Molecular Characterization of Diverse Mechanisms of Mucosal Immunity in Teleost Fishes

by

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Abstract

The mucosal barriers (gill, skin and intestine) of fish constitute the first line of defense against aquatic pathogens invasion. By exploring and targeting fish mucosal immune system, we can rationally design better vaccines and immunostimulants to maximize fish health and prevent infectious diseases. *Flavobacterium columnare*, the causative agent of columnaris disease, causes ulcerative, necrotic, ectopic infection resulting in tremendous losses in farmed fish globally. A recently developed mucosal vaccine has been demonstrated to provide superior protection for channel catfish (*Ictalurus punctatus*) against columnaris. I examined the mechanisms of this protection by comparing transcriptional responses to *F. columnare* challenge in vaccinated and unvaccinated control catfish. Transcriptome profiling at early time points post *F. columnare* infection revealed a basal polarization in vaccinated fish gills and a central role for eosinophilic granular cells. Vaccinated fish were armed with an array of preformed mediators by vaccination, but had lower expression of pro-inflammatory genes after secondary infection.

The rhamnose-binding lectin (RBL1a) was previously identified by our group as a potential mediator of *F. columnare* adhesion and exposure of fish to its carbohydrate ligand, L-rhamnose, prior to challenge decreased pathogen adherence and protected catfish from columnaris mortality. However, rhamnose is prohibitively expensive. Therefore, in my second study, I examined whether rhamnolipids (RLs), an affordable and commercially available alternative, would offer host protection against *F. columnare* similar to that provided by L-rhamnose through feeding trials. Dietary RLs studied here increased susceptibility of channel

catfish to columnaris disease, associated with a robust upregulation of RBL1a expression immediately following challenge and a suppression of mucin and lysozyme production.

In my final project, considering the importance of RBL in fish immunity, I identified four putative RBL genes from Nile tilapia (*Oreochromis niloticus*) and characterized their expression profiles. They were significantly expressed by exposure to *Streptococcus agalactiae*, another important fish pathogen and one of the major causes of streptococcosis in farmed tilapia, in at least one tissue (gill or intestine) or time point. Taken together, my research will expand our knowledge of fish mucosal immunity and contribute to the development of effective immunotherapies for disease prevention.

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Chapter I Introduction and literature review

The immune system is a host defense system comprising a complex organization of cells and molecules with specialized biological structures, roles, and processes within an organism to protect the host from a universe of constantly evolving pathogenic microbes (viral, bacterial and parasitic) and to maintain host homeostasis under environmental stressors [1]. In vertebrates, the immune system consists of systemic compartments in diffuse organs that are dispersed throughout most of the tissues of the body (not confined to the site of infection). The mucosal surfaces represent a dynamic surface interface between the host and the external environment and while concurrently carrying out many other vital physiological processes, they constitute the host's first line of defense against invading pathogens and other exogenous threats [2,3]. Teleost fish species rely more heavily on mucosal barriers than their terrestrial counterparts as they are directly interacting with pathogen-rich aquatic environments and continuously colonized by diverse commensal and pathogenic organisms [4]. During the event of pathogen invasion, mucosal tissues are the first barrier that the pathogen needs to break through in order to establish the infection and the result of this interaction determines the consequence of invasion, which could be either further penetration of the pathogen or blocking of the invasion by the host. This molecular interplay between host and pathogen also governs the severity and chronicity of infection. Therefore, for a sustainable aquaculture, better understanding of host-pathogen interaction is of great importance for developing effective vaccines and efficient preventive strategies to protect fish from infectious disease.

1. Fish mucosal immunity

Fish mucosal surfaces act as physical barriers and function as active immune tissues to surveil the environment and protect the host against pathogen infection [5]. They can also carry out a variety of other critical physiological processes, such as nutrient and oxygen absorption, osmoregulation, environmental sensing, and waste excretion [6]. With the new molecular sequencing techniques, substantial progress has been made in characterization of the mechanisms and pathways of mucosal immunity in many teleost fish species including rainbow trout (O*ncorhynchus mykiss*) [7,8], Atlantic salmon (*Salmo salar*) [9,10], olive flounder (*Paralichthys olivaceus*) [11], Atlantic cod (*Gadus morhu*) [12] turbot (*Scophthalmus maximus*) [13] and catfish (*Ictalurus spp*.) [14–23]. However, there are still numerous significant gaps in our understanding of the cellular actors and detailed information regarding host-pathogen interaction is only available for a small number of fish species with limeted pathogens [24,25].

Same as the systemic immune system, the mucosal immune system of vertebrates can be divided into two subsystems, the innate and adaptive immune systems, which both contain the cellular and humoral immune components [25]. Innate immunity is a fundamental and nonspecific defense system which targets all types of pathogens. Adaptive or acquired immunity is specified for its ability of creating an immunological memory after an initial response to a specific pathogen and driving an enhanced secondary pathogen-specific immune response when the host is attacked by the same pathogen again. The mucosal immune system of fish is primarily comprised of three mucosa-associated lymphoid tissues (MLATs) of gills, skin and intestine, as well as the associated innate and adaptive immune cells and humoral molecules [25]. Over the last decade, many areas of intense research in aquaculture have hinged upon a better understanding of mucosal immunity in aquaculture species.

1.1 Mucosal tissues

As one of the major organs of fish that directly contact and communicate with the surrounding environment, fish gills possess multifunctional physiological roles including respiration, osmoregulation, nitrogenous waste excretion, hormone production [26], and immune response [6]. Gills of teleost fishes have hundreds of filaments and numerous secondary lamellae attached to the arches, with cores of cartilages, connective tissue, blood vessels, nerves, and immune cells [27,28]. For pathogenic bacteria, adhesion to host surfaces is a pre-requisite for colonization and infection and gill epithelium is often considered an easy site for waterborne pathogen adhesion and is the primary site of attack of many infectious agents [6]. A number of pathogens have been reported to attach to or uptake across the gills in fish including *Yersinia ruckeri*, *Flavobacterium columnare*, *Edwardsiella tarda*, *Aeromonas salmonicida*, *Pasteurella piscicida*, *Vibrio anguillarum*, infectious salmon anemia virus (ISAV), the ectoparasite *Neoparamoeba perurans* and the ciliated protozoan *Ichthyophthirius multifiliis* (reviewed by [29]). Bath exposure to live *Y. ruckeri* resulted in an immediate presence of the pathogen in gill mucus and subsequent invasion of the gill epithelium in rainbow trout [30]. *N. perurans* can vegetate at the gill epithelial surface and cause hyperplasia in epithelial cells [29]. *F. columnare* is the causative agent of columnaris disease that causes high mortality in Ictalurid catfish species particularly [31]. Several studies have pointed to the rapid invasive potential of *F. columnare*, particularly virulent isolates, through the mucosal barriers including gill tissue. *F. columnare* can adhere and colonize the gill epithelium within 1 h of challenge and cause local necrosis within 2 h [32,33].

Fish skin is another critical regulatory organ and serves in many important physiological processes, including communication, sensory perception, locomotion, respiration, ion regulation,

excretion, and thermal regulation [2]. Anatomically, teleost skin can be divided into three layers: cuticle or mucous layer, epidermis and dermis (reviewed by [5]). The outermost layer of cells is alive and it retains the capacity to divide. The epidermis is a stratified epithelium (surface, intermediate, and basal layers). The surface layer is a single-celled layer of squamous cells with only minimal quantities of keratin that develops microridges at the outer surface. The intermediate layer of epidermis is composed of various types of cells, including unicellular glands (such as mucous cells and club cells), sensory cells, ionocytes, immune cells, pigment cells, and undifferentiated cells. The basal part of the epidermis is a single-cell layer (basal layer) that tightly links to the dermis. The dermis is composed of a collagenous matrix while the hypodermis contains loosely organized collagen and vasculature. In addition to being a mechanical barrier, it represents a metabolically dynamic tissue. Fish skin is considered the largest immunologically active organ and plays a frontier role in defending host against pathogen invasion [2]. In order to trap and immobilize pathogens before they reach the epithelial layer, skin mucus is continuously secreted and replaced. Some studies have demonstrated that the skin mucus of some fish species is able to inhibit the adhesion of certain pathogenic bacteria. For example, seabream (*Sparus aurata*) skin mucus inhibits the adhesion of *P. piscicida*, *Tenacibaculum maritimum*, and *V. damsela*, while many bacterial pathogens have been reported to have a strong ability to adhere to fish skin mucus, such as *F. columnare* and *A. hydrophila* [5]. Pathogen infection is often associated with changes of mucus in the mucosal tissues. For example, *A. hydrophila* infection rapidly altered the gene expression of mucus to enhance its ability to invade catfish skin [15,16].

Each mucosal tissue contains an important immune component, that is mucus layer. Mucus of a fish provides physical protection by trapping pathogens and preventing their attachment and

invasion to the underlying epithelial surfaces, and also serves as a reservoir for many innate immune factors with antimicrobial activities, such as mucins, lysozymes, lectins, complement proteins, and antimicrobial peptides [34–36].

Fish intestine is a complex multifunctional organ central to digestion and absorption of nutrients, water and electrolyte balance, endocrine regulation, metabolism, immunity, pathogen recognition, and regulation of the intestinal microbiome, therefore, it has been subjected to intense research [37]. The intestinal epithelium is generally composed of a single layer of specialized simple columnar epithelium, lamina propria, blood vessel, nerves, collagenous matrices and immune cells [6]. The intestinal health of fish is of special interest for the fish farming industry for many reasons. Primarily, farmed fish stocked at high densities are susceptible to intestinal infections, with the gut being an important point of entry for pathogens. *E. ictaluri* infection in catfish is typically associated with intestinal barrier destruction [14,38]. Similarly, the intestine is also an important route of infection for *A. salmonicida* and *V. anguillarum* [39]. Additionally, aquaculture fish are typically fed commercial feed pellets, which opens up avenues for manipulating fish health through the incorporation of various feed additives, drugs and vaccines into the feed. Intestine is one of the most important targets of dietary immunostimulants, prebiotics, probiotics and oral vaccines [5]. Finally, the intestinal microbiome is a key component of the mucosal barrier, plays an important role in fish health by stimulating immune response, aiding in nutrient acquisition, and outcompeting opportunistic pathogens [40]. Hence, a comprehensive understanding of the diet-gut interactions, gut microbiome respond to dietary manipulations, and immunoregulatory properties of intestinal epithelium in fish could aid in the development of new effective measure for disease prevention and control in aquaculture.

1.2 Mucosal innate immunity

1.2.1 Humoral innate immunity

The fish mucosal immune system contains a wide variety of innate immune molecules including mucins, lectins, complement proteins, cytokines and antimicrobial peptides (AMPs), lysozyme, protease inhibitors and lytic enzymes, etc. [3,25].

Mucins

Mucins are a family of high-molecular weight glycoproteins saturated with O-linked sugars [41]. To-date, 19 mucins have been identified in human, and their roles in the immune defense have been elucidated [42]. They are one of the most important components in fish mucus. Mucin secretion in skin, gill and intestine responds to complex signals from the environment, host, pathogen, and the commensal microbiome [6]. In common carp (*Cyprinus carpio*), Mucin5B is mostly expressed in the skin, and its expression was up-regulated by administration of β-glucan [43]. Expression patterns of MUC2, MUC5AC, MUC19 and related isoforms in catfish gill were also examined following exposure to the common freshwater pathogen, *F. columnare* [6]. Higher mucin expression was likewise noted in the gills of catfish fed with prebiotics (yeast cell wall components) compared to control fish before and after *F. columnare* challenge [19]. Moreover, following experimental challenge with *A. hydrophila*, microarray analyses revealed differential expression patterns of MUC5AC, MUC5B at early time points in the more susceptible channel catfish but not in blue catfish [15,16]. Similarly, expression changes of MUC2 and MUC5B were observed in the channel catfish intestine following *E. ictaluri* infection [14].

Lectins

Lectins are a diverse group of carbohydrate-binding proteins and glycoproteins that can recognize specific carbohydrate moieties including specific microbial surface glycans [44,45]. They are widely present in both invertebrates and vertebrates, ranging from virus, bacteria, fungi, protista, plants, to animals, including teleost fishes. Vasta et al. (2011) reviewed that fish lectins have key roles in innate immunity, not only for pathogen recognition, but also for additional effector functions such as agglutination and complement- mediated opsonization and killing [46]. They were also found be indirectly involved in the adaptive immune responses in fish, as regulators of adaptive immune responses [46]. C- type lectins (CTLs), F-type lectins (FTLs), galectins, rhamnose-binding lectins (RBLs), mannose-binding lectins (MBLs), pentraxins and intelectins have been identified in diverse teleost fishes [46,47]. However, few studies examined their roles in mucosal tissues. Previous studies by our group have found that lectins, including RBLs, MBLs and galectins were rapidly and strongly regulated in response to a variety of infections in catfish gills, skin, and intestine (reviewed by [6]), pointing to the critical roles of these molecules during the early stages of infection in catfish.

Rhamnose-binding lectins are composed of one or multiple characteristic carbohydrate recognition domains (CRDs) particularly recognizing L-rhamnose or D-galactose without Ca^{2+} dependence [47]. They are crucial elements associated with immune responses to infections and have been characterized from a variety of teleost fishes. Previously, our group characterized the broader RBL family in channel catfish and their expression showed degrees of changes in the mucosal tissues post bacterial infection [48]. Transcriptome profiling in channel catfish gill highlighted a high expression of an RBL at 3 h following *F. columnare* infection [17]. Higher expression of this RBL was observed also in susceptible channel catfish gills under basal

conditions and following infection [18], as well as in fasted catfish gills [49]. Saturation of the RBL with its carbohydrate ligands, L-rhamnose or D-galactose prior to *F. columnare* challenge substantially lowered its expression, columnaris mortality, and pathogen adhesion [49].

Mannose-binding lectins are an important member of the collectin (collagen-containing C-type lectin) family [50]. They are capable of inducing the lectin-dependent complement system and have been identified in many fish species [51–55]. Some MBL members were found to be highly induced in catfish skin following *A. hydrophila* infection [15] or in resistant channel catfish gills compared to susceptible gills following *F. columnare* challenge [18].

Galectins are a family of β-galactoside-binding lectins with multiple roles in embryogenesis, host early development and innate immunity [56]. Broder galectin family characterization and expression profiling have been carried out in channel catfish [57]. Most members were highly expressed in mucosal tissues, but their expression post infection varied depending on both pathogens and tissue types. After *A. hydrophila* infection, galecin-3 was strongly induced at 2 h in channel catfish skin [16] and the expression of galectin-4 was significantly downregulated at 24 h in blue catfish skin [15]. Galectin-4 has been observed to be downregulated by short term deprivation in channel catfish as well [23].

Complement system

The complement system, an important component of the innate immune system, is present in both vertebrates and invertebrates and plays a crucial role in aiding humoral immunity, promotion of inflammatory reactions, elimination of apoptotic and necrotic cells and most importantly, destruction of pathogens [25]. Three main biochemical pathways can activate the complement system in mammals: 1) the classical pathway triggered by antibody binding to the

cell surface, 2) the alternative pathway (independent of antibodies) activated directly by pathogens, and 3) the lectin pathway activated by the binding of a protein complex consisting of MBL in bacterial cells. All three pathways are present in teleost fishes [58]. Moreover, the presence of several complement components (C1, C3, C4, C5, C7, C9, and factor B, D and P) has been demonstrated in mucosal tissues of many teleost fishes (reviewed by [25]). C3 is the central complement molecule of all three pathways and has been identified in the gills and intestine of Atlantic salmon [59], Atlantic halibut (*Hippoglossus hippoglossus*) [60], and Atlantic cod (*Gadus morhua*) [61]. Complement regulatory protein genes and two complement factor genes were characterized in channel catfish [62,63] and some of them were significantly regulated after bacterial infections in mucosal tissues, suggesting their important roles in immune responses to bacterial infection in catfish.

Cytokines

Cytokines include a broad category of small proteins that can mediate cell signaling within the immune system, including chemokines, interleukins (ILs), interferons (IFNs) and some other cytokine families, such as colony stimulating factors, tumor necrosis factors (TNFs) and transforming growth factors (TGFs) [29]. They are secreted by activated immune-related cells upon induction by various pathogens (parasitic, bacterial, or viral components) [64]. However, studies regarding their roles in teleost mucosal sites are still lacking.

Chemokines represent a superfamily of chemotactic cytokines involved in cell mobilization for immune surveillance, inflammation, and development [65]. They have been identified in many fish species, such as zebrafish, catfish and rainbow trout. The genome-wide characterization of chemokine superfamily members has been conducted in channel catfish and

the expression of some members significantly changes in mucosal tissues in response to bacterial infection and/or hypoxic conditions [66,67].

Interleukins are molecules involved in the intercellular regulation of the immune system [68]. In mammals, 35 interleukins are currently described and many direct homologues of them, such as IL-1, IL-2, IL-10 and IL-17 subfamily are present in fish. They can be classified into pro-inflammatory cytokines (such as IL-1β, IL-8, IL-17 and IL-22), and anti-inflammatory cytokines (such as IL-1 receptor antagonist, IL-4, IL-10 and IL-13). Of these, IL-17 is an important mediator of mucosal immune responses and has been demonstrated in several RNA-seq studies [6]. IL-17 ligands and receptors homologues have been characterized in channel catfish, their expression has been profiled in mucosal tissues following bacterial infection [69] and some members can be highly induced at as early as 4 h post-infection, particularly in gill tissues.

Interferons are a family of cytokine mediators critically involved in alerting the cellular immune system in response to viral infection of host cells [70]. They were also demonstrated to have important roles during bacterial and parasite infection [71–73]. IFNs are classified into two main groups, type I IFN and type II IFN. They have been identified in many teleost fishes. Fish IFN responses are mediated by the host pattern recognition receptors and an array of transcription factors including the IFN regulatory factors, such as the suppressor of cytokine signalling (SOCS) molecules [73]. In mammals, the type I IFN has been reported to have a key role in early immune events at the mucosa [72]. IFN stimulation has been observed in catfish gills within 24 hours following *F. columnare* infection [17].

Other humoral factors

Antimicrobial peptides (AMPs) with antimicrobial activity and lysozymes with bacteriolytic activity are both well-studied innate immune components in fishes and are abundant in fish mucus [74]. Alpha-2-macroglubilin can act against pathogen proteases [75], and lytic enzymes, such as cathepsins, may have important roles in fish mucosal immunity but they were explored in few fish species [76,77]. More studies are needed to expand our knowledge of the humoral innate immune components in fish mucosa.

1.2.2 Cellular innate immunity

The main cellular components of the mammalian innate immune system are natural killer cells, mast cells, neutrophils, eosinophils, basophils, macrophages and dendritic cells [25]. In addition to the intrinsic immune system, there are several other cell types that are especially relevant in mucosal tissues of teleost fishes, including epithelial cells, mucus-producing cells, neuroendocrine cells, and neuroepithelial cells [25].

Macrophages and neutrophils are the main phagocytic cell types of the innate immune system and are also present in mucosa-associated lymphoid tissues of teleost fishes [25]. Phagocyte-mediated antimicrobial responses have been widely studied in fish [78–80]. These cells can remove pathogens or particles by production of reactive oxygen species (ROS) during a respiratory burst and/or lysozymes and other hydrolytic enzymes in their lysosomes [58]. In addition, macrophages can produce antibacterial agents, such as nitric oxide (NO) [81]. Neutrophils are the most abundant cells of the innate immune system and are well known to be the first immune cells that arrive at sites of infection or damage [82]. In fish-pathogen interactions, neutrophils play a critical role in the initial defense against pathogens through phagocytosis, release of granule proteins and other antimicrobials, production of ROS, and

furthermore mediating the inflammatory response by recruiting and activating other immune cells [83,84].

Mast cells (MCs) are sometimes referred to as eosinophilic granule cells (EGCs) in fish (EGC/MCs) [85]. EGC/MCs have been identified in many teleost fishes and are often located at the mucosal sites interacting with environment, such as skin, gills and intestine [85–94]. The term EGCs was first introduced in fish to indicate some epidermal cells resembling mast cells morphologically, but with red granules upon staining with hematoxylin and eosin [95]. They were considered to be analogous of mammalian mast cells for their cytochemical and histochemical characteristics, structure and functional similarities [85,86,91,96]. Fish EGC/MCs were demonstrated to have important roles in fish immune defense [85]. They contain a wide range of bioactive compounds, including proteases, histamine, heparin, lysozyme and antimicrobial peptides. EGC/MCs can also react to exposure of parasites, killed bacteria, bacterial products, and toxicants by releasing components and stimulating other immune cells and/or mechanisms [87,90,91,93,97–100]. Although often overlooked, mammalian MCs can also be a very critical piece of the immunological memory, increasing in numbers following primary infection and rapidly preventing and controlling reinfection [101]. This feature demonstrates the potential of using mast cell activators as effective vaccine adjuvants to confer protective immunity against microbial pathogens [102]. Moreover, MCs can stimulate fibroblast proliferation, collagen deposition and wound healing [103–105]. However, these functions need to be confirmed in fish EGCs/MCs.

Epithelial cells are important in fish mucosal defense, as one of the first sensors of commensal and pathogenic organisms. Epithelial cells are active orchestrators of homeostasis, microbial colonization, innate and adaptive immune responses [106]. They may express

pathogen recognition receptors (PRRs), including lectins, nod-like receptors (NLRs) and toll-like receptors (TLRs), be responsible for antigen uptake, and be involved in transport of mucosal Igs (reviewed by [25]). On the other hand, many fish pathogens can target these mucosal epithelial cells causing severe damage, such as *F. columnare*, *E. ictaluri*, *V. anguillarum* and *Amyloodinium ocellatum* [13,14,33,107].

Mucus-producing cells, frequently referred to as goblet cells, are columnar epithelial cells that synthesize and secrete gel-forming mucins, the major components of mucus [6]. They are the dominant mucus cell type in fish intestinal epithelium and are also abundant in fish skin and gills. Consistent with the changes of mucin levels described before, goblet cell numbers vary in response to signals from environment, host and pathogen [6].

Neuroendocrine cells and neuroepithelial cells are also present in the fish mucosal tissues [108–110]. Although it is not well understood yet, it seems that they can interact with the local immune systems and maintain the homeostasis of the immune and other physiological functions through their secreted hormones, neuropeptides or neurotransmitters [111–113].

1.3 Mucosal adaptive immunity

1.3.1 Humoral adaptive immunity

The principle components of the humoral adaptive immune system are the immunoglobulins (Igs). In mammals, IgA is the main Ig involved in mucosal immune responses and immune exclusion of commensals, whereas, there is no IgA found in fish. In teleost fish, three Ig isotypes have been described: IgM, IgD and IgT/IgZ [114–116]. IgM has been reported to be present in several fish mucosal secretions and is involved in responses against several pathogens (reviewed by [114]), although the antibody response intensity varies among fish

species (reviewed by [25]). IgD has been identified in many fish species, including channel catfish, but its role in mucosal immunity remains unclear [114,116–118]. However, the detection of IgD transcripts in vaccinated rainbow trout with a mucosal vaccine and the much higher ratio of IgD to IgM plasma cells in the gills compared to the systemic lymphoid tissues may indicate a role of IgD in mucosal immunity [4]. The third teleost immunoglobulin class, IgT/IgZ, was discovered in 2005, and it has been found in all studied teleost fishes until now, except channel catfish [6]. IgT has recently been shown to behave as the prevalent immunoglobulin in gut mucosal immune responses [114]. IgT, similar to mammalian IgA, is the only teleost Ig isotype with a specialized mucosal function as demonstrated in the gut of rainbow trout [119].

Additionally, comparable to mammalian mucosal surfaces, polymeric immunoglobulin receptor, pIgR is also expressed in the gut mucus of rainbow trout [119], the skin mucus of fugu (*Takifugu rubripes*) [120], and the gill and skin of channel catfish [23]. The pIgR is an epithelial glycoprotein that is involved in the transport of IgA and IgM across mucosal epithelium in mammals [121]. In teleosts, pIgR is associated with IgM and IgT, indicating an evolutionary conservation between fishes and mammals [25].

