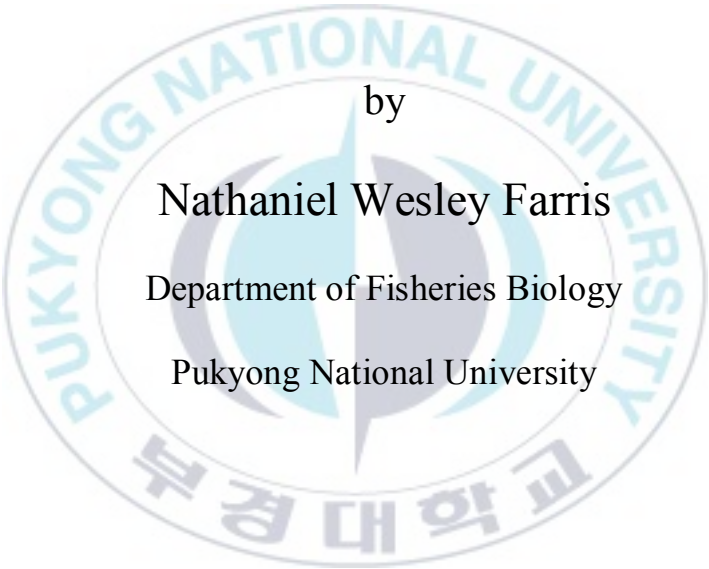
Thesis for the Degree of Master of fisheries science

Effects of different dietary γ-aminobutyric acid levels on growth, immunity and digestive enzyme activity in juvenile olive flounder, *Paralichthys olivaceus*



by

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February 2020

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A thesis submitted in partial fulfillment of the requirements

for the Degree of

Master of Fisheries Science

at the Department of Fisheries Biology, Graduate School

Pukyong National University

February 2020

내이선 패리스 의 수산학석사 학위논문을 인준함

2020 년 2 월 21 일

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A dissertation

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February 21st 2020

Table of Contents

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Abstract

An 8-week feeding trial was conducted to determine an optimal dietary level for γ-Aminobutyric acid (GABA) and its effects in juvenile olive flounder, (*Paralichthys olivaceus*). A total of 630 fish with an average weight of 4.90 ± 0.1 g were stocked by groups of 30 fish into 21, 40 L (0.153 m^2) seawater tanks resulting in a 38% coverage area (0.960 kg/m^2), and assigned randomly to seven triplicate dietary treatment groups; a basal diet without supplemental GABA as a negative control containing 92 mg kg^{-1} endogenous GABA (CON_{92}), a positive control composed of CON_{92} + 4g kg^{-1} oxytetracycline (OTC), and five other diets prepared by adding 50, 100, 150, 200, and 250 mg kg^{-1} GABA to the diet resulting in 154 (GAB_{154}), 229 (GAB_{229}), 282 (GAB_{282}), 327 (GAB_{327}) and 352 mg kg^{-1} (GAB_{352}) respectively (named according to High Performance Liquid Chromatography results). At the end of the trial, results showed that the weight gain (WG) and specific growth rate of fish fed the GAB_{229} and GAB_{282} diets were significantly higher (P<0.05) than those of fish fed the CON_{92}, OTC, GAB_{154}, and GAB_{352} diets. However, there were no significant differences among fish fed the GAB_{229} and GAB_{327} diets. Intestinal amylase activity among fish fed the OTC and GAB_{282} diets had significantly higher enzyme activity than all other GABA supplemented diets. Lysozyme activity among fish fed the GAB_{154}, GAB_{229}, GAB_{282}, and GAB_{327} diets were significantly higher than those of fish fed the CON_{92} diet. At the end of the *Streptococcus iniae* challenge test (day 12) the cumulative percent survival among fish fed the OTC, GAB_{229}, and GAB_{282} diets were significantly higher than all other diets. However, there were no significant differences among fish fed the OTC, GAB_{229}, and GAB_{282} diets. There were

no significant differences among fish fed the GAB_{154}, GAB_{327}, and GAB_{352} diets, yet they had significantly higher cumulative survival than the fish fed the CON_{92} diet. The results of growth, intestinal amylase activity, lysozyme activity, and bacterial challenge indicated that GABA supplementation under the normal stocking density could replace dietary OTC, and improve pathogen resistance as well as growth and intestinal amylase activity in juvenile olive flounder. Based on the quadratic regression (polynomial) analysis of WG, the optimal dietary level could be 236.9 mg kg^{-1}, which results in a supplementation level of 145 mg kg^{-1}.

요약문

치어기 넙치 사료 내 아미노뷰티르산 (Gamma aminobutyric acid, GABA)의 첨가효과를 평가하기 위해 8 주간의 사육실험을 실시하였다. 평균 어체중 4.90 ± 0.1g (mean±SD)인 치어기 넙치를 21 개의 수조 (40L, 0.153 m^2) 에 각 실험구별 30 미씩 무작위로 배치하였다 (0.960kg/m^2). 실험사료는 GABA 가 첨가되지 않은 기초사료 (GABA 92, 기존함량 92mg/kg), 기초사료에 항생제 4g/kg (OTC), 기초사료에 GABA 50 (GAB_{154}), 100 (GAB_{229}), 150 (GAB_{282}), 200 (GAB_{327}), 250 (GAB_{352}) mg/kg 첨가하여 설계하였다. 사육실험 종료 후, 증체율(weight gain)과 일간성장률(specific growth rate)에 있어서 GAB_{229} 와 GAB_{282} 실험구가 CON_{92}, GAB_{154}, GAB_{352}, OTC 실험구에 비해 유의적으로 높게 나타났으나($P<0.05$). GAB_{229} 와 GAB_{327} 실험구간에는 유의적인 차이를 나타내지 않았다($P>0.05$). 아밀라아제 소화효소 분석결과, GAB_{282} 와 OTC 실험구가 타 실험구에 비해 유의적으로 높은 활성을 나타냈다($P<0.05$). Lysozyme activity 에 있어서는 GAB_{154}, GAB_{229}, GAB_{282} 및 GAB_{327} 실험구가 CON_{92} 실험구에 비해 유의적으로 높았으며($P<0.05$), *Streptococococcus iniae* 를 접종하여 12 일간 공격실험을 진행한 결과, OTC, GAB_{229}, GAB_{282} 실험구의 생존율이 타 실험구에 비해 유의적으로 높게 나타났으나($P<0.05$), GAB_{154}, GAB_{327}, GAB_{352} 를 실험구간에는 유의적인 차이가 없었다($P>0.05$). 따라서, WG 을 토대로 한 회귀 분석 (polynomial) 결과 치어기 넙치 사료 내 적정 GABA 첨가량은 145mg/kg (GABA 236)이며, GABA 적정 첨가 시 성장, 소화효소, 면역 및 질병 내병성의 효과뿐만 아니라 항생제 대체가 가능할 것으로 판단된다.

List of Figures

List of Tables

1. Introduction

Olive flounder, also known as Japanese flounder or bastard halibut, is a marine demersal, oceanodromous flat fish species of high market value in East Asia. It is native to the temperate and subtropical waters of the Western Pacific, and can be found from the far eastern coast of Russia down to the warm waters of the South China sea with its highest concentrations occurring in the waters just off the Southern tip of the Korean peninsula, Jeju Island, and the Japanese archipelago. Olive flounder is highly prized for its tender fillet, often served as "sashimi" or "Hwei" in the culinary traditions of Japan and Korea respectively. Even though this species is a very important fishery, aquaculture has replaced the capture industry as the primary source of production over the last 40 years. In fact, while landings of olive flounder have seldom exceeded 11,000 mt since the 1980s, commercial aquaculture production has been in steady growth and development since its inception in the late 1960s in Japan (Harda et al., 1966). While Japan's production peaked in 1997 at 8,583 mt and has entered into a steady decline, Korea's industry sky rocketed in the early 2000s to a height of 54,674 mt in 2009, with the most recent figures being 43,507 mt in 2017 (FAO 2017). As the industry grows, certain problems have begun to manifest themselves such as a need to improve the efficacy of feed formulation, reduction of fishmeal, and improvement in immune functionality to ward off disease. There have been many trials that have focused on a wide array of feed additives to help ameliorate these difficulties. One feed additive that has been receiving increasing interest is γ-Aminobutyric acid (GABA). GABA, also known as 3-Carboxypropylamine, 4-Aminobutanoic acid, or Piperidic acid, is a non-proteogenic amino acid (or non-α amino acids) with the chemical formula $C_4H_9NO_2$ and a molecular weight of 103.12 g mol^{-1}. GABA is synthesized from glutamate via decarboxylation by glutamate decarboxylase (GAD) with vitamin

B_6 in the form of Pyridoxal 5'-phosphate (PLP) as a coenzyme. It's appearance can be described as a beige to light brown powder that is soluble in water and heat stable at temperatures less than 80°C for durations of less than 15 minutes (Khan et al, 2015) and has a melting point of 153-155 °C .

Historically, much of the literature on GABA has focused on its function in brain chemistry, due to it's essential function in the regulation of the nervous system. Thus, for nearly 70 years GABA has been the subject of intense research. GABA has been found to serve many important biochemical functions across all domains of life from single cell organisms to human beings (Hulme et al, 1950; Awapara et al, 1950; Graham et. al, 1970; Fugelli, K. 1970; Morse et al, 1979; Wilkinson et al, 1983; Roseth & Fonnum 1995; Kinnersley & Turano 2000;Watanabe et al, 2002; Finalti et al, 2007; Strandwitz et al, 2019; Temu et al, 2019). One of the most important roles of GABA in vertebrates revolves around its role in the regulation of neuronal excitability and synaptic transmission by inhibition of the action potential (McCormick, D. A. 1989). This is done in concert with glutamate, which serves as the principle excitatory neurotransmitter in what is known as the glutamine-glutamate/GABA cycle (Walls et al, 2015). This makes GABA an important topic of study across many disciplines in the field of biology with promising applications in the field of nutrition.

It has long been believed that oral administration of GABA could produce anxiolytic effects in line with those produced by endogenous GABA production in neural tissue (Abdou et. al, 2006) and even a reduction in systolic blood pressure (Shizuka, F. et al, 2004). Also, since GABA is a metabolite of probiotic bacterial metabolism in the gut, there have been a few important studies to assess its effects on the microbiota and digestive health (Dhakal et. al, 2012; Mika & Fleshner 2016; Strandwitz et al, 2018; Strandwitz et. al, 2019;). So far there have been only a few studies

that have investigated the effects of GABA in the diet of fin fish, yet the results have been promising. For example, in a recent study by Temu et. al, (2019) juvenile Nile tilapia receiving a dose of 144 mg Kg^{-1} resulted in significantly improved weight gain (WG), and with a dose of 197 mg kg^{-1}, significantly improved superoxide dismutase activity (SOD). In this trial the optimal level was calculated to be 158 mg kg^{-1}. Another trial by Wu et. al, (2016), juvenile grass carp fed GABA supplemented feeds also found that growth and SOD activity were significantly increased. In this case the optimal dietary level for juveniles was determined to be 87.5 mg kg^{-1} in the diet. The primary benefits for fin fish appear to be improvements in growth and non-specific immunity, yet the exact mechanism is very hard to determine due to its multiple avenues of action, GABA contents in ingredients, and limited knowledge of its effects in the digestive tract of aquatic organisms. Some have hypothesized that the action could be, at least in part, due to direct effects on the central nervous system GABAergic signaling pathway, however there has been a bit of a controversy as to if GABA can pass through the blood brain barrier (BBB) to any significant extent, or whether or not dietary GABA can produce any noticeable effect once it has successfully crossed the BBB. It is important to note that GABA transporters have been identified in the BBB (Takanaga et. al, 2001), but the efflux of GABA from the brain is approximately seventeen times higher than the influx (Kakee et. al, 2001; Boonstra et. al, 2015). Still, GABAergic receptors have been found in multiple non-neural tissues (Ong & Kerr 1990) and it has been found to serve functions in the immune system, the peripheral nervous system (PNS), and the enteric nervous system (ENS) (Kerr & Ong 1984). It is believed that the interface between the CNS and the PNS, also known as the gut-brain axis, largely via the vagal nerve (Carabotti et. al, 2015), may account for the influence on the nervous system following GABA ingestion despite impediments caused by GABA's BBB impermeability.

