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Comparative immunomodulatory responses and disease resistance of rainbow trout (Oncorhynchus mykiss Walbaum, 1792) juveniles fed selected nutritional compounds

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A dissertation submitted by

Trinh Dinh Khuyen

in fulfilment of the requirements

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Contents

List of abbreviations and units

%	Percentage
/	Per
±	Approximately
× g	Relative centrifugal force or G force
°C	Degree Celsius
µg	Microgram
µl	Microlitre
bp	Base pair
C3	Complement type 3
DNA	Deoxyribonucleic acid
EU	European Union
FAO	Food Agriculture Organization of the United Nations
g	Gram
h	Hour
HDAC	Histone deacetylase
IFN-γ	Interferon-gamma

Ig	Immunoglobulin
IgM	Immunoglobulin M
IL	Interleukin
kDa	KiloDalton
kg	Kilogram
LD	Lethal dose
mg	Milligram
min	Minute
mL	Millilitre
MCH	Major histocompatibility complex
MPO	Myeloperoxidase
NBT	Nitroblue terazolium
OD	Optical density
pH	Measure of the acidity of a solution
RNA	Ribonucleic acid
RBC	Red blood cell
TLR	Toll like receptor
US$	United States of America dollars
USA	United States of America
WBC	White blood cell

Abstract

Rainbow trout is a salmonid species of major aquaculture importance. For the past decades, traditional strategy for fish disease controls in pond culture relied on the use of antibiotics and chemical disinfectants leading to the development of antibiotic-resistant bacterial strains and other problems such as environmental hazards, food safety problems and resistance of pathogens. This study aimed to evaluate the efficiency and investigate the mechanisms of action of different immunostimulating compounds (bovine lactoferrin, inulin, β-glucans, each at two dietary levels) in rainbow trout juveniles as alternatives to reduce the use of antibiotics and control fish diseases.

Bovine lactoferrin at low and high doses (BLf 0.1%, BLf 1%), inulin at low dose (In 0.1%) and low dose of GAS1- β-glucan (GAS1 0.2%) significantly modulated the blood leukocyte cell populations, humoral immune parameters after a short time treatment (35 days for bovine lactoferrin and inulin treatments; 15 days for β-glucans treatment), increased the expression of several immune related genes in spleen and kidney especially after long time of treatment (51 days for bovine lactoferrin and inulin tests; 36 days for β-glucans tests). Further, those compounds improved the fish disease resistance and enhanced the survival rate of rainbow trout juveniles after 14 days of infection with *Aeromonas salmonicida*. Meanwhile, high inulin dose (In 1%), macrogard β-glucan, wild type β-glucan, high dose of GAS 1-β-glucan and the combination diet of the best bovine lactoferrin dose (BLf 1%) + inulin (In 0.1%) + GAS1-β-glucan (GAS1 0.2%) did not improve the specific growth rate of rainbow trout juveniles compared with the control and single compounds used separately. Whereas a significant decrease of several immune gene expression levels of *il-1β*, *tgf-1β*, *il-10*, *cd4-2β*, *mIgM* and *hepcidin* was recorded in macrogard β-glucan, wild type β-glucan and high dose of GAS 1-β-glucan treatments in this study. However, BLf 1%, In 0.1%, GAS 1 0.2% and the combination of those compounds diets significantly increased the survival of rainbow trout juveniles after 14 days of bacterial challenge with *A. salmonicida*. Globally, low dose of inulin, low and high doses of bovine lactoferrin, and low dose of GAS1-β-glucan performed very well as immunostimulants provided orally, through the diet, in rainbow trout juveniles. Validations in field trials are still needed before promoting the use of those immunostimulants in intensive rainbow trout culture practices.

Based on the scientific literature and our own results, we can suggest some putative mechanisms of action of these compounds on the trout immune response: bovine lactoferrin compound may directly affect the fish immune system by facilitating iron absorption and transportation abilities from blood stream to the whole fish immune cells. Meanwhile, enterocytes may facilitate the transportation of β-glucans compounds across the intestinal cell wall into the lymph to interact with macrophages in order to activate immune function. Furthermore, inulin and β-glucans are fermented by Bifidobacterium and lactic acid producing bacteria to produce amount of butyric - a short-chain fatty acid which is reported to improve the fish immune system.

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Namur on 15th July, 2018

Trinh Dinh Khuyen

Chapter 1

General Introduction

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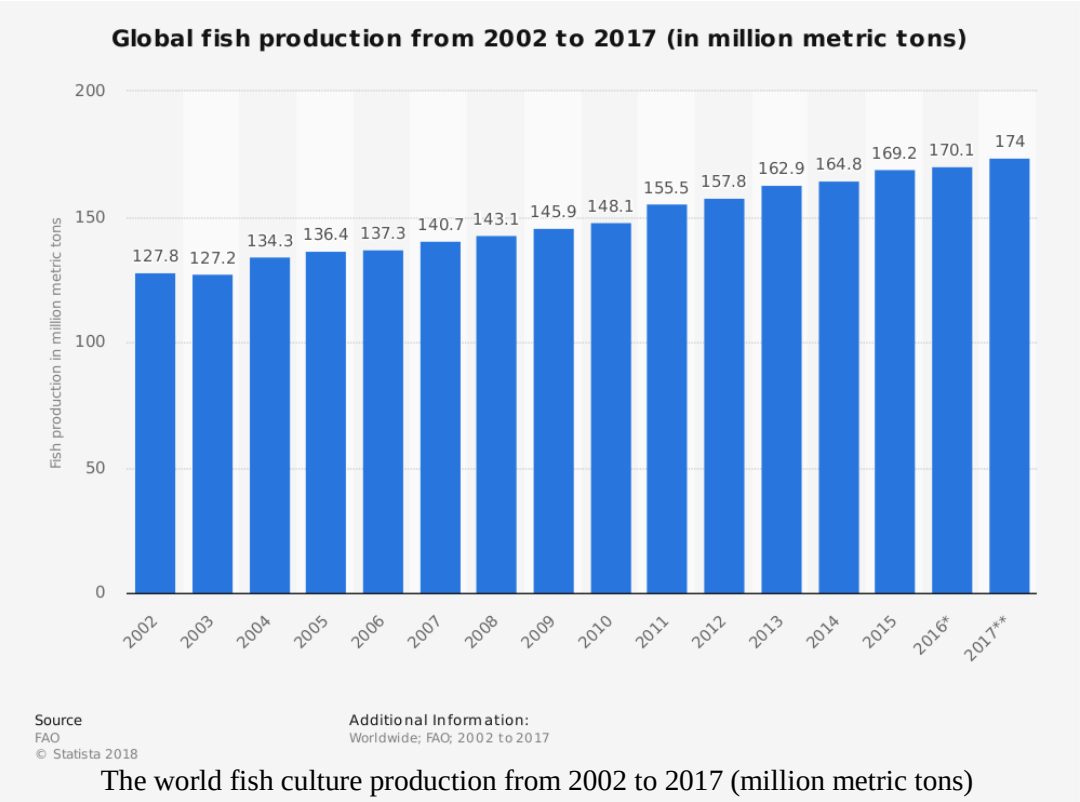
1. Current status of world aquaculture

1.1. World aquaculture production

According to the Food and Agriculture Organization (FAO), aquaculture is understood to mean the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants by applying some form of intervention in the rearing — stocking, feeding and protection from predators — to enhance the production. Aquaculture is now the fastest growing segment of the animal production industry. Fish aquaculture plays an important part in the economy of the aquaculture industry. In 2017, the total fish production of the world was 174 million metric tons (Figure. 1.1) — an increase of 23.7% and 36.2% in comparison with the yield of year 2007 and year 2002 respectively (FAO, 2018).



The world fish culture production from 2002 to 2017 (million metric tons)

Figure 1.1: World fish production from 2002 to 2017 (FAO Statistic, 2018)

The aquaculture industry has provided millions of jobs and billions of US dollars yearly to many countries. Indeed, aquaculture feeds are among the most important drivers for the aquaculture industry. The recent rapid development of the aquaculture industry also requires suitable development of the aquaculture feed industry. The nutritional requirements of target species must be satisfied while maintaining a more sustainable use of feed ingredients and the optimal use of feed for health enhancement and stress resistance (FAO, 2018).

1.2. Rainbow trout culture production

Rainbow trout is one of the world's most important fish species for consumption. According to FAO Statistics 2018, the total world production of the group including rainbow trout, salmon and smelts in 2015 was 3,411,000 tons valued at up to 16.189 million US dollars. Chile, Iran, Turkey and Norway are the most important rainbow trout producers in the world, followed by the EU-countries Denmark, Italy and France. Chile and Norway are the biggest

producers of large trout also known as salmon trout, while Iran and Turkey dominate the freshwater production of portion-sized rainbow trout.

2. Rainbow trout disease and therapeutic treatments

2.1. Diseases of rainbow trout

Both salmon and rainbow trout are susceptible to a number of bacterial and viral diseases as well as a number of stressors related to husbandry, such as handling and transportation, that are of importance at different stages of growth and production [1]. The most severe viral diseases of salmonids are infectious pancreatic necrosis (IPN), viral haemopoietic septicaemia (VHS), infectious haemopoietic necrosis (IHN), pancreas disease (PD), infectious salmon anaemia (ISA), heart and skeletal muscle inflammation (HSMI), and cardiomyopathy syndrome (CMS) [2]. On the other hand, extensive research has begun to demonstrate the efficacy of using pre and probiotics to improve disease resistance and reduce mortality of salmonids. The most important bacterial diseases of salmonids are furunculosis, vibriosis, cold water vibriosis, piscirickettsiosis, enteric redmouth disease (ERM), bacterial kidney disease and winter ulcer [1].

2.2. Therapeutic treatments

During the last decades, a number of approaches have been taken to control diseases including sanitary prophylaxis, disinfection, and chemotherapy. Recently, increased attention has been paid on the use of different types of immunostimulants and vaccination to reduce susceptibility to various stressors and diseases, as well as enhance the overall health of fish [3,4]. However, vaccination method is labour intensive and usually causes the stress to fish, time-consuming and impractical with very small fish. Further, vaccination is seemed to be limited when it's specific to against a typical of disease. Meanwhile, administration of immunostimulants has been reported to increase the leukocytes function and protect the fish to against infection diseases. This method is non-stressful and permits mass administration regardless of fish size.

3. Fish immune system

Fish are constantly exposed to pathogens in their environment. This has led to the evolution of defensive immune systems consisting of a variety of molecules, cells and tissues. The immune system of fish can be divided into non-specific and specific arms, and each of these has a separate role in the destruction and removal of invading pathogens [5]. The immune structure of rainbow trout is shown in figure 1.2.

3.1. Innate immune system

The innate system is the earliest immune mechanism and it is characterised by being non-specific and therefore not dependent upon previous recognition of the surface structures of the invader [6].

3.1.1. Gill

The fish gill is a multifunctional organ including gas exchange, ionoregulation, osmoregulation, acid-base balance, ammonia excretion, hormone production, modification of circulating metabolites and fish immune defence activities [7]. Fish pathogens always spread in the water and the thin respiratory epithelium of the gills represents an obvious entry point

for pathogens. For instance, it has been reported that salmon anaemia virus infection is supposed to be established first in the gills before spreading to other organs [8]. The physical barrier of the fish gills is comprised of the gill epithelium, a glycocalyx layer and a mucus layer. Recent research has indicated that the fish gill is the major organ for antibody secreting cell production following direct immersion immunization [9]. Meanwhile, lymphocyte accumulations have been recognized recently on the caudal edge of the interbranchial septum, at the base of the gill filaments in salmonid fish [10]. Further, the flow cytometry analysis shows that high numbers of T cells in the gills as well as in thymus and intestine and the interbranchial T cells, are embedded in a meshwork of epithelial cells [11].

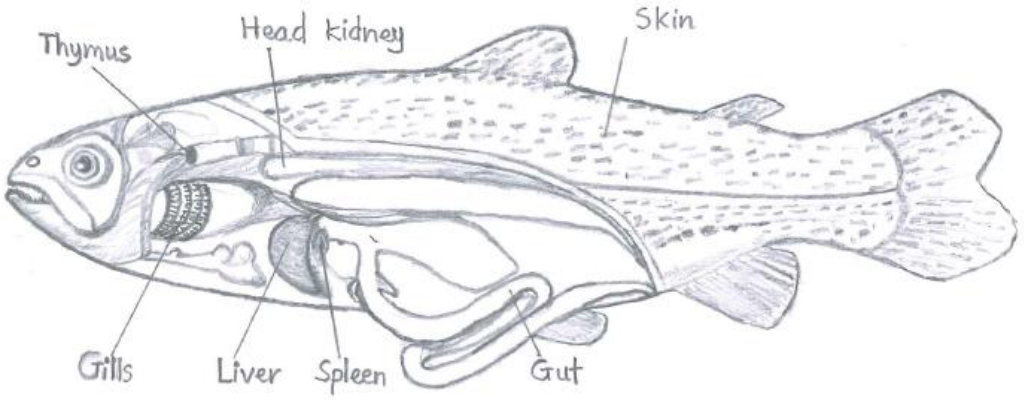


Figure 1.2: Basic immune structure of rainbow trout *Oncorhynchus mykiss*

3.1.2. Skin

The fish body is in contact with many different types of microorganisms via its skin. The integumentary surface is the first barrier against pathogens with mechanisms protecting against pathogen entry [6]. Among the important integumental mechanisms are mucus secretions. The mucus produced by fish skin represents an almost unique first line of defence against foreign invaders. The mucus contains immunoglobulin (IgM), complement protein, lectins, pentraxins, and lysozyme [5]. The mucus of fish skin is an important barrier for fish because (i): it provides the substrate in which antibacterial mechanisms may act (ii): in most fish species the mucus covers most of the external surfaces, and mainly the skin. The production of mucus is significantly increased when subjected to stressing situations, such as chemical aggressions, which induce higher expression and activity of antibacterial agents [6,12].

3.1.3. Thymus

The thymus plays a pivotal role in the development of the adaptive immune system, an important development that separates higher vertebrates from the rest of the animal phyla [13]. The thymus appeared at a very early stage of fish evolution [13]. The structure of the fish thymus is highly variable between species and even within a species [14]. In fresh water fish, the thymus is the first organ to become lymphoid although, prior to this, the kidney can contain hematopoietic precursors, but not lymphocytes. In marine fish, the order in which the major lymphoid organs develop is kidney, spleen and then the thymus [15]. The early

development of the thymus in fish has been investigated in many diverse teleost species and has shown that the development time frame can differ from species to species even when accounting for temperature effects on growth [13]. The rainbow trout embryo already possesses the rudiments of a thymus at 5 days pre-hatching at 14°C [16] while a thymus is only seen at 28 days post-hatching in Atlantic cod [17].

3.1.4. Spleen

The spleen is composed of a system of splenic ellipsoids, melano-macrophages and lymphoid tissue. In most species, ellipsoids are clustered together and organized around the other two components [18]. In mammals, the spleen is the largest secondary immune organ in the body and is responsible for initiating immune reactions to blood-borne antigens and for filtering the blood of foreign material and old or damaged red blood cells. The spleen is also a major secondary lymphoid organ in fish. It contains the same elements as the other vertebrates: blood vessels, ellipsoids, red pulp and white pulp [14]. The pulp occupies the majority of the organ and consists of a reticular cell network supporting blood-filled sinusoids that hold diverse cell populations, including macrophages and lymphocytes. The spleen can also be a major reservoir of disease and there is much interest in trying to understand its role in protection against bacterial infection as well as red blood cell regulation [19]. The populations of lymphocytes and macrophages capable of mounting an immune response are situated close to sites of antigen trapping and often associated with accumulations of melano-macrophages.

3.1.5. Kidney

The kidney in fish is often a Y-shaped organ that lies along the body axis above the swim bladder. The lower part, the long structure situated parallel to the vertebral column, works as a renal system [6]. The active immune part of the head kidney is formed by the two Y arms which penetrate under the gills [14]. The head kidney has a reticulo-endothelial stroma consisting of sinusoidal cells and reticular cells similar to those of mammalian bone marrow [20]. The fish head kidney appears to be the primary organ for antibody production [21]. It is an important endocrine organ, homologous to mammalian adrenal glands and contains aminergic chromaffin and interenal steroidogenic cells [14].

3.1.6. Intestine

The intestines of fish consist of two segments, the small intestine and the large intestine. The intestine plays dual roles in mammals such as digestion or the uptake of nutrients and immune homeostasis. The gut protects the body from potentially harmful microbes and also induces a tolerogenic response to innocuous food, commensals and self-antigens [14]. In many species investigated, the intestine can be subdivided into three segments based on the microscopic anatomy of their mucosa, especially their enterocytes. The enterocytes of the first segment are absorbent. The enterocytes of the second segment are characterized by the presence of large supranuclear vacuoles. An irregular microvilli zone and high pinocytotic activity at the apical part, take up macromolecules. The enterocytes of the third segment have been less studied but are thought to have an osmoregulatory function [22]. Leukocytes are abundantly present in the lamina propria and intestinal epithelium of the fish gut. However, the lack of suitable antibodies in fish has hampered the distinction of subpopulations within the gut-associated lymphoid tissues [14].

3.1.7. Liver

The liver is responsible for protein, carbohydrate and lipid metabolism, bile secretion and detoxification. However, it is also an important immune organ [14]. The liver is the only non-

lymphoid organ able to retain and activate naïve $CD8^+$T cells in an antigen-specific manner and this is associated with the induction of T-cell tolerance [23]. The immune functions of fish liver are still not well understood, but the large impact on immune gene expression after bacterial infection suggests that the fish liver is actively involved in immune defence [24].

3.2. Adaptive immune responses

3.2.1. The humoral adaptive immune responses

3.2.1.1. Immunoglobulin

Immunoglobulin, also known as antibodies, are a large, Y-shaped protein produced mainly by blood cells and is used by the immune system to identify and neutralize pathogens such as bacteria and viruses [25]. The antibody recognizes a unique molecule of the harmful agent, called an antigen, via the variable region [26]. Immunoglobulin is glycoproteins which constitute most of the gamma globulin fraction of the blood protein. They are typically made of basic structural units, each with two large heavy chains and two small light chains [27]. Five classes of heavy chain (H) carboxyl-terminal constant domains are known in mammals that define the IgM, IgG, IgA, IgD and IgE isotypes [28,29]. IgM is the predominant immunoglobulin isotype found in teleost blood/serum while IgD and IgT are found in lesser amounts. In teleost fish, serum IgM is expressed as a tetramer, even IgM monomers have been reported in some fish species. In contrast, serum IgT is expressed as a monomer in rainbow trout serum, and a tetramer in gut mucus [30]. IgT and IgM can be expressed from very early developmental stages in teleost fish. The expression of IgT increases more rapidly when compared with that of IgM, which may suggest that IgT plays a significant role in protecting fish larvae [14]. Three isotypes, IgM, IgD and IgT (also called IgZ in zebrafish) have been identified in almost all studied species belonging to the main orders of teleost fish [14].

3.2.1.2. B cells

The teleost fish appears to contain two or more major subsets of B cells although the presence of the IgT^+ and IgD^+ B cells subset needs to be confirmed in other fish species. Since fish lack IgG, IgE and IgA and as IgT and IgM are not co-expressed in the same B cell, fish only have the ability to make IgM^+IgD^+, IgM^+IgD^-, single IgD^+ and IgT^+ B cells [14]. It has been reported that rainbow trout have IgM^+IgD^+ B cells ($IgM^+/IgD^+/IgT^-$) and IgT^+ B cells ($IgM^-/IgD^-/IgT^+$) [30]. Meanwhile, catfish have IgM^+/IgD^+, IgM^+/IgD^- and IgM^-/IgD^+ subsets in the absence of IgT [31]. The rainbow trout IgT^+ B cells subset represents 16–27% of all B cells in the main systemic lymphoid organs, IgM^+ occupied about 72–83% of all B cells. In the gut, the percentage of IgT^+ and IgM^+ lymphocytes is around 54% and 46% of all B cells respectively [30]. The specific immune response to infection and vaccination has been reported in fish through measurement of IgM responses and IgT responses [14]. Agglutinating or neutralizing antibodies can be enhanced with vaccines against viral, bacterial and parasitic diseases [32,33]. Fish Ig isotypes are compartmentalized into mucosal IgT and systemic IgM in response to pathogen challenge. IgT^+ B cells accumulate in the gut of rainbow trout that survive an infection of an intestinal parasite *Ceratomyxa shasta* [30]. Fish IgM can be present in high concentration in fish serum as natural antibodies, which are believed to have pattern recognition receptor properties [34].

3.2.2. The cellular adaptive immune response

The expression of the co-receptors, CD8 or CD4, that stabilizes the interaction of the T cell receptors (TCR) with major histocompatibility complex (MHC) molecules, thus enhancing

TCR activation through the CD3 tyrosine phosphorylation pathway, two main sets of T cells are distinguished — $CD4^+$ and $CD8^+$ T cells [14]. CD8 marks CTL that recognized antigenic peptides associated with MCH class I molecules on the surface of antigen presenting cells and whose main function is the direct killing of target cells. CD4 marks T helper cells (Th cells) that recognize peptides associated with MHC class II and orchestrates many aspects of the immune response by release of cytokines [14]. It has been reported that the fish T cell primary differentiation takes place in the thymus, where mature T cells migrate to secondary lymphoid organs such as the head kidney and spleen. T cells can also be found in the gills, liver, olfactory pit and gut [35]. The detail of the T cell differentiation processes in fish is less well known. $CD8^+$ and $CD4^+$ mRNAs are highly expressed in the thymus of naïve fish, and in kidney and spleen tissues and a low degree of expression in gill and gut. The expression pattern of these molecules is correlated with the expression of other T cell markers such as LAG3 or TCR. The expression of these markers can be modulated after infection or after in vitro exposure to T cell mitogens [36]. Specific cell-mediated cytotoxicity was first described in catfish, and later in several other fish species [37]. In rainbow trout, specific cell mediated cytotoxicity occurs when leukocytes from viral haemorrhagic septicaemia virus (VHSV)-infected clonal fish are mixed with infected MHC compatible RTG2 fibroblasts [37]. Interestingly, peripheral blood leukocytes from fish immunized with a DNA vaccine encoding the VHSV G protein efficiently lysed VHSV-infected histocompatible targets [38].

4. Immunostimulation and its effects on fish

4.1. Immunostimulants

Immunostimulants are a group of biological and synthetic compounds that enhance the non-specific cellular and humoral defence mechanism in mammals [39]. The biological effects of immunostimulants are highly dependent on the receptors on the target cells recognising them as potential high-risk molecules thus triggering various defence pathways. Therefore, it is important to increase our knowledge of receptor specificity and how the inflammatory processes of the different receptors upon antigen binding are induced [3]. It has been reported that immunostimulants could be grouped according to their sources: bacterial, algae derived, nutritional factors and hormones/cytokines [40].

4.2. Background of the tested immunostimulants compounds

4.2.1. Lactoferrin

Lactoferrin is an 80 kDa iron-binding glycoprotein of the transferrin family, which is involved in many physiological functions, namely in regulation of iron absorption and stimulation of innate and specific immune systems as well as disease resistance [41]. Due to its immunomodulatory properties, BLf may stimulate antibacterial, antifungal or antiviral functions and consequently may induce effective protection against various bacterial and fungal diseases in several species of domestic animals depending on the dose and administration mode [3,4,41,42]. Numerous immunostimulant effects of BLf were reported in fish and it was shown to improve survival rate [43], resistance to diseases and enhance humoral immunity [44,45]. In-vitro studies by Kamilya et al. [46] in catla, *Catla catla,* head kidney leukocytes and by Esteban et al. [47] on head kidney leukocytes of gilthead seabream, *Sparus aurata*, showed that BLf induced changes in innate immune cellular activity, mainly respiratory bursts and natural cytotoxic activities. For example, oral administration at 40 mg/kg body weight/day for 28 days, BLf has been reported to protect red sea bream, *Pagrus major*, against white spot disease [44]. The structure of bovine lactoferrin is indicated in

figure 1.3. Kumari et al. [45] reported that BLf supplement, particularly at the 100 mg level, significantly enhanced serum lysozyme levels, oxidative radical production and the level of protection against *A. hydrophila* challenge in Asian catfish, *Clarias batrachus*.

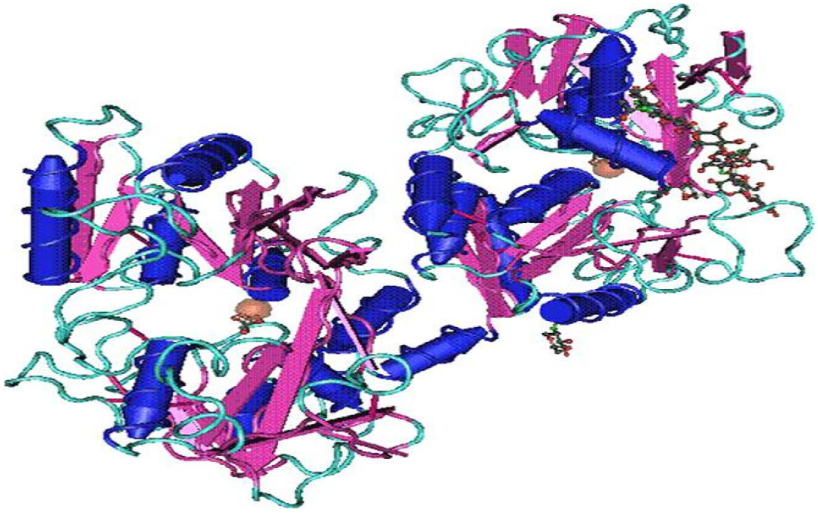


Figure 1.3. Three-dimensional structure of biferric bovine lactoferrin at

a resolution of 2.8 Angstroms [48]

However, it had no influence on the specific immunity of vaccinated Asian catfish [45]. In some experiments, growth performance [43,49], physiological [43] and immunological variables [43,50] were not always equally affected by dietary BLf. For example, in Atlantic salmon, *Salmo salar*, a dietary administration of BLf failed to reduce mortality challenged with *Aeromonas salmonicida* or salmon anaemia virus [50] and [45]. The use of lactoferrin in aquaculture is summarized in table 1.1.

4.2.2. Lipopolysaccharides

The Gram-negative bacterial lipopolysaccharides (LPS), also known as term endotoxins, is a major component of the outer membrane, which plays a very important role in host pathogen interactions with the innate immune system [51]. Lipopolysaccharides are responsible for the lethal effects and clinical manifestations of diseases in humans and animals [52]. Endotoxins are comprised of an outer leaflet or outer membrane like most of Gram-negative bacteria [53], which is a large molecular weight substance with a unique structure and consisting of three regions including an outer polysaccharide region-known as “O” antigen, a branched component of oligosaccharide residues, a polysaccharide core region and an inner fatty acid region. A typical lipopolysaccharide unit showing different regions is indicated in figure 1.4. Lipopolysaccharides or/endotoxins can innumerable biological systems in all living things. It has been found many times to be responsible for the pathogenicity of several diseases caused by Gram-negative bacteria in fish [54]. The innate immunity of fish recognizes the presence of infectious agents for activation of the adaptive process [55,56]. Endotoxin/LPS exerts several clinical signs and symptoms such as hyperaemia or discolouration of mucus membrane, hypotension, acute disruption of gastric functions, improvement in motility of small and large intestine, increases in body temperature, respiratory and heart rate as well as dehydration in sensitive fish [57]. Treated *Escherichia coli* and *Aeromonas salmonicida* endotoxin doses of 80 mg/kg body weight have shown to improve resistance in rainbow trout [58].

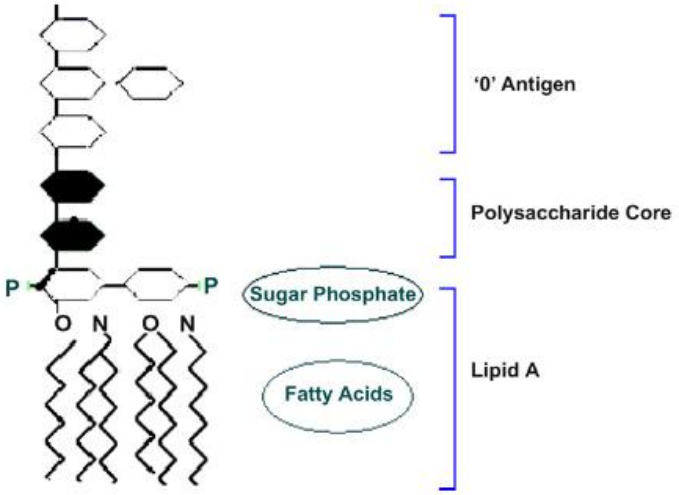


Figure 1.4. A typical lipopolysaccharide unit showing different regions [52]

Indian major carp, *Labeo rohita*, yearlings ranging from 80 to 100g were injected intraperitoneally with 0.5, 1, 2, 5, 10 and 20 endotocxin units/fish dose of endotoxin. Fish resist the endotoxin dose up to 20 endotocxin units/fish and at the lower doses at 1 and 2 endotocxin unit/fish [59]. Furthermore, an in-vivo study of the effects of *Escherichia coli* Lipopolysaccharide on striped catfish (*Pangasianodon hypophalmus*) demonstrated that LPS treatments differed in blood cell count and immune variables such as plasma and spleen lysozyme, and complement immune activity and antibody titer. LPS induced differentially over-expressed immune proteins such as complement component C3 and lysozyme C2 precursor; enhanced innate immune responses [60].

4.2.3. Chitosan

Chitosan is a basic polysaccharide that is a biocompatible and biodegradable polymer having immense structural possibilities for chemical and mechanical modification to generate novel properties and functions in different fields, especially in the biomedical field [61]. The structure of chitosan is indicated in figure 1.5.

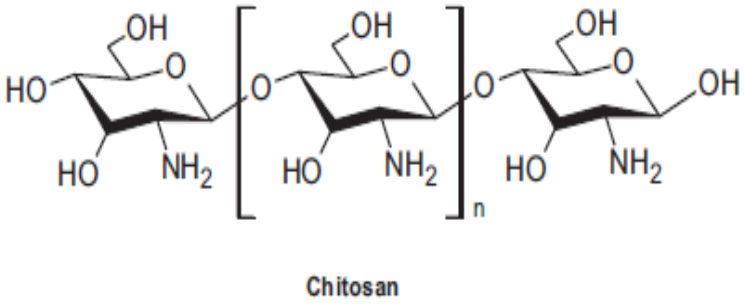


Figure 1.5. Structure of chitosan [61]

It exhibits special properties such as viscosity, solubility in various media, mucoadhesivity, polyoxysalt formation, polyelectrolyte behaviour, ability to form films, metal chelation, optical, structural characteristics and binds antagonistically with microbial and mammalian cells [61,62]. Chitosan plays an important role in the osteoblast formation process, which accounts for bone formation, fungi-static, sperminidal, haemostatic, antitumor and anti-

cholesteremic activities [63]. The hetero-polymer form of chitosan is comprised of glucosamine and acetyl-glucosamine units, which are available in different grades depending on the degree of acetylated moieties. Results from a previous study showed that dietary chitosan nanoparticles protect crayfish (*Procambarus clarkia*) against white spot syndrome virus infection indicating that dietary chitosan nanoparticles effectively improve innate immunity and survival of crayfish challenged with white spot syndrome virus [64]. Feeding 1% chitosan as a preventive/therapeutic regime has shown several remarkably enhanced innate immunological parameters such as increased phagocytic activity/index, NBT, lysozyme activity and ACH50 and increased resistance against *A. hydrophila* [65]. Furthermore, zebrafish (*Danio rerio*) larvae at 5 days post-hatching stage exposed to chitosan nanoparticles (5µg/mL) showed a higher survival rate at 72 h post infection stage against pathogenic *A. hydrophila* compared to control. These results suggest that chitosan nanoparticles could be attributed to stronger immune modulatory properties [66].

4.2.4. Inulin

Inulin is a naturally occurring fructooligosaccharide (FOS) extracted from the chicory root (*Cichorium intybus*) as well as from many other plants (cereals, leeks, onions, garlic, wheat, artichokes and bananas) and belongs to a class of carbohydrates known as fructans [67,68]. Fructans are non-digestible oligosaccharides as the β (2-1) linkage of the fructans cannot be hydrolysed by pancreatic or brush border digestive enzymes in the proximal intestinal tract of human and domestic animals [69]. The chemical structure of inulin is shown in figure 1.6.

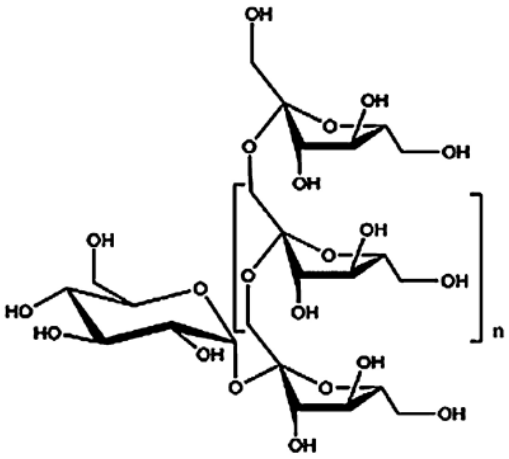


Figure 1.6. Chemical chair structure of inulin [70]

Inulin is fermented in the large intestine or colon [71] by beneficial bifidobacteria and other lactic acid-producing bacteria, enhancing their relative population while selectively inhibiting the growth of pathogenic microorganisms [72,73]. The total serum immunoglobulins, bactericidal activity and anti-protease activity increased in grass carp (*Ctenopharyngodon idella*), when fed inulin diets at 0.2% and 2% for 8 weeks [67]. Similarly, an increase in the serum complement activity, IgM level, leukocyte phagocytic activity and leukocyte respiratory activity was reported in gilthead seabream fed inulin at the same dose for two weeks [74]. In common carp fry (*Cyprinus carpio*), blood respiratory burst activity increased after 7 weeks of feeding treatment with inulin at 5 to 10g/kg [75]. Dietary inulin at 2.5g and 5g/kg increased red blood cells, improved lysozyme activity and complements haemolytic activity (ACH50) of juvenile tilapia *Oreochromis niloticus* [76]. Inulin-enriched diets significantly upregulated mRNA transcripts of immunoglobulin M (IgM) in intestine, head kidney and skin after 6–8 weeks of feeding, but did not show any difference in lysozyme

activity, serum protein or blood glucose of the leopard grouper (*Mycteroperca rosacea*) [77]. Dietary supplementation of inulin has been reported to improve weight gain, specific growth rate, protein efficiency ratio and food conversion rate in grass carp [67]. But, other authors did not find any positive effect on growth performance and survival rate in common carp [75], tilapia [76]. Dietary inulin at 0.5% alone did not show any positive effect on gut microbiota. The combination of 0.5% of inulin and the Gram-positive bacteria *Weissella cibaria* from the family of *Leuconostocaceae* at 7.87 ± 0.2 log CFU g^{-1} induced a higher concentration of lactic acid bacteria and low levels of *Vibrio spp* and *Pseudomonas spp* in the midgut of hybrid surubim *Pseudoplatystoma sp* [78]. The use of inulin in aquaculture is summarized in table 1.2.

4.2.5. Beta glucans

β-glucan is a heterogeneous group of glucose polysaccharides consisting of a backbone of β-(1,3)-link β - D-glucopyranosyl units with β - (1,6)-link side chains of varying length and distribution [79], which are the major structural component of fungi, bacteria, plants, algae, yeast, and mushroom cell walls. The molecular structure of β-glucan is shown in figure 1.7.

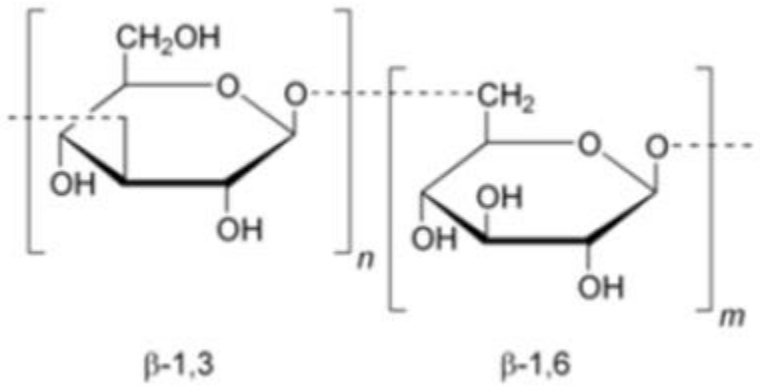


Figure 1.7. Molecular structure of β-glucan [80]

Further, β-glucans are also extracted from the bran of oat, barley, rye, wheat grains and several species of seaweed [81,82]. Numerous immunostimulant effects of β-glucans have been reported in several fish species, for instance: against pathogen of Asian catfish [83,84], common carp [85], catla [86], Nile tilapia [87], rohu (*Labeo rohita*) [88], and zebrafish [79] when infected with *Aeromonas hydrophila*, grass carp [89] infected with grass carp haemorrhagic virus, large yellow croaker (*Pseudosciaena crocea*) infected with *Vibrio harveyi* [90]; and rohu infected with *Edwardsiella tarda* [91]. It has been reported that dietary β-glucans increased antibody titres production of catla-injected with 0.1mL glucan solution of 100µg mL^{-1}/fish of 30g, carp-injected 100-1000µg/fish of 25–30g [19,25]; stimulated an increase of lysozyme activity [90,93,94], macrophage bactericidal activity [79,92], superoxide anion production [83,84,89,92,94]. Dietary β-glucans increased the number myelomonocytic cell population in head kidney of zebrafish at 6h post-challenge with *A. hydrophila* [79], increased the proportion of neutrophil and monocyte cells of carp-injected with 100, 500 and 1000 µg of glucan/fish [92]. However, dietary supplements containing β-glucans administrated to channel catfish (*Ictalurus punctatus*) for 4 weeks did not significantly affect several immune parameters such as plasma lysozyme, bactericidal and haemolytic complement activities, respiratory bursts of phagocytes and the number of lymphocytes found, survival rate in fish infected with *Edwardsiella ictaluri* [95]. Further, dietary

supplements of β-glucans did not affect growth performance of European sea bass (*Dicentrarchus labrax*) [96]. It appears that the immune-stimulating effects of β-glucans on the immune system of fish are universal [97]. Beta glucan is a heterogeneous group of glucose polysaccharides consisting of a backbone of β (1, 3)-link β -D-glucopyranosyl units with β-(1, 6)-link side chains of varying length and distribution [79], but their efficiency may vary according to their source. The most common forms of β-glucans are those comprising D-glucose units with β-1, 3 links. But, yeast and fungal β-glucans contain 1–6 side branches, meanwhile cereal β-glucans contain only β-1, 3 and β-1, 4 backbone bonds [98]. The frequency, location, and length of the side-chains may play a role in immunomodulation. Differences in molecular weight, shape, and structure of β-glucans indicate the differences in biological activity [99]. The use of β-glucans is summarized in table 1.3.

4.2.6. Levamisole

Levamisole is a synthetic anthelminthic drug for animals against stomach, intestinal and lung worms [100]. It is a medication used to treat parasitic worm infections, specifically ascariasis and hookworm infection [101,102]. Levamisole, as a nicotinic acetylcholine receptor agonist, causes the stimulation of parasitic worm muscles, leading to paralysis. Levamisole is a heterocyclic compound that is an effective anthelminthic agent and is also immunoregulatory. The immunoregulatory mode of action is by mimicry of the thymic hormone thymopoietin [103]. The structure of levamisole is shown in figure 1.8.

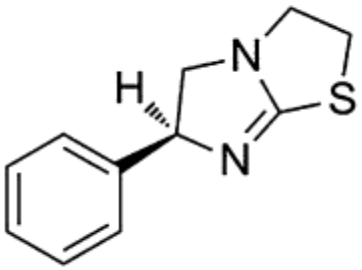


Figure 1.8. Structure of levamisole [104]

Levamisole may affect many components of the immune system including both neutrophils, macrophages, and lymphocytes and its therapeutically important actions are probably targeted at stimulation of phagocytosis and stimulation of regulatory T cells to restore homeostasis in a perturbed immune system [103]. Feeding levamisole at 50, 150, or 450 mg/kg to Asian catfish for 10 days increased the survival rate [105]. Dietary supplements of levamisole hydrochloride at 300 mg/kg have been reported effective in controlling the infection by parasites *Rondonia rondoni* and caused moderate histological changes in pacu liver [106]. Furthermore, administration of dietary levamisole at 300 mg/kg to *clarias fuscus* could be optimum for stimulating nonspecific defence mechanisms and specific immune response against *Acinetobacter lwoffii* [107]. Dietary levamisole has been modulated circulating cortisol levels during the stress response and improved the innate immune response against *A. hydrophila* infection in pacu (*Piaractus mesopotamicus*) [108]. Moreover, dietary supplementation of levamisole at 100 mg/kg significantly enhanced growth and feed efficiency of hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) meanwhile, fish fed a diet of 1000 mg/kg showed chronic toxicity signs of inferior growth, reduced feed intake and feed efficiency [100].

