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Original Article

Dietary supplementation with *Zooshikella marina* improves growth performance, haemato–immunological parameters and disease resistance against *Streptococcus agalactiae* in Nile Tilapia (*Oreochromis niloticus*)

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Abstract

The effects of dietary supplementation with *Zooshikella marina* cells or extract on growth performance, haemato–immunological parameters and resistance against *Streptococcus agalactiae* infection in Nile tilapia (*Oreochromis niloticus*) were studied. In trial I (growth performance experiment), the fish received dietary supplementation of *Z. marina* cells at concentrations of 0.5, 1.0 and 1.5 g/kg in the feed, or extracts at concentrations of 2.5, 5.0 and 7.5 mg/kg, for comparison to fish fed with a control diet for 4 weeks. At 2 and 4 weeks of receiving experimental diets, the Nile tilapia receiving *Z. marina* cells at a concentration of 1.5 g/kg or extracts at 7.5 mg/kg showed significantly better final weight, weight gain, specific growth rate and feed conversion ratio than those in the control group ($p<0.05$). In trial II (immune response and disease resistance experiment), the Nile tilapia received the same experimental diets as in Trial I, also for 4 weeks. At 2 and 4 weeks of treatment, the fish receiving cells at a concentration of 1.5 g/kg or extracts at 7.5 mg/kg had significantly higher haematocrit, phagocytic activity and phagocytic index than the fish in the control group ($p<0.05$). Moreover, all treatments with dietary supplements had significantly lower mortality rate than in the control group ($p<0.05$) when challenged with *S. agalactiae* by immersion method. The present study demonstrated that dietary administration of *Z. marina* cells at a concentration of 1.5 g/kg or extract at a concentration of 7.5 mg/kg improved growth, immune response, as well as disease resistance against *S. agalactiae* in Nile tilapia.

Keywords: Nile tilapia, *Zooshikella marina*, growth, immune response, *Streptococcus agalactiae*

1. Introduction

Nile tilapia (*Oreochromis niloticus*) is an important economic fish cultured worldwide. At present, tilapia culture has rapidly expanded in Thailand due to its high economic value as well as its consumer acceptance. However, farmers have changed their culture practices from an extensive to an intensive culture system in order to meet the growing demand in domestic as well as international markets. As a result, effective farm management practices are essential, particularly in terms of disease prevention. Nowadays, streptococcosis caused by *Streptococcus agalactiae* can be highly devastating

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and is responsible for larger economic losses in tilapia cultures worldwide (Suanyuk, Kong, Ko, Gilbert, & Supamattaya, 2008; Ye *et al.*, 2011; Zamri–Saad, Amal, Siti–Zahrah, & Zulkafli, 2014). At present, *S. agalactiae* is divided into ten serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII and IX) based on the composition of its capsular polysaccharide (Slotved, Kong, Lambertsen, Sauer, & Gilbert, 2007). In Thailand, *S. agalactiae* serotypes Ia, Ib and III have caused serious damage in tilapia, climbing perch (*Anabas testudineus*), and Günther's walking catfish (*Clarias macrocephalus*), when farmed in cages or in earthen ponds (Dangwetngam, Suanyuk, Kong, & Phromkunthong, 2016; Klingklib & Suanyuk, 2017; Klingklib, Suanyuk, Kongkapan, & Tantikitti, 2021; Suanyuk *et al.*, 2008). The clinical signs exhibited by infected fish include lethargy, loss of appetite, erratic swimming, corneal opacity, exophthalmia, and hemorrhaging of the internal organs (Klingklib & Suanyuk, 2017; Suanyuk, Kangheae, Khongpradit, & Supamattaya, 2005; Suanyuk *et al.*, 2008; Suwannasang, Dangwetngam, Issaro, Phromkunthong, & Suanyuk, 2014). Currently, prevention and control of infectious diseases relies mostly on application of antibiotics and chemicals. However, the use of antibiotics is becoming limited due to the impacts on fish performance and welfare, as well as to increased environmental contamination with antibiotic residues and selection for antibiotic resistant bacteria. Recently, many studies have focused on identification and characterization of secondary metabolites with pharmaceutical potential from marine bacteria, including *Pseudoalteromonas rubra* (Setiyono *et al.*, 2020), *Hahella chejuensis* (Lee *et al.*, 2001), *Serratia marcescens* (Lapenda, Silva, Vicalvi, Sena, & Nascimento, 2015) and *Zooshikella ganghwensis* (Yi, Chang, Oh, Bae, & Chun, 2003). *Z. marina* was first isolated from a beach sand sample in India, and the major pigments produced by this bacterium were identified as prodigiosin and cycloprodigiosin (Ramaprasad, Bharti, Sasikala, & Ramana, 2015). Prodigiosin possesses many bioactivities, such as anti–bacterial, immunosuppressive, anti–diabetic, and anti–cancer activities (Yi *et al.*, 2003). Previous studies have indicated that prodigiosin inhibits both Gram–positive and Gram–negative bacteria (Lapenda *et al.*, 2015; Lee *et al.*, 2011). On the other hand, cycloprodigiosin is produced only as a minor component by some marine bacteria (Kawauchi *et al.*, 1997; Lee *et al.*, 2011; Ramaprasad *et al.*, 2015). Although *in vitro* studies on the inhibitory effects of prodigiosin and cycloprodigiosin have been reported, little is known about the *in vivo* effects of cells or extracts from this prodigiosin and cycloprodigiosin producing marine bacterium. Therefore, the purpose of this study was to investigate the *in vivo* effects of cells and extracts from *Z. marina* on the growth performance, immune responses and disease resistance of Nile tilapia against *S. agalactiae* infection. The results from this study will be useful for the fish farmers, helping prevent bacterial diseases in a sustainable way.

