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Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

Carolina Paz Moraleda Chiang



Thesis presented for the degree of Doctor of Philosophy

Genetics and Genomics

The University of Edinburgh

2023

Declaration

I declare,

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Carolina Paz Moraleda Chiang

February 2023

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Abstract

Salmon Rickettsial Syndrome (SRS), caused by the bacterium *Piscirickettsia salmonis*, is one of the most severe infectious diseases threatening the Chilean Atlantic salmon industry. Among the leading causes of mortality and morbidity, SRS significantly affect the seawater production stage, where biomass losses account for a major economic impact.

One potential avenue to tackle SRS is the improvement of host resistance using selective breeding. To accomplish this, insight into the genetic basis of host response, identifying specific genes and pathways involved in this response, and comprehending the potential function these genes have in infection overcome, is valuable.

Consequently, this study aims to identify functional genes and pathways that contribute to genetic host resistance to SRS and investigate the effect of CRISPR/Cas9 knockout on these genes during *P. salmonis* infection.

Candidate genes were identified from a previous *in vivo* large-scale infection study of 2,265 Atlantic salmon smolts injected with *P. salmonis* and genotyped. These data were used to estimate SRS resistance breeding values. Head-kidney and liver samples for RNA-Seq were obtained from 48 individuals at pre-infection, 3 and 9 days post-infection, and tests of differential expression between pre- and post-infection, and between high and low resistance breeding values were performed. From the thousands of differentially expressed genes, enrichment of several KEGG pathways related to immune response such as bacterial internalisation, intracellular trafficking, apoptosis, and inflammasome was observed in both tissues in fish relatively more resistant to infection. A literature review of the biological function of genes in these pathways highlighted the most suitable candidates for functional studies.

Subsequently, five genes related to SRS resistance were successfully edited using a CRISPR/Cas9 Ribonucleoprotein (RNP) transfection to knockout these genes in an Atlantic salmon cell line (SHK-1). An *in vitro* infection challenge model of the knockout and control cell lines with *P.salmonis* was performed to elucidate the impact on cytopathic damage, cell viability and bacterial load during infection. These findings suggest a promising avenue of research into the genetic architecture of host resistance to SRS.

Lay Summary

Salmon Rickettsial Syndrome (SRS), caused by the bacterium *Piscirickettsia salmonis*, is one of the most severe infectious diseases threatening the Chilean Atlantic salmon industry. Among the leading causes of death and severe illness, SRS significantly affect the seawater production stage, where biomass losses account for a major economic impact.

One potential avenue to tackle SRS is the improvement of disease resistance using selective breeding. In this context, disease resistance can be defined as the ability to overcome a bacterial infection. Selective breeding is reproducing animals resistant to SRS to preserve this characteristic in the productive population. Therefore, it is important to know and understand the genetic basis of the host response to infection, identify specific genes and pathways involved in this response, and elucidate the potential function these genes have in infection overcome.

This study aims to identify functional genes and pathways that contribute to genetic host resistance to SRS. To accomplish this, a genome editing technology CRISPR/Cas9 was used to knockout the function of these genes to compare the performance of non-functional cells to functional cells during *P. salmonis* infection. The candidate genes were identified from a previous large-scale infection study of 2,265 Atlantic salmon smolts injected with *P. salmonis* and genotyped. These data were used to estimate SRS resistance breeding values. Tissues from two organs, the liver and head-kidney, were obtained from 48 fishes at the beginning of the experiment, at 3 days and 9 days after infection. RNA-Seq was used to study the differential expression of genes between pre- and post-infected tissues and between high and low resistance breeding values. From the thousands of differentially expressed genes, enrichment of several KEGG pathways related to immune response, such as bacterial internalisation, intracellular trafficking, apoptosis, and inflammasome, was observed in both tissues in fish relatively more resistant

to infection. A further research review of the biological function of genes in these pathways highlighted the most suitable candidates for functional studies.

Five genes related to SRS resistance were successfully edited using a CRISPR/Cas9 Ribonucleoprotein (RNP) transfection to knockout these genes in an Atlantic salmon cell line (SHK-1). An infection challenge of edited knockout cells versus unedited cells infected with *P. salmonis* was used to elucidate the impact on cytopathic damage, cell viability and bacterial load during infection. These findings suggest a promising avenue of research into the genetic architecture of host resistance to SRS.

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List of Abbreviations

16S rRNA	16S ribosomal RNA
23S rRNA	23S ribosomal RNA
ASCs	Antibody-secreting cells
ASK	Atlantic salmon kidney cell line
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BCWD	Bacterial cold-water disease
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like
CBL	E3 ubiquitin-protein ligase CBL-like isoform X1
CDH1	Epithelial cadherin gene
CFD	Cutting frequency determination
CGG	Cumulative genetic gains
CHH	Chinook salmon heart cell line
CHSE-214	Chinook salmon embryo cell line
CHSE-EC	CHSE-214 cell line + EGFP + nCas9
CIK	Carp kidney cells
CMS	Cardiomyopathy syndrome
CO ₂	Carbon dioxide
CPE	Cytopathic effect
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/CRISPR associated effector 9
crRNA	CRISPR RNA
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
Dot/Icm T4SS	Dot/Icm type-four secretion system
dpi	Days post infection
DR	Disease resistance
DSB	Double-strand breaks
ECACC	European collection of authenticated cell cultures

EFSA	European Food Safety Authority
EGFP	Enhanced green fluorescence protein
ELISA	Enzyme-linked immunosorbent assay
EM-90	Piscirickettsia salmonis strain EM-90
EPC	Epithelioma papulosum cyprini cell line
F0	First generation
FAO	Food and Agriculture Organization of the United Nations
FASTA	"FAST-All" text-based format
FBS	Foetal bovine serum
FokI	Restriction endonuclease FokI
GCRV	Grass Carp Reovirus
gDNA	Genomic DNA
GE	Genome editing
GFP	Green fluorescence protein
GMOs	Genetically modified organisms
GS	Genomic selection
GWAS	Genome-wide association studies
HDAD	Host-directed antimicrobial drugs
HDR	Homology-directed repair
ICE	Inference of CRISPR Edits
IDT	Integrated DNA Technologies
IFAT	Immunofluorescence antibody test
IHNV	Infectious hematopoietic necrosis virus
Indel	Insertion or deletion
IPN	Infectious pancreatic necrosis
ISAV	Infectious Salmon Anaemia Virus
JAK/STAT	Janus kinase/signal transducers and activators of transcription
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	Knockout
LF-89	Piscirickettsia salmonis strain LF-89
MAS	Marker-assisted selection
MIT	Massachusetts Institute of Technology

MOI	Multiplicity of infection
mRNA	Messenger RNA
NAE1	NEDD-8 activating enzyme 1
nCas9	Nucleus localised Cas9 protein
NCBI	National Center for Biotechnology Information
NF- κ B	Nuclear factor kappa B
NHEJ	Non-homologous end joining
NLRC3	Protein NLRC3-like
NLRP1	NACHT, LRR and PYD domains-containing protein 1-like
NLRP12	NACHT, LRR and PYD domains-containing protein 12-like
NORM-VET	Norwegian Veterinary Institute
nts	Nucleotides
ORF	Open reading frame
PAM	Protospacer adjacent motif
PBLUP	Pedigree-based Best Linear Unbiased Prediction
PCR	Polymerase Chain Reaction
PCVs	Pathogen-containing vacuoles
PD	Pancreas disease
PUFAs	Polyunsaturated fatty acids
QPCR	Quantitative Polymerase Chain Reaction
QTL	Quantitative trait locus
R ²	Correlation coefficient
RNAi	Interference RNA
RNA-Seq	RNA-sequencing
RNP	Ribonucleoprotein
RT-PCR	Reverse transcription PCR
RVDs	Repeat-variable residues
SERNAPESCA	Servicio Nacional de Pesca y Acuicultura
sgRNA	Single guide RNA
SHK-1	Atlantic salmon head kidney cell line
SLC11A2	Solute carrier family 11 member 2 or DMT1 or NRAMP2
SLC45A2	Solute carrier family 45 member 2 or MATP

SNP	Single nucleotide polymorphism
SpCas9	Streptococcus pyogenes Cas9 nuclease
SRS	Salmon Rickettsial Syndrome
T7ENI	T7 Endonuclease 1
TALEN	Transcription activator-like effector nuclease
TALEs	Transcription activator-like effectors
TGC	Thermal growth coefficient
tracrRNA	Trans-activating CRISPR RNA
TSA	Tryptic soy agar
UN	United Nations
VHSV	Viral hemorrhagic septicemia virus
WBCs	White blood cells
WGD	Whole-genome duplication
WT	Wild type
ZFN	Zinc-finger nuclease
ZFP	Zinc finger protein

Conferences

Conferences Poster Presentation

Moraleda C.P., Robledo D., Gutiérrez A., Del Pozo J., Yáñez J.M., Houston R.D. 2019. Identification of functional genes related to host resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*) using RNA-seq. Aquaculture Europe (AE2019), Berlin, Germany, 7th - 10th October.

Moraleda C.P., Robledo D., Gutiérrez A., Del Pozo J., Yáñez J.M., Houston R.D. 2019. Identification of functional genes related to host resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*) using RNA-seq. 4th International Conference on Integrative Salmonid Biology (ICISB), Edinburgh, UK, 17th - 20th November.

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CHAPTER 1: General Introduction.

1.1. Selective breeding in salmon aquaculture.

The current global human population is 7.7 billion and is expected to rise between 9 and 11 billion within 30 years (Godfray *et al.*, 2010; United Nations, 2019). As the human population grows, the need for high-quality animal protein for human consumption increases proportionately (Gordon, 2018). Thus, this represents difficulty in meeting future global food security demands (FAO, 2020). Aquaculture is a rapidly growing business, with annual production increasing at an average rate of 7.5 percent since 1970. Aquaculture surpassed capture fisheries as the primary source of fish for human consumption in 2018, with a global production of 54.3 million tons of finfish, equating to an estimated value of USD 139.7 billion. Furthermore, overall fish production is forecast to increase from 179 million tons in 2018 to 204 million tons in 2030 (FAO, 2020). Therefore, aquaculture is clearly going to be a critical component of addressing the global food security dilemma.

As a steadily expanding industry, the intensification of aquaculture production raises concerns about its impact. Destruction of habitats, environmental imbalance, disease spread, welfare, and harm to the wildlife species population are significant areas of concern (Ahmed *et al.*, 2019; Jennings *et al.*, 2016). Consequently, intensive research is essential to establish a sustainable and efficient production intensification (FAO, 2020).

A critical component of sustainable intensification in aquaculture is selective breeding for genetic improvement of production traits (Gjedrem & Rye, 2018). The foundation of any organism's genetic improvement programme is selective breeding, in which individuals are chosen to produce offspring with desirable characteristics (Hill, 2001). Terrestrial livestock has

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*).

been domesticated and genetically improved for several millennia, initially through observation and selective mating of chosen parents; recently, tools and methods for objectively measuring genetic gains have been developed (Van Eenennaam, 2017). Global livestock production has increased significantly over the last 60 years; carcass weights increased approximately 30% for chickens and beef cattle due to advances in nutrition, disease control, breeding, and genetics (Thornton, 2010). Additionally, the average annual milk yield per cow increased 5.1 fold from 1924 to 2011, with more than half of this increase attributed to improved genetics (Thornton, 2010). In contrast to terrestrial livestock, modern selective breeding programs support only a relatively small portion of aquaculture production. In 2012, an estimated <10% of global aquaculture production was based on genetically enhanced stocks (Gjedrem, 2012; Gjedrem *et al.*, 2012). Nowadays, the use of genetic technologies has significantly increased this percentage, with over 80% of European aquaculture production (Janssen *et al.*, 2017), and approximately 75% of the top ten species with the highest production volume (finfish, crustacean, and mollusc) benefited from selective breeding programmes (Houston *et al.*, 2020).

Aquaculture comprises an enormously diverse group of finfish and shellfish species, estimated in 543 distinct animal varieties (FAO, 2020). Most farmed aquatic species are either wild-caught or in the early stages of domestication, implying that a significant genetic variation exists for economically valuable traits since the domestication bottlenecks that terrestrial livestock had sustained (Teletchea, 2019) had not been experienced by aquatic species so far (Gratacap *et al.*, 2019; Houston *et al.*, 2020). This key feature is essential, as to be effective, selective breeding requires the presence of genetic variation for the target traits within-species (Hill, 2001). In addition, fish and shellfish species possess a high heritability for economically significant traits and high fecundity with relatively short generation intervals (between 1–4 years) in most species (Gjedrem *et al.*, 2012). Likewise, the

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*).

reproductive biology of finfish and shellfish enables the formation of large nuclear families and the collection of extensive phenotypic data on selection candidates or their close relatives for breeding programmes (Gratacap *et al.*, 2019). Thus, the aforementioned vital characteristics increase aquaculture's potential for significant genetic improvement through high selection intensity and genetic gain without major loss of genetic diversity (Houston *et al.*, 2020).

In salmonid species, selective breeding programmes aimed at genetic improvement have been conducted since the 1970s (Gjedrem *et al.*, 2012). Ever since, high genetic gains have been typical, demonstrating the possibility of cumulative and permanent improvement of important production traits (Houston *et al.*, 2020). The impact of selective breeding in salmonid production programmes has demonstrated cumulative genetic gains (CGG) of 900% in harvest weight and a 200% in thermal growth coefficient (TGC) for farmed rainbow trout (*Oncorhynchus mykiss*) vs. wild rainbow trout (Janssen *et al.*, 2017). Moreover, genetic gain per generation has been estimated to increase by an average of 13% in Atlantic salmon (*Salmo salar*), implying that the growth potential can be doubled in only six generations of selection (Gjedrem & Rye, 2018).

The increasing availability and affordability of genomic tools enable the routine incorporation of molecular genetic markers to improve the prediction of breeding values for target traits (Houston *et al.*, 2020). Two methods can be used to predict breeding values: Marker-assisted selection (MAS) allows selecting breeding individuals based on genetic markers linked to quantitative trait locus (QTL) affecting that trait (Dekkers & Hospital, 2002). Genomic selection (GS) uses genome-wide genetic markers to estimate genomic breeding values to select suitable individuals from a reference population for selective breeding (Houston *et al.*, 2020; Meuwissen *et al.*, 2001). Moreover, the method to use depends on the genetic architecture of the target trait; if a single major-effect locus controls the trait, MAS will be successful (Dekkers &

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*).

Hospital, 2002); however, GS is the preferred method to use if a large number of minor effect loci underpins the genetic variation (Meuwissen *et al.*, 2001, 2013).

Numerous examples arise where the prompt application of MAS resulted in a significant improvement of a production trait. A notable achievement in Atlantic salmon is the identification of a major QTL that accounted for 21% of the phenotypic variation in resistance to infectious pancreatic necrosis (IPN) virus (Houston, Gheyas, *et al.*, 2008; Houston, Haley, *et al.*, 2008; Moen *et al.*, 2009). Other noteworthy achievements are the identification of two crucial QTL explaining half of the total phenotypic variance for cardiomyopathy syndrome (CMS) (Boison, Jingwen Ding, *et al.*, 2019) and one QTL on chromosome 3 significantly associated with resistance to pancreas disease (PD) in Atlantic salmon (Gonen *et al.*, 2015). In addition, critical production traits such as phenotypic age at maturity were influenced by VGLL3 in salmon, explaining between 30% and 40% of the phenotypic variation in this trait (Ayllon *et al.*, 2015; Barson *et al.*, 2015). In rainbow trout, the use of MAS enabled the detection of two major QTL affecting resistance to bacterial cold-water disease (BCWD) in rainbow trout (Liu *et al.*, 2018).

In aquaculture species, genome-wide association studies (GWAS) have revealed that most production-relevant traits are polygenic in nature, implying that a large number of loci with typically minor effects controls these traits (Houston, 2017; Meuwissen *et al.*, 2013). On this subject, the use of GS has been demonstrated to be a suitable and efficient method of utilising DNA marker information for complex traits improvement (Meuwissen *et al.*, 2013). GS aims to estimate the effect of each marker, providing the most accurate estimate of an individual's breeding value. GS assumes that several nearby markers collectively explain the genetic variation associated with a QTL (large or small) (Meuwissen *et al.*, 2013). Typically, genotype and phenotype data from a training population model are used to predict the breeding values of

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selected candidates using only genotype data (Goddard & Hayes, 2007; Meuwissen *et al.*, 2001).

Through the development of the first high-density single nucleotide polymorphism (SNP) array, the incorporation of GS into salmon breeding was possible for the first time in Atlantic salmon (Houston & Macqueen, 2019; Ødegård *et al.*, 2014a), demonstrating its effectiveness in accurately predicting breeding values in breeding programmes (Ødegård *et al.*, 2014a; Tsai *et al.*, 2015). Recently, medium- to high-density SNP arrays have been developed for the majority of important aquaculture species, including: European sea bass (*Dicentrarchus labrax*) (Griot *et al.*, 2021; Peñaloza *et al.*, 2021), channel catfish (*Ictalurus punctatus*) (Liu *et al.*, 2014), rainbow trout (Palti *et al.*, 2015), gilthead seabream (*Sparus aurata*) (Peñaloza *et al.*, 2021) and Nile tilapia (*Oreochromis niloticus*) (Peñaloza *et al.*, 2021). This current availability of single nucleotide polymorphism (SNP) arrays allows to routinely applied GS in breeding programmes with notable benefits in inbreeding control and genetic gains. As previously mentioned, with complex polygenic traits such as resistance to bacterial diseases, GS methods provide a more accurate prediction of breeding values. Multiple studies have demonstrated the value of using genetic markers to improve the accuracy of breeding selection. In gilthead seabream, genetic resistance to the bacterium *Photobacterium damselae*, the causative agent of Pasteurellosis, a substantial improvement in breeding values prediction accuracy using 2b-RAD genotype data was obtained in comparison to the pedigree-based model (Palaïokostas *et al.*, 2016). Similarly, in salmonid species such as rainbow trout, genomic breeding values prediction using DNA (RAD) sequencing and a 57K SNP array for resistance to *Flavobacterium psychrophilum*, the causative agent of BCWD, demonstrated a highest predictive ability compared to pedigree-based estimated breeding values (Vallejo *et al.*, 2016, 2017) (**Table 1.1**). Additionally, genome-wide selection for resistance to *Flavobacterium columnare*, the causative agent of columnaris disease (CD) in rainbow trout, proves an

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*).

increased ability to predict future performance compared to pedigree-based selection (Silva *et al.*, 2019) (**Table 1.1**).

Aquaculture species high fecundity allows the generation of large full- and half-sibling families, advantageous for high-resolution genetic mapping experiments and GWAS studies that further identify genomic regions associated with traits of interest like fillet quality or disease resistance (Fraslin *et al.*, 2022; Houston *et al.*, 2020). Nevertheless, genomic regions typically contain hundreds or thousands of putatively causative variants and dozens of genes, some of which may be non-coding regions influencing transcriptional regulation. Thus, to identify variants and genes that are potentially causal in the genetic variability, incorporating functional genomic techniques with an understanding of the biology of the trait may facilitate the shortlisting (Houston *et al.*, 2020).

1.2. Disease resistance: A target trait to aquaculture.

Disease control poses a significant obstacle to aquaculture production, leading to substantial losses due to high mortality and stunted growth (Houston, 2017). Therefore, salmonid aquaculture success and long-term viability depend heavily on controlling disease propagation (Yáñez, Houston, *et al.*, 2014). Given the nature of salmonid production, exposure to ocean ecosystems and contact with wild species cannot be avoided. Hence, disease prevention through management and biosecurity only represents a formidable challenge (Lafferty *et al.*, 2015). In many finfish species, vaccines and antibiotics are used for the prevention and control of disease outbreaks (Houston, 2017). Nevertheless, effective administration of pharmaceutical tools requires an expensive and inconvenient physical handle, and the outcome is often partially effective (Houston *et al.*, 2020). A prominent example of the impact of disease outbreaks in the aquaculture industry is the catastrophic outbreak of Infectious Salmon Anaemia Virus (ISA) in Chile

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between 2007 and 2010, causing a decrease in the production of Atlantic salmon by over 300.000 tonnes difference between 2005 and 2010 (Asche *et al.*, 2009; Yáñez, Lhorente, *et al.*, 2014).

The criteria for selecting a strategy for disease control is disease-specific. Vaccines may relatively control some pathogens such as ISA virus and salmonid alphavirus (SAV) (Caruffo *et al.*, 2016; Graham *et al.*, 2014; Rozas-Serri, 2022). However, numerous aquaculture diseases rely exclusively upon antibiotics and chemotherapy treatments to control. The extensive utilisation of antimicrobials has been linked to the selection and prevalence of resistant pathogens (Cabello *et al.*, 2013). The emergence of drug-resistant strains of the ectoparasitic copepod sea lice is a prime example (Aaen *et al.*, 2015; Houston, 2017). Moreover, the vast bulk of antimicrobials used in salmonid farming is administered through medicated feed (Miranda *et al.*, 2018). In addition to urinary and faecal excretion, there is an important risk of environmental contamination by the spread of medicated feed (Kemper, 2008). Therefore, developing innovative strategies for disease control is essential to meet the necessities of a growing demand industry.

Doeschl-Wilson *et al.*, (2012) define disease resistance (DR) as the ability of the host to limit the infection by reducing the rate of pathogen replication. In contrast, the opposite constitutes susceptibility. Aquaculture breeders have been interested in DR for nearly three decades, as the first DR-focused breeding programmes began in the early 1990s (Gjøen & Bentsen, 1997). Salmonid species are affected by a broad type of pathogens, including bacteria such as *Flavobacterium psychrophilum*, *Flavobacterium columnare*, *Piscirickettsia salmonis* and *Listonella anguillarum*; viruses such as Infectious pancreatic necrosis virus (IPNV), Infectious hematopoietic necrosis virus (IHNV), Infectious salmon anaemia virus (ISAV), and Salmon pancreas disease virus (SPDV); parasitic agents such as sea lice (*Caligus rogercresseyi* and *Lepeophtheirus salmonis*), and amoebas such

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as *Neoparamoeba perurans* among other agents (**Table 1.1**). Multiple GWAS studies in salmonid species have revealed that the DR trait for bacterial diseases such as *Piscirickettsia salmonis* is typically polygenic in nature (Barría *et al.*, 2018; Barría, Marín-Nahuelpi, *et al.*, 2019; Correa *et al.*, 2015). Hence, GS methods to predict genetic gain have demonstrated an increased reliability versus pedigree-based Best Linear Unbiased Prediction (PBLUP) in Atlantic salmon (Bangera *et al.*, 2017) and rainbow trout (Yoshida *et al.*, 2018). Nevertheless, a negative genetic correlation was found in coho salmon between resistance to *P. salmonis* and harvest weight, two economically important genetic traits. This finding highlights the importance of carefully evaluating traits to incorporate into genetic improvement programmes (Yáñez *et al.*, 2016) (**Table 1.1**). Moreover, a considerable number of studies have shown genetic variation (heritability) for resistance to different infectious diseases in salmonids (**Table 1.1**). This advantageous trait indicates that disease-challenge data obtained from relatives of the selected candidates can be reliably extrapolated to predict DR in a breeding programme (Ødegård *et al.*, 2011; Yáñez, Lhorente, *et al.*, 2014). In order to simulate a natural disease outbreak in the field, disease challenges often include infecting a tagged population of salmon with a viral or bacterial pathogen under standardised environmental conditions (including tank, temperature, feeding, and others). Mortality or survival data are recorded until parameters revert to their initial values (before the pathogen infection). Strong genetic correlations of these traits measured in the field and experimental conditions indicate are suitable indicators of DR in the field setting (Ødegård *et al.*, 2007). Other parameters as bacterial or viral load obtained by QPCR or cell culture, may be used as alternative measures of DR (Yáñez, Houston, *et al.*, 2014). Consequently, disease challenges and GS, combined with other disease preventive and control measures, provide an efficient method for achieving cumulative and permanent advances in breeding resistant stocks (Houston, 2017; Houston *et al.*, 2020).

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*).

Table 1.1. Summary of studies testing genetic variation for disease resistance in salmonid species.

Species	Disease agent	Measurement	Heritability (genetic markers)	Heritability (pedigree)	Reference
Atlantic salmon (<i>Salmo salar</i>)	Sea lice	Lice count	0.33	0.27	Tsai <i>et al.</i> , 2016
		Lice count	0.22	0.27	Tsai <i>et al.</i> , 2016
	<i>Caligus rogercresseyi</i>	Lice count	0.11	0.10	Correa <i>et al.</i> , 2017
	<i>Neoparamoeba perurans</i>	Gill score	0.24	0.25	Robledo <i>et al.</i> , 2018
		Amoebic load	0.25	0.36	Robledo <i>et al.</i> , 2018
		Gill score	0.28	0.32	Boison, Gjerde, <i>et al.</i> , 2019
	<i>P. salmonis</i>	Time to death	0.27	0.18	Bangera <i>et al.</i> , 2017
		Binary survival	0.39	0.26	Bangera <i>et al.</i> , 2017

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Rainbow trout (<i>Oncorhynchus mykiss</i>)	<i>Flavobacterium psychrophilum</i>	Time to death	0.33	0.37	Vallejo <i>et al.</i> , 2017
		Binary survival	0.35	0.35	Vallejo <i>et al.</i> , 2017
		Time to death	0.29	0.31	Vallejo <i>et al.</i> , 2016
		Binary survival	0.45	0.48	Vallejo <i>et al.</i> , 2016
	Infectious pancreatic necrosis virus (IPNV)	Time to death	0.25	0.40	Yoshida <i>et al.</i> , 2019
		Binary survival	0.24	0.35	Yoshida <i>et al.</i> , 2019
	<i>P. salmonis</i>	Time to death	0.45	0.38	Yoshida <i>et al.</i> , 2018
		Binary survival	0.55	0.54	Yoshida <i>et al.</i> , 2018
	Infectious hematopoietic necrosis virus (IHNV)	Time to death	0.23	0.33	Vallejo <i>et al.</i> , 2019
		Binary survival	0.25	0.28	Vallejo <i>et al.</i> , 2019

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	<i>Flavobacterium columnare</i>	Binary survival	0.32	-	Silva <i>et al.</i> , 2019
		Binary survival	0.51	-	Silva <i>et al.</i> , 2019
Coho salmon (<i>Oncorhynchus kisutch</i>)	<i>P. salmonis</i>	Time to death	0.16	-	Yáñez <i>et al.</i> , 2016

Table adapted from Houston *et al.*, 2020

As indicated previously, various studies on salmonid species have attempted to determine the genetic architecture of DR features. Liu *et al.*, (2018) validated the use of MAS for resistance to Bacterial cold-water disease (BCWD) in rainbow trout utilising three SNPs from two key QTL regions on chromosomes 8 and 25. In Atlantic salmon, numerous studies identified important QTLs influencing DR. Two QTLs on chromosomes 27 and 12 explained 7.6% and 31.7% trait variation for Cardiomyopathy syndrome (CMS) (Boison, Jingwen Ding, *et al.*, 2019), one QTL in chromosome 3 explained 23% of within-family variation in Pancreas disease (PD) (Gonen *et al.*, 2015), and a notorious example in the matter is the identification of a major QTL found on chromosome 26, responsible of 80-100% of genetic variation in resistance to Infectious pancreatic necrosis (IPN) (Houston *et al.*, 2008; Moen *et al.*, 2009). Subsequently, the introduction of technology such as high-throughput sequencing permitted the creation of SNP-based genetic assays to predict IPN resistance and obviate disease challenge studies (Houston *et al.*, 2012; Moen *et al.*, 2015). The substantial variation between resistant and susceptible homozygous within and across families and evidence of partial dominance of the resistant allele allowed successful selective breeding for IPNV resistance through family and MAS (Moen *et al.*, 2015; Robledo *et al.*, 2016). Consequentially, in Atlantic salmon selection programmes, the incidence of IPN outbreaks decreased steadily, reaching near-zero mortality in 2015 (Norris, 2017). Additional functional analyses uncovered substantial variation in the gene expression responses of resistant and susceptible salmon to infection. Susceptible fish displayed an exacerbated upregulation of genes associated with inflammation and cytokine activity. In contrast, the resistant fish immune response was moderate, involving up-regulation of the M2 macrophage system (Robledo *et al.*, 2016). Moreover, a potential role for the epithelial cadherin (CDH1) gene in the entry of the virus into host cells has been proposed as the cause of the observed QTL genotype pattern (Moen *et al.*, 2015). Nevertheless, a recent study has implicated the NEDD-8 activating enzyme 1 (NAE1) as a potential candidate for the IPNV resistance QTL,

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highlighted by gene expression analysis. Functional analysis of CRISPR/Cas9 KO and chemical inhibition of NAE1 in Atlantic salmon cell lines demonstrated a significant reduction in IPNV replication vs CDH1 KO cells (Pavelin *et al.*, 2021).

The IPN instance may be an exceptional occurrence since disease resistance is typically a polygenic trait impacted by numerous genes. However, the positive results demonstrate the potential of applying genetic markers to improve the effectiveness and precision of conventional breeding techniques (Norris, 2017). Regarding this, it has been shown that the genetic basis for resistance to the economically significant parasitic disease sea lice is highly polygenic (Correa *et al.*, 2017; Ødegård *et al.*, 2014; Tsai *et al.*, 2016). Therefore, the prediction of sea lice resistance breeding values using GS and genome-wide markers offers a relative advantage of 10–27% over pedigree selection (Correa *et al.*, 2017; Tsai *et al.*, 2016). Moreover, noteworthy findings are three QTLs on chromosomes 3, 18, and 21 in Atlantic salmon, explaining between 7 and 13% of the genetic diversity in resistance to *Caligus rogercresseyi* infestation (Robledo *et al.*, 2019). Additionally, different salmonid species respond to infestations in distinct manners. For instance, coho salmon (*Oncorhynchus kisutch*) exhibit a fast inflammatory response and epithelial hyperplasia resulting in parasite encapsulation and a greater than 90% reduction in lice loads (Fast, 2014). However, Atlantic salmon cannot produce a fully functional immune response and is very susceptible to infestation by sea lice (Fast, 2014). Recently, an international cooperation research project (Scotland, Canada, Norway) awarded a £1.7 million research grant to harness this cross-species variation in sea lice resistance (Fish Farming Expert Editorial, 2022).

1.3. Genome editing in fish species.

Genome editing (GE) is the modification of specifically targeted genomic regions and is considered a highly effective method for assessing gene functions (Yang *et al.*, 2022). Recent developments in genome editing techniques have prompted revolutionary advances in the field of functional genomics. Zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeats associated with the Cas9 protein (CRISPR/Cas9) have enabled efficient and accurate targeted genome editing in a wide variety of organisms, including fishes (Gaj *et al.*, 2013; Zhu & Ge, 2018). This technology, combined with the increasing availability of genome sequencing of fish species, offers enormous potential for functional studies of genes encoding interesting traits (Zhu & Ge, 2018).

ZFN and TALENS technologies utilise designed nucleases comprising sequence-specific DNA-binding domains linked to a non-specific DNA cleavage module. These hybrid nucleases facilitate precise and efficient gene editing by inducing a targeted DNA double-strand break (DSB) that triggers the cellular DNA repair mechanisms, including the error-prone non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Carroll, 2011; Urnov *et al.*, 2010). A ZFN is an engineered endonuclease constituted of a zinc finger protein (ZFP) bound to the cleavage domain of the FokI restriction enzyme. By designing ZFPs with novel sequence specificities, a ZFN can be reconfigured to cleave new targets (Urnov *et al.*, 2010). The cleavage of target DNA requires the dimerization of two ZFNs for the FokI enzyme to cleave the DNA sequence, leading to a DSB at the target sequence (Carroll, 2011). In contrast, TALEs are naturally occurring proteins from the bacteria genus *Xanthomonas*, a plant pathogen. These proteins contain DNA-binding domains composed of 33 to 35 amino acid repeat domains, each of which recognises a single base pair (Deng *et al.*, 2012). Two hypervariable

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amino acids referred to as repeat-variable residues (RVDs) determine TALE specificity. Similar to zinc fingers, modular TALE repeats may be linked to identify contiguous DNA sequences (Gaj *et al.*, 2013). TALEN is advantageous over ZFN because they recognise single nucleotides rather than DNA triplets. This quality simplifies the design process without the restriction of a DNA triplet requirement. TALEN also have shown greater accuracy (H. Kim & Kim, 2014). Although these are advantages, the TALEN system targets a single site at a time, which is a disadvantage if the editing site requires to disrupt a lengthy segment of the genome, such as transposons or promoters (Yang *et al.*, 2022).

CRISPR/Cas9 originated from the adaptive immune system of bacteria and archaea (Horvath & Barrangou, 2010) and was engineered to produce simple, inexpensive, and efficient targeted genome editing (Gratacap *et al.*, 2019). The system combines an endonuclease, the most widely used enzyme derived from *Streptococcus pyogenes* (SpCas9), with an adaptor RNA (**Figure 1.1**). This adaptor is composed of two units, the target genome complementary RNA (crRNA) and the transactivating crRNA (tracrRNA) (Gratacap *et al.*, 2019). Following the annealing of both units, the crRNA identifies the target DNA sequence recognising the presence of a protospacer adjacent motif (PAM). The tracrRNA recruits the Cas9 protein, allowing a targeted DSB endonuclease activity (Jinek *et al.*, 2012). As previously mentioned, two methods are used to repair DSBs (**Figure 1.1**), each of which can be utilised to introduce different types of genome editing. First, the two adjacent strands of DNA can be repaired by an NHEJ route, which is prone to mistakes and generates the insertion or deletion of a few nucleotides. Second, the HDR route can be followed in the presence of a template. This method may be used to insert desired alterations (ranging from a single nucleotide swap to the insertion of an entire chromosomal area) (Doudna & Charpentier, 2014; Mali *et al.*, 2013).

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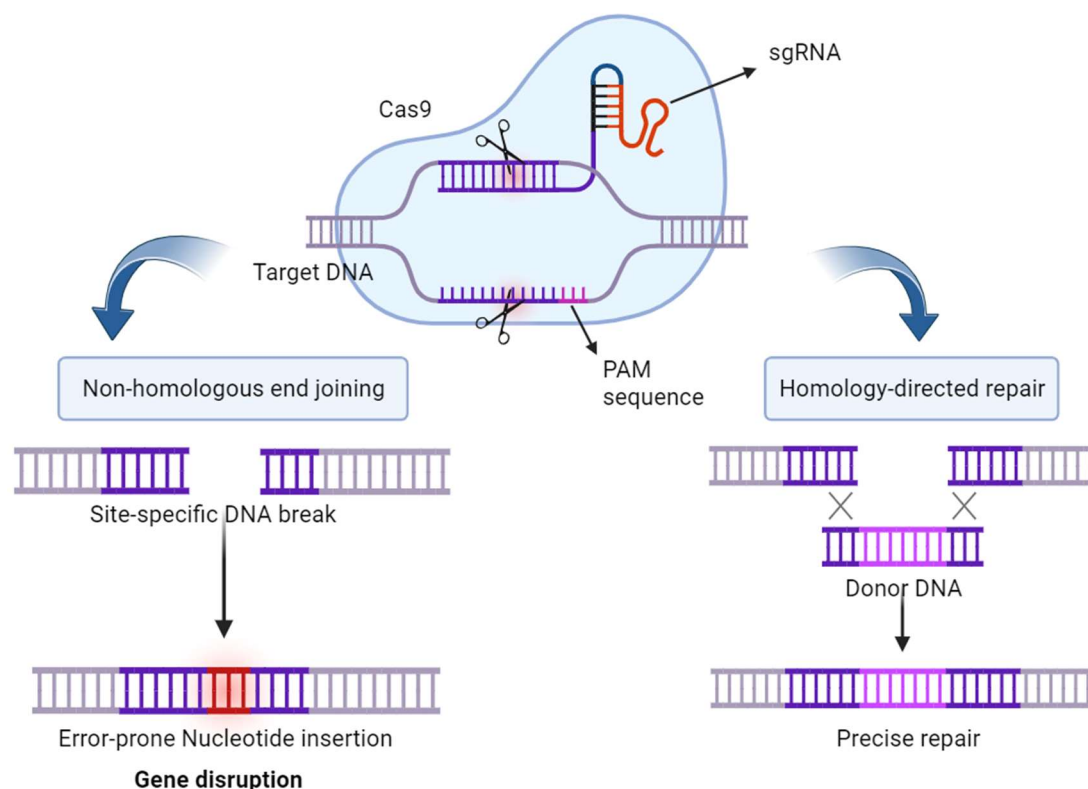


Figure 1.1. CRISPR/Cas9 system components and the induced NHEJ or HDR DNA repair routes. Figure created with BioRender.com.

Genome editing (GE) with CRISPR/Cas9 has dramatically contributed to functional genomic research and enables the rapid genetic improvements of critical features for aquaculture, such as growth, sex, and pigmentation (Yang *et al.*, 2022). Often GE studies are applied in model organisms such as zebrafish (*Danio rerio*) with a proof-of-principle focus (Jao *et al.*, 2013). Typically, targeting genes with an observable phenotype to test the editing success, such as pigmentation (Davis *et al.*, 2021).

Since the first successful evidence of genome editing in Atlantic salmon (Edvardsen *et al.*, 2014), CRISPR/Cas9 has been successfully applied in farmed fish and mollusc species, primarily for gene knockdown and proof of principle studies (Houston *et al.*, 2020). A remarkable milestone in

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CRISPR/Cas9 technology applied to salmonid species is the first gene-specific bi-allelic mutations in the first (F0) generation of Atlantic salmon (Edvardsen *et al.*, 2014). In addition, this technology demonstrated the potential to address challenges in fish farming, such as escapees into wild populations, through the creation of germ-free salmon. Creating sterile animals for aquaculture is a highly desirable feature to preserve the genetic integrity of wild populations and prevent introgression and early maturation (Wargelius *et al.*, 2016). A better understanding of the role played by ELOVL2 in synthesising polyunsaturated fatty acids (PUFAs) has been accomplished by generating a CRISPR-mediated ELOVL2 partial knockout (KO). This model will improve the knowledge of molecular mechanisms in the biosynthesis of PUFA to incorporate vegetable oils in aquafeeds in Atlantic salmon (Datsomor *et al.*, 2019).

Moreover, GE has been used to generate a stable Chinook salmon (*Oncorhynchus tshawytscha*) cell line (CHSE-EC) that overexpresses an integrated EGFP gene and a nucleus localised Cas9 protein (nCas9). The subsequent transient transfection of sgRNA targeting EGFP demonstrated a gene disruption efficacy of 34.6% (Dehler *et al.*, 2016). In summary, using CRISPR/Cas9 to investigate the genes and mutations underpinning economically significant traits is a fundamental objective of aquaculture breeding and genetics research (Houston *et al.*, 2020).

Disease resistance and immunity have been addressed through genome editing. Some studies have developed models to improve our fundamental knowledge of host response to infection in fish (**Table 1.2**). In Rohu (*Labeo rohita*) and Channel catfish, *in vitro* infection challenge models have been developed using CRISPR/Cas9 in stable cell lines. The genes TLR22 (Chakrapani *et al.*, 2016) and TICAM1/RBL (Elaswad *et al.*, 2018) have been successfully knocked out to enable further functional studies. In grass carp kidney cells (CIK), the JAM-A gene was disrupted by the transfection of an all-

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*).

in-one plasmid vector decoding Cas9 and sgRNA simultaneously. Disrupted cells significantly reduced the replication of Grass carp reovirus (GCRV) (Ma *et al.*, 2018). Recently, a notorious example of the potential GE has elucidating disease resistance genes function has demonstrated the importance of NEDD8 Activating Enzyme E1 Subunit 1 (NAE1) gene in the replication and survival of the Infectious pancreatic necrosis virus (IPNV) in Atlantic salmon (Pavelin *et al.*, 2021). In addition, Strømsnes *et al.*, (Strømsnes *et al.*, 2022) have optimised two CRISPR/Cas9 delivery methods (RNP and plasmid) to obtain high editing efficiencies (90 to 100%) in CHSE-214, ASK and SHK-1 cell lines and reported cloned isolation and expansion of edited SHK-1 for the first time.

