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Impacts of Dietary Supplementation of Peptidoglycan Extracted from *Lactobacillus* sp. on the Growth Performance and Resistance to *Streptococcus Agalactiae* of Nile Tilapia

Nguyen Thi Mai[1*], Mai Van Tung[1], Do Hoanh Quan[2], Vu Thi Thanh Huong[3] & Nguyen Thu Hang[4]

[1]Faculty of Fisheries, Vietnam National University of Agriculture, Hanoi 131000, Vietnam
[2]Bio-floc Co., Ltd., Lien Hiep, Phuc Tho 153600, Hanoi, Vietnam
[3]Faculty of Aquaculture, Ha Long University, Quang Ninh 208220, Vietnam
[4]Pharmacology Department, Hanoi University of Pharmacy 111000, Hanoi, Vietnam;

Abstract

The current experiment aimed to determine the effects of peptidoglycan, known as a prebiotic compound, on the growth, feed efficiency, and disease resistance in Nile tilapia. Fish at an initial body weight of 22.6 ± 0.3g were distributed into a 100 L-glass tank system. Peptidoglycans extracted from *Lactobacillus* sp. were added to commercial feed at ratios of 0, 5, and 10 g kg^{-1} diet corresponding to the PG0, PG5, and PG10 treatments, respectively. Fish were then fed at 4% their body weight for four weeks. After a 4-week trial, fish were infected with *Streptococcus agalactiae* at 50% the lethal dose (1.1×10^6 CFU mL^{-1}), and monitored for 14 days. After 2 and 4 weeks of the feeding trial (T2 and T4) and on the second day of the bacterial challenge, fish blood samples were collected for hematological analysis. The results indicated that the dietary supplementation of peptidoglycan induced a positive effect on fish growth performance and the highest value was observed in the PG5 treatment. The lowest value of cumulative mortality was also observed in the PG5-fed fish indicating that the dietary supplementation at 5 g kg^{-1} diet supported the highest resistance to *S. agalactiae*. In conclusion, the beneficial effects of dietary supplementation of peptidoglycan extracted from *Lactobacillus* sp. were recorded on the growth performance and disease resistance in Nile tilapia.

Keywords

Prebiotics, cumulative mortality, disease resistance, peptidoglycan, *Streptococcus agalactiae*

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Correspondence to
ntmai.ntts@vnua.edu.vn

ORCID
Nguyen Thi Mai
https://orcid.org/0000-0001-6754-4385

Introduction

The immune system of fish, including both innate (non-specific) and adaptive (specific) immunology, plays a very important role in

the resistance of fish to pathogenic diseases (Dalmo & Ingebrigtsen, 1997). The innate immune system of fish can be stimulated by various exogenous agents including probiotics, prebiotics, plant extracts, or other bioactive compounds (Nguyen *et al*., 2016, 2021; Nhu *et al*., 2019). These substances can enhance immune parameters such as lysozymes, complement, macrophages, peroxidase activities, and other immune indicators. Among them, prebiotics are considered to be of increasing interest in recent years because of their positive effects on fish immune system stimulation. Prebiotics are indigestible ingredients that selectively and beneficially affect the host by stimulating the growth and/or activity of one or a limited number of bacteria in an animal's intestine (Akhter *et al*., 2015). The immunomodulatory action of prebiotics occurs due to their direct interaction with the immune system, or by enhancing the growth of a synergistic microbiome (Dawood *et al*., 2018). Positive impacts have previously been reported in fish fed dietary prebiotics such as β-glucan, LPS, and lactoferrin (Nguyen *et al*., 2020).

