B-glucan immunostimulation against columnaris in a white sturgeon (Acipenser transmontanus) model

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ABSTRACT

Flavobacterium columnare represent one of the most important bacterial pathogens of cultured sturgeon. However, at present there are no commercially available vaccines to prevent infection and treatment options are limited. β-glucans have been shown to be potent immunostimulants that can provide fish protection against infectious disease. In this study, the effects of dietary β-glucan supplementation on disease susceptibility were examined by exposing 0.3% β-glucan-fed white sturgeon (Acipenser transmontanus) to Flavobacterium columnare in laboratory-controlled challenges. Morbidity and mortality were monitored for 15 days post-challenge (dpc). Additionally, transcript levels for pro-inflammatory cytokines, regulatory cytokines and acute phase proteins (APP) were investigated in the spleen and gills at different time points post-challenge. No evidence of protection was observed in β-glucan-fed fish challenged with the bacteria. Moreover, significantly greater mortalities were observed in β-glucan-fed fish challenged with F. columnare (p<0.05), likely associated with acute inflammatory response as haptoglobin and serotransferrin transcripts in the gills were significantly higher in fish within this group at 1 dpc. Transcript levels for all tested cytokines and APP in the spleen were similar amongst treatment groups. The results from this study suggest that β-glucan supplementation at the concentration and rate investigated provides no-benefit to white sturgeon against F. columnare.

1. Introduction

Sturgeon (Acipenser spp.) aquaculture has become a rapidly expanding industry mainly focused on restocking fry and fingerlings into natural reservoirs and commercialization of caviar and meat for human consumption [1,2]. The main cultured species are white sturgeon (Acipenser transmontanus), Siberian sturgeon (Acipenser baerii), Adriatic sturgeon (Acipenser naccarii) and Russian sturgeon (A. gueldenstaedtii) [3]. Along with reducing pressure on wild-caught fisheries, sturgeon culture is a multi-million dollar industry generating considerable producer revenue and employment opportunities [1–3]. However, with the rapid expansion of intensive sturgeon culture, infectious diseases have also emerged, causing significant challenges to the sturgeon industry, typically resulting in high morbidity and mortality and serious economic losses [3–5].

Flavobacterium columnare is a filamentous Gram-negative bacterium responsible for significant economic losses in several cultured freshwater fish including white sturgeon [6], rainbow trout Oncorhynchus mykiss [7], Atlantic salmon Salmo salar L. and coho salmon O. kisutch

Abbreviations

dpc days post-challenge
Fc F. columnare
FDA U.S. Food and Drug Administration
USDA United States Department of Agriculture
MSA modified-Shieh agar
PBS phosphate buffered saline
FCGM F. columnare growth medium
MS-222 buffered tricaine methanesulfonate
SAA serum amyloid A
TNF-α tumor necrosis factor alpha
TGF-β transforming growth factor beta
IL-17 interleukin 17 predicted protein
IRF8 interferon regulatory factor 8
MHCII major histocompatibility class II
PCR polymerase chain reaction
RT-qPCR reverse transcription quantitative real-time PCR

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2. Materials and methods

Immunostimulants are emerging as an alternative tool to antibiotics and vaccines to combat infectious disease in aquaculture [17]. This includes β-glucans, which are high molecular-weight polysaccharides extracted from D-glucose building blocks that are common components of the cell walls of bacteria, fungi, algae, and plants [18]. As one of the most well-studied immunostimulants, β-glucans are broadly used to improve the health of both domesticated animals and humans [19]. Various studies in aquaculture models have confirmed β-glucans as a potent immunostimulant to enhance the immune defense system of fish and to reduce the spread of infectious diseases [20]. Specifically, β-glucans have been shown to enhance both humoral and cellular immunity inducing short-lived and long-lived effects on the fish immune response resulting in protection against several infectious diseases [21]. Additionally, it has been reported that administration of β-glucans can result in stress-reduction [20], growth enhancement [22] and anti-toxin effects [23] to additionally support food fish production. In sturgeon, feed supplementation with β-glucans have been shown to help prevent losses due to the fungus *Veronaea botryosa* [24,25].

