SUPPLEMENTATION OF ORGANIC ACIDS AND ALGAE EXTRACTS IN AQUAFEEDS: IMMUNOLOGICAL IMPACTS

A Thesis

by

MARIA GUADALUPE MENDOZA-RODRIGUEZ

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

MASTER OF SCIENCE

Chair of Committee,	Delbert M. Gatlin III
Committee Members,	Michael Criscitiello
	Michael Hume
Head of Department,	Michael Masser

December 2013

Major Subject: Wildlife and Fisheries Sciences

ABSTRACT

Two organic acids, polyhydroxybutyrate (PHB) and potassium diformate (KDF) have been researched to only a limited extent with aquatic species but have been shown to have various positive effects on terrestrial animals. Two algae extracts, carrageenan and alginic acid, also have been shown to elicit immunostimulation in some fish. Therefore, the present study was conducted with red drum (*Sciaenops ocellatus*) as a model marine species to study the effects of organic acids and algae extracts as feed supplements by evaluating several humoral immune responses.

Two feeding trials, one of 7-week duration and the other of 3-week were conducted with disease-free juvenile red drum (average initial wt. 2.6±0.2 g and 78.2 ±0.2 g, respectively). Semipurified diets were formulated to be isocaloric and contain 40% crude protein. Experimental diets were produced by supplementing the basal diet with KDF at 0.6%, PHB at 2%, alginic acid at 1% or carrageenan at 0.5% by weight in place of cellulose. Fish were stocked into 110-L aquaria operated as a recirculating system with each diet assigned to three replicate aquaria containing either 15 fish (7-week trial) or 9 fish per aquarium (3-week trial). All fish were fed their respective diets at the same fixed percentage of body weight (initially 6% and gradually reduced to 4% as the fish grew). Body weight was monitored by collectively weighing fish from each aquarium every week.

At the end of each feeding trial, weight gain and feed efficiency were significantly ($P<0.0001$) reduced in fish feed PHB compared to the basal diet and both algae extracts. There were no significant differences in condition indices such as hepatosomatic index (HSI) and intraperitoneal fat (IPF) ratio among fish fed the various diets. Lysozyme activity was

significantly higher in fish fed alginic acid. The greatest phagocytic activity was found in fish fed the diet containing PHB. Total immunoglobulin level was higher in fish fed the diet supplemented with carrageenan. Goblet cell proliferation was greatest in the posterior end of the gastrointestinal tract but not different among dietary treatments. Organic acids and algae extracts evaluated in this study produced variable immunological responses in red drum with carrageenan showing the greatest potential as an immunostimulant.

DEDICATION

I dedicate this thesis to my parents and brother, who always supported me and listened to me talk about fish. Thanks to my friends for helping me get through this.

ACKNOWLEDGEMENTS

I would like to thank Dr. Gatlin for his guidance and patience and my committee members Dr. Hume and Dr. Criscitiello for their assistance and suggestions.

Many thanks to the fish nutrition laboratory students and to the Aquacultural and Research Teaching Facility staff for helping along the way, I could not have done it without your help. Special thanks to Dr. Camilo Pohlenz, Angie Peredo, Donovan Patterson, Icshan Fauzi, Alejandro Velasquez, Alton Burns, Waldemar Rossi Jr., Sergio Castillo, Misael Rosales, Katie Zychowski and Brian Ray for all of the assistance and support throughout my studies. I would also like to thank the Histology Laboratory of the Department of Veterinary Integrative Biosciences at Texas A&M University for the histological processing and Dr. Kevin Conway of the Department of Wildlife and Fisheries Sciences at Texas A&M University for providing the image analysis equipment.

TABLE OF CONTENTS

Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

TABLE Page

INTRODUCTION

With the demand for seafood continuously growing, the production of seafood from aquaculture now has exceeded capture fisheries, and is expected to steadily increase as the world's population continues to expand (FAO, 2010). The greatest increase in aquaculture production is anticipated from intensive production systems in which fish are fed nutritionally complete diets. Intensive aquaculture generally entails high stocking densities, marginal water quality and other stressors that can result in diseases which cause economic loss. Fish reared under intense production conditions can be more susceptible to disease onset. Thus, dietary supplementation of antibiotics has been used in aquaculture and other types of animal production to combat some infectious diseases. Unfortunately, antibiotics have been misused in certain applications, resulting in antibiotic resistance of some bacteria and other environmental problems. Thus, to reduce the use of antibiotics, various immunostimulants including certain feed additives are being sought to increase the resistance of aquatic organisms to infectious agents (NRC 2011). Two such groups of feed additives, organic acids and algae extracts, have emerged as promising supplements.

Organic acids are known to have stimulatory potential to the immune system and have been proven efficient for disease prevention (Midtlyng et al. 1996). Two organic acids which have been researched to only a limited extent are polyhydroxybutyrate (PHB) and potassium diformate (KDF). Polyhydroxybutyrate is a natural short-chain fatty acid monomer. The monomers of PHB are known to act as microbial control agents (Najdegerami et al. 2012). Organic acids thus may influence the microbial composition of the digestive tract in fish, as well as stimulate the immune system. The microbial community plays an important role in the nutrition and health of various fish species (Burr et al. 2005).

Recently, PHB has been shown to protect Artemia (*Artemia franciscana*) nauplii against *Vibrio* infection (Halet et al. 2007). De Schryver et al. (2010) reported that PHB increased survival and weight gain of European sea bass (*Dicentrarchus labrax*). A change in the microbial community of the gastrointestinal (GI) tract also was found in sea bass when presented with 10% of the diet coated with PHB solution (De Schryver et al. 2010).

Potassium diformate is another organic acid salt mixture that has been reported to improve growth and disease resistance in some fish species (Baruah et al. 2007). Recent studies have shown that KDF in tilapia (*Oreochromis sp*.) increased weight gain, feed efficiency and survival against *Vibrio anguillarum* (Ramli et al. 2005, Wing-Keong et al. 2009). De Schryver et al. (2010) also observed higher survival of sea bass larvae when fed KDF particles compared to a basal diet.

Algae polysaccharides such as carrageenan and alginic acid (also known as algin or alginate) are another group of compounds which have been shown to elicit immunostimulation in some fish. Carrageenan is a linearly sulfated polysaccharide derived from red seaweed; whereas, alginic acid is an anionic polysaccharide obtained from brown seaweed. Alginic acid can be found in the intercellular mucilage and cell walls of species such as *Undaria pinnatifida* and *Macrocystis pyrifera* (Chapman and Chapman 1980, Fujiki et al. 1994). Both of these compounds have been shown to effectively improve immunological responses of common carp (*Cyprinus carpio*) (Fujiki and Yano 1997). In previous studies, algin also has been reported to increase immune responses of snakehead (*Channa striata)* (Miles et al. 2001), sea bass (Bagni et al. 2005) and kelp grouper *(Epinephelus brneus)* (Harikrishman et al. 2011). However, additional research is needed to further characterize the effects of algae polysaccharides as feed additives (Bagni et. al 2005, Cheng et al. 2008).

