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Effect of biological additives on Japanese eel (*Anguilla japonica*) growth performance, digestive enzymes activity and immunology

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ABSTRACT

Japanese eel (*Anguilla japonica*) has become a commercially important fish species all over the world. High-density aquaculture has led to congestion and contributed to bacterial infection outbreaks that have caused high mortality. Therefore a 56-days feeding trial was conducted to determine the effects of dietary *Bacillus amyloliquefaciens* (GB-9) and *Yarrowia lipolytica* lipase2 (YLL2) on growth performance, digestive enzymes activity, innate immunity and resistance to pathogens of *A. japonica*. Fish growth performance was significantly affected by dietary YLL2 supplementation but not by GB-9. Fish fed diets with YLL2 at 2.0 g/kg diet in combination of high and low levels of GB-9 (5.0 g/kg and 2.0 g/kg) produced the highest growth. For digestive enzyme, lipase and trypsin activities was promoted by dietary containing YLL2, while amylase activities was increased by dietary containing YLL2, GB-9 single or combination. For innate immunity, the mucus lysozyme activity, leukocytes phagocytosis activity and reactive oxygen species level of skin, peroxidase and lysozyme activity of serum were enhanced in fish fed with GB-9 compared to those in control group ($p < 0.05$). The highest resistance to *Vibrio anguillarum* and *Aeromonas hydrophila* was determined in fish fed with 5.0 g kg^{-1} GB-9 + 2.0 g/kg YLL2. This study demonstrated that GB-9 and YLL2 enhanced non-specific immune defense system of *A. japonica*, providing them with higher resistance to pathogens. The present results suggested that the combination of these supplements could be considered as potential biological additives for aquaculture farmed fish.

1. Introduction

Japanese eel, *Anguilla japonica*, are catadromous fishes, spawning in waters west of the Mariana Islands [1,2]. It is a very important culture species in East Asia due to its high market value, desirable taste and recent supply shortage. The North Equatorial and Kuroshio currents transport the leptocephalus larvae over 4–6 months from the spawning ground to the continental shelf of the Asian countries, Taiwan, China, Korea and Japan [3,4]. They then metamorphose to glass eels before entering coastal waters where they become pigmented elvers in the estuaries. Most of the elvers migrate upstream to the river and grow to become yellow eels. At maturation, yellow eels become silver eels and migrate downstream to spawn and die [3,4]. However, recent studies have indicated that in the year of 2016, the fishing amount of natural Japanese eel has been decreased to 20% of that in 2003. The International Union for Conservation (IUCN) announced the addition of the Japanese eel into the red list of endangered species in 2018. Thus this shortage has recently become a serious problem, leading to extensive studies on techniques for farming the eel artificially.

In recent years, dietary administration of functional biological feed additives has been suggested as an environmental friendly alternative approach to enhance immunity and increase the growth performance of fish [5–12]. Regarding to the Japanese eel, the dietary supplementations also play a significant role in the blood chemistry and non-specific immune responses [13–18].

Bacillus subtilis, one of the most studied probiotics in fish and shrimp, has been reported to have various beneficial properties, including immunostimulant and disease resistance substances [12,19,20]. Several reports suggest that supplemented probiotics can improve growth, immune responses and alleviate fish resistance [21–24]. The most commonly used probiotics in aquaculture are *Bacillus, Lactobacillus* and *Saccharomyces* [20,25]. *Bacillus* sp. is a non-pathogenic

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aerobic gram-positive bacterium that enhances physical condition and GI microbial populations either by oral administration or by oral administration to bacterial water of the fish [7,26]. GB-9 is a non-pathogenic aerobic Gram-positive bacterium belonging to the genus B. subtilis, which produces various bacteriostatic substances as probiotics including antimicrobial proteins, lipopeptides, antibiotics, macrolides, oligopeptidases, polyketides, peptides and polyketides [27–29]. While GB-9 is added to fish diet, it plays a beneficial role in the intestine by inhibiting the growth of harmful intestine bacteria through producing antimicrobial substances, inhibiting the growth of competing bacteria. However, most research evaluate the effect of administering probiotics individually, modulation of immune responses using a combination of lipase and probiotics has rarely been investigated in Japanese eel.

