Fish & Shellfish Immunology 33 (2012) 1008–1015

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Putative roles for a rhamnose binding lectin in *Flavobacterium columnare* pathogenesis in channel catfish *Ictalurus punctatus*

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ARTICLE INFO

Article history:
Received 21 June 2012
Received in revised form
6 August 2012
Accepted 17 August 2012
Available online 31 August 2012

Keywords:
Flavobacterium columnare
Channel catfish
Rhamnose-binding lectin
Columnaris disease

ABSTRACT

Columnaris disease, caused by the bacterial pathogen *Flavobacterium columnare*, continues to be a major problem worldwide and commonly leads to tremendous losses of both wild and cultured freshwater fish, particularly in intensively farmed aquaculture species such as channel catfish. Despite its ecologic and economic impacts, the fundamental molecular mechanisms of the host immune response to this pathogen remain unclear. While *F. columnare* can induce marked pathologic changes in numerous ectopic tissues, the adhesion of *F. columnare* to the gill in particular is strongly associated with pathogen virulence and host susceptibility. Recently, in this regard, using RNA-seq expression profiling we found that a rhamnose-binding lectin (RBL) was dramatically upregulated in the gill of fish infected with *F. columnare* (as compared to naïve fish). Thus, in the present study we sought to further characterize and understand the RBL response in channel catfish (*Ictalurus punctatus*). We first identified two distinct catfish families with differential susceptibilities to columnaris disease; one family was found to be completely resistant while the other was susceptible (0% mortality versus 18.3% respectively, $P < 0.001$). Exclusively, in the susceptible family, we observed an acute and robust upregulation in catfish RBL that persisted for at least 24 h ($P < 0.05$). To elucidate whether RBL play a more direct role in columnaris pathogenesis, we exposed channel catfish to different doses of the putative RBL ligands L-rhamnose and D-galactose, and found that these sugars, protected channel catfish against columnaris disease, likely through competition with *F. columnare* binding of host RBL. Finally, we examined the role of nutritional status on RBL regulation and found that RBL expression was upregulated (>120-fold; $P < 0.05$) in fish fasted for 7 d (as compared to fish fed to satiation daily), yet expression levels returned to those of satiated fish within 4 h after re-feeding. Collectively, these findings highlight putative roles for RBL in the context of columnaris disease and reveal new aspects linking RBL regulation to feed availability.

1. Introduction

Columnaris disease, caused by the Gram-negative bacterium *Flavobacterium columnare*, is one of the oldest known fish diseases in North America, has been a significant problem in many fish species throughout the world and can lead to tremendous economic losses [1,2]. Members of the family Ictaluridae, particularly the economically important foodfish channel catfish (*Ictalurus punctatus)* are especially susceptible to this pathogen [3,4].

Morphologically, *F. columnare* is a long, slender, non-flagellated rod, 0.3–0.7 μm wide and 3–10 μm long, whose cells exhibit gliding motility. Colonies of *F. columnare* on selective agar are flat, yellow, rhizoid, strongly adherent, and spread across the surface forming irregular margins. Based on the site of infection and gross appearance of infected tissues, the disease is commonly known as "saddleback", "fin rot", or "cotton wool disease". A common clinical presentation of the disease is the pronounced erosion and necrosis of external tissues, with the gills typically being a major site of damage [5]. Presumptive diagnosis of columnaris disease is based on the presence of the clinical signs mentioned above and by the cell morphology (e.g., notably columnar aggregates referred to as "haystacks") of *F. columnare* in wet mount preparations of scrapings from infected tissues [6]. The bacterium is capable of entering the blood stream and is routinely isolated from the internal organs; however, internal lesions are poorly described and are often absent or undetectable [7,8]. Columnaris disease may be exacerbated by elevated organic loads in the water, crowded conditions, and excessive handling [9].

The mucosal surfaces of fish such as the gill and skin are important sites of bacterial exposure and host defense mechanisms

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http://dx.doi.org/10.1016/j.fsi.2012.08.018

[10,11]. It has long been hypothesized that observed differences in disease susceptibility between catfish species or strains are due to the differing ability of the host to prevent pathogen attachment and entry at mucosal epithelial sites such as the gill, skin, and gastrointestinal tract [10,12]. In stark contrast to numerous other bacterial pathogens of fish, experimental infection protocols with *F. columnare* are more effective by contact exposure (i.e., immersion) than by injection [13,15]. Accordingly, the ability to adhere to gill tissue or skin is thought to be a critical initial step in the pathogenesis of columnaris disease [14,15]. Interestingly, several *in vitro*, *ex vivo*, and *in vivo* studies have examined *F. columnare* adhesion and have collectively demonstrated that the virulence of different *F. columnare* isolates closely correlates with their respective ability to adhere and/or persist on host tissue [14–17]. Nevertheless, despite the recognized importance of adhesion, the precise mechanisms by which *F. columnare* attaches to target tissues, and the early events facilitating disease progression remain unclear.

