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Effects of Mango Seed (*Mangifera indica*) Powder on Growth Performance, Immune Response, Gut Morphology, and Gene Expression of Nile Tilapia (*Oreochromis niloticus*)

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Abstract: This study explored the effects of mango seed (MS) powder supplementation on the growth, immune response, gene expression, and intestinal morphology of Nile tilapia (*Oreochromis niloticus*) over an 8-week period. A total of 300 Nile tilapia fingerlings (average weight of 15.29 ± 0.05 g) were divided into five treatment groups and fed either a basal diet or one of four experimental diets containing MS powder at concentrations of 10 (MS10), 20 (MS20), 40 (MS40), and 80 (MS80) g kg^{-1}. The results demonstrated that Nile tilapia fed MS-supplemented diets experienced significant improvements ($p < 0.05$) in weight gain (WG), specific growth rate (SGR), and survival rate (SR) compared to the control group (0 g kg^{-1} MS). The MS-treated groups also showed a significant increase ($p < 0.05$) in the height and branching of intestinal villi along the entire length of the intestine, as well as a significantly higher villus-to-crypt depth ratio (V/C), indicating enhanced intestinal health and functionality. Moreover, although MS supplementation did not increase peroxidase activity, it did lead to a significant increase ($p < 0.05$) in the activity of skin mucus and serum lysozyme, along with upregulated gene expression of immune-related (*IL-1*, *IL-8*, and *LBP*) and antioxidant genes (*GST-α*, *GPX*, and *GSR*). Polynomial regression analysis identified an optimal MS dosage of 36.43–45 g kg^{-1} for effectively improving growth, immunity, and immuno-oxidant gene expression in Nile tilapia. These results emphasize mango seed (MS) as a promising natural supplement for improving the diet of Nile tilapia and, potentially, other freshwater fish widely used in aquaculture.

Keywords: mango seed; Nile tilapia; immune response; gene expression; biofloc system

Key Contribution: This study highlights the potential of mango seed powder as a natural feed additive in aquaculture, demonstrating significant improvements in growth performance, immune response, intestinal morphology and gene expression of Nile tilapia. The findings provide valuable insights into the use of agricultural by-products to enhance fish health and support sustainable aquaculture practices.

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1. Introduction

Aquaculture is the fastest-growing sector in global food production [1,2], playing a vital role in feeding the world's increasing population, improving health, reducing poverty, and generating employment and economic opportunities [3]. Nile tilapia (*Oreochromis niloticus*), one of the most farmed freshwater fish, is appreciated for its high nutritional content, which includes proteins, lipids, vitamins, and minerals [4]. Its popularity is further enhanced by its rapid growth, resilience, omnivorous diet, tolerance to low oxygen levels, and ease of farming [5]. However, the rising costs of fish feed and farming systems are becoming significant barriers to its expansion [1]. As global demand for seafood and fish increases, feed has become the most expensive component of aquaculture operations [6]. To address these challenges, aquaculture practices increasingly include the supplementation of animal feeds with plant-derived compounds to enhance the health and productivity of farmed species [1]. This has led to extensive research into natural feed additives, especially for species commonly farmed in aquaculture, such as fish and crustaceans [7]. Among these, agricultural by-products have shown great promise in improving growth performance and health [8]. Fruit by-products have demonstrated significant potential in enhancing fish growth and immunity [9]. Fruit seeds are especially rich in bioactive compounds such as flavonoids, phenols, fatty acids, saponins, sterols, tannins, and carotenoids, all of which are essential for promoting animal health [10].

Mango (*Mangifera indica* L.) is one of the world's most widely grown and consumed tropical fruits, with significant producers including India, China, Thailand, and Mexico [11]. It is consumed in 85 countries, making it the world's second most farmed tropical fruit, with a projected production of 59 million tons in 2022 [12]. Known for its sweet taste and aroma, mango is popular for producing juices, nectars, jams, jellies, and other products [13]. However, processing these foods to extract the pulp generates significant by-products, particularly the peel and seeds, which are often considered waste. The seeds alone make up 10% to 25% of the total fruit weight, with 45% to 85% of the seed composed of the core, depending on the variety [13]. Nutritionally, mango seeds are rich in dietary fiber, vitamins, fatty acids, minerals, and bioactive compounds [14]. These bioactive chemicals, which are secondary metabolites produced by plants in response to stress, work as protective agents because of their antioxidant, antibacterial, and antiviral capabilities [15]. Bioactive chemicals have long been linked to a variety of health advantages, including the prevention and treatment of chronic diseases [16]. Antioxidants have garnered attention for their potential to combat diseases such as cancer, due to their antioxidative effects [17]. Mango by-products from different varieties have been found to contain a range of bioactive compounds, including mangiferin, quercetin, kaempferol, and anthocyanins in the seeds [18], as well as tocopherols like β-carotene, β-cryptoxanthin, and lutein in the peel and paste [19]. Phenolic acids, such as gallic, ellagic, and vanillic acids, are also present in the peel and pulp [20], with gallic acid showing notable antioxidant, anti-inflammatory [21], and hypoglycemic properties [22].

Biofloc technology (BFT) is recognized as one of the most effective aquaculture systems for promoting sustainable development and maintaining a clean environment [23]. BFT systems offer multiple benefits, including enhanced biosecurity, improved feed conversion, better water quality, and more efficient land use [24]. One of the key advantages of BFT is its ability to increase protein utilization efficiency, which improves the feed conversion ratio (FCR) and promotes fish growth, even when dietary protein levels are reduced [25]. This makes BFT particularly suitable for species like tilapia and carps, as well as shrimps such as pink, brine, and Pacific white leg shrimp, which possess digestive systems adapted to efficiently utilize the microbial protein generated within the biofloc [23,26,27]. Although previous research has explored various natural feed additives, there is a lack of studies specifically investigating the use of mango seed powder in fish diets, particularly in Nile tilapia. This knowledge gap highlights the need for novel approaches to integrate agricultural by-products into BFT systems to enhance fish health and productivity [28]. The current study addresses this need by investigating the impact of mango seed powder as a dietary supplement on the growth, intestinal health, innate immunity, and gene expression of Nile tilapia cultured in a BFT system. This approach not only aligns with the principles of sustainable aquaculture, but also seeks to leverage the bioactive compounds found in mango seeds to enhance the overall resilience and well-being of the fish. By integrating agricultural by-products like mango seed powder into BFT systems, this study aims to evaluate the effects of mango seed (*Mangifera indica*) powder on the growth performance, immune response, gut morphology, and gene expression of Nile tilapia (*Oreochromis niloticus*).

