Investigation of growth and immune responses in channel catfish (*Ictalurus punctatus)* following feeding of dietary immunostimulants

by

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Abstract

The commercial farming of channel catfish (*Ictalurus punctatus*) in the United States often faces economic losses due to infectious diseases caused by opportunistic pathogens. Using natural immunostimulants has positively affected aquaculture production, modified hematological and immunological parameters, and enhanced fish response to infectious agents. This study evaluated two immunostimulants, a protease complex (PC) and a humic substance (HS), on the growth, disease resistance, and immune response of channel catfish. In a short-term study, the oral administration of PC and HS enhanced defenses against columnaris disease, *Flavobacterium covae*, resulting in increased lysozyme activity in the blood and upregulation of proinflammatory immune genes in the splenic tissue. Additionally, these immunostimulants provided protection against columnaris disease. The study also revealed that varying protein concentrations in the diet influenced the growth of channel catfish fingerlings over a short feeding period. However, in a long-term study, prolonged supplementation of PC or HS in channel catfish diets led to positive growth but increased mortality was observed. PC supplementation showed potential for long-term use in commercial production systems and improved feed efficiency. On the other hand, HS supplementation enhanced daily growth but had lower biomass gain compared to the control or PC supplementation. Both PC and HS increased lysozyme activity, indicating immune stimulation, but did not significantly affect immune-related genes after long-term feeding. The reduced survival could be attributed to prolonged exposure to specific dosages of PC or HS and potential interactions with parasitic pathogens. These findings suggest dietary immunostimulants like PC and HS can positively impact channel catfish health and disease resistance. However, their long-term use requires optimization to avoid adverse effects and maximize growth and immunostimulatory benefits in large-scale catfish production. Further research is needed to

optimize the inclusion of dietary PC and HS in channel catfish diets and their interactions with pathogens.

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List of Abbreviations

cDNA	Complementary deoxyribose nucleic acid
CPM	Cumulative percent mortality
CP	Crude protein
PC	Protease complex
HS	Humic substance
DNA	Deoxyribonucleic acid
DPI	Days post infection
ESC	Enteric septicemia in catfish
MSA	Modified Sheih agar
MSB	Modified Sheih broth
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RPM	Revolutions per minute
US	United States

Chapter 1:

Investigation of dietary exogenous protease and humic substance on growth, disease resistance to *Flavobacterium covae,* and immune responses in juvenile channel catfish (*Ictalurus punctatus*)

1.1 Abstract

Many bacterial pathogens impact the U.S. catfish industry, and disease control can be challenging for producers. Columnaris disease in channel catfish, *Ictalurus punctatus,* is primarily caused by *Flavobacterium covae* (formerly *F. columnare*). Immunostimulants may enhance nonspecific immune responses and offer an alternative to antibiotic treatments in catfish. Further, dietary protein sources and inclusions are essential to fish health and nutrition and may enhance fish performance in pond culture. The current project evaluated two immunostimulants: a protease complex (PC) and a humic substance (HS) derived from a reed-sedge peat product. A 60-day trial examined the effects of five diets on growth performance, immune response, and resistance to experimental *F. covae* infection in channel catfish. Diets included a high-quality fishmeal diet (32 %; CF32), a high-protein soy-based diet (32 %; C32), a low-protein soy-based diet (28 %; C28; predominately used in industry), a low-protein soy diet supplemented with C28+PC at 175 g metric ton^{-1}, and C28+HS in a low-protein diet at 23 g metric ton^{-1}. Following feeding for 60 d, juvenile channel catfish were sampled for growth performance and baseline health indicators (n=3; body mucus, blood for sera, kidney, and spleen). A subset of fish was then subjected to an immersion-based *in vivo* challenge trial with *F.covae* (ALG-00-530; 10^6 CFU mL^{-1} exposure). At 60d post-initiation, there were no dietary differences in the relative growth rate (P=0.063) or thermal growth coefficient (P=0.055), but the 32% diets generally appeared to perform best. Post-challenge, the C32 group's mortality was higher than the C28+PC (P=0.006) and C28+HS diets (P=0.005). Although not significant, the C28 and CF32 groups also demonstrated higher mortality compared to PC and HS diets. Sera lysozyme concentration was found to increase following pathogen challenge (P<0.001) and in comparison to mock-challenged catfish (P<0.001). Elevated expression levels of pro-inflammatory cytokines *(il-1β, il-8, tnf-α,* and *tgf-β)* were observed at trial midpoint and post-

infection compared to 60d. The C28 treatment was found to have lower *tnf-α* expression than the C28+PC (P = 0.042) and C28+HS (P = 0.042) groups following exposure to *F. covae*. These challenge data suggest that the immunostimulants (PC & HS) in plant-based protein may be beneficial in protecting against *F. covae* when offered in low-protein channel catfish diets.

1.2 Introduction

Catfish aquaculture, which includes channel catfish (*Ictalurus punctatus*) and hybrid catfish (♀ *I. punctatus* X ♂ blue catfish *I. furcatus*), in the top-four producing states of Mississippi, Alabama, Arkansas, and Texas account for 95% of U.S catfish production and processing (USDA-NASS, 2020). As a result, the catfish industry is an essential contributor to the economic development of the southern United States, generating significant economic output and numerous jobs in traditionally poor rural areas (Kaliba and Engle, 2004). Each year, infectious diseases are estimated to cost U.S. catfish producers millions in revenue due to fish loss. Abdelrahman et al. (2023) estimated fish losses at $11.1 million per year based on seven years (2015-2021) of data from West Alabama. Similarly, Peterman and Posadas (2019) estimated that East Mississippi lost $16.9 million in sales and income in 2016. This pattern is observed in all catfish-producing states, including Arkansas and Texas. Intensive commercial catfish farming systems have increased productivity through higher stocking densities and environmental control (Kumar et al., 2016, 2020; Tucker et al., 2014). However, as primary and opportunistic pathogens exploit environmental, genetic, or nutritional deficiencies due to this intensification, the incidence and severity of disease outbreaks increase. Enteric septicemia of catfish (ESC; *Edwardsiella ictaluri*), columnaris disease (including *Flavobacterium covae*, formerly known as *F. columnare* (LaFrentz et al., 2022)), and motile *Aeromonas* septicemia (MAS; *Aeromonas* spp., including virulent *Aeromonas hydrophila*) are primarily responsible for clinical disease diagnostic concerns (Wagner et al., 2002; Plumb and Hanson, 2010).

Flavobacterium covae (including strain ALG-00-530), a newly reclassified species from the formerly known *F. columnare* genetic groups, is a lethal strain that appears more virulent in channel catfish (LaFrentz et al., 2018). Saddleback lesions form along the dorsal midline and extend posterior to the dorsal fin and lateral flanks (Wolke, 1975; Groff & LaPatra, 2000). As a result, the disease is known as "saddleback disease" (Bernardet and Bowman, 2006). Specifically, columnaris disease has contributed to high mortality in farmed fish, with numerous reported cases in channel catfish (Welker et al., 2005;

Klesius et al., 2008; Shoemaker et al., 2008; Soto et al., 2008). Antibiotic therapy, vaccination, and chemical therapeutics are currently used to control columnaris diseases.

Immunostimulants such as glucans (β-glucans) have been used to improve disease resistance, but the results have been inconsistent. In an oral diet application, a specific strain of *Saccharomyces cerevisiae* (Bio- Mos®) composed of the outer cell wall, rich in mannan oligosaccharides, has shown promise in modulating immune responses in rainbow trout (Staykov et al. 2007) and improved survival in channel catfish when challenged with experimental *E. ictaluri* (Peterson et al., 2010). Duncan and Klesius (1996) discovered that processed spirulina, *Spirulina platen*, stimulated nonspecific cellular responses in peritoneal macrophages but did not provide nonspecific protection against *E. ictaluri*. A yeast product protected columnaris susceptibility in channel catfish fingerlings by upregulating immune genes in the gut and surface mucosal layer (Zhao et al., 2015).

Alternative protein sources have been studied, including animal proteins such as bone meal (Ai et al., 2006), poultry by-product meal (Zhou et al., 2011); plant proteins (Chowdhury et al., 2012, 2015; Fuertes et al., 2013; Kader et al., 2012; Silva-Carrillo et al., 2012), and single-cell proteins (Ju et al., 2012). Nonetheless, these protein substitutes have relatively low digestibility compared to fish meals. Dietary enzyme supplementation in fish diets has been shown to improve nutrient utilization in fish. Protease enzymes can improve the overall quality of plant protein ingredients (Dalsgaard et al., 2012; Li et al., 2016; Shi et al., 2016). Dalsgaard et al. (2012, 2016) reported that enzyme supplementation modified nutrient digestibility in rainbow trout (*Oncorhynchus mykiss*) fed a plant protein-based diet. Related results were reported in hybrid tilapia (*Oreochromis niloticus* x *O. aureus*) (Li et al., 2016), gibel carp (*Carassius auratus gibelio*) (Shi et al., 2016), and Pacific white shrimp (*Litopenaeus vannamei*) (Li et al., 2016; Song et al., 2017). Humic-based reed-sedge peat (RSP) extracts have been used as feed supplements in animal diets and various clinical applications. RSP is especially recommended for stimulating piglets and sows' growth and immune system. (Pavlik et al., 2003). The report suggests that the organic derivative, when added to the animal diet, protects the sensitive mucosal layer of the digestive system, stomach, and intestine, thereby decreasing the incidence of enteric illness and diarrhea, particularly in young animals (Trckova et al., 2005). The absence of animal allergy and resistance, ease of administration, and lack of residues in animal products (Kuhnert et al., 1989, 1991; Lange et al., 1996) suggest that RSP may be beneficial to stimulate growth and strengthen fish immune systems.

This study supplemented a protease complex (PC) and a humic substance (HS) in a plant-based protein diet to feed channel catfish (*Ictalurus punctatus*). Following the feeding trial, an experimental *F. covae* challenge trial was conducted to discern the potential immune benefit of the additives against columnaris disease. In addition, we aimed to determine growth performance, immune response, and disease susceptibility in channel catfish fed immunostimulatory feed additives. We hypothesized that high-quality fishmeal would enhance growth performance and overall channel catfish health attributes, and the immunostimulants, PC and HS products would alleviate mortality during pathogen challenge and enhance innate and adaptive immune parameters.

1.3 Materials and Methods

1.3.1. Experimental diet preparation

The five diets formulated for the feeding trial consisted of two controls without additives and three treatment diets. The two control diets contained low crude protein (C28) and high crude protein (C32), respectively, using meat and bone meal (MBM) and soybean meal as primary protein sources. The third diet mirrored the 32 % control diet (C32) but utilized high-quality fishmeal protein (CF32) instead. The fourth diet was the C28 (low crude protein) base diet supplemented with a commercial PC, denoted as C28+PC. The protease complex product used was AG175 by Jefo Nutrition Inc. (Saint-Hyacinthe, QC, Canada) at a 175 g 1000 kg-1 dose level. This product is an alkaline serine endopeptidase produced from bacterial fermentation, has high-temperature stability (Li et al., 2016), and is beneficial in enhancing feedstuff protein digestibility and promoting intestinal gut health (Shi et al., 2016). The fifth diet also used the base C28 diet and incorporated a commercial HS product at a dose of 23 g metric ton^{-1}, and the dietary treatment is referred to as C28+HS. The commercial product was Menefeed MFG 150 by the Kent Nutrition Group (Muscatine, IA, USA) and is a broad-spectrum organic acid extract from freshwater RSP.

All diets were formulated as isonitrogenous and isolipidic (8 %; Table 1). The diets were produced as sinking pellets at the Aquatic Animal Nutrition Laboratory at the School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University (Auburn, AL, USA), using standard procedures for catfish feeds. Briefly, diets were prepared by mixing the pre-ground dry ingredients in a food mixer (Hobart, Troy, OH, USA) for 10–15 minutes. Boiling water was blended into the mixture to obtain a consistency appropriate for pelleting. Diets were then pressure-pelleted using a meat grinder with a 3 mm die. The wet pellets were then placed into a fan-ventilated oven (< 45 °C) overnight to less than 10 % moisture content. Portions of each diet were then ground and sieved before use in the earlier stages of the study and adjusted until the catfish were large enough to handle a 3 mm pellet. All diets were then stored in a freezer at -20 °C until used in the experiment.

1.3.2 Experimental fish and feeding trial

Channel catfish fingerlings were locally obtained from USDA-ARS Aquatic Animal Health Research Unit (Auburn, AL) with an average size of 1.55 ± 0.01 g. They were stocked at 100 fish per tank into 15 rectangular fiberglass aquaria (420 L volume containing ~300 L of water) within a recirculating aquaculture system at Auburn University's E.W. Shell Fisheries Center (Auburn, AL). The system input water used for the trial was dechlorinated municipal and was supplied to the tanks at a flow rate of 3.6 L min^{-1} per aquarium. Dissolved oxygen (DO) concentrations in the aquarium were controlled using air stones in each aquarium (5-7 mg L^{-1}.) Water temperature, pH, ammonia, nitrate, and alkalinity were measured during the feeding period twice daily using a YSI-ProPlus sonde and YSI colorimetric test kits during the trial. The tanks were randomly assigned to five diet groups in triplicate (one hundred fish per tank, 300 fish per group). Feed was distributed until apparent satiation twice a day, in the morning (08:00 h) and late afternoon (16:00 h), and the distributed amounts were recorded. Each feeding consisted of 4 x 15-minute rounds of administering feed and making tank observations to ensure satiation. At 30 and 60 days of feeding, all the fish were counted and group-weighed. Concurrently, ten fish per tank were sampled and anesthetized using 100 mg L^{-1} buffered tricaine methanesulfonate (MS-222, Syndel, Ferndale, WA) for individual weight and length measurements to discern the condition factor. Additionally, three fish per tank were sampled for blood (sera), kidney and spleen tissue (immune gene expression), and mucus samples were collected to investigate lysozyme activity. Growth data were analyzed in three segments: days 1-30, 30-60, and days 1-60. The performance parameters were calculated as the following equations:

Tank biomass gain (TBG, g) = Final tank biomass (g) - Initial tank biomass (g)

Relative growth (RG, %) = 100[final body weight (g)-initial body weight (g)]/initial body weight (g)*

Specific growth rate (SGR, %/day) =100[ln (final body weight(g)) − ln (initial body weight (g))] /rearing period (days)*

Thermal growth coefficient (TGC) = 1000[(final body weight^1/3 (g) - initial body weight^1/3 (g)) / (time (days)* temperature (^{0}C))*

Cumulative consumption (g) = Sum of feed offered from start to end of the study

Feed conversion ratio (FCR) = feed intake (g) / [final body weight (g) − initial body weight (g)]

Fulton's condition factor (K-value) (g[cm^3]$^{-1}$) = 100[final body weight (g)/standard body length (cm)]*

Survival (%) = 100[final quantity/initial quantity]*

Fish samples were obtained from three fish per tank during the growth performance sampling interstation and seven days post-infection (dpi) following the pathogen challenge with *F. covae*. During sampling, skin mucus samples were collected on days 30 and 60 of the feeding trial according to the method by Nhu et al. (2019). Briefly, catfish were anesthetized using 0.1 ppm MS-222 buffered with sodium bicarbonate. Fish surfaces were individually washed with distilled water and then transferred into polyethylene bags containing 1 mL of 1X phosphate-buffered saline (PBS). After 2 min of gentle shaking, mucus was collected, transferred to sterile 1.5 mL microcentrifuge tubes, and centrifuged (1500 × g for 10 min at 4 °C). The collected supernatant was stored at −80 °C until analysis. Similarly, a separate set of three fish per tank at 30 and 60 dpi was anesthetized using MS-222 (250 mg L^{-1}). Blood was drawn from the caudal vein for serum collection using a 1 mL Tuberculin Syringe and 25 G x 5/8-inch needle (Exelint International, CA). Head kidney and spleen tissue pieces were removed and stored in DNA/RNA Shield (Zymo Research Corp, Irvine, CA) before storing at −20 °C until RNA extraction. The blood samples were left to clot overnight at 4 °C before centrifuging at 15,000 × *g* for 5 minutes for sera collection. Sera samples were kept at −80 °C until analysis.

