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Organic Zinc Supplementation Improve Growth Perfomance, Digestive Enzyme Activity, and Antioxidant Response of the Pacu *Colossoma macropomum*

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ABSTRACT

The organic form of zinc (Zn) has high bioavailability and is more readily absorbed than inorganic forms. This study aimed to determine the optimum dietary dosage of organic Zn to enhance the growth performance of pacu (*Colossoma macropomum*). A completely randomized design (CRD) was applied with five dietary treatments containing 0, 20, 40, 80, and 160 mg Zn kg^{-1} feed, each in four replicates. Pacu were stocked at a density of 15 fish per aquarium (200 fish m^{-3}; aquarium size: 50 × 50 × 40 cm; water volume: 75 L), with an initial mean weight of 9.27 ± 0.01 g, and reared for 60 days. Fish were fed to apparent satiation twice daily (09:00 and 17:00). Parameters evaluated included digestive enzyme activities (amylase, lipase, trypsin, chymotrypsin), antioxidant responses (superoxide dismutase [SOD], glutathione peroxidase [GPx], malondialdehyde [MDA]), intestinal histology, health status (lysozyme activity, respiratory burst), growth performance (survival rate, feed intake, feed conversion ratio [FCR], specific growth rate [SGR], protein efficiency ratio [PER], protein retention), and Zn content in organs. Results showed that the 40 mg Zn kg^{-1} diet produced the highest activities of amylase, lipase, trypsin, and chymotrypsin. Antioxidant responses under air stress indicated optimal defense at 20– 40mg Zn kg^{-1}, with high SOD and GPx levels and reduced MDA levels. Zn supplementation also enhanced lysozyme activity and respiratory burst compared to the control. Histological analysis revealed improved intestinal absorption areas in fish fed 20, 40, and 80mg Zn kg^{-1}. Furthermore, Zn supplementation significantly increased Zn deposition in organs ($P< 0.05$). Correlation analysis between SGR and FCR with Zn dosage demonstrated that 40 mg Zn kg^{-1} feed provided the best growth performance. Overall, organic Zn supplementation improved antioxidant defenses, digestive enzyme activity, intestinal health, immune responses, and growth in pacu, with 40mg Zn kg^{-1} identified as the optimal dietary level.

INTRODUCTION

The increasing demand for pacu fish (*Colossoma macropomum*) has driven a rise in its production **(Aida *et al.*, 2020)**. To meet market needs, farmers have adopted intensive farming systems with higher stocking densities **(Fitriadi *et al.*, 2020)**. However, high stocking density increases fish susceptibility to stress caused by environmental changes, which in turn elevates their energy demands **(Klinger & Naylor, 2012)**. Intensive farming also produces large amounts of waste, contributing to oxidative stress in the aquatic environment and promoting the generation of reactive oxygen species (ROS) **(Liang *et al.*, 2016)**.

ROS are natural byproducts of aerobic metabolism, including superoxide anion (O_2^-), hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2) **(Wulandari, 2017)**. Excessive ROS accumulation can damage lipids and proteins, compromising cellular function **(Schieber & Chande, 2014)**. To counteract these effects, fish rely on an enzymatic antioxidant defense system, producing enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) **(Mishra *et al.*, 2015)**. Zinc (Zn) plays a critical role in the formation and activity of these enzymes, making dietary supplementation essential **(Meiler *et al.*, 2021)**.

In addition to antioxidant defense, digestive enzyme activity is vital for nutrient metabolism and absorption in fish. Proteolytic enzymes such as trypsin and chymotrypsin hydrolyze proteins into amino acids required for growth and tissue repair, while amylase converts carbohydrates into simple sugars, and lipase breaks down fats into fatty acids and glycerol. Efficient digestive function ensures optimal nutrient utilization, supporting fish health and growth **(Song *et al.*, 2017; Zafar & Khan, 2024)**. Conversely, impaired digestive activity can limit nutrient absorption, leading to stunted growth, weakened immunity, and greater disease susceptibility.

Organic zinc has higher bioavailability than inorganic zinc because it binds directly with amino acids, enhancing absorption and reducing the effects of antinutritional factors **(Kishawy *et al.*, 2020; Meiler & Kumar, 2021)**. Previous studies have shown that zinc supplementation can improve digestive enzyme performance **(Kumar *et al.*, 2017)**. In addition, it acts as a cofactor in numerous metabolic processes related to antioxidant defense, immune function, growth, and organ development **(Malekpouri *et al.*, 2011)**. Research on different aquaculture species—including the giant gourami (*Osphronemus gourami*), juvenile grouper (*Epinephelus malabaricus*), whiteleg shrimp (*Litopenaeus vannamei*), common carp, snakehead fish (*Channa* sp.), and the Nile tilapia (*Oreochromis niloticus*)—has demonstrated positive effects of zinc supplementation on growth and immunity **(Setiawati *et al.*, 2007; Houng-Yung *et al.*, 2014; Oktaviana, 2018; Liang *et al.*, 2020; Fei *et al.*, 2022; Inarto *et al.*, 2023)**.

