

Research Article

Dietary γ -Aminobutyric Acid (GABA) Promotes Growth and Resistance to *Vibrio alginolyticus* in Whiteleg Shrimp *Litopenaeus vannamei*

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Received 27 December 2021; Revised 11 February 2022; Accepted 25 February 2022; Published 9 March 2022

Academic Editor: Erchao Li

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An 8-week feeding trial was conducted to evaluate the effects of dietary γ -aminobutyric acid (GABA) on growth performance, immune response, and disease resistance for juvenile whiteleg shrimp, *Litopenaeus vannamei*. Five isonitrogenous diets were formulated by supplementing 0 (CON), 50 (GABA₅₀), 100 (GABA₁₀₀), and 300 (GABA₃₀₀) mg of GABA and 4 g of oxytetracycline (OTC) per kilogram of diet. A total of 225 juvenile whiteleg shrimp with an initial weight of 2.97 ± 0.06 g were randomly distributed and reared in 15 aquaria as triplicates. After 8 weeks of the feeding trial, weight gain, specific growth rate, feed efficiency, and protein efficiency ratio of shrimp fed GABA₁₀₀ were significantly higher than those of shrimp fed CON, GABA₅₀, and GABA₃₀₀ diets ($P < 0.05$). However, there were no significant differences among shrimp fed CON, GABA₅₀, GABA₃₀₀, and OTC diets ($P > 0.05$). After nine days of challenge test with *Vibrio alginolyticus*, the average cumulative survival rate of shrimp fed GABA₅₀, GABA₁₀₀, and OTC was significantly higher than that of shrimp fed GABA₃₀₀ and CON diets ($P < 0.05$). These results may suggest that 100 mg dietary GABA supplementation (including endogenous GABA, 175.6 mg/kg diet) per kilogram of diet could be the optimum dietary level to replace antibiotics and improve growth performance and disease resistance in whiteleg shrimp *L. vannamei*.

1. Introduction

Shrimp production is important to global aquaculture growth, and it is the most traded aquaculture product [1]. Whiteleg shrimp, *Litopenaeus vannamei*, is the most abundantly raised shrimp contributing to 83% of global shrimp aquaculture production, of which the majority (82%) is produced in Asian countries [2]. This shrimp species is also

quite resilient to variations in culture salinity and can be raised in both inland and marine water environments. As such, the last ten years has seen an increase of over 51% in *L. vannamei* production. This rapid growth, in addition to the aforementioned reasons, can largely be attributed to their superior growth rate and survival in high-density cultures, low protein requirements, and ability to successfully spawn in commercial hatcheries [3–6].

As with the growth of aquaculture, the intensification of *L. vannamei* is characterized by high stocking densities, which have led to increased feed inputs, resulting in oxidative stress to the shrimp as well as local environmental challenges that occur at the farm level [7, 8]. For instance, during feeding events, dissolved oxygen levels can plummet, leading to greater oxidative stress and concomitant increases in such indicators as superoxide dismutase [9, 10]. These crowding and related stressors lower *L. vannamei* growth rates and impair immune function, thus increasing susceptibility to disease outbreaks and mass mortality events [7, 11, 12]. Furthermore, *L. vannamei* lacks a true adaptive immune system and is therefore incapable of benefiting from interventions such as vaccination, thereby causing an increased usage of antimicrobial treatments [1, 13]. Though many serious shrimp-related pathogens are viral, the threat posed by bacterial pathogens such as *Vibrio* spp. is often mitigated by antibiotic usage for either prophylactic or prescriptive purposes [1, 14]. Though the majority of *Vibrio* spp. are benign, *V. parahaemolyticus*, *V. campbellii*, *V. harveyi*, *V. owensii*, and *V. alginolyticus* have wreaked havoc on the industry in recent years [15, 16]. The excessive use of antibiotics likely stems from farmer's lack of knowledge concerning the hazards related to their abuse such as the natural selection of antimicrobial-resistant strains, inadequate pathogen diagnosis, and ease of availability in some developing countries [17, 18]. This leads to these agents being liberally administered with inadequate information on preparation, dosage, and disease to be treated [18]. The excessive use of antibiotics may pose health threats to both humans and animals alike due to the resulting proliferation of the previously mentioned antimicrobial-resistant pathogens. Sadly, more than 90% of oxytetracycline (OTC) is released into the immediate environment [14]. Because of these serious issues, antibiotics are highly regulated or outright banned in many countries.