Physiologically speaking, the immune system of fish is similar to other vertebrates. They possess various defense mechanisms including physical barriers such as skin and mucous membranes to help prevent pathogen entry. One of the major immune barriers in fish is the gut associated lymphoid tissue (GALT) which consists of lymphoid aggregates and follicles in the intestine (Rombout et al. 1989). All immune cells necessary for an immune response are present in the gut mucosa of teleost species. Mainly, the GALT consists of mucus produced by goblet cells which can serve as a non-specific defense mechanism. This can prevent colonization by fish pathogens. If pathogens do gain entrance, there are innate cellular and humoral immune responses which fish may activate (Uribe et al. 2011). The phagocytic response is developed by immune cells such as macrophages (monocytes) and granular leukocytes such as neutrophils which can engulf and kill invading pathogens. Macrophages also have an important role in antigen-presentation, thus linking the non-specific and acquired immune responses. Humoral immunity is known to be mediated by molecules in the blood and mucosal secretions. Antibody or immunoglobulin (Ig) is the primary effector molecule of humoral immunity and provides specificity. In the present study, there was emphasis on humoral immunity by analyzing properties in blood, intestine and digesta of fish. Fish also possess specific immunity which may be developed against particular pathogens and result in immunological memory. However, specific disease-causing organisms were not investigated in this study.

It was suggested by Dalmo (2005) that strategies to control pathogens should be developed for aquaculture production to reach its full potential. Such strategies should include diet additives for enhancing immune responses of the cultured organism to invading pathogens to thus limit their negative effects.

The red drum (*Sciaenops ocellatus*) is a good model for intensive marine aquaculture and was chosen to evaluate the specific diet additives as means to improve immune responses and disease resistance. Over past decades, red drum has been intensively cultured in Texas as well as other countries such as China and Taiwan, and has become a valuable food and recreational resource. Numerous studies have been conducted focusing on the effects of various immunostimulants on this species, especially nucleotides (Li et al. 2004, 2007) and prebiotic supplements (Burr et al. 2008, Burr et al. 2010, Cheng et al. 2011, Anguiano et al. 2012). However, there are other potential immunostimulants which have not been studied in red drum, such as organic acids and algae extracts. Thus, the present study was conducted with red drum by evaluating several immunological parameters including plasma antiprotease activity, total immunoglobulin concentration and alternative complement pathway activity, as well as goblet cell proliferation.

MATERIALS AND METHODS

The basal diet, which used menhaden fishmeal and soy protein concentrate as the protein sources, was formulated to contain 40% crude protein, 10% fat, and an estimated digestible energy level of 3.3 kcal g^{-1} (Table 1) to satisfy all known nutrient requirements of red drum (Gatlin, 2002). Four experimental diets were prepared by adding 0.6% potassium diformate, 2% polyhydroxybutyrate, 1% alginic acid or 0.5% carrageenan by weight in place of cellulose in the basal diet. The levels of inclusion for each additive were chosen based on optimal responses of other fish species in published studies. Diets were prepared as previously described (Moon and Gatlin 1991), and analyzed for crude protein, lipid, and ash levels using standard procedures (AOAC 1990).

Experiment 1

The experimental diets were evaluated in a 7-week feeding trial at the Texas A&M Aquacultural Research and Teaching Facility, College Station, Texas. A total of 225 disease-free juvenile red drum were stocked as groups of 15 fish in 110-L aquaria with an initial weight of 2.6±1 g. The cultured system was operated as a recirculating system with water quality control (total ammonia nitrogen <0.5 mg L^{-1}) through biological and mechanical filtration. Oxygen was maintained near saturation and water temperature was held at 27 ± 1ºC. Salinity was maintained at 6-8 g L^{-1} using well water and synthetic sea salt (Fritz Industries, Dallas, TX, USA). A 12-h light:12-h dark photoperiod was maintained with fluorescent lights controlled by timers. All groups of fish were fed their respective diets twice daily, 7 days a week at the same fixed percentage of body weight. Body weight was monitored by collectively weighing fish from each aquarium every week.

Table 1. Diet formulations (g/100 g dry weight) containing potassium diformate, polyhydroxybutyrate, alginic acid and carrageenan.

Ingredient[1]	Basal	0.6% KDF	2% PHB	1% Alginic Acid	0.5% Carrageenan
Menhaden Fishmeal[2]	29.7	29.7	29.7	29.7	29.7
Soy Protein Conc.[3]	27.3	27.3	27.3	27.3	27.3
Dextrinized Starch[4]	20.0	20.0	20.0	20.0	20.0
Menhaden Oil[5]	7.10	7.10	7.10	7.10	7.10
Vitamin Premix[6]	3.0	3.0	3.0	3.0	3.0
Mineral Premix[7]	4.0	4.0	4.0	4.0	4.0
Carboxymethyl Cellulose[8]	2.0	2.0	2.0	2.0	2.0
Cellulose[9]	6.9	6.3	4.9	5.9	6.6
Potassium Diformate[10]	0.0	0.60	0.0	0.0	0.0
Polyhydroxybutyrate[11]	0.0	0.0	2.0	0.0	0.0
Alginic Acid[12]	0.0	0.0	0.0	1.0	0.0
Carrageenan[12]	0.0	0.0	0.0	0.0	0.50

[1]All the diets will be formulated to contain 40% crude protein, 10% fat, and 3.3 kcal of digestible energy per gram. Dietary supplements to be evaluated (potassium diformate (KDF), polyhydroxybutyrate (PHB), alginic acid and carrageenan) in place of cellulose.

[2]Special Select, Omega Protein, Houston, TX, USA.

[3]Solae LLC, St. Louis, MO, USA.

[4]MP Biomedicals, Solon, OH, USA.

[5]Omega Protein, Reedville, VA, USA.

[6]Same as Moon and Gatlin (1991).

[7]Same as Moon and Gatlin (1991) but prepared by MP Biomedicals, Solon, OH, USA.

[8]MP Biomedicals, Solon, OH, USA.

[9]US Biochemical Corp., Cleveland, OH, USA.

[10]ADDCON GROUP GmbH, Bonn, Germany.

[11]Goodfellow Cambridge Limited, Huntingdon, England.

