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Effect of Dietary Supplementation of Brewer's Yeast and GroBiotic®-A on Growth, Immune Responses, and Low-Salinity Tolerance of Pacific White Shrimp *Litopenaeus vannamei* Cultured in Recirculating Systems

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Two separate trials were conducted in clean recirculating systems at salinities of 32.9 (optimal) and 2 ppt (low-salinity challenge) to evaluate brewer's yeast and GroBiotic®-A, a commercial prebiotic, as dietary supplements for growth and health management of Pacific white shrimp Litopenaeus vannamei. *The growth-promoting influences of brewer's yeast or GroBiotic®-A previously observed with fish were not demonstrated in these trials with shrimp, when each component was supplemented at 2% or 5% of the diet. No significant dietary effects on hematological and immunological responses—including total hemocyte count, hemolymph protein, phenoloxidase, hemocyte respiratory burst, and clearance efficiency of* Vibrio harveyi*—were observed in shrimp cultured at full strength salinity (30 ppt) in feeding trial 1 after a 10 week period.*

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In a second feeding trial, dietary supplementation of GroBiotic®-A improved survival of shrimp cultured at low-salinity (2 ppt). Although the mechanism(s) for enhanced survival under low-salinity conditions by dietary immunostimulants or prebiotics have not been identified, these observations indicate potential use of prebiotics such as GroBiotic®-A for shrimp cultured in less than optimal environments.

KEYWORDS Pacific white shrimp, Litopenaeus vannamei, *brewer's yeast, GroBiotic®-A, immunostimulant, prebiotic*

INTRODUCTION

Global farmed shrimp production has grown phenomenally into a prosperous industry producing nearly 1.8 million metric tons per year by quantity and $9.3 billion by value, and it is expected to double in the next 20 years (Leung & Engle, 2006). Despite rapid expansion, this industry has been negatively impacted by infectious diseases including viruses, rickettsiae, bacteria, fungi, and parasites, causing billions of dollars in losses (Lightner, 2005). Traditional uses of antibiotics have been criticized due to the potential development of antibiotic-resistant bacteria, presence of antibiotic residues in seafood, destruction of microbial populations in the aquacultural environment, and suppression of the aquatic animals' immune systems.

Potential manipulations of gastrointestinal microflora in aquatic animals by application of dietary probiotics to enhance growth efficiency and disease resistance have attracted extensive attention in recent years (reviewed by Irianto & Austin, 2002; Burr, Gatlin III, & Ricke, 2005). Research on dietary supplementation of probiotics and prebiotics for shrimp is still rather limited (Rengpipat et al., 1998; 2000; 2003), although many reports have been published regarding application of probiotics in the aquatic environment (Irianto & Austin, 2002). Recently, we found dietary supplementation of fructosaccharide can alter gastrointestinal microbiota and enhance shrimp immune response indices including total hemocyte count and hemocyte respiratory burst, probably indicating a potential use of dietary prebiotics for health and growth management of shrimp. In addition, our previous studies (Li & Gatlin, 2004, 2005) have shown that dietary supplementation of the commercial prebiotic GroBiotic®-A beneficially influenced immune responses and diseases resistance as well as growth of hybrid striped bass *Morone chrysops* × *M. saxatilis*. The prebiotic effects of the GroBiotic®-A on shifting microbial composition have been confirmed by *in vitro* fermentation assays (Burr & Gatlin, 2008). Collectively, those previous exciting observations with shrimp and fish prompted the current series of experiments on the dietary prebiotic GroBiotic®-A with shrimp. Because brewer's yeast is one of

the components of GroBiotic®-A and also a recognized immunostimulant for shrimp (Scholz et al., 1999; Burgents, Burnett, & Burnett, 2004; Sritunyalucksana et al., 2005), two levels of brewer's yeast also were evaluated in semi-purified diets fed to shrimp under both ideal and low-salinity culture conditions.

