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Dietary inclusion of chestnut (*Castanea sativa*) polyphenols to Nile tilapia reared in biofloc technology: Impacts on growth, immunity, and disease resistance against *Streptococcus agalactiae*

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ABSTRACT

A feeding trial was carried out to examine the effects of adding chestnut (*Castanea sativa*) polyphenols (CSP) on the growth, skin mucus and serum immune parameters of Nile tilapia (*Oreochromis niloticus*). Five experimental diets with inclusion levels of 0, 1, 2, 4, and 8 g kg^{-1} of CSP were fed to Nile tilapia fingerlings (12.77 ± 0.17 g $fish^{-1}$) during an eight-week trial. Fish were analyzed on the fourth and eighth week to determine the influences of CSP on growth, skin mucus, and serum immune parameters. Challenging test versus *Streptococcus agalactiae* was evaluated at the end of the trial. Fish fed with CSP enriched diets displayed a significant increase ($P \leq 0.05$) in growth and a decline in feed conversion ratio ($P \leq 0.05$). Similarly, skin mucus and serum immune parameters were significantly increased ($P \leq 0.05$) in fish fed CSP with respect to the control. The effects were already evident four weeks after the CSP administration. The disease protection test displayed that the fish's survival rate was significantly higher ($P < 0.05$) in CSP diets over the control. The relative percentage of survival (RSP) was 62.5, 75.0, 58.3, and 37.5 in fish fed diets contained 1, 2, 4, and 8 g kg^{-1} CSP, respectively. The best effect on growth, immune response, and disease resistance were shown in Nile tilapia fed with a diet supplementation of 2 g kg^{-1} CSP.

1. Introduction

As the global seafood market is growing, aquaculture plays an increasing role in the globalized economy [1,2], contributing to more than 50% of the global seafood production [3]. The high demand for animal protein, especially derived from seafood, enforces the aquaculture industry toward intensive practices which may cause stressful conditions to fish, and consequently cause the spread of diseases and infections [4]. Bacterial infections are one of the main obstacles in intensive aquaculture, causing considerable economic losses [5]. Antibiotics and chemotherapeutics are the most common additives employed in aquafeeds to boost the fish immune system and improve the disease resistance. However, the extensive use of antibiotics has led to new strains of antimicrobial-resistant bacteria with consequent negative influence on the development of aquaculture [6]. Since the early years of the 20th century, dozens of studies have been carried out to find safe alternatives to antibiotics and synthetic chemicals such as phytochemicals or plant extracts [7]. The inclusion of plant extracts is considered and excellent strategy to improve growth performances, immune response, and disease resistance of farmed fish [8]. The high content of bioactive molecules such as phenolic compounds explains such properties [9–11].

Phenolic compounds or polyphenols are secondary metabolites produced by plants to defend themselves against the attack of bacteria, viruses, and fungi. More than 8000 different polyphenols have been found, but they are considered to be just 10% of the total polyphenols

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present in nature [12]. What polyphenols have in common is an aromatic ring with at least one hydroxyl group. The number of hydroxyl groups is related to their antioxidant power, which resides much of their health-promoting effect [13,14]. The chestnut (*Castanea sativa*) shows a high level of phenolic compounds in all plant components [15,16]. Several studies have shown that phenolic compounds in the chestnut act positively on health, displaying various pharmacological characteristics, such as anti-inflammatory, immune stimulatory, antiviral, and antioxidant properties [17,18], leading to a decrease in the risk of cardiovascular diseases and cancer in humans [19,20]. Moreover, chestnut extracts have been found to contain compounds with inhibitory effects on bacteria [21,22]. The study of polyphenol application in aquaculture is very recent. An *in vitro* study highlighted the stimulating effect on leucocyte activity in rainbow trout, *Oncorhynchus mykiss* [23], while *in vivo* studies point at beneficial effects on both the immune response and growth parameters in common carp, *Cyprinus carpio* [24] and convict cichlid, *Amatitlania nigrofasciata* [25].