1.3.3. Proximate analyses

Test diets were sent to Midwest Laboratories (Omaha, NE, USA) for proximate analyses. Moisture (AOAC 930.15), crude protein (AOAC 990.03), crude fat (AOAC 2003.05), fiber (acid detergent via ANKOM), and ash (AOAC 942.05) were all determined. Total digestible nutrients (TDN) and digestible energy (DE) were calculated using the online finished feed matrix software at Midwest Laboratories (Table 2). For the calculations, the laboratory used a protein factor (Pf) of 0.73, a fat factor (Ff) of 2, an acid detergent fiber factor (ADFf) of 0.58, and a nitrogen-free extract factor (NFEf) of 0.90. The factors are based on unique feedstuff sample types within the software. The software calculations were based on the following equations (P=protein, F=fat, A=ash, ADF=acid detergent fiber, and NFE=nitrogen-free extract):

$$NFE=100-(P+F+ADF+A)$$

$$TDN=(P*Pf)+(F*Ff)+(NFE*NFEf)+(ADF*ADFf)$$

$$DE=20*TDN/1000$$

1.3.4 Challenge trial with F. covae

Immediately following trial completion at day 60, a subset of fish (pooled across treatment tanks) was transferred to a bio-secure wet laboratory for the *in vivo F. covae* challenge trial. Bacterial culture and an *in vivo* immersion challenge protocol similar to that reported by Wise et al. (2023) were carried out after the feeding trial. *F. covae* (strain ALG-00-530; GenBank Accession #MG516971.1; LaFrentz et al., 2018) was revived from cryostock and recovered on modified Shieh agar (MSA) plate medium at 28 °C for 24 h. Following a colony morphology check, a single colony was picked and used to inoculate 12 mL of modified Shieh broth (MSB) in 50 mL tubes at 28 °C in a shaker incubator at 0.33 x g for 12 h. Six mL of the culture was inoculated in a final volume of 200 mL and incubated at 0.33 x g for 12 h to reach a final OD_{540} of 0.75. The colony forming units per milliliter (CFU mL^{-1}) in the final inoculum was measured using serial dilution and plate counts on MSA at 24 h post-challenge.

For the challenge experiment, 20 fish each were stocked in 30 tanks, and each tank was randomly assigned to one of the five pooled fish groups from the feeding trial. Six tanks were used for each treatment; three were challenged with *F. covae* and three served as mock-challenged controls for each group. Mock-challenged fish were treated with an identical procedure except that they were not exposed to the bacteria but to an equivalent amount of sterile MS broth. The water volume in each 114 L tank was decreased to 10 L. Then, inoculum or sterile control media was poured into the tank in an immersion experiment at a final tank concentration of 5.5 × 10^6 CFU mL^{-1}, which the final culture plate counts determined. The immersion continued for 30 min with continuous aeration, and then the tank flows were resumed and filled to a final volume of 20 L for the trial period. Catfish were fed the respective treatment diet and were checked twice daily for mortality throughout the experiment. Water temperature and DO were maintained at (28 ^oC and 7.5 mg L^{-1}), with flows retained at 0.4 L min^{-1}. Dead fish were collected twice and recorded to determine cumulative mortality. Up to 20% of the daily mortalities from each tank were necropsied, with the kidney and spleen plated on MSA for pathogen confirmation.

1.3.5. Serum and mucus lysozyme activity

The lysozyme activity in the serum and surface mucosa was assayed by the method of Welker et al. (2012). Briefly, the assay is based on the lysis of lysozyme-sensitive Gram-positive bacterium *Micrococcus lysodeikticus* (Worthington Biochemical, Lakewood, NJ) by lysozyme present in sera and mucus samples. A suspension of 0.25 mg mL^{-1} freeze-dried *M. lysodeikticus* was made shortly before use by dissolving it in sodium phosphate buffer (0.04 M Na_2HPO_4, pH 6.0). Sera (10 µL $well^{-1}$, in duplicate) was placed in a microtiter plate, and 250 µL of bacterial cell suspension was added to each well. Hen egg white lysozyme was used as an external standard. The initial and final (after 20 min incubation at 35 ^oC) sample absorbances were measured at 450 nm using a microplate reader (BioTek Synergy HTX, Agilent, Winooski, VT). The rate of reduction in absorbance of samples was translated to lysozyme concentration (µg mL^{-1}) using a standard curve.

1.3.6. RNA extraction, cDNA synthesis and quantitative RT-PCR

RNA was extracted from the spleen and head kidney of the channel catfish using the Quick-RNA MiniPrep Plus kit (Zymo Research Corp.) according to the manufacturer's recommendations. After the extraction, the concentration and quality of the RNA were confirmed with a Nanodrop One^{c} spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA), ensuring the 260/280 ratios were between 1.8 and 2.0. Extracted spleen and kidney RNA samples were diluted to 20 ng μL $^{-1}$ and 50 ng μL $^{-1}$, respectively. According to the manufacturer's instructions, RNA was synthesized into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA). Briefly, each 20 μL reaction contained 2 μL of 10x R.T. buffer, 0.8 μL of 25x dNTP Mix, 2 μL of 10x R.T. random primers, 1 μL of kit Multiscribe reverse transcriptase, 10 μL of template RNA and 4.2 μL of nuclease-free water. cDNA samples were synthesized in a MiniAmp™ plus thermal cycler (Applied Biosystems). Program conditions were set at 25 °C for 10 minutes, 37 °C for 120 minutes, and 85 °C for 5 minutes. After the reaction, the cDNA was stored at -20°C. Quantitative real-time PCR was conducted with QuantStudio 5 Real-Time PCR (Applied Biosystems) using the PowerUp SYBR Green Master Mix (Applied Biosystems). All cDNA samples were diluted to 1.25 ng μL $^{-1}$ prior to the reaction. Each 10 μL reaction consists of 5 μL of master mix, 0.5 μL each of forward and reverse primer (10 μM stock), and 4 μL of diluted sample cDNA. Each PCR was performed with duplicate samples, and the cycling conditions were 2 min at 50 °C, 2 min at 95 °C followed by 15 s at 95 °C, 15 s at 58 °C and 30 s at 72 °C for 40 cycles. In addition, a melt curve analysis was performed after amplification to verify product specificity. Four genes of interest, *il-1*β, *tnf-α*, *il-8*, and *tgf*-β were assessed, and the relative quantification of each gene (i.e., ΔCt) was determined through normalization against two housekeeping genes (*ef-1*β and *actb).* PCR primers used in this study are described in Table 3. Further, the assessed gene from each dietary group was normalized against the low protein control diet (C28). When mock-challenged and challenged expression was compared, the C28 mock-challenged group was used for the inherent comparison. The relative abundance of interested genes was calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

1.3.7. PCR confirmation of reisolated bacteria

Single colonies of unconfirmed *F. covae* isolates from collected mortalities or moribund fish during the bacterial challenge were purely cultured and grown in MSB for 24h and further centrifuged to harvest the cell pellet. According to the manufacturer's instructions, genomic DNA was extracted from the pellet using a Bacterial DNA kit (Omega Bio-Tek, Inc. Norcross, GA). A multiplex PCR developed for previous *F.columnare* genetic groups by LaFrentz et al. (2019) was performed to confirm *F.covae*. PCR was performed in a T100 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA). Each 25 mL PCR reaction contained a 12.5 mL DreamTaq Green master mix (ThermoFisher Scientific), 2 µL of a primer mixture (0.5 µM GG-forward, 0.1 µM GG1-reverse, 0.45 µM GG2-reverse, 0.45 µM GG3-reverse, 0.3 µM GG4-reverse), 5.5 mL of nuclease-free water, and 5.0 mL of extracted DNA from suspected *F. covae* harvested from kidneys or spleens of moribund fish. The *F. covae* AL-02-36^{T} type strain (ATCC # TSD-246; LaFrentz et al. 2018) was run as a positive control and nuclease–free water as a negative control. PCR products (5 µL) were inserted into a 1.5% agarose gel electrophoresis in Tris-acetate-EDTA (TAE) buffer, stained with GelRed (Biotium Inc., Fremont, CA, USA), and product banding was observed with ultraviolet transillumination in a Gel Doc Go imaging system (Bio-Rad Laboratories, Inc.)

1.3.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.51 (GraphPad Software, CA, USA). All fish growth performance data and mortality were analyzed using one-way ANOVA. Assessments of sera and mucus lysozyme activity and gene expression were analyzed using a two-way ANOVA to determine the main effects of diet, time, and interaction in the model (□ = 0.05). Post-challenge sera lysozyme activity and gene expression were also analyzed using a two-way ANOVA to determine the main effects of dietary treatment, challenge, and interaction in the model. Gene expression data were log-transformed prior to analysis. The normality of the model residuals was assessed using a

Shapiro-Wilk test, and variance homogeneity was evaluated using a homoscedasticity plot in conjunction with Spearman's test for heteroscedasticity. Statistical analyses considered tanks as experimental units. Tukey's post hoc testing was conducted when differences were significant ($P<0.05$) to identify which groups were different. All data were expressed as mean ± standard error.

1.4. Results

1.4.1. Growth performance

During the feeding period water quality was maintained at (Mean+SE): Temperature = 27.41 ± 0.02 °C, DO = 6.42 ± 0.05 mg L^{-1}, pH = 8.11 ± 0.03, ammonia = 0.06 ± 0.01 mg L^{-1}, nitrite = 0.35 ± 0.05 mg L^{-1} and alkalinity = 79.21 ± 1.24 mg L^{-1}. Growth performance data are summarized in Table 4. No statistically significant differences in metrics were discerned during the initial 30 days, but we found diet-related treatment differences during the 30-60 d period. The soy-based, 32 % diet (C32) demonstrated a significantly higher biomass gain (356g) than the 28 % variant but was not different from the fishmeal diet (CF32) or immunostimulant-supplemented diets (C28+PC & C28+HS) (P = 0.028). The thermal growth coefficient followed a similar trend (P = 0.027).

The FCR was significantly lower in the CF32 diet compared to the basal control diet C28 but was not significantly different from the CF32, C28+PC, and C28+HS diets (P = 0.040). Concerning cumulative consumption (CC) at the 30-60 d period, C32 had a significantly higher feed intake than CF32 and C28 (P = 0.002). Overall at 60 d, the high protein diets (CF32 & C32) had numerical growth advantages compared to the low protein diets. However, no significant growth metrics were discerned at the end of the feeding trial.

1.4.2. *Pathogen challenge trial*

Day 60 *in vivo* cumulative percent mortality (CPM) data are summarized in Figure 1. During the 8d challenge trial, water quality in the flow-through system was (Mean+SE): Temperature = 28.45 ± 0.19°C and dissolved oxygen = 8.27 ± 0.03. A CPM difference was observed across treatments (P = 0.003). Specifically, the C32 diet displayed higher mortality than the C28+PC (P = 0.006) and the C28+HS (P = 0.005) groups. Although not found to be significantly different, the immunostimulant diets demonstrated a marked reduction in mortality compared to the basal diet groups.

1.4.3. Lysozyme activity

Results from the lysozyme analyses are presented in Figure 2. Sera lysozyme activity within all dietary treatment groups was found to be different when all periods were compared ($P = 0.005$). With the main effect of treatment, CF32 was found to be different from the C28+PC (P=0.015), and the C32 was also different than the C28+PC (P=0.009). Concerning the sampling period, lysozyme activity was similar at 30 d and 60 d, but a significant increase was apparent at 8 dpi ($P < 0.001$). Although not different, diets C28+PC and C28+HS showed a numerical increase in lysozyme at 8 days post-infection in the challenged catfish. The skin mucus lysozyme activity for all dietary treatment groups was not significantly different at 30 d and 60 d ($P = 0.276$), and no interaction was discerned between them in the model ($P = 0.486$). However, there was a significant time improvement in lysozyme activity in body mucus at 60 d ($P = 0.027$). Post-challenge sera lysozyme activity was significantly improved within the bacteria-challenged group compared with the mock control groups ($P = 0.001$), although there with a significant interaction between the two groups ($P = 0.001$). No treatment differences were discerned from the model ($P = 0.084$), although the diets with additives appeared to have elevated lysozyme levels following pathogen exposure.

1.4.4. Immune gene expression

When comparing kidney tissues at the 30, 60, and post-challenge sampling times, there were no treatment effects for *il-1β* ($P = 0.601$), *il-8* ($P = 0.882$), *tnf-α* ($P = 0.676$) or *tgf-β* ($P = 0.954$; Figure 3). Although not different at 30 days of dietary feeding, *il-1β* and *tnf-α* expression were relatively high in the CF32 and C28+PC fish groups. Day 60 expression was lower across all time points, with time as a significant main effect for *il-1β* ($P < 0.001$), *il-8* ($P < 0.001$), *tnf-α* ($P < 0.001$), or *tgf-β* ($P = 0.003$).

Treatment differences were found with gene expression in the splenic tissue for *il-1β* ($P < 0.001$) and *tnf-α* ($P = 0.009$) but not for *il-8* ($P = 0.253$) or *tgf-β* ($P = 0.183$; Figure 4). For *il-1β*, the C28+PC diet was elevated compared to the C28 ($P = 0.006$) and C32 diets ($P = 0.038$) at 30 d. The effect of the protease complex enhancing splenic *il-1β* expression in the C28 diet also was apparent following infection (*P* = 0.021). The *il-1β* gene showed no overall differences in sampling time (*P* = 0.282). Time was a significant main effect for *tnf-α* expression in the spleen (P < 0.001), and there was a depression in overall expression at 60d. Differences between the C28 and C28+PC groups were detected at 30d (*P* = 0.036), and C28 was found to be lower than the C28+PC (*P* = 0.042) and C28+HS (*P* = 0.042) following exposure to *F. covae*. Expression of *il-8* was elevated at 8 dpi compared to 30d (*P* =0.012), and *tgf-β* at 30d was lower than 60 d (*P* = 0.002) or 8 dpi (*P* = 0.002).

Immune gene expression in the kidney did not appear to be impacted by treatment and pathogen exposure (Figure 5). No treatment effects were found for *il-1β* (*P* = 0.330), *il-8* (*P* = 0.175), *tnf-α* (*P* = 0.812), or *tgf-β* (*P* = 0.852) expression. Exposure to *F. covae* also did not influence *il-1β* (*P* = 0.448), *il-8* (*P* = 0.587), *tnf-α* (*P* = 0.587), or *tgf-β* (*P* = 0.310) expression. Only the *il-8* model showed a significant interaction effect following analysis (*P* = 0.046).

When comparing splenic cytokine gene expression for the *F. covae*-infected and mock-challenged fish, there were no treatment effects for *il-1β* (*P* = 0.074), *il-8* (*P* = 0.145) and *tgf-β* (*P* = 0.458; Figure 6). The expression of *tnf-α* did have a significant treatment main effect (*P* = 0.016), with overall differences for the C28 diet and the protease complex (*P*= 0.026) and humic substance (*P* = 0.036) additives. However, differences from Tukey's HSD did not show any treatment-related differences when treatment was compared with respect to exposure. The challenged fish showed overall expression changes for *tnf-α* (*P* = 0.028), *il-8* (*P* = 0.002), and *tgf-β* (*P* = 0.006) when compared to catfish that were not exposed to *F. covae*.

1.5 Discussion

Dietary additives in animal diets have received considerable attention in combating bacterial infection and growth stimulation. Several additives, including probiotics, prebiotics, organic extracts, and exogenous enzymes, have been extensively studied as growth promoters, immunostimulants, and supplements in fishmeal-free diets for fish. Primarily plant-based diets are typical in the industry but contain several poorly digested components; hence, exogenous enzymes to promote endogenous enzyme activity in the fish gut are becoming more common (Shi et al., 2016). Some enzymes commonly evaluated in fish diets include β-glucanase, xylanase, carbohydrase, and protease (Dalsgaard et al., 2012; Yildirim and Turan, 2010; Ghomi et al., 2012; Zamini et al., 2014; Lin et al., 2007). Drew et al. (2005) and Shi et al. (2016) revealed that protease can increase the digestible protein availability in fish diets, thereby causing a reduction in the total protein in the diet. Among other evaluated feed additives in livestock production are humic extracts mined from organic substrates. Reed-sedge peat (RSP) has been a great source of humic compounds, with 60-80% humic, fulvic, ulmic, humates, and humins (Price, 2019). However, the humic substance in RSP has aided growth and boosted the immune system of poultry when supplemented in diets (Arif et al., 2019). Further reports have emphasized the easy application of humic substances and the absence of side effects on animals (Kuhnert et al., 1989, 1991; Lange et al., 1996). This present study is the first, as far as we know, to evaluate the effects of individual dietary supplementation of protease and humic acid extract on growth, disease resistance, and innate immune response in channel catfish.

