Thesis for the Degree of Master of Fisheries Science

Effects of Dietary γ-aminobutyric Acid in Juvenile Nile Tilapia (*Oreochromis niloticus)*

by

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KOICA-PKNU International Graduate Program of Fisheries Science

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치어기 틸라피아 사료내 적정 γ-aminobutyric Acid

Advisor: Prof. Sungchul C Bai

by

Verynice Herman Temu

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Abstract

The experiment was conducted to evaluate the effects of dietary gamma-aminobutyric acid (GABA) in Nile tilapia, *Oreochromis niloticus*. Triplicates of six groups of fish averaging 3.03 ± 0.07g (mean±SD) were randomly distributed in 18 rectangular 40 L volume tanks. A basal diet without GABA supplementation was used as control (CON), and the other five diets were prepared by adding 75 mg GABA (GB_{75}), 75 mg GABA + 50 mg heat-killed *Lactobacillus paracasei* ($GB_{75}L$), 150 mg GABA (GB_{150}), 150 mg GABA + 50 mg heat-killed *Lactobacillus paracasei*, ($GB_{150}L$), and 600mg GABA (GB_{600}) of per kg diet. The results showed weight gain, $GB_{75}L$ was significantly higher than those fish fed CON and

GB_{600} ($P<0.05$), the specific growth rate of fish fed $GB_{75}L$, $GB_{150,}$ and $GB_{150}L$ diets were significantly higher than those fish fed CON and GB_{600} diet ($P<0.05$). SOD of fish fed $GB_{75}L$, $GB_{150}L$ and GB_{600} were significantly higher than fish fed the CON and GB_{75} diets. Challenge test against *Aeromonas hydrophila* showed fish fed diets GB_{75}, $GB_{75}L$ and $GB_{150}L$ were significantly higher on disease resistance than those fish fed GB_{150} and CON. Broken line analysis for weight gain showed that the optimum dietary GB level could be 158 mg kg^{-1} diet in juvenile Nile tilapia.

Keywords: *Oreochromis niloticus*, GABA, *A. hydrophila*, heat-killed *Lactobacillus paracasei*, SOD.

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First and foremost I would like to give thanks for Almighty God for the blessings, graces, and inspirations in leading me to accomplish this study.

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1. Introduction

Increase of human population creates a food crisis and an advantage to the tremendous growth in the aquaculture sector to cover the food demand (mainly protein). Lately, global capture fisheries production was 92 million tons in 2016, while Aquaculture production was 110.2 million tons same year also capita, food fish consumption grew from 9.0kg in 1961 to 20.2kg in 2015 FAO (2018). This led to the increase in production and the intensification of aquaculture with increase great demand for efficient diets which will prevent excess feeds input and deterioration of water quality which contributes to fish stress, serious diseases, and problems into the intensive culture. Antibiotics have been used since they enhance growth performance, treatment of bacterial diseases and improve survival, However, due to practice carries many risks to human health and environment the, have been banned in aquaculture Kümmerer (2009); Berendonk et al. (2015); Limbu et al. (2018). Hence majority of current studies have looked into the modulation of the immune system in fish to prevent disease outbreaks and the possibility of altering nutrition to favor normal growth and enhance fish health Li and Gatlin (2004) one of this innovative are feed additives which are nutritive/nonnutritive components that are complemented in little quantities precisely to improve the quality of fish as the final product, to preserve the physical and chemical quality of the diet or to maintain the quality of the aquatic

environment NRC (2011); Bai et al. (2015). Feed additives increasing growth, digestibility, palatability, stability of feed, feed efficacy and specific and nonspecific immune responses NRC (2011); Ajiboye et al. (2012) Lee et al. (2017).

Gamma-aminobutyric acid (GABA) is a nonprotein amino acid that exists in bacteria, plants, and vertebrates, which is synthesized from glutamate via the enzyme glutamate decarboxylase Martin et al. (1993); Chung et al. (2009). GABA serves as the principal inhibitory neurotransmitter in the central nervous system, the peripheral nervous system, and some non-neuronal tissues Watanabe et al. (2002). It performs by relieving the intensity of stress Dai et al. (2011) and reducing the activity of the nerve cells. Also, GABA can have various nutritional functions such as promoting animal's feed intake, improvement of appetite and nutrient utilization efficiency cherubini et al. (1991). In chicken, GABA supplementation in the diet have shown to alleviate protective effects to heat stress and rise the goblet cells number, enhance productivity, egg quality and serum haptoglobin Zhang et al. (2012); Chen et al. (2013); Park et al. (2015). Dai et al. (2011) reported that GABA decrease heat stress-related depression in broilers. In cows, GABA enhanced feed intake, lactation performance and animal health Wangu et al. (2013). In growing pigs, GABA enhanced feed intake and weight gain Qiu-liang et al. (2009); Li et al. (2015). However, studies reporting the effect of GABA supplementation on aquatic animals are scarce. Xie et al. (2015) reported that 150 mg kg-1 of GABA could improve the growth performance, endocrine hormone and stress tolerance of Juvenile Pacific white shrimp, *Litopenaeus vannamei*, fed low fishmeal diets. Wu et al. (2016), concluded that 87.5 mg kg-1 of dietary

GABA can enhance growth, antioxidative status and feeding-related gene expression in juvenile grass carp, *Ctenopharyngodon idellus*. Also, it was mentioned that 0.25% of GABA decreased the growth and survival of the giant freshwater prawn, *Macrobranchium rosenbergii* Kim et al. (2003) and 1% dietary GABA had no significant effect on the growth of Japanese flounder, *Paralichthys solivaceus* Chettri et al. (2007). There have been no studies regarding the influence of GABA on Nile tilapia *Oreochromis niloticus.*

Nile tilapia is the second most cultured El-Sayed (2002) and one of the most economically important fish species worldwide 4.2 million tons as of 2016 FAO (2018). It has a well-established seedling production with favorable characteristics for culture such as rapid growth, good survival in high-density culture, stress and disease resistance, and high adaptation to a wide range of supplemented nutrients El-Sayed (2006). This fish can digest relatively high amounts of carbohydrates in the diet, and effectively utilize lower-cost feed ingredients (e.g. rice bran, copra meal, and cassava) to completely replace or significantly reduce fishmeal use Jackson et al. (1982); NRC (1993). Therefore, the purpose of this study was to evaluate the effects of dietary gamma-aminobutyric acid on the growth performance, hematology, and non-immune response of juvenile Nile tilapia (*Oreochromis niloticus*).

2. Materials and Methods

2.1. Experimental diets

The powder form of gamma-aminobutyric acid (GABA™) and heat-killed *Lactobacillus paracasei* was provided by the Milae resource ML Co. Ltd. (Seoul, Republic of Korea). GABA bulk which was used as diets supplements in this study was made by calculating the appropriate amount of inclusion following standards of 76.5% purity. Six isonitrogenous and isocaloric diets were formulated based on requirements for Juvenile tilapia for optimal growth (NRC 2011). A basal diet without GABA premix was used as CON (CON) and other five diets were prepared by adding 75 mg GABA (GB_{75}), 75 mg GABA with 50 mg heat-killed *Lactobacillus paracasei* ($GB_{75}L$), 150 mg GABA (GB_{150}), 150 mg GABA with 50mg heat-killed *Lactobacillus paracasei* ($GB_{150}L$) and 600 mg GABA (GB_{600}) per kg of basal diet. Actual dietary GABA contents for CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$ and GB_{600} diets were 96, 144, 189, 197, 242 and 507 mg kg^{-1}, respectively. Tuna by-product, squid liver powder, wheat gluten meal, spray-dried blood meal, and dehulled soybean meal were used as protein sources. Wheat flour and cornstarch were used as carbohydrate sources and soybean oil as a lipid source. Essential vitamins and minerals

were provided by the additional vitamin and mineral premixes. Proximate analyses and ingredient composition of the formulated experimental diets are shown in Table 1.

Procedures for diet preparation and storage were followed as previously described by Bai and Kim (1997). Concisely, diets were prepared by mixing all the dry-powder ingredients in an electric mixer (HYVM-1214, Hanyoung Food Machinery, Republic of Korea), then soybean oil and 30% of filtered tap water were blended to the mixture. The moist mash mixture was made by passing the dough through a screw-type laboratory pelletizing machine (Baokyong Commercial Co., Busan, Korea) with a 2 mm die. The pellets were then air dried for approximately 48 hours at room temperature. After drying, the pellets were broken up using a grinding machine, sieved into the proper diameter size (approximately 10mm), packaged in zip-lock polythene bags, labeled and stored at −20˚C until use for the feeding trial.

Table 1.GABA premix composition (mg/kg).

	Diets (g/kg)					
	CON	**GB_{75}**	**$GB_{75}L$**	**GB_{150}**	**$GB_{150}L$**	**GB_{600}**
GABA (76.5%)	0	0.098	0.098	0.196	0.196	0.784
CMC[1]	0	19.902	19.852	19.804	19.754	19.216
Heat- killed[2]	0	0	0.05	0	0.05	0
GABA Premix	0	20	20	20	20	20
Actual GABA[3]	96	144	189	197	242	507

[1]Carboxymenthl cellulose

[2]*Lactobacillus paracasei*.

[3]Detected GABA analysis from the experimental diets by HPLC grade (Merck)

Table 2. Ingredient composition and proximate analysis of the basal diets of Juvenile Nile tilapia (% dry matter basis).

Ingredients	%
Tuna by-product [1]	17.0
Squid liver powder[1]	3.0
Blood meal[1]	5.0
Soybean meal[2]	30.0
Wheat gluten meal[2]	6.1
Wheat flour[2]	20.0
Cornstarch[2]	10.5
Soybean oil[2]	2.4
Vitamin Premix[3]	1.0
Mineral Premix[4]	1.0
CMC[5]	2.0
Calcium phosphate[6]	2.0
GABA bulk	0.0
Total	100.0
Proximate analysis (% of DM)	
Moisture	8.44
Ash	6.79
lipids	8.64
Protein	36.0

[1]Suhyup feed Co. Uiryeong, Republic of Korea

[2] The feed Co. Goyang, Republic of Korea

[3] contains (as mg/kg in diets) : Ascorbic acid, 300; dl-Calcium panthothenate, 150; Choline bitatrate, 3000; Inositol, 150; Menadione, 6; Niacin, 150; Pyridoxine·HCl, 15; Riboflavin, 30; Thiamine mononitrate, 15; dl-α-tocopherol acetate, 201; Retinyl acetate, 6; Biotin, 1.5; Folic acid, 5.4; B_{12}, 0.06.

[4] contains (as mg kg^{-1} in diets): NaCl, 437; $MgSO_4 \cdot 7H_2O$, 1,380; $NaH_2P_4 \cdot 2H_2O$, 878; $Ca(H_2PO_4)$ $2H_2O$, 1,367; KH_2PO_4, 2,414; $ZnSO_4 \cdot 7H_2O$, 226; Fe-Citrate, 299; Ca-lactate, 3,004; $MnSO_4$, 0.016; $FeSO_4$, 0.0378; $CuSO_4$, 0.00033; Calcium iodate, 0.0006; MgO, 0.00135; $NaSeO_3$, 0.00025.