Table 1.1: Use of lactoferrin in aquaculture

Species	Stimulant/Duration	Route of administration/Doses	Results	Reference
Nile tilapia	Bovine lactoferrin (for 8 weeks)	Oral administration at 0, 0.2g, 0.4g, 0.8g, or 1.6g/kg.	No effect on growth performance or haematological parameters; Improved survival rate in fish fed the 0.8g/kg.	[43]
Gilthead seabream	Human lactoferrin (for 1 week)	Oral administered at 0.1 g/kg.	The inate immune responses although only the cytotoxic activity.	[47]
Atlantic salmon	Bovine lactoferrin for 19 days)	Oral administration at 0.14g/kg feed, and with 0.15 or 1g vitamin C.	Reduced mortality challenged with *A.salmonicida*	[50]
Rainbow trout	Bovine lactoferrin (for 3 days)	Oral administration by intubation at 0.1g/kg	Increased survival rate when challenged with *V. anguillarum* and enhanced resistance against *Streptococcus sp*.	[109]
Rainbow trout	Plasmid DNA, lactoferrin and β-glucan (for 45 min four times with an interval of 1 week)	Bath administration of plasmid DNA, lactoferrin and β-glucan at two different doses (0.1 μM and 1.0 μM).	Enhanced pro-inflammatory genes: *il-1β*, *tnf-α*, *il-6;* And anti-inflammatory cytokines: *il-10* and *tgf-β*.	[110]
Rainbow trout	Bovine lactoferrin (for 8 weeks)	Oral administration 0.1g/kg.	Enhanced lysozyme activity; Haemolytic complement activity and anti-protease.	[111]

Table 1.2: Use of inulin in aquaculture

Species	Stimulant/Duration	Route of administration/Doses	Results	Reference
Great sturgeon	Inulin (for 8 weeks)	Oral administration at 10g, 20g, 30g/kg.	No change of RBC count; Increased of Total WBC count; No change of MCH; Dose dependent alkaline phosphatase decreased	[112]
Gilthead seabream	Inulin (for 2 and 4 weeks)	Oral administration at 10 g/kg.	Increased serums complement activity; IgM level; leukocyte phagocytic activity; leukocyte respiratory burst activity and increased the protection from *P. damselae* challenge.	[74,113,114]
Hybrid surubim	Inulin (for 15 days)	Oral administration at 5g/kg.	Increased lactic acid bacteria; Decreased *Vibrio spp*; Decreased *Pseudomonas spp*; Increased total Ig; No change of serum protein or lysozyme.	[78]
Nile tilapia	Inulin (for 1 and 2 months)	Oral administration at 5g/kg.	Increased Hematocrit; NBT (Superoxide activity); lysozyme activity; and increased the protection from *A. hydrophila challenge*.	[115]

(RBC: Red Blood Cell, WBC: White Blood Cell, MCH: Major Histocompatibility Complex, IgM: Immunoglobulin M, NBT: Nitroblue terazolium)

Table 1.3: Effective dose of various β-glucans used in fish species

Species	Stimulant/Duration	Route of administration/Doses	Results	Reference
Rainbow trout	β-1,3/1,6-glucan (for 15 and 30 days)	Oral administration at 1g, 2g and 5g/kg.	Overdoses of β-glucans and/or prolonged medication may lead to a non-reactive physiological status and consequently leading to a poor immune response.	[116]
Rainbow trout	β-1,3/1,6-glucan (for 37 days)	Oral administration at 2g and 4g/kg.	Decreased expression of pro-inflammatory genes.	[117]
Rainbow trout	β-1,3/1,6-glucan (for 46 days)	Oral administration 2g/kg.	Increasing trend in lysozyme activity (not significant) observed in glucan fed rainbow trout; Increased resistance against challenge with *Ichthyophthirius multifiliis* (white spot disease).	[118]
Rainbow trout	β-1,3-glucan (for 84 days)	Oral administration at 10g/kg.	No effect of β-glucan alone or as adjuvant on survival after *Yersinia ruckerii* challenge; Down-regulation of expression of pro-inflammatory gene, acute phase and lysozyme related genes after challenge.	[119]
Rainbow trout	β-1,3-glucan (for 4 weeks)	Oral administration at 3/kg.	Increased gene expression of *cathelicidins 2* and *il-1β* in gut epithelial cells; Increased number of mucus secreting cells in the intestine.	[120]
Common carp	β-1,3-glucan + LPS	Administered on days 1, 7, and 14 through (intraperitoneal injection, bathing, and oral administration)	Intraperitoneal injection elicited 100% RPS even at the lowest concentration (100 mg β-glucan + 10 mg LPS); Oral administration improved RPS rate of carps at higher concentration (1% β -glucan + 0.25% LPS) Bathing did not improve the RPS	[85]

(Continued on next page)

(Continued table 3)

Koi carp	β-1,3-glucan, (for 56 days)	Oral administration at 1.8g/kg.	Increased white blood cell count (WBC); Increased oxidative burst, lysozyme activity; Phagocytosis, bactericidal activity.	[121]
Zebrafish	β-1,3/1,6-glucan (at 6, 4 and 2 days prior the challenge)	Intraperitoneal injection at three different concentrations (5, 2 and 0.5 mg/mL).	Intraperitoneal injection of 5mg/mL of β-glucan significantly reduced the mortality. Challenge with *A. hydrophila*.	[79]
Labeo rohita	β-glucan yeast (for 53–60 days)	Oral administration at 1g/kg.	Increased bacterial activity; Increased serum phagocytic index; Increased hemagglutination titre.	[88]
Gilthead seabream	β-glucan (for 2 weeks)	Oral administration 1g/kg.	Increased head kidney phagocytic activity; Increased spleen macrophage respiratory burst activity.	[122]
Sea bass	Macrogard or Ergosan (for 4 weeks)	Oral administration of Macrogard glucans 1g/kg or Ergosan at 5g/kg.	Increased serum alternative complement activity and lysozyme activity.	[96]
Large yellow croaker	β-1,3-glucan (for 8 weeks)	Oral administration at 0.9g and 1.8g/kg.	Low dose of β-glucan supplementation (0.9g/kg) significantly enhanced fish growth whereas high supplementation (1.8g/kg) did not affect; No significant differences of alternative complement pathway.	[90]

Overall, there are several methods to fight against fish disease such as vaccination, antibiotics and immunostimulants. Vaccination is used to prevent infectious diseases. However, this method is usually expensive and labour intensive. The application of antibiotics is undesirable since it leads to pathogen resistance and consumer reluctance. Immunostimulants may be able to compensate for the limitations of chemotherapeutics and vaccines. Immunostimulants may be safer than chemotherapeutics and their range of efficacy is wider than vaccination. Further, the combination of immunostimulants with vaccination administration may also increase the potency of immunostimulants and vaccine to fight against fish disease.

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Chapter 2

Objectives and Outline of Thesis

1. Objectives

The objective of this thesis was to evaluate the efficacy of different immunostimulating compounds (inulin, bovine lactoferrin, β-glucans) in rainbow trout juveniles in order to reduce the use and impact of antibiotics and chemicals by alternative method of immunostimulation. For each compound, the mechanisms of action have been investigated through the use of a combination of husbandry, physiological and molecular indicators.

2. Outline of the Thesis

The thesis is subdivided into 8 chapters:

Chapter 1	General Introduction: provide some details, such as the list of topics developed
Chapter 2	Objectives and Outline of the Thesis
Chapter 3	Physiological and immune responses of rainbow trout (*Oncorhynchus mykiss*) juveniles to bovine lactoferrin – Published in Fish and Shellfish Immunology Journal 2017.
Chapter 4	Immune pathway responses of rainbow trout (*Oncorhynchus mykiss*) juveniles to low and high dose of inulin - to be submitted
Chapter 5	Physiological and immune responses of rainbow trout (*Oncorhynchus mykiss*) juveniles to different types and doses of β-glucans, in preparation
Chapter 6	Can a dietary combination of inulin, bovine lactoferrin and β-glucan diets efficiently replace antibiotic in rainbow trout (*Oncorhynchus mykiss*) against bacterial infection by *A. salmonicida*?
Chapter 7	General Discussion
Chapter 8	Conclusions & Perspectives

Some experiment pictures:

Figure 2A: *Oncorhynchus mykiss*; Walbaum, 1792, Source: galleryhip.com

Figure 2B: Treatments tanks

Figure 2C: Flow cytometer

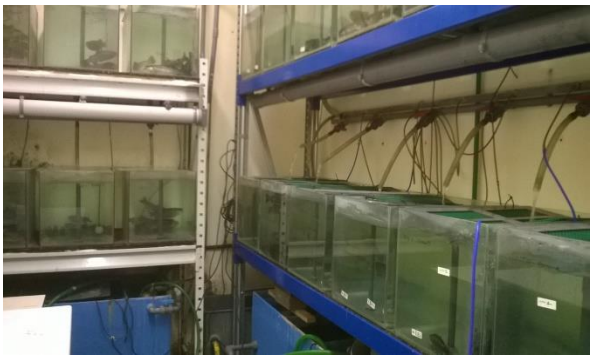

Figure 2D: Bacteria challenge tank system

Figure 2G: Rainbow trout juvenile infected with *A.salmonicida* (bleeding in the root of pectoral fins and anal inflammatory)

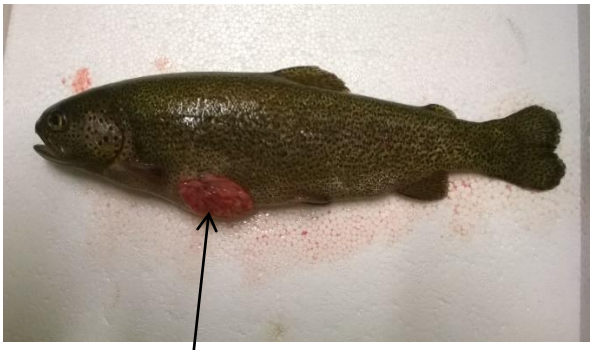

Figure 2E: Rainbow trout juvenile infected with *A.salmonicida* (bacterial infected at particular side)

Chapter 3

Physiological and immune response of juvenile rainbow trout (*Oncorhynchus mykiss*) to dietary bovine lactoferrin

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Abstract

Lactoferrin, a large multifunctional glycoprotein, is involved in many physiological functions such as regulation of iron absorption, stimulation of the innate and specific immune systems of organisms as well as disease resistance. The objective of the present study was to determine the ability of bovine lactoferrin (BLf) at low (0.1%) and high (1%) dose diet to improve the immune status and disease resistance of rainbow trout *Oncorhynchus mykiss* juveniles. BLf diets did not affect specific growth rate (SGR), haematocrit (Ht), splenic index (SI), spleen respiratory burst activity (RBA) as well as humoral (*mIgM*) and neutrophils (*MPO*) gene expression after short term - 35 days (D35) and long term nutrient test - 51 days (D51) of feeding. Both low and high BLf doses induced a significantly higher level of plasma alternative complement activity, plasma total immunoglobulin on D35 and D51, lymphocyte plus thrombocyte cell proportion on D35 and monocyte cell proportion in total blood leukocyte cells on D51 than those of the control. On D51 but not on D35, BLf diets upregulated the expression of inflammatory genes in kidney for *il-1* at the low BLf dose, *il-8* at both BLf doses and *il-6* at the high BLf dose in spleen, and *il-10* at both BLf doses in kidney. Moreover, the expression of T helper (*cd4-2α*; *cd4-2β*) genes was significantly upregulated only on D51 by both BLf doses in both spleen and kidney tissues. After 51 days treatment with BLf diets, juvenile rainbow trout were intraperitoneally injected with *A. salmonicida achromogenes*. The expression of 13 immune genes was evaluated at 44 h post-injection on day 54 (D54). The expression of lysozyme gene was upregulated by high BLf dose after bacterial infection both in spleen and kidney. The expression of *mcsfrα*, *tgf-β1* in spleen and *tgf-β1* in kidney was also modulated by high BLf dose and both BLf doses respectively. Low and high BLf doses enhanced disease resistance of rainbow trout juveniles with the cumulative survival rate of 36% and 38% respectively while those of the control was 19% after 14 days' challenge with bacteria ($p = 0.073$, close to significant difference level). The results indicate that BLf diets activated the humoral immunity, associated to blood leukocyte cells of rainbow trout after short term BLf administration, and the long term BLf administration was necessary for sensitizing other lymphoid organs such as in spleen and kidney. Only after long term test, BLf diets induced significantly higher levels of innate and adaptive immune gene expressions than those of the control. Dietary BLf activated more markedly the expression of innate immune genes than the adaptive ones; this upregulation of some immune genes could explain the higher disease resistance observed in rainbow trout juveniles fed BLf.

Keywords: bovine lactoferrin, rainbow trout (*Oncorhynchus mykiss*), immunity, gene expressions, disease resistance.

1. Introduction

Intensive fish farming practices at high stocking densities combined with limited prophylactic control can suppress the immune defence and consequently result in increased susceptibility to pathogen infection causing major problems in the aquaculture industry [1–3]. During the last decades, a number of approaches have been taken to control diseases including sanitary prophylaxis, disinfection, and chemotherapy with particular emphasis on the use of antibiotics. However, the application of antibiotics and chemicals in culture is often expensive and undesirable since it leads to pathogen resistance and consumer reluctance as well as to the release of these compounds in the surrounding environment [2]. Therefore, the use of antibiotics can decrease the efficiency of some therapeutic and prophylactic treatments. Consequently, limitations of the use of antibiotic treatments in animal production are progressively increasing in several countries without effective alternatives against disease outbreaks, especially in the case of aquaculture production [4].

Recently, increased attention has been paid on the use of different types of immunostimulants (either pre- or probiotics, or a combination of both named synbiotics) to reduce susceptibility to various stressors and diseases, as well as enhance the overall health of fish [5,6]. The beneficial effects of prebiotics are due to by-products derived from the fermentation of intestinal commensal bacteria. Among many health benefits attributed to prebiotics, the modulation of the immune system is one of the most anticipated benefits as well as their ability to stimulate systemic and local immunity [7] which directly enhances innate immune responses including phagocytic activation, neutrophils activation and activation of the alternative complement system, and increased lysozyme activity [8].

Because of its large multifunctional glycoprotein properties, lactoferrin (Lf) is involved in many physiological functions, namely in regulation of iron absorption and stimulation of innate and specific immune system as well as disease resistance [9]. Several Lf products have been characterized and tested, but bovine lactoferrin (BLf) has received much attention for animal prophylaxis. Due to its immunomodulatory properties, BLf may stimulate antibacterial, antifungal or antiviral functions, and consequently may induce effective protection against various bacterial and fungal diseases in several species of domestic animals depending to the dose and administration mode [5,6,9,10]. A number of studies focused on the immunomodulatory potentials of BLf in fish culture, and the available results vary greatly depending on the fish species, dose and administration mode [8-15].

Numerous immunostimulant effects of BLf were reported in fish on which it was shown to improve survival rate [17], resistance to diseases and enhanced humoral immunity [18,19]. In-vitro studies by Kamilya et al. [20] in catla *Catla catla* head kidney leukocytes and by Esteban et al. [21] on head kidney leukocytes of gilthead seabream *Sparus aurata* showed that BLf induced changes in innate immune cellular activity, mainly respiratory burst and natural cytotoxic activities. While applied in vivo by oral administration at 40 mg/kg body weight/day for 28 days, BLf has been reported to protect red sea bream *Pagrus major* against white spot disease [18]. Furthermore, Kumari et al. [19] reported that BLf supplement, particularly at 100 mg level, significantly enhanced serum lysozyme level, oxidative radical production and level of protection against *A. hydrophila* challenge in Asian catfish *Clarias batrachus.* However, it had no influences on the specific immunity of vaccinated Asian catfish [19]. Thus, it appeared that in some experiments, growth performance [17,22] physiological [17] and immunological variables [17,23] were not always equally affected by dietary BLf. For example, in Atlantic salmon *Salmo salar*, a dietary administration of BLf failed to reduce mortality challenged with *Aeromonas salmonicida* or *salmon anaemia virus*

[23] and [19]. Altogether, BLf effects in fish seem to be different depending on the experimental conditions and the species concerned [24]. Apart from the controversial results on the immunomodulatory actions of BLf, the pathways by which BLf may interfere with the immune system are still not well understood [21].

Studies on the potential immunomodulatory effects of BLf in rainbow trout are relatively limited. Twenty three years ago, Sakai et al. [25,26] reported that oral administrated of BLf (100 mg/kg body weight of fish) by intubation for three days resulted in increased survival of rainbow trout juveniles to *Vibrio anguillarum* infection but the relative immune modulation was not well clarified apart from an increase on phagocytic activity of kidney cells. Zhang et al. [27] demonstrated an enhancement of pro-inflammatory cytokines *il-1β*, *tnf-α*, *il-6* and anti-inflammatory cytokines *il-10* and *tgf-β* after bath immunostimulation (combined solution of plasmid DNA, bovine lactoferrin and laminaran β-glucan). Recently, Rahimnejad et al. [28] reported that feeding rainbow trout with a diet supplemented with BLf at 100 mg/kg or higher for 8 weeks enhanced lysozyme activity, haemolytic complement activity and antiprotease activity.

Despite several reports dealing with general effects of BLf on fish immune response and the potential immunomodulation of BLf at systemic level, to our knowledge, few studies investigated the temporal effect of BLf on immunological organs of rainbow trout juveniles. While several publications have studied and reported the effect of BLf on specific immune indicators of trout, in this study we aimed to make a comprehensive evaluation of the time (D35 and D51) and dose effects (low dose at 0.1% diet *vs* high dose at 1% diet) of oral administration of BLf on the overall immune function and disease resistance of trout juveniles including: blood leukocyte analysis, humoral immune parameters and immune related genes expression in relevant immune organs, such as spleen and kidney. Moreover, the disease resistance ability of trout juveniles was tested by applying *A. salmonicida achromogenes* challenge test.

2. Materials and methods

Experimental fish

Feeding trial and bacterial challenge were approved by the local Ethic Committee for Animal Research of the University of Namur, Belgium (Protocol number: 13197 KE). Rainbow trout juveniles (n = 315) were transported from a local fish farm (Hatrival, Belgium) to our facilities and distributed into 15 fibreglass tanks of 100L (35 fish/tank) in a recirculation system. Fish were allowed to acclimate to the new housing conditions for 21 days. During this period, water temperature was maintained at 13.9 ± 1.2°C by a cooling system, oxygen level averaged 11.6 ± 0.7 mg/L (aeration applied), constant photoperiod (Light:Dark ratio = 12:12) and fish were fed 1.5% of fish biomass, twice daily (at 9:00 am and 5:00 pm) with a specific trout diet with crude protein = 48%, crude fat = 15% (Coppens TROCO SUPREME-16, The Netherlands).

Fish diet and experimental design

After acclimation, fish (mean body weight: 73.8 ± 13.6 g) were fed either a control diet (no bovine lactoferrin) or bovine lactoferrin (Friesland Campina DOMO®, Netherlands) enriched diets (0.1% diet or 1.0% diet) for 51 days at 1.5% of fish biomass. All diets were formulated and pelleted in the laboratory (Table 3.1). Three replicate tanks were used for each experimental regime. After 35 days (short term nutrient test) and 51 days (long-term nutrient

test) of feeding, six fish per tank (n = 18 fish per experimental diet) were anesthetized in ethyl 3-aminobenzoate methane sulfonic acid salt (98% purity) (MS-222, Sigma) solution (120 mg/L). Blood was obtained by caudal vein puncture using a heparinized syringe and stored on ice in heparinized tubes. Fish were then euthanized by spinal cord rupture and decapitation.

Table 3.1: Ingredients and proximate composition of the experimental pelleted diet

Ingredients	Diet (g/kg)	Diet (%)	Crude protein (%)	Crude fat (%)	Crude protein in diet	Crude fat in diet
Cod fish meal[a]	350.0	35.00	89.0	4	31.15	1.40
Blood meal[b]	70.0	7.00	87.6	0	6.13	0.00
Wheat gluten[c]	134.0	13.40	80.0	6	10.72	0.80
Cod fish oil[d]	128.0	12.80		100		12.80
Starch[e]	223.6	22.36				
Carboxylmethylcellulose[e]	20.0	2.00				
α- cellulose[e]	42.4	4.24				
Mineral mix[f]	10.0	1.00				
Vitamin mix[g]	10.0	1.00				
Betaine[e]	10.0	1.00				
BHA	1.0	0.10				
BHT	1.0	0.10				
Total CP diet %					48.00	
Total CF diet %						15.00

BHA: butylated hydroxyanisole; BHT: butylated hydroxyl toluene. [a]Cod fish meal provided by SNICK euroingredient NV, Ruddervoorde (Belgium). [b]ActiproHemoglobin, Zwevezele (Belgium). [c]Roquette Freres, Lestrem (France). [d]Sigma-Aldrich, Saint-Luis, MO, (USA). [e]Mosselman SA, Chlin (Belgium). [f]Mineral mix (g kg-1 of mix) was prepared in the lab, from (CaHPO4)2H2O, 727.77; $(MgSO_4)7H_2O$, 127.50; NaCl, 60.00; KCl, 50.00; $(FeSO_4)$7H2O, 25.00; $(ZnSO_4)7H_2O$, 5.50; $(MnSO4)_4H_2O$, 2.54; $(CuSO_4)5H_2O$, 0.78; $(CoSO_4)7H_2O$, 0.48; $(CaIO_3)6H_2O$, 0.29; $(CrCl_3)6H_2O$, 0.13. [g]Vitamin mix was provided by INVE Aquaculture Company.

Spleen and kidney were dissected, part of spleen was immediately homogenized to prepare for spleen respiratory burst activity analysis, the remaining spleen and kidney samples were immediately snap-frozen into liquid nitrogen and finally stored at -80°C until analysis (RT-qPCR immune-related genes). Heparinized blood was immediately analysed for leukocyte populations by flow cytometry, and the remaining volume of blood was then centrifuged at 7500×g for 10 min to collect plasma stored at -80°C until subsequent analysis (lysozyme activity, alternative complement activity, total immunoglobulin content).

Bacterial challenge

In order to evaluate whether BLf has a beneficial effect on disease resistance, we experimentally infected juvenile rainbow trout with a virulent strain of *Aeromonas salmonicida achromogenes* provided by the CER group (Centre d'Economie Rurale, Laboratoire de Pathologie des Poissons, Belgium). Bacteria were cultured in sterile Brain Heart Infusion (BHI, Sigma Aldrich, Saint-Louis, MO, USA) and incubated at 28°C for 24h. A preliminary test infection including various bacterial doses was performed to determine the LD50 CFU needed for virulence on the targeted rainbow trout population (LD50 = 3.1×10^7 CFU/100 g fish body weight) .

On day 52 of treatment, a total of 30 fish from each dietary condition (10 fish x 3 replicate tanks) were anesthetized. Then, fish were intraperitoneally injected with a weight-adjusted dose (3.1×10^7 CFU/100 g of fish body weight) of the freshly prepared *A. salmonicida achromogenes* culture and equally distributed into three 50L-tanks. Fish were confined on animal facility (Biosafety level 2) along the infection assay. They were starved one day before as well as the day of bacterial injection, and then fed the respective experimental diets after infection until the end of the challenge test. At 44h post-injection (D54), a total of 9 fish from each dietary condition (3 fish × 3 replicate tanks) were anesthetized and blood sampled for subsequent immune assays (lysozyme and alternative complement pathway activity). Levels of plasma total Ig content and spleen respiratory burst activity (RBA) were not determined because of limitations in this experiment. Fish were then euthanized, and spleen and kidney were sampled and immediately snap-frozen until carry out the immune gene expression analysis (RT- qPCR).

Blood leukocyte populations

Blood cell populations were analysed at D35 and D51 of dietary treatment by flow cytometry (Flow Activated Cell Sorter Calibur, Flow Cytometry System) according to Inoue et al. [29], later adapted by Mathieu et al. [30]. Due to limitations in this experiment, leukocyte formula was not analysed on D54. Briefly, 10 µl of fresh heparinized blood were mixed with 1950 µl of Hanks Balanced Salt Solution (HBSS, Sigma) and 40 µl of fluorochrome DiOC6 (3,3-dihexyloxacarbocyanine, Molecular Probes, Eugene) diluted 1:10 in ethanol. The tube was mixed gently and incubated at room temperature (RT) for 10 min. The FACS was calibrated with True Count Beads diluted in HBSS, Sigma-Aldrich, Steinheim, Germany). Each blood cell population was identified by its typical location in a FL-1 v. SSC and FSC v. SSC according to Inoue et al. [29] and Pierrard et al. [31]. Four clusters were identified, thrombocyte and lymphocyte were gathered in a same cluster according to Pierrard et al. [31].

Plasma lysozyme activity

Lysozyme activity assay was performed by the turbidimetric method of Siwicki and Studnicka [32], later adapted by Mathieu et al. [33]. Briefly, 7 µL of plasma were added to 130 µL of freshly prepared *Micrococcus luteus* (Sigma-Aldrich, Saint-Louis, USA) solution (0.6 mg/mL of Na_2HPO_4 0.05 M, pH 6.2) in triplicate. Absorbance corresponding to *Micrococcus luteus* lysis was measured at 450 nm for 60 min at regular intervals (5 min). One unit (U) of lysozyme was determined as an absorbance decrease of 0.001 per min.

Plasma haemolytic activity of alternative complement activity

Plasma haemolytic activity of alternative complement activity (ACH50) was assayed following Sunyer and Tort [34], later modified by Milla et al. [35]. Briefly, 10 µL of rabbit red blood cells suspension (RRBC, Biomerieux, Mary-I'Etoile, France) suspended at 3% in veronal buffer was mixed with serial dilutions of plasma (70 µL of total volume). Haemolysis

100% was obtained by adding 60 µL of distillate water to 10 µL of RRBC. Negative control (fresh water) was obtained by adding 60 µL of veronal buffer to 10 µL of RRBC. Samples were incubated 100 min at 27°C and centrifuged (3000 × g, 5 min, 4°C). Then, 35 µL of supernatant was transferred to a new microplate in order to measure the absorbance at 405 nm. The ACH50 value was defined as the reciprocal of the plasma dilution which induced the haemolysis of 50% RRBC.

Plasma total immunoglobulin assay

Analysis of total plasma immunoglobulins content (Ig) is based on a spectrophotometric technique described by Siwicki and Anderson and later adapted by Milla et al. [35] with some modifications. Immunoglobulin was precipitated using 10,000 kDa polyethylene glycol (PEG, Sigma). Plasma was mixed with an equal volume of 12% PEG solution, and shake 150 rpm for 2h at room temperature. After centrifugation at 1000xg for 10 min, the supernatant was collected and assayed for its protein concentration by method of Bradford [36]. Plasma total immunoglobulin content was calculated by subtracting this value from the total protein concentration in the plasma before precipitation with PEG.

Spleen leukocyte respiratory burst activity (RBA)

Just after collection, spleen samples were conditioned in L15 medium (Sigma-Aldrich) at 4°C, and gently pressed through sterilised nylon mesh (40 µm, Dutscher) to obtain leukocyte suspensions. Then, the L15 medium-diluted samples were loaded onto Ficoll gradient (Healthcare, GE). After centrifugation (2500 rpm, 20 min, at 4°C), leukocyte suspensions were collected and washed twice in L15 medium and again centrifuged (1200 rpm, 5 min, 4°C). They were re-suspended in L15 medium and viable leukocytes were adjusted at 10^6 cells/mL before classification of leukocyte populations (lymphocyte, macrophage and granulocyte) by flow cytometry. RBA analysis was performed using flow cytometry method as previously described by Chilmonczyk and Monge [37] with some modifications of Jolly et al. [38]. The RBA test corresponded to an evaluation of intracellular hydrogen peroxide production following cell activation or not with phorbol 12-myristate 13-acetate (PMA). The fluorescence levels of unstimulated and PMA-stimulated cells were determined after 30 min of cell incubation (18°C in the dark) with 2′-7′dichlorofluorescin diacetate (DCFH-DA (5 µM) and DCFH-DA plus PMA (2 $\mu g.mL^{-1}$), respectively. The spleen respiratory burst activities were expressed in stimulation index as the ratio between the mean fluorescence measured in stimulated cells (DCFH-DA + PMA) and the basal mean fluorescence of control (DCFH-DA only).

RNA precipitation and complementary DNA (cDNA) synthesis

Three pools of each experimental condition and time-point were collected to compare their gene expression profiles. Total RNA was extracted individually from the spleen and kidney using TriReagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. The pellet was dried and resuspended in 200 µL of RNase-free water. Total RNA concentration was determined by Nano Drop-2000 spectrophotometer (Thermo Scientific) and the integrity was checked by Experion RNA Std Sens analysis (Bio-Rad Laboratories, Hercules, CA). Total RNA (1µg) was used to synthesise cDNA with iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions.

Table 3.2: Primers used for each gene expression analysis by real-time RT

Genes	Primer sequence (5'-3')	Accession number	Amplicon size
ef-1α	Fw: 5'-ATGCCCCCAAGTTCCTGAAG-3' Rv: 5'-AACAGCAACAGTCTGCCTCA-3'	NM_001124339.1	140
il-1	Fw: 5'-TGAGAACAAGTGCTGGGTCC-3' Rv: 5'-GGCTACAGGTCTGGCTTCAG-3'	NM_001124347.2	148
lysozyme	Fw: 5'-TGCCTGTCAAAATGGGAGTC-3' Rv: 5'-CAGCGGATACCACAGACGTT-3'	NM_001124716.1	152
mIgM	Fw: 5'-AAAGCCTACAAGAGGGAGACCGAT-3' Rv: 5'-AGAGTTATGAGGAAGAGTATGATGAAGGTG-3'	X65263.1	128
mcsf-ra	Fw: 5'-ATCTCCACTCATGGCGACACA-3' Rv: 5'-CATCGCACTGGGTTTCTGGTA-3'	AB091826	177
mpo	Fw: 5'-GCAGAGTCACCAATGACACCA-3' Rv: 5'-ATCCACACGGGCATCACCTG-3'	GBTD01119227	68
il-10	Fw: 5'-CCGCCATGAACAACAGAACA-3' Rv: 5'-TCCTGCATTGGACGATCTCT-3'	NM_001245099.1	105
tgf-b1	Fw: 5'-GCCAAGGAGGTCCACAAGTT-3' Rv: 5'-GTGGTTTTGATGAGCAGGCG-3'	NM_001281366.1	146
cd4-2b	Fw: 5'-AAGCCCCTCTTGCCGAGGAA-3' Rv: 5'-CTCAACGCCTTTGGTACAGTGA-3'	AY899932	108
vapA	Fw: 5'-ATTAGCCCGAACGACAACAC-3' Rv: 5'-CCAACACAATGAAACCGTTG-3'	KP184543.1	148

Gene expression analysis

Real-time PCR assay was carried out in order to analyse the expression pattern of different genes of immunological relevance in spleen and kidney from rainbow trout stimulated with bovine lactoferrin. Samples were taken randomly from three fish of three different tanks for each treatment (n = 9) at D35 and D51 days of feeding with BLf. Samples of three randomly selected fish from each tank were pooled for each treatment (n = 3 fish per each pool). On day 52 of feeding, fish were challenged against *A. salmonicida achromogenes* and 44 hours post-challenge (D54 of treatment) three fish of three different tanks for each treatment (n = 9 fish) were also collected. Samples were also taken using the same strategy from fish fed with basal diet and from non-infected fish (n = 3 fish per each pool). As a housekeeping gene, elongation factor 1 alpha (*ef-1α*) was amplified from all the evaluated samples. The gene expression of pro-inflammatory (*il-1*, *il-6*, *il-8*), macrophages (*mcsfra*), antibacterial (*lysozyme*), humoral (*mIgM*), neutrophils (*mpo*), T-helper (*cd4-1*, *cd4-2α*, *cd4-2β*), cytotoxic T-cells (*cd8*), and anti-inflammatory responses (*il-10*, *tgf-β1*) were evaluated. The list of specific primers used for gene expression analysis is given in Table 3.2. Real-time PCR reactions were carried out with iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories) using a 1:40 dilution of the cDNA for target genes or 1:1000 dilutions for *ef-1α*. Primers for target genes were used at 500 nM. The thermal conditions used were 3 min at 95ºC of pre-incubation, followed by 40 cycles at 95ºC for 30s and 60ºC for 30s. All reactions were performed using ABIprism 7300 (Applied Biosystem) and quantification was done according to the Pfaffl method [39] corrected for efficiency of each primer set. Values for each sample were expressed as normalized relative expression (NRE), calculated in relation to values of control group and normalized against those of the housekeeping gene *ef-1α*. The results are expressed as average of values obtained in all pools from D35 and D51of feeding, and D54 (after bacterial challenge test).

Statistical analysis

Results are presented as mean ± SD. The data were checked for normal distribution and homogeneity of variances by Univariate tests. Humoral immune parameters, blood leukocyte proportions, specific growth rate, mortality, splenic index, haematocrits, and immune genes expression on D35 and D51 were carried on using a two-way ANOVA test. One-way ANOVA was used to test for those parameters on D54 and for data of cumulative mortality over the 14 days of bacterial challenge test. In all statistical analysis tests used, $p < 0.05$ was considered statistically significant. The statistical analysis was performed using the Statistica bio-software (Version 10.0)

3. Results

3.1. Specific growth rate (SGR), haematocrit (Ht) and splenic index (SI)

Fish fed with BLf diets showed a slight increasing trend, although not statistically significant probably due to a high intra-treatment variability, especially on D51 (Table 3). Neither Ht value differed between controls nor BLf-fed fish (Table 3.3). On the other hand, a non-statistically significant decreasing trend was observed in the SI values on D35 in both BLf doses compared to fish fed with control diet, but this was not confirmed on D51 (Table 3). In sum, BLf diets did not modify the SGR, Ht and SI compared to rainbow trout fed with control diet.

Table 3.3: Mean (±SD) values for specific growth rate (SGR), haematocrit and splenic index of trout juveniles fed with 0.1% and 1% BLf diets on day 35th and day 51th of the nutrition test

Times	Parameters	Control	BLf 0.1%	BLf 1%	$F_{-values}$	$P_{-values}$
D35	SGR (%/day)	0.69 ± 0.08	0.77 ± 0.03	0.85 ± 0.17	0.94	0.44
	Haematocrits (%)	33.70 ± 2.5	33.20 ± 1,9	31.80 ± 1.9	0.27	0.88
	Splenic index (%)	0.32 ± 0.14	0.29 ± 0.05	0.22 ± 0.02	1.22	0.35
D51	SGR (%/day)	0.62 ± 0.28	0. 80 ± 0.21	0.75 ± 0.10	0.37	0.69
	Haematocrits (%)	32.30± 3.6	32.80± 1.0	30.30± 3.1	0.58	0.46
	Splenic index (%)	0.21 ± 0.06	0.23 ± 0.05	0.25 ± 0.09	1.42	0.27

3.2. Humoral parameters

3.2.1. Plasma haemolytic activity of alternative complement pathway (ACH50)

Both BLf doses significantly increased plasma haemolytic alternative complement pathway levels ($F = 33.95$, $p = 0.0012$) after 35 and 51 days of BLf administration (Figure 3.1.A). Two days (D54) after bacterial injections, only fish receiving 1% BLf dose displayed significantly higher ($F = 24.98$, $p = 0.0000114$) plasma ACH50 levels comparing to both 0.1% BLf and control diets.

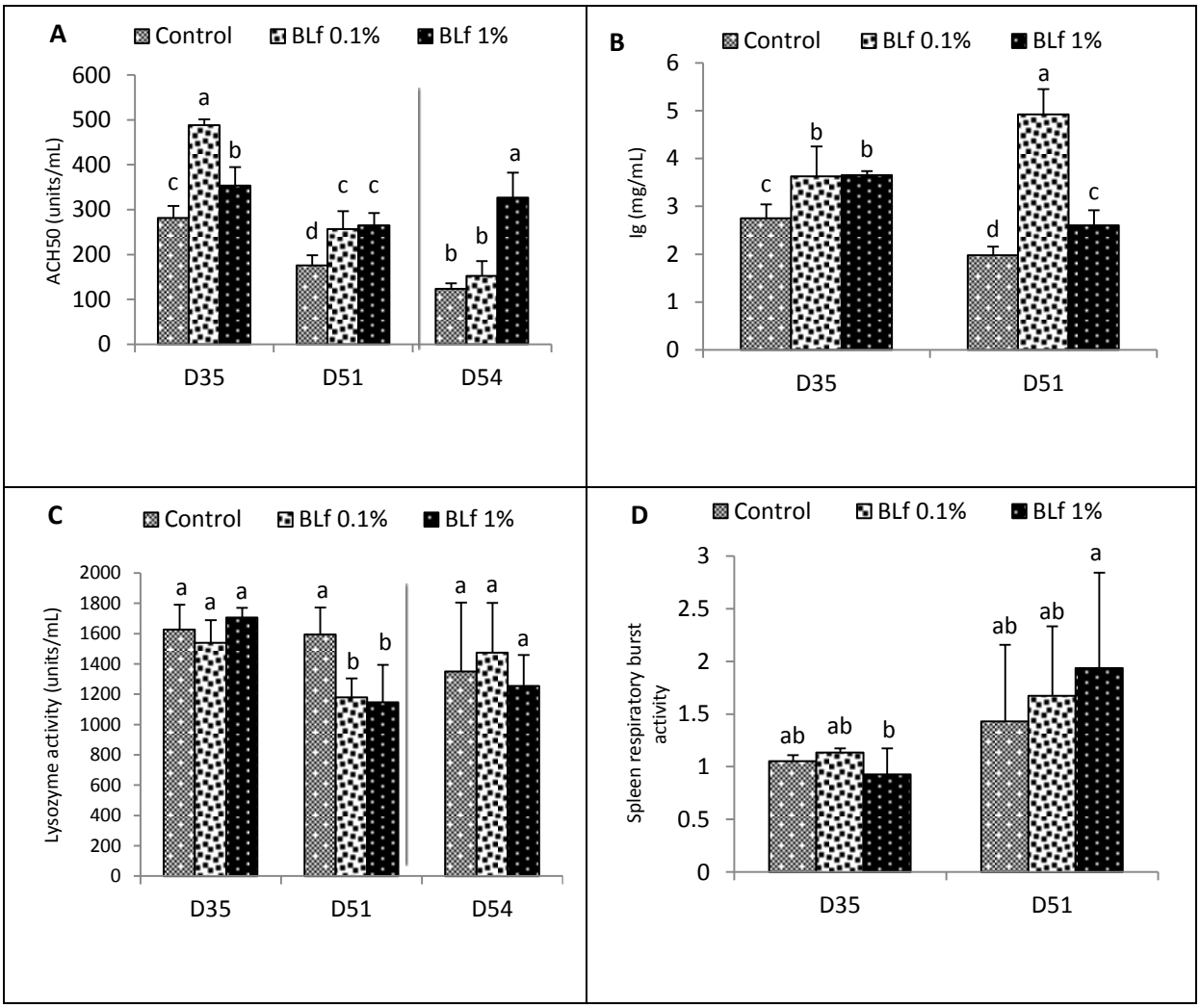


Figure 3.1 (A-D): Effects of dietary BLf administration on humoral parameters of fish sampled during the nutrient test on D35 and D51, or two days after bacterial infection (D54). (A) Plasma haemolytic alternative complement activity, (B) Plasma total immunoglobulins content, (C) Plasma lysozyme activity, (D) Respiratory burst activity (RBA) of spleen macrophage cells. Values are expressed as mean ± SD of 18 fish/diet/day on D35, D51 and 9 fish/diet on D54. For spleen respiratory burst activity (RBA), data are expressed as mean ± SD of 9 fish/diet/day on D35, D51. Statistical differences between dietary treatments and treatment times are indicated by different letters with $p < 0.05$.

3.2.2. Plasma total immunoglobulin (Ig) content

Dietary BLf administration at 35 and 51 days significantly increased levels of plasma total Ig ($F = 33.95$, $p = 0.0012$) compared to control diet (Figure 3.1.B). For fish receiving 0.1% BLf dose, total Ig values peaked of 4.5mg/mL on D51, and were 2.45 times higher than control diet-fed fish.

3.2.3. Plasma lysozyme activity

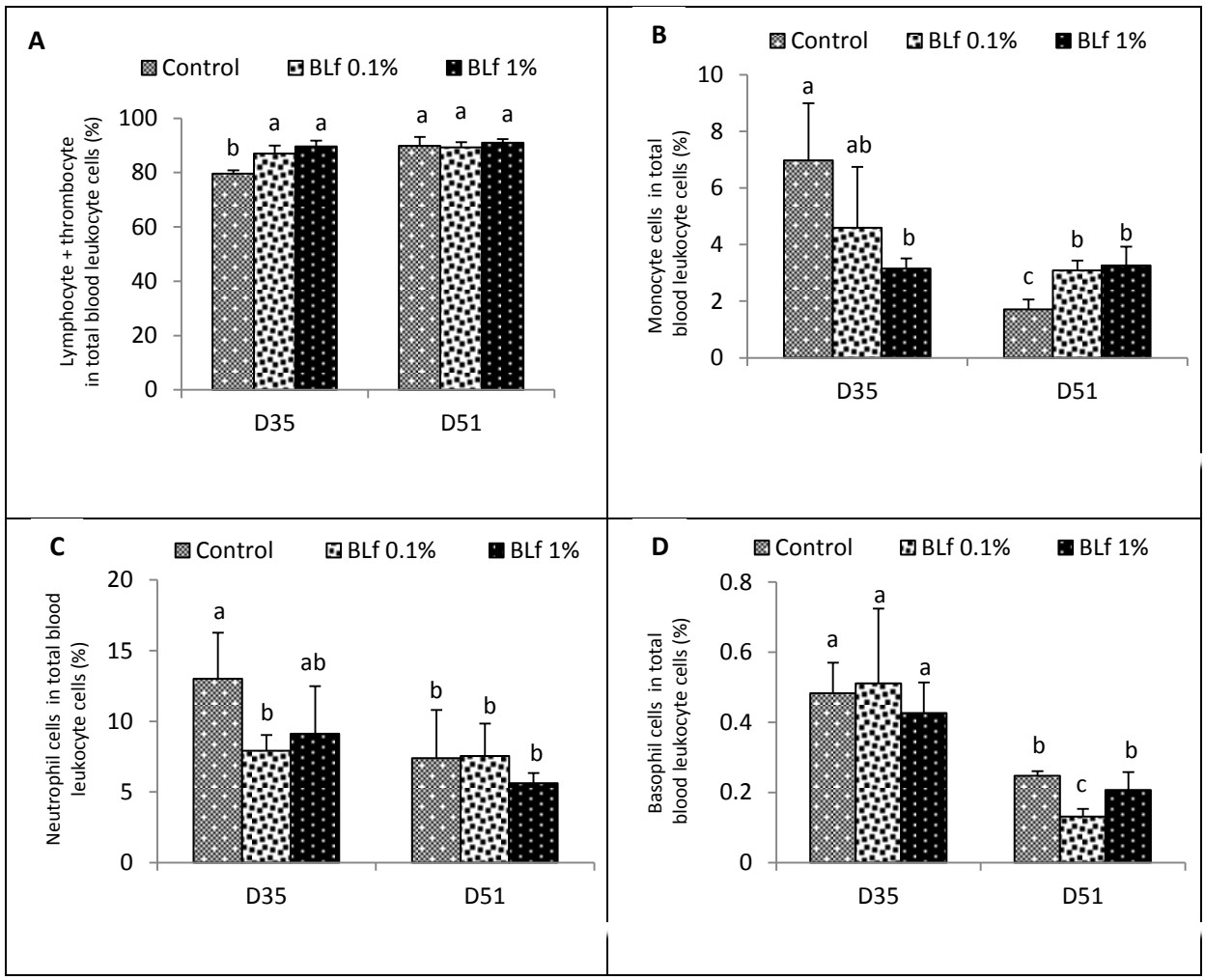
Values of plasma lysozyme activity were not affected by the dietary BLf on D35 but a significant decrease ($F = 17.09$, $p = 0.0013$) was observed on D51 for both doses compared to control diet (Figure 3.1.C). Two days after bacterial infection, no variations on lysozyme activity was observed among treatments.

3.2.4. Spleen leukocyte respiratory burst activity (RBA)

Levels of RBA of spleen lymphocytes did not significantly differ whatever the administration duration as for macrophages (Figure 3.1.D). RBA values of macrophages increased on D51 compared to D35 ($F = 7.41$, $p = 0.018$), especially for fish fed 1% BLf.

3.3. Blood leukocyte cell proportions

Blood leukocytes were composed of high percentages of lymphocytes followed by those of neutrophils, monocytes and basophils (Figure 3.2:A-D). Both BLf doses significantly increased ($F = 6.84$, $p = 0.01$) the proportion of blood lymphocytes on D35 but not on D51 (Figure3. 2A). A significant increase of lymphocyte population was observed on D51 in the control but not in the BLf-treated groups.



Figures 3.2 (A-D): Effects of dietary BLf administration on blood leukocyte proportions of fish sampled during the nutrient test on D35 and D51. (A) Lymphocyte + Thrombocyte proportions, (B) Monocyte proportion, (C) Neutrophil proportion and (D) Basophil proportion in total blood leukocyte cell population. Values are expressed as mean ± SD of 18 fish/diet/day on D35, D51. Statistical differences between dietary treatments and treatment times are indicated by different lower case letters with $p < 0.05$.