2. Materials and Methods

2.1 Ethics statement

This study was performed in accordance with the "Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes", National Research Council, Thailand, and it was approved by the Institutional Animal Care and Use Committee, Prince of Songkla University, under permission numbers Ref. 12/2020 and Ref. AQ016/2022.

2.2 Preparation of *Z. marina* extract

The ethanolic extraction of *Z. marina* isolated from marine sponge was carried out according to the method described previously. Briefly, *Z. marina* cultured on marine agar (MA) was transferred into marine broth (MB) and incubated at 30°C with 200 rpm agitation for 48 h. Bacterial cells were then harvested by centrifugation at 10,000 rpm and 4°C for 10 min, and washed 3 times with 0.85% sterile saline. Bacterial cells were then freeze–dried and cell pellets were repeatedly crushed in 95% ethanol using a mortar and pestle, until the solution was clear and colorless. The suspension was filtered with filter paper and the filtrate was evaporated using a rotary evaporator until a dry red powder was obtained (Puangpee & Suanyuk, 2021). The extract was stored in an amber glass bottle at –20°C until use.

2.3 Preparation of diets

Experimental diet was formulated to be isonitrogenous at 36%, and isolipidic at 8.5%, with fishmeal as the major protein source to satisfy the nutrient requirements of Nile tilapia. Furthermore, soybean meal, wheat flour, fish oil, soybean oil, alpha–starch, a vitamin and mineral mixture, inositol, choline chloride, and di–calcium phosphate were used as ingredients of the basal diet (Table 1). The sinking pellets with a diameter of 2.0 mm were processed using HOBART machine (LEGACY®, OH, USA), oven–dried at 60 °C and stored at −20 °C. Feed samples were analyzed for their proximate chemical compositions, using the procedures of Association of Official Analytical Chemists (AOAC, 1995) (Table 1). Before the experiment, the prepared feed was mixed with *Z. marina* cells and extracts at concentrations of 0.5, 1.0 and 1.5 g/kg and 2.5, 5.0 and 7.5 mg/kg, respectively. The feed pellets supplemented with *Z. marina* cells and extracts were then spray–coated with fish oil to prevent the leaching of *Z. marina* cells and extracts during the feeding process, while the control diet was spray–coated with fish oil only. All experimental diets were then air–dried under sterile conditions in a laminar airflow chamber and stored at −20 °C until use. Prodigiosin content in the diet was quantified spectrophotometrically according to the methods modified from Puangpee and Suanyuk (2021) and Lin *et al*. (2019). Briefly, the pellets were properly diluted with ethanol and crushed using a mortar and pestle. The solution was filtered with filter paper (Whatman, No. 42) and the absorbance at 535 nm was then measured. Prodigiosin content in the sample was calculated using a calibration curve for purified prodigiosin hydrochloride from *S. marcescens* (98% (HPLC), powder, Sigma) as the standard. The actual concentrations of prodigiosin in the seven experimental diets were 0.00±0.00, 0.49±0.01, 0.97±0.01, 1.49±0.02, 2.08±0.05, 4.18±0.04 and 6.59±0.02 mg/kg, respectively.

2.4 Experimental animals

Healthy Nile tilapia with an initial body weight of approximately 15 g were obtained from a commercial fish

Table 1. Composition and proximate analysis of the baseline experimental diet

Ingredient	Experimental diet (g/kg)
Fish meal	300
Soybean de–hulled	300
Wheat flour	292
Fish oil	20
Soybean oil	20
Alpha–starch	50
Vitamin & Mineral mixture[1]	1.5
Inositol	0.5
Choline chloride	1
Di–calcium phosphate	15
Chemical composition (%)[2]	
Protein	35.99±0.13
Lipid	8.51±0.24
Ash	9.65±0.12
Moisture	6.68±0.12

[1]Vitamin & Mineral premix deliver the following in unit/kg diet: Retinal (A) 8,000 IU; Cholecalciferol (D3) 1,500 IU; Tocopherol (E) 100 mg; Menadione sodium bisulfite (K3) 5 mg; Thiamine (B1) 10 mg; Riboflavin (B2) 15 mg; Pyridoxine (B6) 15 mg; Cobalamin (B12) 0.02 mg; Niacin 80 mg; Calcium pantothenate 40 mg; Ascorbic acid (C) 150 mg; Biotin 0.5 mg; Folic acid 4 mg; Cu 5 mg; Fe 30 mg; Zn 40 mg; Mn 25 mg; Co 0.05 mg; I 1 mg; Se 0.25 mg.
[2] Chemical contents are expressed as mean ± SD of triplicates.

farm in Pak Phanang district, Nakhon Si Thammarat province, Thailand. During the acclimatization period, the fish were reared in fiberglass tanks under continuous aeration, and they were fed a commercial diet twice daily to satiation until an individual weight of about 18 g was achieved. Before starting the experiment, test fish were sampled and examined to ensure that they were streptococcosis–free prior to use in the experiment.

