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The effectiveness of forest onion *Eleutherine bulbosa* simplisia to prevent *Streptococcus agalactiae* infection on Nile tilapia *Oreochromis* sp.

Efektivitas simplisia bawang hutan *Eleutherine bulbosa* untuk pencegahan infeksi *Streptococcus agalactiae* pada ikan nila *Oreochromis* sp.

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ABSTRACT

Forest onion potentially acts as an immunostimulant, enhancing the immune response of tilapia to pathogen infection. This study aimed to evaluate the effectiveness of forest onion simplisia on growth performance, immune response, and resistance of tilapia to *Streptococcus agalactiae* infection. The research was divided into in vitro and in vivo tests. In vitro testing was conducted to determine the dose of forest onion extract inhibiting the growth of *S. agalactiae* with seven treatments, namely positive control, negative control, addition of forest onion extract at 0.625, 1.25, 2.5, 5, and 10 mg/mL. In vivo testing to evaluate the administration of forest onion simplisia through feed in enhancing growth performance, immune response, and resistance of tilapia to *S. agalactiae*. Tilapia with an average weight of 7.57 ± 0.26 g were maintained in glass aquariums sized 60×30×30 cm^3 with 15 units at a density of 15 fish/aquarium for 30 days. Experimental treatments included maintaining tilapia with the addition of forest onion simplisia at 12.5, 25, and 50 g/kg feed, negative and positive controls. The results showed that the addition of 12.5 g/kg simplisia significantly increased growth which was significantly different from the other treatments, while the immune response and resistance to *S. agalactiae* in all simplisia treatments showed values that were not different, but significantly different from the control treatment. The conclusion is supplementation of forest onion simplisia at a dose of 12.5 g/kg effectively enhances growth performance, immune response, and resistance of tilapia to *S. agalactiae* infection.

Keywords: forest onion, immunostimulant, Nile tilapia, S*treptococcus agalactiae*

ABSTRAK

Bawang hutan berpotensi sebagai imunostimulan yang dapat meningkatkan respons imun ikan terhadap infeksi patogen. Penelitian ini bertujuan mengevaluasi efektivitas simplisia bawang hutan terhadap kinerja pertumbuhan, respons imun dan resistansi ikan nila terhadap infeksi *Streptococcus agalactiae*. Penelitian terbagi menjadi pengujian *in vitro* dan *in vivo*. Pengujian *in vitro* dilakukan untuk menentukan dosis ekstrak bawang hutan dalam menghambat pertumbuhan bakteri *S. agalactiae* dengan delapan perlakuan yaitu kontrol positif, kontrol negatif, penambahan ekstrak bawang hutan 0,625,1.25, 2,5, 5, dan 10 mg/mL. Adapun pengujian *in vivo* untuk mengevaluasi pemberian simplisia bawang hutan dalam meningkatkan kinerja pertumbuhan, respons imun, dan resistansi ikan nila terhadap *S. agalactiae*. Ikan nila berukuran 7,57 ± 0,26 g dipelihara pada akuarium berukuran 60×30×30 cm^3 sebanyak 15 unit dengan kepadatan 15 ekor/akuarium selama 30 hari. Perlakuan uji meliputi pemeliharaan ikan nila dengan penambahan simplisia bawang hutan 12.5, 25, dan 50 g/kg pakan, kontrol negatif dan positif. Hasil penelitian menunjukkan penambahan simplisia 12,5 g/kg signifikan meningkatkan pertumbuhan yang berbeda nyata dengan perlakuan lainnya, adapun respons imun dan resistansi terhadap *S. agalactiae* pada semua perlakuan simplisia menunjukkan nilai yang tidak berbeda, akan tetapi berbeda nyata dengan perlakuan kontrol. Kesimpulan penelitian ini adalah suplementasi simplisia bawang hutan 12,5 g/kg efektif meningkatkan kinerja pertumbuhan, respons imun, dan resistansi ikan nila terhadap *S. agalactiae*.

Kata kunci: bawang hutan, ikan nila, immunostimulan, *Streptococcus agalactiae*

INTRODUCTION

The Nile tilapia (*Oreochromis* sp.) is a key freshwater aquaculture commodity in Indonesia, characterized by high growth rates, a relatively short cultivation cycle, and adaptability to diverse environmental conditions (Marie *et al*., 2018). The species has been targeted for increased production from 2020 to 2024 (DJPB, 2020). According to the Indonesian Ministry of Marine Affairs and Fisheries, tilapia production in Indonesia exhibited an average annual growth rate of 9.68% between 2018 and 2022. However, production levels have yet to meet the annual targets. In 2022, tilapia production reached 1.6 million tons, falling short of the 1.9 million-ton target (DJPB, 2022). To achieve the expected production goals, strategies for enhancing tilapia cultivation must be reinforced, particularly through the implementation of intensive aquaculture systems.

Intensive aquaculture systems have the potential to improve productivity however, they can also compromise fish health, increasing the risk of disease outbreaks (Rijal *et al*., 2022). Disease emergence in aquaculture is influenced by the interaction of three key factors: host susceptibility due to weakened immune systems, the presence of pathogenic populations, and poor environmental conditions (Hamed *et al*., 2018). One of the most prevalent diseases in tilapia aquaculture is streptococcosis (Wulandari *et al*., 2018), caused by *Streptococcus agalactiae*. This bacterium primarily affects the brain, eyes, kidneys, and other fluid-containing organs (Suhermanto *et al*., 2020), with reported mortality rates reaching 70% (Huang *et al*., 2013). Clinical symptoms typically manifest within 72 hours of *S. agalactiae* infection, including changes in body coloration, hemorrhages, reduced feeding response, C-shaped body curvature, and abnormal swimming behavior (Sa'adah *et al*., 2015).

The conventional approach to controlling *S. agalactiae* infections involves antibiotic administration. However, the widespread use of antibiotics in aquaculture is increasingly restricted due to concerns regarding bacterial resistance (Puspitarani *et al*., 2016) and food safety risks (Gauthier, 2015). Consequently, alternative disease management strategies that are safe, environmentally friendly, and free from antibiotic residues are required. One potential alternative is the use of natural bioactive compounds, such as forest onion (*Eleutherine bulbosa*). Forest onion has been reported to exhibit immunostimulatory (Fauzi *et al*., 2023), antibacterial (Harlita *et al*., 2018), prebiotic and antioxidant (Munaeni *et al*., 2020a), antifungal (Naibaho *et al*., 2023), anti-inflammatory (Hardi *et al*., 2019), and anticancer (Muti'ah *et al*., 2020) properties.

