Full length article

Impacts of pineapple peel powder on growth performance, innate immunity, disease resistance, and relative immune gene expression of Nile tilapia, *Oreochromis niloticus*

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ABSTRACT

An 8-week growth trial was conducted to examine the efficacy of pineapple peel powder (PAPP) on growth rate and immunity of Nile tilapia, *O. niloticus*. Three hundred Nile tilapia (20.91 ± 0.11 g) were fed five diets containing different levels of PAPP at 0, 10, 20, 30 and 40 g kg^{-1} PAPP, respectively. After four and eight weeks of the feeding trial, growth rates, and immune responses were tested. A challenge test using *Streptococcus agalactiae* and relative immune gene expression were performed after eight weeks of PAPP feeding. It was found that skin mucus and serum lysozyme, skin mucus and serum peroxidase, alternative complement, phagocytosis, and respiratory burst activities were significantly increased with the addition of PAPP. The maximum ($P \leq 0.05$) innate immune values were noted in fish fed 10 g kg^{-1} PAPP. Similarly, the up-regulation of *IL1, IL8,* and *LBP* gene expressions were also detected in fish fed PAPP diets, with the maximum value was found in 10 g kg^{-1} PAPP fed fish. The relative percentage of survival (RPS) of *Oreochromis niloticus* after the challenge test were (56.00%, 72.00%, 60.00%, and 44.00%) for the 5, 10, 20 and 40 g kg^{-1} PAPP diets, respectively. Fish fed the 10 g kg^{-1} PAPP supplemented diet achieved the highest ($P < 0.05$) survival rate against *S. agalactiae*. Growth and feed efficiency were outstandingly ($P < 0.05$) enhanced in the PAPP groups. In conclusion, PAPP can be potentially used as a feed additive in Nile tilapia culture under Biofloc system.

1. Introduction

Aquaculture represents a major role in securing the increasing demands for animal protein for human consumption [1,2]. Today, aquaculture represents approximately fifty percent of globalized fishery production and has been accountable for the prominent growth of protein sources [3]. Tilapia is commonly cultivated fish worldwide; due to its rapid growth, acclimation, and high returns [4]. Nevertheless, the intensification and enlargement of fish farming have resulted in a negative effect on water quality, as well as an increased risk of pathogen infection and disease outbreak [5], leading to a reduction of the survival rate of farmed fish [6]. Excessive use of antibiotics and

chemotherapeutics compounds have resulted in the development of antimicrobial-resistant bacteria and accumulated antibiotic residues in farm water and fish tissues, that causes environmental intimidation and alters the microflora arrangement [7,8]. Therefore, searching for safe compounds to manage pathogen microbe infection in aquaculture is needed.

Feed costs represent approximately 80% of total operating costs in aquaculture [9]. To maximize net profits, and enhance growth and immune system, we must minimize feed costs and control disease infection. Through the use of agro-industrial by-products, we can produce high-quality aquafeed and reduce waste output [10], thereby reaching a "zero waste" outcome in both livestock and aquaculture diets [11,12].

Among them, the use of pineapple peel, explored herein, is a viable choice. The global production of pineapple increased to 27 million metric tons in 2017 [13]. Pineapple peel accounts for 29 to 42% of the total weight of the fruit [13], yet is only seldom used for fertilization or compost, or it is simply discarded [14]. However, pineapple by-products contain insoluble fibres, sugars, pectin, proteins, micronutrients, and polyphenols ingredients [15–17]; and are an abundant source of bromelain, a costly blend of proteolytic enzymes with various biological functions in the pharmaceutical, food, and cosmetic sectors [18]. There are also extensive therapeutic benefits, due to the cytotoxic, antidiabetic, antihyperlipidemic, and antioxidant properties present [19–21]. Therefore, consideration as a functional feed additive in aquaculture is appropriate.

Biofloc is another aspect of environmentally friendly and sustainable aquaculture [22]. Biofloc technology is a protocol that combines microorganisms and other organic materials be accessible as fish feed [22–24]. The addition of feed additives to the biofloc reproduce a microorganism community in both the water and fish gut capable of restraining pathogenic strains [25–28]. To date, we are not aware of any past research on the inclusion of pineapple peel powder (PAPP) in Nile tilapia diet cultures within biofloc system. The objectives of the current research, therefore, were to examine the impacts of PAPP on immune parameters and gene expressions; as well as challenging the fish with *Streptococcus agalactiae*, and the growth of *Oreochromis niloticus* in the biofloc system.

2. Materials and methods

2.1. Pineapple peel powder preparation

Fresh pineapple fruit was obtained from a private farm at Chiang Mai, Thailand. Pineapple peel was collected dried at 60 °C for two days. After the dried process, peels were ground using a hammermill, filtered with a sieve (100-mesh) to obtain a powdered form of pineapple peel powder (PAPP), then stored for future use.

2.2. Diets preparation

Five experimental diets (isonitrogenous and isocaloric) were prepared according to the nutrient requirements of this species NRC [29]. The diets (1–5) were supplemented with 0, 10, 20, 30, and 40 g PAPP kg^{-1}, respectively (Table 1). Diets was made following Doan, Hoseinifar, Jaturasitha, Dawood and Harikrishnan [30], then stored in plastic bags at 4°C for future use.

