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

Hien Van Doan[1,2], Seyed Hossein Hoseinifar[3], Korawan Sringarm[1], Sanchai Jaturasitha[1,2], Bundit Yuangsoi[4], Mahmoud A.O. Dawood[5,*], Maria Ángeles Esteban[6], Einar Ringø[7], Caterina Faggio[8]

[1]*Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand*

[2]*Science and Technology Research Institute, Chiang Mai University 239 Huay Keaw Rd., Suthep, Muang, Chiang Mai 50200*

[3]*Department of Fisheries, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan-Iran*

[4]*Department of Fisheries, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand*

[5]*Department of Animal Production, Faculty of Agriculture, Kafrelsheikh University, 33516, Kafrelsheikh, Egypt*

[6]*Fish Innate Immune System Group, Department of Cell Biology & Histology, Faculty of Biology, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, Spain.*

[7]*Norwegian College of Fishery Science, Faculty of Bioscience, Fisheries and Economics, UiT The Arctic University of Norway, Tromsø, Norway*

[8]*Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina Viale Ferdinando Stagno d'Alcontres, 31 98166, S. Agata–Messina, Italy*

***Corresponding author Email:** mahmouddawood55@gmail.com

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

47 ATE especially at 2 g kg^{-1} increased the humoral and mucosal immunity, enhanced
48 growth performance, and offered higher resistance against *S. agalactiae* infection in
49 Nile tilapia.
50
51 **Keywords**: Assam tea extract; Growth performance; Mucosal immunity; Humoral
52 immunity; Disease resistance; Nile tilapia; *S. agalactiae*
53

54 **1. Introduction**

55 Aquaculture is an important sector that provides a valuable and essential protein source
56 for human consumption [1]. Despite being the fastest-growing food production sectors
57 with 5.8 % annual growth rate since 2000 [2], the intensification and extension of the
58 aquaculture industry are subject to disease outbreaks [3]. Antimicrobial substances were
59 extensively used in aquaculture for prophylactic aims and metaphylactic treatments [4,
60 5]. However, controlling the outbreak of aquaculture diseases through antimicrobial
61 substances has led to the emergence of antimicrobial resistance (AMR)
62 pathogens. Recent microbiological and clinical evidence has revealed that antimicrobial
63 resistance genes and bacteria are transferred from both livestock and aquaculture
64 animals to humans [6]. As a natural consequence, alternatives to such antibiotics and
65 chemotherapeutics have been sought out by several researchers within the scientific
66 community. The use of medicinal plants is one of promising means for the prevention
67 and/or treatment of such diseases in aquacultural farming [7, 8]. Due to their cost-
68 effectiveness, biodegradability, and safety; medicinal plants have been widely applied in
69 the aquaculture industry in an attempt to control such diseases. Additionally, they
70 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 hours at 60°C, then ground into fine particles (0.2-mm) for further extraction. Then, 500g of the powdered sample was thoroughly mixed with five litres of ethanol (AR grade; RCI Lab-Scan), and left in the dark, at room temperature, for 72 hours. 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 hours. The pellets were then stored at 4^{o}C for a week.

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 5x5x2 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 liters), 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 $litre^{-1}$.

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 1mL syringe and immediately released into 1.5 mL Eppendorf tubes without anticoagulant. The tubes were then incubated at room temperature for one hour and stored in a refrigerator (4°C) for four hours. After incubation, the samples were centrifuged at 1500g for five minutes 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 15mL tubes, containing 3mL of *Histopaque* (Sigma, St. Louis, MO, USA). These tubes were then centrifuged at 400 g for 30 minutes 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 15mL tubes. After which, 6mL 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 250g for ten minutes 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.

167 [100]. The anesthetized fish (using clove oil at a concentration of 5 mL per 1 litre of
168 water) was placed into the plastic bag containing 10mL of 50mM NaCl, and then gently
169 rubbed inside the plastic for two minutes. The solution was immediately transferred to a
170 15mL sterile tube and centrifuged at 1500g at 4 ˚C for ten minutes (5810R Eppendorf,
171 Engelsdorf, Germany). The supernatant was collected and stored at -80 ˚C until further
172 analysis.
173

174 **2.4.2 Serum and skin mucus lysozyme activities**

175 Serum lysozyme activity was analyzed according to Parry et al. [101]. Briefly, 25µL of
176 undiluted serum and 100µL of skin mucus from each fish was loaded into 96 well plates
177 in triplication; after which, *Micrococcus lysodeikticus* (175µL, 0.3 mg mL^{-1} in 0.1 M
178 citrate phosphate buffer, pH 5.8; Sigma-Aldrich, USA) was added to each well. The
179 contents were rapidly mixed, and any changes in turbidity were measured every 30
180 seconds, for ten minutes, at 540nm, 25 °C, via a microplate reader (Synergy H1,
181 BioTek, USA). The sample's equivalent unit of activity was determined and compared
182 with the standard curve, which was generated from the reduction of OD value *vs.* the
183 concentration of hen egg-white lysozyme ranging from 0-20µl mL^{-1} (Sigma Aldrich,
184 USA), and expressed as µg mL^{-1} serum.
185

186 **2.4.3 Serum and skin mucus peroxidase activities**

187 We calculated the peroxidase activity via the Quade and Roth [31]; and Cordero et al.
188 [32] protocol. Briefly, 5µL of undiluted serum or skin mucus from each fish was placed
189 in the flat bottomed of 96 well plates, in triplication. Then, 45µl of *Hank's Balanced*
190 *Salt Solution* (without Ca^{+2} or Mg^{+2}) was added to each well. Later, 100µL of solution

