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Original article

Immunological parameters of rainbow trout (*Oncorhynchus mykiss*) with *Flavobacterium psychrophilum* gill infection

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Abstract

Flavobacterium psychrophilum, the etiological agent of bacterial coldwater disease and rainbow trout fry syndrome, causes great economic losses in salmonid aquaculture worldwide but the influence of this bacterium on non-specific immunity in fish and the development of disease is uncertain. In our studies rainbow trout were diagnosed with gill infections caused by the bacterium after experiencing mortality in one of the fish farm's tanks. For 6 weeks, we assessed the immunity parameters in affected fish. The activity of lysozyme and ceruloplasmin, as well as the level of total protein and immunoglobulins in fish serum were measured. Also, spleen phagocyte respiratory burst and potential killing activity, as well as head kidney lymphocyte proliferation stimulated by concanavalin A or lipopolysaccharide were determined. Almost all tested immunological parameters except for ceruloplasmin activity were reduced compared to the control group. *F. psychrophilum* seems to have mechanisms that allow it to evade the fish's immune system and suppress the basic non-specific humoral and cellular defense mechanisms of the infected fish.

Keywords: atypical bacterial gill disease, cellular immunity, Flavobacteriaceae, humoral immunity, innate immunity

Introduction

Flavobacteria are considered one of the most important fish pathogens worldwide. Three species, in particular, are responsible for significant losses among farmed and wild fish: *Flavobacterium* (*F.*) *branchiophilum*, *F. columnare*, and *F. psychrophilum* (Wahli and Madsen 2018). *F. psychrophilum* was originally isolated from diseased juvenile coho salmon (*Oncorhynchus kisutch*) in the United States in 1948 (Borg 1948). The first cases of *F. psychrophilum* infections in Europe were reported in France and Germany in the mid-1980s (Bernardet et al. 1988). Subsequently, the disease emerged in all parts of the world (Bernardet and Bowman 2006). Salmonids, like rainbow trout *(Oncorhynchus mykiss*), coho salmon, and ayu (*Plecoglossus altivelis*) are the most susceptible species to *F. psychrophilum* infection (Nematollahi et al. 2003).

Differences in geographic location, bacterial isolate, and host age can result in distinct forms of infection. *F. psychrophilum* is the causative agent of bacterial rainbow trout fry syndrome (RTFS) which manifests as acute bacteremia mainly in small fish and bacterial coldwater disease (BCWD) as a more chronic disease characterized by ulcerative dermatitis in larger fish. Interestingly, it has also been recognized as the cause of necrotic myositis and cephalic osteochondritis affecting rainbow trout (Lumsden et al. 1996, Ostland et al. 1997), and reports about atypical bacterial gill disease occur in a variety of species of farmed salmonids (Ostland et al. 1999). On the other hand, it was also isolated from fish without any clinical signs of disease (Boyacioglu and Akar 2012).

Bacterial gill disease (BGD) is an infection often found in salmonids reared in hatcheries under intensive conditions. The etiological factor is typically *F. branchiophilum*, although other opportunistic Gram-negative bacteria including *F. psychrophilum*, aeromonads, and pseudomonads have been associated with the atypical BGD (Ostland et al. 1999, Rach et al. 2000). *F. psychrophilum* is present in the aquatic environment. It can reproduce in water for up to 4 months (FAO 2020). Previous data have suggested that adhesion to the gill epithelium might be an important initial step in the pathogenesis of infection (Amita et al. 2000). Its adherence is significantly influenced by phenotype, tissue type, and time which may play a crucial role in the pathogenesis (Papadopoulou et al. 2017).

Various research groups have conducted studies to explain the pathogenesis, which has led to significant progress in elucidating the complex interactions between bacterial organisms and their host, but little is still known about the effect of *F. psychrophilum* infection on the immune system of the fish. The present study examined the influence of a natural outbreak of *F. psychrophilum* infection on non-specific humoral and cellular defences mechanisms in rainbow trout. The disclosure of the effect of this bacteria on the immune cells of rainbow trout presents valuable information on pathogenesis, which may help in the development of effective vaccines and other preventive treatments for aquaculture.

Materials and Methods

Mortality of rainbow trout weighing 10-15 g occurred at a local fish farm in one of four tanks at 11±1°C. The fish were kept in plastic tanks with a volume of 1 m^3 with a flow-through water system. They were fed commercial feed twice a day at 1% of body weight. Mortality of the affected fish increased for 7 days. After that, it rapidly decreased. No mortality or disease symptoms were observed in any of the other tanks. Overall losses reached 30% of the stocking in the affected tank. Clinical examination revealed increased mucus secretion, swelling, and necrosis of the gills. No changes were found in internal organs.

