EVALUATION OF THE DAIRY/YEAST PREBIOTIC, GROBIOTIC®-A, IN THE DIET OF JUVENILE NILE TILAPIA, *Oreochromis niloticus*

A Thesis

by

ANJELICA MARIA PEREDO

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

MASTER OF SCIENCE

December 2011

Major Subject: Wildlife and Fisheries Sciences

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ABSTRACT

Evaluation of the Dairy/Yeast Prebiotic, GroBiotic®-A, in the Diet of Juvenile Nile Tilapia, *Oreochromis niloticus*. (December 2011)

Anjelica Maria Peredo, B.S., Texas A&M University

Co-Chairs of Advisory Committee: Dr. Delbert M. Gatlin III
Dr. Alejandro Buentello

Two different feeding trials were conducted to evaluate the effects of dietary supplementation with the dairy/yeast prebiotic GroBiotic®-A (GBA) to Nile tilapia diets. A nutritionally complete basal diet was supplemented with GBA at either 1 or 2% of dry weight, and all three diets were fed to triplicate groups of juvenile fish in two consecutive trials. Trial 1 continued for 8 weeks, while Trial 2 was conducted for 5 weeks to more specifically assess immunological responses, intestinal characteristics and disease resistance of tilapia. At the conclusion of Trial 1, there were no differences in weight gain (WG) or feed efficiency (FE) among fish fed the three diets. However, fish fed the diet with GBA at 2% had significantly increased survival and noticeably elevated levels of plasma lysozyme compared to fish fed the basal diet or the diet with GBA at 1%. Similarly, at the conclusion of Trial 2, WG and FE were unaffected by GBA supplementation; however, fish fed the diet with GBA at 2% also exhibited elevated plasma lysozyme as well as significantly ($P < 0.05$) increased levels of extracellular superoxide anion production (EX-SOAP) by macrophages. Dendrogram analysis of denaturing gradient gel electrophoresis (DGGE) images detected a significantly different

microbial community within the intestine of fish fed the diet with GBA at 2% compared to fish fed the basal diet and diet with GBA at 1%. None of the experimental diets resulted in significant improvements to survival after exposure to *Streptococcus iniae* due to within treatment variability. However, fish fed the diet with GBA at 2% did tend to experience reduced mortality (12.5%) as compared to fish fed the basal diet (35%). Thus, supplementation of GBA at 2% of diet did alter the gut microbiota of tilapia and enhanced immunological responses and disease resistance to *S. iniae.*

DEDICATION

This is for my family

My father- he gave me the tools to build character, patience and integrity

My mother- she kept me sane and grounded during the last two years

My sister- this has been the only thing she hasn't helped me with

To those of you who I am lucky enough to truly call friends…thank you for always being there

To my fickle friend, tilapia- the next one of you I see better have fries next to it.

One last thing, you may not be able to call me "Dr.", but "Master" will work just fine.

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I would like to bring attention to the National Center for Foreign Animal and Zoonotic Disease Defense (FAZD Center) and the Hispanic Leaders in Agriculture and the Environment (HLAE) at Texas A&M University and thank both entities for the financial assistance given to me to pursue my Master's degree. More specifically, I would like to thank Dr. Heather Simmons of the FAZD and Dr. Manuel Piña of HLAE for their unwavering support throughout my graduate program.

I extend my gratitude to my fellow lab mates, Camilo, Brian, Maritza, Dale and Ben, for the countless hours of sampling they endured.

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INTRODUCTION

Over the past decade, as the world's population has continued to increase exponentially, aquaculture has played an integral role in providing a safe and sustainable seafood alternative to commercial capture fisheries. According to the United Nations' Food and Agriculture Organization (FAO), capture fisheries' production levels have remained relatively static over the past several decades; whereas, aquacultural production has continued to increase to meet the world's growing seafood demand, and now contributes over half of the total (FAO 2010). Tilapia, one of the most commonly farmed species of fish, is among the top contributors to the overall production in finfish aquaculture. As of 2008, world aquaculture production of Nile tilapia, alone, was at 2,334,432 tonnes (FAO 2005-2010).

As the demand for seafood from aquaculture continues to grow, production systems will need to become increasingly efficient. However, several factors currently constrain aquaculture production with the single most costly problem being the loss of fish due to disease, which is facilitated by high stocking densities, poor water quality conditions, and improper nutrition. In the past, the heavy and indiscriminate use of antibiotics to combat various diseases in aquaculture led to increased concerns regarding resistant strains of bacterial fish pathogens as well as drug-resistance in microorganisms present in the natural environment. Also worrisome was the escalating presence of antibiotic residues in fish tissues (Alderman & Hastings 1998; Cañada-Cañada *et al*. 2009)

This thesis follows the style of the journal *Aquaculture Nutrition*.

In response to these concerns, significant progress has been made over the past several years regarding the dietary inclusion of various immunomodulators. A variety of feed additives, including non-nutritive immunostimulatory compounds such as microbial cells or cellular fractions from brewer's yeast, β-glucans, peptidoglycans, chitin and oligonucleotides, as well as probiotics or live microbial dietary supplements, also have received more extensive evaluation with fish in recent years (Nakagawa *et al.* 2007; Gatlin & Li 2008). One specific group of non-nutritive compounds that has gained considerable notoriety is that of prebiotics. These substances were first described as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of beneficial bacteria in the gastrointestinal tract (GIT, Gibson & Roberfroid 1995). Due to the increasing amount of prebiotic research conducted in recent years, the definition has been upgraded to distinguish a prebiotic as a selectively fermented ingredient that fosters specific changes, both in the composition and/or activity of the GIT microbiota, which confer benefits upon host well-being and health (Gibson *et al*. 2004).

