AQUAEXCEL

Aquaculture Infrastructures for Excellence in European Fish Research

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Deliverable D7.7

Standardized waterborne challenge with *Flavobacterium psychrophilum* for phenotyping host resistance in trout

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Dissemination Level	
PU Public	**X**
PP Restricted to other programme participants (including the Commission Services)	
RE Restricted to a group specified by the consortium (including the Commission Services)	
CO Confidential, only for members of the consortium (including the Commission Services)	

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Glossary

AQUAEXCEL: Aquaculture Infrastructures for Excellence in European Fish Research

Isogenic lines: the INRA isogenic lines of rainbow trout used in the task were obtained by 2 successive generations of uniparental (gynogenesis) reproduction. Within a line, all individuals are fully homozygous and are genetically identical to each other. Those lines were established and maintained at INRA for several generations.

Summary

Objectives: *Flavobacterium psychrophilum* (Fp) is one of the major bacterial pathogens of salmonid fish in freshwater, causing considerable economic losses worldwide. No commercial vaccine is available so far and antibiotic treatments raise both environmental and consumer health issues. Selection for naturally resistant broodstock seems a promising alternative. However, the knowledge on pathogeny and virulence is still very limited. Injection is the most common route of infection for experimental infection. However, a 'natural' challenge is needed to refine phenotypes in experimental approaches aiming at better understanding mechanisms of infection and pathogeny of Fp, to test vaccine candidates or to design efficient selective breeding programmes that could take benefit of the genetic variability at the different steps of host defense. Bath challenge is likely closer than injection to the natural conditions of infection. However, mortality using a bath challenge is usually low and Fp strains convenient for a bath challenge must combine high level of virulence and good growth ability *in vitro*. There is also practical limitation with waterborne infection when a large number of groups have to be compared, because very large volumes of bacterial culture have to be produced simultaneously. Therefore, the objective was to optimize a waterborne challenge with Fp in trout juveniles.

Rationale: The work was organized in 3 different steps:
-1/ optimization of a repeatable immersion challenge. Harvest and culture conditions of the bacterial inoculum were re-optimized and an efficient Fp isolate was selected after testing 5 isolates responsible of recent severe outbreaks in French farms. An immersion procedure (24h exposure at moderate bacterial concentrations) was designed that produce repeatable results and can be used for large-scale experiments.
-2/ comparison of two routes of infection (injection *vs* immersion). In order to maximise the chance to detect differences among lines according to the route of infection, the comparison was performed using the INRA collection of rainbow trout lines already known to exhibit a wide range of resistance after injection with Fp. Several lines highly susceptible after injection appeared among the most resistant after immersion challenge, a result that points to the key role of external first lines of defense that protect the fish during a waterborne challenge but are overridden by injection. Besides, the identification of lines with opposite resistance/susceptibility paves the way for further genetic or functional studies to better understand and exploit the mechanisms of host defense.
-3/ monitoring early infection kinetics in lines exhibiting differing susceptibility to infection, focussing on mucus characteristics expected to be part of the first line of host defense. A nested PCR test specifically designed was used to monitor the presence of Fp in different tissues (spleen, gill and to a lesser extend brain) from lines exhibiting differing mortality after an immersion challenge. Altogether, the detection of Fp tended to correlate with the presence of mortality within a line. Mucus immune characteristics (lysozyme and IgM content) were investigated. Though differences were recorded among lines in both infected and control fish, no clear relationship with resistance/susceptibility could be identified so far.

Teams involved:
Partner 1: INRA, Animal Genetics and Integrative Biology Research Unit and Molecular Virology and Immunology Research Unit
Partner 3: University of Stirling, Institute of Aquaculture, Aquatic Vaccine Unit

Geographical areas covered: among salmonid fish, rainbow trout is the most affected species by flavobacteriosis. The first users of the procedure for waterborne infection with Fp will be research laboratories that develop experimental approaches. Further development of new tools to control the disease (vaccines, selected broodstock) will benefit all areas that produce rainbow trout and are affected by the disease, i.e. almost all producing countries.

1. Context

Flavobacterium psychrophilum (Fp) is one of the major bacterial pathogens of salmonid fish in freshwater, causing considerable economic losses worldwide. It occurs in all regions of the world where salmonid fish are raised: North America, all European countries, Japan and Korea, Tasmania and Chile. Rainbow trout is the most affected species. Called “Rainbow Trout Fry Syndrome” (RTFS) in Europe, the disease is a haemorrhagic septicaemia, but external lesions are frequent, making fish surviving to outbreaks difficult to market. Transmission is both horizontal and vertical (*in ovo*), which has certainly contributed to the wide dissemination of the disease through the eyed eggs market.