[5]Carboxymenthl cellulose, and can be replaced by GABA bulk for other GABA diets.

[6]$CaHPO_4$ Sigma-Aldrich Korea Yongin Korea

2.2.Experimental fish and feeding trial

The experiment was carried out for 8 weeks at Pukyong National University (Busan, Republic of Korea). Juvenile Nile tilapia were obtained from Feeds and Foods Nutrition Research Centre (FFNRC) Pukyong National University. Two weeks prior to feeding trial all fish were fed to apparent satiation twice per day with a commercial diet (Suhyup feed, Gyeongsangnam-do Republic of Korea) to become acclimatize to experimental conditions and facilities. After the 2-week acclimation, fish were starved for 24 h to measure the initial body weight. A total of 360 fish with the mean weight of 3.03± 0.07g were randomly distributed into each of the18 tanks. Each tank was then randomly assigned as one of the three replicates of the six dietary treatments. Fish were fed twice daily (09:00 and 17:00 h) at a fixed rate of 5% to 5.5% of wet body weight. The feeding trial was conducted using a semi-recirculating system with 18 tanks (37L) receiving filtered fresh water at the rate of 2 L min^{-1} from the center tank. Supplemental aeration was provided by air stones to maintain

the dissolved oxygen 7.5 mg L^{-1} above during the feeding trial. Water temperature and pH during the experiment were maintained at 26 ± 1.6ºC and 7.5 ± 0.3, respectively. Siphoning was conducted two times per day 1 hour after feeding to remove non-eaten feed and feces in the rearing tanks. 50% of water was exchanged every day using pre-heated filtered tap water to cover up the water loss during daily siphoning and evaporation Mortality was also monitored daily and any dead fish was removed, weighed and recorded accordingly. A photoperiod of 12hour light: 12-hour dark was used throughout the experiment.

2.3.Sample collection and analysis

2.3.1. Growth performance

At the end of the feeding trial, fish were starved for 24 hours, and the total number and weight of fish in each tank was determined for calculation of weight gain (WG), feed efficiency (FE), specific growth rate (SGR), protein efficiency ratio (PER) and survival rate (SR). Three fish per tank were randomly sampled, individually weighed, and then dissected to obtain liver and viscera samples for determination of hepatosomaticidex (HSI), Viscerosomatic index (VSI) and condition factor (CF), respectively Okorie et al. (2007); Kim et al. (2014).

Weight gain (WG, %) = (final wt. –initial wt.) × 100/initial wt

Specific growth rate (SGR, % /day) = (loge wt. –loge initial wt.) × 100/days

Feed Efficiency (FE, %) = (wet weight gain/ dry feed intake) 100

Survival rate (%) = (total fish –dead fish) × 100/ total fish

Protein efficiency ratio (PER) = (wet weight gain/ protein intake)

Daily feed efficiency (DFI, %) = specific growth rate × 100/ feed efficiency

Hepatosomatic index (HIS, %) = liver wt. × 100/body wt

Visceral somatic index (VSI, %) = visceral wt. × 100/ body wt

Condition factor = (wet weight / total length^3× 100

2.3.2. Hematological parameters

Three additional fish per tank were randomly captured and anesthetized with ethylene glycol phenyl ether (200 mg L^{-1}) and blood samples were obtained from the caudal vein using 1 ml disposable syringe without anticoagulation. The blood samples were separated by centrifugation (5000×g) for 10 min. Then, the serum was separated and stored at -70 ºC for later analysis of glucose, total glycerol (TG), total cholesterol (TCHO), total protein and Glutamic oxaloacetic transaminase (GOT) measurements by a chemical analyzer (Fuji DRI- CHEM3500i, Fuji Photo Film, LTD)

2.3.3. Non-specific immune responses

Another set of blood sample of the same fish was taken without heparin and allowed to clot for 30 minutes. The serum was then separated by centrifugation at 5000×g for 10 minutes and stored at -70 ºC for the analysis of the non-specific immune responses including superoxide dismutase (SOD) and myeloperoxidase (MPO) activities. MPO was measured according to Quade and Roth (1997). Briefly, 20µL of serum was diluted with HBSS (Hanks balanced salt solution) without Ca_2^+ or Mg_2^+ (Sigma- Aldrich) in a 96-well plate. Then 35µL of 3.3'5.5-tetramethylbenzidine hydrochloride (TMB, 20µL; Sigma- Aldrich) and H_2O_2 (5 mm) were added. The color change reaction was stopped after 2min by adding 35 µL of 4 M sulfuric acid. Finally, the optical density was read at 450 nm in the microplate reader (UV-1800 UV-VIS spectrophotometer, Shimadzu, Japan).

Superoxide dismutase (SOD) activity was measured by the superoxide radical based on reaction inhibition rate of an enzyme with WST-1 (water-soluble tetrazolium dye) substrate and xanthine oxidase using the SOD assay kit (Sigma- Aldrich, 19160) in accordance with the procedure of products. Each endpoint assay was observed by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 effect with superoxide) and after 20 minutes of reaction time at 37ºC. The percent inhibition was normalized by mg protein and expressed as SOD unit/mg.

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2.3.4. Proximate analysis

Analyses of moisture, crude protein lipid, and ash whole body sample and experimental diets were performed using standard methods (AOAC 2005). A sample of diets and fish were dried to constant weights at 105ºC to determine their moisture Content. Ash was determined by incineration at 550ºC, crude lipid was determined by Soxhlet extraction using the Soxtec system 1046 (Tecator AB, Hogans, Sweeden), and crude protein Content was determined by the Kjeldahl method (2300 Autoanalyzer, Foss Tecator AB, Hoganas, Sweeden) (N×6.25) after acid digestion.

2.4.Challenge test

Challenge test a bacterial pathogen, *Aeromonas hydrophila*, was obtained from the Department of Biotechnology, Pukyong National University, Busan, Republic of Korea. Fish (n=9 per tank) were distributed according to their dietary treatment group in the 11L container for the challenge test with no water exchange. Fish were injected anally into the test fish at a dose of 1% volume per body weight of culture suspension of pathogenic *A. hydrophila* containing 2×10^7 CFU/mL. Fish mortality was recorded daily for 5 days.

2.5.Statistical analysis

After confirming normality and homogeneity of variance all data were subjected to one-way analysis of variance (ANOVA) using SAS Program Version 9.4 for Windows to test the effects of the dietary treatment with GABA across the treatment groups. When a significant treatment effect was observed, a Least Significant Difference (LSD) test was used to compare the treatment means. Treatment effects were considered at $P<0.05$ level of significance.

3. Results

3.1.Growth performance

Table 3 growth performance and feed efficiency of Nile tilapia fed on the different experimental diets. At the end of the 8 weeks feeding trial; weight gain (WG) of fish fed diet $GB_{75}L$ were significantly higher than those fish fed on CON and GB_{600} ($P<0.05$). However, there was no significant difference in WG among fish fed diet $GB_{75}L$, $GB_{150,}$ and $GB_{150}L$ ($P>0.05$) graphical presentation of weight gain is as shown in Figure 1.

Broken line linear regression analysis of weight gain indicated the optimum level of GABA meal inclusion for improved performance in Nile Tilapia as 158.7mg kg^{-1} in the diet as shown in Figure 2. **($y = 1.2367x + 354.48$, $R^2=1$).**

Specific growth rate (SGR) of fish fed diet $GB_{75}L$, $GB_{150,}$ and $GB_{150}L$ were significantly higher than those fish fed on CON and GB_{600} ($P<0.05$). However, there was no significant difference in SGR among fish fed diet $GB_{75,}$ $GB_{75}L$, $GB_{150,}$ and $GB_{150}L$ diets ($P>0.05$) graphical presentation of SGR is as shown in Figure 3.

Feed efficiency (FE) and Protein efficiency ratio (PER) of fish fed diet $GB_{75,}$ $GB_{75}L$, GB_{150}, and $GB_{150}L$ were significantly higher than those of fish fed CON and GB $_{600}$ ($P<0.05$). And equally, there was no significant difference in FE and PER among fish fed on $GB_{75,}$ $GB_{75}L$,

GB_{150}, and $GB_{150}L$, the graphical presentation of FE and PER are as shown in Figure 4 and Figure 5 respectively. No significant difference was observed in the survival rate among the group graphical presentation of survival rate is as shown in Figure 6.

There were no significant differences in the hepatosomatic index, Viscerosomatic index and condition factor among the treatment diets.

Table 3. Growth performance of juvenile Nile tilapia fed on the experimental diets for 8 week[1]

	Diets						
	CON	GB_{75}	$GB_{75}L$	GB_{150}	$GB_{150}L$	GB_{600}	Pooled SEM[12]
IBW[2]	3.0	3.0	3.0	3.0	3.0	3.1	0.02
FBW[3]	18.4	19.3	19.7	18.8	18.8	17.8	0.40
WG (%)[4]	473^{d}	533^{bc}	589^{a}	576^{ab}	556^{ab}	483^{cd}	12.2
SGR(%/day)[5]	3.64^{c}	3.84^{ab}	4.00^{a}	3.98^{a}	3.94^{a}	3.67^{bc}	0.04
FE (%)[6]	66.2^{b}	72.3^{a}	72.4^{a}	77.1^{a}	72.6^{a}	65.6^{b}	1.12
PER[7]	1.84^{b}	2.02^{a}	2.02^{a}	2.14^{a}	2.06^{a}	1.77^{b}	0.03
SR (%)[8]	96.67	95.00	93.33	91.67	95.00	98.33	1.28
HSI (%)[9]	1.28	1.19	1.18	0.99	1.36	1.26	0.03
VSI (%)[10]	6.33	5.82	5.50	6.21	5.62	5.28	0.11
CF[11]	1.65	1.73	1.61	1.71	1.73	1.60	0.01

CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg, 75mg, 75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively

[1]Values are means from triplicate groups of fish where values in each row with a different superscript are significantly different (*P<0.05)*. The lack of superscript letter means no significant differences among the treatment diets

[2]IBW = Initial body weight (g)

[3]FBW= Final body weight (g)

[4]WG (%) = Weight gain = (final weight-initial weight) ×100 / initial weight

[5]SGR (% day-1) = Specific growth rate = (ln final weight – ln initial weight) × 100 / days

[6]FE (%) = Feed efficiency = (wet weight gain ⁄ dry feed intake) x 100

[7]PER = Protein efficiency ratio = (wet weight gain/protein intake)

[8]SR (%) = Survival rate

[9]Hepatosomatic index (HSI, %) = liver wt. x 100 / body wt.