Nile tilapia (*Oreochromis niloticus*) is a common warm water species cultured worldwide thanks to its adaptation to intensive farming conditions, stable market price, and large commercial scale, which consequently increased the production fourfold over the last decades [26]. However, intensification of tilapia production exposes the fish to transmittable diseases caused by micro-organisms [27,28]. *Streptococcus spp*. rank among the most frequent pathogens, which causes considerable economic losses in tilapia farming [29]. Although several vaccines have been recently developed and successfully protected against streptococcosis in tilapia, antimicrobial treatment is still commonly applied to deal with the outbreak of diseases in practical farming [30]. Thus, the use of phytochemicals seems to be an attractive strategy to boost the immune system, keeping the use of synthetic chemicals at a low rate [31].

The biofloc technology is considered an alternative approach along with the addition of functional feed-additives in aquafeeds to boost the immune system and deal with the outbreak of infectious diseases in aquaculture [32–38]. The biofloc technology allows stocking fish at high densities and consequently improving productivity [39]. The main advantage of using the biofloc is to maintain the water quality in aquaculture systems using heterotrophic and chemotropic organisms that regulate the quantity of nitrogen and phosphorus substances in the water. The use of biofloc also limits the water renewal and thus reduces the possibility of pathogenic micro-organism introduction [40–44]. Therefore, in the present study, the effects of chestnut, *Castanea sativa* polyphenols on immune response, growth parameters, and disease protection against *S. agalactiae* of Nile tilapia, *Oreochromis niloticus* fingerlings cultured under the indoor biofloc system, have been analyzed.

2. Methodologies

2.1. Diet preparation

One basal diet, only varying in the CSP inclusion rate, was prepared. CSP was added at inclusion rates of 0 g kg^{-1} (CSP0), 1 g kg^{-1} (CSP1), 2 g kg^{-1} (CSP2), 4 g kg^{-1} (CSP4) and 8 g kg^{-1} (CSP8) resulting in to five experimental diets. Diets were manufactured by an extruder machine and stored in the freezer at 4 °C until use. The experimental diets' chemical composition was analyzed according to AOAC [45] and displayed in (Table 1). The polyphenols used in this study were extracted from chesnut by-products (extraction procedure is patent covered), which were kindly provided by SILVATEAM (12080 San Michele Mondovì, Cuneo, Italy). The extracts were in aqueous form with high concentration of tannins [23].

2.2. Experimental setup

Male tilapia fingerlings were obtained from a local farm and given completed feed (CP, 9950) for 60 days. Prior to the feeding trial, fish were acclimatized to the experimental conditions and fed a basal diet twice a day for 14 days. Three hundred fish (mean 12.77 ± 0.17 g) were assembled into 15 tanks, at 20 fish per tank. Five experimental diets were tested according to a completely randomized design. Each diet was administered in to three replicate tanks, and the feeding trial lasted for eight weeks, with the fish being sampled for growth and immune parameters analysis on the mid-term and at the end of the trial. All fish were returned to their tanks after the collection of skin mucus and blood. Challenging test with *S. agalactiae* was performed in all tanks (10 fish $tank^{-1}$). Fish were provided the diets at 3% body weight twice a day. Temperature, pH, and dissolved oxygen were checked daily and maintained within the desirable levels for Nile tilapia throughout the experimental period.

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

Ingredients	Diets (g kg^{-1})				
Fish meal	150	150	150	150	150
Corn meal	200	200	200	200	200
Soybean meal	390	390	390	390	390
Wheat flour	70	70	70	70	70
Rice bran	150	150	150	150	150
Chesnutt polyphenols	0	1	2	4	8
Cellulose	20	19	18	16	12
Soybean oil	5	5	5	5	5
Premix[a]	10	10	10	10	10
Vitamin C[b]	5	5	5	5	5
Proximate composition of the experimental diets (g kg^{-1} dry matter basis)					
Crude protein	306.09	307.89	306.76	308.15	306.99
Crude lipid	61.38	62.01	61.78	62.19	61.88
Fibre	60.61	59.89	59.69	59.57	59.33
Ash	88.17	87.97	87.77	87.38	86.59
Dry matter	908.47	910.78	906.98	907.55	911.23
GE (Cal/g)[c]	4031	4028	4025	4019	4007

[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% 8 g.
[c] GE = Gross energy.