1.3.2 Cellular adaptive immunity

Same to higher vertebrates, the main adaptive immune cells in fish are B-lymphocytes and T-lymphocytes. The main role of B cells in adaptive immunity appears to be the recognition of antigens in their native form and function as antibody-secreting cells (ASC) [25]. Channel catfish possess three B cell subsets: IgM+/IgD+, IgM+/IgD−, and IgM−/IgD+, without any IgT+ B cells identified [122]. Following immunization of catfish against *I. multifiliis* (Ich), a common skin parasite of channel catfish caused by a ciliated protozoan, the numbers of IgM secreting plasma

cells were shown to be higher in the skin of immune versus the non-immune catfish [6]. In rainbow trout, two populations of B cells (IgD+/IgM+/IgT− and IgD−/IgM−/IgT+ B cells) have been characterized. IgM+ B cells are the major population of B cells in the systemic lymphoid immune tissues, but in the gut, IgT+ B cells account for more than half of all the B cells and their percentage increases after a parasite infection in the gut [119]. IgD secreting cells were also identified in rainbow trout skin and they have a higher ratio compared to IgM secreting cells [119]. However, our overall knowledge and studies on B cells in teleost mucosa-associated lymphoid tissues (MALTs) is still limited.

T cells play an essential role in cell-mediated immunity and they are very important in creating tolerance or immunity against pathogens in mucosal surfaces [4]. T cell populations (cytotoxic T cells, helper cells (Th1, Th2 and Th17) and regulatory T cells (Tregs)) in mammals have been described in some teleost fishes. CD4 is the marker of T helper cells, which can acquire Th1, Th2 or Th17 phenotypes depending on their cytokine secretion. Th1/Th17 promote the overexpression of pro-inflammatory genes, while Th2 are likely to promote specific effective humoral responses. In response to *F. columnare* infection, the resistant catfish skewed toward a Th2 phenotype with high IL-4 expression and much lower pro-inflammatory gene expression compared to control fish [19]. Similar results were also witnessed in Atlantic salmon gills and skin [123]. Cytotoxic T cells were defined by expressing CD8 T cell surface molecules and mainly function in clearing pathogen-infected epithelial cells in mucosal barriers [4]. The Tregs are another important subset of T cells, which are found in high numbers in mucosal tissues where they may have cytolytic and immunoregulatory functions [6]. Tregs have been described in some fish species, such as European sea bass (*Dicentrarchus labrax*) [124] and channel catfish [125]. However, we have no information regarding Tregs in mucosal tissues of teleost fishes.

2. Host-pathogen interactions

Host-pathogen interaction is defined as how pathogens sustain themselves within host organisms and whether they cause disease or not [126]. Host-pathogen interaction studies provide information that can help scientists and researchers understand disease pathogenesis, the biology of pathogens, as well as the biology of the host. Pathogens develop a wide range of strategies to attempt to subvert or avoid the host defense system to establish their infection and cause diseases, while at the same time, hosts establish their physical and mucosal barriers and systemic immunity to defend the infection [127]. Consequently, both pathogen virulence and host susceptibility/resistance determine whether or not the infection establishes. Of course, host-pathogen interactions are complex and can favor one or the other depending on many other factors, such as the host age, physiological and immunological status and the environmental changes that also influence the host susceptibility/resistance and/or the virulence of pathogens.

Recently, our group has utilized channel catfish and *F. columnare* as a model for host-pathogen interaction to understand the dynamics of fish mucosal immunity [6,33]. The attachment and entry through fish mucosal surfaces are the initiation steps of infection. Catfish mucus, including antimicrobial immune components, is continually secreted to physically trap and prevent pathogen attachment and invasion. But on the other hand, bacteria can utilize the mucus layer to facilitate the disease processes. Mucus secreted from the skin and gills can potentially promote chemotaxis of *F. columnare*, particularly the highly virulent strains [128]. The adhesion ability of *F. columnare* to the mucosal tissues, as well as the associated mucus coverings, is an important aspect in the pathogenesis of columnaris disease. We revealed a potential lectin-mediator (a rhamnose-binding lectin; RBL1a) of *F. columnare* adhesion [17]. *F.*

columnare was able to induce the expression of catfish RBL genes and bind to RBL through their surface glycoconjugates to mediate their invasion [19,20,49].

After attachment, pathogens can penetrate and disrupt the mucus barriers, secrete virulence factors, and avoid, suppress or subvert the host's immune defense. It has been reported that pathogens can disrupt the cellular junctions present in the epithelium to facilitate invasion of the host. Transcriptomic profiling of channel catfish gills and skin showed rapid and robust alternations in the expression factors related to junctional processes after *F. columnare* challenge [18]. The perturbed expression of the cell junction related genes was also observed in catfish mucosal tissues in response to *E. ictaluri* [14] and *A. hydrophila* [16] infections. Chondroitin AC lyase, proteases, and sialic acid secreted by *F. columnare* are all important virulence factors related to the pathogenesis of columnaris disease [33]. Chondroitin AC lyase degrades the complex polysaccharides in connective tissues, proteases contribute to damaging the tissue or enhancing invasive processes, and sialic acid can inhibit the catfish alternative complement pathway (ACP). Apoptosis is an immune evasion mechanism of some pathogens that can modulate the host immune responses to their advantage, including promoting apoptosis in host immune cells programmed to attack them [24].

RNA-seq approach is a powerful tool for studying infectious diseases and exploring host-pathogen interactions and mechanisms of fish immune responses to these infections [24,129,130]. It utilizes next-generation sequencing technologies to provide transcriptome profiling in a high-throughput and quantitative manner. It facilitates the discovery of novel transcripts and splicing variants independent of previous sequence [131]. The gene signatures gathered from RNA-seq offer insights into strategies the pathogen may be using to evade fish defenses and strategy which resistant fish can employ to gain the upper hand over pathogens.

Ultimately, we may translate these findings into real-world strategies to reduce the incidence of infectious diseases in farmed fish.

3. Exploring mucosal immunity for immunotherapy

Aquaculture has grown rapidly in the last few decades and now provides half of the world's seafood supply. Outbreaks of infectious diseases are major constraints in the development of aquaculture, including in the culture of many commercially important fishes [132]. Antibiotics and chemotherapeutics have long been used to prevent or control bacterial infections in aquaculture. However, for a sustainable development of the aquaculture industry, novel strategies to control bacterial infections are needed. This is reflected in the recent implementation of more strict regulations on the use of antibiotics and chemicals, as they are not environment-friendly, may lead to antibiotic resistance, and may cause food safety issue due to the presence of antibiotic residues in aquaculture products [132]. Therefore, the use of environmentally friendly and effective alternative techniques for disease prevention is necessary.

3.1 Mucosal vaccination

Vaccination is an effective and environmentally friendly strategy used to improve immunity for a specific disease prevention in large-scale commercial fish farming [133]. Types of fish vaccines include bacterins or inactivated agents, live attenuated vaccines, recombinant vaccines or subunit vaccines and DNA vaccines [134]. Vaccine delivery routes in fish commonly include intraperitoneal injection, immersion (dip or bath), or oral administration. Recently, mucosal vaccination has emerged as one of the main areas in fish vaccinology and attracted a lot of research. This has been exacerbated by the growing demand for less labor-intensive

vaccination techniques as alternatives to injectable vaccines that require individual handling of fish which may result in stress-related immunosuppression and handling mortalities. Given that mucosal vaccines are administered by immersion, oral, or bath without the need to handle fish individually, mucosal vaccination offers many advantages to the aquaculture industry: 1) less stressful to fish, 2) less labor-intensive, 3) allows large-scale application, and 4) applicable to small fish [135].

Mucosal vaccines, as antigens, can be taken up by the mucosal epithelia of gills, skin or intestine and possibly evoke local immune responses, or may even stimulate systemic immune reactions [135]. However, there are few commercially available mucosal vaccines for fish at the moment and the only licensed mucosal vaccine against columnaris (named AQUAVAC-COL) did not show effective protection against virulent *F. columnare* isolates [136]. Some of the challenges that limited the development of protective mucosal vaccines include: 1) large dose requirement due to instability of antigens in the mucosal surfaces, 2) absence of mucosal adjuvants to improve the performance of inactivated mucosal vaccines, 3) lack of knowledge regarding measures of mucosal vaccine efficacy and correlates of protection, and 4) reduction of systemic responses due to prolonged exposure to oral vaccination [135].

Adjuvants are defined as a group of structurally heterogeneous compounds able to modulate the intrinsic immunogenicity of an antigen, which function as antigen delivery vehicles and immunostimulants [137]. The traditional adjuvants, such as oil-based adjuvants, mainly influence the fate of the vaccine antigen in time, place, and concentration. Recent work on adjuvants has especially focused on targeting specific immune cell responses [138]. One promising new generation adjuvants are ligands/agonists of different PRRs and different cytokines, such as β-glucans ligands for dectin-1. It has been demonstrated that furunculosis

vaccine supplemented with β-glucans could induce significantly higher protection than vaccines without this adjuvant in Atlantic salmon [139]. Mast cell activators also have been reported as a new class of highly effective vaccine adjuvants, particularly for mucosal vaccines [102], as mast cells are abundant in mucosal tissues and are loaded with secretory granules containing inflammatory mediators (that were recognized as important inducers of the innate immune response, regulators of immune-cell migration and activation, and can be formed between first and second pathogen exposure [140].

Many indicators have been applied for analyses of vaccine success, including cumulative mortality post challenge, specific antibody level and immune-associated gene expression analysis [137]. Antibodies are the classically reported vaccine-induced immune effectors [141–145], while these are imperfect measures in light of the multi-faceted nature of secondary responses to infection at mucosal surfaces. Immune-associated gene expression studies have revealed the participation of immune cells and other factors in the vaccine protection [146–149]. The gene expression analysis, particularly by genome-wide transcriptome sequencing (RNA-seq) is a relevant approach to understand the vaccine-induced immune protection mechanisms. Thorough understanding of the of the molecular mechanisms underlying the mucosal vaccine protection and the features of some critical mediators in the fish mucosal immunity will lead to the development of potentially better mucosal vaccines and adjuvants for the growing aquaculture industry.

3. 2 Immunostimulation

In addition to vaccination, application of immunostimulants to boost or stimulate the immune system of farmed fish is an environmentally friendly, sustainable, and effective

approach for preventing infectious diseases in fish [150]. Immunostimulants are natural or chemical substances that have a modulatory effect upon the immune system. They provide protection to fish mainly by triggering nonspecific immune responses after administration [132]. Immunostimulants include chemical agents such as levamisole, bacterial derivatives such as lipopolysaccharide (LPS) and peptidoglycan (PGN), nucleotides, polysaccharides such as β-glucans and mannan-oligosaccharides (MOS), animal or plant extracts, nutritional factors such as vitamin C, and certain hormones and cytokines (reviewed by [132]). Some of them are pattern-associated molecular patterns (PAMPs) that can be mediated by specific pathogen recognition receptors (PRRs), such as MOS ligand for MBL and β-glucans ligands for dectin-1 [151]. Vallejos-vidal et al. (2016) have reviewed the use of PAMPs as immunostimulants in fish in the past 15 years [151].

Oral administration is the most cost-effective means of utilizing immunostimulants to protect fish in a large-scale aquaculture. Immunostimulants have been used as feed additives for several years to maintain fish health and improve performance in cultured fish [150–153]. Typically, the efficiency of immunostimulants is assessed using pathogen challenges following feeding. The most commonly used immunostimulants in fish are glucans, bacterial/yeast components, and plant extracts/derivatives. They have been used in a wide range of fish species and were found to protect fish from bacterial pathogens including *A. hydrophila*, *A. salmonicida*, *E. ictaluri*, *E. tarda*, *Streptococcus agalactiae*, *S. iniae*, *V. alginolyticus*, *V. anguillarum*, and *V. harveyi* [132]. While the effects of immunostimulants on the systemic immune system have been studies extensively, few researches have been conducted regarding their effects on mucosal immunity of fish [132]. Recently, it has been demonstrated that dietary MOS can offer distal mucosal protection against *F. columnare*, potentially through modulating key mucosal gene

pathways [19]. Another attractive pattern-associated molecular pattern (PAMP), rhamnose has been emerged to protect catfish from columnaris disease by blocking the expression of RBL in gills [49]. Pre-exposure L-rhamnose could decrease columnaris mortality and pathogen adherence via the down-regulation of one RBL gene in gills, suggesting that the provided carbohydrate blocked pathogen adherence via the lectin receptor [49], while no study evaluated its protective effect via feeding in fish.

Rhamnose, a deoxyhexose sugar, is widely found in bacteria and plants but not in humans [154,155]. It has two isomers (L and D) and the former is much more common. L-rhamnose has been found to be a common component of the cell wall and/or capsular polysaccharides of bacteria, such as many Gram-positive species of the genera *Streptococcus*, *Enterococcus* and *Lactococcus*, and some Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Helicobacter pylori* [156,157]. L-rhamnose is often associated with bacterial virulence and viability [156]. It has also been reported to be an important component of analyzed glycopeptide and extracellular polymeric substance (EPS) fractions of *F. columnare* [158,159] suggesting its importance in both cell wall structures and biofilm formation. The relevance of L-rhamnose for bacterial pathogenesis has made its biosynthetic pathway an appealing target for novel therapeutic interventions of many pathogens (reviewed by [156]). The L-rhamnose is prohibitively expensive, limiting its practical application in commercial aquaculture. A potential affordable alternative source of rhamnose such as rhamnolipids may be evaluated and widely used as diet additives with immune-stimulating ability in farmed fish [160].

Rhamnolipids are glycolipid biosurfactants containing rhamnose as the sugar moiety linked to β-hydroxylated fatty acid chains [161]. They can be produced by various bacteria and are also often important for bacterial pathogenicity (reviewed by [162]). RLs have antimicrobial

activity against a number of bacteria [163] and are able to reduce the adhesion and interfere with biofilm formation of some food pathogenic bacteria [164]. As stimulators of human and animal immunity, RLs have been shown to be involved in triggering defense responses and can be described as a new class of microbe-associated molecular patterns MAMPs [163,165]. As surfactant products, RLs have been utilized in a number of industries due to the above mentioned features and their low toxicity and high biodegradability [166]. Mass-produced purified RLs are now commercially available at a low cost [167] and the distal rhamnose group in almost all the homologs remains generally free [168]. Above all, rhamnose-containing RLs are a potential cost-effective source of purified rhamnose and may modulate the fish immune system to protect fish from bacterial infections [160].

5. Dissertation study overview

Columnaris disease, caused by the *F. columnare*, is a significant hindrance to the production of commercially important freshwater fish. A recently developed attenuated vaccine (17-23) for *F. columnare* has been demonstrated to provide superior protection for channel catfish. We were interested in the molecular mechanisms of this protection, so in **chapter II**, high-throughput RNA-seq was utilized to compare the early transcriptional responses to *F. columnare* challenge between vaccinated and unvaccinated juvenile catfish in gill tissues collected pre-challenge (0 h), and 1 h and 2 h post infection. Additionally, our previous study revealed that a rhamnose-binding lectin (RBL1a) can mediate *F. columnare* adhesion and RBL carbohydrate ligand L-rhamnose can effectively protect catfish from columnaris . However, it is prohibitively expensive, and we sought to check the potential of rhamnolipids (RLs) as an alternative cost-effective source of rhamnose, so in **chapter III**, we studied the impact of oral

and waterborne administration of RLs on the susceptibility of channel catfish to columnaris. Regarding the importance of RBL, we want to determine whether Nile tilapia, another commercially important freshwater fish, utilizes the RBL compartment in the same fashion as channel catfish. So, in **chapter IV**, we characterized the sequence feature of tilapia RBLs and detected their expression patterns in mucosal tissues post following experimental infection with *S. agalactiae*, one of the major causes of streptococcosis in farmed tilapia.

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Chapter II More than just antibodies: Protective mechanisms of a mucosal vaccine against fish pathogen *Flavobacterium columnare*

Abstract

A recently developed attenuated vaccine for *Flavobacterium columnare* has been demonstrated to provide superior protection for channel catfish, *Ictalurus punctatus*, against genetically diverse columnaris isolates. We were interested in examining the mechanisms of this protection by comparing transcriptional responses to *F. columnare* challenge in vaccinated and unvaccinated juvenile catfish. Accordingly, 58 day old fingerling catfish (28 days post-vaccination or unvaccinated control) were challenged with a highly virulent *F. columnare* isolate (BGSF-27) and gill tissues collected pre-challenge (0 h), and 1 h and 2 h post infection, time points previously demonstrated to be critical in early host-pathogen interactions. Following RNA-sequencing and transcriptome assembly, differential expression (DE) analysis within and between treatments revealed several patterns and pathways potentially underlying improved survival of vaccinated fish. Most striking was a pattern of dramatically higher basal expression of an array of neuropeptides (e.g. somatostatin), hormones, complement factors, and proteases at 0 h in vaccinated fish. Previous studies indicate these are likely the preformed mediators of neuroendocrine cells and/or eosinophilic granular (mast-like) cells within the fish gill. Following challenge, these elements fell to almost undetectable levels (>100-fold downregulated) by 1 h in vaccinated fish, suggesting their rapid release and/or cessation of synthesis following degranulation. Concomitantly, levels of pro-inflammatory cytokines (IL-1b, IL-8, IL-17) were

induced in unvaccinated fish. In contrast, in vaccinated catfish, we observed widespread induction of genes needed for collagen deposition and tissue remodeling. Taken together, our results indicate an important component of vaccine protection in fish mucosal tissues may be the sensitization, proliferation and arming of resident secretory cells in the period between primary and secondary challenge.

1. Introduction

Disease outbreaks are often the critical factor restraining the sustainable growth of aquaculture for a particular species. While strategies to combat disease should consider the role of production practices, nutrition, and water quality, ultimately, reliable protection is often achieved only through vaccination. Vaccine development against aquaculture pathogens has lagged considerably behind the growth of the industry, often hindered by lack of efficacy and by lack of cost-effective means of inoculation [1,2]. Mucosal vaccination (via bath immersion or via feeding) has several potential advantages over injection routes, reducing fish handling and labor, more closely mimicking natural routes of infection, and triggering fulminant immune responses [3–6]. Our understanding of the mechanisms underlying vaccine-induced protection against disease in fish is also severely limited. In this regard, live attenuated vaccine strains provide several means by which to investigate the basis of pathogen invasion and virulence, host resistance, and immunogenicity. A handful of studies to-date have utilized transcriptomic approaches to examine vaccine-induced gene expression [7–10]. Host genes and pathways differentially expressed following exposure to either a virulent or attenuated isolate can reveal immunosuppressive strategies for pathogenesis [11], potential adjuvant targets [12], critical mediators of host immunity [9,13,14], and/or candidates for marker-assisted selection. In

mammals, similar studies have highlighted the complexity of vaccine-induced responses, with cellular and molecular polarization induced by vaccination extending far beyond rising antibody titers [15–17].

Columnaris disease, caused by the Gram-negative pathogen *Flavobacterium columnare*, is a significant hindrance to the production of commercially important freshwater fish (reviewed by [18]). Characterized by pronounced erosion and necrosis of ectopic tissues including the fin and gills, columaris can cause particularly high mortality in Ictalurid catfish [19], which comprise the largest sector of US aquaculture. Use of antibiotics or chemical treatments in response to columnaris infection has proven either ineffective, unsustainable, or too costly on a commercial basis [18,20]. A commercial live-attenuated vaccine for protection against columnaris in catfish is available in the US [21]. However, research has shown that the commercial vaccine, derived from a genomovar I strain [19], fails to provide adequate protection against more virulent genomovar II *F. columare* strains, due to differences in virulence factors/antigens and rate and persistence of adhesion [22]. Accordingly, the authors have recently described the development and testing of a new genomovar II strain attenuated vaccine (17-23) with superior protection against both genomovars [22,23]. In the present study, we were interested in comparing the transcriptomic profiles of vaccinated (17-23) or unvaccinated catfish fingerlings both prior to and immediately following challenge with a virulent *F. columnare* isolate, time points which have been shown to be critical for adhesion and invasion of *F. columnare* through the fish mucosal barriers, particularly the gills [24-28]. Our results highlight mechanisms of pathogen virulence and host immunity that can potentially be exploited to increase vaccine efficacy and, ultimately, reduce columnaris-related disease mortality.

2. Methods and materials

2.1 *Fish husbandry and experimental vaccination and challenge*

The transcriptomic experiments described here were carried out alongside those in the previously published work [22]. Therefore, detailed protocols of fish husbandry, vaccination, and challenge protocols can be found in the aforementioned citation. Briefly, 21 days post hatch channel catfish fry weighing around 0.05 g were acclimated 9 days prior to vaccination via immersion utilizing an attenuated *F. columnare* genomovar II mutant (17-23). Control fish were reared under the same conditions but immersed in modified Shieh broth instead of vaccine. After 28 days post vaccination, both vaccinated and control fish were challenged by immersion with a highly virulent *F. columnare* genomovar II strain (BGFS-27). The cumulative mortality was recorded and ANOVA analysis was conducted with SAS to determine the statistical significance ($p < 0.001$) of survival rates between vaccinated and control groups over an 8-day challenge period.

2.2 Sample collection, RNA extraction, library construction and sequencing

Gill tissues were collected from each treatment (vaccinated and unvaccinated) at 0 h before infectious challenge and at 1 h and 2 h post infection. Sampled timepoints were chosen based on previous work by our group and others pointing to this period as critical for adhesion and invasion of *F. columnare* [24–28]. At each time point, 10 fish from each tank were sampled and pooled together as a replicate and 3 replicate pools were prepared for each treatment. Fish were euthanized with MS-222 (300 mg/L) prior to the sampling. Equal amounts (approximately 50 mg) of gill tissue were collected from each fish, flash frozen with liquid nitrogen, and stored at -80 ºC until RNA isolation. Total RNA was extracted using the RNeasy Plus Universal Mini Kit

(Qiagen, Germantown, MD) and the concentration and quality were measured on a NanoDrop 2000 instrument (Thermo Scientific, Wilmington, DE). RNA-seq library preparation and sequencing were carried out by the HudsonAlpha Genomic Services Lab (Huntsville, AL, USA). Briefly, TruSeq RNA Sample Preparation Kit (Illumina) was used for cDNA library preparation, following the TruSeq protocol with 2.14–3.25 μg of starting total RNA. The libraries were amplified with 15 cycles of PCR and contained 18 TruSeq indexes within the adaptors. Finally, amplified library yields were 30 μl of 19.8–21.4 ng/μl with an average length of 270 bp, indicating a concentration of 110–140 nM. 18 libraries were clustered in two lanes and sequenced on an Illumina HiSeq 2000 instrument with 100 bp pair end (PE).

2.3 De novo assembly and annotation

Before assembly, raw reads were trimmed by removing adaptor sequences and ambiguous nucleotides. Reads with quality scores less than 20 and length below 30 bp were also trimmed. Then de novo assembly was performed on high quality reads using the Trinity package following three software modules: Inchworm, Chrysalis and Butterfly to reconstruct a large fraction of transcripts [29,30]. Inchworm generated transcript contigs using a greedy extension based on (k-1)-mer (k=25) overlaps After mapping of reads to Inchworm contig bundles, Chrysalis incorporated reads into de Bruijn graphs and then Butterfly processed the individual graphs in parallel, reporting full-length transcripts and paralogous genes. In order to generate a more comprehensive reference transcriptome, sequences generated from both this project and another challenge experiment in the catfish gill (unpublished) were pooled.

The assembled contigs were used as queries against the NCBI zebrafish protein database, the UniProtKB/SwissProt database and the non-redundant (NR) protein database by the

BLASTX program. The cutoff ***E***-value was set at 1e-5 and only the top gene id and name were initially assigned to each contig.