One potential mechanism of attachment may be through lectin-mediated interactions. Lectins can be expressed by bacterial cells, including *F. columnare*, and by host epithelial cells which utilize lectins to bind and agglutinate bacteria by binding to surface glycoconjugates [18]. Decostere et al. [15] reported that treatment of either *F. columnare* bacterial cells or gill tissue with sodium metaperiodate (cleaves the C–C bond between vicinal hydroxyl groups of sugar) or with various sugars significantly reduced bacterial adhesion. They hypothesized that the interaction between a bacterial lectin and its host's carbohydrate receptor explained the reduction. Subsequent studies have shown that catfish mucus from skin and gill promoted chemotaxis of *F. columnare*, that the chemotactic effect was correlated with virulence, and that this effect was inhibited by pretreatment with sodium metaperiodate as well as by D-mannose, D-glucose, and N-acetyl-D-galactosamine [19,20]. Similarly, reports from common carp and catfish include observations of *F. columnare* aggregated within and surrounding mucus pores of the skin and capping tissue of the gill filaments [14,16], suggesting discrete, mucus-dependent areas of pathogen attachment.

Given the consensus that a carbohydrate receptor plays a key role in columnaris adhesive events [15,19], we recently screened host transcript expression signatures of infected catfish gill for candidate genes [11]. Our RNA-seq-based analysis highlighted a rhamnose-binding lectin (RBL) which was by far the most highly up-regulated gene observed in our differentially expressed set, with expression increasing 105-fold by 4 h following infection. This up-regulation had dramatically decreased at 24 h and 48 h, suggesting the importance of this gene during early infection rather than in downstream immune responses. The rhamnose-binding lectin (RBL) family has been identified in over 25 species of fish and its various members have collectively emerged as important effectors in innate immunity [18]. It has been shown that RBLs are a key component in the first line of immune defense and function through recognition of generic protein patterns commonly expressed by various pathogens. In rainbow trout (*Oncorhynchus mykiss*), RBLs were shown to be expressed by numerous cell and tissue types, including those related to the immune system such as spleen, thrombocytes, lymphocytes, monocytes, neutrophils, and mucous (goblet) cells of gills and intestine [21,22]. Agglutination of Gram-negative and Gram-positive bacteria by RBLs is driven by the recognition of L-rhamnose/D-galactose residues of the O-antigen of lipopolysaccharides (LPSs) and lipoteichoic acid (LTA), with binding affinities to smooth type LPS exceeding rough LPS [21–23].

In the present study, we sought to extend these findings by further characterizing the early events of columnaris disease with a specific emphasis on examining the kinetics and magnitude of RBL gene expression within the gill in two families of channel catfish exhibiting different susceptibilities (i.e., susceptible versus resistant) to columnaris disease. We demonstrate that RBL expression is selectively upregulated in the gill of susceptible fish, and that the presence of putative RBL ligands in water, namely L-rhamnose and D-galactose, can strongly protect channel catfish—in a dose dependent fashion—from mortality associated with columnaris disease. Lastly, we provide evidence for nutrition-mediated regulation of expression of RBL transcripts in catfish gill, providing a potential molecular mechanism connecting feeding regimens and columnaris susceptibility.

2. Methods

2.1. Experiment 1 schema and general fish husbandry

The objective of Experiment 1 was to profile the mortality, adhesion kinetics, and RBL transcript expression profiles in two distinct families of channel catfish which we pre-screened and determined to exhibit different susceptibilities to *F. columnare*. Of note, siblings of these respective family members were first revealed to have differential susceptibilities to *Edwardsiella ictaluri*; with susceptibilities being the inverse of columnaris disease (LaFrentz et al. [24]). Channel catfish fingerlings of the two families were stocked into twelve 20-L aquaria, at a density of 20 fish per aquarium. There were six tanks for each family, five of which were challenged with *F. columnare*. At the same timepoint, all challenged tanks received the same dose of *F. columnare* cells, from the same preparative stock. Three of the challenged tanks were utilized for survival data, two tanks for time-course sampling of gill and one non-challenged negative control for each family. Four fish per replicate tank were collected at each sampling timepoint. Fingerlings were 13.9 ± 0.44 g (mean ± SEM). Aquaria contained 10 L of aerated flow-through well water using the "Ultra Low-Flow System" described by Mitchell and Farmer [25]. The flow rate was set to 29 ± 1 ml/min and monitored daily; this rate allows for a natural progression of the disease after challenge in a flow-through environment [25]. Temperature and dissolved oxygen were measured daily with a Wissenschaftlich-Technische Werkstätten (WTW) pH/Oxi 340i/SET meter (Weilheim, Germany). Water temperatures averaged 26.5 ± 0.02 °C and dissolved oxygen averaged 5.81 ± 0.03 mg/L. In Experiments 1 and 2 (see below), Total Ammonia Nitrogen (TAN) concentrations were determined in each tank with a Hach DR/4000V spectrophotometer using the Nessler Method 8038 (Hach Company, Loveland, Colorado) and values averaged 1.46 +/− 0.3 mg/L. In Experiments 1 and 2, an Accumet Basic AB15 pH meter (Fisher Scientific, Waltham, Massachusetts) was used to measure pH (7.5–8.2) during the study. Standard titration methods (APHA 2005) were used to measure total alkalinity (213 mg/L) and total hardness (112 mg/L). In all experiments, fish were maintained on a 12 h dark: 12 h light photoperiod. In Experiments 1 and 2, fish were fed to apparent satiation beginning on day 2 of the study and every day thereafter (35% protein, 2.5% fat; Delta Western, Indianola, Mississippi). At the termination of experiments fish were sacrificed by cervical dislocation.