MATERIALS AND METHODS

Two separate growth trials were conducted with the experimental diets. The basal diet contained 35% protein, 7.8% lipid, and an estimated total and digestible energy level of 15.5 and 14.6 kJ/g, respectively (Table 1). This diet satisfied and/or exceeded all known nutrient requirements of shrimp (D'Abramo, Conklin, & Akiyama, 1997). Partially autolyzed brewer's yeast (Brewtech) and GroBiotic®-A, a commercial prebiotic product, were supplied by International Ingredient Corporation (St. Louis, MO). Two incremental levels (2% and 5% of diet) of GroBiotic®-A or brewer's yeast were added to the basal diet, and other ingredients were adjusted to provide isonitrogenous and isocaloric diets. Experimental diets were processed as described by Gong and colleagues (2000). Briefly, all dry ingredients were mixed thoroughly for at least 40 min. Then, purified lipid was slowly added and mixed for an additional 30 min. Finally, hot water was added to form a dough that was immediately extruded through a 2 mm orifice die using a Hobart A-200 extruder (Hobart, Troy, OH). Extruded diets were dried in an oven at 45°C for 12 h to reduce moisture content to 9.3-9.5%, ground to appropriate sizes for juvenile shrimp, and stored frozen in sealed plastic bags until used.

Specific-pathogen-free *L. vannamei* postlarvae (virus-susceptible Kona strain, PL 05-02, SR052) obtained from the Oceanic Institute in Hawaii were reared at the Texas Agricultural Experiment Station Shrimp Mariculture Project, Texas A&M University System (Port Aransas, TX) at 32 ± 0.6°C, 32.9 ± 0.6‰ salinity and 5.2 ± 0.2 mg dissolve oxygen/l. Shrimp were fed a commercial post-larval diet (45% crude protein and 10% crude lipid, Rangen, Buhl, ID) and supplemented with live *Artemia* nauplii twice daily until the feeding trial began. The growth trial was conducted in 60, 104-L rectangular tanks (bottom area 0.33 m^2) in a recirculating water system. Water exchange rate was maintained at 20.5 ± 2% daily to ensure an optimum environment (ammonia: 0.06 ± 0.02 mg N/l; nitrite: 0.06 ± 0.02 mg N/l; nitrate: 0.43 ± 0.06 mg N/l; pH 8.0). Groups of 17 shrimp of similar size were blotted dry and weighed before being stocked into each tank. The average shrimp weight was 1.68 ± 0.18 g (SD) each. Also, all shrimp from each tank were blotted dry and weighed as a group at the end of the feeding trial. There were ten replicate groups of shrimp for each dietary treatment. No significant difference in shrimp initial weights (either group weight or average weight) was observed among the various dietary treatments. Shrimp were fed the experimental diets 15 times daily using automatic

TABLE 1 Composition and Determined Proximate Composition (As-Fed Basis) of the Experimental Diets in Both Trials[1]

Constituent	Basal	2% Brewer's yeast	5% Brewer's yeast	2% GroBiotic®-A	5% GroBiotic®-A
Menhaden meal[2]	13.6	13.6	13.6	13.6	13.6
Squid muscle[3]	13.6	13.6	13.6	13.6	13.6
Krill meal[3]	9.0	9.0	9.0	9.0	9.0
Soy protein[3]	10.4	9.5	8.2	9.7	8.6
Phospholipid[3]	4.7	4.6	4.6	4.6	4.5
Wheat starch[4]	29.8	28.9	27.5	28.8	27.2
Mineral/vitamin premix[3]	0.50	0.50	0.50	0.50	0.50
Vitamin C, 35% active[3]	0.05	0.05	0.05	0.05	0.05
Diatomaceous earth[4]	0.5	0	0	0.1	0.1
Sodium alginate[5]	2.0	2.0	2.0	2.0	2.0
Sodium chloride[3]	0.4	0.6	0.7	0.6	0.6
Potassium chloride[3]	1.9	1.8	1.7	1.7	1.6
Magnesium oxide[3]	1.7	1.7	1.7	1.7	1.7
Calcium carbonate[3]	1.5	1.3	1.4	1.3	1.4
Dicalcium Phosphorus[4]	6.6	7.0	6.9	7.0	6.9
Sodium hexametaphosphate[3]	1.0	1.0	1.0	1.0	1.0
Cellulose[2]	2.75	2.85	2.55	2.75	2.65
Brewer's yeast[6]	0	2.0	5.0	0	0
GroBiotic®-A[6]	0	0	0	2.0	5.0
Analyzed proximate composition (% as-fed)					
% Moisture	9.3	9.5	9.4	9.3	9.3
% Crude protein	35.3	35.1	35.0	35.4	35.5
% Crude fat	7.7	7.8	7.9	7.7	7.6
% Crude fiber	2.8	2.7	2.7	2.8	2.9
% Total ash	17.2	17.3	17.3	17.2	17.1