Yarrowia lipolytica lipase2 (YLL2) is a typically lipase which has the characteristics of tolerance to the gastrointestinal environment, good biological safety, stable at low pH and superior temperature performance. YLL2 has been reported to have various beneficial properties when supplementing in juvenile fish diets, including reducing the amount of fish oil adding in diet, promoting the secretion of digestive enzymes [30–32]. More interestingly, YLL2 could efficiently hydrolyze the crude fish oil to produce polyunsaturated fatty acids, especially DHA and EPA, which would improve fecundity, egg hatchability, cell viability, immune functions and the overall quality of the fish [33–35].

Our previous research has indicated that dietary supplementation GB-9 and YLL2 has contributed to a significant increase in final weight of Hybrid sturgeon. DHA and EPA concentration, skin mucus lysozyme activity, leukocytes phagocytosis activity and reactive oxygen species level, and serum alternative complement pathway activity, peroxidase and lysozyme activity were proved in Hybrid sturgeon [36]. In order to broaden our horizon about the application of GB-9& YLL2 in aquatic product industry, the present investigation was carried out in Japanese eel (*Anguilla japonica*). In this study, except for the growth performance and other factors described above were determined [36], the digestive enzymes activity, resistance to pathogens after feeding trial, has also been further studied, to explore the relationship between digestive enzyme activity and growth performance, immunity and pathogenic bacteria resistance. In this research, universal relations between biological additives (GB-9& YLL2) and fish were revealed, it can not only constitute a new therapeutic strategy for disease infection, but also play a significant role in sustainable development of fisheries.

2. Materials and methods

2.1. Japanese eel

Japanese eel (18.9 g mean body weight) were reared in out-door cement ponds with 3.0 m^3 volume circulating with a constant flow (50 L/min) of filtered freshwater, the top of ponds were covered with black plastic net film at Hai-ning Riverside farms, China. 600 fish were randomly divided into 6 groups (100 fish per cement pond). The fish were fed twice daily, at 07:00 and 17:00 from May 2018 to July 2018 under natural environment. Aerators were operated to ensure that dissolved oxygen concentration was higher than 5.0 mg/L. Water temperature and pH were maintained at 25–28 °C and pH 7.5–8.5 respectively, during the experimental period. Uneaten feeds were scooped out after 1 h of feeding and cement ponds inside was scrubbed once per week to minimize algal and fungal growth.

2.2. Microorganisms and lipase

GB-9 strain was stored in 20% glycerol in our lab, Zhejiang Science and Technology University, China [29], the GB-9 preparation was 2×10^9 CFU/g, *Yarrowia lipolytica* was prepared by our previous experiment [36].

Table 1
The formulation of experimental groups (g/kg diet).

Ingredients[a]	Control	Group 2	Group 3	Group 4	Group 5	Group 6
Feed[b]	1000	998	998	995	996	993
GB-9	/	/	2.0	5.0	2.0	5.0
YLL2	/	2.0	/	/	2.0	2.0

[a] All diets were produced at Hai-ning Riverside farms, Zhejiang, China.
[b] Feeds was supplied as extruded powder by Fujian Tian Ma Technology Ltd. Co. Fujian, China.

2.3. Diet

For preparation of the experimental diets, the basal diet was supplemented with different levels of GB-9 and YLL2 (Table 1), The YLL2 concentration (2.0 g/kg, 8.25 U/g feed) employed was based on previous studies carried [37,38]. Briefly, GB-9 and YLL2 was added into the normal powder feeds at the desired concentration in the iron bucket, and appropriate water ($W_{mixture}$: W_{water} = 1:1.6) was added. Then the mixture was kneaded into dough and pre-incubation for 1.0 h in order to help hydrolyze the crude lipid in the place away from light.

2.4. Growth performance

The growth performance of Japanese eel fed different experimental diets was calculated at the end of the feeding trial, based on the following formulate:

2.4.1. Determination of SR, WG, SGR, FCR

Survival rate (SR, %) = 100 × number of final fish/number of initial fish;

Weight gain (WG) = final weight (g) - initial weight (g);

Specific growth rate (SGR, % day^{-1}) = 100 × [ln (final body weight) − ln (initial body weight)]/days;

Total amount of the feed consumed (g) = total amount of the fed food − collected uneaten feed/leaching loss rate;

Feed conversion ratio (FCR) = feed given (dried weight)/weight gain (wet weight).