1.5.1 Growth performance

This study observed similar growth metrics in the 32% fishmeal diet (CF32) group and the 32% fishmeal-free (C32) diet group of channel catfish. Specifically, the fishmeal diet did not stimulate growth differently from similar protein concentrations when replaced with MBM through the entire feeding trial period of 60 days. However, numerical improvement was seen in the feed efficiency of the CF32 group with a reduced FCR at 60 d. Inversely, the MBM 32% fed group had better weight gain. This could result from the reduced feed consumption shown by the CF32 group in the second 30 d period (30-60 d). Contrary to our study, Rossi & Davis (2014) found growth reduction in the fishmeal-free control diet. The study reported that growth and survival were negatively affected when dietary fishmeal was wholly replaced with meat and bone meal in a soybean meal-based diet fed to Florida pompano (*Trachinotus carolinus*). Furthermore, Huan et al. (2018) reported significant weight loss and increased FCR in hybrid tilapia when provided with a meat and bone meal diet. They specified that low digestibility and nutrient retention in the fish group fed meat and bone meal affected the overall growth performance. However, our study did not find these growth deficiencies in channel catfish juveniles, which was similar to findings from Hedrick et al. (2005).

Studies on the effect of dietary protease in some cases have been reported to improve growth metrics in aquatic species significantly. In this work, we did not observe significant improvements in channel catfish growth when adding protease to a fishmeal-free (28%) diet. The protease-supplemented diet (C28+PC) numerically improved catfish growth across all calculated metrics compared to the low-protein control (C28) diet in both 30 d periods of this study. Interestingly, the observed enhanced growth metrics shown in the 32% diets were insignificant compared to the 28% diet when supplemented with protease. This result suggests that protease supplementation can maximize the nutrient digestibility of a low-quality protein source in channel catfish compared to a protease-free diet of similar protein sources. This is because protease can hydrolyze protein, break complex amino acids (Shi et al., 2016) and degrade dietary antinutritional compounds (Caine et al., 1998). Previous studies reported improved growth performance in low fish meal diets supplemented by dietary protease in gibel carp (Shi et al., 2016), hybrid tilapia, and Pacific white shrimp (Li et al., 2016). However, these results are inconsistent with our findings as we completely replaced fishmeal with meat and bone meal in the supplemented diet. Like our study, Huan et al., 2018 reported a similar result. They found that dietary protease supplemented in a meat and bone meal diet did not significantly improve the growth and nutrient digestibility in hybrid tilapia.

Previous studies reported that humic acid supplementation in animal diets promotes growth and enhances productivity (Abo-Egla et al., 2011, Arpášová et al., 2016, Rath et al., 2006, Ozturk et al., 2010; Cetin et al., 2011). Shermer et al. (1998) opined that humic acid stabilizes the intestinal microflora and thus ensures improved utilization of nutrients in animal feed. However, the effect of this substance has not been thoroughly examined in fish production. In our study, we fed channel fish a low-protein fishmeal-free diet supplemented with humic acid extract (C28+HS). After 30-60d of feeding, the humic supplementation did not significantly improve weight gain and FCR in channel catfish fingerlings. Our study examined the diets in catfish fingerlings (1-5g), which can be categorized as a starting phase of catfish growth. Interestingly, significant differences in all determined growth metrics were not observed between the humic substance diet (28%) and the high-protein diets (32%). Our findings suggest that humic substance supplementation does not affect feed intake and growth performance in this stage of catfish development. Concerning high-quality fishmeal, no real benefits were discerned for the 32 % protein source (both treatments generally presented the highest tank averages for these performance metrics). These findings agree with Li et al. (2020), where reduced dietary protein concentrations and source changes to porcine or bone meals did not alter growth performance. Thus, this component further provides evidence that producers may not need to spend extra on higher-quality protein sources for this life stage of catfish rearing.

1.5.2 Effects of dietary protease and humic extracts on F. covae infection

Barman et al. (2013) suggested that adding immunostimulants to the diet can be the most cost-effecting method of bacterial infection treatment in aquaculture. In this study, we set an experimental *F.covae* infection via bath to discern the immunostimulatory effects of protease and humic substances in the catfish diet. According to Taklimi et al. (2012), humic acid can provide a protective layer against the penetration of microbes and other toxic substances into the animal intestine. Likewise, exogenous enzymes promoted immune performance and pathogen resistance in aquatic species (Norag et al., 2018; Baruah et al., 2009; Liu et al., 2013, 2016; Song et al., 2017; Zheng et al., 2019).

In our study, after 8 dpi, the protease diet group (C28+PC) and humic substance (C28+HS) significantly decreased the cumulative mortality of channel catfish. In a similar experiment, Norag et al. (2018) found a significant increase in the survival of Nile tilapia (*Oreochromis niloticus*) fed a low-protein protease diet after being infected with *A. hydrophila*. A similar result was also reported in shrimp (Song et al., 2016). Although several inhibitory benefits against pathogenic bacterial development in poultry have been documented (Taklimi et al., 2012; Mutus et al., 2006; Ur Rehman et al., 2016; Ceylan et al., 2003), the health benefits of humic acid in aquatic species have been underexplored. Our study found that supplementing humic substances in a low-protein diet provided resistance to *F. covae* infection. This may be because humic substances affect the microbes' carbohydrates and proteins, destroying the pathogenic viruses and bacteria (Arif et al., 2019). Therefore, individual protease and humic substance supplementation can enhance resistance to *F. covae* in channel catfish.

1.5.3 Effects of protease complex and humic acid substance on channel catfish immunity

The skin mucus of fish plays a vital role in the innate immune system and is integral to protecting from diseases (Olivares-Fuster et al., 2011). Bacteriolytic enzymes detected in serum, mucosal membranes, and leucocytes rich tissues are partly responsible for the nonspecific defense mechanism in most animals (Uribe et al., 2011). The defense activity of lysozyme in fish serum and mucosal tissues has been related to the hydrolyzation of the peptidoglycan of bacterial cell walls resulting in lysis (Uribe et al., 2011). Therefore, the intact structure of fish mucosal tissues functions as the first physiological barrier against pathogen attack. The role of immunostimulants in catfish's innate immunity and bacterial protection has been inconsistent (Dawood et al., 2018). Peterson et al. (2010) found that Bio-Mos did not improve serum lysozyme in channel catfish after dietary trial and post-challenge. Similarly, Welker et al. (2007) evaluated the effect of dietary yeast supplementation in the catfish diet against *E. ictaluri*; they found that dietary YYS supplementation had no impact on lysozyme activities. Similar results were reported by Welker et al. (2011). However, yeast supplementation enhanced lysozyme activity in other aquatic species (Bagni et al., 2000, 2005; Thompson et al., 1995; Ortuño et al., 2001). In this study, sera lysozyme was not found to change with dietary treatments throughout the feeding trial. However, although not significant, lysozyme in catfish sera in both groups fed protease (C28+PC), and humic substance (C28+HS) was numerically enhanced after 8 dpi to *F. covae* compared to the diets without protease or humic substance. This enhancement might explain the higher survival in C28+PC and C28+HS treatment groups within the *F. covae* pathogen challenge. This result also demonstrated a considerable elevation in sera lysozyme in channel catfish after 8 dpi. This indicated the enhanced bactericidal ability of channel catfish during *F. covae* infection.

At the same time, skin mucus taken from the five dietary groups was evaluated for lysozyme activity at 30 d and 60 d of dietary treatment. The epidermal mucus acts as the first barrier to pathogen infection in fish due to the presence of cellular and humoral components (Salinas et al., 2011; Dash et al., 2018). However, lysozyme activity was similar across all dietary groups at both sampling periods, but significant improvement of skin mucus lysozyme activity was observed at 60 d of dietary treatment. These results suggest that the feed additives used in this study did not affect lysozyme activity in channel catfish skin mucus. However, the significant improvement at 60 d may be due to overall stress response (Dash et al., 2018).

1.5.4 Immune gene expression

Epithelial/mucosal barriers and humoral and cellular components have characterized the fish immune system. During pathogen infection, these immune components are essential and considered the first line of defense and essential to a specific defense buildup in fish (Whyte, 2007). However, it is generally agreed that the regulation of inflammation results from a balance between pro- and anti-inflammatory cytokines, which will minimize the adverse effects of the inflammatory processes (Liu et al., 2021). In this sense, numerous pieces of literature have demonstrated inflammatory activities and immune responses in fish head kidney and spleen (Areechon & Plumb, 1983; Kocabas et al., 2002; Yuan et al., 2008; Suresh and Ranganathan, 2008; Pohlenz et al., 2012; Zhao et al., 2013; Castro et al., 2019; Liu et al., 2020). This is because the head kidney, and spleen in teleost are more susceptible to cellular degeneration and toxic by-products during bacterial infection (Areechon & Plumb 1983). However, previous studies have reported immune regulation in the head kidney, and spleen in channel catfish following vaccination and dietary additives. (Pohlenz et al., 2012; Chen et al., 2015).

In this study, *F. covae* infection triggered elevated expression of proinflammatory *il-1β*, *tnf*-α, and *il-8* and antiinflammatory *tgf-β* in the head kidney of channel catfish at 8 dpi when compared to the 60d sampling, which was pre-challenge**.** However, significant treatment differences were not observed within the dietary groups, although supplemented diets (C28+HS and C28+PC) showed an elevation trend. Moreover, significant time differences in gene expression were also apparent in the head kidney during the feeding trial, except for *tnf*-α. The significant downregulation of all genes might be related to stocking stress and acclimatization. Concerning cytokine expression in the splenic tissue, the dietary protease (C28+PC) significantly induced an earlier and higher regulation of *il*-1β in the channel catfish spleen following short-term feeding and post-challenge. Meanwhile, *F. covae* infection did not influence a significant modulation of *il-1β* compared to the mock-challenged catfish group at 8 dpi. Still, the two other proinflammatory cytokines investigated showed an overall increase in expression following the challenge. Although no treatment differences were discerned, there was an overall trend in enhanced expression for the catfish fed diets with additives. Based on the mortality results from the *F. covae* challenge in conjunction with the trends in immune gene expression, protease complexes or humic substances supplementation in channel catfish diets may have influenced macrophage and monocyte activity in defense against *F. covae* infection, but further investigation is required. Similarly, exogenous protease improved the humoral immune system by upregulating immunoglobin M (IgM) in Nile tilapia (Hassaan et al., 2021) and Siberian sturgeon (*Acipenser baerii*; Shekarabi et al., 2022) and Ghodrati et al. (2021). Furthermore, Hassaan et al. (2021) inferred that protease might enhance the macrophages and monocytes, producing cytokine production towards an improved immune system in fish. During the feeding trial component of the current study, the humic substance additive was not overly influential for the expression of proinflammatory genes in the head kidney and spleen of catfish. However, along with C28+PC, the C28+HS fish group showed a somewhat increased expression of proinflammatory cytokines following *F. covae* infection, especially in the kidney tissues. Similar to our findings, Pelusio et al. (2020) reported no significant cytokine gene expression in rainbow trout-fed humic acid after 82 days. However, they found substantial upregulation of *il-8* and *tnf-α* after exposure to high water temperature. Furthermore, Akaichi et al. (2022) found that the dietary inclusion of humic acid in combination with organic acid improved antibody response against infectious bronchitis virus and Newcastle disease virus. This may explain the overall trend of numerical upregulation observed in the kidney tissues of C28+HS-fed catfish following the challenge. Riede et al. (1991) also demonstrated that humic substances could form bio-active complexes ready to bind to the surface of T lymphocytes and natural killing cells, which can regulate their functions and stimulate cytokines production against pathogen infection.

On the contrary, the anti-inflammatory gene *tgf-β* is produced to inhibit excessive immune response activation (Verburg-Van Kemenade et al., 2009). When we evaluated the trend over time, there was upregulated *tgf-β* expression in the spleen across diets following longer-term feeding and after the infection challenge. Thus, the 60d influence may be related to potential overstimulation, and this aligns with a decreased expression level of *tnf*-α at 60 d and a relatively lower expression of *il-1β* in the same splenic tissue. A comparison of the gene expression between infected groups (8 dpi) and mock-infected controls further explained the positive impact of *F. covae* infection on gene regulation. In the spleen, all three out of four examined immune genes were significantly upregulated in channel catfish after 8 dpi, whereas the difference was not as pronounced in the kidney tissue. These results further elucidate the relative expression of cytokines in the head kidney and spleen in response to *F. covae* infection in channel catfish. The study findings suggest that adding dietary protease complexes and humic substances can upregulate inflammatory cytokines in the splenic tissue of channel catfish when faced with pathogen exposure.

1.6 Conclusion

The results of this study offer promising insights into dietary modifications to improve the health of channel catfish. The present study demonstrated that oral administration of a protease complex (C28+PC) and a humic acid substance (C28+HS) could modulate aspects of the immune system of channel catfish, thus enhancing defenses against *F. covae*. Specifically, administering these immunostimulants increased lysozyme activity in the blood, upregulation of proinflammatory immune genes in the splenic tissue, and afforded some protection against columnaris disease. Moreover, our findings indicate that varied protein concentrations (i.e., 28% and 32 % plant-based formulations) can also influence the growth of channel catfish fingerlings over a short feeding period. Future studies should focus on optimizing the use of these feed additives and protein sources to improve the growth and immunostimulatory benefits of channel catfish. Overall, these findings contribute to understanding the potential applications of dietary immunostimulants in catfish aquaculture and highlight the specific potential in modulating the innate immune response against bacterial infections.

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Tables

Table 1. Experimental diet composition of the five formulated diets used in the feeding trials. Ingredient values are presented on a dry matter basis. CF32=32% high-quality fishmeal; C32=32% control soybean-based diet; C28=28% control soybean-based diet; C28+PC=28% protease complex diet; C28+HS=28% humic substance diet.

Ingredients (g kg^{-1})	CF32	C32	C28	C28+PC	C28+HS
Menhaden fishmeal[a]	73.6	0	0	0	0
Meat & bone meal (Apcon 60)[b]	0	80	64	64	64
Soybean meal[c]	420	420	331.8	331.8	331.8
DDGS ANDVANTAGE 40Y[d]	130	130	128	128	128
Menhaden fish oil[a]	40.1	39	34.2	34.2	34.2
Lecithin (soy)[e]	10	10	10	10	10
Corn Starch[f]	63.3	67.5	64.5	64.33	64.48
Corn[g]	230	220	330	330	330
Mineral premix[h]	5	5	5	5	5
Vitamin premix[i]	8	8	8	8	8
Choline chloride[j]	2	2	2	2	2
Rovimix Stay-C 35%[k]	1	1	1	1	1
CaP-dibasic[l]	15	15	15	15	15
Lysine (78.8%)[m]	1	1	5	5	5
Methionine[n]	1	1.5	1.5	1.5	1.5
Mennefeed MFG50[o]	0	0	0	0	0.023
Jefo AG175[p]	0	0	0	0.175	0
Total	1000	1000	1000	1000	1000

[a] Omega Protein Inc., Houston, TX, USA.
[b] Apcon 60Midsouth Milling Co., Memphis, Tennessee, USA
[c] Solvent Extracted Soybean Meal, Auburn University, Auburn, Alabama, USA.
[d] The Andersons, Maumee, OH, USA.
[e] The Solae Company, St. Louis, MO, USA.
[f] MP Biomedicals Inc., Solon, OH, USA.
[g] Faithway Feed Co., Guntersville, AL, USA.
[h] Trace mineral premix (g 100 g^{-1} premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.250; Ferrous sulfate, 4.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 67.964.
[i] Vitamin premix (g kg^{-1} premix): Thiamin HCl, 0.438; Riboflavin, 0.632; Pyridoxine HCl, 0.908; Ca-Pantothenate, 1.724; Nicotinic acid, 4.583; Biotin, 0.211; folic acid, 0.549; Cyanocobalamin, 0.001; Inositol, 21.053; Vitamin A acetate, 0.677; Vitamin D3, 0.116; Menadione, 0.889; dL-alpha-tocoperol acetate, 12.632; Alpha-cellulose, 955.589.
[j] VWR Amresco, Suwanee, GA, USA.
[k] Stay-C® (L-ascorbyl-2-polyphosphate 35% Active C), Roche Vitamins Inc., Parsippany, NJ, USA.
[l] VWR Amresco, Suwanee, GA, USA.
[m] Ajinomoto Heartland Onc
[n] Smartamine ®M; Kemin.
[o] Kent Nutrition Group, IA, USA.
[p] Jefo Nutrition Inc. QC, Canada.