Even though the number of studies in this area are limited among aquatic species, it has attracted the attention of feed manufacturers, perhaps, impart due to its recent success in terrestrial species such as swine and poultry (Chand et al, 2016) ; Li Y.H. et. al, 2015). Due to the recent interest in GABA as a functional feed additive, there is a need to establish the optimal dietary supplementation level of GABA in species important to the aquaculture industry.

An investigation into GABA as a dietary supplement rather than as an endogenous neurotransmitter is important since GABA is found at varying levels in dietary components. A better understanding of natural GABA content will also be important to accessing the origin of dietary effects of various feed ingredients along with taking into account the probiotics that produce GABA as a metabolite. A recent trial in our laboratory showed improved weight gain, flounder growth hormone expression, and non-specific immunity in juvenile olive flounder fed a GABA supplemented diet (276 mg kg^{-1} diet). The results from this trial provided a foundation upon which to determine an optimal GABA level in the present trial. Furthermore, the recent body of work on juvenile olive flounder performed in our lab has provided knowledge to determine an optimal stocking density suitable for the culture tanks used in this trial. Due to the above mentioned beneficial effects of GABA on immunity and growth, it is worthwhile to assess GABA's ability to reduce or replace the use of antibiotics such as oxytetracycline (OTC) in the diet of olive flounder when challenged with a bacterial pathogen.

Therefore a trial was carried out to establish an optimal dietary level for GABA in a practical olive flounder diet, determine its suitability as an antibiotic replacer, and its effects on growth, non-specific immunity, and physiology. This research is important due to GABA's promising applications and beneficial effects in finfish culture and aquafeed formulation.

2. Materials and Methods

Diet preparation and experimental design

Treatment groups were fed one of seven dietary treatments, briefly, a basal diet without GABA as a negative control (CON_{92}), a positive control composed of CON_{92} + 4g kg^{-1} oxytetracycline (OTC), and five other diets prepared by adding 50, 100, 150, 200, and 250 mg kg^{-1} GABA at the expense of wheat flour (Table 1). (GABA was procured from Milae Resources ML Co Ltd, and was analyzed by high pressure liquid chromatography (HPLC) which yielded a purity of 76.5%. All diets were formulated to account for the purity of the GABA used. All dry feed ingredients were combined and mixed using a planetary electric feed mixer electric mixer (HYVM-1214, Hanyoung Food Machinery, Republic of Korea), then fish oil was added slowly until completely homogenized. The feed mix was then moistened with water to approximately 25% of the dry feed weight. The moistened feed mix was then pelletized using a benchtop pelletizer (Baokyong Commercial Co., Busan, Republic of Korea) with a 2mm die which produced uniform strands of feed, broken into smaller pieces, then spread out on paper sheets in a room equipped with a dehumidifier. Feed was left to dry for three days, then stored at -20°C prior to use. Diets were then analyzed at the Feeds and Foods Nutrition Research Center (FFNRC) to determine proximate composition of crude protein, moisture, lipid, and ash.

Analysis of moisture, crude protein, lipid, ash and fiber of the experimental diets were performed using standard methods (AOAC 1995), with carbohydrate content being determined by Bomb Calorimetry (Parr Instrument Company 1351, Co., Illinois, USA). The energy values of nutrients were calculated on the basis of their physiological fuel values, i.e., 3.99 kcal g^{-1}(16.69 kJ g^{-1}) for proteins and carbohydrates, and 9.01 kcal g^{-1} (37.70 kJ g^{-1}) for lipids (Lee and Putnam, 1973). The diet samples and whole fish were dried to a constant weight at 105°C to determine their

moisture content. Ash was determined by incineration at 550°C. The crude lipid content was determined by Soxhlet extraction using the Soxtec system 1046 (Tecator AB, Hoganas, Sweden), and the crude protein content was determined by the Kjeldahl method (N×6.25) after acid digestion (Table 2).

An additional sample of each diet was sent to the National Instrumentation Center for Environmental Management College of Agriculture and Life Sciences at Seoul National University (Seoul 151-742, Korea) where the actual level of dietary GABA in the feed were determined (Table 3) via high pressure liquid chromatography (HPLC).

Experimental fish

The 8-week feeding trial was conducted at the Dept. of Marine Bio Materials & Aquaculture, Pukyong National University (PKNU), (Busan, Republic of Korea). Initially over 2,000 Juvenile olive flounder averaging 2.0 g in weight were purchased from SamBu farm, (Chungcheong province, Republic of Korea) , and brought to PKNU where they were carefully stocked into several 250L tanks, allowed to acclimatize to experimental conditions for 3 weeks, and fed a commercial starter diet to bring them up to the desired average initial weight of approximately 5.0g in weight, at which time they were fasted for one day prior to stocking. The overall behavior and appearance of the fish were noted as being exceptional. A total of 630 fish with an average weight of 4.90 ± 0.1 g were divided into 21 groups of 30 individuals, stocked into 40L (0.153 m^2) tanks with a flow rate of 2 L min^{-1} and supplied with air stones. The stocking density was an average of 147.13 g/tank at 0.962 kg/ m^2 approximating guidelines established by Bai & Lee (2010). This resulted in a starting percent coverage area (PCA) of 38%, as determined by analysis of the average total Body coverage area of the fish during stocking. Water temperature was maintained at approximately 20 ± 1°C (Iwata et al.,1994). Fish were checked several times daily

for mortality, any dead fish were weighed and feed was adjusted according to biomass feeding guidelines provided by the National Institute of Fisheries Science (NIFS). Biomass was calculated from the initial stocking density, then by estimation of growth over an 8 week period which was established in previous trial with the same species and apparatus. The average feed given daily was approximately 3.2% estimated biomass.

Sample collection and data analysis

At the end of the 8-week trial, fish were fasted for 24 hours prior to being moved to a shallow flat bottomed basins of water were the final number and weight of individuals per tank were recorded for calculation of the final weight (FW), weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER), and survival. In addition, when fish were placed in the basins, with standard 2x2cm graph paper, laminated to the bottom, a photograph was taken and later used to analyze the percent coverage area (PCA) of the respective tank. This was to be used as an additional measure of stocking density change from the original. These indices were calculated using the following equations:

WG (%) = (final wt. – initial wt.) × 100/initial wt.
SGR (%/day) = (ln (final wt.) − ln(initial wt.)) × 100/days of feeding
FE (%) = (final wt. – initial wt.) × 100/dry feed intake
PER = (final wt. – initial wt.)/protein intake
Survival rate (%) = (total fish − dead fish) × 100/survival fish
PCA = (total area of flounder blindside in cm^2 / total area of tank bottom in cm^2) x 100

Three fish from each tank were randomly selected, individually weighed, and then dissected to extract the liver and intestines for determination of the hepatosomatic index (HSI) and visceral somatic index (VSI). The same three specimens were kept after measurement and used for determination of whole body proximate composition (Table 5).

For Digestive Enzyme activity analysis, three fish were randomly collected from each treatment group and anesthetized with 2-phenoxyethanol (200 mg L^{-1} for 5–10 min), then intestines were removed, added to an assay buffer provided by each respective enzyme activity colorimetric assay kit (amylase and lipase) in proportions prescribed by the manufacturer (Bio Vision Incorporated CA USA), homogenized, and centrifuged for 10 minutes. The supernatant was then transferred to 1.5 mL microcentrifuge tubes and stored at -20°C prior to colorimetric analysis according to the manufacturer provided instructions.

Three additional fish from each treatment group were captured and then anesthetized as described above. Once fish were sedated, blood samples were taken without heparin and allowed to clot at room temperature for 30 minutes. The serum was then separated by centrifugation at 5000 × g for 10 min and stored at −70°C for the analysis of non-specific immune responses including lysozyme, myeloperoxidase (MPO) and superoxide dismutase (SOD) activities.

Lysozyme activity of the serum was determined by use of a turbidimetric assay using the methods described by Hultmark (1980) with slight modification. Briefly, *Micrococcus lysodeikticus* (0.75 mg mL^{-1}) was suspended in a sodium phosphate buffer (0.1 M, pH 6.4), 200 µL of suspension was then placed in each well of a 96 well microplate, and then 20 µL of serum was added. The reduction in absorbance of the samples were recorded at a wavelength of 570 nm after incubation at room temperature both initially (0 min) and at 30 minutes in a microplate reader (UVM 340, Biochrom, Cambridge, UK). A reduction in absorbance of 0.001 min^{-1} was regarded as one unit of lysozyme activity (Table 7).

MPO activity was measured in accordance with Quade and Roth (1997). Briefly, 20 µL of serum was diluted with HBSS (Hanks Balanced Salt Solution) without Ca_2^+ or Mg_2^+ (Sigma-Aldrich, USA) in a 96-well microplate. Then, 35 µL of 3,3′,5,5′-tetramethylbenzidine

hydrochloride (TMB, 20 mM) (Sigma-Aldrich, USA) and H_2O_2 (5 mM) were added. The color change reaction was stopped after 2 min by adding 35 μL of 4 M sulfuric acid. Finally, the optical density was read at a wavelength of 450 nm in a microplate reader (Table 7).

SOD activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using an SOD Assay Kit (Dojindo Laboratories, Kumamoto, Japan). Each endpoint assay was monitored by absorbance at a wavelength of 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 minutes of reaction time at 37 °C (Table 7).

An additional set of blood samples were collected (the same specimen) from the caudal vein with heparinized syringes and immediately deposited into 1.5 mL microcentrifuge tubes. Plasma was then separated by centrifugation at 5000 × g for 10 min and stored at −70°C for later determination of the blood plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP) and glucose (GLU).

A chemical analyzer (Fuji DRI-CHEM 3500i, Fuji Photo Film, Ltd., Tokyo, Japan) was used to determine the blood plasma levels of glucose (GLU) and total protein (TP) as well as the activities of AST and ALT (Table 8)

A bacterial challenge test was performed by Intraperitoneal injection with $1x10^8$ CFU *Streptococcus iniae* (*S. iniae*) after the end of the feeding trial in accordance with Hasan Md et al. 2018. Briefly, five fish from each dietary treatment groups were fasted 24 hours prior to being sedated by 2-phenoxyethanol, then given an intraperitoneal injection with 100 μl of *S. iniae* (KCTC 3657) using sterile nonheparinized 1.0 mL syringes at a concentration of 1×10^8 CFU mL^{-1} (Hasan Md. et al.2018 Heo et al., 2013), kept in separate tanks without recirculation, and supplied with

airstones. The fish were not fed during the course of the challenge. Tanks were checked twice daily for mortality.

Statistical analysis

All data were analyzed by one-way ANOVA to test for the effects of dietary treatment. When significant differences were found, a Duncan's Multiply Range Test (DMRT) test was used to identify differences among experimental groups. Treatment effects were considered to be significant at a level of $P < 0.05$. All statistical analyses were performed by SPSS 20 (IBM).

3. Results

Growth

At the end of the feeding trial, average WG & SGR of fish fed the GAB_{282} diet were significantly higher ($P < 0.05$) than all other diets, with the exception of the GAB_{229} diet. Fish fed the GAB_{229} & GAB_{282} diets had significantly higher values than fish fed the CON_{92}, OTC, GAB_{154}, and GAB_{352} diets (Table 4; Fig. 1). Yet, there were no significant differences among fish fed the GAB_{229} and GAB_{327} diets. According to quadratic regression analysis (Fig. 2) the possible optimal level for growth is determined to be 236.9 mg kg^{-1}. There were no significant differences with regards to other growth parameters (see Table 4, Figures 1-8).