The knowledge on pathogeny and virulence of Fp is still very limited. No commercial vaccine is available so far and antibiotic treatments are problematic and raise both environmental and consumer health issues. There is increasing evidence that resistance is under genetic control (description of isogenic lines with disparate resistance, heritability estimates, response to experimental selection) and selection for more resistant broodstock appears as a promising alternative to antibiotics to fight the disease.

Many experimental infection models of rainbow trout by Fp have been published. Injection (intramuscular or intraperitoneal) is reliable and the most common infection route. However, injection likely bypasses the first steps of the host defense (external barriers like skin or mucus) and a reproducible bath challenge is needed to better mimic the natural infection. Some trials using the immersion route of infection have been published, but they included mechanical or chemical damaging of the skin prior to infection and consequently did not fully meet the objectives. Hence, optimization of an appropriate immersion challenge is still needed for both research and finalized goals:

- to refine phenotypes in experimental approaches aiming at a better understanding of infection mechanisms and pathogeny of Fp
- to test vaccine candidates
- to design efficient selective breeding programmes that take benefit of the genetic variability at the different steps of host defense mechanisms.

2. Rationale and approach

The work was organized in 3 different steps:

-1/ optimization of a repeatable immersion challenge. This included (1) choosing the most appropriate bacterial isolate and optimizing its growth conditions to prepare the bacterial inoculum (2) testing bath parameters (bacterial concentration, duration of exposure, fish density, etc.) that can be used for different experimental purposes.

-2/ comparison of two routes of infection (injection *vs* immersion) on the resistance of a set of rainbow trout lines known to exhibit a wide range of resistance following an injection challenge. Reranking of lines according to the infection route would point to the key role of external barriers in host defense mechanisms.

-3/ monitoring early infection kinetics in lines exhibiting differing susceptibility to infection, focussing on mucus characteristics (immune or chemotactic properties) expected to be part of the first line of host defense.

3. Teams involved

Partner 1: INRA, Animal Genetics and Integrative Biology Research Unit and Molecular Virology and Immunology Research Unit
Partner 3: University of Stirling, Institute of Aquaculture, Aquatic Vaccine Unit

Partner 1 was in charge of Steps 1 and 2, and supplied tissue and mucus samples to Partner 3 for step 3. Partner 3 was involved in tissue and mucus sampling at INRA facilities where the infectious challenges for Step 3 were performed, and was in charge of Step 3.

4. Detailed work and results

4.1. Optimization of a repeatable immersion challenge

4.1.1. Optimization of growth and harvest conditions of Fp

Several parameters for growth conditions of Fp were re-examined in order to increase the quality and the consistency of the culture to be used for challenge. Criteria for optimization were growth rate, cell morphology and auto-agglutinating properties of the bacteria, culture yield and viability (colony counts). Using 18°C broth culture, improvements were as follows:
- Anacker and Ordal's replaced by TYES enriched with 5% FCS as the broth medium;
- standardization of the inoculum for pre-cultures and cultures
- use of commercial extrapure deionized water;
- orbital shaking increased to 200 rpm to optimize aeration;
The doubling time (about 3 h) and the growth curve were determined, making it possible to evaluate the approximate number of bacteria per mL prior to the challenge (this number was then systematically confirmed by plate enumeration).

4.1.2. Selection of a Fp isolate for immersion challenge

Mortality after a bath challenge is usually lower than after an injection challenge. Thus, the Fp isolate had to exhibit a good balance between high virulence and good growth *in vitro*.

Preliminary tests performed before Aquaexcel started had indicated that the INRA Fp reference isolate (JIP 02/86) could be efficient for immersion challenge. However, this was not confirmed in the trials performed at the beginning of Aquaexcel. Several batches of the original JIP 02/86 isolate were then collected by Partner 1 and tested by Partner 3 on a standard population of rainbow trout to select the most virulent one; the batch maintained by Partner 3 proved to be slightly more virulent. However, when 12 isogenic lines were challenged by bath using this batch in Year 2 by Partner 1, only 2 of them exhibited mortality higher than 10% [using $>2.10^7$ CFU/mL for 1h]. The low virulence of the isolate was further confirmed on the Sy INRA reference strain of trout [0% mortality, 2.10^8 CFU/mL for 2h].

It was thus decided to search for a novel Fp isolate well adapted to immersion challenge. Partner 1 collected 5 novel isolates from recent severe outbreaks in different French farms. The 5 isolates were genetically characterized using MLST (Multi Locus Sequence Typing) and tested for *in vitro* culture. Three of them formed clumps in broth culture, preventing the correct measurement of OD. The two isolates that exhibited homogeneous broth culture were further tested *in vivo* on the Sy

standard trout population. After intramuscular challenge, only strain 1882/11 showed higher virulence than the reference strain JIP 02/86 (Fig. 1A). When used for immersion challenge, strain 1882/11 induced moderate mortality in the Sy population (Fig. 1B) but further tests performed on a subset of 6 isogenic lines (4h immersion, 3.4 10^7 to 4.95 10^7 CFU/m, 0.5-2 g fish) resulted in a wide range of mortality among lines (from less than 10 to 100% mortality). Despite some inconsistency in the ranking of the lines, this result indicated that strain 1882/11 was appropriate to discriminate between lines using bath infection (data not shown).

