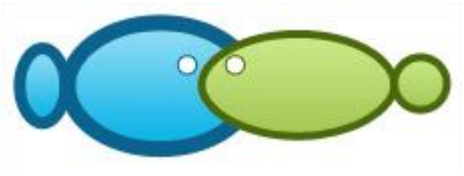

Effect of clove (*Syzygium aromaticum*) powder supplementation on non-specific immune response of hybrid grouper (*Epinephelus fuscoguttatus* ♀×*Epinephelus lanceolatus* ♂) infected with *Vibrio alginolyticus*

[1,2]Inem Ode, [1]Sukenda Sukenda, [1]Widanarni Widanarni, [1]Dinamella Wahjuningrum, [1]Munti Yuhana, [1]Mia Setiawati

[1] Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University, Indonesia; [2] Water Resource Management Study program, Faculty of Fisheries and Marine Sciences, Darussalam Ambon University, Indonesia. Corresponding author: S. Sukenda, Sukenda@apps.ipb.ac.id

Abstract. Grouper is an economically important aquaculture commodity in the global market. The main obstacle of this fish culture development is mortality due to disease. *Vibrio alginolyticus* are Gram-negative bacteria that cause a vibriosis disease in grouper fish. The aim of this study was to evaluate the non-specific immune response of hybrid grouper supplemented with clove powder against the *V. alginolyticus* infection. Two dose levels of clove powder were used, namely 10 and 15 g kg^{-1}. The control treatments without clove powder supplementation contained positive control (infected with *V. alginolyticus*), and the negative control (injected with phosphate buffer saline (PBS)) during the challenge test. Feeding was performed for 14 days, and all test fish were injected intramuscularly with *V. alginolyticus* (10^7 CFU mL^{-1}, 0.1 mL^{-1}), except the negative control treatment. The study results showed that the supplementation of clove powder at 15 g kg^{-1} could enhance the immune response and increase the survival rate significantly higher ($p < 0.05$) than the positive control.
Key Words: disease, hybrid grouper, immunity, *Syzygium aromaticum*, *Vibrio alginolyticus*.

Introduction. Grouper is an economically important aquaculture commodity in the global market. The hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) is the latest grouper species firstly produced in 2007 by the University of Sabah Malaysia and has become extremely popular with high economical value in the Southeast Asia region (Jiang et al 2016). The main obstacle of this fish culture development is mortality due to disease. *Vibrio alginolyticus* are Gram-negative bacteria that cause a vibriosis disease in grouper fish. The frequent handling effort to prevent from this disease is by antibiotic applications, but the negative impact from improper antibiotic doses is bacterial resistance against antibiotics, food security danger, and environmental problems (Manage 2018).

The disease handling effort in aquaculture organisms with herbs can become an alternative replacement for antibiotics due to safe and environmentally-friendly for the aquaculture organisms effective for a sustainable aquaculture. Several previous studies have found that immunostimulants from herbs supplemented in feed can induce the immune responses. Sahu et al (2007) stated that the use of immunostimulants from plant materials could enhance the fish immune response. Lillehoj et al (2018), the plant bioactive components have roles as chemical protectors against microbial infection and immunomodulators by increasing the immune cell proliferation, cytokine modulation, and antibody formation. Anderson (1992) stated that the phytochemical compounds, such as steroids, flavonoids, glycosides, phenols, and tannins, could activate the cellular immune cells by enhancing the cell proliferation that played roles for immunity, such as

macrophages, granulocytes, lymphocyte T and B. When the immunostimulants enter the fish body, macrophages will be induced to produce interleukin that will produce lymphocytes, such as lymphocyte-T and lymphocyte-B, whereas lymphocyte-B becomes more active in producing the antibody, while lymphocyte-T produces interferon that enhances the macrophage capability in producing more lysozymes.

One of the herbs that can potentially be used is clove. Clove (*Syzygium aromaticum*) is one of the most important herbs in traditional medicine, included in Myrtaceae family, and originated from Maluku Islands, Indonesia (Sohilait et al 2018). Several studies have been reported that the supplementation of clove oil in feed can improve the growth of Nile tilapia *Oreochromis niloticus* (Gaber 2000), increase the feed efficiency of striped catfish *Pangasianodon hypophthalmus* (Pratiwi et al 2016), improve the growth performance of common carp *Cyprinus carpio* (Silvianti et al 2016). Adeshina et al (2019) reported a stimulation effect of clove in feed for growth performance, nutrient utilization, and antioxidant capacity on African catfish against the *Aeromonas hydrophilla* infection. Shalaby et al (2011) found increased white-blood cells in albino mice fed with clove oil-mixed feed. Sohilait et al (2018) stated that the eugenol and eugenyl acetate in clove oil had a high antioxidation activity which could be utilized as a hepatos-protector and lipid peroxidation inhibitor. Gaspar et al (2018), eugenol as an antioxidant is considered to inhibit the lipid peroxidation process, both in initiation and propagation stages. The antioxidation activity is suspected to become a cue for increasing the fish growth performance and health condition.

Clove has bioactive compounds, namely 10-13% tannins, terpenoids, glycosides, and 14-21% essential oils. The essential oils contained in clover are eugenol, caryophyllene, furfural, vanillin, methyl salicylate, pyrocatechol, methyl ketone, and valeric aldehydes, eugenin, isoeugenitol, isoeugenitin, eugenitin, tannin, mucilage, sitosterol, estigmaterol, resins, cellulose, pinene, oleanolic acid, and fixed oil (Cortés-Rojas et al 2014). These bioactive compounds are possible to be used as immunostimulants.