Commonly used prebiotics that have been evaluated in various aquatic species include inulin, mannanoligosaccharides (MOS), fructooligosaccharides (FOS), short-chain fructooligosaccharides (scFOS), GroBiotic®-A (GBA), and to a lesser extent, galactooligosaccharides (GOS), xylooligo-saccharides (XOS), arabinoxylooligosaccharides (AXOS), and isomaltooligosaccharides (IMO). Table 1 summarizes several studies in which prebiotics have been evaluated with aquacultured

organisms. Ringø *et al.* (2010) and Yousefian & Amiri (2009) also have recently provided comprehensive reviews of prebiotic use in aquaculture.

Table 1 Summary of prebiotic use in aquaculture

Prebiotic	Dose (g kg^{-1}); duration of trial	Species	Initial Wt. (g)	Response[a]	Reference
Inulin	150; 4 weeks	Arctic charr (*Salvelinus alpines* L.)	218	Intestinal cell damage	Wang & Wang (1997)
	75; 3 weeks	Atlantic salmon (*Salmo salar* L.)	218	→ Intestinal cell damage; ↑ Intestinal growth and relative mass of the gastrointestinal tract	Refstie *et al.* (2006)
	5 and 10; 1 week	Gilthead seabream (*Sparus aurata* L.)	175	Significant inhibition of phagocytosis and respiratory burst in leucocytes	Cerezuela *et al.* (2008)
	20; 1 month	Turbot larvae (*Psetta maxima)*	n/a	↑ Growth rate; Effects on gut microbiota (*Bacillus* and *Vibrio*)	Mahious *et al.* (2006b)
MOS	10; 4 months	Atlantic salmon	200	↓ Oxygen consumption; ↓ Protein and ↑ energy concentration in the whole body	Grisdale-Helland *et al.* (2008)
	2; 4 weeks	Channel catfish (*Ictalurus punctatus*)	16.0	→ Growth performance, hematology, or immune function	Welker *et al.* (2007)
	20 and 40; 67 days	European sea bass (*Dicentrarchus labrax*)	33.7	↑ Growth; → Feed conversion; ↓Lipid vacuolization; ↓ Presence of *Vibrio alginolyticus* on head kidney	Torrecillas *et al.* (2007)
	2; 90 days	Rainbow trout (*Oncorhynchus mykiss*)	30.0	↑ Growth and survival; ↑ Antibody titer and lysozyme activity	Staykov *et al.* (2007)
	2; 8 weeks	Rainbow trout	n/a	↑ Absorptive surface in the posterior gut region; ↑ Microvilli density and length	Dimitroglou *et al.* (2008)

Table 1 continued

Prebiotic	Dose (g kg^{-1}); duration of trial	Species	Initial Wt. (g)	Response[a]	Reference
MOS, contin.	0 and 4; 12 weeks	Rainbow trout	13.2	↑ Growth; ↑ Hemolytic and phagocytic activity; ↑ Mucus weight; ↑Survival against *Vibrio anguillarum*	Rodrigues-Estrada *et al*. (2008)
	0, 2 and 6; 58 days	Hybrid tilapia (*Oreochromis niloticus* × *O. aureus*)	8.1	→ Growth rate; ↑ Survival; ↑ Non-specific immunity	He *et al*. (2003)
	10; 4 weeks	Red drum (*Sciaenops ocellatus*)	10.9	↑ Feed efficiency; ↑ survival following parasitic challenge; ↑ Non-specific immunity	Buentello, *et al*. (2010)
FOS	10; 4 months	Atlantic salmon	200	→ Feed intake, growth or digestibility	Grisdale-Helland *et al*. (2008)
	10; 4 weeks	Red drum	10.9	↑ Non-specific immunity	Buentello, *et al*. (2010)
	0, 2 and 6; 58 days	Hybrid tilapia	57.0	→ Growth rate; ↑ Survival; ↑Non-specific immunity	He *et al*. (2003)
	20; 1 month	Turbot larvae	n/a	↑ Growth rate; Effects on gut microbiota (*Bacillus* and *Vibrio*)	Mahious *et al*. (2006b)
	2.5; 100 days	Soft-shell turtle (*Triortyx sinensis*)	n/a	↑ Growth rate; ↑ SOD activity; ↓Lysozyme activity	Ji *et al*. (2004)
scFOS	0.8 and 1.2; 8 weeks	Hybrid tilapia	5.6	↑ Growth rate, feed intake, feed conversion; → Survival	Hui-Yuan *et al*. (2007)
GBA	10 and 20; 4 (Trial 1) and 7 (Trial 2) weeks	Hybrid striped bass (*Morone chyrsops* × *M. saxatilis*)	91.4 (Trial 1) and 19.7 (Trial 2)	↑ Feed efficiency; ↑ Respiratory bursts; ↑ Resistance against *Streptococcus iniae*	Li & Gatlin (2004)
	10; 6 weeks	Red drum	2.4	→ WG or FE; → Intestinal microbiota	Burr *et al*. (2009)
	10; 4 weeks	Red drum	10.9	↑ Feed efficiency; enhanced WG; ↑ survival following parasitic challenge; ↑ Non-specific immunity	Buentello *et al*. (2010)