2.3. Fish culture setup

Nile tilapia were collected from the Chiang Mai Pathana Farm Co., Ltd, Chiang Mai, Thailand. To minimize stress, experimental fish were stocked in a cage after arrival and fed commercial feed for two months. At the beginning of the trial, 20 fish were selected randomly for a health examination. Three hundred fingerlings (20.91 ± 0.11 g) were randomly dispersed into 15 tanks (20 fish tank^{-1}). The experimental fish in each replicate were hand-fed their respective diets *ad libitum*, at 9.00 a.m. and 5.00 p.m. daily for eight weeks. Starch was used with a C:N ratio of 15:1 to maintain flocs volume [31]. Water quality criteria were monitored daily, and optimum ranges for Nile tilapia were recorded. The records were 27.5 ± 0.8 °C for water temperature, 7.79 ± 0.15 for pH, and 5 mg L^{-1} for DO. Total ammonia-nitrogen (TAN), was 0.02 mg L^{-1} throughout the experiment. Biofloc volume (BFV) was determined, according to Avnimelech and Kochba [31].

2.4. Growth parameters

The fish fasted for 24 h and bulk weight at weeks 4 and 8 post-feeding were assessed according to our previous study [32].

Table 1
The formulation and proximate compositions of experimental diet (g kg^{-1}).

Ingredients	Diets (g kg^{-1})				
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Fish meal	150	150	150	150	150
Corn meal	200	200	200	200	200
Soybean meal	390	390	390	390	378
Wheat flour	70	70	70	70	70
Rice bran	150	150	150	145	142
PPP[a]	0	5	10	20	40
Cellulose	20	15	10	5	0
Soybean oil	5	5	5	5	5
Premix[b]	10	10	10	10	10
Vitamin C[c]	5	5	5	5	5
Proximate composition of the experimental diets (g kg^{-1} dry matter basis)					
Crude protein	302.28	302.62	302.95	303.21	303.32
Crude lipid	61.38	61.44	61.50	60.94	59.15
Fibre	60.61	63.50	66.39	68.50	70.07
Ash	88.17	87.41	86.65	85.44	83.34
Dry matter	991.49	991.51	991.50	991.56	991.64
GE (cal/g)[4]	4,009	4,002	3,979	3,946	3,878

[a] PPP = Pineapple peel powder.
[b] 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.
[c] Vitamin C 98% 5 g.

Parameters of growth performance and feed utilization were calculated based on the following formulae:

Weight gain (g) = $W_2 - W_1$;

Specific growth rate (SGR; %g/day) = 100 [Ln W_2 (g) – Ln W_1 (g)]/T; where W_2 is final weight (g), W_1 is initial weight (g), and T is the experimental period (day);

Feed intake (g feed/fish) = the summation of a diet offered to fish throughout the experiment/fish number;

Feed conversion ratio (FCR) = feed intake/weight gain;

Fish survival (%) = 100 (fish number at final/fish number at initial).

2.5. Samples collection and immunological assays

2.5.1. Skin mucus
Mucus samples were collected from each treatment (three fish per group). Fish were anesthetized with clove oil and smoothly massaged in a bag containing 50 mM NaCl. Subsequently, a sterile tube was used to centrifuge the solution at 1500 g at 4 °C for ten minutes. Afterward, supernatant (500 µL) was collected and kept in a freezer for further analysis.

2.5.2. Serum collection
Clove oil was used to sedate the fish, and samples of blood were collected from the caudal vein using a sterile syringe (1 mL) as described by Van Doan, Hoseinifar, Chitmanat, Jaturasitha, Paolucci, Ashouri, Dawood and Esteban [32]. 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 blood samples were then led to clot at room temperature for one hour, and stored in a refrigerator (4 °C) for four hours. After that, 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.

2.5.3. Leukocytes isolation

One mL of blood was collected to obtain the leukocytes cells needed to measure the phagocytic and respiratory burst activities, as described by Chung and Secombes [33]. Briefly, one milliliter of blood was withdrawn from each fish, at a rate of three fish per replication, and then transferred into 15 mL tubes containing 2 mL of RPMI 1640 (Gibthai). This mixture was then carefully inserted into 15 mL tubes, containing 3 mL of Histopaque (Sigma, St. Louis, MO, USA). These tubes were then centrifuged at 400 g for 30 minutes at room temperature. Upon completion, a buffy coat of leucocytes cells drifted to the top of the Histopaque was carefully collected using a Pasteur pipette, and released into sanitized 15 mL tubes. After which, 6 mL of phosphate buffer solution (PBS: Sigma-Aldrich, USA) was added to each tube and gently aspirated. The cells in these tubes were washed twice by centrifugation at 250g for ten minutes at room temperature to remove any residual Histopaque. The cells obtained were then re-suspended in the PBS and adjusted to the numbers of cells required to evaluate phagocytic and respiratory burst activities.