Understanding the way of phytochemical substances to modulate the immune response can be used as a key to enhance the fish health and reduce the loss due to disease attack. This study aimed to evaluate the non-specific immune response of hybrid grouper supplemented with *S. aromaticum* powder against the *V. alginolyticus* infection.

Material and Method

Clove powder collection and preparation. Dried clove were obtained from the Mardika Market, Ambon City, Maluku Province, Indonesia. The *S. aromaticum* used were identified in the Biology Research Center, Indonesian Institute of Science (No. 1114/IPH.1.01/If.07/XI/2020). *S. aromaticum* were cleaned from the attached dirt, before blending in a blender (Miyako BL-125 GF, Japan) to obtain a simplicial powder.

Bacteria preparation. *V. alginolyticus* bacteria were obtained from Brackish Water Aquaculture Development Center, Jepara. *V. alginolyticus* were isolated from hybrid grouper which had clinical symptoms, namely lesion in mouth and eyes. *V. alginolyticus* were sub-cultured and identified using Gram staining, biochemical test and the polymerase chain reaction (PCR) method using the primers F-gyrB 5'-ATT GAG AAC CCG ACA GAA GCG AAG-3' and R-gyrB 5'-CCT AAT GCG GTG ATC AGT GTT ACT-3' (Zhou et al 2007).

Container and test animal preparations. This study was performed from May to June 2021 in the Laboratory of IPB Fisheries and Marine Observation Station (IFMOS) Ancol. Hybrid groupers were obtained from Brackish Water Aquaculture Development Center, Situbondo, East Java, Indonesia. The fish were acclimatized for 2 weeks in a fiber tank (1000 L) before starting the experiments. The containers used for rearing the fish were glass aquaria with 60 x 40 x 40 cm (70 L) size. Each aquarium was equipped with filter and aerator. The fish used for the experiment averagely had the total length of 10.52±0.56 cm and weight of 20.35±0.45 g, which were stocked at a density of 11 fish

per aquarium. The present study fulfilled ethical requirements, considered the welfare of the test animals, and received prior ethical approval from the IPB University animal ethics commission (ethical approval number 198-2021 IPB).

Water exchange was performed every day at 30%. The water quality parameters measured during the experiment were temperature: 28-30^{o}C, pH: 7.8-8.2, salinity 30 ppt, dissolved oxygen (DO): 4.3-5.2 ppm, and total ammonia nitrogen (TAN): 0.40-0.61 mg L^{-1}.

Treatment and experimental design. This study had four treatments and four replications. Each replication used 11 fish, the total fish used in this study was 176 fish. The treatments used were two dose levels of clove powder, namely 10 g kg^{-1} and 15 g kg^{-1}, and control or without clove powder supplementations, such as positive control (CP) and negative control (CN). The positive control treatment was infected with *V. alginolyticus* pathogen without clover powder supplementation, while the negative control treatment was injected with phosphate buffer saline (PBS) during the challenge test. Diets were produced following the repelletting method. Commercial feed was made into powder, mixed with clove powder according to the treatment dose, then added water 400 mL kg^{-1} feed. Pellets were formed and dried in an oven for 3 hours at 50^{o}C. Pelleted diets were packed in plastic containers labeled following the applied treatments. All treatment diets were analyzed their proximate conditions based on Watanabe (1988) in the Laboratory of Fish Nutrition, Department of Aquaculture, IPB University. The proximate analysis results of the diet treatments are shown in Table 1. Diets were fed twice a day in apparent satiation at 08.00 and 17.00 GMT+7 for 14 days. In the 15-th day, challenge test was performed by injecting the test fish intramuscularly with *V. alginolyticus* in 10^{7} CFU mL^{-1} at 0.1 mL $fish^{-1}$. The negative control treatment was only injected with the PBS. Fish were then reared further for 25 days. The test fish samples were taken at six fish from each treatment on the day before the challenge test, followed by the one, 3, and 7 days post the challenge test for parameter analyses.

Table 1

Proximate analysis of diet treatments

Composition (%)	*Treatment (g kg^{-1} feed)*		
	Control	*10*	*15*
Protein	44.46	42.37	42.93
Lipid	11.30	11.84	12.57
Moisture	9	7.65	9.06
Ash	10	10.55	10.26
Crude fiber	3	3.10	2.34
Nitrogen free extract (NFE)	22.24	24.49	22.84

Note: clove powder used was 10 g kg^{-1}, 15 g kg^{-1}, and control (positive and negative).

Blood parameters. The blood parameter measurements contained the total erythrocytes, total leucocytes, hematocrit, haemoglobin and leucocyte differential, and were performed following the Blaxhall & Daisley (1973) procedures. The fish blood sample was taken using a 1 mL syringe from the vena caudalis and collected in a microtube.

Phagocytic activity. The phagocytic activity was calculated based on Anderson & Siwicki (1995) method. The phagocytic activity test was performed by taking 50 µL blood, which was distributed to a microplate added with 50 µL *Staphylococcus aureus* suspension (10^{7} CFU mL^{-1}) in PBS. Homogenization and incubation at room temperature were performed for 20 minutes. From the mixture, a 5 µL solution was taken for smear sample and dried in the air. The sample was fixated with methanol for 5 minutes and dried, before soaking in Giemsa staining for 15 minutes. This sample was cleaned with a flowing-water and dried, before being observed and counted. The phagocytic activity was calculated based on the 100 phagocytic cells that showed a phagocytic activity. The

phagocytic activity was calculated following the formula: Phagocytic activity (%) = total phagocyting cells/total phagocytic cells x 100.