The bioactive secondary metabolites in forest onion, particularly flavonoids, function as immunomodulators by enhancing neutrophil oxidative activity, cellular phagocytosis, and cytotoxic stimulation (Erjon *et al*., 2022). Additionally, Almarri *et al*. (2023) identified tannins, saponins, steroids, triterpenoids, and phenols in forest onion, which contribute to its dual role as an immunostimulant and antibacterial agent. The application of forest onion in fish disease prevention has been explored in both extract and simplicia forms. Several studies have indicated that the simplicia form is more effective in disease prevention than the extract form.

Munaeni *et al*. (2020b) demonstrated that dietary supplementation with forest onion simplicia at 12.5 g/kg feed was more effective than extract supplementation at 1.25 g/kg in enhancing immune responses, immune-related gene expression, and resistance in Pacific white shrimp (*Litopenaeus vannamei*) against *Vibrio parahaemolyticus* infection. Furthermore, Sudrajat *et al*. (2023) reported that the combination of *Bacillus* sp. NP5 probiotic with forest onion simplicia at 20 g/kg feed positively impacted growth performance, immune response, and disease resistance in Nile tilapia against *Aeromonas hydrophila* infection. Although previous studies have demonstrated the efficacy of forest onion simplicia in inhibiting pathogenic bacteria, its potential application in preventing streptococcosis remains unexplored. Therefore, this study aims to evaluate the effectiveness of forest onion simplicia in enhancing growth performance, hematological and immune responses, and disease resistance in Nile tilapia against *S. agalactiae* infection.

MATERIALS AND METHODS

Test materials

The test animals used in this study were Nile tilapia with an average weight of 7.57 ± 0.26 g, obtained from a fish farmer in Bogor, West Java. The bacteria used was *Streptococcus agalactiae*, sourced from the collection of the Research Institute for Freshwater Aquaculture and Fisheries

Extension, Depok, West Java. The forest onion (*Eleutherine palmifolia*) was obtained from Pontianak, West Kalimantan Province, Indonesia.

Preparation of forest onion

The preparation of forst onion simplicia involved cleaning the bulbs, slicing them thinly, and drying them in an oven at 60°C for 48 hours. The dried forest onion slices were then ground into a powder using a blender to obtain the simplicia. Forest onion extraction was conducted for in vitro testing to determine the effective dosage. The extraction process followed the method described by Munaeni *et al*. (2019), using 96% ethanol at a 1:4 (w/v) ratio, with maceration in an orbital shaker for 24 hours. The first maceration filtrate was filtered using Whatman No. 41 filter paper and re-macerated twice with 96% ethanol. The obtained filtrate was then concentrated using a rotary vacuum evaporator at 40°C. The forest onion extract was stored at -20°C for in vitro testing.

Experimental design

This study employed a completely randomized design (CRD) consisting of two stages. The first stage was an in vitro test to determine the forest onion simplicia dose capable of inhibiting *S. agalactiae* growth. The in vitro test included seven treatments with three replications: media supplemented with forest onion extract at concentrations of 0.625 mg/mL, 1.25 mg/mL, 2.5 mg/mL, 5 mg/mL, and 10 mg/mL, along with a positive control and a negative control.

The second stage was an *in vivo* test to evaluate the effects of forest onion simplicia supplementation through feed on growth performance, hematological and immune responses, and Nile tilapia resistance against *S. agalactiae* infection. The doses used in the *in vivo* test were derived from the in vitro results, with extract conversion into simplicia based on a 10% yield. The yield was determined by measuring the extract weight obtained (5 g) compared to the initial raw material weight (50 g). Thus, the in vivo doses used in this study were 12.5 g/kg, 25 g/kg, and 50 g/kg, along with a positive control and a negative control group.

In vitro test of forest onion

Inhibition zone

The inhibition zone test was conducted using the Kirby-Bauer method. *Streptococcus agalactiae* was inoculated onto the media using a sterile cotton swab. The treatments included a positive control (OTC antibiotic 50 ppm), a negative control (PBS), and forest onion extract at concentrations of 0.625, 1.25, 2.5, 5, and 10 mg/mL. Sterile disc papers were soaked in the extract solution and placed on the surface of BHIA media, which was then incubated for 72 hours at 28°C. The clear zones formed were measured using a caliper.

Minimum inhibitory concentration (MIC)

The MIC test was performed using the broth dilution method as described by Owu *et al*. (2020). Eight BHIB medium tubes were supplemented with forest onion extract at concentrations of 0.625, 1.25, 2.5, 5, and 10 mg/mL. The controls included a negative control (BHIB + PBS) and a positive control (BHIB + *S. agalactiae*). A total of 5 µL of *S. agalactiae* was added to all treatments except the negative control, and the tubes were incubated for 72 hours. The MIC value was determined by visually observing turbidity in the media, and the results were confirmed quantitatively using spectrophotometry at 630 nm to minimize subjectivity. The lowest concentration of forest onion extract that showed no turbidity (bacterial growth) was recorded as the MIC value.

In vivo test of forest onion

Nile tilapia were reared for 30 days in 15 aquarium units, each measuring 60×30×30 cm^3. Each aquarium contained 15 fish with an average weight of 7.57 ± 0.26 g, and strong aeration was provided. Before stocking, the fish underwent a seven-day acclimatization period. Forest onion simplicia was incorporated into the feed for preventive treatment against *S. agalactiae* infection. The simplicia was mixed into the feed using a repelleting method.

One kilogram of finely ground feed was supplemented with forest onion simplicia at the designated treatment doses and 5 g/kg of a commercial binder as an adhesive. The mixture was stirred until evenly distributed, followed by the addition of 30% water and further mixing to achieve homogeneity. The feed was then pelletized and oven-dried at 60°C for two hours. Once dried, the pellets were stored in an airtight container. The fish were fed three times daily at 08:00, 12:00, and 16:00 WIB, following an ad libitum feeding regimen. Water was replaced every three days, and water quality parameters (temperature, DO, and pH) were monitored daily, while nitrite and TAN levels were measured weekly.