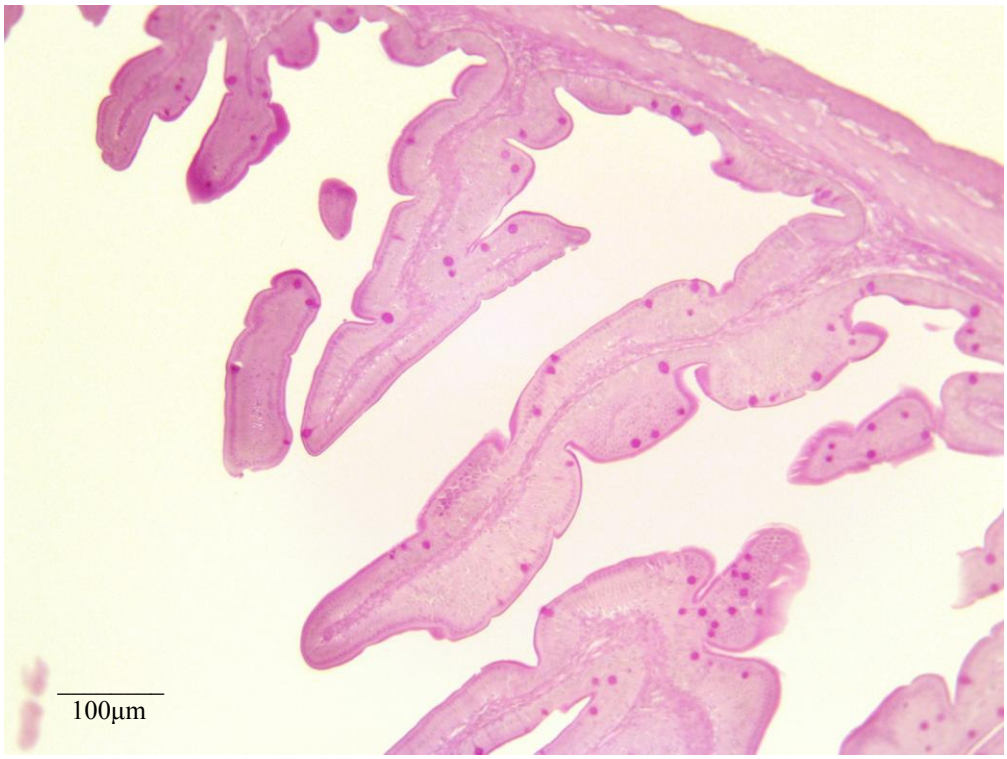
[12]Alfa Aesar, Ward Hill, MA, USA.

At the end of the 7-week trial, all fish were collected from each aquarium, weighed and then anesthetized with tricaine methane sulphonate (MS-222, 150 mg L^{-1}). Three random fish per aquarium were weighed, dissected, and liver and intraperitoneal fat (IPF) were weighed to calculate hepatosomatic index (HSI) ((liver wt/live wt)*100) and IPF ratio ((IPF wt/live wt)*100) values. Nine head kidneys were fixed with L15-2% media for isolation of macrophages and measurement of phagocyte extracellular superoxide anion activity and phagocytic activity (Sealy and Gatlin 2002). Three random fish per aquarium also were homogenized into a composite sample for determining whole-body proximate composition as described by Serrano et al. (1992).

For blood analysis, nine fish per tank were bled with 1-mL syringes previously coated with heparin. Blood neutrophil oxidative radical production was measured in 50-µL aliquots of whole blood mixed in a 96-well microtiter plate with nitroblue tetrazolium (NBT) solution (Li and Gatlin 2003). Plasma was separated from whole blood by centrifugation at 3,000 x *g*. Plasma was frozen and subsequently used to measure plasma antiprotease activity (Ellis 1990), lysozyme activity (Li and Gatlin 2003), total immunoglobulin (Ig) (Siwiki et al. 1994), total plasma protein (Siwiki et al. 1994) and alternative complement pathway activity (Sunyer and Tort 1995).

For histological analysis, three randomly selected fish were taken from each aquarium. The GI tract was dissected from the gastro-pyloric region to the anal region, and tied at both ends. Intestines were fixed in 10% formaldehyde and prepared as described by Anguiano et al. (2012). Slides were stained with PAS stain for goblet cell count. Slides were analyzed under a compound light microscope (Olympus BX40) at 10X. Goblet cells were counted in a total of 10 folds per cross-section, resulting in 60 measured folds per dietary treatment.

Figure 1. Intestinal folds showing goblet cells. (PAS; 10X).



Experiment 2

The basal diet in Experiment 2 was similar to that used in Experiment 1, being formulated to contain 40% crude protein, 10% fat, and an estimated digestible energy level of 3.3 kcal g^{-1} (Table 1). Four experimental diets were prepared and analyzed as in Experiment 1.

The experimental diets were evaluated in a 3-week feeding trial at the same location and under similar conditions as Experiment 1. A total of 135 disease-free red drum was stocked into 110-L aquaria as groups of 9 fish with an initial weight of 78.2 ±0.2 g per fish. The larger size of fish in this feeding trial allowed for larger quantities of tissues to be collected for the various analyses. This feeding trial was conducted using the same procedures as described for Experiment 1. At the end of the 3-week trial, fish were collected from each aquarium and analyzed for the same responses as in Experiment 1.

In addition to the analyses conducted in Experiment 1, samples for denaturing gradient gel electrophoresis (DGGE) were also taken. Samples were collected 6 hours after feeding from two fish per aquarium. Fish were aseptically processed and their gastrointestinal (GI) tract contents were expressed into sterile microcentrifuge tubes and frozen for DGGE analysis. Procedures for DGGE analysis were similar to those of Burr et al. (2010).

Statistical analysis

The data from the feeding trials and immune response assays were subjected to analysis of variance and Tukey's test using the Statistical Analysis System. Differences in treatment means were considered significant at $P<0.05$. All statistical analyses were performed using the SAS® software package (SAS Institute Inc., Cary, NC USA).

RESULTS

Experiment 1

At the end of Experiment 1, weight gain of the juvenile red drum was significantly lower in fish fed 2% PHB compared to the other four diets. Feed efficiency ratio was significantly lower for fish fed diets with 2% PHB and 0.5% carrageenan compared to the basal diet (Table 2). Fish survival in this trial was above 90% and it was not influenced by diets. There were no significant differences in body condition indices including hepatosomatic index (HSI) or intraperitoneal fat (IPF) ratio among fish fed the various diets based on one-way ANOVA.