Few studies have investigated the immune responses of sturgeon to bacterial pathogens or characterized the effects of immunostimulants on systemic response to disease. To date, no study has investigated the effects of immunostimulants on *Flavobacterium columnare* infections in white sturgeon. Therefore, the objectives of this study were to evaluate the protections conferred to white sturgeon fed β-glucans and challenged with *Flavobacterium columnare* and to examine cytokine and acute-phase protein transcript expression in the gills and spleen of white sturgeon after β-glucan immunostimulation in feed.

2. Materials and methods

2.1. Fish and husbandry conditions

A total of 250 white sturgeon fingerling (53.2 ± 15.5 g; 21.6 ± 2.2 cm) (mean ± SD) were obtained from a local producer in Northern California, USA and acclimated to laboratory conditions for two weeks. Ten fish were subjected to clinical examination and bacterial culture was performed on the posterior kidney prior to beginning the experiment to ascertain that the population was non-infected before challenge. Fish (n = 240/tank) were maintained in an outdoor, enclosed, 1000-L freshwater tanks with flow-through, 18 ± 1 °C well water. Fish were maintained in this tank for 30 days and provided a “β-glucan feed” or “normal feed” regimen as described below. After this period, fish were transferred to 130-liter tanks (20 fish/tank, three replicatetanks per treatment) with fresh, flow-through well water at a rate of four liters per minute at 18 ± 2 °C.

2.2. Diets

Fish were fed a basal commercial feed (Skretting, Tooele, UT, USA) at 1% body weight per day. The β-glucan diet included the basal feed supplemented with 0.3% β-glucan (3 g of β-glucan per 1 kg basal diet) derived from the yeast *Saccharomyces cerevisiae* (Macrodarg®, Biorigin, Sao Paulo, Brazil) along with vegetable oil (10 ml/kg food) mixed for 10 min manually. The non-β-glucans diet was prepared by mixing the feed with vegetable oil only [24]. The pellets were dried overnight at room temperature and stored at 4 °C until used. All diets were prepared weekly.

2.3. Bacteria

The *F. columnare* isolate was cultured from an outbreak of columnaris in Lahontan cutthroat trout *Oncorhyncus clarkii henshowi* in California in 2018 and identified as *F. columnare* by de Alexandre Sebastião, et al. [6]. *Flavobacterium columnare* was grown on modified-Shieh agar (MSA) [26] supplemented with tobramycin (1 μg/ml) [27], and incubated at 28 ± 0.5 °C for 48 h.

2.4. Laboratory controlled challenges

All challenges were conducted under protocols approved by the University of California, Davis Institutional Animal Care and Use Committee. Forty-eight hours pre-challenge, *F. columnare* colonies were inoculated into 10 mL of *F. columnare* growth medium (FCGM) broth [28] in a 50 mL conical tube and incubated at 28 °C for 17–18 h with shaking at 180 rpm. The broth culture was then inoculated into 100 mL of FCGM broth in a 500 mL Erlemeyer flask and incubated at 28 °C for ~15–18 h with shaking at 170 rpm until reaching a optical density of 0.901 at 600 nm to produce ~10^7 CFU/mL. The bacterial suspension was diluted ten-fold using PBS and spread on MSA to estimate the number of CFUs used in the *F. columnare* challenge.

