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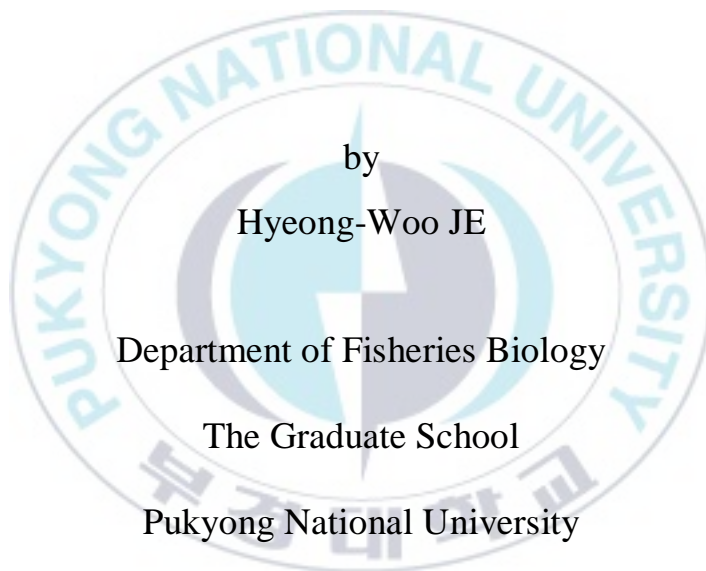
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Thesis for Degree of Master of fisheries science

Effects of dietary gamma aminobutyric acid as a
feed additive in juvenile olive flounder,
Paralichthys olivaceus



February 2021

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additive in juvenile olive flounder, *Paralichthys*
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성장기 넙치의 사료 내 gamma aminobutyric
acid의 첨가 효과

Advisor: Prof. Sungchul C. Bai, Youn Hee Choi

by
Hyeong-Woo JE

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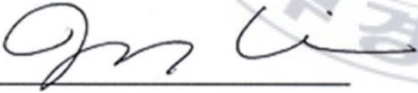


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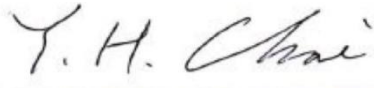
A dissertation
by
Hyeong-Woo JE

Approved by:


Approved as to style and content by:



(Chairman) Jong-Myoung Kim



(Member) Youn Hee Choi



(Member) Sungchul C. Bai

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성장기 넙치의 사료 내 gamma aminobutyric acid의 첨가 효과

제 형 우

부경대학교 대학원 수산생물학과

요 약

지속적인 세계인구의 증가에 발맞춰 양식기술은 고도로 발전하고 있으며, 대량생산을 위한 집약적인 양식방법이 주로 이용되고 있다. 밀폐된 공간에서 사육되는 양식어류는 집약적인 사육환경에 따른 스트레스와 질병 감수성의 증가로 인해 대량 폐사의 위험성이 있다. 따라서 본연구는 고밀도 양식환경에서 대표적인 억제성 신경전달 물질인 gamma aminobutyric acid (GABA)를 사료 첨가체로서 스트레스 저감 및 면역 증진 효과들을 우리나라의 대표적인 어종인 넙치를 대상으로 검증하였다.

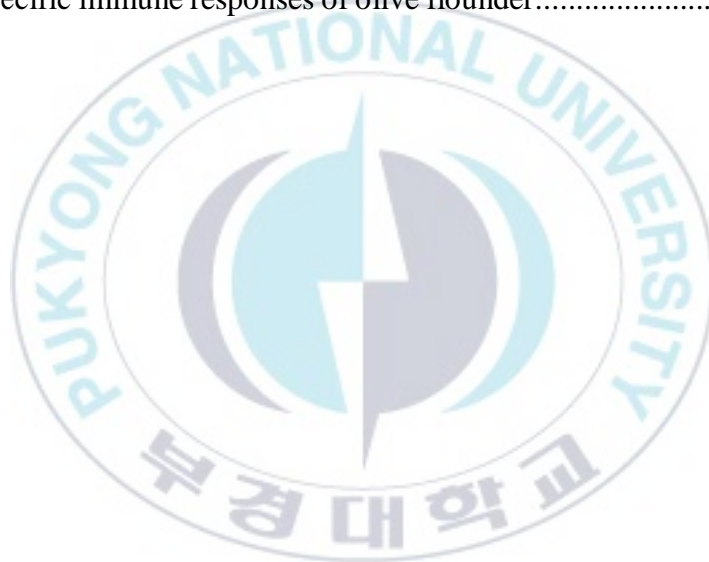
대조구와 3 개 구간의 실험사료를 제작하였으며, 각 실험사료에는 150 mg/kg (GABA₁₅₀), 200 mg/kg (GABA₂₀₀), 250 mg/kg (GABA₂₅₀)의 GABA를 첨가하였으며 실험사료 제작 후 실제 GABA함량을 분석한 결과 63.9 mg/kg (CON), 231.3 mg/kg (GABA₁₅₀), 291.6 mg/kg (GABA₂₀₀), 323.9 mg/kg (GABA₂₅₀)였다. 각 실험사료마다 12.75 g \pm 0.3 g의 넙치를 40L수조에 적정밀도 (Normal density) 20fish/tank와 고밀도 (High density) 40fish/tank로 구분하여 3 반복으로 무작위 배치하였고, 수온 17.5 \pm 1°C, 염분 33 \pm 1 ppt를 유지하였다. 8 주간의 사육실험 후 성장과 사료이용능력, 전어체 일반성분 분석, 혈액학적 분석, 비특이적 면역반응, 공격실험을 실시하였다.

비특이적 면역반응 중 SOD (Superoxide dismutase activity) 분석에서 GABA첨가 실험구가 대조구보다 유의적으로 높은 값을 보여주었지만 밀도구간과 밀도 \times GABA농도 상호작용 유의적인 효과는 없었다($P < 0.05$). MPO (Myeloperoxidase) 분석에서 GABA₂₀₀과 GABA₂₅₀이 유의적으로 높은

값을 보여주었으며 밀도×GABA농도 상호작용 효과가 관찰되었다($P<0.05$). Lysozyme activity에서는 GABA₁₅₀이 CON, GABA₂₀₀, GABA₂₅₀보다 유의적으로 높은 값을 보여주었으며, 밀도, 밀도×GABA농도 상호작용 효과가 없었다($P<0.05$). 병원균인 *Edwardsiella tarda*를 복강에 주사하여 15일간 공격실험을 진행한 결과, GABA₁₅₀, GABA₂₀₀, GABA₂₅₀이 CON보다 유의적으로 높은 누적생존율을 보였다($P<0.05$). 증체율(Weight gain), 일간성장률(Specific growth rate)에서 GABA농도와 밀도×GABA농도 상호작용에 있어서 유의적인 차이를 보이지 않았지만 저밀도 실험구가 고밀도 실험구보다 유의적으로 더 높은 값을 보여주었다 ($P<0.05$). 사료효율(Feed efficiency), 단백질전환효율(Protein efficiency ratio)에 있어서는 전구간에서 유의적인 차이를 보이지 않았다. 전어체 일반성분 분석에서는 전 구간에서 유의적인 차이가 없었다 ($P>0.05$). 혈액학적 분석 중 Cortisol에서 사료와 밀도×GABA농도 상호작용에 있어서는 유의적인 차이를 보이지 않았지만 고밀도 실험구가 유의적으로 높은 값을 나타냈으며 혈액 내 GABA는 밀도 구간과 상관없이 첨가량에 따라 유의적으로 증가하였다($P<0.05$). 따라서 넙치 사육 시 적정 GABA첨가량은 사육 밀도와 상관없이 면역 증진 효과 및 질병 내병성 효과가 있을 것으로 판단된다.

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I. Introduction

The world population is exploding, and food crisis continue to emerge. Accordingly, many scientists and researchers in all fields such as agriculture, livestock, and fisheries are working hard to solve the food crisis that will come. Among them, aquaculture is an emerging industry for food security and plays a very important role as a protein source. In fact, aquaculture production has increased by a 7 percent per year over the past 30 years (CAGR: compound annual growth rate), and in 2018, it represents 115 million tons, and this trend is expected to continue in the future. On the other hand, fishery production has stagnated at about 98 million tons from 2004 to the present (FAO, 2020). The aquaculture industry is developing radically due to the standstill of fishery production and increasing demand for protein and aquatic products in modern society. The aquaculture industry has been changing from a labor-intensive primary industry to a knowledge-intensive fourth industry that utilizes Big Data, AI, and ICT. (Gorbunova, A., et al 2020; Li, P., et al 2009)

What is important in this change is the stocking density of fish. As the aquaculture industry develops, high density culture naturally occurs, and the stress of fish increases due to high-density culture, which increases the fish's disease susceptibility and eventually leads to mass death (Noble, A. C., & Summerfelt, S. T., 1996). For a successful aquaculture industry, the development of ways that can reduce the stress level of fish is essential. However, in the current aquaculture industry, diseases are suppressed by using large amounts of antibiotics, not substances that lower the stress level of fish. Due to the excessive use of these antibiotics, many pathogens have recently become resistant to antibiotics, and in particular, bacteria resistant to multiple antibiotics have emerged (Serrano, P. H., et al 2005). In addition, when antibiotics are added to feed and feeding environment, eventually aquatic products, they remain or accumulate

in the water, damaging the health of end-users, humans, and polluting the aquatic environment due to antibiotics flowing into the natural environment. Therefore, research on antibiotic substitutes and alternatives that can lower the stress level is indispensable.

