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Effects of orally administered immunostimulants on inflammatory gene expression and sea lice (*Lepeophtheirus salmonis*) burdens on Atlantic salmon (*Salmo salar*)

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ABSTRACT

Sea lice (*Lepeophtheirus salmonis*) are the most economically important ectoparasites affecting Atlantic salmon (*Salmo salar*) culture worldwide. In recent years the efficacy of historically successful treatments has been on the decline. As such, a new management strategy for controlling lice infections is a high priority for the salmon farming industry. In this study, we tested the ability of three orally administered immunostimulants to decrease the number of lice successfully infecting Atlantic salmon post-smolts. It was found that the β-glucan (ProVale) fed group actually maintained more sea lice than did the control group (24% increase). However, both the CpG ODN (31–46%) and AllBrew NuPro (11–31%) fed groups showed decreased infection levels when compared to the control group. Histopathological and differential gene expression analyses indicate that localized and systemic inflammatory mechanisms may be transiently altered by these immunostimulatory feeds and may result in increased host resistance to sea lice.

1. Introduction

Aquaculture is one of the world's fastest growing agricultural sectors, with cultured fin fish projected to surpass that of capture fisheries in 2011 (FAO, 2010). Atlantic salmon (*Salmo salar*) accounts for approximately 90% of the total salmonid culture, with an annual worldwide production of approximately 1,000,000 tonnes (FAO, 2012). As with all cases of intensive fish culture, stress and disease can sometimes become problems due to the densities at which the animals are reared. The sea louse, *Lepeophtheirus salmonis*, is the most economically important ectoparasite in Atlantic salmon culture due to the significant pathology it causes to the infected host (Costello, 2009). The economic impact linked to lice infections has been tallied at over 300 million USD annually and has made the therapeutic approaches to dealing with the infestation a lucrative industry (Costello, 2009).

Immunostimulants have been used in human and terrestrial animal health for many decades and, as of late, have become important tools in fish health management programs (Bricknell and Dalmo, 2005; Carrington and Secombes, 2006). The least stressful, and most cost effective way to deliver immunostimulants to fish on a commercial scale is through "in-feed" oral administration (Guselle et al., 2010; Tacchi et al., 2011). Although manipulation of the host's own immune response is currently a common practice in many extensive aquaculture facilities the particular immunostimulant, along with dosage and administration timing, is often species and life-stage specific (Doñate et al., 2010; Tacchi et al., 2011). Identification of immunostimulants, and their respective dosages, showing efficacy against sea lice infection could prove to be a viable mode of controlling lice populations in a commercial culture situation.

The immunostimulating substances contain pathogen-associated molecular patterns (PAMPs) that the vertebrate host recognizes as being foreign by pattern-recognition receptors (PPRs). The PAMPs can contain either molecular or structural features that are commonly found in microbial pathogens, but that are extremely rare in vertebrates (Lui et al., 2010). Signaling through PRRs initiates a cascade of pro-inflammatory cytokines and can lead to increased phagocytosis, bactericidal activity, respiratory burst, cytotoxic abilities, antiviral activity and enhanced activity of the complement cascade (Carrington et al., 2004; Cuesta et al., 2008b; Tacchi et al., 2011). One of the most common immunostimulants used in agriculture and aquaculture is β-glucan. β-Glucans are cell wall carbohydrates that have been extracted from yeast, fungi, or plants (Volman et al., 2008). Yeast β-glucans are the major structural component of yeast cell walls and are found in a β-(1,3)-linked glucose chain arrangement, with a small number of β-(1,6)-linked branches (Guselle et al., 2010). For salmonids, both intraperitoneal administration and oral administration of β-glucans have been shown to enhance resistance to bacterial pathogens and also the microsporidial parasite

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Loma salmonae (Guselle et al., 2010). However, their effectiveness at enhancing resistance to external parasite infections has not been substantiated (Doñate et al., 2010).

Another commonly used PAMP is unmethylated DNA which contains cytosine–phosphate–guanine oligodeoxynucleotide motifs (CpG ODN) (Cuesta et al., 2008a). In vertebrate genomes, CpG motifs are suppressed and methylated, but in bacterial and viral genomes they are prevalent and are commonly unmethylated (Lui et al., 2010). Unmethlyated DNA, containing CpG motifs act as danger signals to the vertebrate host and trigger an immune response (Carrington et al., 2004). Recognition of CpG motifs by host PRRs specifically occurs through engagement of the Toll-like receptor (TLR) 9, which in turn induces antiviral and antibacterial cell signaling (Carrington and Secombes, 2006; Lui et al., 2010; Strandskog et al., 2008). The efficacy of these motifs for control of arthropod ectoparasites is not as yet known.

The efficacy of 3 orally administered immunostimulants (β-glucan (ProVale), CpG ODN, and a soluble yeast fermentation extract-Brewer's yeast compound (AllBrew NuPro)) was tested with regard to their ability to protect Atlantic salmon from infection by *L. salmonis*. We hypothesized that by enhancing systemic, and subsequently localized, inflammatory mechanisms through immunostimulation prior to *L. salmonis* exposure, we would be able to both accelerate and boost Atlantic salmon protective responses to infection by *L. salmonis*. To the best of our knowledge, this is the first time that CpG ODN has been orally administered to any species of fish. It is also, the first time that immunostimulants have been administered orally and the effects on immune gene expression were followed alongside histopathological changes and disease development.