2.4 Identification of differentially expressed contigs

The high quality reads from each sample were mapped onto the assembled Trinity reference using Bowtie software with default parameters. After that the estimation of transcript abundance was performed using RSEM (RNA-Seq by Expectation-Maximization) program. Then the total mapped reads number for each transcript was determined, and furthermore normalized to detect FPKM (fragments per kilobase of exon per million mapped reads). Finally, the Bioconductor edgeR (Empirical analysis of Digital Gene Expression in R) package was used for differential expression analysis based on the read count matrix generated by RSEM package [31]. The read count matrix was normalized in edgeR package by the trimmed mean of M-values (TMM). TMM equates the overall expression levels of genes between samples under the assumption that the majority of them are not differentially expressed. Transcripts with log2 fold change values $\geqslant$ 1.5 and a FDR-corrected p value < 0.05 were included in analysis as differently expressed genes. Contigs with previously identified gene matches were carried forward for further analysis. Functional groups and pathways encompassing the differently expressed genes were identified based on GO analysis. Pathway analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and manual literature review.

2.5 Gene ontology and enrichment analysis

In order to identify overrepresented GO annotations in the differentially expressed gene set compared to the broader reference assembly, GO analysis and enrichment analysis were

performed using Ontologizer 2.0 using the Parent–Child-Intersection method with a Benjammini–Hochberg multiple testing correction [32,33]. GO terms for each gene were obtained by utilizing zebrafish annotations for the unigene set. The difference of the frequency of assignment of gene ontology terms in the differentially expressed genes sets was compared to the overall catfish reference assembly. The threshold was set as FDR-corrected p value < 0.05.

2.6 Experimental validation - qPCR

Twenty four significantly expressed genes with different expression patterns were selected for validation using real time PCR, or qPCR, with gene specific primers designed using Primer 5 software. Primers were designed based on contig sequences (Table 1). Remaining RNA (after RNA sequencing) was used for first strand cDNA synthesis using the qScript™ cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD) according to manufacturer's instructions. The qScript chemistry uses an optimized blend of random and oligo (dT) primers and reverse transcriptase. All the cDNA products were diluted to 250 ng/µl and utilized for the quantitative real-time PCR reaction using the PerfeCta SYBR Green Fastmix (Quanta Biosciences, Gaithersburg, MD) on a CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The thermal cycling profile consisted of an initial denaturation at 95 ºC (30 s), followed by 40 cycles of denaturation at 94 ºC (5 s), an appropriate annealing/extension temperature at 58 ºC (5 s). An additional temperature ramping step was utilized to produce melting curves of the reaction from 65 ºC to 95 ºC. Results were expressed relative to the expression levels of 18S rRNA in each sample using the Relative Expression Software Tool (REST) version 2009 [34]. The biological replicate fluorescence intensities of the control and treatment products for each gene, as measured by crossing-point (Ct) values, were compared and

converted to fold differences by the relative quantification method. Expression differences between groups were assessed for statistical significance using a randomization test in the REST software. The mRNA expression levels of all samples were normalized to the levels of 18S ribosomal RNA gene in the same samples. Test amplifications were conducted to ensure that 18S and target genes were within an acceptable range. A no-template control was run on all plates. qPCR analysis was repeated in triplicate runs (technical replicates) to confirm expression patterns.

Table 1 Primers used for qPCR validation.

Gene	**Forward (5'-3')**	**Reverse (5'-3')**
18S rRNA	GAGAAACGGCTACCACATCC	GATACGCTCATTCCGATTACAG
Beta-microseminoprotein precursor	AAGAGGTCAGTGTTCGTGGGG	GAAAGGCTATTGGCGTTGC
Six-cysteine containing astacin protease 4 precursor	ATTCCCCTCGGTGCTTTG	GTTCCCCAACAGATAGTTCAGC
CD276 antigen precursor，B7-H3	CCTTCACCTGAAGAGGGATATG	AGAGGAAACTCCATCCAGCAC
CD83	AGCAGCAGCAAAGAGGCATA	TAATGTGGCTGCCGGTGAAA
Chymotrypsin-like precursor	GACCGCTCTGCCCATACTGA	TGACACGATTCCGACCTGATA
Complement component C3a	GTCAAGCCCGGTGATGTGA	GGTTCTTGCGGTGATTTGTAG
Fos-related antigen 1	TTTCCAGTGCCAGGACCATC	TCTGCGCCTTTCGAGTTCTT
Galectin-4	CGAAGGGACTACTCCTCAAGAT	ACGCTGTTCAGGGAGGTATG
Gastrotropin	CGACATGGAGACCATCGGAG	AAGTCTCAACCAGCTTCCCG
IgGFc-binding protein isoform X3	ACATCCAGACTCACCTTTCGC	CATAACCCAGCAGACACCAAC
Immunoglobulin M heavy chain	ATATGGAAGGATGCGAGCGG	TTCCCGTTCCAGTCAGAAGC
Inducible nitric oxide synthase form	CTGGCCCGTGTTAATGAGGT	TTGCGTGCATCAAACACCTG
Inter-alpha (globulin) inhibitor H3, inflammation	ACGAAGTGTGGACCCTGTTC	CCGTCTTGGGTAACTCCACC
Interleukin 1, beta	AGGCTTAGAGGAGGTAAAAGAC	CTTATAGTCCTCCTTTGAGGTG
Leukocyte cell-derived chemotaxin 2 precursor	ACGGCTGCGGAAACTATGG	CGTCGAATGGAGCGTAAACC
MHC class II antigen	CGTGGTGCCTGGAGAAGAA	CTCCGGAGAACTCGTCAGTG
Mucin-19-like isoform X1	TCAATCTGGCAATGGAGCA	GCAGTCTTATCACAGCAGTCGT
Mucolipin-1	TTCAGTGGCATGCAGGAGAG	ATGGGCTCCTGAGTTTGGTG
Peptide YY	CGAGGAGCTGGCCAAGTATC	ACCAGGTCAAACCGTCATCC
Proto-oncogene c-Fos	GAAGACCCTGAGCGGAAACA	GTCTCCTGTTGCGGCATTTG
Sodium/glucose cotransporter 1 like	ACAGCCCTCTACACCGTCAC	CCTCGTATCCTCCCACCTTAT
Somatostatin 2 precursor	GACGAGAAACAGAGCAAGCAA	CGCACGATATGACACCGAC
Transferrin	GACTGTGAAACCGGCAGCAC	GCGTAATAGGACGAGGCATCTC

3. Results

3.1. Survival rate of infected fish

Vaccinated fish had significantly lower mortality following virulent *F. columnare* challenge compared with the unvaccinated control group ($p < 0.001$, Fig. 1) as previously reported [22]. After initial mortality, by day 2 no significant additional mortality was recorded in the vaccine treatment, whereas survival continued to decline over the eight day challenge in the unvaccinated control.

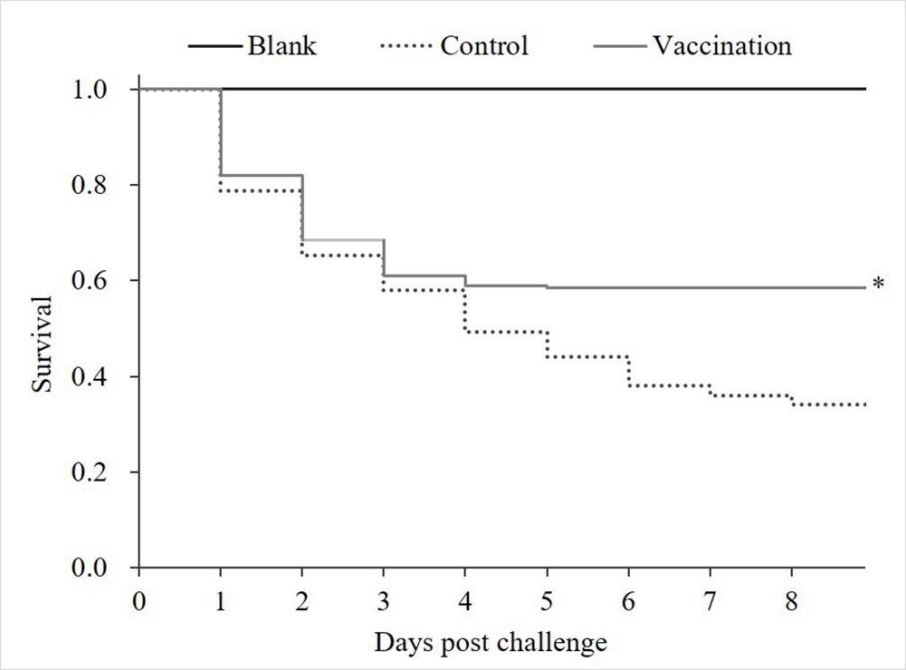


Fig. 1 Summary of the survival rate of vaccinated and control treatment channel catfish after infection with *F. columnare* ($p < 0.001$).

3.2. Sequencing and de novo assembly

Illumina-based RNA-sequencing (RNA-seq) on gill samples from vaccinated and unvaccinated control fish generated greater than 23 million reads for each of the 18 libraries. De

novo assembly of the channel catfish gill transcriptome by Trinity generated 380,180 contigs with N50 contig size of 2,041 bp. Over 85% of reads mapped to the final composite assembly and over 73% of reads mapped in pairs (Table 2). Raw read data were archived at the NCBI Sequence Read Archive (SRA) under the Accession **SRP070957** and this Transcriptome Shotgun Assembly (TSA) project has been deposited at DDBJ/EMBL/GenBank under the Accession **GEHJ00000000**. The version described in this paper is the first version, **GEHJ01000000**.

Table 2 Summary of de novo assembly results of Illumina sequence data from channel catfish gill using Trinity.

Contigs	380180
Large contigs (≥ 1000 bp)	100365
N50 (bp)	2041
Average contig length	980.2
Reads mapped in pairs	73.14%
Reads mapped in final reference (%)	85.56%

3.3. Gene identification and annotation

BLAST-based gene identification was performed to annotate the channel catfish gill transcriptome and inform downstream differential expression analysis. After gene annotation, 105,898 Trinity contigs had a significant BLAST hit against 23,462 unique zebrafish genes (Table 3). A total of 21,826 quality unigenes were identified based on hits to the zebrafish database with the more stringent criteria of BLAST score >100 and *E*-value ⩽ 1e-20. The same BLAST criteria were used in comparison of the Trinity reference contigs with the Uni-Prot and NR databases.

Table 3 Summary of gene identification and annotation of assembled catfish contigs based on BLAST homology searches against various protein databases (Zebrafish, UniProt and NR). Putative gene matches were at *E*-value ⩽ 1e-5. Hypothetical gene matches denote those BLAST hits with uninformative annotation. Quality unigene hits denote more stringent parameters, including score ⩾ 100, *E*-value ⩽ 1e-20.

	Channel catfish		
	Zebrafish	UniProt	NR
Contigs with putative gene matches	105898	97159	110852
Annotated contigs ≥500 bp	94178	88253	96477
Annotated contigs ≥1000 bp	78940	75441	79985
Unigene matches	23462	23749	31330
Hypothetical gene matches	1126	0	2174
Quality Unigene matches	21826	20578	26635

3.4. Identification and analysis of differentially expressed genes-within group

Differential expression in comparison to their own pre-challenge 0 h samples was carried out for the vaccinated and control groups, respectively (Table 4). At 1 h post challenge, 66 and 121 genes were differentially expressed in vaccinated and control samples respectively. By 2 h post challenge, these numbers rose to 166 and 192 genes in the same respective groups. Notably, in vaccinated gill samples, 77% (1 h) and 93% (2 h) of differentially expressed genes were downregulated in comparison with 0 h following virulent challenge. In contrast, in unvaccinated control gill samples, 80% (1 h) and 65% (2 h) of differentially expressed genes were upregulated in comparison with 0 h following virulent challenge. Further indicating the extent of polarization of early responses to *F. columnare* between vaccinated and unvaccinated fish, the differentially expressed gene sets had little to no overlap in membership between treatments. In all cases, less

than 5% of differentially expressed genes were shared among vaccinated and unvaccinated samples.

Table 4 Statistics of differentially expressed genes at early timepoints 1 h and 2 h following *F. columnare* challenge in channel catfish of 28-day vaccination and control groups relative to their pre-challenge (0 h) as control samples respectively. Shared category indicates the number of genes significantly differentially expressed in the same direction in both groups at a given timepoint, while the percentage is the number of shared genes/number of potentially shared genes. Values indicate contigs/genes passing cutoff values of fold change $\geqslant$ 1.5 (FDR-corrected p value < 0.05).

Group	Up-regulated	Down-regulated	Total
Control 1 h vs 0 h	97	24	121
Vaccination 1 h vs 0 h	15	51	66
Shared	4/108	0/75	
Control 2 h vs 0 h	125	67	192
Vaccination 2 h vs 0 h	12	154	166
Shared	2/135	2/119	

3.5. Identification and analysis of differentially expressed genes-between groups

Additional levels of analysis were conducted on the comparisons of differences in gene expression profiles between vaccinated and control fish at pre-challenge 0 h and post *F. columnare* challenge 1 h and 2 h. Designating the unvaccinated group as the control group, a comparison of global transcription levels was made between vaccination and control treatment groups at each timepoint. A total of 532 contigs showed significant differential expression between groups for at least one timepoint following infection. The comparison at pre-challenge 0

h revealed 102 differentially expressed genes, with over 86% of those showing higher expression in vaccinated fish. After *F. columnare* infection, induced expression in unvaccinated fish resulted in 272 and 313 differentially expressed genes at 1 h and 2 h respectively, with the majority of these showing higher expression in unvaccinated controls than in vaccinated samples (Table 5).

Table 5 Statistics of differently expressed genes pre-challenge (0 h) and following *F. columnare* challenge at 1 h and 2 h. Vaccination > Control indicates numbers of genes with significantly higher expression in vaccinated treatment group relative to control treatment group. These genes elsewhere are indicated with positive values. Vaccination < Control indicates numbers of genes with significantly lower expression in vaccinated treatment group relative to control treatment group. These genes elsewhere are indicated with negative values. Values indicate contigs/genes passing cutoff values of fold change $\geqslant$1.5 (FDR-corrected *p* value < 0.05).

Vaccination vs Control	0 h	1 h	2 h
Vaccination > Control (+)	88	82	133
Vaccination < Control (−)	14	190	180
Total	102	272	313

3.6. Enrichment and pathway analysis

The differentially expressed unique genes pre-challenge between groups and post-challenge within groups were then used as inputs to perform enrichment analysis using Ontologizer 2.0 respectively. Terms with *p* value (FDR-corrected) < 0.05 were considered significantly overrepresented and retained as informative for further pathway analysis (Table 6). The GO terms included extracellular region, serine hydrolase activity, collagen, binding and

coagulation. Based on enrichment analysis and manual annotation and literature searches, representative key genes were arranged into 8 broad functional categories: immune response, neuropeptides/hormones, protease/inhibitor, extracellular matrix degradation (ECM)/tissue remodeling, coagulation and fibrinolysis, lipid metabolism and transport and transcription/signaling regulation (Table 7). Putative functional roles and significance of these gene categories/pathways are covered in depth in the Discussion.

Table 6 Summary of GO term enrichment result of significantly expressed genes in channel catfish following *F. columnare* challenge. The differentially expressed genes were analyzed as the study set in analyzing terms in within-group comparison of control treatment (A) and vaccination treatment (B) and between-group comparison (C). P value ≤ 0.05 was considered significant. Count = study term/population term, where population term is the number of genes associated with the term in the population set. Study term is the number of genes associated with the term in the study set.

(A)

GO ID	GO Name	*p*-Value (FDR)	Count
GO:0005488	Binding	3.29E-04	101/6618
GO:0001071	Nucleic acid binding transcription factor activity	7.66E-04	18/639
GO:0009790	Embryo development	1.99E-03	20/753
GO:0005576	Extracellular region	2.10E-03	15/557
GO:0018149	Peptide cross-linking	2.64E-03	2/9
GO:0072376	Protein activation cascade	3.49E-03	2/10
GO:0050896	Response to stimulus	5.46E-03	49/2763
GO:0045165	Cell fate commitment	7.75E-03	5/108
GO:0005102	Receptor binding	9.51E-03	11/330
GO:0009611	Response to wounding	9.71E-03	8/150

(B)

GO ID	GO Name	*p*-Value (FDR)	Count
GO:0005576	Extracellular region	8.69E-21	62/557
GO:0017171	Serine hydrolase activity	2.34E-09	18/110
GO:0051604	Protein maturation	1.34E-06	32/500
GO:0050817	Coagulation	4.13E-06	7/41
GO:0061134	Peptidase regulator activity	7.89E-06	14/82
GO:0019538	Protein metabolic process	8.25E-06	40/1968
GO:0008233	Peptidase activity	8.85E-06	26/413
GO:0005102	Receptor binding	9.30E-05	13/330
GO:0004857	Enzyme inhibitor activity	1.09E-04	14/102
GO:0030234	Enzyme regulator activity	1.31E-04	15/305

(C)

GO ID	GO Name	*p*-Value(FDR)	Count
GO:0005576	Extracellular region	2.36E-17	62/557
GO:0005581	Collagen	9.88E-14	19/52
GO:0005201	Extracellular matrix structural constituent	2.90E-12	19/28
GO:0005198	Structural molecule activity	2.29E-08	33/301
GO:0051604	Protein maturation	1.98E-06	40/500
GO:0061134	Peptidase regulator activity	2.77E-05	18/82
GO:0004857	Enzyme inhibitor activity	1.24E-04	19/102
GO:0008233	Peptidase activity	1.24E-04	36/413
GO:0017171	Serine hydrolase activity	1.41E-04	17/110
GO:0005215	Transporter activity	1.41E-04	49/808

Table 7 Differentially expressed genes in the gills of channel catfish fingerlings within and between 28-day vaccination and control groups before and post *F. columnare* challenge. Within-group comparison, C 1 h and C 2 h indicated log2 fold change of control fish post challenge 1 h and 2 h compared to their pre-challenge 0 h respectively, while V 1 h and V 2 h represented log2 folds of vaccinated fish post vaccination at 1 h and 2 h compared to their pre-challenge 0 h respectively. Between-group comparison, 0 h, 1 h and 2 h indicated log2 folds of vaccinated fish compared to log2 folds of control fish at pre-challenge 0 h and post challenge 1 h and 2 h respectively, and positive values indicate higher expression in vaccinated fish, while negative values indicate higher expression in control fish. Bold values indicate significant fold change (FDR-corrected p value < 0.05). Gene abbreviations are: Mannan-binding lectin serine protease 2, MASP2; Latent-transforming growth factor β-binding protein 1-like, LTBP1; Pancreatic secretory granule membrane major glycoprotein 2, GP2; Insulin-like growth factor binding protein, IGFBP; Glucose-6-phosphatase a, catalytic subunit tandem duplicate 1, G6PCa1; DNA damage-inducible transcript 4-like protein, DDIT4L.

Description	Contig ID	Within group				Between group		
		Control		Vaccination		Vaccination vs control		
		C 1 h	C 2 h	V 1 h	V 2 h	0 h	1 h	2 h
Immune response								
Acidic chitinase-like precursor	comp137478_c1_seq1	0.63	0.23	-3.67	-2.90	-0.52	**-4.82**	-3.66
Beta-microseminoprotein precursor	comp152921_c1_seq1	4.23	**8.28**	-1.92	**-8.04**	**9.54**	3.40	-6.79
CD276 antigen precursor, B7H3	comp153336_c0_seq1	**4.49**	**4.60**	2.04	1.51	1.34	-1.12	-1.76
CD59B glycoprotein-like	comp38571_c0_seq1	0.09	0.74	-2.36	**-5.16**	1.19	-1.26	**-4.72**
CD83	comp145716_c0_seq1	**2.09**	**2.68**	1.01	0.87	0.03	-1.05	**-1.79**
Cell wall-associated hydrolase	comp155080_c0_seq13	**6.34**	**5.29**	3.85	4.19	-0.74	-3.23	-1.84
Complement C1 inhibitor precursor	comp136349_c0_seq1	4.08	**8.50**	-2.11	-7.98	**9.66**	3.48	-6.82
Complement C4-B	comp151516_c0_seq1	0.15	2.21	-2.85	**-5.25**	3.63	0.63	-3.84
Complement component 3-like X1 precursor	comp153246_c0_seq1	9.01	**12.48**	-3.41	-6.82	**14.29**	2.03	-5.08
Complement component 9	comp155791_c0_seq1	2.67	6.84	-1.99	-7.44	**8.55**	3.90	-5.74
Complement component C3a precursor	comp154676_c0_seq1	2.05	**6.07**	-3.09	**-7.29**	**8.40**	3.26	-4.97