2.5.4. Activities of lysozyme in the serum and skin mucus

Lysozyme activity was measured according to Parry, Chandan and Shahani [34] with slight modification [35]. Briefly, 25 µL of undiluted serum and 100 µL of skin mucus from each fish was loaded into 96-well plates in triplication. *Micrococcus lysodeikticus* (175 µL, 0.3 mg mL^{-1} in 0.1 M citrate phosphate buffer, pH 5.8) was then added to each well. The contents were rapidly mixed, and any changes in turbidity were measured every 30 seconds, for five minutes, at 540 nm, 25 °C, via a microplate reader. 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.

2.5.5. Peroxidase, ACH50, phagocytic, and respiratory activity

The peroxidase activity in serum and skin mucus were measured according to the protocol established by Cordero, Cuesta, Meseguer and Esteban [36]. Briefly, 5 µL of undiluted serum or skin mucus from each fish was placed in 96-flat-bottomed-well plates, in triplicate. Then, 45 µl of *Hank's Balanced Salt Solution* (without Ca^{+2} or Mg^{+2}) was added to each well. Afterward, 100 µL of solution (40 ml of distilled water + 10 µL of H$_2$O$_2$, 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, after 30–60 seconds, a 50 µL solution of 2M H$_2$SO$_4$ was 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 through the following equation: Peroxidase activity = [absorbance of the sample] – [absorbance of blank (containing all solution without serum or mucus sample)].

ACH50 was measured according to Yano [37]. Briefly, rabbit red blood cells (R-RBC) were washed twice with PBS by centrifugation at 3000 rpm, with a 0.01M ethylene glycol tetra-acetic acid--magnesium-gelatin veronal buffer (0.01M – EGTA-Mg-GVB). The R-RBC concentration was adjusted to 2 × 10^8 cells mL^{-1} in the 0.01M – EGTA-Mg-GVB buffer. Then, 100 µL of the R-RBC suspension was lysed with 3.4 mL of distilled water. The hemolysate absorbance was measured at 414 nm *vs.* distilled water as a blank, and 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 placed 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 hours with occasional shaking. After incubation, 3.15 mL of cold saline solution (0.85% NaCl) was placed into each tube to stop the reaction. The tube was

centrifuged at 1600 g for 5 minutes. 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.

Phagocytic activity was determined using the protocol of Yoshida and Kitao [38], with slight modification [39]. Briefly, 200 µL 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 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 hours. 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 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.

Respiratory burst activity measured as described by Secombes [40] with minor changes [39]. Briefly, 175 µL PBS cell suspension at a concentration of 6 × 10^6 cells mL^{-1} was 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 two hours 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 five minutes 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 thirty minutes at room temperature. Then, 125 µL of 2N 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 2N KOH and 150 µL with no leucocytes)].

2.6. Gene expression analysis

Relative gene expression was analysed in three fish per treatment using quantitative real-time PCR (qRT-PCR). Total RNA was extracted from 0.5 g of the Nile tilapia's liver samples using TRIzol Reagent (Life Technologies). The quantified and purity of the RNA were assessed by spectrophotometer (NanoDrop one, Thermo Scientific, Wilmington, USA) at 260:280 nm. The total RNA, 8 µL (concentration of 1 µg/µL), were reverse-transcribed to generate first-strand Complementary DNA (cDNA) using Master mix 1 (1 µL of RQ1 DNase 10x reaction Buffer, 1 µL of RQ1 RNAse free DNase, and 1 µL of RQ1 DNase stop solution - Invitrogen), then Master mix 2 [(1 µL of Oligo dT and 1 µL of DNTP's (Deoxynucleotide Mix - Aldrich Sigma)), and finally with Master mix 3 [(4 µL of buffer 5x (5x SSI V Buffer), 1 µL of DTT, 1 µL of DEPC water, and 1 µL of SuperScript IV (SuperScript® IV Reverse Transcriptase) – Invitrogen)]. Candidate genes (Table 2) were chosen for qRT-PCR analysis. For normalizing the expression of the selected genes, 18S rRNA was used as the reference gene. The qRT-PCR was performed in triplicate using 100 ng of cDNA, 300 mM of primers, and iTaq Universal

Table 2
Sequences of primer using in quantitative real-time PCR.

Primer name	Primer sequence (5′-3′)	Target gene	Tm (°C)	Product size (bp)
18S rRNA –F	GTGCATGGCCGTTCTTAGTT	18S rRNA	60	150
18S rRNA -R	CTCAATCTCGTGTGGCTGAA		60	
IL1-F	GTCTGTCAAGGATAAGCGCTG	IL-1	59	200
IL1-R	ACTCTGGAGCTGGATGTTGA		58	
IL8-F	CTGTGAAGGCATGGGTGTG	IL-8	59	196
IL8-R	GATCACTTTCTTCACCCAGGG		58	
LBP-F	ACCAGAAACTGCGAGAAGGA	LBP	59	200
LBP-R	GATTGGTGGTCGGAGGTTTG		59	

SYBR Green supermix (2X) on a CFX Connect™ Real-Time PCR System (BIO-RAD, USA). Thermal cycling conditions were 95 °C for 15 sec (denaturation), 60 °C for 60 sec (annealing), and 95 °C for 15 sec (melt-curve analysis). The relative expression of the different genes was calculated according to the $2^{-\Delta\Delta Ct}$ method [41].