Lysozyme activity. Lysozyme activity test was performed based on Ellis et al (2011), as blood was taken at 150 µL and centrifuged at 3000 rpm speed for 5 seconds. The 10 µL serum was added with 190 µL *Micrococcus lysodeikticus* (0.4 mg mL^{-1} in PBS pH 6.2). The number of lysozyme activity units was calculated based on the absorbance reading derivation for each 0.1 unit mL^{-1} min^{-1} serum. Reading was performed with an ELISA reader at 630 nm wavelength. The reading results were converted using the following formula: Lysozyme concentration (unit mL^{-1}) = $[(OD_{5m} - OD_{20m}) \times 1000] \times [1/(t \times s)]$, where: 1000 = absorbance result conversion (OD) in IU unit, t = time (minutes), s = total serum sample (mL), OD_{5m} = optical density reading at 5-th minute, OD_{20m} = optical density reading at 20-th minute.

Immunity gene-related expressions. The fish immunity gene-related expressions were observed from liver organ, namely interleukin-1β (il1b) and hepcidin (hamp) with a quantitative method of the real-time reverse-transcription-polymerase chain reaction (RT-PCR) using SensiFAST SYBR Hi-ROX Kit enzyme (Bioline) in StepOne™ Real-Time PCR Systems machine (Applied Biosystems) on the day before the challenge test, 1-st day after the challenge test, and 3-rd day after the challenge test. The RNA extraction used the Genezol™ reagent (Geneaid). The cDNA synthesis used Revertra Ace qPCR RT Master Mmix with gDNA Remover reagent (Toyobo, Japan). The primers used for interleukin-1β (il1b) were forward-primer of 5'-ATCATCGCCACACAGAGGTTT-3' and reverse-primer of 5'-TGCCTCACAACCGAACACAT-3' (GenBank accession no. EF582837.1), while primers for Hepcidin (hamp) were forward-primer of 5'-CGTGCTCGCCTTTATTTGC-3' and reverse-primer of 5'-GAGTGTCATCGCTCGCTGC-3' (GenBank accession no. GU988692.1) (Ye et al 2020). The β-actin was determined using the forward-primer of 5'-GGCTACTCCTTCACCACCACA-3' and reverse-primer of 5'-TCTGGGCAACGGAACCTCT-3' (GenBank accession no. AY510710.2) (Ye et al 2020). The RT-PCR was conditioned at 95ºC for 10 seconds, followed by 40 cycles at 95ºC for 10 seconds, 60ºC for 15 seconds, 72ºC for 15 seconds, and melting-curve at 95ºC for 30 seconds. The gene expression quantification was calculated using the $2^{-\Delta\Delta Ct}$ method with β-actin as the reference gene (Livak & Schmittgen 2001).

Survival rate. The survival rate (SR) was determined using the following formula: SR (%) = [(Nt/No) × 100], whereas Nt was the number of living-fish at the end of the study (individuals), and No is the number of living-fish at the beginning of the study (individuals).

Statistical analysis. The data were analyzed qualitatively and quantitatively. Statistical analysis using an analysis of variance was applied to identify the effect treatment, followed by Duncan's continuous test at 5% confidence level using the Statistical Program Software System (SPSS) version 20.0.

Results and Discussion. Hybrid grouper blood parameters before and post challenge test are shown in Figure 1. The dietary supplementation of clove powder could provide significantly ($p < 0.05$) higher total erythrocytes, haemoglobin, and hematocrit values than positive control. The total erythrocytes of hybrid grouper at 10 g and 15 g treatment doses obtained a higher value ($p < 0.05$) than positive control treatment before challenge test and on day 7 after the challenge test. The hemoglobin and hematocrit values of hybrid grouper at 10 g and 15 g treatment doses showed a higher value ($p < 0.05$) than positive control treatment on all observational days after the challenge test. The total leucocytes on day one after the challenge test increased significantly ($p < 0.05$) in positive control treatment and 10 g treatment dose, while negative control treatment and 15 g treatment dose obtained an insignificant different value. On day 3 post challenge test obtained a significant increase of total leucocytes ($p < 0.05$) on all

treatments, except negative control treatment, while the 10 g and 15 g treatment doses continued to increase until the 7-th day, while the positive control treatment decreased.

