environments (Gjerde, 2005). For expensive and difficult traits to measure such as disease resistance or fillet quality, marker-assisted selection (MAS) can be used directly on the selection candidates, which can significantly increase accuracy of selection in salmon breeding programs (Sonesson, 2005). The detection of quantitative trait loci (QTL) can be applied in MAS programs, and each QTL is tested

for its statistical significance (Houston et al., 2008). In contrast, genomic selection (GS) omits significance testing and uses genome-wide genetic markers to accurately estimate selection candidates' breeding values for quantitative traits (Meuwissen et al., 2001). GS has the ability to predict the breeding value of individuals based on their genotype at a large number of markers spread over the genome, achieved using single nucleotide polymorphism (SNP) arrays, which are becoming the most popular genetic markers. SNP arrays are high-quality and convenient genotyping platforms, allowing high-throughput and high-efficiency for genetic research and breeding programs (Zhou et al., 2021). SNP applications include high-density genetic linkage map construction, QTL mapping, GWAS, and GS. Currently, high-density SNP chips are available for several aquaculture species (Table 1.3). Linkage maps show positions of genetic markers on chromosomes. The large number of SNPs discovered, and the development of SNP arrays has enabled the construction of high-density maps, such as for Atlantic salmon using up to 96,000 SNP (Houston et al., 2014) and a 57,000 SNP array in rainbow trout (Palti et al., 2015). Genetic markers associated with phenotypic traits are known as QTL markers. When studying disease resistance, one approach is to map QTLs giving information on the location and the effects of the genes linked to the trait. A whole-genome scan can be conducted to identify QTLs in which large number of markers distributed across the genome are segregated and tested for association with specific phenotypes (Barroso et al., 2008). These advances in genetic improvement tools facilitate MAS, which enables the improvement of important traits, which can be incorporated into salmonids breeding programmes.

**Table 1.3** High-density SNP chips developed for aquaculture species

| Species                                       | References                                      |
|-----------------------------------------------|-------------------------------------------------|
| Atlantic salmon ( <i>Salmo salar</i> )        | Houston et al. (2014) and Yáñez et al. (2016)   |
| Rainbow trout ( <i>Oncorhynchus mykiss</i> )  | Palti et al., (2015)                            |
| Nile tilapia ( <i>Oreochromis niloticus</i> ) | Peñaloza et al. (2020) and Yáñez et al. (2020)  |
| Common carp ( <i>Cyprinus carpio</i> )        | Xu et al. (2014)                                |
| Pacific oyster ( <i>Crassostrea gigas</i> )   | Gutierrez et al. (2017) and Qi et al. (2017)    |
| European flat oyster ( <i>Ostrea edulis</i> ) | Gutierrez et al. (2017)                         |
| European bass ( <i>Dicentrarchus labrax</i> ) | Griot et al. (2021) Peñaloza et al. (2021)      |
| Gilt-head bream ( <i>Sparus aurata</i> )      | Griot, et al. (2021) and Peñaloza et al. (2021) |
| Atlantic cod ( <i>Gadus morhua</i> )          | Poćwierz-Kotus et al. (2015)                    |

In the salmon industry the health status of farmed fish is one of the main factors affecting revenue (Yáñez et al., 2014), and genetic improvement for disease resistance could prevent disease outbreaks. Disease resistance can be recorded by exposing the fish to the pathogen experimentally in enclosed facilities, away from breeding stocks and commercial fish farms and wild populations. Challenge facilities are expensive; hence, often tests are performed with small group of fish for a short period of time. In a challenge, environmental conditions mimic the natural environment but are not the same, meaning that the information arising from challenge is potentially different. In the field, response to selection will depend on the genetic correlation between survival after challenge test and general resistance in commercial farms (Fjalestad et al., 1993). The method by which the fish is exposed to a pathogen will also affect the challenge. Fraslin et al. (2018) observed that infection challenge by injection (intraperitoneal or intramuscular) is a more reproducible challenge than immersion, with high mortalities between 70-90% compared to 30-55% (Garcia et al., 2000; Madsen & Dalsgaard, 1999). Challenge infections with *F. psychrophilum* seem to be more effective when the fish are stressed, for example after being treated with formalin or hydrogen peroxide, suggesting that the external barriers of the fish control the entry of the bacteria (Henriksen et al., 2013).

In salmonids, there is limited information on the genetic architecture of disease resistance, however knowledge on the QTL or genes affecting disease resistance traits is increasing, which will allow an understanding of the biology of immune response in these species (Aslam, Robledo, et al., 2020; Houston et al., 2008b; Karami et al., 2020). Predicting disease resistance is one of the most common desires of aquaculture selective breeding (Aslam, Boison, et al., 2020; Boison et al., 2019; Robledo et al., 2018).

Several studies on salmonids, mainly on rainbow trout, have demonstrated genetic variation for resistance to *F. psychrophilum* infection. Silverstein et al. (2009) showed that resistance to *F. psychrophilum* is a heritable trait ( $h^2 = 0.35$ ) and that improved resistance does not compromise traits selected to improve growth. In contrast Henryon et al. (2005) estimated resistance to *F. psychrophilum* to be a relatively low heritability trait ( $h^2 = 0.07$ ).

Differences in heritability estimates can be attributed to difference in fish size, *F. psychrophilum* strains and challenge conditions, given the lack of a standardised

method to experimentally infect fish. To select for resistant breeds of fish to protect against *F. psychrophilum*, reliable and reproducible challenge models are required.

A family-based selection program to improve resistance was initiated in 2005 at the United States Department of Agriculture National Center for Cool and Cold-Water Aquaculture (Vallejo et al., 2014). Select crosses were made in 2007 and 2009 to evaluate family-based disease survival, challenging the animals with *F. psychrophilum* by infection. From this project, Vallejo et al. (2014) estimated the polygenic heritability of survival status to be 0.45, which is higher than that previously reported for rainbow *F. psychrophilum* resistance in larger breeding populations (Leeds et al., 2010; Silverstein et al., 2009; Henryon et al., 2005). Vallejo et al. (2014) also identified nine major QTLs on seven chromosomes that were significant or highly significant with moderate to large effects of at least 13 % of the total phenotypic variance. Throughout this thesis a polygenic trait will refer to a trait that is controlled by two or more genes (Turelli & Barton, 1994), while an oligogenic trait will refer to a trait with few major effect loci (Barson et al. 2015).

Fraslin et al. (2019) took advantage of a natural *F. psychrophilum* outbreak on a Farm in France to characterize the genetic bases of resistance in a rainbow trout population using the medium-throughput Axiom Trout Genotyping Array (57,501 SNPs markers; Palti et al., 2015). From this study, five QTLs associated with resistance were detected on four different chromosomes with two QTLs on chromosome 7. Two chromosome-wide significant QTL were detected, at 41.61 Mb on Omy7 and at 59.33 Mb on Omy17. Based on GWAS analysis, using single step genomic BLUP (ssGBLUP) which enables combining the pedigree-based relationship matrix with the genomic relationship matrix into a hybrid matrix, Omy7 and Omy17 explained 1.0% and 2.1% of genetic variance respectively. While three minor QTLs were found on Omy3, Omy7 and Omy10. Omy 3 and Omy 10 were previously discovered by Vallejo et al. (2017) and Campbell et al. (2014) respectively, a QTL at 19.34 Mb on Omy7 was firstly reported by Palti et al. (2015) however it was identified as Omy 8.

Due to the samples being collected from a natural outbreak, the newly detected QTLs by Fraslin et al. (2019) may be specific to mechanisms associated with the natural entry of the bacteria, i.e., via the skin and the mucus.

This study confirmed the polygenic inheritance of resistance to *F. psychrophilum* in rainbow trout with a few QTL with moderate effects and a large polygenic background, the heritability of the trait being estimated at 0.34. Two new chromosome-wide

significant QTL and three suggestive QTL were detected, each of them explaining between 1% and 4% of genetic variance.

Knowledge of the biology of resistance to *F. psychrophilum* has been revealed by comparing the transcriptomes of susceptible or resistant fish to *F. psychrophilum* after challenge with the bacterium by injection, however no studies have looked at challenging the fish by immersion, which more closely mimics natural routes of infection, therefore affecting the fish immune response. Additionally, no studies have yet looked at detecting QTL affecting resistance to *F. psychrophilum* in a commercial Atlantic salmon population.

## 1.12 Aims and Objectives

The overall aim of this thesis was to study *F. psychrophilum* infection in rainbow trout and Atlantic salmon resistant and susceptible families, and to investigate the genetic underpinnings of resistance to *F. psychrophilum*.

This aim was achieved by fulfilling the following objectives:

1. To develop an immersion challenge model for *F. psychrophilum* infection of Atlantic salmon and rainbow trout fry; to perform a genome wide association study to identify DNA markers associated to the QTL for resistance to *F. psychrophilum* in Atlantic salmon and rainbow trout from the AquaGen breeding nucleus
2. To develop and validate a SYBR Green qPCR assay to detect and quantify *F. psychrophilum* copies in Atlantic salmon and rainbow trout *F. psychrophilum*-infected head kidney tissue using the newly developed immersion challenge model
3. To characterise antibodies for the detection of *F. psychrophilum* and IgT positive cells in infected Atlantic salmon and rainbow trout tissues
4. Transcriptomic analysis of Atlantic salmon head kidney post-*Flavobacterium psychrophilum* infection using RNA sequencing to identify global changes in gene expression

## **Chapter 2: Development of an Immersion Challenge Model for *Flavobacterium psychrophilum* with the aim to test (or show) resistance to flavobacteriosis in Atlantic salmon and rainbow trout fry**

### **2.1 Introduction**

Flavobacteriosis is a bacterial disease caused by the Gram-negative bacterium *Flavobacterium psychrophilum* (*F. psychrophilum*) that affects freshwater salmonids worldwide (Loch & Faisal, 2018). Rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792), is particularly susceptible to *F. psychrophilum* infection; the disease is known as rainbow trout fry syndrome (RTFS) and can result in high mortalities (up to 70%) and survivors may develop detrimental deformities (Nematollahi et al., 2003). Infection with *F. psychrophilum* is also referred to as bacterial cold-water disease (BCWD) and the bacterium has been isolated from Atlantic salmon (*Salmo salar* L.) in Norway (Nilsen, Johansen, et al., 2011) and Scotland (Anonymous, 2021).

Flavobacteriosis affects the early life-stage fish or the sac fry till the early feeding developmental stage (Waśkiewicz & Irzykowska, 2014), because at this stage the fish is likely to be immunologically and physiologically more vulnerable (Brown et al., 1997).

Given that treatment of *F. psychrophilum* relies on antibiotics administered in the feed (Sundell et al., 2019), selecting for natural resistance to this pathogen offers another possibility for addressing the problems posed by *F. psychrophilum*. To select for resistant fish, or to develop new and more effective vaccines to protect against *F. psychrophilum*, including those delivered orally or by immersion (Bøggwald & Dalmo, 2019; Ghosh et al., 2016), reliable and reproducible challenge models are required. Challenge models for *F. psychrophilum* have long been available for rainbow trout (Cipriano & Holt, 2005; Madsen & Dalsgaard, 1999) and, more recently, have been developed for Atlantic salmon (Bruce et al., 2021; Fredriksen et al., 2016). In these models, typically, naïve fry are infected with a known dose of the pathogen by subcutaneous, intraperitoneal or intramuscular injection, with disease progression then assessed by the onset of clinical signs, morbidities and mortalities. Whilst this

method of delivery to establish the infection provides standardised and reproducible models with generally high mortality, injection does not well reflect the natural route of infection because it bypasses the primary host defences of the skin and mucus (Koshio, 100 2016). Establishing infections by injection is also relatively labour intensive, especially when larger groups of fish are included such as in studies for genetic resistance (Plant & LaPatra, 2011).

Challenge by immersion (i.e., bathing the fish in a suspension of *F. psychrophilum*) is closer to the natural route of infection, however morbidity and mortality rates tend to be more variable, which makes comparisons between experimental groups and studies challenging (Decostere et al., 2000). Indeed, attempts to develop an immersion challenge model for rainbow trout have produced inconsistent results, with some studies documenting only low mortality after challenge (Decostere et al., 2000; Madetoja et al., 2000). Meanwhile, greater mortalities have been achieved by pre-treating the fish prior to challenge with hydrogen peroxide to remove the surface mucus (Henriksen et al., 2013; Hoare et al., 2017) or through physically disrupting the fish skin by scarification (Long et al., 2014). Nevertheless, an immersion challenge model requires large quantities of bacteria and resources needed to culture such large amounts of bacteria, and *F. psychrophilum* is a fastidious and slow-growing organism (Cain & LaFrentz, 2007).

The lack of a reliable and standardised immersion challenge model for *F. psychrophilum* for Atlantic salmon has hampered vaccine development as well as genomic studies (Kiros et al., 2012). Vaccination is one of the main methods used to increase survival and profitability in a large-scale fish production (Mutoloki et al., 2015). However, depending on the type of vaccine and method of delivery, fish can be stressed or suffer from side effects thus reducing their immunity. Moreover, vaccinated fish can still carry the pathogen and become asymptomatic carriers and spread the disease to other fish.

On the other hand, selective breeding programs have been developed for increased host disease resistance in aquaculture and have proven a viable solution to control infectious diseases (Houston et al., 2020; Gjedrem & Rye, 2018). Aquaculture breeding benefits from large family numbers and the ability to routinely monitor and collect morbidities or mortality data from large experimental challenges (Gjedrem & Rye, 2018). Various research groups have been working on family-based rainbow trout selective breeding programs to increase resistance of naïve fish against *F.*

*psychrophilum* (Elaswad & Dunham, 2018; Gjedrem, 2015; Houston et al., 2017; Wiens et al., 2018; Yáñez et al., 2014). For example, Wiens et al. (2018) demonstrated that family-based selection works in rainbow trout and after three generations of artificial selection translated into improved survival following either natural or experiment exposure to *F. psychrophilum*.

In aquaculture, genetic maps, characterisation of traits, inheritance patterns, markers and assembled genomes (for salmonids of commercial importance) have been developed for a wide range of species (Barría et al., 2021; Chatziplis et al., 2020; Lien et al., 2016; Houston et al., 2008) to aid the discovery of variations of alleles affecting specific traits and consequently the identification of DNA sequences underlying phenotypes (Rexroad et al., 2008).

Genetic markers for disease resistance have been identified with a variety of techniques including single nucleotide polymorphisms (SNPs) which are abundant and distributed genome-wide and are the most suitable for high-throughput association studies (Marana et al., 2021; Zhang et al., 2019). High density SNP panels have been developed for Atlantic salmon and rainbow trout and are widely used to better understand disease resistance and facilitate selective breeding (Houston, 2017).

Genome wide association studies (GWAS) and quantitative trait loci (QTL) mapping for economically important traits have been widely conducted to identify relationships between SNP markers covering a large portion of the genome and trait phenotypes, providing an insight into the genetic architecture of the trait. Several GWAS have identified moderate to strong QTLs for disease resistance in rainbow trout and Atlantic salmon, including studies on infectious pancreatic necrosis virus, *Piscirickettsia salmonis* and *Aeromonas salmonicida* in rainbow trout (Barria et al., 2019; Marana et al., 2021). GWAS has been previously performed to study *F. psychrophilum* resistance in rainbow trout (Fraslin et al., 2019; Wiens et al., 2018; Palti et al., 2015) confirming that it is a polygenic trait with a few QTL of moderate effect size. Therefore, it can be hypothesised that if QTLs involved with resistance were found in rainbow trout, the knowledge can be extrapolated to Atlantic salmon breeding for increased resistance to *F. psychrophilum*. Genetic studies will have the potential to assist in the effective and sustainable control of this disease in both the rainbow trout and salmon industries.

Hence, the aim of this present study was to develop an immersion challenge model for *F. psychrophilum* in Atlantic salmon fry based on a previous model developed for rainbow trout (Hoare et al., 2017), and to compare rainbow trout and Atlantic salmon challenges for reliability and reproducibility when establishing the infection with different bacterial isolates. GWASs were performed by AquaGen Norway on *F. psychrophilum* resistance traits in commercial rainbow trout and Atlantic salmon breeding populations from the AquaGen nucleus using the immersion challenge models developed here for *F. psychrophilum*. The results from the GWAS are presented in this thesis, as reference to these results will be made throughout.

## 2.2 Materials and Methods

### 2.2.1 Bacterial isolates, culture media and growth conditions

The *F. psychrophilum* isolates used in this present study were recovered from moribund fry at commercial fish farms in Scotland (Table 2.1). At the farms, spleens were aseptically removed from moribund fish and streaked across plates of modified veggietone agar (MVA), consisting of 5 g/L veggitones GMO-free soya peptone (Oxoid, UK), 0.5 g/L yeast extract (Oxoid, UK), 0.5 g/L magnesium sulphate heptahydrate (Fisher Chemicals, UK), 0.2 g/L anhydrous calcium chloride (Thermo Fisher Scientific, USA), 2 g/L dextrose (Oxoid, UK), and 15 g/L of agar (Oxoid, UK); agar was omitted when liquid medium (MVB) was required. Inoculated agar plates were incubated at 15°C for 72–96 h. Then, predominant representative colonies were confirmed to be *F. psychrophilum* by a nested PCR that targets the 16S *rRNA* gene, as described by Ngo et al. (2017).

The nucleotide sequence of 16S *rRNA* from each bacterial isolate was >98% identical to the corresponding sequence of *F. psychrophilum* JIP02/86 (also known as ATCC 49511, DSM 21280 or CIP 103535; GenBank accession number AM398681.2). This strain was used for comparison because the complete genome sequence is available (Duchaud et al., 2007) and it has been used similarly in previous studies (Ngo et al., 2017). Each isolate was stored long-term on Protect™ beads (Technical Services, Consultants Ltd.) at -70°C. Prior to challenge, isolates were revived by plating on to MVA and incubating at 15°C for 72–96 h.

Then a single representative colony of each isolate was inoculated into 3 mL MVB and incubated at 15°C for 72 h at 150 rpm (starter culture). Following this, the starter culture was inoculated into 27 mL MVB, incubated as before, and finally introduced into 150 mL MVB at 15°C for 24 h at 150 rpm (Figure 2.3A). Prior to challenge, the optical density at 525 nm (OD) of the culture was adjusted with phosphate-buffered saline (PBS, pH 7.3) to 1.0 AU (ca.  $2 \times 10^8$  colony forming units [CFU]/mL), with an accurate CFU/mL value determined by the 10-fold plate dilution method of Miles et al. (1938).

**Table 2.1** Characteristics of the *F. psychrophilum* isolates used for the Atlantic salmon and rainbow trout immersion challenge model.

| Bacterial isolate            | Fish species    | Origin; Year of Isolation | Bacteria culture characteristics                |
|------------------------------|-----------------|---------------------------|-------------------------------------------------|
| <i>F. psychrophilum</i> 6_S  | Atlantic salmon | Scotland; 2018            | Agglutination, yellow, circular, and convex     |
| <i>F. psychrophilum</i> 18_S | Atlantic salmon | Scotland; 2018            | Agglutination, yellow, circular, and convex     |
| <i>F. psychrophilum</i> 19_5 | Rainbow trout   | Scotland; 2019            | Non-agglutination, yellow, circular, and convex |

## 2.2.2 Experimental animals

Atlantic salmon ( $1.8 \pm 0.237$  g) and rainbow trout ( $1.5 \pm 0.209$  g) fry were obtained from AquaGen Norway (ca. 500 and 200 full-sib families, respectively; 5-10 fish/family) and transported to the research facility at Veso Viken (Namsos, Norway) where the challenge trials were performed. The fish were acclimated for 14 days in 120-L glass fibre tanks with well-aerated flow-through freshwater. Oxygenation was maintained at  $>70\%$  in effluent water (0.8 L/kg/min). The water in the tanks was maintained at  $12 \pm 1^\circ\text{C}$  and a photoperiod of 12:12 light:dark was in operation. The fish were fed a commercial Atlantic salmon feed (Skretting AS, Norway) continuously by automatic feeder at a rate of 2% bodyweight per day. The *F. psychrophilum*-free status of the fry prior to the challenge was determined by streaking head kidney and spleen samples of ten fish on to MVA and incubating at  $15^\circ\text{C}$  for 72–96 h.

## 2.2.3 Immersion challenges

### 2.2.3.1 Preliminary challenges

Preliminary challenges were conducted to determine the bacterial isolates and concentration to use for the main challenge. Four groups of Atlantic salmon fry ( $n=30$  in each tank; ca. 2.4 g) and one group of rainbow trout ( $n=15$ ; ca. 1.8 g) were starved for 24 h prior to challenge.

The fry were immersed in hydrogen peroxide (200 mg/L) for 1 h in aerated 4-L tanks with water flow stopped. Immediately thereafter, groups of Atlantic salmon fry were placed in replicate 4-L tanks containing  $2 \times 10^6$  or  $2 \times 10^7$  CFU/mL of either bacterial isolate (*F. psychrophilum* 18\_S or *F. psychrophilum* 6\_S) (Figure 2.1). Meanwhile, rainbow trout fry were placed in a 4-L tanks at  $2 \times 10^7$  CFU/mL of *F. psychrophilum* 19\_5. The challenges for both fish species were performed for 4 h at  $12 \pm 1^\circ\text{C}$ , with water flow stopped (Figure 2.2). Thereafter, the water flow was returned to normal, and the fish were fed as described in Section 2.2.2.

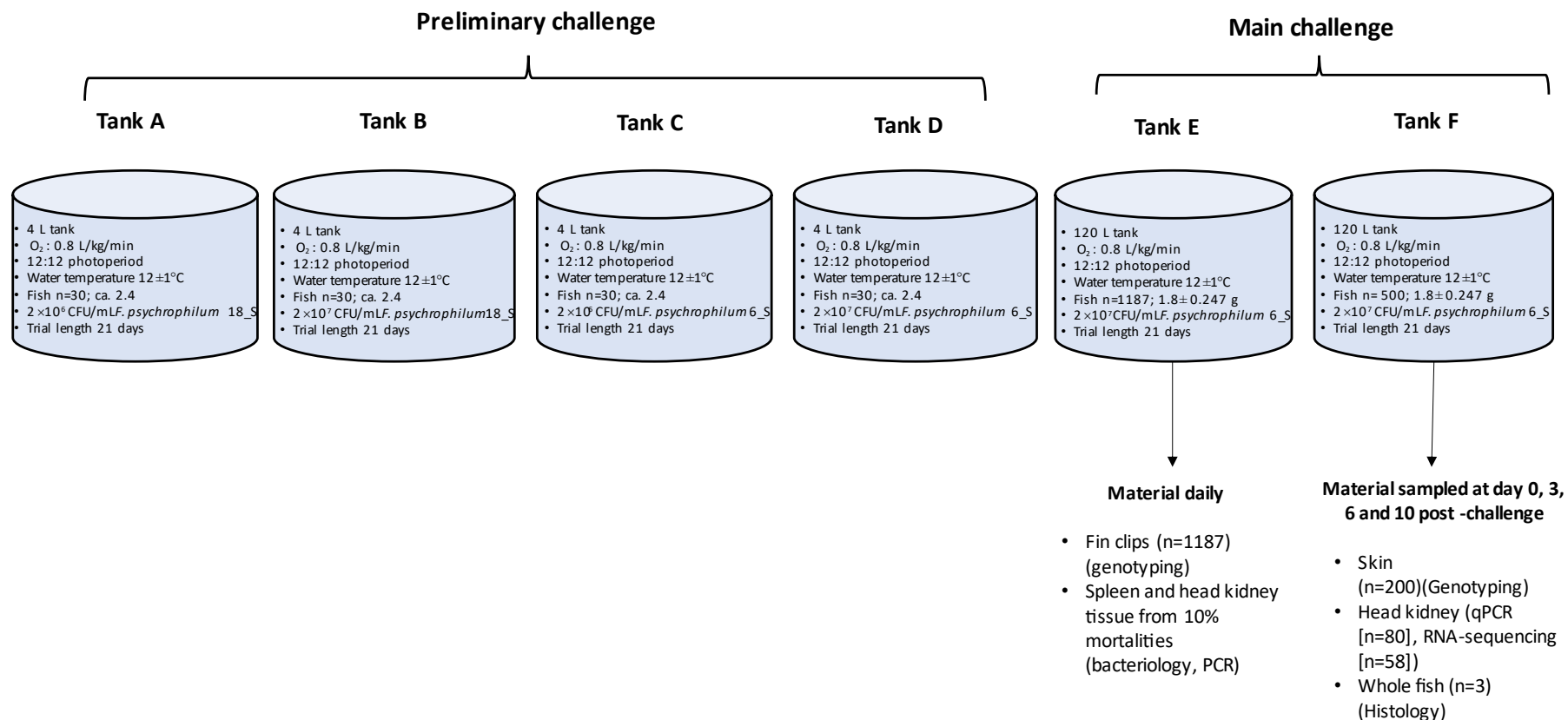
Morbidities and mortalities were recorded each day until the end of the trial (21 days for Atlantic salmon and 31 days for rainbow trout). Moribund fish were euthanised with an overdose of benzocaine chloride (Sykehusapoteket Oslo, Ullevål, Norway) and recorded as dead.

Head kidney and spleen were removed aseptically and plated across MVA for 10% of the mortalities. The agar plates were incubated as described in Section 2.2.1 before a representative colony from each plate resembling *F. psychrophilum* was analysed by PCR as described in Section 2.2.3 to confirm its identity (Figure 2.3D).

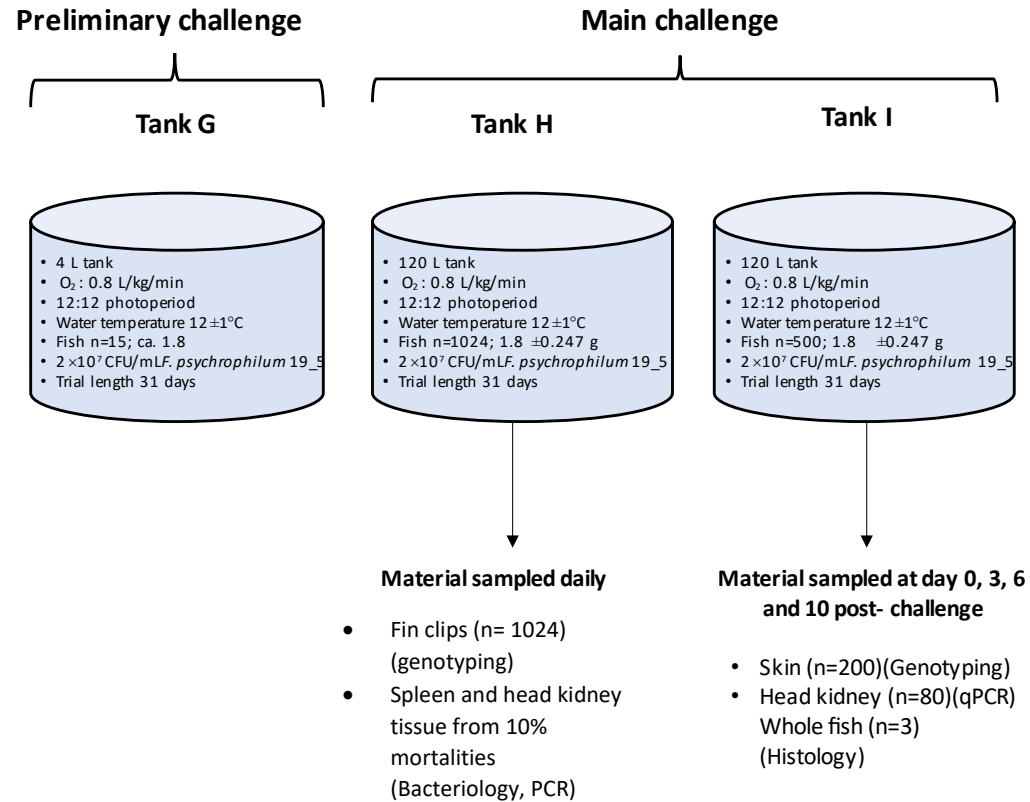
### **2.2.3.2 Main challenge**

The main challenge was performed for Atlantic salmon ( $1.8 \pm 0.237$  g) in two tanks, a main challenge tank (n=1,187) and a sampling tank (n=500); and rainbow trout ( $1.8 \pm 0.247$  g;) fry also in two tanks, a main tank (n=2,100) and a sampling tank (n=500). All the fish that had been starved for 24 h prior to the challenge. The fish were placed into four separate 90-L aerated tanks containing hydrogen peroxide (200 mg/L) for 1 h (Figure 2.3B and Figure 2.3C). Then the fish were returned to the 120-L glass fibre acclimation tanks. Water flow was stopped in each tank and the water volume reduced to 54 L. For each challenge, 6 L of bacteria was added to the tank water to give a final concentration of  $2 \times 10^7$  CFU/mL. Atlantic salmon were challenged with *F. psychrophilum* 6\_S while rainbow trout were challenged with *F. psychrophilum* 19\_5 (Figure 2.1 and Figure 2.2).

For rainbow trout, due to technical issues, the challenge was terminated after 1.5 h, while for Atlantic, after 4 h the water flow recommenced as per the protocol, and the fish were fed as described in Section 2.2. Morbidities and mortalities were recorded each day until the end of the trial (21 days post challenge [dpc] for Atlantic salmon and 31 dpc for rainbow trout).



**Figure 2.1** Schematic representation of the Atlantic salmon preliminary and main immersion challenges tanks, parameters (tank size, oxygen levels, photoperiod regime, water temperature), number of animals challenged, bacterial isolate and dosage used, trial length and material sampled.



**Figure 2.2** Schematic representation of the rainbow trout preliminary and main immersion challenges tanks, parameters (tank size, oxygen levels, photoperiod regime, water temperature), number of animals challenged, bacterial isolate and dosage used, trial length and material sampled

#### **2.2.4 Two-round nested PCR to confirm mortalities due to *F. psychrophilum* infection**

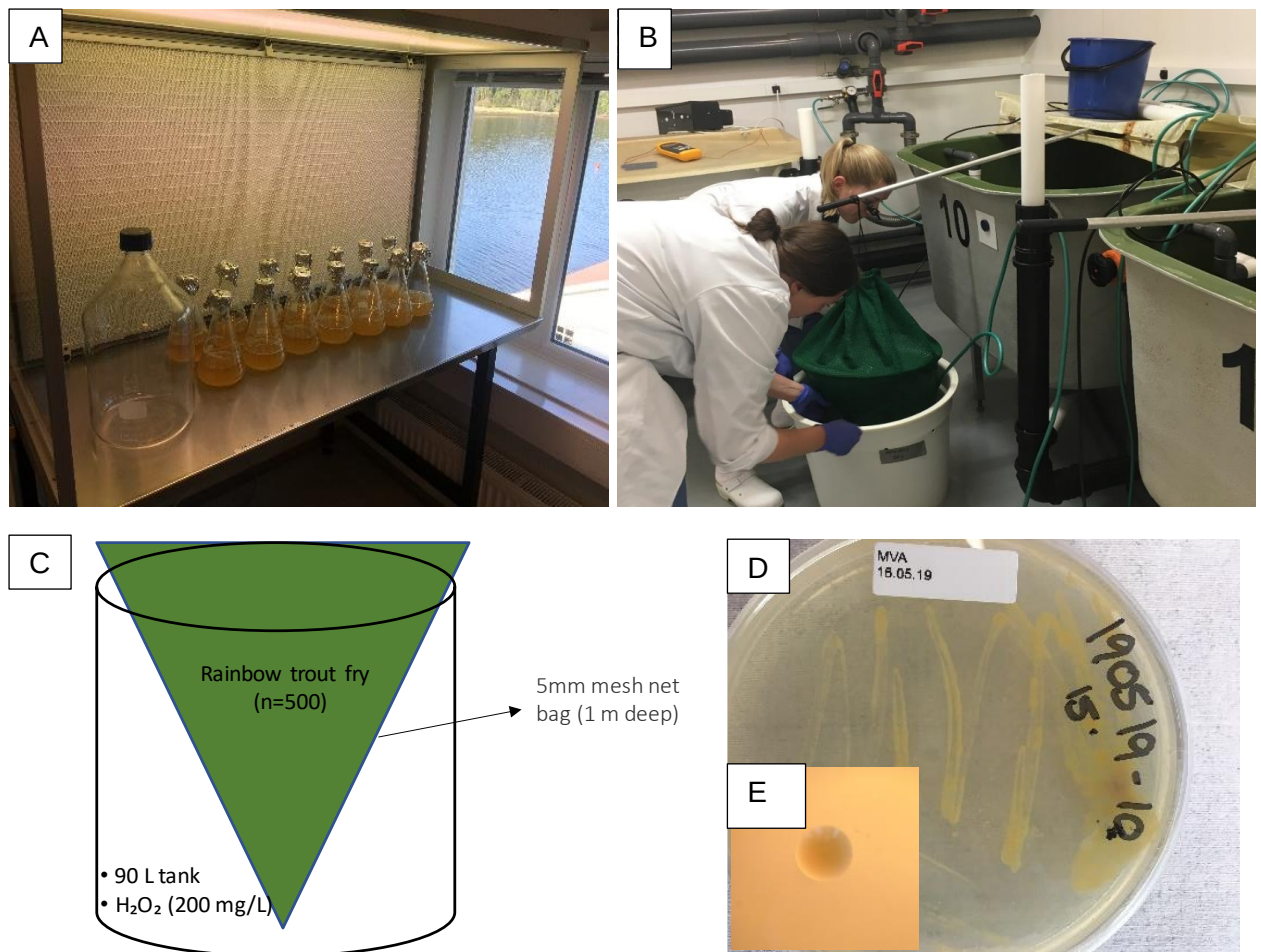
From the main challenges, the spleen of 10% of the mortalities were streaked on MVA and single representative colonies were inoculated in 3 mL MVB for 72 h at 15°C with continuous shaking at 150 rpm. Following incubation, a cell pellet was collected by centrifugation. The cell pellet was washed in 1 mL of STE buffer, consisting of 0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, and resuspended in 100 µL of 10 mM Tris-HCl and 1 mM EDTA (pH 8.0 (TE) buffer). Tubes were incubated at 95°C for 15 min, and the cell suspension was then cooled on ice. Cellular debris was removed by centrifugation. The DNA pellet was air dried and 40 µL of TE buffer (10 mM Tris; 1 mM EDTA) was added to dissolve the DNA at 4°C overnight.

The concentration of the DNA was measured using a Nanodrop ND-1000 Spectrophotometer UV7Vis (Thermo Scientific, Courtaboeuf, France) (Figure 1). DNA samples were standardised to 50 ng/µL using Milli-Q distilled water. The supernatant was used as DNA template for PCR typing (Bartie et al., 2012). PCR was performed in a final reaction volume of 15 µL using 16 S conserved bacteria primers: 20 F (5'-CCTACGGGNGGCWGCAG-3') and 1500 R (5'-GACTACHVGGGTATCTAATCC-3') primers (Herlemann et al., 2011). Each reaction consisted of 10 µM of each of forward and reverse primers, 2X My-Taq PCR Master Mix (Thermo Scientific, Courtaboeuf, France), nuclease-free water (Thermo Scientific, Courtaboeuf, France), and 1.0 µL of DNA template.

Each assay also included a positive control, *F. psychrophilum* JIP02/86 (also known as ATCC 49511, DSM 21280 or CIP 103535; GenBank accession number AM398681.2), and a negative control (nuclease-free water). Cycling conditions consisted of 95°C for 3 min, followed by 35 amplification cycles of denaturation of DNA at 95°C for 15 s, annealing of primers at 45°C for 30 s and extension at 72°C for 45 s in a Light Cycler<sup>TM</sup> 96 (Roche, Germany).

The PCR product was then used as template for the second PCR round for the *F. psychrophilum* -specific PCR. PCR was performed in a final reaction of 15 µl, using 1 µl of PCR product (template), 0.2 µM primers PSY1 (5'-CGATCCTACTTGCGTAG-3') and PSY2 (5'-GTTGGCATCAACACACT-5') and made up to volume with nuclease free water. The PCR was carried out at 95°C for 3 min, and for 25 cycles at 95°C for 15 s, 56°C for 15 s and 72°C for 30 s. *F. psychrophilum*-positive samples and the positive control were expected to yield a *F. psychrophilum* specific 1089 bp product (Toyama et al., 2011).

DNA quantity was measured using a Nanodrop ND-1000 Spectrophotometer UV7Vis and DNA purity was determined with the 260/280 and 260/230 ratios. The quality and integrity of total DNA was determined by agarose gel electrophoresis using a 1% gel with 0.6 µL ethidium bromide (5 mg/mL). The samples (50 ng/µl) relative molecular weight was compared against a 1 µl of Lambda HindIII ladder (Thermo Scientific, Courtaboeuf, France) on 1% agarose gel run at 50 V for 10 min and then 80 volts for 30 min. The gel was visualised using the SYNGENE UV scanner (transilluminator).



**Figure 2.3** Rainbow trout (n=1024) main immersion challenge with isolate *F. psychrophilum* 19\_5. A) Cultivation of bacteria in MVB (ca. 6 L), B) Rainbow trout fry treated with hydrogen peroxide (200 mg/L) in a 90 L tank for 1 h. C) Schematic diagram of what is happening in the tank in Figure 3B, in which the animals are treated with hydrogen peroxide to strip off the mucus to expose the fish to the bacteria D) Head kidney swab specimen from moribund rainbow trout, MVA plate showing clear growth of *F. psychrophilum*-like colonies E) Magnified *F. psychrophilum* colony isolated from head kidney swab specimen on MVA.

### 2.2.5 Genotyping

Moribund or dead fish were collected twice a day from the tanks and fin clips were sampled for DNA extraction. DNA extraction was performed by Vaxxinova Norway. DNA from all challenged individuals (Atlantic salmon n= 1187; rainbow trout n= 1024), were genotyped using a custom 70Kv1 SNP (Atlantic salmon) or Omy50k SNP (rainbow trout) Affymetrix Axiom array chip developed by AquaGen Norway.

### 2.2.6 Genome-wide association study and heritability

Genomic relationship matrices were constructed using the GCTA software (Yang et al. 2014). Heritability ( $h^2$ ) of resistance to *F. psychrophilum* was inferred using the restricted maximum likelihood (*--reml*) method implemented in GCTA, which uses SNP-based relatedness estimates to calculate the proportion of phenotypic variance explained by a set of genotyped SNPs (Yang et al. 2011). Genetic heritability was estimated using the binary measurement survival for both the Atlantic salmon and rainbow trout studies. The genome wide association study (GWAS) was performed using the following linear mixed animal model implemented in GCTA program with the functions “*--mlma-loco*” with the option “*--mLma-no-preadj-covar*” (Yang et al., 2014). This algorithm conducts a mixed linear model based association analysis with the chromosome, on which the candidate SNP is located, excluded from calculating the genetic relationship matrix (GRM). The model is:

$$y = \mu + Xb + g + e$$

Where  $y$  is the phenotype vector (the binary trait survival),  $\mu$  is the mean term,  $X$  is the SNP genotype matrix,  $b$  is the additive genetic effect of the tested SNP,  $g$  is the accumulated effect of all SNPs except those on the chromosome where the candidate SNP is located, and  $e$  is the residual.

### 2.2.7 Statistical analyses

Kaplan-Meier survival curves were generated using Excel and the log rank tests were performed using R package survminer (Version 0.4.9; Kassambara et al., 2017) and xfun (Version 0.30; Xie et al., 2018). Log rank tests were used to compare differences in percentage survival for each fish species for the different isolates and bacterial

challenge concentrations. This statistical test was also used to compare the preliminary and main challenges for each fish species at the same bacterial concentration. P-values  $<0.05$  were considered to indicate a statistically significant difference.

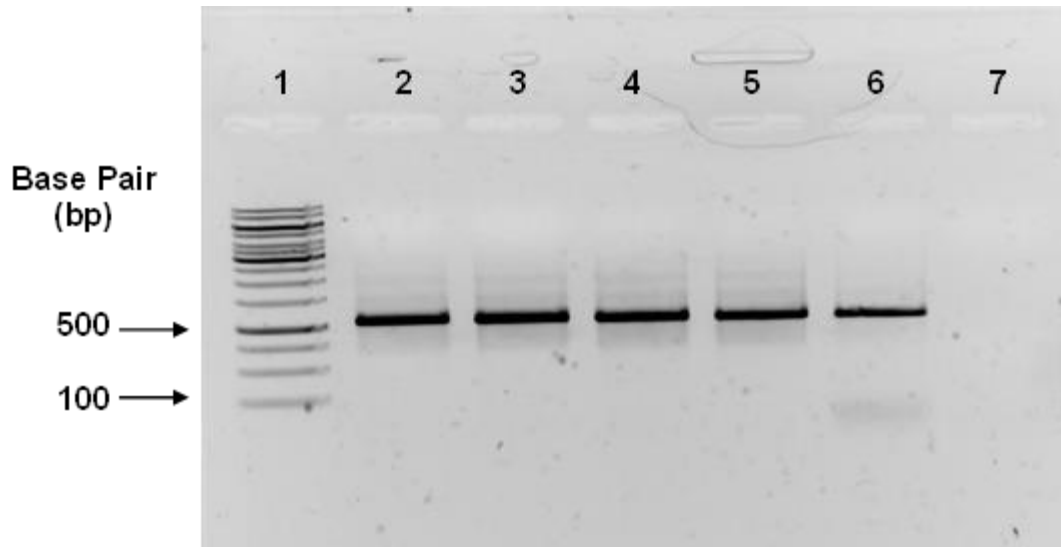
### **2.2.8 Ethical approval**

All experimental procedures with live fish were carried out in accordance with the Norwegian Food Safety Authority guidelines (FOTS ID 15478 and 23188) and were approved by the Animal Welfare and Ethical Review Body of the University of Stirling, UK.

## 2.4 Results

### 2.4.1 Bacterial isolates

The Atlantic salmon and rainbow trout *F. psychrophilum* isolates used in this present study were recovered from moribund fry at commercial fish farms in Scotland (Table 2.1). The spleens from these moribund animals were streaked in MVA and representative colonies were confirmed to be *F. psychrophilum* by a nested PCR that targets the 16S *rRNA* gene, as described by Ngo et al. (2017). Agarose gel electrophoresis (Figure 2.4) was used to visualise the PCR results. All bacteria isolates used for the Atlantic salmon and rainbow trout challenges were confirmed *F. psychrophilum* positive. The nucleotide sequence of 16S *rRNA* from each bacterial isolate was >98% identical to the corresponding sequence of *F. psychrophilum* JIP02/86 (Table 2.2).



**Figure 2.4** Agarose gel (1%) used to confirm the presence of *F. psychrophilum* from representative colonies recovered from moribund animals sampled from natural outbreaks of flavobacteriosis in Atlantic salmon and rainbow trout using a nested PCR that targets the 16S rRNA gene. From left to right the wells were filled with 100 bp gene ruler (position 1), Atlantic salmon isolate 6\_S and 18\_S (position 2 and 3), rainbow trout isolate 19\_5 and 365a (position 4 and 5), positive control (position 6), and a non-template control (NTC) (position 7).

**Table 2.2** Top 100 sequences producing significant alignments when blasting nucleotide sequence of 16S rRNA from each bacterial isolate in BLASTn.

| Description                         |                                                             | Query Cover | E value | Percentage Identity |
|-------------------------------------|-------------------------------------------------------------|-------------|---------|---------------------|
| <i>Flavobacterium psychrophilum</i> | gene for 16S rRNA, partial sequence, strain: FPC814         | 86%         | 0       | 98.53%              |
| <i>Flavobacterium psychrophilum</i> | strain CH27 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.52%              |
| <i>Flavobacterium psychrophilum</i> | strain CH21 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.52%              |
| <i>Flavobacterium psychrophilum</i> | strain CH14 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.52%              |
| <i>Flavobacterium psychrophilum</i> | strain FLpH 004/09 16S ribosomal RNA gene, partial sequence | 86%         | 0       | 98.52%              |
| <i>Flavobacterium psychrophilum</i> | strain FPCH3 16S ribosomal RNA gene, partial sequence       | 86%         | 0       | 98.52%              |
| <i>Flavobacterium psychrophilum</i> | strain FPCH1 16S ribosomal RNA gene, partial sequence       | 86%         | 0       | 98.52%              |
| <i>Flavobacterium psychrophilum</i> | strain CH16 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.45%              |
| <i>Flavobacterium psychrophilum</i> | strain CH22 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> | strain CH09 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> | strain FPCH6 16S ribosomal RNA gene, partial sequence       | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> | strain FPCH4 16S ribosomal RNA gene, partial sequence       | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> | strain CH31 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> | strain CH30 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> | strain CH29 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> | strain CH26 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> | strain CH25 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> | strain CH24 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> | strain CH23 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> | strain CH20 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> | strain CN6 chromosome, complete genome                      | 87%         | 0       | 98.38%              |
| <i>Flavobacterium psychrophilum</i> | strain 5 genome                                             | 87%         | 0       | 98.38%              |
| <i>Flavobacterium psychrophilum</i> | strain MH1 genome                                           | 87%         | 0       | 98.38%              |
| <i>Flavobacterium psychrophilum</i> | strain PG2, complete genome                                 | 87%         | 0       | 98.38%              |
| <i>Flavobacterium psychrophilum</i> | strain VQ50 chromosome                                      | 87%         | 0       | 98.38%              |
| <i>Flavobacterium psychrophilum</i> | strain 3 chromosome                                         | 87%         | 0       | 98.38%              |

|                                     |                                                   |     |   |        |
|-------------------------------------|---------------------------------------------------|-----|---|--------|
| <i>Flavobacterium psychrophilum</i> | strain 950106-1/1, complete genome                | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R18 chromosome                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R14 chromosome                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R12 chromosome                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R17 chromosome                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R9 chromosome, complete genome      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R11 chromosome                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R16 chromosome, complete genome     | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R13 chromosome                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R8 chromosome                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R7 chromosome                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R15 chromosome                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R10 chromosome                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R5 chromosome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R2 chromosome                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R1 chromosome                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R9 chromosome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R8 chromosome, complete genome | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R7 chromosome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-C1 chromosome, complete genome      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R6 chromosome                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R3 chromosome                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R5 chromosome                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R4 chromosome                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R4 chromosome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-C1 chromosome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R2 chromosome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R3 chromosome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R5 chromosome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R1 chromosome 160401_1_5N_R1   | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | FPG101, complete genome                           | 87% | 0 | 98.38% |

|                                     |                                                                           |     |   |        |
|-------------------------------------|---------------------------------------------------------------------------|-----|---|--------|
| <i>Flavobacterium psychrophilum</i> | strain CSF259-93, complete genome                                         | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain v4-33, complete genome                                             | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain V4-28 genome                                                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain V4-24, complete genome                                             | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain V2-20 genome                                                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain V1-20 genome                                                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain V3-5, complete genome                                              | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain CN38 chromosome, complete genome                                   | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain CN46 chromosome, complete genome                                   | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | JIP02/86 complete genome                                                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain AQ_Fpsy_001_2012_A4215 16S ribosomal RNA gene,<br>partial sequence | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-F21 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-F22 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-F33 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-F32 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-F30 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-D15 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-D10 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain P30-2B/09 chromosome                                               | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain P15/8B-11 chromosome                                               | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain P7-7B/10 chromosome                                                | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S11B chromosome                                                | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain K9/00 chromosome                                                   | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S11A chromosome                                                | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S10 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-F16 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-F27 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S9 chromosome                                                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 030522-1/1 chromosome                                              | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5M chromosome                                             | 87% | 0 | 98.38% |

|                                     |                                                               |     |   |        |
|-------------------------------------|---------------------------------------------------------------|-----|---|--------|
| <i>Flavobacterium psychrophilum</i> | strain 990512-1/2A chromosome                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 141127-1/2N chromosome                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 010418-2/1 chromosome                                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-F15 chromosome                                     | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain F164 chromosome                                        | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-G1 chromosome                                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-R7 chromosome, complete genome                     | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N chromosome, complete genome                | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain strain 950106-1/1 chromosome, complete genome          | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPRT1 chromosome, complete genome                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6 chromosome, complete genome                     | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain CSF 259-93 16S ribosomal RNA gene, partial<br>sequence | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain OSU THCO2-90 genome assembly, chromosome:              | 87% | 0 | 98.30% |

### 2.4.2 Experimental animals

The *F. psychrophilum*-free status of the animal prior to the challenge, was determined by streaking head kidney and spleen samples of ten fish on to MVA and all were negative for *F. psychrophilum* colonies after incubation.

### 2.4.3 Preliminary challenges

The preliminary challenge of Atlantic salmon fry showed that both *F. psychrophilum* 18\_S and *F. psychrophilum* 6\_S were virulent in a dose-dependent manner, with first mortalities observed at 2 dpc for *F. psychrophilum* 6\_S at  $2 \times 10^7$  CFU/mL and 4 dpc for *F. psychrophilum* 18\_S at  $2 \times 10^7$  CFU/mL; no mortalities occurred in any group after 3 dpc (Figure 2.5). *F. psychrophilum* 6\_S was significantly more virulent at the higher concentration ( $\chi^2=5.77$  dof=1,  $p<0.05$ ). Colonies recovered from plating Atlantic salmon tissues across MVA were suspected to be *F. psychrophilum* for 71.6% to 100% of the fish sampled (Table 2.4). A representative colony from each culture plate was confirmed to be *F. psychrophilum* by PCR, with all the colonies examined testing positive by this analysis.

Meanwhile, the preliminary challenge in rainbow trout showed that *F. psychrophilum* 19\_5 at  $2 \times 10^7$  CFU/mL was highly virulent and resulted in 81.5% mortality after 21 dpc (Figure 2.6). Since the only isolate used in the preliminary challenge was *F. psychrophilum* 19\_5, no statistical comparison can be made (Table 2.3).

### 2.4.4 Main challenge

The main challenge was carried out using  $2 \times 10^7$  CFU/mL of *F. psychrophilum* 6\_S and *F. psychrophilum* 19\_5 for the Atlantic salmon and rainbow trout challenges, respectively. The survival for Atlantic salmon and rainbow trout at the end of the main challenges was 81.2% (at 21 dpc) and 37.9 % (at 28 dpc), respectively (Figure 2.7). Similar to the preliminary challenges, no external lesions were observed on dead or moribund fish. Colonies suspected to be *F. psychrophilum* were detected by culture for 79.0% and 93.3% of Atlantic salmon and rainbow trout tissues plated across MVA, respectively. PCR of a representative colony from each culture plate was performed and those tested were positive by this analysis in each case. There were no significant differences in percentage survival of Atlantic salmon between the preliminary and

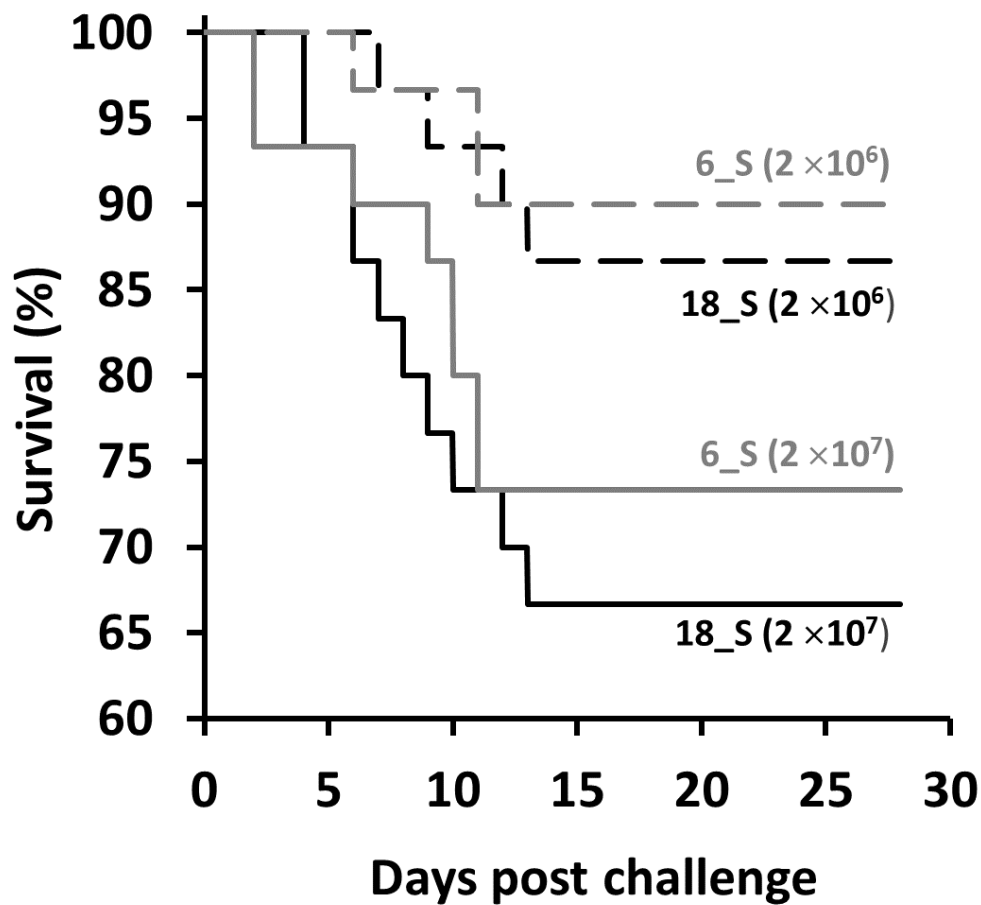
main challenges for *F. psychrophilum* 6\_S ( $\chi^2= 2$ , dof=1,  $p=>0.05$ ), however there was a significant difference between the rainbow trout preliminary and main challenges using *F. psychrophilum* 19\_5 ( $\chi^2= 36.1$ , dof=1,  $p<0.05$ ) performed at the same doses, (Table 2.4).