2.7. Challenge test

The *S. agalactiae* was isolated from diseased tilapia in Northern Thailand and identified, then characterized by Gram staining and biochemical test. Detailed preparation of *S. agalactiae* was described in the previous study of Van Doan, Hoseinifar, Khanongnuch, Kanpiengjai, Unban, Van Kim and Srichaiyo [42]. Briefly, *S. agalactiae* was cultured in Tryptic Soy Broth and incubated at 30 °C for 24 hours in a rotation shaker at a speed of 110 rpm. A 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 hours. The sub-cultures were raised in duplicate under similar conditions for the experiment. Growth was evaluated through optical density of 560 nm (0.75% NaCl was used to adjust bacterium concentration). The plates were counted in the Tryptic Soy Agar, and the calibration curves were calculated by measuring the OD of each consecutive one-half dilution series, in triplicate, 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).

Ten fish were randomly selected from each tank to participate in the challenge test. After setup, fish were injected intraperitoneally with 0.1 mL of 0.85% normal saline solution containing 10^7 CFU mL^{-1} of *S. agalactiae* as described in Wang, Gan, Cai, Wang, Yu, Lin, Lu, Wu and Jian [43]. Fish were monitored for disease symptoms, and dead fish

were removed daily for two weeks. The relative percentage of survival (RPS) was calculated for each treatment through the following equation Amend [44]:

RPS = (1- % mortality in vaccinated/% mortality in control) × 100.

2.8. Data analysis

Data were processed via the SAS software program SAS [45], using one-way variance analysis (ANOVA) and Duncan's multiple range test. Different mean values ($P < 0.05$) and other observations are displayed as mean ± SD.

3. Results

The inclusion of PAPP over eight weeks resulted in ($P < 0.05$) an enhancement of growth variables (Table 3), most notably in the 10 g kg^{-1} PAPP group. No significant ($P > 0.05$) differences were observed between the 5, 20, and 40 g kg^{-1} PAPP groups, nor were there any statistical ($P > 0.05$) variations in survival among the PAPP groups. The PAPP-fed fish also showed greater ($P < 0.05$) FCR values, the highest being attributed to the 10 g kg^{-1} PAPP group (Table 3).

Table 4 shows the effect of the experimental diets on skin mucus lysozyme and peroxidase activities. The findings showed that dietary incorporation of PAPP resulted in increased lysozyme and peroxidase activities ($P \leq 0.05$) after 4 and 8 weeks of feeding. The maximum rates of the skin mucus immune values were observed in the 10 g kg^{-1} PAPP group ($P \leq 0.05$); whereas fish fed a diet without the inclusion of PAPP recorded the lowest levels of lysozyme and peroxidase activities.

The impacts of PAPP on immune parameters are shown in Table 5. The results indicate that the addition of 10 g kg^{-1} of PAPP produced the highest levels of ($P \leq 0.05$) of serum lysozyme, peroxidase, alternative complement, phagocytosis, and respiratory burst activities *vs.* the other treatments (Table 5). Fish fed the 0 g kg^{-1} and 40 g kg^{-1} PAPP diets recorded the lowest values of these parameters ($P \leq 0.05$), though their differences were not statistically significant ($P \geq 0.05$). Likewise, no significant variations were noticed between the 5 g kg^{-1} and 20 g kg^{-1} PAPP groups ($P \geq 0.05$). The dietary inclusion of 10 g kg^{-1} of PAPP up-regulated the IL1, IL8, and LBP gene expressions *vs.* the control and other supplemented groups (Fig. 1).

The survival rates in all PAPP diets increased ($P < 0.05$) to 63.33% (Diet 2), 76.68% (Diet 3), 66.67% (Diet 4), and 53.33% (Diet 5) *vs.* the control (16.67%), as shown in Fig. 2. The relative percent survival (RPS) of tilapia in Diets 2–5 were 56.00%, 72.00%, 60.00%, and 44.00%, respectively (Fig. 2). Fish fed 10 g kg^{-1} PAPP produced ($P < 0.05$) the highest RPS and highest resistance towards *S. agalactiae* (Fig. 2).