Despite these advances, no studies to date have evaluated the effects of organic zinc supplementation on growth performance, digestive enzyme activity, and antioxidant responses in pacu. This gap highlights the need to explore organic zinc's potential role in enhancing both physiological and health-related traits in this economically important species.

MATERIALS AND METHODS

1. Experimental diets

The formulation and proximate composition of the experimental diets are shown in Table (1). A completely randomized design (CRD) was applied, consisting of five dietary treatments with four replicates each. The diets were supplemented with organic Zn at levels of 0, 20, 40, 80, and 160mg kg^{-1} feed (designated as Zn0, Zn20, Zn40, Zn80, and Zn160, respectively).

All coarse dry ingredients were finely ground using a grinder and thoroughly mixed to form a homogenous mixture. The dough was processed into 2mm pellets using multifunctional spiral extrusion equipment. Pellets were oven-dried at 50°C for 3h, sealed in plastic bags, and stored at 25°C until use.

The feed preparation was conducted at the Laboratory of Nutrition, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University. The organic zinc source used in this study was Amaze-ZINC®, produced by PT. Aquacell Indo Pasifik.

Table 1. Formulation and proximate composition of the experimental diets (% dry matter)

	Experimental diets				
	Zn 0	Zn 20	Zn 40	Zn 80	Zn 160
Fish Meal	4,00	4,00	4,00	4,00	4,00
Poultry Meat Meal	7,00	7,00	7,00	7,00	7,00
Corn Gluten Meal	5,00	5,00	5,00	5,00	5,00
Wheat Flour	20,120	20,098	20,076	20,031	19,942
Soy Bean Meal	22,61	22,61	22,61	22,61	22,61
Meat Bone Meal	7,00	7,00	7,00	7,00	7,00
Wheat Pollard	19,59	19,59	19,59	19,59	19,59
Cassava Flour	5,00	5,00	5,00	5,00	5,00
Fish Oil	1,80	1,80	1,80	1,80	1,80
Crude Palm Oil	1,80	1,80	1,80	1,80	1,80
Mineral Mix Zinc Free	4,72	4,72	4,72	4,72	4,72
Vitamin Mix	1,35	1,35	1,35	1,35	1,35
Organic Zn (9% Zn)	0,000	0,022	0,044	0,089	0,178
Proximate composition(%)					
Dry matter	90,25	90,27	90,27	90,05	90,55
Protein	32,92	33,41	32,95	33,01	33,33
Lipid	7,86	7,37	7,94	7,37	7,51
Ash	8,37	8,54	8,41	9,10	8,90
Zn (mg Zn kg^{-1})	74,64	92,69	110,59	156,12	222,96

2. Experimental fish and feeding trial

Pacu fish were obtained from a private fish farm in Bogor, West Java, Indonesia. Prior to the experiment, fish were acclimatized in 121.5L aquaria until deemed healthy and suitable for use. They were then transferred to experimental tanks measuring 50 × 50 × 40 cm with a water height of 30cm (75 L). Each tank was stocked with 15 fish (200 fish m^{-3}), with an initial average body weight of 9.27 ± 0.01g. A total of 20 aquaria were used, each covered with mesh lids to prevent jumping.

Water for the experimental system was supplied from a sedimentation tank. Each aquarium was equipped with continuous aeration and heaters to maintain a water temperature of approximately 30°C. Fish were hand-fed to apparent satiation twice daily (09:00 and 17:00) for 60 days. Uneaten feed, if present, was siphoned 30min after feeding, dried, and weighed to calculate feed intake.

Water quality was managed by replacing 80% of the water every two days. Parameters monitored included temperature, pH, dissolved oxygen (DO), and total ammonia nitrogen (TAN). Temperature was measured twice daily (08:30 and 16:30) using a thermometer; pH was measured daily with a pH meter; DO was measured weekly using a DO meter; and TAN was measured every 14 days in the laboratory. The observed ranges were: temperature 28.7– 31.2°C, DO 5.3– 5.9mg L^{-1}, pH 6.8–7.7, and TAN 0.02–0.08mg L^{-1}.

To minimize handling stress, fish were fasted overnight prior to weighing or sampling.