Challenge test

The challenge test was conducted after 30 days of rearing with forest onion simplicia supplementation. The test was performed in aquariums of 60×30×30 cm^3, where 10 tilapia per unit were intramuscularly injected with *S. agalactiae* at a bacterial density of 10^6 CFU/mL (0.1 mL/fish). During the challenge test, the fish were fed commercial feed without forest onion supplementation, following the same feeding schedule as before. Water quality was maintained through daily monitoring of temperature, DO, and pH, and water was changed every three days.

Data collection timeline

Growth performance measurements were conducted at the beginning (H0) and the end (H30) of the rearing period after feeding with forest onion simplicia. Hematology and immune response assessments were performed at the start (H0) and end (H30) of the rearing period, followed by post-infection observations of *S. agalactiae* at H+1, H+3, H+7, and H+10 after the challenge test. Enzyme activity and total bacterial & lactic acid bacterial abundance were measured on H30, while histopathology was observed on H+3 post-challenge.

Parameter of observation

This study examined growth performance, enzyme activity, total bacterial and lactic acid bacterial abundance, hematology and immune response, histopathology of kidney, brain, and eye organs, and Nile tilapia resistance against *S. agalactiae* infection.

Growth performance

The growth performance of Nile tilapia was observed from the start to the end of the 30-day experiment. Growth performance parameters included final weight, specific growth rate (Zokaeifar *et al*., 2012), feed conversion ratio (Ho *et al*., 2017), and survival rate (Sari *et al*., 2017).

Enzyme activity

Enzyme activity was assessed on day 30 after forest onion simplicia administration. Measurements were conducted at the Nutrition Laboratory, Faculty of Fisheries and Marine Science, IPB University. Nile tilapia intestinal samples were aseptically collected from one fish and placed in a sample bottle (0.1 g per sample). Samples were stored in a freezer at 0°C to 4°C until testing. Lipase activity was measured according to Yanbo and Zirong (2006), while amylase and protease activities were assessed following Bergmeyer *et al*. (1983).

Total bacterial and lactic acid bacterial (LAB) abundance

Bacterial abundance was determined using the plate count method. The entire intestine of one fish was aseptically collected and placed in a sample bottle (0.1 g per sample). The intestine was homogenized in 0.9 mL phosphate-buffered saline (PBS). A 0.1 mL homogenized sample was serially diluted, and 0.05 mL of each dilution was spread on tryptic soy agar (TSA) for total gut bacteria and de Man, Rogosa, Sharpe Agar (MRSA) for total LAB. The plates were incubated at room temperature for 24 hours, and bacteria were quantified using the total plate count (TPC) method.

Hematology and immune response

Hematological parameters were measured at the beginning and end of the rearing period with forest onion simplicia administration and post-challenge at days 1, 3, 7, and 10. Three fish per treatment were sampled. Before blood sampling, fish were anesthetized to minimize stress. Post-challenge observations lasted up to 10 days to obtain comprehensive data on immune system recovery and fish health following *S. agalactiae* infection. Hematological parameters included total erythrocytes, total leukocytes (Blaxhall & Daisley, 1973), hemoglobin (Wedemeyer & Yatsuke, 1977), and hematocrit (Anderson & Siwicki, 1995). Immune response parameters included phagocytic activity (Anderson & Siwicki, 1995), lysozyme activity, and respiratory burst (Divyagnaneswari *et al*., 2007).

Histopathology

Histopathological changes in the brain, kidney, and eye were analyzed post-challenge with *S. agalactiae*. The analysis was conducted qualitatively and quantitatively. Qualitative observations involved aseptic organ sampling on day 3 post-challenge (H+3). The target organs were preserved in 10% neutral buffer formalin (NBF) for 24 hours, then trimmed to 2 mm thickness and fixed. Dehydration was performed using graded absolute alcohol solutions (70%, 80%, 90%, and 95%) for two hours each.

Dehydrated tissues were cleared with xylene for two hours, embedded in paraffin, and sectioned

at 5 µm thickness using a microtome. Sections were mounted on glass slides and stained with hematoxylin-eosin (HE). The stained slides were examined under a microscope to identify tissue damage, such as necrosis, inflammatory cell infiltration, and morphological changes. Quantitative analysis assessed the percentage of damaged areas in the brain, kidney, and eye. Each slide was examined under five random fields of view at 400× magnification, and organ damage was scored according to the modified method of Wolf *et al*. (2015) in Table 1.

Nile tilapia resistance against *S. agalactiae* infection

The resistance of Nile tilapia to *S. agalactiae* infection was determined based on the survival rate, which was calculated on day 10 post-challenge with *S. agalactiae*. The survival rate was determined using the formula by Sari *et al*. (2017).

Data analysis

The measured parameter data were analyzed using Microsoft Excel 2019. Normality and homogeneity tests were performed before statistical analysis. Data were then analyzed using analysis of variance (ANOVA) with a 95% confidence interval in SPSS 26. If significant differences were detected (P<0.05), further analysis was conducted using Duncan's multiple range test.

RESULTS AND DISCUSSION

Results

In vitro test

This study investigated the inhibitory potential of forest onion extract against *S. agalactiae* growth, as assessed by the inhibition zone test presented in Table 2. The results demonstrated the formation of inhibition zones at all tested concentrations of forest onion extract, indicating its ability to suppress *S. agalactiae* growth. The highest inhibition zone was observed at a concentration of 10 mg/mL, which was significantly different from the inhibition zones produced by the 0.625, 1.25, and 2.5 mg/mL concentrations, as well as the control. The MIC test results revealed the lowest concentration of forest onion extract that inhibited bacterial growth, with 1.25 mg/mL identified as the minimum concentration capable of inhibiting *S. agalactiae* growth. This concentration showed a significant difference compared to the control, as well as the 0.625, 5, and 10 mg/mL concentrations of forest onion extract (Table 3).

Table 1. Scoring the percentage of fish organ damage.

Score	Persentage of damage	Notes
0	<20%	Normal
1	20-40%	Mild damage
2	40-60%	Moderate damage
3	60-80%	Severe damage
4	>80%	Extreme damage

Table 2. Inhibition zone test results of forest onion extract against *Streptococcus agalactiae* growth.

Concentration of Forest onion	Inhibition zone diamater (cm)
KN	0.000 ± 0.000^{a}
KP	0.000 ± 0.000^{a}
0.625 mg/mL	1.030 ± 0.051^{b}
1.25 mg/mL	1.160 ± 0.050^{c}
2.5 mg/mL	1.733 ± 0.121^{d}
5 mg/mL	2.110 ± 0.100^{e}
10 mg/mL	2.700 ± 0.031^{f}

Note: Superscript letters indicate significant differences (P<0.05), with KN (negative control, PBS), KP (positive control, OTC 50 ppm), and treatments consisting of forest onion extract at concentrations of 0.625, 1.25, 2.5, 5, and 10 mg/mL.

