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Characteristics of *Aeromonas salmonicida*, a rainbow trout pathogen, and early signatures of host immune response

Charakterystyka *Aeromonas salmonicida*, patogenu pstrąga tęczowego, i wczesne oznaki odpowiedzi immunologicznej gospodarza

Doctoral thesis

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Content

1 List of abbreviations

2D GE	2D Gel Electrophoresis
ADP	Adenosine diphosphate
AexT	ADP-ribosyltransferase toxin
ANOVA	Analysis of Variance
APC	Antigen Presenting Cells
ATT	Attenuated strain
BLAST	The Basic Local Alignment Search Tool
Ca	Calcium
cfu	Colony forming unit
cRNA	Complementary RNA
DC	Dendritic cells
DHR123	Dihydrorhodamine 123
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ECP	Extracellular Proteins
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
EST	Expressed Sequence Tag
FACS	Fluorescence-Activated Cell Sorting
FAO	Food and Aquaculture Organization
FBS	Fetal Bovine Serum
Fe	Iron
FEAP	The Federation of European Aquaculture Producers
FES	Feature Extraction Software
FITC	Fluorescein isothiocyanate
FSC-H	Forward Scatter-Height
GISD	Global Invasive Species Database
GRASP	Genomics Research on Atlantic Salmon Project
HKL	Head kidney leukocytes
hpi	Hours post infection
HV	Highly virulent strain
i.p.	Intraperitoneal
ID	Identification
IFN	Interferon
IgM	Immunoglobulin M
IgT	Immunoglobulin T
IS	Insertion Sequences
KEGG	The Kyoto Encyclopedia of Genes and Genomes
LB	Luria Broth
LPS	Lipopolysaccharide
mAb	Monoclonal antibodies

MACS	Magnetic-activated cell sorting
MHC	Major histocompatibility complex
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	The National Center for Biotechnology Information
NCCCWA	National Centre for Cool and Cold-Water Aquaculture
NGS	Next-Generation Sequencing
NK cells	Natural killer cells
nr database	Non-redundant database
ORF	Open Reading Frame
Ox	Oxygen limited condition
PAI	Pathogenic Island
PAMP	Pathogen Associated Molecular Pattern
pASA	Plasmid ASA
PBS	Phosphate-Buffered Saline
pcDNA	plasmid DNA
PCR	Polymerase chain reaction
PEL	Peritoneal leukocytes
PFA	Paraformaldehyde
PMA	Phorbol 12- myristate 13-acetate
PRR	Pathogen Recognition Receptor
RAG	Recombination activating genes
RIN	RNA integrity number
RNA	Ribonucleic acid
RPE	R-Phycoerythrin
rRNA	Ribosomal RNA
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SSC-H	Side Scatter-Height
TCA	Trichloroacetic acid
TE	Tris-EDTA
TLR	Toll-like receptor
TNF	Tumor Necrosis Factor
tRNA	Transporting RNA
T3SS	Type Three Secretion System
VAMP	Virulence Associated Molecular Pattern
WT	Wild type

2 Introduction. Immunity of rainbow trout against pathogen *Aeromonas salmonicida*, its mechanisms and impact

2.1 Rainbow trout habitats around the world

Rainbow trout (*Oncorhynchus mykiss)* is the freshwater fish, although sea-run populations, often known as steelhead, also exist in some areas. Any population of *O. mykiss* is, however, capable of migrating to or surviving in the sea. *O. mykiss* tends to thrive better in lakes than in streams or rivers, although large fish are often present in remote waters [1]. The species prefers well-oxygenated, clean, fresh water. The optimal temperature for growth is 17 °C, the preferred temperature for spawning is around 10 - 12 °C, and it tolerates temperature in the range of 0 °C to 25 °C.

Despite it spreads worldwide, rainbow trout has only limited native range of habitat (Figure 1). It lives in the Eastern Pacific Ocean and the freshwater, west of the Rocky Mountains, from northwest Mexico (including extreme northern Baja, California), to the Kuskokwim River, Alaska. Additionally, it lives in northern places of Pacific Rim – from Kamchatka to Alaska region. It is native in the areas of the Peace and Athabasca rivers east of the Rocky Mountains [2].

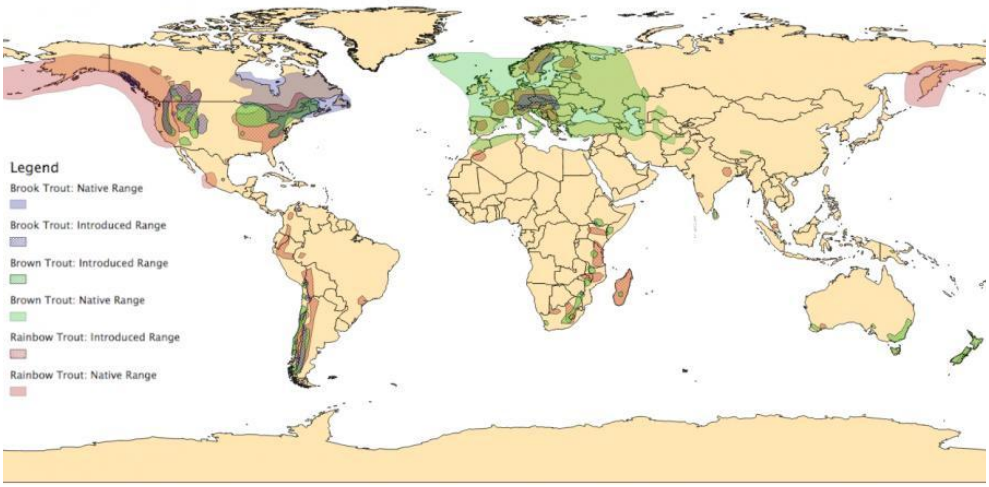

**Figure 1. Native and introduced ranges of rainbow trout (*Oncorhyncus mykiss) (red),* Brook trout *(Salvelinus fontinalis) (green)* and Brown trout (*Salmo trutta) (blue)* [3].

The species is flexible and adaptable to new habitats, including hatcheries, lakes, rivers, ponds, and artificial impoundments. The populations have also been sustained by continuous releases and escapes from hatcheries and farms. Nevertheless, successful reproduction in nature is rare [4]. Over the time *Oncorhynchus mykiss* has been introduced into worldwide aquaculture for food, culture and recreation purposes [5]. It was sent to Germany from Michigan in 1882 and dispersed to

various hatcheries. According to European Union Statistical Office rainbow trout was one of the second most produced fish species in terms of tons in 2022. It was also the most profitable fish in 2022 (Figure 2).

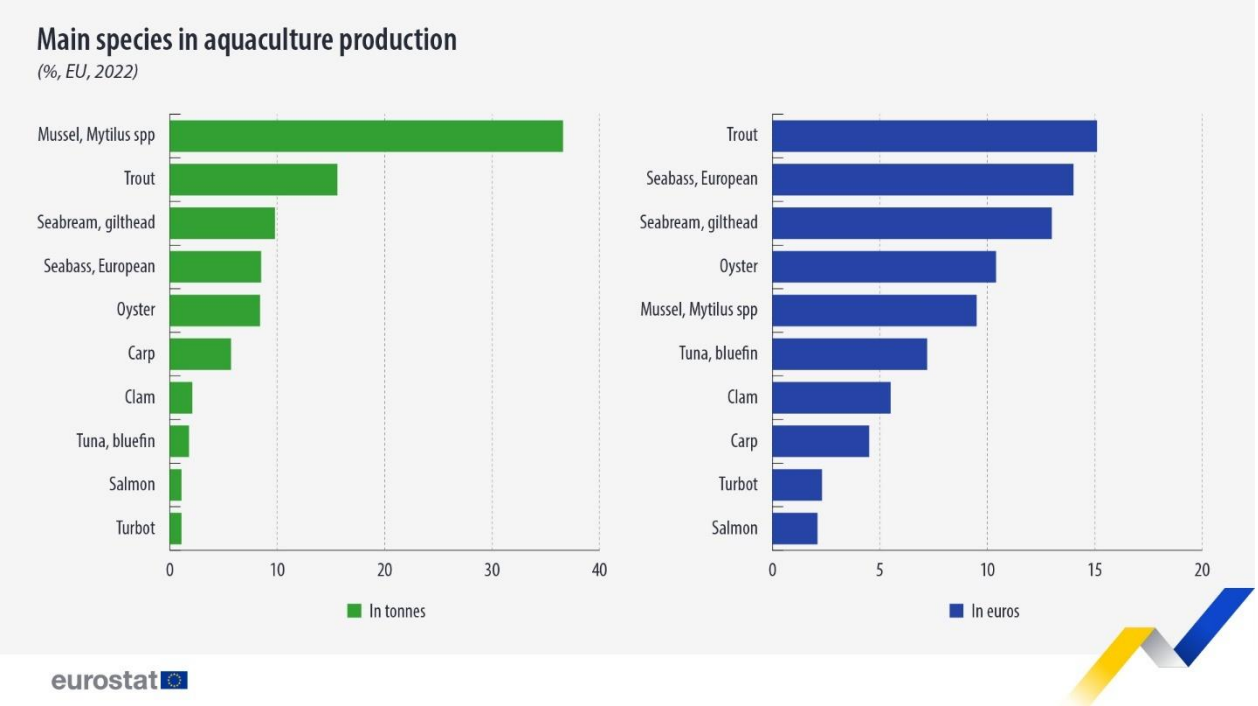

Figure 2. Main species in European Union aquaculture production in tonnes live weight (left) percent shares in euro (right) in 2022. In both terms rainbow trout is placed in top species (Source: EUROSTAT: fish_aq2a).

In 2022 total aquaculture production yielded approximately 1.1 million tons of aquatic organisms of total value €4.8 billion. The main EU producers in 2022 were Spain (25 %), France (17 %), Greece (13 %) and Italy (12 %) making 56 % of production. Production covered mainly finfish and mollusks under controlled conditions. Nevertheless, rainbow trout was the most valuable farmed species accounting of 15 % of total production (Figure 2). Its production is exceptional. While there is a high specialization in EU countries as for species production, rainbow trout was farmed in twenty-two countries and 51 % of farmed weight of trout production was in three countries: France, Denmark, Italy. Fish were farmed in fresh water (80 % of total), in tanks, but also in seawater of the Atlantic, Northeast area.

The production of trout in Poland is measured annually by EUROSTAT, FAO, State Statistics and PTBA (Polish Trout Breeders Associations) and ranges from 15 395 tones (EUROSTAT) to 22 400 tones PTBA) in 2019. Values ranges due to different methodology. State statistics data are collected by Inland Fisheries Institute, which is in line with Eurostat methodology, whereas estimation of

production volume of PTBA data is based on annual surveys of amount of consumed feed from Trout Service (EUROSTAT, 2019).

Rainbow trout was the main farmed species in Poland which covers 86 % of production. The other species belong to Arctic char *(Salvelinus alpinus)*, brook trout *(Salvelinus fontinalis),* spring trout *(Salmo trutta m. fario) and* cross between the Arctic char and brook trout Production is mostly concentrated in Northern parts of Poland in West Pomeranian and Pomeranian where trouts are farmed in domestic farms in flow through facilities or in recirculating aquaculture systems (RAS).

RAS aquacultures are the modern and innovative approach towards fish farming in Europe (Figure 3). It combines modern knowledge of biology, engineering, information technology and environmental science [6]. It originates from Japanese purification systems from 1960s. Since then, the technology was developing and putting pressure of automation, parameter monitoring of good water quality, less wastewater discharge but still stays expensive and energy consuming.

The main benefits of RAS technology are that it requires lesser amounts of water which produces less waste and environmental pollution. Additionally, environmental conditions are kept on stable level due to high automatization therefore fish production of possible 365 day in year. Importantly, small farm surface demands facilitate high production levels, profitability, and high growth rate but also higher densities of fish in tanks affecting animals negatively.

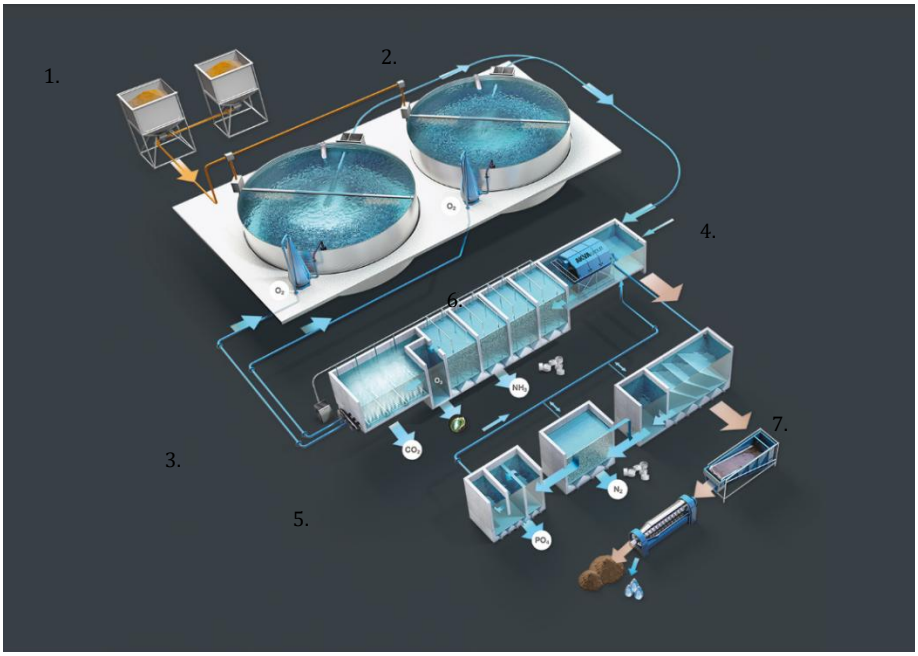

Figure 3. RAS technology. Example of device chain proposed by AKVAgroup. 1 – feeding system, 2 – fish tanks and fish handling, 3 – oxygenation, 4 – mechanic filters, 5 – CO2 degassing, 6 – biofilters, 7 – Zero Water Concept (ZWC) (https://www.akvagroup.com/).

In fish industry using RAS technology, crowding of animals (loading rate – [kg/L/min]) increased temperature, amount of oxygen in the necessarily intensive culture environment are well-known factors to increase the risk of infectious disease outbreaks in modern aquaculture [7,8]. The elevated density (kg/m^3), which is more than one thousand times higher than under natural conditions, has also a negative impact on fish behavior and final product quality (e.g. fin erosion and skin abrasion) [9–11]. Annual acute and chronic losses due to infectious disease outbreaks in aquaculture can impose significant costs on productivity and, therefore, to profit [12]. Good manufacture practices formulated in FAO guidelines aim to prevent it and are the best line of defense. The list of rules is long and covers on one hand fisheries management such as the use of improved husbandry/management practices, water disinfection, and its biological control, genetically resistant stocks. on the other hand, supplements, vaccines, nonspecific immunostimulants, probiotics and prebiotics, medicinal plant products, antimicrobial compounds are the best solutions in control of infectious diseases of fish [13]. Simple actions such as daily removing dead fish from the pond to avoid disintegration or closing their system in case of neighboring farm disease outbreak at first play a crucial role in outbreak spread prevention [7]. Nevertheless, intensification of production in aquaculture requires a better management of input and waste products. So far there is no unified guideline specifying mandatory parameters and their levels or fluctuations that should be monitored in RAS. Each farmer decides what parameters should be followed. Currently, the water quality is the most important parameter in aquaculture management. Among suggested are physical parameters, organic and biological contaminants, and pathogens. Physical parameters such as pH, dissolved oxygen level, temperature, salinity, turbidity should be monitored in real time to prevent production losses. In the ideal range water quality can improve fish growth and reduce pathogens occurrence [14,15]. Organic compounds or particulate feed matter can be a substrate for bacteria growth and disease spread [16]. This increases the ecological impact on the local environment and eventually the potential for the spread of pathogens [17,18]. A single approach is not sufficient to control the outbreak. Combination of many strategies can bring desired effects. It is crucial to provide complete information to producers and build awareness about those factors that contribute to the losses based on scientific evidence.

2.1.1 **Antibiotics, probiotics, and vaccines**

One of the solutions to the pathogen outbreak is the usage of antibiotics. In previous years it was an easy and effective, but overused solution to control bacterial diseases [19,20]. There are three main purposes why antibiotics were so desired in fish production: therapeutic use to treat sick animals; prophylactic use to prevent infection in animals; as growth promoters to improve feed utilization and production [21–25]. Nevertheless, an excessive amount of usage leads to uncontrolled

resistance of pathogenic bacteria. What is more, initially positive prophylactic supplementing of water with antibiotic lead to the development of bacteria resistance [21,22,26,27]. Another issue is that antibiotics persist in the environment and may reach human organism with a food chain because they cannot be degraded [28,29]. Moreover, many studies indicate that antimicrobial treatment changed the composition of bacteria in the aquatic environment surrounding aquaculture and an increased number of antibiotic-resistant bacteria [22,30–32]. Eventually, it contributes to the ineffectiveness of antibiotic usage and susceptibility not only of animals but also humans to infections.

Due to the problems with resistant strains appearing after the treatment, other alternative methods have been taken into consideration. Probiotic treatment is currently investigated and has a positive effect on fish health and recovery during infection [33]. According to latest definition, it is described as "Live microorganisms that when being administered in appropriate doses, confer a benefit to the health of the host" [34]. Most of them are the isolates of intestinal microbiota. It has been proven that gut microbiota has evolutionary stable composition and in case of fish it is harbored by more than 1 x 10^8 bacteria per gram, mainly dominated by *Acinetobacter* sp., *Aeromonas* sp., *Flavobacterium* sp., *Lactococcus* sp., *Pseudomonas* sp., *Bacteroides* sp., *Clostridium* sp. and *Fusobacterium* sp. [35–38]. Enhancing the proper gut bacteria composition and supporting probiotic species eliminates the chances of pathogenic bacterial colonization and gut penetration [39]. Introducing a correct fish diet rich in feed ingredients that promote the fish immune system, and their gut microbiome may help to prevent harsh outbreaks of disease [38]. Additionally, it supports the induction (education) and development of the immune system during the host growth. For example, feeding with *Carnobacterium* sp. augments the immune response upon challenge with *Aeromonas salmonicida and Yersinia ruckeri*, increasing the phagocytic activity, respiratory burst, and lysozyme activity [40]. Similarly, trout fed with three freeze-dried bacterial species (*Lactobacillus ramnosus, Enterococcus faecium* and *Bacillus subtilis*) or with *Lactococcus lactis, Lactobacillus sakei* and *Leuconostoc mesenteroides* exhibit enhanced production of superoxide anion and the activity of the alternative complement pathway [41–43]. Moreover, lactic bacteria can reduce the growth of *A. salmonicida* [44] and pre-treatment with *Lactobacillus delbrueckii* subsp. *lactis* can prevent the destructive effects of *Aeromonas* sp. *on the* healthy intestinal barrier in *vitro* [45]. Number of in *vivo* trials proved also the significance of probiotic microbiota where fry or fingerlings were fed with viable *Carnobacterium* sp., *Bacillus* sp. *and Aeromonas sobria and* even with formaldehyde inactivated *Vibrio fluvialis*, *Aeromonas hydrophila, and Carnobacterium* sp. *and* it reduced the mortality after challenge with *Aeromonas salmonicida* [46–48].

Another solution used to maintain fish in good health are vaccines. They are an effective way of controlling diseases caused by *Aeromonas salmonicida, Aeromonas hydrophila*, *Vibrio anguillarum* or *Flavobacterium psychophilum* [49,50]. First protective vaccine was created and published against furunculosis in 1942 [51]. Following, a number of immersion and injection vaccines was used but not always provided the protection [52,53]. Till now the best protection against fish diseases is guaranteed by vaccines delivered by intraperitoneal injection with polyvalent vaccines protecting simultaneously against most pathogens [54]. There are many vaccines of different composition: killed or attenuated pathogens, DNA, recombinant vectors, genetically modified bacteria, or protein subunits [50,55–58]. Moreover, their delivery is highly dependent on various external conditions such as fish species, immune system condition, history of diseases in aquaculture system (seasons), farming technology – mechanization, environment, cost of benefit / loss ratio [50]. Yet, none of them assures complete protection [58,59].

2.1.2 **Furunculosis in trout caused by *A. salmonicida***

Despite having prevention and cure solutions such as probiotics and vaccines, one of severe problems in fisheries around the world remains furunculosis. It inflicts heavy losses in aquaculture [60]. The name of the disease derives from the presence of "blisters" or furuncles on the surface of chronically infected salmonids [60,61]. The infection causes high mortality in fish within a few (3 - 7) days after symptoms appear [62]. Fish may show lethargic swimming or swimming just below the surface, loss of appetite, respiratory distress or jumping from the water. Common pathological signs observed during the infection are furuncles (or boils) involving skin and/or muscle, progressing to crater lesions (usually restricted to the subacute or chronic phase in adult salmon), hemorrhages on the skin, mouth, fin bases, in muscles and internal organs, darkening of body color and pale gills, bloody discharge from nares and/or vent, enlarged spleen and focal necrosis of the liver, stomach filled with mucus, blood and sloughed epithelial cells [61,63,64].

The furunculosis infectious agent, *Aeromonas salmonicida* ssp. *salmonicida,* belongs to the Aeromonadaceae family, genus *Aeromonas* [65]. These Gram-negative, rod-shaped, non-motile bacteria are the etiological agent of a disease called furunculosis in farmed and wild salmonids. It was firstly isolated from brown trout and described by Emmerich and Weibel in 1894 [66]. Four subspecies have been described within the species of *A. salmonicida*, i.e., *A. salmonicida* ssp. *salmonicida*, *achromogenes*, *masoucida, pectynolytica and smithia* [67]. *A. salmonicida* ssp. *salmonicida* causes disease of typical form, and other subspecies contribute to the atypical form of the disease. Other bacteria subspecies, *A. salmonicida* ssp. *achromogenes*, *masoucida, pectynolytica and smithia* contribute to atypical form of the disease such as ulcer disease (atypical furunculosis) of trout and goldfish

(*Carassius auratus*) [68,69] [70,71], erythrodermatitis of common carp (*Cyprinus carpio*) [72] [73] and systemic infections among several other warm water and marine species.

There are many *A. salmonicida* isolates and all have various levels of virulence. *Aeromonas salmonicida* ssp*. salmonicida* is one of the seasonal pathogens which cause outbreaks in salmonids during summertime (July - August). Depending on the age, health, and fish species the disease exists in different forms. The difficulty in investigating *A. salmonicida* infection mechanisms originates also from the fact that its virulence is unstable [74]. Major virulent factors (effector proteins and type III secretion system – T3SS) are localized on large plasmids which may be lost or rearranged during the culture treatment as shown by recent studies [75,76].

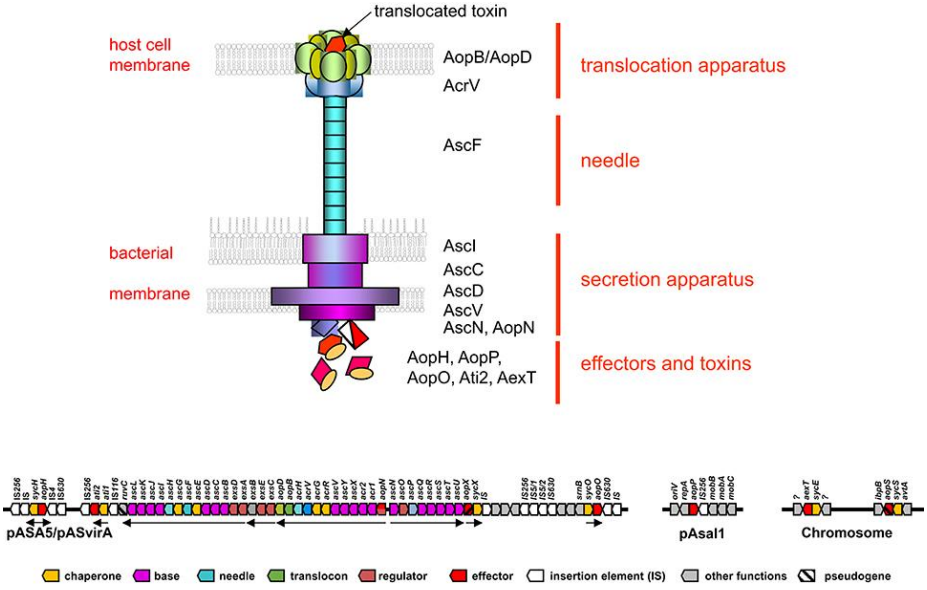

Figure 4. Type three secretion system of *Aeromonas salmonicida* almonicida. (Source: Frey and Origgi 2016).

Type III secretion system (T3SS) has also been proven to highly contribute to establishing the infection [77,78]. T3SS is a complicated structure characteristic to Gram-negative bacteria, anchored in the bacterial cell membrane (Figure 4). Researchers suggest that it evolutionary origins from flagella apparatus which after generations lost the motility and gained ability to secrete proteins [79]. T3SS commonly occurs in many pathogenic bacteria such as *Yersinia* sp. [80,81], *Salmonella* sp. [82], *Shigella* sp. [83,84], enteropathogenic and enterohaemorrhagic *Escherichia coli* [85–87] and *Pseudomonas aeruginosa* [88].

In *A. salmonicida* T3SS enables translocation of effector proteins, such as AexT, AopH, Ati2. AopP, AopO, AopN, and toxins directly to the cytosol of the host cell [89,90]. The one mostly studied is the ADP-ribosylating toxin AexT. It has a severe impact on the integrity of cellular cytoskeleton [91]. Effector proteins are affecting the actin cytoskeleton and interfere with signaling pathways [80,92–94].

As reported by Stuber et al, once rearrangements in T3SS-encoding plasmid appear, they stay permanent. Moreover, the plasmid is strain – dependent. While strain *A. salmonicida* strain A449 does not lose its virulence after incubation in liquid medium, the rearrangements appear when it is grown as colonies on agar medium [75]. Alteration of virulence factors and rearrangement of pAsa5 plasmid is caused by the growth of *Aeromonas salmonicida* under stressful conditions [95]. It seems that the occurrence of atypical strains of *A. salmonicida* and highly unstable genome both complicate the image of the infection process.

Among others, several virulent factors are in focus of extensive research. Firstly, the A-layer protein protects the bacteria against the host's specific and nonspecific defense mechanisms [96,97]. It promotes the adherence of bacteria to host cells and protects them from proteolytic activities of host enzymes [76,98]. Additionally, lipopolysaccharide (LPS), an endotoxin unique to Gram-negative bacteria, causes systemic inflammation (endotoxemia and septic shock) by a release of lipid from lysed bacteria which then stimulates the immune system via TLR4 signaling pathway [99,100].

2.2 Infection as an environmental niche homed by the bacteria

The armory of pathogenic microorganisms represents an extremely complex system. Researchers established repeating patterns such as protein composition and structures or specific interaction that bacteria use for invading and spreading in a host [101,102]. Components of bacterial cell membranes, toxins, enzymes and adhesins are all factors contributing to taking control of the host organism by pathogens. Behavioral patterns such as intracellular lifestyle of bacteria (*Salmonella* sp., *Shigella* sp., *Mycobacterium* sp.) [103–105], blocking immune responses (*Salmonella* sp.) [106–108], destroying or dysregulation defense mechanisms are other examples how bacterial pathogens escape the host defense and struggle for survival [106,109].

To devise effective tools promising to improve rainbow trout's health in aquaculture in a timely and cost-effective fashion, it is essential to identify key biological targets, such as antigens, host immune factors, and stress response genes. Systematic dissection of the interactions between host aquatic animals and pathogens with genomics, proteomics, and other biotechnological approaches help us to define targets for anti-pathogen interventions [110]. Such approach is useful also in practice. Scientists by expanding basic knowledge about biology of disease go deep to the nature of the problem and build awareness in the aquaculture industry. By directly controlling environmental conditions, monitoring the current situation in facility, and providing animals the optimal living conditions fish producers indirectly control diseases and prevent future outbreaks.

3 Aim of the thesis

The main aim of this study is to characterize the pathogen *Aeromonas salmonicida* repertoire of virulence factors to search for new interaction ways with host rainbow trout. High-throughput tools were used to effectively scan known and unknown genes and transcripts of *A. salmonicida and* define those bacterial genes that products interact with the host in the early time points of infection. This work focuses on virulence-related molecular patterns (VAMPs) as agents permitting the immune system to differentiate self from non-self of bacterial origin and potentially causing the disease.

The aims presented above were fulfilled by following tasks:

1. Genome sequencing and comparison of bacterial genomes between strains of high and low virulence, and the reference strain. Experiments included sequencing different strains of bacteria *Aeromonas salmonicida* regarding their virulence to rainbow trout. The gene sequences determining the high virulence were identified in highly virulent strain and compared to sequences in wild type and avirulent strain.
2. Analysis of expression levels of *A. salmonicida* genes linked to invasion and infection adapting to levels of (i) iron, (ii) calcium, (iii) oxygen and (iv) elevated temperature using microarray.
3. The candidate genes selection by products encoding proteins capable to interact with the host. There, the selection was done by two criteria (i) being capable to cause infection and (ii) being recognized by the innate immune system.
4. First signatures of bacteremia in blood are visualized by extracellular proteins impact on erythrocytes.
5. Cellular response analysis during stimulation with dead and alive bacteria. Innate immune response was measured by oxidative burst levels assessment during head kidney leukocytes stimulation in vitro.
6. Monitoring serum capabilities of controlling bacteria growth.
7. An expression panel of TLR molecules was analyzed after *A. salmonicida* antigen stimulation in vivo.

4 Genome of Aeromonas salmonicida

4.1 Introduction

Genes and genomes are directly or indirectly responsible for the phenotypes of bacteria [111]. Understanding genomes' evolution in bacteria and the related changes in bacterial phenotypes helped understanding of both bacterial virulence and their resistance to antibiotics [111–113]. Thus, the genomes appeared reliable source of insights into bacteria virulence whose encoded components can be detected in bacterial genomes based on sequencing and systems biology analysis.

Only four *Aeromonas salmonicida* subspecies are documented in literature, yet among these four, multiple pathological isolates exhibit varying virulence against fish [114–117]. In this case, it is highly recommended to analyze collectively many strains simultaneously and compare all genes available, meaning to explore the "pan-genome" within this species. From the definition of Lapierre and Gogarten the pan-genomes is divided into a larger group of so-called "character genes" characterizing a group of organisms and so-called "accessory genes" typical for individuals or a few genomes [118]. There are many pan-genomic studies lead currently that aim to explain the evolutionary impact of horizontal gene transfer (HTG) [119–122], develop vaccines against pathogenic strains [123–125] and to characterize new strains in metagenomics samples [126].

Among research of many *Aeromonas* species, Tekedar et al. studied the pan-genome of *Aeromonas veronii* pathotypes (phenotypes related to pathogenicity) showing their genomic variations [127]. Similar research done by Awan et al. considered pan-genomic analysis of *Aeromonas hydrophila* [128]. Interspecies studies of Ghatak et al. applied comparative approach in pan-genomic analysis and based on these in silico analysis they suggested a pathogenic potential of three *Aeromonas* species: *A. hydrophila*, *A. veronii* and *A. caviae* [129] thus providing the benefit of pan-genomic approach. *The* latest research of Vasquez et al. aimed to compare typic and atypic strains of *Aeromonas salmonicida* and the first genome of *A. salmonicida* ssp. *salmonicida* A449 was sequenced and annotated in 2008 by Reith et al. In addition to genome, the report included also characterization of extrachromosomal DNA, encoded in five plasmids, out of which two large plasmids pAsa4 and pAsa5 [130] appeared virulent and three, pAsa1. pAsa2 and pAsa3 [131], the cryptic ones. It was the first fully annotated and completed genome of *A. salmonicida* ssp. *salmonicida*. Aside from this, the second genome of *A. salmonicida* strain 01-B526 was published in 2012 [132]. In parallel to chromosomal genomes' annotation, other studies focused on extrachromosomal DNA, with plasmid sequencing and annotation aimed to explain bacterial pathogenicity of *A. salmonicida* strain JF2267 [78,133]. In 2009 Najimi et al. found next plasmid, pAsa6 in the genome of *A. salmonicida* coding one of the effector

proteins of T3SS, AopH [134]. Its genetic content and architecture share many similarities to pAsa5. Recently, the study of Tanaka et al. revealed another plasmid – pAsa9 [116]. Its structure implied that its genomic rearrangements originate from insertion sequences (IS). During recent years, the interest in the *A. salmonicida* pathogenicity studies has been growing.