2.2. Experiment 2 schema

The objective of the second experiment was to investigate the interactions between pathogen and host with regards to ligand–lectin interactions. A generic population of channel catfish fingerlings were stocked at a rate of 30 per tank with 18 tanks in the study. In triplicate tanks, either L-rhamnose or D-galactose were added to tanks at rates of 0 mM, 20 mM, and 40 mM. Fish were exposed to the sugars for 15 min prior to bacterial challenge. At the

same timepoint, all challenged tanks received the same dose of *F. columnare* cells, from the same preparative stock. For each sugar, a single tank was exposed to the 40 mM concentration, but not challenged with the bacterium as a negative control. As an additional negative control, a single tank of unchallenged fish containing no sugar was used. The same aquaria system was employed and data were collected as described in Experiment 1. Water temperatures averaged 26.5 +/− 0.16 °C and dissolved oxygen averaged 5.8 +/− 0.24 mg/L.

2.3. Experimental challenge

Tanks receiving a bacterial challenge in both Experiments 1 and 2 were exposed to *F. columnare* isolate LV-359-01 (a genomovar II isolate as determined by employing the methods of Arias et al. [4]) which was previously demonstrated to produce mortality from columnaris disease in the described system [26]. The isolate was retrieved from a −80 °C freezer and streaked on Ordals' medium [27]; 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 (FCGM; [28]). This suspension was incubated at 28 °C for 24 h and was used to inoculate 1 L of FCGM. The inoculated 1 L broth was incubated for up to 24 h at 28 °C in an orbital shaker incubator set at 200 rpm; when the bacterial growth reached an absorbance of 0.70 at 550 nm (approximately 4.0 E^{10} bacteria/mL), the flask was removed and placed on a stir plate at room temperature. A 10 mL sample was removed from the broth for serial dilution and colony forming unit (CFU) data. In both experiments, 150 mL of bacterial suspension was added to tanks receiving bacterial challenges.

2.4. Bacteriology

Fish were observed daily for clinical signs associated with columnaris disease. Fish unable to maintain neutral buoyancy were considered moribund and removed for sampling. Fish were not fed the first day after challenge, but offered food on day 2 and throughout the rest of the study. Dead and moribund fish were removed from the tanks daily and samples were taken from the necrotic lesions and cultured on selective cytophaga agar (SCA; [26]) containing 5 μg/ml neomycin sulfate and 200 U/ml polymyxin B. This selective media has previously been shown to be effective in inhibiting all bacterial species tested except *Flavobacterium* spp. and *Streptococcus* spp. [7]. If present, a maximum of three moribund or dead fish were sampled from each tank daily. Cultures were incubated at 28 °C for 48 h, and then scored as being positive or negative for growth based on colony morphology (i.e., flat, yellow, rhizoid colonies). Animal care and experimental protocols were approved by the Stuttgart National Aquaculture Research Center Institutional Animal Care and Use Committee and conformed to Agricultural Research Service Policies and Procedures 130.4 and 635.1.

2.5. Quantitative PCR for F. columnare quantification

Water samples were collected from each tank at 15 min following bacterial challenge for bacterial dose confirmation. Briefly, 1 ml of water was removed from each tank, centrifuged, and DNA was extracted as described below. For all experiments, there were no differences in challenge dose between tanks, which averaged 1.6 E^{08} CFU/mL. Fish were also sampled at 0 h, 1 h, and 2 h to assess bacterial adherence to gill; these timepoints were selected based on the findings of Olivares-Fuster et al. [16]. A section of the left second gill arch was taken (approximately 50 mg) from each fish for qPCR analysis. Samples were stored at −80 °C until DNA extraction. DNA extractions were performed according to the manufacturer's directions using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, California). Water samples were extracted with the same kit following the Gram Negative Bacteria protocol, which includes a centrifugation step (7500 rpm for 10 min) to harvest the cells prior to lysis.

The extracted template DNA (comprised of catfish genomic and bacterial DNA) was used for pathogen detection, identity confirmation, and quantification utilizing the primers of Panangala et al. [29] which were FcFp [5′-CCTGTACCTAATTGGGGAAAAGAGG-3′], FcRp [5′-CGGTTATGGCCTTGTTTATCATAGA-3′] and FAM labeled probe [5′-ACAACAATGATTTTGCAGGAGGAGTATCTGATGGG-3′]. This primer and fluorescent probe set targets a region of the chondroitin AC lyase gene of *F. columnare*. Primers and FAM labeled probe were obtained from Applied Biosystems Incorporated (Foster City, California). Quantitative polymerase chain reaction (qPCR) assays were performed on a Lightcycler 480 Real Time PCR system (Roche Applied Science, Indianapolis, Indiana). All samples were run in duplicate. A standard (containing 1.0×10^4 *F. columnare* cells) and a control without extracted template (no-template control) were included on each plate; the standard was used as a positive control and to validate the standard curve within each run. Reactions included 500 nM of forward (FcFp) and reverse (FcRp) primers, 250 nM labeled probe, 1 μL template DNA, BioRad 2× master mix (Bio-Rad Laboratories, Hercules, California), and molecular grade water were added to give 20 μl total reaction volumes. Template for tissue samples contained genomic catfish and bacterial DNA and ranged from 300 to 380 ng/ul using a nanodrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware). The initial DNA denaturation step was 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s and then 60 °C for 30 s. These data were calculated using a Roche Lightcycler 480 software macro for absolute quantification. A standard curve was applied using the software which had been generated from bacterial samples grown in broth then serially diluted and counted from 10^8–10^2. The PCR efficiency was 1.99 and the r^2 value for the standard curve was 0.98, similar to the results generated by Panangala et al. [29]. The equation describing the standard curve line was $y = -3.472 \times +47.94$.