[10]Visceralsomatic index (VSI, %) = Viscera wt. x 100/ body wt

[11]Condition factor = (wet weight/total length^3) x100

[12]Pooled standard error of mean: SD/ √n

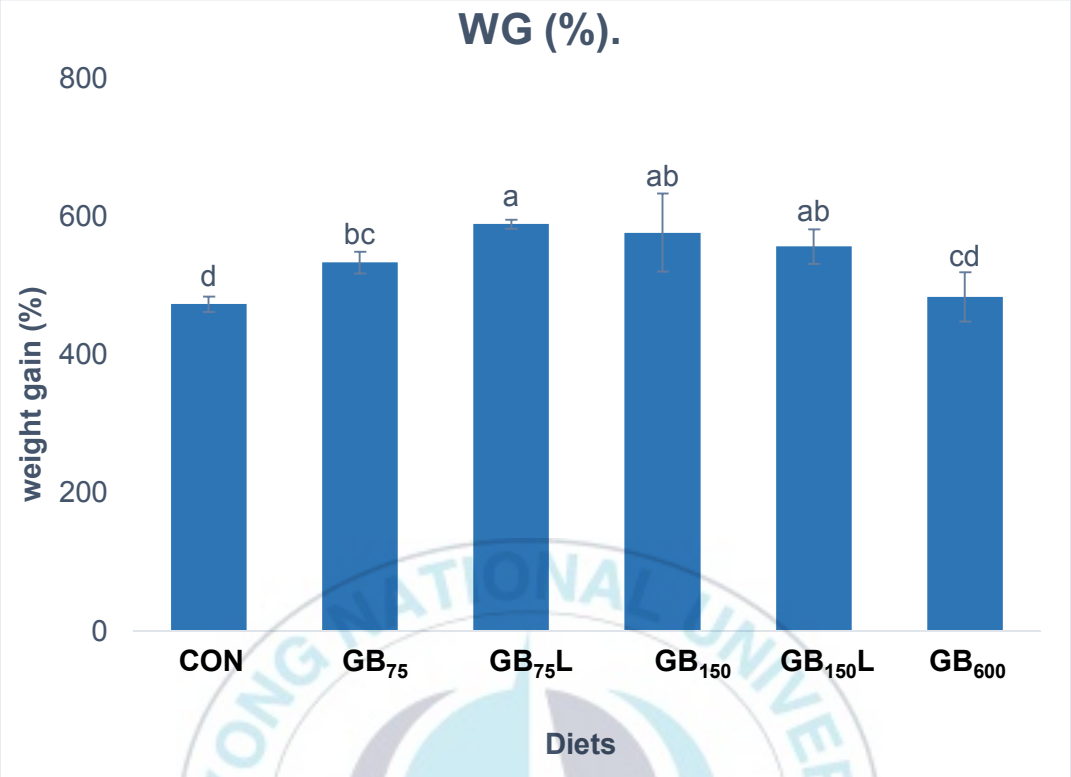

Fig. 1 Weight gained by the juvenile Nile tilapia fed on the experimental diets for 8 weeks. CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg, 75mg, 75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively.

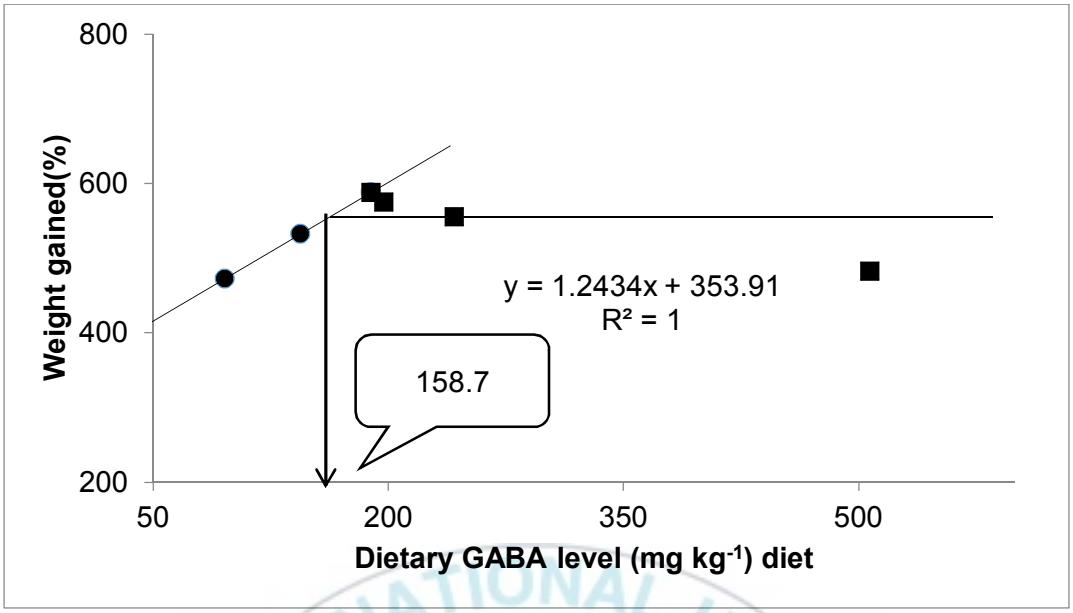

Fig. 2. Broken line analysis of weight gain in juvenile rock bream fed the different dietary GABA levels for 8 weeks. Each point represents the mean of three triplicate group.

CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg, 75mg, 75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively

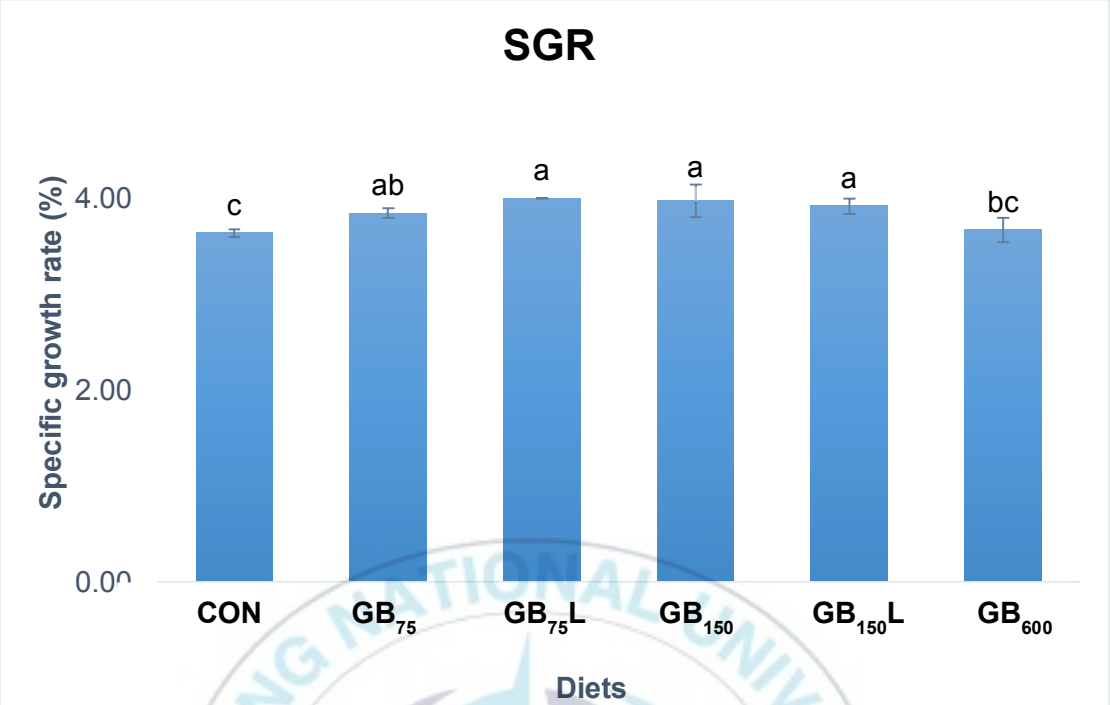

Fig. 3. The specific growth rate of the juvenile Nile tilapia fed on the experimental diets for 8 weeks.

CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg, 75mg,75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively.

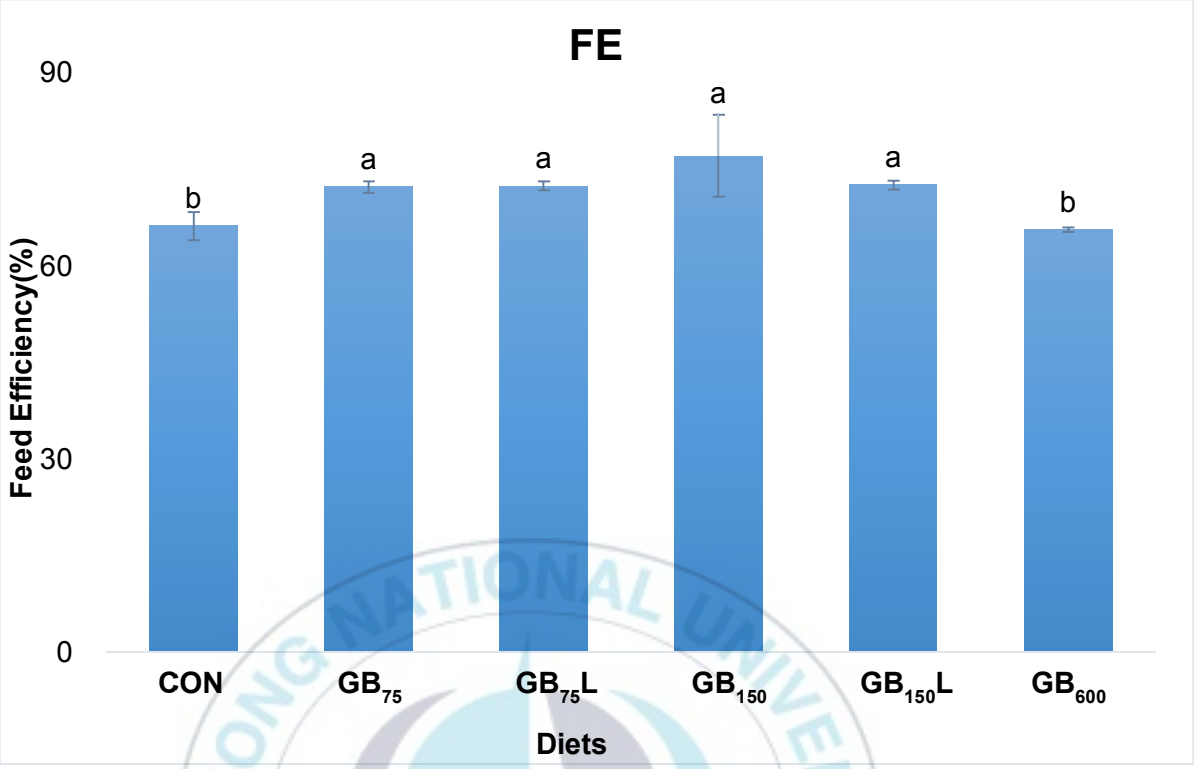

Fig. 4. Feed efficiency of the juvenile Nile tilapia fed on the experimental diets for 8 weeks. CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg, 75mg, 75mg with 50mg heat killed (*Lactobacillus paracasei*),150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively.