Table 2. Analyzed ingredient proximate composition of experimental protein sources (dry matter basis) used in the feeding trial. CF32=32% high-quality fishmeal; C32=32% control soybean-based diet; C28=28% control soybean-based diet; C28+PC=28% protease complex diet; C28+HS=28% humic substance diet. NE=net energy, DE=digestible energy, TDN=total digestible nutrients, ME=metabolizable energy.

Proximate	CF32	C32	C28	C28+PC	C28+HS
Crude protein (%)	35.30	36.70	33.10	32.40	32.70
Crude lipid (%)	8.58	8.87	8.77	8.69	8.87
Ash (%)	6.66	6.84	6.00	5.88	6.04
Fiber (%)	4.60	5.50	5.20	4.90	5.60
NE (lactation) (Mcal lbs^{-1})	0.90	0.90	0.91	0.91	0.91
NE (maint.) (Mcal lbs^{-1})	0.94	0.94	0.95	0.96	0.95
NE (gain) (Mcal lbs^{-1})	0.62	0.62	0.63	0.63	0.63
DE (Mcal lbs^{-1})	1.72	1.71	1.74	1.74	1.74
ME (Mcal lbs^{-1})	1.53	1.52	1.55	1.56	1.55
Sulphur	0.42	0.40	0.36	0.36	0.37
Total Phosphorus	1.10	1.17	1.06	1.06	1.08
Total Potassium	1.39	1.34	1.16	1.17	1.17
Total Magnesium	0.22	0.23	0.20	0.20	0.21
Total Calcium	1.05	1.26	1.01	1.00	1.10
Total Sodium	0.13	0.11	0.09	0.09	0.10
Total Iron	231.00	180.00	162.00	161.00	166.00
TDN (%)	86.00	85.60	87.00	87.20	87.00
Moisture (%)	7.04	6.88	6.40	6.35	6.14

Table 3. Channel catfish primers used in the study for qPCR gene expression and confirmation of *F. covae.*

	Forward Sequence	Reverse Sequence	Ref.
actb	CCGTGACCTGACTGAATACC	GCCCATCTCCTGCTCAAAG	1
ef-1a	GTTGAAATGGTTCCTGGCAA	TCAACACTCTTGATGACACCAAC	2
il-1b	GTGTAAGCAGCAATCCAGTCA	CAAGCACAGAACAGTCAGGTAT	3
il-8	TGGGGAGAAGGAAAAGCAGAG	TGAACAGGAGGCACTTGGAGG	4
tnf-b	GGCCTCTACTTCGTCTAC	GCAGCAGCTTCTCGTCCAT	3
tgf-b	GAAACATCCCAGCACCTCCA	GCCAAGCAAACAACGGCTAA	5
GG-fwd	ACRGGRGATAAAGCAGAASA	-	6
GG1-rev	-	GACTTTTGTGTTGAAACGG	6
GG2-rev	-	AAGAAAATAGGGGAGAGG	6
GG3-rev	-	CAAGTTTCGTTATGATGAGG	6
GG4-rev	-	TCCAAAAAGTCCGMAATC	6

[1]Hao et al., 2021
[2]Jiang et al., 2017
[3]Wang et al., 2021
[4]Jiang et al., 2016
[5]Moreira et al., 2017
[6]LaFrentz et al., 2019

Table 4. Growth performance results from days 1-30, 30-60, and 1-60. CF32=32% high-quality fishmeal; C32=32% control soybean-based diet; C28=28% control soybean-based diet; C28+PC=28% protease complex diet; C28+HS=28% humic substance diet. TBG=tank biomass gain; RG=relative growth; SGR=specific growth rate; TGC=thermal growth coefficient; K-value=Fulton's condition factor; CC=cumulative consumption (feed administered); FCR=feed conversion ratio. All data presented are the mean ± SE (SE=Standard error of the mean for all) of three replicate tanks. Different letters denote significantly different dietary treatments, as discerned by Tukey's HSD.

Performance Parameter	**CF32**	**C32**	**C28**	**C28+PC**	**C28+HS**	***P*-value**
Days 1-30						
Initial weight (g)	1.54±0.30	1.5±0.01	1.54±0.02	1.57±0.01	1.57±0.02	*0.557*
Final weight (g)	4.59±0.11	4.56±0.06	4.33±0.12	4.63±0.16	4.68±0.20	*0.504*
Initial biomass (g)	152±2	154±1	154±2	157±1	157±2	*0.357*
TBG (g)	307±13	302±6	280±12	306±15	290±10	*0.446*
RG (%)	198±12	196±5	182±8	195±9	199±14	*0.772*
SGR (% d^{-1})	3.64±0.13	3.61±0.05	3.46±0.09	3.60±0.10	3.64±0.15	*0.757*
TGC	0.61±0.02	0.61±0.01	0.58±0.02	0.61±0.02	0.62±0.03	*0.671*
K-value	1.87±0.03	1.79±0.18	1.87±0.04	2.20±0.24	1.87±0.02	*0.310*
CC (g)	301±0	298±2	301±0	301±1	301±0	*0.443*
FCR	0.98±0.04	0.99±0.01	1.08±0.04	0.99±0.05	1.04±0.04	*0.375*
Survival (%)	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	99.7±0.3	*0.452*
Days 30-60						
Mid weight (g)	4.38±0.13	4.39±0.19	4.44±0.09	4.16±0.02	4.08±0.18	*0.328*
Final weight (g)	11.25±0.10	11.50±0.14	10.65±0.10	10.62±0.08	10.63±0.51	*0.082*
Mid biomass (g)	219±7	219±10	222±5	208±1	204±9	*0.321*
TBG (g)	343±2^{ab}	356±3^{a}	311±1^{b}	323±4^{ab}	328±18^{ab}	*0.028*
RG (%)	173±17	169±9	149±9	163±8	177±17	*0.746*
SGR (% d^{-1})	3.14±0.07	3.22±0.11	2.92±0.04	3.12±0.03	3.19±0.08	*0.085*
TGC	0.74±0.01^{ab}	0.76±0.02^{a}	0.68±0.01^{b}	0.72±0.01^{ab}	0.73±0.02^{ab}	*0.027*
K-value	2.06±0.12	2.02±0.04	1.76±0.04	2.09±0.18	2.28±0.10	*0.088*
CC (g)	339±1^{bc}	377±5^{a}	352±4^{c}	357±7^{ac}	366±4^{ac}	*0.002*
FCR	0.99±0.00^{a}	1.06±0.02^{ab}	1.13±0.01^{b}	1.10±0.00^{ab}	1.12±0.06^{ab}	*0.040*
Survival (%)	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	*1.000*
Days 1-60						
RG (%)	631±17	645±14	593±6	575±2	578±32	*0.063*
SGR (% d^{-1})	3.31±0.04	3.35±0.03	3.23±0.01	3.18±0.00	3.19±0.08	*0.063*
Survival (%)	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	99.7±0.3	*0.452*
TGC	0.66±0.01	0.67±0.01	0.64±0.00	0.63±0.00	0.63±0.02	*0.055*

Figures

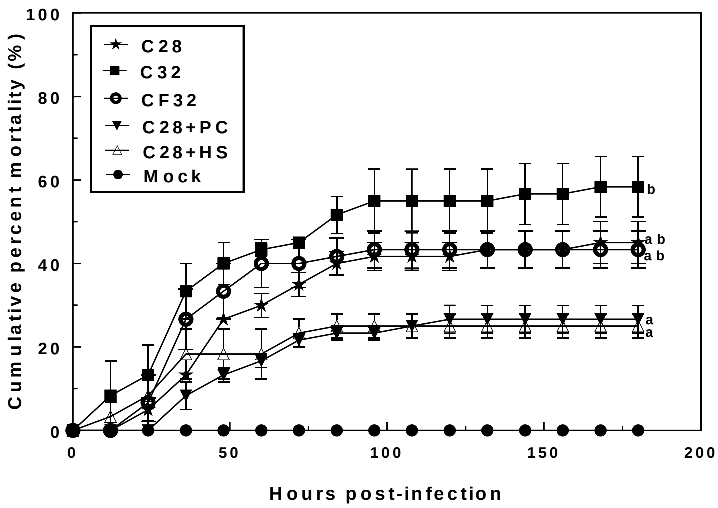

Figure 1. Cumulative percent mortality from *F. covae* infection over the entirety of the trial (8 days). Each treatment group had three tanks (*n*=3). Error bars represent the standard error of the mean for each day. CF32=32% high-quality fishmeal; C32=32% control soybean-based diet; C28=28% control soybean-based diet; C32=32% control soybean-based diet; CF32=32% high-quality fishmeal; C28+PC=28% protease complex diet; C28+HS=28% humic substance diet

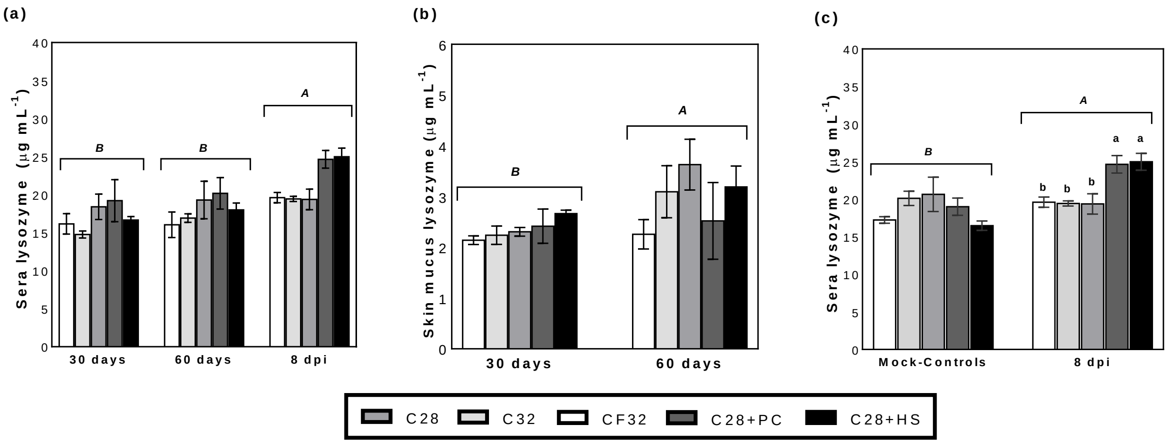

Figure 2. Lysozyme activity (mg mL^{-1}) from **a)** sera at 30 days, 60 days pre-challenge, and 8 days post-infection; **b)** body mucus of sampled fish at 30 days and 60 days of feeding pre-challenge.; and **c)** sera of infected and mock-control fish at 8 days post-challenge. Capital letters indicate significant differences (*P* > 0.05) in activity between sampling times, and lowercase letters represent significance within dietary treatment groups. All data presented are the mean ± SE (SE=Standard error of the mean for all) of three replicate tanks. C28=28% control soybean-based diet; C32=32% control soybean-based diet; CF32=32% high-quality fishmeal; C28+PC=28% protease complex diet; C28+HS=28% humic substance diet.

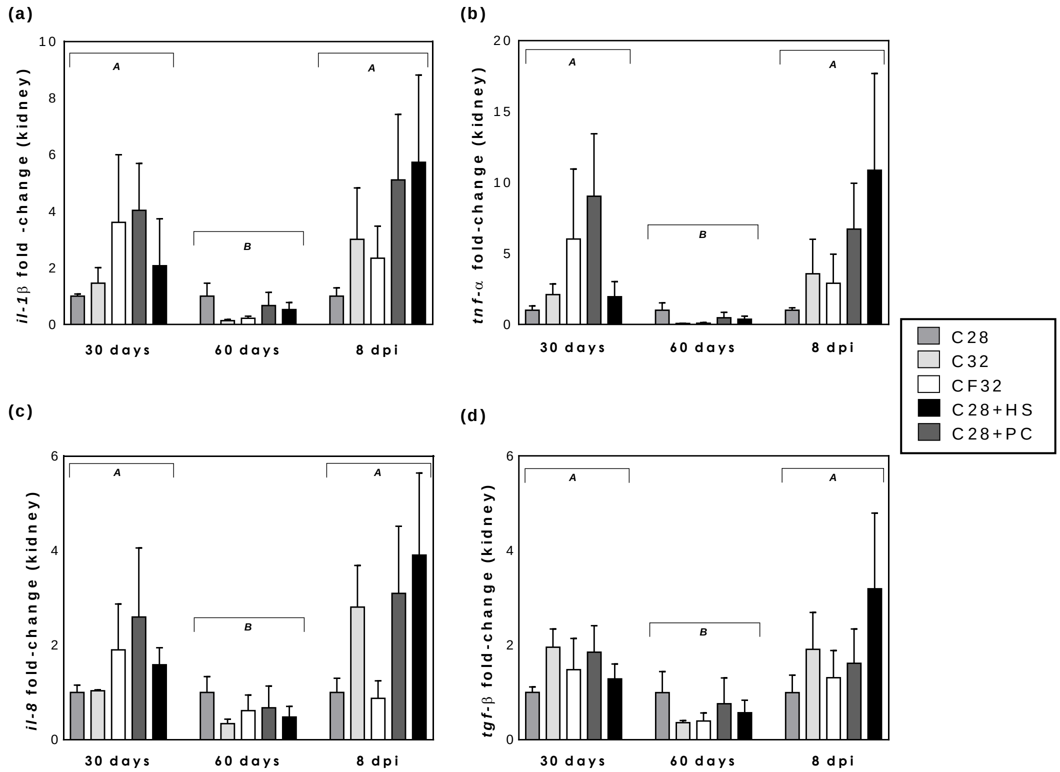

Figure 3. a) *il-1β* expression (fold change) was evaluated from extracted head kidney at 30 days, 60 days, and 8 days post-infection. **b)** *il-8* expression (fold-change) was evaluated from extracted head kidney at 30 days, 60 days, and 8 days post-infection. **c)** *tnf-α* expression (fold-change) was evaluated from extracted head kidney at 30 days, 60 days, and 8 days post-infection. **d)** *tgf-β* expression (fold-change) was evaluated from extracted head kidney at 30 days, 60 days, and 8 days post-infection. Capital letters indicate significant differences in quantity between sampling times, and lowercase letters represent significance within dietary treatment groups. All data presented are the mean ± SE (SE=Standard error of the mean for all) of three replicate tanks. C28=28% control soybean-based diet; C32=32% control soybean-based diet; CF32=32% high-quality fishmeal; C28+PC=28% protease complex diet; C28+HS=28% humic substance diet. Gene expression data were first normalized to *actb* and *ef1a* and then adjusted to the C28 dietary treatment group for comparison ($2^{-\Delta\Delta Ct}$ methodology). The analysis was performed on log-transformed data.

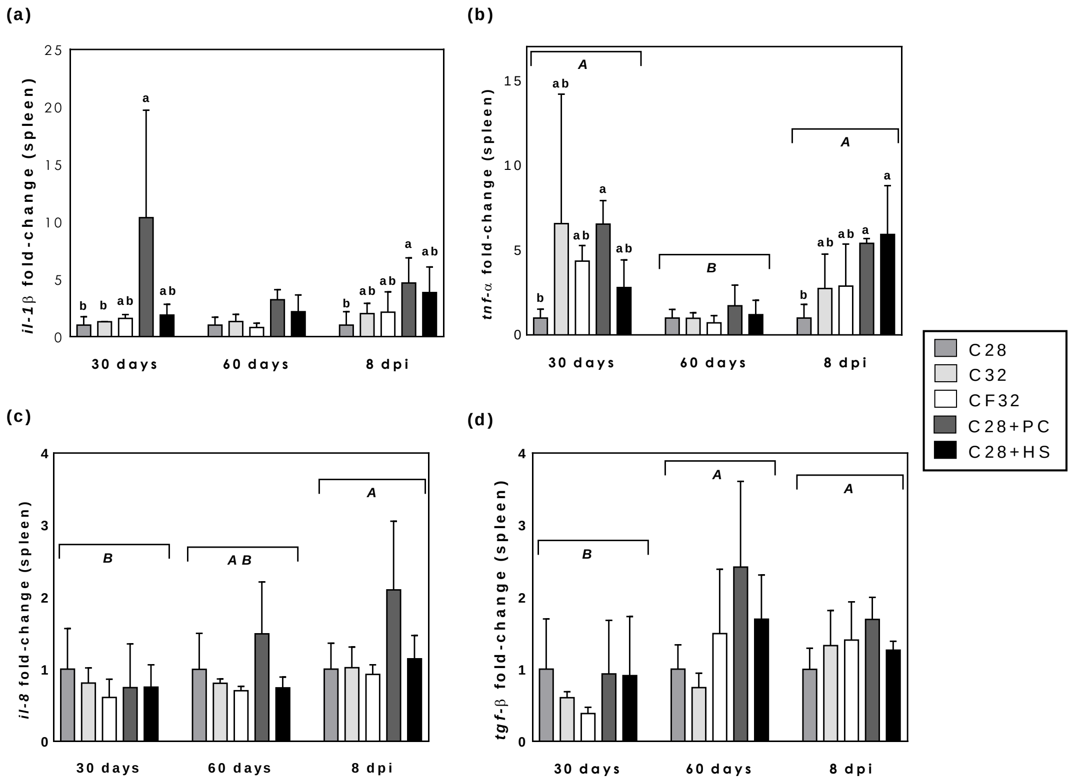

Figure 4. a) *il-1β* expression (fold change) was evaluated from extracted spleen at 30 days, 60 days, and 8 days post-infection. **b)** *il-8* expression (fold-change) was evaluated from extracted spleen at 30 days, 60 days, and 8 days post-infection.). **c)** *tnf-α* expression (fold-change) was evaluated from extracted spleen at 30 days, 60 days, and 8 days post-infection. **d)** *tgf-β* expression (fold-change) was evaluated from extracted spleen at 30 days, 60 days, and 8 days post-infection. Capital letters indicate significant differences in quantity between sampling times, and lowercase letters represent significance within dietary treatment groups. All data presented are the mean ± SE (SE=Standard error of the mean for all) of three replicate tanks. C28=28% control soybean-based diet; C32=32% control soybean-based diet; CF32=32% high-quality fishmeal; C28+PC=28% protease complex diet; C28+HS=28% humic substance diet. Gene expression data were first normalized to *actb* and *ef1a* and then adjusted to the C28 dietary treatment group for comparison ($2^{-\Delta\Delta Ct}$ methodology). The analysis was performed on log-transformed data.