[1]The chemical analyses of protein ingredients calculations were performed as specified by Siccardi (2006).
[2]Omega Protein Corp., Houston, TX. Contained 68.9% crude protein, 23.6% ash, and 4.64% (dry-weight basis).
[3]Zeigler Brothers Corp., Gardners, PA. Squid muscle contained 91.4% crude protein, 4.2% ash, and 5.63 Kcal/g (dry-weight basis). Krill meal contained 70.2% crude protein, 12.2% ash, and 5.19 Kcal/g (dry-weight basis). Soy protein contained 51.6% crude protein, 7.4% ash, 4.42 Kcal/g (dry-weight basis).
[4]MP Biomedicals, Cleveland, OH.
[5]NutraSweet-Kelco Co., Chicago.
[6]International Ingredient Corp., St. Louis, MO.

feeders. The feeding rate was maintained as 2.5 g feed/shrimp/week (0.357 g feed/shrimp/d), so that the shrimp were fed to slight excess. Uneaten feed, fecal waste, and molted exuviae were removed in the morning before the next daily feed ration was placed into the automatic feeders. At the end of the sixth week of feeding, shrimp were counted and weighed for determination of survival and growth. Shrimp from six tanks out of the original ten replicate tanks per treatment were randomly chosen and maintained in the same culture system for an additional 4 weeks at the Texas A&M University System Shrimp Mariculture Project (Port Aransas, TX) prior to immunological sampling.

At the end of week 10, six shrimp from each treatment (one shrimp from each of six replicate tanks) were bled as described by Liu and colleagues (2004) for determination of total hemocyte count (THC), respiratory burst of hemocytes, and total hemolymph protein. Briefly, hemolymph (approximately 200 μL) was withdrawn from the ventral sinus of each shrimp into a 1 mL sterile syringe (25 gauge) containing 0.5 mL of anticoagulant solution (trisodium citrate 30 mM, sodium chloride 0.34 M, EDTA 10 mM, pH 7.55, osmolality 780 mOsm kg^{-1}). The quantity of hemolymph was precisely measured by weighing the syringes prior to and after bleeding. Fifty μL of hemolymph-anticoagulant mixture were mixed with 50 μL of trypan blue and placed in a hemacytometer for counting hemocytes. Hemocytes from individual shrimp were separated by centrifugation at 300 × *g* for 10 min, and respiratory burst of hemocytes was determined following the procedure described by Liu and colleagues (2004). Supernatant fractions from hemocyte separation were used for hemolymph protein determination by using a serum protein kit (Sigma-Aldrich Corp., St. Louis, MO, USA). An additional six shrimp from each treatment (one shrimp from each of six replicate tanks) were bled for phenoloxidase determination by following the procedure described by Hernández-López, Gollas-Galván, and Vargas-Albores (1996), and Liu and colleagues (2004).

A bacterial clearance efficiency test was conducted by following the procedure described by Sritunyalucksana and colleagues (2005) with minor modification. Briefly, a *Vibrio harveyi* isolate was ordered from the American Type Culture Collection (Manassas, VA), and grown in marine broth (Difco 2216) at 30°C overnight. Bacterial concentration was estimated by optical density. Because a preliminary experiment showed that a high dosage (1×10^8 cells/mL) of *V. harveyi* induced rapid mortality of shrimp, a lower dosage (2×10^7 cells/mL) was used to assess bacterial clearance. One hundred μL of bacterial suspension was injected intramuscularly between the 3rd and 4th segment under the exoskeleton. Six shrimp from each treatment (one shrimp from each of six replicate tanks) were injected and confined in a 20 L container with sufficient airflow for 1 h. Then, hemolymph was withdrawn as described above and serially diluted in artificial seawater. Fifty μL of each dilution were dropped on a thiosulphate, citrate, bile-salt agar (TCBS), a *Vibrio* selective agar. The culture plates were incubated at 30°C for 48 h to obtain bacterial counts.

