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Effect of Clove Powder *Syzygium aromaticum* Supplementation on Growth and Health Status of Cantang Grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) in Floating Net Cage

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ABSTRACT Cantang grouper is a hybrid grouper which is one of the main grouper species cultivated in Indonesia. This study aimed to evaluate the growth and health status of cantang grouper in floating net cages supplemented with clove powder. This study used a completely randomized design with three treatments, each with three replications. Two levels of clove powder dose ,10 and 15 g kg^{-1}, and one treatment without clove powder dose (control) were used were. Feed treatment using the repelletting method. Parameters observed were growth, survival rate, levels of malondialdehyde and superoxide dismutase enzyme, blood parameters, phagocity activity, total bacterial count (TBC) and total vibrio count (TVC). The results showed that the treatment of clove powder supplementation in cantang grouper feed for 30 days able to increase the growth, survival rate, activity of the enzyme superoxide dismutase (SOD), and total lymphocyte. The application of clove powder in the feed did not significantly affect the total erythrocytes, leukocytes, hematocrit, phagocytic activity, total bacterial count (TBC) and total vibrio count (TVC) of hybrid grouper in floating net cages. This study concludes that administration of clove powder at a dose of 15 g kg^{-1} feed able to significantly increased the fish growth, total lymphocyte, and survival rate of cantang grouper in floating net cages.

Keywords: Cantang grouper; clove; feed supplement; fish health; growth

INTRODUCTION

Cantang grouper is a hybrid grouper that has now become one of the most widely cultivated grouper species using a floating net cage in Indonesia. An indicator of success in fish farming is the achievement of fast growth and a high survival rate to increase production value of fish. The feed provides an important nutrient input for optimal grouper growth. One of the ways to improve the quality of cultured fish feed is by adding natural ingredients that function as feed additives in feed formulations to increase fish productivity. According to Lillehoj *et al*. (2018), natural ingredient supplements in feed can stabilize gut microbiota and reduce microbial toxic metabolites in the gut, reduce oxidative stress, and increase antioxidant activity, thereby having a positive impact on growth and immunity. The use of natural ingredients as an alternative to feed supplements because it has biodegradable and environmentally friendly properties, and are easy to find.

One natural ingredient that has the potential to be used is clove. Cloves contain bioactive compounds such as 10-13% tannins, terpenoids, glycosides, and 14-21% essential oils. The essential oil compounds are *eugenol*, *caryophyllene*, *furfural*, *vanillin*, *methyl salicylate*, *pyrocatechol*, *methyl ketone* & *valeric aldehydes*, *eugenin*, *isoeugenol*, *isoeugenitin*, *eugenitin*, *tannin*, *mucilage*, *sitosterol*, *stigmasterol*, *resins*, *cellulose*, *pinene* & fixed oil (Rojas *et al*., 2014). Cloves in the field of fisheries are widely used as anesthetics. Several studies have stated that the use of clove oil in certain doses as an anesthetic is safe and effective for fish. Roubach *et al*. (2005) stated that the addition of clove oil as an anesthetic agent in *Colossoma macropomum* at a dose of 65 mgL^{-1} was safe and effective. Okey *et al*. (2018) stated that the addition of clove powder at a concentration of 80-140mg/l was a safe and ideal anesthetic for African catfish *Clarias gariepinus*. Clove oil applied at a concentration of 75 ppm was safe for *Carassius auratus* and did not indicate any hematological changes (Abdolazizi *et al*., 2011). Javahery *et al*. (2012), eugenol is the main compound in cloves which is rapidly absorbed and metabolized after oral administration, and it is almost completely excreted in the urine within 24 h with no apparent ill effects in fish. Sohilait *et al*. (2018), *eugenol* and *eugenyl acetate* contained in clove oil have high antioxidant activity so they can be used as hepato-protective and lipid peroxidase inhibitory activities. Antioxidant activity is thought to be a trigger for improving fish growth and health performance.

