Fish and Shellfish Immunology 93 (2019) 428–435

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Effects of Assam tea extract on growth, skin mucus, serum immunity and disease resistance of Nile tilapia (*Oreochromis niloticus*) against *Streptococcus agalactiae*

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ARTICLE INFO

Keywords:
Assam tea extract
Growth performance
Mucosal immunity
Humoral immunity
Disease resistance
Nile tilapia
S. agalactiae

ABSTRACT

The present study aimed to assess the possible effects of Assam tea (*Camellia sinensis*) extract (ATE) on growth performances, immune responses, and disease resistance of Nile tilapia, *Oreochromis niloticus* against *Streptococcus agalactiae*. Five levels of ATE were supplemented into the based diet at 0, 1, 2, 4, and 8 g kg^{-1} feed of Nile tilapia fingerlings (10.9 ± 0.04 g initial weight) in triplicate. After four and eight weeks of feeding, fish were sampled to determine the effects of the tea supplements upon their growth performance, as well as serum and mucosal immune responses. A disease challenge using *S. agalactiae* was conducted at the end of the feeding trial. Fish fed ATE revealed significantly improved serum lysozyme, peroxidase, alternative complement (ACH50), phagocytosis, and respiratory burst activities compared to the basal control fed fish ($P < 0.05$). The mucus lysozyme and peroxidase activities were ameliorated through ATE supplementation in the tilapia diets. Supplementation of ATE significantly ($P < 0.05$) enhanced final body weight, weight gain, and specific growth rate; while a decreased feed conversion ratio was revealed at 2 g kg^{-1} inclusion level, after four and eight weeks. Challenge test showed that the relative percent survival (RSP) of fish in each treatment was 33.33%, 60.00%, 83.33%, 76.68%, and 66.68% in groups fed 0, 1, 2, 4, and 8 g kg^{-1}, respectively. In summary, diets supplemented with ATE especially at 2 g kg^{-1} increased the humoral and mucosal immunity, enhanced growth performance, and offered higher resistance against *S. agalactiae* infection in Nile tilapia.

1. Introduction

Aquaculture is an important sector that provides a valuable and essential protein source for human consumption [1]. Despite being the fastest-growing food production sectors with 5.8% annual growth rate since 2000 [2], the intensification and extension of the aquaculture industry are subject to disease outbreaks [3]. Antimicrobial substances were extensively used in aquaculture for prophylactic aims and metaphylactic treatments [4,5]. However, controlling the outbreak of aquaculture diseases through antimicrobial substances has led to the emergence of antimicrobial resistance (AMR) pathogens. Recent microbiological and clinical evidence has revealed that antimicrobial resistance genes and bacteria are transferred from both livestock and aquaculture animals to humans [6]. As a natural consequence, alternatives to such antibiotics and chemotherapeutics have been sought out by several researchers within the scientific community. The use of

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https://doi.org/10.1016/j.fsi.2019.07.077
Received 29 April 2019; Received in revised form 22 July 2019; Accepted 26 July 2019
Available online 27 July 2019

medicinal plants is one of promising means for the prevention and/or treatment of such diseases in aquacultural farming [7,8]. Due to their cost-effectiveness, biodegradability, and safety; medicinal plants have been widely applied in the aquaculture industry in an attempt to control such diseases. Additionally, they provide more extended protection periods than synthetic drugs, which have shorter recovery rates [9,10]. It is well-documented that numerous types of medicinal plants contain the antioxidant properties which can delay or prevent oxidative damage, and thereby play an essential role in disease prevention [7,11,12].

Assam tea (*Camellia sinensis*) leaves (Assam, CTC, India) have been used as traditional medicine for health benefit since ancient times [13]. The leaves contain many bioactive compounds; such as polyphenols, nitrogenous compounds, caffeine, vitamins, inorganic elements, and carbohydrates, and lipids [14–16]. Previous studies have demonstrated the beneficial impacts of Assam tea integrated diets on bone density, cognitive functions, kidney stones, and dental caries in both human and animals [15,17]. In aquaculture, the positive effects of tea and its derivatives on growth, antioxidant defense, blood chemistry, and enhancement of immune systems and protection against pathogens were observed in studies of olive flounder (*Paralichthys olivaceus*) [18]; rainbow trout (*Oncorhynchus mykiss*) [19–21], and grey mullet (*Mugil cephalus*) [22].

Nile tilapia (*Oreochromis niloticus*), remains one of the most commonly cultured fish species worldwide, due to their natural breeding, tolerance to varied environments and diseases, fast growth, and high market demand [23,24]. Global tilapia production has developed rapidly in recent decades, reaching approximately 6.3 million tons in 2018 [25]. However, it faces significant challenges due to the infection of *Streptococcus* spp., *Vibrio* spp., *Aeromonas hydrophila*, and *Flavobacterium* spp. Among the pathogens, *Streptococcus agalactiae* is one of the most severe bacteria. The mortality rate up to 95% have been recorded in Thailand's hot season, causing significant losses, both economically and in terms of market availability the tilapia farming industry [26]. *S. agalactiae* has developed in the most damaging impediment to the expansion of the tilapia industry worldwide [27,28]. The present study, therefore, addresses and evaluates the possible effects of Assam tea extract on the growth function, skin mucus immune response, serum immunity, and resistance to *S. agalactiae* of Nile tilapia fingerlings.