2.6. Nutritional regulation of rhamnose-binding lectins in channel catfish

To examine the role of nutritional status on rhamnose-binding lectin transcript expression, juvenile channel catfish (42.2 ± 5.6 g) were randomly distributed (30 per tank) among three 600 L tanks with forced air aeration and flow-through well water at 24.8 ± 0.8 °C, pH 7.7, and dissolved oxygen of 7.4 ± 0.3 mg/L. Fish were then subjected to one of three treatments. Fish in treatment group 1 were fed to satiation three times daily with a standard catfish ration (35% protein, 2.5% fat; Delta Western, Indianola, Mississippi) for 7 d. In treatment 2, fish were withheld feed for 7 d. In treatment 3 fish were withheld feed for 7 d, subsequently fed to satiation, and at 4 h following the introduction of food, fish were sacrificed. All fish were sacrificed on day 7 and portions of the gill, skin, and intestine were isolated from equivalent locations on each fish and stored in RNALater (Ambion® Brand Products, Life Technologies, Grand Island, New York) at −80 °C until RNA extraction.

2.7. Rhamnose-binding lectin transcript expression

Extracts were analyzed by reverse transcriptase qPCR to investigate changes in the RBL expression during exposure to the bacteria and following different feeding regimens. For the columnaris challenge (experiment 1), fish were sampled at 0 h, 1 h, 2 h, 4 h, 8 h, and 24 h. A section of the left second gill arch was taken

(approximately 50 mg) from each fish for analysis. Three individual fish from each family were used for RBL analysis. For the feeding experiment, three pools (five fish each) of tissue were generated from each timepoint (fed, fasted, fasted and refed). In all experiments, equal amounts of tissue (approximately 50 mg) were collected from each fish within a pool. Tissue samples were collected from the gill, skin, and intestine at each timepoint (for fasted and fed studies). Samples were immediately placed in 5 mL RNAlater (Ambion®) and stored at −80 °C until extractions could be completed at the end of the study. Samples were homogenized with mortar and pestle in the presence of liquid nitrogen. Extractions were performed according to the manufacturer's directions using an RNeasy Kit (Qiagen, Valencia, California). Expression was examined as previously described [10,11]. Briefly, extracted RNA (500 ng) was reverse-transcribed into cDNA with the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California) according to the manufacturer's protocol. cDNA was diluted in nuclease-free water prior to QPCR. Catfish RBL primers, designed based on RNA-seq contig sequence, were (5′ to 3′): Upper—ATCCTCAGTTCTCAGACC; Lower—CAGTTCAGTGTAGCAGTA CTTG. Real-time PCR was performed in a total volume of 20 μl with cycling conditions: 94 °C for 5 s, followed by 40 cycles of 94 °C for 5 s, 60 °C for 5 s (fluorescence measured), and a disassociation curve profile of 65 °C–95 °C for 5 s/0.5 °C increment. Reactions were run on a CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, California) using the SsoFast™ EvaGreen® Supermix. Results were expressed relative to the expression levels of 18S rRNA in each sample using the Relative Expression Software Tool (REST) version 2009 [30]. The biological replicate fluorescence intensities of the control and treatment products for each gene, as measured by crossing-point (*C*t) 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. A no-template control was run on all plates. Ct values are given in Supplementary Table 1. QPCR analysis was repeated in triplicate runs (technical replicates) to confirm expression patterns.

2.8. Statistical analysis

Survival data was analyzed with SigmaPlot 11 (San Jose, California) using Kaplan–Meier Log Rank Survival Analysis and all pair-wise multiple comparisons used the Holm–Sidak method with adjusted *P* values. Treatment effects were considered significant at $P \leq 0.05$. The RTQ-PCR expression data were analyzed using a one-way ANOVA, with a post-hoc Tukey test for significant differences at $P \leq 0.05$.

3. Results

3.1. Clinical signs in disease challenge tests

Moribund fish in challenged tanks displayed signs consistent with a *F. columnare* infection. Fish were lethargic, and gross pathologies were typical; the skin of moribund fish initially had discrete depigmented areas that became multifocal and diffuse, often encompassing most of the body, as the infection progressed. Necrotizing dermatitis and cutaneous sloughing exposed the underlining muscle in severe cases. The gills had focal and multifocal necrotizing branchitis with yellowish pigment. Severely frayed fins were common as the infection progressed. No internal gross pathology was observed. Notably, *F. columnare* was cultured from at least one tissue from all fish sampled. Cultures were considered positive if at least one colony matched the colony morphology of *F. columnare*.