**Table 2.3** Table showing the different statistical comparisons made in the preliminary and main challenge trials.

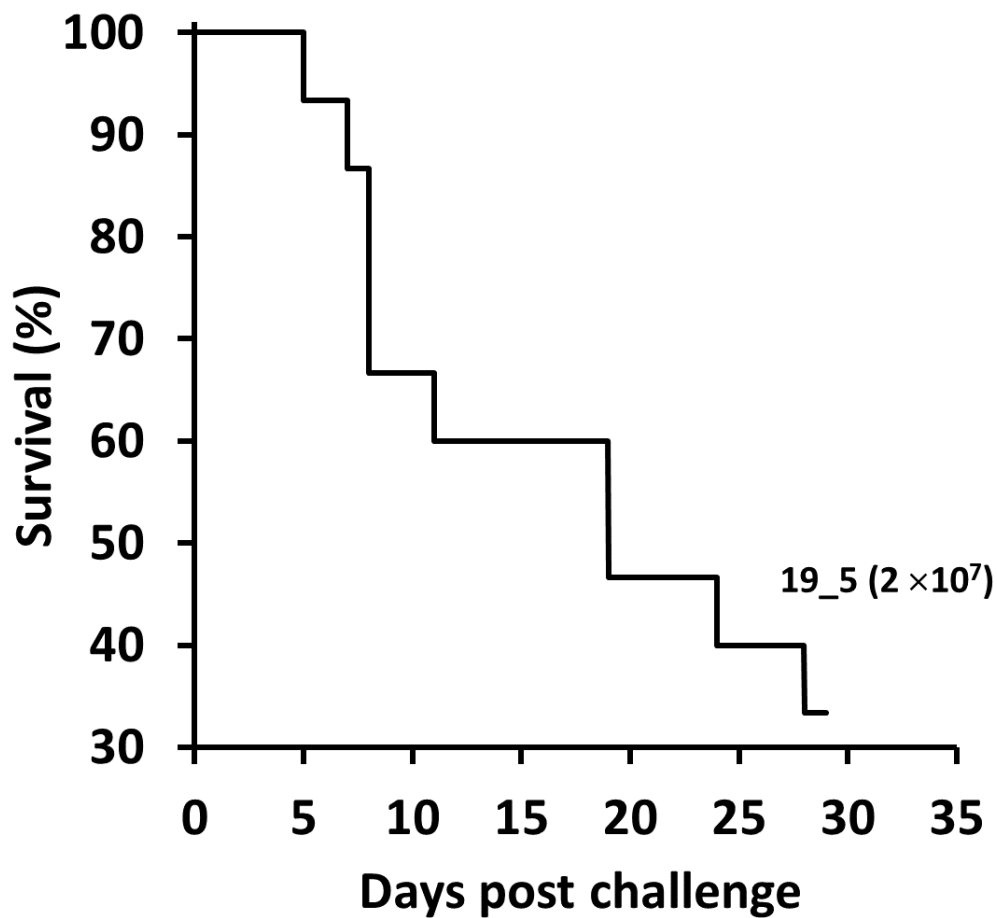
|                       | Statistical comparison                                                                                                                                | Statistical Results              |
|-----------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------|
| Preliminary challenge | <u>Bacterial dose:</u>                                                                                                                                |                                  |
|                       | <i>F. psychrophilum</i> 6_S at $2 \times 10^6$ CFU/mL<br>vs<br><i>F. psychrophilum</i> 6_S at $2 \times 10^7$ CFU/mL                                  | $\chi^2=5.77$ dof=1, $p<0.05$    |
| Main Challenge        | <u>Percentage survival in Atlantic salmon preliminary and main challenge trials:</u>                                                                  |                                  |
|                       | <i>F. psychrophilum</i> 6_S at $2 \times 10^7$ CFU/mL (preliminary study)<br>vs<br><i>F. psychrophilum</i> 6_S at $2 \times 10^7$ CFU/mL (main study) | $\chi^2= 2$ , dof=1, $p=>0.05$   |
|                       | <u>Percentage survival in Atlantic salmon and rainbow trout main challenge trials:</u>                                                                |                                  |
|                       | <i>F. psychrophilum</i> 6_S at $2 \times 10^7$ CFU/mL<br>vs<br><i>F. psychrophilum</i> 19_5 at $2 \times 10^7$ CFU/mL                                 | $\chi^2= 36.1$ , dof=1, $p<0.05$ |

**Table 2.4** Outline of the preliminary and main challenges with *F. psychrophilum* in Atlantic salmon and rainbow trout, including the bacterial isolates used and their origins, the challenge concentrations, and percentage of sampled mortalities where *F. psychrophilum* was recovered by bacteriological culture and confirmed by PCR.

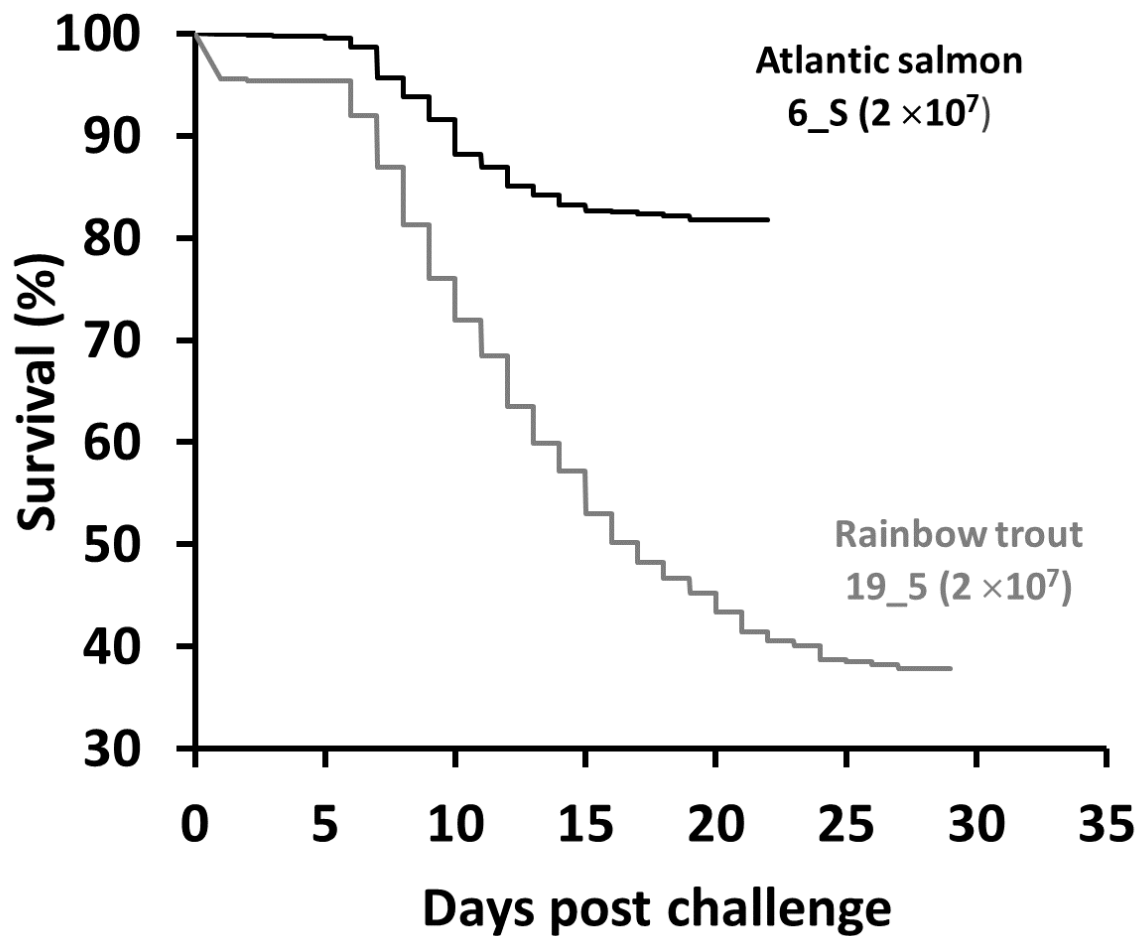
|                           | Tank number<br>(volume; number<br>of fish) | Bacterial isolate            | Fish species    | Bacterial challenge<br>concentration<br>(CFU/mL) | Proportion of fish tested that<br>yielded <i>F. psychrophilum</i><br>colonies (%) |
|---------------------------|--------------------------------------------|------------------------------|-----------------|--------------------------------------------------|-----------------------------------------------------------------------------------|
| Preliminary<br>challenges | A (4 L; n=30)                              | <i>F. psychrophilum</i> 6_S  | Atlantic salmon | $2 \times 10^6$                                  | 72.0                                                                              |
|                           | B (4 L; n=30)                              | <i>F. psychrophilum</i> 6_S  | Atlantic salmon | $2 \times 10^7$                                  | 100.0                                                                             |
|                           | C (4 L; n=30)                              | <i>F. psychrophilum</i> 18_S | Atlantic salmon | $2 \times 10^6$                                  | 71.6                                                                              |
|                           | D (4 L; n=30)                              | <i>F. psychrophilum</i> 18_S | Atlantic salmon | $2 \times 10^7$                                  | 79.0                                                                              |
| Main challenge            | E (120 L; n=1,187)                         | <i>F. psychrophilum</i> 6_S  | Atlantic salmon | $2 \times 10^7$                                  | 79.0                                                                              |
|                           | F (120 L; n=50)                            | <i>F. psychrophilum</i> 6_S  | Atlantic salmon | $2 \times 10^7$                                  | 93.3                                                                              |
|                           | G (4 L; n=15)                              | <i>F. psychrophilum</i> 19_5 | Rainbow trout   | $2 \times 10^7$                                  | 80.0                                                                              |
|                           | H (120 L; n=500)                           | <i>F. psychrophilum</i> 19_5 | Rainbow trout   | $2 \times 10^7$                                  | 93.3                                                                              |



**Figure 2.5** Kaplan-Meier survival curves of Atlantic salmon in the preliminary challenge with two isolates derived from Atlantic salmon (*F. psychrophilum* 6\_S and *F. psychrophilum* 18\_S) at two concentrations, specifically  $2 \times 10^6$  and  $2 \times 10^7$  CFU/mL. Fish with a mean mass of  $2.4 \pm 0.237$  g ( $n=30$  per treatment group) were exposed to hydrogen peroxide (200 mg/L) for 1 h prior to immersion challenge with the bacteria for 4 h.

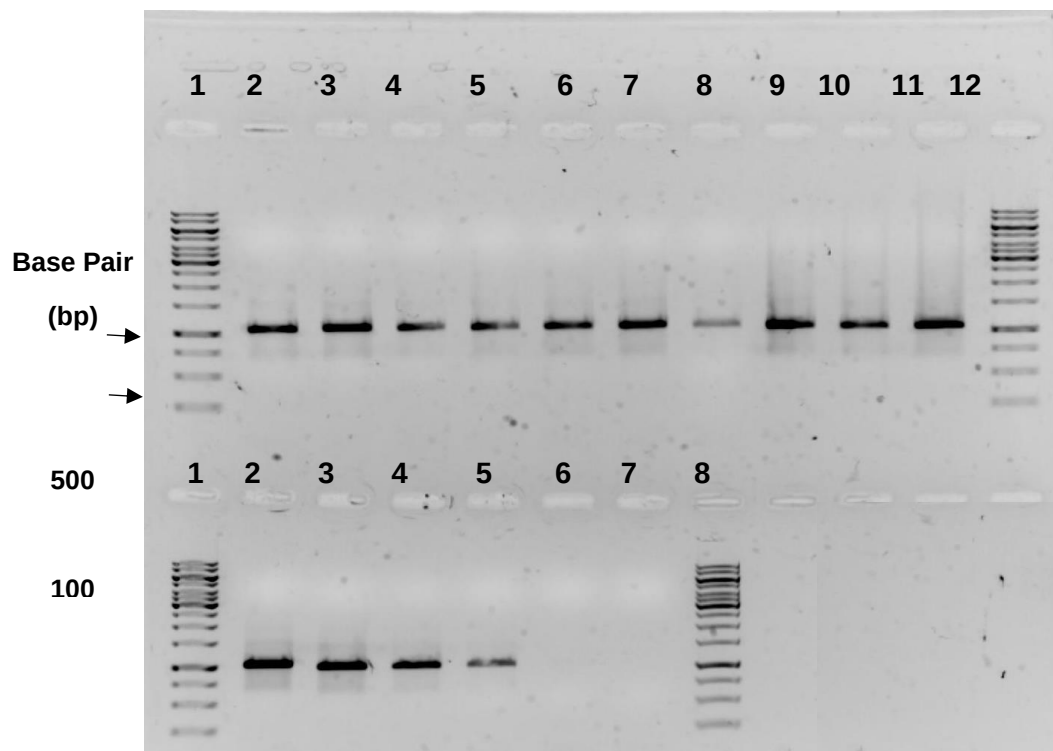


**Figure 2.6** Kaplan-Meier survival curves of rainbow trout in the preliminary challenge with one isolate derived from rainbow trout (*F. psychrophilum* 19\_5) at  $2 \times 10^7$  CFU/mL. Fish (n=15) with a mean mass of  $1.8 \pm 0.247$  g; were exposed to hydrogen peroxide (200 mg/L) for 1 h prior to immersion challenge with the bacteria for 4 h.



**Figure 2.7** Kaplan-Meier survival curves of Atlantic salmon in the main challenge with *F. psychrophilum* 6\_S (for Atlantic salmon) and *F. psychrophilum* 19\_5 (for rainbow trout) at  $2 \times 10^7$  CFU/mL. Atlantic salmon ( $n=1,187$ ;  $1.8 \pm 0.237$ g) and rainbow trout ( $n=1,024$ ;  $1.8 \pm 0.247$  g;) were exposed to hydrogen peroxide (200 mg/L) for 1 h prior to immersion challenge with the bacteria for 4 h (Atlantic salmon) or 1.5 h (rainbow trout).

During the main Atlantic salmon and rainbow trout challenges, the spleen of 10% of the mortalities were streaked of MVA and a representative colony from each plate resembling *F. psychrophilum* was analysed by two-round nested PCR (Figure 2.8)



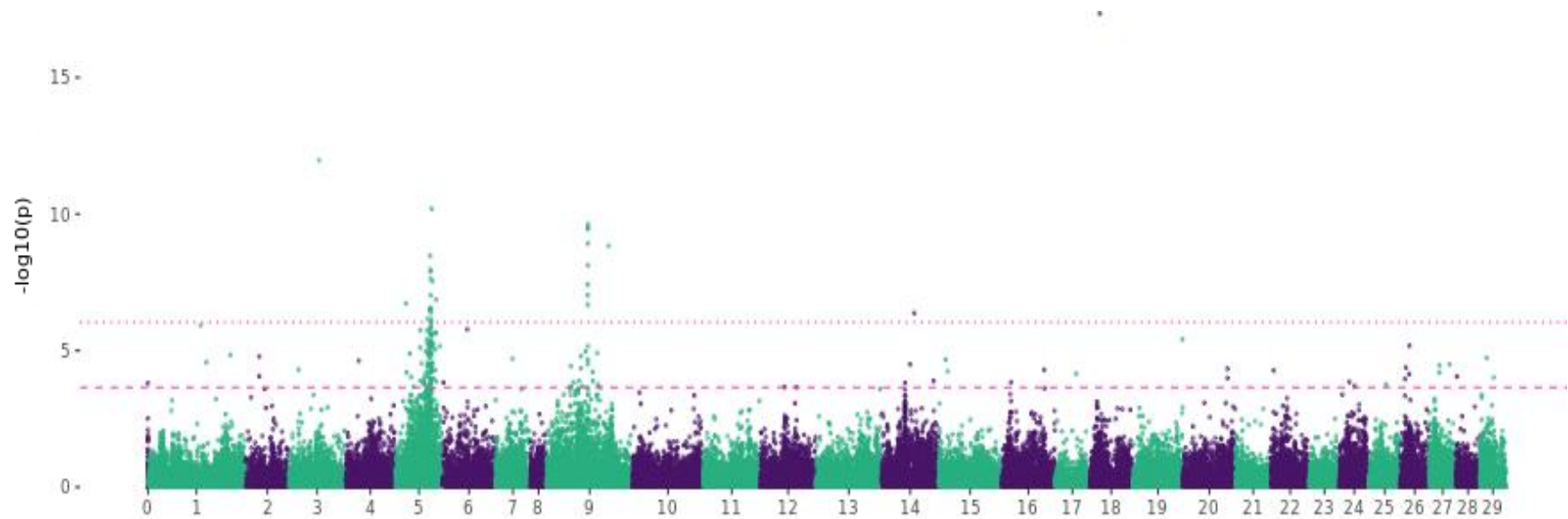
**Figure 2.8** *F. psychrophilum* nested PCR used to confirm representative colonies recovered from moribund or deceased fish during the 2019 Atlantic salmon main immersion challenge trial. Top lane: 100-bp gene ruler (position 1 and 12), positive control (position 2), samples 2-11 (position 3-11); bottom lane: 100bp gene ruler (position 1 and 8), samples 12-15 (position 2-5) and NTC (position 6 and 7).

### 2.4.3 Heritability and GWAS

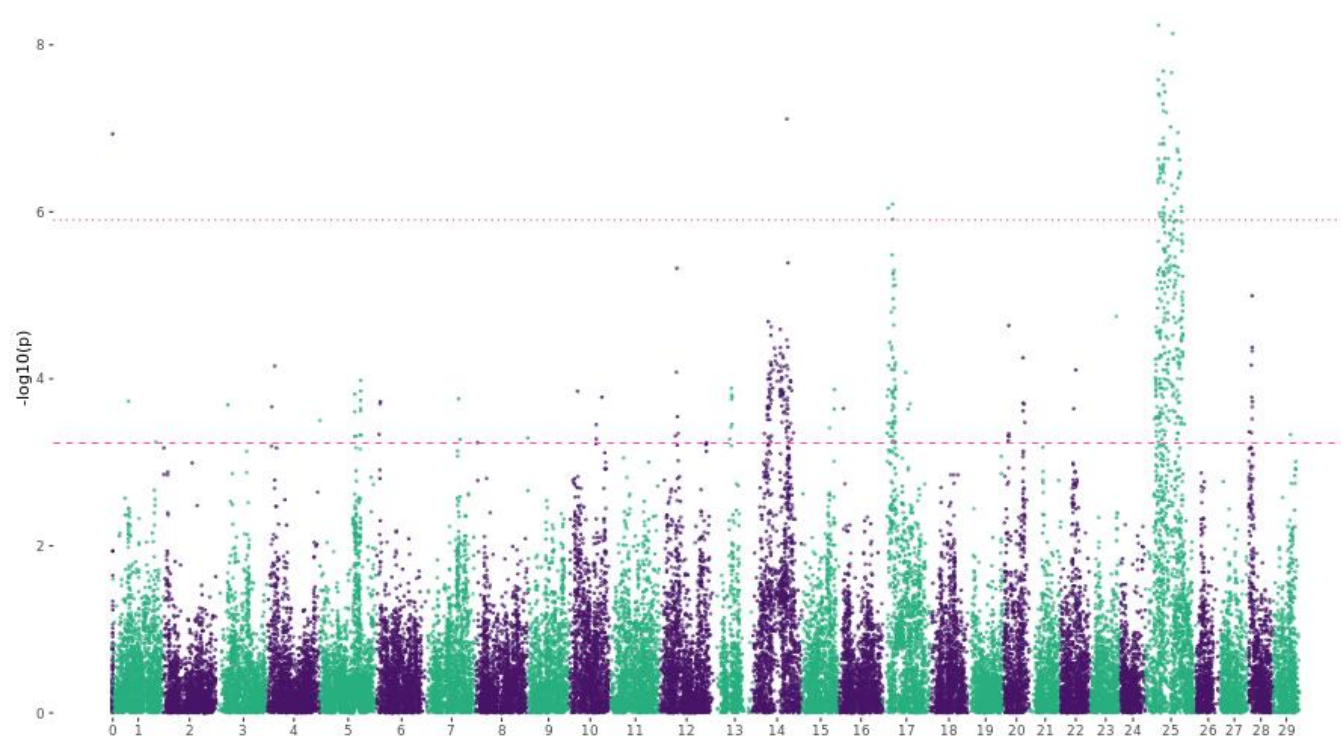
Heritability measures the relative importance of hereditary and environmental influences on the development of a specific quantitative trait, and it is important to take this into consideration when looking at disease resistance. The genetic heritability was determined using the binary measurement survival and the results estimated moderate heritability for *F. psychrophilum* survival for both Atlantic salmon and rainbow trout,  $h^2 = 0.173 \pm 0.047$  and  $h^2 = 0.37 \pm 0.04$  respectively. The moderate heritability in both Atlantic salmon and rainbow trout suggest that selection for the trait can be based upon the individual's own level of performance, which allows a rapid rate of improvement.

GWAS are used to assess the genetic architecture of a phenotypic trait by genome partitioning, and to identify genes associated with a particular disease (or trait). Using this method, the entire genome of a large number of Atlantic salmon and rainbow trout fry infected with *F. psychrophilum* was studied to find genetic variations associated with this bacterial disease. In Atlantic salmon, the GWAS identified 21 variants. The Manhattan plot for survival (Fig. 2.9), as a result of the GWAS, indicates two strong QTLs located on chromosome 5 and chromosome 9 with most significant variants being located on these two regions (chromosome 9  $n=6$  and chromosome 5  $n=14$ ) (Figure 2.9). Chromosome 5 explained 5% and chromosome 9 explained 8% of the phenotypic variation.

In rainbow trout, the GWAS identified hundreds of variants, and the Manhattan plot for survival (Fig. 2.10), highlights several QTLs with larger effects discovered on chromosome 14, chromosome 17, and chromosome 25, however it was expected to find QTLs located on chromosome 3 and chromosome 8, which have been previously discovered by AquaGen (data not available) and by other research groups (Palti et al. 2015; Fraslin et al. 2018) (Figure 2.10).



**Figure 2.9** Manhattan plots for genome-wide association analysis of *F. psychrophilum* resistance in Atlantic salmon. The figure shows the  $-\log_{10}(p\text{-value})$  of the test statistics for each SNP plotted against the physical positions of the markers on the chromosomes. The two lines in the panel indicate significance threshold after accounting for multiple testing using either: Bonferroni correction (dotted line) or False Discovery Rate correction (dashed line). Figure produced by Fabian Grammes (Senior Scientist, AquaGen Norway).



**Figure 2.10** Manhattan plots for genome-wide association analysis of *F. psychrophilum* resistance in rainbow trout. The figure shows the  $-\log_{10}(p)$ -value of the test statistics for each SNP plotted against the physical positions of the markers on the chromosomes. The two lines in the panel indicate significance threshold after accounting for multiple testing using either: Bonferroni correction (dotted line) or False Discovery Rate correction (dashed line). Figure produced by Fabian Grammes (Senior Scientist, AquaGen Norway).

## 2.5 Discussion

Flavobacteriosis caused by the Gram-negative, psychrophilic bacterium *F. psychrophilum* affects rainbow trout and Atlantic salmon, especially at the fry stage when the fish are too small to be vaccinated by injection. To address the need for a reliable challenge model that can underpin future disease mitigation efforts, an immersion challenge model for *F. psychrophilum* in Atlantic salmon fry was developed for the first time. The development of a standardised and reproducible immersion model for *F. psychrophilum* for rainbow trout and Atlantic salmon allows a better understanding of this pathogen, transmission dynamics, virulence, and potential disease treatments.

In the present Chapter, an immersion challenge model for *F. psychrophilum* in rainbow trout was developed based on a previous study by Hoare et al. (2017) and this model was then successfully applied for the first time to Atlantic salmon fry. A rainbow trout challenge was conducted as a point of comparison to evaluate reliability and reproducibility when establishing the infection with different isolates of the bacterium. The immersion challenge models were reproducible for rainbow trout, as evidenced by the non-significant differences in percentage survival between the preliminary and the main challenges for Atlantic salmon ( $p > 0.05$ ). For the preliminary challenge the fish was immersed in the bacteria for 4 h, while in the main rainbow trout challenge, the fish was immersed in the bacteria just for 1.5 h due to technical reasons. A similar study using the *F. psychrophilum* immersion challenge model developed here did not find a significant difference between the preliminary and main challenged rainbow trout percentage survival (Macchia et al., 2022).

Several studies have attempted to develop a *F. psychrophilum* water borne challenge model for salmonids that is effective and reproducible, but attempts have produced inconsistent results in terms of mortalities (Cipriano & Holt, 2005; Henriksen et al., 2013; Long et al., 2014; Madsen & Dalsgaard, 1999). Previous experimental research on *F. psychrophilum* infection of salmonids, including Atlantic salmon and rainbow trout, has relied on inducing mortality via intramuscular injection of the bacteria (Fredriksen et al., 2016). However, injection delivery bypasses the physical and immune barriers of the skin and the mucosa that play an important role in protecting the fish against infection (Koshio, 2016). Long et al. (2014) performed an immersion

challenge and observed that mortality was significantly greater in fish that had been scarred compared to those that had not been pre-treated in this way (81.5% vs. 19.4%). Similar mortality rates have been reported where fry were compromised with an incision below the dorsal fin prior to challenge by immersion (Madetoja et al., 2000). These findings suggest that disruption of the skin surface facilitates the entering of the host and the subsequent establishment of the infection for *F. psychrophilum*. Moreover, Hoare et al. (2022) highlighted the importance of rainbow trout mucus during immersion challenge studies with *F. psychrophilum*. In particular, greater levels of actins were observed in skin mucus of rainbow trout fry challenged by immersion compared to injection delivery, and the authors suggest these may have a role in mucosal immunity by potentiating the macrophage response to pathogens (Hoare et al., 2022). In this present study, the fish were pre-treated with hydrogen peroxide prior to bacterial challenge to remove the surface mucus and expose the skin to the bacteria. This approach has been used previously to establish the effective immersion challenge model for rainbow trout (Henriksen et al., 2013; Hoare et al., 2017). In addition to serving as a barrier, rainbow trout and Atlantic salmon have bactericidal enzymes and other antimicrobial compounds in the surface mucus (Ángeles Esteban, 2012; Kelly & Salinas, 2017; Sprague & Desbois, 2021). Furthermore, Fast et al. (2002) observed that the Atlantic salmon mucus exhibits protease and lysozyme activities and abundant mucous cells are found in the epidermis. Hence, when designing this challenge model for Atlantic salmon, the pre-treatment was used to strip the mucus prior to challenge with a large dose of *F. psychrophilum* ( $2 \times 10^7$  CFU/mL) in an attempt to ensure that an infection would be established.

Despite the lack of external lesions in moribund or deceased fish, bacterial re-isolation rates were relatively high (e.g., 90.1% from rainbow trout and 79% from Atlantic salmon in the main challenge) and this suggests that the infection was systemic in nature. This result is in line with reports on natural outbreaks, where Nilsen et al. (2011) and Rimstad (2019) observed systemic infection with *F. psychrophilum* in rainbow trout hatcheries. *F. psychrophilum* can be difficult to isolate from fish tissues and this may explain why the bacterium was not recovered by culture from each fish sampled (Cepeda et al., 2004).

Of course, further studies involving more tanks and replication, including mock-challenged control groups, are needed to be completely satisfied with the reproducibility of this model, but the present observations are promising. Notably, biomass density can influence pathogen transmission, host stress levels and mortality rates (Williams et al., 2002; Tort 2011), for Atlantic salmon. The lack of significant differences between the preliminary and main challenges suggests the biomass density differences between the trials had little or no effect on overall mortality. This observation is consistent with Klung et al. (2021) who observed stocking density had no significant effect on the stress response and disease susceptibility of juvenile rainbow trout when infected with infectious haematopoietic necrosis virus.

Furthermore, there remains scope to improve this challenge model, for example by determining a lower threshold for the bacterial dose that would still establish an infection reliably, particularly if this led to the development of more classical external clinical signs indicative of surface infection.

The results presented here describe a novel immersion infection model which leads to a satisfactory high level of mortality required for assessment of an intervention in both rainbow trout and Atlantic salmon studies. However, with respect to lethal infection in Atlantic salmon fry, survival was relatively high and it would be highly desirable to attempt to reduce this further for some applications, including vaccine efficacy assessments where >60% mortality is recommended (Amend, 1981).

One logistical issue with carrying out immersion challenges with *F. psychrophilum* is the large quantity of bacteria that is needed for the challenge and consequent difficulties with washing and preparing the bacteria, including preventing contamination and maintaining high viability. With these large quantities of bacteria, maintaining the bacterial culture pure is essential to preserve enough viable bacteria for challenge and retain its virulence (Lagier et al., 2015). Additionally, *F. psychrophilum* is a fastidious, slow-growing organism that requires specialised media for optimal growth in the laboratory (Cain & LaFrentz, 2007) and could potentially become non-viable during the washing steps. In this present study, washing steps for the bacteria were not performed, meaning extracellular products were also present for the challenges, and there is some evidence that these may enhance the efficacy of an immersion challenge (Lapatra et al., 2010), though this requires further study to confirm. This Chapter demonstrated that it is safe and more effective for both Atlantic salmon and rainbow trout to remove the washing step and immerse the fish directly in

unwashed bacterial culture, however, to fully justify this statement, further studies comparing the immersion challenge using washed and un-washed bacteria could strengthen these results.

Virulence in *F. psychrophilum* is poorly understood and differences in virulence of these bacterial isolates is possibly due to the genotypes of the isolates used (Castillo et al., 2021; Sundell et al., 2019). Conducting tests with isolates of different serotypes would help to determine the extent of the *F. psychrophilum* isolates for which this newly developed model is useful, whilst providing a platform to understanding differential virulence of strains.

The research in this Chapter presented the first published GWAS analysis of *F. psychrophilum* resistance in commercial Atlantic salmon fry with a 70k high-density SNP chip (Figure 6.9 and Figure 6.10) as well as the first reproducible immersion challenge model for *F. psychrophilum* in Atlantic salmon fry, which should emulate a more natural route of infection. From these challenge studies there is a confidence that the correct traits have been measured since, bacteriology confirmed presence of *F. psychrophilum* colonies in the morbidities/mortalities and no *F. psychrophilum* colonies were recovered from animals prior to the challenge.

Resistance against *F. psychrophilum* was a moderately heritable trait in both Atlantic salmon ( $h^2 = 0.173 \pm 0.047$ ) and rainbow trout ( $h^2 = 0.37 \pm 0.04$ ). In Atlantic salmon, the genetic architecture of the trait appears to be oligogenic with two QTLs located on chromosomes 5 and 9. In contrast, in rainbow trout searches for QTL associated with resistance to *F. psychrophilum* have been identified, however with varying consistency (Fraslin et al., 2019; Vallejo et al., 2014, 2016). In the present study, several large QTLs were found on chromosomes 14, 17 and 25, and for this study the focus was on two QTLs on chromosome 3 and 8, chromosomes which have been previously discovered by AquaGen Norway (data not available) and it was therefore expected to see larger peaks on these two chromosomes. The lack of strong peaks on chromosome 3 and 8 could be as the result of the bacteria isolate used in the study or the experimental design itself. The challenge trials used different bacterial isolates which varied in terms of virulence. *F. psychrophilum* is a highly heterogeneous bacterium (Calvez et al., 2021), hence it is possible that the bacterial strains could have affected the QTLs.

Lee et al. (2022) has previously shown that resistance to bacterial wilt in Chili Pepper, *Capsicum annuum* L., occurred differently depending on the *Ralstonia solanacearum* isolate used to infect the plants, therefore the *F. psychrophilum* isolates used in this Chapter can all have a different effect on mortalities.

Nevertheless, the present Chapter determined that the lack of consistency of QTLs for resistance to *F. psychrophilum* indicate that the trait in rainbow trout is of polygenic nature, or this could also be as a result of differences in power of GWAS.

Based on what has been found in rainbow trout (Fraslin et al., 2018; Vallejo et al., 2014, 2017), where resistance to *F. psychrophilum* is a polygenic trait, it was expected to find a similar situation in Atlantic salmon, specifically multiple peaks on various chromosomes with small effects. Examination of the genetic architecture of traits related to host response to pathogens is an important approach for gaining understanding of the host-pathogen interactions in fish, giving information on the position in the genome of genetic variants and the effects that are associated with the trait of interest.

## 2.6 Conclusion

In conclusion, this is the first study to develop a reliable and reproducible immersion challenge model for *F. psychrophilum* in Atlantic salmon and rainbow trout fry. This challenge model provides the means to investigate host-pathogen interactions and allows for the evaluation of new therapies and preventative measures such as resistant fish breeds and vaccines. The GWASs gave an insight into the genetic architecture of resistance traits in both Atlantic salmon and rainbow trout highlighting the differences in QTLs. To explore the results of this chapter further, it would be of interest to look at whether the QTLs discovered are the same across the species, and synteny of QTL regions and fine mapping of the top SNPs will need to be performed. This present study allowed the selection for *F. psychrophilum* resistant Atlantic salmon eggs which are currently available from AquaGen (<https://www.aquagen.no/>). Due to the nature of the trait being polygenic in rainbow trout, the existing *F. psychrophilum* resistant rainbow trout eggs products based on the discovered QTLs in this study are still being investigated. Further studies are still required to identify the causative genes and variants underlying these newly discovered QTLs to identify and elucidate the mechanisms of resistance. Meanwhile salmonid aquaculture success still relies on

biosecurity and tools that allow the detection of the bacteria in the early stages. The next Chapter will look at the development of a specific, sensitive, and cost-effective method for detection of *F. psychrophilum*, which could be applied as a point-of-care diagnostic tool for monitoring the spread of flavobacteriosis in Atlantic salmon and rainbow trout fry in fish farm facilities.

## **Chapter 3: SYBR Green qPCR assay for *F. psychrophilum* detection in Atlantic salmon and rainbow trout tissues**

### **3.1 Introduction**

During the past decade flavobacteriosis has become one of the main bacterial diseases affecting mainly hatchery-reared rainbow trout fry and fingerlings and, in more recent years, Atlantic salmon fry in many parts of Europe (Sommerset et al., 2021; Starliper, 2011; Yatabe et al., 2018). Diagnosis of *Flavobacterium psychrophilum* (*F. psychrophilum*) is a challenging issue, due to the nature of this bacterium being a fastidious microorganism, and in recirculating aquaculture systems (RAS), due to its biofilm forming properties (Lewin et al., 2020). Different *F. psychrophilum* strains are known to attach themselves to and can form biofilms on the living surfaces of fish bodies, spleen, and eggs (Nematollahi et al., 2003; Strepparava et al., 2012; Vatsos et al., 2001) as well as on non-living surfaces such as polystyrene surfaces (Högfors-Rönholm et al., 2015). Bacteria in biofilms have the ability to evade the host defence systems by remaining dormant and hidden from the immune system and may cause local tissue damage which can cause an acute infection later (Vestby et al., 2020). These adaptations of the biofilm can make it difficult to diagnose the bacteria, because the biofilm is localised in a specific part of the fish which is not targeted for the diagnosis (Vestby et al., 2020). There are a range of available methods to detect *F. psychrophilum* depending on the laboratory seeking to identify this bacterium including culture-dependent techniques (Avendaño-Herrera et al., 2020; Pérez-Sancho et al., 2017), and non-culture methods such as antigen based (Crump et al., 2001), polymerase chain reaction (PCR)- based (Ngo et al., 2018), quantitative PCR (qPCR)-based (Orieux et al., 2011; Strepparava et al., 2014), and, more recently, droplet digital PCR (Lewin et al., 2020).

These techniques have a number of challenges in their application, for instance *F. psychrophilum* identification can be protracted due to the slow growth of the bacteria (Avendaño-Herrera et al., 2020), it can be labour intensive to culture (Crump et al., 2001), there is a prerequisite need for skilled staff with experience in growing this bacterium (Lewin et al., 2020) and in PCR-based diagnostic techniques, the reaction is highly influenced by reaction inhibitors (Ngo et al., 2018). Additionally, conventional diagnostic techniques can be highly costly (Strepparava et al., 2014) (Table 3.1).

**Table 3.1** Techniques used to identify bacteria.

| Technique              | Advantages                                                                                                                                                                                                                             | Disadvantages                                                                                                                                                                                                         |
|------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Biochemical            | <ul style="list-style-type: none"> <li>- 16S rRNA for bacterial identification circumvents the need for bacteria cultivation (Rizal et al., 2020)</li> <li>- High sensitivity with appropriate media (Dickson et al., 2014)</li> </ul> | <ul style="list-style-type: none"> <li>- Costly laboratory setup and reagents</li> <li>- Lack of techniques validations (Rizal et al., 2020)</li> </ul>                                                               |
| Antigen based          | <ul style="list-style-type: none"> <li>- Simple procedure and easy to perform</li> <li>- High specificity and sensitivity</li> <li>-</li> </ul>                                                                                        | <ul style="list-style-type: none"> <li>- Labour intensive and expensive antibodies</li> <li>- High possibility of a false positive/negative result</li> <li>- Antibody instability (Sakamoto et al., 2018)</li> </ul> |
| PCR- based             | <ul style="list-style-type: none"> <li>- The PCR procedure is faster than those of cultures</li> <li>- Higher positivity rates and sensitivity than culture and stains for both bacteria and fungi (Journal et al., 2019)</li> </ul>   | <ul style="list-style-type: none"> <li>- Time- and resource-consuming</li> <li>- Require post-PCR analysis (Smith &amp; Osborn, 2009)</li> </ul>                                                                      |
| Quantitative PCR-based | <ul style="list-style-type: none"> <li>- Greater sensitivity and enables discrimination of gene numbers across a wider dynamic</li> </ul>                                                                                              | <ul style="list-style-type: none"> <li>- Requires separate priming reactions for each target at the same time.</li> </ul>                                                                                             |

|             |                                                                                                                                                                                                                                                                      |                                                                                              |
|-------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------|
|             | range (Smith & Osborn, 2009)                                                                                                                                                                                                                                         | - Wasteful if only limited amount of material (DNA/RNA) are available (Bustin & Nolan, 2004) |
| Droplet PCR | <ul style="list-style-type: none"> <li>- Absolute quantification of target DNA copies without running standard curves</li> <li>- Greater (10%) precision in quantification</li> <li>- Reduced consumable and equipment costs (Bio-Rad Laboratories, 2022)</li> </ul> | - False positive results (Kojabad et al., 2021)                                              |

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Due to the superficial location of the bacteria on the fish, the low bacterial concentration in tissues and the slow growth of this bacteria, the early stages of the infection are often overlooked, leading to false negative results (Rangdale et al., 1996). At the fry stage, the fish are most vulnerable to this pathogen (Wahli & Madsen, 2018), therefore, there is a need for a rapid, specific, and cost-effective method for detection of *F. psychrophilum* in fish tissues.

In the past few years, PCR methods have been developed for the detection and diagnosis of *F. psychrophilum* infections (Madetoja & Wiklund, 2002; Wiklund et al., 2000). PCR is a powerful genetic technique with a key role in making multiple copies of a segment of DNA and can amplify a specific DNA target from a mixture of DNA molecules, however it also requires post-PCR procedures, such as agarose gel visualisation, that take more time and can lead to contamination and false positive results (Heid et al., 1996). qPCR is the result of evolution in PCR method and allows the quantification of nucleic acids in a sample before amplification by PCR. qPCR has been widely used to increase the sensitivity of methods for detection and quantification of DNA, including bacterial gene sequences thus by interface bacterial cells. The chemistry consists of special fluorescent dye and probes in the PCR including DNA-binding dyes, such as SYBR Green I, and hydrolysis probes such as TaqMan. When SYBR Green I binds to the minor groove of double-stranded DNA, it emits fluorescence, the greater the concentration of DNA in the reaction leads to a greater amount of bound dyes and thus greater signal from SYBR Green I (Tajadini et al., 2014). Therefore, this method is relatively inexpensive and improves the sensitivity of detection and quantification of bacteria (Carelli et al., 2007). SYBR Green has proved to be a sensitive and specific method for the identification of different microorganisms such as *Aeromonas salmonicida* (Fernández-Álvarez et al., 2016) and nervous necrotic virus (Oliveira et al., 2021). Hydrolysis probes, such as TaqMan Probes, are sequence-specific dually fluorophore-labelled DNA oligonucleotides that increase the assay specificity because only sequence-specific amplifications are measured (BioRad Laboratories, 2021). One fluorophore is known as the quencher, while the other is known as the reporter. On the same short oligonucleotides, the quencher and reporter are in proximity and during amplification, the quencher absorbs the reporter's signal, the oligonucleotide is broken apart by DNA polymerase 5'-nuclease activity, and the reporter and quencher are separated, allowing the reporter's fluorescent signal

to be liberated (BioRad Laboratories, 2021). Nevertheless, the precision of hydrolysis probes is comparable to that of SYBR Green I (Tajadini et al., 2014). Due to its high sensitivity, probe-based qPCR methods have been developed to detect low concentrations of *F. psychrophilum* DNA from rainbow trout tissues (Orioux et al., 2011), however Strepparava et al. (2014) developed a Taqman quantitative PCR to detect *F. psychrophilum* from water and fish tissue samples with detection limits of  $10^3$  gene copies per reaction. While highly specific, probe-based assays are more expensive to use and can be challenging to optimise (Smith & Osborn, 2009), and the approximate cost of reagents for a single reaction is £0.63 if a probe is used, while the cost is of £0.43 if SYBR is used (LGC Biosearch Technologies, 2022). An alternative assay design would be to use fluorescent intercollating dyes such as SYBR Green I, which would decrease the running costs especially when working with large number of samples, during genetic resistance studies where large number of fish/families are included (Plant & LaPatra, 2011).

In *Flavobacterium* spp., the  $\beta'$  DNA-dependent RNA polymerase (*rpoC*) is a single copy gene, and this gene has been used to assess phylogenetic relationships and mutation rates in different genera and species. The *rpoC* gene has been shown to be more variable at the interspecific level than the *16S rRNA* gene (Battesti & Bouveret, 2009; Chelo et al., 2007; Morse et al., 1996). Each bacterial cell can have a variable number of *16S rRNA* genes copies, including *F. psychrophilum* which has six copies, therefore it can make it difficult to accurately quantify the number of bacteria in a sample. Several qPCR assays have been described, which rapidly and quantitatively detect the *16S rRNA* gene sequence (del Cerro et al., 2002; Orioux et al., 2011). However, interpretation of the assay results is complicated due to the six *16S rRNA* gene copies (Duchaud et al., 2007) that can vary in sequence (Soule et al., 2005), and the reported off-target amplification from non-*F. psychrophilum* bacteria (Orioux et al., 2011). As a result, targeting a single copy gene such as the *rpoC* allows a more accurate quantification of the pathogen, where one gene copy corresponds to one bacterial cell (Morillo et al., 2003). Strepparava et al. (2014) successfully developed a Taqman quantitative assay based on the single copy gene *rpoC*, to detect and quantify *F. psychrophilum* in samples with low pathogen concentrations.

In Chapter 2, Atlantic salmon (n=500) and rainbow trout (n=500) fry (from the sampling tanks), from the AquaGen breeding nucleus were challenged with *F. psychrophilum*

6\_S and *F. psychrophilum* 19\_5 respectively with the newly developed *F. psychrophilum* immersion challenge model. The fish challenged were expected to have some degree of resistance and susceptibility to *F. psychrophilum*. Fish were genotyped in Chapter 2 and these included heterozygous (Y/N) and homozygous (Y/Y and N/N) fish. The genome wide association studies (GWASs) performed following these challenges determined that two major QTLs responsible for resistance to flavobacteriosis were discovered in Atlantic salmon on chromosome 5 and 9; while several peaks were discovered in rainbow trout, however no QTLs were observed on chromosome 3 and 8, which in a previous study by AquaGen (data not available) large QTLs were discovered on these chromosomes, and hence this chapter focused on chromosome 3 and 8 for the analysis.

The aim of the current study was to develop and validate a more cost-effective and rapid qPCR assay using SYBR Green I based on a single-copy gene, the *rpoC* gene, for detection and quantification of *F. psychrophilum* in Atlantic salmon and rainbow trout head kidney tissues after immersion challenges with *F. psychrophilum*. This Chapter also aims to investigate whether there is a relationship between fish genotype and bacterial abundance in rainbow trout and Atlantic salmon head kidney after exposure to *F. psychrophilum*.

## 3.2 Materials and methods

### 3.2.1 Bacterial isolates

The *F. psychrophilum* isolates used in this present study were *F. psychrophilum* 6\_S and *F. psychrophilum* 19\_5 isolated from Atlantic salmon and rainbow trout, respectively (Chapter 2, Table 2.1). The isolates were plated on MVA and cultured on MVB as described in Chapter 2, Section 2.2.1.

Five *F. psychrophilum* isolates (*F. psychrophilum* 6\_S, *F. psychrophilum* 19\_5, *F. psychrophilum* 469, *F. psychrophilum* 468, *F. psychrophilum* JIP02/86) and other bacteria genera, including *Aeromonas salmonicida* sub. *salmonicida*, *Francisella orientalis*, *Vibrio anguillarum*, *Escherichia coli*, *Yersinia ruckeri*, *Edwardsiella piscicida* were used to test the specificity of the SYBR Green *F. psychrophilum* qPCR assay. All the *Flavobacterium* strains including *Flavobacterium hydatis* were cultured from stock cultures in Protect™ beads on MVA plates. The agar plates were incubated at 15°C for 48-72 h. After incubation, growth and purity of the colonies were checked and then a loop-full of bacteria from each plate was inoculated into MVB in a shaking incubator (Kuhner, Switzerland) at 15°C for 48-72 h. Colonies of *A. salmonicida*, *E. coli* and *Y. ruckeri* were grown on Tryptic soy agar (TSA, Oxoid, UK) and incubated at 22°C for 48 h, then inoculated into Tryptic soy broth (TSB, Oxoid, UK) and incubated at 22°C for 48 h in a shaking incubator. Strains of *F. orientalis* were grown on cysteine heart agar media (Difco, USA) with 1% bovine haemoglobin (Becton Dickinson BBL, USA) and incubated for 72 h at 28°C then inoculated into modified Mueller Hinton broth (MMHB) with 2% IsovitaleX and 0.1% glucose (Difco, USA). Strains of *V. anguillarum* were cultured on marine agar (Difco, USA) at 28°C for 48 h and then incubated in TSB for 24 h at 28°C.

### 3.2.2 Experimental animals

Atlantic salmon (n= 500;  $1.8 \pm 0.207$  g) and rainbow trout fry (n=500;  $1.5 \pm 0.209$  g) were obtained from AquaGen Norway and transported to the research facility at Veso Viken, Norway. Upon arrival at the facility, the fish were acclimatised, and their *F. psychrophilum* -free status was examined using a nested PCR method targeting the *16S rRNA* gene, as described in Section 2.2.3.4. All experimental procedures with live fish were carried out in accordance with the Norwegian Food Safety Authority

guidelines and were approved by the Animal Welfare and Ethical Review Body of the University of Stirling, UK.

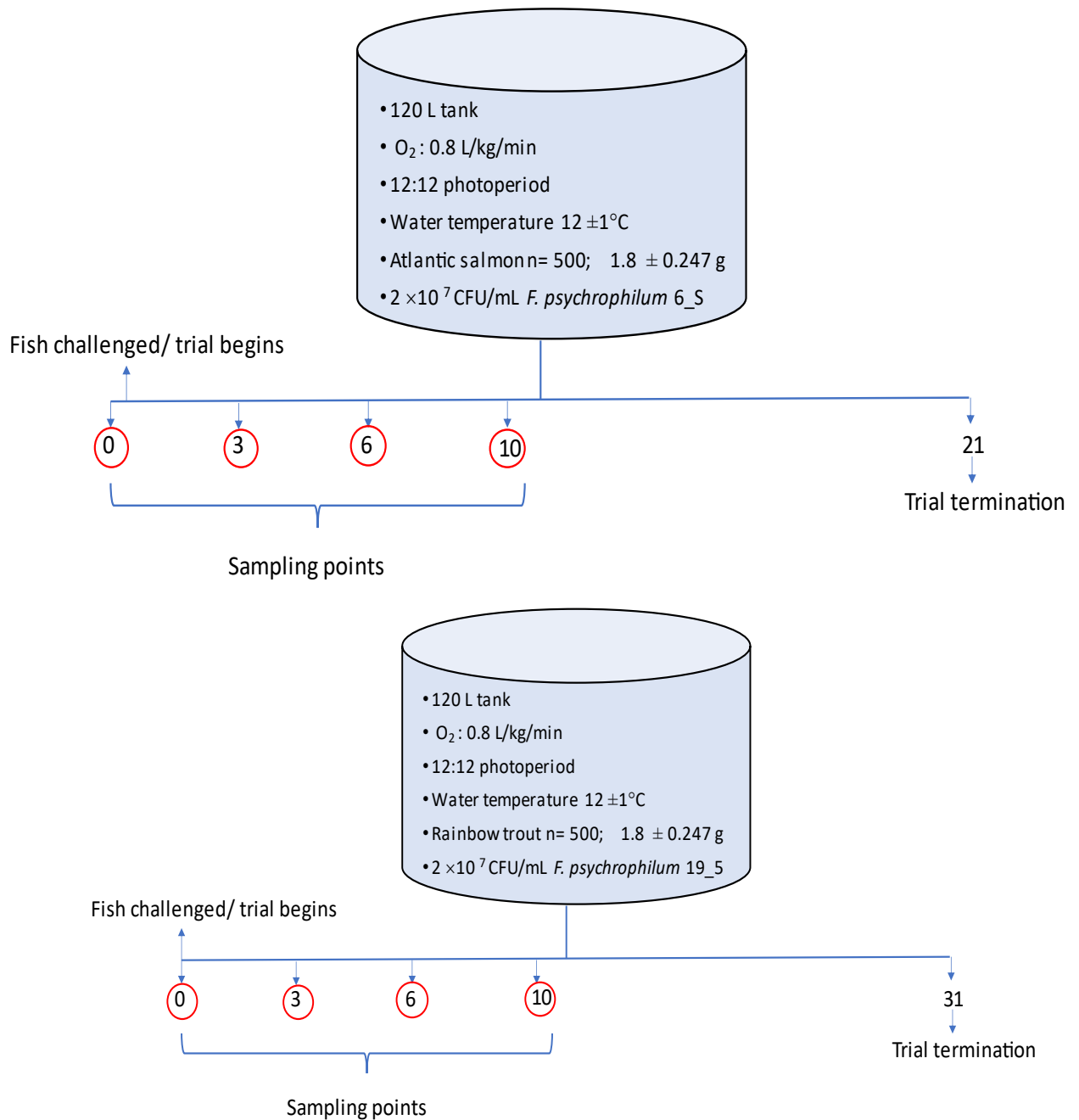
### 3.2.3 Sampling tank challenge

The material used for the present study came from the challenge described in Section 2.2.3.2. Briefly, in separate tanks, 500 Atlantic salmon fry ( $1.8 \pm 0.237$  g) and 500 rainbow trout fry ( $1.8 \pm 0.247$  g) had been starved for 24 h prior to the challenge. The fish were placed into two separate 90-L aerated tanks containing hydrogen peroxide (200 mg/L) for 1 h. Then the fish were returned to the 120-L glass fibre acclimation tanks. Water flow was stopped in each tank and the water volume reduced to 54 L. For each challenge, 6 L of bacteria was added to the tank water to give a final concentration of  $2 \times 10^7$  CFU/mL. Atlantic salmon were challenged with *F. psychrophilum* 6\_S while rainbow trout were challenged with *F. psychrophilum* 19\_5 (Figure 2.1 and Figure 2.2).

For rainbow trout, the fish were challenged for 1.5 h, while Atlantic salmon were challenged for 4 h. Morbidities and mortalities were recorded each day until the end of the trial (21 days post challenge [dpc] for Atlantic salmon and 31 dpc for rainbow trout).

### 3.2.4 Sampling

Fifty fish in total were randomly selected using a sampling net at 0 (n= 50), 3 (n=50), 6 (n=50) and 10 (n=50) dpc, and then euthanised with an overdose of benzocaine chloride (Sykehusapoteket Oslo, Ullevål, Norway). The head kidney tissues were sampled at each time point for quantification of bacterial load by qPCR and for transcriptomic analysis (Chapter 5). For Atlantic salmon, at 0 dpc the fish are considered control fish, given that sampling occurred prior to the immersion challenge; however, for the rainbow trout challenge there was not a control fish group and the fish sampled at 0 dpc have been exposed to *F. psychrophilum* for 1.5 h (Figure 3.1). Tissues were dissected out and immediately submerged in 5 volumes (1.5 mL) of RNA later (Sigma, UK) and stored at -80°C until further use.



**Figure 3.1** Schematic representation of the challenge trials, time at which the challenges started, sampling time points and challenge termination point (Atlantic salmon: 21 dpc and rainbow trout: 31 dpc).

### 3.2.5 Bacterial isolates and DNA extraction

Purified genomic DNA from the different bacterial cultures was extracted by a modified salt precipitation method (Bartie et al., 2020; Syaifudin et al., 2019). Bacteria was cultured in 3 mL of MVB for 48 h as described in Section 3.2.1 and then 1 mL of the bacteria was harvested by centrifugation at  $10,000 \times g$  and washed with 1 mL of STE buffer (10mM Tris-HCl; 1mM EDTA; 0.1 M NaCl pH 8.0). The cell pellet was placed into 300  $\mu$ L SSTNE lysis solution (0.3 M NaCl, 50 mM Tris base, 0.2 mM EDTA pH 8.0, 0.2 mM EGTA, 0.5 mM spermidine, 0.25 mM spermine, and 0.1% SDS) containing 1.5  $\mu$ L proteinase K (10 mg/mL) and incubated at 55°C overnight. Following lysis, the samples were heated at 70°C for 15 min to inactivate proteinase K, cooled to room temperature (RT) and 5  $\mu$ L RNaseA (2 mg/ml; Thermo Fisher Scientific; Courtaboeuf, France) added, mixed by inversion, and incubated at 37°C for 1 h. On ice, 180  $\mu$ L 5 M NaCl were added, causing a precipitation, and the samples were centrifuged twice at  $21,000 \times g$ .

The supernatant was collected, placed in a fresh Eppendorf tube, and centrifuged at  $21,000 \times g$  for 10 min at room temperature (RT). An equal volume of isopropanol was added to the supernatant and the contents then mixed by inversion followed by centrifugation at  $21,000 \times g$  for 10 min at RT. Then the pellet was washed twice in 70% ethanol. Samples were centrifuged at  $20,000 \times g$  for 10 min at RT, the supernatant discarded, and the DNA pellet air-dried for 5-10 min. The DNA pellet was hydrated in 35  $\mu$ L TE buffer (10 mM Tris, 1 mM EDTA pH 8.0) overnight at 4°C.

The quality and integrity of DNA was measured using a Nanodrop ND-1000 Spectrophotometer UV/Vis. The purity of the DNA was determined with the 260/280 and 260/230 ratios. If the 260/280 ratio was around 1.8 and the 260/230 ratio was between 2.0 and 2.2 the DNA was considered of sufficient quality for use in downstream processing. Since spectrophotometry cannot discriminate between RNA and DNA or presence of contaminants (proteins) in the samples, a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies) was used to quantify DNA accurately when DNA was used for downstream applications such as sequencing, genotyping, and qPCR analysis.

The quality of total DNA was determined by agarose gel electrophoresis using a 1% gel with 0.5 µg/mL of ethidium bromide. In an Erlenmeyer flask, 0.6 g of agarose (Thermo Scientific; Courtaboeuf, France) was added to 60 mL of ×0.5 TAE (40 mM Tris-acetate, 1 mM EDTA) (Thermo Scientific; Courtaboeuf, France) and mixed by swirling. The agarose/buffer mix was melted by heating in a microwave. At 30 s intervals, the flask was removed from the microwave and swirled to mix the contents. This step was repeated until the agarose was completely dissolved. Once the solution was cooled, ethidium bromide (EtBr) to a concentration of 0.5 µg/mL was added. The gel tray was placed into the casting apparatus and the appropriate comb were placed into the gel mould to create the wells. The molten agarose was then poured into the gel mould and allowed to set at RT,

DNA samples (50 ng/ µL) and 1 µL of Lambda HindIII ladder (Thermo Scientific; Courtaboeuf, France) were loaded into pre-cast wells in the gel and a current was applied. The gel was run at 50V for 10 min then 80V for 30 min. The gel was visualised under UV trans-illumination (Amersham Imager 680, GE Healthcare) (Figure 3.3). Each DNA sample was standardised to 50 ng/µL with nuclease-free water and stored at -20°C.