2. Materials and methods

2.1. Preparation of medicinal plants

The Assam tea (*C. sinensis*) leaves were collected from Bann Phang Ma O, Chiang Dao District, Chiang Mai, Thailand (720 MSL). The tea leaves were then oven-dried for 48 h at 60 °C, then ground into fine particles (0.2-mm) for further extraction. Then, 500g of the powdered sample was thoroughly mixed with 5 L of ethanol (AR grade; RCI Labscan), and left in the dark, at room temperature, for 72 h. After that, the supernatant was filtered using a Whatman No. 41 filter paper. The resulting solution was then evaporated to dried under reduced pressure condition (40 °C), via a rotary evaporator (Büchi, Flawil, Switzerland). Samples were then labeled and stored at (−20 °C for 1 month) until use.

2.2. Dietary preparation

Adjustments to the basal diet were determined according to the previous study of Van Doan et al. [29]; which had been proven suitable for tilapia. Pellets were made using an extruder pellet machine and subsequently stored in polyethylene bags at 4 °C. The proximate composition of the experimental diets quantified following AOAC [30] method comprised the percentage of crude protein, crude lipid, crude ash, and crude fibre (Table 1). For diets preparation, the Assam tea extracted powder at different concentrations was dissolved in distilled water and sprayed into the pellets, and then thoroughly mixed. Assam tea (*C. sinensis*) extract (ATE) was supplemented into the based diet at 0, 1, 2, 4, and 8 g kg^{-1} feed (Diet 1, Diet 2, Diet 3, Diet 4 and Diet 5, respectively) of Nile tilapia fingerlings in triplicate. The mixture was coated using fish oil (Premer Co., LTD), then dried in room temperature for 24 h. The pellets were then stored at 4 °C for a week.

Table 1
The formulation and proximate composition of Assam tea extraction experiment (g kg^{-1}).

Ingredients	Diets (g kg^{-1})
Fish meal	270
Corn meal	200
Soybean meal	270
Wheat flour	60
Rice bran	150
Cellulose	30
Soybean oil	5
Premix[a]	10
Vitamin C[b]	5
Proximate composition (g kg^{-1} dry matter basis)	
Crude protein	322.06
Crude lipid	74.75
Fibre	52.48
Ash	106.68
Dry matter	817.80
GE (cal/g)[c]	4,105

[a] Vitamin and trace mineral mix supplemented as follows (IU kg^{-1} or g kg^{-1} diet): retinyl acetate 1,085,000 IU; cholecalciferol 217,000 IU; D, L-a-tocopherol acetate 0.5 g; thiamin nitrate 0.5 g; pyridoxine hydrochloride 0.5 g; niacin 3 g; folic 0.05 g; cyanocobalamin 10 g; Ca pantothenate 1 g kg^{-1}; inositol 0.5 g; zinc 1 g; copper 0.25 g; manganese 1.32 g; iodine 0.05 g; sodium 7.85 g.
[b] Vitamin C 98% 5 g.
[c] GE = gross energy.

2.3. Experimental design

Nile tilapia (*O. niloticus*) (mono-sex) fingerlings were bought from the Chiang Mai Pathana Farm Co., Ltd., Chiang Mai. Upon arrival, fish were distributed in 5 × 5x2 meter cages and fed commercial pellets (CP, 9950) for two months. A control diet was administrated bi-weekly in preparation for the present experiment. Before the start of the experiment, ten fish were randomly selected to check the health status through observation of body surface, gills and internal organs under a microscope to confirm that the tested fish are free of the common diseases, parasites and disorders. A total of 300 healthy fingerlings, weighing 10.9 ± 0.04 g $fish^{-1}$ were placed into 15 glass tanks (150 L), comprising 20 fish per tank. A Completely Randomised Design (CRD) with five groups (three replications) was applied for eight weeks. Growth rates, weight gain, specific growth rate, feed conversion ratio as well as immune responses to tilapia were computed 4 and 8 weeks after feeding. Eight weeks after feeding, ten fish were randomly retrieved from each replication and challenged with the *S. agalactiae*. Experimental diets were provided *ad libitum* two times per day at 8:30 a.m. and 5:30 p.m., the water temperature was 28 ± 1 °C, and pH maintained a range of 7.75 ± 0.05. The dissolved oxygen was fixed at no less than 5 mg L^{-1}.

2.4. Immune response

2.4.1. Serum, leukocytes, and mucus collection

Serum was prepared using blood collected from four fish per replication (group 1). Blood (1 mL) was collected via the caudal vein of each fish using a 1 mL syringe and immediately released into 1.5 mL Eppendorf tubes without anticoagulant. The tubes were then incubated

at room temperature for 1 h and stored in a refrigerator (4 °C) for 4 h. After incubation, the samples were centrifuged at 1500g for 5 min at 4 °C, and the anticipated serum was gathered using a micro-pipette and stored at - 80 °C for further evaluation.