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Table 1.2. Summary of studies using genome editing to address improvement of disease resistance.

Species	Pathogen/ Disease	Edited gene	Notable features	References
Rohu (<i>Labeo rohita</i>)	Double-stranded RNA viruses, bacteria, and parasites such as fish lice.	TLR22	A carp model system was developed to elucidate the impact of TLR22 disruption in infection challenges.	Chakrapani <i>et al.</i> , 2016
Grass carp (<i>Ctenopharyngodon idellus</i>)	Grass Carp Reovirus (GCRV)	JAM-A	KO-JAM-A grass carp kidney cells reduced GCRV infection for two different genotypes.	Ma <i>et al.</i> , 2018
Channel catfish (<i>Ictalurus punctatus</i>)	Model of study	TICAM1/RBL	Successful KO the TICAM1 and RBL gene as a model for genome editing in channel catfish	Elaswad <i>et al.</i> , 2018
American alligator <i>Alligator mississippiensis</i> Knock-In Channel catfish (<i>Ictalurus punctatus</i>)	Model of study	CAMP	Successful Knock-In of the alligator's cathelicidin gene in channel catfish genome.	Simora <i>et al.</i> , 2020

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Olive flounder (<i>Paralichthys olivaceus</i>)	Viral haemorrhagic septicaemia virus (VHSV)	MAF1	PoMaf1 gene disruption increased the VHSV glycoprotein (G) mRNA levels during VHSV infection	Kim <i>et al.</i> , 2021
Atlantic salmon (<i>Salmo salar</i>)	Infectious pancreatic necrosis virus (IPNV)	NAE1	KO-NAE1 cells demonstrated a substantial decrease in IPNV replication.	Pavelin <i>et al.</i> , 2021
Atlantic salmon (<i>Salmo salar</i>) Chinook salmon (<i>Oncorhynchus tshawytscha</i>)	Infectious salmon anaemia virus (ISAV)	CR2/MMP9	CRISPR/Cas9 RNAP and plasmid editing optimisation, successful clonal isolation and expansion of edited SHK-1 cell line.	Strømsnes <i>et al.</i> , 2022

As previously mentioned, genomic technologies (*in silico*) have significantly contributed to improving scientific breeding programmes and identifying naturally occurring disease-resistant alleles in a target population. Genome editing can be employed as a component of pipelines to uncover causal genes and variants controlling a target trait previously highlighted by genome-wide screening and gene expression approaches (Gratacap *et al.*, 2019) (**Figure 1.2**). The development of *in vitro* models of study based on genome editing of fish cell lines is a valuable strategy to complement the *in vivo* research. *In vitro* gene editing in cell lines could improve the optimisation of methods, reducing cost, time and the number of animals required prior to *in*

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*).

vivo testing. Furthermore, the *in vitro* approach should be used in CRISPR/Cas9 editing experiments to determine the outcomes of performing a deletion since genome modifications may result in deadly or undesirable outcomes affecting fish welfare (Strømsnes *et al.*, 2022). Additionally, cell line model optimisation, for instance, enables an efficient evaluation of multiple CRISPR/Cas9 delivery strategies against crucial target genes and allows the selection of highly prioritised targets for further *in vivo* studies (Strømsnes *et al.*, 2022). Merging *in silico*, *in vitro*, and *in vivo* approaches has a remarkable potential to genetically improve disease resistance and other performance parameters in aquaculture species (Gratacap *et al.*, 2019; Houston *et al.*, 2020).

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*).

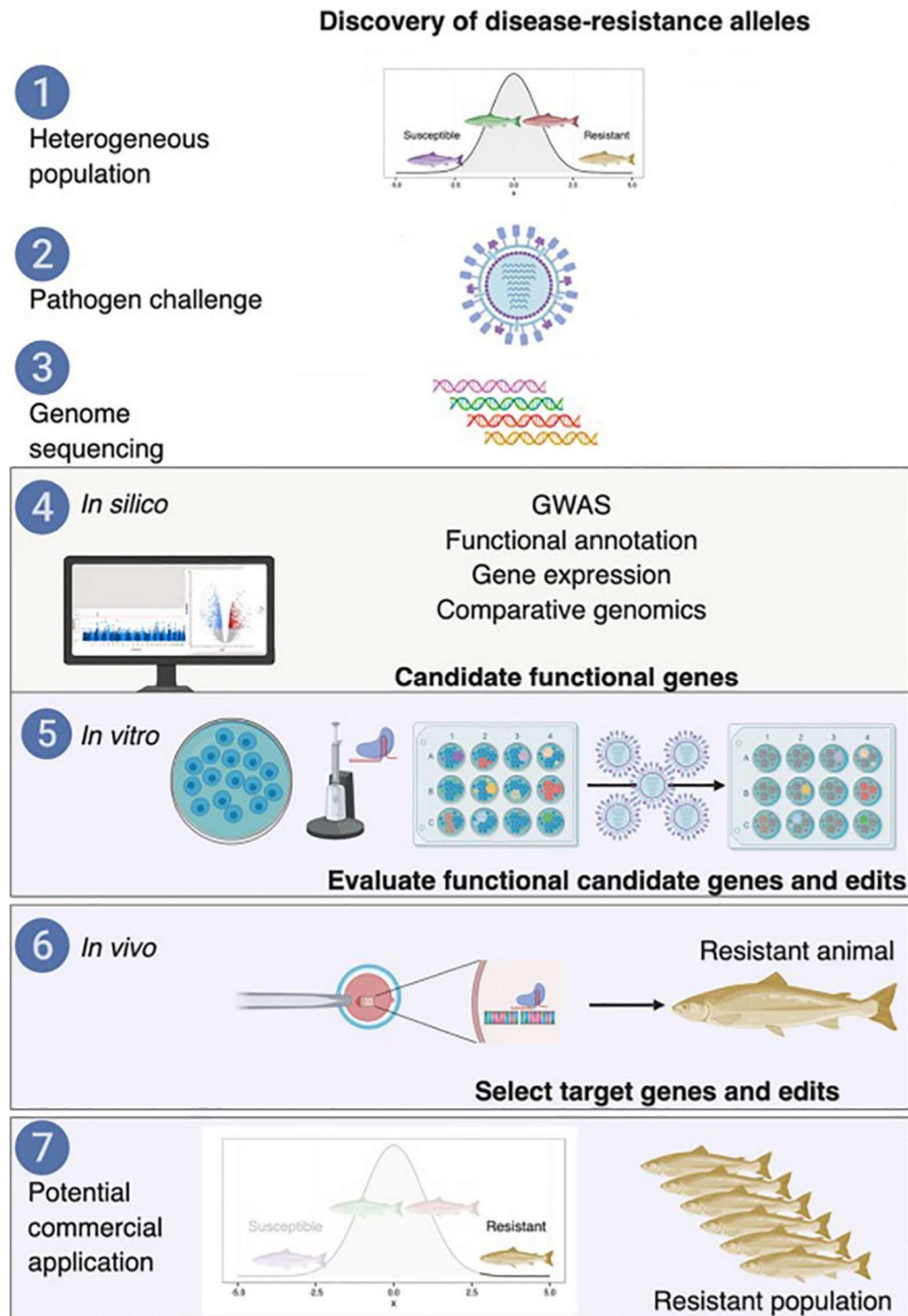


Figure 1.2. Roadmap of genome editing applications in aquaculture breeding to accelerate genetic improvement. 1) Initiate with a diverse population of fish; 2) infection challenge with a target pathogen; 3) identify resistant and susceptible animals through sequencing and/or genotyping; 4) combined functional and

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comparative genomics, such as large-scale genome-wide association study (GWAS) and RNA-sequencing, to detect naturally occurring disease resistance alleles; 5) the putative functional genes identified move forward to in vitro 6) and in vivo testing and characterization; 7) resulting in a breed of animals with significantly enhanced disease resistance. Figure modified from Gratacap et al., 2019.

1.4. *Piscirickettsia salmonis* and Salmon Rickettsial Syndrome (SRS): A major threat to aquaculture production.

Piscirickettsiosis, or Salmonid Rickettsial Syndrome (SRS), is a septicemic bacterial infection affecting salmonid species. It has been positioned as the most significant infectious disease affecting the salmon farming industry in Chile due to the large economic losses produced by morbidity and mortality since first isolated in 1989 (Rozas-Serri, 2022). The causative agent is the facultative intracellular bacterium *Piscirickettsia salmonis* capable of infecting three salmonid species of economic importance, Atlantic salmon, coho salmon and rainbow trout (Rozas & Enriquez, 2014). SRS outbreaks occur at the seawater stage, where economic losses concerning biomass are highest. Indirect losses through reduced growth rates and early harvests exacerbate the impact on production sustainability (Ramirez et al., 2015). In 2021, SRS constituted 49.4% of mortality related to infectious diseases and 11.2% of total mortalities in Atlantic salmon production (SERNAPESCA, 2021a).

1.4.1. Epidemiology.

Although penetration through intact skin and gills by *P. salmonis* has been reported as the cause of infection in experimental conditions (Rozas-Serri et al., 2017; Smith et al., 1999), the entire picture of the infectious disease

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*).

epidemiology and transmission dynamics is not fully understood (Bravo *et al.*, 2020; Miranda *et al.*, 2018). Studies on the subject indicated that disease transmission occurs horizontally in fresh and seawater (Cvitanich *et al.*, 1991; Lannan & Fryer, 1994) in the absence of vectors or direct contact fish to fish (Almendras *et al.*, 1997). In addition, vertical transmission occurs from infected parents to fry (Larenas *et al.*, 2003), and *P. salmonis* can survive in seawater for 14 to 40 days (Lannan & Fryer, 1994; Olivares & Marshall, 2010). Wild animal reservoirs are a concerning subject as in recent studies; the bacterium has been described in wild fish and mussels surrounding salmon farms (Fryer & Hedrick, 2003; Larenas *et al.*, 2019; Quintanilla *et al.*, 2021).

SRS outbreaks are geographically distributed between the Los Lagos and Aysen regions in Chile (SERNAPESCA, 2021a). An acute rise in reports is observed from January to April when water temperatures rise between 8 to 18°C in Los Lagos and reach 15°C in Aysen (SERNAPESCA, 2021a). Naturally occurring outbreaks typically start 6 to 12 weeks after smolts are transferred to seawater (Cvitanich *et al.*, 1991; Fryer *et al.*, 1992). Other salmon-producing nations such as Norway, Ireland, Canada, and Scotland have also reported SRS (Brocklebank *et al.*, 1993; Grant *et al.*, 1996; Olsen *et al.*, 1997; Otterlei *et al.*, 2016; Rodger & Drinan, 1993). It has been identified as an emerging global concern due to the increase in reports of non-salmonid species such as Nile tilapia (*Oreochromis niloticus*), white seabass (*Atractoscion nobilis*), and European sea bass (*Dicentrarchus labrax*) over the past few years (Arkush *et al.*, 2005; Mauel *et al.*, 2007; Zrnčić *et al.*, 2021).

1.4.2. Pathological findings.

Multiple organs are affected by this bacterial infection, described as a septicemic disease (Almendras *et al.*, 1997; Arkush *et al.*, 2005; Rozas & Enriquez, 2014), which is the reason why pathological signs and lesions are presented in a broad spectrum of systemic failures (**Table 1.3**). Multiple grey-white to yellow subcapsular nodules are the most prevalent gross pathology finding in organs of deceased fish (**Figure 1.3 A**) (Almendras *et al.*, 2000; Arkush *et al.*, 2005; Cvitanich *et al.*, 1991; Fryer & Hedrick, 2003; Mauel & Miller, 2002; Olsen *et al.*, 1997; Rozas-Serri *et al.*, 2017). These typical lesions are detected in the liver, kidney, spleen, and intestine. However, pathological changes may also be found in the brain, heart, ovaries, and gills, as severe multifocal necrosis and inflammation and moderate lesion in the cardiac, pancreatic, and ovarian tissues (Almendras *et al.*, 2000; Cvitanich *et al.*, 1991; Fryer & Hedrick, 2003; Mauel & Miller, 2002). Granulomas of macrophages and neutrophils, combined with perivascular infiltration, are the most frequent microscopic findings (**Figure 1.3 B**) (Rozas-Serri, 2022). Different variables may influence the intensity of pathological signs and lesions findings, fish species, bacterial strain, route of infection, infection duration, water salinity and temperature (Almendras *et al.*, 2000; Meza *et al.*, 2019; Rozas-Serri *et al.*, 2017).

Table 1.3. Summary of pathological findings during SRS infection.

Clinical presentation	Pathological findings	References
Behavioural changes	Anorexia, lethargy, erratic swimming, respiratory distress	Almendras <i>et al.</i> , 1997, 2000
External findings	Pale gills. Darkening in skin pigmentation, perianal and periorcular haemorrhages, abdominal petechia, and shallow haemorrhagic ulcers. Bilateral exophthalmia and ulcerative stomatitis.	Almendras <i>et al.</i> , 1997; Fryer & Hedrick, 2003; Lannan & Fryer, 1994; Mauel & Miller, 2002; Rozas & Enriquez, 2014
Internal macroscopic lesions	Grey-white to yellow subcapsular nodules in the liver, kidney, and spleen. Petechia, and ecchymosis on the serosa surface of the pyloric caeca, swim bladder, and caudal intestine. Ascites, peritonitis, overall pallor and diffuse swelling.	Almendras <i>et al.</i> , 2000; Cvitanich <i>et al.</i> , 1991; Lannan & Fryer, 1994; Rozas & Enriquez, 2014
Microscopic lesions	Hepatocytes with multifocal necrosis, diffuse infiltration of inflammatory cells. Multifocal and Coalescing granulomas: central necrosis with bacterial presence, surrounded by different stages of macrophages, neutrophils, putative dendritic cells, and natural killer cells entirely encircled by lymphocytes (putative T- and B-cells).	Evensen, 2016; Rozas-Serri, 2022

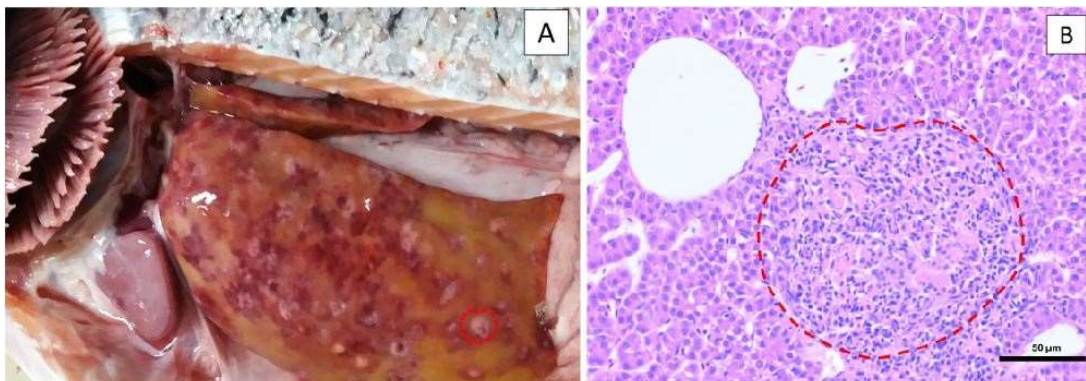


Figure 1.3. Pathological lesions in the liver of Atlantic salmon during SRS infection. A) Pale liver with a circular grey–white nodule of approximately 1-6 mm in diameter characteristic of *P. salmonis* infection. **B)** Histopathological image of a multifocal and coalescing granuloma. The dashed red line delineates the borders of a focal granuloma. Bar. 50 µm. Figure modified from Rozas-Serri, 2022.

1.4.3. Diagnostic, treatment and control strategies.

The primary diagnosis of SRS is the presence of clinical signs and gross pathology findings (Rozas & Enriquez, 2014; Yáñez *et al.*, 2012). Confirmation of *P. salmonis* infection must be validated by molecular diagnostic methods upon the initial detection (Lannan & Fryer, 1994; Larenas *et al.*, 2003; Makrinos & Bowden, 2017). Bacterium isolation by cell or media culture techniques is considered one of the best detection methods as it provides strain information and potential treatment options (Mauel & Miller, 2002). However, isolating *P. salmonis* from the field can be difficult due to contamination with environmental microorganisms and the need for specially formulated media (Mauel & Miller, 2002). Conventional PCR, RT PCR, and indirect fluorescent antibody tests are more accurate and sensitive than culture procedures (Corbeil & Morrison, 2019; Karatas *et al.*, 2008; Makrinos & Bowden, 2017; Mauel *et al.*, 1999). Other detection methods to specifically identify *P. salmonis* are immunofluorescence (IFAT) (Lannan & Fryer, 1994; Larenas *et al.*, 2003), enzyme-linked immunosorbent assay (ELISA) (Aguayo

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et al., 2002), and in situ hybridization assay (Venegas *et al.*, 2004). Direct observation techniques, such as Gram, Giemsa or methyl blue staining, are helpful for the fast detection of the bacterium within host cells (Fryer *et al.*, 1992; Lannan & Fryer, 1994).

Antibiotics are the most frequent treatment for SRS, administered through medicated feed or intraperitoneal injection. The primarily used drug is florfenicol and secondly oxytetracycline, with 97.4% and 1.48%, respectively, of the total reported in 2021 (SERNAPESCA, 2021b). In the past decade, the excessive use of antibiotics in the Chilean salmon industry has become a significant problem (Higuera-Llantén *et al.*, 2018; Lozano-Muñoz *et al.*, 2021; Miranda *et al.*, 2018; Rozas & Enriquez, 2014; Rozas-Serri, 2022), for instance during 2021, Norway reported the use of 0.953 tonnes of antimicrobials vs 463.4 tonnes used in the same year in Chile (NORM-VET, 2021; SERNAPESCA, 2021b). The primary cause of concern is the proliferation of antimicrobial-resistant strains and the impact on the aquatic environment surrounding salmon farms (Higuera-Llantén *et al.*, 2018; Lozano-Muñoz *et al.*, 2021). Strikingly, an estimated 93.5% of the total volume of antimicrobials used in 2021 was for SRS treatment, adding a further troublesome issue to salmon farming sustainability (SERNAPESCA, 2021b).

Prevention strategies include improving biosecurity and good husbandry practices, managing stress factors, reducing fish density in cages and vaccination (Rozas & Enriquez, 2014). Multiple vaccines have been developed to control SRS outbreaks. Although some vaccines have demonstrated efficacy under experimental conditions, commercial vaccine's efficacy has proved inadequate protection against *P. salmonis* in field conditions (Evensen, 2016; Figueroa *et al.*, 2022; Rozas & Enriquez, 2014).

1.4.4. *Piscirickettsia salmonis* bacteria.

Piscirickettsia salmonis is a facultative intracellular, gram-negative, aerobic, pleomorphic coccoid, non-motile bacterium. Recognized as the first fish-pathogen rickettsia-like microorganism, despite its rickettsial-like characteristics (basis of morphology and replication site), molecular phylogenetic analysis based on 16S rRNA gene sequencing has classified it as a gammaproteobacteria, family *Piscirickettsiaceae* (Fryer *et al.*, 1992; Fryer & Hedrick, 2003; Rozas & Enriquez, 2014). The gammaproteobacteria class has included the genera of *Francisella*, *Coxiella* and *Legionella*, which constituents share an intracellular nature (Fryer & Hedrick, 2003). These pleomorphic marine bacteria are mainly coccoid in shape, and their size ranges between 0.5 to 1.8 µm in diameter (Almendras *et al.*, 1997; Fryer & Hedrick, 2003; Otterlei *et al.*, 2016).

Genetic variability analysis of the 16S rRNA and 23S rRNA genes (ITS 16S-23S) of isolates from three distinct geographical hosts supports the existence of two genogroups, LF-89 and EM-90 (Mauel *et al.*, 1999). These strain's pangenome contain 148 unique proteins for LF-89 and 273 for EM-90 (Nourdin-Galindo *et al.*, 2017), and differences in salmon species susceptibility and geographical distribution have been described (Bohle *et al.*, 2014).

P. salmonis replicates through binary fission within membrane-bound cytoplasmic vacuoles in permissive fish cell lines such as Chinook salmon embryo (CHSE 214), epithelioma papulosum cyprini (EPC), and Chinook salmon heart (CHH), leading to a characteristic cytopathic effect (CPE) (**Figure 1.4**) (Makrinos & Bowden, 2017; Mauel & Miller, 2002). In addition, this bacterium infects lymphoid cells such as macrophages, monocytes, and nonlymphoid cells (McCarthy *et al.*, 2008; Rojas *et al.*, 2009). Since 2007, multiple cell-free liquid and solid media have been developed for *P. salmonis*

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culture (Contreras-Lynch *et al.*, 2017; Henríquez *et al.*, 2013; Mauel *et al.*, 2008; Mikalsen *et al.*, 2008; Yáñez *et al.*, 2013; Yáñez *et al.*, 2012).

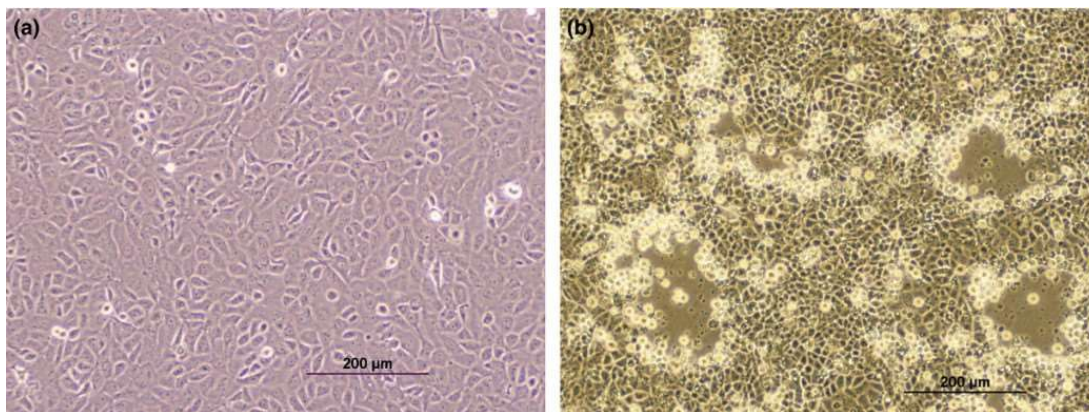


Figure 1.4. Cytopathic effect (CPE) of CHSE-214 cell line infected with *P. salmonis* LF-89. A) Uninfected CHSE-214 cell line. B) CPE in CHSE-214 cell line at 10 dpi with *P. salmonis* strain LF-89. Bar. 200 μm. Figure obtained from Makrinos & Bowden, 2017.

1.5. Hallmarks of *P. salmonis* infection.

In the context of this introduction, "hallmarks of *P. salmonis* infection" refers to the several biological pathways exploited by the bacterium to infect and replicate in host cells, bacterial virulence factors, and strategies to modulate the host's immune response or cell cycle to promote bacterial survival.

1.5.1. The host: salmonids immune system.

Teleosts (bony fishes) evolution diverted from mammals approximately 320 - 350 million years ago (Glasauer & Neuhauss, 2014); however, their immune system shares numerous components comparable to mammals (Semple & Dixon, 2020). Among the similarities is the existence of two major branches, the innate and adaptative immune systems, present in both groups.

Nevertheless, a crucial difference in teleosts is the limitations of the adaptative system in terms of delayed initiation and limited antibodies repository versus mammals (Semple & Dixon, 2020). These characteristics rely on the burden of prevention and clearance of pathogen agents on the innate system (Ye *et al.*, 2011). Numerous studies indicate that salmonids possess all of the distinctive aspects of innate immunity described in mammals. These include physical barriers such as skin and mucous membranes; humoral factors like the complement and cytokines (Grayfer & Belosevic, 2012); pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) (Álvarez *et al.*, 2017; Arnemo *et al.*, 2014; Chen *et al.*, 2004; Laing *et al.*, 2008); and cellular components such as macrophages, monocytes, neutrophils, basophils and eosinophils (Niimi & Lowe-Jinde, 1984; Pérez-Stuardo *et al.*, 2019, 2020). Hence, the innate immune system responds promptly to invading pathogens and tissue injury. However, it cannot offer targeted and specific protection against pathogens or establish an enduring immunological memory.

The adaptive immune system in salmonids, similar to mammalian models, designates lymphocytes as the adaptive immune cells of fish. T and B lymphocytes are the exclusive cell types that can identify and react selectively to an antigen pattern, and both cell types differentiate on the type of receptor expressed on the cellular surface (T or B cell receptor). Teleosts lymphocytes represent 83 to 90% of the total white blood cells (WBCs), while mammalian lymphocytes represent between 20 to 40% of WBCs (Gordeev *et al.*, 2022; Niimi & Lowe-Jinde, 1984). Antibodies secretion in fishes is described to happen in antibody-secreting cells (ASCs), which are considered early stages of B cells (Ye *et al.*, 2011). Three distinct antibody classes have been described, namely immunoglobulin M (IgM), immunoglobulin D (IgD), and immunoglobulin T (IgT), categorised based on variations observed in their constant region (Sunyer, 2013). IgM is the predominant immunoglobulin found in the serum of bony fishes, playing a crucial role in providing systemic

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*).

immunity (Parra *et al.*, 2015). IgD is an antibody whose precise role has not been fully described, even within mammalian species (Sunyer, 2013). Finally, IgT is an antibody isotype that has been identified exclusively in teleosts. It has been described as playing a role in the mucosal immunity of fish (Hansen *et al.*, 2005).

1.5.2. Host immune evasion and intracellular trafficking modulation.

In recent years, significant knowledge has been generated on the different routes *P. salmonis* use to infect host cells. The bacterium is a highly adaptable microorganism observed in numerous host cells, including hepatocytes and macrophages in the liver, kidney, spleen, and blood tissues (Almendras *et al.*, 2000; McCarthy *et al.*, 2008). However, macrophages are the primary target cells in which this bacterium may live and proliferate (McCarthy *et al.*, 2008; Rojas *et al.*, 2009). Colocalization studies and fluorescence microscopy observation have suggested that clathrin-mediated endocytosis is essential for the bacterial internalisation process (Ramirez *et al.*, 2015), and further transcriptomic modulation studies confirmed these findings (Valenzuela-Miranda *et al.*, 2016). It has been observed that following entering macrophages via phagocytosis, *P. salmonis* exploits the cell actin monomers, inducing cytoskeleton disorganisation to produce tridimensional vacuoles denominated pathogen-containing vacuoles (PCVs). These structures play a pivotal role in bacterial replication and evading the lysosomal degradation pathway during the later stages of infection (Ramirez *et al.*, 2015; Rojas *et al.*, 2009; Rozas-Serri, 2022; Valenzuela-Miranda & Gallardo-Escárate, 2018). Modulation of clathrin-mediated endocytosis is a well-established entryway for numerous intracellular bacteria and viruses (Latomanski & Newton, 2019; Levicán-Asenjo *et al.*, 2019; Veiga & Cossart, 2005).

One of the most remarkable strategies developed by *P. salmonis* is the modulation of the host cell intracellular trafficking, facilitated by a virulence factor that encodes a Dot/Icm type-four secretion system (T4SS) present in the bacterium genome (Cortés *et al.*, 2017; Gomez *et al.*, 2013; Labra *et al.*, 2016; Zúñiga *et al.*, 2020). The bacterium infects and replicates within the cytoplasmic vacuoles of macrophages and leukocytes, minus the usual CPE observed in infected epithelial cell lines (Rojas *et al.*, 2009). The Dot/Icm T4SS enables bacteria to translocate proteins into host cells and modulate host pathway activity (Schroeder, 2018) considered a fundamental virulence factor of *Legionella pneumophila* and *Coxiella burnetii*, involved in their intracellular survival and replication (Segal *et al.*, 2005). PCVs are demonstrated to inhibit the fusion with the host lysosomal compartment by altering its pH. This change disrupts the PCV endosome maturation as a strategy to avoid bacterial clearance and ensure *P. salmonis* survival (Pérez-Stuardo *et al.*, 2019, 2020). In addition, the coding sequence of ClpB and BipA/TypA genes has been identified in *P. salmonis*' genome (Isla *et al.*, 2014). These well-characterised virulence factors are essential for the replication and persistence of *Francisella tularensis* and *Pseudomonas aeruginosa* (Meibom *et al.*, 2008; Neidig *et al.*, 2013). Moreover, a significative overexpression of ClpB and BipA/TypA induced by early *P. salmonis* infection in SHK-1 cells suggests the bacterium may use these factors to adapt to the hostile intracellular conditions in macrophages and facilitate its replication (Isla *et al.*, 2014).

1.5.3. Cytoskeleton reorganisation.

The activation of the cytoskeleton is implicated in numerous innate immune response pathways, such as pathogen recognition, phagocytosis, cell-cell signalling, cell migration, and secretion (Mostowy & Shenoy, 2015). Moreover, substantial changes in actin components have been observed during the infection phase of intracellular bacteria such as *Legionella*

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pneumophila, *Coxiella burnetii*, and *Listeria monocytogenes* (de Souza Santos & Orth, 2015; Liu *et al.*, 2017; Meconi *et al.*, 2001; Yoshikawa *et al.*, 2009). Correspondingly, major disruptions in the cytoskeletal structure of infected cells are modulated by *P. salmonis* altering actin filaments, tubulins, myosins, and septins in the host cell (Pulgar *et al.*, 2015; Ramirez *et al.*, 2015). These components have been demonstrated to participate in PCVs assembly and bacterial survival.

1.5.4. Apoptosis inhibition.

Transcriptomic studies have described a significant delay in protein transport, antigen processing, vesicle trafficking, and autophagy during *P. salmonis* infection. In contrast, it also promotes cell survival and inhibits apoptosis (Rozas-Serri *et al.*, 2018; Tacchi *et al.*, 2011). Díaz *et al.*, (2017) observed a temporal pattern of apoptosis modulation by *P. salmonis* infection. During early infection in macrophages, the bacterium inhibits the apoptotic process to promote bacterial survival, replication and transport inside phagocytes; however, in late stages, macrophage apoptosis is no longer inhibited, facilitating bacterial exit to continue the infection cycle.

1.5.5. Iron deprivation.

Transcriptional studies highlighted the essential role of iron in *P. salmonis* survival, finding gene expression differences between Atlantic salmon families with different susceptibilities. Fishes capable of limiting iron availability to the bacterium had improved survival, suggesting that iron deprivation represents a mechanism of innate immunity and resistance against *P. salmonis*. (Pulgar *et al.*, 2015). In addition, iron deficiencies induced by using iron chelators in cell culture negatively affected bacterial proliferation and limited the infection process (Díaz *et al.*, 2021). Further *in vivo* studies

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corroborated the protective capacity of iron chelators as host-directed antimicrobial drugs (HDAD), reducing mortality by 35% in rainbow trout infected with *P. salmonis* (Caruffo *et al.*, 2020).

1.5.6. Inflammasome.

The inflammasome is an intracellular sensing system that plays an essential role in the innate immune response to infection. A wide range of microorganisms activates it, initiating a cascade of receptors and sensor recruitment resulting in the activation of Caspase-1 and later IL-1 β , a potent inducer of the inflammatory response (**Figure 1.5**) (Guo *et al.*, 2015; Swanson *et al.*, 2019). Previous studies have found orthologs genes encoding inflammasome components present in teleost (Álvarez *et al.*, 2017; Huising *et al.*, 2004; Koussounadis *et al.*, 2004; Wang *et al.*, 2020; Zou & Secombes, 2016). Recently, Caspase 1-like activity capable of activating IL-1 β in response to infection with *Francisella noatunensis*, has been reported in leukocytes of zebrafish (Vojtech *et al.*, 2012), and evidence of a functioning zebrafish DrNLRP1 inflammasome was confirmed to play an essential function in the antibacterial immune response (Li *et al.*, 2018). In Atlantic salmon, two distinct isoforms of SsNLRC3 have been described to play different roles, as only one isoform was over-expressed during the early stages of *P. salmonis* infection (Pontigo *et al.*, 2020). These findings suggest that inflammasome activation may prove significant in the innate immune response against *P. salmonis* infection, inducing pyroptosis and proinflammatory cytokine production.

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*).

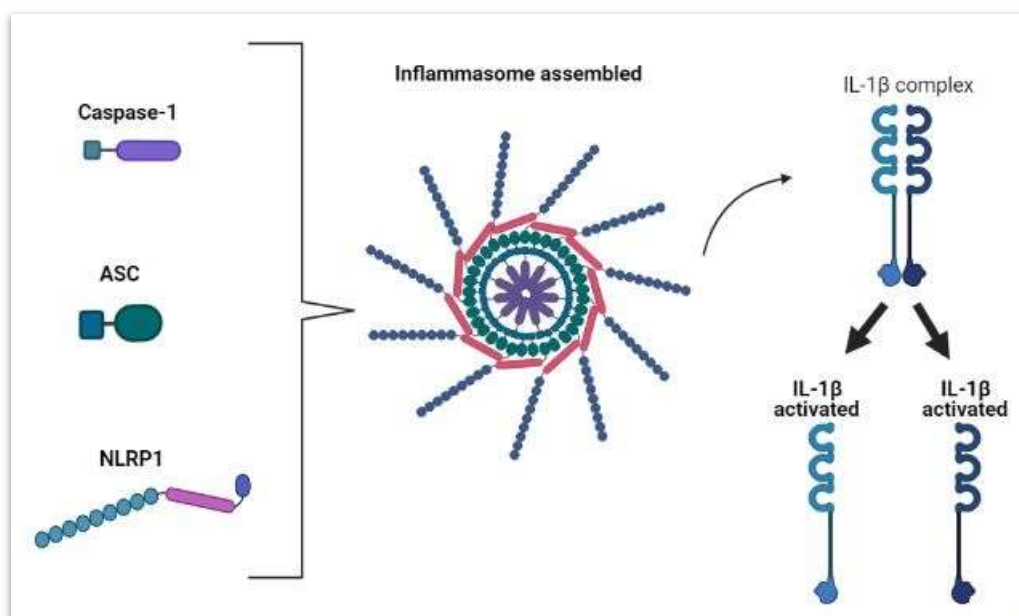


Figure 1.5. Inflammasome components and IL-1 β activation. Caspase-1 protease, Apoptosis-associated speck-like protein containing a C-terminal caspase recruit domain (ASC), and NLR family, pyrin domain containing 1 (NLRP1) cytosolic sensor participate in the classical inflammasome assembly to activate IL-1 β . Figure created with BioRender.com

1.6. Genetic resistance to SRS.

In the last ten years, multiple studies have approached the host resistance to *P. salmonis*. The main highlights concord that there is a significant genetic variation for the resistance trait ranging between 0.11 to 0.45 for Atlantic salmon (Bassini *et al.*, 2019; Correa *et al.*, 2015; Yáñez *et al.*, 2013; Yáñez, Lhorente, *et al.*, 2014), 0.38 to 0.62 in rainbow trout (Yoshida *et al.*, 2018), and 0.16 for coho salmon (Yáñez *et al.*, 2016). These findings are important since the variability of the trait is essential to further improvement by selective breeding (Hill, 2001). Moreover, genome-wide association studies (GWAS) on Atlantic salmon (Correa *et al.*, 2015), rainbow trout (Barría, Marín-Nahuelpi, *et al.*, 2019), and coho salmon (Barría *et al.*, 2018) revealed that resistance to *P. salmonis* is a polygenic trait with numerous markers

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contributing to a small part of the genetic variance, and the absence of a single QTL influencing the genetic variance. However, a QTL region may contain hundreds of genes and strategies to narrow the candidates underlying genetic resistance are necessary.

A valuable complementary approach is to evaluate differentially expressed genes during key moments of infection using transcriptional studies such as RNA sequencing and contrasting those findings with the scientific literature about the pathogen and critical milestones of the host immune response. Finally, a thorough strategy to identify causative genes is using genome editing technologies, such as CRISPR/Cas9 knockout, aiming to disrupt a targeted gene's biological function and evaluate its role in the infection process (Gratacap *et al.*, 2019). This comprehensive method may highlight critical genes and pathways influencing the resistance to SRS trait and comprise a valuable contribution to developing specific and effective control methods for this main threat to aquaculture sustainability.

1.7. Aims and objectives.

Salmon Rickettsial Syndrome (SRS), caused by the fish pathogen *Piscirickettsia salmonis*, is the most problematic infectious disease affecting Chilean salmon farming. Chile is positioned as the second-largest producer of Atlantic salmon, and SRS represents a significant threat to economic sustainability in Chile and globally. Developing new and effective strategies to control this threat is essential, and increasing the natural host resistance is a promising avenue. This increase in host resistance can be achieved through selective breeding, augmented with genomics, or potentially via genome editing for target resistance genes. However, the specific genes underlying genetic resistance and their utility as potential targets for genome editing are unknown. This study took a holistic approach to unravel the genetic makeup of resistance to SRS in Atlantic salmon using transcriptomic studies and quantitative trait locus mapping to identify the most relevant genes and pathways up-regulated during infection, cross-reference those with literature on immune response to SRS, and interrogate these genes' role in the host response using a cell line model.

The overall research aim was to develop an *in vitro* model using CRISPR/Cas9 to study the functional consequences of KO candidate genes for resistance to SRS in the Atlantic salmon cell line SHK-1. Therefore, the experimental objectives of this thesis are:

i) to investigate the genetic and functional basis of host resistance to SRS and find suitable target genes contributing to host survival and infection overcoming.

ii) to generate a reliable and effective CRISPR/Cas9 system method to knockout candidate genes for resistance to SRS in the Atlantic salmon cell line SHK-1.

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iii) to interrogate the functional impact of knocked-out genes during *P. salmonis* infection challenge and characterise the physiological effect on bacterial survival and cell mortality.

CHAPTER 2: Elucidating the genetic and functional basis of host resistance to SRS.

This chapter is based on the published paper:

Carolina P. Moraleda, Diego Robledo, Alejandro P. Gutiérrez, Jorge Del-Pozo, José M. Yáñez and Ross D. Houston. 2021. Investigating mechanisms underlying genetic resistance to Salmon Rickettsial Syndrome in Atlantic salmon using RNA sequencing. BMC Genomics 22, 156. <https://doi.org/10.1186/s12864-021-07443-2>

The original document is included within this chapter's body of work, along with an introduction, conclusion, and supplementary data.

2.1. Introduction.

Salmon Rickettsial Syndrome (SRS) is the leading cause of pathogen-related mortality in the Chilean salmon industry (SERNAPESCA). This disease is caused by the gram-negative bacterium *Piscirickettsia salmonis*, a contagious and hard-to-control pathogen. Numerous attempts to control the spread and impact of SRS, including vaccine development, antibiotics, and biosecurity management, have demonstrated only limited efficacy under field conditions (Evensen, 2016; Figueroa *et al.*, 2022; Rozas & Enriquez, 2014; Rozas-Serri, 2022). Multiple biological strategies employed by the bacterium to modulate and evade host mechanisms for infection control are primarily responsible for the difficulty of disease control (Alvarez *et al.*, 2016; Díaz *et al.*, 2017; Gomez *et al.*, 2013; Rozas-Serri, 2022). Improving our understanding of the genetic and functional aspects of the host-pathogen interaction, such as the process of entry into host cells, intracellular replication, virulence mechanisms, and genetic variation in host response, is necessary for the

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development of novel strategies to tackle SRS (Evensen, 2016; Rozas-Serri, 2022).