3. Growth parameters

The nutritional performance was evaluated in terms of initial individual weight (W_0) and the final individual weight (Wt), final biomass (Bt), final individual weight (Wt), survival rate (SR), feed intake (FI), feed conversion ratio (FCR), specific growth rate (SGR), protein retention (PR), lipid retention (LR), and average daily growth (ADG). Growth performance was measured using the following formulas:

SR (%) = [initial fish count / final fish count] x 100

FCR = total feed intake (g) / [final total fish biomass (g) − initial total fish biomass (g)]

SGR (%) = [In (final weight (g)) − In (initial weight (g)) / maintenance period (days)] × 100

Protein Retention (%) = [final biomass protein (g) − initial biomass protein (g)] / total protein intake (g) × 100

Lipid Retention (%) = [final biomass lipid (g) − initial biomass lipid(g)] / total lipid intake (g) × 100

ADG (g/day) = (final weight – initial weight) / maintenance period (days)

4. Sample collection

At the initial stocking, 10 pacu were randomly sampled to determine baseline proximate body composition for growth performance evaluation. At the end of the trial, fish were fasted overnight to empty their stomachs. All fish were netted, and the biomass of each aquarium was recorded. Subsequent samples were collected as follows:

- **Final proximate analysis:** Two fish per aquarium.
- **Histology:** One fish per aquarium; intestines were fixed in neutral buffered formalin (BNF).
- **Zn content:** Three fish per aquarium; liver, intestine, and muscle were collected.
- **Digestive enzymes:** Three fish per aquarium; intestine samples were collected.
- **Health status:** Two fish per aquarium; blood was drawn from the caudal vein using a 1 mL syringe for lysozyme activity and respiratory burst assays.

Anesthesia was administered using clove oil (200μL L^{-1} water) following **Syahidah *et al.* (2019)**. Organ samples for digestive enzyme and antioxidant assays were stored at −80°C, while those for proximate analysis and Zn content were stored at −20°C until testing.

5. Histological examination

Intestinal fragments (~0.5 cm) were preserved in 10% BNF for 72h, then dehydrated in graded ethanol (70, 80, 90, 95, 99, and 100%; 2h each). Samples were cleared with xylene (40min) and infiltrated with paraffin (30min at 60°C). Sections were cut with a microtome, floated on warm water (35°C), dried for 24h, and mounted on slides. Tissues were stained with hematoxylin and eosin following the methods of **Fischer *et al.* (2008)** and **Titford (2009)** to visualize cellular and tissue structures.

6. Biochemical biomarkers

Antioxidant biomarkers including superoxide dismutase (SOD), glutathione peroxidase (GPx), and malondialdehyde (MDA) were measured from liver tissue. Storage and assay procedures followed the instructions provided in the Elabscience® ELISA Kit.

7. Air-exposure challenge

On the final day of the trial, three fish per aquarium were subjected to an air-exposure challenge. Fish were netted and exposed to open air for 3min, then dissected to obtain liver samples. These were analyzed for SOD, GPx, and MDA activity, following the protocols of **Arends *et al.* (1999)** and **Wardani *et al.* (2020)**.

8. Statistical analysis

Data were processed using Microsoft Excel 2019 and analyzed in SPSS version 26.0. Normality and homogeneity of variance were tested prior to analysis. One-way analysis of variance (ANOVA) was performed at a 95% confidence level ($P< 0.05$) to evaluate treatment effects. When significant differences were detected, Duncan's multiple range test was applied to determine pairwise differences among treatments.

RESULTS

1. Growth performance

The growth performance of pacu fed diets with varying Zn levels is summarized in Table (2). Fish were maintained for 60 days under uniform environmental conditions across treatments and replicates. Dietary Zn supplementation significantly affected growth performance compared with the control diet lacking Zn ($P< 0.05$).

Growth was assessed based on initial individual weight (W_0) and final individual weight (W_t), as well as final biomass (B_t), specific growth rate (SGR), protein retention (PR), lipid retention (LR), and average daily growth (ADG). These parameters increased with Zn supplementation up to 40mg kg^{-1} feed, after which they declined at higher dosages (80– 160mg kg^{-1}; $P< 0.05$).

Feed conversion ratio (FCR) was significantly lower ($P< 0.05$) in fish fed 20 and 40mg kg^{-1} Zn diets compared with other treatments, indicating more efficient feed utilization at moderate Zn levels. Notably, the final individual weight (W_t) of fish receiving 40 mg kg^{-1} Zn was ~24% higher than that of fish in the unsupplemented control group.

These results suggest that dietary organic Zn supplementation improves pacu growth performance, with an optimal level at 40mg kg^{-1} feed.