Intestinal digestive enzyme activity

As for intestinal digestive enzyme activity, only amylase activity presented a clear trend with significant results (Table 6, Figures 12 and 13). Results show that the OTC and GAB_{282} diets had significantly higher enzyme activity than all other diets. With regards to lipase enzyme activity, there was a trend similar to that of amylase, however values were not distinct enough to breech the level of statistical significance in all diets except for the positive control (OTC diet) (Table 6).

Organosomatic indices

There were no significant differences found amongst any group in terms of FE, VSI, HSI, Survival at the end of the feeding trial (Table 4, Figures 4, 5, 6, and 7) .

Non-specific immunity

Average lysozyme activity of fish the GAB_{154}, GAB_{229}, GAB_{282}, and GAB_{327} diets were significantly higher ($P<0.05$) than the CON_{92} and all other diets, with exception to the OTC diet. Furthermore, there were no significant differences between the CON_{92}, OTC and GAB_{352} diets (Table 7, Fig. 11). There were no significant differences found amongst any group in terms of SOD and MPO between any treatment groups.

Blood Chemistry

At the end of the feeding trial there were no significant differences in blood serum AST, ALT, GLU, or TP levels (Table 8).

Challenge Test

Percent survival of all fish fed a GABA supplemented diet was significantly better than CON_{92} by the 10th day of the challenge test with the greatest survival found among the OTC, GAB_{229}, and GAB_{282} diets by day 12 (Fig. 18).

Tables and Figures

Table 1. Formulation of experimental diets

	Diets g kg^{-1}						
Ingredients	**CON_{92}**	**GAB_{154}**	**GAB_{229}**	**GAB_{282}**	**GAB_{327}**	**GAB_{352}**	**OTC**
Sardine FM	250.00	250.00	250.00	250.00	250.00	250.00	250.00
Anchovy FM	250.00	250.00	250.00	250.00	250.00	250.00	250.00
Soybean meal[b]	150.00	150.00	150.00	150.00	150.00	150.00	150.00
Wheat flour[b]	130.00	129.93	129.87	129.80	129.74	129.67	126.00
Squid Liver Powder[a]	40.00	40.00	40.00	40.00	40.00	40.00	40.00
Meat & Bone meal	50.00	50.00	50.00	50.00	50.00	50.00	50.00
Poultry BP	40.00	40.00	40.00	40.00	40.00	40.00	40.00
Fish oil	42.00	42.00	42.00	42.00	42.00	42.00	42.00
Lecithin	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Betaine	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Taurine	5.00	5.00	5.00	5.00	5.00	5.00	5.00
MCP	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Mineral mix[c]	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Vitamin mix[d]	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Choline	3.00	3.00	3.00	3.00	3.00	3.00	3.00
GAB (76.5%)	0.00	**0.0654**	**0.1307**	**0.1961**	**0.2614**	**0.3268**	0.0000
Oxytetracycline HCl	--	--	--	--	--	--	4.00
Total	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00

[a] Suhyup feed Co. Uiryeong, Republic of Korea.
[b] The feed Co. Goyang, Republic of Korea.
[c] 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.
[d] Contains (as mg/kg in diets): NaCl, 437; $MgSO_4 \cdot 7H_2O$, 1380; $NaH_2P_4 \cdot 2H_2O$, 878; $Ca(H_2PO_4) \cdot 2H_2O$, 1367; KH_2PO_4, 2414; $ZnSO_4 \cdot 7H_2O$, 226; Fe-Citrate, 299; Ca-lactate, 3004; $MnSO_4$, 0.016; $FeSO_4$, 0.0378; $CuSO_4$, 0.00033; Calcium iodate, 0.0006; MgO, 0.00135; $NaSeO_3$, 0.00025.
[e] $CaHPO_4$. Sigma-Aldrich Korea Yongin, Korea.

Table 2. Proximate analysis (dry matter basis) of the experimental diets [1].

Diet	Moisture %	Protein %	Lipid %	Ash %
CON_{92}	8.22^{ns}	51.93^{ns}	9.92^{ns}	12.03^{ns}
GAB_{154}	8.18	52.12	10.14	12.40
GAB_{229}	8.73	52.20	10.21	12.05
GAB_{282}	8.74	51.83	9.88	12.26
GAB_{327}	8.60	51.97	10.39	12.54
GAB_{352}	9.65	51.56	9.93	12.00
OTC	8.74	52.03	9.40	12.06
Pooled SEM[2]	0.11	0.07	0.12	0.14

[1] Values are mean of duplicate samples. Values with different letters within the same row are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT).
[2]Pooled SEM: SD/√n.

Table 3. Actual percentage of GABA according to HPLC for the experimental diets[1].

Diet	CON_{92}	GAB_{154}	GAB_{229}	GAB_{282}	GAB_{327}	GAB_{352}	OTC
GABA %	0.009236	0.015387	0.022927	0.028179	0.032668	0.035228	0.010299
GABA mg kg^{-1}	92.36	153.87	229.27	281.79	326.68	352.28	102.99

[1] Values are mean of duplicate samples.
Diet names have been changed to reflect the actual dietary level of GABA.HPLC performed at the National Instrumentation Center for Environmental Management College of Agriculture and Life Sciences at Seoul National University (Seoul 151-742, Korea)

Table 4. Growth performance, feed efficiency, organosomatic indices, survival, and fPCA [1].

Diets	IBW[2]	WG[3]	SGR[4]	FE[5]	VSI[6]	HSI[7]	Survival (%)[8]	fPCA[9*]
CON_{92}	4.86^{ns}	482.28^{c}	3.04^{c}	120.64^{ns}	1.50^{ns}	0.87^{ns}	97.8^{ns}	103.28^{ns}
GAB_{154}	4.96	480.16^{c}	3.03^{c}	119.06	1.48	0.73	96.7	99.91
GAB_{229}	4.94	530.75^{ab}	3.17^{ab}	130.36	1.45	0.65	93.3	105.64
GAB_{282}	4.97	544.63^{a}	3.21^{a}	127.67	1.44	0.72	90.0	101.24
GAB_{327}	4.90	492.51^{bc}	3.07^{bc}	112.87	1.50	0.75	88.9	93.46
GAB_{352}	4.91	483.62^{c}	3.04^{c}	113.62	1.56	0.86	90.0	95.71
OTC	4.81	475.04^{c}	3.02^{c}	115.82	1.67	0.79	94.4	97.87
Pooled[10] SEM	0.02	6.35	0.02	1.77	0.03	0.02	1.00	1.37

[1] Values are mean of triplicate samples. Values with different letters within the same row are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT).
[2] Initial body weight (g).
[3] Weight gain (%) = (final wt. – initial wt.) × 100 / initial wt.
[4] Specific growth rate (%) = (ln final weight − ln initial weight) × 100 / d.
[5] Feed efficiency (%) = wet weight gain × 100 / dry feed intake.
[6] Viscerosomatic index (VSI) = 100 × viscera weight (g)/body weight (g)
[7] Hepatosomatic index (%) = liver weight × 100 / body weight.
[8] Survival rate (%) = (survival fish − dead fish) × 100/survival fish
[9] Final Percent Coverage Area (%) = total ventral surface of fish in cm^2 / total tanks bottom area x 100. * initial PCA = 38%
[10] Pooled SEM: SD/$\sqrt{n}$.

Table 5. Whole-body proximate compositions (%, DM) of juvenile olive flounder [1].

Diet	Moisture	Protein	Lipid	Ash
CON_{92}	2.28^{ns}	71.43^{ns}	11.53^{ns}	15.51^{ns}
GAB_{154}	2.14	71.48	12.07	16.81
GAB_{229}	1.90	70.26	10.90	16.33
GAB_{282}	2.67	70.52	12.17	15.92
GAB_{327}	2.64	71.55	12.12	15.37
GAB_{352}	2.41	71.59	11.58	16.25
OTC	2.75	71.34	10.87	15.94
Pooled SEM[2]	0.10	0.26	0.24	0.18

[1] Values are mean of duplicate samples. Values with different letters within the same row are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT).
[2] Pooled SEM: SD/√n.

Table 6. Enzyme activity of fish fed the experimental diets[1].

Diet	Amylase mU L^{-1}	Lipase mU μL^{-1}
CON_{92}	1723.2^{b}	40.32^{b}
GAB_{154}	998.6^{c}	46.42^{b}
GAB_{229}	1926.1^{b}	45.15^{b}
GAB_{282}	2947.8^{a}	76.76^{a}
GAB_{327}	1631.4^{b}	46.97^{b}
GAB_{352}	1969.6^{b}	56.00^{b}
OTC	2626.6^{a}	100.87^{a}
Pooled SEM[2]	139.19	5.44

[1] Values are mean of duplicate samples. Values with different letters within the same row are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT).
[2]Pooled SEM: SD/$\sqrt{n}$.

Table 7. Non-specific immune responses of juvenile olive flounder[1].

Diet	SOD[2] (% inhibition)	MPO[3] (absorbance)	Lysozyme (U mL^{-1})
CON_{92}	90.84^{ns}	4.59^{ns}	0.61^{c}
GAB_{154}	78.31	3.68	1.06^{ab}
GAB_{229}	85.01	4.46	1.21^{ab}
GAB_{282}	85.35	3.89	1.39^{a}
GAB_{327}	83.51	4.41	1.32^{ab}
GAB_{352}	86.21	3.68	0.60^{c}
OTC	92.00	3.75	0.97^{bc}
Pooled SEM[5]	**2.71**	**0.18**	**0.08**

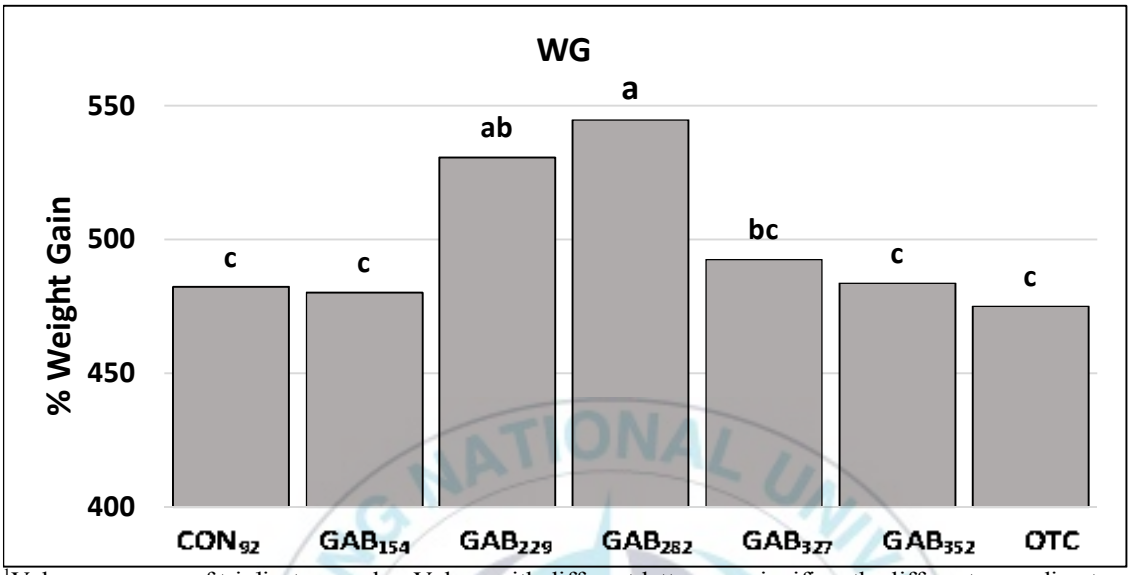
[1] Values are mean of triplicate samples. Values with the different letters within the same row are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT).
[2] Superoxide dismutase (% inhibition).
[3] Myeloperoxidase (absorbance).
[4] Lysozyme activity
[5]Pooled SEM: SD/$\sqrt{n}$.