Gamma-Aminobutyric acid (GABA) used in this experiment is a natural substance present in bacteria, plants, and animals, and is synthesized from glutamic acid inside the synaptic presynaptic of inhibitory nerve cells (Abe, T., & Takeya, K., 2005; Arumugam, K., et al., 1997; Bown, A. W., et al., 1997). Glutamic acid decarboxylase (GAD) makes GABA by removing carboxyl groups from glutamic acid. Although it is a kind of amino acid in terms of molecular structure, it is not used for protein synthesis (Dhakal, R., et al., 2012; Fenalti, G., et al., 2007; Walls, A. B., et al., 2015). Its chemical formula is $C_4H_9NO_2$ and its mass is 103.120 g / M. The synthesized GABA is stored in synaptic vesicles, released to the synaptic cleft, and binds to the Gaba receptor ($GABA_A$ receptor & $GABA_B$ receptor) located on the posterior synaptic surface to activate the receptor (Watanabe, M., et al., 2002). $GABA_A$ receptor is a ligand-gated ion channel that passes chloride ions when GABA binds. In the developed brain, the concentration of chloride ions outside neurons is higher than inside, so when $GABA_A$ receptor opens, chloride ions enter the neurons, which induces hyperpolarization of neurons, suppressing excitability and suppressing the occurrence of action potentials (Ong, J., & Kerr, D. I., 1990; McCormick, D. A. 1989). $GABA_B$ receptor is a G protein-associated receptor that does not directly act as an ion channel, but it inhibits the excitability of nerve cells by indirectly controlling various ion channels such as potassium ion channel through G protein signaling. When there is a problem in the production of glutamic acid decarboxylase (GAD) and synthesizes GABA, the amount of GABA in the brain decreases, which causes a decrease in the activity of inhibitory synapses. Neurons that are not normally inhibited are overexcited, which in turn leads to abnormal neuronal activity (Abdou, A. M., et al). In mammals, when there is a problem with GABA synthesis, it has been reported that schizophrenia and bipolar

disorder occur (Bach, B. et al., 2009; Bongiani, F. et al., 2006; Boonstra, E. et al., 2015; Hsu, Y. T., et al., 2018)

GABA induces stable metabolic processes and immune and antioxidant responses by inhibiting overactive neurons in stressful situations. Because of this effect, it is a feed additive used in the poultry industry and livestock industry for various purposes such as stress reduction, immunity enhancement, antioxidant status, and promotion of feeding (Al Wakeel, R. A et al., 2017; Li, Y. H., et al., 2015). Although it is a feed additive that has not been researched largely in fish, studies on feed with GABA are also being conducted in aquaculture diets. According to previous research (Chand, N., et al., 2016; Kim, S. K., et al., 2003; Wu, F., et al., 2016; Xie, S. W., et al., 2017 and Temu, V., et al., 2019) GABA has also been proven the positive effect in fish. In Juvenile grass carp, 87.5 mg/kg of GABA in feed had a positive effect on growth and antioxidant ability. Likewise, dietary GABA at 158.7 mg/kg in Nile tilapia showed an effect on growth, feed utilization ability, and homeostasis ability. In whiteleg shrimp, 150 mg/kg of GABA showed an effect on growth and antioxidant capacity. However, there were no research studies in any stress conditions.

Therefore, this study was conducted to evaluate the growth performance and feed utilization ability, hematological analysis, non-specific immune response and disease resistance that occurs during normal and high-density culture by adding different concentrations of GABA in feed for olive flounder, *Paralichthys olivaceus* as a major domestic species.

II. Material and method

Experimental design and diets

Anchovy fish meal, soybean meal, squid liver meal, meat and bone meal, and poultry by-products were used as the main protein sources for the experimental feed, and marine fish oil was used as a lipid source, and flour and cellulose were used for the shape of the feed, and sufficient minerals and vitamins were supplied (NRC., 2011). For this, a commercial premix was added. Choline, taurine, betaine, lecithin, and monocalcium phosphate were used as other additives, and the composition of the feed source is shown in Table.1. All feed sources were put in a mixer (HYVM-1214, Hanyoung Food Machinery, Rep. Korea), mixed well, and feed was prepared using a pellet machine (SMC-12, SUN Engineering, Rep. Korea), dried at room temperature for 48 hours, and stored in a freezer at -20°C. GABA was used in powder form obtained from Mirae Resources company (Seoul, Rep. Korea) and showed a purity of 76.5% GABA as a result of analysis with high performance liquid chromatography (HPLC). In order to evenly add GABA, which is a very small amount of the experimental feed, it was mixed with cellulose for 72 hours using a ball mill machine (PL-BM5L, POONG LIM Trading., co. Rep. Korea) to make cellulose containing 40,000 mg/kg of GABA. After that, four experimental diets were made by adding 0 (CON), 150 (GABA₁₅₀), 200 (GABA₂₀₀) and 250 (GABA₂₅₀) mg of GABA per kg of diet. The HPLC analysis showed 63.92, 231.30, 291.59 and 323.90 mg/kg of actual GABA content in CON, GABA₁₅₀, GABA₂₀₀ and GABA₂₅₀ diets, respectively Table. 2 and Table. 3.

Experimental fish and feeding trial

The olive flounder used in this experiment was transported from SAMWOO Aqua hatchery (Boryeong, Chungcheongnam-do, Rep. Korea) to the Laboratory of Feeds and Foods Nutrition Research Center (FFNRC) at Pukyong National University. Before starting the experiment, acclimation phase was carried out by mixing and supplying commercial feed and CON for 3 weeks in a 2000 L tank. After the acclimation phase, olive flounder with an average weight of 12.75 ± 0.5 g were reared in a 40 L square tank and 20 fish at the normal density and 40 fish at the high density. Each experimental tank was controlled so that the amount of water flow was 2.0 L/min by semi-recirculating system, and 150 % water was exchanged every day. The water temperature was set to $17.5 \pm 1^\circ\text{C}$, and the salinity of the water was maintained at 33 ± 1 ppt. For sufficient oxygen supply, an air stone was installed in each tank to keep the dissolved oxygen content at 6~7 ppm, and feed was supplied by dividing 3~4% of the fish's body weight twice a day.

Sample collection and analysis

Growth performance

After the 8 weeks culture experiment was over, the total weight of the fish in each tank was measured after fasting for 24 hours in order to investigate the growth performance and feed efficiencies. The equations for WG, FE, SGR and PER analysis are as follows.

$$\text{Weight gain (WG, \%)} = [\text{final weight} - \text{initial weight}] \times 100 / \text{initial weight}$$

$$\text{Feed Efficiency (FE, \%)} = [\text{wet weight gain} / \text{dry feed intake}] \times 100$$

$$\text{Specific growth rate (SGR, \% / day)} = [\log_e \text{ final weight} - \log_e \text{ initial weight}] \times 100 / \text{days}$$

$$\text{Protein efficiency Ratio (PER)} = \text{Wet weight gain} / \text{Protein intake}$$

Proximate analysis

Proximate analysis was performed according to the standard AOAC (2005) method, Moisture and ash were measured by a sample of constant weight for 24 hours in a dry oven at 105°C and burning it in a muffle furnace at 500°C for 3 hours. For the analysis of crude lipid and crude protein of feed and whole fish, the sample was frozen at -20°C for 24 hours, and then made into a powder form using a freeze-drying machine (Advantage 2.0, VirTis, New York, USA). Afterwards, crude protein was analyzed using the Auto Kjeldahl system (Buchi B-324/435/412, Switzerland; Metrohm 8-719/806, Switzerland) by Kjeldahl nitrogen determination (Nitrogen \times 6.25). (Tecator AB, Sweden). In addition, the actual GABA content of the experimental feed was analyzed using HPLC by requesting the National Instrumentation Center for Environmental Management College of Agriculture and Life science at Seoul National University (Seoul 151-742, Rep. Korea).

Hematological parameters

After eight weeks of feeding trial, 6 experimental fish were randomly selected for each tank, and blood was collected from the caudal vein using a non-heparinized 1mL syringe. In order to analyze the amount of GABA remaining in the blood, blood was collected 24 hours after feeding. Blood was centrifuged (5000 \times g) for 10 minutes, and the serum was retaken and immediately put at -70°C. Glutamic oxaloacetic transaminase (GOT), Glutamic pyruvic transaminase (GPT), and Glucose were Fuji DRI-CHEM 3500i (Fuji Photo Film Ltd, Tokyo, Japan.) was used.

Cortisol ELISA kit (ENZO Life Sciences Inc., New York, USA; ADI-900-071) was used to analyze cortisol in blood, and carried out according to the manufacturer's standard protocol. The kit for the quantitative measurement of cortisol uses a monoclonal antibody to cortisol to bind, in a competitive manner, cortisol in a sample or an alkaline phosphatase molecule which has cortisol covalently attached to it. After a simultaneous incubation at room temperature the

excess reagents are washed away and substrate is added. After a short incubation time the enzyme reaction is stopped and read on a microplate reader at 405 nm. The measured optical density is used to calculate the concentration of cortisol.

To analyze GABA in blood, QuickDetect™ GABA ELISA Kit (Biovision Inc., San Francisco, USA; E4455-100) was used, and the analysis method of use was carried out according to the manufacturer's standard protocol. In addition, blood was collected and analyzed exactly 24 hours after feeding to determine the amount of GABA in the blood.

Non-specific immune response

Lysozyme activity was measured using the absorbance of the microplate technique proposed by Ellis (1999). For this measurement, a bacterial suspension was prepared by mixing 0.2 mg of *Micrococcus lysodeikticus* (Sigma) for each sample in 1 mL sodium citrate buffer (0.02M, pH 5.52). Prepare serum dilution by mixing 30 μ L of serum and 270 μ L of sodium citrate buffer (0.02M, pH 5.52). A 180 μ L of bacterial suspension was placed into each 96-well plate, and 20 μ L serum dilution was subsequently added and is directly incubated thereafter at room temperature for 30 min. The decrease in absorbance was then recorded at 450 nm at 0, 30 and 60 min in a microplate reader (Sunrise TECAN, Männedorf, Switzerland). The active unit of lysozyme was defined as the amount of enzyme showing a decrease in absorbance of 0.001 per minute.