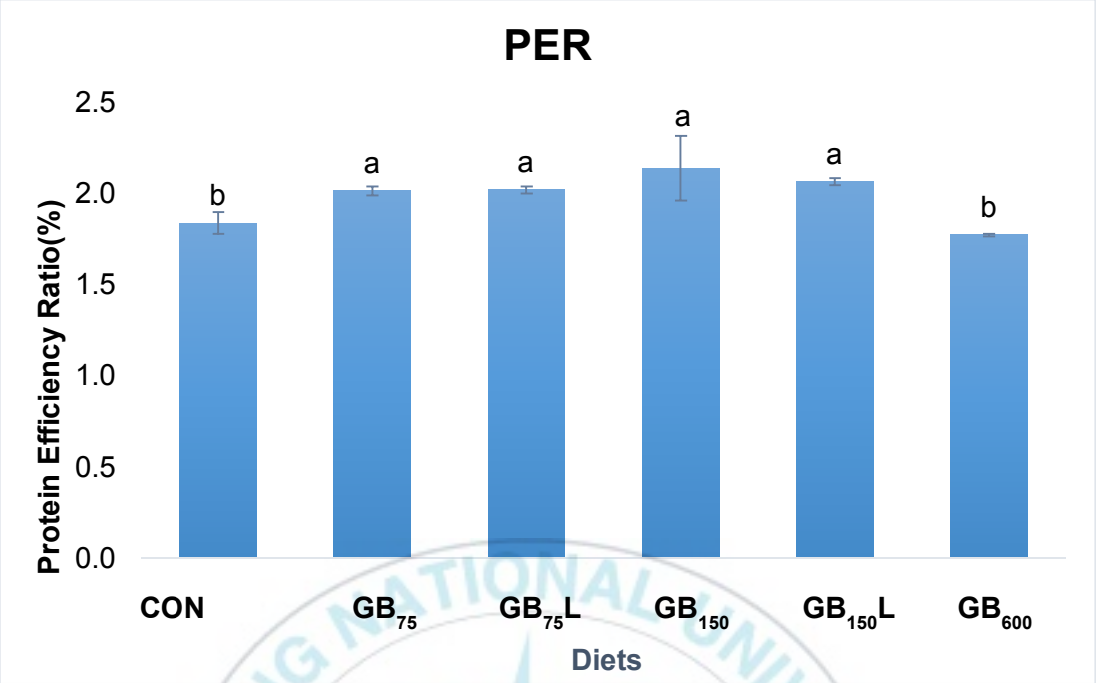

Fig. 5. Protein efficiency ratio of the juvenile Nile tilapia fed on the experimental diets for 8 weeks.

CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg,75mg,75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively.

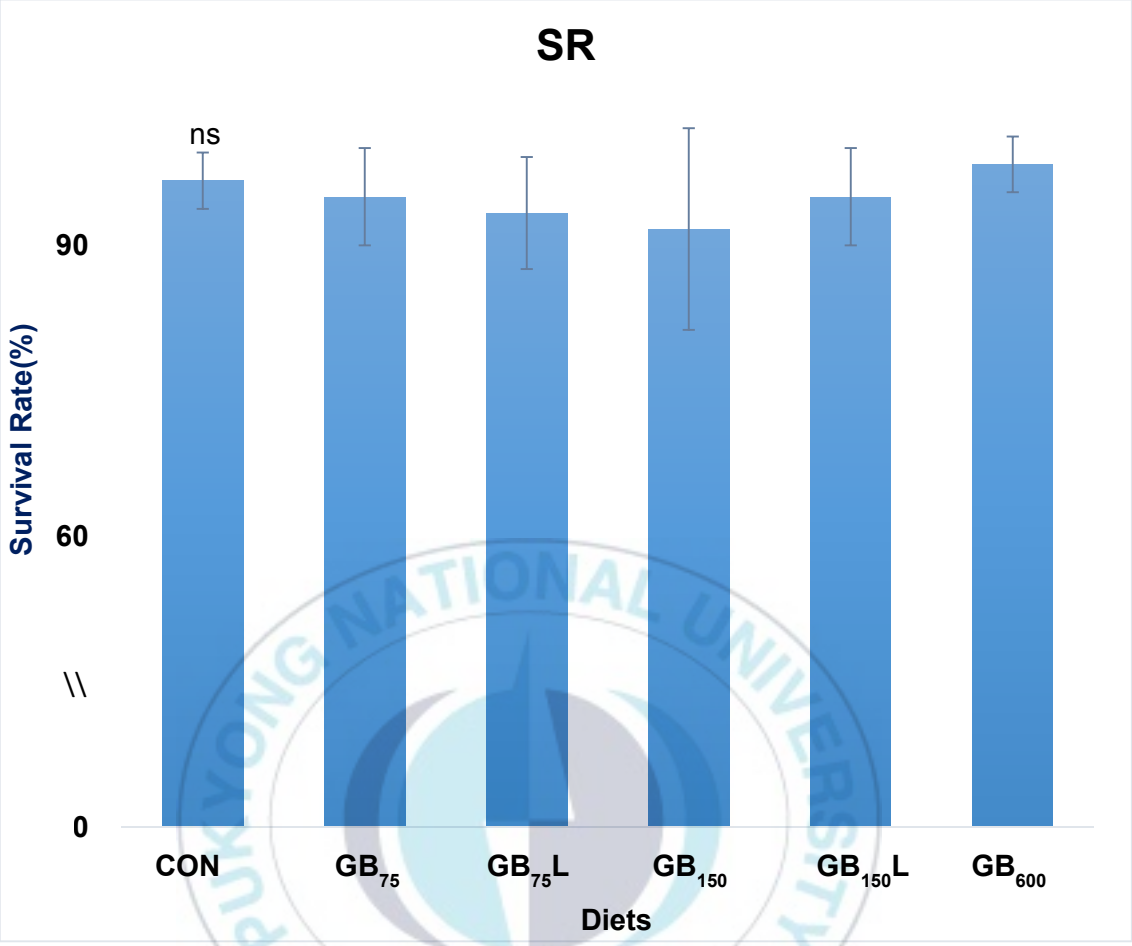

Fig. 6. The survival rate of the juvenile Nile tilapia fed on the experimental diets for 8 weeks

CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg,75mg,75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively.

3.2. Proximate body composition

Table 4 and Figure 7-9 shows the content of crude protein, crude lipid, ash and moisture in all diet at the end of t 8 weeks feeding trial. GABA could not significantly enhance crude protein, lipids, and moisture content (*P>0.05*). However, there was a significant difference in whole body ash content as GB_{75L} had a significantly higher content than fish fed GB_{150} (*P<0.05*). Although was no significant difference in the whole-body ash content among fish fed on CON, GB_{75}, GB_{150} $GB_{150}L$ and GB $_{600}$ (*P> 0.05*)

Table 4 the whole-body proximate composition of juvenile Nile tilapia fed on the experimental diets for 8 weeks[1] (% of wet matter basis).

Content (%)	Diets						
	CON	GB_{75}	$GB_{75}L$	GB_{150}	$GB_{150}L$	GB_{600}	Pooled SEM[2]
Crude Protein	15.6[ns]	15.9	15.2	15.6	15.2	15.8	0.15
Crude Lipids	6.5[ns]	6.2	6.5	5.9	6.1	7.0	0.21
Crude Ash	4.8[ab]	4.7[ab]	4.9[a]	4.2[b]	4.6[ab]	4.5[ab]	0.08
Moisture	72.4[ns]	73.1	72.5	72.3	72.8	72.5	0.24

[1]Values are means from triplicate groups of fish where values in each row with a different superscript are significantly different (*P<0.05)*. The lack of superscript letter means no significant differences among the treatment diets

[2] The Pooled standard error of mean: SD/√n.

CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg,75mg,75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively.

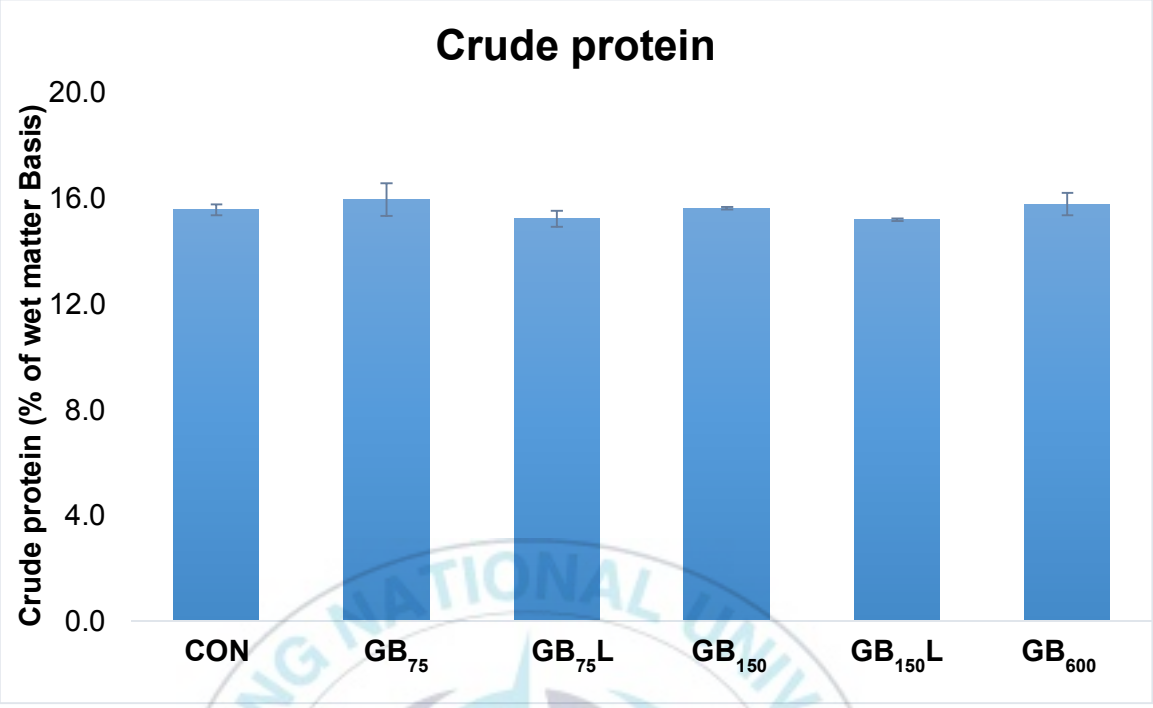

Fig. 7. Whole – body protein content (% of wet matter basis) of juvenile Nile tilapia fed on the experimental diets for 8 weeks.

CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg,75mg,75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively.