Research on the use of cloves in fish feed has been conducted by several previous studies, including Gaber (2000), the use of clove oil at a dose of 8 mg 100 g^{-1} feed can increase body weight and feed efficiency of tilapia *Oreochoromis niloticus*. Pratiwi *et al*. (2016) the use of clove oil at a dose 10 mg 100 g-1 increase the feed efficiency, protein and lipid retention of *Pangasianodon hypophthalmus*. Silvianti *et al*. (2016) the addition of clove oil at a dose of 100 mg to 100 g^{-1} feed can improve the growth performance of *Cyprinus carpio*. The use of clove oil at a dose of 100 mg to 100 g of feed can improve the

growth performance of *Colossoma macropomum* (Putri *et al*., 2016). Adeshina *et al*. (2019) found of clove extract at a dose 15 g/kg diet can improve growth performance and nutrient utilization of African catfish.

Several research results have shown that feed additives added to feed function as immunostimulants that can increase the resistance of fish and shrimp to infectious diseases by increasing nonspecific immune responses while increasing fish growth (Pais *et al*., 2008). The use of plants as an alternative to feed supplements, because it has biodegradable and environmentally friendly properties, and is easy to find. This study aimed to evaluate the growth and health status of hybrid grouper in floating net cages supplemented with clove powder. The results of this study are expected to be an alternative solution to the use of cloves as a feed supplement that can increase the growth and health status of cantang grouper.

MATERIALS AND METHODS

Time and place

This research was conducted from August to September 2021 at the floating net cage on Kelapa Dua island, Kepulauan Seribu Regency, and the Aquatic Organism Health Laboratory of IPB University.

Materials

The tool used is net cages (1x1x1.7 m), blender (*Miyako BL-125 GF, Japan*), spectrophotometer, centrifuge, analytical balance, syringe, dissecting set, petri dish, *erlenmeyer*, micropipette, serological pipettes, test tubes, loop needle, bunsen, autoclave, *eppendorf* tube, hemocytometer, microscope and water quality measuring instrument. The ingredients used were cantang grouper (average weight size of 13.5±0.95 g and the average length size of 8.9±0.04 cm), dry cloves, commercial pellets, Abcan kit test, TCBS media, SWC media, SOD enzyme, PBS, and EDTA.

Methods

Research design

This study used a completely randomized design containing three treatments and three replications. The treatments used contained two levels, namely clove powder doses at 10 and 15 g kg^{-1}, and control without clove powder supplementation. Feeding was performed twice a day for 30 days with satiation at 08.00 and 16.00 GMT+7.

Feed test preparation

Dried clove was obtained from Mardika Market, Ambon, Maluku Province. Cloves were cleaned from the attached substance before were blended in a blender machine (*Miyako BL-125 GF, Japan*) to obtain a powder. Feeds were produced following the repelletting method. Commercial feed is 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^{0}C. Pelleted diets were packed in plastic containers labelled following the applied treatments.

Growth

The Daily growth rate was measured at the end of the study using a formula based on Hopkins (1992):

$$DRG = \left[\sqrt[t]{\frac{Wt}{Wo}} - 1\right] \times 100$$

DGR = Daily growth rate (%); Wt = Final average weight (g); Wo = Initial average weight (g); t = Time (day).

Survival rate

The survival rate (SR) was determined using the following formula:

$$SR\,(\%) = \frac{\text{Final number of fish}}{\text{Initial number of fish}} \times 100$$

Malondialdehyde (MDA) levels and superoxide dismutase (SOD) activity

Malondialdehyde (MDA) assay was done using Lipid Peroxidation Test kit (Abcan, UK), 0.3 g of liver tissue, and EDTA. a 2-5 ml of chloroform-methanol was added to a faded liver tissue that previously has been removed from the EDTA. Samples were then centrifuged using a centrifuge at 3000 rpm for 15-20 minutes. The supernatant was taken and put into a sterile test tube and added with 200 L of test solution, and shake until the mixture was reddish yellow or red. The mixture was read by spectrophotometry at 550 nm. Level of MDA was calculated as follows:

MDA level= absorbance x 100 x 0.0113 [µMol/L].

SOD assay was conducted using SOD Test kit (Abcan, UK). A total of 0.3 g of the liver was added to 10 ml of 10 M sulfuric acid and centrifuged. The supernatant was taken and put into a sterile tube, and added with 20 L of the test solution (20 L of SOD Assay Buffer + 50 L of SOD enzyme solution in 10 ml of cold PBS). Allow 20 minutes, and stop the reaction by adding 50 L BHT. Read on spectrophotometry at 550 m. SOD activity was canculated based on a formula (absorbance x 70.16/ total protein in the sample). The total protein sample was calculated by adding 20 L of PA solution to 10 M in 100 ml of sulfuric acid, followed by homogenization, addition of 50 l of the mixture to the sample supernatant, and absorbance check a wavelength of 450 m. the total protein samples (gram protein) were design as the results from the equation (absorbance x 6. 11).