2.4.2. Concentration of DHA and EPA

In the total lipid extract, concentration of DHA and EPA of Japanese eel muscle was determined. Total lipids were extracted and measured through dichloromethane method [39,40]. Fatty acid methyl esters were prepared by acid-catalyzed transmethylation of total lipids using boron trifluoride-methanol [41], and were analyzed in a Thermo Scientific Trace GC Ultra gas chromatograph (USA). The chromatograph was equipped with a Restek-Rtx-225, 60.0 m × 250 μm capillary column. Nitrogen was used as carrier gas (1.4 mL min^{-1}) and the thermal gradient was a constant temperature of 100 °C for 13 min, 100–180 °C at 10 °C min^{-1}, 180–200 °C at 1 °C min^{-1} and 200–230 °C at 4 °C min^{-1}. Injector and flame ionization detector temperatures were 270 °C and 280 °C, respectively. The fatty acid concentration of Japanese eel was determined with artificial fatty acid as a standard [36].

2.5. Digestive enzyme activity

Digestive enzyme (amylase, lipase and trypsin) activities were assayed at the end of the feeding experiment. The whole digestive tract and hepatopancreas were thoroughly homogenized in ice cold distilled water and centrifuged at 15000 g for 45 min at 4 °C. The supernatant was used as a crude enzyme source. Amylase activity was analyzed by the starchhydrolysis method [42]. Lipase activity was determined using the previous method [43]. Trypsin was determined using *N*-a-benzoyl-DL-arginine-4-nitroanilide hydrochloride as a substrate [44]. Specific activities of all digestive enzymes were calculated as U/mg protein.

2.6. Measurement of innate immunity

At the end of the feeding trial (56-days), 10 randomly selected fish from each group were selected for the immunological assays. The skin mucus was collected by scrapping the mucus off the eel's skin by clean slides. Briefly, The mucus samples were centrifuged to harvest supernatant after processing and stored at −80 °C for further use [45,46]. Whole blood samples were obtained from the caudal vein of each specimen with 10.0 mL syringe. After clotting at 4 °C, each sample was centrifuged (5000 g, 25 min, 4 °C) and the serum was collected and frozen at −80 °C until further analysis. Leukocytes were isolated from peripheral blood with 10.0 mL syringe (containing anticoagulant), each sample was centrifuged (100 g, 10 min, 4 °C) to remove erythrocytes. Leukocytes were extracted following previous method [45,46]. The protein concentration of skin mucus and serum was assayed with BCA method.

2.6.1. Reactive oxygen species level (ROS level)

Leukocyte were adjusted to the concentration of 3×10^6 cells/mL (V = 1.0 mL) with PBS buffer solution, the activity oxygen fluorescent probe was added until the final concentration reached 2.0 μM, incubated on rotary equipment for 50 min at room temperature without light [47,48]. After cells were washed three times with PBS, the fluorescence intensity was detected on the flow cytometer (BD-FACSAria II). Setting the assay with the parameters of PE-Texas Red-A, the average fluorescence intensity (MFI) of P1 PE-Texas Red-A was analyzed as the result.

2.6.2. Lysozyme activity (LZM)

The method described by Parry et al. was used for determination of lysozyme activity [41]. The lysozyme activity of mucus and serum was measured and expressed as μg/mL.

2.6.3. Peroxidase activity (POD)

Peroxidase activity was measured with peroxidase assay kit [49]. The optical density was read at 420 nm in a cuvette (optical path = 1.0 cm). Standard samples without serum or skin mucus were used as blanks. Peroxidase activity was defined as follows:

$$\text{Peroxidase}-\text{activity (U/mL)} = \frac{OD - ODblank}{12 \times \text{Optical path(1.0cm)}} \times \frac{Total\ \text{reaction volume(mL)}}{Sample\text{(mL)}} \div \text{Reaction time} \times \text{Diluting factor} \times 1000$$

2.6.4. Phagocytosis activity

Phagocytic activity was defined as the percentage of cells with one or more ingested fluorescent beads within the phagocytic cell population [50]. All samples (leukocytes) were analyzed in a flow cytometer (BD-FACSAria II) with an argonion laser adjusted to 488 nm. The quantitative study of the flow cytometric results was made using the statistical option of the Lysis Software Package (BD-FACSAria II) [51].

2.7. Resistance to pathogenic bacteria

After feeding with biological activity additives for 56-days, 40 fish were selected from each group to assay the resistance to pathogenic bacteria in other prepared water tanks following the previous dietary administration. It is reported that *Vibrio anguillarum* could produce exotoxin lethal to eel [52], *Aeromonas hydrophila* could cause septicemia and ascites of eel [53]. The most common pathogen in Japanese eel aquaculture, both *Vibrio anguillarum* and *Aeromonas hydrophila* were chosen as the challenging bacteria respectively in this experiment. The *V. anguillarum* (ATCC strain 19264) was incubated in 2261E medium for 36 h at 28 °C, *A. hydrophila* (ATCC strain 7966T) was incubated in nutrient broth for 24 h at 30 °C, then both were diluted with PBS buffer (50 mM, pH 7.5) to the final concentration of 1×10^8 cells/mL, the 10 μL dilution was intraperitoneally injected in fish (20 fish challenged with *V. anguillarum*, 20 fish challenged with *A. hydrophila*). The challenged fish were observed for 10 d for recording of the daily mortalities [8].