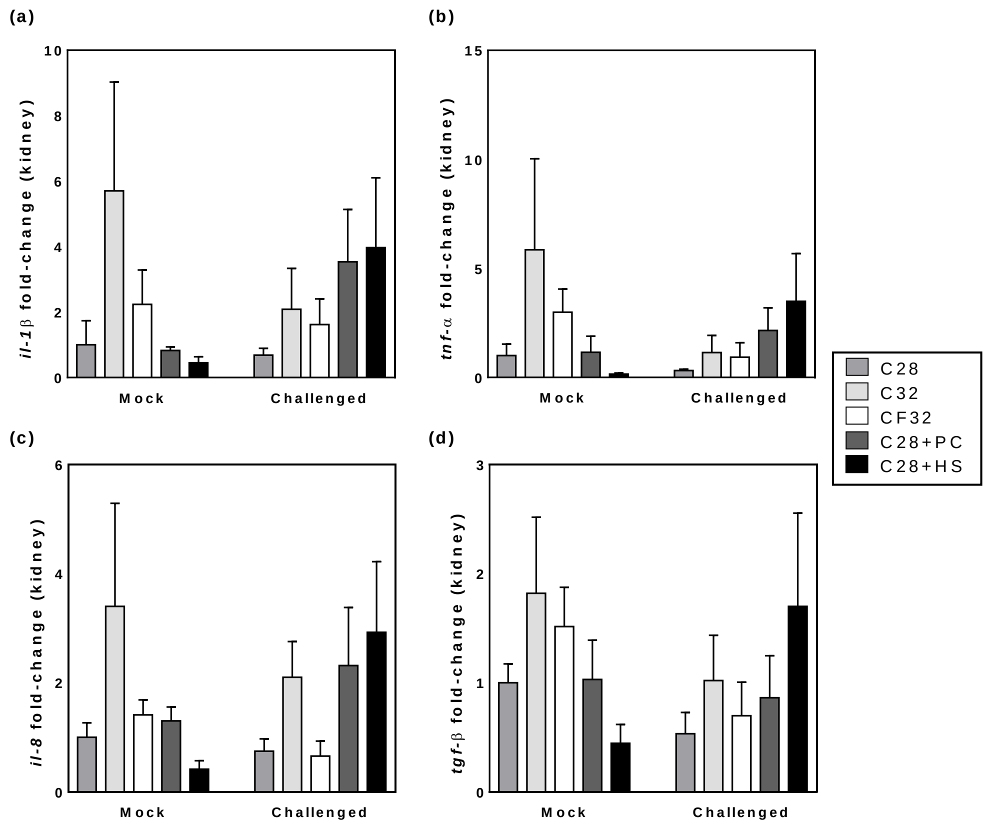

Figure 5. a) *il-1β* expression (fold change) was evaluated from extracted head kidney of the mock-control and infected group (8dpi). **b)** *il-8* expression (fold-change) was evaluated from extracted head kidney of the mock-control and infected group (8dpi). **c)** *tnf-α* expression (fold-change) was evaluated from extracted head kidney of the mock-control and infected group (8dpi). **d)** *tgf-β* expression (fold-change) was evaluated from extracted head kidney of the mock-control and infected group (8dpi). Capital letters indicate significant differences in quantity between sampling times, and lowercase letters represent significance within dietary treatment groups. All data presented are the mean ± SE (Standard error of the mean for all) of three replicate tanks. C28=28% control soybean-based diet; C32=32% control soybean-based diet; CF32=32% high-quality fishmeal; C28+PC=28% protease complex diet; C28+HS=28% humic substance diet. Gene expression data were first normalized to *actb* and *ef1a* and then adjusted to the mock-control C28 group for comparison ($2^{-\Delta\Delta Ct}$ methodology). The analysis was performed on log-transformed data.

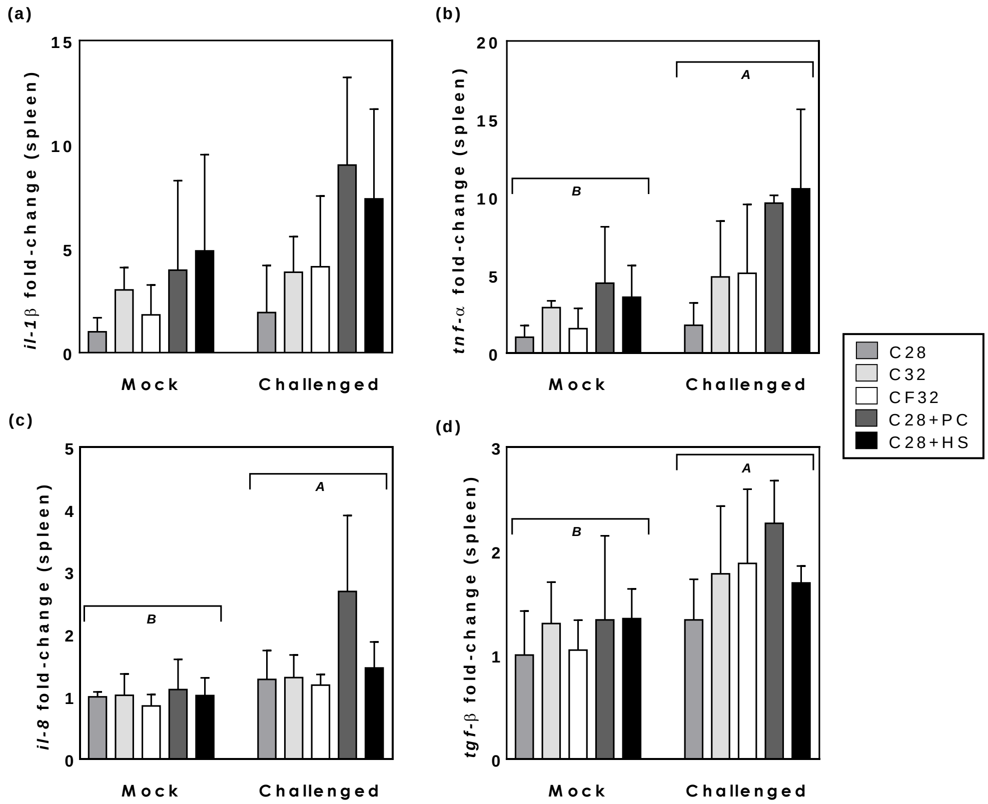

Figure 6. a) *il-1β* expression (fold change) was evaluated from the extracted spleen of the mock-control and infected group (8dpi). **b)** *il-8* expression (fold-change) was evaluated from the extracted spleen of the mock-control and infected group (8dpi). **c)** *tnf-α* expression (fold-change) was evaluated from the extracted spleen of the mock-control and infected group (8dpi). **d)** *tgf-β* expression (fold-change) was evaluated from the extracted spleen of the mock-control and infected group (8dpi). Capital letters indicate significant differences in quantity between sampling times, and lowercase letters represent significance within dietary treatment groups. All data presented are the mean ± SE (SE=Standard error of the mean for all) of three replicate tanks. C28=28% control soybean-based diet; C32=32% control soybean-based diet; CF32=32% high-quality fishmeal; C28+PC=28% protease complex diet; C28+HS=28% humic substance diet. Gene expression data were first normalized to *actb* and *ef1a* and then adjusted to the mock-control C28 group for comparison ($2^{-\Delta\Delta Ct}$ methodology). The analysis was performed on log-transformed data.

Chapter 2:

Long-term administration of exogenous dietary protease and humic substance on immunity, growth, and survival of channel catfish (*Ictalurus punctatus*) over-wintered in an in-pond raceway system

2.1 Abstract

Numerous studies have demonstrated the effects of dietary immunostimulants on growth and immune response in fish species. However, most existing research has focused on short-term applications, and the effects of continuous administration of certain dietary immunostimulants to fish species still need to be clarified. In our previous investigation, dietary protease complex (PC) and humic substance (HS) improved growth and feed utilization after 30 days. They enhanced innate immunity and disease resistance when supplemented differently in channel catfish (*Ictalurus punctatus*) diets after 60 days in an indoor system. However, continuous use of immunostimulants may up-regulate the immune system or cause tolerance or immunosuppression. Consequently, this study aimed to evaluate the long-term effects of PC or HS dietary inclusion on the growth, survival, and innate immunity indices in channel catfish raised in commercial rearing systems. A 270-day continuous feeding trial was conducted in nine in-pond raceway systems (IPRs), each with 238 sub-adult channel catfish with treatments triplicated. Raceways were randomly assigned to one of three 28% crude protein diet treatments: a control diet (C28), a PC diet (C28 + PC), or an HS diet (C28 + HS). Growth sampling was performed every 30 days, and the final growth metrics, innate immune response, and gene expression were evaluated at the end of the 270-day feeding period. The results showed that the C28 + PC group exhibited a lower feed conversion ratio than fish offered C28 (P = 0.048), indicating improved efficiency of feed utilization. Conversely, fish offered C28 + HS had lower biomass gain than those reared on the C28 (P = 0.025). Fulton's K-factor was higher in the C28 +

HS group than C28 + PC ($P = 0.033$) and control ($P = 0.002$) groups. Control (C28) had higher survival rates than both the C28 + PC ($P = 0.001$) and C28 + HS ($P < 0.001$) groups. Sera lysozyme activity was enhanced in the C28 + PC group compared to both the C28 + HS ($P = 0.004$) and C28 groups ($P < 0.001$). Similarly, the C28+HS group exhibited elevated sera lysozyme activity compared to the C28 group ($P = 0.007$). Immune-related genes from the spleen and head kidney did not have differential expression. However, a trend in the head kidney suggested slight downregulation of *il-1β*, *il-8*, and *tnf-α* genes in the C28+HS group. These findings demonstrated that including PC or HS in diets could improve growth and enhance the non-specific immune response of channel catfish in raceway systems used as grow-out systems. However, long-term exposure to these dietary immunostimulants may lead to heightened immune regulation and, ultimately, reduced survival.

2.2 Introduction

Channel catfish (*Ictalurus punctatus*) and hybrid catfish (♀ *I. punctatus* X ♂ blue catfish *I. furcatus*) production remains the dominant contributor to the overall aquaculture output in the United States, accounting for more than 52% of the total production volume (NOAA Fisheries, 2020). Adopting intensive production methods, such as split ponds and intensive aeration, has facilitated high fish productivity, allowing for multiple batch stockings and increased stocking densities in production systems (Kumar et al., 2018). Despite these advancements, the intensification of commercial catfish aquaculture has also introduced challenges, such as disease outbreaks, that hinder the achievement of increased stocking densities (Abdelrahman et al., 2017; Wagner et al., 2002). Overcrowding and immune system alterations induced by high stocking densities have made fish populations susceptible to environmental pathogen infections. However, disease outbreaks in commercial catfish production have resulted in substantial economic losses, \$16.9 million in East Mississippi in 2016 (Peterman & Posadas, 2019) and \$11.1 million annually in West Alabama (Abdelrahman et al., 2022) due to reduced survival rates and weight loss caused by poor feeding behavior (Plumb & Hanson, 2010). As a result, disease management is crucial in commercial catfish farming to maintain optimal fish health, reduce economic losses, and sustain the industry's growth.

Feed additives, when used as immunostimulants, have been a non-stressful method of enhancing the fish immune system and allowing mass application regardless of fish size (Wang et al., 2016). In addition, dietary additives may decrease culture costs by improving feed efficiency through enhanced digestibility of alternative protein sources used to replace fishmeal as the sole protein source in fish feed (Drew et al., 2005; Shi et al., 2016; Hassaan et al., 2019). The use of exogenous protease enzyme supplementation has been reported to be beneficial to growth performance in tilapia *(Oreochromis niloticus; O niloticus x O. aureus)* (Adeoye et al., 2016; Wu et al., 2020; Lin et al., 2007; Huan et al., 2018), white shrimp *(Litopenaeus vannamei)* (Li et al., 2016; Song et al., 2017), Japanese seabass *(Lateolabrax japonicus)* (Huang et al., 2020) and gibel carp *(Carassius auratus gibelio)* (Shi et al., 2016). Hassaan et al. (2021) and Wu et al. (2020) have also reported improved intestinal microbial growth and immune responses in tilapia following protease treatment. Commercial protease product (AG175; Jefo Nutrition Inc. Ca) is a fermentation by-product with reports of high-temperature stability (Li et al., 2016), which was beneficial in boosting feedstuff protein digestibility and promoting intestinal gut health (Shi et al., 2016).

Humic-based reed-sedge peat (RSP) extracts have been used as feed supplements in animal diets and various clinical applications. RSP is especially recommended for stimulating the growth and the immune system of piglets and sows. (Pavlik et al., 2003). The report suggests that the organic derivative, when added to the animal diet, protects the sensitive mucosal layer of the digestive system, stomach, and intestine, thereby decreasing the incidence of enteric illness and diarrhea, particularly in young animals (Trckova et al., 2005). The absence of animal allergy and resistance, ease of administration, and lack of residues in animal products (Kuhnert et al., 1989, 1991; Lange et al., 1996) suggest that RSP may be beneficial to stimulate growth and strengthen fish immune systems. While these functional feed additives may be crucial to the aquaculture's success, however, it has been understood that the timing, dose, and length of administration can influence the performance of dietary additives in fish species (Ortuno et al., 2000; Galindo-Villegas et al., 2006; Misra et al., 2006; Wang et al., 2016).

In our previous study, oral administration of an exogenous protease complex product (PC) and a commercial humic substance (HS) improved the non-specific immunity of juvenile channel catfish reared at indoor facilities, providing significant resistance against *Flavobacterium covae* in an *in vivo* challenge trial (Oladipupo et al., 2023). Although insignificant, the supplementation of protease complex and humic substance in our study also improved body weight gain compared to a non-supplemented diet after a 30d feeding period. However, we do not know the effect of these immunostimulants when continuously fed to channel catfish, especially in outdoor commercial production systems faced with multiple environmental pathogens. More specifically, exploring the long-term administration of dietary PC and HS on catfish growth and survival in grow-out applications will provide information on using these additivities in commercial catfish culture. Hence, this study investigated the effects of the prolonged application of dietary PC and HS on the immunity, growth, and survival of sub-adult channel catfish reared over winter in a commercial culture system. We hypothesized that dietary-stimulated catfish would have better growth, enhanced immunity, and survival during a grow-out production period than those fed a commercial control diet.