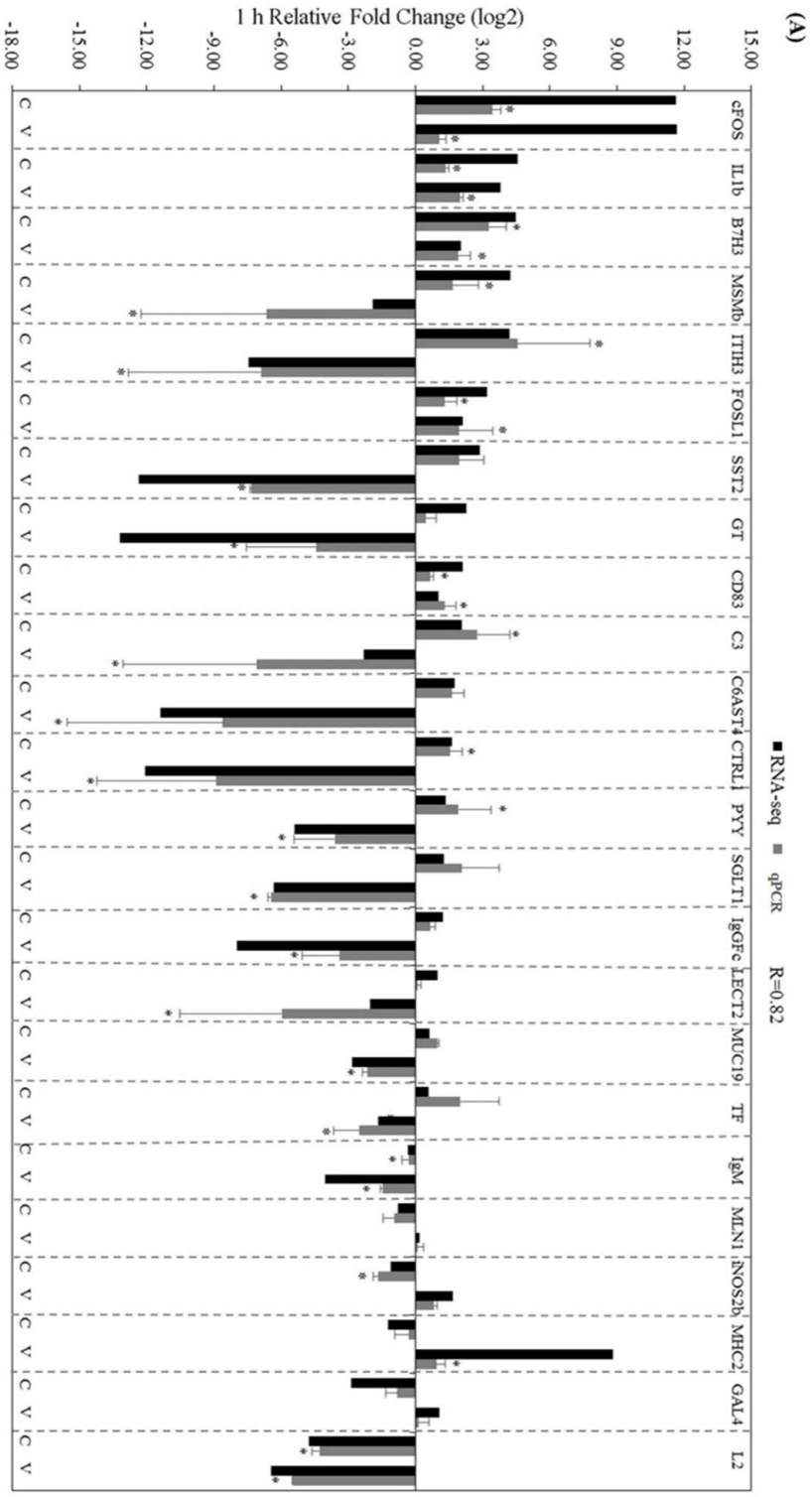
Description	Contig ID	Within group				Between group		
		Control		Vaccination		Vaccination vs control		
		C 1 h	C 2 h	V 1 h	V 2 h	0 h	1 h	2 h
Complement component C7	comp149743_c2_seq1	0.16	1.29	-2.57	**-5.08**	2.20	-0.52	-4.17
Complement factor B precursor	comp57153_c0_seq1	1.26	4.95	-3.28	**-7.16**	**6.26**	1.72	**-5.86**
Complement factor H	comp153633_c1_seq5	6.27	9.04	-5.24	**-12.15**	**12.36**	1.00	-8.83
Complement factor H like 4 precursor	comp123588_c0_seq1	3.24	**7.68**	-2.20	**-7.17**	**9.17**	3.74	-5.69
GP2	comp155117_c0_seq12	**-13.34**	-1.31	-0.73	-1.44	0.29	**12.89**	0.15
Hepcidin	comp131129_c0_seq1	-0.16	2.52	-2.35	**-5.14**	4.10	1.91	-3.57
Hyaluronan synthase 1	comp122767_c0_seq1	**5.35**	**4.91**	1.33	0.59	0.21	**-3.80**	**-4.12**
IgGFc-binding protein isoform X3	comp154220_c0_seq4	1.22	1.91	**-7.99**	-2.22	0.25	**-8.96**	-3.89
IgGFc-binding protein-like	comp150688_c0_seq1	0.69	-3.84	-4.18	**-5.56**	0.31	-4.56	-1.42
Immunoglobulin M heavy chain	comp129637_c0_seq1	-0.35	0.97	**-4.03**	-3.37	2.90	-0.78	-1.44
Interleukin 1, beta	comp146085_c0_seq4	**3.01**	**4.64**	1.87	1.50	0.14	-1.01	**-3.01**
Interleukin 17c precursor	comp131088_c2_seq2	2.26	**3.87**	2.09	1.97	-0.26	-0.42	**-2.16**
Interleukin 26 precursor	comp130802_c0_seq2	3.36	**4.77**	6.17	4.29	-2.62	0.22	-2.94
Interleukin 8	comp67304_c0_seq1	**2.12**	**2.14**	1.43	0.98	-0.05	-0.74	-1.22
Interleukin-12 beta	comp117325_c0_seq1	**2.96**	**3.91**	**2.61**	2.09	-0.31	-0.66	**-2.14**
Leukocyte cell-derived chemotaxin 1	comp123804_c0_seq1	0.42	0.49	0.20	0.66	**2.23**	**2.02**	**2.40**
Leukocyte cell-derived chemotaxin 2 precursor	comp65549_c0_seq1	0.99	3.28	-2.03	**-6.28**	4.77	1.75	-4.80
Macrophage migration inhibitory factor gene	comp151073_c5_seq1	3.78	**7.62**	-3.71	**-8.12**	**8.84**	1.36	-6.91
MASP2 precursor	comp137876_c0_seq1	2.14	6.17	-1.68	-6.46	**7.49**	3.68	-5.15
Mannose-specific lectin	comp152092_c1_seq1	0.81	4.59	-2.14	**-6.35**	6.13	3.19	-4.82
MHC class II antigen	comp152387_c8_seq11	-1.22	0.68	**8.81**	**8.46**	**-8.55**	1.49	-0.60
Microfibrillar-associated protein 4 precursor	comp151066_c0_seq1	1.63	**7.53**	-5.40	**-8.47**	**8.29**	1.26	**-7.72**
Multidrug resistance-associated protein 9	comp153040_c2_seq3	0.20	2.37	-3.19	**-10.80**	3.93	0.54	-9.17
TNF receptor superfamily member 11B	comp153876_c1_seq2	**2.68**	**3.14**	1.13	0.81	0.25	**-1.31**	**-2.09**
TNF receptor superfamily member 12A	comp154334_c3_seq1	**1.59**	**1.78**	0.58	0.36	0.55	-0.47	-0.88
TNF receptor superfamily, member a precursor	comp129254_c1_seq3	**1.68**	1.48	0.34	0.46	0.27	-1.08	-0.75
TNF superfamily, member 2	comp142980_c0_seq1	**2.28**	**3.96**	0.26	0.42	1.16	-0.85	**-2.39**
Toll-interacting protein	comp133551_c0_seq1	2.14	**2.97**	0.87	0.85	0.62	-0.66	-1.51
Protease/inhibitor								
Alpha-2-HS-glycoprotein (cystatins)	comp33971_c0_seq1	2.50	6.37	-1.93	**-8.15**	**9.49**	5.06	-5.03
Alpha-2-macroglobulin	comp155313_c0_seq2	6.58	10.29	-3.35	**-12.77**	**12.97**	3.20	-10.08
Aminopeptidase N-like	comp145750_c1_seq1	1.57	-3.18	-4.65	**-5.02**	1.10	**-5.12**	-0.74
Carboxypeptidase A precursor	comp134662_c0_seq1	1.79	3.73	**-7.11**	**-5.55**	4.36	-4.54	**-4.92**
Carboxypeptidase A4 precursor	comp123189_c0_seq1	1.39	3.58	**-6.18**	**-5.36**	4.47	-3.10	**-4.48**
Carboxypeptidase B isoform X1	comp99364_c0_seq1	1.88	3.99	**-8.55**	**-5.78**	4.21	**-6.23**	**-5.57**
Chymotrypsin B1 precursor	comp36248_c0_seq1	1.72	3.86	**-9.70**	**-5.88**	4.60	**-6.83**	-5.14
Chymotrypsin-like elastase family 2A	comp121567_c0_seq1	3.81	3.54	**-6.75**	**-4.80**	4.91	**-5.66**	-3.44
Chymotrypsin-like precursor	comp99261_c0_seq2	1.63	4.12	**-12.06**	**-9.64**	4.60	**-9.25**	**-9.15**
Elastase 2 like precursor	comp155428_c0_seq1	1.80	3.56	**-8.44**	**-5.80**	3.87	**-6.37**	**-5.50**
Elastase 3 like isoform X1	comp67057_c0_seq1	1.79	3.84	**-8.95**	**-5.73**	4.49	**-6.26**	**-5.09**
Inter-alpha-trypsin inhibitor heavy chain H3	comp149396_c2_seq4	4.18	5.91	**-7.45**	-6.49	**7.80**	-3.82	-4.61

Description	Contig ID	Within group				Between group		
		Control		Vaccination		Vaccination vs control		
		C 1 h	C 2 h	V 1 h	V 2 h	0 h	1 h	2 h
Protein AMBP precursor (protease inhibitor)	comp152621_c1_seq1	0.76	3.10	-1.80	**-4.35**	**4.47**	1.91	-2.99
Separin isoform X1	comp154807_c0_seq11	9.93	11.81	-4.78	-4.47	**13.86**	-0.69	-2.48
Serine protease inhibitor Kazal-type 2-like	comp66252_c0_seq1	-0.21	0.33	**-4.72**	**-4.62**	1.52	-2.99	-3.44
Serine proteinase inhibitor A1 precursor	comp112247_c0_seq1	2.99	**7.21**	-2.30	**-8.22**	**9.65**	4.36	-5.78
Serpin clade A member 1 precursor	comp124410_c1_seq1	3.63	8.11	-2.22	-6.25	**10.06**	4.37	-4.38
Six-cysteine containing astacin protease 4 X1	comp115492_c0_seq1	1.36	3.31	**-9.12**	-5.41	3.15	**-7.34**	**-5.57**
Trypsinogen precursor	comp99592_c0_seq1	1.92	3.93	**-10.03**	**-6.55**	3.91	**-8.06**	**-6.57**
Neuropeptides/hormones								
Glucagon b precursor	comp148986_c0_seq1	0.93	2.33	**-9.52**	**-9.33**	4.00	-6.61	-7.60
Glucagon isoform 1 precursor	comp148986_c0_seq4	3.30	3.21	-9.58	**-9.40**	6.02	-7.01	-6.53
Insulin preproprotein	comp118534_c0_seq1	1.44	3.38	**-9.07**	**-8.77**	5.73	-4.81	-6.42
IGFBP1a precursor	comp134968_c0_seq2	2.20	3.00	-1.93	**-3.15**	**3.13**	-1.01	**-3.03**
IGBP1b precursor	comp153568_c1_seq3	1.76	5.94	-5.10	**-12.29**	6.39	-0.47	**-11.78**
Peptide Y-like	comp45942_c0_seq1	1.34	0.16	**-5.41**	**-4.71**	1.72	**-5.04**	-3.16
Somatostatin precursor	comp146543_c7_seq1	2.34	4.42	**-7.64**	**-5.64**	**6.75**	-3.24	-3.32
Somatostatin 2 precursor	comp72300_c0_seq1	2.86	3.98	**-12.37**	**-7.64**	6.53	**-8.86**	-5.10
ECM/tissue remodeling								
Angiotensinogen precursor	comp98596_c0_seq1	1.80	-3.35	6.34	**-7.30**	**7.46**	2.31	-6.18
Collagen alpha-1(I) chain precursor	comp149768_c0_seq12	-1.30	-0.02	0.14	0.67	**2.48**	**3.92**	**3.16**
Collagen alpha-1(II) chain precursor	comp122468_c1_seq1	0.65	1.01	0.21	0.72	**2.69**	2.26	**2.39**
Collagen alpha-1(II) chain precursor	comp122468_c0_seq1	0.30	0.42	0.26	0.41	**2.51**	**2.47**	**2.49**
Collagen alpha-1(II) chain precursor	comp122468_c1_seq2	0.43	0.56	0.33	0.87	**2.49**	**2.38**	**2.79**
Collagen alpha-1(XI) chain	comp144688_c0_seq4	-0.26	0.26	0.04	0.75	**2.35**	**2.65**	**2.83**
Collagen alpha-2(IX) chain precursor	comp150091_c1_seq3	1.01	0.37	0.15	0.98	**3.96**	**3.11**	**4.56**
Collagen alpha-3(IX) chain	comp140959_c0_seq1	0.41	0.64	0.16	0.52	**2.31**	**2.05**	**2.18**
Collagen Type 1 Alpha-2	comp149389_c2_seq4	0.78	0.67	0.71	1.25	**3.82**	**3.75**	**4.39**
Collagen Type 2 Alpha 1a	comp121602_c0_seq1	0.53	0.71	0.42	1.04	**2.47**	**2.37**	**2.79**
LTBP1	comp153253_c4_seq1	-0.54	**-8.13**	**8.25**	**8.69**	**-8.20**	0.61	**8.61**
Coagulation and fibrinolysis								
Alpha-2-antiplasmin-like (serpin)	comp144853_c0_seq1	1.30	5.17	-2.86	**-7.10**	6.57	2.42	-5.71
Coagulation factor V precursor	comp144579_c0_seq1	1.72	5.84	-2.66	-6.18	**7.24**	2.87	-4.79
Coagulation factor VIII precursor	comp130330_c0_seq1	0.56	2.60	-2.17	**-5.40**	4.53	1.80	-3.47
Coagulation factor X isoform X1	comp112609_c0_seq1	1.66	4.56	-2.49	**-6.64**	**5.87**	1.72	-5.34
Fibrinogen 1 precursor	comp118814_c0_seq1	0.83	4.56	-2.35	**-6.83**	**6.88**	3.70	-4.52
Fibrinogen beta chain precursor	comp114675_c0_seq1	3.14	**7.92**	-2.93	**-8.22**	**9.46**	3.39	**-6.69**
Fibrinogen gamma polypeptide precursor	comp131675_c1_seq1	1.48	5.11	-2.93	**-7.82**	**7.03**	2.63	-5.91
Heparin cofactor 2 isoform X1	comp156319_c0_seq1	1.56	5.76	-1.97	**-7.24**	**7.59**	4.07	-5.42
Histidine-rich glycoprotein	comp129028_c0_seq1	2.15	5.72	-2.89	**-8.15**	**8.88**	3.84	-5.00
Histidine-rich glycoprotein-like	comp146616_c0_seq1	3.78	**7.97**	-2.02	**-8.01**	**9.82**	4.03	-6.17
Plasma kallikrein B1 precursor	comp152468_c0_seq4	2.53	7.02	-2.38	**-10.52**	8.61	3.73	-8.88
Plasminogen precursor	comp138094_c0_seq1	2.82	**7.32**	-2.26	**-7.86**	**8.46**	3.38	**-6.73**

Description	Contig ID	Within group				Between group		
		Control		Vaccination		Vaccination vs control		
		C 1 h	C 2 h	V 1 h	V 2 h	0 h	1 h	2 h
Vitronectin/S protein (protease inhibitor)	comp152319_c6_seq1	3.00	7.04	-2.32	**-7.73**	**8.81**	3.49	-5.97
Metabolism								
14 kDa apolipoprotein	comp99411_c0_seq1	3.09	6.21	-1.74	**-8.08**	**9.87**	5.03	-4.43
Amylase-3 protein precursor	comp154109_c1_seq1	3.02	**5.60**	**-7.48**	**-6.87**	**6.26**	-4.24	**-6.22**
Apolipoprotein A-Ib precursor	comp122577_c0_seq1	3.07	**7.09**	-1.75	**-8.03**	**9.52**	4.71	-5.60
Apolipoprotein A-IV-like	comp137778_c0_seq1	3.62	**8.24**	-2.65	**-8.37**	**8.98**	2.71	**-7.64**
Apolipoprotein A-IV-like precursor	comp154172_c1_seq4	0.51	-4.98	-6.68	**-11.43**	1.04	-6.15	-5.34
Apolipoprotein C-I	comp116700_c0_seq1	3.03	6.56	-2.32	**-8.05**	**7.50**	2.16	**-7.12**
Apolipoprotein E precursor	comp132831_c0_seq1	1.26	-6.16	**-8.72**	**-7.51**	1.26	**-8.73**	-0.10
Bile salt-activated lipase	comp138207_c0_seq1	1.38	3.75	**-7.40**	**-5.44**	4.26	-4.51	**-4.94**
Fatty acid-binding protein 10-A, liver basic	comp67061_c0_seq1	2.72	5.40	-2.02	**-7.19**	**9.79**	5.06	-2.81
Fatty acid-binding protein, intestinal	comp99508_c0_seq1	0.98	-6.96	**-11.11**	**-9.97**	2.32	**-9.81**	-0.71
Gastrotropin	comp155606_c0_seq1	2.26	0.03	**-13.18**	**-12.99**	**13.19**	-2.25	-0.01
G6PCa1	comp142170_c1_seq1	2.63	7.33	-2.88	**-7.67**	**8.18**	2.68	-6.82
Lipase member H precursor	comp154545_c4_seq6	2.06	3.40	**-10.88**	**-10.69**	3.45	-9.65	**-10.57**
Lipoprotein lipase-like isoform X2	comp154545_c4_seq1	0.82	1.25	**-10.75**	**-10.57**	3.11	-8.63	-8.64
Lipoyl synthase, mitochondrial	comp154144_c1_seq11	**11.20**	**10.70**	2.07	-0.08	6.41	-2.55	-4.44
Phospholipase A2	comp38076_c0_seq1	1.28	3.42	**-6.42**	**-5.98**	5.01	-2.69	-4.40
Phospholipase B1, membrane-associated-like	comp141555_c0_seq1	-0.38	-0.43	**-7.38**	**-6.53**	5.54	-1.47	-0.57
Apoptosis/transcriptional regulation								
B-cell translocation gene 3	comp114235_c0_seq1	**2.06**	**1.89**	0.83	0.43	0.32	-0.92	-1.15
BCL2-like14 (apoptosis facilitator)	comp137940_c0_seq3	**1.80**	**2.33**	0.38	0.67	0.10	-1.32	**-1.56**
DDIT4L	comp144694_c0_seq1	**2.53**	**2.42**	1.35	0.95	0.14	-1.04	-1.34
Early growth response 2a	comp150365_c0_seq1	**2.61**	**1.95**	1.53	0.88	0.26	-0.83	-0.82
Early growth response protein 3	comp150314_c4_seq1	**3.65**	**3.49**	1.85	1.02	0.59	-1.21	**-1.89**
EGR1 binding protein 1	comp137571_c0_seq1	**2.72**	**2.44**	1.31	1.23	0.16	-1.25	-1.07
EGR1 binding protein 2	comp145531_c3_seq1	1.70	**2.02**	1.00	0.69	-0.15	-0.85	-1.48
Fos-related antigen 1	comp66786_c0_seq1	**3.18**	**3.96**	2.12	1.15	-0.22	-1.28	**-3.04**
Growth differentiation factor 15	comp79157_c0_seq1	**2.55**	**2.40**	0.75	0.31	0.80	-1.01	-1.30
Hdr protein	comp150650_c0_seq7	**2.74**	**3.36**	1.58	1.55	0.74	-0.42	-1.07
Immediate early response 2	comp153682_c5_seq1	**1.62**	**1.89**	0.72	0.62	0.41	-0.50	-0.87
Jun B proto-oncogene b	comp152893_c0_seq10	**2.60**	**2.99**	1.24	0.94	0.55	-0.81	-1.51
KAISO-like zinc finger protein	comp151787_c4_seq15	**-10.25**	**-10.00**	-3.90	-3.73	-6.17	0.00	-0.01
Krueppel-like factor 16	comp153700_c12_seq2	**5.20**	**5.24**	2.32	1.75	1.48	-1.40	**-2.02**
Krueppel-like factor 4	comp145530_c8_seq1	**2.78**	**2.04**	1.51	0.56	0.22	-1.05	-1.26
Neural orphan nuclear receptor NOR1	comp151006_c2_seq2	**3.06**	**3.03**	1.81	1.31	-0.26	-1.50	**-1.99**
Proto-oncogene c-Fos	comp149932_c5_seq33	**11.64**	**8.87**	**11.67**	0.00	0.02	0.23	**-8.66**
Transcription factor jun-B	comp114446_c1_seq1	**1.99**	**2.48**	1.52	0.76	-0.31	-0.78	**-2.03**
Transcription factor Sox-2	comp155092_c2_seq2	**1.66**	**1.40**	0.49	0.14	0.41	-0.77	-0.86

3.7. Validation of RNA-seq profiles by qPCR

In order to validate the differentially expressed genes identified by RNA-Seq, we selected 24 genes for qPCR confirmation, selecting from those with differing expression patterns and from genes of interest based on functional enrichment and pathway results. Samples from control and vaccinated channel catfish (with three replicate sample pools per timepoint) were used for qPCR. Melting-curve analysis revealed a single product for all tested genes. Fold changes from qPCR were compared with the RNA-seq expression analysis results. As shown in Fig. 2, qPCR results were significantly correlated with the RNA-seq results at each timepoint (average correlation coefficient 0.82 and 0.87 respectively; p-value < 0.001).


(A)
1 h Relative Fold Change (log2)
15.00
12.00
9.00
6.00
3.00
0.00
-3.00
-6.00
-9.00
-12.00
-15.00
-18.00
RNA-seq
qPCR
R=0.82
cFOS
IL1b
B7H3
MSMb
ITIH3
FOSL1
SST2
GT
CD83
C3
C6AST4
CTRL1
PYY
SGLT1
IgGFc
LECT2
MUC19
TF
IgM
MLN1
iNOS2b
MHC2
GAL4
L2
C
V

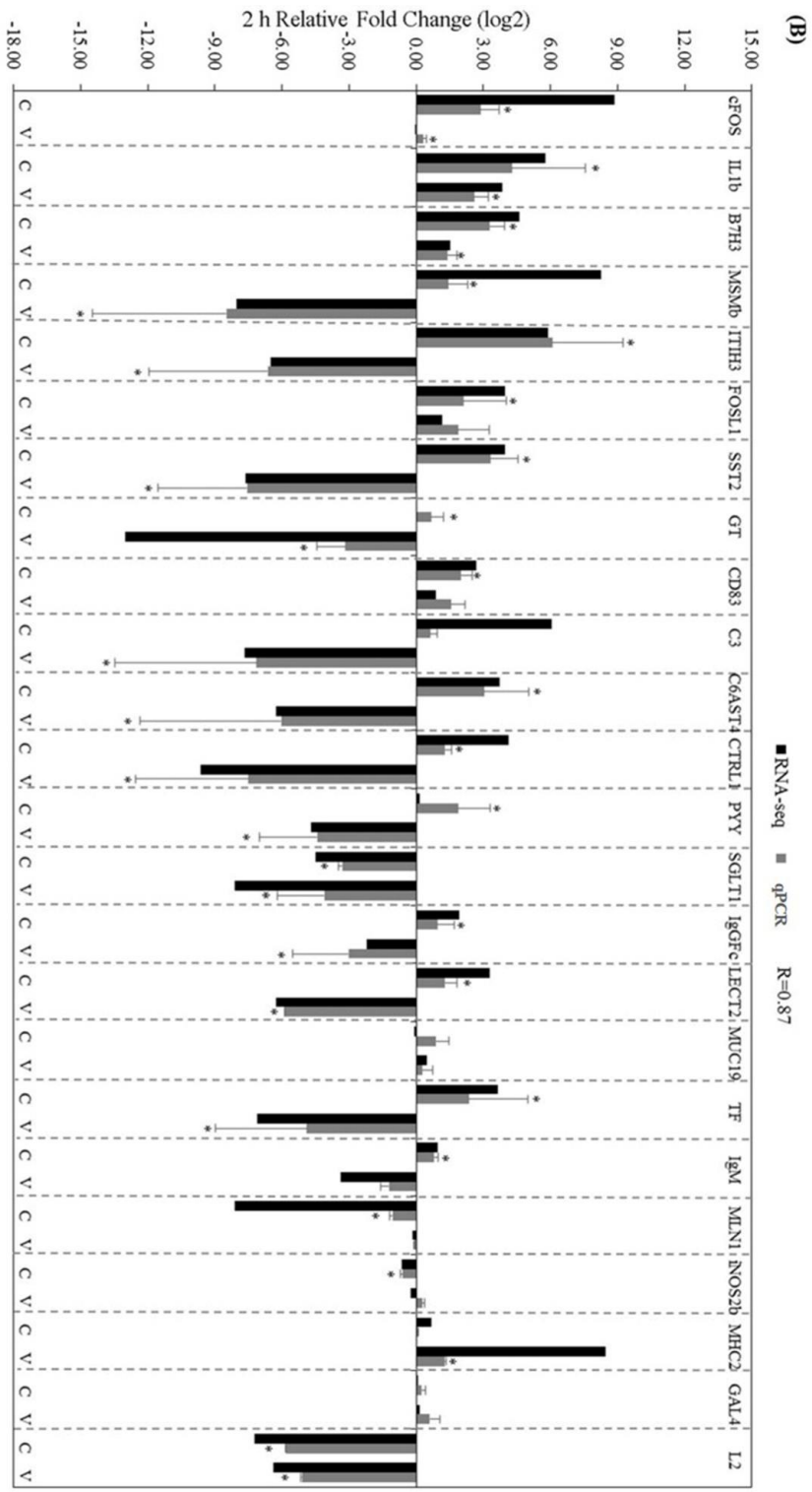


Fig. 2 Comparison of relative fold changes (log2) between RNA-seq and qPCR results in vaccination treatment (V) and control treatment (C) channel catfish post-challenge at 1 h (A) and

2 h (B) compared to their pre-challenge 0 h respectively. Gene abbreviations: Proto-oncogene c-Fos, cFOS; Interleukin 1, beta, IL1b; CD276 antigen precursor, B7-H3; Beta-microseminoprotein precursor, MSMb; Inter-alpha (globulin) inhibitor H3, inflammation, ITIH3; Fos-related antigen 1, FOSL1; Somatostatin 2 precursor, SST2; Gastrotropin, GT; Cluster of Differentiation 83, CD83; Complement component 3a, C3; Six-cysteine containing astacin protease 4 precursor, C6AST4; Chymotrypsin-like precursor, CTRL1; Peptide YY, PYY; Sodium/glucose cotransporter 1 like, SGLT1; IgGFc-binding protein isoform X3, IgGFc; Leukocyte cell-derived chemotaxin 2 precursor, LECT2; Mucin-19-like isoform X1, MUC19; Transferrin, TF; Immunoglobulin M heavy chain, IgM; Mucolipin 1.1, MULN1; Nitric oxide synthase 2b, inducible, iNOS2b; MHC class II antigen, MHC2; Galectin-4, GAL4; Lactose-binding lectin l-2, L2.

