

**EVALUATION OF VARIOUS DIETARY SUPPLEMENTS AND STRATEGIES
TO ENHANCE GROWTH AND DISEASE MANAGEMENT OF HYBRID
STRIPED BASS *Morone chrysops* × *M. saxatilis***

A Dissertation

by

PENG LI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2005

Major Subject: Nutrition

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ABSTRACT

Evaluation of Various Dietary Supplements and Strategies to Enhance Growth and Disease Management of Hybrid Striped Bass *Morone chrysops* × *M. saxatilis*.

(December 2005)

Peng Li, B. A., Ocean University of China

Chair of Advisory Committee: Dr. Delbert M. Gatlin, III

The US hybrid striped bass (*Morone chrysops* × *M. saxatilis*) industry has been negatively impacted by infectious diseases because there are very few approved drugs and vaccines. Therefore, a series of experiments was conducted to explore the potential use of various dietary supplements including autolyzed brewers yeast, the commercial prebiotic GroBiotic[®]-A, oligonucleotides and levamisole for improvement of hybrid striped bass growth, immunity and resistance to disease caused by various pathogenic bacteria.

In two trials with brewers yeast, fish fed diets supplemented with yeast at 2% generally showed enhanced weight gain and feed efficiency compared with those fed a basal diet. Brewers yeast also positively influenced resistance to *S. iniae* infection. In addition, results of immune response assays demonstrated that brewers yeast can be administered for relatively long periods without causing immunosuppression.

GroBiotic[®]-A (Grobiotic[™] AE) also resulted in significantly enhanced weight gain, innate immune responses and resistance of juvenile hybrid striped bass to *S. iniae* infection. An additional experiment with sub-adult fish showed significantly reduced mortality of fish fed a diet supplemented with GroBiotic[®]-A at 2% when subjected to an

in-situ *Mycobacterium marinum* challenge. This is the first report of positive effects from dietary prebiotics for fish health management, although many fundamental questions should be pursued further.

Dietary supplementation of a commercial oligonucleotide product (Ascogen P[®]) at 0.5% of the diet was shown to enhance resistance of hybrid striped bass against *S. iniae* infection and increased their neutrophil oxidative radical production. However, the effect on growth was marginal.

Dietary levamisole supplementation at a low level (100 mg/kg) enhanced the growth and feed efficiency of juvenile hybrid striped bass. However, an elevated dosage (1000 mg/kg diet) strongly suppressed growth, feed intake and feed efficiency. Hypothesized beneficial influences, including antibody production and resistance to *S. iniae* and *A. hydrophila* were not substantiated. Although dietary levamisole increased fish macrophage respiratory burst, an *in vitro* study failed to show a direct effect on cultured macrophages.

This suite of studies demonstrated the potential use of some dietary supplements to enhance hybrid striped bass production. Thus, immunonutrition represents a valuable strategy to apply in aquaculture.

DEDICATION

To my parents Zhongshan, Huan and my wife Xiaoxue.

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CHAPTER I

INTRODUCTION

Aquaculture has grown at an average rate of 8.9 percent per year since 1970 and has become the fastest growing sector of food production in the world (FAO, 2004; Subasinghe, 2005). In 2002, total world aquaculture production was reported to be 51.4 million tons by quantity and \$60 billion by value (FAO, 2004). It was estimated that the aquatic products generated from aquaculture by 2050 will be between 177.9 (assuming growing fisheries at 0.7% per year) and 209.5 (assuming stagnating fisheries) million tons per year (Wijkström, 2003). Despite the encouraging trends, several constraints may have negative impact on the growth of aquaculture. Diseases are one of these primary limiting factors (FAO, 2004). Although global economic losses from aquaculture diseases have not been compiled, disease reports from many regions of the world have been increasing with advances in the live aquatic animal trade (reviewed by Subasinghe, 2005).

Current methods for prevention and treatment of infectious aquatic diseases include a limited number of government-approved antibiotics and chemotherapeutics and limited vaccines that can be used to compliment environmental management. However, use of antibiotics has been seriously criticized for development of antibiotic-resistant bacterial strains (FAO, 2002), potential impact on the non-target bacterial communities in the environment (Stickney, 1997) and risk of antibiotic residues in aquaculture products that are harmful to antibiotic-intolerant human consumers (FAO, 2002). In addition,

This dissertation follows the style of *Aquaculture*.

numerous studies have shown that most antibiotics including FDA-approved oxytetracycline can suppress the natural immunity of fish, increasing the risk of infection from non-bacterial pathogens such as viruses, fungi and parasites (Rijkers et al., 1980, 1981; Siwicki et al., 1989; Taffaella et al., 1999, Luden et al., 1999, 2002). Because vaccines are usually delivered via injection instead of dietary supplementation or immersion, tremendous amounts of human labor are required to deliver the vaccines and there is associated handling stress on the fish that may offset the efficacy of the vaccines to a great extent. While considerable effort in vaccine development has continued, vaccines against some severe infectious diseases are still unavailable.

1. Fish Immune System

Traditionally, the immune system of fish is divided into two primary components, innate and adaptive. The innate immune system consists of physical barriers including scales, mucus, gills and epidermis; immunocytes including phagocytic cells, non-specific cytotoxic cells, endothelial cells and a wide variety of humoral components such as transferrin, lysozyme, complement, protease inhibitors, natural antibodies, lectins, pentraxins, cytokines, chemokines and antimicrobial peptides from internal secretion of various tissues and cell types (reviewed by Magnadóttir, 2005). The adaptive immune responses are dependent on the activities of B lymphocytes and T lymphocytes, which function in specific immunoglobulin production and cytotoxic activities and immunomodulation via cytokines (Shoemaker et al., 2001).

Increasing evidence on the essential interaction between innate and adaptive immune systems has emerged in recent years. Innate immunity has been demonstrated to

be essential in activation of acquired immunity (Fearon and Locksley, 1996; Fearon, 1997). The activation of innate recognition components, through the stimulation of phagocytes, produces cytokines and chemokines that activate the complement system and various cell receptors and stimulating T-, B- and antigen- presenting cells (reviewed by Lo et al., 1999), which have been tentatively confirmed in fish (Dixon and Stet, 2001). A recent study (Acosta et al. 2005) showed that vaccination could enhance the inducible nitric oxide responses of macrophages from gilthead seabream, suggesting an important role of immunological memory in innate immune response.

Considerable progress has been made in molecular immunology of aquatic animals and many immune genes including cytokines, receptors and humoral proteins have been sequenced. Although the homology of these proteins among various species may be low and restrict the application of molecular tools across species (Secombes, 2002), it is becoming increasingly feasible to evaluate environmental factors, especially dietary components, via quantitatively measuring expression of genes associated with growth, metabolism and health, at least for some model fishes such as rainbow trout, gilthead seabream and channel catfish.

2. Overview of Dietary Strategies for Fish Health Management

Prepared diets not only provide the essential nutrients that are required for normal physiological functioning but also may serve as a medium by which fish receive certain compounds that may alter endocrine activity, immunity and other physiological responses (Gatlin, 2002; Li et al., 2005). The research pertaining to dietary modulation of immunity

and disease resistance has been conducted for over 15 years and encouraging progress has been made. Major dietary strategies are summarized as follows:

1. Fortification with immunonutrients. It is known that some nutrients have specific functions in immune responses, such as arginine as the substrate of nitric oxide production (reviewed by Wu and Morris, 1998), glutamine for energy (Wu et al., 1991), nucleotides for cell proliferation and immunogene expression (reviewed by Li and Gatlin, 2005), as well as vitamins C and E for protecting phagocytes from oxidative stress and destruction (Bendich, 1990). Elevated supplementation of vitamin C has been shown to enhance immune responses and disease resistance in various fish species (Ai et al., 2004; Lin and Shiau, 2005).
2. Supplementation of immunostimulants and metabolism-modifying chemicals. There has been heightened attention in recent years to the use of natural microbial products in aquafeeds which may enhance the immune responses and disease resistance of aquacultured fishes by altering the signaling of immunocytes (reviewed by Sakai, 1999; Gatlin, 2002). The most extensively studied immunostimulant is β -glucans, constituents of yeast cell walls. Studies with various products such as peptidoglycan, lipopolysaccharide, bacterial fermentation products (FK-650) and herbal extracts also have shown promise as dietary supplements for disease management in aquaculture. Many synthetic drugs also have been used in terrestrial animal production for modification of the somatogenic axis toward rapid growth and enhanced feed efficiency. Up-regulation of the immune system by anabolic hormones such as growth hormone (GH) and insulin-like growth factor (IGF-1) has been confirmed in fishes (Harris and

Bird, 2000). Therefore such drugs may be important for development of new dietary strategies in aquaculture, although current research seems limited to levamisole.

3. Supplementation of gut microflora-altering components. Diet supplementation with live microorganisms such as bacteria or yeast to alter composition of the gastrointestinal tract of fish has been shown to enhance growth as well as reduce disease risk of various species (reviewed by Gatescoupe, 1999; Irianto and Austin, 2002). The evidence of beneficial effects of probiotics gave rise to the concept of prebiotics, which are defined as non-digestible feed ingredients which beneficially affect the host by selectively stimulating growth and activating health-promoting bacteria in the intestinal tract. However, potential use of prebiotics in aquaculture has not been pursued.
4. Passive immunization using pathogen-specific antibody supplementation to the diet. Pathogenic bacteria-specific Ig Y can be isolated from egg yolk produced by laying hens vaccinated with killed fish pathogenic bacteria. The antibody could be transferred to fish by oral administration, indicating a novel method in prevention of certain diseases. Successful reports on anti-*Vibrio* and anti-*Yersinia* Ig Y in rainbow trout (Lee et al., 2000; Arasteh et al., 2004) as well as anti-*Edwardsiella* Ig Y in Japanese eel (Gutierrez et al, 1993) have shown promise of passive immunity as a dietary supplementation strategy for enhancement of fish health.

3. Hybrid striped bass

Hybrid striped bass culture began in the late 1980s and has rapidly developed into a major aquaculture industry in the US. The production in the US was 5.2 million

kilograms in 2003 and was projected to be 6.4 million kilograms in 2004 (Calberg, personal communication). Although a wide range of pathogenic organisms has been documented (Plumb, 1997), several bacteria such as *Streptococcus iniae*, *Aeromonas hydrophila* and *Mycobacterium marinum* are posing major threats to this industry in the US. The economic loss from *S. iniae* infection alone was estimated as \$2 million annually (Ostland, 2003). Because there is no therapeutic agent approved by the US Food and Drug Administration or any commercial vaccines specifically for hybrid striped bass, research on dietary modulation of immunity and disease resistance is of special relevance. Sealey and Gatlin (2002a, b) demonstrated that dietary anti-oxidative vitamins (ascorbic acid and α -tocopherol) *in vivo* and *in vitro* modulated respiratory burst of hybrid striped bass macrophages; however, this beneficial influence did not lead to enhanced resistance against *S. iniae* (Sealey and Gatlin, 2002c). Therefore, further research on dietary supplementation strategies in hybrid striped bass is needed, not only to provide insights concerning interactions between nutrition and physiological responses but also to provide practical solutions to reduce basic risk from infectious diseases for the hybrid striped bass industry.

The objectives of the proposed research were:

1. To evaluate autolyzed brewers yeast, a commercial prebiotic, nucleotides and levamisole as individual dietary supplements for growth and disease management of hybrid striped bass.
2. To explore potential influences of dosage and long-term administration of these supplements.

3. To examine the *in vitro* effects of some soluble supplements such as levamisole on hybrid striped bass macrophage function.

CHAPTER II

EVALUATION OF BREWERS YEAST (*Saccharomyces cerevisiae*) AS A FEED SUPPLEMENT FOR HYBRID STRIPED BASS (*Morone chrysops* × *M. saxatilis*)*

1. Introduction

Hybrid striped bass production is considered to be the fastest growing segment of the U.S. aquaculture industry over the past decade and is poised to become a global seafood delicacy in the 21st century (Harrell and Webster, 1997; Kohler, 2000). However, one major constraint to hybrid striped bass aquaculture is suboptimal production efficiency stemming from intrinsically high sensitivity to various stressors and susceptibility to infectious agents during normal aquacultural production. Although viral diseases are not a primary threat to fish culture activity thus far, heavy economic loss may be caused by other pathogenic organisms, including bacteria, fungus and protozoan ectoparasites (Kohler, 2000).

In recent years, there have been growing concerns about the adverse effects of the bacterium *Streptococcus iniae* in the aquaculture of many economically important marine and freshwater fish species including rainbow trout (Eldar and Ghittino, 1999), Nile tilapia (Bowser et al., 1998), hybrid tilapia (Perera et al., 1997), yellowtail (Kaige et al., 1984), Japanese flounder (Nguyen et al., 2001a, b), hybrid striped bass (Sealey and Gatlin, 2002a), red drum (Eldar et al., 1999; Colorni et al., 2002), rabbitfish *Siganus*

* Reprinted from Aquaculture, Vol 219, Li, P., Gatlin, D. M. III, Evaluation of brewers yeast (*Saccharomyces cerevisiae*) for a feed supplement for hybrid striped bass (*Morone chrysops* × *M. saxatilis*), pp. 681-692, Copyright 2003, with permission from Elsevier Science.

(Yuasa et al., 1999) and even wild fishes (Colorni et al., 2002). This bacterium may cause heavy losses from mortality, reduced growth and unmarketable appearance in various fish species. Unfortunately, hybrid striped bass is one of the most susceptible fish to *S. iniae* infection. *S. iniae* is also associated with acute cellulitis in humans. Although some reports suggest that the fish and human *S. iniae* might be genetically different (Dodson et al., 1999), all reported occurrences of the human disease were associated with puncture wounds or abrasions and handling of infected fish or contaminated water (Greenlees et al., 1998). Therefore, an effective preventive strategy is not only needed to limit economical loss in aquaculture, but also to protect the health of aquaculturists and fish processing workers.

In aquaculture, traditional methods for treating infective pathogens include a limited number of government-approved antibiotics and chemotherapeutics. However, the disadvantages such as marginal effectiveness and high cost are obvious (Sealey and Gatlin, 2001). These treatments also may cause the accumulation of chemicals in the environment and/or fish, thus posing potential threats to consumers and the environment. An alternative strategy, besides vaccine development, is nutritional modulation of immune responses and disease resistance of aquaculture species. Research on the subject of nutritional modulation, especially evaluation of natural extracts or synthetic compounds, which may enhance the immune responses and disease resistance of hybrid striped bass, is still in its infancy. In the United States, because there is no therapeutic approved by the U.S. Food and Drug Administration specifically for hybrid striped bass, research on this topic is of special urgency.

Yeast by-products from the brewing industry are natural diet additives that have been shown to positively influence non-specific immune responses (Siwicki et al., 1994 and Anderson et al., 1995) as well as growth (Rumsey et al., 1991; Oliva-Teles and Goncalves, 2001) of some fish species. However, yeast products have not been investigated with hybrid striped bass. In addition, doses and time of administration have been recognized to have important effects on immunostimulant function, and efficacy of oral administration of immunostimulants has been reported to decrease over time (Sakai, 1999). The present study was conducted to determine the effects of graded levels of brewers yeast *Saccharomyces cerevisiae* on growth performance, body composition and resistance to *S. iniae* infection in hybrid striped bass. In addition, the efficacy of long-term oral administration of brewers yeast was explored by comparing various immune responses.

2. Materials and Methods

2.1. Experiment 1

An initial feeding trial was conducted to evaluate growth performance of hybrid striped bass fed graded levels of brewers yeast. The basal diet of Keembiyehetty and Gatlin (1997), which utilized menhaden meal as the protein source, was modified to contain 40% protein, 10% lipid and an estimated digestible energy level of 3.5 kcal/kg (Table 1). This diet satisfied and/or exceeded all known nutrient requirements of hybrid striped bass (Gatlin, 1997) or other warmwater fishes (National Research Council, 1993). Dried brewers yeast (Brewtech[®]) was supplied by International Ingredient Corporation (St. Louis, MO, USA). Three incremental levels (1%, 2% and 4% of diet) were added to

Table 1. Composition of experimental diets in experiment 1

Constituent	Basal Diet	Yeast Supplementation (%)		
		1	2	4
Ingredient (% dry weight)				
Menhanden fish meal ^a	59.0	59.0	59.0	59.0
Dextrin ^b	22.5	22.5	22.5	22.5
Menhaden oil ^a	3.7	3.7	3.7	3.7
Mineral premix ^c	3.0	3.0	3.0	3.0
Vitamin premix ^c	4.0	4.0	4.0	4.0
Carboxymethyl cellulose ^b	2.0	2.0	2.0	2.0
Cellulose ^b	5.8	4.8	3.8	1.8
Brewers yeast ^d	0	1	2	4
Proximate analysis (% dry matter)				
Dry matter	88.5	92.6	92.4	92.5
Crude protein (N×6.25)	39.8	40.8	40.7	42.1
Crude lipid	11.2	10.4	10.8	10.4
Ash	14.2	14.0	14.5	14.4

^a Omega Protein Corporation, Reedville, VA. Menhaden fish meal contained 67.8%

protein and 10.7% lipid at a dry-weight basis.

^b US Biochemical Corp., Cleveland, OH.

^c Same as Gaylord and Gatlin (2000)

^d International Ingredient Corporation, St. Louis, MO. Brewers yeast contained 50.7% crude protein and 2% crude lipid (dry weight basis).

the control diet in place of cellulose. Procedures for diet preparation and storage were as previous described (Rawles and Gatlin, 1998).

Juvenile hybrid striped bass (*Morone saxatilis*×*M. chrysops*) were obtained from a commercial supplier (Keo Fish Farm, Keo, AR) and maintained indoors at the Texas A&M University Aquacultural Research and Teaching Facility prior to the feeding trial. Fish were then graded by size and groups of 10 fish with a total weight of 253±5 g/group were stocked into 110-l aquaria. The basal diet was fed to all fish in 110-l aquaria during a 1-week conditioning period. Water flow rate were maintained at approximately 650 ml/min via a recirculating system, which maintained water quality through mechanical and biological filtration (Sealey and Gatlin, 2002b). Salinity was maintained at 2.5–3.5‰ using well water and synthetic sea salt (Fritz Industries, Dallas, TX, USA). Low-pressure electrical blowers provided aeration via air stones and maintained dissolved oxygen (DO) levels at or near saturation. Water temperature was at 26±1 °C throughout the trial and a 12-h light:12-h dark photoperiod was maintained with fluorescent lights controlled by timers.

Each diet was fed to fish in triplicate groups at 3% of body weight daily, except during the last week of the trial, in which feeding rate reduced to 2.5% of body weight. The feeding trial was conducted for 6 weeks. Group weights of fish in each aquarium were obtained weekly and feed amounts adjusted accordingly. At the end of both feeding trials, three representative fish from each aquarium were anesthetized with tricaine methane sulfonate (MS-222), and approximately 0.5 ml of blood was collected from the caudal vasculature using a 1-ml syringe and 27-gauge needle for hematocrit determination.

2.2. Experiment 2

The second feeding trial was conducted to further evaluate growth responses of hybrid striped bass fed graded levels of brewers yeast as well as some of their immune responses. The basal diet formulation was the same as one in Experiment 1. Three incremental levels of dried brewers yeast (1%, 2% and 4% of diet) were added to the basal diet and cellulose, menhaden meal and menhaden oil were adjusted to provide isonitrogenous and isolipidic diets (Table 2).

Prior to initiation of this feeding trial, juvenile hybrid striped bass obtained from Keo Fish Farm were subjected to a 2-week conditioning period to adjust to standardized regimes in a recirculating culture system consisting of 38-l aquaria (Gaylord and Gatlin, 2000). Water was maintained in the system at 25 ± 1 °C and was provided to each aquarium at a rate of 500 ml/min. Salinity was maintained to 1.5–2‰. Optimal water quality ($DO\geq 6$ mg/l, total ammonia nitrogen ≤ 0.3 mg/l) was maintained by biofiltration and aeration as in Experiment 1. Groups of 15 juvenile hybrid striped bass weighing approximately 9.7 g/fish were stocked into individual aquaria such that initial weight averaged 142 ± 5 g/group. Each experimental diet was fed to four replicate groups of fish for 8 weeks. All groups were fed their respective diets at the same fixed rate (initially 5% of body weight per day and gradually reduced to 3%). This rate was adjusted each week to maintain a level approaching apparent satiation. Fish were fed in the morning and evening, 7 days each week. Growth and feed efficiency were monitored weekly by collectively weighing each group of fish.

At the end of this feeding trial, three representative fish from each aquarium were anesthetized with MS-222, and blood collected as previously described for Experiment 1.

Table 2. Composition of experimental diets in experiment 2

Constituent	Basal Diet	Yeast Supplementation (%)		
		1	2	4
Ingredient (% dry weight)				
Menhaden fish meal ^a	57.9	57.2	56.4	55.0
Dextrin ^b	24.9	25.1	25.0	24.9
Menhaden oil ^a	2.4	2.4	2.5	2.7
Mineral premix ^c	3.0	3.0	3.0	3.0
Vitamin premix ^c	4.0	4.0	4.0	4.0
Carboxymethyl cellulose ^b	2.0	2.0	2.0	2.0
Cellulose ^b	5.8	5.3	5.1	4.4
Brewers yeast ^d	0	1.0	2.0	4.0
Proximate analysis (% dry matter)				
Dry matter	89.6	88.5	88.6	88.8
Crude protein (N×6.25)	40.4	40.5	40.7	40.7
Crude lipids	9.6	9.0	9.5	9.2
Ash	14.2	14.4	14.4	14.2

^a Omega Protein Corporation, Reedville, VA. Menhaden fish meal contained 69% protein and 13.2% lipid at a dry-weight basis.

^b US Biochemical Corp., Cleveland, OH.

^c Same as Gaylord and Gatlin (2000)

^d International Ingredient Corporation, St. Louis, MO. Brewers yeast contained 50.7% crude protein and 2% crude lipid (dry weight basis).

After a sample of whole blood was taken for hematocrit determination, serum was isolated by centrifugation ($3000\times g$ for 5 min) and kept at $-80\text{ }^{\circ}\text{C}$ for lysozyme assay (Engstad et al., 1992). After bleeding, the fish were frozen for whole body composition analysis (Rawles and Gatlin, 1998).

An additional 30 fish previously fed each of the experimental diets for a total of 9 weeks were exposed to an estimated LD_{50} dose of *S. iniae*. A virulent isolate of *S. iniae* originally obtained from the brain of an infected tilapia (*Oreochromis* sp.) was biochemically identified and provided by the Texas Veterinary Medicine Diagnostic Laboratory. The bacterial suspension was prepared according to the method described by Sealey and Gatlin (2002b) and diluted to a concentration of 2.6×10^5 CFU/ml in fresh well water. Thirty fish from each dietary treatment, pooled separately in mesh baskets, were immersed in the bacterial suspension for 2 h. After bacterial exposure, the 30 fish from each dietary treatment were divided into three groups of 10 and placed into 38-l aquaria in an isolated culture system that received a constant supply of well water at $25\pm 1\text{ }^{\circ}\text{C}$. Fish continued to be fed their respective diets to apparent satiation twice daily and mortality was monitored for 2 weeks. The brains of dead fish were streaked on modified selective agar (Nguyen and Kanai, 1999) to confirm death from *S. iniae*.

After the second feeding trial and diseases challenge, additional fish continued to be fed each experimental diet to apparent satiation for a total of 16 weeks. After this extended feeding trial period, four fish from each treatment were bled from the caudal vasculature. Neutrophil oxidative radical production was determined following the procedure described by Siwicki et al. (1994). Absorbance was converted to Nitro Blue Tetrazolium (NBT) units based on a standard curve of NBT diformazan per milliliter of

blood. Serum samples were separated as described above for lysozyme assay (Engstad et al., 1992). Also, after 16 weeks, eight fish per treatment were euthenized and head kidney samples were taken for macrophage isolation and assay of extracellular and intracellular superoxide anion. This assay followed the procedure of Sealey and Gatlin (2002a). The amount of extracellular superoxide anion was calculated from the formula: nmol superoxide anion/well=(Δ absorbance after 60 min \times 100)/6.3 (Pick and Mizel, 1981).

Data from both feeding trials and the bacterial challenge were subjected to analysis of variance and Duncan's multiple range test using the Statistical Analysis System (SAS, 1985). Differences in treatment means were considered significant at $P<0.05$.