2.3 Materials and Methods

2.3.1 *Culture System*

This study was conducted in nine, 4 m^3 in-pond raceways (IPRs) installed on a 0.4 ha, traditional, earthen pond at the E.W. Shell Fisheries Center, Auburn University, Auburn, Alabama (Fig. 7). The culture system consisted of individual raceways attached to a floating wooden bridge and a 203.2mm airline that ran through a connecting bridge. The earthen pond was supplied by watershed runoff and rain, and each raceway was installed to allow water to flow through using an airlift system. Each raceway was made with PVC reinforced with galvanized iron and PVC floats for suspension. A 50.8mm airline was sub-channeled perpendicular to the major airline, diffusing air directly to each raceway and simultaneously promoting water flow. Primary air supply to the systems was generated by three 1.5hp single-stage regenerative blowers (Sweetwater, Aquatic Ecosystems, Inc., Apopka, FL, USA) stationed at the pond levee. Temperature, DO concentration, and pH were measured *in situ* using a YSI-ProPlus sonde at 8:00h and 16:00h before feeding. Water samples were collected weekly and analyzed for total ammonia nitrogen, nitrite nitrogen, alkalinity, and hardness using YSI colorimetric test kits.

2.3.2 *Experimental fish, feed and feeding trial*

Subadult channel catfish with an average body weight (mean ± SE) of 200±12 g initially reared in earthen ponds at the E.W. Shell Fisheries Center, Auburn, Alabama, were stocked and retained in the nine raceways for two weeks before experimental feeding to acclimate to the raceway structure. The three diets formulated for the feeding trial consisted of a control diet without additives and two treatment diets. The control diet (C28) is a 28% crude protein commercial channel catfish diet (SouthFresh Feeds, Demopolis, AL, USA). The second diet was supplemented with protease complex at 175 g ton^{-1} (C28+PC), while the third was supplemented with humic substance (C28+HS). The protease complex (PC) product (AG175; Jefo Nutrition Inc., QC, Canada) is an alkaline serine endopeptidase produced from bacterial fermentation. Humic substance product (MFG50; Kent Nutrition Muscatine, IA, USA) is a broad-spectrum organic acid extract from freshwater reed-sedge peat. All three diets were extruded as 8 mm floating pellets according to the commercial extrusion process used by SouthFresh Feeds.

Fish in the IPRs were randomly assigned to three diet groups in triplicate (two hundred and thirty-four fish per tank, 702 per group). Feed was distributed until the fish reached apparent satiation twice (8:00h and 16:00h) daily based on the response and health condition for 270 days. Feed distributed in individual raceway were recorded daily. Daily system maintenance includes scrubbing mesh screens of individual raceways to remove debris and grasses, mortality check, and carcass removal. The weight and number of mortalities were recorded to estimate total biomass. Moribund or freshly dead fish were assessed for clinical signs and necropsied for disease diagnosis. All treatment recommendations commonly used and accepted at Auburn University, such as potassium permanganate and formalin, were followed to treat external parasites following diagnostics. The application of potassium permanganate (3ppm) and formalin (200ppm) through a 1 h bath was performed within all raceways, and dissolved oxygen was monitored throughout the treatment.

Fish were sampled every 30 days of feeding for individual weight and length measurements to discern the average body weight and condition factor. Twenty-five fish were randomly sampled and anesthetized using 100 mg L^{-1} buffered tricaine methanesulfonate (MS-222, Syndel, Ferndale, WA, USA). After measurement, fish were allowed to recover in a 60 L bucket filled with pond water before returning to their respective raceway tanks. The monthly estimated biomass in each IPR unit was adjusted by subtracting mortalities from the number of fish stocked and multiplying that value by the average body weight (ABW). The estimated final tank biomass over time of each IPR unit was calculated by adding the estimated biomass in each RW with the total biomass of mortality (Bruce et al., 2021). A weighted average and biomass from the initial day (ABW at Day 0) to sample day X (e.g., ABW at Day 90) were used to determine growth parameters at each sampling time.

At 270 d, individual raceway tanks were harvested, group-weighed, and counted. Five fish per tank were also sampled for blood (serum) to investigate serum lysozyme activity or kidney and spleen tissue for immune gene expression. Similarly, the final harvested biomass from each raceway was added to the respective mortality biomass to discern the final tank biomass. Growth and survival data were analyzed from 1 d to 270 d. The performance parameters were calculated as the following equations:

Tank biomass gain (TBG, g) = Final raceway biomass (g) - Initial raceway biomass (g)

Weight gain (WG, %) = 100[final body weight (g)-initial body weight (g)]/initial body weight (g)*

Thermal growth coefficient (TGC) = 1000[(final body weight$^{1/3}$ (g) - initial body weight$^{1/3}$ (g)) / (time (days)* temperature (^{0}C))*

Growth per day (GPD, g) = [Final weight gain (g) / Final quantity] / time (days)

Cumulative consumption (g) = Sum of feed offered from start to end of the study

Feed conversion ratio (FCR) = feed intake (g) / [final body weight (g) − initial body weight (g)]

*Fulton’s condition factor (K-value) (g [mm^3]$^{-1}$*10,000) = 100*[final body weight (g)/standard body length (cm)]*

Survival (%) = 100[final quantity/initial quantity]*

2.3.3 *Proximate analyses*

Test diets were sent to Midwest Laboratories (Omaha, NE, USA) for proximate analyses. Moisture (AOAC 930.15), crude protein (AOAC 990.03), crude fat (AOAC 2003.05), fiber (acid detergent via ANKOM), and ash (AOAC 942.05) were all determined. Total digestible nutrients (TDN) and digestible energy (DE) were calculated using the online finished feed matrix software at Midwest Laboratories (Table 8). For the calculations, the laboratory used a protein factor (Pf) of 0.73, a fat factor (Ff) of 2, an acid detergent fiber factor (ADFf) of 0.58, and a nitrogen-free extract factor (NFEf) of 0.90. The factors are based on unique feedstuff sample types within the software. The software calculations were based on the following equations (P=protein, F=fat, A=ash, ADF=acid detergent fiber, and NFE=nitrogen-free extract):

$$NFE=100-(P+F+ADF+A)$$

$$TDN=(P*Pf)+(F*Ff)+(NFE*NFEf)+(ADF*ADFf)$$

$$DE=20*TDN/1000$$

2.3.4 *Serum lysozyme activity*

The lysozyme activity in the serum was assayed by the method of Welker et al. (2012). Briefly, the assay is based on lysozyme-sensitive Gram-positive bacterium *Micrococcus lysodeikticus* (Worthington Biochemical, Lakewood, NJ, USA) lysis by lysozyme present in serum samples. A suspension of 0.25 mg mL^{-1} freeze-dried *M. lysodeikticus* was made shortly before use by dissolving it in sodium phosphate buffer (0.04 M Na_2HPO_4, pH 6.0). Serum (10 µL $well^{-1}$, in duplicate) was placed in a microtiter plate, and 250 µL of bacterial cell suspension was added to each well. Hen egg white lysozyme was used as an external standard. The initial and final (after 20 min incubation at 35 ^{o}C) sample absorbances were measured at 450 nm using a microplate reader (BioTek Synergy HTX, Agilent, Winooski, VT, USA). The rate of reduction in absorbance of samples was translated to lysozyme concentration (μg mL^{-1}) using a standard curve.

2.3.5 *RNA extraction, cDNA synthesis and quantitative RT-PCR*

RNA was extracted from the spleen and head kidney of the channel catfish using the Quick-RNA MiniPrep Plus kit (Zymo Research Corp.) according to the manufacturer's recommendations. After the extraction, the concentration and quality of the RNA were confirmed with a Nanodrop One^{c} spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA), ensuring the 260/280 ratios were between 1.8 and 2.0. Extracted spleen and kidney RNA samples were diluted to 20 ng μL $^{-1}$ according to the manufacturer's instructions, and RNA was synthesized into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). Briefly, each 20 μL reaction contained 2 μL of 10x R.T. buffer, 0.8 μL of 25x dNTP Mix, 2 μL of 10x R.T. random primers, 1 μL of kit Multiscribe reverse transcriptase, 10 μL of template RNA and 4.2 μL of nuclease-free water. cDNA samples were synthesized in a MiniAmp^{TM} plus thermal cycler (Applied Biosystems). Program conditions were set at 25 °C for 10 minutes, 37 °C for 120 minutes, and 85 °C for 5 minutes. After the reaction, the cDNA was stored at -20°C. Quantitative real-time PCR was conducted with QuantStudio 5 Real-Time PCR (Applied Biosystems) using the PowerUp SYBR Green Master Mix (Applied Biosystems). All cDNA samples were diluted to 1.25 ng μL $^{-1}$ prior to the reaction. Each 10 μL reaction consists of 5 μL of master mix, 0.5 μL each of forward and reverse primer (10 μM stock), and 4 μL of diluted sample cDNA. Each PCR was performed with duplicate samples, and the cycling conditions were 2 min at 50 °C, 2 min at 95 °C followed by 15 s at 95

°C, 15 s at 58 °C and 30 s at 72 °C for 40 cycles. In addition, a melt curve analysis was performed after amplification to verify product specificity. Four genes of interest, *il-1β*, *tnf-α*, *il-8*, and *tgf-β* were assessed, and the relative quantification of each gene (i.e., ΔCt) was determined through normalization against two housekeeping genes (*ef-1α* and *actb)*. PCR primers used in this study are described in Table 9. Further, the assessed gene from each dietary group was normalized against the control diet (C28). The relative abundance of interested genes was calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

2.3.6 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 9.51 (GraphPad Software, CA, USA). Monthly fish growth performance and mortality data observed over time were analyzed using Two-way repeated measures ANOVA to determine the main effect of diet, time, and interaction (α = 0.05). Final growth parameters at harvest (270 d), serum lysozyme activity, and gene expression in kidney and spleen tissue were assessed using a one-way ANOVA (α = 0.05). Gene expression data were log-transformed prior to analysis. The normality of the model residuals was assessed using a Shapiro-Wilk test, and variance homogeneity was evaluated using a homoscedasticity plot in conjunction with Spearman's test for heteroscedasticity. Statistical analyses considered tanks as experimental units. Tukey's post hoc testing was conducted when differences were significant (P<0.05) to identify which groups were different. All data were expressed as mean ± standard error.

2.4 Results

2.4.1 *Growth Performance and Survival*

As shown in Table 5, water quality variables from this study were compared with the typical commercial catfish culture conditions. Dissolved oxygen, pH, total ammonia nitrogen, and nitrite were within the catfish range for pond culture. The low alkalinity and hardness were likely related to the water supply and pond bottom but still within an acceptable range (Tucker & Robinson 1990).

Dietary supplementation with PC and HS affected overall catfish survival (P>0.001). The control group without dietary additive supplementation (C28) showed a significantly higher survival rate than the supplemented diets C28+PC (P > 0.001) and C28+HS (P > 0.001) after 270 d of dietary treatment. Diagnostic examinations performed on sick and moribund fish revealed mainly inflamed gills caused by the monogenean parasite *Datylogyrus spp* and isolation of *Aeromonas hydrophila* from the kidney and spleen. Additionally, trophonts of *Ichthyophthirius multifiliis* were occasionally found on skin samples, leading to the application of formalin and potassium permanganate for parasite control.

Total biomass gain (TBG), growth per day (GPD), feed conversion ratio (FCR), thermal growth coefficient (TGC), Fulton's condition factor (K-factor) as well as survival were calculated and presented in Table 6. The control group (C28) had significantly higher biomass gain than C28+HS (P=0.025). However, the fish receiving a protease-supplemented diet (C28+PC) had significantly lower FCR than the fish offered the control diet (C28) (P=0.048) but insignificant compared to the C28+HS fish group (P = 0.136). Growth per day was not significantly different across the dietary groups (P = 0.061). However, biologically, the higher increase observed in both C28+PC and C28+HS compared to C28, as shown in Table 6, is significant, as daily growth in the PC and HS groups was twice that of the control group. No significant difference in TGC among the groups was detected at the end of this study (P = 0.776). A significantly varied cumulative consumption (CC) was observed among the dietary group at the end of this study (P > 0.001). Tukey's comparison test revealed that the C28 group had significantly higher cumulative consumption than C28+HS (P > 0.001) and C28+PS (P = 0.005). Also, C28+PC had significantly higher consumption than C28+HS (P = 0.040). Fulton's K-factor is used to evaluate the fish's weight condition and nutritional status based on length and weight (Fulton, 1911). As shown in Table 6, dietary group C28+HS had a significantly higher K-factor than C28+PC (P = 0.033) and C28 (P = 0.002).

2.4.2 *Lysozyme Activity*

Results from the lysozyme analyses are presented in Fig. 8. Sera lysozyme activity within all dietary treatment groups was significantly different (P > 0.001) when analyzed at 270 d. Specifically, the protease-supplemented diet (C28+PC) had the most sera lysozyme activity, which was considerably higher than the control (C28) (P = 0.007) and humic diet (C28+HS) (P = 0.004). Similarly, sera lysozyme in the C28+HS fish group significantly increased compared to C28 (P = 0.005). This result indicated a significant increase in sera lysozyme in the catfish group fed immunostimulant diets compared to those without both immunostimulants.

2.4.3 *Immune gene expression*

Immune gene expression in the head kidney, and spleen did not appear to be impacted by the dietary treatment after 270 d, as shown in Fig. 9 and 10. For pro-inflammatory cytokines in the head kidney, no significant difference was observed in the expression of *il-1β* (P = 0.718), *il-*

8 (*P* = 0.339), or *tnf-α* (*P* = 0.683). Similarly, in the splenic tissue, the expressions of *il-1β* (*P* = 0.828), *il-8* (*P* = 0.828), and *tnf-α* (*P* = 0.978) were not different among the three dietary treatments. In addition, no significant expression of *tgf-β* was discerned in the head kidney (*P* = 0.154) or spleen (*P* = 0.842) among the dietary group after 270 d.

Although this study did not find significant expression of the selected cytokines in both the head kidney and spleen due to protease complex or humic substance dietary inclusion, a numerical fold-change pattern was observed in both immune tissues. Pro-inflammatory cytokines (*il-1β*, *il-8*, and *tnf*-α) expressed in the head kidney were observed to be similarly expressed in the control (C28) and protease-supplemented diet (C28+PC) but down-regulated in the humic-supplemented diet (C28+HS) group. However, the expression of *tgf-β* in the head kidney of the C28+HS fish group was improved and similarly regulated compared to C28. In contrast, the C28+HS fish group had improved regulation of *il-Iβ*, *il-8*, *tnf-α*, and *tgf-β* in the spleen tissue compared with C28.

2.5 Discussion

The use of natural immunostimulants has improved aquaculture production, positively impacting fish farming and modifying the biological response of some hematological and immunological parameters (Mohammadi et al., 2020; Jami et al., 2019; Mona et al., 2015) and enhancing the response of fish to infectious agents (Heidarieh et al., 2013). In our previous study, incorporating a protease complex (PC) and humic substance (HS) in the diet of juvenile channel catfish resulted in improved growth and enhanced non-specific immunity against *F. covae* infection. However, concerns have been raised about the continuous use of immunostimulants, as they may up-regulate the immune system or cause adverse effects like tolerance or immunosuppression. Consequently, existing trials have often been too short (3-5 weeks) to provide conclusive results for large-scale production systems. Therefore, it is necessary to rigorously test the long-term effects of the oral application of PC and HS on a large-scale production system throughout an entire crop cycle.

2.5.1 *Effects on growth and survival of channel catfish*

Positive growth occurred in the C28+PC and C28+HS groups after 270 d of feeding. However, it is noteworthy that the C28 exhibited a significantly higher survival rate than the PC and HS groups. This suggests that prolonged supplementation of PC or HS may lead to immunosuppression while promoting further growth in channel catfish. The increased mortality observed in the supplemented groups could be attributed to the application duration, inclusion dose, and potential interactions between both. We have provided some insights into possible mechanisms and hypotheses underlying this effect.

The dietary inclusion of PC resulted in a significantly reduced feed conversion ratio (FCR) compared to the control group, indicating improved feed efficiency within this dietary group. Similarly, the daily growth was highest in C28+PC. This study confirms the potential for long-term administration of PC in commercial production systems, building upon our previous findings that demonstrated growth improvement in juvenile catfish after 30 days of PC supplementation in a low-protein diet. Prior studies have also reported positive growth and enhanced nutrient utilization in fish species when continuously fed exogenous protease. For example, significant improvements in FCR and weight gain were observed in hybrid tilapia (*Oreochromis niloticus x O. aureus)* (Lin et al., 2007) and Nile tilapia *(Oreochromis niloticus)* raised in outdoor in-concrete pens (Hassaan et al., 2021) when fed a PC-supplemented diet for 84 days. Comparable results were observed in gibel carp over the same feeding duration (Shi et al., 2016). Notably, the extended application of protease has not been reported to affect fish growth performance adversely. Contrary reports (Adeoye et al., 2016; Dias et al., 2014) have shown no growth effects in fish when PC was supplemented in high protein diets. They concluded that the non-effect of protease in a relatively higher crude protein diet could be because of the non-beneficial effects of digestible protein when the level exceeds the requirement for fish maintenance and growth.