Samples for bacteriological tests were taken from the changed gills, kidney and spleen. They were inoculated on two media: tryptone yeast extract salts (TYES, Sigma-Aldrich) and tryptic soy agar (TSA, Sigma--Aldrich). Agar plates were incubated at 18°C for 5 days. Further identification of bacterial isolates that formed yellow colonies on TYES agar was made. The morphology of the cells was examined in preparation stained using the Gram method. The production of catalase was determined by using 3% hydrogen peroxidase. The cytochrome oxidase test was performed using a ready-made reagent (BactiDrop Oxidase, Remel). The production of flexirubin pigment was examined by observing the change in colony color after KOH application. Classical test tube methods were used for carbohydrate fermentation tests. Other biochemical and enzymatic properties of the isolates were tested using the API20 E, API 20NE and API Zym kits (bioMérieux).

For 6 weeks at 7-day intervals after the onset of initial mortality material for testing was collected from 10 randomly selected fish from the affected tank and 10 healthy fish. Blood for the study was collected from the tail vein and after centrifugation, the blood serum was used for the determination of humoral immune parameters. Spleen and head kidney were collected to assess cellular immune parameters after isolation of immunocompetent cells.

Ethical review and approval was waived for this study since the samples were taken during the veterinary examination of the animals on behalf of the breeder.

Table 1. Kinetics of non-specific humoral immune parameters in serum of *Flavobacterium psychrophilum*-infected (FPI) and control (C) rainbow trout (n=10, * p≤0.05).

	Group	Week					
		1	2	3	4	5	6
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
LSM [mg/L]	C	22.0 ± 1.5	26.0 ± 2.5	25.5 ± 2.0	27.5 ± 3.5	23.5 ± 4.5	21.5 ± 2.0
	FPI	12.5 ± 3.5*	5.9 ± 1.5*	4.5 ± 0.5*	9.5 ± 2.4*	15.5 ± 3.5*	20.8 ± 2.4
Cp [UI]	C	56.0 ± 5.3	58.0 ± 5.9	61.0 ± 6.1	60.0 ± 6.3	58.0 ± 4.9	59.0 ± 5.8
	FPI	85.0 ± 8.5*	115.0 ± 16.4*	124.0 ± 13.5*	105.0 ± 10.6*	85.0 ± 9.6*	62.0 ±6.5
T-P [g/L]	C	40.8 ± 3.5	42.7 ± 3.8	43.2 ± 4.1	44.5 ± 4.2	45.3 ± 4.6	45.9 ± 4.2
	FPI	32.5 ± 3.4*	30.8 ± 3.2*	31.2 ± 3.6*	36.8 ± 3.6*	40.6 ± 3.8*	44.4 ± 5.6
T-Ig [g/L]	C	10.2 ± 2.5	16.5 ± 2.0	16.0 ± 2.5	14.0 ± 3.0	13.5 ± 1.8	12.0 ± 1.6
	FPI	6.5 ± 1.5*	6.0 ± 1.2*	6.8 ± 1.4*	8.5 ± 2.5*	10.9 ± 1.8*	11.5 ± 1.8

LSM – lysozyme activity, Cp – ceruloplasmin activity, T-P – total protein level, T-Ig – total immunoglobulin level

Humoral immune parameters

The lysozyme activity (LSM) was measured by turbidimetric assay. The serum ceruloplasmin activity was determined using the spectrophotometric method. The total protein level (T-P) was determined using the biuret method. Double determinations were averaged to calculate OD values. The spectrophotometric method adapted for fish was used to determine the total serum immunoglobulin level (T-Ig). This method requires precipitating the immunoglobulins out of the serum. Mean OD values were calculated by averaging duplicate determinations. Total serum Ig levels were calculated by subtracting supernatant OD values from those of total protein. The details are described by Terech-Mejewska et al. (2016).

Isolation of lymphocytes

Lymphocytes for the tests were isolated from the fish spleen and head kidney. The spleen and head kidney of each fish were removed aseptically and pressed through a 60-µm nylon mesh. Single-cell suspensions were obtained for isolating individual cells using Gradisol L (Aqua-Medica, Łódź, Poland) gradient and were then centrifuged at 400 g for 45 min at 4°C as described by Siwicki and Cossarini-Dunier (1990). The cells were suspended in RPMI-1640 medium containing 10% fetal calf serum (FCS, Sigma-Aldrich) and 1% antibiotic-antimycotic solution (Sigma-Aldrich), and then dispensed into 96-well plates and cultured/ /incubated at 24°C and used for the following assays.