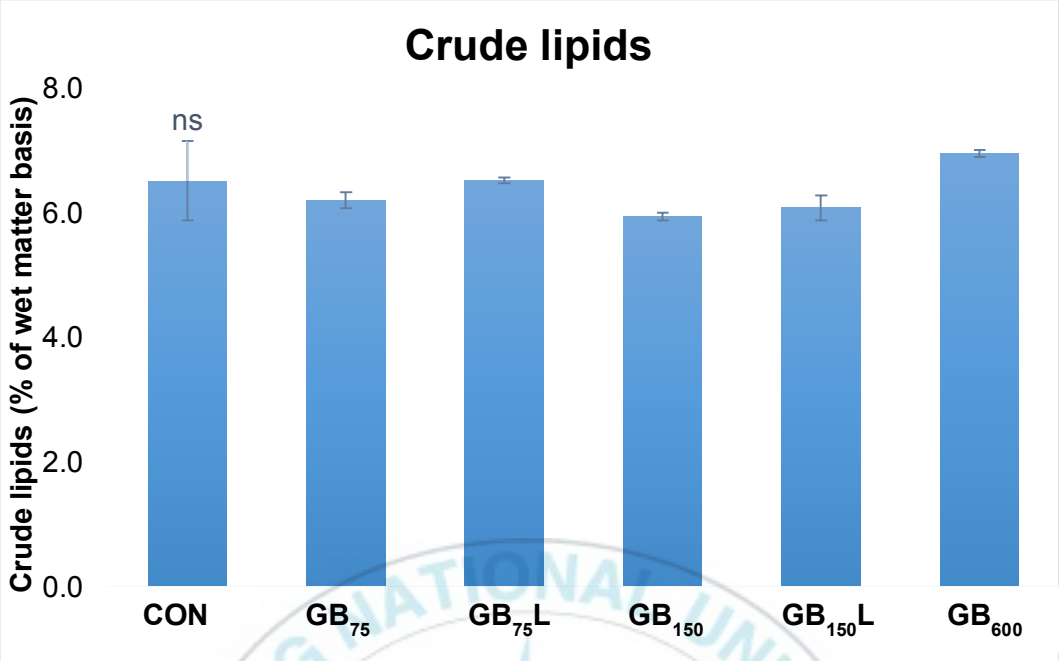

Fig. 8. Whole – body lipids content (% of wet matter basis) of juvenile Nile tilapia fed on the experimental diets for 8 weeks.

CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg,75mg,75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively

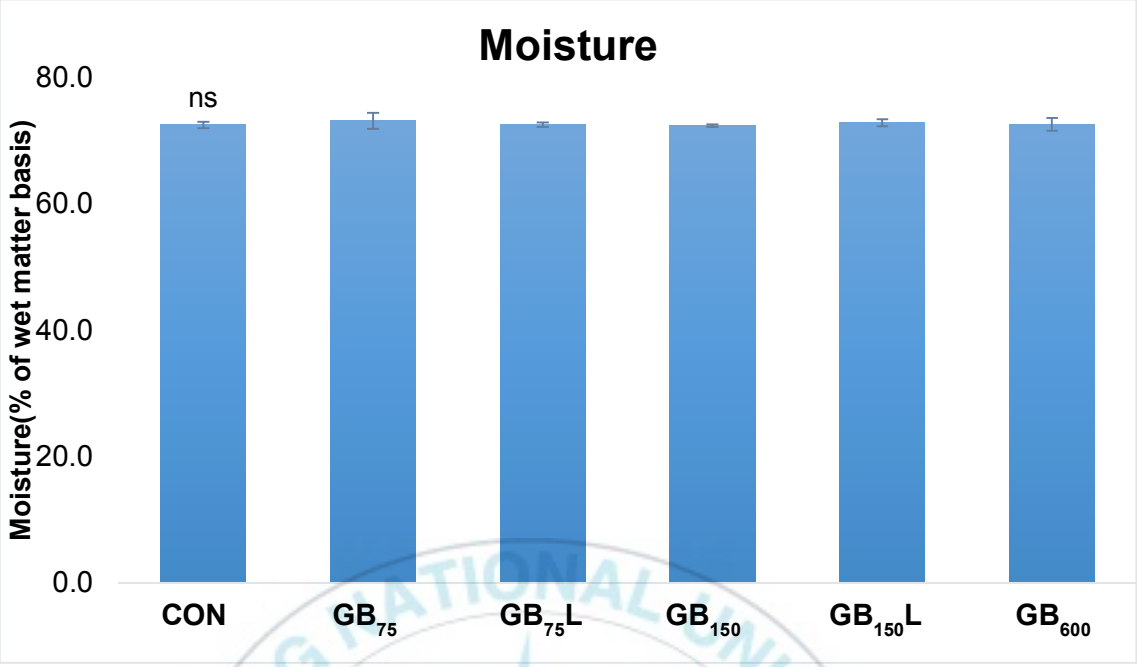

Fig. 9 Whole – body moisture content (% of wet matter basis) of juvenile Nile tilapia fed on the experimental diets for 8 weeks.

CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg,75mg,75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively

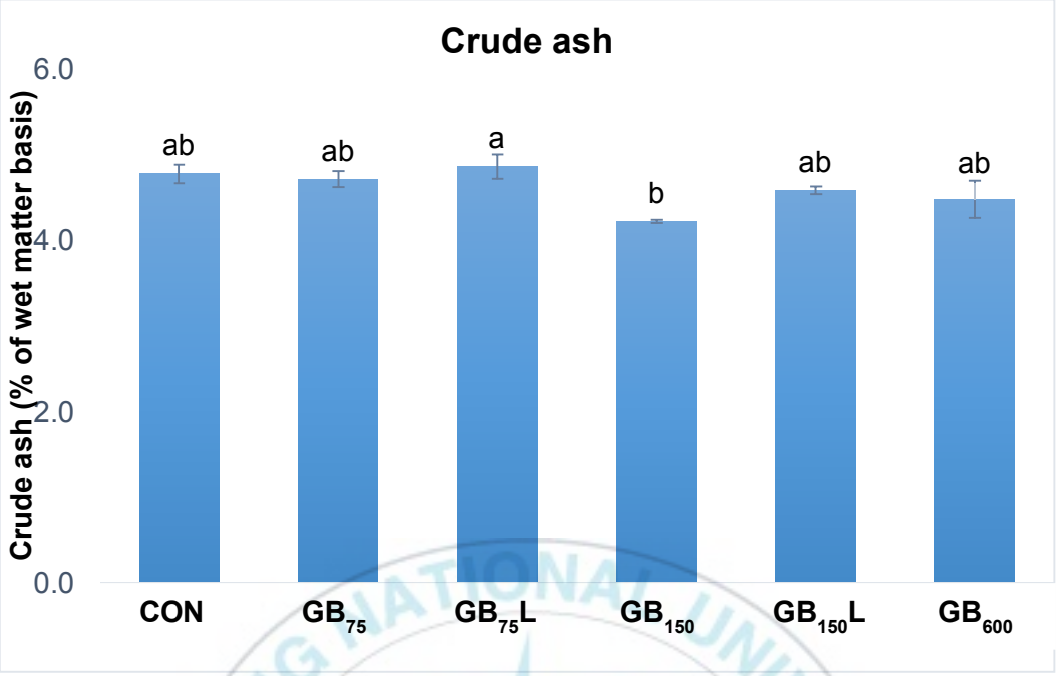

Fig. 10. Whole – body ash content (% of wet matter basis) of juvenile Nile tilapia fed on the experimental diets for 8 weeks.

CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg,75mg,75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei)* and 600 mg in the diet, respectively.

3.3. Hematological parameters

Hematological parameters of juvenile Nile tilapia fed the experimental diets for 8 weeks are shown in Table 5. Glutamic oxaloacetic transaminase (GOT) levels of fish fed diet GB_{75} and $GB_{75}L$ were significantly lower than those of fish fed on diets GB_{150} and GB_{600} ($P<0.05$). However, there were no significant differences between fish fed CON, GB_{75}, $GB_{75}L$ and $GB_{150}L$ diets ($P>0.05$). The graphical presentation of GOT levels across the treatment groups is as shown in Figure 11.

Fish fed diet $GB_{75}L$ had significantly higher total protein than those of fish fed CON, GB_{75}, GB_{150}, and $GB_{150}L$ diets ($P<0.05$). However, there were no significant differences in total protein levels among fish fed diets $GB_{75}L$ and GB_{600} ($P>0.05$). The graphical presentation of total protein levels across the treatment groups is as shown in Figure 12.

The total glycerol (TG) of fish fed CON, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} diets were significantly higher than those of fish fed the GB_{75} diet ($P<0.05$). However, there were no significant differences in total glycerol levels in fish fed GB_{75} and $GB_{150}L$ diets ($P>0.05$). The graphical presentation of TG levels across the treatment groups is as shown in Figure13

Fish fed diets CON, GB_{75}, $GB_{75}L$, GB_{150}, and $GB_{150}L$ had a significantly higher level of plasma glucose than those of fish fed diet GB_{600} ($P<0.05$) However, there were no significant differences in plasma glucose than those of fish fed diet CON, $GB_{75}L$, GB_{150}, $GB_{150}L$ and GB_{600} ($P>0.05$). The graphical presentation of Total plasma glucose level across the treatment groups is as shown in Figure 14.

There were no significant differences in total cholesterol among fish fed all the experimental diets (P>0.05). The graphical presentation of total cholesterol levels across the treatment groups is as shown in Figure 15.

Table 5. Biochemical parameters of juvenile Nile tilapia fed on the experimental diets for 8 weeks[1]

	Diets						Pooled SEM[3]
	CON	**GB_{75}**	**$GB_{75}L$**	**GB_{150}**	**$GB_{150}L$**	**GB_{600}**	
GOT (U.L)[2]	58.7^{bc}	46.7^{c}	43.3^{c}	70.0^{ab}	63.0^{abc}	84.5^{a}	4.2
T-protein (g/dl).	3.17^{bc}	2.83^{c}	3.63^{a}	3.20^{b}	3.20^{b}	3.45^{ab}	0.1
T-Glycerol (mg/dl).	262^{a}	108^{b}	225^{a}	214^{a}	191^{ab}	208^{a}	15
Glucose (mg/dl).	49.3^{ab}	57^{a}	50^{ab}	49^{ab}	50^{ab}	40^{b}	1.8
T-cholesterol. (mg/dl).	193	178	195	177	202	176	4.6

[1]Values are means from triplicate groups of fish where values in each row with a different superscript are significantly different (*P<0.05)*. The lack of superscript letter means no significant differences among the treatment diets.
[2]GOT (U/L): Glutamatic oxaloacetic transaminase
The [3]Pooled standard error of mean: SD/√n.