Blood analysis of neutrophil oxidative radical production showed no significant differences among fish fed the various diets. Analysis done on plasma components such as lysozyme activity, antiprotease activity, total protein and alternative complement pathway also did not show any significant differences among fish fed the various diets (Table 3). However, total Ig count was significantly higher in diets fed 1% alginic acid and 0.5% carrageenan (Table 3). Cells isolated from the head kidney were used for extracellular superoxide anion and phagocytic activity analyses. Superoxide anion production did not show any significant difference among treatments; however, phagocytosis levels were significantly higher in macrophages from fish fed the diet supplemented with PHB at 2% (Table 3).

Histological analysis of the gastrointestinal tract (GIT) from fish fed the various diets revealed no significant differences related to diet or the three cross-sections measured. When comparing the GIT tract of fish from all dietary treatments, goblet cell counts were significantly higher in the posterior end of the GIT compared to the anterior end (Table 4).

Table 2: Weight gain, survival, feed efficiency, hepatosomatic index (HSI), intraperitoneal fat (IPF) ratio, and moisture, ash, protein and lipid of whole-body tissues from red drum fed a basal diet or diets supplemented with 0.6% KDF, 2% PHB, 1% alginic acid and 0.5% carrageenan in experiment 1[1].

Dietary Treatment	Weight Gain (% of initial weight)	Feed Efficiency (g gain/g diet fed)	Survival (%)	HSI (% of fresh weight)	IPF Ratio (% of fresh weight)	Moisture (%)	Ash (%)	Protein (%)	Lipid (%)
Basal	969^{A}	0.88^{A}	91.1	3.22	1.13	23.69	3.72	15.01	5.39
0.6% KDF	878^{A}	0.85^{AB}	95.6	3.21	1.01	23.88	3.76	16.05	4.44
2% PHB	730^{B}	0.79^{B}	100.0	3.30	1.10	23.40	3.92	15.33	4.58
1% Alginic Acid	961^{A}	0.88^{A}	93.3	3.19	1.08	23.54	3.71	14.86	4.85
0.5% Carrageenan	925^{A}	0.83^{AB}	100.0	3.38	1.31	23.92	3.55	15.52	4.92
ANOVA									
P-value	0.0039	0.0073	0.1705	0.8350	0.6829	0.9402	0.8012	0.2272	0.1897
Pooled S.E.	15.6049	0.0063	1.2570	0.0587	0.0658	0.2310	0.0924	0.1608	0.1195

[1]Values for weight gain, feed efficiency and survival are means of three replicate groups; whereas, condition indices and body composition were obtained from three fish in each of three replicate aquaria. Values in a row that do not have the same superscript are significantly ($P<0.05$) different.

Table 3: Blood neutrophil oxidative radical production (NBT), serum lysozyme activity analysis, plasma antiprotease activity, total plasma protein, total immunoglobulin, alternative complement pathway, phagocyte extracellular superoxide anion and phagocytic activity from red drum fed a basal diet or diets with 0.6% KDF,2% PHB, 1% alginic acid and 0.5% carrageenan in experiment 1[1].

Dietary Treatment	NBT (mg/ml)	Lysozyme Activity (units/l)	Plasma Antiprotease (430 nm)	Total Plasma Protein (mg/ml)	Total Immunoglobulin (mg/ml)	Alternative Complement Pathway (μ/ml)	Extracellular Superoxide Anion (550 nm)	Phagocytic Activity (510 nm)
Basal	9.33	439	88.2	40.0	4.57^{AB}	628	1.70	0.22^{AB}
0.6% KDF	8.82	372	84.6	32.4	3.90^{B}	725	1.97	0.12^{C}
2% PHB	9.61	461	83.7	21.9	5.63^{AB}	235	1.48	0.25^{A}
1% Alginic Acid	9.43	406	85.6	32.0	9.18^{AB}	528	1.89	0.11^{C}
0.5% Carrageenan	9.65	456	85.6	34.7	14.70^{A}	312.3	1.91	0.12^{BC}
ANOVA								
P-value	0.3568	0.9231	0.5722	0.3847	0.0422	0.6306	0.9438	0.0239
Pooled S.E.	0.1337	35.9	0.85	2.72	1.04	113.8	0.2106	0.0137

[1]Values for NBT are means of three replicate groups; whereas, values for lysozyme activity, plasma antiprotease, total plasma protein and total immunoglobulin were obtained from composite plasma samples of nine fish in each of three replicate aquaria. Values for extracellular superoxide anion and phagocytic activity were obtained from pooled head kidney samples from nine fish in each of three replicate aquaria. Values in a row that do not have the same superscript are significantly ($P<0.05$) different.

Table 4. Goblet cell proliferation count from red drum fed a basal diet or diets with 0.6% KDF, 2% PHB, 1% alginic acid and 0.5% carrageenan in experiment 1[1].

Intestinal section	Dietary treatments					P-value	Pooled S.E.
	Basal	0.6% KDF	2% PHB	1% Alginic Acid	0.5% Carrageenan		
Proximal	7.0	6.0	6.7	6.3	6.3	0.9690	0.48
Middle	8.3	7.7	9.3	9.0	8.3	0.9143	0.12
Distal	13.0	13.0	14.3	13.7	12.7	0.9051	0.60

Count	Intestinal Section			P-value	Pooled S.E.
	Proximal	Middle	Distal		
All treatmetns	6.5^{C}	8.5^{B}	13.3^{A}	0.0001	0.2862

[1]Values are means of two fish (10 fold measurements for each fish) from each of three replicate groups. Values in a row that do not have the same superscript are significantly ($P<0.05$) different.

Experiment 2

At the end of the second feeding trial, weight gain and feed efficiency ratio of advanced red drum juveniles was not affected by diet (Table 5). There were no significant differences in intraperitoneal fat (IPF) ratio; however, hepatosomatic index (HSI) ratio was significantly higher in fish fed diets with 2% PHB and 0.5% carrageenan compared to that of fish fed the basal diet and KDF. Survival of red drum in this experiment was significantly higher for fish fed KDF and carageenan compared to that of fish fed the basal diet and KDF (Table 5).

Blood analysis of neutrophil oxidative radical production showed no significant differences among fish fed the various diets. Analysis of plasma responses such as plasma antiprotease, total Ig count, total plasma protein and alternative complement pathway also did not show any significant differences among fish fed the various diets. However, lysozyme activity was significantly higher in fish fed diets supplemented with 1% alginic acid, 2% PHB and 0.5% carrageenan compared to that of fish fed the basal diet (Table 6). Macrophages isolated from the head kidney were analyzed for superoxide anion production and phagocytosis, but neither response showed significant differences among fish fed the various diets (Table 6).

Histological analysis of the GIT from fish fed the various diets revealed that only goblet cell numbers in the posterior portion of the GIT was significantly increased by the diet supplemented with alginic acid (Table 7). There were no significant differences in the anterior and middle portions of the GIT. When comparing the GIT tract as a whole, independent of diet, goblet cell counts were significantly higher in the posterior portion of the GIT compared to the anterior and middle portions as observed in Experiment 1 (Table 7).