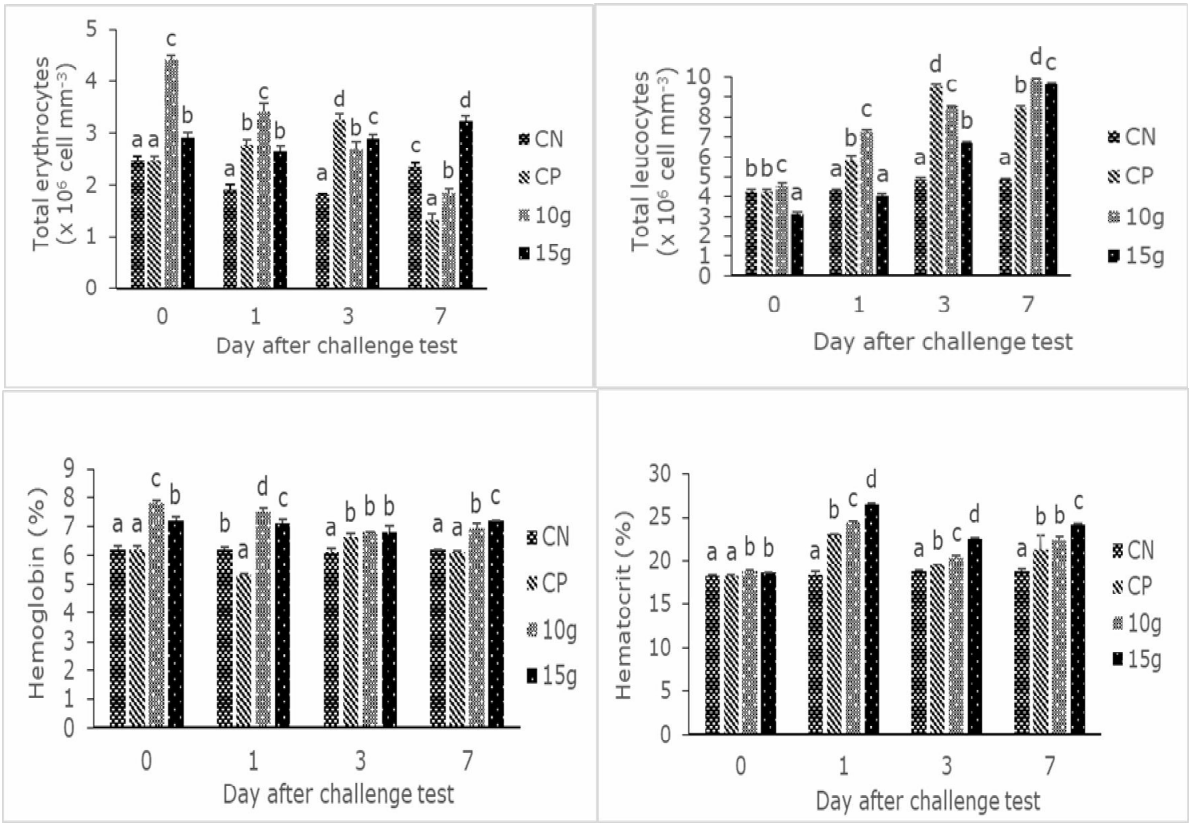

Figure 1. Total erythrocytes, total leucocytes, hemoglobin, and hematocrit of hybrid grouper before challenge test (0), on day one, 3, and 7 post challenge test on negative control (CN), positive control (CP), 10 g kg^{-1}, and 15 g kg^{-1} clove powder treatments. Values were presented as mean±SD. Values in the same bar having different superscript letters are significantly different ($p < 0.05$; Duncan's test).

Leucocyte differential. The leucocyte differential observation contained total lymphocytes, neutrophils, and monocytes of hybrid grouper before and after the challenge test and are shown in Figure 2. The lymphocyte percentage on the day before challenge test in negative control, positive control, and 15 g treatments were insignificantly different. The lymphocyte percentage after challenge test obtained a significant difference at 10 g and 15 g treatments, compared to the positive control treatment. The neutrophil percentage was insignificantly different before and after the challenge test. On day one, the neutrophil percentage increased significantly ($p < 0.05$) at 10 g and 15 g treatments, compared to the control treatment. On day 3, the neutrophil percentage increased significantly ($p < 0.05$) on the positive control treatment and started to decrease on day 7. The neutrophil percentage at 10 g treatment increased after challenge test until the 7-th day. The monocyte percentage decreased on all treatments after the challenge test.

The non-specific immune system is an important way for fish to attack pathogens. The haematological parameters are widely used to evaluate health status, stress and disease conditions of fish. The supplementation of clove powder in this study produced a better hematological profile before the challenge test and on day 7 post challenge test than the positive control treatment. Several spices supplemented in fish diets can alter the hematological profile and increased fish welfare. Iheanacho et al (2007) found that ginger at an inclusion level of 1000 mg/35 L increased the total erythrocytes, hematocrit, hemoglobin, and lymphocytes on juvenile *Clarias gariepinus*. A similar condition was also reported by Talpur (2014), that Asian seabass fed with peppermint *Mentha piperita* supplemented diet at 5 g kg^{-1} diet dose could increase the total erythrocytes, leucocytes,

and hemoglobin significantly ($p < 0.05$), followed by increased total leucocytes and hemoglobin, although the total erythrocytes decreased.