3.2. Two catfish families exhibit different susceptibilities to columnaris disease

In experiment 1, over the duration of the 7 day study, no mortality was observed in the resistant family, while 11 out of 60 (18.3%) fish died in the susceptible family ($P < 0.001$; Fig. 1). This rate of mortality was consistent with that previously published with the Ultra-Low-Flow system [25]; a challenge system designed to reliably reproduce columnaris disease with the kinetics and severity of mortality approximating columnaris disease epizootics. The mortality observed in the susceptible family of fish occurred on days 2–5 post-challenge.

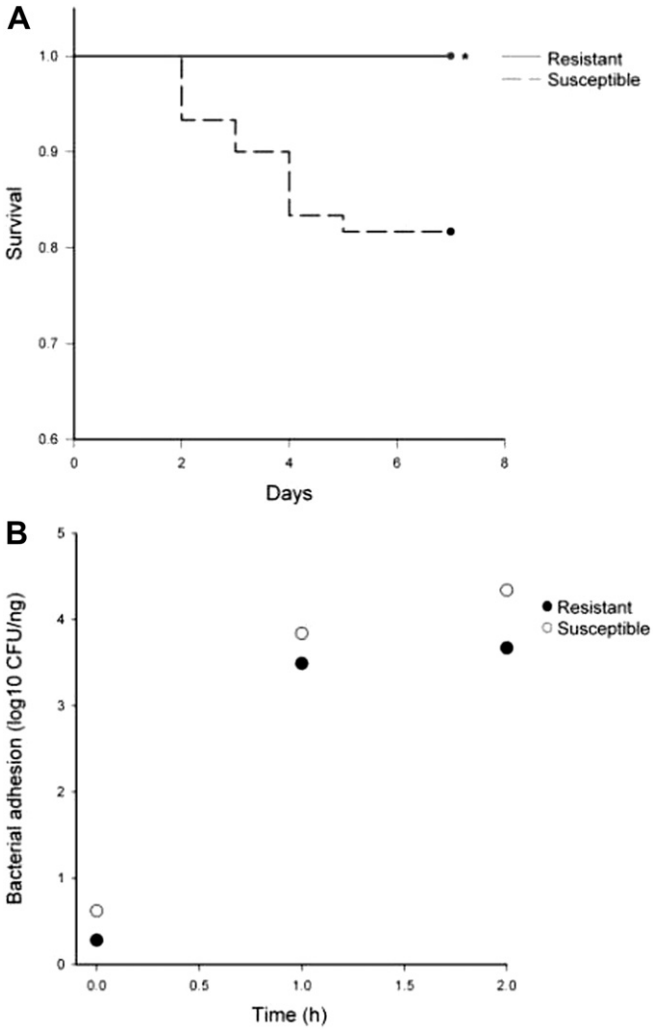

Fig. 1. Two channel catfish families exhibit different susceptibilities to columnaris disease and show disparate bacterial adhesion trends. A.) Kaplan–Meier survival analysis plot demonstrating that one family of channel catfish tested experienced no mortality (termed resistant) in a 7 day challenge study, while in the second family tested 11 out of 60 (18.3%) fish died in a 7 d period (termed susceptible; asterisk denotes statistical significance, $P < 0.05$). Results are from three replicate tanks containing 20 fish per tank. No mortality was observed in unchallenged tanks from either family (not shown). B.) Quantitative PCR (specific for chondroitin AC lyase gene) results showing mean bacterial adhesion (log10 CFU/ng) on gill tissue at just before challenge, and 1 h and 2 h post-challenge.

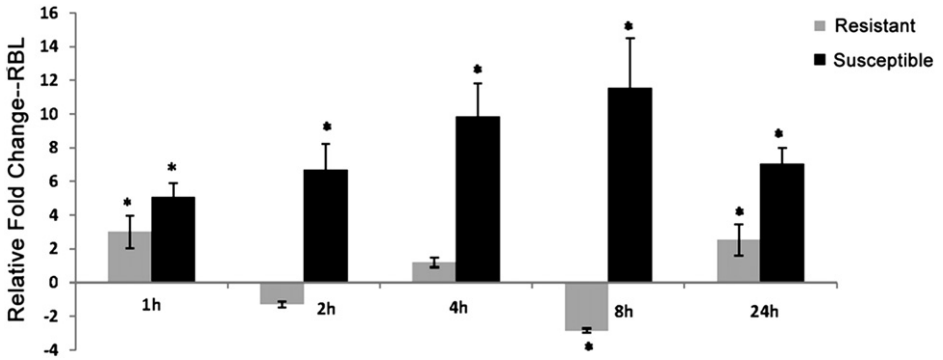

Fig. 2. Mean fold change (+/− SE) of transcript expression of catfish rhamnose-binding lectin (RBL) at five timepoints in gill following *F. columnare* challenge. Individuals ($n = 3$ from each family at each timepoint) from two families with known patterns of susceptibility to columnaris were assayed for expression levels. Fold changes are expressed as relative to uninfected control samples from a given family, normalized against 18S rRNA expression levels. * indicates significant ($P < 0.05$) fold at a given timepoint relative to 0 h.

