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Dietary Astaxanthin and Betaine Modulate Digestive Function and Intestinal Morphometry in Salinity-Stressed Nile Tilapia

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ABSTRACT

Chronic exposure to elevated salinity can alter gastrointestinal function in Nile tilapia (*Oreochromis niloticus*). This study evaluated dietary astaxanthin (ASX), betaine (BET), and their combination during 40 days at 25 ppt using five treatments with three replicate aquaria: freshwater control (FC), salinity control (SC), ASX (150 mg kg^{-1} diet), BET (5 g kg^{-1} diet), and ASX + BET. Protease did not differ significantly among the five treatments (one-way analysis of variance (ANOVA), $p = 0.161$), whereas amylase ($p = 0.046$) and lipase ($p = 0.013$) did. A secondary 2 × 2 factorial ANOVA of the four saline groups showed no significant factorial effects on protease. ASX significantly affected amylase [$F(1,8) = 10.16$, $p = 0.013$], while the ASX × BET interaction was not significant ($p = 0.077$). Lipase showed a significant ASX × BET interaction [$F(1,8) = 10.67$, $p = 0.011$] without significant main effects. Salinity reduced villus length from 221.79 ± 34.26 to 105.00 ± 3.17 µm and width from 82.22 ± 2.26 to 54.79 ± 3.05 µm. Villus length showed significant ASX [$F(1,8) = 65.43$, $p < 0.001$], BET [$F(1,8) = 19.19$, $p = 0.002$], and interaction effects [$F(1,8) = 19.43$, $p = 0.002$]; villus width showed a significant ASX effect [$F(1,8) = 20.63$, $p = 0.002$] and interaction [$F(1,8) = 5.72$, $p = 0.044$]. Thus, combined supplementation produced endpoint-specific interactions rather than a uniform synergistic benefit, while ASX showed the most consistent main effects across amylase and intestinal morphometry.

1. INTRODUCTION

Global aquaculture production reached a record 130.9 million tonnes in 2022, underscoring the growing role of aquatic foods in global food systems [1]. Among cultured finfish species, Nile tilapia (*Oreochromis niloticus*) is particularly important for its rapid growth rate, efficient feed conversion, high reproductive output, and broad environmental tolerance. These traits have traditionally supported intensive production in freshwater systems. However, mounting competition for freshwater resources, land-use pressures, and the progressive salinization of inland and coastal areas have driven the expansion of tilapia farming into brackish-water environments [2]. While Nile tilapia is euryhaline, its physiological tolerance is not unlimited: prolonged exposure to elevated salinity imposes substantial osmoregulatory and metabolic costs that can exceed the species' adaptive capacity [3]. From a sustainability perspective, nutritional strategies that help tilapia maintain function in brackish or otherwise marginal waters could broaden production options where freshwater is scarce, reduce pressure on freshwater resources, and strengthen aquaculture adaptation to salinization and climate-related water constraints [2, 4].

The intestine occupies a central role in salinity acclimation, functioning simultaneously as a digestive organ and a principal site of water and electrolyte processing in seawater-acclimated teleosts [5-7]. Consequently, salinity-related disturbance may manifest as altered digestive enzyme activity, disrupted epithelial organization, and changes in mucosal fold dimensions [8, 9]. Protease, amylase, and lipase activities serve as functional indices of protein, carbohydrate, and lipid hydrolysis, respectively, while villus or mucosal-fold length and width offer structural indicators of absorptive surface development [8]. Elevated salinity has previously been linked to abnormal intestinal histology in Nile tilapia [8, 9], though the magnitude of this response is modulated by salinity level, exposure duration, dietary composition, fish size, and broader culture conditions [8].

Functional dietary additives are being investigated as practical means to support gastrointestinal stability under environmental stress. Astaxanthin (ASX) is a xanthophyll carotenoid used in aquaculture feeds for pigmentation and for its biological activity at cellular membranes. Current evidence associates dietary ASX with antioxidant, immunomodulatory, and stress-related responses in aquatic animals, although responses vary with source, dose, species, and physiological context [10]. Studies with other lipid-soluble antioxidants in

tilapia also show that improved digestive-enzyme activity can coincide with better intestinal fold organization, supporting the value of jointly assessing functional and structural gut endpoints [11]. Nevertheless, an antioxidant-mediated mechanism cannot be inferred from intestinal measurements alone.