*Flavobacterium columnare* challenge was performed by immersion as in Soto, et al. [29] with some modifications. *Flavobacterium columnare* suspension was inoculated into tank water to obtain a challenge concentration of 1.1 × 10^6 CFU/mL for 2 h in static conditions with aeration. Two hours post challenge, the flow of water into the tanks was resumed. The *F. columnare* control group was immersed with sterile FCGM broth and treated in a similar manner. Morbidity and mortality were recorded daily for 15 days following *F. columnare* challenge. Fish presenting with two or more clinical signs of poor body condition, skin ulceration, hyperemia, loss of balance, lethargy, anorexia, scale protrusion, and/or exophthalmia were euthanized using 500 mg/L of buffered MS-222. Gills of 3–5 moribund and dead fish per tank were cultured to verify the cause of mortality.

At 1, 5 and 10 days post-challenge (dpc), two fish per tank (six fish per group) were euthanized with MS-222 and ~30 mg of gill and spleen were collected. Tissues were immediately preserved in RNAlater (Qia-Gen) and stored at −80 °C until analysis.

At the end of the challenge, nine survivors (3 fish/tank) were cultured from each treatment group to determine carrier status. Posterior kidney and gills were streaked on MSA plates and incubated for 48 h at 28 °C. *Flavobacterium columnare* was morphologically identified as golden-yellow, flat, rhizoid colonies with irregular margins, tightly adherent to the agar.

2.5. Transcript expression analysis using quantitative reverse transcriptase PCR (RT-qPCR)

Quantitative analysis of haptoglobin, serotransferrin, serum amyloid A (saa), tumor necrosis factor alpha (tnfa), transforming growth factor beta (tgfb), interleukin 17 predicted protein (il17), interferon regulatory factor 8 (irf8), and major histocompatibility class II (mhcII) transcript expression in the gills and spleen tissues was investigated using RT-qPCR.

[8], tilapia (*Oreochromis* spp) [9], and channel catfish *Ictalurus punctatus* [10]. *Flavobacterium columnare* strains have been assigned into four different genetic groups (GG) likely representing distinct bacterial species with some fish-host association [11]. Recently, LaFrentz, et al. [12] identified that these four GG of *F. columnare* represent four different species with the names *F. columnare*, *F. covae* sp. nov., *F. davisi* sp. nov., and *F. oreochromis* sp. nov., representing genetic groups 1, 2, 3, and 4, respectively. Columnaris disease has been described as both a primary and secondary infection [13]. Generally, fry and fingerling are most sensitive to columnaris infection and clinical signs include frayed fins or fin rot, necrotic lesions on the mouth and gills and depigmented ‘sad-dleback’ lesions on the skin [14].

Currently, few effective vaccines and antibiotics are available to combat columnaris in cultured fish. Additionally, development of antimicrobial resistant bacterial strains and the high costs and low efficacy that have been associated with immunization and treatment highlight the need for other prophylactic tools against these infectious diseases [15,16].

Microbial resistant bacterial strains and the high costs and low efficacy of the fungus *S. cerevisiae* [9] , and channel catfish *Ictalurus punctatus* [10]. Currently, few effective vaccines and antibiotics are available to combat columnaris in cultured fish. Additionally, development of anti-microbial resistant bacterial strains and the high costs and low efficacy that have been associated with immunization and treatment highlight the need for other prophylactic tools against these infectious diseases [15,16].

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2.6. Histopathology

Tissues from six F. columnare challenged fish that died between two and five dpf in the glucan-fed group and six fish that died from the normal-fed group were preserved in 10% buffered formalin and submitted for histopathologic analysis. Additionally, six survivors from each challenged group and three fish from each control group were euthanized at the end of the experiment for histological analysis. Sections of gill, heart, liver, spleen, and cranial kidney were collected from each fish and processed routinely, sectioned at 5 μm, and stained with hematoxylin and cosin (H&E). Tissues were examined blindly by a single pathologist. Based on preliminary examination of tissues, each organ was semi-quantitatively scored for the amount of inflammatory infiltrates, cellular necrosis, and bacteria (Table 2).