In vivo test

Growth performance and enzyme activity

The growth performance and enzyme activity of Nile tilapia after 30 days of forest onion supplementation are presented in Table 4. The highest weight gain was observed in the 12.5 g/kg treatment group at 28.03 ± 0.17 g, which was significantly different from other treatments. This result positively correlated with the specific growth rate (SGR), where the highest SGR was also recorded in the 12.5 g/kg treatment group at 10.86 ± 0.02%, significantly higher than the other treatments (P<0.05). During the rearing period, the lowest feed conversion ratio (FCR) was observed in the 12.5 g/kg treatment at 1.19 ± 0.04, while the highest FCR was recorded in the control group.

Survival rates across all treatments remained at 100.00 ± 0.00%. Growth performance positively correlated with enzyme activity. Enzyme activity in the 12.5, 25, and 50 g/kg treatments was significantly higher compared to the control (P<0.05). Protease activity in the 12.5 g/kg and 25 g/kg treatments was significantly higher (P<0.05) than in the control but did not differ significantly from the 50 g/kg treatment. Similarly, amylase and lipase activities in the 12.5 g/kg and 25 g/kg treatments were significantly higher (P<0.05) than in the control group.

Total bacterial abundance and lactic acid bacteria (LAB)

The abundance of total bacteria and lactic acid bacteria after 30 days of administering forest onion simplicia is presented in Figure 1. The total bacterial count after cultivation with forest onion simplicia administration showed higher results (P<0.05) compared to the control treatments. The highest total bacterial abundance was found in the 12.5 g/kg simplicia treatment, at 8.93 ± 0.02 log CFU/g, which was significantly different from the control treatment (P<0.05), as well as from the 25 g/kg and 50 g/kg simplicia treatments. As for lactic acid bacteria, the 12.5 g/kg simplicia

Table 3. MIC test results of forest onion extract against *Streptococcus agalactiae* growth.

Concentration of Forest onion	*S. agalactiae* (OD 630 nm)
KN	0.000 ± 0.000^{a}
KP	1.747 ± 0.079^{f}
0.625 mg/mL	1.255 ± 0.024^{e}
1.25 mg/mL	0.598 ± 0.022^{d}
2.5 mg/mL	0.490 ± 0.024^{d}
5 mg/mL	0.112 ± 0.005^{bc}
10 mg/mL	0.161 ± 0.017^{ab}

Notes: Superscript letters indicate significant differences (P<0.05), with KN (negative control: media + PBS), KP (positive control: media + *S. agalactiae*), and treatments consisting of forest onion extract at concentrations of 0.625, 1.25, 2.5, 5, and 10 mg/mL.

Table 4. Growth performance and enzyme activity of Nile tilapia supplemented with forest onion simplisia in feed during 30 days of rearing.

Parameters	K-	K+	12.5	25	50
W0 (g)	7.62 ± 0.09^{a}	7.55 ± 0.39^{a}	7.62 ± 0.17^{a}	7.56 ± 0.47^{a}	7.54 ± 0.29^{a}
Wt (g)	18.84 ± 0.39^{a}	18.89 ± 0.09^{a}	28.03 ± 0.17^{d}	26.08 ± 0.47^{c}	22.07 ± 0.29^{b}
SGR (%)	9.40 ± 0.08^{a}	9.42 ± 0.02^{a}	10.86 ± 0.02^{d}	10.60 ± 0.11^{c}	9.99 ± 0.47^{b}
FCR	1.91 ± 0.07^{d}	1.89 ± 0.02^{d}	1.19 ± 0.04^{a}	1.34 ± 0.03^{b}	1.70 ± 0.03^{c}
SR (%)	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
Protease (IU/mL)	0.020 ± 0.002^{a}	0.020 ± 0.002^{ab}	0.040 ± 0.004^{c}	0.030 ± 0.002^{c}	0.030 ± 0.002^{bc}
Amilase (IU/mL)	1.350 ± 0.003^{a}	1.450 ± 0.230^{a}	2.510 ± 0.250^{b}	2.260 ± 0.080^{b}	2.130 ± 0.350^{ab}
Lipase (IU/mL)	0.020 ± 0.001^{a}	0.020 ± 0.002^{a}	0.040 ± 0.002^{c}	0.040 ± 0.020^{c}	0.030 ± 0.001^{b}

Notes: Initial weight (W_0), final weight (W_t), specific growth rate (SGR), feed conversion ratio (FCR), survival rate (SR). Different superscript letters indicate significant differences (P<0.05). Negative control (K-), positive control (K+), forest onion simplicia at doses of 12.5, 25, and 50 g/kg.

treatment had 5.77 ± 0.04 log CFU/g, which was not significantly different from the 25 g/kg simplicia treatment but was significantly different from the control treatment and the 50 g/kg simplicia treatment.

Hematology and immune response

The administration of forest onion simplicia affects the hematological levels, immune response, and resistance of Nile tilapia to *S. agalactiae* infection. The total erythrocyte count (TE) at H0 was (2.28 ± 0.076 $\times 10^6$ cells/mm³), and it increased at H30 during the cultivation period with the simplicia treatment, showing a more significant increase compared to the control treatment (P<0.05). The TE decreased at H+1 and H+3 after the challenge test. The TE at H+10 showed an increase from H+7, with the TE values between simplicia treatments showing no significant difference, but they were significantly different from the control treatment (P<0.05) (Figure 2).

Hematocrit (HC) at the start of the cultivation was 17.10 ± 0.88%. The administration of forest onion simplicia increased HC levels in the 50 g/kg simplicia treatment (22.34 ± 1.22%), which was significantly different from the control treatment. The HC value decreased after the challenge test at H+1 and H+3, then increased at H+7 and H+10, with the HC in the simplicia treatment showing a significantly different value compared to the positive control (P<0.05) (Figure 3). The hemoglobin (Hb) level at H0 was 7.33 ± 0.29 g%, then increased at H30 with the highest Hb level in the 25 g/kg simplicia treatment at 9.73 ± 0.07 g%. The Hb level in Nile tilapia decreased after the challenge test at H+1 and H+3, then increased at H+7 and H+10 post-challenge.