Table 5. Weight gain, survival, feed efficiency, hepatosomatic index (HSI), intraperitoneal fat (IPF) ratio, and moisture, ash, protein and lipid of whole-body tissues from red drum fed a basal diet or diets with 0.6% KDF, 2% PHB, 1% alginic acid and 0.5% carrageenan in experiment 2[1].

Dietary Treatment	Weight Gain (% of initial weight)	Feed Efficiency (g gain/g diet fed)	Survival (%)	HSI (% of fresh weight)	IPF Ratio (% of fresh weight)	Moisture (%)	Ash (%)	Protein (%)	Lipid (%)
Basal	294	0.81	85.2^{B}	1.80^{AB}	0.28	23.04	3.25	70.95	4.36
0.6% KDF	291	0.63	96.3^{A}	1.76^{B}	0.26	23.40	3.55	69.90	3.92
2% PHB	186	0.61	85.2^{B}	2.21^{AB}	0.32	23.08	3.35	70.47	3.71
1% Alginic Acid	242	0.58	92.6^{AB}	2.02^{AB}	0.36	24.46	3.73	70.52	4.52
0.5% Carrageeenan	281	0.52	100.0^{A}	2.38^{A}	0.32	24.03	3.88	69.75	4.20
ANOVA									
P-value	0.0911	0.3045	0.0343	0.0271	0.3973	0.4371	0.8984	0.9956	0.4217
Pooled S.E.	12.4691	0.0405	1.4814	0.0560	0.0165	0.2738	0.2294	1.0457	0.1413

[1]Values for weight gain, feed efficiency and survival are means of three replicate groups; whereas, condition indices and body composition were obtained from three fish in each of three replicate aquaria. Values in a row that do not have the same superscript are significantly ($P<0.05$) different.

Table 6. Blood neutrophil oxidative radical production (NBT), serum lysozyme activity analysis, plasma antiprotease activity, total plasma protein, total immunoglobulin, alternative complement pathway, phagocyte extracellular superoxide anion and phagocytic activity from red drum fed a basal diet or diets with 0.6% KDF,2% PHB, 1% alginic acid and 0.5% carrageenan in experiment 2[1].

Dietary Treatment	NBT (mg/ml)	Lysozyme Activity (units/l)	Plasma Antiprotease (430 nm)	Total Plasma Protein (mg/ml)	Total Immunoglobulin (mg/ml)	Alternative Complement Pathway (μ/ml)	Extra cellular Superoxide Anion (550 nm)	Phagocytic Activity (510nm)
Basal	5.89	231.48^{C}	83.36	33.03	3.47	455.542	2.08	0.059
0.6% KDF	6.27	294.44^{BC}	72.55	33.47	1.90	442.136	2.07	0.060
2% PHB	7.01	377.78^{AB}	76.81	30.87	2.74	485.572	1.57	0.064
1% Alginic Acid	6.40	429.63^{A}	78.85	33.37	1.51	478.831	2.79	0.059
0.5% Carrageenan	6.86	383.33^{AB}	81.03	26.11	2.12	544.646	2.59	0.056
ANOVA								
P-value	0.5463	0.0046	0.4962	0.4932	0.4327	0.9929	0.4677	0.9127
Pooled S.E.	0.2250	12.9365	1.9431	1.4630	0.3365	73.6269	0.2192	0.0028

[1]Values for NBT are means of three replicate groups; whereas, values for lysozyme activity, plasma antiprotease, total plasma protein and total immunoglobulin were obtained from pooled plasma samples of three fish in each of three replicate aquaria. Values for extracellular superoxide anion and phagocytic activity were obtained from nine head kidneys of four fish in each of three replicate aquaria. Values in a row that do not have the same superscript are significantly ($P<0.05$) different.

Table 7. Goblet cell proliferation count from red drum fed a basal diet or diets with 0.6% KDF, 2% PHB, 1% alginic acid and 0.5% carrageenan in experiment 2[1].

Intestinal section	Dietary treatments					P-value	Pooled S.E.
	Basal	0.6% KDF	2% PHB	1% Alginic Acid	0.5% Carrageenan		
Proximal	12.3	12.3	12.0	13.3	12.3	0.9517	0.5577
Middle	16.0	15.0	15.0	14.7	14.0	0.8981	0.6359
Distal	15.0^{B}	19.7^{AB}	15.0^{B}	25.3^{A}	20.0^{AB}	0.0084	0.7630

Count	Intestinal Section			P-value	Pooled S.E.
	Proximal	Middle	Distal		
Goblet cell count	12.7^{C}	14.9^{B}	19.7^{A}	0.0001	0.4814

[1]Values are means of two fish (10 fold measurements for each fish) from each of three replicate groups. Values in a row that do not have the same superscript are significantly ($P<0.05$) different.

DISCUSSION

Two organic acids, polyhydroxybytyrate (PHB) and potassium diformate (KDF) as well as two algae extracts, alginic acid and carrageenan, were evaluated in this study to assess the potential immunological impacts of each when used as dietary supplements for red drum. It was hypothesized that supplementation of all four ingredients might potentially improve growth rates and immunological responses compared to fish fed a basal diet containing all nutrients at required levels. However, growth enhancement was not observed in the present study with red drum. Instead, diets containing 0.6% KDF, 1% alginic acid and 0.5% carrageenan supported similar weight gain as fish fed the basal diet; however, fish fed the diet with 2% PHB had significantly lower weight gain in both of the experiments. De Schryver et al. (2010) reported that PHB increased weight gain in European sea bass. Siberian sturgeon (*Acipenser baerii*) fed 2% PHB showed no difference in weight gain or feed efficiency compared to fish fed the basal diet (Najdegerami et al. 2011).

In the second experiment of the current study, red drum fed diets supplemented with carrageenan and KDF had higher survival than fish fed the basal diet or PHB. In previous studies, alginic acid and carrageenan have resulted in 100% survival of brown-marbled grouper (*Epinephelus fuscoguttatus*) compared to 75% survival of those fed a basal diet (Cheng et al. 2008). In another study, tilapia fed diets with 2% KDF and exposed to *Streptococcus agalactiae,* had improved disease resistance compared to fish fed the basal diet (Wing-Keong et al. 2009). In contrast to results of the current study with red drum, higher survival of sea bass larvae was observed for fish fed PHB particles in comparison with a basal diet (De Schryver et al. 2010).