(contains 40ml of distilled water + 10µL 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 seconds), a solution of 50µl of 2M H_2SO_4 was then immediately added to each well. The optical density was then read at 450nm 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, 200µL of leucocyte cell suspensions (2×10^6 cells mL^{-1}) were loaded on coverslips and incubated at room temperature for two hours. After incubation, the coverslips were washed with 3mL of RPMI-1640 to remove any non-adherent cells. Then, a solution of 200µL 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 hours. The coverslips were then rewashed with 3mL 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 ten seconds. 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, 175µL PBS cells suspension at a concentration of 6×10^6 cells mL^{-1} were loaded into the 96 well plates in triplication. Then, 25µL of nitro blue tetrazolium (NBT) at a concentration of 1mg mL^{-1} was added to each well and incubated the solution for two hours at room temperature. Later, the supernatant was carefully discarded from each well, and 125µL of 100% methanol was then added into each well for five minutes to fix the cells. After that, 125µL of 70% methanol $well^{-1}$ were added into each well, twice, for clean-up. The plates were then dried for thirty minutes at room temperature. Then, 125µL of 2N KOH and 150µL of DMSO were added to each well. Afterward, the plates were measured at 655nm 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 125µL of 2N 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 0.01M

238 ethylene glycol tetra-acetic acid-magnesium-gelatin veronal buffer (0.01M – EGTA-
239 Mg-GVB) for twice. The R-RBC concentration was adjusted to 2×10^8 cells mL^{-1} in
240 0.01M – EGTA-Mg-GVB buffer. Then 100 µL of the R-RBC suspension was lysed
241 with 3.4 mL of distilled water. Hemolysate absorbance was measured at 414 nm *vs.*
242 distilled water as a blank and was adjusted to reach 0.740.
243 For the ACH50 test, 100 µL of serum was diluted with 400 µL of 0.01M-EGTA-Mg-
244 GVB, and serial two-fold dilution was conducted. The tubes were performed on ice to
245 retard the reaction of complement until all tubes were prepared. Consequently, 100 µL
246 of R-RBC suspension was loaded into each tube and incubated at 20^{o}C for 1.5 hours
247 with occasional shaking. After incubation, 3.15 mL of cold saline solution (0.85%
248 NaCl) was placed into each tube to stop the reaction, and then the tube was centrifuged
249 at 1600 g for 5 minutes. After centrifugation, 100 µL of supernatant in each dilution was
250 loaded into 96-well plate and read at 414 nm. The degree of hemolysis was calculated
251 by dividing the corrected absorbance 414 value by the corrected absorbance 414 of the
252 100% hemolysis control. The degree of hemolysis and the serum volume were plotted
253 on a log-log paper. The volume of serum that gave 50% hemolysis was used for
254 calculating the ACH50 using the formula: ACH50 (units/ml) = 1/K x r x ½.
255 Where K is the amount of serum giving 50% hemolysis, r is the reciprocal of the serum
256 dilution, and ½ is the correction factor. The assay was performed on a ½ scale of the
257 original method.
258
259 **2.5 Challenge test**
260 The *S. agalactiae* were isolated from diseased tilapia in Northern Thailand. It was
261 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 ºC for 24 hours 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 ºC for 24 hours. 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 0.1mL 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 [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 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 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

405 metabolites by phagocytes are vital factors in limiting the spread of diseases in fish [66].
406 Phagocytosis is an essential cellular immune system component in fish [72-74]. Its role
407 is to assist fish to avoid pathogen attacks more efficiently by recognizing the existing
408 pathogens and to limit their spread and progress [75]. Through the increase of
409 phagocytosis, the present study has revealed that ATE promotes immune responses and
410 provides greater tolerance against infectious pathogens. Similar to our result, a
411 significant increase in respiratory burst and phagocytosis activities were recorded in
412 grey mullet fed *C. sinensis* [22]. Although the precise mechanisms in which *C. sinensis*
413 tea stimulate immune responses in fish is not elucidated yet, it might be attributable to
414 the presence of some bioactive compounds, such as catechins, flavonols, flavanononones,
415 phenolic acids [76-79]. Polyphenols are a diverse group of naturally occurring
416 substances with a wide range of biological functions. Many polyphenols, such as
417 catechin can control immunological reactions by regulating pro-inflammatory cytokines
418 and chemokines or by affecting the activity of immune cells [80, 81]. Moreover, a
419 recent study showed that polysaccharide isolated from *C. sinensis* not only significantly
420 stimulated interleukin (IL)-6 and IL-12 production but also enhanced tumoricidal
421 activity against Yac-1 tumor cells in mice. Additionally, intravenous administration of
422 GTE-II significantly stimulated natural killer (NK) cytotoxicity against Yac-1 tumor
423 cells [82].
424 It is now clear that ATE can be used as an immunostimulant in tilapia aquaculture. It is
425 observed, herein, the decrease in tilapia mortality from *S. agalactiae* through dietary
426 inclusion of ATE. The significant increase in disease resistance may be due to the
427 elevation in mucosal and serum immunity. It has been reported that mucosal immunity
428 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

453 channel catfish, olive flounder, and black rockfish. Tea has a high fiber content which
454 may negatively affect the feed efficiency of fish, and growth performance accordingly
455 [18]. Li et al. [89] reported that fish are capable of consuming up to 23 % total dietary
456 fibre before showing a decline in growth rate. High levels of tea have been shown to
457 reduce weight by increasing both the metabolic rate and energy expenditures while
458 decreasing the digestibility of ingredients; because of its content some antinutritional
459 factors, such as of tannins, catechin monomers, and caffeine [92-97]. Tea polyphenols
460 have been found to exert their influence upon the emulsion interface, interacting with
461 digestive enzymes to decrease feed utilization and WG [98]. However, the exact nature
462 of these compounds remains unclear and requires further study.
463 To conclude, the present study revealed that ATE supplementation might potentially
464 activate the humoral, mucosal, and cellular immune mechanisms; generate disease
465 resistance to *S. agalactiae* and improve growth rate and feed utilization.
466

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473

474 **Compliance with Ethical Standards**

475 **Conflict of interest**

476 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).