4. Discussion

Successful vaccination strategies via mucosal routes are needed for the continued growth of aquaculture in the face of persistent/mounting pathogen threats. However, several gaps in our understanding prevent practical implementation of vaccination programs. Among these is a lack of knowledge regarding measures of mucosal vaccine efficacy and correlates of protection [35]. While antibody titers in response to vaccination are often used as a correlate of protection, these measures are imperfect in light of the known compartmentalization in functional roles of antibody types (mucosal vs. systemic), and the multi-faceted nature of secondary responses to infection at mucosal surfaces, including interactions between immune and neuroendocrine systems [3].

Functional genomics (transcriptomics) approaches when applied to vaccination using attenuated pathogens can provide insights on several levels. Comparisons between host expression responses induced more strongly by virulent strains relative to attenuated strains may highlight mechanisms of virulence, immune evasion, and barrier disruption. Conversely, responses unique to or skewed toward higher differential expression (DE) in vaccinated fish may represent signatures indicative of protective host immunity, components of secondary responses, and shifts in circulating cell types due to prior exposure.

The most pronounced dynamic pattern of differential expression when comparing vaccinated and control samples was one of: A) higher basal expression (pre-challenge) of a set of genes in vaccinated fish relative to control, followed by B) downregulation or repression of transcription of these and other genes soon after secondary exposure (challenge) by *F. columnare* concomitant with C) a rise of pro-inflammatory mediators uniquely in the unvaccinated control (Table 7). Based on this striking expression pattern and its component parts, we hypothesize that much of the observed signature may be the result of vaccine-induced polarization toward pre-formed mediators of immunity, and, following challenge, the rapid release of these stored enzymes, cytokines, and other immune effectors from the primed mucosal surface. This is accompanied by the rapid cessation of gene synthesis from these cells, while naïve (non-vaccinated) fish begin to mount a less-effective primary response, vulnerable to the evasive and manipulative strategies of *F. columnare*. As outlined in detail below, we speculate that mast-like cells (eosinophilic granular cells; EGCs), may play a central role in the observed patterns and be at the center of vaccine-induced protection against *F. columnare* in the catfish gill. While relatively little study has been conducted on fish EGCs, they are known to present in increased numbers in the gills and intestines of previously infected fish, where they have been observed to

degranulate [36], to be associated with responses to proliferative gill disease in channel catfish [37], and to be an important component of the cellular response to *F. columnare* in carp [24]. Below, we discuss gene components underlying these observations.

Prior to experimental challenge (0 h), a number of genes with functions in immunity, proteases and their inhibitors, extracellular matrix formation and remodeling, coagulation and fibrinolysis, and metabolism showed significantly higher expression in vaccinated fish than in unvaccinated controls (Table 7). These genes likely represent cellular and molecular changes which polarized the gill in the 28 days following vaccination towards surveillance and rapid responses to a secondary exposure. A non-significant trend toward higher expression of IgM was observed in vaccinated fish at this timepoint, whereas no changes in IgD (previously reported in gill-associated B cell populations) were seen [38–40]. A number of complement factors had higher expression in vaccinated fish (C3a, C9, C4B, CFB, CFH). The complement factors have well-established roles in immune surveillance, opsonization, and activation of cell types including mast cells, which in turn can also synthesize C3 and C5 [41,42]. Complement is well established to aid humoral immunity, including lowering the threshold for FcR activation and suppressing inhibitory signals [43–45]. Higher complement levels, therefore, likely reflect the enhanced readiness of vaccinated mucosal surfaces to respond to secondary exposure. As with other groups discussed below, complement factor expression declined markedly by 2 h post challenge in vaccinated fish, while the same factors showed induced expression in unvaccinated controls at the same timepoint.

Among other immune genes following this pattern of higher basal expression post vaccination, was macrophage migration inhibitory factor (MIF). MIF plays pivotal roles in regulating systemic and local inflammatory responses and is stored intracellularly by circulating

leukocytes [46] as well as by mast cells [47]. Following cellular activation, MIF serves as pro-inflammatory cytokine. Mast-cell secreted MIF has recently been shown to stimulate fibroblast proliferation and collagen production [48], a process also evident in the expression signatures of catfish gill (below).

Microfibrillar-associated protein 4 (MFAP4), one of a large gene family in catfish [49] and commonly noted for its association with immune responses in catfish gills [26,28,50], also displayed the aforementioned pattern, with induction post-challenge in control fish and downregulation in vaccinated fish. Interestingly, MFAP4 is known both as a macrophage marker in zebrafish [51,52], as well as a biomarker of fibrosis [53].

A number of both proteases and their inhibitors showed a similar trend of higher basal levels in vaccinated fish followed by rapid downregulation or cessation of synthesis. One of the most prominent among these was the broad-spectrum protease inhibitor alpha-2-macroglobulin (A2M). A higher basal pool of A2M would be predicted to both inhibit *F. columnare* proteases, but also prevent damage from catfish secreted proteases, based on its protective roles reported in a wide array of aquatic species [54,55]. Beyond A2M, proteases including aminopeptidases, chymotrypsin, carboxypeptidase, elastases, and trypsinogens were higher in vaccinated fish at time 0 (Table 7). Proteases are the main protein component of mast cell/eosinophilic granular cells, where, as preformed mediators, they are first responders, released within seconds to minutes following pathogen recognition [56]. Indeed, mammalian studies have demonstrated that mast cells can alter their transcription and storage of preformed mediators after initial infection to prevent and control reinfection, an often overlooked form of immunological memory [57]. Further cellular and transcriptional studies are needed in fish to establish how primary infection may alter the contents and localization of mast-like cells.

A number of neuropeptides and hormones appeared to be co-regulated with the aforementioned immune and protease responses (Table 7), including glucagon, insulin preprotein, peptide Y-like, and somatostatins. Among these, somatostatins deserve further scrutiny. Somatostatins are neuropeptide hormones with a broad range of functions, including control of appetite and digestion, however, they are now appreciated to play roles in immunity as well [58–61] where they guard against damaging airway and intestinal inflammation. Somatostatins are secreted by neuroendocrine (NE) cells and are believed, along with other neuropeptides, to play a role in regulating closely-associated mast cells [62], serving to mitigate their otherwise damaging effects. They also downregulate the secretion of the pro-inflammatory cytokines, IL-1b and IL-8 [63], both of which were upregulated after challenge in unvaccinated fish, but not vaccinated fish (Table 7). Little is known about the roles of somatostatin in infections in teleost fish. However, they are found in fish NE cells [64] and the regulatory relationship between NE and EGCs also appears to be conserved [65].

Mast cells in mammals are also associated with roles in extracellular matrix (ECM) deposition and collagen synthesis [66]. While these phenomena have been often linked with fibrotic diseases in these species [67], we have found evidence previously that higher collagen and fibroblast expression in catfish is correlated with survival to *F. columnare* [28]. Here, we identified higher collagen expression (multiple types and isoforms) both before and after challenge in vaccinated fish. As well, critical mediators of fibroblast activation (according to mammalian models), angiotensin and latent TGF-binding protein (LTBP1), were highly differentially expressed at the examined time points [68,69]. Relatedly, a group of genes with roles in the coagulation and fibrinolysis stages of wound healing showed higher expression in vaccinated fish and the same pattern of rapid downregulation after secondary challenge noted

above. These included coagulation factors and fibrinogens (Table 7; [70]). Interestingly, these ECM elements have been reported to interact with mast cells, contributing to their adhesion and activation [71]. Taken together, these signatures are suggestive of a pattern of collagen maturation and ECM remodeling between vaccination and secondary exposure that appears to be protective [72].

A number of genes involved in lipid transport and metabolism were higher at 0 h in vaccinated fish gill. As with other groups, most were downregulated sharply after challenge. They included apolipoproteins, lipases, and fatty acid binding proteins (Table 7). Circulating lipoproteins can be protective against endotoxin, associating with and neutralizing LPS [73]. Of note, lipoproteins have also been reported to modulate Th2 allergic immune responses [74], of which similarities have been reported in *F. columnare* responses [28]. The rapid downregulation of these elements following challenge in vaccinated fish, while their levels generally rose in unvaccinated samples, may be indicative of a primed, rapid response.

In contrast to the patterns described above, unvaccinated fish expression profiles were dominated by induction of pro-inflammatory immune factors and transcriptional factors (Table 7). These included IL-1b, IL-8, IL-17, IL-26, IL-12b, and TNF superfamily members. On the other hand, glycoprotein 2 (GP2), a marker of M cells in mammals [75], a key mucosal immunomodulator [76] and commonly induced in catfish mucosal tissues [50], was potently repressed at 1 h post infection in unvaccinated fish, potentially indicating evasion of early immune-surveillance mechanisms. Unvaccinated fish also appeared to begin transcriptional programs soon after infection that were unperturbed in vaccinated fish. These included *jun* and *fos* elements of AP-1 transcription, potentially linked to early pro-apoptotic signaling [77]. Upregulation of other transcriptional factors and signaling molecules, such as immediate early

genes, has been linked to pathogen-induced changes in cell status and maturation conducive to infection [78].

To summarize, our findings here indicated that the 28 d intervening period between mucosal vaccination and secondary *F. columnare* challenge was sufficient to dramatically polarize responses of vaccinated fish relative to unvaccinated controls. Basally higher expression of proteases, neuropeptides, complement factors, and ECM elements in vaccinated fish was followed up rapid (1-2 h) downregulation of these same elements upon re-exposure. While additional histological and in-vitro studies are clearly needed to confirm, we suggest that EGCs (mast-like cells) are likely a major contributor to the observed expression pattern in whole gill tissue. While relatively unexamined in fish, mast cells have been reported as critical mediators of vaccine-induced clearance of pathogens [79] and are increasingly targeted by vaccine adjuvants [80-81]. Further examination of these cell types in aquaculture species may be a fruitful avenue for research as we seek to develop more effective mucosal vaccines.

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Chapter III Impact of oral and waterborne administration of rhamnolipids on the susceptibility of channel catfish (*Ictalurus punctatus*) to *Flavobacterium columnare* infection

Abstract

Flavobacterium columnare is the causative agent of columnaris disease and causes tremendous morbidity and mortality of farmed fish globally. Previously, we identified a potential lectin-mediator (a rhamnose-binding lectin; RBL1a) of *F. columnare* adhesion and showed higher RBL1a expression in susceptible channel catfish under basal conditions and following infection. Exposure of challenged fish to the carbohydrate ligand L-rhamnose just prior to a challenge substantially decreased columnaris mortality and pathogen adherence via the down-regulation of RBL1a. While highly effective in protecting fish from columnaris, L-rhamnose is prohibitively expensive, underscoring the need for alternative cost-effective sources of rhamnose for disease control. One such alternative may be microbially produced glycolipid compounds termed rhamnolipids (RLs), which feature abundant L-rhamnose moieties and are readily available from commercial sources. In the present study, we examined whether commercially available RLs (administered either by immersion or via feed) would function similarly to L-rhamnose in affording host protection against *F. columnare*. A four-week feeding trial with basal and RL top-coated diets (basal diet + RLs) was conducted in channel catfish fingerlings. Surprisingly, columnaris challenges revealed significantly lower survival following the 10 d challenge period in RL diet fed fish *when compared with* the basal treatment group ($p < 0.001$). In fish fed RLs, we observed a rapid and large-scale upregulation of RBL1a immediately after

challenge combined with a suppression of mucin and lysozyme transcripts. Similarly, fish that were briefly pre-exposed to RLs by immersion and then challenged exhibited lower survival as compared to unexposed fish during a 4 d trial. In conclusion, RLs do not represent an alternative to rhamnose as an experimental treatment for protecting catfish from columnaris mortality. Further research is needed to find other affordable and efficacious alternative sources of L-rhamnose.

1. Introduction

Flavobacterium columnare, the causative agent of columnaris disease, is an opportunistic pathogen that, while part of the normal environmental and host-associated microbiota [1], can, under certain conditions, cause significant losses in several aquaculture species worldwide including catfish, tilapia, and trout [2–4]. The importance of biofilm formation and adhesion in *F. columnare* virulence has been highlighted by a number of studies [5–8]. A potential lectin-mediator of *F. columnare* adhesion (a rhamnose-binding lectin; RBL1a) was recently identified by RNA-seq analyses of early fish host responses to colonization of channel catfish (*Ictalurus punctatus*) gill tissue [9]. Subsequent studies revealed higher RBL1a expression basally and following infection in *F. columnare* susceptible fish and in fish subjected to short-term feed deprivation when compared to resistant and fed controls, respectively [10,11]. Addition of the ligand L-rhamnose to fish tank water rapidly decreased RBL1a expression, decreased bacterial adherence, and lessened subsequent *F. columnare* mortality, suggesting that the provided carbohydrate blocked pathogen adherence via the lectin receptor [10]. Indeed, rhamnose has been found to be an important component of analyzed glycopeptide and extracellular polymeric

substance (EPS) fractions of *F. columnare* [12,13] suggesting its importance in both cell wall structures and biofilm formation for the pathogen.

Pre-exposure of fish to rhamnose moieties, therefore, may be beneficial in saturating otherwise high-jacked receptors and in priming host immune responses toward recognition of an important pathogen-associated molecular pattern (PAMP) [14]. However, L-rhamnose is prohibitively expensive, limiting its practical application in commercial aquaculture. A potential affordable alternative source of rhamnose may be microbially produced rhamnolipids [15].

Rhamnolipids (RLs) are glycolipid surfactants produced by a number of bacterial species, but best studied from another opportunistic pathogen, *Pseudomonas aeruginosa*. RLs are amphiphilic molecules typically composed of mono- or di- L-rhamnoses linked through a beta-glycosidic bond to hydroxyacyl moieties (mostly from C8 up to C12) [16–18]. The distal rhamnose group in almost all the homologs remains generally free [19] and rhamnose-containing RLs have been reported as a potential cost-effective source of purified rhamnose [15].

Rhamnolipids, like the sugar from which they derive their name, have been shown to play a number of roles in bacteria, many of which are linked to pathogenicity. They are known to serve as virulence factors in *P. aeruginosa,* modulating barrier function *in-vitro* in human respiratory epithelium and thereby promoting early infiltration [20]. Their synthesis is regulated by quorum-sensing operons [21,22] and contribute in *P. aeruginosa* to biofilm formation, where they regulate cell surface hydrophobicity and modify adhesive interactions facilitating surface-associated migration of bacteria and initial microcolony formation [23,24]. As a surfactant product, RLs are utilized in a number of industries due to their low toxicity, high biodegradability, strong antimicrobial properties and their ability to disrupt biofilms of a wide range of pathogens [21,25,26]. Given their growing use in bioremediation and industrial

processes [27] mass-produced purified (HPLC/MS grade) RLs are now commercially available at a low cost.

Beneficial manipulation of immunity via feed delivery is the most cost-effective means to protect fish in large-scale pond-based aquaculture. In this vein, a recent study in catfish found that addition of mannan oligosaccharides (MOS) to the diet could offer distal mucosal protection against *F. columnare*, potentially through modulating key mucosal gene pathways [28]. Here, we examined whether RLs (administered either by immersion or via feed) would function similarly to L-rhamnose in affording protection against *F. columnare*.

2. Methods and materials

2.1 Rhamnolipid (RL) feeding experiment

2.1.1 Fish husbandry and diet composition

The basal diet, AquaMax Starter 300 was a commercial product formulated by Purina Mills to meet the known nutrient requirements of catfish. A rhamnolipid solution with 90% purity and 5% (w/v) concentration purified from *P. aeruginosa* (AGAE Technologies, LLC) was mixed with the basal diet at a 1% (v/w) ratio to result in a diet with final concentration of 0.045 g RL per 100 g feed. Given that no previous dosing information was available, a relatively low concentration was selected based on the dosing of immunostimulants and prebiotics in fish diets (reviewed by [29]). The mixed diet was dried for one day in ambient air and then stored at -20 °C.

Pond-reared channel catfish fingerlings (n = 240) were randomly divided into basal feed and RL feed groups with 8 replicates for each group. All the fish were cultured in 36 L volume aquariums with aerated flow-through water at a density of 15 fish per aquarium. After 10 d

acclimation (fed with basal diet), fish were fed twice daily (8:00 am, 4:00 pm) for 4 weeks with either the basal diet or RL diet. The total amount of feed fed per day was set at 3% of the fish weight. Fish were weighed upon initiation of the trial and every two weeks thereafter. Water quality was checked twice each day to maintain established parameters. YSI Pro20 (YSI, Inc., Yellow Springs, Ohio) was used to measure water temperature (27.14 ± 0.24 °C) and dissolved oxygen (6.02 ± 0.71 mg/L). Tetra EasyStrips aquarium test strips were used to measure the ammonia concentration (less than 0.5), pH (7.41 ± 0.23) and alkalinity (90 ppm). All animal protocols during this study were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC).

2.1.2 Bacterial challenge

The *F. columnare* used for challenge was a virulent genomovar II BGFS-27 strain isolated from catfish by Olivares-Fuster & Arias (2011) [30] and stored in a -80 °C freezer. The bacteria were first recovered on plate medium and then a single colony was chosen and inoculated in modified Shieh (MS) broth for 24 h at 28 °C in a shaker incubator at 100 rpm [3031]. The colony forming units per milliliter (CFU/mL) in the final culture were measured by plate counting.

After four weeks of the above feeding trial, fish were moved to a different set of tanks, acclimated, and both fed the basal diet for two days in order to minimize potential carryover effects of the RL in the water or feces of the fish prior to challenge. Five tanks of fish from each feeding group were exposed to *F. columnare* by immersion for 30 min at a final concentration of 1.6×10^6 CFU/mL. Sham-challenge (control group) followed an identical procedure using MS broth inoculum without bacteria for the remaining three tanks of fish from each treatment group.

After challenge, fish were returned to the previous tanks with the same water conditions. Fish in each treatment were monitored daily for signs of disease and mortality over a 10-day period.

2.1.3 RNA extraction and qPCR analysis

Gill tissues were collected before (0 h) and after bacterial challenge (2 h and 8 h). At each time point, 9 fish from each treatment were randomly selected and divided into 3 replicate pools (3 fish each) respectively. Fish were euthanized with MS 222 at 300 mg/L (buffered with sodium bicarbonate) and then equal amounts (approximately 50 mg) of tissue from each fish within the replicates were collected and flash frozen in liquid nitrogen and stored at -80 °C until RNA isolation.

Prior to RNA extraction, samples were removed from the -80 °C freezer and ground with sterilized mortar and pestle in the presence of liquid nitrogen to a fine powder. Total RNA was extracted from tissues using the RNeasy Plus Universal Mini Kit (Qiagen) following manufacturer's instructions. A gDNA elimination step was included for the removal of genomic DNA. Immediately after RNA isolation, the total RNA integrity was visualized using 1.5% agarose gel electrophoresis (28S:18S ≈ 2). The concentration and purity both were measured on NanoDrop 2000 (Thermo Scientific) and all the extracted samples had A260/280 and A260/230 ratios of approximately 2.0. First strand cDNA was synthesized by qScript™ cDNA Synthesis Kit (Quanta Biosciences) according to manufacturer's instructions. The qScript chemistry uses the optimized blend of random and oligo (dT) primers and reverse transcriptase is a mixture of an engineered MMLV RT and a ribonuclease inhibitor protein. All the cDNA products were diluted to 250 ng/µl and utilized for the quantitative real-time PCR reaction using the PerfeCta

SYBR Green Fastmix (Quanta Biosciences) on a CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA).

The expression of rhamnose-binding lectin (RBL1a) [32], mucin 2 (MUC2), mucin 19 (MUC19), c-type lysozyme (LYC) and g-type lysozyme (LYG) were detected by real time PCR. Gene specific primers were designed using Primer Premier 5 software and are listed in Table 1. The thermal cycling profile consisted of an initial denaturation at 95 °C (for 30 s), followed by 40 cycles of denaturation at 94 °C (5 s), an appropriate annealing/extension temperature 58 °C (5 s). An additional temperature ramping step was utilized to produce melting curves of the reaction from 65 °C to 95 °C for 5s. Results were expressed relative to the expression levels of 18S rRNA in each sample using the Relative Expression Software Tool (REST) version 2009 [33]. The biological replicate fluorescence intensities of the control and treatment products for each gene, as measured by crossing-point (Ct) values, were compared and converted to fold differences by the relative quantification method. Expression differences between groups were assessed for statistical significance using a randomization test in the REST software. The mRNA expression levels of all samples were normalized to the levels of 18S ribosomal RNA gene in the same samples. Test amplifications were conducted to ensure that 18S and target genes were within an acceptable range. A no-template control was run on all plates. qPCR analysis was repeated in triplicate runs (technical replicates) to confirm expression patterns.

Table 1 Sequences of primers used for qPCR. Gene abbreviations are: RBL1a, rhamnose-binding lectin 1a; mucin 2, MUC2; mucin 19, MUC19; c-type lysozyme, LYC; g-type lysozyme, LYG.

Gene	Forward (5'-3')	Reverse (5'-3')
18S	GAGAAACGGCTACCACATCC	GATACGCTCATTCCGATTACAG
RBL1a	GTGATGTCCAAAGACTCACGTG	GGTCGGGGTTGCCAAGTAAATC
MUC2	TGCAGAAGAACCAGAAAGAT	TTTTGGCAGTCTGTTAAGGT
MUC19	TCAATCTGGCAATGGAGCA	GCAGTCTTATCACAGCAGTCGT
LYC	GATGGATCAACGGACTATG	CTGTCTCACTATGGTCTTG
LYG	CATCGGAAATAACAGCCAAG	TCTCTGGATATAATGCCTGC

2.2 Rhamnolipid (RL) exposure experiment

2.2.1 Fish husbandry and RL exposure

Channel catfish fingerlings (Stuttgart strain; approximately 10 g in mass) were reared indoors at the USDA/ARS Harry K. Dupree Stuttgart National Aquaculture Research Center (SNARC; Stuttgart, Arkansas) on a standard, basal diet (AquaMax). Twelve experimental tanks were randomly assigned to one of three groups, with four replicate tanks per group: RL alone; *F. columnare* alone; RL + *F. columnare*; and control (no RL or *F. columnare*). Prior to stocking into the experimental tanks, fish in the RL treatment group were exposed to 0.1 ml/L RL in 15 L of aerated water for 15 min and then transferred briefly (approximately 2 min) into 15 L of fresh water as a rinsing step to prevent the transfer of residual RL into the experimental tanks. To account for any handling effects, untreated fish were treated in identical fashion, but were not exposed to RL at any time point. Fish (50 individuals per tank) were then stocked into the experimental system featuring twelve 18 L aquaria supplied with 10 L of flow-through well water. Temperature and dissolved oxygen were measured daily with an YSI Pro20 (YSI, Inc., Yellow Springs, Ohio). Flow rates ranged from 28-30 ml/min, the mean dissolved oxygen concentration was 8.4 ± 0.03 mg/L and water temperatures ranged from 25.1 to 25.7 °C. Total

ammonia nitrogen (TAN) concentrations were monitored daily in each tank with a Hach DR/4000V spectrophotometer using the Nessler Method 8038 (Hach Company, Loveland, Colorado). Standard titration methods (APHA 2012) were used to measure total alkalinity (210 mg/L) and total hardness (114 mg/L).