Peptidoglycan is one of the major components of the gram-positive bacteria cell wall (McDonald *et al*., 2005). This substance has been documented as a prebiotic compound as well as an immunostimulant in mammals and several aquatic species (Zhou *et al*., 2006; Casadei *et al*., 2015; Pan *et al*., 2015). Pan *et al*. (2015) indicated that dietary supplementation of peptidoglycan (Chemoforma Ltd., Augst, Switzerland) at 0.18 g/kg in black tiger shrimp (*Penaeus monodon*) enhanced the fish's immune responses including the total haemocyte count, phenoloxidase, and respiratory burst activities. Moreover, a significant improvement in growth performance was also demonstrated. Other studies carried out on fish such as rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) (Casadei *et al*., 2015) and Japanese flounder (*Paralichthys olivaceus* Temminck & Schlegel, 1846) (Zhou *et al*., 2006) also documented the beneficial effects of dietary peptidoglycan (EWOS and extracts from *Bifidobacterium* sp., respectively) on fish growth, disease resistance, and immune responses. However, the results were still limited and no results have been reported in Nile tilapia.

Nile tilapia (*Oreochromis niloticus*) is a freshwater fish with many outstanding advantages compared to other species due to its filet quality, fast growth rate, and ability to adapt to various rearing conditions. Therefore, tilapia farming is increasing in terms of both scale and farming area. As with many other economic fish species, intensive fish farming is always accompanied by problems of low growth and disease outbreaks. To mitigate fish diseases, farmers usually use chemical and antibiotic products to treat the aquatic animals and environment; however, these solutions are often accompanied by adverse effects on the environment and consumer health due to residues of these products. Consequently, the use of solutions to increase the resistance of aquatic animals in general and of Nile tilapia in particular is very necessary. Among the pathogens that cause serious diseases in tilapia, gram-positive *Streptococcus* sp. bacteria are considered to be the main cause of the high fish mortality rate in Nile tilapia farms (Abuseliana *et al*., 2010; Alazab *et al*., 2022).

Based on the above arguments, the current study was carried out to estimate the effects of dietary supplementation with a prebiotic compound derived from beneficial gram-positive bacteria on the growth, feed utilization efficiency, cellular immunity, and resistance against *Streptococcus agalactiae* in Nile tilapia.

Materials and Methods

The protocols of the feeding trial and challenge test were approved by the Vietnam National University Animal Ethics Committee (T2022-14-53).

Fish

Juveniles of monosex male Nile tilapia were collected from a local hatchery farm (Golden Fish Farm Dung Quat, Thanh Mien, Hai Duong, Vietnam) and acclimatized in the Aquaculture Nutrition and Feed wet-lab, Faculty of Fisheries, Vietnam National University of Agriculture (VNUA) for 14 days. During this period, fish

were fed with commercial feed (35% crude protein, Agrifeed). The healthy and disease-free fish were then collected for the experiment.

Diet preparation

Peptidoglycans derived from *Lactobacillus* sp. (Bio-floc Ltd. Co., Vietnam) in powder form were suspended in a constant volume of distilled water (10mL per 100g feed) at various concentrations of 0, 5, and 10 g kg^{-1} diet corresponding to the PG0, PG5, and PG10 groups, respectively. Each homogenous solution was then mixed with commercial feed. Experimental feeds were air-dried at room temperature for 15 minutes and prepared daily.

Feeding trial

Nile tilapia juveniles with an initial average weight of 22.6 ± 0.3g were allocated into the experimental tanks of 100L at a density of 25 fish per tank. Fish were fed the commercial feed supplemented with/without peptidoglycan twice a day at a ration of 4% body weight for 28 days. Each experimental group was replicated three times. The tank system was maintained with continuous circulation and aeration. The environmental parameters of temperature (27-29ºC), oxygen (6-7 mg L^{-1}), pH (7-8), nitrites (< 0.05 mg L^{-1}), and NH_3/NH_4^+ (< 0.05 mg L^{-1}) were maintained to the suitable requirements of Nile tilapia. The experimental tanks were siphoned daily and about 20% of the water volume was renewed. Fish in each tank were weighed weekly to monitor their growth rate and to regulate the daily feed amount. After 28 days of the feeding period, the fish in each tank were counted and weighed to calculate the survival rate and husbandry parameters. Moreover, the intestinal indices, namely the gut length and the weights of the liver, viscera, and gut, were also recorded and calculated. The formulas used followed those cited in the study of Nguyen *et al*. (2022):

