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Supplementation of Lactic Acid Bacteria in Feed Induced Non-Specific Immune Response of Tiger Grouper

(SUPLEMENTASI BAKTERI ASAM LAKTAT PADA PAKAN MERANGSANG TANGGAP KEBAL NON SPESIFIK IKAN KERAPU MACAN)

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ABSTRACT

Lactic acid bacteria (LAB) have an ability to enhance innate responses in fish. The aim of this study was to study the effect of three LAB isolates (Kerapu Situbondo Usus: KSBU 12C, KSBU 5Da, and KSBU 9 on non-specific immune responses of tiger grouper (*Epinephelus fuscoguttatus*). The fish were fed diet supplemented with each of the bacterial isolates at final concentration of 10^8 CFU mL^{-1} twice every day. The haematology parameters (haematocrit, leucocyte count, heterophils/neutrophils, monocytes, lymphocytes), phagocytic activity and total LAB were measured every seven days. After 28 days, the fish were challenged with *Vibrio alginolyticus*. During the feeding period, the haematocrit values of fish fed with LAB isolates were higher than those of control group, and values ranged from 24.67±8.33 % to 40.50±7.09 %. The leucocyte counts increased, mainly after being challenged with *V. alginolyticus*, and the values ranged from 40.33 ±6.03 × 1000 cell mL^{-1} to 139.33±15.57 × 1000 cell mL^{-1}. With the exception of lymphocytes, proportion of heterophils and monocytes, and phagocytic activity were higher in fish fed with LAB isolates than those of control. The presence of LAB in the gastrointestinal tract of fish fed with LAB resulted in higher haematology parameters and phagocytic activity in comparison to fish fed control diet. In conclusion, supplementation of LAB in diet could enhance non-specific immune response of tiger grouper.

Key words: Tiger grouper, lactic acid bacteria, haematocrit, leucocyte, phagocytic activity.

ABSTRAK

Bakteri asam laktat (BAL) mempunyai kemampuan untuk meningkatkan tanggap kebal bawaan pada ikan. Tujuan penelitian ini adalah untuk mempelajari efek tiga isolat BAL (Kerapu Situbondo Usus: KSBU 12C, KSBU 5Da, dan KSBU 9) terhadap tanggap kebal non-spesifik ikan kerapu macan (*Epinephelus fuscoguttatus*). Ikan diberi pakan yang disuplementasi dengan masing-masing isolat bakteri pada konsentrasi akhir 10^8 CFU mL^{-1} dua kali sehari. Parameter hematologi (hematokrit, total leukosit, heterofil/netrofil, monosit, limfosit), aktivitas fagositosis dan total BAL diukur setiap tujuh hari. Setelah 28 hari, ikan diuji tantang dengan *Vibrio alginolyticus*. Selama pemberian pakan, nilai hematokrit ikan yang diberi pakan dengan isolat BAL lebih tinggi daripada ikan kontrol, dan nilainya berkisar dari 24,67±8,33% sampai 40,50±7,09%. Total leukosit ikan, terutama setelah diuji tantang dengan *V. alginolyticus* meningkat, dan nilainya berkisar dari 40,33±6,03 × 1000 sel mL^{-1} sampai 139,33±15,57 × 1000 sel mL^{-1}. Kecuali limfosit, proporsi heterofil dan monosit dan aktivitas fagositosis ikan yang diberi pakan dengan isolat BAL lebih tinggi daripada ikan kontrol. Adanya BAL pada saluran pencernaan ikan yang diberi pakan yang disuplementasi BAL menyebabkan tingginya nilai parameter hematologi dan aktifitas fagositosis yang lebih tinggi pada ikan yang diberi pakan BAL dibanding ikan yang diberi pakan kontrol. Dengan demikian dapat disimpulkan bahwa suplementasi BAL pada pakan ikan dapat meningkatkan respon imun nonspesifik ikan kerapu macan.

Kata kunci: kerapu macan, bakteri asam laktat, hematokrit, leukosit, aktivitas fagositosis.