3. Results

3.1. Experiment 1

In Experiment 1, hybrid striped bass fed the diets supplemented with 1% and 2% Brewtech dried brewers yeast had up to 20% more weight gain compared to fish fed the basal diet during the course of the feeding trial (Table 3). However, fish fed the diet supplemented with 4% brewers yeast had weight gain similar to fish fed the basal diet and responses of fish fed the various diets were not significantly different. One replicate group of fish fed the control diet also was excluded from analysis because of mortality experienced during weighing due to toxicity from net disinfectant. Survival and hematocrits of fish in all other replicates were high and not affected by diet (Table 3).

Table 3. Performance of hybrid striped bass fed diets containing various amounts of dried brewers yeast for 6 weeks in experiment 1¹

Dietary brewers yeast (%)	Weight gain (% of initial wt ²)	Feed efficiency (g gain/g feed)	Survival (%)	Hematocrit (%)
0	86	0.41	85	37.7
1	104	0.54	96.7	44.4
2	98	0.51	93.3	39.3
4	85	0.48	100	37.6
Pr > F ³	0.26	0.15	0.24	0.28
pooled se	8.51	0.038	3.97	5.23

¹Values represent means of three replicate groups except the basal diet that had two replicate groups.

² Fish initially averaged 25.3 g.

³ Significance probability associated with the F statistic

3.2. Experiment 2

In Experiment 2, weight gain, feed efficiency and survival of fish fed all experimental diets were excellent (Table 4). In this experiment, however, dietary effects on weight gain tended to be significant at $P < 0.1$, and fish fed the diets with 1% and 4% brewers yeast had the highest gain. Hematocrits of fish fed the various diets were not significantly affected by diet. Serum lysozyme was highly variable in hybrid striped bass in Experiment 2 (Table 4), with no significant effects of dietary yeast supplementation observed. Whole body composition of hybrid striped bass was not significantly affected by the dietary supplementation of brewers yeast in Experiment 2 (Table 5).

3.3. *S. iniae* challenge

The controlled exposure of hybrid striped bass to a virulent strain of *S. iniae* at the end of the feeding trial in Experiment 2 resulted in limited mortality after the challenge (Fig. 1). Fish fed diets with 2% and 4% Brewtech brewers yeast did not experience mortality, while 20% and 10% mortality was observed in fish fed the control diet and 1% brewers yeast supplemented diet, respectively, although differences in survival were not statistically significant.

3.4. Immune responses after long-term administration

After 16 weeks of feeding the diet in Experiment 2, neutrophil oxidative radical production, serum lysozyme, intracellular and extracellular superoxide anion of head kidney phagocytic cells were tested (Table 6). Significant differences ($P < 0.01$) in

Table 4. Performance of hybrid striped bass fed diets containing various amounts of dried brewers yeast for 8 weeks in experiment 2¹

Dietary brewers yeast (%)	Weight gain (% of initial wt)	Feed efficiency (g gain/g feed)	Survival (%)	Hematocrit (%)	Lysozyme (10 ³ Units/L)
0	396	0.91	98.3	44.6	939
1	418	0.96	98.3	49.4	811
2	388	0.93	98.3	44.1	811
4	413	0.93	93.3	45.8	712
Pr > F ²	0.10	0.19	0.24	0.36	0.50
pooled se	8.14	0.013	3.97	2.08	165

¹ Values represent means of four replicate groups.

² Fish initially averaged 9.7g.

³ Significance probability associated with the F statistic.

Table 5. Body composition of hybrid striped bass fed diets containing various amounts of dried brewers yeast for 8 weeks in experiment 2¹

Dietary brewers yeast (%)	Moisture (%)	% Fresh weight		
		Protein	Lipid	Ash
0	68.9	17.1	8.4	4.1
1	69.2	17.2	7.8	4.3
2	68.1	17.6	7.8	4.5
4	69.4	17.1	8.3	4.2
Pr > F ²	0.512	0.057	0.516	0.250
pooled se	0.14	0.37	0.16	0.064

¹Values represent means of composite samples of three fish from each of four replicate groups

except the basal diet and 2% brewers yeast supplementation group that had three replicate groups.

²Significance probability associated with the F statistic.

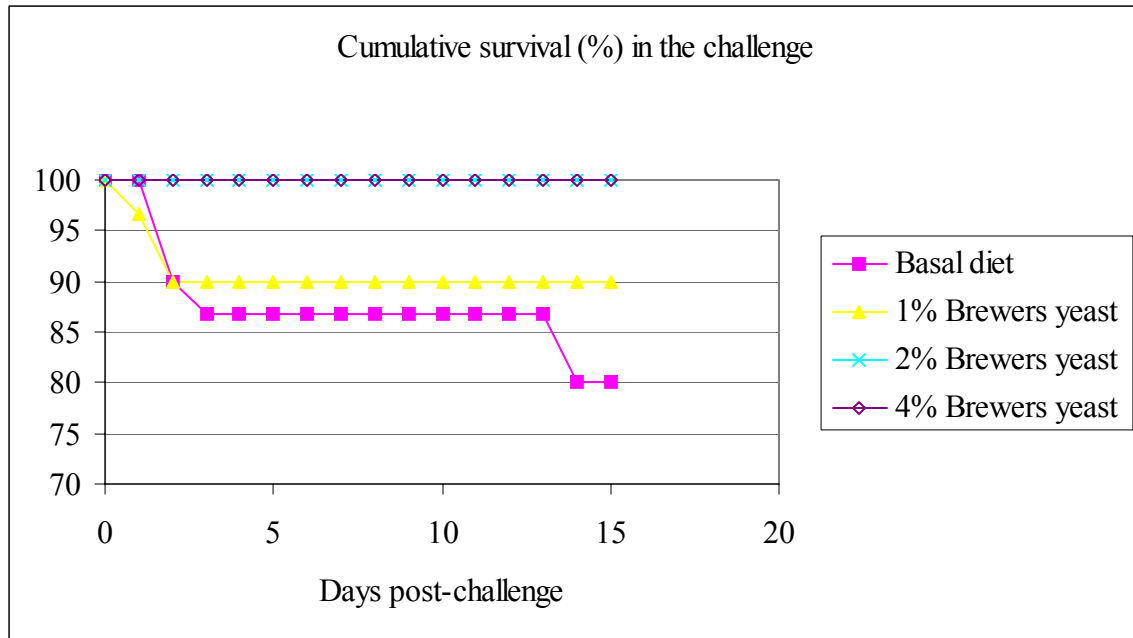


Fig. 1. Percent cumulative survival rate of hybrid striped bass fed incremental levels of brewers yeast (1, 2 and 4%) for 9 weeks and subsequently exposed to *S. iniae* bath ($P = 0.1$). Symbols represent means of three replicate tanks per treatment. Pooled SE was 5.77.

neutrophil oxidative and extracellular superoxide anion of head kidney phagocytic cells were observed among fish fed control diet and yeast-supplemented diets.

4. Discussion

Single cell proteins, including yeast and bacteria, have been viewed as promising substitutes for fishmeal in fish diets. Researchers have evaluated the nutritional value of brewers yeast *S. cerevisiae* in lake trout (Rumsey et al., 1990), rainbow trout (Rumsey et al., 1991) and sea bass (Oliva-Teles and Goncalves, 2001) by comparing growth performance, feed efficiency, liver uricase and nitrogen retention. Based on these studies, brewers yeast could replace up to 25–50% of fish meal protein without adversely affecting growth of these species. In the present study, brewers yeast was evaluated for its potential as an immunostimulant at relatively low inclusion levels.

In Experiment 1, the fish were not in optimal condition as reflected in chronic, low-level mortality (as high as 15%). Dietary supplementation of brewers yeast, especially at 1%, tended to improve the growth and health of fish in that experiment. In Experiment 2, weight gain, feed efficiency and survival of fish fed all diets were excellent. The fishes' high state of health and optimal environmental conditions may have limited potential expression of dietary effects on fish performance.

Hemocrits of fish fed the various diets in Experiments 1 and 2 were variable, but within normal ranges (Hrubec et al., 2001; Sealey and Gatlin, 2002c) and highest for fish fed 1% brewers yeast. Differences in fish size may have accounted for some of the differences in hematocrit values observed between experiments. Serum lysozyme, which

Table 6. Blood neutrophil oxidative radical production (NBT test), serum lysozyme, intracellular superoxide anion production of head kidney macrophages and extracellular superoxide anion production of hybrid striped bass after long-term (16 week) oral administration of graded levels of brewers yeast in experiment 2¹

Dietary brewers yeast (%)	NBT test (mg ml ⁻¹)	Lysozyme (unit ml ⁻¹)	Intracellular superoxide anion (O.D. at 620 nm)	Extracellular superoxide anion (nmol O ₂)
0	1.99 ^c	1246.7	0.897	2.679 ^c
1	2.59 ^{ab}	1073.3	1.039	3.694 ^b
2	3.09 ^a	965	1.293	4.511 ^{ab}
4	2.31 ^{bc}	1155	1.091	4.86 ^c
Pr > F ²	0.004	0.19	0.466	0.006
pooled se	0.083	0.013	0.062	0.123

¹Values in a column that do not have the same superscript are significantly different at P ≤ 0.05 based on Duncan's multiple range test.

²Significance probability associated with the F statistic.

is one measure of non-specific immunity, was highly variable in hybrid striped bass in both experiments. The average lysozyme levels in the present study were within the range reported by Sealey and Gatlin (2002c).

Although Streptococciosis is attracting more attention, information on effect of nutrition on resistance to this disease is limited. Matsuyama et al. (1992) observed intraperitoneal injection of β -1,3-glucan derived from *Schizophyllum commune* and *Sclerotium glaucanicum* could significantly enhance the percent survival of yellowtail after *Streptococcus* sp. challenge. However, for the present, few strategies other than vaccination have been proven to enhance resistance to *S. iniae* infection of any fish species. There is some evidence that antibiotic treatment has been ineffective (Klesius et al., 2000). After exposure to *S. iniae* in Experiment 2, hybrid striped bass fed diets with 2% and 4% brewers yeast did not experience mortality, while 20% mortality was observed in fish fed the control diet. Constant disease signs were observed including dermal hemorrhages around the mouth, base of fins and anus, erratic swimming, dark skin pigmentation and slow acceptance or refusal of feed, which are similar to those of tilapia described by Perera et al., 1994 and Perera et al., 1997 and Evans et al. (2000). However, noticeable better feeding and less dark skin pigmentation were generally observed in fish fed diets with 2% and 4% yeast supplementation.

Some reports (Matsuo and Miyazano, 1993; Sakai, 1999) have indicated that long-term oral administration of immunostimulants to fish may induce immunosuppression. To determine this phenomenon in hybrid striped bass, after 16 weeks of feeding, non-specific immune responses including blood neutrophil oxidative radical production, serum lysozyme, extracellular and intracellular superoxide anion of

head kidney macrophages were tested. Significant differences ($P < 0.01$) in blood neutrophil oxidative radical production and extracellular superoxide anion of head kidney phagocytic cells were observed among fish fed the basal diet and yeast-supplemented diets. These results confirm early reports (Siwicki et al., 1994; Anderson et al., 1995) on effects of dietary brewers yeast on immune responses including neutrophil oxidative radical production and serum immunoglobulin level in rainbow trout. No significant differences of serum lysozyme and intracellular superoxide anion of phagocytic cells were observed among fish. The intracellular superoxide anion and extracellular superoxide anion of head kidney phagocytic cells of fish fed the basal diet were observed to be similar to that of Sealey and Gatlin, 2002b and Sealey and Gatlin, 2002c.

Brewers yeast is a source of nucleic acids and polysaccharides including glucans. β -1,3-glucans have been recognized to effectively enhance immune functions of many aquaculture species including African catfish (Yoshida et al., 1995), Atlantic salmon (Engstad et al., 1992), rainbow trout (Jorgensen et al., 1993; Siwicki et al., 1994) and shrimp *Penaeus monodon* (Thanardkit et al., 2002). β -1,3-Glucan is generally viewed as the factor in brewers yeast with a known immunological mechanism (Gannam and Schrock, 2001). Sakai et al. (2001) reported that the nucleotides from brewers yeast RNA were capable of enhancing the phagocytic and oxidative activities of kidney phagocytic cells, serum lysozyme in common carp as well as resistance to *Aeromonas hydrophila*. Burrells et al. (2001) also reported that dietary nucleotides, extracted from brewers yeast, could enhance resistance to various pathogenic infections in Atlantic salmon. However, the extent to which RNA in brewers yeast contributes to the beneficial influences of

dietary brewers yeast on immune responses and resistance to *S. iniae* infection of hybrid striped bass is not clear.

It was concluded that brewers yeast positively influenced growth performance and feed efficiency of hybrid striped bass as well as resistance to *S. iniae* infection. In addition, results of immune response assays demonstrate that brewers yeast can be administered for relatively long periods without causing immunosuppression.

CHAPTER III

DIETARY BREWERS YEAST AND THE PREBIOTIC GROBIOTIC™AE INFLUENCE GROWTH PERFORMANCE, IMMUNE RESPONSES AND RESISTANCE OF HYBRID STRIPED BASS (*Morone chrysops*×*M. saxatilis*) to *Streptococcus iniae* INFECTION*

1. Introduction

Hybrid striped bass production is considered to be the fastest growing segment of the U.S. aquaculture industry over the past decade and has spread to several countries and regions in Europe and Asia (Harrell and Webster, 1997; Hiney et al., 2002). It is known that most of the common disease-causing organisms associated with various aquacultured fish also may affect cultured hybrid striped bass (Plumb, 1997). In recent years, *Streptococcus iniae* has become one of the most threatening pathogenic organisms in hybrid striped bass aquaculture and caused about \$ 2 million of economic loss to USA hybrid striped bass producers in 2002 (Ostland, 2003). Although positive results have come from experimental treatment of this disease in tilapia with oxytetracycline (Darwish et al., 2002) and in hybrid striped bass with enrofloxacin (Stoffregen et al., 1996), these drugs are still under investigation and currently not approved by the U.S. Food and Drug Administration to treat bacterial diseases of hybrid striped bass.

Proper nutrition has long been recognized as a critical factor in promoting normal

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growth and sustaining health of fish. Prepared diets not only provide the essential nutrients that are required for normal physiological functioning but also may serve as the medium by which fish receive other components that may affect their health (Gatlin, 2002). Although the concept of functional feeds is novel to the aquaculture industry, it represents an emerging new paradigm to develop diets that extend beyond satisfying basic nutritional requirements of the cultured organism. For hybrid striped bass, dietary requirements for the most essential nutrients have been established to provide balanced diets than satisfy metabolic needs (Gatlin, 1997; Webster, 2002); however, research on optimization of diets to enhance health is still in its infancy. Probiotics, live microbes that may serve as dietary supplements to improve the intestinal microbial balance have received some attention in aquaculture (Gatesoupe, 1999; Irianto and Austin, 2002). Evidence of the beneficial effects of probiotics gave birth to the concept of prebiotics (Gibson and Roberfroid, 1995; Teitelbaum and Walker, 2002), which are defined by Gibson and Roberfroid (1995) as “a nondigestible food ingredient which beneficially affects the host by selectively stimulating the growth of and/or activating the metabolism of one or a limited number of health-promoting bacteria in the intestinal tract, thus improving the host’s intestinal balance”. Examples of prebiotics include mannan oligosaccharides (White et al., 2002), lactose (Szilagyi, 2002), as well as oligofructose and inulin (Teitelbaum and Walker, 2002). Information pertaining to prebiotics in aquaculture is extremely limited.

Brewers yeast (*Saccharomyces cerevisiae*) is a natural product from the brewing industry that contains various immunostimulating compounds such as β -glucans, nucleic acids as well as mannan oligosaccharides, and has been used as a diet additive for various

animals. It has been observed to be capable of enhancing immune responses (Siwicki et al., 1994; Ortuño et al., 2002) as well as growth (Lara-Flores et al., 2002) of various fish species and thus may serve as an excellent health promoter for fish culture. Li and Gatlin (2003) established the beneficial effects of partially autolyzed brewers yeast on immune responses of hybrid striped bass and resistance to *Streptococcus iniae* infection.

Grobiotic™AE is a commercial prebiotic mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products. The present study was conducted to determine the effects of graded levels of the prebiotic, Grobiotic™AE, on hybrid striped bass growth performance, immune responses and resistance to *Streptococcus iniae* infection as compared with brewers yeast.

2. Materials and Methods

2.1 Experimental Diets

The basal diet of Keembiyehetty and Gatlin (1997), which utilized menhaden fish meal as the protein source, was modified to contain 40% protein, 10% lipid and an estimated digestible energy level of 3.5 kcal/kg (Table 7). This diet satisfied and/or exceeded all known nutrient requirements of hybrid striped bass (Gatlin, 1997; Webster, 2002) or other warmwater fishes (National Research Council, 1993). Partially autolyzed brewers yeast (Brewtech®) and Grobiotic™AE, a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products, were supplied by International Ingredient Corporation (St. Louis, MO, USA). Two incremental levels (1 and 2% of diet) of Grobiotic™AE and brewers yeast were added to the basal diet and cellulose, menhaden meal and menhaden oil were adjusted to provide isonitrogenous and

Table 7. Composition of experimental diets in feeding trials 1 and 2

Constituent	Basal diet	1% Brewers yeast	2% Brewers yeast	1% Grobiotic™AE	2% Grobiotic™AE
<i>Ingredient (% dry weight)</i>					
Menhaden fish					
meal ^a	59.2	58.2	57.5	58.7	58.3
Dextrin ^b	25.0	25.0	22.5	24.5	23.9
Menhaden oil ^a	4.4	4.5	4.5	4.4	4.4
Mineral premix ^c	4.0	4.0	4.0	4.0	4.0
Vitamin premix ^c	3.0	3.0	3.0	3.0	3.0
Carboxymethyl					
cellulose ^b	2.0	2.0	2.0	2.0	2.0
Cellulose ^b	2.4	2.3	4.5	2.4	2.4
Brewers yeast ^e	0	1.0	2.0	0	0
Grobiotic™AE ^e	0	0	0	1.0	2.0
<i>Analyzed proximate composition (% dry matter)^f</i>					
Dry matter	92.1	92.7	92.6	92.1	90.4
Crude protein	40.8	41.9	41.8	39.2	40.5
(N×6.25)					
Crude lipid	8.5	9.9	10.3	8.7	8.6
Ash	14.5	14.4	15.6	14.4	13.9

^a Omega Protein Corporation, Reedville, VA. Menhaden fish meal contained 67.6% protein and 9.5% lipid on a dry-weight basis.

^b US Biochemical Corp., Cleveland, OH.

^c Same as Gaylord and Gatlin (2000).

^d International Ingredient Corporation, St. Louis, MO. Contained 50.7% crude protein and 2% crude lipid (dry-weight basis).

^e International Ingredient Corporation, St. Louis, MO. Contained 30.0 % crude protein and 1.2 % crude lipid (dry-weight basis).

^f means of two analyses.

isolipidic diets (Table 7).

2.2 Feeding trial 1

Juvenile hybrid striped bass (*Morone chrysops* × *M. saxatilis*) were obtained from a commercial supplier (Keo Fish Farm, Keo, AR) and maintained indoors at the Texas A&M University Aquacultural Research and Teaching Facility prior to the feeding trial. Fish were then graded by size and groups of 13 fish with a total weight of 91.4 ± 4.3 g per group were stocked into 110-l aquaria. The basal diet was fed to all fish during a 2-wk conditioning period. Water flow rate was maintained at approximately 650 ml/min via a recirculating system which maintained adequate water quality (total ammonia nitrogen ≤ 0.6 mg/l) through biological and mechanical filtration (Li and Gatlin, 2003). Salinity was maintained at 2.5-3.5 ‰ using well water and synthetic sea salt (Fritz Industries Inc., Dallas, TX). Low pressure electrical blowers provided aeration via air stones and maintained dissolved oxygen (DO) levels at or near saturation. Water temperature was controlled by ambient air and remained at 26 ± 1 °C throughout the trial. A 12 h light: 12h dark photoperiod was maintained with fluorescent lights controlled by timers. Each experimental diet was fed to five replicate groups of fish for 7 weeks. All groups were fed their respective diets at the same fixed rate (initially 5% of body weight per day and gradually reduced to 3%). This rate was adjusted each week to maintain a level approaching apparent satiation. Fish were fed in the morning and evening, 7 days each week. Growth and feed efficiency were monitored weekly by collectively weighing each group of fish.

2.3 Feeding trial 2

A second feeding trial was conducted to further evaluate immune responses and disease resistance of hybrid striped bass after feeding experimental diets for 4 weeks. Groups of 17 juvenile hybrid striped bass weighing approximately 19.7 g/fish were stocked into individual aquaria such that initial fish weight averaged 334 ± 3 g/group. These fish were raised in the same culture system as feeding trial 1. Each experimental diet was fed to three replicate groups of fish for 4 weeks. All groups were fed their respective diets at the same fixed rate (initially 4% of body weight per day and gradually reduced to 3.5%). Growth and feed efficiency were monitored weekly by collectively weighing each group of fish.

2.4 Sample collection and analysis

At the end of the second feeding trial, two representative fish from each aquarium were anesthetized with tricaine methane sulfonate (MS-222), and approximately 0.5 ml of blood was collected from the caudal vasculature using a 1-ml syringe and 27-gauge needle. Whole blood neutrophil oxidative radical production was determined as described by Siwicki et al. (1994). Absorbance was converted to Nitro Blue Tetrazolium (NBT) units based on a standard curve of NBT diformazan/ml blood. Serum lysozyme activity was determined by turbidimetric assay as described by Jørgensen et al. (1993). A lysozyme activity unit was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min^{-1} . Three fish from each aquarium also were euthanized and head kidney samples were pooled for macrophage isolation and assay of extracellular and intracellular superoxide anion. This assay followed the procedure of Secombes (1990), as

modified by Sealey and Gatlin (2002a). The amount of extracellular superoxide anion was calculated from the formula of Pick and Mizel (1981).

2.5 Bacterial challenge

At the end of the second trial, an additional 30 fish previously fed each experimental diet were exposed to an estimated LD₅₀ dose of *Streptococcus iniae*. Before inoculation, this isolate of *S. iniae* was injected into hybrid striped bass to improve virulence, then reisolated by the Texas Veterinary Medical Diagnostic Laboratory. This isolate was grown in brain-heart infusion broth (EM Science, Darmstadt, Germany) in a shaking bath at 27 °C overnight as described by Sealey and Gatlin (2002b). The concentration of bacterial suspension was determined by the serial plate count method and diluted to 9.3×10^5 CFU/ml in fresh well water. Thirty fish from each treatment (10 fish from each aquarium of each treatment), pooled separately in mesh baskets, were immersed in the bacterial suspension for 2 hours. After bath exposure, 30 fish from each dietary treatment were divided into three groups of 10, and placed into 38-l flow-through aquaria in an isolated culture system. Water temperature was maintained at 27 ± 1 °C with immersion heaters. Fish continued to be fed their respective diets to apparent satiation twice daily, and mortality was monitored for 19 days. The brains of both dead fish and surviving fish were streaked on modified selective agar to determine infection status and confirm death from *S. iniae* (Nguyen and Kanai, 1999).

2.6 Statistics

Data from the feeding trials, immune response assays and the bacterial

challenge were subjected to analysis of variance and Duncan's multiple-range test using the Statistical Analysis System. Differences in treatment means were considered significant at $P < 0.05$.

3. Results

3.1 Feeding trial 1

After the 7-week feeding period, enhanced weight gain was generally observed in fish fed diets supplemented with 1 and 2% GrobioticTMAE compared to those fed the basal diet, although only feed efficiency was significantly ($P < 0.05$) improved (Table 8). Survival during feeding trial 1 was high and no significant differences were observed among treatments.

3.2 Feeding trial 2

No significant differences in growth, feed efficiency and survival were observed in fish fed experimental diets after 4 weeks. Neutrophil oxidative radical production of fish fed brewers yeast and GrobioticTMAE tended ($P = 0.15$) to be higher than that of fish fed the basal diet (Table 9). Serum lysozyme was highly variable, with no significant differences observed among the various dietary treatments (Table 9). Intracellular superoxide anion production of head kidney macrophages was not affected by the dietary factors in this experiment; however, extracellular superoxide anion production was observed to be significantly higher in fish fed diets with brewers yeast and 1% GrobioticTMAE (Table 9).

Table 8. Performance of hybrid striped bass fed diets containing various amounts of dried brewers yeast and Grobiotic™AE for 7 weeks in feeding trial 1^a

Diet	Weight gain (% of initial wt ^b)	Feed efficiency (g gain/g feed)	Survival (%)
Basal	388	0.93 ^b	92.3
1% Brewers yeast	388	0.95 ^{ab}	100
2% Brewers yeast	404	0.95 ^{ab}	93.8
1% Grobiotic™AE	420	1.0 ^a	95.4
2% Grobiotic™AE	405	1.0 ^a	96.9
Pr > F ^c	0.45	0.05	0.28
pooled se	5.97	0.21	1.20

^a Values represent means of five replicate groups. Values in a column that do not have the same superscript are significantly different at $P \leq 0.05$ based on Duncan's multiple range test.