Treatments with the addition of forest onion simplicia resulted in Hb values that did not differ significantly among simplicia treatments but were significantly different from the positive control treatment (P<0.05) (Figure 4). The total leukocyte count (TL) of Nile tilapia at the start

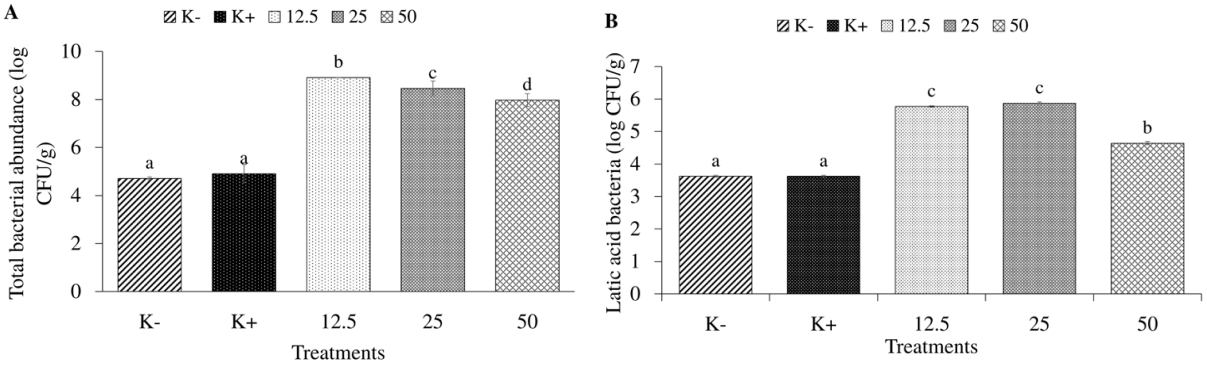

Figure 1. Total bacterial abundance (A) and lactic acid bacteria in the intestines of Nile tilapia (B) after 30 days with the addition of forest onion simplicia. Different superscript letters indicate significant differences (P<0.05).

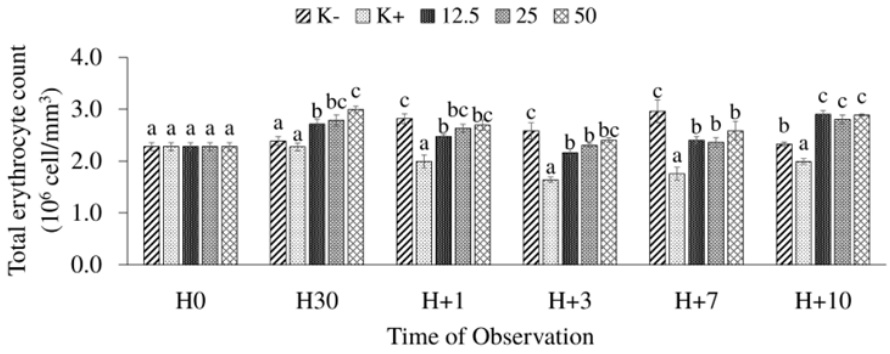

Figure 2. Total erythrocyte count of Nile tilapia before and after the *S. agalactiae* challenge test. Different superscript letters indicate significant differences (P<0.05).

of the cultivation was 2.59 ± 0.11 ×10⁴ cells/mm³. The administration of forest onion resulted in an increase in total leukocytes after 30 days of cultivation. After the challenge test, TL increased at H+1 and H+3, with the TL values in the simplicia treatments being significantly different from the control treatment (P<0.05), but there were no significant differences between the simplicia treatments (P>0.05). After the challenge test at H+7 and H+10, TL values decreased in all forest onion simplicia treatments, which were significantly different from the control (P<0.05) (Figure 5).

The AF value at the start of the cultivation was 40.36 ± 0.77%. After 30 days of cultivation, the forest onion simplicia treatment showed significantly higher values compared to the control treatment (P<0.05). After the challenge

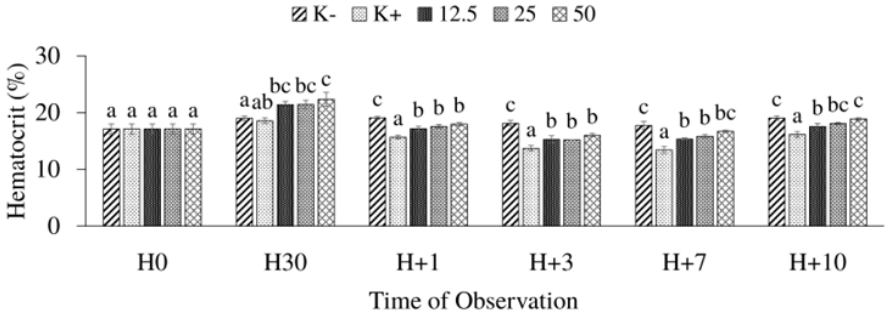

Figure 3. Total hematocrit of Nile tilapia before and after the *S. agalactiae* challenge test. Different superscript letters indicate significant differences (P<0.05).

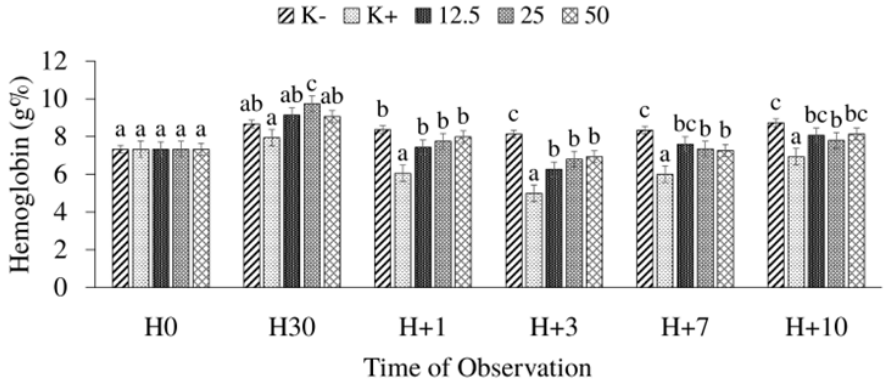

Figure 4. Total hemoglobin of Nile tilapia before and after the *S. agalactiae* challenge test. Different superscript letters indicate significant differences (P<0.05).