Table 8. Blood Chemistry of juvenile olive flounder[1]

Diet	AST[2] U L^{-1}	ALT[3] U L^{-1}	GLU[4] mg dL^{-1}	TP[5] g dL^{-1}
CON_{92}	16.00[ns]	5.00 [ns]	17.33 [ns]	3.27 [ns]
GAB_{154}	13.33	4.33	22.00	3.03
GAB_{229}	15.33	5.00	22.00	3.43
GAB_{282}	15.33	5.00	24.33	3.87
GAB_{327}	17.67	4.33	23.33	4.23
GAB_{352}	16.67	6.00	19.67	3.83
OTC	15.67	4.33	29.00	3.97
Pooled SEM[6]	**0.72**	**0.16**	**1.70**	**0.17**

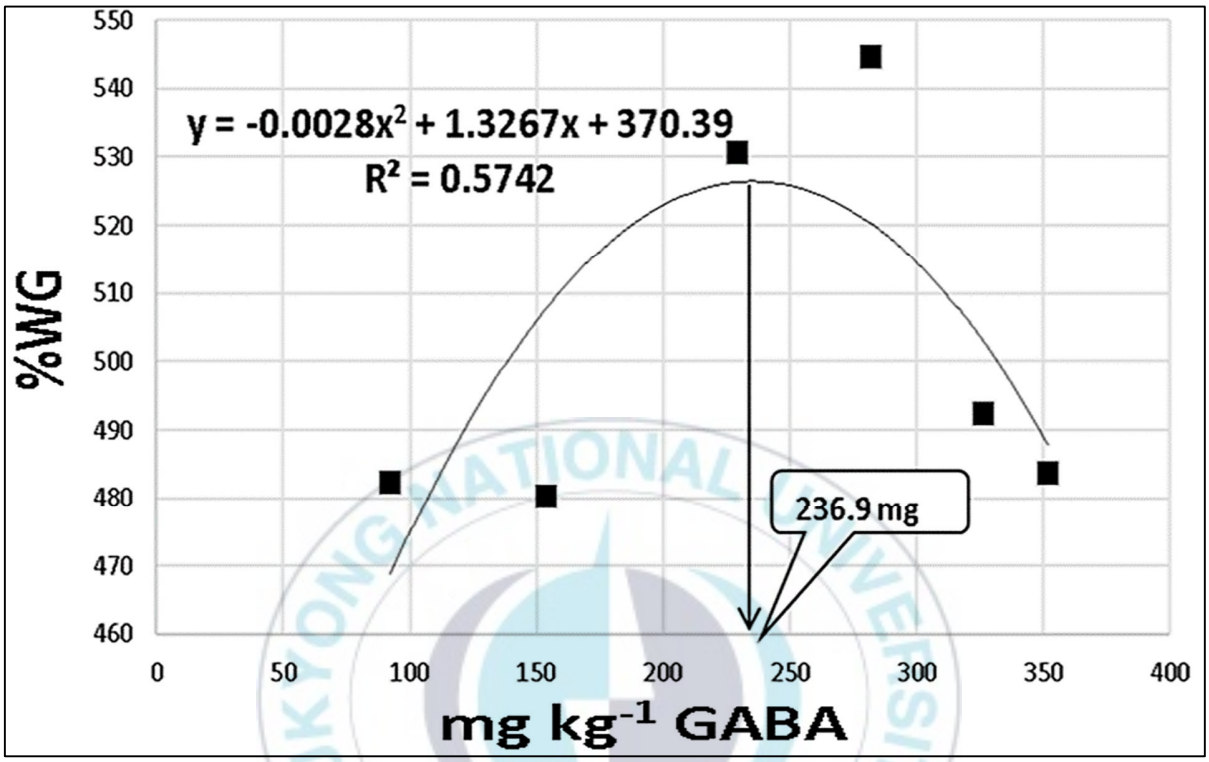
[1] Values are mean of triplicate samples (Fuji DRI-CHEM 3500i, Fuji Photo Film, Ltd., Tokyo, Japan). Values with different letters within the same row are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT).
[2]AST (U L^{-1}): Aspartate aminotransferase.
[3]ALT (U L^{-1}): Alanine aminotransferase.
[4]GLU (mg/dl): Serum Glucose.
[5]TP (g/dl): Serum Total Protein.
[6]Pooled SEM: SD/$\sqrt{n}$.

Figure 1. Percent Weight Gain (%WG) of juvenile olive flounder fed the experimental[1]



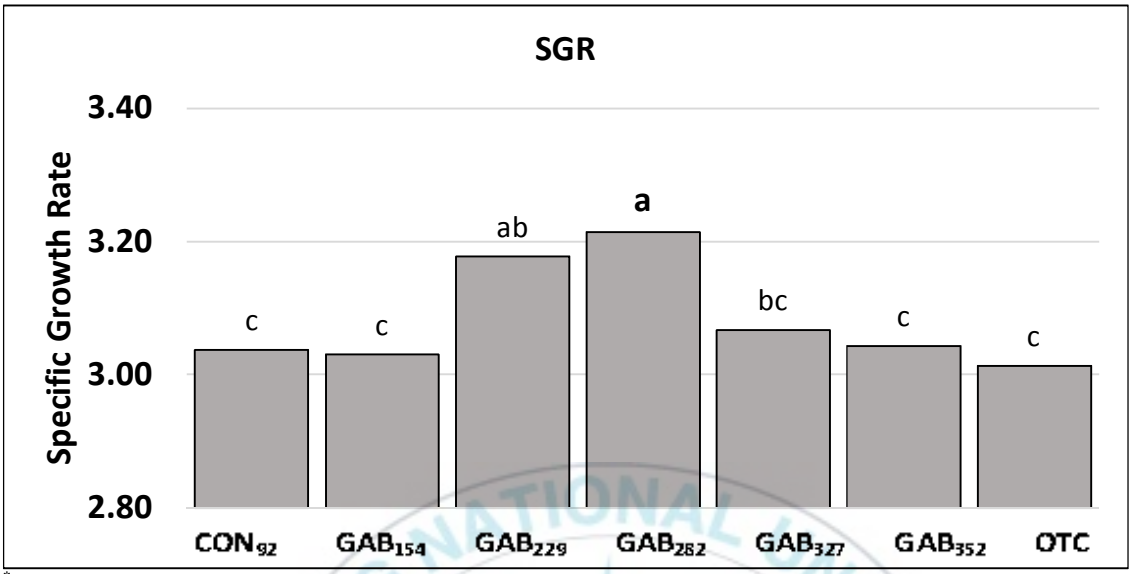
[1]Values are mean of triplicate samples. Values with different letters are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT). For information about growth see table 4.

Figure 2. Optimal level of dietary GABA in mg/kg according to WG by polynomial analysis in Juvenile olive flounder.



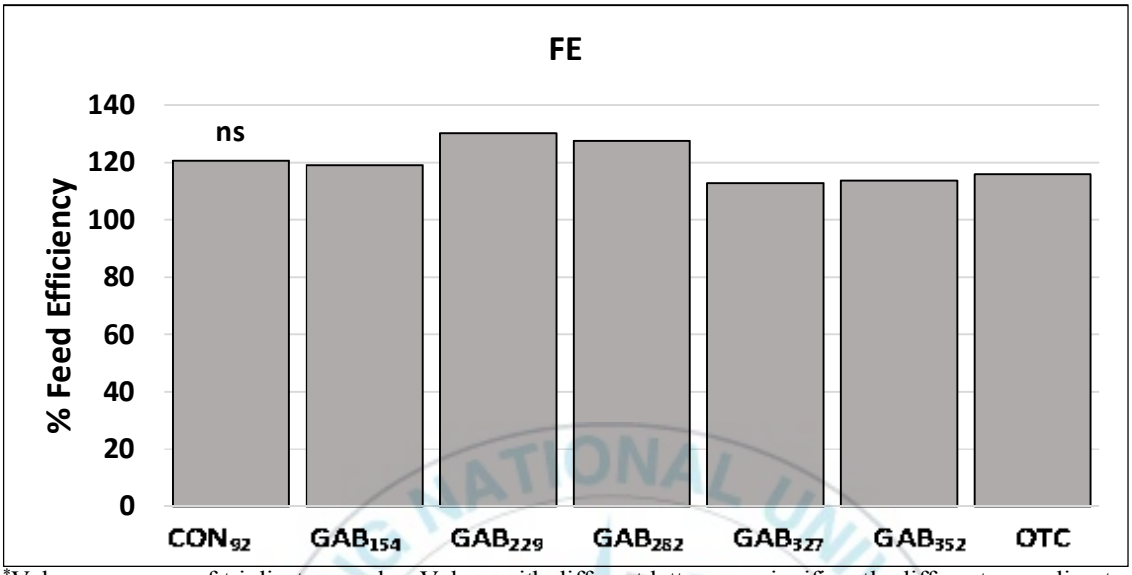
*OTC has been excluded in this analysis.
For information about growth see table 4 and figure 1.

Figure 3. Specific Growth Rate



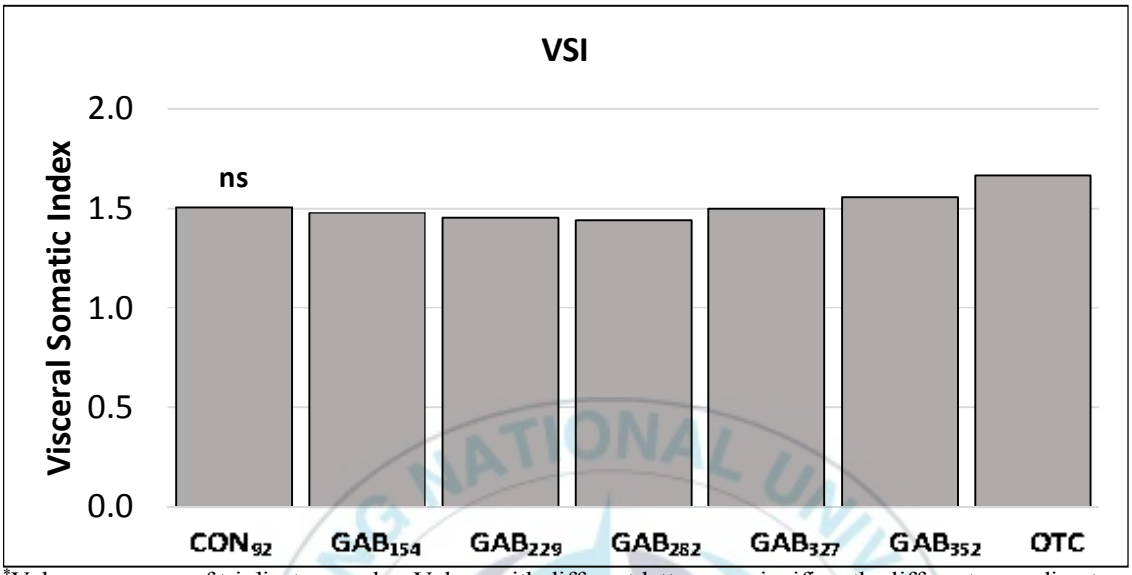
*Values are mean of triplicate samples. Values with different letters are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT). For information about the nonspecific immunity, see table 4.