A potential alternative to the use of antibiotics is feed additives. Feed additives are components of the diet formulation, whether nutritive or otherwise, that are incorporated into the feed in relatively small quantities for purposes other than fulfilling the nutrient requirements of the target species. Such purposes may include but not be limited to enhancement of growth, immunity, pigmentation, feed stability, and final product properties [19, 20]. Numerous studies [21–23] have demonstrated the positive effects of feed additives on the growth rate, survival, immune response, and disease resistance in fish and shrimp.

γ -Aminobutyric acid (GABA) is a four-carbon non-essential amino acid and widely known as the principal inhibitory neurotransmitter in the animal central nervous system (CNS). It is also regarded as an interkingdom signaling molecule and serves a myriad of functions across all kingdoms of life ranging from plants and bacteria to invertebrate and vertebrate animals [24]. Recently, it has gained importance as a feed additive [25–27]. Lactic acid bacteria and yeast are the most important producers of GABA due to their commercial availability and scalability [20]. GABA is synthesized from glutamate (Glu) in the neurons via glutamate decarboxylase (GAD) and can play an important role in fish growth enhancement, immune responses, and feed efficiency [28].

Despite the progress made with the evaluation of the effects of other feed additives on various fish species, there is a scarcity of information on the effects of dietary GABA.

Though there has been a resurgence of interest in GABA in the context of aquaculture nutrition, it has long been known for its ability to improve reproduction and other health parameters in shellfish. In Pacific oyster (*Crassostrea gigas*), 10 μ mol/L inhibited nitric oxide synthase (NOS) activity and proinflammation cytokines in hemocytes [29]. In the case of red abalone (*Haliotis rufescens*), GABA induces planktonic larvae to settle and undergo metamorphosis when administered at 1–2 μ mol/L [30, 31]. It has also been shown to improve reproduction in rotifer (*Brachionus plicatilis*) reared in stressful culture conditions at 87.5 mg/L. These trials involved the administration of GABA directly to the water. Though not considered a dietary supplementation, these early trials point to GABA's usefulness in aquaculture. With regard to dietary supplementation in fish, to date, there have only been a handful of trials evaluating GABA as a feed additive. Wu et al. [32] demonstrated that GABA supplementation in the diet of juvenile grass carp (*Ctenopharyngodon idella*) significantly improves specific growth rate (SGR) and modulates NPY and ghrelin gene expression. Analysis of their results indicates that 87.5 mg/kg GABA could be the optimal supplemental level. Temu et al. [33] found that supplementation with GABA significantly improved growth and modulated both aspartate aminotransferase (AST) and superoxide dismutase activity (SOD) in juvenile Nile tilapia (*Oreochromis niloticus*) with a 158 mg/kg determined to be an optimal level in the diet. GABA has also been shown to modulate the immune response and improve survival against bacterial challenge. In a trial by Bae et al. [34], juvenile olive flounder (*Paralichthys olivaceus*) given a GABA supplementation of 158 mg/kg GABA led to significantly improved growth and enhanced villi length, trypsin activity, and disease resistance against *Edwardsiella tarda*. In a similar trial, also involving juvenile *P. olivaceus*, Farris et al. [35] found that in addition to improved growth, supplementation with 100 and 150 mg/kg led to increased lysozyme and amylase activity and disease resistance against *Streptococcus iniae*. In this trial, an optimal supplementation level of 237 mg/kg was determined based on the total GABA content of the experimental diets (supplemental as well as endogenous to the feed). Furthermore, GABA has been implicated in the modulation of inflammatory pathways as well as modulation of the intestinal microbiome in juvenile turbot (*Scophthalmus maximus*) [36] at a supplementation level of 160 mg/kg GABA. Feeding trials assessing GABA supplementation in crustaceans have been much more limited than is the case with fish. So far, there have only been a few trials evaluating GABA's effects. In a recent trial assessing GABA in Chinese mitten crab (*Eriocheir sinensis*) by Zhang et al. [37], it was found that GABA works to modulate gene expression within orexigenic/anorexigenic pathways which indicates GABA's ability to affect satiation. As for shrimp, to the best of our knowledge, the only study on the effect of GABA on *L. vannamei* was carried out by Xie et al. [26] which reported that GABA was effective against ammonia stress and can improve growth performance in low-fishmeal diets when given 150 mg/kg

GABA. Therefore, the present study is aimed at evaluating the effects of different dosages of dietary GABA on growth, hematological parameters, nonspecific immune responses, and disease resistance of *L. vannamei*. Comparisons were also made with a commercial antibiotic (OTC).