Weight gain (WG, %) = 100 × (FBW – IBW)/IBW

Specific growth rate (SGR, %/day) = 100 × (ln FBW - ln IBW)/T

Daily weight gain (DWG, g/fish/day) = (FBW - IBW)/T

(where FBW and IBW are the final and initial body weights, respectively and T is the days of the feeding period)

Protein efficiency ratio (PER) = fish weight gain/protein consumption

Feed conversion ratio (FCR) = consumable feed amount (dried weight)/fish weight gain (wet weight)

Survival rate (%) = 100 × (final fish number/initial fish number)

Hepatosomatic index (HSI, %) = 100 × liver weight/fish body weight

Gastro-somatic index (GaSI, %) = 100 × gut-weight/fish body weight

Visceral somatic index (VSI, %) = 100 × visceral weight/fish body weight

Bacterial challenge

Streptococcus agalactiae bacteria isolated from diseased Nile tilapia were identified and stored at -20ºC in the Department of Environment and Aquatic Diseases, Faculty of Fisheries, Vietnam National University of Agriculture (VNUA), and used for bacterial infection. Briefly, *S. agalactiae* was cultured in an NB (nutrient broth) medium at 28°C for 48h. The bacterial solution was then centrifuged at 5000×g for 5min. to collect the bacteria. The bacteria were then suspended in 0.85% physiological saline and diluted to a concentration of 1.1×10^6 CFU mL^{-1} (LD_{50}). The LD_{50} used followed the results published in the study by Sherif *et al*. (2022) and was tested with local fish before conducting the bacterial challenge.

On day 29 of the experiment, a batch of 30 fish per experimental group was subjected to the bacterial challenge with *S. agalactiae* at a dose of LD_{50} (0.1mL per fish). These fish were then distributed to an isolated tank system of 100L. Infected fish were monitored for 14 days and the number of dead fish was recorded daily. Dead fish with pathological symptoms caused by *S. agalactiae* in tilapia as described in Pretto-giordano *et al*. (2010) and Zhang (2021) were dissected immediately and re-isolated to confirm the cause of death. The liver and head kidney tissue samples were firstly Gram stained and

observed under the microscope. A small amount of each tissue sample was then put in the TSA medium to culture the bacteria. An isolated colony was used for Gram staining to determine the pathogenic bacteria. The pathogenic bacteria samples were then identified by the colony and bacterium form and biochemical kit. The external and internal morphology of the dead fish were also observed.

Sample collection and analysis

After 14 (T2) and 28 days (T4) of the feeding trial, and on the second day of the bacterial challenge (day 29), blood samples were collected to analyze the hematology parameters. The hematological parameters, namely total red blood cell count (RBC), total white blood cell count (WBC), lymphocytes, monocytes, neutrophils, and hematocrit (HCT), were analyzed according to the manufacturer's procedures using veterinary analyzers (URIT-3000 VETPLUS).

Data analysis

Mean values were checked for homogeneity by a univariate test, and the data were then subjected to a one-way analysis of variance (one-way ANOVA) using the replicate tank as the statistical unit for the husbandry and fish mortality variables (n = 3). Two-way analysis of variance (two-way ANOVA) using the replicate samples for each experimental condition as the statistical unit for the other parameters (n = 9) was then carried out, followed by a LSD post-hoc test. Comparisons between the data of the feeding trial and bacterial challenge were subjected to T-Test. Differences among treatments were considered significant at *P*-value <0.05. All data were analyzed using the statistical package of STATISTICA 10.0 software.

Results

Husbandry parameters and intestinal indices

After the 28-day feeding trial, the growth performance factors of daily weight gain (DWG, 1.07-1.21 g/day/fish), specific growth rate (SGR, 3.02-3.27%/day), and weight gain (WG, 132.83-149.83 %); the feed utilization efficiencies of feed intake (FI, 1.10-1.21) and feed conversion rate (FCR, 1.08-1.16); and the survival rate (100%) were calculated (**Table 1**). Accordingly, significant differences were observed only in the growth parameters. Specifically, the highest growth rate value was recorded in fish fed on the PG5 diet and the lowest was found in the PG0 group (P <0.05). The average weight gain of the PG5-fed fish (149.83%) was higher than that of the PG0-fed group (132.83%) (P <0.05) but comparable to the PG10-fed group. Moreover, the low value of FCR and the absolute value of the survival rate demonstrated that the rearing conditions, namely the commercial feed and environmental parameters, in the current experiment were suitable for Nile tilapia.