In the low-salinity experiment, another supply of specific-pathogen-free *L. vannamei* postlarvae (virus susceptible Kona strain, SR052) were obtained from the Oceanic Institute in Hawaii and reared at the Texas Agricultural Experiment Station Shrimp Mariculture Project, Texas A&M University System (Port Aransas, TX) at 30 ± 1°C, 35‰ salinity, and 5.2 ± 0.2 mg dissolve oxygen/l. These shrimp were then acclimated to 2‰ salinity over 7 days. The growth trial was conducted in 40, 32 L square tanks (bottom area 0.09 m^2) at 2‰ salinity in an indoor recirculating water

system. Water exchange was maintained at 10% every other day to ensure an optimum environment. Groups of five shrimp of similar size were blotted dry and weighed before being stocked into each tank. The average initial weight was 0.16 ± 0.01 g per shrimp. There were ten replicate groups of shrimp for each dietary treatment. Due to space limitations in the experimental system, the 2% brewer's yeast treatment was excluded from this experiment. This feeding trial was conducted under a very similar regime as described in feeding trial 1, but was terminated after 20 days due to mortality of greater than 40% for shrimp fed the basal diet. Due to limited shrimp size, no immune assays or bacterial clearance tests were conducted in this experiment.

All the data were analyzed using one-way ANOVA, following by Duncan's multiple-range test by SPSS. Probability values less than 0.05 were taken to indicate statistical significance.

RESULTS

Growth and Survival

In this feeding trial, because of the high quality of animals, experimental diets, and environmental conditions, shrimp fed experimental diets demonstrated superior growth performance (averaging 2.1 g weight gain/wk) as shown in Table 2. Survival of shrimp in all treatments during the feeding trial was approximately 97.5% without significant ($P < 0.05$) dietary influences.

Immune Responses and Bacterial Clearance

The immune response assays failed to show significant differences among shrimp fed diets with different amounts of brewer's yeast or GroBiotic®-A (Table 3). However, shrimp fed the GroBiotic®-A supplemented diets tended ($P < 0.1$) to have higher total hemocyte counts when compared to

TABLE 2 Growth Performance of Shrimp Fed the Experimental Diets for 6 Weeks[1]

Diet	Final weight (g)	Weight gain (g)	Feed efficiency	Survival (%)
Basal	14.1 ± 0.3	12.4 ± 0.3	0.79 ± 0.02	95.9 ± 1.5
2% Brewer's yeast	14.2 ± 0.3	12.5 ± 0.3	0.82 ± 0.02	98.2 ± 0.9
5% Brewer's yeast	14.0 ± 0.2	12.3 ± 0.2	0.80 ± 0.01	98.2 ± 0.9
2% GroBiotic®-A	14.2 ± 0.2	12.5 ± 0.2	0.80 ± 0.01	96.5 ± 1.8
5% GroBiotic®-A	14.1 ± 0.3	12.4 ± 0.3	0.81 ± 0.02	97.7 ± 1.3
$P > F^2$	0.976	0.976	0.841	0.635

[1]Means of ten replicate aquaria containing 17 shrimp each. Initial weight of individual shrimp averaged 1.68 g.
[2]Significance probability associated with the F statistic.

TABLE 3 Physiological Responses of Shrimp Fed Various Experimental Diets for 10 Weeks[1]

Diet	Total hemocyte count (million cells/ml)	Hemolymph phenoloxidase (absorbance/50 µl hemolymph)	Respiratory burst (absorbance/ 10 mg hemolymph)	Hemolymph protein (g/100 g hemolymph)	Log_{10} (bacterial count)/g hemolymph
Basal	15.0 ± 2.3	0.04 ± 0.01	0.026 ± 0.007	9.34 ± 0.39	5.7 ± 0.3
2% Brewer's yeast	15.4 ± 2.5	0.03 ± 0.01	0.027 ± 0.004	9.43 ± 0.80	5.6 ± 0.5
5% Brewer's yeast	19.8 ± 2.2	0.06 ± 0.02	0.029 ± 0.006	8.63 ± 0.39	6.3 ± 0.4
2% GroBiotic®-A	21.9 ± 3.9	0.05 ± 0.01	0.036 ± 0.007	9.22 ± 0.55	6.3 ± 0.2
5% GroBiotic®-A	21.8 ± 2.6	0.07 ± 0.01	0.031 ± 0.005	8.91 ± 0.37	5.4 ± 0.4
$P > F^2$	0.288	0.359	0.757	0.784	0.347
Pooled S. E.	2.768	0.014	0.006	0.490	0.367

[1]Values represent mean ± standard error of six replicate shrimp from each treatment.
[2]Significance probability associated with the F statistic.

shrimp fed the basal diet (Table 3). The bacterial clearance rate of individual shrimp varied tremendously and no significant dietary effect was observed (Table 3).

Survival in the Low-Salinity Trial

In the low-salinity feeding trial, significant differences in shrimp survival at low-salinity (2‰) were observed (Figure 1). Survival of shrimp fed diets supplemented with 5% brewer's yeast and 2% GroBiotic®-A averaged 72%, respectively, and was significantly higher ($P < 0.05$) than shrimp fed the basal diet (average 53%). Dietary effects on weight gain were not significant.