As mentioned in chapter 1, improving the host's natural resistance by selective breeding is a promising approach to long-term control of this disease (Gratacap *et al.*, 2019; Houston, 2017; Houston *et al.*, 2020). Using disease challenge experiments, multiple authors have demonstrated significant genetic variation for resistance to *P. salmonis* in Atlantic salmon ($h^2 = 0.11$ to 0.41) (Correa *et al.*, 2015; Yáñez *et al.*, 2013; Yáñez, Lhorente, *et al.*, 2014). However, the susceptibility or resistance of a host is polygenic in nature (Barría, Marín-Nahuelpi, *et al.*, 2019; Correa *et al.*, 2015), and therefore typically mediated by the action of numerous genes. Nonetheless, genomic selection has demonstrated to increase the accuracy of breeding value prediction for multifactorial traits in commercial aquaculture breeding programmes (Houston *et al.*, 2020; Zenger *et al.*, 2019). Using genomic information improved the accuracy of breeding value predictions for SRS resistance by up to 30% compared to pedigree approaches (Bangera *et al.*, 2017).

Previous research has studied Atlantic salmon's host response to infection using microarrays and RNA sequencing. The main findings suggest that *P. salmonis* modulates several host metabolic pathways to evade immune response clearance, achieve persistence and replication (Alvarez *et al.*, 2016; Ramirez *et al.*, 2015; Rojas *et al.*, 2009). These pathways include the iron deprivation system, cytoskeletal reorganisation, pro-inflammatory cytokine response, interference with protein transportation and vesicle trafficking (Alvarez *et al.*, 2016; Gomez *et al.*, 2013; Machuca & Martinez, 2016; Pulgar *et al.*, 2015; Ramirez *et al.*, 2015). However, the connection between genetic variation in resistance and functional mechanisms remains elusive.

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The main aims of this chapter are to i) evaluate the genetic architecture of resistance to SRS using a large *in vivo* Atlantic salmon infection challenge from an industrial breeding programme, ii) to improve our understanding of the intricate network of the host response to infection, and ii) to discover pathways and candidate genes contributing to host genetic resistance to SRS for further functional studies.

2.2. Statement of authorship.

All data presented is the first author's own work, except for the indicated in the further detailed statement.

Carolina P. Moraleda: Background, literature review, data analysis, data interpretation, drafted manuscript, figures 1, 3 and 5, KEGG pathway enrichment analysis, immune pathways prioritization, literature review of key biological processes, gene network analysis, candidate genes shortlisting, editing and formatting.

Diego Robledo: Background, methodology, Genome-wide association analysis (GWAS), RNA-Seq analysis, network correlation analysis, figures 2, 4 and 6, drafted manuscript and editing.

Alejandro P. Gutiérrez: Molecular biology experiments, sample processing, DNA and RNA extraction.

Jorge Del Pozo: Drafted manuscript and editing.

José M. Yáñez: Sample collection, concept, experimental design, funding acquisition, and project administration.

Ross. D. Houston: Abstract, concept and experimental design, supervision, funding acquisition, project administration, drafted manuscript and editing.

2.3. Original document.

RESEARCH ARTICLE

Open Access



Investigating mechanisms underlying genetic resistance to Salmon Rickettsial Syndrome in Atlantic salmon using RNA sequencing

Carolina P. Moraleda¹, Diego Robledo¹, Alejandro P. Gutiérrez¹, Jorge del-Pozo¹, José M. Yáñez^{2*} and Ross D. Houston^{1*}

Abstract

Background: Salmon Rickettsial Syndrome (SRS), caused by *Piscirickettsia salmonis*, is one of the primary causes of morbidity and mortality in Atlantic salmon aquaculture, particularly in Chile. Host resistance is a heritable trait, and functional genomic studies have highlighted genes and pathways important in the response of salmon to the bacteria. However, the functional mechanisms underpinning genetic resistance are not yet well understood. In the current study, a large population of salmon pre-smolts were challenged with *P. salmonis*, with mortality levels recorded and samples taken for genotyping. In parallel, head kidney and liver samples were taken from animals of the same population with high and low genomic breeding values for resistance, and used for RNA-Sequencing to compare their transcriptome profile both pre and post infection.

Results: A significant and moderate heritability ($h^2 = 0.43$) was shown for the trait of binary survival. Genome-wide association analyses using 38 K imputed SNP genotypes across 2265 animals highlighted that resistance is a polygenic trait. Several thousand genes were identified as differentially expressed between controls and infected samples, and enriched pathways related to the host immune response were highlighted. In addition, several networks with significant correlation with SRS resistance breeding values were identified, suggesting their involvement in mediating genetic resistance. These included apoptosis, cytoskeletal organisation, and the inflammasome.

Conclusions: While resistance to SRS is a polygenic trait, this study has highlighted several relevant networks and genes that are likely to play a role in mediating genetic resistance. These genes may be future targets for functional studies, including genome editing, to further elucidate their role underpinning genetic variation in host resistance.

Keywords: SRS, Aquaculture, Genetics, Genomics, RNA-Seq, Disease, Salmon, Breeding, GWAS

* Correspondence: jmayanez@uchile.cl; ross.houston@roslin.ed.ac.uk

²Faculty of Veterinary and Livestock Sciences, University of Chile, Santiago, Chile

¹The Roslin Institute and Royal (Dick) School of Veterinary Sciences, The University of Edinburgh, Edinburgh, UK



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Background

Finfish aquaculture is a fast-growing industry with a worldwide production of 54.3 million tonnes during 2018, corresponding to an estimated value of USD 139.7 billion [1]. Atlantic salmon (*Salmo salar*) comprises 4.5% of global finfish trade, and demand for salmon has grown steadily since 2010 [1]. However, the expansion of salmon aquaculture has been associated with a concurrent increase in the occurrence and impact of infectious diseases, which can cause major welfare and production challenges. One of the most serious of these diseases is Salmon Rickettsial Syndrome (SRS), caused by the Gram-negative bacterium *Piscirickettsia salmonis*, which can cause severe morbidity and mortality in salmonid species. SRS is particularly problematic for salmon aquaculture in Chile, the world's second largest producer, and is responsible for 47.5% of the mortality due to infectious diseases and 10.9% of the total mortality in Atlantic salmon production [2]. The morbidity and mortality caused by SRS occur at the seawater stage, where economic losses in relation to biomass are highest. The direct losses through mortality are exacerbated by indirect losses through reduced growth rates and premature harvests [3]. SRS has also been reported in other salmon-producing countries such as Norway, Ireland, Canada and Scotland [4–8]. Several strategies for SRS control have been developed, such as vaccination, antibiotics and biosecurity measures, however, they have shown only partial efficacy under field conditions [3]. Development of novel strategies to control SRS requires improved knowledge of the genetic and functional aspects of *P. salmonis* host-pathogen interaction, such as the process of entry into host cells, intracellular replication, virulence mechanisms, and genetic variation in host response [3].

A promising avenue to mitigate the impact of SRS in Atlantic salmon aquaculture is to improve SRS disease resistance traits through selective breeding. This is possible due to naturally occurring genetic variation (heritability) for disease resistance, which has been observed in other infectious diseases impacting farmed populations of farmed salmonids [9–11]. Significant additive genetic variation for resistance to SRS has been found in various farmed populations, with family mortality levels ranging from 5 to 82% and heritability estimates from 0.11 to 0.41 [12, 13]. The genetic architecture of resistance to SRS has been studied using genome-wide association studies (GWAS) in populations of different salmonid species, suggesting that SRS resistance is a polygenic trait [14, 16]. For such traits, genomic selection has been shown to be effective in increasing accuracy of breeding value prediction in commercial aquaculture breeding programmes [17, 18]. In the case of SRS resistance, the use of genomic information was

shown to improve prediction accuracy by up to 30% compared to pedigree approaches [19].

While selective breeding and genomic selection for improved resistance to SRS can be performed without knowledge of the mechanisms underlying genetic resistance, understanding these mechanisms is a major goal for aquaculture research [20]. Such information can yield novel disease treatment and mitigation options, including possible targets for vaccination and therapeutics. Furthermore, knowledge of functional genes and polymorphisms can be applied in functionally-enriched genomic selection, which can further improve prediction accuracy relative to the use of anonymous markers [21]. Finally, putative causative genes and variants can be targeted by CRISPR/Cas genome editing, initially to confirm their role, and ultimately to edit broodstock to carry resistant variants pending a suitable regulatory environment [20].

P. salmonis infects and replicated in salmonid macrophages, and stimulates a significant innate immune response together with an oxidative defence response [22, 23]. The host response to infection in Atlantic salmon has been assessed in a number of studies using microarrays and RNA-Sequencing. Their findings suggest that *P. salmonis* modulates the pro-inflammatory cytokine response, the iron deprivation system and the cytoskeletal reorganization, and interferes with protein transportation and vesicle trafficking to evade immune response, increase persistence and aid replication [24, 25]. This may reflect a strategy of the bacteria to evade the adaptive immune response and modify cell-autonomous immunity [24]. However, while gene expression differences between families with different levels of resistance have been examined using microarrays [25], the functional mechanisms underpinning genetic variation in resistance to SRS remain poorly understood.

Therefore, the aims of this study were i) to evaluate the genetic architecture of SRS resistance in a large Atlantic salmon population from a commercial breeding programme, ii) to improve our understanding of the molecular basis of host response, and iii) to discover functional genes and pathways contributing to host genetic resistance to SRS.

Results

Genetics of resistance to SRS

A large-scale *P. salmonis* injection challenge was performed on a population of salmon pre-smolts from a commercial breeding programme with fish distributed evenly across three tanks. The challenge was terminated after 47 days, and there were a total of 756 mortalities and 1509 survivors, corresponding to an average mortality rate of 33%. The challenged fish started to die 17 days post-challenge, and mortality rate was consistent across

the three tanks (Fig. 1a). The estimated heritability of mortality as measured on the binary scale was 0.43 ± 0.04 .

The genome-wide association analysis revealed a poly-genic architecture for the trait of resistance to SRS, although a few SNPs reached the suggestive level of significance [p -value $< 2.18 \times 10^{-5}$] (Fig. 1b). These SNPs were situated on chromosomes 1, 2, 12 and 27, indicative of putative QTL on these chromosomes. However, no single SNP explained more than 1% of the genetic variation in resistance to SRS.

Transcriptomic response to SRS infection

To examine the transcriptomic response to infection, 48 fish were euthanized and sampled pre-challenge, 3 days post-challenge and 9 days post-challenge from the same tank (total $n = 144$). Head kidney and liver samples were obtained from each animal and stored in RNAlater at 4°C for 24 h, and then at -20°C until RNA extraction. A total of 133 samples were then selected for RNA sequencing (74 liver and 59 head kidney samples; Supplementary file 1) based on (i) high and low Estimated breeding values (EBVs) for resistance to SRS, and (ii) RNA quality. An average of ~40 M reads per sample were produced using RNA Sequencing of the head kidney and liver samples collected at 3 and 9 days post-challenge. Hierarchical clustering of all the samples using gene expression data clustered head kidney and liver separately, as expected (Fig. 2a). Principal Component Analysis was performed in each tissue separately to assess the sample clustering within tissue. Liver samples showed a clear separation between controls and the 9 days post infection samples, with the samples from 3 days post infection falling in between and showing a significant overlap with the other two groups (Fig. 2b). In the case of head kidney, the infected samples clustered

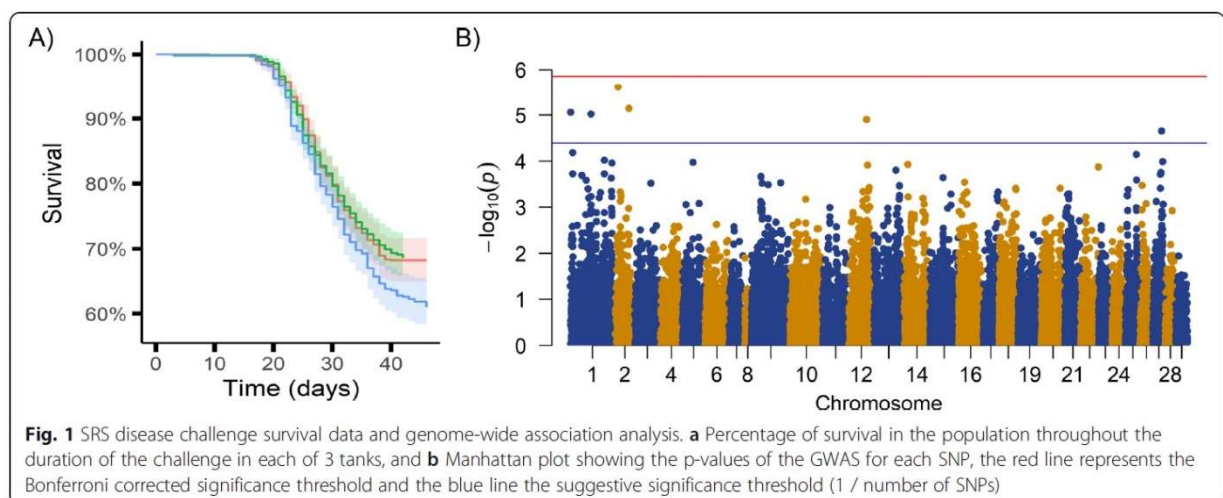
separately from controls, but a clear separation between 3 and 9 days post infections was not observed (Fig. 2c).

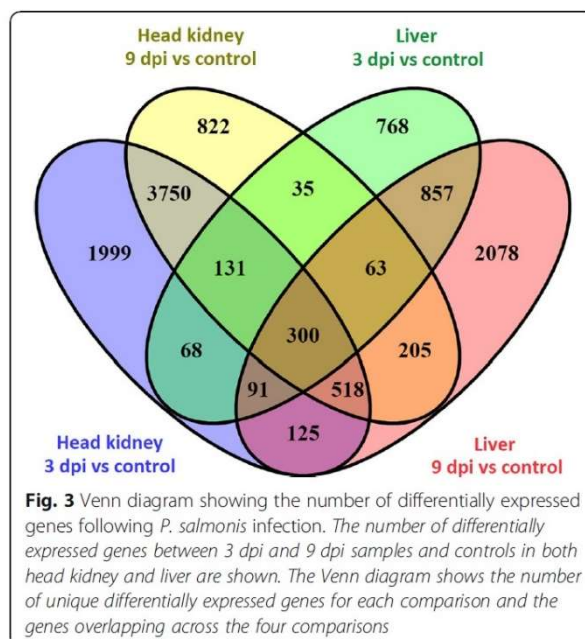
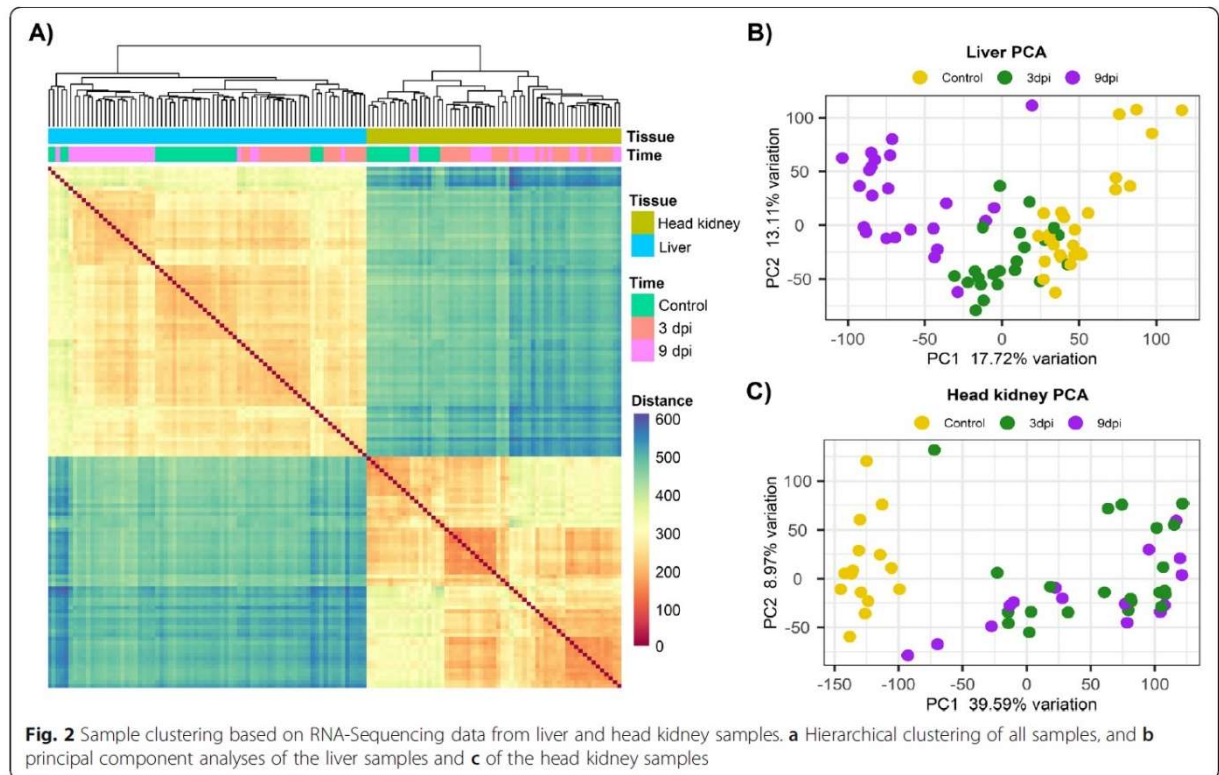
Differential expression analyses between controls and infected samples highlighted a very large number of differentially expressed genes (10 K to 20 K per comparison, False Discovery Rate - FDR p -value < 0.05), which was expected considering the high statistical power associated with the large sample size in this experiment. To facilitate downstream analyses and interpretation, only genes with FDR p -value < 0.001 , normalized mean expression > 10 reads, and absolute $\log_2FC > 1$ were retained for downstream analyses. This resulted in 2000 to 7000 differentially expressed genes in each comparison, with a moderate overlap between time points, especially in head kidney (Fig. 3, Supplementary file 2). Several innate immune genes had altered expression in response to SRS, including interleukins, tumor necrosis factor related genes, caspases and interferon genes (Fig. 4).

Between 15 and 55 KEGG pathways were enriched for differentially expressed genes in the four comparisons (Fig. 5, Supplementary file 3). Generally, immune pathways such as cytokine-cytokine receptor interactions, apoptosis, and Toll-like receptor signaling showed enrichment for gene upregulation in both organs, albeit more strongly in head kidney than liver at 3dpi. TNF signaling and bacterial invasion of epithelial cells were only enriched for upregulated genes in head kidney, while evidence for *Staphylococcus aureus* infection and phagosome upregulation was liver-specific. Energy metabolism pathways showed evidence for downregulation in both organs, including glycolysis / gluconeogenesis or fatty acid degradation (Fig. 5).

Signatures of resistance to SRS

SRS resistance breeding values for all the RNA-Seq animals were estimated according to the linear mixed

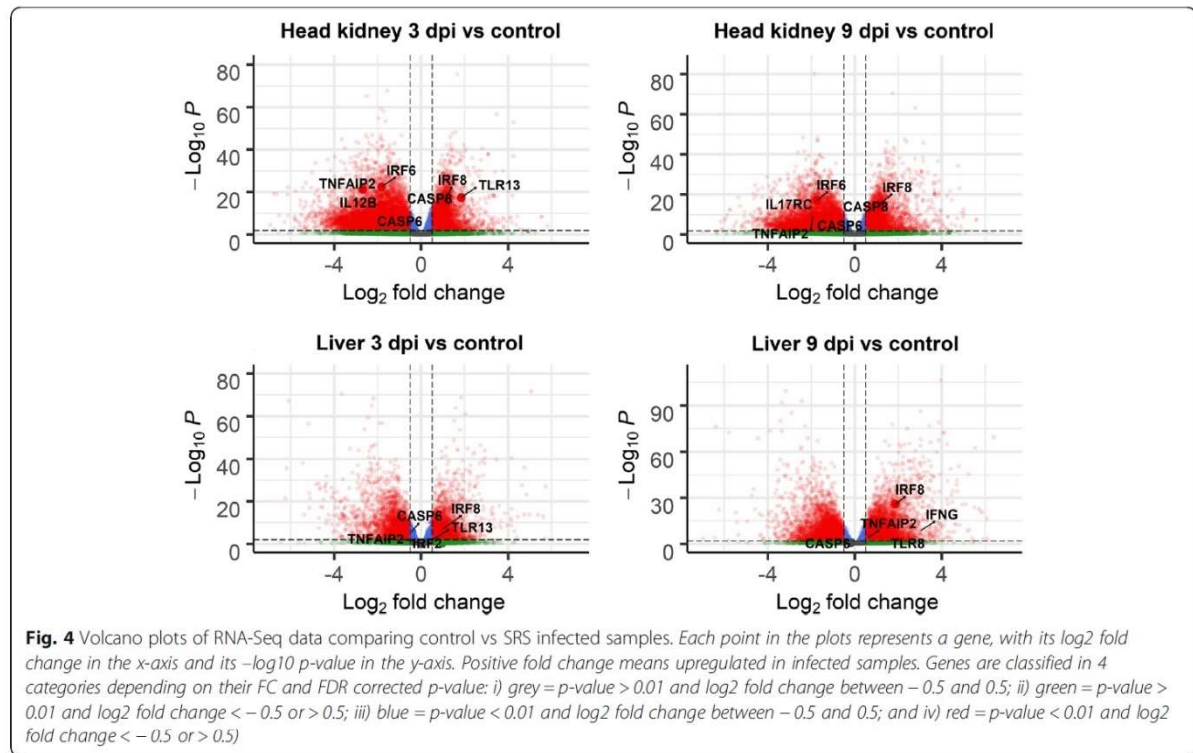




model described in the methods. To investigate the association between gene expression and resistance to SRS, a network correlation analysis was performed. Head kidney and liver transcriptomes clustered into 31 and 22 putative gene networks respectively, with each network containing between 25 and 7000 genes. The correlation between the SRS resistance EBVs at each time point and average network gene expression (Supplementary Figure 1) revealed significant associations for 7 and 2 gene networks in head kidney and liver, respectively ($|r| > 0.45$, $p < 0.001$; Supplementary file 4), suggesting that these networks may play a functional role in defining host resistance to SRS. KEGG enrichment analysis of the gene networks associated with resistance revealed genes involved in the apoptotic processes, such as BCL2L1, ITP3 and BNIP3, in the Cytoskeletal reorganization pathway such as SPTB, and in Bacterial invasion and Intracellular trafficking such as CBL and RAB9A (Fig. 6).

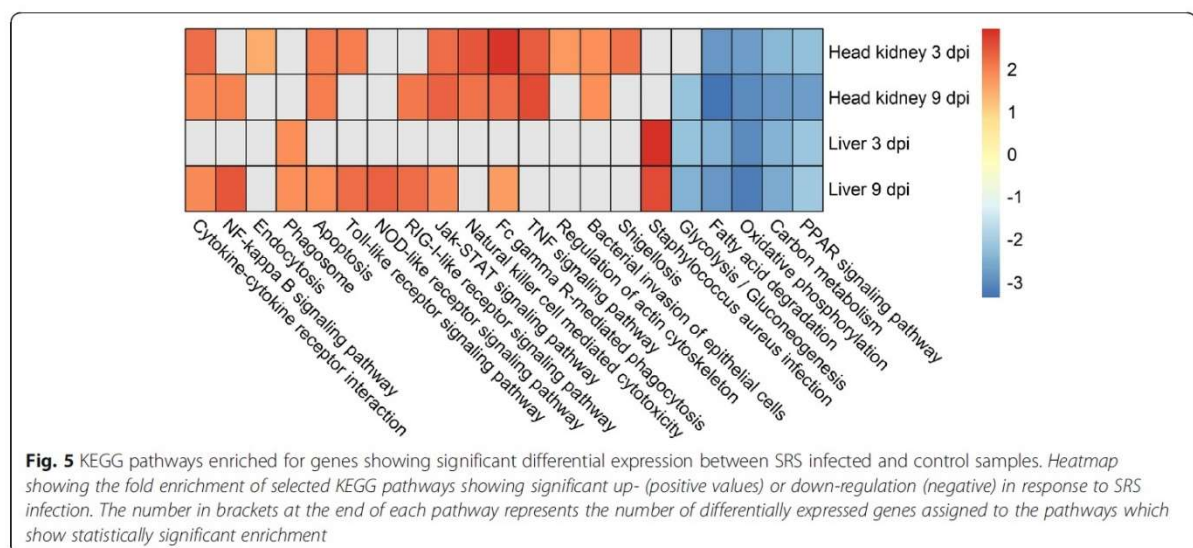
Discussion

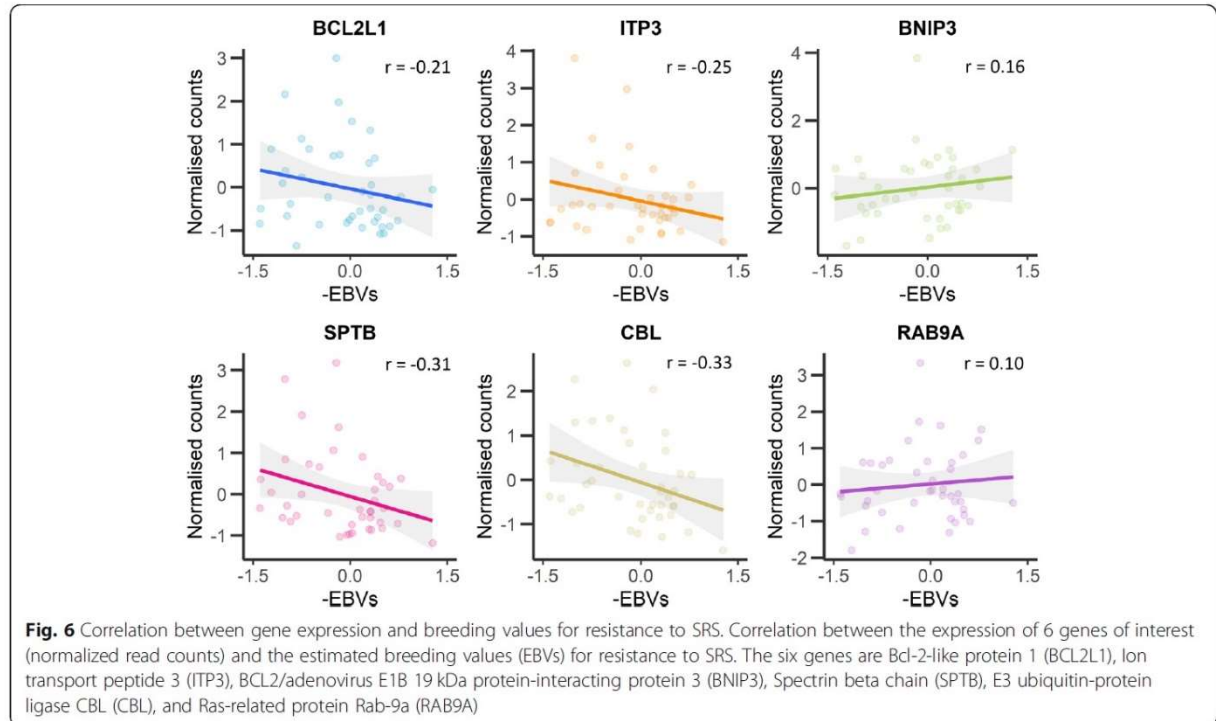
Improving our understanding of the functional basis of genetic resistance and host response to SRS in Atlantic salmon is valuable for the development of new strategies of disease control. To this end, this large-scale study has provided further evidence for significant heritability of host resistance to SRS, and suggested that the genetic architecture of resistance



is polygenic in nature. Furthermore, RNA-Sequencing of liver and head kidney samples from SRS-challenged salmon pre-smolts highlighted a large-scale up-regulation of immune pathways and down-regulation of energy metabolic pathways compared to controls.

Resistance to SRS in the population studied herein had a moderate level of genetic control, with a heritability estimate of 0.43 (binary survival). This estimate is towards the upper limit of those reported in previous studies for Atlantic salmon, which ranged between 0.11 and 0.41 [12, 26, 27], and is also similar to those reported for





resistance to SRS in rainbow trout (ranging between 0.38 and 0.54) [13, 28], but somewhat higher to the values found in coho salmon (ranging between 0.16 to 0.31) [29, 30]. The genetic variation in resistance to SRS appears to be polygenic in nature, without any significant major QTL, and suggestive QTLs on only four chromosomes. This polygenic architecture was also reported in previous studies [14, 16]. Chromosomes 1 and 12 have also been found harbouring genomic regions associated with resistance to SRS in previous studies carried out in a different Atlantic salmon population, raising the possibility the QTL are the same [14, 15]. The putative QTL found herein on chromosomes 2 and 27 identified here differ from previous studies, which can be explained by differences in disease challenge conditions (discussed below), different genetic background between populations and the polygenic nature of the trait. The use of whole-genome resequencing could also result in the discovery of additional QTL not in linkage disequilibrium with the genetic markers used in these studies. Nonetheless, the moderate heritability and polygenic architecture of resistance to SRS in Atlantic salmon make this trait an ideal candidate for genomic selection in salmon breeding programmes, which has proved to be an efficient method to select for resistance to SRS and other diseases with a polygenic background in salmon [19, 31–34]. However, it should be noted that the intraperitoneal injection model used for SRS

challenges could have significant impact on the interpretation of the trait of genetic resistance. The route of entry for *P. salmonis* is via epithelial tissues (skin and gills) [35], and the pattern of infection observed in intraperitoneal injections differs from that of cohabitation infections [36], which is consistent with the barrier of epithelial tissues against bacterial infections [37]. The intraperitoneal injection bypasses this, and therefore it is to be expected that only part of the mechanisms of genetic resistance are being captured. For this reason, benchmarking genetic resistance measured in the laboratory injection challenge with mortality levels observed in the field is an important consideration [38].

SRS infected animals showed major transcriptional differences compared to uninfected controls in both the head kidney and the liver, involving the differential expression of thousands of genes, similarly to previous studies that also reported a significant gene expression modulation in liver and head kidney in response to SRS [25, 39, 40]. Two factors may have contributed to the large number of significant differentially expressed genes in this study, the large sample size (over 30 samples per comparison) and the use of time zero controls. This experimental design (with lack of time-matched controls) means that we may have captured not only the response to the bacteria, but also the response to the intraperitoneal injection and associated stress. Therefore, the results are likely to correspond to the response to SRS,

and the consistency of the results with previous knowledge on SRS infection and other intracellular bacteria support this. Several important innate immune response pathways were up-regulated in both organs, such as Apoptosis, NOD-like receptor signalling, NF-kappa B signalling and Bacterial invasion of epithelial cells (Fig. 5). Likewise, several energy metabolism pathways are down-regulated in response to the infection, probably as a result of diversion of cellular resources towards immune response, as has been suggested in previous studies of macrophage cell lines response to *P. salmonis* infection [22]. The integration of the transcriptomic response to infection and the gene network analysis to identify signatures of resistance to SRS allowed us to identify four key biological processes that seem to be important for the outcome of the infection: i) cytoskeleton reorganization, ii) apoptosis, iii) bacterial invasion and intracellular trafficking, and iii) the inflammasome.

Cytoskeleton reorganization

Genes and pathways related to cytoskeleton reorganization featured heavily in the lists of differential expression genes in response to infection. The cytoskeleton plays an active role in the innate immune response: cytoskeletal activation is involved in pathogen detection, phagocytosis, cell-cell signalling, cell migration, and secretion [41]. Furthermore, major disruptions in actin components have been described during the infection process of intracellular bacteria such as *Legionella pneumophila*, *Coxiella burnetii* and *Listeria monocytogenes* [42–45]. Similarly, *P. salmonis* modulates the cytoskeleton by inducing actin depolymerization [46], which results in cytoskeletal reorganization [24]. This is consistent with our results, where several cytoskeleton associated genes showed high correlation with estimated breeding values for resistance. A notable example is the Rho-associated coiled-coil kinase 1 (ROCK1; $r = 0.27$), a serine/threonine kinase downstream effector of the Rho family, described as an essential regulator of actin cytoskeleton [47]. ROCK kinases participate in the bacterial invasion of *Coxiella burnetii* in human cells, and the use of ROCK inhibitors during infection hampered the bacterial internalization process [48]. Furthermore, genes highly correlated with SRS susceptibility such as SPTB ($r = -0.57$) and SEPTIN3 ($r = -0.42$) are cytoskeleton constituents that participate in protein linking (SPTB [49];) and GTP-binding (SEPTIN3 [50];), respectively. This high correlation of these genes with susceptibility may be explained by the availability of actin in these structures, which is a target for modulation by the bacterium during cytoskeletal depolymerisation, and therefore disrupting

this modulation of the cytoskeleton may be a strategy to increase resistance to SRS.

Apoptosis and cell survival promotion

Apoptosis is a programmed cell-death mechanism essential to development and maintenance of homeostasis [51]; but induction of apoptosis has also been observed during bacterial and viral infection, hampering microbial replication and dissemination [52]. Intracellular bacteria actively modulate cellular apoptosis to enable their replication within the cells [53]. Previous studies suggest that *P. salmonis* modulates the apoptotic process of the host as a strategy to ensure intracellular survival [24, 54]. In line with this, apoptotic genes and pathways were heavily modulated during SRS infection in the current study. Furthermore, the expression of two different inhibitors of apoptosis, BCL2L1 ($r = -0.21$) and ITP3 ($r = -0.25$), was negatively correlated with resistance to SRS. BCL2L1 inhibits caspase-1 activation by interfering with NLRP1 oligomerization, a key component of the inflammasome immune response [55], and ITP3 has an anti-apoptotic effect in mammalian cancer cells [56]. In contrast, apoptosis promoting genes, such as BNIP3 ($r = 0.16$) [57, 58] and Bim (BCL2L11 $r = 0.18$) [59], were positively correlated with genetic resistance. These findings support the hypothesis that apoptosis is initiated as a host strategy to mitigate pathogen dissemination, which is subverted by SRS to promote cell survival and bacterial replication.

Bacterial invasion and intracellular trafficking

The intracellular environment provides diverse advantages to pathogens, for example protection against humoral and complement-mediated host defence mechanisms, and availability of nutrients and direct access to metabolic pathways to modulate in their favour. In order to establish an intracellular infection, pathogens utilise a wide range of mechanisms for internalization and survival [60]. Once inside host cells, *P. salmonis* is capable of establishing intracellular infections, and replicate in macrophages within cytoplasmic vacuole-like structures [61]. In *P. salmonis*, this is facilitated by a virulence factor that encodes a type-four secretion system (T4SS) [22, 62]. The Dot/Icm T4SS allows bacteria to translocate proteins into host cells, and manipulate host pathways [63]. In *P. salmonis*, this may involve modulation of the host cell intracellular trafficking, leading to disrupted phagosome-lysosome pathogen clearance [62]. Interestingly, in this study key genes participating in intracellular trafficking such as RAB1B ($r = 0.24$) and RAB9A ($r = 0.10$) are positively correlated with genetic resistance to SRS. RAB1B is a Rab protein modulated by *Legionella pneumophila* Dot/Icm T4SS effectors to recruit endoplasmic reticulum-derived vesicles to establish

bacterial replication vacuoles [64]. Conversely, RAB9A is involved in the transport between endosome vesicles and the trans Golgi network [65], and is interrupted by *Salmonella enterica* SifA effector to attenuate the lysosomal activity in *Salmonella* containing vacuoles (SCV) [66]. In the current study a strong negative correlation was found between the gene CBL ($r = -0.33$) and resistance to SRS, suggesting that *P. salmonis* virulence factors may target this gene to facilitate bacterial internalization. Furthermore, E3 ubiquitin-protein ligase CBL-like isoform X1 (CBL) was found in chromosome 2, located in the most significant QTL region for resistance to *P. salmonis* infection. Interestingly, *Listeria monocytogenes*, another intracellular bacteria, expresses surface proteins to modulate host proteins like Met and CBL and hijack the clathrin-dependent endocytosis process [67], and previous studies indicate that *P. salmonis* internalization process is mediated by clathrin endocytosis [46].

Inflammasome

Another interesting result was the large number of genes differentially expressed in response to infection involved in the inflammasome. The inflammasome is an intracellular sensing system activated by a broad range of microorganisms that has a pivotal role in the innate immune response to infection [68]. Activation of the inflammasome initiates a signalling cascade that culminates in caspase-1 expression and maturation of the proinflammatory cytokine IL-1 β [69]. Numerous studies suggest that genes participating in the inflammasome assembly may be conserved in teleost fish [70, 71]. Moreover, gene activation of inflammasome associated components such as NLRP1, ASC and caspase-1 has been described in response to bacterial infection in zebrafish (*Danio rerio*) and turbot (*Scophthalmus maximus*) [72, 73]. In the current study, genes involved in the activation of the inflammasome had higher expression on average in resistant fish, suggesting that overexpression of this pathway could be protective during SRS infection. The expression of NLRP1, a sensor that initiates the inflammasome response, is significantly positively correlated with genetic resistance ($r = 0.20$). NLRP1 is a NOD-like receptor (NLR) that detects pathogen molecules and triggers the activation of effector caspases (caspases 1, 4, 5 and 11) [72]. Similarly, NLRC3 is another component of the inflammasome positively correlated with resistance ($r = 0.31$). While in humans it has been described as an inhibitor of the innate immune response through the inhibition of NF- κ B activity [71], in teleosts NLRC3 expression is significantly increased in mucosal tissue after exposure to bacteria, implying an involvement in the early immune response [74, 75]. In contrast, NLRP12 ($r = -0.4774$) is a regulator of inflammation which acts

as a suppressor of pro-inflammatory cytokines interfering with the NF- κ B pathway [76], and therefore its negative correlation with genetic resistance suggests that the activation of the inflammasome pathway is beneficial in response to SRS. In summary, these findings suggest that the activation of the inflammasome pathway is important for a successful immune response against *P. salmonis*.

Conclusions

This study highlights a significant genetic component to SRS resistance in Atlantic salmon, underpinned by a polygenic architecture. The RNA-Sequencing comparison of control and infected fish identified a major signature of host response evident in both head kidney and liver tissues. When comparing this response between individual fish of high and low resistance breeding values, several interesting gene expression networks were identified that correlate with genetic resistance. These include genes related to cytoskeleton, apoptosis and cell survival, bacterial invasion/intracellular trafficking, and the inflammasome. Considering the scale and complexity of the transcriptomic response observed in salmon challenged with *P. salmonis*, and the lack of any significant QTL associated with host resistance, the potential mechanisms leading to genetic resistance are likely to be heterogeneous and vary across different families and individuals. However, the pathways and genes highlighted by this study are potential candidates for functional studies, and downstream applications in salmon production. For example, strategies to increase resistance to the bacteria can focus on disrupting its modulation of cellular homeostasis (i.e. cytoskeleton or apoptosis) or on boosting the immune processes that prevent or restrain the infection (i.e. inflammasome). Such strategies may include CRISPR/Cas knockout or modulation in cell line models, or ultimately in vivo to interrogate the impact of perturbation of the identified genes on genetic resistance.