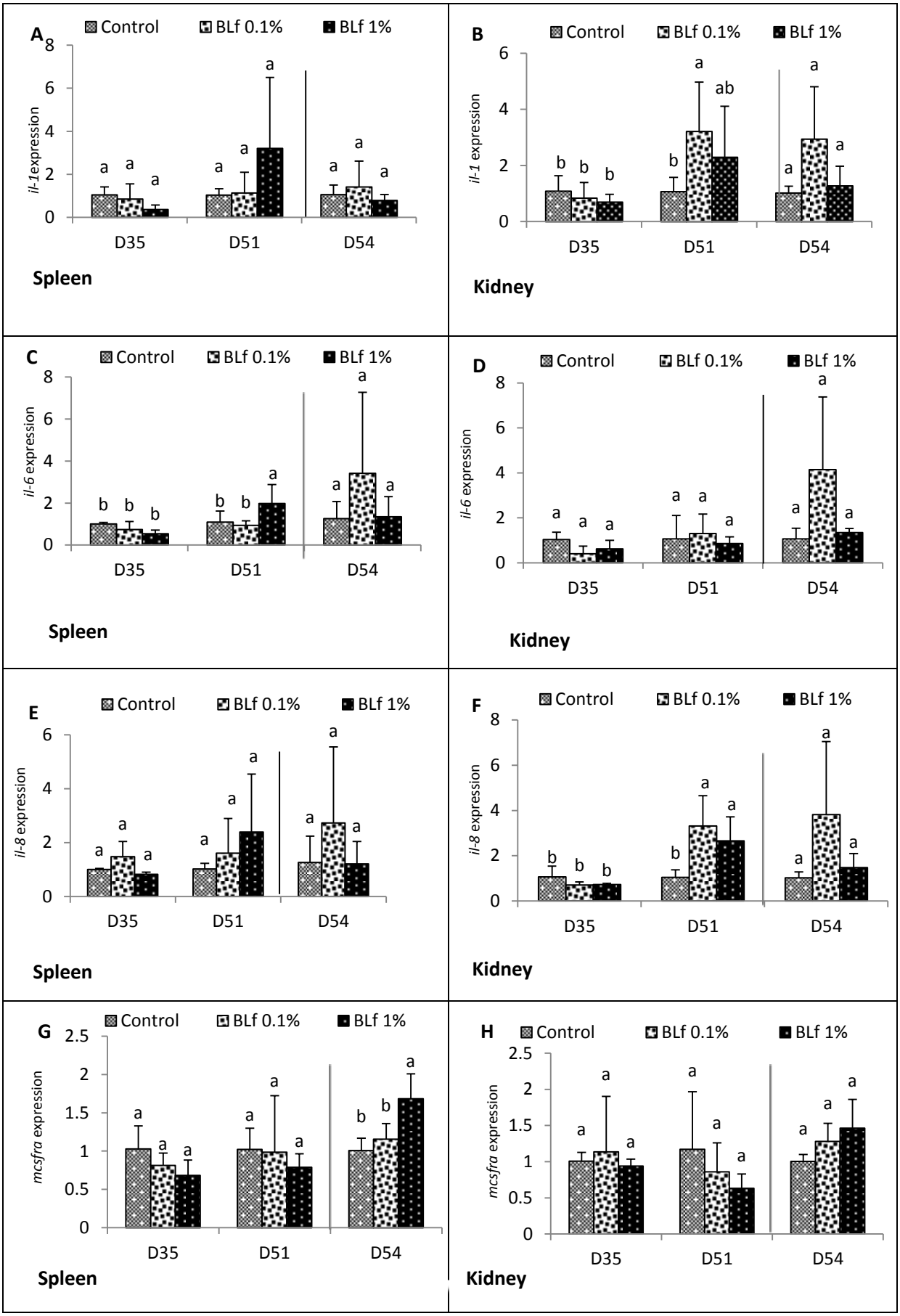
The high BLf dose induced a significant decrease ($F = 16.86$, $p = 0.00145$) in the percentages of monocyte cells on D35. Importantly, a decrease ($F = 9.75$, $p = 0.003$) on control- and 0.1% BLf-fed fish, but not on 1% BLf-fed fish (which remained at the same level as D35) was observed on D51 (Figure 3.2B). The percentages of neutrophil cells decreased ($F = 6.63$, $p =$

0.024) in fish fed with low BLf dose on D35 but not on D51 (Figure 3.2C). Proportion of basophil cells was not affected by BLf doses on D35, but a significant decrease (F = 51.29, p = 0.0000114) was observed on D51 after the low BLf dose treatment (Figure 3.2D). All data concerning leukocyte populations showed positive effects of both BLf diets on lymphocyte populations at the expense of a decrease in neutrophil and basophil populations.

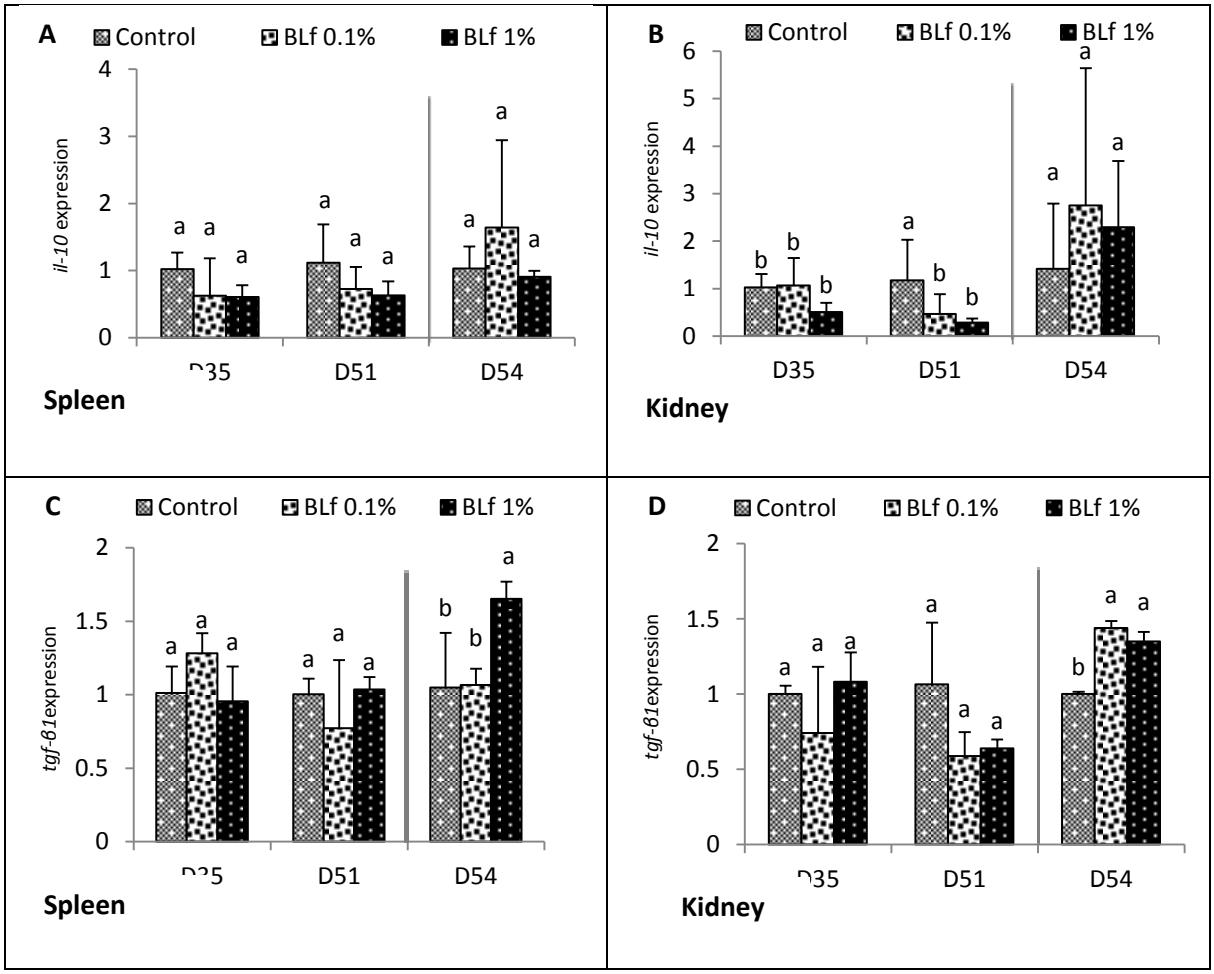
3.4. Immune gene expression

3.4.1. Pro- and anti-inflammatory gene expression

The expression levels of both pro-inflammatory (*il-1*, *il-6*, *il-8*, *mcsfra*,) and anti-inflammatory (*tgf-β1*, *il-10*) genes were evaluated in spleen and kidney (Table 3.4).There were no statistical changes were observed in fish sampled after short-term BLf administration on D35 whatever the tested dose. However, the expression of pro-inflammatory genes was significantly affected by both doses in kidney or spleen (Figure 3.3a & 3.3b) after the long-term BLf administration on D51. Indeed, in kidney tissue, the low BLf dose highly increased level of *il-1* expression (F = 6.45, p = 0.025; Figure 3.3a: B), and both doses upregulated *il-8* expression (F = 35.81, p = 0.00006; Figure 3.3a: F) but no changes were observed two days (D54) after bacterial infection for the expression of these two genes. Level of *mcsfra* expression did not differ on D51 but was significantly increased (F = 6.58, p = 0.03) by the high BLf dose on D54 in spleen samples (Figure 3.3a: G). Concerning anti-inflammatory process, level of *tgf-β1* expression was not affected by both BLf doses on D51 but was upregulated by both BLf doses (F = 78.67, p = 0.000049) on D54 (Figure 3.3b: D). In spleen, only the high BLf dose upregulated *il-6* expression on D51 (F = 6.65, p = 0.024) but no significant effect of both BLf doses was observed on D54 (Figure 3.3a: C). Moreover, both BLf doses down-modulated *il-10* expression (F = 5.13, p = 0.02) on D51 but no significant effect was observed two days after the bacterial challenge on D54 (Figure 3.3b: B) and Table 3.4). In contrast, level of *tgf-β1* expression was not affected by both BLf doses on D35 and D51 but was significantly increased (F = 6.33, p = 0.03) by the high BLf dose on D54 (Figure 3.3b: C). Therefore, long-term feeding of a high BLf dose seemed to improve the rainbow trout immune defence by upregulating the expression of pro- and anti-inflammatory gene expression, especially for *tgf-β1* and *mcsfra* genes.


A
Control
BLf 0.1%
BLf 1%
il-1expression
D35
D51
D54
Spleen
B
il-1 expression
Kidney
C
il-6 expression
Spleen
D
Kidney
E
il-8 expression
Spleen
F
Kidney
G
mcsfra expression
Spleen
H
Kidney

Figures 3.3a (A-H): Effects of dietary BLf on pro- inflammatory gene expressions in spleen and kidney of fish sampled during the nutrient test on D35 and D51, or two days after the bacterial challenge (D54). A, B, C, D, E, F, G and H are expression levels of *il-1*, *il-6*, *il-8*, *mcsfra* in spleen and kidney, respectively. Relative transcript (mRNA) levels were determined by real-time RT-PCR and normalized by the arithmetic mean of *ef-1α* expression. Values are expressed as mean ± SD of 3 pool samples per experimental condition (3 fish per pool sample). Statistical differences between dietary treatments and treatment times are indicated by different lower case letters $p < 0.05$.



Figures 3.3b (A-D): Effects of dietary BLf on anti- inflammatory gene expressions in spleen and kidney of fish sampled during the nutrient test on D35 and D51, or two days after the bacterial challenge (D54). A, B, C and D are expression levels of *il-10* and *tgf-β1* in spleen and kidney, respectively. Relative transcript (mRNA) levels were determined by real-time RT-PCR and normalized by the arithmetic mean of *ef-1α* expression. Values are expressed as mean ± SD of 3 pool samples per experimental condition (3 fish per pool sample). Statistical differences between dietary treatments and treatment times are indicated by different lower case letters $p < 0.05$.

3.4.2. Expression of T-helper and mIgM genes

For adaptive immune functions, expression levels of four T-helper isoform genes (*cd4-1*, *cd4-2α*, *cd4-2β*, *cd8*) and *mIgM* gene were determined in kidney and spleen, but only *cd4-2α* and *cd4-2β* expressions were markedly affected by the BLf diets (Table 3.4). In kidney, a trend of decrease was already observed in *cd4-2α* and *cd4-2β* expression after the short-term BLf administration on D35 (Figure 3.4: B & D). Then, a marked down-regulation was induced by

both BLf doses for *cd4-2α* (F = 4.64 and p = 0.032) and *cd4-2β* (F = 6.80, p = 0.01) expression on D51. In spleen, the same trend of decrease in expression levels of the two genes was also observed on D35, while *cd4-2α* expression was significantly decreased by the high BLf dose (F = 6.15, p = 0.014) on D51 as did both doses for *cd4-2β* expression (F = 9.12, p = 0.0039; Figure 3.4: A & C). Two days after bacterial infection (D54), the expression levels of all targeted adaptive genes were not affected by both BLf diets.

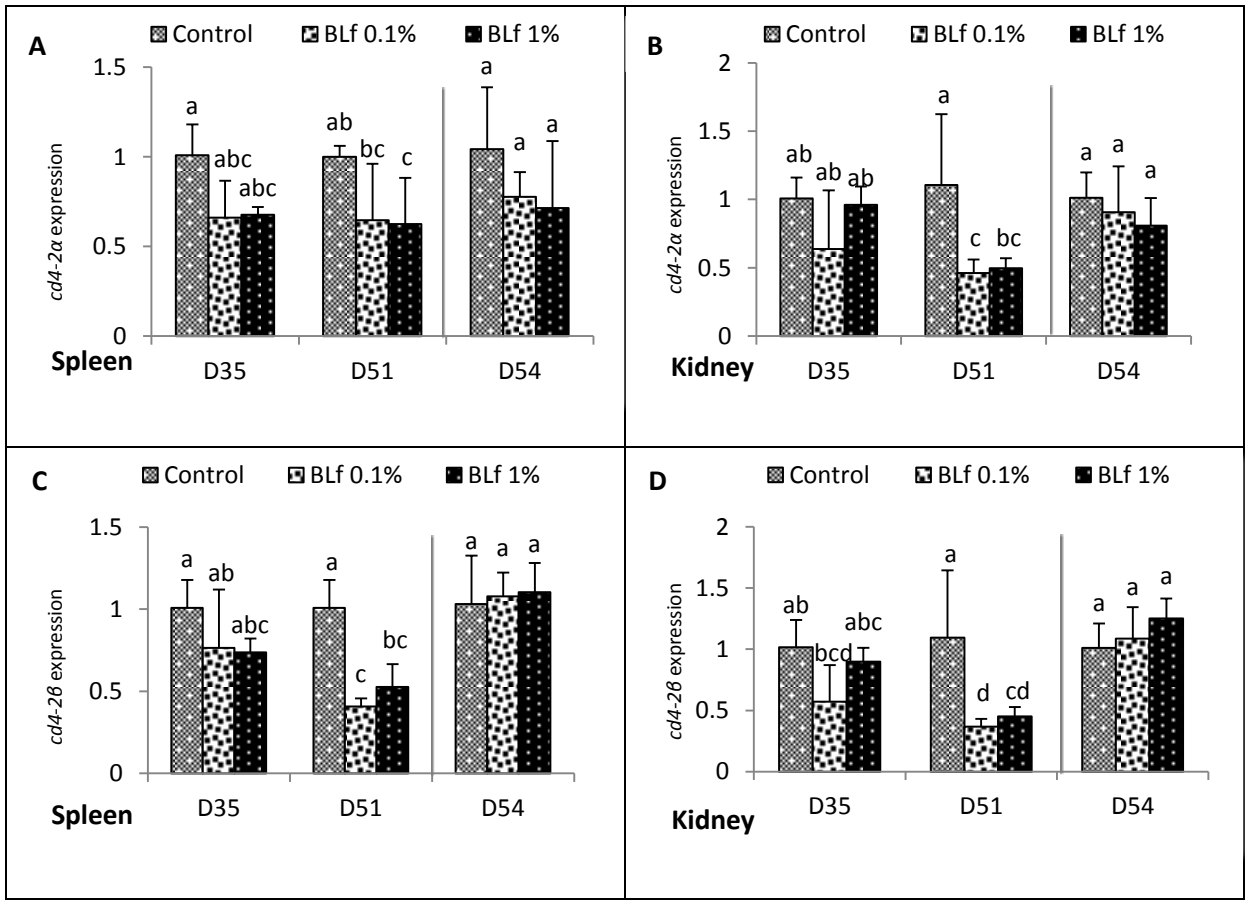


Figure 3.4 (A-D): Effects of dietary BLf on T-helper gene expressions (*cd4-2α, cd4-2β*) in spleen or kidney of fish sampled during the nutrient test on D35 and D51, or two days after the bacterial challenge (D54). (A) *cd4-2α* gene expression in spleen, (B) *cd4-2α* gene expression in kidney, (C) *cd4-2β* gene expression in spleen, (D) *cd4-2β* gene expression in kidney. Relative transcript (mRNA) levels were determined by real-time RT-PCR and normalized by the arithmetic mean of ef-1α gene expressions. Values are expressed as mean ± SD of 3 pool samples per experimental condition (3 fish per pool sample). Statistical differences between dietary treatments and treatment times are indicated by different lower case letters $p < 0.05$).

3.4.3. Antibacterial gene expressions

Expression of two anti-bactericidal genes (*C-type lysozyme and mpo*) was evaluated in kidney and spleen on D35, 51, and D54, but only C-type lysozyme gene expression was affected by BLf diets (Table 4). In spleen and in kidney, both BLF doses did not significantly affect lysozyme expression during the nutrition test. However, an upregulation of lysozyme expression was induced by the high BLf after bacterial infection on D54 in both kidneys (F = 9.66, p = 0.013; Figure 3.5B) and spleens (F = 8.12, p = 0.019; Figure 3.5A).

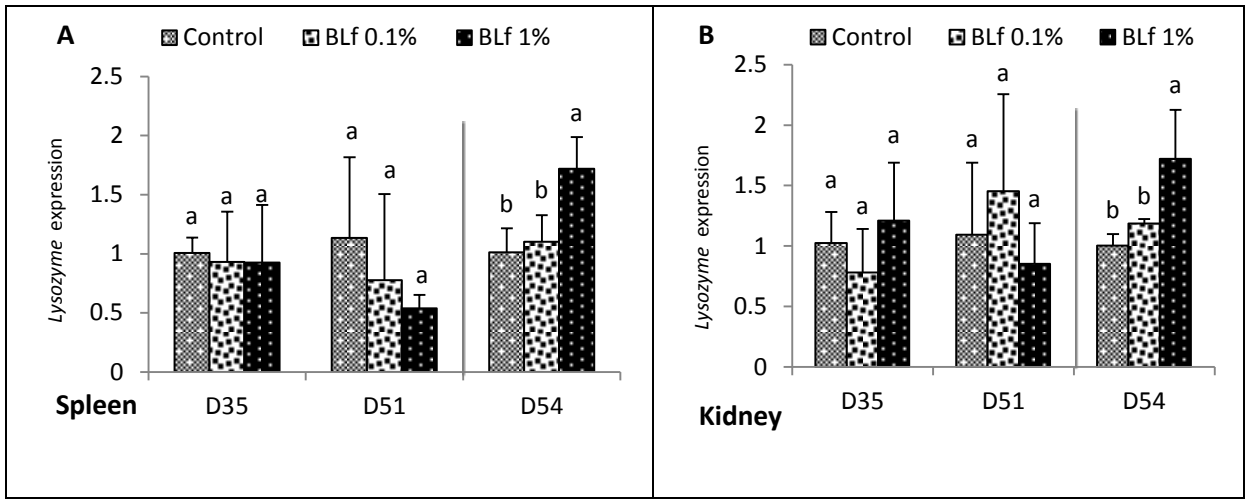
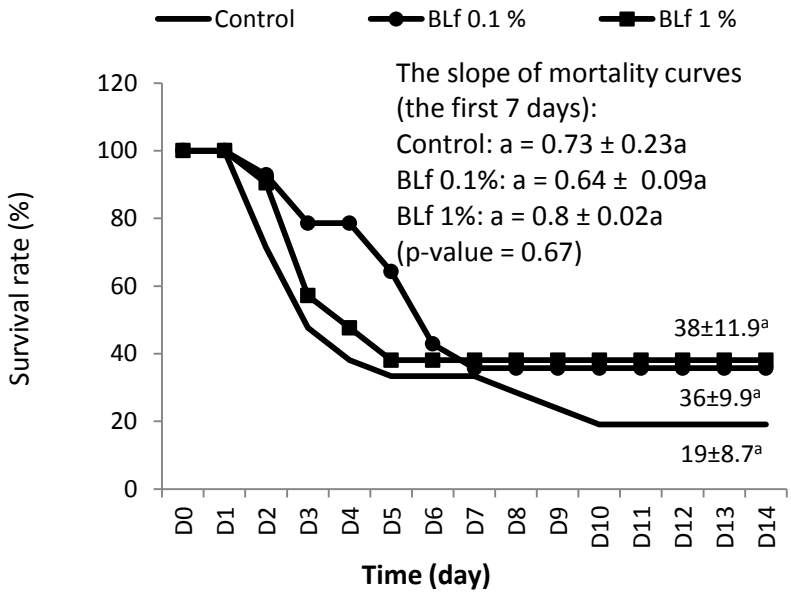


Fig 3.5 (A-B): Effects of dietary BLf on C-type lysozyme gene expression in spleen or kidney of fish sampled during the nutrient test on D35 and D51, or two days after the bacterial challenge (D54). (A) lysozyme gene expression in spleen, (B) lysozyme gene expression in kidney. Relative transcript (mRNA) levels were determined by real-time RT-qPCR and normalized by the arithmetic mean of ef-1α gene expressions. Values are expressed as mean ± SD of 3 pool samples per experimental condition (3 fish per pool sample). Statistical differences between dietary treatments and treatment times are indicated by different lower case letters ($p < 0.05$).

3.5. Disease resistance

During the first week of infection, mortality increased earlier and more rapidly in trout fed with control diet than in fish fed with BLf diets, especially in those receiving the low BLf dose (Figure 3. 6). Then, mortalities stopped around the 8th day after bacteria injections in all BLf groups but not in control. Thus, survival rate was higher in BLf treated fish (36% or 38% for BLf 0.1% or BLf 1%) than in controls (19%) 14 days after bacterial injections, with values at close to statistical significance level ($F = 4.587$, $p = 0.073$). Regarding values of VapA expression in spleen and kidney, the effectiveness of bacterial infection was already evidenced two days after bacterial injections since the presence of VapA was detected in 5 of 18 pool samples. These VapA positive pool samples were detected only in pool samples from controls and fish fed 0.1% BLf, indicating that 1% BLf was more protective against *A. salmonicida* infection.



Figures 3.6: Survival rate profile in rainbow trout juveniles fed with 0.1% or 1% BLf and challenged with *A. salmonicida achromogenes* (3.1×10^7 CFU/100 g fish body weight) for 14 days. Statistical differences between dietary treatments are indicated by different lower case letters ($p < 0.05$).

4. Discussion

4.1. Influence of BLf on growth, haematocrit and splenic index

In the present study, rainbow trout juveniles were fed with BLf at low and high doses and fish were sampled after 35 and 51 days of feeding. No significant effect was observed for SGR whatever the BLf dose or administration duration. This result corroborates previous reports revealing no significant effect of BLf on growth performance of rainbow trout [28], Nile tilapia [17], orange spotted grouper [22], and Siberian sturgeon [24]. However, Kakuta [40] reported a significantly higher growth rate of goldfish *Carassius auratus* after 28 days of dietary BLf treatment. It was hypothesized that the differences in growth effects of BLf dietary administration is related to the interaction between BLf and diet nutrients [22], species specificity such as the absorption capacity of BLf, but also to experimental context [28]. Based on the present growth results, it seems that some stimulation of growth rate by BLf would be evidenced if more experimental replicates would be available, since high intra-treatment variability was observed. Dietary BLf supply had no significant effect on Ht and SI values as previously reported for some haematological features of other fish species [28].

4.2. Influence of BLf diets on humoral and cellular immune parameters

In this study, BLf did not induce a ubiquitous action on activities and gene expressions of immune effectors but some significant effects were observed depending on the dose, the administration duration, the interaction with bacterial infection or lymphoid organ. Indeed, both BLf diets induced no marked effects on lysozyme activity and RBA during the nutrition

test, or after bacterial infection. Neither Lygent et al. [23] and Welker et al. [27] found an effect of dietary BLf on plasma lysozyme activity response in Atlantic salmon or Nile tilapia. In agreement with the present results, Esteban et al. [21] indicated that human lactoferrin has a direct but weak effect on gilthead seabream innate immune response by enhancing respiratory burst activity at 100 mg kg^{-1}feed for 2 weeks. Moreover, BLf did not alter the expression of lysozyme gene expression either in spleen or in kidney during the nutrition test, however high BLf dose upregulated lysozyme expression after bacterial challenge on D54 in spleen and kidney organs. Perhaps an increase in the level of lysozyme gene expression after bacterial injection might be explained by the fact that lysozyme, a glycoside hydrolase is present in cytoplasmic granules of the macrophages and polymorphonuclear neutrophils, and was activated by *A. salmonicida*. Lysozyme was reported to damage bacterial cell walls by catalysing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins [41].

In contrast, it is interesting to notice that BLf diets significantly increased plasma ACH50 activity on D35 and D51. Moreover, ACH50 values were significantly increased by the high BLf dose after bacteria injections on D54. Complement activity plays a major role in the innate immune response such as to destroy the cell surface membranes of pathogens by creating pores and opsonising pathogens for destruction by an enhanced uptake of phagocytes and mediating through ligand-receptor interactions between the surfaces of the two cells [42]. Rahimnejad et al. [28] also reported significantly higher haemolytic complement activity of rainbow trout juveniles fed with 100 and 400 mg BLf kg^{-1} diet for 6 weeks than that of control, but no effect of BLf at 50 and 200 mg kg^{-1}doses after the same time of administration. In the contrary, other studies reported no significant effects of dietary BLf on haemolytic complement activity of Atlantic salmon *Salmo salar* [23], Siberian sturgeon [24], Asian catfish [19] and Nile tilapia [17]. Therefore, it appears that the response of ACH50 to BLf may be species specific, dose and/or time-dependent. As in the case of ACH50 profile, both BLf treatments induced a significantly higher amount of plasma total immunoglobulin (Ig) after short- or long-term administration. The production of antibodies is the main function of the humoral immune system [43], and the synchronized increase in ACH50 level and plasma total Ig content in the present study ascertain that dietary BLf powerfully stimulated the synergic interactions between some innate and specific functions in rainbow trout.

In the present study, BLf diets also modulated the proportion of leukocyte populations with an increase in the proportion of lymphocytes on D35 and also the monocytes on D51. A decrease in other leukocyte cell types was also observed depending to the BLf dose or administration duration. These results indicate that dietary BLf may differentially activate or down-regulate some immune functions associated to the different types of leukocyte cells. Our finding corroborates the previous report that the number of blood granulocyte and lymphocyte cells of red sea bream *Pagrus major* were significantly increased in BLf treated fish [44]. Sakai et al. [26] reported that BLf administered by intubation activated some immune functions on rainbow trout leukocytes, such as phagocytosis and production of superoxides. In mammals, the production and function of neutrophils and monocytes were directly regulated by lactoferrin [45]. Perhaps, BLf contains some important glycoprotein growth factors which regulate blood leukocyte proliferation and maturation, such as interleukins, stem cell factor, colony stimulating factors, granulocyte-macrophage colony stimulating factor, transforming growth factor beta, stromal cell derived factor-1 which stimulated granulocyte formation and activated on progenitor cells. Furthermore, *cd4-2α* and *cd4-2β* gene expression results confirmed that the feeding with both low and high BLf doses induced a down-regulation of *cd4-2α*, *cd4-2β* gene expressions in both spleen and kidney after long-term administration on

D51. *Cd4*$^{+}$ T cells are involved in mediating immune response through the secretion of specific cytokines and activate different cells of innate immune system, B-lymphocytes, and cytotoxic T cells [46]. *Cd4* is a type I membrane glycoprotein with four extracellular Ig –like domains (D1-D4), a transmembrane domain, and a short cytoplasmic domain [47]. Lactoferrin is known to be involved in differentiation of T-helper and development of cell polarization [48]. Here, the decrease in the expression level of these immune genes could be due to a negative control of T-cells themselves as at short-term an increase in lymphocyte population is observed and then after long-term no more difference remaining between control and BLf fed fish. Though, our findings suggest that both BLf diets may improve the immune status of rainbow trout by increasing lymphocyte proportion at the expense of a decrease in neutrophil and basophil populations.

Our results also show that dietary BLf could regulate the expression of some pro-inflammatory immune genes produced in particular by lymphocytes. Indeed, low BLf dose upregulated *il-1* in kidney as well as high BLf dose enhanced *il-6* expression in spleen, while both BLf doses induced a significant up-regulation of *il-8* in kidney. Interleukin 1 plays an important role in the regulation of immune and inflammatory processes by participating in the stimulation of growth and proliferation of T and B lymphocytes, macrophages and vascular endothelial cells [49]. A previous study also showed that bovine lactoferrin-treated rainbow trout fry displayed upregulation of pro-inflammatory cytokines *il-1β* and *il-6* 24h post-bath in the high dose of 1.0 µM treated groups [27]. On the other hand, *il-8* belongs to CXC chemokines and is mainly involved in chemotaxis of immune cells towards the site of wound or infection [50]. Concomitant with the upregulation of pro-inflammatory genes, we observed that anti-inflammatory *il-10* was down-regulated by both BLf diets after long-term treatment. Such down-regulation of *il-10* by dietary BLf has been observed in intestine of mice [51]. In our study, it appears that dietary BLf could stimulate rainbow trout's inflammatory response by modulating gene expression in a time-dependent manner and being effective only after a long-term administration. After bacterial infection, because of high vary of pro-inflammatory gene expression data in the low BLf dose treated group leading to non-statistically significant difference of the expression of pro-inflammatory genes were recorded. However, the anti-inflammatory gene *tgf-β1* was significantly upregulated in spleen and kidney of fish treated with BLf diets. Transforming growth factor-β (*tgf-β*) has been shown to play an essential role in the suppression of inflammation, regulating T cells, inhibiting the proliferation of immune cells as well as cytokine production via Foxp3-dependent and-independent mechanisms [52]. *Tgf-β* can suppress T-cells proliferation by inhibiting of production of interleukin-2, a lymphokine known to potently activate T cells, NK cells [53]. In this study, BLf had no significant effect on *mcsfra* gene expression whatever the dose on D35 and D51, the administration duration or the organ, except for a stimulatory action at the high dose in spleen after bacterial infection on D54. Finally, the effect of BLf on immune gene expression was only observed after long-term administration with no clear difference in kidney and spleen before and after bacterial infection, indicating a similar sensitivity to BLf in these two organs. A recent study indicated that lymphoid organs of rainbow trout may display differences in their sensitivity to some immunostimulants, such as a higher sensitivity in spleen than in kidney in the case of β-glucans [54], but the experimental context should also be considered.

Table 3.4: Summary results of the analysis of rainbow trout immune parameters after short- and long- term nutrient test with low (0.1%) and high (1%) dose of bovine lactoferrin in the diets

Immune parameters		**Organs**	**BLf 0.1%**			**BLf 1%**		
			D35	**D51**	**D54**	**D35**	**D51**	**D54**
Humoral immune parameters	haemolytic activity of complement	blood plasma	↑×1.73	↑×1.25	NS	↑×1.46	↑ ×1.51	↑×2.64
	total immunoglobulin		↑×1.39	↑×1.40	NA	↑× 2.49	↑× 1.31	NA
	lysozyme activities		NS	↓×1.35	NS	NS	↓ ×1.39	NS
Macrophage activity	respiratory burst activity	fresh spleen	NS	NS	NA	NS	NS	NA
Blood leukocyte populations	lymphocyte + thrombocyte	fresh blood	↑× 1.09	NS	NA	↑×1.12	NS	NA
	monocyte		NS	↑× 1.76	NA	↓×2.21	↑×1.91	NA
	neutrophils		NS	NS	NA	NS	NS	NA
	basophils		NS	↓×1.88	NA	NS	NS	NA
Immune gene expression	*il-1*	spleen tissue	NS	NS	NS	NS	NS	NS
	il-6		NS	NS	NS	NS	↑×1.82	NS
	il-8		NS	NS	NS	NS	NS	NS
	lysozyme		NS	NS	NS	NS	NS	↑×1.69
	mIgM		NS	NS	NS	NS	NS	NS
	mcsf-ra		NS	NS	NS	NS	NS	↑×1.68
	mpo		NS	NS	NS	NS	NS	NS
	il-10		NS	NS	NS	NS	NS	NS
	tgf-β1		NS	NS	NS	NS	NS	↑× 1.58
	cd4-1		NS	NS	NS	NS	NS	NS
	cd4-2α		NS	NS	NS	NS	↓× 2.65	NS
	cd4-2β		NS	↓×2.5	NS	NS	↓×1.92	NS
	cd8		NS	NS	NS	NS	NS	NS
Immune gene expression	*il-1*	kidney tissue	NS	↑×3.0	NS	NS	NS	NS
	il-6		NS	NS	NS	NS	NS	NS
	il-8		NS	↑×3.20	NS	NS	↑× 2.57	NS
	lysozyme		NS	NS	NS	NS	NS	↑× 1.72
	mIgM		NS	NS	NS	NS	NS	NS
	mcsf-ra		NS	NS	NS	NS	NS	NS
	mpo		NS	NS	NS	NS	NS	NS
	il-10		NS	↓× 2.54	NS	NS	↓× 4.17	NS
	tgf-β1		NS	NS	↑× 1.43	NS	NS	↑× 1.35
	cd4-1		NS	NS	NS	NS	NS	NS
	cd4-2α		NS	↓× 2.39	NS	NS	↓× 2.24	NS
	cd4-2β		NS	↓×3.02	NS	NS	↓×2.42	NS
	cd8		NS	NS	NS	NS	NS	NS

Significant level at $P < 0.05$, NS: non-significant, NA: no data, ↑ indicated the increase in fold change of the value, ↓ indicate the decrease in fold change of the value.

4.3. Influence of BLf diets on disease resistance

Concerning bacteria resistance, both BLf doses increased the rainbow trout ability to resist the pathogen. The upregulation of *mcsfra* expression at the high BLf dose was observed in spleen two days after bacterial infection (D54) together with the increasing trend (although not significant) in the expression of pro-inflammatory cytokines and lysozyme after the challenge with *A. salmonicida* could explain the higher survival of fish fed with BLf compared to those fed with control diet. The modulation of these genes and also the upregulation of *tgf-β1* could

favour the resolution of infection on BLf-treated rainbow trout. The cumulative survival showed that fish fed with 1% BLf displayed the highest survival after bacterial challenge. Importantly, only in fish fed 1% BLf no VapA expression was detected two days after the bacterial challenge which supports the possibility that 1% BLf diet could play a more protective effect than 0.1% BLf diet in rainbow trout. Several studies had already shown the ability of BLf treatments to increase the bacterial resistance of rainbow trout [25], Asian catfish [19] or giant fresh water prawn [55]. Our results show that the improvement of disease resistance may be more related to the modulation of innate immune functions by dietary BLf, but regulation of some adaptive immune compounds (plasma immunoglobulins and expression of *cd4*$^{+}$ genes) observed on D51 may also account for such immune defence. Previous studies showed that the increased bacterial resistance after BLf treatment is due to the enhanced functions of non-specific immune system as well as the ability BLf to take up the Fe^{3+} ion, limiting of this nutrient by bacteria at the infection site and inhibiting the growth of these microorganisms as well as the expression of their virulence factors [56]. Arnold et al. [57] reported that apo-lactoferrin (non-depleted Lf) had a direct bactericidal effect on *Streptococcus mutans*, *S. salivarius*, *S. mitior*, *V. cholerae* and *Pseudomonas aeruginosa*, although iron-saturated lactoferrin (holo-Lf) did not show these activities. Though, lactoferrin could also directly interact with the bacterial surface and could damage the external membrane of Gram-negative bacteria through an interaction with lipopolysaccharide [58]. The positively charged N-terminus of lactoferrin may prevent the interaction between LPS and the bacterial cation (Ca^{2+} and Mg^{2+}), causing a release of LPS from the cell wall, an increase in the membrane's permeability and ensuring damage to the bacteria [59]. Regarding the use of BLf as feed additive as in the present study, it has been recently demonstrated that gastric and intestinal digestion does not alter Lf functional properties, namely its potential for producing negatively charged peptides or amino acids [60].

5. Conclusions

In this study, both BLf doses 0.1% and 1% positively affected the rainbow trout immune system in a time/dose dependant manner. Dietary BLf supplemented to trout juveniles feed at 0.1% and 1% BLf differentially stimulated the immune system, in temporal terms. At short term, the immune system of juvenile rainbow trout rapidly responded to BLf diets (after 35 days of treatment) by modifying the proportion of different blood leukocyte cell types such as lymphocyte cells, thrombocyte cells, monocyte cells, neutrophil cells in total blood leukocyte cells (100%) on D35 as well as D51. This has led to a change of humoral immune parameters such as antibody production Ig as well as ACH50 activity in this study. At long term, BLf diets stimulated leukocyte cells to transcribe immune genes in specific organs such as spleen and kidney. The significant different immune response of the trout immune system between D35 and D51 found its basis in the absence of change as induced by the BLf diets, in 13 immune genes expression levels in spleen and kidney organs on D35. Meanwhile, the immune system was significantly modulated by increasing pro-inflammatory (*il-1*, *il-6*, *il-8*, *mcsfra*) and T-helper genes (*cd4-2α*, *cd4-2β*) on D51, anti-inflammatory (*tgf-β1, il-10*) and lysozyme genes on D54 by both of dietary BLf doses. The changes in immune genes expression in juvenile rainbow trout were only manifest after long term-BLf exposure either unchallenged (D51) or challenged (D54). Finally, both low and high BLf doses could protect juveniles rainbow trout to a limited extent in a 14 day *Aeromonas salmonicida* challenge (with $p_{\text{-value}} = 0.073$ close to significant difference level).

Conflict of interest

The authors have declared that no competing interests exist.

Acknowledgments

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Chapter 4

Immune pathway responses of rainbow trout (*Oncorhynchus mykiss*) juveniles to low and high dietary doses of inulin

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Abstract

Inulin is used as a prebiotic additive in feed nutrition of fish and known to modulate immune functions and improve fish resistance to infections. The objective of the present study was to describe the immune pathway responses of rainbow trout *Oncorhynchus mykiss* juveniles fed low (0.1%) and high (1%) doses of inulin after mid (35 days, D35)- or long (51 days, D51)-term feeding period, and to determine the resistance of fish to bacterial infection. Inulin diets did not affect specific growth rate, haematocrit and splenic index as well as plasma lysozyme activity on D35 or D51. Low inulin dose significantly induced high plasma activity levels of alternative complement (ACH50) on D35 and D51, total immunoglobulins (Ig) and spleen macrophage respiratory burst on D51. Both inulin doses affected differentially the proportions of leukocyte populations on D35 or D51. No significant effects on immune gene expression in spleen or kidney were observed whatever the inulin dose on D35. In contrast, high inulin dose upregulated the expression of some inflammatory genes, namely *il-1*, *mcsfra* in spleen and *il-8* in kidney on D51. On D51, both inulin doses stimulated anti-inflammatory gene (*tgf-β1*) expression in kidney. Moreover, the expression of T helper (*cd4-2β*) gene was significantly down-regulated only on D51 by low inulin dose in kidney. Both low and high inulin doses did not affect *il-6*, *il-10*, *cd4-1*, *cd4-2α*, *mpo*, *mIgM* and *cd8* genes in spleen and kidney after mid and long term inulin test. After 51 days of feeding, trout were intraperitoneally injected with *Aeromonas salmonicida achromogenes*, and both inulin doses significantly upregulated *il-8* in kidney at 44 h-post-injection (D54). The low inulin dose significantly enhanced disease resistance with cumulative survival of 48% compared to 19% and 7% for the control and high inulin dose, respectively. These results indicated that dietary inulin activated the humoral immunity by acting differentially on leukocyte populations after mid- or long- term administration. But only long-term administration influenced other immune pathways by inducing a differential regulation in the expression of some immune genes in kidney and spleen, mostly related to innate immunity functions. Low inulin dose seemed better effective for enhancing bacterial resistance in relation to a highest humoral immune status and upregulation of the expression of pro- and anti-inflammatory immune genes in spleen and kidney.

Keywords: inulin, rainbow trout (*Oncorhynchus mykiss*), immunity, gene expressions, disease resistance.

1. Introduction

Intensive practices of aquaculture production combined with other factors including overcrowding, handling, temperature change and poor water quality may create a state of stress leading to immunosuppression in fish [1], boosting the susceptibility to infectious diseases [2–4], and may cause an increase of disease outbreaks resulting in low or total loss of aquaculture production [5]. During the last decades, different approaches have been investigated to control diseases including sanitary prophylaxis, disinfection, and chemotherapy with a particular emphasis on the use of different families of antibiotics. However, application of antibiotics can lead to the development of antibiotic-resistant bacterial strains and cause many other problems such as environmental hazards, food safety problems and resistance of pathogens; and can also adversely affect the health status of fish [6]. Moreover, in the past, sub-therapeutic doses of antibiotic have been often added to aquatic feeds to promote growth, and this has further contributed to drug resistance [7]. For these reasons, the prophylactic use of antibiotic treatments in aquaculture has resulted in a ban of most compounds in Europe and stringent regulations on the application of antibiotics in the United States and other countries [8]. This situation and recent restrictions on the use of antibiotics have promoted the use of prebiotics and probiotics (or a combination of both named synbiotics) as significant alternatives to antibiotics and there is an increasing interest in aquaculture in using these additives to prevent and/or control fish diseases [9–11].

Prebiotics are selectively fermentable ingredients that lead to specific changes in the composition and/or activity of the gastro-intestinal microflora, with resulting benefits for the host's well-being and health [12]. Among many health benefits attributed to prebiotics, the modulation of the immune system is one of the most anticipated benefits, through the enhancement of various innate immune responses including phagocytic activation, increase of neutrophils, activation of the alternative complement system, and increased lysozyme activity [13]. Although prebiotics are now widely used in human and animal foodstuffs, the modes of action in aquatic animals are still poorly understood [14,15].

Inulin is a naturally occurring fructooligossacharide (FOS) extracted from the chicory root (*Cichorium intybus*) as well as from many other plants (cereals, leeks, onions, garlic, wheat, artichokes and bananas) and belongs to a class of carbohydrates known as fructans [8,16]. Fructans are non-digestible oligosaccharides as the β (2-1) linkage of the fructans cannot be hydrolysed by pancreatic or brush border digestive enzymes in the proximal intestinal tract of human and domestic animals [17]. It is fermented in the large intestine or colon [18] by beneficial bifidobacteria and other lactic acid-producing bacteria, enhancing their relative population while selectively inhibiting the growth of pathogenic microorganisms [19,20]. Due to these properties, inulin may stimulate antibacterial functions, and consequently may induce effective protection against various bacterial diseases in several species of domestic animals, depending on the dose and administration mode. In fish, the available results on the immunomodulatory potentials of inulin are contradictory and the modes of action of inulin are not yet well described.

It has been reported that the use of diets enriched with inulin at different doses and durations influenced the innate and acquired immune system of different aquatic species. The total serum immunoglobulins, bactericidal activity and anti-protease activity increased in grass carp (*Ctenopharyngodon idella*), when fed inulin diets at 0.2% and 2% for 8 weeks [16]. Similarly, increase of the serum complement activity, IgM level, leukocyte phagocytic activity and leukocyte respiratory activity was reported in gilthead seabream (*Sparus aurata*) fed inulin at the same dose for two weeks [6]. In common carp fry (*Cyprinus carpio*), blood

respiratory burst activity increased after 7 weeks of feeding treatment with inulin at 5 to 10g kg^{-1} [21]. Dietary inulin at 2.5g and 5g kg^{-1} increased red blood cells, improved lysozyme activity, alternative complements haemolytic activity (ACH50) of juvenile tilapia (*Oreochromis niloticus*) [22]. Inulin-enriched diets significantly upregulated mRNA transcripts of immunoglobulin M (IgM) in intestine, head kidney and skin after 6-8 weeks of feeding but did not show any difference in lysozyme activity, serum protein or blood glucose of Leopard grouper (*Mycteroperca rosacea*) [23]. Dietary supplementation of inulin has been reported to improve weight gain, specific growth rate, protein efficiency ratio and food conversion rate in grass carp [16]. On the other hand, other authors did not find any positive effect on growth performance and survival rate in common carp [21], tilapia [22]. Dietary inulin at 0.5% alone did not show any positive effect on gut microbiota, meanwhile the combination of 0.5% of inulin and the Gram-positive bacteria *Weissella cibaria* from the family of *Leuconostocaceae* at 7.87 ± 0.2 log CFU g^{-1} induced a higher concentration of lactic acid bacteria and low levels of *Vibrio spp* and *Pseudomonas spp* in the midgut of hybrid surubim (*Pseudoplatystoma* sp) [24]. Altogether, the results obtained in aquatic species with dietary inulin seem to differ depending on experimental conditions, species and treatment mode.