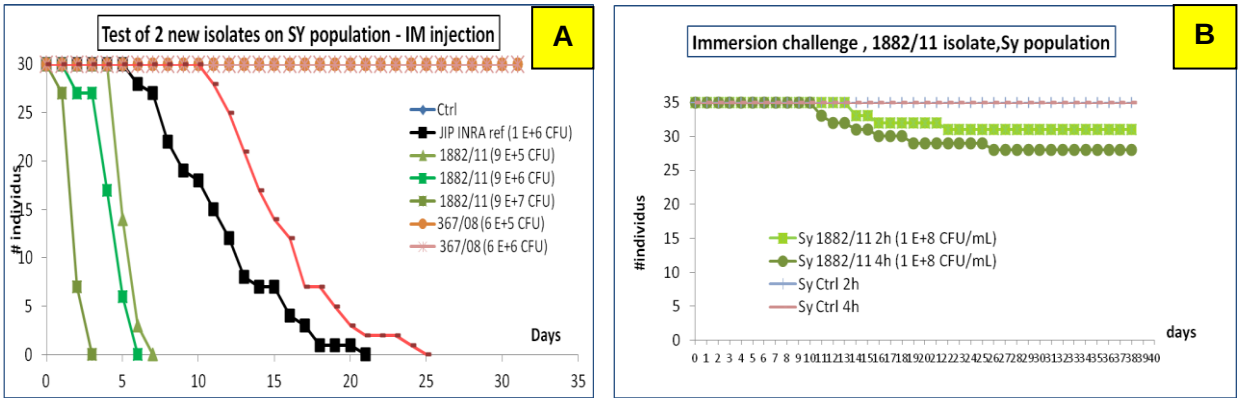

Figure 1: test of virulence of new Fp isolates after intramuscular injection (A) and immersion challenge (B) on the Sy standard population. No mortality was observed after immersion challenge with strains JIP 02/86 and 367/08 (not shown)

4.1.3. Duration of exposure for efficient and tractable immersion challenge

First trials were performed using short duration challenges (i.e., a few hours). A 4-h challenge seemed most effective than a 2-h challenge (Fig. 1B). However, very high bacterial concentrations (around 10^8 CFU/mL) were required to reach significant mortality following such short duration challenges which represented a practical limitation to compare a large number of lines because very large volumes of bacterial culture had to be produced simultaneously. In order to by-pass this problem, further experiments were performed to test the feasibility and efficiency of bath challenges of longer duration (i.e., 24 h).

A preliminary calibration was performed to monitor the water quality in aquaria during 24h after stopping the water flow. Temperature was kept constant (10-11°C) using a refrigeration system and a vigorous aeration maintained oxygen concentration at 10mg/L. However, increased concentration of NH_4^+ was identified as the critical point. In order to limit the concentration below the 1mg/L threshold, the biomass was limited to ~10g fish/L, and fish were fasted 48h before beginning the challenge. In parallel, tests were performed to control the viability of the bacteria when suspended in aquarium water (instead of culture medium): viability was good, and a slight bacterial growth was even recorded after 24h.

After the bath challenge procedure was established, two replicated tests were performed with the Sy population using a range of bacterial concentrations in order to determine efficient doses. Results are shown in Table 1. They indicate that 24-h challenges can induce mortality of the same magnitude than short duration challenges using much lower bacterial concentrations (reduction by 10 to 100 fold). Though there was no clear dose effect, highest mortalities were observed with concentrations around 10^6 or 10^7 CFU/mL.

Concentration	Test 1	Test 2
10^4 CFU/mL	8%	18%
10^5 CFU/mL	8%	10%
10^6 CFU/mL	20%	15%
10^7 CFU/mL	10%	25%

Table 1: mortalities(%) recorded after 24h duration immersion challenge according to the bacterial concentration. Tests were performed with 40 fish of the Sy population per aquarium; mean body weight 1.2g; 5 L water/aquarium; biomass: 45 to 55g/aquarium. The two tests were independent (performed on 2 different days, using 2 different bacterial cultures)

4.2. Comparison of the effect of the two routes of infection

Comparison of the injection and immersion routes of infection was performed with a set of INRA isogenic lines of rainbow trout. Those lines were known to exhibit a wide spectrum of susceptibility to strain JIP 02/86 after intra-muscular injection, which made them a relevant experimental material for this purpose. Indeed, large differences among lines increase the chances to observe a possible re-ranking according to the route of infection.