INTRODUCTION

Vibriosis is dominant bacterial disease found in groupers, mainly in between dry to wet seasons in addition to Viral Nervous Necrotic (VNN) infection. Vibriosis is a disease caused by an infection with Gram negative bacteria of the *Vibrio* genus which causes systemic infection on fish. *Vibrio alginolyticus* and *V. anguillarum* were the main causative agents of vibriosis found in groupers (Nitimulyo *et al*., 2005; Desrina *et al*., 2006). Conventional approaches to control diseases with chemicals including use of antimicrobial drugs, pesticides, and disinfectants (Gomez-Gil *et al*., 2000). Unfortunately, the abuse of such antimicrobials in disease prevention and growth promotion leads to the evolution of resistant strains of bacteria (Esiobu *et al*., 2002).

Different studies with different approaches have been performed to increase immunity of tiger grouper larvae against viral and bacterial infections. Outer membrane protein (OMP) of *V. alginolyticus* was reported more immunogenic than the whole cells on tiger grouper (Desrina *et al*., 2007). Alkaloid bioactive substances of jellyfish, *Bougeinvillia* sp., increased phagocytic activity, macrophage, total leucocyte, differential leucocyte (lymphocyte, monocyte, neutrophils) and plasma protein of tiger grouper (Andayani, 2011).

Manipulation of the gut microbiota through dietary supplementation of beneficial microbe(s), usually referred as probiotics, is a novel approach not only from nutritional point of view but also as an alternate viable therapeutic modality to overcome the adverse effects of antibiotics and drugs (Nayak, 2010). Modulating the immune system is one of the most commonly purported benefits of probiotics. Unlike other animals, probiotics modulate various immunohaematology parameters in teleosts. Probiotics interact with the immune cells such as mononuclear phagocytic cells (monocytes, macrophages) and polymorphonuclear leucocytes (neutrophils) and NK cells to enhance innate immune responses. Like higher invertebrates, certain probiotics can enhance the number of erythrocytes, granulocytes, macrophages, and lymphocytes in different fish (Irianto and Austin, 2002; Kumar *et al*., 2008).

Lactic acid bacteria, a group of Gram positive and catalase negative bacteria producing lactic acid, have been intensively studied and applied as probiotics in aquaculture. In tilapia (*Oreochromis niloticus*) a two weeks feeding of *Lactobacillus rhamnosus* significantly stimulated the phagocytic activity (Pirarat *et al*., 2006). *Enterococcus faecium* MC13 has protected the post larvae of shrimp *Panaeus monodon* against *parahaemolyticus*, and feeding of *Streptococcus phocae* P180 enhanced the survival of the larvae when challenged with *V. harveyi* (Swain *et al*., 2009).

Lactic acid bacteria have been isolated from the intestine of tiger grouper, and have been evaluated the antagonistic potency against *V. alginolyticus* (Nursyirwani *et al*., 2011). However, the effect on immune response has not been investigated. The present study was therefore designed to investigate the non-specific immune responses of tiger grouper during four weeks feeding of diet supplemented with the lactic acid bacteria isolates and after being challenged with *V. alginolyticus*.

RESEARCH METHODS

Fish Experiment

Tiger grouper, *E. fuscoguttatus* (500 fish of 33.91 ± 5.77 g weight and 11.59 ± 0.83 cm length) were obtained from Brackishwater Aquaculture Development Center (BADC) Jepara, Indonesia. The fish were acclimatized for two weeks in a re-circulating system in two 500-L circular fiber tanks in the Laboratory of Environmental Health and Fish Diseases of BADC, All tanks were equipped with an air-filter. During the experiment, 50% of the sea water was renewed daily to maintain the water quality monitored as: dissolved oxygen, DO (4.31 ± 0,27 mgL^{-1}), temperature (26.61±0.03°C), salinity (31.17±0.58 ppt), and pH (7.64±0.07).