Serum isolated from fish in each experimental section. Using the SOD assay kit (Sigma-Aldrich, 191600), according to the manufacturer's instructions, the inhibition rate of the enzyme was calculated as a percentile with WST-1 (Water soluble tetrazolium dye) and xanthine oxidase. Each sample was reacted in a 37°C incubator for 20 minutes, and then absorbance was measured at a wavelength of 450 nm (absorbance of the wavelength to measure the color displayed by the reaction of WST-1 and reactive oxygen) in microplate reader

(Sunrise TECAN, Männedorf, Switzerland). The inhibition rate was expressed in units of SOD activity per mg protein.

Measurement of myeloperoxidase (MPO) activity was determined as accorded by Quade and Roth (1997) along with the modification made by Park et al. (2016). Broadly 20 μL of serum was diluted with 80 μL of HBSS (Hanks Balanced Salt Solution) without Ca^{2+} or Mg^{2+} in 96-well plates and was consequently added with 35 μL of 3, 3', 5, 5' tetramethylbenzidine hydrochloride (TMB, 20 mM) (Sigma Aldrich) and H_2O_2 (5 mM). After addition of 35 μL of 4 M sulfuric acid, absorbance was measured at 450 nm wavelength after 2 min incubation in a 37°C incubator.

Challenge test

In the Challenge test with *Edwardsiella ictaluri* was incubated at 27°C for 24 to 48 hours in BHI broth medium and then suspended in sterile distilled water at 1×10^7 CFU/mL. After injecting 0.1 mL of suspension into the peritoneal cavity of 15 fish randomly selected for each experimental feed, the survival rate of olive flounder was investigated according to elapsed time without feeding, and the experimental group and the control group were compared and analyzed.

Statistical analysis

The arrangement of the experimental tank was completely random, and the growth and analysis results were statistically analyzed by two-way ANOVA using the IBM SPSS 26 statistics program. When a significant difference was observed, a Least Significant Difference (LSD) test was used to compare means. The significance differential level was set to $P < 0.05$.

III. Results and discussions

Growth performance

After 8 weeks of feeding trial, weight gain (WG, %), specific growth rate (SGR, %/day), feed efficiency (FE, %) and protein efficiency ratio (PER) are measured. As a result, there was no significant difference according to the GABA level. In addition, there was no significant interaction effect between GABA level and Density in all experimental groups. However, the normal-density group showed significantly higher values than the high-density group in WG and SGR ($P < 0.05$). In the results of this experiment, dietary GABA did not reduce the stocking density stress, so the result of WG and SGR was significantly higher at the normal density group. Compared with previous experiment conducted on olive flounder in our laboratory, there was a significant difference in growth performance at a specific GABA level (Farris. et al., 2020; Bae et al., 2020), and the same results were obtained for other fish species (Nile tilapia, grass carp and whiteleg shrimp). This showed contrary results to my experiment. (Temu, V., et al., 2019; Xie, S. W., et al., 2017; Wu, F., et al., 2016) For the reason, the stocking density that is too high compared to the carrying capacity that the culture system in the laboratory can handle is considered to have influenced the normal-density group as well. Therefore, if designing such an experiment in the future, another culture system should be introduced to minimize the influence between density groups. But there was no significant difference in feed utilization abilities such as FE and PER. This is because GABA is converted through GABA transaminase and glutamine synthetase into glutamate and glutamine, which affects the TCA cycle, and affects the glial cells that provide energy to neurons, thereby reducing glucose oxidation in oxidative metabolism. Because it reduces, it affects energy metabolism. (Golam et al., 2007; Patel, A. B., et al., 2005; Carabotti, M., et al., 2015; Strandwitz, P., 2018; Strandwitz, P., et al., 2019) Therefore, it is considered that there was no significant difference

in feed utilization. In addition, we could see the effect of GABA in tilapia, white shrimp, and grass carp with the same results. However, the exact pathways for inhibitory neurotransmitters for energy metabolism have not yet been identified. (Temu, V., et al., 2019; Xie, S. W., et al., 2017; Wu, F., et al., 2016) The results refer to the Table 4 and Figure 1, 2, 3, 4.

Proximate analysis

After 8 weeks of feeding trial, there was no significant difference in GABA level and Density in all experimental group. There was also no significant interaction effect of GABA level and Density ($P<0.05$). The results refer to the Table 5.

Hematological parameters

Hematological analysis is an important indicator of the health and physiological condition of fish. (Magnadóttir, B., et al., 2006; Maita et al., 2007) After 8 weeks of feeding trial, glutamic oxalacetic transaminase (GOT), glutamic pyruvate transaminase (GPT) and glucose (GLU) were no significant difference in GABA level and Density in all experimental group. Also, there was no significant interaction effect of GABA level and Density ($P>0.05$). GOT (=aspartate aminotransferase) and GPT (=alanine aminotransferase) are enzymes present in hepatocytes and are mainly released into the blood when hepatocytes are damaged. As a result of GOT and GPT analysis, there was no significant difference in all experimental groups, which is a good indicator of the safety of GABA as a feed additive. (Bucher, F & Hofer, R., 1990; Vaglio, A & Landriscina, C., 1999) In addition, Blood cortisol level was no significant difference in the GABA level and interaction effect, however high-density group showed significantly higher value than normal-density group ($P<0.05$). Blood GABA content was significantly increased according to GABA intake without Density and interaction effects ($P<0.05$). Analysis of GABA content in blood to determine the amount of GABA actually

digested after ingestion showed a result that significantly increased with the amount consumed without the density group. This means dietary GABA is well absorbed in olive flounder (Fugelli, K., et al., 1970). The content of cortisol in blood is a representative stress indicator. In case of chronic stress, the interrenal cells of the head kidney are stimulated through the HPI axis to promote cortisol secretion, and metabolic reactions are promoted, thereby increasing the concentration of glucose in the blood. As a result of the analysis, the high-density group showed significantly higher cortisol value compared to the normal-density group, which is judged that dietary GABA did not show the direct effect of reducing stocking density stress. However, broiler chicken, pig and cow showed the effect of reducing temperature stress. (Tang, J., & Chen, Z., 2016; Li, Y et al., 2015; Wang, D. M., et al., 2013) This means dietary GABA plays an important role in temperature stress rather than density stress, and it is necessary to proceed as a follow-up study. In addition, glucose was no significant difference in all experimental group, which is believed to be maintained at a similar level of blood glucose by insulin, thyroid hormone and other hormones, which control blood glucose level in the body. (Tort, L., 2011; Peter, M. S., 2011). The results refer to the Table 6 and Figure 5, 6, 7, 8, 9.

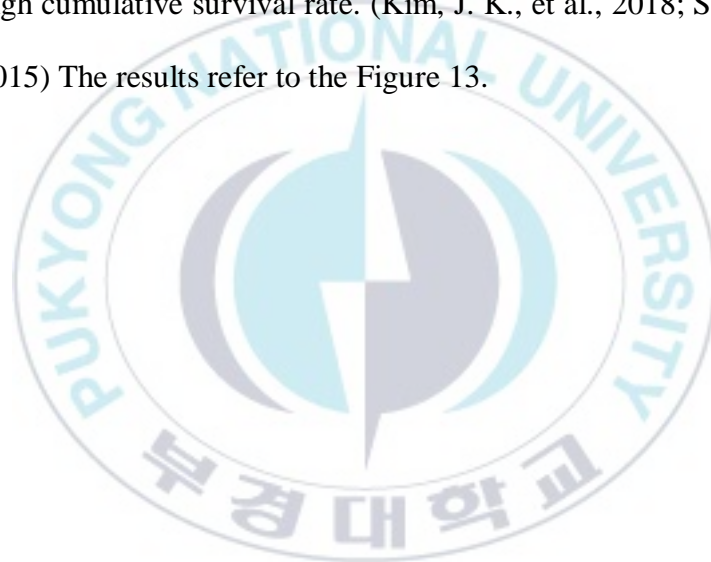
Non-specific immune response

A non-specific immune response is the innate immune system that reacts primarily in the body when exposed to dangerous substances. The reaction to external substances or abnormal substances (infectious substances, chemical / physical damage) occurs immediately and non-selectively, thus acting as an important indicator of the immune response. (Delves, P. J., et al., 2017; Abbas AK et al., 2014.) After 8 weeks of feeding trial, there were no significant differences according to density in lysozyme activity, myeloperoxidase activity (MPO, absorbance) and superoxide dismutase (SOD, % inhibition) ($P>0.05$). However, there was a significant difference in the GABA level. Lysozyme activity of fish fed GABA₁₅₀ was

significantly higher values than those of fish fed CON, GABA₂₀₀ and GABA₂₅₀, and there was no interaction effect ($P>0.05$). MPO of fish fed GABA₂₀₀ and GABA₂₅₀ were significantly higher values than those of fish fed CON and GABA₁₅₀, and there were also interactions effect ($P<0.05$). SOD of fish fed GABA₂₅₀ and GABA₁₅₀ were significantly higher values than those of fish fed CON and there was no significant difference compare to fish fed GABA₂₀₀, and there was no interaction effect ($P>0.05$). SOD is an enzyme that acts as an antioxidant defense mechanism in almost all of cell exposed to oxygen. As a result of the analysis, significantly higher values were obtained in the experimental group to which GABA was added. It was found that GABA decrease antioxidant stress and increases the activity of antioxidant enzymes in fish as found in humans, rats, and broilers, similar to those found in whiteleg shrimp and Nile tilapia. MPO is an enzyme mainly contained in neutrophils. It catalyzes the reaction to produce hypochlorous acid (HOCl) from hydrogen peroxide (H₂O₂) and chloride ion (Cl⁻), and has antibacterial and antiviral effects. As a result of the analysis, significantly higher values were found in fish fed GABA₂₀₀ and GABA₂₅₀. Lysozyme activity is an enzyme that destroys the cell wall of bacteria and plays an important part in the innate immune system. As a result of the analysis, fish fed GABA₁₅₀ has a significantly higher value. This confirmed that the treatment with GABA or GABAergic drugs is related to the results of recent studies that improve macrophage maturation, autophagy activation, antibacterial response to bacterial infection, and enhance the host's innate immune response (Keshav, S., et al., 1991). In summary, it could be seen that the innate immune response was enhanced in the experimental group to which GABA was added without the rearing density. However, different trends were shown depending on the amount of GABA added to the feed, so the amount of GABA added according to the fish species and size should be identified. The results refer to the Table 7 and Figure 10, 11, 12.