2. Materials and Methods

2.1. Experimental Design and Diets. Presented in Table 1 is the formulated experimental diets and proximate composition analysis. To evaluate the dietary γ -aminobutyric acid (GABA) as a feed additive in whiteleg shrimp, five experimental diets were formulated to be isonitrogenous and isocaloric. Diets were supplemented with 0 (CON), 50 (GABA_{50}), 100 (GABA_{100}), and 300 (GABA_{300}) mg of GABA and 4 g of oxytetracycline (OTC) per kilogram of diet. The actual GABA content of each experimental diet was 62.4 (CON), 102 (GABA_{50}), 175 (GABA_{100}), 248 (GABA_{300}), and 57.4 (OTC) mg/kg diet. Ingredients such as fishmeal have some endogenous GABA content, and therefore, diets CON and OTC show some endogenous GABA composition. The composition of the GABA content in each experimental diet was evaluated and analyzed using high-performance liquid chromatography (HPLC, Sykam, Eresing, Germany) following the procedure of Lee et al. [38]. 20 g of each diet was ground and extracted with 100% of MeOH ($3 \times 100 \text{ mL}$) for sample preparation. The content of GABA was measured by a reverse-phase column with a gradient elution program (water : acetonitrile = 90 : 10 to 0:100 for 40 min). The condition of UV detection was 280 nm. For the preparation of the diets, ingredients were ground to powder using a mechanical grinder and then mixed using an electronic industrial mixer (Hanyoung Food Machinery, Gyeonggi-do, Republic of Korea). The fish oil was added into the mixture and mixed until homogenized. Additionally, 30% of water was added into the mixture. Thereafter, the moistened mixture was passed through a 0.2 cm die pelleting machine (Shinsung, Seoul, Republic of Korea), dried with a dehumidifier, and stored at a temperature of 4°C until the feeding trials.

2.2. Experimental Shrimp and Feeding Trials. The experimental juvenile whiteleg shrimp, *Litopenaeus vannamei*, was acquired from a commercial farm (Pal-ttak shrimp farm, Go-seong city, Republic of Korea) and acclimated in 250 L rectangular tanks at the FFNRC (Feeds & Foods Nutrition Research Center, Pukyong National University, Busan, Rep. Korea) for two weeks. After the acclimation period, 15 juvenile whiteleg shrimp with an average weight of $2.97 \pm 0.06 \text{ g}$ (mean \pm SD) were randomly distributed into 15 aquaria as triplicates. Each aquarium was supplied with filtered seawater in a semirecirculating system during the experiments. The inlet water flow was maintained at 1.3 L/min at a water temperature of $28 \pm 1.0^\circ\text{C}$. Dissolved oxygen was maintained at 6~7 mg/L, and salinity was 33 ppt. The whiteleg shrimp were fed three times per day at 5~7% of wet body weight during the 8 weeks of experiment. Mortality was checked every day, and dead shrimp were removed directly. The feeding ratio was recalculated after the removal of dead shrimp to allow for balance in feeding. Aquarium

TABLE 1: Ingredient composition and proximate analysis of the basal diet for juvenile whiteleg shrimp (% dry matter basis).

Ingredients	%
Sardine fish meal ¹	30.0
Soybean meal ¹	20.0
Blood meal ¹	3.00
Wheat gluten meal ¹	5.00
Squid liver powder ¹	5.00
Wheat flour ²	14.0
Corn starch ¹	8.00
Fish oil ¹	4.00
Calcium phosphate ³	2.50
Vitamin premix ⁴	2.00
Mineral premix ⁵	2.00
Cholesterol ³	0.50
Lecithin ¹	2.00
Cellulose ¹	2.00
Total	100
Proximate composition (% dry matter basis)	
Crude protein	40.7
Crude lipid	8.81
Crude ash	8.28
Moisture	9.61

¹The Feed Co., Goyang, Rep of Korea. ²Samhwa Flour Mills Co., Seoul, Rep of Korea. ³Sigma-Aldrich Korea, Yongin, Rep of Korea. ⁴Vitamin premix (as mg/kg premix): A, 1000000 IU; D, 200000 IU; E, 10000; B1, 2000; B6, 1500; B12, 10; C, 10000; calcium pantothenic acid, 5000; nicotinic acid 4500; B-biotin 10; choline chloride, 30000; inositol, 5000. ⁵Mineral premix (as g/kg premix): ferrous fumarate, 12.50; manganese sulfate, 11.25; dried ferrous sulfate, 20.0; dried cupric sulfate, 1.25; cobaltous sulfate, 0.75; zinc sulfate KVP, 13.75; Calcium iodate, 0.75; magnesium sulfate, 80.20; aluminum hydroxide, 0.75.

tanks were maintained by careful cleaning and daily removal of feces by siphoning.