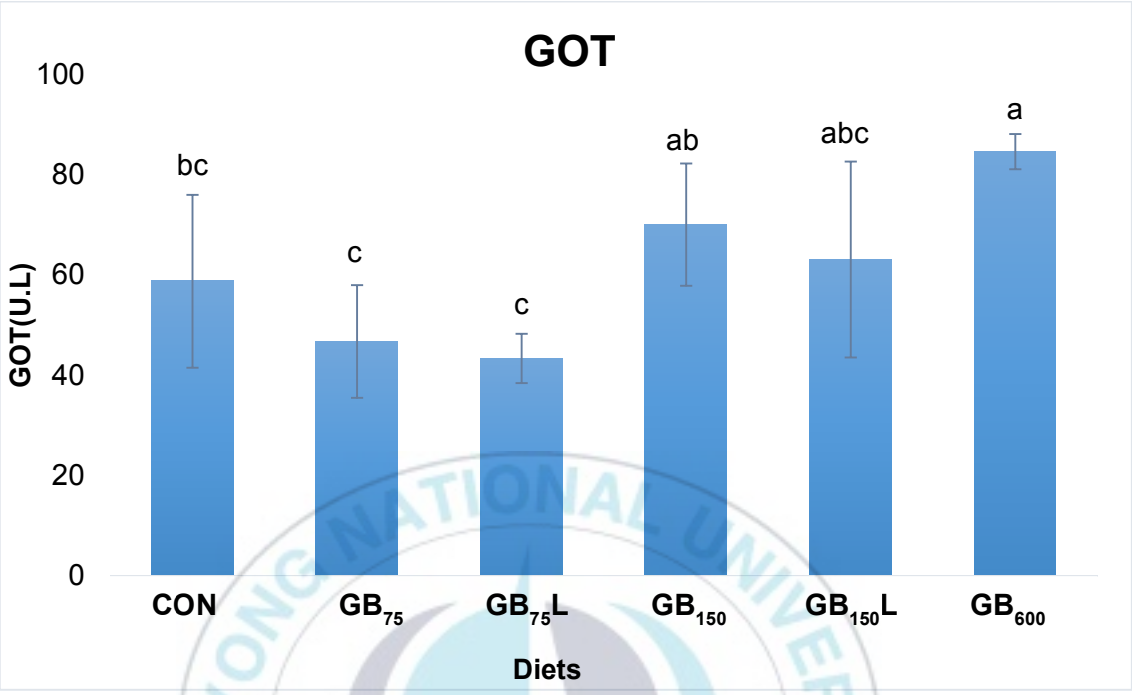

Fig. 11. Glutamic oxaloacetic transaminase (GOT) activity (% dry matter basis) of juvenile Nile tilapia fed on the experimental diets for 8 weeks.

CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg,75mg,75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively.

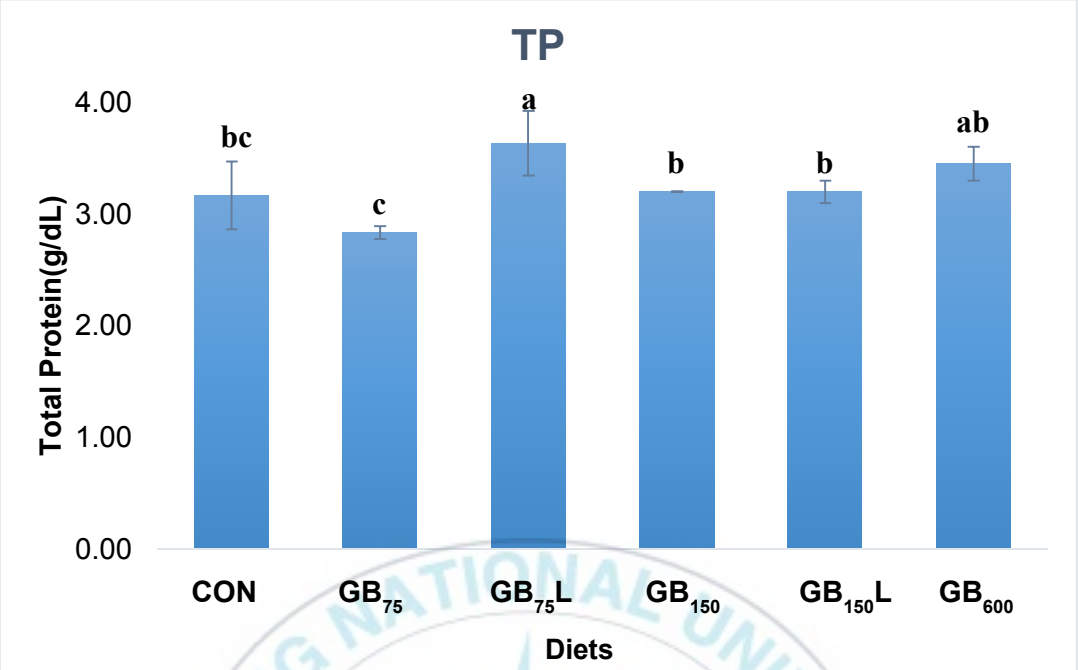

Fig. 12. The total Protein level (% dry matter basis) of juvenile Nile tilapia fed on the experimental diets for 8 weeks.

CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg,75mg,75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively.

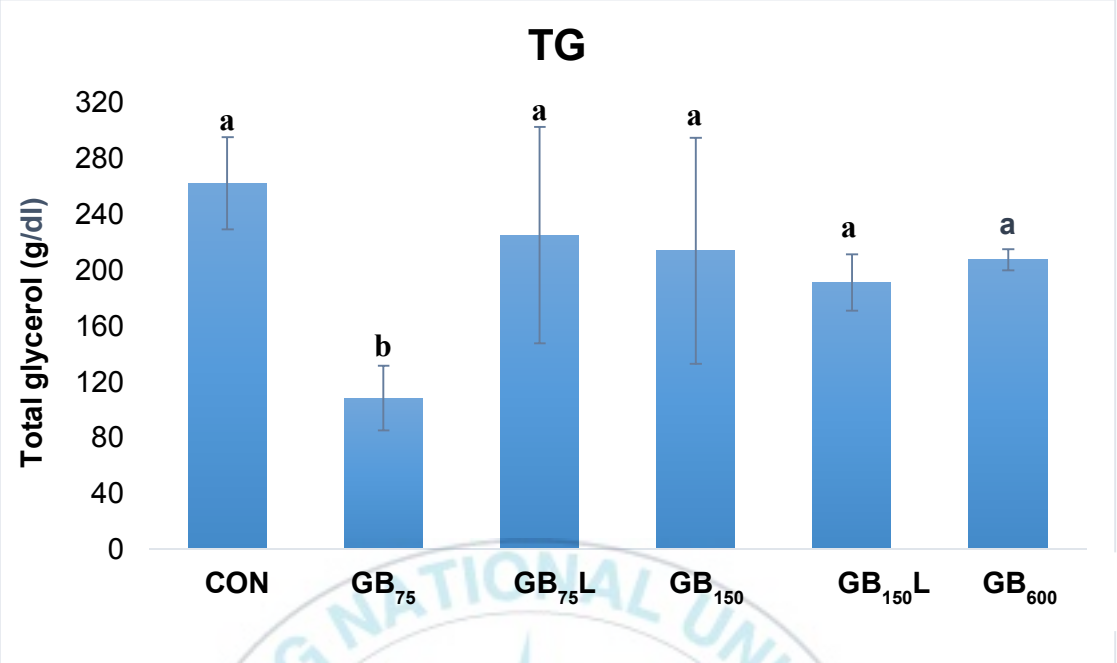

Fig. 13. Total glycerol level (% dry matter basis) of juvenile Nile tilapia fed on the experimental diets for 8 weeks.

CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg,75mg,75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively.

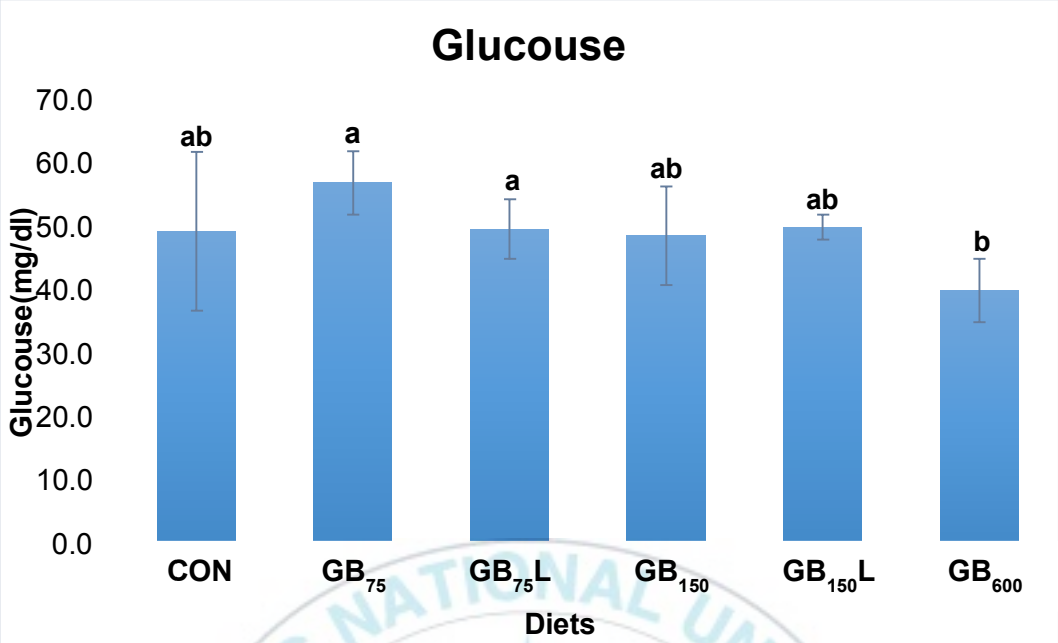

Fig. 14. The total plasma glucose level (% dry matter basis) of juvenile Nile tilapia fed on the experimental diets for 8 weeks.

CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg,75mg,75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively.