2.5 Trial I: Growth performance experiment

Eight hundred and forty Nile tilapia were used in this study. The experimental fish were randomly distributed into twenty–four aquaria (50 cm width × 120 cm length × 50 cm height) each containing 200 L dechlorinated water. Seven treatment groups of fish (40 fish each × 3 replicates) were fed with different dietary levels of *Z. marina* cells and extracts as follows: T1 (Control diet, without supplemented *Z. marina* cells or extracts), T2 (*Z. marina* cells 0.5 g/kg), T3 (*Z. marina* cells 1.0 g/kg), T4 (*Z. marina* cells 1.5 g/kg), T5 (*Z. marina* extracts 2.5 mg/kg), T6 (*Z. marina* extracts 5.0 mg/kg) and T7 (*Z. marina* extracts 7.5 mg/kg) to satiation twice daily at 8.30 a.m. and 4.30 p.m. During the experiment, the water quality parameters temperature, total alkalinity, pH, dissolved oxygen, ammonia, and nitrite were analyzed by standard methods (Boyd & Tucker, 1992).

At 2 and 4 weeks of the feeding trial, all the fish in each tank were fasted for 24h, and then counted and weighed after being anesthetized with 0.05 mL/L clove oil (Hamackova, Kouril, Kozak, & Stupka, 2006). The weight measurements and fish counts were used to calculate weight gain, specific growth rate, feed conversion ratio, and survival rate (Maniat, Ghotbeddin, & Ghatrami, 2014). The growth performance indicators were calculated as follows:

Weight gain (%) = [(final weight – initial weight) / initial weight] × 100

Specific growth rate (%/day) = [(Ln of final weight – Ln of initial weight) / t (time interval in days)] × 100

Feed conversion ratio = feed intake (as fed basis) / weight gain

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

2.6 Trial II: Immune responses and disease resistance

2.6.1 Experimental fish and feeding trials

Nine hundred and sixty Nile tilapia with an average weight of 18.86±2.99 g were used in this study. The experimental fish were randomly distributed into twenty–four aquaria (50 cm width × 120 cm length × **5**0 cm height) each containing 200 L dechlorinated water. Eight treatment groups of fish (40 fish each × 3 replicates) were fed with different dietary levels of *Z. marina* cells and extracts as follows: T1 and T2 (Control diet, without supplemented *Z. marina* cells or extracts), T3 (*Z. marina* cells 0.5 g/kg), T4 (*Z. marina* cells 1.0 g/kg), T5 (*Z. marina* cells 1.5 g/kg), T6 (*Z. marina* extracts 2.5 mg/kg), T7 (*Z. marina* extracts 5.0 mg/kg) and T8 (*Z. marina* extracts 7.5 mg/kg) to satiation twice daily at 8.30 a.m. and 4.30 p.m. for 4 weeks.

2.6.2 Haemato–immunological parameters

At 2 and 4 weeks of the feeding trial, 3 fish from each replication (9 fish/treatment) were randomly sampled and anaesthetized with clove oil. The blood was collected from caudal vein/artery for haemato–immunological analyses. Red blood cells and white blood cells were counted using a Bright–Line™ Haemacytometer (Hausser Scientific, USA) under a compound microscope, haemoglobin level was measured colorimetrically by determining the formation of cyanmet–haemoglobin, and haematocrit was examined using heparinized micro–haematocrit capillary tube (Vitrex, Denmark) following a method of Suwannasang *et al.* (2014). The nitroblue tetrazolium (NBT) reduction was assayed using the modified method of Stasiak and Baumann (1996). For the quantification of serum protein, immunoglobulin and lysozyme, fish blood was centrifuged at 5,000 rpm for 10 min at 4°C, and the supernatant (serum) was transferred into a new microcentrifuge tube. Total serum protein was quantified colorimetrically following the method of Lowry, Rosebrough, Farr, and Randall (1951). Immunoglobulin was analyzed from total serum protein with subtraction of non–immunoglobulin protein, by the method of Siwicki, Anderson and Rumsey (1994). Lysozyme was measured based on a turbidimetric microplate assay using *Micrococcus lysodeikticus* (Sigma, USA) suspension as a substrate (Demers & Bayne, 1997). For determination of the phagocyte responses to foreign substances, head kidney leucocytes were isolated using an aseptic technique. Phagocytic activity, phagocytic index, and average bead ingested per cell were measured following the

methods of Klingklib *et al.* (2021).