2.3. Immunity analysis

2.3.1. Mucus and serum collection

Three fish per tank were collected for mucus collection. They were anesthetized by clove oil and located in a plastic bag comprising 10 mL of NaCl. Fish were gently massaged, and the solution was transferred into a sterilized tube and centrifuged for 10 min. After that, the supernatant was collected and kept in the freezer until further analysis. Three fish from each tank were used to collect serum as described [46]. Briefly, 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 1500 g for 5 min at 4 °C, and the anticipated serum was gathered using a micro-pipette and stored at - 80 °C for further evaluation.

2.3.2. Blood leucocyte preparation

Leukocytes were isolated according to the protocol described [47]. The protocol design was optimized according to Ref. [46]. Briefly, 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 this, 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 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.

2.3.3. Lysozyme activity

Lysozyme activity was measured following the procedure [48] using undiluted serum and mucus. 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.

2.3.4. Serum and skin mucus peroxidase activities

Peroxidase activity was measured as described by Ref. [49]. The procedure was optimized according to Ref. [46]. 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].

2.3.5. Complement activity (ACH50)

ACH50 was measured by Refs. [50] procedure, and the protocol optimized as described by Ref. [46]. 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.

2.3.6. Phagocytosis measurement

Phagocytosis was measured according to the procedure described [51] and the protocol optimized according to Ref. [46]. Briefly, 200 μL of leucocyte cell suspensions (2×10^6 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) was calculated through the following equations: PI = (Number of phagocytized beads divided by the number of phagocytizing leukocytes) *100.

2.3.7. Respiratory burst

The respiratory burst was measured according to Ref. [52] procedure, and the protcol was optimized according to Ref. [46]. 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. Challenge test

Ten fish per tank were accidently captured for challenging test versus *S. agalactiae* following the published procedure [53], and the relative percentage of survival (RPS) was determined as per Amend [54]. 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 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* [55]. 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 [54]: RPS = (1- % mortality in vaccinated/% mortality in control) × 100.

2.5. Growth parameters

Growth parameters and survival rate were recorded according to Ref. [46] on the fourth and eighth weeks after experimental feeding. Growth parameters were calculated using following equations: Specific growth rate (SGR %) = 100 x (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) x 100.

2.6. Statistical analysis

One way anova (ANOVA) and Duncan's Multiple Range Test were used to measure the significant differences among treatments using SAS Computer software [56]. Treatment effects were considered significant at ($P \leq 0.05$).

3. Results

3.1. Skin mucosal parameters

Skin mucus lysozyme (SMLA) and skin mucus peroxidase (SMPA) activities were higher ($P \leq 0.005$) in fish fed diets containing CSP compared to those fed with the control diet (Table 2). The maximum SMLA and SMPA were detected in fish fed CSP2 followed by CSP4, CSP1, CSP8, and CSP0 ($P < 0.05$; Table 2).

3.2. Serum immunity

Serum lysozyme (SL) quantities varied significantly across treatments (Table 3). Fish fed CSP enriched diets showed improved SL values ($P \leq 0.05$) over the control. The highest value was documented in the CSP2. However, no variations in ($P \geq 0.05$) SL amounts were noted in fish fed CSP1, CSP4, and CSP8) ($P > 0.05$; Table 3).

Table 3 states that the highest serum peroxidase (SP) values were detected in fish fed CSP enriched diets over the control. The maximum amounts were observed in the CSP2, followed by CSP1, CSP4, and CSP8, while the control diet recorded the lowest level ($P \leq 0.05$) of SP. No meaningful variations among CSP1, CSP4, and CSP8 were observed ($P > 0.05$; Table 3).

Regarding complement activity (ACH50), CSP inclusion in the diet generated a significant boost of ACH50 in fish fed CSP2 when compared with the fish fed CSP1, CSP4, and CSP8 ($P < 0.05$). At the end of the trial, the lowest ACH50 value was recorded in fish fed the control diet, and the highest in fish fed CSP2 ($P > 0.05$; Table 3).

For phagocytosis (PI), the CSP inclusion in the diets improved the PI ($P \leq 0.05$) in comparison to the control diet (Table 3). Fish fed CSP2 recorded the maximum PI value over the control and inclusion diets. No differences ($P \geq 0.05$) of PI values were observed in fish fed CSP1, CSP4, and CSP8 after 4 weeks and CSP1 and CSP2 after 8 weeks of feeding.