The difficulty in investigating *A. salmonicida* infection mechanisms comes from the fact that its virulence is unstable. Many *A. salmonicida* isolates demonstrate diverse levels of virulence after exposure to different environments [135]. This variability of virulence is connected to major virulent factors (among others: effector proteins such as AexT and T3SS) which are localized on large labile plasmids and may be lost or rearranged after passaging in fish or during the culture treatment [75,136]. This *A. salmonicida* ability to change was proven in *vitro* in recent studies in which just one passage through the fish was sufficient to extremely increase virulence [115,137]. The ability to change virulence so rapidly causes significant problem for researchers working on an efficient vaccine against *A. salmonicida and* furunculosis. Additionally, although the bacteria's natural habitat is waterborne, *Aeromonas* sp. can also be found in the microbiota of fish intestine [38,138]. It may indicate that host-pathogen interactions are crucial in shaping the diverse responses of both habitats' organisms. Finding the gene candidate that would be both stable and ubiquitous in *A. salmonicida* pathological strains is a key to an effective vaccine. Nonetheless, the research should not be limited only to known virulent proteins because other proteins may also participate, even when indirectly, in the infection. Next-generation sequencing (NGS) and comparative analysis of closely related bacterial genomes facilitate assembling the *A. salmonicida* pan-genome. NGS and annotating the sequence-to-virulence terms together improve understanding of microbial diversity, host-pathogen interactions, pathogenesis, and evolution of the microbes at previously unavailable pace and precision. Such a broadly correlated study can either address bacteria family, their genus or be focused on species.

Here, our study of pan-genome aims to contribute to the delineation of *A. salmonicida* evolution, identifying the gene loss or gene acquisition, the event of attenuation process or their pathological mechanisms, also to discover new virulence factors and novel virulence mechanisms.

The genome composition potentially describes (in)directly the pathogen lifestyle, growth conditions or even adaptation to environmental niches. Genome sequencing of strains that originate one from another allows identification of the variations between them, facilitated by overlapping sequences and a common ancestor – strains genomes compose one interrelated system. Previous studies on genomes of *A. salmonicida* focused on the most challenging parts of the genome like

plasmids and their contribution to virulence [115]. Instead, our study aims to get the larger picture of fish-pathogen interaction evolution.

Critical issue we address is the behavior of bacteria as a pathogen and their infection strategies with question: How and why do *A. salmonicida* changes its genomic content to convert from a symbiont to a virulent pathogen? What are the triggering factors of this change? Are there any common virulence factors contributing to the disease or, rather each strain possesses its unique ones? on the other hand, from the perspective of the host immune defenses, it should be clarified in which way the immune system of fish distinguishes between non-pathogenic commensals and virulent pathogens. Answering those questions would bring us closer to develop a satisfactory vaccine against furunculosis not only in rainbow trout but also in other fish species prone to the disease caused by *A. salmonicida*.

To address these issues, in our work, the genome content of three strains of *A. salmonicida* with documented virulence was analyzed and subsequently focused on the genes determining the virulence of each of these strains. Further analysis allowed to segregate genes depending on the levels of virulence and to identify those responsible for the infection initiation and progression.

4.2 Materials and methods

4.2.1 **Bacteria strains and culture conditions**

All three strains of bacteria *Aeromonas salmonicida* ssp. *salmonicida* was kindly provided by J. Frey, University Bern, Switzerland. *A. salmonicida* JF2267 (WT) was isolated from an arctic char (*Savelinus alpinus*) [139,140]. Strain JF5054 (HV) was reisolated from infected moribund trout after animal challenge [141].The isogenic mutant JF3239 (ATT) created from JF2267 strain was not virulent inducing no mortality at $1x10^5$ cfu / fish. In the long term the bacteria were stored in cryocultures in 15 % glycerol - LB broth at -70 °C. Before each experiment, the bacteria were inoculated on LB agar plates under sterile conditions and incubated for 48 h at 18 °C. A single colony was used to inoculate LB broth. Bacteria were cultured at 18 °C for another 24 h.

4.2.2 **Biochemical characterization of bacteria**

API 20 NE test (BioMérieux, France) was chosen for the biochemical identification of non-fastidious, non-enteric gram-negative rods (e.g. *Pseudomonas* sp., *Acinetobacter* sp., *Flavobacterium* sp., *Moraxella* sp., *Vibrio* sp., *Aeromonas* sp., etc.) because it gives fast and reliable results covering also a good amount of biochemical tests. It consists of twenty microtubes with dehydrated substrates that bacteria utilize while growing. It consists of eight conventional biochemical tests and twelve different

carbon source assimilation tests. In case of conventional tests after inoculation because of bacteria metabolism the color of substrate changes either spontaneously or by addition of reagents. Assimilation tests results are observed as an increase of opaque turbidity. The reactions are read according to the Reading Table (Table 1.) provided by the manufacturer and the identification is obtained by referring to the identification software on the BioMérieux website.

The test was prepared according to manufacturer's instruction. In brief, 24 h culture of *Aeromonas salmonicida* JF2267 strains: WT, ATT and HV were cultured on agar plates for 48 h at 18 °C. Distilled water was distributed equally on the bottom of the tray of API 20 NE test stripe to create humid chamber. Colonies of identical morphology were prepared as a sterile saline suspension with a turbidity equivalent of 0.5 McFarland. Names of test tubes ordered as on the stripe are presented in the table below (Table 1). Test tubes of marked NO_3 to PNPG were inoculated with bacteria suspension. The remaining cell suspension was suspended in API AUX Medium. Test tubes and cupules from GLU to PAC were filled with the new suspension. Mineral oil was added on top of GLU, ADH and URE test tubes to create anaerobic conditions. The API strips were incubated at 18 °C. After 24 h the NIT 1 and the NIT 2 reagents were added to the NO_3 cupule. The James reagent was added to TRP test. Assimilation tests were observed for opaque cupule 24 h and 48 h after inoculations indicating growth of bacteria. All results were collected in a table creating numerical profile and obtained numbers were used in the manufacturer's software that allowed identification of the species (BioMérieux, France). API test was prepared in duplicates for each strain.

Table 1. List of tests of API 20 NE stripe. Each test in the stripe represented one biochemical reaction which in total descibed biochemical pattern of characterised bacteria giving the user a code identifying the species.

TESTS	ACTIVE INGRIEDIENTS	QTY (mg/cup.)	REACTIONS/EMZYMES	RESULTS	
				NEGATIVE	POSITIVE
NO_3	Potassium nitrate	0.136	Reduction of nitrates to nitrites	NIT 1 + NIT 2 / 5 min	
				Colorless	Pink, red
			Reduction of nitrates to nitrogen	Zn / 5 min	
				Pink	Colorless
TRP	L-tryptophan	0.20	Indole production	James / immediate	
				Colorless	Pink
GLU	D-glucose	1.92	Fermentation	Blue to green	Yellow
ADH	L-arginine	1.90	Arginine dihydrolase	yellow	Orange / pink / red
URE	Urea	0.76	Urease	yellow	Orange / pink / red
ESC	Esculin ferric citrate	0.56 0.072	Hydrolysis (β-glucosidase) (esculin)	yellow	Grey / brown / black
GEL	Gelatin (bovine origin)	0.60	Hydrolysis (protease) (gelatine)	No pigment diffusion	Diffusion of black pigment
PNPG	4-nitrophenyl-β, D-galactopyranoside	0.22	B-galactosidase (para-nitrophenyl-β, D-galactopyranosidase	Colorless	yellow
GLU	D-glucose	1.56	Assimilation of glucose	transparent	opaque
ARA	L-arabinose	1.40	Assimilation of arabinose	transparent	opaque
MNE	D-mannose	1.40	Assimilation of mannose	transparent	opaque
MAN	D-mannitol	1.36	Assimilation of mannitol	transparent	opaque
NAG	N-acetyl-glucosamine	1.28	Assimilation of N-acetyl-glucosamine	transparent	opaque
MAL	D-maltose	1.40	Assimilation of maltose	transparent	opaque
GNT	Potassium gluconate	1.84	Assimilation of potassium gluconate	transparent	opaque
CAP	Capric acid	0.78	Assimilation of capric acid	transparent	opaque
ADI	Adipic acid	1.12	Assimilation of adipic acid	transparent	opaque
MLT	Malic acid	1.56	Assimilation of malate	transparent	opaque
CIT	Trisodium citrate	2.28	Assimilation of trisodium citrate	transparent	opaque
PAC	Phenylacetic acid	0.80	Assimilation of phenylacetic acid	transparent	opaque
OX	Oxidase test	-	Cytochrome oxidase	No change	Gas production

4.2.3 **Genome sequencing**

Preparation of bacterial DNA

Bacterial DNA was isolated using modified chloroform / isoamyl alcohol method with modifications [142]. In brief, bacteria from 24 h culture were collected from agar plates by rinsing with TE buffer. The suspension was washed once using TE buffer. Cells were lysed 30 min at 37 °C using lysozyme (10 mg / ml). Cell disruption was enhanced by adding 10 % of SDS and incubation in water bath in 50 °C for 10 min. Bacterial proteins solution was precipitated with 5 M sodium perchlorate reaching final concentration of 1 M sodium perchlorate. Sodium perchlorate breaks up protein - DNA interactions. Each sample was shaken in hand for 2 min with 24:1 chloroform / isoamyl alcohol in room temperature and centrifuged 15 000 rpm for 10 min in room temperature to separate protein from DNA. At the end of this step, three phases were observed. The organic phase, which was at the bottom and contained chloroform, the middle phase at the boarder of organic and water phase contained insoluble proteins, and the water phase contained DNA. The aqueous phase was transferred to a new tube, washed by gentle inverting the tube with ice-cold 96 % ethanol to precipitate DNA and centrifuged 15 000 rpm for 10 min. The supernatant was removed and DNA pellet dried in the air. Bacterial DNA was suspended in TE buffer and incubated 5 min in 65 °C to dissolve precipitated DNA. Yield and purity of isolated DNA were tested on the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). The DNA for each bacteria sample was stored at -20 °C until the next-generation sequencing.

Next-generation sequencing (NGS)

Nucleotide sequence data was generated by pyrosequencing with a 454 GS FLX Genome Sequencer (Roche Diagnostics, Germany) in the Laboratory for NGS and Microarray Diagnostics in Friedrich Loeffler Institute, Germany as described previously [143,144]. Briefly, *A. salmonicida* DNA, fragmented enzymatically, were separated by agarose gel electrophoresis, and purified using the ZymocleanTM Gel DNA Recovery Kit (Zymo Research Corporation, USA) prior to library preparation. dsDNA libraries were prepared according to published protocols according to Rapid Library Preparation protocols, Roche [145]. In brief, 1 µg of bacterial DNA was diluted in 100 µl TE buffer and transferred to Nebulizer cup. DNA fragmentation was followed by binding to library capture beads and recovery of the single-stranded template DNA library. These libraries were used for sequencing in a picotiter plate according to the manufacturer's instructions. Obtained reads were trimmed, assembled and created contigs served for further analysis in Newbler Metrix software, Roche.

Analysis. Comparison of genomic sequences. Mapping and de novo assembly

In course of sequencing three sets of reads from three strains had to be processed and analyzed according to the needs as follows. It was aimed to compare the general content of the *A. salmonicida* JF2267 genome in relation to A449 strain (Figure 5). The goal was to identify differences in gene content between the different virulent strains WT, ATT, HV and connect them to pathogenicity. The published genome of *A. salmonicida* A449 served as an annotation help providing the gene names in homology to the sequence [130,131]. Therefore, reads from sequencing were mapped onto *A. salmonicida* A449 strain genomic and plasmid sequences in the Geneious 10.0.6 software, Dotmatics [146]. Created set of data helped to identify present or absent genes as well as genes displaying lower homology (Figure 5 – left side of analysis tree). The genes found with any identity were grouped in the table (supplementary material). The reads that were left unmapped were excluded from further analysis.

De novo assembly of JF2267 reads (Figure 5 - right side of the analysis tree) aimed to find new genes belonging to *Aeromonas salmonicida* pan-genome. Out of contigs assembled from *A. salmonicida* JF2267 reads the ORFs were predicted and then searched against database using the Geneious 10.0.6 software [146]. In brief, the annotations of genes from A449 strain were transferred on the contig sequences of each library with the 25 % similarity threshold. Following, the ORFs were found using following custom settings in Geneious software. Using bacterial transcription code ATG, GTG and TTG start codons were searched including interior ORFs and assuming that start and stop codons are outside of the sequence. Out of these only those ORFs that did not overlap with transferred annotation were selected and NCBI Refseq_protein database was searched using BLAST tool [147]. Unassembled reads were grouped in a table and excluded from further analysis (supplementary material).

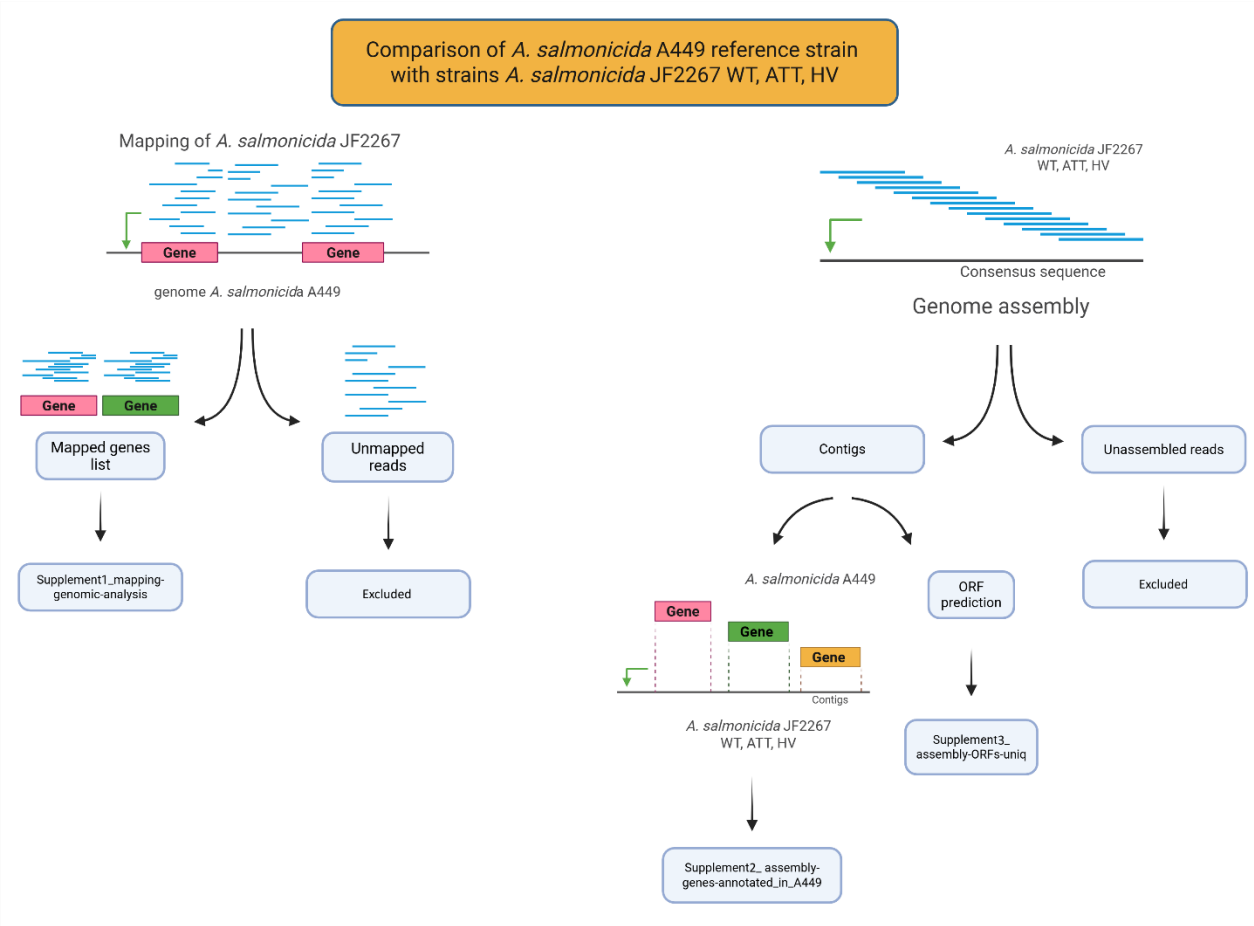

Figure 5. Analysis plan of sequencing results showing the different reads processing approaches.
The left side of the tree ("Mapping") shows the analysis process in search of gene homology and identity with the help of *A. salmonicida* A449 strain. Reads from JF2267 sequencing were mapped on the A449 genome to compare similarity of JF2267 sequence to A449 published genes.
The right side of analysis ("Genome assembly") represent the pipeline for finding unknown genes of JF2267, not found in A449 strain. Reads were assembled into contigs using Geneious Software and genes from *A. salmonicida* A449 were annotated on the sequence. The predicted ORFs were the most interesting that did not match with the annotated genes as potential newly found genes. Detailed description in the text. Complete results of such analysis are gathered in supplementary material.

4.3 Results

4.3.1 **Biochemical characterization**

The metabolic activity of the bacteria was tested on API stripe assay consisting of a set of biochemical tests. According to the BioMérieux identification system *Aeromonas salmonicida* ssp. *salmonicida* was identified for all three strains with 99.99 % accuracy (Table 2). The test readouts were collected after 24 h and 48 h since the output information at other time points in several specific tubes was unsure. The tests for WT and ATT were identical whereas in case of HV strain two different results for NAG and GNT tests were observed (marked in red in the table). NAG test represented assimilation of N-Acetyl-Glucosamine of which HV strain showed slowed down assimilation. Similar case was with fermentation of glucose by ATT strain which appeared only after 48 h of incubation. Interestingly, HV strain was able to assimilate potassium gluconate in contrast to WT and ATT that could not do it. This gives HV strain the advantage in growth possibilities. The rest of test the results were compatible with the identification table provided by the manufacturer (last row in Table 2).

Table 2. Result of API20 NE biochemical tests for *A. salmonicida* JF2267 WT, ATT, HV strains marked as negative (-) or positive (+) outcome. Except tests: NO3 and TRP: first row in each of the strain result row represents readout after 24 h, second row in each of the strain result row represents readout after 48 h. Results in red font represent aberrations.

API 20 NE	WT		ATT		HV		BioMérieux reference readout for *A. salmonicida*
	24h	48h	24h	48h	24h	48h	
NO3	+		+		+		+
TRP	-		-		-		-
Glu	+	+	-	+	+	+	50 %
ADH	-	-	-	-	-	-	35 %
URE	-	-	-	-	-	-	-
ESC	+	+	+	+	+	+	+
GEL	+	+	+	+	+	+	+
PNPG	-	-	-	-	-	-	-
Glu	+	+	+	+	+	+	+
Ara	-	-	-	-	-	-	-
Mne	-	-	-	-	-	-	-
Man	+	+	+	+	+	+	+
Nag	+	+	+	+	-	+	+
Mal	+	+	+	+	+	+	+
GNT	-	-	-	-	-	+	+
CAP	-	-	-	-	-	-	-
API	-	-	-	-	-	-	-
MLT	+	+	+/-	+	+/-	+	+
CIT	-	-	-	-	-	-	-
PAC	-	-	-	-	-	-	-

4.3.2 JF2267 strains 454 sequencing

The different biological activity of each strain raised a question which genomic composition was responsible for the observed different activity in *vivo* and in *vitro*. Therefore, the genomes of all three strains were analyzed by NGS (Table 3). Whole analysis was performed on raw data firstly in the form of reads and then after assembly – contigs. The genome sizes of three strains were following: *A. salmonicida* JF2267 WT strain - 4.79 Mb, *A. salmonicida* JF2267 ΔascV - ATT strain – 4.83 Mb, *A. salmonicida* JF5054 - HV strain - 4.87 Mb. Among these three strains WT was the smallest and HV strain the largest based on genome size.

Reads assembly builds up three sets of contigs. Whereas number of contigs for WT and ATT were similar (284 and 263 respectively), the number of contigs for HV strain was outstanding reaching 1,241 contigs (Table 3). Although the number of contigs was quite high in general, they were all more fragmented as the maximal contig length reached 190,894 bp when in case of WT and ATT it was 302,284 bp and 312,139 bp, respectively. After the assembly we also got a set of unassembled reads which were later processed and compared to BLAST database.

Table 3. General information about results from reads assembly in Geneious Software. Genome sizes of sequenced strains were similar to each other. The HV strain was the most shattered into small contigs. Reads assembled into contigs were analysed for the gene content. *A. salmonicida* A449 published genes were found aside with undescribed ORFs.

Assembly	Genome size (bp)	Contigs	Max contig length	Min contig length	Transferred genes	ORFs	Reads used in the assembly	Reads not used in the assembly
WT	4,794,714	284	302,284 bp	36 bp	11,412	239,692	416,247	1,001
ATT	4,828,460	263	312,139 bp	33 bp	11,297	240,887	327,004	775
HV	4,867,058	1241	190,894 bp	42 bp	12,078	265,214	255,525	3,296

4.3.3 Sequence comparison

Next step of analysis covered gene comparison based on different processing of reads. The aim was not only to have a deeper look into the genomic content of each strain but also to define those genes that differ in all three strains which may decide about the virulence of each strain.

Mapping. Virulence genes in focus

Mapping reads of *A. salmonicida* JF2267 strain in Geneious software on published A449 strain aimed to annotate gene content in JF2267 by their name and function (Figure 5). For this analysis, we set the threshold of 50 % consensus above which genes were considered as present ones in JF2267 strain for all analysis. Strain JF2267 appeared to be quite homogenous as for chromosomal sequence (Supplement1_mapping-genomic-analysis /WT, /ATT, /HV spreadsheets). Out of 4690 genomic genes 3581 genes had exactly the same sequence in all three strains. In contrast to *A. salmonicida* A449 and its 4,320 chromosomal genes, all three JF2267 strains missed five chromosomally located genes (Table 4). Interestingly, more differences were observed in the composition of plasmids 4 (175 genes in A449 strain) and 5 (168 genes in A449). Genes from plasmid five in HV strain were best preserved in the same number whereas in case of wild type and attenuated strains they missed 67 and 39 genes, respectively. Sequencing proved that all JF2267 strains

contained equally 148 genes from plasmid 4 whereas strain A449 was reported to have 175 genes within the analogous plasmid. Finally, small cryptic plasmids in all JF2267 strains had the same number of genes as in A449 strain. Detailed information can be found in supplementary material (Supplement1_mapping-genomic-analysis /diff-identities).

Table 4. Distribution of genes in *A. salmonicida* JF2267 in contrast to A449 strain. The differences between gene numbers was marked in red. In the last row the number of hypothetical ORFs are mentioned. Main differences were found in case of two plasmids pAsa4 and pAsa5 where some of genes could not have been identified.

A. salmonicida genome	JF2267			A449
	Wild type	Attenuated	Highly virulent	
Chromosome	4,315	4,315	4,315	4,320
pAsa1	10	10	10	10
pAsa2	8	8	8	8
pAsa3	7	7	7	9
pAsa4	148	148	148	175
pAsa5	101	129	168	168
Sum	4,589	4,617	4,656	4,690
Hypothetical ORFs*	**235,702**	**236,997**	**261,874**	

Missing chromosomal genes encoded IS3 and IS630 family transposases, ribosomal RNA, tRNAs, phage proteins and hypothetical proteins (Supplement1_mapping-genomic-analysis /missing genes). Genes that were found in JF2267 strains based on the *A. salmonicida* A449 annotation database were used for further analysis. They were collected in the form of a table showing the differences in the presence of specific genes among *A. salmonicida* JF2267 strains (Supplement1_mapping-genomic-analysis /missing genes). In this way it was possible to count the number of unique genes in each strain, genes common only for two strains and genes present in all three strains (Figure 6). All the bacteria strains had 4,584 genes in common originating either from chromosome or plasmids. Moreover, there were 34 genes of *A. salmonicida* A449 that could not have been assigned to any genomes of JF2267 strain. HV strain had 34 exclusive genes whereas WT and ATT strain had neither unique genes nor common for each other. What is more, WT and ATT strains shared both 5 and 33 gene sequences common with HV, respectively.

Table 5. Gene identity comparison between *A. salmonicida* JF2267 strains based on annotations from A449 published genome. Most genes of highly virulent strains had the highest identity to the A449 strain in comparison to other WT and ATT strains.

A. salmonicida	Gene No. In JF2267		
Identity	**Wild type**	**Attenuated**	**Highly virulent**
100 %	3,950	3,895	4,085
50 % -100 %	639	722	571
<50 %	42	23	9
0 %	59	50	25

Among all mentioned 4,690 genes annotated in three JF2267 strains 3,950 genes in WT, 3,895 genes in ATT strain and 4,085 genes in HV strain were 100 % identical (Table 5). ATT strain had the most while HV strain the fewest genes of identity lower than 100 %. In JF2267 WT, ATT, HV, respectively, 42, 23, 9 genes annotated in A449 strain were below 50 % of consensus sequence. Additionally, 59, 50 and 29 *A. salmonicida* A449 genes were not found at all in JF2267 sequences in WT, ATT, and HV, respectively. Altogether, attenuated strain had the highest number of 745 genes with identity lower than 100 %. Nevertheless, for all three strains the differences oscillated between 90.00 % - 99.99 % consensus.

In order to present *A. salmonicida* JF2267 genomes in more detailed way some genes were selected that were reported to participate in *Aeromonas* sp. or in other species pathogenesis (Table 6 and Table 7). The criteria to consider a specific gene were as follows: the gene was supposed to be putatively virulent or contributing to virulence of *Aeromonas salmonicida*. Chromosomal genes were well preserved, had high coverage and identity to the published strain whereas more discrepancies appeared in plasmid genes. Not only plasmid genes could be found only partially but also identity was lower than expected. Complete sets of genes can be found in Supplementary data.

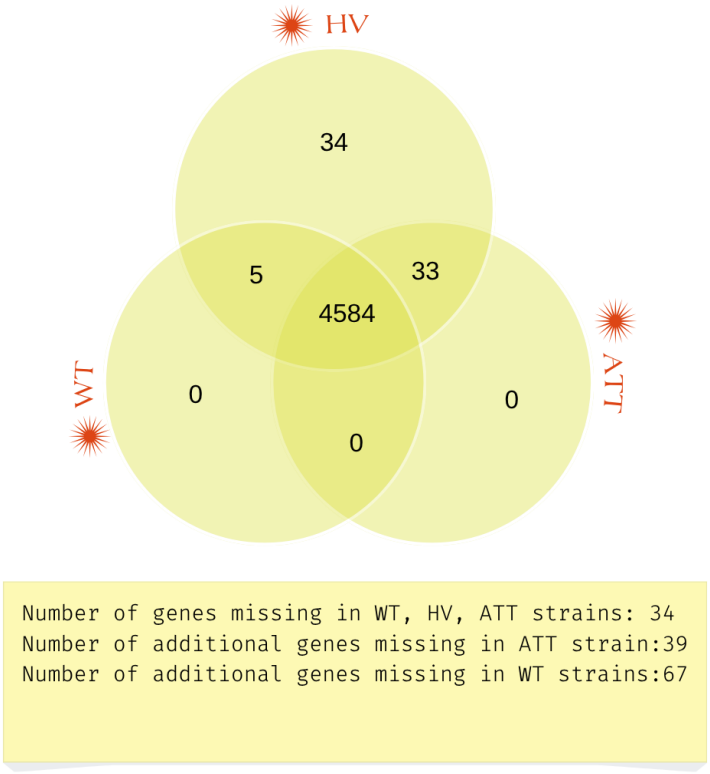

Figure 6. Correlation of genes within *A. salmonicida* JF2267 strains. Venn diagram represents the genes uniquely present in each of the strain and also the genes that the strain had in common. Number of genes originating from A449 strain missing in JF2267 strain was placed below the diagram.

Table 6. Sample comparison of chosen *plasmid* genes from *A. salmonicida* strain A449 showing gene differences between the reference genes and sequenced strains. Identity of genes is given in percentages. Genes were selected by protein product which is interesting from the pathogenicity point of view.

Locus tag in A449	Transferred From	Identity			Gene product
		WT	ATT	HV	
ASA_RS22570	NC_009349	99.21 %	99.21 %	99.21 %	type VI secretion protein
ASA_RS22445	NC_009349	100.00 %	100.00 %	100.00 %	ferredoxin
ASA_RS22925	NC_009350	0.00 %	0.00 %	100.00 %	type III secretion protein, YscG family
ASA_RS22930	NC_009350	0.00 %	0.00 %	100.00 %	EscF/YscF/HrpA family type III secretion system needle major subunit
ASA_RS22935	NC_009350	0.00 %	0.00 %	100.00 %	EscE/YscE/SsaE family type III secretion system needle protein co-chaperone
ASA_RS23015	NC_009350	0.00 %	0.00 %	100.00 %	type III secretion protein
ASA_RS23025	NC_009350	0.00 %	0.00 %	100.00 %	type III secretion chaperone SycN
ASA_RS23030	NC_009350	0.00 %	0.00 %	100.00 %	SepL/TyeA/HrpJ family type III secretion system gatekeeper
ASA_RS23055	NC_009350	0.00 %	0.00 %	100.00 %	type III secretion system protein
ASA_RS23000	NC_009350	0.00 %	13.68 %	99.65 %	type III secretion protein
ASA_RS23035	NC_009350	0.00 %	38.79 %	99.89 %	type III secretion protein
ASA_RS22970	NC_009350	0.00 %	94.87 %	100.00 %	ExsE protein
ASA_RS22775	NC_009350	0.00 %	100.00 %	100.00 %	conjugal transfer protein TraL
ASA_RS22780	NC_009350	0.00 %	100.00 %	100.00 %	conjugal transfer protein TraE
ASA_RS23665	NC_009350	0.00 %	100.00 %	100.00 %	conjugal transfer protein TrbI
ASA_RS22910	NC_009350	10.56 %	0.00 %	100.00 %	EscJ/YscJ/HrcJ family type III secretion inner membrane ring protein
ASA_RS23075	NC_009350	10.58 %	26.13 %	100.00 %	EscU/YscU/HrcU family type III secretion system export apparatus switch protein
ASA_RS22940	NC_009350	13.93 %	28.66 %	100.00 %	EscD/YscD/HrpQ family type III secretion system inner membrane ring protein
ASA_RS22770	NC_009350	15.71 %	100.00 %	100.00 %	TraA fimbrial protein precursor
ASA_RS22880	NC_009350	22.18 %	0.00 %	99.93 %	endonuclease
ASA_RS22960	NC_009350	30.07 %	54.53 %	100.00 %	AraC family transcriptional regulator
ASA_RS22945	NC_009350	32.55 %	0.00 %	100.00 %	EscC/YscC/HrcC family type III secretion system outer membrane ring protein
ASA_RS22955	NC_009350	32.72 %	0.00 %	100.00 %	ExsD protein
ASA_RS23010	NC_009350	33.88 %	26.72 %	100.00 %	EscV/YscV/HrcV family type III secretion system export apparatus protein
ASA_RS22800	NC_009350	36.51 %	100.00 %	100.00 %	type IV secretion system protein TraC
ASA_RS23045	NC_009350	58.66 %	0.00 %	100.00 %	type III secretion protein
ASA_RS22915	NC_009350	62.65 %	0.00 %	100.00 %	EscI/YscI/HrpB family type III secretion system inner rod protein

Table 7. Comparison of chosen *chromosomal* genes from *A. salmonicida* strain A449 showing differences between the reference genes and the sequenced strains. Identity of genes is given in percentages. Genes were selected by their protein product which is interesting from the pathogenic point of view.