Preparation of Lactic Acid Bacteria Isolates

Three isolates of lactic acid bacteria (KSBU 12C, KSBU 5Da and KSBU 9) examined in this study was isolated from the intestine of tiger grouper, and had been characterized based on morphological and physiological properties and biochemical reactions in earlier study (Nursyirwani *et al*., 2011). The LAB isolates (KSBU) were named based on the natural source, where K stands for *Kerapu* (tiger grouper); SB for *Situbondo* waters in East Java, Indonesia; and U for *usus* (intestine). Each of the isolates was grown in de Man, Rogose, and Sharpe (MRS, Merck) broth (pH 5.7) at 30°C for 48 hours. The cells were harvested by centrifugation (Eppendorf

centrifuge 5810R) at 15.000 × *g* for 15 min), washed twice with phosphate buffer saline (PBS) and re-suspended in the same buffer. The number of bacteria (10^9 CFU ml^{-1}) was adjusted to McFarland standard number 4.

Prepation of Experimental Feed

Commercial grouper feed (MarinXcel 5011, produced by PT. Cargill Indonesia) was used as the basal diet for the supplementation of bacterial isolates. The probiotic diets were prepared with cells re-suspended in 5 mL of PBS to 10^9 CFU mL^{-1} and mixed with an equal volume of fish oil. The emulsion was applied to achieve a viable dose equivalent to 10^8 CFU g^{-1} of feed.

Culture of Pathogen

Bacterial pathogen, *V. alginolyticus* provided by the Microbiology Laboratory of BADC Jepara, was cultured in tryptic soy broth (TSB, Oxoid), centrifuged and the pellets was re-suspended in PBS to adjust the bacterial suspension approximately to 10^9 CFU mLl^{-1} for the challenge test.

Experimental Design

The experiment was conducted as a randomized completely block design (Gomez and Gomez, 1995) with four treatments: (1) fish fed basal diet (control), (2) fish fed diet mixed with isolate of KSBU 12C, (3) fish fed diet mixed with isolate of KSBU 5Da; and (4) fish fed diet mixed with isolate of KSBU 9. Each treatment had three replicates of 25 fish each. Fish were fed two times daily at 3% body weight per day with a 50% water change every day, during 28-day feeding trials. After the feeding trial, 10 fish of control and treatments were challenged intraperitoneally (i.p.) with 0.1 mL of the pathogen (*V. alginolyticus*) suspension. During the first 24h-postinfection, water was not renewed to ensure the infection. The immune response and survival rate of fish were observed at 0 (before treatment), 7, 14, 21, 28^{th} day of feeding and 35^{th} day (7 days after the challenge test).

Blood Sample Collection

Three fish from each treatment were randomly sampled at 7, 14, 21, 28, and 35^{th} day of rearing for the blood analysis. Blood sample was collected (following the method of Anderson and Siwicki (1995) individually, after anesthetizing the fish by immersion in clove oil solution (0.1 mL clove oil in 5 L water) for two minutes, from the caudal vein with a 27-gauge needle, 1-mL syringe, and was removed to a microtube added with 10% ethylene diamine tetracetic acid (EDTA) as anticoagulant.

Haematology Parameters

The percent volume of erythrocytes in fish blood (haematocrit counts) was determined following the method of Anderson and Siwicki (1995) by using heparinized haematocrit capillary tubes (Brand, Wertheim) and Haemokrit Centrifuge (Hettich) at 1000 rpm for five minutes. Leucocyte counts was performed following the procedure of Schaperclaus (1991) by using counting chamber (*improved* Neubauer). A total of 100 cells was counted with this means, and the heterophil, monocyte, and lymphocyte were expressed as fractional component.

Phagocytic Activity

Phagocytic activity of blood heterophil and monocyte was determined following the method of Anderson and Siwicki (1995). *Staphylococcus aureus* used as cell being phagocytized was cultured in 5 mL of TSB medium. The bacterial cells are harvested by centrifugation and washed twice with PBS. The bacterial count was determined using the McFarland standard to adjust the bacterial suspension approximately at density of 10^9 CFU mL^{-1}. One mL of the bacterial suspension is added with 9 ml of PBS to reach bacterial concentration 10^8 CFU mL^{-1}.

