EFFECTS OF DIETARY SUPPLEMENTATION OF IMMUNOSTIMULANTS AND PROBIOTICS ON IMMUNE RESPONSE AND DISEASE RESISTANCE IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Authorization to Submit Thesis

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Abstract

Flavobacterium psychrophilum is the etiological agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS). Current prevention strategies are limited and disease treatment primarily relies on antibiotics. As concerns about antibiotic use increase so too does the search for alternative treatment and prevention methods.

In this study, testes meal from pink salmon, *Oncorhynchus gorbuscha*, testes collected from Alaskan seafood processing byproducts was supplemented in diets to determine effects on growth and immune function of rainbow trout, *Oncorhynchus mykiss*. Three diets were formulated for this study: a diet containing 5% testes meal, a diet containing a β-glucan immunostimulant (Macrogard) and a control diet. Each diet was fed to triplicate groups of juvenile rainbow trout for 12 weeks, at which time they were challenged with *F. psychrophilum*. No significant differences in growth were observed. Intermittent increases in phagocytic activity were observed in fish fed the testes meal. At 12 weeks; however, the phagocytic activity was significantly lower in fish fed the testes meal and Macrogard diets. Fish fed the testes meal and Macrogard diets also had significantly lower ($P < 0.05$) antibody titers at 8 weeks post challenge. These findings suggest suppression of the innate immune response following long term application impaired the adaptive immune response.

To further investigate potential feed additive benefits, this study also examined the long term use of the probiotics *Enterobacter* sp.strains C6-6 and C6-8 in combination with an attenuated *F. psychrophilum* vaccine. In the first challenge, rainbow trout fry were fed a diet top-coated with C6-6, C6-8 or a combination of the two from first feeding and challenged at 54 days post hatch with a virulent strain of *F. psychrophilum*. Following

challenge there were no significant differences in the cumulative percent morality (CPM) between any of the treatments, suggesting long term feeding of these probiotic strains may have limited benefits. For the second challenge, fish fed the same probiotic treatments as above were vaccinated with a live attenuated *F. psychrophilum* vaccine by 90 second immersion and subsequently challenged with virulent *F. psychrophilum*. In the second challenge, relative percent survival values were lower than expected, potentially due to high mortality in the challenge. However, fish fed C6-6 prior to vaccination had significantly lower mortality following challenge. This suggests that feeding with this probiotic strain prior to vaccination may be beneficial. The reason for this enhanced efficacy is not clear, but this could represent a more focused management strategy for BCWD.

In an attempt to gain a baseline understanding of the potential mechanisms of protection of C6-6, a final study was conducted to determine if injection of the bacteria into rainbow trout reduced mortality and resulted in cross-protection due to *F. psychrophilum* infections out to 28 days. Fry received an intraperitoneal injection of phosphate buffered saline (negative control), supernatant from a C6-6 culture, formalin killed C6-6 or live C6-6 and were subsequently challenged with *F. psychrophilum* 7 days and 28 days post injection. In the challenge occurring 7 days post injection, mortality was significantly reduced ($P < 0.05$) in fish injected formalin killed and live C6-6 compared to the negative control. In the challenge 28 days post injection high variability within treatments was observed and as a result there was no significant reduction in mortality. Interestingly, at both time points significant reduction in mortality rate was observed in all treatments following log rank survival curve analysis when compared to the negative controls. At 28 days post injection, fish receiving formalin killed and live C6-6 also had significantly increased cross-reactive

antibodies to *F. psychrophilum*. This was unexpected, but indicates that both innate and adaptive responses are stimulated by *Enterobacter sp.* C6-6, and that benefits observed following use of this probiotic are in part linked to immune enhancement.

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Dedication

I would like to dedicate this work to my parents Rick and Peggy Fehringer. Without their unending love and support this project would not have been possible.

Table of Contents

List of Figures

Chapter 2: Partial replacement of fish meal with salmon testes meal from Alaskan seafood processing byproducts: effects on growth and immune function of rainbow trout, *Oncorhynchus mykiss* (Walbaum)

Chapter 3: The effects of probiotic feeding and vaccination on survival of rainbow trout (*Oncorhynchus mykiss*) challenged with *Flavobacterium psychrophilum*

Chapter 4: The probiotic (*Enterobacter* sp. C6-6) elicits a protective response to rainbow trout (*Oncorhynchus mykiss*) following injection and challenge with *Flavobacterium psychrophilum*.

List of Tables

Chapter 2: Partial replacement of fish meal with salmon testes meal from Alaskan seafood processing byproducts: effects on growth and immune function of rainbow trout, *Oncorhynchus mykiss* (Walbaum)

Chapter 3: The effects of probiotic feeding and vaccination on survival of rainbow trout (*Oncorhynchus mykiss*) challenged with *Flavobacterium psychrophilum*

Chapter 1: Review of Literature

Immunostimulants

Immunostimulant use has become widely accepted in the aquaculture industry (Sakai, 1999). Initially an immunostimulant was defined as a compound that targeted cells of the mononuclear phagocyte system (Seljelid, 1990). However, Bricknell and Dalmo (2005) redefined an immunostimulant as "a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens." As such, the Bricknell and Dalmo (2005) definition removed the requirement for a specific mode of action in order for a compound to be defined as an immunostimulant. Immunostimulants, unlike vaccines, act on the innate immune system and therefore, do not provide any immunological memory or long lasting protection. However, because immunostimulants enhance non-specific defense mechanisms they can promote protection against a number of different pathogens simultaneously (Sakai, 1999).

Types of Immunostimulants

Unlike previous definitions, Sakai (1999) divided immunostimulants into groups based upon their source: bacterial, algae derived, animal derived, nutritional factors, and hormones/cytokines. Improvements in immune response and disease resistance of fishes have been reported following exposure to a variety of compounds including, vitamin C (Verlhac et al., 1996 ; Ortuño et al., 1999), vitamin E (Wise et al., 1993), lipopolysaccharide (Knight et al., 1998 ; Ilievet al., 2005), alginate (Conceicao et al., 2004) glucans (Bagni et al., 2005; Bonaldo et al., 2007 ; Refstie et al., 2010), yeast cells (Reyes-Becerril et al.,

2010), nucleotides (Sakai et al., 2001; Leonardi et al., 2003 Tahmasebi-Kohyani et al., 2011), and chitin (Anderson and Siwicki, 1994; Kawakami et al., 1998).

Glucans

The most common immunostimulants in aquaculture are β-1.3 and β-1.6 glucans (Ringø et al., 2011). Glucans are polysaccharides most commonly isolated from bacterial cell wall, mushrooms, algae, cereal grains, and yeast (Zeković et al., 2005). The immunomodulatory effects of glucans were first noted in mammals, where administration induced haemopoiesis, enhanced innate immunity, and increased pathogen resistance (Di Luzio, 1985). In aquaculture, glucans have been shown to increase disease resistance and innate immunity in a number of species, including; sea bass, *Dicentarchus labrax*, (Bagni et al., 2000; Bagni et al., 2005; Bonaldo et al., 2007), rainbow trout, *Oncorhynchus mykiss*, (Jørgensen and Robertsen, 1995; Thompson et al., 1995; Ogier et al., 1996; Sealey et al., 2008), and Atlantic salmon, *Salmo salar*, (Robertsen et al., 1990; Engstad et al., 1992; Refstie et al., 2010) among others. Glucans have also been shown to increase resistance to a number of different bacterial and viral pathogens including but not limited to *Aeromonas hydrophila* (Wang and Wang, 1997; Selvaraj et al., 2005; Kumari and Sahoo, 2006), *Edwardsiella tarda* (Yano et al., 1989; Sahoo and Mukherjee, 2002; Misra et al., 2006a), *Vibrio anguillarum* (Robertsen et al., 1990; Skjermo, et al., 1995; Skjermo and Berg, 2004;), infectious hematopoietic necrosis virus (Sealey et al., 2008), and white spot syndrome virus (Chang et al., 1999; Sajeevan et al., 2009). Glucans are also capable of affecting numerous components of the innate and adaptive immune system including antibody titers (Chen and Ainsworth, 1992; Aakre et al., 1994; Kamilya et al., 2006), complement activity (Engstad et al., 1992; Misra et al., 2006), lysozyme (Engstad et al., 1992; Jørgensen and Robertsen,

1995; Gopalakannan and Arul, 2010) and lymphocyte activity (Chen and Ainsworth, 1992; Siwicki et al., 1994; Jørgensen and Robertsen, 1995; Yoshida et al. 1995; Sahoo and Mukherjee, 2001).

Nucleotides

Nucleotides are the base components of RNA and DNA and provide essential physiological functions such as, mediating energy metabolism and cell signaling (Carver and Walker, 1995; Cosgrove, 1998). Nucleotide nutrition with respect to immunity in fishes received little attention until Burrells et al. (2001a) reported immune and disease resistance modifications following dietary supplementation of nucleotides. Burrells et al. (2001a) supplemented a traditional rainbow trout and Atlantic salmon diet with nucleotides. Following 3 weeks of feeding and subsequent exposure to sea lice, *Lepeophtheirus salmons*, fish fed the nucleotide diet had significantly fewer lice attached ten days after exposure compared to the control diet. In a similar study, Burrells et al. (2001b) examined the effects of dietary nucleotides on the effects of vaccination, sea water transfer, growth rates, and physiology of Atlantic salmon. Fish that were fed a diet supplemented with nucleotides and then vaccinated against *Aeromonas salmonicida* had significantly higher antibody titers and significantly reduce mortality following *A. salmonicida* challenge than fish fed the control diet. Fish fed the nucleotide diet also had significantly lower plasma chloride levels three weeks after transfer to salt water and significant increases in growth five weeks post salt water transfer.

After the initial reports of the effects of nucleotides on growth and immunity, numerous other studies were conducted and have reported similar benefits of dietary nucleotide supplementation. Sakai et al. (2001) reported significant increases in phagocytic

activity, serum lysozyme, and complement activity as well as a reduction in the number of pathogenic bacteria in tissues following challenge with *Aeromonas hydrophila* in carp, *Cyprinus carpio*, fed a diet containing yeast nucleotides. Similarly, Li et al. (2004) fed hybrid striped bass, *Morone chrysops* x *Morone saxatilis*, a diet supplemented with yeast RNA for eight weeks and reported increases in neutrophil oxidative radical production and enhanced survival following *Streptococcus iniae* challenge. Leonardi et al. (2003) reported increases in the number of circulating B lymphocytes, increased resistance to infectious pancreatic necrosis virus, and reduced plasma cortisol in rainbow trout administered a nucleotide supplemented diet for 120 days. Finally, Tahmasebi-Kohyani et al. (2011) reported improvements in final weights, feed conversion rations, and specific growth rates in rainbow trout after feeding a nucleotide supplemented diet, as well as increases serum complement and lysozyme levels.

The mechanism by which dietary nucleotides influence growth and immunity is not fully understood. There are three ways that nucleotides are provided to most tissues: *de novo* synthesis, the salvage pathway and through the diet (Carver and Walker, 1995). Under normal metabolic circumstances, aquaculture diets are to thought contain sufficient amounts of nucleotides (Cosgrove, 1998). However, during times of stress, rapid growth, or rapid cell division dietary levels may be insufficient to meet the increased metabolic demands; thus, forcing cells to resort to the more energetically expensive *de novo* synthesis. Increased dietary supplementation of nucleotides may allow cells to forgo *de novo* synthesis and operate more efficiently (Carver and Walker, 1995; Burrells et al., 2001a).

Probiotics

The most widely accepted definition of a probiotics is taken from Fuller (1987) and states that a probiotic is a cultured product or live microbial feed supplement which beneficially affects the host by improving intestinal balance. Alternatively, Gram et al. (1999) proposed that a probiotic is any live microbial supplement which beneficially affects the host animal by improving its microbial balance and is not necessarily dependent on feed. However, this definition still indicates direct action of the probiotic on the host. For the purpose of aquaculture, Moriarty (1999) suggested that the definition be expanded to include microorganisms applied to or naturally occurring in the rearing environment. Thus, in aquaculture the definition has expanded to include any organism that is beneficial to the health of the host regardless of application method (Irianto and Austin, 2002). A large variety of microorganism have been examined for their potential as probiotics including both gram-negative, gram positive bacteria, bacteriophages, yeasts, and unicellular algae (Irianto and Austin 2002).

Mode of action

The mode of action for most probiotics has not been investigated (Irianto and Austin 2002). However, Balcazar et al. (2006) identified the five most common modes of action for probiotics: competitive exclusion, source of nutrients and enzymatic contribution to digestion, influence on water quality, enhancement of the immune response, and antiviral effects.

Competitive exclusion

It is well known that bacteria are capable producing antimicrobial compounds; therefore, interactions between probiotics and a potentially pathogenic bacteria are common

(Balcazar et al., 2006). Competitive exclusion by probiotics can occur in many ways, including, competition for adhesion sites and nutrient sources. Vine et al. (2004) reported that probiotic strains isolated from clown fish (*Amphiprion percula*) were capable of displacing the pathogenic *Vibrio alginolyticus* in fish mucus. Similarly, Verschuere et al. (1999) reported that protection of *Artemia* sp. by various probiotic strains required administration of live probiotics and hypothesized that competition for energy sources and adhesion sites was the mode of action. Probiotic strains have also been shown to produce compounds directly inhibitory to pathogenic bacteria. Gram et al. (1999) found that supernatant of the probiotic *Pseudomonas fluorescens* grown in iron limited conditions inhibited the growth of *V. anguillarum* and hypothesized iron binding siderophores produced by the probiotic to be the mode of action. Chythanya et al. (2002) reported that *Pseudomonas* I2 produced a low molecular weight antibacterial compound inhibitory to pathogenic *Vibrio* species.

Source of nutrients and enzymatic contribution to digestion

Intestinal microflora are well known for the ability to influence digestion, and strains of probiotics have shown to further increase these benefits (Irainato and Austin 2002). For example, Ramiraz and Dixon (2003) found that intestinal bacteria of oscars, *Astronotus ocellatus*, angelfish, *Pterophyllum scalare*, and southern flounder, *Paralichthys lethostigma*, were capable of breaking down normally indigestible compounds such as chitin, cellulose, and collagen to a form suitable for the host. Additionally, application of the probiotics *Bacillus subtilis* and *Lactobacillus acidophilus* have been reported to increase weight gain in tilapia, *Oerochromis niloticus*, and attributed to effects on the digestibility of feed (Aly et al., 2008).