Challenge test

After 8 weeks of feeding trial, challenge test with pathogenic bacteria (*Edwardsiella tarda* 1×10^7 CFU/mL) for 15 days showed significantly higher cumulative survival rate for those of fish fed GABA₁₅₀, GABA₂₀₀, and GABA₂₅₀ diets compared to these of fish fed CON diet ($P < 0.05$). But there is no fully established mechanism for the interaction of the nervous and immune systems. In particular, it has not been studied mainly in fish. However, according to a recently published paper, the effect of enhancing immunity through macrophages and autophagy through in vivo GABA and GABAergic is gradually revealed. In the end, it is judged that it showed a high cumulative survival rate. (Kim, J. K., et al., 2018; Sasai, M et al., 2017; Auteri, M et al., 2015) The results refer to the Figure 13.



Tables and figures

Table 1. Formulation of experimental diets

Ingredients	Diets %			
	CON	GABA ₁₅₀	GABA ₂₀₀	GABA ₂₅₀
Anchovy FM¹	50.00	50.00	50.00	50.00
Soybean meal¹	15.00	15.00	15.00	15.00
Wheat flour²	13.70	13.83	13.70	13.58
Squid Liver Powder¹	4.00	4.00	4.00	4.00
Meat & Bone meal¹	4.00	4.00	4.00	4.00
Poultry by product¹	4.00	4.00	4.00	4.00
Fish oil¹	4.00	4.00	4.00	4.00
Lecithin¹	0.50	0.50	0.50	0.50
Betaine¹	1.00	1.00	1.00	1.00
Taurine¹	0.50	0.50	0.50	0.50
Mono calcium phosphate³	0.50	0.50	0.50	0.50
Mineral mix⁴	1.00	1.00	1.00	1.00
Vitamin mix⁵	1.00	1.00	1.00	1.00
Choline³	0.30	0.30	0.30	0.30
Cellulose¹	0.50			
Cellulose (included 40,000 ppm of GABA)⁶	0.000	0.375	0.500	0.625
Total	100.00	100.00	100.00	100.00

¹The Feed Co., Seoul, Rep Korea

²Samhwa Flourmils Co., Seoul, Rep Korea

³Sigma-Aldrich Korea Co., Seoul, Rep Korea

⁴Mineral 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; Cancium iodate, 0.75; Magnesium sulfate, 80.20; Aluminum Hydroxide, 0.75

⁵Vitamin premix (as mg/kg premix): A, 1000000 IU; D, 200000 IU; E, 10000; B1, 2000; B6, 1500; B12, 10; C, 10000; Calcium pantotenic acid, 5000; Nicotinic acid 4500; B-Biotin 10; Choline chloride, 30000; Inositol, 5000.

⁶Milae Bioresources Co., Seoul, Rep Korea

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added; GABA₂₅₀: GABA 250 mg/kg added

Table 2. proximate analysis (dry matter basis) of the experimental diets¹

Diets (%)	CON	GABA ₁₅₀	GABA ₂₀₀	GABA ₂₅₀
Moisture	7.65 ^{ns}	7.16	7.90	7.95
Protein	52.90 ^{ns}	52.10	52.30	52.00
Lipid	10.19 ^{ns}	10.20	10.13	10.09
Ash	12.48 ^{ns}	12.60	12.42	12.42

¹Values are means from triplicate groups where the values in each row with different superscripts are significantly different. ($P < 0.05$)

ns: no significant different

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added; GABA₂₅₀: GABA 250 mg/kg added



Table 3. Actual percentage of GABA according to HPLC for the experimental diets

Diet	CON	GABA₁₅₀	GABA₂₀₀	GABA₂₅₀
GABA (%)	0.006392	0.02313	0.029159	0.03239
GABA (mg/kg)	63.92	231.30	291.59	323.90

GABA: Gamma aminobutyric acid

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added;

GABA₂₅₀: GABA 250 mg/kg added



Table 4. Whole-body proximate composition (as is %) of olive flounder¹

Density	ND				HD				Nested ANOVA, P-value		
Diet	CON	GABA ₁₅₀	GABA ₂₀₀	GABA ₂₅₀	CON	GABA ₁₅₀	GABA ₂₀₀	GABA ₂₅₀	Density	GABA	D×G
Moi ²	79.6 ^{ns}	80.3	79.7	79.0	79.3	79.1	79.4	79.7	0.154	0.623	0.620
C. Pro ³	15.2 ^{ns}	14.6	15.2	15.4	15.2	15.6	15.3	14.9	0.064	0.406	0.898
C. Lip ⁴	1.17 ^{ns}	1.18	1.00	1.06	1.16	1.16	1.19	1.13	0.370	0.729	0.177
C. Ash ⁵	3.54 ^{ns}	3.84	3.87	3.81	4.04	3.71	3.89	3.89	0.109	0.643	0.623

¹Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different. ($P<0.05$)

²Moi = Moisture

³C. Pro = Crude Protein

⁴C. Lip = Crude Lipid

⁵C. Ash = Crude Ash

ND: Normal density; HD: High density

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added; GABA₂₅₀: GABA 250 mg/kg added

Table 5. Growth performance and feed utilization¹

Density	ND				HD				Nested ANOVA, P-value		
Diet	CON	GABA ₁₅₀	GABA ₂₀₀	GABA ₂₅₀	CON	GABA ₁₅₀	GABA ₂₀₀	GABA ₂₅₀	Density	GABA	D×G
WG²	145.21 ^a	149.03 ^a	135.66 ^a	139.48 ^a	129.07 ^b	120.93 ^b	122.97 ^b	123.26 ^b	<0.001	0.292	0.325
SGR³	1.87 ^a	1.90 ^a	1.78 ^a	1.82 ^a	1.73 ^b	1.65 ^b	1.67 ^b	1.67 ^b	<0.001	0.302	0.348
FE⁴	84.10 ^{ns}	82.27	73.40	81.80	77.40	73.30	70.70	77.13	0.070	0.213	0.891
PER⁵	1.59 ^{ns}	1.58	1.42	1.57	1.46	1.41	1.37	1.48	0.067	0.324	0.896

¹Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different. ($P<0.05$)

²Weight gain (WG, %) = [final weight - initial weight] × 100 / initial weight

³Specific growth rate (SGR, % / day) = [loge final weight - loge initial weight] × 100 / days

⁴Feed Efficiency (FE, %) = [wet weight gain / dry feed intake] × 100

⁵Protein efficiency Ratio (PER) = Wet weight gain / Protein intake

ns: no significant different

ND: Normal density; HD: High density

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added; GABA₂₅₀: GABA 250 mg/kg added

Table 6. Blood Chemistry of olive flounder (Biochemical characteristics)¹

Density	ND				HD				Nested ANOVA, P-value		
Diet	CON	GABA ₁₅₀	GABA ₂₀₀	GABA ₂₅₀	CON	GABA ₁₅₀	GABA ₂₀₀	GABA ₂₅₀	Density	GABA	D×G
GOT²	17.7 ^{ns}	18.3	19.0	18.7	16.7	16.0	16.7	16.0	0.053	0.959	0.936
GPT³	6.7 ^{ns}	7.0	6.0	6.3	6.7	5.0	6.3	6.0	0.188	0.605	0.149
GLU⁴	12.7 ^{ns}	13.0	11.7	13.3	12.0	14.3	16.0	11.7	0.379	0.560	0.145
Cortisol⁵	2.6 ^b	1.7 ^b	1.8 ^b	1.7 ^b	4.2 ^a	5.9 ^a	5.8 ^a	5.0 ^a	0.002	0.980	0.739
GABA⁶	166 ^a	220 ^b	239 ^c	302 ^d	175 ^a	214 ^b	247 ^c	293 ^d	0.790	<0.001	0.059

¹Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different. ($P<0.05$)

²GOT: Glutamic oxaloacetic transaminase (U L⁻¹)

³GPT: Glutamic pyruvic transaminase (U L⁻¹)

⁴GLU: Glucose (mg dL⁻¹)

⁵Cortisol: Cortisol in blood (ng mL⁻¹)

⁶GABA: gamma aminobutyric acid in blood (pg mL⁻¹)

ns: no significant different

ND: Normal density; HD: High density

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added; GABA₂₅₀: GABA 250 mg/kg added

Table 7. Non-specific immune responses of olive flounder¹

Density	ND				HD				Nested ANOVA, P-value		
Diet	CON	GABA ₁₅₀	GABA ₂₀₀	GABA ₂₅₀	CON	GABA ₁₅₀	GABA ₂₀₀	GABA ₂₅₀	Density	GABA	D×G
SOD²	16.24 ^b	23.15 ^a	18.78 ^{ab}	21.79 ^a	12.66 ^b	21.09 ^a	18.22 ^{ab}	22.05 ^a	0.462	0.046	0.906
MPO³	1.45 ^b	1.20 ^b	1.87 ^a	1.44 ^a	1.23 ^b	1.44 ^b	1.40 ^a	1.81 ^a	0.818	0.015	0.006
Lysozyme⁴	0.15 ^b	0.39 ^a	0.10 ^b	0.25 ^b	0.17 ^b	0.39 ^a	0.26 ^b	0.14 ^b	0.542	<0.001	0.520

¹Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different. ($P<0.05$)

²SOD: Superoxide dismutase activity (% inhibition)