2. Materials and Methods

2.1. Mango Seed Powder Production

Mature mango fruits were procured from a local market in Mueang Chiang Mai, Chiang Mai Province, Thailand. The fruits were peeled, and the pulp was removed to separate the seeds from the kernels. The seeds were then thoroughly washed under tap water to ensure cleanliness. Afterward, the cleaned seeds were chopped into small pieces and dried in a hot air oven at 60 °C for three days, reducing their moisture content to approximately 10%. The dried seeds were finely ground into mango seed (MS) powder and stored at 4 °C for future use. The composition and bioactive compound analysis of the mango seed powder were conducted at the Food Innovation and Packaging Center, Faculty of Agro-Industry, Chiang Mai University, with the results detailed in Tables 1 and 2.

Table 1. Proximate composition of mango seed powder used in the experiment.

Test Items	Results
Dry matter	95.30
Ash	2.28
Crude fiber	5.48
Crude protein	5.5
Ether extract	6.84
Nitrogen-free extract	75.2

Table 2. Bioactive compounds of mango seed powder used in the experiment.

Test Items	Results	Methods
DPPH (IC50) (mg/mL)	0.21 ± 0.00	[29]
ABTS+ (mg TE/g)	5.38 ± 2.47	[29]
FRAP (mg TE/g)	64.98 ± 0.41	[29]
Total flavonoid content (mg CE/g)	4.40 ± 0.18	[30]
Total phenolic content (mg GAE/g)	36.97 ± 0.54	[30]

2.2. Dietary Treatments

The control diet was formulated following the protocols established in our previous study [31]. The inclusion levels of mango seed (MS) powder used in this study were based on the recommendations from a previous study [32]: 10 g kg^{-1} (MS10), 20 g kg^{-1} (MS20), 40 g kg^{-1} (MS40), and 80 g kg^{-1} (MS80). The detailed composition and ingredient proportions for these experimental diets are presented in Table 3. To prepare the feed, all ingredients were thoroughly mixed, and oil and distilled water were added to form a cohesive dough. The dough was extruded into pellets, which were subsequently dried at 60 °C to achieve a moisture content of 10% and then stored in sealed bags at 4 °C until use.

Table 3. Formulation and proximate analysis of the experimental diets (g kg^{-1}).

Ingredients	Experimental Diets (g kg^{-1})				
	MS0	**MS10**	**MS20**	**MS40**	**MS80**
Fish meal	200	200	200	200	200
Soybean meal	390	390	390	390	390
Corn meal	150	150	150	150	150
Rice bran	150	145	140	125	90
Wheat flour	70	70	70	70	70
Binder	20	15	10	5	0
Soybean oil	2	2	2	2	2
MS [a]	0	10	20	40	80
Premix [b]	10	10	10	10	10
Vitamin C98%	8	8	8	8	8
Proximate composition of the experimental diets (g kg^{-1})					
Dry matter	98.87	98.13	98.21	98.18	98.31
GE (Kcal/kg)	4231.5	4224.6	4218.9	4227.4	4224.6
Crude protein	32.14	32.27	32.09	32.33	32.18
Ash	8.04	7.98	7.99	8.1	8.08
Fiber	3.98	3.78	3.86	3.91	3.79
Crude lipid	3.01	3.11	3.09	2.98	3.00

[a] MS: mango seed powder. [b] Vitamin and trace mineral mix supplemented as follows (IU kg^{-1} or g kg^{-1} diet): cholecalciferol (217,000 IU), retinyl acetate (1,085,000 IU), thiamine nitrate (0.5 g), folic acid (0.05 g), Ca pantothenate (1 g kg^{-1}), D, L-a-tocopherol acetate (0.5 g), inositol (0.5 g), pyridoxine hydrochloride (0.5 g), sodium (7.85 g), niacin (3 g), zinc (1 g), copper (0.25 g), manganese (1.32 g), cyanocobalamin (10 g), and iodine (0.05 g).

2.3. Proximate Analysis of Experimental Diets

The nutritional composition of each diet was analyzed using standardized procedures as described by the Association of Official Analytical Chemists (AOAC) [33]. The dry matter (DM) content was determined by oven-drying the samples at 105 °C for six hours (AOAC 2001.12). The nitrogen content was measured using the Kjeldahl digestion and titration method, and crude protein was calculated by multiplying the nitrogen content by 6.25 (AOAC 954.01). Ash content was assessed by incinerating the samples in a muffle furnace at 550 °C (AOAC 923.03). Fiber content was determined through the enzymatic–gravimetric method (AOAC 978.10). Lipid content was analyzed using the acid hydrolysis method, followed by Soxhlet extraction (AOAC 920.85). The gross energy (GE) was estimated using the formula $5.72 \times CP + 9.50 \times EE + 4.79 \times CF + 4.17 \times NFE$, based on the analyzed concentrations (g/kg DM) of CP (crude protein), EE (ether extract), CF (crude fiber), and NFE (nitrogen-free extract) [34].

2.4. Biofloc (BF) Water Formation and Regulation

Three weeks prior to the experiment, tanks were prepared to cultivate biofloc (BF) as the inoculum source. To establish the biofloc water, each tank was supplemented with 2 g of fish feed, 400 g of salt, 5 g of dolomite, and 5 g of molasses (local market, Chiang Mai, Thailand). The carbon-to-nitrogen (C: N) ratio was maintained at 15:1 throughout the experiment by adding molasses (containing 40% carbon) as the carbon source, in accordance with protocols from a previous study [35]. Molasses were added once daily, two hours after feeding. The C: N ratio was calculated based on the residual nitrogen levels in each tank and the nitrogen contribution from the diet.

Water parameters, including temperature, pH, and dissolved oxygen, were monitored using the HI98196 m (Hanna Instruments, Strada Hanna NUSFALAU, Salaj, Romania). The observed values were 27.50 ± 0.55 °C for temperature, 7.97 ± 0.06 for pH, and 5.05 ± 0.04 mg L^{-1} for dissolved oxygen. To maintain floc levels below 10 mL per tank, molasses and probiotics (PondPlus, Bayer, Bayer AG, Leverkusen, Germany) were periodically added. Ammonia (NH_3) concentrations were measured with an HI96733 m (Hanna Instruments, Romania) and controlled to remain below 0.10 mg L^{-1}. To sustain optimal water quality, 5% of the water in each tank was replaced weekly with fresh, clean water.

2.5. Study Setup

Male Nile tilapia fingerlings were sourced from Chiang Mai Pattana Farm and acclimated on a commercial diet for 30 days, followed by a control diet for an additional two weeks. Before initiating the feeding trial, the health status of 20 fish was assessed by examining their external bodies, gills, and internal organs. A total of 300 fish, with an average weight of 15.29 ± 0.05 g, were evenly distributed into 15 aerated tanks (150 L capacity each) at a stocking density of 20 fish per tank. The experiment was conducted using a completely randomized design (CRD) with three replicates per treatment. During the eight-week trial, the fish were fed the experimental diets to apparent satiation twice daily, at 8:30 a.m. and 4:30 p.m.