Blood sample with anticoagulant EDTA (1:10) was filled in haematocrit capillary tubes, and centrifuged at 1000 rpm for five minutes. 50 µL of puffy quote contained polymorphonucleic (PMN) cells was placed in microtiter plate well, mixed well with 50 µL of *S. aureus* suspension for one minute, and incubated under room temperature for 45 minutes. Five µL of the mixture was placed on a glass slide to make a smear, air-dried, fixed with methanol for five minutes, and air-dried at room temperature. The slide was stained with 10% Giemsa solution for 30 minutes, and washed with distilled water. Number of phagocytic cells was observed and counted within 100 cells under microscope (1000 x).

Enumeration of LAB from Fish Intestines

Total lactic acid bacteria (LAB) in the intestines of tiger grouper was counted every seven days during the 28-days of the probiotic feeding. The bacterial counts were determined by plate counting on MRS agar (Merck)+$CaCO_3$.

Three fish for each treatment and time were sampled for this study.

Statistical Analysis

Statistical analysis using one-way analysis of variance (Statistical Analysis System, SAS, version 9.1.3) was performed to find significant difference at the level of P <0.05 on various parameters between treated and control trials.

RESULTS and DISCUSSION

Haematology Parameters

The haematocrit values of fish fed LAB isolates were higher than that of control, and the value increased until 21 days feeding (Fig. 1). The values declined at 28 days and 35 days of observation. Fish fed with isolate KSBU 5Da performed the highest haematocrit value (40.50±7.09%), and the lowest value was found in fis fed control diet (24.67±8.33%). However, the haematocrit values was not significantly different among the treatment, but significantly different (P<0.05) was observed between days of observation.

The leucocyte counts found in this study ranged from 40.33±6.03 × 1000 cell mL^{-1} to 139.33±15.57 × 1000 cell mL^{-1} (Fig. 2). Although there was not significant difference, the leucocyte counts increased until 14 days. Fish fed with isolate KSBU 9 showed the highest leucocyte counts (87.33±31.07 × 10^3 cell mL^{-1}), and the lowest counts was found in fish fed control diet (63.00±27.22 × 10^3 cell mL^{-1}). The leucocyte counts declined until day 28th. However, at the 35 days, the counts increased after being cessation of the probiotics and challenged with *V. alginolyticus*. Similar finding has been reported on leucocyte numbers of rainbow trout, *Oncorhynchus mykiss* after feeding with Gram positive isolate A1-6 for 14 days, which was higher (14.3±1.7 × 10^6 cell mL^{-1}) than the controls (13.3±1.0 × 10^6 cell mL^{-1}) (Irianto and Austin, 2002).

There were significant difference in the proportion of heterophil, monocyte, and lymphocyte in leucocyte between fish fed diet supplemented with LAB isolates and those of control fish (Fig. 3). The values of heterophil, monocyte, and lymphocyte ranged from 7.18±0.84% - 17.3±2.00%, 11.52 ± 0.87% - 24.43 ± 4.80%, and 60.6 ± 8.47% - 81.03 ± 0.75%, respectively. Fish fed with isolate KSBU 12C performed the highest heterophil value at 35 days although the difference was not significant between the days of observation. While, the highest monocyte value was performed by fish fed with isolate KSBU 9. The differences were statistically significant (P<0.05) between the four treatments and days of observation. On contrary, the lymphocyte value was higher in control than fish fed with different LAB isolates, and was significantly different (P<0.05) between the days of observation.

Data on haematology parameters of tiger grouper observed in this study were in the range of values in marine cultured tiger grouper studied by Johnny *et al*, (2003), where the haematocrit (PVC) value was 31.5%, and the leucocyte counts (57.6 × 10^3 cells mL^{-1}) consisted of 4.2% heterophils, 2.5% monocytes, 68.3% lymphocytes, and 25% trombocytes. These findings indicate that leucocyte cells are important in the cellular response to microbial invasion. The main cells involved in phagocytosis in fish are neutrophils and macro-phages (Secombes and Fletcher, 1992). These cells remove bacteria mainly by the production of reac-tive oxygen species during a respiratory burst. In addition, neutrophils possess myeloperoxidase in their cytoplasmic granules, which in the presence of halide and hydrogen peroxide kills bacteria by halogenation of the bacterial cell wall. Moreover, these cells have lysozymes and other hydrolytic enzymes in their lysosomes (Fischer *et al*., 2006).