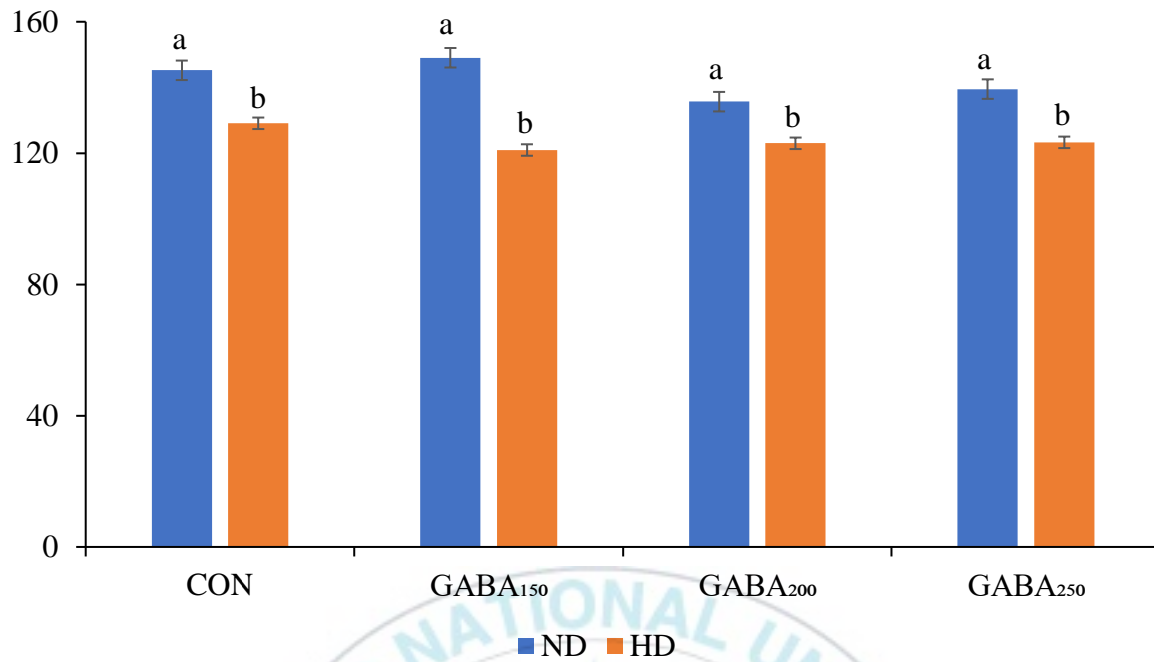
³MPO: Myeloperoxidase (OD at 450nm)

⁴Lysozyme: Lysozyme activity (U mL⁻¹)

ND: Normal density; HD: High density

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added; GABA₂₅₀: GABA 250 mg/kg added

Fig 1. Weight gain (WG, %)¹



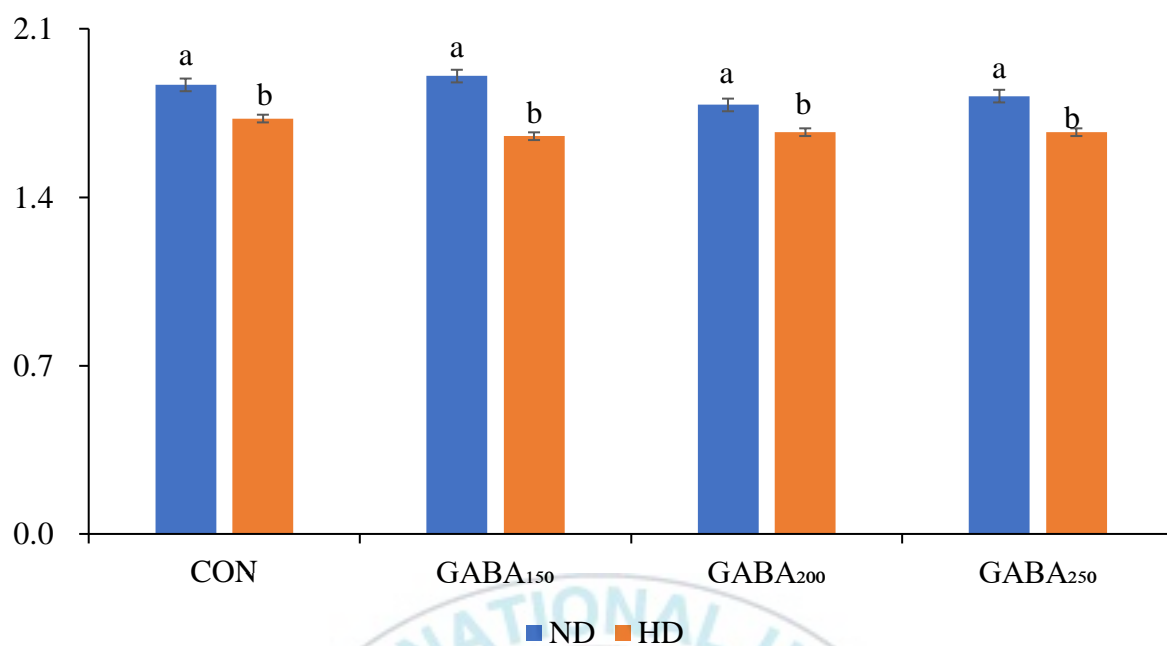
Nested ANOVA, P-value		
Density	GABA	D×G
<0.001	0.292	0.325

¹Values are mean of triplicate samples. Values with different letter are significantly different according to two-way ANOVA ($P=0.05$) and post-hoc test (LSD).

ND: Normal density; HD: High density

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added; GABA₂₅₀: GABA 250 mg/kg added

Fig 2. Specific growth rate (SGR, %/day)¹



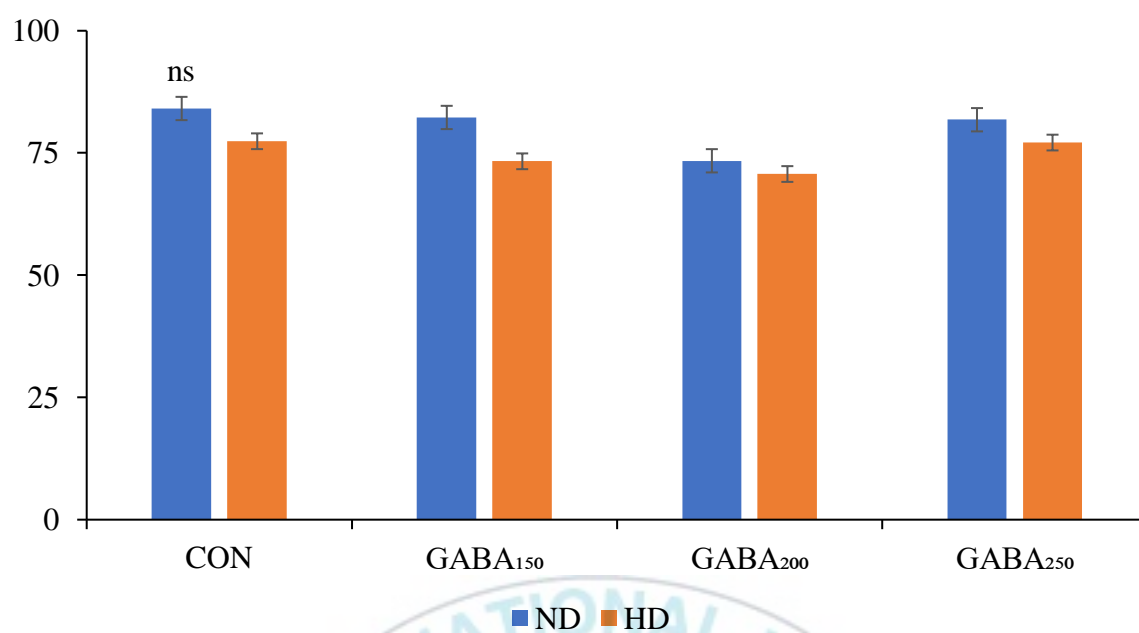
Nested ANOVA, P-value		
Density	GABA	D×G
<0.001	0.302	0.348

¹Values are mean of triplicate samples. Values with different letter are significantly different according to two-way ANOVA ($P=0.05$) and post-hoc test (LSD).

ND: Normal density; HD: High density

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added; GABA₂₅₀: GABA 250 mg/kg added

Fig 3. Feed efficiency (FE, %)¹



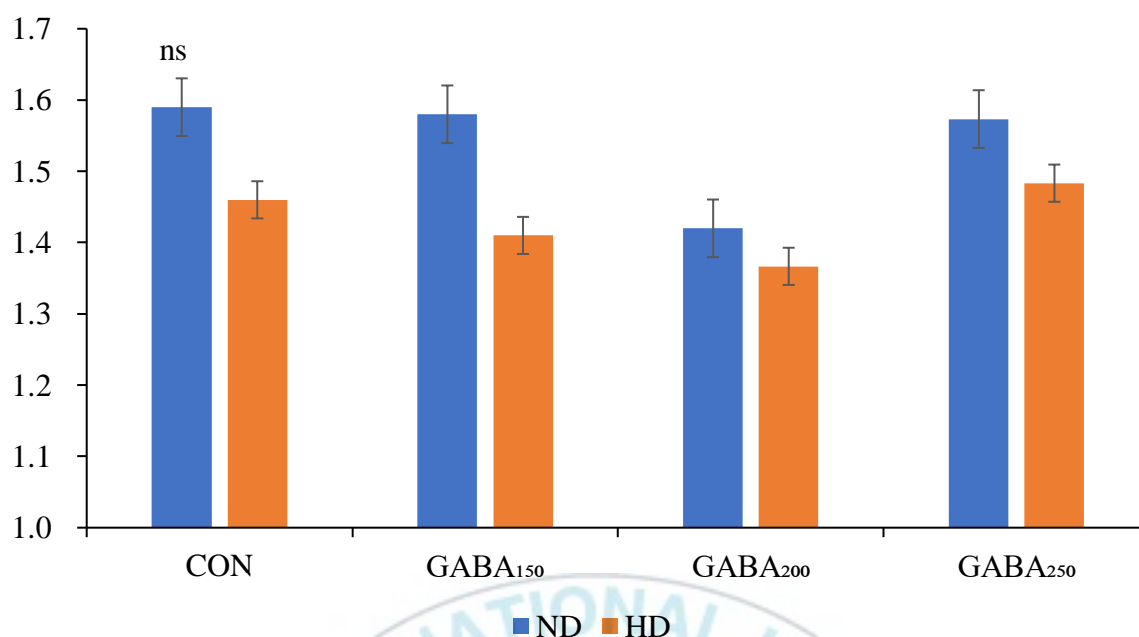
Nested ANOVA, P-value		
Density	GABA	D×G
0.070	0.213	0.891

¹Values are mean of triplicate samples. Values with different letter are significantly different according to two-way ANOVA ($P=0.05$) and post-hoc test (LSD).