Materials and methods

Experimental design

Two thousand two hundred-sixty-five Atlantic salmon pre-smolts (average weight 135 ± 47 g) from 96 full sibling families from the breeding population of Aqualnovo (Salmones Chaicas, Xth Region, Chile) were experimentally challenged with *Piscirickettsia salmonis* (strain LF-89) in 3×7 m³ tanks. Fish had been vaccinated for Flavobacterium, IPNV (Alpha Ject Flavo + IPN) and ISAV (Alpha Ject Micro 1-ISA), and prior to the challenge animals were tested for ISAV, IPNV, *Renibacterium salmoninarum*, *Flavobacterium psychrophilum* and Mycoplasma by (q) PCR, and for bacterial contamination by culture in TSA, TSA + salt, and *Piscirickettsia*

salmonis agar at 18 °C and 35 °C. Fish were intraperitoneally injected with 0.2 mL of a 1/2030 dilution of *P. salmonis*. This dose was expected to cause a population-level mortality of close to 50%, based on a pre-challenge of 300 fish from the same families challenged with different doses of the bacteria. The main challenge was terminated when daily mortality returned to baseline levels (i.e. negligible mortality), which occurred 47 days after the start of the challenge. Caudal fin clips were taken from all mortality and survivor fish for future DNA extraction and genotyping.

For RNA sequencing, 48 fish were sampled pre-challenge, 3 days post-challenge and 9 days post-challenge from the same tank, for a total of 144 fish. Head kidney and liver samples were obtained from each animal and stored in RNAlater at 4 °C for 24 h, and then at - 20 °C until RNA extraction.

Genotyping and imputation

DNA was extracted from the fin clips of the challenged fish using a commercial kit (Wizard Genomic DNA Purification Kit, Promega), following the manufacturer's instructions. All samples were genotyped with a panel of 968 SNPs (Supplementary file 5) chosen as a subset of the SNPs from a medium density SNP array [77] using Kompetitive Allele Specific PCR (KASP) assays (LGC Ltd., UK). A population containing full-siblings of the challenged animals had previously been genotyped with a SNP panel of 45,818 SNPs ($n = 1056$, [77]; Supplementary file 5), and the experimental population was imputed to ~46 K SNPs using FImpute v.2.2 [78]. This strategy was selected due to its cost-effectiveness compared with high-density genotyping or whole-genome resequencing. Imputation accuracy was estimated by 10-fold cross validation, masking all SNPs except the 968 SNP panel for 10% of the 1056 genotyped full-sibs, and then assessing the correlation between the true genotypes and the imputed genotypes for the remainder of the SNPs. All imputed SNPs showing imputation accuracy below 80% were discarded. The average imputation accuracy for the 39,416 SNPs retained (Supplementary file 5) was of 95%. Further details about the low-density SNP panel and imputation methods can be found in Robledo et al. (2019) [79]. The imputed genotypes were then filtered and removed according to the following criteria: SNP call-rate < 0.9, individual call-rate < 0.9, FDR rate for high individual heterozygosity < 0.05, identity-by-state > 0.95 (both individuals removed), Hardy-Weinberg equilibrium p -value < 10^{-6} , minor allele frequency < 0.01. After filtering 38,028 markers and 2345 fish remained for the downstream analyses.

Estimation of genetic parameters

The phenotype of resistance to SRS was measured as binary survival, recording mortalities as 0 and survivors

as 1. Genetic parameters for SRS resistance were estimated using the genomic relationship matrix (**G**-matrix) to model the additive genetic relationship between animals in ASReml 4.1 [80] using the following linear mixed model:

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

where **y** is a vector of observed phenotypes, μ is the overall mean of phenotype records, **b** is the vector of fixed effects which includes tank as factor and weight at the start of the challenge as covariate, **a** is a vector of additive genetic effects distributed as $\sim N(0, G\sigma^2_a)$ where σ^2_a is the additive (genetic) variance, **G** is the genomic relationship matrix. **X** and **Z** are the corresponding incidence matrices for fixed and additive effects, respectively, and **e** is a vector of residuals. The identity-by-state genomic relationship matrix (**G**) was calculated using the GenABEL R package ("gkins" function [81]); kinship matrix [82], multiplied by two and inverted.

Single-SNP genome-wide association study

The single-SNP GWAS was performed using the GenABEL R package [81] by applying the mmscore function [83], which accounts for the relatedness between individuals applied through the GenABEL [81] genomic kinship matrix [82]. Significance thresholds were calculated using a Bonferroni correction where genome-wide significance was defined as 0.05 divided by number of SNPs [84] and suggestive as 1 / number SNPs.

RNA extraction and sequencing

For all the 288 head kidney and liver samples, a standard TRI Reagent RNA extraction protocol was followed. Briefly, approximately 50 mg of tissue was homogenized in 1 ml of TRI Reagent (Sigma, St. Louis, MO) by shaking using 1.4 mm silica beads, then 100 μ l of 1-bromo-3-chloropropane (BCP) was added for phase separation. This was followed by precipitation with 500 μ l of isopropanol and posterior washes with 65–75% ethanol. The RNA was then resuspended in RNase-free water and treated with Turbo DNase (Ambion). Samples were then cleaned up using Qiagen RNeasy Mini kit columns and their integrity was checked on Agilent 2200 Bioanalyzer (Agilent Technologies, USA). A total of 133 samples were selected for RNA sequencing (74 liver and 59 head kidney samples; Supplementary file 5) based on their EBVs for resistance to SRS and RNA quality. Thereafter, the Illumina Truseq mRNA stranded RNA-Seq Library Prep Kit protocol was followed directly. Libraries were checked for quality and quantified using the Bioanalyzer 2100 (Agilent), before being sequenced on 16 lanes of the Illumina HiSeq 4000 instrument using 75

base paired-end sequencing at Edinburgh Genomics, UK. Raw reads have been deposited in NCBI's Sequence Read Archive (SRA) under BioProject accession number PRJNA669807.

Read mapping

The quality of the sequencing output was assessed using FastQC v.0.11.5 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Quality filtering and removal of residual adaptor sequences was conducted on read pairs using Trimmomatic v.0.38 [85]. Specifically, Illumina specific adaptors were clipped from the reads, leading and trailing bases with a Phred score less than 20 were removed and the read trimmed if the sliding window average Phred score over four bases was less than 20. Only reads where both pairs were longer than 36 bp post-filtering were retained. Trimmed reads were then pseudoaligned against the Atlantic salmon reference transcriptome (ICSASG_v2 Annotation Release 100 [86];) using kallisto v0.44.0 [87].

Differential expression

Transcript level expression was imported into R v3.6 [88] and summarised to the gene level using the R/tximport v1.10.1 [89]. Gene count data were used to estimate differential gene expression using the Bioconductor package DESeq2 v.3.4 [90]. Briefly, size factors were calculated for each sample using the 'median of ratios' method and count data was normalized to account for differences in library depth. Next, gene-wise dispersion estimates were fitted to the mean intensity using a parametric model and reduced towards the expected dispersion values. Finally a negative binomial model was fitted for each gene and the significance of the coefficients was assessed using the Wald test. The Benjamini-Hochberg false discovery rate (FDR) multiple test correction was applied, and transcripts with $FDR < 0.001$, normalized mean read counts > 10 and absolute \log_2 fold change values (FC) > 1 were considered differentially expressed genes. Hierarchical clustering and principal component analyses were performed to visually identify outlier samples, which were then removed from the analyses. The R packages "pheatmap", "PCAtools" and "EnhancedVolcano" were used to plot heatmaps, PCAs and volcano plots, respectively. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were carried out using KOBAS v3.0.3 [91]. Briefly, salmon genes were annotated against KEGG protein database [92] to determine KEGG Orthology (KO). KEGG enrichment for differentially expressed gene lists was tested by comparison to the whole set of expressed genes in the corresponding tissue using Fisher's Exact Test (genes with mean normalized count values > 10). KEGG pathways with ≥ 5 DE genes assigned and showing a Benjamini-

Hochberg FDR corrected p -value < 0.05 were considered enriched for differential expression.

Network correlation analysis

Network correlation analyses were performed in R v3.6 [88] using the WGCNA package v1.69 [93]. Read counts after variance stabilizing transformation in DESeq2 [90] were used as measure of gene expression. Co-expression networks were then built using a power of 10, and clusters of genes were grouped into different color modules, allowing a minimum of 25 genes per module. Correlation between network summary profiles and external traits was quantified, and network trait associations showing $|r| > 0.45$ and $p < 0.001$ were considered significant. Thereafter, Kegg enrichment analyses were performed for the significantly associated networks using KOBAS 3.0.3 [91] as described above.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12864-021-07443-2>.

Additional file 1: Supplementary File 1. Phenotypes of all the samples used in the RNA-Seq experiment

Additional file 2: Supplementary File 2. Lists of differentially expressed genes in control vs 3 and 9 dpi samples in head kidney and liver

Additional file 3: Supplementary File 3. Lists of KEGG pathways showing significant enrichment in the lists of differentially expressed genes for control vs 3 and 9 dpi samples in head kidney and liver

Additional file 4: Supplementary File 4. Composition of the WGCNA network modules and correlation between the expression of each gene and resistance to SRS (gEBVs)

Additional file 5: Supplementary File 5. SNPs in the high-density SNP array, in the low-density SNP panel, and in the imputed dataset after QC

Additional file 6: Supplementary Figure 1. Correlation between different phenotypes and the gene expression pattern of the WGCNA networks in head kidney and liver

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Authors' contributions

RH and JY were responsible for the concept and design of this work. AG performed the molecular biology experiments. CM and DR performed data analysis and interpretation. CM, DR, RH, JP drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

RNA sequencing raw reads have been deposited in NCBI's Sequence Read Archive (SRA) under BioProject accession number PRJNA669807.

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*).

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Ethics approval and consent to participate

The challenge experiments were performed under local and national regulatory systems and were approved by the Animal Bioethics Committee (ABC) of the Faculty of Veterinary and Animal Sciences of the University of Chile (Santiago, Chile), Certificate N° 01–2016, which based its decision on the Council for International Organizations of Medical Sciences (CIOMS) standards, in accordance with the Chilean standard NCh-324-2011.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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2.4. Conclusions.

This chapter used a holistic approach to studying the genetic basis of host resistance and host-pathogen interactions for SRS, highlighting several relevant genes and pathways underlying resistance. A GWAS mapped chromosomal regions associated with host resistance, and no major QTL was identified, confirming the multifactorial architecture of the trait described in prior research (Barría, Marín-Nahuelpi, *et al.*, 2019; Correa *et al.*, 2015). RNA-seq was used to investigate the host transcriptomic response to *P. salmonis* infection and identified up- and down-regulated genes at critical time points during the infection in both tissues, the head-kidney and liver. Comparing this response between animals with high and low resistance breeding values revealed various gene expression networks correlating with genetic resistance (**Figure 2.1**). A KEGG pathway enrichment analysis of the correlated networks and an extensive review of immune pathways identified in the scientific literature as important in *P. salmonis* infection were identified, such as cytoskeleton reorganisation, apoptosis and cell survival, bacterial invasion/intracellular trafficking and the inflammasome.

Moreover, a comprehensive literature review of the most recent information about the bacterium infection, replication, and survival strategies resulted in a list of candidate genes implicated in resistance or susceptibility to infection by *P. salmonis* (**Table 2.1**). Limitations of the study comprise the nature of the bacterial infection. The intraperitoneal infection used differs from the natural method of infection described for *P. salmonis* as direct contact and tissue penetration (Almendras *et al.*, 1997; Smith *et al.*, 1999). This study prioritised the infection of all individuals simultaneously as the study comprises time-of-infection as a differential expression variable, which requires every individual to be infected at the same time to be comparable. This limitation affects the expression of genes and pathways of immune response that may be implicated in the bacterial colonisation of the host. Thus, excluding those pathways implicates that SRS resistance traits related to bacterial colonisation

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are not evaluated in this study. Future studies on the subject are suggested to include the natural method of infection to evaluate the impact of these pathways in SRS resistance and infection overcome. This chapter's findings present numerous opportunities for future functional studies and potentially downstream applications in the salmon industry. Using functional studies such as CRISPR/Cas9 knockout editing in Atlantic salmon cell lines could provide a valuable *in vitro* model of study. Through contrasting the differences between functional and non-functional genes during *P. salmonis* infection, crucial information on the biological function of the candidate genes in SRS resistance may be obtained. Potential applications include modulation of cellular homeostasis to limit bacterial proliferation or enhancement of immune-mediated processes involved in infection (i.e. inflammasome).

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2.5. Supplementary data.

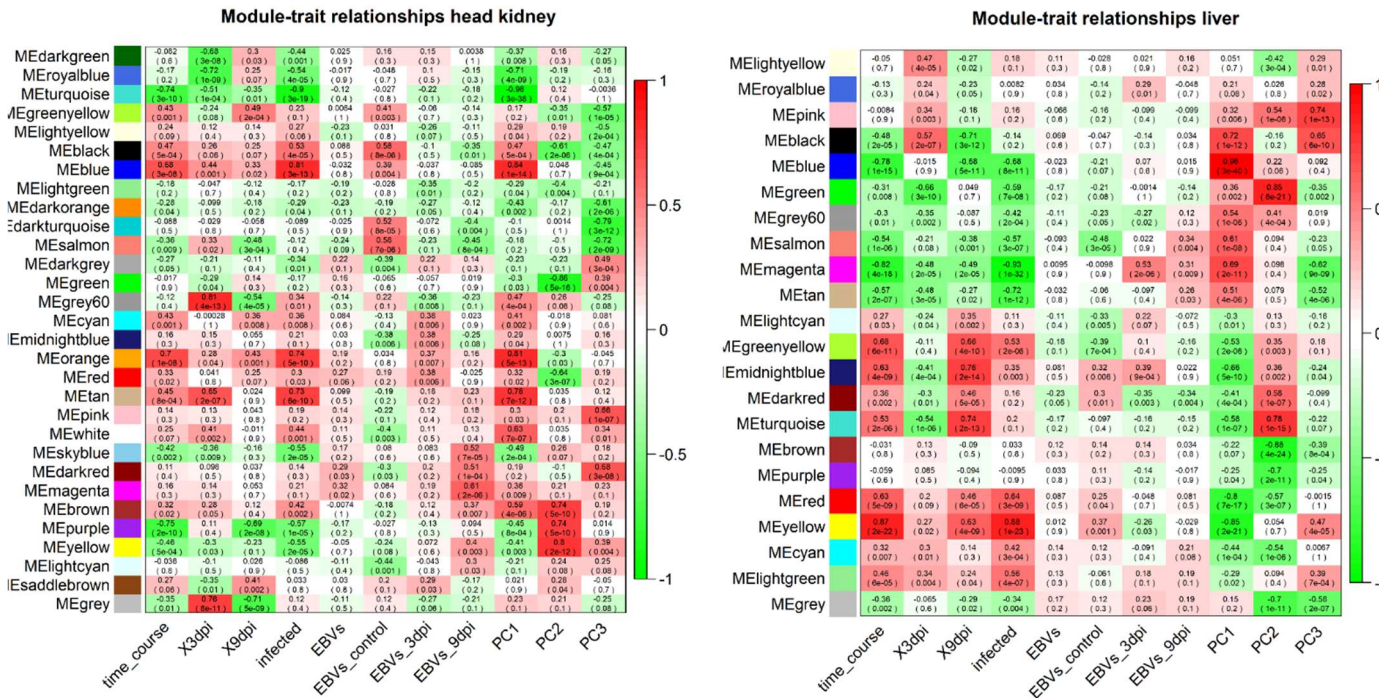


Figure 2.1: Correlation between different phenotypes and the gene expression pattern of the WGCNA networks in head-kidney and liver. Gene expression networks are represented by multiple colour modules on the left, where 31 networks were highlighted in the head-kidney and 22 in liver tissue. Positive (red) or negative (green) correlation is represented in shaded boxes according to correlation values described on the right.

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Table 2.1: List of candidate genes correlated with disease resistance to *P. salmonis*.

Gene symbol	Gene name	ID	Pathway	R	Susceptibility/Resistance	Function	Ref
ROCK1	rho-associated protein kinase 1-like isoform X2	106569322	Cytoskeleton reorganisation	0.27	Resistance	Essential regulator of actin cytoskeleton	Salinas <i>et al.</i> , 2015
SPTB	spectrin beta chain, erythrocytic-like isoform X1	106602627	Cytoskeleton reorganisation	-0.57	Susceptibility	Cytoskeleton constituents: protein linking	Liem, 2016
SEPTIN3	neuronal-specific septin-3-like	106589417	Cytoskeleton reorganisation	-0.42	Susceptibility	Cytoskeleton constituents: GTP-binding	Dolat <i>et al.</i> , 2014
BCL2L1	bcl-2-like protein 1	106582883	Apoptosis and cell survival promotion	-0.21	Susceptibility	Inhibitors of apoptosis	Banga <i>et al.</i> , 2007; Faustin <i>et al.</i> , 2009; Shi & Kehrl, 2019
ITPR3	inositol 1,4,5-trisphosphate receptor type 3-like	106572092	Apoptosis and cell survival promotion	-0.25	Susceptibility	Inhibitors of apoptosis	Rezuchova <i>et al.</i> , 2019
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like	106560822	Apoptosis and cell survival promotion	0.16	Resistance	Apoptosis promoting	Cai <i>et al.</i> , 2017; Zhang & Ney, 2009

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BCL2L11	bcl-2-like protein 11	106608126	Apoptosis and cell survival promotion	0.18	Resistance	Apoptosis promoting	Dai & Grant, 2015
RAB1B	ras-related protein Rab-1B	106577519	Bacterial invasion and intracellular trafficking	0.24	Resistance	Stablish bacterial replication vacuoles	Kagan <i>et al.</i> , 2004; Müller <i>et al.</i> , 2012
RAB9A	ras-related protein Rab-9A-like	106575287	Bacterial invasion and intracellular trafficking	0.10	Resistance	Transport between endosome vesicles and the trans Golgi network	Shapiro <i>et al.</i> , 1993
CBL	E3 ubiquitin-protein ligase CBL-like isoform X1	106577440	Bacterial invasion and intracellular trafficking	-0.33	Susceptibility	Bacterial internalisation facilitator	Qingjun <i>et al.</i> , 2014; Veiga & Cossart, 2005
NLRP1	NACHT, LRR and PYD domains-containing protein 1-like	106592095	Inflammasome	0.20	Resistance	Activation of effector caspases	Chavarría-Smith & Vance, 2015; Li <i>et al.</i> , 2018

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NLRC3	protein NLRC3-like	106597076	Inflammasome	0.31	Resistance	Early immune response	Gao <i>et al.</i> , 2016; Hou <i>et al.</i> , 2017; Pontigo <i>et al.</i> , 2020
NLRP12	NACHT, LRR and PYD domains-containing protein 12-like	106571215	Inflammasome	-0.48	Susceptibility	Suppressor of pro-inflammatory cytokines of the NF-κB pathway	Tuncer <i>et al.</i> , 2014

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The following supplementary material comprises a list of large-size data files available online at <https://doi.org/10.1186/s12864-021-07443-2>

Supplementary File 1. *Phenotypes of all the samples used in the RNA-Seq experiment.*

Supplementary File 2. *Lists of differentially expressed genes in control vs 3 and 9 dpi samples in head-kidney and liver.*

Supplementary File 3. *Lists of KEGG pathways showing significant enrichment in the lists of differentially expressed genes for control vs 3 and 9 dpi samples in head-kidney and liver.*

Supplementary File 4. *Composition of the WGCNA network modules and correlation between the expression of each gene and resistance to SRS (gEBVs).*

Supplementary File 5. *SNPs in the high-density SNP array, in the low-density SNP panel, and in the imputed dataset after QC.*

CHAPTER 3: CRISPR/Cas9 knockout of candidate genes in Atlantic salmon cell line SHK-1.

3.1. Introduction.

An improved understanding of the genetic and functional basis of host resistance to SRS was obtained in the preceding chapter. Several relevant genes and pathways were synthesised into a short list of interesting candidates for functional studies. The research described in this chapter evaluates the phenotypic changes caused by the selective knockout (KO) of the function of one or more genes to decipher its effective role in a controlled cell-based or *in vivo* model (Graham & Root, 2015). As discussed in chapter 1, CRISPR/Cas9 has become the genome-disturbing technique of choice for evaluating gene function due to its ease of use and application (Campenhout *et al.*, 2019). In addition, the technology enables precise gene KO, activation, or inhibition, as well as targeted deletion and insertion of genomic DNA, which is advantageous compared to other genome editing techniques such as ZFNs and TALENs (Knott & Doudna, 2018). Moreover, CRISPR/Cas9 can achieve complete functional KO, offering the potential to generate robust and uniform phenotypes instead of varying degrees of functional loss as with interference RNA (RNAi) (Graham & Root, 2015). However, technical and biological factors may significantly influence the outcome of CRISPR/Cas9 gene KO. Several variables, such as the determination of the target locus, the design of the sgRNA, an effective delivery method of the CRISPR/Cas9 components, and the incidence of off-target deletions and unwanted mutations, have the potential to influence the outcome of functional KO experiments (Campenhout *et al.*, 2019).

3.1.1. Determination of the target locus and sgRNA design.

To effectively achieve a loss of function through genome KO, it is necessary to choose the target sites carefully. Although a thorough understanding of protein structure and functional domains is not required for a successful KO, targeting functional protein domains has recently been shown to increase the proportion of loss-of-function mutations and decrease the number of in-frame variants retaining functionality (He *et al.*, 2019; Shi *et al.*, 2015). In addition, genome-editing experiments designed to generate KOs should target exons shared by all gene transcript variants. This approach may be applied to exons conserved between all gene families (Hyams *et al.*, 2018). Moreover, sites that code for amino acids near the N-terminus of the protein should be avoided to limit the use of an alternative ATG downstream of the annotated start codon. Similarly, targeting sites that code for amino acids near the C' terminus of the protein decreases the likelihood of creating a non-functional allele since the protein translated may remain functional (Doench, 2018).

In order to generate an effective frame-shift mutation in the target site, two important variants should be considered for the sgRNA design, the on-target efficiency and the potential off-target binding. The latest studies indicate that the primary sequence of the sgRNA and the intrinsic properties of the target site are significant determinants of its on-target activity properties (Doench *et al.*, 2016; Doench, 2018; Graham & Root, 2015; Wu *et al.*, 2014). Numerous computational strategies for scoring sgRNAs to predict cleavage efficiency and specificity have been developed. The majority of scores are empirical or trained using experimental datasets and are implemented using various computational techniques (Li *et al.*, 2022). Effective computational methods facilitate the *in silico* design of specific sgRNAs with a prediction of their efficiency. Currently, available sgRNA design tools include CHOPCHOP, E-CRISP, CRISPR RGEN tools and CRISPOR (Li *et al.*, 2022).

3.1.2. Off-target editing.

A principal advantage of CRISPR/Cas9 genome editing is the generation of edits in particular target sites of the host genome due to the specificity of the sgRNA component (Knott & Doudna, 2018). Nevertheless, the off-target binding of the sgRNA and generation of unintended/nonspecific modifications to the genome is a possible outcome due to the sgRNA tolerance to nucleotide mismatches (Wu *et al.*, 2014). Unspecific sgRNA binding is a crucial aspect of functional studies because mutations may result in unexpected effects on the host, such as a change in transcription factors, gene silencing, or gene KO (Hallerman, 2021; Kosicki *et al.*, 2018).

In previous studies of CRISPR/Cas9 teleost genome editing, a high percentage of embryo mortality (Elaswad *et al.*, 2018) and malformation (Simora *et al.*, 2020) has been attributed to off-target mutations. Nonetheless, the off-target effects can be minimised through careful design of the guide RNA (Blix *et al.*, 2021; Hallerman, 2021), and multiple methods have been developed to predict the occurrence of off-target effects. Several studies on teleost genome editing utilise the alignment-based method, which compares the sgRNAs to the specie's genome assembly and evaluates the occurrence of off-target sequences (Datsomor *et al.*, 2019; Edvardsen *et al.*, 2014; Gratacap, Regan, *et al.*, 2020; Ma *et al.*, 2018; Stundl *et al.*, 2022). In addition, the aforementioned sgRNA design tools can predict off-target annealing using a score-based method in which a penalty matrix is created based on the number and position of the mismatches between sgRNA and the contrasted species genome assembly (Hsu *et al.*, 2013). A score for each sgRNA is given based on its potential off-target sites. The cutting frequency determination (CFD) (Doench *et al.*, 2016), and the Massachusetts Institute of Technology (MIT) score ranks between 0 to 100 (Hsu *et al.*, 2013). In both methods, the higher score numbers correspond to fewer possibilities of off-target binding. Diverse teleost genome editing studies have utilised the following tools to

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minimise off-target effects: Cas-OFFinder (Jin *et al.*, 2020), CRISPRscan (Elaswad *et al.*, 2018), and CRISPOR (Gratacap, Jin, *et al.*, 2020).

3.1.3. Delivery method of the CRISPR/Cas9 components.

Another essential variable to accomplish a successful CRISPR/Cas9 KO is how the CRISPR/Cas9 components are delivered. The delivery system generally consists of two components; the cargo and the delivery vehicle (Lino *et al.*, 2018). Three main types of cargo have been used in teleost genome editing; plasmid DNA constructs encoding the Cas9 protein and the sgRNA (Edvardsen *et al.*, 2014; Ma *et al.*, 2018; Zhu *et al.*, 2019), mRNA encoding Cas9 protein together with the sgRNA (Datsomor *et al.*, 2019; Jin *et al.*, 2020; Li *et al.*, 2023), and the direct delivery of the ribonucleoprotein (RNP) complex (Cas9 protein and sgRNA) (Elaswad *et al.*, 2018; Gratacap, Jin, *et al.*, 2020; Liu *et al.*, 2018). Regarding the delivery vehicle, three different approaches have been reported to successfully deliver the CRISPR/Cas9 cargo. The most used physical delivery is the microinjection of early stage embryos (Hallerman, 2021). This method is widely used in *in vivo* studies due to the external nature of teleost reproduction, which facilitates genome edits near the one-cell stage of development (Gratacap *et al.*, 2019). However, in *in vitro* studies, delivery by transfection has demonstrated high efficiency in medaka (*Oryzias latipes*) (Liu *et al.*, 2018), grass carp (*Ctenopharyngodon idellus*) (Ma *et al.*, 2018), chinese giant salamander (*Andrias davidianus*) (Ma *et al.*, 2018), Chinook salmon (*Oncorhynchus tshawytscha*) (Dehler *et al.*, 2016) and Atlantic salmon (*Salmo salar*) (Gratacap, Jin, *et al.*, 2020) immortalised cell lines. Finally, delivery mediated by viral vectors (lentivirus and/ or adenovirus) has been successfully used in zebrafish (*Danio rerio*), medaka (Kawasaki *et al.*, 2009), and Chinook salmon (Gratacap, Regan, *et al.*, 2020).

3.1.4. Whole-genome duplication events.

Effective use of CRISPR gene editing to assess gene function requires knowledge and strategic targeting of specific gene paralogues, which can be challenging in complex genomes. Whole-genome duplication (WGD) is a duplication of the genome that results in an additional set of all genes found in numerous finfish lineages, including salmonids. WGD may result in subfunctionalization when duplicated genes retain the function of the source gene, neofunctionalization when duplicated genes acquire a new function distinct from the source, or non-functionalization when duplicated genes lose their function entirely (Blix *et al.*, 2021). Salmonids had undergone four WGD events (Ss4R), and the presence of paralogues genes is crucial to consider in genome editing studies for functional KO, as it may be required to target all paralogues genes to achieve the desired phenotype (Gratacap, Regan, *et al.*, 2020). Studies on the expression of IGFBP-2 in rainbow trout (*Oncorhynchus mykiss*) indicate the importance of targeting both IGFBP-2 gene duplicates to disrupt protein expression completely, as the functional paralogue may nullify the effect of the intended mutation (Cleveland *et al.*, 2018). In contrast, studies in which single-copy genes such as SLC45A2 and TYR were edited exhibited no paralogue disruption (Edvardsen *et al.*, 2014; Wargelius *et al.*, 2016).

In chapter 2, a comprehensive examination of the transcriptomic host response to *P. salmonis* infection revealed several genes underlying SRS resistance. In this chapter, an *in vitro* CRISPR/Cas9 KO model was developed to investigate the potential role of these genes in the infection process.

The overarching objective of this chapter is to optimise a CRISPR/Cas9 method for editing the Atlantic salmon cell line SHK-1 in a reliable and reproducible manner. Within this work, three aspects of the CRISPR/Cas9 methodology were assessed: i) to design efficient sgRNAs to target the potential candidate genes associated with SRS resistance; ii) to explore the

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potential off-target edits in the Atlantic salmon genome after CRISPR/Cas9 KO, and iii) to evaluate the efficiency of CRISPR/Cas9 RNP complex electroporation for genome editing in the SHK-1 cell line.

3.2. Material and Methods.

3.2.1. Experimental design.

Differential expression (DE) analysis of up-regulated genes during a large-scale *in vivo* infection challenge with the bacterium *Piscirickettsia salmonis* yielded five candidate genes putatively associated with disease resistance: NLRP1, NLRP12, SLC11A2, BNIP3 and CBL. These genes are involved in key processes during this host-pathogen interaction, namely the inflammasome, intracellular trafficking, apoptosis and bacterial internalisation.

An Atlantic salmon immortalized cell line Salmon Head Kidney 1 (SHK-1) was transfected by electroporation with a CRISPR/Cas9 RNP complex designed to knockout (KO) the function of these five genes (**Figure 3.1 A**). Additionally, one gene involved in pigmentation and non-related to immune response, SLC45A2, was also knocked out and used as an “edited-control”. SHK-1 cells were incubated with 60 µL of the broad-spectrum antibiotic Kanamycin Sulphate (50 mg/mL) for three days before transfection to avoid pre-existent bacterial contamination. After transfection, cells were maintained without antibiotics for two days, and subsequently, a 1% Penicillin-Streptomycin (Gibco, Waltham, USA) antibiotic was added to the media and incubated at 20°C until 80% confluency. Cells were split and grew until passage three, when enough cells were available for the infection challenge (**Figure 3.1 B**).

One week after transfection, the DNA of the edited cell lines was extracted to test editing efficiency. The target region was amplified by PCR

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and the amplicons were sequenced by Sanger sequencing (GATC/Eurofins, Germany). The chromatogram obtained was analysed using the Inference of CRISPR Edits (ICE) tool by Sinthego to obtain the editing efficiency and KO score (**Figure 3.1 C**).

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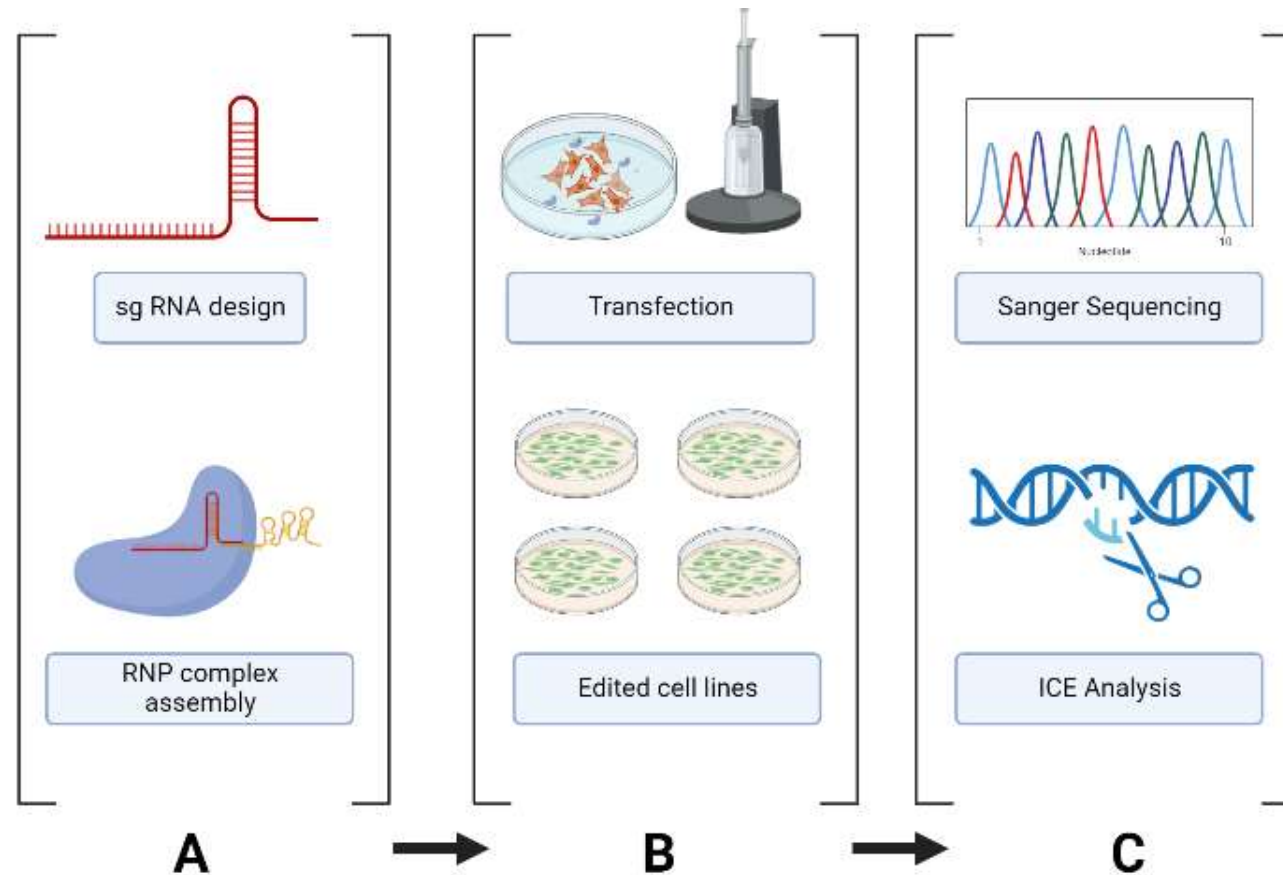


Figure 3.1: Diagram representing the experimental workflow of CRISPR/Cas9 RNP genome editing. A) sgRNA design, B) delivery of the Cas9/gRNA RNP complex, and C) the assessment of editing efficiency. Figure created with BioRender.com.

3.2.2. SHK-1 cell line and growth conditions.

The cell line Salmon Head Kidney 1 (SHK-1) is a macrophage-like cell line that originated from Atlantic salmon head-kidney cells and was obtained from the European collection of authenticated cell cultures (ECACC) General cell collection (97111106). The SHK-1 cells were cultured in growth media Leibovitz L-15 (Sigma-Aldrich, St. Louis, USA) supplemented with 40 μ M β -Mercaptoethanol (Gibco, Waltham, USA), 5% Foetal Bovine Serum (FBS) (Gibco, Waltham, USA), and 1% Penicillin-Streptomycin (Gibco, Waltham, USA) and maintained at 20°C without CO₂. Cultured cells were seeded at 10,000 cells/cm² and were split in 1:2 at 80% confluence, adding 0.05% Trypsin/EDTA (Thermo Fisher Scientific) for 5 minutes.

3.2.3. Design and synthesis of sgRNA.

The crRNA targeting NLRP1, NLRP12, SLC11A2, BNIP3, and CBL were designed using the online tool CRISPOR (version 4.99, <http://crispor.tefor.net/>) and Benchling (<https://benchling.com>). SLC45A2 crRNA was obtained from Gratacap *et al.*, (2020). crRNA custom design and tracrRNA were acquired from Integrated DNA Technologies (IDT) (<https://eu.idtdna.com/pages>). The most suitable guide sequences were determined using CRISPOR by entering a < 2000 bp target exon sequence (FASTA) in the input sequence box. The reference genome “*Salmo salar* – Atlantic salmon NCBI GCF_000233375.1 (ICSASG_v2)” was selected. Ultimately, the Protospacer Adjacent Motif (PAM) “20bp-NGG-Sp Cas9, SpCas9-HF1, eSpCas9 1.1.” was used. crRNA candidate sequences were ranked according to the highest CFD specificity score (Tycko *et al.*, 2019), MIT specificity score, and the lowest off-target for mismatches next to PAM (Concordet & Haeussler, 2018) in the reference genome. Two crRNA sequences were selected for each gene, and their editing efficiency was evaluated by ICE analysis (Synthego Inc.). sgRNAs with indel rate over 80%

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and knockout score over 50% were selected for genome editing. Primers to amplify the sgRNA segments were selected from CRISPOR, and their specificity was confirmed with the primer design tool of NCBI primer BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

3.2.4. Cas9/sgRNA RNP complex assembly.

In order to assemble the sgRNA, a crRNA and tracrRNA hybridization process was made combining 1 μ L of crRNA (100 μ Mol, IDT) with 1 μ L of tracrRNA (100 μ M, IDT), then incubated at 95°C for 5 min and cooled down to room temperature. Subsequently, 2 μ L of Cas9 (20 μ M, NEB, Ipswich, USA) was added to obtain the Cas9/sgRNA RNP complex assembly. The mixture was incubated for 15 min at room temperature and chilled on ice until use (Gratacap, Jin, *et al.*, 2020).

3.2.5. Cas9/sgRNA RNP complex electroporation in SHK-1 cells.

Delivery of the assembled Cas9/sgRNA RNP complex into the SHK-1 cells was achieved by electroporation using a Neon Transfection System (Invitrogen). 1 μ L of Cas9/sgRNA RNP complex was diluted in 3 μ L of Opti-MEM (Gibco, Waltham, USA) reduced serum media and mixed with 10^5 cells/mL of SHK-1 cells suspended in 10 μ L of Opti-MEM (Gibco, Waltham, USA), for a final volume of 14 μ L. The mixture was electroporated using the Neon pipette and 10 μ L tips for 3 pulses at 1600 V for 10 ms. Electroporation conditions were determined using the standard simple optimisation protocol suggested by the manufacturer using a plasmid expressing GFP (**Figure 3.4**) (Gratacap, Jin, *et al.*, 2020). The electroporated cells were dispensed in a 12-well plate with 2 mL of Leibovitz L-15 (Sigma-Aldrich, St. Louis, USA) fresh media without antibiotics. Three replicates were seeded per edited target gene, and two control samples of transfected SHK-1 without Cas9/sgRNA RNP complex and non-transfected SHK-1 were used. Transfected cells were

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incubated at 20°C without CO₂, and 48 hours post-transfection 20 µL/well of Penicillin-Streptomycin (Gibco, Waltham, USA) was added. Cultured cells were split 1:2 at 80% confluency adding 0.05% Trypsin/EDTA (Thermo Fisher Scientific) for 5 minutes. Seven days post-transfection, one replicate sample per transfected cell line was used for genomic DNA isolation.