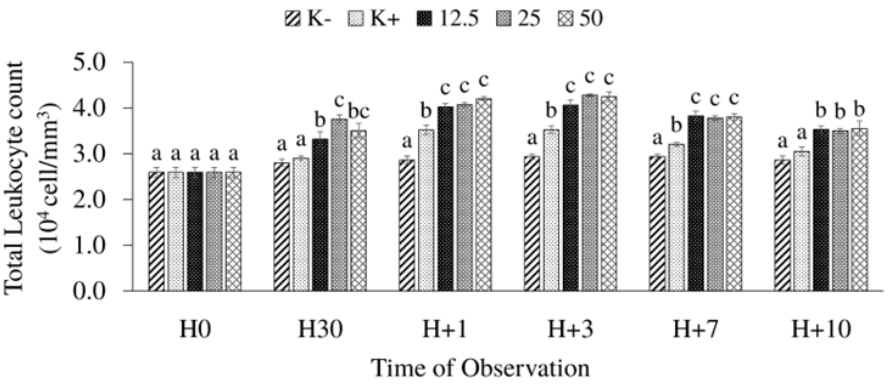

Figure 5. Total leukocyte count of Nile tilapia before and after the *S. agalactiae* challenge test. Different superscript letters indicate significant differences (P<0.05).

test, AF increased at H+1 and H+3, then decreased at H+7 and H+10 post-challenge. Treatments with the addition of simplicia showed significantly different values from the control treatment, but there were no significant differences among the simplicia treatments (Figure 6).

Respiratory burst (RB) values increased after 30 days of cultivation with the simplicia treatment, showing significantly different values compared to the control (P<0.05). The RB value further increased after the challenge test at H+1 and H+3, then decreased at H+7 and H+10. The forest onion simplicia treatment showed significantly different results compared to the positive control (P<0.05) (Figure 7). Lysozyme activity (LA) at the start of the cultivation was 13.60 ± 1.05 U/mL.

After 30 days of cultivation, the LA value showed a significant increase compared to the control treatment. The LA value continued to increase after the *S. agalactiae* challenge test at H+1 and H+3. A decrease in LA values occurred at H+7 and H+10. Treatments with the addition of forest onion simplicia showed higher LA values compared to the positive control (P<0.05) (Figure 8).

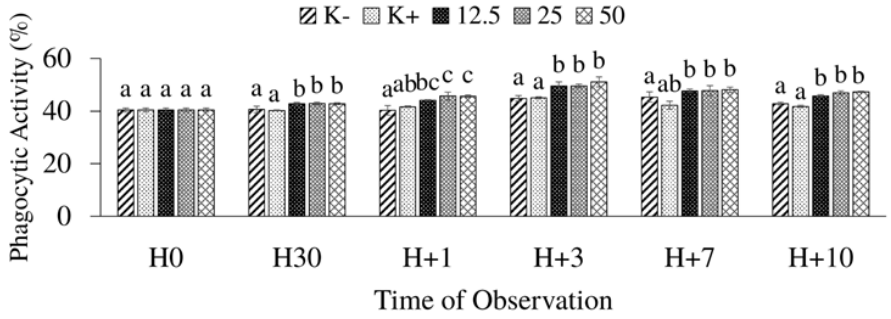

Figure 6. Phagocytic activity of Nile tilapia before and after the *S. agalactiae* challenge test. Different superscript letters indicate significant differences (P<0.05).

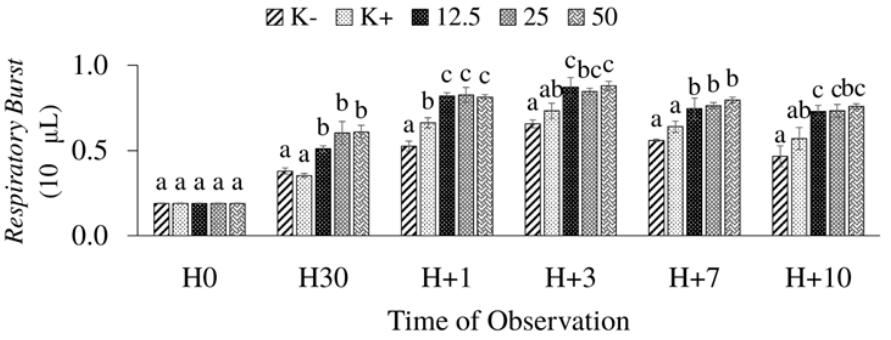

Figure 7. Respiratory burst activity of Nile tilapia before and after the *S. agalactiae* challenge test. Different superscript letters indicate significant differences (P<0.05).

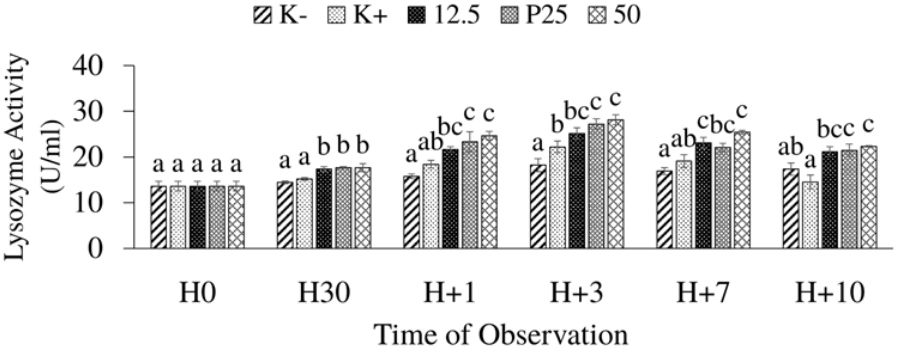

Figure 8. Lysozyme activity of Nile tilapia before and after the *S. agalactiae* challenge test. Different superscript letters indicate significant differences (P<0.05).

Histopathology

Histopathological observation of the kidney, brain, and eye organs showed moderate damage in the positive control group, with the tissue structure starting to change significantly and potentially leading to a decline in tissue function. In contrast, all forest onion supplementation groups showed mild damage, with tissue structure and function remaining relatively normal, though some minor changes were observed under the microscope. This was supported by the results showing the highest percentage of damage in the positive control group for the kidney, brain, and eye organs, with values of 47.88 ± 2.19%, 44.24 ± 3.19%, and 45.09 ± 0.70%, respectively, which were significantly different from the negative control group and the forest onion supplementation groups.