Enhancement of the immune response

Probiotics have also been reported to have direct effects on the immune system of fishes. Salinasa et al. (2008) reported increases in phagocytic activity and cytotoxic activity following administration of *Lactobacillus delbrüeckii* ssp. lactis or *B. subtilis* or a combination of both strains to gilthead seabream, *Sparus aurata*. Similarly, Son et al. (2009) reported increases in alternative complement activity, serum lysozyme, phagocytic activity, and respiratory burst activity of grouper, *Epinephelus coioides*, fed the probiotic *Lactobacillus plantarum*. Finally, Panigrahi et al. (2004) reported increases in serum lysozyme, phagocytic activity, and complement activity in rainbow trout following feeding of the probiotic strain of *Lactobacillus rhamnosus.*

Bacterial coldwater disease

Flavobacterium psychrophilum is the etiological agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS). The disease was first described by Davis (1946) as peduncle disease following epizootics in rainbow trout, based upon pathology affecting the peduncle and caudal fins. The etiological agent was not isolated until 1948 following an outbreak in coho salmon, *Oncorhynchus kisutch* (Borg, 1948). When originally isolated the bacteria was incorrectly believed to be a myxobacterium and classified as *Cytophaga psychrophila* (Borg, 1948). Recent molecular analysis has allowed for reassignment of the bacteria to its current classification of *F. psychrophilum*. (Bernardet et al., 1996).

Pacha (1968) described *F. psychrophilum* as a gram-negative, aerobic, filamentous rod 1.5 to 7.5 µm long and 0.75 µm wide. Alternatively, Bernadart and Kerouault (1989) described the length of the bacterium as ranging between 3 and 10 µm long, however,

morphology may vary depending on age and culture conditions (Kondo et al., 2001). Gliding motility is a common feature of *F. psychrophilum*, however, some strains have been reported to lack this feature (Pacha, 1968; Bernardet and Kerouault, 1989; Schmidtke and Carson, 1995). Growth of *F. psychrophilum* occurs between 4 and 30^{0}C and cultures are typically maintained on triptone yeast extract salts (TYES) or *Cytophaga* media (Holt et al., 1993; Cain and LaFrentz, 2007). Under laboratory conditions when grown on TYES, *F. psychrophilum* forms yellow colonies approximately 3 mm in diameter with thin spreading margins (Cipriano and Holt, 2005). Standard laboratory conformation of *F. psychrophilum* utilizes culture techniques and serological or microtiter agglutination tests.

Epizoology

F. psychrophilum has a worldwide distribution, and likely affects all species of salmonids (Nematollahi et al., 2003). However, coho salmon and rainbow trout are particularly susceptible (Davis 1946; Holt et al., 1993). The pathogen has also been detected in Japanese eel, *Auguilla japonica*, (Izumi et al., 2005), common carp (Lehmann et al., 1991), ayu, *Plecoglossus altivelis*, (Lee and Heo, 1998), perch, *Perca fluviatilis*, (Madetajo et al., 2002), and roach, *Rutilis rutilis* (Madetajo et al., 2002). Disease outbreaks in salmonids can result in mortality ranging from 5-85% and are largely dependent on fish species and size (Barnes and Brown, 2011). Clinical signs of *F. psychrophilum* infections largely depend on species and size. Most commonly infection manifests as skin and muscle lesions particularly in the peduncle region (Cipriano and Holt, 2005). Lesions on the anterior dorsal fin, the lateral side, near the vent, and on the lower jaw are also common (Holt et al., 1993). Additionally, fish can exhibit spinal deformities, erratic swimming, distended abdomens, exophthalmia, enlarged spleen, dark coloration, and petichial

hemorrhaging of the organs (Cipriano and Holt, 2005). Epizooitics in salmonids are most common between the temperatures of 4 and 10^0C but have been reported up to 18^0C (LaFrentz et al., 2004; Cipriano and Holt, 2005).

Transmission

Horizontal transmission of *F. psychrophilum* via waterborne contact is well established (Madesen and Dalsgaard, 1999). *F. psychrophilum* has been found in the skin mucus, connective tissue of the gills, and opercula of fish (Nematollahi et al., 2003). Live fish are reported to shed up to 1×10^7 cells/fish/hr and shedding rates are even higher in dead fish (Madetajo et al., 2002; Nilsen et al., 2011). The portal of entry for *F. psychrophilum* remains unclear; however, abrasions of the skin and mucus increase infection rates among fish challenged by bath or cohabitation (Madetoja et al., 2000).

Vertical transmission of *F. psychrophilum* is also likely (Brown et al., 1997; Taylor 2004). *F. psychrophilum* has been associated with salmonid milt (Holt, 1993; Ekman et al., 1999), ovarian fluid (Madsen and Dalsgaard, 1999), and both the surface and inside eggs (Brown et al., 1997; Taylor 2004).

Treatment and prevention

There are currently no commercial vaccines available for BCWD although numerous attempts have been made using; killed cells (Lafrentz et al., 2002), outer membrane proteins (Rahman et al., 2002; Dumetz et al., 2006; Crump et al., 2007; Dumetz et al., 2007), and live strains (LaFrentz et al., 2008; Lorenzen et al., 2010).

Without a readily available vaccine, antibiotics are currently the most effective method for treating BCWD outbreaks. Currently, there are two antibiotics licensed for use against *F. psychrophilum*, oxyctetracycline and florfenicol; however, antibiotic resistance to

oxyctetracycline has been reported. Bruun et al. (2000) evaluated 387 *F. psychrophilum* isolates from Danish farms and reported 75% of the strains were resistant to oxyctetracycline while no isolates were resistant to florfenicol. Kum et al. (2008) evaluated 20 *F. psychrophilum* isolates and reported 75% were resistant to oxyctetracycline and 25% were resistant to florfenicol. In the United States, florfenicol is sold only under a Veterinary Feed Directive (VFD) while oxyctetracycline does not require a VFD (U.S Fish and Wildlife Service 2011).

Probiotics for bacterial coldwater disease

Despite the lack of a commercial vaccine and increasing concerns with antibiotics, only a few attempts have been made to identify alternative management strategies for *F. psychrophilum* infections. Nikoskelainen et al. (2001) and Balcazar et al. (2007) evaluated a number of different lactic acid bacteria for their ability to inhibit *F. psychrophilum* growth *in vitro* and observed no inhibition with any of the isolates. Strom-Bestor and Wiklund (2011) reported that *Pseudomonas* sp. MSB1 was able to limit the growth of *F. psychrophilum in vitro*; however, no *in vivo* trials were conducted. Another *Pseudomonas* species, M174, has also been reported as inhibitory to *F. psychrophilum in vitro* and is hypothesized to act through siderophore production. This probiotic also exhibited promise *in vivo* in rainbow trout following *F. psychrophilum* challenge. Following oral administration of M174 and subsequent challenge fish receiving the probiotic had increased survival compared to those receiving a diet without probiotics. Finally, M174 was shown to increase the respiratory burst activity of kidney macrophages (Korkea-aho et al., 2011). Additionally, Burbank et al. (2012) isolated 318 bacterial strains from the gastrointestinal tracts of rainbow trout. From these 318 isolates 24 were capable of *in vitro* inhibition of *F. psychrophilum.* These strains

were then tested *in vivo* and two candidate probiotics emerged; *Enterobacter* sp. strain C6-6 and *Enterobacter* sp. strain C6-8 were both found to significantly reduce mortality in rainbow trout challenged with *F. psychrophilum* (Burbank et al., 2011).

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Chapter 2: Partial replacement of fish meal with salmon testes meal from Alaskan seafood processing byproducts: effects on growth and immune function of rainbow trout, *Oncorhynchus mykiss* (Walbaum)

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Running title: Supplementing diets with testes meal

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Abstract

This study investigated growth and immune function effects following partial replacement of fish meal with dried testes meal from pink salmon (*Oncorhynchus gorbuscha*) testes collected from Alaskan seafood processing byproducts. It was hypothesized that the dietary nucleotide content of testes meal may provide an immunostimulatory effect in rainbow trout (*O. mykiss*). Three diets were formulated and used for this study; a fish meal-based control diet, a Macrogard diet, and an experimental diet containing 5% testes meal. Triplicate groups of juvenile rainbow trout (8.65 ± 0.13 g $fish^{-1}$) were fed each diet for 12 weeks, at which time they were challenged with *Flavobacterium psychrophilum* (the causative agent of coldwater disease). No significant differences in feed conversion ratio or specific growth rates were observed. Kidney macrophage (phagocytic) activity was significantly ($P < 0.05$) increased in fish fed the testes meal at weeks 2 and 10 when compared to fish fed the fish meal or Macrogard diets. At 12 weeks; however, this activity was significantly lower ($P < 0.01$) in fish fed either the Macrogard or the testes meal diets compared to fish fed the fish meal diet. Following pathogen challenge, fish fed either the Macrogard or testes meal diets had significantly lower ($P < 0.05$) antibody titers at 8 weeks post challenge. The findings suggest impaired macrophage activity at the time of challenge resulted in suppressed adaptive responses due to inadequate antigen presentation. Taken together, it appears that testes meal replacement can stimulate some innate responses; however, prolonged feeding of this or glucan based diets may be counterproductive and affect specific antibody development.

Keywords: Testes meal, immunostimulant, immunosuppression, rainbow trout

Introduction

The administration of dietary immunostimulants in aquaculture has been identified as a valid preventative measure in the management of a wide range of diseases (Sakai, 1999). Immunostimulants are naturally occurring compounds that alter the host's immune system in a way that increases its resistance to disease (Bricknell and Dalmo, 2005). According to Sakai (1999), immunostimulants can be divided into subgroups based upon their origin and include bacterial derivatives, animal and plant derivatives, dietary compounds, hormones and cytokines.

Nucleotides are the precursors of DNA synthesis and are essential for most cellular processes. Despite their importance, nucleotides have traditionally been considered non-essential nutrients as deficiencies are rarely seen (Li and Gatlin, 2006). While not traditionally considered immunostiumulants, dietary nucleotides have received increased attention in recent years. Burrells et al., (2001a) first reported the non-specific immunostimulatory benefits of exogenous dietary nucleotides in fish after feeding rainbow trout (*Oncorhynchus mykiss*) a diet containing supplemental nucleotides. Results also showed that survival was increased following challenge with *Vibro anguillarum*. Burrells et al., (2001b) also reported that dietary supplementation of exogenous nucleotides reduced stress parameters and increased weight gain after vaccination and salt water transfer of Atlantic salmon (*Salmo salar*). Following these initial studies numerous others have reported benefits of dietary nucleotides. A variety of innate and adaptive immune parameters show changes when nucleotides are added to the diet including phagocytic activity (Sakai et al., 2001; Li et al., 2004), serum lysozyme (Sakai et al., 2001; Tahmasebi-Kohyani et al.,

2011; Jarmołowicz et al., 2012;) and antibody levels (Low et al., 2003; Tahmasebi-Kohyani et al., 2011).

The positive immune stimulation exhibited in fish fed diets containing dietary nucleotides has lead to an increased effort to find additional sources of nucleotides for dietary supplementation. Plante et al. (2008) reported that meal from pink salmon (*O. gorbuscha*) testes has a nutrient profile similar to fish meal and up to ten times the purine content of commercial fish meals, and suggested that addition of salmon testes meal to a diet may provide an immunostimulatory effect. In most reported studies dietary nucleotides are added to diets in a purified form that contains only nucleotides. Therefore, the specific objectives of this study were to: (1) determine the effects on growth of rainbow trout fed a diet containing 5% testes meal, (2) determine if testes meal replacement is capable of stimulating increases in lysozyme or macrophage activity and (3) determine if feeding a diet containing testes meal increases survival or effects antibody development following bacterial challenge with *Flavobacterium psychrophilum*.

Methods

Diets

Three diets were formulated for use in this study. A commercial-type diet (fish meal diet) containing no supplemental immunostimulant served as a negative control. A positive control diet (Macrogard diet) was formulated by adding the known β-glucan based immunostimulant Macrogard® at a rate of 2 g kg^{-1} diet according to the manufacturer's directions to the fish meal diet. The experimental diet (testes meal diet) was formulated to contain 5% testes meal through replacement of an equilivant amount of anchovy meal from the fish meal diet with testes meal. Diets were produced at the University of Idaho's

Hagerman Fish Culture Experiment Station using a compression pellet mill without steam. Diets were air dried for 48 hours to a moisture content of <10% and stored at -20^{0}C until use. All diets were formulated to be isonitrogenous and isocaloric and contain 420 g crude protein kg^{-1} diet and 147 mJ kg^{-1} diet (Table 2.1). Salmon tests meal contains approximately 79.9% protein, 3.0% lipid and 12.8% ash and was obtained from pink salmon (*O. gorbuscha*) testes collected in the Alaskan fishery (Plante et al., 2008). Samples of each diet were analyzed for proximate composition (moisture, protein and fat) using AOAC (1990) procedures. Briefly, samples were dried in a convection oven at 105 °C for 12 h to determine moisture level. Dried samples were finely ground by mortar and pestle and analyzed for crude protein (total nitrogen × 6.25) using a LECO FP-428 nitrogen analyzer (LECO Instruments, St. Joseph, MI). Crude lipid was analyzed using an ANKOM XT15 acid hydrolysis and extraction apparatus (ANKOM Technology, Macedon, NY) with petroleum ether as the extracting solvent. Energy content of samples was determined using a Parr Adiabatic Calorimeter (Parr Instruments, Moline, IL).

Feeding

Rainbow trout with mean initial weight of 8.65 ± 0.13 g were obtained from the University of Idaho's Hagerman Fish Culture Experiment Station. Fish were maintained in nine 225 L tanks with 70 fish tank^{-1}. Each tank was supplied with 5 L min^{-1} of 15°C dechlorinated municipal water in a flow through system with a 12:12 hour day night cycle. Diets were randomly assigned to triplicate tanks and fish were fed to apparent satiation twice daily for 12 weeks. Length and weights were recorded from 20 randomly selected fish from each tank every two weeks. All growth indices were determined by averaging total

biomass for individual tanks. Feed conversion ratio (FCR) and specific growth rates were calculated according to Cho (1992):

$$FCR = \text{total feed fed/weight gain (wet gain)}$$

$$SGR = 100 \ (\ln \text{mean final weight} - \ln \text{mean initial weight}) / \text{duration}$$

Innate immunity sampling

To determine effects of the diets on serum lysozyme and kidney macrophage phagocytic activity, blood and kidney tissue were collected from 8 fish $tank^{-1}$ at weeks 0, 2 and 4 and from 4 fish $tank^{-1}$ at weeks 6, 8, 10 and 12. Due to the size of fish, blood and kidney samples were pooled (two fish $pool^{-1}$) to produce 4 samples in weeks 0, 2 and 4. In weeks 6, 8, 10 and 12, fish were larger allowing for individual samples to be collected. Prior to sampling, fish were euthanized with an overdose of tricaine methane sulfonate (100 mg L^{-1}, MS-222; Argent Chemicals, Redmond, WA). All experimental protocols were approved in advance by the University of Idaho's Institutional Animal Care and Use Committee (protocol 2011-21).