### 3.2.6 Tissue DNA Extraction

Genomic DNA was extracted from randomly selected Atlantic salmon head kidney tissues across the different time points, (0 dpc [n=10], 3 dpc [n=18], 6 dpc [n=18], 10 dpc [n=24]) and rainbow trout head kidney (0 dpc [n=7], 3 dpc [n=15], 6 dpc [n=15], 10 dpc [n=16]) for detection and quantification of *F. psychrophilum* load. Due to the small size of the tissues, DNA was extracted using AllPrep DNA/RNA Mini Kit (QIAGEN, Germany) following the manufacturer's instructions. Briefly, less than 5 mg of frozen head kidney was placed into 2 mL microcentrifuge tubes (Alpha Laboratories, UK) and 350 µL of Buffer RLT (contains a high concentration of guanidine isothiocyanate, which supports the binding of RNA to the silica membrane) and 3.5 µL β-mercaptoethanol were added to the tissue and subsequently homogenised with a pellet pestle (Merck, Germany). The lysate was centrifuged at 21,000 × g for 3 min and the supernatant was placed in an AllPrep DNA spin column and centrifuged at 8,000 × g for 30 s. The flow-through was stored at 4°C and then used for RNA extraction (Chapter 5). To the AllPrep DNA spin column, 500 µl Buffer AW1 (stringent

wash with low concentration of guanidine hydrochloride) was added and centrifuged at  $8,000 \times g$  for 15 s. This step was repeated with the addition of 500  $\mu$ L Buffer AW2 (Tris-based ethanol solution to remove salts).

After centrifugation the AllPrep DNA spin column was placed in a 1.5 ml collection tube and 50  $\mu$ L Buffer EB (10 mM Tris-Cl, pH 8.5; preheated to 70°C) were added to the centre of the column. The tube was incubated at RT for 5 min and then centrifuged at  $8,000 \times g$  for 1 min. The flowthrough was placed in the AllPrep DNA spin column, incubated, and then centrifuged at  $8,000 \times g$  for 1 min to improve DNA yield. DNA quality and quantity were determined as described in section 3.2.5. Samples were standardised to 350ng/ $\mu$ L with nuclease-free water (Thermo Scientific; Courtaboeuf, France) and stored at -20°C.

### **3.2.7 Tissue DNA Extraction for Genotyping**

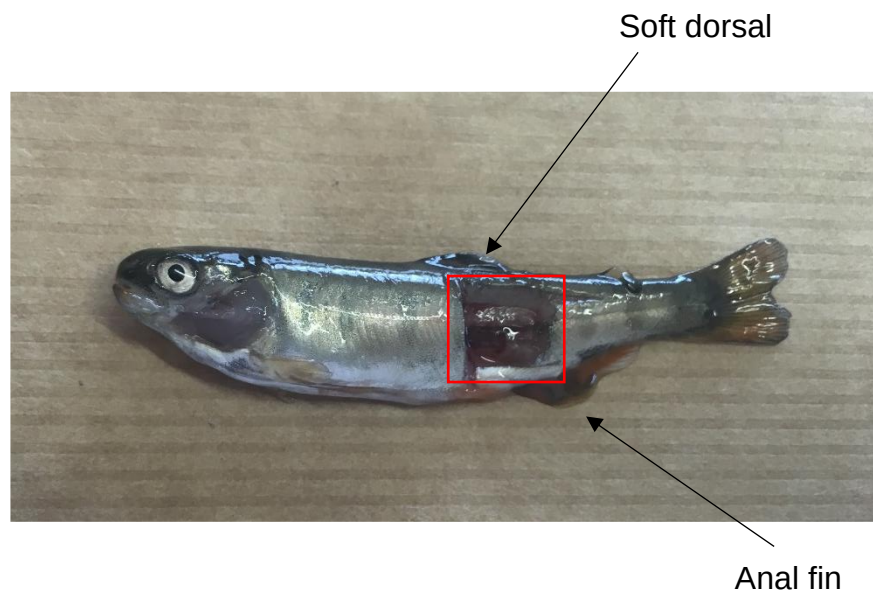
To determine if there is a correlation between fish genotypes and *F. psychrophilum* infection, genotyping was performed. For genotyping, DNA was isolated differently than the DNA extraction used in section 3.2.6. The head kidney used in this present study were too small to extract DNA for genotyping, hence skin tissues were used instead, and DNA was extracted with a different DNA extraction technique, description to follow.

Genomic DNA was extracted from Atlantic salmon skin tissue samples (n=70) and rainbow trout skin tissue samples (n=53) (using DNeasy Blood & Tissue extraction kit – Spin columns (QIAGEN, Germany) following the manufacturer's instructions with slight modifications. Briefly, ca. 5 mg of skin (muscle tissue was scraped off the skin with a scalpel), taken from the bottom of the soft dorsal fin and the top of the anal fin (Figure 3.2), was cut up into small pieces and placed in a microcentrifuge tube containing 180  $\mu$ L of Buffer ATL (sodium dodecyl sulphate) and 20  $\mu$ L proteinase K (600mA/ml). The tubes were incubated at 56°C overnight. Following lysis, the samples were vortexed for 15 s and 200  $\mu$ L Buffer AL (guanidinium chloride) and 200  $\mu$ L ethanol (96-100%) were added to the samples and mixed by vortex for 30 s. The lysate including any precipitate was then pipetted into DNeasy Mini spin columns and centrifuged at  $6,000 \times g$  for 1 min. The flow-through was discarded and 500  $\mu$ L buffer AW1 (which contains stringent wash with low concentration of quinidine) was added

to the column and centrifuged for 1 min at  $6,000 \times g$ . Following centrifugation 500  $\mu\text{L}$  buffer AW2 (Tris-based ethanol solution to remove salts) was added to the DNeasy Mini spin columns and centrifuged at  $20,000 \times g$  for 3 min to dry the DNeasy Mini spin column membranes.

The DNeasy Mini spin columns were placed into a clean Eppendorf and the DNA was eluted with 50  $\mu\text{L}$  nuclease-free water (preheated at  $50^\circ\text{C}$ ) and incubated at RT for 5 min. The tubes were centrifuged at  $6,000 \times g$  for 1 min. This latter elution step was repeated to increase the overall DNA yield. The concentration of DNA samples was measured using a Nanodrop ND-1000 Spectrophotometer UV/Vis.

DNA samples were standardised to 50  $\text{ng}/\mu\text{L}$  with nuclease-free water and stored at  $-20^\circ\text{C}$ .



**Figure 3.2** The red box illustrates the location of skin tissue dissected (from the bottom of the dorsal fin until the bottom of the anal fin) from rainbow trout fry for DNA extraction for genotyping.

### **3.2.8 *Flavobacterium psychrophilum* PCR**

Previously published primers designed against the *rpoC* gene sequence were used to quantify bacterial abundance in each tissue sample by PCR according to Strepparava et al. (2014).

PCR was carried out in a final volume of 15  $\mu\text{L}$  with 50 ng /  $\mu\text{L}$  DNA template, whilst the master mix per sample consisted of 7.5  $\mu\text{L}$  2x MyTaq HS Mix (Bioline), 0.2  $\mu\text{M}$  of forward (Eurofins) and 0.2  $\mu\text{M}$  reverse (Eurofins) primers, 5.9  $\mu\text{L}$  of Milli-Q and 1.0  $\mu\text{L}$  of sample or control at 50 ng/ $\mu\text{L}$ .

Milli-Q water was used as a no template control (NTC) and the positive control was JIP02/86 (also known as ATCC 49511, DSM 21280 or CIP 103535; GenBank accession number AM398681.2).

The following thermal cycling conditions were used in a TProfessional Basic Thermocycler gradient (Biometra, Germany): initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 95°C for 15 s, annealing at 54.5°C for 15 s, and elongation at 72°C for 30 s. The final cycle was followed by extension at 72°C for 7 min. The PCR amplicons were visualised by gel electrophoresis, as described in section 2.2.4, and the amplified was expected at ~164bp.

### 3.2.9 SYBR Green quantitative PCR (qPCR)

The qPCR assay developed and validated in this present study was designed against the single copy *rpoC* gene of *F. psychrophilum* using the primers designed by Strepparava et al. (2014) to rapidly detect and quantify bacterial load in Atlantic salmon and rainbow trout head kidney tissues.

PCR amplicons produced using these primers were sent for sequencing to make sure that the amplicon produced was the desired product, using the local nucleotide alignment tool “BLAST” (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). These criteria were applied for the qPCR primers used in this study.

The qPCR was carried out in a final volume of 10 µL containing 0.3 µM each of forward and 0.3 µM reverse (Eurofins) primer, 5.0 µL of Luminaris colour HiGreen qPCR master mix (the master mix includes Thermo Scientific Hot Start Taq DNA polymerase, uracil-DNA glycosylase (UDG) and dNTPs in an optimized PCR buffer; Thermo Scientific), 2.9 µL of Milli-Q and 1.5 µL of DNA template at 120 ng/µL. Milli-Q water was used as NCT and the positive control template was JIP02/86.

The following thermal cycling conditions were used in a TOptical Thermocycler (Biometra): initial denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s, annealing at 54.5°C for 30 s, and elongation at 72°C for 30 s.

Bacterial load in the head kidney tissues were normalised against the relative abundance of *EF1-α* housekeeping gene using Pfaffl (2001) calculation methods. The *EF1-α* primers used for this study were designed by Ytteborg et al. (2010) and had previously been tested by Vera et al. (2017) and were specific in both Atlantic salmon and rainbow trout. To confirm the primer specificity, samples were run on the *EF1-α* qPCR, and the amplicons were visualised by agarose gel electrophoresis as described in Section 3.2.5. For this assay, the same *F. psychrophilum* qPCR assay recipe was used with the exception of the type of primers used, i.e., *EF1-α* primers (Table 3.2). Milli-Q water was used as NTC, and the positive control was DNA extracted from Atlantic salmon head kidney (DNA was extracted as described in Section 3.2.6).

The reactions were run through the following thermal cycles: initial denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30

sec and elongation at 72°C for 30 s. For both assays a melt curve analysis was performed from 60°C to 95°C at 0.1 °C/s melt rates to confirm a single product was amplified in each well, and the number of cycles used were chosen according to Strepparava et al. (2014) and Vera et al. (2017).

**Table 3.2** Primers used for qPCR of Atlantic salmon and rainbow trout *EF1- $\alpha$*  and *rpoC* genes.

| Gene                           | Oligo sequence                                                                      | Annealing temperature (°C) | Amplicon size (bp) | Reference                 |
|--------------------------------|-------------------------------------------------------------------------------------|----------------------------|--------------------|---------------------------|
| <i>EF-1<math>\alpha</math></i> | F: 5'-CACCACCGGCCATCTGATCTACAA-'3<br>R: 5'-TCAGCAGCCTCCTTCTCGAACTTC-'3              | 60                         | 78                 | Ytteborg et al. (2010)    |
| <i>rpoC</i>                    | R:5'- GAAGATGGAGAAGGTAATTTAGTTGATATT-'3<br>F:5'-CAAATAACATCTCCTTTTTTCTACAACCTTGA-'3 | 54.5                       | 164                | Strepparava et al. (2014) |

### 3.2.10 Preparation of Standard Curve

Isolates *F. psychrophilum* 6\_S and *F. psychrophilum* 19\_5 were used for the construction of the standard curve (STC) for the Atlantic salmon and rainbow trout qPCR (*rpoC* gene), respectively; while rainbow trout and Atlantic salmon head kidney tissue were used for the generation of the *EF1-α* qPCR assay STC.

For the *rpoC* gene qPCR, the isolates were cultured, and DNA was extracted as described in Section 3.2.5. The DNA was amplified using *F. psychrophilum*-specific PCR (Strepparava et al., 2014). The PCR product was cleaned (QIAquick PCR Purification Kit, Qiagen). The quality and quantity of the DNA was measured by spectrophotometry and gel electrophoresis (Section 3.2.5). The cleaned PCR product was sent for sequencing to Eurofins Genomics, and the DNA sequencing results were examined using the manufacturer's guide and the FASTA output was blasted at National Library of Medicine (NCBI) to determine the matching to the open database. The PCR product was confirmed to be amplifying the *rpoC* gene, and as follows, the PCR products were used to generate two different qPCR STC.

To determine the number of copies of *F. psychrophilum* DNA, the total amount of DNA measured was divided by  $1.797 \times 10^{-7}$  pg [the weight of one *rpoC* fragment (164 bp)] as determined by Strepparava et al. (2014). The result was an estimate of the number of gene copies in 1 µl of purified product.

For the *EF1-α* gene qPCR, DNA was extracted from Atlantic salmon and rainbow trout head kidney as described in Section 3.2.6. The DNA was amplified using Vera et al. (2017) protocol and the PCR product was cleaned (QIAquick PCR Purification Kit, Qiagen). The quality and quantity of the DNA was measured by spectrophotometry and gel electrophoresis (Section 3.2.5). The cleaned PCR product was sent for sequencing to Eurofins Genomics, and the DNA sequencing results were examined using the manufacturer's guide and the FASTA output was blasted in NCBI to determine the matching to the open database. The PCR product was confirmed to be amplifying the *EF1-α* gene, and as follow the PCR products were used to generate two different qPCR STC, to analyse in separate assays the Atlantic salmon and rainbow trout head kidney.

Both standard curves for the *rpoC* and *EF1-α* genes, in two technical repeats, were generated with PCR product concentrations ranging from  $10^1$  to  $10^7$  copies of *F. psychrophilum* DNA  $\mu\text{l}^{-1}$  and  $10^1$  to  $10^7$  copies of Atlantic salmon DNA  $\mu\text{l}^{-1}$  using 10-fold serial dilutions in Lambda TE buffer. The initial concentration of the standard curve was 120 ng/ $\mu\text{L}$  measured using a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies). The linear regression equation was determined for the standard curve as:  $y = mx + c$ , where  $y$  is the dependent variable,  $m$  is the slope,  $x$  is the independent variable and  $c$  is the y-intercept. Efficiency ( $E$ ) of the stand curve determined using the formula of Kubista et al. (2006):

$$Efficiency (\%) = \left( 10^{\frac{-1}{Slope}} - 1 \right) \times 100$$

Melt curves were checked to ensure specific product amplification as described in section 3.2.9.

### 3.2.11 Genotyping

All Atlantic salmon individuals ( $n=70$ ) were genotyped using a custom 70Kv1 SNP Affymetrix Axiom array chip (AquaGen) and all the rainbow trout individuals ( $n=53$ ) were genotyped using a custom Omy50k SNP Affymetrix Axiom array chip (AquaGen) as described in Chapter 2. Each fish used for the analysis of this Chapter was genotyped to determine whether there was a correlation between fish genotype and bacterial abundance in the head kidney tissues. In this Chapter, N/N represents the 'susceptible' genotype, Y/Y the 'resistant' genotype, and Y/N the heterozygous genotype (Appendix, Table 1 and 2).

## 3.3 Statistical analysis

Microsoft Excel 2019 was used to arrange the data for analysis and to calculate a semi-log regression of the qPCR by plotting the cycle threshold (Ct) against the molecules detected of the target gene *rpoC* or the housekeeping gene *EF1-α* DNA dilutions ( $10^7:10^1$  copies/  $\mu\text{L}$ ). Melt curves were extrapolated from TOptical Thermocycler (Biometra) after completion of each qPCR assays to determine that the product amplified produced a single peak. In both fish species, bacterial load in the head kidney tissues were normalised against the relative abundance of *EF1-α* housekeeping gene using Pfaffl (2001) calculation methods.

In R, the linear model (*lm*) function (Knowles et al., 1991) was used to determine if there was any significant association in bacterial abundance in the animals' head kidney regardless of the day post challenge (without including day 0) and the fish genotype. The *lm* function was used to determine whether there was a significant relationship between the variables, the *lm* function was applied to a formula that described the variable bacterial abundance by the variable days post challenge. P-values ( $p$ )  $<0.05$  were considered to indicate a statistically significant difference. The plots used to illustrate the qPCR results were produced in R using ggplot2 (Murrell, 2005).

## 3.4 Results

### 3.4.1 qPCR primers sensitivity, efficiency, and specificity

Primers targeting the *rpoC* gene of *F. psychrophilum* isolated from rainbow trout and salmon were screened for specificity by PCR and then amplicon sequencing and were then confirmed to amplify the *rpoC* gene.

After blasting a sequence of the PCR product (Table 3.3) the sequence produced significant alignments with 100 sequences, all of which were *F. psychrophilum* strains and they all have an expectation value (E-value) of 0.0. These results suggest very high similarity between the amplicon sequenced and the *F. psychrophilum* strains.

All the *F. psychrophilum* isolates amplified a product at ~164bp (Figure 3.3), while the non-*F. psychrophilum* isolates were not amplified by the *F. psychrophilum* developed assay (Figure 3.4).

*EF1-a* primer specificity was tested on DNA samples extracted from two Atlantic salmon head kidney, one randomly selected rainbow trout head kidney and Atlantic salmon liver extracted by Vera et al. (2017) (Figure 3.5) and these samples (n=4) all amplified a product at ~ 78 bp, which is what was expected and previously reported by Vera et al. (2017).

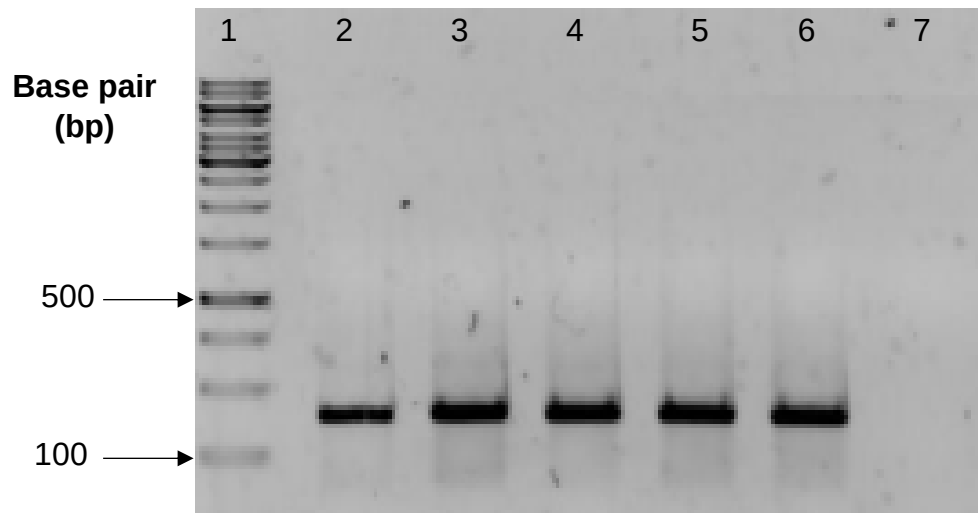
**Table 3.3** BLASTn results obtained from the NCBI website of the top 100 sequences producing significant alignments when blasting the PCR product produced following the *F. psychrophilum* PCR assay targeting the *rpoC* gene of *F. psychrophilum*

| Description                                                                                     | Query Cover | E value | Percentage Identity |
|-------------------------------------------------------------------------------------------------|-------------|---------|---------------------|
| <i>Flavobacterium psychrophilum</i> gene for 16S rRNA, partial sequence, strain: FPC814         | 86%         | 0       | 98.53%              |
| <i>Flavobacterium psychrophilum</i> strain CH27 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.52%              |
| <i>Flavobacterium psychrophilum</i> strain CH21 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.52%              |
| <i>Flavobacterium psychrophilum</i> strain CH14 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.52%              |
| <i>Flavobacterium psychrophilum</i> strain FLpH 004/09 16S ribosomal RNA gene, partial sequence | 86%         | 0       | 98.52%              |
| <i>Flavobacterium psychrophilum</i> strain FPCH3 16S ribosomal RNA gene, partial sequence       | 86%         | 0       | 98.52%              |
| <i>Flavobacterium psychrophilum</i> strain FPCH1 16S ribosomal RNA gene, partial sequence       | 86%         | 0       | 98.52%              |
| <i>Flavobacterium psychrophilum</i> strain CH16 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.45%              |
| <i>Flavobacterium psychrophilum</i> strain CH22 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> strain CH09 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> strain FPCH6 16S ribosomal RNA gene, partial sequence       | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> strain FPCH4 16S ribosomal RNA gene, partial sequence       | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> strain CH31 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> strain CH30 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> strain CH29 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> strain CH26 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> strain CH25 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> strain CH24 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> strain CH23 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> strain CH20 16S ribosomal RNA gene, partial sequence        | 86%         | 0       | 98.44%              |
| <i>Flavobacterium psychrophilum</i> strain CN6 chromosome, complete genome                      | 87%         | 0       | 98.38%              |
| <i>Flavobacterium psychrophilum</i> strain 5 genome                                             | 87%         | 0       | 98.38%              |
| <i>Flavobacterium psychrophilum</i> strain MH1 genome                                           | 87%         | 0       | 98.38%              |
| <i>Flavobacterium psychrophilum</i> strain PG2, complete genome                                 | 87%         | 0       | 98.38%              |
| <i>Flavobacterium psychrophilum</i> strain VQ50 chromosome                                      | 87%         | 0       | 98.38%              |

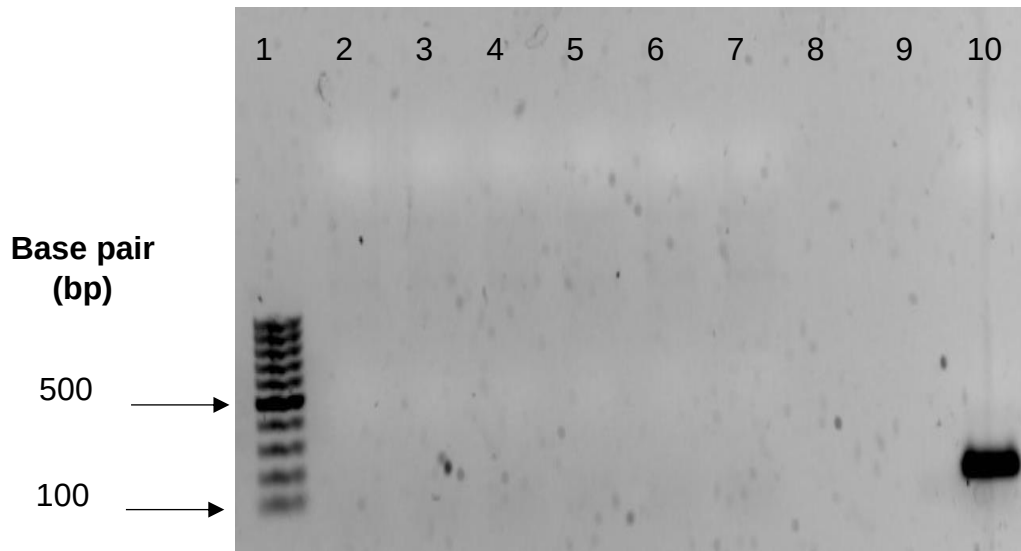
|                                     |                                                   |     |   |        |
|-------------------------------------|---------------------------------------------------|-----|---|--------|
| <i>Flavobacterium psychrophilum</i> | strain 3 chromosome                               | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 950106-1/1, complete genome                | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R18 chromosome                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R14 chromosome                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R12 chromosome                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R17 chromosome                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R9 chromosome, complete genome      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R11 chromosome                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R16 chromosome, complete genome     | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R13 chromosome                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R8 chromosome                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R7 chromosome                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R15 chromosome                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R10 chromosome                      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R5 chromosome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R2 chromosome                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R1 chromosome                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R9 chromosome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R8 chromosome, complete genome | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R7 chromosome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-C1 chromosome, complete genome      | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R6 chromosome                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R3 chromosome                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R5 chromosome                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6-R4 chromosome                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R4 chromosome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-C1 chromosome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R2 chromosome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R3 chromosome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R5 chromosome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N-R1 chromosome 160401_1_5N_R1   | 87% | 0 | 98.38% |

|                                     |                                                                           |     |   |        |
|-------------------------------------|---------------------------------------------------------------------------|-----|---|--------|
| <i>Flavobacterium psychrophilum</i> | FPG101, complete genome                                                   | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain CSF259-93, complete genome                                         | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain v4-33, complete genome                                             | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain V4-28 genome                                                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain V4-24, complete genome                                             | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain V2-20 genome                                                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain V1-20 genome                                                       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain V3-5, complete genome                                              | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain CN38 chromosome, complete genome                                   | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain CN46 chromosome, complete genome                                   | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | JIP02/86 complete genome                                                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain AQ_Fpsy_001_2012_A4215 16S ribosomal RNA gene,<br>partial sequence | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-F21 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-F22 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-F33 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-F32 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-F30 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-D15 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-D10 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain P30-2B/09 chromosome                                               | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain P15/8B-11 chromosome                                               | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain P7-7B/10 chromosome                                                | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S11B chromosome                                                | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain K9/00 chromosome                                                   | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S11A chromosome                                                | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S10 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-F16 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-F27 chromosome                                                 | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S9 chromosome                                                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 030522-1/1 chromosome                                              | 87% | 0 | 98.38% |

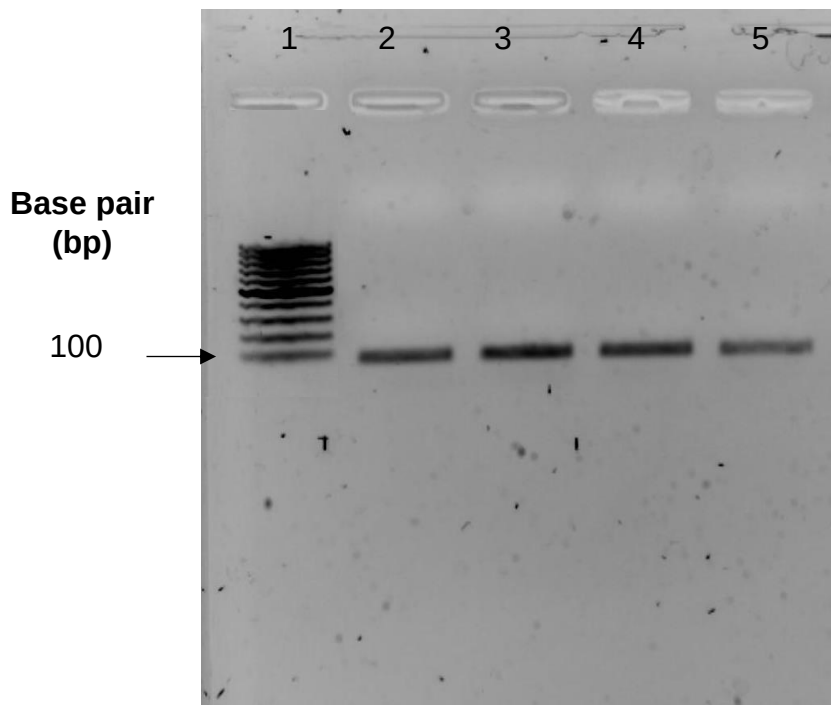
|                                     |                                                            |     |   |        |
|-------------------------------------|------------------------------------------------------------|-----|---|--------|
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5M chromosome                              | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 990512-1/2A chromosome                              | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 141127-1/2N chromosome                              | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 010418-2/1 chromosome                               | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-F15 chromosome                                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain F164 chromosome                                     | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-G1 chromosome                                   | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-R7 chromosome, complete genome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain 160401-1/5N chromosome, complete genome             | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain strain 950106-1/1 chromosome, complete genome       | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPRT1 chromosome, complete genome                   | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain FPS-S6 chromosome, complete genome                  | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain CSF 259-93 16S ribosomal RNA gene, partial sequence | 87% | 0 | 98.38% |
| <i>Flavobacterium psychrophilum</i> | strain OSU THCO2-90 genome assembly, chromosome: chr       | 87% | 0 | 98.30% |



**Figure 3.3** *F. psychrophilum* PCR to test the primers, targeting the *rpoC* gene, specificity on different *F. psychrophilum* isolates at 50ng/  $\mu$ L. Samples were run in agarose gel (1%). From left to right: 100bp GeneRuler Ladder (position 1). *F. psychrophilum* 6\_S (position 2), *F. psychrophilum* 19\_5 (position 3), *F. psychrophilum* 469 (position 4), *F. psychrophilum* 468 (position 5), *F. psychrophilum* JIP02/86 (position 6) and NTC (position 7). PCR product is expected at ~ 164bp.



**Figure 3.4** *F. psychrophilum* PCR to test the primers, targeting the *rpoC* gene, specificity on different non- *F. psychrophilum* bacteria genera isolates at 50ng/  $\mu$ L Samples were run on agarose gel (1%). From left to right: 100bp GeneRuler Ladder (position 1), *Aeromonas salmonicida* sub. *salmonicida* (position 2), *Francisella orientalis* (position 3), *Vibrio anguillarum* (position 4), *Escherichia coli* (5), *Yersinia ruckeri* (6), *Edwardsiella piscicida* (7) NTC position (8 and 9) and *F. psychrophilum* JIP02/86 (position 10). PCR product is expected at ~ 164bp.

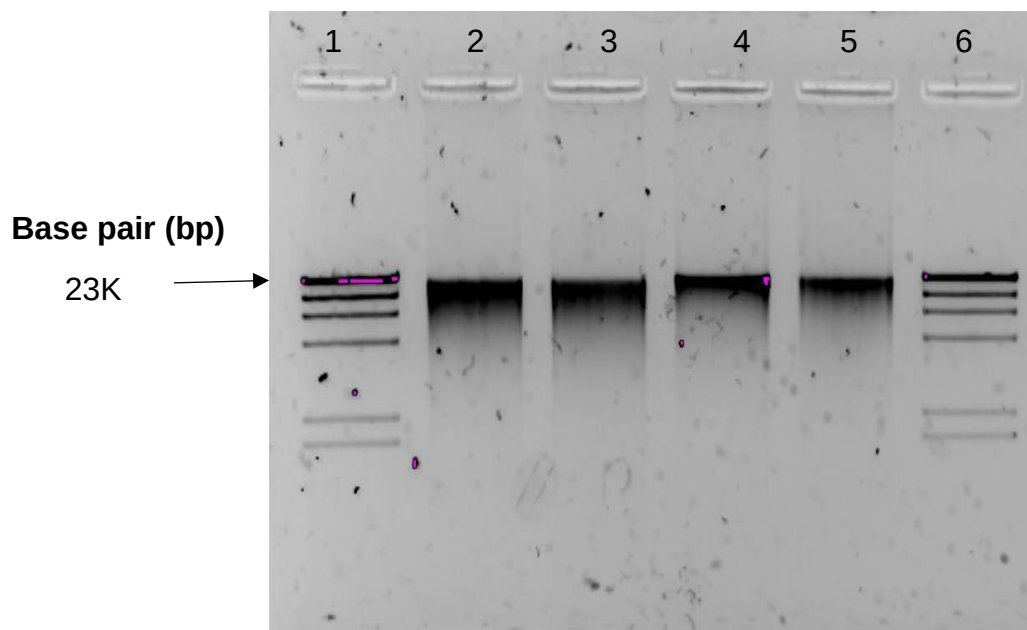


**Figure 3.5** PCR analysis run to test *EF1-a* primers specificity on DNA samples extracted from two Atlantic salmon head kidney randomly selected from 0 dpc (position 2 and 3), and one randomly selected rainbow trout head kidney from 0 dpc at 50ng/  $\mu$ L. Samples were run in agarose gel (1%). From left to right: 100-bp GeneRuler Ladder (position 1), Atlantic salmon head kidney (position 2), Atlantic salmon head kidney (position 3), rainbow trout head kidney (position 4), Atlantic salmon liver extracted by Vera et al. (2017) (position 5). PCR product is expected at  $\sim$  78 bp

### 3.4.2 Tissue DNA Extraction

DNA was extracted from Atlantic salmon head kidney (n= 70) and rainbow trout head kidney (n=53) using AllPrep DNA/RNA Mini Kit (QIAGEN, Germany), and the integrity and quality of the DNA was determined by agarose gel electrophoresis (Figure 3.6) and a band was expected at 23 Kbp.

All DNA samples were standardised to 350ng/μL with nuclease-free water and used for the qPCR analysis to detect and quantify *F. psychrophilum* abundance in Atlantic salmon and rainbow trout head kidney tissues at the different time points, day 0 (control fish only for Atlantic salmon; rainbow trout exposed to the bacteria by immersion for 1.5 h), day 3, day 6 and day 10.



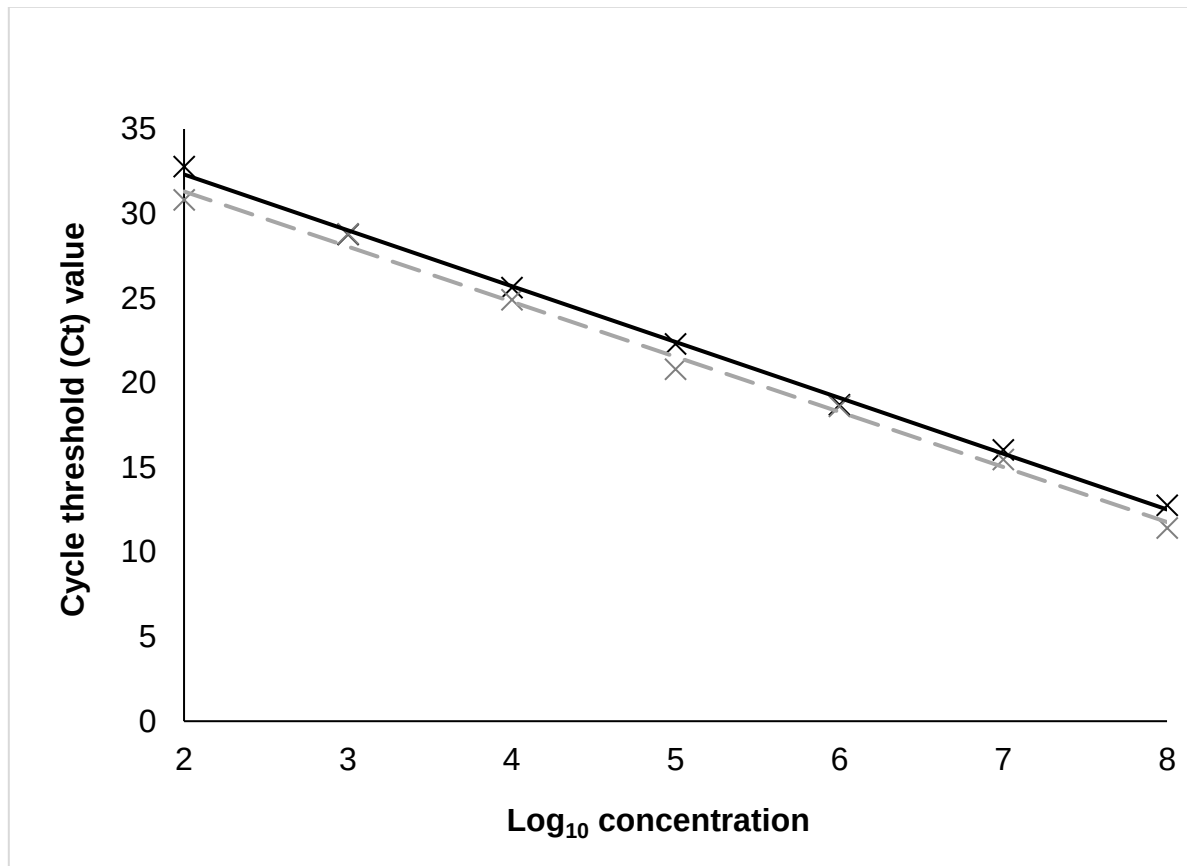
**Figure 3.6** Agarose gel (1%) used to check DNA integrity and quality. Lambda HindIII ladder (Position 1 and 6), Atlantic salmon head kidney day 0 post-challenge (position 2), Atlantic salmon head kidney day 3 post-challenge (position 3), rainbow trout head kidney day 6 post-challenge (position 4), and rainbow trout head kidney day 10 post-challenge (position 5).

### 3.4.2 qPCR standard curve

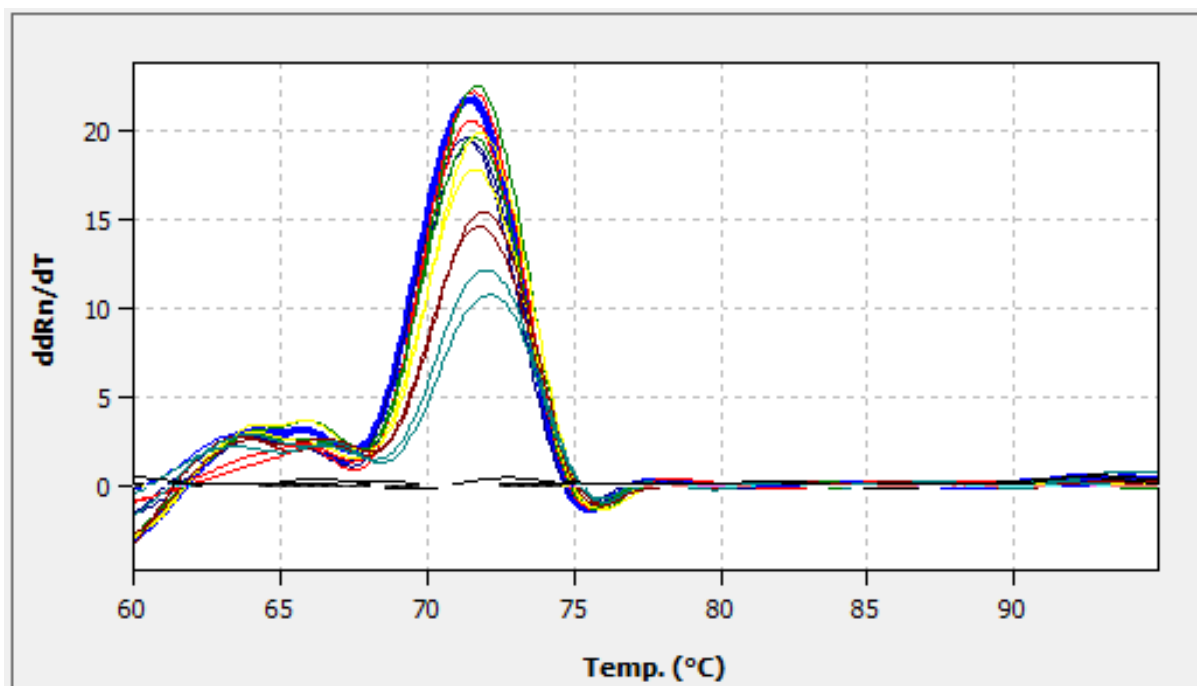
All the qPCR standards and sample runs met the criteria defined in the methods, in which: the cycle threshold (Ct) values were inverse to the amount of target nucleic acid in the STC (Pfaffl, 2001). The standard curve developed using seven serial dilutions of *F. psychrophilum* isolate 6\_S and *F. psychrophilum* 19\_5 both ranged from  $10^1$  to  $10^7$  *rpoC* gene copies and generated a slope of  $-3.30$  ( $R^2 = 0.998$ ), indicating an efficiency of 100 % (*F. psychrophilum* 6\_S) and  $-3.25$  ( $R^2 = 0.994$ ), indicating an efficiency of 101% (*F. psychrophilum* 19\_5) (Figure 3.7).

A melt curve was generated to see that the amplicon produced for each STC, which had a single specific peak, indicating that the correct product was amplified (Figure 3.8). The cleaned-up amplicons of the *rpoC* fragment were therefore used for all the successive quantifications (qPCR) of *F. psychrophilum* in head kidney tissues samples from the challenge as standards.

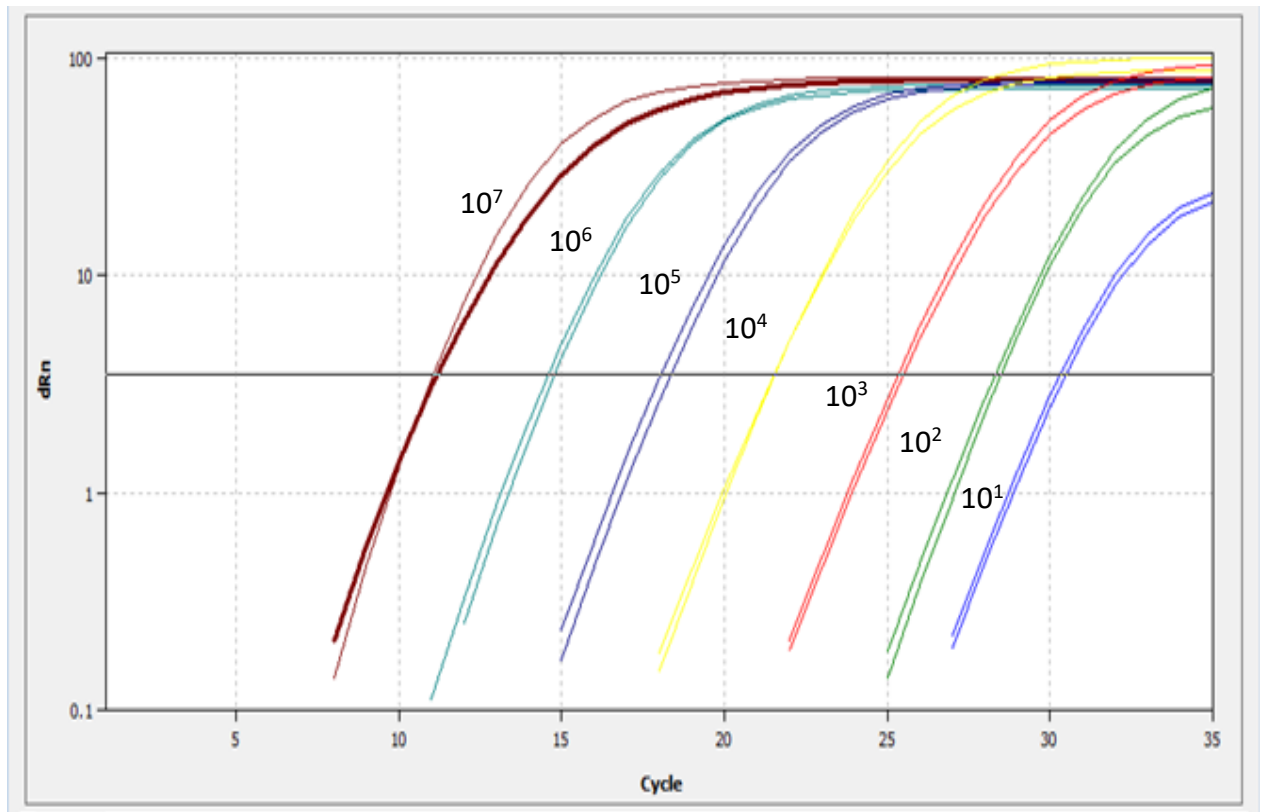
The quantification limit was  $10^3$  gene copies per reactions which was determined from the number of cycles (Figure 3.9).



**Figure 3.7** qPCR (*rpoC*) standard curve developed using seven serial dilutions of isolate *F. psychrophilum* 6\_S (black line) and *F. psychrophilum* 19\_5 (grey dotted line). Standards range in concentration from  $10^1$  to  $10^7$  theoretical number of isolate *F. psychrophilum* 6\_S (x-axis). The Ct was within threshold when values were between 15 and 35 (y-axis). For Atlantic salmon ( $n=3$ ,  $p>0.05$ ) the slope was of  $-3.30$  ( $R^2 = 0.998$ ), indicating an efficiency of 100 % and for rainbow trout ( $n=3$ ,  $p>0.05$ ) the slope was  $-3.25$  ( $R^2 = 0.994$ ), indicating an efficiency of 101%



**Figure 3.8** Representative image of melt curve for qPCR, targeting the *rpoC* gene in *F. psychrophilum* 6\_S, standard curve developed using seven serial dilutions of isolate *F. psychrophilum* 6\_S. Standards range in concentration from  $10^1$  to  $10^7$  with all demonstrating a melting point of ca. 72°C showing a single product being amplified.

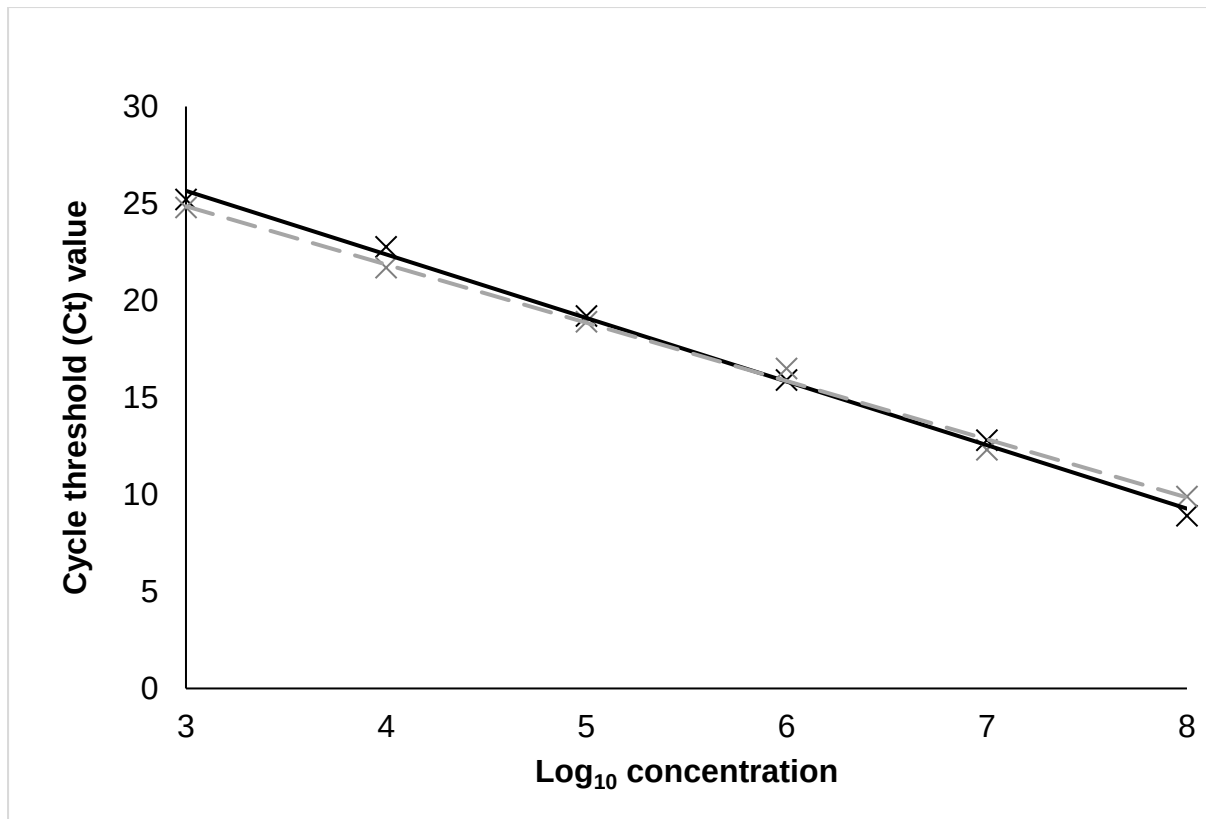


**Figure 3.9** Representative image of performance of *F. psychrophilum* specific SYBR Green qPCR amplifying the *rpoC* gene using seven serial dilutions of isolate *F. psychrophilum* 6\_S STC. The amplification curves (in duplicates) show the fluorescence development over time using a dilution range of  $10^1$  to  $10^7$  copies/  $\mu\text{L}$  of the *F. psychrophilum* DNA standard.

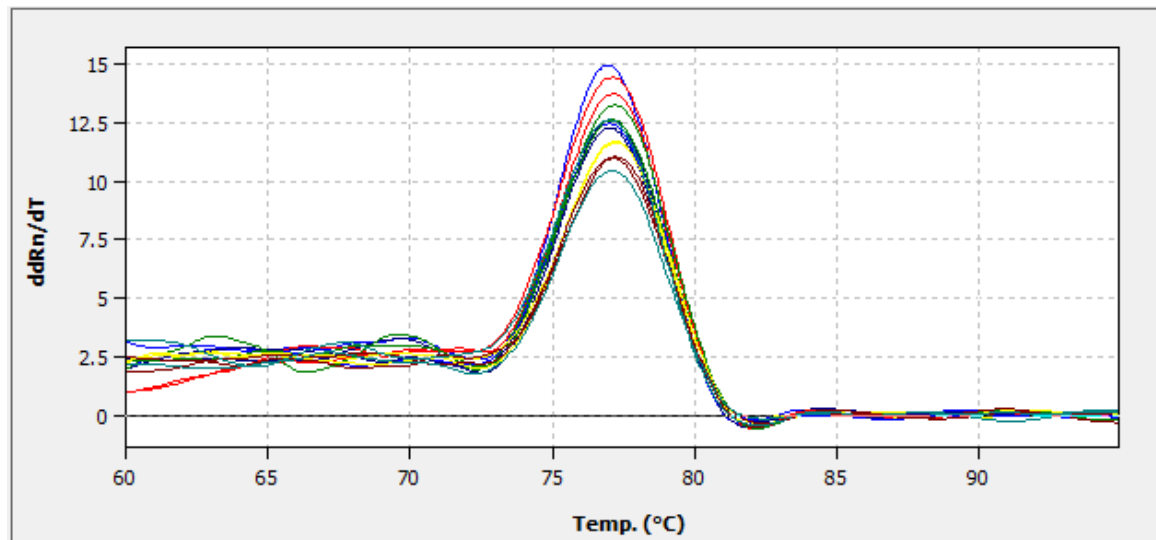
Bacterial load in the head kidney tissues were normalised against the relative abundance of *EF1-α* housekeeping gene.

Atlantic salmon and rainbow trout head kidney tissues DNA was amplified and cleaned-up for use as template for the STC development. The standard curve developed using seven serial dilutions of Atlantic salmon head kidney tissue and rainbow trout. Both STC ranged from  $10^2$  to  $10^7$  *EF1-α* gene copies and generated a slope of -3.20 ( $R^2 = 0.997$ ), indicating an efficiency of 103 % (Atlantic salmon head kidney) and -3.33 ( $R^2 = 0.997$ ), indicating an efficiency of 100% (rainbow trout head kidney) (Figure 3.10).

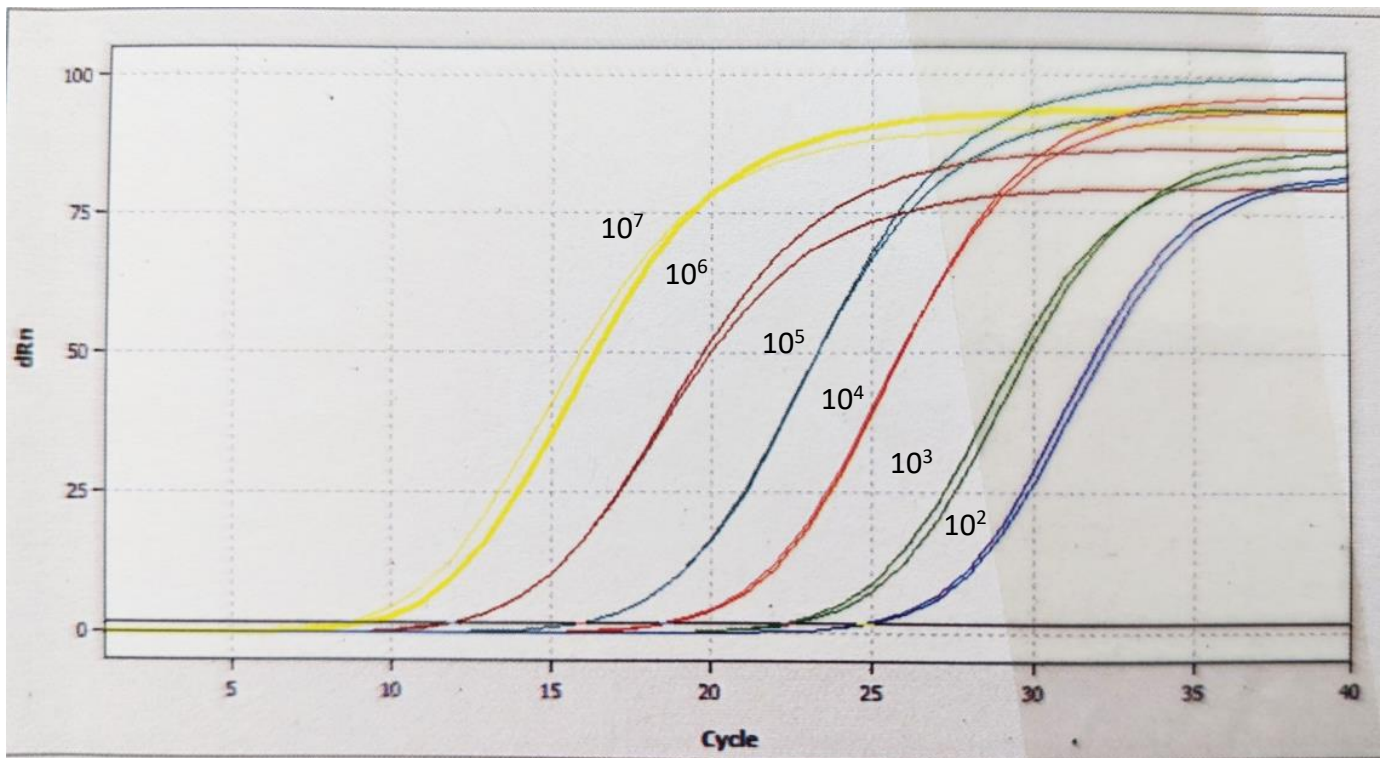
A melt curve was generated to see that the amplicon produced for each STC, which had a single specific peak, indicating that the correct product was amplified (Figure 3.11). The cleaned-up amplicons of the *rpoC* fragment were therefore used for all the successive quantifications (qPCR) of *F. psychrophilum* in head kidney tissues samples from the challenge as standards. The quantification limit was  $10^3$  gene copies per reaction which was determined from the number of cycles (Figure 3.12).



**Figure 3.10** qPCR (*EF1-α* gene) standard curve developed using seven serial dilutions of Atlantic salmon head kidney DNA (black line) and rainbow trout head kidney DNA (grey dotted line). Standards range in concentration from  $10^2$  to  $10^7$  theoretical number of isolate *F. psychrophilum* 6\_S (x-axis). The Ct was within threshold when values were between 15 and 35 (y-axis). For Atlantic salmon (n=3,  $p>0.05$ ) the slope was of  $-3.20$  ( $R^2 = 0.997$ ), indicating an efficiency of 103 % and for rainbow trout (n=3,  $p>0.05$ ) the slope was  $-3.33$  ( $R^2 = 0.997$ ), indicating an efficiency of 100%



**Figure 3.11** Representative image of melt curve for qPCR, targeting the *EF1- $\alpha$*  gene in Atlantic salmon head kidney, standard curve developed using seven serial dilutions of isolate *F. psychrophilum* 6\_S. Standards range in concentration from  $10^2$  to  $10^7$  with all demonstrating a melting point of ca. 78°C showing a single product being amplified.