Betaine (BET), or trimethylglycine, acts as a compatible organic osmolyte and methyl-group donor [12]. By contributing to intracellular osmotic balance without the disruptive effects associated with high concentrations of inorganic ions, BET could help preserve protein conformation and cellular hydration during osmotic challenge [12, 13]. Dietary BET has been linked to immune, antioxidant, metabolic, and intestinal responses in fish, but outcomes are strongly context-dependent and do not establish that BET will improve every digestive endpoint under saline culture [14]. Moreover, combining BET with ASX is mechanistically plausible but does not necessarily generate additivity or synergy.

Previous research have examined nutritional interventions for tilapia exposed to salinity, including essential oils, proline, synbiotics, biofloc-derived ingredients, and modified feeding strategies [15, 16]. However, evidence remains limited regarding the individual and combined effects of ASX and BET on digestive-enzyme activity and quantitative intestinal morphometry during chronic exposure to 25 ppt. The novelty of the present work is not the independent use of either additive, both of which have been studied previously, but the side-by-side evaluation of their individual and combined response profiles under the same chronic salinity challenge using paired functional and structural gastrointestinal endpoints. This design can indicate whether combined supplementation broadens the response profile, but it does not by itself establish statistical synergy or antagonism. This distinction is important because preservation of intestinal function may determine whether a stress-mitigation strategy ultimately translates into effective nutrient utilization.

The present study therefore evaluated intestinal protease, amylase, and lipase activities, villus length and width, and qualitative intestinal morphology in juvenile Nile tilapia fed ASX, BET, or both, during a 40-day exposure to 25 ppt. We hypothesized that unsupplemented salinity exposure would alter digestive-enzyme profiles and reduce villus dimensions, and that dietary supplementation would attenuate these responses. We further tested whether the combined treatment produced a response exceeding that of either additive alone.

2. MATERIALS AND METHODS

2.1 Experimental diets

Five experimental diets were prepared from a commercial floating pellet containing approximately 33% crude protein. The treatments were: freshwater control (FC), maintained at 0 ppt and fed the unsupplemented basal diet; salinity control (SC), maintained at 25 ppt and fed the basal diet; ASX, maintained at 25 ppt and fed ASX at 150 mg kg^{-1} diet; BET (BET), maintained at 25 ppt and fed BET at 5 g kg^{-1} diet; and the combined treatment (ASX + BET), maintained at 25 ppt and receiving both inclusion levels. ASX containing 5% active Asx was supplied by Yangge Biotech, and BET hydrochloride (≥98% purity) was supplied by Shandong Sunwin Biotech. The inclusion levels were selected a priori from published Nile tilapia feeding studies rather than from a dose-response optimization in the present experiment. An ASX level of 150 mg kg^{-1} has been evaluated within graded-dose studies in Nile tilapia and associated with beneficial physiological responses, whereas 5 g kg^{-1} BET has been tested in Genetically Improved Farmed Tilapia (GIFT) Nile tilapia and was associated with favorable growth and feed-conversion responses [17, 18].

Supplements were applied by surface coating. For each 100-g batch, the required additive was dispersed or dissolved in distilled water to a final concentration of 100 mL kg^{-1} feed, with lignosulfonate at 5 g kg^{-1} as a binder. The solution was sprayed onto pellets during continuous mixing. Coated pellets were dried at 40 °C for 4 h and subsequently coated with squid oil at 10 g kg^{-1} diet. Diets were stored in airtight polyethylene bags at 4 °C, and fresh batches were prepared every 10 days. The diets contained 31.51–31.88% crude protein, 6.31–6.79% crude lipid, and 7.72–8.70% ash on a dry-weight basis.

2.2 Fish, acclimation, and experimental design

A total of 150 healthy juvenile Nile tilapia (*Oreochromis niloticus*) originating from a single spawning cohort were obtained from the Sumber Pasir Aquaculture Laboratory, Universitas Brawijaya, Indonesia. Prior to the experiment, fish were visually examined to ensure normal swimming behavior and the absence of external signs of disease or injury. At the beginning of the trial, mean initial body weight was comparable among treatments and ranged from 4.29 to 4.77 g. Fish assigned to the saline treatments were gradually acclimated to the target salinity to minimize acute osmotic stress. Salinity was increased by approximately 5 ppt every 48–72 h using technical-grade sodium chloride dissolved in dechlorinated freshwater. Each subsequent increase was performed only after fish resumed normal feeding behavior and no mortality had been observed for at least 24 h. Fish in the FC remained at 0 ppt throughout the acclimation period. During acclimation, fish were maintained in aerated 500-L fiberglass tanks and fed the commercial basal diet at approximately 4% body weight day^{-1}, divided into two daily meals.