There are several reports on the general effects of inulin on rainbow trout immune response and on the potential immunomodulation of inulin at a systemic level, but the physiological pathways by which inulin may affect the immunocompetence status of rainbow trout are not well described yet and, to our knowledge, few studies investigated the temporal effects of inulin on immune responses. Ortiz et al. [25] evaluated the effects of inulin or FOS-containing diets (5g and 10g kg^{-1}) and reported a significant increase of body weight gain, gross energy and Ca content in rainbow trout. Moreover, inulin reduced the intestinal population of *Vibrio spp* in the distal region of trout gut. Recently, Yarahmadi et al. [26] reported in rainbow trout that commercial fermentable fibre Vitacel® (a pure raw fibre, mainly consisting of cellulose and hemicellulose), such as inulin, significantly upregulated *lysozyme* and *TNFα* gene expression and remarkably elevated innate immune parameters including serum lysozyme, ACH50, bactericidal activity and agglutination antibody titer. Apart from these studies, there are, however, very few publications investigating the effects of inulin on the immune system of rainbow trout juveniles.

In this study, we aimed to conduct a comprehensive evaluation of mid-and long-term (D35 and D51) and dose effects (low dose at 0.1% diet *vs* high dose at 1% diet) of oral administration of inulin on the overall immune function of trout juveniles analysing blood leukocyte cells, humoral immune parameters and immune related genes expression in relevant immune organs, such as spleen and kidney. Moreover, the disease resistance of trout juveniles was tested by applying a bacterial challenge test based on the intraperitoneal injection of *Aeromonas salmonicida achromogenes*.

2. Materials and methods

Experimental fish

Feeding trial and bacterial challenge were carried out in agreement with the European and Belgian national legislations on animal welfare (Protocol number: 13197 KE). Rainbow trout juveniles (n = 315) were transported from a local fish farm (Hatrival, Belgium) to UNamur facilities and distributed into 9 fibreglass tanks of 100L (35 fish/100L/tank) in a recirculation system. Fish were allowed acclimating to the new housing conditions for 21 days. During this period, water temperature was maintained at 13.9 ± 1.2°C by a cooling system, oxygen level

averaged 11.6 ± 0.7 mg/L (aeration applied), constant photoperiod (Light: Dark ratio = 12:12) and fish were fed 1.5% of fish biomass, twice daily (at 9:00 am and 5:00 pm) with a specific trout diet (Coppens TROCO SUPREME-16, The Netherlands, crude protein = 48%, crude fat = 15%).

Table 4.1: Ingredients and proximate composition of the experimental pelleted diet

Ingredients	Diet (g/kg)	Diet (%)	Crude protein (%)	Crude fat (%)	Crude protein in diet	Crude fat in diet
Cod fish meal[a]	350.0	35.00	89.0	4	31.15	1.40
Blood meal[b]	70.0	7.00	87.6	0	6.13	0.00
Wheat gluten[c]	134.0	13.40	80.0	6	10.72	0.80
Cod fish oil[d]	128.0	12.80		100		12.80
Starch[e]	223.6	22.36				
Carboxylmethylcellulose[e]	20.0	2.00				
α- cellulose[e]	42.4	4.24				
Mineral mix[f]	10.0	1.00				
Vitamin mix[g]	10.0	1.00				
Betaine[e]	10.0	1.00				
BHA	1.0	0.10				
BHT	1.0	0.10				
Total CP diet %					48.00	
Total CF diet %						15.00

BHA: butylated hydroxyanisole; BHT: butylated hydroxyl toluene. [a] Cod fish meal provided by SNICK euroingredient NV, Ruddervoorde (Belgium). [b]ActiproHemoglobin, Zwevezele (Belgium). [c] Roquette Freres, Lestrem (France). [d] Sigma-Aldrich, Saint-Louis, MO, (USA). [e]Mosselman SA, Chlin (Belgium). [f] Mineral mix (g kg^{-1} of mix) was prepared in the lab, from $(CaHPO_4)2H_2O$, 727.77; $(MgSO_4)7H_2O$, 127.50; NaCl, 60.00; KCl, 50.00; $(FeSO_4)7H_2O$, 25.00; $(ZnSO_4)7H_2O$, 5.50; $(MnSO_4)4H2O$, 2.54; $(CuSO_4)5H_2O$, 0.78; $(CoSO_4)7H_2O$, 0.48; $(CaIO_3)6H_2O$, 0.29; $(CrCl_3)6H_2O$, 0.13. [g]Vitamin mix was provided by INVE Aquaculture Company.

Fish diet and experimental design

After acclimation, fish (mean body weight: 73.8 ± 13.6 g) were fed either a control diet (no inulin) or inulin (Beneo - Orafti® - Rue L. Maréchal 1 - B - 4360 Oreye, Belgium) enriched diets (0.1% or 1.0% of the diets) for 51 days at 1.5% of fish biomass/day. All diets were formulated and pelleted in the laboratory (Table 4.1).

Three replicate tanks were used for each experimental regime. After 35 days (mid-term nutrient test, D35) and 51 days (long-term nutrient test, D51) of feeding, six fish per tank (18 fish per experimental diet) were anesthetized in ethyl 3-aminobenzoate methane sulfonic acid salt (98% purity, MS-222, Sigma) solution (120 mg/L). Blood was obtained by caudal vein puncture using a heparinized syringe and stored on ice in heparinized tubes. Fish were then euthanized by overdose of MS-222 before decapitation. Spleen and whole kidney were dissected, part of spleen was immediately homogenized after dissection to prepare for spleen respiratory burst activity analysis, the remaining spleen and kidney samples were immediately snap-frozen into liquid nitrogen and finally stored at -80°C until analysis (RT-qPCR immune-related genes). Heparinized blood was immediately analysed for leukocyte populations by flow cytometry, and the remaining volume of blood was then centrifuged at 7500×g for 10 min to collect plasma stored at -80°C until subsequent analysis (lysozyme activity, alternative complement activity, total immunoglobulin content).

Bacterial challenge

In order to evaluate whether inulin has a beneficial effect on disease resistance, rainbow trout juveniles were experimentally infected with a virulent strain of *Aeromonas salmonicida achromogenes* provided by the CER group (Centre d'Economie Rurale, Laboratoire de Pathologie des Poissons, Belgium). Bacteria were cultured in sterile Brain Heart Infusion (BHI, Sigma Aldrich, Saint-Louis, MO, USA) and incubated at 28°C for 24h. A preliminary test infection including various bacterial doses was performed to determine the LD50 CFU of the targeted rainbow trout population (LD50 = 3.1×10^7 CFU/100g fish body weight).

On day 52 of treatment, a total of 30 fish from each dietary condition (10 fish × 3 replicate tanks) were anesthetized. Then, fish were intraperitoneally injected with a weight-adjusted dose (3.1×10^7 CFU/100 g of fish body weight) of the freshly prepared *A. salmonicida achromogenes* culture and equally distributed into three 50L-tanks. Fish were confined on animal facility (Biosafety level 2) along the infection assay. They were starved one day ahead of infection as well as on the day of bacterial injection, and then fed the respective experimental diets until the end of the challenge test. At 44h post-injection (D54), a total of 9 fish from each dietary condition (3 fish × 3 replicate tanks) were anesthetized and blood was sampled for subsequent immunological assays (lysozyme and alternative complement pathway activity). Levels of plasma total Ig content and spleen RBA were not determined because of limitations in this experiment. Fish were then euthanized, and spleen and kidney were sampled and immediately snap-frozen until immune gene expression analysis (RT-qPCR).

Blood leukocyte populations

Blood cell populations were analysed at D35 and D51 of dietary treatment by flow cytometry (Flow Activated Cell Sorter Calibur, Flow Cytometry System) according to Inoue et al. [27], later adapted by Mathieu et al. [28]. Briefly, 10 µl of fresh heparinized blood were mixed with 1950 µl of Hanks Balanced Salt Solution (HBSS, Sigma) and 40 µl of fluorochrome DiOC6 (3,3-dihexyloxacarbocyanine, Molecular Probes, Eugene) diluted 1:10 in ethanol. The tube was mixed gently and incubated at room temperature (RT) for 10 min. The FACS was calibrated with True Count Beads diluted in HBSS, Sigma-Aldrich, Steinheim, Germany). Each blood cell population was identified by its typical location in a FL-1 v. SSC and FSC v. SSC according to Inoue et al. [27] and Pierrard et al. [29]. Four clusters, (lymphocyte+thrombocyte, monocytes, neutrophil and basophil), were gathered in a same cluster according to Pierrard et al. [29].

Plasma lysozyme activity

Lysozyme activity assay was performed by the turbidimetric method of Siwicki and Studnicka [30], later adapted by Mathieu et al. [31]. Briefly, 7 µL of plasma were added to 130 µL of freshly prepared *Micrococcus luteus* (Sigma-Aldrich, Saint-Louis, USA) solution (0.6 mg/mL of Na_2HPO_4 0.05 M, pH 6.2) in triplicate. Absorbance corresponding to *Micrococcus luteus* lysis was measured at 450 nm for 60 min at regular intervals (5 min). One unit (U) of lysozyme was determined as an absorbance decrease of 0.001 per min.

Plasma haemolytic activity of alternative complement activity

Plasma haemolytic activity of alternative complement activity (ACH50) was assayed following Sunyer and Tort [32], later modified by Milla et al. [33]. Briefly, 10 µL of rabbit red blood cells suspension (RRBC, Biomerieux, Mary-I'Etoile, France) suspended at 3% in veronal buffer was mixed with serial dilutions of plasma (70 µL of total volume). Haemolysis (100%) was obtained by adding 60 µL of distillate water to 10 µL of RRBC. Negative control (fresh water) was obtained by adding 60 µL of veronal buffer to 10 µL of RRBC. Samples were incubated 100 min at 27°C and centrifuged (3000×g, 5 min, 4°C). Then, 35 µL of supernatant was transferred to a new microplate in order to measure the absorbance at 405 nm. The ACH50 value was defined as the reciprocal of the plasma dilution which induced the haemolysis of 50% RRBC.

Plasma total immunoglobulin assay

Analysis of total plasma immunoglobulin content (Ig) was based on a spectrophotometric technique described by Siwicki and Anderson and later adapted by Milla et al. [33] with some modifications. Immunoglobulin was precipitated using 10,000 kDa polyethylene glycol (PEG, Sigma). Plasma was mixed with an equal volume of 12% PEG solution, and shake 150 rpm for 2h at room temperature. After centrifugation at 1000×g for 10 min, the supernatant was collected and assayed for its protein concentration by the method of Bradford [34]. Plasma total immunoglobulin content was calculated by subtracting this value from the total protein concentration in the plasma before precipitation with PEG.

Spleen leukocyte respiratory burst activity (RBA)

Just after collection, spleen samples were conditioned in L15 medium (Sigma-Aldrich) at 4°C, and gently pressed through sterilised nylon mesh (40 µm, Dutscher) to obtain leukocyte suspensions. Then, the L15 medium-diluted samples were loaded onto Ficoll gradient (Healthcare, GE). After centrifugation (2500 rpm, 20 min, at 4°C), leukocyte suspensions were collected and washed twice in L15 medium and again centrifuged (1200 rpm, 5 min, 4°C). They were re-suspended in L15 medium and viable leukocytes were adjusted at 10^6 cells/mL before classification of leukocyte populations (lymphocyte, macrophage and granulocyte) by flow cytometry. RBA analysis was performed using flow cytometry method as previously described by Chilmonczyk and Monge [35] with some modifications of Jolly et al. [36]. The RBA test corresponded to an evaluation of intracellular hydrogen peroxide production following cell activation or not with phorbol 12-myristate 13-acetate (PMA). The fluorescence levels of unstimulated and PMA-stimulated cells were determined after 30 min of cell incubation (18°C in the dark) with 2′-7′dichlorofluorescin diacetate (DCFH-DA (5 µM) and DCFH-DA plus PMA (2 $\mu g.mL^{-1}$), respectively. The spleen respiratory burst activities were expressed in a stimulation index as the ratio between the mean fluorescence measured in stimulated cells (DCFH-DA + PMA) and the basal mean fluorescence of control (DCFH-DA only).

RNA precipitation and complementary DNA (cDNA) synthesis

Three pools of each experimental condition and time-point were collected to compare their gene expression profiles. Total RNA was extracted individually from the spleen and kidney using TriReagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. The pellet was dried and resuspended in 200 µL of RNase-free water. Total RNA concentration was determined by Nano Drop-2000 spectrophotometer (Thermo Scientific) and the integrity was checked by Experion RNA Std Sens analysis (Bio-Rad Laboratories, Hercules, CA). Total RNA (1µg) was used to synthesise cDNA with iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions.

Gene expression analysis

Real-time PCR assay was carried out in order to analyse the expression pattern of different genes of immunological relevance in spleen and kidney from rainbow trout stimulated with inulin. Samples were taken randomly from three fish of three different tanks for each treatment (n = 9) at D35 and D51 days of feeding with inulin. Samples of three randomly selected fish from each tank were pooled for each treatment (n = 3 fish per each pool). On day 52 of feeding, fish were challenged against A. salmonicida achromogenes and 44 h post-challenge (D54 of treatment) three fish of three different tanks for each treatment (n = 9 fish) were also collected. Samples were also taken using the same strategy from fish fed with basal diet and from non-infected fish (n = 3 fish per each pool). As a housekeeping gene, elongation factor 1 alpha (*ef-1α*) was amplified from all the evaluated samples. The gene expression of pro-inflammatory (*il-1, il-6, il-8*), antibacterial (lysozyme), humoral (*mIgM*), macrophages (*mcsfra*), neutrophils (mpo), T-helper (*cd4-1, cd4-2α, cd4-2β*), cytotoxic T-cells (*cd8*), and anti-inflammatory responses (*il-10, tgf-β1*) were evaluated. The list of specific primers used for gene expression analysis is given in Table 2. Real-time PCR reactions were carried out with iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories) using a 1:40 dilution of the cDNA for target genes or 1:1000 dilutions for *ef-1α*. Primers for target genes were used at 500 nM. The thermal conditions used were 3 min at 95ºC of pre-incubation, followed by 40 cycles at 95ºC for 30s and 60ºC for 30s. All reactions were performed using ABIprism 7300 (Applied Biosystem) and quantification was done according to the Pfaffl method [37] corrected for efficiency of each primer set. Values for each sample were expressed as normalized relative expression (NRE), calculated in relation to values of control group and normalized against those of the housekeeping gene *ef-1α*. The results are expressed as average of values obtained in all pools from D35 and D51of feeding, and D54 (after bacterial challenge test).

Table 2: Primers used for each gene expression analysis by real-time RT

Genes	Primer sequence (5'-3')	Accession number	Amplicon size
ef-1α	Fw: 5'-ATGCCCCCAAGTTCCTGAAG-3' Rv: 5'-AACAGCAACAGTCTGCCTCA-3'	NM_001124339.1	140
il-1	Fw: 5'-TGAGAACAAGTGCTGGGTCC-3' Rv: 5'-GGCTACAGGTCTGGCTTCAG-3'	NM_001124347.2	148
lysozyme	Fw: 5'-TGCCTGTCAAAATGGGAGTC-3' Rv: 5'-CAGCGGATACCACAGACGTT-3'	NM_001124716.1	152
mIgM	Fw: 5'-AAAGCCTACAAGAGGGAGACCGAT-3' Rv: 5'-AGAGTTATGAGGAAGAGTATGATGAAGGTG-3'	X65263.1	128
mcsf-ra	Fw: 5'-ATCTCCACTCATGGCGACACA-3' Rv: 5'-CATCGCACTGGGTTTCTGGTA-3'	AB091826	177
mpo	Fw: 5'-GCAGAGTCACCAATGACACCA-3' Rv: 5'-ATCCACACGGGCATCACCTG-3'	GBTD01119227	68
il-10	Fw: 5'-CCGCCATGAACAACAGAACA-3' Rv: 5'-TCCTGCATTGGACGATCTCT-3'	NM_001245099.1	105
tgf-β1	Fw: 5'-GCCAAGGAGGTCCACAAGTT-3' Rv: 5'-GTGGTTTTGATGAGCAGGCG-3'	NM_001281366.1	146
cd4-2β	Fw: 5'-AAGCCCCTCTTGCCGAGGAA-3' Rv: 5'-CTCAACGCCTTTGGTACAGTGA-3'	AY899932	108
vapA	Fw: 5'-ATTAGCCCGAACGACAACAC-3' Rv: 5'-CCAACACAATGAAACCGTTG-3'	KP184543.1	148

Statistical analysis

Results are presented as mean ± SD. Data were checked for normal distribution and homogeneity of variances by Univariate tests. Humoral immune parameters, blood leukocyte proportions, specific growth rate, mortality, splenic index, haematocrits, and immune gene expressions on D35 and D51 were carried on using a two-way ANOVA test. One-way ANOVA was used to test for those parameters on D54 and for data of cumulative mortality over the 14 days of bacterial challenge test. In all statistical analysis tests used, $p < 0.05$ was considered statistically significant. The statistical analysis was performed using the Statistica bio-software (version 10.0)

3. Results

3.1. Specific growth rate (SGR), haematocrit (Ht) and splenic index (SI)

Fish fed inulin diets showed a slightly increasing SGR, although not statistically significant, probably due to a high intra-treatment variability, especially on D51 (Table 4.3).

Table 4.3: Mean (±SD) values for specific growth rate (SGR), haematocrit and splenic index of trout juveniles fed with 0.1% (In 0.1%) and 1% (In 1%) inulin diets on day 35th and day 51th of the feeding test

Times	Variables	Control	In 0.1%	In 1%	$F_{-values}$	$P_{-values}$
D35	SGR (%/day)	0.69 ± 0.11	0.83 ± 0.08	0.81 ± 0.03	1.04	0.40
	Haematocrit (%)	33.70 ± 2.5	31.70 ± 1.9	32.90 ± 1.9	0.62	0.56
	Splenic index (%)	0.32 ± 0.14	0.22 ± 0.04	0.20 ± 0.04	1.90	0.22
D51	SGR (%/day)	0.62 ± 0.28	0. 89 ± 0.13	0.85 ± 0.19	1.46	0.30
	Haematocrit (%)	32.30± 3.6	30.90± 0.3	28.0.9± 1.3	1.84	0.23
	Splenic index (%)	0.21 ± 0.06	0.19 ± 0.10	0.18 ± 0.01	0.20	0.82

Ht values did not differ between controls and inulin-fed fish (Table 3). On the other hand, a non-statistically significant decreasing trend was observed in the SI values on D35 and D51 in both inulin doses compared to fish fed control diet (Table 4.3).

3.2. Humoral immune variables

Only the low dose of inulin significantly increased plasma haemolytic alternative complement pathway (ACH50) levels ($F = 15.56$, $p = 0.0004$) after 35 and 51 days of inulin administration (Figure 4.1: A). But two days (D54) after bacterial injection, there were no significant differences of plasma ACH50 levels between treatments and control groups.

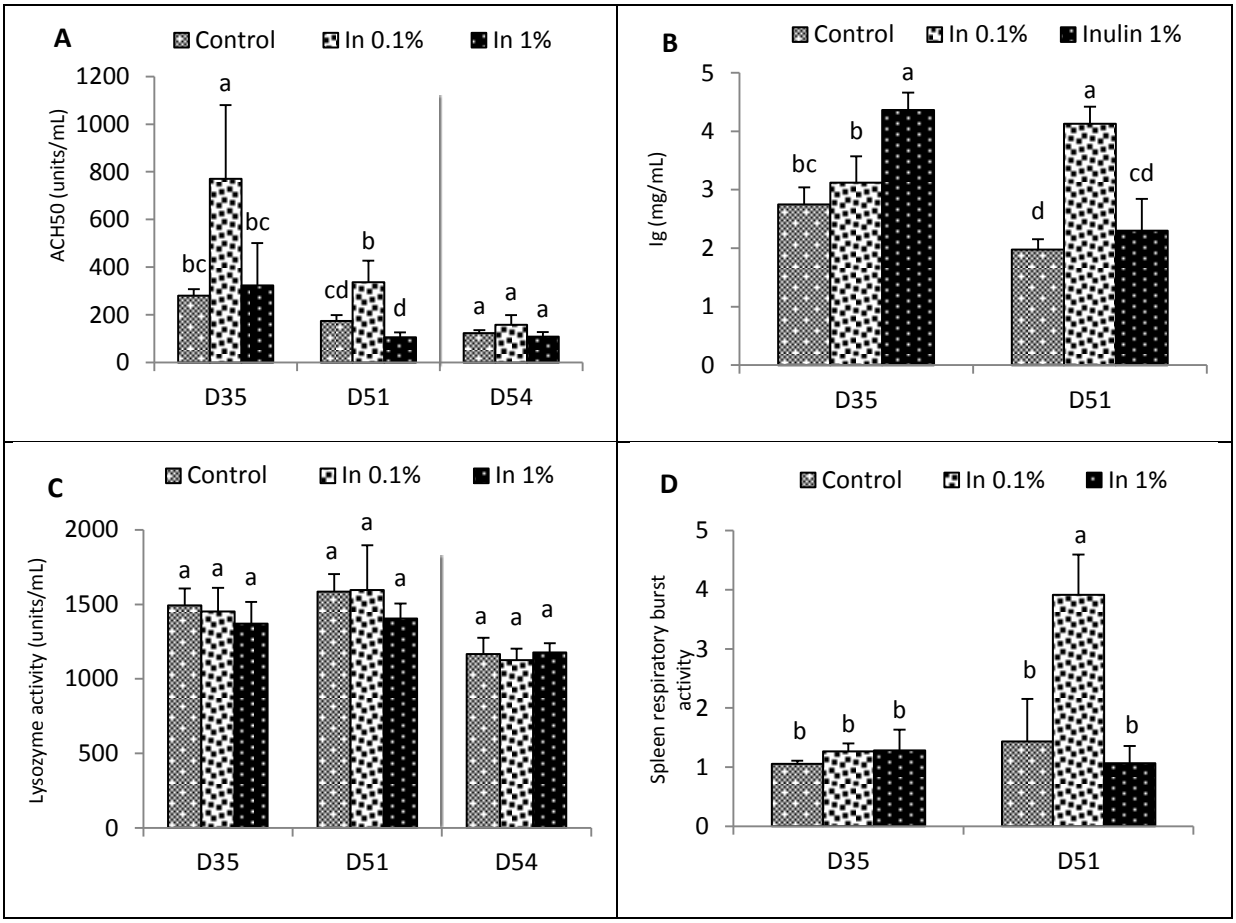


Figure 4.1 (A-D): Effects of dietary inulin administration on humoral parameters of fish sampled during the nutrient test on D35 and D51, or two days after bacterial infection (D54). (A) Plasma haemolytic alternative complement activity (ACH50), (B) Plasma total immunoglobulin's content, (C) Plasma lysozyme activity, (D) Respiratory burst activity (RBA) of spleen macrophage cells. Values are expressed as mean ± SD of 18 fish/diet/day on D35, D51 and 9 fish/diet on D54. For spleen RBA, Plasma ACH50 data are expressed as mean ± SD of 9 fish/diet/day on D35, D51. Statistical differences between dietary treatments and treatment times are indicated by different letters with $p < 0.05$.

High inulin dose on D35 and low dose on D51 significantly increased levels of plasma total Ig ($F = 12.81$, $p = 0.003$) compared to control diet (Figure 4.1: B).

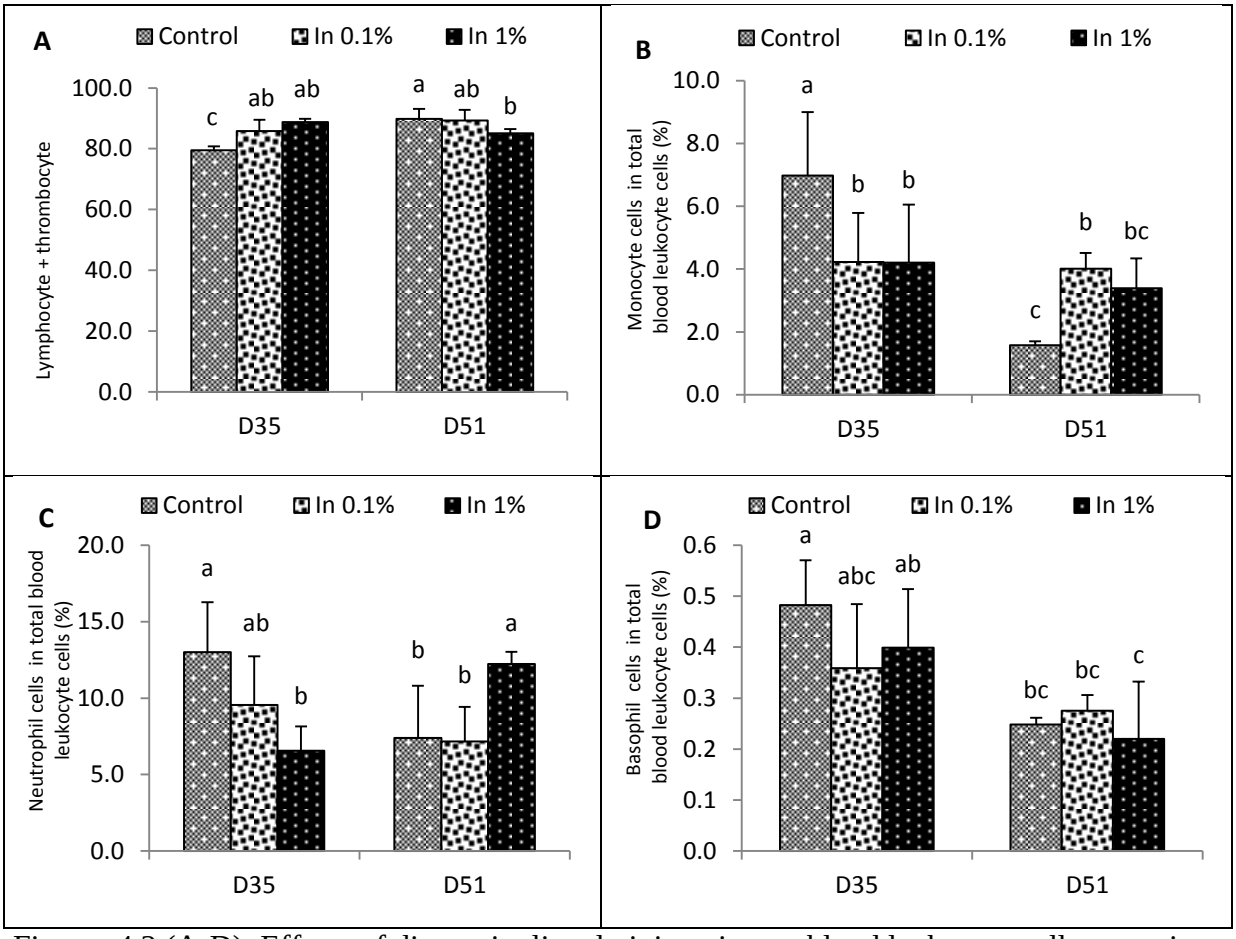
Values of plasma lysozyme activity were not affected by the dietary inulin on D35 and D51 for both doses, but tended to decrease after bacterial challenge (D54) in all experimental conditions even if they did not statistically differ (Figure 4.1: C).

Levels of RBA of spleen lymphocytes (as for macrophage cells) did not significantly differ whatever the inulin administration dose on D35 (Figure 4.1: D). However, RBA values of macrophages from fish fed the low dose of inulin significantly increased on D51 compared to the control and the high dose of inulin ($F = 18.73$, $p = 0.0002$).

3.3. Blood leukocyte cell proportions

Blood leukocytes were composed of high percentages of lymphocytes followed by those of neutrophils, monocytes and basophils (Figure 4.2: A-D). Both inulin doses significantly

increased (F = 7.48, p = 0.01) the proportion of blood lymphocytes on D35. On D51, a significant decrease (F = 7.4, p = 0.018) of lymphocyte population appeared in the high dose-treated groups in association to an increase in the control fish (Figure 4.2: A). Both inulin doses induced a significant decrease (F = 6.54, p = 0.01) in the percentages of monocytes on D35, but on D51, a decrease (F = 11.23, p = 0.005) was observed in control fish not in treated ones (Figure 2: B). As for monocytes, the percentages of neutrophils in fish fed high inulin dose decreased (F = 7.44, p = 0.007) on D35 (Figure 4.2: C), but an increase was observed on D51 in association to a decrease control fish. The proportion of basophils was not affected by inulin doses on D35 and D51, and only a temporal trend of decrease was observed on D51, especially in control fish and fish fed high inulin dose (F = 14.19, p = 0.002; Figure 4.2: D). All data concerning leukocyte populations showed both positive and negative effects of both inulin diets on the proportions of leukocyte populations.



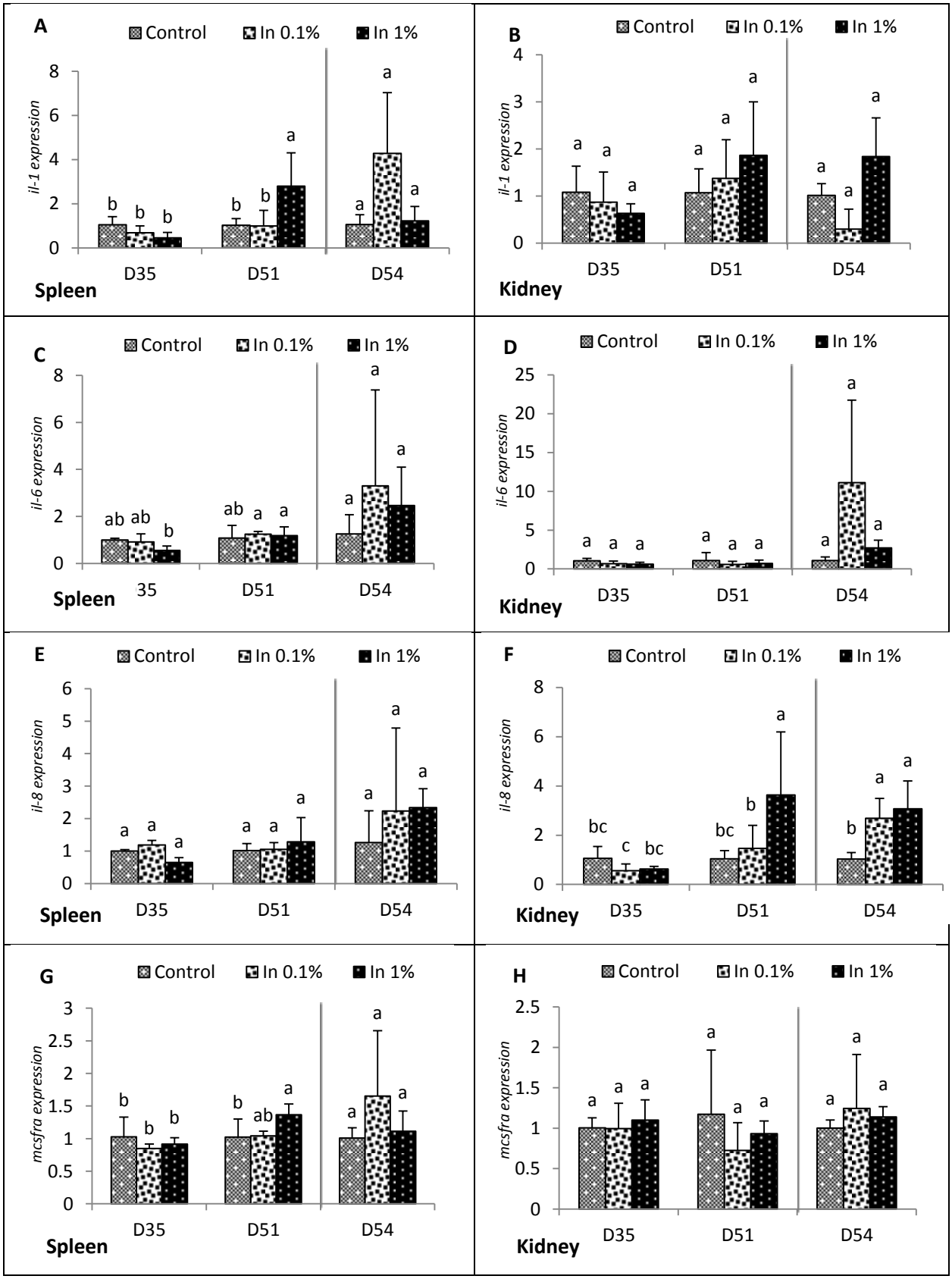
Figures 4.2 (A-D): Effects of dietary inulin administration on blood leukocyte cell proportions of fish sampled during the nutrient test on D35 and D51. (A) Lymphocyte + thrombocyte cells proportions, (B) Monocyte cell proportion, (C) Neutrophil cell proportion and (D) Basophil cell proportion in total blood leukocyte cells population. Values are expressed as mean ± SD of 18 fish/diet/day on D35, D51. Statistical differences between dietary treatments and treatment times are indicated by different lower case letters with $p < 0.05$.

3.4. Immune gene expression

3.4.1. Pro- and anti-inflammatory gene expressions

The expression levels of both pro-inflammatory (*il-1, il-6, il-8, mcsfra*) and anti-inflammatory (*tgf-β1, il-10*) genes were evaluated in spleen and kidney but no statistical changes were

observed in fish sampled after mid-term inulin administration (D35) whatever the tested dose, while the expression of some genes was up- or down- regulated on D51 and D54 (Figure 4.3).



Figures 4.3 (A-H): Effects of dietary inulin on pro- inflammatory gene expressions in spleen and kidney of fish sampled during the nutrient test on D35 and D51, or two days after the bacterial challenge (D54). A, B, C, D, E, F, G and H are expression levels of *il-1*, *il-6*, *il-8*, *mcsfra* in spleen and kidney, respectively. Relative transcript (mRNA) levels were determined by real-time RT-PCR and normalized by the arithmetic mean of *ef-1α* expression. Values are expressed as mean ± SD of 3 pool samples per experimental condition (3 fish per pool sample). Statistical differences between dietary treatments and treatment times are indicated by different lower case letters $p < 0.05$.

In spleen, only the high inulin dose significantly upregulated the expression levels of *il-1* ($F = 5.74$, $p = 0.01$, Figure 4.3: A) and mcsfra ($F = 5.64$, $p = 0.03$, Figure 4.3: G) genes on D51. But after bacterial challenge on D54, the highest values for expression levels of all the tested pro-inflammatory genes and the inflammatory *tgf-β1* were observed in fish fed low inulin dose although no statistical differences were calculated due to high intra-treatment variability (Figure 4.3: A-C-E-G and Figure 4.4: A). In kidney tissue, only *il-8* expression level was statistically increased by the high inulin dose on D51 ($F = 13.57$, $p = 0.003$), and both inulin doses significantly upregulated the expression level of this gene two days after bacterial infection on D54 ($F = 9.158$, $p = 0.021$; Figure 4.3: F). In contrast, the *tgf-β*1expression level was significantly decreased by both inulin doses on D51 ($F = 11.2$, $p = 0.0058$), but a trend of increase was observed after bacterial infection on D54 (Figure 4.4: B). No dose effect was observed for other pro- and anti- inflammatory gene expressions on D51 and D54 in kidney, except for a trend of increase in il-6 values by low inulin dose after bacterial infection on D54.

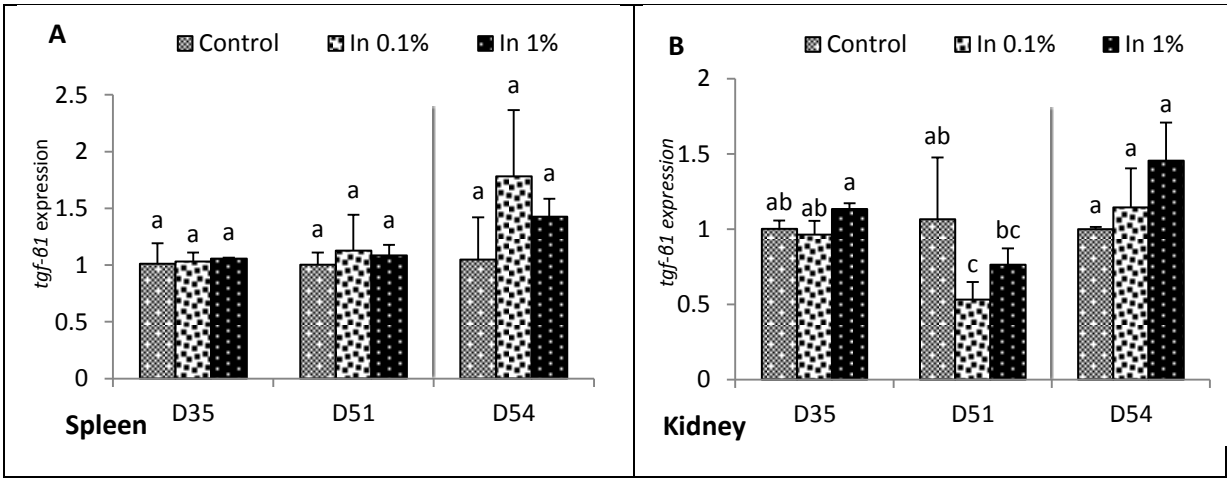


Figure 4.4 (A-B): Effects of dietary inulin on anti- inflammatory gene expressions in spleen and kidney of fish sampled during the nutrient test on D35 and D51, or two days after the bacterial challenge (D54). A, B are expression levels of in spleen and kidney, respectively. Relative transcript (mRNA) levels were determined by real-time RT-PCR and normalized by the arithmetic mean of *ef-1α* expression. Values are expressed as mean ± SD of 3 pool samples per experimental condition (3 fish per pool sample). Statistical differences between dietary treatments and treatment times are indicated by different lower case letters $p < 0.05$.

3.4.2. Expression of T-helper, cytotoxic T - cells and mIgM genes

For adaptive immune functions, expression levels of four T-helper isoform genes (*cd4-1, cd4-2α, cd4-2β*), cytotoxic T - cells (*cd8*) and *mIgM* genes were determined in kidney and spleen samples, and no statistical changes were observed in fish sampled after mid-term inulin administration (D35) whatever the tested dose. The levels of *cd4-1, cd4-2α* and *mIgM*

expression also showed no significant change in both spleen and kidney after 51 days and after bacterial challenge on D54. Both inulin doses induced a down-regulation of *cd4-2β* expression on D51 in kidney, especially by the low inulin dose (F = 4.54, p = 0.03, Figure 5: B), however this dose induced a trend of increase after bacterial infection on D54.

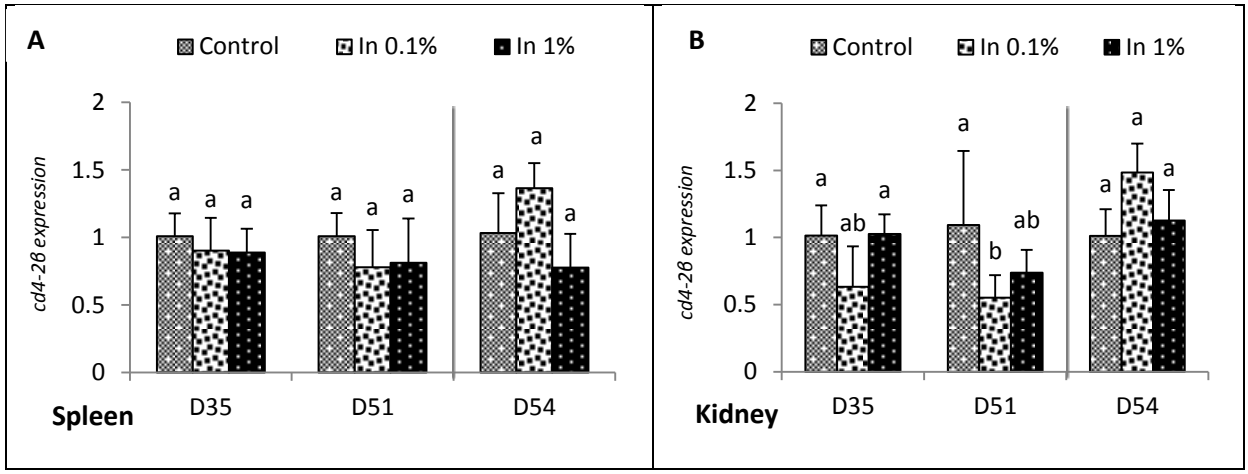


Figure 5 (A-B): Effects of dietary inulin on T-helper gene expressions (*cd4-2β*) in spleen or kidney of fish sampled during the nutrient test on D35 and D51, or two days after the bacterial challenge (D54). (A) *cd4-2β* gene expression in spleen, (B) *cd4-2β* gene expression in kidney samples. Relative transcript (mRNA) levels were determined by real-time RT-PCR and normalized by the arithmetic mean of *ef-1α* gene expressions. Values are expressed as mean ± SD of 3 pool samples per experimental condition (3 fish per pool sample). Statistical differences between dietary treatments and treatment times are indicated by different lower case letters $p < 0.05$).

3.4.3. Antibacterial gene expressions

Expression of two antibacterial genes (C-type lysozyme and mpo) was evaluated in kidney and spleen on D35, 51, and D54, but only C-type lysozyme gene expression was affected by inulin diets (Table 4.4). In spleen, both inulin doses did not significantly affect lysozyme gene expression during the nutrition; nonetheless, a trend of decrease was observed on D51 for both doses (Figure 4.6: A). In kidney, values for lysozyme expression were the lowest in fish fed low inulin dose on D51 (F = 4.85, p = 0.02; Figure 4.6: B). After the bacterial infection (D54), low dietary dose inulin induced an upregulation of lysozyme gene expression in both organs (spleen and kidney tissues), but no significant differences were observed between treatments due to large intra-treatment variability.

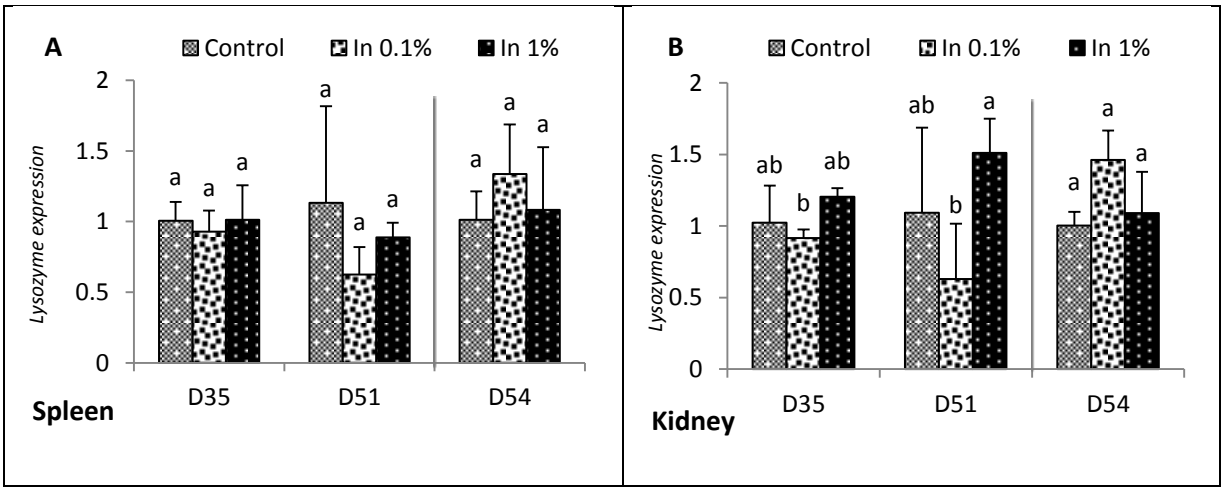


Figure 4.6 (A-B): Effects of dietary inulin on C-type lysozyme gene expression in spleen or kidney of fish sampled during the nutrient test on D35 and D51, or two days after the bacterial challenge (D54). (A) lysozyme gene expression in spleen, (B) lysozyme gene expression in kidney. Relative transcript (mRNA) levels were determined by real-time RT-PCR and normalized by the arithmetic mean of *ef-1α* gene expressions. Values are expressed as mean ± SD of 3 pool samples per experimental condition (3 fish per pool sample). Statistical differences between dietary treatments and treatment times are indicated by different lower case letters ($p < 0.05$).