2.7. Statistical analysis

Survival was analyzed using SigmaPlot 11.0, Kaplan-Meier Survival Analysis. Multiple comparisons were performed using the Holm-Sidak method. Histological scores for each variable were compared between groups and between tissues using a one-way ANOVA. Transcript expression differences between the different treatments at each time point, and within the same treatment at different time points were compared using a two-way ANOVA and Tukey’s multiple comparisons test. Histologic scoring and transcript expression analysis was performed using GraphPad Prism 9 (San Diego California, USA).

### Table 2

| Observed Tissue Change | Scoring 1 (Mild) | Scoring 2 (Moderate) | Scoring 3 (Severe) |
|------------------------|-------------------|----------------------|-------------------|
| Inflammatory cell infiltrates | Not present | Infiltration by ≤ 10 mononuclear cells within a 20x field. | Infiltration of 11–50 mononuclear cells within a 20x field. |
| Cellular necrosis | Not present | Rare pyknosis and/or karyorrhexis of cells affecting ≤ 5% of the entire tissue. | Pyknosis and/or karyorrhexis of cells affecting 5–50% of the entire tissue. |
| Bacteria | Not present | Bacteria are present within ≤ two inflammatory cells within a 40x field. | Bacteria are present within 3–5 inflammatory cells and/or in a single extracellular aggregates within a 40x field. |

### Table 1

| Gene | Oligo name | Primer sequences (5′–3′) | Refs. |
|------|------------|--------------------------|-------|
| beta-actin | Sturact60F | CATGGTCAACCACGTGGGATGAC | Roy, et al. [31] |
| elongation factor | Sturact125R | ACAGCACTCCTAATGTAAGAGGT | Roy, et al. [31] |
| | EF | GGACTCCACTGAGCCACCT | Akbarzadeh, et al. [32] |
| | R | GGTTGACGGCCATCTCCTTG | Akbarzadeh, et al. [32] |
| haptoglobin | AcHp-3′ F | ACAGTCCTCTGCTGAGGTCAC | Soto, et al. [33] |
| | AcHp-3′ R | ATGGACCTATGGATGACG | Soto, et al. [33] |
| | AcSTF-1 M-F | TCTGCTACGTCATTGGCTTC | Soto, et al. [33] |
| | AcSTF-1 M-R | TCTGCTACGTCATTGGCTTC | Soto, et al. [33] |
| | AcMHC-IIb | ATGGAAGCGTCGATTTCTTG | Soto, et al. [33] |
| | AcMHC-IIb F | ATGGAAGCGTCGATTTCTTG | Soto, et al. [33] |
| | AcMHC-IIb R | ATGGAAGCGTCGATTTCTTG | Soto, et al. [33] |
| Tissue Change | Scoring 1 (Mild) | Scoring 2 (Moderate) | Scoring 3 (Severe) |
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| Inflammatory cell infiltrates | Not present | Infiltration by ≤ 10 mononuclear cells within a 20x field. | Infiltration of 11–50 mononuclear cells within a 20x field. |
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| Bacteria | Not present | Bacteria are present within ≤ two inflammatory cells within a 40x field. | Bacteria are present within 3–5 inflammatory cells and/or in a single extracellular aggregates within a 40x field. |
moribund fish sampled. Cultures did not yield *F. columnare* from the gills of the surviving fish (*n* = 36) in all four treatment groups at 15 dpc.

Similar transcript levels of *tnfa*, *tgfb*, *il17*, *irf8*, and *mhcII* were quantified in the gills of control and treatment groups at 1 dpc, 5 dpc, and 10 dpc (Fig. 2). However, haptoglobin, *saa* and serotransferrin transcripts were significantly higher in β-glucan-fed fish challenged with *F. columnare* when compared to glucan-fed non-exposed control fish at 1 dpc (*p* < 0.05, two-way ANOVA followed by Tukey’s multiple comparisons test). Associated with peak of morbidity and mortality in normal-fed fish challenged with *F. columnare*, higher expression of the acute-phase proteins haptoglobin, *saa* and serotransferrin were observed 5 dpc; however, only serotransferrin transcript level was significantly greater to those quantified in glucan-fed fish challenged with *F. columnare* (Fig. 2). By 10 dpc, the transcript number of most cytokines had decreased to similar levels of control treatments; however, transcript levels of *tnfa* significantly increased in β-glucan-fed fish challenged with *F. columnare* (Fig. 2). Within the spleen, higher expression of *saa*, serotransferrin, *tnfa*, *il17*, *irf8* and *mhcII* transcripts were observed 5 dpc when compared to 1 and 10 dpc, although most were not significantly different (Fig. 3).