Blood Phagocytic Activity

There was a significant difference and increase in the phagocytic activity of blood between the tiger grouper fed diet supplemented with LAB isolates (KSBU 12C, KSBU 5Da, KSBU 9) and fish fed control diet (Fig. 4). The increase was observed until 14 days on fish fed the LAB isolates (34.5 ± 11.46%, 38.33% ± 7.78%, and 51.5 ± 3.5%; P < 0.05, respectively), and was higher than that of control (22.25 ± 3.47%). With the exception to fish fed with KSBU 12C, the activity declined after cessation of feeding with probiotic-containing diets and injection of pathogen *V. halginolyticus* until days 35th (48.67 ± 14.74%, 49.00 ± 5.20%, 43.33 ± 7.77%, and 28.00 ± 4.00%; P < 0.05). The difference in phagocytic activity was not statistically significant between the days of observation.

Stimulation of phagocytic activity by probiotics has been found in other grouper species. Using grouper *Epinephelus coioides*, feeding with *Lactobacillus plantarum* at 10^{6-10} CFU kg^{-1} after

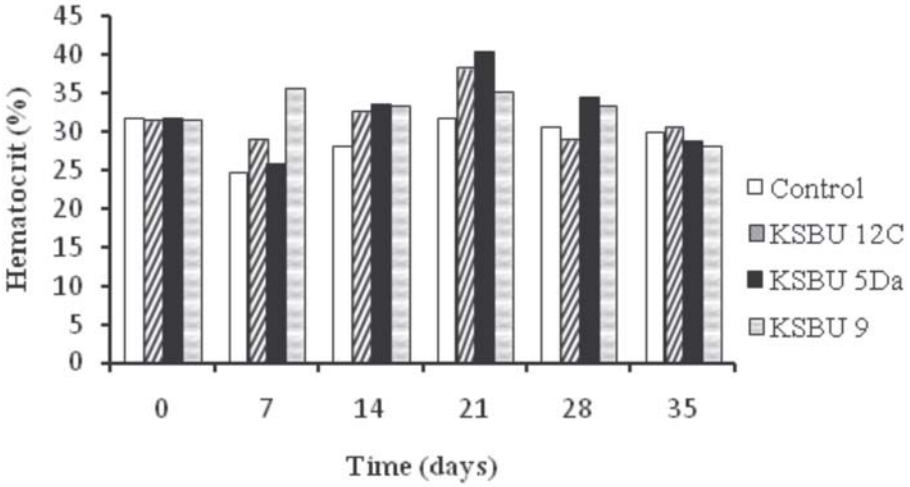

Fig. 1. Haematocrit value (%) of tiger grouper during feeding with lactic acid bacteria (up to 28 days) and after challenging with *V. alginolyticus* (days 28-35); *error bars* indicate standard error. Data (mean of triplicate samples ± standard error) at the same sampling time with different letters are significantly different (P<0.05).