Table 1 continued

Prebiotic	Dose (g kg-1); duration of trial	Species	Initial Wt. (g)	Response[a]	Reference
GBA, contin.	20; 16 weeks	Hybrid striped bass	64.5	↑ Growth performance, ↑ Resistance against *Mycobacterium marinum*	Li & Gatlin (2005)
	20; 16 weeks	Golden shiner (*Notemigonus crysoleucas*)	1.06	↑ Resistance against *Flavobacterium columnare*	Sink *et al*. (2007)
	20; 10 weeks	Golden shiner	0.46	→ Survival; ↑Resistance against *Flavobacterium columnare*	Sink & Lochmann (2008)
	10; 3 weeks	Red drum	500	↑ Protein, lipid and organic ADC values	Burr *et al*. (2008)
	10; 4 weeks	Red drum	10.9	↑ FE; ↑ enhanced weight gain; ↑ Survival following *Amyloodinium ocellatum*; ↑ non-specific immune responses	Burr *et al.* (2009)
	10 and 20; 8 weeks	Hybrid striped bass	34.4	→ WG or FE	Burr *et al*. (2010)
	20 ; 9	Rainbow trout	14.3	→ WG or FE; →antibody levels	Sealey *et al*. 2007
XOS	0, 0.15, 2.1 and 3.2; 45 days	Crucian carp (*Carassius auratus gibelio)*	17.0	↑ Growth; → Survival; ↑ Enzymatic activity	Xu *et al*. (2009)

[a]Arrows indicate an increase (↑), decrease (↓), or no change (→) in the response

Previous studies with various fish species in our laboratory revealed that prebiotics have the capacity to afford a range of beneficial effects including improved performance indices (Li & Gatlin 2005; Buentello *et al.* 2010; Zhou *et al.*2010), increased immunological responses (Buentello *et al.* 2010; Zhou *et al.*2010), changes to the intestinal morphology (Zhou *et al.*2010), and, most notably, enhanced survival following disease challenge against various bacterial (Li & Gatlin 2005) and parasitic

(Buentello *et al.* 2010) pathogens. In addition to the previously stated prebiotic benefits, these compounds may also favor the proliferation of beneficial bacteria (in lieu of potentially pathogenic microorganisms) within the intestine. Such effects have been demonstrated in various animals, including poultry (Spring *et al.* 2000; Patterson & Burkholder 2003), swine (Smiricky-Tjardes *et al.* 2003; Konstantinov *et al.* 2004), and various fish species (Burr *et al.* 2008; 2010).

Additionally, GBA supplementation has been shown to improve the nutrient value of plant feedstuffs, enhance non-specific immune responses and increase disease resistance in fish (Li & Gatlin, 2004; 2005; Sealey *et al*. 2007; Burr *et al*. 2008; Buentello *et al*. 2010). Therefore, the objective of the current study was to evaluate the responses of Nile tilapia (*Oreochromis niloticus)* to graded levels of the dairy/yeast prebiotic GBA, with respect to performance indices, selected non-specific immune responses, intestinal microbiota and histology, as well as disease resistance following a controlled bacterial challenge.

MATERIALS AND METHODS

An initial 8-week feeding trial (Trial 1) and a 5-week follow-up trial (Trial 2) were conducted at the Texas A&M University Aquacultural Research and Teaching Facility (ARTF). Prior to the start of both feeding trials, all fish were fed a conditioning diet twice daily (a.m. and p.m.) for 2 weeks. During this conditioning phase the fish became acclimated to the culture system and other environmental conditions.

Diet Formulation

A nutritionally complete basal diet was formulated to contain approximately 35% crude protein on a dry-matter basis. Experimental diets were formulated by supplementing the basal diet with GBA at 1% or 2% of dry weight in place of cellulose (Table 2). The resulting three diets were evaluated in both trials.

All diets were prepared at the ARTF as previously described by Li and Gatlin (2004). Dry ingredients were mixed in a V-mixer followed by the blending of oil and water with the dry ingredients in a food mixer. Then 3-mm pellets were prepared using a commercial blender with a meat grinding attachment. Each diet was crumbled to a size small enough to be consumed by the fish.

Culture System and Feeding Trials

Both feeding trials were conducted in a closed, recirculating system consisting of 110-L glass aquaria. Salinity was maintained at 2 ppt by mixing a synthetic sea salt blend and sodium chloride with well water. Water quality was maintained within acceptable levels for tilapia using mechanical and biological filtration, and supplemental aeration was provided to each aquarium via an airstone to achieve oxygen levels near air

saturation. Water temperature was maintained at 26 ± 1°C by conditioning ambient air, and a 12-h light:12-h dark photoperiod was achieved with fluorescent lights controlled by timers.

Table 2 Composition of experimental diets (% dry weight)

	Diet		
Ingredient (%)	**Basal**	**1% GBA**	**2% GBA**
Menhaden fish meal[1]	10.0	10.0	10.0
Soybean meal, dehulled[2]	45.0	45.0	45.0
Dextrin[3]	25.0	25.0	25.0
Menhaden oil[1]	2.0	2.0	2.0
Vitamin premix[4]	3.0	3.0	3.0
Mineral premix[4]	4.0	4.0	4.0
Carboxymethyl cellulose[3]	2.0	2.0	2.0
CaPO4, dibasic[5]	1.0	1.0	1.0
Corn oil[3]	1.4	1.4	1.4
Casein[3]	4.6	4.6	4.6
Celufil[3]	2.0	1.0	0.0
GroBiotic-A[6]	0.0	1.0	2.0
Proximate composition (%)[7]			
Dry matter	88.3	86.2	87.8
Crude protein	38.2	38.7	39.3
Crude lipid	5.9	5.7	6.2
Ash	8.7	8.6	8.9