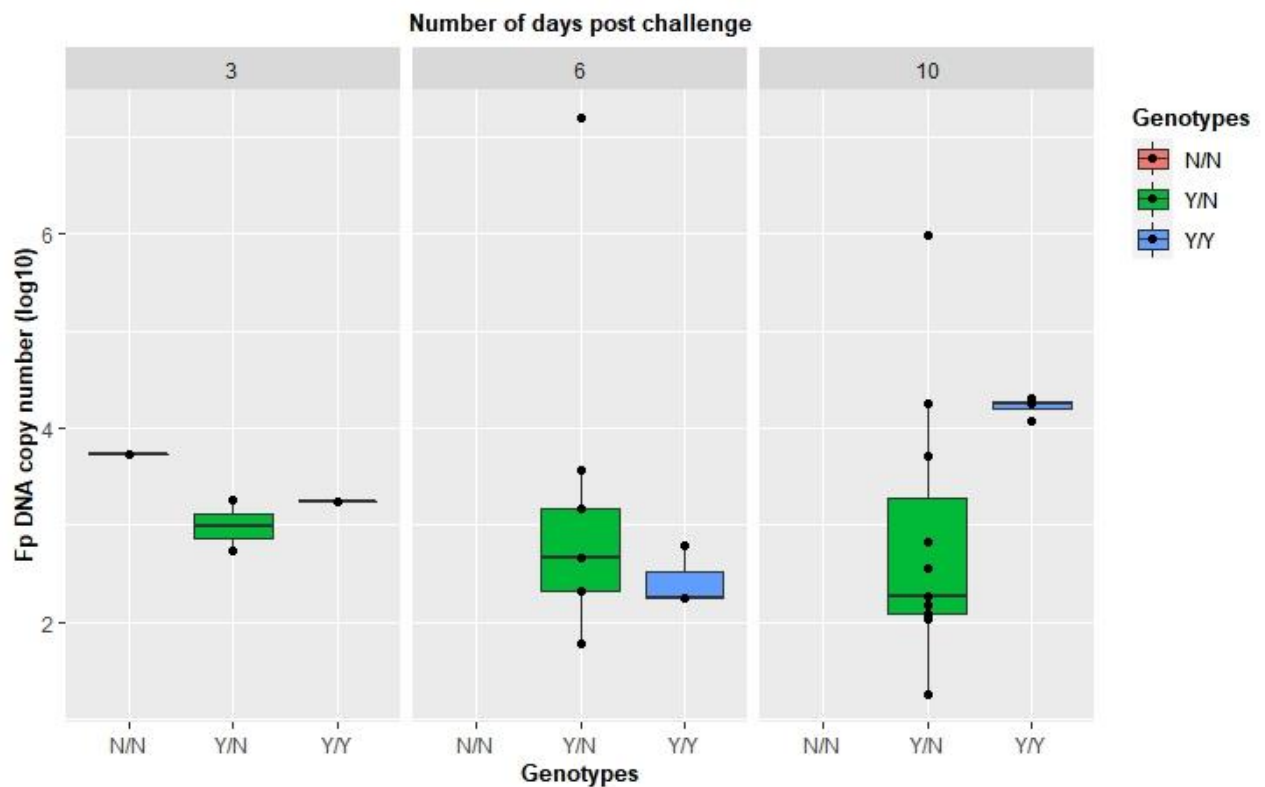
**Figure 3.12** Representative image of performance of SYBR Green qPCR amplifying the *EF1- $\alpha$*  gene using seven serial dilutions of Atlantic salmon head kidney. The amplification curves (in duplicates) show the fluorescence development over time using a dilution range of  $10^2$  to  $10^7$  copies/  $\mu$ L of Atlantic salmon head kidney DNA.

### 3.4.3 SYBR Green qPCR assay for *F. psychrophilum* detection in Atlantic salmon and rainbow trout tissues

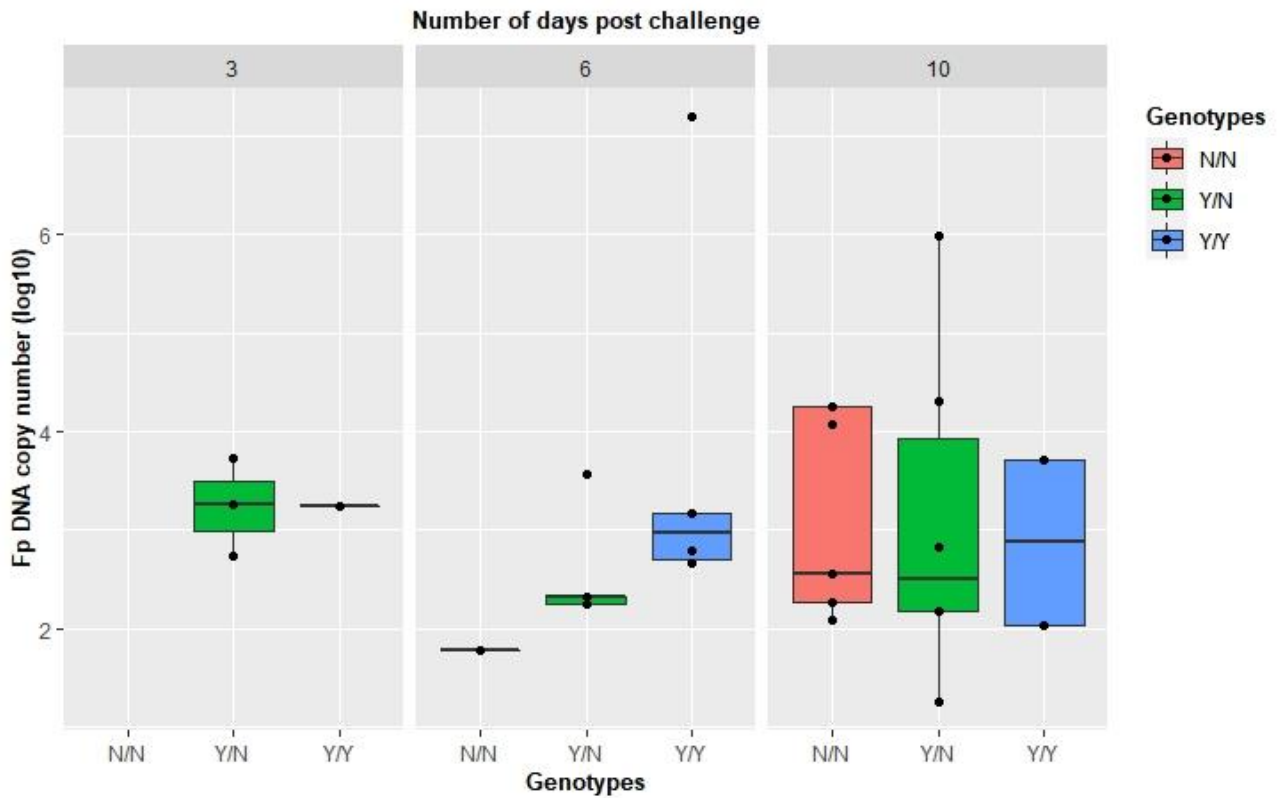
This newly developed SYBR Green qPCR assay, based on the primers designed on the single copy gene *rpoC* (Strepparava et al., 2014), was used to detect and quantify *F. psychrophilum* abundance in Atlantic salmon and rainbow trout fry head kidney infected with isolates *F. psychrophilum* 6\_S and *F. psychrophilum* 19\_5, respectively, using samples derived from the immersion challenge model described in Chapter 2.

In Atlantic salmon, the samples run on the qPCR (n=70), showed that at different time points post challenge (0, 3, 6 and 10 dpc) *F. psychrophilum* was detected in animals with mainly the heterozygous genotype (Y/N) or the resistant (Y/Y) genotype, with the exception of 10 dpc, where the highest *F. psychrophilum* abundance was recorded in the fish with the susceptible (N/N) genotype on chromosome 9 (Figure 3.13); while *F. psychrophilum* was detected in all the animals genotypes across the time points associated with chromosome 5, with the exception of the lack of detection of the bacteria at 3 dpc in the animals with the susceptible genotype (Figure 3.14). There was not a significant association between bacterial abundance regardless of the day post challenge, without including day 0, associated with chromosome 9 ( $R^2 = 0.005$ ,  $F(1,120) = 0.588$ ,  $p = 0.447$ ) or associated with chromosome 5 ( $R^2 = 0.006$ ,  $F(1,120) = 0.721$ ,  $p = 0.397$ )

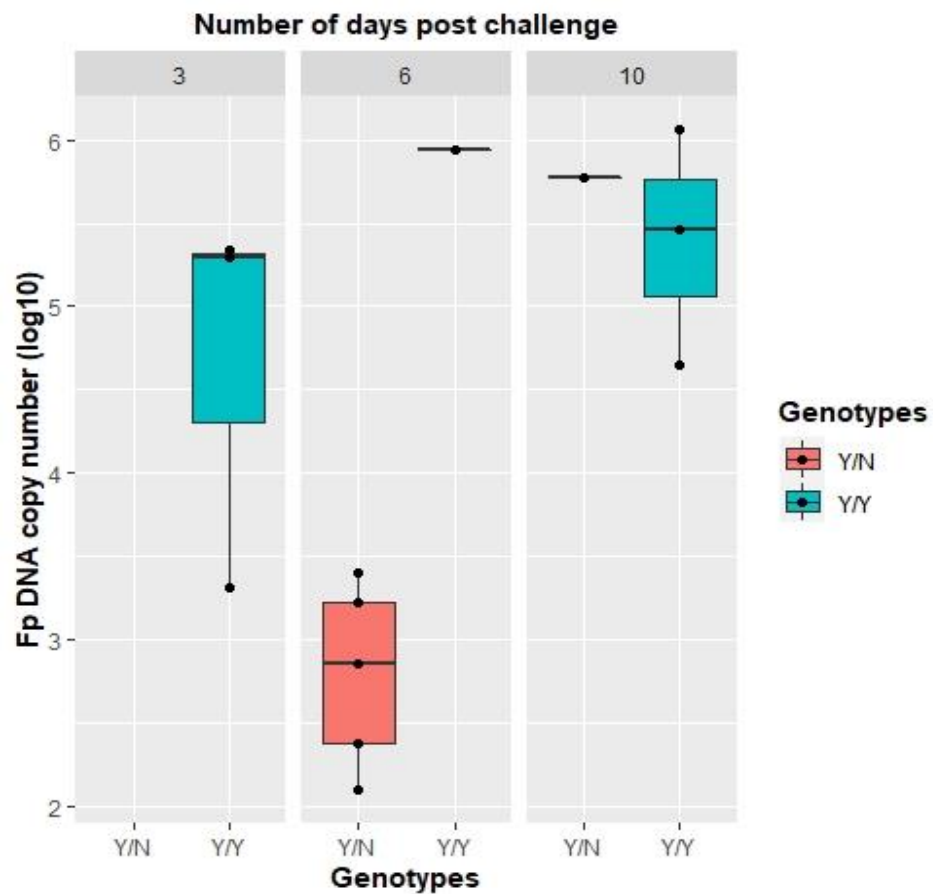
In rainbow trout (n= 53), *F. psychrophilum* was not detected in any animal with the susceptible genotype (N/N) on chromosome 3 and. At 6 dpc, the resistant genotype (Y/Y) had the highest copy number (abundance) of *F. psychrophilum* DNA in the head kidney (Figure 3.15). Similarly, on chromosome 8, *F. psychrophilum* was detected in animals with the susceptible genotype only 3 dpc at a low abundance (3.3 *F. psychrophilum* DNA copy number) (Figure 3.16). At 10 dpc, the highest abundance of *F. psychrophilum* DNA was observed, regardless of the fish genotype, except for the susceptible genotype where no bacteria were detected. For rainbow trout there is a significant association between bacterial abundance regardless of the day post challenge, without including 0 dpc, on chromosome 3 ( $R^2 = 0.517$ ,  $F(1,24) = 25.66$ ,  $p < 0.05$ ) or chromosome 8 ( $R^2 = 0.514$ ,  $F(1,24) = 25.44$ ,  $p < 0.05$ ).



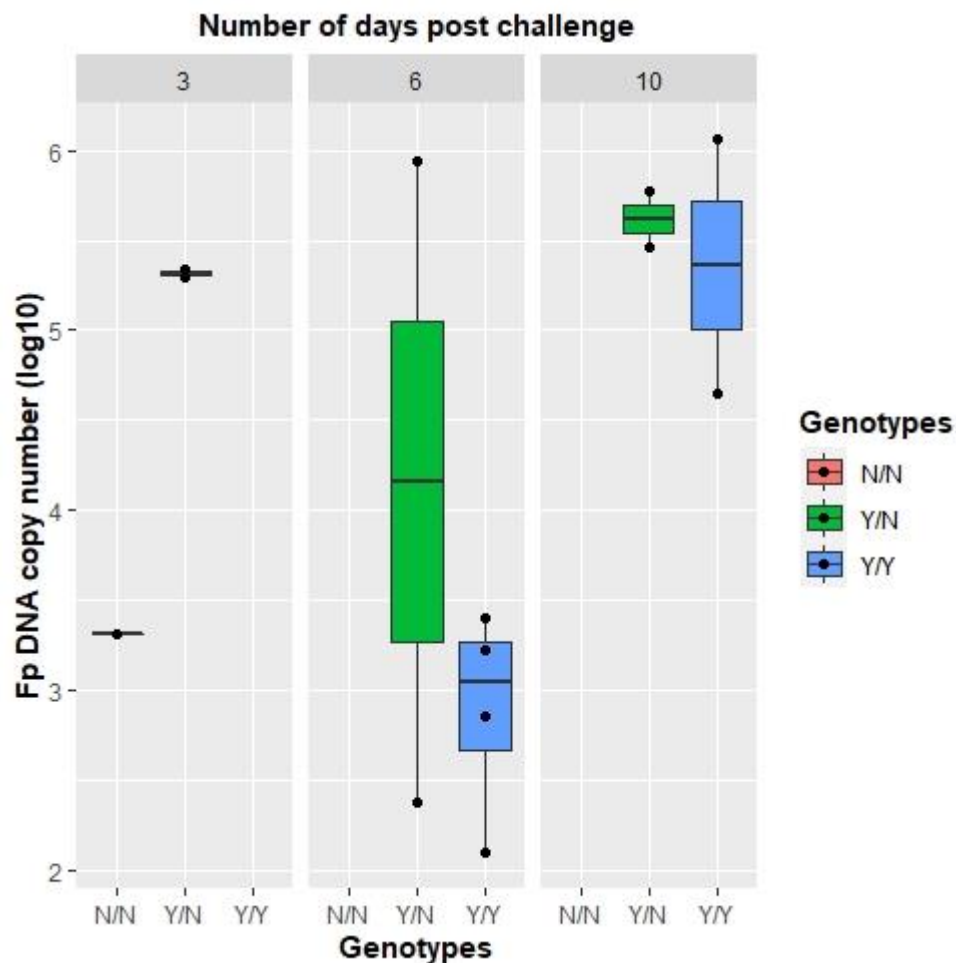
**Figure 3.13** *F. psychrophilum* DNA copy number ( $\log_{10}$ ) in Atlantic salmon head kidney challenged by immersion with *F. psychrophilum* 6\_S. Fish were sampled at different time points post-challenge (i.e., 0 [n=10], 3 [n=18], 6 [n=18] and 10 [n=24] dpc) and qPCR was used to detect and quantify bacterial abundance in the head kidney tissues of Atlantic salmon with different genotypes on chromosome 9 at the different time points. Day 0 is not represented in the figure as no detection was observed. N/N represents the ‘susceptible’ genotype, Y/Y the ‘resistant’ genotype and Y/N the heterozygous genotype. The boxplot shows how the data are distributed, the top edge of the box represents the upper quartile, the bottom edge the lower quartile, and the horizontal line in the box represents the median value. There is not a significant difference in bacterial load between fish genotypes on day 3, 6 and 10 ( $p > 0.05$ ), while there is a significant difference between 0 vs 3, 6, 10 dpc regardless of the fish genotype ( $p < 0.05$ ).



**Figure 3.14** *F. psychrophilum* DNA copy number (log<sub>10</sub>) in Atlantic salmon head kidney challenged by immersion with *F. psychrophilum* 6\_S. Fish were sampled at different time points post-challenge (i.e., 0 [n=10], 3 [n=18], 6 [n=18] and 10 [n=24] dpc) and qPCR was used to detect and quantify bacterial abundance in the head kidney tissues of Atlantic salmon with different genotypes on chromosome 5 at the different time points. Day 0 is not represented in the figure as no detection was observed. N/N represents the 'susceptible' genotype, Y/Y the 'resistant' genotype and Y/N the heterozygous genotype. The boxplot shows how the data are distributed, the top edge of the box represents the upper quartile, the bottom edge the lower quartile, and the horizontal line in the box represents the median value. There is not a significant difference in bacterial load between fish genotypes on day 3, 6 and 10 ( $p > 0.05$ ), while there is a significant difference between 0 vs 3, 6, 10 dpc regardless of the fish genotype ( $p < 0.05$ ).



**Figure 3.15** *F. psychrophilum* DNA copy number (log<sub>10</sub>) in rainbow trout head kidney challenged by immersion with *F. psychrophilum* 19\_5. Fish was sampled at different time points post-challenge (i.e., 0 dpc [n=7], 3 dpc [n=15], 6 dpc [n=15], 10 dpc [n=16]) and qPCR was used to detect and quantify bacterial abundance in the head kidney tissues with different genotypes on chromosome 3 at the different time points. Day 0 is not represented in the figure as no detection was observed. Y/Y represents the ‘resistant’ genotype and Y/N the heterozygous genotype. Bacteria were not detected in the fish with the susceptible genotypes. The boxplot shows how the data are distributed, the top edge of the box represents the upper quartile, the bottom edge the lower quartile, and the horizontal line in the box represents the median value. There is not a significant difference in bacterial load between fish genotypes on day 3, 6 and 10 ( $p > 0.05$ ), while there is a significant difference between 0 vs 3,6,10 dpc regardless of the fish genotype ( $p < 0.05$ ).



**Figure 3.16** *F. psychrophilum* DNA copy number (log<sub>10</sub>) in rainbow trout head kidney challenged by immersion with *F. psychrophilum* 19\_5. Fish was sampled at different time points post-challenge (i.e., 0 dpc [n=7], 3 dpc [n=15], 6 dpc [n=15], 10 dpc [n=16]) and qPCR was used to detect and quantify bacterial abundance in the head kidney tissues with different genotypes on chromosome 8 at the different time points. Day 0 is not represented in the figure as no detection was observed. N/N represents the 'susceptible' genotype, Y/Y the 'resistant' genotype and Y/N the heterozygous genotype. There is not a significant difference in bacterial load between fish genotypes on day 3, 6 and 10 ( $p > 0.05$ ), while there is a significant difference between 0 vs 3, 6, 10 dpc regardless of the fish genotype ( $p < 0.05$ ).

### 3.5 Discussion

Diagnosis of *F. psychrophilum* is a challenging issue and in this Chapter, a more cost-effective and rapid qPCR assay using SYBR Green I targeting the single copy gene *rpoC* of *F. psychrophilum* for detection and quantification of the bacteria in rainbow trout and Atlantic salmon head kidney tissues was developed and validated. Atlantic salmon and rainbow trout fry from the AquaGen breeding nucleus were challenged with *F. psychrophilum* 6\_S and *F. psychrophilum* 19\_5, respectively. Based on the GWAS described in Chapter 2, the genetic architecture of the trait resistance against *F. psychrophilum* in Atlantic salmon appears to be oligogenic with two QTLs located on chromosome 5 and 9 in Atlantic salmon, and in rainbow trout, with multiple peaks identified in the GWASs including on chromosome 3 and chromosome 8. In a previous study by AquaGen, two strong QTLs were identified on chromosome 3 and chromosome 8 (data not available) and as a result, the study in this Chapter, focused on the rainbow trout genotypes based on chromosome 3 and chromosome 8. Nevertheless, if the data from this chapter were to be re-analysed, it would be important to look at the new relevant QTLs that were discovered for the study population.

The present study shows that the SYBR Green qPCR assay developed is specific and able to detect and quantify *F. psychrophilum* in rainbow trout and Atlantic salmon head kidneys with a confident level of detection of  $10^3$  copies/  $\mu\text{L}$  of *F. psychrophilum* DNA. The use of a single copy gene (*rpoC*) allowed the more accurate detection and quantification of the pathogen, given that one bacterial gene is one bacterial cell (Morillo et al., 2003). When testing the primers targeting the *rpoC* gene, amplification was present when analysing different *F. psychrophilum* isolates, including *F. psychrophilum* 6\_S, *F. psychrophilum* 19\_5, *F. psychrophilum* 469, *F. psychrophilum* 468, *F. psychrophilum* JIP02/86, while amplification was absent in non-target bacterial isolates such as *A. salmonicida* sub. *salmonicida*, *F. orientalis*, *V. anguillarum*, *E. coli*, *Y. ruckeri*, *E. piscicida*, and all the negative control templates (nuclease free water).

From the results of the current study, there was not a significant difference between bacterial abundance regardless of the day post challenge, without including 0 dpc, on both chromosome 5 and chromosome 9. However, in rainbow trout when looking at fish carrying different genotypes at chromosome 3 and chromosome 8 there was a significant difference between bacterial abundance regardless of the day post

challenge, without including 0 dpc ( $p < 0.05$ ). These results suggest that in rainbow trout genotype could affect fish susceptibility to *F. psychrophilum*, however for Atlantic salmon, there is not a significant correlation between genotype and bacteria abundance in the head kidney tissues ( $p > 0.05$ ). The genetic association with susceptibility and association at the QTL level is not simple and to further explore the mechanisms of resistance it is important to look at the genetic architecture particularly at the transcriptomic level, looking at possible expression QTL (eQTL). eQTL associated genomic and transcriptomic data sets from the same individuals to identify loci that affect mRNA expression. By linking SNPs to changes in gene expression, eQTL can be useful for annotating GWAS variants (Jung et al., 2020). Similar results were observed by Marancik & Wiens (2013) as they did not observe significant difference in bacterial loads in experimental challenge of rainbow trout selectively bred for divergent bacteria cold water disease (BCWD) susceptibilities. Marancik & Wiens (2013) suggested that for acute mortalities, bacterial burden may not directly influence survival differences between genetic lines.

In terms of detection and quantification of bacterial DNA in the head kidney, in Atlantic salmon the mean *F. psychrophilum* DNA copy number ( $\log_{10}$ ) detected was below 4.5 copies/  $\mu\text{L}$ , while in rainbow trout the mean *F. psychrophilum* DNA copy number ( $\log_{10}$ ) detected was below 6 copies/ $\mu\text{L}$ . This low bacterial load, could suggest that the immersion challenge caused a systemic infection in the fish infected, rather than the infection being localised in the head kidney. This is not surprising, as the immersion challenge model used for the present study mimics the more natural route of infection of this bacterial disease, and it has been previously reported that in the wild flavobacteriosis, especially in rainbow trout, has been associated with systemic infection (Nilsen, Olsen, et al., 2011). Hoare et al. (2022) highlighted the importance of the route of infection in challenge studies with *F. psychrophilum*, which is suspected to have a mucosal route of entry. Many challenge experiments in fish are conducted using intraperitoneal or intramuscular injection, however, they bypass the primary natural barriers of mucus and epithelium, thereby promoting an 'unnatural' infection. Rainbow trout and Atlantic salmon mucosal immunity could have an important role in suppressing the bacteria. New technology has allowed the visualization of bacterial *V. anguillarum* spread in a zebrafish model (Schmidt et al., 2017), revealing the tissues and organs where bacteria were detected differed significantly depending on the route

of infection. In the study by Schmidt et al. (2017), as with the present *F. psychrophilum* study in this Chapter, significantly higher numbers of bacteria were needed to cause infection and morbidity/mortality by the mucosal route than by injection, revealing how effective the mucosal barriers of fish are to prevent infection. Similarly, (Hoare et al., 2019) determined that when the fish is challenged by injection, the disease is established in a shorter length of time compared to when it is challenged by immersion (Hoare et al., 2019). Similar results were also observed by Li et al. (2021), who conducted a study with rainbow trout (25 g), injected the fish with two isolates of *F. psychrophilum*, and after 24 h post infection kidney tissues were sampled and tested by qPCR, and an average of  $1.40 \times 10^3$  copies/  $\mu$ l were detected in moribund or dead fish.

One shortcoming of this study is that both Atlantic salmon and rainbow trout fry were sampled, and these fish did not exhibit any typical gross pathology associated with flavobacteriosis. From the qPCR results, it was not possible to find an effect of fish genotype on bacterial load, however this could be down to the sampling regime used in this study. If the experiment was to be repeated, sampling would be better if targeted at mortalities, rather than random sampling, and it would be important to compare healthy fish against moribund/dead fish.

In the present study, it has proven to be particularly challenging to extract DNA from the head kidney of the fry, due to their relatively small size of the fish (< 2 g), however the developed and validated qPCR is a very sensitive technique which does not require large amounts of DNA. These results highlighted the importance of the development of this qPCR assay to analyse small tissue samples especially when diagnosing this pathogen in fry, vulnerable to this bacterial disease (Wahli & Madsen, 2018). In this study, due to the small size of the fry sampled, *F. psychrophilum* infection was investigated solely in head kidney tissues. The *F. psychrophilum* life cycle is intricately involved with spleen and head kidney macrophages (Semple et al., 2020). In a previous study, rainbow trout head kidney and spleen macrophages have shown to induce strong reactive oxygen species production when infected with *F. psychrophilum*, and head kidney macrophages seem to be able to kill a greater percentage of *F. psychrophilum* strains compared to spleen macrophages (Nematollahi et al., 2003). Since spleen macrophages are less antimicrobially active than head kidney macrophages when exposed to *F. psychrophilum* infection, they

form a “hot spot” where bacteria can reside. This could explain the relatively low detection of *F. psychrophilum* in Atlantic salmon and rainbow trout head kidney tissues. If this study was to be repeated, it would be interesting to compare bacterial load in both lymphoid organs which are both macrophage centres, with key immune-related roles (Kumar et al., 2018).

### **3.6 Conclusions**

In conclusion, this study has shown that this newly develop SYBR Green qPCR assay targeting the *rpoC* gene is a reliable and specific diagnostic tool to detect and quantify *F. psychrophilum* infections in fish tissues, especially when the fish tested is small. This qPCR assay could be applied as a point-of-care diagnostic tool for monitoring the spread of flavobacteriosis. These results also underline that the genetic association with susceptibility and association at the QTL level is not simple and easy. This Chapter suggests that the protection against *F. psychrophilum* in both rainbow trout and Atlantic salmon is likely to be due to induction of the mucosal immune response, in particular immunoglobulin T (IgT) and this will be the focus of Chapter 4.

## **Chapter 4: Characterisation of antibodies for the detection of *Flavobacterium psychrophilum* and IgT positive cells in infected Atlantic salmon and rainbow trout fry**

### **4.1 Introduction**

Fish in culture are susceptible to a wide range of infectious diseases in the form of bacterial, fungal, viral, and parasitic agents. These pathogens pose key challenges to the aquaculture industry, and result in considerable economic losses worldwide (Adams et al., 2005; Mekasha & Linke, 2021). Effective disease control is fundamental to stop the spread of infectious diseases within aquatic farming systems. Effective health management programmes, including proper management and husbandry practice, efficient biosecurity and hygiene methods are key standards. Improved resistance to diseases through vaccination and breeding programmes can help reduce and control diseases at farm sites (Doeschl-Wilson et al., 2021). However, rapid and diagnosis and surveillance is also essential for disease monitoring, allowing appropriate action to be taken readily before they became significant problems for the farmers. A variety of methods are available to detect pathogens in the aquatic environments, including immunological and molecular methods (Reta et al., 2020). Serodiagnosis, including immunofluorescent antibody technique (IFAT), whole-cell agglutination, and enzyme linked immunosorbent assay (ELISA), which allow rapid bacterial diagnosis directly from infected fish (Naqid et al., 2016).

Monoclonal antibodies (mAbs) can be used in place of polyclonal sera to increase the precision of identification of pathogens. Monoclonal antibodies (mAbs) recognise specific antigens and are produced with hybridoma technology (Zaroff & Tan, 2019) allowing a limitless supply. In aquaculture, the development of mAbs to bacterial fish pathogens has provided useful tools to rapidly immuno-diagnose and control diseases. IFAT and ELISA protocols have been used to detect a variety of infectious diseases including bacterial kidney disease, mycobacteriosis and proliferative kidney disease (Adams et al., 1995).

This technology can be labour-intensive, costly and during the fusion of antibody-expressing B lymphocytes and myeloma cells, antibodies lose their diversities.

Nevertheless, mAbs recognise a single antigenic determinant (epitope) and this restricted reactivity allows for more specific identification of the organism of interest. mAbs are highly reproducible and scalable (unlimited production source) and the antigen or immunogen does not need to be pure (Siddiqui, 2010). Development of specific, sensitive, and rapid diagnostic methods, including serotyping, are key to detect the various stages of disease (Shankar et al., 2000). At present, laboratories use conventional biochemical methods to diagnose bacterial fish pathogens, which are tedious, expensive, and not sensitive enough to discriminate between heterogenous isolates. mAbs complement these approaches providing rapid and sensitive methods of pathogen detection (Austin, 2019).

Several mAbs have been developed for the diagnosis of a variety of fish pathogens such as *Vibrio harveyi* (Phianphak et al., 2005), *Aeromonas hydrophila* (Delamare et al., 2002) and *Renibacterium salmoninarum* (Adams et al., 1995). However, validated mAbs do not exist for *Flavobacterium psychrophilum*, a major pathogen of salmonids (Nilsen et al., 2011; Starliper, 2011). *F. psychrophilum* can be detected in infected fish using a combination of methods (Christiansen et al., 2014; Nilsen et al., 2014; Strepparava et al., 2014), including polymerase chain reaction (PCR), quantitative PCR (qPCR) (e.g., Chapter 3), ELISA, IFAT, immunohistochemistry (IHC), sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting.

ELISA, a widely used technique in aquaculture, is an assay that can be developed in different formats (direct, indirect, sandwich-direct, indirect- and competitive) for detection of the presence of either antigens/pathogens (sandwich ELISA) or host antibodies in the serum following infection or vaccination (indirect ELISA) (Adams et al., 1995; Li et al., 2019). IFAT is a semi-quantitative, sensitive, and rapid test which can be used for the detection of antibodies in serum samples. If specific antibodies are present in the serum, they react with specific antigens in tissue or bacterial smears which are then detected by fluorescein-labelled anti-species antibodies and a signal is observed using a fluorescence microscope (Messenger & Rupprecht, 2015). This technique presents advantages such as simple methodology, inexpensiveness, less labour-intensive especially when IFAT is performed on bacterial smears and can be applied on tissues to observe organ lesions, which can be used as an effective screening test for large number of samples (Gao et al., 2018).

SDS-PAGE is one of the least expensive and most commonly used techniques to analyse antibodies for purity (Kirley & Norman, 2018). SDS-PAGE is commonly used to obtain high resolution separation of complex proteins mixtures. The method includes initial denaturation of component proteins with an anionic detergent that binds to them, giving all proteins a negative charge proportional to their molecular mass. Following this step, the samples undergo electrophoresis through a porous acrylamide gel matrix that separates the proteins according to their molecular mass (Nowakowski et al., 2014).

Following SDS-PAGE, western blotting analysis can be performed. In western blotting, proteins that have been separated by SDS-PAGE are transferred to a membrane which produces a band for each protein, and the membrane is then incubated with labelled antibodies specific to the protein of interest (Mahmood & Yang, 2012). One of the important features of this technique is the highly specific interaction between an antibody and its corresponding epitope. The antigen, usually a protein or peptide, is the target of the antibody. The precise point of interaction is between a small region of the antigen, an epitope, and the recognition sites found on the arms of the antibody molecule. In a western blot the antibodies which detect the target protein can be either polyclonal (a mixed pool of immunoglobulin [Ig] molecules that bind to several different epitopes) or monoclonal (clonal Ig that bind to a single epitope within a target antigen) (Mishra et al., 2017).

Rabbit polyclonal antibodies (pAbs) have been used for the rapid detection of *F. psychrophilum* in infected tissues, and these exhibit low-levels of cross-reactivity with other *Flavobacterium* species (Lorenzen & Olesen, 1997). Antigenic composition of *F. psychrophilum*, observed by immunogold transmission electron microscopy, showed that anti-*F. psychrophilum* antibodies are predominantly localised on the cell surface. To discriminate these antigens further, western blotting was used and a major antigen with an apparent molecular mass ~16 kDa was identified. This antigen was identified as low-molecular-mass lipopolysaccharide (LPS) (Crump et al., 2001). A high molecular-weight-mass LPS O-antigen was detected at ca. 70-200 kDa (Crump et al., 2001). Faruk et al. (2000) has previously produced mAbs against whole cell preparations of two *F. psychrophilum* strains: B97026P1 (virulent) and 32/97 (non-virulent). However cross-reactivity with several non-*F. psychrophilum* species was observed, and by ELISA some of the isolates examined were non-detectable making

these mAbs unsuitable as diagnostic probes. This is possibly due to *F. psychrophilum* being an heterogenous bacteria with several host-specific serotypes.

The aim of the present Chapter was to characterise antibodies for the detection of *F. psychrophilum* in formalin fixed tissue and to investigate *F. psychrophilum* infection in Atlantic salmon and rainbow trout tissues. The first objective of this Chapter was to re-clone mAbs previously produced against whole cell preparation of the two *F. psychrophilum* strains B97026P1 and 32/97 (non-virulent) at the University of Stirling by Faruk et al. (2000) and characterise the mAbs using ELISA, IFAT, SDS-PAGE and immunoblotting protocols. The second objective of this study was to detect and quantify *F. psychrophilum* cells in infected Atlantic salmon and rainbow trout tissues using IHC. The third, and final objective of the present Chapter was to determine whether mucosal immune responses, in particular IgT, are induced in *F. psychrophilum* challenged Atlantic salmon and rainbow trout tissues.

## 4.2 Materials and Methods

### 4.2.2 Cloning of mAbs

Five anti- *F. psychrophilum* mAbs (9G7, 32, 5A9, 5 and 8) were used in this study and these were previously produced by Faruk et al. (2002). Briefly, the hybridoma was removed from liquid nitrogen storage, thawed at room temperature (RT) for ca. 30 min and placed in a Corning™ Falcon™ 15 mL Conical Centrifuge Tube (Fisher Scientific; UK) with 10 mL of Dulbecco's Modified Eagles Medium (DMEM) with supplements [L-Glutamine 200 mM, 100 mM Sodium pyruvate, 10.000 µ/mL penicillin, 10% foetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO)] (ThermoFisher Scientific; UK). The solution was then centrifuged on a bench centrifuge (Merck; Germany) at RT at 200 × *g* for 7 min.

Following centrifugation, the DMEM layer was carefully removed by pipetting, leaving the FCS layer untouched. Fresh DMEM+10% foetal calf serum (FCS) (2 mL) was added to the tube, and the cells were resuspended by gently pipetting the solution (2 mL) into a 24-well cell culture (VWR International) and incubated at 37°C in 5% CO<sub>2</sub> (Richmond Scientific; UK). After 48-72 h, the culture growth was inspected using an inverted light microscope (Olympus LS; UK) and 2 mL of media was removed from each well without disturbing the cells and topped up with 2mL of fresh medium (DMEM+10% FCS). After 48-72 h further incubation, 150 µL of culture was removed and transferred into a fresh 24-well cell culture plate (VWR International) containing 1.5 mL of fresh media (DMEM with supplements, as described above) and 0.05% sheep red blood cells. After 7 days, the supernatant was removed from each well showing good growth and transferred into one bijoux container (Alpha Laboratories, UK) and stored at -20°C until further use.

### 4.2.1 Bacterial cultivation

All the bacterial isolates used in this study were stored in Protect Beads (Technical Service Consultants Ltd) and were cultured as follows. Briefly, for *F. psychrophilum* isolates one bead was placed in 3 mL MV broth culture (5 g/L Veggitones GMO-free soya peptone [Oxoid, UK], 0.5 g/L yeast extract [Oxoid, UK], 0.5 g/L magnesium sulphate heptahydrate [Fisher Chemicals, UK], 0.2 g/L anhydrous calcium chloride [Thermo Fisher Scientific, USA], 2 g/L dextrose [Oxoid, UK]) for 48-72 h at 15°C (150 rpm). The tubes were centrifuged twice at 3,000 × *g* for 10 min, the supernatant

discarded, the cell pellet was re-suspended in PBS (0.02 M Phosphate, 0.15 M NaCl; pH 7.2) and the optical density of the cell suspension adjusted to 1.0 at OD<sub>525 nm</sub> using a spectrophotometer (Biotek Synergy HT, USA). Aeromonadaceae, Vibrionaceae and Enterobacteriaceae isolates were cultured from Protect Beads as above.

For Aeromonadaceae and Vibrionaceae, one bead of each species was placed in Tryptone Soya Broth (17 g/L casein peptone (pancreatic), 2.5 g/L dipotassium hydrogen phosphate, 2.5 g/L glucose, 5 g/L sodium chloride, 3 g/L soya peptone (papain digest.) [TSB: Sigma- Aldrich; United states]) for 24 h and 48 h respectively at 25°C (150 rpm).

For *Yersinia ruckeri*, one bead was placed in 3 mL *Yersinia* enrichment broth base (30 gm/L tryptone soya broth, 6 gm/L yeast extract [ThermoFisher Scientific; UK]) and incubated at 25°C for 48 h with continuous shaking at 150 rpm. Each culture was centrifuged, and the bacterial pellet washed twice in PBS, before the optical density was measured at OD<sub>525 nm</sub> for *F. psychrophilum* isolates and OD<sub>600 nm</sub> for all the other bacterial genera equivalent to ca.  $1 \times 10^8$  CFU/mL.

**Table 4.1** List of bacterial species cultured for the characterisation of the anti-*F. psychrophilum* mAbs

| Isolate                             | Strain ID                                              |
|-------------------------------------|--------------------------------------------------------|
| <i>Flavobacterium psychrophilum</i> | IoA_240                                                |
|                                     | IoA_350                                                |
|                                     | IoA_469                                                |
|                                     | <i>F. psychrophilum</i> 6_S,                           |
|                                     | <i>F. psychrophilum</i> 19_5,<br>Fp. <sup>T</sup> 1947 |
| <i>Flavobacterium hydatis</i>       | NCIMB 2215                                             |
| <i>Flavobacterium aquatile</i>      | NCIMB 8694                                             |
| <i>Aeromonas salmonicida</i>        | NCIMB 1002                                             |
| <i>Yersinia ruckeri</i>             | NCIMB 2194                                             |
| <i>Vibrio anguillarum</i>           | NCIMB 6                                                |
| <i>Aeromonas salmonicida</i>        | NCIMB 1002, MT 423                                     |

### 4.2.3 Characterisation of anti-*F. psychrophilum* mAbs by IFAT

The ability of the anti-*F. psychrophilum* mAbs 9G7, 32, 5A9, 5 and 8 to detect a variety of *F. psychrophilum* strains in bacterial smears was examined by IFAT. As a positive control the mAbs were also tested on a section from a skin lesion of a rainbow trout known to be positive for *F. psychrophilum* (Hoare et al., 2017). For the positive control slides, the paraffin slides were dewaxed and rehydrated with two successive xylene baths (Sigma-Aldrich, United States) of 5 min each, followed by immersion in 100% ethanol (Sigma-Aldrich, United States) for 5 min and 70% ethanol for 3 min. Following this step, the slides were incubated with chilled 98% acetone (Sigma-Aldrich, United States).

In duplicates, a single colony of *F. psychrophilum* 6\_S, *F. psychrophilum* 19\_5, IoA\_469, IoA\_240, IoA\_350, *F. psychrophilum* 1947 (Fp. <sup>T</sup> 1947), *F. hydatidis* or *Y. ruckeri* were selected and their smears were treated with either mAb 9G7, mAb 32, anti-KHV mAb, anti-*F. psychrophilum* pAb 151 or normal rabbit serum. mAbs 9G7 and mAb 32 were chosen for this study because these mAbs showed the best production during cloning.

All the steps for IFAT were carried out in a humid chamber at RT (Hybaid, Wash Sleeve). A single colony of *F. psychrophilum* was spread in a very thin film on the surface of a microscope slide (VWR), a drop of chilled acetone was added on the smear and air dried for 5 min to fix the bacteria on the slide. To block endogenous peroxidase activity, the slides were covered with 3% H<sub>2</sub>O<sub>2</sub> in methanol (Fisher Scientific, UK) for 10 min. The slides were washed three times for 5 min in phosphate buffered saline (PBS) then blocked with 2% bovine serum albumin (BSA; Sigma-Aldrich, Germany) in PBS for 15 min. The BSA was tapped off and the slides were blocked with 15% goat serum (Sigma-Aldrich, Germany) for 15 min. The slides were carefully rinsed with PBS and placed in a PBS bath for a further 5 min. The slides were incubated for 1 h with either the mAb (1:200 in PBS), PBS (negative control) or an isotype matched mAb not expected to cross react (anti-koi herpesvirus). The slides were washed in PBS as above and incubated with anti-mouse IgG (whole molecule)–FITC antibody produced in goat (1:200 in PBS; Sigma-Aldrich, United States) for 30 min. The slides were washed three times in PBS for 5 min and stained with DAPI (Sigma-Aldrich, United States) for 10 min for nucleic acid staining. Slides were cover

slipped and stored in the dark at 4°C until they were examined for fluorescence under oil immersion with a fluorescence microscope (Olympus FL) at 100× objective lens.

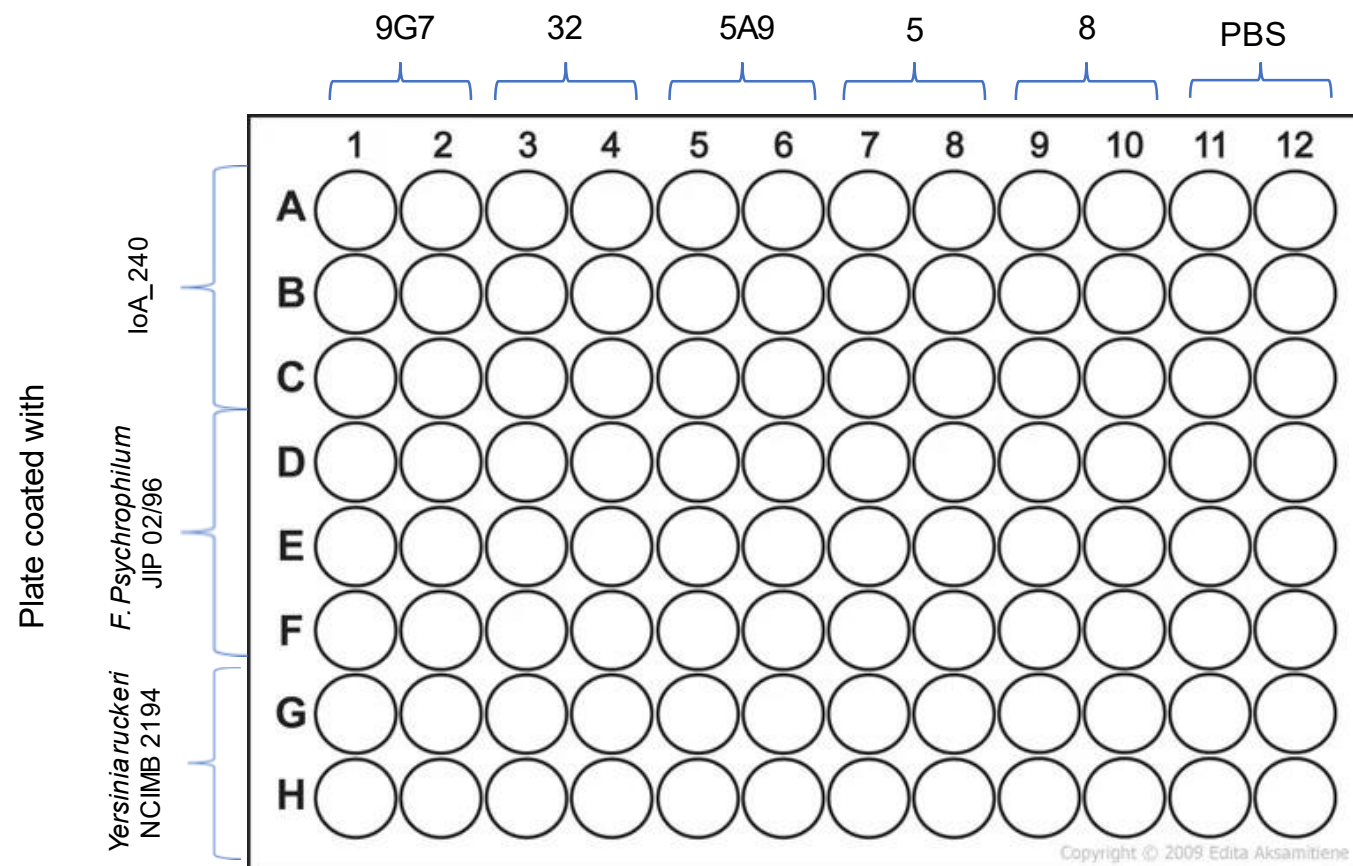
#### 4.2.4 Specificity of mAbs tested by direct ELISA

The five mAbs that were cloned for this study (9G7, 32, 5A9, 5 and 8) were tested by ELISA, to test their specificity and cross-reactivity, against different *F. psychrophilum* strains: two rainbow trout strains (*F. psychrophilum* 240, JIP 02/86), one isolate from Coho salmon (Fp<sup>T</sup> 1947) and six Atlantic salmon *F. psychrophilum* strains (*F. psychrophilum* 220, *F. psychrophilum* 235, *F. psychrophilum* 18\_S, *F. psychrophilum* 171/07, and *F. psychrophilum* 62). Moreover, the specificity of the mAbs was tested against other bacterial fish pathogens, namely *A. salmonicida* (NCIMB 1002 and MT 423), *Y. ruckeri* (NCIMB 2194) and *V. anguillarum* (Table 4.1).

For the ELISA, a 96-well ELISA plate (Fisher Scientific, UK) was coated with 50 µL of 0.05% poly-L-lysine (Sigma Aldrich, United States) in coating buffer (Sigma Aldrich, United States) and incubated for 1 h at room temperature (RT). The wells were washed three times with low-salt wash buffer (LSWB; 0.2 M Tris, 0.5 M NaCl and 0.5% Tween-20, pH 7.3) and 100 µL of each bacterium resuspended in PBS to OD<sub>525 nm</sub> = 1.0 (equivalent to ca. 1 ×10<sup>8</sup> CFU/mL) was added to each well and the plates incubated at 4°C overnight.

To fix the bacteria 50 µL 0.05% (v/v) glutaraldehyde (Sigma-Aldrich, United States) in PBS was added to each well and the plate incubated for 20 min at RT. The plate was washed three times with low-salt wash buffer (LSWB), blocked by adding 250 µL of 3% (w/v) milk powder (Marvel, Premier Foods Group Ltd, UK) in distilled water to each well, and the plate incubated for 2 h at RT. The plate was washed three times with LSBW and incubated with 100 µL/well of the different mAbs to test (neat supernatant of mAb: 9G7, 32, 5A9, 5, 8) or PBS (negative control) was added to the plate and incubated for 1 h at RT (Figure 4.1). The plate was washed five times with high-salt wash buffer (HSWB; 0.2 M Tris, 5 M NaCl; 10% Tween-20; pH 7.7) and at the final wash the plate was incubated for 5 min at RT. One hundred microlitres of goat anti-mouse IgG-HRP conjugate (diluted to 1:4000 in conjugate buffer: 1% BSA in LSBW; Sigma-Aldrich) was added to each well and the plate was incubated for 1 h at RT. Each well was washed with high-salt wash buffer (HSWB) as described above and 100 µL/ well of 42 mM 3,3' 5,5' tetramethylbenzidine (TMB) in substrate buffer (150

μL chromogen/ 15 μL substrate buffer (1:2 acetic acid in distilled water [dH<sub>2</sub>O]) was added. The reaction was stopped with 50 μL/well 2 M sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) (Fisher Scientific, UK) and the plates read with a spectrophotometric plate reader (Biotek Synergy HT, USA) at OD<sub>450 nm</sub>.



**Figure 4.1** Diagram used to show a representative ELISA assay plate layout. In this representative plate, the 96-well plate was coated with loA\_240, *F. psychrophilum* JIP 02/96 and *Y. ruckeri* NCIMB 2194. The plate was then incubated with 100  $\mu$ L/well of the mAb 9G7, 32, 5A9, 5, 8 and PBS (negative control) for 1 h at RT.

#### **4.2.5 1D SDS PAGE (1-DE) and silver staining of *F. psychrophilum* isolates**

*F. psychrophilum* IoA\_240, *F. psychrophilum* IoA\_350, Fp.<sup>T</sup> 1947, *F. hydatidis* NCIMB 2215, *F. aquatile* NCIMB 8694, *V. anguillarum* NCIMB 6, *A. salmonicida* NCIMB 1002 and *A. salmonicida* MT 423 were cultured as described in Section 4.2.1. The bacterial suspensions were centrifuged for 15 min at 3000 × *g*. Bacterial pellets were washed with PBS and adjusted to OD=1 at 525 nm (*F. psychrophilum* isolates) or 600 nm (other species). The solutions were centrifuged for 10 min at 8000 × *g*. The pellet was resuspended in 100 µL of 2 × sample buffer (250 mM Tris-HCl, 30% glycerol, 10% SDS, 0.5 M dithiothreitol, 0.2% bromophenol blue) and boiled for 15 min. Once cooled the samples were centrifuged at 300 × *g* for 10 min.

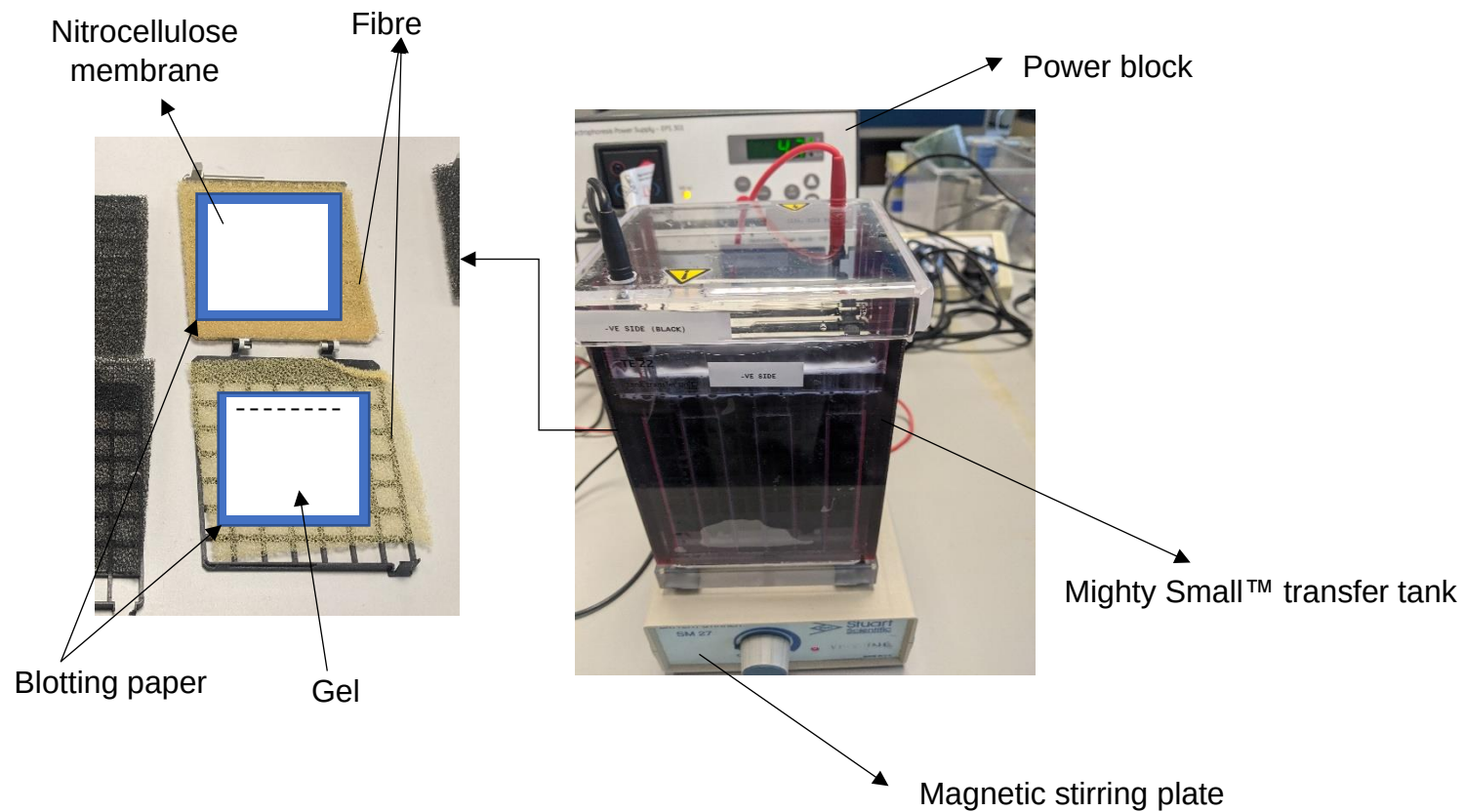
The supernatant was stored in a new Eppendorf tube and 10 µL of the samples were loaded onto 4-15% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad Laboratories, 2022). To the gel was added a 5-µL Precision Plus Protein Standard (Bio-Rad Laboratories, Inc). The gel was run at 130 V (power pack: Cambridge Scientific; Massachusetts) for 90 min and then silver stained using a ProteoSilver™ Silver Stain Kit according to the manufacturer's instructions (Sigma Aldrich, United States). The gels were run in duplicates for western blotting analysis.

#### **4.2.6 Western blot**

Electrophoresis was performed as described in Section 4.2.5. The separated proteins were transferred to a nitrocellulose membrane (Amersham™ Protran™, Merck KGaA, Germany). Filter paper (Whatman filter paper No.1, Camlab Ltd) was cut to the size of the protein gel (Section 4.2.5) soaked in transblot buffer (21.6 g/L glycine, 4.54 g/L Tris base, 100 mL dH<sub>2</sub>O and 200 mL 100% methanol; pH 8.3) before being assembled in the blotting apparatus (Mighty Small™ transfer tank; VWR). The protein gel/blot was run at 60 V for 35 min to blot the proteins from the gels onto the membranes on a magnetic stirring plate at ca. 360 rpm to keep the transfer buffers homogeneous (Stuart Equipment; UK)(Figure 4.2). The nitrocellulose membrane was removed, washed for 5 min in Tris Buffered Saline (TBS:10 mM Tris base, 0.5 M NaCl pH 7.5), and blocked in tris-buffered saline (TBS) with 5% (w/v) dried skimmed milk (Marvel, Premier Foods Group Ltd, UK) (50 mL/blot) for 1 h at 4°C.

The membrane was rinsed three times with tween-tris-buffered saline (TTBS) buffer (2.42 g/L Tris base; 58.48 g/L NaCl; pH 7.5, 0.05% [v/v] Tween-20) on a Gyro-Rocker (Stuart Scientific, UK) and incubated with 10 mL of neat mAb supernatant/membrane (mAbs 32, 9G7 or 5A9) or TBS (negative control) and incubated overnight at 4°C. The blot was washed three times in TTBS for 5 min each wash and incubated with anti-mouse-horseradish peroxidase (HRP; 1:200 in TBS; Sigma Aldrich: AP160P).

Then the blot was washed three times in TTBS as described above, followed by 1 min in TBS, and developed using a 4CN substrate kit (Vector, ImmPACTTM DAB). Once the bands had materialised (ca. 2 min), the reaction was stopped using dH<sub>2</sub>O (5 min) and left to dry. The blots were analysed using Amersham Imager 680 (GE Healthcare, United States) with the Fluorescence Trans-UV or Fluorescence Epi-RGB option.