3.5. Disease resistance

During the first week of infection, mortality increased earlier and more rapidly in trout fed high inulin dose and control diet than in fish fed low inulin diets (Figure 4.7). The mortalities stopped on the 6th day after bacterial injections in low inulin dose groups but were still observed on the 8th and 10th days post-infection for high inulin dose and control groups, respectively. Therefore, survival rate was significant higher in fish fed low inulin dose (48%) than in controls (19%) and high inulin dose (7%) 14 days after bacterial injection ($F = 15.10$, $p = 0.007$), whereas no significant differences were observed between high inulin dose and control groups. Based on VapA expression in spleen and kidney, the effectiveness of bacterial infection was not really detectable two days after bacterial injections since the presence of VapA was detected in only 1 of 18 pool samples. This VapA positive pool sample was detected from control dietary only.

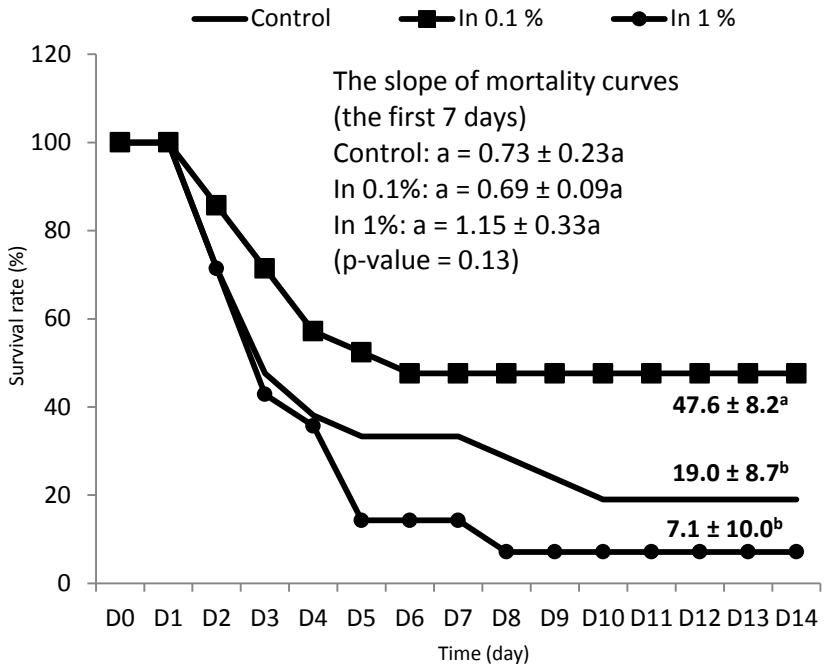


Figure 4.7: Survival rate profile in rainbow trout juveniles fed with 0.1% or 1% inulin and challenged with Aeromonas salmonicida achromogenes (3.1×10^7 CFU/100g fish body weight) for 14 days. Statistical differences between dietary treatments and treatment times are indicated by different lower case letters ($p < 0.05$).

4. Discussion

4.1. Influence of inulin on growth, haematocrit, splenic index and leukocyte populations

In this study, rainbow trout juveniles were fed inulin at low and high doses (0.1% and 1% diets), and fish were sampled after 35 and 51 days of feeding. Specific growth rate (SGR) of fish fed both inulin doses seemed to be higher than those of control fish, but because of high intra-group variability, no significant differences were recorded whatever the administration duration. This result corroborates previous reports revealing no significant effects of inulin on growth performance of Nile tilapia fingerlings (feeding diets of 2.5 and 5 g inulin/kg for 4 weeks) [38], common carp (feeding diets of 5 and 10g inulin/kg for 7 weeks) [39], and white-leg shrimp (feeding diets of 1.25 to 10 g inulin/kg for 62 days) [40]. In contrast, Ortiz et al. [25] reported that rainbow trout fed with inulin or FOS-containing diets (5 and 10g kg^{-1}) exhibited significant increase of body weight gain. Mo et al. [16] reported a significantly higher growth rate of grass carp after 8 weeks feeding with dietary inulin at 0.2% or 2%. Dietary inulin supplementation (5 to 20g kg^{-1}) for 60 days had a significant effect on SGR in Asian seabass [41], and synergistic effect of the combination of inulin and *Lactobacillus sakei* on weight gain was reported in leopard grouper [23]. All these results demonstrate a high variability in growth response after dietary inulin treatment, probably based on species specificity related to differences of digestive tract morphology and functionality between the tested species, dose and duration of feeding period, but also to experimental conditions such limited experimental units in the current study.

In the present study, dietary inulin supply had no significant effects on haematocrit (Ht) and splenic index (SI) values as previously reported for some haematological features of hybrid surubim Pseudoplatystoma (feeding 0.5% inulin for 15 days) [24]. But inulin diets significantly modulated the proportions of leukocyte populations after 35 and 51 days of inulin treatment. In line with our results, the combination diet of 0.5% inulin with the bacteria *Weissella cibaria* (7.87 ± 0.2 log CFU g-1) increased erythrocytes and reduced circulating neutrophils of hybrid surubim [24]. Dietary inulin and Jerusalem artichoke (*Helianthus tuberosus*) fed for 8 weeks increased red blood cell numbers of Nile tilapia [22]. In contrast, increasing dietary inulin from 0% to 1, 2 and 3% significantly decreased the mean values of white blood cells, red blood cells and haematocrit in great sturgeon [42]. The positive effect of dietary inulin on the proportions of leukocyte populations in fish could be explained by a stimulatory action of inulin supplement on the anaerobic fermentation processes which increases the production of intestinal short-chain fatty acids (SCFA), especially enhanced butyric acid levels, as observed in mammals [44-48]. In fish, very few studies report the kind of end products obtained after the fermentation of prebiotic like inulin. However, an in vitro study performed on fish bacteria mono-cultures revealed the production of SCFA [43]. Moreover, Hoseinifar et al. [44] demonstrated that the administration of galacto-oligosaccharide (GOS) could increase the presumptive autochthonous lactic acid bacteria (LAB) in *O. mykiss*' gut and in turn, could lead to a higher fermentation of prebiotics. Fish fed inulin can displayed comparable cell stimulation as for mammals since in vitro studies have demonstrated that Tuna (*Thunnus tonggol*) cells from different gastrointestinal sections added inulin and gastric microflora can produce various short fatty acids with high amount of butyric acid [45]. In rats, one of the end product of this fermentation, the butyric acid, was reported to enhance epithelial proliferation in distant intestinal segments [46], a promoter of intestinal regulatory T cells [47]. Moreover, butyric acid is an important energy (ATP) source for cells lining the mammalian colon [48], increased energy production and cell proliferation [49]. On the other hand, butyric acid is also a histone deacetylase (HDAC) inhibitor, such as

HDAC1, HDAC2, HDAC3 and HDAC8, which inhibits the function of histone deacetylase enzymes leading to deacetylase [50]. The decrease of HDCA causes the loss of structures of chromatin due to the decrease of electrostatic attraction between histone and DNA [50].

4.2. Influence of inulin diets on humoral immune parameters

The present study demonstrates differential actions of dietary inulin on humoral immune parameters. Indeed, it is interesting to notice that the low dose of inulin significantly increased plasma alternative complement activity (ACH50) on D35 and D51, plasma total immunoglobulin (Ig) and spleen respiratory burst activity on D51. Meanwhile, the high dose of 1% inulin in diet significantly increased only plasma Ig content on D35. Alternative complement activity plays a major role in the innate immune response such as to destroy the cell surface membranes of pathogens by creating pores and opsonising pathogens for destruction by an enhanced uptake of phagocytes and mediated through ligand-receptor interactions between the surfaces of the two cells [51]. Apart from the latter immune function, our results demonstrated that low inulin dose may potentiate the production of antibodies such as by increasing the production of total circulating immunoglobulins or activate the inhibition of bacterial development by stimulating the spleen respiratory burst activity. In agreement with these results, inulin dietary administration of 10g kg^{-1} fed to gilthead seabream for two weeks stimulated the serum complement activity, IgM level as well as leukocyte respiratory burst activity [6]. Dietary inulin at 0.2% and 2% feeding to grass carp for 8 weeks showed significant increase in total serum immunoglobulin [16], while 5g kg^{-1} feeding to Nile tilapia fingerlings from 5 to 12 weeks increased total immunoglobulin and alternative complement activity [38]. However, Hoseinifar et al. [21] shown that dietary inulin 2% feeding to common carp fry for 60 days did not stimulate ACH50 and Ig content in blood plasma. Moreover, dietary inulin 0.5% did not increase the amount of Ig content in blood plasma of hybrid surubim after 15 days of feeding. In this study, both inulin diets induced no marked effects on plasma lysozyme activity during the nutrition test or after bacterial infection. In line with the present results, Hoseinifar et al. [21], Mourino et al. [24] and Ahmdifar et al. [42] did not find any effect of dietary inulin on plasma lysozyme activity response in common carp fry, hybrid surubim and great sturgeon juveniles (Huso huso). However, Tiengtam et al. [22] reported that inulin supplementation at 5g kg^{-1} improved lysozyme activity in juvenile Nile tilapia. Similarly, dietary inulin 1% feeding after 45 days increased lysozyme activity of juvenile common carp [52]. Therefore, it appears that the response of humoral immune parameters to inulin may be species specific and dose dependent. Our results showed that low inulin doses may be more immunomodulatory in rainbow trout than high doses since more immune functions were stimulated, especially after a long-term administration. The production of antibodies is one of the main functions of the humoral immune system [53] and the synchronized increase in ACH50 level and plasma total Ig content in the present study ascertain that dietary inulin powerfully stimulated the coordinated interactions between some innate and specific functions in rainbow trout.

4.3. Influence of inulin on immune gene expressions

In the present study, dietary inulin did not induce a ubiquitous action on the expression of genes encoding for immune functions, but some significant effects were observed depending on the dose, the administration duration, and the interaction with bacterial infection or tested organs (spleen and kidney). The effect of dietary inulin on immune gene expressions was time-dependent because no statistical changes were observed in fish sampled after mid-term inulin administration (D35) whatever the tested dose, while the expression of some genes was up- or down- regulated after a long-term administration on D51 and after bacterial challenge on D54. As for type-C lysozyme gene, while no effects were observed on D35 during the

nutrient test, low inulin dose induced a slight down-regulation in the kidney tissues on D51. But, two days (D54) after bacterial challenge, no significant effect of inulin doses on the lysozyme expression gene was observed as for plasma lysozyme activity, so no obvious effect of dietary inulin on lysozyme function can be confirmed. Lysozyme was reported to damage bacterial cell walls by catalysing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins [54].

Table 4.4: Summary results of the analysis of rainbow trout immune parameters after mid- and long- term nutrient test with low (0.1%) and high (1%) dose of inulin in the diets

Immune parameters		**Organs**	**In 0.1%**			**In 1%**		
			D35	**D51**	**D54**	**D35**	**D51**	**D54**
Humoral immune parameters	haemolytic activity of complement	blood plasma	↑× 2.73	↑×1.92	NS	NS	NS	NS
	total immunoglobulin		NS	↑× 1.58	NA	↑× 2.09	NS	NA
	lysozyme activities		NS	NS	NS	NS	NS	NS
Macrophage activity	respiratory burst activity	fresh spleen	NS	↑×2.73	NA	NS	NS	NA
Blood leukocyte populations	lymphocyte + thrombocyte	fresh blood	↑×1.08	NS	NA	↑×1.11	↓×1.05	NA
	monocyte		↓×1.66	↓×1.66	NA	↑×2.5	NS	NA
	neutrophils		NS	NS	NA	↓×1.96	↑×1.64	NA
	basophils		NS	NS	NA	NS	NS	NA
Immune gene expression	*il-1*	spleen tissue	NS	NS	NS	NS	↑×2.7	NS
	il-6		NS	NS	NS	NS	NS	NS
	il-8		NS	NS	NS	NS	NS	NS
	lysozyme		NS	NS	NS	NS	NS	NS
	mIgM		NS	NS	NS	NS	NS	NS
	mcsf-ra		NS	NS	NS	NS	↑×1.33	NS
	mpo		NS	NS	NS	NS	NS	NS
	il-10		NS	NS	NS	NS	NS	NS
	tgf-ß1		NS	NS	NS	NS	NS	NS
	cd4-1		NS	NS	NS	NS	NS	NS
	cd4-2α		NS	NS	NS	NS	NS	NS
	cd4-2ß		NS	NS	NS	NS	NS	NS
	cd8		NS	NS	NS	NS	NS	NS
Immune gene expression	*il-1*	kidney tissue	NS	NS	NS	NS	NS	NS
	il-6		NS	NS	NS	NS	NS	NS
	il-8		NS	NS	↑×2.62	NS	↑×3.51	↑×3.0
	lysozyme		NS	NS	NS	NS	NS	NS
	mIgM		NS	NS	NS	NS	NS	NS
	mcsf-ra		NS	NS	NS	NS	NS	NS
	mpo		NS	NS	NS	NS	NS	NS
	il-10		NS	NS	NS	NS	NS	NS
	tgf-ß1		NS	↓×1.06	NS	NS	NS	NS
	cd4-1		NS	NS	NS	NS	NS	NS
	cd4-2α		NS	NS	NS	NS	NS	NS
	cd4-2ß		NS	↓×1.98	NS	NS	NS	NS
	cd8		NS	NS	NS	NS	NS	NS

Significant level at $P< 0.05$, NS: non-significant, NA: no data, ↑ indicated the increase in fold change of the value, ↓ indicate the decrease in fold change of the value.

Our results showed better that dietary inulin can regulate the expression of some pro-inflammatory immune genes. Indeed, high inulin dose upregulated *il-1β* in spleen on D51 as well as high inulin dose enhanced *il-8* in the kidney and mcsfra expression in spleen on D51. Interleukin 1β plays an important role in the regulation of immune and inflammatory

processes by participating in the stimulation of growth and proliferation of T and B lymphocytes, macrophages and vascular endothelial cells [55]. In a previous study on the effect of dietary inulin, *Bacillus subtilis* and microalgae on intestinal gene expression also showed that inulin can upregulate intestinal gene expression in the gilthead seabream such as pro-inflammatory genes (*il-8*), β-actin, occludin and transport protein genes [56]. Pro-inflammatory gene *il-8* belongs to CXC chemokines and is mainly involved in chemotaxis of immune cells towards the site of wound or infection [57]. In our study, it appears that dietary inulin could stimulate rainbow trout's inflammatory response by modulating gene expression in a time-dependent manner and being effective only after a long-term administration. After bacterial infection, only pro-inflammatory gene *il-8* and *tgf-β1* were significantly upregulated in kidney of fish treated with inulin diets. Transforming growth factor-β (*tgf-β*) has been shown to play an essential role in the suppression of inflammation, regulating T cells, inhibiting the proliferation of immune cells as well as cytokine production via Foxp3-dependent and-independent mechanisms [58]. *Tgf-β* can suppress T-cells proliferation by inhibiting of production of interleukin-2, a cytokine known to potently activate T cells, NK cells [59].

Regarding genes encoding for adaptive immune function, our results showed that dietary inulin had no significant effects on expression of membrane immunoglobulin M gene (*mIgM*) and *cd4-1* and *cd8* genes. However, low inulin dose induced a down-regulation of *cd4-2β* gene expressions in kidney after long-term administration on D51, but such effect was not observed after bacterial infection. $Cd4^+$ T and $cd8^+$ T cells are involved in mediating immune response through the secretion of specific cytokines and activate different cells of innate immune system, B-lymphocytes, and cytotoxic T cells [60]. *Cd4* is a type I membrane glycoprotein with four extracellular Ig-like domains (D1-D4), a transmembrane domain, and a short cytoplasmic domain [61]. It has been described that trout *cd4-2* has two Ig-like domains and both *cd4-1* and *cd4-2* molecules containing the cytoplasmic motif that binds Lck protein [62–64]. The decrease in the expression level of this immune gene could be due to a negative action of T-cells themselves. Inulin treatments indeed increased the proportion of lymphocytes after short-term exposition. However, after long-term exposure, high (1%) inulin dose diet significantly decreased the lymphocyte proportion in total leukocyte populations, which may reflect a regulation of lymphocyte production.

Results from the present study showed a differential temporal sensitivity to inulin treatment among the tested immune gene functions. Indeed, no marked effect was observed on the expressions of all the 13 immune genes tested in spleen or kidney tissues sampled after mid-term nutrient test (D35). The effect of inulin on immune gene expression was only observed after long-term administration with no clear differences in kidney and spleen before and after bacterial infection, indicating a potentiation of the inulin immunomodulatory action along the dietary duration as recently demonstrated for other types of immunostimulants such as bovine lactoferrin in the same fish species [65]. It also worth noting that inulin showed a similar sensitivity in the tested two immune organs. Previous studies indicated that the immune organs of rainbow trout may display differences in their sensitivity to immunostimulation, such as a higher sensitivity in spleen than in kidney in the case of β-glucans [66], but the experimental context should also be considered.

4.4. Influence of inulin diets on disease resistance

The result concerning the infection test using *A. salmonicida* achromogenes revealed that low inulin dose (0.1% diet) increased rainbow trout resistance to pathogens. Indeed, the cumulative survival showed that fish fed with low inulin displayed the highest survival after bacterial challenge. Importantly, in fish fed 0.1% and 1% inulin no VapA expression was

detected two days after the bacterial challenge, which supports the possibility that inulin diets could protect rainbow trout juveniles after injected with bacteria. In agreement with our results, Ibrahem et al. [67] reported that survival rate of Nile tilapia was significantly increased in group supplemented with inulin (5g kg^{-1}) after one or two months of exposure compared to the control. Further, inulin diets decreased the prevalence of WSSV in treated shrimp [40]. In the present study, the improvement of disease resistance may be related to modulation of innate immune functions by dietary inulin, but also to upregulation of some adaptive immune compounds (total plasma immunoglobulin and expression of *cd4+* genes) observed on D51. Especially, the trend of highest expression levels of *il-1, il-6, il-8, mcsfra* and *tgf-β1* cytokines observed in spleen of fish fed low inulin dose two days after bacterial injection on D54 may indicate a highest pro- and anti-inflammatory response which could allow better bacteria resistance. The fact that low inulin dose provided a better protection against pathogens and supported a significantly higher survival rate than the control and high inulin dose groups may also be explained by the fermentation of inulin by *Bifidobacterium spp* and lactic acid producing bacteria which increase short-chain fatty acids (SCFA) such as lactic and butyric acids. Those SCFAs such as an energy metabolite to synthesis ATP, G protein coupled receptor (GPCR) activator and a histone deacetylase (HDAC) inhibitor [50]. Previous study has been reported that SCFAs such as butyric acid exert antimicrobial activity in humans by the antimicrobial peptide LL-37 [68] and the benefits of butyrate depend on the amount and type of fats in the diet and the duration of the treatments [49]. As for mammals, it was recently demonstrated that dietary inulin increased lactic acid bacteria and *Bifidobacterium spp* but decreased pathogen *Vibrio spp* in Nile tilapia [69]. Hoseinifar et al. [70] also showed that feeding rainbow trout with another prebiotic remarkably increased resistance against *Streptococcus iniae*. In our study, the low protection against bacterial pathogen induced by the high dietary inulin dose (1%) may be explained by too high level of butyric acid production in the intestinal gut. Such a potential non-linear dose response should be further investigated as this might have very practical consequences.

5. Conclusions

In this study, dietary inulin supplemented at 0.1% and 1% inulin differentially stimulated the trout immune system depending on the time course. After mid-term inulin feeding (D35), the immune system of rainbow trout juveniles differentially responded to the two tested inulin doses by modifying the proportions of different blood leukocyte cell types such as lymphocytes, thrombocytes, monocytes and neutrophils. This led to a better humoral immune status in fish fed the low inulin dose on D51 as evidenced by highest levels of spleen respiratory burst activity and plasma ACH50 activity as well as total plasma Ig content. Also, the significantly different response of trout immune system between D35 and D51 found its basis in the absence of change of immune gene expression levels, as for the 13 genes assessed in spleen and kidney on D35. On D51, the immune system was significantly modulated as shown by the increase of pro-inflammatory genes (*il-1, mcsfra*) and decrease of T-helper genes (*cd4-2β*) and anti-inflammatory (*tgf-β1*) genes. Pro- and anti-inflammatory immune response after bacterial injection seemed the highest in spleen of fish fed low inulin dose, even if both doses increased pro-inflammatory gene expression in kidney. Therefore, such better immune pathway responses can explain the better protection for trout juveniles by low inulin dose as shown by the significant increase of the survival rate after 14 days of bacterial challenge, when compared with control and high inulin dose.

Conflict of interest

The authors have declared that no competing interests exist.

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Chapter 5

Physiological and immune pathway responses of rainbow trout (*Oncorhynchus mykiss*) juveniles to different types of β-glucans

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Abstract

β-glucans are immunostimulants used by the aquaculture industry to enhance the immune response of fish, but their efficiency may vary according to their source origin. The objective of the present study was to describe the immune pathway responses of rainbow trout *Oncorhynchus mykiss* juveniles fed low (0.2%) and high (0.5%) doses of three β-glucan types (Macrogard, GAS1 and Wild type-β-glucan) after short (15 days, D15) or long (36 days, D36) term feeding period, and to determine the resistance of fish to bacterial infection. β-glucan diets did not affect specific growth rate, mortality and splenic index as well as spleen respiratory burst activity on D15 or D36. Low dose of GAS1-β-glucan (G0.2%) significantly induced high plasma lysozyme activity on D15, total immunoglobulins on D36, increased of membrane protein *Toll-like receptor 2* gene expression (*TLR_2*) in both spleen and kidney organs, enhanced *Myeloperoxidase* gene expression (*MPO*) in kidney at 35h after bacterial injection. Dietary M0.5%, G0.2%, G0.5%, W0.2% and W0.5% significantly upregulated *Cathelicidin* (*CATH1*) gene expression on D15 in kidney tissues. However, a significant decrease of the expression levels of *il-1β* of M0.5% D36 and G0.2% D39 in spleen, *tgf-1β of G0.2% in spleen on D39, il-10 of G0.2% in spleen on D39 and WT0.2% in spleen on D39, cd4-2β, mIgM* of G05%, WT0.2% in spleen on D15 and *Hepcidin* of M0.5% and WT0.5% in spleen on D15, G0.2% and WT0.2% in kidney on D36 was observed. Regarding disease resistance, low and high dose of GAS1-β-glucan (G0.2% and G0.5% diet) significant increased rainbow trout juvenile resistance to the infection of *Aeromonas salmonicida Achromogenes*. The cumulative survival after 14 days bacterial challenge was the highest for fish fed G0.2 (43%) in relation to a high humoral immune status and upregulation of the expression of *TLR_2*, *CATH1* and *MPO* immune genes in spleen and kidney on two days after bacterial injection (D39). Dietary supplemented at 0.2% of GAS1-β-glucans was more effective in stimulating the trout immune system than MacroGard and WT-β-glucan. Which induced a better humoral immune status in fish fed the low β-glucan dose (G0.2%) on D15 and D36 and highest levels of plasma lysozyme activity on D15, total plasma Ig content on D36. Further, dietary GAS1-β-glucan at low dose (G0.2%) significantly modulated the immune system of rainbow trout juvenile through the increase of humoral (*mIgM*) in kidney, membrane protein (*TLR_2*) both in spleen and kidney, neutrophils (*MPO*) in spleen, *CATH1* in spleen at 35h after bacterial injection (D39). Therefore, such better immune pathway responses could explain the better protection for trout juveniles by low dose of GAS1-β-glucan as shown by the significant increase of the survival rate after 14 days of bacterial challenge, when compared with control and other β-glucan doses.

Keywords: beta glucans, rainbow trout (*Oncorhynchus mykiss*), immunity, gene expressions, disease resistance.

1. Introduction

Diseases are major constraints to sustainable aquaculture production, especially for intensive aquaculture systems [1,2]. Indeed, fish might often be exposed to stressful conditions, eventually becoming more susceptible to microbial infections [3]. For the past few decades, the traditional strategy for fish disease control in pond cultures relied on the use of antibiotics and chemical disinfectants. However, application of antibiotics can lead to the development of antibiotic-resistant bacterial strains and cause many other problems such as environmental hazards, food safety problems and increasing resistance of pathogens. The use of antibiotics can also adversely affect the health status of the fish [4]. Further, sub-therapeutic doses of antibiotic have been often added to aquatic feeds to promote growth, and this has further contributed to drug resistance [5]. Therefore, the prophylactic use of antibiotic treatments in aquaculture has resulted in a very limited use or a ban of most compounds in top aquaculture production countries in Europe, and stringent regulations on the application of antibiotics in the United States [6]. This situation and recent restrictions on the use of antibiotics have promoted the use of prebiotics and probiotics (or a combination of both named synbiotics) as significant alternatives to antibiotics and there is an increasing interest in aquaculture in using these additives to prevent and/or control fish diseases [7–9].

β-glucan is a heterogeneous group of glucose polysaccharides consisting of a backbone of β (1,3)-link β-D-glucopyranosyl units with β -(1,6)-link side chains of varying length and distribution [10]. Among immune-stimulant agents, β-glucans are the most important compounds used in fish culture for the defence of fish against pathogens [11,12]. β-glucans are a major structural component of fungi, bacteria, plants, algae, yeast, and mushroom cell walls. One of the most common sources of β-glucans is derived from the cell wall of baker's yeast *Saccharomyces cerevisiae*. Further, β-glucans are also extracted from the bran of oat, barley, rye, wheat grains and several species of seaweed [13,14]. It has been reported that β-glucans bind to specific cell surface receptors of macrophages and neutrophilic granulocytes that promote the enhancement of an organism's protective activity against infection through the activation of leukocytes, phagocytic activity, inflammatory cytokines, chemokines, reactive oxygen free radicals, increase the activity of antioxidant enzymes, and initiate the development of adaptive immunity and elimination and killing of microorganisms [15].

Numerous immunostimulant effects of β-glucans have been reported in several fish species, for instance: against pathogen of Asian catfish (*Clarias batrachus*) [16, 17], common carp (*Cyprinus carpio*) [18], catla (*Catla catla*) [19], Nile tilapia (*Oreochromis niloticus*) [20], rohu (*Labeo rohita*) [21], and zebrafish (*Danio rerio*) [10] when infected with *Aeromonas hydrophila*, grass carp (*Ctenopharyngodon idella*) [22] infected with grass carp haemorrhage virus, large yellow croaker (*Pseudosciaena crocea*) infected with *Vibrio harveyi* [23]; and rohu infected with *Edwardsiella tarda* [24]. It has been reported that dietary β-glucans increased antibody titre production of catla-injected with 0.1mL mushroom glucan solution of 100µg mL^{-1}/fish of 30g, carp-injected 100-1000µg/fish of 25-30g [19,25]; stimulated an increase of lysozyme activity [23,26,27], macrophage bactericidal activity [10,25], superoxide anion production [16,17,22,25,27]. Moreover, dietary β-glucans increased the number myelomonocytic cell population in head kidney of zebrafish at 6h post challenge with *A. hydrophila* [10], increased the proportion of neutrophil and monocyte cells of carp-injected with 100, 500 and 1000 µg of glucan/fish [25]. However, the effects of β-glucans on fish immune system functions can be variable depending on the frequency, location and length of the side-chains, which may play a role in immunomodulation. Differences in molecular weight, shape and structure of β-glucans indicate the differences in biological activity [38], among others, fish species and administration route. For instance, diets containing β-glucans

administrated to channel catfish (*Ictalurus punctatus*) for 4 weeks did not significantly affect several immune parameters such as plasma lysozyme, bactericidal and haemolytic complement activities, respiratory burst of phagocytes and the number of lymphocytes found, survival rate in fish infected with *Edwardsiella ictaluri* [28]. Dietary β-glucans did not affect growth performance of European sea bass (*Dicentrarchus labrax*) [29]. It appears that the immune-stimulating effects of β-glucans on the immune system of fish are universal [30].

The potential immunomodulatory of β-glucans in rainbow trout has been reported in several publications. Djordjevic et al. [31] showed that dietary β-1,3/1,6-glucans (level 0.2% and 0.4% diets for 37 days) decreased the expression of genes involved in acute inflammatory reactions to the inflammatory agent while major parts of the immune response remained un-changed. Lauridsen et al. [32] reported that dietary β-1,3/1,6-glucans (0.2% diets, for 46 days) fed rainbow trout increased resistance against challenge with *Ichthyophthirius multifiliis* (white spot disease). Dietary administration of yeast cells (*Saccharomyces cerevisiae*) for 30 days (5×10^7 CFU yeast cells/g) treated with beta-mercaptoethanol significantly promoted the growth performance, enhanced the resistance against *Yersinia ruckeri* [33]. Moreover, Skov et al. [34] reported that dietary β-1,3-glucans from *Euglena gracilis* at (1% diets, 1% biomass day^{-1} for 84 consecutive days) stimulated down-regulation of expression of pro-inflammatory genes whilst, no effect of β-1,3-glucans diets on survival after *Yersinia ruckeri* challenge. Schemitt et al. [35] reported that dietary β-glucans fed rainbow trout (at level 0.3% diets, 1% biomass day^{-1}, for 4 weeks) increased gene expression of *cathelicidins 2* and *il-1β* in gut epithelial cells and the number of mucus secreting cells in the intestine. The study of Ghaedi et al. [36] concluded that brood fish fed the diet with 0.2% β-glucans exhibited the highest white blood cell value, ACH_{50}, total Ig and IgM levels and lysozyme activity. However, no significant differences in survival rates (from fertilization to swim-up stage) were found among dietary treatments. Ji et al. [37] showed that dietary β-glucans (at 0.2% diets) improved specific growth rate, weight gain, feed efficiency and survival rate after challenge with *A. salmonicida*. Recently, Douxfils et al. [38] found that overdoses of β-glucans and/or prolonged medication can lead to a non-reactive physiological status and, consequently, to a poor immune response. Beta glucan is a heterogeneous group of glucose polysaccharides consisting of a backbone of β (1, 3)-link β -D-glucopyranosyl units with β-(1, 6)-link side chains of varying length and distribution [27], but their efficiency may vary according to their source origin. The most common forms of β-glucans are those comprising D-glucose units with β-1, 3 links. But, yeast and fungal β-glucans contain 1–6 side branches, while cereal β-glucans contain only β-1, 3 and β-1, 4 backbone bonds [37]. The frequency, location, and length of the side-chains may play a role in immunomodulation. Differences in molecular weight, shape, and structure of β-glucans indicate the differences in biological activity [38].

In this study, we aimed to conduct a comprehensive evaluation of short- and long-term (D15 and D36) and dose effects (low dose at 0.2% diet *vs* high dose at 0.5% diet) of oral administration of different types of beta-glucan to compare the effects between the commercial Macrogard and pure β-glucans (GAS1 and WT- β-glucan) extracted by laboratory of Aquaculture and Artemia Reference Centre (ARC, UGent) on the overall immune functions of trout juveniles analysing blood leukocyte cells, humoral immune parameters and immune related gene expression in relevant immune organs, such as the spleen and kidneys. Moreover, the disease resistance of trout juveniles was tested by applying a bacterial challenge test based on the intraperitoneal injection of *Aeromonas salmonicida achromogenes*.

2. Materials and methods

Experimental fish

Feeding trial and bacterial challenge were carried out in agreement with the European and Belgian national legislation on animal welfare (Protocol number: 13197 KE). Rainbow trout juveniles (n = 315) were transported from a local fish farm (Hatrival, Belgium) to UNamur facilities and distributed into 9 fibreglass tanks of 100L (35 fish/100L/tank) in a recirculation system. Fish were allowed to acclimate to the new environment for 21 days.

Table 5.1: Ingredients and proximate composition of the experimental pelleted diet

Ingredients	Diet (g/kg)	Diet (%)	Crude protein (%)	Crude fat (%)	Crude protein in diet	Crude fat in diet
Cod fish meal[a]	350.0	35.00	89.0	4	31.15	1.40
Blood meal[b]	70.0	7.00	87.6	0	6.13	0.00
Wheat gluten[c]	134.0	13.40	80.0	6	10.72	0.80
Cod fish oil[d]	128.0	12.80		100		12.80
Starch[e]	223.6	22.36				
Carboxylmethylcellulose[e]	20.0	2.00				
α- cellulose[e]	42.4	4.24				
Mineral mix[f]	10.0	1.00				
Vitamin mix[g]	10.0	1.00				
Betaine[e]	10.0	1.00				
BHA	1.0	0.10				
BHT	1.0	0.10				
Total CP diet %					48.00	
Total CF diet %						15.00

BHA: butylated hydroxyanisole; BHT: butylated hydroxyl toluene. [a] Cod fish meal provided by SNICK euroingredient NV, Ruddervoorde (Belgium). [b]Actipro Hemoglobin, Zwevezele (Belgium). [c] Roquette Freres, Lestrem (France). [d] Sigma-Aldrich, Saint-Louis, MO, (USA). [e]Mosselman SA, Chlin (Belgium). [f] Mineral mix (g kg^{-1} of mix) was prepared in the lab, from $(CaHPO_4)2H_2O$, 727.77; $(MgSO_4)7H_2O$, 127.50; NaCl, 60.00; KCl, 50.00; $(FeSO_4)7H_2O$, 25.00; $(ZnSO_4)7H_2O$, 5.50; $(MnSO_4)4H_2O$, 2.54; $(CuSO_4)5H_2O$, 0.78; $(CoSO_4)7H_2O$, 0.48; $(CaIO_3)6H_2O$, 0.29; $(CrCl_3)6H_2O$, 0.13 g. [g]Vitamin mix was provided by INVE Aquaculture Company.

During this period, water temperature was maintained at 13.9 ± 1.2°C by a cooling system, oxygen level averaged 11.6 ± 0.7 mg/L (aeration applied), constant photoperiod (Light : Dark ratio = 12:12) and fish were fed 1.5% of fish biomass, twice daily (at 9:00 am and 5:00 pm) with a specific trout diet (Coppens TROCO SUPREME-16, The Netherlands, crude protein = 48%, crude fat = 15%).

Fish diet and experimental design

After acclimation, fish (mean body weight: 44.5 ± 3.0 g) were fed either a control diet (no β-glucans) or diets containing three types of β-glucans: a commercial mixture of β-glucans, MO (Macrogard-Biorigin-Brasil), and two yeast β-glucan products, namely GAS1 GAS1-β-glucans (GAS1) and Wild type-β-glucans (WT) extracted by the Laboratory of Aquaculture & Artemia Reference Center (ARC) of Ghent University, Belgium). For each type of β-glucans, two doses were tested (0.2% or 0.5% of the diet) for 36 days at 1% of fish biomass/day. All diets were formulated and pelleted in the laboratory of the University of Namur (Table 5.1). Three replicate tanks were used for each experimental regime. After 15 days (short-term nutrient test, D15) and 36 days (long-term nutrient test, D36) of feeding, six fish per tank (18 fish per experimental diet) were anesthetized in ethyl 3-aminobenzoate methane sulfonic acid salt (98% purity, MS-222, Sigma) solution (120 mg/L). Blood was obtained by caudal vein puncture using a heparinized syringe and stored on ice in heparinized tubes. Fish were then euthanized by overdose of MS-222 (240 mg/L) before decapitation. Spleen and whole kidney were dissected, a part of spleen was immediately homogenized after dissection to prepare for spleen respiratory burst activity analysis. The remaining spleen and kidney samples were immediately snap-frozen in liquid nitrogen and finally stored at -80°C until analysis (RT-qPCR immune-related genes). Heparinized blood was immediately analysed for leukocyte populations by flow cytometry, and the remaining volume of blood was then centrifuged at 7500 × *g* for 10 min to collect plasma stored at -80°C until subsequent analysis (lysozyme activity, alternative complement activity, total immunoglobulin content).

Bacterial challenge

In order to evaluate whether β-glucan has a beneficial effect on disease resistance, rainbow trout juveniles were experimentally infected with a virulent strain of *Aeromonas salmonicida achromogenes* provided by the CER group (Centre d'Economie Rurale, Laboratoire de Pathologie des Poissons, Belgium). Bacteria were cultured in sterile Brain Heart Infusion (BHI, Sigma Aldrich, Saint-Louis, MO, USA) and incubated at 28°C for 24h. A preliminary test infection including various bacterial doses was performed to determine the LD50 CFU of the targeted rainbow trout population (LD50 = 3.1×10^7 CFU/100g fish body weight).

On day-52 of treatment, a total of 30 fish from each dietary condition (10 fish × 3 replicate tanks) were anesthetized. Then the fish were intraperitoneally injected with a weight-adjusted dose (3.1×10^7 CFU/100 g of fish body weight) of the freshly prepared *A. salmonicida achromogenes* culture and equally distributed into three 50L-tanks. The fish were confined at the animal facility (Biosafety level 2) along the infection assay. They were starved one day ahead of infection as well as the day of bacterial injection, and then fed the respective experimental diets until the end of the challenge test. At 35h post-injection (D54), a total of 9 fish from each dietary condition (3 fish × 3 replicate tanks) were anesthetized and blood was sampled for subsequent immunological assays (lysozyme and alternative complement pathway activity). Levels of plasma total Ig content and spleen RBA were not determined because of limitations in this experiment. Fish were then euthanized, and spleen and kidney were sampled and immediately snap-frozen until immune gene expression analysis (RT-qPCR).

Blood leukocyte populations

Blood cell populations were analysed at D15 and D36 of dietary treatment by flow cytometry (Flow Activated Cell Sorter Calibur, Flow Cytometry System) according to Inoue et al. [39], later adapted by Mathieu et al. [40]. Briefly, 10 µl of fresh heparinized blood were mixed with 1950 µl of Hanks Balanced Salt Solution (HBSS, Sigma) and 40 µl of fluorochrome DiOC6 (3,3-dihexyloxacarbocyanine, Molecular Probes, Eugene) diluted 1:10 in ethanol. The tube

was mixed gently and incubated at room temperature (RT) for 10 min. The FACS was calibrated with True Count Beads diluted in HBSS, Sigma-Aldrich, Steinheim, Germany). Each blood cell population was identified by its typical location in an FL-1 v. SSC and FSC v. SSC according to Inoue et al. [39] and Pierrard et al. [41]. Four clusters were identified, thrombocyte and lymphocyte cells were gathered in the same cluster according to Pierrard et al. [41].

Plasma lysozyme activity

Lysozyme activity assay was performed by the turbidimetric method of Siwicki and Studnicka [42], later adapted by Mathieu et al. [43]. Briefly, 7 µL of plasma were added to 130 µL of freshly prepared *Micrococcus luteus* (Sigma-Aldrich, Saint-Louis, USA) solution (0.6 mg/mL of Na_2HPO_4 0.05 M, pH 6.2) in triplicate. Absorbance corresponding to *Micrococcus luteus* lysis was measured at 450 nm for 60 min at regular intervals (5 min). One unit (U) of lysozyme was determined as an absorbance decrease of 0.001 per min.

Plasma haemolytic activity of alternative complement activity

Plasma haemolytic activity of alternative complement activity (ACH50) was assayed following Sunyer and Tort [44], later modified by Milla et al. [45]. Briefly, 10 µL of rabbit red blood cell suspension (RRBC, Biomerieux, Mary-I'Etoile, France) suspended at 3% in veronal buffer was mixed with serial dilutions of plasma (70 µL of total volume). Haemolysis (100%) was obtained by adding 60 µL of distillate water to 10 µL of RRBC. Negative control (fresh water) was obtained by adding 60 µL of veronal buffer to 10 µL of RRBC. Samples were incubated 100 min at 27°C and centrifuged (3000×g, 5 min, 4°C). Then, 35 µL of supernatant was transferred to a new microplate in order to measure the absorbance at 405 nm. The ACH50 value was defined as the reciprocal of the plasma dilution that induced the haemolysis of 50% RRBC.

Plasma total immunoglobulin assay

Analysis of total plasma immunoglobulin content (Ig) was based on a spectrophotometric technique described by Siwicki and Anderson and later adapted by Milla et al. [45] with some modifications. Immunoglobulin was precipitated using 10,000 kDa polyethylene glycol (PEG, Sigma). Plasma was mixed with an equal volume of 12% PEG solution and shake 150 rpm for 2h at room temperature. After centrifugation at 1000×g for 10 min, the supernatant was collected and assayed for its protein concentration by the method of Bradford [46]. Plasma total immunoglobulin content was calculated by subtracting this value from the total protein concentration in the plasma before precipitation with PEG.

Spleen leukocyte respiratory burst activity (RBA)

Just after collection, spleen samples were conditioned in L15 medium (Sigma-Aldrich) at 4°C, and gently pressed through sterilised nylon mesh (40 µm, Dutscher) to obtain leukocyte suspensions. Then, the L15 medium-diluted samples were loaded onto Ficoll gradient (Healthcare, GE). After centrifugation (2500 rpm, 20 min, at 4°C), leukocyte suspensions were collected and washed twice in L15 medium and again centrifuged (1200 rpm, 5 min, 4°C). They were re-suspended in L15 medium and viable leukocytes were adjusted at 10^6 cells/mL before classification of leukocyte populations (lymphocyte, macrophage and granulocyte) by flow cytometry. RBA analysis was performed using the flow cytometry method as previously described by Chilmonczyk and Monge [47] with some modifications of Jolly et al. [48]. The RBA test corresponded to an evaluation of intracellular hydrogen

peroxide production following cell activation or not with phorbol 12-myristate 13-acetate (PMA). The fluorescence levels of unstimulated and PMA-stimulated cells were determined after 30 min of cell incubation (18°C in the dark) with 2′-7′dichlorofluorescin diacetate (DCFH-DA (5 µM) and DCFH-DA plus PMA (2 $\mu g.mL^{-1}$), respectively. The spleen respiratory burst activities were expressed in a stimulation index as the ratio between the mean fluorescence measured in stimulated cells (DCFH-DA + PMA) and the basal mean fluorescence of control (DCFH-DA only).

RNA precipitation and complementary DNA (cDNA) synthesis

Three pools of each experimental condition and time-point were collected to compare their gene expression profiles. Total RNA was extracted individually from the spleen and kidney using TriReagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. The pellet was dried and resuspended in 200 µL of RNase-free water. Total RNA concentration was determined by Nano Drop-2000 spectrophotometer (Thermo Scientific) and the integrity was checked by Experion RNA Std Sens analysis (Bio-Rad Laboratories, Hercules, CA). Total RNA (1µg) was used to synthesise cDNA with iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions.

Gene expression analysis

Real-time PCR assay was carried out in order to analyse the expression pattern of different genes of immunological relevance in spleen and kidney from rainbow trout stimulated with β-glucan. Samples were taken randomly from three fish of three different tanks for each treatment (n = 9) at D15 and D36 days of feeding with different doses and types of β-glucan. Samples of three randomly selected fish from each tank were pooled for each treatment (n = 3 fish per each pool). On day 37 of feeding, fish were challenged against *A. salmonicida achromogenes* and 35h post-challenge (D39 of treatment) three fish of three different tanks for each treatment (n = 9 fish) were also collected. Samples were also taken using the same strategy from fish fed with basal diet and from non-infected fish (n = 3 fish per each pool). As a housekeeping gene, elongation factor 1 alpha (*ef-1α*) was amplified from all the evaluated samples. The gene expression of pro-inflammatory (*il-1*), macrophages (*mcsfra*), antibacterial (*Lysozyme, Hepcidin*), humoral (*mIgM*), membrane protein (TLR_2), neutrophils (*mpo*), Cathelicidin (*CATH1*), T-helper (*cd4-2β*), and anti-inflammatory responses (*il-10*, *tgf-β1*) were evaluated. The list of specific primers used for gene expression analysis is given in Table 2. Real-time PCR reactions were carried out with iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories) using a 1:40 dilution of the cDNA for target genes or 1:1000 dilutions for *ef-1α*.