3.2.6. Sanger-Sequencing of the edited target region.

In order to assess the editing efficiency and confirm the sequence disruption of each edited candidate gene, genomic DNA (gDNA) was extracted from edited cell lines and unedited controls using the kit Dynabeads DNA DIRECT (Invitrogen), following the manufacturer's instructions. Initially, 10⁴ cells/gene were suspended in DNA-free PBS (pH 7.2–7.6), and in the final step gDNA was eluted off the Dynabeads (Invitrogen) by incubation at 65°C for 5 min. Subsequently, gDNA samples were subjected to PCR amplification of the target region. A total volume of 25 µL composed of 12.5 µL Q5 High-Fidelity 2X Master Mix (NEB), 2 µL of gDNA, 1.25 µL of each forward and reverse primer (**Table 3.1**) and 8 µL of nuclease-free water. Thermal cycling conditions were:

Step	Temperature	Time
Denaturation	98°C	30 sec
Amplification 33 cycles	98°C	10 sec
	Optimal annealing temperature (Table 3.1)	20 sec
	72°C	25 sec
Extension	72°C	2 min
Hold	4°C	Until use

From the amplified mixture, 5 µL were used to run an agarose gel (1.5%) to confirm amplification and correct amplicon size, and 20 µL of the remaining volume was purified using the AMPure XP PCR purification kit (Agencourt) according to the manufacturer protocol. The purified amplicon DNA

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concentration was measured using a NanoDrop (ThermoFisher Scientific) spectrophotometer. Amplicon samples were diluted to 12 ng/μL in DNase-free water and mixed with 5 μM of the specific forward or reverse primer (**Table 3.1**). The final mixture was sent to GATC/Eurofins (Germany) and processed by their Sanger sequencing service.

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Table 3.1: List of primers used for sequencing of candidate's gene target region.

Primer pair	Exon		Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temp (°C)	Amplicon length (bp)	Primer for sequencing
ppNLRP1	Exon 2 XM_014183399.1		GTCGTTGTGTGGACTCTGTGA	ACCTCGTAGAACTGGTGTCTTG	67	302	Forward
ppNLRP12	Exon 3 XM_014144004.1		CAAGTCTGGAATTTGTTATGCTC CT	TTCCCAACACCAGATAGTACGTT	59	328	Forward
ppSLC11A2	Exon 3 XM_014132553.1		ACAGATGGCTTGTCATGGGA	TAGATCCTGGGGGTCATAGGC	60	404	Reverse
ppBNIP3- Ex1	Exon 1 XM_014124158.1		ATGGAGCAAACCCGTGTAGG	AGGAAGACGATACAGCATGGC	62	410	Forward
ppBNIP3- Ex2	Exon 2 XM_014124158.1		TCCCACTGTACATGAAGGATGT	AGTGTTAGAGCCTGACACCTG	61	207	Forward
ppCBL	Exon 2 XM_014155413.1		GCTGCACTGAACAGATCCCA	GTTATGGTCCGGCCCTTGTT	65	471	Forward
ppSLC45A2 (ALBINO)	Exon 6 XM_014129320.1		CAATCACAGGTGGGAAAAGGGC	GAGGGTACTGACCTCCTCCTCA	67	528	Reverse

3.2.7. T7 endonuclease I (T7EI) assay.

A thorough assessment of the potential off-target editing of NLRP12 sgRNA1 was performed in the intronic region NC_027314.1 (XM_014145619.1/XM_014145618.1/XM_014145617.1/XM_014145616.1/NM_001139785.1) suggested by CRISPOR. gDNA from NLRP12-KO cells and unedited SHK-1 cells was extracted seven days post-transfection using Dynabeads DNA DIRECT (Invitrogen) kit. NLRP12-KO gDNA was amplified with two sets of primer pairs (pp). pp Off-target-T7 was designed to amplify a 993 bp size amplicon from the potentially affected locus by an unintended edit of NLRP12 sgRNA1. Similarly, pp NLRP12-T7 was designed to amplify a 918 bp size amplicon from the sgRNA1 target site in the NLRP12 gene as a positive control. gDNA from NLRP12-KO cell line was amplified with both sets of primers, and SHK-1-WT gDNA was amplified with pp Off-target-T7 as a negative control. A PCR was used to amplify both amplicons in a total volume of 50 µL composed of 25 µL Q5 High-Fidelity 2X Master Mix (NEB), 2 µL of gDNA (100 ng), 2.5 µL of each forward and reverse primer (**Table 3.2**) and 18 µL of nuclease-free water. The following cycling conditions were used for the T7EI assay:

Step	Temperature	Time
Denaturation	98°C	30 sec
Amplification	98°C	5 sec
35 cycles	Optimal annealing temperature (Table 3.2)	10 sec
	72°C	20 sec
Extension	72°C	2 min
Hold	4°C	Until use

Amplified amplicons were purified using the AMPure XP PCR purification kit (Agencourt). DNA concentration was estimated using a NanoDrop (ThermoFisher Scientific) spectrophotometer. 200 ng of the purified product

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was mixed with 2 μ L of 10X NEBuffer (NEB), and nuclease-free water completed 19 μ L of reaction volume. A hybridization step was carried out using the following conditions:

Step	Temperature	Ramp rate	Time
Denaturation	95°C		5 min
Annealing	95 - 85°C	-2°C/sec	
	85 - 25°C	-0.1°C/sec	
Hold	4°C		Until use

Finally, 1 μ L of T7 Endonuclease I (NEB) was added to the hybridization product and incubated at 37°C per 15 min. 20 μ L of the final product was resolved in agarose gel (1.5%) to confirm the size of the digested products.

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Table 3.2: Primers pairs used for T7ENI editing assessment of NLRP12 off-target region.

Primer pair	Target site	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temp (°C)	Amplicon length (bp)		
					Total	Small	Big
pp Off-target-T7	Intron NC_027314.1	TGCTCAAAAAGGGATGGACCC	TTGGGGGCATATCCATCTGGT	63	993	299	671
pp NLRP12-T7	Exon 3 XM_014144004.1	AACAGATCGGGTTCGACCTT	TCCCAACACCAGATAGTACGTTG	61	918	215	680

3.2.8. Genome editing efficiency by Inference of CRISPR Edits (ICE) Analysis.

Chromatograms obtained from Sanger sequencing (in .ab1 file format) were analysed using the online tool ICE Analysis from Synthego (version 3, <https://ice.synthego.com/>). Differences between CRISPR/Cas9 edited sequences and the unedited controls were assessed by analysing the indels resulting from SpCas9 induced double-stranded DNA breaks. Editing efficiency expressed as indel % (percentage of cells estimated to contain DNA cuts) and knockout score (proportion of indels that cause a frameshift mutation) were the parameters used to select the optimal gRNAs for successful genome editing of candidate genes.

3.3. Results.

3.3.1. Optimisation of CRISPR/Cas9 system for SHK-1 cell line editing.

3.3.1.1. CHSE-EC cell line editing and promoters efficiency in CHSE-214 versus SHK-1 cell line.

Preliminary work on CRISPR/Cas9 editing of salmonid cell lines was carried out in the Chinook salmon cell line CHSE-EC (Dehler *et al.*, 2016), kindly provided by the Institute of Biological and Environmental Sciences at the University of Aberdeen. This cell line has an integrated EGFP gene and a nucleus Cas9 protein (nCas9) overexpression, which allows editing by transfection of a sgRNA targeting EGFP and a straightforward evaluation of editing efficiency by fluorescence microscopy observation (**Figure 3.2 A**) and fluorescence signal quantification. Flow cytometry quantified a 32.3% EGFP signal loss between CHSE-EC cells and CHSE-EC + sgRNA transfected cells

(**Figure 3.2 B**). These results suggested that the CRISPR/Cas9 method used by Dehler *et al.*, (2016) could be adapted to edit SHK-1 cells, transfecting a plasmid construct encoding Cas9 and EGFP. Nevertheless, this idea was discarded as efficient promoters to express the plasmid construct in SHK-1 cells were not found (**Figure 3.3**).

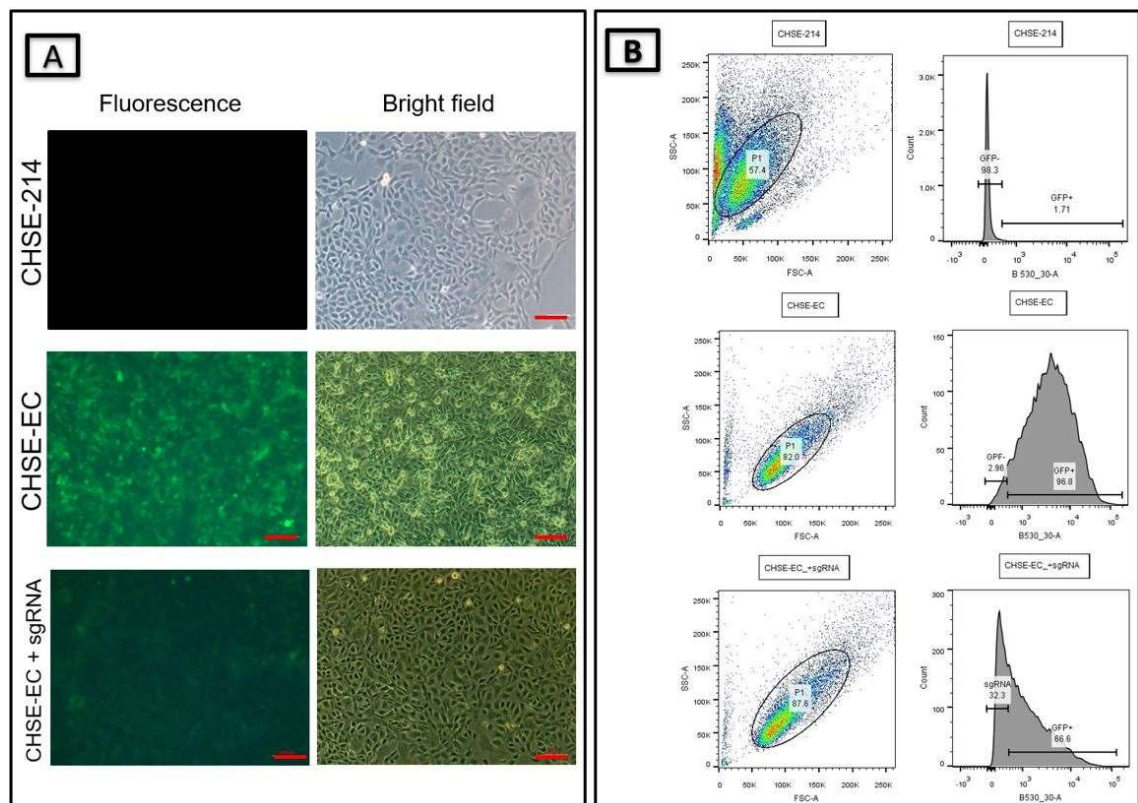


Figure 3.2: CRISPR/Cas9 editing of the EGFP + nCas9 salmonid cell line CHSE-EC. **A)** fluorescence microscopy images of unedited CHSE-214 cells, CHSE-EC cells and CHSE + sgRNA transfected cells. Bar 100 μ m. **B)** Flow cytometer quantification of EGFP fluorescence between unedited CHSE-214 cells, CHSE-EC cells and CHSE + sgRNA transfected cells.

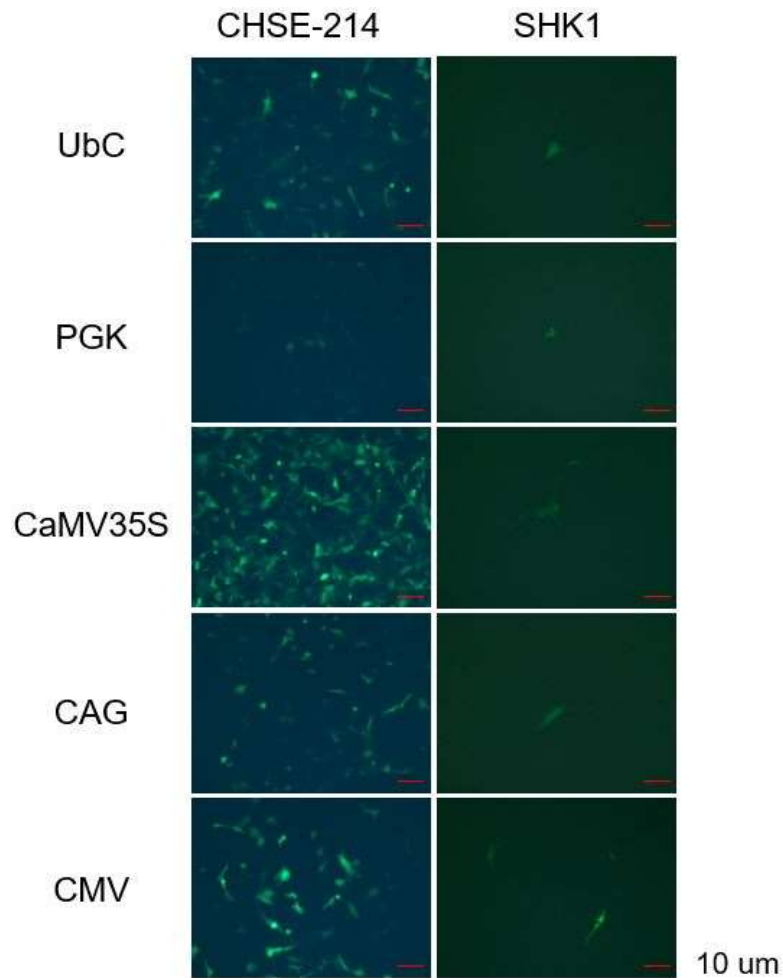


Figure 3.3: Promoters efficiency in CHSE-214 cell line versus SHK-1 cell line.

UbC: ubiquitin C promoter; *PGK*: murine phosphoglycerate kinase promoter; *CaMV35S*: cauliflower mosaic virus 35S promoter; *CAG*: cytomegalovirus enhancer + chicken beta-actin promoter; and *CMV*: cytomegalovirus promoter. Bar 10 μm.

3.3.1.2. Electroporation settings optimisation in SHK-1 cell line.

A high mortality post-transfection in CHSE-214 and SHK-1 cells was observed after optimising the transfection protocol according to the manufacturer's guidance (Neon Transfection System - Invitrogen). This problem was addressed after replacing the buffer R with Opti-MEM (Gibco, Waltham, USA). The modified protocol was carried according to the manufacturer's instruction in 23 different settings, transfecting a plasmid

expressing GFP (Gratacap, Jin, *et al.*, 2020) in SHK-1 cells, and the best results were obtained using 3 pulses at 1600 V for 10 ms (**Figure 3.4**).

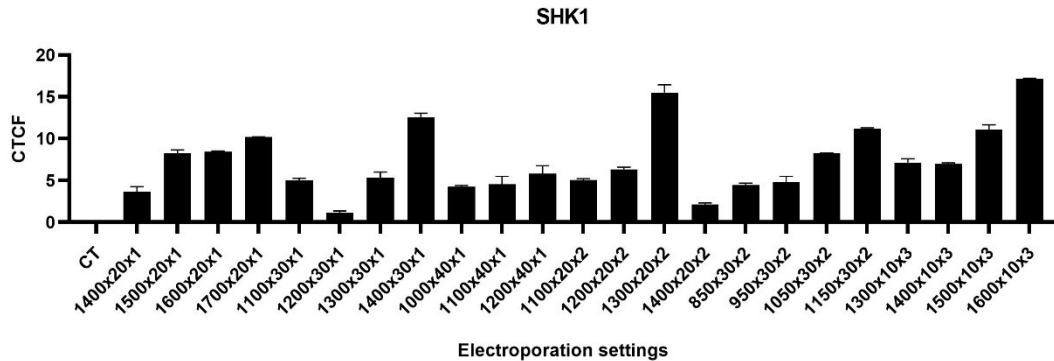


Figure 3.4: Optimisation of SHK-1 cells electroporation protocol. CTCF: corrected total cell fluorescence. Volt x time (ms) x pulses.

3.3.1.3. Candidate genes selection.

Candidate genes selected for CRISPR/Cas9 genome edit were obtained from a large *in vivo* Atlantic salmon infection challenge aimed to dissect the genetic architecture of resistance to SRS, described in chapter 2 (**Table 2.1**). The following criteria highlighted these genes as relevant in response to SRS. **i)** genes flanking the single nucleotide polymorphisms (SNPs) that surpassed the suggestive significance level in the GWAS mapping chromosomal regions associated with host resistance. **ii)** genes significantly up or downregulated at different time points during *P. salmonis* infection, identified through RNA-seq. **iii)** genes present in gene networks associated with resistance to SRS, and **iv)** genes meeting the above criteria and/or highlighted by the scientific literature about *P. salmonis* infection or host immune response to infection (**Table 3.3**). In addition, genes which function, as described in the scientific literature, are essential for metabolic pathways or cell cycle were dismissed to avoid mortality unrelated to bacterial infection. Thus, all genes participating in the cytoskeleton composition and intracellular trafficking of essential metabolites (**Table 2.1**) were obviated from the final list (**Table 3.3**). The gene SLC45A2 was included

as an editing control. This gene has been successfully edited in previous studies and used widely as a visible marker for *in vivo* genome editing (Edvardsen *et al.*, 2014; Gratacap, Regan, *et al.*, 2020). SLC45A2 is involved in pigmentation; thus, no changes in the host response to the infection in a cell line are expected.

Table 3.3. Final list of candidate genes for CRISPR/Cas9 genome editing and selection criteria.

Gene	Pathway	Number of gene copies in the genome *	Selection criteria
NLRP1	Inflammasome	20	ii, iii and iv
NLRP12	Inflammasome	22	ii, iii and iv
SLC11A2	Lysosome; Ferroptosis	3	ii, iii and iv
BNIP3	Apoptosis and cell survival promotion	7	ii, iii and iv
CBL	Bacterial invasion and intracellular trafficking	4	i, ii and iv

* Reference genome: NCBI GCF_000233375.1 ICSASG_v2

3.3.1.4. Design of gRNA and determination of the editing site.

The sgRNAs were designed using the CRISPOR tool (version 4.99, <http://crispor.tefor.net/>). A list of possible sgRNAs was obtained by introducing the target exon sequence in the CRISPOR tool and ranking the potential editing sites according to the predicted specificity scores (MIT and CFD) and predicted efficiency scores. The sgRNAs selected were required to score an MIT and CFD over 50, and no identical target sites in the Atlantic salmon

reference genome (NCBI GCF_000233375.1 ICSASG_v2) (**Table 3.4**). In addition, potential off-target and mismatch were considered during sgRNA selection and will be described in detail in section 3.3.1.6 of this chapter. Two sgRNAs per gene were selected, but their editing efficiency was tested one by one; thus, if the first sgRNA's efficiency is over an 80% indel rate and a 50% knockout index, then the second guide was not tested.

The NLRP1 sgRNA was designed to target exon 2, the largest early exon to contain sgRNA sites scoring over 50, disturbing the PYR domain of the protein (**Figure 3.5**). For NLRP12, the targeted exon was exon 3, using the same criteria as NLRP1 (**Figure 3.6**). Limited information about the protein structure for SLC11A2 in teleost was found. Consequently, the exon selected was exon 3, considered an early exon far enough from a potential second initial codon and early enough to disrupt the synthesis of a functional protein (**Figure 3.7**). For BNIP3, no sgRNAs with efficiency scoring over 50 were found in exons 2-5, and the only sgRNAs scoring over 50 was in exon 1. Due to the risk of off-target effects in the genome, three sgRNA were tested, two in exon 2 and one in exon 1 (**Figure 3.8**). Finally, CBL's sgRNAs target exon 2, the largest exon of the 13-exon gene, aiming to disrupt the 4H domain of the protein (**Figure 3.9**).

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

Table 3.4. List of sgRNAs specificity score and predicted efficiency score.

Gene	Exon	Guide	Guide Sequence	PAM	Specificity Score		Predicted Efficiency	
					MIT	CFD	Doench	Mor-Mateos
NLRP1	Exon 2	sgRNA1	GAGGAATTGCCTCTAAGAGG	AGG	93	95	64	54
	Exon 2	sgRNA2	CTCAGAATACCTCCTCTTAG	AGG	78	88	43	34
NLRP12	Exon 3	sgRNA1	AGGAGGCCAGCCCTGCAAAG	AGG	85	92	67	61
SLC11A2	Exon 3	sgRNA1	TGGGGTTATCAAGACCACCC	AGG	93	96	71	56
BNIP3	Exon 2	sgRNA1	TTTAACAATGGCAACAGTGT	TGG	53	85	51	41
	Exon 2	sgRNA2	TGTTGGCTCACCAGCACATG	GGG	47	95	61	59
	Exon 1	sgRNA3	CGAACGATCGCGACGCAAAG	GGG	100	100	61	56
CBL	Exon 2	sgRNA1	ATCGGCTGTGTACCAACCCC	AGG	93	97	53	65

*MIT: Massachusetts Institute of Technology; CFD: Cutting frequency determination (Doench et al., 2016; Moreno-Mateos et al., 2015)

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

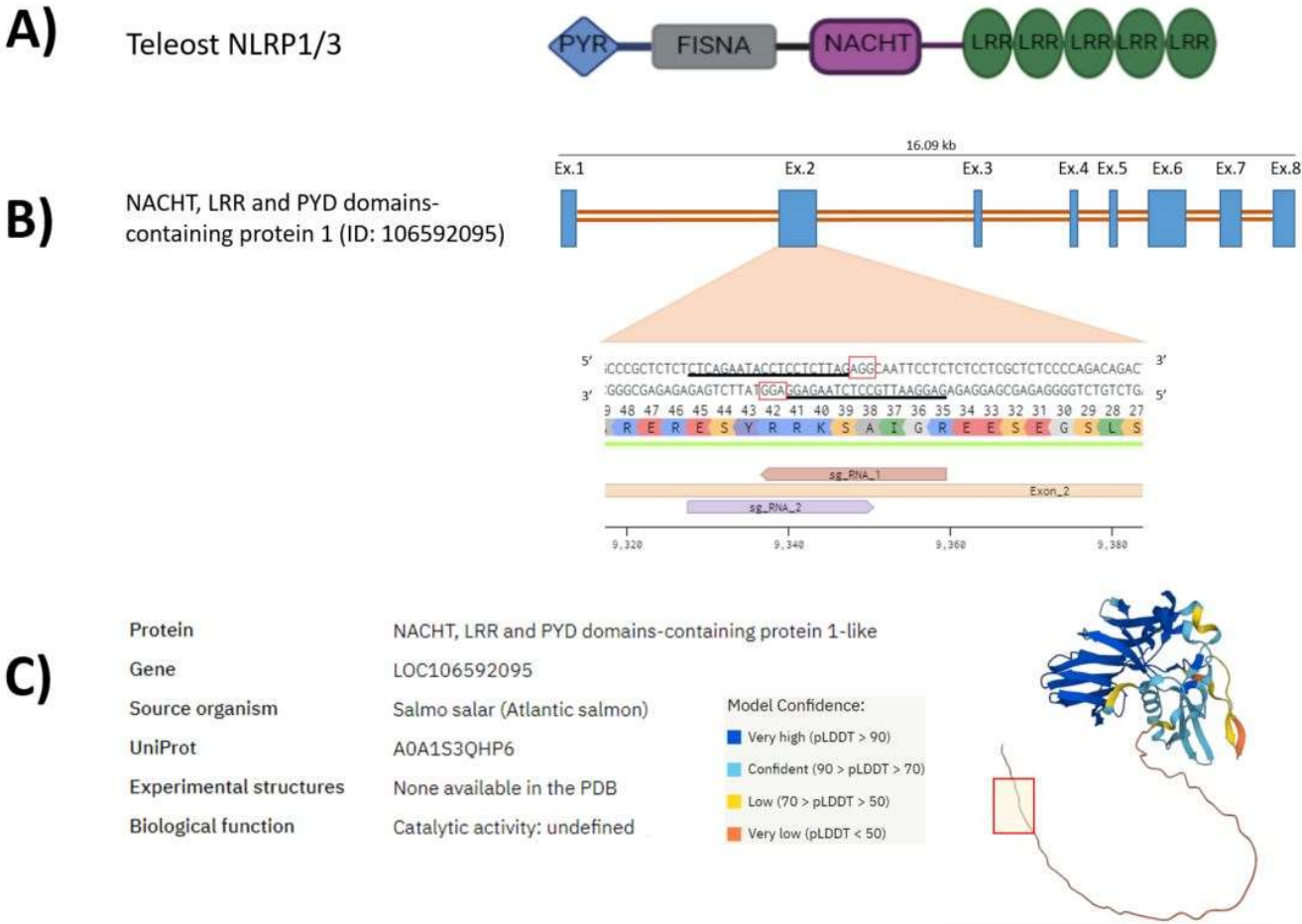


Figure 3.5. Target sites of NLRP1 gene from the Atlantic salmon reference genome (ICSASG_v2 GCF_000233375.1). **A)** Schematic representation of the Nod-like receptor family's protein structure in teleost. **B)** Genomic structure of Atlantic salmon NLRP1 gene (106592095) located in chromosome 4 (ssa04). sgRNA1 and sgRNA2 are located in the second exon. The target site sequence is underlined in black, and the PAM region is boxed in red. **C)** AlphaFold predicted protein 3D structure showing the target site region boxed in red and filled in yellow (Jumper et al., 2021. <https://alphafold.ebi.ac.uk/>). pLDDT= per-residue confidence score (0 to 100).

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

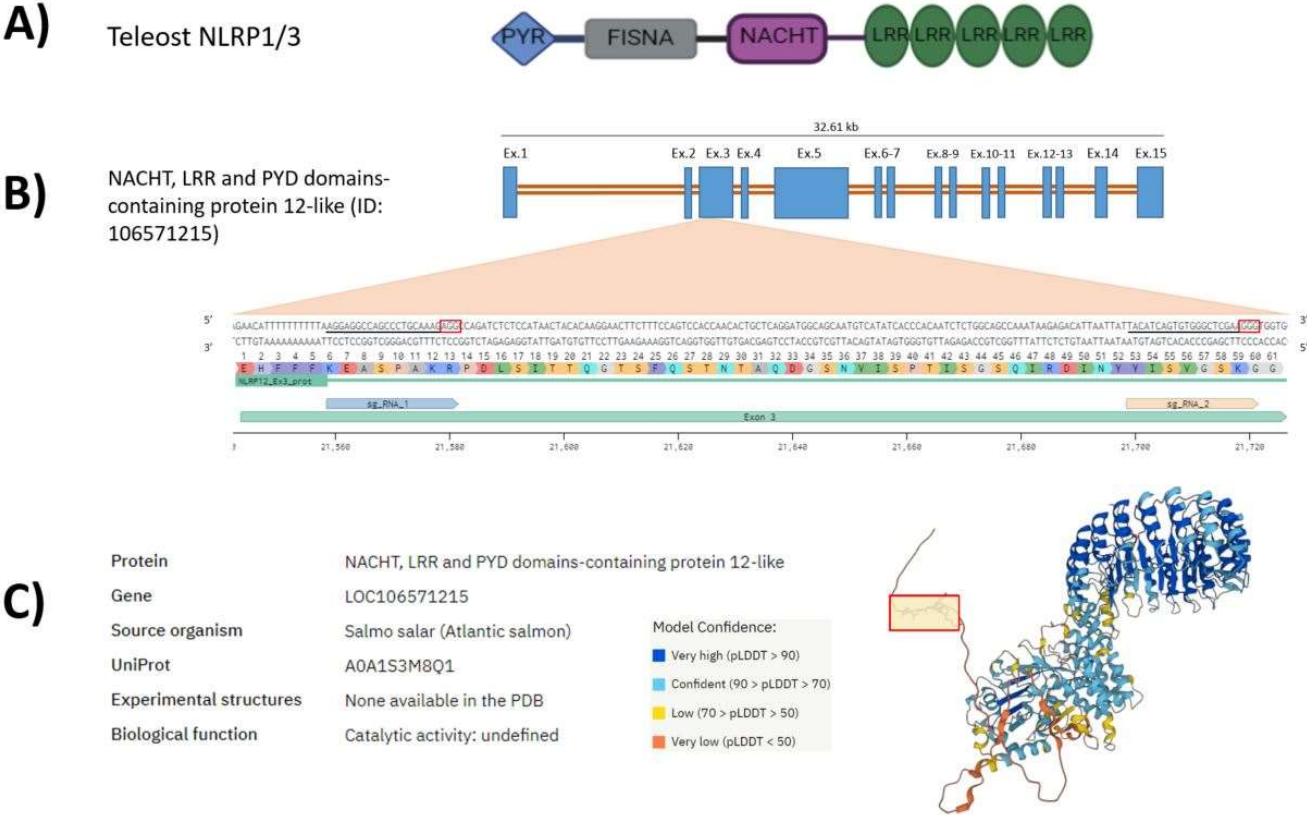
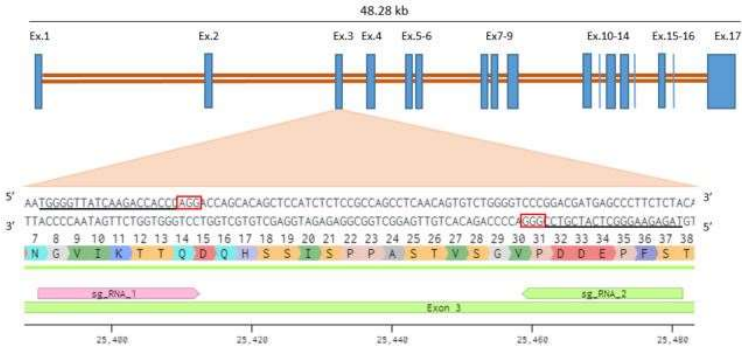


Figure 3.6. Target sites of NLRP12 gene from the Atlantic salmon reference genome (ICSASG_v2 GCF_000233375.1). **A)** Schematic representation of the Nod-like receptor family's protein structure in teleost. **B)** Genomic structure of Atlantic salmon NLRP12 gene (106571215) located in chromosome 15 (ssa15). sgRNA1 and sgRNA2 are located in the third exon. The target site sequence is underlined in black, and the PAM region is boxed in red. **C)** AlphaFold predicted protein 3D structure showing the target site region boxed in red and filled in yellow (Jumper et al., 2021. <https://alphafold.ebi.ac.uk/>). pLDDT= per-residue confidence score (0 to 100).

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A)

Natural resistance-associated
macrophage protein 2-like(ID:
106565428)



B)

Protein	natural resistance-associated macrophage protein 2-like
Gene	LOC106565428
Source organism	Salmo salar (Atlantic salmon)
UniProt	A0A1S3LC20
Experimental structures	None available in the PDB
Biological function	Not available.

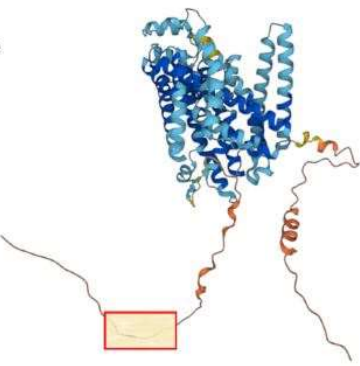
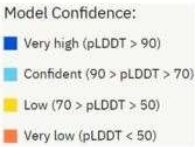


Figure 3.7. Target sites of SLC11A2 gene from the Atlantic salmon reference genome (ICSASG_v2 GCF_000233375.1).

A) Genomic structure of Atlantic salmon SLC11A2 gene (106565428) located in chromosome 12 (ssa12). sgRNA1 and sgRNA2 are located in the third exon. The target site sequence is underlined in black, and the PAM region is boxed in red. **B)** AlphaFold predicted protein 3D structure showing the target site region boxed in red and filled in yellow (Jumper et al., 2021).

<https://alphafold.ebi.ac.uk/>.

pLDDT= per-residue confidence score (0 to 100).

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

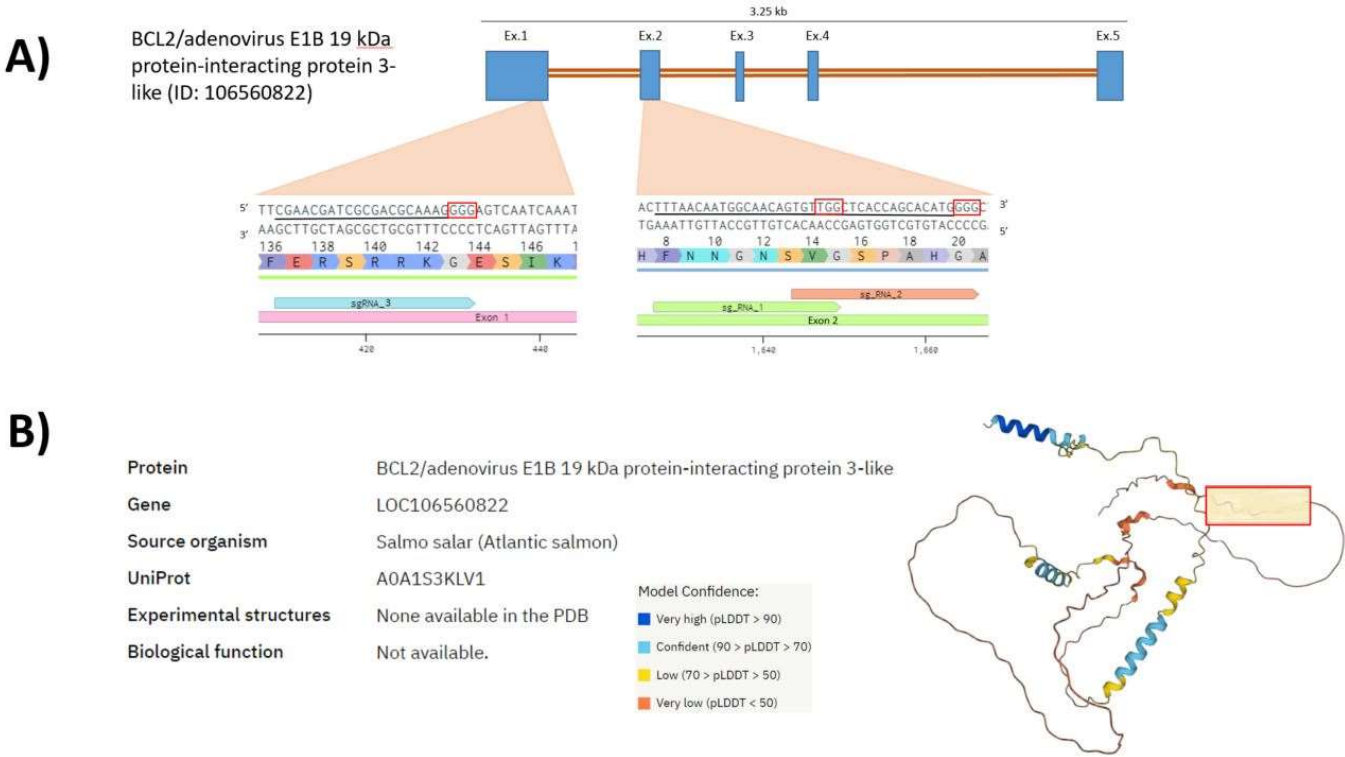


Figure 3.8. Target sites of BNIP3 gene from the Atlantic salmon reference genome (ICSASG_v2 GCF_000233375.1). **A)** Genomic structure of Atlantic salmon BNIP3 gene (106560822) located in chromosome 1 (ssa01). sgRNA1 and sgRNA2 are located in the second exon, and sgRNA3 is located in the first exon. The target site sequence is underlined in black, and the PAM region is boxed in red. **B)** AlphaFold predicted protein 3D structure showing the target site region boxed in red and filled in yellow (Jumper et al., 2021. <https://alphafold.ebi.ac.uk/>). pLDDT= per-residue confidence score (0 to 100).

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

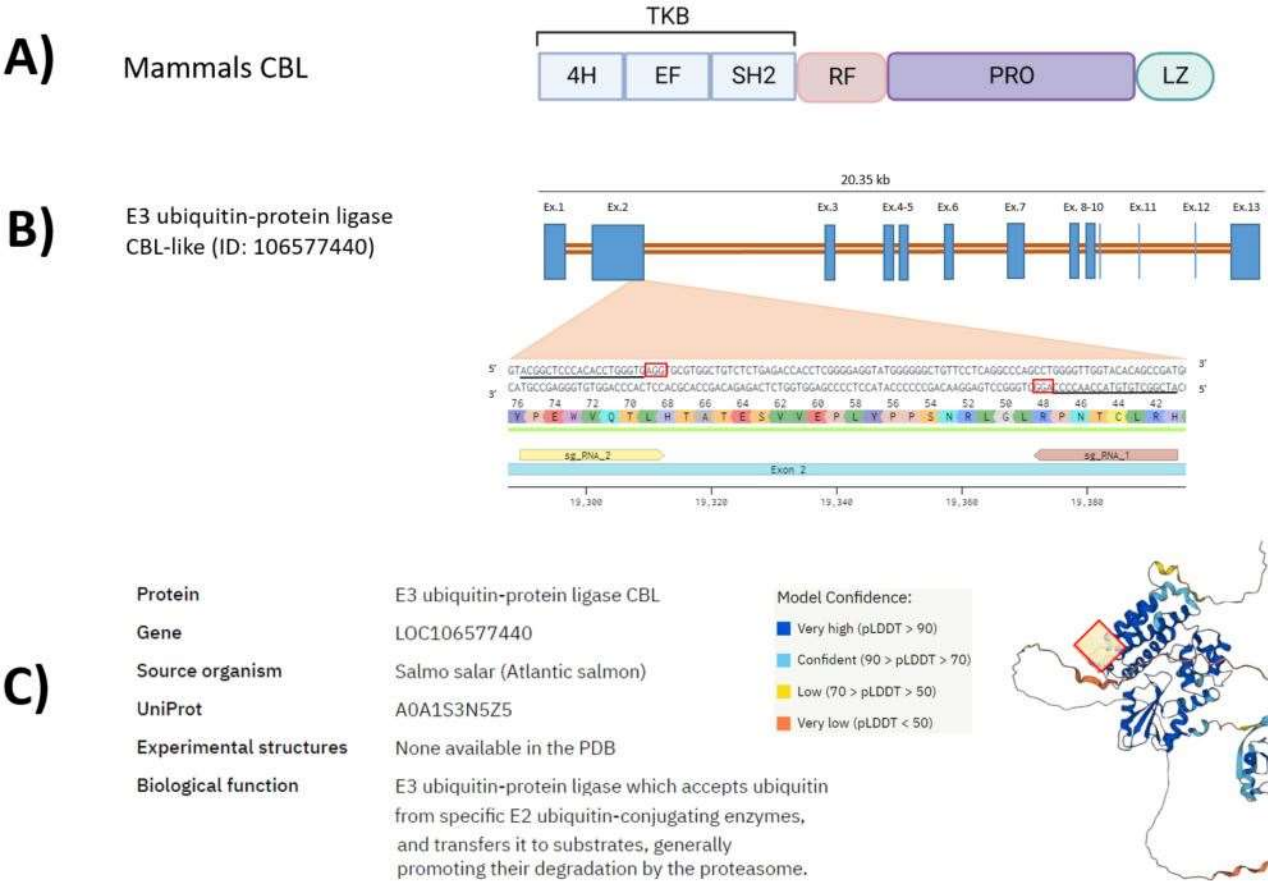


Figure 3.9. Target sites of CBL gene from the Atlantic salmon reference genome (ICSASG_v2 GCF_000233375.1). **A)** Schematic representation of the CBL protein structure in mammals. **B)** Genomic structure of Atlantic salmon CBL gene (106577440) located in chromosome 2 (ssa02). sgRNA1 and sgRNA2 are located in the second exon. The target site sequence is underlined in black, and the PAM region is boxed in red. **C)** AlphaFold predicted protein 3D structure showing the target site region boxed in red and filled in yellow (Jumper et al., 2021. <https://alphafold.ebi.ac.uk/>). pLDDT= per-residue confidence score (0 to 100).

3.3.1.5. Primer design and optimisation.

Primers to amplify the target editing region were designed using the CRISPOR “PCR primer” option for each sgRNA (**Figure 3.10**). The specificity of the primer pairs was confirmed using the online tool NCBI primer BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The optimal annealing temperature was determined by a gradient temperature PCR (**Figure 3.11**).