Table 2. Growth performance of pacu fish fed with organic Zn supplementation with different doses for 60 days

Parameter	Experimental diets				
	Zn 0	Zn 20	Zn 40	Zn 80	Zn 160
B_0 (g)	145,97±0,11	145,97±0,18	145,95±0,10	146,05±0,07	146,04±0,12
B_t (g)	681,85±37,64^d	808,60±8,41^b	850,80±23,03^a	728,48±25,68^c	686,68±24,64^d
W_0 (g)	9,73±0,01	9,73±0,01	9,73±0,01	9,74±0,00	9,74±0,01
W_t (g)	45,46±2,51^d	53,91±0,56^b	56,72±1,54^a	48,57±1,71^c	45,78±1,64^d
SR (%)	100±0,00	100±0,00	100±0,00	100±0,00	100±0,00
FI (g)	749,38±16,23^b	771,55±16,99^b	800,50±14,66^a	767,84±11,52^b	747,73±14,42^b
FCR	1,40±0,08^b	1,16±0,02^a	1,14±0,04^a	1,32±0,07^b	1,38±0,05^b
SGR (%)	2,57±0,09^c	2,85±0,02^a	2,94±0,04^a	2,68±0,06^b	2,58±0,06^c
ADG (g day^{-1})	0,60±0,04^d	0,74±0,01^b	0,78±0,03^a	0,65±0,03^c	0,60±0,03^d
PER	2,41±0,14^b	2,85±0,04^a	2,96±0,10^a	2,55±0,07^b	2,40±0,08^b
PR (%)	33,69±2,63^b	46,15±3,16^a	46,87±3,17^a	35,02±0,42^b	27,14±1,90^c
LR (%)	50,85±2,37^c	72,86±4,74^a	75,39±2,29^a	61,13±3,58^b	52,84±7,18^c

[1] Initial Biomass (B_0), Final Biomass (B_t), Initial Individual Weight (W_0), Final Individual Weight (W_t), Survival Rate (SR), Feed Intake (FI), Feed Conversion Ratio (FCR), Specific Growth Rate (SGR), Average Daily Growth (ADG), Protein Efficiency Ratio (PER), Protein Retention (PR), Lipid Retention (LR)

[2] Different letters in the same row indicate significant differences among treatments ($P<0.05$). The values are presented as means ± standard deviation.

2. Digestive enzyme activity

The effects of dietary Zn supplementation on digestive enzyme activities in pacu are shown in Table (3). Supplementation with 20– 40mg Zn kg^{-1} feed significantly enhanced digestive enzyme activities compared with other treatments ($P< 0.05$).

Among the treatments, 40mg Zn kg^{-1} feed produced the highest enzymatic activities ($P< 0.05$), with marked increases in amylase, lipase, chymotrypsin, and trypsin. Overall, enzyme activity exhibited a positive trend from 0 to 40mg Zn kg^{-1} feed, followed by a decline at higher inclusion levels (80– 160mg Zn kg^{-1} feed).

These findings indicate that moderate dietary Zn supplementation optimizes digestive enzyme function in pacu, with 40mg Zn kg^{-1} identified as the most effective level.

Table 3. Digestive enzyme activity of pacu fish fed with diets supplemented with different doses of organic Zn for 60 days

Parameter	Experimental Diets				
	Zn0	Zn20	Zn40	Zn80	Zn160
Amylase (mU/ml)	531,52±40,49[c]	882,69±167,26[ab]	1003,63±7,91[a]	971,94±71,76[a]	754,08±27,97[b]
Lipase (mU/ml)	23,25±4,39[b]	41,27±3,63[a]	48,25±5,33[a]	37,79±5,33[a]	14,53±10,66[b]
Chymotrypsin (mU/ml)	2,95±0,21[b]	3,45±0,44[a]	3,49±0,55[a]	3,16±0,38[ab]	2,68±0,12[b]
Trypsin (mu/ml)	2,63±0,20[c]	3,99±0,23[ab]	4,06±0,37[a]	3,97±0,32[ab]	3,47±0,29[b]

[1] Different letters in the same row indicate significant differences among treatments ($P<0.05$). The values are presented as means ± standard deviation.

3. Antioxidant response and health status

The testing of antioxidant response parameters in pacu fish fed diets with different Zn dosages is presented in Table (4). The addition of Zn at doses ranging from 20 to 40mg kg^{-1} feed resulted in significantly higher outcomes ($P<0.05$) compared to other treatments for both SOD and GPx parameters. The MDA parameter test results showed significantly lower outcomes ($P<0.05$) at doses of 20 to 40mg kg^{-1} feed compared to the control and highest doses. The testing of health status of Zn levels in pacu fish fed diets with different Zn dosages is presented in Table (5). The Respiratory Burst (RB) values showed an increasing trend with increasing Zn dosage. The best lysozyme activity values were significantly ($P<0.05$) demonstrated in the treatments with Zn doses of 40 to 80mg kg^{-1} feed, while other treatments showed lower and statistically non-significant results ($P>0.05$).