Cellular immune parameters

The metabolic activity of spleen phagocytes was measured by determining the size of the intracellular respiratory burst activity (RBA) after phorbol myristate acetate (PMA, Sigma-Aldrich) stimulation. The phagocyte-killing activity (PKA) was determined spectrophotometrically after stimulation with *Aeromonas hydrophila*. The proliferative response of head kidney lymphocytes (MTT) was determined using the colorimetric assay in the presence of mitogens – concanavalin A (ConA) as a T-cell mitogen or lipopolysaccharide from *Serratia marcescens* (LPS) as a B-cell mitogen (both mitogens purchased from Sigma-Aldrich). The details are described by Terech-Majewska et al. (2016).

Statistical analysis

Mean values and standard deviations from pooled experiments were used for comparisons among groups. Data are reported as means ± SE. Student's t-test was used to determine the significant difference in immunological parameters between the groups. All calculations were determined to be significant at p≤0.05.

Results

A comparison of the non-specific humoral parameters is presented in Table 1. Analysis of the results show that the activity of serum lysozyme was decreased in the fish from the group infected with *F. psychrophilum* for 5 weeks in comparison with the control group, reaching the lowest level in the 3rd week after the mortality occurred. Total protein and immunoglobulin levels were also decreased in the serum of infected fish compared with the control group, but the lowest value occurred in the 2nd week of the study and then began to increase. On the other hand, ceruloplasmin activity increased for 5 weeks after the initial mortality, reaching the highest level at week 3. A comparison of the innate cellular defense parameters in rainbow

Table 2. Kinetics of non-specific cellular immune parameters of *F. psychrophilum*-infected (FPI) and control (C) rainbow trout (n=10, * p<0.05).

	Group	Week					
		1	2	3	4	5	6
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
RBA	C	0.41 ± 0,05	0.43 ± 0.05	0.45 ± 0.03	0.47 ± 0.05	0.50 ± 0.06	0.44 ± 0.04
	FPI	0.21 ± 0.05*	0.19 ± 0.03*	0.18 ± 0.04*	0.20 ± 0.04*	0.29 ± 0.05*	0.41 ± 0.08
PKA	C	0.52 ± 0.03	0.54 ± 0.04	0.56 ± 0.05	0.56 ± 0.07	0.58 ± 0.06	0.51 ± 0.08
	FPI	0.23 ± 0.05*	0.20 ± 0.04*	0.18 ± 0.05*	0.19 ± 0.06*	0.22 ± 0.05*	0.49 ± 0.05
MTT (ConA)	C	0.45 ± 0.08	0.47 ± 0.06	0.49 ± 0.05	0.49 ± 0.03	0.48 ±0.07	0.47 ± 0.02
	FPI	0.25 ± 0.04*	0.18 ± 0.02*	0.19 ± 0.06*	0.19 ± 0.04*	0.29 ± 0.08*	0.43 ± 0.06
MTT (LPS)	C	0.38 ± 0.05	0.39 ± 0.05	0.40 ± 0.05	0.44 ± 0.04	0.40 ± 0.05	0.41 ± 0.06
	FPI	0.27 ± 0.05*	0.20 ± 0.03*	0.19 ± 0.04*	0.19 ± 0.05*	0.24 ± 0.04*	0.38 ± 0.05

RBA – respiratory burst activity of the spleen phagocytes, PKA – potential killing activity of the spleen phagocytes, MTT – proliferative response of head kidney lymphocytes stimulated by concanavalin A (ConA) or lipopolysaccharide (LPS)

trout is presented in Table 2. Analysis of the results show that respiratory burst activity and potential killing activity of spleen phagocytes decreased in fish from the *F. psychrophilum*-infected group with the lowest value in the 2nd week of the study. Similarly, the proliferation of B lymphocytes, as well as proliferation of T cells isolated from the head kidney was reduced in fish from the group in which mortality occurred. All parameters did not differ statistically between the groups in the 6th week of the study.

Discussion

F. psychrophilum is a ubiquitous Gram-negative fish pathogen worldwide. The range of variability in the diseases that can be caused by this bacterium is notable. The symptoms of infection differ depending on the fish species, the characteristics of the bacterial strain or the geographical area. Mortality associated with *F. psychrophilum* infections also varies depending on the form of infection, water temperature, and developmental stage of the host. In a typical form of infection it can be as high as 90% (Nilsen et al. 2011), while it is usually much lower in gill infections (Brocklebank 1999).