Following acclimation, fish were fasted for 24 h and then randomly distributed among 15 glass aquaria measuring 80 × 40 × 30 cm, with three replicate aquaria per treatment and ten fish per aquarium. Fish were allowed to adapt to the experimental aquaria for three days before the 40-day feeding trial commenced. Experimental diets were provided twice daily at 08:00 and 16:00 h at approximately 4–5% of body weight day^{-1}. Feed allowance was recalculated every seven days based on group biomass estimation to maintain an appropriate feeding level throughout the experiment.

2.3 Rearing conditions and water quality

Each aquarium received continuous aeration. A static-renewal system was used, with approximately 30% of the water replaced every two days using preconditioned water at the corresponding salinity and ambient temperature. Salinity was checked after water exchange using a hand-held refractometer. Temperature, dissolved oxygen, and pH were measured regularly, and inorganic nitrogen compounds were monitored with colorimetric test kits. Across the trial, temperature ranged from 22.7 to 28.9 °C, pH from 6.02 to 7.20, dissolved oxygen from 4.25 to 6.03 mg L^{-1}, ammonia from 0 to 0.05 mg L^{-1}, nitrite from 0 to 0.50 mg L^{-1}, and nitrate from

0 to 250 mg L^{-1}. Saline treatments were maintained at 25 ppt and FC at 0 ppt. The upper nitrate reading indicates episodes of nitrogen accumulation. Because all aquaria followed the same water-renewal and monitoring protocol, nitrate was not a deliberately manipulated treatment factor; nevertheless, background nitrate exposure could have contributed to physiological stress and is therefore considered a potential experimental limitation.

2.4 Intestinal sampling and crude-enzyme extraction

At the end of the trial, fish were fasted for 48 h to reduce residual digesta. Fish selected for sampling were anaesthetized with clove oil at 100 mg L^{-1} and euthanized by destruction of the medulla oblongata under deep anaesthesia. The intestine was removed and gently rinsed. Approximately 2–3 g of intestinal tissue was collected, and a 1-g portion was combined with 2 mL ice-cold distilled water. The mixture was vortexed and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was transferred to a clean microtube and maintained at 4 °C until analysis. One analytical value was generated for each replicate aquarium (n = 3 per treatment).

2.5 Digestive-enzyme assays

2.5.1 Protease activity

Protease activity was determined using casein as the substrate. Crude intestinal enzyme extract was mixed with 2% casein prepared in 0.05 M phosphate buffer (pH 7.0) and incubated at 37 °C for 10 min. The enzymatic reaction was terminated by adding 0.4 M trichloroacetic acid to precipitate undigested protein. After centrifugation, the resulting supernatant was reacted with Na_2CO_3 and Folin–Ciocalteu reagent. Absorbance was measured spectrophotometrically at 660 nm. Protease activity was calculated using a tyrosine standard curve and expressed as U mL^{-1} of crude enzyme extract.

2.5.2 Amylase activity

Amylase activity was measured using soluble starch as the substrate. Crude enzyme extract was incubated with 5% soluble starch in buffer containing 20 mM NaCl at 37 °C for 15 min. The reducing sugars released during starch hydrolysis were quantified using 1% 3,5-dinitrosalicylic acid reagent. Absorbance was measured at 540 nm, and amylase activity was calculated from a maltose standard curve and expressed as U mL^{-1}.

2.5.3 Lipase activity

Lipase activity was determined using a 10% olive-oil emulsion as the substrate. Crude enzyme extract was incubated with the substrate at 37 °C and 150 rpm for 15 min. The reaction was terminated with 95% ethanol. Released fatty acids were quantified by titration with 0.005 M NaOH using phenolphthalein as the endpoint indicator. Enzyme activity was corrected against the corresponding blank and expressed as U mL^{-1}.

2.6 Intestinal histology and morphometry

Intestinal tissue was fixed in 10% neutral-buffered formalin. Samples were dehydrated through graded ethanol (70, 80, 85, and 100%), cleared in xylene, infiltrated with paraffin at 50–60 °C, embedded, and sectioned at 5 μm. Sections were mounted on glass slides, dried, stained with haematoxylin for 10 min and eosin for 5 min, and mounted with Entellan. Slides were examined by light microscopy at 400× magnification.