2. Material and methods

2.1. Fish husbandry

All experimental protocols for the use of fish followed the guidelines provided by the Canadian Council on Animal Care (2005; www.ccac.ca/Documents/Standards/Guidelines/Fish.pdf) and were submitted for review and approval to the UPEI Animal Care Committee (UPEI Animal Care Protocol #10-014).

Atlantic salmon smolts were obtained from a commercial production facility in Pennfield, New Brunswick, Canada. The fish were transported to the Atlantic Veterinary College aquatic holding facility at the University of Prince Edward Island (UPEI) in Charlottetown, Prince Edward Island, Canada. Fish were held in circular flow-through tanks containing 250 L of 11 °C (10.6–11.1 °C) freshwater for the first 3 weeks as acclimatization. The system was then switched to saltwater (Instant Ocean®, Cincinnati, OH) recirculation over the course of 7 days, resulting in a final salinity of 33 ppt. Fish were held for a further 2 weeks to acclimatize to the saltwater. During this period, fish were fed a control diet at 1% body weight/day, divided over 2 feeds. Fish were kept on a light:dark cycle of 14 h:10 h. At the start of the study, fish weighed 60.8 ± 9.5 g (mean ± SD), and by the end of the study the average weight was 135.5 ± 3.0 g (mean ± SD).

2.2. Feed production

The 5 treatment groups in the study were: control feed (uninfected), control feed (infected with *L. salmonis*), ProVale™ (400 g/1000 kg feed; infected with *L. salmonis*), CpG ODN 1668 (20 g/1000 kg feed; infected with *L. salmonis*), and ABN (4 kg/1000 kg AllBrew + 1 kg/tone NuPro; infected with *L. salmonis*). All feeds were produced by Northeast Nutrition Ltd., Truro, NS. The base feed (also used as the control feed throughout the study) was Northeast Nutrition Signature 2.5 mm salmon feed. CpG ODN (Sigma, MO) and ProVale (Stirling Products, PE) components were dissolved in water and then applied to the feed as a top coat with ethanol. The AllBrew and NuPro combination (ABN) (Alltech Inc., Nicholasville, KY) was milled directly into a separate mixture of the base diet formulation. An ethanol treated control feed was not included in the study as the top-coated components were applied weeks prior to the start of the study, giving ample time for ethanol evaporation. Any residual traces of ethanol would have been dispersed when the feed came in contact with water at the time of oral administration.

2.3. Sea lice culture and infection

Gravid female *L. salmonis* were collected from cage cultured Atlantic salmon in New Brunswick, Canada, and transported back to the laboratory. They subsequently had their egg strings removed and maintained in aerated, 13 °C ± 1.0 °C saltwater collected from the culture site until the nauplii hatched and molted into copepodids (~9–10 days). Fish were infected following a modified method of Mustafa et al. (2000). Approximately, 15 copepodids/fish were added to each of the infected tanks for 6 h under conditions of heightened aeration (i.e. O_2 maintained > 8 mg/L). The control tanks were subjected to the same conditions but without the addition of copepodids. Before the recirculation system was turned back on, a 100 μm mesh was placed over the inflow of the control uninfected tanks to prevent copepodid exposure at any point during the trial. This procedure for sea lice infection was carried out 3 times over the course of the study (25, 39 and 41 days after the fish were given treated feed). Multiple, low level challenges were administered because they better mimic field conditions than do large, single dose exposures. In sea cages, salmon are repeatedly exposed to small numbers of infective copepodids. Single exposures to large numbers of infective lice induce a disease state, and provide little information about the interactions that take place when parasites are present at lower abundances (Wagner et al., 2008). Additionally, large, single pulse infections lead to increased settlement of lice on the gills, an artifact not seen in the wild or cage culture (Wagner et al., 2008).

2.4. Study design

Following the saltwater acclimatization period, tanks of 30 fish were randomly assigned to each treatment group (i.e. control uninfected, control infected, CpG ODN, ProVale and ABN), with each treatment fed to duplicate tanks. Just prior to the administration of treated feed, a T(0) sampling of 2 fish/tank (n = 20) was taken. These measurements were used as a normalization factor for future gene expression analysis. Fish were kept on treatments for 7 weeks. T(1) sampling (n = 12/group) occurred after 2 weeks, and prior to *L. salmonis* exposure. T(2) sampling (n = 12/group) occurred after 5 weeks on treated feed and 10 days after the initial lice exposure. T(3) sampling (n = 12/group) occurred after 7 weeks on treated feed and 22 days after the initial infection and 10 days post final exposure (dpfe). T(4) (n = 12/group) sampling was 29 days after the initial infection and 17 dpfe, and 1 week off of treated feed. A final sampling of all groups except ProVale™ was done after 37 dpfe to determine final lice counts and stages on each fish, without any gene expression or histopathological analysis (Fig. 1).

2.5. Sampling procedure

All control and treatment groups were sampled (n = 12/treatment) at each of the 5 time points. Feed was withheld for 24 h prior to sampling, and fish were euthanized with an overdose of tricaine methanesulfonate (MS-222: 250 mg/L; Syndel Laboratories Ltd., BC) and blood was sampled within minutes of immobilization. At each of the sampling points post-infection (T 2–4), the number of the lice found on each fish was recorded. A subset of lice (n = 25/group/time) was also placed in 10% neutral buffered formalin (NBF) for future staging. Spleen, intestine, anterior kidney and