2.6. Growth Performance

The growth performance and survival rate of Nile tilapia were determined using formulas outlined in a previous study [36].

2.7. Innate Immune Parameter Analyses

2.7.1. Skin Mucus and Serum Collection

Skin mucus samples were obtained from Nile tilapia (two fish per replication) using a modified method from [31]. In brief, three fish were randomly selected from each tank, anesthetized with clove oil, and gently massaged to extract mucus, which was transferred into sterile tubes using 10 mL of NaCl solution. The samples were centrifuged at 1500× *g* for 10 min at 4 °C, and 1 mL of supernatant was stored at −20 °C for later analysis. Blood serum was collected following the procedure outlined by Van Doan et al. [37].

A 1 mL blood sample was collected from the caudal vein using a syringe and transferred to 1.5 mL Eppendorf tubes (Eppendorf, Hamburg, Germany) without anticoagulant. After coagulating at room temperature for one hour, the samples were centrifuged at 1500× *g* for 5 min at 4 °C. The serum was then extracted with a micropipette, stored in Eppendorf tubes, and frozen at −80 °C for later analysis.

2.7.2. Lysozyme and Peroxidase Activities

Serum and mucus lysozyme levels were determined following the method of Parry Jr, Chandan and Shahani [38], with modifications from a previous study [39].

The methodology of Quade and Roth [40] was slightly modified to assess peroxidase levels in serum and skin mucus [41].

2.8. Relative Immune- and Antioxidant-Gene Expression Study

Collection of Tissues, RNA Isolation, cDNA Synthesis and qRT-PCR Analysis

To assess immune and antioxidant gene expression, six fish per tank were sampled. After anesthesia, 0.5 g of liver and intestinal tissues were collected, preserved in TRIzol Reagent (Life Technologies, Carlsbad, CA, USA), and homogenized with pellet pestles (Sigma-Aldrich: St. Louis, MO, USA). The homogenates were incubated at room temperature for 5 min, followed by the addition of 100 µL chloroform and a 2 min incubation. Samples were centrifuged at 12,000× *g* for 15 min at 4 °C, and the RNA-rich aqueous phase was purified using the PureLink™ RNA Mini Kit (Invitrogen, Waltham, MA, USA), as per the manufacturer's instructions. RNA quantity and purity were checked using a NanoDrop™ One spectrophotometer (Thermo Scientific, Waltham, MA, USA). A total of 1000 ng of RNA was reverse-transcribed into cDNA with the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Primer sequences for target genes are listed in Table 4. Quantitative real-time PCR (qRT-PCR) was performed in triplicate on a CFX96 Touch Real-Time PCR System (Bio-Rad, USA) using 100 ng of cDNA, primers, and iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Thermal cycling followed a previously described protocol [39], and gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method [42].

Table 4. Primers used for quantitative RT-PCR in this study.

Target Gene	Sequence (5′–3′)	T_m (°C)	Product Size (bp)	Reference
18S rRNA	F: GTGCATGGCCGTTCTTAGTT R: CTCAATCTCGTGTGGCTGAA	60	150	XR_003216134
IL-1	F: GTCTGTCAAGGATAAGCGCTG R: ACTCTGGAGCTGGATGTTGA	59	200	XM_019365844
IL-8	F: CTGTGAAGGCATGGGTGTG R: GATCACTTTCTTCACCCAGGG	59	196	NM_001279704
LBP	F: ACCAGAAACTGCGAGAAGGA R: GATTGGTGGTCGGAGGTTTG	59	200	XM_013271147
GST-α	F: ACTGCACACTCATGGGAACA R: TTAAAAGCCAGCGGATTGAC	60	190	NM_001279635
GPX	F: GGTGGATGTGAATGGAAAGG R: CTTGTAAGGTTCCCCGTCAG	60	190	NM_001279711
GSR	F: CTGCACCAAAGAACTGCAAAR: CCAGAGAAGGCAGTCCACTC	60	172	XM_005467348

2.9. Histological Screening of Intestinal Samples

Intestinal tissue samples from three fish per group were fixed in 10% neutral buffered formalin, dehydrated through graded ethanol, cleared with xylene, and embedded in paraffin. Sections 5 µm thick were cut using a rotary microtome (Leica 2025, Wetzlar, Germany) and stained with Hematoxylin and Eosin (H&E) for histological analysis [43]. Microscopic examination was performed using a CX 43 light microscope (Olympus, Hachioji-shi, Tokyo, Japan) equipped with an E620 digital camera (Olympus, Hachioji-shi, Tokyo, Japan) for imaging.

2.10. Statistical Analysis

Data normality was evaluated using the Shapiro–Wilk test. ANOVA was conducted to analyze differences among treatments, followed by the Least Significant Difference (LSD) test at a 95% confidence level. Statistical analysis was performed with the "agricolae" package (version 1.3-7) in R and JMP Pro Version 15.1.0 (SAS Institute Inc., Cary, NC, USA). The optimal MS level was identified using polynomial regression analysis [44], with a significance threshold of $p \leq 0.05$.

3. Results

3.1. Growth Performance

The growth performance and feed utilization of Nile tilapia fed with mango seed (MS)-supplemented diets were evaluated over 4 and 8 weeks (Table 5). Initial weights were consistent across all groups, with no significant differences at the start. After 4 weeks, fish in the MS10 and MS20 groups exhibited significantly higher FW, WG, and SGR compared to the control group ($p < 0.05$). By the end of the 8-week period, all MS-supplemented groups maintained superior growth performance, though the differences were not statistically significant. The FCR also improved in the MS-supplemented groups, with the MS10 group showing a significantly better FCR after 4 weeks ($p < 0.05$) and the MS40 group optimizing FCR by 8 weeks. Survival rates (SRs) were high across all groups, with the MS20 and MS40 groups achieving 100% survival after 8 weeks, significantly higher than the MS80 group ($p < 0.05$). Polynomial regression analysis determined the optimal dietary inclusion level of mango seed powder to be approximately 39 g kg^{-1} for FW, WG, SGR, and FCR (Figure 1).

Table 5. Growth performances and feed utilization of Nile tilapia after 4 and 8 weeks feeding with mango seed diets.