Villus length and width were measured in micrometres from digital micrographs. Villus length was defined as the distance from the mucosal base to the villus apex, while width was measured across the villus body. The mean value for each replicate aquarium was used as the biological observation. In addition to morphometry, sections were examined qualitatively for overall fold organization, continuity, and relative shortening or broadening. No formal lesion index, blinded semiquantitative pathology score, epithelial-integrity score, fold count, or goblet-cell density assessment was applied. Consequently, qualitative observations were treated as descriptive support and not as an independent inferential endpoint.

2.7 Statistical analysis

The aquarium was treated as the experimental unit (n = 3). Results are reported as mean ± standard deviation. Normality was evaluated using the Shapiro–Wilk test and homogeneity of variance using Levene's test. For the overall comparison among the five predefined treatments (FC, SC, ASX, BET, and ASX + BET), data were analysed by one-way analysis of variance (ANOVA), followed by Duncan's multiple-range test only when the overall ANOVA was significant. In addition, because the four groups maintained at 25 ppt (SC, ASX, BET, and ASX + BET) formed a balanced 2 × 2 structure, a secondary two-way factorial ANOVA was performed with dietary ASX (absent/present) and BET (absent/present) as fixed factors. The factorial model tested the main effects of ASX and BET and the ASX × BET interaction. The FC was excluded from the factorial analysis because it differed in environmental salinity and therefore did not constitute a factorial treatment combination. The factorial analysis complemented, rather than replaced, the overall treatment-wise comparison. Statistical significance was accepted at $p < 0.05$. Given the limited biological replication of three aquaria per factorial cell, interaction effects were interpreted conservatively. Analyses were performed using IBM SPSS Statistics.

3. RESULTS AND DISCUSSION

3.1 Digestive enzyme responses to dietary astaxanthin and betaine under chronic salinity stress

Protease activity did not differ significantly among the five treatments [$F(4,10) = 2.060$, $p = 0.161$]. Mean activity was 2.72 ± 0.38 U mL^{-1} in FC, 2.26 ± 0.27 U mL^{-1} in SC, 2.34 ± 0.34 U mL^{-1} in ASX, 2.06 ± 0.31 U mL^{-1} in BET, and 2.23 ± 0.11 U mL^{-1} in ASX + BET (Table 1). Because the overall ANOVA was not significant, no treatment-specific post-hoc differences were inferred for protease. The lower numerical values in the saline groups relative to FC should therefore be interpreted cautiously and do not demonstrate a statistically supported suppression or restoration of proteolytic activity.

Amylase activity differed among treatments [$F(4,10) = 3.585$, $p = 0.046$]. SC showed the lowest mean activity (0.67 ± 0.05 U mL^{-1}), approximately 33% below FC (1.00 ± 0.19 U mL^{-1}). Activities were 0.91 ± 0.08, 0.85 ± 0.08, and 0.90 ± 0.09 U mL^{-1} in ASX, BET, and ASX + BET, respectively.

Duncan's test indicated that SC was significantly lower than FC, ASX, and ASX + BET, whereas BET was intermediate. Thus, the ASX-containing treatments maintained amylase activity at approximately 91% of the freshwater-control level.

Table 1. Intestinal digestive-enzyme activities of juvenile Nile tilapia fed astaxanthin (ASX), betaine (BET), or their combination during 40 days of chronic salinity exposure

Parameter	Treatment				
	FC	SC	ASX	BET	ASX + BET
Protease (U mL^{-1})	2.72 ± 0.38	2.26 ± 0.27	2.34 ± 0.34	2.06 ± 0.31	2.23 ± 0.11
Lipase (U mL^{-1})	0.11 ± 0.05^{a}	0.25 ± 0.08^{b}	0.11 ± 0.05^{a}	0.19 ± 0.05^{ab}	0.28 ± 0.05^{b}
Amylase (U mL^{-1})	1.00 ± 0.19^{b}	0.67 ± 0.05^{a}	0.91 ± 0.08^{b}	0.85 ± 0.08^{ab}	0.90 ± 0.09^{b}

Note: Values are mean ± SD (n = 3 aquaria). Different letters within a row indicate significant differences according to Duncan's multiple-range test ($p < 0.05$); post-hoc grouping was not applied to protease because the overall one-way ANOVA was not significant. FC = freshwater control, SC = salinity control.