ND: Normal density; HD: High density

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added; GABA₂₅₀: GABA 250 mg/kg added

Fig 4. Protein efficiency Ratio (PER) ¹



Nested ANOVA, P-value		
Density	GABA	D×G
0,067	0.324	0.896

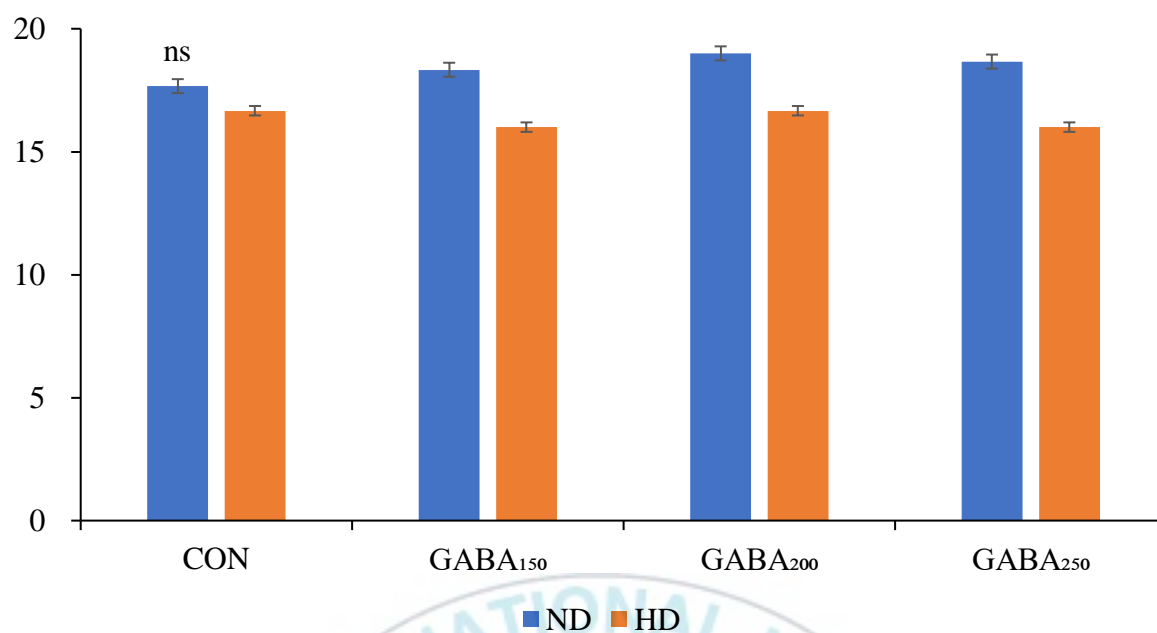
¹Values are mean of triplicate samples. Values with different letter are significantly different according to two-way ANOVA ($P=0.05$) and post-hoc test (LSD).

ND: Normal density; HD: High density

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added;

GABA₂₅₀: GABA 250 mg/kg added

Fig 5. Glutamic oxalacetic transaminase (GOT, U mL⁻¹)¹



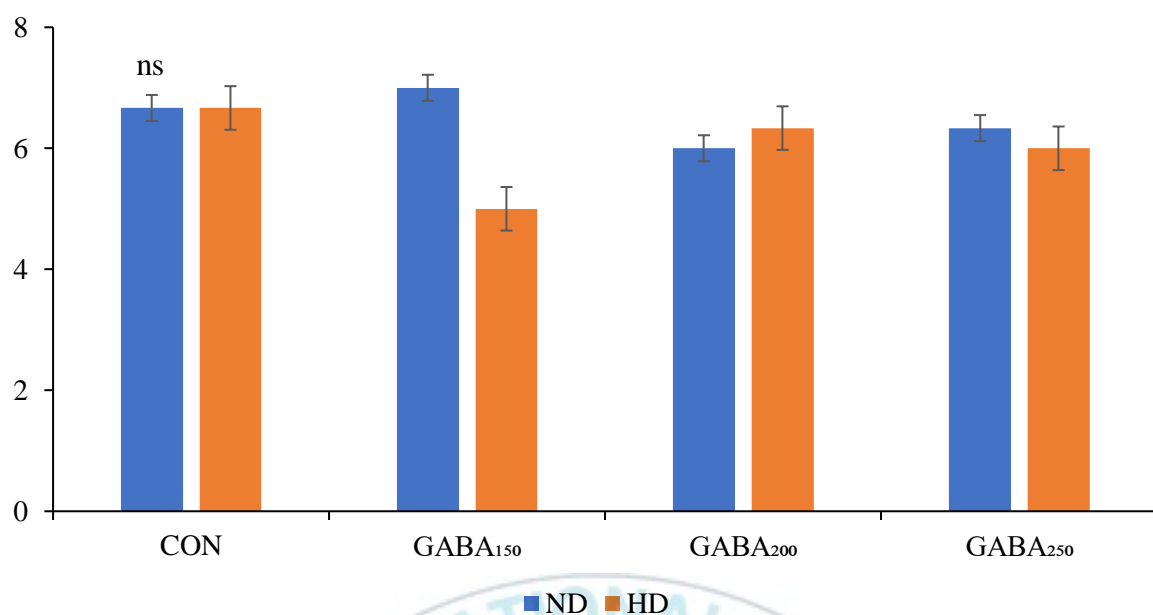
Nested ANOVA, P-value		
Density	GABA	D×G
0.053	0.959	0.936

¹Values are mean of triplicate samples. Values with different letter are significantly different according to two-way ANOVA ($P=0.05$) and post-hoc test (LSD).

ND: Normal density; HD: High density

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added; GABA₂₅₀: GABA 250 mg/kg added

Fig 6. Glutamic pyruvate transaminase (GPT, U mL⁻¹)¹



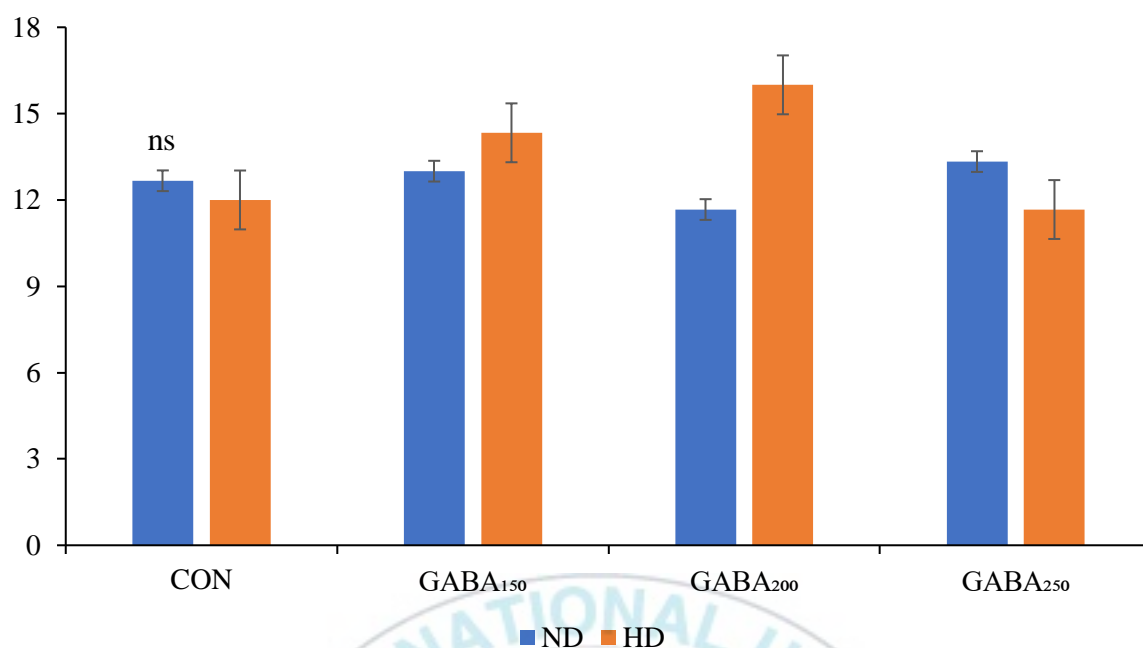
Nested ANOVA, P-value		
Density	GABA	D×G
0.188	0.605	0.149

¹Values are mean of triplicate samples. Values with different letter are significantly different according to two-way ANOVA ($P=0.05$) and post-hoc test (LSD).