References

[1] A.G. Murray, L.A. Munro, The growth of Scottish salmon (*Salmo salar*) aquaculture 1979–2016 fits a simple two-phase logistic population model, Aquaculture 496 (2018) 146-152.

[2] FAO, The State of World Fisheries and Aquaculture 2018 - Meeting the Sustainable Development Goals. Rome, in: F.a.A. Organiszation (Ed.) Rome, Italy, 2018.

[3] D. Yi, T. Reardon, R. Stringer, Shrimp aquaculture technology change in Indonesia: Are small farmers included?, Aquaculture 493 (2018) 436-445.

[4] F.C. Cabello, Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment, Environmental Microbiology 8(7) (2006) 1137-44.

[5] P. Smith, 7 - Antibiotics in aquaculture: reducing their use and maintaining their efficacy, in: B. Austin (Ed.), Infectious Disease in Aquaculture, Woodhead Publishing2012, pp. 161-189.

[6] J.J.T.r.o.a.r. O'Neill, Antimicrobials in agriculture and the environment: reducing unnecessary use and waste, (2015) 1-44.

[7] E. Awad, A. Awaad, Role of medicinal plants on growth performance and immune status in fish, Fish & Shellfish Immunology 67 (2017) 40-54.

500 [8] P.A. Subramani, R.D. Michael, Chapter 4 - Prophylactic and Prevention Methods
501 Against Diseases in Aquaculture, in: G. Jeney (Ed.), Fish Diseases, Academic
502 Press2017, pp. 81-117.
503 [9] C.-C. Wu, C.-H. Liu, Y.-P. Chang, S.-L. Hsieh, Effects of hot-water extract of
504 Toona sinensis on immune response and resistance to Aeromonas hydrophila in
505 Oreochromis mossambicus, Fish & Shellfish Immunology 29(2) (2010) 258-263.
506 [10] J. Yostawonkul, S. Kitiyodom, S. Kaewmalun, K. Suktham, N. Nittayasut, M.
507 Khongkow, K. Namdee, U.R. Ruktanonchai, C. Rodkhum, N. Pirarat, S. Surassmo, T.
508 Yata, Bifunctional clove oil nanoparticles for anesthesia and anti-bacterial activity in
509 Nile tilapia (*Oreochromis niloticus*), Aquaculture 503 (2019) 589-595.
510 [11] E. Awad, D. Austin, A. Lyndon, A. Awaad, Possible effect of hala extract
511 (*Pandanus tectorius*) on immune status, anti-tumour and resistance to *Yersinia ruckeri*
512 infection in rainbow trout (*Oncorhynchus mykiss*), Fish & Shellfish Immunology 87
513 (2019) 620-626.
514 [12] M. Ekor, The growing use of herbal medicines: issues relating to adverse reactions
515 and challenges in monitoring safety, 4(177) (2014).
516 [13] A. Gomes, P. Datta, A. Sarkar, S.C. Dasgupta, A. Gomes, Black tea (Camellia
517 sinensis) extract as an immunomodulator against immunocompetent and
518 immunodeficient experimental rodents, Oriental Pharmacy and Experimental Medicine
519 14(1) (2014) 37-45.
520 [14] D.C. Chu, L.R. Juneja, General chemical composition of green tea and its infusion,
521 (1997) 13-22.
522 [15] C. Cabrera, R. Artacho, R. Giménez, Beneficial Effects of Green Tea—A Review,
523 Journal of the American College of Nutrition 25(2) (2006) 79-99.

524 [16] M.G. Sajilata, P.R. Bajaj, R.S. Singhal, Tea Polyphenols as Nutraceuticals,
525 Comprehensive Reviews in Food Science and Food Safety 7(3) (2008) 229-254.
526 [17] V. Crespy, G. Williamson, A review of the health effects of green tea catechins in
527 in vivo animal models, 134(12) (2004) 3431S-3440S.
528 [18] S.H. Cho, S.-M. Lee, B.H. Park, S.-C. Ji, J. Lee, J. Bae, S.-Y. Oh, Effect of dietary
529 inclusion of various sources of green tea on growth, body composition and blood
530 chemistry of the juvenile olive flounder, *Paralichthys olivaceus*, Fish Physiology and
531 Biochemistry 33(1) (2007) 49-57.
532 [19] N. Sheikhzadeh, K. Nofouzi, A. Delazar, A.K. Oushani, Immunomodulatory
533 effects of decaffeinated green tea (*Camellia sinensis*) on the immune system of rainbow
534 trout (*Oncorhynchus mykiss*), Fish & Shellfish Immunology 31(6) (2011) 1268-1269.
535 [20] S. Nootash, N. Sheikhzadeh, B. Baradaran, A.K. Oushani, M.R. Maleki
536 Moghadam, K. Nofouzi, A. Monfaredan, L. Aghebati, F. Zare, S. Shabanzadeh, Green
537 tea (*Camellia sinensis*) administration induces expression of immune relevant genes and
538 biochemical parameters in rainbow trout (*Oncorhynchus mykiss*), Fish & Shellfish
539 Immunology 35(6) (2013) 1916-1923.
540 [21] J. Thawonsuwan, V. Kiron, S. Satoh, A. Panigrahi, V. Verlhac, Epigallocatechin-3-
541 gallate (EGCG) affects the antioxidant and immune defense of the rainbow trout,
542 Oncorhynchus mykiss, Fish Physiology and Biochemistry 36(3) (2010) 687-697.
543 [22] S. Kakoolaki, P. Akbary, M.J. Zorriehzahra, H. Salehi, A. Sepahdari, M.
544 Afsharnasab, M.R. Mehrabi, S. Jadgal, *Camellia sinensis* supplemented diet enhances
545 the innate non-specific responses, haematological parameters and growth performance
546 in *Mugil cephalus* against *Photobacterium damselae*, Fish & Shellfish Immunology 57
547 (2016) 379-385.