Condition indices of red drum fed the various diets in the two feeding trials showed only limited difference. The HSI values of fish in experiment 2 were highest for red drum fed diets containing PHB and carrageenan. The reason for this is not readily apparent but may be related to composition of the liver. In a similar study, HSI was not affected in red hybrid tilapia fed diets which were supplemented with KDF (Wing-Keong et al. 2009).

Immunity is the inherent ability to recognize foreign living and non-living agents. Fish have developed non-specific and acquired immune mechanisms, both of which contain humoral components. In the present study, lysozyme activity, which assists in the degradation of both extracellular and intracellular proteins, was higher in red drum fed the diets with 2% PHB, 1% alginic acid and 0.5% carrageenan in experiment 2. Although red drum in that experiment were exposed to the assigned diets for less time than in experiment 1, they also were older. In previous studies factors such as physiological age has been studied and considered to make a difference in the immunocompetence of fish (Gislason et al. 1994). Juvenile sea bass and kelp grouper fed algin showed significantly higher lysozyme activity (Bagni et al. 2005; Harikrishman et al. 2011) as did grouper fed carrageenan (Cheng et al. 2008) which is consistent with the observations in the present study with red drum. Phagocytic activity measures cells that are capable of identifying, ingesting and destroying microbes. Usually, concentrations of phagocytic cells are found in the head kidney and involved in antigen trapping and immune responses (Agius and Roberts 2003). This phagocytic activity was higher in red drum fed diets with 2% PHB in experiment 1. The phagocytic activity measurement seemed to be a reliable indicator of the immunostimulatory response to the feed supplements. Although PHB increased phagocytic activity in the present study, it has mainly been studied as a growth promoter and enhancer of the intestinal

bacterial community (Mahious et al. 2006; Zhou et al. 2007), with limited research done on humoral immunity. Even though red drum fed PHB in experiment 1 had a lower growth rate, they had a higher phagocytic activity. Previously, it has been found that in grouper both alginic acid and carrageenan showed higher phagocytic activity and lysozyme activity (Cheng et al. 2008) but increased phagocytic activity was not observed with red drum in the current study. Alginic acid also has been reported to increase phagocytic activity and resistance against *Edwardsiella tarda* in common carp (Fujiki and Yano 1997).

Total plasma protein of red drum was not elevated by the dietary factors in either experiment. However, total immunoglobulin was significantly elevated in experiment 1. Diets supplemented with alginic acid and carrageenan both increased immunoglobulin count. Plasma immunoglobulins are a major component of the humoral immune system. Until recently there has been a general belief that IgM was the only functional immunoglobulin in fish; however, IgD (Edholm et al. 2010) and IgT/IgZ (Danilova et al. 2005; Hansen et al. 2005) are now known to be functional immunoglobulins in teleosts. In the present study, the total Ig count included all of the possible immunoglobulins found in red drum. Based on these results, it can be inferred that the two algae extracts tested increase immunoglobulin production of red drum.

The alternative pathway of complement activity is a powerful non-specific defense mechanism, protecting fish from a wide range of potentially invasive organisms such as bacteria, fungi, viruses, and parasites (Muller-Eberhard 1988). There have been limited studies on alternative complement pathway (ACP) in fish. In the present study, 50% lysis of the red blood cells was found as a measure of ACP activity. Compared to human sera

(Sunyer and Tort 1995), red drum ACP was much higher. The titration of carp ACP has been reported at high values as well (Matsuyama et al. 1988). In a study with carp, sodium alginate did not affect ACP over a narrow range of doses. Based on this study, the supplemented dosages may not have been high enough to stimulate ACP.

Goblet cells secrete high-molecular-weight glycoproteins called mucins (Forstner 1978). The mucus layer keeps the epithelium moist, acts as a lubricant, traps microbes and aids the expulsion of micro-organisms (Basset et al. 2003). Higher goblet cell numbers also relate to increase protection and lubrication for fecal exclusion (Dai et al. 2007). Histochemical analysis on goblet cells is possible using PAS staining because these mucous cells contain neutral mucosubstances which are PAS positive (Khojasteh et al. 2009). Mucins contain O-linked oligosaccharides which act similar to polysaccharides. Based on this study, perhaps the polysaccharides found in alginic acid triggered goblet cell proliferation in the GIT as this dietary treatment showed increased goblet cell numbers. Also, when comparing cell counts of red drum fed the different dietary supplements, there were no significant differences in experiment 1; however, alginic acid significantly enhanced the proliferation of cells in the posterior section of the gut in experiment 2. The differences observed between the two experiments were possibly due to the size of the fish and exposure time. Based on this study, it can be inferred that alginic acid enhances goblet cell production more readily than KDF, PHB or carrageenan. However, when fish were exposed for more than 3 weeks, KDF, PHB and carrageenan enhanced goblet cell production as much as alginic acid. Comparing cell counts along the entire GIT, higher numbers were found in the posterior portion of the GIT and lower in the anterior portion in experiment 1 as well as in experiment 2. Similar results were found in *Acanthopagrus latus*, with goblet cells increasing towards the posterior of the

GIT (Salamat et al. 2011). Conversely, in a study of channel catfish (*Ictaluri punctatus*) fewer cells were found in the posterior section (Hebert et al. 2002).

The supplements used in the present study have been used in other animals as well. A study done in chickens with KDF supplementation showed no significant effect on growth or weight gain. However, KDF changed the microbial composition of the upper part of the digestive tract (Mikkelsen et al. 2009). It has been previously discussed by Mroz (2005) that the supplementation of organic acids may enhance nutrient utilization of animals by manipulating pH of the GIT and buffering of the diet. No signs of increased nutrient utilization such as improved weight gain or feed efficiency were observed in the present study with red drum.

The other group of immunostimulant evaluated in the present study was algae extracts, which have also been evaluated with different animal species. Previously, alginic acid was found to enhance the IgA response and stimulate IgG in calves (Bowersock et al. 1998). Carrageenan has been widely used in human foods due to its strong protein-binding capacities (FAO 1965). Studies have shown carrageenan might function as a topical microbicide for human papilloma virus, and human immunodeficiency virus (Gonzales et al. 1987).

CONCLUSION

Feed additives such as organic acids and algae extracts are considered to be promising alternatives as immunostimulants to prevent disease as opposed to treating fish with antibiotics after they become diseased, as these compounds have been shown to have the potential to increase survival, disease resistance and growth performance in several fish species. Based on the present study, both groups studied supported good growth of red drum when compared to a basal diet meeting all of the known nutrient requirements of this species. Some of the immunological aspects studied, such as lysozyme, total Ig, phagocytic activity and goblet cell count, were enhanced by the supplementation of these additives. Further research will be completed in evaluating the effects of these various supplements on microbial composition and histometric measurements of the GIT in red drum. Once that information is obtained, it will provide a thorough characterization of the effects of these diet supplements on red drum. Organic acids and algae extracts evaluated in this study produced variable immunological responses in red drum with carrageenan showing the greatest potential as an immunostimulant.