^b Fish initially weighed 7.0 ± 0.3 g each.

^c Significance probability associated with the F statistic

Table 9. Neutrophil oxidative production (NBT test), serum lysozyme, extracellular and intracellular superoxide anion production of head kidney macrophages of hybrid striped bass fed experimental diets in feeding trial 2^a

Diet	NBT test (mg ml ⁻¹) ^b	Lysozyme (10 ³ Units/L) ^b	Extracellular superoxide anion (nmol O ₂) ^c	Intracellular superoxide anion (O.D. at 620 nm) ^c
Basal	1.20	473	7.21 ^c	1.28
1% Brewers yeast	1.40	515	11.22 ^a	1.55
2% Brewers yeast	1.58	472	10.55 ^a	1.66
1% Grobionic™ AE	1.28	448	9.71 ^{ab}	1.79
2% Grobionic™ AE	1.31	640	8.34 ^{bc}	1.59
P>F ^d	0.15	0.728	0.0008	0.67
pooled se	0.045	43.778	0.274	0.163

^a Values in a column that do not have the same superscript are significantly different at P≤0.05 based on Duncan's multiple range test.

^b Means of six samples of individual fish.

^c Means of three samples of pooled head kidney cells from 3 fish from each aquarium of the same treatment.

^d Significance probability associated with the F statistic.

3.3 Bacterial challenge

The disease challenge with live *S. iniae* resulted in approximately 50% mortality of fish fed the basal diet after 19 days. Survival of fish fed diets containing Grobiotic™AE or brewers yeast was significantly ($P < 0.01$) higher than fish fed the basal diet after the same period (Fig. 2). The moribund fish showed typical symptoms of *S. iniae* including extremely erratic swimming, cloudiness in eyes, hemorrhages around mouth and base of fins, dark pigmentation and slow acceptance or refusal of feed. No *S. iniae* was isolated from the brain of surviving fish, while colonies of *S. iniae* were isolated from dead fish.

4. Discussion

The concept of prebiotics arose from the observation that inulin and oligofructose selectively stimulate the growth of bifidobacteria, which are considered to be beneficial to human health (reviewed by Blaut, 2002; Teitelbaum and Walker, 2002). Other substances including mannan oligosaccharides (White et al., 2002) and lactose (Szilagy, 2002) also have been reported to possess prebiotic functions in humans and/or terrestrial animals. Such information on prebiotics in aquatic organism is very limited. Olsen et al. (2001) observed that a diet supplemented with 15% inulin caused harmful effects to Arctic charr. However, their previous studies (Ringø et al., 1998; Ringø and Olsen, 1999) showed that dietary fatty acids and carbohydrates altered the bacterial flora of the gastrointestinal tract of fish. In this present study, the commercial prebiotic, Grobiotic™AE, significantly enhanced feed efficiency, immunological responses and resistance of hybrid striped bass to *Streptococcus iniae* infection. The beneficial influence

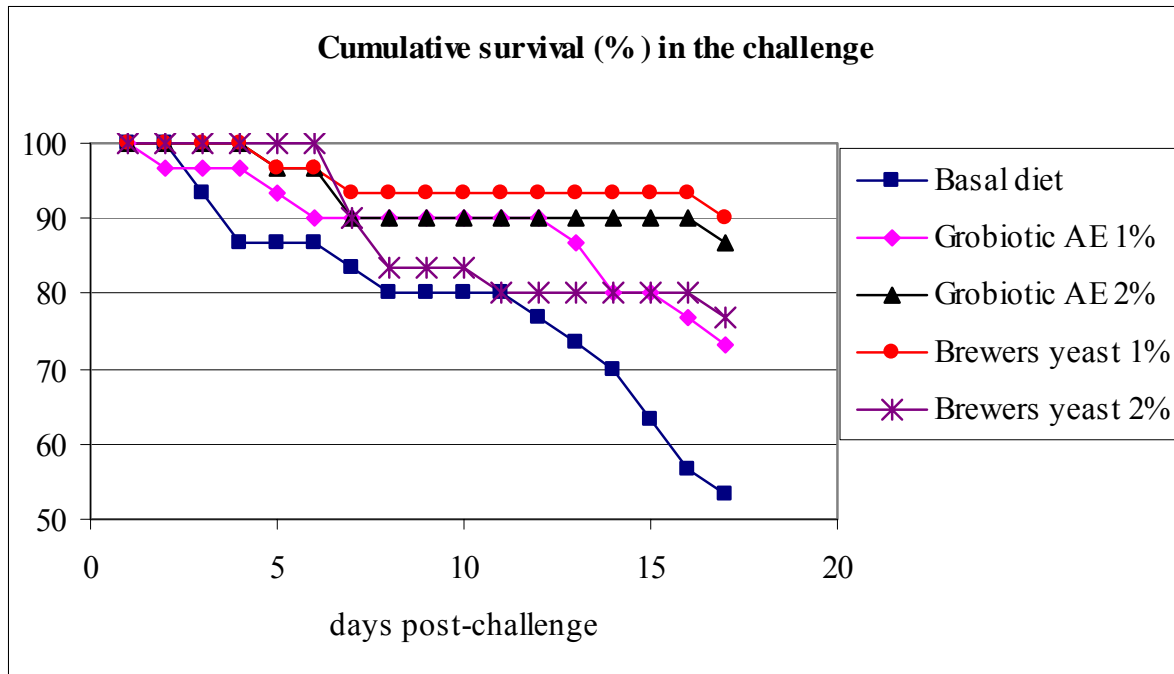


Fig 2. Percent cumulative survival rate of hybrid striped bass fed incremental levels of GrobioticTMAE and brewers yeast (1 and 2%) for 4 weeks and subsequently exposed to *S. iniae* bath ($P < 0.01$). Symbols represent means of three replicate tanks per treatment. Pooled SE was 3.10.

of Grobiotic™AE on growth was possibly due to alteration of the intestinal microflora by mannan oligofructose, lactose or other carbohydrates from the dairy ingredient, partially autolyzed yeast and/or dried fermentation products; however, a detailed study intestinal microbiology is needed. Lactic acid bacteria have been considered beneficial residents of the fish's intestinal ecosystem by producing bacteriocins, which inhibit growth of certain fish pathogens and thus positively affect the host's microflora (Ringo et al., 1998). Some reports have shown that lactose, as well as other prebiotic ingredients, may promote the maintenance of lactic acid-producing bacteria and prevent the expansion of potential pathogens in certain human diseases (Szilagyi, 2002). However, the hypothesis needs to be tested by further studies in fish.

Brewers yeast has been recognized to have potential as a substitute for live food in the production of certain fish (Nayar et al., 1998) or as a potential replacement for fish meal (Rumsey et al., 1991; Oliva-Teles and Goncalves, 2001). As a protein feedstuff, brewers yeast has been included in commercial diet formulations for several fish species, including salmonids (National Research Council, 1993). The cell walls of yeast also may provide very important non-nutritive compounds that may benefit fish health, including mannose polymers covalently linked to peptides (mannoprotein), glucose polymers (glucans), chitin in minor amounts (Cabib et al., 1982) as well as nucleic acids (Rumsey et al., 1992). The beneficial influence of glucans has been demonstrated with various fish species (Verlhac et al., 1998; Sahoo and Mukherjee, 2002; Couso et al., 2003). Enhanced immunological responses including respiratory burst also have been reported for dietary chitin (Esteban et al., 2001). According to Rumsey et al. (1992) and Cabib et al. (1982), yeast cells provide about 7.7% crude glucan and 1% chitin. It is known that glucan is

capable of enhancing innate immune responses, including respiratory burst of head kidney macrophages, serum complement activity and serum lysozyme (Engstad et al., 1992; Jørgensen et al., 1993) when administered by injection. However, the increase of serum lysozyme was not observed in fish orally administered glucan or chitin (Verlhac et al., 1998; Esteban et al., 2001; Ortuño et al., 2002), which agrees with results of the present study. Serum lysozyme activity of hybrid striped bass in the present study varied greatly among individual fish. A similar observation was reported in a previous study with hybrid striped bass after 8 and 16 week of feeding with diets supplemented with brewers yeast (Li and Gatlin, 2003). Jorgensen et al. (1993) observed rainbow trout injected with 1% β -1,3 -glucan showed significantly enhanced extracellular superoxide anion production of head kidney cells. Results of the present study showed that extracellular superoxide anion production of head kidney macrophages of hybrid striped bass also could be activated by oral administration of glucan and/or chitin from brewers yeast.

Nucleic acids, accounting for 12-20% of the total nitrogen of brewers yeast, are found primarily in the purine and pyrimidine bases of nucleoproteins (Rumsey et al., 1992). Rumsey et al. (1992) observed that supplementation of RNA extract from brewers yeast elevated the level of hepatic nucleic acids in rainbow trout and assumed that dietary nucleic acids could be incorporated into the tissue pool. However, detailed information on digestion and absorption of nucleic acids by fish is still limited. In recent years, a growing number of reports have indicated that oral administration of nucleotides is capable of enhancing immune responses and/or diseases resistance in several fish species, including hybrid striped bass (Burrells et al., 2001; Sakai et al., 2001). The extent to

which hybrid striped bass are capable of utilizing nucleic acids from brewers yeast and their contribution to enhancement of immune responses and disease resistance needs to be investigated further. In the present study, Grobiotic™AE and brewers yeast influenced immune response in very similar ways. The specific mechanism(s) by which these compounds exerted their beneficial influence remains unknown, although intestinal micro flora is recognized as a major determinant for the development of the immune system (Blaut, 2002).

Disease associated with *S. iniae* represents a serious health and economic problem in cultured fish species, with an annual economic loss estimated at greater than \$150 million world wide (Buchanan et al., 2003). In North America, the hybrid striped bass industry has been particularly impacted (Buchanan et al., 2003). In the present study, survival of hybrid striped bass fed brewers yeast and Grobiotic™AE after *S. iniae* challenge was significantly higher than fish fed the basal diet. This demonstrates the potential of dietary strategies in reducing the risk of disease, which is generally associated with aquaculture. Recent progress in identification of virulence genes of *S. iniae* and development of gene vaccines against this pathogenic bacterium for hybrid striped bass would contribute greatly to reducing its impact in aquaculture (Buchanan et al., 2003). Grobiotic™AE and brewers yeast also hold promise of promoting fish health and resistance to infection from other pathogenic organism. Fattal-German and Bizzini (1992) observed the antiviral property of oral administration of live yeast cells in mice. This phenomenon is also worth exploring in fish as well as challenging with other pathogens in future. With the increasing concerns about use of antibiotics in aquaculture, various pre- and pro-biotics that have shown potential as alternative treatments

(Nikoskelaiken et al., 2001; reviewed by Irianto and Austin, 2002) should receive further consideration. How to utilize dietary strategies and prebiotics to maximize the efficiency of probiotics is a promising subject and more research is warranted.

CHAPTER IV

EVALUATION OF THE PREBIOTIC GROBIOTIC[®]-A AND BREWERS YEAST AS DIETARY SUPPLEMENTS FOR SUB-ADULT HYBRID STRIPED BASS (*Morone chrysops* × *M. saxatilis*) CHALLENGED IN SITU WITH *Mycobacterium marinum**

1. Introduction

Rapid growth and disease resistance of aquacultured organisms are two of the most important concerns. Traditionally, antibiotics have been supplemented in aquafeeds for prevention and/or treatment of bacterial disease of aquatic animals. It has been reported that antibiotics may enhance growth and feed efficiency by killing intestinal microflora and thus increasing amino acid utilization by the host in some animal species (Rawles et al., 1997). However, the use of antibiotics may pose threats such as development of bacterial strains that are more resistant to antibiotic treatment, or the occurrence of antibiotic residues in cultured organisms for human consumers (FAO, 2002). Increasing concerns of antibiotic use have resulted in a ban on subtherapeutic antibiotic usage in Europe and the potential for a ban in the United States and other countries (Patterson and Burkholder, 2003). These alterations in policy may impact aquaculture and therefore prompt interest in developing alternative strategies for disease control. Beside vaccine development, dietary supplements including probiotics,

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prebiotics and immunostimulants have received heightened attention. A rapidly expanding body of literature has been established that many intestinal microbial species may have beneficial influences on the performance of fish (reviewed by Irianto and Austin, 2002), and dietary composition is capable of influencing the intestinal microflora of fishes (Ringø et al., 1998; Ringø and Olsen, 1999). However, development of prebiotics, classified as “nondigestible food ingredients that beneficially affect the host by stimulating growth and/or activity of a limited number of bacteria in the intestine”, is in its infancy with fishes, compared to the progress that has been made in development of prebiotics for poultry (Patterson and Burkholder, 2003). In a previous evaluation of the commercial prebiotic, GroBiotic[®]-A, a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products, significantly enhanced feed efficiency of juvenile hybrid striped bass was observed (Li and Gatlin, 2004a), although the dynamics of the intestinal microflora was not defined in that study. Supplementation of this prebiotic also enhanced respiratory burst of head kidney leucocytes and resistance against *Streptococcus iniae* infection; however, the interpretation of these beneficial influences was complicated by the presence of brewers yeast, which is generally considered to be an immunostimulant for fishes (Siwicki et al., 1994; Ortuño et al., 2002; Li and Gatlin, 2003; Rodríguez et al., 2003).

The hybrid striped bass is an important fish for U.S. aquaculture, but it is affected by several pathogenic bacteria such as *Streptococcus iniae*, *Aeromonas hydrophila* and *Mycobacterium marinum* (reviewed by Plumb, 1997). With limited availability of approved therapeutic compounds and the inconvenient and costly administration of vaccines, this fish has been used in our laboratory as a model for investigating the

interaction between diet and disease resistance. Besides *Streptococcus iniae*, the prevalence of mycobacteria in wild striped bass and cultured hybrid striped bass has attracted increased attention (Gauthier et al., 2003; Overton et al., 2003). Although mycobacteriosis is generally chronic, this disease may cause severe infection and high cumulative mortality in closed, recirculating systems (Plumb, 1997). In addition, this bacterium is reported to be capable of surviving under many adverse environmental conditions, including low temperature, and may cause infection on the extremities of humans (Plumb, 1997; Mediel et al., 2003). A recent report of the presence of mycobacteria in frozen seafood also raised concern about the safety of such seafood for human consumers (Mediel et al., 2003). Currently, effective treatment of this disease is very limited as reported for other fish species (Colorini et al., 1998). Therefore, the present study was conducted to explore growth performance and non-specific immune responses of sub-adult hybrid striped bass fed the dietary prebiotic, GroBiotic[®]-A and brewers yeast under conditions of chronic exposure to *Mycobacterium marinum*.

2. Materials and Methods

2.1 Experimental diets

The basal diet was formulated from practical feedstuffs obtained at a commercial feed mill (Rangen Inc. Angleton, TX) to contain 40% protein, 10% lipid and an estimated digestible energy level of 3.5 kcal/kg (Table 10). This diet satisfied and/or exceeded all known nutrient requirements of hybrid striped bass (Gatlin, 1997; Webster, 2002) or other warmwater fishes (National Research Council, 1993). Partially autolyzed brewers yeast (Brewtech[®]) and GroBiotic[®]-A, a mixture of partially autolyzed brewers yeast,

Table 10. Composition of experimental diets

Constituent	Basal diet	1% Brewers yeast	2% Brewers yeast	2% GroBiotic [®] - A
<i>Ingredient (% dry weight)</i>				
Menhaden fish meal ^a	34.7	34.4	34	34
Soybean meal ^a	32.6	32.3	31.9	31.9
Wheat flour ^a	31.3	31	30.7	30.7
Menhaden fish oil ^b	5	5	5	5
Salt ^a	1.1	1.1	1.1	1.1
Mineral/vitamin premix ^a	0.3	0.3	0.3	0.3
Brewers yeast ^c	0	1	2	0
GroBiotic [®] -A ^c	0	0	0	2
<i>Analyzed proximate composition (% dry matter) ^e</i>				
Moisture	4.3	3.5	6.0	4.4
Crude protein (N×6.25)	41.2	38.6	39.1	40.8
Crude lipid	9.0	9.1	9.0	9.1
Ash	9.5	9.7	9.5	9.6

^a Rangen Inc. Angleton, TX.

^b Omega Protein Corporation, Reedville, VA.

^c International Ingredient Corporation, St. Louis, MO. Contained 51.0 % crude protein and 1.1 % crude lipid (dry-weight basis).

^d International Ingredient Corporation, St. Louis, MO. Contained 35.2 % crude protein, 1.7 % crude lipid and ~53% simple and complex carbohydrates including oligosaccharide (dry-weight basis).

^e Means of two analyses.

dairy ingredient components and dried fermentation products, were supplied by International Ingredient Corporation (St. Louis, MO, USA). Two incremental levels (1 and 2% of diet) of brewers yeast and 2% of GroBiotic[®]-A were added to the basal diet (Table 10). In addition, 5% menhaden fish oil (Omega Protein Corp., Reedville, VA) was added prior to extrusion processing as described by Rawles and Gatlin (2000) to produce neutrally buoyant 5-mm pellets. Diets were stored in sealed bags in a temperature-controlled room at 22 ± 2 °C until fed.

2.2 Feeding trial

Juvenile hybrid striped bass (*Morone chrysops* × *M. saxatilis*) were obtained from a commercial supplier (Keo Fish Farm, Keo, AR) and maintained in an earthen pond at the Texas A&M University Aquacultural Research and Teaching Facility prior to the feeding trial. Three fish were randomly obtained from this population and analyzed by the Texas Veterinary Medical Diagnostic Laboratory (TVMDL) to confirm they were pathogen-free by routine culture and histopathology. The culture system consisted of 16, 1134-l round fiberglass tanks connected as a closed recirculating system to a settling chamber and biological filter. This system had held hybrid striped bass with a severe mycobacteria infection shortly before the present feeding trial. Fish were seined from the pond, transported to the culture system and fed the basal diet to apparent satiation twice per day for a 2-week conditioning period. Fish were then graded by size and 12 groups of 50 fish averaging 64.5 g each with a total weight of 3225 ± 142 g (mean ± SD) per group were stocked into individual tanks and four groups of 50 fish averaging 118 g with a total weight of 5900 ± 81 g (mean ± SD) per group were stocked in the remaining tanks,

according to a randomized complete block design. Water flow rate was maintained at approximately 2 l/min via a recirculating system which maintained adequate water quality through biological and mechanical filtration. Salinity was maintained at 2-3‰ using well water and synthetic sea salt (Fritz Industries Inc., Dallas, TX). Low pressure electrical blowers provided aeration via air stones and maintained dissolved oxygen (DO) levels between 4 and 6 mg/l. Water temperature was controlled by conditioning the ambient air in the building and remained at 26 ± 1 °C throughout the trial. A 12 h light: 12h dark photoperiod was maintained with fluorescent lights controlled by timers.

Each experimental diet was fed to three groups of small fish and one group of large fish for 16 weeks. All groups were fed to apparent satiation in the morning and evening, 7 days each week. Growth and feed efficiency were monitored monthly by collectively weighing each group of fish. Weight gain was expressed as the increase in total cumulative biomass per tank. After week 16, a chronic mycobacterial infection became severe and was confirmed by the TVMDL to be caused by *Mycobacterium marinum* by biochemical analysis. At that, fish were fed to apparent satiation once per day for additional 5 weeks. Mortality was monitored twice daily during this period. All procedures were approved by the Animal Care and Use Committee of Texas A&M University.

2.3 Sample collection and analysis

At the end of the feeding trial, three apparently healthy fish (no obvious skin lesions and visceral granulomas) from each tank were anesthetized with tricaine methane sulfonate (MS-222), and approximately 2 ml of blood was collected from the caudal

vasculature using a 3-ml syringe and 23-gauge needle. These representative fish were euthanized and head kidney samples were pooled for macrophage isolation and assay of respiratory burst of head kidney leukocytes. The assay of extracellular and intracellular superoxide anion followed the procedure of Secombes (1990), as modified by Sealey and Gatlin (2002a). The amount of extracellular superoxide anion was calculated by the formula of Pick and Mizel (1981). Whole blood neutrophil oxidative radical production was determined as described by Siwicki et al. (1994). Absorbance was converted to nitro blue tetrazolium (NBT) units based on a standard curve of NBT diformazan/ml blood. Plasma was separated as previously described (Li and Gatlin, 2003) and lysozyme activity was determined by a turbidimetric assay (Jørgensen et al., 1993). A lysozyme activity unit was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min^{-1} at pH 6.1. Serum peroxidase was analyzed as described by Rodríguez et al. (2003).

2.4 Statistics

Data from the feeding trial, immune response assays and the bacterial challenge were subjected to analysis of variance according to a randomized complete block design using a significant level of $P \leq 0.05$. If a significantly main effect was observed, treatment means were separated using the LSD comparison test. All statistical analysis was conducted with Statistix[®] Analytical Software (Tallahassee, FL).

3. Results

3.1 Growth performance

Generally, fish fed the diets supplemented with brewers yeast and GroBiotic[®]-A had better growth performance during the 16-week feeding trial (Table 11). After 4 weeks of feeding, fish fed 2% brewers yeast had a significantly ($P < 0.05$) higher weight gain than fish fed the basal diet and the diet supplemented with 2% GroBiotic[®]-A. After 12 weeks, fish fed 1 and 2% brewers yeast and 2% GroBiotic[®]-A had significantly higher weight gain than fish fed the basal diet, and feed efficiency showed a similar trend. At the end of the 16-week period, fish fed 2% brewers yeast and GroBiotic[®]-A had the highest biomass, which tended to be significant ($P = 0.11$). Feed efficiency of fish fed 2% brewers yeast was significantly greater than the other treatments.

3.2 Immune response assays

No significant effects of the various diets were observed on neutrophil oxidative radical production, serum lysozyme and intracellular superoxide anion production by head kidney macrophages of sub-adult hybrid striped bass after the 16-week period (Table 12). However, fish fed 1 and 2% brewers yeast had a significantly ($P < 0.01$) higher serum peroxidase level than fish fed the basal diet and the diet supplemented with 2% GroBiotic[®]-A diet. The extracellular superoxide anion production of head kidney macrophages of fish fed the basal diet was significantly higher compared to fish fed diets supplemented with 1% brewers yeast and 2% GroBiotic[®]-A (Table 12).

Table 11. Weight gain (g gain/tank) and feed efficiency (g gain/g feed) of hybrid striped bass fed diets containing various amounts of dried brewers yeast and GroBiotic® -A^a

Diet	4 week		8 week		12 week		16 week	
	Weight gain	Feed efficiency	Weight gain	Feed efficiency	Weight gain	Feed efficiency	Weight gain	Feed efficiency
Basal	3298 ^a	1.11	6257	1.02	8168 ^a	0.89	9237	0.78 ^a
1% Brewers yeast	3550 ^{ab}	1.24	6734	1.04	9052 ^b	0.93	9349	0.76 ^a
2% Brewers yeast	3574 ^b	1.21	6880	0.95	9385 ^b	0.97	9876	0.82 ^b
2% GroBiotic® -A	3242 ^a	1.12	6693	1.03	9322 ^b	0.96	9878	0.78 ^a
ANOVA, Pr ≥ F ^b	0.04	0.33	0.19	0.87	0.05	0.13	0.11	0.02
Pooled SE	160.3	0.08	348.8	0.07	413.2	0.04	307.5	0.02

^a Values represent means of four replicate groups with three replicates of small fish initially averaging 64.5 g/fish and one replicate of large fish averaging 118 g/fish. Values in a column that do not have the same superscript are significantly different at P ≤ 0.05 based on LSD comparison test.

^b Significance probability associated with the F statistic

Table 12. Neutrophil oxidative production (NBT test), serum lysozyme and extracellular and intracellular superoxide anion production of head kidney macrophages of hybrid striped bass fed experimental diets for 16 weeks^a

Diet	NBT test (mg ml ⁻¹) ^b	Lysozyme (10 ³ units/l) ^b	Peroxidase (O. D. at 450 nm) ^b	Extracellular superoxide anion (nmol O ₂ ⁻) ^c	Intracellular superoxide anion (O.D. at 620 nm) ^c
Basal	2.50	2323	0.428 ^a	4.50 ^a	0.597
1% Brewers yeast	2.40	2224	0.729 ^b	3.77 ^b	0.529
2% Brewers yeast	2.48	2752	0.706 ^b	4.31 ^{ab}	0.706
2% GroBiotic® -A	2.41	2124	0.497 ^a	3.89 ^b	0.742
ANOVA, Pr ≥ F ^d	0.49	0.13	0.01	0.05	0.85
Pooled SE	0.10	186	0.059	0.34	0.193

^a Values in a column that do not have the same superscript are significantly different at P ≤ 0.05 based on LSD comparison test.