2.6.3 *In vivo* immersion challenge test

At 2 and 4 weeks of the feeding trial, seven of the eight treatment groups (T2–T8) were challenged with *S. agalactiae* serotype Ia while the remaining treatment group (T1) served as a not challenged control. The combined effect of high temperature and hypoxia on immersion challenge with *S. agalactiae* was conducted in this study using the modified method of He, Li, Li, and Li (2021). Briefly, *S. agalactiae* grown at 30°C for 18 h on tryptic soy agar (TSA) were scraped using a sterile loop, resuspended and adjusted to 10^8 CFU/mL with phosphate buffer saline (PBS). Subsequently, 10 fish from each replication were transferred from culture tanks to their assigned challenge tanks containing *S. agalactiae* suspension. After 2 h of exposure, the fish were moved from the challenge tank to glass tanks (45 cm width x 90 cm length x 45 cm height) containing 100 L dechlorinated water under stress condition of an elevated temperature (32±0.5°C) combined with low dissolved oxygen (DO) (2.05±0.53 ppm). The experiment was carried out in triplicate and results were compared to two control groups, i.e., T1 as non–challenged control group where the experimental fish were immersed in PBS, and T2 as a challenged control group where the experimental fish were immersed in *S. agalactiae* suspension. Clinical signs and mortality were recorded for 14 days. The tissue of the brain obtained from all the dead fish was inoculated onto TSA to re–isolate bacteria and verify the streptococcal infection.

2.7 Statistical analysis

Data are reported as means ± standard deviations. Percentage data were subjected to arcsine transformation prior to an analysis of variance. Significant differences in growth performance, haemato–immunological parameters and cumulative mortalities were analyzed using one–way analysis of variance (ANOVA) and differences between treatments were analyzed using Duncan's multiple range test and were considered significant at $p<0.05$.

3. Results and Discussion

3.1 Trial I: Growth performance experiment

After 2 weeks of the feeding trial, the fish receiving dietary supplementation with 1.5 g/kg cells or 7.5 mg/kg extract had significantly higher final weight and weight gain than the control group ($p<0.05$). Moreover, the fish receiving cells or extracts at any concentration had significantly better specific growth rate and feed conversion ratio than the control group ($p<0.05$, Table 2). Similarly, after 4 weeks of the feeding trial, fish receiving cells or extract at any concentration showed significantly better final weight, weight gain, specific growth rate, and feed conversion ratio than the control group ($p<0.05$). No significant difference was found in survival rate between the actual treatments and the control after 2 or 4 weeks of the feeding trial ($p>0.05$, Table 2). Water quality during the experiment was at an acceptable level consistently, with temperature from 27.80±0.40°C to 28.20±0.36°C, total alkalinity from 22.67±9.81 mg/L to 28.33±9.81 mg/L, pH from 7.17±0.06 to 7.40±0.17, dissolved oxygen from 5.57±0.15 mg/L to 6.13±0.06 mg/L, and total ammonia were below 0.1 mg/L. In this study, experimental diets were prepared to contain 36% protein and 8.5% fat, which was apparently similar as in Afuang, Siddhuraja, and Becker (2003) who prepared the experimental diets for tilapia to contain 36.2% crude protein and 8.9% crude lipid. The growth of Nile tilapia fed with the control diet supports that diet as suitable for these experiments. Furthermore, Nile tilapia fed 1.5 g/kg cells or 7.5 mg/kg extract showed significantly better growth performance than in the control group at 2 or 4 weeks, indicating that *Z. marina* cells and extracts improved fish growth. This is consistent with Kim, Harikrishnan, Kim, Balasundaram, and Heo (2010), who demonstrated that olive flounder (*Paralichthys olivaceus*) fed *Zooshikella* sp. cells had higher weight gain than the fish

Table 2. Growth performance of Nile tilapia receiving dietary supplementation at different levels of *Z. marina* cells or extracts

Treatment	Initial weight (g/fish)	Final weight (g/fish)	Weight gain (%)	Specific growth rate (%/day)	Feed conversion ratio	Survival rate (%)
2 weeks of feeding						
T1: Control	19.01±0.16^{ns}	23.87±0.52^{a}	34.61±1.46^{a}	0.86±0.01^{a}	1.49±0.64^{b}	100±0.00^{ns}
T2: 0.5 g/kg ZC	18.84±0.01^{ns}	23.89±0.55^{a}	38.18±1.60^{ab}	0.98±0.05^{b}	1.32±0.55^{a}	100±0.00^{ns}
T3: 1.0 g/kg ZC	18.80±0.03^{ns}	24.46±0.89^{ab}	37.78±1.61^{ab}	1.02±0.10^{b}	1.30±0.45^{a}	100±0.00^{ns}
T4: 1.5 g/kg ZC	18.82±0.04^{ns}	24.69±0.64^{b}	39.90±2.84^{b}	1.04±0.11^{b}	1.28±0.46^{a}	100±0.00^{ns}
T5: 2.5 mg/kg ZE	18.89±0.03^{ns}	24.22±0.97^{ab}	38.39±2.89^{ab}	1.03±0.04^{b}	1.33±0.49^{a}	100±0.00^{ns}
T6: 5.0 mg/kg ZE	18.84±0.02^{ns}	24.48±0.58^{ab}	39.04±1.92^{ab}	1.04±0.05^{b}	1.30±0.54^{a}	100±0.00^{ns}
T7: 7.5 mg/kg ZE	18.93±0.13^{ns}	24.72±0.44^{b}	40.26±3.01^{b}	1.06±0.06^{b}	1.25±0.50^{a}	100±0.00^{ns}
4 weeks of feeding						
T1: Control	19.01±0.16^{ns}	26.78±0.42^{a}	45.17±1.97^{a}	1.26±0.11^{a}	1.62±0.46^{b}	100±0.00^{ns}
T2: 0.5 g/kg ZC	18.84±0.01^{ns}	28.96±0.13^{bc}	52.89±3.32^{b}	1.55±0.03^{b}	1.45±0.45^{a}	100±0.00^{ns}
T3: 1.0 g/kg ZC	18.80±0.03^{ns}	29.08±0.56^{bc}	55.55±3.87^{b}	1.58±0.08^{b}	1.45±0.43^{a}	100±0.00^{ns}
T4: 1.5 g/kg ZC	18.82±0.04^{ns}	29.78±0.41^{c}	58.24±3.25^{b}	1.68±0.08^{b}	1.40±0.41^{a}	100±0.00^{ns}
T5: 2.5 mg/kg ZE	18.89±0.03^{ns}	28.35±0.55^{b}	52.90±4.46^{b}	1.51±0.09^{b}	1.49±0.44^{a}	100±0.00^{ns}
T6: 5.0 mg/kg ZE	18.84±0.02^{ns}	29.24±0.19^{bc}	55.00±3.33^{b}	1.57±0.06^{b}	1.46±0.42^{a}	100±0.00^{ns}
T7: 7.5 mg/kg ZE	18.93±0.13^{ns}	29.86±0.18^{c}	56.25±4.92^{b}	1.65±0.06^{b}	1.43±0.50^{a}	100±0.00^{ns}