### 3.2. Histopathology

Histopathologic changes were consistent between mortalities from β-glucan and normal-fed challenged groups with no significant differences in semi-quantified scores for inflammatory infiltrates, cellular necrosis, or bacterial infiltration of the tissues. The most common finding in mortalities was a moderate to severe, diffuse, necrotizing interstitial nephritis (7/12, 57.4%) and a mild to moderate, multifocal to diffuse necrotizing branchitis (6/12, 58.3%) rarely associated with extracellular filamentous bacteria consistent with *F. columnare* in H&E stained sections. The severity of cellular necrosis and bacterial infiltrates were significantly higher in both kidney and gill compared to liver, heart, and spleen (*p* = 0.03) (Fig. 4). One β-glucan-fed mortality had a mild, multifocal lymphoplasmacytic myocarditis. The levels of inflammatory infiltrates and tissue necrosis were significantly higher in challenged fish compared to recovered and non-challenged-control fish (*p* = 0.001). Tissues from fish that were challenged but recovered from infection were relatively uneventful. The only finding was a mild,...
multifocal, branchial hyperplasia and fusion in one normal-fed challenged fish.

4. Discussion

Results of this study showed no evidence of improved protection in fish fed 0.3% β-glucan supplemented feed for 30 days and challenged with _Flavobacterium columnare_. Moreover, significantly greater mortalities associated with higher gill transcript levels of several acute phase proteins were observed in glucan-fed fish challenged with _F. columnare_ compared to the control diet of normal-fed fish. Although β-glucan administration has been shown to improve protection in fish against a number of pathogens, this is not the first study to demonstrate negative outcomes associated with β-glucan supplementation [34]. These data highlight that immunomodulation of fish requires a more in-depth understanding at both the basic and applied level before they can be used consistently to improve fish health.

There are a number of potentially overlapping factors that may have resulted in the outcomes of this study. This includes sturgeon-specific response to immunostimulation, route of administration, and the study’s environmental parameters. Twenty-one day supplementation of β-glucan has been shown to provide protection in white sturgeon challenged with _Veronaea botryosa_, a systemic fungal disease resulting in phaeohyphomycosis [24]. This indicates effective immunomodulation does occur in sturgeon. The dosing timeline was shorter than the 30-day feeding trial described herein which suggests shorter immunostimulation periods may be more optimal for white sturgeon. However, it is difficult to directly compare results as systemic fungal infection may require different protective immune responses to combat _V. botryosa_ compared to that associated with a largely mucosal bacterial infection such as columnaris. Alternative β-glucan administration routes may provide different levels of protection in mucosal tissues. Specifically, immersion administration of β-glucans has been found to increase wound healing in carp skin [35] and provide surface protection to chum.
salmon against Saprolegnia spp. [36]. This is similar to immersion vaccination in fish which has been found to induce more localized mucosal immune responses compared to systemic responses elicited with vaccine that is distributed by feed or injection [37]. There is also evidence that feeding β-glucan in fish held at cooler water temperatures stimulates greater immunomodulatory effects of some immune factors [38]. Along with exploration of various administration routes, the effects of environmental parameters during the feeding and challenge time periods warrant further investigation.