Serum lysozyme

Blood was collected using heparinized capillary tubes after severing of the caudal artery, transferred to 1.5 mL centrifuge tubes and allowed to clot overnight at 4 °C. The clotted samples were centrifuged at 15 000 x g for 5 min. Serum was then transferred to clean 1.5 mL centrifuge tubes and stored at -80 °C until needed.

Serum lysozyme levels were determined using a turbidimetric assay described by (Korkea-aho et al., 2011). Briefly, 10 µL of serum was added to triplicate wells of a flat bottom 96-well plate, 190 µL of 0.04 mol sodium phosphate buffer (SPB) containing 0.2 mg mL^{-1} of lyophilized *Micrococcus luteus* was then added to all wells and gently mixed.

Absorbance was measured at 540 nm after 1 and 5 min using a multiplate reader (BioTek® PowerWave™ Micro-plate Spectrophotometer, Winooski, VT). One unit of lysozyme activity is defined as the amount of serum required to cause a decrease in absorbance of 0.001 min^{-1} and is reported as units mL^{-1} serum.

Phagocytic activity

Kidney macrophages were isolated according to the methods of Brunt and Austin (2006). Briefly, the head kidney was removed and homogenized in 1:10 w/v of RPMI 1640 containing 1 μg 100 mL^{-1} oxyctetracycline, 0.2 mg 100 mL^{-1} heparin and 2% fetal bovine serum (FBS). The homogenate was forced through a 100 μm nylon mesh using a vacuum to remove tissue debris. The filtrate was then layered onto a Percoll gradient containing 34%/51% Percoll in Hank's balanced salt solution in a 5 mL centrifuge tube and centrifuged at 400 x g for 25 min at 4 °C. The macrophage enriched interface was then collected.

Phagocytic activity was determined using the methods described by Panagrahi et al. (2004). The above macrophage enriched cell suspension was adjusted to 1 x 10^6 cells mL^{-1} in RPMI 1640 containing 1 μg 100 mL^{-1} oxyctetracycline, 0.2 mg 100 mL^{-1} heparin and 0.1% FBS using a hemocytometer. Following adjustment 1 mL of the cell suspension was added to a glass microscope slide. Cells were allowed to adhere for 1 hr at room temperature, after which all non-adherent cells were removed by gentle washing with the above RPMI 1640 media. After washing, 1 mL of RPMI 1640 with 0.1% FBS media containing 1 x 10^7 0.8 μm latex beads mL^{-1} (Sigma, St. Louis, MO) was added to each slide and incubated for 1 hr at room temperature. Cells were then fixed in methanol and stained with Diff Quick according to the manufactures directions. To determine the phagocytic activity, 200 cells per slide were observed under a microscope to count and determine the

presence/absence of phagocytised beads within cells. Phagocytic activity was determined as the number of cells containing at least one bead/number of cells counted.

Challenge

Following the feeding and innate immunity experiment, remaining fish were challenged with a virulent *F. psychrophilum* strain (CSF 259-93) (Crump et al. 2001) and monitored for 8 weeks to assess antibody development. For the challenge, fish were divided into 4 tanks per treatment with 25 fish $tank^{-1}$. Duplicate tanks per treatment received a 100 µl intraperitoneal injection of 4.92×10^5 cfu $fish^{-1}$ *F. psychrophilum* and duplicate tanks per treatment were injected with sterile phosphate buffed saline (PBS) (mock infected). *F. psychrophilum* was cultured at 15°C for 72 hours in triptone yeast extract salts (TYES) broth and harvested by centrifugation at 1600 x g for 15 min at 15^0C. The supernatant was decanted and the pellet re-suspended in sterile PBS to an optical density of 0.185 at 525nm. Colony forming units (cfu) mL^{-1} were determined by the drop plate method (Chen et al. 2003). Mortalities were recorded for 28 days and at least 20% of mortalities per day were examined and *F. psychrophilum* re-isolation was attempted by inoculating spleen, liver and kidney on TYES.

Enzyme linked immunosorbant assay

To determine anti-*F. psychrophilum* antibody titers, an enzyme linked immunosorbant assays (ELISA) was used according to methods outlined in LaFrentz et al. (2002) with some modifications. Prior to challenge, blood was collected from naïve fish (2 per tank) using a 21 gauge needle and syringe via caudle puncture and allowed to clot overnight at 4°C. The clotted samples were centrifuged at 15 000 x g for 5 min and the serum removed and pooled into one sample to serve as a negative control for the ELISAs.

Blood was collected at 4 and 8 weeks post challenge from 5 randomly selected fish tank^{-1} and prepared and stored as above. All serum was stored at -80 °C until needed. Whole cell antigen was prepared for coating ELISA plates according to methods describe in LaFrentz et al. (2002). Briefly, *F. psychrophilum* was grown for 72 hours to log phase in 40 mL of TYES broth at 15^{0}C. Cultures were centrifuged at 4300 x g for 15 min at 15^{0}C, the supernatant was then discarded and the pellet re-suspended in 15 mL of carbonate coating buffer containing 0.05% sodium azide. Protein concentration was determined using a Micro BCA Protein Assay according to the manufacturer's instructions (Pierce, Rockford, IL, USA); all antigen was used the same day it was prepared.

Immulon II high binding 96 well plates (Thermo Labsystems, Franklin, MA) were coated with 100 ul well^{-1} antigen diluted to 0.1 µg mL^{-1} in carbonate coating buffer and allowed to bind overnight at 4^{0}C. Plates were then washed 3x with PBS containing 0.05% Tween-20 (PBST). The coated plates were blocked with PBS containing 5% non-fat dry milk for 1 h at room temperature. Plates were again washed as above, serum samples diluted in PBS containing 0.02% sodium azide (PBS-AZ) were added in duplicate and serially diluted in doubling dilutions (100 µL well^{-1}). Negative and positive controls diluted 1:200 in PBS-AZ were added to each plate in duplicate and incubated overnight at 15^{0}C. Following incubation, plates were washed as above and 100 µL of mouse monoclonal anti-trout IgM antibody (MAb 1.14; DeLuca, Wilson & Warr 1983) diluted 1:400 in PBS-T containing 0.1% non-fat dry milk was added to each well and incubated at room temperate for 1 h. Plates were again washed and 100 µL horseradish peroxidase conjugated goat anti-mouse immunoglobulin (Calbiochem, San Diego, CA) diluted 1:5000 in PBS-T containing 0.1% non-fat dry milk was added to each well and incubated for 1 hour at room temperature.

Plates were again washed and 50 µL of ABTS peroxidase substrate and H_2O_2 (Kirkegaard and Perry Laboratories, Inc., Gaitherburg, MD) in the ratio of 1:1 was added to each well and incubated for 15 min at room temperature. After incubation the reaction was stopped by the addition of 50 µL of deionized water containing 1% w/v sodium dodecyl sulphate (Bio-Rad, Hercules, CA). The optical density of the wells was read at 405 nm. Positive ELISA titers were defined as the reciprocal of the highest dilution showing an optical density at least two standard deviations greater than the negative control.

Statistical Analysis

All data were analyzed using one way analysis of variance ANOVA with pair-wise comparisons using Tukey's post-hoc test when appropriate. Following challenge cumulative percent mortality was calculated by and analyzed by tank. All immune assay results and weights obtained from individual fish were averaged by tank and statistical analysis was preformed using individual tanks as replicates and analyzed between treatments within weeks. Survival curves for a treatment as a whole were compared using a log rank survival curve analysis after confirming there were no significant differences between tanks within a treatment (GraphPad Prism, version 5.04 for windows, GraphPad Software, San Diego CA, USA). Differences were considered significant at $P \leq 0.05$.

Results

Growth

There were no significant differences among diets in final weight, overall FCR or overall SGR (Table 2.2). However, fish fed the Macrogard diet had significantly higher average weight, 52.63 ± 2.11 g, compared to fish fed the fish meal diet, 48.36 ± 2.12 ($P \leq 0.05$), and fish fed the testes meal diet, 45.74 ± 1.55 ($P \leq 0.01$), at week 8 ($F_{2,6} = 33.12$).

Fish fed the Macrogard diet also had significantly higher weights, 70.52 ± 2.62, compared to fish fed the testes meal diet, 66.51 ± 1.67 ($P \leq 0.05$), at week 10 ($F_{2,6} = 6.97$). There were no significant differences between the weights of fish fed the testes meal or the fish meal diets within any week (Figure 2.1).

Serum Lysozyme

There were no significant differences ($P > 0.05$) in the serum lysozyme activity among any diets within any week. Serum lysozyme levels were highest for all diets in week 2 and lowest in week 4. Lysozyme levels ranged from 67 ± 11.59 to 205 ± 32.44 units/ml in fish fed the fish meal diet, from 66 ± 3.84 to 160 ± 31.69 units/ml in fish fed the testes meal diet and 76 ± 12.57 to 201 ± 52.26 units/ml in fish fed the testes meal diet (Figure 2.2).

Phagocytic Activity

There was a significant increase in phagocytic activity of the fish fed the testes meal diet, 36.30 ± 2.33%, compared to fish fed the fish meal diet, 29.54 ± 0.58% ($P \leq 0.05$) or the Macrogard diet, 27.88 ± 1.00 % ($P \leq 0.05$), in week 2 ($F_{2,6} = 8.377$). Significant increases were also observed in week 10 in fish fed the testes meal diet, 37.71 ± 3.33%, compared to fish fed the fish meal diet, 28.67 ± 5.50% ($P \leq 0.05$), or the Macrogard diet, 30.50 ± 0.88% ($P \leq 0.05$) ($F_{2,6} = 5.79$). At week 12 the testes meal-fed fish, 22.75 ± 1.23% ($P \leq 0.01$) and fish fed the Macrogard diet, 21.71 ± 1.15% ($P \leq 0.05$) had significantly lower phagocytic activity than fish fed the fish meal diet 45.65 ± 2.25% ($F_{2,6} = 22.36$) (Figure 2.3).

Challenge Mortality

Survival was high following challenge. Survival ranged from 68.3 ± 3.2% in fish fed the Macrogard diet and 71.7 ± 3.8% in fish fed the fish meal diet. There were no significant

differences in survival or log rank survival curves among any of the dietary treatment groups (Figure 2.5).

Enzyme linked immunosorbant assay

Antibody titers were low and not significantly different between fish fed any of the diets at 4 weeks following *F. psychrophilum* challenge. Titers ranged from 813 ± 206 in fish fed the testes meal diet to 947 ± 265 in fish fed the Macrogard diet. In week 8, titers in the fish meal fed fish increased to 1493 ± 430 and were significantly higher ($F_{2,6} = 8.06$) than titers of fish fed the testes meal diet 346 ± 71 ($P \leq 0.01$) and fish fed the Macrogard diet 413 ± 107 ($P \leq 0.05$) (Figure 2.4).

Discussion

Inclusion of testes meal in a rainbow trout diet had no significant effect on FCR, SGR or weight of fish fed this diet in relation to fish fed a fish meal control diet at any point during the study. This suggests that partial replacement of fish meal using processing byproducts is possible without sacrificing fish growth or performance.

The immunostimulant effects of testes meal were not dramatic, but intermittent increases in phagocytic activity of macrophages were observed in fish during weeks 2 and 10. This increased activity agrees with other studies using purified exogenous nucleotides that have shown an effect on innate cellular responses. Li et al. (2004) noted increases in neutrophil oxidative radical production in hybrid striped bass (*Morone chrysops* X *Morone saxatilis*) after 8 weeks of feeding a diet containing supplemental dietary nucleotides. Similarly, significant increases in extracellular superoxide anion production by red drum (*Sciaenops ocellatus*) kidney macrophages were reported by Cheng et al. (2011) after feeding a diet supplemented with 1% nucleotides for 6 weeks. Unlike most studies in which

exogenous nucleotides are provided through purified sources, in the preset study dietary nucleotide levels were increased through the addition of unpurified nucleotides to the diet. Interestingly, other studies have noted direct effects on immune function associated with fish testes and milt. Pedersen et al. (2003) reported increases in Atlantic salmon head kidney macrophages activation four days after injection of histone like proteins isolated from Atlantic salmon milt fractions. Similarly, Pedersen et al. (2004) reported that oral administration of cod milt proteins increased survival of Atlantic cod fry following challenge with *Vibrio anguillarum.* Further, Pedersen et al. (2006) reported that cationic proteins derived from fish milt were able to increase the antiviral response in Atlantic salmon leukocytes when administered in conjunction with oligodeoxynucleotides containing cytosine-guanosine motifs. The authors also suggested that the cationic proteins alone may have a direct effect on the type I INF activity of leukocytes. Based on the findings of these studies it is likely that increases in macrophage activity observed in the present study are not a result of increased dietary nucleotides alone. Instead, immune stimulation observed in fish fed the testes meal diet may be due to a number of different stimulatory compounds associated with testes meal.