For respiratory burst activity (RB), CSP inclusion in the diet increased the RB ($P \leq 0.05$; Table 3) with the highest value detected in fish fed CSP2, while fish fed CSP8 and the control group recorded the lowest quantities of RB ($P \leq 0.05$). No notable changes in RB were found across CSP1 and CSP4, and CSP0 and CSP8 ($P > 0.05$; Table 3).

3.3. Challenge test

Fish fed CSP enriched diets showed a greater survival rate ($P \leq 0.05$, Fig. 1) compared to the control. Fish fed CSP2 had the highest survival rate, followed by those fed diets CSP1, CSP4, CSP8, and CSP0, which recorded the lowest survival rate (Fig. 1). The RPS values were 62.5, 75.0, 58.3, and 37.5% in CSP1, CSP2, CSP4 and CSP8, respectively (Fig. 1).

3.4. Growth parameters

Growth parameters are shown in Table 4. Fish fed CSP enriched diets grew significantly more ($P \leq 0.05$) than those fed the control diet with the highest growth rate being observed in fish fed diet CSP2. However, there were no variations in the SGR and WG of fish fed CSP1, CSP4, and CSP8 ($P > 0.05$) were observed. The best FCR was observed in fish fed CSP2 compared to the other groups ($P < 0.05$), while the fish fed control diet attained the worst FCR values. No significant differences were found on the survival rate of fish fed the control tested diet ($P \geq 0.05$) (Table 4).

4. Discussion

One of the major challenges in the modern aquaculture industry is the occurrence of potential disease outbreaks [26,30]. Antibiotics and chemotherapeutics were used intensively in aquaculture to protect bacterial infection and diseases [57–61]. However, antibiotic use causes unfavourable effects on human health and the ecosystem through its accumulation and distribution of antibiotics in the body and water [62,63]. Over the past century, scientists have paid great attention to find eco-friendly and safe alternatives, such as phytogenics, to enhance immune response and prevent disease outbreak [64,65].

Physiologically, skin mucus acts as a first defense of immune barriers against pathogens infection [66–68]. In the present study inclusion of chestnut polyphenols at 2 g kg^{-1} improved SMLA and SMPA enzyme activity. The current findings are consistent with previous research conducted in different fish species, such as in common carp and convict cichlid fed chestnut polyphenol enriched diets [24,25]. The positive effects of chestnut in boosting the immune system could be due to the mucosa-associated lymphoid tissues (MALT), which consist of different mechanisms as SALT, GIALT, and GALT. They contain different types of immune cells, such as T, B cells, and goblet cells that can trigger gastrointestinal immunity [69]. Another reason for chestnut to enhance the immune system is the presence of different bioactive molecules, such as polyphenols and flavonoids with antioxidant properties that stimulate the production of antibodies and antioxidant enzymes [70–72]. It is known that polyphenols are the most abundant chemicals in phytoextracts [73] and are widely recognized as boosters of the innate immune

Table 2
Skin mucus lysozyme and peroxidase activities of *O. niloticus* after 4 and 8 weeks feeding with experimental diets: CSP0 (0 - control), CSP1 (1 g kg^{-1}), CSP2 (2 g kg^{-1}), CSP4 (4 g kg^{-1}), and CSP8 (8 g kg^{-1}). Different letter in a row denote significant difference ($P < 0.05$).

		CSP0	CSP1	CSP2	CSP4	CSP8
4 weeks	**SMLA**	1.69 ± 0.13^{c}	2.66 ± 0.11^{b}	3.31 ± 0.06^{a}	2.68 ± 0.11^{b}	2.34 ± 0.06^{b}
	SMPA	0.08 ± 0.008^{c}	0.12 ± 0.006^{b}	0.21 ± 0.008^{a}	0.13 ± 0.01^{b}	0.09 ± 0.005^{c}
8 weeks	**SMLA**	3.36 ± 0.35^{d}	5.03 ± 0.10^{bc}	6.81 ± 0.18^{a}	5.31 ± 0.17^{b}	4.49 ± 0.12^{c}
	SMPA	0.12 ± 0.008^{c}	0.19 ± 0.005^{b}	0.33 ± 0.02^{a}	0.17 ± 0.008^{bc}	0.13 ± 0.01^{dc}

SMLA (μg mL^{-1}) = Skin mucus lysozyme activity.
SMPA (μg mL^{-1}) = Skin mucus peroxidase activity.