2.8. Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) to test the difference between a serious of feeding. Multiple comparisons among means were conducted using Duncan's procedure when significant difference was identified. Difference was regarded as significant when $p < 0.05$. All statistical analyses were performed by SPSS 18.0 software package.

3. Result

3.1. Growth performance

The growth performances of Japanese eel fed the diets containing alternative additives over a period of 56-days. There was a statistically significant increase of Group 2, 5, 6 in the specific growth rate (SGR), weight gain (WG), final weight (FW) compared with that of control ($p < 0.05$; Table 2). However, no significant differences in these parameters were observed between group 5 and group 6, group 3 and group 4. The concentration of DHA and EPA of Japanese eel has increased significantly in dietary supplementation with YYL2 single or combined with GB-9, was shown in Table 2.

3.2. Digestive enzyme (amylase, lipase and trypsin) activities

The amylase enzyme activity of Group 5, 6 was significantly higher than that of the other experimental groups and that of the Control group was the lowest ($p < 0.05$; Fig. 1).

The lipase activity is summarized in Fig. 1. The lipase activity observed in the group 2, 5, 6 was significantly higher than that of experimental groups 3, 4 and control group ($p < 0.05$).

The trypsin activity in the group 2, 5, 6 was significantly higher

Table 2
Effect of dietary on growth performance and feed utilization of Japanese eel fed experimental diets for 56-days.

Index	Control	Group 2	Group 3	Group 4	Group 5	Group 6
IW (g)	18.9 ± 0.3	18.8 ± 0.3	19.1 ± 0.1	18.7 ± 0.3	18.8 ± 0.4	19.1 ± 0.3
FW (g)	31.5 ± 2.9^b	34.1 ± 1.2^a	31.2 ± 1.5^b	31.4 ± 1.8^b	35.2 ± 1.4^a	35.5 ± 1.1^a
WG (g)	12.4 ± 2.4^c	15.3 ± 1.8^b	12.9 ± 1.6^c	12.7 ± 1.5^c	16.4 ± 1.7^a	16.6 ± 1.3^a
SGR (%)	0.91 ± 0.02^c	1.06 ± 0.04^b	0.87 ± 0.09^c	0.93 ± 0.05^c	1.12 ± 0.11^a	1.11 ± 0.07^a
FCR	1.62 ± 0.12^a	1.31 ± 0.07^c	1.56 ± 0.11^b	1.58 ± 0.02^b	1.23 ± 0.05^c	1.21 ± 0.09^c
DHA (%)$^\alpha$	1.47 ± 0.03^c	2.08 ± 0.02^b	1.66 ± 0.04^c	1.69 ± 0.02^c	2.24 ± 0.04^a	2.27 ± 0.05^a
EPA (%)$^\beta$	0.79 ± 0.09^c	1.21 ± 0.05^b	0.85 ± 0.03^c	0.82 ± 0.04^c	1.26 ± 0.02^a	1.32 ± 0.02^a

IW = initial weigh fish-1; FW = Final weight fish-1; SGR = Specific growth rate fish-1; FCR = Feed conversion ratio.
Data are mean ± SEM. Means not bearing the same superscript letters in the same row are significantly different ($p < 0.05$).
DHA (%)$^\alpha$ means the concentration of DHA in Japanese eel muscle; EPA(%)$^\beta$ means the concentration of EPA in Japanese eel muscle.