4.2.1. Immersion challenge of the isogenic lines

Fifteen lines were produced and reared at INRA facilities (Jouy-en-Josas). The genetic status of lines was controlled using a set of microsatellite markers. The 24h immersion challenge was performed when fish weighted around 2-3g, according to the procedure defined previously. In order to minimize weight differences among lines, infection was performed at two different periods: the 11 heaviest lines were infected in May, and the 4 remaining (+ 3 lines included in the first challenge as control) were infected one month later. For each infection period, 2 replicated immersion challenges were performed a few days apart (for each replicate: one line/aquarium, 30 to 50 fish per aquarium and per line). For infection, the volume of each aquarium was adjusted according to the mean weight of the line (10 or 15L) to meet the recommended density (see above). Infectious doses were 2.5 10^5 to 1.7 10^6 CFU/mL (bath 1) and 3 10^5 to 8.8 10^5 CFU/mL (bath 2) at the beginning of the challenge (T0) and 4 10^5 to 2.8 10^6 CFU/mL (bath 1) and 1.7 to 6.5 10^6 CFU/mL (bath 2) at the end of the challenge (T24). Altogether, the challenge was highly repeatable, and lines exhibited a wide spectrum of resistance/susceptibility (0 to 100% survival) (see fig. 2A and 2B).

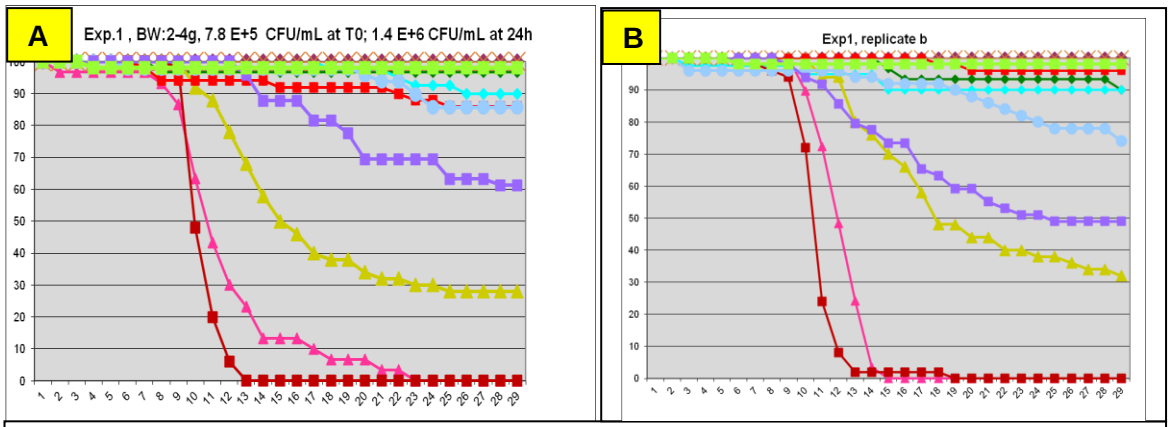

Figure 2: cumulative survival curves of isogenic lines after 24h immersion challenge. The figure illustrates the results of the challenge performed in May (11 lines). A and B correspond to the 2 replicated infections.

4.2.2. Intramuscular challenge of the lines

The 15 lines were infected intra-muscularly 2 months after the immersion challenge (3-10g). Several weeks prior to the challenge, fish were individually tagged with internal transponder (Tiny, Biolog ID), so that fish from all lines were mixed into a single tank for challenge. As for immersion challenge, 2 replicated infections were performed one day apart. Fish were anaesthetized and received an intramuscular injection of 50µL [7 10^2 CFU/fish (challenge 1); 1.5 to 4 10^2 CFU/fish (challenge 2)]. As illustrated in Fig. 3, overall mortality was higher than for immersion challenge, and many lines appeared as highly susceptible (100% mortality).

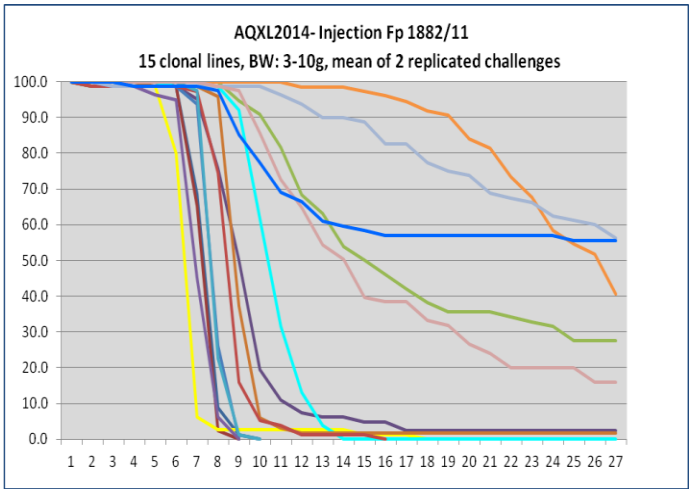

Figure 3: cumulative survival curves of isogenic lines after intramuscular injection. For clarity of the figure, only the mean of the 2 replicated challenges is represented.