Table 1. Growth performance, feed utilization, and survival of fish fed on diets supplemented with or without peptidoglycan for 28 days

Parameters	Experimental groups		
	PG0	PG5	PG10
IBW (g/fish)	22.5 ± 0.1	22.7 ± 0.3	22.5 ± 0.5
FBW (g/fish)	52.3^{a} ± 0.9	56.7^{c} ± 1.2	54.6^{b} ± 0.2
DWG (g/fish/day)	1.07^{a} ± 0.03	1.2^{c} ± 0.0	1.1^{b} ± 0.0
SGR (%/day)	3.0^{a} ± 0.1	3.3^{c} ± 0.1	3.2^{b} ± 0.1
WG (%)	132.8^{a} ± 5.1	149.8^{b} ± 4.5	142.3^{ab} ± 4.7
FI (g/fish/day)	1.1 ± 0.1	1.2 ± 0.0	1.2 ± 0.0
FCR	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
Survival rate (%)	100	100	100

Note: PG0, PG5, and PG10 were the commercial feed supplemented with peptidoglycan at 0, 5, and 10 g kg^{-1} diet, respectively. IBW: initial body weight, FBW: final body weight, DWG: daily weight gain, SGR: specific growth rate, WG: weight gain, FI: feed intake, FCR: feed conversion rate. The values with a common letter denote non-significant differences (P >0.05).

The intestinal indices of visceral somatic index (VSI), hepatosomatic index (HSI), and gastro-somatic index (GaSI), and relative gut length are presented in **Table 2**. The influence of the utilization and duration of dietary peptidoglycan was expressed in the GaSI variable (P <0.05) while the VSI, HSI, and relative gut length were not different. Specifically, the GaSI indices observed in the PG5- and PG10-fed fish at T2 were higher than those measured at T0 whereas no differences were recorded for the PG0-fed fish compared to T0.

Haematological indices

The hametological indices at T2, T4, and after the bacterial challenge are presented in **Figure 1** for total white blood cells (WBC, ranging from 62.8 to 69.0×10^9/mL; 71.5 to 88×10^9/mL; and 77.7-81.9×10^9/mL, respectively), lymphocytes (LYM, 28.2-29.1×10^9/mL; 29.7-33.0×10^9/mL; and 30.0-32.0×10^9/mL), monocytes (MID, 22.6-24.5×10^9/mL; 25.5-30.9×10^9/mL; and 25.9-28.4×10^9/mL), granulocytes (GRAN, 12.1-15.4×10^9/mL; 16.3-24.1×10^9/mL; and 18.8-21.5×10^9/mL), total red blood cells (RBC, ~1.0 ×10^{12}/mL; 1.1-1.4×10^{12}/mL; and 1.3-1.4×10^{12}/mL), and hematocrite (HCT, 13.8-14.6 %; 15.9-20.4%; and 19.3-19.8%). Differences were only found in the RBC variable after two weeks (T2) of the feeding trial (P <0.05) indicating the influence of the dietary supplementation of peptidoglycan on this parameter (**Figure 1**). Specifically, the value of RBC in PG5-fed (1.4×10^{12}/mL) fish was higher

Table 2. Intestinal indices of fish fed on the diet supplemented with or without peptidoglycan for 14 (T2) and 28 days (T4)