ZC= *Z. marina* cells; ZE= *Z. marina* extracts
Values within the same column with different superscripts are significantly different ($p<0.05$).

receiving the control diet. Similarly, Nile tilapia fed with experimental diet containing 0.48x10^6 CFU/g *Bacillus licheniformis* and 1.0% yeast extract showed the highest final body weight, weight gain, specific growth rate, best feed conversion ratio, protein efficiency ratio and protein productive value (Hassaan, Soltan, & Ghonemy, 2014).

3.2 Trial II: Immune responses and disease resistance

3.2.1 Haemato–immunological parameters

After 2 weeks of the feeding trial, the fish receiving dietary supplementation with 1.5 g/kg cells had significantly higher red blood cell, white blood cell, haemoglobin, haematocrit and NBT reduction than the control group ($p<0.05$). Moreover, the fish receiving dietary supplementation with 7.5 mg/kg of extract had significantly higher haemoglobin, haematocrit and serum protein than in the control treatment ($p<0.05$). No significant differences were found in immunoglobulin and lysozyme between the actual treatments and the control groups ($p>0.05$, Table 3). Besides, the fish receiving supplementation with 1.5 g/kg cells or 7.5 mg/kg extract had significantly higher phagocytic activity and phagocytic index than the control group ($p<0.05$, Figure 1a–b). No significant difference was found in average bead ingested per cell in comparison to the control groups ($p>0.05$, Figure 1c).

After 4 weeks of the feeding trials, the fish receiving 1.5 g/kg cells in the feed had significantly higher white blood cell, haemoglobin, haematocrit and serum protein than the control group ($p<0.05$). Also, fish fed with dietary supplementation of 7.5 mg/kg extract had significantly higher red blood cell, haematocrit and serum protein than the control group ($p<0.05$). No significant differences were found in NBT reduction, immunoglobulin and lysozyme between the actual treatments and the control groups ($p>0.05$, Table 4). In addition, the fish receiving 1.5 g/kg cells or 7.5 mg/kg extract had significantly higher phagocytic activity and phagocytic index than the control groups ($p<0.05$, Figure 2a–b). No significant difference was found in average bead ingested per cell between the treatments and the control groups ($p>0.05$, Figure 2c).

Table 3. Haemato–immunological parameters of Nile tilapia fed with dietary supplementation at different levels of *Z. marina* cells or extract for 2 weeks

Treatment	RBC (x10^{9}cell/mL)	WBC (x10^{7}cell/mL)	Haemoglobin (g/dL)	Haematocrit (%)	NBT reduction (OD_{630})	Serum protein (mg/mL)	Immunoglobulin (mg/mL)	Lysozyme (µg/mL)
T1: Control–1	2.18±0.35^a	6.72±1.21^a	3.93±0.33^a	24.47±1.97^a	1.23±0.22^a	24.31±2.54^a	0.98±0.21^{ns}	5.12±0.44^{ns}
T2: Control–2	2.22±0.32^a	6.35±0.90^a	3.46±0.95^a	24.25±2.74^a	1.17±0.21^a	25.28±3.35^a	0.96±0.63^{ns}	4.80±1.00^{ns}
T3: 0.5 g/kg ZC	2.37±0.21^{ab}	7.77±0.66^{ab}	4.14±1.26^a	26.74±5.22^{ab}	1.39±0.19^{ab}	27.23±2.41^{ab}	1.73±1.04^{ns}	5.25±1.25^{ns}
T4: 1.0 g/kg ZC	2.39±0.17^{ab}	7.81±0.69^{ab}	5.44±0.75^b	27.32±2.80^{ab}	1.41±0.22^{ab}	28.43±3.79^{ab}	1.84±1.48^{ns}	5.25±0.19^{ns}
T5: 1.5 g/kg ZC	2.56±0.29^b	8.47±2.04^b	5.83±0.87^b	29.50±4.76^b	1.73±0.58^b	28.71±1.30^{ab}	1.89±0.54^{ns}	6.05±0.85^{ns}
T6: 2.5 mg/kg ZE	2.30±0.24^{ab}	6.81±1.57^{ab}	5.05±0.83^b	27.94±3.34^{ab}	1.39±0.21^{ab}	27.43±1.18^{ab}	1.60±1.53^{ns}	5.32±2.05^{ns}
T7: 5.0 mg/kg ZE	2.42±0.15^{ab}	6.86±1.42^{ab}	5.58±0.64^b	28.42±2.20^b	1.44±0.30^{ab}	27.49±2.37^{ab}	1.76±1.68^{ns}	5.80±0.73^{ns}
T8: 7.5 mg/kg ZE	2.41±0.19^{ab}	6.94±1.15^{ab}	5.72±0.43^b	29.25±3.65^b	1.55±0.34^{ab}	30.30±4.70^b	2.71±0.80^{ns}	6.53±2.70^{ns}