Following the 8-week feeding trial, the growth performance parameters such as weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER), and survival of shrimp from each tank were measured and calculated according to the following equations [39]:

$$\begin{aligned}
 \text{WG (\%)} &= [\text{final weight (g)} - \text{initial weight (g)}] \times 100 / (\text{initial weight (g)}), \\
 \text{SGR (\%/day)} &= 100 \times \frac{(\ln \text{final weight (g)} - \ln \text{initial weight (g)})}{\text{days}}, \\
 \text{FE (\%)} &= (\text{final weight (g)} - \text{initial weight (g)}) \times \frac{100}{\text{feed ration (g)}}, \\
 \text{PER} &= \frac{\text{wet weight gain (g)}}{\text{protein intake (g)}}, \\
 \text{Survival (\%)} &= 100 \times \left(\frac{\text{final number of shrimp}}{\text{initial number of shrimp}} \right).
 \end{aligned} \tag{1}$$

2.3. Sample Collection and Analysis

2.3.1. Proximate Composition Analysis. Proximate composition of ingredients, experimental diets, and whole-body

shrimp carcass was measured according to the Association of Official Analytical Chemists [40]. Samples for this analysis were grounded and freeze-dried (Advantage 2.0, VirTis, New York, USA) for 72 hours. Total moisture and crude ash contents were measured by drying to constant weight at 105°C for 24 h and combustion at 550°C in a muffle furnace for 3 h, respectively. Nitrogen content ($N \times 6.25$) was measured using acid digestion followed by the Kjeldahl method (2300 Autoanalyzer, Foss Tecator AB, Hoganas, Sweden) to analyze crude protein. Crude lipid was measured by using Soxtec system 1046 (Tecator AB, Hoganas, Sweden).

2.3.2. Hemolymph Analysis. At the end of experiment, shrimp were fasted for 1 day and the hemolymph was extracted. Hemolymph was taken from the ventral sinus in the first abdominal segment using a 1 mL syringe (without anticoagulation) and centrifuged at $3000 \times g$ for 10 minutes to separate the serum. Then, the serum was stored at a temperature of -70°C . Hemolymph biochemical parameters such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and glucose (GLU) were measured by a chemical analyzer (Fuji DRI-CHEM 3500i, Fuji Photo Film Ltd., Tokyo, Japan). Antioxidant enzyme such as superoxide dismutase (SOD), nonspecific immune responses such as myeloperoxidase (MPO), and lysozyme activities were also analyzed using the sampled hemolymph. SOD was determined using an assay kit (Enzo ADI-900-157, Enzo Life Sciences, Inc.) following the manufacturer's instructions. This method relies on inhibition against water-soluble tetrazolium dye and determination of SOD enzyme activity. Samples were incubated for 20 minutes at 37°C using a multiwell spectrophotometer, and absorbance was monitored at 450 nm. Lysozyme activity was determined by the reaction against *Micrococcus lysodeikticus* and spectrophotometric (Sunrise TECAN, Männedorf, Switzerland) analysis at 530 nm absorbance. Myeloperoxidase (MPO) value was analyzed following the procedure of Quade et al. [41]. Briefly, hemolymph (20 μL); Hanks Balanced Salt Solution (HBSS), 3, 3, 5, 5' tetramethylbenzidine dihydrochloride (TMB, 20 mM; Sigma-Aldrich); H_2O_2 (5 mM); and 4 M sulphuric acid were diluted in a 96-well plate. The color changes were measured at 450 nm in a microplate reader (Sunrise TECAN, Männedorf, Switzerland).

2.3.3. Challenge Test. After the feeding trial, *Vibrio alginolyticus* suspension was prepared to conduct a nine-day bacterial challenge test according to Tseng et al. [42]. The concentration of the pathogen after being cultured was adjusted to $2 \times 10^6 \text{ CFU/mL}$ using 0.85% NaCl. Five randomly selected shrimp from each tank were redistributed in 15 tanks (10 L capacity) in a nonrecirculating system to perform the challenge test. The challenge test was conducted in triplicate by injecting 20 μL of the bacterial solution into the ventral sinus of the cephalothorax. The water temperature was maintained at $28 \pm 0.5^{\circ}\text{C}$ (mean \pm SD) with aeration of the challenge test tanks. Shrimp were not fed during the challenge test. Dead shrimp were removed, and daily mortality was recorded.