[1] OmegaProtein Corporation, Houston, TX, USA
[2] Rangen Inc., Angleton, TX, USA
[3] US Biochemical Corporation, Cleveland, OH, USA
[4] Same as Moon and Gatlin (1991)
[5] Fisher Scientific, Pittsburgh, PA, USA
[6] International Ingredient Corp., St. Louis, MO, USA
[7] Means of two replicate analysis per sample expressed on a dry-matter basis

Trial 1. For Trial 1, fish were obtained from Til-Tech Aquafarm in Robert, LA. This company produces all-male progeny of genetically male tilapia (GMT) using the "Y-Y male technology" developed by Fishgen Ltd. at the University of Swansea in Wales. A total of 225 fish with an initial weight of 4 to 5 g/fish were randomly divided into nine tanks. Twenty-five individuals were then graded by size and stocked into each aquarium such that their collective weight differed by ≤ 5%.

Each diet was randomly assigned to three replicate tanks, with fish in each aquarium receiving their respective diet initially at a rate of 6% of body weight (BW) per day divided into two feedings. The feeding rate was gradually reduced equally among all treatments over the course of the trial to 4% BW to maintain a rate close to apparent satiation without overfeeding. Fish in each aquarium were collectively weighed once every week to monitor weight gain and adjust the feed ration accordingly. This feeding trial continued for an 8-week period following the methodologies established by similar studies conducted with tilapia (He *et al.* 2003; Hui-Yuan *et al.* 2007).

Trial 2. Trial 2 was conducted to more specifically evaluate the immunological responses and disease resistance of tilapia fed the same experimental diets. Mixed sex fish for Trial 2 were obtained from Simaron Freshwater Fish, Inc., Hempstead, TX. A total of 270 (6 to 7 g/fish) disease-free juvenile fish were randomly divided into nine aquaria. Similar group weights were achieved by sorting the fish so as to ensure limited variation among aquaria (≤ 5%). Each experimental diet was provided to fish in three randomly assigned aquaria and weekly weight gain was monitored as previously

described. After 5 weeks of feeding, fish were subjected to a controlled challenge with *Streptococcus iniae*. This time interval was chosen based on previous studies conducted with GBA (Li & Gatlin 2004) and Nile tilapia (Samrongpan *et al.* 2008) which included a disease challenge component.

Bacterial challenge. Prior to the actual challenge, a previously obtained isolate of *Streptococcus iniae* was propagated in brain-heart infusion (BHI, Becton, Dickinson and Company, Sparks, MD) and passed through two fish to assure virulence. A preliminary LD_{50} was conducted in order to ascertain the proper concentration of colony forming units (CFU) needed to achieve 50% survival 1 week after disease exposure. Following the 5-week feeding period in Trial 2, 40 fish from each dietary treatment were randomly selected and subjected to the pathogenic challenge. These fish received an intraperitoneal injection (~0.6 mL/10 g BW) of BHI broth inoculated with virulent bacteria. After injection, fish were transferred to an isolated recirculating system and stocked at a rate of 20 fish/tank, with two replicate aquaria per dietary treatment. Based on the LD_{50} assay, the concentration of *S. iniae* was targeted to be approximately 5.37 $x10^7$ CFU/mL and this concentration was confirmed via replicate plate counts. Fish mortality was then monitored for 21 days following the injection.

Immune responses. At the end of Trial 1 three fish from each tank were humanely euthanized using tricaine methane sulfonate (MS-222, 200 mg/mL; Western Chemical Inc., Ferndale, WA, USA) to collect various biological samples. Blood was collected

from the caudal vasculature using heparinized 1-mL syringes in order to assay selected non-specific immune responses which would indicate the state of immune system function at the time of sampling. A portion of the whole-blood was used to determine neutrophil oxidative radical production with nitro blue tetrazolium (NBT) as described by Siwicki *et al.* (1994). Plasma was then separated by centrifuging the remaining blood at 3,000 × *g* for 10 min and then kept at -80°C until lysozyme activity was determined according to the assay of Jorgensen *et al.* (1993). The same procedures were undertaken following the conclusion of Trial 2, but only two fish per aquarium were sampled.

At the conclusion of Trial 2, in addition to the two immunological assays described above, superoxide anion production from kidney macrophages also was determined as described by Secombes (1990) with modifications in order to further assess the level of immune system function. Head and trunk kidneys from all remaining fish in each treatment (7 to 10 fish per aquaria) were dissected and placed into separate 15-mL vials containing 5-10 mL of Leibovitz (L-15) media with L-glutamine (Sigma-Aldrich Co., St. Louis, Mo). Macrophage isolation and estimation (Buentello & Gatlin 1999) as well as the detection of extracellular and intracellular superoxide anion production (Sealey & Gatlin 2002) were then assessed.