548 [23] M. Etyemez, J.L. Balcazar, Isolation and characterization of bacteria with
549 antibacterial properties from Nile tilapia (*Oreochromis niloticus*), Research in
550 Veterinary Science 105 (2016) 62-64.
551 [24] W.-L. Guo, H.-W. Deng, F. Wang, S.-F. Wang, Z.-H. Zhong, Y. Sun, X.-F. Chen,
552 J.-H. Wang, Y.-C. Zhou, *In vitro* and in vivo screening of herbal extracts against
553 *Streptococcus agalactiae* in Nile tilapia (*Oreochromis niloticus*), Aquaculture 503
554 (2019) 412-421.
555 [25] FAO, Globefish Highlights (A quarterly update on world seafood markets), in:
556 F.a.A.O.o.t.U. Nations (Ed.) 2019.
557 [26] G. Liu, J. Zhu, K. Chen, T. Gao, H. Yao, Y. Liu, W. Zhang, C. Lu, Development of
558 Streptococcus agalactiae vaccines for tilapia, Diseases of Aquatic Organisms 122(2)
559 (2016) 163-170.
560 [27] L. Guangjin, Z. Jielian, C. Kangming, G. Tingting, Y. Huochun, Yongjie Liu, Z.
561 Wei, L. Chengping, Development of *Streptococcus agalactiae* vaccines for tilapia,
562 Diseases of Aquatic Organisms 122(8) (2016) 163.
563 [28] A. Laith, M.A. Ambak, M. Hassan, S.M. Sheriff, M. Nadirah, A.S. Draman, W.
564 Wahab, W.N.W. Ibrahim, A.S. Aznan, A. Jabar, Molecular identification and
565 histopathological study of natural *Streptococcus agalactiae* infection in hybrid tilapia
566 (*Oreochromis niloticus*), 10(1) (2017) 101.
567 [29] H. Van Doan, S.H. Hoseinifar, C. Faggio, C. Chitmanat, N.T. Mai, S. Jaturasitha,
568 E. Ringø, Effects of corncob derived xylooligosaccharide on innate immune response,
569 disease resistance, and growth performance in Nile tilapia (Oreochromis niloticus)
570 fingerlings, Aquaculture 495 (2018) 786-793.

571 [30] AOAC, Official methods of analysis of AOAC International, 16th edition. Volume
572 1, AOAC International, Arlington, 1995.
573 [31] M.J. Quade, J.A. Roth, A rapid, direct assay to measure degranulation of bovine
574 neutrophil primary granules, Veterinary Immunology and Immunopathology 58(3-4)
575 (1997) 239-48.
576 [32] H. Cordero, A. Cuesta, J. Meseguer, M.A. Esteban, Changes in the levels of
577 humoral immune activities after storage of gilthead seabream (*Sparus aurata*) skin
578 mucus, Fish and Shellfish Immunology 58 (2016) 500-507.
579 [33] T. Yano, Assay of hemolytic complement activity, in: J.S. Stolen, T.C. Fletcher,
580 D.P. Anderson, S.C. Hattari, A.F. Rowley (Eds.), Techniques in Fish Immunology, SOS
581 Publications, New Jersey, 1992, pp. 131-141.
582 [34] H. Van Doan, S.H. Hoseinifar, C. Khanongnuch, A. Kanpiengjai, K. Unban, V.
583 Van Kim, S. Srichaiyo, Host-associated probiotics boosted mucosal and serum
584 immunity, disease resistance and growth performance of Nile tilapia (Oreochromis
585 niloticus), Aquaculture 491 (2018) 94-100.
586 [35] B. Wang, Z. Gan, S. Cai, Z. Wang, D. Yu, Z. Lin, Y. Lu, Z. Wu, J. Jian,
587 Comprehensive identification and profiling of Nile tilapia (*Oreochromis niloticus*)
588 microRNAs response to *Streptococcus agalactiae* infection through high-throughput
589 sequencing, Fish & Shellfish Immunology 54 (2016) 93-106.
590 [36] D.F. Amend, Potency testing of fish vaccines., Developments in Biological
591 Standardization 49 (1981) 8.
592 [37] SAS, SAS Institute Inc, SAS Campus Drive, Cary, NC USA 27513-2414, 2003.
593 [38] P.D. Gupta, T.J. Birdi, Development of botanicals to combat antibiotic resistance,
594 Journal of Ayurveda and Integrative Medicine 8(4) (2017) 266-275.