3.3. *F. columnare* rapidly adheres to the gills of susceptible fish

At 1 h, susceptible fish had higher levels of bacteria adhered to gill (3.9 ± 0.48 $\log_{10}$ CFU/ng; mean ± SEM) as compared to resistant fish (3.5 ± 0.25 $\log_{10}$ CFU/ng). Similarly, at 2 h, susceptible fish had a greater number of bacteria adhered to gill (4.3 ± 0.48 log10 CFU/ng) as compared to resistant fish (3.6 ± 0.35 $\log_{10}$ CFU/ng). Despite having greater than a half log more bacteria adhered to gill in susceptible fish, these differences failed to reach statistical significance due to large sample variability.

3.4. Catfish rhamnose-binding lectin is strongly upregulated in fish susceptible to columnaris disease

Expression of RBL transcripts was strongly upregulated in gill samples at all timepoints following infection in fish from the susceptible family, while expression levels among resistant fish manifested only minimal changes with the exception of at 1 h and 8 h post-infection (Fig. 2). Basal expression levels between individuals from either family differed significantly ($p < 0.05$), with 5.35-fold higher expression in susceptible individuals at 0 h.

3.5. Rhamnose-binding lectin ligands L-rhamnose and D-galactose reduce mortality by F. columnare

We next sought to determine whether the blocking of rhamnose-binding lectins *in vivo* could have a protective function against columnaris disease. Indeed, when added to water 15 min before challenge, L-rhamnose reduced *F. columnare*-induced mortality in a dose-dependent fashion (Fig. 3). Over the course of 5 d, in the positive control fish treatment (not treated with any RBL ligand) 31 out of 90 fish died (all values are across three replicate tanks containing 30 fish per tank), while only 16 out of 90 fish died in the 20 mM L-rhamnose ($P < 0.05$) treatment and 10 out of 90 fish died in the 40 mM L-rhamnose treatment ($P < 0.05$). Similarly, D-galactose provided protection against mortality with 10 deaths out of 90 fish in the 20 mM D-galactose and 13 out of 90 fish died in the 40 mM D-galactose groups (Fig. 4).

3.6. Rhamnose-binding lectin transcript expression in catfish gill is acutely regulated by feeding status

Given the known importance of feeding regimen/nutritional status on columnaris disease development (see Discussion), and the effective reduction of early *Flavobacterium columare* mortality following addition of RBL ligands, we investigated whether RBL levels are responsive to feed availability. A dramatic significant upregulation of RBL transcripts in the gill of fasted fish (>120-fold, $p < 0.05$) was observed (Fig. 5). Expression levels returned to close to those of the continuously fed control following a short period of refeeding (fasted + 4 h), indicating a sensitive regulation of expression based on feed availability. RBL levels in other mucosal tissues (skin and intestine) did not show similar large fluctuations in expression, with only a modest changes of expression in the intestine of fasted fish, and non-significant changes in all other groups (Fig. 5).

4. Discussion

F. columnare ("columnaris") outbreaks are an annual threat to freshwater aquaculture operations worldwide, often brought on by routine handling or crowding stress, changes in water quality parameters, or primary infections by parasites or bacterial pathogens. Despite its widespread impact, our understanding of *F. columnare* infectious processes remains limited. In the present work, we examined potential roles for a rhamnose-binding lectin (RBL) recently identified in transcriptomic studies of catfish gill following columnaris infection [11]. Our results, building on

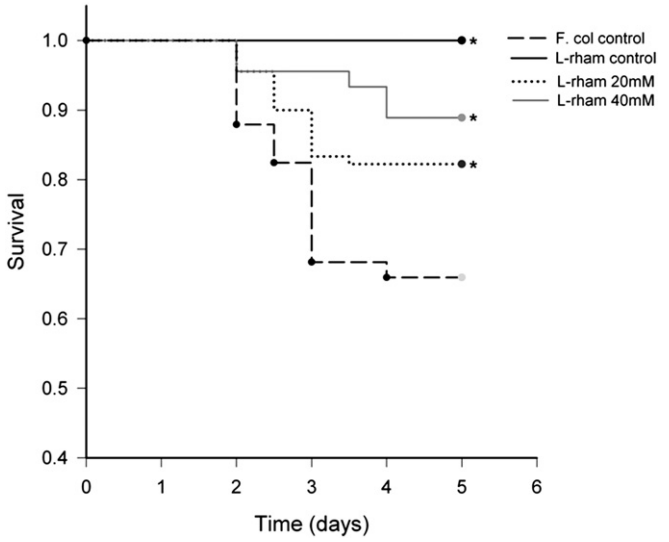

Fig. 3. Kaplan–Meier survival analysis plot of challenged untreated fish (F. col control), unchallenged fish treated with 40 mM L-rhamnose (L-rham control), and challenged fish treated with 20 mM (L-rham 20 mM) or 40 mM L-rhamnose (L-rham 40 mM). For L-rhamnose treatments, fish were exposed to indicated concentrations for 15 min before bacterial challenge. * indicates significant ($P < 0.05$) from the F. col control.