ZC=*Z. marina* cells; ZE=*Z. marina* extracts; RBC=Red blood cell; WBC=White blood cell
Values within the same column with different superscripts are significantly different ($p<0.05$).

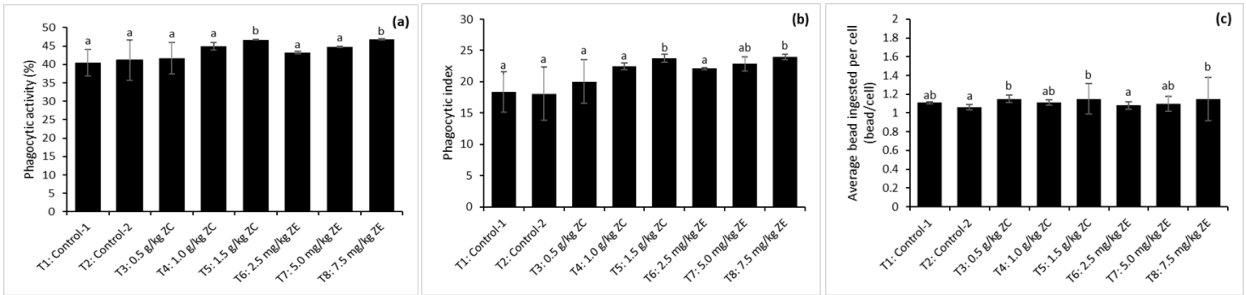

Figure 1. Phagocytic activity (a), phagocytic index (b), and average bead ingested per cell (c) of Nile tilapia fed with dietary supplementation at different levels of *Z. marina* cells (ZC) or *Z. marina* extracts (ZE) for 2 weeks. Values with different letters are significantly different ($p<0.05$).

Table 4. Haemato–immunological parameters of Nile tilapia fed with dietary supplementation at different levels of *Z. marina* cells or extract for 4 weeks

Treatment	RBC ($x10^9$cell/mL)	WBC ($x10^7$cell/mL)	Haemoglobin (g/dL)	Haematocrit (%)	NBT reduction (OD_{630})	Serum protein (mg/mL)	Immunoglobulin (mg/mL)	Lysozyme (µg/mL)
T1: Control–1	2.40±0.08^{a}	6.62±1.34^{a}	5.80±0.41^{a}	28.03±0.37^{a}	1.05±0.19^{ns}	26.48±1.53^{a}	3.16±3.46^{ns}	6.80±0.53^{ns}
T2: Control–2	2.34±0.12^{a}	6.66±0.74^{a}	5.75±0.74^{a}	27.98±0.38^{a}	1.06±0.34^{ns}	26.20±1.50^{a}	2.97±2.68^{ns}	5.60±0.93^{ns}
T3: 0.5 g/kg ZC	2.38±0.13^{a}	6.65±1.26^{a}	6.56±0.91^{ab}	29.50±2.97^{ab}	1.13±0.39^{ns}	29.76±4.15^{ab}	3.19±2.35^{ns}	5.80±2.31^{ns}
T4: 1.0 g/kg ZC	2.49±0.31^{ab}	7.63±0.72^{ab}	6.58±0.78^{ab}	30.11±4.82^{ab}	1.13±0.45^{ns}	29.97±1.91^{ab}	4.73±2.71^{ns}	6.60±2.14^{ns}
T5: 1.5 g/kg ZC	2.58±0.31^{ab}	7.82±1.13^{b}	6.94±1.01^{b}	32.33±4.20^{b}	1.35±0.46^{ns}	31.72±4.38^{b}	5.25±2.73^{ns}	7.47±1.21^{ns}
T6: 2.5 mg/kg ZE	2.52±0.22^{ab}	6.63±0.71^{a}	6.67±1.10^{ab}	29.93±1.98^{ab}	1.15±0.21^{ns}	30.34±4.83^{ab}	5.17±0.99^{ns}	6.57±1.65^{ns}
T7: 5.0 mg/kg ZE	2.56±0.15^{b}	6.72±0.55^{a}	6.70±0.58^{ab}	32.44±2.77^{b}	1.16±0.09^{ns}	30.79±2.35^{ab}	5.24±2.72^{ns}	6.93±0.90^{ns}
T8: 7.5 mg/kg ZE	2.66±0.08^{b}	7.70±0.89^{ab}	6.80±0.66^{ab}	32.93±3.48^{b}	1.20±0.13^{ns}	31.51±2.68^{b}	6.00±2.77^{ns}	6.94±1.30^{ns}

ZC=*Z. marina* cells; ZE=*Z. marina* extracts; RBC=Red blood cell; WBC=White blood cell
Values within the same column with different superscripts are significantly different ($p<0.05$).