595 [39] J.M.G. Beltrán, C. Espinosa, F.A. Guardiola, M.Á. Esteban, In vitro effects of
596 *Origanum vulgare* leaf extracts on gilthead seabream (*Sparus aurata* L.) leucocytes,
597 cytotoxic, bactericidal and antioxidant activities, Fish & Shellfish Immunology 79
598 (2018) 1-10.
599 [40] N. Van Hai, The use of medicinal plants as immunostimulants in aquaculture: A
600 review, Aquaculture 446(0) (2015) 88-96.
601 [41] Y.-b.-p. Zhang, Y.-b. Zhou, B.-y. Sang, X.-c. Wan, Y.-o. Yang, J.-l. Zhang, T.L.
602 Welker, K. Liu, Effect of dietary Chinese tea on growth performance, disease resistance
603 and muscle fatty acid profile of channel catfish (*Ictalurus punctatus*), Aquacult Int 23(2)
604 (2015) 683-698.
605 [42] F.A. Guardiola, A. Cuesta, M. Arizcun, J. Meseguer, M.A. Esteban, Comparative
606 skin mucus and serum humoral defence mechanisms in the teleost gilthead seabream
607 (*Sparus aurata*), Fish & Shellfish Immunology 36(2) (2014) 545-551.
608 [43] M.A.O. Dawood, S. Koshio, M. El-Sabagh, M.M. Billah, A.I. Zaineldin, M.M.
609 Zayed, A.A.E.-D. Omar, Changes in the growth, humoral and mucosal immune
610 responses following β-glucan and vitamin C administration in red sea bream, *Pagrus*
611 *major*, Aquaculture 470 (2017) 214-222.
612 [44] F.A. Guardiola, C. Porcino, R. Cerezuela, A. Cuesta, C. Faggio, M.A. Esteban,
613 Impact of date palm fruits extracts and probiotic enriched diet on antioxidant status,
614 innate immune response and immune-related gene expression of European seabass
615 (*Dicentrarchus labrax*), Fish Shellfish Immunol 52 (2016) 298-308.
616 [45] S.S. Giri, V. Sukumaran, S.C. Park, Effects of bioactive substance from turmeric
617 on growth, skin mucosal immunity and antioxidant factors in common carp, *Cyprinus*
618 *carpio*, Fish & Shellfish Immunology 92 (2019) 612-620.

619 [46] S.H. Hoseinifar, A. Sohrabi, H. Paknejad, V. Jafari, M. Paolucci, H. Van Doan,
620 Enrichment of common carp (*Cyprinus carpio*) fingerlings diet with *Psidium guajava*:
621 The effects on cutaneous mucosal and serum immune parameters and immune related
622 genes expression, Fish & Shellfish Immunology 86 (2019) 688-694.
623 [47] T.Q. Nhu, B.T. Bich Hang, L.T. Bach, B.T. Buu Hue, J. Quetin-Leclercq, M.-L.
624 Scippo, N.T. Phuong, P. Kestemont, Plant extract-based diets differently modulate
625 immune responses and resistance to bacterial infection in striped catfish
626 (*Pangasianodon hypophthalmus*), Fish & Shellfish Immunology 92 (2019) 913-924.
627 [48] C.M.A. Caipang, Nutritional impacts on fish mucosa: immunostimulants, pre- and
628 probiotics, in: E.P. Benjamin H. Beck (Ed.), Mucosal Health in Aquaculture, Academic
629 Press, London, 2015.
630 [49] D. Gomez, J.O. Sunyer, I. Salinas, The mucosal immune system of fish: the
631 evolution of tolerating commensals while fighting pathogens, Fish & shellfish
632 immunology 35(6) (2013) 1729-1739.
633 [50] I. Salinas, Y.A. Zhang, J.O. Sunyer, Mucosal immunoglobulins and B cells of
634 teleost fish, Developmental and Comparative Immunology 35(12) (2011) 1346-1365.
635 [51] D. Parra, F.E. Reyes-Lopez, L. Tort, Mucosal Immunity and B Cells in Teleosts:
636 Effect of Vaccination and Stress, Frontiers in Immunology 6 (2015) 354.
637 [52] E. Vallejos-Vidal, F. Reyes-López, M. Teles, S. MacKenzie, The response of fish
638 to immunostimulant diets, Fish & Shellfish Immunology 56 (2016) 34-69.
639 [53] D.P. Anderson, Immunostimulants, adjuvants, and vaccine carriers in fish:
640 Applications to aquaculture, Annual Review of Fish Diseases 2(Supplement C) (1992)
641 281-307.

642 [54] R. Amarowicz, F. Shahidi, A rapid chromatographic method for separation of
643 individual catechins from green tea, Food Research International 29(1) (1996) 71-76.
644 [55] M. Donà, I. Dell'Aica, F. Calabrese, R. Benelli, M. Morini, A. Albini, S. Garbisa,
645 Neutrophil Restraint by Green Tea: Inhibition of Inflammation, Associated
646 Angiogenesis, and Pulmonary Fibrosis, 170(8) (2003) 4335-4341.
647 [56] K. Osada, M. Takahashi, S. Hoshina, M. Nakamura, S. Nakamura, M. Sugano, Tea
648 catechins inhibit cholesterol oxidation accompanying oxidation of low density
649 lipoprotein in vitro, Comparative Biochemistry and Physiology Part C: Toxicology &
650 Pharmacology 128(2) (2001) 153-164.
651 [57] M. Toda, S. Okubo, H. Ikigai, T. Suzuki, Y. Suzuki, Y. Hara, T. Shimamura, The
652 Protective Activity of Tea Catechins against Experimental Infection by *Vibrio cholerae*
653 O1, Microbiology and immunology 36(9) (1992) 999-1001.
654 [58] B. Magnadóttir, Innate immunity of fish (overview), Fish & Shellfish Immunology
655 20(2) (2006) 137-151.
656 [59] R. Harikrishnan, C. Balasundaram, M.-S. Heo, Influence of diet enriched with
657 green tea on innate humoral and cellular immune response of kelp grouper (*Epinephelus*
658 *bruneus*) to Vibrio carchariae infection, Fish & Shellfish Immunology 30(3) (2011)
659 972-979.
660 [60] M.C.H. Holland, J.D. Lambris, The complement system in teleosts, Fish &
661 Shellfish Immunology 12(5) (2002) 399-420.
662 [61] H. Boshra, J. Li, J.O. Sunyer, Recent advances on the complement system of
663 teleost fish, Fish & Shellfish Immunology 20(2) (2006) 239-262.
664 [62] A.E. Ellis, Immunity to bacteria in fish, Fish & Shellfish Immunology 9(4) (1999)
665 291-308.