Locus tag in A449	Transferred From	Identity			Gene product
		WT	ATT	HV	
ASA_RS00385	NC_009348	100.00 %	100.00 %	100.00 %	ferritin
ASA_RS01885	NC_009348	100.00 %	100.00 %	100.00 %	flagellar MS-ring protein
ASA_RS02450	NC_009348	100.00 %	100.00 %	100.00 %	bacterioferritin
ASA_RS03160	NC_009348	100.00 %	100.00 %	100.00 %	hemolysin D
ASA_RS04475	NC_009348	100.00 %	100.00 %	100.00 %	chitinase
ASA_RS21110	NC_009348	100.00 %	99.93 %	100.00 %	ADP-ribosyltransferase
ASA_RS04510	NC_009348	99.96 %	100.00 %	100.00 %	TonB-dependent receptor
ASA_RS06015	NC_009348	100.00 %	100.00 %	100.00 %	hemerythrin
ASA_RS06315	NC_009348	99.88 %	99.76 %	99.88 %	Zn-dependent protease
ASA_RS06705	NC_009348	99.74 %	99.71 %	99.71 %	acriflavine resistance protein B
ASA_RS06940	NC_009348	100.00 %	100.00 %	100.00 %	heme exporter protein CcmB
ASA_RS06950	NC_009348	100.00 %	100.00 %	100.00 %	heme exporter protein D
ASA_RS07260	NC_009348	99.95 %	100.00 %	100.00 %	secretin
ASA_RS07505	NC_009348	99.76 %	100.00 %	100.00 %	flagellar basal body rod protein FlgC
ASA_RS08165	NC_009348	100.00 %	100.00 %	100.00 %	iron transporter FeoA
ASA_RS08170	NC_009348	99.96 %	99.96 %	99.96 %	ferrous iron transporter B
ASA_RS09165	NC_009348	100.00 %	100.00 %	100.00 %	ferrochelatase
ASA_RS09270	NC_009348	99.44 %	99.55 %	99.91 %	permease
ASA_RS10155	NC_009348	99.89 %	100.00 %	100.00 %	lipase
ASA_RS11075	NC_009348	100.00 %	100.00 %	99.78 %	permease
ASA_RS11620	NC_009348	100.00 %	100.00 %	100.00 %	heme lyase subunit CcmF
ASA_RS12620	NC_009348	100.00 %	100.00 %	100.00 %	aerotaxis receptor Aer
ASA_RS13470	NC_009348	100.00 %	100.00 %	100.00 %	ecotin
ASA_RS14240	NC_009348	100.00 %	99.95 %	100.00%	hemolysin
ASA_RS14540	NC_009348	100.00 %	100.00 %	100.00 %	pilin
ASA_RS14790	NC_009348	99.77 %	99.77 %	99.77 %	azurin
ASA_RS14960	NC_009348	100.00 %	100.00 %	100.00 %	hemolysin secretion protein D
ASA_RS15685	NC_009348	100.00 %	100.00 %	100.00 %	zinc metalloprotease
ASA_RS16560	NC_009348	100.00 %	100.00 %	100.00 %	heme utilization protein HutZ
ASA_RS16570	NC_009348	100.00 %	100.00 %	100.00 %	hemin receptor
ASA_RS17580	NC_009348	99.81 %	99.90 %	99.73 %	endonuclease
ASA_RS18870	NC_009348	100.00 %	100.00 %	100.00 %	peroxiredoxin
ASA_RS19370	NC_009348	99.93 %	99.93 %	99.93 %	aerolysin
ASA_RS19475	NC_009348	99.93 %	99.93 %	99.93 %	protease TldD

Finally, based on the collation above, the genes important for the virulence were selected and presented in Table 8. The genes 'important for virulence' were defined as those present in HV and / or WT strains but absent in ATT strain. This assumption is because bacteria need active genes in their genome to be used to stay pathogenic. The results were grouped into sets of genes. Genes in group #1 were defined as responsible for increased virulence of HV strain and #2 as genes responsible for moderate virulence that WT strain also exhibits. Group #3 included examples of genes contributing to virulence that can be found also in strain ATT. Group #4 included genes of function other than virulence predicted according to division assumptions. Interestingly, plasmid encoded genes were present in both groups. Furthermore, genes present in HV strain were mainly 100 % identical with A449 genes whereas there were much more differences in genes from WT compared to A449 strain. The identity was lower but also the coverage was decreased indicating partially missing genes. However, the reasons whether it was technical, or biological issue cannot be explained now and need more detailed investigation.

Table 8. Genes unique for HV or HV and WT strain were mentioned as those that may be important for virulence as they are only present in HV or HV and WT strain. Percent of identity was calculated based on annotation from *A. salmonicida* A449. Genes were grouped based on the identity in three strains in a following way: 1. Presumably responsible for high virulence: not detected in WT and ATT but found in HV, 2. Presumably responsible for moderate virulence: missing in ATT and low identity in WT and high in HV strain. 3. Contributing to virulence: high identity in HV and moderate in WT and ATT. 4. Function other than virulence: missing only in WT, low identity in ATT but high in HV.

Group	Locus tag in A449	Transferred From	Identity with A449			Gene product
			WT	ATT	HV	
1	ASA_RS22885	NC_009350	0.00 %	0.00 %	100.00 %	ati2 chaperone
	ASA_RS22890	NC_009350	0.00 %	0.00 %	100.00 %	transposase
	ASA_RS22905	NC_009350	0.00 %	0.00 %	100.00 %	type III secretion protein
	ASA_RS22920	NC_009350	0.00 %	0.00 %	100.00 %	RNA-binding protein
	ASA_RS22925	NC_009350	0.00 %	0.00 %	100.00 %	type III secretion protein, YscG family
	ASA_RS22930	NC_009350	0.00 %	0.00 %	100.00 %	EscF/YscF/HrpA family type III secretion system needle major subunit
	ASA_RS22935	NC_009350	0.00 %	0.00 %	100.00 %	EscE/YscE/SsaE family type III secretion system needle protein co-chaperone
	ASA_RS22975	NC_009350	0.00 %	0.00 %	100.00 %	glycosyl transferase
	ASA_RS23015	NC_009350	0.00 %	0.00 %	100.00 %	type III secretion protein
	ASA_RS23020	NC_009350	0.00 %	0.00 %	100.00 %	type III secretion protein
	ASA_RS23025	NC_009350	0.00 %	0.00 %	100.00 %	type III secretion chaperone SycN
	ASA_RS23030	NC_009350	0.00 %	0.00 %	100.00 %	SepL/TyeA/HrpJ family type III secretion system gatekeeper
	ASA_RS23055	NC_009350	0.00 %	0.00 %	100.00 %	type III secretion system protein
	ASA_RS23085	NC_009350	0.00 %	0.00%	100.00 %	hypothetical protein
2	ASA_RS23065	NC_009350	53.93 %	0.00 %	100.00 %	EscS/YscS/HrcS family type III secretion system export apparatus protein
	ASA_RS23045	NC_009350	58.66 %	0.00 %	100.00 %	type III secretion protein
	ASA_RS22915	NC_009350	62.65 %	0.00 %	100.00 %	EscI/YscI/HrpB family type III secretion system inner rod protein
	ASA_RS23100	NC_009350	64.28 %	0.00 %	100.00 %	hypothetical protein
	ASA_RS22910	NC_009350	10.56 %	0.00 %	100.00 %	EscJ/YscJ/HrcJ family type III secretion inner membrane ring protein
	ASA_RS22880	NC_009350	22.18 %	0.00 %	99.93 %	endonuclease
	ASA_RS23060	NC_009350	27.06 %	0.00 %	100.00 %	ssaR
	ASA_RS22945	NC_009350	32.55 %	0.00 %	100.00 %	EscC/YscC/HrcC family type III secretion system outer membrane ring protein

	ASA_RS22955	NC_009350	32.72 %	0.00 %	100.00 %	ExsD protein
	ASA_RS22900	NC_009350	37.99 %	0.00 %	100.00 %	type III secretion system protein
	ASA_RS22950	NC_009350	3.05 %	0.00 %	100.00 %	type III secretion system chaperone, YscB family
3	ASA_RS23075	NC_009350	10.58 %	26.13 %	100.00 %	EscU/YscU/HrcU family type III secretion system export apparatus switch protein
	ASA_RS22940	NC_009350	13.93 %	28.66 %	100.00 %	EscD/YscD/HrpQ family type III secretion system inner membrane ring protein
	ASA_RS23040	NC_009350	29.46 %	27.77 %	100.00 %	fliI
	ASA_RS23010	NC_009350	33.88 %	26.72 %	100.00 %	EscV/YscV/HrcV family type III secretion system export apparatus protein
	ASA_RS23050	NC_009350	40.92 %	27.52 %	100.00 %	AscP protein
	ASA_RS23095	NC_009350	49.40 %	4.30 %	100.00 %	transposase
	ASA_RS23090	NC_009350	65.92 %	49.44 %	100.00 %	transposase
4	ASA_RS23000	NC_009350	0.00 %	13.68 %	99.65 %	type III secretion protein
	ASA_RS22980	NC_009350	0.00 %	22.52 %	100.00 %	hypothetical protein
	ASA_RS23080	NC_009350	0.00 %	25.44 %	100.00 %	hypothetical protein
	ASA_RS22995	NC_009350	0.00 %	30.66 %	100.00 %	hypothetical protein
	ASA_RS22990	NC_009350	0.00 %	31.35 %	100.00 %	CesD/SycD/LcrH family type III secretion system chaperone
	ASA_RS23035	NC_009350	0.00 %	38.79 %	99.89 %	type III secretion protein
	ASA_RS23070	NC_009350	0.00 %	45.63 %	100.00 %	EscT/YscT/HrcT family type III secretion system export apparatus protein

In search of new genes

New genes were searched with the help of published *A. salmonicida* ssp. *salmonicida* strain A449. As the settings of annotation transfer were customized the consensus sequence had much more annotated genes than expected (Table 9). Some genes were overlapping in the same loci, but they had different identity. It gave a false impression of vast number of hits 11,412 in WT, 11,297 in ATT and 12,078 genes in HV strain. All genes are presented in the table in supplementary material (Supplement2_assembly-genes-annotated_in_A449, Supplement3_assembly-ORFs-uniq).

Table 9. Outcome summary of gene annotation transfer. Reads and new ORFs searched by Geneious Software.

Assembly	Transferred genes	ORFs	Used reads	Unused reads
WT	11,412	239,692	416,247	1,001
ATT	11,297	240,887	327,004	775
HV	12,078	265,214	255,525	3,296

In this way, for example, a gene in contig 14 was identified as a A449 gene of locus tag ASA_RS17685 transferred with 100 % identity to consensus sequence (Table 10). However, this identity was lower in case of other genes. Moreover, many annotations from A449 database could be assigned to the same sequence with different identity. For example, in contig 16 there is a gene sequence annotated from A449, and two different gene loci tags were assigned to this sequence ASA_RS00115 and ASA_RS00035. Nevertheless, having assigned two different loci to one gene sequence meant in most cases that the gene appeared in many repeats in the genome and locus tag defined the positions of the annotation on A449 genomic sequence.

Table 10. Example of genes annotated on assembled sequences. Not only one annotation was found for single sequence but more with varying identity. It was mainly related to the repeating sequence of annotated genes in A449 sequence.

Sequence Name	Type	locus_tag	Direction	db_xref	Transferred From	Transferred Similarity
Contig 18	gene	ASA_RS02400	reverse	GeneID:4996574	NC_009348	39.71 %
Contig 4	gene	ASA_RS12400	forward	GeneID:4995354	NC_009348	43.42 %
Contig 80	gene	ASA_RS23130	forward	GeneID:23570285	NC_009350	71.43 %
Contig 107	gene	ASA_RS00085	reverse	GeneID:1496852	NC_004925	85.84 %
Contig 56	gene	ASA_RS21995	forward	GeneID:4999049	NC_009349	96.20 %
Contig 8	gene	ASA_RS22610	reverse	GeneID:4999083	NC_009349	96.30 %
Contig 1	gene	ASA_RS15600	reverse	GeneID:4996672	NC_009348	99.92 %
Contig 1	gene	ASA_RS16220	forward	GeneID:4998176	NC_009348	99.92 %
Contig 14	gene	ASA_RS17685	forward	GeneID:4995383	NC_009348	100.00 %
Contig 14	gene	ASA_RS17695	forward	GeneID:4995385	NC_009348	100.00 %
Contig 14	gene	ASA_RS17770	forward	GeneID:4995318	NC_009348	100.00 %
Contig 16	gene	ASA_RS00115. ASA_RS00035	reverse	GeneID:1496846. GeneID:1496839	NC_004924. NC_004923	95.44 % -> 75.06 %
Contig 7	gene	ASA_RS03855. ASA_RS03865. ASA_RS03875. ASA_RS03885. ASA_RS20740. ASA_RS21355. ASA_RS04450. ASA_RS04455. ASA_RS04460. ASA_RS04465. ASA_RS00265. ASA_RS04125. ASA_RS16120	reverse	GeneID:4995075. GeneID:4995629. GeneID:4996726. GeneID:4998385. GeneID:4998060. GeneID:4998566. GeneID:4995664. GeneID:4996916. GeneID:4996920. GeneID:4997323. GeneID:4994662. GeneID:4998576. GeneID:4995058	NC_009348	96.20 % -> 62.66 %
Contig 56	gene	ASA_RS21950. ASA_RS15275	reverse	GeneID:4999036. GeneID:4994952	NC_009349. NC_009348	97.26 % -> 80.59 %
Contig 7	gene	ASA_RS03860. ASA_RS03850. ASA_RS03870. ASA_RS03880. ASA_RS02335. ASA_RS04345. ASA_RS05860. ASA_RS05870. ASA_RS05890. ASA_RS00270. ASA_RS02320. ASA_RS02330. ASA_RS04350. ASA_RS12270. ASA_RS10925. ASA_RS12265	reverse	GeneID:4994655. GeneID:4996568. GeneID:4997627. GeneID:4998444. GeneID:4996052. GeneID:4996570. GeneID:4994867. GeneID:4997629. GeneID:4998603. GeneID:4994969. GeneID:4996058. GeneID:4996375. GeneID:4997962. GeneID:4998787. GeneID:4998088. GeneID:4995425	NC_009348	98.70 % -> 60.06 %
Contig 6	gene	ASA_RS11870. ASA_RS15305	reverse	GeneID:4994833. GeneID:4994956	NC_009348	100.00 % -> 98.59 %

Contig 17	gene	ASA_RS15305. ASA_RS11870	reverse	GeneID:4994956. GeneID:4994833	NC_009348	100.00 % -> 98.59 %
Contig 3	gene	ASA_RS01480. ASA_RS15165	reverse	GeneID:4995502. GeneID:4995725	NC_009348	100.00 % -> 98.67 %
Contig 17	gene	ASA_RS15165. ASA_RS01480	forward	GeneID:4995725. GeneID:4995502	NC_009348	100.00 % -> 98.67 %
Contig 29	gene	ASA_RS05865. ASA_RS05875. ASA_RS05885. ASA_RS05880. ASA_RS05945	forward	GeneID:4994866. GeneID:4995736. GeneID:4998586. GeneID:4994891. GeneID:4998349	NC_009348	100.00 % -> 98.67 %

The ORFs in all three libraries were identified using custom settings in Geneious software. All putative ORFs were identified. The settings enabled to predict ORFs within already annotated sequence including the ORFS located in the beginning or end of the contig sequence. Geneious software identified 239,692 for WT, 240 887 for ATT and 265,214 for HV strain ORFs overlapping with each other and annotated A449 genes. Selected ORFs that completely did not overlap with annotated genes were further searched in database to prove its existence. By depletion of ORF number we obtained 10,048 and 10,301 of ORFS from WT and ATT strains and 15,471 of ORF from HV strain. The results due to its size are presented in supplementary material (Supplement3_assembly-ORFs-uniq).

4.4 Discussion

Here, for the first time, we comparatively analyze three genomes of *A. salmonicida* ssp. *salmonicida* strains JF2267 with different virulence levels documented by Vanden Bergh [114,137]. The important fact that is fundamental for this research is that all strains belong to *A. salmonicida* genus but at the same time provide with different virulence levels. Why? The wild type strain isolated from Arctic char in nature exhibits moderate virulence, whereas highly virulent strain is an effect of animal challenge studies. Attenuation effect in *vitro* of *Aeromonas salmonicida* was also mentioned by Ishiguro [148]. Here, in *vitro* attenuation through genetic manipulations was a reason for T3SS effect studies of Burr and Vanden Bergh.

Till now the evolutionary concept may suggest the existence of quasi-species which are better described in viruses [149,150] yet there are reports about bacteria quasi-species for example in *Helicobacter pylori* [151]. Quasi-species introduced by Eigen are defined as a population of viruses with a huge genome variation rate related to mutations within [152]. In case of bacteria, the repetitive extragenic palindromic doublets forming hairpins (REPINs) can be used as the quasi-species marker related to genetic element called transposases, the self-replicating and mutating DNA fragments [153]. Its identification demands sophisticated methods. Techniques used in this research were insufficient to assess the virulence levels of the bacteria. Furthermore, after the elimination of the weak and attenuated subpopulation, they can recolonize and dominate the environment with higher and better visible virulence capabilities. The other explanation considers the horizontal gene transfers among mobile elements like plasmid or the influence of transposons [133,154]. Both theories cause *Aeromonas salmonicida* virulence origin needs detailed analysis.

In this research, the bacteria were firstly characterized and identified positively that all three strains WT, ATT and HV belong to *Aeromonas salmonicida* ssp. *salmonicida*. API20 NE test proved that the bacteria belong to *A. salmonicida* ssp. *salmonicida*. Their metabolism activities are modulated slightly which could be seen on the API20NE biochemical test. Attenuated strain showed no significant differences in comparison with the wild type bacteria. Interestingly, only HV strain was able to assimilate and metabolize gluconate, a precursor for the pentose phosphate pathway which may be directly related to the virulence as it is in case of *Pectobacterium carotovorum*, a gram-negative phytopathogen causing soft rot and the degradation of pectin [155]. Additional activity may lead to better nutrients acquisition and survival. Yet, the topic requires further investigation.

Next-generation sequencing and comparative analysis of bacterial genome gives good opportunity to understand not only the microbial diversity and describe it as pan-genome, but also

host–pathogen interactions, pathogenesis, and evolution of the microbes. Bacterial genomes can be described as one pan-genome which is composed of the core set of genes and dispensable genes [121,156,157]. This idea groups all genes from all bacterial species into one huge common genome and assigns each gene to one of those three groups. The core genome (ca. 250 gene families) includes genes responsible for basic aspects of species biology (translation, replication, energy hemostasis, etc.) whereas dispensable genes determine species diversity. Different literature sources divide dispensable genes into character genes (ca. 7900 gene families) which contribute to colonization, antibiotic resistance or survival in the environment and probably infinite number of accessory genes mainly of unknown function used to distinguish strains and serotypes (Figure 7) [118,156]. For the use of this study, the broad approach can be limited to bacteria family, genus, or species to assess for example the pan-genome of *Aeromonas salmonicida*. As observed in pathogenic bacteria, the genome composition describes directly the pathogen lifestyle, adaptation to niches or growth conditions.

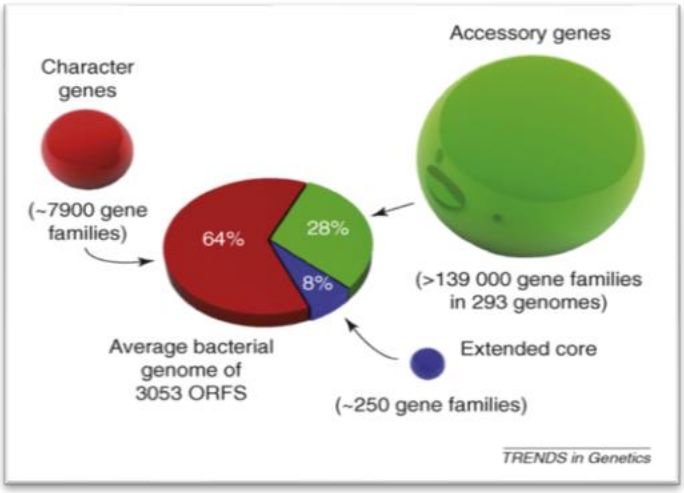

Figure 7. Bacterial pan-genome. Each gene found in any bacteria belongs to one of three gene sets: core, character or accessory genes[118].

To characterize and select the genes responsible for described responses of host cells, the bacteria genome sequencing was performed. Obtained genomes of three strains had similar genome size of 4.7 Mb similar to the *A. salmonicida* A449 strain. The HV strain genome appeared to be the longest out of all three suggesting presence of additional sequence. As reported by Najimi et al., it may belong to the plasmid pAsa6 discovered in 2009 [134]. The three-strain-comparison characterized the genes that are present in all strains with 100 % identity to A449 and those genes are characteristic only for *Aeromonas salmonicida* ssp. *salmonicida*, what called the core genome for the purpose of this

thesis. Moreover, we could determine the genes uniquely present in each of the strain belonging to dispensable group of genes defining strain individual behavior described by Medini [156].

A. salmonicida ssp. *salmonicida* strain A449 was needed only as a baseline to which we can refer our assembly and mapping analysis to obtain annotations. Part of HV sequences was not found in WT and ATT strain. The search for ORFs in silico helped to find the genes present in JF2267 strain and absent in A449. Some of the ORFs were purely computationally designed and did not appear in the NCBI database and were excluded from analysis. However, we were interested in the ORFs found in NCBI RefSeq Protein database. Those ORFs composed our dispensable genome of *A. salmonicida* JF2267 characteristic only for our three strains. Furthermore, they give a good starting point for the analysis in the direction of a new virulent gene of *A. salmonicida* search.

To fulfil the main point of the hypothesis, differences in the genome were related to the levels of virulence exhibited by each strain. It helped to address precisely virulence factors to a group of genes present in HV strain and absent in ATT one. Mapping of A449 genes on JF2267 strains showed that chromosome of *A. salmonicida* is quite stable whereas plasmid genes are highly variable, plastic, and unstable which was also reported by Daher and Brown [133,158]. Not only have they lower percentage of identity to the reference but also some of them are absent partially or completely in one, two or three JF2267 strains simultaneously. It was found that HV strain preserved most of the genes annotated in plasmid 5 of A449 strain. This could suggest that main virulence factors are localized in the plasmids determining differences in virulence. This fact was also discussed in the work of Vanden Bergh where the secretome of JF2267 was analyzed in contrast to the T3SS deficient attenuated JF2747 strain [115]. Moreover, small cryptic plasmids obviously may also contribute to the diversification of virulence and adaptive traits of bacteria [159].

Detailed analysis of gene composition of three genomes proved that during the culture and passages some genes get lost. After the revision of all, several genes having documented contribution to virulence on other bacterial species have been grouped in the table report. It shows that among others the chromosomal genes responsible for iron acquisition (hemin receptor, heme exporter protein D and CcmB, ferrichrome receptors, heme iron utilization proteins hutZ) are conserved which was also mentioned by Najimi [160]. Moreover, genes encoding aerolysin B - *Aeromonas* sp. cytolytic toxin described by Bernheimer and Avigad [161] or ecotin (virulence-associated serine protease inhibitor described in *Yersinia pestis* [162,163]) reveal no variations in sequence. Similarly, there is no difference in the sequence of AexT protein which is one of the main chromosomally encoded virulent factors as reported by Braun [164]. This suggests that they can be grouped as genes coding proteins responsible for basic virulence common for all three strains. What is more, they were found in all

three JF2267 strains which indicates that they do not influence directly on a different level of virulence [165].

In the case of *A. salmonicida* loss of virulence was described and investigated many times. Firstly, it was related to the loss of virulent plasmid 5 encoding T3SS responsible for infections firstly described by Burr [77,166,167]. Later, it was found that not a plasmid loss appears, but genomic rearrangements related to insertions sequences and transposase activity [74,133,154]. Such a gene insertion sequence-related rearrangements may be caused by culture in stressful conditions, including growth at temperatures above 20 °C [75,78,90,168]. It was proved that the presence of insertion sequences (IS) and high transposases activity is giving the bacteria high chances for survival in unfavorable conditions by adjusting their metabolic and pathogenic activity. Our results prove this research and show that plasmid sequences exhibit high plasticity because of not complete gene loss in plasmid 4 and 5. Such rearrangements in plasmid genomic composition and additional sequence in HV strain mentioned above may indicate that JF2267 strain possesses also the sixth plasmid in its genome as mentioned by Najimi [134]. Moreover, the results are supported by the recent research of Tanaka who showed that plasmids of strains A449. 01-B526 and 01-B516 show different gene loss profile. Additionally, it may be a starting point for a discussion of the influence of IS on JF2267 and virulence of WT, ATT and HV strains which was not yet analyzed [116].

The answer to the question where the virulence of each strain comes from lays in the genome of each strain and not only in its content but also the usage meaning by the gene regulation. All genome modifications during the culture and encountering other organisms such as the host affect the bacterial genome and force it to optimize its usage [169–171]. For that reason, among others, the mechanism of horizontal gene transfer is used equipping the bacteria in foreign genetic material that can be used during the infection process [119,157]. In the comparable manner insertion elements and transposases are actively modulating the bacteria transcriptome by silencing or activating different genes [74,134,154,172]. It indicated that not only gaining of genes may contribute to general outcome but also the gene loss plays an equally crucial role in virulence as proved for *Shigella* sp. and *Escherichia* sp. [74,173]. In this research, highly virulent JF2267 was observed to possess more genes than attenuated and wild type strains which might determine its extreme virulence. It cannot be forgotten that not only genomic composition is vital for strain virulence. There are many more types of machinery on higher levels (regulation of transcription, protein folding, post-translational modification like hydroxylation, methylation, citrullination, acetylation, phosphorylation, and more) responsible for obtaining properly folded [83,174] and active malicious proteins which will be discussed in the second part of this thesis.

Nonetheless, genome research is the first that is logical to investigate in search of the basic difference in bacteria strain pathological behavior. Significant finding about the virulence of three strains JF2267 was gathered in Table 8. To specifically name crucial genes, the selection was limited to two cases: when genes are present in WT and HV strain defining moderate virulence and when genes are present only in HV strain and absent in WT and ATT defining increased virulence. The other possibilities were not important for this study from the point of view of pathogenesis. They can be found in supplementary data. All genes from two groups belonged to the plasmid genes which suggest that chromosomal genes of the *A. salmonicida* may play the secondary role in virulence. In the first place the virulent may be determined by mobile elements of bacteria genome – plasmids. In this thesis, it is only the first indication, and the results need further analysis on transcriptomic and proteomic levels. For example, a functional analysis should be held to prove the importance of selected genes for the infection.

In this thesis, the limit was not only virulent genes encoding proteins that directly harm host cells in *vitro* which can be translated to in *vivo* but the research is addressed to a broader spectrum of genes that also facilitate survival in the host. As mentioned by Kamada, pathogens are considered as negative factors inducing disease [175]. This is just another niche of bacteria dramatic colonization and competition between resident microbes and pathogens for nutrient sources. Moreover, it is affected by the expression of pathogens' virulence factors and by the nutritional requirements of both populations. These dynamics can steer the survival, colonization, and clearance of pathogens in the gut keeping up with the disease or getting healed. In the topic of pathogenicity, scientists should stay flexible and open-minded not to omit important points. Defining genes as virulent gives us only a frame which one can follow but we cannot forget that bacteria genome usage is an extensive network with multiple nods and exceptions may exist. Moreover, the proper functioning of a pathogen depends on all of them as bacteria survival depends on it.

5 Transcriptome of Aeromonas salmonicida

5.1 Introduction

The etiology of disease, the pathogen and the infection mechanisms is required as the first need for cognition to produce a cure for the disease. Many approaches to explain *A. salmonicida* mechanism of infection has been taken, starting from molecular description of virulent genes like a – layers, hemolysins, type III and type IV secretion systems [75,95,160,176,177] through microarray analysis [178] and finishing on 2D GE analysis of bacterial proteome [169,179]. The point of the disease is the interaction between the host and the pathogen. Not only fish species and its susceptibility matter as presented by Korytář [62] but also the pathological type of the bacteria itself being more or less virulent [115]. Yet before starting those functional studies, it is highly advisable to study each interaction side separately. Finding virulent proteins contributing to the progress of the disease is a crucial point for interaction studies. However, in this work "virulent proteins" means all proteins that contribute to the disease including those proteins forming a metabolic network facilitating the fish inner environment. The animal challenges indicate that bacteria can start killing a fish within 72 h [62]. The adaptive immune system does not respond with such a speed so it must be the innate immune response. It is believed that they are the main target in the innate recognition of the danger.

Each previous study described in literature was focused only on the chosen part of bacterial genomics, transcriptomics or proteomics that mostly was related strictly to virulence factors. The analysis considered mainly characterization of genes and proteins like: S layer proteins [180], hemolysins [181,182], exotoxins [183], exoenzymes [91], type three secretion system, structural [166] and effector proteins [91,95,184,185]. Those are all well-known factors that appear also in other fish and human pathogens [160,186]. Yet, the complete network and cooperation of all those single virulence factors have not been described. Moreover, the research was based on knowledge from other species and conserved virulence factors described already in *Aeromonas* sp. or in other species. Thus, for the explanation of infection process, it is important to keep in mind the role of the host and what is recognized from bacteria by hosts innate immune system and what a single virulent protein in course of interaction does.

The mechanisms of *A. salmonicida* infection are not yet fully described and many questions are not answered. It is highly demanded to elaborate on the method that covers known and unknown yet not described aspects of its pathogenesis. The whole genome of one of the isolates, strain A449. has been annotated and published until now [130,178]. *A. salmonicida* is extremely flexible bacterial species

which is explained among others by the presence of many transposons [130], insertion elements and unstable plasmids [116,133,159137].

In the previous part of this thesis, a raw genome of JF2267 strain has been presented with all its modifications. However, it gave only the insight into the plasticity of genomic sequence and evolutionary explanation of levels in virulence. It was shown that the absence of genes may influence the levels of virulence but there are also other levels of gene expression – transcription, translation, post-translational modifications – that create fully functional protein. Studying only the bacteria genome does not give any functional information. To narrow down the number of genes directly involved in infection it is necessary to study the transcriptome of *A. salmonicida* as the secondary level of regulation of virulent protein production.

In the present study, the influence of abiotic conditions on *A. salmonicida* transcriptome is evaluated by exposing bacteria to selected conditions in *vitro*. Culture medium was modified by limiting the access to crucial components to mimic the possible situations that bacteria may encounter during trout infections. The goal was not to describe fully all responses of the transcriptome, although this is the starting point of analysis, but to identify the genes that are possibly involved in the virulence of the strain JF2267 and choose the best candidates for interaction studies with rainbow trout immune cells.

5.2 Materials and methods

5.2.1 **Bacteria culture. Growth curve**

Bacteria (1 x 10^6 cells) were cultured in LB broth at 18 °C for which served as control. Supplementing LB broth with 10 mM EGTA and 120 mM 2, 2’ bipyridyl created calcium and iron deficiency conditions, respectively. Anaerobic conditions were created by culturing bacteria on LB broth covered with mineral oil. Inducing the temperature stress required cultivating bacteria at 23 °C.

Growth curves of *A. salmonicida* were obtained by monitoring cell number / ml in LB broth for all conditions: 1) control culture in 18 °C, 2) supplemented with 10 mM EGTA (iron limitation), 3) supplemented with 120 mM 2, 2' bipyridyl (calcium limitation), 4) LB broth covered with mineral oil on top (anaerobic conditions), 5) LB broth incubated in 23 °C (extreme temperature). Samples were prepared in triplicates. The bacteria were counted every 2 – 4 h on hemocytometer – Neubauer chamber in early time points, later twice and once per day. Experiment was repeated four times.

$$G = \frac{t}{3.3 * log\, b/B}$$

$$G - generation\ time$$
$$t - time\ interval[h][min]$$
$$B - number\ of\ bacteria\ at\ the\ beginning\ of\ a\ time\ interval$$
$$b - number\ of\ bacteria\ at\ the\ end\ of\ the\ time\ interval$$

Figure 8. Generation time was calculated for log phase according to the pattern above.