Table 4. Antioxidant response of pacu fish fed with different dietary treatments for 60 days

Parameter	Experimental Diets				
	Zn0	Zn20	Zn40	Zn80	Zn160
SOD (% inhibition)	72,53±2,55^{b}	82,37±3,15^{a}	83,64±1,80^{a}	74,63±3,20^{b}	67,62±2,46^{c}
GPx (% inhibition)	25,79±1,94^{bc}	29,72±2,55^{ab}	29,74±1,76^{ab}	31,05±3,99^{a}	24,99±1,57^{c}
MDA (nmol/mL)	6,53±0,41^{b}	4,01±0,85^{a}	4,35±0,31^{a}	5,03±0,72^{a}	6,60±0,42^{b}

[1] *Superoxide dismutase* (SOD), *Glutathione Peroxidase* (GPx), *Malondialdehyde* (MDA)

[2] Different letters in the same row indicate significant differences among treatments (P<0.05). The values are presented as means ± standard deviation.

Table 5. Health status of pacu fish fed with different dietary treatments for 60 days

Parameters	Experimental Diets				
	Zn0	Zn20	Zn40	Zn80	Zn160
RB (OD)	0,44±0,03^{a}	0,51±0,07^{ab}	0,58±0,11^{b}	0,61±0,06^{b}	0,74±0,05^{c}
LA (U/mL)	217±9,87^{c}	239±7,02^{b}	277±15,53^{a}	289±8,08^{a}	215±6,11^{c}

[1] *Respiratory Burst* (RB), *Lysozyme Activity* (LA)

[2] Different letters in the same row indicate significant differences among treatments (P<0.05). The values are presented as means ± standard deviation.

4. Intestinal histology

Representative histological images of pacu intestines under different Zn supplementation treatments are shown in Fig. (1), and morphometric measurements are summarized in Table (6). Villi height was significantly greater (P< 0.05) in fish fed 40 mg Zn kg^{-1} feed compared with all other treatments. Villi width was also significantly higher (P< 0.05) at Zn supplementation levels of 20– 80mg kg^{-1} feed compared to the control and highest dose group (160mg kg^{-1}).

Overall, dietary Zn supplementation at 20– 40mg kg^{-1} feed produced the most favorable intestinal morphometric characteristics, suggesting improved absorptive surface area and nutrient assimilation efficiency.

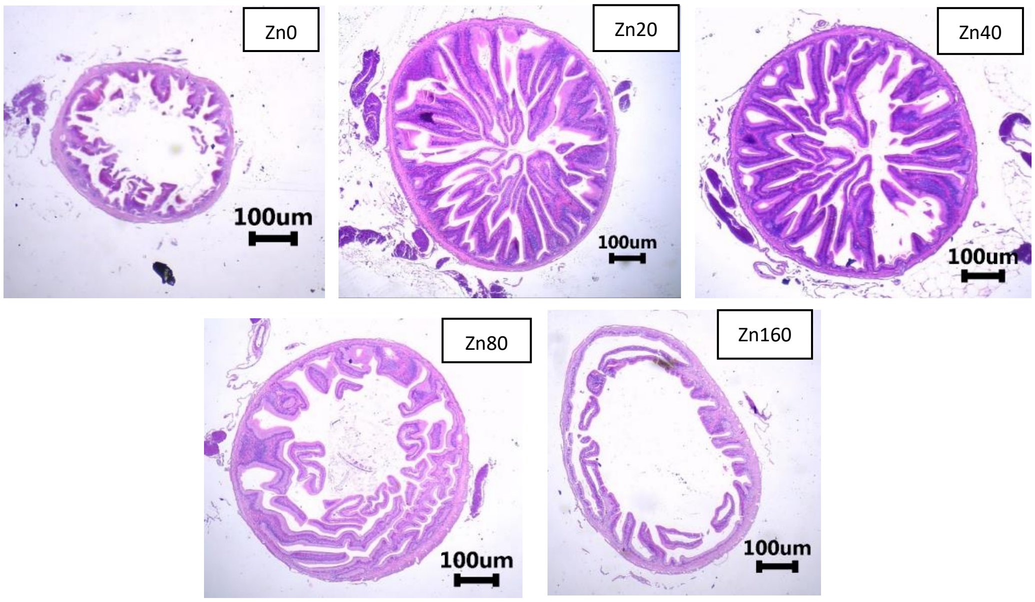

Fig. 1. Intestinal histology of pacu fish treated with organic Zn supplementation in the feed

Table 6. Villi size of pacu fish fed with different dietary treatments for 60 days

Parameter	Experimental Diets				
	Zn0	Zn20	Zn40	Zn80	Zn160
VH (µm)	124,48±17,20^{c}	323,35±64,92^{a}	245,02±37,05^{b}	142,39±35,74^{c}	106,72±14,68^{c}
VW (µm)	24,61±5,12^{c}	82,86±12,76^{a}	55,01±16,13^{b}	47,86±10,40^{b}	35,55±4,18^{bc}

[1] *Villi height (VH)*; *Villi width (VW)*

[2] Different letters in the same row indicate significant differences among treatments (*P*<0.05). The values are presented as means ± standard deviation.