4.2.3. Effect of the route of infection

Analyses of the performances of all lines according to the route of infection allowed identification of 3 groups of lines:

- lines that are resistant whatever the route of infection. Such lines do not tell much about any possible protective role of external barriers (skin, mucus) during immersion challenge.
- lines that are highly susceptible after both types of infection. For such lines, no protective effect of external barriers is expected.
- lines that are highly susceptible after IM injection and whose relative rank is improved (i.e., less susceptible) after immersion challenge. Figure 4 illustrates this situation for 2 lines. Such a re-ranking points to external first lines of defense that protect the fish during a waterborne challenge but are overridden after IM infection.

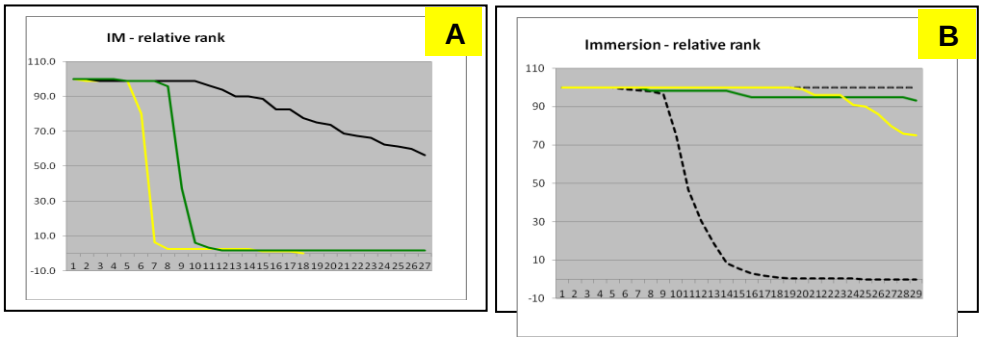

Figure 4: example of reranking of 2 lines (yellow and green) after immersion challenge (A) vs intramuscular (B) challenge. For each route of infection, black curves are the survival cumulative curves of the most extreme lines within the 15 tested lines. In A, the yellow line is the most susceptible one.

4.3. Early infection kinetics and mucus characteristics

4.3.1. Design of a specific nested PCR assay to detect Fp

Many different species of *Flavobacterium* co-exist in fish farms and it is important to establish which of them are indeed Fp. Such a detection tool is also useful to monitor the presence of Fp in lines with differing resistance to infection. A specific nested PCR test was developed to allow quantification of the bacterial loads by measuring the copy numbers of Fp DNA in relation to insulin growth factor (IGF-1) as reference (Figure 5).

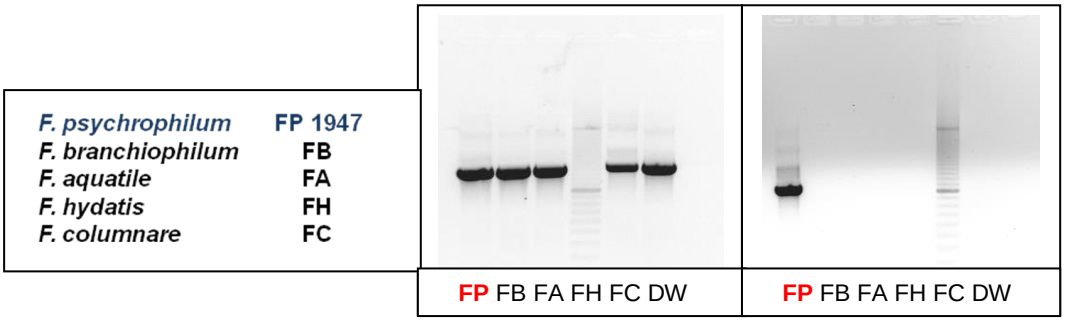

Figure 5: Development of a nested PCR assay specific of Flavobacterium psychrophilum (left: PCR1; right: PCR2) - Sensitivity: 5 fg DNA/PCR tube

4.3.2. Early infection kinetics

A survey of 165 tissues (spleen, gill, brain) sampled from 6 isogenic lines infected by immersion (using strain JIP 02/86) was performed to monitor the presence of Fp. The overall mortality was low in most lines (no mortality in several lines) but reached 21% in the most susceptible line. Altogether, the detection of Fp tended to correlate with the presence of mortality within a clonal line (n=17 in 3 lines); conversely samples from 3 lines exhibiting no mortality, rarely tested positive for Fp (n=2). The majority of PCR positive samples were found to originate from the spleen (n=15) and to a lesser extent from gill samples (n=4). No Fp was detected in the brain. Interestingly, the line with the lowest prevalence of Fp was one of those likely to have efficient protective external barriers.