The time of sampling for the microarray was estimated based on the growth curve.

5.2.2 Sample preparation for microarray hybridization

After the time of culture, the bacteria were centrifuged in 8,000 rpm, 10 min. The bacteria RNA was stabilized in RNA Protect Bacteria reagent (Qiagen Inc., Germany). Total RNA was isolated using RNeasy Mini Kit (Qiagen Inc., Germany) with on-column DNA digestion according to the manufacturer's instruction. Traces of genomic DNA were removed by RNase-free DNase (Qiagen Inc., Germany) treatment for 15 minutes. Yield and purity of isolated RNA dissolved in nuclease-free water (Roche, Germany) were evaluated on the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Samples were stored at -20 °C.

All samples were prepared in triplicates and cultured 24 h and 48 h at 18 °C.

Table 11. Culture conditions modifications to induce different transcriptomic answer of *A. salmonicida*.

Condition	Sample ID	Time point	Number of sample replicates
Calcium limitation	Ca 24 h	24 h	3
	Ca 48 h	48 h	3
Iron limitation	Fe 24 h	24 h	2
	Fe 48 h	48 h	3
Anaerobic conditions	Ox 24 h	24 h	2
	Ox 48 h	48 h	3
Elevated temperature	RT 24 h	24 h	3
	RT 48 h	48 h	3

5.2.3 Microarray

The oligonucleotide probes (length between 45 - 60 bp) were designed using the genome sequence of *Aeromonas salmonicida* JF2267 highly virulent strain with a help of Miltenyi Biotech, Germany. Three replicates of probes were designed for each gene. The control group consisted of housekeeping genes described in *A. salmonicida* and *E. coli.*

The quality of samples was checked via Agilent 2100. Bioanalyzer platform (Agilent Technologies, USA). An RNA Integrity Number (RIN) was generated to check integrity and overall quality of total RNA samples. RNA samples with the RIN number > 6 are of sufficient quality and was approved for gene expression profiling experiments. Since the samples LB-ox_24h_3 and LB-Fe_24h_3 revealed results that did not fulfil the QC Criteria (RIN>6) these samples were excluded from further analysis.

RNA amplification to sRNA

For the linear T7-based amplification step, 200 ng of each total RNA sample was used. All samples were labelled with Cy3 dye. To produce Cy3-labelled cRNA, the RNA samples were amplified and labelled using the Agilent Quick Amp Labelling Kit (Agilent Technologies, USA) following the manufacturer's protocol that was adapted for prokaryotic RNA (several tests were performed to establish this protocol). Yields of cRNA and the dye incorporation rate were measured with the ND-1000 Spectrophotometer (NanoDrop Technologies, USA).

Microarray hybridization

The hybridization procedure was performed according to the Agilent 60-mer oligo microarray processing protocol using the Agilent Gene Expression Hybridization Kit (Agilent Technologies, USA). Briefly, 600 ng Cy3-labelled fragmented cRNA in hybridization buffer was hybridized overnight (17 h, 65 °C) to Agilent Whole Aeromonas Genome Oligo Microarrays 8x60K (custom design) using Agilent's recommended hybridization chamber and oven. In order to avoid position / batch effects the samples were randomly distributed on the different microarrays. Finally, the microarrays were washed once with the Agilent Gene Expression Wash Buffer 1 for 1 min at room temperature followed by a second wash with preheated Agilent Gene Expression Wash Buffer 2 (37 °C) for 1 min. The last washing step was performed with acetonitrile.

Readout: scanning, image analysis

Fluorescence signals of the hybridized Agilent Microarrays were detected using Agilent's Microarray Scanner System (Agilent Technologies, USA). The Agilent Feature Extraction Software (FES) was used to read out and process microarray image files. The software determines feature intensities (including background subtraction), rejects outliers, and calculates statistical confidence. For determination of differential gene expression FES derived output data files were further analyzed using the Rosetta Resolver gene expression data analysis system (Rosetta Biosoftware, USA).

5.2.4 **Analysis**

The signal intensities from the single-experiment raw data lists were normalized by dividing the intensity values by their median. The ratios were calculated by dividing sample signal intensity through control signal intensity. The intensity profiles of the control samples from 24 h and 48 h were merged respectively and compared to the single intensity profiles of each replicate of the samples of interest as well as to their merged profiles. The results of the ratio experiments were visualized in a double-log scatter plot. A gene list with all normalized sample/control log10 ratios and fold changes, sequence descriptions, p-values, etc. referred to as gene ratio list (of all genes) was exported using the Resolver Software. Putative candidate genes with a fold change > 2 and p-value < 0.01 were summarized in one pre-selected gene list.

5.2.5 **Gene selection**

After the analysis provided by Miltenyi Biotech, genes having a fold change higher than two and p-value lower than 0.01 were preselected. Out of those, only those genes being positively expressed and meeting chosen criteria were selected. The criteria for genes were set as follows: the gene must encode outer membrane proteins, secreted proteins, receptors, virulent proteins or proteins interacting with outer environment. The criteria aimed to select those genes that fit the definition of a PAMP or VAMP.

TreeMap

The Seed and RegPrecise databases were searched for comparative analysis of transcriptomic data. Preselected genes were classified by their function and belonging to certain transcription factor regulon. By using Voronoi tree maps, a visualization method for hierarchical structures, all genes assigned by regulon and function were shown in a hierarchically organized and space-filling manner [187,188]. The two-dimensional plane is divided into subareas according to the space-filling approach, which are then subdivided into subsub-areas of the subrules and so forth. The polygons on the last level represent all functionally assigned genes.

Pathogenic islands

Pathogenic islands were identified within the contig sequence in order to characterize genome composition of *A. salmonicida* JF2267 strain. The database has been searched using computational tool Island Viewer which integrates three different methods: IslandPick, IslandPath DIMOB and SIGI-HMM [189]. Pathogenic islands from *A. salmonicida* ssp. *salmonicida* A449 have been compared

with pre-selected genes by the locus tag from genome of *A. salmonicida* ssp. *salmonicida* JF2267. In that way, it was possible to identify the genes localized within the specific pathogenic island.

5.3 Results

High throughput analytical methods were applied in order to characterize the pathogenic *Aeromonas salmonicida* JF2267. Furthermore, the molecules responsible for primary interaction with the host immune system were found using methods covering whole bacteria metabolome interactions.

5.3.1 **Growth curve**

Growth curve was established to assess growth speed of *A. salmonicida and* its generation time (Figure 9). It was also necessary to estimate the sampling time points for the experiment with microarray. The stationary phase for each condition started around 30 h of cultivation. However, comparison of growth curve between different conditions revealed several differences in the growth speed. The bacteria grown under basic conditions grew with similar speed as bacteria with limited oxygen or higher temperature. The shortest generation time of 2.23 h was during growth in higher temperature. Generation time in basic and anaerobic conditions were almost the same and reached 2.72 h and 2.68 h respectively. The anaerobic conditions limited slightly the growth and generation time is elongated to 2.53 h. Culturing bacteria on iron and calcium deficient medium restricted the growth significantly and elongate the generation time to 6.84 h and 4.30 h, respectively. Moreover, the largest cell number that was reached during stationary phase was limited by the growth under iron and calcium deficient conditions (Supplement4_growthCurve)

For further experiments time points of 24 h as log phase and 48 h as stationary phase were chosen.

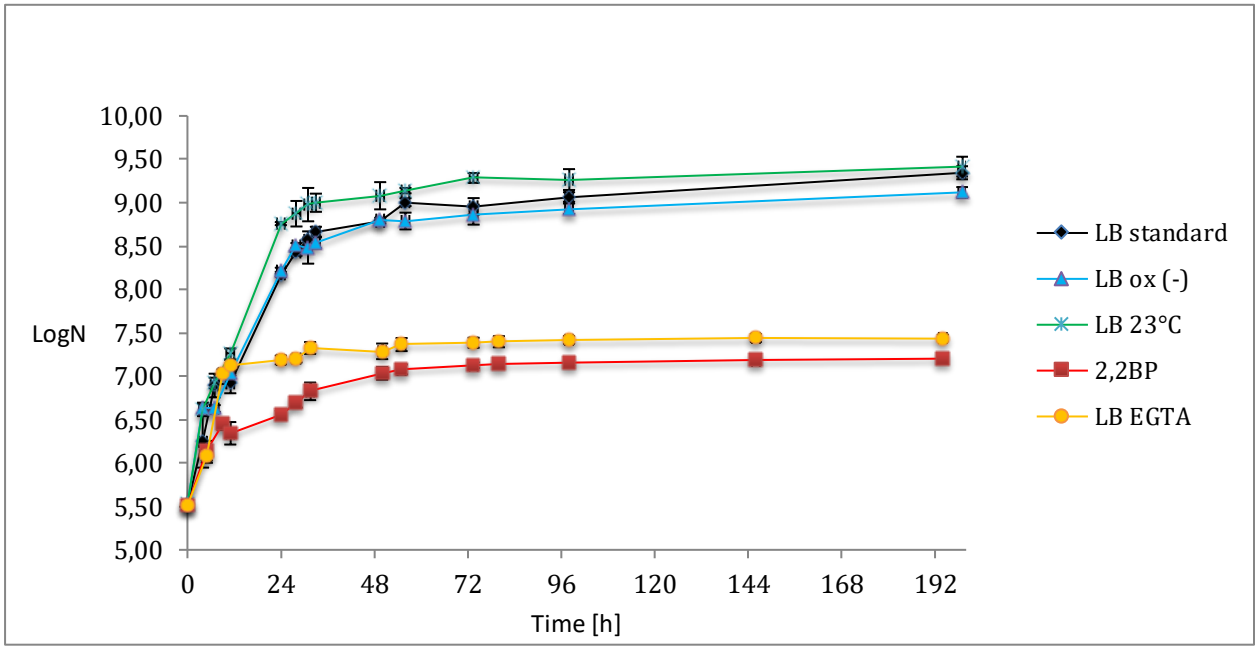

Figure 9. Growth curve of *Aeromonas salmonicida* JF2267. Conditions were modified in order to assess the sampli time point and to obtain the generation time in different growth conditions.

5.3.2 Microarray analysis

Microarray analysis was used to investigate the impact of environmental conditions on transcriptome of *A. salmonicida*. It aimed to scan the whole transcriptome of *Aeromonas salmonicida in* search of genes being important for the initial recognition by innate immune system of the trout in the first days of the disease. The genes coding proteins responsible for the direct contact with the host immune system are in focus.

Statistics

The results of the ratio intensity experiments can be visualized in a double-log scatter plot. Example of representative double-log scatter plot for the ratio intensity experiment is visualized in Figure 10. Other plots are attached in supplementary data. The output data of the Agilent Feature Extraction software included gene lists with the complete raw data sets. Furthermore, the signal intensities from the single-experiment raw data lists are normalized by dividing the intensity values by their median. These normalized signal intensities are joined to a common table the single experiment normalized data list. This list includes in addition to the normalized intensity values the

feature gIsPosAndSignif which shows a value equals 1 that the signal intensity is positive and significant above the background and a value equals 0 that the signal intensity is not positive and significant above the background. The Resolver Software allows the export of a gene list with all normalized sample/control log10 ratios and fold changes, sequence descriptions, p-values, etc., referred to as gene ratio list (Supplement5_microarrayQC).

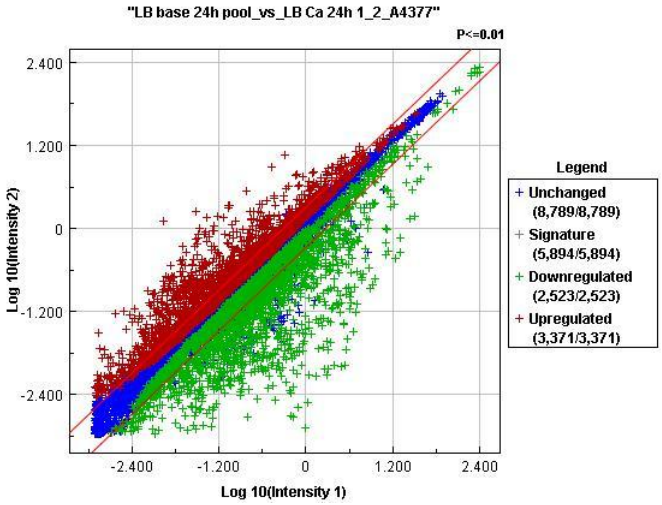

Figure 10. Scatter plot of signal intensities of all spots as an example the data of one microarray. The signal intensities of each feature represented by a dot are shown in double logarithmic scale. X-axis: control-log signal intensity; y-axis: sample-log signal intensity. Red diagonal lines define the areas of 2-fold differential signal intensities. Blue cross: unchanged genes. Red cross: significantly upregulated genes (p-value < 0.01). Green cross: significantly downregulated genes (p-value < 0.01). Grey cross: summary of significantly up- and downregulated signatures. Plots for other culture conditions are presented in supplementary material.

Microarray

First step was the selection of genes with minimum 2-fold change and p-value < 0.01 genes regulated in different conditions were plotted on VENN diagrams showing usage of genes after 24 h and 48 h. In general, limitation of iron caused regulation of 1,566 genes after 24 h and 1,724 genes after 48 h of incubation which was the highest change in regulation. In case of calcium limited conditions in the beginning only 509 genes were regulated and then it dramatically increased to 1,322 genes after 48 h (Figure 11). The trend of increasing regulation remained similar for both conditions. It was opposite in case of anaerobic conditions and increased temperature. More genes were regulated after first 24 h than after 48 h of culture. Nevertheless, in case of anaerobic conditions

regulation of only 246 genes was changed and decreased to 103 genes. Higher temperature induced the regulation 1,199 genes after 24 h of culture followed by 906 genes regulated after 48 h.

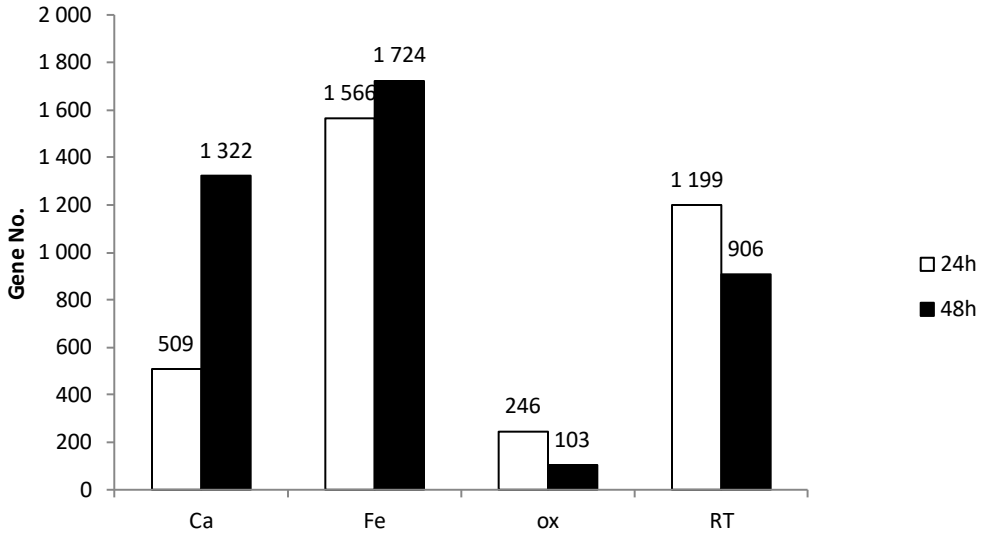

Figure 11. Number of genes differentially regulated in 24 h and 48 h in modified conditions. Conditions: Ca – calcium limitation, Fe – iron limitation, ox – anaerobic condtions, RT – elevated temperature, 23 °C. White bars represent readout after 24 h culture. Black bars represent readout after 48 h culture.

To find out what is the distribution of genes regulation in each condition, the results were combined for each time point in two pictures separately. The results were presented in VENN diagrams showing the sum of positively and negatively regulated genes. Diagrams depict the complex nature of analysis showing the number of genes regulated in one, two, three and all conditions together (Figure 12). Most interesting genes were those uniquely regulated in one condition. Conditions with limited calcium and iron were the most important in the experiment. Here, 784 genes were uniquely expressed in iron limited condition after first 24 h of incubation which slightly decreases to 553 genes after 48 h. Limitation of calcium changed the RNA expression of 92 genes after 24 h and 437 genes after 48 h. It is worth noticing that there was a distinct set of genes common for both conditions changing from 158 genes to 478 genes after 24 h and 48 h, respectively. On the contrary, oxygen limitation did not change the expression of many genes. There were only 38 and 11 genes regulated in 24 h and 48 h time point. Increased temperature induced expression of 601 and 174 genes uniquely expressed in 24 h and 48 h time point. What is more, there was high number of genes expressed in both iron and calcium limited condition.

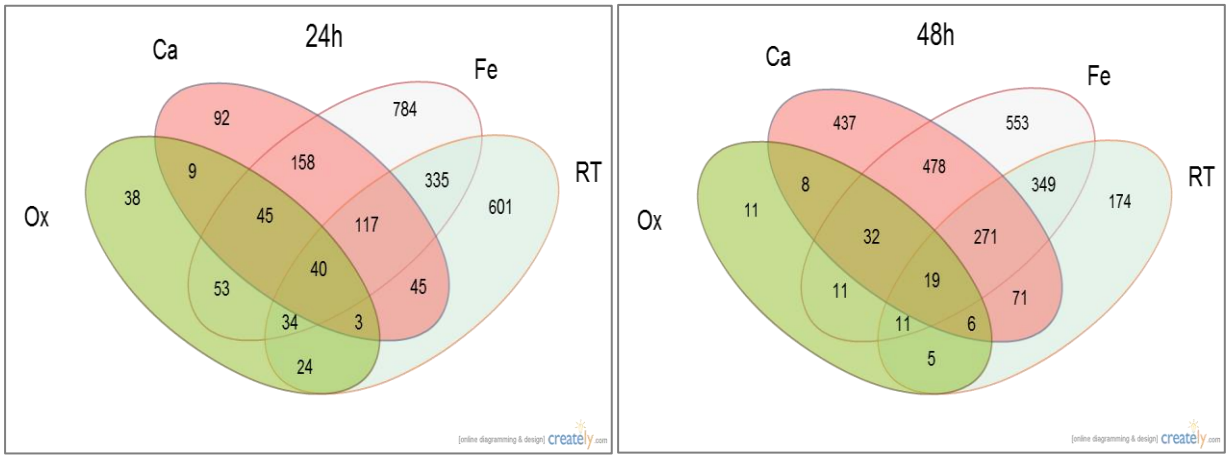

Figure 12. Number of genes expressed in modified conditions. Oxygen (green ellipse) limitation does not influences gene expression significantly due to anaerobic capabilities of bacteria to survive. Increased temperature (grey ellipse) changes gene expression quite highly. However, iron (white ellipse) and calcium (red ellipse) shortage have most influence of gene regulation.

5.3.3 Selection of genes according to defined criteria: outer membrane bound, secreted, receptors, virulent, interacting with outer environment

Next step of analysis included selection of genes putatively virulent that are either localized on bacterial cell membrane or secreted to the environment. Each step of analysis helped to establish a table of genes (Table 12). After selection of genes with minimum 2-fold change and p-value <0.01 genes regulated in different conditions were plotted on VENN diagrams showing usage of genes after 24 h and 48 h. From each condition the genes were selected that were reported in literature to contribute to virulence in pathogenic bacteria. Among many genes that were regulated during the stimulation only several were selected for further analysis. Sequence of genes marked in grey were analyzed and could be used in further studies. Information from KEGG database was collected in pdf files in supplementary data.

Table 12. Selected genes important for the virulence of *A. salmonicida* JF2267. Selection was based on their protein product. Each gene has its equivalent in *A. salmonicida* A449 described by locus tag. Regulation fold change was marked in yellow for each modified condition. Genes were grouped by the condition the expression occurred: Ca – calcium limitation, Fe – iron limitation, Ox – anaerobic culture, RT – elevated temperature, 23 °C. Different numbers of genes in each group is random.

Gene name	Protein product	Accession number	Locus tag	Fold change	P-value
Ca24h					
exsE	ExsE protein	gi|4999232	ASA_P5G062	13.305	0.00011
aopD	AopD protein	gi|4999234	ASA_P5G064	7.633	0.00014
flp1	flp pilin	gi|4996409	ASA_2915	7.627	2.89E-16
Ca48h					
hycI	hydrogenase 3 maturation protease	gi|4997007	ASA_1808	19.941	0
asoA	serine transport-like protein asoA	gi|4996673	ASA_3137	15.618	0
-	pyruvate ferredoxin/flavodoxin oxidoreductase	pid|142850940	ASA_1140	10.639	1.02E-32
flp1	flp pilin	gi|4996409	ASA_2915	10.224	1.02E-33
pspA	phage shock protein A	gi|4997131	ASA_0547	9.270	0
flpA	flp pilus prepilin peptidase	gi|4996408	ASA_2914	8.613	0
pspG	phage shock protein G	gi|4997798	ASA_0549	8.101	0
Fe24h					
nirB	nitrite reductase (NAD(P)H), large subunit	gi|4997971	ASA_0196	24.591	8.54E-13
fstB	ferric siderophore receptor B	gi|4998503	ASA_4368	15.256	0
-	outer membrane protein	gi|4995121	ASA_1265	14.710	0
flpA	flp pilus prepilin peptidase	gi|4996408	ASA_2914	13.522	0
fdx	Fdx protein	gi|4999007	ASA_2606	13.003	6.78E-23
flp1	flp pilin	gi|4996409	ASA_2915	11.350	0
fstB	ferric siderophore receptor B	gi|4998503	ASA_4368	9.940	0
Fe48h					
entC	isochorismate synthase	gi|4995589	ASA_1838	100.000	0
entE	2.3-dihydroxybenzoate-AMP ligase	gi|4995590	ASA_1839	100.000	0
entB	isochorismatase	gi|4995591	ASA_1840	100.000	0
flpA	flp pilus prepilin peptidase	gi|4996408	ASA_2914	100.000	0
flp1	flp pilin	gi|4996409	ASA_2915	100.000	0
fstB	ferric siderophore receptor B	gi|4998503	ASA_4368	100.000	0
hupA	outer-membrane heme receptor	pid|142852987	ASA_3328	100.000	0
-	hemin receptor	gi|4998299	ASA_2695	99.102	0
ptrB	protease II	gi|4998300	ASA_2696	92.195	0
hutZ	heme iron utilization protein	gi|4996380	ASA_3332	70.857	0
fstC	colicin receptor	gi|4995601	ASA_1850	64.189	0
bfd	bacterioferritin-associated ferredoxin	gi|4995141	ASA_0466	42.818	0
-	ferrichrome-iron receptor	gi|4997707	ASA_2341	40.545	0
-	outer membrane ferric siderophore receptor	gi|4997825	ASA_3883	36.854	0
dsdX	DsdX permease	gi|4996027	ASA_2939	31.139	0
fhuA	hydroxamate-type ferric siderophore receptor	pid|142853957	ASA_4363	22.973	0
bfr	bacterioferritin	gi|4995139	ASA_0467	12.336	0

fdx	Fdx protein	gi|4999007	ASA_2606	10.242	0
Ox 24h					
pulA	pullulanase	gi|4998483	ASA_0628	6.294	1.08E-09
chb	chitobiase	gi|4998436	ASA_2826	4.914	6.69E-08
ox 48h					
hyfA	hydrogenase 4 Fe-S subunit	gi|4997014	ASA_1815	3.851	4.83E-08
fstC	colicin receptor	gi|4995601	ASA_1850	2.272	0
-	protease	gi|4997544	#NV	2.203	1.50E-12
RT 24h					
lasA	protease LasA	gi|4994721	ASA_1287	38.909	0
hutG	formimidoylglutamase	gi|4996311	ASA_3954	13.369	1.77E-35
tnaA	tryptophanase	gi|4995086	ASA_2330	13.354	6.30E-16
prpB	methylisocitrate lyase	gi|4997510	ASA_2041	13.354	0
hutI	imidazolonepropionase	gi|4996312	ASA_3955	11.622	0
argH	argininosuccinate lyase	gi|4996911	ASA_0582	9.539	0
RT 48h					
rafY	glycophorin	gi|4996011	ASA_0475	10.053	0
crG	AcrG protein	pid|142856333	ASA_P5G068	9.266	0.00042
	ROK family protein	gi|4995983	ASA_0476	7.204	0
traL	TraL protein	pid|142856141	ASA_P4G157	6.919	0
traE	sex pilus assembly	pid|142856142	ASA_P4G158	6.341	0

5.3.4 **TreeMap**

Functional analysis of genes expression was carried out using Voronoi TreeMaps, a novel visualization method for hierarchical structures. Genes were categorized by their biological process and regulon affiliation. To see the pattern of expression, the Seed database was searched for *Aeromonas salmonicida* strain A449 genes and their affiliation to specific metabolic process. Processes were categorized into three levels and the fourth level were single genes (Figure 13). Images from Figure 14 in better resolution are provided in supplementary material. Not all results from TreeMaps will be discussed here. Only most outstanding groups and trends were pointed out.

Following, the dynamic of transcriptome adjustments affected by abiotic conditions was shown by grouping genes using different criteria. Calcium and iron chelators caused most dramatic changes in gene regulation. Unfortunately, there is no clear pattern for up or down regulation of genes related to pathways. In particular, calcium limitation downregulated after 24 h genes from TCA cycles, succinate and respiratory dehydrogenase, glycerol, and glycerol 3 phosphate uptake pathways (Figure 14). All mentioned pathways were weakly upregulated in 48 h time point. Upregulation of flagella and iron acquisition pathways is most visible after 24 h and it stayed similar in 48 h time point. What is more, 48 h of such stimulation upregulated even more genes from different pathways

of RNA, DNA and protein metabolism, cell wall and capsule production that were faintly regulated in 24 h time point. TCA cycle pathway was down regulated also in both time points in iron limitation conditions. However, TreeMap image of gene regulation in iron limited condition showed more discrepancies (Figure 14). In general, there were more genes strongly regulated. Down regulated genes in 24 h belonged also to, glycerol and glycerol-3-phosphate uptake and respiration pathways. Interestingly, there was always downregulation of membrane transport genes, especially type III secretion system, type VI secretion system and type IV pilus gene pathways.

Figure 13. Levels of Voronoi treeMap based on metabolic process of *A. salmonicida* created from the Seed database. Each square represents one level of gene grouping by function from top left – the highest, to bottom right – the lowest on single gene names. The high quality images are in supplementary material (Supplement7_treemaps).

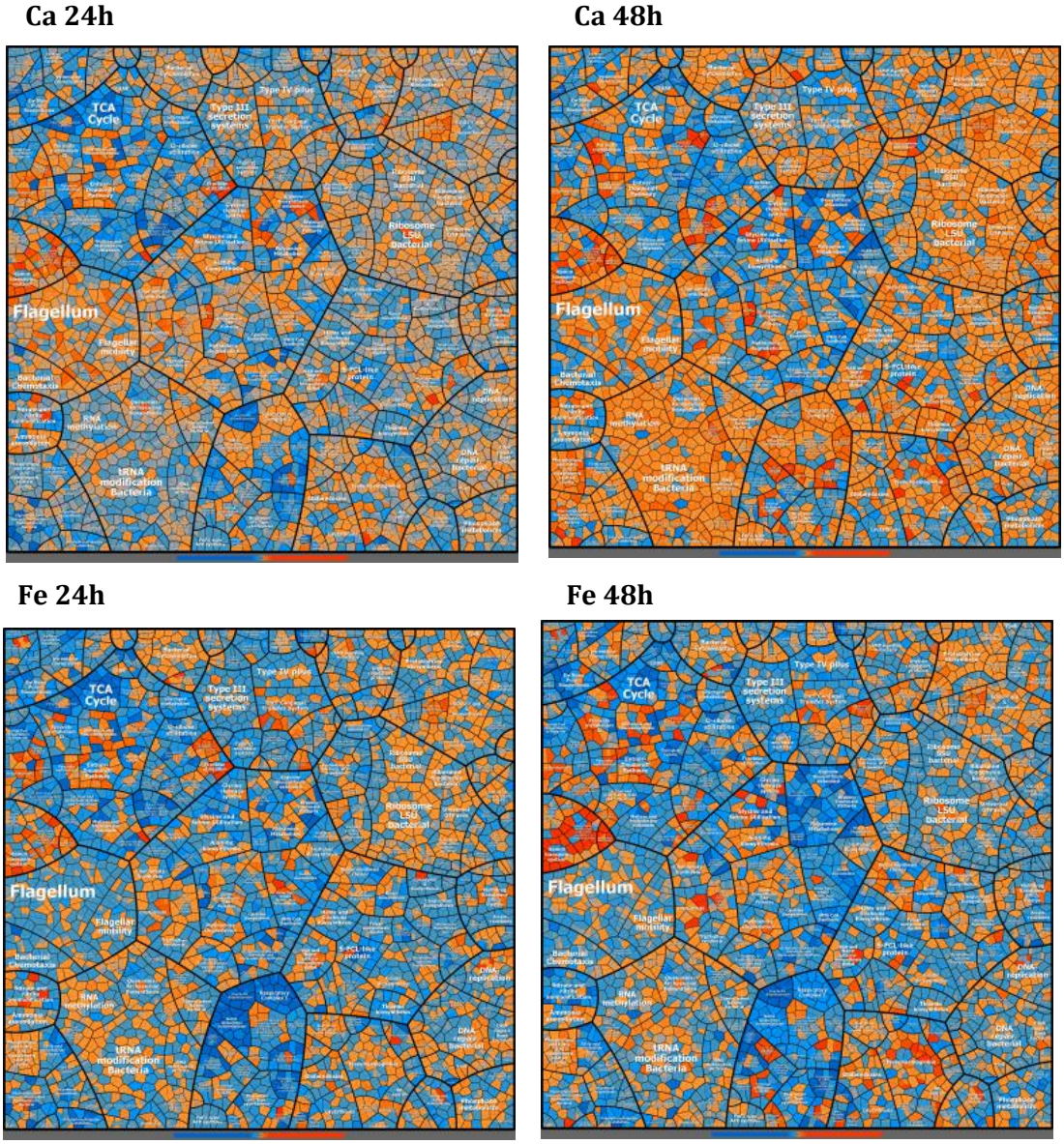

Figure 14. Gene expression of *A. salmonicida in the* form of Voronoi treeMaps for calcium(Ca) and iron (Fe) limited conditions after 24 h and 48 h culture. Transcriptional data were mapped by using a gradient colour scale from blue for repression to orange for incresed expression. The intensity of each colour represents the greatness of regulation. The high quality images are in supplementary material (Supplement7_treemaps).