Table 3
Serum immunity of *O. niloticus* after 4 and 8 weeks feeding with experimental diets: CSP0 (0 - control), CSP1 (1 g kg^{-1}), CSP2 (2 g kg^{-1}), CSP4 (4 g kg^{-1}), and CSP8 (8 g kg^{-1}). Different letter in a row denote significant difference ($P < 0.05$).

		CSP0	CSP1	CSP2	CSP4	CSP8
4 weeks	**SL**	5.15 ± 0.22^{c}	6.67 ± 0.29^{b}	8.44 ± 0.17^{a}	6.18 ± 0.03^{b}	6.52 ± 0.33^{b}
	SP	0.13 ± 0.01^{c}	0.20 ± 0.005^{b}	0.28 ± 0.02^{a}	0.19 ± 0.008^{b}	0.18 ± 0.005^{b}
	ACH50	133.09 ± 2.34^{c}	155.86 ± 3.54^{b}	180.23 ± 5.05^{a}	159.47 ± 3.09^{b}	139.43 ± 1.38^{c}
	PI	1.64 ± 0.03^{c}	2.17 ± 0.06^{b}	2.75 ± 0.05^{a}	2.25 ± 0.05^{b}	2.08 ± 0.09^{b}
	RB	0.07 ± 0.008^{c}	0.12 ± 0.01^{b}	0.19 ± 0.008^{a}	0.12 ± 0.008^{b}	0.01 ± 0.005^{bc}
8 weeks	**SL**	7.24 ± 0.24^{c}	9.27 ± 0.28^{b}	11.37 ± 0.51^{a}	9.15 ± 0.35^{b}	8.71 ± 0.30^{b}
	SP	0.21 ± 0.01^{d}	0.33 ± 0.005^{a}	0.44 ± 0.02^{a}	0.29 ± 0.008^{bc}	0.27 ± 0.005^{c}
	ACH50	166.33 ± 2.97^{c}	195.91 ± 6.50^{b}	257.49 ± 5.07^{a}	195.31 ± 7.17^{b}	167.21 ± 15.02^{b}
	PI	2.32 ± 0.07^{d}	3.20 ± 0.11^{ab}	3.52 ± 0.17^{a}	3.00 ± 0.08^{bc}	2.74 ± 0.04^{dc}
	RB	0.17 ± 0.01^{d}	0.24 ± 0.01^{b}	0.34 ± 0.02^{a}	0.22 ± 0.005^{bc}	0.18 ± 0.006^{cd}

SL = Serum lysozyme activity (μg mL^{-1}); SP = Serum peroxidase activity (μg mL^{-1}); ACH50 = Alternative complement activity (units mL^{-1}); PI = Phagocytosis activity (bead $cell^{-1}$); RB = Respiratory burst activity (OD655).

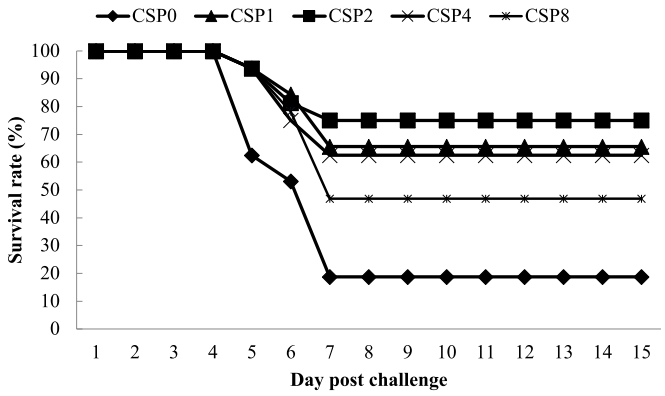

Fig. 1. Survival rate of *O. niloticus* after feeding with experimental diets: CSP0 (0 - control), CSP1 (1 g kg^{-1}), CSP2 (2 g kg^{-1}), CSP4 (4 g kg^{-1}), and CSP8 (8 g kg^{-1}). Different letter in a row denote significant difference ($P < 0.05$).

system [71].