The enzyme data indicate selective rather than uniform digestive adjustment under hyperosmotic conditions. In saline water, the teleost intestine participates not only in nutrient digestion but also in drinking-water processing, ion transport, and water absorption [19]. Sustained salinity exposure may therefore modify the intestinal ionic and physicochemical environment and redistribute metabolic resources among digestive and osmoregulatory processes. In the present study, the statistically supported one-way response was a reduction in amylase, whereas the numerical decrease in protease was not significant. Salinity has previously been reported to affect intestinal morphology, nutrient digestibility, and digestive-enzyme activities in Nile tilapia [8, 9]. Accordingly, these enzyme changes are best interpreted as salinity-associated modulation of digestive physiology; direct effects on digestive efficiency would require digestibility or nutrient-flux measurements.

Lipase activity also differed among treatments [$F(4,10) = 5.501$, $p = 0.013$]. FC and ASX had the lowest means (both 0.11 ± 0.05 U mL^{-1}), BET was intermediate (0.19 ± 0.05 U mL^{-1}), and SC and ASX + BET had higher activities (0.25 ± 0.08 and 0.28 ± 0.05 U mL^{-1}, respectively). Duncan's test separated FC and ASX from SC and ASX + BET, with BET intermediate. The approximately 2.3-fold increase in SC relative to FC argues against generalized suppression of digestive-enzyme activity by salinity. Instead, it suggests enzyme-specific regulation, potentially reflecting altered reliance on lipid-derived energy, substrate availability, or luminal conditions during osmotic acclimation. Because lipid digestibility, bile secretion, and metabolic flux were not measured, higher lipase activity cannot be equated with improved lipid digestion and is more appropriately interpreted as a compensatory or adaptive response.

The divergent responses of protease, amylase, and lipase show that digestive enzymes should not be interpreted as a single coordinated response. Their activities depend on dietary composition, intestinal region, feeding status, substrate availability, and metabolic demand. Thus, the present enzyme profile is consistent with selective metabolic adjustment under salinity stress rather than uniform enhancement or suppression of digestion. Similar studies in Nile tilapia indicate that digestive enzymes may respond independently and may not change in parallel with intestinal morphology or growth [9, 11].

The significant amylase response in ASX-containing groups is consistent with better maintenance of carbohydrate-hydrolysing activity under salinity exposure, whereas protease did not show a statistically significant treatment effect. ASX is a membrane-associated xanthophyll with antioxidant and immunomodulatory properties that may help preserve cellular and enzymatic stability during stress [20]. However, antioxidant status, membrane integrity, and molecular signaling were not measured here; therefore, these mechanisms remain plausible explanations rather than demonstrated causal pathways.

The factorial analysis of the four saline-water groups clarified which responses were associated with the individual additives and which depended on their combination (Table 2). Protease showed no significant main effect of ASX [$F(1,8) = 0.703$, $p = 0.426$], no main effect of BET [$F(1,8) = 0.975$, $p = 0.352$], and no ASX × BET interaction [$F(1,8) = 0.075$, $p = 0.792$]. For amylase, ASX had a significant main effect [$F(1,8) = 10.155$, $p = 0.013$], whereas the BET main effect [$F(1,8) = 3.525$, $p = 0.097$] and ASX × BET interaction [$F(1,8) = 4.116$, $p = 0.077$] were not significant. For lipase, neither the ASX main effect [$F(1,8) = 0.668$, $p = 0.437$] nor the BET main effect [$F(1,8) = 2.669$, $p = 0.141$] was significant, but the ASX × BET interaction was significant [$F(1,8) = 10.669$, $p = 0.011$]. This interaction was endpoint-specific: lipase was low with ASX alone (0.11 ± 0.05 U mL^{-1}) but high in ASX + BET (0.28 ± 0.05 U mL^{-1}), while SC itself also showed elevated lipase (0.25 ± 0.08 U mL^{-1}). Consequently, the interaction indicates dependence of the ASX response on BET presence but does not establish a generalized synergistic improvement in digestion.