Leucocyte was isolated from fish's blood following the method described by Chung and Secombes [99]. One milliliter of blood was withdrawn from each fish, at a rate of four fish per replication, and then transferred into 15 mL tubes containing RPMI 1640 (2 mL) (Gibthai). This mixture was then carefully inserted in the 15 mL tubes, containing 3 mL of *Histopaque* (Sigma, St. Louis, MO, USA). These tubes were then centrifuged at 400 g for 30 min at room temperature. Upon completion, buffy coat of leucocytes cells drifted to the top of the Histopaque was carefully collected using a Pasteur pipette, and released into a sanitized 15 mL tubes. After which, 6 mL of phosphate buffer solution (PBS: Sigma-Aldrich, USA) was added to each tube and gently aspirated. The cells in these tubes were washed for twice by centrifugation at 250*g* for 10 min at room temperature, to remove any residual *Histopaque*. The obtaining cells were then re-suspended in the PBS and adjusted to the numbers of cells requires to evaluate phagocytic and respiratory burst activities.

Skin mucus collection from another group of four fish per replication (group 2), or twelve fish per experimental group, was conducted using the method of Miandare et al. [100]. The anesthetized fish (using clove oil at a concentration of 5 mL per 1 L of water) was placed into the plastic bag containing 10 mL of 50 mM NaCl, and then gently rubbed inside the plastic for 2 min. The solution was immediately transferred to a 15 mL sterile tube and centrifuged at 1500 g at 4 °C for 10 min (5810R Eppendorf, Engelsdorf, Germany). The supernatant was collected and stored at −80 °C until further analysis.

2.4.2. Serum and skin mucus lysozyme activities

Serum lysozyme activity was analyzed according to Parry et al. [101]. Briefly, 25 μL of undiluted serum and 100 μL of skin mucus from each fish was loaded into 96 well plates in triplication; after which, *Micrococcus lysodeikticus* (175 μL, 0.3 mg mL^{-1} in 0.1 M citrate phosphate buffer, pH 5.8; Sigma-Aldrich, USA) was added to each well. The contents were rapidly mixed, and any changes in turbidity were measured every 30 s, for 10 min, at 540 nm, 25 °C, via a microplate reader (Synergy H1, BioTek, USA). The sample's equivalent unit of activity was determined and compared with the standard curve, which was generated from the reduction of OD value *vs.* the concentration of hen egg-white lysozyme ranging from 0 to 20 μl mL^{-1} (Sigma Aldrich, USA), and expressed as μg mL^{-1} serum.

2.4.3. Serum and skin mucus peroxidase activities

We calculated the peroxidase activity via the Quade and Roth [31]; and Cordero et al. [32] protocol. Briefly, 5 μL of undiluted serum or skin mucus from each fish was placed in the flat bottomed of 96 well plates, in triplication. Then, 45 μl of *Hank's Balanced Salt Solution* (without Ca^{+2} or Mg^{+2}) was added to each well. Later, 100 μL of solution (contains 40 ml of distilled water + 10 μL of H_2O_2, 30%; Sigma Aldrich + one pill of 3,3′,5,5′-tetramethylbenzidine, TMB; Sigma Aldrich) was then added to each well. When the reaction color turned blue (30–60 s), a solution of 50 μl of 2 M H_2SO_4 was then immediately added to each well. The optical density was then read at 450 nm via a microplate reader (Synergy H1, BioTek, USA). Samples not containing serum or skin mucus were considered to be blanks. A single unit was defined as the amount which produces an absorbance change, expressed as units (U) mL^{-1} of serum or mucus following the equation: Peroxidase activity = [absorbance of the sample] – [absorbance of blank containing all solution without serum or mucus sample].

2.4.4. Phagocytic activity

Phagocytosis activity was measured via the procedure specified in Yoshida and Kitao [102]. Briefly, 200 μL of leucocyte cell suspensions (2 x 10^6 cells mL^{-1}) were loaded on coverslips and incubated at room temperature for 2 h. After incubation, the coverslips were washed with 3 mL of RPMI-1640 to remove any non-adherent cells. Then, a solution of 200 μL of fluorescence latex beads with a concentration of 2×10^7 of beads (mL^{-1}) (Sigma-Aldrich, USA) was placed into each coverslip and incubated again at room temperature for 1.5 h. The coverslips were then rewashed with 3 mL of RPMI- 1640 to remove any non-phagocytized bead. After washing, the coverslips were then fixed with methanol, and stained with Diff-Quik staining dye (Sigma-Aldrich, USA) for 10 s. After staining, a wash of PBS (pH 7.4) removed any excessive stains. The washed coverslips were allowed to dry at room temperature and then attached to the slides with Permount (Merck, Germany). The number of phagocyte cells per 300 adhered cells was later counted microscopically. The phagocytic index (PI) and phagocytic rate (PR%) were calculated through the following equations:

PI = (Number of phagocytized beads divided by the number of phagocytizing leukocytes) *100

PR = (Number phagocytizing leukocytes divided by the number total cells count) *100