In the present study, no significant increases in immune parameters in fish fed the Macrogard diet were observed. It is not clear why no such changes were observe and this is contrary to the majority of studies examining the effects of dietary glucan when some form of immunostimulation is typically noted (Bagni et al., 2005; Misra et al., 2006; Siwicki et al., 2010). Despite the lack of observed immune stimulation in fish fed the Macrogard diet, there was evidence that prolonged feeding led to immunosuppression in fish fed the Macrogard as well as the testes meal diets. After 12 weeks, kidney macrophage phagocytic

activity of fish fed these diets was significantly lower when compared to fish fed the fish meal diet. When antibody response was evaluated, fish receiving the Macrogard and testes meal diets exhibited significantly lower antibody titers than fish meal fed fish by 8 weeks post *F. psychrophilum* challenge. Traditionally, researchers have cautioned that long term administration or high doses of immunostimulants such as β-glucans may result to tolerance or immunosuppresion (Bricknel and Dalmo 2005). Immunostimulants such as β-glucans act on specific innate cellular receptors, continuous stimulation of these receptors can induce a negative feedback response and result in reversion to a baseline or depressed immune state (Sakia 1999; Bricknell and Dalmo 2005). This effect is most commonly noted in innate immune cells. For example, Yoshida et al. (1995) reported that the number of NBT-positive cells in African catfish (*Clarias gariepinus*) fed diets supplemented with oligosachrides or glucans peaked after 12 and 30 days respectively and returned to baseline after 45 days. Similarly, Misra et al. (2006) reported increases in the phagocytic index of rohu (*Labeo rohita*) fingerlings fed diets containing 500 mg kg-1 B-glucan for 42 days followed by a decrease to levels below that of the control diet after 56 days of feeding. Finally, Jørgensen and Robertsen (1995) found that *in vitro* incubation of Atlantic salmon macrophages with 10 $\mu g\ mL^{-1}$ and 50 $\mu g\ mL^{-1}$ yeast glucan had a negative or inhibitory effect, respectively. Phagocytic and antigen presenting innate immune cells such as macrophages are critical in stimulating the adaptive immune response (Magnadóttir 2006). As such, decreases in the activity of these innate immune cells is likely correlated to poor antigen presentation and depressed antibody production as observed in fish fed the Macrogard and testes meal diets.

While concerns of tolerance and immune suppression are common with traditional immunostimulants they are rarely noted with the administration of dietary nucleotides. This

is likely due to the method in which nucleotides are thought to interact with the immune system. Unlike traditional immunostimulants, nucleotides are not known to directly stimulate immune cell receptors. Instead, dietary nucleotides are thought to provide cells an energetically efficient way of obtaining the nucleotides needed for optimal function. Nucleotides are provided to the body in three ways: de novo synthesis, the salvage pathway and through the diet. Immune cells are incapable of de novo nucleotide synthesis and require that all nucleotides are provided by the diet or through the more energetically expensive salvage pathway. During times of rapid cellular division such as infection, an exogenous source of nucleotides may improve the function of immune cells by allowing them to forgo the salvage pathway and thus operate more efficiently (Carver and Walker, 1995).

As such, it is unlikely that the additional nucleotides supplied by the testes meal were responsible for the observed immunosuppression. This is further evidence that components of testes meal other than nucleotides are capable of affecting the immune system. If these components are capable of stimulating the immune system through direct activation of cellular receptors there is the potential for tolerance and immune suppression noted with long term administration of traditional immunostimulants. However, further study into the mechanism associated with immune stimulation induced by milt and testes meal components is required.

In summary, addition of testes meal to a trout diet had no negative effect on the growth of the fish and was capable of stimulating periodic increases in the phagocytic activity of kidney macrophages when compared to the fish meal control or a Macrogard supplemented diet. Therefore, testes meal can be considered a functional ingredient for

salmonids. However, prolonged feeding a diet containing testes meal may not be advisable as depressed antibody development following pathogen challenge was observed in fish fed this as well as the Macrogard diet. If fed periodically, the partial replacement of fish meal with testes meal may provide benefit for aquaculture, but further research is necessary to better define the use of such products and how they may serve to decrease pressure on existing fish meal supplies.

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Table 2.1 Ingredient composition (g kg^{-1}) and nutrient profiles of experimental diets

INGREDIENT	Testes Meal	Fish Meal	Macrogard
Anchovy meal	150.0	200.0	200.0
Salmon testes meal	50.0	0.0	0.0
Soy protein conc.	156.0	177.0	177.0
Wheat gluten	20.0	10.0	10.0
Wheat flour	259.6	172.6	172.6
Fish oil	176.0	172.0	172.0
Vitamin C (Stay-C)	2.0	2.0	2.0
Choline	6.0	6.0	6.0
TM premix[1]	1.0	1.0	1.0
Vitamin premix[2]	8.0	8.0	8.0
Permapel	15.0	15.0	15.0
Corn gluten, yellow	100.0	180.0	180.0
Dicalcium phosphate	30.0	30.0	30.0
Lysine	16.4	16.4	16.4
Methionine	5.0	5.0	5.0
Taurine	5.0	5.0	5.0
Macrogard	0	0	2.0
Proximate Composition			
Fat (g/100g) dry wt	21.08	21.33	21.35
Protein (g/100g) dry wt	45.95	47.38	49.98
Cal. MJ/g	23.35	23.49	23.63

[1] Trace mineral premix supplied the following per kg diet: Cu (as $CuSO_4.5H_2O$), 1.54; I (as KIO_3),10; Mn (as $MnSO_4$), 20; Zn (as $ZnSO_4.7H_2O$), 75,

[2] Vitamin premix supplied the following per kg diet: vitamin A (vitamin A palmitate, stabilized), 13,200 IU; vitamin D_3 (stabilized), 880 IU; vitamin E (DL-α-tocopheryl acetate), 704 IU; menadione sodium bisulfate complex, 22g; D-calcium pantothenate, 21.5; pyridoxine (pyridoxine HCl), 61.6g; riboflavin, 105.6g; niacinamide, 440g; folic acid, 17.6g; thiamin (thiamin mononitrate), 70.4g; biotin, 88 µg; vitamin B12, 5.5ug

Table 2.2 Feed conversion ratio, specific growth rate, initial and final weights of rainbow trout fed a control diet, a diet supplemented with Macrogard or a diet containing testes meal for 12 weeks. Data are presented as mean ± S.E.M.

	Fish Meal	Macrogard	Testes Meal
Initial weight (g)	8.56±0.24	8.84±0.04	8.55±0.22
Final Weight (g)	82.26±5.11	95.9±5.29	92.48±3.77
FCR	0.97±0.06	0.96±0.06	1.08±0.07
SGR	2.83±0.10	2.83±0.07	2.70±0.04

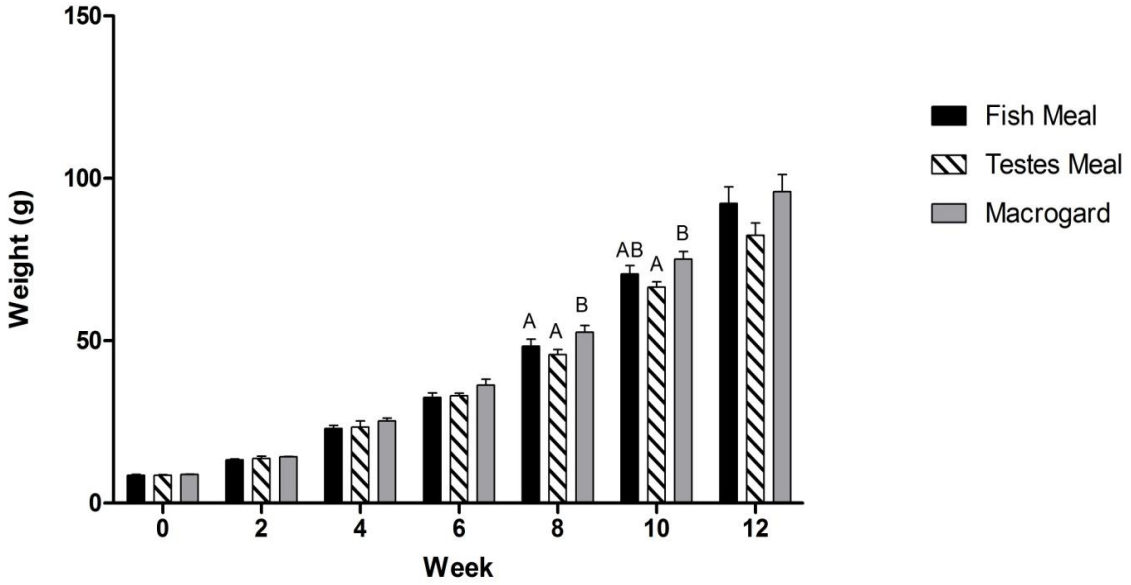

Figure 2.1 Weight of rainbow trout fed a control diet, a diet supplemented with β-glucan or a diet containing testes meal. Data are presented as mean ± S.E.M. Different letters indicate a significant difference ($P \leq 0.05$) between treatments within a week.

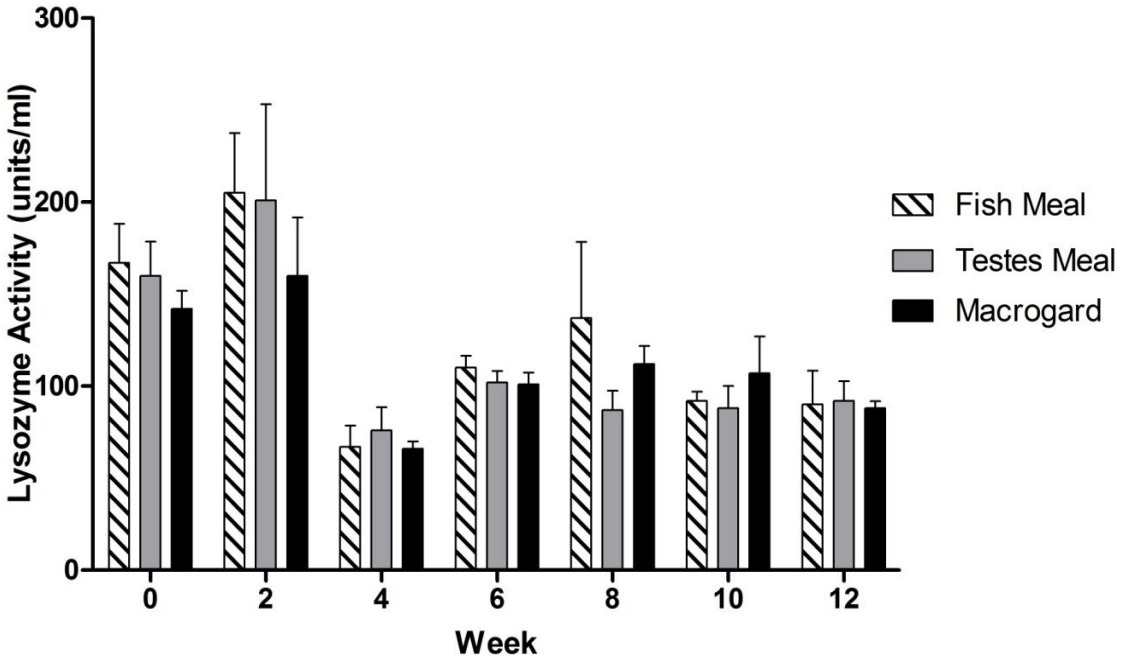

Figure 2.2 Serum lysozyme activity of rainbow trout fed fish meal, Macrogard and testes meal diets. Data presented as mean ± S.E.M

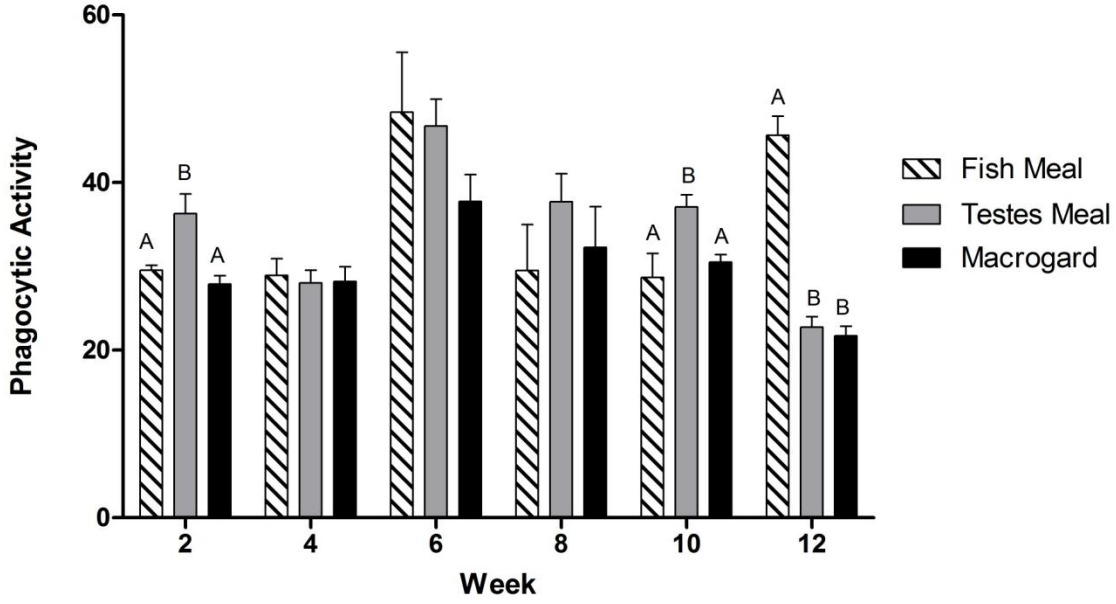

Figure 2.3 Phagocytic activity of rainbow trout kidney macrophages fed fish meal, Macrogard and testes meal diets. Data presented as mean ± S.E. Different letters indicate significant difference ($P \leq 0.05$) between diets within a week.

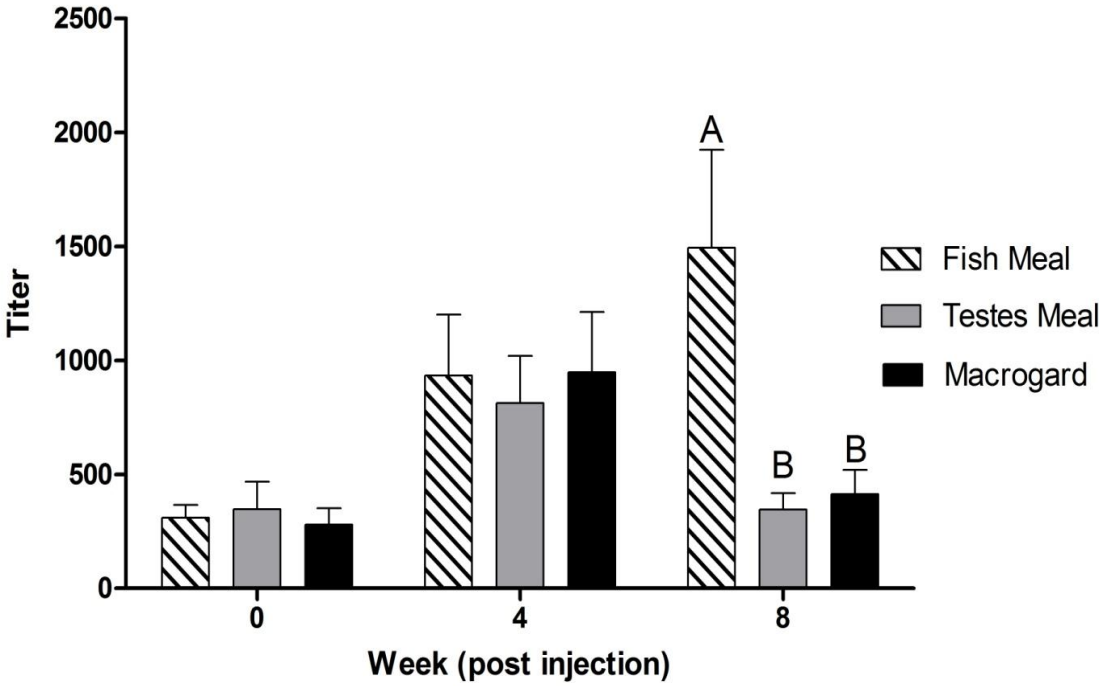

Figure 2.4 Serum antibody titers of rainbow trout fed fish meal, Macrogard and testes meal diets for 12 weeks prior to injection with *F. psychrophilum*. Data presented as mean ± S.E.M. Different letters indicate significant difference ($P \leq 0.05$) between diets within a week.