Figure 4. Feed Efficiency (%)



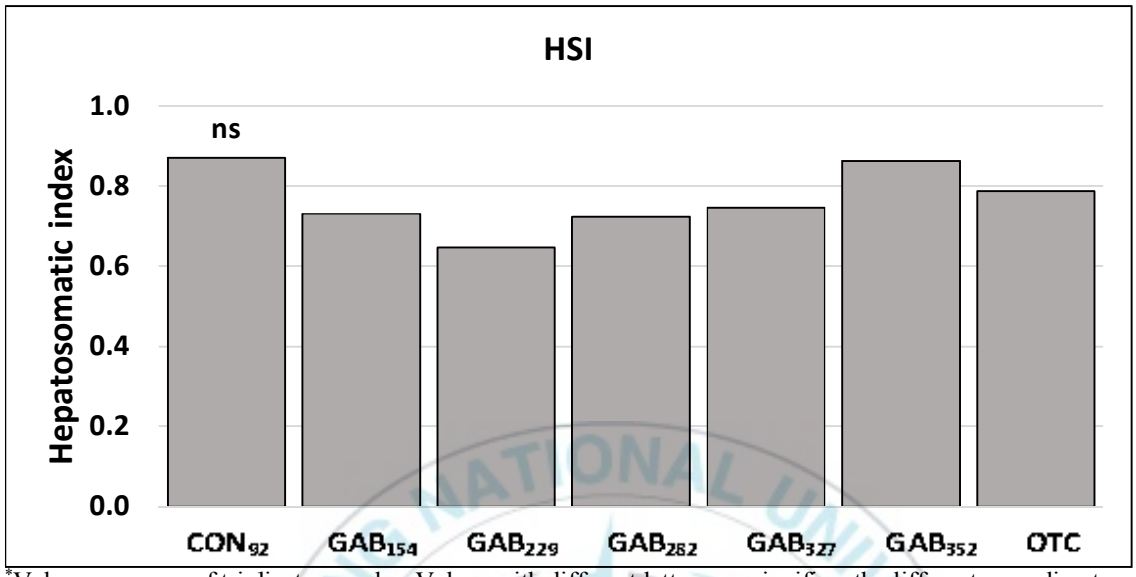
*Values are mean of triplicate samples. Values with different letters are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT). For information about growth, see table 4.

Figure 5. Visceral Somatic Index (VSI)



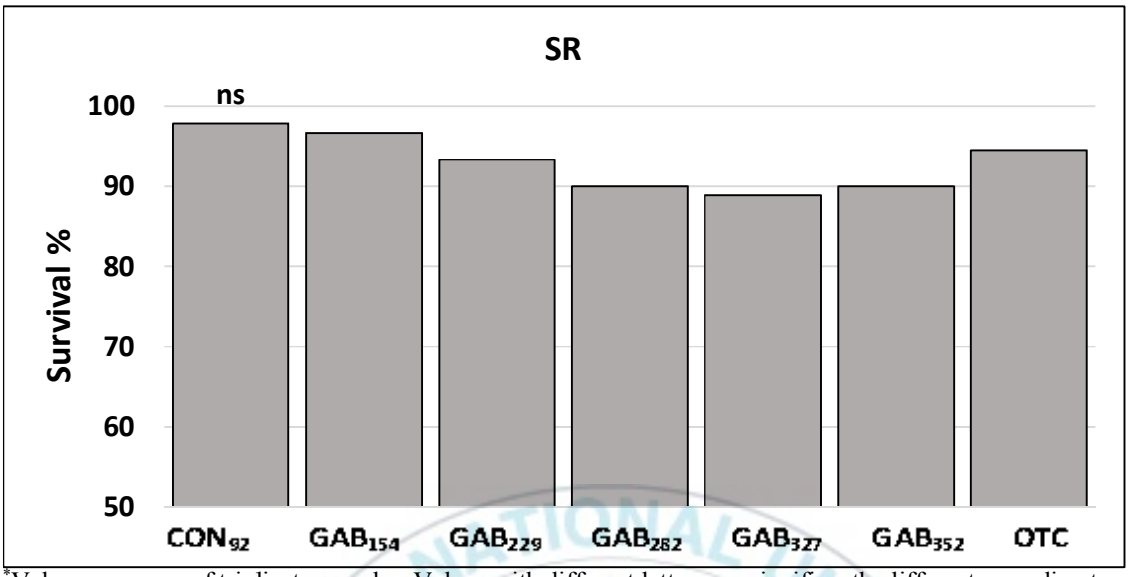
*Values are mean of triplicate samples. Values with different letters are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT). For more information about growth, see table 4.

Figure 6. Hepatosomatic Index (HSI)*



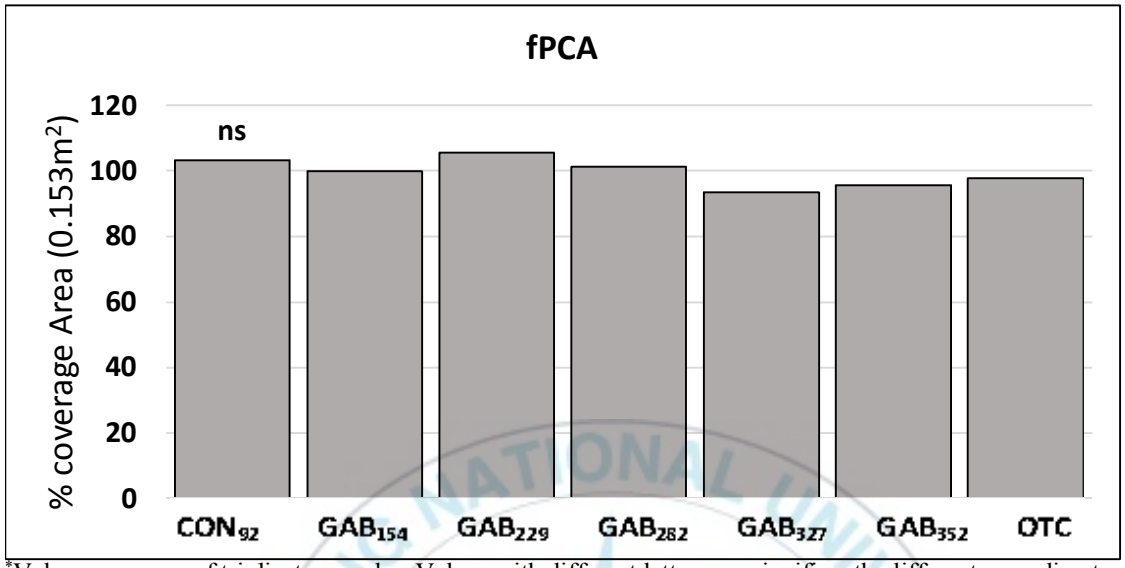
*Values are mean of triplicate samples. Values with different letters are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT). For more information about growth see table 4.

Figure 7. Survival (%)



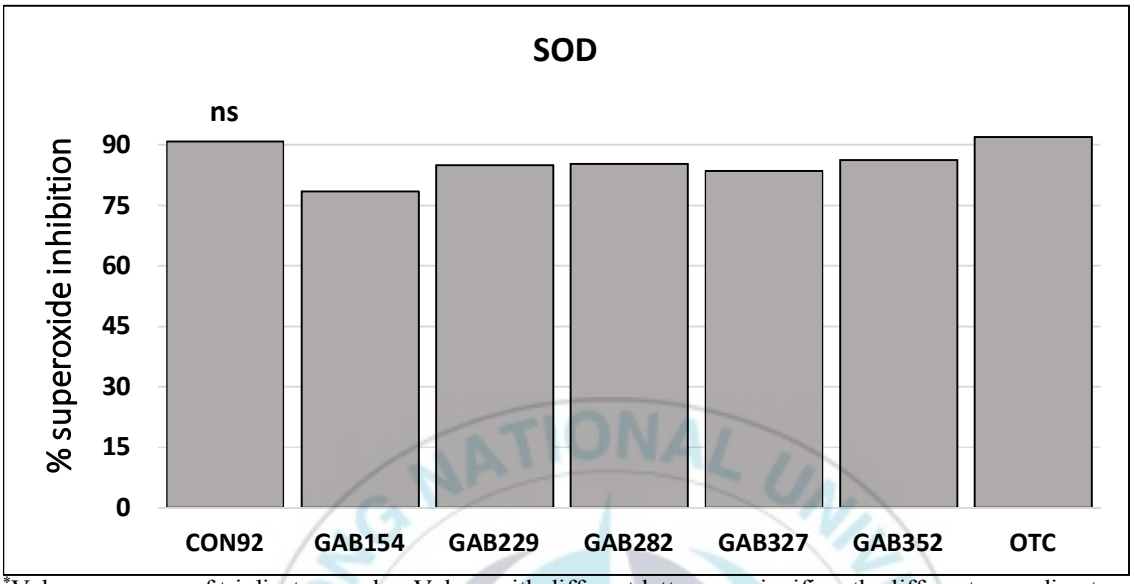
*Values are mean of triplicate samples. Values with different letters are significantly different according to one-way ANOVA (*P*=0.05) and Duncans Multiple Range test (DMRT). For information about growth, see table 4.

Figure 8. final Percent Coverage Area*



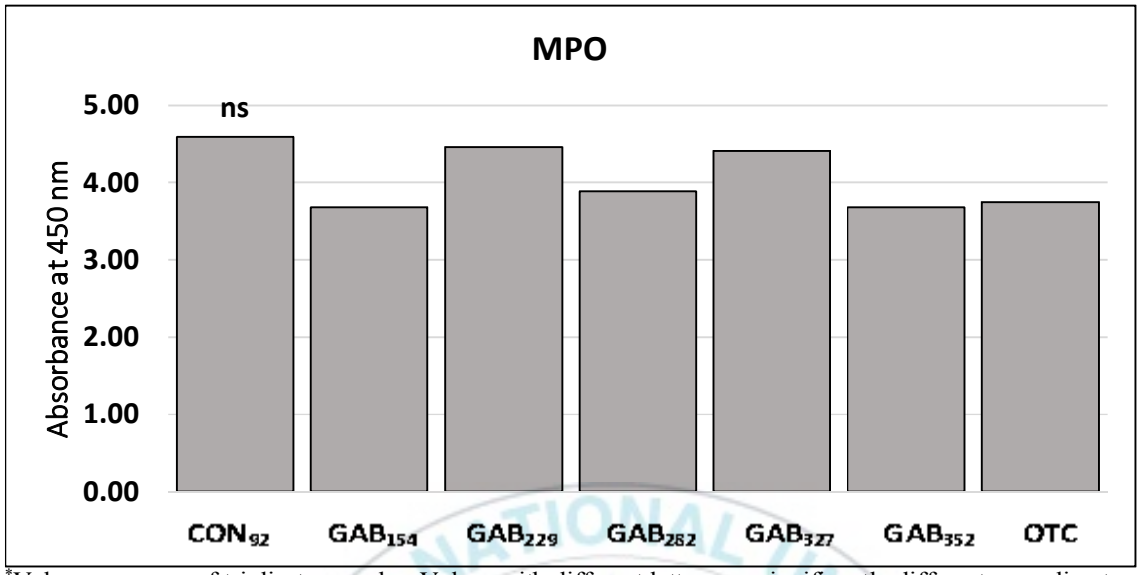
*Values are mean of triplicate samples. Values with different letters are significantly different according to one-way ANOVA (*P*=0.05) and Duncans Multiple Range test (DMRT). For information about growth, see table 4.