4.3.3. Skin mucus characteristics

Standard operating procedures were used for skin mucus collection, protein quantification, total IgM quantification and measuring lysozyme. Mucus characteristics were examined for 4 lines chosen among 11 lines challenged by immersion with Fp strain JIP 02/86 (2 replicated aquaria of 40 fish per line). Mucus samples were collected from infected fish (150-200g, IM injection, JIP 02/86) at 3 time points (Day 0 = control, Day 1 and Day 4 post-infection). Significant differences among lines were found for lysozyme and total protein concentration in control fish (before infection) (Figures 6 and 7). Lysozyme activity, total protein and total IgM levels all decreased in mucus following challenge whatever the line (Figures 6, 7 and 8). Changes of mucus activity across time depended on the line (lysozyme and total protein significantly decreased in a resistant line - no mortality -, and total IgM significantly decreased in the most susceptible line). No clear link between mucus characteristics and resistance has been identified so far.

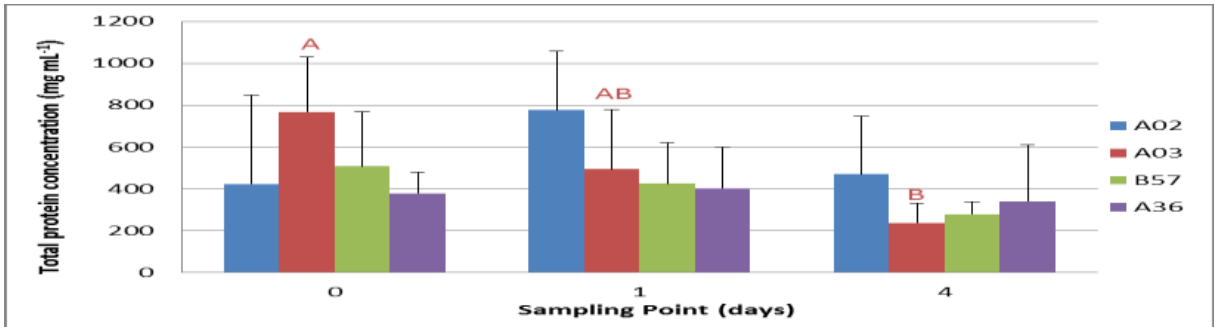

Figure 6: Total protein concentration of mucus from four clonal lines (A02, A03, B57, A36) of rainbow trout following injection with the F. psychrophilum isolate JIP02/86. Statistical significant difference, where P-values <0.05, is indicated by different letter.

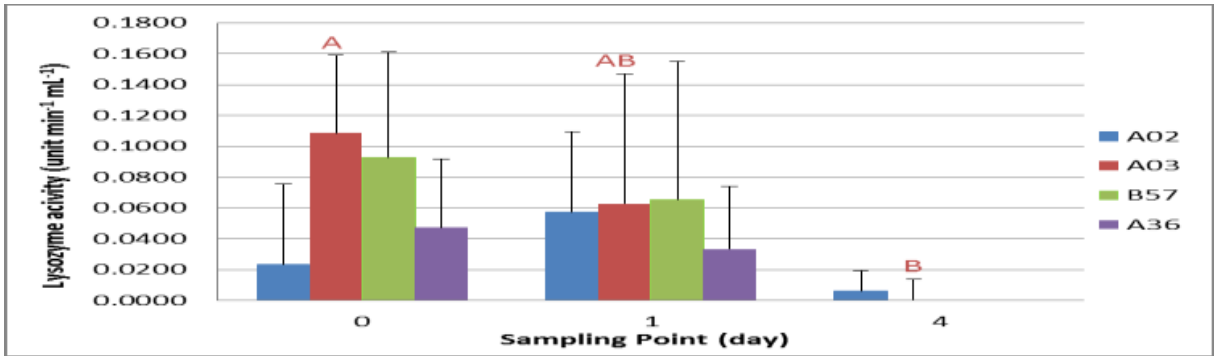

Figure 7: Lysozyme activity of mucus from four clonal lines (A02, A03, B57, A36) of rainbow trout following injection with the F. psychrophilum isolate JIP02/86. Statistical significant difference, where P-values <0.05, is indicated by different letter.