5. Zinc content in organs

Testing of zinc levels in pacu fish organs fed diets with different Zn dosages showed significant differences. Addition of Zn at doses ranging from 20 to 40mg kg^{-1} feed resulted in significantly higher outcomes (*P<0.05*) compared to other treatments in the liver, intestines, and muscles. The test results are presented in Table (7).

Table 7. Zn content in the organs of pacu fish fed with different treatments for 60 days

Parameter	Experimental Diets				
	Zn0	Zn20	Zn40	Zn80	Zn160
Liver (mg/kg)	9,35±0,39^{b}	15,44±0,46^{a}	15,27±1,54^{a}	13,94±1,45^{a}	10,02±2,17^{b}
Intestine (mg/kg)	9,48±1,41^{cd}	16,40±1,73^{a}	15,17±3,41^{ab}	12,46±1,35^{bc}	8,17±2,07^{d}
Muscle (mg/kg)	0,68±0,28^{c}	6,50±0,96^{a}	5,70±0,58^{ab}	5,87±0,81^{ab}	4,29±1,20^{b}

[1] Different letters in the same row indicate significant differences among treatments (*P*<0.05). The values are presented as means ± standard deviation.

DISCUSSION

Histological examination of the intestine is a vital physiological indicator for evaluating intestinal health status **(Xu *et al.*, 2023)**. In this study, the intestinal villi of pacu fed with organic Zn supplementation at 40mg kg^{-1} diet exhibited greater height and width compared to without organic Zn supplementation. This finding aligns with that of **Xu *et al*. (2023)**, who reported that tilapia fed with organic Zn had taller and wider villi compared to those fed with Zn inorganic and nanoparticle forms. These results are further supported by **Inarto *et al.* (2023)**, proving that supplementation of organic Zn within the range of 20 to 40 mg kg^{-1} diet resulted in a larger absorption area. **Yang *et al.* (2022)** also proved that Zn dosage of 40mg kg^{-1} diet exhibited the highest intestine absorption area, surpassing other treatments and dosages. These findings collectively underscore that organic Zn supplementation in feed enhances intestinal absorption area.

Intestinal histology can be assessed through tight junctions **(Buckley & Turner, 2018)**, which are primarily located in the apical areas of cells and play a crucial role in ion and fluid diffusion, defense functions, and cell proliferation **(Garcia *et al.*, 2018)**. The intestine's ability to absorb nutrients is influenced by villi size; larger villi increase the surface area for absorption, facilitating nutrient uptake and digestion **(Mohammady *et al.*, 2021)**. Proper dosage of Zn supplementation enhances cell proliferation and inhibits apoptosis **(Diao *et al.*, 2021)**. Conversely, excessive dosage can lead to reduced surface area of intestinal villi, indicating negative effects due to Zn toxicity exceeding optimal limits. Toxicity from excessive Zn accumulation can result in intestinal damage, such as goblet cell formation, hemorrage, cell death, necrosis, degeneration, and villi shortening and fusion **(Khan *et al.*, 2022)**.

Digestive enzymes are crucial components of the digestive tract in fish. Activity of these enzymes and the absorptive capacity of the intestine determines dietary nutrients utilized by fish **(Zhao *et al.*, 2016)**. In this study, Zn supplementation with dose of 40 mg kg^{-1} feed showed the highest activity of digestive enzymes, such as amylase, lipase, trypsin, and chymotrypsin. This finding is consistent with **Muralisankar *et al.* (2015)**, who reported that the activity of digestive enzymes (amylase, lipase, and protease) in giant freshwater prawns increased when fed with 60mg kg^{-1} feed of Zn supplementation. Similarly, **Inarto *et al.* (2023)** observed enhanced activity of amylase, lipase, trypsin, and chymotrypsin in the Nile tilapia fed with 40mg kg^{-1} feed of organic Zn.