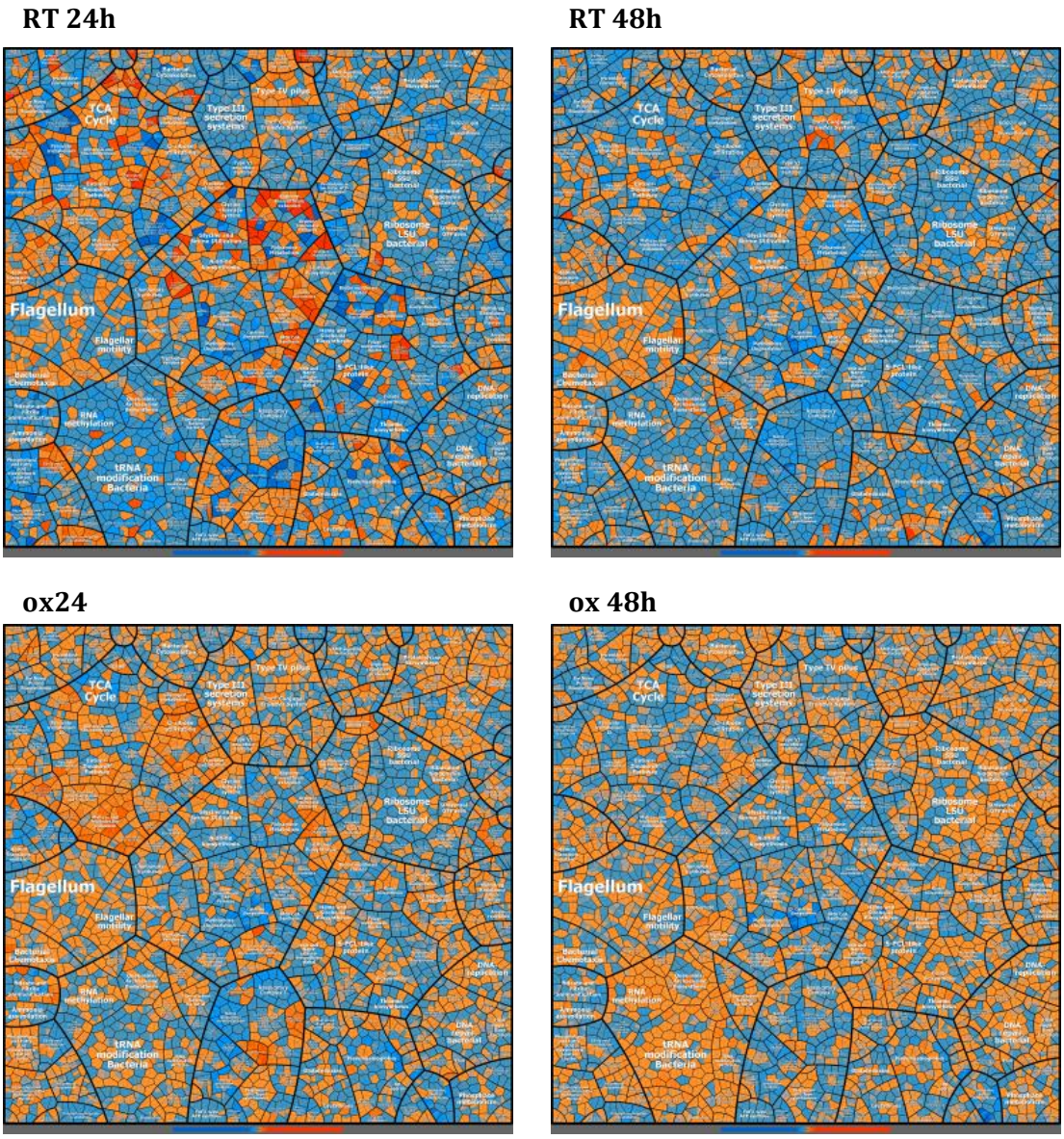

Figure 15. Gene expression of *A. salmonicida in the* form of Voronoi treeMaps for oxygen limited conditions (ox) and increased temperature (RT) after 24 h and 48 h culture. Transcriptional data were mapped by using a gradient colour scale from blue for repression to orange for incresed expression. The intensity of each colour represents the greatness of regulation. The high quality images are in supplementary material (Supplement7_treemaps).

Unfortunately, the regulon database seemed more limited in genes. Description of metabolic processes in *A. salmonicida* was better described and covered higher number of genes. Clustering genes by their affiliation to regulon better explains the regulation strategies of the bacteria (Figure 16).

The most significant varieties were noticed in case of iron and calcium limitation (Figure 17). Genes regulated by Fur regulon were mainly upregulated after 24 h and 48 h. Furthermore, it reached higher levels after 48 h. Regulation of genes by FruR regulon appeared after 24 h and decreased overtime in following 24 h in both conditions. In case of bacteria cultured in room temperature the most outstanding positive regulation was observed after 24 h for genes regulated by ArgR, LiuR and HutC regulon. Negatively regulated genes comprised of regulons: BirA, PhnR1. SgaR, NarP. In this case 48 h culture of bacteria seemed to come back to natural balance and the gene regulation was weaker. Similarly, induction of anaerobic conditions hardly influenced the expression of all annotated genes as compared with other conditions (Figure 18).

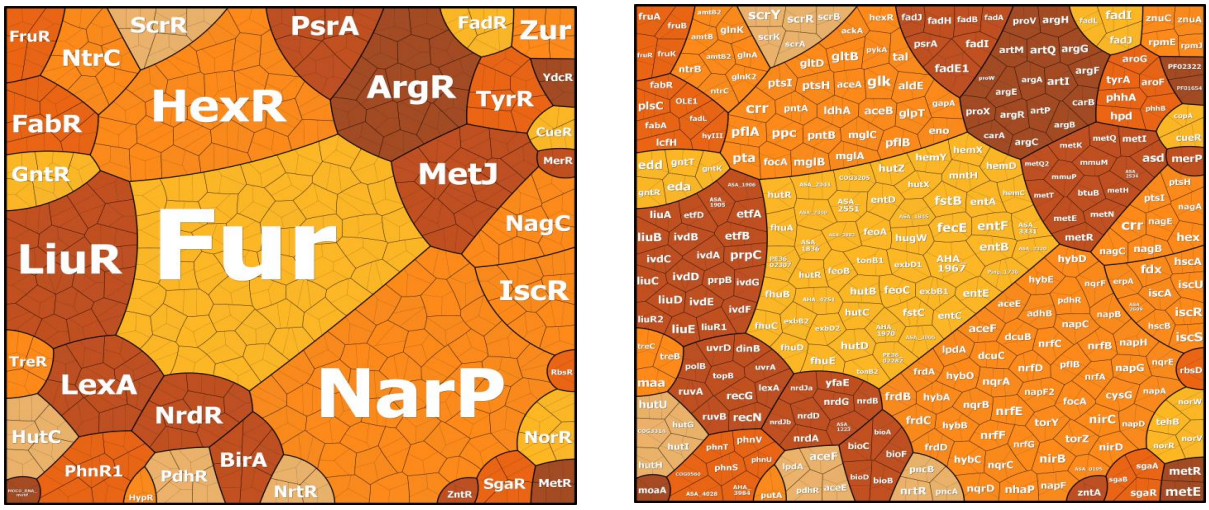

Figure 16. General representation of regulons of *A. salmonicida*. General (left) and detailed (right) levels of Voronoi treeMap based on regulon of *A. salmonicida* created from the RegPrecise database.

In case of the other regulon the outstanding result gave the regulation of Arg regulon. It showed a change from positive to negative regulation during calcium and iron depletion from the first to the second day of analysis whereas high upregulation was observed after 24 h culture in increased temperature. However, anaerobic conditions did not have such profound effect on those genes.

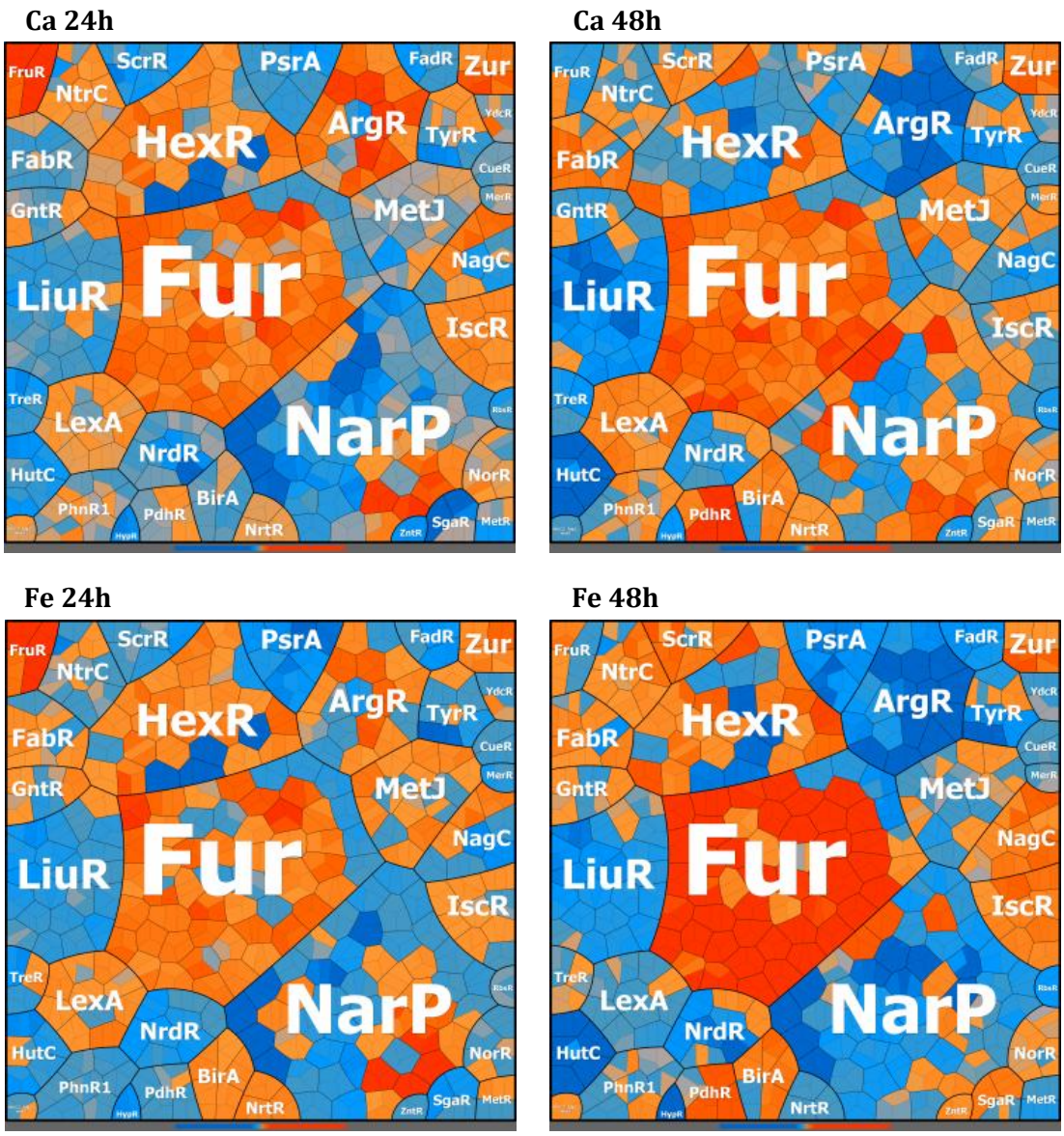

Figure 17. Regulation of genes in calcium (top) and iron (bottom) limitation conditions after 24 h and 48 h described by treemap of differentially regulated *A. salmonicida* ssp. *salmonicida* JF2267 genes. Genes were mapped according to regulon operator obtained from regprecise.lbl.org website. Blue or orange tiles represent the repression and increased expression, respectively. The intensity of each colour represents the greatness of regulation. The high quality images are in supplementary material (Supplement7_treemaps).

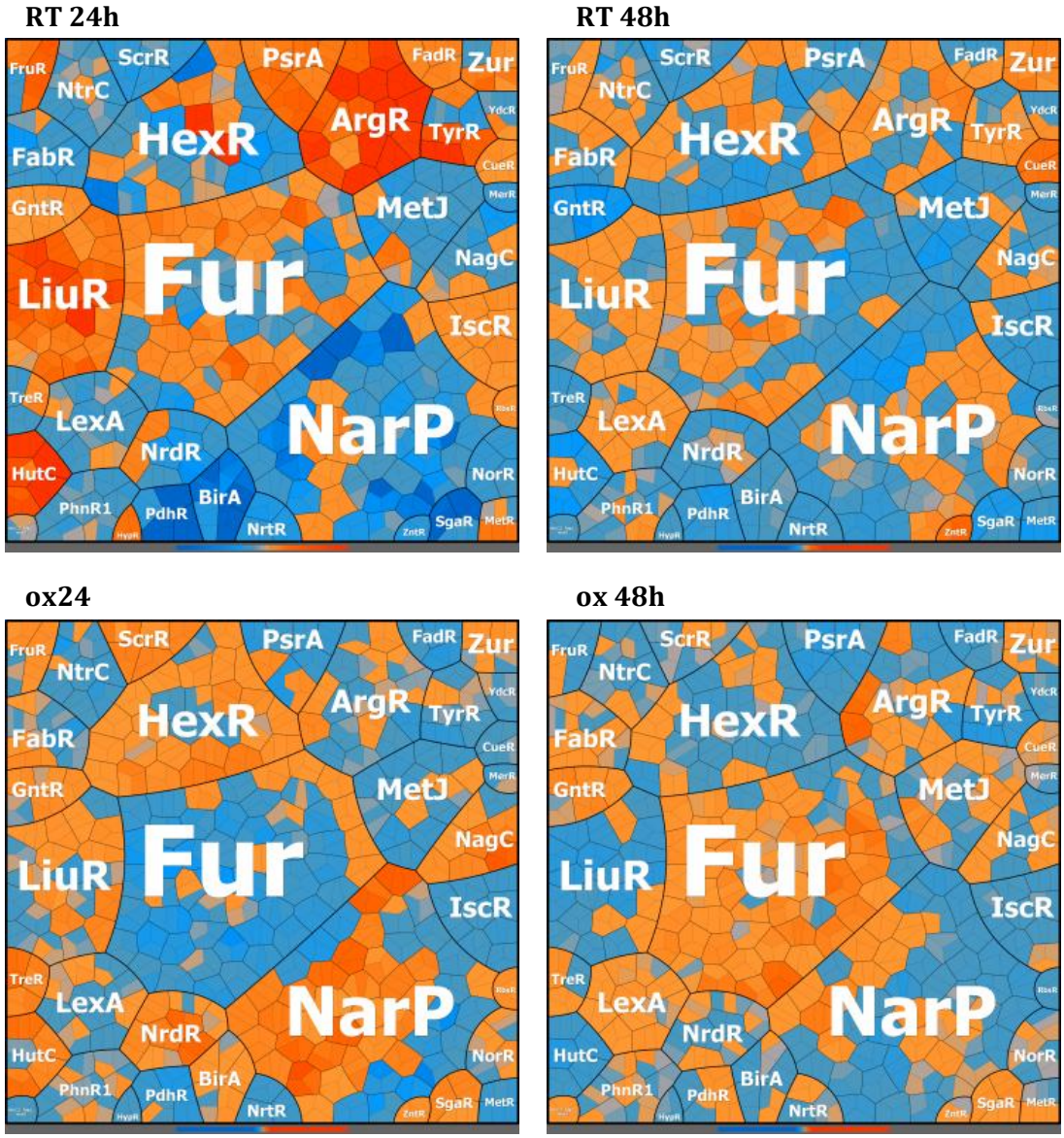

Figure 18. Regulation of genes in higher temperature (top) and anaerobic (bottom) condition after 24 h and 48 h described by treemap of differentially regulated *A. salmonicida* ssp. *salmonicida* JF2267 genes. Genes were mapped according to regulon operator obtained from regprecise.lbl.org website. Blue or orange tiles represent the repression and increased expression, respectively. The intensity of each colour represents the greatness of regulation. The high quality images are in supplementary material (Supplement7_treemaps).

5.3.5 **Pathogenic islands**

Whole list of pathogenic islands (PAI) reported for *A. salmonicida* A499 has been downloaded from Island Viewer website. The database has been searched using computational tool Island Viewer which integrates three different methods: IslandPick, IslandPath DIMOB and SIGI-HMM. PAI from *A. salmonicida* ssp. *salmonicida* A449 has been compared with pre-selected genes by their Locus Tag. In that way it was possible to identify the genes localized within the specific PAI. The analysis of microarray results showed that out of 347 possible genes found in A449 strain there are only 283 genes identified for JF2267 strain (Supplement6_microarray-summary /PAI_selection).

Genes selected from the full list of genes found in PAIs (supplementary data) are depicted in the table (Table 13). Its regulation was not outstanding. Except non-ribosomal peptide synthetase module, RNA polymerase sigma factor $FeCI_2$ and ferric siderophore receptor B it does not exceed 9-fold upregulation and 5-fold downregulation. Other not presented data cover mainly hypothetical proteins. Interestingly, the genes encoding protein building lateral flagella were 2 to 3-fold positively regulated after 24 h and 48 h of incubation.

Table 13. Selected genes from pathogenic islands.

Accessions	Gene Symbol	Description	Ca_24h	Fe_24h	ox_24h	RT_24h	Ca_48h	Fe_48h	ox_48h	RT_48h
gi|4995908	fecI	RNA polymerase sigma factor FecI	4.97	9.00	n.d.	n.d.	6.15	100.00	n.d.	2.04
gi|4995910	-	non-ribosomal peptide synthetase module	n.d.	n.d.	n.d.	-2.22	n.d.	97.98	n.d.	n.d.
gi|4995911	-	non-ribosomal peptide synthetase module	2.56	n.d.	n.d.	n.d.	3.43	100.00	2.00	n.d.
gi|4998503	fstB	ferric siderophore receptor B	n.d.	9.94	n.d.	n.d.	n.d.	100.00	n.d.	n.d.
gi|4994868	fliF	flagellar MS-ring protein	2.83	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
gi|4994897	lfiH	lateral flagellar assembly protein FliH	3.21	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
gi|4995019	lafK	two-component system lateral flagellar response	3.98	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
gi|4995734	fliP	flagellar biosynthesis protein FliP	n.d.	n.d.	n.d.	-2.03	2.08	n.d.	n.d.	n.d.
gi|4995864	lfiR	lateral flagellar biosynthetic protein LfiR	n.d.	n.d.	n.d.	n.d.	2.09	n.d.	n.d.	n.d.
gi|4996475	lfiN	lateral flagellar motor switch protein; lfiN	n.d.	n.d.	n.d.	-2.70	2.35	n.d.	n.d.	n.d.
gi|4996534	lfhA	lateral flagellar biosynthesis protein	n.d.	n.d.	n.d.	n.d.	2.09	n.d.	n.d.	n.d.
gi|4996678	lfiI	lateral flagellar FliI-like assembly ATPase	2.79	n.d.	n.d.	n.d.	2.40	n.d.	n.d.	n.d.
gi|4997040	lfiE	lateral flagellar hook basal body protein; LfiE	3.56	n.d.	n.d.	n.d.	2.14	n.d.	n.d.	n.d.
gi|4997975	lfiJ	lateral flagellar protein; LfiJ	2.72	n.d.	n.d.	n.d.	2.28	n.d.	n.d.	n.d.
gi|4998265	fliG	flagellar motor switch protein G	3.48	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
gi|4998379	lfiM	lateral flagellar motor switch protein; LfiM	n.d.	n.d.	n.d.	-2.60	2.31	n.d.	n.d.	n.d.
gi|4997275	lafF	LafF	n.d.	n.d.	n.d.	n.d.	2.22	n.d.	n.d.	n.d.
gi|4995023	flgE	flagellar hook protein FlgE	2.25	n.d.	n.d.	n.d.	2.65	n.d.	n.d.	n.d.
gi|4997204	lfgD	Unknown	n.d.	n.d.	n.d.	n.d.	3.18	n.d.	n.d.	n.d.
gi|4997903	flgC	flagellar basal body rod protein FlgC	n.d.	n.d.	n.d.	n.d.	3.67	n.d.	n.d.	n.d.
gi|4998375	lfgF	lateral flagellar basal-body rod protein LfgF	n.d.	n.d.	n.d.	n.d.	2.83	n.d.	n.d.	n.d.
gi|4998468	lfgH	lateral flagellar L-ring protein; LfgH	n.d.	n.d.	n.d.	n.d.	2.36	n.d.	n.d.	n.d.
gi|4998939	lfgG	lateral flagellar basal-body rod protein LfgG	2.22	n.d.	n.d.	n.d.	2.75	n.d.	n.d.	n.d.
gi|4998986	lafX	LafX	n.d.	n.d.	n.d.	n.d.	2.01	n.d.	n.d.	n.d.
gi|4995422	cspD	cold shock domain-containing protein CspD	-3.44	-5.00	n.d.	7.16	-4.53	-5.12	n.d.	n.d.

n.d. – not defined

5.4 Discussion

Three factors such as host, pathogen and environment decide about the disease. Environment is where all nutrients are acquired. All bacteria require energy to grow and replicate. Non-pathogenic bacteria obtain it from the environment or the host in a symbiotic manner. Pathogens transiently derive their nutrients and energy parasitically or destructively from their host organism. Damaging the host cells or killing allows catabolizing many macromolecules which are accomplished by the synthesis of a set of virulence determinants. Therefore, it is not surprising that nutrient availability regulates also the virulent capabilities of bacteria.

To some extent, the environment is the factor that can be controlled in fish farms (temperature, water salinity, etc.). Nevertheless, it still affects both the host and pathogen. As shown by growth curve establishment experiment, the generation time changes with the medium composition which can also suggest that bacteria immediately adapt to the new situation by compensating their metabolism speed and protein composition [190,191]. Additionally, when the infection occurs the bacteria switch on the machinery allowing them not only to gain necessary nutrients from fish body but also facilitate survival and counter host defense mechanisms with the help of moonlighting proteins [169,192–195]. on the other hand, host innate immune defense protects in the first days of infection the host and minimizes the risk of damages done by the bacteria that may lead to death [62]. With its help, the adaptive arm of the fish immune system acts to prevent long term harm. The balance between them must be kept so that the fish stay in good health. If the balance is shifted to bacteria side the disease appears.

Bacterial transcriptome activity after the distraction of the balance was in focus of this part of the thesis. However, due to the complexity of the in *vivo* model, the initial limitations had to be introduced by elimination or modulation of single environmental condition to see which factors contribute most to disease.

Therefore, in *vitro* conditions had limited variables to a minimum and yet were adjusted to that they mimic a state what bacteria may encounter in fish body to see the changes in transcriptome. The conditions stayed artificial and the whole experiment was kept in *vitro*. Moreover, the selection of conditions was dictated by literature indications. The main and most important conditions of this experiment were the limitation of calcium and iron. Those two elements obviously play a role in virulence of *A. salmonicida in vivo* as to establish infection bacteria needs them to survive within a host, defeat host defense and grow. It seems that fish cells demand a high amount of calcium for their basic metabolism. As mentioned by Burr limitation of iron leads to the production of siderophores

that enable the bacteria iron acquisition from host proteins like transferrin or ferritin using higher affinity feature [196–199]. Furthermore, calcium is an element that is necessary also in cell metabolism for example in proliferation process [200–202]. on the other hand, its depletion affects the production of AexT and T3SS in many gram-negative bacteria such as *Pseudomonas* sp., *Yersinia* sp., *A. salmonicida* strain JF2267 and *A. hydrophila* AH-3 [95,203–205]. Strikingly, our study showed no change in expression of AexT gene in any of tested conditions. Similarly, other strains, for example, *A. salmonicida* A229 and *A. hydrophila* SSU also do not induce the expression of T3SS components and AexT in calcium depleted medium [95,206]. This finding opens new fields for research and the question 'why' may be answered by a massive analysis of *Aeromonas* sp. pan-genome as mentioned in Part 4.

On the contrary, anaerobic conditions served as a control as it is known that *A. salmonicida* is facultative anaerobe [207]. Nonetheless, the reason of choosing this condition was the existence of anaerobic bacteria in fish gut including *Aeromonas* sp. as one of natural microbiota [38,208–212]. The elevated temperature of 23 °C was applied to test the genomic plasticity and confirm the results of Stuber about plasmid loss or rearrangements [75,133]. Moreover, as most of the outbreaks occur in the summertime in which bacteria have less oxygen in the water due to increased temperature it was a good opportunity to investigate also this aspect of infections. At the same time, it was an extreme condition which bacteria had to deal with.

As for the technique, the microarray seemed to be the best solution to cover as much as possible of bacteria transcriptome and clearly see the complexity of interactions. To recent knowledge, it was the first time such a complex cDNA microarray was created to investigate the whole *A. salmonicida* transcriptome. Previous research focuses on clinical isolates and differences between them on the DNA level [178]. Additionally, DNA microarray performed by Nash et al. covers only virulent genes from strain A449 strain that in this case served only as a reference base yet here the microarray was composed of genes from whole genome of JF2267 strain which broadens the spectrum of analysis by those not yet annotated ORFs that could not have been found in A449 strain.

It seems that limitation of iron strongly affects the bacteria as the gene regulation was the highest among all 4 conditions which lead to growth restriction what was observed in the growth curve. The literature suggestions of component concentration were followed here, yet the growth restriction was at first striking suggesting that the chosen concentration was too high. Calcium limitation similarly regulated the same number of genes. However, the kinetic pattern was opposite, and more genes were responding in the first 24 h rather than after 48 h. Genes regulated in higher temperature responded faster than during limitation of calcium. This could be explained in the way that bacteria used the remaining non-chelated calcium from the medium which caused a delay in gene

regulation as a response to harsh conditions. On the other hand, increased temperature caused rapid genes expression after the first 24 h and it decreased after 48 h whereas lack of oxygen does not change the gene expression so greatly. This indicated that this is a natural and common condition of *Aeromonas salmonicida* growth and as for facultative anaerobe there were not so many genes involved in adjusted to new conditions. What is more, obviously changing respiration way from aerobic to anaerobic does not require many genes to be regulated.

The next step of the analysis showed that there are genes specifically expressed in a single condition. ORFs detected in bacteria DNA sequence were added to microarray yet due to the lack of detailed description they were skipped in further analysis. Genes that were regulated in all conditions were also not that important for a given hypothesis as the one uniquely expressed due to their potential in the artificial infection model. As expected, bacteria reacted most rapidly in the iron-depleted condition in both time points which indicates the importance of iron in bacterial metabolism.

Microarray output data are always complex and cover many aspects of a single problem. Using Voronoi TreeMaps the relationship between single regulated genes was described and presented in the form of a network. Gene functions were the first and most intuitive categories applied for Voronoi TreeMaps. The aim was to answer the question of which proteins functionally related respond to the four conditions. However, the results were more confusing than explaining the nature of the bacterial response to stimulation. There was a mixture of genes that were up- and down-regulated in most of the function clusters and it was impossible to see the complete networking between all genes adding also the time as an additional dimension. There were slight trends of upregulation visible for flagella motility, protein biosynthesis, cell wall and capsule clusters in calcium depleted condition. As expected, iron acquisition and metabolism cluster were clearly upregulated in iron and calcium limited conditions. The images of Voronoi TreeMaps clustered by function covered many genes yet they were unclear to read. On the other hand, due to the regulation on various levels, the contribution of genes having a different function is normally required to optimally adjust to the change. Some genes may act as either positive or negative regulators of transcription influencing the general outcome.

Another way to analyze indirectly functional network of regulated genes was clustering by regulon. Here, with the help of RegPrecise database, it was found out that regulation of transcription is mainly mediated by the ferric uptake regulator protein Fur [213]. Fur regulon was also the one best described in literature. Fur protein in the presence of iron acts as the transcription repressor. Fur protein with ferrous iron forms is a complex which binds regulatory DNA (Fur-boxes) and by this inhibits the transcription of genes involved in iron acquisition. Iron starvation is a signal for

pathogenic bacteria indicating in most cases that they are inside the host which initiates the production of virulent proteins [214–216]. Depletion of iron causes activation of Fur regulated genes which is widely documented in gram negative and positive bacteria including human pathogens such as *Neisseria* sp., *Salmonella enterica* sv. Typhimurium, *Helicobacter pylori, Bacillus subtilis* [217–220]. For example, calcium and iron regulate the expression of T3SS in *Vibrio parahaemolyticus* [221]. Presumably, the basic mechanisms of iron acquisition in *Aeromonas salmonicida* stay similar yet it requires further detailed investigations. The results showed a general tendency of gene upregulation to the limitation of iron a bit weaker after 24 h and stronger in 48 h time point which also was confirmed by Hirst [222]. In contrast to what Najimi has shown, some genes (hutA, asbD, asbE) of Fur regulon could not be identified which indicates high gene (regulon) diversity [160]. Moreover, less distinct but noticeable upregulation was observed for calcium limitation which can be explained by the fact that although EGTA has a high affinity for Ca^{2+}, it can also chelate other ions as Fe^{3+} [221,223].

Gene response of Arg regulon indicates its high sensitivity to condition change. Research done in gram negative and gram-positive bacteria shows that it might be important for pathogenesis. For example, Ding and Christie showed in *Agrobacterium tumefaciens* that arginine is important for flagellation and chemotaxis [224]. Similarly, arginine impact on virulence in *Staphylococcus aureus* was investigated by Zhu [225]. Other bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Laribacter hongkongensis*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Legionella pneumophila*, and *Mycobacterium bovis* were also investigated for its metabolism of arginine and importance for virulence[226–233]. Regulation of arginine production and its acquisition by bacteria is important as from the host side this amino acid regulates many immune processes. Both sides compete for it. Arginine-derived nitric oxide also plays a significant role in inflammation and immunity affecting most immune cells, including T cells [234]. As proved for human and mice, it has a direct effect on cell proliferation and T-cell activity in *vivo* and in *vitro* [235,236]. Moreover, macrophages use L-arginine as a major substrate for many of their functions [237]. Furthermore, phagocytosis by neutrophils and adhesion of polymorphonuclear cells is protective and enhanced [238]. The importance of arginine is *Aeromonas* sp. pathogenesis has not yet been described, yet many studies present the metabolism on arginine and its importance for growth [239,240]. The results were unpredictable as it was expected that genes from Arg regulon should be upregulated in iron or calcium limited conditions because they imitate the environment in the host, yet it was opposite.

The question that could not be answered here was whether acquisitions of PAI can transform attenuated bacteria into a pathogen as the regulation of PAI localized genes was not outstanding. It would require further investigation. The evolution driving forces are mainly point mutations,

genomic rearrangements and horizontal gene transfer. While point mutations are considered as slow progress in evolution, the rearrangements and horizontal genes transfer can quickly generate new variants of the same species what could be seen in previous part of this thesis. As they encode longer DNA sequences, in most cases operons of virulent genes are horizontally transferred between species. Pathogenic islands first mentioned by Kaper and Hacker were firstly described in *E. coli*. [241]. Later many other PAIs were described and direct contribution in species such as *Yersinia* sp., *Salmonella* sp., *Shigella* sp. *Vibrio* sp., *Pseudomonas* sp. was proved [242]. With the help of bioinformatics *Aeromonas* sp. PAIs were also detected in A449 strain and partially in JF2267 as the equivalents.

Finding pathogenic island affiliation was another feature that helped to localize the important genes in the first days of infection. Unfortunately, many genes belonging to PAI of *A. salmonicida* were gone or if they were present then the regulation was not that outstanding as expected. It looks that many genes of PAI were missing yet some islands stayed untouched in comparison to PAI described in the database. One of the theories suggested by Blum says that PAI deletion is a way of virulence regulation [243] and that by limiting the genomic size pathogens can be more effective during the infection. It is widely known that *A. salmonicida* is non-motile bacteria and the reason for lack of motility was also proved by Merino [244]. As mentioned by Erhardt flagella proteins have their homologues in T3SS and it was shown in other bacterial species effector proteins might be secreted via flagella system instead of T3SS [245–247]. Worth noticing is that there is a regulation of flagella genes on the transcriptomic level. Whether the single proteins are produced should be still tested by any proteomic method. Nevertheless, Vanden Bergh did not find any functional lateral and polar flagella in *Aeromonas salmonicida* proteome [115]. Yet, it gives a field for interesting speculations because there is a positive regulation of TLR5 in rainbow trout peritoneal cells during the infection which is the main receptor for bacterial flagella as it was shown by Korytář [62]. Therefore, it is possible that there may be a selective individual substitution of T3SS homologue proteins with flagella one being recognized by TLR5 during the infection. Another possibility is that TLR5's recognition spectrum is higher than researchers expected or TLR5 may recognize conserved motives in T3SS proteins.