The growth-promoting effects of humic substances (HS) have been previously documented in aquatic animals (Fierro-Coronado et al., 2018; Nakagawa et al., 2009; Kodama et al., 2007), but their potential as dietary additives for aquaculture remains underexplored. In this study, channel catfish fed with HS for 270 days exhibited higher percent weight gain (WG), growth per day (GPD), and improved feed conversion ratio (FCR) compared to the control group (C28). Although the observed differences were not statistically significant in this study, the results suggest that HS supplementation may enhance catfish growth in continuous application. Similar findings have been reported in pigs by Wang et al. (2008), who suggested that the effects of HS may be influenced by the duration of administration, with long-term supplementation potentially yielding more prominent growth effects. Weinreich et al. (2002) also highlighted the potential benefits of including HS in the later stages of broiler diets for improved growth performance. However, the C28+HS group in this study had significantly lower biomass gain than the fish fed C28+PS (protease complex supplementation) and the control group (C28). This discrepancy could be attributed to the significantly lower survival rate observed in the C28+HS. Although Eren et al. (2000) reported significant weight gain in broilers only after 42 days of HS supplementation, whereas our research spanned a continuous dietary treatment period of 270 days. In support, Stepchenko et al. (1991) found a significant weight gain in chickens fed the Menefeed® humate diet from 8 to 12 weeks, but the significant effect did not persist beyond 12 weeks.

The reduced survival observed in the C28+HS and C28+PC groups may be attributed to the prolonged exposure of catfish to high dietary PC or HS. This finding aligns with Bailey et al. (1996), who observed increased mortality but also higher body weight after 42 days of including 5 g kg^{-1} Menefeed® humate in the diet of broilers. Conversely, Teravita (2004) reported a 50% increase in poultry survival with improved live weight after 35 days of HS supplementation.

Some studies have reported the susceptibility of fish to parasites under conditions of dietary overstimulation. For instance, a high inclusion dose of 5.0% glucan increased the susceptibility of rainbow trout to *I. multifiliis* (Jaafar et al., 2011). Similarly, oral inclusion of humic acid (100 mg kg^{-1} body weight) in mice induced structural and numerical chromosomal abnormalities in intestinal cells (Bernacchi et al., 1996). Furthermore, studies have suggested that the potential bio-toxicity of humic substances may be associated with their structural composition and diversity (Meinelt et al., 2007; Steinberg et al., 2007). However, the structural components of the humic product used in this study were not analyzed, and it is believed that not only the dosage and duration of application but also the structural components may interact with disease susceptibility in the oral application of immunostimulants (Raa. 1996; Lieke et al., 2020).

In commercial catfish systems, Southworth et al. (2006a) reported an average survival rate of 24% to 36% for catfish grown in traditional ponds using multiple-batch conditions. Conversely, under single-batch conditions, the same research group (Southworth et al., 2006b) found higher survival rates of 67% to 83%. Similar to our study, Brune et al. (2004) observed survival rates as low as 25% when catfish were over-wintered in a partitioned aquaculture system, with the cause of mass mortality attributed to proliferative gill disease. This current study showed a generally high FCR (>3.0), which is linked to low survival rates, which is consistent with the findings of Ruane et al. (1977) and Lewis and Wehr (1976). However, Brune et al. (2004) stated that catfish could be successfully grown in commercial-scale systems with an FCR of less than 1.8.

2.5.2 *Effects on innate immunity*

The immune system is pivotal in the immune response of fish (Tort et al., 2003; Rauta et al., 2012), and the presence of bacteriolytic enzymes in serum, mucosal membranes, and leukocyte-rich tissues contributes to the nonspecific defense mechanism (Uribe et al., 2011; Ángeles Esteban. 2012). Lysozyme, found in fish serum, has the potential to enhance the innate defense mechanism by enzymatically breaking down the peptidoglycan present in bacterial cell walls, resulting in their lysis (Zapata-Vívenes et al., 2021; Wang et al., 2009). Several studies have highlighted the beneficial effects of oral additives in stimulating an increase in the microbiocidal capacity of fish, enabling them to protect themselves against pathogens effectively. Consequently, it may be necessary to consider dietary supplementation with immunostimulants to enhance disease resistance in fish species (Dawood et al., 2019)

Although studies have examined the immune potential of humic substances (HS) and protease complexes (PC), they have mainly focused on short-term applications (Yasar et al., 2002; TeraVita, 2004; Islam et al., 2005; Hassaan et al., 2019), including our previous study. However, the implications of continuously administering these immunostimulants over a long-term period remain understudied. In this study, channel catfish were fed diets containing PC or HS, resulting in increased lysozyme activity in the sera after a 270-day feeding trial compared to the control group (C28). Similar findings were reported by Gao et al. (2017b), who observed elevated lysozyme activity and IgM content in juvenile loach *(Paramisgurnus dabryanus*) following 60 days of dietary feeding, with increasing inclusion dosage of HS. In another study, high oral inclusion of fulvic acid, a derivative of HS, improved lysozyme activity and phagocytosis in juvenile rainbow trout *(Oncorhynchus mykiss)* (Lieke et al., 2021). Interestingly, our previous study showed that a lower dose of HS (0.003%) did not affect lysozyme activity in juvenile catfish after 60 days of dietary inclusion but demonstrated a significant increase in response to *F. covae*. However, in this current study with a higher dose (0.25%), significantly elevated lysozyme activity was observed after 270 days of dietary treatment. The dosage effect of immunostimulants on lysozyme activity is consistent with findings by Jaafar et al. (2011) and Adloo et al. (2015). Hassaan et al. (2021) reported elevated levels of hemoglobin, hematocrit, serum protein, immunoglobulin M (IgM), lysozyme, and phagocytic parameters in Nile tilapia fed a diet supplemented with exogenous protease and probiotics. Similar results were obtained when protease was supplemented with malic acid (Hassaan et al., 2020). However, studies have not been thoroughly performed to explore the immune potential of dietary exogenous protease.

Despite the observed increase in sera lysozyme levels, catfish offered PC and HS had significantly lower survival rates in this trial. In some previous catfish studies, the elevated immune response observed due to dietary immunostimulants did not protect against bacterial pathogens (Duncan & Klesius, 1996; Welker et al., 2007; Welker et al., 2011). However, Welker et al. (2007) inferred that enhanced immune function does not always correlate with increased protection against diseases. Although the mortalities in this current trial are primarily attributed to parasitic infections, it is hypothesized that parasitic pathogens may be able to evade the lethal effects of protective enzymes from innate protection. Alternatively, the gills and skin of the immunostimulated fish may have become immune-privileged sites (IPSs) due to heightened immune protection of more vulnerable areas with limited regenerative capacity. As a result, these sites may become preferential sites for parasite colonization (Sitjà-Bobadilla, 2008).

2.5.3 *Immune gene expression*

The epithelial and mucosal barriers and humoral and cellular components of the fish immune system work together to provide an initial defense response and develop specific defenses (Cain & Swan, 2010; Riera Romo et al., 2016). Inflammation triggers the release of various cytokines in lymphoid organs, including important pro-inflammatory cytokines like IL-1β, interferons, tumor necrosis factor-α, and anti-inflammatory cytokines such as transforming growth factor-β (TGF-β) (Shaikh. 2011; Zanotti et al., 2002). These cytokines play crucial roles in regulating both innate and acquired immune responses. Previous studies have suggested that the inclusion of humic substances (HS) in animal feed could potentially create a protective film on the gastrointestinal tract's mucus epithelium, offering protection against infectious agents and toxins (Arif et al., 2018; Islam et al., 2005) Also, Ehsani and Torki. (2010) inferred that exogenous enzymes might enhance the proliferation of macrophages and monocytes, and resultant cytokine production improved immune response in fish. However, no significant expression of immune-related genes was observed in the present study after long-term HS or PC feeding (270 days).

In the head kidney, pro-inflammatory genes *(il-1β, tnf-α, and il-8)* were downregulated in the C28+HS group compared to the control (C28) and C28+PC groups. Conversely, in the spleen, the C28+HS group exhibited improved expression of *il-1β, tnf-α*, and *il-8* compared to the control. However, the expression of *tgf-β* was similar in the head kidney, and spleen across all three diets. Exogenous protease supplementation has also been shown to enhance the humoral immune system by upregulating immunoglobulin M (IgM) in Nile tilapia (Hassaan et al., 2021) and Siberian sturgeon *(Acipenser barrii)* (Ghodrati et al., 2021). However, in those feeding studies, PC was co-supplemented with probiotics. Overall, while HS or PC did not significantly regulate immune-related genes after dietary treatment in this study, including both immunostimulants may positively impact the immune system in response to biological stressors, as observed in previous studies. Our previous study observed similar insignificant trends in the head kidney within the groups after 30d and 60d of feeding. However, despite no treatment differences, significant downregulation was observed in the pro-inflammatory genes at 60 d. The significant time differences suggested that the upregulation at 30 d compared to 60 d is likely due to stocking stress and acclimation. Following infection with *F. covae*, the inflammatory genes showed significant upregulation, particularly in the group supplemented with humic substances (HS), compared to the 60-day time point.

In another study involving rainbow trout *(Oncorhynchus mykiss)*, no significant differences in immune gene expression were observed after 82 days of feeding on organic substances. However, significant upregulation of *il-8* and *tnf-α* occurred when the fish were exposed to high water temperatures (Pelusio et al., 2020). Similar results were reported in Atlantic cod *(Gadus morhua)* exposed to short-term overcrowding (Caipang et al., 2008). Yarahmadi et al. (2016) also found a significant density-dependent downregulation of *il-1β* and *il-8*, with the lowest levels observed in the highest stocking density group. The differences observed between the present study and the mentioned findings can be attributed to the fact that short-term stressors (acute) and long-term stressors (chronic) have distinct effects on the fish immune system (Nardocci et al., 2014). These results highlighted the complex nature of stress and its impact on gene expression in fish. In a study conducted by Sanmiguel and Rondon (2016) on laying hens, adding 0.1% and 0.2% HS resulted in an improved phagocytic index after 8 and 30 days of administration. However, by day 60, the phagocytic index significantly decreased compared to the control group. This finding suggests that the effects of HS on the immune system may also depend on the duration of administration or the dosage used. In the current study, we did not collect samples at different time points, which could have provided insights into the potential inflammatory activity during the trial.

Our result implies that there might not have been persistent stressors over time to trigger a significant inflammatory process that could compromise the functional activity of the kidney and spleen at the end of this trial. Additionally, the production of anti-inflammatory cytokines aims to inhibit excessive immune responses (Verburg-Van Kemenade et al., 2009). Since no signs of inflammatory activity were observed after 270 d, the lack of significant *tgf-B* expression is likely. Overall, these findings suggest that the duration of administration can influence the impact of HS or PC on the immune system, and the absence of prolonged stressors may have contributed to the neutral results observed in this study.

2.6 *Conclusion*

In conclusion, this study demonstrated that prolonged protease complex (PC) or humic substances (HS) supplementation in channel catfish diets resulted in positive growth and increased mortality. The PC supplementation improved feed efficiency and showed potential for long-term administration in commercial production systems. HS supplementation enhanced daily catfish growth but had lower biomass gain than fish offered the control or PC diet. Both PC and HS significantly increased lysozyme activity in the sera, indicating immune stimulation, but did not significantly influence immune-related genes after long-term feeding. The low survival could be attributed to the culture system, prolonged exposure to the specific dosages of PC or HS used in the study and the potential interactions with parasitic pathogens. This finding is relevant to large-scale catfish production and highlights the need for further investigation in improved commercial rearing systems and optimizing the inclusion of dietary PC and HS in channel catfish diet.

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Tables

Table 5. Water quality variables measured for 270 days were compared with optimum concentrations for channel catfish production. Values represent the monthly average from all raceways. SE=Standard error of the mean for all.

Water quality variable	Min	Max	Mean ± SE	**Optimum level***
Dissolved oxygen (mg L^{-1})	7.18	12.18	9.46 ± 0.06	5-15
Temperature (^{o}C)	11.06	29.03	19.40 ± 1.95	27-29
pH	7.7	8.6	8.3 ± 0.1	6-9
TAN (mg L^{-1})	0.00	0.02	0.01 ± 0.0	0 as unionized ammonia
Nitrite-N (mg L^{-1})	0.00	0.01	0.00 ± 0.0	0
Total alkalinity (mg L^{-1})	16	53	29 ± 4	20-400
Total hardness (mg L^{-1})	25	49	35 ± 3	20-400

*Tucker and Robinson (1990)

Table 6. Growth performance results from days 1-270 of the growth trial. C28=28% control diet; C28+PC=28% Jefo protease complex diet; C28+HS=28% MFG50 humic substance inclusion diet. TBG=tank biomass gain; RG=relative growth; GPD=growth per day; TGC=thermal growth coefficient; K-value=Fulton's condition factor; CC=cumulative consumption (feed administered); FCR=feed conversion ratio. All data presented are the mean ± SE (SE=Standard error of the mean for all) of three replicate tanks. Different letters denote significant differences in performance parameters between dietary treatments, as discerned by Tukey's HSD.

Performance Parameter	**C28**	**C28+HS**	**C28+PC**	***P*-value**
Initial weight (g)	211 ± 4	204 ± 3	203 ± 5	*0.470*
Final weight (g)	395 ± 13	388 ± 16	399 ± 4	*0.860*
TBG (g)	35,816 ± 2,491[a]	26,201 ± 678[b]	34,258 ± 487[a]	*0.022*
RG (%)	87 ± 7	90 ± 10	97 ± 4	*0.731*
GPD (g d^{-1})	0.66 ± 0.05	1.29 ± 0.22	1.32 ± 0.09	*0.061*
TGC	0.26 ± 0.02	0.27 ± 0.02	0.28 ± 0.01	*0.776*
K-value	1.79 ± 0.04[b]	2.13 ± 0.03[a]	1.94 ± 0.03[b]	*0.003*
CC (g)	130,035 ± 3,351[a]	92,145 ± 2,695[c]	106,665 ± 1,159[b]	>*0.001*
FCR	3.66 ± 0.15[b]	3.52 ± 0.06[ab]	3.12 ± 0.07[a]	*0.049*
Survival (%)	84.73 ± 0.60[a]	34.45 ± 5.19[b]	40.76 ± 2.38[b]	>*0.001*

Table 7. Growth performance results were observed from monthly sampling over 270 d of the growth trial. C28=28% control diet; C28+PC=28% Jefo protease complex diet; C28+HS=28% MFG50 humic substance inclusion diet. TBG=tank biomass gain; RG=relative growth; GPD=growth per day; TGC=thermal growth coefficient; K-value=Fulton's condition factor; CC=cumulative consumption (feed administered); FCR=feed conversion ratio. All data presented are the mean ± SE (SE=Standard error of the mean for all) of three replicate raceway tanks. T x T=Treatment x Time interaction. Different letters within a column denote significantly different dietary treatments, as discerned by Tukey's HSD