666 [63] R.E. Engstad, B. Robertsen, E. Frivold, Yeast glucan induces increase in lysozyme
667 and complement-mediated haemolytic activity in Atlantic salmon blood, Fish &
668 Shellfish Immunology 2(4) (1992) 287-297.
669 [64] G. Jeney, D.P. Anderson, Glucan injection or bath exposure given alone or in
670 combination with a bacterin enhance the non-specific defence mechanisms in rainbow
671 trout (*Oncorhynchus mykiss*), Aquaculture 116(4) (1993) 315-329.
672 [65] J. Lamas, A.E. Ellis, Atlantic salmon (*Salmo salar*) neutrophil responses to
673 *Aeromonas salmonicida*, Fish & Shellfish Immunology 4(3) (1994) 201-219.
674 [66] A. Rodríguez, M.Á. Esteban, J. Meseguer, Phagocytosis and peroxidase release by
675 seabream (*Sparus aurata* L.) leucocytes in response to yeast cells, The Anatomical
676 Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology 272A(1)
677 (2003) 415-423.
678 [67] S.T. Solem, J.B. Jørgensen, B. Robertsen, Stimulation of respiratory burst and
679 phagocytic activity in Atlantic salmon (*Salmo salar* L.) macrophages by
680 lipopolysaccharide, Fish & Shellfish Immunology 5(7) (1995) 475-491.
681 [68] M. Abdel-Tawwab, M.N. Monier, S.H. Hoseinifar, C. Faggio, Fish response to
682 hypoxia stress: growth, physiological, and immunological biomarkers, (2019) 1-17.
683 [69] V. Aliko, M. Qirjo, E. Sula, V. Morina, C. Faggio, Antioxidant defense system,
684 immune response and erythron profile modulation in gold fish, Carassius auratus, after
685 acute manganese treatment, Fish & Shellfish Immunology 76 (2018) 101-109.
686 [70] T. Miyazaki, A simple method to evaluate respiratory burst activity of blood
687 phagocytes from Japanese flounder, 33(3) (1998) 141-142.
688 [71] T.-H. Lee, F. Qiu, G.R. Waller, C.-H. Chou, Three new flavonol galloylglycosides
689 from leaves of *Acacia confusa*, Journal of Natural Products 63(5) (2000) 710-712.

690 [72] X. Zhang, H. Fan, Q. Zhong, Y.-c. Zhuo, Y. Lin, Z.-z. ZENG, Isolation,
691 identification and pathogenicity of Streptococcus agalactiae from tilapia, 5 (2008) 772-
692 779.
693 [73] M.A. Burgos-Aceves, A. Cohen, Y. Smith, C. Faggio, Estrogen regulation of gene
694 expression in the teleost fish immune system, Fish & Shellfish Immunology 58 (2016)
695 42-49.
696 [74] E.R. Lauriano, S. Pergolizzi, G. Capillo, M. Kuciel, A. Alesci, C. Faggio,
697 Immunohistochemical characterization of Toll-like receptor 2 in gut epithelial cells and
698 macrophages of goldfish *Carassius auratus* fed with a high-cholesterol diet, Fish &
699 Shellfish Immunology 59 (2016) 250-255.
700 [75] R. Harikrishnan, J.-S. Kim, M.-C. Kim, C. Balasundaram, M.-S. Heo, *Prunella*
701 *vulgaris* enhances the non-specific immune response and disease resistance of
702 *Paralichthys olivaceus* against *Uronema marinum*, Aquaculture 318(1–2) (2011) 61-66.
703 [76] V.P. Pereira, F.J. Knor, J.C.R. Vellosa, F.L. Beltrame, Determination of phenolic
704 compounds and antioxidant activity of green, black and white teas of *Camellia sinensis*
705 (L.) Kuntze, Theaceae, Revista Brasileira de Plantas Medicinais 16 (2014) 490-498.
706 [77] M.-K. Lee, H.-W. Kim, S.-H. Lee, Y.J. Kim, G. Asamenew, J. Choi, J.-W. Lee, H.-
707 A. Jung, S.M. Yoo, J.-B. Kim, Characterization of catechins, theaflavins, and flavonols
708 by leaf processing step in green and black teas (*Camellia sinensis*) using UPLC-DAD-
709 QToF/MS, European Food Research and Technology 245(5) (2019) 997-1010.
710 [78] L.-Z. Lin, P. Chen, J.M. Harnly, New Phenolic Components and Chromatographic
711 Profiles of Green and Fermented Teas, Journal of Agricultural and Food Chemistry
712 56(17) (2008) 8130-8140.