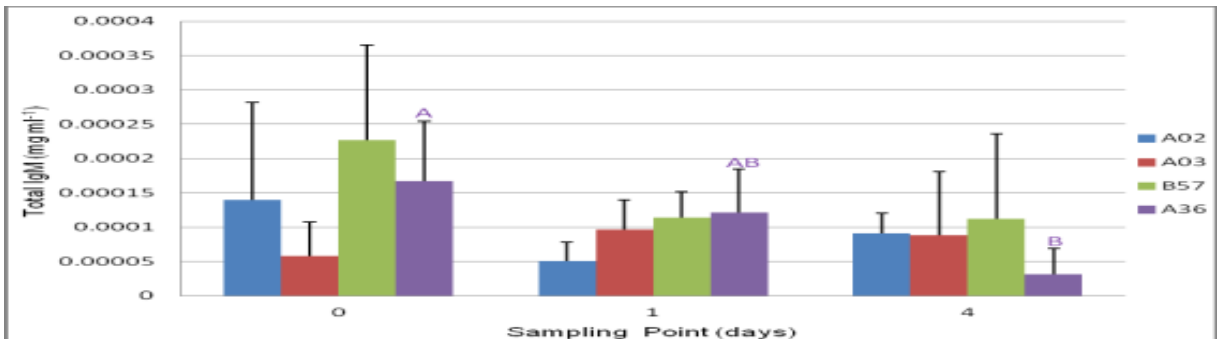

Figure 8: Total IgM of mucus from four clonal lines (A02, A03, B57, A36) of rainbow trout following injection with the F. psychrophilum isolate JIP02/86. Statistical significant difference, where P-values <0.05, is indicated by different letter.

The levels of total IgM and Lysozyme were also measured in the plasma over time for each of the fish clonal lines and these were compared to levels in the mucus (Figures 9 and 10). At Day 0 (pre-injection), while no statistically significant differences were found between the total IgM in mucus and plasma for the two susceptible clonal lines (B57 and A36),statistically significant differences were found between the total IgM in mucus and plasma in the two resistant clonal lines (A02 *P*=0.02; A03 *P*=0.000). In these two resistant clonal lines (A02, A03), there appears to be a parallel pattern of activity in mucus and plasma IgM while the susceptible clonal lines (B57, A36) appear to show an opposing pattern of activity (plasma levels of total IgM increasing as mucus levels of total IgM decreases).

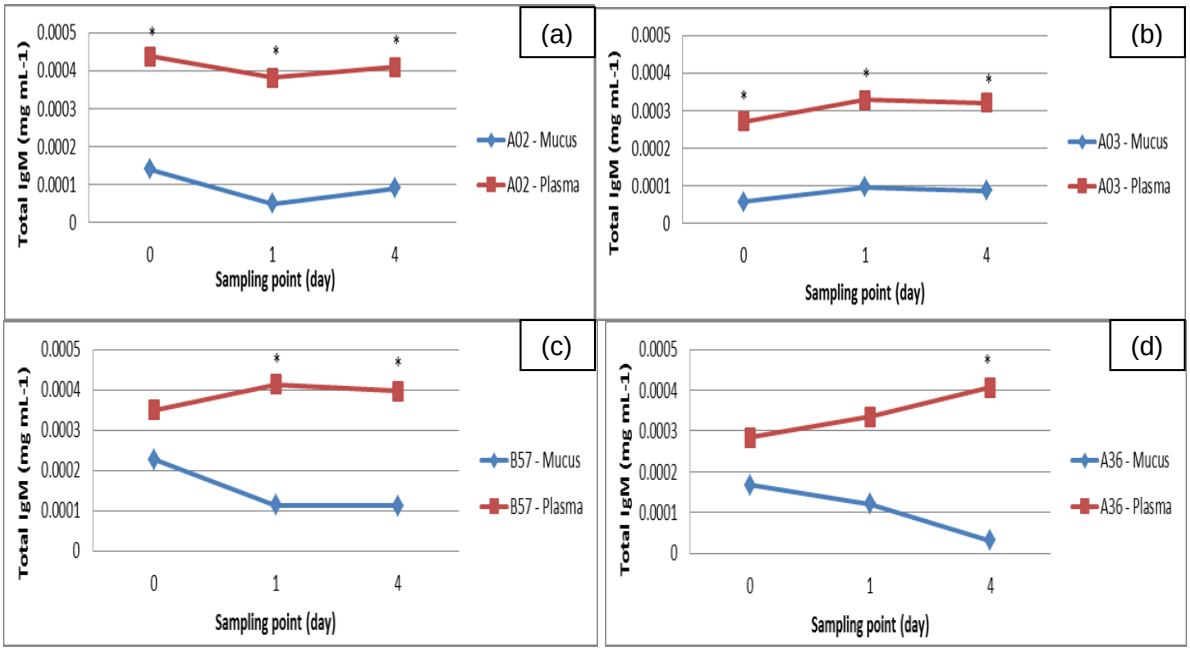

Figure 9: Total IgM of mucus and plasma from (a) clonal line A02 (b) clonal line A03 (c) clonal line B57 and (d) clonal line A36 (pre and post-injection). Statistical significance, where P-value <0.05, is indicated by asterisk (*).