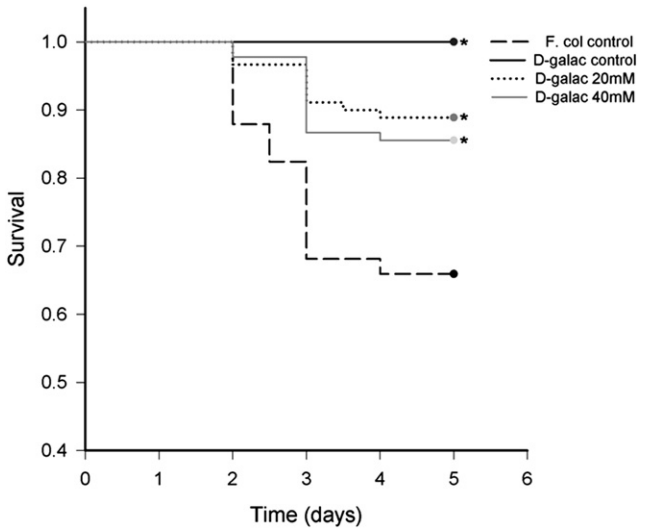

Fig. 4. Kaplan–Meier survival analysis plot of challenged untreated fish (F. col control), unchallenged fish treated with 40 mM D-galactose (D-galac control), and challenged fish treated with 20 mM (D-galac 20 mM) or 40 mM D-galactose (D-galac 40 mM). For D-galactose treatments, fish were exposed to indicated concentrations for 15 min before bacterial challenge. * indicates significant ($P < 0.05$) from the F. col control.

previous evidence for lectin-mediated host–pathogen interactions [15,19], indicate heightened RBL responses in catfish susceptible to columnaris, the effective use of RBL ligands to reduce columnaris mortality, and the regulation of RBL transcript expression by feed availability. Collectively, these findings represent a putative mechanism for RBL action in the context of nutrition and disease and will drive future studies to determine the breadth and scope of RBL functions.

In the present study, the use of differentially susceptible families of catfish to model the disease demonstrates that susceptibility to columnaris disease may be due, in some part, to early transcriptional changes in RBL transcript expression. RBLs are viewed as important innate effectors situated at critical interfaces of environment and host, such as mucosal epithelium where they are believed to contribute to immune protection. Alternatively, we show here that RBL transcript levels are higher under homeostatic conditions and are markedly upregulated in susceptible fish following exposure to *F. columnare*. Moreover, when putative RBL ligands were added to the water as *in vivo* competitive agonists, strong protection against columnaris disease was provided. Taken together and in stark contrast to the classical immunoprotective roles of RBL, we contend that *F. columnare* may 'hijack' RBL activity as a means by which to initiate disease in channel catfish. While the innate immune response evolved to generate rapid and robust inflammatory responses to cope with microbial insults, it is no surprise that many pathogens have evolved to avoid detection altogether, or to immunoedit/immunosculpt effector responses to improve chances of pathogen survival [31]. More specifically, with regards to *F. columnare* this dramatic RBL upregulation could represent an inappropriate or overexuberant response that was mounted with intentions to clear the pathogen, yet instead served to facilitate or exacerbate columnaris disease. Interestingly, this very same RBL upregulation may, in some part, have conferred the protection seen by siblings derived from this identical family when