Digestive enzymes are essential for the hydrolysis of nutrients such as proteins, polypeptides, amino acids, lipids, and various carbohydrates during digestion. Enzymes like amylase, lipase, and protease are secreted into the lumen of digestive organs such as the stomach and intestines. Enzymes on the intestinal membrane (carbohydrase, peptidase, and protease) are bound to microvilli. Carbohydrate digestion requires amylase, lipid digestion requires lipolytic enzymes like lipase, and protein digestion requires proteolytic enzymes like protease, trypsin, and chymotrypsin. Chymotrypsin, a digestive enzyme found in pancreatic tissue, is secreted into the duodenum. It can hydrolyze amides, esters, and peptides at the carboxyl side of proteins. The inactive form of chymotrypsin (chymotrypsinogen) is activated by trypsin. Chymotrypsin transforms into intermediate products such as α-chymotrypsin, γ-chymotrypsin, δ-chymotrypsin, and π-chymotrypsin before finally becoming chymotrypsin A. The intermediate products formed depend on the concentration and reaction of trypsin **(Friedman & Fernandez-Gimenez, 2023)**. Trypsin primarily cleaves peptides at the carboxyl side of amino acids such as lysine and arginine, whereas chymotrypsin predominantly cleaves peptide bonds at the carboxyl side of aromatic amino acids like tyrosine, tryptophan, and phenylalanine **(Solovyev *et al.*, 2023)**. These amino acids are then absorbed by the intestines and distributed by the blood to various organs and tissues **(Halver & Hardy, 2002)**.

The rate of zinc absorption highly affected by the source of zinc itself. For example, ZnO and $ZnSO_4$, which are inorganic zinc, usually used in feed supplementation. On the other hand, organic zinc pairs zinc with an organic ligand, such as amino acids, peptides, or proteins, all of which have higher bioavailability compared to inorganic zinc **(Star *et al.*, 2012; Świątkiewicz *et al.*, 2014)**. Zinc bioavailability is limited, because they are competing with other minerals and also inhibited by antagonistic substances present in the body **(Lönnerdal, 2000; Sauer *et al.*, 2017)**. Therefore, selecting sources of zinc that can be absorbed without interference from other substances is very crucial. Unlike inorganic zinc, which requires transporters, organic zinc can be carried by aminio acids **(Gao *et al.*, 2014; Sauer *et al.*, 2017)**. This alternative pathway for zinc transport is a major factor contributing to the higher bioavailability of organic zinc compared to inorganic zinc **(De Grande *et al.*, 2020)**. Despite the higher bioavailability of organic zinc, its use remains limited due to its relatively high cost **(Zhao *et al.*, 2014)**.

Research findings indicate that pacu fish fed with Zn supplementation within the dose range of 20 to 40 mg kg^{-1} feed experienced increased Zn levels in the liver, intestine, and muscle compared to fish that did not receive Zn supplementation. The result was in line with **Liang *et al.* (2020)** cited in **Ling *et al.* (2020)**, which demonstrated that fish fed with organic Zn supplementation accumulated more Zn in organs, including the intestine. Zinc transport is regulated by Zn transporters, specifically the ZnT, ZIP, and MTF1 families **(Kambe *et al.*, 2015)**. Organic Zn significantly influences the regulation of gene expression related to Zn transport. The efflux of Zn from cells is mediated by ZnT1 **(Nishito & Kambe, 2019; Krall *et al.*, 2020)**. Cytoplasmic Zn homeostasis is maintained by the regulation of the efflux of Zn from the cytoplasm with the help of ZnT4 and ZnT5, which can be found in the cytoplasmic membrane **(Kambe *et al.*, 2015)**. On the other hand, Zn importer, such as ZIP1, ZIP4, ZIP5, ZIP8, and ZIP14 can be found in the plasma membrane, facilitating the transport of zinc from extracellular space into the cell **(Kambe *et al.*, 2015; Nagamatsu *et al.*, 2022)**. This study found that Zn supplementation in feed increased Zn levels in the intestine, liver, and muscle. However, when the administered dose exceeded the optimal range, Zn levels in these organs decreased. The research by **Xu *et al.* (2023)** explains that organic Zn can upregulate the expression of ZIP family genes to facilitate Zn uptake into cells, leading to increased Zn accumulation in organs. Conversely, when Zn intake is excessive, the expression of ZnT family genes plays a role in Zn efflux to maintain Zn homeostasis. This is supported by **Chen *et al.* (2017)**, who reported that excess Zn increases the expression of ZnT5 and ZnT7 genes, which help reduce Zn toxicity within cells.

Cellular Zn homeostasis is largely governed by the uptake and efflux of Zn through specific transporters, as well as the sequestration of Zn by carrier proteins. The absence of specific sites for Zn production and storage underscores the importance of supplementing Zn through feed at appropriate doses to ensure Zn homeostasis, thereby enabling the body to maintain and support various physiological processes **(Bonaventura *et al.*, 2015)**.

The results of this study indicate an increase in SOD and GPx activities and a decrease in MDA activity in pacu fish fed with Zn supplementation at a dose of 40mg kg^{-1}. This finding is consistent with the study by **Yu *et al.* (2021)**, postulating that the Zn supplementation at 35mg kg^{-1} of feed exhibited an increase in SOD activity and led to a decrease in MDA activity. However, when the Zn dose exceeded the optimal limit, there was a reduction in SOD and GPx activities and an increase in MDA activity. This suggests a toxic effect from excessive Zn consumption by the fish. Excessive Zn exposure can lead to a decline in SOD and GPx activities due to metal binding and -SH group interactions on the enzymes. Moreover, the decrease in SOD and GPx activities may also result from higher ROS consumption following prolonged Zn exposure **(Eyckmans *et al.*, 2011; Wang *et al.*, 2020)**.