2.4.5. Respiratory burst

The calculation of the respiratory burst activity of blood leucocytes, followed by the protocol of Secombes [103]. Briefly, 175 μL PBS cells suspension at a concentration of 6×10^6 cells mL^{-1} were loaded into the 96 well plates in triplication. Then, 25 μL of nitro blue tetrazolium (NBT) at a concentration of 1 mg mL^{-1} was added to each well and incubated the solution for 2 h at room temperature. Later, the supernatant was carefully discarded from each well, and 125 μL of 100% methanol was then added into each well for 5 min to fix the cells. After that, 125 μL of 70% methanol $well^{-1}$ were added into each well, twice, for clean-up. The plates were then dried for 30 min at room temperature. Then, 125 μL of 2 N KOH and 150 μL of DMSO were added to each well. Afterward, the plates were measured at 655 nm via microplate-reader (Synergy H1, BioTek, USA), according to the following: Spontaneous O^{2-} production = (absorbance NBT reduction of the sample) – [(absorbance of blank (containing 125 μL of 2 N KOH and 150 μL with no leucocytes)].

2.4.6. Alternative complement pathway activity (ACH50)

Calculation of ACH50 has followed the method of Yano [33]. Briefly, rabbit red blood cells (R-RBC) were washed with PBS by centrifugation at 3000 rpm, and in 0.01 M ethylene glycol tetra-acetic acid-magnesium-gelatin veronal buffer (0.01 M − EGTA-Mg-GVB) for twice. The R-RBC concentration was adjusted to 2×10^8 cells mL^{-1} in 0.01 M − EGTA-Mg-GVB buffer. Then 100 μL of the R-RBC suspension was lysed with 3.4 mL of distilled water. Hemolysate absorbance was measured at 414 nm *vs.* distilled water as a blank and was adjusted to reach 0.740.

For the ACH50 test, 100 μL of serum was diluted with 400 μL of 0.01M-EGTA-Mg-GVB, and serial two-fold dilution was conducted. The tubes were performed on ice to retard the reaction of complement until all tubes were prepared. Consequently, 100 μL of R-RBC suspension was loaded into each tube and incubated at 20 °C for 1.5 h with occasional shaking. After incubation, 3.15 mL of cold saline solution (0.85% NaCl) was placed into each tube to stop the reaction, and then the tube was centrifuged at 1600 g for 5 min. After centrifugation, 100 μL of supernatant in each dilution was loaded into 96-well plate and read at 414 nm. The degree of hemolysis was calculated by dividing the corrected absorbance 414 value by the corrected absorbance 414 of the 100% hemolysis control. The degree of hemolysis and the serum volume were plotted on a log-log paper. The volume of serum that gave 50% hemolysis was used for calculating the ACH50 using the formula: ACH50 (units/ml) = 1/K x r x ½.

Where K is the amount of serum giving 50% hemolysis, r is the reciprocal of the serum dilution, and ½ is the correction factor. The

assay was performed on a ½ scale of the original method.

2.5. Challenge test

The *S. agalactiae* were isolated from diseased tilapia in Northern Thailand. It was identified and characterized by Gram staining and biochemical test. Detailed preparation of *S. agalactiae* was described in the previous study of Van Doan et al. [34]. Briefly, *S. agalactiae* was cultured in Tryptic Soy Broth and incubated at 30 °C for 24 h in the rotation shaker at a speed of 110 rpm. The sub-culture was obtained from the stock. Then, 5 mL of the stock solution was transferred into a 50 mL flask contained Tryptic Soy Broth and incubated at 30 °C for 24 h. The sub-cultures were raised in duplicate under similar conditions for the experiment. Growth was evaluated by the optical density of 560 nm (0.75% NaCl was used to adjust bacterium concentration) and then using plate counting in Tryptic Soy Agar. The calibration curves, relating optical density (OD) at 560 nm with plate counts, were collected by measuring the OD of consecutive one-half dilution series with triplicate each, before determining the cell density by classic plate count methods (10^7 CFU mL^{-1} of *S. agalactiae* = 0.8465 OD + 1.6187, $R^2 = 0.91$).

Eight weeks post-feeding, ten fish from each tank (group 3) were randomly retrieved for testing. The fish were intraperitoneally injected with 0.1 mL of 0.85% saline solution containing 10^7 CFU ml^{-1} of *S. agalactiae* [35]. The clinical sign and lesion of disease were observed, and dead fish were removed daily. We computed the tilapia's mortality rates, in percentages, for each treatment, 15 days after the challenge; as well as the relative percentage of survival (RPS), through the following equation of Amend [36]: RPS = (1- % mortality in vaccinated/% mortality in control) × 100.

2.6. Growth performance

At 4 and 8 weeks after feeding, growth performance and survival rate of the fish (20 fish per replication) were measured using the following equations: Specific growth rate (SGR %) = 100 × (ln final weight - ln initial weight)/total duration of experiment; Feed conversion ratio (FCR) = feed given (dried weight)/weight gain (wet weight); Survival rate (%) = (final fish number/initial fish number) × 100.