All above mentioned analysis gave a possibility to fulfil the aim of this thesis which is to define the new bacterial virulence factors in *A. salmonicida* required to establish an infection that was not described earlier in *Aeromonas* sp. but in other bacterial species. Presumably, those factors should be recognized by the trout innate immune system as the infection process progresses very fast and first symptoms are visible after 2-3 days as reported by Korytář [62]. That brought one to the conclusion that the virulence factors, that the fish have to face in the first place, have to be either secreted to the environment or localized on the outer side of the cell membrane to be visible for immune cells and

the amount of gene transcripts has to increase. Alternative criteria were also other reported in literature virulence genes of irrelevant localization since dead cells or cells lysed in course of immune defense free the cytoplasmic connect that can also contain ligands for immune receptors. Genes fitting those criteria were selected and identified as a set of receptors localized on the surface (ferric siderophore receptor, hemin receptor, colicin receptor) or secreted proteins (protease II, isochorismatase, bacterioferritin, LasA protease). Surface receptors were suggested as they may contribute to virulence by optimizing environmental conditions and facilitating survival inside the host. As reported in *E. coli* ferric siderophore receptor fhuA transports colicin M, microcin 25. TonB-dependent ferrichrome transport, albomycin, and rifamycin CGP 483 [248]. Similarly, outer membrane heme receptor is overexpressed in iron-limited conditions suggesting importance in an iron acquisition which was also shown by Najimi [160]. Comparably isochorismatase entB, which is an enzyme involved in the biosynthesis of the catechol siderophore enterobactin in *E. coli* showed increased regulation [249]. Thus, some of the genes will be further investigated in functional interaction experiments to see whether they are responsible for the stimulation of the innate immune system.

To sum up, the world of infectious bacteria seems to be on one hand broadened each day by scientists but on the other hand, there are still many things unknown that need better and more detailed description. Most interesting is the fact of interaction and recognition of pathogen by the immune system. The model of bacteria of the same origin but different virulence gave one a chance to answer questions about differences between apathogenic and pathogenic bacteria. As it is known, even commensals possess in their genomic repertoire some features (LPS, DNA, RNA, surface layer proteins) that appear also in pathogenic strains of the same species. It seems then that in course of evolution host developed tolerance to them up to some levels and there are many yet not described factors contributing to the disease. In this thesis, it was shown that a large number of genes was regulated by single change of environmental condition. Depletion of one component from the medium that is important in pathogenesis induced not only metabolic genes but also those responsible for the infection process. The selection methods applied here helped to limit the number of presumably virulent genes and make a choice for functional studies. This study not only helped to identify them but also showed the approach and unique perspective of research that can be applied to other interaction studies.

6 Rainbow trout innate immune response in early stages of infection with *A. salmonicida of* different virulence profiles.

6.1 Introduction

Aeromonas salmonicida and rainbow trout interaction has been studied for years putting pressure on trout immune response and bacteria serving as stimulus agent. Different environments, different conditions affect not only fish but also bacteria which adapt their infection profile. Each strain apart from common features has its unique characteristics of infecting fish. This massive group of different *Aeromonas salmonicida* isolates could be seen in bacteriological literature.

Researchers evaluate bacteria environmental isolates in search for new virulence features, bacteria sensing strategies [250], new vaccine or probiotic supplementing solutions [251] and try to characterize bacteria by various phenotype or genotype traits. Starting from describing biochemical characteristics and brown pigment production in typical *A. salmonicida* environmental isolates by Wiklund et al or Hanninen and Koski [252,253] through more sophisticated and detailed genomic transcriptomic and proteomic analysis of single virulent molecules or mechanisms such as siderophore production [254], superoxide dismutase impact, *Aeromonas* sp. exoenzyme T (AexT) impact of cytoskeleton [255], type three secretion system function and structure [256] and finishing on advanced global study in comparative genomics [257,258] or microbiome effect of infections giving the overview of whole mechanism of interaction and response. All those projects give insight only in part of the story.

Interaction study between rainbow trout and *A. salmonicida* as pathogen is also such a part of selected research. As observed before, furunculosis develops fast. Korytář et al. has shown that the first days of infection are crucial for immune system response [259]. Innate immune system senses danger signals through a set of germ-line encoded pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are one group of ubiquitously expressed receptors in immune cells recognizing conserved molecular motifs known as pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) such as LPS, peptidoglycan, lipoteichoic acids, non-methylated CpG motifs, single and double stranded DNA and RNA. Firstly, described in Drosophila TLRs are typical type I transmembrane proteins localized either on the surface of innate cells or in intracellular vesicles such as endosomes or lysosomes. They are composed of three domains: N-terminal ectodomain of leucine rich repeats (LRR) motifs, transmembrane domain and a cytoplasmatic Toll/Interleukin (IL)-1 Receptor (TIR) domain [260]. LRR are diverse, long horseshoe shaped 20 – 30 amino acid chains and are responsible for sensing

the PAMP whereas TIR domain transfers the signal to downstream cascades activating acquired immunity signaling pathways.

TLRs are common in vertebrates and invertebrates' lineages. To date, at least twenty-five functional TLR have been identified in vertebrates, at least 13 TLRs in mammals, 22 TLRs in fish [260]. Teleost fish TLRs include mammalian orthologs (TLR1, TLR2, TLR3, TLR5, TLR7, TLR8, TLR9) and fish-specific (soluble TLR5, TLR14, TLR19, TLR20, TLR21, TLR22, TLR23-25) [260–262]. Not all listed TLRs have been reported in all fish species. Not all fish TLRs have their ligand and function identified and their specificity is in most case presumed based on transcriptional studies.

In the presented study the model was expanded to show the early immune signatures of trout to *A. salmonicida* infection and immunization using bacteria of different virulence profile. Early innate immune response was in focus to answer the question what happens shortly before fish start to die from furunculosis and what is the influence of bacteria strain in whole infection. Innate response of erythrocytes and myeloid fraction was assessed by stimulation with bacteria having different virulence profiles. The first recognition system including TLR signaling or complement response starting the chain reaction of immune defenses are investigated here. First signatures of bacteremia in fish blood are presented by extracellular proteins impact on erythrocytes.

6.2 Materials and methods

6.2.1 **Fish strains and handling**

The commercially available rainbow trout (*Oncorhynchus mykiss*) Steelhead TCO (Tacoma, USA) and Born lines were obtained from the Landesforschungsanstalt für Landwirtschaft und Fischerei, Born, Germany and Binnenfischerei Mecklenburg GmbH Schwerin in Frauenmark, Germany.

For this study animals were kept in 1000 l tanks at 15 °C and 12 h day/night light period. Fish were held at 15 °C in 400 l tanks in a partially recirculating water system and were fed with commercial dry pellets. Fish were at age of one to two years during sampling. During each fish handling a dose of benzocaine was used as anesthetic agent. Fish were euthanized by overdose (100 mg / l) of benzocaine (Sigma, Germany). Section of each fish as documented in images below (Figure 19).

Figure 19. Rainbow trout section.

Plan of experiments below was included in Figure 20.

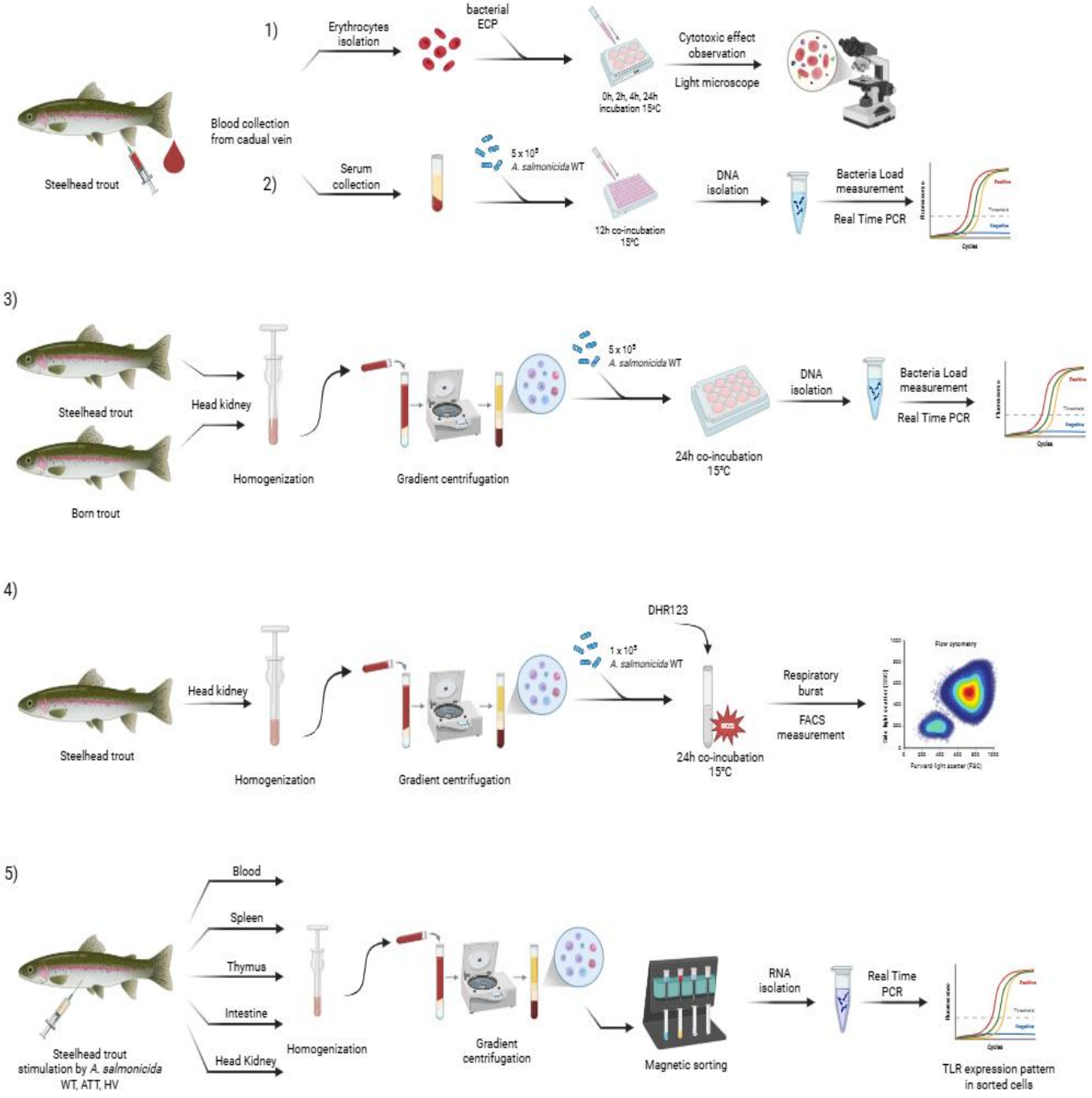

Figure 20. Plan of interaction experiments of *A. salmonicida and* rainbow trout immune cells. 1 – Bacteria ECP influence on fish erythrocytes, 2 – Serum influence on bacteria growth, 3 – Bacteria growth in culture with head kidney leukocytes, 4 – Respiratory burst in head kidney leukocytes after incubation with a *salmonicida*, 5 – TLR expression pattern in leukocytes subpopulations.

6.2.2 **Primary cell culture preparation**

Blood (1 ml) was collected from the caudal vein using a heparinized syringe and immediately diluted in cold medium (Mixed Iscove's DMEM/Ham's F12 (Gibco)) at a ratio of 1:5. Head kidney, spleen and thymus isolated from fish were homogenized with Potter-Elvehjem homogenizers to prepare single cell suspension in 5 ml of ice cold PBS containing 5 mM EDTA. Middle section of intestine was removed from fish aseptically. Intestine was cut along, food content was removed, intestine was washed with ice cold PBS and homogenized in 5 ml of cold PBS with 5 mM EDTA with Potter-Elvehjem homogenizers. The obtained cell suspensions were layered onto an isotonic Percoll gradient (Biochrom AG) (r = 1.075 g / ml) and centrifuged at 1,800 rpm (650 g) for 40 minutes. Cells at the Percoll / medium interphase were collected, washed with PBS with 5 mM EDTA, suspended in corresponding volume of ice cold medium (blood, spleen) or PBS-EDTA (head kidney, thymus, intestine) to the final concentration of 5 x 10^6 cells / ml and kept on ice until further preparation. Erythrocytes that passed an isotonic Percoll gradient and pelleted in the bottom of the tube were collected and washed with PBS-EDTA, suspended in corresponding volume of medium to the final concentration of 1 x 10^7 cells / ml and kept on ice until further preparation.

6.2.3 **Bacteria inactivation**

The bacteria *Aeromonas salmonicida* ssp. *salmonicida* strain JF2267 WT, ATT, HV was obtained from professor. Joachim Frey, University of Bern, Switzerland. Bacteria were cultured in LB medium at 18 °C for 24 h before each further experiment. After the culture *A. salmonicida* JF2267 WT strain was inactivated in 1.5 % PFA for 1 h. Afterwards, 10 µl of bacteria suspension was plated on LB agar to prove successful inactivation. Bacteria suspension in concentration of 1.6 x 10^9 cells / ml were frozen in 1 ml aliquots and kept at -20 °C. Prior to the usage, the bacteria were thawed and diluted to the needed concentration in sterile PBS.

6.2.4 **Bacteria ECP influence on fish erythrocytes**

A. salmonicida JF2267 WT was cultured in LB broth at 18 °C for 48 h. After this time, the bacteria were spined down at 7500 rpm for 10 min. Supernatant was collected and pH was optimized to 7.2 with 2N NaOH. Filtered supernatant (sterile ECPs) (filter 0.22 µm) was stored at -20 °C for further use. Erythrocytes were prepared as described in 6.2.2.

Culture six-well plates were inoculated with 5 x 10^5 cells in 500 µl medium. Sterile ECP from the bacteria culture were added in the concentration of 1 %, 5 %, 10 %, 25 %, 50 % and 100 %. Erythrocytes without ECP served as negative control. The cell morphology was observed under microscope after 0 h, 2 h, 4 h and 24 h co-incubation in 15 °C from inoculation. The experiment was repeated twice. Minimum five images were taken from each sample.

6.2.5 **Serum influence on bacteria growth**

Blood (5 ml) collected from naive Steelhead trout (n = 2) was transferred to sterile Falcon tubes. To prepare serum, samples were allowed to clot by leaving them undisturbed at room temperature overnight. After 24 h samples were centrifuged 2000 rpm for 10 min. Resulting supernatant (the serum fraction) was transferred to new tube aseptically. Samples were used immediately after preparation. Aliquots of serum (1 ml) were stored in -20 °C. Serum samples (100 µl) were loaded on 96 well plate in duplicates and were inoculated with 5 x 10^5 cells of bacteria *A. salmonicida* JF2267 WT strain. Samples were incubated in 15 °C for 12 h. After this time bacteria were collected to fresh tube for DNA isolation. Wells were additionally washed with TE buffer. Bacteria were centrifuged 10 min, 8000 rpm. Supernatant was discarded and 180 µl ALT buffer was added. Following, bacterial DNA was isolated according to manufacturer's protocol from DNeasy Mini Kit, Qiagen. Bacterial growth levels were assessed by Real Time PCR based on growth curve measuring gybB housekeeping gene amount in bacteria 10-fold dilutions from 1 x 10^2 to 1 x 10^7 cells. In brief, Real Time PCR was preformed using SensiFAST™ SYBR® No-ROX Kit (Bioline, Germany) on thermocycler CFX96 (BioRad, Germany). The reaction was held in 20 µl volume using: 2 µl of total DNA; 2.5 pmol of forward and reverse primers (Forward: 5'-TCATCATGACTGTGCTGCAC-3', Reverse: 3'-ATGGTCAGCAACAGCTTGT-5'), 10 µl of SYBR Green mix. The PCR reaction parameters consisted of 1 cycle of 95 °C for 3 minutes, 40 cycles of (95 °C for 5 sec; 60 °C for 10 sec; 72 °C for 20 sec), 80 °C final elongation 5 sec. In the end melting curve analysis was also performed to verify the quality of the PCR product and specific product amplification. Each sample was measured in duplicate. Calculation of bacteria growth in sample was based on Ct values with reference to growth curve. Data were presented as the mean values from each replicate. The statistical significance was calculated by one-way ANOVA.

6.2.6 **Bacteria growth in culture with trout head kidney leukocytes.**

Head kidney leukocytes from Steelhead trout (n = 1) and Born trout were prepared as described previously in 6.2.2. Only for the purpose of this experiment the more resistant trout line was used to test the response of head kidney leukocytes and underline its importance of interaction of this cell population. Prepared cells (5 x 10^6 cells / well) were transferred to 2 ml of culture medium (Mixed Iscove's DMEM/Ham's F12 (Gibco)) on 12 well plate in duplicate and were inoculated with 72 h culture of bacteria *A. salmonicida* JF2267 WT (5 x 10^5 cells / well). Mixture of leukocytes and bacteria was incubated for 12 h at 18 °C. Bacteria cells cultured in medium alone served as a control. After this time supernatant was collected and attached leukocytes with bacteria were removed by trypsin treatment for 5 min at 37 °C. Following activity of an enzyme was blocked by washing wells with medium containing 10 % FBS. Samples containing bacteria and cells were centrifuged 10 min, 1,800 rpm in room temperature. Pellet

without the supernatant was taken for DNA isolation using DNeasy Mini Kit, Qiagen according to manufacturer's protocol. The statistical significance was calculated by one-way ANOVA.

6.2.7 Phagocytosis assay in fish leukocytes

The ability to induce phagocytosis was assessed in naïve Steelhead leukocytes to characterize the bacterial phenotype differences. Respiratory burst was measured using FagoFlowEx (Exbio, Czech Republic) which is based on the measurement of respiratory burst of macrophages, granulocytes after their stimulation with the bacteria. During the process of bacteria ingestion, phagocytes activate the NADPH oxidase producing reactive oxidative intermediates (respiratory burst). Resulting hypochlorite ions inside phagocytes strongly oxidize dihydrorhodamine 123 (DHR123) into fluorescent rhodamine 123 which is detected in FITC channel (525 nm) by flow cytometer FACSCalibur (Becton Dickinson, Germany). A positive control sample is stimulated using 10 µl PMA (Phorbol 12 – myristate 13 - acetate, Exbio, Czech Republic) of concentration 30 µg / ml which activates respiratory burst of granulocytes without adhesion and ingestion of the pathogen.

The test was performed according to manufacturer's instruction with slight modifications. Head kidney leukocytes (5 x 10^5 cells) and bacteria (5 x 10^5 cells) were incubated together in medium Iscove's DMEM / Ham's F12 (Gibco) at 18 °C for 0.5 h; 1.5 h and 2.5 h. The cells without bacteria served as the negative control. The positive control was the cells incubated with PMA. The fluorescent reagent DHR123 (10 µl) was added to all the samples 30 min before measuring on FACS to develop fluorescence. Shortly before measurement cells were washed and suspended in PBS-EDTA buffer. The fluorescence of Rhodamine 123. produced by oxidation of DHR123 was detected in FITC channel (525 nm). Measurements of FASC were collected on forward – side scatter (FSC-SSC) plots with specific gating approach determining myeloid and lymphoid fraction as presented in Figure 21.

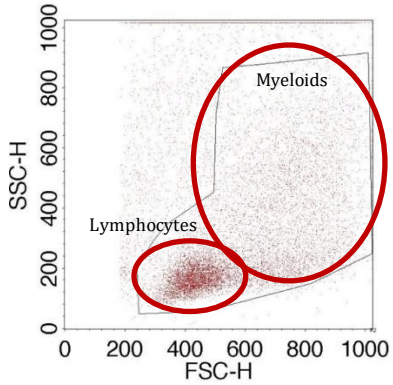

Figure 21. FACS plot showing forward and side scatter defining size and granularity of fish head kidney leukocytes. Main cell populations (lymphocytes and myeloids) gated during FACS meassurement are marked in ellipse red rings.

6.2.8 *A. salmonicida* stimulation experiment of different leukocytes subpopulations

Bacteria stimulation of rainbow trout

Bacteria *A. salmonicida* strain JF2267 WT, ATT and HV were thawed from the stock culture and diluted in sterile PBS to the concentration of 1 x 10^6 cells / ml. Rainbow trout (Steelhead TCO, n = 3) was stimulated with inactivated bacteria by a single intraperitoneal injection (i.p.) containing 200 µl of 1 x 10^6 cell / ml. Control fish received the i.p. Injection of 200 µl of PBS. Leukocytes from thymus, spleen, head kidney, blood and gut were isolated aseptically after 24 h poststimulation as described in 6.2.2. Suspension of cells from each organ was pooled together due to expected low cell count after sorting.

Magnetic sorting of leukocyte subpopulation

Cell subpopulations were isolated by magnetic sorting using specific antibodies. Magnetic cell sorting was applied using Mini Macs on-column cells separation according to manufacturer's protocol (Miltenyi Biotec, Germany). Briefly, 1 x 10^7 leukocytes isolated from each organ was centrifuged 1600 rpm, 10 min. Pellet was suspended in PBS and stained for 30 min in 4 °C with primary mouse antibody specific for selected cell subpopulation (Table 14).

Table 14. Antibodies used for magnetic sorting.

Antibody combinations for sorting	Protein recognition	Specificity	Selected organ	Reference
mab 1.14	IgM	IgM+ B cells	Head kidney	DeLuca et al., 1983
mab 1.14	IgM	IgM+ B cells	Blood	DeLuca et al., 1983
mab D11 / mab 21	n.d.	Subset of myeloid cells	Blood	Castro et al. 2013 Korytář et al. 2013
mab D11 / mab D30	n.d.	Thymocytes, T cells, subset of B cells, myeloid cells	Spleen	Korytář et al., 2010
mab 42	CD41/42 like membrane glycoprotein	Thrombocytes	Spleen	Koellner et al., 2004
mab CD8	CD8	Cytotoxic T cells	Thymus	Takizawa et al., 2011
mab CD8/ mab 89	CD8 / CD40	Mucosal T cells	Intestine	Thi et al., 2013

n.d – not defined.

Cells were washed twice with PBS, each time centrifuged 1,600 rpm, 10 min and labelled with secondary Anti-Mouse IgM Microbeads (Miltenyi Biotec, Germany). All procedures were performed on ice. Magnetically labelled cells were sorted on MiniMacs cell sorter on MS Columns (Miltenyi Biotec, Germany). Following the washing of columns, positive fractions of cells were bound to columns using the magnetic field on MACS Separator. After removing of the magnetic

field, the positive fraction of cells was eluted from the column to the fresh tube. Purity of negative and positive fractions was assessed on FACSCalibur (Becton Dickinson, Germany). Magnetically sorted cells were used further for genetic analysis. RNA from positive fractions was isolated by on-column purification using RNeasy Mini kit (Qiagen, Germany) according to manufacturer's protocol. RNA was used for further analysis of set of pathogen receptors listed in Table 15.

Table 15. Distribution and function of selected receptors in cells and tissues.

Receptor	Cells / Tissue expression	Function	Literature
TLR 1	Spleen, head kidney leukocytes	Forms heterodimers with TLR2. recognizes lipopeptides expressed by distinct species of bacteria	Palti et a., 2010
TLR 3	Leukocytes from liver, intestine, spleen, anterior and trunk kidney tissues	Antiviral response, response to Poly (I:C)	Rodriguez et al. 2003
TLR 5	Monocyte, macrophages, intestinal epithelium	Sensing of flagellin, Fish soluble Toll-like receptor TLR5 amplifies human, TLR5 response via physical binding to flagellin	Tsujita et al. 2006. Tsukada et al. 2005
TLR 7	B cells, plasma cells, dendritic cells, spleen, gills, liver	Sensing of single stranded RNA	Palti et al. 2010. Lee et al. 2013
TLR 8a1	Monocyte, macrophages, gills, spleen, head kidney	Sensing of single stranded RNA	Palti et al. 2008. Lee et al. 2013
TLR 8a2	Monocyte, macrophages, gills, spleen, head kidney	Sensing of single stranded RNA	Palti et al. 2008. Lee et al. 2013
TLR 9	Monocyte, macrophages, B cells	Sensing unmethylated CpG DNA	Palti et a., 2010
TLR 19	spleen, head kidney, gut, brain leukocytes (widespread among tissues)	Sensing peptidoglycan, flagellin, intracellular TLR	Shan et al. 2021
TLR 20	blood leukocytes, gut, intracellular	unknown, ligand	Pietretti et al. 2014
TLR 21	spleen, head kidney, and gills, liver, muscle	Unmethylated CpG-oligodeoxynucleotides	Palti et al., 2006; Qi et al., 2013, Kileng et al., 2011
TLR 22a1	peripheral blood leukocytes, spleen leukocytes, head kidney leukocytes	Sensing LPS	Xiao et al. 2011
TLR 22a2	peripheral blood leukocytes, spleen leukocytes, head kidney leukocytes	Sensing LPS	Xiao et al. 2011

Real Time PCR. TLR expression in leukocytes subpopulations from different organs

Total stimulation of sorted cells was assessed by Real Time PCR using SensiFAST™ SYBR® No-ROX Kit (Bioline, Germany). PCR amplification was performed on thermocycler CFX96 (BioRad, Germany). The reaction was held in 10 µl volume using: 1 µl of total RNA; 2.5 pmol of forward and reverse primers; 5 µl of SYBR Green mix. Primers are listed in supplementary data The PCR reaction parameters consisted of 1 cycle of 95 °C for 3 minutes, 40 cycles of (95 °C for 10 sec; 60 °C for 20 sec; 72 °C for 25 sec). In the end melting curve analysis was also performed to verify the quality of the PCR product and specific product amplification. Total expression was verified by pcDNA plasmid positive control.

6.3 Results

6.3.1 Bacterial ECP cytotoxicity observed in erythrocytes

Incubation of erythrocytes with increasing concentration of extracellular proteins (ECP) revealed their pathological effect of cells. It was the first level of interaction and pathological effect of bacteria secreted proteins on trout cells. ECP concentrations higher than 10 % modified the cell morphology and contributed to cell death. Changes from oval and round shape into lemon-like were observed in cell treated with 25 %, 50 % and 100 % solution of ECPs shortly after 20 min of incubation (Figure 22). Interestingly, those concentrations were high enough to kill erythrocytes through lysis after 24 h which was observed as decreasing cell number in the well and creation of ghost cells (Figure 23). However, lower amounts of ECP were still able to affect cell morphology.

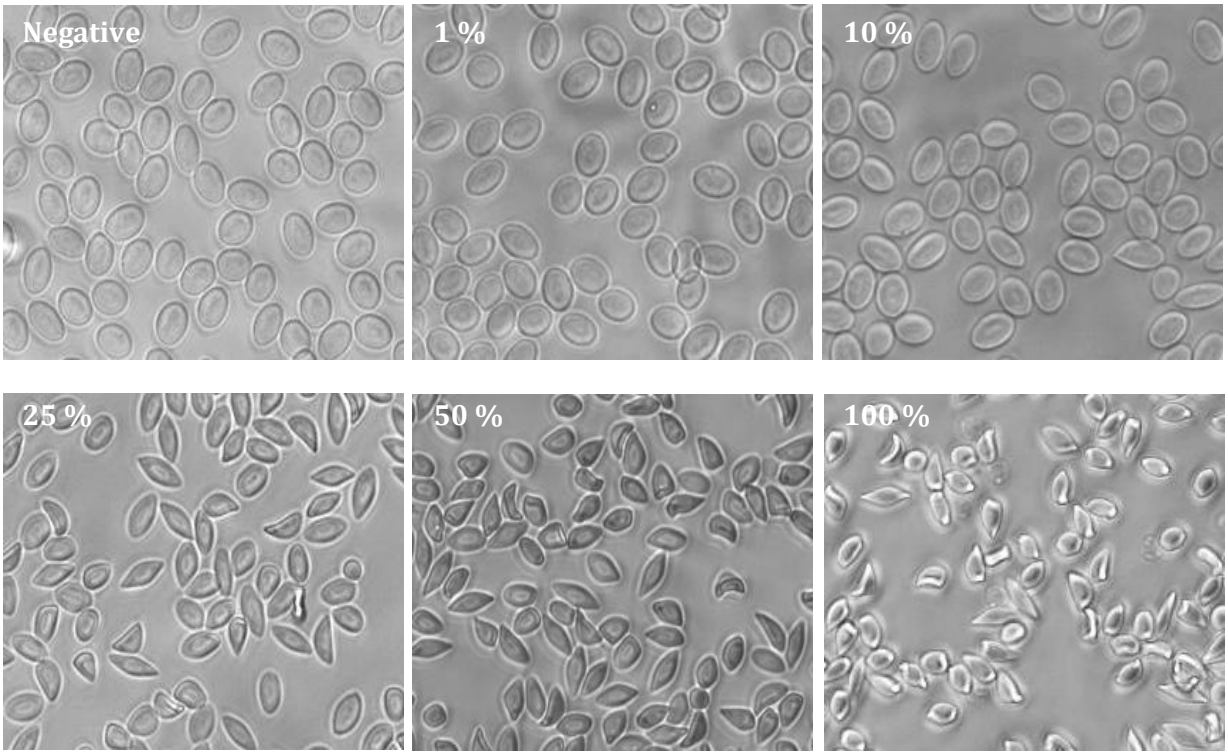

Figure 22. Extracellular proteins concentration impact on trout cells (erythrocytes) after 20 min of incubation. Morphology change of erythrocytes from oval to spindal, deformed and in the end degraded is one of toxicity traits of ECPs. Highest impact is noticed for concentrations > 25 %.

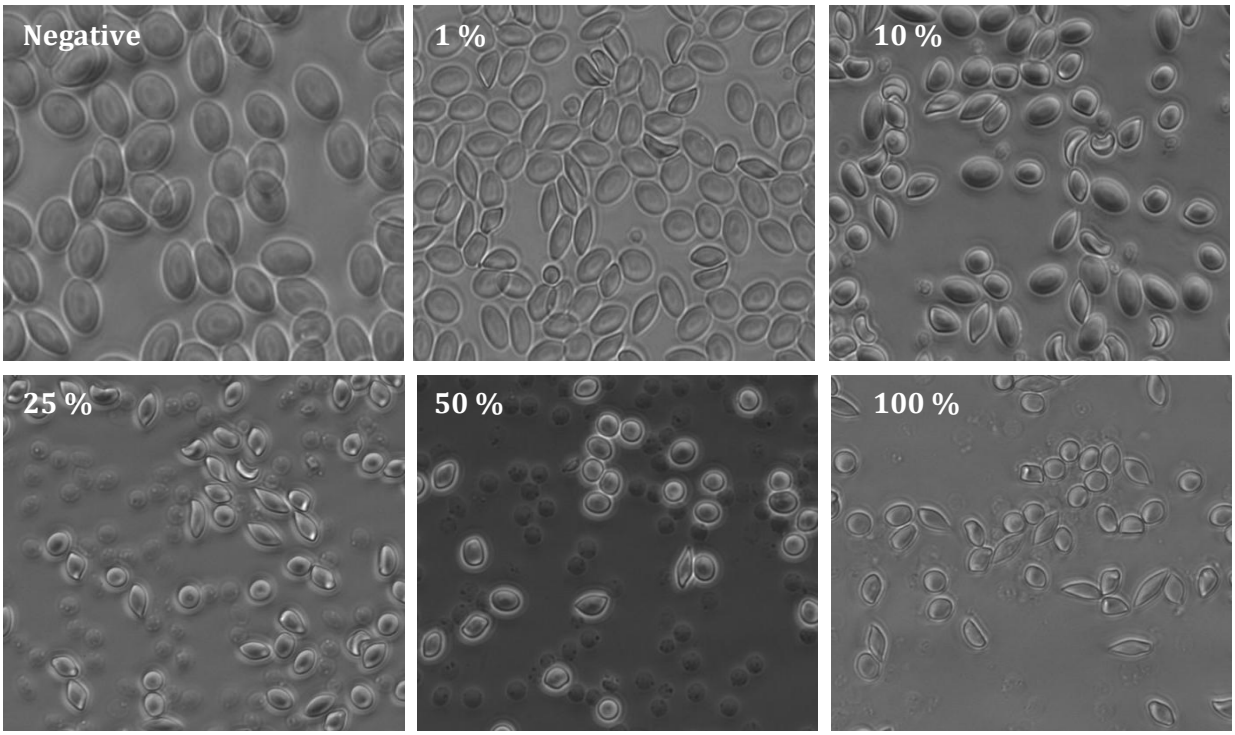

Figure 23. Extracellular proteins concentration impact on trout cells (erythrocytes) after 24 h of incubation. ECPs in high (> 25 %) concentration lyse erythrocytes completely.

6.3.2 Serum inhibits bacteria growth

To check the serum antimicrobial capabilities to restrict bacteria growth during infection we performed a test measuring the bacteria cell number in basic culture medium or in serum originating from Steelhead trout. After 24 h of culture a sudden almost two-fold decrease in bacteria cell number was observed for serum samples Figure 24.