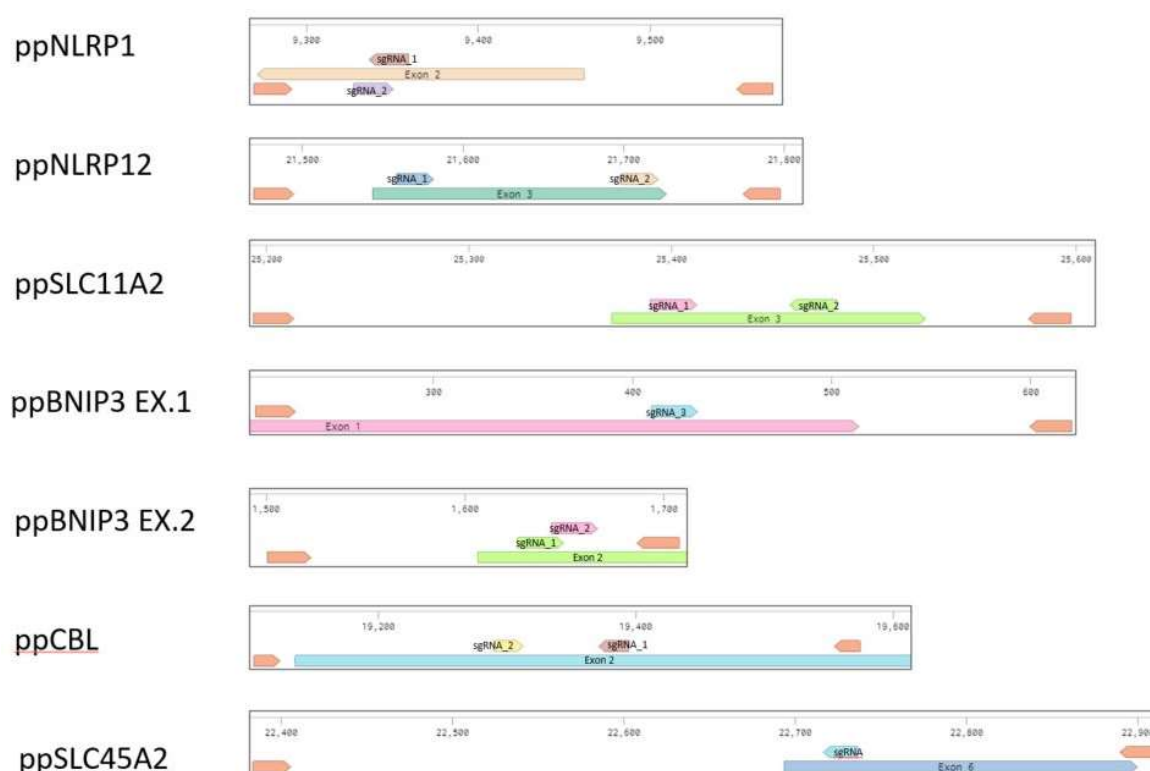


Figure 3.10. Primer pair (pp) location. Schematic figure of ppNLRP1, ppNLRP12, ppSLC11A2, ppBNIP3 exon 1, ppBNIP3 exon 2, ppCBL and ppSLC45A2 location in the target region. Primer sequences and amplicon length, can be found in Table 2.1.

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

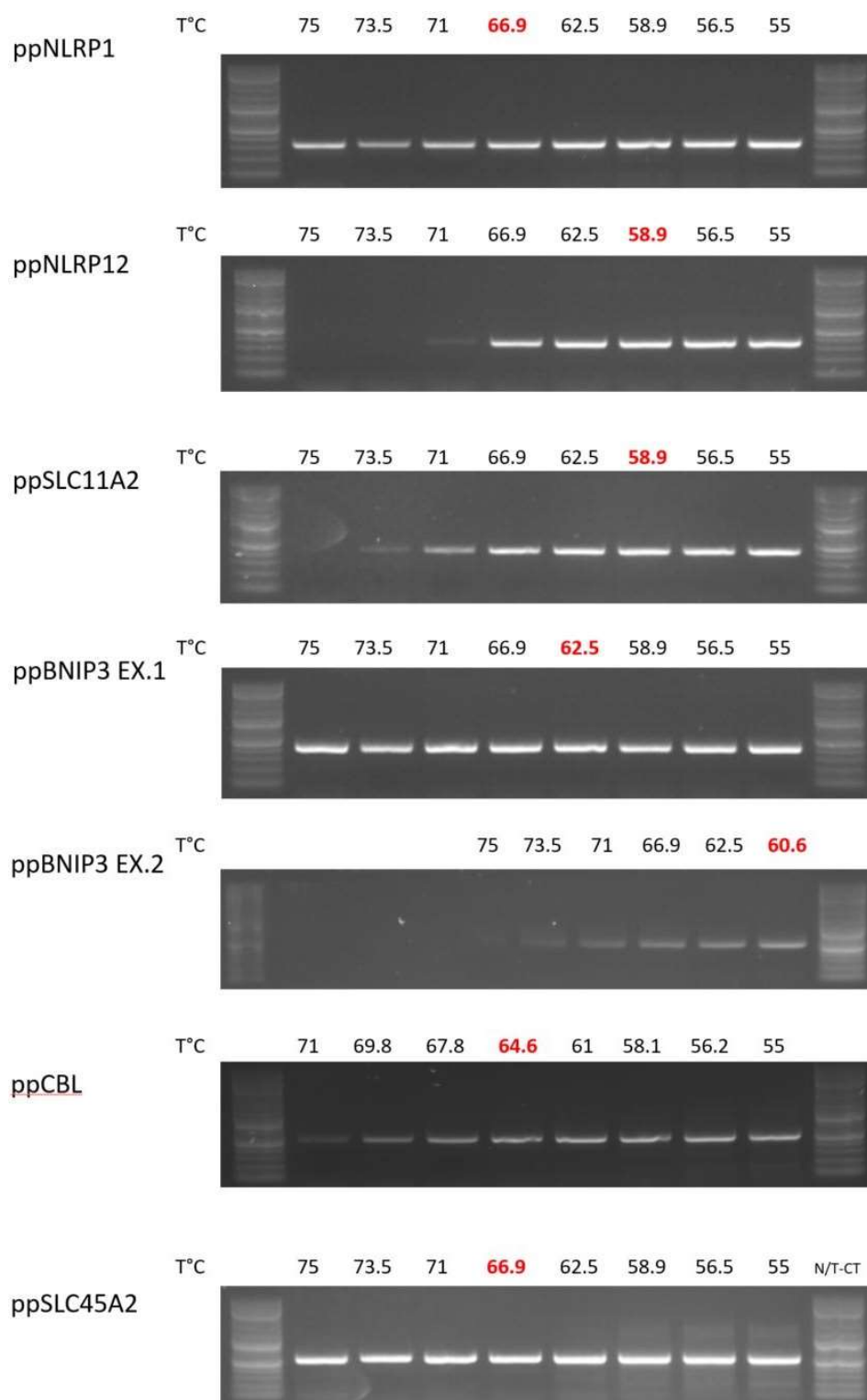


Figure 3.11. Gradient temperature optimisation for primers pairs. The optimal annealing temperature for ppNLRP1 and ppSLC45A2 is 67°C, for ppNLRP12 and SLC11A2 is 59°C, for ppBNIP3 exon 1, ppBNIP3 exon 2 and ppCBL is 62, 61 and 65°C respectively.

3.3.1.6. Potential off-target effects.

In order to minimise the potential off-target effects of the CRISPR/Cas9 editing in the genome, the selected sgRNAs had three or more nucleotides (nts) mismatch with the predicted off-target sequence. In addition, the selected sgRNA sequences had no more than 12 nucleotides matching the seed sequence adjacent to the protospacer adjacent motif (PAM) region. This feature was addressed by the CRISPOR tool with an “off-target for mismatches next to PAM score”, where CFD and MIT numbers indicate the probability of cleavage considering the mismatches of the guide versus the seed sequence (Haeussler *et al.*, 2016). In this study, all selected sgRNA met the abovementioned requirements (**Table 3.5; 3.7 to 3.9**), except for the sgRNA1 utilised to modify the NLRP12 gene (**Table 3.6**). The reason for including this sgRNA was the high CFD and MIT specificity scored (**Table 3.4**) and the three mismatches versus the predicted off-target sequence that met the criteria. In order to evaluate the possible unintended editing, a T7EI assay was tested to visually assess the predicted off-target region, using the edited target region in exon 3 of the NLRP12 sequence as an edited control. Based on the absence of fractions of nuclease-specific cleavage products and the visualization of just one undigested 993 bp segment, it was concluded that no unintended edits were found in the intron region (XM_014145619.1/XM_014145618.1/XM_014145617.1/XM_014145616.1/NM_001139785.1) (**Figure 3.12**).

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

Table 3.5. Potential off-target sites for NLRP1 sgRNA2.

Target site	Mismatch	Potential Off-target Sequence	Off-target score:		Position:
			CFD	MIT	
intergenic:XM_014208400.1-XM_014208399.1	4	guide: CTCAGAAT <u>ACCTCCTCTTAG</u> AGG off-target: AGCAGAAG <u>ACCCCCTCTTAG</u> AGG	0.58	0.70	NC_027306.1:45 537285- 45537307:-
exon:XM_014125176.1	5	guide: CTCAGAAT <u>ACCTCCTCTTAG</u> AGG off-target: CCCAGAAC <u>ACCCCCTCTAAG</u> CGG	0.54	0.15	NC_027309.1:88 556534- 88556556:+
exon:XM_014183986.1	4	guide: CTCAGAAT <u>ACCTCCTCTTAG</u> AGG off-target: ATCAGAAG <u>ACATCCTCTTAA</u> AGG	0.52	0.39	NW_012348582. 1:8137-8159:+
intergenic:XM_014201034.1/XM_014201032.1/ XM_014201037.1/XM_014201036.1/XM_01420 1033.1-XM_014201196.1	5	guide: CTCAGAAT <u>ACCTCCTCTTAG</u> AGG off-target: TTCAGAGT <u>ACCTCCTCCTAA</u> GGG	0.47	0.18	NC_027304.1:60 461753- 60461775:+
exon:LOC106560224	5	guide: CTCAGAAT <u>ACCTCCTCTTAG</u> AGG off-target: CTCAGAGC <u>ACCTTCTCTTAA</u> TGG	0.46	0.17	NC_027309.1:32 094850- 32094872:+

* Grey-shaded sequences are mismatches, and underlined sequences are the seed sequence of 12 nts closer to PAM.

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Table 3.6. Potential off-target sites for NLRP12 sgRNA1.

Target site	Mismatch	Potential Off-target Sequence	Off-target score:		Position:
			CFD	MIT	
intron:XM_014145619.1/XM_014145618.1/XM_014145617.1/XM_014145616.1/NM_001139785.1	3	guide: AGGAGGCCAGCCCTGCAAAG AGG off-target: GGTAGACCAGCCCTGCAAAG AGG	0.5	1.45	NC_027314.1:74 954171- 74954193:-
exon:LOC106581303	4	guide: AGGAGGCCAGCCCTGCAAAG AGG off-target: AGGAGGCTAGCACAGCAAAG TGG	0.38	0.19	NC_027319.1:68 121810- 68121832:+
intron:XM_014176464.1	4	guide: AGGAGGCCAGCCCTGCAAAG AGG off-target: AGGAAACCAGCCCTGTAAAG GGG	0.26	0.29	NC_027325.1:37 989796- 37989818:+
intergenic:Gap-XM_014185852.1	4	guide: AGGAGGCCAGCCCTGCAAAG AGG off-target: AGGAACACTGCCCTGCAAAG AGG	0.26	0.33	NW_012352845. 1:3784-3806:+
intron:XM_014200079.1/XM_014200078.1/XM_014200077.1	5	guide: AGGAGGCCAGCCCTGCAAAG AGG off-target: CAGTGGGCAGCCCTGCAAAG CGG	0.22	0.93	NC_027304.1:40 430454- 40430476:+

* Grey-shaded sequences are mismatches, and underlined sequences are the seed sequence of 12 nts closer to PAM.

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

Table 3.7. Potential off-target sites for SLC11A2 sgRNA1.

Target site	Mismatch	Potential Off-target Sequence	Off-target score:		Position:
			CFD	MIT	
intron:XM_014201212.1/XM_014201211.1	4	guide: TGGGGTTAT <u>CAAGACCACCC</u> AGG off-target: TGGGAGTATCAAA <u>ACCATCC</u> AGG	0.29	0.07	NC_027304.1:62 064385- 62064407:-
intergenic:XM_014177423.1/XM_014177422.1/ XM_014177424.1/XM_014177426.1/XM_01417 7425.1/XM_014177427.1-XM_014177421.1	5	guide: TGGGGTTAT <u>CAAGACCACCC</u> AGG off-target: TGGAGTAATCAGGACCACCA CGG	0.28	0.22	NC_027326.1:90 37537- 9037559:+
exon:XM_014140755.1	4	guide: TGGGGTTAT <u>CAAGACCACCC</u> AGG off-target: TGGGGGTGACAAGACCATCC AGG	0.25	0.11	NC_027313.1:33 488930- 33488952:+
intron:XM_014123275.1	5	guide: TGGGGTTAT <u>CAAGACCACCC</u> AGG off-target: AGGGGTAA <u>CCTAGACCACCC</u> TGG	0.25	0.33	NC_027309.1:38 506804- 38506826:+
intergenic:XM_014166384.1/XM_014166382.1- XR_001323391.1/XM_014166397.1/XM_01416 6391.1/XM_014166390.1/XM_014166389.1/XM _014166388.1/XM_014166387.1/XM_01416638 5.1	5	guide: TGGGGTTAT <u>CAAGACCACCC</u> AGG off-target: GGGGGTTATCAAAACCAACA TGG	0.24	0.05	NC_027321.1:14 171338- 14171360:-

* Grey-shaded sequences are mismatches, and underlined sequences are the seed sequence of 12 nts closer to PAM.

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

Table 3.8. Potential off-target sites for BNIP3 sgRNA3.

Target site	Mismatch	Potential Off-target Sequence	Off-target score:		Position:
			CFD	MIT	
intron:XM_014157587.1	5	guide: CGAACGAT <u>CGCGACGCAAAG</u> GGG off-target: CCATCGAT <u>CGCGAAGCAAAA</u> AGG	0.14	0.10	NC_027318.1:23 529854- 23529876:+
intergenic:XM_014177085.1/XM_014177084.1- XM_014177538.1/XM_014177537.1/XM_01417 7535.1/XM_014177534.1/XM_014177533.1	5	guide: CGAACGAT <u>CGCGACGCAAAG</u> GGG off-target: CGAACGAT <u>CGGGTCACCAAG</u> TGG	0.01	0.03	NC_027326.1:79 09770-7909792:-
intergenic:Gap-LOC106592610	5	guide: CGAACGAT <u>CGCGACGCAAAG</u> GGG off-target: TGAACCAT <u>CGCCAGGCAAAG</u> TGG	0.00	0.07	NW_012348527. 1:1467-1489:-

* Grey-shaded sequences are mismatches, and underlined sequences are the seed sequence of 12 nts closer to PAM.

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

Table 3.9. Potential off-target sites for CBL sgRNA1.

Target site	Mismatch	Potential Off-target Sequence	Off-target score:		Position:
			CFD	MIT	
exon:XM_014201567.1	3	guide: ATCGGCTGT <u>GTACCAACCCC</u> AGG off-target: ACCAGCTGTGTACCAACCTC AGG	0.36	0.96	NC_027304.1:68 080886- 68080908:+
intergenic:XM_014146043.1/XM_014146042.1- XM_014146042.1	5	guide: ATCGGCTGT <u>GTACCAACCCC</u> AGG off-target: AGGGGCTCAGTACCAACCCC TGG	0.24	0.82	NC_027314.1:69 358052- 69358074:+
intron:XM_014177989.1	5	guide: ATCGGCTGT <u>GTACCAACCCC</u> AGG off-target: ATCAGCTGAGCACCAACCAC GGG	0.24	0.17	NC_027326.1:21 428846- 21428868:+
intron:XM_014149849.1	5	guide: ATCGGCTGT <u>GTACCAACCCC</u> AGG off-target: ATACGCTGTGAACCGACCCC TGG	0.22	0.22	NC_027315.1:53 641014- 53641036:-
intergenic:XM_014133113.1/XM_014133112.1- XM_014133631.1/XM_014133630.1/XM_01413 3628.1/XM_014133629.1	4	guide: ATCGGCTGT <u>GTACCAACCCC</u> AGG off-target: TTCGACTGTATCCCAACCCC AGG	0.21	0.65	NC_027311.1:74 775056- 74775078:-

* Grey-shaded sequences are mismatches, and underlined sequences are the seed sequence of 12 nts closer to PAM.

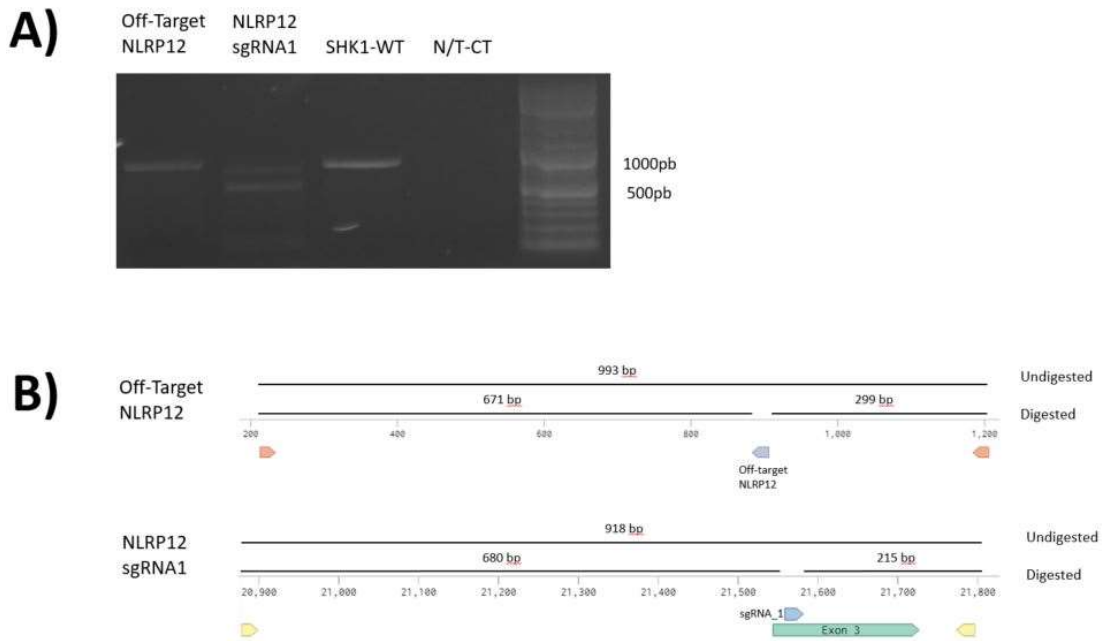


Figure 3.12. T7 endonuclease I (T7EI) assay of sgRNA1 NLRP12 off-target region (NC_027314.1). **A)** Off-target NLRP12, NLRP12 sgRNA1 in exon 3 as positive control, unedited SHK-1-WT as negative control and no-template control. **B)** Schematic figure of the expected digested and undigested amplicon lengths. Primer sequences and annealing temperature, can be found in Table 3.2.

3.3.1.7. Genome editing confirmation.

The selected crRNAs were annealed to form a sgRNA and following to assemble the CRISPR/Cas9 RNP complex. The RNP complex was electroporated in Atlantic salmon SHK-1 cells, and seven days post-transfection, samples were taken for gDNA extraction and amplified by PCR with the optimised primers. Purified PCR products were Sanger sequenced, and the ICE analysis of edited cells was made by contrasting edited samples with unedited SHK-1 cells. sgRNAs reaching over 80% indel rates and 50% knockout index were selected to create the CRISPR/Cas9 edited cell lines. For NLRP1, the first sgRNA1 tested reached a 45% indel rate and a 45% knockout score, while the second sgRNA2, which reached a mean of 95 and 94%, respectively (**Figure 3.13 A**). sgRNA1 and sgRNA2 for BNIP3 did not meet the

indel and knockout score requirements. In comparison, sgRNA3 reached a mean of 85% indel rate and 84% knockout index (**Figure 3.13 B**). NLRP12, SLC11A2, CBL and SLC45A2 sgRNA1 reached over 80% indel rate (88, 93, 88 and 93%, respectively), meeting the selection requirements (**Figure 3.13 C**) depicts the final selected sgRNAs for each gene.

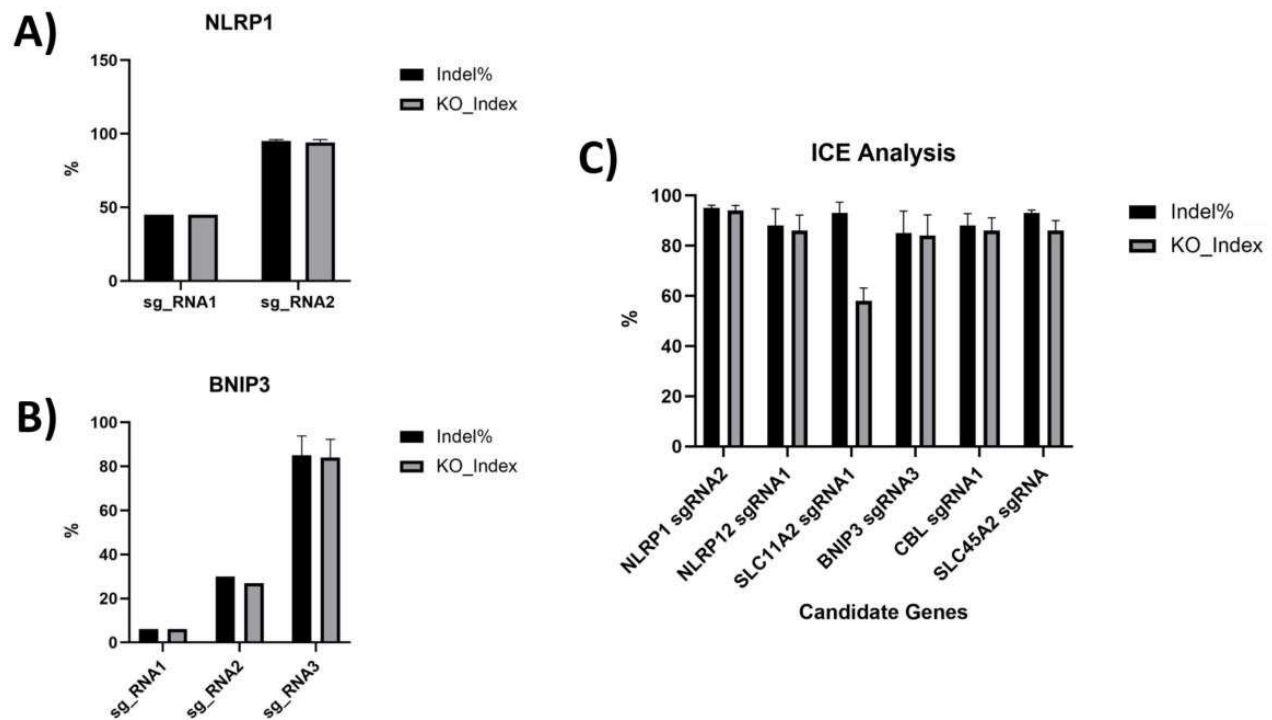


Figure 3.13. ICE Analysis of CRISPR/Cas9 edited candidate genes. **A)** Indel rate and knockout index of NLRP1 sgRNA1 vs sgRNA2, **B)** indel rate and knockout index of BNIP3 sgRNA1 vs sgRNA2 vs sgRNA3, and **C)** indel rate and knockout index of the selected sgRNAs for candidate genes CRISPR/Cas9 genome editing. Data were presented as mean \pm SD with at least three biological replicates.

The knockout index is estimated based on the relative contributions of the different types of mutations present and their relative abundance. It indicates what percentage of the contributing indels are liable to cause a KO of the targeted gene. In this study NLRP12, CBL and SLC45A2 sgRNA1 scored over 80% knockout index (86% each). In contrast, SCL11A2 sgRNA1 was the only guide scoring a 58% knockout index, a tight gap from the minimum requirements. **Figures 3.14 to 3.18** displays the chromatograms of the target sequence sites and the profiles of the different types of mutations and their relative contributions.

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

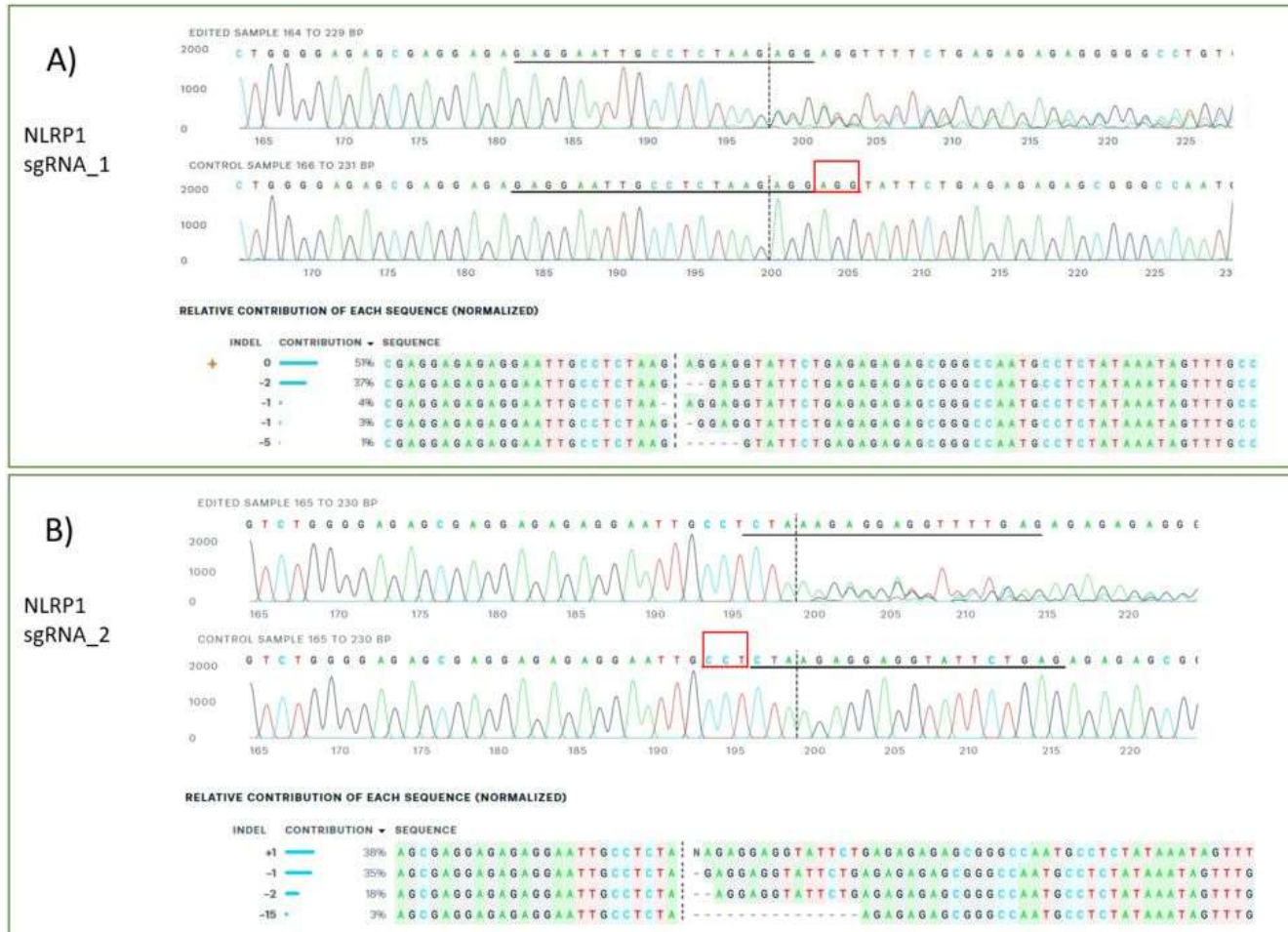


Figure 3.14. Chromatograms from the NLRP1 target region edited by CRISPR/Cas9 and relative contribution of each mutant sequence. The target site sequence is underlined in black, and the PAM region is boxed in red. Dash sign means deletion, and N means inserted nucleotides or substitution. A) sgRNA1, and B) sgRNA2.

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

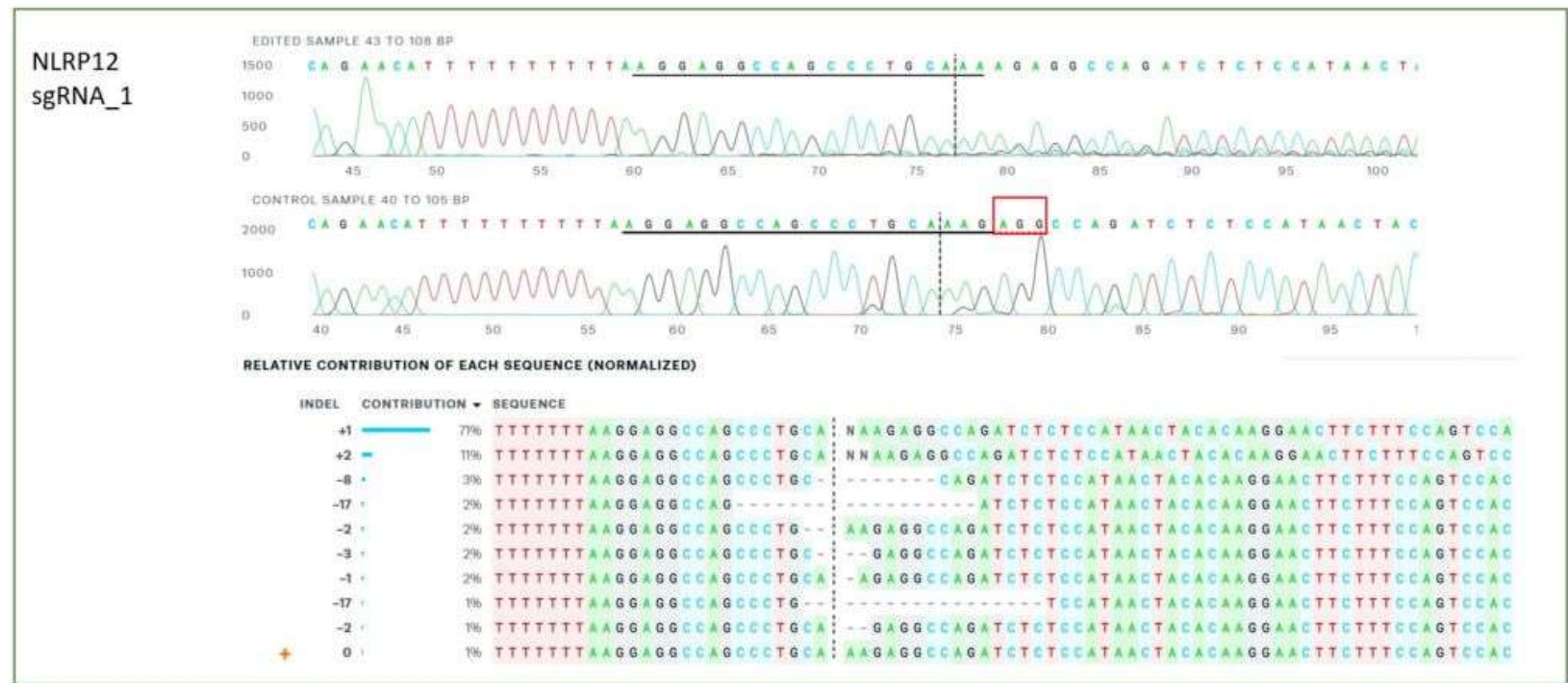


Figure 3.15. Chromatogram from the NLRP12 target region edited by CRISPR/Cas9 and relative contribution of each mutant sequence. The target site sequence is underlined in black, and the PAM region is boxed in red. Dash sign means deletion, and N means inserted nucleotides or substitution.

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

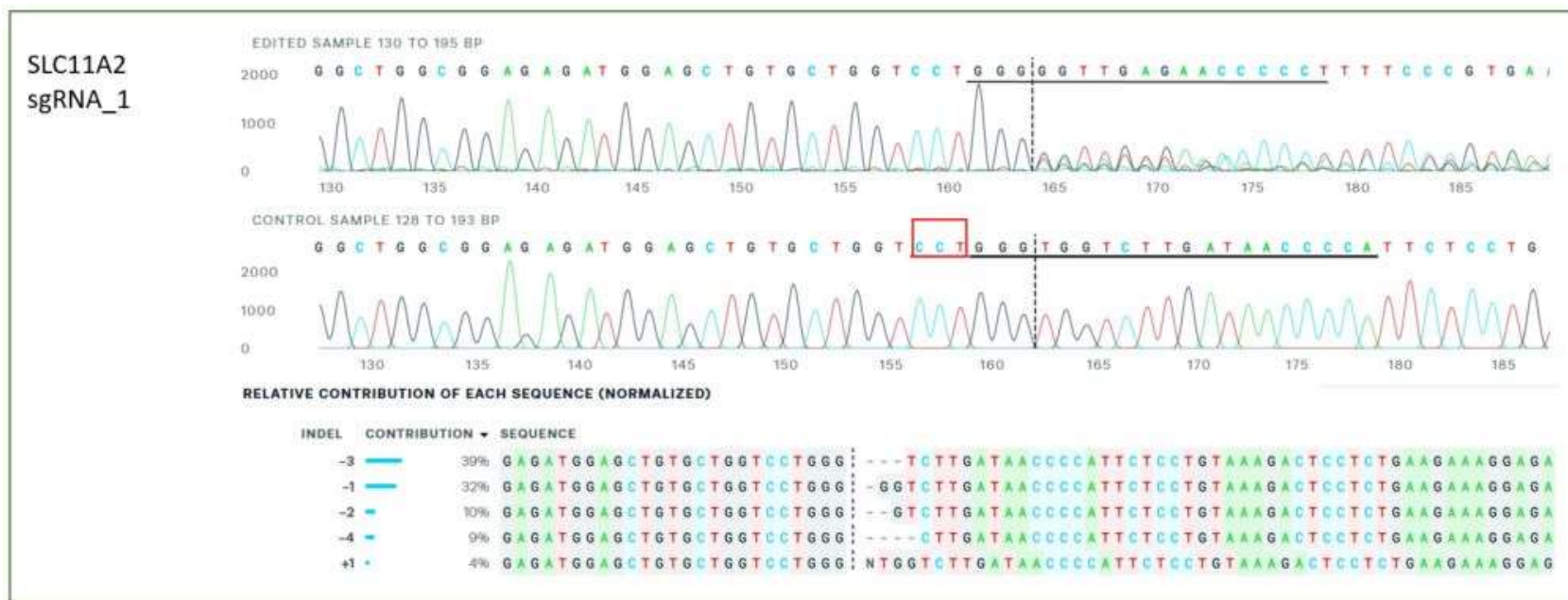


Figure 3.16. Chromatogram from the SLC11A2 target region edited by CRISPR/Cas9 and relative contribution of each mutant sequence. The target site sequence is underlined in black, and the PAM region is boxed in red. Dash sign means deletion, and N means inserted nucleotides or substitution.

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

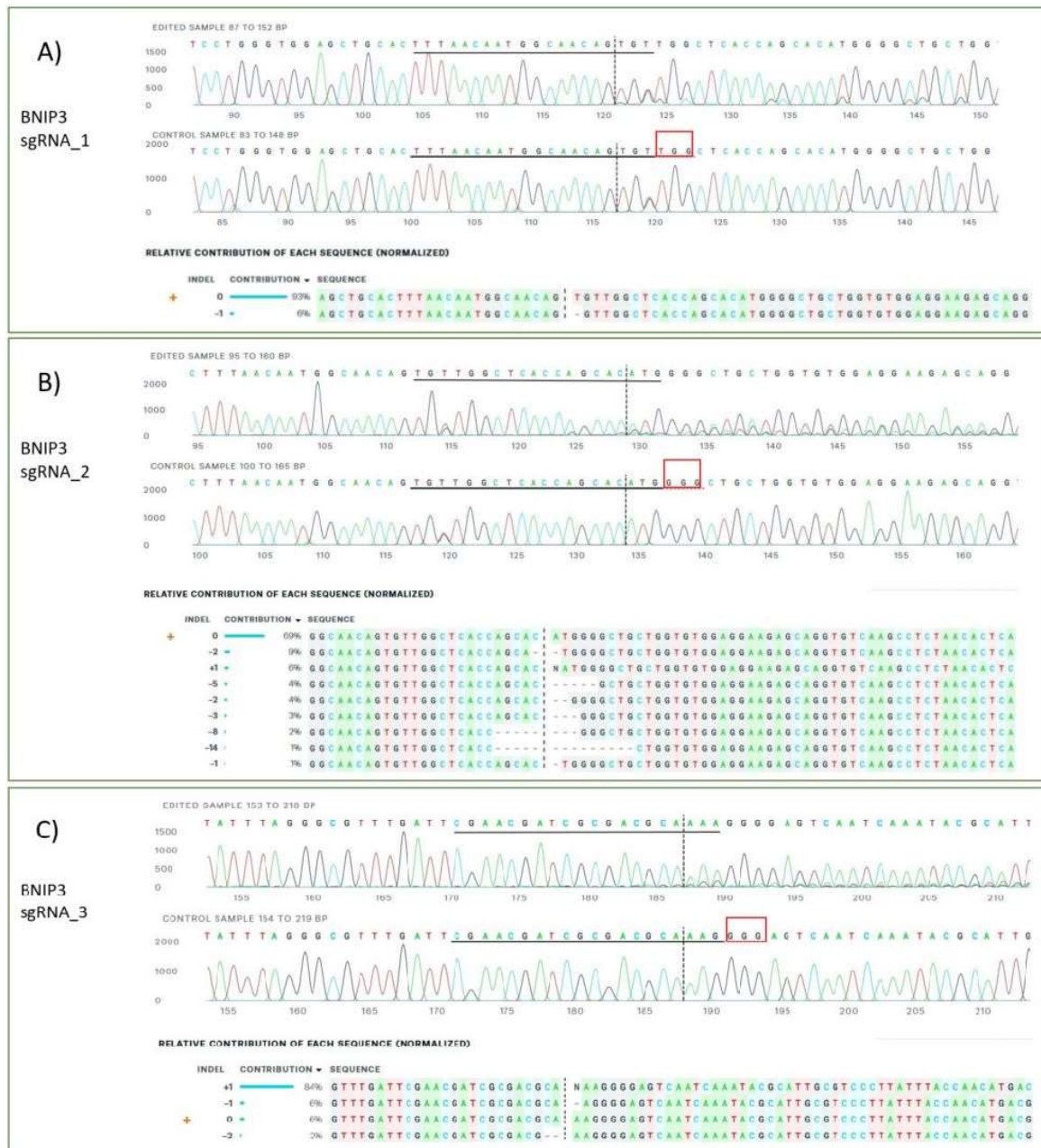


Figure 3.17. Chromatograms from the BNIP3 target region edited by CRISPR/Cas9 and relative contribution of each mutant sequence. The target site sequence is underlined in black, and the PAM region is boxed in red. Dash sign means deletion and, N means inserted nucleotides or substitution. **A)** sgRNA1, **B)** sgRNA2, and **C)** sgRNA3.