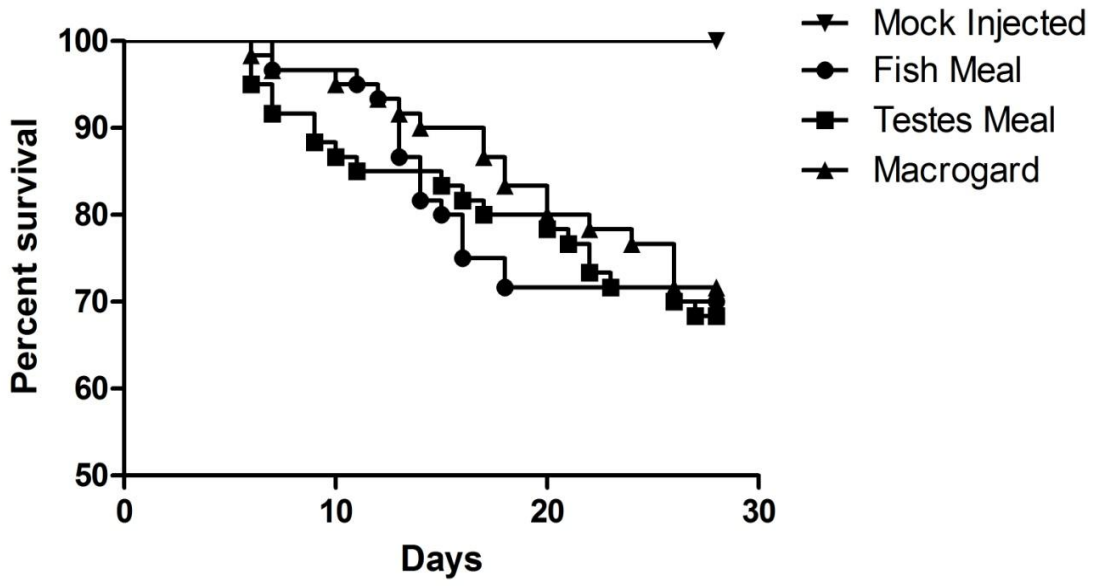

Figure 2.5 Survival of rainbow trout following intraperitoneal injection of 4.92×10^5 cfu $fish^{-1}$ *Flavobacterium psychrophilum* (CSF 259-93). Fish were fed a fish meal diet, a diet containing testes meal or a diet containing Macrogard.

Chapter Three: The effects of probiotic feeding and vaccination on survival of rainbow trout (*Oncorhynchus mykiss*) challenged with *Flavobacterium psychrophilum*

Abstract

In this present study, we evaluated the effects of combining probiotics and vaccination in controlling bacterial coldwater disease (BCWD) caused by *F. psychrophilum* infections. Two *Enterobacter* strains (C6-6 and C6-8) were fed to fish alone or in combination for 40 days and survival following *F. psychrophilum* challenge was evaluated prior to or following vaccination with a live attenuated *F. psychrophilum* vaccine (CSF 259-93 B.17). This study consisted of two pathogen challenges with virulent *F. psychrophilum*. In the first challenge, rainbow trout, *Oncorhynchus mykiss,* fry were fed a diet top coated with menhaden oil (negative control) C6-6, C6-8, or a combination of the two at first feeding. At a size of approximately 1 gram, fry were injection challenged with 25 µL of 1.40×10^7 CFU mL^{-1} live *F. psychrophilum*. Following challenge, no significant differences were found in cumulative percent morality (CPM) and mortality rates did not differ between treatments. However, both probiotic strains were shown to colonize the gastrointestinal tract and were isolated out to at least 36 days post feeding in all fish examined. In the second challenge, fish fed the same probiotic treatments as above were then vaccinated with the live attenuated *F. psychrophilum* strain at approximately 1 gram. Fish were then injection challenged with live virulent *F. psychrophilum* 4 weeks after booster vaccination. Challenge mortality was high in all groups and relative percent survival (RPS) values ranged from 5.4% in fish fed with C6-8 and vaccinated to 23.6% in fish fed C6-6. Only fish fed C6-6 + vaccination had significantly reduced CPM, which corresponded directly to the log rank survival curve analysis showing reduced mortality over fish fed the oil + mock

vaccination treatment. Results from this study indicate that C6-6 and C6-8 are effective colonizers of the gut of rainbow trout, and that C6-6 may provide additional benefit for BCWD management when combined with vaccination.

Introduction

Flavobacterium psychrophilum is a gram negative bacteria and the causative agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (Nematollahi et al., 2003). BCWD is responsible for high economic losses in the salmonid industry of the Pacific Northwest and has been considered to be one of the most important bacterial pathogens in aquaculture worldwide (Barnes and Brown, 2011). Mortality rates from *F. psychrophilum* outbreaks can range from 5-85%, and coho salmon, *Oncorhynchus kisutch*, rainbow trout and steelhead, *Oncorhynchus mykiss*, are particularly susceptible (Barnes and Brown 2011). There is currently no commercially available vaccine and treatment of BCDW has been limited to antibiotic treatments; however, increasing antibiotic resistance has been observed (Michel et al., 2003; Soule et al., 2005; Akinbowale et al., 2007; Del Cerro et al., 2010).

With the emergence of antibiotic resistance strains of *F. psychrophilum* and the lack of a commercially available vaccine, alternative management and prevention strategies have been proposed. One such technique receiving attention is the use of probiotics (Burbank et al., 2011; Korkea-aho et al., 2011; Ström-Bestor and Wiklund, 2011). C6-6 and C6-8 are *Enterobacter* strains originally isolated from the gastrointestinal tract of healthy rainbow trout. These probiotic strains have shown promise through *in vitro* inhibition of *F. psychrophilum* (Burbank et al., 2012). Each probiotic has also been shown to significantly

reduce mortality of rainbow trout fry due to BCWD following oral administration and challenge (Burbank et al., 2011).

While there are currently no commercially available *F. psychrophilum* vaccines there have been numerous attempts to develop an efficacious vaccine (Högfors-Rönnholm et al., 2008; LaFrentz et al., 2002; Plant et al., 2009; Rahman et al., 2000; Rahman et al., 2002). Recently, a live attenuated *F. psychrophilum* strain (CSF 259-93 B.17) has shown promis and is currently undergoing field safety trials (U.S. Patent No. 7740864). Initial laboratory immunization trials of this B.17 vaccine via injection or immersion in rainbow trout resulted in a relative percent survival (RPS) of 45% for both immunization methods (LaFrentz et al., 2008). More recently, Long et al. (2013) reported an RPS of 46% in coho salmon vaccinated via immersion.

It has been hypothesized that early administration of probiotics to fish during susceptible stages and prior to immunecompetence would be beneficial, and that following this with a vaccination would be a viable management strategy. To address this and other questions, three objectives were developed for this study. The first objective was to determine if simultaneous administration of C6-6 and C6-8 was capable of providing benefits beyond those provided by each probiotic alone. At this time the mode of action of C6-6 and C6-8 are unknown; however, it is unlikely that the mode of action for each probiotic is identical. Therefore, we hypothesized that dual administration of the probiotics would have increased benefits to the host. The most severe BCWD outbreaks occur in fish less than 2 grams (Nematollahi et al., 2003). Rainbow trout are not completely immunocompetent at hatch and are unable to mount a humoral response capable of immunological memory until about 8 weeks after hatch (Tatner, 1986). With this in mind,

the second objective was to determine if administration probiotics at first feeding could provide early protection until the fish are immunocompetent and if additional benefit was evident following vaccination of these groups 1 gram. Both probiotics were originally isolated from the gut of rainbow trout and Burbank et al. (2012) demonstrated that they were capable of growth in the presence of bile. Thus, suggesting that colonization through feeding may be possible and provide protection after feed administration has ceased. Therefore, our third objective was to determine if these probiotics could effectively colonize the gut and remain viable after feeding of the probiotics had stopped.

Methods

Fish

Rainbow trout (Walbaum) were received as eyed eggs from the Utah Division of Wildlife Resources and incubated in upwelling incubators supplied with 15^0C de-chlorinated municipal water until hatch. Following hatching the fry were maintained in four separate flow-through troughs supplied with 4 L min^{-1} of 15^0C de-chlorinated municipal water.

Probiotics feeding

Probiotic bacterial culture and coating of feed followed methods described in Burbank et al. (2011). Briefly, frozen stocks of probiotic bacteria were thawed and grown to log phase in tryptic soy (TS) broth at 15^0C for 24 h. The log phase culture was then centrifuged at 1600 x g for 15 min at 15^0C, the supernatant was removed and the pellet resuspended in a volume of menhaden oil equal to the volume of removed supernatant. The oil or oil and probiotic mixture was then top coated onto feed at 1:10 (v/w). Probiotics were coated at a rate of 10^6 to 10^8 colony forming units (CFU) g^{-1} feed. For the combined

treatments equal parts from each probiotic suspensions were combined and mixed thoroughly after resuspending in menhaden oil and then top coated as above. Fish were fed feed coated with menhaden oil (negative control), C6-6, C6-8 or C6-6 and C6-8 (combined). Probiotic feeding began at first feeding and continued until fry reached an average weight of 1.0 g (54 days) at which point they were transitioned to a standard trout diet (Rangen EXTR 450 #2 crumble) or challenge and remained on their respective diet.

Probiotic gut persistence

Following transition to a standard trout diet, fry were killed to determine the length of time the probiotics were able to persist in the gut. Five fish from each treatment were sampled every 3 days for 36 days. Bacteria were removed and isolated from the gut according to the methods of Spanggaard et al. (2000). Fish were euthanized with an overdose of tricaine methane sulfonate 100 mg L^{-1} (MS-222; Argent Chemicals, Redmond, WA). The intestine was aseptically removed from the pyloric caeca to the anus; the contents were expelled by squeezing, the intestine was opened with a scalpel and then rinsed with sterile phosphate-buffered saline (PBS). The intestine was then placed in a stomacher bag containing 2 mL sterile PBS and homogenized. The homogenate (200 µL) was then plated on TS agar and allowed to grow for 48 hours at 15^{0}C. After incubation colonies with morphology similar to that of C6-6 or C6-8 were subcultured on TS agar until isolation was achieved. Following isolation, colonies were gram stained, and the oxidase and catalase reactions determined. Those matching the probiotics for all tests were selected for sequencing. Following isolation 16s rRNA sequencing was performed to confirm bacterial identification according to the method described in (Burbank et al., 2011). DNA was extracted using a DNeasy Kit (Qiagen, Valencia, CA) according to the manufactures

direction for gram-negative bacteria. A complete ORF of 16s rDNA was amplified in a PCR reaction containing 10 µL high fidelity master mix (Bio-Rad, Hercules, CA), 1uL forward primer (001F: 5′-aattgaagagtttgatcatggctca-3′), 1 µL reverse primer (1518R: 5′-aaggaggtgatccaNccRca-3′), 4uL PCR water (sigma, USA) and 1 uL of DNA template. PCR cycling conditions were as follows; initial denaturation at 98 °C for 30 sec followed by thirty cycles of 98 °C for 10 sec, 55 °C for 30 sec and 72 °C for 45 sec. The final extension was performed at 72 °C for 10 min. A negative control containing no DNA was also placed in the thermocycler concurrently with the DNA templates. The PCR products were cleaned using ExoSAP-IT (Affymetrix, Santa Clara, CA) according to the manufactures directions and submitted to Amplicon Express (Pullman, WA) for sequencing. Sequences were then compared to the known 16s sequences for C6-6 and C6-8 using GenBank.

Vaccination

After 7 days of feeding the standard diet without probiotics, fish were vaccinated with the commercially produced *F. psychrophilum* vaccine (supplied by Neogen, Lansing, MI). Following vaccination, fish were transferred to eight 225 L tanks with 600 fish $tank^{-1}$ each probiotic treatment was represented by 2 tanks. Vaccination was carried out following removal of the adipose fin with a scalpel after anesthesia with MS-222 (100 mg L^{-1}). After fish recovered they were immersed in a 1:10 dilution of the vaccine in tank water or 1:10 dilution of sterile triptone yeast extract salts (TYES) in tank water as a mock vaccination for 90 sec. Vaccination dose was determined according to the methods of Chen et al. (2003) and was 6.83 x 10^6 CFU mL^{-1} after dilution. Fish were held in separate tanks by treatment for two weeks at which time they were administered a booster vaccination or mock

vaccination using the same methods a primary vaccination without adipose fin clip. Booster vaccination dose was determined to be 1.6 x 10^7 CFU mL^{-1} after dilution.

Enzyme linked immunosorbant assay

To determine anti-*F. psychrophilum* antibody titers following vaccination, an enzyme linked immunosorbant assays (ELISA) was conducted. Prior to vaccination, blood was collected using 0.1 mL haematocrit tubes from naïve fish (50 fish per probiotic treatment) by severing the caudal peduncle and allowed to clot overnight at 4°C. The clotted samples were centrifuged at 15 000 x g for 5 min and serum collected and pooled by treatment to serve as a negative control for the ELISAs. Blood was collected immediately prior to vaccination and biweekly thereafter for 12 weeks. Blood was collected from 50 fish $treatment^{-1}$ at each time point, blood from ten fish was pooled to produce 5 samples per treatment and serum was collected as described above. All serum samples were stored at -80°C until needed. ELISAs were conducted according to the methods of LaFrentz et al. (2002). A positive ELISA titer was defined as the reciprocal of the highest dilution showing an optical density at least two times greater than the negative control.