Denaturing gradient gel electrophoresis. Following the conclusion of Trial 1, digesta from the intestines of three fish per tank was pooled into a sterile, DNase-free microcentrifuge tube and immediately frozen in liquid nitrogen. The digesta was then used to determine possible differences in the microbial communities within the GIT

according to the procedures described by (Hume *et al.* 2003). Genomic DNA was isolated from ~0.2 g of thawed digesta with QIAGEN's QIAamp® DNA Mini Kit (Valencia, CA, USA), and 250 ng of template DNA per sample were combined Polymerase Chain Reaction (PCR) was conducted by combining bacteria-specific PCR primers (Burr *et al*. 2010) targeting the conserved regions flanking the variable V3 region of 16S rDNA, with Jump Start Red-Taq Ready Mix (Sigma Chemical Company, St. Louis, MO, USA), bovine serum albumin, to help stabilize the reaction, and 250 ng of template DNA, per sample collected, and run according to the following thermocyler (PTC-200 Peltier Thermal Cycler; MJ Research, MJ Research, Inc., Waltham, MA) program: 1) denaturation at 94.9° C for 2 min; 2) subsequent denaturation at 94.0° C for 1 min; 3) annealing at 67.0° C for 45 sec; -0.5° C per cycle (touchdown to minimize spurious by-products (Don *et al*. 1991; Wawer & Muyzer 1995)); 4) extension at 72.0° C for 2 min; 5) repeat steps 2 to 4 for 17 cycles; 6) denaturation at 94° C for 1 min; 7) annealing at 58.0° C for 45 sec; 8) repeat steps 6 to 7 for 12 cycles; 9) extension at 72.0° C for 7 min; 10) 4.0° C holding temperature. Denaturing gradient gel electrophoresis (DGGE) was conducted using the following modification: 1 µL of PCR product from each of three tanks per treatment was pooled accordingly and added to 3 µL of 2X loading buffer, then loaded onto a corresponding well of an 8% polyacrylamide gel [(vol/vol) acrylamide-bisacrylamide ratio of 37:5:1 (Bio-Rad, Richmond, CA, USA) with a 35% to 60% urea-formamide gradient (100% was 7 M urea and 40% deionized formamide) using a DCode System (Bio-Rad, Hercules, CA, USA) with 0.5x TAE buffer (20 mM Tris (pH 7.4), 10 mM sodium acetate, 0.5 M EDTA) at 59° C for 17 h at

60 V. Gels were stained with SYBR Green I (Sigma). The same techniques were applied at the conclusion of Trial 2 pooling the digesta of two fish per aquarium.

The analysis of band pattern relatedness was determined with Molecular Analysis Fingerprinting software (v 1.6; Bio-Rad, Hercules, CA). This analysis is based on the Dice similarity coefficient (SC) and the un-weighted pair group method using arithmetic averages for clustering. Comparisons between sample band patterns was expressed as a percentage SC (SC > 95% means two populations are identical; SC ≤ 95 and ≥ 80 means two populations are similar; SC < 80 means two populations are significantly different).

Histology. After the conclusion of Trial 1, a single intestine from one fish per tank was injected with Davidson's fixative solution (acetic acid:95% ethanol:formaldehyde:H_2O at a 1:3:2:3 ratio) and fixed for 24 h, after which it was transferred to a 70% ethanol solution for long-term storage. Segments (~1 cm length) of proximal, middle and distal intestine were sliced, embedded in paraffin and transverse sections were processed to 4-μm slides and stained with hematoxylin and eosin. The slides were examined under a light microscope (Olympus, Bx60, Center Valley, PA, USA) equipped with a digital camera (Donpisha, XC-003P) and VGA 460 Osteomeasure software (Osteometrics, Decatur, GA) for image acquisition. Electronic images were further analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA) for assessing dimensions of intestinal folds, enterocytes and microvilli in different enteric sections (10

measurements per section per fish). The same technique was applied to fish from Trial 2, using the intestines of two fish from each aquarium.

Statistical analysis. Analysis of variance (ANOVA) was used to determine significant ($P < 0.05$) dietary effects with regard to the performance indices, immunological assays and histological measurements. All statistical analyses were performed using JMP 9 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Trial 1

Performance indices. Weight gain and feed efficiency values of juvenile Nile tilapia were similar across all three dietary treatments (Table 3); however, at the end of the 8-week feeding trial, fish fed the diet containing 2% GBA showed significantly ($P < 0.05$) increased survival.

Immunological responses. Selected non-specific immune responses are shown in Table 3. Neutrophil oxidative radical production (NBT) was not significantly ($P > 0.05$) affected by prebiotic supplementation. Lysozyme activity in plasma tended to increase with dietary GBA supplementation, although statistical significance was not achieved.

Table 3 Performance indices and non-specific immune responses of Nile tilapia fed experimental diets for 8 weeks (Trial 1)[*]

Diet	WG[§] (%)	FE (g gain/g fed)	Survival (%)	NBT[‡] (U mg^{-1} protein)	Lysozyme[‡] (U ml^{-1})
Basal	670	0.66	90.7^{ab}	5.08	79.0
1% GBA	704	0.65	86.9^{b}	4.94	90.0
2% GBA	669	0.65	98.7^{a}	5.06	111.7
***P*> F[†]**	0.95	0.98	0.04	0.81	0.26
PSE	87.6	0.03	2.56	0.16	12.81

[*] Values represent the means of three replicate aquaria unless otherwise noted. Values in the same column with different superscript letters are significantly different ($P < 0.05$)

[§] Weight gain (WG) as a percent of initial weight

[†] Significance probability associated with the *F* statistic

[‡] Means of nine fish per dietary treatment

Intestinal microbial analysis. Dendrogram analysis (Figure 1) showed that the microbial communities found within fish fed the experimental diets were very similar (SC = 94.7%), being almost identical to each other. The microbial composition of digesta from fish fed the basal diet was very similar to that obtained from fish fed both of the GBA-supplemented diets (SC = 91.0%), indicating that GBA supplementation did not significantly affect the microbial composition within the GIT of juvenile Nile tilapia at the time of sampling.