Figure 9. Superoxidedismutase activity (% inhibition)*



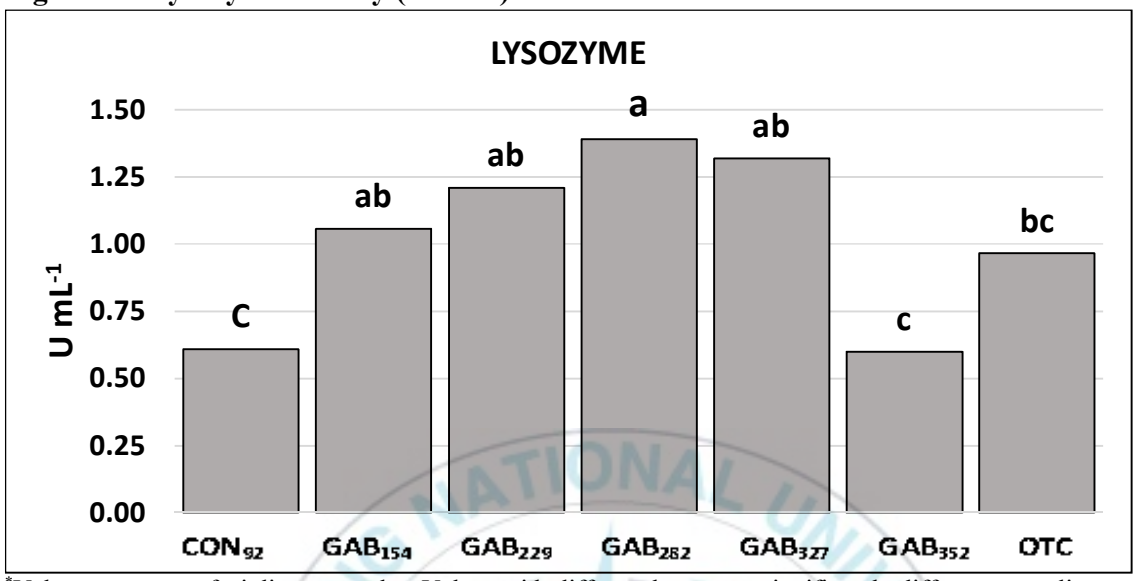
*Values are mean of triplicate samples. Values with different letters are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT). For information about the nonspecific immunity, see table 7.

Figure 10. Myeloperoxidase absorbance (450nm)



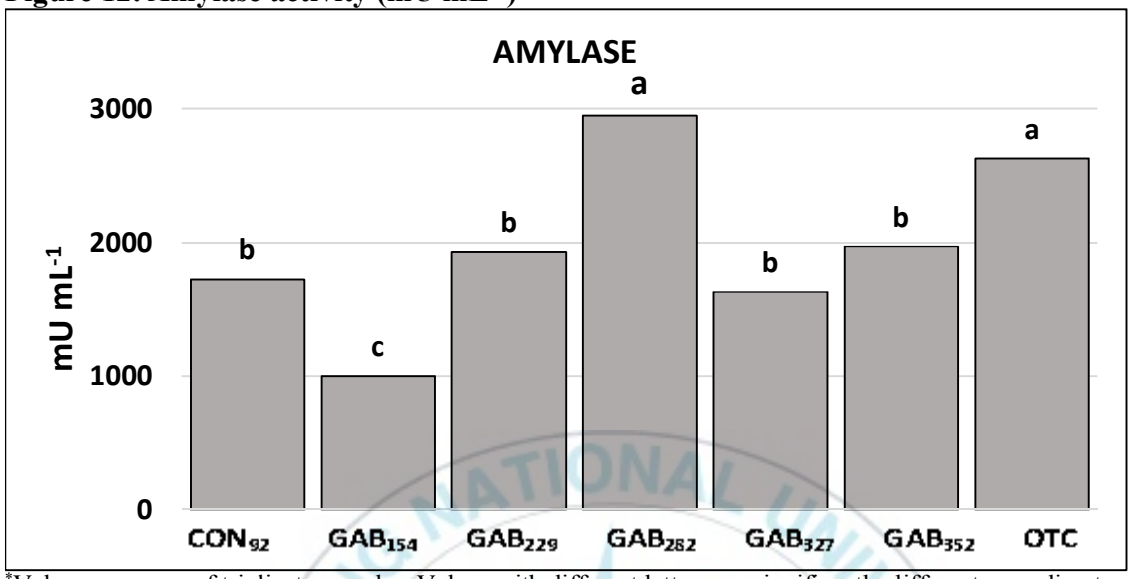
*Values are mean of triplicate samples. Values with different letters are significantly different according to one-way ANOVA (*P*=0.05) and Duncans Multiple Range test (DMRT). For information about the nonspecific immunity, see table 7.

**Figure 11. Lysozyme activity (U mL^{-1})*



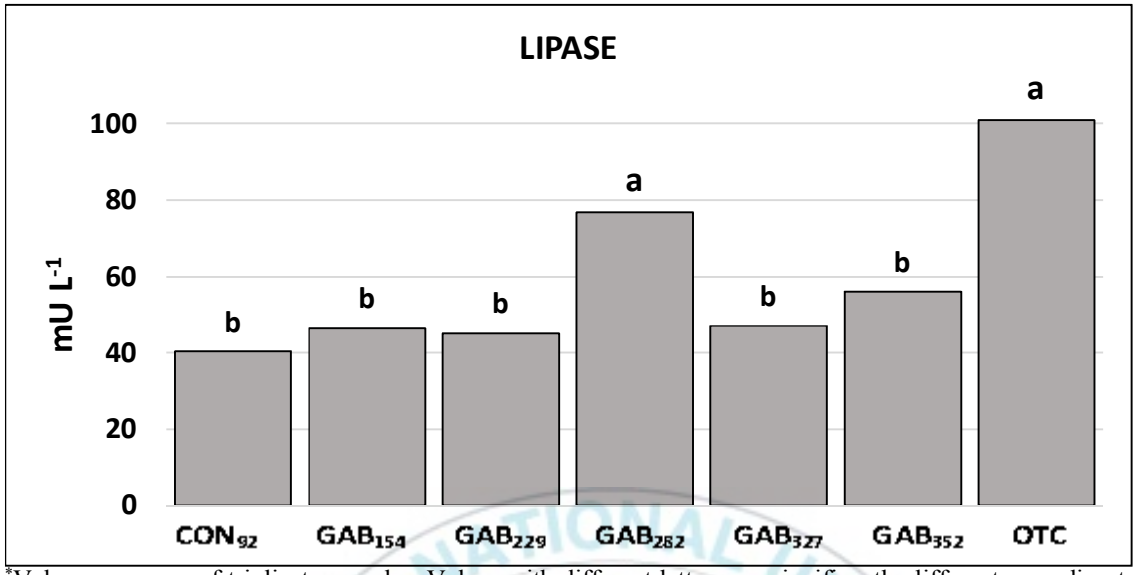
*Values are mean of triplicate samples. Values with different letters are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT). For information about the nonspecific immunity, see table 7.

**Figure 12. Amylase activity (mU mL^{-1})*



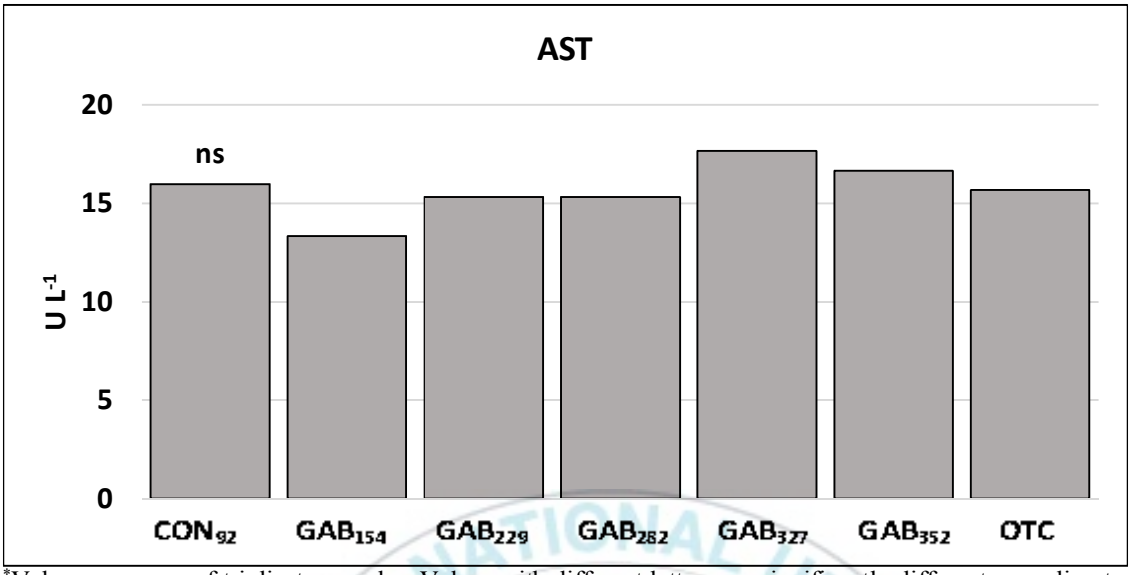
*Values are mean of triplicate samples. Values with different letters are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT). For information about digestive enzyme activity, see table 6.

Figure 13. Lipase activity (U mL^{-1})*



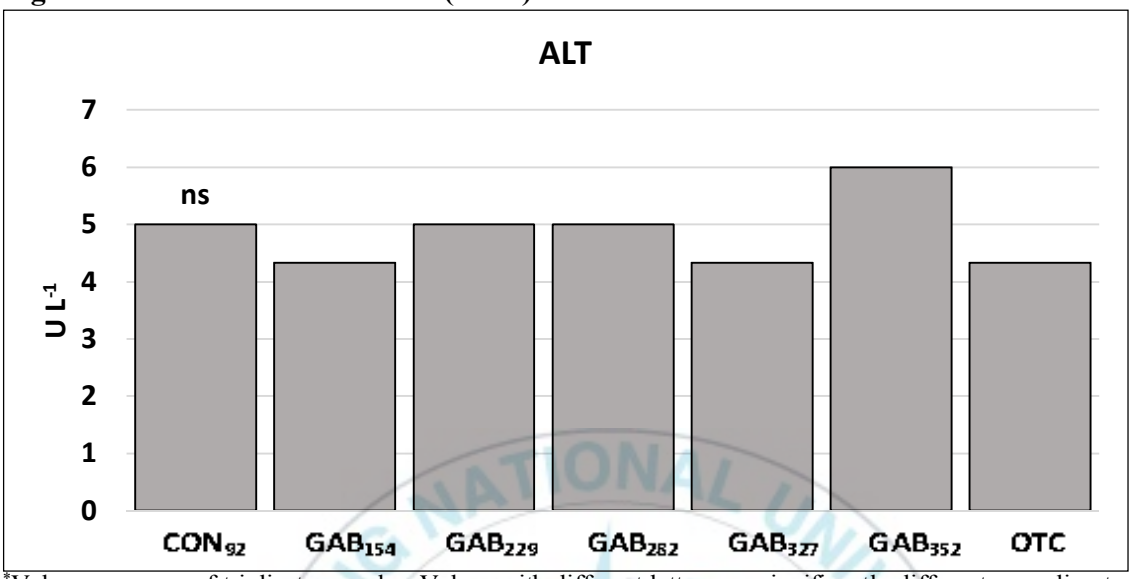
*Values are mean of triplicate samples. Values with different letters are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT). For information about digestive enzyme activity, see table 6.

Figure 14. Aspartate transaminase (U L^{-1}) *



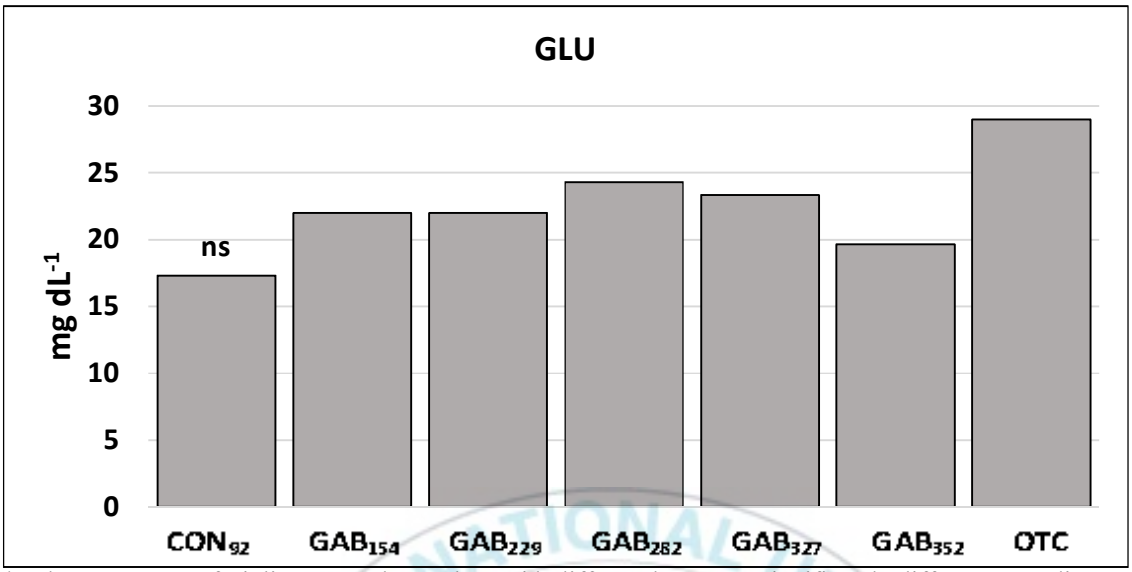
*Values are mean of triplicate samples. Values with different letters are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT). For information about blood chemistry, see table 8.