2.7. Statistical analysis

After testing and confirming the normality of the data through using Kolmogorov- Smirnov test. We analyzed the significant differences among treatment given the application of one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test) via the SAS Computer Program [37]. Significant different mean values ($P < 0.05$) and other data are displayed as means ± standard deviation.

3. Results

3.1. Mucosal immune response

The supplemental ATE diets resulted in significant ($P < 0.05$) improvements skin mucus lysozyme and peroxidase activities *vs.* the control diet after eight weeks post-feeding (Table 3). Improved values of SMLA and SMPA were found in the fish fed 2 g kg^{-1} ATE, but no significant ($P > 0.05$) differences were observed in fish fed 1 and 2 g kg^{-2} ATE, and between fish fed 4 and 8 g kg^{-2} ATE ($P > 0.05$; Table 3).

3.2. Serum immune responses

We observed the variations in serum immunity activities between the control and the supplemented ATE groups (Table 2). Dietary supplementation of ATE resulted in considerably higher SL ($P < 0.05$) compared with that of the control fed fish after four- and eight-weeks post-feeding. Similarly, SP, ACH50, PI, and RB significantly improved in the fish fed the ATE diets compared to those fed the control diet ($P < 0.05$). The highest values were recorded in the 2 g kg^{-1} ATE concerning the control and other supplemented groups ($P < 0.05$; Table 2). Nonetheless, no significant ($P > 0.05$) differences were revealed among the 1, 4, and 8 g kg^{-1} ATE supplemented diets, and no significant ($P > 0.05$) differences in RB were displayed between 1 and 2 g kg^{-1} ATE (Table 2).

3.3. Disease resistance challenge

We calculated the survival rates for 15 days after injection of *S. agalactiae*, which was conducted eight weeks post-feeding. The findings revealed that the survival rates of fish given the ATE inclusion diets were significantly higher than that of the control treatment (33.33%) by 60.00% (Diet 2), 83.33% (Diet 3), 76.68% Diet 4, and 66.68% (Diet 5) ($P < 0.05$, Fig. 1). The appearance of dead fish revealed typical *S. agalactiae* infected clinical sign and lesion; including erratic swimming, loss of appetite, darkness, exophthalmia, pair-fins basal haemorrhage, and pale liver. Based on the survival rates, the relative percent survival (RSP) of fish in each treatment was 40.00%, 75.00%, 65.00%, and 50.00% in Diet 2 through 5, respectively. The highest RPS value and resistance to *S. agalactiae* were detected in fish fed the 2 g kg^{-1} ATE diet, which was significantly ($P < 0.05$) higher when than that of the control treatment and other supplemented diets (Fig. 1).

3.4. Growth performance

After four- and eight-weeks post-feeding, dietary inclusion of ATE resulted in significantly ($P < 0.05$) improved the specific growth rate (SGR), weight gain (WG), and final weight (FW); compared with the control treatment (Table 4). The highest values of SGR, WG, and FW were a result of the 2 g kg^{-1} ATE, four weeks post-feeding (Table 4). However, there were no significant ($P > 0.05$) differences in the parameters of each of the dietary inclusions of ATE at eight weeks post-feeding (Table 4). The 2 g kg^{-1} ATE diet produced the lowest feed conversion ratio (FCR), the control diet scored the highest value. Significantly ($P < 0.05$) improved FCR was displayed in fish fed the 2 g kg^{-1} ATE diet, in comparison with both the control and other supplementary groups (Table 4). However, no significant ($P > 0.05$) differences in FCR were found in the 1, 4, and 8 g kg^{-1} ATE diets. Similarly, no significant difference was present in the survival rates among treatments after eight weeks post-feeding (Table 4).

4. Discussion

The impending emergence of antimicrobial bacteria has forced the scientific community to reevaluate the use of alternative, natural treatments, which can stimulate immunity and enhance antioxidant capabilities [38,39]. Medicinal plants have been proven to have a positive effect on growth performance, immune systems, and diseases resistance of fish and shellfish [7,39,40]. The scientific community, therefore, has been searching for suitable feed additives that can improve both the immune systems and general wellbeing of fish. To the best of our knowledge, there is no study has been conducted to judge the possibility of supplementing ATE on the growth rate, mucosal and serum immunities, and resistance of Nile tilapia (*O. niloticus*) to *S. agalactiae*. Tea (*Camellia sinensis*) has been found to possess antioxidative and anticarcinogenic properties, which have been attributed to the monomer polyphenolic compounds which may help in improving the health status and the growth performance of fish [41].

Skin mucus is a crucial element of innate immunity, and represents the first defensive stand against invading microorganisms, as it contains a diverse range of non-specific and specific immune factor which create a physio-chemical barrier that protects fish against infectious pathogens

Table 2
Serum immunity of (mean ± S.E., $n=4$) of *O. niloticus* after 4 and 8 weeks feeding with experimental diets containing different levels of Assan tea.

		Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
4 weeks	SL	4.49 ± 0.22^{c}	6.33 ± 0.19^{b}	8.03 ± 0.16^{a}	6.57 ± 0.34^{b}	6.38 ± 0.29^{b}
	SP	0.11 ± 0.008^{c}	0.15 ± 0.003^{b}	0.18 ± 0.005^{a}	0.15 ± 0.006^{b}	0.16 ± 0.005^{b}
	ACH50	132.04 ± 4.30^{c}	150.37 ± 5.01^{b}	182.08 ± 5.24^{a}	163.83 ± 3.85^{b}	160.40 ± 4.87^{b}
	PI	1.42 ± 0.05^{c}	2.39 ± 0.08^{a}	2.42 ± 0.11^{a}	2.05 ± 0.04^{b}	2.03 ± 0.06^{b}
	PR	47.28 ± 1.84^{c}	83.33 ± 1.53^{a}	91.78 ± 1.25^{a}	70.22 ± 3.40^{b}	71.10 ± 3.42^{b}
	RB	0.04 ± 0.005^{c}	0.08 ± 0.005^{a}	0.09 ± 0.008^{a}	0.08 ± 0.006^{a}	0.07 ± 0.008^{b}
8 weeks	SL	7.14 ± 0.35^{c}	8.88 ± 0.24^{b}	10.82 ± 0.21^{a}	9.16 ± 0.36^{b}	8.49 ± 0.49^{b}
	SP	0.16 ± 0.008^{c}	0.21 ± 0.01^{b}	0.26 ± 0.005^{a}	0.23 ± 0.006^{b}	0.22 ± 0.005^{b}
	ACH50	166.68 ± 4.86^{c}	211.85 ± 4.39^{b}	256.32 ± 9.46^{a}	212.71 ± 6.61^{b}	210.78 ± 6.13^{b}
	PI	1.97 ± 0.09^{c}	2.42 ± 0.04^{b}	2.87 ± 0.09^{a}	2.48 ± 0.07^{b}	2.51 ± 0.08^{b}
	PR	56.22 ± 2.92^{c}	87.22 ± 3.39^{a}	85.56 ± 4.26^{a}	67.33 ± 1.81^{b}	66.50 ± 2.15^{b}
	RB	0.12 ± 0.02^{b}	0.15 ± 0.01^{a}	0.17 ± 0.01^{a}	0.17 ± 0.01^{a}	0.16 ± 0.01^{a}

Different letter in a row denote significant difference ($P < 0.05$).
SL = Serum lysozyme activity (μg mL^{-1}); SP = Serum peroxidase activity (U mL^{-1}); ACH50 = Alternative complement activity (units ml^{-1}); PI = Phagocytosis activity (bead cell^{-1}); PR = Phagocytosis rate (%); RB = Respiratory burst activity (U mL^{-1}).

[42–44]. The present study revealed that the administration of supplementary ATE created remarkable boosts of mucus lysozyme and peroxidase activities. As far as we know, there is no available information about the effects of *C. sinensis* skin mucus immune response in fish. However, significantly enhanced skin mucosal immune response has been reported in common carp (*Cyprinus carpio*) [45,46] and striped catfish (*Pangasianodon hypophthalmus*) [47]. It is known that mucosal immunity can be boosted by dietary administration of prebiotics, probiotics, and medicinal plants [48]. As immunological sites, skin-associated lymphoid tissues, (SALT), gill-associated lymphoid tissues (GIALT), and gut-associated lymphoid tissues (GALT) can ascend a robust immune response against pathogenic bacteria [49,50]. At an immunologically level, GALT is assembled of granulocytes, macrophages, lymphocytes, and plasma cells, as well as T and B cells. These cells, along with epithelial cells, goblet cells, and neuroendocrine cells, can generate and control gut immune responses [51,52]. Nonetheless, the exact mechanism in which ATE affected skin mucus immune response needs further investigations.

Several humoral and cellular immune parameters within this study exhibited significant enhancements activity after four and eight weeks on feed supplemented with ATE. Incorporation of functional feed additives in the diet is helping more significant number of fishes consume an adequate amount of tea extract, with low-cost and minimal effort [53]. Tea contains a considerable amount of catechins, which are anti-inflammatory, anti-bacterial, anti-angiogenic, anti-oxidative, and anti-viral [54–57]. ATE is widely accepted as a medicinal herb around the globe; however, their properties as an effective immunostimulant or a natural substance against *S. agalactiae* has not been studied in fish. Lysozyme represents a vital defense component which is responsible for the lysis of pathogenic bacteria [58]. In this study, fish fed with ATE demonstrated significantly enhanced lysozyme activity, similar to previous studies in grouper, *Epinephelus bruneus* [59]; rainbow trout (*O. mykiss*) [19], grey mullet (*M. cephalus*) [22]; in which heightened lysozyme activity was presented in fish fed tea supplemented diets. Alternative complement activity has been proven to be one of the most