2.3.4. Statistical Analysis. After analysis of every parameter, data were checked for normality and homogeneity of variances then analyzed by one-way ANOVA test using SAS program (SAS Institute, Inc. Cary, NC, USA) to analyze the differences of each treatment group. A Fisher Least Significant Difference (LSD) test was used to compare means whenever a significant difference is observed in the ANOVA table. Treatment effects were considered significant at the confidence level of $P < 0.05$.

3. Results

3.1. Growth Performance and Body Composition. The growth and survival of juvenile whiteleg shrimp fed different levels of GABA and OTC for eight weeks are shown in Table 2. The findings show that shrimp fed GABA₁₀₀ had significantly higher final body weight, weight gain, feed efficiency, protein efficiency ratio, and specific growth rate in comparison to shrimp fed CON, GABA₅₀, and GABA₃₀₀ diets ($P < 0.05$). However, there were no significant differences between GABA₁₀₀ and OTC diets ($P > 0.05$). Furthermore, as shown in Table 3, whole-body composition analysis of shrimp fed the five experimental diets did not show significant differences in crude protein, crude lipid, moisture, and ash contents ($P > 0.05$).

3.2. Hemolymph Biochemical Parameters. As shown in Table 4, there were no significant differences among treatment groups in terms of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and glucose ($P > 0.05$).

3.3. Antioxidant Enzyme and Nonspecific Immune Responses. The antioxidant enzyme activity and nonspecific immune responses of shrimp fed diets with different GABA levels and OTC are shown in Table 5. At the end of the eight-week feeding trial, there were no significant differences in superoxide dismutase (SOD), lysozyme activity, and myeloperoxidase (MPO) among the different treatments ($P > 0.05$).

3.4. Challenge Test. The cumulative survival rate of juvenile whiteleg shrimp after nine days of challenge test with *Vibrio alginolyticus* is presented in Figure 1. According to these results, shrimp fed GABA₅₀, GABA₁₀₀, and OTC had significantly higher cumulative survival than those of shrimp fed GABA₃₀₀ and CON diets ($P < 0.05$). There was no significant difference in cumulative survival of whiteleg shrimp fed GABA₅₀ and GABA₁₀₀ diets ($P > 0.05$). Also, there was no significant difference between shrimp fed CON and GABA₃₀₀ diets after nine days of the challenge test ($P > 0.05$).

4. Discussion

This study evaluated the effects of different dosages of dietary GABA on growth, survival, whole-body proximate composition, hematology, nonspecific immune responses, and disease resistance of juvenile *L. vannamei* reared under laboratory conditions. The findings of the present study indicated that the addition of dietary GABA at 100 mg/kg has a positive effect on weight gain, specific growth rate, feed efficiency, and protein efficiency ratio, indicating that GABA

TABLE 2: Growth performance of juvenile whiteleg shrimp fed the five experimental diets for 8 weeks¹.

	CON	GABA ₅₀	GABA ₁₀₀	GABA ₃₀₀	OTC
IBW ²	2.93 ± 0.07	2.98 ± 0.04	2.93 ± 0.07	2.98 ± 0.10	2.98 ± 0.04
FBW ³	10.1 ± 0.53 ^b	10.3 ± 0.41 ^b	11.8 ± 0.32 ^a	10.5 ± 0.40 ^b	11.1 ± 0.71 ^{ab}
WG ⁴	244 ± 22.6 ^b	247 ± 15.6 ^b	304 ± 17.4 ^a	252 ± 20.7 ^b	273 ± 28.6 ^{ab}
SGR ⁵	2.20 ± 0.12 ^b	2.22 ± 0.08 ^b	2.49 ± 0.08 ^a	2.24 ± 0.10 ^b	2.35 ± 0.14 ^{ab}
FE ⁶	56.0 ± 5.19 ^b	56.6 ± 3.57 ^b	69.6 ± 3.98 ^a	57.7 ± 4.75 ^b	62.5 ± 6.56 ^{ab}
PER ⁷	1.38 ± 0.13 ^b	1.39 ± 0.09 ^b	1.70 ± 0.10 ^a	1.43 ± 0.12 ^b	1.54 ± 0.16 ^{ab}
Survival ⁸	88.9 ± 10.2	97.8 ± 3.85	93.3 ± 0.00	88.9 ± 13.9	91.1 ± 3.85

¹Data are means ± SD of triplicate groups of shrimp. Values in each row with different superscripts are significantly different. ²IBW: initial body weight (g).