	MS0	MS10	MS20	MS40	MS80	*p*-Value
IW (g)	15.32 ± 0.05 ^{a}	15.23 ± 0.03 ^{a}	15.33 ± 0.03 ^{a}	15.30 ± 0.05 ^{a}	15.28 ± 0.03 ^{a}	0.9785
FW (g)						
4 weeks	39.61 ± 1.67 ^{b}	42.77 ± 1.33 ^{a}	41.86 ± 1.15 ^{a}	41.37 ± 0.86 ^{ab}	40.81 ± 0.07 ^{ab}	0.0224
8 weeks	86.54 ± 5.86 ^{a}	91.70 ± 3.95 ^{a}	91.72 ± 0.56 ^{a}	91.87 ± 3.3 ^{a}	87.99 ± 3.14 ^{a}	0.675
WG (g)						
4 weeks	24.29 ± 1.64 ^{b}	27.53 ± 1.33 ^{a}	26.53 ± 1.12 ^{a}	26.07 ± 0.88 ^{ab}	25.53 ± 0.06 ^{ab}	0.0312
8 weeks	71.47 ± 0.89 ^{a}	76.47 ± 3.84 ^{a}	76.38 ± 0.53 ^{a}	76.57 ± 3.35 ^{a}	72.07 ± 3.12 ^{a}	0.747
SGR (%/day)						
4 weeks	3.16 ± 0.13 ^{b}	3.44 ± 0.10 ^{a}	3.35 ± 0.09 ^{a}	3.32 ± 0.08 ^{ab}	3.27 ± 0.00 ^{ab}	0.0412
8 weeks	2.88 ± 0.12 ^{a}	2.99 ± 0.07 ^{a}	2.98 ± 0.01 ^{a}	2.99 ± 0.07 ^{a}	2.90 ± 0.06 ^{a}	0.8210
FCR						
4 weeks	0.78 ± 0.05 ^{b}	0.89 ± 0.04 ^{a}	0.85 ± 0.06 ^{ab}	0.83 ± 0.01 ^{ab}	0.82 ± 0.05 ^{ab}	0.0156
8 weeks	0.73 ± 0.06 ^{a}	0.82 ± 0.12 ^{a}	0.71 ± 0.03 ^{a}	0.70 ± 0.02 ^{a}	0.78± 0.02 ^{a}	0.1456
SR (%)						
4 weeks	96.67 ± 2.89 ^{a}	100.0 ± 0.00 ^{a}	98.33 ± 2.89 ^{a}	96.33 ± 2.89 ^{a}	96.67 ± 2.89 ^{a}	0.2143
8 weeks	96.67 ± 2.89 ^{ab}	95.00 ± 5.00 ^{ab}	96.67 ± 3.21 ^{a}	96.33 ± 2.75 ^{a}	91.67 ± 7.64 ^{b}	0.0297

MS: Mango seed, IW: initial fish weight, FW: final fish weight, WG: weight gain, SGR: specific fish growth rate, FCR: feed conversion ratio, SR: survival rate. Different letters in the same row represent significant differences ($p < 0.05$).

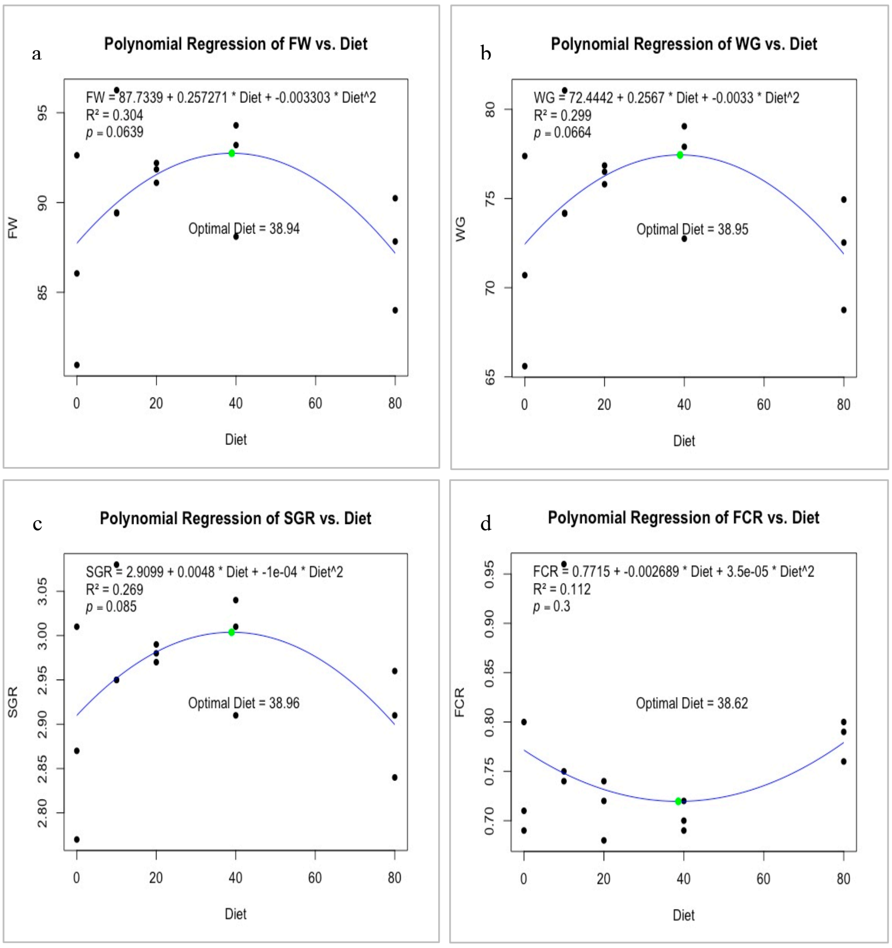
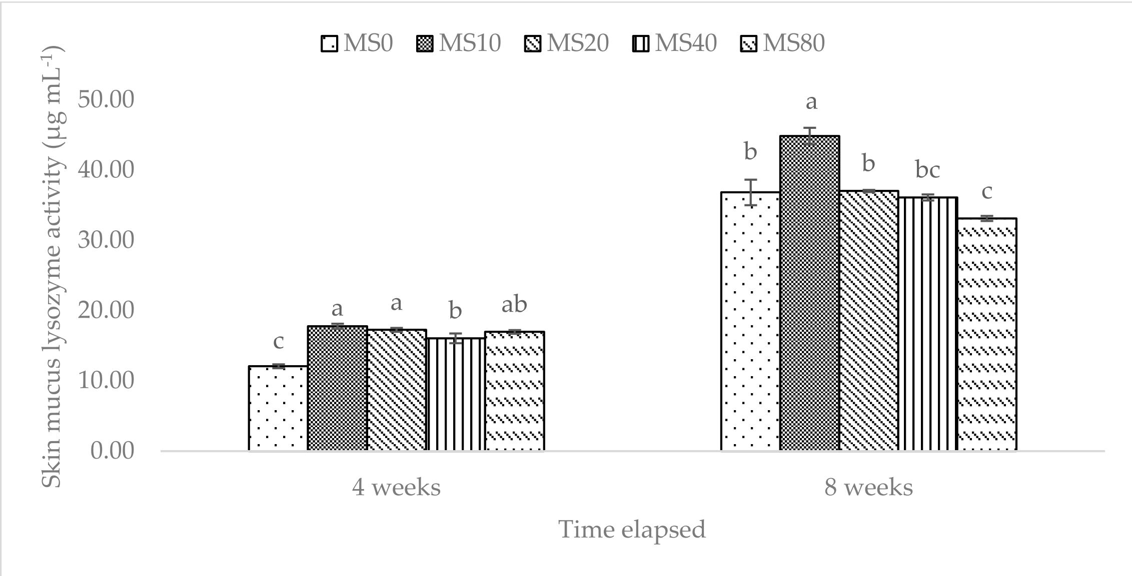
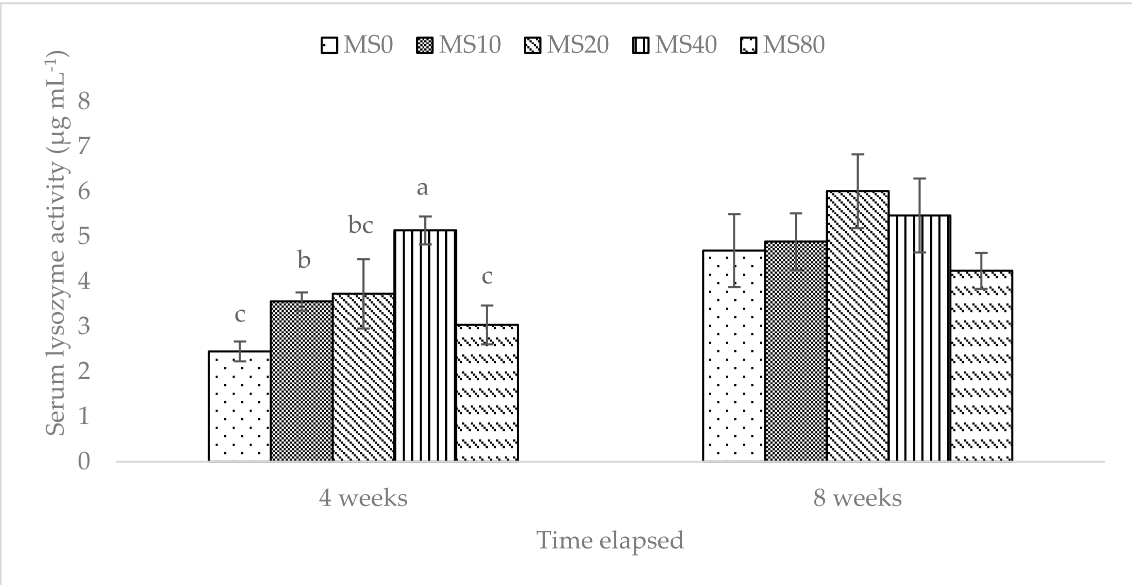