LITERATURE CITED

Agius, C. and R.J. Roberts. 2003. Melano-macrophages centers and their role in fish pathology. Journal of Fish Diseases 26:499-509.

Anguiano, M., C. Pohlenz, A. Buentello and D.M. Gatlin III. 2012. The effects of prebiotics on the digestive enzymes and gut histomorphology of red drum (*Sciaenops ocellatus*) and hybrid striped bass (*Morone chrysops x M. saxatilis*). British Journal of Nutrition 1-7.

Association of Official Analytical Chemists (AOAC). 1990. Official methods of analysis of the AOAC, Vol 2. K.C Helrich. Cab Direct, Arlington, Virginia, USA.

Bagni M., N. Romano, M.G. Finoia, L. Abelli, G. Scapigliati, P.G. Tiscar, M. Sarti, and G. Marino. 2005. Short- and long-term effects of a dietary yeast β-glucan (Macrogard) and alginic acid (Ergosan) preparation on immune response in sea bass (*Dicentrarchus labrax*). Fish and Shellfish Immunology 18:311-325.

Baruah K., N.P. Sahu, A.K. Pal, K.K. Jain, D. Debnath and S.C. Mukherjee. 2007. Dietary microbial phytase and citric acid synergistically enhances nutrient digestibility and growth performance of *Labeo rohita* (Hamilton) juveniles at sub-optimal protein level. Aquaculture Research 38:109-120.

Basset C., J. Holton, R. O'Mahony and R. Roitt. 2003. Innate immunity and pathogen-host interaction. Vaccine 21:12-23.

Bowersock T.L., H. HogenEsch, S. Torregrosa, D. Borie, B. Wang, H. Park and K. Park. 1998. Induction of pulmonary immunity in cattle by oral administration of ovalbumin in alginate microspheres. Immunology Letters 60:37-43.

Burr G., D. Gatlin III and S. Ricke. 2005. Microbal ecology of the GI tract of fish and the potential application of prebiotics and probiotics in finfish aquaculture. Journal of the World Aquaculture Society 36:425-436.

Burr G., M. Hume, W.H. Neill and D.M Gatlin III. 2008. Effects of prebiotics on nutrient digestibility of soybean-meal-based diets by red drum (*Sciaenops ocellatus*). Aquaculture Research 39:1680-1686.

Burr G., M. Hume, S. Ricke, D. Nisbet and D.M. Gatlin III. 2010. In vitro and in vivo evaluation of the prebiotics Grobiotic®-A, inulin, mannanoligosaccharide, and galactooligosaccharide on the digestive microbiota and performance of hybrid striped bass (*Morone chrysops x Morone saxatilis*). Microbial Ecology 59:187-198.

Chapman V.J. and D.J. Chapman. 1980. Seaweeds and their uses. 3rd ed: Chapman and Hall. London, England.

Cheng A.C., Y.Y. Chen and J.C. Chen. 2008. Dietary administration of sodium alginate and κ-carrageenan enhances the innate immune response of brown-marbled grouper *Epinephelus fuscoguttatus* and its resistance against *Vibrio alginolyticus*. Veterinary Immunology and Immunopathology 121:206-215.

Cheng Z., A. Buentello and D.M Gatlin III. 2011. Dietary nucleotides influence immune responses and intestinal morphology of red drum (*Sciaenops ocellatus*). Fish and Shellfish Immunology 30:143-147.

Dai, X., M. Shu and W. Fang. 2007. Histological and ultrastructural study of the digestive tract of rice field eel, *Monopoterus albus*. Applied Ichtyology 23:177-183.

Dalmo R.A. 2005. Ontogeny of the fish immune system. Fish and Shellfish Immunology 19:395-396.

Danilova, N., J. Bussmann, K. Jekosch and L.A. Steiner. 2005. The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. Nature Immunology 6:295-302.

De Schryver P., A.K. Sinha, P.S. Kunwar, K. Baruah, N. Boon, W. Verstraete, G. De Boeck and P. Bossier. 2010. Poly-β-hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European sea bass, *Dicentrarchus labrax*. Applied Microbiology and Biotechnology 86:1535-1541.

Edholm, E.S., E. Bengten, J.L. Stafford, M. Sahoo, E.B Taylor, N.W. Miller and M. Wilson. 2010. Identification of two IgD+ B cell populations in channel catfish, *Ictalurus punctatus*. Journal of Immunology 185:4082-4092.

Ellis, A.E. 1990. Serum antiproteases in fish. Techniques in Fish Immunology (ed. Stolen, J.S., Fletcher, T.C., Anderson, D.P., Roberson, B.S. and van Muiswinkel, W.B.). SOS Publications. Fair Haven, New Jersey, USA.

Food and Agriculture Organization of the United States (FAO) 2010. The State of World Fisheries and Aquaculture. Publishing Policy and Support Branch, Office of Knowledge Exchange, Research and Extension. Rome, Italy.

Food and Agriculture Organization of the United States (FAO) 1965. Agar and carrageenan manual. Forstner J.F. 1978. Intestinal mucins in health and disease. Digestion 17:234-263. Publishing Policy and Support Brance, Office of Knowledge Exchange, Research and Extension. Rome, Italy.

Fujiki K, H. Matsuyama and T. Yano. 1994. Protective effect of sodium alginates against bacterial infection in common carp, *Cyprinus carpio L.* Journal of Fish Diseases 17:349-355.

Fujiki K and T. Yano. 1997. Effects of sodium alginate on the non-specific defense system of the common carp (*Cyprinus carpio*). Fish and Shellfish Immunology 17:417-427.

Fujiki, S.D and N.M. Yano. 1997. Effects of k-Carrageenan on the non-specific defense system of carp (*Cyprinus carpio*). Fisheries Science 63:934-938.

Gatlin III, D.M. 2002. Red drum *Sciaenops ocellatus*. Pages 147-158 in C.D. Webster and C. Lim, editor. Nutrient requirements and feeding of finfish for aquaculture. CAB Publishing. Wallingford, Oxford, UK.

Gislason G, R.E. Olsen and E. Ringo. 1994. Lack of growth stimulating effect of lactate on Atlantic salmon, *Salmo salar.* Aquaculture and Fisheries Management 25:861-862.

Gonzales M.E., B. Alarcon and L. Carrasco. 1987. Polysaccharides as antiviral agents: antiviral activity of carrageenan. Antimicrobial Agents and Chemotherapy 31:1388-1393.