Table 2: Primers used for each gene expression analysis by real-time RT

Genes	Primer sequence (5'-3')	Accession number	Amplicon size
ef-1α	Fw: 5'-ATGCCCCCAAGTTCCTGAAG-3' Rv: 5'-AACAGCAACAGTCTGCCTCA-3'	NM_001124339.1	140
il-1	Fw: 5'-TGAGAACAAGTGCTGGGTCC-3' Rv: 5'-GGCTACAGGTCTGGCTTCAG-3'	NM_001124347.2	148
lysozyme	Fw: 5'-TGCCTGTCAAAATGGGAGTC-3' Rv: 5'-CAGCGGATACCACAGACGTT-3'	NM_001124716.1	152
mIgM	Fw: 5'-AAAGCCTACAAGAGGGAGACCGAT-3' Rv: 5'-AGAGTTATGAGGAAGAGTATGATGAAGGTG-3'	X65263.1	128
mcsf-ra	Fw: 5'-ATCTCCACTCATGGCGACACA-3' Rv: 5'-CATCGCACTGGGTTTCTGGTA-3'	AB091826	177
mpo	Fw: 5'-GCAGAGTCACCAATGACACCA-3' Rv: 5'-ATCCACACGGGCATCACCTG-3'	GBTD01119227	68
il-10	Fw: 5'-CCGCCATGAACAACAGAACA-3' Rv: 5'-TCCTGCATTGGACGATCTCT-3'	NM_001245099.1	105
tgf-β1	Fw: 5'-GCCAAGGAGGTCCACAAGTT-3' Rv: 5'-GTGGTTTTGATGAGCAGGCG-3'	NM_001281366.1	146
cd4-2β	Fw: 5'-AAGCCCCTCTTGCCGAGGAA-3' Rv: 5'-CTCAACGCCTTTGGTACAGTGA-3'	AY899932	108
vapA	Fw: 5'-ATTAGCCCGAACGACAACAC-3' Rv: 5'-CCAACACAATGAAACCGTTG-3'	KP184543.1	148

Primers for target genes were used at 500 nM. The thermal conditions used were 3 min at 95ºC of pre-incubation, followed by 40 cycles at 95ºC for 30 s and 60ºC for 30 s. All reactions were performed using ABIprism 7300 (Applied Biosystem) and quantification was done according to the Pfaffl method [49] corrected for efficiency of each primer set. Values for each sample were expressed as normalized relative expression (NRE), calculated in relation to values of the control group and normalized against those of the housekeeping gene *ef-1α*. The results are expressed as an average of values obtained in all pools from D15 and D36 of feeding, and D39 (after bacterial challenge test).

Statistical analysis

Results are presented as mean ± SD. Data were checked for normal distribution and homogeneity of variances by Univariate tests. Humoral immune parameters, blood leukocyte proportions, specific growth rate, mortality, splenic index, haematocrits, and immune gene expressions on D15 and D36 were carried on using a two-way ANOVA test. One-way ANOVA was used to test results of D39 and data of cumulative mortality over the 14 days of the bacterial challenge test. In all statistical analysis tests used, $p < 0.05$ was considered statistically significant. The statistical analysis was performed using the Statistica bio-software (version 10.0).

3. Results

3.1. Specific growth rate (SGR), mortality and splenic index (SI)

Fish fed different β-glucan types showed no significant differences of SGR in comparison with the control diet either on day 15 (D15) or day 36 (D36) (Table 3). Mortality rate did not differ between controls and β-glucan treatments (Table 3). Furthermore, no significant changes in SI values were observed on D15 and D36 in all β-glucan diets compared to control diet (Table 3).

Table 3: Mean (±SD) values for specific growth rate (SGR), mortality and splenic index (SI) of trout juveniles fed with low (0.2 %) and high (0.5 %) doses of different beta glucan types on day 15 and day 36 of the feeding test. Statistical differences between dietary treatments are indicated by different lower case letters ($p < 0.05$).

Times	Variables	Control	Macrogard beta glucan		GAS1 beta glucan		Wild type beta glucan		$F_{-values}$	$P_{-values}$
			M0.2%	M0.5%	G0.2%	G0.5%	W0.2%	W0.5%		
D15	SGR (%/day)	1.53 ± 0.33	1.46 ± 0.26	1.72 ± 0.32	1.56 ± 0.32	1.32 ± 0.37	1.80 ± 0.01	1.60 ± 0.18	1.497	0.254
	Mortality (%)	3.8 ± 6.6	4.8 ± 8.2	2.9 ± 4.9	4.8 ± 5.9	1.0 ± 1.6	4.8 ± 5.9	1.0 ± 1.6	0.233	0.958
	Splenic index (%)	0.11± 0.02	0.11 ± 0.02	0.13 ± 0.04	0.12 ± 0.03	0.12 ± 0.02	0.11 ± 0.01	0.11 ± 0.03	0.214	0.966
D36	SGR (%/day)	1.58 ± 0.06	1.54 ± 0.10	1.73 ± 0.35	1.51 ± 0.08	1.75 ± 0.34	1.36 ± 0.34	1.54 ± 0.06	0.224	0.965
	Mortality (%)	3.3 ± 5.8	0.0 ± 0.0	1.1 ± 1.9	1.3 ± 2.2	1.1 ± 1.9	3.6 ± 3.5	1.1 ± 2.0	0.586	0.736
	Splenic index (%)	0.16 ± 0.03	0.16 ± 0.04	0.18 ± 0.03	0.18 ± 0.02	0.17 ± 0.01	0.16 ± 0.04	0.17 ± 0.00	0.438	0.841

3.2. Humoral immune variables

On D15, a high dose of Macrogard (M0.5%) significantly increased alternative complement pathway (ACH50) levels ($F = 5.114$, $p = 0.000117$) in comparison with control diet. On D36, a high dose of wild type β-glucan (W0.5%) significantly stimulated ACH50 level in comparison with all other diets (accept for M0.2%) while low dose of GAS1 β-glucan (G0.2%) significantly reduced it compared to all other diets (except for G0.5%) ($F = 5.114$, $p = 0.000117$). However, two days after bacterial injection (D39), there was no significant difference of plasma ACH50 level between treatments and control groups (Figure 5.1: A).

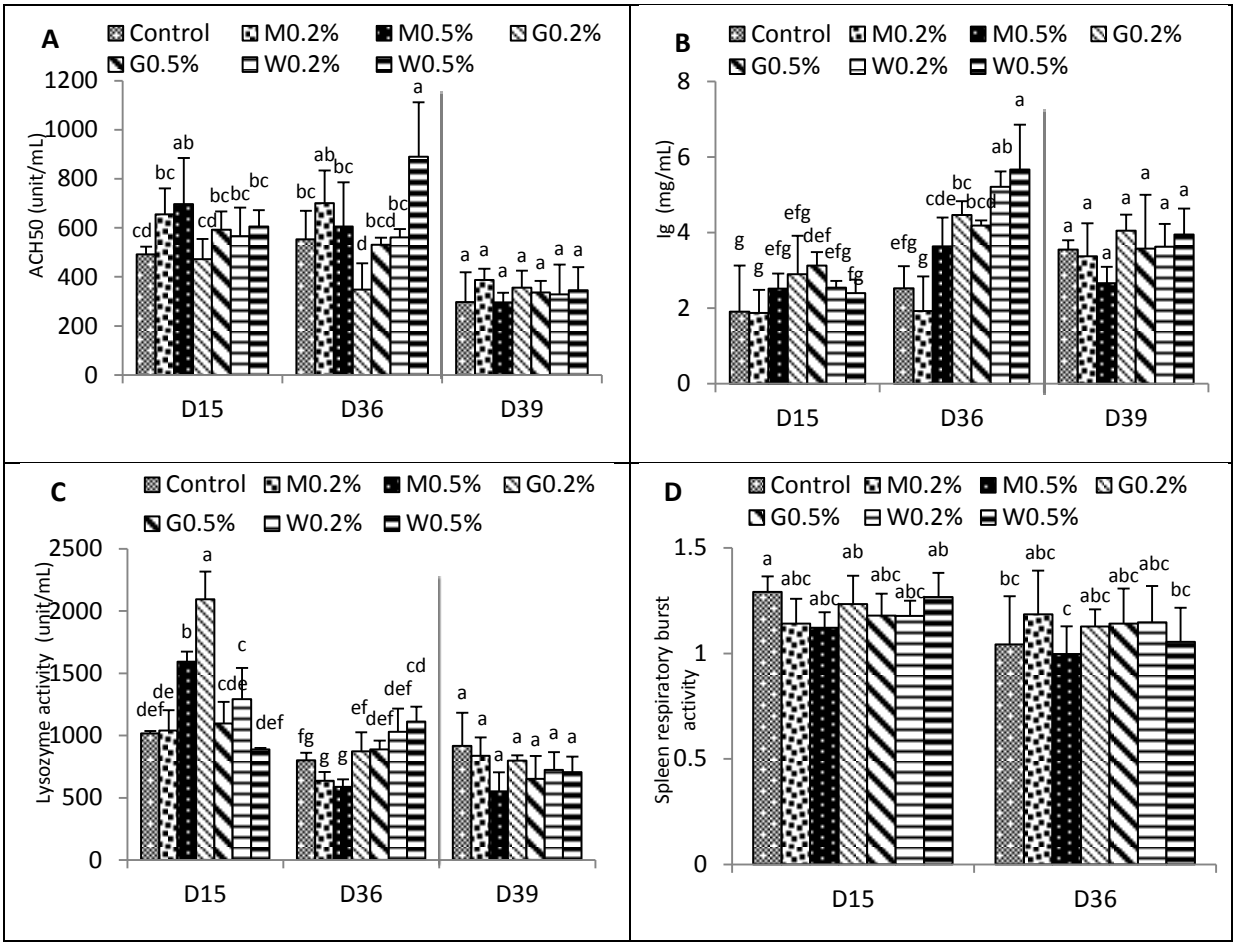


Figure 5.1 (A–D): Effects of dietary beta glucan administration on humoral parameters of fish sampled during the nutrient test on D15 and D36, or two days after bacterial infection (D39). (A): Plasma haemolytic alternative complement activity, (B): Plasma total immunoglobulin's content, (C): Plasma lysozyme activity, (D): Respiratory burst activity (RBA) of spleen macrophage cells. Values are expressed as mean ± SD of 18 fish/diet/day on D15, D36 and 9 fish/diet on D39. For spleen respiratory burst activity (RBA), Plasma haemolytic alternative complement activity (ACH50) data are expressed as mean ± SD of 9 fish/diet/day on D15, D36. Statistical differences between dietary treatments and treatment times are indicated by different letters with $p < 0.05$.

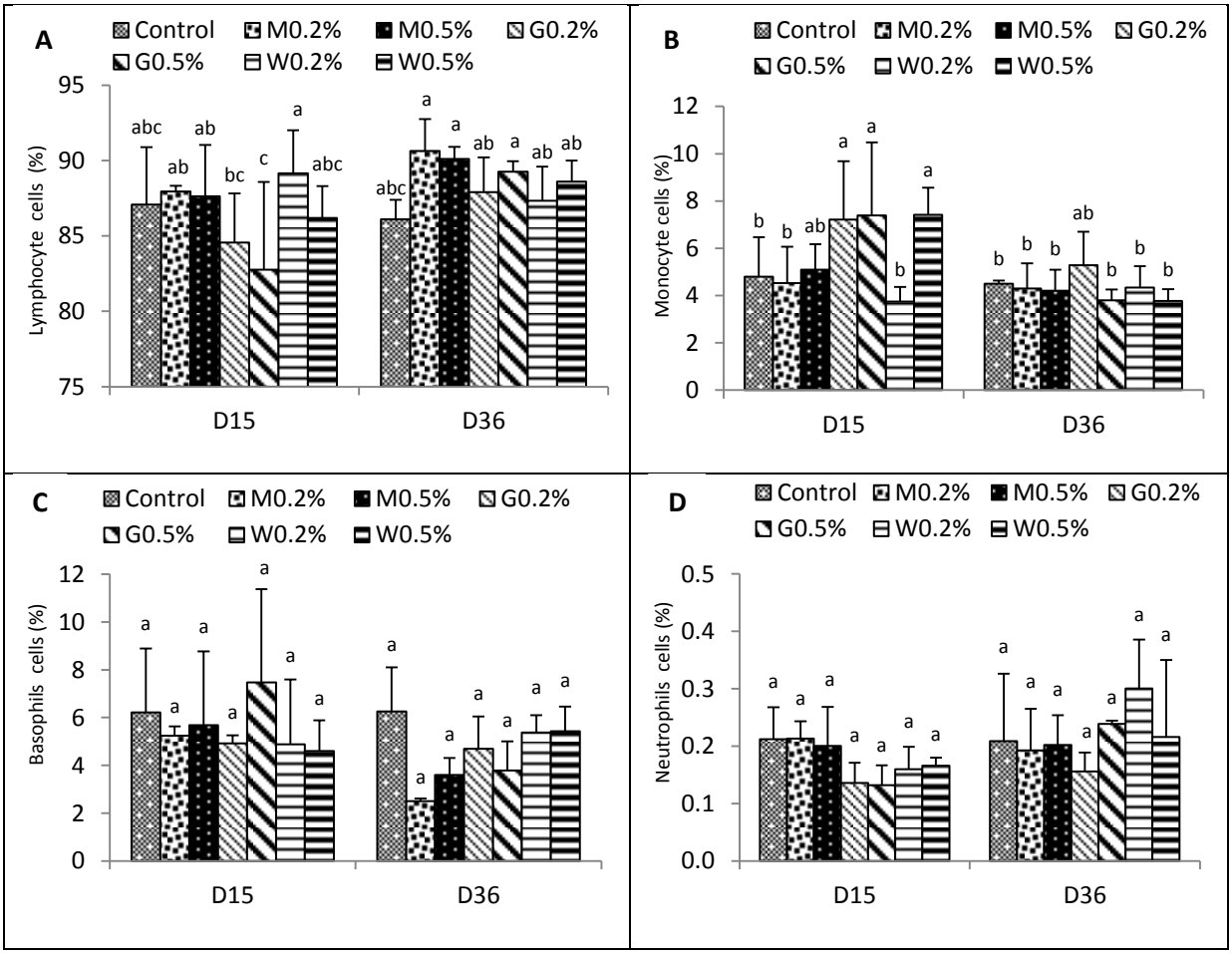
The diet supplemented with GAS1 0.5% increased the amount of total Ig in comparison with the control and M0.2% on D15. On D36, both low and high dose of GAS1 and WT-β-glucan doses significantly elevated the levels of plasma total Ig ($F = 8.781$, $p = 0.00002$) compared to the control diet (Figure 5.1: B).

Concerning lysozyme parameters, on D15, the highest dose M0.5% significantly improved its activity compared to the control diet and M0.2%. On the contrary, for GAS1 and Wild type β-glucan, the lowest doses (*i.e*., G0.2% and W0.2%) were best to improve lysozyme activity in comparison with the control ($F = 14.12$, $p = 0.0000024$) and their respective high dose (Figure 5.1: C). At D36, only W0.5% succeeded in significantly increasing lysozyme activity compared to the control.

Finally, levels of respiratory burst activity (RBA) of spleen lymphocytes (as for macrophage cells) did not significantly differ whatever the β-glucan administration doses and types on D15 and D36 (Figure 5.1: D).

3.3. Blood leukocyte cell proportions

Blood leukocytes were composed of high percentages of lymphocytes followed by those of neutrophils, monocytes and basophils (Figure 5.2: A-D). Lymphocyte proportion was significantly higher in W0.2% in comparison with both doses of GAS1 β-glucan, meanwhile dietary G0.2%, G0.5% and W0.5% doses significantly increased ($F = 10.31$, $p = 0.0033$) the proportion of blood monocyte cells on D15. However, there were no significant differences of basophil and neutrophil cell proportions in total blood leukocyte cells between the treatment and control groups.



Figures 5.2 (A-D): Effects of dietary beta glucan administration on blood leukocyte cell proportions of fish sampled during the nutrient test on D15 and D36. (A): Lymphocyte + thrombocyte cells proportions, (B): Monocyte cells proportion, (C): Neutrophil cells

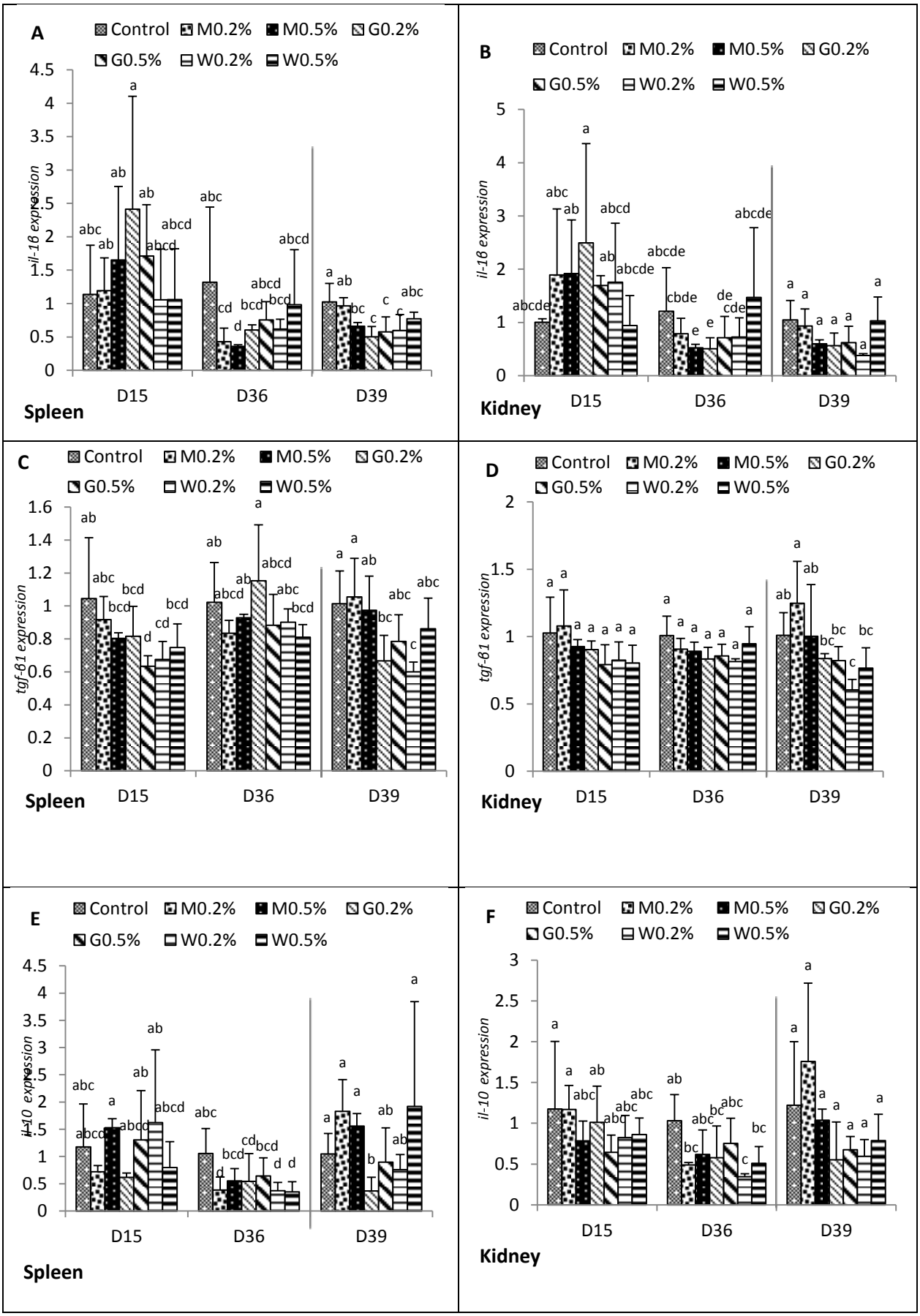
proportion and (D): Basophil cells proportion in total blood leukocyte cells population. Values are expressed as mean ± SD of 18 fish/diet/day on D15, D36. Statistical differences between dietary treatments and treatment times are indicated by different lower case letters with $p < 0.05$.

3.4. Immune gene expression

3.4.1. Pro- and anti-inflammatory gene expressions

Expression levels of both pro-inflammatory (*il-1β, mcsfra*) and anti-inflammatory (*tgf-β1, il-10*) genes were evaluated in spleen and kidney organs of fish sampled after short-term (D15) or long-term β-glucan administration, also the expression of some immune genes was recorded on D39 - after bacterial challenge (Figure 5.3). In the spleen, a high dose M0.5% induced a significant down regulation of *il-1β* gene expression levels on D36 compared to the control group ($F = 13.83$, $p = 0.000886$, Figure 3: A). A high dose G0.5% and a low dose W0.2% significantly decreased ($F = 6.933$, $p = 0.013$, Figure 5.3: C) *tgf-β1* gene expression on D15 in comparison with the control. Furthermore, dietary M0.2%, W0.2% and W0.5% significantly decreased *il-10* gene expression on D36 ($F = 15.34$, $p = 0.000525$; Figure 5.3: E). On D39, after bacterial challenged, M0.5%, G0.2%, G0.5% and W0.2% diets significantly reduced *il-1β* gene expression compared to the control ($F = 3.561$, $p = 0.0235$; Figure 5.3: A). Meanwhile, G0.2% and W0.2% diets down-regulated *tgf-β1*gene expression ($F = 2.877$, $p = 0.0484$; Figure 5.3: C) in comparison with the control group and G0.2% significantly decreased *il-10* gene expression ($F = 2.841$, $p = 0.050$; Figure 5.3: E).

In the kidney, only W0.2% diet significantly reduced *il-10* gene expression on D36 ($F = 8.359$, $p = 0.0073$; Figure 5.3: F). After bacterial challenge, only W0.2% diet significantly repressed *tgf-β1*gene expression compared to the control ($F = 2.897$, $p = 0.0473$; Figure 5.3: D) meanwhile, there was no change of *il-1β and il-10* gene expression of all type β-glucan diets after bacteria injected on D36 (Figure 5.3: B and F). There was no change of *mcsfra* gene expression in both spleen and kidney organs on D15, D36 and D39 after β-glucan treatments.

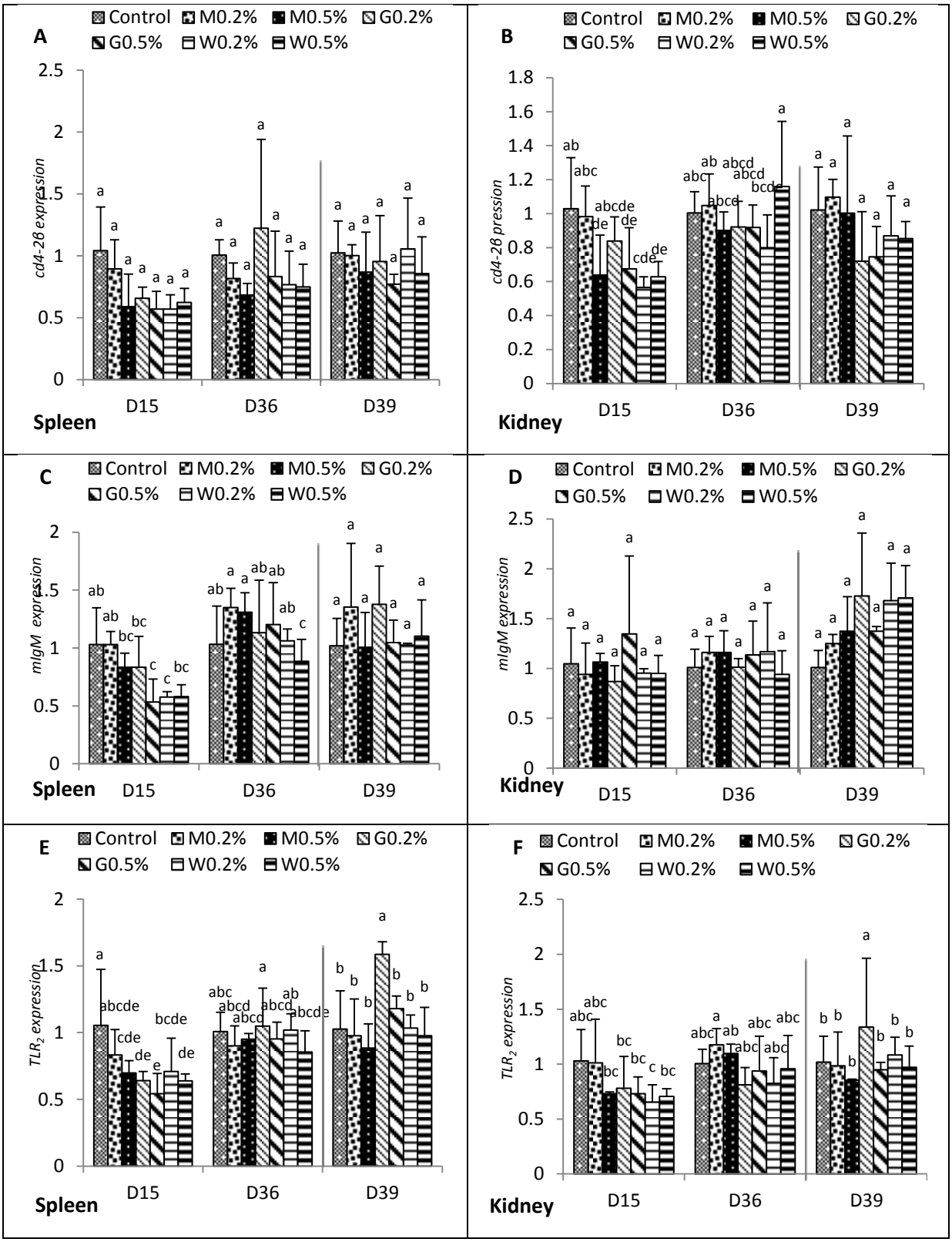


Figures 5.3 (A-H): Effects of dietary β-glucan on pro- inflammatory gene expressions in spleen and kidney of fish sampled during the nutrient test on D15 and D36, or two days after

the bacterial challenge (D39). A, B, C, D, E, and F are expression levels of *il-1β*, *tgf-β1,* and *il-10* in spleen and kidney, respectively. Relative transcript (mRNA) levels were determined by real-time RT-PCR and normalized by the arithmetic mean of *ef-1α* expression. Values are expressed as mean ± SD of 3 pool samples per experimental condition (3 fish per pool sample). Statistical differences between dietary treatments and treatment times are indicated by different lower case letters $p < 0.05$.

3.4.2. Expression of T-helper, mIgM, complements C_3 and TLR_2 genes

The expression levels of T-helper genes (cd4-2β), humoral (mIgM), complement C3 and Toll like receptor (*TLR2*) genes were determined in spleen and kidney samples. Beta glucan diets did not display any change of complement C3 gene expression in spleen and kidney tissues after 15 and 36 days of β-glucan administration. In the spleen, G0.5% and W0.2% diets significantly reduced mIgM gene expression on D15, while only W0.5% diet significantly down regulated mIgM gene expression on D36 ($F = 2.638$, $p = 0.0372$; Figure 5.4: C).



Figures 5.4 (A-F): Effects of dietary beta glucan on T-helper (*cd4-2β*), humoral (*mIgM*) and membrane protein (TLR_2) genes expressions in the spleen and kidney of fish sampled during the nutrient test on D15 and D36, or two days after the bacterial challenge (D39). A, B, C, D, E, and F are expression levels *of cd4-2β*, *mIgM*, TLR_2 in spleen and kidney, respectively. Relative transcript (mRNA) levels were determined by real-time RT-PCR and normalized by the arithmetic mean of *ef-1α* expression. Values are expressed as mean ± SD of 3 pool

samples per experimental condition (3 fish per pool sample). Statistical differences between dietary treatments and treatment times are indicated by different lower case letters $p < 0.05$.

No changes were observed in fish sampled after bacteria injection (D39). For *TLR_2*, only the low dose (G0.2%) of GAS1 β-glucan significantly stimulated its gene expression in comparison with control and other β-glucan diets in spleen on D15 ($F = 4.377$, $p = 0.0107$; Figure 5.4: E). There was no change of *cd4-2β* gene expression in the spleen tissues after 15 days, 36 days and bacterial injection on D39.

In the kidneys, M0.5%, G0.5%, W0.2% and W0.5% diets repressed *cd4-2β* gene expression compared to the control on D15 ($F = 10.538$, $p = 0.00302$; Figure 5.4: B). In addition, after bacterial challenge, only the low dose (G0.2%) of GAS1 β enhanced *TLR_2* gene expression in comparison with the control and other β-glucan diets ($F = 3.761$, $p = 0.044$; Figure 5.4: F). There was no change of *mIgM* gene expression in the kidney tissues after 15 days, 36 days and bacterial injection on D39.

3.4.3. Antibacterial gene expressions

Expression of antibacterial genes (*C-type lysozyme, Hepcidin, Cathelicidin and Myeloperoxidase*) were evaluated in the kidney and spleen on D15, D36 and D39. In the spleen, at D15, the high dose M0.5% appeared to increase lysozyme expression compared to the low dose M0.2%. But, M0.5% diet induced a significant decrease of lysozyme gene expression on D36 compared those of D15 ($F = 5.487$, $p = 0.0264$; Figure 5.5a: A). A diet of G0.2%, G0.5%, W0.2% and W0.5% caused a significant raise of *Hepcidin* gene expression on D36 in comparison with the respective levels of those genes on D15 for the same diet ($F = 21.133$, $p = 0.000833$; Figure, 5.5a: C). Moreover, M0.5%, G0.2% and G0.5% repressed *Cathelicidin* gene expression on D36 in the comparison to those of D15 ($F = 18.886$, $p = 0.000165$; Figure 5.5b: A).

In the kidneys, a diet of M0.5%, G0.2%, G0.5% and W0.2% induced a decrease of lysozyme gene expression on D36 in comparison with those levels of D15 ($F = 25.346$, $p = 0.000252$; Figure 5.5a: B).

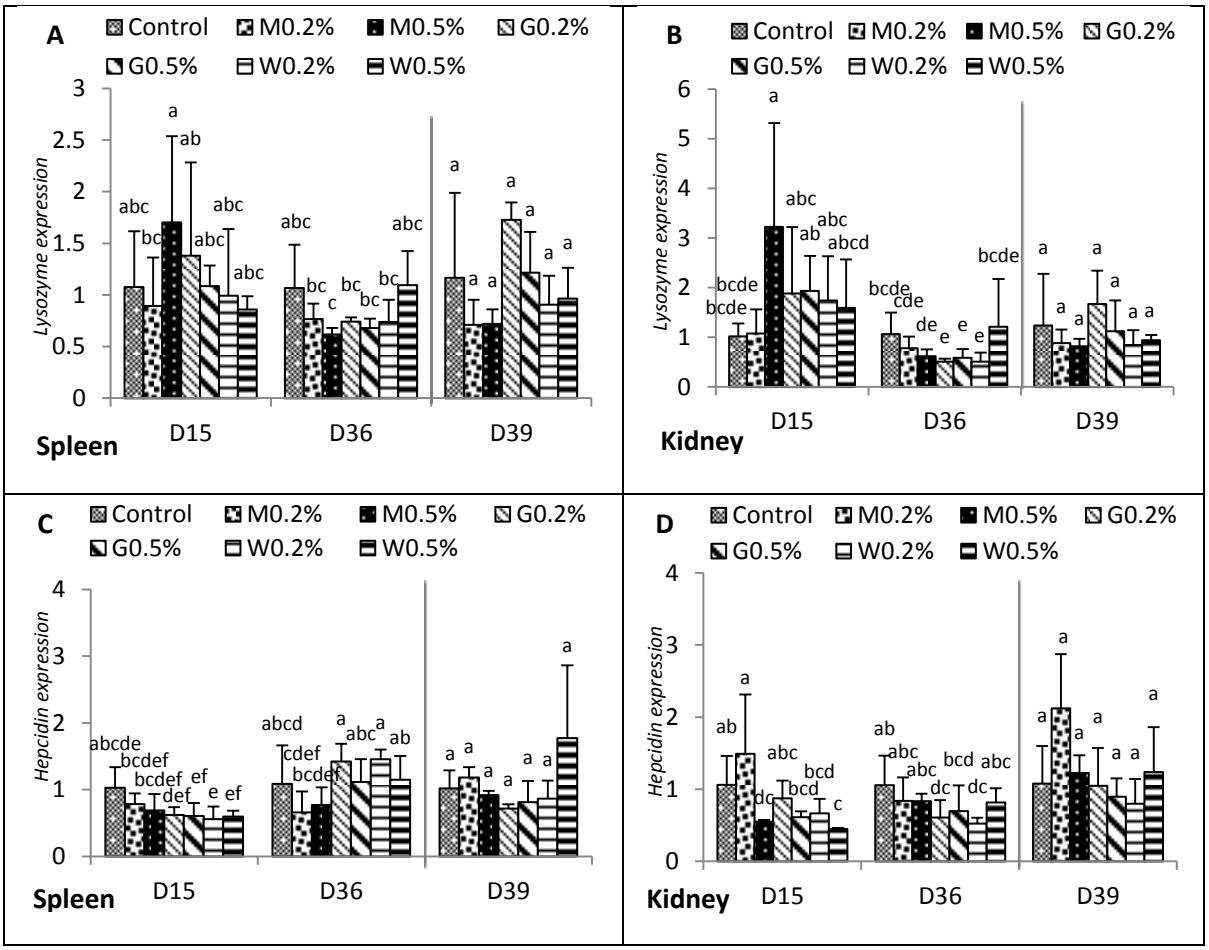


Figure 5.5a (A-D): Effects of dietary beta glucans on *C-type lysozyme* and *Hepcidin* genes expression in the spleen or kidney of fish sampled during the nutrient test on D15 and D36, or two days after the bacterial challenge (D39). (A): *lysozyme* gene expression in spleen, (B): *lysozyme* gene expression in kidney, (C): *Hepcidin* gene expression in spleen, (D): *Hepcidin* gene expression in kidney. Relative transcript (mRNA) levels were determined by real-time RT-PCR and normalized by the arithmetic mean of *ef-1α* gene expressions. Values are expressed as mean ± SD of 3 pool samples per experimental condition (3 fish per pool sample). Statistical differences between dietary treatments and treatment times are indicated by different lower case letters ($p < 0.05$).

Moreover, M0.5% and W0.5% diets down regulated *Hepcidin* gene expression on D15 compared to the control, while G0.2% and W0.2% also induced a significantly decreased of *Hepcidin* gene expression on D36 compared to the control ($F = 3.241$, $p = 0.0151$; Figure 5.5a: D). On the contrary, M0.5%, G0.2%, G0.5%, W0.2% and W0.5% diets significantly stimulated *Cathelicidin* gene expression compared to the control on D15 ($F= 4.024$, $p = 0.00496$), but those levels were significantly decreased on D36 compared to D15 ($F = 53.335$, $p = 0.000000595$; Figure 5.5b: B). Finally, the low dose of GAS1 β-glucan (G0.2%) significantly enhanced Myeloperoxidase (*MPO*) gene expression after bacterial injection on D39 ($F = 4.349$, $p = 0.01101$; Figure 5.5b: D).

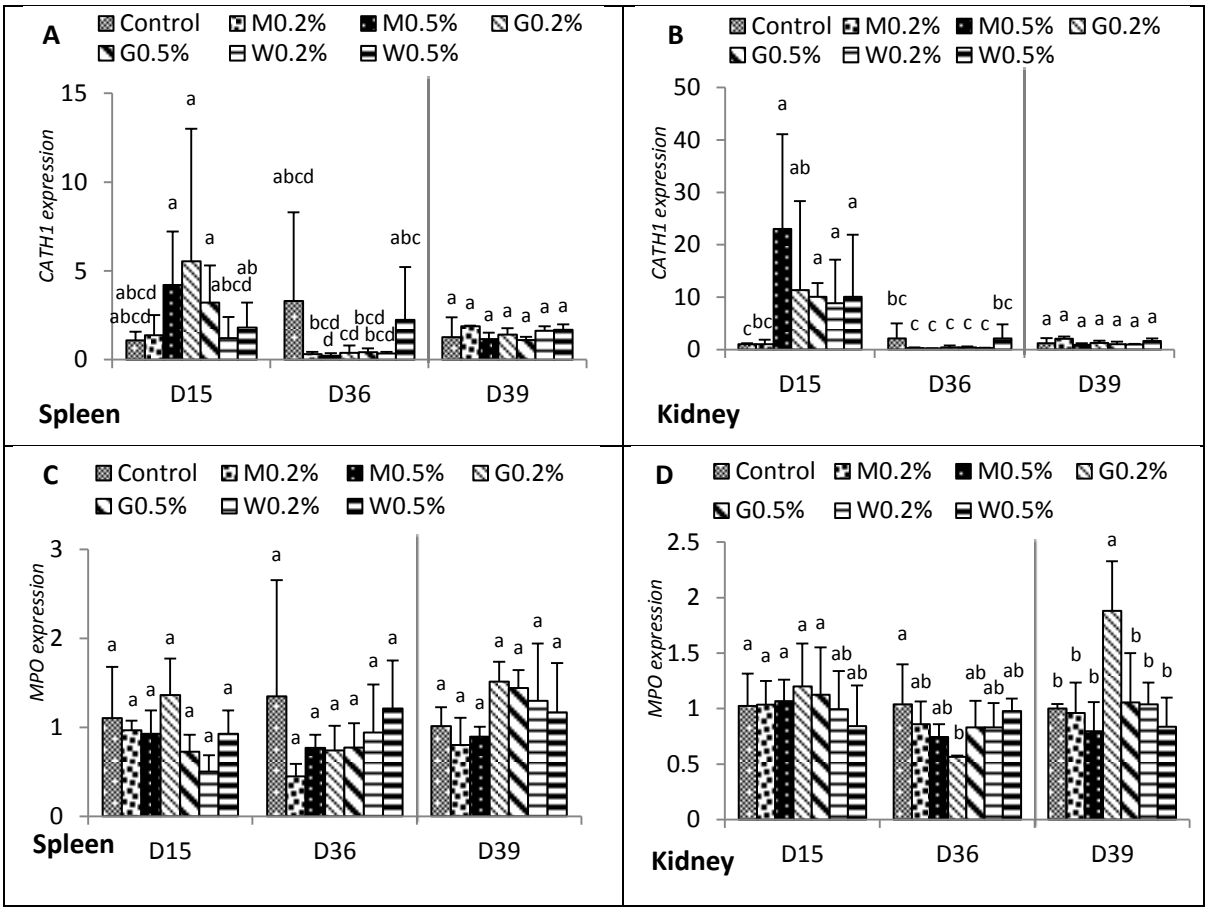


Figure 5.5b (A-D): Effects of dietary beta glucans on *Cathelicidin* and *Myeloperoxidase* (*MPO*) genes expression in spleen or kidney of fish sampled during the nutrient test on D15 and D36, or two days after the bacterial challenge (D39). (A): *CATH1* gene expression in spleen, (B): *CATH1* gene expression in kidney. (C): *MPO* gene expression in spleen, (D): *MPO* gene expression in kidney. Relative transcript (mRNA) levels were determined by real-time RT-PCR and normalized by the arithmetic mean of *ef-1α* gene expressions. Values are expressed as mean ± SD of 3 pool samples per experimental condition (3 fish per pool sample). Statistical differences between dietary treatments and treatment times are indicated by different lower case letters ($p < 0.05$).

3.5. Disease resistance

The mortality rate increased earlier and more rapidly in trout fed with the control and most of β-glucan diets during the first week of infection, except for fish fed with low dose of GAS1 β-glucan (G0.2%) (Figure: 5.6).

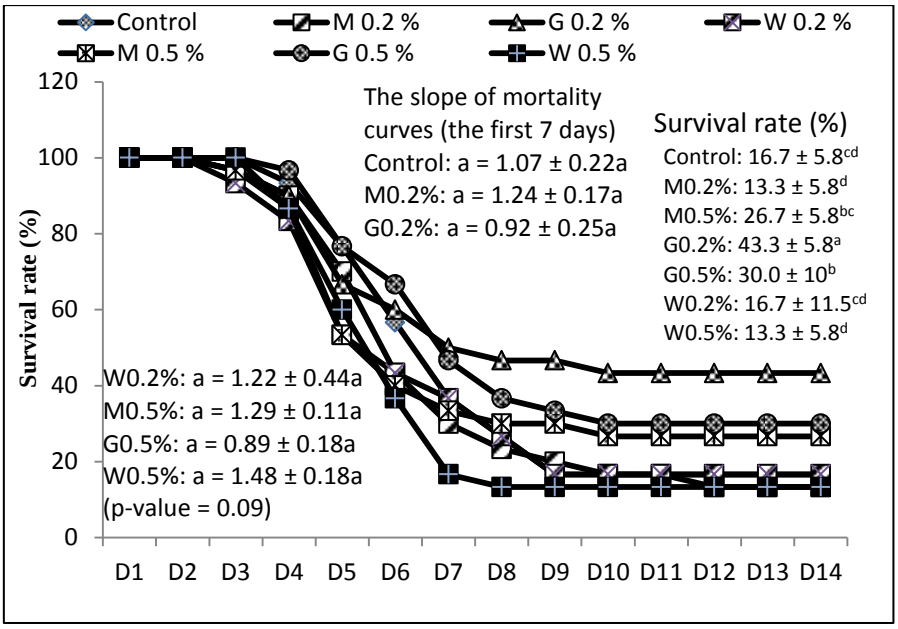


Figure 5.6: Survival rate profile in rainbow trout juveniles fed with different types of beta glucan at low (0.2% diets) or high doses (0.5% diets) and challenged with *Aeromonas salmonicida achromogenes* (3.1×10^7 CFU/100g fish body weight) for 14 days. Statistical differences between dietary treatments and treatment times are indicated by different lower case letters ($p < 0.05$).

The mortality of fish fed with G0.2% was reduced from day 5 and stopped on the 10^{th} day after bacterial injections but other β-glucan diets were stopped later between the10^{th} and 12^{th} day post-infection. The survival rates were significantly higher in fish fed G0.2% (43.3%) and G0.5% (30.0%) than in controls (16.7%) 14 days after bacterial injection ($F = 6.5$, $p = 0.0019$; Figure: 5.6), whereas no significant differences were observed between M0.2%, M0.5%, W0.2% and W0.5% and control groups.

4. Discussion

4.1. Influence of β-glucan types on growth, mortality, splenic index and leukocyte populations

In this study, rainbow trout juveniles were fed with a control diet or enriched diets with Macrogard-β-glucan, GAS1- β-glucan and WT-β-glucan at low (0.2% diet) and high doses (0.5% diet), and fish were sampled after short- (15 days) and long-term (36 days) feeding. After 15 days and 36 days of treatment, β-glucan diets did not significantly modify specific growth rate (SGR), mortality or splenic index of treated fish in comparison with the control group. This result corroborates previous reports revealing no significant effects of dietary yeast β-glucan (Macrogard) or lginic acid (Ergosan) on survival, growth performances of sea bass [29] or growth performance of Asian catfish [16]. A diet containing commercial whole-cell yeast subcomponents (Macrograd: 1g/kg; Bio-Mos Aqua Grade: 2g/kg; Betagard A: 0.1g/kg and Levucell SB20: 0.1g/kg) trial for 4 weeks did not affect to growth performance of channel catfish (*Ictalurus punctatus*) [28]. In contrast, Tukmechi et al. [33] reported that dietary yeast cells (*Saccharomyces cerevisiae*) treated of beta- mercaptoethanol fed rainbow trout for 30 days significantly promoted growth performances. Dietary β-glucan at 0.1% and 0.2% diets (fed rainbow trout of 293.0 ± 18.3g/fish, for 42 days) significantly increased specific growth rate, weight gain and feed efficiency [37]. Moreover, dietary β-1,3/1,6-

glucans (0.1% diet, isolated from *Laminarina digitata*) increased growth performance of gilthead seabream (*Sparus aurata*) after 4 weeks of feeding [50]. A diet containing 10g kg^{-1} β-1,3-glucans (derived from *Schizophyllum commune*) fed grass spawn (*Penaeus monodon*) juvenile increased survival rate after 20 days of feeding [51], whilst dietary β-glucans (Macrogard, at level 0.1%, 0.2% and 0.3% β-glucans in diets) fed Persian sturgeon (*Acipenser persicus*) for 6 weeks significantly increased growth performance but not survival rate [52]. Furthermore, the positive effects of dietary β-glucan on growth/survival rate of aquatic animals have been reported in koi (*Cyprius carpio koi*) [53], in Nile tilapia [54] and in sea cucumber (*Apostichopus japonicas*) [55]. Huu et al. [56] demonstrated that feeding dietary β-glucans to pompano fish (*Trachinotus ovatus*) at low doses (0.05% and 0.1% diets) after 8 weeks significantly increased the growth and survival rates, while the high doses at 0.2% and 4% had no effect. Moreover, in their study on the effects of dietary β-1,3 glucan on innate immune responses of large yellow croaker, Ai et al. [23], indicated that low β-glucan supplementation (0.09%) after 8 weeks significantly enhanced the fish growth whereas high supplementation (0.18%) did not. In summary, to date numerous studies have reported positive and negative effects of β-glucans in aquatic animals on specific growth rate. However, those effects vary depending on doses and treatment times of β-glucans. The origin of β-glucan does not seem to exert a marked effect. To our knowledge, β-glucans may affect specific growth rate of fish through indirect activity by promoting the development of a beneficial community of bacteria in the posterior gut, which gives the fish a healthy digestive system and inhibits the disease bacteria in the intestine of the fish. In our studies, dietary β-glucans at 0.2% and 0.5% diets levels did not affect rainbow trout growth performance. This might be explained by a too high concentration of β-glucans in the diets causing a high level of butyric acid by the anaerobic fermentation processes in the guts. But, butyric acid is also a histone deacetylase inhibitor, which can affect the number of structures of chromatin due to the decrease of electrostatic attraction between histone and DNA [57].