Table 3
Growth performances and feed utilization of the Nile tilapia fed different levels of dietary PAPP: Diet 1 (control), Diet 2 (5 g kg^{-1} PAPP), Diet 3 (10 g kg^{-1} PAPP), Diet 4 (20 g kg^{-1} PAPP), and Diet 5 (40 g kg^{-1} PAPP). Data assigned with different letters denote significant difference in a row (P < 0.05).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
IW (g)	20.85 ± 0.13	20.93 ± 0.12	20.90 ± 0.13	20.87 ± 0.12	20.98 ± 0.03
FW (g)					
4 weeks	61.68 ± 0.62[c]	67.90 ± 0.30[b]	71.51 ± 0.66[a]	68.11 ± 0.29[b]	66.95 ± 0.40[b]
8 weeks	125.08 ± 4.51[c]	135.68 ± 4.77[b]	151.74 ± 4.40[a]	136.66 ± 0.97[b]	131.84 ± 2.84[bc]
WG (g)					
4 weeks	40.83 ± 0.57[c]	46.97 ± 0.35[b]	50.61 ± 0.61[a]	47.24 ± 0.35[b]	45.97 ± 0.38[b]
8 weeks	104.23 ± 4.51[c]	114.75 ± 4.79[b]	130.84 ± 4.45[a]	115.79 ± 0.95[b]	110.86 ± 2.84[bc]
SGR					
4 weeks	3.62 ± 0.03[c]	3.92 ± 0.02[b]	4.10 ± 0.02[a]	3.94 ± 0.02[b]	3.87 ± 0.02[b]
8 weeks	2.99 ± 0.02[c]	3.11 ± 0.06[b]	3.30 ± 0.05[a]	3.13 ± 0.01[b]	3.06 ± 0.04[bc]
FCR					
4 weeks	1.19 ± 0.01[a]	1.08 ± 0.005[b]	1.04 ± 0.008[c]	1.08 ± 0.003[b]	1.10 ± 0.003[b]
8 weeks	1.29 ± 0.01[a]	1.25 ± 0.01[b]	1.21 ± 0.01[c]	1.26 ± 0.002[b]	1.26 ± 0.01[b]
SR (%)					
4 weeks	100	100	100	100	100
8 weeks	95 ± 0.01	96.68 ± 1.44	98.33 ± 1.44	96.68 ± 1.44	96.68 ± 1.77

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

Table 4

Skin mucus lysozyme and peroxidase activities of *O. niloticus* after 4 and 8 weeks feeding with experimental diets: Diet 1 (0 - control), Diet 2 (5 g kg^{-1} PAPP), Diet 3 (10 g kg^{-1} PAPP), Diet 4 (20 g kg^{-1} PAPP), and Diet 5 (40 g kg^{-1} PAPP). Different letters in a row denote significant difference ($P < 0.05$).

		Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
4 weeks	SMLA	1.38 ± 0.08[c]	1.93 ± 0.04[b]	2.29 ± 0.07[a]	2.03 ± 0.03[b]	1.50 ± 0.07[c]
	SMPA	0.06 ± 0.003[c]	0.11 ± 0.005[b]	0.17 ± 0.01[a]	0.11 ± 0.003[b]	0.08 ± 0.005[c]
8 weeks	SMLA	2.18 ± 0.06[c]	2.96 ± 0.07[b]	3.48 ± 0.14[a]	2.78 ± 0.05[b]	2.32 ± 0.11[c]
	SMPA	0.12 ± 0.005[c]	0.18 ± 0.005[b]	0.24 ± 0.02[a]	0.16 ± 0.008[b]	0.12 ± 0.003[c]

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

Table 5

Serum immunity of *O. niloticus* after 4 and 8 weeks feeding with experimental diets: Diet 1 (0 - control), Diet 2 (5 g kg^{-1} PAPP), Diet 3 (10 g kg^{-1} PAPP), Diet 4 (20 g kg^{-1} PAPP), and Diet 5 (40 g kg^{-1} PAPP). Different letters in a row denote significant difference ($P < 0.05$).

		Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
4 weeks	SL	5.04 ± 0.14[c]	6.20 ± 0.07[b]	7.44 ± 0.17[a]	6.08 ± 0.03[b]	5.13 ± 0.06[c]
	SP	0.12 ± 0.008[c]	0.19 ± 0.005[b]	0.25 ± 0.02[a]	0.19 ± 0.003[b]	0.16 ± 0.006[bc]
	ACH50	158.27 ± 4.84[c]	186.19 ± 3.32[b]	229.70 ± 12.10[a]	189.47 ± 3.09[b]	172.77 ± 5.5[bc]
	PI	1.55 ± 0.05[c]	2.04 ± 0.03[b]	2.45 ± 0.06[a]	2.08 ± 0.03[b]	1.71 ± 0.07[c]
	RB	0.11 ± 0.008[c]	0.17 ± 0.01[b]	0.22 ± 0.009[a]	0.16 ± 0.008[b]	0.10 ± 0.005[c]
8 weeks	SL	6.81 ± 0.28[c]	8.20 ± 0.08[b]	9.37 ± 0.18[a]	8.14 ± 0.06[b]	7.37 ± 0.08[c]
	SP	0.19 ± 0.008[c]	0.25 ± 0.008[b]	0.31 ± 0.01[a]	0.26 ± 0.01[b]	0.21 ± 0.008[c]
	ACH50	189.21 ± 4.42[c]	223.57 ± 8.62[b]	295.49 ± 6.55[a]	228.98 ± 5.91[b]	187.21 ± 3.00[c]
	PI	2.12 ± 0.03[c]	2.41 ± 0.07[b]	2.81 ± 0.06[a]	2.34 ± 0.06[b]	2.11 ± 0.02[c]
	RB	0.15 ± 0.008[c]	0.20 ± 0.005[b]	0.27 ± 0.008[a]	0.21 ± 0.01[b]	0.16 ± 0.005[c]