	Initial weight (g)	Final weight (g)	TBG (g)	RG (%)	GPD (g d^{-1})	TGC	K-value	CC (g)	FCR	Observed Mortality (%)
Days 1-30										
C28	211.4 ± 4.1	266.4 ± 10.5	13,051 ± 1,486	25.90 ± 2.50	1.83 ± 0.21^{b}	0.55 ± 0.05^{b}	2.02 ± 0.22^{ab}	26,562 ± 340	2.11 ± 0.23^{b}	0.3 ± 0.1
C28+HS	204.4 ± 3.1	266.7 ± 4.5	14,645 ± 1,580	30.60 ± 3.56	2.07 ± 0.22^{ab}	0.63 ± 0.07^{ab}	2.40 ± 0.05^{b}	22,971 ± 403	1.64 ± 0.22^{a}	0.8 ± 0.3
C28+PC	202.8 ± 4.7	274 ± 10.8	16,685 ± 1,558	34.98 ± 2.55	2.35 ± 0.22^{a}	0.71 ± 0.05^{a}	1.78 ± 0.37^{a}	25,371 ± 251	1.55 ± 0.12^{a}	0.6 ± 0.3
Days 1-60										
C28	211.4 ± 4.1	314.7 ± 10.0^{b}	24,240 ± 2,769^{b}	49.11 ± 6.27^{b}	1.71 ± 0.19^{b}	0.53 ± 0.06^{b}	1.92 ± 0.04	60,278 ± 1,550^{a}	2.56 ± 0.20^{b}	1.0 ± 0.1
C28+HS	204.4 ± 3.1	327.6 ± 11.7^{ab}	28,269 ± 1,550^{b}	60.12 ± 3.33^{ab}	2.07 ± 0.14^{b}	0.62 ± 0.03^{b}	2.02 ± 0.04	47,797 ± 1,905^{b}	1.70 ± 0.03^{a}	4.1 ± 1.3
C28+PC	202.8 ± 4.7	359.7 ± 2.4^{a}	36,676 ± 1,762^{a}	77.76 ± 5.37^{a}	2.62 ± 0.12^{a}	0.77 ± 0.04^{a}	2.07 ± 0.03	50,955 ± 467^{b}	1.40 ± 0.07^{a}	2.1 ± 0.7
Days 1-90										
C28	211.4 ± 4.1	350.7 ± 10.6	32,407 ± 2,369^{b}	66.06 ± 5.46^{b}	1.55 ± 0.11^{b}	0.50 ± 0.03^{b}	2.21 ± 0.09	78,985 ± 2,914^{a}	2.46 ± 0.11^{b}	2.4 ± 0.3
C28+HS	204.4 ± 3.1	373.0 ± 4.5	37,821 ± 829^{ab}	82.52 ± 0.88^{ab}	2.05 ± 0.05^{a}	0.60 ± 0.03^{ab}	2.47 ± 0.01	60,763 ± 1967^{b}	1.61 ± 0.74^{a}	13.7 ± 3.6
C28+PC	202.8 ± 4.7	387.7 ± 5.0	42,687 ± 254^{a}	91.34 ± 2.19^{a}	2.10 ± 0.03^{a}	0.65 ± 0.01^{a}	2.53 ± 0.03	67,805 ± 682^{b}	1.59 ± 0.02^{a}	5.0 ± 1.5
Days 1-120										
C28	211.4 ± 4.1	366.9 ± 12.8^{b}	36,179 ± 2,623^{b}	73.63 ± 5.46^{b}	1.30 ± 0.09^{b}	0.45 ± 0.03^{b}	2.46 ± 0.02^{ab}	93,552 ± 2,710^{a}	2.62 ± 0.15^{b}	2.4 ± 0.3
C28+HS	204.4 ± 3.1	378.9 ± 7.9^{ab}	38,888 ± 585^{b}	85.34 ± 1.25^{ab}	2.13 ± 0.09^{a}	0.51 ± 0.01^{ab}	2.09 ± 0.02^{b}	70,447 ± 2,263^{c}	1.81 ± 0.05^{a}	14.1 ± 3.7
C28+PC	202.8 ± 4.7	407.7 ± 8.8^{a}	47,224 ± 1,321^{a}	101.19 ± 4.02^{a}	1.75 ± 0.07^{b}	0.58 ± 0.02^{a}	2.70 ± 0.04^{a}	79,072 ± 798^{b}	1.68 ± 0.06^{a}	5.6 ± 1.6

Days 1-150										
C28	211.4 ± 4.1	381.6 ± 4.1	39,589 ± 170^{b}	80.63 ± 1.53^{b}	1.14 ± 0.00	0.44 ± 0.00	2.33 ± 0.04^{b}	98,468 ± 2,912^{a}	2.49 ± 0.08^{b}	2.4 ± 0.3^{b}
C28+HS	204.4 ± 3.1	401.6 ± 7.0	44,028 ± 3,254^{ab}	96.67 ± 5.30^{ab}	1.62 ± 0.08	0.50 ± 0.02	2.37 ± 0.16^{b}	72,697 ± 2,223^{c}	1.52 ± 0.14^{a}	16.1 ± 3.8^{a}
C28+PC	202.8 ± 4.7	408.3 ± 3.2	47,630 ± 1,926^{a}	101.69 ± 5.50^{a}	1.46±0.06	0.52±0.02	1.84 ± 0.11^{a}	82,622 ± 585^{b}	1.70 ± 0.09^{a}	6.3 ± 1.7^{ab}
Days 1-180										
C28	211.4 ± 4.1	428.8 ± 13.5^{b}	50,548 ± 2.885^{b}	102.97 ± 6.37^{b}	1.21 ± 0.07	0.47 ± 0.02	2.66 ± 0.03	103,735 ± 2,928^{a}	2.06 ± 0.08^{b}	2.4 ± 0.3^{b}
C28+HS	204.4 ± 3.1	433.1 ± 12.2^{b}	49,633 ± 1,976^{b}	111.80 ± 3.93^{b}	1.47 ± 0.08	0.50 ± 0.01	2.59 ± 0.08	75,882 ± 2,163^{c}	1.54 ± 0.07^{a}	21.0 ± 3.6^{a}
C28+PC	202.8 ± 4.7	483.6 ± 9.8^{a}	64,117 ± 1,798^{a}	138.72 ± 5.77^{a}	1.62 ± 0.06	0.59 ± 0.02	2.31 ± 0.19	87,072 ± 412^{b}	1.36 ± 0.05^{a}	7.7 ± 1.1^{b}
Days 1-210										
C28	211.4 ± 4.1	442.9 ± 14.1	53,872 ± 2,624	109.43 ± 3.35	1.11 ± 0.05	0.43 ± 0.01	2.39 ± 0.05	113,102 ± 3,329^{a}	2.11 ± 0.10^{b}	2.9 ± 0.3^{c}
C28+HS	204.4 ± 3.1	443.1 ± 12.7	53,299 ± 1,939	116.77 ± 5.14	1.54 ± 0.07	0.44 ± 0.02	2.51 ± 0.09	82,985 ± 2,011^{c}	1.57 ± 0.09^{a}	30.1 ± 4.7^{a}
C28+PC	202.8 ± 4.7	445.7 ± 8.6	58,927 ± 688	119.85 ± 0.86	1.40 ± 0.03	0.45 ± 0.00	2.53 ± 0.11	94,898 ± 760^{b}	1.61 ± 0.03^{a}	15.8 ± 1.9^{b}
Days 1-240										
C28	211.4 ± 4.1	371.2 ± 8.6	37,466 ± 1,965^{b}	75.74 ± 4.38	0.70 ± 0.04^{b}	0.27 ± 0.01	2.15 ± 0.07	120,335 ± 3,285^{a}	3.23 ± 0.09^{b}	6.7 ± 0.0^{b}
C28+HS	204.4 ± 3.1	369.8 ± 8.9	41,118 ± 3,280^{b}	81.25 ± 7.18	1.41 ± 0.18^{a}	0.28 ± 0.02	2.27 ± 0.05	87,262 ± 2,212^{c}	2.16 ± 0.18^{a}	45.5 ± 9.6^{a}
C28+PC	202.8 ± 4.7	373.1 ± 8.6	50,050 ± 1,062^{a}	84.01 ± 2.51	1.40 ± 0.08^{a}	0.29 ± 0.01	2.23 ± 0.01	99,815 ± 649^{b}	2.00 ± 0.05^{a}	37.0 ± 2.3^{a}
Days 1-270										
C28	211.4 ± 4.1	395.0 ± 12.7	35,815 ± 2,491^{a}	87.06 ± 6.69	0.66 ± 0.05^{b}	0.26 ± 0.02	1.79 ± 0.04	130,035 ± 3,351^{a}	3.66 ± 0.1^{b}	15.3 ± 0.6^{b}
C28+HS	204.4 ± 3.1	387.7 ± 16.2	26,201 ± 678^{b}	90.04 ± 9.97	1.28 ± 0.22^{a}	0.27 ± 0.02	2.13 ± 0.03	92,145 ± 2,695^{c}	3.52 ± 0.06^{ab}	65.5 ± 5.2^{a}
C28+PC	202.8 ± 4.7	399.2 ± 4.2	34,258 ± 487^{a}	97.13 ± 4.22	1.32 ± 0.09^{a}	0.28 ± 0.01	1.94 ± 0.03	106,665 ± 1,159^{b}	3.12 ± 0.08^{a}	59.2 ± 2.4^{a}
Treatment		0.151	0.016	0.031	0.002	0.010	0.288	<0.001	<0.001	0.008

Time	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
T x T	0.155	0.003	0.136	0.250	0.142	0.007	<0.001	0.027	<0.001

Table 8. Analyzed proximate ingredient composition of experimental protein sources (dry matter basis) used in the feeding trial. C28=28% control; C28+PC=28% protease complex diet; C28+HS=28% humic substance diet. NE=net energy, DE=digestible energy, TDN=total digestible nutrients, ME=metabolizable energy.

Proximate	**C28**	**C28+PC**	**C28+HS**
Crude protein (%)	26.0	31.2	31.1
Crude lipid (%)	1.86	4.66	4.31
Ash (%)	7.25	6.67	6.27
Fiber (%)	13.7	11.0	11.2
NE (lactation) (Mcal lbs^{-1})	0.71	0.76	0.73
NE (maint.) (Mcal lbs^{-1})	0.73	0.79	0.76
NE (gain) (Mcal lbs^{-1})	0.49	0.52	0.51
DE (Mcal lbs^{-1})	1.37	1.46	1.41
ME (Mcal lbs^{-1})	1.24	1.30	1.26
Sulphur	0.27	0.30	0.28
Total phosphorus	0.92	1.08	1.00
Total potassium	1.32	1.50	1.46
Total magnesium	0.32	0.38	0.37
Total calcium	0.62	0.69	0.57
Total sodium	0.05	0.08	0.07
Total iron	206	176	177
TDN (%)	68.6	72.8	70.5
Moisture (%)	8.98	8.33	10.76

Table 9. Channel catfish primers used in the study for qPCR gene expression.

	Forward Sequence	Reverse Sequence	Reference
actb	CCGTGACCTGACTGAATACC	GCCCATCTCCTGCTCAAAG	1
ef-1a	GTTGAAATGGTTCCTGGCAA	TCAACACTCTTGATGACACCAAC	2
il-1b	GTGTAAGCAGCAATCCAGTCA	CAAGCACAGAACAGTCAGGTAT	3
il-8	TGGGGAGAAGGAAAAGCAGAG	TGAACAGGAGGCACTTGGAGG	4
tnf-a	GGCCTCTACTTCGTCTAC	GCAGCAGCTTCTCGTCCAT	3
tgf-b	GAAACATCCCAGCACCTCCA	GCCAAGCAAACAACGGCTAA	5

[1]Hao et al., 2021
[2]Jiang et al., 2017
[3]Wang et al., 2021
[4]Jiang et al., 2016
[5]Moreira et al., 2017

Figures

Figure 7. In-pond raceway system at E.W Shell Fisheries station, Auburn, AL.

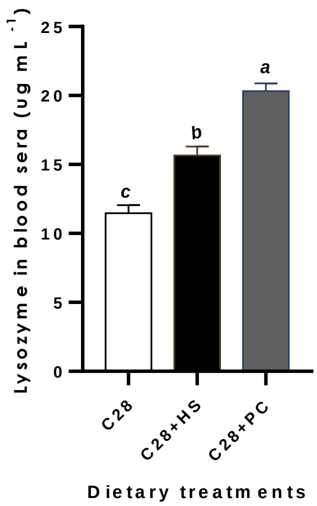

Figure 8. Lysozyme activity (mg mL^{-1}) from serum collected at 270 days. Letters represent significance within dietary treatment groups. All data presented are the mean ± SE (SE=Standard error of the mean for all) of three replicate tanks. C28=28% control diet; C28+PC=28% protease complex diet; C28+HS=28% humic substance diet.

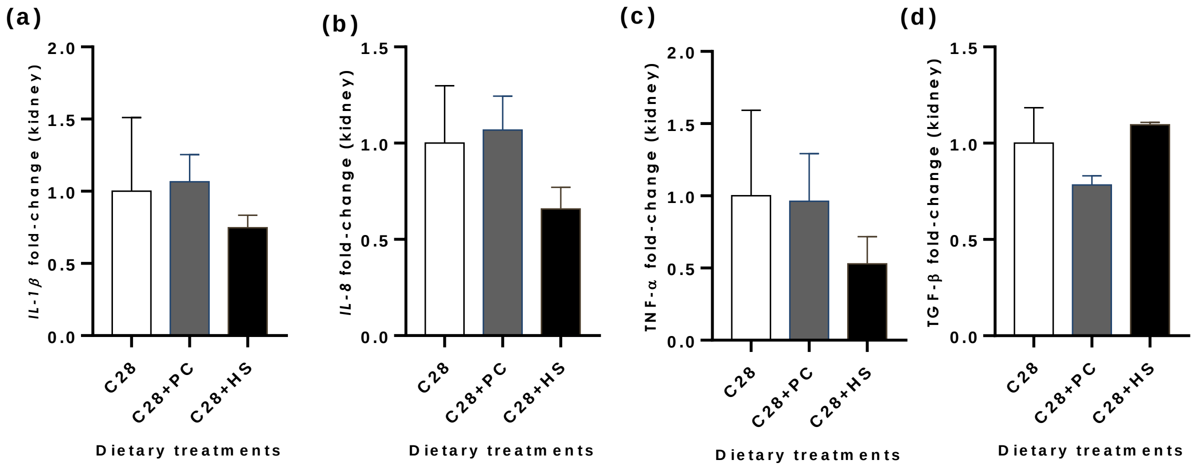

Figure 9. a) *il-1β* expression (fold-change) was evaluated from extracted head kidney at 270 days. **b)** *il-8* expression (fold-change) was evaluated from the extracted head kidney. **c)** *tnf-α* expression (fold-change) was evaluated from the extracted head kidney. **d)** *tgf-β* expression (fold-change) was evaluated from the extracted head kidney. All data presented are the mean ± SE (SE=Standard error of mean for all) of three replicate tanks. C28=28% control diet; C28+PC=28% protease complex diet; C28+HS=28% humic substance diet. Gene expression data were first normalized to *actb* and *ef1a* and then adjusted to the C28 dietary treatment group for comparison ($2^{-\Delta\Delta Ct}$ methodology). The analysis was performed on log-transformed data.

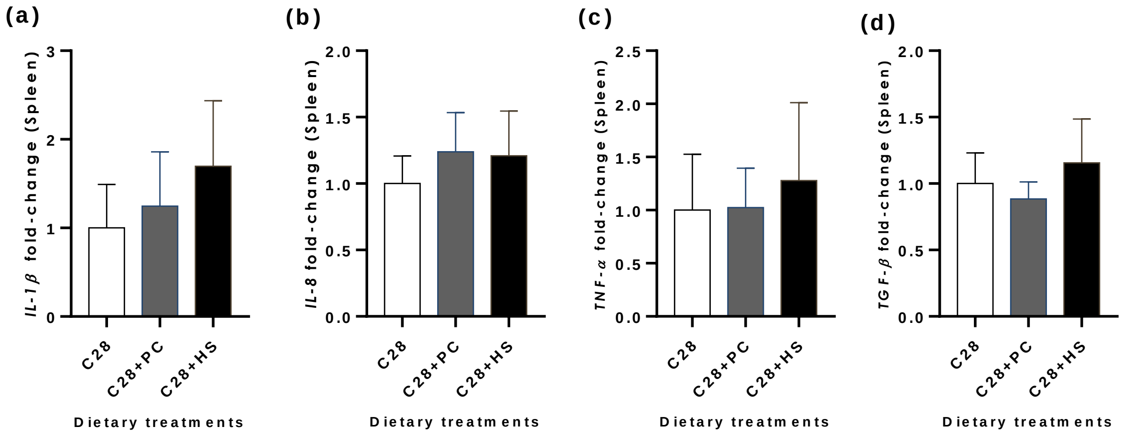

Figure 10. a) *il-1β* expression (fold-change) was evaluated from extracted spleen at 270 days. **b)** *il-8* expression (fold-change) was evaluated from the extracted spleen. **c)** *tnf-α* expression (fold-change) was evaluated from the extracted spleen. **d)** *tgf-β* expression (fold-change) was evaluated from the extracted spleen. All data presented are the mean ± SE (SE=Standard error of mean for all) of three replicate tanks. C28=28% control diet; C28+PC=28 % protease complex diet; C28+HS=28% humic substance diet. Gene expression data were first normalized to *actb* and *ef1a* and then adjusted to the C28 dietary treatment group for comparison ($2^{-\Delta\Delta Ct}$ methodology). The analysis was performed on log-transformed data.