^b Means of three individual fish from each of four replicate tanks.

^c Means of composite samples of head kidney cells from three fish in each of four replicate tanks.

^d Significance probability associated with the F statistic.

3.3 Bacterial challenge

The survival of fish in the feeding trial decreased over time as the severity of the mycobacterial infection increased. This *in situ* mycobacterial challenge resulted in approximately 25% mortality over the 21-week period. Fish fed the diets with 1% brewers yeast and 2% GroBiotic[®]-A had enhanced survival ($P < 0.05$) at the 8-, 12- and 16-week intervals (Fig. 3). At the end of the 21-week period, survival of fish fed 2% GroBiotic[®]-A was significantly higher than fish fed the basal diet and brewers yeast supplemented diets (Fig. 3). Typical signs of mycobacterial infection including granulomas in the spleen, head kidney and a pale granulomatous liver with rough granular surface after necropsy as well as ulcerations and hemorrhaging in the skin were observed in moribund fish. The cause of mycobacteria was confirmed by the TVMDL via bacterial isolation and histopathology.

4. Discussion

Mycobacterial species have been known for years to be capable of infecting many fish species in both fresh water and seawater (Frerichs, 1993; Plumb, 1997; dos Santos et al., 2002). Striped bass and hybrid striped bass may be one of the most susceptible species based on increasing case reports in recent years (Gauthier et al., 2003; Harms et al., 2003; Overton et al., 2003). Although the presence of mycobacteria is rather common in wild fish populations, it has become one of the most serious infections in intensive, recirculating culture systems (Plumb, 1997). Besides the economic need for developing anti-mycobacteriosis strategies, the chronic progression of this disease provided an opportunity to explore growth and immune responses of hybrid striped bass fed different

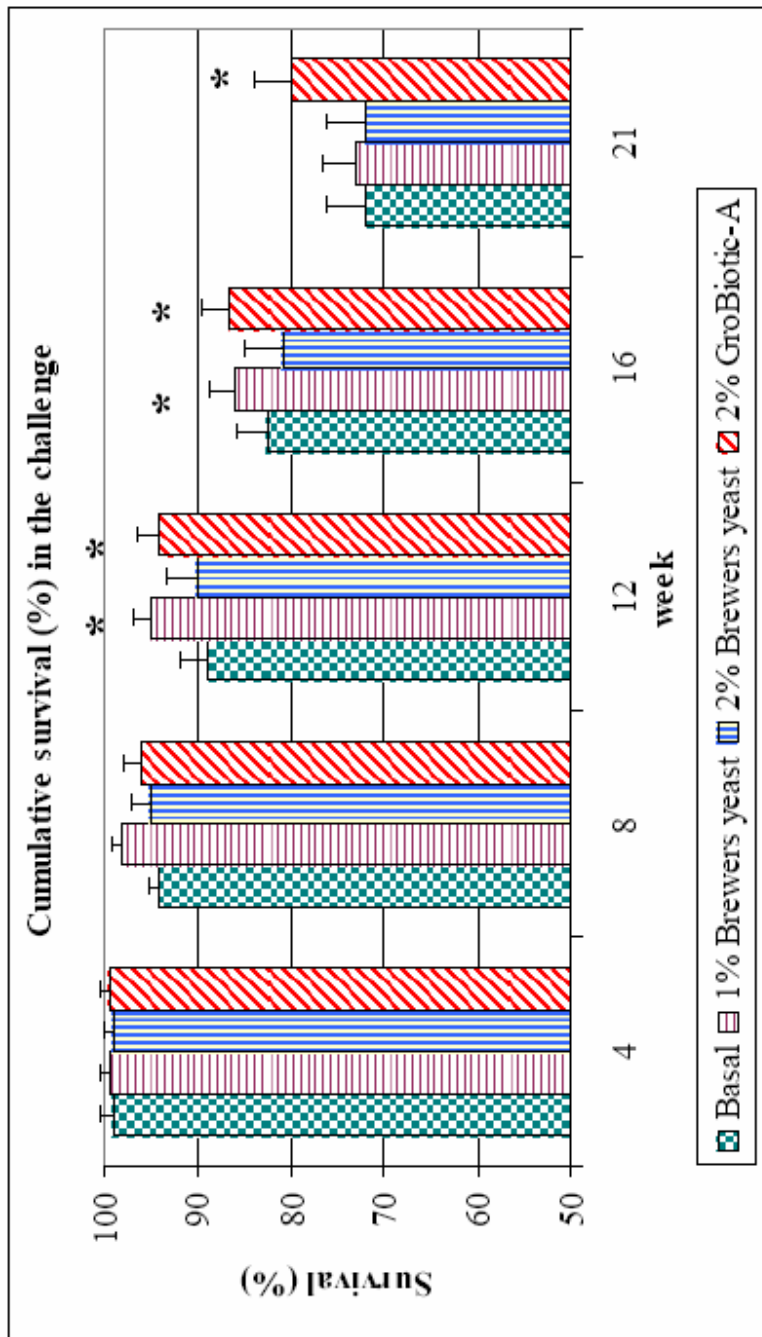


Fig 3. Percent cumulative survival of hybrid striped bass fed 2% of GroBiotic[®]-A and brewers yeast (1 % and 2%) for 4, 8, 12, 16 and 21 weeks and exposed to *Mycobacterium marinum*. Bars represent means of four replicate tanks per treatment. Treatments with an asterisk (*) had a significantly ($P < 0.05$) higher survival than treatments without an asterisk at each special time interval.

immunostimulants. Because water has long been recognized as a primary source for mycobacterial transmission (Goslee and Wolinsky, 1976; dos Santos et al., 2002; Gauthier et al., 2003), chronic exposure to mycobacteria-contaminated water was employed in this trial to mimic a natural infection and to prevent possible excessive mortality from acute inflammation, although experimental mycobacterial infections traditionally have been conducted by intramuscular (Wolf and Smith, 1999) or intraperitoneal injections (Colorni et al., 1998; Gauthier et al., 2003), which have been used for research on pathology and immunology associated with this disease.

Increased weight gain and feed efficiency were generally observed in hybrid striped bass fed diets supplemented with partially autolyzed brewers yeast and GroBiotic[®]-A at each sampling time (4, 8, 12 and 16 weeks). However, variation within treatments complicated the interpretation of these data. Fish fed the diet supplemented with 2% brewers yeast had consistently and significantly better growth performance throughout the feeding trial compared to fish fed the diets. To the best of our knowledge, this is the first time to report that dietary supplementation of inactivated or autolyzed brewers yeast could serve as growth enhancer under certain conditions such as chronic infection, although biologically-active brewers yeast had been reported to serve as a probiotic and enhanced growth of tilapia (Lara-Flores et al., 2002). Feed efficiency of every dietary treatment decreased over time in the present study. It is speculated that this reduction was correlated with the severity of the mycobacterial infection. With the progression of the mycobacterial infection, inflammation was induced and immune responses were upregulated accordingly. Increased expense for growth would be expected as a consequence of dramatically increased requirement for amino acids to

synthesize differentiated proteins for immune functions, substrates for nitric oxide production (arginine) and energy for macrophages and lymphocytes (glutamine). It also was noted that weight gain of fish fed all experimental diets decreased between week 12 and 16. Although progress of mycobacteriosis could possibly suppress feed intake and increase the cost of growth, overaccumulation of metabolic wastes such as ammonia (up to 10.6 mg/l) and nitrite (up to 1.2 mg/l) may have contributed more to the undermined feed intake and subsequently reduced growth, because the concern of spreading this bacterium through effluent restricted the exchange of water between system and environment.

A previous study in our laboratory showed that feeding a diet supplemented with brewers yeast for 16 weeks could increase blood neutrophil oxidative radical production and extracellular superoxide anion production of head kidney leucocytes of juvenile hybrid striped bass (Li and Gatlin, 2003). This present study failed to support our previous observation, possibly due to the masking effect of inflammation induced by mycobacterial infection, if no difference in age/size responses to immunostimulation by dietary supplements was involved. At the end of 16 weeks, over 50% of hybrid striped bass in the trial were observed to have skin ulcerations and hemorrhages, suggesting a high prevalence of mycobacterium in the present study. Although only apparently healthy fish were sampled for immune response assays, mycobacteria-infected fish sometimes showed rather normal appearances (Gauthier et al., 2003). This uncertainty in infection status compromised the conclusiveness of data from the immune response assays. Fish fed diets supplemented with 1 or 2% brewers yeast for 16 weeks had a significantly higher serum peroxidase level compared to fish fed the basal diet and diet supplemented

with GroBiotic[®]-A. Rodríguez et al. (2003) observed that 6 weeks, but not 2 or 4 weeks of feeding a cell-wall modified yeast (33% glucan, 56% mannoproteins and 11% chitin) decreased serum peroxidase level of gilthead sea bream. The effect of dietary brewers yeast on serum peroxidase warrants further investigation. Intracellular superoxide anion production of head kidney leucocytes and serum lysozyme were not affected by dietary treatments in the present study. However, it is noted that the serum lysozyme measured in the present study was dramatically higher than all the values previously published for hybrid striped bass (Sealey and Gatlin, 2002b; Li and Gatlin, 2003, 2004; Jaramillo and Gatlin, 2004; Li et al., 2004). Although serum lysozyme level of hybrid striped bass is influenced by genetic polymorphisms (Wang and Gatlin, unpublished data) and variation in genetic makeup of fish may contribute to the phenomenon, it is speculated that mycobacteriosis stimulated serum lysozyme level of hybrid striped bass in the present study. Chen et al. (1998) reported that injection of the extracellular product of *Mycobacterium* sp induced an elevation in serum lysozyme level. Although pathology of mycobacteriosis is well defined, the immunological responses associated with mycobacterial infection are still limited. Bartos and Sommer (1981) and Harms et al. (2003) reported responses of cellular immunity and transforming growth factor- β responses associated with mycobacteriosis, respectively, but further investigation of potential interactions among dietary strategies, immune responses and mycobacterial infection is warranted.

In the present study, dietary supplementation of prebiotic GroBiotic[®]-A significantly enhanced survival of hybrid striped bass during the *in situ* mycobacterial challenge, suggesting a potential use of this prebiotic in aquaculture. Based on knowledge

acquired from human and terrestrial animals, prebiotics are usually most effective against enteric diseases. It is known that ingestion of feed also is a port of entry for mycobacteria in some fish species including snakehead (Chinabut et al., 1990). This could possibly be a factor contributing to the positive response associated with the GroBiotic[®]-A supplement; however, efforts to characterize intestinal microbiology of fish in the present study failed. Research on prebiotics for human use since 1995 has established that some bacterial species are health-promoting and are capable of selectively utilizing non-digestible dietary components in the colon such as inulin and lactose (Manning and Gibson, 2004). Lactobacilli also have been reported to be probiotics for fishes (reviewed by Irianto and Austin, 2002). Positive influences of GroBiotic[®]-A on growth performance and disease resistance of hybrid striped bass in the present study showed desirable influences of prebiotics for aquaculture.

CHAPTER V

DIETARY OLIGONUCLEOTIDE INFLUENCES IMMUNE RESPONSES AND RESISTANCE OF HYBRID STRIPED BASS (*Morone chrysops* × *M. saxatilis*) TO *Streptococcus iniae* INFECTION*

1. Introduction

Hybrid striped bass (*Morone chrysops* × *M. saxatilis*) production is considered to be the fastest growing segment of the U.S. aquaculture industry over the past decade and has spread to several countries and regions in Europe and Asia (Harrell and Webster, 1997; Hiney et al., 2002; Sealey and Gatlin, 2002c). This fish is intrinsically sensitive to various stressors and subsequently susceptible to infectious agents during normal aquacultural production (Plumb, 1997). In recent years, there have been growing concerns about the adverse effects of the bacterium *Streptococcus iniae* in the aquaculture of many economically important marine and freshwater fish species (Li and Gatlin, 2003). This bacterium has become one of the most threatening pathogenic organisms of the hybrid striped bass culture industry and is estimated to cause about \$ 2 million in economic loss in the USA every year attributable to fish mortality, poor growth and feed conversion, as well as the expense of therapeutic treatment which usually occurs multiple times (Ostland, 2003). Although positive results have come from experimental

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treatment of this disease in tilapia with oxytetracycline (Darwish et al, 2002) and hybrid striped bass with enrofloxacin (Stoffregen et al, 1996), these drugs are still under investigation and there are currently no antibiotics approved by the U.S. Food and Drug Administration to treat bacterial diseases of hybrid striped bass. Therefore, immunonutrition may be of great promise in prevention of infectious diseases of hybrid striped bass such as streptococciosis and thus warrants investigation.

Nucleotides and their metabolites have received heightened attention in recent years as potential immunomodulators. They play key roles in numerous essential physiologic functions, including encoding genetic information, mediating energy metabolism and signal transduction (Carver and Walker, 1995; Aggett et al., 2003). It is generally assumed that the requirement for nucleotides can be met by endogenous metabolic pathways of synthesis and salvage. However, this is now being challenged by reports involving humans and terrestrial animals, which have shown that dietary nucleotides are capable of enhancing cell-mediated immunity, lymphocyte proliferation, interleukin (IL)-2 production, and improving host resistance to bacterial infection. In recent years, beneficial influences of oral administration of nucleotides on immune functions, vaccine efficiency, or disease resistance also have been demonstrated in fish such as Atlantic salmon (*Salmo salar*) (Burrells et al., 2001a, b), coho salmon (*Oncorhynchus kisutch*) (Burrells et al., 2001a), rainbow trout (*Oncorhynchus mykiss*) (Burrells et al., 2001a), common carp (*Cyprinus carpio*) (Sakai et al., 2001) and hybrid tilapia (*O. niloticus* × *O. aureus*) (Ramadan et al., 1996). However, information on hybrid striped bass is not available to date. Dietary brewers yeast has been proven to be capable of enhancing immune responses of hybrid striped bass as well as resistance to

Streptococcus iniae infection (Li and Gatlin, 2003). However, the extent to which nucleotides and RNA from brewers yeast contributed to the positive influence remains unclear. Therefore, the present study was conducted to determine the effects of dietary nucleotides on specific and non-specific immune responses of hybrid striped bass and resistance to *Streptococcus iniae* infection. The efficacy of long-term oral administration of nucleotides also was explored by comparing various immune responses.

2. Materials and Methods

2.1. Diets

The basal diet, which utilized menhaden fish meal as the protein source, contained 40% protein, 10% lipid and an estimated digestible energy level of 3.5 kcal/g (Table 13). This diet satisfied and/or exceeded all known nutrient requirements of hybrid striped bass (Gatlin, 1997) or other warmwater fishes (NRC, 1993). An oligonucleotide mixture of guanosine, adenosine, uridine and cytidine isolated from a strain of *Saccharomyces* (brewers yeast) was supplied by Ascogen P[®] (Canadian Bio-Systems Inc., Calgary, Alberta, Canada) and was supplemented at the manufacturer's recommended rate of 0.5% to the basal diet. Cellulose, menhaden meal and menhaden oil were adjusted to provide isonitrogenous and isolipidic diets. Procedures for diet preparation and storage were as previously described (Rawles and Gatlin, 1998).

2.2.1. Feeding trial 1

An initial feeding trial was conducted to evaluate growth performance, non-specific immune responses as well as disease resistance of hybrid striped bass fed the two

Table 13. Composition of diets

Constituent	Basal	Nucleotide-Supplemented
Ingredient (% dry weight)		
Menhaden fish meal ^a	57.9	57.7
Dextrin ^b	24.9	24.9
Menhaden oil ^a	2.4	2.4
Mineral premix ^c	4.0	4.0
Vitamin premix ^d	3.0	3.0
Carboxymethyl cellulose ^b	2.0	2.0
Cellulose ^b	5.8	5.5
Ascogen P [®] ^e	0	0.5

^a Omega Protein Corporation, Reedville, VA. Menhaden fish meal contained 69.0% protein and 13.2% lipid at a dry-weight basis.

^b US Biochemical Corp., Cleveland, OH.

^c Contains (as g/kg): Ca(C₆H₁₀O₆)·5H₂O, 348.49; Ca(H₂PO₄)₂·H₂O, 136.0; FeSO₄·7H₂O, 5.0; MgSO₄·7H₂O, 132.0; K₂HPO₄, 240.0; NaH₂PO₄·H₂O, 88.0; NaCl, 45.0; AlCl₃·6H₂O, 0.15; KI, 0.15; CuSO₄·5H₂O, 0.5; MnSO₄·H₂O, 0.7; CoCl₂·6H₂O, 1.0; ZnSO₄·7H₂O, 3.0; Na₂SeO₃, 0.011.

^d Contains (as g/kg): ascorbic acid, 50.0; dl-calcium pantothenate, 5.0; choline chloride, 36.2; inositol, 5.0; menadione sodium bisulfite, 2.0; niacin, 5.0; pyridoxine·HCl, 1.0; riboflavin, 3.0; thiamine mononitrate, 0.5; dl- α -tocopheryl acetate (250 IU/g), 8.0; vitamin A acetate (500,000 IU/g), 0.2; biotin, 0.05; cholecalciferol (1 μ g = 40 IU), 0.002; folic acid, 0.18; vitamin B₁₂, 0.002; cellulose, 819.93.

^e Canadian Bio-System Inc., Calgary, Alberta, Canada. Ascogen P[®] contained 34.9% crude protein and 5.0 % crude lipid (dry-weight basis).

diets. Prior to initiation of this feeding trial, juvenile hybrid striped bass obtained from Keo Fish Farm (Keo, AR) were subjected to a 2-week conditioning period to adjust to standardized regimes in a recirculating culture system consisting of 38-l aquaria. Groups of 15 juvenile hybrid striped bass weighing approximately 9.7 g/fish were stocked into individual aquaria such that initial weight averaged 142 ± 5 g/group. The basal and experimental diets were both fed to four replicate groups of fish for 8 weeks. All groups were fed their respective diets at the same fixed rate (initially 5% of body weight per day and gradually reduced to 3%) which approached apparent satiation. Growth and feed efficiency were monitored weekly by collectively weighing each group of fish.

2.2.2. Preparation of blood and serum

At the end of this feeding trial, three representative fish from each aquarium were anesthetized with an overdose of MS-222 and blood collected from the caudal vasculature. After a sample of whole blood was taken for hematocrit determination, serum was isolated by centrifugation ($3000 \times g$ for 5 min) and kept at -80 °C. After bleeding, the fish were frozen for whole-body composition analysis (Rawles and Gatlin, 1998).

2.2.3. Lysozyme assay

Serum lysozyme activity was determined by turbidimetric assay as described by Jorgensen et al. (1993a). A lysozyme activity unit was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min^{-1} .

2.2.4. Preparation of bacterial suspension and bath challenge

An additional 30 fish previously fed each of the experimental diets for a total of 9 weeks were exposed to an estimated LD₅₀ dose of *Streptococcus iniae*. A virulent isolate of *S. iniae* originally obtained from the brain of an infected tilapia (*Oreochromis* sp.) was biochemically identified and provided by the Texas Veterinary Medicine Diagnostic Laboratory. The bacterial suspension was prepared according to the method described by Sealey and Gatlin (2002c) and diluted to a concentration of 2.6×10^5 CFU/ml in fresh well water. Thirty fish from each dietary treatment, pooled separately in mesh baskets, were immersed in the bacterial suspension for 2 hours. After bacterial exposure, the 30 fish from each dietary treatment were divided into three groups of 10, and placed into 38-l aquaria in an isolated culture system that received a constant supply of well water at 25 ± 2 °C. Fish continued to be fed their respective diets to apparent satiation twice daily, and mortality was monitored for 2 weeks. Thereafter, a secondary bacterial exposure was conducted on surviving fish with an increased bacterial concentration of 1.5×10^6 CFU/ml following the procedure described by Nguyen et al. (2001a, b). The mortality was monitored for 3 weeks. The brains of both dead fish and surviving fish were streaked on modified selective agar to determine infection status and confirm death from *S. iniae* (Nguyen and Kanai, 1999).

2.3.1. Feeding trial 2

A second feeding trial was conducted to further evaluate resistance of hybrid striped bass fed nucleotides to *Streptococcus iniae* infection as well as specific immune responses. Groups of 13 juvenile hybrid striped bass weighing approximately 7.1 g/fish

were stocked into individual aquaria such that initial weight averaged 92 ± 5 g/group. Each diet was fed to fish in four replicate groups initially at 6% of body weight and gradually reduced to 2.5% of body weight over the course of the trial such that fish were continuously fed at a rate approaching apparent satiation. The feeding trial was conducted for 6 weeks. After the feeding trial, 12 fish per treatment (three fish per aquarium) were bled and serum was separated for lysozyme as described above and serum protein analyzed using Sigma Kit 690-A (Sigma, St. Louis, MO).

2.3.2. *Neutrophil oxidative radical production assay*

Neutrophil oxidative radical production was determined following the procedure described by Siwicki et al. (1994). Absorbance was converted to Nitro Blue Tetrazolium (NBT) units based on a standard curve of NBT diformazan/ml blood.

2.3.3. *Bacterial challenge*

An additional 30 fish previous fed each diet for a total 7 weeks were exposed to *S. iniae* at a concentration of 8.4×10^5 CFU/ml in fresh well water following the previously described procedure. Fish continued to be fed their respective diets to apparent satiation once daily and mortality was monitored for 3 weeks.

2.3.4. *Specific immune function sampling*

Eight surviving fish from each treatment were chosen randomly and injected with *S. iniae* intraperitoneally 3 weeks after first exposure to *S. iniae*. The bacterial suspension was prepared by the procedure described by Sealey and Gatlin (2002c) at a concentration

of 6×10^6 CFU/ml. Three fish fed the basal diet died within 10 days after immunization but all fish fed the nucleotide-supplemented diet survived. All the surviving fish were bled at day 10 after immunization and serum was separated as previously described. Slide agglutination and microtitration agglutination were used to test for antibody presence in the serum following the procedure described by Roberson (1991). A positive reaction was described by the presence of a button with fuzzy edges; whereas, a negative reaction consisted of a round precipitate with clearly defined circular margins (Sealey and Gatlin, 2002a). Antibody titer was expressed as the reciprocal log base 2 of the highest dilution demonstrating a positive reaction.

2.4.1. Feeding trial 3

A separate feeding trial was conducted to further evaluate the efficacy of long-term administration (16 weeks) of nucleotides on immune functions. Fifteen fish weighing approximately 10 g/fish were stocked into each of two 38-l aquaria in the same system as Trial 1 and fed either the basal or nucleotide-supplemented diets. During the first 8 weeks, both groups of fish were fed their respective diets twice daily at the same fixed rate (initially 5% of body weight per day and gradually reduced to 3%). In the second 8 weeks, both groups of fish were fed once daily to apparent satiation. After 16 weeks of feeding, four fish from each treatment were bled. Neutrophil oxidative radical production and serum lysozyme were determined as previous described. Eight fish per treatment were euthenized and head kidney samples were taken for leukocyte isolation.

2.4.2. Leukocyte isolation and superoxide anion production assay

This assay followed the procedure described by Secombes (1991) with minor modifications by Sealey and Gatlin (2002a). The amount of extracellular superoxide anion was calculated from the formula: nanomoles superoxide anion per well = (Δ absorbance after 60 min \times 100)/6.3 (Pick and Mizel, 1981).

2.5. Statistics

Data from all feeding trials and bacterial challenges were subjected to Student's *t*-test using the Statistical Analysis System (SAS, 1985). Differences in treatment means were considered significant at $P < 0.05$.

3. Results

3.1. Feeding trial 1

In trial 1, no significant differences were observed in weight gain or feed efficiency and survival of fish fed the basal diet and nucleotide-supplemented diet (Table 14) and survival averaged 97% over the 8-week period. Hematocrit and serum lysozyme averaged 46.4% and 923 units/L, respectively, with no significant effects of dietary nucleotide supplementation observed. Whole-body composition (moisture, crude protein, lipid and ash) also was not significantly affected by dietary supplementation of nucleotides. Generally lower mortality was observed in fish fed the nucleotide-supplemented diet after initial *S. iniae* exposure; however, in the secondary challenge, significant differences in mortality between fish fed the basal diet and the nucleotide-supplemented diet were evident (Table 14). No *S. iniae* was isolated from the brain of

Table 14. Mortality of hybrid striped bass after two separate challenges with *Streptococcus iniae* in trial 1¹

Diet	Weight gain (% of initial wt) ²	Feed efficiency (g gain/g feed)	Mortality in first challenge (%)	Mortality in second challenge (%)
Control	396	0.91	20	83.81 ^a
Nucleotide- supplemented	388	0.90	3.3	52.22 ^b
p³	0.583	0.63	0.189	0.017
pooled se	5.58	0.008	4.303	3.269

¹ Values in a column that do not have the same superscript are significantly different at $P \leq 0.05$.