It is essential to understand certain aspects of the infection process. This is one of the key areas of aquaculture research, especially concerning economically important species such as rainbow trout. In many cases, the immune response of fish must respond in a multifactorial manner to resist infection. Pathogen recognition is crucial for the initiation of the host's innate immune response through multiple signaling pathways, such as increased lysozyme production, which contributes to pathogen eradication (Janeway and Medzhitov 2002).

The level and activity of lysozyme in the serum is an important indicator of the innate immunity of fish. It has antibacterial and antiviral properties (Saurabh and Sahoo 2008) and is present in fish mucus, serum, and tissues rich in leukocytes. It has also been identified in monocytes and neutrophils (Murray and Fletcher 1976), which are likely to be the source of lysozyme in the serum. In our study, lysozyme activity was suppressed for 5 weeks after the onset of mortality compared to the control group. Siwicki et al. (2004) reported a reduction in serum lysozyme activity in rainbow trout infected with *F. psychrophilum* compared to uninfected fish for 10 days. A decrease in lysozyme and bactericidal activity was also observed by Zahran et al. (2014) in Nile tilapia *(Oreochromis niloticus*) after infection with *F. columnare*. Similar results were observed in juvenile common carp (*Cyprinus carpio*) (Hassanien et al. 2023). Such results may be an indication of a specific ability of flavobacteria to inactivate the defense mechanisms of fish. It is known that some bacteria have evolved highly specific protein lysozyme inhibitors as part of their defense against the action of lysozyme. The first inhibitor was discovered in *Escherichia coli* in 2001. Since then, several other lysozyme-specific inhibitors have been identified in various bacteria. There is increasing evidence that these inhibitors contribute to bacterial symbiosis or pathogenesis, enabling them to evade host immune defenses (Vanderkelen et al. 2023).

In addition to its antibacterial activity, lysozyme promotes phagocytosis by directly activating phagocytic cells or indirectly by opsonic action. During infection, phagocytic cells eliminate pathogens through a series of antimicrobial responses. The major phagocytes in teleosts are monocytes and macrophages, granulocytes, and dendritic cells (Esteban et al. 2015).

The effectiveness of these innate immune responses is critical to the immune outcome. Previous studies of respiratory burst activity of kidney leukocytes from resistant and susceptible rainbow trout lines infected with *F. psychrophilum*, showed that high activity was observed only on days 0 and 1, followed by a significant decrease in activity which returned to pre-infection levels after 4 weeks (Semple et al. 2018). Our results showed very similar tendencies with a reduction of the respiratory burst and potential killing activity of splenic phagocytes and lymphocyte proliferation in the kidney. Leukocyte proliferation and phagocytic activity in peripheral blood were also reduced in juvenile carp during *F. columnare* infection (Hassanien et al. 2023). In different studies, live *F. psychrophilum* was shown to significantly reduce the survival rate of the monocyte/macrophage-like cell line (RTS11). A significant percentage of cells died and many cells became more diffuse, indicating that the bacteria can produce virulence factors to evade host immune cells (Semple et al. 2020). Decostere et al. (2001) found that after infection, viable bacteria remain in splenic phagocytes, which may protect them from humoral defense mechanisms, and possibly prevent activation of the immune system. Extensive degeneration of spleen tissue was also found (Rangdale et al. 1999). Such settlement of organs responsible for immunity may result in the body's ability to mobilize an anti-infective response. Several pathogens in mammals actively kill immune cells, thereby preventing the host from responding effectively to control the infection. This strategy is often effective for the pathogen, but few similar virulence factors have been observed in aquatic diseases.

Besides major myeloid and lymphoid tissues such as the head kidney and spleen, rainbow trout have immunoglobulin cells distributed throughout their body. They are produced as membrane-bound receptors on B-cells or in a secreted form, known as antibodies. Compared to mammals, fish produce immunoglobulins at a slower and weaker rate. The antibodies produced have lower affinity and are not as diverse (Randelli et al. 2008). In teleosts, three immunoglobulin isotypes, IgM, IgT, and IgD, are present. IgM is the most abundant antibody type, thought to be involved in systemic and mucosal defense (Quddos and Zwollo 2021). The correlation between host specificity and bacterial virulence was studied by Nagai and Nakai (2011), indicating that isolates showed varying pathogenicity depending on the fish species. *F. psychrophilum* isolates from ayu were pathogenic to this species, but not to trout. Meanwhile, other isolates from salmonids and cyprinids were not pathogenic to ayu, indicating different host-pathogen interactions in *F. psychrophilum* infection and diversified antibody production. Studies also indicated that immune responses differ between resistant and susceptible genetic lines within one species of fish, with bacterial load exerting a significant influence on immune response (Langevin et al. 2012, Marancik et al. 2015, Lee et al. 2023). In studies conducted by Orieux et al. (2013), fish naturally infected with *F. psychrophilum* did show decreased plasma IgM titers compared to healthy fish, suggesting that this may be due to suppression of the immune response in diseased fish. Our results showed very similar tendencies regarding total immunoglobulin level. During infection, the expression of the IgT gene in the spleen, head-kidney and gills of farmed fish was also reduced (Orieux et al. 2013, Henriksen et al. 2015a, 2015b). Despite the presence of specific antibodies that could be detected a few days after infection or immunization, the antibody titer was undetectable (Ma et al. 2023).