Figure 1 Dendrogram analysis of the microbial community in the intestine of tilapia fed experimental diets in Trial 1

Histology. An overview of the histological measurements from Trial 1 is shown in Table 4. Fish fed the basal diet had significantly increased fold height in the proximal intestine. Fish fed diets supplemented with 1% GBA experienced significantly increased total enterocyte height in the middle section of the intestine and significantly increased microvilli height in the distal intestine when compared to fish fed the basal and 2% GBA diets. Fish fed 2% GBA diets experienced significantly increased fold height in the distal section of the intestine when compared to the other diets.

Table 4 Histomorphometric measurements (μm) of the proximal, middle and distal sections of intestine (Trial 1)*

Diets	Basal	1% GBA	2% GBA	ANOVA	
Proximal				***P*> F**	**PSE**
Fold height	334.1[a]	295.6[b]	307.2[b]	0.02	9.55
Total enterocyte height	40.3	42.9	44.2	0.13	1.35
Microvilli height	1.9	2.0	2.2	0.06	0.09
Middle					
Fold height	191.7	168.0	159.0	0.12	11.56
Total enterocyte height	32.5[b]	37.9[a]	34.5[b]	0.01	1.18
Microvilli height	2.1	2.4	2.1	0.08	0.10
Distal					
Fold height	112.4[b]	119.7[b]	177.7[a]	0.00	11.04
Total enterocyte height	42.9	43.6	41.7	0.45	1.07
Microvilli height	1.7[b]	2.1[a]	1.9[ab]	0.01	0.07

* Data represent means of 10 independent measurements from one fish per aquarium (three per dietary treatment). Values in the same row with different superscript letters are significantly different ($P < 0.05$)

Trial 2

Immunological responses. At the end of the 5-week period in Trial 2, values for neutrophil oxidative radical production were relatively similar for fish fed the different diets (Table 5). Lysozyme production tended to increase with the amount of GBA in the diet, with fish fed the 2% GBA diet having the highest numerical value, although statistical significance was not reached (Table 5). Extracellular super oxide anion production (EX-SOAP) of fish fed the diet containing 2% GBA was significantly higher than that of fish fed the basal diet. Intracellular superoxide anion (IN-SOAP) was

numerically highest in fish fed the diet with GBA at 2%, but no statistical significance was attained.

Intestinal microbial analysis. The dendrogram analysis (Figure 2) of samples obtained from fish in Trial 2 showed two distinct groups of microbial communities brought about by the different experimental diets. The microbiota found in the digesta of fish fed the basal and 1% GBA diets were remarkably similar (SC = 92.5%). However, fish fed the 2% GBA diet showed a very different microbial community based on the DGGE analysis (SC = 71.9%).

Histology. Histological measurements from Trial 2 are summarized in Table 6. Fish fed the basal diet experienced significantly increased microvilli height in the proximal intestine, as well as significantly increased fold height in the middle section of the intestine. Fish fed the 1% GBA diet experienced significantly increased total enterocyte height in the middle section of the intestine as well as significantly increased fold height in the distal intestine. Fish fed the 2% GBA diet only experienced statistically significant increases in fold height in the proximal intestine.

Bacterial challenge. Fish fed the diet supplemented with 2% GBA experienced noticeably higher survival during the bacterial challenge when compared to fish fed the basal and 1% GBA diets (Figure 3). However, due to within treatment variability, a *P*-value of only 0.33 was achieved.

Table 5 Selected non-specific immune responses of tilapia fed experimental diets for 5 weeks (Trial 2)

Diet	NBT* (U mg^{-1} protein)	Lysozyme* (U ml^{-1})	EX-SOAP‼ (nmol O_2^-)	IN-SOAP† (OD)
Basal	7.16	61.7	0.29^{b}	0.18
1% GBA	6.67	76.7	0.63^{ab}	0.19
2% GBA	6.83	81.7	0.75^{a}	0.24
***P*> F**	0.87	0.81	0.04	.20
PSE	0.67	22.49	0.09	.02

* Means of two fish from each of the three replicate aquaria per dietary treatment. Values in the same column with different superscript letters are significantly different ($P < 0.05$)

‼ Extracellular superoxide anion production

† Intracellular superoxide anion production, optical density at 620 nm

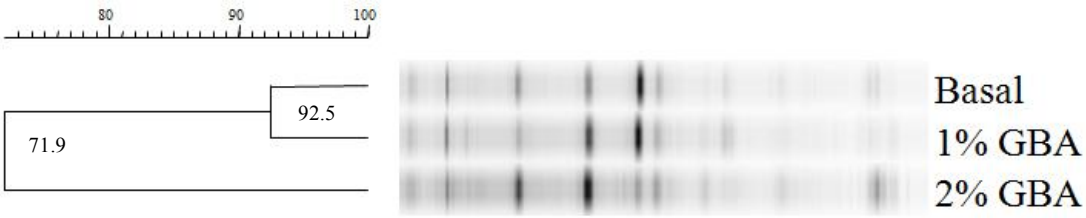

Figure 2 Dendrogram analysis of the microbial community in the intestine of tilapia fed experimental diets in Trial 2

Table 6 Histomorphometric measurements (μm) of the proximal, middle and distal sections of intestine (Trial 2)*

Diet	Basal	1% GBA	2% GBA	ANOVA	
Proximal				***P*> F**	**PSE**
Fold height	321.2[a]	315.2[a]	272.9[b]	0.00	8.30
Total enterocyte height	38.1	37.6	36.5	0.29	0.71
Microvilli height	1.9[a]	1.8[ab]	1.7[b]	0.03	0.05
Middle					
Fold height	227.4[a]	182.3[b]	180.0[b]	0.00	8.07
Total enterocyte height	29.6[a]	33.2[b]	32.2[a]	0.00	0.71
Microvilli height	2.0	2.0	2.0	0.81	0.07
Distal					
Fold height	150.3[b]	174.4[a]	148.0[b]	0.01	6.51
Total enterocyte height	36.3[a]	32.7[b]	35.6[a]	0.01	0.93
Microvilli height	2.4	2.3	2.3	0.69	0.08