Blood parameters and phagocytic activity

The fish blood sample was taken using a 1 ml syringe from the vena caudalis and collected in a microtube. The blood parameter measurements containing the total erythrocytes, total leucocytes, hematocrit, hemoglobin, and differential leucocyte count were performed following the Blaxhall & Daisley (1973) procedures. The phagocytic activity was calculated based on Anderson & Siwicki (1993) method. The phagocytic activity was calculated following the formula:

$$\text{Phagocytic activity}\,(\%) = \frac{\text{Total phagocyting cells}}{\text{Total phagocytic cells}} \times 100$$

Total bacterial count (TBC) and total vibrio count (TVC) in Kidney

The test fish were dissected and the kidneys were taken, then mashed. After that, 0.1 g was taken and homogenized with 0.9 mL PBS solution, then spread on SWC agar media (to calculate TVC) and thiosulfate citrate bile salt sucrose (TCBS) agar (to calculate TBC). It was then incubated at 28°C for 24 hours. After 24 hours, the number of bacterial colonies formed on the media was counted. The total bacteria were calculated using the spread plate count method with the following formula:

$$\Sigma \text{bacteria} = \frac{N}{\Sigma \text{Speared}} \times \frac{1}{f}$$

Σ bacteria = Number of bacterial cells (CFU/g); N = Number of bacterial colonies; f = Diluent factor.

Water Quality

Water quality measurements are carried out once a week at 07.00, 12.00 and 16.00 GMT+7, measurements include temperature, salinity, pH and DO.

Statistical analysis

The study data were analyzed qualitatively and quantitatively. The statistical analysis uses a one-way analysis of variance (one-way ANOVA) with the *statistical program software system* (SPSS) *ver 20.0*. All treatments with a significant difference ($p<0.05$), were subsequently analyzed with Duncan's multiple range test.

RESULTS AND DISCUSSION

Growth

The results of observations of the growth of cantang grouper for 30 days is shown in Table 1. Feed treatment with supplementation clove powder at a dose 15 g/kg^{-1} feed was able to increase the length and weight gain significantly ($P<0.05$) compared to the control.

Table 1. Growth of cantang grouper for 30 days.

Parameter	Treatment clove powder dose (g kg^{-1})		
	0 (Control)	5	10
Initial body weight (g)	13.57±0.95	13.65±0.71	13.9±0.91
Final body weight (g)	21.70±0.73^{a}	22.28±0.82^{ab}	24.08±1.42^{b}
Initial body length (cm)	8.95±0.0^{4}	8.92±0.15	9.01±0.08
Final body length (cm)	10.50±0.08^{a}	10.45±0.47^{a}	10.63±0.31^{b}
Daily Growth Rate (DGR) (%/day)	1.57±0.21^{a}	1.64± 0.06^{a}	1.82±0.04^{a}

*Values are presented as mean ± standard deviation. Different letters in the same line show a significantly different effect ($P<0.05$).

The highest daily growth rate of cantang grouper in this study was 1.82 (%/day) at a dose of 15g/kg clove powder. He *et al*. (2021) obtained the specific growth rate of cantang grouper of 0.82-1.18% after the addition of Chinese herbs *Pyrola calliantha* and *Verbena officinalis* into the feed. Abdel-Rahman *et al*. (2020) obtained a specific growth rate of tilapia of 1.20%/day on a supplement with 3% clove powder.

Survival rate

The survival rate of cantang grouper for 30 days showed that the feed with clove powder supplementation gave a better survival rate than the control (Figure 1). The results of statistical tests showed that the two doses of clove statistical tests showed that the two doses of clove powder had a significant effect ($P<0.05$) on the survival value of cantang grouper. The results of previous studies showed that the addition of clove oil with a concentration of 3% (equivalent to 3 g/100 g) in tilapia feed, could increase the survival rate after being challenged with *Lactococcus garvieae* (Rattanachaikunsopon & Phumkhachorn, 2009). Clove extract in feed was able to increase the survival rate of African catfish after being challenged with *Aeromonas sobria* (Ghaly *et al*., 2015).