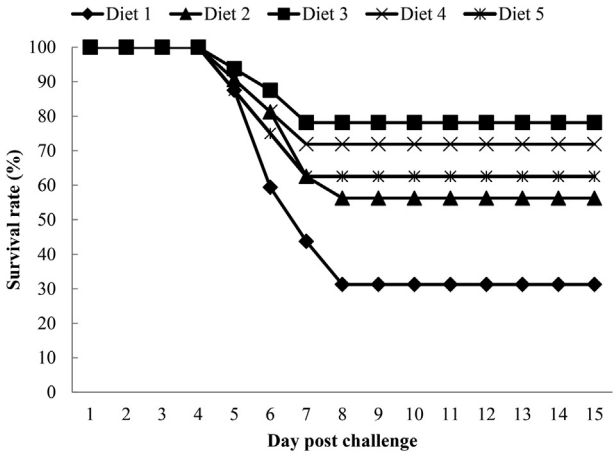

Fig. 1. Survival rate of *O. niloticus* fed different experimental diets ($n=30$) containing different levels of Assan tea during 15 days challenge with *S. agalactiae*.

significant methods of removing pathogenic bacteria from fish [60,61]. Furthermore, its activation as an independent alternative complement pathway can be achieved through immunostimulants [62–64]. The present study has shown that ATE can increase this type of alternative complement activity in both weeks four and eight, through the recommended ATE supplementary diets. This result is consistent with the work of Harikrishnan et al. [59]; in which the oral administration of tea in grouper enhanced the alternate complement activity. Fish neutrophils contain various phagocytic, bactericidal, respiratory burst, and peroxidase activities [52,65–67]. Evaluation of the neutrophil function is necessary for the assessment of the general health of fish [68,69]. It is determined, herein, that the administration of all ATE doses appreciably enhanced serum peroxidase activity and respiratory burst activity after four and eight weeks. Similarly, in grouper and rainbow trout fed with a tea supplemented diet, peroxidase activity also rose after four

Table 3
Skin and mucus lysozyme and peroxidase activities (mean ± S.E., $n=4$) of *O. niloticus* after 4 and 8 weeks feeding with experimental diets containing different levels of Assan tea.

		Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
4 weeks	SMLA	1.12 ± 0.03^{c}	1.68 ± 0.09^{b}	2.65 ± 0.17^{a}	1.88 ± 0.20^{b}	1.61 ± 0.06^{b}
	SMPA	0.04 ± 0.005^{c}	0.10 ± 0.005^{a}	0.10 ± 0.005^{a}	0.06 ± 0.003^{b}	0.07 ± 0.005^{b}
8 weeks	SMLA	2.93 ± 0.21^{c}	4.63 ± 0.26^{b}	5.39 ± 0.16^{a}	4.49 ± 0.18^{b}	4.61 ± 0.19^{b}
	SMPA	0.08 ± 0.005^{c}	0.15 ± 0.003^{a}	0.16 ± 0.003^{a}	0.13 ± 0.003^{b}	0.12 ± 0.008^{b}

Different letter in a row denote significant difference ($P < 0.05$).
SMLA (μg mL^{-1}) = Skin mucus lysozyme activity; SMPA (U mL^{-1}) = Skin mucus peroxidase activity.

Table 4
Growth performances and feed utilization of *O. niloticus* after 4 and 8 weeks feeding with experimental diets containing different levels of Assan tea.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
IW (g)	10.83 ± 0.04	10.83 ± 0.04	10.87 ± 0.03	10.90 ± 0.07	10.88 ± 0.03
FW (g)					
4 weeks	33.19 ± 0.35^{c}	35.26 ± 0.45^{bc}	38.61 ± 0.59^{a}	36.27 ± 1.05^{ab}	36.34 ± 0.41^{ab}
8 weeks	72.16 ± 1.78^{b}	77.71 ± 0.36^{a}	80.43 ± 1.05^{a}	77.65 ± 0.59^{a}	76.49 ± 0.69^{a}
WG (g)					
4 weeks	22.36 ± 0.31^{c}	24.43 ± 0.42^{bc}	27.75 ± 0.62^{a}	25.37 ± 1.00^{b}	25.46 ± 0.39^{b}
8 weeks	61.32 ± 1.81^{b}	66.87 ± 0.32^{a}	69.57 ± 1.07^{a}	66.75 ± 0.52^{a}	65.61 ± 0.66^{a}
SGR (%)					
4 weeks	3.73 ± 0.02^{c}	3.94 ± 0.03^{b}	4.23 ± 0.06^{a}	4.00 ± 0.08^{b}	4.02 ± 0.03^{b}
8 weeks	3.16 ± 0.05^{b}	3.28 ± 0.003^{a}	3.33 ± 0.03^{a}	3.27 ± 0.003^{a}	3.25 ± 0.01^{ab}
FCR					
4 weeks	1.54 ± 0.009^{a}	1.48 ± 0.005^{b}	1.46 ± 0.004^{c}	1.48 ± 0.006^{b}	1.49 ± 0.003^{b}
8 weeks	1.58 ± 0.005^{a}	1.52 ± 0.005^{b}	1.50 ± 0.003^{b}	1.52 ± 0.01^{b}	1.52 ± 0.008^{b}
SR (%)	97	98	99	97	98

Different letter in a row denote significant difference ($P < 0.05$).
dIW (g) = Initial weight; FW (g) = Final weight; WG (g) – Weight gain; SGR (%) = Specific growth rate; FCR = Feed conversion ratio; SR (%) = Survival rate.