Challenge bacterial culture

Challenge culture was prepared by growing *F. psychrophilum* at 15 °C for 72 hrs in TYES broth and harvesting by centrifugation at 1600 x g for 15 min at 15^0C. The supernatant was removed and the pellet re-suspended in sterile PBS to an optical density of 0.1 at 525nm. Dosage (CFU mL^{-1}) was determined by the drop plate method.

Probiotic challenge

Rainbow trout fry (mean weight 1.1g) from the probiotic fed groups were challenged with virulent *F. psychrophilum* CSF 259-93 prior to vaccination. The fish continued to

receive their respective probiotic treatment for the duration of the challenge. Triplicate groups of 25 fish treatment^{-1} received a sub-cutaneous injection along the dorsal midline with 25 µL of 1.40 x 10^7 CFU mL^{-1}. One group of 25 fish treatment^{-1} was injected with 25 µL sterile PBS as mock infected controls. Mortalities were recorded for 28 days and at least 20% of mortalities per day were examined and *F. psychrophilum* re-isolation was attempted by inoculating spleen, liver and kidney on TYES. Cumulative percent mortality (CPM) was calculated.

Vaccine challenge

Fry (mean weight 3.4 g) were challenged with virulent *F. psychrophilum* strain (CSF 259-93) by subcutaneous injection. Challenge dose was prepared as above 4 weeks after booter immunization. Triplicate groups of 25 fish treatment^{-1} were injected along the dorsal midline with 25 µL of 3.65 x 10^7 CFU mL^{-1} suspension of *F. psychrophilum* CSF 259-93 suspended in sterile PBS. One group of 25 fish treatment^{-1} was injected with 25 µL sterile PBS as mock infected controls. Final challenge dose was 1.46 x 10^5 CFU fish^{-1}. The RPS was compared to the mock vaccinated oil fed group. The RPS was calculated according to Amend (1981):

RPS=[1-(% mortality of vaccinated fish/ % mortality of non-vaccinated fish)] x 100

Statistical Analysis

CPM was calculated for each tank and antibody titers were averaged by tank with tanks as replicates. CPM and antibody titers analyzed using one way analysis of variance (ANOVA) with pair-wise comparisons using Tukey's post-test when appropriate. Antibody titers were compated between treatments within weeks. Survival curves were analyzed by treatment after confirming there were no significant differences between tanks within

treatmetns using a log rank survival curve analysis (GraphPad Prism, version 5.04 for windows, GraphPad Software, San Diego CA, USA). Differences were considered significant at $P \leq 0.05$.

Results

Gut persistence

Identification of probiotics from gut samples was first attempted on isolates taken at 1 and 36 d post probiotic feeding. At least one isolate from each fish at each time point contained a 100% match to the Genbank sequence for the fed probiotic. In the combined treatment, both C6-6 and C6-8 were isolated and confirmed by sequencing. At all other isolates intermediate time points *Enterobacter* C6-6 or C6-8 were confirmed based on cell and colony morphology as well as catalase and oxidase reactions consistent for these strains. No probiotics were isolated from fish fed the oil treatment

Enzyme linked immunosorbant assay

Antibody titers following vaccination remained below the detectable limit (< 50) for all groups at all sampling points.

Probiotic challenge

In the probiotic challenge, CPM ranged from 30.0 ± 12.2%% in the C6-8 group to 50.7 ± 6.7% in the oil group. CPM for the C6-6 and combined groups were 40.6 ± 7.0% and 39.2 ± 1.7% respectively. There was no mortality in mock infected groups. There were no significant differences in CPM or survival curve analysis survival between any of the infected groups (Figure 3.1).

Vaccine challenge

CPM and RPS values for all groups in the vaccine challenge are presented in Table 1. Overall, CPM was very high and ranged from 68.6% in the C6-6 + vaccination treatment to 89.8% in the Oil + mock vaccination treatments. There was a significant difference ($P \leq 0.05$) in CPM and mortality rate following survival curve analysis between the oil + mock vaccination and the C6-6 + vaccination treatments ($F_{7,16} = 2.31$). There were no other significant differences in CPM (Figure 3.2). RPS ranged from 23.6% in the C6-6 + vaccination group and 5.4% in the C6-8 + vaccination group. RPS for the oil + vaccination and the combined + vaccination groups were 7.6% and 15.4% respectively (Table 3.1).

Discussion

In the present study, colonization and persistence of probiotics in gut samples was confirmed following feeding. Isolation and identification of the probiotics immediately after probiotic feeding ceased indicated successful passage of live probiotics through the stomach and into the intestine. Isolation and identification of the probiotics 36 days after probiotic feeding ceased indicated that the probiotics are not only able to successfully pass to the intestine but also colonize and persist in the gut. This is not surprising as both probiotics were originally isolated from the gastrointestinal tract of rainbow trout (Burbank et al., 2012). What is not known, is the exact mode of action of these probiotics play and what level of colonization would be required to provide long term health benefits. In the probiotic challenge, no significant improvements in survival of the fish fed the probiotics was observed. This was unexpected as Burbank et al. (2011) demonstrated that these probiotics are capable of reducing mortality when fed just prior to and during an *F. psychrophilum* challenge. Similar to our study, Merrifield et al. (2010) reported variability

between studies of the effects *of Bacillus subtilis, B. licheniformis and Enterococcus faecium* in rainbow trout. The authors suggest that factors such as physiological status of the fish, rearing conditions, and probiotic interaction with indigenous gut microbiota can play a role in the effects of the probiotic. One notable difference between this and previous studies with C6-6 and C6-8 is the length of time that the probiotics were administered prior to challenge. Burbank et al. (2011) reported that feeding the probiotics for 7 days prior to and for the duration of the challenge was most effective. In the present study, fish were fed the probiotics from first feeding (40 days prior to challenge) and throughout the challenge. It is possible that these probiotics provide benefit by enhancing immunity and that prolonged feeding somehow led to tolerance and suppressed this effect, or that fish lacked immunocompetence and were unable to elicit such responses. This is only speculative, but stimulatory effects of probiotics on innate immune parameters including, phagocytic activity, respiratory burst activity, lysozyme activity, complement activity, cytokines, and gut immunity have been widely reported (reviewed by Nayak 2010). Sharifuzzaman and Austin (2009) administered the probiotic *Kocuria* SM1 to rainbow trout for 4 weeks and noted the greatest benefits to cellular and humoral immune response, as well as, survival following challenge occurred after 2 weeks of probiotic feeding and decreased thereafter with increasing administration periods. Furthermore, Nayak (2010) suggests that increases in innate immunity observed in the short term may be due to adjuvant like effects. Thus, long term probiotic feeding may allow for the development of immune tolerance and a return to baseline immune parameters reducing their effectiveness. Without further study on the mode of action of these probiotics it remains unclear why limited benefit was observed in the probiotic challenge. Additionally, due to the limited success of the probiotic

challenge, we are unable to determine the benefits of a combined supplementation with C6-6 and C6-8.

In the vaccine challenge, no significant reduction in mortality of groups fed the probiotics without vaccination was observed. Again, this is not entirely unexpected as fish in the vaccine challenge had not been exposed to the probiotics for 7 weeks prior to challenge. Again, Burbank et al. (2011) reported the greatest improvements in survival when fish were fed the probiotics just prior to and during challenge. As reported above we were able to isolate probiotics from the gut out to 36 days after the feeding of probiotics ceased; however, beyond this point their ability to persist in the gut is unknown. Even if these probiotics persisted in the gut for extended periods following feeding, the level of colonization may be below what is needed to provide a measurable health benefit.

In the present study the highest RPS value observed was 23.6%. LaFrentz et al. (2008) and Long et al. (2013) reported RPS values of 45% and 46%, respectively, using a similar vaccine, although the high mortality observed in the present study likely overwhelmed the fish and therefore limited the effectiveness of the vaccine and probiotics. In the present study, antibody titers following vaccination were below detection levels. Castro et al. (2008) examined the effects of an inactivated *Edwardsiella tarda* vaccine in turbot (*Scophthalmus maximus*) via injection and 60 second immersion. The injection vaccination resulted in significant increases in antibody titers and survival following challenge. However, fish vaccinated via immersion had no detectable increases in antibody titers and mortality was very similar to un-immunized fish following challenge. Similarly, Akhlaghi et al. (1996) reported no increases in antibody titer or improvements in survival of fish immersion vaccinated for 30 seconds with inactivated *Streptococus* sp. and

subsequently challenged with *Streptococus* sp. but reported significant improvements in survival in fish vaccinated via injection and subsequently challenged. However, immersion immunization with this vaccine has proven effective in previous studies (LaFrentz et al. 2008, Long et al. 2013). These studies have also reported significant increases in antibody titers following immunization. However, our vaccination method differed in that fish were exposed to a lower vaccination dose for a much shorter duration (90 sec versus 1hr), and fish were smaller in size. In LaFrentz et al. (2008) rainbow trout were immunized by immersion in 1.4×10^8 CFU mL^{-1} followed by a booster immunization 4 weeks later with 9.7×10^7 CFU mL^{-1} for 1 hour. In Long et al. (2013) coho salmon were immunized via immersion in 5.3×10^7 CFU mL^{-1} followed by a booster vaccination 4 week later with 5.3×10^6 CFU mL^{-1} for 1 hour. Thus, it is possible that the lower vaccination dosage, shorter exposure time, and immune status of 1 g fish limited the effects of the vaccine on antibody response and survival in light of high challenge mortality.

Despite the lack of expected effect on CPM and RPS with the probiotics or vaccine alone, we did observe a significant reduction in CPM of the C6-6 + vaccine treatment. The improved survival observed in this group appears promising especially when considering the high challenge mortality. The most likely modes of action for probiotics as reported by Fuller (1987) are; stimulation of humoral and/or cellular immune response, alteration of microbial metabolism by the increase or decrease of relevant enzyme levels, or competitive exclusion in which the probiotic alters the activity of the pathogen by production of inhibitory compounds or by competition for nutrients, space or oxygen. The observed benefit of the C6-6 + vaccine treatment suggests that C6-6 may have acted as an adjuvant by stimulating the immune system. The success of combining compounds that stimulate the

immune system and vaccines is well documented (Ramadan et al., 1994; DeBaulny et al., 1996; Figueras et al., 1998; Kawakami,et al., 1998; Burrells et al. 2001;). Further study on the mode of action of C6-6 would be required to confirm any adjuvant effect.

In conclusion, this study showed that the probiotics C6-6 and C6-8 are capable of surviving and colonizing the gut of rainbow trout for at least 36 days post probiotic feeding. Despite lower than expected RPS values following vaccination, it was shown that supplementation of feed with C6-6 prior to vaccination with a live attenuated *F. psychrophilum* vaccine CSF 259-93B.17 improve survival of trout following *F. psychrophilum* challenge.

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Table 3.1 Cumulative percent mortality (CPM) and relative percent survival RPS (mean ± SEM) following challenge with 25 µL of 3.65 x 107 CFU mL-1 *Flavobacterium psychrophilum* of rainbow trout fry after feeding of probiotics and vaccination with live *F. psychrophilum* CSF 259-93B.17. CPM was significantly different ($P < 0.05$) between the oil + mock vaccination and C6-6 + vaccination treatments.

Treatment	**CPM**	**RPS**
Oil + mock vaccination	89.8 ± 2.0	-
C6-6 + mock vaccination	74.0 ± 9.5	-
C6-8 + mock vaccination	73.7 ± 7.0	-
Combined + mock vaccination	85.5 ± 1.2	-
Oil + vaccination	83.0 ± 7.0	7.6
C6-6 + vaccination	68.6 ± 7.2	23.6
C6-8 + vaccination	85.0 ± 2.1	5.4
Combined + vaccination	76.0 ± 1.2	15.4

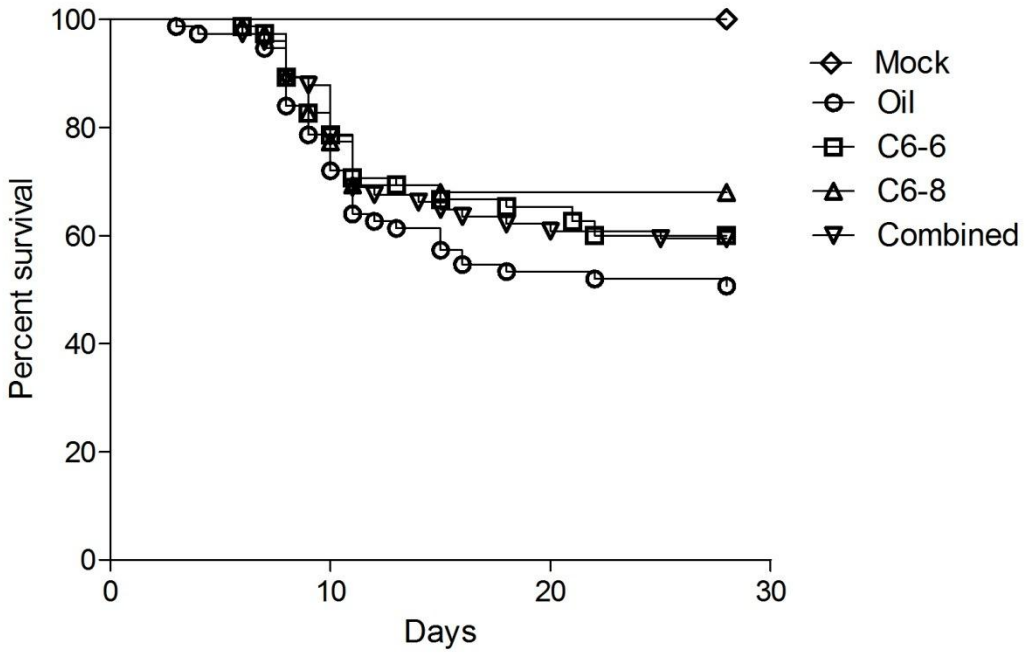

Figure 3.1 Survival curve of rainbow trout fry fed feed coated with the probiotics C6-6, C6-8, C6-6 + C6-8 or oil (negative control) at first feeding for 54 d. Fry were then challenged with 25 µL of 1.40 x 10^7 CFU mL^{-1} *Flavobacterium psychrophilum*.