713 [79] M. Jeszka-Skowron, A. Zgoła-Grześkowiak, R. Frankowski, *Cistus incanus* a
714 promising herbal tea rich in bioactive compounds: LC–MS/MS determination of
715 catechins, flavonols, phenolic acids and alkaloids—A comparison with *Camellia*
716 *sinensis*, Rooibos and Hoan Ngoc herbal tea, Journal of Food Composition and Analysis
717 74 (2018) 71-81.
718 [80] N. Yahfoufi, N. Alsadi, M. Jambi, C. Matar, The immunomodulatory and anti-
719 inflammatory role of polyphenols, Nutrients 10(11) (2018) 1618.
720 [81] Z.S. Wen, Y.L. Xu, X.T. Zou, Z.R. Xu, Chitosan nanoparticles act as an adjuvant
721 to promote both Th1 and Th2 immune responses induced by ovalbumin in mice, Marine
722 drugs 9(6) (2011) 1038-1055.
723 [82] H.-R. Park, D. Hwang, H.-J. Suh, K.-W. Yu, T.Y. Kim, K.-S. Shin, Antitumor and
724 antimetastatic activities of rhamnogalacturonan-II-type polysaccharide isolated from
725 mature leaves of green tea via activation of macrophages and natural killer cells,
726 International Journal of Biological Macromolecules 99 (2017) 179-186.
727 [83] C.A. Iregui, J. Comas, G.M. Vásquez, N. Verján, Experimental early pathogenesis
728 of *Streptococcus agalactiae* infection in red tilapia *Oreochromis* spp, Journal of Fish
729 Diseases 39(2) (2016) 205-215.
730 [84] M. Abdel-Tawwab, M.H. Ahmad, M.E.A. Seden, S.F.M. Sakr, Use of green tea,
731 *Camellia sinensis* L., in practical diet for growth and protection of Nile tilapia,
732 *Oreochromis niloticus* (L.), against *Aeromonas hydrophila* infection, Journal of the
733 World Aquaculture Society 41(SUPPL. 2) (2010) 203-213.
734 [85] Y. Yoda, Z.-Q. Hu, T. Shimamura, W.-H. Zhao, Different susceptibilities of
735 *Staphylococcus* and Gram-negative rods to epigallocatechin gallate, Journal of Infection
736 and Chemotherapy 10(1) (2004) 55-58.

737 [86] S.H. Hoseinifar, S. Yousefi, G. Capillo, H. Paknejad, M. Khalili, A. Tabarraei, H.
738 Van Doan, N. Spanò, C. Faggio, Mucosal immune parameters, immune and antioxidant
739 defence related genes expression and growth performance of zebrafish (*Danio rerio*) fed
740 on *Gracilaria gracilis* powder, Fish & Shellfish Immunology 83 (2018) 232-237.
741 [87] G. Rashidian, S. Bahrami Gorji, M.N. Farsani, M.D. Prokić, C. Faggio, The oak
742 (*Quercus brantii*) acorn as a growth promotor for rainbow trout (*Oncorhynchus mykiss*):
743 growth performance, body composition, liver enzymes activity and blood biochemical
744 parameters, (2018) 1-11.
745 [88] J. Huang, Y. Zhang, Y. Zhou, Z. Zhang, Z. Xie, J. Zhang, X. Wan, Green Tea
746 Polyphenols Alleviate Obesity in Broiler Chickens through the Regulation of Lipid-
747 Metabolism-Related Genes and Transcription Factor Expression, Journal of Agricultural
748 and Food Chemistry 61(36) (2013) 8565-8572.
749 [89] S. Li, I.M.Y. Tse, E.T.S. Li, Maternal green tea extract supplementation to rats fed
750 a high-fat diet ameliorates insulin resistance in adult male offspring, The Journal of
751 nutritional biochemistry 23(12) (2012) 1655-1660.
752 [90] M.H. Li, D.F. Oberle, P.M. Lucas, Effects of dietary fiber concentrations supplied
753 by corn bran on feed intake, growth, and feed efficiency of channel catfish, North
754 American Journal of Aquaculture 74(2) (2012) 148-153.
755 [91] J.H. Weisburger, F.-L. Chung, Mechanisms of chronic disease causation by
756 nutritional factors and tobacco products and their prevention by tea polyphenols, Food
757 and Chemical Toxicology 40(8) (2002) 1145-1154.
758 [92] W. Yong Feng, Metabolism of green tea catechins: an overview, 7(7) (2006) 755-
759 809.

760 [93] S. Frejnagel, M. Wroblewska, Comparative Effect of Green Tea, Chokeberry and
761 Honeysuckle Polyphenols on Nutrients and Mineral Absorption and Digestibility in
762 Rats, Annals of Nutrition and Metabolism 56(3) (2010) 163-169.
763 [94] S. Klaus, S. Pültz, C. Thöne-Reineke, S. Wolfram, Epigallocatechin gallate
764 attenuates diet-induced obesity in mice by decreasing energy absorption and increasing
765 fat oxidation, International Journal Of Obesity 29 (2005) 615.
766 [95] N. Ota, S. Soga, A. Shimotoyodome, S. Haramizu, M. Inaba, T. Murase, I.
767 Tokimitsu, Effects of Combination of Regular Exercise and Tea Catechins Intake on
768 Energy Expenditure in Humans, Journal of Health Science 51(2) (2005) 233-236.
769 [96] T. Unno, C. Osada, Y. Motoo, Y. Suzuki, M. Kobayashi, A. Nozawa, Dietary Tea
770 Catechins Increase Fecal Energy in Rats, Journal of nutritional science and
771 vitaminology 55(5) (2009) 447-451.
772 [97] A. Shimotoyodome, S. Haramizu, M. Inaba, T. Murase, I. Tokimitsu, Exercise and
773 green tea extract stimulate fat oxidation and prevent obesity in mice, 37(11) (2005)
774 1884-1892.
775 [98] P. Bandyopadhyay, A.K. Ghosh, C. Ghosh, Recent developments on polyphenol–
776 protein interactions: effects on tea and coffee taste, antioxidant properties and the
777 digestive system, Food & function 3(6) (2012) 592-605.
778 [99] Chung, S. and Secombes, C.J., 1988. Analysis of events occurring within teleost
779 macrophages during the respiratory burst. Comparative Biochemistry and Physiology
780 Part B: Comparative Biochemistry, 89(3), pp.539-544.
781 [100] Miandare, H.K., Farvardin, S., Shabani, A., Hoseinifar, S.H. and Ramezanpour,
782 S.S., 2016. The effects of galactooligosaccharide on systemic and mucosal immune