Figure 15. Alanine transaminase (U L^{-1}) *



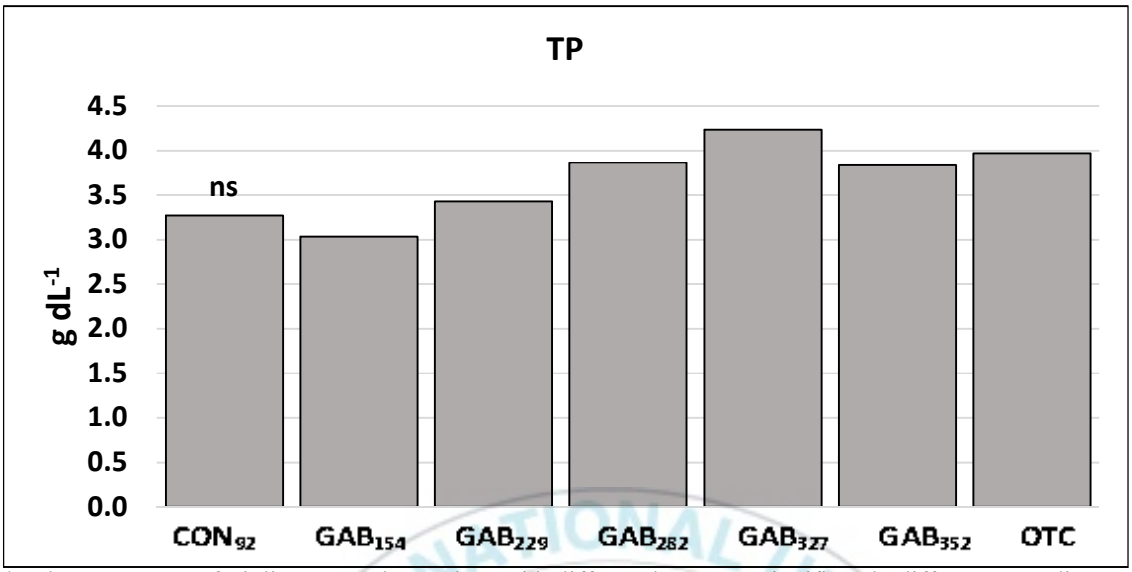
*Values are mean of triplicate samples. Values with different letters are significantly different according to one-way ANOVA (*P*= 0.05) and Duncans Multiple Range test (DMRT). For information about blood chemistry, see table 8.

Figure 16. Glutamate (mg dL^{-1})*



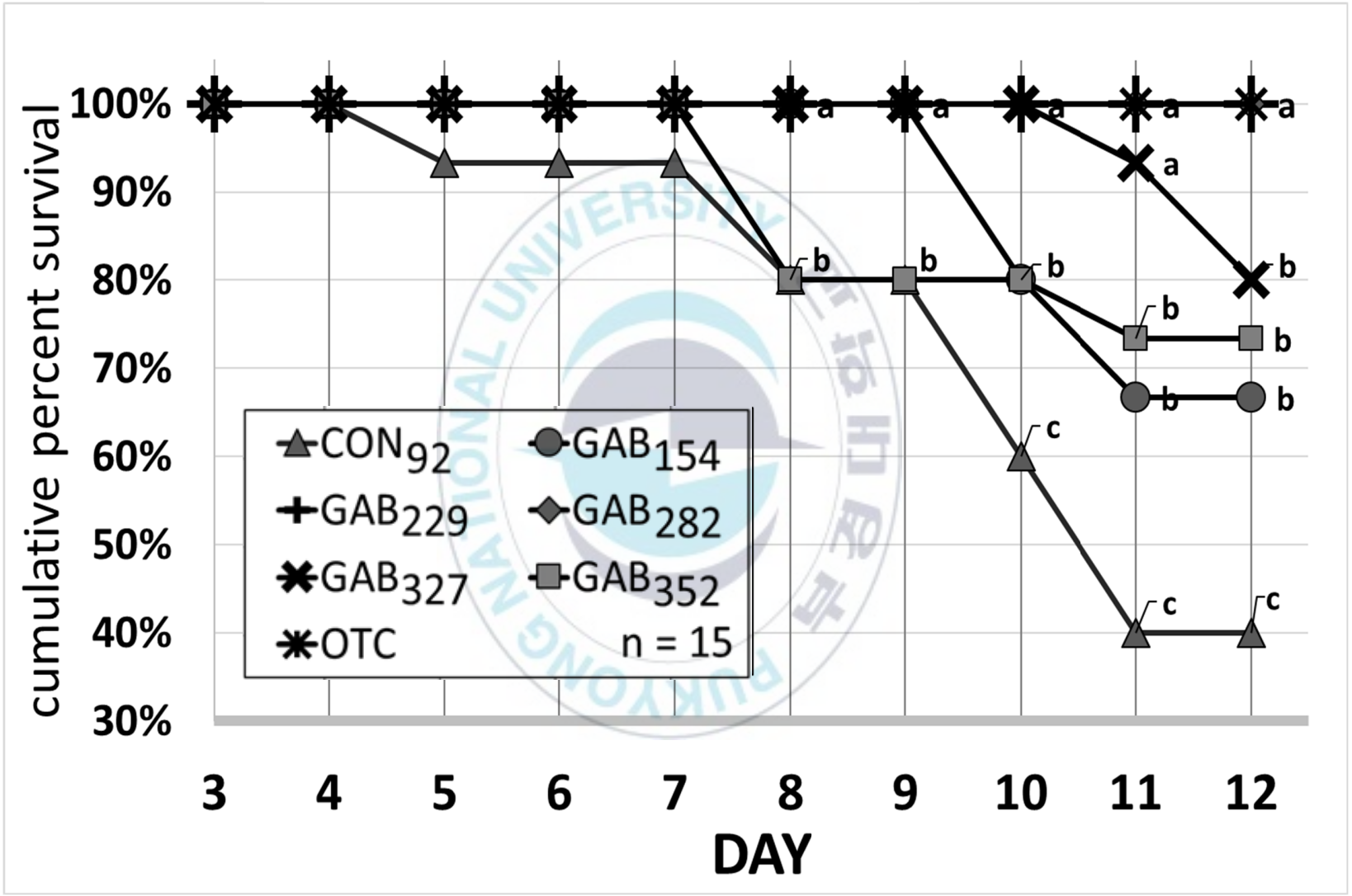
*Values are mean of triplicate samples. Values with different letters are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT). For information about blood chemistry, see table 8.

Figure 17. Total Protein (g dL^{-1})*



*Values are mean of triplicate samples. Values with different letters are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT). For information about blood chemistry, see table 8.

Figure 18 . Challenge Test: 1x10^8 CFU *Streptococcus iniae*[*]



[*] Values are mean of triplicates of five fish: n=15. Values with different letters are significantly different according to one-way ANOVA (P=0.05) and Duncans Multiple Range test (DMRT) according to the day.

4. Discussion

Growth

In this experiment WG and SGR were significantly improved with the GAB_{229} and GAB_{282} diets compared to the control, with the GAB_{282} diet being significantly higher than all other diets except the GAB_{229} diet. In terms of growth, similar results were found in experiments assessing an optimal level for GABA with grass carp and tilapia (Wu, F. et al, 2016; Temu, V. et al, 2019). Though statistical analysis finds no difference between GAB_{229} and GAB_{282}, the values of the GAB_{282} diet are higher, and one can hypothesize that significant differences could arise if a longer trial were undertaken, or if different stressors were employed. One reason for the improvement in WG could come from the documented effects that GABA has on the expression of growth hormone. (Powers et al, 2008). This shows that supplementation of the diet with GABA within a range of 229 - 282mg kg^{-1} has clear effects on WG. Analysis of the results indicates that a dietary level of 237 mg kg^{-1} based on quadratic regression (polynomial) analysis could be the optimum in this diet at this life stage for olive flounder, and, as will be elaborated, can improve growth, digestive amylase, lysozyme activity, and disease resistance as well.

The reason for the decrease in benefit as supplementation level increase may be multifaceted. But one possible reason is due to an inhibition of feeding as evidenced in a trial by Kim S.K. et al, (2003). In their trial, also using juvenile *Paralichthys olivaceus*, fish fed diets containing 1000 mg kg^{-1} GABA had an inhibitory effect on feeding. Thus, in the present trial an inverse relationship between higher levels of dietary inclusion and growth can be observed. It stands to reason, (when considering WG) (Table 4), that a dietary nearly three times the level of the GAB_{327} diet, the first diet showing a significant decrease in growth performance which is above the GAB_{282} diet level,

that a continued downward trend would be observed in this experiment as well if such high doses were utilized.

Intestinal digestive enzyme activity

Digestive enzymes are an important class of enzymes that break down nutrients such as carbohydrates, proteins, and fats from complex polymeric structures into smaller, more digestible constituents. In this trial, we assessed the activity of amylase and lipase in the intestine of juvenile olive flounder. Our results showed that the OTC and GAB_{282} diets had significantly higher enzyme activity than all other diets.

Non-specific immunity

Non-specific immunity, also known as innate immunity, is a fundamentally important defense mechanism in fish. In fish, non-specific immunity is much more important in the case of fish, which are poikilothermic, than acquired immunity which is far less robust than in warm-blooded vertebrates. The benefit of non-specific immunity is that it is almost instantaneous (Magnadóttir, B. 2006). There are several enzymes involved in the non-specific immune response which are important in the study of fish; among these are SOD, MPO, and lysozyme. In this trial, only lysozyme activity was significantly different. lysozyme activity was significantly improved in all GABA supplemented diets as well as the OTC diet. GABA's effects on lysozyme in this trial may be due to an increase in GABA associated macrophage activation (Kim J.K. et al, 2018). The increase in macrophage activation is then made evident by improved lysozyme activity (Keshav et al, 1991).

Our results differed in terms of SOD and MPO significance from a preceding, currently unpublished trial in our lab, assessing GABA at a concentration of 277 mg kg^{-1} in Juvenile olive flounder, which showed significantly higher results with regards to SOD activity. The reason for

differences in these two trials may stem from differences in the overall health condition of the specimens, temperature, stocking density, and formulation of the basal diet. Nonetheless, a trend of non-specific immune up-regulation in the case of GABA supplemented feeds is an important result. In Nile tilapia (*Oreochromis niloticus*) Temu et al. (2019) found that fish fed a dietary level of 158 mg kg^{-1} also had a significantly increase in SOD activity when compared to other diets.

Challenge Test

Pathogens have wreaked havoc on the olive flounder industry in recent years. Production in Korea peaked at 54,574 MT in 2009, but shortly thereafter fell into a sharp decline, down to the level of 36,921 MT in 2014. This hit to production was largely due to the outbreak of pathogens amongst olive flounder operations; a 32% decline. (Hasan Md et al., 2018; FAO 2019). One bacterial pathogen that is of special concern to olive flounder culture is that of *S. iniae*. *S. iniae* is a Gram-positive bacteria that has become a serious threat in aquaculture species. Therefore, it was chosen to assess the disease resistance of the olive flounder in this experiment. Our results show that cumulative percent survival among all fish fed a GABA supplemented diet was significantly higher than the CON_{92} diet by the 10th day, with the greatest distinction found among the OTC, GAB_{229}, and GAB_{282} diets by the end of the 12th day. Since there were no significant differences between the afformentioned diets, this indicates that GABA could replace OTC in the diet of juvenile olive flounder.