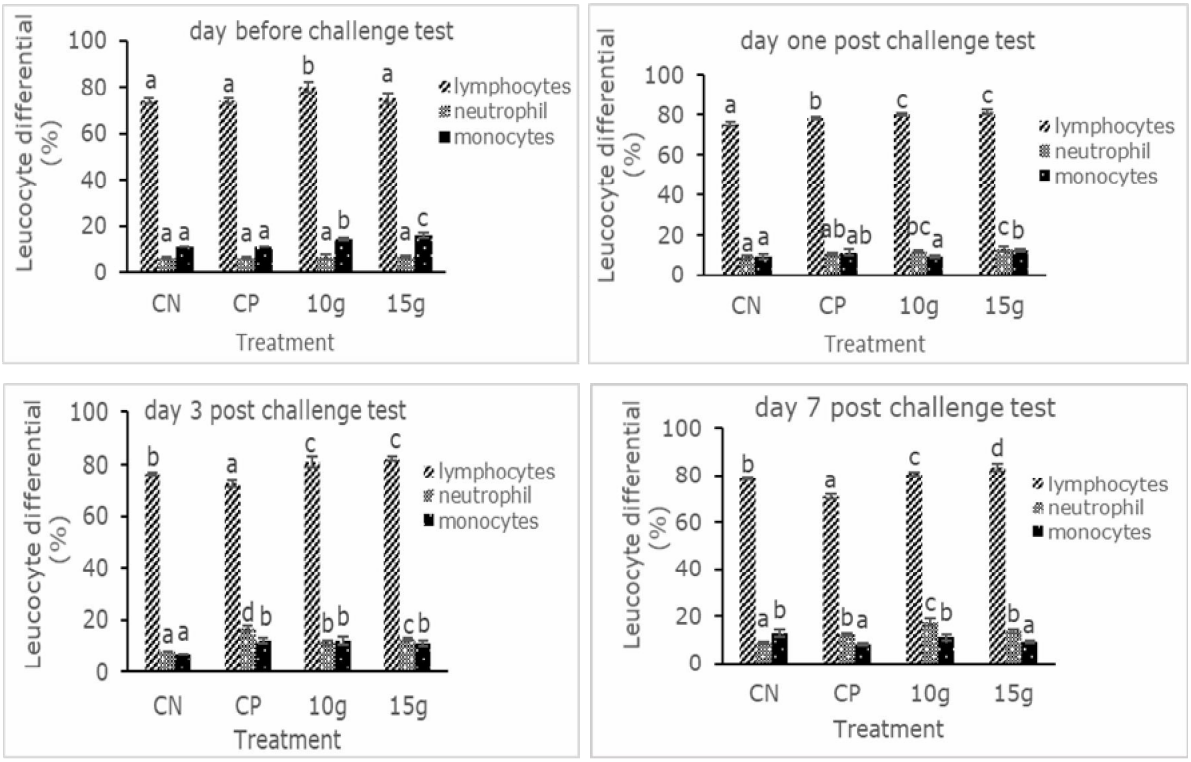

Figure 2. Leucocyte differential (lymphocytes, neutrophils, and monocytes) of hybrid grouper before and after challenge test on negative control (CN), positive control (CP), 10 g kg^{-1}, and 15 g kg^{-1} clove powder treatments. Values were presented as mean±SD. Values in the same bar having different superscript letters are significantly different ($p < 0.05$; Duncan's test).

Phagocytic and lysozyme activities. Phagocytic and lysozyme activities before challenge test and post challenge test are presented in Figure 3.

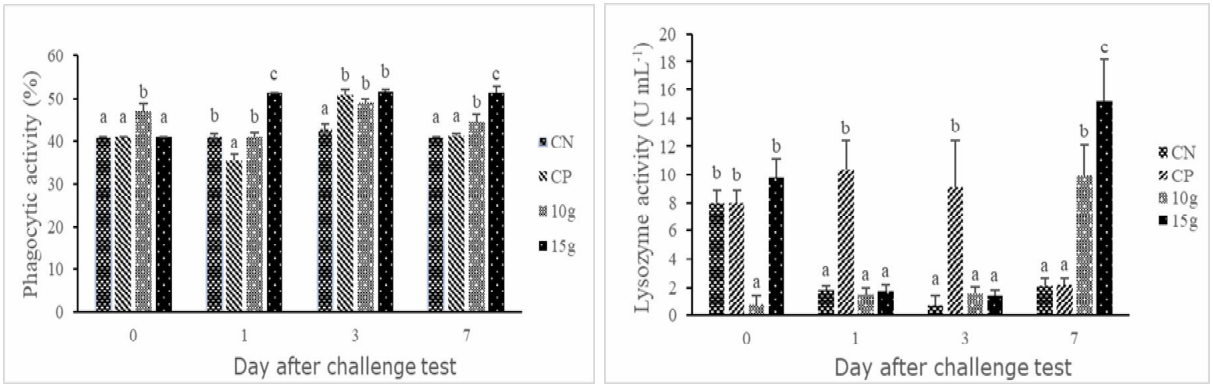

Figure 3. Phagocytic and lysozyme activities before challenge test (0), on the first day, 3, and 7 after challenge test on negative control (CN), positive control (CP), 10 g kg^{-1}, and 15 g kg^{-1} clove powder treatments. Values were presented as mean±SD. Values in the same bar having different superscript letters are significantly different ($p < 0.05$; Duncan's test).

Phagocytic activity before the challenge test on negative control, positive control, and 15 g kg^{-1} clove powder treatments were insignificantly different, but showing a significant difference with 10 g kg^{-1} clove powder treatment. On the first day after the challenge test, the phagocytic activity increased significantly ($p < 0.05$) at 15 g kg^{-1} clove powder

treatment. The phagocytic activity on day 3 post challenge test was insignificantly different for positive control, 10 g kg^{-1}, and 15 g kg^{-1} clove powder treatments, but showing a significant difference on negative control treatment. On day 7 post challenge test, the phagocytic activity was insignificantly different on negative and positive control treatments, but significantly different on 10 g kg^{-1} and 15 g kg^{-1} clove powder treatments. The lysozyme activity increased significantly ($p < 0.05$) on clove powder dietary supplementation treatments compared to the control treatments post 7 days of challenge test.