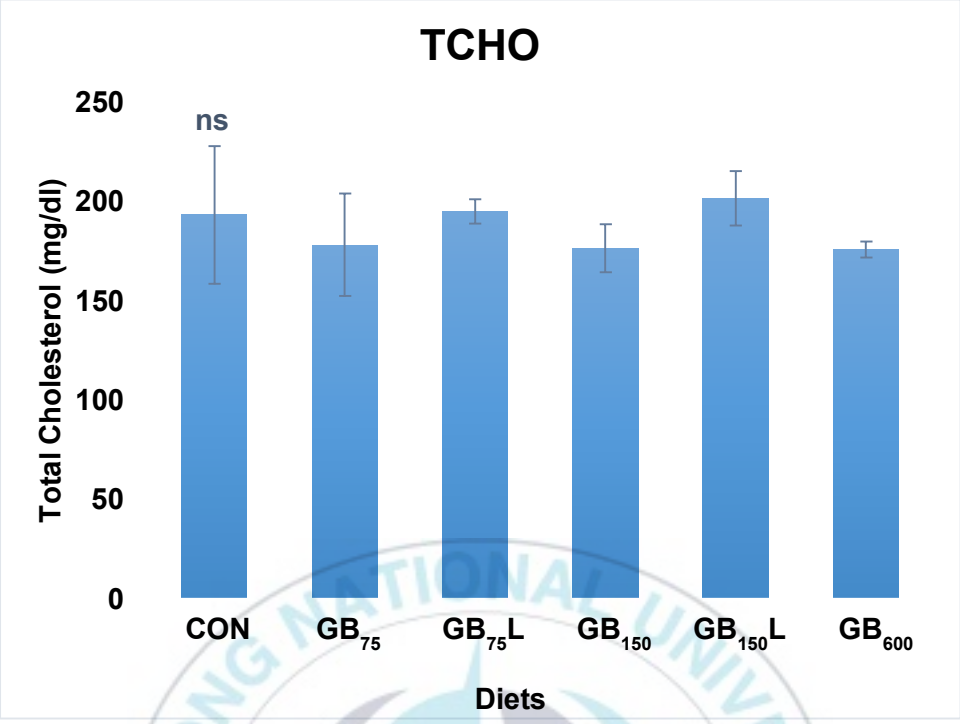

Fig. 15. A total cholesterol level (% dry matter basis) of juvenile Nile tilapia fed on the experimental diets for 8 weeks.

CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg,75mg,75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively.

3.4.Non-specific immunity

The non- specific immune response of the fish fed on the experimental diets for 8 weeks are as shown in table 6. Superoxide dismutase (SOD) inhibition in juvenile Nile tilapia of fish fed $GB_{75}L$, $GB_{150}L$ and GB_{600} were significantly higher than those fish fed the CON and GB_{75} diets ($P < 0.05$), however, there were no significant differences among fish fed $GB_{75}L$, GB_{150} and GB_{600} and among fish fed GB_{75} and GB_{150} diets. The graphical presentation of Superoxide dismutase (SOD) inhibition in juvenile Nile tilapia across the treatment groups is as shown in Figure 16.

There was no significant difference in myeloperoxidase (MPO) in Juvenile Nile tilapia across the treatment groups. The graphical presentation on myeloperoxidase (MPO) activity in juvenile Nile tilapia is as shown in Figure 17.

Table 6. Non-specific immune responses of juvenile Nile tilapia fed on the experimental diets for 8 weeks[1].

	Diets						
	CON	GB_{75}	$GB_{75}L$	GB_{150}	$GB_{150}L$	GB_{600}	Pooled SEM[4]
SOD[2]	53.94^{d}	71.62^{c}	88.35^{ab}	80.25^{bc}	97.68^{a}	87.96^{ab}	3.65
MPO[3]	1.84	2.22	2.38	2.10	1.61	1.70	0.07

[1]Values are means from triplicate groups of fish where values in each row with a different superscript are significantly different (*P<0.05)*. The lack of superscript letter means no significant differences among the treatment diets

[2]SOD (% superoxide inhibition): Superoxide dismutase

[3]MPO (absorbance at 450 nm): Myeloperoxidase. [4]Pooled standard error of mean: SD/√n.

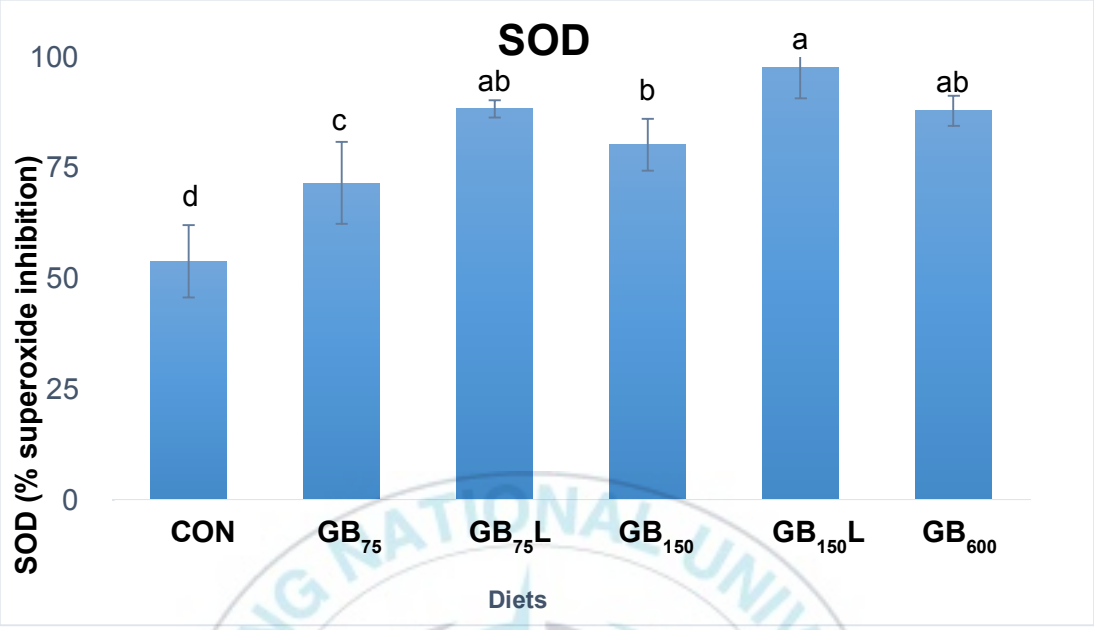

Fig. 16. Superoxide dismutase (SOD)% superoxide inhibition of juvenile Nile tilapia fed on the experimental diets for 8 weeks.

CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg,75mg,75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively.

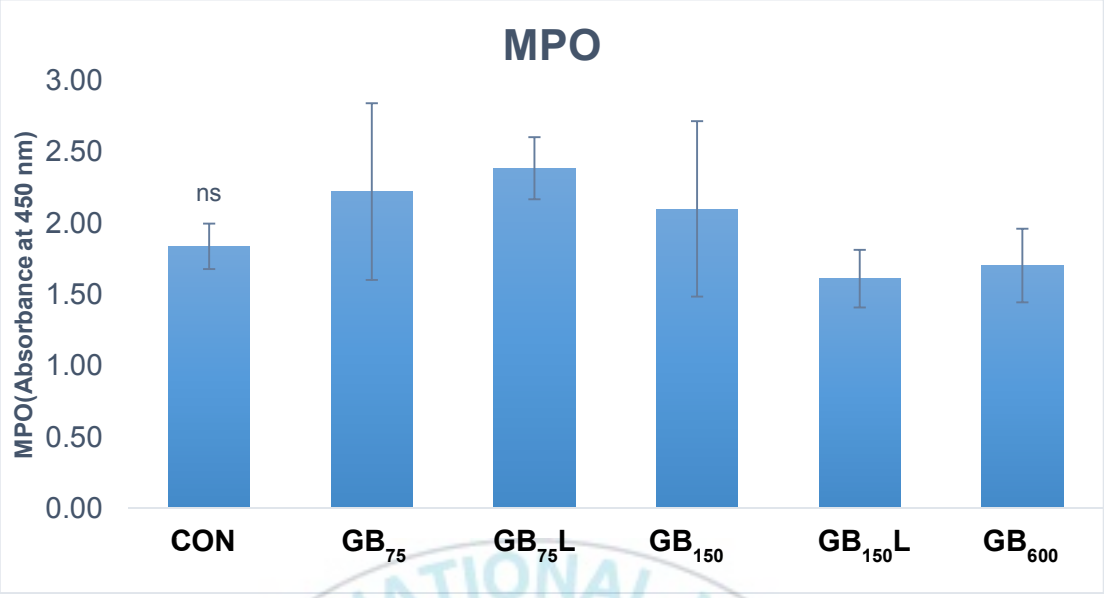

Fig. 17. Myeloperoxidase (MPO (absorbance at 450 nm) in juvenile Nile tilapia fed on the experimental diets for 8 weeks.

CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg, 75mg, 75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively.

3.5. Bacterial challenge test

The cumulative survival rate in juvenile Nile tilapia fed on the experimental diets challenged with *Aeromonas hydrophila* for 5 days is shown in Figure 19. During *Aeromonas hydrophila* challenge test, the first mortality occurred on day 1 after injection of bacteria. The cumulative survival rate of fish fed diet GB_{75}, $GB_{75}L$, and $GB_{150}L$ was significantly higher than those of fish fed CON and GB_{150} diets ($P<0.05$). However, there was no significant difference in cumulative survival rates on fish fed GB_{75}, $GB_{75}L$, $GB_{150}L$, and GB_{600} and among GB_{150} and GB_{600} diets after 5 days of challenge test.

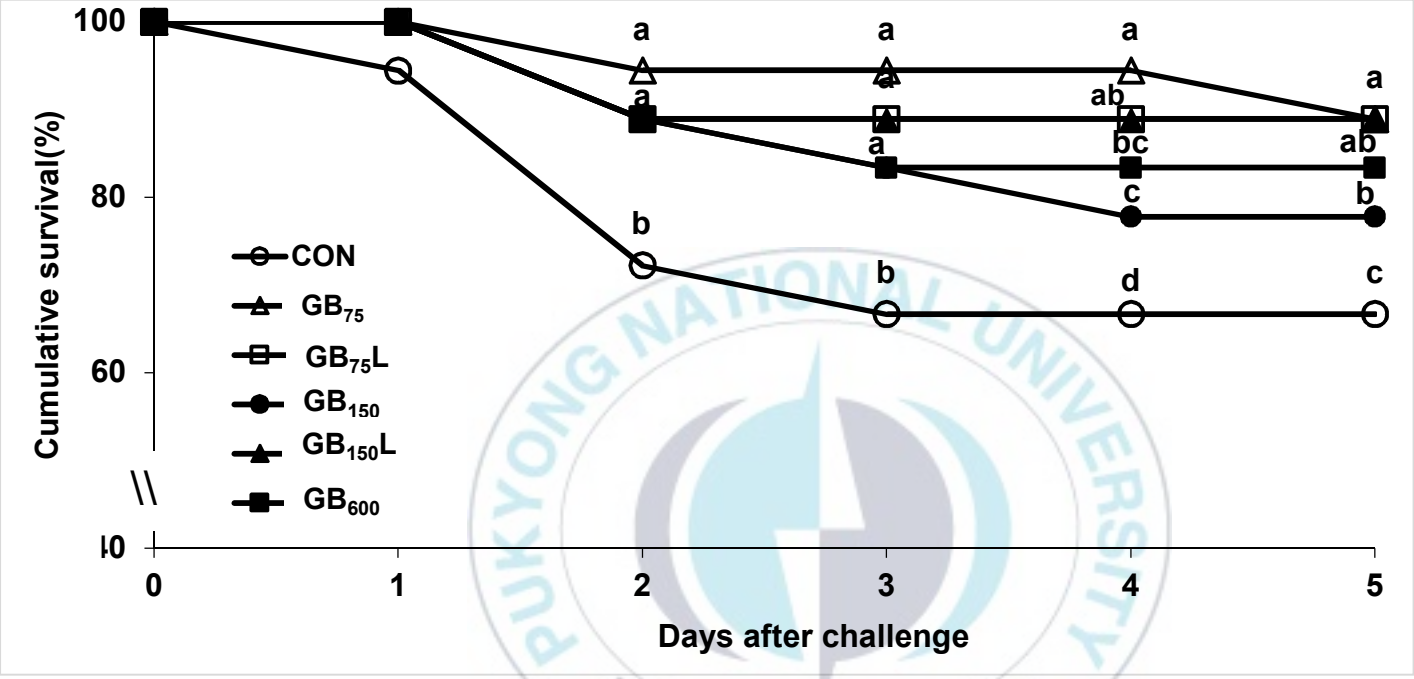

Fig. 18. Cumulative survival rate after injection of Aeromonas hydrophila of juvenile Nile tilapia fed on the six experimental diets as observed for 5 days. CON, GB_{75}, $GB_{75}L$, GB_{150}, $GB_{150}L$, and GB_{600} represent diets with GABA meal supplementation level of 0mg,75mg,75mg with 50mg heat killed (*Lactobacillus paracasei*), 150mg, 150mg with 50mg heat killed (*Lactobacillus paracasei*) and 600mg in the diet, respectively.