Variables	Sampling						
	T0	T2			T4		
		PG0	PG5	PG10	PG0	PG5	PG10
VSI (%)	9.1 ± 1.5	9.7 ± 1.0	11.3 ± 1.4	11.2 ± 1.7	9.6 ± 1.7	9.8 ± 0.7	9.7 ± 1.8
HSI (%)	1.8 ± 0.7	1.9 ± 0.4	2.0 ± 0.6	2.0 ± 0.4	2.0 ± 0.4	2.1 ± 0.3	2.1 ± 0.4
GaSI (%)	4.8^{a} ± 1.2	6.3^{ab} ± 1.3	7.7^{b} ± 1.5	7.3^{b} ± 2.2	5.7^{ab} ± 1.4	5.7^{ab} ± 0.5	5.9^{ab} ± 1.4
Relative gut length (%)	416.4 ± 58.6	574.0 ± 95.3	525.9 ± 97.5	531.1 ± 97.1	477.7 ± 93.5	465.8 ± 63.5	530.3 ± 112.0

Note: PG0, PG5, and PG10 were the commercial feed supplemented with peptidoglycan at 0, 5, and 10 g/kg diet, respectively. VSI: Visceral somatic index. HSI: Hepatosomatic index. GaSI: Gastro-somatic index. T0, T1, and T2 were the samplings at day 0, 14th, and 28th days of the feeding trial, respectively. The values with a common letter denote non-significant differences (P >0.05).

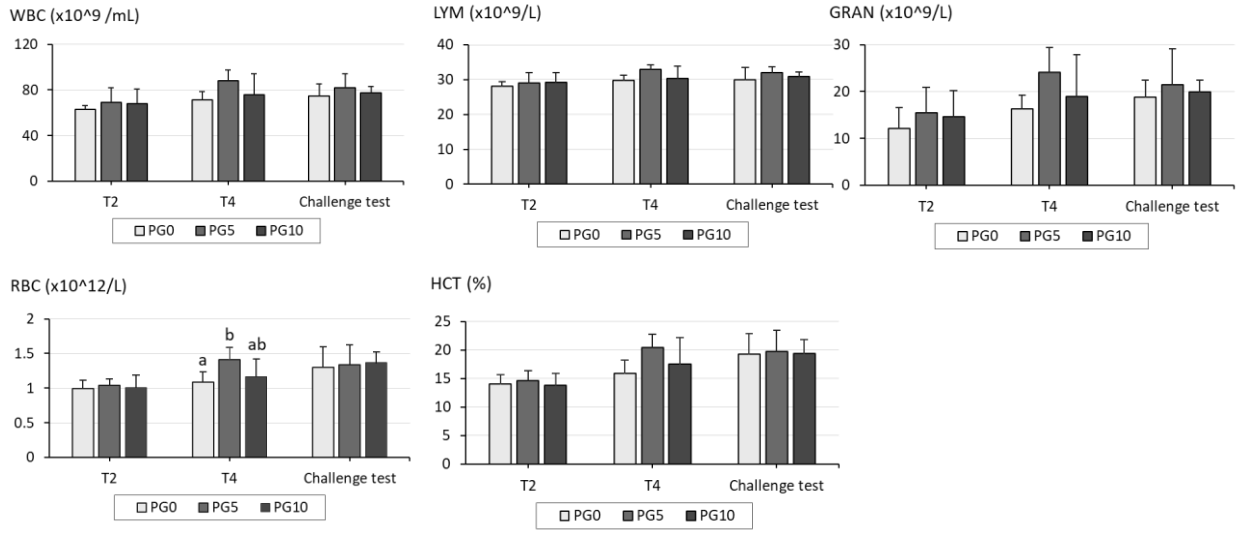

Figure 1. Hematological parameters in fish fed on the diet supplemented with (PG5, PG10) or without peptidoglycan (PG0) at T2, T4, and after the bacterial challenge. WBC: white blood cells, LYM: lymphocytes, GRAN: granulocytes, RBC: red blood cells, HCT: hematocrit

(P <0.05) than that observed in the control (1.1×10^{12}/mL) and comparable to the PG10-fed ones (1.2×10^{12}/mL).