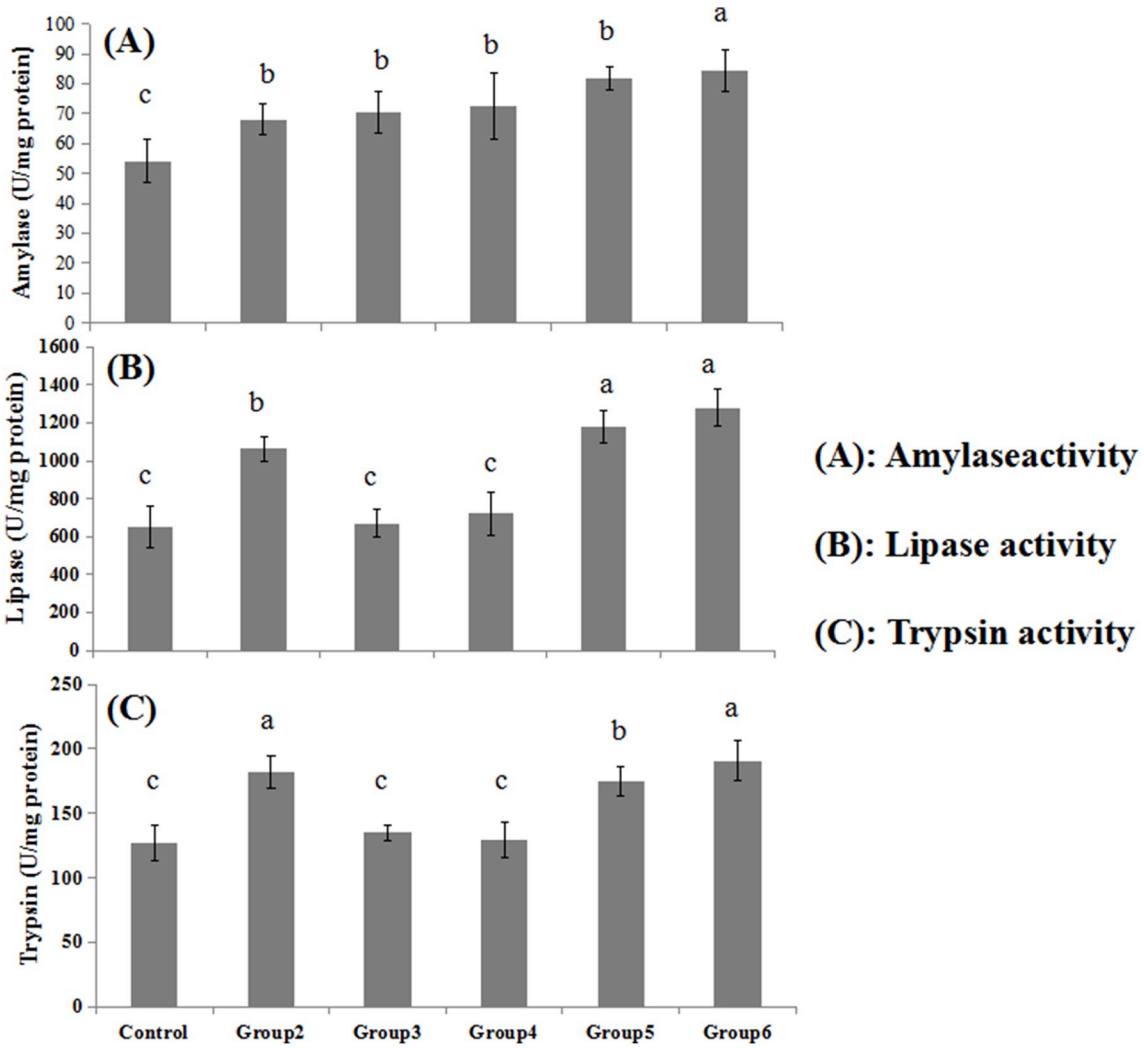

Fig. 1. The amylase activity, lipase activity and trypsin activity in Japanese eel after fed with additives for 56-days. Control Group (0-control); Group 2 (2.0 g/kg YLL2); Group 3 (2.0 g/kg of GB-9); Group 4 (5.0 g/kg of GB-9); Group 5 (2.0 g/kg YLL2 + 2.0 g/kg of GB-9; Group 6 (2.0 g/kg YLL2 + 5.0 g/kg of GB-9). Values are expressed as mean ± SE (n = 3). Different letters above bars indicate significant differences between groups ($p < 0.05$).

than that in the control group and the other experimental groups. The lowest trypsin activity was determined in the group 3, 4 and control group, Fig. 1 ($p < 0.05$).

3.3. Mucosal lysozyme activity assays

The mucosal immunity of Japanese eel fed on different additives was evaluated after 56-days of the treatment. The result showed that lysozyme activity significantly increased following 56-days of the feeding trial with the GB-9 and YLL2 diets compared to the control diet (Table 3). The highest values 11.4 ± 0.2 (μg/mL) and 12.0 ± 0.3 (μg/mL) were recorded for fish fed supplements in group 5 and 6.