The enzyme superoxide dismutase (SOD) plays an important role in the disintegration of free radicals by converting them into hydrogen peroxide (H_2O_2). Low SOD activity in stressed fish can lead to the accumulation of reactive oxygen species (ROS), ultimately resulting in mortality **(Phull *et al.*, 2018)**. Glutathione peroxidase (GPx) is an intracellular enzyme vital for breaking down hydrogen peroxide (H_2O_2) into water (H_2O) and lipid peroxides into alcohols, primarily within the mitochondria and cytosol. This process protects cells from oxidative stress (Ighodaro and Akinloye 2018). Zinc (Zn) acts as antioxidant agent **(Gamoh & Rink, 2017)** as it serves as cofactor for Zn-superoxide dismutase, protecting cells from free radicals **(Oteiza, 2012)**. However, excessive Zn levels can generate an overproduction of ROS **(Wang *et al.*, 2020)**. The impact of elevated ROS is inducing oxidative stress and damaging biological molecules, which leads to lipid peroxidation, DNA adduct formation, and amino acid modifications. This aligns with the findings of **Wang *et al.* (2020)**, who reported that the Nile tilapia fed with Zn-enriched lemna exhibited high Zn^{2+} accumulation, reduced SOD and GPx activities in the liver, and increased MDA levels, indicative of lipid peroxidation. Lipid peroxidation can be used as biomarker to measure oxidative stress and indicate oxidative stress degree within tissues. Increased MDA activity suggests heightened oxidative stress due to excessive Zn and significant lipid peroxidation in the liver. Therefore excessive Zn exposure causes production of ROS because of insufficient antioxidant capacity **(Wu *et al.*, 2014)**.

Fish have various defense mechanisms to address harmful threats such as pathogen attacks and stress through their immune system. The activity of respiratory burst (RB) in phagocytes produces reactive oxygen species (ROS) which are essential for nonspecific immunity and the antioxidant response **(Bu *et al.*, 2019)**. In this defense process, RB activity level indicates bactericidal capability of phagocytic cells **(Zhang *et al.*, 2021)**. Phagocytosis itself serves as an index to measure the body's defense ability against pathogens, as phagocytic cells attack pathogens using RB activity. Additionally, lysozyme, an important hydrolytic enzyme for the nonspecific immune system, plays a key role in fish defense against pathogens. It directly enhances phagocytic activity by hydrolyzing the mucopolysaccharides in bacterial cell walls **(Ragland and Criss 2017)**.

The results of this study indicate a trend of increased respiratory burst (RB) activity in treatments with Zn supplementation ranging from 20 to 80mg kg^{-1} of feed. This finding is consistent with the research conducted by **Kishawy *et al.* (2020)** and **Inarto *et al.* (2023)**, which found that the Nile tilapia fed with Zn supplementation at a dose of 40mg kg^{-1} of feed experienced increased lysozyme and RB activity. **Mondal *et al.* (2020)**, also reported RB activity in rohu fish fed with Zn supplementation. Additionally, this study found an increase in lysozyme activity in treatments with Zn at 40 and 80mg kg^{-1} of feed.

This study demonstrates that pacu fish fed with Zn supplementation at a dose of 40mg kg^{-1} exhibited enhanced growth compared to fish not receiving Zn supplementation. This finding aligns with various studies conducted on the rainbow trout **(Shahpar & Johari, 2019)**, rohu **(Thangapandiyan & Monika, 2020)**, catfish **(Mahboub *et al.*, 2020)**, and tilapia **(Ghazi *et al.*, 2021)**. Research over the past decade has shown that the Zn requirements for different fish species vary **(Gajula *et al.*, 2011; Sauliute *et al.*, 2020; Wei *et al.*, 2020)**. Fish deficient in Zn intake experience stunted growth **(Song *et al.*, 2017)**, increased mortality, and cataracts. Conversely, excessive Zn absorption can inhibit performance of reproduction and growth, as well as utilization of other minerals, leading to disruptions in various physiological and biochemical processes and within the body, ultimately resulting in oxidative damage and ion regulation disturbances **(Yu *et al.*, 2021)**.

CONCLUSION

Organic Zn supplementation through feed enhances growth performance, digestive enzyme activity, antioxidant response, intestinal villi area, health status, and organ Zn content in pacu fish. Based on the conducted research, the optimal treatment for pacu involves Zn supplementation at 40mg kg^{-1}.

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