In the present study, low and high doses of GAS1-β-glucan (G0.2%, G0.5%) and a high dose of WT- β-glucan (W0.5%) significantly increased monocyte cell proportions on D15, but not on D36 in comparison with those of the control fish. However, there was no difference in lymphocyte, basophil and neutrophil cell proportions of all diets on D15 and D36 compared with those of the control. Selvaraj et al. [18] confirmed that injection of 100 µg β-glucan + 10 µg LPS (lipopolysaccharide)/individual-carp of 20–30g/fish increased total blood leukocyte counts, neutrophils and monocytes. Moreover, dietary β-glucan, mannan oligosaccharides (MOS) and their combinations fed sea cucumber after 29 days of treatment significantly increased total coelomocytes count, phagocytosis and superoxide [55]. Ghaedi et al. [36] reported that dietary 0.2% β-glucans fed brood rainbow trout for two months exhibited significantly higher of white blood cell value than control. Dietary 0.1% β-glucans (Hang Zhou Bio-Technology Co., Ltd. China) fed Nile tilapia for 21 days stimulated an increase of total lymphocyte count in blood sample [20]. Huu et al. [56] described that β-glucans at 0.2 - 0.4% diet increased red blood count, total leukocytes, both lymphocyte and monocyte count in pompano fish after 8 weeks feeding.

4.2. Influence of β-glucan diets on humoral immune parameters

The present study demonstrated differential actions of β-glucan dietary on humoral immune parameters. Indeed, it is interesting to notice that the high dose of Macrogard β-glucan significantly increased plasma alternative complement activity (ACH50) on D15 and the high dose of Wild type β-glucan significantly increased ACH50 level on D36. Meanwhile, a significant decrease of ACH50 of low dose of GAS1 β-glucan was observed on D36. Alternative complement activity plays a major role in the innate immune response to destroy the cell surface membranes of pathogens by creating pores and opsonising pathogens for

destruction by an enhanced uptake of phagocytes and mediated through ligand-receptor interactions between the surfaces of the two cells [58]. In agreement with our results, dietary alginic acid and glucan (0.1% diet) fed for 15 days significantly elevated the serum complement activity of sea bass [26], β-glucan diets at 1%, 2% and 3% stimulated an increase of ACH50 of Persian sturgeon [52]. Moreover, Ghaedi et al. [36] stated that dietary 0.2% β-glucan fed brood rainbow trout modulated a significantly increase of ACH50 after three months of treatments. In contrast, administration of dietary β-glucan at 0.1% diet in feed did not affect ACH50 in Asian catfish [16]. Dietary β-glucans at level of 0.09% and 0.18% diets fed large yellow croaker did not affect ACH50 after 8 weeks of feeding [23]. Dietary β-1,3-glucan, chitosan or raffinose fed koi (Cyprius carpio koi) for 8 weeks did not change ACH50 value [53]. Further, Selvaraj et al. [25] revealed that different concentrations of β-glucan were administered to test on carp on day 1, 3 and 5 through different routes (intraperitoneal injection, bathing and oral administration) however, ACH50 was not affected by β-glucan administration by any of three routes. Further, suggesting that different effects of β-glucan on ACH50 of rainbow trout seem to be depending on types of β-glucans, doses and treatment time of this immunostimulant compounds.

Low and high doses of GAS1 β-glucan and wild type β-glucan significantly increased plasma total immunoglobulin (Ig) on D36. In addition, high dose of Macrogard (M0.5%), low dose of GAS1 (G0.2%) and low dose of wild type β-glucan significantly stimulated plasma lysozyme activity on D15 and high dose of wild type β-glucan (W0.5%) increased of lysozyme activity on D36.

Table 4: Summary results of the analysis of rainbow trout immune parameters after short- and long- term nutrient test with low (0.2%) and high (0.5%) of different types and doses of β-glucan in the diets

Immune parameters		Organs	Macrogard-Beta glucan			GAS 1-Beta glucan			WT-Beta glucan		
			D15 (Low/high dose)	D36 (Low/high dose)	D39 (Low/high dose)	D15 (Low/high dose)	D36 (Low/high dose)	D39 (Low/high dose)	D15 (Low/high dose)	D36 (Low/high dose)	D39 (Low/high dose)
Humoral immune parameters	haemolytic activity of complement	blood plasma	NS/↑1.4	NS/NS	NS/NS	NS/NS	↓1.6/NS	NS/NS	NS/NS	NS/↑2.6	NS/NS
	total immunoglobulin		NS/NS	NS/NS	NS/NS	NS /↑1.4	↑1.8 /↑1.7	NS/NS	NS/NS	↑2.1/ ↑2.3	NS/NS
	lysozyme activities		NS/↑1.6	NS/NS	NS/NS	↑2.1/NS	NS/NS	NS/NS	↑1.3/NS	NS/↑1.4	NS/NS
Macrophage activity	respiratory burst activity	fresh spleen	NS/NS	NS/NS	NA	NS/NS	NS/NS	NA	NS/NS	NS/NS	NA
Blood leukocyte populations	lymphocyte + thrombocyte	fresh blood	NS/NS	NS/NS	NA	NS/NS	NS/NS	NA	NS/NS	NS/NS	NA
Immune gene expression	monocyte	Spleen tissue	NS/NS	NS/NS	NA	↑1.5/↑1.5	NS/NS	NA	NS/↑1.5	NS/NS	NA
	neutrophils		NS/NS	NS/NS	NA	NS/NS	NS/NS	NA	NS/NS	NS/NS	NS/NS
	basophils		NS/NS	NS/NS	NA	NS/NS	NS/NS	NA	NS/NS	NS/NS	NS/NS
	il-1		NS/NS	NS/↓3.8	NS/↓1.6	NS/NS	NS/NS	↓2.0/↓1.6	NS/NS	NS/NS	↓1.7/ NS
	Complement C3		NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS
	lysozyme		NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS
	mIgM		NS/NS	NS/NS	NS/NS	NS/↓1.9	NS/NS	NS/NS	↓1.8/ NS	NS/↓1.2	NS/NS
	MCSFRA		NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS
	MPO		NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS
	il-10		NS/NS	↓2.8/ NS	NS/NS	NS/NS	NS/NS	↓2.9/ NS	NS/NS	↓2.8/↓3.0	NS/NS
	tgf-β1		NS/NS	NS/NS	NS/NS	NS/↓1.7	NS/NS	↓1.53/ NS	↓1.56/ NS	NS/NS	↓1.7/ NS
	Hepcidin		NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS
	CATH1		NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS
	Cd4-2 β		NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS
	TLR2		NS/↓1.5	NS/NS	NS/NS	↓1.7 /↓1.9	NS/NS	↑1.5/NS	↓1.5/↓1.7	NS/NS	NS/NS

Significant level at $P < 0.05$, NS: non-significant, NA: no data, ↑ indicated the increase in fold change of the value, ↓ indicate the decrease in fold change of the value.

Table 4: Summary results of the analysis of rainbow trout immune parameters after short- and long- term nutrient test with low (0.2%) and high (0.5%) of different types and doses of β-glucan in the diets (Continued).

Immune parameters		Organs	Macrogard-Betan glucan			GAS 1-Beta glucan			WT-Beta glucan		
			D15 (Low/high dose)	D36 (Low/high dose)	D39 (Low/high dose)	D15 (Low/high dose)	D36 (Low/high dose)	D39 (Low/high dose)	D15 (Low/high dose)	D36 (Low/high dose)	D39 (Low/high dose)
Immune gene expression	*il-1*	kidney tissue	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS
	Complement C3		NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS
	lysozyme		NS/↑ 1.8	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS
	mIgM		NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS
	MCSFRA		NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS
	mpo		NS/NS	NS/NS	NS/NS	NS/NS	↓ 1.8/ NS	↑ 1.9/NS	NS/NS	NS/NS	NS/NS
	il-10		NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	↓ 3.0/NS	NS/NS
	tgf-β1		NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	↓ 1.7/ NS
	Hepcidin		NS/↓ 2.0	NS/NS	NS/NS	NS/NS	↓ 1.7/ NS	NS/NS	NS/↓ 2.4	↓ 2.0/ NS	NS/NS
	CATH1		NS/↑ 23	NS/NS	NS/NS	↑ 11.3/↑ 10	NS/NS	NS/NS	↑ 8.8 /↑ 10	NS/NS	NS/NS
	Cd4-2β		NS/↓ 1.6	NS/NS	NS/NS	NS/ ↓ 1.5	NS/NS	NS/NS	↓ 1.8 /↓ 1.6	NS/NS	NS/NS
	TLR2		NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	↑ 1.3/NS	NS/NS	NS/NS	NS/NS

Significant level at $P < 0.05$, NS: non-significant, NA: no data, ↑ indicated the increase in fold change of the value, ↓ indicated the decrease in fold change of the value.

Several studies have shown that β-glucans could stimulate lysozyme activity. For examples, dietary β-glucan at 0.2% and 0.3% increased of lysozyme activity on Persian sturgeon juvenile [52], rainbow trout [32], Asian catfish [16], and increased lysozyme and respiratory burst activity in large yellow croaker [23]. Dietary Ergosan (0.5%) and Macrogard (0.1%) feeding for 30 days significantly increased serum lysozyme activity in sea bass [29]. Moreover, 0.2% dietary β-glucan fed to brood rainbow trout for two months significantly increased lysozyme activity, total Ig and IgM compared to the control [36].

4.3. Influence of β-glucan on immune gene expressions

The effect of dietary β-glucans on rainbow trout juvenile immune gene expression in this study was investigated through the analysis of various immune genes (13 genes) in spleen and kidney tissues. Interestingly, a significant increase in membrane protein gene (*TLR_2*) was observed in fish treated with GAS1β-glucan at low dose (G0.2%) in both spleen and kidney organs at 35h after bacterial injection. Moreover, dietary GAS1β-glucan at low dose (G0.2%) significantly increased *Myeloperoxidase* gene expression (*MPO*) at 35h after bacterial injection. Furthermore, dietary M0.5%, G0.2%, G0.5%, W0.2% and W0.5% modulated a significantly increase of *Cathelicidin* (*CATH1*) gene expression on D15 in kidney tissues. An increase of antibacterial lysozyme gene in M0.5% diet on D15 was also observed in kidney organs. This result supports that the effects of β-glucans on rainbow trout juvenile immune functioning can be dependent on the source of β-glucans, doses and administration route. On the other hand, a decrease of several immune genes' expression levels of *il-1β, il-10*, *cd4-2β, mIgM* and *hepcidin* gene in spleen and kidney tissues after the fish was fed dietary β-glucan was observed in this study. The results indicate that a decrease in most of the immune related genes was observed in both spleen and kidney tissues after a short time (D15), a long time (D36) or at 35h after bacterial injection (D39). In agreement with our results, Falco et al. [59] reported that treatment with dietary β-glucan (Macrogard) administered daily to carp (6 mg/kg body weight) for 14 days prior to infection in generally down-regulated the expression of all measured genes when compared to their corresponding control. Further, after bacterial injection, a significant down-regulation of *tnf-α1*, *tnf-α2*, *il-1β*, and *il-6* in infected fish fed β-glucan was observed in common carp. It is noted that low dose of β-glucan (0.05-0.1% diets, fed for 8 weeks) has positive effects on pompano fish [56], but high dose of β-glucan (at 0.5 mg/mL combined zymosan) decreased respiratory burst activity of carpet shell clam (*Ruditapes decussatus*) and Mediterranean mussel (*Mytilus galloprovincialis*) [60]. Furthermore, it has been reported that overdoses of β-glucan and/or prolonged medication can lead to non-reaction physiological status of rainbow trout [38]. The decrease of several immune genes expression in this study may be explained by the high doses of β-glucan used in this experiment leading to high levels of butyric acid production in the guts by the fermentation of bifidobacterium and lactic acid producing bacteria. On the other hand, butyric acid is also a histone deacetylase (HDAC) inhibitor, such as HDAC1, HDAC2, HDAC3 and HDAC8, which inhibits the function of histone deacetylase enzymes leading to deacetylase [57]. The decrease of HDCA causes the loss of structures of chromatin due to the decrease of electrostatic attraction between histone and DNA [57].

4.4. Influence of β-glucan diets on disease resistance

The results concerning resistance against *A. salmonicida achromogenes* revealed that low and high doses of GAS1-β-glucan (G0.2% and G0.5% diet) significantly increased rainbow trout juvenile resistance to pathogens. Indeed, the cumulative survival showed that fish fed with G0.2% displayed the highest survival with 43.3%, followed by G0.5% with 30% and fish fed

control diet at 16.7% after bacterial challenge. Meanwhile, there was no difference in survival rate for fish fed low and high doses of Macrogard β-glucan, Wild type (WT)-β-glucan diets in comparison to those of the control. Numerous studies have reported the immunomodulation potential of β-glucan in fish disease resistance and the available results vary greatly depending on the fish species, doses and administration modes. The protection of significantly high disease resistance of fish fed G0.2% and G0.5% may be related to the modulation of innate immune functions by dietary GAS1-β-glucan, but also to upregulation of some immune compounds (plasma lysozyme activity D15, total plasma immunoglobulin on D36, blood monocyte cell proportion on D15) as well as a significant increase of TLR_2 gene expression on D39 in both spleen and kidney organs, lysozyme gene expression on D15 in kidney, *CATH1* gene expression on D15 in kidney and *MPO* gene expression on D39 in kidney organs. For instance, dietary β-glucan (0.05, 0.1 and 0.2% in diets) significantly increased the survival rate of rainbow trout injected with *A. salmonicida* [37] and *Y.ruckeri* [33], enhanced the resistance of koi infected with *A.veronii* [53], enhanced significantly the survival of zebrafish against *A.hydrophila* infection [10], increased the survival rate of catfish (*Pangasianodon hypothalamus*) against *Edwardsiella tarda* [61]; increased the survival rate of grass carp infected with grass carp haemorrhage virus [22]. Also, oral administration of the combination dietary β-glucan (100 µg β-glucan + 10 µg LPS) could enhance resistance of carp against *A. hydrophila* [18]. In contrast, dietary β-glucans (0.1, 0.2 and 0.3% in diets) had no significant effect on survival of juvenile Persian sturgeon [52]. Administration of dietary β-1,3-glucan from *Eulena gracilis* (1% in diet) had no effect on the survival rate of unvaccinated or vaccinated rainbow trout with *Y. ruckeri* after 70 or 84 days of treatments [34].

5. Conclusions

In this study, a diet supplemented with 0.2% of GAS1-β-glucans was more effective in stimulating the trout immune system than MacroGard and WT-β-glucan. After short-term β-glucan feeding (D15), the immune system of rainbow trout juveniles differentially responded to the two β-glucan doses tested by modifying the proportions of different blood leukocyte cell types such as monocytes. This led to a better humoral immune status in fish fed the low β-glucan dose (G0.2%) on D15 and D36 as evidenced by the highest levels of plasma lysozyme activity on D15 and a high level of total plasma Ig content on D36. The low and high doses of Macrogard-β-glucan and WT-β-glucan induced a decrease of several immune gene expression levels such as *il-1β* on D36; *tgf-β1* on D15, *il-10* on D15 and D36, *cd4-2β*, *mIgM*, TLR_2, *Hepcidin* on D15 and *Lysozyme* gene on D36. On the other hand, dietary GAS1-β-glucan at low doses (G0.2%) significantly modulated the immune system of rainbow trout juveniles through the increase of humoral (*mIgM*) in kidney, membrane protein (TLR_2) both in spleen and kidney, neutrophils (*MPO*) in spleen, and *CATH1* in spleen at 35h after bacterial injection (D39). Therefore, such enhanced immune pathway responses could explain the better protection for trout juveniles by low doses of GAS1-β-glucan as shown by the significant increase of the survival rate after 14 days of bacterial challenge, when compared with control and other β-glucan doses.

Conflict of interest

The authors have declared that no competing interests exist.

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Chapter 6

Can a dietary combination of inulin, bovine lactoferrin and β-glucan efficiently replace antibiotics in rainbow trout (*Oncorhynchus mykiss*) against bacterial infection by *Aeromonas salmonicida*?

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Abstract

Lactoferrin, a large multifunctional glycoprotein, is involved in many physiological functions such as iron absorption, stimulation of innate and specific immune system of organisms. Inulin is used as prebiotics additive in fish feed nutrition while, β-glucans are are known as immunostimulants to modulate the immune functions and improve fish resistance to infections. The objective of the present study was to evaluate the immune responses of rainbow trout *Oncorhynchus mykiss* juveniles fed a dietary combination of inulin 0.1% (In0.1%), bovine lactoferrin 1% (BLf 1%) and GAS1-β-glucan 0.2% (GAS1 0.2%) and compare them with a dietary supply of In0.1%, BLf1%, GAS1 0.2% alone. After a 38-day feeding period, the resistance of fish to bacterial infection was determined by an injection of fish with *Aeromonas salmonicida achromogenes.* The different dietary treatments did not affect significantly the specific growth rate, mortality, splenic index, peroxidase and plasma lysozyme activities on D38. On the other hand, all dietary treatments activated the humoral immunity by acting differentially on lymphocyte + thrombocyte cell proportions. After challenge with *A. salmonicida* all treated diets significantly enhanced disease resistance of fish to infection than in the control groups. All immunostimulants stimulated a slighly higher survival rates than those of antibiotic treatment suggesting the possibility of replacement of antibiotics by alternative methods such as immunoprohyxis.

Keywords: Bovine lactoferrin, inulin, GAS1-β-glucan, rainbow trout (*Oncorhynchus mykiss*), immunity, disease resistance.

1. Introduction

Infectious diseases are a major problem in aquaculture causing a heavy loss to fish farmers [1]. The traditional strategy for fish disease control in pond culture used to rely mostly on the use of antibiotics and chemical disinfectants. However, the use of chemotherapeutic agents such as antibiotics might lead to the development of drug resistant organisms and cause many other problems such as environmental hazards, food safety problems and resistance of pathogens [2,3]. The use of antibiotics can also adversely affect the health status of fish [4]. Therefore, there is very limited prophylactic use of antibiotics in aquaculture and a ban of most compounds is in place in almost all top countries involved in aquaculture production [5]. This situation and recent restrictions on the use of antibiotics have promoted the use of prebiotics and probiotics (or a combination of both named synbiotics) as significant alternatives to antibiotics and there is an increasing interest in aquaculture in using these additives to prevent and/or control fish diseases [6–8].

Lactoferrin is involved in many physiological functions, namely in regulation of iron absorption and stimulation of innate and specific immune systems as well as disease resistance [9]. Due to its immunomodulatory properties, bovine lactoferrin (BLf) may stimulate antibacterial, antifungal or antiviral functions, and consequently may induce effective protection against various bacterial and fungal diseases in several fish species, depending on the dose and administration mode [7–10]. Several studies have focused on the immunomodulatory potentials of BLf in fish culture and the available results vary greatly depending on fish species, dose and administration mode [8–15]. Inulin is a naturally occurring fructooligossacharide (FOS) extracted from the chicory root (*Cichorium intybus*) as well as from many other plants (cereals, leeks, onions, garlic, wheat, artichokes and bananas) and belongs to a class of carbohydrates known as fructans [5,17]. Fructans are non-digestible oligosaccharides as the β (2-1) linkage of the fructans cannot be hydrolysed by pancreatic or brush border digestive enzymes in the proximal intestinal tract of human and domestic animals [18]. It is fermented in the large intestine or colon [19] by beneficial bifidobacteria and other lactic acid-producing bacteria, enhancing their relative population while selectively inhibiting the growth of pathogenic microorganisms [20,21]. Due to these properties, inulin may stimulate antibacterial functions, and, consequently, may induce effective protection against various bacterial diseases in several species of domestic animals, depending on the dose and administration mode. Beta glucan is a heterogeneous group of glucose polysaccharides consisting of a backbone of β-(1,3)-link β-D-glucopyranosyl units with β-(1,6)-link side chains of varying length and distribution [22]. Among immune-stimulants agents, β-glucans are the most important compounds used in fish culture for the defence against pathogens [23,24]. Beta glucans are a major structural component of fungi, bacteria, plants, algae, yeast and mushroom cell walls. One of the most common sources of β-glucan is derived from the cell walls of baker'yeast *Saccharomyces cerevisiae*. Further, β-glucans are also extracted from the bran of oat, barley, rye, wheat grains and several species of seaweed [25,26]. β-glucans bind to specific cell surface receptors of macrophages and neutrophilic granulocytes that promote the enhancement of an organism's protective activity against infection through the activation of leukocytes, phagocytic activity, inflammatory cytokines, chemokines, reactive oxygen free radicals, increasing the activity of antioxidant enzymes, initiating the development of adaptive immunity and elimination and killing of microorganisms [27].

Studies on the potential immunomodulatory effects of bovine lactoferrin, inulin and β-glucan have been reported in several fish species in which BLf diets was shown to improve survival rate [28], resistance to diseases and enhanced humoral immunity [29,30]. BLf induced

changes respiratory burst and natural cytotoxic activities in gilthead seabream *Sparus aurata*. BLf has been reported to protect red sea bream *Pagrus major* against white spot disease [29]. BLf supplement, particularly at 100 mg level, significantly enhanced serum lysozyme level, oxidative radical production and the level of protection against *A. hydrophila* challenge in Asian catfish *Clarias batrachus*. Dietary supplements enriched with inulin increased total serum immunoglobulins, bactericidal activity and anti-protease activity increased in grass carp (*Ctenopharyngodon idella*) [17]; increased the serum complement activity, IgM level, leukocyte phagocytic activity and leukocyte respiratory activity was reported in gilthead seabream [4], increased blood respiratory burst activity in common carp fry (*Cyprinus carpio*) [31]; Improved lysozyme activity, alternative complements haemolytic activity (ACH50) of juvenile Nile tilapia (*Oreochromis niloticus*) [32]. The, numerous immunostimulant effects of β-glucans have been reported in several fish species, for instance: against pathogen of Asian catfish [33,34], common carp [1], catla (*Catla catla*) [35], Nile tilapia [36], rohu (*Labeo rohita*) [37], and zebrafish (*Danio rerio*) [22] when infected with *Aeromonas hydrophila*, grass carp [38] infected with grass carp haemorrhage virus, large yellow croaker (*Pseudosciaena crocea*) infected with *Vibrio harveyi* [39]; and rohu infected with *Edwardsiella tarda* [40].

The available results on the immunomodulatory potentials of bovine lactoferrin, inulin in fish are somewhat contradictory and the modes of action of inulin are not yet well described. For example, BLf had neither influence on the specific immunity of vaccinated Asian catfish [30], nor effects on growth performance [28,41], physiological [28] and immunological variables [28,42]. In Atlantic salmon *Salmo salar*, a dietary administration of BLf failed to reduce mortality challenged with *Aeromonas salmonicida* or salmon anaemia virus [42]. Further, dietary inulin did not provide any positive effect on growth performance and survival rate in common carp [31] and tilapia [32]. Dietary supplementation of β-glucans did not influence the growth performance of European sea bass (*Dicentrarchus labrax*) [43] or the plasma lysozyme, bactericidal and haemolytic complement activities, respiratory burst of phagocytes and the proportion of lymphocytes found in channel catfish (*Ictalurus punctatus*) infected with *Edwardsiella ictaluri* [44].

Supplemental dietary inulin alone did not show any positive effect on gut microbiota, while the combination of inulin and Gram-positive bacteria *Weissella cibari*a induced a higher concentration of lactic acid bacteria and low levels of *Vibrio spp* and *Pseudomonas spp* in the midgut of hybrid surubim (*Pseudoplatystoma sp)* [45]. Altogether, the results obtained in aquatic species with dietary bovine lactoferrin, inulin and β-glucans seem to differ depending on experimental conditions, species and treatment mode.

In this study, we aimed to conduct a comprehensive evaluation of the effects of a dietary combination of inulin, bovine lactoferrin and beta glucan on the overall immune function of trout juveniles analysing blood leukocyte cells and humoral immune parameters. Moreover, the disease resistance of trout juveniles was tested by applying a bacterial challenge test based on the intraperitoneal injection of *Aeromonas salmonicida achromogenes*.

2. Materials and methods

Experimental fish

Feeding trial and bacterial challenge were carried out in agreement with the European and Belgian national legislation on animal welfare (Protocol number: 13197 KE). Rainbow trout juveniles (n = 315) were transported from a local fish farm (Hatrival, Belgium) to UNamur

facilities and distributed into 9 fibreglass tanks of 100L (35 fish/100-L tank) in a recirculation system. Fish were allowed to acclimate to the new environment for 21 days. During this period, water temperature was maintained at 13.9 ± 1.2°C by a cooling system. The oxygen level averaged 11.6 ± 0.7 mg/L (aeration applied), constant photoperiod (Light : Dark ratio = 12:12) and fish were fed 1.5% of fish biomass, twice daily (at 9:00 am and 5:00 pm) with a specific trout diet (Coppens TROCO SUPREME-16, The Netherlands, crude protein = 48%, crude fat = 15%).

Table 4.1: Ingredients and proximate composition of the experimental pelleted diet

Ingredients	Diet (g/kg)	Diet (%)	Crude protein (%)	Crude fat (%)	Crude protein in diet	Crude fat in diet
Cod fish meal[a]	350.0	35.00	89.0	4	31.15	1.40
Blood meal[b]	70.0	7.00	87.6	0	6.13	0.00
Wheat gluten[c]	134.0	13.40	80.0	6	10.72	0.80
Cod fish oil[d]	128.0	12.80		100		12.80
Starch[e]	223.6	22.36				
Carboxylmethylcellulose[e]	20.0	2.00				
α- cellulose[e]	42.4	4.24				
Mineral mix[f]	10.0	1.00				
Vitamin mix[g]	10.0	1.00				
Betaine[e]	10.0	1.00				
BHA	1.0	0.10				
BHT	1.0	0.10				
Total CP diet %					48.00	
Total CF diet %						15.00

BHA: butylated hydroxyanisole; BHT: butylated hydroxyl toluene. [a] Cod fish meal provided by SNICK euroingredient NV, Ruddervoorde (Belgium). [b]ActiproHemoglobin, Zwevezele (Belgium). [c] Roquette Freres, Lestrem (France). [d] Sigma-Aldrich, Saint-Louis, MO, (USA). [e]Mosselman SA, Chlin (Belgium). [f] Mineral mix (g kg^{-1} of mix) was prepared in the lab, from $(CaHPO_4)2H_2O$, 727.77; $(MgSO_4)7H_2O$, 127.50; NaCl, 60.00; KCl, 50.00; $(FeSO_4)7H_2O$, 25.00; $(ZnSO_4)7H_2O$, 5.50; $(MnSO_4)4H_2O$, 2.54; $(CuSO_4)5H_2O$, 0.78; $(CoSO_4)7H_2O$, 0.48; $(CaIO_3)6H_2O$, 0.29; $(CrCl_3)6H_2O$, 0.13 g. [g]Vitamin mix was provided by INVE Aquaculture Company.

Fish diet and experimental design

After acclimation, fish (mean body weight: 115.5 ± 30.5 g) were fed either a control diet (without immunostimulants), antibiotic treatment (Control diet + 20 mg of Flumiquil 50%/kg) or dietary inulin 0.1% (In0.1%); bovine lactoferrin 1% (BLf 1%); GAS1-β-glucan 0.2% (GAS1 0.2%) or the combination diet of (In0.1% + BLf 1% + GAS1 0.2%) for 38 days at 1% of fish biomass/day (Bovine lactoferrin-Friesland Campina DOMO®, Netherlands; Inulin-

Beneo-Orafti® -Rue L. Maréchal 1-B-4360 Oreye, Belgium; GAS1-β-glucan was extracted by the Laboratory of Aquaculture & Artemia Reference Center (ARC) — Faculty of Bioscience Engineering — Blok F, Ghent University, Coupure Links 653, B-9000 Gent, Belgium). All diets were formulated and pelleted in the laboratory (Table 4.1). Three replicate tanks were used for each experimental regime. After 38 days of feeding, six fish per tank (18 fish per experimental diet) were anesthetized in ethyl 3-aminobenzoate methane sulfonic acid salt (98% purity, MS-222, Sigma) solution (120 mg/L). Blood was obtained by caudal vein puncture using a heparinized syringe and stored on ice in heparinized tubes. Heparinized blood was immediately analysed for leukocyte populations by flow cytometry, and the remaining volume of blood was then centrifuged at 7500×g for 10 min to collect plasma which was stored at -80°C until subsequent analysis (lysozyme activity, peroxidase activity).

Bacterial challenge

In order to evaluate whether the combination (Inulin 0.1% + Bovine lactoferrin 1% + GAS1-β-glucan 0.2%) diet has a beneficial effect on disease resistance, rainbow trout juveniles were experimentally infected with a virulent strain of *Aeromonas salmonicida achromogenes* provided by the CER group (Centre d'Economie Rurale, Laboratoire de Pathologie des Poissons, Belgium). Bacteria were cultured in sterile Brain Heart Infusion (BHI, Sigma Aldrich, Saint-Louis, MO, USA) and incubated at 28°C for 24h. A preliminary test infection including various bacterial doses was performed to determine the LD50 CFU of the targeted rainbow trout population (LD50 = 3.1×10^7 CFU/100g fish body weight).

On day 39 of treatment, a total of 30 fish from each dietary condition (10 fish × 3 replicate tanks) were anesthetized. Then, fish were intraperitoneally injected with a weight-adjusted dose (3.1×10^7 CFU/100 g of fish body weight) of the freshly prepared *A. salmonicida achromogenes* culture and equally distributed into three 50L-tanks. Fish were confined in the animal facility (Biosafety level 2) along the infection assay. They were starved one day ahead of infection as well as on the day of bacterial injection, and then fed the respective experimental diets until the end of the challenge test. At 35h post-injection (D54), a total of 9 fish from each dietary condition (3 fish × 3 replicate tanks) were anesthetized and blood was sampled for subsequent immunological assays (lysozyme activity, peroxidase activity).

Blood leukocyte populations

Blood cell populations were analysed at D15 and D36 by flow cytometry (Flow Activated Cell Sorter Calibur, Flow Cytometry System) according to Inoue et al. [46], later adapted by Mathieu et al. [47]. Briefly, 10 µl of fresh heparinized blood were mixed with 1950 µl of Hanks Balanced Salt Solution (HBSS, Sigma) and 40 µl of fluorochrome DiOC6 (3,3-dihexyloxacarbocyanine, Molecular Probes, Eugene) diluted 1:10 in ethanol. The tube was mixed gently and incubated at room temperature (RT) for 10 min. The FACS was calibrated with True Count Beads diluted in HBSS, Sigma-Aldrich, Steinheim, Germany). Each blood cell population was identified by its typical location in a FL-1 v. SSC and FSC v. SSC according to Inoue et al. [46] and Pierrard et al. [48]. Four clusters were identified, thrombocyte and lymphocyte cells were gathered in a same cluster according to Pierrard et al. [48].

Plasma lysozyme activity

Lysozyme activity assay was performed by the turbidimetric method of Siwicki and Studnicka [49], later adapted by Mathieu et al. [50]. Briefly, 7 µL of plasma were added to 130 µL of freshly prepared *Micrococcus luteus* (Sigma-Aldrich, Saint-Louis, USA) solution

(0.6 mg/mL of Na_2HPO_4 0.05 M, pH 6.2) in triplicate. Absorbance corresponding to *Micrococcus luteus* lysis was measured at 450 nm for 60 min at regular intervals (5 min). One unit (U) of lysozyme was determined as an absorbance decrease of 0.001 per min.

Peroxidate activity

The total peroxidate activity present in serum was measured according to Quade and Roth [51]. Fifteen microliters of serum were diluted with 135 µL of HBSS without Ca^{2+} or Mg^{2+} in flat-bottomed 96-well plates. Then, 50 µL of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB) (Sigma) and 5 mM H_2O_2 (both substrates of peroxidase and prepared daily) were added. The color-change reaction was stopped after 2 min by adding 50 µL of 4 M sulphuric acid (H_2SO_4). The optical density (OD) was read at 450 nm in plate reader. Standard samples without serum were also analysed. The peroxidate activity, units/mL serum, was determined defining as one unit the peroxidate that produces an absorbance change of 1 OD.

Statistical analysis

Results are presented as mean ± SD. Data were checked for normal distribution and homogeneity of variances by Univariate tests. Humoral immune parameters, blood leukocyte proportions, specific growth rate, mortality, splenic index and immune gene expressions on D15 and D36 were carried on using a two-way ANOVA test. One-way ANOVA was used to test for those parameters on D39 and for data of cumulative mortality over the 14 days of bacterial challenge test. In all statistical analysis tests used, $p < 0.05$ was considered statistically significant. The statistical analysis was performed using the Statistica bio-software (Version 10.0).

3. Results

3.1. Specific growth rate (SGR), mortality and splenic index (SI)

On day 38, fish fed inulin 0.1%, bovine lactoferrin 1%, GAS1-β-glucan 0.2% alone or a combination of these three compounds did not display any differences of growth and survival rates, and splenic index (SI) value in comparison with those of the control group (Table 3).

Table 3: Mean (±SD) values for specific growth rate (SGR), mortality and splenic index (SI) of trout juveniles fed with inulin 0.1%, BLf 1%, GAS1 0.2% and the combination diets after 38 days of feeding test.

Variables	Control diet	Inulin (In 0.1%)	Bovine lactoferrin (BLf1%)	GAS1-β-glucan (GAS1 0.2%)	In0.1% +BLf1%+GAS1 0.2%	F- values	P- values
SGR (%/day)	0.71 ± 0.12	0.80 ± 0.12	0.84 ± 0.14	0.82 ± 0.03	0.76 ± 0.12	0.656	0.635
Mortality (%)	4.1 ± 3.5	2.0 ± 1.7	2.0 ± 3.5	2.0 ± 1.7	6.1 ± 3.1	1.234	0.356
Splenic index (%)	0.23 ± 0.04	0.25 ± 0.14	0.37 ± 0.06	0.30 ± 0.08	0.34 ± 0.09	1.457	0.285

Statistical differences between dietary treatments are indicated by different lower case letters ($p < 0.05$).

3.2. Humoral immune variables

Values of plasma lysozyme and plasma peroxidase activity were not affected by the dietary supplementation of inulin 0.1%, bovine lactoferrin 1%, GAS1-β-glucan 0.2% and the combination of those compounds diet compared to control diet (Figure 1: A and B).

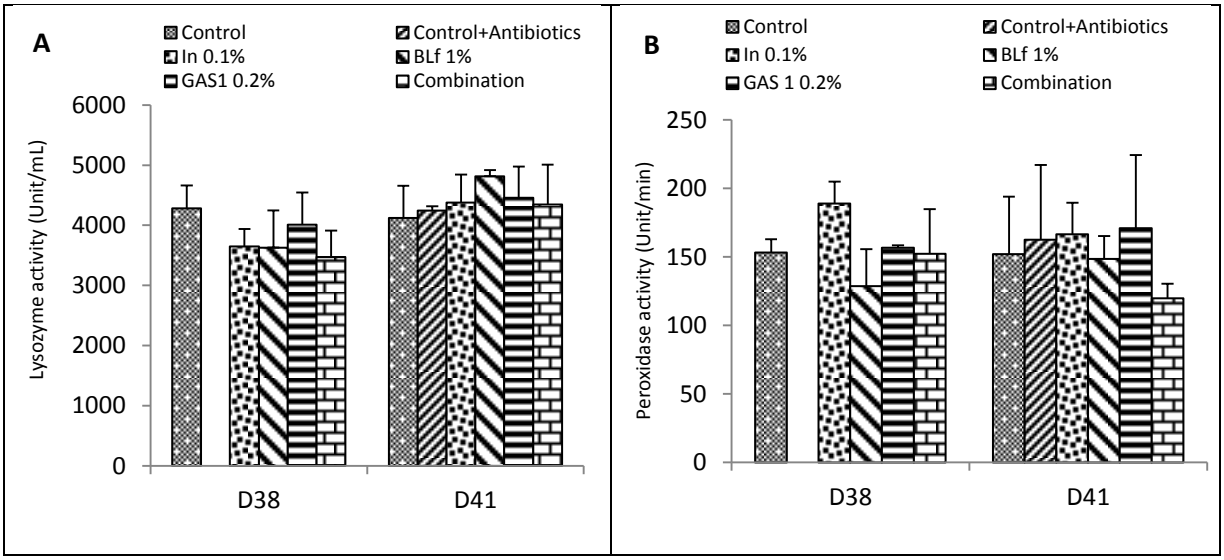

Figure 1 (A-B): A: Plasma lysozyme activity; B: Peroxidase activity of fish fed with dietary inulin 0.1%, bovine lactoferrin 1%, GAS1 β-glucan 0.2% and the combination (In 0.1%+BLf 1%+GAS1 0.2%) after 38 days of feeding test and after bacterial injection on D41.

3.3. Blood leukocyte cell proportions

Blood leukocytes were composed of high percentages of lymphocytes followed by those of neutrophils, monocytes and basophils (Figure 2: A-D). Dietary In 0.1%, BLf 1%, GAS1 0.2% and the combination of the three compounds significantly increased ($F = 3.489$, $p = 0.0495$) the proportion of lymphocyte + thrombocyte cells on day 38 and at 36h after bacterial injection on D41 ($F = 4.616$, $p = 0.0153$). Meanwhile, there were no significant differences of monocyte, basophil and neutrophil proportions in total blood leukocyte cells between treatment and control groups.

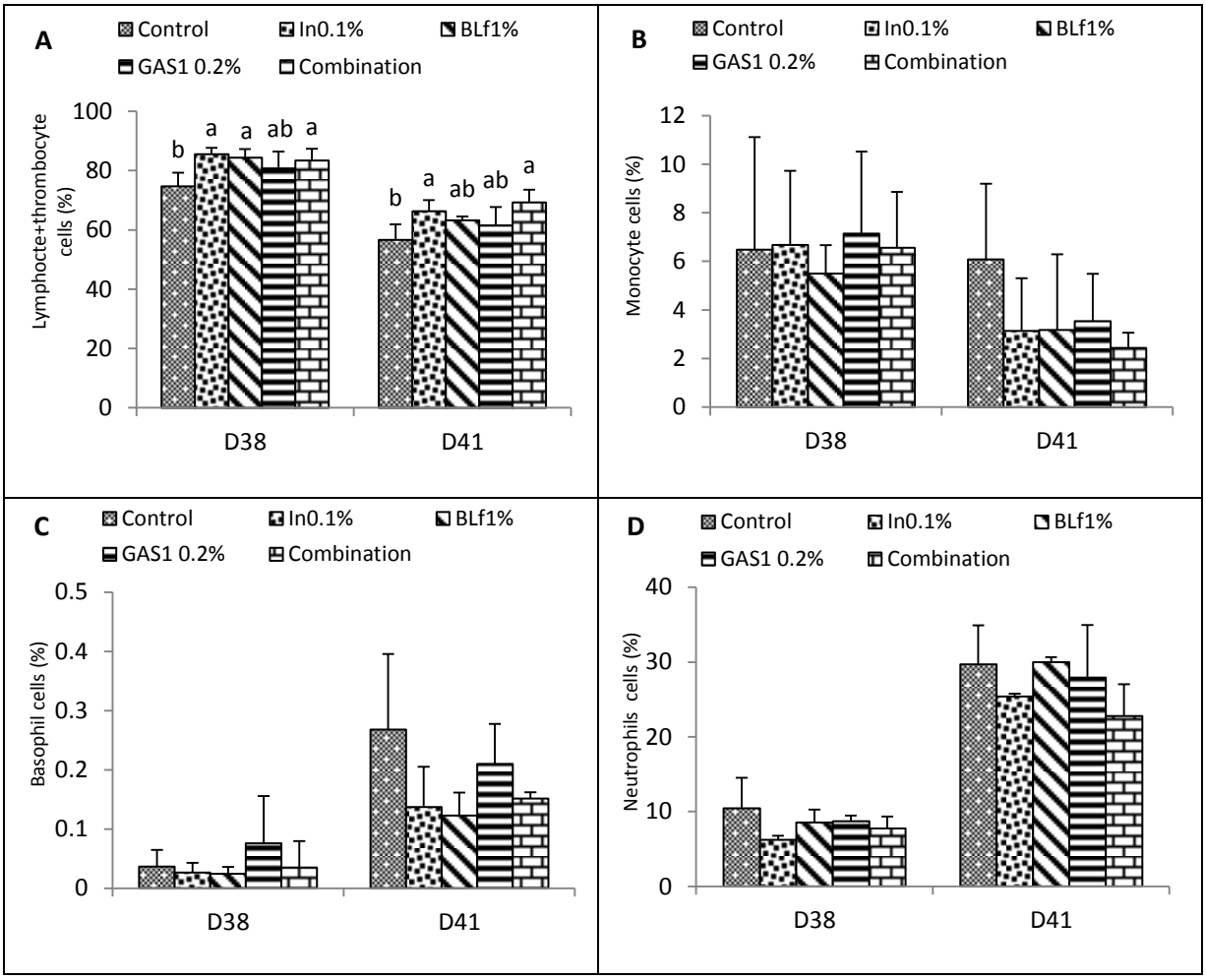

Figure 2 (A-D): (A): Lymphocyte + thrombocyte cells proportions, (B): Monocyte cells proportion, (C): Neutrophil cells proportion and (D): Basophil cells proportion of total blood leukocyte cell population. Values are expressed as mean ± SD of 18 fish/diet/day on D38. Statistical differences between dietary treatments and treatment times are indicated by different lower case letters with $p < 0.05$.

3.5. Disease resistance

The figure 3 shows that the survival rates of fish in the control and treated diet groups decreased from day 2 after bacterial injection. However, survival rates rapidly decreased in trout fed the control diet and continued until day 14 after bacterial injection (Figure 3). In0.1%, BLf 1%, GAS1 0.2% and the combination of these compounds supported a significantly higher survival rate compared to the control groups ($F = 3.215$ $p = 0.0452$). However, there were no significant differences of survival rates between fish fed the 3 compounds alone or in combination, and no significant differences between these treatments and fish fed with antibiotic treatment (Control diet supplemented with Flumiquil).