Cumulative mortality after challenge to *Streptococcus agalactiae*

The cumulative mortality rates observed in the experimental groups after 14 days of challenge with *S. agalactiae* are presented in **Figure 2**. Accordingly, the highest mortality was found in the PG0-fed fish (63.3 ± 11.6%) while the PG5-fed (33.3 ± 15.3%) and PG10-fed groups (33.3 ± 11.5%) displayed lower values (P <0.05). Dead fish were observed beginning on the 5th day of bacterial infection in the PG0-fed group and the highest mortality was observed on the 6th day of the challenge test. On the other hand, the dead fish in the PG5 and PG10-fed groups were found from the 6th day of the bacterial challenge and the mortality increased gradually. From the 10th day, no more dead fish were observed in the peptidoglycan-fed groups while the cumulative mortality continued increasing until the 13th day of the challenge test.

The results of the fish observations after bacterial infection are shown in **Figure 3**. Specifically, the external and internal morphologies of the dead fish showed the specific symptoms of disease caused by *S. agalactiae* such as having a body dark, one or both eyes bulging and cloudy, and hemorrhages in the fins and operculum. The abdominal cavity contained a lot of fluid, and the intestines were bleeding and contained air bubbles. There was also fluid in the abdomen. Moreover, the Gram-stained samples of head kidney also showed the blue bacteria (**Figure 3-D**). The colonies isolated from the dead fish were round, convex in the middle, cream colored, and about 1mm in size (**Figure 3-E**). Stained bacteria showed that *S. agalactiae* is a Gram (+), circular, chain-linked bacteria with some being discrete (**Figure 3-F**).

Discussion

Fish growth, feed utilization, and survival

The current study reported the results about the influences of dietary supplementation of peptidoglycan in Nile tilapia for the first time. The previous data were pricipally reported in shrimps (Itami *et al*., 1998; Purivirojkul *et al*., 2006), rainbow trout (Casadei *et al*., 2013), sea cucumber (Zhang *et al*., 2014), Japanese flounder (Zhou *et al*., 2006), and common carp (Yin & Yang, 2022). The obtained results demonstrate the interesting application of this kind of bioactive compound in this economic species. The fish growth performances in the experimental groups fed with dietary supplementation of peptidoglycan were higher

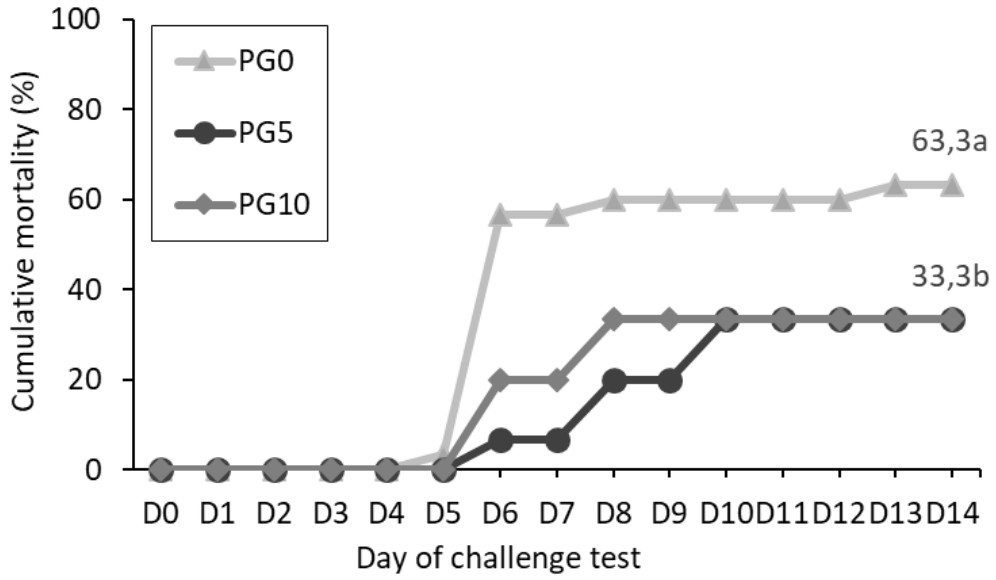

Figure 2. Cumulative mortality (%) of experimental fish after 14 days of bacterial challenge