Halet D., T. Defoirdt, H. Vervaeren, N. Boon, T. Van de Wiele, P. Sorgeloos, P. Bossier and W. Verstraete. 2007. Poly-beta-hydroxybutyrate-accumulating bacteria protect gnotobiotic *Artemia franciscana* from pathogenic *Vibrio campbellii*. FEMS Microbiology Ecology 60:363-369.

Hansen, J.D., E.D. Landis and R.B. Phillips. 2005. Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: implications for a distinctive B cell developmental pathway in teleost fish. Proceedings of the National Academy of Sciences 102:6919-6924.

Harikrishman R., M.C. Kin, J.S. Kim, Y.J. Han, I.S. Jang, C. Balasundaram and M.S. Heo. 2011. Immunomodulatory effect of sodium alginate enriched diet in kelp grouper

Epinephelus brneus against *Steptococcus inae*. Fish and Shellfish Immunology 30:543-549.

Hebert, P., A.J. Ainsworth and B. Boyd. 2002. Histological enzyme and flow cytometric analysis of channel catfish intestinal tract immune cells. Developmental and Comparative Immunology. 26:53-62.

Khojasteh, S.M., F. Sheikhzadeh, D. Mohammadnejad and A. Azami. 2009. Histological, histochemical and ultrastrutural study of the intestine of rainbow trout (*Oncorhynchus mykiss*). World Applied Sciences Journal 6:1525-1531.

Li, P, and D.M. Gatlin III. 2003. Evaluation of brewers yeast (*Saccharomyces cerevisiae*) as a feed supplement for hybrid striped bass (*Morone chrysops x M. saxatilis*). Aquaculture 219:681-692.

Li, P., D.H. Lewis and D.M. Gatlin III. 2004. Dietary supplementation of oligonucleotides from yeast RNA influence immune responses and resistance of hybrid striped bass (*Morone chrysops* X *M. saxalitis*) to *Streptococcus inae* infection. Fish and Shellfish Immunology 16:561-569.

Li, P., D.M. Gatlin III and W.H. Neill. 2007. Dietary supplementation of a purified nucleotide mixture transiently enhanced growth and feed utilization of juvenile red drum, *Sciaenops ocellatus*. Journal of the World Aquaculture Society 38:281-286.

Mahious, A.S., F.J. Gatesoupe, M. Hervi, R. Metailler and F. Ollevier. 2006. Effects of dietary inulin and oligosaccharides as prebiotics for weaning turbot, *Psetta maxima*. Aquaculture International 14:219-229.

Matsuyama, H., K. Tanaka, M. Nakao and T. Yano. 1988. Characterization of the alternative complement pathway of carp. Developmental and Comparative Immunology 12:403-

408.

Midtlyng P.J., L.J. Reitan, A. Lillehaug and A. Ramstad. 1996. Protection, immune responses and side effects in atlantic salmon (*Salmo salar* L.) vaccinated against furunculosis by different procedures. Fish and Shellfish Immunology 6:599-613.

Mikkelsen L.L., J.K. Vidanarachchi, C.G. Olnood, Y.M. Bao, P.H. Selle and M. Choct. 2009. Effect of potassium diformate on growth performance and gut microbiota in broiler chickens challenged with necrotic enteritis. British Poultry Science 50:66-75.

Miles D.C., J. Polchana, J.H. Lilley, S. Kanchnankhan, K.D. Thompson and A. Adams. 2001. Immunostimulation of striped snakehead *Channa striata* against epizootic ulcerative syndrome. Aquaculture 195:1-5.

Moon, H.Y. and D.M. Gatlin III. 1991. Total sulfur amino acid requirement of juvenile red drum, *Sciaenops ocellatus*. Aquaculture 95:97-106.

Mroz, Z. 2005. Organic acids as potential alternatives to antibiotic growth promoters for pigs. Advances in Pork Production 16:169-182.

Muller-Eberhard H.J. 1988. Molecular organization and function of the complement system. Annual Review of Biochemistry 57:321-347.

Najdegerami, E.H., T. Tran, T. Defoirdt, M. Marzorati, P. Sorgeloos, N. Boon and P. Bossier. 2012. Effects of poly-β-hydrobutyrate (PHB) on Siberian sturgeon (*Acipenser baerii*) fingerlings performance and its gastrointestinal tract microbial community. Microbial Ecology 79:29-33.

National Research Council. 2011. Nutrient requirements of fish and shrimp. The National Academies of Press. Washington, DC. USA.

Ramli N., U. Heindl and S. Sunanto. 2005. Effects of potassium-diformate on growth perfomance of tilapia challenged with *Vibrio anguillarum*. Abstract, World Aquaculture 2005, Bali, Indonesia.

Rombout, J.H.W.M., H.E. Bot and J.J. Taverne-Thiele. 1989. Immunological importance of the second gut segment of carp. II Characterization of mucosal leucocytes. Journal of Fish Biology 35:167-178.

Salamat, N., R. Darbeh, N.E. Majd and A.R. Safahieh. 2011. Histological aspects of gut associated lymphoid tissue in *Acanthopagrus latus*. Global Veterinaria 7:381-385.

Sealy, W.M. and D.M. Gatlin III. 2002. 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. Journal of Nutrition 132:748-755.

Serrano, J.A., G.R. Nematipour and D.M. Gatlin III. 1992. Dietary protein requirement of the juvenile red drum *Sciaenops ocellatus* and relative use of dietary carbohydrate and lipid. Aquaculture 101:283-291.

Siwicki A.K., D. Anderson, and G.L. Rumsey. 1994. Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis. Veterinary Immunology and Immunopathology 41: 125-139.

Sunyer, J.O. and L. Tort. 1995. Natural hemolytic and bactericidal activities of sea bream *Sparus aurata* serum are effected by the alternative complement pathway. Veterinary Immunology and Immunipathology 45:333-345.

Uribe, C., H. Folch, R. Enriquez and G. Moran. 2011 Innate and adaptive immunity in teleost fish: a review. Veterinarni Medicina 56:486-503.

Wing-Keong, N.G., C. Koh, K. Sudesh and A. Siti-Zahrah. 2009. Effects of dietary organic acids on growth, nutrient digestibility and gut microflora of red hybrid tilapia, *Oreochromis sp.* and subsequent survival during a challenge test with *Streptococcus agalactiae*. Aquaculture Research 40:1490-1500.

Zhou, Z.G., Z.K. Ding, and L.V. Huiyuan. 2007. Effects of dietary short-chain fructooligosaccharides on intestinal microflora, survival, and growth performance of juvenile white shrimp, *Litopenaeus vannamei*. Journal of the World Aquaculture Society 38:296-301.