Serum immunity has a significant impact on fish immune response [74]. Lysozyme enzyme and peroxidase activities are responsible for decomposing the pathogens and playing a critical role in oxidative stress [75,76]. In the present study, a fish-fed diet containing 2 g kg^{-1} chestnut increased the lysozyme and peroxidase activities compared to other treatments. These results show a clear parallel with previous researches [24,25], who reported that fish fed polyphonels extract from chestnut by-products significantly enhanced lysozyme and peroxidase activities. It seems strange to note an increase in ROS in the leukocytes of *Oreochromis niloticus* fingerlings regarding respiratory burst activity. An *in vitro* experiment carried out using blood and intestinal leukocytes of *Oncorhynchus mykiss* showed that chestnut polyphenols exerted an immunomodulatory activity increasing both phagocytosis and superoxide production [23]. Likely, oxygen species, including anion superoxide are produced as a consequence of phagocytosis in fish leukocytes [77,78]. In the current research, fish fed 2 g kg^{-1} chestnut enhanced the phagocytic ability. The present result was in agreement with previous studies [58,79]. In terms of the complement activity, the present study found that fish fed 2 g kg^{-1} chestnut improved the complement activity. The present results are clear parallel with previous trials conducted in gilthead seabream, *Sparus aurata* [80,81]; in rainbow trout, *O. mykiss* [82], and tilapia, *O. niloticus* [83]. Thus, chestnut mechanisms to improve fish immunity could be attributed to the presence of bioactive molecules as chlorogenic acid and gallic acid [23,84,85]. Another reason for the improvement of the immune response in fish fed diets contain medical plants could be attributed to activate different immune mechanisms of protection, such as phagocytic, respiratory burst, complement activity, lysozyme activity, and antiprotease activity [86,87].

The incorporation levels of medicinal plants in aquafeeds are controlled by their effect on growth performance and feed efficiency. The polyphenols used in this study were extracted from the by-products of the chestnut supply chain. The extracts are in aqueous form with high concentration of tannins [23]. Although tannins have been long regarded as antinutritional factors that negatively affect growth and feed conversion ratio when administered to farmed animals [88], other studies demonstrate that their inclusion in the diet is accompanied to beneficial effects [89]. Here we show that their administration for eight weeks to juvenile tilapia improved growth performances, but the effect could be seen even after four weeks. This outcome is in agreement with a study carried out on the juvenile carp *Ciprinus carpio*, fed for eight weeks

Table 4
Growth performances and feed utilization (mean ± SE) of *O. niloticus* after 4 and 8 weeks feeding with experimental diets: CSP0 (0 - control), CSP1 (1 g kg^{-1}), CSP2 (2 g kg^{-1}), CSP4 (4 g kg^{-1}), and CSP8 (8 g kg^{-1}). Different letter in a row denote significant difference ($P < 0.05$).

	CSP0	CSP1	CSP2	CSP4	CSP8
IW (g)	12.73 ± 0.11	12.75 ± 0.08	12.78 ± 0.04	12.80 ± 0.13	12.80 ± 0.07
FW (g)					
4 weeks	41.01 ± 0.27^{c}	42.90 ± 0.30^{b}	48.18 ± 0.41^{a}	43.11 ± 0.29^{b}	41.95 ± 0.40^{bc}
8 weeks	95.86 ± 1.88^{c}	102.78 ± 3.55^{b}	117.90 ± 5.06^{a}	103.09 ± 3.33^{b}	95.83 ± 0.46^{c}
WG (g)					
4 weeks	28.28 ± 0.23^{d}	30.15 ± 0.27^{bc}	35.39 ± 0.37^{a}	30.31 ± 0.17^{b}	29.15 ± 0.33^{cd}
8 weeks	83.12 ± 1.89^{c}	90.03 ± 3.50^{b}	105.01 ± 4.99^{a}	90.29 ± 3.25^{b}	83.03 ± 0.44^{c}
SGR (%)					
4 weeks	3.90 ± 0.03^{c}	4.05 ± 0.02^{b}	4.42 ± 0.02^{a}	4.05 ± 0.01^{b}	3.96 ± 0.0^{c}
8 weeks	3.36 ± 0.01^{c}	3.48 ± 0.05^{b}	3.70 ± 0.07^{a}	3.48 ± 0.05^{b}	3.57 ± 0.01^{c}
FCR					
4 weeks	1.18 ± 0.006^{a}	1.12 ± 0.009^{b}	1.02 ± 0.005^{c}	1.13 ± 0.005^{b}	1.16 ± 0.004^{a}
8 weeks	1.37 ± 0.02^{a}	1.29 ± 0.04^{b}	1.17 ± 0.03^{c}	1.27 ± 0.05^{b}	1.36 ± 0.03^{a}
SR (%)					
4 weeks	100	100	100	100	100
8 weeks	93.33 ± 1.44	96.67 ± 1.44	98.33 ± 1.44	96.67 ± 1.44	96.67 ± 1.77