783 response, growth performance and appetite related gene transcript in goldfish
784 (*Carassius auratus* gibelio). Fish & shellfish immunology, 55, pp.479-483.
785 [101] Parry Jr, R.M., Chandan, R.C. and Shahani, K.M., 1965. A rapid and sensitive
786 assay of muramidase. Proceedings of the Society for Experimental Biology and
787 Medicine, 119(2), pp.384-386.
788 [102] Yoshida, T. and Kitao, T., 1991. The opsonic effect of specific immune serum on
789 the phagocytic and chemiluminescent response in rainbow trout, *Oncorhynchus mykiss*
790 phagocytes. Fish Pathology, 26(1), pp.29-33.
791 [103] Secombes, C.J., 1990. Isolation of salmonid macrophages and analysis of their
792 killing activity. Techniques in fish immunology., pp.137-154.

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[1]	10
Vitamin C[2]	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)[3]	4,105

[1]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.

[2]Vitamin C 98% 5 g.

[3]GE = gross energy.

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}).

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 ($\mu g\ mL^{-1}$) = 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).

IW (g) = Initial weight; FW (g) = Final weight; WG (g) – Weight gain; SGR (%) = Specific growth rate; FCR = Feed conversion ratio; SR (%) = Survival rate.

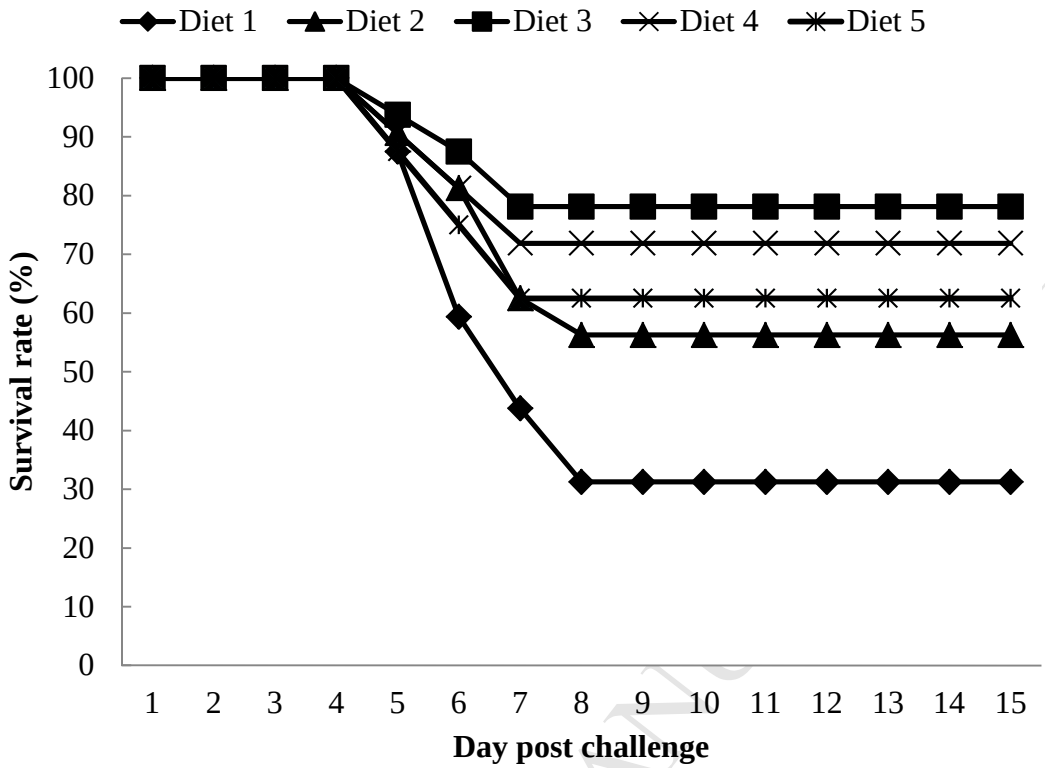

Figure 1. Survival rate of *O. niloticus* fed different experimental diets (*n=30*) containing different levels of Assan tea during 15 days challenge with *S. agalactiae*.

Highlights

- Dietary inclusion of Assam tea (*Camellia sinensis*) extract (ATE) significantly improved growth rate and reduced the feed conversion ratio.
- Significant enhances in the skin mucus and serum immunity were found in Nile tilapia fed ATE.
- Significant reduction in mortality was observed in Nile tilapia fed ATE against *Streptococcus agalactiae*.
- Supplementation of ATE at 2 g kg^{-1} is recommended for better growth performance, immune response and resistance against *S. agalactiae* challenge.