Resistance of Nile tilapia to S. agalactiae

The survival rate (SR) of Nile tilapia after infection with *S. agalactiae* in all forest onion simplicia treatments showed better values than

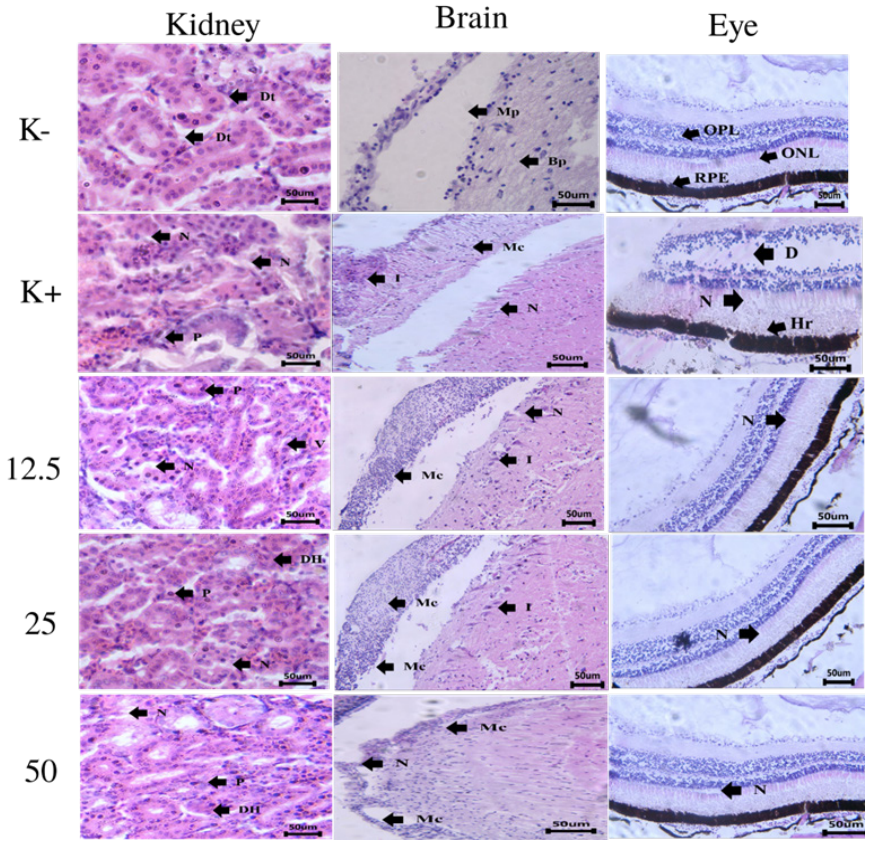

Figure 9. Histopathology of the kidney, brain, and eye of Nile tilapia at H+3 post-infection with *S. agalactiae*. Staining was done using hematoxylin and eosin (H&E); distal tubules (Dt); necrosis (N); pronephritis (P); vacuolization (V); hydropic degeneration (HD); primitive meninx (MP); brain parenchyma (BP); mononuclear cells (Mc); leukocyte infiltration (I); outer plexiform layer (OPL); outer nuclear layer (ONL); retinal pigmented epithelium (RPE); degeneration (D); retinal hyperpigmentation (Hr).

Table 5. Scoring of the percentage of tissue damage in the kidney, brain, and eye organs of Nile tilapia after the *S. agalactiae* challenge test.

Treatments	Kidney (%)	Brain (%)	Eye (%)
K-	12.89 ± 1.06 [a]	10.36 ± 0.36 [a]	11.99 ± 0.89 [a]
K+	47.88 ± 2.19 [c]	44.24 ± 3.19 [c]	45.09 ± 0.70 [c]
12.5	27.34 ± 1.00 [b]	29.02 ± 1.25 [b]	28.46 ± 1.60 [b]
25	26.64 ± 0.68 [b]	28.09 ± 0.89 [b]	26.54 ± 0.60 [b]
50	24.33 ± 2.24 [b]	25.14 ± 0.20 [b]	25.26 ± 2.21 [b]

Notes: Different superscript letters indicate significant differences (P<0.05). Negative control (K-), positive control (K+), forest onion simplicia doses of 12.5, 25, and 50 g/kg.

the positive control treatment. The simplicia 12.5 g/kg treatment had an SR of 73.33 ± 3.33%, the 25 g/kg treatment had an SR of 76.66 ± 6.66%, and the 50 g/kg treatment had an SR of 83.33 ± 3.33%. The positive control group had the lowest SR at 46.66 ± 3.33%. Statistical analysis showed no significant differences between the simplicia doses of 12.5, 25, and 50 g/kg, but there were significant differences compared to the positive control group (Figure 10).

Discussion

The administration of forest onion into feed has demonstrated positive effects in preventing *S. agalactiae* infection in Nile tilapia. In vitro antimicrobial assays revealed clear inhibition zones at all tested concentrations of forest onion extract. Furthermore, the minimum inhibitory concentration (MIC) test indicated that the lowest concentration capable of inhibiting *S. agalactiae* growth was 1.25 mg/mL of forest onion extract. The antibacterial properties of forest onion are attributed to its ability to disrupt *S. agalactiae* cell membranes, interfere with cellular metabolic functions, and induce cell lysis, ultimately leading to bacterial death (Munaeni *et al*., 2019). In addition, secondary metabolites such as flavonoids, phenols, saponins, and steroids present in forest onion contribute to bacterial inhibition through various mechanisms.

In vivo experiments involving the dietary supplementation of forest onion simplisia for 30 days demonstrated enhanced growth performance in Nile tilapia. The final body weight, specific growth rate (SGR), and feed conversion ratio (FCR) at a supplementation level of 12.5 g/kg feed were significantly improved compared to other treatments. The increase in SGR is likely due to phytochemicals such as flavonoids, alkaloids, and saponins, which enhance nutrient absorption by interacting with the digestive and metabolic systems, thereby promoting fish growth (Adeniyi *et al*., 2021). The lowest FCR value at the 12.5 g/kg supplementation level indicates improved feed utilization efficiency. However, at a higher supplementation level (50 g/kg), a reduction in SGR was observed, possibly due to the pharmacological effects of certain forest onion compounds, such as tannins, which at high concentrations interfere with digestive enzyme activity and impair nutrient absorption, thereby negatively affecting metabolism and growth (Li *et al*., 2020).