**Figure 4.2** Diagram illustrating the western blotting apparatus used for this study and how to assemble the protein gel in the cast. For the purpose of the illustration the blotting paper, protein gel and nitrocellulose membrane are not to size, however blotting paper and nitrocellulose membrane need to be cut to the size of the protein gel during before being assembled in the blotting apparatus.

## 4.2.7 2D SDS-PAGE

### 4.2.7.1 Preparation of bacterial pellets for 2D gel electrophoresis

Two isolates (*F. psychrophilum* 240 and Fp<sup>T</sup> 1947), with different serotypes, previously investigated at the University of Stirling, Institute of Aquaculture (IoA) were selected for the present Chapter. These isolates were grown on MVB for 48-72 h at 15°C (150 rpm) as described in Section 4.2.1. Extraction of whole-cell proteins from these two isolates were performed according to Pang et al. (2010). In brief, cells were harvested by centrifugation at 5000 × g at 4°C for 10 min and washed twice in ice-cold wash buffer (10 mmol/L Tris–HCl, pH 8.0, 5 mmol/L magnesium acetate) and resuspended in lysis buffer containing 7 mol/L urea, 2 mol/L thiourea, 1% IPG (immobilized pH gradient strips) buffer (pH 4–7), 4% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 1% DTT, with 1% protease inhibitor mix (GE Healthcare, Uppsala, Sweden), and 1% nuclease mix (GE Healthcare) just before use. The cell suspensions were incubated on ice for 2 h, and insoluble cellular debris removed by centrifugation at 15,000 × g at 4°C for 1 h. The supernatants were stored in aliquots at –70°C until use.

### 4.2.7.2 Protein quantification and clean up

The protein samples were quantified using a 2-D Quant Kit following the manufacturer's instructions (GE Healthcare, United States). Briefly, a standard curve (prepared with 10-fold dilutions from 0 µg to 50 µg) was prepared using 2 mg/mL bovine serum albumin. To quantify the samples, 10 µL of each sample was placed in separate Eppendorf tubes, 500 µL precipitation solution was added to each sample and the standard curve, before tubes were vortexed (30 s) and incubated for 3 min at RT. Co-precipitation solution (500 µL) was added to the tubes and the contents mixed briefly by inversion. The tubes were centrifuged twice at 10,000 × g for 5 min and the supernatants discarded. A solution of copper (100 µL) and dH<sub>2</sub>O (400 µL) was added to each tube and then the contents vortexed (15 s) to dissolve the precipitated protein. One millilitre of working colour reagent was added to each tube, and mixed by inversion, before incubating for 15 min at RT. Three hundred microliters of each sample and standard curve samples were placed individually in a 24-well plate reader (VWR) and the absorbance read at 480 nm using water as reference. Each protein sample was then cleaned using a Clean-up kit (GE Healthcare, United States)

following the manufacturer's instructions with slight modification. Briefly, 100 µg of protein (40 µL) sample was transferred to a microcentrifuge tube and 300 µL of precipitate solution were added to the tubes, mixed by inversion, and incubated on ice for 15 min. Then, 300 µL of co-precipitant were added to the mixture, mixed by vortexing (15 s) and centrifuged twice at  $12,000 \times g$  for 5 min and the supernatant was discarded. Without disturbing the pellet, 40 µL of co-precipitant was added on top of the pellet and incubated on ice for 5 min. The wash was removed carefully with a pipetted and 25 µL of dH<sub>2</sub>O was added to the pellet and vortexed (10 s). Then 1 mL of cold wash buffer and 5 µL of wash additive were added and the tubes vortexed until the pellet had dissolved. The tubes were incubated at -20°C for 30 min the centrifuged at  $12,000 \times g$  for 5 min and the pellet was air dried (< 5 min). The proteins were dissolved in 500 µL of lysis buffer, 5 µL of protein inhibitor and 5 µL of nuclease inhibitor.

The pellet was resuspended in rehydration buffer (7 M Urea, 2 M Thiourea, 2% (w/v) CHAPS, 0.002% (v/v) or 1% bromophenol blue (Abcam, United States), 40 mM dithiothreitol (DDT; Sigma, UK) containing 0.5% (v/v) IPG buffer pH 4-7 (GE Healthcare, United States) and centrifuged at  $12,000 \times g$  for 1 min and 100 µL aliquots of the protein solution were stored at -70°C until further use.

#### **4.2.7.3 2D SDS PAGE (2-DE)**

Bacterial protein solutions were thawed on ice and mixed with a vortex for 1 min. Sixty micrograms of protein was passively rehydrated on Immobilized pH Gradient Strips (IPG strips) (7 cm, pH 4-7; Bio-Rad, United States) in an IPG Box (GE Healthcare, United States) for 19-20 h at RT. The first-dimensional isoelectric focusing (IEF) and second-dimensional SDS-gel electrophoresis were performed according to the Amersham Biosciences manual (GE Healthcare, 2010).

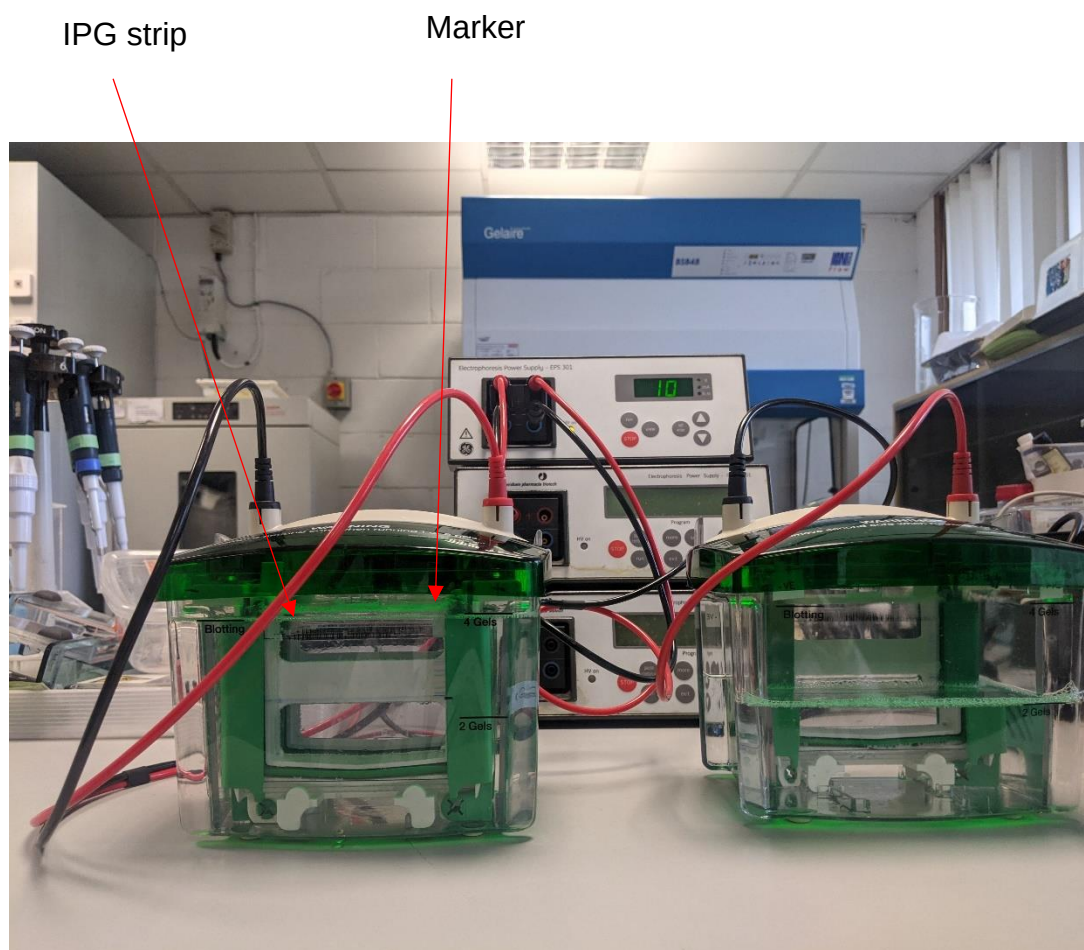
Separation of protein in the first-dimension was performed using an IPGphor Ettan isoelectric focusing system (Amersham, Pharmacia, Biotech, United States) in a 7-cm Ettan IPGphor cup-loading manifold (GE, Healthcare Biosciences). The manifold was filled with 1.2 mL of Immobiline drystrip cover fluid (ThermoScientific, UK) and then closed with a coverslip. The IPG strips were run at 20°C at an incremental program consisting of 300 V for 30 min, 1000 V for 30 min, 5000 V for 90 min and 5000 V for 25 min, with a constant current of 40-50 mA/strip. After the programme, the strips were

washed carefully with dH<sub>2</sub>O and equilibrated in a two-step- equilibration protocol (denaturation and alkylation) on a rocking platform (Stuart Scientific, Gyro Rocker) at 10 rpm for 15 min per step as follows. First, the strips were equilibrated in equilibration buffer I (6 M urea, 75mM Tris-HCl pH 8.8, 29.3 % (v/v) glycerol, 2% (w/v) SDS, 0.002% bromophenol blue, 1% (w/v) DDT) following by a washing step in dH<sub>2</sub>O. For the third step, the strip was equilibrated in equilibration buffer II (6 M urea, 75mM Tris-HCl pH 8.8, 29.3 % (v/v) glycerol, 2% (w/v) SDS, 0.002% bromophenol blue, 2.5% (w/v) DDT) and then the strip was washed with dH<sub>2</sub>O.

The second-dimension protein separation was done using a 12% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, United States). The gels were assembled in the same electrophoresis system used in the 1D PAGE step, then the IPG strips were inserted on top of the gel and sealed with 100 µL of warm agarose sealing solution (25 mm Tris base, 192 mM glycine, 0.1% SDS, 0.5% agarose, 0.002% bromophenol blue). A filter paper soaked with 5 µL of precision protein dual colour molecular weight standard (Bio-Rad, United States) was added to the anodic (positive) end of the strip. An extra 100 µL of warm agarose sealing solution was added to fix the strip and the filter paper with the marker attached to the gel. The gels were run at 100 V for 90 min (Figure 4.3). The gels were then fixed for 15 min in 100 mL fixative solution (40 mL 100% ethanol + 10 mL glacial acetic acid + 50 mL dH<sub>2</sub>O), then rinsed three times, 1 min/wash.

Finally, the gels were stained overnight with 30 mL Bio-Safe™ Coomassie Stain (Bio-Rad, United States). The next day the gels were de-stained for 3 h with dH<sub>2</sub>O and then stored in 0.02% sodium azide (Sigma-Aldrich, United States) at 4°C. Western blot was performed in the duplicate gel as described in Section 4.2.6.

The gel images were analysed using Amersham Imager 680 (GE Healthcare, United States) with the Fluorescence Trans-UV or Fluorescence Epi-RGB option and the spots were analysed with a naked eye from a single observer.



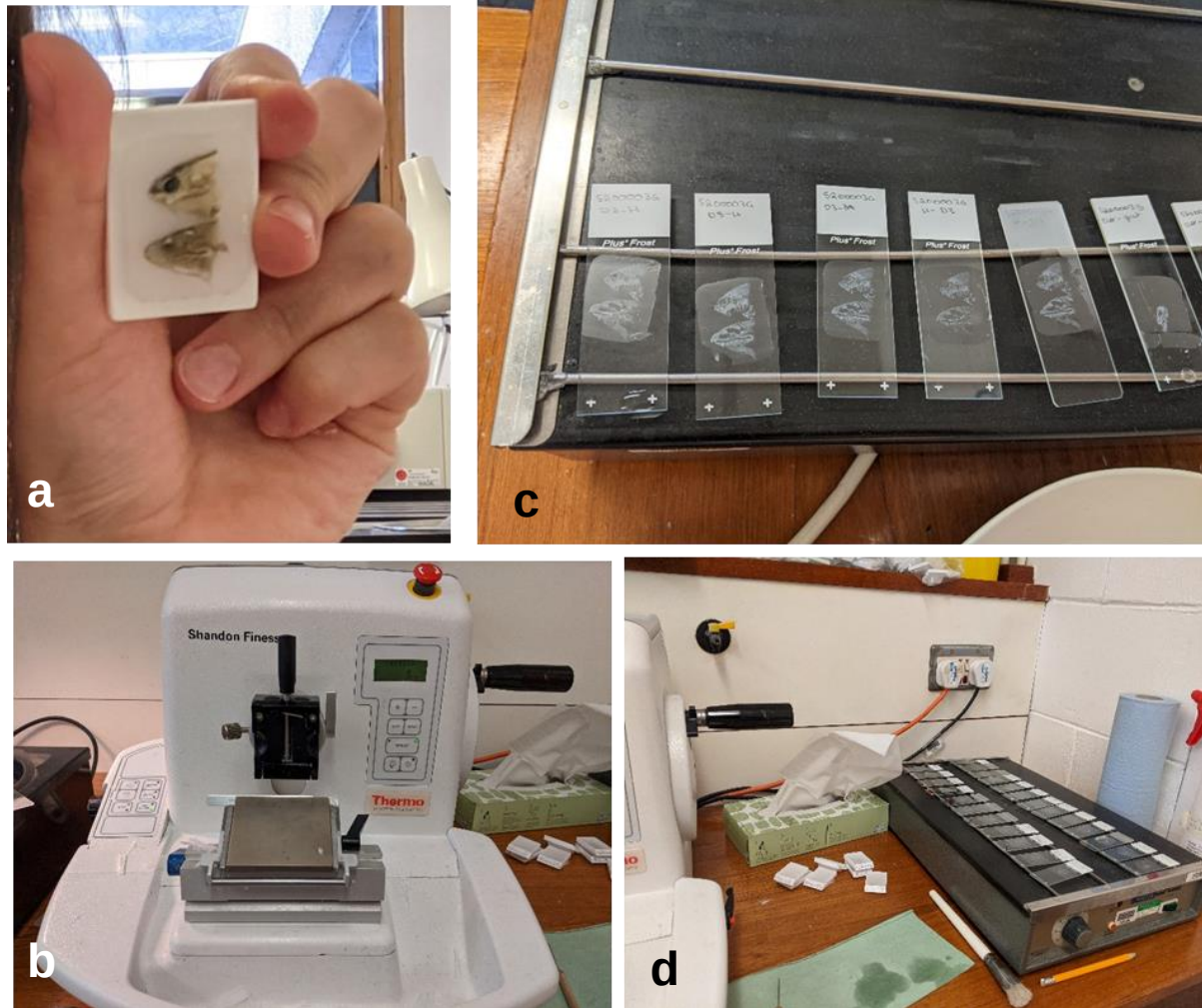
**Figure 4.3** Image illustrating the 2D SDS PAGE set up. The IPG strips were inserted on top of the gel, and a precision protein dual colour molecular weight standard (marker) was added to the anodic end of the strip and finally sealed with warm agarose sealing solution. The gels were run at 100 V for 90 min.

#### 4.2.8 Histopathological processing

Following the experimental challenge described in Chapter 3.2.4, in which Atlantic salmon and rainbow trout fry were challenged with *F. psychrophilum* 6\_S and *F. psychrophilum* 19\_5 respectively, fish (n=10/day post challenge [dpc]) were sampled at random from the tanks with a sampling net at 0, 3, 6 and 10 dpc and euthanised with an overdose of benzocaine chloride (Sykehusapoteket Oslo, Ullevål, Norway). In the Atlantic salmon challenge, fish sampled at 0 dpc are control fish, while for rainbow trout, due to a technical issue, the fish sampled at 0 dpc were sampled 1.5 h after the fish was challenged with *F. psychrophilum* 19\_5.

Briefly, the abdominal cavity was opened aseptically to expose the organs and fixed in 10% neutral buffered formalin (Sigma Aldrich, United States) for subsequent histological processing. For histology, the tissues were secured in individual cassettes before being processed overnight in the Shandon Citadel 2000 tissue processor (ThermoFisher Scientific, United States). The tissues were fixed in formalin and then dehydrated through a series of graded alcohol washes (alcohol 70%, 90%, 100%), xylene baths and paraffin wax at 60°C (20 h) following the manufacturer's instructions (Thermo Fisher Scientific, 2009). At the end of the programme, the tissues were embedded in paraffin wax in moulds before being left to set on a cold plate (Ra Lamb, Hilditch Group) until hardened.

The blocks were trimmed using a microtome (Shandon Finess, Thermo ELECTRON CORPORATION) set to a thickness of 20 µm until the whole tissue was exposed (Figure 4.4. [b]). Once trimmed, the blocks were left to soak in dH<sub>2</sub>O for 30 min and then placed face down on the cold plate for 5 min prior to sectioning. Sections were cut using the microtome set to a thickness of 5 µm and floated onto microscope slides (Figure 4.4 [c]) (Solmedia Ltd) before drying at 60°C on a heated slides dryer (Akribis Scientific Limited) (Figure 4.4[d]) and then overnight at 60°C (Windsor Incubator, Akribis Scientific Limited).



**Figure 4.4** Histopathology processing. Figure 4.4 (a) tissue embedded using paraffin wax; (b) microtome used for sectioning the paraffin blocks (c); heated slide dryer used to dry the sectioned tissues (i.e., representative image of head tissues) on positive charged slides for antigen retrieval analysis.

#### **4.2.9 Detection of *F. psychrophilum* in Atlantic salmon and rainbow trout tissues by immunohistochemistry using anti-*F.***

##### ***psychrophilum* mAbs**

Three Atlantic salmon and rainbow trout per time point (0, 3, 6 and 10 dpc) were randomly selected for the detection of *F. psychrophilum* using mAbs in tissues (gut, gill, head kidney and skin). As a positive control, a section from a skin lesion of a rainbow trout known to be positive for *F. psychrophilum* was used. Tissue sections were dewaxed, dehydrated (two successive xylene baths of 5 min each, followed by immersed in 100% ethanol for 5 min and 70% ethanol for 3 min) and incubated with 3% hydrogen peroxide in methanol for 10 min at 22°C to block endogenous peroxidases. Slides were washed three times with TBST and then submerged in 350 mL of retrieval buffer (tri-sodium citrate, pH 8.5) before microwaving at 900 W for 2 min to provide access for the antibody to any target proteins within each tissue sample.

Non-specific binding sites were blocked with 2% BSA (Fisher BioReagents, Germany) in TBS for 15 min followed by normal goat serum (Sigma-Aldrich, United States) diluted 1:10 in TBS for 15 min at RT. To detect *F. psychrophilum* antigens, 50–100 µL of neat mAb 9G7 or mAb 32 was placed onto each tissue section, or TBS was added to the tissue section (negative control) and incubated for 2 h at room temperature in a humid chamber. Slides were washed three times with TBST and then incubated with anti-mouse-HRP (1:200; Sigma-Aldrich, United States - 12-349) for 1 h, and washed again.

To visualise any reaction, slides were incubated for 10 min with DAB solution (10x concentrated; Sigma-Aldrich, United States). To stop the reaction, slides were immersed in tap water, followed by counterstaining in Mayer's haematoxylin 'Z' stain (CellPath, United Kingdom) for 5 min, and then rinsed in running tap water until the water ran clear. Then the slides were rehydrated in 70% ethanol for 3 min, 100% ethanol for 5 min, and twice in xylene for 5 min. Once rehydrated the slides were mounted using Pertex mounting medium (Histo-Line Laboratories, Italy) and a cover slip was added to allow microscopic examination.

#### **4.2.10 Detection of *F. psychrophilum* in tissues by immunohistochemistry with pAb 151**

Three Atlantic salmon and rainbow trout per time point (0, 3, 6 and 10 dpc) were randomly selected for the detection of *F. psychrophilum* using pAb 151 in the different tissues. Tissue sections were dewaxed, dehydrated and blocked with hydrogen peroxide as described in section 4.3.9. Slides were then washed three times with TBS and non-specific binding sites blocked with 2% BSA (Fisher BioReagents, Germany) in TBS for 15 min followed by normal goat serum (Sigma-Aldrich, United States) diluted 1:10 in TBS for 15 min at RT. For the detection of *F. psychrophilum* antigen, 50–100 µL of anti-*F. psychrophilum* polyclonal antibody (1:200) (Ngo et al., 2018) was placed onto the tissue sections and incubated for 2 h at RT in a humid chamber. Slides were washed as above and incubated with anti-rabbit-HRP (1:200) for 1 h. Once again, slides were then washed three times with TBS.

To visualise the reaction, slides were incubated for 10 min with DAB solution (Sigma-Aldrich, United States). To stop the reaction the slides were immersed in tap water, followed by counterstaining in Mayer's haematoxylin 'Z' stain (CellPath, United Kingdom) for 5 min and then held under running tap water until the water ran clear. The slides were then rehydrated in 70% ethanol for 3 min, 100% ethanol for 5 min and twice in xylene for 5 min.

Once rehydrated the slides were mounted using Pertex mounting medium (Histo-Line Laboratories, Italy) and cover slipped. The slides were observed under a light microscope (Olympus LS) and the pAb staining was assessed by a single researcher (Valeria Macchia) and blind checked by another researcher (Dr Rowena Hoare).

#### **4.2.11 Immunohistochemistry for detection of IgT positive cells in tissue**

Three fish for each species for each sampling point were randomly selected for the detection of IgT positive cells in tissues by IHC. This protocol was performed according to section 4.2.9. For the IgT positive cells detection, 50–100 µL of trout anti-IgT monoclonal antibody (1:15) (ADL Ltd.) was applied to the tissue sections in a humid chamber and incubated overnight at 4°C. For the negative controls, slides were incubated with TBS or an irrelevant antibody (koi herpesvirus; ADL Ltd.).

The positive control was a section from a skin lesion of a rainbow trout known to be positive for *F. psychrophilum*. Then the tissues were incubated with anti-mouse-HRP (Agilent Technologies, United States) for 30 min at RT. The slides were visualised with AEC-Plus Substrate-Substrate (Agilent Technologies, United States) and immersed in tap water to stop the reaction.

The slides were counterstained in Mayer's haematoxylin 'Z' stain (CellPath, United Kingdom) for 4min and then rehydrated as described in Section 4.2.9. Lastly, the slides were mounted with Aquamount (Vector Laboratories, Inc), cover slipped and stored in the dark.

#### **4.2.12 Ethical approval**

All experimental procedures with live fish were carried out in accordance with the Norwegian Food Safety Authority guidelines and were approved by the Animal Welfare and Ethical Review Body of the University of Stirling, UK.

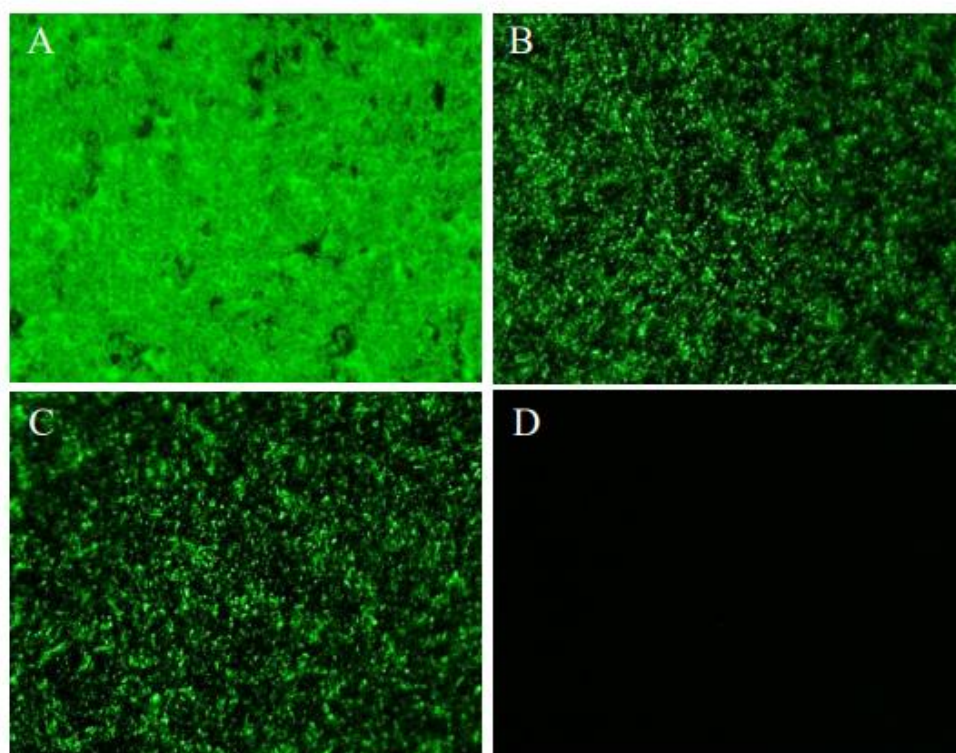
## 4.3 Results

### 4.3.1 Cloning of mAbs

The anti- *F. psychrophilum* mAbs used in this study were produced against isolate *F. psychrophilum* B97026PI and isolate *F. psychrophilum* 32/97 by Faruk et al. (2002). For the purpose of this study, five mAbs (9G7, 32, 5A9, 5 and 8) were successfully cloned and characterised by serological, immunohistochemical and proteomic methods as described in the next sections.

### 4.3.2 Characterisation of anti-*F. psychrophilum* mAbs by IFAT

A single colony of *F. psychrophilum* 6\_S, *F. psychrophilum* 19-5, IoA\_469, IoA\_240, IoA\_350, Fp.<sup>T</sup> 1947, *F. hydatidis* or *Y. ruckeri* were selected and their smears were treated with either mAb 9G7, mAb 32, anti-KHV mAb, PBS (negative control), anti-*F. psychrophilum* pAb 151 (positive control) or normal rabbit serum (serum control) and stained using anti-mouse or anti-rabbit FITC (Sigma Aldrich, United States- AP106F). When the smear was stained with pAb 151, an extensive fluorescent stain was observed across the bacterial smear as illustrated in Figure 4.5 (A), indicating the presence of *F. psychrophilum* cells. When treated with the mAbs, a specific fluorescent signal was observed as shown in Figure 4.5 (B) and Figure 4.5 (C), while no signal was detected when the smear was treated with anti-KHV mAb illustrated in Figure 4.5 (D)

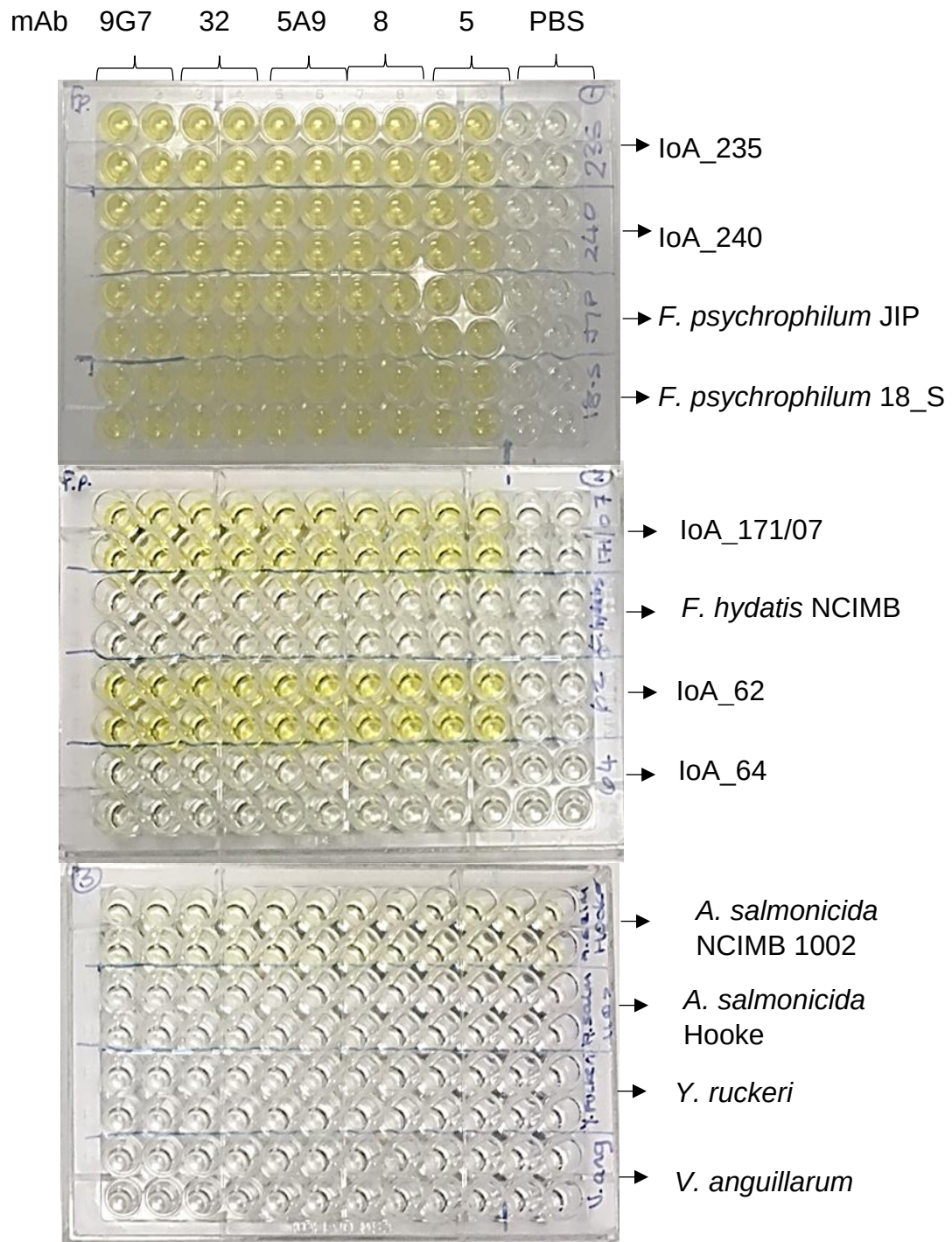


**Figure 4.5** Representative image of characterisation of anti-*F. psychrophilum* mAbs and pAbs on isolate *F. psychrophilum* 240 by IFAT. The bacterial smear was incubated with (A) anti-*F. psychrophilum* pAb 151 (B) mAb 9G7, (C) mAb 32 and (D) anti-KHV mAb (negative control).

### 4.3.3 Specificity of mAbs tested by direct ELISA

Five mAbs (9G7, 32, 5A9, 5 and 8) were tested by ELISA against different *F. psychrophilum* strains, two rainbow trout isolates (IoA\_240 and *F. psychrophilum* JIP 02/86), one isolate from Coho salmon (Fp.<sup>T</sup> 1947) and six Atlantic salmon *F. psychrophilum* strain (IoA\_220, IoA\_235, *F. psychrophilum* 18\_S, IoA\_171/07, IoA\_62).

To confirm that the mAbs did not cross-react with other bacteria, the mAbs were tested against non-*F. psychrophilum* isolates, including *F. hydatidis* NCIMB 2215, *A. salmonicida* (Hooke- As.H), *A. salmonicida* NCIBM 1102- (As), *Y. ruckeri* NCIBM 2194- (Yr) and *V. anguillarum* (Va). The mAbs did not react with all *F. psychrophilum* isolates examined by ELISA, and they appeared to differ in their patterns of reactivity. mAb 9G7 and mAb 32 reacted with all the *F. psychrophilum* isolates with the exception of Fp.<sup>T</sup> 1947; mAb 5A9 only reacted with *F. psychrophilum* IoA\_240; and finally mAb 5 and mAb 8 reacted *F. psychrophilum* IoA\_240, IoA\_220 and Fp. <sup>T</sup> 1947 (Table 4.2). With the exception of *A. salmonicida* NCIBM 1102 in which a weak reaction was observed, all the other bacterial genera did not react with any of the anti-*F. psychrophilum* mAbs (Figure 4.6).



**Figure 4.6** Representative image of characterisation of anti-*F. psychrophilum* mAbs (9G7, 32, 5A9, 8 and 5) by ELISA. mAbs specificity was tested in duplicates on different *F. psychrophilum* isolates (n=7), *F. hydatidis* (n=1), *A. salmonicida* isolates (n=2), *Y. ruckeri* (n=1) and *V. anguillarum* (n=1).

**Table 4.2** Characterisation of anti-*F. psychrophilum* mAbs (9G7, 32, 5A9, 8 and 5) by ELISA. mAbs specificity was tested in duplicates on different *F. psychrophilum* isolates (n=7), *F. hydatidis* (n=1), *A. salmonicida* isolates (n=2), *Y. ruckeri* (n=1), *V. anguillarum* (n=1) and PBS. The ELISA plates were read with a spectrophotometer and the cut-off point was determined to be 3 × average negative control (PBS, OD: 0.364). OD >0.7-1.0 (++) . OD > 0.4- 0.6 (+). No reactivity=negative reactions (-).

| mAb | Bacterial Strains       |                                      |            |         |         |                                 |            |        |                                   |                                 |                            |                                 |                                  |
|-----|-------------------------|--------------------------------------|------------|---------|---------|---------------------------------|------------|--------|-----------------------------------|---------------------------------|----------------------------|---------------------------------|----------------------------------|
|     | <i>F. psychrophilum</i> |                                      |            |         |         |                                 |            | Others |                                   |                                 |                            |                                 |                                  |
|     | IoA_240                 | <i>F. psychrophilum</i><br>JIP 02/86 | Fp. T 1947 | IoA_220 | IoA_235 | <i>F. psychrophilum</i><br>18_S | IoA_171/70 | IoA_62 | <i>F. hydatidis</i><br>NCIMB 2215 | <i>A. salmonicida</i> MT<br>423 | <i>A. salmonicida</i> 1102 | <i>Y. ruckeri</i><br>NCIMB 2194 | <i>V. anguillarum</i><br>NCIMB 6 |
| 9G7 | ++                      | ++                                   | -          | +       | ++      | ++                              | ++         | ++     | -                                 | -                               | -                          | -                               | -                                |
| 5A9 | ++                      | Nd                                   | -          | -       | -       | -                               | nd         | nd     | -                                 | -                               | -                          | -                               | -                                |
| 32  | ++                      | ++                                   | -          | +       | ++      | ++                              | ++         | ++     | -                                 | -                               | -                          | -                               | -                                |
| 8   | +                       | Nd                                   | +          | ++      | -       | -                               | nd         | nd     | -                                 | -                               | -                          | -                               | -                                |
| 5   | ++                      | Nd                                   | ++         | ++      | -       | -                               | nd         | nd     | -                                 | -                               | -                          | -                               | -                                |

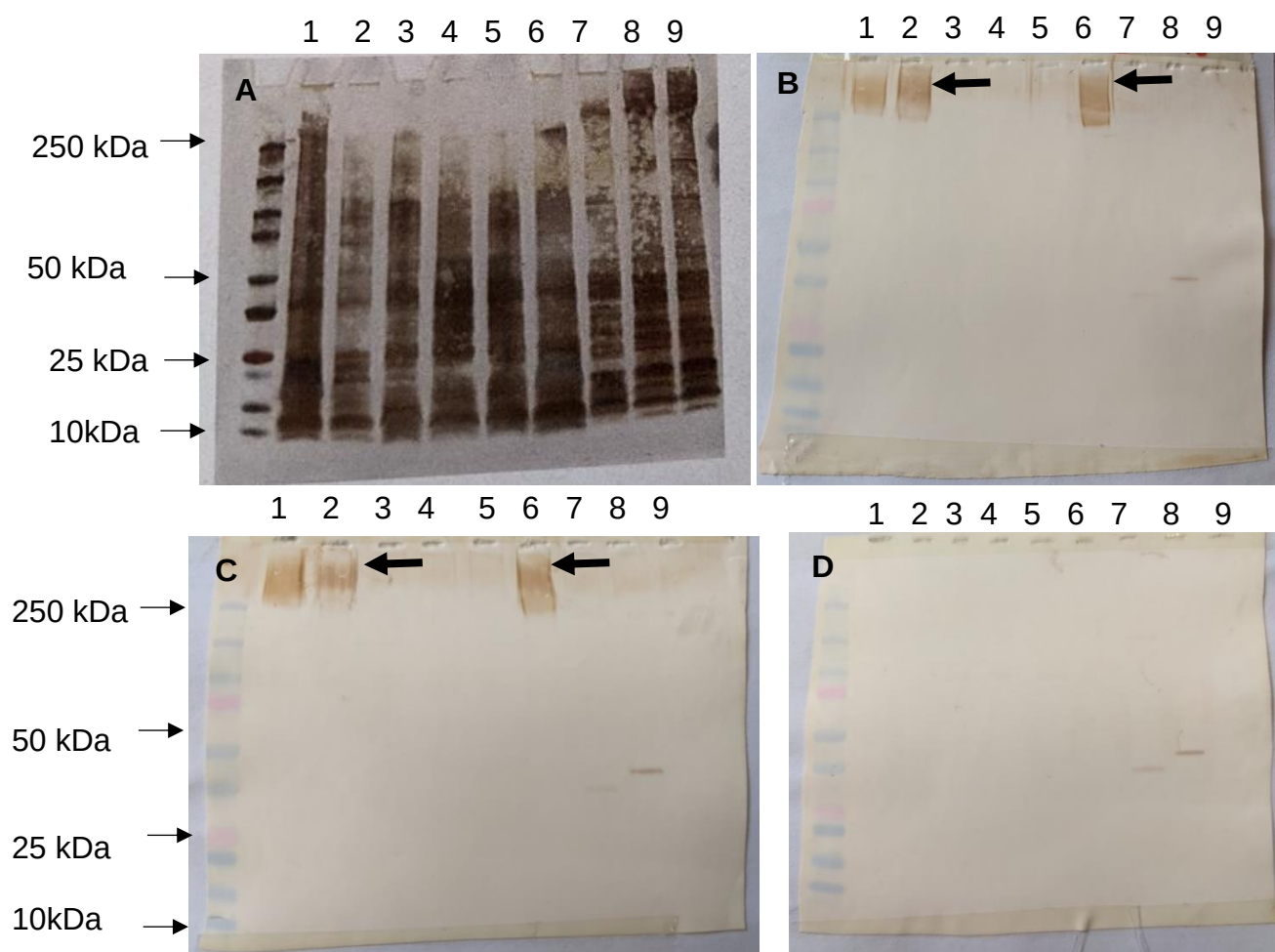
#### **4.3.4 1D SDS PAGE (1-DE), silver staining and western blotting of *F. psychrophilum* isolates**

Whole cell lysates of strains *F. psychrophilum* IoA\_240, IoA\_350, Fp. <sup>T</sup> 1947 and *F. hydatis*, *F. aquatile*, *F. psychrophilum* 18\_S, *V. anguillarum*, *A. salmonicida* NCIMB 1002 and *A. salmonicida* MT 423, were treated with proteinase K were analysed by SDS PAGE, silver stained and western blotted.

By western blot a dominant smear at > 250 kDa on the gels was observed when testing mAb 9G7 and mAb 32 on *F. psychrophilum* isolates IoA\_240, IoA\_350 and *F. psychrophilum* 18\_S (Table 4.3).

There was no cross reaction of mAb 9G7 or 32 with *F. hydatis*, *F. aquatile*, *V. anguillarum*, and *A. salmonicida* isolates, nor any reaction in the blot incubated with TBS (negative control) at > 250 kDa. However there seem to be two non-specific bands at ca. 37 kDa on *A. salmonicida* isolates when incubated with mAb 9G7 and 32, and TBS (Figure 4.7)

Overall, the blots indicate that both mAb 9G7 and mAb 32 recognised *F. psychrophilum* 240, 350 and 18\_S and the arrows indicate high intensity bands at > 250 kDa. However, the mAbs did not recognise Fp. T 1947 and the other bacterial genera, and the blot incubated with TBS did not show any reaction.



**Figure 4.7** 1D-SDS PAGE and western Blotting was performed on different strains of *F. psychrophilum* isolates. (A) Whole cell lysate treated with proteinase K of the following bacterial strains IoA\_240 (lane 1), IoA\_350 (lane 2), Fp.<sup>T</sup> 1947 (lane 3) and *F. hydatis* (lane 4), *F. aquatile* (lane 5), *F. psychrophilum* 18\_S (lane 6), *V. anguillarum* (lane 7), *A. salmonicida* (NCIMB 1002) (lane 8) and *A. salmonicida* (MT 423)(lane 9) were separated by SDS-PAGE and stained with silver stain. Western blot was performed following SDS-PAGE, the gels were blotted onto nitrocellulose membranes and incubated with anti-*F. psychrophilum* monoclonal antibodies (B) mAb 9G7, (C) mAb 32 and (D) TBS (negative control blot).

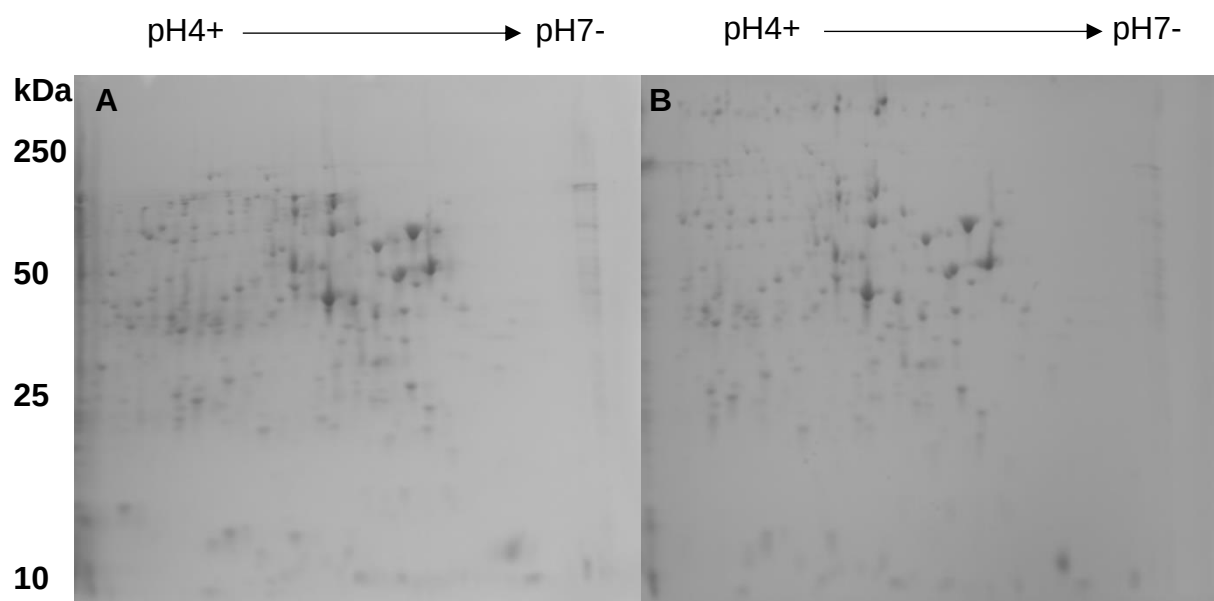
**Table 4.3** Western blotting of the different bacterial species tested by 1D SDS PAGE to determine reactivity of mAb 9G7 and mAb 32 with different bacterial species and isolates to investigate cross reactivity with non-*F. psychrophilum* species. The staining intensity to the band at >250 kDa was determined by a single observer: (+++) very strong band, (++) strong band, (+) weak band, (-) no reaction.

| Lane | Sample                           | Staining with<br>mAb 32 | Staining with<br>mAb 9G7 |
|------|----------------------------------|-------------------------|--------------------------|
| 1    | Marker (10-250 kDa)              |                         |                          |
| 2    | IoA_240                          | +++                     | +++                      |
| 3    | IoA_350                          | ++                      | +++                      |
| 4    | Fp. <sup>T</sup> 1947            | -                       | -                        |
| 5    | <i>F. hydatidis</i> NCIMB 2215   | -                       | -                        |
| 6    | <i>F. aquatile</i> NCIMB 8694    | -                       | -                        |
| 7    | <i>F. psychrophilum</i> 18_S     | +++                     | +++                      |
| 8    | <i>V. anquillarum</i>            | -                       | -                        |
| 9    | <i>A. salmonicida</i> NCIMB 1002 | -                       | -                        |
| 10   | <i>A. salmonicida</i> MT 423     | -                       | -                        |

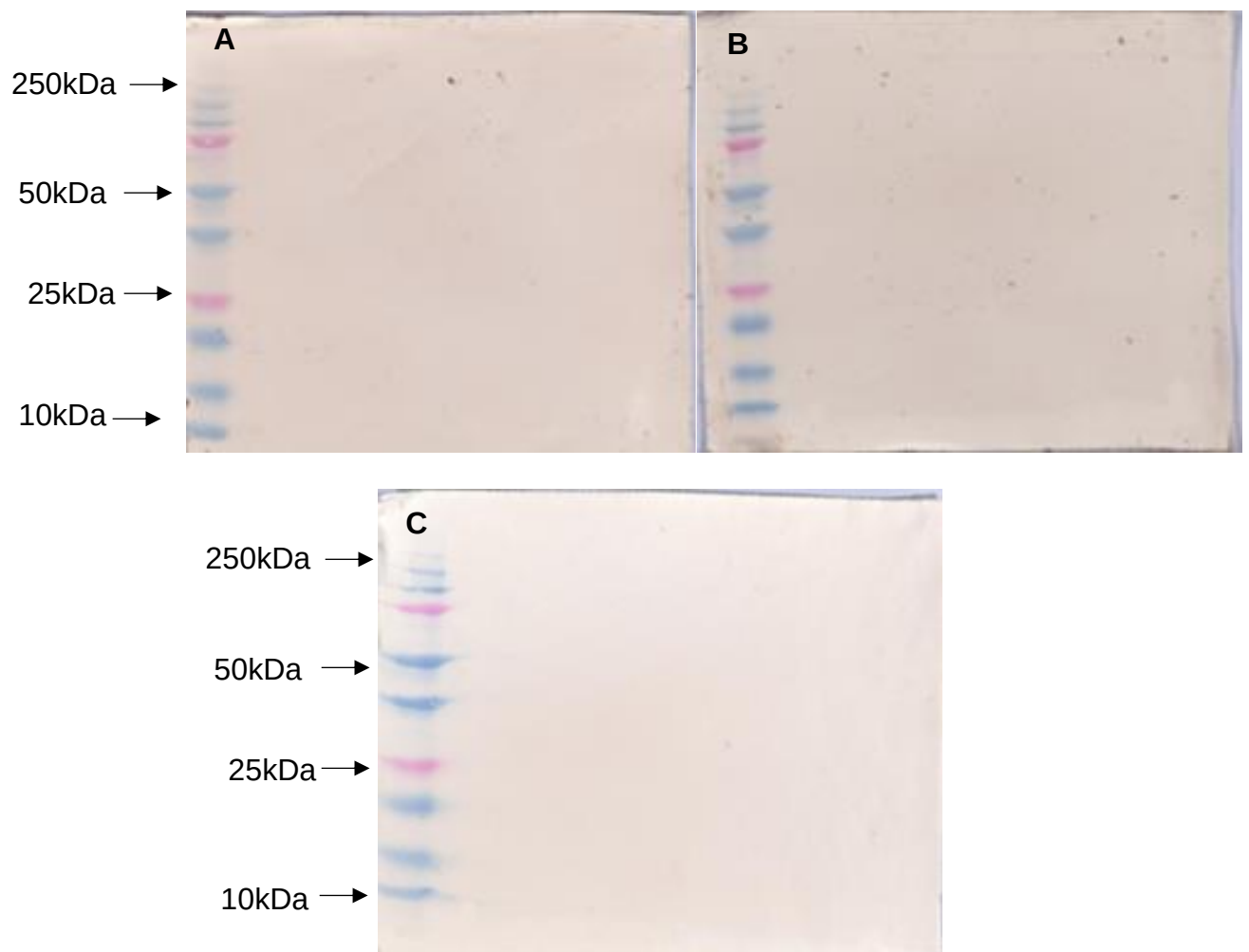
#### **4.3.5 2D SDS PAGE, silver staining and western blotting of *F. psychrophilum* isolates**

Whole cell proteins were extracted from *F. psychrophilum* IoA\_240 and the reference Fp. <sup>T</sup> 1947, and 2D gel electrophoresis was used to analyse the isolates proteins (Figure 4.8). High similarity in distribution of the protein spots were observed by eye between replicate gels as well as similar distribution of spot between the two different *F. psychrophilum* isolates. The gels were transferred into a western blot and incubated with either mAb 9G7, mAb 32 or TBS (negative control).

No bands were visualised in any of the immunoblots meaning that neither mAb 9G7 or mAb 32 recognised the protein expressed in the 2D gels. The spots that can be observed in the immunoblots (Figure 4.9) are carry over of the 2D gel electrophoresis as they do not correspond to any of the spots observed in the 2D gels (Figure 4.8).



**Figure 4.8** Representative 2D gels electrophoresis of the protein profile of the whole cell lysate of two *F. psychrophilum* isolates (A) IoA\_240 and (B) Fp. <sup>T</sup> 1947 (60 µg protein). Duplicate gels were run for each treatment group as technical replicates. The gels were stained with Bio-Safe™ Coomassie Stain and the marker used was Precision Plus Protein Dual Colour Molecular Weight Standard Marker (10-250 kDa).



**Figure 4.9** Western blot analysis of *F. psychrophilum* strains. Representative 2D Western blot gels of *F. psychrophilum* isolates 240 separated by 2D SDS-PAGE and then blotted onto nitrocellulose and reacted with mAb 9G7 (A) and mAb 32 (B) and TBS- negative control (C). These western blots were performed in duplicates. Precision Plus Protein Dual Color Molecular Weight Standard Marker (10-250 kDa) was used.

#### **4.3.6 Detection of *F. psychrophilum* in Atlantic salmon and rainbow trout tissues by IFAT**

Atlantic salmon and rainbow trout fry tissues (n=3/time point) were incubated with anti-*F. psychrophilum* mAb 9G7, mAb 32, anti- KHV mAb (irrelevant control) or TBS (negative control) and stained in DAPI. A smear of *F. psychrophilum* 18\_S (positive control) reacted with anti-*F. psychrophilum* mAb 9G7, mAb 32 giving a strong fluorescent signal, however the smear did not react with anti- KHV mAb.

The positive control slide, an authenticated skin lesion of *F. psychrophilum* infected rainbow trout (Hoare et al., 2017) and the Atlantic salmon and rainbow trout tissues did not react with any of the mAbs and did not react with the anti-KHV mAb (irrelevant control).

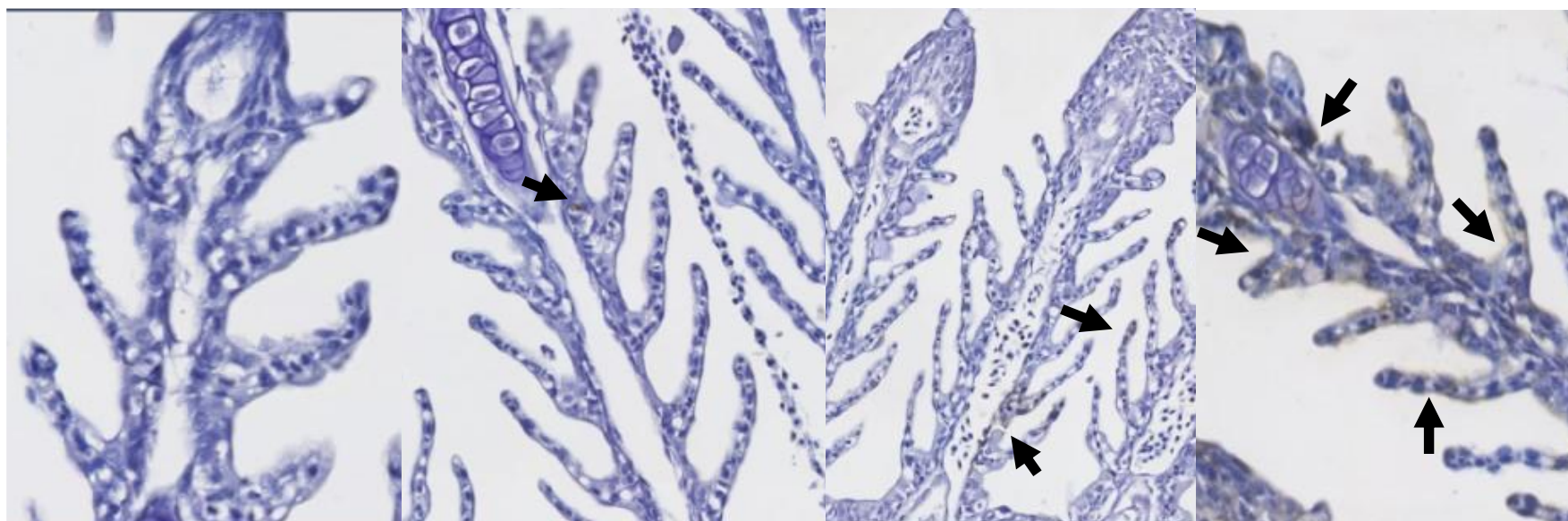
From these results, it can be seen that mAb 9G7, mAb 32 do not work in IHC to detect *F. psychrophilum* in infected tissues. When staining the same slide with the anti- *F. psychrophilum* pAb 151, extensive positive staining was observed in the epithelial layer of the skin, suggesting the presence of *F. psychrophilum* cells. The positive control section showed no staining when incubated with the irrelevant anti-KHV mAb.

#### **4.3.7 Immunohistochemistry for detection of *F. psychrophilum* in Atlantic salmon and rainbow trout tissues**

Atlantic salmon and rainbow trout tissues including gut, gill, head kidney and skin, sampled at the different time points, were also tested by IHC using the anti-*F. psychrophilum* pAb 151 to determine the presence of *F. psychrophilum* cells. The slides were then analysed under the microscope and the infection in the tissues was scored 0-3, shown for gills in Figure 4.10.

In Atlantic salmon, staining by IHC for *F. psychrophilum* was observed in the skin epithelium from 3 dpc around the mouth, and *F. psychrophilum* cells were detected around the eyes at 6 dpc and 10 dpc (4.11). Positive staining for *F. psychrophilum* was observed from 3 dpc on the gills and gut tissues of Atlantic salmon (Figure 4.12) and rainbow trout (Figure 4.13). Positive staining for *F. psychrophilum* was seen in the head kidney by 3 dpc, however staining becomes stronger on day 6 and 10 dc. And no staining was observed in the 0 dpc tissues (Table 4.4).

In rainbow trout staining by IHC with pAb 151 (Table 4.5) for *F. psychrophilum* was observed in the gill, gut, head kidney and in the skin epithelium from 0 dpc (i.e., 1.5h post- challenge). In rainbow trout 0 dpc, is not a true day 0 because the fish was sampled hours post challenge, rather than before the challenge like the Atlantic salmon fish were sampled above. In 1/3 of the fish sampled at day 0, there is a high detection of *F. psychrophilum* in the gut. In the gills, there seems to be an increase of bacterial detection over time, while in the gut and head kidney there was no difference in staining over time post challenge (Table 4.5). In the skin the highest detection of *F. psychrophilum* cells was detected at 3 dpc and 10 dpc, and the lowest detection on 10 dpc.