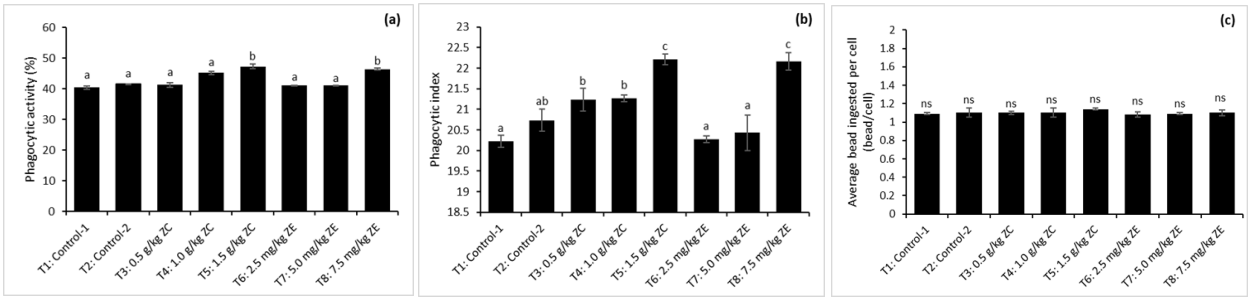

Figure 2. Phagocytic activity (a), phagocytic index (b), and average bead ingested per cell (c) of Nile tilapia fed with dietary supplementation at different levels of *Z. marina* cells (ZC) or *Z. marina* extracts (ZE) for 4 weeks. Values with different letters are significantly different ($p<0.05$).

The use of the haemato–immunological parameters red blood cell, white blood cell, haemoglobin, haematocrit, and serum protein to monitor fish growth and health has proven to be effective and repeatable (Esmaeili, 2021). Better growth performance of Nile tilapia with any of the supplemented diets (cells or extract from *Z. marina*) in the present study support this aspect. Basically, red blood cells are in charge of transporting oxygen; white blood cells are involved in both specific and non–specific immune responses; haemoglobin is in charge of aerobic metabolism, which involves binding oxygen, dissolving gas, and transporting them to the tissues; haematocrit is in charge of viscosity, which is beneficial for health; and serum proteins are involved in a wide range of functions, including maintaining osmotic pressure, pH, transporting various metabolites, and interacting with the immune system (Esmaeili, 2021). Decreased haemato–immunological parameters were reported in Nile tilapia infected with *S. agalactiae* (Suanyuk *et al*., 2005). The results from the current study are, however, consistent with a previous study in that Nile tilapia fed with dietary supplementation of 1g/kg *Aspergillus oryzae*, 1g/kg β–glucan, and 0.5 g/kg *A. oryzae* + 0.5 g/kg β–glucan exhibited significantly higher haematocrit, hemoglobin, white blood cells, red blood cells and phagocytic activity than those in the control group ($p<0.05$) while NBT reduction and phagocytic index increased significantly ($p<0.05$) in fish fed 1 g/kg *A. oryzae* and 0.5 g/kg *A. oryzae* + 0.5 g/kg β–glucan over the control (Dawood, Eweedah, Moustafa, & Shahin, 2020). Similarly, highest values of haemoglobin, haematocrit, red blood cell and white blood cell counts were observed in Nile tilapia fed with $0.48x10^6$ CFU/g *B. licheniformis* and 0.5% yeast extract (Hassaan *et al*., 2014). The results from the present study indicate that dietary supplementation with *Z. marina* cells at a concentration of 1.5 g/kg or with the extract at a concentration of 7.5 mg/kg can stimulate the innate immune system in Nile tilapia.

3.2.2 *In vivo* immersion challenge assay

A variety of infection methods including injection, immersion, oral administration, and cohabitation has been used to infect tilapia with *S. agalactiae* (He *et al*., 2021; Mian, Godoy, Leal, Yuhara, Costa, & Figueiredo, 2009; Rodkhum, Kayansamruaj, & Pirarat, 2011; Soto, Zayas, Tobar, Illanes,

Yount, Francis, & Dennis, 2016; Suanyuk *et al*., 2008; Suwannasang *et al*., 2014). The route of immersion infection closely resembles natural infection because gill, skin, and gastrointestinal tract are the three major entry routes of pathogens in fish (Ling, Wang, Lim, & Leung, 2001; Wu, Zhang, Lin, Hao, Wang, Zhang, & Li, 2021). However, the immersion challenge could not induce a similarly high mortality rate as an injection challenge (Soto *et al*., 2016). Hence, the combined effects of high temperature and hypoxia as synergistic stressors on immersion challenge with *S. agalactiae* were employed in this study, using the modified method of He *et al.* (2021). After 2 weeks of the feeding trial, the cumulative mortalities of experimental fish exposed to *S. agalactiae* were 6.67, 10.00 and 3.33% for the fish fed with dietary supplementation of 0.5, 1.0 and 1.5 g/kg cells; and the mortalities were 13.33, 10.00 and 3.33% in fish fed with dietary supplementation of 2.5, 5.0 and 7.5 mg/kg extract. These were significantly lower ($p<0.05$) than in the infected control group (36.67%). No mortality was observed in the non–challenged control group (Figure 3). After 4 weeks of the feeding trial, the cumulative mortality of experimental fish exposed to *S. agalactiae* was 0.00% when receiving dietary supplementation with cells at any concentration; and the cumulative mortalities were 16.67, 10.00 and 0.00% in fish receiving 2.5, 5.0 and 7.5 mg/kg extract, whereas a significantly higher cumulative mortality of 36.67% was recorded for the infected control group ($p<0.05$). No mortality was found in the non–challenged control group (Figure 4).