Figure 1. Quadratic relationships and polynomial regression analyses ($p < 0.05$) were used to evaluate the effects of dietary mango seed (MS) powder levels on the final body weight (**a**), weight gain (**b**), specific growth rate (**c**), and feed conversion ratio (**d**) of Nile tilapia after eight weeks. Data are presented as means ± SE.

3.2. Skin Mucus and Serum Immunities

The effects of mango seed (MS) powder supplementation on lysozyme and peroxidase activity in Nile tilapia were assessed in both skin mucus and serum after four and eight weeks of feeding (Figure 2). In skin mucus, lysozyme activity significantly increased in the MS10, MS20, and MS40 groups compared to the control (MS0) after four weeks ($p < 0.05$), with the MS10 group maintaining this elevated activity after eight weeks. However, the MS80 group showed a significant decrease in lysozyme activity by the end of the study. In serum, lysozyme activity was significantly higher in the MS10 and MS40 groups at four weeks, but no significant differences were observed at eight weeks. Peroxidase activity in both skin mucus and serum remained consistent across all treatment groups, with no significant differences at either time point ($p > 0.05$).





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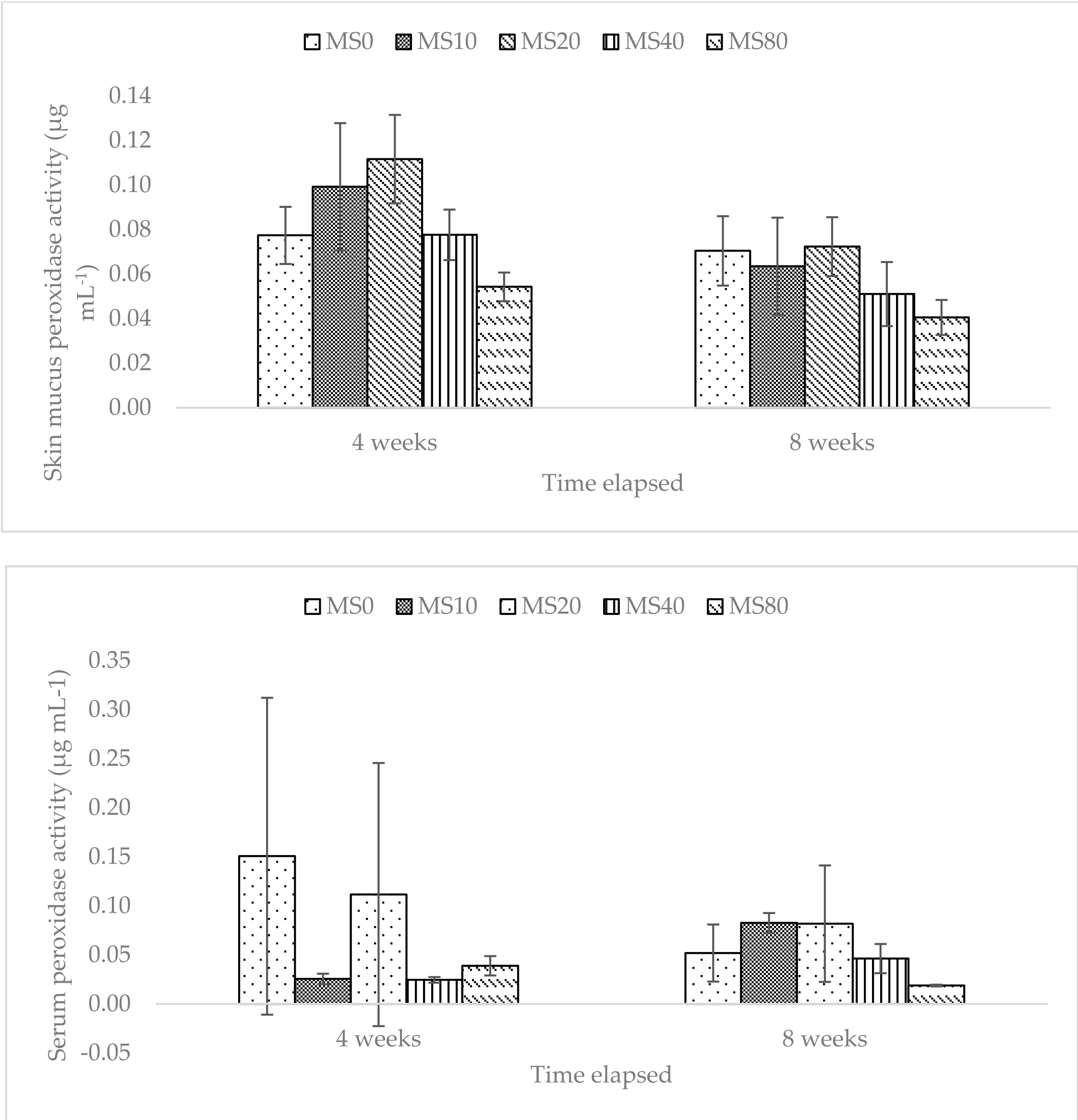