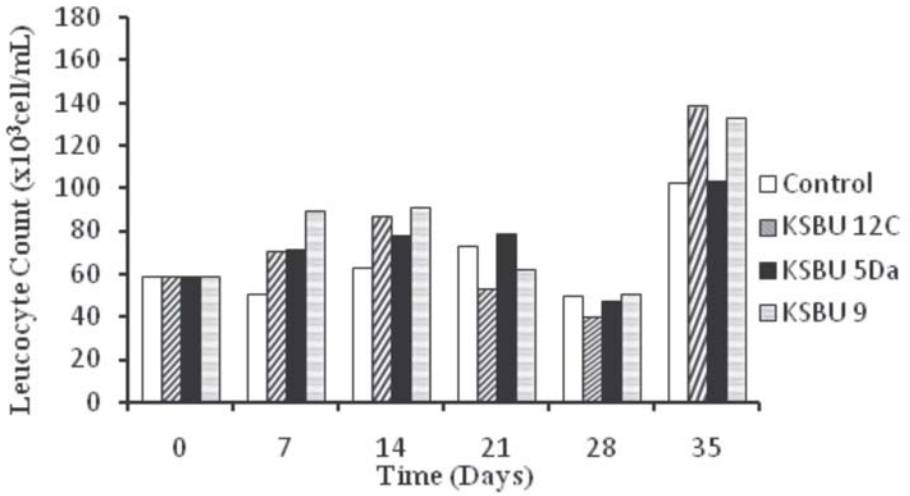

Fig. 2. Leucocyte counts (%) of tiger grouper during feeding with lactic acid bacteria (up to 28 days) and after challenging with *V. alginolyticus* (days 28-35); *error bars* indicate standard error. Data (mean of triplicate samples ± standard error) at the same sampling time with different letters are significantly different (P<0.05).

four weeks of feeding leads to significantly higher in phagocytic activity than those of fish fed the control diet (Son *et al*., 2009). Phagocytic activity of kelp grouper (*bruneus*) fed with diet enriched with mushroom *Phellinus linteus* extract after challenge with or without *V. anguillarum*, *V. harveyi*, and *V. alginolyticus* significantly higher when compared to control group (Harikrishnan *et al*., 2011). In present study, the phagocytic activity of tiger grouper fed diet supplemented with each of the LAB isolates was different during the feeding period. Fish fed diet supplemented with isolate KSBU 9 performed the highest phagocytic activity during 21 days feeding trial. However, at days 28 and 35 the highest phagocytic activity was found in fish fed diet supplemented with isolate KSBU 5Da. These findings indicate that stimulation of phagocytic activity in groupers depends on species of probiotics enriched in the diet.