The supplementation of clove powder in this study could enhance the immune response, such as phagocytic and lysozyme activities in hybrid grouper before and after the challenge test. Haghighi & Rohani (2013) found that the rainbow trout *Oncorhynchus mykiss* fed with 1% ginger *Zingiber officinale* rhizome showed a significantly increased phagocytic activity of leucocytes compared to the control treatment. Xu et al (2020) found that the supplementation of 20 g kg^{-1} Chinese herbal medicine increased the lysozyme activity significantly in *Lateolabrax japonicus*. Lillehoj et al (2018) stated that the plant bioactive components played a role as chemical protector against microbial infections and as immunomodulator by enhancing the immune cell proliferation, cytokine modulation, and antibody response.

Hepcidin (hamp) and interleukin-1β (IL-1β). The dietary supplementation of clove powder obtained significantly higher ($p < 0.05$) hepcidin (*hamp*) and interleukin-1β (IL-1β) gene expression values than positive control (Figure 4). The *hamp* and IL-1β genes were expressed before challenge test until on day 3 post challenge test. The *hamp* gene expression on 10 g kg^{-1} clove powder treatment obtained a significantly higher value ($p < 0.05$) than other treatments, either before or on day 3 post challenge test. The significantly highest IL-1β gene expression ($p < 0.05$) was obtained from the 15 g kg^{-1} clove powder treatment, either before or on day 3 post challenge test.

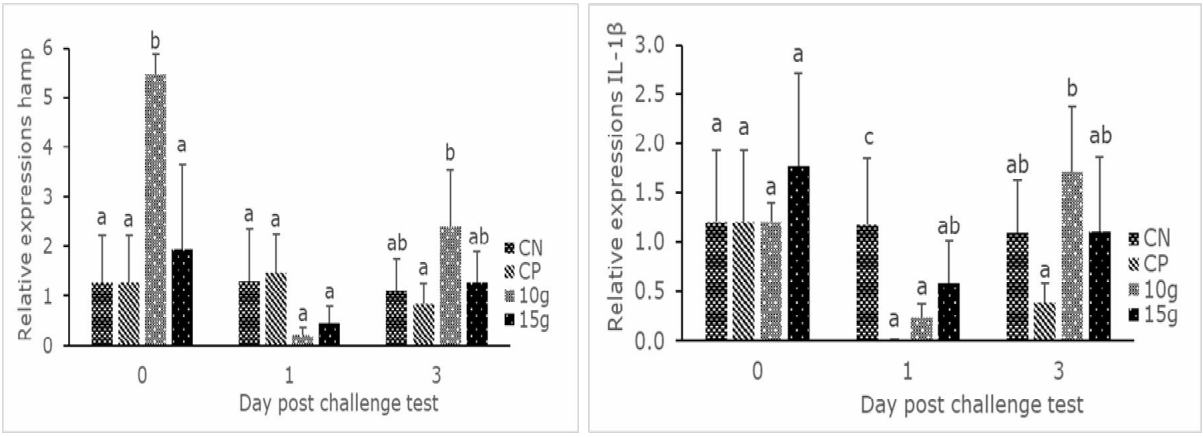

Figure 4. Hepcidin (*hamp*) and interleukin 1 β (IL-1β) gene expressions on hybrid grouper. Values were presented as mean±SD. Values in the same bar having different superscript letters are significantly different ($p < 0.05$; Duncan's test).

The interleukin-1β (IL-1β) is a proinflammatory cytokine that facilitates the inflammatory response caused by microbial invasion, tissue lesions, and immunological reactions (Liao et al 2018). Hepcidin is an important component in carrier body immune system as fish immunity against bacteria (Zhou et al 2020). The supplementation of clove powder in this study could increase the gene expressions of interleukin-1β and hepcidin before challenge test and on day 3 post challenge test, compared to the positive control treatment. Several previous studies have reported that the use of herbal ingredients can increase the expression of immune genes. Bilen et al (2019) found that banana plant powder could increase the IL-1β gene expression. Giri et al (2015) found that *Labeo rohita* fish fed with guava leaf powder supplemented diet could highly enhance the IL-1β gene expression.

Survival rate. The survival rate of hybrid grouper after 10 days of challenge test with *V. alginolyticus* obtained a better value on clove powder supplementation treatments than positive control treatment (Figure 5).

In this study, high immune response, immune gene expression, and survival rate in clove powder treatment obtained several effects that could induce the immune response and immunity-related gene expressions in hybrid grouper.

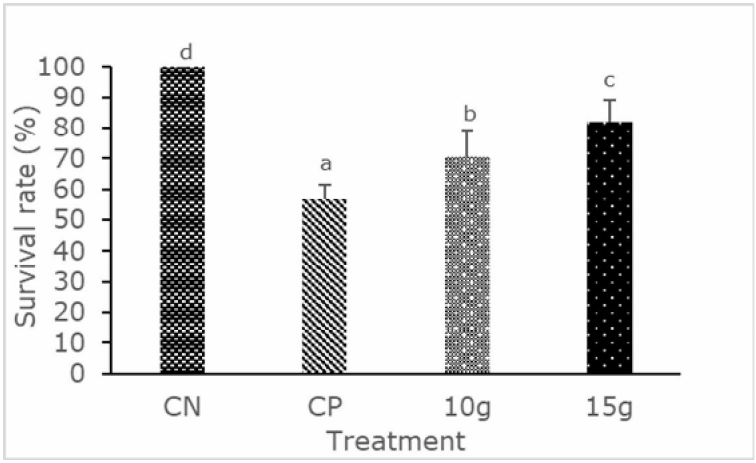

Figure 5. Survival rate of hybrid grouper after 10 days of challenge test. Data (average±SD), different letters over each treatment bar indicate a significant difference ($p < 0.05$; Duncan's test).