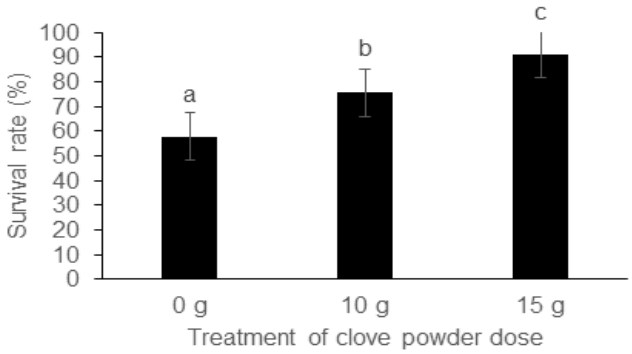

Figure 1. The survival of the cantang grouper during the study.
*Data (average±SD), different letters over each treatment bar indicate a significant difference ($p < 0.05$; Duncan's test).

Malondialdehyde (MDA) levels and superoxide dismutase (SOD) activity

Levels of malondialdehyde (MDA) and superoxide dismutase (SOD) in the liver of cantang grouper after 30 days showed that the levels of malondialdehyde (MDA) were lower in the control compared to the clove powder treatment, while the activity of the enzyme superoxide dismutase (SOD) in the 10 g clove powder treatment significantly increased ($p<0.05$) compared to the control (Figure 2 & 3). The increase in SOD value is thought to be due to the effect of antioxidant activity from feed containing clove powder but has not been able to reduce MDA levels. Chakraborty & Hancz (2011), suggest that various kinds of chemical compounds in herbal plants have antioxidant effects that can help organisms deal with (oxidative) stress by damaging free radicals. The antioxidant effect improves the general physiological condition of fish.

Figure 2. Levels of malonaldehyde (MDA) in the liver of cantang grouper.
*Data (average±SD), different letters over each treatment bar indicate a significant difference ($p < 0.05$; Duncan's test).

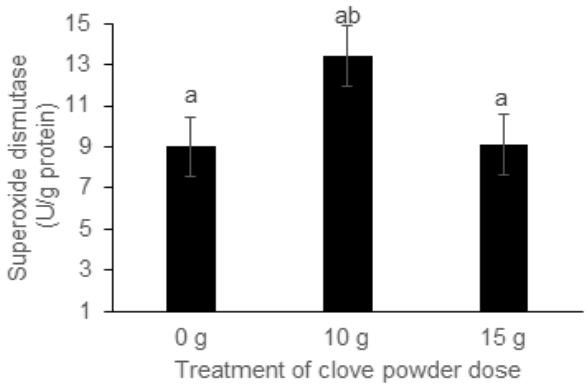

Figure 3. Levels of superoxide dismutase (SOD) in the liver of cantang grouper.

*Data (average±SD), different letters over each treatment bar indicate a significant difference ($p < 0.05$; Duncan's test).

Blood parameters

Blood parameters are an approach that can be used to analyze the health status of fish. The addition of clove powder in the feed gave the results of blood parameters that were not significantly different for all treatments, but for the hemoglobin parameter, the clove powder treatment at a dose of 10 g kg^{-1} gave significantly higher results than the control and a dose of 15 g kg^{-1} (Table 2).

Table 2. Blood parameters of cantang grouper.

Parameter	Treatment clove powder dose (g kg^{-1})		
	0 (Control)	10	15
Total erythrocytes (10^6sel/mm^3)	2.49±1.11^a	2.42±0.53^a	2.82±0.53^a
Total leucocytes (10^4sel/mm^3)	3.14±0.71^a	4.38±0.74^a	3.26±0.75^a
Haemoglobin (%)	6.49±0.61^b	8.61±0.79^c	4.29±1.04^a
Hematocrit (%)	16.99±1.21^a	16.91±1.35^a	14.65±0.82^a
Phagocytic activities (%)	47.88±1.83^a	46.66±1.52^a	49.22±2.33^a

*Values are presented as mean ± standard deviation. Different letters in the same line show a significantly different effect ($P<0.05$),

availability of oxygen in the blood. A feed with 15 g clove powder decreased the hematocrit count. According to Setiawati *et al*. (2017) different amounts of blood hematocrit are influenced by environmental factors and physiological conditions of fish.