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).
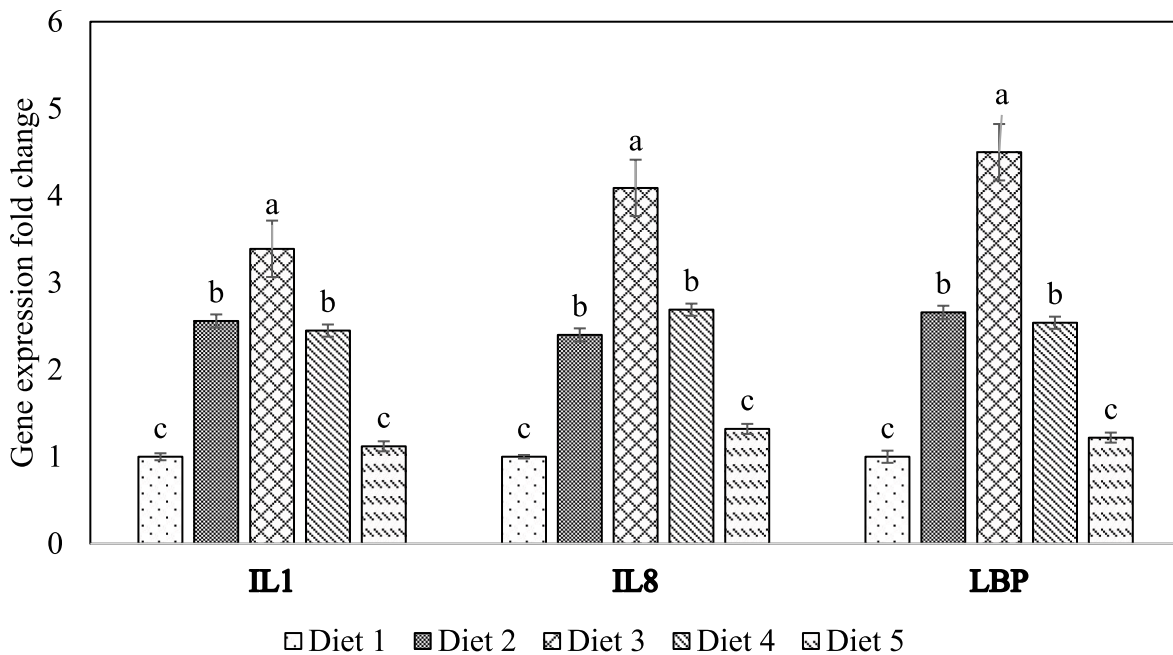

Fig. 1. Effect of pineapple peel powder (PAPP) on genes expression; *IL1*, *IL-8*, and *LBP* of Nile tilapia after feeding with experimental diets: Diet 1 (0 - control), Diet 2 (5 g kg^{-1} PAPP), Diet 3 (10 g kg^{-1} PAPP), Diet 4 (20 g kg^{-1} PAPP), and Diet 5 (40 g kg^{-1} PAPP). Different superscript letters indicate significant differences between groups ($p < 0.05$).

4. Discussion

Utilization of agricultural by- and co-products, such as fruits and vegetables in animal feed not only reduces the environmental issues but also increases the farmers' income [46]. This practice complies with the recent legislation, which requires the food industry to find successful ways to use these residues [47,48]. Therefore, the present study was carried out to evaluate the effects of pineapple peel powder on growth performance, immune response, disease resistance, and relative immune gene expression of Nile tilapia.

The results indicate that growth response improved remarkably ($P \leq 0.05$) in the PAPP groups, particularly with the fish fed the 10 g kg^{-1} PAPP treatment. These results parallel those of Yuangsoi, Klahan, Charoenwattanasak and Lin [49], who found that Nile tilapia fed diets containing pineapple waste extract resulted in the optimal nutrient digestibility and growth response (p ≤ 0.05). Wiszniewski, Jarmołowicz, Hassaan, Mohammady, Soaudy, Łuczyńska, Tońska, Terech-Majewska, Ostaszewska and Kamaszewski [50] found that Sterlet fed diets
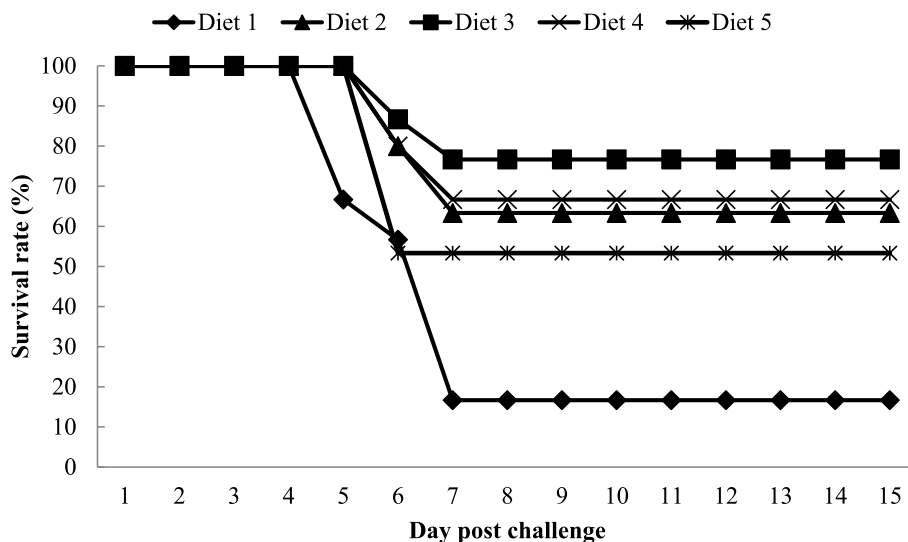
Fig. 2. Survival rate of *O. niloticus* after feeding with experimental diets: Diet 1 (0 - control), Diet 2 (5 g kg^{-1} PAPP), Diet 3 (10 g kg^{-1} PAPP), Diet 4 (20 g kg^{-1} PAPP), and Diet 5 (40 g kg^{-1} PAPP).