Digestive enzyme activity and bacterial abundance are positively correlated with improved growth performance and immune response in fish. The supplementation of forest onion simplisia at 12.5 g/kg resulted in the highest levels of digestive enzyme activity, total bacterial abundance, and lactic acid bacteria populations. Lactic acid bacteria play a crucial role in fermentation, producing various metabolic products and organic acids that alter the gut environment, which, in turn, enhances digestive enzyme activity. Additionally, lactic acid bacteria secrete specific enzymes that break down complex molecules into simpler forms, facilitating digestion (Virdis *et al*., 2021). The increase in total bacterial and lactic acid bacteria abundance is attributed to the presence of prebiotic compounds such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), and inulin in forest onion, which promote the growth of beneficial bacteria (Munaeni *et al*., 2020a).

Fish health status was evaluated by assessing hematological parameters. After 30 days of forest onion supplementation, total erythrocyte (TE), hematocrit (Hc), and hemoglobin (Hb) levels were elevated, indicating improved physiological conditions. Increased TE suggests enhanced

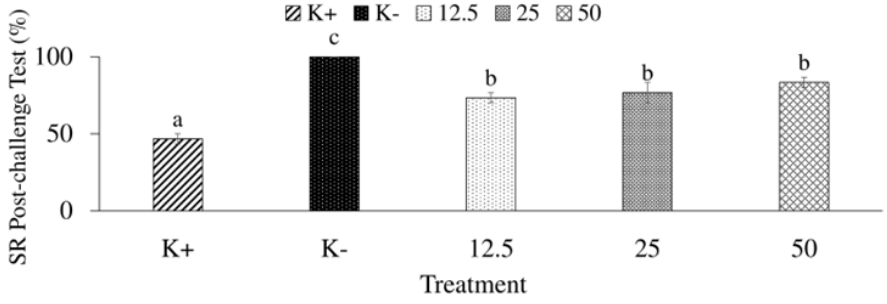

Figure 10. Resistance of Nile tilapia after the *S. agalactiae* challenge test. Data with different letters indicate significant differences (P<0.05). Negative control (K-), positive control (K+), forest onion simplicia doses of 12.5, 25, and 50 g/kg.

oxygen transport capacity, while elevated Hc and Hb levels reflect improved erythrocyte volume and oxygen-binding capacity, respectively. Although these hematological improvements were not significantly different among the supplementation treatments, they suggest the potential benefits of forest onion in supporting fish health. Following bacterial challenge with *S. agalactiae*, TE, Hc, and Hb levels declined on days 1 and 3 post-infection but recovered by days 7 and 10.

The initial decline in these parameters is likely due to erythropoiesis inhibition caused by inflammatory cytokine production, such as interleukins and tumor necrosis factor (TNF), which suppress erythropoietin synthesis, a key hormone for red blood cell production. Additionally, the hemolytic nature of *S. agalactiae* leads to erythrocyte destruction and hemoglobin release into plasma, reducing Hb levels (Coates & Decker, 2017). The subsequent recovery of hematological parameters suggests physiological adaptation and restoration of erythropoiesis (Sirimanapong *et al*., 2018). Leukocytes play a critical role in non-specific immune responses by producing antibodies and exhibiting phagocytic activity. After 30 days of dietary forest onion supplementation, total leukocyte (TL) counts were higher than those in the control group, indicating an immunostimulatory effect (Aluta *et al*., 2021).

This finding aligns with Fauzi *et al*. (2023), who reported increased leukocyte counts in Nile tilapia following forest onion supplementation. TL levels peaked on day 3 post-infection with *S. agalactiae*, suggesting immune activation against the invading pathogen (Pervaiz *et al*., 2022). Phagocytic activity (PA) increased significantly on days 1 and 3 post-infection in both the forest onion-supplemented and positive control groups, with the supplementation group exhibiting superior PA. This enhancement is attributed to the antimicrobial properties of forest onion, which are mediated by allinase enzyme activity, leading to the production of allicin, a potent antimicrobial and antioxidant compound that enhances immune defense against bacterial infections (Fauziah *et al*., 2015). Similar findings were reported by Fransira and Yanuhar (2023), who observed increased PA in Nile tilapia infected with Pseudomonas fluorescens following forest onion supplementation.

Respiratory burst (RB) activity, an indicator of reactive oxygen species (ROS) production by immune cells, significantly increased following *S. agalactiae* infection, with the highest levels observed on day 3 post-infection. These findings are consistent with Munaeni *et al*. (2020b), who reported increased RB activity in Pacific white shrimp (Litopenaeus vannamei) following forest onion supplementation and Vibrio parahaemolyticus infection. RB activity positively correlates with TL and PA, as leukocytes generate ROS during phagocytosis to eliminate pathogens (Sujono *et al*., 2022). Lysozyme activity (LA), which reflects macrophage activation and bacterial cell wall degradation, increased following *S. agalactiae* infection, indicating enhanced bactericidal activity.

The decline in LA on days 7 and 10 post-infection suggests that the fish had passed the critical phase of infection and were undergoing recovery (Nasution *et al*., 2018). Beyond its effects on hematological and immune responses, *S. agalactiae* infection impacted tissue integrity and survival rates. Histopathological analysis of the kidney, brain, and eye revealed moderate tissue damage in the positive control group, whereas fish in the forest onion-supplemented groups exhibited only mild damage. This finding suggests that forest onion enhances immune defense mechanisms, minimizing tissue damage associated with *S. agalactiae* infection.

These results align with Fauzi *et al*. (2023), who reported that forest onion supplementation mitigated liver and kidney damage in fish infected with Aeromonas hydrophila. The improved resistance of Nile tilapia to *S. agalactiae* infection is attributed to the immunomodulatory effects of forest onion metabolites, which enhance immune responses and promote higher survival rates. In conclusion, dietary supplementation with forest onion simplisia at an optimal dose of 12.5 g/kg enhances growth performance, immune responses, and disease resistance in Nile tilapia against *S. agalactiae* infection. These benefits are mediated through improved nutrient absorption, modulation of gut microbiota, and enhancement of innate immune functions. Further investigations on the specific mechanisms of action and long-term effects of forest onion supplementation are warranted to optimize its application in aquaculture.

CONCLUSION

Supplementation of forest onion simplicia at a dose of 12.5 g/kg through feed is effective in enhancing growth performance, hematological

parameters, immune response, and resistance of Nile tilapia against *Streptococcus agalactiae* infection.

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