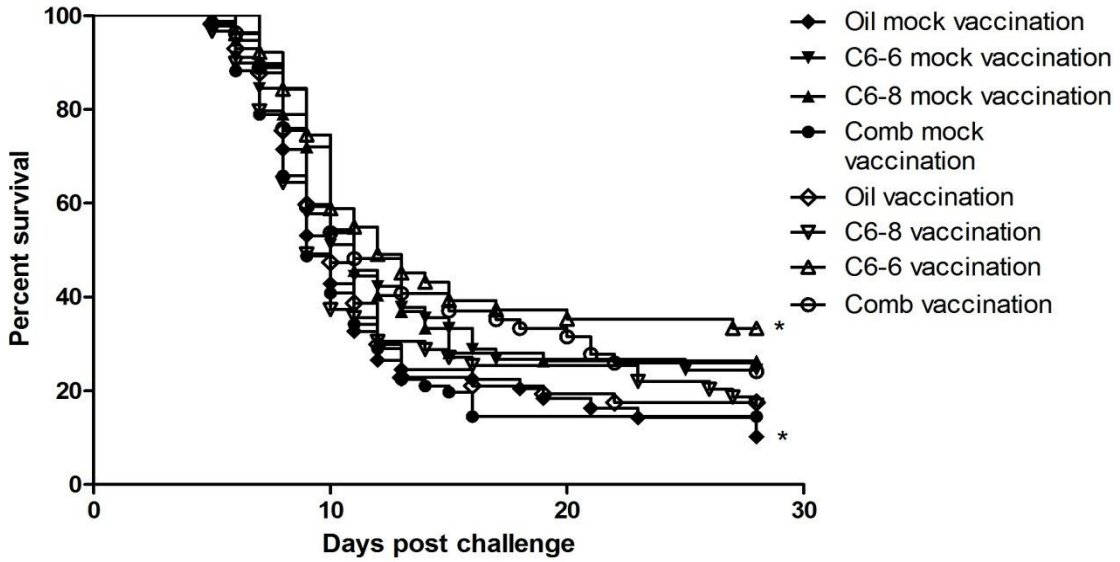

Figure 3.2 Survival curves of rainbow trout fry following challenge with *Flavobacterium psychrophilum* after feeding of probiotics and vaccination with live *F. psychrophilum* CSF 259-93 B.17. Significant differences ($P < 0.05$) between treatments in log rank survival. Significant differences ($P < 0.05$) between treatments in log rank survival curves are noted with (*).

Chapter 4: The probiotic (*Enterobacter* sp. C6-6) elicits a protective response to rainbow trout (*Oncorhynchus mykiss*) following injection and challenge with *Flavobacterium psychrophilum*.

Abstract

To determine potential mechanisms of cross-protection against *F. psychrophilum* infection, a study was conducted where *Enterobacter* sp. (strain C6-6) was delivered to rainbow trout, *Oncorhynchus mykiss*, via injection. Groups of fry (1.3 ± 0.1g) received either an intraperitoneal injection of phosphate buffered saline (PBS) (negative control), supernatant from a C6-6 culture, formalin killed C6-6, or live C6-6 and were subsequently challenged with *F. psychrophilum* 7 days or 28 days post injection. Log rank survival analysis showed significant reduction in mortality of fish receiving any of these treatments at both 7 and 28 days post injection. Injection of live C6-6 resulted in the lowest mortality at either time point and cumulative mortality at 7 days post injection was significantly reduced ($P < 0.05$) in fish receiving formalin killed (62.7 ± 7.4%) or live *Enterobacter* C6-6 (48 ± 6.9%) when compared to the negative controls (92 ± 2.3%). At 28 days post injection of C6-6, fish receiving the formalin killed or live C6-6 had significantly increased antibody titers to *F. psychrophilum*. This was not expected and indicates that protection observed at 28 days could be in part due to a cross protective adaptive response. Results suggests that disease protection following delivery of this naturally occurring bacterium is at least in part dependent on enhanced immune function in fish.

Introduction

Flavobacterium psychrophilum is the causative agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome. High loses from *F. psychrophilum* infections

have resulted in BCWD becoming a worldwide concern in aquaculture facilities (Barnes and Brown, 2011). Losses are highest in young fish and most salmonids are susceptible to *F. psychrophilum* infections. Mortality tends to be highest in rainbow trout and steelhead (*Oncorhynchus mykiss*) as well as coho salmon (*O. kisutch*). Currently treatment of BCWD is limited to antibiotics. However, reliance on antibiotics has become problematic with the emergence of antibiotic resistant *F. psychrophilum* strains (Schmidt et al., 2000; Del Cerro et al., 2010).

Recently, naturally occurring gut bacteria have been identified and appear to show promise as an alternative prevention and treatment strategy for *F. psychrophilum* infections (Burbank et al., 2011; Korkea-aho et al., 2011; Ström-Bestor and Wiklund, 2011). Traditionally, probiotics are administered through feeding or for aquaculture they may be added to the water (Irianto and Austin, 2002). In fact, an early and wildly accepted definition by Fuller (1987) defines probiotics as a cultured or live microbial feed supplement that beneficially affects the host by improving its intestinal balance. However, a more recent definition by Salminen et al. (1999) removed the requirement that a probiotic be fed or even be a live culture and defines it as any microbial preparation or component of microbial cells with a beneficial effect on the health of the host. A recently identified probiotic (*Enterobacter sp.* C6-6) has been shown to reduce mortality of fish following probiotic feeding and challenge with *F. psychrophilum* (Burbank et al. 2011), but the reasons for such protection are unclear. In this study, C6-6 was further evaluated to gain baseline knowledge of protection mechanisms.

The *Enterobacter* sp. strain C6-6 was originally isolated from the gastrointestinal tract of rainbow trout. In recent trials, Burbank et al. (2011 and 2012) demonstrated that this

strain was able to significantly reduce mortality in rainbow trout following dietary administration and subsequent challenge with *F. psychrophilum* as well as inhibit *F. psychrophilum* growth *in vitro*. However, dietary administration of C6-6 and its ability to control *F. psychrophilum* infections has been variable and appears to depend on challenge severity (unpublished results). Since the mechanisms associated with protection for this bacterium are unclear, this study was designed to further increase our understanding of C6-6 and how it may act to provide benefit. Therefore, rainbow trout fry were injected with C6-6 culture supernatant, formalin killed C6-6 or live C6-6 and then challenged with *F. psychrophilum*. Protection following pathogen challenge and development of cross-reactive antibodies were then determined.

Methods

Fish

Rainbow trout fry (1.3 ± 0.1g) were obtained from the Aquaculture Research Institute at the University of Idaho and held in 190 L tanks prior to experiments. Fish were maintained on 15^0C single pass de-chlorinated municipal water throughout the study. Fish were administered a 25 µL intraperitoneal injection with one of four treatments: sterile phosphate buffered saline (PBS) (negative control), C6-6 culture supernatant, formalin killed C6-6 or live C6-6. To prepare the supernatant treatment 25 mL of C6-6 was cultured in tryptic soy (TS) broth for 48 hrs at 15^0C following inoculation with frozen stock C6-6. Fish injected with the live C6-6 treatment received 2.1 x 106 CFU $fish^{-1}$. The culture was centrifuged at 4300 x g for 15 min at 15^0C and the supernatant collected. The supernatant was filter sterilized using a 0.22 µm filter and stored at 4^0C for 48 hrs. To confirm the supernatant contained no viable cells 100 µL was plated on TS agar, incubated for 48 hrs at

15^0C and monitored for growth. The formalin killed treatment was prepared by inoculating 50 mL of TS broth with frozen stock C6-6 and growing for 48 hrs at 15^0C. The culture was killed by the addition of formalin to a final concentration of 1% and incubating overnight on a stir plate at 15^0C. The killed culture was harvested by centrifugation at 4300 x g for 15 min, the supernatant was removed and the pellet washed in sterile PBS. The culture was centrifuged again as above and resuspended in PBS to an optical density (OD) at 525 nm of 0.1 and stored for 48 hrs at 4^0C until use. To confirm there were no viable bacteria, 100 µL was plated on TS agar and incubated for 48 hours at 15^0C and monitored for growth. The live C6-6 treatment was prepared by growing C6-6 for 48 hrs at 15^0C in TS broth. The culture was then adjusted to an OD of 0.1 at 525 nm using sterile TS broth and use immediately. Colony forming units (CFU) were determined according to the drop plate method of Chen et al. (2003).

Challenge preparation

F. psychrophilum strain CSF 259-93 was cultured at 15^0C for 72 hours in triptone yeast extract salts (TYES) broth and harvested by centrifugation at 1600 x g for 15 min at 15^0C. The supernatant was removed and the pellet re-suspended in sterile PBS to an OD at 525 nm of 0.1 for the challenge 7 d after C6-6 injection and an OD of 0.15 for the challenge 28 days after C6-6 injection. CFU were determined by the drop plate method.

Experimental design

Fish were challenged with a virulent *F. psychrophilum* strain CSF 259-93 at 7 and 28 days post injection of the C6-6 treatments. For the challenge 7 days after treatment, triplicate tanks of 25 fish per treatment received a 25 µL subcutaneous injection of 3.43 x 10^5 CFU $fish^{-1}$ or 25 µL sterile PBS (mock infected). Mortalities were removed daily and

recorded for 21 days. At least 20% of mortalities per day were examined and *F. psychrophilum* re-isolation was attempted by inoculating spleen, liver, and kidney on TYES agar. The challenge 28 days post C6-6 injection utilized the same methods as above except fish were monitored for 28 days after challenge. Fish in the second challenge received 5.6 x 10^5 CFU $fish^{-1}$.

Enzyme linked immunosorbant assay

An enzyme linked immunosorbant assay (ELISA) for the detection of serum antibodies reactive to *F. psychrophilum* was conducted according to LaFrentz et al. (2002). Prior to C6-6 injection, blood was collected using 0.1 mL haematocrit tubes from 150 fish by severing the caudal peduncle and allowed to clot overnight at 4 °C. The clotted samples were centrifuged at 15 000 x g for 5 min and the serum removed. Sera was pooled and served as a negative control for the ELISA. Blood was collected immediately prior to the challenges and from survivors of the 28 day challenge. Prior to the challenges blood was collected from 50 fish per treatment and pooled to produce 5 pools of 10 fish. Serum was produced as above. After completion of the challenge 28 days post C6-6 injection, blood was collected from up to 5 surviving fish from each tank and pooled by tank to obtain 3 samples per treatment. All serum samples were stored at -80 °C until needed. Antibody titers were defined as the reciprocal of the highest dilution showing an optical density at least two times greater than the negative control.

Statistical analysis

Cumulative percent mortality (CPM) was calculated for each tank and antibody titers for individual fish were averaged by tank with tanks as replicates. CPM and antibody titers analyzed using one way analysis of variance (ANOVA) with pair-wise comparisons using

Tukey's post-test when appropriate. Antibody titers were compared between treatments within weeks. Survival curves were analyzed by treatment after confirming there were no significant differences between tanks within treatmetns using a log rank survival curve analysis (GraphPad Prism, version 5.04 for windows, GraphPad Software, San Diego California USA). Differences were considered significant at $P \leq 0.05$.

Results

Challenge

Fish injected with the live C6-6 treatment received 2.1×10^6 CFU fish^{-1}. No bacterial growth was observed on the plates inoculated with the supernatant or formalin killed bacteria. The CPM for the challenge 7 days after C6-6 injection was higher than expected with the PBS injected fish highest CPM at 92% and fish injected with live C6-6 having the lowest CPM at 48%. CPM for fish injected with supernatant and formalin killed C6-6 were 73.3% and 62.7%, respectively. There were significant differences in CPM between the PBS injected treatment and the formalin treatment ($P \leq 0.05$) and the PBS injected treatment and live C6-6 treatment ($P \leq 0.01$) ($F_{3,8} = 8.67$). There were significant differences ($P < 0.05$) in the log rank survival analysis between the PBS injected groups and all other groups and between the supernatant and live C6-6 injected groups for the challenge 7 days post C6-6 injection (Figure 4.1).

CPM for the challenge 28 days after injection of C6-6 treatments was highest in the PBS injected group, 73.6 ± 9.7%, and lowest in the live C6-6 injected group 29.2 ± 12.28%. CPM values for the fish injected with supernatant and formalin killed C6-6 were 65.0 ± 6.9% and 53.3 ± 11.67%, respectively. Due to increased variability between tanks ANOVA results for CPM were not significant between any of the treatments for this challenge.

However, significant differences ($P < 0.05$) were observed following log rank survival curve analysis between the live C6-6 treatments and all other treatments and between the PBS and formalin killed treatments (Figure 4.2).

Enzyme linked immunosorbant assay

Antibody titers were below the detectable limit (<50) for all treatments at 7 days after C6-6 injection. However, 28 days post C6-6 injection positive antibody titers of 40 ± 10, 200 ± 55 and 400 ± 0 were observed in the supernatant, formalin killed and live C6-6 injected groups respectively ($F_{3,8} = 16.45$) (Figure 4.3). There were significant differences between the PBS injected treatment and the formalin killed treatment ($P < 0.01$), between the PBS injected treatment and live C6-6 treatment ($P < 0.01$) and between the live and formalin killed treatments ($P < 0.05$). Antibody titers from survivors of the challenge 28 days post C6-6 injection were 1837 ± 705 for the PBS injected group, 800 ± 400 for the supernatant group, 1600 ± 267 for the formalin killed C6-6 group and 533 ± 133 for the live C6-6 injected group. There were no significant differences ($P > 0.05$) in antibody titers between survivors of any groups at this time point.

Discussion

This study has shown that injection of formalin killed C6-6 or live C6-6 can significantly reduce mortality rainbow trout following challenge with *F. psychrophilum*. This was especially evident when challenged 7 days post C6-6 injection. Survival curve analysis also showed significant differences in fish injected with all other treatments at 7 days post injection and in the formalin killed and live C6-6 treatments at 28 days when compared to PBS injected fish.