Conclusions. The supplementation of clove powder could enhance the non-specific immune response of hybrid groupers against *V. alginolyticus* infection. The 15 g kg^{-1} treatment was the best dose for clove powder due to capable of inducing the immune response and increasing the survival rate of hybrid groupers post challenge test. This study demonstrates the properties of clove (*S. aromaticum*) as an antibacterial agent, which allows its use as an alternative to antibiotics in diseases caused by *V. alginolyticus*.

Acknowledgements. This research was partially supported by BPPDN scholarship No. B/67/D.D3/KD.02.00/2019 from Ministry of Education, Culture, Research and Technology, of Republic Indonesia. The authors are very grateful to the Laboratory of Fisheries and Marine Observation Station (IFMOS) and the laboratory staff of aquatic organism health in the IPB University for facility support. We would like to thank Ms. Dian Novita Sari, Ms. Maulina Agriandini, Mrs. Muzna Toatubun, Mrs. Valenzia Enzeline, Mr. Lutfi, Mr. Dendy Hidayatullah and Ms. Naula for their excellent help for this study.

Conflict of interest. The authors declare that there is no conflict of interest.

References

Adeshina I., Jenyo-Oni A., Emikpe B. O., Ajani E. K., Abdel-Tawwab M., 2019 Stimulatory effect of dietary clove, *Eugenia caryophyllata*, bud extract on growth performance, nutrient utilization, antioxidant capacity, and tolerance of African catfish, *Clarias gariepinus* to *Aeromonas hydrophila* infection. Journal of the World Aquaculture Society 50(2):390-405.

Anderson D. P., 1992 Immunostimulants, adjuvants, and vaccine carriers in fish: applications to aquaculture. Annual Review of Fish Disseases 2:281-307.

Anderson D. P., Siwicki A. K., 1995 Basic haematology and serology for fish health programs. Paper Presented in Second Symposium on Diseases in Asia Aquaculture "Aquatic Animal Health and the Environmental", Phuket Thailand 25-29th October 1993, pp. 185-202.

Bilen S., Sirtiyah A. M. A., Terzi E., 2019 Therapeutic effects of beard lichen, *Usnea barbata* extract against *Lactococcus garvieae* infection in rainbow trout *Oncorhynchus mykiss*. Fish and Shellfish Immunology 87:401-409.
Blaxhall P. C., Daisley K. W., 1973 Routine haematological methods for use with fish blood. Journal of Fish Biology 5(6):771-781.
Cortés-Rojas D. F., Fernandes de Souza C. R., Pereira Oliveira W., 2014 Clove (*Syzygium aromaticum*): a precious spice. Asian Pacific Journal of Tropical Biomedicine 4(2):90-96.
Ellis R. P., Parry H., Spicer J. I., Hutchinson T. H., Pipe R. K., Widdicombe S., 2011 Immunological function in marine invertebrates: responses to environmental perturbation. Fish and Shellfish Immunology 30(6):1209-1222.
Gaber M., 2000 Growth response of nile tilapia fingerlings (*Oreochromis niloticus*) fed diets containing different levels of clove oil. Egyptian Journal of Aquatic Biology and Fisheries 4(1):1-18.
Gaspar E. M., Duarte R., Santana J. C., 2018 Volatile composition and antioxidant properties of clove products. Biomedical Journal of Scientific and Technical Research 9(4):1-7.
Giri S. S., Sen S. S., Chi C., Kim H. J., Yun S., Park S. C., Sukumaran V., 2015 Effect of guava leaves on the growth performance and cytokine gene expression of *Labeo rohita* and its susceptibility to *Aeromonas hydrophila* infection. Fish and Shellfish Immunology 46(2):217-224.
Haghighi M., Rohani M. S., 2013 The effects of powdered ginger (*Zingiber officinale*) on the haematological and immunological parameters of rainbow trout *Oncorhynchus mykiss*. Journal Medicinal Plant and Herbal Therapy Research 1(1):8-12.
Iheanacho S. C., Ogunji J. O., Ogueji E. O., Nwuba L. A., Nnatuanya I. O., Ochang S. N., Mbah C. E., Usman I. B., Haruna M., 2017 Comparative assessment of ampicillin antibiotic and ginger (*Zingiber officinale*) effects on growth, haematology and biochemical enzymes of *Clarias gariepinus* juvenile. Journal of Pharmacognosy and Phytochemistry 6(3):761-767.
Jiang S., Wu X., Luo Y., Wu M., Lu S., Jin Z., Yao W., 2016 Optimal dietary protein level and protein to energy ratio for hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) juveniles. Aquaculture 465:28-36.
Liao C. L., Zhang G. R., Zhu D. M., Ji W., Shi Z. C., Jiang R., Fan Q. X., Wei K. J., 2018 Molecular cloning and expression analysis of interleukin-1β and interleukin-1 receptor type I genes in yellow catfish (*Pelteobagrus fulvidraco*): responses to challenge of *Edwardsiella ictaluri*. Comparative Biochemistry and Physiology Part B 223:1-15.
Lillehoj H., Liu Y., Calsamiglia S., Fernandez-Miyakawa M. E., Chi F., Cravens R. L., Oh S., Gay C. G., 2018 Phytochemicals as antibiotic alternatives to promote growth and enhance host health. Veterinary Research 49(1):76.
Livak K. J., Schmittgen T. D., 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔcT method. Methods 25(4):402-408.
Manage P. M., 2018 Heavy use of antibiotics in aquaculture: emerging human and animal health problems - a review. Sri Lanka Journal of Aquatic Sciences 23(1):13-27.
Pratiwi N., Jusadi D., Nuryati S., 2016 [Utilization of clove oil *Syzigium aromaticum* to improve feed efficiency on striped catfish *Pangasianodon hypophthalmus* (Sauvage, 1876)]. Jurnal Ikhtiologi Indonesia 16(3):233-242. [in Indonesian]
Sahu S., Das B. K., Mishra B. K., Pradhan J., Sarangi N., 2007 Effect of *Allium sativum* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. Journal of Applied Ichthyology 23(1):80-86.
Shalaby S. E. M., Magd El-Din M., Abo-Donia S. A., Mettwally M., Attia Z. A., 2011 Toxicological impacts of essential oils from *Eucalyptus globulus* and clove *Eugenia caryophyllus* on albino rats. Polish Journal of Environmental Studies 20(2):429-434.
Silvianti T., Jusadi D., Nuryati S., 2016 [The supplementation of clove oil *Syzygium aromaticum* in the diet to improve the growth performance of common carp *Cyprinus carpio* Linnaeus 1758]. Jurnal Ikhtiologi Indonesia 16(2):211-225. [in Indonesian]