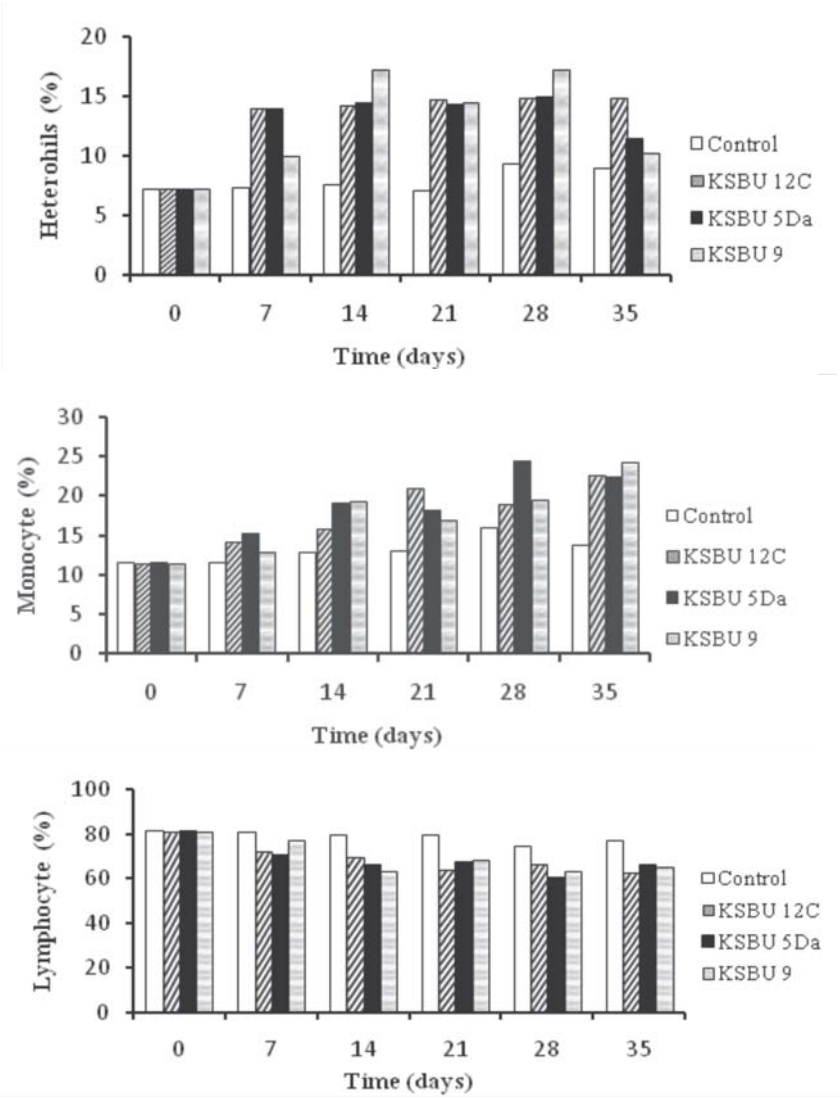

Fig. 3. Proportion (%) of netrophils (a), monocyte (b) and lymphocyte (c) in blood of tiger grouper during feeding with lactic acid bacteria (up to 28 days) and after challenging with *V. alginolyticus* (days 28-35); *error bars* indicate standard error. Data (mean of triplicate samples ± standard error) at the same sampling time with different letters are significantly different (P<0.05).

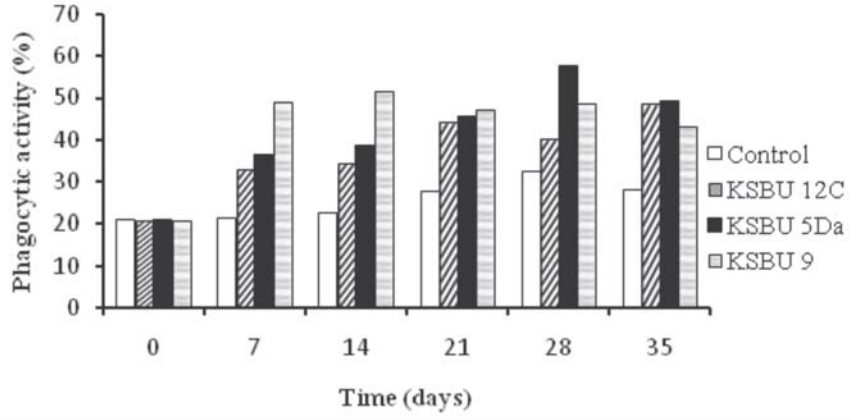

Fig. 4. Phagocytic activity of tiger grouper during feeding with lactic acid bacteria (up to 28 days) and after challenging with *V. alginolyticus* (days 28-35); *error bars* indicate standard error. Data (mean of triplicate samples ± standard error) at the same sampling time with different letters are significantly different (P<0.05).