³FBW: final body weight (g). ⁴WG: weight gain (%) = [(final wt.–initial wt.) × 100]/initial wt. ⁵SGR: specific growth rates (%) = [(log_e final wt.–log_e initial wt.) × 100]/days. ⁶FE: feed efficiency ratios (%) = (wet weight gain/dry feed intake) × 100. ⁷PER: protein efficiency ratio = (wet weight gain/protein intake).

⁸Survival (%) = [(total fish – dead fish) × 100]/total shrimp.

TABLE 3: Whole-body proximate composition (% wet matter) of juvenile whiteleg shrimp fed the five experimental diets for 8 weeks¹.

	CON	GABA ₅₀	GABA ₁₀₀	GABA ₃₀₀	OTC
Crude protein	18.4 ± 0.30	18.3 ± 0.67	18.2 ± 0.53	18.7 ± 0.15	18.3 ± 0.76
Crude lipid	2.64 ± 0.15	2.84 ± 0.06	2.77 ± 0.11	2.85 ± 0.07	2.82 ± 0.09
Crude ash	3.55 ± 0.23	3.65 ± 0.16	3.66 ± 0.08	3.75 ± 0.07	3.72 ± 0.22
Moisture	75.4 ± 0.65	76.2 ± 0.90	77.3 ± 0.85	75.7 ± 1.37	75.3 ± 1.31

¹Data are means ± SD of triplicate groups of shrimp.

TABLE 4: Hemolymph biochemical analysis of juvenile whiteleg shrimp fed the five experimental diets for 8 weeks¹.

	CON	GABA ₅₀	GABA ₁₀₀	GABA ₃₀₀	OTC
AST ²	117 ± 5.03	123 ± 7.23	119 ± 4.58	118 ± 7.64	114 ± 3.06
ALT ³	1.87 ± 0.45	2.20 ± 0.46	2.03 ± 0.51	2.13 ± 0.29	2.10 ± 0.30
Glucose ⁴	66.4 ± 2.12	65.5 ± 3.16	63.9 ± 0.50	66.5 ± 2.38	68.0 ± 2.83

¹Data are means ± SD of triplicate groups of shrimp. ²AST: aspartate aminotransferase (units/liter). ³ALT: alanine aminotransferase (units/liter). ⁴Glucose, GLU (mg/dL).

TABLE 5: Antioxidant enzyme and nonspecific immune response of juvenile whiteleg shrimp fed the five experimental diets for 8 weeks¹.

	CON	GABA ₅₀	GABA ₁₀₀	GABA ₃₀₀	OTC
SOD ²	92.4 ± 1.34	92.2 ± 0.87	92.8 ± 0.74	93.7 ± 1.93	94.3 ± 2.27
Lysozyme ³	0.73 ± 0.07	0.69 ± 0.04	0.72 ± 0.02	0.73 ± 0.09	0.73 ± 0.03
MPO ⁴	3.53 ± 0.28	3.73 ± 0.12	3.81 ± 0.30	3.66 ± 0.21	3.78 ± 0.11

¹Data are means ± SD of triplicate groups of shrimp. ²SOD: superoxide dismutase (% inhibition). ³Lysozyme activity (U/L). ⁴MPO: myeloperoxidase (absorbance).

could be effective in increasing the growth of whiteleg shrimp. This corroborates with studies on whiteleg shrimp *L. vannamei* [26], pharaoh cuttlefish *Sepia pharaonis* [43], Jian carp *Cyprinus carpio* var. *Jian* [44], and olive flounder *Paralichthys olivaceus* [34, 35] on the usage of dietary GABA as a feed additive. GABA is an inhibitory neurotransmitter that has shown to reduce stress indices in animals [45–47]. The ability of GABA to control the neural excitation in animals affects the metabolism, thus, improving the growth performance [48]. The supplementation of diets with GABA has shown to increase feed intake and improve growth performance in cows and protect organs in broilers [49, 50]. Increased fish growth is attributed to effective feeding effi-

ciency which can be improved by dietary glutamine [51]. Based on the previous studies and the results obtained in the present study, it can be inferred that GABA can improve the growth performance of reared animals. However, the effect of GABA on growth is highly dependent on the level of inclusion. The effects of GABA appear to be dosage-dependent. Reference [32] found that the addition of GABA to grass carp diet increased growth rate, superoxide dismutase, and total antioxidant level with an increased GABA level from 50 to 150 mg/kg. This is why at 200 mg/kg, there was a reduction in the mentioned parameters. These authors suggested that a dosage of 85 mg/kg dietary GABA is optimum for juvenile grass carp. A high level of GABA inclusion also