² Fish initially averaged 9.7 g.

³ Significance probability associated with the *t* statistic.

surviving fish, while colonies of *S. iniae* were isolated from dead fish.

3.2. Feeding trial 2

Fish fed the nucleotide-supplemented diet tended ($P < 0.1$) to have greater weight gain than those fed the basal diet (Table 15). No differences were observed in feed efficiency, survival or serum protein concentration (Table 15). Significant enhancement in blood neutrophil oxidative radical production of fish fed the nucleotide-supplemented diet was observed (Table 15). During the 3 weeks after bath challenge with *S. iniae*, 40% mortality was observed in fish fed the basal diet, while only 13.3% mortality occurred in fish fed the nucleotide-supplemented diet ($P < 0.1$). After the booster injection, five out of eight fish fed the basal diet and all 8 fish fed the nucleotide-supplemented diet survived. Although strong agglutination responses were observed only in fish fed the nucleotide-supplemented diet, the antibody titers were not significantly different because no detectable levels of antibody were observed in some fish fed each diet (Table 15).

3.3. Feeding trial 3

Blood neutrophil oxidative radical production, extracellular and intracellular superoxide anion production of head kidney leukocytes and serum lysozyme were measured after 16 weeks of feeding each diet. No significant differences in these responses were observed between fish fed the two diets (Table 16).

4. Discussion

Nucleotides have been generally considered as non-essential nutrients because deficiency signs have not been observed (Carver and Walker, 1995). It is now known that

Table 15. Weight gain, feed efficiency, survival, neutrophil oxidative production (NBT test), serum protein, mortality in bacterial challenge and antibody titer of hybrid striped bass fed control diet and nucleotide-supplemented diet in trial 2¹

Diet	Weight gain (% of initial wt) ²	Feed efficiency (g gain/g feed)	Survival (%)	NBT test (mg ml ⁻¹)	Serum Protein (mg/ml)	Mortality in challenge	Antibody titer (log ₂) ³
Control	389	0.93	92.3	2.14 ^b	50.8	40	0.80
Nucleotide- supplemented	433	0.93	86.5	2.45 ^a	48.8	13.3	2.63
P ⁴	0.095	0.951	0.278	0.011	0.686	0.091	0.330
pooled se	8.093	0.014	1.713	0.023	1.84	3.68	0.511

¹ Values in a column that do not have the same superscript are significantly different at $P \leq 0.05$ based on Student *t*-test.

² Fish initially averaged 7.1 g.

³ Means of titers of 5 fish fed the basal diet and 8 fish fed the nucleotide-supplemented diet.

⁴ Significance probability associated with the *t*-statistic.

Table 16. Blood neutrophil oxidative radical production (NBT test), serum lysozyme, intracellular superoxide anion production of head kidney macrophages and extracellular superoxide anion production of hybrid striped bass after long-term (16 week) oral administration of nucleotides

Diet	NBT test (mg ml ⁻¹)	Lysozyme (unit ml ⁻¹)	Intracellular superoxide anion (O.D. at 620 nm)	Extracellular superoxide anion (nmol O ₂)
Control diet	1.99	1247	0.328	2.679
Nucleotide diet	2.10	1217	0.149	3.439
P ¹	0.769	0.811	0.146	0.191
Pooled se	0.118	47.970	0.029	0.138

¹ Significance probability associated with the *t*-statistic.

tissues such as intestinal mucosa, bone marrow hematopoietic cells, lymphocytes and the brain have limited capacity for de novo nucleotide synthesis and depend on the supply by the salvage pathway (Yamauchi et al. 2002). Therefore, Yamauchi et al. (2002) raised the hypothesis that the endogenous supply of nucleotides may not be adequate for optimal functioning, especially of the immune system, under stressful conditions such as sepsis and trauma, and thus a dietary supply may be of particular significance. Aquacultural production undoubtedly may create compromising environments for the target organism, where stressors caused by handling, grading, transporting, poor water quality, overcrowding and diseases are typically common. It appears reasonable to supplement exogenous nucleotides to the normal diets of aquacultured organisms, although information pertaining to the synthesis and metabolism of nucleotides in lower vertebrates such as fish and invertebrates such as crustaceans is extremely limited to date. Although the nutritional value of nucleotides to humans and other animals is still in some dispute, the results from studies on fish appear to be rather consistent. To our best knowledge, all published results in fish including this present study showed that oral administration of nucleotides enhanced host resistance to pathogens (Burrells et al., 2001a, b; Sakai et al., 2001). However, the mechanism(s) behind this beneficial influence is not clear.

In this present study, neutrophil oxidative radical production of hybrid striped bass fed the nucleotide-supplemented diet was significantly higher than that of fish fed the basal diet after 8 weeks. Matsumoto et al. (1995) observed that a mixture of nucleosides and nucleotides increased the peripheral neutrophil number in mice. Although some reports showed that dietary nucleotides can increase macrophage function

in the rat (Carver, 1994), the respiratory burst of macrophages in fish did not show consistent results. In present study and that of Burrells et al. (2001a), no enhancement of respiratory burst head kidney macrophage was observed. However, Sakai et al. (2001) observed that oral administration of nucleotides resulted in enhanced phagocytic and NBT responses in kidney phagocytic cells of common carp for more than 10 days post-treatment. In that study (Sakai et al., 2001), serum lysozyme activity and serum complement of common carp orally administered nucleotides were significantly higher than that of fish fed a basal diet. In the present study, serum lysozyme appeared to be unaffected by dietary nucleotides at administration intervals of 7, 8 and 16 weeks.

Burrells et al. (2001a) reported beneficial effects of dietary nucleotides when challenging salmonids with infectious salmon anaemia virus, *Vibrio anguillarum*, *Piscirickettsia salmonis* and sea lice. They hypothesized that dietary nucleotides are capable of enhancing the potential of the immune system in general to mount greater and more rapid specific responses, as compared to the primarily non-specific capacity of phagocytes induced by glucan. This hypothesis was supported by other reports on Atlantic salmon (Burrells et al., 2001a) and hybrid tilapia (Ramadan et al., 1994) fed nucleotide-supplemented diets. Atlantic salmon were reported to have an enhanced efficacy of vaccination based on comparing antibody titers and survival rate (Ramadan et al., 1994). Similarly, a marked rise in the geometric mean of antibody titers after vaccination was observed in tilapia (Ramadan et al., 1994). In addition, dietary nucleotides have been observed to modulate gene expression (Sanchez-Pozo and Gil 2002), a phenomenon also confirmed in fish by Low et al. (2003). However, the way in which dietary nucleotides modulate gene expression during development of adaptive

immunity is still not clear. In the present study, the most significant enhancement in survival of hybrid striped bass fed nucleotide-supplemented diet was observed after *S. iniae* reexposure. It is possible that dietary nucleotides contribute to the development of the specific immune response. In Trial 2, antibody titer also tended to be higher in hybrid striped bass fed the nucleotide-supplemented diet. This result also supports the hypothesis of Jyonouchi et al. (1993, 1994) and Navarro et al. (1996) that dietary nucleotides exert the greatest impact on the immune system by modulating Ig production.

It is generally recognized that dose and timing have important effects on the efficacy of immunostimulants. Because most publications on nucleotide supplementation to fish have used patented commercial products, information pertaining to concentration and ratio of various types of nucleotides are generally unavailable. Therefore, it is very difficult to quantitatively estimate or compare the effects of supplemented nucleotides on the immune responses of various fish species. In the present study with hybrid striped bass, no significant differences in blood neutrophil oxidative radical production, or intracellular and extracellular superoxide anion production of head kidney leukocytes were observed after 16 weeks of feeding the nucleotide-supplemented diet while neutrophil oxidative radical production of fish fed this diet was significantly higher than that of fish fed the basal diet after 8 weeks. This may have been due to difference in the timing of administration of the nucleotide-supplemented diet or changes in the requirement for exogenous nucleotides as a function of fish size.

The study of Ramadan and Atef (1991) showed that dietary nucleotides significantly enhanced weight gain of tilapia. However, this phenomenon was not noticeable in all of the feeding trials of the present study, although higher weight gain

tended to be observed in fish fed the nucleotide-supplemented diet in Trial 2.

It is concluded that dietary oligonucleotides positively influenced non-specific immune response and resistance of juvenile hybrid striped bass to *S. iniae*. However, there are gaps in our knowledge about exogenous nucleotides in fish concerning absorption, metabolism, modulation of immunoglobulin production, age/size-related response and appropriate doses or time of intakes. Therefore, further investigation in these areas is warranted.

CHAPTER VI

EVALUATION OF LEVAMISOLE AS A DIET SUPPLEMENT FOR GROWTH AND HEALTH MANAGEMENT OF HYBRID STRIPED BASS (*Morone chrysops* × *M. saxatilis*)

1. Introduction

For decades, antibiotics have been extensively used to treat illness, prevent infections or promote growth in animal production. However, concerns on the part of the scientific community and general public about development of antibiotic-resistant bacterial strains are strongly challenging the use of antibiotics in agriculture (FAO, 2002). These concerns have been heightened by the occasional cases of human death from antibiotic-resistant bacteria reported in recent years (Ferber, 2000). Therefore, the potential uses of existing antibiotics and approval of new ones for aquaculture may be limited. Research on interactions between nutrition and immunity and the development of alternatives to antibiotics that may keep fish healthy such as probiotics, prebiotics and immunomodulators is receiving heightened attention in aquaculture nutrition and aquafeed research.

Hybrid striped bass is an important economic fish species in the USA. Although a wide range of pathogenic organisms in hybrid striped bass have been described (Plumb, 1997), the major pathogens threatening this industry are several bacteria, especially *Streptococcus iniae*, *Aeromonas hydrophila* and mycobacterial species (Ostland, 2003). Because disease prevention and treatment strategies such as use of vaccines and drugs are

currently limited in hybrid striped bass aquaculture due to regulatory constraints or excessively burdensome administration protocols, extensive effort has been expended in screening a variety of recognized immunonutrients and immunostimulants to develop practical dietary immunotherapies for hybrid striped bass against those bacteria (Sealey and Gatlin, 2002a; Li and Gatlin, 2003; 2004; Li et al., 2004; Jaramillo and Gatlin, 2004).

Levamisole is a synthetic antihelminthic drug for protecting terrestrial animals against stomach, intestinal and lung worms (JECFA, 1991). It was the first drug reported to increase the functions of cellular immunity in healthy laboratory animals (Renoux, 1980). This phenomenon was confirmed with rainbow trout in the late 1980s (Siwicki, 1989). Levamisole also has been shown with several aquatic species to be a potent immunostimulant in modulation of leukocyte cytotoxic activity (Cuesta et al., 2002), phagocytosis ((Mulero et al., 1998; Findlay and Munday, 2000), respiratory burst (Siwicki, 1989; Mulero et al., 1998), antibody response (Jeney and Anderson, 1993; Cuesta et al., 2004) and macrophage-activating factor (Mulero et al. 1998a). Levamisole has been reported to be capable of enhancing resistance to pathogenic bacteria such as *Vibrio anguillarum* (Kajita et al., 1990), *Aeromonas hydrophila* (Baba et al., 1993), *Paramoeba* sp. (Findlay et al., 2000; Munday and Zilberg, 2003), *P. fluorescent* (Baruah and Prasad, 2001) *Edwardsiella tarda* (Sahoo and Mukherjee, 2002), *Photobacterium damsela* (Leano et al., 2003) and nematodes such as *Anguillicola crassus* (Geets et al., 1992). This drug does not have direct effects on bacteria, viruses and fungi (Renoux, 1980); therefore, it mainly serves as an immunotherapy and the possibility of resistance development by pathogenic bacteria is rather minimal. Because levamisole can be effectively delivered via dietary supplementation, it would be much more feasible for

industry use, compared to injection and immersion. Besides the immunostimulating influence and potential use in fish health management, levamisole also was recognized to enhance growth of gilthead sea bream (Mulero et al., 1998), carp (Siwicki and Korwin-Kossakowski, 1988) and several terrestrial animals (Cabaj et al., 1995), although the administration protocols including dose and time still have not been fully defined. A previous study with hybrid striped bass failed to demonstrate a growth-enhancing effect of dietary levamisole, probably because of limited supplemental levels (Li et al. 2004b). In addition, some research showed that administration of levamisole via immersion or intraperitoneal injection can modulate adaptive immunity and antibody production (Jeney and Anderson, 1993; Midtlyng et al., 1996), although efficacy of levamisole as an adjuvant on vaccination has been debated (Morrison et al., 2000, 2001). Administration of levamisole through dietary supplementation has been reported to enhance antibody response to hepatitis B vaccination in humans (Kayatas, 2002); however, research on possible effects on antibody production in fish is currently very limited to the best of our knowledge. This experiment was designed to explore the effects of dietary levamisole on growth performance, innate and adaptive immunity and resistance of hybrid striped bass to *S. iniae* and *A. hydrophila* infection.

2. Materials and Methods

2.1 Experimental Diets

The basal diet, which utilized menhaden fish meal as the protein source, was formulated to contain 40% protein, 10% lipid and an estimated digestible energy level of 3.5 kcal/g (Table 17). This diet satisfied and/or exceeded all known nutrient requirements

Table 17. Dietary composition and macronutrient profile analysis

Constituents	Levamisole (mg kg ⁻¹ diet)				
	0	100	250	500	1000
<i>Ingredient (% dry weight)</i>					
Menhanden fish					
meal ^a	59.2	59.2	59.2	59.2	59.2
Dextrin ^b	25.0	25.0	25.0	25.0	25.0
Menhaden oil ^a	4.4	4.4	4.4	4.4	4.4
Mineral premix ^c	3.0	3.0	3.0	3.0	3.0
Vitamin premix ^c	4.0	4.0	4.0	4.0	4.0
Carboxymethyl					
cellulose ^b	2.0	2.0	2.0	2.0	2.0
Cellulose ^b	2.4	2.39	2.375	2.35	2.3
Levamisole	0	0.01	0.025	0.05	0.1
<i>Analyzed proximate composition (% dry matter) ^d</i>					
Dry matter	91.9	90.0	89.8	91.8	89.7
Crude protein	41.2	41.8	41.7	41.2	40.8
(N×6.25)					
Crude lipid	10.2	10.8	10.7	11.0	10.5
Ash	16.0	15.9	16.1	14.6	16.1

^a Omega Protein Corporation, Houston, TX. Menhaden fish meal contained 67.6% protein and 9.5% lipid on a dry-weight basis.

^b US Biochemical Corp., Cleveland, OH.

^c Same as Li et al. 2004a.

^d Means of two analyses.

of hybrid striped bass (Webster, 2002). Levamisole (Sigma, St. Louis, MO, USA) was added to the diet at 100, 250, 500, 1000 mg/kg diet in place of equal amounts of cellulose. Procedures for diet preparation and storage were as previously described by Webb and Gatlin (2003). Experimental diets were analyzed for moisture, crude protein, crude lipid and ash following established procedures (Webb and Gatlin, 2003) to confirm formulated composition.

2.2 Feeding trial 1

The first feeding trial was conducted to evaluate incremental levels of dietary levamisole on growth performance, non-specific immune responses as well as resistance of hybrid striped bass to *S. iniae* infection. Prior to initiation of this feeding trial, juvenile hybrid striped bass obtained from Keo Fish Farm (Keo, AR) were subjected to a 2-week conditioning period, to adjust to standardized regimes in a recirculating culture system consisting of 110-l aquaria. Water flow rate was maintained at approximately 650 ml/min via a recirculating system which maintained adequate water quality (total ammonia nitrogen ≤ 0.6 mg/l) through biological and mechanical filtration. Salinity was maintained at 2.5-3.5 ‰ using well water and synthetic sea salt (Fritz Industries Inc., Dallas, TX). Low pressure electrical blowers provided aeration via air stones and maintained dissolved oxygen (DO) levels at or near saturation. Water temperature was controlled by ambient air and remained at 26 ± 1 °C throughout the trial. A 12 h light: 12h dark photoperiod was maintained with fluorescent lights controlled by timers. Groups of 14 hybrid striped bass weighing approximately 40.6 g/fish were stocked into individual aquaria such that initial weight averaged 565 ± 12 g/group. The basal and

experimental diets were fed to three replicate groups of fish for 3 weeks. All groups were fed their respective diets at the same fixed rate (initially 3% of body weight per day and gradually reduced to 2%), which approached apparent satiation. Growth and feed efficiency were monitored weekly by collectively weighing each group of fish.

2.3 Sample collection and innate immune response assays

At the end of this feeding trial, three representative fish from each aquarium were anesthetized with an overdose of MS-222 and blood collected from the caudal vasculature. After a sample of whole blood was taken for hematocrit determination, serum was isolated by centrifugation ($3000 \times g$ for 5 min) and kept at -80°C . Head kidney macrophages were isolated by following the procedure described by Secombes (1990) with minor modifications as described by Sealey and Gatlin (2002b). Briefly, head kidneys from the three fish per tank were removed, pooled and finely ground with a tissue homogenizer. The homogenate was pushed through 100- μm mesh and layered on a 34%/51% Percoll gradient. After being centrifuged for 30 min at $400 \times g$ at 4°C , cells were collected and washed with Hank's balanced salt solution twice. The cells were counted and viability was determined by trypan blue to be more than 95%.

Serum lysozyme activity was determined by turbidimetric assay as described by Jørgensen et al. (1993). A lysozyme activity unit was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min^{-1} at $\text{pH} = 6.1$. Serum peroxidase was analyzed by following the procedure described by Rodriguez et al. (2003). Extracellular and intracellular superoxide anion production of head kidney macrophages was determined following the procedure of Secombes (1990). The amount of extracellular

superoxide anion was calculated from the formula: nanomoles superoxide anion per well = $(\Delta \text{ absorbance after 60 min} \times 100)/6.3$ (Pick and Mizel, 1981).

2.4. *S. iniae* challenge

After 4 weeks of feeding upon the experimental diets, representative fish were exposed to an estimated LD₅₀ dose of *Streptococcus iniae*. The isolate of *S. iniae* was kindly supplied by the Texas Veterinary Medical Diagnostic Laboratory (TVMDL) and confirmed by polymerase chain reaction. This isolate was grown in brain-heart infusion broth (EM Science, Darmstadt, Germany) in a shaking bath at 29 °C overnight. The concentration of bacterial suspension was determined by the serial plate count method and diluted to 2.6×10^6 CFU/ml in fresh well water. Twenty-four fish from each treatment (8 fish from each triplicate aquarium), pooled separately in mesh baskets, were immersed in the bacterial suspension for 2 hours. After bath exposure, the fish from each dietary treatment were divided into three groups of 8, and placed into 38-l flow-through aquaria in an isolated culture system. Water temperature was maintained at 27 ± 1 °C with immersion heaters. Fish continued to be fed their respective diets to apparent satiation twice daily, and mortality was monitored for 3 weeks. The brains of both dead fish and surviving fish were streaked on modified selective agar to determine infection status and confirm death from *S. iniae* (Nguyen and Kanai, 1999). Infected fish were counted as having suffered mortality.

2.5. Feeding trial 2 and *A. hydrophila* challenge

The second feeding trial was conducted to explore the effect of dietary levamisole on resistance of hybrid striped bass to *A. hydrophila* infection. Prior to initiation of this feeding trial, juvenile hybrid striped bass obtained from Keo Fish Farm (Keo, AR) were subjected to a 2-week conditioning period to adjust to standardized regimes in a recirculating culture system consisting of 38-l aquaria. Water quality and environmental factors were maintained as described above. Groups of 18 (18.4 ± 0.4 g/fish, mean \pm S. D.) hybrid striped bass were stocked into individual aquaria. Basal and experimental diets with the same graded levels of levamisole as in trial 1 were fed to two replicate groups of fish for 3 weeks. All groups were fed their respective diets at the same fixed rate (initially 3% of body weight per day and gradually reduced to 2%), which approached apparent satiation. At the end of 3 weeks, *A. hydrophila* (ATCC 49140) was obtained from ATCC (Manassas, VA, USA) and was grown in brain-heart infusion broth (EM Science, Darmstadt, Germany) in a shaking bath at 26 °C overnight. The bacteria were centrifuged and resuspended in PBS. Because the experimental infection model for *A. hydrophila* had not been established with hybrid striped bass, an estimated LD₅₀ established with goldfish (Rahman et al., 2001), which has the most similar environmental requirements as hybrid striped bass was employed in the present study. Fish were anesthetized by MS-222 and 0.2 ml of 1×10^7 CFU/ml bacterial suspension was injected intraperitoneally. Fish were delivered to the isolated flow-through system consisting of 38-l aquaria and equally distributed as 12 fish in each of three aquaria per treatment. . Water temperature was maintained at approximate 25 °C by ambient air. Salinity of water was 0.5 ‰. The morbidity and mortality were monitored three times per

day for 30 days, although no mortality was observed after 20 days post-challenge. Fish were fed their originally assigned diets once per day during this period. Moribund fish were randomly sent to the TVMDL to confirm death from *A. hydrophila* infection.

2.6. Feeding trial 3 and assays of specific antibody titer against *S. iniae*

The third feeding trial was conducted to determine the effect of dietary supplementation of levamisole on serum antibody titer against *S. iniae*. Groups of 8 hybrid striped bass weighing approximately 36.6 ± 0.5 g/fish (mean \pm S. D.) were stocked into individual 38-l aquaria. The basal and levamisole-supplemented diets were fed to two replicate groups of fish for 3 weeks. All groups were fed their respective diets at the same fixed rate (initially 3% of body weight per day and gradually reduced to 2%) which approached apparent satiation. At the end of the 3-wk feeding, ten fish of similar size (approximately 56 g/fish) from each treatment were immunized following the procedure established by Sealey and Gatlin (2002). Briefly, each fish was injected intraperitoneally (IP) with 4×10^8 formalin-killed *Streptococcus iniae* cells/fish. Fish were given a booster IP injection of the same dose 10 days post-immunization. Fish continued to be fed their respective diets twice daily to apparent satiation. Surviving fish were bled 7 days after the booster IP injection. Serum was separated following the procedure described above and agglutination antibody titers were determined by microtitration agglutination as described by Roberson (1990). A positive reaction was described by the presence of a button with fuzzy edges; whereas, a negative reaction consisted of a round precipitate with clearly defined circular margins (Sealey and Gatlin, 2002). Antibody titer

was expressed as the reciprocal log base-2 of the highest dilution demonstrating a positive reaction.

All animal procedures used in this study were approved by the Texas A&M University Animal Care and Use Committee.

2. 7. *Statistics*

Data from the feeding trials, immune response assays and the bacterial challenge were subjected to analysis of variance and Duncan's multiple-range test using the Statistical Analysis System. Differences in treatment means were considered significant at $P < 0.05$.

3. **Results**

3.1 *Growth performance (feeding trial 1)*

After the 3-week feeding period in feeding trial 1, enhanced weight gain was generally observed in fish fed the diets supplemented with low concentrations (<500 mg/kg) of levamisole (Table 18). Dietary supplementation of levamisole at 100 mg/kg significantly ($P < 0.05$) enhanced growth and feed efficiency by approximately 10%, compared to fish fed the basal diet (Table 18). However, fish fed the diet supplemented with 1000 mg levamisole/kg diet showed growth retardation, reduced feed intake and feed efficiency. Survival during feeding trial 1 was high and no significant differences were observed among treatments (Table 18).

The growth responses generated from feeding trial 2 and 3 showed a similar trend

Table 18. Performance of hybrid striped bass fed diets containing various levels of levamisole for 3 weeks in feeding trial 1^a

Levamisole (mg/kg)	Growth (% increase)	Feed efficiency (g gain/ g feed)	Survival (%)
0	50.2 ^y	0.84 ^{yz}	97.6
100	55.2 ^x	0.93 ^x	97.6
250	53.4 ^{xy}	0.90 ^x	100
500	52.4 ^{xy}	0.87 ^{xy}	97.6
1000	46.6 ^z	0.78 ^{yz}	97.6
P	0.014	0.004	0.903
Pooled. S. E.	1.412	0.020	2.128

^a Values represent means of five replicate groups. Values in a column that do not have the same superscript are significantly different at $P \leq 0.05$ based on Duncan's multiple range test.

^b Fish initially weighed 40.6 ± 0.6 (mean \pm 0.6) g each.