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

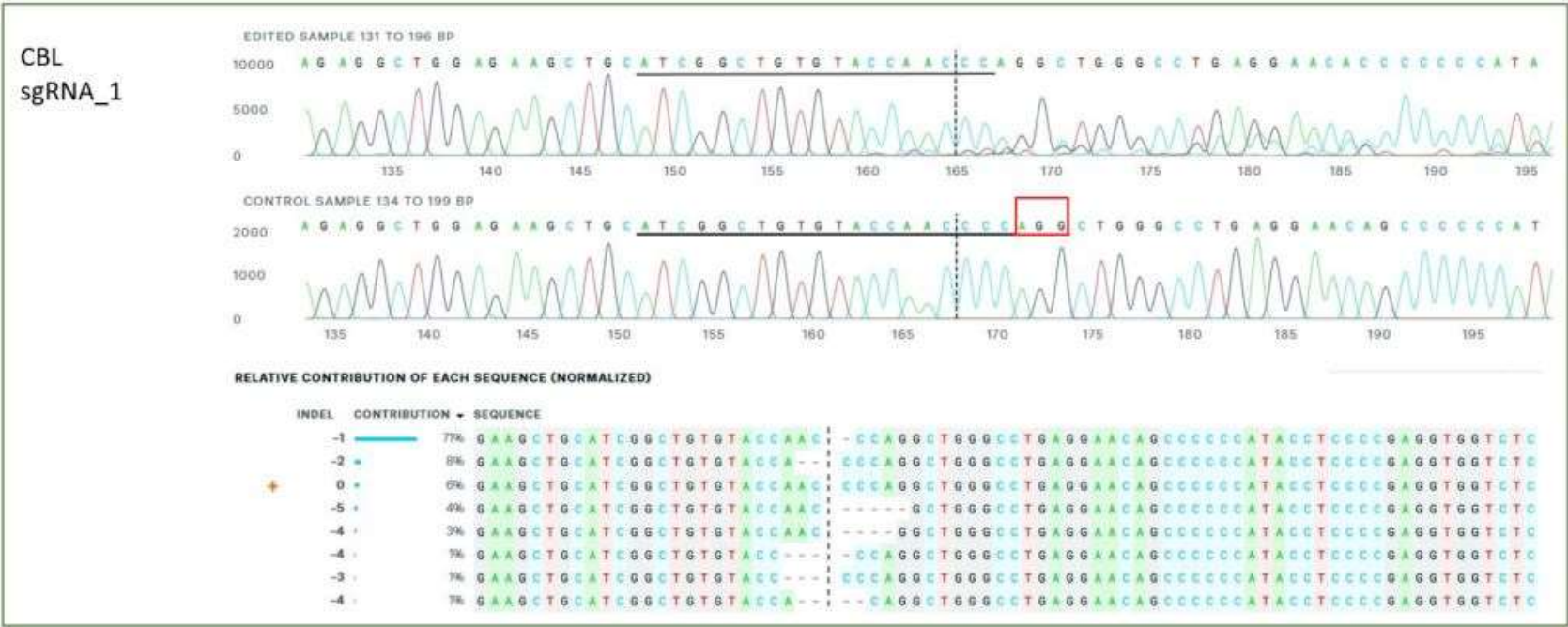


Figure 3.18. Chromatogram from the CBL target region edited by CRISPR/Cas9 and relative contribution of each mutant sequence. The target site sequence is underlined in black, and the PAM region is boxed in red. Dash sign means deletion, and N means inserted nucleotides or substitution.

3.3.2. Grow and maintenance of edited cell lines.

Edited cell lines were incubated at 20°C without CO₂ until growth and multiplication reached an 80% confluency. Two days post-editing, a 1% Penicillin-Streptomycin antibiotic was added, and samples for editing confirmation were taken seven days after RNP complex electroporation. A maximum of three passages were used to avoid changes in the edited population rates for unedited population overgrowth.

3.4. Discussion.

In this chapter, a genome editing method using CRISPR/Cas9 was developed to KO candidate genes in the Atlantic salmon cell line SHK-1.

3.4.1. sgRNAs design

A series of steps had to be achieved for successful genome editing; the first was to design efficient and specific sgRNAs to target the candidate genes and induce functional loss. Three principal considerations were taken to design the guides; i) the location of the target site, ii) the cleavage specificity and minimising off-target probability, and iii) the efficiency of the CRISPR/Cas9 editing.

3.4.1.1. Location of the target sites.

A considerable hurdle to determining the editing site was gathering information about the functional domains of the protein. While some genes are well described in mammalian studies, the teleosts structure is not well characterised. NLRP1 and NLRP12 are members of the Nod-like receptors (NLR) family (Tuncer *et al.*, 2014). The NLRP1 functional domains are well-characterised in zebrafish, and an effector PYR domain in the N-terminus of

the protein is described as critical to inflammasome activation (Li *et al.*, 2018; Morimoto *et al.*, 2021). In contrast, NLRP12 structure has not been characterised in teleosts; however, phylogenetical analysis has demonstrated the presence of the PYR domain in the medakas (*Oryzias latipes*) NLRP12 (Morimoto *et al.*, 2021). In order to interrupt the functional domain PYR, sgRNAs were designed to target exon 2 in NLRP1 and exon 3 in NLRP12. In contrast, scarce information about the structure of SLC11A2 was available. It is described as a transmembrane protein constituted by 12 domains in rainbow trout (Cooper *et al.*, 2007), sharing similar architecture with the mammalian SLC11 family. The determination of the target site was addressed by targeting an early exon 3 aiming to cause an early frameshift mutation (Doench, 2018). No characterisation of the CBL domains in teleosts was found in the scientific literature. Nevertheless, the protein domains of CBL are described as highly conserved from nematodes to humans (Qingjun *et al.*, 2014), comprising three N-terminus domains common to the CBL family 4H, EF and SH2 (Qingjun *et al.*, 2014). The guides were designed to target exon 2, the largest exon of the 13-exon gene, aiming to disrupt the early 4H domain of CBL. Initially, the site location for BNIP3 disruption was exon 2, following the same criteria as SLC11A2. Nevertheless, the sgRNAs found in exons 2 to 5 had low predicted specificity, with only sgRNA1 scoring over 50 (53 MIT) (**Table 3.4**), but with a low editing efficiency (6% indel rate) (**Figure 3.13 B**). Thus, sgRNAs suitable to meet the requirements were found only in exon 1.

3.4.1.2. Cleavage specificity and minimising off-target probability.

The design of sgRNAs was made using the CRISPOR tool (version 4.99, <http://crispor.tefor.net/>) due to its flexible and intuitive platform offering a selection of sgRNAs for a given DNA sequence, ranked in base on predicted efficiency scores (MIT and CFD) and estimating potential off-target binding sites in the Atlantic salmon genome (*Salmo salar* – NCBI GCF_000233375.1 ICSASG_v2) (Li *et al.*, 2022). In addition, previous studies reported high

genome editing efficiency using this tool in salmonid cell lines (Gratacap, Jin, *et al.*, 2020; Gratacap, Regan, *et al.*, 2020). In this study, all sgRNAs selected for genome editing scored above 70 in CFD and MIT specificity, which was well above the score of 50 recommended by the CRISPOR tool developers (Concordet & Haeussler, 2018), minimising the probability of off-target cleavage and unintended edits in the genome. In addition, an off-target identification based on the number of mismatches of the guide on the Atlantic salmon genome was carried out (CRISPOR conducts a whole-genome search identifying similar sequences to the sgRNA and provides a list of the predicted off-target sites according to the number of mismatches and the nucleotide position). Despite the evidence that guide cleavage is tolerant to even five nucleotide mismatches (Zheng *et al.*, 2017), the position and number of these mismatches have been demonstrated to be important in the off-target cleavage efficiency (Cho *et al.*, 2014; Jinek *et al.*, 2012; Wu *et al.*, 2014). The threshold utilised in this study required the selected guides to have no identical sequences in the genome other than the intended target site. In addition, at least three nts mismatches with the potential off-target site and at least one mismatch in the 12 nts seed sequence closer to the PAM region. Only the NLRP12 sgRNA1 did not meet the mentioned criteria; thus, a T7EN1 assay was carried out to evaluate the possibility of unintended edits in the predicted off-target site. No unintended edits were found using this approach.

A previously documented difficulty of sgRNA design in salmonid cell lines is the challenges presented by the WGD events (Ss4R) due to the presence of duplicated genes in salmonid genomes. As a result, specificity and efficiency predictions for sgRNA selection are inconsistent due to the similarity of target sites in different chromosomes within the genome (Blix *et al.*, 2021; Gratacap, Regan, *et al.*, 2020). In the present work, the BNIP3 sgRNA design met some difficulties related to the lack of specific guides scoring over 50 MIT and CFD in exons 2 to 5. The low specificity of the sgRNAs is due to the multiple off-target sites found in chromosome 18, where a highly similar copy of BNIP3 is

located. This hurdle influenced the selection of a sgRNA3 in exon 1, which increased the risk of editing too close to the protein's N-terminus and the potential use of an alternate ATG start codon. Consequently, the protein may remain functional and active (Doench, 2018). In this study, the KOs targeted only the candidate genes highlighted in the previous chapter study; hence, the guides were designed to avoid KOs in duplicated genes. Another alternative could have been to KO both paralogues, considering the potential functional similarities. However, here we aimed to discern the impact of the candidate KO function on the infection.

3.4.1.3. Efficiency of the CRISPR/Cas9 editing.

In complement to the specificity score given by the CRIPOR tool, a score that predicts the guide cleavage performance is provided. Similar to the specificity score, the greater the efficiency score, the highest probability of cleavage at that position. Nevertheless, the CRISPOR developers mention the inaccuracy of this prediction (Doench *et al.*, 2016; Moreno-Mateos *et al.*, 2015). Regardless of the high-efficiency rates reported in this work, substantial variations were observed in the editing efficiency of sgRNAs targeting the same or nearby sites, such as sgRNA1 and sgRNA2 targeting the NLRP1 gene (**Figure 3.5**). Although both guides targeted the same location and overlapped by 14 nts, the editing performance of sgRNA2 was higher than sgRNA1 (45% versus 95% indel rate, respectively) (**Figure 3.13 A**). The intrinsic features of the editing site, such as chromatin or epigenetic states (Uusi-Mäkelä *et al.*, 2018), transcription activity, and GC content, may explain this discrepancy (Doench *et al.*, 2016; Jao *et al.*, 2013; Wu *et al.*, 2014). Additional knowledge is required to understand the fundamental mechanisms of target site recognition, cleavage, and interactions between sgRNA and Cas9 nuclease (Jao *et al.*, 2013). A pragmatic strategy involving testing multiple sgRNAs to target intended sites is advised.

3.4.2. CRISPR/Cas9 RNP complex delivery.

The first successful genome edit with CRISPR/Cas9 in a salmon cell line (Chinook salmon embryo cell line CHSE-214) reported a 34.6% editing efficiency. By transfecting a plasmid construct encoding a nuclear version of Cas9 (nCas9n), Dehler *et al.* (2018) generated a cell line stably expressing Cas9 and EGFP. The present study tested this approach in SHK-1 cells, but it required finding efficient promoters to express Cas9, sgRNA and selection markers. Preliminary results showed marked differences in promoters' expression efficiency between CHSE214 and SHK-1 cells (**Figure 3.3**); thus, constructing a transient plasmid encoding CRISPR/Cas9 products was challenging. Previous studies have reported difficulties editing genes in fish cell lines (Gratacap, Jin, *et al.*, 2020; Q. Liu *et al.*, 2018; Strømsnes *et al.*, 2022) principally during transfection of the CRISPR/Cas9 products, the slow growth and metabolism versus mammalian cells, low-temperature culture conditions affecting the nuclease function, and the challenging development of clonal-edited cell lines (Collet *et al.*, 2018; Gratacap, Jin, *et al.*, 2020; Xiang *et al.*, 2017). During this study, similar challenges were met in selecting the best approach to edit SHK-1 cell line. An important hurdle was to minimise the high mortality post-transfection, requiring optimisation of the electroporation settings (**Figure 3.4**) and replacing the use of buffer R with Opti-MEM (Gibco, Waltham, USA).

Gratacap *et al.* (2020) was the first study reporting a high efficiency (over 90% indel rate) of genome editing in multiple salmonid cell lines, including SHK-1, using a CRISPR/Cas9 RNA transfection method. This technique eliminates the necessity to construct transient expression plasmids and obtain functionally efficient promoters for sgRNA and Cas9 expression. Moreover, viral vector approaches, such as lentivirus, have reported low editing efficiency in SHK-1 cells (Gratacap, Regan, *et al.*, 2020). In the present study, high editing efficiency was accomplished using the RNP protocol described by

Gratacarp *et al.* (2020). Validation of this protocol conditions concurred with the electroporation settings optimisation in the present study (1600 V x 10 ms x 3 pulses) (**Figure 3.4**). Essential aspects such as RNA complex concentration and crRNA annealing conditions were maintained. However, the cell concentration was modified to 10^5 SHK-1 cells/mL to improve confluency conditions. Accordingly, a recent study reported high-efficiency mutation rates (90 to 95%) using the RNP editing approach in CHSE 214 and ASK cell lines from Chinook and Atlantic salmon, respectively (Strømsnes *et al.*, 2022). This outcome agrees with Gratacarp *et al.* and the present study. Nevertheless, the same research reported successful genome editing in the SHK-1 cell line (100% efficiency targeting the CR2 gene) using a plasmid construct encoding Cas9, sgRNA and a puromycin resistance gene. The contrasting results may be explained by the transfection settings used, as the mentioned preliminary results were obtained before the SHK-1 protocol optimisation.

3.5. Conclusions.

In conclusion, this study developed a CRISPR/Cas9 genome editing method that produces a highly efficient mutation rate (> 80%) and knockout index (>50%) in a consistent and reproducible manner. Moreover, this approach can potentially induce genome modifications in SHK-1 cells to disrupt the phenotype of candidate genes related to SRS resistance for further functional studies.

CHAPTER 4: Evaluating the physiological impact of the candidate's genes knockout using an *in vitro* infection challenge model.

4.1. Introduction.

In chapter 3, a consistent CRISPR/Cas9 method for genome editing in SHK-1 Atlantic salmon cells provided five modified cell lines featuring disrupted genes hypothesised to be implicated in SRS resistance. In order to study the effects of functional loss in the cell biological processes and infection development, an *in vitro* model of infection for functional studies was implemented in this chapter.

Functional studies investigate the role and function of specific genes in biological processes. These studies aim to determine how a particular gene contributes to an organism's development, growth, and function (Li *et al.*, 2020). Functional studies can be conducted using various techniques, including gene knockout (KO), genome editing and gene expression analyses (Gasperskaja & Kučinskis, 2017). Gene KO studies investigate the phenotypic alterations generated by the selective KO of the function of one or more genes to identify the underlying causes of a determinate trait (Graham & Root, 2015). *In vitro* CRISPR/Cas9 editing in cell lines can improve the optimisation of genome editing methods in a controlled setting and an approachable manner, reducing the time, cost, and number of animals required for *in vivo* experiments. In addition, the *in vitro* approach is required as an initial screening of potential undesired outcomes of genome editing, allowing for pinpointing off-target deletions without affecting the animal welfare (Strømsnes *et al.*, 2022). Furthermore, initial optimisation in cell line models enables testing multiple delivery strategies for CRISPR/Cas9 components and prioritising the target

selection, improving the design strategy before animal testing validation (Strømsnes *et al.*, 2022).

4.1.1. Candidate genes.

4.1.1.1. NACHT, LRR and PYD domains-containing protein 1 (NLRP1).

The inflammasome is a cytosolic protein complex with an essential function in the innate immune system activating pro-inflammatory proteins and receptors. It comprises signalling-sensing proteins such as NLRP1, ASC and Caspase-1 that recognise microbial infection patterns and trigger the complex assembly (Chavarría-Smith & Vance, 2015). Within mammalian species, the NLRP1 inflammasome is one of the best-characterised inflammasomes. Moreover, limited information exists about the function of this inflammasome in non-mammalian animals such as teleosts (Li *et al.*, 2018). Diverse homologs of the inflammasome components have been found encoded in fish species' genomes. Studies in zebrafish (*Danio rerio*) have found *DrCaspase-A* and *DrCaspase-B* homologs participating in the cleavage of a pro-*DrIL-1 β* and final activation of *DrIL-1 β* in response to the fish bacteria *Francisella noatunensis* infection (Vojtech *et al.*, 2012). Moreover, the activation of a classical inflammasome assembly has been demonstrated by *DrNLRP1*, promoting the aggregation of *DrASC* and consequently activating a mature form of IL-1 β (Li *et al.*, 2018). These findings suggest that the inflammasome-mediated immune response is present in teleosts and can play an important role in overcoming infection. The NLRP1 protein is a member of the NACHT-domain and leucine-rich-repeat-containing family (NLR) and acts as the sensor component of the inflammasome activation (Li *et al.*, 2018). The members of the NLR family are highly variable in vertebrate species comprising 23 proteins in humans, 34 in mice (Franchi *et al.*, 2009) and more than 400 proteins reported in the zebrafish genome (Howe *et al.*, 2021). In teleosts, the structural characterisation of NLRP1 comprises a pyrin domain (PYD), a fish-specific

NACHT-associated domain (FISNA), a central NACHT domain, and a leucine-rich repeat domain (LRR) (Chuphal *et al.*, 2022). This structure architecture is similar to the mammalian NLRP1s (Li *et al.*, 2018).

4.1.1.2. NACHT, LRR and PYD domains-containing protein 12 (NLRP12).

NLRP12 is a member of the NLR family and is among the first described NLR proteins whose role is still debated (Tuncer *et al.*, 2014). According to their functions, NLRs can be classified into four broad categories: transcriptional transactivators, NF- κ B and MAPK activators, inflammasome activators, and inflammatory signalling inhibitors (Lich *et al.*, 2007). As NLRP12 expression is confined to immune cells and down-regulated in response to pathogen products and inflammatory cytokines, it has been hypothesised that this protein has a crucial role as a potent mitigator of inflammation (Normand *et al.*, 2018). In addition, canonical and non-canonical NF- κ B and ERK activation pathways are inhibited by NLRP12 (Arthur *et al.*, 2007), thereby promoting bacterial tolerance (Normand *et al.*, 2018). No description of the functional role or structure of NLRP12 was found in teleosts. However, phylogenetic studies have revealed the existence of a PYR domain in the NLRP12 gene of the medakas (*Oryzias latipes*) NLRP12 (Morimoto *et al.*, 2021), sharing architecture structures with NLRP1.

4.1.1.3. Natural resistance-associated macrophage protein 2 (SLC11A2).

The SLC11A1 and SLC11A2 genes are members of the SLC11 family of transmembrane divalent metal transporters. SLC11A1 has been implicated in pathogen resistance by playing a pivotal role in mice's immune defence against multiple bacterial infections, including *Leishmania*, *Mycobacteria*, and *Salmonella* (Gomes & Appelberg, 1998; Skamene *et al.*, 1998). It is located

almost exclusively in the membrane of late endosomes and lysosomes of immune cells transporting iron out of phagosomes (Gruenheid *et al.*, 1997). In contrast, SLC11A2 has been described as an iron transport in the intestinal tract and transferrin-bound transport (Feng *et al.*, 1996; Vidal *et al.*, 1993). SLC11A1 has never been identified in teleosts and is assumed to have been lost during early vertebrate evolution (Neves *et al.*, 2011). Numerous SCL11 homologs have been found in teleost, such as rainbow trout (*Oncorhynchus mykiss*) (Cooper *et al.*, 2007; Dorschner & Phillips, 1999), channel catfish (*Ictalurus punctatus*) (Chen *et al.*, 2002), and zebrafish (Donovan *et al.*, 2002). Additionally, a significant role in the immune function of fish has been described in red sea bream (*Pagrus major*) (Chen *et al.*, 2004), Striped Bass (*Morone saxatilis*) (Burge *et al.*, 2004), and European sea bass (*Dicentrarchus labrax*) (Neves *et al.*, 2011), been significantly overexpressed during *Mycobacterium*, *Vibrio anguillarum*, and *Photobacterium damsela* spp. infection (Burge *et al.*, 2004; Chen *et al.*, 2004; Neves *et al.*, 2011). This evidence suggests that regardless of the absence of SLC11A1 in teleost fish, one of the duplicated SLC11A2 paralogues has assumed its immunological functions (Neves *et al.*, 2011). Although the extensive research about SLC11A2 in fishes, the structural architecture of the protein is poorly characterised in teleosts. However, it has been described as a transmembrane protein containing 12 domains sharing similar structure with the SLC11A2 protein in mammals (Chen *et al.*, 2004; Neves *et al.*, 2011).

4.1.1.4. BCL2/Adenovirus E1B 19 KDa Protein-Interacting Protein 3 (BNIP3).

The Bcl-2 family proteins function as essential cell death regulators and participate in various biological processes, such as carcinogenesis and immunological responses, by modulating the permeability of the mitochondrial outer membrane (Cai *et al.*, 2017; Yuan *et al.*, 2016). This family is composed of pro-apoptotic and anti-apoptotic members. BNIP3 is classified as a pro-

apoptotic BH3-only protein from the Bcl-2 family and is the only BH3-only protein capable of inducing apoptosis and mitochondrial autophagy (mitophagy) (Cai *et al.*, 2017). This protein can be incorporated into the mitochondrial outer membrane through transmembrane domains, triggering mitochondrial permeabilization and impairing the potential of the mitochondrial membrane (Bocharov *et al.*, 2007). In teleosts, it has been described that BNIP3 induce mitophagy under hypoxia conditions in channel catfish (Yuan *et al.*, 2016), and environmental stimuli such as methionine levels and hypoxia induce DNA methylation of BNIP3 as a metabolic programming response in rainbow trout (Veron *et al.*, 2018; Wu *et al.*, 2017). In addition, infections by *Edwardsiella ictaluri* and *Flavobacterium columnare* in channel catfish induced down-regulation of BH3-only proteins such as BNIP3, suggesting a bacterial modulation of these genes to decrease the cell apoptotic activity (Yuan *et al.*, 2016). Consequently, BNIP3 overexpression during Singapore grouper iridovirus (SGIV) infection in Orange spotted grouper (*Epinephelus coioides*) indicates it may play a role in the immune response to infection in teleost fish (Cai *et al.*, 2017).

4.1.1.5. E3 Ubiquitin-Protein Ligase CBL (CBL).

The CBL gene is classified as a proto-oncogene and encodes a RING finger E3 ubiquitin-protein ligase enzyme required for recruiting substrates for degradation by the proteasome (Magnifico *et al.*, 2003). It has been reported to play an essential function in modulating innate immune responses in host defence against pathogens by setting the threshold for T-cell activation and regulating peripheral T-cell tolerance (Naramura *et al.*, 2002; Shamim *et al.*, 2007). Some microorganisms, such as *Listeria monocytogenes*, produce virulence factors such as InlB, which can modulate CBL ubiquitin ligase to hijack the cell endocytic mechanism for bacterial internalisation and invasion (Veiga & Cossart, 2005). The structural characterisation of CBL is well described in the scientific literature for mammal species; in contrast, no

information about teleosts CBL structure was found. However, the protein domains of CBL are reported as being highly conserved from invertebrates to mammals (Qingjun *et al.*, 2014) containing three N-terminus domains 4H, EF, and SH2; two central helical linkers (L) and ring finger (RF) domains, and a variable C-terminus proline-rich motifs (PR) bind to SH3 domain (Qingjun *et al.*, 2014).

4.1.2. Screening methods to evaluate host response and infection progression.

Cytopathic effect (CPE).

Cytopathic effects (CPE) are characterised by morphological alterations in the host cell induced by viral infection. These changes occur when the infected cell synthesises viral components and experiences typical biochemical and morphological alterations (Albrecht *et al.*, 1996). CPEs can be a useful diagnostic tool for virologists in detecting an animal or human infection, as some viruses produce characteristic CPEs (Albrecht *et al.*, 1996). Although CPEs are described in viral infections, some intracellular pathogens, such as intracellular bacteria, can induce metabolic and structural modulations, inducing alterations in the host cell (Eisenreich *et al.*, 2019). Several bacterial and host cell processes are required to establish a sustained intracellular bacterial life cycle, namely: internalisation by the host cell, formation of specific pathogen-containing vacuoles (PCVs), avoidance of the host defence mechanisms, reprogramming of the host cell metabolism, and bacterial metabolic adaptability. (Ribet & Cossart, 2015). Ultimately, these metabolic changes translate into CPEs as rounding of the cells, structural alterations by cytoskeleton organisation, and mortality (Albrecht *et al.*, 1996).

Cell viability.

Cell viability is a cell-based assay frequently used for screening groups of substances or treatments to evaluate the possible impact on cell proliferation or direct cytotoxic effects that ultimately lead to cell death (Kamiloglu *et al.*, 2020; Riss *et al.*, 2004). Cell viability refers to the cell's ability to survive and propagate under determined conditions. In the context of infection, this assay is used to ascertain the host cells' response to the invading pathogen (Zhang *et al.*, 2016). Various cell viability assays are available to study compounds and treatments of different natures. A broad classification separates these tests into five groups; dye exclusion, colourimetric, fluorometric, luminometric, and flow cytometric assays. The ideal cell viability assay should be accurate, reliable, reproducible, time and cost-effective, and not interfere with infection (Kamiloglu *et al.*, 2020).

Bacterial load.

Bacterial load refers to quantifying bacteria present in a given sample, such as blood, tissue or culture. The most widely used method for bacterial quantification is RT-PCR measuring the copies of a DNA region defined by specific primers (Kralik & Ricchi, 2017). This method is highly efficient, fast, reliable, sensitive, accurate and specific as long as the standard procedures are optimised (Kralik & Ricchi, 2017). The relationship between bacterial load and the severity of infection has been reported in numerous studies where patients with severe disease cases presented significantly higher levels of bacterial load. These studies included *Meningococcus*, *Mycobacterium*, and *Staphylococcus aureus* (Hackett *et al.*, 2002; Nieman *et al.*, 2022; Sabiiti *et al.*, 2020). In addition, bacterial load has been described as an indicator of disease resistance in coho salmon (*Oncorhynchus kisutch*) (Barría, Doeschl-Wilson, *et al.*, 2019) and Atlantic salmon (Dettleff *et al.*, 2015; Pulgar *et al.*, 2015), where resistant families presented significative lower bacterial loads than susceptible families. These findings suggests the abundance of bacteria in an infection process indicates successful invasion and bacterial proliferation.

The main aim of this chapter is to investigate the consequences of functional loss of the CRISPR/Cas9 edited cell lines developed in chapter 3 during *P. salmonis* infection. To accomplish this, three steps were followed to characterise the physiological effect of the candidate genes on host response and *P. salmonis* infection: i) to develop an *in vitro* infection challenge model of edited cell lines with *P. salmonis* bacterium. ii) to determine the impact on the host response by measuring cell survival and assessing differences in the progression of cytopathic effect (CPE) of the infected cell lines. iii) to contrast the differences in bacterial load of edited cell lines and unedited controls during *P. salmonis* infection.

4.2. Material and Methods.

4.2.1. Experimental design.

In order to evaluate the impact of gene KO on the bacterial infection process, an *in vitro* infection model comparing the unedited SHK-1 cell line to the successfully modified cell lines obtained in chapter 3 was developed. NLRP1-KO, NLRP12-KO, SLC11A2-KO, BNIP3-KO, CBL-KO and SLC45A2-KO were infected with *P. salmonis* P-013 strain LF-89 using an MOI of 80:1 (bacteria:cell) and cultured at 20°C. Cytopathic effect (CPE) and time to cell damage were assessed at 0, 5, 9 and 14 dpi. Variations in cell viability were determined by ATP quantification of metabolically active cells at 4, 8 and 12 dpi using CellTiter-Glo 2.0 (Promega, Madison, WI). Finally, differences in the infection outcome were gauged by bacterial load quantification tested at 0, 4, 8 and 12 dpi. Three controls were used to compare differences in the infection process: one "unedited control" SHK-1-WT, one "transfection control" comprising of electroporated SHK-1 cells without CRISPR/Cas9 RNA complex, and one "edited-control" consisting of an unrelated to immune-response edited cell line, SLC45A2-KO. Two biological replicates per infected

Genome editing of candidate genes related to disease resistance to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

cell line were tested using the same infection conditions (cell density, MOI, edited cell lines, controls, and technical replicates) (**Figure 4.1**).

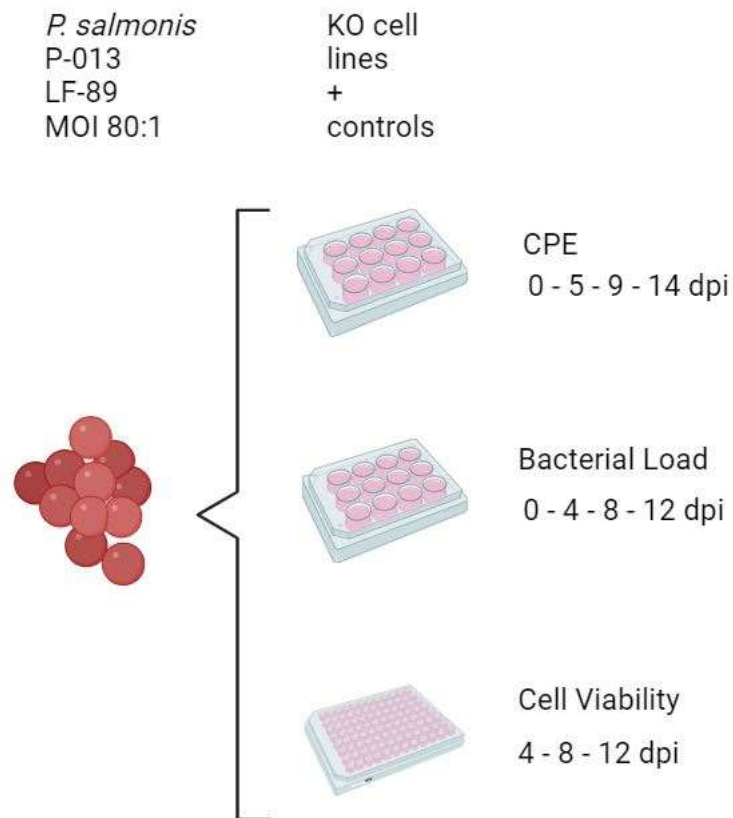


Figure 4.1: Schematic representation of the *in vitro* infection challenge. Edited cell lines *NLRP1-KO*, *NLRP12-KO*, *SLC11A2-KO*, *BNIP3-KO*, *CBL-KO* and *SLC45A2-KO* were infected with *P. salmonis* P-013 strain LF-89. Figure created with BioRender.com.

4.2.2. Edited cell lines.

In this experiment, the edited cell lines NLRP1-KO, NLRP12-KO, SLC11A2-KO, BNIP3-KO, CBL-KO and SLC45A2-KO were seeded at a density of 2×10^3 cells/well in a 96-well plate to estimate cell viability and 4×10^4 cells/well in a 12-well plate to determine CPE and bacterial load. Cells were counted using the TC20 Automated Cell Counter (BioRad), and cell viability was addressed by recognising alive cells using trypan blue dye and using multiple focal planes. Three passages after transfection were sufficient to produce the required number of cells for the infection challenge. Cells were cultivated for 24 h before infection in 1.5 mL of Leibovitz (L-15) media (Sigma-Aldrich, St. Louis, USA) supplemented with 40 μ M β -Mercaptoethanol (Gibco, Waltham, USA) and 5% Foetal Bovine Serum (FBS) (Gibco, Waltham, USA) without antibiotics.

4.2.3. Bacteria, growth conditions and maintenance.

The bacteria *Piscirickettsia salmonis* P-013 (strand LF-89), P-0163, P-0182 and P-068 (strand EM-90), used in this study, were kindly donated by Henning Sørum from the Norwegian University of Life Sciences (NMBU). It was cultured at 20°C for six days on a specially formulated agar containing 40 g of Tryptic soy agar (TSA) (Merck) and 15 g of Sodium chloride (MP Biomedicals) dissolved in 900 mL of distilled water. This mixture was sterilized in an autoclave for 15 min at 121°C and cooled down at room temperature until reaching 45°C. Moreover, 50 mL of defibrinated sheep blood (E&O Labs, Bonnybridge, Scotland) was added and slowly heated to 75°C in a water bath. Finally, 10 g of D-glucose (Sigma-Aldrich, St. Louis, USA), 1 g of L-cysteine hydrochloride (Merck), and 50 mL of FBS (Gibco, Waltham, USA) were added aseptically (Contreras-Lynch *et al.*, 2017). For long-term storage, the bacterium was cryopreserved at – 80°C in 400 μ L of a 50% Glycerol solution

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(Sigma-Aldrich, St. Louis, USA), 400 µL of L-15 media (Sigma-Aldrich, St. Louis, USA), and 200 µL of FBS (Gibco, Waltham, USA). *P. salmonis* colonies were aseptically collected from the agar plate and diluted in 1 mL of L-15 media without antibiotics. The following formula was used to quantify the bacteria in a Petroff-Hausser (Hausser Scientific, Horsham, USA) haemocytometer:

$$\text{Number of bacterium (mL)} = \text{Cells counted} * \text{Dilution factor} * 2 \times 10^7$$

Where **Cells counted** is the average cell count in 6 central squares, **Dilution factor** is the factor by which the stock solution is diluted, and **2x10⁷** corresponds to 25 groups of 16 small squares (25*16) * 50 cells depth in 1000 mm³ (50.000). A maximum passage three was used for infection in the edited cell lines.

4.2.4. Bacterial Identification.

4.2.4.1. PCR 16S rRNA gene amplification.

A polymerase chain reaction (PCR) to amplify the *P. salmonis* 16S ribosomal RNA (rRNA) gene was performed to confirm the strain used in this study. Two primers targeting distinct segments of the 16S rRNA sequence were used. The primer PS2A2 amplifies all strains of *P. salmonis*, while the primer PS3AS amplifies strain LF-89 exclusively (Mauel & Giovannoni, 1996) (**Table 4.1**). The custom oligos were obtained from IDT, and the bacterial DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the instructions provided by the manufacturer. The positive control used to corroborate LF-89 amplification was DNA extracted from the commercially acquired *P. salmonis* VR-1361 from the American Type Culture Collection (ATCC, USA) (**Table 4.2**). Amplification was performed using 12.5 µL Q5 High-Fidelity 2X Master Mix (NEB), 2 µL of *P. salmonis* DNA, 1.25 µL of each

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forward and reverse primer, and 8 µL of nuclease-free water totalling 25 µL.

Thermal cycling conditions consisted of:

Step	Temperature	Time
Denaturation	98°C	30 sec
Amplification	98°C	10 sec
33 cycles	Optimal annealing temperature (Table 4.1)	20 sec
	72°C	25 sec
Extension	72°C	2 min
Hold	4°C	Until use

Finally, the PCR product was run on a 1.5% agarose gel stained with SYBR Safe DNA stain (Thermo Fisher) for DNA imaging in a UV transilluminator Gel Dock.

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Table 4.1: Primers to amplify all strains of *P. salmonis* and specifically the LF-89 strain.

Primer	Strain	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temp (°C)	Amplicon length (bp)	Reference
ppPS2A2	All strains	CTAGGAGATGAGCCCGCGTTG	GCTACACCTGAAATTCCACT	59	467	Mauel & Giovannoni, 1996
ppPS3AS	LF-89	CTAGGAGATGAGCCCGCGTTG	TCCCGAAGGCACTTCCGCA	71	816	Mauel & Giovannoni, 1996

Table 4.2: *P. salmonis* isolates used in this study.

Identification Name	Origin	Strain	Use
P-013	NMBU	LF-89	Infection challenge
P-163	NMBU	EM-90	Control EM-90
P-182	NMBU	EM-90	Control EM-90
P-068	NMBU	EM-90	Control EM-90
VR-1361	ATCC (USA)	LF-89	Control LF-89/ Plasmid insert

4.2.4.2. Gram Stain.

In addition to confirming the *P. salmonis* P-013 LF-89 strain using PCR amplification, the purity of the *P. salmonis* culture was determined using gram staining. *P. salmonis* is a gram-negative coccoid bacterium with a diameter of approximately 0.5 to 1.8 μm . This experiment's positive control was the certified *P. salmonis* VR-1361 from the American Type Culture Collection (ATCC, USA). According to the provider, these bacteria was expanded by infecting a Chinook salmon embryo cell line (CHSE-214) cultured at 20°C until a CPE of 100% was achieved between 14 and 16 days post-inoculation. Supernatant media was centrifuged 4 min at 2500 rpm to separate cell debris from the media. The separated supernatant was centrifuged for 15 minutes at 13000 rpm to concentrate the media-free bacteria. Media was eliminated using a pipette, and the cell pellet was smeared and heat-fixed in a microscope slide. *P. salmonis* P-013 (NMBU, Norway) was isolated aseptically from a specially formulated agar plate (**section 4.2.3.**), diluted in 200 μL of Leibovitz (L-15) media (Sigma-Aldrich, St. Louis, United States), and 5 μL were smeared and heat-fixed on a microscope slide. Crystal violet solution (CellPath) was used to stain fixed samples for one minute before rinsing them with distilled water. Afterwards, samples were flooded for one minute with an iodine solution (Sigma-Aldrich, St. Louis, United States) and then rinsed with distilled water. Subsequently, samples were flooded for 3 seconds with decolourizer (a 25% acetone / 75% isopropanol solution) and then rinsed with distilled water. Finally, samples were flooded for 30 seconds with safranin solution (Sigma-Aldrich, St. Louis, USA), rinsed with distilled water, and dried at room temperature. The samples were mounted in resin medium and dried for 24 hours at room temperature. A Nikon Ni-U transmitted light microscope (Nikon, UK) was used to examine stained P-013 and VR-1361 control slides. Images were taken using an Axiocam 503 high-resolution colour camera and Zen software.

4.2.5. Cell Viability.

CellTiter-Glo 2.0 (Promega, Madison, WI) was utilised to quantify cellular ATP levels to determine cell viability. This protocol was based on the manufacturer's recommended conditions and adapted to the metabolism of the SHK-1 cell line (Gratacap, Jin, *et al.*, 2020). At 4, 8, and 12 dpi, infected KO cell lines and controls were compared to uninfected KO cell lines and controls. At each time point, media was collected and discarded. Cells were then washed with 100 μ L of 1X sterilised PBS, followed by a 30-minute incubation with 120 μ L/well of CellTiter-Glo 2.0 solution in 1X sterilised PBS (1:10 dilution). Plates were shielded from light (aluminium foil cover) and launched at 30 revolutions per minute. Consequently, 100 μ L/well were transferred to a flat bottom white 96-well plate (Greiner Bio-One, Austria), and luminescence was captured by a Cytation 3 imaging reader using the Gen 5 software V3.0 (BioTek, Winooski, USA).

4.2.6. Cytopathic effect.

In order to observe variation in the progression of pathological damage caused by *P. salmonis* infection, microscopic imaging was performed on infected KO cell lines, infected controls, uninfected KO cell lines and uninfected controls at 0, 5, 9, and 14 dpi. Images were captured using the Zeiss Axiovert 25 inverted microscope with an Axiocam 503 high-resolution colour camera and processed using Zen software.