Phagocytic activity is a non-specific cellular immune response. A feed with 15 g clove powder resulted in higher phagocytic activity. Chakraboty & Hancz (2011), suggested that the phytochemicals contained in herbs can increase phagocytic activity, complement system activity, and antibody production.

The leukocytes differential observations of cantang grouper included the number of lymphocytes, neutrophils, and monocytes (Figure 4). Lymphocytes are the most common leukocyte cells found in fish leukocytes, followed by neutrophils and monocytes (Sonida *et al*., 2014). Lymphocyte cells play a major role in the specific immune system and consist of B cells, T cells, and natural killer (NK) cells. Lymphocytes have a role as a provider of immune substances for body defense. Neutrophils protect the host against bacterial infection by secreting proteolytic enzymes and lysozyme to destroy bacteria. Meanwhile, monocytes will increase if there is an interaction that interferes with the condition of the fish, such as an infection caused by bacteria (Utami *et al*., 2013). The lymphocyte value in the feed with clove powder increased significantly ($p<0.05$) compared to the control, it is suspected that cloves in the feed can function as an immunomodulator that stimulates the immune response mechanism. The number of neutrophils was not significant in all treatments, while the number of monocytes in the feed treatment with clove powder was significantly ($p<0.05$) lower than the control.

Erythrocytes function as carriers of oxygen and nutrients to all parts of the body of an organism. Erythrocytes carry O_2 to the tissue and release CO_2 and protons produced during the metabolism process (Sakuragui *et al*., 2019). Feed treatment with clove powder dose 15 g was able to increase the number of erythrocyte cells compared to the control. Elimanafe *et al*. (2013) suggested that the number of erythrocyte cells in seawater fish ranged from 1 x 10^6 cells/mm^3 to 3 x 10^6 cells/mm^3. White blood cells in fish play an important role in the defense system (immunity) due to stress, inflammation, and infectious diseases. The addition of clove powder in the feed was able to increase leukocytes compared to the control. Shalaby *et al*. (2011) also found an increase in the number of white blood cells as a defense response of albino rats after the addition of clove oil to the feed.

Treatment of 15 g of clove powder resulted in low hemoglobin levels in cantang grouper, the decrease in hemoglobin was thought to be caused by stress or bacterial infection from the environment. Syawal *et al*. (2011) suggested that a decrease in hemoglobin levels indicates that the fish are stressed or infected, which reduces the

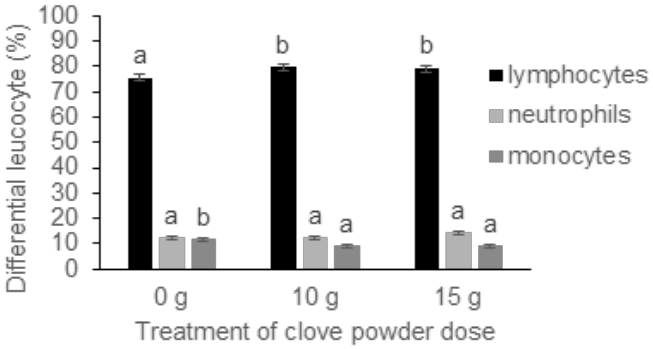

Figure 4. Differential Leucocyte (lymphocytes, neutrophils, and monocytes) of cantang grouper.

*Data (average ± SD), different letters over each treatment bar indicate a significant difference ($P< 0.05$; Duncan's test).

The lower number of monocytes is thought to be because the lymphocytes have succeeded in eliminating the antigen from the host's body.