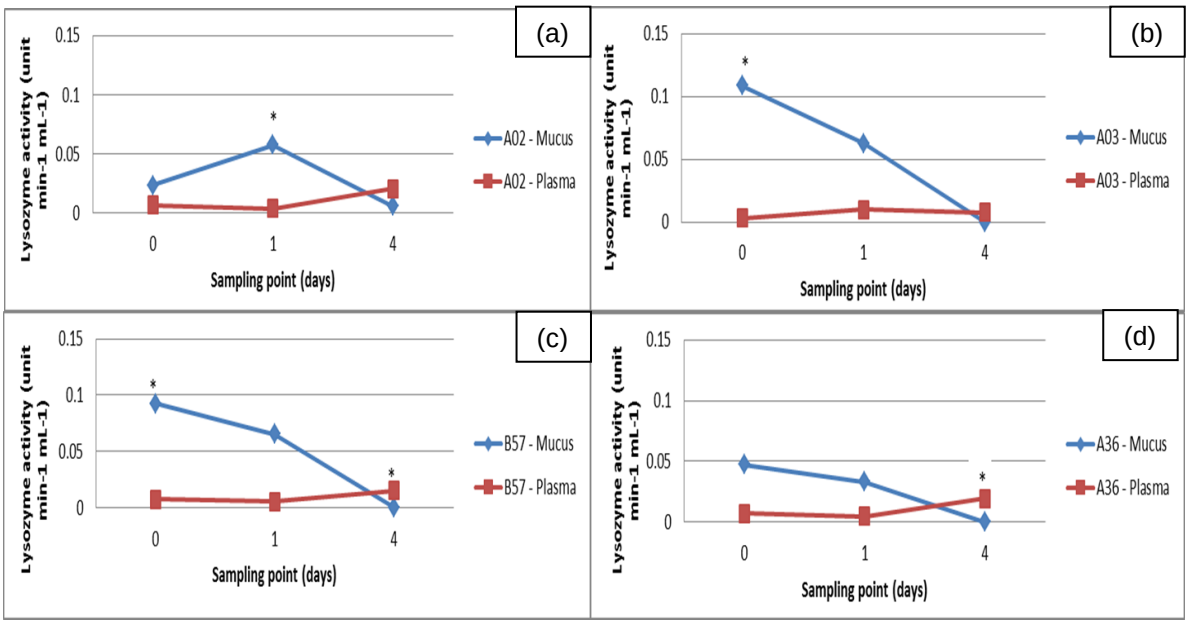

Figure 10: Lysozyme activity of mucus and plasma from (a) clonal line A02 (b) clonal line A03 (c) clonal line B57 and (d) clonal line A36 (pre- and post-injection). Statistical significance, where P-value <0.05, is indicated by asterisk (*).

A second set of mucus samples was collected from these four clonal lines as well as another 10 clonal lines following infection with Fp strain 1882/11. These are currently being analysed by 1D gel electrophoresis, divided into 24 bands, digested and analysed by mass spectrometry (LC-MS/MS). This will provide detailed information on any differences in the mucus components between resistant and susceptible lines.

Outcomes

- **A repeatable immersion procedure** was designed, that can be applied to large-scale experimental challenges. Though overall mortality is still moderate in the Sy standard population (around 20%), it seems appropriate for application in different studies such as estimation of genetic parameters.

- A **specific nested PCR** test was designed to monitor the presence of Fp for a range of purposes (diagnostic, monitoring of infection kinetics)

- **Immersion challenge points to an external first line of defense (likely mucus and skin), which constitutes a key feature** to better understand pathogeny and host defense. However, the role of mucus in the resistance/susceptibility is not fully elucidated so far.

- **Trout lines with a range of response according to the route of infection were identified** that can be used in further studies. For instance, highly susceptible lines will be useful to test vaccine candidates or attenuated bacterial mutants. Resistant *vs* susceptible lines constitute a relevant tool for functional and genetic analyses and have been proposed as experimental material in other ongoing projects.

- **Aquaexcel results served as the foundation for 2 new ongoing projects**. The waterborne challenge procedure and the characterized trout lines and their crosses are being used in a 4-year French joint research-industry project on “Selective breeding of farmed fish for resistance to diseases” (Re-sist- project) and in the EU Fishboost project.

Dissemination:

- Publication 1: Cross effects of bacterial inoculum and route of infection on resistance to Fp in rainbow trout clonal lines
- Publication 2: Mucus characteristics in trout lines exhibiting differing resistance to waterborne infection with *F. psychophilum*