^c Significance probability associated with the F statistic.

as in feeding trial 1, although the growth enhancement was not significant at $P < 0.05$ based on only duplicate groups of fish. However, growth retardation and depressed feed intake and feed efficiency resulting from an overdose of levamisole (1000 mg/kg) were confirmed (data not shown).

3.2 Select innate immune response assays (feeding trial 1)

The average hematocrit of juvenile hybrid striped bass was relative high in the present study without significant dietary influences (Table 19). Although the average serum peroxidase level of fish fed the levamisole-supplemented diet was noticeably higher than fish fed the basal diet, it was not statistically significant (Table 19). Serum lysozyme of fish fed 1000 mg levamisole/kg diet tended to be lower compared with that of fish fed the other diets (Table 19). Fish fed the diet with 250 mg levamisole/kg had significantly ($P < 0.05$) higher intracellular superoxide anion production than fish fed other diets; however, the extracellular superoxide anion production was not significantly influenced by dietary supplements (Table 19).

3.3 *S. iniae* challenge (feeding trial 1)

The disease challenge with live *S. iniae* resulted in approximately 50% mortality of fish fed the basal diet after 19 days. The relative percent survival (RPS) for fish fed 100, 250, 500 and 1000 mg levamisole/kg diet were 43.7, 50.0, 62.5 and -25, respectively. The moribund fish showed typical signs of *S. iniae* infection including extremely erratic swimming, cloudiness of the eyes, haemorrhages around mouth and base of fins, dark pigmentation and slow acceptance or refusal of feed. Because of the large variance,

Table 19. Haematological and immune responses of hybrid striped bass fed graded levels of levamisole in feeding trial 1^a

Levamisole (mg/kg)	Extracellular superoxide anion (nmol O ₂) ^b	Intracellular superoxide anion (O.D. at 620 nm) ^b	Hematocrit (%) ^c	Serum Lysozyme (unit ml ⁻¹) ^c	Serum Peroxidase (O. D. at 450 nm) ^c
0	3.81	0.88 ^z	55.2	367.8	0.85
100	3.45	0.81 ^z	50.9	417.8	1.17
250	3.19	1.16 ^y	53.2	354.4	1.25
500	3.47	0.91 ^z	54	408.9	1.36
1000	3.23	0.83 ^z	54.8	236.7	1.16
P	0.68	0.042	0.782	0.392	0.254
Pooled. S. E.	0.323	0.064	2.57	70.57	0.156

^a Values in a column that do not have the same superscript are significantly different at $P \leq 0.05$ based on Duncan's multiple range test.

^b Means of three samples of pooled head kidney cells from 3 fish from each aquarium of the same treatment.

^c Means of nine samples of individual fish.

which possibly resulted from subsequent infection from cohabitation instead of immersion, the influence of dietary levamisole on survival of hybrid striped bass after challenge with *S. iniae* cannot be demonstrated in this study (Fig. 4a).

3.4 A. *hydrophila* challenge (feeding trial 2)

The IP injection resulted in cumulative mortality of approximately 40% over the 20 days post-challenge (Fig. 2). Mortality was monitored for 30 days and no mortality was observed after 20 days. The bacteria caused a rapid infection and mortality began 48 hours after exposure. Infected fish did not show noticeable signs of septicemia, although all the challenged fish showed sluggish movement and slow acceptance or refusal of diet, as well as reddening of the skin. The relative percent survival (RPS) for fish fed 100, 250, 500 and 1000 mg levamisole/kg diet were 21.6, 18.7, 33.8 and -2.3, respectively. Possibly because of the infection from cohabitation in individual aquarium, the variation in mortality among replicate tanks was high. Fish fed the levamisole-supplemented diet for 3 weeks (less than 500 mg/kg provide exact level) failed to show significantly improved survival than fish fed the basal diet.

3.5 Agglutinating antibody titers against *S. iniae* (feeding trial 3)

After booster injection with formalin-killed *S. iniae*, agglutinating antibody was detected in most of the fish (approximately 82%). The variation among individual fish across dietary treatment was large and no significant differences were observed (Fig. 4c).

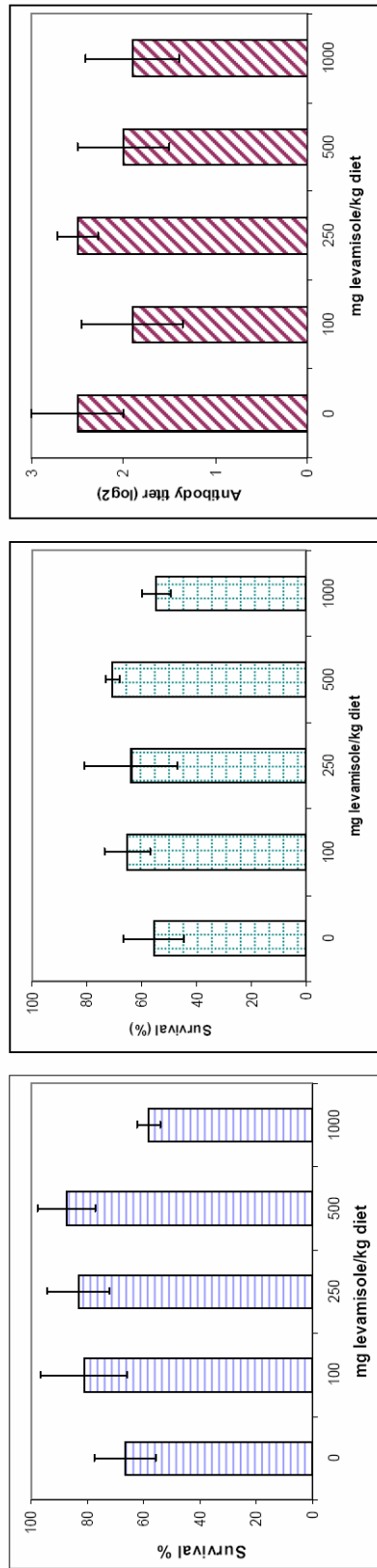


Fig. 4a. Percent cumulative survival of hybrid striped bass fed incremental levels of levamisole (0, 100, 250, 500 and 1000 mg/kg) for 4 weeks and subsequently exposed to *S. iniae* by immersion. Symbols represent means and standard errors of three replicate tanks per treatment ($P=0.36$, Pooled SE = 11.4).

Fig. 4b. Percent cumulative survival of hybrid striped bass fed incremental levels of levamisole (0, 100, 250, 500 and 1000 mg/kg) for 3 weeks and subsequently exposed to *A. hydrophila* by intraperitoneal injection. Symbols represent means and standard errors of three replicate tanks per treatment. ($P=0.77$, Pooled SE = 10.1).

Fig. 4c. Agglutinating antibody titer against *S. iniae* in hybrid striped bass fed incremental levels of levamisole (0, 100, 250, 500 and 1000 mg/kg) for 3 weeks and subsequently exposed to formalin-killed *S. iniae* by intraperitoneal injection. Symbols represent means and standard errors of ten replicate individual fish per treatment. ($P=0.774$, Pooled SE = 0.5).

4. Discussion

Fish growth and disease resistance are two primary concerns in aquaculture. A wide variety of antibiotics and other antibacterial agents, antiparasitic drugs, ionophores as well as anabolic and xenobiotic agents have been screened for growth control of terrestrial animals (reviewed by Beermann, 1989); however, current knowledge in this area with respect to aquatic animals is very limited. Because the growth rate of fish is generally slower than farmed terrestrial animals, growth regulation by dietary manipulation should be a prioritized topic in aquaculture research. Levamisole is one of the most recognized immunostimulants for aquaculture (reviewed by Sakai, 1999). It has been shown to enhance growth of carp (Siwicki and Korwin-Kossakowski, 1988) and gilthead sea bream (Mulero et al., 1998), by immersion and short-term (10 days) oral administration, respectively. To the best of our knowledge, a detailed feeding protocol to induce rapid fish growth by levamisole has not been reported. In the present study, juvenile hybrid striped bass fed low doses of levamisole (100-250 mg/kg) had significantly enhanced feed efficiency, and growth increased by approximately 10% after 3 weeks of feeding. This confirmed the growth-promoting effects of levamisole reported by other researchers and also may represent a new strategy to enhance industrial production. Based on mammalian research, levamisole can be extensively and rapidly metabolized by the liver such that residues are very low (Kayatas, 2002). This characteristic would be very suitable for use in hybrid striped bass culture in addition to the low cost and low effective dosage of this compound. The present study also confirmed the previous observation that excessive use of levamisole (1000 mg/kg) resulted in growth depression and reduction of feed efficiency (Li et al., 2004b), although

the possible influence of levamisole overdose on immunity and disease resistance was not demonstrated. Regardless the significant benefits conferred by feeding levamisole-supplemented diets for 3-weeks, information pertaining to long-term administration and potential tissue accumulation of levamisole is still needed to guarantee its safe and optimal use. In addition, the mechanism by which levamisole regulates growth and possibly influences the hypothalamus–pituitary gland–liver axis is important to characterize for potentially maximizing the benefit of levamisole; therefore, this warrants further study.

It has been shown that levamisole is capable of enhancing immune responses and/or reducing losses from bacteria and/or parasitic infections in many cultured species including carp (Siwicki, 1989; Baba et al., 1993), gilthead sea bream (Mulero et al., 1998), rainbow trout (Siwicki et al., 1989; Kajita et al., 1990; Jeney and Anderson, 1993), Atlantic salmon (Findlay and Munday 2000; Findlay et al., 2000; Morrison et al., 2001) rohu (Sahoo and Mukherjee, 2002), eel (Geets et al., 1992), cobia (Leano et al., 2003) and giant prawn (Baruah and Prasad, 2001). In the present study, only intracellular superoxide anion production of head kidney cells of fish fed the diet supplemented with 250 mg levamisole/kg was significantly higher than that of fish fed the other experimental diets. Because respiratory burst of phagocytic cells is recognized as an important defensive mechanism of fish and other animals, this enhancement may result in resistance to certain pathogens. Because both *S. iniae* and *A. hydrophila* are not intracellular pathogens, challenge with other pathogens such as viruses and intracellular bacterial species may be important to fully define the potential use of dietary levamisole to enhance fish health management. Based on the results from controlled challenges with

S. iniae and *A. hydrophila* after 3 weeks of feeding levamisole-supplemented diets, the influence on resistance to infection of these two bacterial species was marginal. Because dose and timing are important factors influencing the efficacy of immunostimulants (Sakai, 1999), other administration protocols should be investigated thoroughly before a definite conclusion can be drawn concerning the effectiveness of levamisole in protecting against *S. iniae* and *A. hydrophila* infection. In addition, although dietary effects on hematocrit are not significant, the hematocrit of all treatments in the present study were higher than any published data on hybrid striped bass (Sealey and Gatlin, 2002a; Li and Gatlin, 2003; Li et al., 2004a). Because high hematocrit (over 50%) is sometimes considered as an indicator of stress (Barton, 2000), this phenomenon may complicate the interpretation of lack of dietary difference in resistance to the two bacteria species, even though no obvious stressor can be traced in the present study. The serum peroxidase and lysozyme levels were within a range that have been reported (Sealey and Gatlin, 2002; Li and Gatlin, 2003; 2005; Jaramillo and Gatlin, 2004) and were not affected by dietary supplementation of levamisole. Because levamisole has been hypothesized to influence immunity by changing cytokine production such as interferon, interleukin 1 and 2 (Redondo et al., 1987; Kimball et al., 1991), measurement of responsive cytokine production may provide insight to optimal use of levamisole in aquafeeds.

Administration of levamisole by immersion and injection has been reported to be capable of enhancing specific antibody production in rainbow trout (Jeney and Anderson, 1993), Atlantic salmon (Midtlyng et al., 1996) and rohu (Sahoo et al., 1999), respectively. Sahoo and Mukherjee (2002) also reported dietary levamisole (5 mg levamisole/kg/day for every 3 days) restored specific immunity of rohu depressed by Aflatoxin B₁, but did

not influence healthy fish. The agglutinating antibody titer of juvenile hybrid striped bass against *S. iniae* failed to show significant or noticeable dietary effects in the present study, which is consistent with the observations made by Sahoo and Mukherjee (2002). The variance among individual fish fed the same diets in the present experiment was rather high, which lessens the conclusiveness of this result. Because possible influences of genetic polymorphisms on fish antibody responses are not fully defined, the genetic background of fish for experiments usually has not been strictly controlled. In addition to possible genetic variation, a recent study showed that antiserum should be stored in -4°C instead of -73°C to maintain biological activity (Nitzan et al., 2003) and suggested a significant modification of traditional protocol. These uncertainties need to be clarified to provide a definitive answer pertaining to the potential use of dietary levamisole as an adjuvant for vaccination against *S. iniae*.

CHAPTER VII

EXCESSIVE DIETARY LEVAMISOLE SUPPRESSES GROWTH PERFORMANCE OF HYBRID STRIPED BASS (*Morone chrysops*×*M. saxatilis*), AND ELEVATED LEVAMISOLE IN VITRO IMPAIRS MACROPHAGE FUNCTION*

1. Introduction

Traditionally, antibiotics have been the most important chemotherapeutics in aquaculture for prevention and treatment of bacterial diseases. However, the use of antibiotics in aquaculture may pose threats such as development of bacterial strains that are more resistant to antibiotic treatment, or the occurrence of antibiotic residues in farmed fish for human consumers (FAO, 2002). Based on these two considerations, the potential uses of existing antibiotics and approval of new ones for aquaculture have been very limited. Alternatively, immunostimulants hold promise as alternative strategies against infectious aquatic diseases and have received heightened attention in recent years (Gatlin, 2002). One such compound, levamisole, an antihelminthic drug commonly used with terrestrial livestock, has been shown with several aquatic species to be a potent immunostimulant in modulation of T-cell function (Renoux, 1980), cytotoxic activity of leukocytes (Cuesta et al, 2002a), phagocytosis (Mulero et al., 1998a), respiratory burst

* Reprinted from *Aquaculture Research*, Vol 35, Li, P., Wang, X., Gatlin, D. M. III, Excessive dietary levamisole suppresses growth performance of hybrid striped bass (*Morone chrysops*×*M. saxatilis*), and elevated levamisole in vitro impairs macrophage function, pp. 1380-1383, Copyright 2004, with permission from Blackwell Publishing.

(Mulero et al., 1998a; Li and Gatlin unpublished data) and macrophage-activating factor (Mulero et al., 1998a). It also has been reported to enhance resistance to amoebic gill disease of Atlantic salmon (Findlay et al, 2000; Munday and Zilberg, 2003) and swimbladder nematode infection of eel (Geets et al., 1992) as well as improve growth performance when administered orally (Mulero et al., 1998a) or by water bath (Siwicki and Korwin-Kossakowski, 1988). Thus, levamisole has shown potential as a dietary supplement for some fish species including hybrid striped bass, an important aquacultured fish in the United States. However, based on studies with terrestrial animals, levamisole can cause acute and chronic toxicity when dose and administration time are not proper (JECFA, 1991). Toxicity of levamisole to Atlantic salmon during sea water bath exposure was recently described (Munday and Zilberg, 2003). However, the toxic level of dietary levamisole in fish is not fully defined. Therefore, the present study was conducted to determine effects of dietary levamisole on growth performance of hybrid striped bass and *in vitro* macrophage response to different levels of levamisole.

2. Materials and Methods

2.1. In-vivo feeding trial

The basal diet was composed of 59.2% menhaden fish meal (Special Select[®], Omega Protein Corporation, Reedville, VA, USA), 4.4% menhaden fish oil (Omega Protein Corporation, Reedville, VA, USA), 25% dextrin, 2% carboxymethyl cellulose, 2.4% cellulose (US Biochemical Corp., Cleveland, OH, USA), 3% vitamin premix and 4% mineral premix. This diet was formulated to contain 40% protein, 10% lipid and an estimated digestible energy level of 3.5 kcal/g. The vitamin and mineral premix

formulation was described by Li et al. (2004). This diet satisfied and/or exceeded all known nutrient requirements of hybrid striped bass (Webster, 2002) or other warmwater fishes (NRC, 1993). Levamisole (Sigma, St. Louis, MO, USA) was added to the basal diet in place of equal amounts of cellulose to provide either 500 or 1000 mg/kg diet.

Prior to initiation of this feeding trial, juvenile hybrid striped bass obtained from Keo Fish Farms (Keo, AR) were subjected to a 2-week conditioning period to adjust to standardized regimes in a recirculating culture system consisting of 110-l aquaria. Groups of 20 hybrid striped bass weighing approximately 6.5 g/fish were stocked into individual aquaria such that initial weight averaged 130.4 ± 1.6 g/group (mean \pm s. e.). Salinity was maintained at 2.0 ± 0.5 ppt. The basal and experimental diets were all fed to three replicate groups of fish for 4 weeks. All groups were fed their respective diets twice daily at the same fixed rate (Table 20) to maintain a level approaching apparent satiation. Growth and feed efficiency were monitored weekly by collectively weighing each group of fish.

2.2. In-vitro study

Twenty hybrid striped bass maintained on a commercial diet (Rangen, Inc., Angleton, TX, USA) in earthen ponds were captured and subjected to conditioning (25 ± 1 °C, 2.5 ppt salinity) in a 1100-l recirculating tank for 1 week before isolating head kidney cells. Twenty fish were killed by pithing and head kidneys were removed and pooled. The macrophages were prepared following the procedure described by Secombes (1990) with minor modifications as described by Sealey and Gatlin (2002). Cell viability was determined to be greater than 95% by trypan blue (0.4%) exclusion. A 100- μ l aliquot

of cell suspension with 2×10^6 cells was added to each well of three, 96-well plates. Various levels (0, 1, 10, 100, 1000 $\mu\text{g ml}^{-1}$) of levamisole were added to L-15 media (Cellgro[®], Herndon, VA, USA) with 0.1 % fetal calf serum (Hyclone[®], Logan, UT, USA), 100 unit ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin (Sigma, St. Louis, MO, USA). Each levamisole dose was added to four replicate wells in each of the three 96-well plates. Plates were incubated for 24, 48 and 72 h at room temperature. Media were changed every 24 h in plates incubated for 48 and 72 h. Intracellular superoxide anion production was determined by the procedure of Secombes (1990). All data were subjected to analysis of variance and Duncan's multiple-range test using the Statistical Analysis System (SAS, 1985). Differences in treatment means were considered significant at $P < 0.05$.

3. Results and Discussion

The growth trial showed significantly suppressed growth and feed efficiency of juvenile hybrid striped bass after 3 weeks of consuming the diet with 1000 mg levamisole/kg (approximately 51.6 mg/kg body weight daily), while this trend became noticeable after 2 weeks of feeding (Table 20). Although side-effects of levamisole-adjuvanted vaccine including mild pathology of gills (Morrison, Nowak & Carson 2001) and suppressed antibody titer (Morrison, Nowak & Carson 2000) have been reported, the chronic toxicity of dietary levamisole to hybrid striped bass observed in this present study is the first report in aquaculture to the best of our knowledge. JECFA (1991) reported in a 30-day feeding trial 50 mg levamisole/kg body weight daily resulted in moderate reduction in weight gain of rats. This chronic toxicity level of dietary levamisole is very

Table 20. Cumulative weight gain (% of initial weight) and feed efficiency (g gain/g feed) of hybrid striped bass fed diets containing various amounts of levamisole for 4 weeks ^{1,2}

Response variable	Dietary levamisole (mg/kg)	Week			
		1	2	3	4
Weight gain (%)	0	39.6 ± 1.1	91.2 ± 0.9	166.6 ± 1.5 ^a	205.5 ± 2.8 ^a
	500	40.9 ± 1.5	93.4 ± 2.5	167.4 ± 3.4 ^a	209.6 ± 3.6 ^a
	100	39.7 ± 0.8	85.4 ± 1.5	145.4 ± 2.5 ^b	176.0 ± 2.9 ^b
P ³		0.511	0.097	0.023	0.012
Feed efficiency	0	1.13 ± 0.2	0.86 ± 0.1	1.07 ± 0.2 ^a	0.96 ± 0.2 ^a
	500	1.17 ± 0.3	0.87 ± 0.2	1.03 ± 0.2 ^a	0.95 ± 0.2 ^a
	100	1.13 ± 0.1	0.79 ± 0.2	0.92 ± 0.2 ^b	0.83 ± 0.2 ^b
P ³		0.474	0.072	0.011	0.004

¹ Values represent means ± standard error of three replicate groups. Values in a column that do not have the same superscript are significantly different at $P \leq 0.05$ based on Duncan's multiple range test.

² Feeding rate during weeks 1, 2, 3, 4 was the same for each diet at 5, 6, 4.5 and 4% of body weight per day.

³ Significance probability associated with the F statistic.

similar to that observed in the present study. This study failed to show beneficial effects of levamisole on growth based on the 4-week trial duration. This result is not conclusive due to limited supplementation levels and administration time; therefore, additional research is warranted. One very recent study (Leano et al., 2003) showed 2-week feeding with 1000 mg levamisole/kg diet enhanced non-specific immunity and disease resistance of cobia (*Rachycentron canadum*) fingerlings without any noticeable effects on growth. This may indicate a species-specific difference in levamisole tolerance.

The *in-vitro* study showed 1000 µg levamisole/ml noticeably and significantly ($P < 0.0001$) suppressed superoxide anion production after 24-h incubation (Fig. 5), possibly attributable to reduced cell viability or availability of intracellular NADPH oxidase (Fig. 5). Even 100 µg levamisole/ml suppressed macrophage function after 48-h incubation. The suppressive effect of levamisole on head kidney cells was confirmed to be dose- and time- dependent. Siwicki and Cossarini- Dunier (1990) reported the suppressive effects of levamisole (100-200 µg/ml) on phagocytosis of carp spleen and head kidney macrophages. The *in-vitro* toxicity level of levamisole to hybrid striped bass kidney leukocytes in the present study is similar with those reported in the carp study. Mulero et al. (1998b) reported that greater than 10 µg levamisole/ml could kill seabream leucocytes. Results from the present study showed that kidney cells of hybrid striped bass could tolerate this level without an obvious decrease in cell viability. Siwicki et al. (1990) reported enhanced respiratory burst of carp intact spleen after incubation with 5 µg levamisole/ml. In a related study (Siwicki and Cossarini- Dunier, 1990), carp macrophage function was enhanced when incubated with 0.8 – 6.2 µg levamisole/ml. However, the present study and that of Mulero et al. (1998b) failed to show the beneficial influences

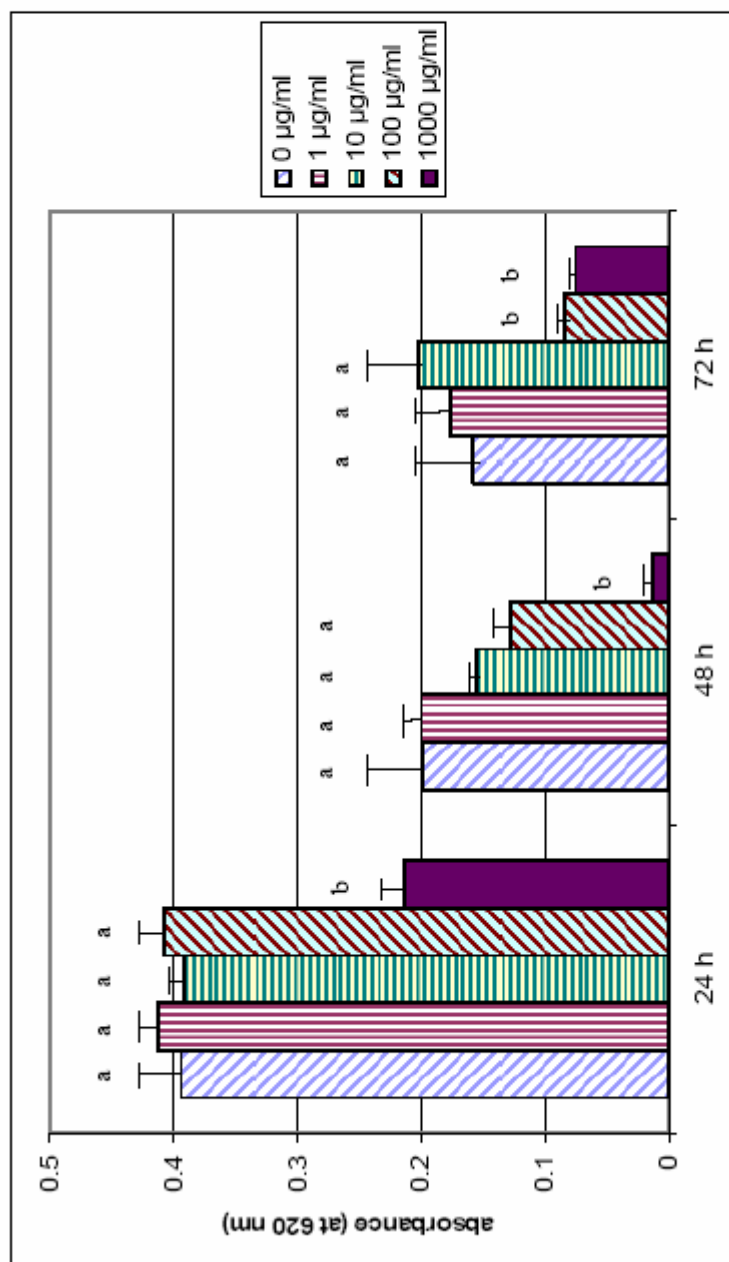


Fig. 5. Intracellular superoxide anion production of head kidney cells in media with various levels of levamisole (0, 1, 10, 100, 1000 $\mu\text{g ml}^{-1}$) at various incubation intervals (24, 48 and 72 h).

of levamisole on respiratory burst of head kidney cells of hybrid striped bass or githead sea bream. Mulero et al. (1998b) hypothesized enhanced production of macrophage activating factors by stimulation with levamisole influenced macrophage responses. Additional research is warranted to determine the effects of levamisole on growth and immunity of hybrid striped bass by optimizing levamisole dosage and timing of administration.