Figure 2. Lysozyme and peroxidase activities in skin mucus and serum of Nile tilapia were measured after 4 and 8 weeks of feeding with mango seed powder at 0 (MS0, control), 10 (MS10), 20 (MS20), 40 (MS40), and 80 (MS80) g/kg. Data represent the mean ± SE from three replicates. Groups with different letters show significant differences ($p < 0.05$), while "ns" indicates no significant difference. Statistical analysis was performed using ANOVA.

3.3. Immune-Related and Antioxidant Gene Expression

The relative gene expression of immune-related (*IL-1*, *IL-8*, *LBP*) and antioxidant-related (*GPX*, *GST-α*, *GSR*) genes in Nile tilapia was analyzed in both liver and intestinal tissues after feeding with diets containing varying concentrations of mango seed (MS) powder (Figure 3). In liver tissue, the MS10 diet resulted in the highest expression levels of all measured genes, with significant upregulation compared to the control group (MS0). As the concentration of MS increased to MS20 and MS40, gene expression levels remained elevated, but were lower than in the MS10 group. The MS80 group exhibited significantly reduced gene expression, with some genes showing no difference from the control. A similar pattern was observed in the intestinal tissue, where the MS10 diet again led to the highest gene expression levels, followed by a gradual decline in the MS20 and MS40 groups, and the lowest expression levels were found in the MS80 group.

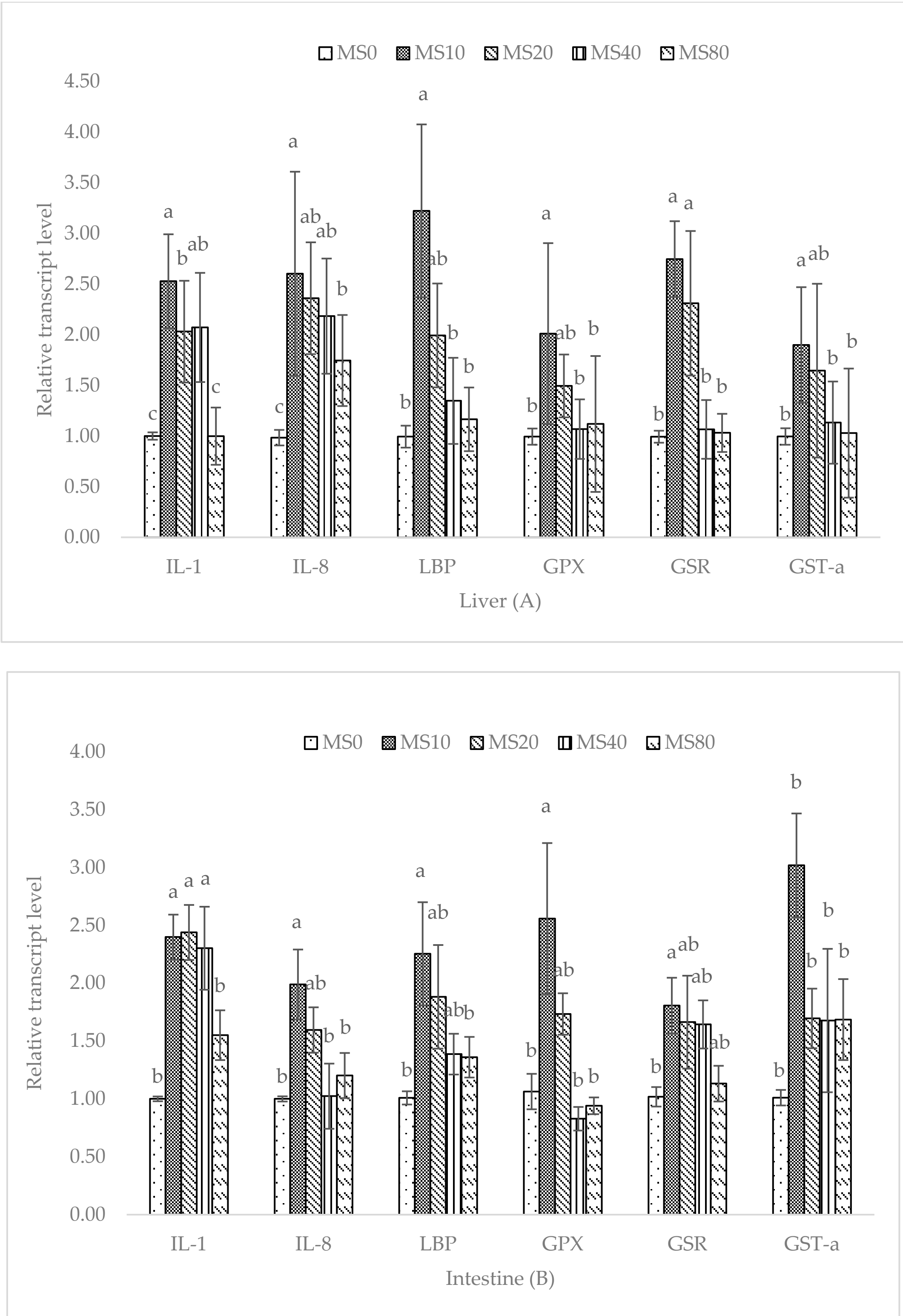

Figure 3. Relative expression levels of immune-related genes (IL-1, IL-8, and LBP) and antioxidant-related genes (GPX, GST-α, and GSR) in the liver (**A**) and intestinal tissues (**B**) of Nile tilapia (n = 6) were assessed after feeding diets containing 0 (MS0, control), 10 (MS10), 20 (MS20), 40 (MS40), and 80 (MS80) g/kg. Data are presented as means ± SE from three replicates. Groups with different letters indicate significant differences ($p < 0.05$). Statistical analysis was conducted using ANOVA.