ND: Normal density; HD: High density

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added; GABA₂₅₀: GABA 250 mg/kg added

Fig 7. Glucose (GLU mg dL⁻¹)¹



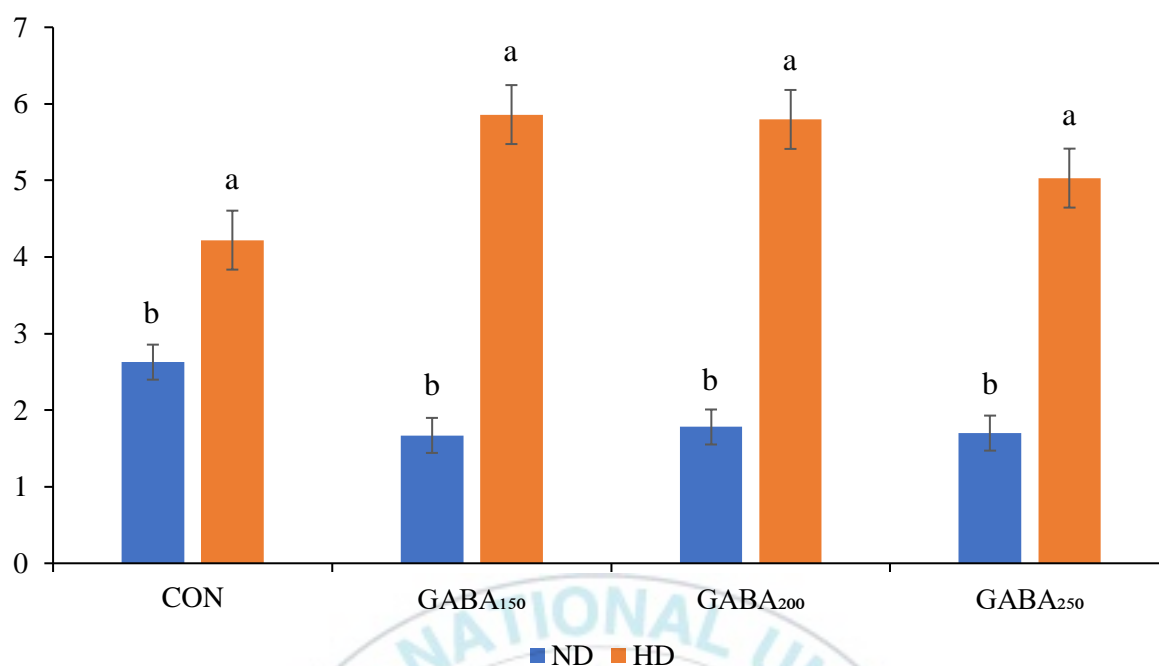
Nested ANOVA, P-value		
Density	GABA	D×G
0.379	0.560	0.145

¹Values are mean of triplicate samples. Values with different letter are significantly different according to two-way ANOVA ($P=0.05$) and post-hoc test (LSD).

ND: Normal density; HD: High density

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added; GABA₂₅₀: GABA 250 mg/kg added

Fig 8. Cortisol (ng mL⁻¹)¹



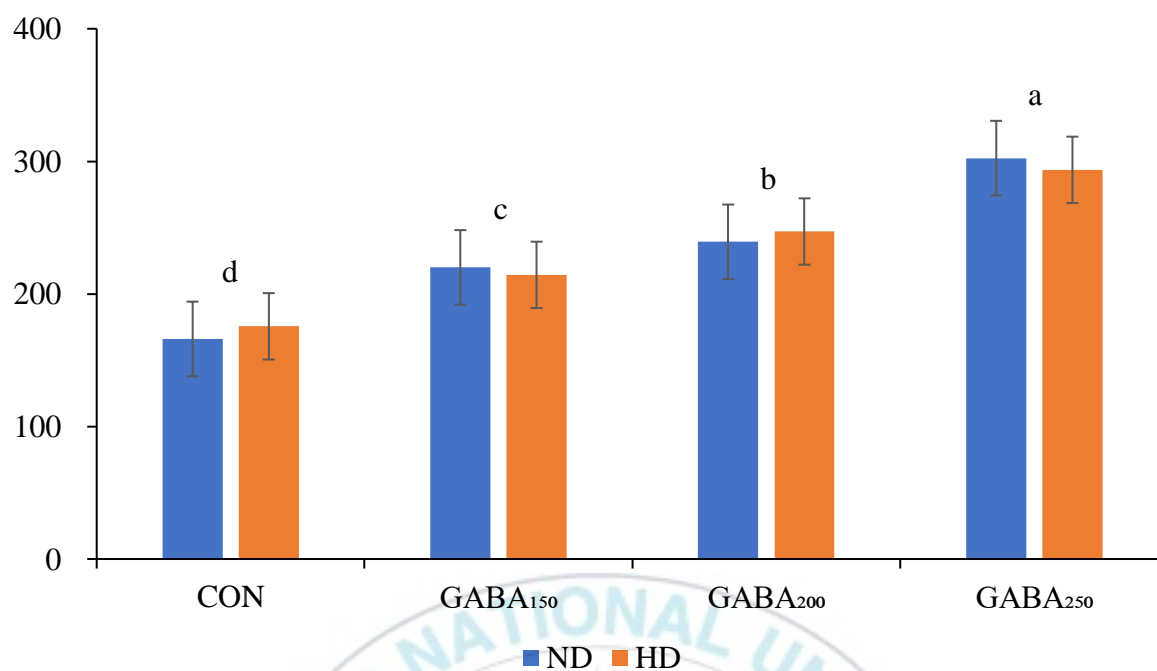
Nested ANOVA, P-value		
Density	GABA	D×G
0.002	0.980	0.739

¹Values are mean of triplicate samples. Values with different letter are significantly different according to two-way ANOVA ($P=0.05$) and post-hoc test (LSD).

ND: Normal density; HD: High density

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added; GABA₂₅₀: GABA 250 mg/kg added

Fig 9. GABA in blood (pg mL^{-1})¹



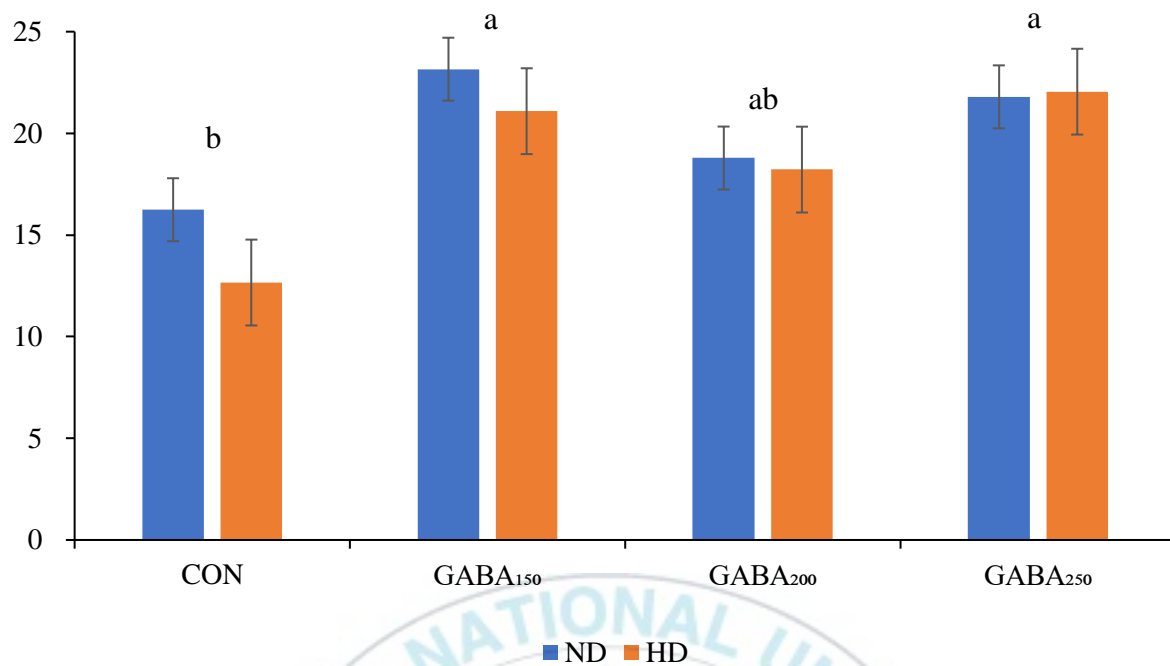
Nested ANOVA, P-value		
Density	GABA	D×G
0.790	0.000	0.059

¹Values are mean of triplicate samples. Values with different letter are significantly different according to two-way ANOVA ($P=0.05$) and post-hoc test (LSD).

ND: Normal density; HD: High density

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added; GABA₂₅₀: GABA 250 mg/kg added

Fig 10. Superoxide dismutase activity (SOD, % inhibition)¹



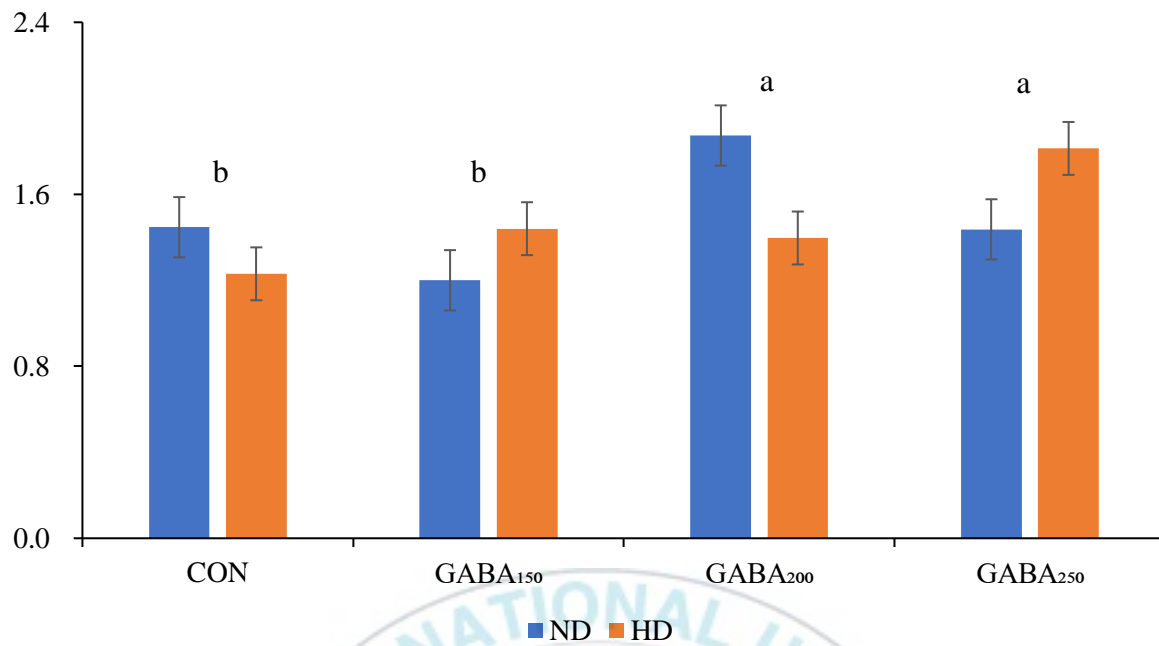
Nested ANOVA, P-value		
Density	GABA	D×G
0.462	0.046	0.906

¹Values are mean of triplicate samples. Values with different letter are significantly different according to two-way ANOVA ($P=0.05$) and post-hoc test (LSD).