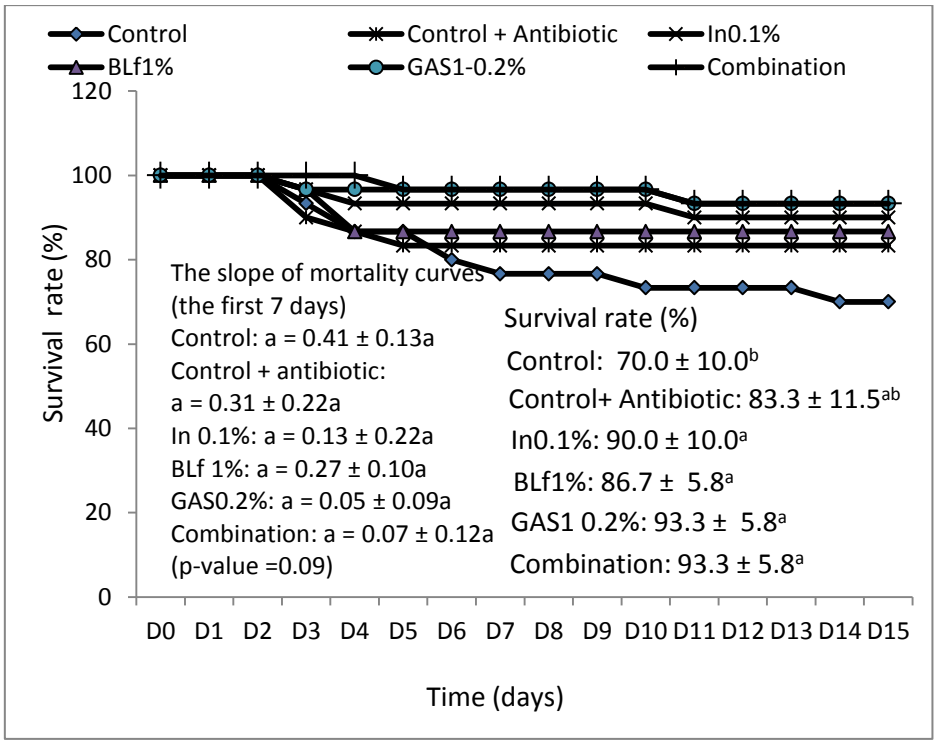

Figure 3: Survival rate profile in rainbow trout juveniles fed with inulin 0.1%, bovine lactoferrin 1%, GAS1 β-glucan 0.2% and the combination (In 0.1% + BLf 1% + GAS1 0.2%) and challenged with *A. salmonicida achromogenes* (3.1×10^7 CFU/100g fish body weight) for 14 days. Statistical differences between dietary treatments and treatment times are indicated by different lower case letters ($p < 0.05$).

4. Discussion

4.1. Influence of inulin, bovine lactoferrin, GAS1-β-glucans and the combination of the three compounds on growth, mortality, splenic index and leukocyte populations

In this study, inulin 0.1%, bovine lactoferrin 1%, GAS1-β-glucan 0.2% and the combination of these three compounds did not induce any significant differences of specific growth rate, mortality and splenic index in comparison with those of the control. These results corroborate previous reports revealing no significant effects of inulin on growth performance of Nile tilapia fingerlings [52], common carp [53], and white-leg shrimp [54]. Bovine lactoferrin diet did not influence the growth performance of rainbow trout [55], Nile tilapia [28], orange spotted grouper [41], and Siberian sturgeon [56]. No significant effects of dietary yeast β-glucan and alginic acid on survival, growth performances of sea bass [43] or Asian catfish [33]. Moreover, diet containing commercial whole-cell yeast subcomponents fed for 4 weeks did not affect the growth performance of channel catfish (*Ictalurus punctatus*) [44]. However, Mo et al. [17] reported a significantly higher growth rate of grass carp after 8 weeks feeding with dietary inulin at 0.2% or 2%. Dietary inulin supplementation (5 to 20g kg^{-1}) for 60 days significantly affected SGR in Asian seabass [57], a synergistic effected of the combination of inulin and *Lactobacillus sakei* on weight gain was reported in leopard grouper [58]. Kakuta [59] reported a significantly higher growth rate of goldfish *Carassius auratus* after 28 days of dietary BLf treatment. Further, it is indicated that a diet of yeast cells fed to rainbow trout for 30 days, promoted the growth [60], β-glucan at 0.1% and 0.2% diets fed for 42 days increased SGR, weight gain and feed efficiency of rainbow trout [61]. Dietary β-1,3/1,6-glucans

increased growth of gilthead seabream after 4 weeks [62], 10g kg^{-1} β-1,3-glucans fed grass spawn (*Penaeus monodon*) juvenile increased survival rate after 20 days [63] whilst, dietary β-glucans (Macrogard) fed Persian sturgeon (*Acipenser persicus*) for 6 weeks increased growth but not survival rate [64]. Moreover, dietary β-glucan increased growth/survival rate of koi [65], Nile tilapia [66] sea cucumber (*Apostichopus japonicas*) [67]. Huu et al. [68] demonstrated that feeding a diet of β-glucans to pompano fish (*Trachinotus ovatus*) at low doses (0.05% and 0.1% diets) for 8 weeks significantly increased the growth and survival rates while the high doses at 0.2% and 4% had no effect. A low dose diet of β-1,3-glucan (0.09%) fed for 8 weeks enhanced the growth whereas a high dose (0.18%) did not affect the innate immune response of large yellow croaker[39]. In this study, a diet of β-glucans at 0.2% and 0.5% diets levels did not affect SGR of rainbow trout, which may be explained by too high β-glucan doses. This is in agreement with previous studies confirming that overdoses of β-glucan and/or prolonged medication can lead to a non-reactive physiological status and consequently, to poor immune response [69].

All immunostimulants diets and the combination of compounds diets stimulated a significant increase of lymphocyte + thrombocyte cell proportions on D38, and after bacterial injection (D41) in comparison with those of the control. However, there was no difference of monocyte, basophil and neutrophil cell proportions of all diets on D38 and D41 compared with those of the control. Selvaraj et al. [1] reported that fish injected with 100 µg β-glucan + 10 µg LPS/individual-carp of 20–30g/fish increased total blood leukocyte counts, neutrophils and monocytes. A diet of β-glucan, mannan oligosaccharide (MOS) and their combinations fed to sea cucumber after 29 days increased total coelomocytes count, phagocytosis and superoxide [67]. Ghaedi et al. [70] reported that a diet of 0.2% β-glucan fed brood rainbow trout for two months exhibited significantly higher white blood cell value than control. A diet of 0.1% β-glucan (Hang Zhou Bio-Technology Co., Ltd. China) fed to Nile tilapia for 21 days stimulated an increase of total lymphocyte count in a blood sample [36]. Huu et al. [68] described that β-glucan increased red blood count at 0.2% β-glucan in diets, total leukocytes at 0.05%-0.02% β-glucan in diets, increased both lymphocyte and monocyte count in pompano fish after 8 weeks fed 0.1%-0.4% β-glucan in diets.

4.2. Influence of inulin, bovine lactoferrin, GAS1-β-glucans and the combination of those compounds on plasma lysozyme and peroxidase activity

Dietary inulin of 0.1%, BLf 1%, GAS1 0.2% and the combination of these compounds in the other diets did not affect plasma lysozyme and peroxidase activity on D38 or after bacterial injection on D41. In agreement with our results, Hoseinifar et al. [31], Mourino et al. [45] and Ahmdifar et al. [71] did not find any effect of dietary inulin on plasma lysozyme activity response in common carp fry, hybrid surubim and great sturgeon juveniles (*Huso huso*). Neither Lygent et al. [42] nor Welker et al. [27] found any effect of dietary BLf on plasma lysozyme activity response in Atlantic salmon or Nile tilapia. In contrast, Tiengtam et al. [32] reported that inulin supplementation at 5g kg^{-1} improved lysozyme activity in juvenile Nile tilapia. Similarly, dietary inulin 1% feeding after 45 days increased lysozyme activity of juvenile common carp [72]. Previous studies on large yellow croaker fish indicated that serum lysozyme activity significantly increased with the increase of dietary glucan, and fish fed the diet with high glucan had significantly higher lysozyme activity compared with low glucan. Dietary Ergosan (0.5%) and Macrogard (0.1%) feeding for 30 days significantly increased serum lysozyme activity in sea bass [43], 0.2% dietary β-glucan fed to brood rainbow trout for two months significantly increased lysozyme activity, total Ig and IgM compared to the control [70]. Administration at 0.1% of yeast β-glucan to Asian catfish significantly enhanced lysozyme level [33].

4.3. Influence of inulin, bovine lactoferrin, GAS1-β-glucans and the combination of these compounds on disease resistance

Concerning bacterial resistance, the cumulative survival showed that fish fed with In 0.1%, BLf 1%, GAS1 0.2% and the combination of these compounds diets (In 0.1% + BLf 1% + GAS1 0.2%) displayed a significantly higher survival rate after bacterial challenge in comparison with the control. Meanwhile, no significant difference of survival rates was observed between treated diets and control + antibiotics diets or between control and control + antibiotics diets. Several studies have already shown the ability of inulin treatments to increase the survival rate of Nile tilapia when supplemented with inulin (5g kg^{-1}) after one or two months [73] or/and inulin diets decreased the prevalence of WSSV in treated shrimp [54]. Bovine lactoferrin diets have been reported to increase the bacterial resistance of rainbow trout [74], Asian catfish [30] or giant fresh water prawns [75]. Regarding beta glucans, Ji et al. [61] reported that dietary β-glucan (0.05, 0.1 and 0.2% in diets) significantly increased the survival rate of rainbow trout compared to the control after being injected with *A. salmonicida*. Moreover, dietary β-glucans is reported to enhance the resistance of koi infected with *A.veronii* [65] and increase the disease resistance of rainbow trout against *Y.ruckeri* [60]. A single injection of 5 mg mL^{-1} of β-glucan 6 days before challenge significantly enhanced the survival of zebrafish against *A. hydrophila* infection [22]. Nano scale β-glucan (500 µg/mL) increased the survival rate of zebrafish against *Edwardsiella tarda* [76]. Further, dietary β-glucans increased the survival rate of catfish (*Pangasianodon hypophthalmus*) against *Edwardsiella tarda* [77]; increased the survival rate of grass carp infected with grass carp hemorrhage virus [38] or oral administration of the combination diet of β-glucan (100 µg β-glucan + 10 µg LPS) enhanced resistance of carp against *A. hydrophila* [1]. In contrast, administration of dietary β-1,3-glucan from Eulena gracilis (1% in diet) had no effect on the survival rate of unvaccinated or vaccinated rainbow trout with *Y. ruckeri* after 70 or 84 days of treatments [78]. In the present study, the improvement in disease resistance of fish fed with In 0.1%, BLf 1%, GAS1 0.2% and the diets of a combination of these compounds (In 0.1% + BLf 1% + GAS1 0.2%) may be related to the ability of BLf to take up the Fe^{3+} ion, limiting this nutrient by bacteria at the infection site and inhibiting the growth of these microorganisms as well as the expression of their virulence factors [79]. Further, the fermentation of inulin by *Bifidobacterium spp* and lactic-acid producing bacteria, which increase short-chain fatty acids (SCFA) such as lactic and butyric acids, could enhance the fish's immune ability to fight against bacterial infection. As well, an increase of blood lymphocyte + thrombocyte cell proportion was observed on D38 and D41 in this study.

5. Conclusions

Oral administration of the combination (In 0.1%, BLf 1%, GAS1 0.2%) diet and the alone diets such as In 0.1%, BLf 1%, GAS1 0.2% after a 38-day administration term significantly increased the lymphocyte + thrombocyte cell proportions on D38 and at 36h after bacterial injection D41. Further, all treated diets significantly induced a higher survival rate of rainbow trout juveniles after 14 challenges with *A. salmonicida* than the control.

Conflict of interest

The authors have declared that no competing interests exist.

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Chapter 7

General Discussion

1. Rainbow trout: *A Model Species of Study*

Rainbow trout (*Oncorhynchus mykiss*) is an economically important fish and suitable experimental organism in many fields of biology such as genome evolution [1], pathogenesis [2]; liver cancer induction by environmental chemicals and development of strategies for chemoprevention [3]; and for toxicology studies [4], aromatase inhibition [5] as well as nutrition and nutrient metabolism studies [6]. In genome evolution studies, rainbow trout is a salmonid specific whole-genome duplication, which has been the most studied of the teleost fish [1]. Indeed, study of genomic resources (e.g., linkage maps) was used to evaluate genome conservation between rainbow trout and three teleost models, medaka, stickleback and zebrafish. Moreover, in the research of *Loma salmonae* pathogen, gill tissues from experimentally infected rainbow trout used to transmit the parasite to other trout. The results indicated that *L. salmonae* can have a complete life cycle in trout and produce viable spores [2]. Rainbow trout have also been utilized as a model for human cancer. This species demonstrates a remarkable sensitivity to mycotoxin and potent human liver carcinogen, aflatoxin B1 [3]. It has been reported that there is more mechanistic toxicology research in the rainbow trout than in any other fish species. Furthermore, the trout has a number of advantages as a model system compared to the smaller laboratory fish because of its relatively large size allowing physiological manipulations and experimentation [4]. According to Hahn et al. [4] fish have close evolutionary relationships with mammals, thus the mechanisms discovered in fish are more likely to be relevant for humans therefore, using fish as biomedical models that will allow the discovery of fundamental principles that can be extrapolated to humans. The present study has been carried out within the framework of the AquaStress (P7/31) *Aquatic systems under multiple stress: a new paradigm integrating aquaculture and ecotoxicology research* funded by the Belgian Science Policy Office (BELSPO). In order to contribute to the understanding of how to increase the resistance of farmed fish to diseases, the main focus of this research is to study how improving the nutrient quality can prevent the effects of bacterial stress. Specifically, the research focused on the differential immunomodulatory pathways of various compounds that have been proposed for prophylactic strategies of humans and domestic mammals but not of aquaculture fish. Therefore, we selected rainbow trout juveniles as a relevant species and development stage in this study.

2. Route of Administration

It has been reported that injection of immunostimulants into fish might enhance the function of leukocytes and improve the ability to fight against pathogens. But, this method is labour intensive, time-consuming, impractical with very small fish, and usually causes stress to the fish. The immersion method can also be applied but the results varied greatly depending on the experimental conditions. It has been reported that carp immersed in levamisole solution at a concentration of 10µg/mL for 24h activated phagocytic activities, chemotactic abilities, increased the production of active oxygen of head kidney phagocytes, and increased ability to fight against *A. hydrophila*. This positive effect lasted over 2 weeks [7]. Further, rainbow trout immersed with glucans or chitosan showed increased protection against *A. salmonicida* after 3 days of treatment, but no effects were recorded after 14 days [8]. Dietary administration of immunostimulants has been reported as a promising administration method to increase the leukocyte functions and protect against infectious diseases in various fish species including rainbow trout. This method is non-stressful and permits mass administration regardless of fish size. For instance, Sakai et al. [9,10] reported that oral administration of

BLf (100 mg/kg body weight of fish) by intubation for three days resulted in increased survival of rainbow trout juveniles to *Vibrio anguillarum* infection but the relative immune modulation was not well clarified apart from an increase in phagocytic activity of kidney cells. Recently, Rahimnejad et al. [11] reported that feeding rainbow trout a diet supplemented with BLf at 100 mg/kg or higher for 8 weeks enhanced lysozyme activity, haemolytic complement activity and antiprotease activity. Moreover, Ortiz et al. [12] evaluated the effects of inulin or FOS-containing diets (5g and 10g kg^{-1}) and reported a significant increase in body weight gain, gross energy and Ca content in rainbow trout. Further, Lauridsen et al. [13] reported that dietary β-1,3/1,6-glucans (0.2% diets, for 46 days) fed to rainbow trout increased resistance against the challenge with *Ichthyophthirius multifiliis* (white spot disease). Dietary administration of yeast cells (*Saccharomyces cerevisiae*) for 30 days treated with beta-mercaptoethanol significantly promoted the growth performance and enhanced the resistance against *Yersinia ruckeri* [14]. Oral administration of bovine lactoferrin has been reported to protect red sea bream *Pagrus major* against white spot disease [15], enhanced resistance to *Aeromonas hydrophila* infection in giant freshwater prawns [16], stimulated a high survival rate of Nile tilapia juveniles [17]. Furthermore, the oral administration method of inulin has been reported to increase serum immunoglobulins, bactericidal activity and anti-protease activity in grass carp [18]. The increase of serum complement activity, IgM level, leukocyte phagocytic activity and leukocyte respiratory activity were reported in gilthead seabream [19]. Blood respiratory burst activity increased after 7 weeks of feeding treatment with inulin at 5 to 10g kg^{-1} in common carp fry [20]. Moreover, the effects of β-glucans have been reported in several fish species, for instance, against pathogens of Asian catfish [21,22], common carp [23], catla [24], Nile tilapia [25], rohu [26], and zebrafish [27] when infected with *Aeromonas hydrophila*, grass carp [28] infected with grass carp haemorrhagic virus, large yellow croaker infected with *Vibrio harveyi* [29]; and rohu infected with *Edwardsiella tarda* [30].

3. Treatment Duration and Immunostimulant Doses

The effects of administration duration and doses in aquaculture are very important issues. Before beginning an experiment, we have to decide the treatment time as well as select the location we use the long-term or short-term administration of immunostimulants. However, the administration time of immunostimulants to fish is still unclear and no fixed rules for the administration model treated time or dose for aquaculture species are available. It may depend on many biological factors such as species, size of fish (fry, fingerling, juvenile or mature fish), physiological and disease status and water temperature. It seems rational to use high dose levels over a short-time experiment, and low dose levels over a long-time experiment, but the available results do not follow the same logic and vary depending on the immunostimulant molecule. Oral administration of bovine lactoferrin did not exhibit any change in immune gene expression after a 15-day test in rainbow trout juveniles. Meanwhile, after 51 days, there was a remarkable change of pro-inflammatory genes (*il-1*, *il-6*, *il-8*); T-helper genes (*cd4-2α*, *cd4-2β*) and anti-inflammatory genes (*tgf-β1*, *il-10*) in spleen and kidney tissues [31]. Moreover, oral administration of inulin to rainbow trout juveniles did not present any change of immune gene expression after a short-term test (15 days). While, inulin stimulated a significant change of *il-1*, *il-10*, *mcsfra* in spleen and kidney tissues after a long-term test (51 days). Qinghui et al. [29] reported that a low dose of β-1, 3 glucan supplementation (0.09%) significantly enhanced fish growth, phagocytosis percentage, respiratory burst activity and low cumulative mortality after bacterial challenge whereas high supplementation (0.18%) did not increase growth rate. Meanwhile, the high dose of beta glucan (0.18%) significantly decreased phagocytosis percentage and respiratory burst activity after 8-week treatments.

4. Effect of Bovine Lactoferrin, Inulin and β-glucans in Diets on Immune Responses and Disease Resistance of Rainbow Trout Juveniles

Bovine Lactoferrin:

Bovine lactoferrin in diets did not affect the specific growth rate of fish, but remarkably affected the blood leukocyte populations, blood plasma complement, total immunoglobulin, and lysozyme activity as well as upregulated several immune relative genes after long-term treatment (D51). Both low (0.1%) and high (1%) doses of bovine lactoferrin remarkably increased the survival rates of rainbow trout juveniles after 14 days injected with *A. salmonicida*. However, no effects of bovine lactoferrin were obtained on immune genes after the short-term treatment (D35). Bovine lactoferrin compound has many functions such as antibacterial, antifungal, antiviral, antiparasitic, antitumor and anti-inflammatory. The most important function of bovine lactoferrin is the iron absorption and transcription even though the responses of rainbow trout on iron absorption pathways were not examined in this study. Lactoferrin is secreted by some acinar cells, stored in secondary granules of neutrophil cells and represented in various secretory fluids, such as milk, saliva, tears and nasal secretions [32]. The results from the current study may explain that bovine lactoferrin has positive effects to rainbow trout functioning because it's iron absorption and transportation ability from the blood stream to the fish body immune cells leading to a better immune functional development. In our study, the significant different immune response of the trout immune system between D35 and D51 found its basis in the absence of change as induced by the BLf diets, in 13 immune genes expression levels in spleen and kidney organs on D35. Meanwhile, the immune system was significantly modulated by increasing pro-inflammatory (*il-1*, *il-6*, *il-8*, *mcsfra*) and T-helper genes (*cd4-2α*, *cd4-2β*) on D51, anti-inflammatory (*tgf-β1, il-10*) and lysozyme genes on D54 by both dietary BLf doses. The first activation of bovine lactoferrin in rainbow trout juveniles may stimulate the changes in blood leukocyte populations leading to the change of humoral immune parameters such as blood plasma complement and antibody production (Ig) on D35. The blood leukocyte cell formation, development, differentiation and migration from blood stream to specific organs such as the spleen and kidney may need a necessary treatment time to process, therefore 51 days — a long-term bovine lactoferrin treatment in this study was necessary to modulate a significant increase of several immune relative genes.

Inulin:

Inulin was proposed as a prebiotic additive in feed nutrition of fish since it is known that it can modulate the immune functions and improve fish resistance to infections. In this study, inulin diets did not affect specific growth rates, but an inulin diet induced significantly high plasma activity levels of alternative complement (ACH50) on D35 and D51, total immunoglobulins (Ig) and spleen macrophage respiratory burst on D51. Inulin diets affected different proportions of leukocyte populations on D35 or D51. Over a long-time treatment (51 days), inulin diets significantly affected the immune gene expression in spleen or kidney whereas no significant effects of inulin on immune gene expression were observed after a short-term treatment whatever the inulin doses on D35. It has been reported that inulin is a naturally occurring fructooligosaccharide extracted from the chicory root as well as from other plants and belongs to a class of carbohydrates known as fructans [18,33]. Fructans are non-digestible oligosaccharides as the β (2-1) linkage of the fructans cannot be hydrolysed by pancreatic or brush border digestive enzymes in the proximal intestinal tract of human and

domestic animals [34]. In the current study, we can speculate that inulin affected the fish immune system of rainbow trout indirectly by the fermentation activities of *Bifidobacterium spp* and lactic acid producing bacteria, which increase short-chain fatty acids such as lactic and butyric acids, even if such processes were not studied. The significantly different response of the trout immune system between D35 and D51 found its basis in the absence of change of immune gene expression levels for the 13 genes assessed in spleen and kidney on D35. On D51, the immune system was significantly modulated as shown by the increase of pro-inflammatory genes (*il-1, mcsfra*) and decrease of T-helper genes (*cd4-2β*) and anti-inflammatory (*tgf-β1*) genes. Pro- and anti-inflammatory immune response after bacterial injection seemed the highest in the spleen of fish fed low inulin dose, even if both doses increased the pro-inflammatory *il-8* gene expression in kidneys. Therefore, these better immune pathway responses can explain the better protection for trout juveniles by low inulin dose as shown by the significant increase in the survival rate after 14 days of bacterial challenge, when compared with the control and high inulin dose. We do not find any different effect of inulin on immune gene expression between spleen and kidney organs of rainbow trout juveniles after short- and long-term experiments. Regarding the effect of inulin in diets on gut microbiota, because of large number of tasks on this project, we did not analyse gut microbiota in this study. However, it has been reported that inulin is a fermentable fibre (*prebiotic*) that provides a substrate for microbiota within the large intestine, increasing the faecal bulk and producing short-chain fatty acids [35], enhancing the growth and activities of bacteria and inhibiting growth or activities of certain pathogenic bacteria [36]. Because inulin provides a substrate to develop beneficial bacteria such as bifidobacteria and lactic acid producing bacteria leading to competition between the beneficial bacterial and pathogenic bacteria in order to reduce the microbiota pathogen agents.

Beta Glucans:

Beta glucans are immunostimulant compounds used by the aquaculture industry to enhance the immune response of fish. It is a heterogeneous group of glucose polysaccharides consisting of a backbone of β (1, 3)-link β -D-glucopyranosyl units with β-(1, 6)-link side chains of varying length and distribution [27], but their efficiency may vary according to their source origin. The most common forms of β-glucans are those comprising D-glucose units with β-1, 3 links. But, yeast and fungal β-glucans contain 1–6 side branches, meanwhile cereal β-glucans contain only β-1, 3 and β-1, 4 backbone bonds [37]. The frequency, location, and length of the side-chains may play a role in immunomodulation. Differences in molecular weight, shape, and structure of β-glucans indicate the differences in biological activity [38]. In this study, low doses (0.2% in diet) of newly extracted β-glucans (GAS 1 β-glucans) significantly and positively affects the immune system of rainbow trout by increasing the monocyte proportion, plasma lysozyme activity on D15, total antibody production (Ig) on D36 as well as increasing several immune genes such as *mIgM* in the kidney, TLR_2 in the spleen and kidney, *MPO* in the spleen, *CATH1* in the spleen at 35h after bacteria injected. Further, low doses of GAS1-β-glucan (GAS1 0.2%) displayed a significantly higher survival rate for rainbow trout juveniles than the control after 14 days challenged with *A. salmonicida*. Meanwhile, low and high doses of Macrogard β-glucan and WT β-glucan induced a decrease in several immune gene expression levels such as *il-1β* on D36; *tgf-β1* on D15, *il-10* on D15 and D36, *cd4-2β*, *mIgM*, TLR_2, *hepcidin* on D15 and *lysozyme* gene on D36. In this study, we propose that the newly extracted GAS 1 β-glucans induced a better activation of the immune system of rainbow trout by two ways: (i) enterocytes facilitate the transportation of β-glucans compounds across the intestinal cell wall into the lymph to interact with macrophages in order to activate immune functions [39]; (ii) β-glucans are better fermented by bifidobacterium and lactic acid producing bacteria to produce more butyric acid — a short-chain fatty acid that

improves the fish immune system [40]. Indeed, dietary GAS1-β-glucan at low doses (G0.2%) significantly modulated the immune system of rainbow trout juveniles through the increase of humoral (*mIgM*) in kidney, membrane protein (TLR_2) both in the spleen and kidney, neutrophils (*MPO*) in the spleen, and *CATH1* in the spleen at 35h after bacterial injection (D39). Improved immune pathway responses could explain the better protection for trout juveniles by low dose of GAS1 β-glucan as shown by the significant increase of the survival rate after 14 days of bacterial challenge, when compared with the control and other β-glucan doses.

5. Impacts of Immunostimulant Compounds on Pellet Feed Price, Toxicity and Environmental Pollution

Bovine Lactoferrin:

In this study, we received bovine lactoferrin (BLf) as a gift from Campina DOM Company of Netherlands. The company welcomed us to test their BLf product, but they did not tell us the price. We have checked the price of bovine lactoferrin from Sigma Company and it is quite expensive, €74.40/10MG (L9507) to €257.0/50MG (L9507), that is to say €5.14–€7.44/mg or €5140–€7440/g. So, we believe that the use of BLf in aquaculture feed may significantly increase the price of pellet feed, but this price needs to be compared with another analytical supplier that may be more affordable. Luckily, our results demonstrated that only low doses may be required to stimulate the fish immune functions. Further studies are recommended to determine the most efficient lactoferrin dose. To our knowledge, there is no publication report about environmental pollution caused by lactoferrin as well as no toxic factors released in the surrounding environment relevant to lactoferrin compound, which is an advantage compared to use of antibiotics or prophylactic agents. In fact, lactoferrin is a natural multifunctional protein that is found in human milk and cow's milk. Especially high concentrations of lactoferrin come from the first milk (colostrum) of humans. The concentration of lactoferrin in human milk varies from 7 g/L in the colostrum to 1 g/L in mature milk. In the cow, the concentration in mid-lactation milk is very much lower 0.1 g/L [32]. So, it is now limited in production, which means the price is still high placing a barrier to the ability to use this compound in real aquaculture production.

Inulin:

In this study, we received inulin as a gift from Beneo - Orafti® Company - Rue L. Maréchal 1 - B - 4360 Oreye, Belgium. Inulin is a naturally occurring fructooligossacharide (FOS) extracted from the chicory root (*Cichorium intybus*) as well as from many other plants (cereals, leeks, onions, garlic, wheat, artichokes and bananas) and belongs to a class of carbohydrates known as fructans [18,33]. Fructans are non-digestible oligosaccharides as the β (2-1) linkage of the fructans cannot be hydrolysed by pancreatic or brush border digestive enzymes in the proximal intestinal tract of human and domestic animals [34]. It is fermented in the large intestine or colon [41] by beneficial bifidobacteria and other lactic acid producing bacteria, enhancing their relative population while selectively inhibiting the growth of pathogenic microorganisms [42,43]. We investigated the effect of inulin at low 0.1% and high 1% doses in the diets for 51 days at 1.5% of fish body weight/day. In the frame work of this experiment, we found that an inulin diet at 0.1% has much greater positive effects on rainbow trout immune system, and induced a significantly higher survival rate than the control after bacterial infection. Meanwhile, a high dose of inulin at 1% in the diet did not show significant survival protection after 14 days' challenge with bacterial infection. To our knowledge, there

is no publication about the toxicity or pollution of inulin in the surrounding environment. The price of inulin on the market varies according to the sources, types of inulin in the production. The approximate price of 1 kg inulin is around €50 (Some typical inulin products can be higher priced while some inulin products are lower priced) Because of the effort of the company to lower the price of inulin products, inulin can be used in pellet food production in order to improve rainbow trout immune ability against pathogen infection.

Beta Glucans:

In this study, we received two types of a pure, newly extracted β-glucans (GAS 1 and Wild Type β-glucan) as a gift from the laboratory by the University of Gent, Belgium and the commercial Macrogard β-glucans as a gift from Biorigin Company, Rua XV de Novembro, 865-Lençois Paulista-SP-Brasil. β-glucans are heterogeneous groups of glucose polysaccharides consisting of a backbone of β-(1,3)-link β-D-glucopyranosyl units with β-(1,6)-link side chains of varying length and distribution [27]. β-glucans are a major structural component of fungi, bacteria, plants, algae, yeast, and mushroom cell walls. But, GAS 1-β-glucans and Wild Type β-glucans are extracted from baker's yeast cell walls that have long β-1,6 branching and are insoluble in water [38], fungus β-glucan has short β-1,6 branching, and are insoluble in water [44] meanwhile, other β-glucans extracted from the bran of cereal and bacteria has no branching and are soluble in water [44]. Regarding toxicity, Túrmina et al. [44] reported that sub-chronic treatment of β-(1;6)-glucans (lasiodiplodan, 50 mg/kg of mouse body weight daily for 28 days) did not generate haematological and histopathological alterations leading to signs of toxicity in healthy mice independent of gender. Further, Chen et al. [45] showed that Sprague Dawley rats (12/sex/group) administered (gavage) mushroom β-glucan at dose levels of 0, 500, 1000 and 2000 mg/kg body weight/day for 90 days, did not result in any toxicologically significant treatment-related changes in clinical observations, ophthalmic examinations, body weights, body weight gains, feed consumption, haematology, serum chemistry parameters or terminal necropsy. Moreover, Delaney et al. [46] reported that there were no adverse effects on general condition and behaviour, growth, feed and water consumption, feed conversion efficiency, red blood cell and clotting potential parameters, clinical chemistry values, and organ weights. Necropsy and histopathology findings revealed no treatment-related changes in any organ evaluated. To date, we have not found any publication about the toxic effects of β-glucans on fish as well as β-glucans causing environmental pollution. However, it was reported that overdoses of β-glucans and/or prolonged medication can lead to a non-reactive physiological status and poor immune response [47]. Concerning the price, the price of β-glucans is very high, depending on the variety of glucans, production companies, sources and type of production. β-glucan production on the market can be in tablets or powder. In the pharmacy, the price of β-glucans is from €65–€237.8/gram (€6,500–€238,000/kg). So unfortunately, the use of β-glucans in aquaculture feed could significantly increase the price of pellet feed, but other glucan compounds of technical quality instead of analytical quality, may be more affordable. Moreover, low doses over a suitable treatment time are required.

6. Fish Immune Response Targets and Experimental Sampling Organs

Fish are free-living organisms from the embryonic stage of life in their aquatic environment. Therefore, fish rely on their innate immune system from the early stages of embryogenesis [48]. The components of the innate immune response are divided into physical, cellular, humoral factors, and cellular receptor molecules that are soluble in plasma and other body fluids. The lymphoid organs found in fish include the thymus, spleen and kidney. Immunoglobulins are the principal components of the immune response against pathogenic

organisms. The kidney in teleost fish is the equivalent of the bone marrow in vertebrates and is the largest site of haematopoiesis [49]. In rainbow trout, the kidney is well developed after hatching and it mainly produces red blood cells, granulocytes [48]. In fish, the spleen is a major secondary lymphoid organ. It contains the blood vessels, ellipsoids, red pulp and white pulp. The pulp consists of a reticular cell network supporting blood-filled sinusoids that hold diverse cell populations, including macrophages and lymphocytes [50]. Based on the research protocols of this study, we planned to collect fresh blood samples, blood plasma and spleen and kidney tissues to analyse for several immune parameters and the expression of several immune-related genes. However, other immune relevant organs such as gut, liver and integumentary surface have also been well documented, and they should be taken into account. According to Secombes et al. [50], leukocytes are abundantly present in the lamina propria and intestinal epithelium of the fish gut including lymphocytes, macrophages, eosinophilic and neutrophilic granulocytes. Furthermore, fish have a local mucosal defence in the gut and produce a local immunoglobulin response [51,52]. Mulder et al. [53] reported that a classic profile of pro-inflammatory cytokine upregulation was observed in the proximal intestine of rainbow trout infected with *A. salmonicida* by bath challenge, as determined by semi-quantitative RT-PCR. Moreover, high ratios of $cd8\alpha^{+}$ cells are found in trout intestine as well as in thymus and gill, but have a relatively low abundance in the head kidney, spleen and blood [54]. Regarding the liver, it has been reported that this organ is responsible for protein, carbohydrate and lipid metabolism and detoxification. However, the fish liver has a large impact on immune gene expression after bacterial infection [55] suggesting that the fish liver is actively involved in immune defence [50]. On the other hand, the mucosa-associated lymphoid tissue in teleost fish (gut, skin and gill) is an important barrier in fish, and covers most of the external surface, especially the skin. Surface mucus provides a substrate which contains many immune molecules including antibacterial agents [56], anti-viral components [57] and intelectins [58]. Mucus secreted by goblet cells reduces the ability of bacteria to adhere to underlying enterocytes [59]. It has been reported that mucus is significantly increased when fish are subjected to a stressful situation [50]. Altogether, several organs of fish can be used for sampling. However, the criteria used to select the sampling organs in fish depend on the objectives and design of the study.

7. Toll-Like Receptors and Their Roles in Fish Fed Immunostimulant Diets

Toll-like receptors (TLRs) are a class of proteins that play a role in the innate immune system. They are single, membrane-spanning, non-catalytic receptors expressed on sentinel cells such as macrophages, dendritic cells or natural killer cells, cells of the adaptive immunity (T and B lymphocytes) and non-immune cells (epithelial, endothelial cells and fibroblasts) that recognize structurally conserved molecules derived from microbes. Since, these microbes have reached physical barriers such as the skin or intestinal tract mucosa, they are recognized by TLRs that activate immune responses [60]. It has been reported that β-glucans bind to specific cell surface receptors of macrophage, neutrophil, granulocytes and modulated the fish immune system by identifying invasive or disseminated fungal infections [61–63]. The effects of β-glucans have been reported to help Asian catfish fight against pathogens [21,22], common carp [23], catla [24], Nile tilapia [25], rohu [26], and zebrafish [27] when infected with *A. hydrophila*, grass carp [28] infected with grass carp *haemorrhagic virus*, large yellow croaker infected with *Vibrio harveyi* [29]; and rohu infected with *E. tarda* [30]. Lauridsen et al. [13] reported that dietary β-1,3/1,6-glucans (0.2% diets, for 46 days) given to rainbow trout increased resistance against the challenge with *Ichthyophthirius multifiliis*. Furthermore, dietary administration of yeast cells (*Saccharomyces cerevisiae*) for 30 days (5×10^7 CFU/g of yeast cells) treated with beta-mercaptoethanol significantly enhanced the resistance against *Yersinia ruckeri* [14]. TLRs activate pathways through a variety of TIR-domain-containing

adaptor proteins responsible for the cell signalling cascade activation, such as MyD88 and TRIF, which activate transcription factors (NF-kB, IRFs) and mitogen-activated protein kinases (MAPKs) to promote inflammatory response modulating innate and adaptive immune responses [64]. Several studies in mammals have shown that some plants and algae compounds can down regulate mRNA expression [65–67], induce TLRs [68,69], suppress the TLR/NK-kB signalling pathway [70], enhance TLR9 and IRF7 activation inducing IFN-β protein expression [71] and elicit the M1 and M2 macrophage responses through the TLR/NF-kB signalling pathway [72]. In teleost fish, at least there are 17 TLRs that are identified with some homology to mammals, and some are unique for teleosts [73]. However, TLRs also demonstrate a very large set of distinct features and diversity in fish species [74]. In the last decade, increased attention has been paid to the use of different sources of prebiotics as immunostimulant dietary supplements in fish to reduce susceptibility to various stressors and diseases, as well as enhance the overall health in fish [75,76]. The effects of plant, herbal and algae extracts have been recorded in a number of publications. However, in most cases, the mechanisms responsible for the physiological pathways in fish are poorly described and still not well known [77]. Ryu et al. [72] reported that oleuropein — a primary phenolic compound present in olive leaves — suppressed the release of pro-inflammatory cytokines such as interleukin-1β (*il-1β*) and interleukin-6 (*il-6*), inhibited the translocation of p65 by suppressing phosphorylation of inhibitory kappa B-α (IκB-α), decreased activation of ERK1/2 and JNK, inhibited LPS-stimulated NO generation, reduced inflammatory responses by inhibiting TLR and MAPK signalling in a zebrafish model. The immunostimulant administration method through diet is easy to follow recognition, identification and response to pathogen associated molecular patters (PAMPs), which can be used to modulate immunological activity. The most commonly used PAMPs in fish immunostimulant diets are lipopolysaccharide (LPS), peptidoglycan (PGN) and β-glucans [78]. Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria. It is recognized by TLR4 and forms a complex with co-receptors lymphocyte antigen 96 (MD2), and CD14 on the cell surface that serves as the main LPS-binding components and leads to activation of multiple signalling components and the subsequent production of pro-inflammatory cytokines [79]. In our study, GAS 1 β-glucan has a much more beneficial affect on rainbow trout immune function than Macrogard and WT β-glucan. The low dose of GAS 1 β-glucan (G0.2%) significantly modulated the immune system of rainbow trout juveniles through the increase of humoral (*mIgM*) in kidney, membrane protein (TLR_2) both in spleen and kidney, neutrophils (*MPO*) in spleen, *CATH1* in spleen at 35h after bacterial injection (D39), protected a higher survival rate of rainbow trout juveniles after 14 days of bacterial challenge. The efficiency of β-glucans may vary according to the source. But, yeast and fungal β-glucans contain 1–6 side branches, meanwhile cereal β-glucans contain only β-1, 3 and β-1, 4 backbone bonds [37]. The frequency, location, and length of the side-chains may play a role in immunomodulation. Differences in molecular weight, shape, and structure of β-glucans indicate the differences in biological activity [38].

8. Immunostimulant Diets Using Pathogen Associated Molecular Patterns (PAMPs)

Pathogen associated molecular pattern recognition is mediated by pathogen recognition receptors (PRRs) that are structurally and functionally conserved between vertebrates, invertebrates and plants and allow the host to distinguish among different classes of pathogens in order to mount an appropriate immune response [80]. These immune responses and activities are mediated through the induction of inflammatory cytokines, chemokine and co-stimulatory molecules that facilitate leukocyte recruitment to the site of the infection,

activation of adaptive immunity and activation of antimicrobial effectors [81]. The most commonly used PAMPs in immunostimulant diets are β-glucans. This major structural component of yeast and fungal cell walls is widely used in fish diets such as the commercial production of Biosaf, DVAQUA, Ecoactiva, Ergosan, Fibosel, MacroGard, and VitaStim, Chitin, LPS, mannan-oligosaccharides (MOS), PGN, and yeast extract [78]. The effects of β-glucans have been reported to help Asian catfish fight against pathogens [21,22], common carp [23], catla [24], Nile tilapia [25], rohu [26], and zebrafish [27] when infected with *A. hydrophila*, grass carp [28] infected with grass carp *haemorrhagic virus*, large yellow croaker infected with *Vibrio harveyi* [29]; and rohu infected with *E. tarda* [30]. Lauridsen et al. [13] reported that dietary β-1,3/1,6-glucans (0.2% diets, for 46 days) given to rainbow trout increased resistance against challenge with *Ichthyophthirius multifiliis*. Furthermore, dietary administration of yeast cells (*Saccharomyces cerevisiae*) for 30 days (5×10^7 CFU/g of yeast cells) treated with beta-mercaptoethanol significantly enhanced the resistance against *Yersinia ruckeri* [14]. The reactions to viral, bacterial, parasitic and fungal pathogens vary according to the pathogen-associated molecular patterns (PAMPs) on the surface of the invader [82]. In fish, phagocytic cells produce reaction oxygen species upon stimulation by PAMPs such as zymosan, β-glucan. Specific knowledge on the reaction pathways in macrophage host cells may be obtained by dissecting expression of central genes in inflammation. This also elucidates whether immunostimulants mimicking different pathogens initiate distinctive reactions and production of specific effector molecules targeting different pathogen types [83]. In our studies, dietary GAS1-β-glucan at low dose (G0.2%) significantly modulated the immune system of rainbow trout juveniles through the increase of humoral (*mIgM*) in kidney, membrane protein (TLR_2) both in spleen and kidney, neutrophils (*MPO*) in spleen, *CATH1* in spleen at 35h after bacterial injection (D39), which led to a higher survival rate for rainbow trout compared to the control. It has been confirmed that, a diet supplemented with 0.2% of GAS 1 β-glucan was more effective in stimulating the trout immune system than Macrogard and WT β-glucan.

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Chapter 8

Conclusions and Perspectives

1. Conclusions

Oral administration of different types of immunostimulant diets such as bovine lactoferrin, inulin and β-glucans has several positive effects in rainbow trout juvenile's immune functions for instance, changing the blood leukocytes population, humoral immune parameters, immune related genes and bacterial resistance. The results obtained from this study have demonstrated that the application of immunostimulants is a valuable method for disease prevention in rainbow trout culture, especially in rainbow trout juvenile production.

The low dose of inulin (0.1% diet) showed positive effect to rainbow trout immune functioning with the highest survival (46%) after bacterial injection for 14 days. But, higher dose of inulin level at 1% diet is recorded for some negative effects to rainbow trout immune system with a significantly decline of the survival rate (7%) after bacterial injection 14 days.

Both low and high doses of bovine lactoferrin (BLf 0.1% and BLf 1% diet) are positive effects to rainbow trout immune functioning. Bovine lactoferrin diets modulated the change of blood leukocytes population, humoral immune parameters, immune related genes and bacterial resistance. Those doses protect survival rate of rainbow trout juveniles after 14 days injected with *A. salmonicida*.

Regarding of treatment times, bovine lactoferrin and inulin stimulated a significantly increase of several immune-related genes after 51 days (long term nutrients test) but did not present any change of immune genes expression on day 15. This recommended that inulin and bovine lactoferrin are much more times and doses dependent.

Low dose of GAS1-β-glucan (GAS1 0.2%) has been showed the best compound effecting on rainbow trout immune system and bacterial resistance. This confirmed that the effects of β-glucans are much dependent on β-glucan types and doses.

The combination dietary bovine lactoferrin (BLf 1%), inulin (In 0.1%) and GAS1-β-glucan (GAS1 0.2%) showed the positive effects on rainbow trout immune system and significantly protect a higher survival rate than the control experiments. This established that combination dietary those immunostimulants is efficiently replacing antibiotic in rainbow trout juveniles against bacterial infection by *A. salmonicida*.

2. Perspectives

The fermentation process of inulin and β-glucans in large intestinal may produce several types of short-chain fatty acids (especially, butyric acid level) which has been reported to effect on fish immune . Therefore, it would interest to measure the butyric acid levels in fish intestinal to confirm the mechanism activity of inulin in the fish diets.

The large number of leukocyte cells of the fish are located in the posterior gut where contributed a huge number of bacteria in gut contents. Therefore, it may possible to sample the gut tissues to analysis of leukocyte cell populations, respiratory burst activity, especially to analysis for the expression of immune related genes.

Numerous studies have been tested the effects of β-glucans on fish immune system for instance: Asian catfish, common carp, catla, Nile tilapia, rohu, and zebrafish, grass carp, large yellow croaker, rohu…). The effects of β-glucans on fish immune system also have been

reported to be doses dependent. It would interest to test the effects of β-glucans on fish immune system at low doses (< 0.2% β-glucan in diets) to clarify the function of β-glucans at the low doses.

To our knowledge, the first effect activities of bovine lactoferrin, inulin and β-glucan may modulate the blood leukocyte cells formation and differentiation (formation, development and migration). Therefore, it would interest to study on how effect of this immunostimulants on the differentiation and migration of leukocyte cells between blood stream, spleen and kidney organs.

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