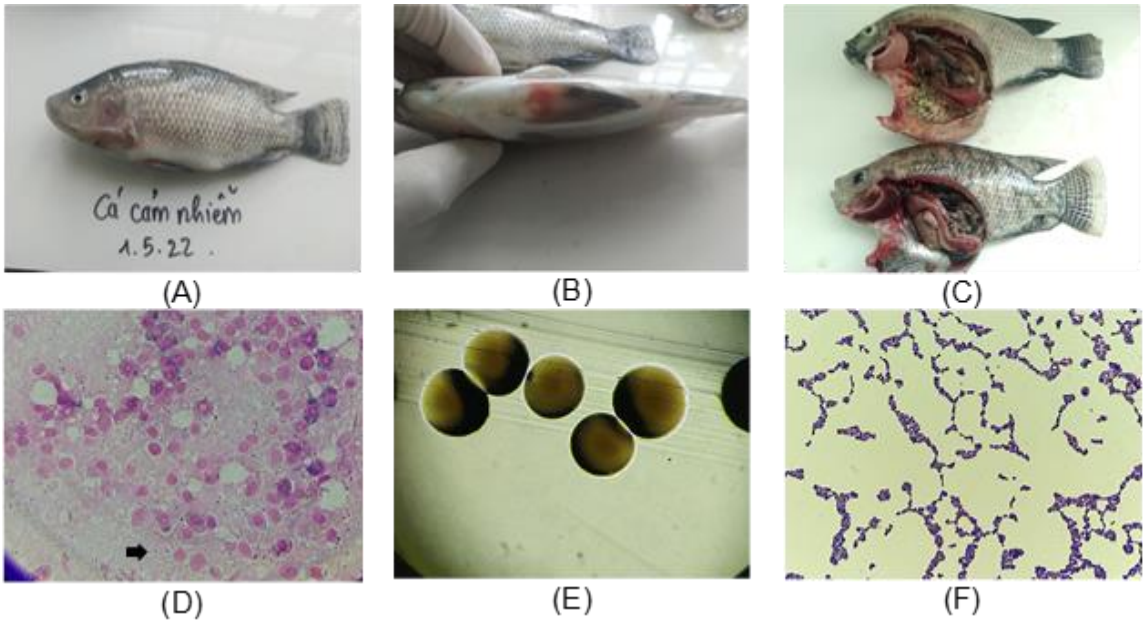

Figure 3. Images of dead fish after bacterial infection including the external and internal morphologies (A, B, and C), stained kidney (D), colony morphology (E), and gram-stained bacteria (F)

than those in the control indicating the beneficial effects of peptidoglycan on fish growth. Peptidoglycan is known as a prebiotic compound (Davani-Davari *et al*., 2019) and its benefits in aquatic animals have been previously documented (Rohani *et al*., 2022). These positive effects could be explained by the changes in the bacterial population in the digestive tube in which the prebiotic supported the activities of beneficial bacteria (Baumgärtner *et al*., 2022). Dietary supplementation of peptidoglycan as a prebiotic typically modulates the endogenous flora in the gastrointestinal tract by influencing enzyme activity. The secretion of digestive enzymes can be enhanced in the intestines of fish by the intake of dietary prebiotics. Dietary prebiotics are initially responsible for modulating the favourable intestinal microflora that plays a major role during the secretion of digestive enzymes, especially amylase (Munir *et al*., 2016).