**Clear- 0**

No sign of infection

**Light- 1**

Light brown staining  
on the filament  
epithelium

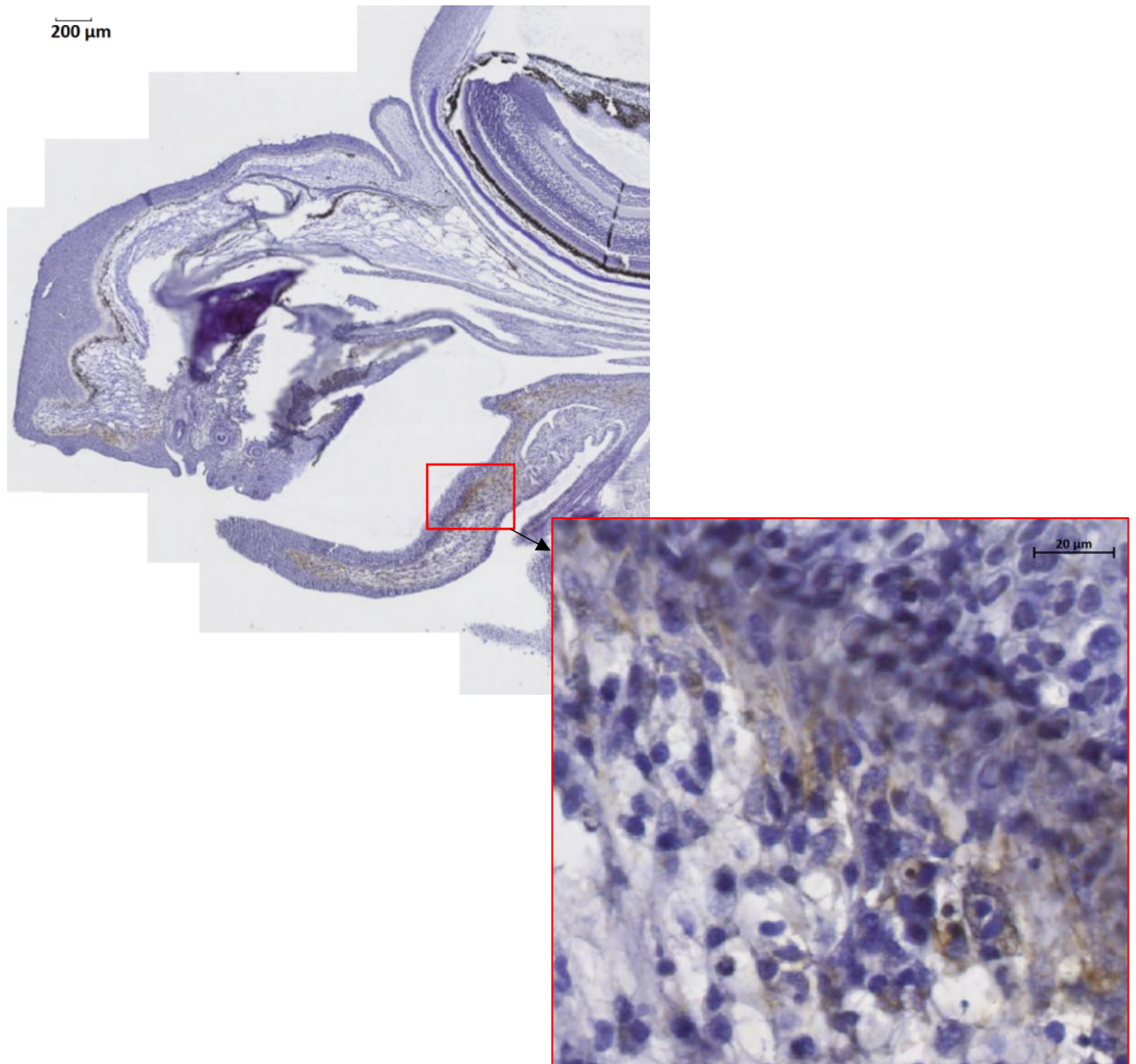
**Moderate- 2**

Brown staining on  
the filament  
epithelium and  
secondary lamellae

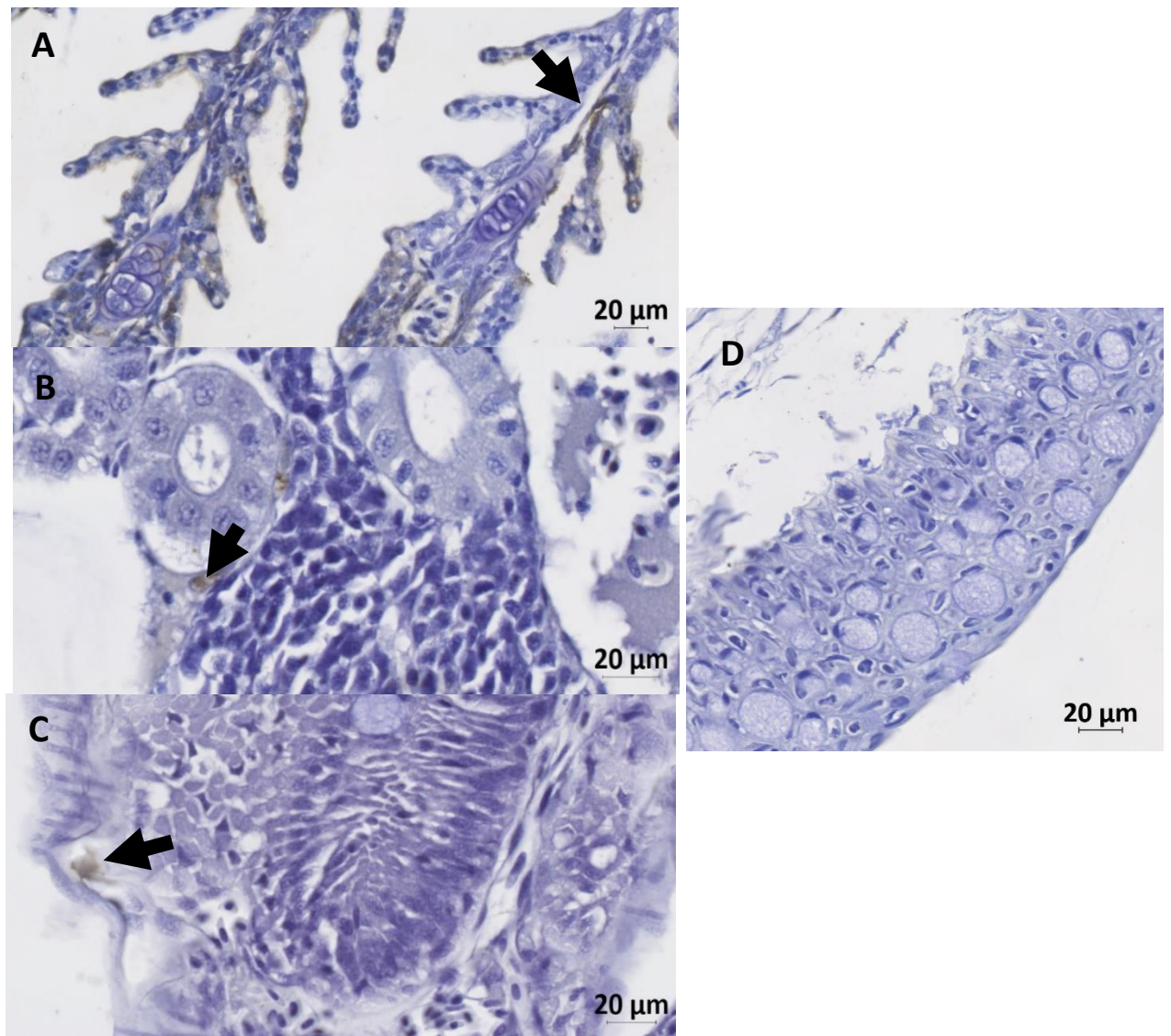
**Advanced- 3**

More intense brown  
staining on the filament  
epithelium and secondary  
lamellae

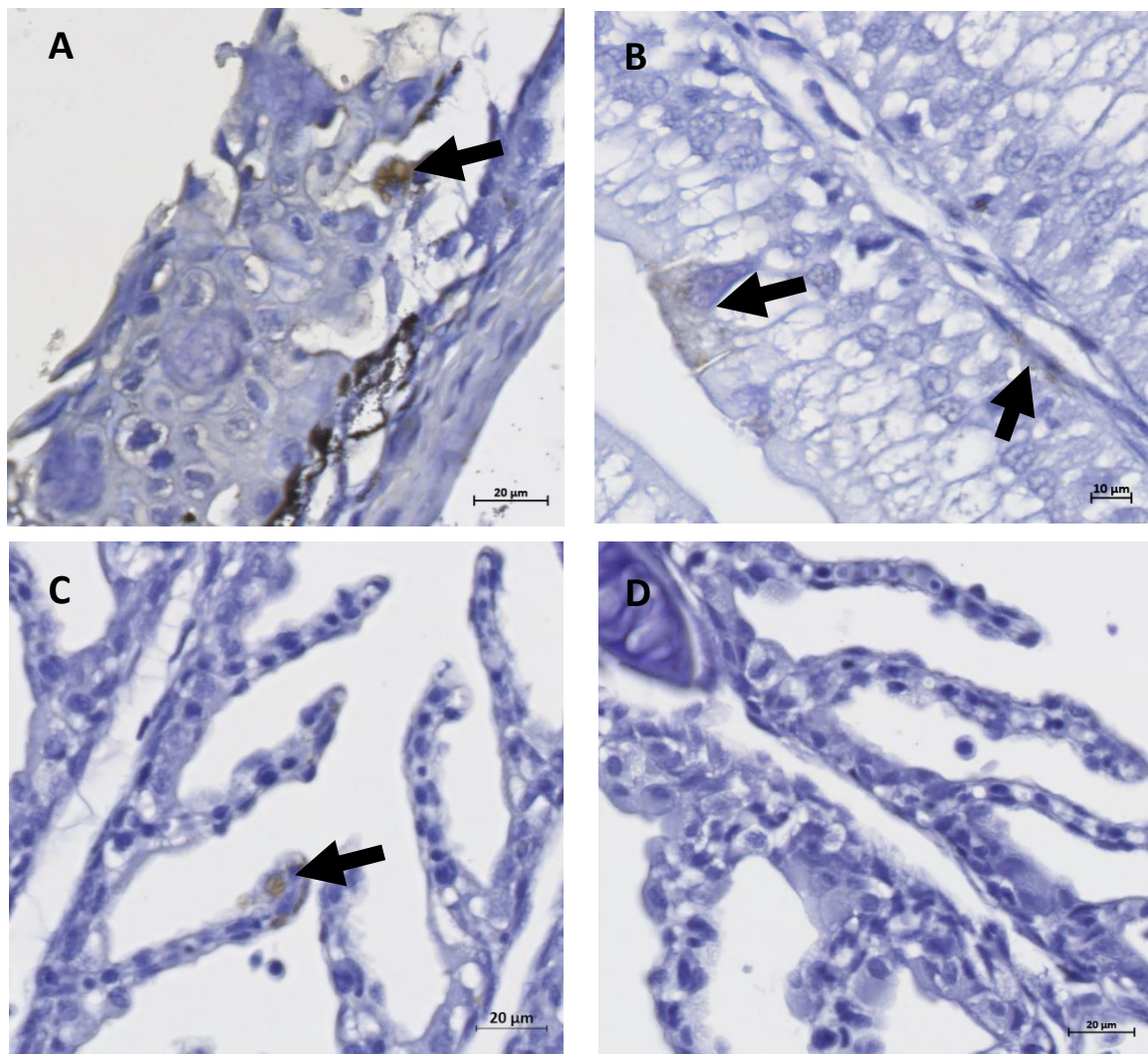
**Figure 4.10** Representative visual scoring of *F. psychrophilum* infection in Atlantic salmon gills following IHC with anti-*F. psychrophilum* pAb 151. Brown staining indicates presence of *F. psychrophilum* colonies.



**Figure 4.11** Immunohistochemical detection of antigen uptake following *F. psychrophilum* immersion challenge in Atlantic salmon in the skin epithelium at 3 dpc around the mouth. Detection of *F. psychrophilum* cells using anti- *F. psychrophilum* pAb 151 (size of specimens: 200 μm and magnification to 20 μm). Detection and quantification of cells were carried out by an individual observer (Valeria Macchia).



**Figure 4.12** Immunohistochemical detection of antigen uptake following *F. psychrophilum* immersion challenge in different tissues of Atlantic salmon. Detection of *F. psychrophilum* cells using anti- *F. psychrophilum* pAb 151. The black arrows point to the brown staining (*F. psychrophilum* colonies) in the different tissues (A) the gills, (B) the head kidney o and (C) the gut (mid-intestine) of an Atlantic salmon sampled 3 dpc (size of specimens: 20 µm). Figure 4.12 (D) represents a negative control slide of Atlantic salmon skin at 3dpc. Detection and quantification of cells were carried out by an individual observer (Valeria Macchia).



**Figure 4.13** Immunohistochemical detection of antigen uptake following *F. psychrophilum* immersion challenge in different tissues of Atlantic salmon. Detection of *F. psychrophilum* cells using anti- *F. psychrophilum* pAb 151. The black arrows point to the brown staining (*F. psychrophilum* colonies) in the different tissues (A) the skin, (B) the gut (mid-intestine) and (C) the gills of a rainbow trout sampled 3 dpc (size of specimens: 10  $\mu$ m and 20  $\mu$ m). Negative control slide of gill tissues(D). Detection and quantification of cells were carried out by an individual observer (Valeria Macchia).

**Table 4.4** Detection of *F. psychrophilum* cells by IHC using anti- *F. psychrophilum* pAb 151. Brown staining intensity (indicating the presence of *F. psychrophilum* cells) in Atlantic salmon tissues (gills, skin, gut) and head kidney sampled from 3 fish per time point was assessed by a single researcher (Valeria Macchia) and blind checked by another researcher (Dr Rowena Hoare). The two scorers gave the same score. Scoring scale 0= clear, 1= light, 2= moderate and 3= advanced (Figure 4.10).

| Sampling Day | Fish Number | Histology Reference number | Gills | Skin | Gut | Head Kidney |
|--------------|-------------|----------------------------|-------|------|-----|-------------|
| 0            | 1           | S20002-A                   | 0     | 0    | 0   | 0           |
| 0            | 2           | S20002-B                   | 0     | 0    | 0   | 0           |
| 0            | 3           | S20002-C                   | 0     | 0    | 0   | 0           |
| 3            | 1           | S20002-F                   | 2     | 2    | 1   | 1           |
| 3            | 2           | S20002-G                   | 0     | 0    | 1   | 1           |
| 3            | 3           | S20002-J                   | 1     | 0    | 0   | 0           |
| 6            | 1           | S20002-O                   | 2     | 1    | 1   | 0           |
| 6            | 2           | S20002-N                   | 1     | 0    | 2   | 2           |
| 6            | 3           | S20002-L                   | 0     | 3    | 1   | 2           |
| 10           | 1           | S20002-P                   | 1     | 0    | 0   | 0           |
| 10           | 2           | S20002-Q                   | 1     | 2    | 2   | 1           |
| 10           | 3           | S20002-S                   | 2     | 1    | 0   | 2           |

**Table 4.5** Detection of *F. psychrophilum* cells by IHC using anti- *F. psychrophilum* pAb 151 in rainbow trout. Day 0 represents 1.5h post-challenge. Brown staining intensity (indicating the presence of *F. psychrophilum* cells) in rainbow trout tissues (gills, skin, gut) and head kidneys sampled from 3 fish per time point was assessed by a single researcher (Valeria Macchia) and blind checked by another researcher (Dr Rowena Hoare). The two scorers gave the same score. Scoring scale 0= clear, 1= light, 2= moderate and 3= advanced. (Figure 4.10).

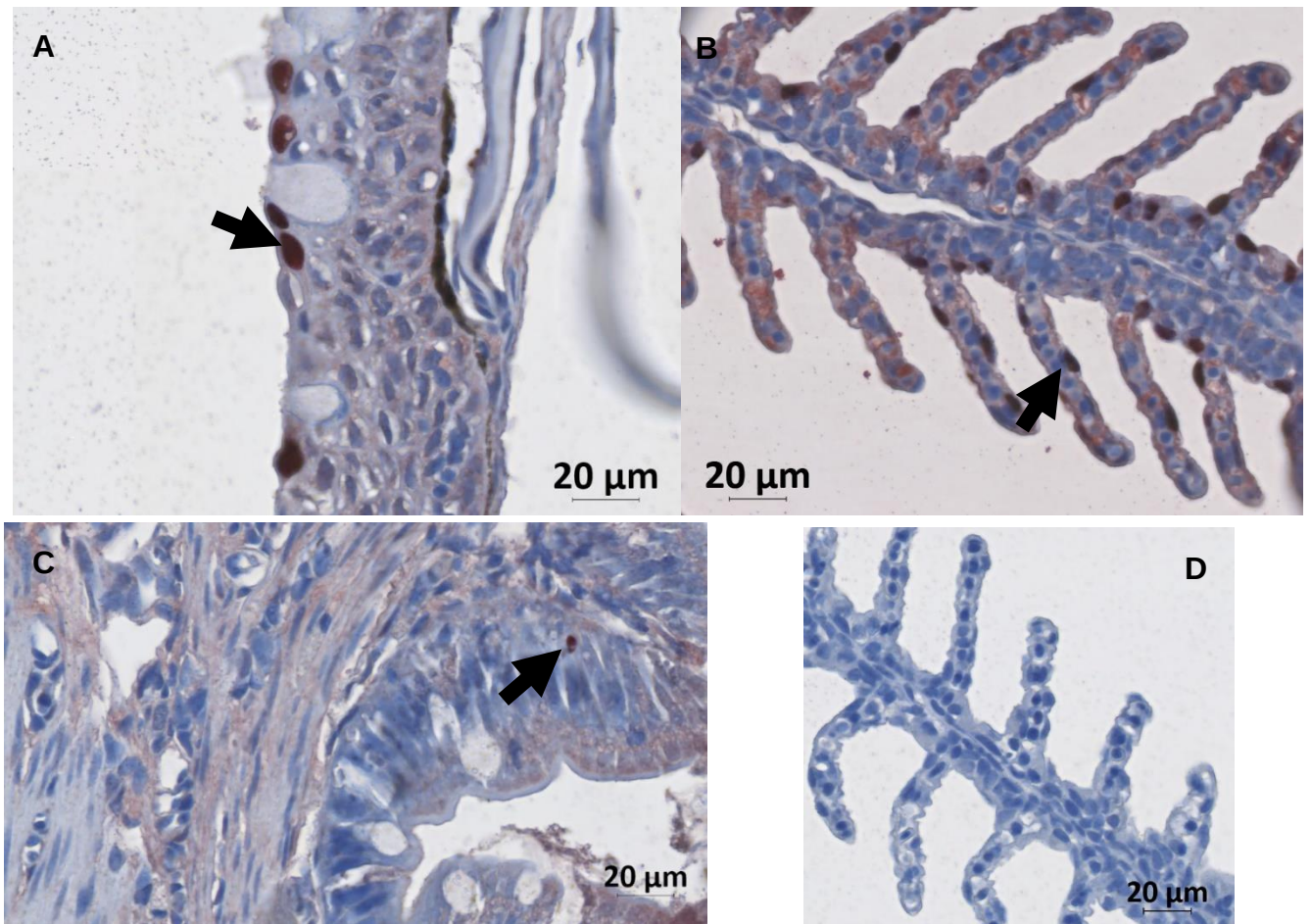
| Sampling Day | Fish Number | Histology Reference Number | Gills | Skin | Gut | Head Kidney |
|--------------|-------------|----------------------------|-------|------|-----|-------------|
| 0            | 1           | S20002-A                   | 1     | 0    | 1   | 1           |
| 0            | 2           | S20002-B                   | 1     | 2    | 1   | 0           |
| 0            | 3           | S20002-C                   | 1     | 0    | 3   | 0           |
| 3            | 1           | S20002-F                   | 1     | 1    | 2   | 0           |
| 3            | 2           | S20002-G                   | 2     | 3    | 0   | 0           |
| 3            | 3           | S20002-J                   | 2     | 0    | 1   | 2           |
| 6            | 1           | S20002-O                   | 1     | 1    | 1   | 0           |
| 6            | 2           | S20002-N                   | 2     | 2    | 2   | 1           |
| 6            | 3           | S20002-L                   | 3     | 1    | 2   | 1           |
| 10           | 1           | S20002-P                   | 2     | 1    | 1   | 1           |
| 10           | 2           | S20002-Q                   | 3     | 0    | 1   | 0           |
| 10           | 3           | S20002-S                   | 1     | 0    | 2   | 0           |

#### **4.2.9 Immunohistochemistry for detection of IgT positive cells in Atlantic salmon and rainbow trout tissues**

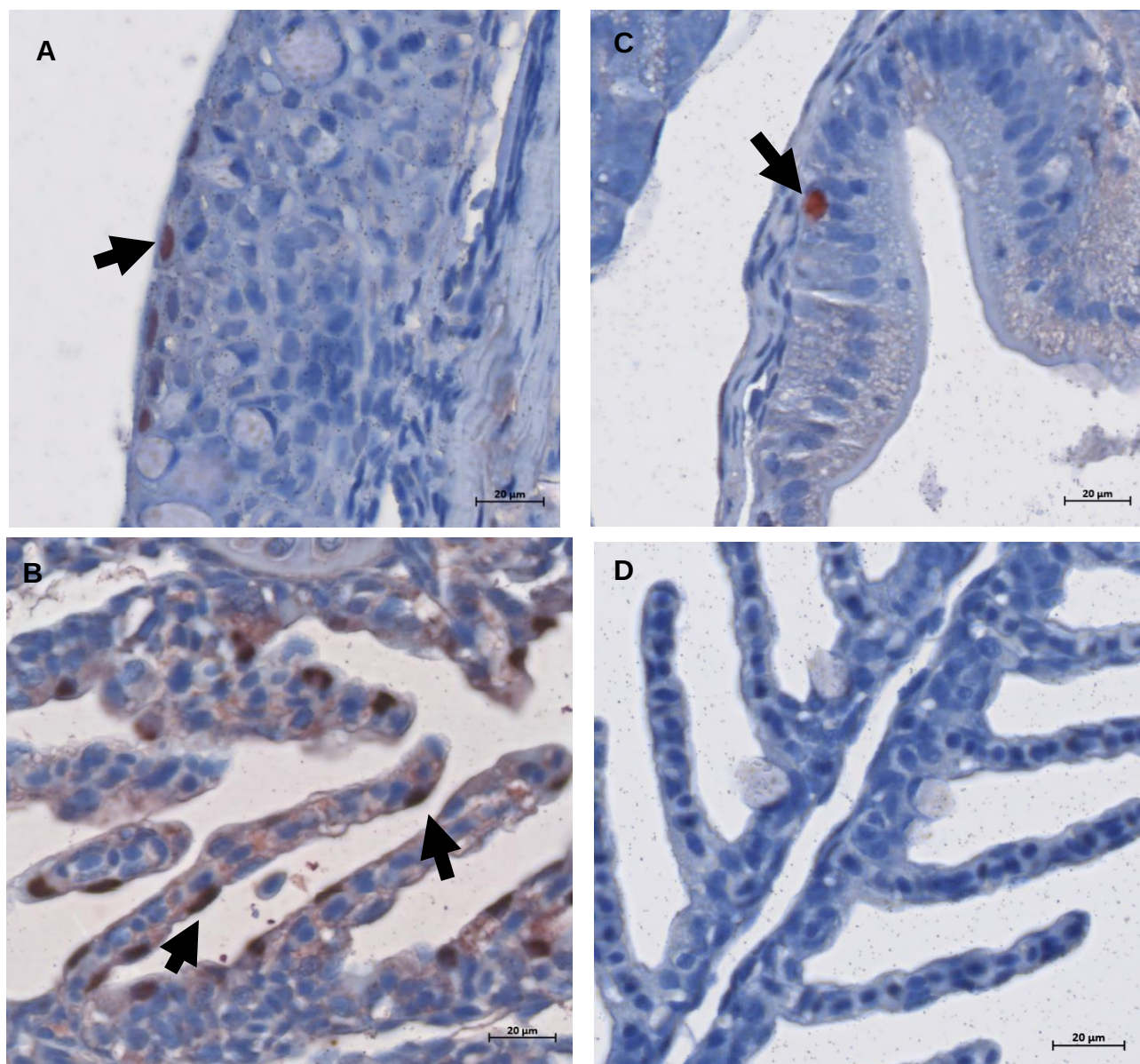
Anti-IgT monoclonal antibody (ADL Ltd.) successfully detected IgT positive cells in Atlantic salmon and rainbow trout tissues (Figure 4.14 and Figure 4.15).

In Atlantic salmon tissues, IgT positive cells were detected at 0 dpc. In the gut there was no significant difference in the average number of IgT positive cells over the time points (Figure 4.16). Compared to the other mucosal tissues, the gills had the highest number of IgT positive cells, and there is a significant difference in detection between 0 dpc and the other time points ( $p \leq 0.01$ ) and 10 dpc vs the other time points ( $p \leq 0.01$ ). In the spleen, the average number of IgT positive cells increased over time, although this was not significant ( $p > 0.05$ ).

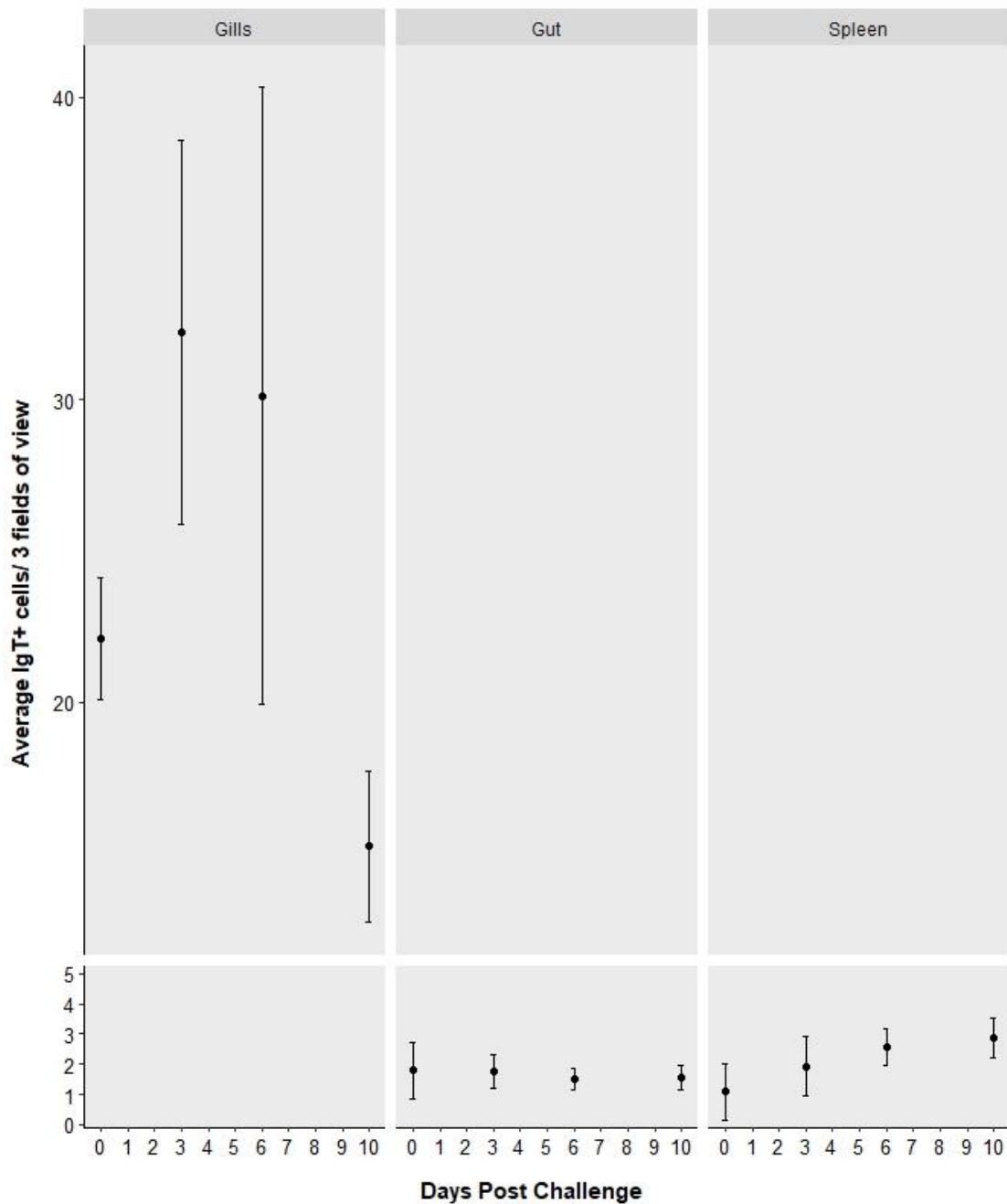
In rainbow trout tissues, IgT positive cells were also detected at 0 dpc. Regardless of the tissue, there was no significant difference in numbers of IgT positive cells over the time points ( $p > 0.05$ ) (Figure 4.17). The highest mean level of detection of IgT positive cells was 55 cells in the gills at 10 dpc. There were no evident trends over the time points.



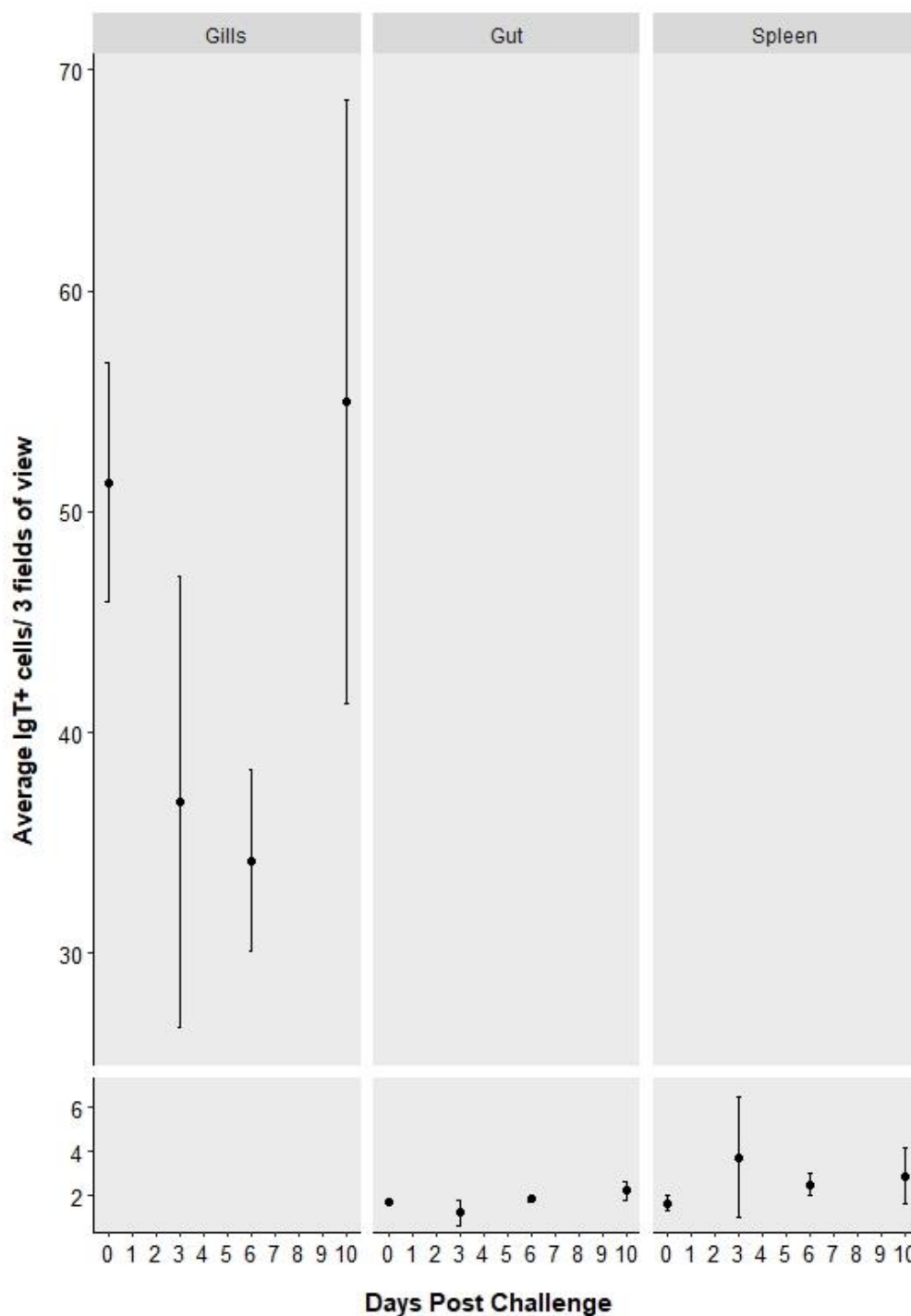
**Figure 4.14** Immunohistochemical detection of IgT<sup>+</sup> cells in mucosal organs and spleen following immersion challenge with *F. psychrophilum*. Representative image of a magnified Atlantic salmon skin (A), gill (B) and (C) gut tissues (specimen size: 20 μm) at 3 dpc. Figure 4.1.4 (D) negative control representing Atlantic salmon gill at 3dpc (specimen size: 20 μm). In the organs, IgT positive cells were identified as oval dark red spots. Detection and quantification of cells were carried out by an individual observer (Valeria Macchia).



**Figure 4.15** Immunohistochemical detection of IgT<sup>+</sup> cells in mucosal organs and spleen following immersion challenge with *F. psychrophilum*. Representative image of a magnified rainbow trout skin (A), gill (B) and (C) gut tissues, (D) negative control- gill tissue (specimen size: 20 µm) at 3 dpc. In the organs, IgT positive cells were identified as oval dark red spots. Detection and quantification of cells were carried out by an individual observer (Valeria Macchia).



**Figure 4.16** Box plot representing the mean number of IgT positive cells (n= 3 fish per time point) detected in 3 fields of view by an individual observed in *F. psychrophilum* infected Atlantic salmon mucosal tissues (gills, gut) and spleen sampled at 0 (n=3), 3 (n=3), 6 (n=3) and 10 (n=3) dpc. The bars represent the standard deviations.



**Figure 4.17** Box plot representing the mean number of IgT positive cells (n= 3 fish per time point) detected in 3 fields of view by an individual observed in *F. psychrophilum* infected rainbow trout mucosal tissues (gills, gut) and spleen sampled at 0 (n=3), 3 (n=3), 6 (n=3) and 10 (n=3) dpc. The bars represent the standard deviations.

## 4.4 Discussion

To prevent and contain flavobacteriosis effective health management strategies including rapid detection of pathogens is desired (Adams et al., 1995). In aquaculture, the application of biotechnology has allowed the development and improvement of immunodiagnostic and molecular technologies and there are a variety of methods available to detect pathogens in fish and the aquatic environment (Adams & Thompson, 2011). Histology and histopathology are routinely used in disease diagnostics and the different immunological methods used in fish diagnostics allow the specific detection of pathogens without first having to isolate the pathogens. mAbs are the main standardised reagents used for these methods (Adams & Thompson, 2008).

Monoclonal antibodies 9G7, 32, 5A9, 5 and 8 were produced against whole cell preparations of both a virulent (B97026PI) and an avirulent (32/97) isolate of *F. psychrophilum* (Faruk et al., 2000). In the current study, these mAbs were cloned and characterised by IFAT, ELISA, IHC, 1D SDS PAGE, 2D SDS PAGE, and Western blot. The mAbs were tested against a variety of *F. psychrophilum* isolates as well as other bacterial strains to compare their reactivity and test their specificity. When testing the mAbs by IFAT and ELISA, mAbs 9G7 and mAb 32 gave the most promising results having reacted to all the *F. psychrophilum* tested, except for *F. psychrophilum* type strain 1947, while not reacting with *F. hydatidis*, *A. salmonicida*, *Y. ruckeri* and *V. anguillarum* isolates. The mAbs were produced against *F. psychrophilum* 32/97 which belongs to the Th serotype, the most dominant *F. psychrophilum* serotype in the UK (Ngo et al., 2017). Ngo et al. (2017) determined that the *F. psychrophilum* type strain 1947 belongs to the minor serotype (Fp. <sup>T</sup>), which could explain the non-reactivity of the mAbs against *F. psychrophilum* 1947, as the mAbs may recognise different surface antigens than the reference Fp. <sup>T</sup> strain *F. psychrophilum* 1947. Different serotypes of *F. psychrophilum* have been isolated from salmon species, with Chinook salmon and coho salmon isolates having a higher genetic diversity compared to isolates collected from rainbow trout, which could further explain why not all the mAbs used in this study reacted with all the *F. psychrophilum* isolates used in this study (Van Vliet et al., 2016). IFAT is a widely used technique in fish diagnostics because it is simple, sensitive, and rapid to perform, and results can be obtained within 2 h of taking the sample. This technique is used to detect pathogens in sample cultured from infected fish, but it could be used to visualise bacteria or viruses directly in formalin-

fixed tissue section, which is extremely useful to use when the pathogen is difficult to culture (Miles et al., 2003). ELISA on the other hand has the advantage of detecting and quantifying the pathogen of the host antibody response, but this assay is less useful for sub-clinical infections due to the sensitivity limits (Adams & Thompson, 2011).

Given that mAb 9G7 and mAb 32 showed good reactivity and specificity, these were further tested by western blot (following 1D SDS PAGE) for their specificity in recognising *F. psychrophilum*. By western blotting a dominant smear at > 250 kDa was observed on isolates IoA\_240, IoA\_350 and *F. psychrophilum* 18\_S and there was no cross reaction of mAb 9G7 or 32 with *F. hydatidis*, *F. aquatile*, *V. anguillarum* and *A. salmonicida* isolates. As the samples were treated with proteinase K for SDS page, the high molecular weight that the mAbs recognise is most likely the carbohydrate chain of the bacterial isolates, located on the slime layer (Crump et al., 2001). Whole-cell proteins of two isolates of *F. psychrophilum* with different serotypes, IoA\_240 and Fp. <sup>T</sup> 1947, run on a 2D SDS PAGE showed, by eye, high similarity in distribution of the protein spots between replicate gels, meaning high reproducibility in the technique used, and high similarity between the two *F. psychrophilum* isolates, suggesting similar proteins profiles between isolates. The gels were further visualised by western blot and coated with mAb 9G7 and mAb 32, however both mAbs did not recognise the bacterial carbohydrates, which could have been removed by the processing of the samples for the 2D gels. For 2D-SDS PAGE, during the clean-up stage of proteins, carbohydrates are removed with a protein precipitation solution containing trichloroacetic acid and acetone (GE Healthcare, United States). Western blotting has been previously used by (Adams & Thompson, 2011) to serotype *F. psychrophilum* isolates.

The second part of the study was to determine the route of antigen uptake in Atlantic salmon by immunohistochemistry, following exposure of the fish to *F. psychrophilum* with the newly developed immersion challenge model developed in Chapter 2. IHC is simple to perform and has the advantage of being able to visualise the pathology associated with the infection using a light microscope. This technique is less sensitive than IFAT, however antigen retrieval can be used to help expose and improve the detection of the antigens (Jung et al., 2001). In the current study, mAb 9G7 and mAb 32 were used for the IHC to determine the level of infection of the fish over the course

of the trial, however no staining was detected after incubation with the mAbs. The mAbs did not detect any *F. psychrophilum* colonies in the authenticated positive control slide (skin tissue sampled from moribund rainbow trout following a confirmed *F. psychrophilum* infection). Formalin (10%) was used to fix the tissue, and formalin is a cross-linking type of fixative, forming methylene bridges between proteins within the tissue. Cross-linking reaction adversely alters the structure of tissue proteins and resulting in loss of antigenicity (Montero, 1991). As a result, heat induced antigen retrieval was used to improve the detection of antibody staining of formalin fixed paraffin embedded tissue sections. This technique is used to expose the epitopes; however, it can cause tissue damage (Krenacs et al., 2010) which could have had a result possibly damaged the antigens, resulting in lack of detection of *F. psychrophilum* colonies by the mAbs.

Due to these inconsistent results, an anti- *F. psychrophilum* pAb (pAb 151) was used to detect and quantify the level of *F. psychrophilum* infection in Atlantic salmon and rainbow trout tissues following challenge. Mucosal tissues, head kidney and spleen tissues were subject to immunohistochemistry to detect the antigen following immersion challenge. From the tissues analysed, positive staining was evident in the skin tissues, especially surrounding the mouth and the gills lamellae. This could suggest that the mucus may have trapped the antigen, hence retaining the bacteria. Despite the protocol used, formalin fixation does not damage the mucus layer (Cohen et al., 2012). Uptake of antigen through the gut was also evident 3 dpc by staining of enterocytes. Previous studies on uptake of *F. psychrophilum* in rainbow trout demonstrated similar tissue-specific antigen detection in tissues following immersion challenge (Hoare et al., 2019). In a study looking at Atlantic salmon experimentally infected with *F. psychrophilum* using scanning electron microscopy, it was observed in the fins that the bacteria were embedded in the mucus layer, while in the absence of mucus the bacteria burrowed into the fin rays (Martínez et al., 2004).

In Chapter 2, high survival rate was observed in Atlantic salmon fry challenged by immersion, and this could be partially due to non-specific immune mechanisms and induction of the mucosal antibody IgT. As evidenced in the IHC, IgT positive cells were observed in mucosal tissues. In gills and spleen tissues the number of IgT cells increases over time, however in the gills by 10 dpc there was a sudden decrease of IgT cells. In contrast in the gut, the number of IgT cells is constant over time. In the

gut of teleost, a variety of immune cells are present including antigen presenting cells (APCs) such as monocytes, macrophages in the lamina propria and intra-epithelial lymphocytes (Rombout Jan et al., 2011). In zebrafish, *Danio rerio*, challenged by immersion with *V. anguillarum*, revealed that APCs in the gut have active responses in antigen recognition (Liu et al., 2014).

In salmonids, IgT is a specialised immunoglobulin specialised for gut and skin mucosal immunity (Hordvik, 2015; Zhang et al., 2010) and it performs a similar function to IgA in mammals during parasitic infection (Tongsri et al., 2020). The highest level of IgT detection between the different mucosal tissues was in the gill, specifically in the secondary lamellae. Pre-treatment of rainbow trout with hydrogen peroxide influences the immune response and postpones healing of gill tissue (Henriksen et al., 2015), which could explain the high level of detection of IgT positive cells in this tissue. This is the first detection of Atlantic salmon IgT cells by IHC using trout anti-IgT monoclonal antibody, and the IgT isotype successfully reacted with both Atlantic salmon and rainbow trout tissues. IgT cells play a central role in the adaptive responses of rainbow trout fish gill mucosa against bacterial infections such as *Flavobacterium columnare* (Tongsri et al., 2020). In this past work, IgT positive cells were scattered and localised on the primary lamellae of gill trout while a few were present in the secondary lamellae of survivor fish. In the current study, visually healthy fish were sampled at the different time points and IgT positive cells in these fish were mainly located in the secondary lamellae of the gills, which was expected, as IgT responses are primarily confined to mucosal surfaces, and bacteria can stimulate the production of IgT in gills mucus (Buonocore et al., 2017; Tongsri et al., 2020).

## 4.5 Conclusion

In this present study, several mAbs produced against *F. psychrophilum* isolate B97026PI and isolate 32/97 were successfully characterised by IFAT, ELISA, 1D- SDS PAGE, however further work is required to characterise the mAbs by 2D-SDS PAGE. During IFAT, when applying these mAbs on *F. psychrophilum* smears, strong fluorescent signals were observed, however when these mAbs are used to detect *F. psychrophilum* in infected/moribund tissues, no reaction was observed possibly due to the technique used to expose the epitopes. In contrast, the pAb successfully reacted with the *F. psychrophilum* antigens in the infected/moribund tissues, and anti-*F. psychrophilum* pAb 151 was used for the IHC to detect and quantify *F. psychrophilum*.

cells in Atlantic salmon and rainbow trout tissues. Finally, this is the first study looking at cross reactivity between rainbow trout and Atlantic salmon IgT, and anti-trout IgT monoclonal antibody was successfully used to detect and quantify IgT positive cells in infected Atlantic salmon and rainbow trout tissues across the different time points (Figure 4.10).

Sometimes, immunological techniques are unable to detect a pathogen, due to the low amount of pathogen in the sample, or the pathogen's antigens have been altered and cannot be recognised by the antibody, either by being desaturated during sample processing or because of the pathogen life cycle in the host (Adams & Thompson, 2011). Generally, molecular techniques have an increased sensitivity over immunological methods as they are not affected by changes or inappropriate expression of antigens (DNA or RNA) rather than proteins.

Nevertheless, this is the first study using IHC to detect and quantify *F. psychrophilum* and IgT positive cells in Atlantic salmon tissues following immersion challenge with *F. psychrophilum*. A limitation of this study is that Atlantic salmon and rainbow trout fry were sampled at random at the different time points and the fish did not have any sign of the infection. To strengthen the results, in future studies, the fish sampled for these immunology studies should be moribund or diseased fish. Additionally, given that this thesis is looking at understanding the genetic resistance to *F. psychrophilum* in Atlantic salmon and rainbow trout, the fish sampled would have benefitted from genotyping to determine possible differences in immunological responses between resistant and susceptible genotypes to this bacterial infection, which can be addressed in a follow up study.

Further studies should look at the level of detection of IgT and other immunoglobulins in tissues as well as fish serum, to better understand the role of immunoglobulins in fish mucosal and systemic immunity after challenge by immersion with *F. psychrophilum*, a predominantly mucosal disease. This is the first study highlighting the immune responses elicited in Atlantic salmon tissues after *F. psychrophilum* infection, however, to better understand the mechanisms of resistance to this bacterial disease, it is essential to analyse the transcriptome of resistant and susceptible rainbow trout and Atlantic salmon, and this forms the focus of Chapter 5.

# **Chapter 5: Transcriptomic Analysis of Atlantic salmon head kidney post-*Flavobacterium psychrophilum* infection using RNA sequencing to identify global changes in gene expression**

## **5.1 Introduction**

*F. psychrophilum* causes diseases in salmonids which are difficult to manage due to new pathogenic strains that have been globally isolated in the aquaculture sector (Van Vilet et al., 2016). Selective breeding of resistance to *F. psychrophilum* has been previously investigated in rainbow trout and it is an effective approach to mitigate this bacterial disease to reduce economic losses and improve animal welfare (Vallejo et al., 2022).

Previous research has shown that genetic variation for *F. psychrophilum* in rainbow trout can be used for family-based sib selection (Leeds et al., 2010; Silverstein et al., 2009), and to increase survival in fish farms (Wiens et al., 2018). Family-based selection cannot, however, exploit within-family genetic variation; therefore within-family genetic information can improve the accuracy of genetic merit prediction and include within-family variation. Genomic selection (GS) uses genetic markers that cover the whole genome to calculate genomic estimated breeding values of selection candidates and has been used for farmed aquatic animal breeding (Houston et al., 2020; Robledo et al., 2018), including increasing accuracy of selection for flavobacteriosis in rainbow trout (Vallejo et al., 2017).

The genetic architecture of resistance to *F. psychrophilum* has been studied in rainbow trout using genome-wide association studies (GWAS) in different populations and these studies have suggested that rainbow trout fry syndrome (RTFS) resistance is a polygenic trait (Fraslin et al., 2018; Marancik et al., 2015; Palti et al., 2015; Vallejo et al., 2014; Langevin et al., 2012). These results demonstrated that genetic variation for flavobacteriosis in rainbow trout can be used to improve the responses to disease challenge under laboratory conditions though selection and genetic gain could be obtained after one generation (Leeds et al., 2010). Several quantitative trait loci (QTL) with moderate effect have been detected, including QTLs on chromosome 5 and 9

discovered in Chapter 2 and explaining 5% and 8% of the phenotypic variation respectively, which opens a promising avenue for genetic improvement of resistance (Table 5.1).

**Table 5.1** Quantitative trait loci discovered for resistance to *Flavobacterium psychrophilum* in rainbow trout

| QTL identified | Study                |
|----------------|----------------------|
| Omy1           | Vallejo et al. 2014  |
|                | Wiens et al. 2013    |
| Omy2           | Vallejo et al. 2014  |
| Omy3           | Fraslin et al. 2018  |
|                | Vallejo et al. 2014  |
|                | Vallejo et al. 2017  |
| Omy4           | Campbell et al. 2014 |
| Omy5           | Vallejo et al. 2014  |
|                | Palti et al. 2015    |
|                | Chapter 2            |
| Omy6           | Campbell et al. 2014 |
|                | Vallejo et al. 2014  |
|                | Wiens et al. 2013    |
| Omy7           | Wiens et al. 2013    |
| Omy8           | Campbell et al. 2014 |
|                | Vallejo et al. 2014  |
|                | Wiens et al. 2013    |
|                | Palti et al. 2015    |
| Omy9           | Campbell et al. 2014 |
|                | Chapter 2            |
| Omy10          | Fraslin et al. 2018  |
|                | Palti et al. 2015    |
| Omy11          | Wiens et al. 2013    |

|       |                        |
|-------|------------------------|
|       | Palti et al. 2015      |
| Omy12 | Vallejo et al. 2014    |
|       | Wiens et al. 2013      |
| Omy13 | Vallejo et al. 2014    |
|       | Palti et al. 2015      |
| Omy14 | Wiens et al. 2013      |
| Omy15 | Palti et al. 2015      |
| Omy16 | Campbell et al. 2014   |
| Omy17 | Campbell et al. 2014   |
| Omy19 | Vallejo et al. 2014    |
|       | Wiens et al. 2013      |
| Omy20 | Vallejo et al. 2014    |
| Omy24 | Vallejo et al. 2014    |
| Omy25 | Vallejo et al. 2014    |
|       | Palti et al. 2015      |
|       | Wiens et al. 2013      |
|       | Mathiessen et al. 2023 |
| Omy26 | Fraslin et al. 2018    |
| Omy29 | Fraslin et al. 2018    |

Heritability defines how much of the differences in phenotypes between animals is due to genetic factors and represents an important genetic parameter in selective breeding programs (Gjedrem & Baranski, 2010). Heritability to resistance to *F. psychrophilum* in rainbow trout has been previously studied, and found to be moderate (Henryon et al., 2005; Silverstein et al., 2009; Fraslin et al., 2018). Linkage or association studies have been used to assess *F. psychrophilum* infection in rainbow trout and QTL associated with resistance have been discovered (Wiens et al., 2013; Vallejo et al., 2014; Palti et al., 2015; Vallejo et al., 2017). For the first time, the role of epistatic interactions, how gene interactions affect phenotypes, between resistance-associated QTL was evidenced (Fraslin et al., 2018). Langevin et al. (2012), Marancik et al. (2014) and Fraslin et al. (2018) tested whether the differentially expressed genes were located close to the proposed QTLs, and 49 genes were located close or within the

95% confidence intervals of the QTLs. Among these genes, 14 had functions involved in resistance controlled by the QTLs, and Fraslin et al. (2018) identified a list of potential candidate immune genes involved in resistance. These can be classified into four functional categories: (1) bacterial sensors and damage associated molecular pattern (DAMP) molecules (*cd209* and other c-type *Lectin-4*, *TLR-2*; and *hmgb3*); (2) inflammatory factors (*STEAP-4*; *il-1r2*; and *drtp1*); (3) effectors killing bacteria (*c3* and *hamp*) or affecting the host (*collagenase/mmp13*); and (4) interferon-stimulated genes (ISG) (*vig2*, *ifi44*, and *ifitm*).

A promising avenue to decreasing the incidence of *F. psychrophilum* infections in Atlantic salmon aquaculture could therefore be to determine whether *F. psychrophilum* resistance is heritable in Atlantic salmon, and if major QTLs for resistance to this bacterial disease can be discovered, which could be used to increase resistance of aquaculture stocks to *F. psychrophilum* through selective breeding. Additional to the value of selective breeding programs, knowledge of the interaction between Atlantic salmon and *F. psychrophilum* can help devise more effective prevention and treatment strategies. This can be achieved by investigating and analysing the different stages of infection, the mechanisms used by the pathogen to invade and proliferate in the host. This is where bioinformatics plays a large role in expanding our knowledge in this field (Ranganathan et al., 2019). RNA sequencing (RNA-seq) analysis allows the identification of putative resistance genes and markers and can lead to the discovery of candidate functional variants (Robledo et al., 2017). RNA-seq has become the tool of choice for conducting transcriptional profiles, replacing microarray studies, to estimate changes in gene expression levels (Wang et al., 2008). RNA-seq has a wider dynamic range to quantify differentially expressed genes (DEGs). RNA-seq may generate more insight into mechanisms of resistance to *F. psychrophilum*, giving an integrated view of the host response to bacterial infection, which in turn can highlight specific genes, pathways and networks involved in the host-bacteria interaction. RNA-seq analysis can quantify gene expression levels within a single RNA sample but also detect DE genes across treatments or conditions (Koch et al., 2018).

In Chapter 2, a genome wide association study (GWAS) was conducted with Atlantic salmon fry from the AquaGen breeding nucleus with and challenged by immersion with *F. psychrophilum*. The results from Chapter 2, highlighted that resistance to *F. psychrophilum* in Atlantic salmon is an oligogenic trait with two strong QTLs, on

chromosome 5 and 9, which explained 5% and 8% of phenotypic variation. The regions between chromosome 5 and 9 in Atlantic salmon and the QTLs previously discovered by AquaGen in rainbow trout on chromosome 3 and 8 have been identified as non-orthologous. A strong peak on chromosome 25, was discovered in the rainbow trout study in Chapter 2 and was also validated by a recent study by Mathiessen et al. (2023) in rainbow trout. An orthology analysis should be run on this newly discovered QTL on chromosome 25.

*F. psychrophilum* appears to have interactions with the spleen and head kidney macrophages of rainbow trout (Nematollahi et al., 2003; Semple et al., 2020), therefore the head kidney is a suitable tissue of interest for transcriptomic analysis to get a better understanding of the genetic and functional aspects of Atlantic salmon immunity during infection with *F. psychrophilum*.

The aim of this Chapter is to analyse the Atlantic salmon head kidney transcriptome in response to *F. psychrophilum* infection using RNA-seq analysis: to characterise the transcriptomic response to *F. psychrophilum* infection in head kidney of Atlantic salmon and unravel possible genes and pathways that play a role in disease resistance; and to compare the gene expression data of this Chapter with two similar transcriptomic studies of *F. psychrophilum* infection in rainbow trout.

## 5.2 Methods

### 5.2.1 Experimental challenge

In this Chapter Atlantic salmon fry were challenged and sampled as described in Chapter 3 section 3.2.3. The challenge included fish sampled ( $n=50/\text{time point}$ ) at random at 0, 3, 6, 10 days post challenge (dpc) and stored in RNAlater (Sigma, UK) as described in Chapter 3 section 3.2.4.

### 5.2.2 RNA extraction and sequencing

RNA was extracted from randomly selected Atlantic salmon fry ( $n=58$ : 0,  $n=10$ ; 3,  $n=16$ ; 6,  $n=15$ ; 10;  $n=17$  dpc) head kidney tissues using AllPrep DNA/RNA Mini Kit (QIAGEN, Germany) following the manufacturer's protocol (QIAGEN, 2022). The unbalanced number of samples analysed in this experimental chapter, was associated with the cost of the analysis. Briefly, approximately 0.5 mg of tissue was homogenised in 350  $\mu\text{l}$  of buffer RLT (QIAGEN, Germany) with a pellet pestle (Merck, Germany). The lysate was centrifuged at  $21,000 \times g$  for 3 min and the supernatant was placed in an AllPrep DNA spin column and centrifuged at  $8,000 \times g$  for 30 s. The AllPrep DNA spin column was kept in the fridge for genomic DNA extraction and to the flow-through was added 250  $\mu\text{l}$  of 100% ethanol and this was mixed by gentle pipetting. The sample was transferred into an RNeasy spin column placed in a 2 mL collection tube and centrifuged for 15 s at  $8000 \times g$  and flow-through was discarded. To the RNeasy column, 700  $\mu\text{l}$  Buffer RW1 was added and centrifuged for 15 s at  $8000 \times g$  to wash the spin column membrane. Twice the RNeasy spin column membrane was washed with 500  $\mu\text{l}$  Buffer RPE and centrifuged for 15 s at  $8000 \times g$ . Finally, 35  $\mu\text{l}$  RNase-free water was added directly to the spin column membrane, incubated for 5 min on ice and then centrifuge for 1 min at  $8000 \times g$  to elute the RNA.