Table 2. Summary of two-way factorial analysis of variance (ANOVA) for dietary astaxanthin (ASX), betaine (BET), and their interaction in Nile tilapia maintained at 25 ppt

Parameter / Effect	F (p)
Protease-ASX	0.703 (0.426)
Protease-BET	0.975 (0.352)
Protease-ASX × BET	0.075 (0.792)
Amylase-ASX	10.155 (0.013)
Amylase-BET	3.525 (0.097)
Amylase-ASX × BET	4.116 (0.077)
Lipase-ASX	0.668 (0.437)
Lipase-BET	2.669 (0.141)
Lipase-ASX × BET	10.669 (0.011)
Villus length-ASX	65.429 (<0.001)
Villus length-BET	19.188 (0.002)
Villus length-ASX × BET	19.429 (0.002)
Villus width-ASX	20.628 (0.002)
Villus width-Bet	2.268 (0.171)
Villus width-ASX × BET	5.718 (0.044)

Note: F-values have df = 1,8. Bold values indicate significant effects ($p < 0.05$).

3.2 Intestinal villus length and width under chronic salinity stress

To further evaluate intestinal structure under chronic salinity stress, villus length and width were measured after 40 days (Figure 1). Villus length differed significantly among treatments [$F(4,10) = 18.494$, $p < 0.001$]. SC had the shortest villi (105.00 ± 3.17 μm), a 52.7% reduction relative to FC (221.79 ± 34.26 μm). Villus length was 240.56 ± 25.55, 200.33

± 5.03, and 240.26 ± 26.91 µm in ASX, BET, and ASX + Bet, respectively. Duncan's test showed that SC had the lowest villus length, BET showed an intermediate value, whereas FC, ASX, and ASX + BET formed the highest statistical group.

Villus width also differed significantly among treatments [$F(4,10) = 29.942$, $p < 0.001$]. FC had the greatest width (82.22 ± 2.26 µm), SC the lowest (54.79 ± 3.05 µm), and ASX, BET, and ASX + BET showed intermediate widths of 68.29 ± 2.86, 62.38 ± 3.65, and 66.56 ± 3.83 µm, respectively. Duncan's test indicated that each supplemented group had wider villi than SC but remained narrower than FC. In the factorial analysis of the saline groups, villus length showed significant main effects of ASX [$F(1,8) = 65.429$, $p < 0.001$] and BET [$F(1,8) = 19.188$, $p = 0.002$], together with a significant ASX × BET interaction [$F(1,8) = 19.429$, $p = 0.002$]. Villus width showed a significant ASX main effect [$F(1,8) = 20.628$, $p = 0.002$], no significant BET main effect [$F(1,8) = 2.268$, $p = 0.171$], and a significant ASX × BET interaction [$F(1,8) = 5.718$, $p = 0.044$]. These interactions show that the effect of one additive depended on the presence of the other, but the pattern was not uniformly beneficial or additive. Representative histological sections were consistent with the quantitative measurements, showing shorter and narrower villi in SC and better-preserved dimensions in supplemented groups (Figure 2).

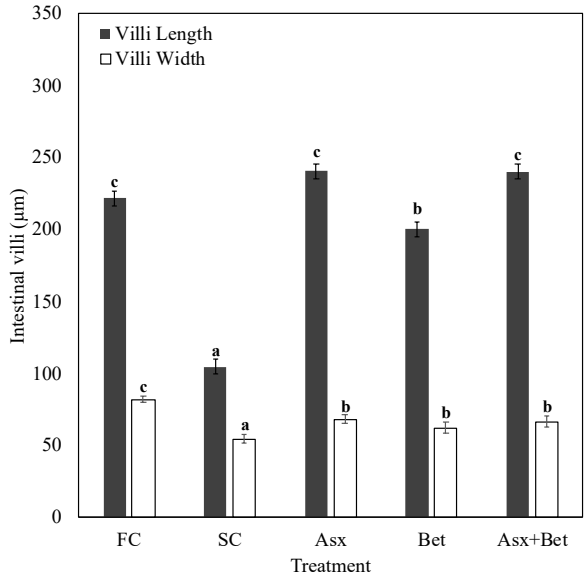

Figure 1. Intestinal villus length and width across treatments: Freshwater control (FC), salinity control (SC), astaxanthin (ASX), betaine (BET), and ASX + BET

Note: Different letters within each morphometric parameter indicate significant differences among treatments according to Duncan's multiple-range test ($p < 0.05$).