2.2.2 Bacterial challenge

Immediately after stocking into the experimental tanks, fish were exposed to *F. columnare* isolate LV-359-01, an isolate which was previously shown to produce consistent mortality in the challenge system [34,35]. The isolate was retrieved from the -80 °C freezer and streaked on Ordal's medium [36]. After 48 h, the isolate was dislodged from the agar using a sterile cotton swab and inoculated into 5 mL of *F. columnare* Growth Medium [37]. This suspension was incubated at 28 °C for 24 h, and the 5 mL starter culture was used to inoculate 1 L of FCGM. The inoculated broth was incubated for 24 h at 28 °C in an orbital shaker incubator at 200 rpm. When the bacterial growth reached an absorbance of 0.70 at 550 nm (approximately 4.0×10^{10} colony forming units [CFU]/ml), the flask was removed and placed on a stir plate at room temperature. A 10 mL aliquot was removed for serial dilution and CFU enumeration. In all challenges, 100 mL of bacterial suspension was added to tanks receiving the challenge dose (5.0×10^{8} CFU/ml in the tanks), and equivalent volumes of sterile FCGM broth was added to the control tanks.

Fish were observed daily for clinical signs associated with columnaris disease. Fish unable to maintain neutral buoyancy were categorized as a mortality, and were removed for necropsy or bacteriological sampling.

2.3 Statistical analysis

Weight gain was analyzed with SAS® software package (SAS Institute Inc., Cary, NC USA) using one-way analysis of variance to determine significant differences ($p < 0.05$) among treatments. Survival data was analyzed using SigmaPlot 11 (San Jose, California) by Kaplan-Meier Log Rank Survival Analysis and all pair-wise multiple comparisons used the Holm-Sidak method. Each experiment was analyzed separately using "tank" as the fixed effect and "replicates" as the random effect.

3. Results and discussion

3.1 RL feeding trial

There were no significant differences in weight gain observed between the RL diet and basal diet fed fish at the study initiation or weeks 2 or 4 (Table 2, $p > 0.05$). Both gained approximately 10 g during the first 2 weeks, and 20 g during the last 2 weeks (Table 2). No mortality was observed during the 4 week feeding trial.

Table 2 Aquaria-based weight response of channel catfish over a 4 week feeding trial with basal diet (Basal) and rhamnolipid diet (RL).

Treatments	Initial weight (g, mean ± SE)	Week 2 weight (g, mean ± SE)	Week 4 weight (g, mean ± SE)
Basal	23.35 ± 1.29	33.95 ± 3.31	54.82 ± 2.71
RL	21.65 ± 1.41	30.73 ± 2.34	51.22 ± 3.23
p-value	0.49	0.40	0.43

3.2 Divergent survival rate and RBL1a expression in RL feeding experiment

After *F. columnare* infection, statistically significant differences were observed in survival curves between the RL diet and basal diet treatments (Fig. 1A, $p < 0.001$). No mortality was observed in the first three days following challenge. Beginning on day four, survival of the RL diet group steadily diminished, eventually reaching 46% by day 10. Mortality in basal-diet fed fish was minimal, with 96% survival at day 10. We hypothesize that pre-exposure of the farm-reared fingerlings to *F. columnare* significantly boosted their resistance to subsequent challenge. Nevertheless, the result indicated the ability of dietary RL exposure to significantly exacerbate *F. columnare* mortality in an otherwise resistant population of fish.

We additionally examined the expression of RBL1a [32] before and soon after *F. columnare* challenge in both the RL and basal diet fish (Fig. 1B, Table 3). Compared to the basal diet fed fish, prior to challenge (0 h) RBL1a had 298-fold lower expression in RL fish, indicating that RLs do indeed perturb the expression of the rhamnose-binding lectin. However, after challenge, expression of RBL1a rose steadily in the RL-fed group but declined in the basal diet fish, resulting in significantly higher expression in RL-fed fish at 2 h and 8 h (Table 3; Fig. 1B). Elsewhere, we have observed induction of RBL1a in the early hours following *F. columnare* infection to be indicative of susceptibility [9,11] while lower baseline expression was associated with disease resistance [11]. Here, while dietary administered RL appeared to suppress RBL1a expression prior to challenge, bacterial exposure rapidly reversed this pattern, accompanied by dramatically higher mortality. The pleiotropic nature of RL [24] suggests the possibility that RL feeding perturbed the mucosa and/or innate immune response of catfish.

Table 3 qPCR analysis of gene expressions in gills of rhamnolipid diet (RL) and basal diet (Basal) fed channel catfish immediately prior to challenge (0 h) and post *F. columnare* infection at 2 h and 8 h. The results are expressed as mean ± SE of fold changes and bold number indicates statistical significance at $p < 0.05$. Gene abbreviations are: RBL1a, rhamnose-binding lectin 1a; mucin 2, MUC2; mucin 19, MUC19; c-type lysozyme, LYC; g-type lysozyme, LYG.

Within groups	RBL1a	MUC2	MUC19	LYC	LYG
Basal 2 h vs Basal 0 h	**-261.68±85.52**	1.5±0.21	**2.19±0.37**	-1.52±0.15	**2.70±0.50**
Basal 8 h vs Basal 0 h	**-202.09±50.35**	1.19±0.12	**4.56±0.66**	1.14±0.11	1.59±0.24
RL 2 h vs RL 0 h	**140.96±15.56**	**-3.25±0.43**	**-3.51±0.17**	**-4.36±0.61**	**-2.83±0.46**
RL 8 h vs RL 0 h	**48.48±15.41**	-1.9±0.32	**-3.34±0.70**	1.99±0.74	**-2.10±0.23**

Between groups	RBL1a	MUC2	MUC19	LYC	LYG
RL 0 h vs Basal 0 h	**-298.16±87.11**	-1.68±0.60	**-4.03±0.72**	**-2.86±0.35**	**-2.89±0.50**
RL 2 h vs Basal 2 h	**54.65±11.23**	**-8.2±1.33**	**-40.19±5.18**	**-8.22±1.00**	**-4.91±0.32**
RL 8 h vs Basal 8 h	**162.18±37.34**	**-3.37±0.61**	**-47.27±7.32**	-1.59±0.35	**-3.89±0.41**

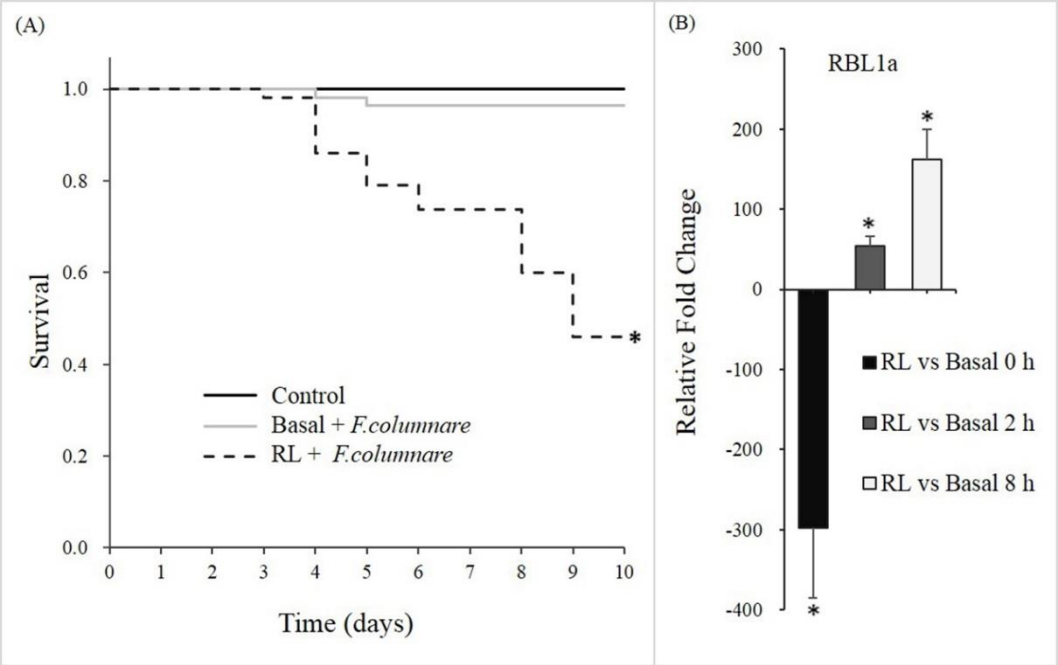

Fig. 1 Impact of rhamnolipid additive on survival rate and RBL1a (rhamnose binding lectin 1a) expression of channel catfish in response to virulent *F. columnare* infection. Asterisks indicated the statistical significance at $p < 0.05$.

(A) Survival analysis of channel catfish fed with rhamnolipid diet (RL) and basal diet (Basal) during 10-day infection with virulent *F. columnare*.

(B) qPCR analysis of RBL1a expression in gills between RL and Basal fed channel catfish before challenge 0 h and post *F. columnare* infection at 2 h and 8 h.

3.4 Expression profiles of Mucin and Lysozyme genes

We examined, therefore, the expression of several important constituents of mucosal immunity in catfish [11,28]. qPCR analyses were carried out on mucin 2 (MUC2), mucin 19 (MUC19), c-type lysozyme (LYC), and g-type lysozyme (LYG). In almost all cases, expression was significantly ($p < 0.05$) lower in RL-fed fish than in fish fed the basal diet, both prior to and following *F. columnare* challenge (Fig. 2, Table 3). These signatures were opposite to those

found to be associated with reduced *F. columnare* mortality following MOS supplementation [28]. RL feeding may have disrupted mucin dynamics, reducing mucus production and accompanying bacterial shedding as has been reported in some mammalian studies [38]. Furthermore, lower lysozyme expression levels may indicate impaired recruitment and/or function of gill-associated macrophages [39,40], again heightening susceptibility to *F. columnare*. Taken together, these results indicated that any beneficial effects achieved prior to bacterial exposure via RBL1a saturation were outweighed by the negative impacts of RL on broader mucosal immunity.

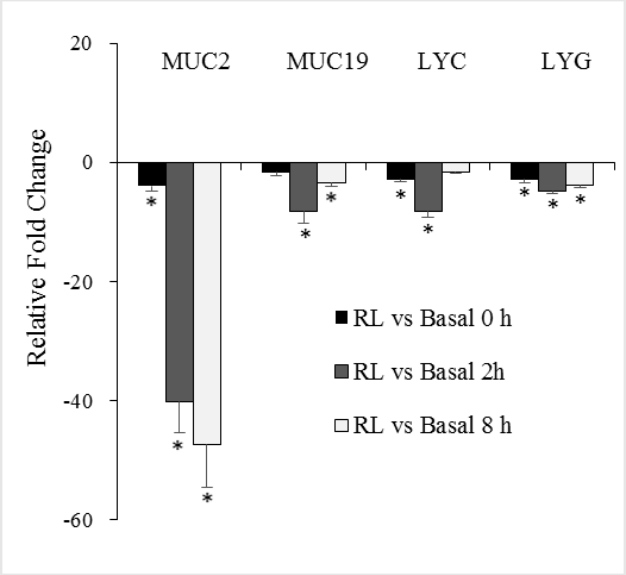

Fig. 2 qPCR analysis for expression of mucin and lysozyme genes in gills between rhamnolipid diet (RL) and basal diet (Basal) fed channel catfish before challenge 0 h and post *F. columnare* infection at 2 h and 8 h. Asterisks indicated the statistical significance at $p < 0.05$. Gene abbreviations are: mucin 2, MUC2; mucin 19, MUC19; c-type lysozyme, LYC; g-type lysozyme, LYG.

3.3 Survival rate of RL immersion exposure experiment

Both control and RL exposed fish experienced mortality due to the *F. columnare* challenge; however, a much lower survival rate was observed in RL exposed fish with more than 50% of fish dying within 24 hours post infection, while only 8% mortality occurred in untreated fish during this same period (Fig. 3). At the termination of the study 4 d post-challenge, cumulative mortality was significantly ($p < 0.001$) different between the two groups with 41% in the untreated and 64% in the RL-exposed group. The precise mechanism driving this difference in susceptibility is unclear; however, in addition to their likely interactions with the RBL compartment, RLs have been shown to exhibit some cytotoxicity to host cells, such as damaging cell membranes and inhibiting epithelial ion transport [18] and modulating the barrier function in the human airway [20]. Alternatively, in rodent models topical administration of RLs has shown to enhance wound healing [41], while in vitro studies suggest that RLs could have clinical value in promoting keratinocyte proliferation [42]. It is important to note here, that fish exposed to rhamnolipid yet not challenged with *F. columnare* experienced no mortality. Taken together with findings from the feeding trial, it appears that RLs are not inherently toxic to catfish as no overt or macroscopic side effects were observed in either study in the absence of a columnaris challenge. Nevertheless, RL exposure could have induced some histological or other cellular or biochemical damage that could have heightened permissivity to columnaris colonization of the catfish host.

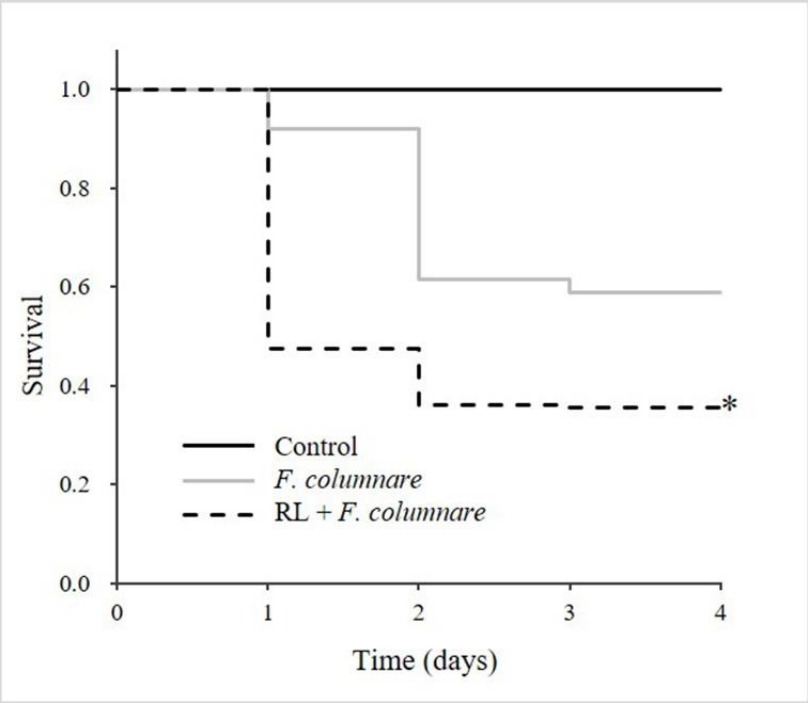

Fig. 3 Cumulative survival rate of rhamnolipid (RL) exposed and normal channel catfish after challenge with virulent *F. columnare* ($p < 0.001$).

Currently lacking among aquaculture species is a commercially available columnaris vaccine that offers robust protection throughout a production cycle. While RLs exposure failed to exert a therapeutic effect, these compounds may offer a means to enhance immersion-based vaccine delivery or uptake by preconditioning the mucosal surfaces prior to vaccination. Indeed, *Pseudomonas*-derived RLs were shown to heighten neutrophil chemotaxis [43] and also prime monocytes/macrophages and augment their oxidative burst response [44]. Further investigation is needed to determine whether RLs could similarly prime cellular effectors in catfish and other relevant warmwater cultured finfish.

4. Conclusions

Based on the above results, we conclude that RLs are not suitable candidates for replacement of native L-rhamnose for mitigating columnaris infections. Although the two experiments were conducted in different facilities with different catfish populations and *F. columnare* isolates, both demonstrated heightened *F. columnare* mortality in RL-exposed fish. Unexpectedly, dietary RLs increased the susceptibility of channel catfish to *F. columnare* infection, presumably by inducing a robust upregulation of RBL1a immediately following a challenge coupled with a suppression in mucin and lysozyme production. The RL immersion experiment confirmed and extended the negative effects of RL exposure. Further research is needed to find other cost-effective alternative sources of rhamnose.

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Chapter IV L-Rhamnose-binding lectins (RBLs) in Nile tilapia, *Oreochromis niloticus*: Characterization and expression profiling in mucosal tissues

Abstract

Rhamnose-binding lectins (RBLs) are crucial elements associated with innate immune responses to infections and have been characterized from a variety of teleost fishes. Given the importance of RBL in teleost fishes, we sought to study the diversity and expression profiles of RBLs in an important cultured fish, Nile tilapia (*Oreochromis niloticus*) following experimental infection with *Streptococcus agalactiae*, a major cause of streptococcosis in farmed tilapia. In this study, four predicted RBL genes were identified from Nile tilapia and were designated as OnRBL3a, OnRBL3b, OnRBL3c, and OnRBL3d. These OnRBLs were composed of two tandem-repeated type five carbohydrate recognition domains (CRDs), classified as type IIIc, and all clustered together phylogenetically. OnRBL-CRDs shared conserved topology of eight cysteine residues, characteristic peptide motifs of -YGR- and -DPC- (or -FGR- and -DTC-), and similar exon/intron organization. OnRBLs had the highest expression in immune-related tissues, gills, intestine or liver. However, the changes of OnRBL expression in the gills and intestine at 2 h, 4 h and 24 h post *S. agalactiae* challenge were modest, suggesting that tilapia may not mediate the entry or confront the infection of *S. agalactiae* through induction of RBL genes. The observed expression pattern may be related to the RBL type and CRD composition, *S. agalactiae* pathogenesis, the accessibility of ligands on the bacterial surface, and/or the species of fish.

OnRBLs characterized in this study were the first RBL members identified in Nile tilapia and their characterization will expand our knowledge of RBLs in immunity.

1. Introduction

Lectins are a group of carbohydrate-binding proteins widely present in organisms ranging from viruses and bacteria to plants and animals, and are involved in multiple biological processes, such as development and immune response [1–3]. Various lectins including C-type lectins (CTLs), F-type lectins (FTLs), galectins (formerly S-type lectins), rhamnose-binding lectins (RBLs), mannose-binding lectins (MBLs), pentraxins and intelectins have been identified in diverse teleost fishes [2–5]. Each category contains their characteristic peptide motif carbohydrate recognition domain (CRD), through which lectins can bind to specific microbial surface glycans, resulting in pathogen recognition and thus triggering immune defense [2,6–8]. RBLs are composed of one or multiple characteristic CRDs particularly recognizing L-rhamnose or D-galactose without Ca^{2+} dependence [1,9]. The approximately 100 amino acid-long CRD contains two characteristic peptide motifs, YGR in N-terminal and DPC in C-terminal, and displays four disulfide bridges through eight highly conserved cysteine residues resulting in the characteristic topology with a unique structural α/β fold [1,10–12].

RBLs have been reported in more than 25 teleost species and mainly identified in fish ovarian cells, eggs and immune-related tissues such as spleen and mucosal tissues (skin, gills and intestine) [1,13-18]. RBLs have been proposed to recognize and agglutinate bacteria and participate in the innate immune response. European sea bass (*Dicentrarchus labrax*) RBL was reported to recognize and agglutinate microbial pathogens and facilitate their phagocytosis [17].

Steelhead trout (*Oncorhynchus mykiss*) RBLs exhibit antibacterial activities by recognizing and binding the lipopolysaccharide and lipoteichoic acid of bacterial surfaces [18]. Expression of RBLs from chum salmon (*O. keta*) can be induced by their putative natural ligand globotriaosylceramide (Gb3) in response to inflammatory stimuli, following enhanced phagocytosis and release of pro-inflammatory cytokines [19]. Recently, our group reported that a RBL (IpRBL1a) can mediate *F. columnare* adhesion and showed higher expression in the gills of susceptible channel catfish under basal conditions and following infection [20–24]. Prior saturation of the RBL with its ligands, L-rhamnose or D-galactose, lowered its expression and substantially decreased *F. columnare* adhesion and channel catfish mortality following experimental challenges [20,21].

Nile tilapia (*Oreochromis niloticus*) is an important cultured freshwater fish contributing around US $7 billion to aquaculture worldwide, with increasing annual production [25,26]. However, tilapia are extremely susceptible to infection by *Streptococcus agalactiae*, a Gram-positive coccus bacterium which is the causative agent of Group B *Streptococcus* (GBS) [27,28]. Although, *S. agalactiae* outbreaks in Nile tilapia farms have been reported worldwide and cause serious mortality [29-32], the gaps in our understanding of the bacterial entry route and infection mechanisms are numerous. Considering the important role of RBL in teleost fish immunity, particularly RBLs recent characterization in channel catfish, we were interested in studying RBL diversity and expression profiles in Nile tilapia following bacterial infection [33]. In the present study, we identified four RBL genes in tilapia and investigated their expression patterns in mucosal tissues including the gills and intestine following exposure to *S. agalactiae*. Our results provide insight for further functional characterization of RBL members in tilapia, particularly the putative roles of RBL during streptococcal infection.

2. Methods and materials

2.1 Sequence identification and analysis

Putative Nile tilapia RBL genes were identified from online transcriptome databases using RBL sequences from other fish speces as queries utilizing the tBLASTn program with a cutoff *E*-value of $1e^{-5}$. The available RBL sequences were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/) and the animal species involved were listed in section 2.2. Transcriptome assemblies of Nile tilapia (from fish embryos and adult tissues of brain, eye, heart, blood, kidney, liver, testis and ovary) were downloaded from DDBJ/EMBL/GenBank under the accessions of GAID00000000, GBAO00000000, GBAR00000000, GBAY00000000, GBAZ00000000, GBBU00000000, GBBX00000000, GBCP00000000, GBDB00000000 and GBDC00000000. Nile tilapia RBL ESTs (from larva fish and adult tissues of skin, gills, stomach, testis, olfactory epithelium and gonads) were also retrieved from NCBI as queries to search against transcriptome assemblies using BLASTN with a cutoff *E*-value of 1e-5. The identified transcripts, as well as those Nile tilapia RBL ESTs were screened and assembled into longer putative Nile tilapia RBL genes using EGassembler (http://www.genome.jp/tools/egassembler/). These genes were translated using Open Reading Frame (ORF) Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and then further verified by BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the NCBI non-redundant (NR) protein database. Their conserved domains were detected using both NCBI conserved domain architecture (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and the simple modular architecture research tool SMART (http://smart.embl-heidelberg.de/). Multiple sequence alignment and identity analyses were carried out using ClustalX-2.1 and MAT-GAT 2.0, respectively. Signal analysis was performed by SignalP 4.0

(http://www.cbs.dtu.dk/services/SignalP/), and N-glycosylation site was determined using NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/). Additionally, RBL transcripts were aligned and compared to the Nile tilapia genome sequences (ASM185804v2) using NCBI's Splign program (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi) to gain the exon-intron organization and genomic structure that aided verification of the sequence accuracy of RBLs in Nile tilapia.