The RNA integrity was checked on Agilent 2100 Bioanalyzer (Agilent Technologies, USA). All samples had RNA integrity number (RIN) values  $> 8$ . RNA-seq libraries were prepared using TruSeq Stranded mRNA Library Prep Kit (Illumina AS, San Diego, CA, USA), following the manufacturer's instructions. The libraries were subsequently sequenced using 100-bp single-end high-throughput mRNA sequencing on an Illumina NextGen (Illumina AS, USA) at the Norwegian Sequencing Centre (Oslo,

Norway). Single-end RNA-Seq was used instead of pair-end RNA-Seq as it was an economical alternative.

### 5.2.3 Atlantic salmon RNA-seq study

Read sequences (.fastq) were processed using the *bcbio-nextgen* pipeline (<https://github.com/bcbio/bcbio-nextgen>). In brief: Raw reads were quality trimmed using *atropos* (Didion et al., 2017) before aligning the trimmed reads to the Atlantic salmon genome (GCF\_000233375.1) (Lien et al., 2016) using *STAR* (Dobin et al., 2013). The resulting .bam files were subsequently used to generate raw gene counts using *featureCounts* (v1.4.4) (Liao et al., 2014) using the ENSEMBL *Salmo salar* Annotation Release 99 (available for download at [http://ftp.ensembl.org/pub/release-99/gff3/salmo\\_salar/Salmo\\_salar.ICSASG\\_v2.99.gff3.gz](http://ftp.ensembl.org/pub/release-99/gff3/salmo_salar/Salmo_salar.ICSASG_v2.99.gff3.gz) ).

#### 5.2.3.1 Differential expression

Prior to differential expression the whole RNA-seq dataset was clustered using principal component analysis (PCA). The PCA simplifies the complexity in high-dimensional data, which are common in this type of study, while retaining the trends and patterns (Lever et al., 2017). A PCA using the log CPM of the top 13,000 genes was performed (Gress et al., 2018). PCA plots were created using the R package *factoextra* (<http://www.sthda.com/english/rpkgs/factoextra/>).

Gene counts produced by *STAR* were read into R and subjected to differential expression analysis using the *edgeR* R/Bioconductor package (Robinson et al., 2010). *edgeR* uses the negative binomial (NB) distribution to model the read counts for each gene in each sample. The NB model can be extended with quasi-likelihood (QL) methods to account for gene-specific variability from both biological and technical sources (Chen et al., 2016). Under the QL framework, the NB dispersion trend described the overall biological variability across all genes, and gene-specific variability above and below the overall level. In the QL approach, the individual (tagwise) NB dispersions are not used. The estimation of QL dispersions was performed using the *glmQLFit* function. Differential gene expression was inferred between control (Day 0) and infected head kidney samples. A false discovery rate (FDR) threshold of  $p\text{-value} \leq 0.05$  was set to determine significance (Benjamini & Hochberg, 1995).

The Bioconductor packages *BiocGenerics* (Huber et al., 2015), *AnnotationDbi* (Pagès et al., 2022) and *SparseM* (Koenker, 2021) were used to perform Gene Ontology (GO)

enrichment analyses in R. GO term annotation for the Atlantic salmon transcriptome was obtained through *Ensembl BioMart* (Kinsella et al., 2011). A GO term was considered enriched, the probability of seeing x number of genes out of the total number of genes in the filtered set detected by the RNA-Seq data annotated to a particular GO Term, if it showed a  $p$ -value  $< 0.001$ . An enrichment  $p$ -value was calculated by comparing the observed frequency of an annotation term with the frequency expected by chance (Tipney & Hunter, 2010).

#### **5.2.4 Cross-species comparison**

*F. psychrophilum* infection has been previously reported in rainbow trout, specifically to determine differentially expressed genes in infected and naïve rainbow trout from resistant and susceptible families (Marancik et al., 2015; Langevin et al., 2012). Improvements in the rainbow trout genome annotation means that the studies by Marancik et al. (2015) and Langevin et al. (2012) deserved to be re-analysed using the rainbow trout genome Omyk\_1.0 (GCA\_002163495.1) for the analysis (Pearse et al., 2019).

##### **5.2.4.1 Re-analysis: Langevin et al. 2012**

The microarray dataset from Langevin et al. (2012) was downloaded from Gene Expression Omnibus (accession: GSE35448). All microarray probes were re-mapped using *bwa* (Li & Durbin, 2009). From 37,394 microarray probes 14,843 could be mapped back to the rainbow trout transcriptome Omyk\_1.0. The microarray data were re-analysed using the *limma* R/Bioconductor package (Table 5.2) (Smyth, 2005). In brief, background corrected spot intensity signals (*gProcessedSignal*) were filtered according to the following criteria provided by the Feature Extraction software: *glsPosAndSignif*, *glsFeatNonUnifOL*, *glsWellAboveBG* and *ControlType*. All control probes were removed from the data as well as probes that were flagged in more than 10 samples. Signals from flagged spots were set as missing before normalising expression values across arrays using quantile normalisation (QN). QN assumes that all samples have similar distribution regardless of sample class to eliminate or minimise technical variability (Zhao et al., 2020). Missing values were subsequently imputed using the method based on K Nearest Neighbours implemented in the R/Bioconductor package *impute* (Troyanskaya et al., 2001). The data was then  $\log_2$  transformed before estimating differential expression between infected and control

samples using *limma*. Similarly, Langevin et al. (2012) imported scanner output files using *read.maimages*, a function of the *limma* package. *Limma* functions were used to perform the differential expression analysis. Raw p-values were adjusted using Benjamini-Hochberg procedure to decrease the false discovery rate, to prevent incorrect rejection of the true null hypotheses (Benjamini & Hochberg, 1995).

#### **5.2.4.2 Re-analysis: Marancik et al. 2015**

Fastq files from the RNA-seq study of Marancik et al. (2015) were obtained via ENA (accession PRJNA259860) and *atropos* (Didion et al., 2017) has been used to trim the sequencing reads to increase the quality and reliability of the read, before being mapped against the rainbow trout Omyk\_1.0 transcriptome (GCA\_002163495.1) using *Salmon* (v0.10.2) (Patro et al., 2017). Quantification files were read into R using the R/Bioconductor package *tximport* and the data were normalized and filtered before estimating differential expression between samples from *F. psychrophilum* and phosphate-buffered saline (PBS) injected fish using *edgeR* (Robinson et al., 2010). Marancik et al. (2015) did map RNA-seq reads to both the *F. psychrophilum* CSF259-93 genome sequence (GenBank accession CP007627.1) as well as to the rainbow trout genome sequence, accession CCAF000000000 (Berthelot et al., 2014) and generated separate host/bacterial counts in each library. Transcript models were subjected to Blast2GO sequence annotation pipeline with the default parameters applied in the blast, mapping, and annotation steps using a local Blast2GO database. NCBI non-redundant protein sequence (nr) database was used as the reference in the blast step (<https://www.ncbi.nlm.nih.gov/refseq/about/nonredundantproteins/>).

#### **5.2.4.3 Comparative gene expression analysis**

Ortholog information for the genes from Atlantic salmon and rainbow trout were extracted from Ensemble using the BioMart data mining tool (Herrero et al., 2016). Differential expression results of the re-analysed *O. mykiss* studies (Langevin et al., 2012; Marancik et al., 2015) were used to identify the differentially expressed up/down regulated genes that could be connected via orthology to an Atlantic salmon gene to define gene-sets. Subsequent gene set enrichment analysis was performed using the *roast* function of the *edgeR* package (Wu et al., 2010) (Figure 5.2).

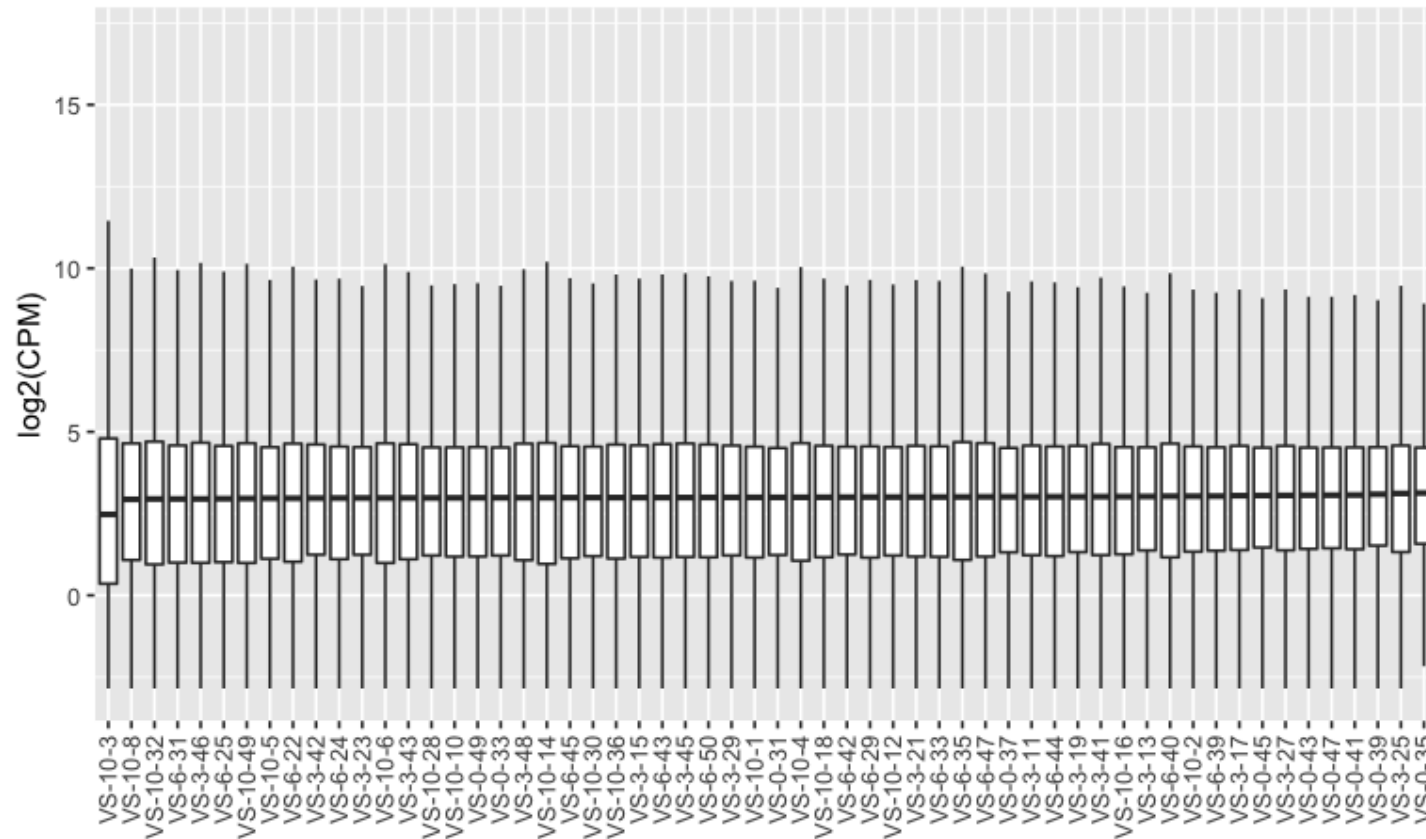
## 5.3 Results

### 5.3.1 Sampling and Sequencing

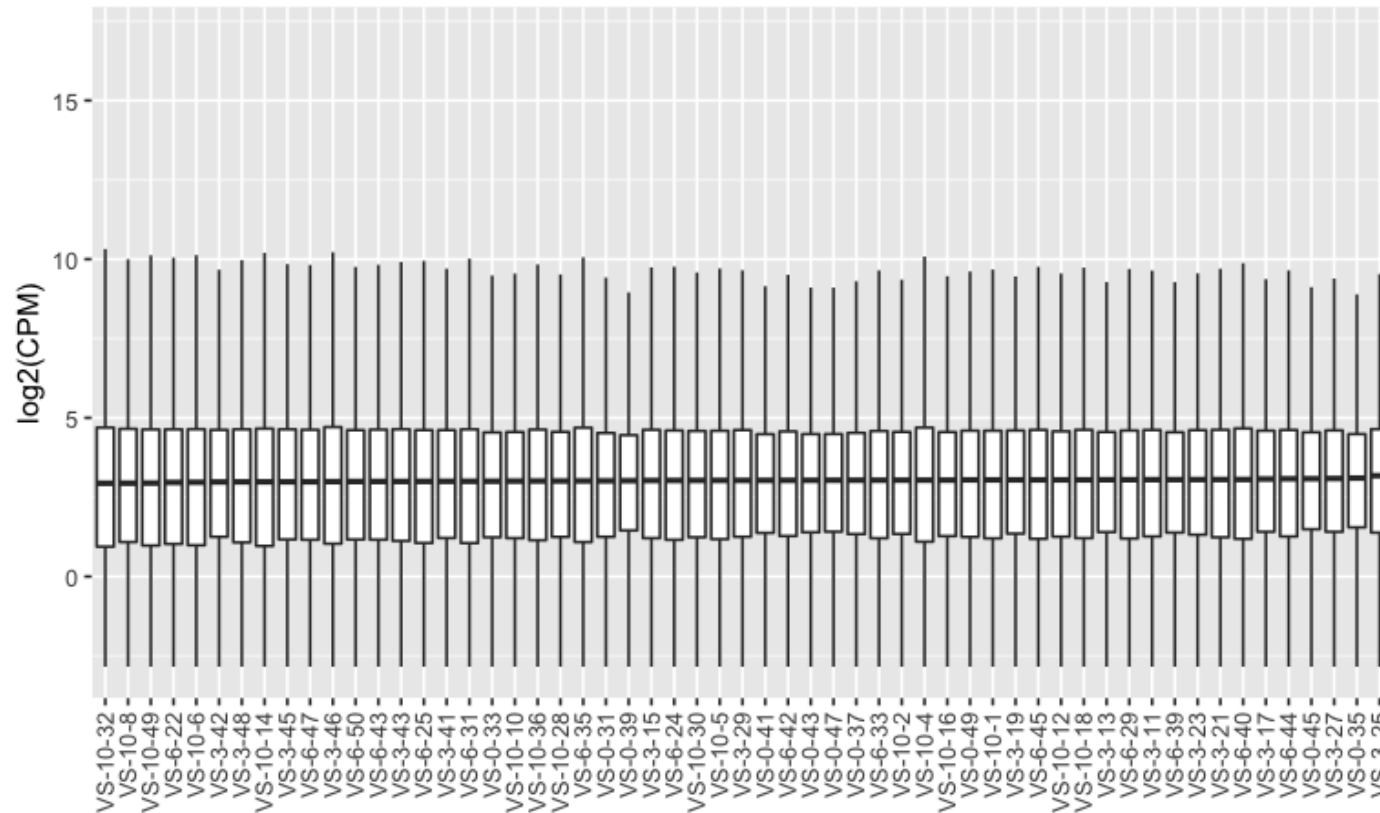
A total of 500 fish belonging to the AquaGen commercial breeding programme were challenged with *F. psychrophilum* isolate 6\_S ( $2 \times 10^7$  CFU/mL), and killed (n=50) for sampling at days 0 (pre-challenge control fish), 3, 6 and 10.

RNA-seq was performed on the head kidney of 58 randomly sampled animals (0, n=10; 3, n=16; 6, n=15; 10, n=17 dpc) and the data using the RNA-seq workflow described in Table 5.2. The read counts were analysed using R and resulted in 81,667 filtered genes. To filter lowly expressed genes, it was chosen to retain genes that were expressed at a counts-per-million (CPM) > 1 in at least 25% of the samples. Hence, the number of genes used for interpretation was reduced to 26,700.

After filtering, the data was normalised using the *calcNormFactors*, which was used to calculate scaling factors to convert raw library sizes into effective library sizes, and the data was then visualised using a box plot (Figure 5.1). Figure 5.1 showed that one sample was an outlier (VS\_Day10\_Sample3), and a duplicate sample was identified and as such they were both removed from the data and the data was once again checked using a box plot (Figure 5.2). The final dataset comprised of 56 head kidney samples from 56 individuals.



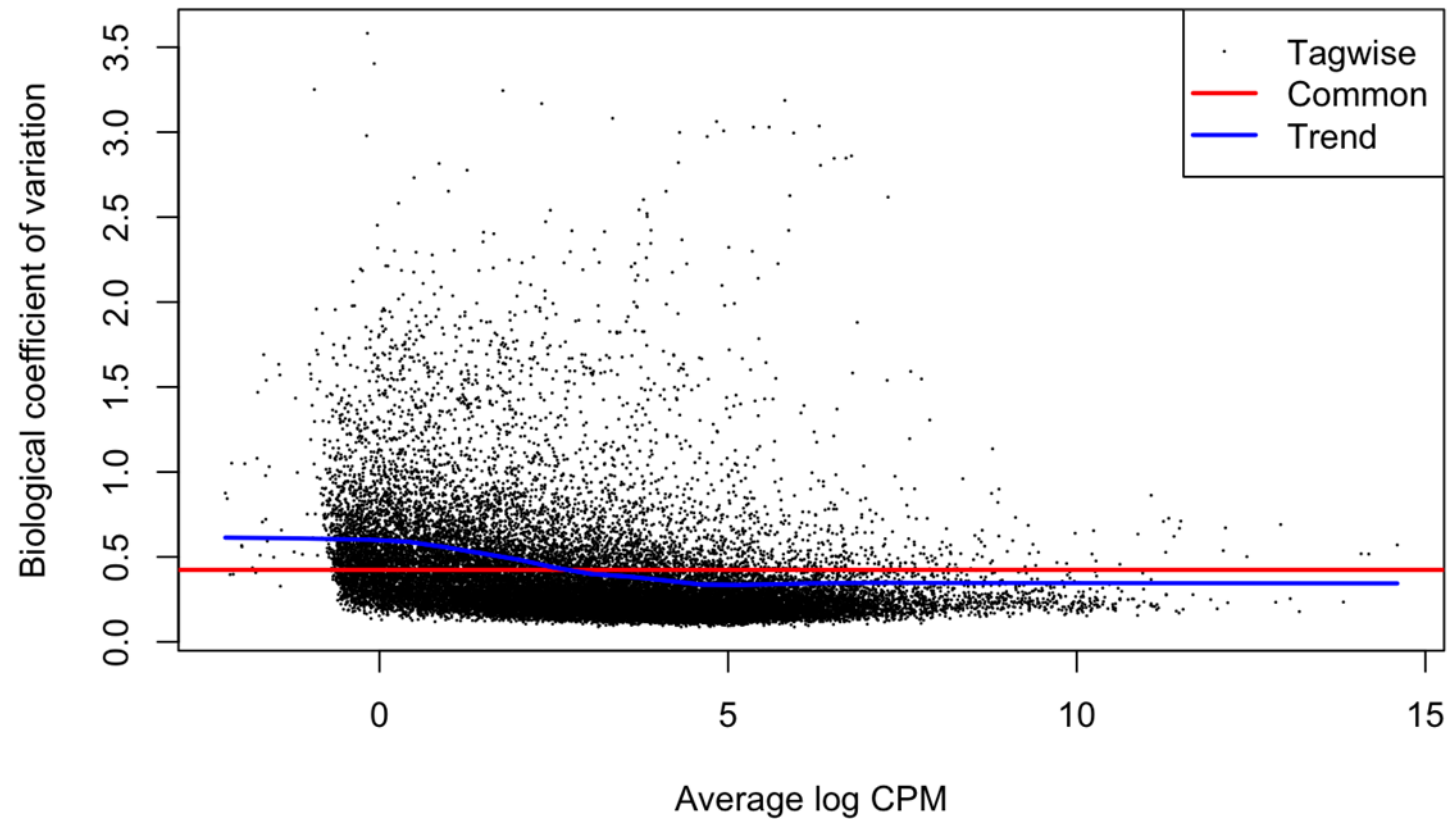
**Figure 5.1** Boxplots of log-count per million (log-CPM) values showing expression distributions of filtered unnormalised data for each sample. The line dividing the box represents the median of the data and top and bottom of the box shows the upper and lower quartiles respectively. The samples are organised by their median, and the whiskers show the highest and lowest values, and it is possible to see that one sample (VS-10-3) is an outlier.



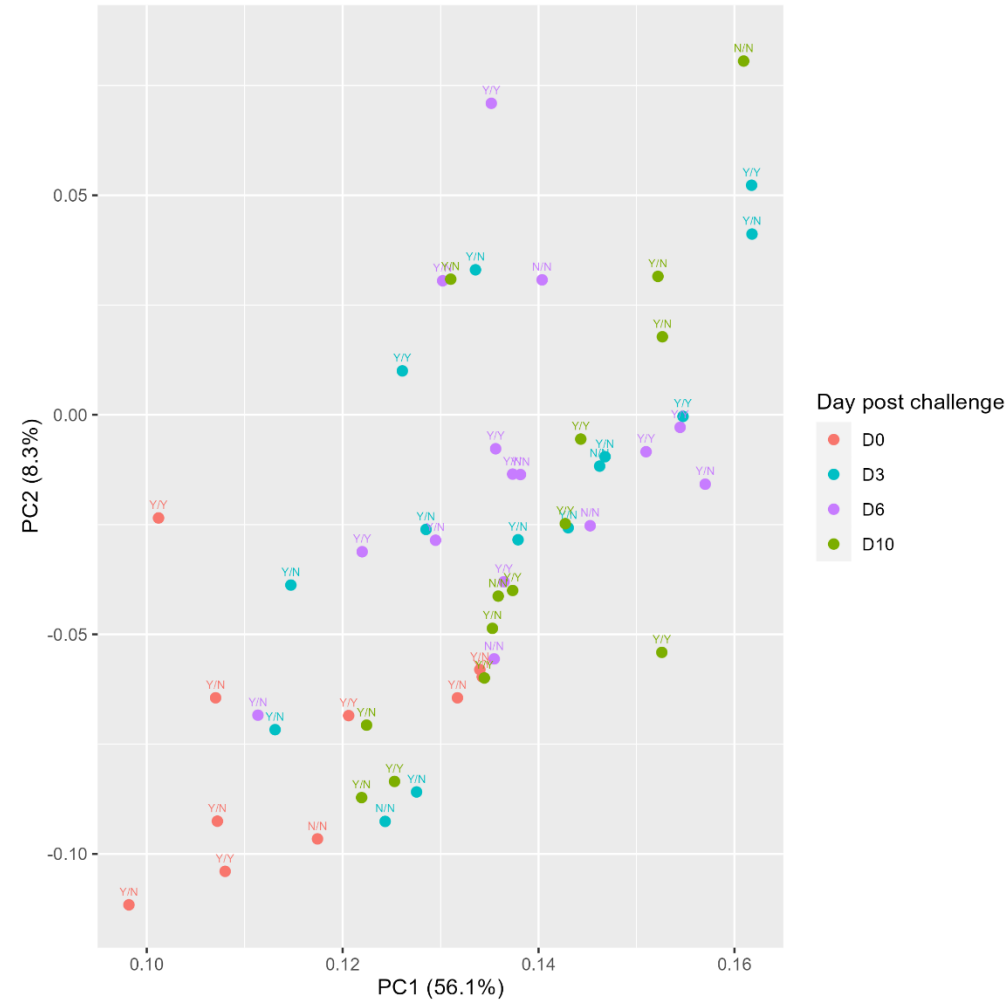
**Figure 5.2** Boxplots of log-CPM values showing expression distributions of filtered and Trimmed Mean of M-values (TMM) normalised data for each sample. The line dividing the box represents the median of the data and top and bottom of the box shows the upper and lower quartiles respectively. The samples are organised by their median, and the whiskers show the highest and lowest values, the outlier sample (VS-10-3) was removed from the dataset as well as the duplicate samples

Differential expression (DE) analysis was then performed using normalised read count data to discover quantitative changes in expression levels of all the genes between experimental groups. A model matrix was constructed including the contrasts (i.e., days post challenge and genotypes at the QTLs on chromosome 5 and chromosome 9, see Appendix). edgeR uses the negative binomial (NB) distribution to model the read counts for each gene in each sample. The dispersion parameter of the NB distribution accounts for variability between biological replicates. edgeR estimated an empirical Bayes moderated dispersion for each individual gene (tagwise). It also estimated a common dispersion, which was a global dispersion estimate averaged over all genes (common), and a trended dispersion where the dispersion of a gene is predicted from its abundance (trend).

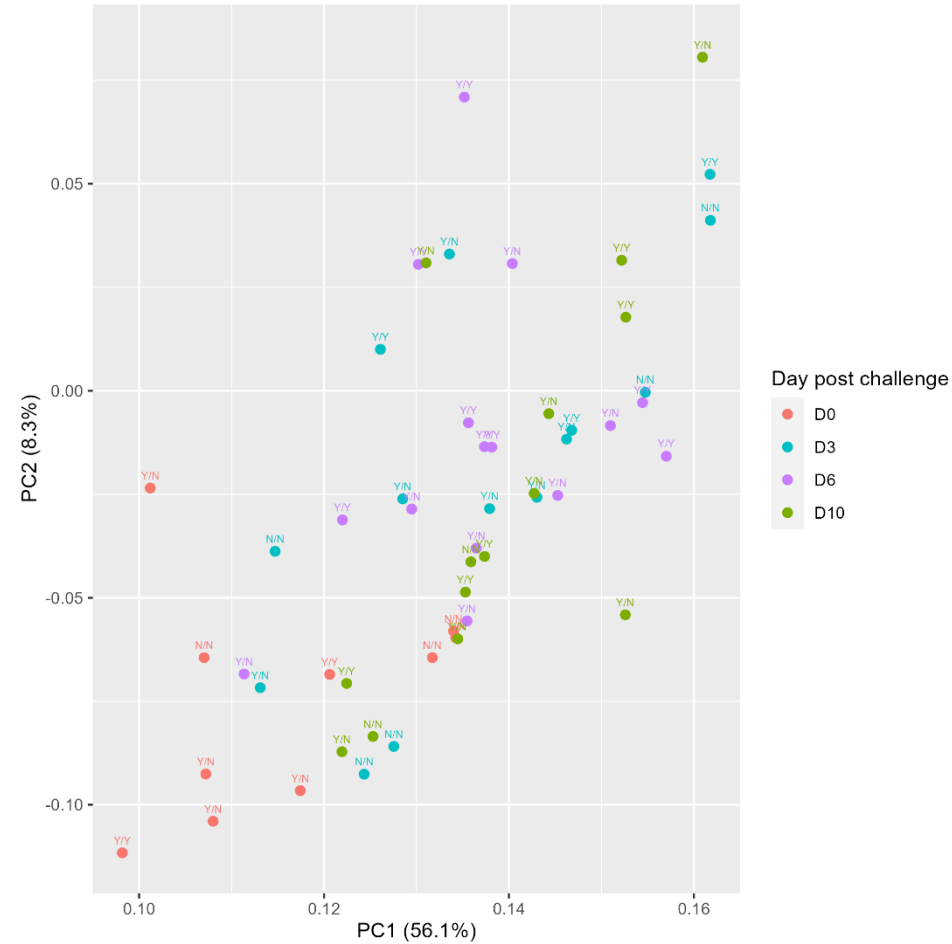
As illustrated from Figure 5.3, the asymptotic value (red line) for the BCV is below 0.5, and it is clear that as the average log CPM increases the biological coefficient of variation decreases. The top 13,000 gene expressed from the RNA-seq samples (n=52, six samples removed from the analysis) analysed were clustered using PCA, and the PCA displayed a clearer tendency trend of separation between control fish (n=10) vs infected fish (n=42) (Figure 5.4[a] and 5.4[b]). However, PCA analysis showed a weak tendency of separation between the different time points post challenge (day 3 vs day 6 vs day 10) and there was not a clear separation between resistant and susceptible genotypes (Figure 5.4). As a result, the RNA-seq analysis of this Chapter focused on the comparison between control (day 0) and infected Atlantic salmon head kidney transcriptomes.



**Figure 5.3** Scatterplot of the biological coefficient of variation (BCV) against the average abundance of each gene ( $n= 26,700$ ). The vertical axis of the BCV plot shows square-root estimates of the common, also known as biological coefficient of variation (BCV), trended and tagwise negative binomial (NB) dispersions. The NB dispersions tend to be higher for genes with very low counts.



**Figure 5.4 (a)** Principal component analysis (PCA) plot of the top 13,000 genes of Atlantic salmon head kidney samples infected with *F. psychrophilum*. The samples were collected at day 0 (control fish), day 3, 6 and 10 post challenge. RNA-seq samples (n=52) clustered according to their gene expression. The labels on the points indicate the fish genotypes (i.e. Y/Y, Y/N and N/N) on chromosome 5.

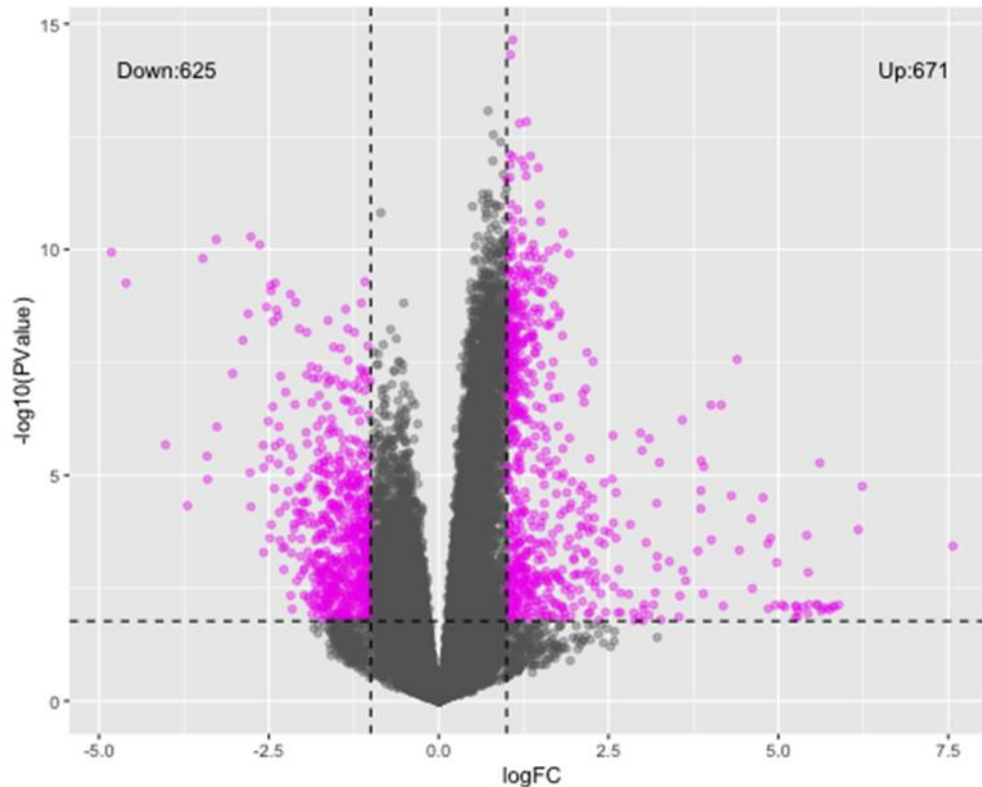


**Figure 5.4 (b)** Principal component analysis (PCA) plot of the top 13,000 genes of Atlantic salmon head kidney samples infected with *F. psychrophilum*. The samples were collected at day 0 (control fish), day 3, 6 and 10 post challenge. RNA-seq samples (n=52) clustered according to their gene expression. The labels on the points indicate the fish genotypes (i.e. Y/Y, Y/N and N/N) on chromosome 9.

The Atlantic salmon genome (ICSASG\_v2; Genbank accession GCF\_000233375.1) was uploaded in R and used to make Genomic Ranges (Granges) for all the genes and an FDR cut-off of 0.05 was applied to determine all the DE genes.

The quasi-likelihood negative binomial generalized log-linear model was used to determine the number of DEGs in the contrast (control [D0]) vs infected Atlantic salmon. In the infected group, 625 genes were down regulated whilst 671 were up regulated. To find up-regulated and down-regulated genes, a log fold change (logFC)  $> 1$  indicates up-regulation of a gene and logFC  $< -1$  indicates down-regulation of a gene. Volcano plots were used to show the statistical significance vs the fold change, and genes with an FDR  $< 0.05$  and a log fold change (logFC)  $> 1$  were considered to be differentially expressed (Figure 5.5).

*Granges* was also used to identify genes that were within 2 Mb of the QTLs on chromosome 5 (n= 99) and chromosome 9 (n=53), and it was determined that there are more DE genes close to the QTL on chromosome 5 than expected by chance (p=0.029; Fisher's Exact Test).



**Figure 5.6** Volcano plot for differential gene expression (n=26,700). Scattered points represent genes: the x-axis is the log fold change (logFC) which represents the difference between the levels of expression for each gene between the control vs infected animals, whereas the  $-\log_{10}$  of the p-value for the contrast: Infected (sampling points after D0) vs control. Dashed lines indicate cut-offs for significance Volcano plot. Genes with an  $FDR < 0.05$  and  $\log FC > 1$  were considered to be differentially expressed (pink dots). The most statistically significant genes are towards the top of plot, the most up regulated genes are towards the right, the most down regulated genes are towards the left.

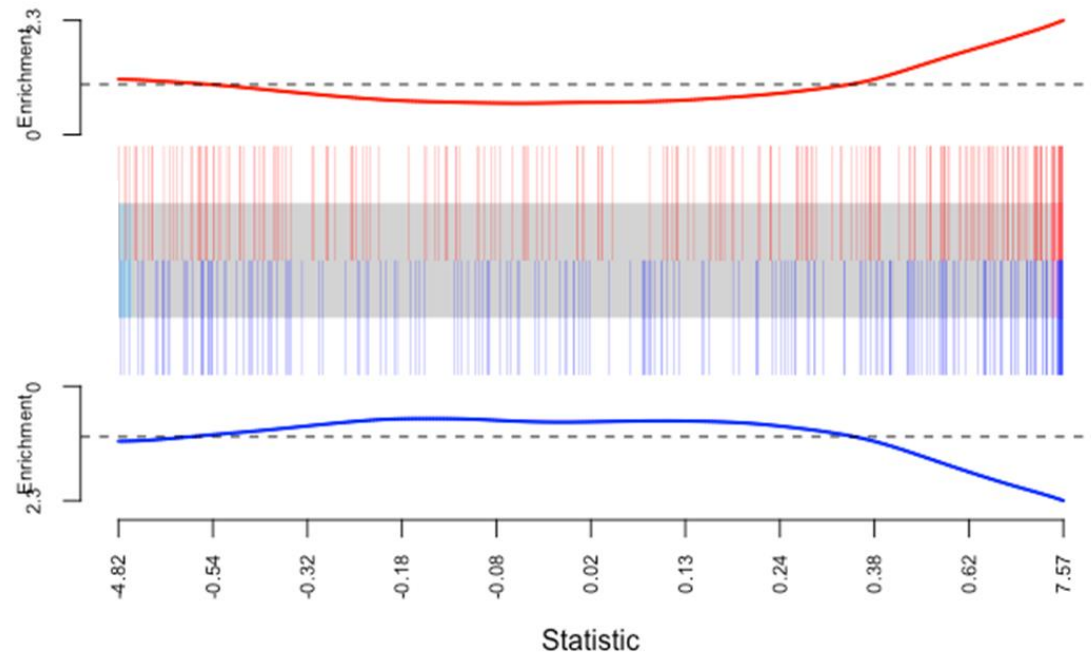
### 5.3.2 Differential expression and GO enrichment analysis

Differential expression between control (day 0) and infected Atlantic salmon head kidney resulted in 1,296 DE genes in the infected group (following multiple-testing correction, FDR p-value < 0.05). Of these genes a similar number of up-regulated and down-regulated genes was found (n = 671 vs. n= 625) (Figure 5.5).

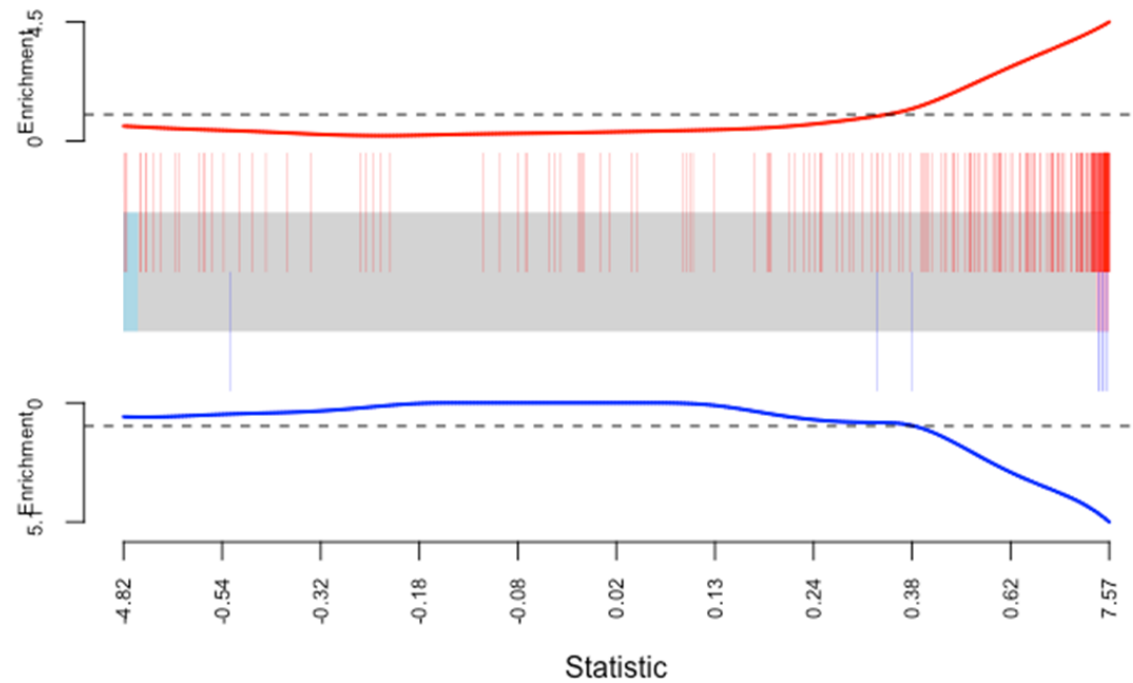
The Langevin et al. (2012) study was re-analysed and the Atlantic salmon and rainbow trout orthologs were added to the metadata, and 717 DE genes were identified between infected and control animals. Of these DE genes, 570 DE were orthologous genes to Atlantic salmon, and 294 DE genes were up regulated. Similarly, Marancik et al. (2015) data was re-analysed and the Atlantic salmon and rainbow trout orthologs were added to the metadata. A total of 3,148 genes were DE, and of these genes 250 genes were orthologous to Atlantic salmon, and 241 DE genes were up regulated.

To test for similarities in the response to *F. psychrophilum* infection between rainbow trout and Atlantic salmon, Atlantic salmon genes were identified, orthologous to the rainbow trout genes that were identified to be DE in the two studies transcriptomic studies by Langevin et al. (2012) and Marancik et al. (2015). These genes were tested if they showed enrichment in the present Atlantic salmon study.

As shown by the barcode enrichment plot in Figure 5.6, for the DE genes identified by Langevin et al. (2012), a significant enrichment of the DE up regulated genes (red lines) was found, while the DE down regulated genes (blue lines) were for the most part not down regulated in the present study of Atlantic salmon. Similarly, the barcode plot for Marancik et al. (2015) (Figure 5.7) shows a significant enrichment of the DE up regulated genes (red lines), while the very few DE down regulated genes (blue lines) were for the most part not down regulated in the present study.



**Figure 5.7** Barcode plot of up regulated gene sets (red bars, top of the plot) and down regulated gene set (blue bar, bottom of plot) of the Langevin et al. (2012) data against the DE genes identified in the Atlantic salmon study (n=717). The curve (or worm) above the barcode shows the relative local enrichment of the bars in each part of the plot. The dotted horizontal line indicates neutral enrichment. All genes are ranked from left to right by increasing log-fold change for the contrast and the genes within the gene set are represented by vertical bars, forming the barcode-like pattern.



**Figure 5.8** Barcode plot of up regulated gene sets (red bars, top of the plot) and down regulated gene set (blue bar, bottom of plot) of the Marancik et al. (2015) data against the DE genes identified in the Atlantic salmon study (n=250). The curve (or worm) above the barcode shows the relative local enrichment of the bars in each part of the plot. The dotted horizontal line indicates neutral enrichment. The dotted horizontal line indicates neutral enrichment. All genes are ranked from left to right by increasing log-fold change for the contrast and the genes within the gene set are represented by vertical bars, forming the barcode-like pattern.

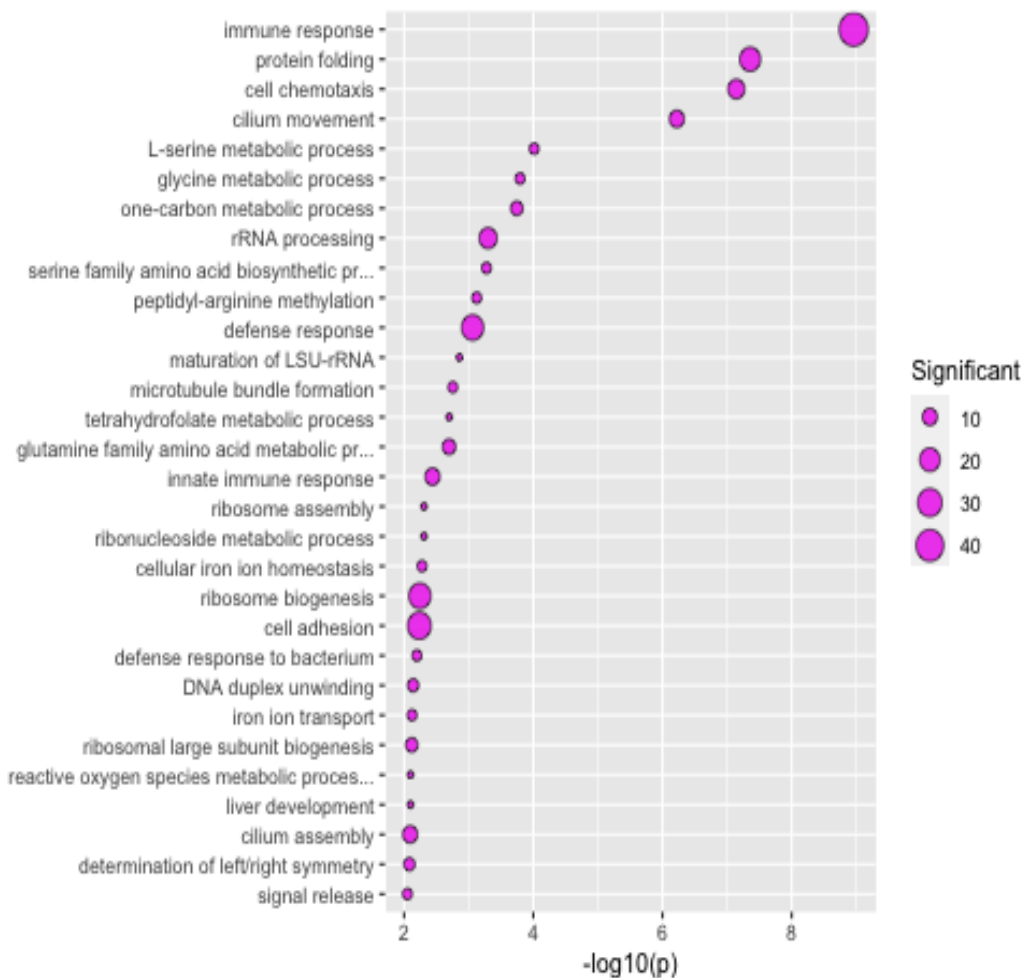
To gain a better overall view of the results, a Gene Ontology (GO) enrichment analysis was performed. Figure 5.9, illustrates significant GO terms irrespective of being up or down regulated. “Immune response” is the most enriched GO term, containing 36 genes, “cell chemotaxis” was also a significantly enriched GO term containing 11 genes and finally “iron cell homeostasis” which contained 4 genes. In the head kidney, several GO terms were clearly over-represented among the DEGs, and these included “immune response”, “negative regulation of apoptotic process”, “cellular amino acid metabolic process” and “response to cytokine” (Figure 5.9).

Amongst the immune-response related transcripts showing differential expression in the head kidney was Interleukin 1 beta (*IL-1 $\beta$* ) and Interleukin 8 (*IL-8*), which had a Log<sub>2</sub> fold change value– (logFC) of 3.9, and logFC= 2.5 respectively in the infected animals, which is strongly upregulated (5.10A). Tumor necrosis factor alpha (*TNF- $\alpha$* ) was also upregulated (logFC= 1.08) in Atlantic salmon head kidney. Furthermore, Toll-like receptor 8 (*TLR8*) was upregulated in Atlantic salmon head kidney (logFC= 0.75) (Figure 5.9). The toll-like receptor 3 (*TLR3*) and toll-like receptor 22 (*TLR22*) were also significantly upregulated (logFC= 0.74 and logFC= 1.12) (Figure 5.9). Finally, another important factor of innate immunity that was up regulated in infected Atlantic salmon head kidney was bactericidal permeability-increasing protein-like (BPI) (logFC= 0.93) (Figure 5.9).

Figure 5.10 (B) illustrates highly upregulated transcript involved in cell chemotaxis in Atlantic salmon head kidney tissues was C-C motif chemokine 4 like (*CCL4*) (logFC= 4.77). C-C motif chemokine 19-like (*CCL19*) was also highly upregulated in this present study (logFC= 3.86). C-C motif chemokine 8 (*CCL8*) and C-X-C motif chemokine 11-like (*CXL11*) were also moderately upregulated cytokines genes (logFC= 2.20 and logFC=1.59 respectively) and complement component 3 (C3) was also upregulated in the current study (logFC= 0.33).

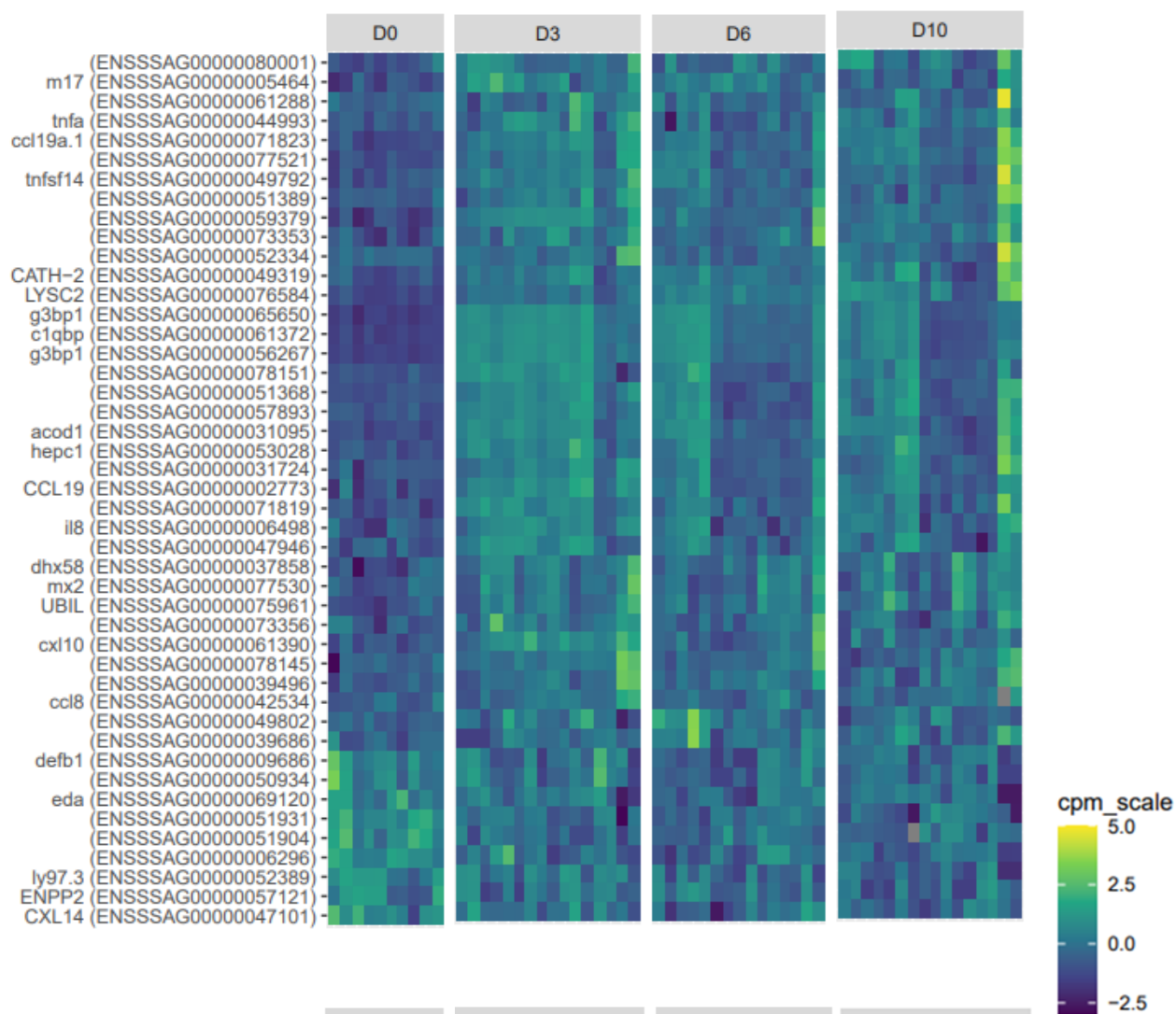
Figure 5.10(E) shows that amongst the cellular iron homeostasis-related transcripts showing differential expression in the head kidney was hepcidin-1, which was highly upregulated in infected animals (logFC= 6.17). Finally, differential expression was observed between ferritin isoforms in Atlantic salmon head kidney. There was a moderate up regulation in Ferritin-M chain (logFC= 1.52) and a down regulation of

Ferritin H-chain and Ferritin L-chain (logFC= -1.08 and logFC=-0.44 respectively) (Figure 5.10).

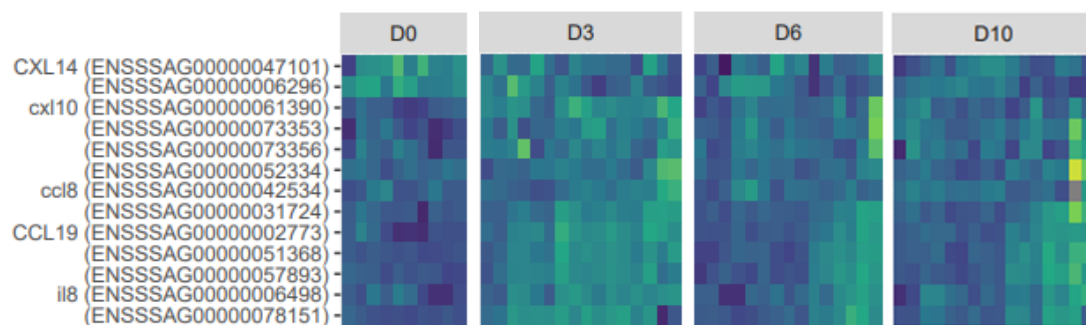


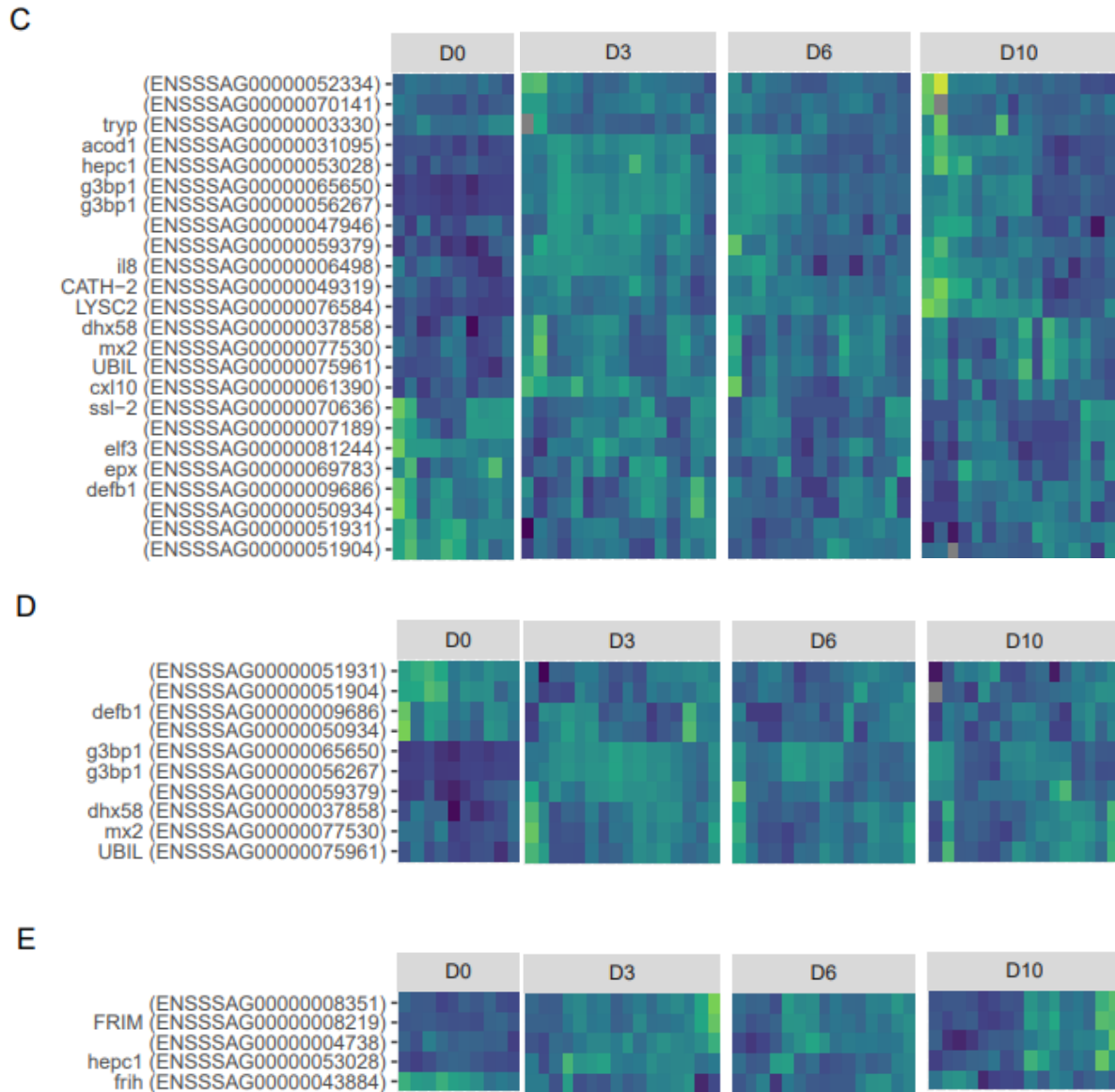
**Figure 5.9** Dot plot visualising significant GO enrichment analysis results (FDR < 0.05). The y-axis displays the different (biological process) GO terms, while the x-axis shows the  $-\log_{10}$  of the Elim p-value (testing for enrichment of the corresponding GO term within the DE genes). The size of the dots corresponds to the number of DE genes associated with each GO term.