Kim *et al*. (2010) revealed that the cumulative mortality was low in infected fish receiving dietary supplementation with a high level of *Zooshikella* sp. cells. Resistance of the experimental fish against *S. agalactiae* infection observed in this current study may be due to prodigiosin and cycloprodigiosin that inhibit pathogenic bacteria. The antibacterial activity of prodigiosin is due to its ability to pass through and damage the cell membrane, as well as its capacity for inhibiting target enzymes involved in DNA replication, such as topoisomerase IV and DNA gyrase (Berlanga, Ruiz, & Hernandez–Borrell, 2000; Darshan & Manonmani, 2016), as well as potentially interfering with certain steps in the pathogenic process by decreasing invasion into the fish target organ (Puangpee & Suanyuk, 2021). In addition, the enhancement of haemato–immunological parameters by dietary supplementation with *Z. marina* diets observed in this study may be an important factor reducing mortality and protecting the fish against *S. agalactiae* infection.

During challenge test, Nile tilapia receiving the control diet and infected with *S. agalactiae* (T2) exhibited erratic swimming, an increased opercular movement, darkening skin, eye opacity, eye exophthalmia, and ascites. Few clinical signs were observed in some fish that received dietary supplementation with cells or extract from *Z. marina*. *S. agalactiae* was re–isolated from the brain of all dead fish indicating that the deaths were caused by streptococcal infection. This is consistent with several reports that fish infected with *S. agalactiae* exhibited similar symptoms typical of streptococcosis (Klingklib & Suanyuk, 2017; Suanyuk *et al*., 2008; Suwannasang *et al*., 2014). However, it should be noted that the environmental factors may play an important role, equal to or dominant over the host and pathogenic factors, as regards disease pathogenesis (Chen & He, 2019).

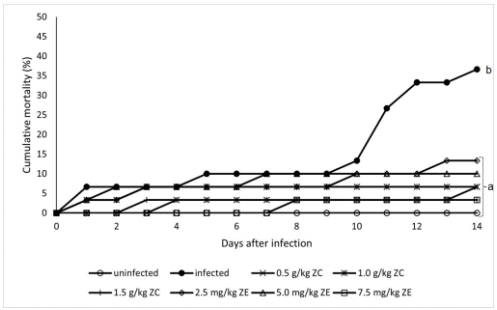

Figure 3. Cumulative mortality of Nile tilapia fed with dietary supplementation at different levels of *Z. marina* cells (ZC) or *Z. marina* extracts (ZE) for 2 weeks before experimental challenge with exposure to *S. agalactiae*. Values with different letters are significantly different ($p<0.05$).

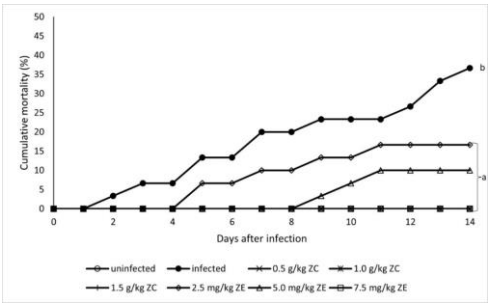

Figure 4. Cumulative mortality of Nile tilapia fed with dietary supplementation at different levels of *Z. marina* cells (ZC) or *Z. marina* extracts (ZE) for 4 weeks before experimental challenge with exposure to *S. agalactiae*. Values with different letters are significantly different ($p<0.05$).

The accumulated mortality of tilapia immersion challenged with *S. agalactiae* at concentration of 10^8 CFU/mL was highest in the group maintained at 33°C followed by 30°C and 25°C. (Rodkhum *et al*., 2011). Moreover, He *et al*. (2021) reported a high mortality in tilapia infected with *S. agalactiae* by immersion challenge when exposed to an elevated temperature (33.5±0.5°C) and hypoxia (2.0±0.5 mg/l dissolved oxygen) indicating that improper environmental factors such as elevated water temperature and hypoxia play important roles in disease pathogenesis.

4. Conclusions

In summary, *Z. marina* cells and extracts promote the growth of Nile tilapia. In addition, the enhancement of innate immune parameters by *Z. marina* cells and extracts is a possibly important factor reducing mortality and protecting the fish against *S. agalactiae* infection. Furthermore, the dietary administration of *Z. marina* cell at a concentration of 1.5 g/kg or of the extract at a concentration of 7.5 mg/kg could be used to promote growth, stimulate immune responses, as well as to protect the fish against streptococcosis caused by *S. agalactiae* infection.

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