3.4. Leukocytes immunological response

To elucidate the cellular immune response, phagocytosis rates and ROS levels were determined between control group and supplemented groups after 56-days feed trail. It has been reported that phagocytosis plays an important role in the host-defense mechanisms and contributes to inflammation and the immune response. Phagocytosis activity was

Table 3
Skin mucus lysozyme activity, phagocytosis rates, ROS levels, serum mucus lysozyme activity and peroxidase activity of Japanese eel after 56-days of feeding trial fed on different diets.

	Control	*Group 2*	*Group 3*	*Group 4*	*Group 5*	*Group 6*
Skin mucus lysozome activity (μg/mL)	7.9 ± 0.3^{c}	8.2 ± 0.4^{c}	9.1 ± 0.2^{b}	9.5 ± 0.3^{b}	11.4 ± 0.3^{a}	12.0 ± 0.3^{a}
Phagocytosis rates (%)	17.9 ± 1.3^{c}	18.2 ± 1.5^{c}	29.1 ± 1.2^{b}	33.5 ± 1.4^{a}	30.2 ± 1.6^{b}	35.2 ± 1.3^{a}
ROS Level (1000MFI)	2.9 ± 0.2^{c}	4.2 ± 0.3^{b}	5.1 ± 0.5^{b}	5.5 ± 0.2^{b}	5.2 ± 0.6^{b}	6.2 ± 0.6^{a}
Serum mucus lysozome activity (μg/mL)	4.9 ± 0.2^{c}	4.8 ± 0.3^{c}	6.1 ± 0.4^{b}	8.5 ± 0.3^{b}	6.5 ± 0.5^{b}	9.0 ± 0.3^{a}
Peroxidase activity (U/mL)	7.0 ± 0.2^{c}	7.5 ± 0.2^{c}	8.2 ± 0.2^{b}	9.5 ± 0.2^{a}	7.6 ± 0.2^{c}	9.9 ± 0.2^{a}

*Control Group (0-control); Group 2 (2.0 g/kg YLL2); Group 3 (2.0 g/kg of GB-9); Group 4 (5.0 g/kg of GB-9); Group 5 (2.0 g/kg YLL2 + 2.0 g/kg of GB-9; Group 6 (2.0 g/kg YLL2 + 5.0 g/kg of GB-9). Values are expressed as mean ± SE (n = 3). Different letters above bars indicate significant differences between groups ($p < 0.05$).

analyzed by flow cytometry, which showed that the phagocytic percentage of Japanese eel leukocytes fed on different supplemented diets was promoted with varying degrees compared with that of the control (Table 3), indicating that YLL2 and GB-9 enhanced the phagocytosis activity of leukocytes. Reactive oxygen species (ROS) is of fundamental importance for host defense and cellular signaling [54]. As shown in (Table 3), ROS levels showed that Japanese eel treated with supplement in diets increased as compared to control.

3.5. Serum immunological response

To elucidate the serum immunological response, peroxidase activity, lysozyme activity, were observed between control group and supplemented groups after 56-days feed trail.

Peroxidase may play an important role in increasing host defenses against pathogens [55]. Lysozyme is an important vertebrate defense factor against invasive microorganisms [40]. Lysozymes lysis Gram-positive bacteria and kill Gram-negative bacteria, except opsonin which promotes phagocytosis [56]. To determine the effect of supplement diet in immunity, peroxidase activity, lysozyme activity were performed in serum of Japanese eel respectively. Results (Table 3) showed that YYL2 and GB-9 induced the upregulation of peroxidase, lysozyme, compared with that of control, Among the serum immune response, lysozyme activity was the most affected by dietary supplementation, which doubled comparing with that of control group.

3.6. Resistance to V. anguillarum and A. hydrophila

Relative survival rates of different experimental fish groups challenged with *V. anguillarum* and *A. hydrophila* are illustrated in Fig. 2 and Fig. 3. The relative survival rate of Japanese eel challenged with *V. anguillarum* was significantly higher in group 6 than in the control and the other experimental groups. The survival rate of the group 2 was similar to that of the control group (Fig. 2). Moreover, the highest survival rate of Japanese eel challenged with *A. hydrophila* was also determined in group 6. Fish in the group 2 had similar survival rates as those in the control group (Fig. 3).