A



B





**Figure 5.10** Heatmaps, showing the scaled gene expression for the DE genes annotated to the GO terms: **A:** Immune response GO:0006955; **B:** cell chemotaxis GO:0060326; **C:** defence response GO:0006952; **D:** innate immune response GO:0045087; **E:** cellular iron GO:0006879. In the diagram each column (n=56) represents an individual animal (i.e. head kidney) divided according to the day post challenge. The scale represents row normalised gene expression (expression mean cpm / standard deviation), the lighter the colour in the scale (i.e. yellow), the higher the expression of the gene). The lightest colour (i.e. yellow) in the scale represent the highly expressed genes, and the darkest colour (i.e. dark blue) represent the low expression genes. On the left-hand side of the graphs it can be noted that some genes have not been annotated yet.

### 5.3.3 Cross-species comparison

The rainbow trout transcriptomic studies were re-analysed and in the Langevin et al. (2012) study 717 DE genes were identified between infected and control animals, and 3,148 genes were DE in Marancik et al. (2015). The differential expression results of the re-analysed studies were used to identify the up/down regulated genes that could have been connected via orthology to an Atlantic salmon gene, to define gene-sets.

From Langevin et al. (2012) study 570 DE were orthologous genes to Atlantic salmon, and 294 of these DE genes were up regulated. From Marancik et al. (2015) study, 250 genes were orthologous of Atlantic salmon, and of these DE genes, 241 were up regulated.

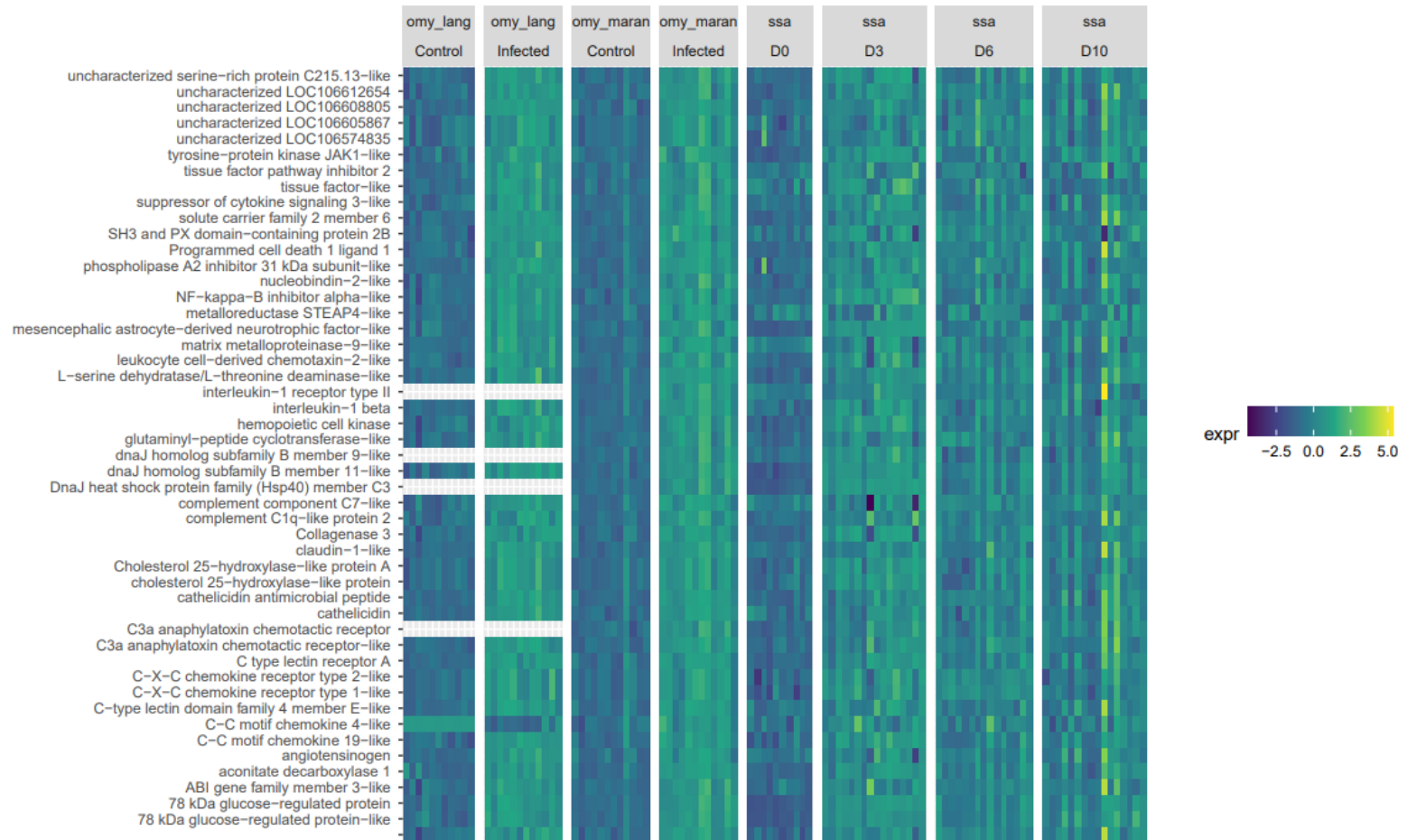
Subsequent gene set enrichment analysis was performed using the *roast* function of the *edgeR* package, setting the significance criteria ( $p\text{-value} < 0.01$ ). The transcriptional response between Atlantic salmon and rainbow trout are similar, and when joining the data from the three different studies, 72 orthologous genes were identified between the current Atlantic salmon RNA-seq study and Langevin et al. (2012) microarray study and Marancik et al. (2015) RNA-seq study in rainbow trout.

Of these 72 DE genes, 67 genes were up regulated across the three studies, 2 genes were up regulated in the rainbow trout studies and down regulated in the Atlantic salmon study, 2 genes were down regulated in the rainbow trout studies and up regulated in the Atlantic salmon study, and finally one gene was down regulated in Langevin et al. (2012) and up regulated in Marancik et al. (2015) and the current Atlantic salmon study. However, no down regulated genes were commonly identified between the studies (Table 5.2).

The 72 genes that were significantly expressed across the three different studies were divided into two main categories by GO analysis: “cellular iron homeostasis” and “immune response”. The common genes identified across the different studies are represented in Figure 5.10, where the scale represents the normalised gene expression scaled for each study.

**Table 5.2** Common DE genes identified across the Langevin et al. (2012), Marancik et al. (2015) and the Atlantic salmon transcriptomic studies.

| <b>Rainbow trout Study<br/>Langevin et al. (2012)</b> | <b>Rainbow trout study<br/>Marancik et al. (2015)</b> | <b>Atlantic salmon<br/>Study<br/>Present study</b> | <b>DE genes<br/>(n)</b> |
|-------------------------------------------------------|-------------------------------------------------------|----------------------------------------------------|-------------------------|
| Down regulated                                        | Down regulated                                        | Up regulated                                       | 2                       |
| Down regulated                                        | Up regulated                                          | Up regulated                                       | 1                       |
| Up regulated                                          | Up regulated                                          | Down regulated                                     | 2                       |
| Up regulated                                          | Up regulated                                          | Up regulated                                       | 67                      |



**Figure 5.11** Heatmap showing the scaled gene expression for the commonly DE genes expressed in rainbow trout following *F. psychrophilum* infection in Langevin et al. (2012) and Marancik et al. (2015) studies and in *F. psychrophilum* infected (sampling points after D0) vs control Atlantic salmon head kidney. The scale represents normalised gene expression (scaled for each gene within each study), with the lightest colour (i.e., yellow) representing the highly expressed genes, and the darkest colour (i.e. dark blue) representing the low expression genes. In Figure 5.10 each column represents a study (i.e., rainbow trout\_ Langevin et al. (2012) [omy\_lang]; rainbow trout\_Marancik et al. (2015) [omy\_maran]; Atlantic salmon [ssa]) and the experimental group (i.e., control vs infected animals).

## 5.4 Discussion

Improving our understanding of the functional basis of genetic resistance and host response to *F. psychrophilum* in Atlantic salmon is valuable for the development of new strategies of disease control. This is the first transcriptome study profiling the response of Atlantic salmon head kidney to *F. psychrophilum* infection *in vivo*. Animals in this study were challenged by immersion with *F. psychrophilum* to mimic a more natural route of infection, and therefore this study possibly targets specific resistance mechanisms more likely to be associated with natural infection, which are overlooked when the fish is infected by injection and the first line of natural defence has been bypassed. In this Chapter, the transcriptional responses during *F. psychrophilum* infection were analysed in control (day 0, n= 10) vs infected fish (sampling points after day 0, n= 46).

The result of this study highlights that the gene expression response of Atlantic salmon to *F. psychrophilum* involves up-regulation of many different components of the immune system (inflammatory response, cytokine production, component activation, tumor necrosis factor and interleukins) as well as tissue repair regulators. The comparison of control (day 0) vs infected animals highlighted enrichment of pathways related to immune response, cell chemotaxis and iron homeostasis. A cross-species study was also performed to compare transcriptional responses in Atlantic salmon to *F. psychrophilum* infection with two transcriptomic studies conducted on rainbow trout, as well as to identify orthologous genes, homologous genes present in different species resulting from direct transmission from a common ancestor (Fang et al., 2010). This present study expands on the work on *F. psychrophilum* by Langevin et al. (2012) and Marancik et al. (2015) who described differential regulation of select genes by microarray and RNA-seq in the anterior head kidney and whole rainbow trout, respectively. Both studies showed an increase in pro-inflammatory cytokines, anti-bacterial effectors, and matrix metalloproteases. Marancik et al. (2015) provided further evidence for upregulation of immune-relevant factors including *TLR5*, *leptin*, *haptoglobin*, and *C-type lectin* and genes associated with adaptive, physiologic, structural, and intracellular process, which have not been identified before. These rainbow trout transcriptomic studies were re-analysed using the most recent rainbow trout genome, and the differentially expressed genes were compared between the different studies. This Chapter highlighted that there were 72 commonly identified

orthologous genes between Atlantic salmon and rainbow trout, of which 67 genes were up regulated. It was expected that a larger number of orthologous genes would have been identified given that Atlantic salmon and rainbow trout are so closely related (Danzmann et al., 2005), and both species were challenged with the same bacteria. These results suggest that the response to *F. psychrophilum* in Atlantic salmon and rainbow trout are similar, however there are some differences which could be associated to the two QTLs that they were found in Atlantic salmon in comparison to the multiple QTLs that were found in rainbow trout (Chapter 2). To date, comparative genomic studies of QTL regions between salmonids have mostly focused on discovering evolutionary similarities in the genetic basis of body and sex related traits, including growth, development, and sexual differentiation (Woram et al., 2003; Reid et al., 2015). This is the first study that aimed at comparing genomic regions associated with resistance to *F. psychrophilum* in both Atlantic salmon and rainbow trout. The results suggest that resistance might be mediated by genes related to immune response, cell chemotaxis and iron homeostasis.

#### **5.4.1 Differentially expressed genes related to immune response**

Cytokines, including interferons (IFNs), interleukins (ILs), tumor necrosis factors (TNFs), colony-stimulating factors, and chemokines, have the ability to stimulate T and B cells and antigen presenting cells, aiding the communication between innate and acquired immune systems in fish (Deng et al., 2022). In rainbow trout, it has been previously observed that higher cytokine abundance in the spleen was correlated with lower *F. psychrophilum* load in the spleen (Yin et al., 2021). In the present study, the transcript levels of *IL-1 $\beta$*  and *IL-8* were higher in the infected Atlantic salmon. *IL-8* is produced by various cells, including macrophages, when inflammation is induced (Zlotnik & Yoshie, 2012). Similar results were observed by Deng et al. (2022), who found that the transcript levels of *IL-1 $\beta$ 1*, *IL-1 $\beta$ 2*, *IL-6*, and *IL-8* continuously increased in the spleen of fish postexposure to *F. psychrophilum*. Similarly, in Atlantic salmon infected by amoebic gill disease, transcriptomic analysis illustrated increase in *IL-1 $\beta$*  in gill tissues in infected animals elucidating *IL-1 $\beta$*  involvement in inflammation in affected tissue (Morrison et al., 2012). Tumor necrosis factor alpha (*TNF- $\alpha$* ) was highly expressed in Atlantic salmon head kidney and this cytokine can trigger its own production as well as that of several interleukins such as *IL-1* and *IL-8* which were also

up regulated in this study, indicating its role in triggering cytokine production and inflammation (Kayama et al., 1997; Wollenberg et al., 1993).

Toll-like receptors (TLRs) play a crucial role in the innate immune system by recognising pathogen-associated molecular patterns (PAMPs) derived from foreign pathogens and self-derived molecules derived from damaged cells, known as damage-associated molecules patterns (DAMPs). TLRs signal recruit specific adaptor molecules (*MyD88* and *TRIF*) which initiate signal transduction pathways that activate the transcription factors NF- $\kappa$ B, IRFs or MAP kinases which determine the innate immune responses by regulating the expression of cytokines, chemokines and type I IFNs (Kawasaki & Kawai, 2014). TLRs play an integrated role as a first line of defence against invading pathogens, where they contribute to the inflammation, immune cell regulation, survival, and proliferation of leukocytes.

Pattern recognition receptor (PRRs), including TLRs, C-type lectin receptor (CLRs) and scavenger receptors (SRs) are important in teleosts to develop a higher specialisation in innate immunity after recognising the pathogens (Deng et al., 2022). Toll-like receptor 8 (*TLR8*), which is located in the endoplasmic reticulum (ER), lysosomes, endosomes and endolysosomes plays an important role in innate immunity (Blasius & Beutler, 2010). In the present Chapter, *TLR8* was up regulated in Atlantic salmon head kidney and similar results were observed in Atlantic salmon infected with salmon alphavirus subtype 3 (Skjæveland et al., 2009). In the present Chapter, the toll-like receptor 3 (*TLR3*) was also up regulated and when this receptor is activated, it leads to pro-inflammatory cytokine secretion (Alexopoulou et al., 2001). Mice deficient in *TLR3* had a 1000-fold augmentation of viral load in the spleen (Tabeta et al., 2004). Another up regulated toll-like receptor toll-like receptor 22 (*TLR22*), which is a cell surface surveillance receptor mostly found in teleosts and it is characterised for detecting extracellular long dsRNA and bacteria (Paria et al., 2018). In orange spotted grouper, *Epinephelus coioides*, challenged with *Vibrio alginolyticus*, *TLR22* has been reported upregulated in the spleen by Ding et al. (2012), and similarly, upregulation of *TLR22* mRNA expression was reported in channel catfish, *Ictalurus punctatus*, and rohu, *Labeo rohita*, liver exposed to *Edwardsiella ictaluri* and *Aeromonas hydrophila*, respectively (Samanta et al., 2014; Zhang et al., 2013). These results clearly indicate that *TLR22* expression can be stimulated by several bacteria.

Another important factor of innate immunity that was upregulated in infected Atlantic salmon head kidney was bactericidal permeability-increasing protein-like (BPI). This glycoprotein is known to possess immunoregulatory effects and plays a significant role in antibacterial and antiviral defence (Sun & Sun, 2016). Sun & Sun (2016) found that tongue sole, *Cynoglossus lingua*, infected with megalocytivirus in the presence of BPI exhibited significantly reduced viral load in the tissue, suggesting BPI inhibitory effects on viral replication. C type lectin receptor A (CLR-A) was up regulated in the three studies after *F. psychrophilum* infection. CLR-A plays an important role in pathogen pattern recognition, specifically carbohydrate structures (Yang et al., 2015). Gram-negative bacteria, such as *F. psychrophilum*, have a cell wall composed of peptidoglycan surrounded by lipopolysaccharide (LPS), phospholipids and proteins (MacLean et al., 2001), therefore CLR-A can be activated upon bacterial recognition, which can explain its upregulation in the present Chapter.

One of the most well-known CLRs is mannose-binding lectin (MBL) which is also part of the complement system, and when this binds to an appropriate carbohydrate or acetylated patterns of microorganisms, it forms MBL-associated serine proteases (MAPSs) complexes which cause the cleavage and deposition of component C3 into C3b, which then leads to lysis and killing of pathogenic bacteria (Degn et al., 2012). Host immune cells have developed negative regulatory mechanisms such as suppressor of cytokine signalling (SOCS) proteins to control inflammatory reactions caused by prolonged exposure to LPS (Duncan et al., 2017). SOCS3, which was up regulated in the cross-species study, has a fundamental role in LPS inflammation by targeting multiple cytokines signalling cascades (Duncan et al., 2017). SOCS3 upregulation contributes to *Th2* down regulation of interferon responses, hence increased disease severity (Sahay et al., 2009). In fish, SOCS gene expression is strongly controlled, and different members have different kinetic response to the same stimulants. In rainbow trout, SOCS3 in head kidney macrophages has the ability to reduce respiratory burst activity, NO production, and bactericidal activity (Zhang et al., 2011). SOCS3 expression in infected macrophages and DCs prevented IL-6-mediated inhibition of *IL-12* secretion, and stimulation of CD4<sup>+</sup> cell-dependent IFN- $\gamma$  expression (Carow & Rottenberg, 2014). SOCS are of the most important regulators of these pathways (Yoshimura et al., 2007). Cathelicidins (CATH) are antimicrobial peptides involved in the suppression of bacterial infections, eliminating the bacteria by

disrupting the cell membrane (Maier et al., 2008). In rainbow trout, CATH-1 was found to be expressed after bacterial challenge with *Vibrio anguillarum*, *Aeromonas salmonicida*, *Escherichia coli* and *Photobacterium damsela* (Chang et al., 2006). Similarly, cathelicidin in Arctic charr, *Salvelinus alpinus*, was up regulated due to infection of the fish with *Aeromonas salmonicida* ssp. *achromogenes*. Five days post-infection, cathelicidin RNA levels increased, suggesting its role not only in the initial but also prolonged immune response (Maier et al., 2008). Cathelicidins also have the potential to directly and indirectly regulate different cell types involved in inflammatory processes and in host defence against pathogens, participating in the neutralisation of bacterial endotoxins, blocking the interaction between the lipoglycan and the lipopolysaccharide binding protein (Dürr et al., 2006). In zebrafish, *Danio rerio*, infected with ten Gram-negative bacteria, the fish-derived cathelicidins *CATH\_BRALE* and *codCath1* have been shown to inhibit the bacteria-induced hyperactivation of the immune system and excessive production of pro-inflammatory cytokines, thus reducing mortality rate (Dürr et al., 2006). These results indicate that cathelicidins were activated in response to *F. psychrophilum* infection to induce membrane permeabilization and cell membrane disruption of the foreign pathogen, which is consistent with previous observations and existing data.

Hemopoietic cell kinase (HCK), a member of the Src family of non-receptor tyrosine kinases (NRTKs), was also up regulated across the cross-species study. HCK is highly expressed in macrophages and is involved in various inflammatory responses involving LPS-induced Tnfa expression (English et al., 1993; Scholz et al., 2000). HCK activates the NLRP3 inflammasome via interaction with NLRP3 and promotion of the apoptosis-associated speck-like protein with a caspase activation and recruitment domain (CARD) oligomerization (Kong et al., 2020). Within tumor-associated macrophages, HCK stimulates podosome formation (actin-rich, adhesive structures on the surface of monocytic myeloid cells) and facilitates extracellular matrix degradation, which triggers immune and epithelial cell invasion (Poh et al., 2015).

#### **5.4.2 Differentially expressed genes related to cell chemotaxis**

Cytokines, including ILs, IFNs and haematopoietic growth factors activate the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathways triggering downstream effects in responsive cells. This explains the up regulation of

tyrosine-protein kinase JAK1-like in the present study, which is involved in the IFN- $\alpha$ /beta/gamma signal pathway, phosphorylates STAT and activates STAT signalling through the activation of other JAK kinases related to signalling receptors (Hellgren et al., 1999). Other important cytokines that were differentially expressed in the cross-species study of the present Chapter were chemokines, including *CCL4* which was highly upregulated. Similar results were observed in orange-spotted grouper, in which *CCL4* has shown to have chemotactic activity for peripheral blood leukocytes, with the ability to up-regulate the transcription of *NF- $\alpha$ 1*, *TNF- $\alpha$ 2*, *IFN- $\gamma$* , *Mx*, *T-bet* and both CD8- $\alpha$  and  $\beta$  chains, illustrating how this gene also induces an inflammatory response (Hsu et al., 2013). Several cytokine genes have been highly expressed, and these are produced by macrophages, neutrophils, dendritic cells (DC) and natural killer cells (Chen et al., 2018). In peripheral lymphoid tissues, *CCL19* directs *CCR7* expression in B cells, naïve T cells and DCs to activate adaptive immunity (Bachmann et al., 2006). *CCL19* has been studied in several fish species and the mRNA expression of this gene is generally up regulated upon pathogen infection (Chen et al., 2018). There are three isoforms of *CCL19*, and these are significantly up regulated after infection with *Flavobacterium columnare* and *CCL19* effectively decreases viral and bacterial numbers in tissues and promotes cytokines and antiviral genes (Chen et al., 2013).

Complement system can be activated to help the immune system to effectively destroy foreign pathogens. In the present Chapter C3 was up regulated, and similar results have been observed in rainbow trout infected with *F. psychrophilum*, where C3 was significantly up regulated at day 3 and 7 post-infection (Deng et al., 2022) and in grass carp experimentally exposed to *Aeromonas hydrophila* (Meng et al., 2019). Head kidney tissues can assist in the synthesis of C3 for combating bacterial infections (Meng et al., 2019). C3 can generate an excess of C3a (the anaphylactic peptide) and as a result recruit and activate circulating macrophages and effector cells, which would then trigger an inflammatory response (Zhou, 2012). These results suggest that the complement system was activated and may contribute to the Atlantic salmon response post- *F. psychrophilum* infection and could therefore explain the low bacterial load detected in Atlantic salmon head kidney by quantitative polymerase chain reaction (qPCR) (Chapter 3) and by immunohistochemistry (IHC) (Chapter 4).

Leukocyte cell-derived chemotaxin-2-like (*LECT2*), another multifunctional cytokine which plays an important function in innate immunity, was upregulated as a result of *F. psychrophilum* infection in both Atlantic salmon and rainbow trout studies. The expression levels of *LECT2* have been reported to be relatively high in the head kidney of Nile tilapia, *Oreochromis niloticus*, following *Streptococcus agalatae* infection (Li et al., 2021), and *LECT2* improved the phagocytic activity of head kidney-macrophages, demonstrating the involvement of this gene in the immune response against a pathogen. CLR is the receptor on *LECT2*, and *LECT2*, during infection, has the ability to upregulate the inflammatory factors *IL-1  $\beta$*  and *IL-10* (Li et al., 2021). In fish, it has also been reported that during bacterial challenge, *TLR2* is considerably upregulated in the head kidney by *LECT2*, and *TLR2* recognises PAMPs and induces the production of cytokines (Lieschke & Trede, 2009).

Cholesterol 25-hydroxylase (*CH25H*) is an INF-induced gene that catalyses the oxidation of cholesterol to 25-hydroxylase (25HC) (Zhang et al., 2019a), and this gene was up regulated in the cross-species comparison study. *CH25H* plays an important role in regulating cholesterol and sterol biosynthesis through nuclear receptors and sterol response element-binding proteins. Studies have shown that *CH25H* inhibits the replication of various viruses, including Porcine Reproductive and Respiratory Syndrome virus and Zika virus (Ke et al., 2017; C. Li et al., 2017). Zhang et al., (2019) found that orange-spotted grouper *CH25H* levels were significantly up regulated during infection with Singapore grouper iridovirus (SGIV) and red-spotted grouper nervous necrosis virus (RGNNV) infection, and that *CH25H* is induced by PAMPs and play an important role in the innate response against these viruses regulating the IFN immune and proinflammatory responses.

#### **5.4.3 Differentially expressed genes related to iron homeostasis**

Iron is a fundamental micronutrient for most living cells and biological availability can be limited under certain physiological conditions affecting cell growth and survival. Pathogenic bacteria have developed mechanisms to sequester iron from their hosts cells, however some hosts have developed nutritional immunity to prevent pathogenic bacteria from obtaining this nutrient (Hood & Skaar, 2012; Skaar & Raffatellu, 2015). Pathogenic bacteria use two common mechanisms to obtain iron from the invaded

hosts, including synthesis of iron siderophores and direct use of heme groups (Richard et al., 2019). The siderophores have high affinity for iron and can outcompete sequestration mechanisms from the host including lactoferrin, transferrin and haem (Wilson et al., 2016).

Amongst the cellular iron homeostasis-related transcripts showing differential expression in the head kidney was *hepcidin-1*, which was highly expressed in infected Atlantic salmon. The iron metabolism and antimicrobial peptide precursor *hepcidin-1* inhibits release of iron from intracellular macrophage pools and is a precursor of an antimicrobial effector (Díaz et al., 2021). In fish, hepcidin plays an important role in response to fish pathogen infection or increased circulating iron loads, and increased hepcidin levels will sequester circulating iron (Rodrigues et al., 2006). Under iron overload conditions, which can result during an infection, high hepcidin levels can be observed (Rodrigues et al., 2006) and, as a result, hepcidin can enhance iron uptake and retention by reticuloendothelial cells, causing ferroprotein to be internalised and blocking the unique pathway of iron transfer from the enterocytes to the plasma (Ganz, 2005). Wei et al. (2018) showed that *hepcidin-1* has a protective effect on grass carp response to *F. columnare* by regulating both the iron distribution and immune gene expression.

Ferritin is a protein with the role of iron sequestration and storage and protein (Muhoberac & Vidal, 2019) and in this study differential expression between the ferritin isoforms in the head kidney was observed, suggesting that *F. psychrophilum* interfered with the iron metabolism and immune response of the host.

The analysis of orthologous proteins provided few characterised proteins; therefore, further investigations are needed to better annotate genes and generate advances in the elucidation of the genetics behind resistance to this bacterial disease in salmonids. The variability between the studies investigated, are likely to be due to differences in experimental design including challenge route, bacterial isolate, bacterial dose, tissue sampled, species studied, and microarray vs transcripts count quantification and analysis. However, even with these differences in methodology, the three studies demonstrated a strong immune response in *F. psychrophilum* challenged fish with differences in the transcriptome of resistant and susceptible fish, associated with immune relevant genes (Figure 5.9)

If the Atlantic salmon work was to be repeated, it should include mock-challenged control groups to be completely satisfied with the observation made in this Chapter, specifically the up and down regulated genes. Another limitation of this study is that the animals used for the RNA-seq, were randomly selected at different points post-challenge, and did not have any typical signs of flavobacteriosis, which could explain in part why the PCA did not show strong separation between the different time points post challenge (i.e., 3 vs 6 vs 10 dpc), therefore in future studies, moribund fish should be analysed. Additionally, the lack of separation in the PCA could be possibly reflecting the variation in the individual response to exposure to *F. psychrophilum*.

Given that this is the first RNA-seq study looking at responses to *F. psychrophilum*, in Atlantic salmon, the gene expression analysis was compared with previous rainbow trout studies, however with the newly developed immersion challenge model (Macchia et al., 2022), more research can be focused on *F. psychrophilum* infection in Atlantic salmon, using different bacterial isolates and analysing different tissues. Nevertheless, this Chapter present the first study to compare Atlantic salmon and rainbow trout responses to *F. psychrophilum* infections, and it highlighted that most of the orthologous genes were up regulated, therefore, further investigations are needed to better annotate genes and generate advances in the elucidation of the genetics behind resistance to *F. psychrophilum*. Future genomics-based studies using *F. psychrophilum* are needed to expand on this knowledge and the molecular pathways involved in response to this bacterial disease. Integrating multiple approaches will lead to a better understanding of pathogen-host immune response interactions. The differentially expressed genes identified and the potential pathways associated to the immune defese against *F. psychrophilum* can provide an insight into the critical immune responses that need to be considered when developing and assessing the effectiveness of a novel vaccine against this mucosal bacterium. For example, Eslamloo et al. (2022) used RNA-Seq to better understand the transcripts and molecular mechanisms underlying the response of Atlantic salmon to *Moritella viscosa* infection and developed qPCR assays for several *M. viscosa*-responsive transcripts which can now be used as valuable biomarkers for monitoring Atlantic salmon response to this bacterial disease.

## 5.5 Conclusion

This was the first report on the transcriptomic profiling of Atlantic salmon head kidney response to *F. psychrophilum* undertaken to understand the complex interactions between host and pathogen. The results of the present Chapter showed activation of pathways associated with chemokine receptor, inflammatory cytokine activity, complement molecules, PRRs, TLR which regulated immune responses, cell chemotaxis and iron homeostasis. For the first time, the response to *F. psychrophilum* was compared between rainbow trout and Atlantic salmon, and the commonly differentially expressed orthologous genes identified, suggesting that there are similar mechanisms that may regulate *F. psychrophilum* in these two salmonid species, however further investigation is needed to explain the differences in responses to this bacterial disease. Overall, this Chapter presents novel information about resistance to *F. psychrophilum* in both Atlantic salmon and rainbow trout, providing an overview of gene expression and possible biological processes and molecular pathways involved in head kidney responses to *F. psychrophilum*, and relevant immune-related candidate genes were identified.

## Chapter 6: General Discussion

Farmed salmonids represent a precious food resource with an estimated global value of £12.7 billion and is the fastest increasing food producing industry (Barasa et al., 2022), however, one of the major challenges facing intensive farming of salmonids is infectious disease (Yáñez et al., 2014). *F. psychrophilum* causes rainbow trout fry syndrome (RTFS) also known as cold-water bacterial disease (CWBD), a salmonid disease that is responsible for considerable losses in rainbow trout farming industries worldwide. To date, much of the research regarding treatment options, including vaccine development, has focused on rainbow trout, however Atlantic salmon are also affected, and *F. psychrophilum* causes septicaemia accompanied with necrotic myositis in this species (Nilsen et al., 2011). Currently, the most common and effective treatment for this pathogen in farmed fish is antimicrobial therapy, such as use of florfenicol, however there is evidence of antibiotic-resistance (Ngo et al., 2017). A commercial oil-based injectable vaccine against *F. psychrophilum* is available to Atlantic salmon farmers in Chile (ALPHA JECT® IPNV-Flavo), however, this vaccine must be delivered by injection, which is not suitable for the fry which is when the fish are most vulnerable to this pathogen (Wahli & Madsen, 2018). To address this issue, the selection of families of fish with resistance or decreased susceptibility to this pathogen offers another possibility for addressing the problems posed by *F. psychrophilum*, reducing economic losses and improving animal welfare (Vallejo et al., 2022; Fraslin et al., 2020; Vallejo et al., 2017; Wiens et al., 2013). These studies have used genetic approaches, specifically quantitative trait loci (QTL) mapping or genome-wide association studies to dissect the genetic architecture of resistance to infection in rainbow trout, and they have pointed toward adaptive mechanisms of resistance to *F. psychrophilum* or towards innate or intrinsic mechanisms. Nevertheless, the underlying mechanisms remain unknown. The advantage of these genome wide association studies (GWAS) is that they enabled the characterisation of the genetic architecture of the trait in each population (Uffelmann et al., 2021).

The first objective of this thesis was to develop and evaluate a reliable and reproducible immersion challenge model for *F. psychrophilum* in rainbow trout and Atlantic salmon (Macchia et al., 2022), which now allows host-pathogen interactions studies to evaluate new therapies, and preventative measures such as resistant fish

breeds. For the first time, Atlantic salmon, and rainbow trout fry from the AquaGen breeding nucleus were challenged with the newly developed challenge model and a GWAS study was then conducted (Chapter 2). A smaller number of fish was challenged under the same conditions, and Atlantic salmon and rainbow trout fry were sampled at 0, 3, 6 and 10 days post challenge (dpc). A sensitive, specific, and inexpensive SYBR Green quantitative polymerase chain reaction (qPCR), designed on the single copy gene  $\beta'$  *DNA-dependent RNA polymerase (rpoC)* was developed, validated, and used to detect and quantify *F. psychrophilum* load from Atlantic salmon and rainbow trout head kidney tissues at the different points post challenge (Chapter 3). *F. psychrophilum* is a mucosal disease, and since immunoglobulin T (IgT) is involved in mucosal immunity in teleost (Tadiso et al., 2011), this is the first study to optimise and report the use of rainbow trout IgT to detect IgT positive cells in Atlantic salmon and rainbow trout mucosal tissues (gills, skin, gut) and head kidney tissues sampled at the different time points by immunohistochemistry (IHC). Additionally, *F. psychrophilum* antibodies were characterised using different immunological techniques, and these antibodies were used to detect and quantify *F. psychrophilum* in the same mucosal and head kidney tissues (Chapter 4). Finally, the RNA-seq analysis of the head kidney transcriptome response to *F. psychrophilum* in Atlantic salmon was analysed. This is the first report looking at gene ontology enrichment analysis to reveal significantly expressed genes involved in the immune response, cell chemotaxis and iron homeostasis in Atlantic salmon head kidney after exposure to *F. psychrophilum*. Additionally, a cross-species study compared this Atlantic salmon RNA-seq study results with transcriptomic studies by Langevin et al. (2012) (microarray study) and Marancik et al. (2015) (RNA-seq study) on *F. psychrophilum* infected rainbow trout to gain a better understanding of the genes/pathways enriched when Atlantic salmon and rainbow trout are exposed to *F. psychrophilum* and to compare the responses in between the two salmonids (Chapter 5). The present thesis provides a platform for further research leading to the identification and elucidation of the mechanisms of resistance to this mucosal disease and provides a pathway towards the selection of *F. psychrophilum* resistant Atlantic salmon strains.

The aim of Chapter 2 was to develop an immersion challenge model for *F. psychrophilum* in Atlantic salmon fry based on a previous model developed for rainbow trout (Hoare et al., 2017), and to compare rainbow trout and salmon challenges for

reliability and reproducibility when establishing the infection with different bacterial isolates (Figure 2.4 and Figure 2.5). The challenge successfully mimicked the natural route of infection of this bacterial disease, and despite the lack of external lesions in moribund or deceased fish, re-isolation rates of *F. psychrophilum* were high and this suggests that the infection was systemic in nature (Table 2.3). These results were not surprising, as in naïve rainbow trout, *F. psychrophilum* infection has been reported to be systemic (Nilsen et al., 2011). From the GWAS it was concluded that resistance against *F. psychrophilum* is a moderately heritable trait in both Atlantic salmon and rainbow trout (Figure 2.9 and Figure 2.10). Similarly to what has been already found in rainbow trout (Fraslin et al., 2018; Vallejo et al., 2014, 2017), this study determined that resistance to *F. psychrophilum* is a polygenic trait, and several consistent small QTLs were identified, however no peaks were identified on chromosome 3 and 8, on which it was expected to detect peaks with significant effects, given that AquaGen has previously found two large-effect QTLs on these chromosomes (data not available). In contrast, in Atlantic salmon, the genetic architecture of the trait appears to be oligogenic, meaning almost duogenic, with two strong QTLs located on chromosome 5 and 9. There was a pressing need for a reliable and reproducible *F. psychrophilum* immersion challenge model that more closely mimicked the natural route of infection (Macchia et al., 2022), and this challenge model now provides the means to investigate host and pathogen interactions and allows for the evaluation of new therapies and preventative measures such as resistant fish breeds and vaccines.

The aim of Chapter 3 was to detect and quantify *F. psychrophilum* in Atlantic salmon and rainbow trout head kidney tissues challenged in Chapter 2 and sampled at different time points using the newly developed SYBR Green qPCR assay to determine whether there was a relationship between fish genotype and bacterial load in head kidney tissues. However, the results highlighted that the genetic association with susceptibility and association at the QTL level is not simple. Low bacterial abundance was detected in the head kidney by qPCR (Figure 3.10 - Figure 3.14), and the lack of external lesions and typical signs of flavobacteriosis in the fish analysed, support once again the suggestion that the immersion challenge caused a systemic infection in both Atlantic salmon and rainbow trout fry. Overall, the newly developed qPCR was specific, sensitive, and cost-effective and as a result this molecular

technique could be applied by fish farms as a point-of-care diagnostic tool for monitoring *F. psychrophilum* infections.

In Chapter 3, it was observed that *F. psychrophilum* infection was not localised in the head kidney, and given that the animals were challenged by immersion, it is suggested that protection against *F. psychrophilum* is likely to be due to induction of mucosal immune response. As a result, in Chapter 4 the levels of specific antibodies, specifically IgT, in mucosal tissues and head kidney of Atlantic salmon and rainbow trout challenged by *F. psychrophilum* were evaluated. A second aim of this chapter was to clone and characterise *F. psychrophilum* monoclonal antibodies (mAbs) using an enzyme-linked immunosorbent assay (ELISA), immunofluorescence antibody test (IFAT), and sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE). *F. psychrophilum* monoclonal antibodies (mAbs) and polyclonal antibody (pAbs) were then applied to detect and quantify *F. psychrophilum* cells in infected Atlantic salmon and rainbow trout tissues using IHC. Monoclonal antibodies have been produced against several bacterial and viral fish pathogens antigens and can be used as biomarkers in aquaculture as useful tools for the rapid immune-diagnosis and control of disease (Adams et al., 1995). The majority of the mAbs investigated in this present study showed good reactivity and specificity to the antigen of *F. psychrophilum*. The mAbs however did not detect *F. psychrophilum* in infected Atlantic salmon and rainbow trout tissue, possibly due to the antigen retrieval technique used to expose the epitopes. If the monospecific binding site is affected by the IHC technique, the mAb cannot recognise the epitopes, and this requires further investigation to develop specific mAbs. As a result, due to these inconsistent results, anti-*F. psychrophilum* pAb 151 was used to detect and quantify *F. psychrophilum* colonies in tissues (Figure 4.10). Bacterial colonies were detected in the epithelial layer of the skin, especially surrounding the mouth and gill lamellae in both infected Atlantic salmon and rainbow trout. However, to overcome the lack of signal when using mAb to detect *F. psychrophilum* by IHC, it would be useful to pool and test multiple mAbs of desired specificities on different infected tissues (Lipman et al., 2005). IgT positive cells were detected in tissues, with the highest detection in the secondary lamellae of the gills. Similarly, Dong et al. (2020) suggested that IgT functions as an important mucosal Ig in teleost nasopharynx-associated lymphoid tissue (NALT), providing local adaptive immunity during bacterial infection. Overall, this was the first

successful study using IHC to detect and quantify *F. psychrophilum* and IgT positive cells in Atlantic salmon tissues following immersion challenge with *F. psychrophilum* (Figure 4.11 and 4.12). The IHC results, complement the qPCR results, as detection of the bacteria in the head kidney was low. In regards with IgT positive cells detection in the spleen, similar results have been reported by Rowena et al., (2017) in rainbow trout spleen vaccinated against *F. psychrophilum*. To understand the mechanism of resistance to this mucosal disease, next it was essential to analyse the transcriptome of resistant and susceptible animals.

The aim of Chapter 5 was to improve our understanding of the functional basis of genetic resistance and host response to *F. psychrophilum* in Atlantic salmon. This is valuable for the development of new strategies of disease control. To characterise the transcriptomic response to *F. psychrophilum* infection in Atlantic salmon head kidney an RNA-sequencing (RNA-seq) study was conducted to compare infected and non-infected Atlantic salmon to *F. psychrophilum*, and this unravelled possible genes and pathways that take part in disease resistance. In fish, the head kidney has hematopoietic functions, involved in phagocytosis, antigen processing, immune memory, and production of immunoglobulin M (IgM) (Geven & Klaren, 2017). The results of Chapter 5 showed that the gene expression response of Atlantic salmon to *F. psychrophilum* involves up regulation of many different components of the immune system, including inflammatory response, cytokine production, component activation, tumor necrosis factor and interleukins, as well as tissue repair regulators (Figure 5.8). The results from this study demonstrate that the bacterial infection induced a marked response with modulation of proinflammatory molecules, and similar responses have been induced in teleosts by many different infectious pathogens (Castro et al., 2015). There is a complex interaction between inflammation on the infecting pathogen and on the survival of the host. Additionally, the transcriptomic data from this study was cross compared with two previous studies on *F. psychrophilum* infection in rainbow trout (Marancik et al., 2015; Langevin et al., 2012). Chapter 5 not only represents the first RNA-seq study on *F. psychrophilum* infected Atlantic salmon head kidney, but also the first comparison between Atlantic salmon and rainbow trout, and orthologous genes identified when these salmonids are exposed to *F. psychrophilum* (Figure 5.10). The inflammatory response begins when the bacteria is recognised by PAMPs, which then trigger downstream release of pro-cytokines, enabling the host to respond

promptly to the infection, and several genes involved in iron homeostasis, including *STEAP-4* and matrix metalloproteinase, are expressed in Atlantic salmon and rainbow trout infected with *F. psychrophilum*. These results were expected as they reflected a general process from pathogen recognition to the inflammatory responses, to the activation of transcription factors which dictate the outcome of the innate immune responses. A more recent study by Deng et al. (2022) on the transcriptome analysis of rainbow trout spleen post-*F. psychrophilum* infection identified upregulation or downregulation of pattern recognition, acute-phase response, complement cascade, inflammatory cytokine, and apoptosis, similar responses observed in the current study. These results provide new insights into the molecular mechanism of the immune response to *F. psychrophilum* infection and are a valuable resource for future research on the prevention and control of this bacterium during salmon culture. The differentially expressed genes identified herein can be used as biomarkers for future studies aimed at development of new treatments, vaccines, or prevention strategies. These data can also be cross-referenced with high power genome-wide association studies to help prioritize putative causative genes and variants that have potential to improve genomic selection programs for genetic improvement of resistance to Atlantic salmon diseases.

In regard to the challenge model, for further studies, more tanks and replication should be included, mock-challenged control groups should be included to be completely satisfied with the results of this thesis. An immersion challenge is considered a more natural way of infection that more closely mimics real-life conditions, however a main disadvantage in the models is that it typically leads to a longer period of time to develop signs of the disease, increasing the costs of the study (Birkbeck et al., 2004). Challenge by intraperitoneal injection could shorten the time to develop signs of disease and reduced time and costs (Meza et al., 2019), therefore comparing the two types of experimental challenges with the same experimental conditions, could help decide on the optimum model to use for *F. psychrophilum* infection. As demonstrated in this thesis, the lack of external signs of the disease could suggest that the infection was systematic in both species, explaining the relatively low *F. psychrophilum* abundance in the head kidney measured by qPCR and IHC, however the lack of clinical signs of the disease could also be associated with the model used to challenge the fish. When looking at the immune responses to this bacterial disease, a large number of the mucosal antibody IgT was found in mucosal tissues by IHC, however

more abundantly around the gills, suggesting that the gills may have trapped the bacteria. During the challenge, water was not sampled from the challenge tanks, however findings indicate that surviving fish can shed the bacteria during the course of the infection (Madetoja et al. 2000, 2002), and additional sources of bacteria, such as biofilm, could have harboured other aquatic microbes which could have influenced the study (Vestby et al., 2020). Fine mapping of loci associated with host resistance provided candidates for further testing, and potentially enabling marker-assisted selection. Comparing gene expression profiles between resistant and susceptible genetic background, this transcriptomic study has contributed to an understanding of gene pathways determining Atlantic salmon response to *F. psychrophilum*. The next step would be to add functional assays to expression and genetic approaches to have a better understanding of resistance mechanisms. Knock-out approaches could be developed to complement these analyses and validate putative candidate genes critical for resistance, and it has been widely used in the medical research community, mainly on mice (Hall et al., 2009). Integrating multiple approaches will lead to a better understanding of pathogen-host immune response interactions. One main limitation with breeding programs to improve disease resistance is the costs associated with this approach. When using genomic selection, individual animals are genotyped for single nucleotide polymorphism (SNP) markers, usually several thousand or tens of thousands, and this technology remains relatively expensive (Griot et al., 2021). In a breeding programme, the genomic breeding values are predicted based on the phenotypes recorded on challenged relatives, and disease challenge is preferred to take place in a dedicated facility to control the infection process to record daily mortality, thus making disease resistance phenotyping expensive (Griot et al., 2021). Additionally, future studies, should focus on the use of modern technologies to advance this research further, for example the use of single cell sequencing which could be used to identify cell-specific gene responses associated with *F. psychrophilum* responses. Single cell transcriptomics involves the global profiling of gene expression in individual cells or nuclei (Daniels et al., 2023). The outcome of infectious disease challenge is the result of immune cell actions, the responses of these cells to pathogens, triggering immune cell activation and differentiation. Single cell transcriptomic can allow information on such processes (Papalexi & Satija, 2018). In Atlantic salmon, single cell transcriptomics has been used to investigate the liver cell atlas in response to *A. salmonicida*, which revealed unique hepatocyte states

associated with infections (Taylor et al. 2022). The work presented in this thesis identified regions associated with resistance to *F. psychrophilum* in Atlantic salmon and rainbow through GWAS. Identifying functional candidate genes related to trait variation through a comparative genomic approach, based on exploring orthologous genes within the associated regions between species. The comparative genomic analyses could be performed to show regions of synteny between chromosomes associated with resistance in Atlantic salmon and rainbow trout, to identify potential homologous regions that are associated with the trait and share similarities between chromosomes from both species. The examined populations could share homologous regions harbouring genes controlling resistance, which could suggest similar genomic regions involved in the regulation of resistance in the two species. Comparative genomics and orthology analysis can help predict functional variants based on sequence conservation (Emms & Kelly, 2015). The identification of functional variants will require functional studies such as genome editing of a specific allele to assess consequences for the trait of interest in cell culture or/and in the whole animal system (Houston et al. 2020). CRISPR-Cas9 genome-editing technique can be the potential solution to preventing diseases for aquaculture sustainability, including flavobacteriosis, by rapidly introducing favourable changes to the genome (Gratacap et al. 2019). Genome editing has been successfully used to investigate disease resistance in Grass carp to reovirus infection (Ma et al. 2018). CRISPR/Cas9 gene editing was used to knockout the DNA sequence of the grass carp Junctional Adhesion Molecule-A (gcJAM-A) and evaluated in vitro resistance against various grass carp reovirus (GCRV) genotypes. Knocking out the gcJAM-A gene resulted in reduction in (GCRV) infection.

Overall, this work provides a better understanding of the immune system and the defence mechanisms of Atlantic salmon to *F. psychrophilum*, and this thesis has updated knowledge on *F. psychrophilum* infection in rainbow trout, which are both important steps forward towards preventing *F. psychrophilum* infections in farmed rainbow trout Atlantic salmon, improving prospects for rearing healthy animals to provide a sustainable source of food for the ever-growing global population

## Appendix

**Table 1.** Resistant and susceptible genotypes on chromosome 5 and 9 of Atlantic salmon individuals used for qPCR analysis (Chapter 3) and RNA-seq analysis (Chapter 5)

| Sample id | Flavo_Chromosome 5 | Flavo_Chromosome 9 |
|-----------|--------------------|--------------------|
| VS-0-23   | N/N                | Y/N                |
| VS-0-27   | Y/N                | N/N                |
| VS-0-33   | Y/N                | N/N                |
| VS-0-35   | Y/Y                | Y/N                |
| VS-0-39   | Y/N                | Y/Y                |
| VS-0-41   | Y/Y                | Y/Y                |
| VS-0-43   | Y/N                | Y/N                |
| VS-0-45   | Y/N                | N/N                |
| VS-0-47   | Y/Y                | Y/N                |
| VS-0-49   | Y/Y                | Y/N                |
| VS-3-13   | Y/N                | Y/N                |
| VS-3-15   | Y/Y                | N/N                |
| VS-3-19   | Y/N                | N/N                |
| VS-3-21   | N/N                | Y/N                |
| VS-3-23   | N/N                | N/N                |
| VS-3-25   | Y/N                | N/N                |
| VS-3-27   | Y/Y                | Y/Y                |
| VS-3-29   | Y/N                | Y/N                |
| VS-3-41   | Y/N                | Y/N                |
| VS-3-42   | Y/N                | Y/N                |
| VS-3-43   | Y/N                | N/N                |
| VS-3-45   | Y/N                | Y/Y                |
| VS-3-46   | Y/Y                | N/N                |
| VS-3-47   | Y/Y                | Y/N                |
| VS-3-48   | Y/Y                | Y/Y                |
| VS-3-49   | Y/Y                | Y/N                |
| VS-3-50   | Y/N                | Y/N                |
| VS-3-7    | N/N                | Y/N                |
| VS-6-22   | Y/N                | Y/Y                |
| VS-6-24   | N/N                | Y/N                |
| VS-6-25   | Y/Y                | Y/Y                |
| VS-6-27   | Y/Y                | Y/N                |
| VS-6-28   | Y/Y                | Y/N                |
| VS-6-31   | Y/Y                | Y/N                |
| VS-6-32   | Y/Y                | Y/N                |
| VS-6-33   | N/N                | Y/Y                |
| VS-6-34   | N/N                | Y/N                |

|          |     |     |
|----------|-----|-----|
| VS-6-35  | N/N | Y/N |
| VS-6-37  | Y/N | N/N |
| VS-6-39  | Y/N | Y/N |
| VS-6-41  | Y/N | Y/N |
| VS-6-42  | Y/Y | Y/Y |
| VS-6-44  | Y/N | Y/Y |
| VS-6-45  | Y/Y | Y/N |
| VS-6-46  | Y/N | Y/Y |
| VS-6-47  | Y/N | Y/Y |
| VS-10-1  | Y/N | Y/Y |
| VS-10-10 | Y/Y | Y/Y |
| VS-10-12 | Y/Y | N/N |
| VS-10-14 | N/N | Y/N |
| VS-10-16 | N/N | N/N |
| VS-10-18 | Y/N | Y/N |
| VS-10-2  | Y/N | Y/Y |
| VS-10-20 | Y/N | Y/Y |
| VS-10-28 | Y/Y | Y/N |
| VS-10-3  | N/N | Y/N |
| VS-10-30 | Y/N | Y/N |
| VS-10-32 | N/N | Y/Y |
| VS-10-34 | Y/N | Y/N |
| VS-10-36 | Y/Y | Y/N |
| VS-10-38 | N/N | Y/Y |
| VS-10-4  | N/N | Y/N |
| VS-10-40 | N/N | Y/N |
| VS-10-42 | Y/N | Y/N |
| VS-10-44 | Y/N | Y/N |
| VS-10-44 | Y/Y | Y/N |
| VS-10-46 | Y/Y | Y/N |
| VS-10-5  | Y/Y | Y/N |
| VS-10-6  | Y/N | Y/Y |
| VS-10-8  | Y/Y | Y/N |

**Table 2.** Resistant and susceptible genotypes on chromosome 3 and 8 of rainbow trout individuals used for qPCR analysis (Chapter 3) and RNA-seq analysis (Chapter 5)

| Sample id | Flavo_Chromosome 3 | Flavo_Chromosome 8 |
|-----------|--------------------|--------------------|
| VT-0-2    | Y/N                | Y/Y                |
| VT-0-34   | Y/N                | Y/Y                |
| VT-0-35   | Y/N                | Y/N                |
| VT-0-36   | Y/Y                | Y/Y                |
| VT-0-37   | N/N                | Y/Y                |
| VT-0-39   | N/N                | Y/N                |
| VT-0-40   | Y/N                | Y/N                |
| VT-0-41   | N/N                | Y/N                |
| VT-0-5    | Y/Y                | N/N                |
| VT-0-6    | Y/Y                | Y/Y                |
| VT-0-9    | Y/N                | Y/N                |
| VT-10-1   | Y/N                | Y/Y                |
| VT-10-10  | N/N                | N/N                |
| VT-10-2   | Y/Y                | Y/Y                |
| VT-10-3   | Y/N                | Y/Y                |
| VT-10-30  | N/N                | Y/Y                |
| VT-10-32  | Y/N                | Y/N                |
| VT-10-33  | N/N                | Y/N                |
| VT-10-34  | Y/N                | N/N                |
| VT-10-35  | Y/N                | Y/N                |
| VT-10-36  | Y/N                | N/N                |
| VT-10-37  | Y/N                | Y/Y                |
| VT-10-39  | Y/N                | Y/Y                |
| VT-10-4   | Y/Y                | Y/N                |
| VT-10-40  | Y/N                | Y/N                |
| VT-10-41  | Y/N                | Y/N                |
| VT-10-42  | N/N                | Y/N                |
| VT-10-43  | Y/N                | N/N                |
| VT-10-5   | N/N                | Y/Y                |
| VT-10-6   | Y/N                | Y/Y                |
| VT-10-7   | Y/N                | Y/N                |
| VT-10-8   | Y/N                | Y/Y                |
| VT-10-9   | Y/Y                | Y/Y                |
| VT-3-1    | N/N                | Y/Y                |
| VT-3-10   | Y/N                | Y/N                |
| VT-3-12   | Y/N                | Y/Y                |
| VT-3-21   | Y/N                | Y/Y                |
| VT-3-23   | Y/Y                | Y/N                |
| VT-3-24   | Y/N                | N/N                |
| VT-3-25   | Y/Y                | Y/N                |
| VT-3-28   | Y/N                | Y/Y                |
| VT-3-3    | Y/Y                | Y/Y                |
| VT-3-4    | Y/Y                | Y/N                |
| VT-3-41   | Y/Y                | N/N                |

|         |     |     |
|---------|-----|-----|
| VT-3-43 | N/N | Y/Y |
| VT-3-44 | Y/N | Y/N |
| VT-3-46 | Y/Y | Y/Y |
| VT-3-6  | Y/Y | Y/Y |
| VT-3-7  | Y/N | N/N |
| VT-3-8  | N/N | Y/Y |
| VT-3-9  | Y/Y | Y/N |
| VT-6-1  | Y/N | Y/Y |
| VT-6-10 | Y/N | Y/N |
| VT-6-2  | Y/N | Y/N |
| VT-6-21 | Y/Y | Y/N |
| VT-6-23 | Y/N | Y/Y |
| VT-6-24 | Y/N | N/N |
| VT-6-25 | N/N | Y/Y |
| VT-6-26 | Y/N | Y/Y |
| VT-6-27 | Y/N | Y/Y |
| VT-6-28 | Y/N | Y/Y |
| VT-6-29 | Y/N | Y/N |
| VT-6-3  | Y/N | Y/Y |
| VT-6-31 | N/N | Y/N |
| VT-6-32 | N/N | Y/Y |
| VT-6-4  | Y/N | Y/Y |
| VT-6-47 | Y/N | Y/Y |
| VT-6-48 | Y/N | Y/N |
| VT-6-49 | Y/Y | N/N |
| VT-6-50 | N/N | Y/Y |
| VT-6-6  | N/N | Y/N |
| VT-6-7  | Y/N | N/N |
| VT-6-8  | Y/N | Y/Y |
| VT-6-9  | Y/Y | Y/N |

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