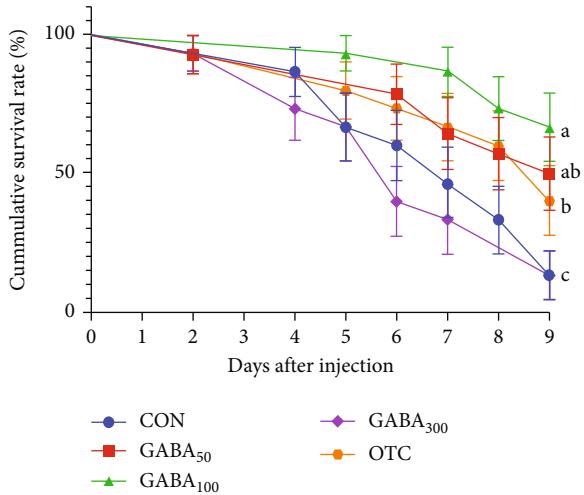


FIGURE 1: Cumulative survival rate of juvenile whiteleg shrimp fed five different experimental diets for 8 weeks and challenged with *Vibrio alginolyticus* (2×10^6 CFU/mL) for 9 days. Data points with different letters are significantly different ($P < 0.05$).

decreased the growth rate of the giant freshwater prawn, *Macrobrachium rosenbergii* [52]. The addition of 150 mg/kg of dietary GABA was suitable for whiteleg shrimp fed with a low-fishmeal diet [26]. These authors asserted that there is a reduction in growth at GABA levels greater than 150 mg/kg, and this validates the findings of our study as the addition of 300 mg/kg of dietary GABA also showed a reduction in growth. This suggests that there is a “dose-effect response” in the usage of dietary GABA in aquafeed [32]. This is likely because GABA has been linked to an increase in feed intake when administered in optimal levels. In a very recent trial by Zhang et al. [37], Chinese mitten crabs that were given an optimal dosage of GABA (84–89 mg/kg) had significantly upregulated orexigenic neural signal-related genes which resulted in an increase in feed intake and molting frequency.

In our study, no significant differences were observed in the whole-body composition analysis of whiteleg shrimp fed diets with different levels of GABA. It was previously shown that dietary glutamine and glutamate, which are precursors of GABA, did not influence whole-body proximate composition of Nile tilapia [53]. Also, the body composition of juvenile grass carp was not influenced by the addition of dietary gamma-aminobutyric acid in the diet [32]. However, Pohlenz et al. [54] noted a significant difference in the lipid content composition of channel catfish (*Ictalurus punctatus*) fed glutamine. A similar result was also reported by Zhao et al. [51] on the addition of supplementary glutamine in the diet of Jian carp. These authors found significant differences in protein and lipid content of fish carcasses compared to the control diet. The inconsistency in the results obtained by previous studies and the present study might have been due to the difference in size and species. Also, shrimp have lower lipid content compared to freshwater and marine fish species; therefore, changes in whole-body lipid content might be negligible [55].

Hemolymph biochemical parameters such as AST and ALT are important indicators of hepatopancreas (the equivalent of the liver in vertebrates) health and function. The variations of these enzymes in hemolymph can indicate hepatopancreas damage and are used to diagnose disease in animals [56, 57]. Hemolymph glucose is also an indicator of stress response and is regulated by crustacean hyperglycemic hormone in shrimp [58]. Chang et al. [59] demonstrated that shrimp glucose levels increase after hypothermal stress. In the present study, there were no significant differences in shrimp hemolymph AST, ALT, and glucose levels fed diets with different dietary GABA levels and OTC. This could indicate that our experimental conditions were optimized enough that the effects of GABA seemed negligible on hemolymph biochemical parameters. The same results were observed by Farris et al. [35] when olive flounders were fed different levels of GABA. Further investigations on the effects of GABA on hemolymph biochemical parameters under suboptimal conditions are required.