CHAPTER VIII

CONCLUSIONS

Six studies were conducted with hybrid striped bass to evaluate various dietary supplements including autolyzed brewers yeast, the commercial prebiotic GroBiotic[®]-A, oligonucleotides and levamisole in terms of their effects on growth, immunological responses and disease resistance.

In the first study with brewers yeast, enhanced weight gain and feed efficiency were generally observed in fish fed the diets supplemented with yeast at 2% compared to the basal diet in both trials. In the second trial, body composition of whole fish, hemocrit and serum lysozyme levels were observed to be within normal ranges and not influenced by yeast supplementation. After 9 weeks of feeding in the second trial, exposure to *S. iniae* resulted in no mortality and reduced signs of disease in fish fed diets supplemented with 2% and 4% brewers yeast, while 20% mortality was observed in fish fed the control diet ($P=0.1$). Blood neutrophil oxidative radical production, extracellular and intracellular superoxide anion production of head kidney macrophages and serum lysozyme were measured after 16 weeks of feeding each diet, in the second trial. Fish fed the diet with 2% brewers yeast had significantly ($P<0.01$) higher blood neutrophil oxidative radical and extracellular superoxide anion production of head kidney macrophages than control fish. Based on the results of the first study, it was concluded that brewers yeast positively influenced growth performance and feed efficiency of hybrid striped bass as well as resistance to *S. iniae* infection. In addition, results of immune response assays

demonstrated that brewers yeast can be administered for relatively long periods without causing immunosuppression.

The second study compared the effects of dietary supplementation of a commercial prebiotic GroBiotic[®]-A and brewers yeast on the performance of juvenile hybrid striped bass. Enhanced growth performance was generally observed in juvenile fish fed the diets supplemented with GroBiotic[®]-A or brewers yeast compared to the basal diet after 7 weeks of feeding in trial 1. Significantly higher ($P<0.05$) feed efficiency was observed in fish fed diets supplemented with 1% and 2% GroBiotic[®]-A. After 4 weeks of feeding in trial 2, growth and feed efficiency were not significantly affected by the various dietary treatments, although some immunological responses were altered. Neutrophil oxidative radical anion production and intracellular superoxide anion production of head kidney macrophages tended to be higher in fish fed diets supplemented with brewers yeast or GroBiotic[®]-A, while extracellular superoxide anion production of head kidney macrophages from fish fed diets with 1% and 2% brewers yeast and 1% GroBiotic[®]-A was significantly ($P<0.01$) higher than that of fish fed the basal diet. All groups of fish fed brewers yeast and GroBiotic[®]-A showed significantly ($P<0.01$) enhanced survival (73.3–90%) after bath exposure to *Streptococcus iniae* compared with fish fed the basal diet (53.3%). These data substantiated that GroBiotic[®]-A and a partially autolyzed brewers yeast can serve as functional feedstuffs in the diet of hybrid striped bass by enhancing growth performance and immunological responses.

The third study was to evaluate the dietary supplementation of GroBiotic[®]-A and brewers yeast in the diet of sub-adult hybrid striped bass. It confirmed previous observations of positive effects of these supplements on growth of juvenile hybrid striped

bass. At the end of 16 and 21 weeks of the feeding trial, fish fed 2% brewers yeast had significantly higher feed efficiency than those fed the other diets. An *in situ* mycobacterial challenge employed in this experiment resulted in overall cumulative mortality of approximately 25%. Fish fed 2% GroBiotic[®]-A had significantly ($P < 0.05$) enhanced survival (80%) compared with those fed the other treatments (72–73%) at the end of 21 weeks. It is concluded that dietary supplementation of 2% GroBiotic[®]-A showed moderate but significant protection against mycobacterial infection. Dietary supplementation of partially autolyzed brewers yeast also may enhance growth performance under chronic infection of mycobacteria. Future studies should include targeting the dynamics of intestinal microbiology in response to prebiotics, screening for beneficial probiotics and developing novel prebiotics and symbiotics (a probiotic together with a prebiotic) for health management of hybrid striped bass and other fish species.

The fourth study failed to show significant differences in growth performance between hybrid striped bass fed basal and oligonucleotide-supplemented diets. Body composition of whole fish, hematocrit and serum lysozyme levels were observed to be within normal ranges and not influenced by dietary nucleotides. However, neutrophil oxidative radical production of fish fed the nucleotide-supplemented diet was significantly ($P=0.011$) higher than in fish fed the basal diet. Significantly ($P<0.05$) enhanced survival after exposure to *S. iniae* also was generally observed in fish fed the nucleotide-supplemented diet. In addition, fish fed the nucleotide-supplemented diet tended to have a higher antibody response based on microtitration agglutination; however, the difference was not statistically significant because of high variation among individual fish. Long-term (16 weeks) administration of oligonucleotides in trial 3 of this

study failed to show enhancement of immune responses between treatments. It is concluded that dietary oligonucleotides positively influenced immune responses and resistance of juvenile hybrid striped bass to *S. iniae* infection.

The fifth study showed dietary supplementation of levamisole at 100 mg/kg diet significantly ($P<0.05$) enhanced growth and feed efficiency of hybrid striped bass, compared with fish fed the basal diet. However, fish fed a diet supplemented with 1000 mg levamisole/kg diet showed chronic toxicity signs such as inferior growth, reduced feed intake and depressed feed efficiency. Although the extracellular superoxide anion production of head kidney cells, hematocrit, serum lysozyme and peroxidase were not significantly affected by dietary treatments, intracellular superoxide anion production of head kidney macrophages of fish fed 250 mg levamisole/kg diet was significantly ($P<0.05$) higher than that of fish fed the other four diets. The hypothesized benefits of dietary levamisole supplementation in reducing mortality from infection of pathogenic bacteria including *S. iniae* and *A. hydrophila* were not observed in two separate 3-week feeding trials and disease challenges in the present study. Agglutinating antibody titers of individual fish were highly variable and not significantly influenced by dietary levamisole at the tested levels.

The sixth study confirmed the growth-suppressing effect of excessive use of levamisole in the diet of hybrid striped bass. An *in-vitro* trial with hybrid striped bass macrophages failed to demonstrate a direct influence of levamisole on superoxide anion production; however, elevated levamisole level in the medium significantly suppressed the respiratory burst and cell viability. These observations suggested proper

administration of immunostimulants such as levamisole is essential for beneficial influences and additional studies with this compound are needed.

Although the concept of functional feeds is novel to the aquaculture industry, it represents an emerging new paradigm to develop diets that extend beyond satisfying basic nutritional requirements of the cultured organism. Observations with hybrid striped bass have shown that research in this area is worthy of further pursuit and may positively impact aquacultural production. Further work pertaining to understanding the mechanism(s) associated with the dynamics of hormones and cytokines as well as expression of immune genes responsible for these beneficial dietary influences should be pursued.

REFERENCES

- Acosta, F., Real, F., Ellis, A. E., Tabraue, C., Padilla, D., Ruiz de Galarreta, C. M., 2005. Influence of vaccination on the nitric oxide response of gilthead seabream following infection with *Photobacterium damsela* subsp. *Piscicida*. *Fish Shellfish Immunol.* 18, 31-38.
- Adamek, Z., Hamackova, J., Kouril, J., Vachta, R., Stibranyiova, I., 1996. Effect of Ascogen probiotics supplementation on farming success in rainbow trout (*Oncorhynchus mykiss*) and wels (*Silurus glais*) under conditions of intensive culture. *Krmiva (Zagreb)* 38, 11–20.
- Aggett, P., Leach, J. L., Rueda, R., MacLean, W. C., 2003. Innovation in infant formula development: a reassessment of ribonucleotides in 2002. *Nutrition* 19, 375-384.
- Ai, Q., Mai, K., Zhang, C., Xu, W., Duan, Q., Tan B., Liufu, Z., 2004. Effects of dietary vitamin C on growth and immune response of Japanese seabass, *Lateolabrax japonicus*. *Aquaculture* 242, 489-500.
- Anderson, D.P., Siwicki, A.K., Rumsey, G.L., 1995. Injection or immersion delivery of selected immunostimulants to trout demonstrate enhancement of nonspecific defense mechanisms and protective immunity. In: Shariff, M., Arthur, J.R. and Subasinghe, R.P., (Eds.), *Diseases in Asian Aquaculture: II. Fish Health Section*, Asian Fisheries Society, Manila, pp. 413–426.
- Arasteh, N., Aminirissehei, A. H., Yousif, A. N., Albright, L. J., Durance, T. D., 2004. Passive immunization of rainbow trout (*Oncorhynchus mykiss*) with chicken egg yolk immunoglobulins (IgY). *Aquaculture* 231, 23-26.

- Baba, T., Watase, Y., Yoshinaga, Y., 1993. Activation of mononuclear phagocyte function by levamisole immersion in carp. *Nippon Suisan Gakk.* 59, 301-307.
- Barton, B. A., 2000. Stress. In: Stickney, R.R., (Ed), *Encyclopedia of Aquaculture*. Wiley, New York, USA, pp. 892–898.
- Bartos, J. M., Sommer, C. V., 1981. In vivo cell mediated immune response to *M. tuberculosis* and *M. salmoniphilum* in rainbow trout (*Salmo gairdneri*). *Dev. Comp. Immunol.* 5, 75-83.
- Baruah, N. D., Prasad, K. P., 2001. Efficacy of levamisole as an immunostimulant in *Macrobrachium Rosenbergii* (De man). *J. Aquacult. Trop.* 16:149-158.
- Beermann D. H., 1989. Status of current strategies for growth regulation. In: Campion, D. R., Hausman, G. J., Martin, R. J. (Eds.), *Animal Growth Control*. Plenum Press, New York, USA, pp. 377-396.
- Blaut, M., 2002. Relationship of prebiotics and food to intestinal microflora. *Eur. J. Nutr.* 41, 11S-16S.
- Bowser, P.R., Wooster, G.A., Getchell, R.G., Timmons, M.B., 1998. *Streptococcus iniae* infection of tilapia *Oreochromis niloticus* in a recirculation production facility. *J. World Aquac. Soc.* 29, pp. 335–339.
- Buchanan, J. T., Stannard, J. A., Westerman, M. E., Ostland, V. E., Van Olst, J. C., Carlberg, J. M., Nizet, V., 2003. Transposon mutagenesis and the identification of virulence genes in the aquaculture pathogen *Streptococcus iniae*. *Book of Abstracts Aquaculture America 2003*, 18 Febuary-20 Feburary, 2003, Louisville, KY, USA.

- Burrells, C., William, P. D., Forno, P. F., 2001a. Dietary nucleotides: a novel supplement in fish feeds 1. Effects on resistance to diseases in salmonids. *Aquaculture* 199, 159-169.
- Burrells, C., William, P. D., Southage, P. J., Wadsworth, S. L., 2001b. Dietary nucleotides: a novel supplement in fish feeds 2. Effects on vaccination, salt water transfer, growth rate and physiology of Atlantic salmon. *Aquaculture* 199, 171-184.
- Cabaj, W., Stankiewicz, M., Jonas, W. E., Moore, L. G., 1995. Levamisole and its influence on the immune responses of lambs. *Vet. Res. Commun.* 19, 17-26.
- Cabib E., Roberts, R., Bowers, B., 1982. Synthesis of the yeast cell wall and its regulation. *Annu. Rev. Biochem.* 51, 763-793.
- Carver, J. D., 1994. Dietary nucleotides: cellular immune, intestinal and hepatic system effects. *J. Nutr.* 124, S144–S148.
- Carver, J. D., Walker, W. A., 1995. The role of nucleotides in human nutrition. *J. Nutr. Biochem.* 6, 58-72.
- Chinabut, S., Limsuwan, C., Chanratchakool, P., 1990. Mycobacteriosis in the snakehead, *Channa striatus* (Fowler). *J. Fish Dis.* 13, 531-535.
- Chen, S. C., Yoshida, T., Adams, A., Thompson, K. D., Richards, R. H., 1998. Non-specific immune response of Nile tilapia, *Oreochromis nilotica* (*Oreochromis niloticus*), to the extracellular products of *Mycobacterium* spp. and to various adjuvants. *J. Fish Dis.* 21, 39-46.
- Colorni, A., Avtalion, R., Knibb, W., Berger, E., Colorni, B., Timan, B., 1998. Histopathology of sea bass (*Dicentrarchus labrax*) experimentally infected with

- Mycobacterium marinum* and treated with streptomycin and garlic (*Allium sativum*) extract. *Aquaculture* 160, 1-17.
- Couso, N., Castro, R., Magariños, B., Obach, A., Lamas, J., 2003. Effect of oral administration of glucans on the resistance of gilthead seabream to pasteurellosis. *Aquaculture* 219, 99-109.
- Cuesta, A., Meseguer, J., Esteban, M. A., 2002. Levamisole is a potent enhancer of gilthead sea bream natural cytotoxic activity. *Vet. Immunol. Immunopathol.* 89, 169-174.
- Cuesta, A., Meseguer, J., Esteban, M. A., 2004. Total serum immunoglobulin M are affected by immunomodulators in sea bream (*Sparus aurata* L.). *Vet. Immunol. Immunopathol.* 101, 203-210.
- Darwish, A. M., Rawles, S. D., Griffin, B. R., 2002. Laboratory efficacy of oxytetracycline for the control of *Streptococcus iniae* infection in blue tilapia. *J. Aquat. Animal Hlth.* 14, 184-190.
- Dixon, B., Stet, R. J. M., 2001. The relationship between major histocompatibility receptors and innate immunity in teleost fish. *Dev. Comp. Immunol.* 25, 683–700.
- Dodson, S.V., Maurer, J.J., Shotts, E.B., 1999. Biochemical and molecular typing of *Streptococcus iniae* isolated from fish and human cases. *J. Fish Dis.* 22, 331–336.
- dos Santos, N. M. S., do Vale, A., Sousa, M. J., Silva, M. T., 2002. Mycobacterial infection in farmed turbot *Scophthalmus maximus*. *Dis. Aquat. Org.* 52, 87-91.
- Eldar, A., Ghittino, C., 1999. *Lactococcus garvieae* and *Streptococcus iniae* infections in rainbow trout *Oncorhynchus mykiss*: similar, but different diseases. *Dis. Aquat. Org.* 36, 227–231.

- Eldar, A., Perl, S., Frelier, P.F., Bercovier, H., 1999. Red drum *Sciaenops ocellatus* mortalities associated with *Streptococcus iniae* infection. Dis. Aquat. Org. 36, 121–127.
- Engstad, R. E., Robertsen, B., Frivold, E., 1992. Yeast glucan induces increase in activity of lysozyme and complement-mediated haemolytic activity in Atlantic salmon blood. Fish Shellfish Immunol. 2, 287-297.
- Esteban, M. A., Cuesta, A., Ortuño, J., Meseguer, J., 2001. Immunomodulatory effects of dietary intake of chitin on gilthead seabream (*Sparus aurata* L.) innate immune system. Fish Shellfish Immunol. 11, 303-315.
- Evans, J.J., Shoemaker, C.A., Klesius, P.H., 2000. Experimental *Streptococcus iniae* infection of hybrid striped bass *M. chrysops*×*M. saxatilis* and tilapia *Oreochromis niloticus* by nares inoculation. Aquaculture 189, 197–210.
- FAO, 2002. Antibiotics residue in aquaculture products. The state of world fisheries and aquaculture 2002. Rome, Italy. pp 74-82.
- FAO, 2004. The state of world fisheries and aquaculture 2004. Rome, Italy. pp 14-17.
- Fattal-German, M., Bizzini, B., 1992. Assessment of the anti-viral effect of a short-term oral treatment of mice with live *Saccharomyces cerevisiae* cells. Dev. Biol. Standard. 77, 115-120.
- Fearon, D. T., Locksley, R. M., 1996. The instructive role of innate immunity in the acquired immune response. Science 272, 50-54.
- Fearon, D. T., 1997. Seeking wisdom in innate immunity. Nature 388, 323-324.
- Ferber, D., 2000. Superbugs on the hoof? Science 288, 792-794.

- Findlay, V. L., Munday, B. L., 2000. The immunomodulatory effects of levamisole on the nonspecific immune system of Atlantic salmon, *Salmo salar* L. J. Fish Dis. 23, 369-378.
- Findlay, V. L., Zilberg, D., Munday, B. L., 2000. Evaluation of levamisole as a treatment for amoebic gill disease of Atlantic salmon, *Salmo salar* L. J. Fish Dis. 23, 193-198.
- Frerichs, G. N., 1993. Mycobacteriosis: nocardiosis. In: Inglis, V., Roberts, R. J., Bromage, N. R. (Eds). Bacterial Diseases of Fish. Blackwell, Oxford, UK, pp 219-233.
- Gannam, A.L. and Schrock, R.M., 2001. Immunostimulants in fish diets. In: Lim, C., Webster, C.D., (Eds.) Nutrition and Fish Health, Haworth Press, Binghamton, NY, USA. pp. 235–266.
- Gatesoupe, F. J., 1999. The use of probiotics in aquaculture. Aquaculture 180, 147-165.
- Gatlin, D. M. III., 1997. Nutrition and feeding of striped bass and hybrid striped bass. In: Harrell, R. M. (Ed.), Striped Bass and Other Morone Culture, Elsevier, Amsterdam, Netherlands. pp. 235-251.
- Gatlin, D. M. III., 2002. Nutrition and fish health. In: Halver, J. E., Hardy, R. W. (Eds). Fish Nutrition. Academic Press, San Diego, CA, USA. pp 671-702.
- Gauthier, D. T., Rhodes, M. W., Vogelbein, W. K., Kator, H., Ottinger, C. A., 2003. Experimental mycobacteriosis in striped bass *Morone saxatilis*. Dis. Aquat. Org. 54, 105-117.

- Gaylord, T.G., Gatlin, D. M. III., 2000. Effects of dietary carnitine and lipid on growth and body composition of hybrid striped bass *Morone chrysops* x *M. saxatilis*. *Fish Physiol. Biochem.* 22, 297-302.
- Geets, A., Liewes, E. W., Ollevier, F. 1992. Efficacy of some anthelmintics against the swimbladder nematode *Anguillicola crassus* of eel *Anguilla anguilla* under saltwater conditions. *Dis. Aquat. Org.* 13, 123-128.
- Gibson, G. R., Roberfroid, M. B., 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr.* 125, 1401-1412.
- Goslee, S.C., Wolinsky, E., 1976. Water as a source of potentially pathogenic mycobacteria. *Am. Rev. Respir. Dis.* 113, 287-292.
- Greenlees, K.J., Machado, J., Bell, T., Sundlof, S., 1998. Food borne microbial pathogens of cultured aquatic species. *Vet. Clin. North Am.* 14, 101–112.
- Gutierrez, M. A., Miyazaki, T., Hatta, H., Kim, M., 1993. Protective properties of egg yolk IgY containing anti-*Edwardsiella tarda* antibody against paracolo disease in the Japanese eel, *Anguilla japonica*. *J. Fish Dis.* 16, 113–122.
- Harms, C. A., Howard, K. E., Wolf, J. C., Smith, S. A., Kennedy-Stoskopf, S., 2003. Transforming growth factor- β response to mycobacterial infection in striped bass *Morone saxatilis* and hybrid tilapia *Oreochromis* spp. *Vet. Immunol. Immunopathol.* 95, 155-163.
- Harrell, R. M., Webster, D. W., 1997. An overview of *Morone* culture. In: Harrell, R. M. (Ed.), *Striped Bass and Other Morone Culture*, Elsevier, Amsterdam, Netherlands pp. 1-9.

- Harris, J. H., Bird, D. J., 2000. Modulation of the fish immune system by hormones. *Vet. Immunol. Immunopathol.* 77, 163-176.
- Hiney, M., Rodger, H., Bricknell, I., Casburn, P., Mulcahy, D., 2002. Aquaculture development and regulation: incompatibility or harmony. *Bull. Eur. Assoc. Fish Pathol.* 22, 178-184.
- Hrubec, T. C., Roberson, J. L., Smith, S. A., 1997. Effects of temperature on hematological and serum biochemical profiles of hybrid striped bass (*Morone chrysops*×*M. saxatilis*). *Am J. Vet. Res.* 58, 126-130.
- Hrubec, T.C., Smith, S.A., Robertson, J.L., 2001. Age-related changes in hematology and plasma chemistry values of hybrid striped bass *Morone chrysops*×*M. saxatilis*. *Vet. Clinic. Pathol.* 30, 8–15.
- Irianto, A., Austin, B., 2002. Probiotics in aquaculture. *J. Fish Dis.* 25, 633-642.
- Jaramillo, F., Jr., Gatlin, D. M. III., 2004. Comparison of purified and practical diets supplemented with or without β -glucan and selenium on resistance of hybrid striped bass to *Streptococcus iniae* infection. *J. World Aquacult. Soc.* 35, 245-252.
- JECFA, Joint FAO/WHO Expert Committee on Food Additives, 1991. Levamisole. *WHO Food Additive Series* 27, 75-101.
- Jeney, G., Anderson, D. P., 1993. Enhanced immune response and protection in rainbow trout to *Aeromonas salmonicida* bacterin following prior immersion in immunostimulants. *Fish Shellfish Immunol.* 3, 51-58.
- Jørgensen, J.B., Lunde, H., Robertsen, B., 1993a. Peritoneal and head kidney cell response to intraperitoneally injected yeast glucan in Atlantic salmon. *J. Fish Dis.* 16, 313–325.

- Jørgensen, J. B., Sharp, J. E., Secombes, C. J., Robertsen, B., 1993b. Effect of a yeast-cell-wall glucan on the bactericidal activity of rainbow trout macrophages. *Fish Shellfish Immunol.* 3, 267-277.
- Jyonouchi, H., Zhang, L., Tomita, Y., 1993. Studies of immunomodulating actions of RNA/nucleotides: RNA/Nucleotides enhance *in vitro* immunoglobulin production by human peripheral blood mononuclear cells in response to T-dependent stimuli. *Pediatr. Res.* 33, 458-465.
- Jyonouchi, H., Zhang-Shanbhag, L., Tomita, Y., Yokoyama, H., 1994. Nucleotide-free diet impairs T-helper cell functions in antibody production in response to T-dependent antigens in normal C57B1/6 mice. *J. Nutr.* 124, 475-481.
- Keembiyehetty, C. N., Gatlin, D. M. III, 1997. Dietary threonine requirement of juvenile hybrid striped bass. *Aquacult. Nutr.* 3, 217-221.
- Kaige, N., Miyazaki, T. and Kubota, S.S., 1984. The pathogen and histopathology of vertebral deformity in cultured yellowtail. *Fish Pathol.* 19, 173-179.
- Kajita, Y., Sakai, M., Atsuta, S., Kobayashi, M., 1990. The immunomodulatory effects of levamisole on rainbow trout, *Oncorhynchus mykiss*. *Fish Pathol.* 25, 93-98.
- Kayatas, M., 2002. Levamisole treatment enhances protective antibody response to hepatitis B vaccination in hemodialysis patients. *Artif. Org.* 26, 492-496.
- Kimball, E. S., Clark, M. C., Schneider, C. R., Persico, F. J., 1991. Enhancement of *in vitro* lipopolysaccharide-stimulated interleukin-1 production by levamisole. *Clin. Immunol. Immunopathol.* 58, 385-398.