4. Discussion

According to the latest report, The Japanese eel population, has declined drastically in recent years, presumably due to a combination of overfishing and habitat loss or changing water conditions in the ocean interfering with spawning and the transport of their leptocephali. Japanese eel, just like other fish species in commercial aquaculture, is usually reared in enclosed spaces and efforts have been made to increase productivity per unit space [57]. This development has led to overcrowding, which tends to adversely affect the immune system and become more susceptible to disease [58]. Thus, developing the safe and sustainable alternative of veterinary antibiotics in aquaculture health management has received heighten attention [59]. However, the study of *Bacillus* and exogenous lipase supplement in Japanese eel is limited.

For immunity proving part, *Bacillus* sp. in fish can increase disease resistance by stimulating cellular and humoral immune functions, including phagocytic activity, lysozyme activity and peroxidase activity [5,60]. Peroxidase as well as Phagocytosis play an important role in the host-defense mechanisms and contributes to innate immunity [51,61,62]. During phagocytosis, lysozyme activates phagocytosis, phagocytic cells produce a high degree microbiocidal reactive oxygen specificity (ROS) and attack invading pathogens [10,61,63]. In this study, lysozyme activity of skin mucus and serum, peroxidase activity of serum are both greatly enhanced, when GB-9 is added at 10^7 CFU/g (group 4) or 4×10^6 CFU/g (group 3). Our data also evidenced ROS level and phagocytic activity of Japanese eel, which fed with GB-9 increased significantly compared with that of control. Interestingly, all data in this study showed the high level of GB-9 supplementation contributed to a higher effect on immunity than that of low level. Regarding to the survival rate, Zhang et al. demonstrated that resistance of juvenile ovate pompano against *Vibrio vulnificus* improves after 8-week B. *subtilis* dietary administration [64]. In this study, the supplementation level of GB-9 has a positive correlation with the survival rate. The high survival rate may be attributable to the higher level of skin mucus lysozyme activity, the higher level of phagocytosis activity and ROS, which could help Japanese eel promote the leukocyte migration and eliminate the pathogenic bacterium.

Considering effectiveness of growth performance, it has been reported that supplementing lipase can improve the fat digestion, *Bacillus*

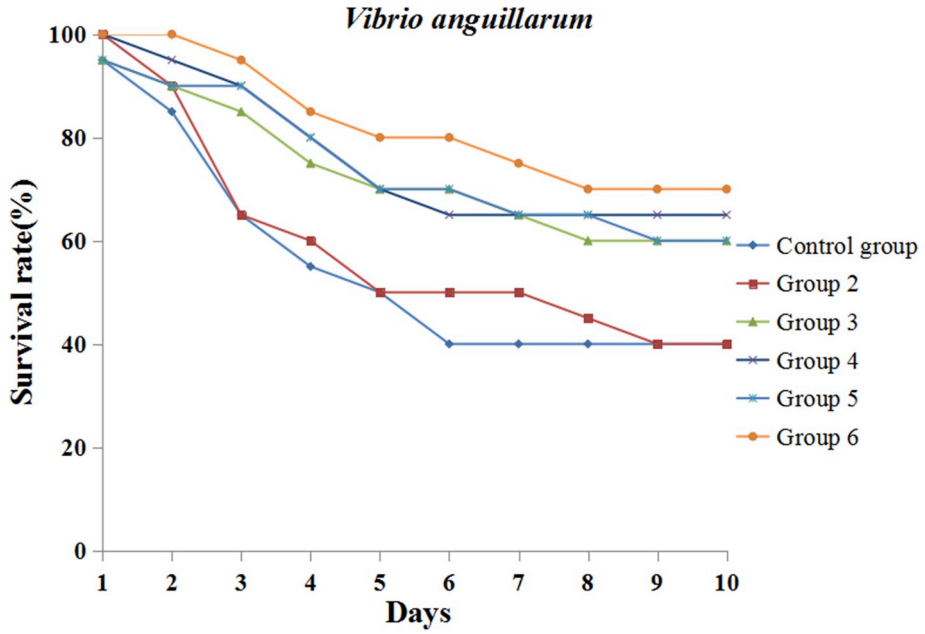

Fig. 2. Survival rates of Japanese eel fed with different diets challenged with *V. anguillarum*: Control Group (0-control); Group 2 (2.0 g/kg YLL2); Group 3 (2.0 g/kg of GB-9); Group 4 (5.0 g/kg of GB-9); Group 5 (2.0 g/kg YLL2 + 2.0 g/kg of GB-9; Group 6 (2.0 g/kg YLL2 + 5.0 g/kg of GB-9).