2.2. Phylogenetic analysis

Types of CRD of RBL were determined following the methods described by Ogawa et al. (2011) [1] using phylogenetic analysis with RBL CRDs previously identified from fish and some other animals, as described below. Multiple protein sequences were aligned using ClustalW program. RBL-CRD sequences of AML from Amazon molly (*Poecilia Formosa*), CSL from chum salmon (*O. keta*), DlRBL from European sea bass (*D. labrax*), ElRBL from northern pike (*Esox lucius*), HkRBL from spotted seahorse (*Hippocampus kuda*), IfRBL from blue catfish (*I. furcatus*), IpRBL from channel catfish (*I. punctatus*), LsRBL from humphead snapper (*Lutjanus sanguineus*), PFL1 from ponyfish (*Leiognathus nuchalis*), SAL from amur catfish (*Silurus asotus*), SHL from snakehead (*Channa argus*), SsRBL form Atlantic salmon (*Salmo salar*), STL from steelhead trout (*O. mykiss*), TBL from far-east dace (*Tribolodon brandtii*), WCL from white-spotted char (*Salvelinus leucomaenis*), CiRBL from vase tunicate (*Ciona intestinalis*), PPL1 from winged pearl oyster (*Pteria penguin*), SpRBL from purple sea urchin (*Strongylocentrotus purpuratus*), SUEL from sea urchin (*Anthocidaris crassispina*) and MnLat-1 from mouse (*Mus musculus*) were chosen and retrieved from NCBI databases. Furthermore, the full-length amino acid sequences of RBLs of the above mentioned animal species plus zebrafish

(*Danio rerio*), shishamo smelt (*Spirinchus lanceolatus*) and Pacific oyster (*Crassostrea gigas*) were included to construct a full-length phylogenetic tree using MEGA 6.0. Based on full-length amino acid sequence alignments, phylogenetic analyses were performed using the neighbor-joining method [34] with 10,000 bootstrapping replications, Poisson correction model, and 95% partial deletion to remove gaps.

2.3 Fish husbandry and bacterial challenge

Nile tilapia (approximately 300 g and 5-6 months old) were reared in 110 L volume aquaria supplied with aerated flow-through water (28 ±1 ^{o}C) at the USDA Aquatic Animal Health Research Unit (Auburn, Alabama) for 1 week prior to challenge. A virulent isolate of *S. agalactiae* LADL-05-108A (capsular type Ib) characterized by Shoemaker et al. [35] was used for bacterial challenge. The isolate was cultured in 500 ml tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD, USA) for 24 h at 28 ^{o}C in a shaking incubator at 150 rpm. After incubation, the optical density (OD) of the bacterial culture was 0.94 at wavelength of 540 nm measured on an Ultrospec 2100 pro spectrophotometer (Biochrom Ltd., Cambridge, England). The average number of CFU per milliliter (CFU/mL) of bacteria were enumerated by standard plate counting (in triplicates). Only plates (or replicate plates from the same dilution) with 30-300 colonies were counted. The average concentration of the original culture was 3.12×10^9 CFU/mL. For the immersion challenge suspension, 500 ml *S. agalactiae* suspension was mixed with 50 L water in the challenge tank, resulting in a final concentration of about 3×10^7 CFU/mL. Three tanks of fish, each with 20 fish, were exposed to *S. agalactiae* by immersion for 1 h in the challenge tank. After the challenge, these fish were returned to the previous tanks. After 24 h post challenge, 5 tissues including brain, gills, kidney, spleen, and liver from 5

individual fish were collected and cultured on blood agar plates for reisolation of *S. agalactiae*. The presence of *S. agalactiae* was confirmed by the characteristic colony morphology, followed by specific PCR [36].

2.4 *Sample collection and RNA extraction*

Seven tissues, including brain, gills, fins, intestine, kidney, liver, and spleen were collected at time 0 h and after bacterial challenge at 2 h, 6 h, and 24 h (time exclude the 1 h immersion period). Timepoints were chosen based on recent reports that have isolated *S. agalactiae* from skin and internal organs of immersion infected tilapia as early as 30 min (dermis, muscle and liver) and all organs samples were positive by 4 h (dermis, muscle, liver gill and nare) [37] as well as recent gene expression studies in tilapia post *S. agalactiae* infection [38-40]. Additionally, given our observation of early modulation of catfish RBL expression [22-24], we wished to make comparisons with similar timepoints in tilapia. At each time point, 15 fish were randomly selected and divided into 3 replicate pools (5 fish each). Fish were euthanized with MS 222 at 300 mg/L (buffered with sodium bicarbonate), and then equal amounts (approximately 50 mg) of tissue from each fish within replicates were collected, flash frozen in liquid nitrogen, and stored at -80 ^{o}C until RNA isolation. Prior to RNA extraction, samples (all healthy tissues at 0 h, and gills and intestine tissues after challenge) were removed from the -80 °C freezer and ground with sterilized mortar and pestle in the presence of liquid nitrogen to a fine powder. Total RNA was extracted from tissues using the RNeasy Plus Universal Mini Kit (Qiagen, Germantown, MD, USA) following manufacturer's instructions. The integrity of isolated RNA was examined and visualized using 1.5% agarose gel electrophoresis. The concentration and quality were

measured on a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) and all extracted samples had A260/280 and A260/230 ratios of approximately 2.0.

2.5 Real-time PCR analysis

Gene-specific primers were designed using Primer Premier 5 software and are listed in Table 1. Nile tilapia 18S rRNA gene was used as a housekeeping gene [41]. First strand cDNA was synthesized by qScript™ cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA) according to manufacturer's instructions. The qScript chemistry uses an optimized blend of random and oligo (dT) primers and reverse transcriptase. All cDNA products were diluted to 250 ng/μl for the following quantitative real-time PCR analysis. A no-template control was run on each plate.

Table 1 Primer sequences of OnRBL genes and reference gene 18S rRNA.

Gene	Forward (5'-3')	Reverse (5'-3')	Product length
18S rRNA*	GGACACGGAAAGGATTGACAG	GTTCGTTATCGGAATTAACCAGAC	111
OnRBL3a	TGGTCCTTTAGCCCACTTCC	GGTTTCTGCATCTGTCCTTCC	136
OnRBL3b	GCTTACTACGGACGCCTTGA	TCTCAGCGACTTTGGGAGTG	167
OnRBL3c	AGCAGGGAACCGTGGACAT	GGTGACTTGCAGGTAAACAGG	147
OnRBL3d	CCATGTCATCCACGTCTACC	TGTCGCCAAGCATTCCAC	182

*18S rRNA primers were obtained from [41].

Three RNA samples from healthy and infected tissues (only gills and intestine post infection) at each time point were analyzed for gene expression. Tilapia skin tissue was excluded for examination post infection as it has been reported that the possibility of the invasion of *S. agalactiae* in tilapia through intact skin is low [37,42], and the scales may also affect the

infection in skin. Real-time PCR reaction used the PerfeCta SYBR Green Fastmix (Quanta Biosciences; Gaithersburg, MD, USA) on a CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The thermal cycling profile consisted of an initial denaturation at 95 ^{o}C (30 s), followed by 40 cycles of denaturation at 94 ^{o}C (5 s), and an appropriate annealing/extension temperature at 58 ^{o}C (5 s). An additional temperature ramping step was utilized to produce melting curves of the reaction from 65 ^{o}C to 95 ^{o}C.

Relative Expression Software Tool (REST) version 2009 was used for qPCR data analysis [43]. Briefly, the relative expression ratio (fold change) of a target gene was computed according to the mathematical model, Ratio = $(E_{\text{target}})^{\Delta CP}{}_{\text{target(control – sample)}}/(E_{\text{ref}})^{\Delta CP}{}_{\text{ref(control – sample)}}$. E is the PCR efficiency and the default value 2.0 was used for both target gene (target) and reference gene (ref) in this experiment. $\Delta CP_{\text{control – sample}}$ is the difference (*Δ) of* the crossing point (Ct) values of a control vesus a treatment sample. The 18S ribosomal RNA gene was used as the non-regulated reference gene. For the expression analysis in healthy tissues, the tissue with the highest Ct value (lowest expression) for each gene was set as the control to calculate the relative expression values in other tissues. For infected tissues, the Ct value of healthy gills or intestine (prior to challenge, 0 h) was set as the control to calculate the relative expression values in gills and intestine at 2 h, 6 h and 24 h post infection, respectively. Subsequently, the expression differences of the investigated gene between groups were tested for significance using Pair Wise Fixed Reallocation Randomization Test, a permutation test based on randomization of the data (10000 randomizations) at the statistical significance level of $p < 0.05$ [43].

3 Results and discussion

3.1 Identification of Nile tilapia RBL genes

After screening Nile tilapia transcriptome databases, four different transcripts and putative amino acids sequences of the predicted Nile tilapia RBL genes (OnRBLs) were identified. The ORF length of each OnRBL gene was similar (220-233 amino acid residues) as they all contained two conserved CRDs (**Table 2**). The structure and composition of CRDs are the key factors for RBL classification [1]. Animal RBL CRDs have been classified into 7 groups, allowing the identification of 13 types of RBL genes according to the composite of CRD structure [1]. In this study, phylogenetic tree analysis revealed that all N-terminal OnRBL-CRDs (OnRBL3a-N, OnRBL3b-N, OnRBL3c-N and OnRBL3d-N) belonged to the clade of type 5 CRDs (CRD5) (**Fig. 1**). The C-terminal CRDs of OnRBLs (OnRBL3a-C, OnRBL3b-C and OnRBL3c-C) were also clustered together with several identified type 5 CRDs (LsRBL-C, SHL-C and DlRBL-C) [1,17,44], with high bootstrap support. However, the bootstrap value of OnRBL3d-C with the other C-terminal RBL-CRDs were low. When combining the result of the full-length phylogenetic tree (discussed later), we believe all C-terminal CRDs of OnRBLs should be classified as CRD5. Therefore, the two tandem-repeated characteristic domains (in N-C orientation) of OnBRLs were CRD5-CRD5, the same structure as LsRBL from sea horse, DlRBL from European sea bass and SHL from snakehead [1,17,44]. Therefore, the four predicted Nile tilapia RBLs were classified as type IIIc and named as OnRBL3a, OnRBL3b, OnRBL3c and OnRBL3d, respectively (**Table 2**).

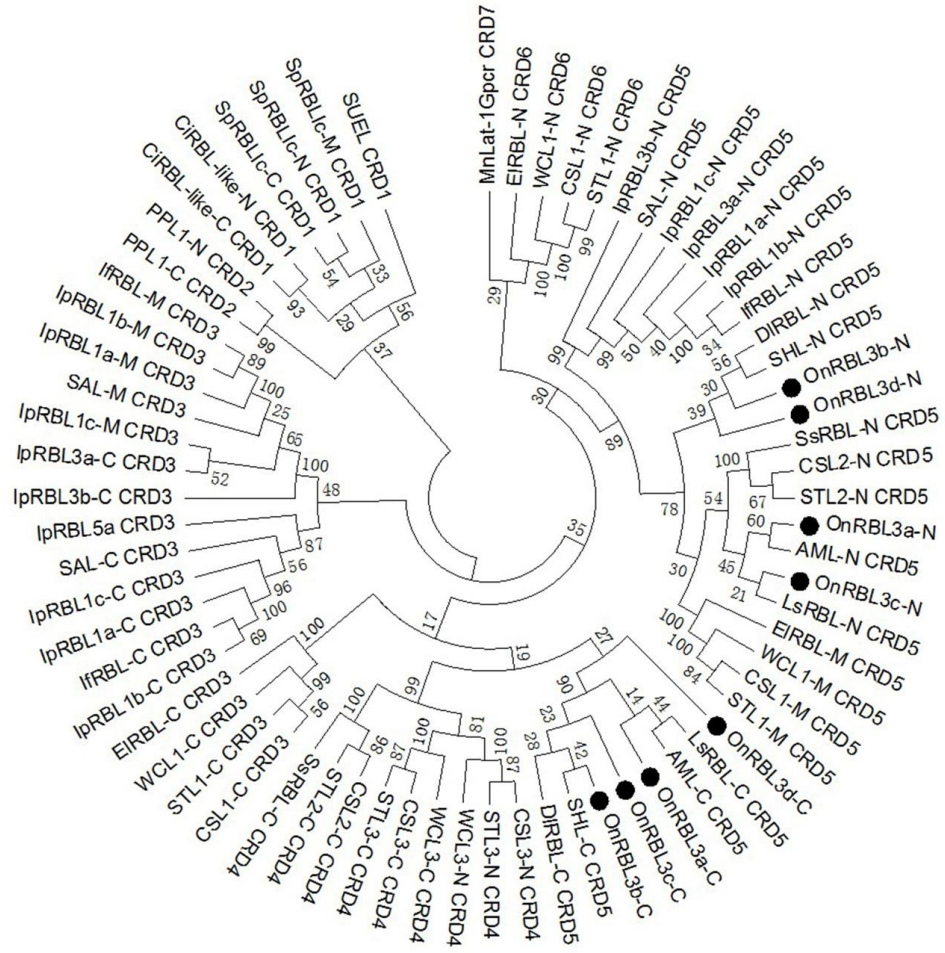


Fig. 1 CRD identification of Nile tilapia (*O. niloticus*) RBLs through phylogenetic classification conducted by a neighbor-joining method in MEGA 6.0. N, M, and C represent N-terminus, Middle, and C-terminus in each CRD, respectively. Dark circles represent CRDs of identified Nile tilapia predicted RBLs. Seven groups of CRDs of RBL family characterized by Ogawa et al. (2011) were included. Abbreviations/species origins: AML (*P. Formosa*), CSL (*O. keta*), DlRBL (*D. labrax*), ElRBL (*E. lucius*), HkRBL (*H. kuda*), LsRBL (*L. sanguineus*), IfRBL (*I. furcatus*), IpRBL (*I. punctatus*), SAL (*S. asotus*), SHL (*C. argus*), STL (*O. mykiss*), SsRBL (*S. salar*), TBL (*T. brandtii*), WCL (*S. leucomaenis*), PFL1 (*L. nuchalis*), CiRBL (*C. intestinalis*), PPL1 (*P. penguin*), SpRBL (*S. purpuratus*), SUEL (*A. crassispina*) and MnLat-1 (*M. musculus*).

Table 2 The sequence features of four predicted RBLs of Nile tilapia (*O. niloticus*). The OnRBL features include their GenBank accession numbers, location on chromosome (Chr), the length of each cDNA transcript, ORF, 5'UTR and 3'UTR, the signal peptide cleavage and N-glycosylation sites, and CRD numbers of each OnRBL. ">" means the 3'UTR may be longer than the detected length.

Name	GenBank Accession no.	Chr	Location	Transcripts (bp)	ORF (aa)	5' UTR (bp)	3' UTR (bp)	Signal peptide	N-glycoslation sites	# of CRDs
OnRBL3a	KY559399	2	22636761-22640678	1357	233	337	> 318	26/27: ARA-GP	70 NKSG	2
OnRBL3b	KY559400	20	1232016-1240091	946	220	41	> 242	23/24: ADA-SM	no	2
OnRBL3c	KY559401	20	17726668-17729110	1044	225	39	> 327	24/25: VVS-TE	118 NYTC	2
OnRBL3d	KY559402	20	34870975-34874058	802	233	24	> 76	no	no	2

Additionally, the phylogenetic tree illustrated that animal CRDs were clustered according to the CRD type but not obviously differentiated among species (**Fig. 1**), which was consistent with several previous studies [1,17,44,45]. The CRD5 group was the largest among the seven RBL-CRD groups as revealed by Ogawa et al. (2011) [1] and its members can be further separated based on different positions (N, M, or C-terminus). N-terminal CRD5 (CRD-N) sequences were clustered together and formed an independent second major clade, separate from C-terminal CRD5 (CRD5-C) sequences, while CRD5-M clade had higher match scores to CRD5-N than to CRD5-C (**Fig. 1**).

Bearing in mind the important role of CRDs in RBL characterization, additional comparisons of OnRBL CRDs with some other key published RBLs were conducted. To make

the comparison concise, we only included all the type IIIc RBLs and each of the other types of RBLs listed in **Table 3**. Each deduced RBL-CRD was 95-98 residues. Multiple sequence alignment illustrated that they contained highly conserved topology of eight cysteine residues and two characteristic peptide segments, -YGR- and -DPC-, with the exception of OnRBL3d containing -FGR- and -DTC- (**Fig. 2**). In channel catfish, IpRBL3b also contains -FGR- but not -YGR- [45]. In contrast, CSL1 from chum salmon and STL1 from steelhead trout have neither of these two conserved segments [46,47]. For sequence identity analysis, OnRBL CRDs (CRD-N or CRD-C) in the same gene shared lower identities with each other, but a higher identity to the same terminal CRDs from other OnRBL genes (**Table 4**). For example, OnRBL3b CRD-N shared 51.5% identity to OnRBL3b CRD-C but shared higher identities (73-79%) to N-terminal CRDs of OnRBL3a, 3c, and 3d. OnRBL CRDs also shared higher identities to same terminal CRDs from DlRBL, SHL, IpRBL, and CSL. The identity percentage results were consistent with the phylogenetic tree of RBL-CRDs (same terminal CRDs have higher match scores; **Fig. 1**).

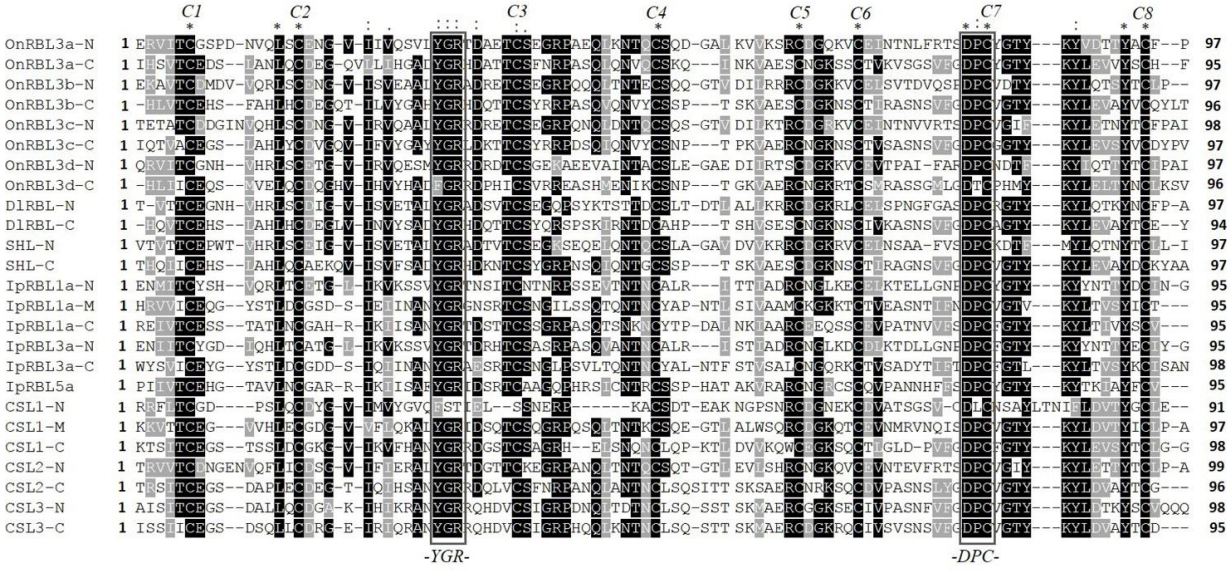


Fig. 2 Alignment of amino acids sequences of RBL CRDs. Abbreviations/species origins: CSL (*O. keta*), DlRBL (*D. labrax*), OnRBL (*O. niloticus*), IpRBL (*I. punctatus*) SHL (*C. argus*), and. N, M, and C represent N-terminus, Middle, and C-terminus in each CRD, respectively. Multiple alignments were achieved by CLUSTALX program. Eight cysteine residues (C1–C8) engaged in disulfide-bond pairs were noted on the top. Conserved motifs of YGR and DPC are enclosed in rectangles and indicated at the bottom. Note: CSL1-N, OnRBL3c-N and OnRBL3d-C lack characteristic RBLs motifs.

Table 3 RBL type and CRD composition of the four OnRBLs and some key published RBLs from other fish species. Abbreviations/species origins: SAL (*S. asotus*), IpRBL (*I. punctatus*), CSL (*O. keta*), STL (*O. mykiss*), WCL (*S. leucomaenis*), DlRBL (*D. labrax*), SHL (*C. argus*) and OnRBL (*O. niloticus*). CRD group was phylogenetically classified, and the number of CRD groups was denoted in the boxes.

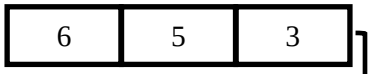
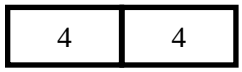
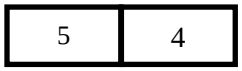
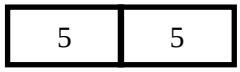
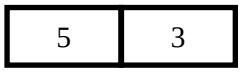
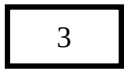
RBL	Type/CRD composition	Order	References
SAL/ IpRBL1a/IpRBL1b/IpRBL1c	Ia 	Siluriformes	[10] [45]
CSL1/STL1/WCL1	II 	Salmoniformes	[14] [15] [16]
CSL3/STL3/WCL3	IIIa 		[14] [15] [16]
CSL2/STL2	IIIb 		[14] [15]
DlRBL/ SHL/ OnRBL3a/OnRBL3b/ OnRBL3c/OnRBL3d	IIIc 	Perciformes	[17] [44] This paper This paper
IpRBL3a/IpRBL3b	IIIg 	Siluriformes	[45]
IpRBL5a	Va 		

Table 4 Percentage of amino acid identity of each OnRBL CRD from Nile tilapia (*O. niloticus*) compared to CRDs from their internal domains, other OnRBLs, and other fish species. Abbreviations/species origins: DlRBL (*D. labrax*), SHL (*C. argus*), IpRBL (*I. punctatus*) and CSL (*O. keta*).

	OnRBL							
	3a-N	3a-C	3b-N	3b-C	3c-N	3c-C	3d-N	3d-C
OnRBL3a-N								
OnRBL3a-C	55.7							
OnRBL3b-N	75.3	56.7						
OnRBL3b-C	54.6	82.3	51.5					
OnRBL3c-N	74.0	55.0	79.0	51.0				
OnRBL3c-C	57.7	75.3	54.6	84.5	53.0			
OnRBL3d-N	70.1	53.6	73.2	51.5	66.0	50.5		
OnRBL3d-C	51.5	66.7	50.5	69.8	52.0	68.0	47.4	
DlRBL-N	62.9	49.5	67.0	44.8	62.0	49.5	57.7	43.8
DlRBL-C	48.5	68.4	50.5	74.0	50.0	70.1	51.5	58.3
SHL-N	69.1	53.6	74.2	47.4	68.0	52.6	67.0	48.5
SHL-C	51.5	72.2	53.6	81.4	55.0	74.2	51.5	69.1
IpRBL1a-N	69.1	56.8	62.9	50.0	57.0	47.4	59.8	53.1
IpRBL1a-M	50.5	55.8	46.4	63.5	46.0	52.6	43.3	53.1
IpRBL1a-C	53.6	62.1	52.6	62.5	50.0	56.7	49.5	52.1
IpRBL3a-N	67.0	56.8	61.9	52.1	59.0	50.5	57.7	53.1
IpRBL3a-C	50.0	54.1	44.9	55.1	48.0	49.0	43.9	49.0
IpRBL5a	55.7	53.7	53.6	59.4	54.0	57.7	49.5	52.1
CSL1-N	57.1	46.3	60.2	46.9	59.0	45.4	53.1	46.9
CSL1-M	71.1	61.9	72.2	55.7	71.0	60.8	57.7	56.7
CSL1-C	48.5	60.2	42.3	58.2	39.0	57.1	45.4	53.1
CSL2-N	77.8	57	73.7	54.5	80.0	55.6	65.7	54.5
CSL2-C	50.5	64.2	54.6	69.8	58.0	66.0	51.5	59.4
CSL3-N	52.6	55.6	58.8	63.0	56.0	58.0	49.5	59.0
CSL3-C	52.0	66.7	54.0	64.6	51.0	66.0	47.0	60.4

After OnRBL CRD identification and classification, the full-length amino acid sequences of RBL family were used to conduct a neighbor-joining phylogenetic tree analysis to determine the identities and the phylogenetic relationships of predicted Nile tilapia RBLs with RBLs of other animal species (**Fig. 3**). Here, all predicted Nile tilapia RBLs were clustered in the same clade, as well as another five type IIIc RBLs from Amazon molly, European sea bass, humphead

snapper, snakehead,and spotted seahorse (**Fig. 3**). Except for the spotted seahorse, all the other fish in this clade belong to the order Perciformes. Similarly, closely related species, such as catfish (*I. furcatus, I. punctatus* and *P. asotus*) in the order Siluriformes, and salmonids (*O. keta*, *O. mykiss* and *S. leucomaenis*) in the order Salmoniformes were also clustered together, respectively (**Fig. 3**). Most zebrafish (*D. rerio*) RBLs (seven in ten) were clustered into one clade with high bootstrap support and no membership from other species, consistent with previous findings [45]. Different from the phylogenetic tree and identities of RBL-CRDs, the phylogenetic tree of full-length RBLs was consistent with their phylogenetic species relationships. The same or related species of RBL sequences had higher match scores and shared similar RBL types (**Fig. 3** and **Table 3**).