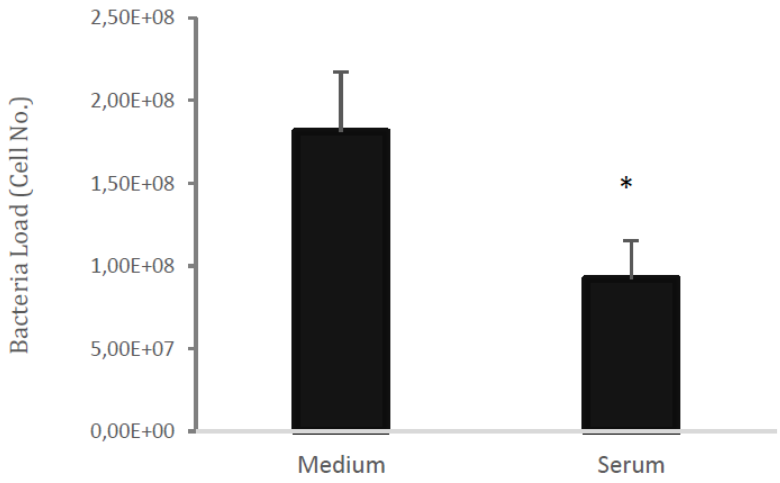

Figure 24. Bacteria growth restricted by Steelhead rainbow trout serum after 24 h of culture. Significant changes counted by one-way ANOVA are marked with (*) for $p \leq 0.05$.

6.3.3 **Bacteria growth is controlled by trout head kidney leukocytes**

After testing antimicrobial effect of humoral part of innate immune system, the ability of immune cells of both trout lines, head kidney leukocytes (HKL) were cultivated with viable *A. salmonicida*. After 12 hours of incubation a significant difference was measured: HKL from robust Born trout impeded the growth about 50 % (Figure 25). In comparison with the growth on LB medium which served as a control the growth with cells was repressed yet more efficient in preventing the growth were Born trout head kidney leukocytes than the Steelhead one.

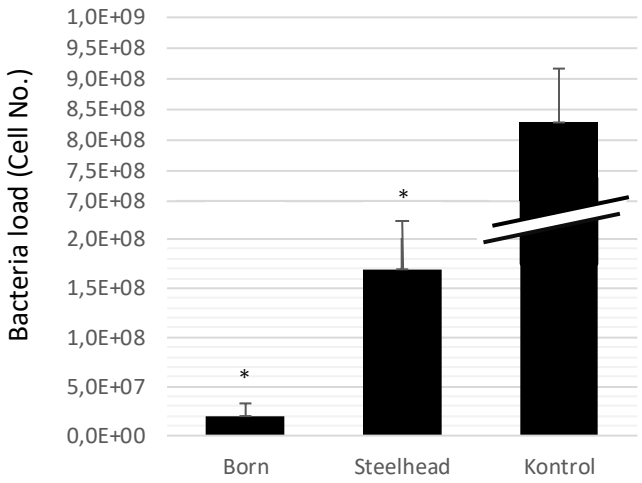

Figure 25. Inhibitory activity of head kidney leukocytes for *A. salmonicida* JF2267 growth.

6.3.4 **Respiratory burst is a signal for bacteria phagocytosis in trout leukocytes**

In the next step the influence of bacteria on fish head kidney leukocytes was measured. The ability to induce phagocytosis in leukocytes by three different bacteria strains was checked by testing the oxidative burst induction abilities. To see in *vitro* difference between the WT, HV and ATT strain viable bacteria strain were used. The population of PMA serving as positive control stimulated myeloid cells responded in all cases with more that 58 % of myeloid cells and it was the strongest after 2 h and 4 h of culture. Moreover, in all cases myeloid cells had the highest response among both the types of cells measured. Their response trend depended on the stimulus and was not changing in time.

As shown on Figure 26 viable *A. salmonicida* ATT strain induced the highest respiratory burst reaching maximum ~55 % positive cells after 4 h and had fluctuating trend while cells stimulated with HV strain showed almost no response with maximum ~2.93 % and throughout all time points stayed low. *A. salmonicida* WT bacteria were able to induce response of cells, yet it decreased gradually from ~30 % of cell population having its peak of ~52 % of cells after 30 min

to ~18 % of responding cells after 4 h. Surprisingly, the response of inactivated bacteria stimulated cells had altogether opposite pattern (Figure 27). Inactivation of bacteria reverses their capability to evoke respiratory burst. Oxidative burst in the ATT bacteria strain also decreased from 40 % to 18 % in first 2 h of experiment yet it rose in the last time point up to 36 % of cells responding in oxidative burst. Interestingly, HV strain stimulated myeloid fraction of cells the strongest and consistently kept them on the level of 34 – 52 % of activated cells and it was the highest among all in each time point. After stimulation with WT the population of leukocytes with respiratory burst was stably decreasing from 18 % to 6 % of cells.

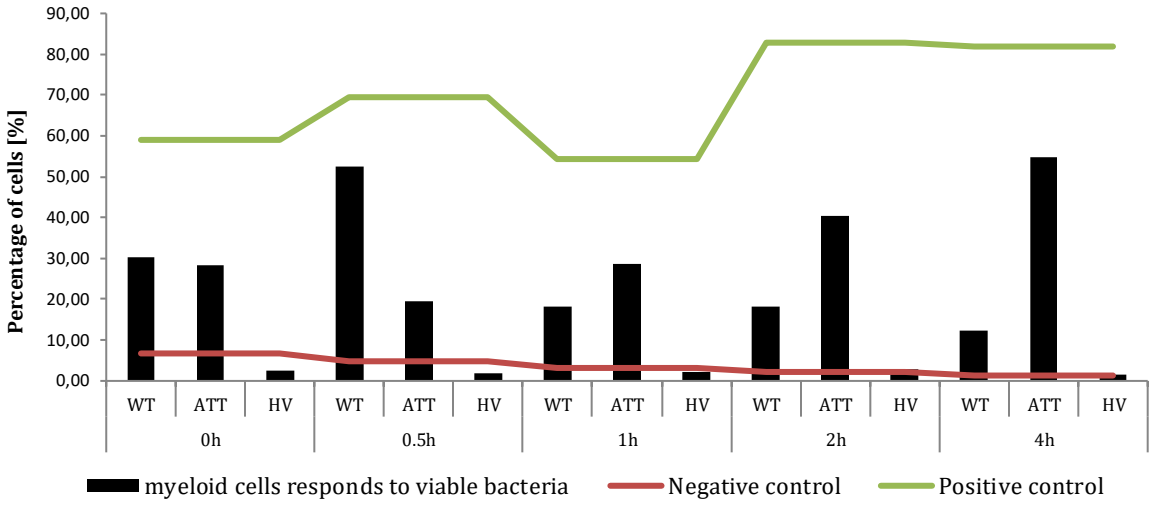

Figure 26. Phagocytosis assay on head kidney leukocytes (myeloid fraction) measured as a level of respiratory burst in cells using live bacteria for stimulation. WT – wild type, ATT– attenuated, HV – highly virulent strains.

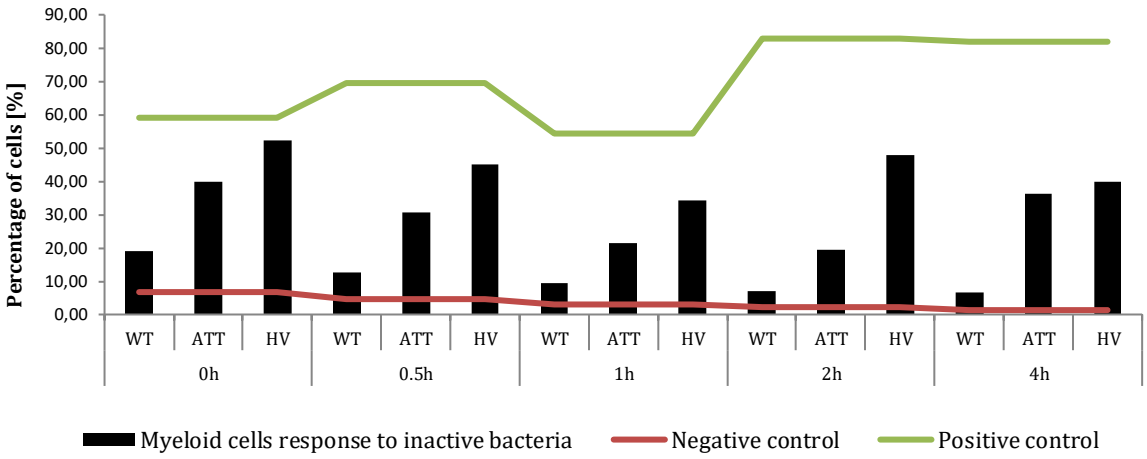

Figure 27. Results of phagocytosis assay on head kidney leukocytes measured as a level of respiratory burst in cells using inactivated bacteria for stimulation. The highly virulent strain induced the strongest while wild type strain the weakest phagocytosis. WT – wild type, ATT– attenuated, HV – highly virulent strains.

The subpopulation of lymphocytes responded as well (Figure 26). In lymphocytes, same as for myeloid cells, highly virulent strain did not evoke respiratory burst while attenuated strain of *A. salmonicida* induced the highest levels of respiratory burst.

Lymphocytes responded much weaker even after stimulation for 4 h. The cells responded hardly more than 2 % after the stimulation with viable bacteria of any strain (Figure 28). However, there was a similar trend as for myeloid cells showing highest oxidative burst in lymphocytes after stimulation by wild type and lowest by highly virulent strain. In contrast to viable bacteria, the capability of inactivated ones to induce respiratory burst was again reversed. Inactivated HV bacteria caused higher respiratory burst in lymphocytes reaching 4 - 6% of total by HV strain. The reversal effect was observed in both myeloid and lymphoid cells. (Figure 29) (Supplement8_phagocytosis).

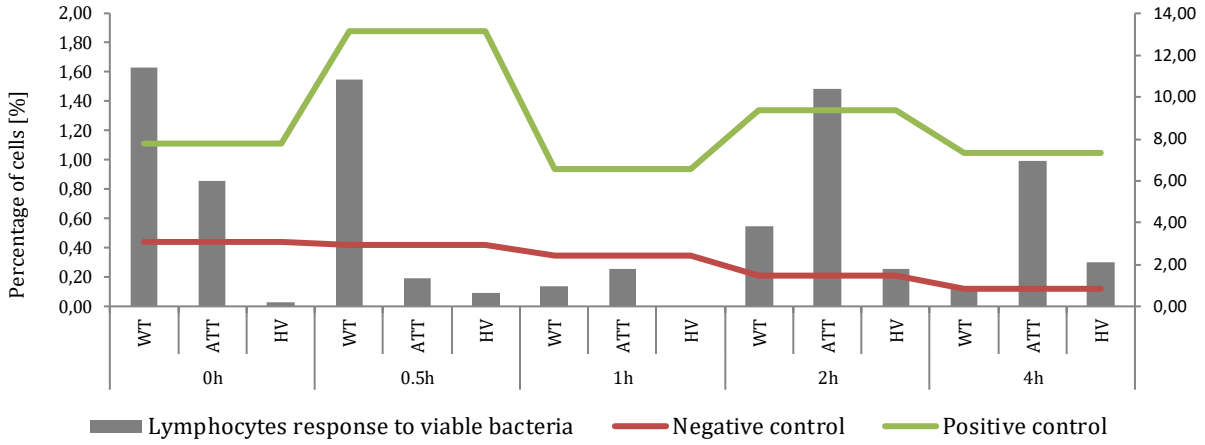

Figure 28. Phagocytosis assay on head kidney leukocytes (lymphoid fraction) measured as a level of respiratory burst in cells using <u>live</u> bacteria for stimulation (left axis). WT – wild type, ATT– attenuated, HV – highly virulent strains. Right axis depicts values for positive control (green).

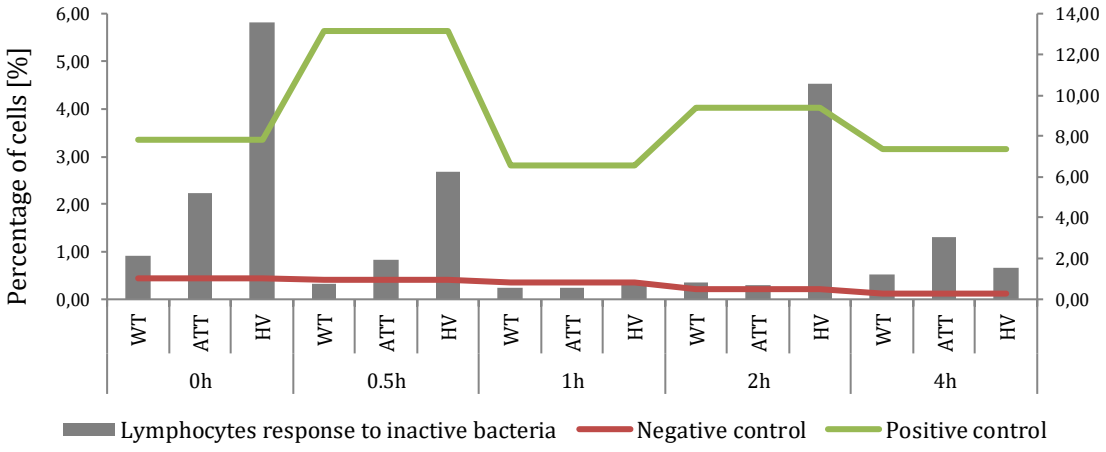

Figure 29. Results of phagocytosis assay on head kidney leukocytes (lymphoid fraction) measured as a level of respiratory burst in cells using <u>inactivated</u> bacteria for stimulation. The highly virulent strain induced the strongest while attenuated strain the weakest phagocytosis (left axis). WT – wild type, ATT– attenuated, HV – highly virulent strains. Right axis depicts values for positive control (green).

6.3.5 TLR expression in leukocytes subpopulations from different organs

The organs for cell sorting were picked for the analysis according to literature predictions of containing most significant toll-like receptors for the immune response. Average expression ratio of TLR panel in blood, head kidney, spleen, thymus, gut in response to *A. salmonicida* stimulation was compared to PBS treatment by their fold change (FC). Generally, the expression patterns did not change strongly except for several most outstanding genes.

Compared to the control group, TLR7, TLR8a1/2, TLR9 showed significant upregulation in B cells in blood following HV stimulation (FC = 4.72, 5.93/7.57, 3.54 respectively). This upregulation was also observed with attenuated strain stimulation yet to a lesser extent (FC = 3.36, 3.67/4.69, 2.57 respectively). TLR1, TLR3, TLR5, TLR20, TLR22a1, TLR22a2 expression level hardly changed after wild type stimulation. B cell had also downregulated TLR21 across all condition (Figure 30).

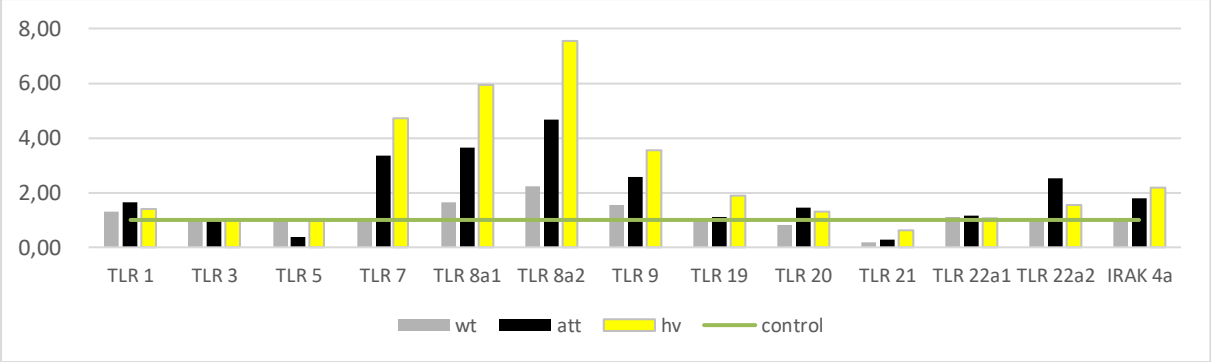

Figure 30. TLR expression in B cells in blood after *A. salmonicida* stimulation.

In contrast to B cells from blood, the overall expression was on lower level. B cells in head kidney had increased regulation of TLR1, TLR2, TLR5, TLR7, TLR9, TLR8a1, TLR21 (FC between 1.53 – 2.87) after highly virulent strain stimulation. TLR19, TLR20, TLR22a1, TLR22a2 and IRAK4a have expression on the level of control samples. Attenuated strain decreased the stimulation below FC = 1 in majority of genes, except from TLR21 (FC = 2.09) and TLR22a2 (FC = 1.51) (Figure 31).

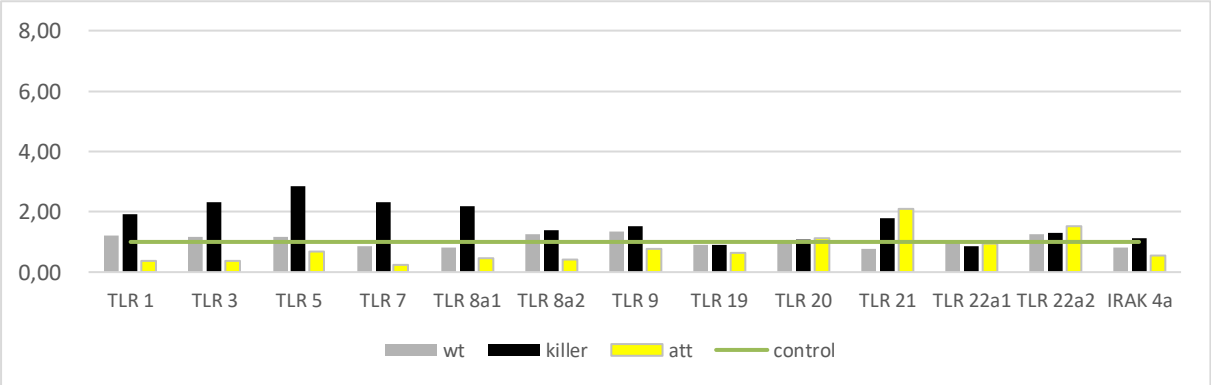

Figure 31. TLR expression in B cells in head kidney after *A. salmonicida* stimulation.

Myeloid fraction in head kidney has increased transcripts levels in case of TLR1, TLR3, TLR5, TLR7 (highest FC = 3.82), TLR8a1, TLR19 after HV stimulation. *A. salmonicida* ATT strain positively regulated only TLR7 (the highest TLR response for this condition FC = 4.32), TLR19, TLR20, TLR22a1. Interestingly, TLR22a1 was upregulated after WT and ATT stimulation (FC = 3.8 and 2.70 respectively), opposite to stimulation of HV strain that underwent downregulation (FC = 0.24). TLR21 after each stimulation was downregulated (FC between 0.14 – 0.26). Wild type bacteria stimulation increased also expression of TLR1, TLR7, TLR9, TLR22a2 (Figure 32).

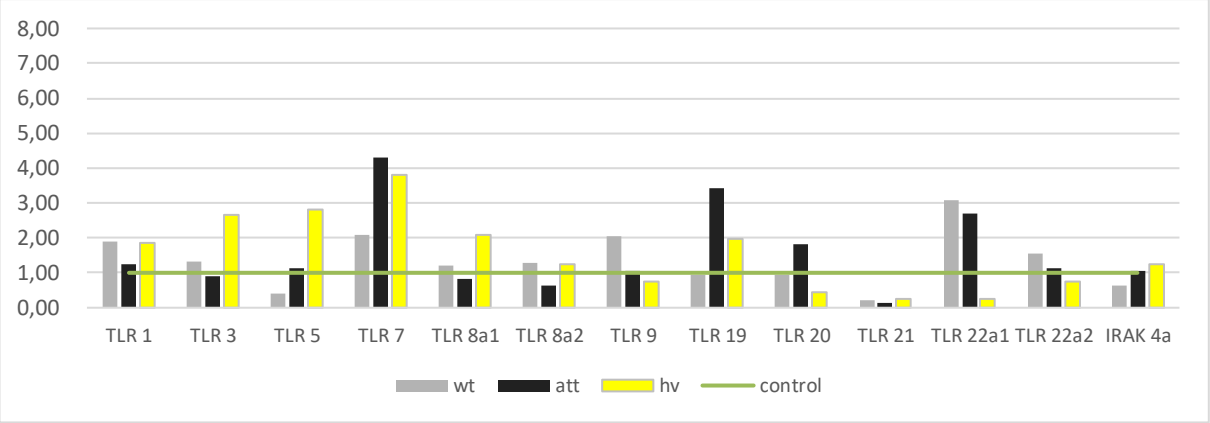

Figure 32. TLR expression in myeloid cells in head kidney after *A. salmonicida* stimulation.

Attenuated strain induced highest response in TLR5 (FC = 5.46) and IRAK4a (FC = 2.89) in spleen myeloid fraction. However, TLR3, TLR7, TLR8a1 showed reduced levels of expression comparing to control. Comparably to myeloids in head kidney, TLR7 was strongly upregulated (FC = 3.89) after HV stimulation. TLR5.TLR8a1, TLR8a2, TLR21 exhibited also high expression in response to HV presence. *A. salmonicida* WT strain was also to induce upregulation only in case of TLR5, TLR8a2 and TLR21 (FC = 1.69 – 2.07) (Figure 33) (Supplement9_TLR-analysis).

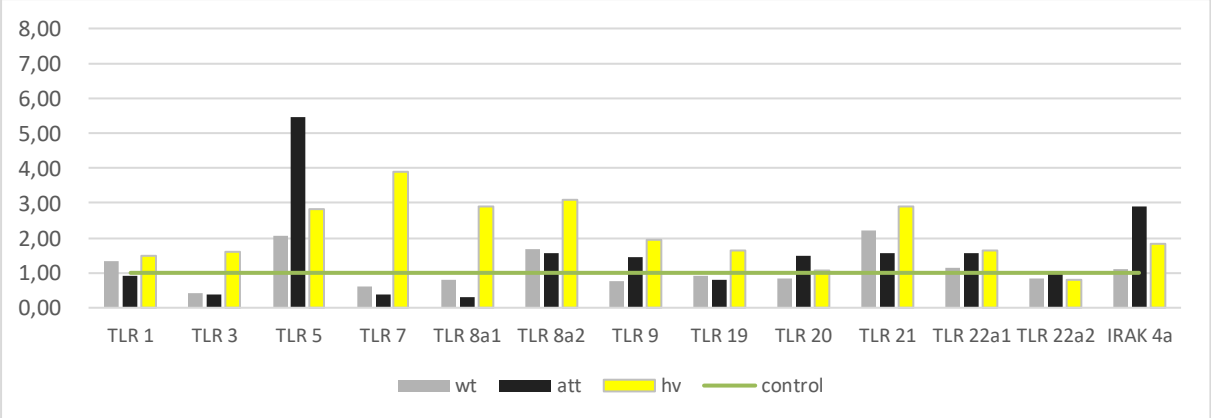

Figure 33. TLR expression in myeloid cells in spleen after *A. salmonicida* stimulation.

After 24 h incubation, levels of TLR5, TLR19, TLR 22a1 in T cells from spleen were close to control sample expression. Transcripts levels of TLR1 (FC = 5.31), TLR3 (FC = 5.11), TLR7 (FC = 5.22), TLR8a1 (FC = 3.38), TLR8a2 (FC = 5.93), TLR9 (FC = 3.58), IRAK4a (FC = 4.60) was

strongly increased after HV stimulation. Here, stimulation by HV strain also resulted in upregulation of TLR1, TLR3, TLR7, TLR8a1, TLR8a2, TLR9 (FC > 1.77). ATT strain upregulated only TLR1, TLR8a2, TLR22a2 (Figure 34).

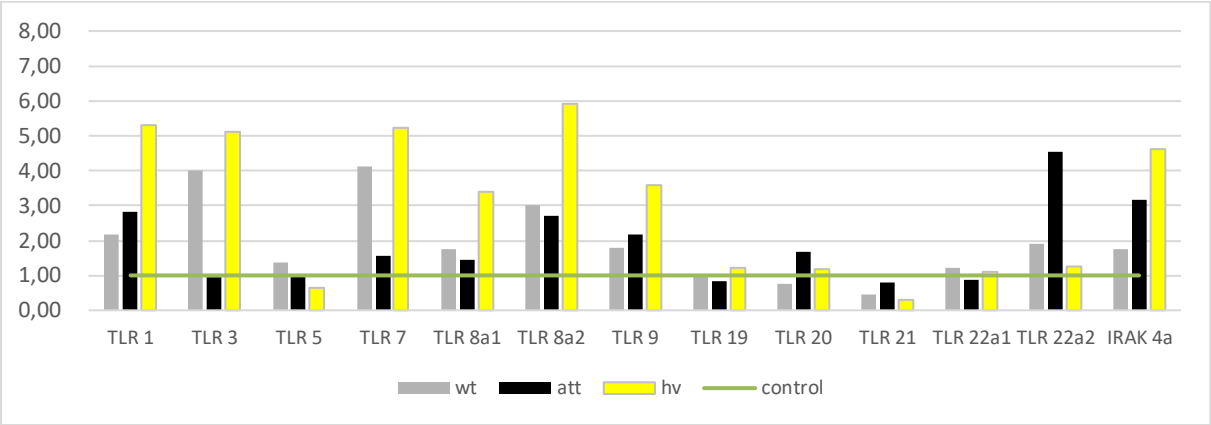

Figure 34. TLR expression in pan T cells in spleen after *A. salmonicida* stimulation.

Mucosal T cells in gut showed higher upregulation of TLR1, TLR8a1 and TLR22a2 after HV stimulation. However, TLR8a2 exhibited increased transcripts levels after stimulation with ATT strain (FC = 3.31). Other TLRS (TLR5, TLR7, TLR9, TLR19, TLR21, TLR22a1, IRAK4a had expression levels close to controls (Figure 35).

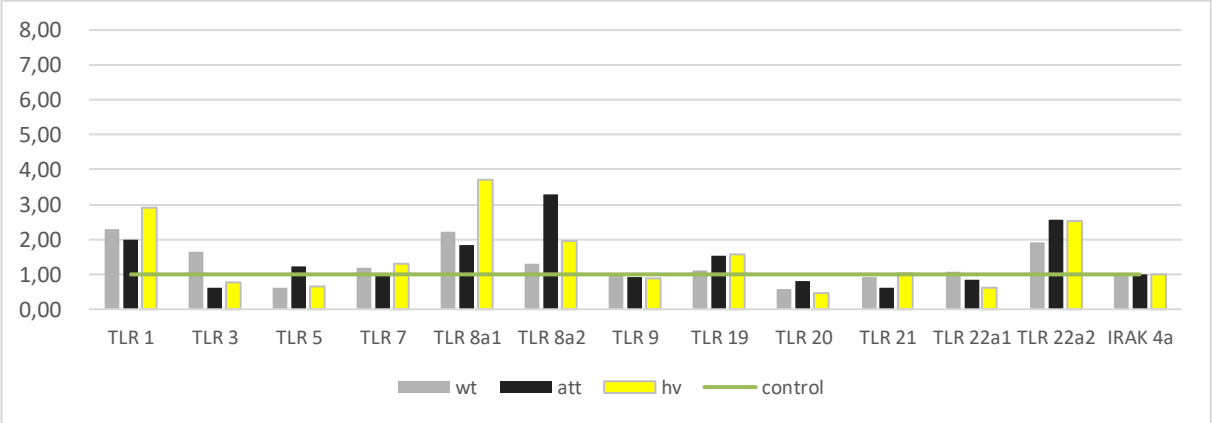

Figure 35. TLR expression in T helper cells in gut after *A. salmonicida* stimulation.

Cytotoxic T cell in thymus were very mildly stimulated in each group. There was hardly any change in expression in TLR7, TLR8a1, TLR8a2, TLR9, TLR20, TLR21, TLR22a1, IRAK4a transcripts. The most visible stimulation involved TLR1 (FC = 3.70 – 4.63) for each strain and TLR19 stimulation by HV strain (FC = 6.04). TLR3, TLR5 expression levels were below controls. *A. salmonicida* HV strain caused downregulation of TLR22a2 gene. (Figure 36).

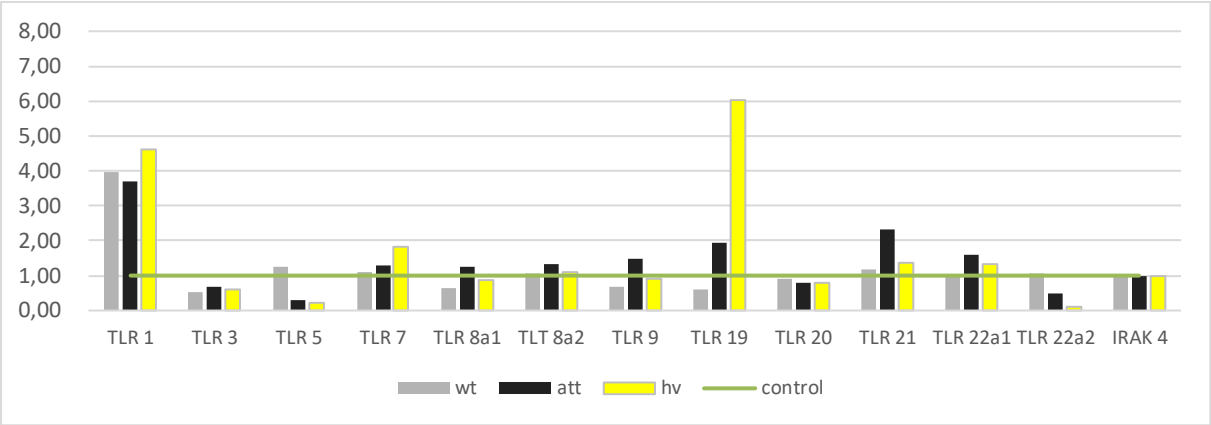

Figure 36. TLR expression in cytotoxic T cells in thymus after *A. salmonicida* stimulation.

Generally, TLR expression levels in spleen thrombocytes were decreased or similar to controls. The only outstanding expression was noted for TLR5 (FC = 2.43 – 2.96) for each type of stimulation. After stimulation with *A. salmonicida* HV and ATT strain TLR1, TLR3, TLR8a1, TLR8a2, TLR21 expression levels the decreased (Figure 37).

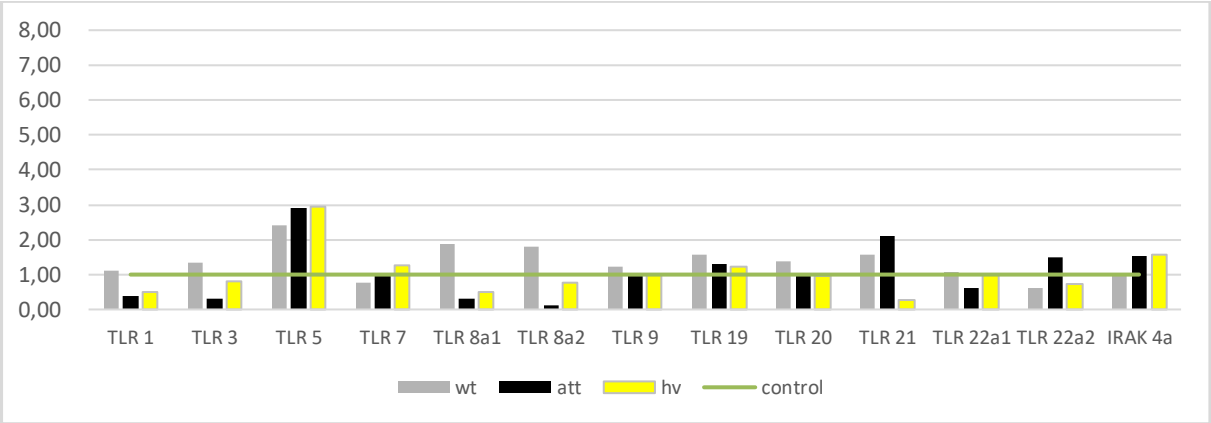

Figure 37. TLR expression in thrombocytes in spleen after *A. salmonicida* stimulation.