with chestnut tannins [24]. On the contrary, tannin administration to the rainbow trout *Oncorhynchus mykiss* [90], and sea bass *Dicentrarchus labrax* [91], negatively influenced growth parameters and feed conversion ratio. Such a discrepancy may be attributable to the concentration of tannins employed. In the seabass [91] and trout [90], studies the tannin inclusion percentage ranged from 1 to 3% of the diet. In the present study and previous feeding trial carried out on carp [24], the tannin percentage was quite lower, ranging from 0.05 to 0.8%. The existence of beneficial effects provided by polyphenol interactions with animal tissues is sustained by numerous studies [92]. However, the negative effects of polyphenols cannot be neglected. Consequently, caution should be considered when the diet is integrated with polyphenols beyond an upper limit that should be carefully evaluated since it may change with the species, likely a reflection of differences in fish species' ability to metabolize or utilize them. The effects of phytochemicals, and thus polyphenols, on weight gain promotion in animals, may be due to a constellation of factors, such as improving digestibility and availability of nutrients, resulting in an increased feed conversion ratio and a higher protein synthesis [93,94].

Among the occurring diseases in intensive fish farming, bacteria are the most prevalent pathogens in cultured fish [95–97]. *Streptococcus* are common bacterial pathogens causing infectious diseases in farmed tilapia, responsible for high mortality, poor meat quality yield, and consequent severe economic losses [30,98]. Polyphenols have been found to have *in vitro* antimicrobial properties [99]. Their use in aquaculture as alternative to antibiotics is prompted by the hypothetical difficulty of bacteria developing resistance against phytochemicals [100]. The present results indicated that fish treated with chestnut polyphenols showed significant greater survival rates compared to the controls. The present results suggested that CSP able to protect Nile tilapia against *S. agalactiae.* It has been chestnut burs extracts with tannins, mainly ellagitannins and glycoside flavonols, as well as ellagic acid and chestanin able to inhibit *Alternaria alternate, Fusarium solani,* and *Botrytis cinereal* [101]. Quave, Lyles, Kavanaugh, Nelson, Parlet, Crosby, Heilmann and Horswill [102] indicated that chesnut leaf extracts rich in ursene and oleanene derivatives could block *Staphylococcus aureus* virulence and pathogenesis without detectable resistance. However, further investigations are required to clearify the effects of chestnut polyphenols in inhibition bacteria infection in fish.

In conclusions, chestnut polyphenols are promising phytochemicals and important source of therapy in fish culture because of their growth promotion, immunostimulant, antioxidant and antimicrobiological properties. They may represent a valid and environmentally safe alternative to antibiotics in aquaculture.

Ethical approval

The research was conducted in compliance with Chiang Mai University recommendations against the use of animals for research purposes.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

CRediT authorship contribution statement

Hien Van Doan: Conceptualization, Funding acquisition, Investigation, Methodology. **Seyed Hossein Hoseinifar:** Writing - original draft. **Tran Quang Hung:** Data curation. **Chompunut Lumsangkul:** Formal analysis. **Sanchai Jaturasitha:** Software. **Ehab El-Haroun:** Writing - review & editing. **Marina Paolucci:** Resources, Writing - review & editing.

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