Total bacterial count (TBC) and total vibrio count (TVC) in the Kidney

The addition of clove powder to the feed resulted in the total bacterial count and total vibrio count which were not significantly different from the control (Table 3). The dose of clove powder used in this study was not able to significantly reduce total vibrio in the kidneys. Even though according to Hu et al. (2018). eugenol compounds in cloves demonstrate the inhibitory activity of Gram-negative and Gram-positive bacteria, as their mechanisms are associated with the biofilm synthesis, virulence factor gene expression, migration, and adhesion inhibitions. Rattanachaikunsopon & Phumkhachorn (2009), stated that antimicrobial power depends on the source of cloves and bacterial strains. Some types of bacteria are sensitive when exposed to certain essential oils so that they produce different responses. steroids, and essential oils. Rifat *et al*. (2008), stated that cloves contain the main active compound, namely eugenol. Eugenol is an essential oil, the difference in feed can stimulate the central nervous system, to increase appetite and consumption of nutrients. The presence of essential oils can also stimulate the production of digestive juices which produce an appropriate pH for digestive enzymes, such as peptinases, resulting in an increase in digestive enzyme activity, which affects energy conversion and digestion of food substances. According to Sohilait *et al*. (2015) eugenol and eugenyl acetate contained in clove oil have high antioxidant activity. Ogata *et al*. (2000) stated that the antioxidant activity of eugenol is thought to trigger an increase in growth performance and fish health.

The survival of fish depends on the adaptability of fish to food and the environment, fish health status, stocking density, and water quality that is sufficient to support growth. The use of clove powder at a dose of 15 g in this study significantly increased the differential leukocyte (lymphocyte) value and the survival of cantang grouper. It is suspected that a dose of 15 g can act as an immunostimulant. Ode *et al*. (2022) supplementing with clove powder in feed was able to significantly increase the non-specific immune response and survival of cantang grouper after being challenged with Vibrio alginolyticus CFU 10^7 mL^{-1}. Feed additives from plant materials can function as immunostimulants which can increase the non-specific immune response of fish when disease infections occur. In addition, feed additives can also act as feed attractants which can increase fish growth (Pais *et al*., 2008).

Table 3. Total bacterial count (TBC) and total vibrio count (TVC) in the kidney.

Parameter	Treatment clove powder dose (g kg^{-1})		
	Control	10	15
TBC (Log 10 CFU/g)	5.07±0.41[a]	5.05±0.46[a]	5.05±0.65[a]
TVC (Log 10 CFU/g)	2.64±0.25[a]	2.60±0.25[a]	2.57±0.27[a]

*Values are presented as mean ± standard deviation. Different superscript letters in the same line show a significantly different effect (P<0.05).

Water quality

The water quality parameters measured during the study are shown in Table 4. The results of water quality measurements compared with water quality data based on the Indonesian National Standard (2014) show that the water quality parameters at the study site are in the normal range and are suitable for the life of cantang grouper.

Table 4. Water quality parameters.

Parameter	Value	SNI 8036.2 (2014)
Temperature (°C)	28 - 29	28 – 32
Salinity (Ppt)	31 - 32	24 – 33
pH	7.9 - 8.1	7.5 – 8.5
DO (mg/L)	6 - 6.5	Minimum 4 ppm

Cultivating fish with floating net cage systems in marine waters often faces several obstacles, namely oceanographic dynamics and fluctuating water quality so that fish have the potential to be stressed and exposed to disease (De *et al*., 2019). Environmental changes can cause stress to fish, and increase the potential for bacterial populations in waters, including pathogenic bacteria (Tammi *et al*., 2015). In this study, the use of clove powder at a dose of 15 g was able to significantly increase growth, differential leukocytes (lymphocytes) and survival of cantang grouper. According to Reverter (2014), plant extracts have been reported to have various activities such as anti-stress, growth-promoting, appetite stimulation, hepatoprotective effects, increased immunostimulation and anti-pathogenic properties in fish farming because they contain many active compounds such as alkaloids, terpenoids, tannins, saponins, glycosides, flavonoids, phenolics,

CONCLUSIONS AND RECOMMENDATION

Conclusions

The administration of clove powder dose of 15 g kg^{-1} feed was able to significantly increased growth, differential leukocyte (lymphocyte) and survival rate of cantang grouper in floating net cages.

Recommendation

This study has demonstrated the potential of cloves as an alternative to feed supplements. However, further research is needed to find the optimum dose of clove powder as a feed supplement and its effect on the growth and health status of fish.

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AUTHOR'S CONTRIBUTIONS

IO: performed the experiment, analyzed the data, and wrote the first version of the manuscript. SS: designed the study and wrote the first version of manuscript and approved for publication. WW: reviewed the first version of the manuscript, and approved for publication. DW: reviewed the first version of the manuscript, and approved for publication. MY: reviewed the first version of the manuscript, and approved for publication. MS: reviewed the first version of the manuscript, and approved for publication.

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