The effectiveness of β-glucans has also been shown to be affected by the concentration, frequency and duration of administration. Positive protective responses have been achieved in salmonids against Aeromonas hydrophila with β-glucan dosage ranges between 0.1% [39] and 0.5% [40]. Although the 0.3% supplementation dosage used in this study falls within this range, species-specific, fish life stage, and infection pathogenesis may influence the protection received [41]. Decreased effectiveness of β-glucans has been associated with inappropriate treatment duration leading to stress induction and immunosuppression [15]. It has been hypothesized that extended β-glucans administration downregulates the immune system by negative feedback regulation [42]. In rainbow trout, fish supplemented with 0.2% β-glucans for 15 days showed higher expression of immune-related genes of il1β and il10 in spleen, tgfβ in kidney and HSP70 in gills as compared to treatments fed the same diet for 30 days [43]. In addition, Amphan, et al. [44] suggested that optimal frequency of feeding β-glucans to tilapia is every-other-week to provide better protection against A. hydrophila in comparison with continuous feeding for 2 and 4 weeks.

Branchial and splenic levels of haptoglobin, serotransferrin, saa, cathelicidin, tnfα, il17, irf8, tgfβ and mhcII were chosen to investigate pro-inflammatory, regulatory, innate and adaptive immune responses within this challenge model [24, 43]. Although transcript levels of most inflammatory mediators were similar between treatments, transcript expression of acute phase proteins were higher in the gills of β-glucan-fed fish challenged with F. columnare at one dpc compared to other treatments. Serum amyloid A and haptoglobin are some of several acute-phase proteins generated early in the initial immune response to infection [45,46] which coincides with the one dpc increase observed in this study. Previous studies have showed increasing levels of haptoglobin expression after bacterial infection suggesting an important role preventing iron loss as a hemoglobin scavenger [47]. Similarly, saa’s role early in infection as pro-inflammatory regulator is well studied in mammals and fish [46,48]. Serotransferrin plays a crucial role in regulating availability of iron during infection and immunostimulation of macrophages and other inflammatory responses [39]. Increased acute phase protein transcript levels are consistent with trends found in V. botryosa infected white sturgeon compared to non-exposed controls 6 weeks post-challenge [24] suggesting sustained responses of these inflammatory mediators during infection.

The association between high acute phase protein transcript expression and increased mortality in β-glucan-fed fish challenged with F. columnare suggests that the supplementation regimen may have contributed to an excessive initial inflammatory response within gills contributing to mortality. Although immune response overstimulation has not been well characterized in fish, this phenomenon is well described in mammals [49]. Necrotizing branchiitis is a predominant histopathologic finding which is consistent with what is described during natural infections as a leading contributor to morbidity and mortality in fish [14].

Haptoglobin, saa and serotransferrin levels were not simultaneously increased in the gill and spleen which may suggest that β-glucan regulation of cytokine expression is organ-dependent. For example, Falco, et al. [50] described up-regulation of tnfα2 transcript expression in the head kidney and concurrent down-regulation of tnfα2 transcript expression in the gut of A. salmonicida infected fish. These findings indicate that organ-specific responses to β-glucan may be important for optimal immune response, especially with pathogens such as F. columnare that have a predilection for the gills and other mucosal sites.

5. Conclusion

This study suggests feed supplementation with β-glucans at the concentration and rate investigated has no benefit to help prevent losses due to F. columnare in cultured white sturgeon. Interestingly, significantly greater mortalities were observed in fish immunostimulated and challenged with F. columnare, associated with a significant increase in the acute inflammatory mediators haptoglobin, saa, and/or serotransferrin transcripts in the gills. Future research investigating various β-glucan supplemental protocols to explore alternate dosages and feeding duration of β-glucans are warranted for examine their use in the management of these important pathogens and to optimize husbandry within the white sturgeon industry. Furthermore, the combinational use of β-glucans with one or more immunostimulators in fish diet can be considered to boost the white sturgeon immune system.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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