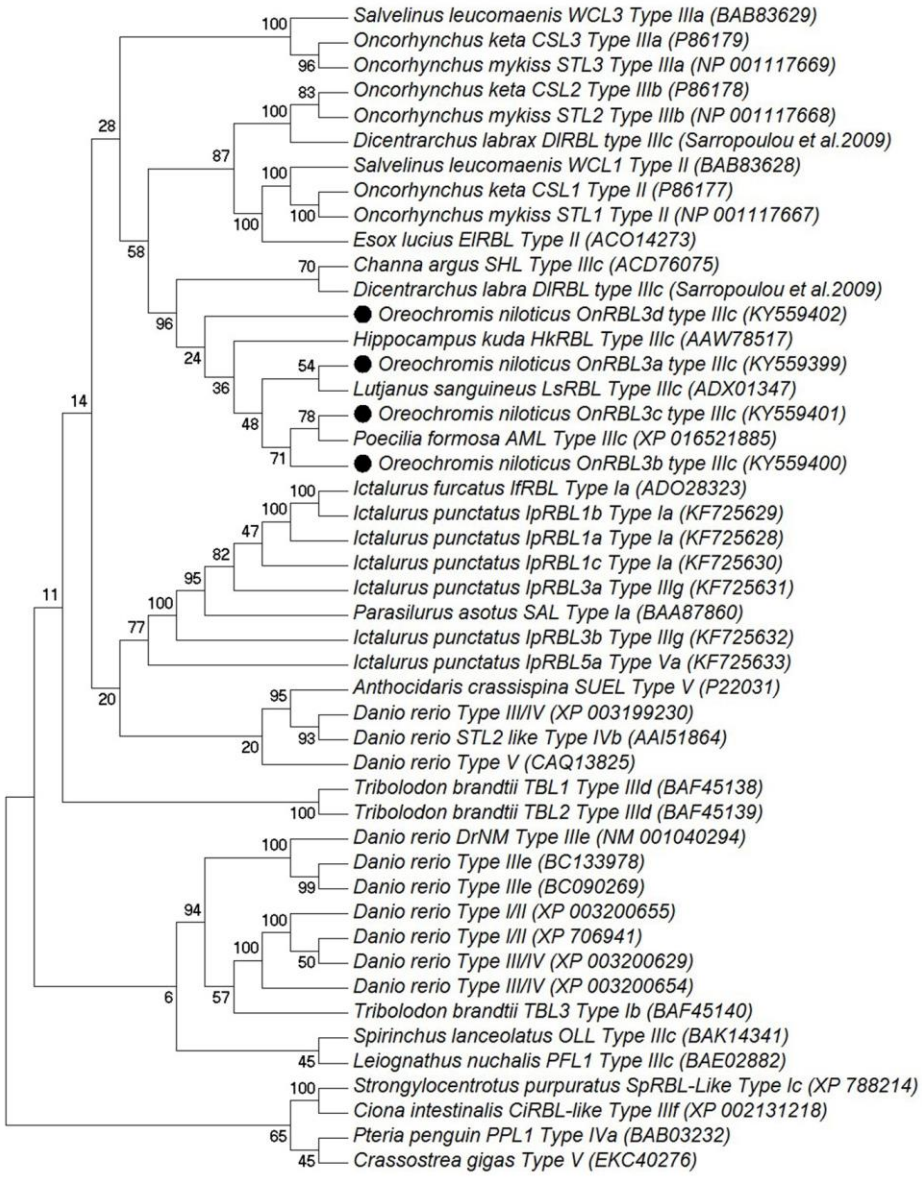


Fig. 3 Phylogenetic tree of RBL family. The phylogenetic tree was constructed based on full-length amino acid sequences of RBL genes from selected fish species and some other animals, using the neighbor-joining method in MEGA 6.0. Dark circles indicate characterized Nile tilapia (*O. niloticus*) predicted RBL genes.

All four nucleotide and putative amino acid sequences of Nile tilapia RBLs were submitted to NCBI GenBank with accession numbers **KY559399 - KY559402**. The features of OnRBL genes including the length of cDNA, 5' untranslated region (UTR), 3' UTR and amino acid of each ORF, the position of the signal peptide and N-glycosylation sites, and the number of CRDs of each OnRBL are summarized in **Table 2**.

3.2 Nile tilapia RBL genomic structure

In order to better understand the organization of OnRBL in the genome, we examined the genomic architecture of Nile tilapia RBLs and compared their exon/intron organization with published RBL genes. Four identified OnRBL transcripts were aligned to Nile tilapia genome (ASM185804v2) and revealed that OnRBL3a contained 10 exons and was located on chromosome 2, while the others contained 9 exons and were located on chromosome 20 but in different regions without overlaps (**Table 2** and **Fig. 4**). The phylogenetic analysis, sequence identities and their location on chromosome indicated these OnRBLs were different unigenes.

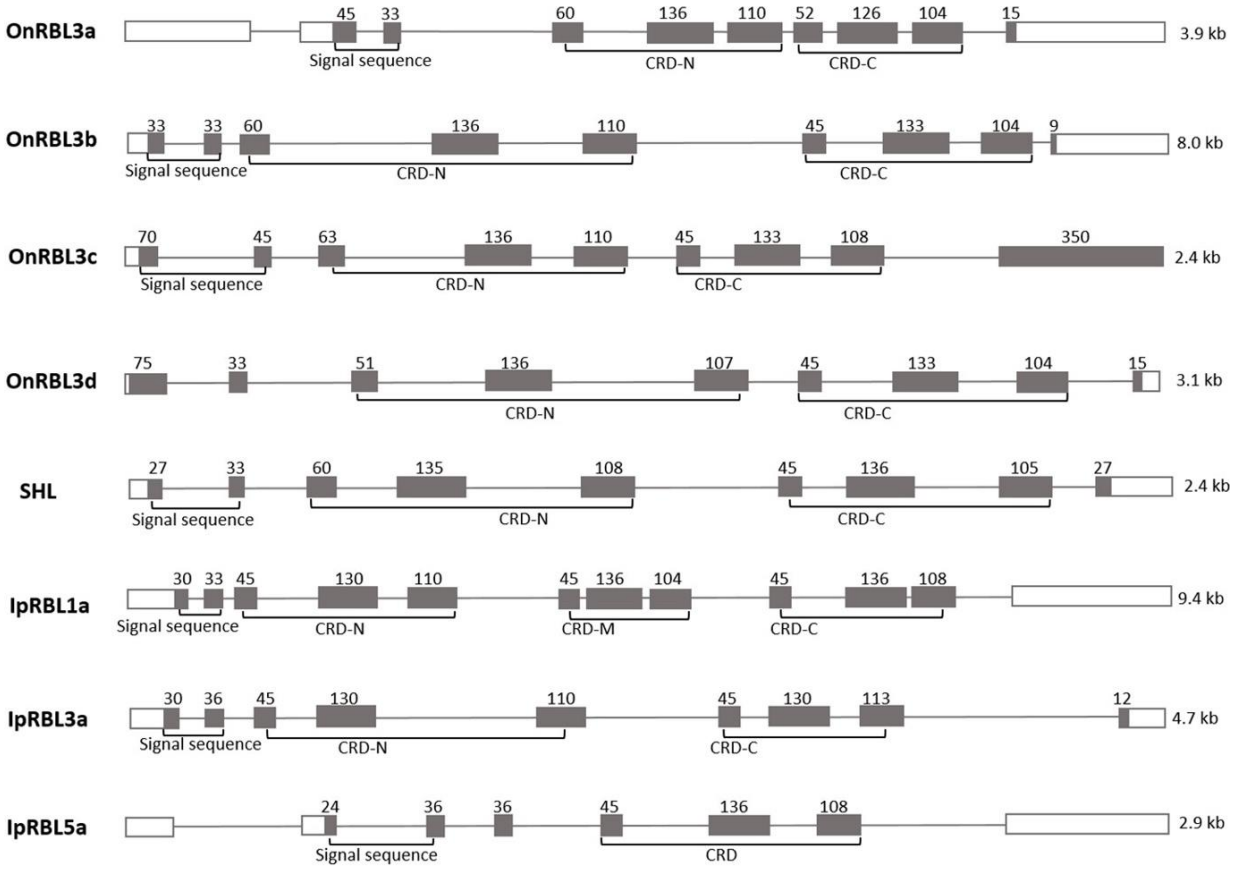


Fig. 4 Comparison of genome schematic depiction of four OnRBL genes from Nile tilapia (*O. niloticus*) with SHL from snakehead (*C. argus*) and IpRBL from channel catfish (*I. punctatus*).

Using the Splign program to align OnRBL transcripts to Nile tilapia genome, we also obtained their genomic structures. Each OnRBL CRD was encoded by three exons and each exon had a comparable size between different RBL CRDs. The first exon of OnRBL CRD-N was 45, 51 or 60 bp, the second was 136 bp, and the last exon was 110bp. The first exon of OnRBL CRD-C was 45 or 52 bp, the second was 126 or 133 bp, and the last one was 104 or 108 bp (**Fig. 4**). Considering that genome sequences of chum salmon, steelhead trout and white-spotted char listed in **Table 3** were not available, and DlRBL gene could not be successfully mapped to the European sea bass scaffolds, we did not use them to compare the exon/intron organization with

OnRBLs. RBL-CRDs of channel catfish and snakehead were composed of 3 exons, and the size of exons was comparable to that of OnRBL-CRDs (**Fig. 4**). These results suggested a high degree of conservation of RBL genes among these three species in exon/intron organization, indicating that RBL may share similar tandem-repeated structure and common ancestry. Additionally structural patterns indicate that RBL genes may evolve from exon shuffling and gene duplication [44].

3.3 Basal tissue expression of OnRBL genes

We performed real-time qPCR analysis to evaluate the basal expression of four OnRBL genes of healthy Nile tilapia in seven tissues: brain, gills, fin, intestine, kidney, liver, and spleen. Fold change was calculated for a given tissue relative to the corresponding tissue with the lowest level of expression per gene (set at 1) and normalized to that of the 18S rRNA gene. As shown in **Fig. 5**, these OnRBLs showed different tissue distributions. OnRBL3a was significantly expressed at higher levels in mucosal sites, gills (250-fold; $p < 0.05$) and fin (47-fold; $p < 0.05$), but expressed at lower levels in classical immune-related tissues such as kidney, liver, and spleen. OnRBL3b had the highest relative expression in liver (11269-fold), followed by intestine (25-fold), and relatively low expression in brain (4.75-fold), kidney (4.58-fold), and spleen (4.57-fold), and lowest expression in gills and fins. OnRBL3c expression was significantly expressed in the intestine (146-fold; $p < 0.05$) and spleen (4.7-fold; $p < 0.05$), whereas OnRBL3d was strongly expressed in liver (6450-fold), followed by the kidney (5.48-fold) and gills (5.24-fold). Taken together, OnRBLs appeared to be highly expressed in immune-related tissues, gills, intestine or liver of Nile tilapia. RBLs have been reported to be mainly expressed in reproductive cells and immune-related and barrier tissues, including gills, intestine and spleen [1]. Almost all

RBLs of channel catfish had the highest expression in skin, intestine, liver or trunk kidney [45]. Snakehead SHL had high expression in liver and intestine [44], steelhead trout STL1 was detected in liver [46,47] and a RBL identified from ayu (*Plecoglossus altivelis*) also had high expression in liver [48]. The distribution of RBL in immune-related tissues may indicate their potential roles in innate immune defense.

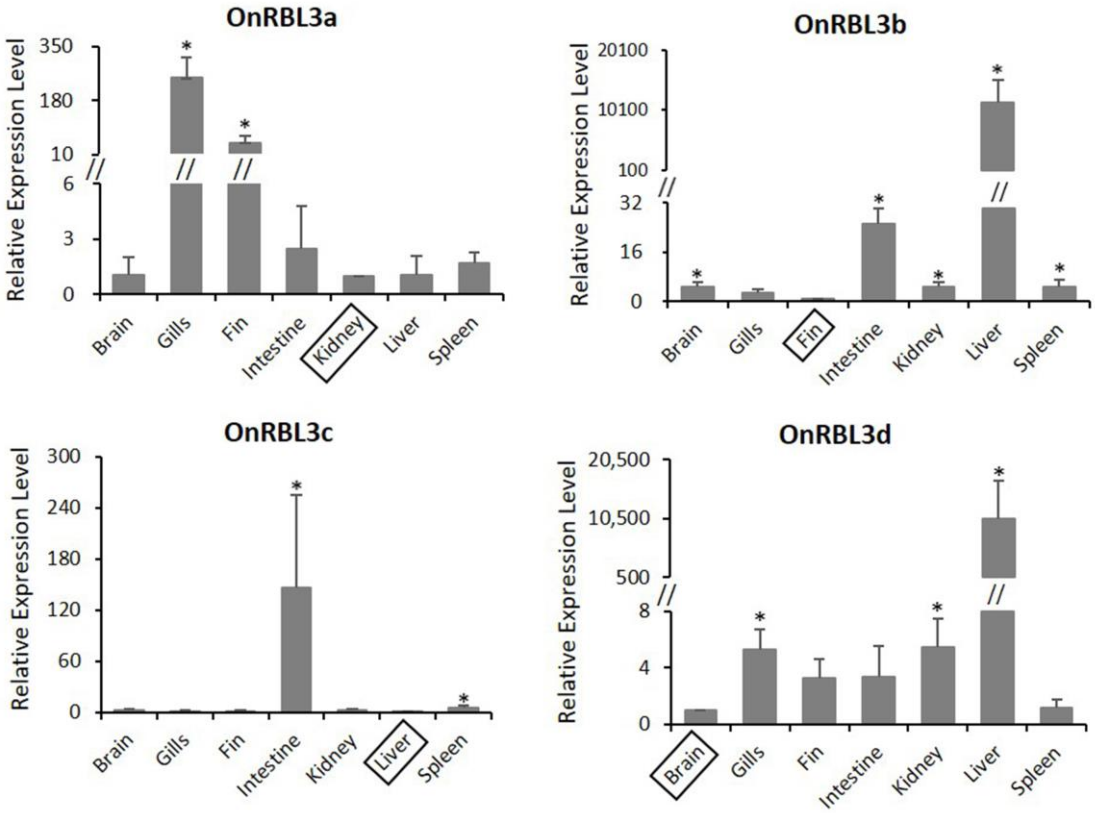


Fig. 5 Basal tissue expression of predicted RBLs in healthy Nile tilapia (*O. niloticus*) by real time PCR. The tissue with the lowest Ct values for each gene was set as control and labeled with a rectangle. Fold change was calculated at a given tissue relative to the expression of the corresponding control tissue (set at 1) and normalized to that of the 18S rRNA. The results are expressed as mean ± SE and the asterisks indicate statistical significant difference at $p < 0.05$.

3.4 Expression profile of OnRBL genes after challenge with S. agalactiae

The mucosal immune system constitutes the first line of host defense against pathogen invasion, particularly for teleost fish as they are directly and continuously exposed to pathogen-rich aquatic environments [49]. Despite the well-characterized roles of RBLs in fish oocyte maturation as well as in innate immunity as pattern recognition receptors, there are few studies focusing on their roles in mucosal immune responses during bacterial infection. Previously, we revealed a dramatic early upregulation (> 100-fold) of IpRBL1a in the channel catfish gills after *F. columnare* infection, and IpRBL1a expression was inversely correlated with host resistance to columnaris disease [20-24]. In order to explore the potential role of Nile tilapia RBL in mucosal tissues after bacterial infection, and further confirm whether tilapia utilizes RBLs in the same manner as channel catfish, we examined the expression profile of OnRBLs in mucosal sites (gills and intestine) within 24 hours following immersion challenge by S. *agalactiae*.

At 24 h post infection, we observed clinic signs of streptococcosis, such as hemorrhage in gills, enlargement of spleen, and softening of liver and kidney. *S. agalactiae* was successfully reisolated from brain, gills, kidney, spleen, or liver from five fish as shown in **Table 5**. Gene expression patterns of OnRBLs were different between gills and intestine. In gills, OnRBL3a was significantly downregulated by 5.82-fold at 24 h post infection (**Fig. 6A**; $p < 0.05$). Conversely, OnRBL3b was significantly induced by 2.51-fold at 6 h post challenge ($p < 0.05$). Both OnRBL3c and OnRBL3d were not significantly differentially expressed relative to unchallenged control at all time points in gills. In the intestine, significant changes in expression were detected in OnRBL3a at 24 h (4.76-fold), OnRBL3c at 24 h (-4.00-fold) and OnRBL3d at 2 h (-5.42-fold) post infection, respectively (**Fig. 6B**; $p < 0.05$).

Table 5 Twenty four hour culture results from five Nile tilapia (*O. niloticus*) sampled on blood agar to confirm infectivity of the immersion exposed tilapia.

	Fish Number (+/-) [a]				
Tissue type	1	2	3	4	5
Gill	-	+	+	+	+
Brain	+	-	-	-	+
Kidney	+	+	-	-	-
Liver	+	-	+	-	+
Spleen	+	-	-	-	+

[a] + = positive result and - = negative result of the sampled organ.

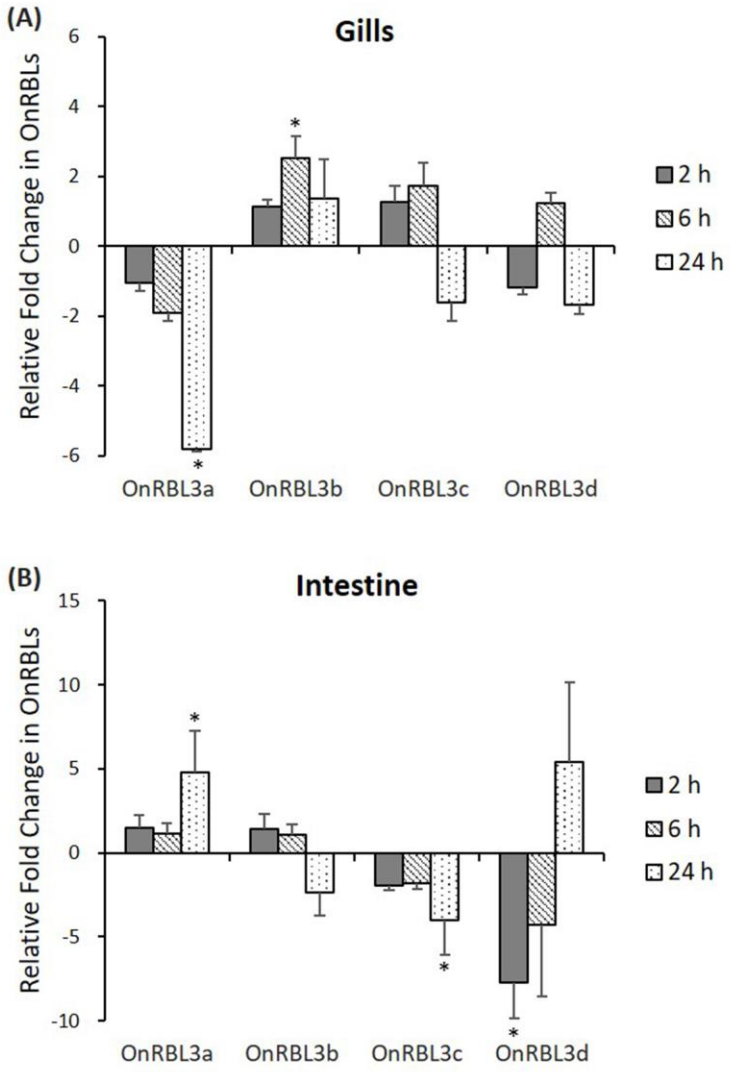


Fig. 6 Real-time PCR analysis of Nile tilapia (*O. niloticus*) predicted RBL gene expression following *S. agalactiae* infection at 2 h, 6 h and 24 h in mucosal gill and intestine tissues. Fold change was calculated at each post infection timepoint relative to control (0 h) and normalized by the changes in 18S rRNA gene. The results are expressed as mean ± SE and asterisks indicate statistical significant difference at $p < 0.05$.

While significant changes in differential expression were observed, the changes in OnRBL expression post infection were relatively modest compared to IpRBL1a in response to bacterial infection in channel catfish. In our previous work, the dramatically high upregulation of RBL was only observed in type Ia RBL, composed of CRD5-CRD3-CRD3, but not in other types IIIg (CRD5-CRD3) and Va (CRD3) RBLs [40]. In contrast, all OnRBLs are type IIIc with the composition of CRD5-CRD5 (**Table 3**; **Fig. 1**). Thus, the different expression patterns of OnRBLs may be associated with the varied CRD composition in Nile tilapia, therefore altering downstream physiological responses. On the other hand, *F. columnare* is a Gram-negative bacterium and RBL is a potential mediator of its adhesion in the case of columnaris disease [20,22], resulting in pronounced erosion and necrosis of mucosal tissues including the fins and gills [50]. In contrast, *S. agalactiae* is a Gram-positive bacterium and the precise entry mechanism(s) are unclear [37]. Streptococcal disease can cause prominent clinical signs in the internal tissues, including encephalitis and meningitis, splenitis, polyserositis and myocarditis [29,31]. One explanation for the limited OnRBL response against *S. agalactiae* could be that the sugar ligand(s) are not highly expressed or accessible on the *S. agalactiae* surface. Group B *Streptococcus* capsular polysaccharides (CPS) are highly terminally sialylated [51,52]. Sialic acid is a major component of host glycoproteins and it is thought that capsular sialic acid allows GBS to evade host immune responses through molecular mimicry [53]. The GBS capsule is composed of repeating subunits of glucose, galactose, N-acetylglucosamine and N-acetyl neuraminic acid, polymerized in a serotype-specific sequence [54]. All GBS serotypes share a terminal alpha 2,3-linked N-acetylneuraminic acid (sialic acid) that is identical to sialic acid found on human cells [55]. Although each serotype displays the same terminal alpha 2,3 linkage of sialic acid, Carlin et al. (2007) suggest the way in which the repetitive subunit is polymerized

into the CPS, types Ia and Ib will contain the highest density of sialic acid over a given length of CPS with one sialic acid residue for every two 2 monosaccharides [55]. The numerous sialic acid residues may limit the availability of the RBL to bind to galactose and thus the modest upregulation of OnRBLs.

4. Conclusions

Here we identified and characterized four predicted RBL genes from Nile tilapia transcriptomes, the first report of this important gene family in the species. Thorough domain structure and phylogenetic analyses allowed us to classify and name these predicted RBLs as well as draw comparisons across fish and shellfish species. Despite encoding the same CRD structure, the predicted tilapia RBLs manifested diverse expression patterns, both basally, and following challenge with *S. agalactiae*, indicating that they may function in varying contexts and respond to a wide range of stimuli. While the expression of each of the RBLs was observed to be significantly induced or repressed by *S. agalactiae* exposure in at least one tissue or timepoint, these changes were relatively modest when compared with those reported in other host/pathogen scenarios. However, our work should enable future characterization of tilapia RBL functions in other homeostatic and immunological processes where they may prove to play additional, and, perhaps, larger roles.

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