At 7 days post injection, differences in the survival curve analysis indicate that all treatments were able to positively affect the survival of the challenged fish. Burbank et al. (2012) demonstrated that live C6-6 as well as C6-6 supernatant is able to inhibit *F. psychrophilum* growth *in vitro*. This suggests that protection in the C6-6 supernatant groups may be due to extracellular compounds inhibitory to *F. psychrophilum*. Administration of live or killed probiotics has also been shown to simulate the innate immune system (Panigrahia et al., 2004; Picchiettiet al., 2009; Sharifuzzaman and Austin, 2010; Korkea-aho et al., 2011). Additionally, Arijo et al. (2008) reported that injections with extracellular cellular components from probiotic *Vibrio* sp. increased the serum lysozyme activity of rainbow trout. It appears likely that treatments in the present study are capable of stimulating the innate immune system either through subcellular components in the culture supernatant or host interaction with the bacterial cell. This explanation is supported by the limited amount of time between injection of the C6-6 treatments as well as the lack of a detectable antibody response prior to the initial challenge (7 days post C6-6 injection). However, it is not known what innate immune parameters were responsible for the observed protection since this was not specifically evaluated in this study.

Due to the limited duration of a typical innate immune response it is unlikely that this persisted out to 28 days post C6-6 injection. Increases in specific antibody titers observed 28 days after injection of formalin killed and live C6-6 suggest that a cross-reactive adaptive response to *F. psychrophilum* may be important. These increased antibody titers were not observed in the fish injected with the supernatant and survival of these fish was also not significantly different from the control at this time point; thus, suggesting a potential role of cross-reactive antibodies. Production of specific antibodies has been

positively correlated to protection in *F. psychrophilum* infections (Rahman et al., 2002; Madetoja et al., 2006;). LaFrentz et al. (2008) demonstrated that even relatively low titers, similar to those observed in the present study, are capable of conferring protection against *F. psychrophilum* infections. Therefore, it is likely that increased antibody titers observed prior to the second challenge (28 days post C6-6 injection) played an important role in protection of the formalin killed and live C6-6 injected groups.

Following administration of probiotics, increased general antibody production has been reported. Korkea-aho et al. (2012) reported significant increase in total serum immunoglobulin levels after feeding *Pseudomonas* M162 to rainbow trout. Similarly, Al-Dohail et al. (2009) observed increases in total immunoglobulin after feeding the probiotic *Lactobacillus acidophilus* to African catfish, *Clarias gariepinus.* Finally, Panigrahi et al. (2005) observed increases in the total immunoglobulin levels of rainbow trout after feeding the probiotic *Lactobacillus rhamnosus.* However, the production of specific cross-reactive antibodies following probiotic administration is not widely reported. Arijo et al. (2008) reported that injection of formalin killed *Vibrio* sps. resulted in increased antibodies reactive to *Vibrio harveyi* in rainbow trout. However, these probiotics were closely related to the pathogen of interest. This is not the case in the present study and raises the question of whether the observed antibody titers are a result of specific antigens shared between C6-6 and *F. psychrophilum* or are due to general immunoglobulin increases.

It is interesting that significant antibody titers were detected by 4 weeks post C6-6 injection. LaFrentz et al. (2008) did not observe significant increases in specific antibody titers in rainbow trout maintained under similar environmental conditions until 8 weeks post injection with a live attenuated *F. psychrophilum* strain. Similarly, Madetoja et al. (2006)

did not observe significant increases in antibody titers until 6 weeks post injection of rainbow trout with formalin killed or heat inactivated *F. psychrophilum*.

Although this study provides insight on how immunity may be affected by C6-6 administration, it also shows that fish can be significantly protected from BCWD following injection delivery. When taken together with earlier feeding studies on these probiotics, it appears that immunity is important and that this *Enterobacter* strain may serve as an "alternate" vaccine. Obviously, injection is not a preferred delivery method for rainbow trout aquaculture, but it may be possible to incorporate a combination of other mass vaccination methods such as immersion and feeding and see similar benefits. Additional studies and delivery strategies are needed to determine the extent and duration of protection over the long term.

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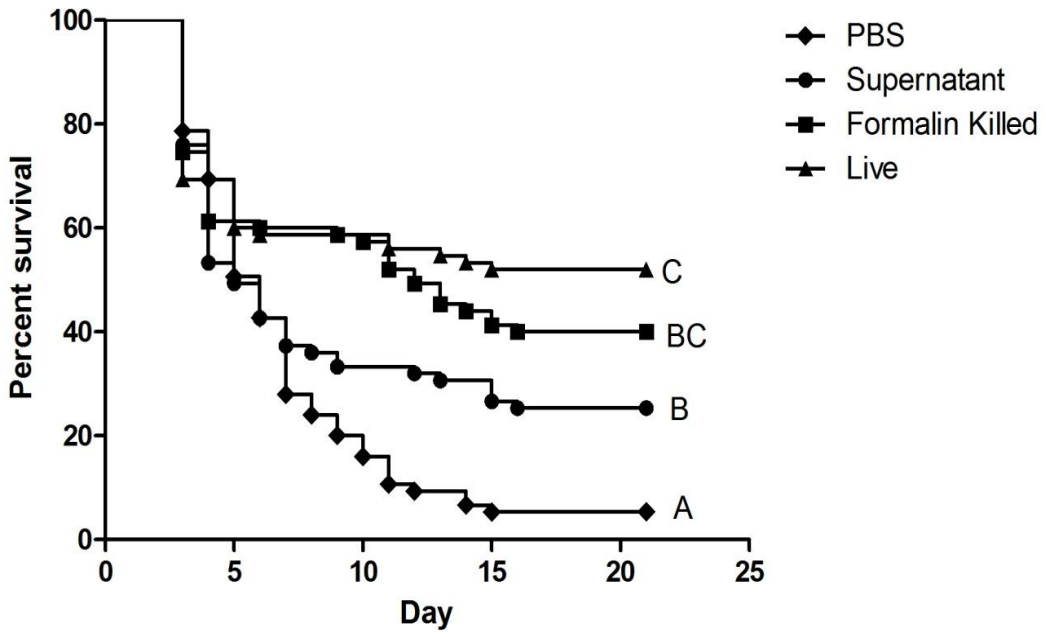

Figure 4.1 Survival curves of rainbow trout fry challenged with *F. psychrophilum* 7 days after i.p. injection with PBS, supernatant from C6-6, formalin killed C6-6, or live C6-6. Different letters indicate significant differences ($P < 0.05$) in survival curves.

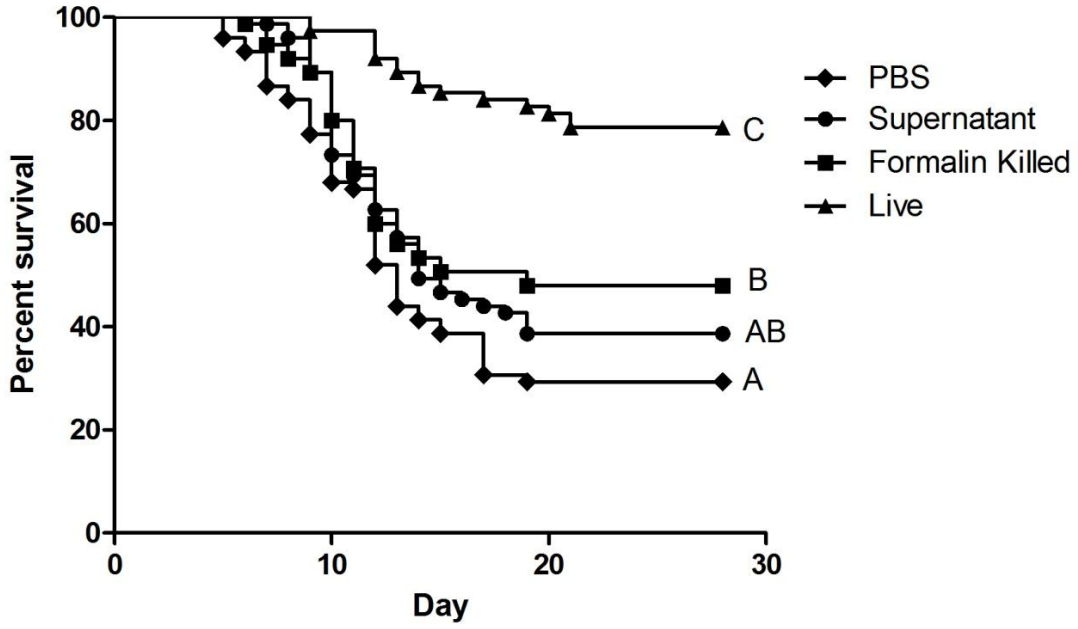

Figure 4.2. Survival curves of rainbow trout fry challenged with *F. psychrophilum* 28 days after i.p. injection with PBS, supernatant from C6-6, formalin killed C6-6, or live C6-6. Different letters indicate significant differences ($P < 0.05$) in survival curves.

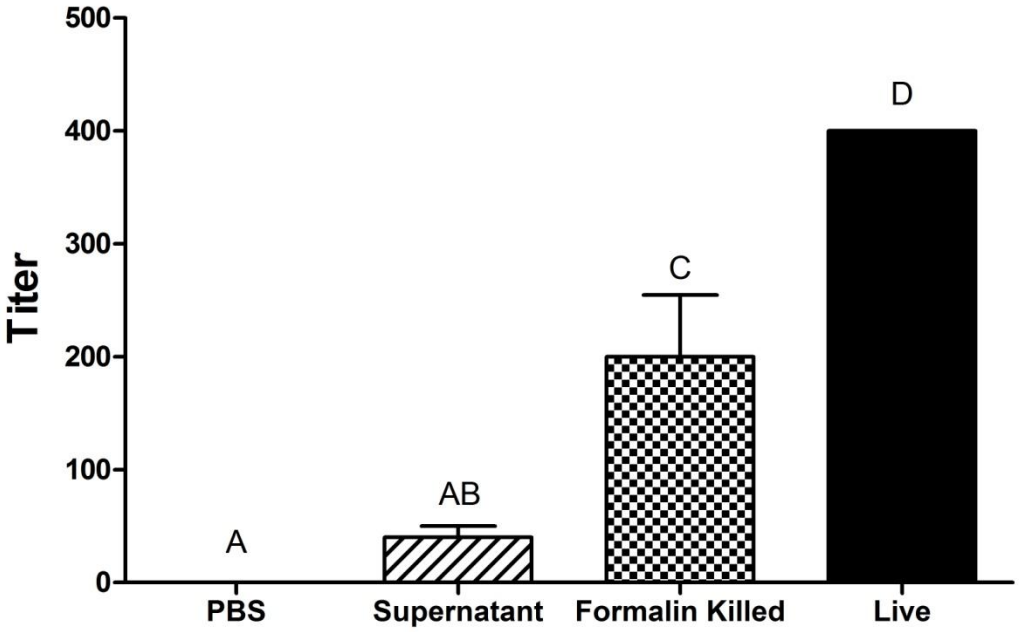

Figure 4.3 Antibody titers of rainbow trout 28 days after injection with PBS, C6-6 supernatant, formalin killed C6-6 or live C6-6. Different letters indicate significant differences ($P < 0.05$) in antibody titer.

General Discussion

This study identified testes meal as a possible partial replacement of fish meal in trout diets. The inclusion of testes meal in the diet had no significant effect on feed conversion ratio, specific growth rate, or final weight of the fish when compared to a control diet. This suggests that partial replacement of fish meal using processing byproducts is possible without sacrificing fish growth or performance.

The testes meal diet was also capable of inducing intermittent increases in phagocytic activity suggesting at least some capability as an immunostimulant. This increased activity agrees with the studies of Li et al. (2004) and Cheng et al. (2011) that demonstrated the effects of dietary nucleotides on innate cellular responses. However, this study also demonstrated a potential drawback of any immunostimulant, immunosuppression. Following long term exposure to a testes meal diet fish exhibited suppressed phagocytic activity after 12 weeks of feeding and lower antibody titers following challenge with *Flavobacterium psychrophilum* when compared to a control diet. Immune suppression with long term exposure to immunostimulants has been widely reported and this study parallels observations by Yoshida et al. (1995), Jørgensen and Robertsen (1995) and Misra et al. (2006). Despite this, if fed periodically the partial replacement of fish meal with testes meal may provide a benefit to the aquaculture industry through the reduction of fish meal in diets.

This study also served to further our knowledge of recently described probiotic bacterial strains *Enterobacter* C6-6 and C6-8. We were able to successfully demonstrate the probiotics' ability to survive in the gut as well as their ability to colonize and persist in the gut for at least 36 days. We also identified potential limitations of these probiotics. When the probiotics were administered for 54 days and fish were subsequently challenged with *F.*

psychrophilum, the influence of the probiotics appeared to be limited and no improvements in survival were observed. This result is similar to those reported by Sharifuzzaman and Austin (2009) and Nayak (2010) in which long term administration of probiotics decreased their influence on the immune system and the reduced effects on survival following challenge. However, without further investigation into the mode of action of these probiotics we are unsure as to the reasons for limited success in this challenge. The present study also reported limited success when combining the probiotics with vaccination. The studies of LaFrentz et al. (2008) and Long et al. (2013) reported relative percent survival (RPS) values of 45% and 46% respectively using a similar vaccine while the highest RPS achieved in our study was 23.6% in the group administered C6-6 and the vaccine. However, mortality in our challenge was very high potentially overwhelmed the effects of the probiotics and vaccine. In this study fish were vaccinated for a much shorter time than in LaFrentz et al. (2008) and Long et al. (2013) and may have also limited the potential of the vaccine. Despite low RPS values fish administered C6-6 and the vaccine did have significantly increased survival when compared to fish administered an oil and mock vaccination treatment. Thus, the combination of feeding C6-6 prior to vaccination may prove to be a beneficial strategy in managing *F. psychrophilum* infections.

Finally, this study demonstrated the ability of live or formalin killed C6-6 administered via injection to protect against *F. psychrophilum* infections in rainbow trout. Fish injected with formalin killed or live C6-6 had significantly lower mortality than the negative control 7 days after C6-6 injection. It appears that the protection observed in this challenge was due to stimulation of the innate immune system, although the mechanisms were not investigated. Interestingly, fish challenge 28 days after injection of the formalin

killed and live C6-6 also demonstrated significantly improved curves. However, due to the length of time between C6-6 injection and challenge it is unlikely that the protection observed at this time point was related to innate immunity. Fish in these treatments also had significantly increased antibodies reactive to *F. psychrophilum*. Thus suggesting protection was conferred through stimulation of the adaptive immune system and demonstrated that C6-6 is capable of stimulating the production of antibodies cross reactive to *F. psychrophilum*. These results are particularly interesting as increases in antibody titers in this study were also observed sooner than in studies which administered *F. psychrophilum* (Madetoja et al. (2006); LaFrentz et al. (2008). The rapid adaptive immune response and observed protection may indicate the potential for C6-6 to be used as an alternative vaccine; however, further study is needed to determine the duration and extent of protection in the long term.

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