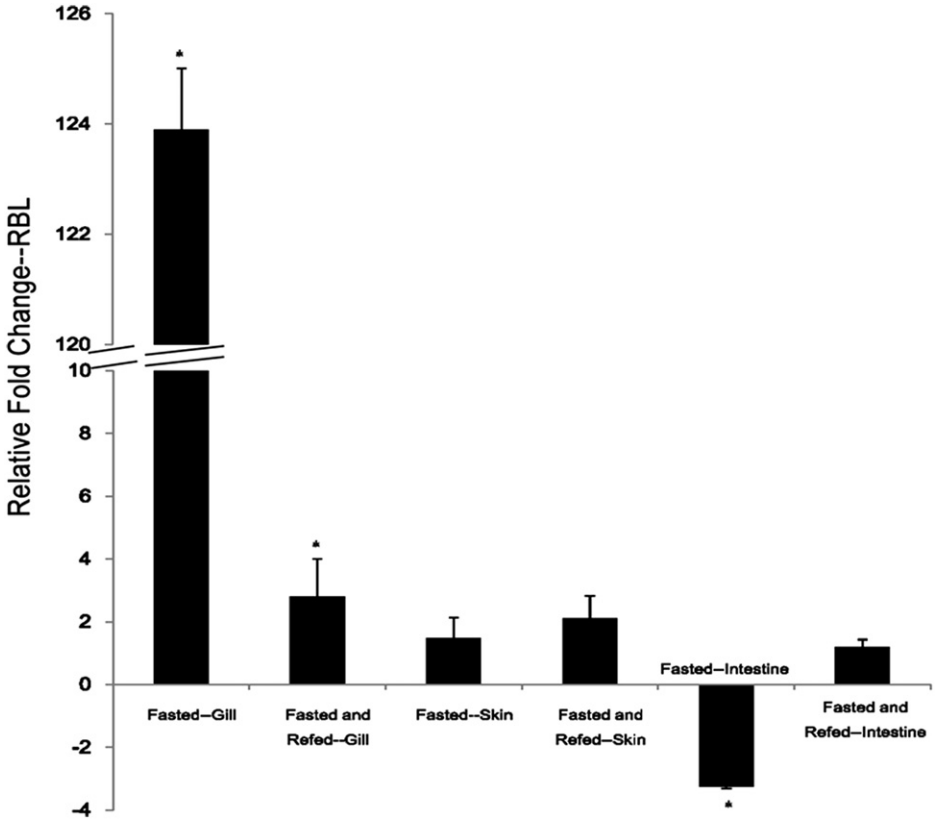

Fig. 5. Mean fold change (+/− SE) of transcript expression of catfish rhamnose-binding lectin (RBL) in fasted (7 d) and fasted and refed (7 d + 4 h) in three mucosal tissues. Fold changes are expressed as relative to fed control samples from a given tissue, normalized against 18S rRNA expression levels. * indicates significant ($P < 0.05$) change relative to fed control within a given tissue.

challenged with *E. ictaluri*, the causative organism of enteric septicemia of catfish (ESC) (data not shown; personal communication, Brian Peterson, Catfish Genetics Research Unit, Stoneville, Mississippi). Ongoing studies in our laboratory are examining the potential role of RBL in the context of ESC. Clearly, future studies are needed to more broadly characterize patterns of lectins on both host and pathogen during early adhesive/invasive timepoints.

The manipulation of feeding regimens represents a simple intervention to cope with disease outbreaks in production settings, and therefore, has been one of the most common strategies in the US catfish industry for managing infectious disease. However, research on the impact of feed availability has suggested differing approaches for the two most common bacterial diseases, ESC and columnaris. In the former, studies by Wise et al. [32] demonstrated that feeding concomitant with *E. ictaluri* exposure produced the highest rates of mortality. Withholding feed after challenge reduced mortalities by close to 50%. Alternatively, feed deprivation of catfish for a 4 week period before and a 2 week period after *F. columare* challenge resulted in significantly higher mortality than was observed in fed groups [33,34]. These contrasting results are particularly noteworthy in light of the aforementioned report [24], which shows an inverse correlation between susceptibility to *E. ictaluri* and *F. columnare*. Indeed, as described previously, the families utilized in the present study, upon screening for ESC resistance, were identified to have differing columnaris susceptibilities. Given the importance of feed availability to both diseases, we asked whether short-term fasting could modulate expression levels of RBL. While we are aware of no previous reports linking host lectin regulation and nutritional status in vertebrates, recent studies of invertebrate mucosal lectins have shown significant (>1000-fold) upregulation of expression of these molecules following feed deprivation [35,36]. We observed a 123-fold increase in transcript expression of RBL in catfish gill, following a 7 d period of fasting. This increase may have the effect of multiplying attachment sites for *F. columnare*, thereby increasing susceptibility to disease, in accordance with the observations of Shoemaker et al. [34], a study which emphasized the importance of continuous feeding. The rapid down-regulation of RBL levels (from fasted levels) following initial re-feeding indicates the likely sensitivity of RBL production to feed-derived carbohydrate ligands. Future studies should determine the consequences of differing feeding regimens for ESC and columnaris disease outcomes in the context of RBL expression patterns.

Notably, RBL transcript expression, while present in skin and intestine, showed little evidence of dietary regulation in those tissues. This result may indicate that catfish RBL assumes unique roles in gill (perhaps conserved from its filter-feeding invertebrate ancestors) or that the timing or nature of samples collected did not allow a comprehensive view of RBL expression patterns. Further work to map lectin (and other immune factor) expression in these mucosal tissues during feeding and fasting is underway using RNA-seq technology.

Several additional avenues and alternative explanations need to be explored in order to validate the proposed model. We assume here that transcript levels are representative of similar increases in expressed protein or higher protein activity, but this remains to be validated. Direct binding of catfish RBL and *F. columnare* remains unproven and observed effects may be due to secondary pathways of which RBL may be a member. Use of RBL sugar ligands may have broad impacts on both host and pathogen receptors and mediate attachment and host responses on several levels. Host expression profiles in the gill and sugar ligand effects for *F. columnare* may not be key determinants of disease progression following infection with the enteric pathogen *E. ictaluri*. Lastly, the model requires validation in additional families and strains and species of catfish which differ in their susceptibility to *F. columnare*.

5. Conclusion

While evidence for lectin-mediated interactions between host fish and *F. columnare* has been building for over a decade, candidate receptors had been lacking. Here, results from our initial examination of catfish RBL transcript levels and RBL ligand blocking ability, in the context of bacterial challenge and feed deprivation, are supportive of a critical, multi-faceted role for mucosal lectins in immunonutritional regulation. Further elucidiation of molecular mechanisms governing the long-observed interplay between feeding status and pathogen susceptibility will be vital for the development of immunoprotective diets and updated feeding strategies for aquaculture.

Acknowledgements

The authors would like to thank Brian Peterson for providing fish and Matt Barnett for his technical assistance and expertise. We would also like to thank S. Adam Fuller, Marty Riche, and Huseyin Kucuktas for carefully reviewing the manuscript. This study was funded by the USDA/ARS under project number 6225-31630-006-00. The USDA is an equal opportunity provider and employer. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2012.08.018.

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