3.4. Intestinal Morphology

The histological analysis of Nile tilapia intestines after 8 weeks of mango seed (MS) supplementation revealed significant improvements in several key parameters (Table 6). Villus height (VH) and villus width (VW) were markedly increased in the MS-supplemented groups, with the MS20 and MS40 groups showing the most substantial enhancements. Crypt depth (CD) was significantly reduced across all MS groups, with the MS80 group exhibiting the lowest CD. Consequently, the villus ratio (VH:CD) was significantly elevated in all MS-supplemented groups, with the highest ratios observed in the MS40 and MS80 groups (Figure 4). These histological improvements suggest that MS supplementation positively impacts intestinal structure, with the most pronounced effects seen at the 20 g kg^{-1} and 40 g kg^{-11} supplementation levels.

Table 6. Effect of dietary MS supplementation on intestinal histological parameters of Nile Tilapia after 8 weeks.

	MS0	MS10	MS20	MS40	MS80	SEM	*p*-Value
Villus height (VH)	3417.39 ^{c}	4087.06 ^{ab}	4224.12 ^{a}	4230.45 ^{a}	3943.28 ^{b}	32.94	<0.001
Villus width (VW)	631.48 ^{ab}	612.06 ^{b}	657.87 ^{a}	659.89 ^{a}	602.35 ^{b}	5.14	<0.001
Crypt depth (CD)	207.11 ^{a}	129.78 ^{b}	113.12 ^{b}	99.25 ^{bc}	96.10 ^{c}	3.86	<0.001
Villus: Crypt ratio (VH: CD)	17.57 ^{d}	33.93 ^{c}	39.36 ^{b}	44.50 ^{a}	42.72 ^{ab}	0.98	<0.001

Groups with different superscript letters indicate significant differences ($p < 0.05$).

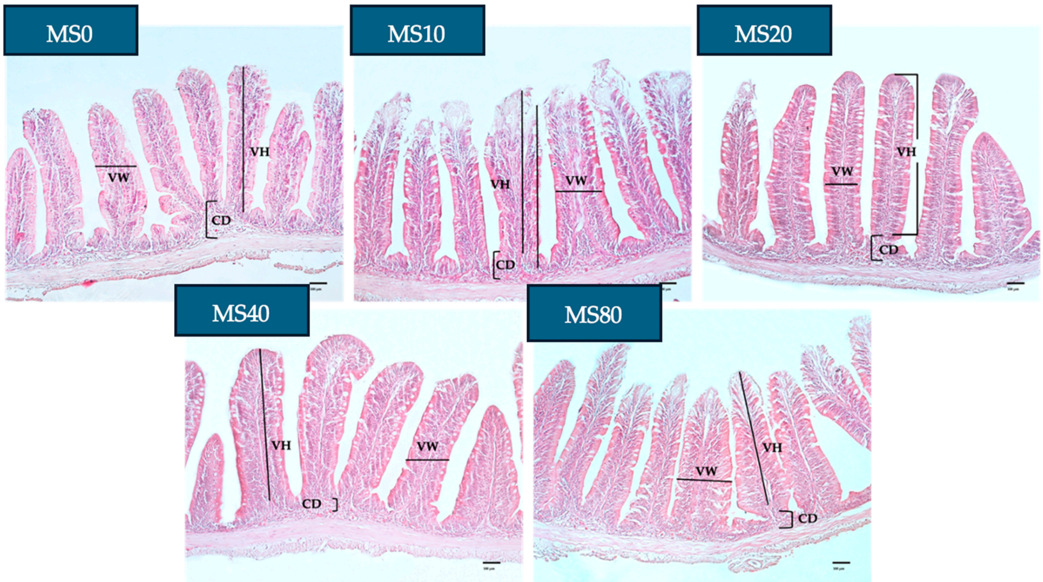

Figure 4. The intestine photomicrograph of Nile tilapia, O. niloticus tested with MS. VH: villus height, VW: villus width, CD: crypt depth. Stain H&E, scale bar = 100 μm.

4. Discussion

As the demand for aquaculture continues to rise, there is an increasing need to enhance the growth and immune system of farmed aquatic species [45]. Traditionally, antibiotics have been used to address these challenges. However, the overuse of antibiotics has led to concerns about antibacterial resistance and residual contamination, which not only jeopardize fish health, but also pose significant food safety risks [46]. In response, the use of agricultural by-products as feed additives has gained attention as a sustainable alternative [47]. These by-products can reduce the reliance on antibiotics while simultaneously improving growth and nutrient utilization in farmed species, which are critical factors for the success of aquaculture production [48].

Growth performance is a critical parameter for farmers and aquaculture practices, making it one of the most important factors to measure in feeding studies. Our results demonstrated that mango seed (MS) supplementation positively influenced growth performance in Nile tilapia, leading to significant improvements in FBW, WG, and SGR. Regression analysis suggests that the optimal amount of mango seed powder to supplement the basal diet ranges from 36.43 to 45 g kg^{-1}. This finding is consistent with the study [32], which showed that red hybrid tilapia (*O. niloticus* × *O. mossambicus*) exhibited improved growth performance when fed a diet containing 10% mango seed meal (MSM), though higher doses above 25% reduced growth. Similarly, Falaye, Shakiru Okanlawon, Salimata and Martha [49] reported that optimal growth and feed conversion ratio (FCR) in Nile tilapia were achieved when 25% of their diet was replaced with mango seed kernel meal at a rate of 7.67 g kg^{-1}. Recent studies have further confirmed that the dietary inclusion of seed powders can enhance growth performance in various fish species, including Nile tilapia [41,50–52], rainbow trout [53,54], and European sea bass [55]. The improvement in growth can be attributed to the high polyphenol content in mango seeds. These bioactive compounds may enhance feed palatability and stimulate the secretion of digestive enzymes, leading to increased feed intake [52]. Polyphenols from mango seeds have been recognized as effective growth promoters in various animals [56,57]. Furthermore, mango seeds are a rich source of fatty acids, including oleic, palmitic, and stearic acids, which together account for approximately 15% of the total seed weight [58]. These fatty acids have been shown to enhance growth performance in fish by providing essential energy sources and supporting various physiological functions critical for growth and development [59]. However, it is noteworthy that the MS80 group experienced a significant decline in growth performance after 8 weeks of feeding. Similar findings have been observed in Nile tilapia fed chia seed [52] and makiang seed [41], as well as in rainbow trout fed lupin seed meal [54]. Beriso and Tesfaye [60] have highlighted the fact that although mango seeds are inexpensive and provide high metabolizable energy, they also contain anti-nutritional factors such as tannins, which can be challenging for animals to digest, potentially explaining the reduced growth observed at higher supplementation levels. The initial significant improvement in the growth performance of Nile tilapia with mango seed powder supplementation at 4 weeks, followed by a lack of significant differences at 8 weeks, can be explained by several factors. Firstly, fish may exhibit an early positive response to the new dietary additive due to enhanced palatability, but this effect may diminish as they adapt to the diet, leading to a plateau in growth [61]. Additionally, the prolonged use of higher doses of mango seed powder may have introduced anti-nutritional factors such as tannins, phytates, and saponins, which can interfere with nutrient absorption and digestion [62]. Over time, these compounds might inhibit digestive enzyme activity, reducing the overall growth benefits. Furthermore, compensatory growth by the control group could also contribute to this, as the fish may have experienced a period of rapid catch-up growth, minimizing the differences between groups by the 8-week mark. Finally, while bioactive compounds in mango seed powder initially enhanced digestive and immune functions, prolonged supplementation may have shifted energy allocation towards immune maintenance or detoxification processes, rather than growth [63]. Collectively, these factors suggest that while mango seed powder can boost short-term growth, its long-term efficacy may be influenced by the dosage and duration of supplementation.