containing 10 and 20 g kg^{-1} bromelain produced the best growth rate and feed utilization. Furthermore, Deka, Sahu and Jain [51] found that the specific growth rates and protein efficiency ratios of *Labeo rohita* increased significantly in diets containing 25% pineapple waste. In the same context, Rahman and Yang [52] found that growth performance, weight gain, and feed conversion ratio increased significantly in chicken fed diets supplemented with leaf powder (PLP). The authors explained that the bioactive compounds present in PLP modified the gut microbiome, leading to enhanced nutrient digestion and assimilation. The present findings may be attributed to several mechanisms: i) The acceptable flavour and palatability of pineapple [53]; ii) Pineapple peel contains high amount of phenolic compounds, such as gallic acid and ferulic acid, which can improve growth performance of fish and livestock [54–57]; iii) Bromelain supplementation significantly improved villus length and mucosal folds, boosted the enzymatic activity of lipase and pepsin, and enhanced gut tissue and intestinal absorptive cells, which consequently improved nutrients absorption and digestion [50, 58,59]; iv) Pineapple chemical nutrients as carbon and other elements act as enlargement medium for the lactobacillus bacteria [60,61]. Interestingly, significantly lower growth performances were found in fish fed high dose of PAPP. Similar results were observed in rabbits fed pineapple peel [62] and pig fed pineapple by-products [63]. This may be attributable to the high fibre contents in pineapple by-products. Fibre can reduce the digestion of other dietary components, including proteins and soluble carbohydrates, that lead to the decreased digestibility and absorption of nutrients and energy [63,64]. These effects are linked to the soluble nutrient coating ability of fiber and to the physiological changes triggered by fibre diets that influence the movement and the solubility of the gut digesta, limiting the penetration of endogenous enzymes in nutrients [65,66].

Improving the immune systems (innate and adaptive immune responses) has been a target goal for feed additive supplementation in aquaculture [67]. The present investigation found that dietary inclusion of pineapple peel powder (PAPP) significant enhanced skin mucus lysozyme and peroxidase activities of Nile tilapia, with the highest values observed in fish fed 10 g kg^{-1} PAPP. As far as we know, no current studies have evaluated the effects of PAPP on fish skin mucus immune response. However, research in similar studies has supported our results; such as the significantly enhanced mucosal immunity found in common carp, *Cyprinus carpio* fed orange peels derived pectin [68], convict cichlid, *Amatitlania nigrofasciata* fed polyphenols from agricultural by-products [69], and in rainbow trout, *Oncorhynchus mykiss* fed olive, *Olea europea* waste [70]. For serum immunity, the present study found that the dietary incorporation of PAPP significantly enhanced serum lysozyme, serum peroxidase, complement, phagocytosis, and respiratory burst activities of Nile tilapia. The results are in agreement with previous studies of African catfish, *Clarias gariepinus* fed doum palm fruit powder [71]; Nile tilapia, *Oreochromis niloticus*, and African catfish, *Clarias gariepinus* fed dehydrated lemon peels [72], and Nile tilapia, *O. niloticus* fed watermelon rind powder [73]. In terms of relative immune gene expressions, the up-regulation of *IL1* and *IL8* genes were examined in fish fed the PAPP diets, with the maximum variables found in the 10 g kg^{-1} PAPP fed fish. IL-1β and IL-8 are essential cytokines related to a fish's immune system that facilitates the response to bacterial infections [74–76]. Similar findings were noticed in Nile tilapia, *Oreochromis niloticus* fed fenugreek seeds powder [77]; rohu, *Labeo rohita* fed banana, *Musa acuminata* peel flour and lemon peel enriched diet [78,79]; gilthead seabream, *Sparus aurata* fed dehydrated lemon peel [80], and Nile tilapia fed *Withania somnifera*, *Nigella sativa* oil and ciprofloxacin, *Echinacea purpurea* extract and/or vitamin C [81–83]. Lipopolysaccharide binding protein (LBP) is a soluble acute-phase protein, which plays a vital role in lipopolysaccharide signaling and non-specific immune response [84,85]. The LBP gene-encoded protein is linked to the acute-phase immunologic response to gram-negative pathogenic bacteria [86]. In fish, LBP can enhance innate and adaptive host's immunity [87,88]. In the present study, the inclusion of 10 g kg^{-1} PAPP significantly enhanced the *LBP* gene expression of Nile tilapia. As far as we are aware, there exists no information regarding the effects of PAPP on LBP gene expression in fish. However, previous studies have suggested that LBP preserves the immunity of Crucian carp following immunization with Aeromonas hydrophila [86], and stimulates growth and immunity in Atlantic salmon [89]. While the mechanism in which PAPP stimulates immune responses in Nile tilapia has not yet been explained, it may be due to the bioactive substances present in the PAPP. Pineapple contains various compounds with pharmacological properties, such as bromelain and ferulic acid, which could stimulate immune response of fish [50,56,57]. Recently, Campos, Coscueta, Vilas-Boas, Silva, Teixeira, Pastrana and Pintado [61] demonstrated that pineapple peel powder could promote the proliferation of beneficial bacteria and production of short chain fatty acids (SCFAs) in human. SCFAs play a potential role in many cellular processes, such as gene expression, differentiation, chemotaxis, proliferation and apoptosis [90, 91]. Interestingly, higher levels of PAPP administration did not significantly affect the immune parameters. Similar results were observed in Rohu, *Labeo rohita* fed banana peel flour [78]. It has been reported that higher concentrations of immunostimulants supplemented for a long