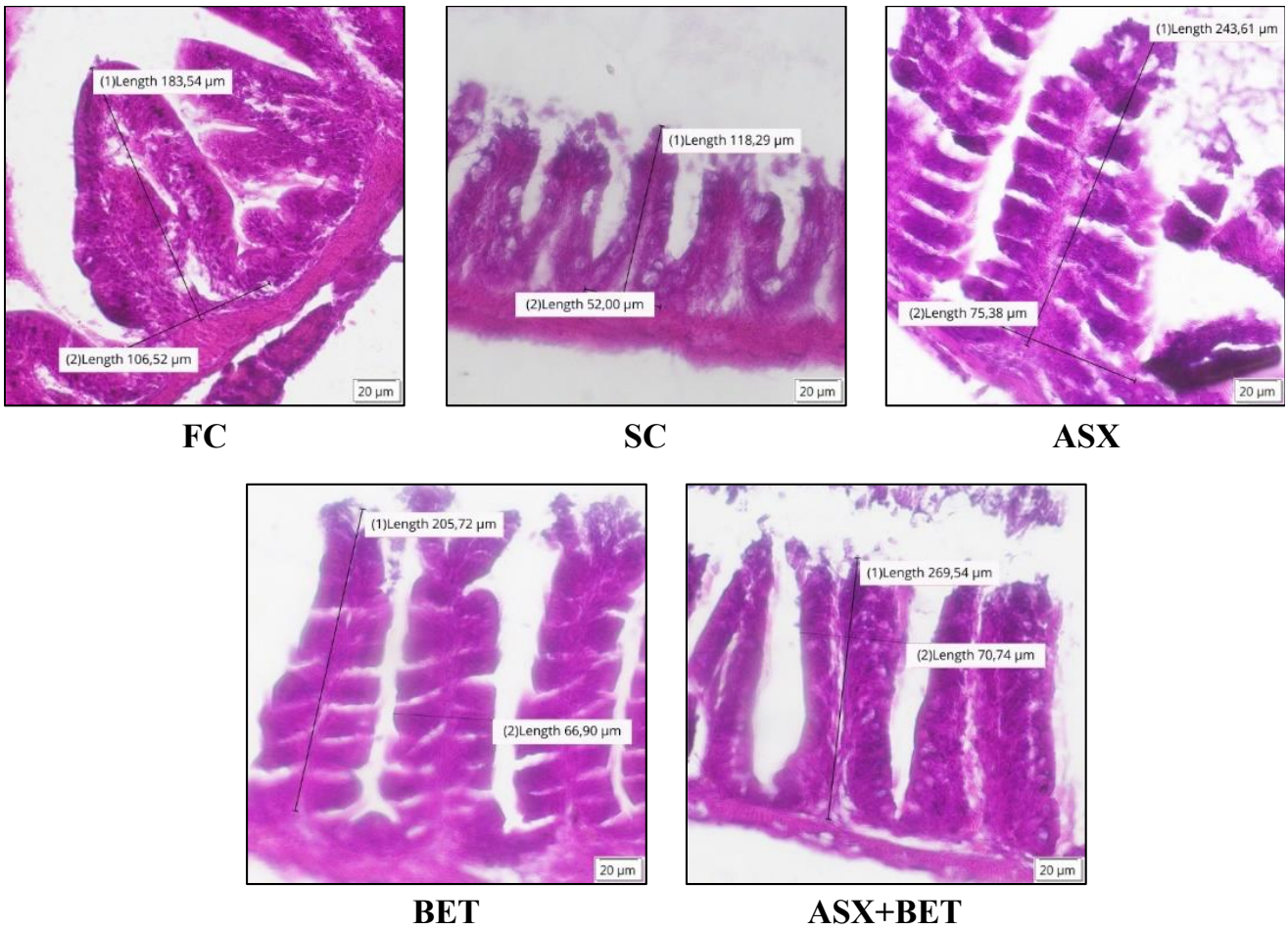

Figure 2. Representative intestinal histology of freshwater control (FC), salinity control (SC), astaxanthin (ASX), betaine (BET), and ASX + BET groups

Note: Measurement lines indicate villus length and width. Scale bar = 20 µm.

The simultaneous reduction in villus length and width in SC indicates that chronic exposure to 25 ppt adversely affected intestinal structure. Under saline conditions, Nile tilapia must allocate additional physiological resources to osmoregulation,

potentially altering intestinal energy use and tissue maintenance. Comparable salinity-related changes in intestinal histology have been reported in Nile tilapia, although their severity depends on salinity level, exposure duration, acclimation protocol, diet, and fish size [4, 15]. Longer and wider villi increase the geometric surface available for interaction with digesta and are generally consistent with improved absorptive potential. However, villus dimensions alone do not directly quantify nutrient absorption, which also depends on digestive-enzyme activity, enterocyte function, membrane transporters, blood supply, and epithelial permeability.