In some studies, not only a decrease in the level of total immunoglobulins in infected fish was observed, but also a decrease in the total protein level, which is consistent with our results (Plant 1997). It is suggested that the amount of total protein after infection depends on the genetic line of the trout. Both resistant and susceptible lines showed significant changes in total protein level after infection, with the values in the susceptible line deviating more from the reference range. A decrease in total protein and albumin level was one of the earliest pathophysiological changes detectable as soon as the first day after infection (Marancik et al. 2014). These analytes are emphasized as early biomarkers of disease because of the relatively early changes in total protein and albumin in infected fish. Similar protein changes have been described in juvenile common carp infected with *F. columnare* (Hassanien et al. 2023) and in rainbow trout infected with *Aeromonas* spp., a mixture of *Aeromonas* and *Streptococcus* spp., and *Cryptobia* sp. (Rehulka et al. 2005). Further studies are needed to identify causative factors, but total protein and albumin levels have been linked to the nutritional status of fish, suggesting that anorexia during infection may contribute to low values in the serum.

Ceruloplasmin is another part of the innate humoral response. In addition to its role in maintaining homeostasis, it is also considered an important acute-phase protein that is activated by the host immune system during stressful conditions. Ceruloplasmin is thought to function in several defense-related processes that include limiting the spread of infectious agents, repairing tissue damage, killing microbes and other potential pathogens (Sahoo et al. 2013). Our results showed an increase in ceruloplasmin levels which corresponds with the results of other authors. An increase in cerulo-

plasmin levels was found in catfish (*Ictalurus punctatus*) during *Edwardsiella ictaluri* infection (Liu et al. 2011), *Saprolegnia* spp. infection in Atlantic salmon (*Salmo salar*) (Roberge et al. 2007), and *Aeromonas hydrophila* infection in rohu (*Labeo rohita*) (Sahoo et al. 2013). This molecule is thought to be partly responsible for the transport of copper to sites in target tissues, where copper may play an important role in fish defense against various forms of stress. *In vitro* experiments indicated that the copper (I) complex has antibacterial activity against *F. psychrophilum*, at non-cytotoxic concentrations (Aldabaldetrecu et al. 2020). *In vivo* use of the copper (I) complex added as a dietary supplement against *F. psychrophilum* infection in rainbow trout also showed antibacterial effects (Aldabaldetrecu et al. 2022). Since the bacterium is sensitive to copper, it is suggested that high concentrations of ceruloplasmin, which transports these ions, may be a good strategy to prevent the spread of infection.

In vertebrates, many host defense mechanisms control the microbiota and, in most cases, effectively prevent the development of disease. The immune system is also thought to play an important role in rainbow trout resistance to *F. psychrophilum*, but the mechanisms are only partially understood. Although bacterial pathogens are single-celled organisms, their behavior is far from predictable. The co-evolution of pathogenic bacteria and hosts has led to the development of complex and efficient methods to overcome innate and adaptive immune mechanisms. There are many examples of pathogens that have developed protective strategies against the vertebrate immune system. Immune evasion strategies use tactics such as modulating cell surfaces, releasing proteins that inhibit or degrade host immune factors, and even mimicking host molecules (Bizzell 2018). This also includes actively degrading antimicrobial proteins, inhibiting phagocyte oxidative bursts by producing detoxifying enzymes, and preventing the assembly of enzymatic components involved in immune responses (Grayfer et al. 2014).

Our results extend the current knowledge of the immune response of rainbow trout to *F. psychrophilum* infection and suggest that this bacterium may belong to a group capable of evading the protection of the immune system by suppressing its basic cellular and humoral parameters. At the same time, they highlight the need for further research into bacterial-host interactions.

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