- Klesius, P.H., Shoemaker, C.A., Evans, J.J., 2000. Efficacy of single and combined *Streptococcus iniae* isolate vaccine administered by intraperitoneal and intramuscular routes in tilapia *Oreochromis niloticus*. *Aquaculture* 188, 237–246.
- Kohler, C.C., 2000. Striped bass and hybrid striped bass culture. In: Stickney, R.R., (Ed.), *Encyclopedia of Aquaculture*, Wiley, New York, USA, pp. 898–907.
- Lara-Flores, M., Olvera-Novoa, M. A., Guzmán-Méndez, B. E., López-Madrid, W., 2002. Use of the bacteria *Streptococcus faecium* and *Lactobacillus acidophilus*, and the yeast *Saccharomyces cerevisiae* as growth promoters in Nile tilapia (*Oreochromis niloticus*). *Aquaculture* 216, 193-201.
- Leano, E. M., Guo, J., Chang, S., Liao, I. C., 2003. Levamisole enhances non-specific immune response of cobia (*Rachycentron canadum*) fingerlings. *J. Fish. Soc. Taiwan*. 30, 321-330.
- Lee, S. B., Mine, Y., Stevenson, R. M. W., 2000. Effects of hen egg yolk immunoglobulin in passive protection of rainbow trout against *Yersinia ruckeri*. *J. Agric. Food Chem.* 48, 110-115.
- Li, P., Gatlin, D. M. III, 2003. Evaluation of brewers yeast (*Saccharomyces cerevisiae*) as a feed supplement for hybrid striped bass (*Morone chrysops*×*M. saxatilis*). *Aquaculture* 219, 681-692.
- Li, P., Lewis, D. H., Gatlin, D. M. III., 2004a. Dietary oligonucleotide influences immune responses and resistance of hybrid striped bass (*Morone chrysops*×*M. saxatilis*) to *Streptococcus iniae* infection. *Fish Shellfish Immunol.* 16, 561-569.
- Li, P., Wang, X., Gatlin, D. M. III., 2004b. Excessive dietary levamisole suppresses growth performance of hybrid striped bass (*Morone chrysops*×*M. saxatilis*), and

- elevated levamisole in vitro impairs macrophage function. *Aquacult. Res.* 35, 1380-1383.
- Li, P., Gatlin, D. M. III., 2005a. Nucleotide nutrition in fish: current knowledge and future applications. *Aquaculture* in press.
- Li, P., Gatlin, D. M. III., 2005b. Evaluation of the prebiotic GroBiotic®-A and brewers yeast as dietary supplements for sub-adult hybrid striped bass (*Morone chrysops*×*M. saxatilis*) challenged in situ with *Mycobacterium marinum*. *Aquaculture* 248, 197-205.
- Lin, M. F., Shiau, S. Y., 2005. Dietary L-ascorbic acid affects growth, nonspecific immune responses and disease resistance in juvenile grouper, *Epinephelus malabaricus*. *Aquaculture* 244, 215-221.
- Lo, D., Feng, L., Carson, M. J., Crowley, M., Pauza, M., Nguyen, A., 1999. Integrating innate and adaptive immunity in the whole animal. *Immunol Rev* 169, 225–239.
- Low, C., Wadsworth, S., Burrells, C., Secombes, C. J., 2003. Expression of immune genes in turbot (*Scophthalmus maximus*) fed a nucleotide-supplemented diet. *Aquaculture* 221, 23-40.
- Lunden, T., Miettinen, S., Linnström, L.G., Lilius, E.M., Bylund, G., 1999. Effect of florfenicol on the immune response of rainbow trout (*Oncorhynchus mykiss*). *Vet. Immunol. Immunopathol.* 67, 317-325.
- Lunden, T., Lilius, E.M., Bylund, G., 2002. Respiratory burst activity of rainbow trout (*Oncorhynchus mykiss*) phagocytes is modulated by antimicrobial drugs. *Aquaculture* 207, 203-212.

- Magnadóttir, B., 2005. Innate immunity of fish (overview). *Fish Shellfish Immunol.* 20, 137-151.
- Manning, T. S., Gibson, G. R., 2004. Prebiotics. *Best Prac. Res. Clin. Gastroenterol.* 18, 287-298.
- Matsumoto, Y., Adje, A. A., Yamauchi, K., Kise, M., Nakasone, Y., Shinegawa, Y., 1995. Mixture of nucleosides and nucleotides increases bone marrow cell and peripheral neutrophil number in mice infected with methicillin-resistant *Staphylococcus aureus*. *J. Nutr.* 125, 815–822.
- Matsuo, K., Miyazano, L., 1993. The influence of long-term administration of peptidoglycan on disease resistance and growth of juvenile rainbow trout. *Nippon Suisan Gakk.* 59, 1377–1379.
- Matsuyama, H., Mangindaan, E.P., Yano, T., 1992. Protective effect of schizophyllan and scleroglucan against *Streptococcus* sp. infection in yellowtail *Seriola quinqueradiata*. *Aquaculture* 101, 197–203.
- Mediel, M. J., Rodriguez, V., Codina, G., Martin-Casabona, N., 2003. Isolation of mycobacteria from frozen fish destined for human consumption. *Appl. Environm. Microbiol.* 66, 3637-3638.
- Midtlyng, P.J., Reitan, L.J., Speilberg, L., 1996. Experimental studies on the efficacy and side-effects of intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. *Fish Shellfish Immunol.* 6, 335–350.
- Morrison, R. N., Nowak, B. F., Carson, J. 2000. The effect of levamisole as an adjuvant on humoral immune response of Atlantic salmon , *Salmo salar* L. *Bull. Eur. Assoc. Fish Pathol* 20, 101-105.

- Morrison, R. N., Nowak, B. F., Carson, J. 2001. The histopathological effects of a levamisole-adjuvanted *Vibrio anguillarum* vaccine on Atlantic salmon *Salmo salar* L. *Aquaculture* 195, 23-33.
- Mulero, V., Esteban, M. A., Munoz, J., Meseguer, J., 1998a. Dietary intake of levamisole enhances the immune response and disease resistance of the marine teleost gilthead seabream, *Sparus aurata* L. *Fish Shellfish Immunol.* 8, 49-62.
- Mulero, V., Esteban, M. A. and Meseguer, J., 1998b. *In vitro* levamisole fails to increase seabream (*Sparus aurata* L.) phagocyte functions. *Fish Shellfish Immunol.* 8, 315-318.
- Munday, B. L., Zilberg, D., 2003. Efficacy of, and toxicity associated with, the use of levamisole in sea water to treat amoebic gill disease. *Bull. Eur. Ass. Fish Pathol.* 23, 3-6.
- National Research Council, 1993. Nutrient Requirements of Fish. National Academy Press, Washington, DC, USA, pp 114.
- Navarro, J., Ruiz-bravo, A., Jimenez-Varela, M., Gil, A., 1996. Modulation of antibody-forming cell and mitogen-driven lymphoproliferative responses by dietary nucleotides in mice. *Immunol. Lett.* 53, 141.
- Nayar, S., Hegde, S., Rao, P. S., Sudha, P., 1998. Live organisms as feed in aquaculture. *Infofish Intern.* 4, 36-39.
- Nguyen, H. T., Kanai, K., 1999. Selective agars for the isolation of *Streptococcus iniae* from Japanese flounder, *Paralichthys olivaceus*, and its cultural environment. *J. Appl. Microbiol.* 86, 769-776.

- Nguyen, H.T., Kanai, K., Yoshikoshi, K., 2001a. Experimental *Streptococcus iniae* infection in Japanese flounder *Paralichthys olivaceus*. Fish Pathol. 36, 40–41.
- Nguyen, H.T., Kanai, K., Yoshikoshi, K., 2001b. Immunohistochemical examination of experimental *Streptococcus iniae* infection in Japanese flounder *Paralichthys olivaceus*. Fish Pathol. 36, 169–178.
- Nikoskelainen, S., Salminen, S., Bylund, G., Ouwehand, A. C., 2001. Characterization of the properties of human and dairy derived probiotics for prevention of infectious diseases in fish. Appl. Environm. Microbiol. 67, 2430-2435.
- Nitzan, S., Shwartsburd, B., Heller, E. D. 2003. The effect of storing hybrid bass (*Morone saxatilis* × *M. chrysops*) sera at freezing temperatures on antibody titers. Fish Shellfish Immunol. 15, 341-347.
- Oliva-Teles, A., Goncalves P., 2001. Partial replacement of fishmeal by brewers yeast *Saccaromyces cerevisiae* in diets for sea bass *Dicentrarchus labrax* juveniles. Aquaculture 202, 269-278.
- Olsen, R. E., Myklebust, R., Kryvi, H., Mayhew, T. M., Ringø, E., 2001. Damaging effect of dietary inulin on intestinal enterocytes in Arctic charr (*Salvelinus alpinus* L.). Aquacult. Res. 32, 931-934.
- Ortuño, J., Cuesta, A., Rodríguez, A., Esteban, M. A., Meseguer, J., 2002. Oral administration of yeast, *Saccharomyces cerevisiae*, enhances the cellular innate immune response of gilthead seabream (*Sparus aurata* L.). Vet. Immunol. Immunopathol. 85, 41-50.

- Overton, A. S., Margraf, F. J., Weedon, C. A., Pieper, L. H., May, E. B., 2003. The prevalence of mycobacterial infections in striped bass in Chesapeake Bay. *Fish. Manage. Ecol.* 10, 5, 301-308.
- Ostland, V., 2003. The importance of approved antibiotics for the treatment of systemic bacterial infections of hybrid striped bass. Presented at World Aquaculture 2003, 18 February-20 February, 2003, Louisville, KY, USA.
- Patterson, J. A., Burkholder, K. M., 2003. Application of prebiotics and probiotics in poultry production. *Poultry Sci.* 82, 627-631.
- Perera, R.P., Johnson, S.K., Collins, M.D., Lewis, D.H., 1994. *Streptococcus iniae* associated with mortality of *Tilapia nilotica* × *T. aurea* hybrids. *J. Aquat. Anim. Hlth* 6, 335–340.
- Perera, R.P., Johnson, S.K., Lewis, D.H., 1997. Epizootological aspects of *Streptococcus iniae* affecting tilapia in Texas. *Aquaculture* 152, 25–33.
- Pick, E., Mizel, D., 1981. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic immunoassay reader. *J. Immunol. Meth.* 46, 211-226.
- Plumb, J. A., 1997. Infectious diseases of striped bass. In: Harrell, R. M. (Ed.), *Striped Bass and Other Morone Culture*, Elsevier, Amsterdam, Netherlands. pp. 271-313.
- Rahman, M. H., Suzuki, S., Kawai, K., 2001. The effect of temperature on *Aeromonas hydrophila* infection in goldfish *Carassius auratus*. *J. Appl. Ichthol.* 17, 282-285.
- Ramadan, A., Atef, M., 1991. Effect of the biogenic performance enhancer (Ascogen “S”) on growth rate of tilapia fish. *Acta Vet. Scand.* 87, S304-306.

- Ramadan, A., Afifi, N. A., Moustafa, M., Samy, A. M., 1994. The effect of ascogen on the immune response of tilapia fish to *Aeromonas hydrophila* vaccine. Fish Shellfish Immunol. 5, 159-165.
- Rawles, S. D., Gatlin, D.M. III., 1998. Carbohydrate utilization in striped bass *M. chrysops* × *M. saxatilis*. Aquaculture 161, 201-212
- Rawles, S.D., Gatlin III, D.M., 2000. Nutrient digestibility of common feedstuffs in extruded diets for sunshine bass *M. chrysops* × *M. saxatilis*. J. World Aquacult. Soc. 31, 570-579.
- Rawles, S. D., Kocabas, A., Gatlin, D. M. III, Du, W. X., Wei, C. I., 1997. Dietary supplementation of Terramycin and Romet-30 does not enhance growth of channel catfish but does influence tissue residues. J. World Aquacult. Soc. 28, 392-401.
- Renoux, G., 1980. The general immunopharmacology of levamisole. Drugs 19, 89-99.
- Redondo, J. M, Lopen-Guerrero, Frenso, M., 1987. Potentiation of Interleukin-2 by levamisole and levamisole imidazole. Immunol. Lett. 14, 111-116.
- Rijkers, G. T., Teunissen, A. G., Oosterom, R. V., Muiswinkel, W. B. V., 1980. The immune system of cyprinid fish. The immunosuppressive effect of the antibiotic oxytetracycline in carp (*Cyprinus carpio* L.). Aquaculture 19, 177-189.
- Rijkers, G. T., Oosterom, R. V., Muiswinkel, W. B. V., 1981. The immune system of cyprinid fish. Oxytetracycline and the regulation of humoral immunity in carp (*Cyprinus carpio*). Vet. Immunol. Immunopathol. 2, 281-290.
- Ringø, E., Olsen, R. E., 1999. The effect of diet on aerobic bacterial flora associated with intestine of Arctic charr, *Salvelinus alpinus* L. J. Appl. Microbiol. 86, 22-28.

- Ringø, E., Bendiksen, H. R., Gausen, S. J., Sundsfjord, A., Olsen, R. E., 1998. The effect of dietary fatty acids on lactic acid bacteria associated with the epithelial mucosa and from faecalia of Arctic charr, *Salvelinus alpinus* L. J. Appl. Microbiol. 85, 855-864.
- Roberson, B. S. 1990. Bacterial agglutination. In: Stolen, J. S., Fletcher, T. C., Anderson, D. P., Roberson, B. S., van Muiswinkel, W. B. (Eds.), Techniques in Fish Immunology. SOS Publications, Fair Haven, NJ, USA. pp. 81–86.
- Rodríguez, A., Cuesta, A., Ortuño, J., Esteban, M. A., Meseguer, J., 2003. Immunostimulant properties of a cell wall-modified whole *Saccharomyces cerevisiae* strain administered by diet to seabream (*Sparus aurata* L.). Vet. Immunol. Immunopathol. 96, 183-192.
- Rumsey, G. L., Winfree, R. A., Hughes, S. G., 1992. Nutritional values of dietary nucleic acids and purine bases to rainbow trout. Aquaculture 108, 97-110.
- Rumsey, G. L., Kinsella, J. E., Shetty, K. J., Hughes, S. G., 1991. Effect of high dietary concentration of brewer's dried yeast on growth performance and liver uricase in rainbow trout (*Oncorhynchus mykiss*). Anim. Feed Sci. Tech. 33, 177-183.
- Sahoo, P. K., Mukherjee, S. C., 2002. The effect of dietary immunomodulation upon *Edwardsiella tarda* vaccination in healthy and immunocompromised Indian major carp, *Labeo rohita*. Fish Shellfish Immunol. 12, 1-16.
- Sahoo, P. K., Mohanty, J., Mukherjee, S. C., 1999. The effect of three immunostimulators on haematological parameters and immunity level in rohu (*Labeo rohita*) fingerlings. J. Aquacult. Trop. 14, 127–135.

- Sakai, M., 1999. Current research status of fish immunostimulants. *Aquaculture* 172, 63-92.
- Sakai, M., Taniguchi, K., Mamoto, K., Ogawa, H., Tabata, M., 2001. Immunostimulant effects of nucleotide isolated from yeast RNA on carp, *Cyprinus carpio* L. *J. Fish Dis.* 24, 433-438.
- Sanchez-Pozo, A., Gil, A., 2002. Nucleotides as semiessential nutritional components. *Br. J. Nutr.* 87, S135–S137.
- SAS Institute, 1985. SAS User's Guide: Statistics, Version 5 Edition. Cary, NC, USA. pp. 956.
- Sealey, W.M., Gatlin III, D.M., 2001. Overview of nutritional strategies affecting the health of marine fish. In: Lim, C., Webster, C.D., (Eds.), *Nutrition and Fish Health*, Haworth Press, Binghamton, NY, USA, pp. 103–118.
- Sealey, W. M., Gatlin, D. M., III., 2002a. Dietary vitamin C and vitamin E interact to influence growth and tissue composition of juvenile hybrid striped bass *Morone chrysops* x *M. saxatilis* but have limited effects on immune responses. *J. Nutr.* 132, 748-755.
- Sealey, W. M., Gatlin, D. M. III., 2002b. *In vitro* manipulations of vitamin C and vitamin E concentrations alter intracellular O₂⁻ production of hybrid striped bass (*Morone chrysops* x *M. saxatilis*). *Fish Shellfish Immunol.* 12, 131-140.
- Sealey, W.M. and Gatlin III, D.M., 2002c. Dietary supplementation of vitamin C and/or vitamin E before or after experimental infection with *Streptococcus iniae* has limited effects on survival of hybrid striped bass. *J. Aquat. Anim. Hlth* 14, 165–174.

- Secombes, C. J., 1990. Isolation of salmonid macrophages and analysis of their killing activity. In: Stolen, J. S., Fletcher, T. C., Anderson, D. P., Roberson, B. S., van Muiswinkel, W. B. (Eds.), *Techniques in Fish Immunology*. SOS Publications, Fair Haven, NJ, USA. pp. 137-154.
- Secombes, C. J., 2002. Cloning and bioactivity of fish cytokines and their relevance to disease resistance. *Bull. Eur. Ass. Fish Pathol.* 22, 110-116.
- Shoemaker, C. A., Klesius, P. H., Lim, C., 2001. Immunity and disease resistance in fish. In: Lim, C., Webster, C. D. (Eds.), *Nutrition and Fish Health*. Haworth Press, Binghamton, NY, USA, pp. 149-162.
- Siwicki, A. K., 1989. Immunostimulating influence of levamisole on nonspecific immunity in carp, *Cyprinus carpio*. *Dev. Comp. Immunol.* 13, 87-91.
- Siwicki, A. K., Korwin-Kossakowski, M., 1988. The influence of levamisole on the growth of carp (*Cyprinus carpio* L.) larvae. *J. Appl. Ichthyol.* 4, 178-181.
- Siwicki, A. K., Cossarini-Dunier, M., 1990. Effect of levamisole on the lymphocyte and macrophage activity in carp *Cyprinus carpio*. *Annales de Recherches Veterinaires* 21, 95-100.
- Siwicki, A. K., Anderson, D. P., Dixon, O. W., 1989. Comparisons on nonspecific and specific immunomodulation by oxolinic acid, oxytetracycline and levamisole in salmonids. *Vet. Immunol. Immunopathol.* 23, 195-200.
- Siwicki, A. K., Anderson, D. P., Dixon, O. W., 1990. *In vitro* immunostimulation of rainbow trout, *Oncorhynchus mykiss* spleen cells with levamisole. *Dev. Comp. Immunol.* 14, 231-237.

- Siwicki, A. K., Anderson, D. P., Rumsey, G. L., 1994. Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis. *Vet. Immunol. Immunopathol.* 41, 125-139.
- Stickney, R. R., 1997. Offshore mariculture. In: Bardach J. E. (Ed.), *Sustainable Aquaculture*. John Wiley & Sons, Inc., New York, NY, USA, pp. 53-86.
- Stoffregen, D. A., Backman, S. C., Perham, R. E., Bowser, P. R., Babish, P. R., 1996. Initial disease report of *Streptococcus iniae* infection in hybrid striped (sunshine) bass and successful therapeutic intervention with the fluoroquinolone antibacterial enrofloxacin. *J. World Aquacult. Soc.* 27, 420-434.
- Subasinghe, R. P., 2005. Epidemiological approaches to aquatic animal health management: opportunities and challenges for developing countries to increase aquatic production through aquaculture. *Prev. Vet. Med.* 67, 117-124.
- Szilagyi, A., 2002. Lactose- a potential prebiotic. *Aliment Pharmacol. Ther.* 16, 1591-1602.
- Taffaella, C., Novoa, B., Alvares, J.M., Figueras, A., 1999. In vivo and in vitro effect of oxytetracycline treatment on the immune response of turbot, *Scophthalmus maximus* (L.). *J. Fish Dis.* 22, 271-276.
- Teitelbaum, J. E., Walker, W. A., 2002. Nutritional impact of pre-and probiotics as protective gastrointestinal organisms. *Annu. Rev. Nutr.* 22, 107-138.
- Thanardkit, P., Khunrae, P., Suphantharika, M. and Verduyn, C., 2002. Glucan from spent brewer's yeast: preparation, analysis and use as a potential immunostimulants in shrimp feed. *World J. Microbiol. Biotechnol.* 18, 527-539.

- Verlhac, V., Obach, A., Gabaudan, J., Schüep, W., Hole, R., 1998. Immunomodulation by dietary vitamin C and glucan in rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.* 8, 409-424.
- Webb, K. A., Gatlin, D. M. III., 2003. Effects of dietary protein level and form on production characteristics and ammonia excretion of red drum *Sciaenops ocellatus*. *Aquaculture* 225, 17-26.
- Webster C. D., 2002. Hybrid striped bass In: Webster C. D., Lim, C. (Eds.). *Nutrient Requirements and Feeding of Finfish for Aquaculture*. CABI Publishing, Wallingford, U. K. pp 327-343.
- White, L. A., Newman, M. C., Cromwell, G. L., Lindemann, M. D., 2002. Brewers dried yeast as a source of mannan oligosaccharides for weanling pigs. *J. Anim. Sci.* 80, 2619-2628.
- Wijkström, U. N., 2003. Short and long-term prospects for consumption of fish. *Vet. Res. Comm.* 27, 461S-468S.
- Wolf, J. C., Smith, S. A., 1999. Comparative severity of experimentally induced mycobacteriosis in striped bass *Morone saxatilis* and hybrid tilapia *Oreochromis spp.* *Dis. Aquat. Org.* 38, 191-200.
- Yamauchi, K., Hales, N. W., Robinson, S. M., Niehoff, M. L., Ramesh, V., Pellis, N. R., 2002. Dietary nucleotides prevent decrease in cellular immunity in ground-based microgravity analog. *J. Appl. Physiol.* 93, 161–166.
- Yoshida, T., Kruger, R., Inglis, V., 1995. Augmentation of non-specific protection in African catfish, *Clarias gariepinus* (Burchell), by the long-term oral administration of immunostimulants. *J. Fish Dis.* 18, 195–198.

Yuasa, K., Kitancharoen, N., Kataoka, Y., Al-Murbyaty, F.A., 1999. *Streptococcus iniae*, the causative agent of mass mortality in rabbitfish *Siganus canaliculatus* in Bahrain. *J. Aquat. Anim. Hlth.* 11, 87–93.

APPENDIX



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6 September 2005

Peng Li
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 Via email lipeng@tamu.edu

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AQUACULTURE, Vol 219, No 1-4, 2003, pp 681-692, Li et al: "Evaluation of brewers..."

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AQUACULTURE, Vol 249, o 1-4, 2005, pp 197-205, Li et al: "Evaluation of the ..."

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FISH AND SHELLFISH IMMUNOLOGY, Vol 16, No 5, 2004, pp 561-569, Li et al: "Dietary oligonucleotides ..."

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Refereed Publications:

- Li, P., Gatlin, D. M. III., 2003. Evaluation of brewers yeast (*Saccharomyces cerevisiae*) as a feed supplement for hybrid striped bass (*Morone chrysops* × *M. saxatilis*). *Aquaculture* 219, 681-692.
- Li, P., Lewis, D. H., Gatlin, D. M. III., 2004. Dietary oligonucleotides from yeast RNA influences immune responses and resistance of hybrid striped bass (*Morone chrysops* × *M. saxatilis*) to *Streptococcus iniae* infection. *Fish and Shellfish Immunology* 16, 561-569.
- Li, P., Gatlin, D. M. III., 2004. Dietary brewers yeast and the prebiotic GroBiotic[®]-A influence growth performance, immune responses and resistance of hybrid striped bass (*Morone chrysops* × *M. saxatilis*) to *Streptococcus iniae* infection. *Aquaculture* 231, 445-456.
- Li, P., Wang, X., Hardy, R. W., Gatlin, D. M. III., 2004. Nutritional value of fisheries by-catch and by-product meals in the diet of red drum (*Sciaenops ocellatus*). *Aquaculture* 236, 485-496.
- Li, P., Gatlin, D. M. III., 2005. Evaluation of the prebiotic GroBiotic[®]-A and brewers yeast as dietary supplements for sub-adult hybrid striped bass (*Morone chrysops* × *M. saxatilis*) challenged *in situ* with *Mycobacterium marinum*. *Aquaculture* 248, 197-205.
- Li, P., Bur, G. S., Goff, J. B., Whiteman, K. W., Davis, K. B., Vega, R. R., Neill, W. H., Gatlin, D. M. III., 2005. A preliminary study on the effects of dietary supplementation of brewers yeast and nucleotides, singularly or in combination, on juvenile red drum (*Sciaenops ocellatus*). *Aquaculture Research* 36, 1120-1127.
- Li, P., Gatlin, D. M. III., 2005. Nucleotide nutrition in fish: current knowledge and future applications. *Aquaculture in press*.