weeks of feeding [19,59]. Respiratory burst, through stimulation by foreign agents, has been found to increase the oxidation levels in phagocytes, and are considered to be a crucial factor in the general defense mechanisms in fish [70,71]. The creation of respiratory burst activities and reactive oxygen metabolites by phagocytes are vital factors in limiting the spread of diseases in fish [66]. Phagocytosis is an essential cellular immune system component in fish [72–74]. Its role is to assist fish to avoid pathogen attacks more efficiently by recognizing the existing pathogens and to limit their spread and progress [75]. Through the increase of phagocytosis, the present study has revealed that ATE promotes immune responses and provides greater tolerance against infectious pathogens. Similar to our result, a significant increase in respiratory burst and phagocytosis activities were recorded in grey mullet fed *C. sinensis* [22]. Although the precise mechanisms in which *C. sinensis* tea stimulate immune responses in fish is not elucidated yet, it might be attributable to the presence of some bioactive compounds, such as catechins, flavonols, flavanonones, phenolic acids [76–79]. Polyphenols are a diverse group of naturally occurring substances with a wide range of biological functions. Many polyphenols, such as catechin can control immunological reactions by regulating pro-inflammatory cytokines and chemokines or by affecting the activity of immune cells [80,81]. Moreover, a recent study showed that polysaccharide isolated from *C. sinensis* not only significantly stimulated interleukin (IL)-6 and IL-12 production but also enhanced tumoricidal activity against Yac-1 tumor cells in mice. Additionally, intravenous administration of GTE-II significantly stimulated natural killer (NK) cytotoxicity against Yac-1 tumor cells [82].

It is now clear that ATE can be used as an immunostimulant in tilapia aquaculture. It is observed, herein, the decrease in tilapia mortality from *S. agalactiae* through dietary inclusion of ATE. The significant increase in disease resistance may be due to the elevation in mucosal and serum immunity. It has been reported that mucosal immunity plays a vital role in protection *Oreochromis* spp. against *S. agalactiae* infection [83]. Similar to the present result, Abdel-Tawwab et al. [84] observed that the inclusion of green tea in Nile tilapia diet presented corresponding decreases in fish mortality. Sheikhzadeh et al. [19] indicated that green tea enhanced serum lysozyme and bactericidal activities against *Yersinia ruckeri* in rainbow trout. A recent study indicated that dietary administration of *C. sinensis* significant reduced the mortality percentage of grey mullet against *Photobacterium damselae* [22]. Although the precise mechanism in which Assam tea extract increased disease resistance of Nile tilapia against *S. agalactiae* is not clarified yet, it may be because of the presence of biological compounds in *C. sinensis*. It was found that dietary supplemented with polyphenols from *C. sinensis* revealed anti-bacterial effects and inhibited the *Staphylococcus* sp., *Clostridium botulinum*, *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumonia*, and *Salmonella* [85].

Growth performance and feed conversion ratio are essential parameters need to judge the potential use of feed additives in aqua-feed [86,87]. The present study determined that the dietary supplement of 2 g kg^{-1} ATE significantly improved the WG and SGR of Nile tilapia, while concurrently reducing FCR; which was consisted with the conclusions of Zhang et al. [41] and Huang et al. [88]. They reported that tea addition increased growth-related parameters while decreasing the feed conversion ratio. It has been demonstrated that the dietary inclusion of tea improves WG and FCR by dietary tea is related to improved metabolic parameters or utilization of nutrients, and the activation of the functionality of intestinal flora [89–91]. Significant decreases in growth rates and feed utilization were present in the higher doses of tea (4 and 8 g kg^{-1}) within this study. Zhang et al. [41], Huang et al. [88] and Cho et al. [18]; also determined that adding higher levels of tea resulted in decreased WG and feed utilisation in the diets of channel catfish, olive flounder, and black rockfish. Tea has a high fiber content which may negatively affect the feed efficiency of fish, and growth performance accordingly [18]. Li et al. [89] reported that fish are capable of consuming up to 23% total dietary fibre before showing a decline in growth rate. High levels of tea have been shown to reduce weight by increasing both the metabolic rate and energy expenditures while decreasing the digestibility of ingredients; because of its content some antinutritional factors, such as of tannins, catechin monomers, and caffeine [92–97]. Tea polyphenols have been found to exert their influence upon the emulsion interface, interacting with digestive enzymes to decrease feed utilization and WG [98]. However, the exact nature of these compounds remains unclear and requires further study.

To conclude, the present study revealed that ATE supplementation might potentially activate the humoral, mucosal, and cellular immune mechanisms; generate disease resistance to *S. agalactiae* and improve growth rate and feed utilization.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflicts of interest.

Ethical approval

The study was performed following the guidelines on the use of animals for scientific purposes (Chiang Mai University).

Acknowledgements

The authors wish to the thank National Research Council of Thailand and the Functional Food Research Center for well-being, Chiang Mai University, Chiang Mai, Thailand for their financial assistance; as well as the staffs at Central and Biotechnology Laboratories, Faculty of Agriculture, Chiang Mai University for their kind support with the data analysis process.

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