4.2.7. Bacterial Load.

4.2.7.1. Gentamicin protection assay.

To precisely quantify intracellular bacteria, a gentamicin protection assay was developed to eliminate external bacteria and prevent the overestimation

of intracellular bacteria (Pérez-Stuardo *et al.*, 2019). Edited cell lines infected with *P. salmonis* were tested at 0, 4, 8 and 12 dpi. Media was collected and discarded at each time point. Infected cells were then incubated for 1 hour at RT with 1 mL/well of gentamicin (100 µg/mL) (Merck) dissolved in 1X sterile PBS media. Gentamicin is a broad-spectrum aminoglycoside antibiotic which has poor penetration into eukaryotic cells; hence, it does not affect intracellular bacterial growth (VanCleave *et al.*, 2017). Gentamicin containing media was collected and discarded, followed by three washes using 500 µL of 1X sterile PBS to maximize extracellular bacterial elimination. Then, cells were lifted, adding 0.05% Trypsin/EDTA (Thermo Fisher Scientific) and incubated at RT for 5 minutes. Lifted cells were centrifuged at 2500 rpm for 4 min, and trypsin supernatant was carefully eliminated with a pipette. The remaining pellet was resuspended in 200 µL of sterilized 1X PBS and stored at - 20°C until use.

The DNeasy Blood & Tissue Kit (Qiagen) was used for genomic DNA extraction following the manufacturer's instructions. Samples were stored at - 20°C in 100 µL of elution buffer for further QPCR bacterial load quantification.

4.2.7.2. Quantification curve.

A standard curve to quantify *P. salmonis* was developed as a relative calibrator. The *P. salmonis* 16S rRNA gene was amplified by conventional PCR using a set of primers to target a segment unique to *P. salmonis* (Karatas *et al.*, 2008). 1.25 µL of each primer 16sRNA Fw (5' AGG-GAG-ACT-GCC-GGT-GAT-A 3') and 16sRNA Rev (5' ACT-ACG-AGG-CGC-TTT-CTC-A 3') were mixed with 12.5 µL Q5 High-Fidelity 2X Master Mix (NEB), 2 µL of *P. salmonis* VR-1361 (ATCC, USA) genomic DNA, and 8 µL of nuclease-free water totalling 25 µL. Thermal cycling conditions consisted of:

Step	Temperature	Time
Denaturation	98°C	30 sec
Amplification	98°C	10 sec
33 cycles	59°C	20 sec
	72°C	25 sec
Extension	72°C	2 min
Hold	4°C	Until use

An agarose gel (1.5%) was used to confirm amplification and correct amplicon size (150 bp). The PCR product was extracted from the gel and purified using the QIAquick Gel Extraction kit (Qiagen), following the manufacturer's instructions. The purified amplicon was ligated using the NEB PCR Cloning kit (New England Biolabs) and transformed into NEB 10-beta Competent *E. coli* (New England Biolabs). Plasmid DNA was extracted using the Wizard® Plus SV Minipreps DNA Purification Systems (Promega). Correct insert of the target sequence and orientation was confirmed by Sanger sequencing GATC/Eurofins (Germany) chromatogram compared to the *P. salmonis* 16S rRNA genome reference (NCBI: U36941) using the nucleotide BLAST tool. The standard curve was generated by preparing seven 10-fold dilutions of the plasmid stock containing 10^7 copies using the online tool DNA Copy Number and Dilution Calculator (Thermo Fisher Scientific). The efficiency of the curve was maintained between 90 and 110%, and the correlation coefficient (R^2) was kept ≥ 0.99 . Finally, three technical replicates were used per curve dilution.

4.2.7.3. QPCR.

Quantification of the bacterial load was performed on an ABI 7500 Fast Dx Real-Time PCR Instrument (Applied Biosystems) mixing 10 µL of Brilliant III SYBR Green QPCR Master Mix (Agilent Technologies), 0.5 µL of each forward and reverse primer 16sRNA, 0.3 µL of diluted reference dye (1:500), 7.7 µL of nuclease-free water, and 1 µL of samples DNA totalling 20 µL.

Amplification conditions were one cycle of 95°C for 3 sec, 40 cycles of 95°C for 5 sec followed by 60°C for 12 sec, and a final cycle of 95°C for 15 sec. Two technical replicates were used per sample. Bacterial load quantification was obtained by extrapolating the standard curve 16S rDNA copies with the samples cycle threshold (Ct). The results are presented as copies of 16S rDNA/ μ L.

4.2.8. Statistical analysis.

This study presents statistical data as mean \pm standard deviation of two biological replicates per gene. Graphics were made using GraphPad Prism Software (version 9.4.1, <https://www.graphpad.com>).

4.3. Results.

4.3.1. *In vitro* model of *P. salmonis* infection in CRISPR/Cas9 KO cell lines.

An *in vitro* model of *P. salmonis* infection was developed comprising the edited cell lines NLRP1-KO, NLRP12-KO, SLC11A2-KO, BNIP3-KO, CBL-KO, the controls SHK-1-WT (unedited control), electroporated SHK-1 cells without ribonucleoprotein complex (RNP) (transfection control), and an unrelated to immune-response SLC45A2-KO cell line (edited control); were infected with *P. salmonis* P-013 strain LF-89 using a multiplicity of infection (MOI) of 80:1 (bacteria: cell) and cultured at 20°C. The bacterial strain was confirmed by PCR amplification with specific primers targeting a 16S rRNA segment conserved for the LF-89 strain (**Figure 4.2**). The controls used were the certified LF-89 strain VR-1361 from the American Type Culture Collection (ATCC, USA) and the EM-90 strain isolates P-182, P-163, and P-068. Further bacterial identification using gram stain confirmed the observation of gram-

negative coccus bacteria, with sizes ranging between 0.1 to 1.8 μm of diameter approx. in VR-1361 and P-013 samples (**Figure 4.3**). Two biological replicates (independent experiments) were conducted using identical conditions, namely cell density, MOI, edited cell lines, controls, and three technical replicates, with a notable exception in control SLC45A2-KO, which is absent in one biological replicate. This difference was caused by environmental contamination of the SLC45A2-KO cell line before the experiment's seeding. The infection challenges investigated the variances in CPE progression, bacterial load and cell survival.

4.3.1.1. PCR.

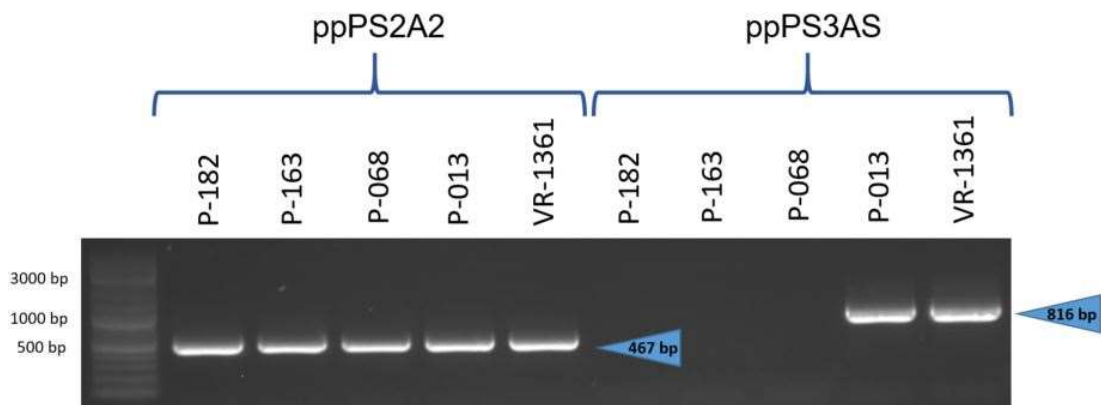


Figure 4.2. PCR identification of *P. salmonis*. *ppPS2A2* amplified all strains of *P. salmonis* (amplicon size 467 bp), and *ppPS3AS* amplified only the LF-89 strain (amplicon size 816 bp).

4.3.1.2. Gram stain.

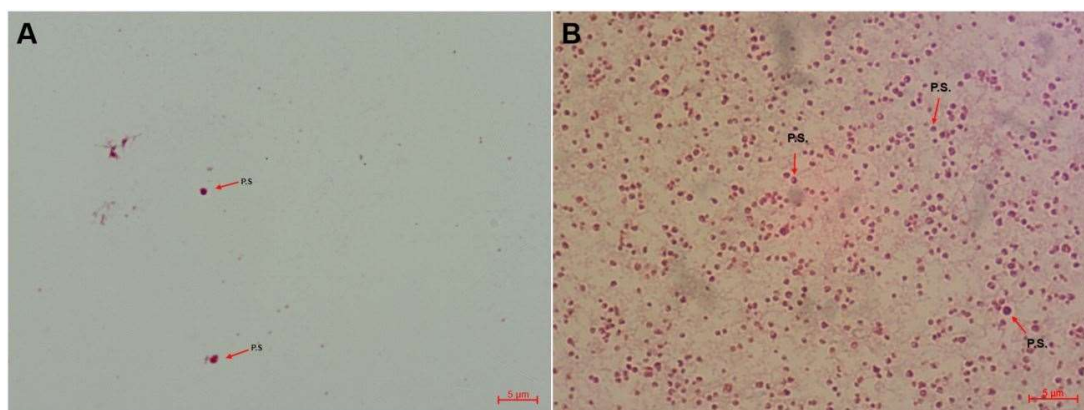


Figure 4.3. *P. salmonis* strain LF-89 Gram stain. A) *P. salmonis* VR-1361 from the American Type Culture Collection (ATCC, USA) collected from CHSE-214 infected cells 16 dpi. **B)** *P. salmonis* P-013 (NMBU, Norway) collected from agar plate. Red arrows show *P. salmonis* bacterium (P.S.). X100 magnification. Bar 5 μm.

4.3.2. Host response to infection.

4.3.2.1. Cytopathic effect (CPE).

No differences in the progression of the canon CPE described for *P. salmonis* infection (Smith *et al.*, 2015) were observed in edited cell lines or controls during infection (**Figure 4.4**). The first observations of pathogen-containing vacuoles (PCVs) occurred at 5 dpi, and mild detached mortality was observed in all edited cell lines and controls. At 9 dpi PCVs are observable in approx. 40 to 50% of the cells; increased attached and detached mortality is observed in edited cell lines and controls. At 14 dpi, extensive vacuolization affected approx. 70 to 80% of the cells, and extensive attached and detached mortality affected 50 to 60% of the cells in edited cells and controls.

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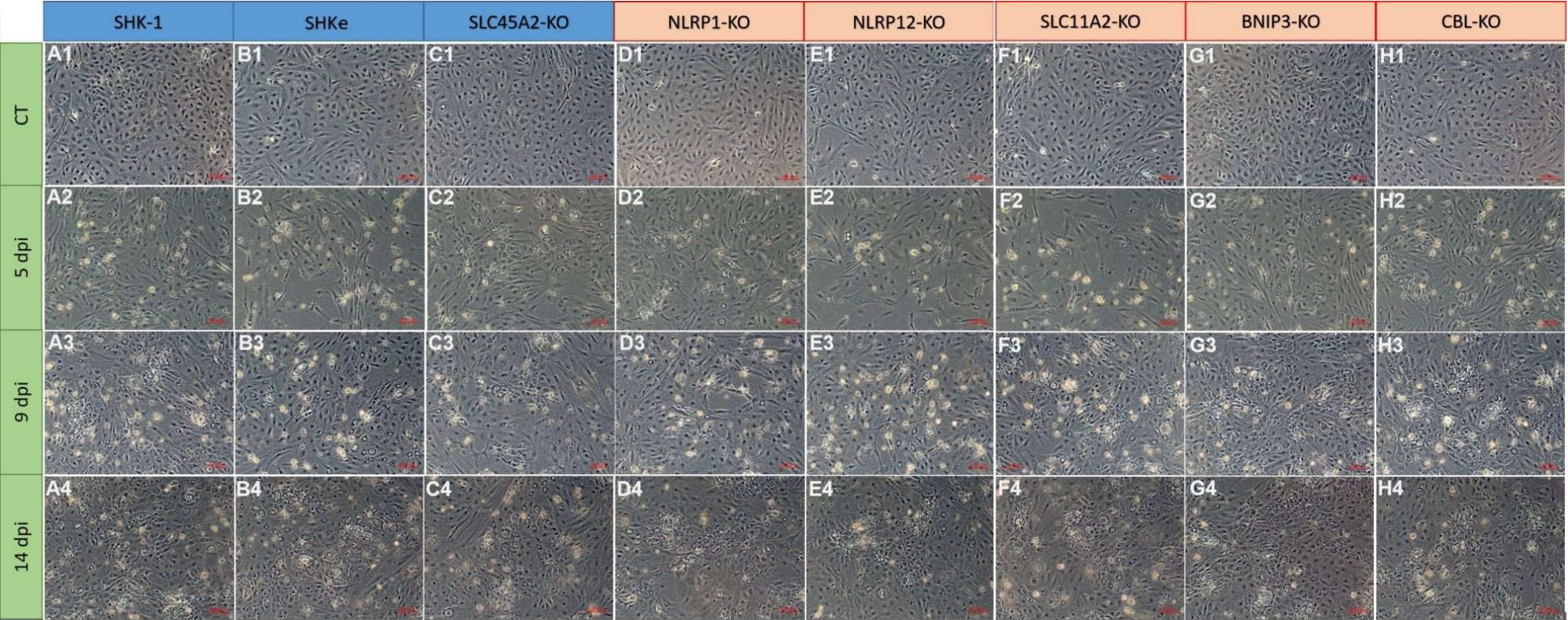


Figure 4.4. Cytopathic effect (CPE) of CRISPR/Cas9 KO cell lines and unedited controls during *P. salmonis* infection. A-C columns displays unedited SHK-1, SHK-1 electroporated (SHKe), and the edited control (SLC45A2-KO, in theory unrelated to *P. salmonis* infection). D-H column displays CRISP/Cas9 KO cell lines. Row 1 displays uninfected cell lines at 9 days after the start of the experiment. Row 2 *P. salmonis* infection after 5 days, Row 3 *P. salmonis* infection after 9 days, and Row 4 *P. salmonis* infection after 14 days. Bar 100 µm.

4.3.2.2. Cell viability.

Due to time contingencies and bacterial availability, only two biological replicates (independent experiments) were performed for this experiment. According to Blainey *et al.*, (2014), a minimum of three biological replicates are necessary to detect statistically significant differences between groups with adequate statistical power. Consequently, the results presented cannot infer statistical significance, but tendencies in the results can be analysed and discussed. No more than a 14.3% difference in cell survival was found at 4 dpi, and 13.8% at 8dpi. This rate difference cannot infer a noticeable survival between KO and the control group, as the maximum variance between biological replicates is 12.1%.

In contrast, at 12 dpi, NLRP12-KO presented the lowest survival rate in the group, scoring 57.9% cell survival. This value constitutes an approximate -16% difference with SLC11A2, the following lowest survival rate, and a -27% difference with the average control rate of 85% (SHK-1, SHKe and SLCA45A2 mean). These differences may suggest that at 12 dpi NLRP12-KO cells struggle to overcome *P. salmonis* infection. However, more research is necessary to confidently infer that NLRP12 may play a role in resistance to *P. salmonis* (**Figure 4.5**). An average 10% variance was found between biological replicates at 8 dpi. This finding indicates that further optimisation of the CellTiter-Glo 2.0 collection method is necessary.

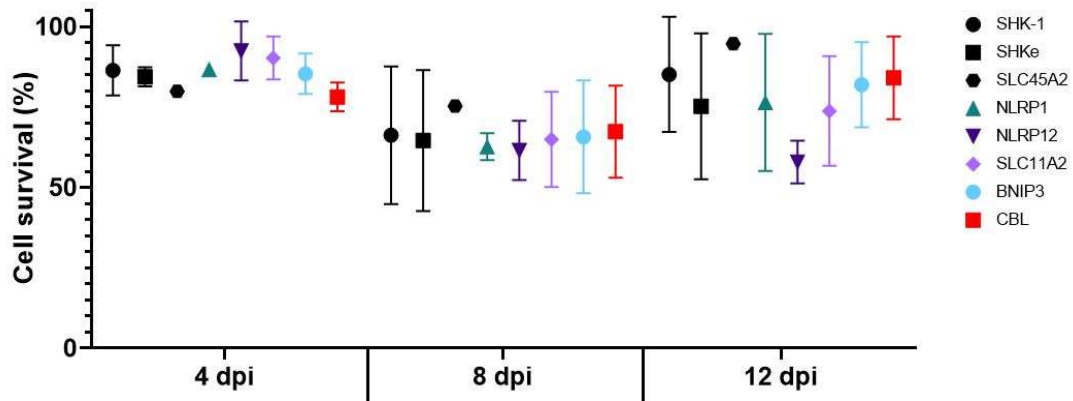


Figure 4.5. Cell viability progression of CRISPR/Cas9 KO cell lines and unedited controls during *P. salmonis* infection. Data were analysed using GraphPad Prism (version 9.4.1) and presented as mean \pm SD values of two biological replicates per experiment.

4.3.3. *P. salmonis* bacterial load.

As previously mentioned, no significant difference between KO cells and controls may be inferred due to the number of biological replicates used. However, descriptive statistics indicate that BNIP3-KO showed the highest bacterial load at 4 dpi (3.4×10^4 copies/ μ L) and at 8 dpi (3.5×10^4 copies/ μ L) versus other KO cell lines and the average control (2.1×10^4 copies/ μ L at 4dpi and 2.5×10^4 copies/ μ L at 8dpi). In contrast, NLRP12-KO showed the lowest bacterial load value at 8 dpi (1.9×10^4 copies/ μ L) and at 12 dpi (2.7×10^4 copies/ μ L), which represents a 41.5% reduction of bacterial copies versus the average control at 12 dpi (4.6×10^4 copies/ μ L). Worth mentioning that at 12 dpi all KO cell lines showed a lower bacterial count (NLRP1-KO: 3.0×10^4 copies/ μ L; NLRP12-KO: 2.7×10^4 copies/ μ L; SLC11A2-KO: 3.5×10^4 copies/ μ L; BNIP3-KO: 2.7×10^4 copies/ μ L; and CBL-KO: 2.8×10^4 copies/ μ L) than the average control cell lines (4.6×10^4 copies/ μ L) (**Figure 4.6**). These findings indicate that further research should study the impact of these genes in later stages of disease progression post-12 dpi. Vast differences in variance between biological replicates were found in every time point studied with a

maximum of 2.0×10^4 copies/ μ L variance in CBL-KO 0 dpi. These findings indicate that further optimisation of the gentamicin protection assay (**section 4.2.7.1.**) method is necessary to minimise the impact of media bacteria in the bacterial load quantification.

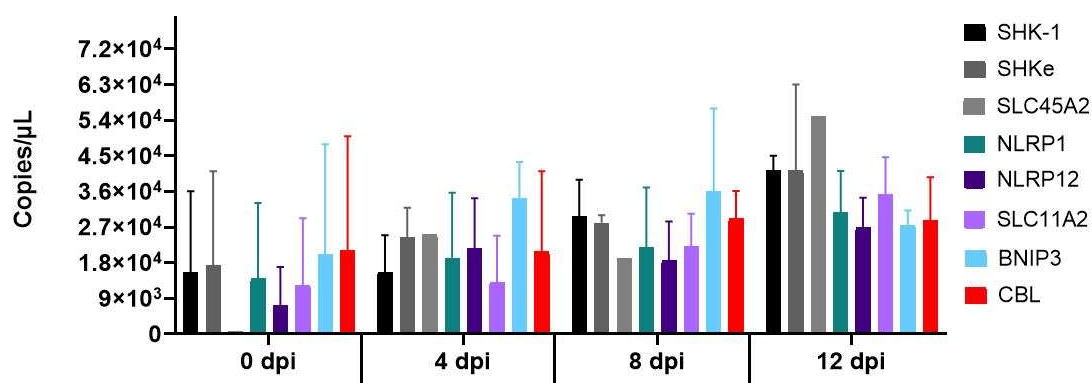


Figure 4.6. Bacterial load progression of *P. salmonis* infection. Data were analysed using GraphPad Prism (version 9.4.1) and presented as mean \pm SD values of two biological replicates per experiment.

4.4. Discussion.

The impact of functional loss of the edited cell lines NLRP1-KO, NLRP12-KO, SLC11A2-KO, BNIP3-KO, and CBL-KO during *P. salmonis* infection was assessed in this chapter. To achieve this, an *in vitro* model was developed contrasting the differences in the infection process between the edited cell lines and experimental controls.

Due to the diversity of biological functions to be assessed in the five edited cell lines, a strategy to pinpoint variance in the infection process using screening tests instead of evaluating individual functional changes was implemented. Three screening tests were chosen to evaluate host response to infection and bacterial proliferation: CPE, cell viability and bacterial load.

P. salmonis is an intracellular bacteria capable of infecting and replicate in numerous continuous cell lines. This attribute has contributed to characterise a canon progression of CPE since first isolated in Chinook salmon embryo cell line (CHSE-214) in 1990 (Fryer *et al.*, 1992). In addition, numerous studies have described the CPE produced in the Atlantic salmon ASK (Ortiz-Severín *et al.*, 2021; Smith *et al.*, 2015) and SHK-1 cell lines (Caruffo *et al.*, 2020; Díaz *et al.*, 2017; Ortiz-Severín *et al.*, 2021; Zúñiga *et al.*, 2020). The CPE induced by *P. salmonis* is characterised by the observation of pathogen-containing vacuoles (PCVs) in the cytoplasm of permissive cells between 3 to 5 dpi, attached and/or detached mortality observable at 3 to 5 dpi, and the progression of these observations affects a 100% of the cellular monolayer at 14 to 20 dpi (Díaz *et al.*, 2017; Smith *et al.*, 2015). Differences in the CPE progression have been reported when external conditions or treatments impaired the bacterial proliferative capacity; for instance, nutrient restrictions (Caruffo *et al.*, 2020; Díaz *et al.*, 2017), infection in macrophages cells (Ortiz-Severín *et al.*, 2021), and bacterial virulence loss (Valenzuela-Miranda *et al.*, 2020). Observation of CPE in the cells is considered a qualitative test where the result is the presence or absence of the canon CPE lessons. This test may be complemented using cell quantification by manually counting affected cells in a given area or using imaging software such as ImageJ. The present study observed no differences in the canon CPE progression between edited cell lines and controls. These results may suggest that the functional loss in edited cell lines has no noticeable impact on the CPE progression or that the changes' variances are too subtle to be detected by a qualitative test as CPE observation, and further quantitative tests are required to estimate the real impact of the KO genes have in the infection.

Determination of cell viability is a quantitative test used to estimate the impact on cell capacity to proliferate or survive a treatment or condition, such as drug treatments, potential cytotoxic chemicals, and pathogen infection. The study results highlighted differences between cell survival in the NLRP12-KO

edited cell line and controls at 12 dpi. The NLRP12-KO cell line revealed a 27% reduced survival rate versus the average control of 85%, while other KO cell lines showed differences of no more than 13.8% versus controls. However, more research is necessary to determine the impact of the NLRP12 gene's functional loss in cell survival to infection. The wide variance between replicates indicates that the method used should be optimised to improve replicability. Cell viability assays have been optimised to evaluate cell mortality in SHK-1 and ASK cell lines in previous studies (Caruffo *et al.*, 2020; Gratacap, Regan, *et al.*, 2020; Ortiz-Severín *et al.*, 2021; Schiøtz *et al.*, 2009; Zúñiga *et al.*, 2020) and two main protocols have been described for infection survival assays in SHK-1, namely Alamar Blue (Ortiz-Severín *et al.*, 2021; Zúñiga *et al.*, 2020) and Cell Titer Blue (Schiøtz *et al.*, 2009). CellTiter-Glo, the protocol used in this study, was optimised to evaluate cell survival to puromycin treatment (Gratacap, Jin, *et al.*, 2020) in the SHK-1 cell line. This difference may explain the inconsistency in results between experimental replicates. Further studies should evaluate the impact of the chemical agent used in the cell mortality assessment during bacterial infection. Another subject for optimisation is the initial cell seeding and the variance between biological replicates in the well plates. Some samples presented a marked difference in variability between biological replicates than the rest of the samples, with cell viability rates ranging between 12 (CBL-KO at 8 dpi) to 2.1% (SHKe at 4 dpi). An optimisation of the initial cell seeding count and consistency between biological replicates can be achieved by using an automated cell counting system and multichannel pipette for seeding. Finally, SHK-1 cells have demonstrated to be sensitive to overgrowth conditions; hence, long-lasting studies should optimise the seeding density to avoid mortality unrelated to infection in the later sampling.

Bacterial load results revealed that BNIP3-KO presented the highest bacterial count at 4 and 8 dpi. Conversely, NLRP12-KO showed a lower count at 8 and 12 dpi, with a 41.4% reduction of bacterial copies versus the average

control at 12 dpi. Vast differences between experimental replicates indicate that further method optimisation is needed. In addition, the variance between biological replicates may be explained by faults in the gentamicin protection assay protocol. Excess bacteria in the media after sample collection may interfere with quantifying intracellular bacteria and generate wide differences between biological replicates. Another measure to improve replicability and consistency is increasing the biological and technical replicates per experiment.

Bacterial load is arguably one of the most used techniques to evaluate *P. salmonis* infection. Applied in multiple studies using real-time-PCR is considered the hallmark of *P. salmonis* infection evaluation (Caruffo *et al.*, 2020; Olivares & Marshall, 2010; Pulgar *et al.*, 2015; Zúñiga *et al.*, 2020). The limitation of this technique is the inability to differentiate between viable and dead bacteria; thus, particular emphasis on eliminating the free-media bacteria and collecting only intracellular bacteria should be taken. Intracellular bacteria like *P. salmonis* cannot survive in non-enriched media, such as the L-15 media used in this experiment. Therefore, applying an early gentamicin protection assay at 3 or 4 dpi instead of at sampling collection may improve the variability between biological samples in the later time-points sampling (Zúñiga *et al.*, 2020).

Despite the results requiring optimisation to improve consistency between experimental replicates, the results may provide an interesting preliminary insight into the functional role played by these candidate genes in *P. salmonis* infection. NLRP12 and BNIP3 are valuable targets for additional studies to identify their biological function in the host response to infection.

NLRP12 is an inflammation inhibitor in mammals (Normand *et al.*, 2018) and a mitigator of NF- κ B pathway activation (Arthur *et al.*, 2007; Lich *et al.*, 2007). Therefore, further research should explore the activation of NF- κ B

pathways in NLRP12-KO cell lines during *P. salmonis* infection. Intracellular bacteria such as *P. salmonis* and *Legionella pneumophila* possess a Dot/Icm type-four secretion system (T4SS) used to translocate bacterial effectors interrupting the host cellular signalling to promote bacterial proliferation and survival (Banga *et al.*, 2007; Cortés *et al.*, 2017; Gomez *et al.*, 2013). BNIP3 is an apoptosis and mitophagy inducer used as a target for *L. pneumophila* Dot/Icm T4SS, to inhibit cell death to allow bacterial replication (Banga *et al.*, 2007).

Although no statistical significance differences may be inferred from these results, the KO cell lines obtained constitute a valuable model for studying the impact of gene functional loss in multiple key infection pathways. BNIP3-KO and CBL-KO cell lines may be used to study the impact of apoptosis modulation and bacterial internalisation of *P. salmonis*. NLRP1-KO cell line may be used to study the inflammasome activation and induced maturation of IL-1 β . It has been described in zebrafish an important involvement of NLRP1 in antibacterial innate immunity (Li *et al.*, 2018). A possible avenue to study the NLRP1 inflammasome activation in Atlantic salmon is to explore the maturation and expression of IL-1 β or the recruitment of inflammasome components (NLRP1, ASC and Caspase-1) in infected NLRP1-KO cell lines.

To the best of my knowledge, this is the first report of the gene NLRP12 to play a role in Atlantic salmon response to *P. salmonis* infection. Further assessment is required to confirm their functional effect on gene resistance to SRS.

4.5. Conclusions.

This study's results suggest that the gene NLRP12 may be functional in the host response to *P. salmonis* infection. NLRP12-KO demonstrated decreased cell viability at 12 dpi and lower bacterial load at 8 and 12 dpi.

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However, these results should be optimised to improve consistency and replicability, and further research is necessary to specify these genes' functional role in *P. salmonis* infection process.

CHAPTER 5: Discussion and final remarks.

5.1. General Discussion.

Salmon Rickettsial Syndrome (SRS) is the most complex infectious disease afflicting Chilean salmon farming and seriously threatens the industry's economic viability. New effective strategies to control this disease are needed, and strengthening the natural resistance of the host to SRS is a possible route.

The present research aimed to develop an *in vitro* model to study the functional impact of genes related to SRS resistance in the infection process. To accomplish that, this thesis comprised several experimental chapters intended to develop an effective and reliable genome editing method to knockout (KO) genes related to SRS resistance. The second chapter uses transcriptomic studies and quantitative trait locus mapping to screen for relevant genes and pathways up-regulated during infection and shortlisted candidate genes by cross-referencing with the scientific literature on the immune response to SRS. In the third chapter, a CRISPR/Cas9 method to efficiently KO genes in the Atlantic salmon cell line SHK-1 was developed. Finally, the fourth chapter interrogates the functional impact of these genes in cell survival and bacterial replication in an *in vitro* model of infection.

5.1.1. Unravelling the genetic basis of SRS.

A large-scale infection challenge in Atlantic salmon revealed significant genetic variance of host resistance to SRS. Genome-wide association analyses showed no major QTL explaining the genetic variance. However, four suggestive QTLs in chromosomes 1, 2, 12 and 27 were identified, implying that resistance to SRS is polygenic in nature. These findings agree with

previous studies reporting a polygenic architecture of the trait (Barría, Marín-Nahuelpi, *et al.*, 2019; Correa *et al.*, 2015). In addition, chromosomes 1 and 12 have been previously described as containing genomic regions implicated in SRS resistance (Correa *et al.*, 2015; Yáñez *et al.*, 2019). However, chromosomes 2 and 27 have been implicated in the trait for the first time in this study. RNA-sequencing of liver and head kidney revealed the differential expression of several thousand genes between control and infected samples and substantial upregulation of immune pathways and downregulation of energy metabolic pathways. In addition, numerous networks significantly correlated with SRS resistance breeding values were found, implying their contribution mediating genetic resistance, namely, cytoskeleton reorganisation, apoptosis, bacterial invasion and intracellular trafficking, and the inflammasome. An extensive literature review of scientific knowledge about host immune response to *P. salmonis* and bacterial proliferation shortlisted suitable candidate genes related to resistance to SRS. Expanding our understanding of the functional basis of genetic resistance and host response to SRS in Atlantic salmon is essential to developing innovative disease control strategies

5.1.2. Gene editing for *P. salmonis* infection studies in Atlantic salmon.

A highly efficient CRISPR/Cas9 editing method for KO candidate genes related to SRS resistance was developed, obtaining over an 80% mutation rate (Indel rates) in the Atlantic salmon continuous cell line SHK-1. Five edited cell lines were obtained, NLRP1-KO, NLRP12-KO, SLC11A2-KO, BNIP3-KO and CBL-KO. The optimisation of this system implied the selection of the candidate genes based on their involvement in networks associated with resistance to SRS and their location flanking single nucleotide polymorphisms (SNPs), surpassing the suggestive significance level in chromosome 2. In addition, the sgRNA design and determination of the target region in each candidate gene

were based on functional domains and proximity to the N-terminus of the protein avoiding in-frame mutations. Finally, caution was taken in designing sgRNAs to minimise the unintended editing off-target by selecting only guides with no identical sequences in the reference genome other than the intended target site and at least three nts mismatches within the 12 nts seed sequence. This method has successfully induced genome mutations in SHK-1 cells and was used to implement *in vitro* models of KO cell lines for SRS resistance for functional studies.

5.1.3. Functional impact of candidate genes.

An *in vitro* infection challenge of edited cell lines and controls with the bacterium *P. salmonis* strain LF-89 MOI 80:1 (bacteria: cell) was developed. Two biological replicates were conducted under the same cell density conditions, MOI, edited cell lines, controls, and three technical replicates. Cytopathic effect (CPE), cell viability and bacterial load were assessed by contrasting differences between KO cell lines and controls during infection. No differences in CPE progression were reported between edited cell lines and controls. However, NLRP12-KO showed a 27% reduced cell viability at 12 dpi and the lowest bacterial load at 8 and 12 dpi versus control cell lines, suggesting that the functional loss of NLRP12 may limit the infection progress by activating cell death. In addition, the RNA-Seq results in chapter 2 revealed that the NF- κ B signalling pathway was significantly up-regulated in liver and head kidney tissues during *P. salmonis* infection (**Figure 5**, Moraleda *et al.*, 2021), and NLRP12 had a negative correlation ($r = -0.48$, **Table 2.1**) with genetic resistance to SRS (Moraleda *et al.*, 2021). These findings agree with previous studies in mammals that indicate NLRP12 is a negative regulator of inflammation, suppressing NF- κ B activity (Arthur *et al.*, 2007; Lich *et al.*, 2007; Normand *et al.*, 2018). The NF- κ B activation and subsequent activation of the JAK/STAT signalling pathway is an innate immune response induced by recognising bacterial components and plays a crucial role in the defence

against infection (Normand *et al.*, 2018). Altogether, evidence suggests that NLRP12 may play a role in promoting *P. salmonis* tolerance; however, these results are a prelude to ongoing studies to establish the actual impact NLRP12 has in SRS resistance.

Additionally, all KO cell lines showed lower bacterial loads than the average control, highlighting the importance of studying late stages of infection after 12 dpi. The high variability of outcomes in experimental replicates indicates that the method requires optimisation and increasing biological and technical replicates should be implemented to improve the reliability of these results.

5.2. Limitations of the study.

Large-scale infection models are valuable tools to improve our knowledge of the host response to infection; however, infecting a whole population of animals has inherent challenges, such as choosing the delivery method. The intraperitoneal injection model used in chapter 2 was selected over the natural mode of infection due to the need to infect a whole population in a given time and to ensure a similar exposure to the bacterium in every individual. This mode of infection may affect the interpretation of the genetic resistance trait, as several genes playing crucial roles in the contagious process will not be assessed by this method of infection. In addition, the natural route of entry of *P. salmonis* is through epithelial tissues of skin and gills (Almendras *et al.*, 1997; Smith *et al.*, 1999), and vast differences have been described in the pattern of infection of intraperitoneal injection versus cohabitation infection (Dash *et al.*, 2018; Ødegård *et al.*, 2011). Therefore, only a part of the genetic resistance mechanisms was expected to be captured in this study.

An important consideration for functional gene assessment is the sgRNA design strategy. As previously mentioned, limited information about the functional domains of the candidate genes was found in Atlantic salmon. Therefore, determining the editing site was restricted to targeting an early exon to disrupt the whole open reading frame (ORF), avoiding in-frame mutations. However, alternative starting codons may be present in any exon, and the likelihood of obtaining truncated proteins that conserve functionality is not entirely estimated in Atlantic salmon gene editing methods. Because of this, targeting functional domains with CRISPR/Cas9 edits is essential to obtain strong functional gene assessment models of study, as targeting functional domains improves the probability of loss function (He *et al.*, 2019; Shi *et al.*, 2015). Focusing further research on characterising the functional domains of Atlantic salmon proteins is a vital precursor for gene editing KO models developing, as structural domain variances may occur within teleost species; for instance, NLRP12' PYR domain is present in Japanese medaka (*Oryzias latipes*) but absent in zebrafish (*Danio rerio*) (Morimoto *et al.*, 2021). In addition, complex genomes such as Atlantic salmon contain several gene paralogues due to whole-genome duplication (WGD) events commonly occurring in fishes. These copies may conserve the function of edited genes or perform partial functionality (Campenhout *et al.*, 2019; Gratacap, Regan, *et al.*, 2020). In this research, only genes highlighted by the differentially expressed study in chapter 2 were edited; hence, gene paralogues were excluded to discern the candidates' impact in the functional assessment. Exploring a complete KO of the target gene and paralogues is suggested for future studies.

Finally, a critical limitation of the gene KO model of study is the variance of phenotypic outcomes of genetic mutations (Rossi *et al.*, 2015). This may be explained by the gene KO not disrupting the protein synthesis; hence, function conservation. Alternatively, paralogues' genes may fulfil the functional loss (Rossi *et al.*, 2015). These findings highlight the necessity of a functional loss

assessment that evaluates the protein synthesis before evaluating the phenotypical loss impact. In this study, high rates of KO cells were obtained (over 80%); however, due to time constraints, no test was carried out to evaluate the protein translation. Future studies are recommended to confirm the protein deletion using mRNA expression, enzyme activity assays or protein expression analysis (for instance, Western blot).

5.3. Future studies.

Numerous networks and genes implicated in mediating resistance to SRS were highlighted in this study. These genes constitute attractive targets for further functional research, such as genome editing, to elucidate their physiological function in the variation of the host resistance trait. Nonetheless, the moderate heritability and polygenic nature of the trait found in chapter 2, constitute a suitable candidate for genomic selection in salmon breeding programmes. This method has proven to be an effective strategy for selecting salmon for resistance to SRS and other traits with a polygenic background (Bangera *et al.*, 2017; Correa *et al.*, 2017; Ødegård *et al.*, 2014b; Robledo *et al.*, 2018; Tsai *et al.*, 2016).

This study first reports NLRP12 as gene putatively related to SRS resistance. Although the results obtained require optimisation to improve consistency and reliability between replicates, the gene highlighted by this study constitute a valuable tool for further functional studies, and represents a potentially interesting avenue of future research. The developed cell lines can be used as a model of study to assess numerous pathways related to the host response to SRS. Further research is critical to improving our understanding of the biological process implicated in *P. salmonis* infection. For instance, BNIP3-KO cell lines may be used to improve our knowledge of the biological process that leads to apoptosis during infection. In addition, it may give

valuable insights into the possible modulation by the bacterium to ensure bacterial proliferation, as described by other intracellular bacteria such as *Legionella pneumophila* (Banga *et al.*, 2007). Similarly, CBL-KO cell model may provide valuable information about the endosome path of bacteria internalisation (Veiga & Cossart, 2005). Finally, NLRP1-KO and NLRP12-KO are valuable models for studying essential immune pathways, such as the inflammasome (Li *et al.*, 2018) and the NF- κ B signalling pathway (Arthur *et al.*, 2007; Normand *et al.*, 2018).

Finally, genetically modified organisms (GMOs), such as CRISPR/Cas9 edited animals, are subjected to thorough safety assessments by the European Food Safety Authority (EFSA) to be commercialised in the Europe Union. While genetic engineering in domestic animals requires regulatory approval, there is growing evidence that methodological optimisation to improve gene editing precision will address the safety issues related to gene editing. It is still very early days to accomplish regulations and public acceptance of genome editing technologies as a conventional method for genetic improvement. However, markets for CRISPR-edited products will eventually open; for instance, two growth-enhanced CRISPR/Cas9 modified fish species, tiger puffer (*Takifugu rubripes*) and a red sea bream (*Pagrus major*), have been approved in Japan for commercialisation ('Japan Embraces CRISPR-Edited Fish', 2022).

5.4. General Conclusion.

The holistic methodology applied in this research work demonstrated enormous potential to advance our understanding of the genetic bases for host resistance to SRS. Genome-wide mapping and differential expression studies applied to a large-scale *P. salmonis* infection challenge fast-forwarded identifying candidate genes related to SRS resistance. The further application of genome editing to develop an *in vitro* model of infection provided an optimal

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strategy to characterise candidate genes functionally. Altogether, this data introduces a valuable prelude to ongoing studies and emphasise the relevance of exploring genetic resistance as a valuable avenue to control SRS.

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