* Data represent means of 10 independent measurements from 2 fish per aquarium (six per dietary treatment). Values in the same row with different superscript letters are significantly different ($P < 0.05$)

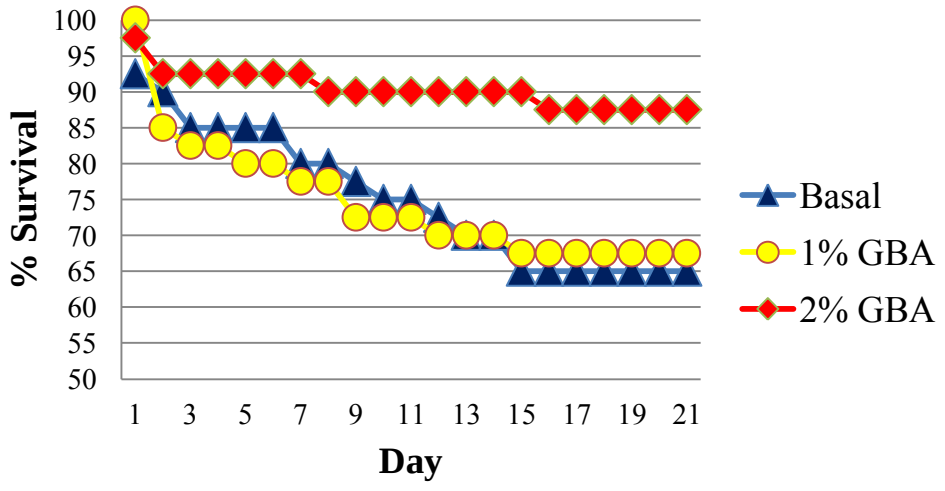

Figure 3 Survival of fish challenged with *Streptococcus iniae*

DISCUSSION

In the current study, GBA supplementation into a 35% crude protein diet was unable to significantly affect WG or FE of Nile tilapia. These results are in stark contrast to a recently published study also supplementing GBA to Nile tilapia where a significant increase in both WG and FE were reported for fish fed a diet containing 29% crude protein and GBA (Zheng *et al*. 2011). The higher protein concentration in the former trial may have negated any beneficial effects on WG and FE, since Zheng *et al*. (2010) failed to see any difference in the WG or FE of fish fed diets containing GBA when compared to another control diet used in that study with a higher crude protein level (33%).

Nevertheless, such intraspecific, as well as interspecific discrepancies are fairly common in prebiotic studies (Ringø *et al*. 2010) and may be largely attributed to initial differences in the composition of intestinal microbiota, although this has not been tested in most studies. Certain members of the microbiota are more apt to utilize certain prebiotic substrates over others (Cummings *et al*. 2001; Sanz *et al*. 2006) and the initial presence or absence of these bacteria will affect the overall outcome. Differences in the size or age of fish (Li & Gatlin 2004; 2005) used in prebiotic studies may also have an impact due to the degree of establishment, or lack thereof, with respect to a stable microbiota (Burr *et al*. 2009; Nayak 2010). Differences in dietary ingredients across studies may also be responsible for the differences observed. Despite the variation in and between trials, prebiotic supplementation has been proven to increase WG (Mahious *et al*. 2006a; 2006b; Zhou *et al*. 2007; Rodrigues-Estrada *et al*. 2008; Xu *et al*. 2009) and

FE (Li & Gatlin 2004; Hui-Yuan *et al*. 2007; Li *et al*. 2007; Zhou *et al*. 2007) in several species of aquatic organisms. In contrast to the reported inconsequential impact GBA had on the survival of hybrid striped bass (Li & Gatlin 2004) and red drum (Buentello *et al*. 2010), in the current study, fish fed the diet containing 2% GBA had significantly higher trial survival than the other two dietary treatments (Table 3). Similar increases in survival throughout the length of the trial have been demonstrated in hybrid tilapia (He *et al*. 2003), and rainbow trout (Staykov *et al*. 2007) fed diets supplemented with FOS and MOS, respectively. It is important to point out the majority of research regarding GBA has been conducted on carnivorous, rather than herbivorous or omnivorous fish (Table 1; Ringø *et al*. 2010)

The most notable outcome of prebiotic supplementation, in general, is changes brought about to the intestine, both morphologically and microbiologically. Changes to the morphology of the intestine may be attributed to the production of short-chain fatty acids through the microbial fermentation of prebiotic substances. A study in which short-chain fatty acids were added to the media resulted in the inhibition of epithelial cell proliferation of caecal tissue *in vitro* (Sakata 1987), while *in vivo* studies have demonstrated stimulated intestinal cell proliferation in rats and pigs (Sakata 1984; Kien *et al*. 2007). This led to the implication that the proliferative effects of short-chain fatty acids are indirect (Sakata 1987). Proof of short-chain fatty acids production from GBA has been demonstrated by Burr *et al.* (2010) in red drum, but no such data exists for Nile tilapia. The ambiguous histological data (Tables 4 & 6) may point to the fact that short-chain fatty acid production, or the undefined indirect mechanism, is limited or non-

existent, particularly in the distal intestine which is the primary site of prebiotic action (Gibson & Roberfroid 1995). However, what is clear is that dietary supplementation of GBA at 2% did not result in the expected increase in microvilli height seen in other prebiotic studies involving different fish species (Yilmaz *et al*. 2007; Salze *et al*. 2008; Zhou *et al*. 2010). In fact, it seems that GBA supplementation had marginally detrimental effects (Tables 4 & 6). However, further research is warranted in order to establish a volatile fatty acid profile and attempt to elucidate the indirect mechanisms that may affect intestinal morphology of Nile tilapia fed diets supplemented with GBA.