Though the reason for the improved cumulative survival in GABA supplemented diets may lie in mechanisms beyond the scope of the parameters analyzed in this trial, improved lysozyme activity lends an improvement in the overall immune status, the actual means of improved bacterial resistance are a topic within a new frontier of research; the microbiome and the gut-brain axis. Yet the benefits of GABA to the immune system can be clearly demonstrated in the results of the

challenge test in which all diets with GABA supplementation resulted in improved survival compared to the control (CON_{92}). A mechanism for pathogen resistance may be gleaned from a recent paper about GABA's effects on microbial defense in which it was shown that it has antimicrobial effects via modulation of macrophage activity via GABAAR-Ca^{2+}- AMP-activated protein kinase signaling (Kim J.K. et al, 2018). In this trial GABAergic signaling was linked to autophage enhancement resulting in the infected host's protection against intracellular bacterial infections. Though the antimicrobial defense offered by GABA is relatively unstudied, in this research, treating macrophages with GABA or other GABAergic agents resulted in an increase in autophage activation leading to phagosomal maturation, and thus an improved antimicrobial response. Additionally, research was done recently on the gut microbiome in which Strandwitz et al, (2019) found that GABA was essential for the growth of a newly discovered bacterial (KLE1738). Lastly, as far as the gut-brain axis, Bravo et al, (2011) found that *Lactobacillus rhamnosus* was able to alter GABA levels in the brain of mice. Future trials accessing the effects of GABA and other metabolites on the composition of the microbiome and its influence on the gut-brain axis will be very important going forward. The results of the current trial are encouraging for GABA's prospects as an important feed additive. To our knowledge this is the first trial to access GABA's effects on survival to bacterial challenge in a marine finfish. Also, in this trial juvenile olive flounder were used. There may likely be different results with adult or broodstock. This is likely due to the fact that GABA is known to play different roles at very early stages of development, especially during embryonic stages (Hsu, Y.T. et al, 2018). Thus, there are opportunities to assess dietary effects in adult olive flounder.

5. Conclusion

In this trial the optimal level of dietary GABA was determined to be 236.9 mg kg^{-1} according to the quadratic regression model based on WG. Results from digestive enzyme and lysozyme activity and the *S. iniae* challenge test fell in line with the results of WG. It is also very important to note that the results for the OTC diet were either statistically indistinguishable, or in the case of WG significantly lower than the best diet (GAB_{282}). This shows that GABA can effectively replace OTC in the diet of juvenile olive flounder. Given the affordability of GABA, it's relatively small inclusion proportional to other ingredients, and efficacy, GABA is likely to gain continued attention, and thus merits further research. A following trial will assess the effects of the optimal GABA levels established in this experiment on juvenile olive flounder under stress from a much higher stocking density. GABA is related to a rapidly expanding body of research on microbial metabolites and other feed additives that will continue to develop. 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. Thus future research should focus on elucidating this system and developing novel methods and markers for accessing its involvement in fish metabolism. This will require a multifaceted examination of genetic expression, microbial flora, endocrinological and neurological parameters which will necessitate an interdisciplinary approach. This trial however, will provide a good baseline for future research in this species and life stage.

6. Acknowledgement

I would first like to say thank you to the thesis committee members' professors Jeonghwan Park, Jeongmyong Kim, and Sungchul C. Bai as well as to the rest of the faculty of the department of Fisheries Biology at Pukyong National University: Professors Dong soo Kim, Chang hoon Kim, Yoon kwon Nam, Seung pyo Gong, and Youn hee Choi.

Also, I would like to give a very special thanks to professor Sungchul C. Bai for giving me the amazing opportunity to study at PKNU as well as my lab mate who mades it all possible: Jinho Bae, Seonghun Won, Ali Hamidoghli, Wonsuk Choi, Jangmin Ok, Euihyun Jang, Joohyeon Jung, Seunghan Lee, Haham Kim, Jinhye Park, and Verynice Temu.

Last, but not least, to my beloved family who shaped me and made me the man I am today: Everett C. Farris 3rd (Dad), Everett C. Farris 2nd (Papa), Nancy Farris (Nanna), Mary-Elizabeth Farris (sister), Christine L. Morgan (Mom), James Morgan (Grampy), Mozella Morgan (Granny), Paul W. Hewitt (brother), and Junggyu Lee & Shihyun Kim (나의 한국인 부모).

This study was supported by the Feeds and Foods Nutrition Research Center (FFNRC), Pukyong National University, Busan, Rep. of Korea. This research was financially supported by the National Research Foundation of Korea, (NRF-2018K1A3A1A39088080) as well as material contributions by MILAE Bioresources, Korea.

O Lord, how manifold are your works! In wisdom have you made them all; the earth is full of your creatures.

Here is the sea, great and wide, which teems with creatures innumerable, living things both small and great.

מָה־רַבּוּ מַעֲשֶׂיךָ יְהוָה כֻּלָּם בְּחָכְמָה עָשִׂיתָ מָלְאָה הָאָרֶץ קִנְיָנֶךָ׃

זֶה הַיָּם גָּדוֹל וּרְחַב יָדָיִם שָׁם־רֶמֶשׂ וְאֵין מִסְפָּר חַיּוֹת קְטַנּוֹת עִם־גְּדֹלוֹת׃

(Psalm 104:24-25)

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Appendix (Raw Data)

Diet	WG	FBW	FCR	FE	PER	SGR	SR (%)
CON_{92}	455.68	27.41	1.33	112.69	2.17	2.96	96.67
	507.23	29.55	1.45	125.43	2.42	3.11	96.67
	483.92	27.83	1.44	123.79	2.38	3.04	100.00
GAB_{154}	459.28	27.78	1.25	106.30	2.04	2.97	90.00
	459.18	27.40	1.38	117.41	2.25	2.97	100.00
	522.00	31.10	1.54	133.47	2.56	3.15	100.00
GAB_{229}	498.97	30.15	1.42	122.58	2.35	3.09	90.00
	540.00	32.00	1.55	135.18	2.59	3.20	96.67
	553.27	31.36	1.52	133.30	2.55	3.24	93.33
GAB_{282}	514.23	29.89	1.42	123.48	2.38	3.13	93.33
	568.45	33.64	1.55	137.05	2.64	3.28	93.33
	551.20	32.56	1.39	122.48	2.36	3.23	83.33
GAB_{327}	480.16	28.81	1.29	111.01	2.14	3.03	90.00
	490.17	29.31	1.27	109.68	2.11	3.06	86.67
	507.19	28.96	1.36	117.92	2.27	3.11	90.00
GAB_{352}	478.46	28.92	1.25	107.91	2.09	3.03	86.67
	482.61	27.97	1.43	122.28	2.37	3.04	96.67
	489.80	29.08	1.28	110.66	2.15	3.06	86.67
OTC	488.19	28.04	1.31	113.20	2.18	3.05	90.00
	462.20	27.38	1.35	115.03	2.21	2.98	96.67
	474.71	27.59	1.39	119.22	2.29	3.01	96.67

Diet	HSI	VSI	AST	ALT	GLU	TP
CON_{92}	0.72	1.53	17	5	14	3.6
	0.87	1.63	16	5	18	3.2
	1.02	1.36	15	5	20	3.0
GAB_{154}	0.72	1.50	13	4	18	2.5
	0.77	1.48	16	5	31	3.1
	0.70	1.45	11	4	17	3.5
GAB_{229}	0.64	1.40	21	6	18	4.2
	0.65	1.43	14	5	19	2.9
	0.65	1.53	11	4	29	3.2
GAB_{282}	0.83	1.48	17	5	28	4.6
	0.71	1.37	17	5	16	3.8
	0.63	1.47	12	5	29	3.2
GAB_{327}	0.79	1.58	13	5	18	2.9
	0.75	1.56	25	4	27	5.8
	0.70	1.36	15	4	25	4.0
GAB_{352}	0.67	1.45	22	6	30	5.2
	1.10	1.66	14	7	15	3.6
	0.82	1.56	14	5	14	2.7
OTC	0.74	1.76	20	3	22	2.8
	0.76	1.39	14	5	14	4.0
	0.86	1.85	13	5	51	5.1

Diet	SOD (% inhibition)	MPO (absorbance)	LYSOZYME U mL^{-1}	AMYLASE mU mL^{-1}	LIPASE mu uL^{-1}
CON_{92}	92.48	4.27	0.50	1563.77	37.45
	94.65	6.02	0.63	1708.70	28.31
	85.39	3.47	0.70	1897.10	55.19
GAB_{154}	63.56	3.86	1.00	549.28	65.03
	96.10	3.65	1.40	1447.83	31.66
	75.27	3.53	0.77	998.55	42.56
GAB_{229}	78.31	4.00	1.23	2317.39	36.40
	105.21	6.02	1.10	2230.43	53.91
	71.51	3.37	1.30	1230.43	45.15
GAB_{282}	69.20	4.32	1.47	2882.61	94.58
	98.41	3.75	1.63	3013.04	45.54
	88.43	3.60	1.07	2947.83	90.17
GAB_{327}	70.79	6.02	1.13	1723.19	83.23
	95.66	3.63	1.43	1578.26	21.37
	84.09	3.60	1.40	1592.75	36.31
GAB_{352}	90.46	3.78	0.83	1926.09	56.00
	90.17	3.64	0.57	2013.04	38.79
	78.02	3.63	0.40	1969.57	73.21
OTC	80.33	3.78	1.07	2230.43	100.87
	108.97	3.81	0.73	2824.64	104.96
	86.70	3.67	1.10	2824.64	96.78

Challenge Test: 1x10^8 CFU *Streptococcus iniae**

Day	CON_{92}	GAB_{154}	GAB_{229}	GAB_{282}	GAB_{327}	GAB_{352}	OTC
1	**0**	**0**	**0**	**0**	**0**	**0**	**0**
1.5	**0**	**0**	**0**	**0**	**0**	**0**	**0**
2	**0**	**0**	**0**	**0**	**0**	**0**	**0**
2.5	**0**	**0**	**0**	**0**	**0**	**0**	**0**
3	**0**	**0**	**0**	**0**	**0**	**0**	**0**
3.5	**0**	**0**	**0**	**0**	**0**	**0**	**0**
4	**0**	**0**	**0**	**0**	**0**	**0**	**0**
4.5	**1**	**0**	**0**	**0**	**0**	**0**	**0**
5	**1**	**0**	**0**	**0**	**0**	**0**	**0**
5.5	**1**	**0**	**0**	**0**	**0**	**0**	**0**
6	**1**	**0**	**0**	**0**	**0**	**0**	**0**
6.5	**1**	**0**	**0**	**0**	**0**	**0**	**0**
7	**1**	**0**	**0**	**0**	**0**	**0**	**0**
7.5	**3**	**0**	**0**	**0**	**0**	**2**	**0**
8	**3**	**0**	**0**	**0**	**0**	**3**	**0**
8.5	**3**	**0**	**0**	**0**	**0**	**3**	**0**
9	**3**	**0**	**0**	**0**	**0**	**3**	**0**
9.5	**6**	**2**	**0**	**0**	**0**	**3**	**0**
10	**6**	**3**	**0**	**0**	**0**	**3**	**0**
10.5	**9**	**5**	**0**	**0**	**1**	**4**	**0**
11	**9**	**5**	**0**	**0**	**1**	**4**	**0**
11.5	**9**	**5**	**0**	**0**	**2**	**4**	**0**
12	**9**	**5**	**0**	**0**	**3**	**4**	**0**

*Numbers represent cumulative deaths according to each day