The supplementation of dietary glutamine in farmed fish has been shown to improve not only growth performance but also immune responses [28]. Shrimp are often cultured in highly intensive systems, thereby increasing their susceptibility to high ammonia accumulation from excessive feeding. Oxidative stress can be caused by ammonia or nitrite accumulation through the production of reactive oxygen species [60]. Liang et al. [61] asserted that nonspecific immune response of antioxidant enzymes such as SOD and glutathione peroxidase can eliminate oxidative stress. However, the accumulation of nitrites reduces the levels of these antioxidant enzymes. The addition of glutamine, a precursor of GABA, improved the intestinal antioxidant capacity in grass carp at various levels [62]. The dietary supplementation and the injection of GABA at different dosages significantly increased the total superoxide dismutase and catalase (CAT) activity in *L. vannamei* [26] and Chinese mitten crab, *Eriocheir sinensis* [63]. However, in the present study, the nonspecific immune response parameters were not significantly influenced by the dietary GABA levels. This is similar to a previous study on supplementation of dietary GABA in olive flounder, *Paralichthys olivaceus* [34], and in the inclusion of dietary glutamine in half-smooth tongue sole [45]. Also, Farris et al. [35] tested graded levels of dietary GABA in flounder diet and reported no significant effects on SOD activity. Differences with regard to this parameter in previous studies might be due to variables such as species, developmental stage, feed formulations, or GABA source. The mechanism of action of dietary GABA and its effects on nonspecific immune responses are not well-known. Therefore, further studies are required to understand the effects of this feed additive on immune responses.

It has been suggested that the addition of dietary GABA can enhance the immune system by improving the gut microbiota and T-cell pathway [51]. The improvement of gut health of whiteleg shrimp could improve the immune system and resistance against pathogenic bacteria. Zhao et al. [51] suggested that the addition of GABA improves the effectiveness of *Enterococcus faecium* probiotic. This can help the culture organisms to fight against pathogenic

bacteria. *Vibrio alginolyticus* is a gram-negative, rod-shaped bacterium that has been known to infect *Litopenaeus vannamei* leading to poor growth performance, anorexia, and death [64]. Disease caused by this bacterium could be treated with antibiotics; however, it has been evidenced that most antibiotics cannot destroy every strain of *V. alginolyticus* [65]. Increased stress as a result of intensive culture may increase the susceptibility of shrimp to disease and lead to increased dopamine levels. An increase in the dopamine level has been shown to increase the vulnerability of *Penaeus monodon* to *Photobacterium damsela* pathogen [66]. In the present study, when challenged with *Vibrio alginolyticus*, *L. vannamei* fed diets supplemented with 100 mg of GABA had a significantly higher cumulative survival in comparison to the OTC diet. This is in an agreement with the previous studies regarding the pharmaceutical effects of GABA, which reveal that GABA could inhibit the activity of *Vibrio para-haemolyticus*. The mechanism of action could be attributed to the ability of GABA to suppress dopamine [67].

5. Conclusions

Conclusively, the results from this study indicated that the supplementation of GABA improved the growth performance and disease resistance of *L. vannamei*, which may have resulted from increased feeding efficiency and reduced stress. This means GABA can be considered an effective functional feed additive in the prevention of disease infection and also be a potential antibiotic replacer. Our findings suggested that the supplementation of dietary GABA at 100 mg/kg could be the optimum level in whiteleg shrimp diet.

Data Availability

The data used to support the findings of this study are available but may only be shared after reasonable request from the corresponding author and the permission of the funding institutes.

Conflicts of Interest

The authors have no declaration of interest.

Authors' Contributions

Jinho Bae was responsible for the conceptualization, methodology, investigation, data curation, formal analysis, writing the original draft, and project administration. Ali Hamidoghli was responsible for the validation, writing the original draft, review, and editing. Nathaniel W. Farris and Olumide Samuel Olowe were responsible for writing the original draft and validation. Wonsuk Choi was responsible for the methodology, data curation, and formal analysis. Seunghan Lee was responsible for the investigation and software. Seonghun Won was responsible for the validation and resources. Mihyang Ohh was responsible for funding acquisition. Seunghyun Lee was responsible for the supervision, writing, review, and editing. Sungchul C. Bai was responsible

for the supervision, project administration, writing, review, and editing.

Acknowledgments

This study was financially supported by Milae Bioresources Co., Ltd (CD20191588), 5LF, D-dong, 26, Beobwon-ro, 9-gil, Songpa-gu, Seoul, Republic of Korea, and Feed & Foods Nutrition Research Center at Pukyong National University, Busan, Republic of Korea.

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