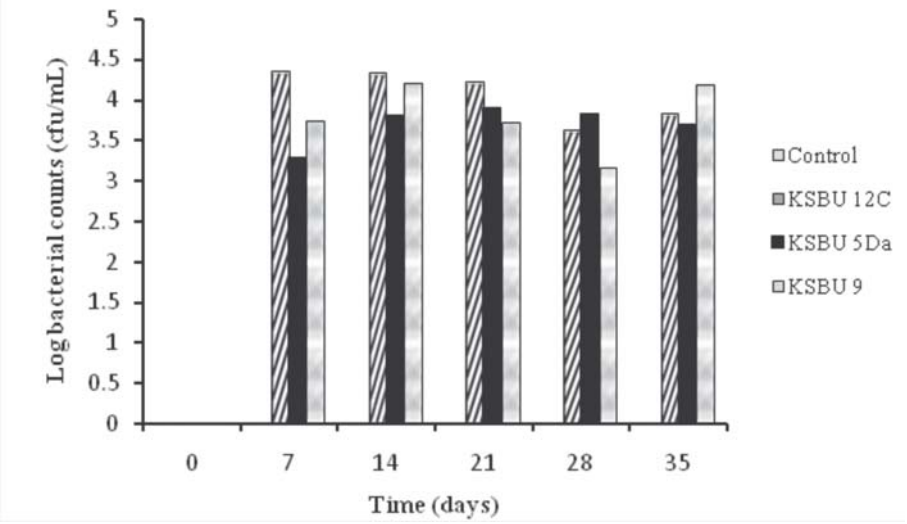

Fig. 5. Total LAB counts in the intestine of tiger grouper during 28 days feeding supplemented with lactic acid bacteria isolates, and after being challenged with *V. alginolyticus* (days 28-35). Data (mean of triplicate samples ± standard error) at the same sampling time with different letters are significantly different (P<0.05).

Phagocytic activity is responsible for early activation of the inflammatory response before antibody production, and plays an important role in antibacterial defense. Probiotics can effectively trigger the phagocytic cells in host and enhancement of phagocytic activity (El-Ezabi *et al*., 2011). Stimulation of phagocytic activity by LAB isolates has been found in several fish species, such as in rainbow trout (*Oncorhynchus mykiss*) by *L. rhamnosus* JCM 1136 (Panigrahi *et al*., 2004) and in nile tilapia (*Oreochromis niloticus*) by *L. plantarum* (El-Ezabi *et al*., 2011). Lactic acid bacteria might mediate the phagocytic activity through modulation of cell surface molecules that are involved in bacterial uptake by leucocytes (Panigrahi *et al*., 2005). Enhancement of non-specific immune response of tiger groupers obtained in the present study might be due to the presence of LAB in intestine of fish fed diet supplemented with LAB isolates.

Total LAB Counts

Before the trial, lactic acid bacteria (LAB) were not detectable in intestines of tiger grouper. However, after the supplementation of each LAB isolate in diet, the bacteria was found in the fish intestines. In comparison, fish control did not reveal the presence of LAB in the intestine during or after the feeding period (Fig. 5). The bacterial counts increased from day 7 to day 14, then slightly decrease until 28 days feeding trials. The values ranged from 3.18±0.22 (Log cfu mL^{-1}) found in fish fed diet with isolate KSBU 9 at day 28 until 4.36±0.66 (Log cfu mL^{-1}) found in fish fed diet with isolate KSBU 12C at day 7. At day 35, after being cessation of the LAB isolates, the bacteria were still found in the fish intestine.

The ability of LAB to survive in the fish intestine was in general agreement with previous studies. The number of viable lactobacilli in the posterior intestines of grouper *Epinephelus coioides* fed with *L. plantarum* ranged from $(5.5 \pm 2.6) \times 10^3$ to $(3.0 \pm 0.6) \times 10^4$ cfu $(g\ gut)^{-1}$ after 2 weeks of feeding, and increased from $(2.2 \pm 0.3) \times 10^7$ to $(2.2 \pm 0.1) \times 10^8$ cfu $(g\ gut)^{-1}$ after 4 weeks of feeding (Son *et al.,* 2009). In an experiment with rainbow trout, maximal populations of *Carnobacterium* sp. ($7.4 \times 10^6\ g^{-1}$ of intestine) were reached after feeding for 28 days. However, these levels decline rapidly after cessation of feeding with the probiotic-containing diet, and could not be isolated from intestine six days later (Robertson *et al*., 2000).

Conclusion

In present study, the lactic acid bacteria isolates (KSBU 12C, KSBU 5Da and KSBU 9) supplemented in diet could enhance the hematocrit value, leucocyte count, percentage of neurophils and monocytes, and phagocytic activity of tiger grouper. Higher haematology parameters and phagocytic activity were due to the presence of LAB in the fish intestine in comparison to fish fed control diet.

RECOMMENDATION

The effect of LAB supplementation on other immune responses such as antiproteases, lysozyme and respiratory burst activities, and competitive exclusion ability of each LAB isolate on pathogenic *Vibrio* need to be investigated. Phenotype and genotype characterizations of the LAB isolates are required before application in aquaculture.

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