periods often lead to immunosuppression [78].

S. *agalactiae* seriously affects the aquaculture industry worldwide and causes vast economic damage [92]. Resistance against Streptococcus infection is a key objective in aquaculture and tilapia farming. In the present study, disease resistance improvement was also found in fish fed PAPP, with the highest value found in fish fed 10 g kg^{-1} PAPP. As far as we know, there is no study on the effects of PAPP on fish disease resistance. Similarly, Van Doan, Lumsangkul, Hoseinifar, Hung, Stejskal, Ringø, Dawood and Esteban [35] determined that the dietary administration of watermelon rind significantly increased disease resistance in Nile tilapia, *O. niloticus*. Improvement in disease resistance to *Aeromonas hydrophila* were also reported in barramundi, *Lates calcarifer* fed *Citrus depressa Hayata* leaf meal [93]. It was further reported that pineapple peel extract is capable of inhibiting several pathogens, such as *B. cereus, S. aureus, S. typhimurium,* and *E. coli* [94]. These findings may be due to the association of bromelain with gastrointestinal secretion signaling pathways, which include 3′:5′-cyclic monophosphatase adenosine, 3′:5′-cyclic monophosphatase guanosine, and calcium-dependent signaling cascades. Bromelain also possesses an anti-adhesive ability that prohibits microbes from adhering to different glycoprotein receptors in the gastrointestinal tract [95].

Biofloc technology plays a key role in reducing feed efficiency and enhancing the health status of aquaculture species [24,96,97]. Several studies have proven that the addition of feed additives into biofloc system could stimulate growth rate, immune response, and disease resistance [26,35,98,99]. Similar findings were recorded in Nile tilapia fed PAPP diets. PAPP is a good source of polyhydroxybutyrate [100], which provide a valuable addition of carbon into biofloc system [101]. Moreover, pineapple by-products exhibit probiotic properties, that can be commercially applied to new functional food formulations [14]. Kishawy, Sewid, Nada, Kamel, El-Mandrawy, Abdelhakim, El-Murr, Nahhas, Hozzein and Ibrahim [98] recorded that prebiotic inclusion to the biofloc system caused a lactic acid bacterial proliferation in the water and gastrointestinal tract, and regulated the immunity and tolerance to *Aeromonas hydrophila*; as well as increased the survival rate and growth of Nile tilapia. Additionally, prebiotic inclusion into the biofloc system can trigger heterotrophic bacteria to absorb the inorganic nitrogen via altering the water C:N ratio, thereby leading to higher microbial protein production for fish and increase water quality [102, 103].

In summary, the current study has concluded that dietary treatment of PAPP produces beneficial effects on growth efficiency, disease resistance, immunity, and related gene expression of Nile tilapia culture under the biofloc system.

Ethical approval

Animal use protocol followed the guideline of Chiang Mai University (No. AQ005/2561[02/2561-10-05]).

CRediT authorship contribution statement

Hien Van Doan: Conceptualization, conducting experiment, review, and editing. **Seyed Hossein Hoseinifar:** Methodology. **Ramasamy Harikrishnan:** Data computation. **Trisadee Khamlor:** Gene expression, Formal analysis. **Montri Punyatong:** Sample collection. **Wanaporn Tapingkae:** Pineapple peel preparation. **Morteza Yousefi:** Data curation. **Jorge Palma:** Validation. **Ehab El-Haroun:** Writing – original draft.

Declaration of competing interest

No competing interests are reported.

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