4. Discussion

One of the main goals of sustainable aquaculture development is to reduce the dependency of aquafeed industry on expensive fish meal. In this regard, a great deal of research has been done to replace or reduce the amount of fish meal in aquafeed (Montoya-Camacho et al. (2018). But it should be considered that potential alternative protein sources, as compared to fish meal, may lack some of the major nutrients required by the target species. One of the methods to overcome this problem is to supply the limiting nutrients through feed additives in the diet Dawood. (2017). GABA is one of the nutrients relatively abundant in fish meal but deficient in plant proteins Rezaei et al. (2013); Xie et al. (2015) Results of the present study demonstrated that supplementation of 75 mg GABA with heat-killed *Lactobacillus paracasei* (diet $GB_{75}L$) can improve WG and SGR as compared to the control group. This is consistent with previous results which 150 mg kg^{-1} GABA enhanced the growth in Pacific white shrimp, *Litopenaeus vannamei* fed low fishmeal diets (Xie et al. 2015). It has been shown that *Lactobacillus* spp. bacteria can convert L-glutamate to GABA and the isolation of specific strains has been studied for industrial purposes (Dhakal et al., 2012). The results observed in the present study could be attributed to the fact that *Lactobacillus paracasei* boosts the GABA levels in Nile tilapia diets. In this study, optimum inclusion level of GABA derived from broken line analysis of weight gain for

Juvenile Nile tilapia was 158.7 mg kg^{-1} diet. Wu et al. (2016), reported that optimal GABA supplementation level in grass carp is 87.5 mg kg^{-1} for improvement of growth performance. Results from all these authors suggested that improvement of growth performance with presence of optimal GABA could be related to higher feed intake and feed utilization performance. In this experiment, it was clearly substantiates that GABA can potentially convey improvement on FE and PER in which fish fed 75, 75 with 50 *Lactobacillus paracasei*, 150, and 150 with 50 *Lactobacillus paracasei* mg kg^{-1} GABA had higher FE and PER as compared with the CON and 600 mg supplemented group. In agreement with our results, it was shown that high dietary GABA supplementation (1000 mg in the diet) did not improve growth performance in Japanese flounder Kim et al. (2003). The reasons for this counter-effect are not clear and more studies are required in this regard.

Haematological parameters are good indicators for fish physiological and health status (Magnadottir, 2006; Maita et al., 2007). Glutamatic oxaloacetic transaminase (GOT) is a sensitive index mainly indicating the liver health (Fan at al., 2015). Serum GOT results from this study showed significantly lower values for fish fed GB_{75} and $GB_{75}L$ diets compared to CON, GB_{150} and GB_{600} diets. This could be attributed to the stress relieving property of GABA, improved liver health and less GOT release to the blood. Previously it was shown that 150 mg GABA kg^{-1} diet significantly increased the survival of Pacific white shrimp against NH_3 stress (Xie et al. 2015).

Superoxide dismutase (SOD) activity has been used as a non-specific immune parameter indicating the fish health status. SOD is an important enzyme in antioxidant defense in

nearly all living cells exposed to oxygen. SOD interchangeably catalyzes the dismutation (or partitioning) of the superoxide (O_2^-) radical into either ordinary O_2 molecule or less reactive H_2O_2 Fridovich (1995). In the present study the superoxide dismutase activity of fish fed $GB_{75}L$, $GB_{150}L$ and GB_{600} diets were significantly higher than those fish fed the CON and GB_{75} diets ($P<0.05$). Similar to these results, higher SOD activities were observed by increased GABA supplementation in the diet of rats Deng et al. (2013) and cows Wang et al. (2013). Also, SOD activities and the antioxidant capacity increased with GABA supplementation up to 150 mg kg^{-1} diet for pacific white shrimp (Xie et al. 2015) and 100 mg kg^{-1} for grass carp Wu et al. (2016). The mechanism behind these observations could be related to the absorption of reactive intermediates from lipid peroxidation and scavenging properties of GABA for reactive carbonyl compounds Deng et al. (2010).

In the present study, significantly higher disease resistance against the *Aeromonas hydrophila* was in fish groups supplemented with GABA and was low in diet with on basal diets. This might be subjective to GABA ability of on improving antioxidant capacity and reduce oxidative damage which improving the feed utilization and growth Wu et al. (2016).

In conclusion GABA enhanced the growth performance, hematological parameters and non-specific immune responses of juvenile Nile tilapia (*Oreochromis niloticus*). Additional dietary heat-killed *Lactobacillus paracasei* could have had a synergistic effect with lower dietary GABA or boosted the GABA level to the required amounts.

On the basis of broken line analysis of weight gain, the optimum dietary GABA level for juvenile Nile tilapia (*Oreochromis niloticus*) could be 158 mg kg^{-1} diet.

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Appendix

Growth performance of juvenile Nile tilapia fed the experimental diets for 8 weeks

Diets	Reps	WG (%)	SGR (%/day)	FE (%)	PER	DFI (%)	Survival (%)
CON	1	462.10	3.60	64.04	1.78	5.62	100.00
	2	484.25	3.68	68.36	1.90	5.38	95.00
	3	473.17	3.64	66.20	1.84	5.49	95.00
GB_{75}	1	515.31	3.79	73.13	2.04	5.18	90.00
	2	542.78	3.88	72.35	2.02	5.36	95.00
	3	541.37	3.87	71.36	1.99	5.43	100.00
$GB_{75}L$	1	588.80	4.00	72.44	2.02	5.52	90.00
	2	582.39	4.00	71.74	2.00	5.58	90.00
	3	595.22	3.99	73.14	2.04	5.46	100.00
GB_{150}	1	576.43	3.98	77.12	2.14	5.15	100.00
	2	519.92	3.80	70.74	1.96	5.37	95.00
	3	632.94	4.15	83.50	2.32	4.97	80.00
$GB_{150}L$	1	530.89	3.84	71.88	2.04	5.34	90.00
	2	556.33	3.92	72.59	2.06	5.40	100.00
	3	581.77	4.00	73.29	2.08	5.46	95.00
GB_{600}	1	464.87	3.61	65.61	1.77	5.50	100.00
	2	460.37	3.59	65.33	1.76	5.50	95.00
	3	524.40	3.82	65.96	1.78	5.79	100.00

Proximate composition of juvenile Nile tilapia fed the experimental diets for 8 weeks

Diets	Reps	Moisture	Crude Ash (%)	Crude lipid (%)	Crude Protein (%)
CON	1	72.66	4.54	5.51	15.97
	2	72.89	4.94	6.25	14.84
	3	71.74	4.85	7.78	15.90
$GABA_{75}$	1	74.12	4.69	6.21	16.47
	2	73.56	5.02	5.53	15.62
	3	71.64	4.44	6.86	15.75
$GABA_{75}L$	1	73.36	4.41	6.00	13.91
	2	71.15	5.58	6.88	15.58
	3	72.92	4.59	6.68	16.18
$GABA_{150}$	1	74.17	4.21	5.80	15.01
	2	72.66	4.38	4.38	15.49
	3	70.07	4.08	7.62	16.36
$GABA_{150}L$	1	73.11	4.54	6.91	15.62
	2	73.16	4.76	5.79	15.25
	3	72.15	4.45	5.52	14.71
$GABA_{600}$	1	72.41	4.19	6.34	15.80
	2	72.94	4.75	7.21	15.35
	3	72.21	4.50	7.30	16.21

Non-specific immune responses of juvenile Nile tilapia fed the experimental diets for 8 weeks

Diets	Reps	MPO (absorbance at 450 nm)	SOD (% superoxide inhibition)
CON	1	2.021	52.61
	2	1.739	62.65
	3	1.747	46.56
$GABA_{75}$	1	2.051	74.63
	2	1.704	79.01
	3	2.909	61.24
$GABA_{75}L$	1	2.137	90.34
	2	2.546	88.35
	3	2.468	86.35
$GABA_{150}$	1	1.603	76.05
	2	2.787	77.85
	3	1.901	86.86
$GABA_{150}L$	1	1.525	97.68
	2	1.841	104.64
	3	1.466	90.73
$GABA_{600}$	1	1.403	87.96
	2	1.881	91.37
	3	1.817	84.55

Hematological parameters of juvenile Nile tilapia fed the experimental diets for 8 weeks

Diets	Reps	GOT (U/I)	GPT (U/I)	TP (g/dl)	TG (mg/dI)	Glucose (mg/dl)	TCHO (mg/dl)
CON	1	45.0	7.0	3.1	245.0	62.0	191
	2	78.0	7.0	2.9	241.0	49.0	160
	3	53.0	5.0	3.5	300.0	37.0	229
$GABA_{75}$	1	37.0	5.0	2.8	130.0	62.0	208
	2	59.0	5.0	2.8	84.0	52.0	160
	3	44.0	5.0	2.9	111.0	57.0	167
$GABA_{75}L$	1	49.0	5.0	3.8	301.0	55.0	198
	2	41.0	6.0	3.3	146.0	46.0	188
	3	40.0	6.0	3.8	228.0	48.0	199
$GABA_{150}$	1	56.0	5.0	3.2	171.0	55.0	186
	2	76.0	5.0	3.2	307.0	51.0	181
	3	78.0	5.0	3.2	163.0	40.0	163
$GABA_{150}L$	1	62.0	5.0	3.2	199.0	52.0	187
	2	83.0	5.0	3.1	168.0	50.0	214
	3	44.0	1.0	3.3	206.0	48.0	204
$GABA_{600}$	1	84.5	5.0	3.5	207.5	40	176
	2	88.0	5.0	3.6	200.0	45	180
	3	81.0	5.0	3.3	215.0	35	172