6.4 Discussion

The main aim of immune cells is to determine self from non-self. By sampling the resident microbiota host immune cells learn the patterns through receptors and bind relevant ligands. In this way, cells can respond to the infections and develop the immunological memory for further infections. The innate immune cells (e.g., antigen-presenting cells like dendritic cells, macrophages, and granulocytes) are responsible for the sampling of environment and presenting the variety of antigens to the adaptive arm of the immune system or simply destroy foreign material without specificity.

Interaction between the pathogen and fish host is always a complex picture with many varying factors. This study uses the model where bacteria with different virulence profile gives a field to explore the mechanism of infection.

The trout in fish farms are swimming for most of the time in overcrowded tanks. This is a reason for interrupting the skin continuity in a form of scratches or bites. Pathogens living in the water have then good access to enter the fish organism and the blood flow. Dominant subpopulation in blood belongs to erythrocytes (1×10^6 cells / mm^3 – 5×10^6 cells / mm^3) that are obviously vulnerable to bacterial extracellular proteins [263]. Within those proteins we can find among others: lipases, hemolytic enzymes, proteases, nucleases, T3SS effector proteins. The experiment has proved the impact of bacterial products leading to erythrocytes death. This experiment did not test bacteria interaction with erythrocytes though. It was constructed in a way to test the harshest conditions of bacteria effector proteins in contact with cells. It can be found in literature though that erythrocytes play a role in immune response, signaling and pathogen clearance in sepsis in human [264] and in animal infections [265,266]. Erythrocytes in rainbow trout are nucleated with a full machinery of genes to respond to antigens including active transcriptionally nucleus (decondensed chromatin), Golgi apparatus. endoplasmic reticulum also including the presence of PRR transcripts such as TLR3 or TLR9 [267].

A study where fish erythrocytes were incubated with bacterial extracellular proteins showed that ECP in the dose dependent manner interacted or even killed erythrocytes in a noticeably short time. Here, *A. salmonicida* showed high hemolytic activities as it was also described by Năcescu and Santos [254,268,269]. During culture with bacteria, secreted proteins firstly changed erythrocytes morphology from round to spindle-shaped and eventually burst them releasing the cytoplasmic content to the environment and leaving only cell ghosts shortly after 24 h. Ghosts cells left after pathogen interference was also noted by Teorell and Kwant [270,271]. As mentioned there the origin of it is an intravascular hemolysis, hemoglobin is dispersed through leakage holes in membrane. As mentioned by [272] this phenomenon may also be a part of erythrocytes immune defense. What is more, the ECPs influence is so huge that cells respond rapidly with morphology change within the first minutes after encountering the ECPs. The concentration of more than 20 % relatively is lethal to them yet it is hard to assess what the ECPs concentration in nature is. Although, the lethal effects in the initial phase of the disease are much slower, fish start dying after the third day that the ECP concentration increases gradually and cannot be higher than 1 %. In contrast, as shown by Valderrama et al. salmon erythrocytes can be invaded by *A. salmonicida* as the first stage of infection getting the useful source of iron [273]. In trout, the intracellular stage was not seen yet. Nevertheless, it has already been shown that trout erythrocytes are important immune cells as they express cytokine-like factor following fungal infections and functional pattern recognition receptor [267]. That brings a conclusion that erythrocytes elements are one of players in bacteria clearance and signaling whilst first contact with bacteria.

Serum is another part of blood derived of clotted erythrocytes. The experiment in this part examined the importance of blood serum and its complement proteins for the defense abilities of viable bacteria. Many studies have described the serum content and behavior from the antibacterial aspect [274]. Trout serum has been tested against bacteria such as *Vibrio anguillarum* or *Flavobacterium psychrophilum* [275–277]. It was shown in this study that trout serum had an inhibitory effect of bacteria growth. The growth inhibition may be particularly important for interaction with erythrocytes. The less bacteria circulating, the less effector proteins to damage cells.

The main immune factor composing the serum are complement proteins circulating in blood. The complement system and the presence of specific antibodies are the part of primary defenses in fish. They make the pathogen more sensible to phagocytosis at the same time activating classical and/or alternative complement cascades for bacteria destruction and/or presentation [278–280]. Complement system is a reason for direct bactericidal effect and bridge interaction between phagocytes and bacteria [281,282]. In poikilothermic teleost fish, both complement activation pathways were described by Miles and Trust long time ago [283–286]. Coating of the pathogen with specific antibodies (opsonization) is necessary for best ingestion and clearance as shown by Griffin in case of the phagocytosis of *Yersinia ruckeri* and rainbow trout leukocytes [278]. In this model the Steelhead trout used in the experiment has never encountered *A. salmonicida* before to produce memory antibodies against *A. salmonicida and* that is why it was assumed that the classical pathway of complement was not triggered. The direct ability to kill A. *salmonicida* was not tested in the experiment so it needs further, more detailed experiments. Such a response of immune system to bacteria may lead to antigenic presentation and bacteria uptake by professional phagocytes. Following experiment focused on the organ that produces most of professional phagocytes – head kidney or anterior kidney located posterior to the cranium [287].

Head kidney is the central hematopoietic organ involved in host immune defenses in fish. It is the equivalent of bone marrow in mammals from function and morphology. The head kidney plays also a major endocrine role in the secretion of cortisol, the major glucocorticoid and mineralocorticoid in fish [288–291]. Thus, it combines the neuro-immuno-endocrine milieu in normal and pathological states. Head kidney leukocytes (macrophages, monocytes, granulocytes) which differentiate there, are composed mainly of cells with phagocytic and antimicrobial activities [292,293]. From its biology, their activity is not specific, yet they play a vital role in the initiation of the adaptive immune response. The myeloid fraction of leukocytes in myeloid phase of infection in peritoneum comes from head kidney [62]. This study proves also that the head kidney leukocytes have a direct inhibitory influence on bacteria growth in culture. The growth of *A. salmonicida* WT with leukocytes originated from Born trout was twice more repressed than with Steelhead

leukocytes indicating more efficient managing the unwanted presence of bacteria in cell proximity which was also seen in *vivo* after trout lines comparison[259]. This activity by nature is non-specific yet can be strengthened by the presence of the complement which was tested in the previous experiment using only serum for bacteria culture. How bacteria interact with macrophage increasing or repressing phagocytic mechanism still needs elucidation. Nevertheless, following experiment emphasizes the role of pathogenic type of bacteria in the innate immune response.

Phagocytosis, the type of endocytosis, is one of innate processes used by different cells to clear pathogens from environment. Cells able to phagocytose such as macrophages, dendritic cells, granulocytes, B cells are called professional phagocytes. After activation of phagocytes enhanced by natural antibodies or complement the bacteria are engulfed into the cell's vacuole forming the phagosome. Lysosomes full of lytic enzymes fuse with phagosome creating phagolysosome and breaks down the pathogen. Waste is released outside the cell by exocytosis. Additionally, antigen presentation occurs by providing antigenic ligands to initiate activation of adaptive immune response in a form of clonal expansion of B cells and T cells.

It is long known that the killing process requires the release of oxygen and nitrogen free radicals via respiratory burst [294,295]. As reviewed by Roos et al., the activation of phagocytic cells activates the NADPH oxidase enzyme which converts molecular oxygen into superoxide which is the precursor of hydrogen peroxide and hypochlorous acid [296]. ROS are generated by the PMN granule enzyme myeloperoxidase[297]. ROS additionally facilitate the release of PMN granule enzymes and peptides that both demonstrate antimicrobial activity[298,299].

A. salmonicida phenotypic characteristic was achieved by interaction experiment between bacteria *A. salmonicida* JF2267 WT, ATT, HV strains of different virulence and trout head kidney cells. Head kidney leukocytes are composed mainly of phagocytic cells (monocytes, macrophages, dendritic cells, neutrophils, B cells). Korytář has shown that during the early infection mainly head kidney myeloid cells colonize the peritoneum supporting the peritoneal leukocytes in the clearance of the bacteria [62]. His result was proved in *vitro* in this study. Head kidney leukocytes were split during analysis into a subpopulation of myeloid and lymphoid fraction. It was clearly shown that the major response was built by the myeloid fraction whereas the lymphocytes responded minimally showing also general lack of ability to oxidative burst.

It is not a surprise that myeloid cells are able to phagocytose foreign material as this is their main innate feature. Increased respiratory burst is a sign of bacteria uptake and phagolysosome creation for further antigen digestion and MHC II presentation for adaptive immune response. However, the viable HV strain inhibited nearly whole respiratory burst in cells as not more than 3 % of fish myeloid cells responded suggesting barely any ingestion nor phagocytosis. Strong repression in response to viable bacteria is contrasted to high respiratory

burst after stimulation with inactivated bacteria. This setup can indicate that HV bacteria actively produce and secrete factors that prevent phagocytosis. As investigated by Chriss and Seyfert production of reactive oxygen species was suppressed also by *Neisseria gonorrhoeae*. Type III secretions system proteins are one of the candidate effectors that may participate in this pathway. Previous results show the over expression of several T3SS proteins in response to calcium and iron limitation conditions, additionally being absent on WT and ATT genome.

The attenuation of the strain enabled trout leukocytes to clear the threat from the environment and in both cases (dead or alive bacteria), the oxidative burst remained on relatively elevated levels. Obviously, viable bacteria produce blockers of respiratory burst in HV comparing to attenuated or wild type strain. This situation is common not only in animal kingdom but also in plants where secreted effectors block the metabolic coenzyme NAPH inhibiting the transfer of electrons to the NADPH oxidases (RBOHs) responsible for ROS generation [300].

The pattern was observed for each time point. At the same time, *A. salmonicida* WT manifested the ongoing effect on leukocytes in time. Inactivation only moderately decreased respiratory burst as if the bacteria pathogenic activity is weaker. As expected, surface of the bacteria cells still was triggering uptake and production of ROS through activation of NADPH oxidase.

The same general reverse pattern during dead – viable comparison for all three bacteria strains could be observed for lymphocyte subpopulation but not on such prominent level as for myeloid fraction. The minimal response of lymphocytes came presumably from a small fraction of phagocytic B cells. This assumption can be supported as, the same as mammalian ones, fish B cells, display also the phagocytic activity towards pathogen[301]. Moreover, bacterial recognition and uptake through C3a/C5a receptors, Fc receptor and TLR9 in phagocytic B cells in teleost has already been described which confirms our results [302–304].

To conclude, the mechanism of phagocytosis suppression in this experiment is yet unknown as the bacteria may e.g. inhibit the cellular metabolism, lead it to apoptotic pathways, stay unrecognized by PRR or enter the cells but inhibit the oxidative burst as it is in case of *Yersinia pseudotuberculosis* [305]. Comparison of all three responses within a time of 4 h implies that on one hand the composition of bacteria themselves, mainly the content of cellular membrane contributes to phagocytosis as in case of inactivated bacteria but on the other hand their metabolites take a significant part, as proved the response of cells to viable *A. salmonicida* HV strain. Whether it is elevated phagocytosis (ATT strain) or inhibited one (HV) of viable bacteria, it all depends qualitatively on the composition of produced effector proteins. Nevertheless, it is clearly visible that more virulent strain is more active towards leukocytes than any other.

TLR expression pattern provided the insight into the receptor response on various cell types originating from different organs. Thanks to magnetic cell sorting it was possible to separate specific cell subpopulations and check overall RNA expression levels in pure cell subsets after stimulation with three differently virulent strains. Overall, the results of this study can be discussed with the results of Brietzke who covered a set of rainbow trout TLRs in an infection challenge experiment. His results suggest that the is no fine candidate out of tested TLR that respond to infection[306]. The advantage of our study is the direct approach of expression analysis of TLRs in different cell subpopulation targeting single cell subset.

Although the expression change was in most cases low comparing to expression in leukocytes from uninfected fish, there were for each cell subpopulation outstanding receptors noticed. Highly virulent strain mostly affected cells expression, comparing to attenuated and wild type strain. Injection of bacteria to peritoneum induced highest response of B cells from blood as the closest to center of infection. It suggests that there may be possible ligand recognition of already digested bacteria inside the cells. In contrast, B cells residing in head kidney did not have a chance to react within 24 h after stimulation.

Despite many studies showing the activation stage in myeloid cells the subset isolated from head kidney presented low expression of all TLRs. Sarais and Korytář discussed in their study the huge influx of myeloid cells to peritoneum during infection to promote first phase of inflammation [259,307]. It is highly probable that the cells that stayed did not participate directly in bacteria clearance from the body and were not in their active state. Nevertheless, more studies should be performed to get the better overview.

Moving on to specific TLR expression, Palti showed that TLR1 is expressed highly in spleen and anterior kidney, followed by posterior kidney which confirms results in this study [308]. Expression analysis indicate that during stimulation with *A. salmonicida* pan T cells from spleen respond with TLR1 upregulation. Compared to WT and attenuated strain, highly virulent bacteria were the most responsive and regulation was the most distinct from all three strains. Previous published study showed that tissue distribution with analysis of cell specificity help to understand the biological processes behind [309]. TLR1 obviously participates in immune response to *A. salmonicida* infection by the recognition of bacterial antigens on T cells. TLR1 following formation of a dimer with TLR2 recognizes peptidoglycans and lipoproteins of bacteria origin. Comparing this result with transcriptional study of *A. salmonicida* it was possible to select bacterial regulated candidate genes for TLR1 recognition such as toxR-regulated lipoprotein, TagA (ASA_3321) which expression increased 11.73 after 24 h of culture in elevated temperature. TagA is a virulence factor of *Aeromonas hydrophila* also reported in *Escherichia coli* O157:H7 [310–312] and *Vibrio cholerae* [313]. It provides increased serum resistance, increased complement inhibition and erythrocytes lysis towards human specimens what was also observed in this

research[312]. Second molecule being candidate for TLR1 ligand is Bor lipoprotein gene (ASA_2755) which responded during calcium limitation after 48 h (FC_{Ca_48h} = 2.62). As mentioned in literature, it also promotes lysogenic bacterial resistances to serum complement killing in *E. coli* [314]. It can also suggest the *Aeromonas salmonicida* phage-origin contribution to infection. Final example is the outer membrane lipoprotein (ASA_4258), with FC_{Ca_48h} = 2.25 after 48 h of calcium starvation that could possibly be recognized by TLR1 causing its upregulation. The proof for the gene's selection requires further investigations and this should be candidate genes for future TLR1 interaction studies.

Rainbow trout TLR3 is mostly involved in antiviral response [315]. Stimulation experiments of rainbow trout TLR3 by dsRNA using poly inosinic: cytidylic (I:C) and infectious hematopoietic necrosis virus (IHNV) treatments described by Rodriguez showed the increased expression of this endosomal PRR. However, no stimulation by bacteria *Yersinia ruckeri* was observed [316]. TLR3 in mammals is a strong activator of type I interferon promoting hyperinflammatory responses recorded during sepsis [315]. Therefore, it is expected that gram-negative bacteria would not stimulate it. Surprisingly, the myeloid subpopulation exhibited only a mild response, contrary to current scientific understanding that macrophages and dendritic cells are the primary cell subsets expressing TLR3 [317]. Moreover, our study shows the regulation of TLR3 expressed in pan T cells in spleen being stimulated by wild type and highly virulent *A. salmonicida*. Attenuated strain had no effect on TLR3 expression in pan T cells. This may suggest that dsRNA of unknown (either host or bacterial) origin stimulated T cells or TLR3 has additional not yet described ligand. In nature dsRNA is only common for viruses and bacteriophages infecting *A. salmonicida* [318,319]. Together with previous conclusion we could support here the hypothesis about *Aeromonas salmonicida* phage infection. Focusing on the function of this stimulation, studies of Suresh and Jannuzzi suggest that TLR3 is a negative regulator of immune response in mammals [315,320,321]. In this model the hypothesis for teleost fish needs further experiments.

Following, the expression of TLR5 recognizing flagellin was very distinct in myeloid subset in spleen and a bit weaker in thrombocytes. Recent studies consider tissue from different lymphatic organs such as spleen, thymus, intestine, and liver expression analysis which does not give the information which cell type precisely respond to pathogen [306,322]. Here, we focus on purified cells what is an invaluable advantage out of this research. Bacteria expression study from previous part of this thesis show that there is an elevated expression of several flagella component proteins during 24 h to 48 h culture with limited calcium access. The bacteria transcripts from chapter 5 code proteins contributing to flagella structures such as flagellar hook protein Felger, lateral flagellar assembly protein Filho, lateral flagellar motor switch protein, lateral flagellar biosynthetic protein, lateral flagellar hook basal body protein, lateral

flagellar basal-body rod protein. Those molecules can the possible *A. salmonicida* ligands for TLR5 receptor.

Mammalian TLR7, TLR8a_1 and TLR8a_2 recognize viral single stranded RNA (ssRNA). They are localized intracellularly in endoplasmic reticulum (ER) and endosomes[323]. Recognition of ssRNA by TLR7 and TLR8a_1 and TLR8a_2 highly likely may come from degraded bacteria remains in endosomes. Reported in mammalian monocytes, neutrophils, dendritic cells TLR8 is required for recognition of bacteria species including *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Enterococcus faecalis* after stimulation as mentioned by Nunes [324]. In contrast, in this thesis we report for the first time that TLR7 and TLR8a_1 and TLR8a_2 transcripts are present in B lymphocytes in blood, and they respond to bacteria presence. Additionally, the highest expression comes from highly virulent strain, second highest surprisingly is the attenuated *A. salmonicida*, and wild type stimulates B cell very weakly. TLR response to stimulation is like observed respiratory burst of lymphocytes after inactive bacteria stimulation suggesting more passive TLR triggering from dead bacteria particles. Furthermore, published results indicate that spleen transcripts express TLR7, TLR8a1, TL8a2 more abundantly as mentioned by Palti and Lee [325,326]. This study shows as well that among tested cells only T cells responded in spleen to bacteria stimulation. Research conducted by Qian in yellow croaker prove the expression in TLR7 and TLR8 kidney and spleen tissues which also proves our results [327]. Highly virulent strain gave the highest stimulation response in contrast to wild type and attenuated strain. Splenic myeloid cells responded weaker, only to highly virulent strain showing that mostly lymphocytes are the main receptor bearing cell subset. Nevertheless, it can be concluded that in rainbow trout B- and T- lymphocytes carry TLR7, TLR8a1 and TLR8a2 receptors. Receptor presence is also tissue specific. As mentioned, presence of bacteria in the body may result in direct PAMP – PRR interaction but also it is worth to remember that endogenous molecules may also act as TLR ligands (for example chromatin complexes release from damaged cells) [328,329]. Thus, TLR7and TLR8a_1 and TLR8a_2 molecules require more detailed study.

TLR9 is a receptor localized inside the cellular compartments recognizing unmethylated CpG in bacteria DNA [322]. Reported in literature TLR9 can be found on different cell types such as B lymphocytes, dendritic cells, monocytes, macrophages [330] Our study shows that TLR9 is only expressed in splenic pan T cells. Expression in other subpopulations was recorded on control levels. Like other TLRs highly virulent strain has the biggest impact on TLR9 response.

As mentioned by Ji TLR19 is one of teleost specific TLRs recognizing dsRNA [331]. Till now there is limited knowledge about its behavior in bacterial infection. TLR19 was described in carp [332], zebra fish [333], Atlantic salmon [334], This study shows that cytotoxic T cells bear the TLR19 receptor and respond to highly virulent *A. salmonicida*. Additionally, there is a noticeable upregulation of myeloid cells in head kidney in response to attenuated bacteria. This proves the

previous observations of Shan who tested TLR19 expression in different organs after stimulation with *Aeromonas hydrophila*. The response is remarkably similar with no outstanding peaks.

Although there is few research published regarding TLR21 expression in yellowtail [335], carp[336] , Atlantic salmon [334], we noticed very weak response only from myeloid fraction of leukocytes from spleen after bacterial infection. Here, only one time point was used for the analysis and the research show that TLR21 expression is visible better in later hours post inoculation. That does not exclude this receptor from investigation, yet it would be worth to see how the receptor reacts in more time points in spleen in all available cell subsets.

In this part, the kinetics of the disease in initial stages was characterized with a focus on rainbow trout innate response. We have shown that the first signal and non-specific immune response are the most important because not only they clear non-self-material out of the site of infection but also pass the information about the foreign presence to the adaptive immune system. However, our TLR expertise showed that is the case of *Aeromonas* stimulation the TLR expression is not significantly changed. There must be a way that the immune system solved the sampling of the environment and detecting the *A. salmonicida*. The alternative feasible way was the signaling through thrombocytes from blood, yet there is no known time from where bacteria reach blood flow from peritoneum. We had indications that thrombocytes are important during the direct antibody-producing B cells stimulation (data not shown). Nevertheless, we believe that there must be system equal in efficacy and speed to TLR signaling not yet fully understood nor described.

7 Conclusions

Based on the results from the whole research it has been concluded:

1. Differences in strain genomic composition of bacteria *Aeromonas salmonicida* ssp. *salmonicida* occur and have a phenotypic impact on virulence of bacteria towards rainbow trout.
2. Plasmid gene content is more flexible than core and more prone to culture conditions influence.
3. Growth conditions may affect the genomic composition of bacteria, so it is important to consider them as one of factors affecting gene usage and presumably progress of infection.
4. Change in single culture condition (temperature, iron, calcium, oxygen) affects group of genes by regulon or a function and may contribute to bacterial infection through transcriptional regulation of a whole network of genes.
5. Transcriptomic analysis indicating presence of genes coding individual proteins belonging to T3SS in highly virulent strain (but not in wild type and attenuated strain) suggests that T3SS is one of major key players during *A. salmonicida* infection in rainbow trout.
6. Bacteria extracellular proteins show dose depended toxic activity towards erythrocytes causing their death withing hours.
7. Ingestion of living bacteria cells of *A. salmonicida* highly virulent strain by phagocytic leukocytes is associated with drastic reduction of oxidative burst, in contrast to dead bacteria cells that did not restrict this process. This may suggest presence of active bacterial mechanisms modeling cellular activity in host.
8. During bacterial stimulation TLRs expression depends on TLR type as well as on investigated cell subpopulation. The most responsive TLRs are present in T lymphocytes and myeloid cells of spleen, B lymphocytes in blood and myeloid cells in head kidney. Prevalent response type was expression increase of selected receptors after stimulation of highly virulent strain of *A. salmonicida*.

8 General conclusion

During the infection process it is not only about the existence and regulation of virulent genes but also other genes responsible for metabolic processes, nutrient acquisitions that overall decide about metabolic fitness and infection process giving bacteria advantage over that trout immune response.

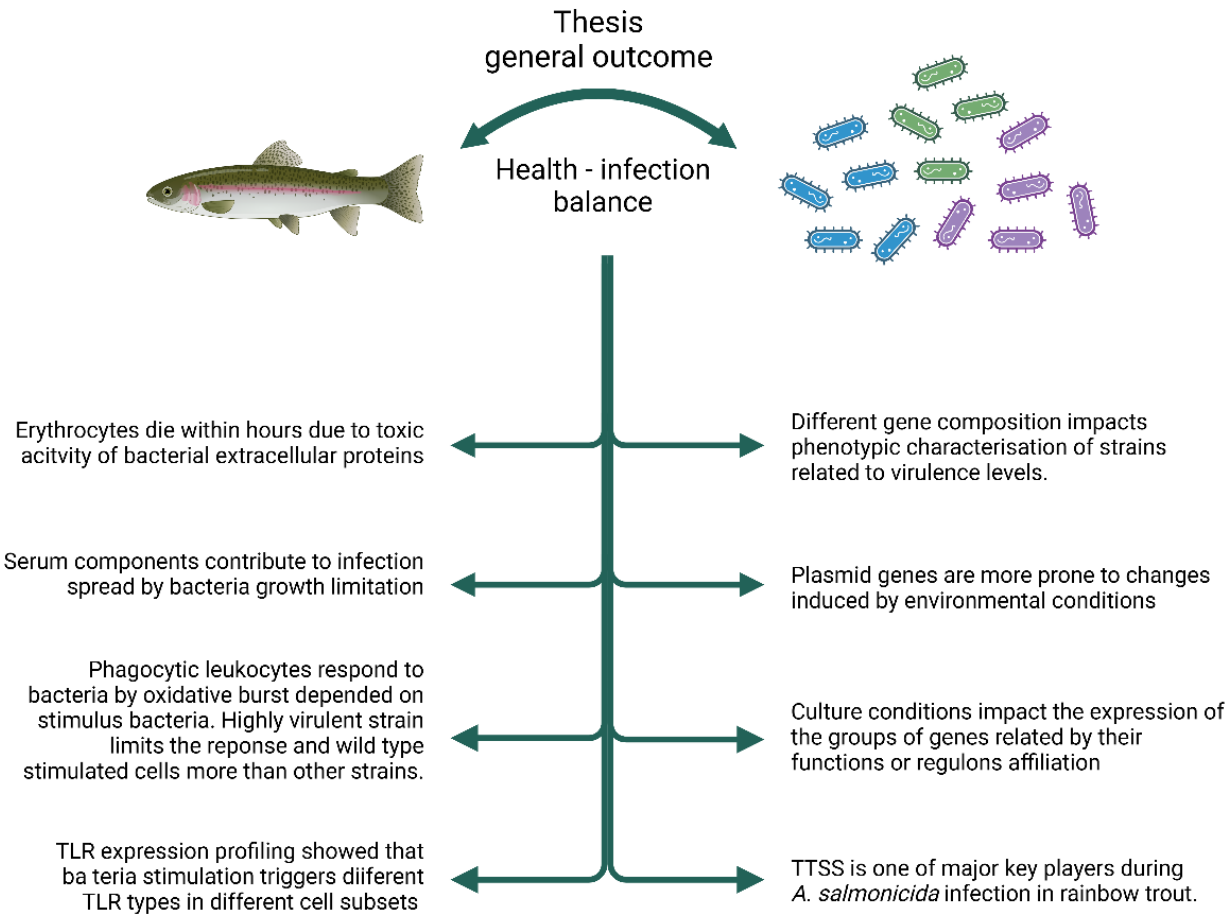

Figure 38. Thesis general outcome summarizing fish and bacteria activities during infection process.

9 Abstract

Rainbow trout is a highly valuable aquaculture species due to its rich nutritional profile, including high-quality proteins, unsaturated fatty acids, vitamins, and minerals. It is the second most widely farmed fish species worldwide, and its immune system that adapted to both freshwater and marine environments, represents an important model from both evolutionary and economic perspectives. However, intensive aquaculture has led to the emergence of numerous diseases causing major economic losses. One of them is furunculosis, caused by the opportunistic pathogen *Aeromonas salmonicida*, which is difficult to study due to its unstable genome and high pathogenic flexibility, substantially linked to plasmid content.

In this study we addressed the early infection mechanisms of *A. salmonicida in* rainbow trout. To uncover the mechanisms underlying distinct immunity profiles, we analyzed early infection stages at the whole-fish level using high-throughput workflows and systems biology tools to study both host and pathogen, as one interdependent system. We assumed that the host (fish) serves as a niche for the pathogen (bacterium) and their interactions are dynamic process through which *A. salmonicida* adapts to new environments. Using bacterial strains that displayed different levels of virulence despite nearly identical genotypes, we compared bacterial genomes of high and low virulent isolates to a reference strain.

In the first part the whole genome analyses demonstrated that even though we have one bacteria species, the genomic composition varied in sequence which affects the virulence towards the host. The unique genes for certain isolate and the genes having lower consensus were found also in a group of genes participating in infection process. In the second part the transcriptomic study identified several factors significantly contributing to infection on both sides — pathogen (virulence factors) and host (receptors responding to bacterial factors). The type III secretion system was identified as a key determinant of infection progression, although focusing on a single virulence molecule proved misleading. Transcriptomic analysis revealed that the process to induce infection is very complex and it is a huge interconnected network of pathways such as arginine pathway or iron and flagella regulation. Both known and previously uncharacterized genes were described to be involved in the early stages of infection. Particular emphasis was placed on virulence-associated molecular patterns (VAMPs), which play a crucial role in enabling the host immune system to distinguish self from non-self components of bacterial origin, thereby shaping the course of infection and disease development.

In the last part of this thesis the fish cellular responses were investigated showing cytotoxicity effect on erythrocytes, bacteria limited growth in culture with head kidney leukocytes known being first sentinels of pathogen and also respiratory burst study in myeloid and lymphoid fractions during bacteria stimulation. The latter demonstrated that myeloid fraction is mainly responsible to bacteria first recognition and clearance but there is a small subset of lymphoid cells that are also able to perform respiratory burst. Finally, molecular study measuring expression of TLR receptors during stimulation show that it is dependent on the leukocyte cell type, time after exposure and TLR receptor ligands they recognize. The study shows how complex defense mechanism the pathogen has to face during infection and explains the reasons for such flexibility of the species.

In summary, this study provides a comprehensive characterization of the virulence factors of *Aeromonas salmonicida*, offering new insights into its interaction mechanisms with the rainbow trout host.

10 Abstract in polish (streszczenie)

Pstrąg tęczowy (*Oncorhynchus mykiss*) jest istotnym gatunkiem w akwakulturze ze względu na wysoką wartość odżywczą, obejmującą białka wysokiej jakości, nienasycone kwasy tłuszczowe, witaminy i minerały. Stanowi drugi pod względem hodowli gatunek ryb na świecie, a jego układ odpornościowy, który jest przystosowany zarówno do środowisk słodkowodnych, jak i morskich, jest cenionym modelem z perspektywy ewolucyjnej i ekonomicznej. W dzisiejszych czasach intensywna hodowla sprzyja jednak rozwojowi chorób, w tym furunkulozy wywoływanej przez oportunistyczną bakterię *Aeromonas salmonicida*, którego badanie jest utrudnione ze względu na niestabilny genom i wysoką elastyczność patogenną, silnie powiązaną z zawartością plazmidów.

Celem niniejszej pracy była analiza mechanizmów wczesnej infekcji *A. salmonicida* u pstrąga tęczowego. Badania prowadzone były z zastosowaniem wysokoprzepustowych metod i narzędzi biologii systemów, traktujących gospodarza i patogenu jako współzależny system. Porównano szczepy bakteryjne o różnym stopniu wirulencji, mimo niemal identycznych genotypów, z genomem szczepu referencyjnego, aby zidentyfikować czynniki determinujące wirulencję. Analizy genomowe wykazały istotne różnice w sekwencjach, w tym obecność genów unikalnych dla poszczególnych izolatów oraz zmienność w grupach genów uczestniczących w procesie infekcji. Badania transkryptomiczne ujawniły czynniki przyczyniające się do infekcji po stronie patogenu (czynniki wirulencji). System sekrecyjny typu III został zidentyfikowany jako kluczowy determinant postępu infekcji, a analiza transkryptomiczna wykazała złożoną, sieciową naturę szlaków molekularnych, takich jak szlak argininy, regulacja żelaza czy regulacja rzęsek. Szczególną uwagę poświęcono również molekularnym wzorcom związanym z wirulencją (VAMP), które umożliwiają gospodarza odróżnienie elementów własnych od bakteryjnych, kształtując przebieg infekcji i rozwój choroby.

Dalsze badania komórkowe wykazały cytotoksyczny wpływ bakterii na erytrocyty, ograniczenie wzrostu bakterii w hodowli z leukocytami nerki przedniej oraz zróżnicowaną odpowiedź frakcji mieloidalnej i limfoidalnej w procesie wybuchu tlenowego. Analiza ekspresji receptorów TLR wykazała, że odpowiedź zależy od typu komórki leukocytarnej, czasu po ekspozycji oraz rodzaju ligandów. Wyniki pracy ukazują złożoność mechanizmów obronnych gospodarza oraz elastyczność patogenu w procesie infekcji.

Podsumowując, praca dostarcza kompleksowej charakterystyki czynników wirulencji *Aeromonas salmonicida*, opisując zarówno geny dotychczas znane, jak i wcześniej niecharakteryzowane, istotne w wczesnych stadiach infekcji oraz otwiera pole do dalszych badań przedstawionego modelu badawczego.

11 Literature

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