Sohilait H. J., Kainama H., Nindatu M., 2018 Chemical composition and antibacterial activity of the essential oils from different parts of *Eugenia caryophylata* Thunb grown in Amboina Island. Internation Journal of Organic Chemistry 8(2):229-239.
Talpur A. D., 2014 *Mentha piperita* (peppermint) as feed additive enhanced growth performance, survival, immune response and disease resistance of Asian seabass, *Lates calcarifer* against *Vibrio harveyi* infection. Aquaculture 420-421:71-78.
Watanabe T. 1988. Fish nutrition and mariculture. Department of Aquatic Bioscience, Tokyo University of Fisheries, Japan International Cooperation Agency (JICA), 223 pp.
Xu A., Shang-Guan J., Li Z., Gao Z., Huang Y. C., Chen Q., 2020 Effects of dietary Chinese herbal medicines mixture on feeding attraction activity, growth performance, nonspecific immunity and digestive enzyme activity of Japanese seabass (*Lateolabrax japonicus*). Aquaculture Reports 17:100304.
Ye G., Dong X., Yang Q., Chi S., Liu H., Zhang H., Tan B., Zhang S., 2020 A formulated diet improved digestive capacity, immune function and intestinal microbiota structure of juvenile hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) when compared with chilled trash fish. Aquaculture 523:735230.
Zhou S., Hou Z., Li N., Qin Q., 2007 Development of a SYBR Green I real-time PCR for quantitative detection of *Vibrio alginolyticus* in seawater and seafood. Journal of Applied Microbiology 103(5):1897-1906.
Zhou S., Tu X., Pang H., Hoare R., Monaghan S. J., Luo J., Jian J., 2020 A T3SS regulator mutant of *Vibrio alginolyticus* affects antibiotic susceptibilities and provides significant protection to *Danio rerio* as a live attenuated vaccine. Frontiers in Cellular and Infection Microbiology 10:183.

Received: 18 March 2022. Accepted: 14 July 2022. Published online: 08 September 2022.
Authors:
Inem Ode, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University (Bogor Agricultural University), Raya Agatis St., Dramaga, Bogor, 16680 West Java, Indonesia, e-mail: inemarfanode@apps.ipb.ac.id
Sukenda Sukenda, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University (Bogor Agricultural University), Raya Agatis St., Dramaga, Bogor, 16680 West Java, Indonesia, e-mail: Sukenda@apps.ipb.ac.id
Widanarni Widanarni, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University (Bogor Agricultural University), Raya Agatis St., Dramaga, Bogor, 16680 West Java, Indonesia, e-mail: widanarni@apps.ipb.ac.id
Dinamella Wahjuningrum, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University (Bogor Agricultural University), Raya Agatis St., Dramaga, Bogor, 16680 West Java, Indonesia, e-mail: dinamellawa@apps.ipb.ac.id
Munti Yuhana, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University (Bogor Agricultural University), Raya Agatis St., Dramaga, Bogor, 16680 West Java, Indonesia, e-mail: muntiyu@apps.ipb.ac.id
Mia Setiawati, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University (Bogor Agricultural University), Raya Agatis St., Dramaga, Bogor, 16680 West Java, Indonesia, e-mail: miasetia@apps.ipb.ac.id

How to cite this article:
Ode I., Sukenda S., Widanarni W., Wahjuningrum D., Yuhana M., Setiawati M., 2022 Effect of clove (*Syzygium aromaticum*) powder supplementation on non-specific immune response of hybrid grouper (*Epinephelus fuscoguttatus* ♀×*Epinephelus lanceolatus* ♂) infected with *Vibrio alginolyticus*. AACL Bioflux 15(5):2282-2291.