The highest value of fish growth was observed in the PG5-fed fish suggesting that the optimal rate of dietary supplementation of peptidoglycan was 5 g kg^{-1} diet. The supplementation of peptidoglycan at the ratio of 10 g/kg induced a higher value of fish growth compared to the PG0-fed group, but lower than that observed in the PG5-fed group. Other studies have also reported that the supplementation of peptidoglycan at a high dose (at 8 or 16 g kg^{-1}) did not induce beneficial effects on the growth performance of aquatic animals (Zhou *et al*., 2006). Moreover, regarding the results of intestinal indices, no differences were found in the visceral index (VSI) or relative gut length but the GaSI was influenced by the dietary supplementation of peptidoglycan after two weeks of the feeding trial. Specifically, the values of GaSI in the PG5 and PG10-fed fish were higher than that in PG0 group, suggesting the use of peptidoglycan increased the thickness of the digestive tube in Nile tilapia. This result may have supported the digestive and absorbtion processes in the experimental fish, inducing better growth performances in these groups. The higher number of red blood cells in the PG5-fed fish compared to the PG0-fed ones also induced a higher oxygen level in the fish blood which in turn induced higher growth performance in the fish (Ali *et al*., 2022).

The results showed that no differences were found in the feed efficiencies or survival rates among the experimental groups indicating that peptidoglycan did not support the improvement of these indicators. The enhancements of feed

utilization have usually been reported in fish fed on a diet supplemented with probiotics. In this case, besides the beneficial bacterial strains, these products also contain the enzymes derived by these bacteria that support the digestion of feed (Hemarajata and Versalovic, 2013; Valdes *et al*., 2018; Wang *et al*., 2021). On the other hand, in the current study, the prebiotics supplemented in the feed may have only supported the development of the bacterial population in the fish digestive tube. However, in combination with the results of fish growth, the PG-fed groups had better overall efficiencies. The survival rate in the present research remained at 100% in all of the treatments indicating the experimental conditions were suitable for Nile tilapia. However, the supplementation of peptidoglycan induced the preservation of fish survival in the bacterial challenge as discussed in the section below.

Fish resistance against *S. agalactiae*

The lower values of cumulative mortality of the fish fed on the diet supplemented with peptidoglycan compared to the control confirmed the support of peptidoglycan in the protection of fish from bacterial disease. In the current study, peptidoglycan was used as an immunostimulant compound to stimulate the immune system of experimental fish, and therefore, the cumulative mortalities found in the PG5 and PG10-fed fish were lower than the PG0-fed ones. Similar results were reported in shrimp (Purivirojkul *et al*., 2006; Pan *et al*., 2015) and Japanese flounder *Paralichthys olivaceus* (Zhou *et al*., 2006). Moreover, peptidoglycan was extracted from the cell wall of positive gram bacteria (*Lactobacillus* sp.) that is in the same bacterial group as *S. agalactiae*; thereby, in this case, the peptidoglycan played a role as an exogenous agent from the positive bacterial group, which the fish immune system recognized, and stimulated the immune system to remove it from the fish body (Lin *et al*., 2019). Consequently, the dietary supplementation of peptidoglycan could boost the resistance of the experimental fish against *S. agalactiae*. Furthermore, peptidoglycan from probiotic lactobacilli has been shown to possess multiple biological activities including immunomodulatory, anti-tumor, and anti-infection effects (Huang *et al*., 2020).

Another explanation may concern the indirect effects on the intestinal microbiota in the experimental fish. If the peptidoglycans used as prebiotics were to be introduced, they would stimulate the beneficial bacteria in the fish intestine system and would compete with the pathogenic bacteria which would be unable to enter the fish body through the digestive tube (Wang *et al*., 2021). Interestingly, the mortalities after 14 days of the challenge test in PG5 and PG10 were similar suggesting the immunostimulatory ability of dietary supplementation at a concentration of 5 g kg^{-1} diet was comparable to the higher dose of dietary supplementation at 10 g kg^{-1} diet. This result is very significant in applied aquaculture.

Conclusions

Through the obtained results, we conclude that the growth performance, total red blood cells, and resistance against *Steptococcus agalactiae* in Nile tilapia were improved by dietary supplementation of peptidoglycan, and the optimal dose was 5 gram per kilogram diet. Based on the results obtained from the present study, experiments to test the effects of peptidoglycan supplementation at lower doses are proposed to find the most efficient concentration to apply in practical aquaculture. Moreover, the analyses of other immune indicators including humoral immune parameters or expression of genes involved in the immune system should be supplemented to improve the results of future studies.

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