The study revealed that mango seed (MS) powder supplementation had a significant impact on the lysozyme activity in the skin mucus and serum of Nile tilapia, particularly at moderate inclusion levels (MS10, MS20, and MS40). The observed increase in lysozyme activity, a key component of the fish's innate immune system, suggests that mango seed powder can enhance the fish's ability to ward off pathogens. Notably, the MS10 group maintained elevated lysozyme activity even after eight weeks, indicating the sustained immunomodulatory benefits of mango seed at this concentration. Mango seed powder has already been shown to be able to increase the presence of lysozyme in the blood serum of Nile tilapia [64], as well as the blood serum of rohu [65], and a similar effect was detected in the blood serum and skin mucus of Nile tilapia fed makiang and garden cress seeds [41,50], and rainbow trout fed medicinal plant seeds [53]. However, the significant decrease in lysozyme activity in the MS80 group by the end of the study suggests that excessive supplementation may have a detrimental effect, possibly due to the presence of anti-nutritional factors such as tannins, which are known to be present in mango seeds [66]. In terms of peroxidase activity, the consistent peroxidase activity observed across all treatment groups suggests that while mango seed supplementation may enhance lysozyme activity, it does not significantly influence peroxidase activity. Peroxidase activity, which plays a role in the oxidative burst response, may not be as sensitive to dietary changes as lysozyme activity, or it may require different types or concentrations of bioactive compounds to be significantly affected. This finding is consistent with previous studies reported in European sea bass (*Dicentrarchus labrax*) fed okra leaves [67], Nile tilapia (*Oreochromis niloticus*) fed makiang seed [41], and rainbow trout (*Oncorhynchus mykiss*) fed tributyrin [68]. Regarding gene expression, this study demonstrated that mango seed (MS) powder supplementation had a significant impact on the expression of immune-related (*IL-1*, *IL-8*, *LBP*) and antioxidant-related (*GPX*, *GST-α*, *GSR*) genes in the liver and intestinal tissues of Nile tilapia. The highest gene expression levels were observed in fish fed with the MS10 diet, indicating that moderate supplementation with mango seed powder most effectively enhances both immune and antioxidant responses in these tissues. As the concentration of MS increased to MS20 and MS40, gene expression levels remained elevated but were lower than those observed in the MS10 group, suggesting a dose-dependent response. In the MS80 group, gene expression was significantly reduced, with some genes showing no difference from the control, indicating that excessive supplementation may have a suppressive effect on these important physiological processes. On the other hand, the antioxidant properties of these compounds can stimulate the expression of genes involved in the detoxification of reactive oxygen species (ROS), such as *GPX, GST-α*, and *GSR*, providing cellular protection against oxidative stress. These findings are consistent with previous studies reported in Nile tilapia fed neem and makiang seeds [41,51]. The enhancement of immune response may also be attributed to several possible mechanisms. First, the strong antioxidant properties of polyphenols and flavonoids in mango seeds [13] likely reduce oxidative stress, preserving immune resources and supporting sustained lysozyme production [69]. Dietary polyphenols influence the composition of gut microbiota, while intestinal microbes metabolize polyphenols, producing bioactive compounds in the process [70], thereby enhancing overall immune function. The anti-inflammatory effects of mango seed bioactive compounds could also play a role in maintaining a balanced immune response, preventing chronic inflammation that might otherwise impair immune effectiveness [71]. Additionally, mango seed is a rich source of vitamin C and E [66], which are well-known for their immunostimulatory effects, promoting the production and function of immune cells and enhancing the overall immune response [72,73].

While the intestine is primarily recognized as the main site for nutrient absorption in fish, it also plays a crucial role as an immune organ, capable of preventing intestinal endotoxins and bacterial invasion [74]. Damage to the intestine has been linked to various immune system disorders, weakened disease resistance, decreased appetite, and even slower growth [75]. With this in mind, we examined the villus height, villus width, crypt depth, and villus-to-crypt ratio in Nile tilapia to determine whether the positive effects of mango seed powder on growth and the immune system are also associated with improvements in intestinal anatomy. Our study revealed a significant increase in villus height across all experimental diets compared to the control (MS0). Although we did not observe a corresponding increase in villus width, the increased villus height is still a promising outcome. Greater villus height and width enhance the contact area between the intestine and nutrients, facilitating better nutrient absorption [76]. Crypt depth, which is related to the renewal of intestinal epithelial cells, showed varied results: an increase in depth for the MS10 and MS20 groups, and a decrease for the MS40 and MS80 groups. Shallow crypts indicate slower renewal of epithelial cells [75], suggesting that while appropriate doses of mango seed powder benefit intestinal anatomy, excessive amounts can have adverse effects. Nevertheless, all MS-supplemented diets resulted in an increased villus-to-crypt ratio, which is a positive outcome. A higher ratio indicates stronger digestive absorption capacity and supports both digestion and the immune system [77]. These findings suggest that mango seed powder, when used in appropriate dosages, can significantly enhance intestinal health, thereby supporting overall growth and immunity in Nile tilapia [77].

5. Conclusions

In summary, mango seed powder is a promising natural feed additive for Nile tilapia, enhancing growth, immune response, intestinal health, and gene expression. An optimal dose of 36.43–45 g kg^{-1} improved key growth metrics and immune function, while promoting better nutrient absorption and intestinal structure. This study supports the use of agricultural by-products as sustainable alternatives to synthetic additives in aquaculture.

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Institutional Review Board Statement: The animals used in this study were cared for in accordance with ethical treatment guidelines. All experimental procedures were approved by the Chiang Mai University Animal Care and Use Committee (AAALAC guidelines) on 31 January 2022 (Approval No. RAGIACUC001/2565).

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Data Availability Statement: Data are contained within the article will be uploaded to public repositories upon acceptance of the manuscript

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