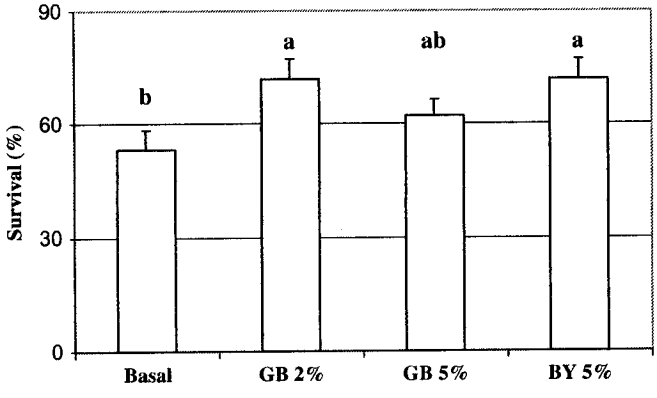

FIGURE 1 Cumulative survival (%) of shrimp fed diets supplemented with brewer's yeast (BY) or GroBiotic®-A (GB) for 20 days in the low-salinity (2 ppt) environment of trial 2 (n = 10). P = 0.04.

DISCUSSION

Potential use of dietary probiotics and prebiotics for shrimp has received very limited attention to date. Our recent study showed that supplementation of short-chain fructooligosaccharide can change shrimp intestinal microbiota and immunity (Li et al., 2007). Dietary supplementation of GroBiotic®-A has been shown to enhance growth and feed efficiency of hybrid striped bass (Li & Gatlin, 2004; 2005). However, the growth-promoting effect of this prebiotic was not proven by the feeding trial with shrimp in the present study. Because the feeding trial was conducted in a very clean recirculating system with UV sterilizer, the culture environment may have had a limited microbial load. In addition, the digestive physiology and feeding behavior of shrimp is different from most fish species.

Except for hemolymph protein, the variations in immune response parameters among individual shrimp, which were rather high in the present trial, may have masked possible dietary effects. Phenoloxidase is considered an important humoral defense component for crustaceans such as shrimp (Söderhäil & Cerenius, 1992; Chen, Huang, & Song, 2004). Although several microbial derivatives such as fugal β-glucan (Chang et al., 2003) and peptidoglycan (Wang, Song, & Huang, 2004) have been shown to significantly enhance phenoloxidase production, similar influences were not reported in studies with brewer's yeast (Scholz et al., 1999). The timing of administration is considered an important factor in immunostimulant therapy (Sakai, 1999), although this aspect has not been sufficiently researched. However, Chang and colleagues (2002) reported that the immunostimulatory enhancement of *P. monodon* broodstock induced by β-1, 3 glucan peaked at day 24 after initiating dietary exposure and subsequently decreased to pre-feeding levels at the end of the 40-d feeding trial. Results of that study were in agreement with an early study by Sung, Kou, and Song (1994), which showed immersion delivery of glucan only conferred short-term protection for *P. monodon* against *Vibrio* infection. Sritunyalucksana et al. (2005) observed the *P. monodon* fed 4% yeast extract for 4 wks had significantly higher hemocyte count and bacterial clearance efficiency than shrimp fed a commercial control diet. However, the present study failed to confirm those observations possibly because of the prolonged feeding. Further evaluation of the potential timing of administration on immunostimulant therapy is warranted.

Potential use of dietary supplementation strategies to enhance environmental tolerance of aquatic animals has aroused increased interest worldwide. For example, Burrells et al. (2001) found dietary supplementation of nucleotides can enhance growth of Atlantic salmon after being transferred to salt water. Glycine-enrichment has been shown to significantly enhance survival of oysters after being transferred to freshwater from seawater (Takeuchi, 2007). However, benefits of dietary prebiotic or probiotic

supplementation to enhance tolerance to environmental changes have not been reported with any aquatic species to the best our knowledge. The low-salinity challenge in the present study suggested that dietary supplementation of GroBiotic®-A and brewer's yeast improved survival of shrimp cultured at low-salinity. These observations might indicate the potential use of prebiotics such as GroBiotic®-A for shrimp cultured in compromised environments. The mechanism of how the prebiotic supplement influenced the osmoregulatory capacity of shrimp in these trials is not known, but further research is warranted.

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