Despite the inconclusive morphological data seen in this study as a whole, and the lack of change in microbial community seen in the initial trial, inclusion of GBA at 2% in the follow-up trial was the only dietary treatment able to significantly alter the intestinal microbiota (Figure 2). Although the exact nature of this shift was not identified, it may be safe to assume, as a preliminary hypothesis, that it was towards the accumulation of more beneficial bacteria, rather than pathogenic, based on the overall results of the study. Similar beneficial shifts have been reported with this prebiotic, inulin, MOS, and GOS in hybrid striped bass (Burr 2007) and with GBA in red drum as well (Burr et al. 2008). A possible explanation for the lack of change demonstrated in the initial trial may be due to an initially high level of beneficial bacteria already being present in the intestine of these fish. This is plausible, considering the fact that when the microbial communities of the control treatments from both trials were run on a single gel and then analyzed using DGGE, they shared a similarity coefficient of < 80%, signifying that the two different populations of fish used in each trial did not start off with the same

microbial community composition. Differences within species, such as this is not unfathomable, considering that the initial colonization and subsequent establishment process of the enteric microbiota can be greatly affected by the initial rearing conditions, which in the two populations of fish used in this study where geographically different (Nayak 1990).

In the current study, fish fed the diet containing GBA at 2% exhibited significantly increased EX-SOAP. Increases in lysozyme and IN-SOAP were generally seen as the percent inclusion of GBA increased, but no statistical significance was reached. The significant increase in EX-SOAP is not surprising, given that GBA contains constituents of dairy ingredient components, dried fermentation products, and autolyzed brewers' yeast. The brewers' yeast component contains β-glucans and nucleotides, which have been shown to induce immunological responses in fish (Li & Gatlin 2006; Dalmo & Bogwald 2008). Previous studies involving prebiotic supplementation to fish also have been shown to enhance similar aspects of the non-specific immune system, such as plasma lysozyme activity, neutrophil oxidative radical production, IN-SOAP, and EX-SOAP (Li & Gatlin 2004; 2005; Buentello *et al*. 2010; Zheng *et al*. 2011).

What might have the greatest impact, in terms of increasing aquaculture production, is how each of the above components may have worked in concert to increase the ability of Nile tilapia to ultimately resist disease. Fish fed the diet containing GBA at 2% experienced noticeably higher survival than fish fed either of the other two diets in feeding Trial 1and after the controlled disease challenge in Trial 2.

Since lysozyme is most effective against gram-positive bacteria and readily increases when bacteria is present, the elevated levels seen in this study (Table 5) may have allowed the lysozyme to increase to such a level as to partially control *S. inaie* when introduced into the fish (Saurabh & Sahoo 2008). However, since lysozyme activity was not assayed following the conclusion of the challenge, this hypothesis cannot be verified. The significant increase associated with the production of EX-SOAP would have likely been another aspect of the immune system working to keep the infection under control and reduce mortality, especially because *S. iniae* has been shown to display anti-phagocytic properties which would render IN-SOAP of macrophages useless (Agnew & Barnes 2007).

The drastic difference in challenge survival in the current study could indicate that GBA inclusion above 1% is required to evoke any noticeable change in survival. This theory is supported by the research conducted by Zheng *et al*. (2011), where GBA included at a level over 1% resulted in the highest survival following a disease challenge with *Aeromonas hydrophila*. Numerous other studies have demonstrated the ability of GBA to increase survival following bacterial challenges with golden shiners exposed to *Flavobacterium columnare* (Sink et al. 2007; 2008; 2010), and hybrid striped bass exposed to *Aeromonas hydrophila* and *Streptococcus iniae* (Li and Gatlin 2004; 2005). Supplementation of GBA into the diet of rainbow trout has also proven to be effective in increasing survival when challenged with infectious hematopoietic virus (Sealey et al. 2007). Further research is warranted in order to continue characterizing the effects of

GBA supplementation in the diets of various fish species commonly cultured in aquaculture production systems.

SUMMARY

The link between gut health and the overall well-being and disease resistance of an animal is now starting to inundate the aquaculture industry as a possible solution to increased mortality associated with a decrease in optimal environmental conditions and subsequent disease outbreaks. Through numerous studies, both *in vitro* and *in vivo*, prebiotics have been shown to trigger a beneficial shift in intestinal microbiota, leading to the cascade of advantageous effects previously mentioned.

The present study highlighted the ability of GBA supplementation to beneficially affect several aspects of host physiology when supplemented into the diet of juvenile Nile tilapia. Although GBA supplementation was unable to increase the weight gain or feed efficiency in the current study, it resulted in the establishment of a significantly different microbial community within the intestine, coupled with the up regulation of several immune responses; ultimately resulting in considerably decreased mortality following a bacterial challenge with the aquatic pathogen *S. iniae*. Other studies supplementing GBA have reported similar findings, but further research is required regarding the microbial characterization of tilapia intestine and other fish species in order to gain a better understanding of the direct and indirect effects garnered by GBA supplementation.

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