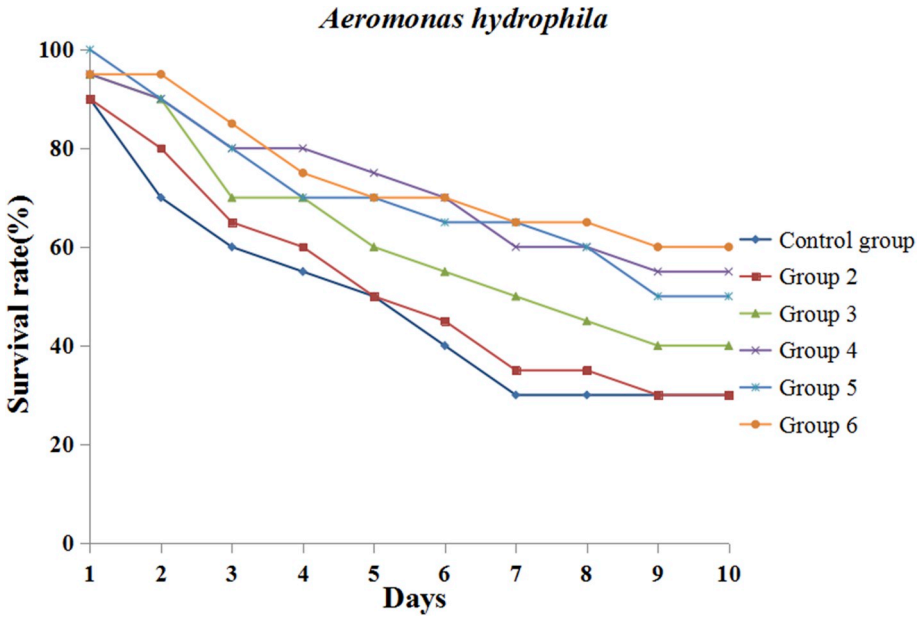

Fig. 3. Survival rates of Japanese eel fed with different diets challenged with *A. hydrophila*: Control Group (0-control); Group 2 (2.0 g/kg YLL2); Group 3 (2.0 g/kg of GB-9); Group 4 (5.0 g/kg of GB-9); Group 5 (2.0 g/kg YLL2 + 2.0 g/kg of GB-9; Group 6 (2.0 g/kg YLL2 + 5.0 g/kg of GB-9).

exo-enzymes are very efficient at metabolizing a large variety of carbohydrate, lipids, and proteins [6,65]. Fat digestion can produce fatty acids in the intestines of mammals and fish. It has been evidenced that dietary medium-chain fatty acids may affect bacterial metabolites and thus affect the intestinal health of weaned piglets [66]. Therefore, exogenous lipase supplementation may affect the fish's gut by raising fat digestion to improve health levels, which requires further investigation [67]. In this research,GB-9 diet supplements (high or low level) can significantly increase the amylase activities of Japanese eel while both lipase and trypsin activities were rarely fluctuated. This may result from GB-9 secretes amylase with a high level, the improvement in digestive tract enzyme activities maybe partially due to enzymes synthesized by the bacteria. However, the proportion of enzymes contributed by bacteria cannot be assayed since the probiotic may also stimulate the production of endogenous enzymes in the fish [21,68]. The addition of exogenous lipase YLL2 into diets can significantly promote the amylase, lipase and trypsin activity in the whole digestive tract and hepatopancreas, improve the growth performance of fish. This result demonstrates that the growth performance of Japanese eel may has a positive correlation with its digestive enzyme activity, which needs further investigation. Besides the DHA&EPA concentration in Japanese eel muscle has also been increased after feeding with YYL2, which revealed that YYL2 could help fish hydrolyze lipid in the feed.

As expected, supplementation of GB-9 and YLL2 combination improved the growth performance, enhanced digestive enzyme activity and promoted the immune stimulation of Japanese eel than supplementation of GB-9 or YLL2 singularly in this study. This synergistic action might be due to the high concentration of DHA and EPA hydrolyzed by YYL2 that improved the poor establishment of the GB-9 in the gastrointestinal tract of Japanese eel and might have promoted the growth of GB-9 [69], However, the detail mechanism is unclear, warrants further investigation.

In short, current research demonstrated that dietary supplementation of YLL2 with GB-9 can significantly increase the growth performance, promote digestive enzyme activity, innate immunity and survival rate of Japanese eel. This study may constitute a new strategy for fish feeding supplementation of exogenous lipase and *Bacillus*. However, the precise mechanism of how YLL2 and GB-9 stimulate growth digestive enzyme activity and immune system of Japanese eel is not clarified as yet and further investigation on this aspect is needed.

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