ND: Normal density; HD: High density

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added; GABA₂₅₀: GABA 250 mg/kg added

Fig 11. Myeloperoxidase (MPO, absorbance at 450 nm) ¹



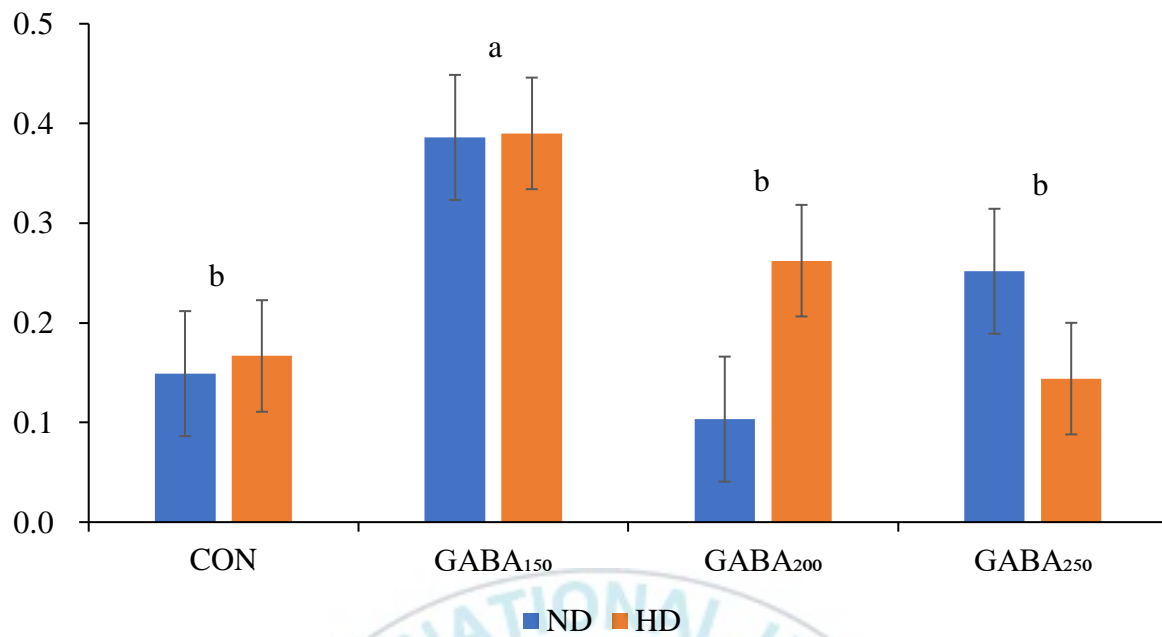
Nested ANOVA, P-value		
Density	GABA	D×G
0.818	0.015	0.006

¹Values are mean of triplicate samples. Values with different letter are significantly different according to two-way ANOVA ($P=0.05$) and post-hoc test (LSD).

ND: Normal density; HD: High density

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added; GABA₂₅₀: GABA 250 mg/kg added

Fig 12. Lysozyme activity (U mL^{-1})¹



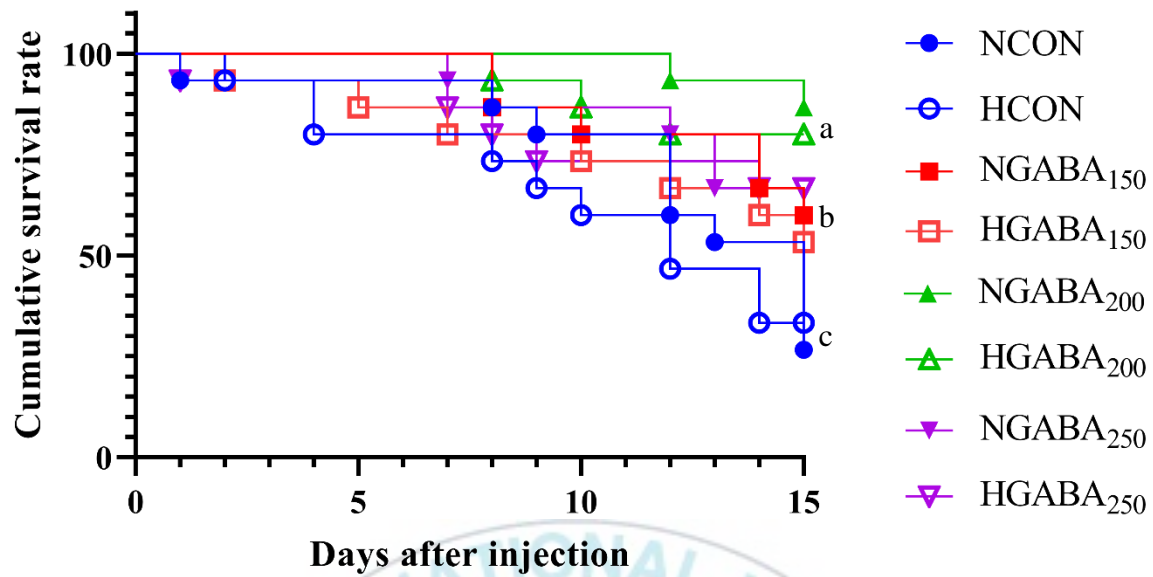
Nested ANOVA, P-value		
Density	GABA	D×G
0.542	<0.001	0.052

¹Values are mean of triplicate samples. Values with different letter are significantly different according to two-way ANOVA ($P=0.05$) and post-hoc test (LSD).

ND: Normal density; HD: High density

CON: Control; GABA₁₅₀: GABA 150 mg/kg added; GABA₂₀₀: GABA 200 mg/kg added; GABA₂₅₀: GABA 250 mg/kg added

Fig 13. Challenge Test with *Edwardsiella tarda* (1×10^7 CFU/mL)¹



Nested ANOVA, P-value		
Density	GABA	D×G
1,000	<0.001	0.698

¹Cumulative survival percentage of olive flounder fed four different experimental diet and two different stocking density for 8 weeks and challenged with *Edwardsiella tarda* (1×10^7 CFU/mL) for 15 days.

NCON: Normal Density + CON diet

HCON: High Density + CON diet

NGABA₁₅₀: Normal Density + GABA₁₅₀

HGABA₁₅₀: High Density + GABA₁₅₀

NGABA₂₀₀: Normal Density + GABA₂₀₀

HGABA₂₀₀: High Density + GABA₂₀₀

NGABA₂₅₀: Normal Density + GABA₂₅₀

HGABA₂₅₀: High Density + GABA₂₅₀

IV. Conclusions

Rearing density has always been an important topic in the aquaculture industry. Efforts to create high production per area are ongoing and various aquaculture technologies are developing. However, the high stocking density increases the risk of mass mortality as the stress on fish increases, low growth performance, and disease susceptibility increases. In order to prevent this, it is necessary to develop feed additives to reduce stress, promote immune response and disease resistance. The gamma aminobutyric acid used in this experiment is an inhibitory neurotransmitter and is a feed additive already used in livestock industries such as poultry, pig and cow farming. Therefore, this study was conducted to see the effect of enhancing fish growth and immunity by adding dietary GABA under density stress. These results indicated that dietary gamma aminobutyric acid (GABA) did not affect olive flounder growth performance but could have beneficial effect on immune responses and disease resistance. However, in the non-specific immune response, it showed a positive effect according to the different GABA level. It is necessary to experiment by dividing the GABA level more accurately in future experiments. Lastly, as mentioned in the body of this paper, the Gut-brain axis and microbiota is a model that helps to establish explanations for the beneficial effects found not only in GABA, but other microbial metabolites in the gut of fish. Though this system provides an excellent basis upon which to assert a mechanism for GABA's systemic effects, the scope of the current trial focuses primarily on GABA's general effects on growth and immunity that can be assessed by using tests common to short term feeding trials with juvenile fish. Therefore, in future studies, it is necessary to observe the exact activity of neurons or immune cells, and also to study other environmental stresses such as temperature stress.

V. References

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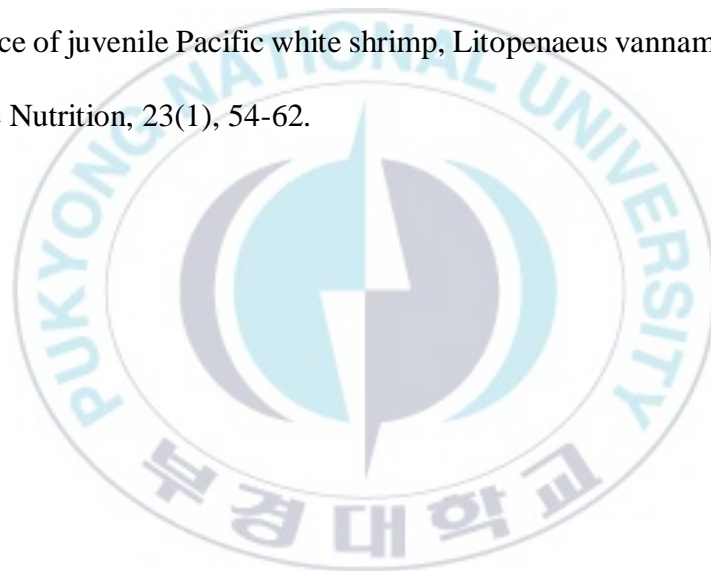
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