ASX produced the most consistent factorial main-effect pattern, with significant effects on amylase activity, villus length, and villus width. In contrast, neither the one-way nor factorial analysis supported a significant effect on protease. ASX is a membrane-associated xanthophyll with recognized antioxidant and immunomodulatory properties in aquatic animals [20]. These properties could contribute to intestinal stability during environmental stress, but the present study did not measure oxidative biomarkers, membrane integrity, or molecular pathways; therefore, these mechanisms remain plausible rather than demonstrated. Similar associations among antioxidant-oriented dietary supplementation, digestive-enzyme activity, and intestinal structure have been reported in Nile tilapia [8, 9].

BET was associated with a significant main effect on villus length, but not on villus width, protease, amylase, or lipase in the factorial analysis. This pattern suggests that its contribution under the present conditions was more evident for selected structural responses than for digestive-enzyme activity. As a compatible organic osmolyte and methyl donor, BET may help stabilize cellular hydration and macromolecular function during hyperosmotic exposure. Dietary BET has previously improved villus dimensions in Nile tilapia, although the response varies with dose, chemical form, basal diet, and stress context [21]. No direct osmotic or molecular biomarker was measured in the present study to confirm the mechanism.

The raw-data factorial results require a nuanced interpretation of the combined ASX + BET treatment. Significant ASX × BET interactions occurred for lipase activity, villus length, and villus width, but not for protease or amylase. Statistical interaction does not itself demonstrate biological synergy. For villus length, ASX alone and ASX + BET yielded virtually identical means (240.56 and 240.26 μm), while villus width was numerically lower in ASX + BET (66.56 μm) than in ASX alone (68.29 μm). Thus, the significant morphometric interactions reflect non-additive response patterns rather than superior performance of the combination. For lipase, activity was 0.11 ± 0.05 U mL^{-1} with ASX alone and 0.28 ± 0.05 U mL^{-1} with ASX + BET, but SC also had high lipase activity (0.25 ± 0.08 U mL^{-1}). The combined treatment should therefore be described as producing endpoint-specific interactive responses, not a uniformly superior or synergistic gastrointestinal effect.

Several limitations constrain interpretation of these findings. Biological replication was limited to three aquaria per treatment, reducing the precision and statistical power of both treatment-wise and factorial estimates, particularly interaction terms. Histological assessment was restricted to villus length, villus width, and descriptive observations; no lesion scoring, inflammatory assessment, epithelial-integrity index, fold count, or goblet-cell density was performed. Growth performance, survival, feed utilization, immune responses, long-term culture performance, antioxidant biomarkers, and molecular pathways were not evaluated, so mechanistic and practical production inferences remain hypothesis-generating. The factorial analysis was limited to a single inclusion level of each additive and therefore cannot establish dose dependence or biological synergy. Finally, nitrate occasionally reached 250 mg L^{-1}; although husbandry procedures were common across treatments, background nitrogen accumulation may have influenced fish physiology and cannot be completely excluded as a confounding stressor.

4. CONCLUSIONS

This study evaluated intestinal protease, amylase, and lipase activities together with villus length and width in juvenile *Oreochromis niloticus* maintained at 25 ppt for 40 days and fed ASX, BET, or their combination. Analysis of the raw aquarium-level data showed that protease did not differ significantly among the five treatments and had no significant factorial main or interaction effects. Amylase differed among treatments, with a significant main effect of ASX, whereas lipase showed a significant ASX × BET interaction without significant main effects. Salinity markedly reduced villus length and width. Factorial analysis showed significant ASX and BET main effects and an ASX × BET interaction for villus length, while villus width showed a significant ASX main effect and ASX × BET interaction. The combined response was therefore endpoint-dependent rather than uniformly additive. Importantly, ASX + BET did not exceed ASX alone for villus length or width, and elevated lipase activity in the combination occurred against a similarly elevated value in the SC; thus, the data do not support a generalized claim of biological synergy. ASX showed the most consistent main effects across amylase and intestinal morphometry. Larger factorial dose-response studies with greater replication and production, immune, and mechanistic endpoints are required before practical or synergistic benefits can be established.

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REFERENCES

[1] FAO. (2024). FAO report: Global fisheries and aquaculture production reaches a new record high. https://www.fao.org/asiapacific/news/news-detail/FAO-Report-Global-fisheries-and-aquaculture-production-reaches-a-new-record-high-/en.

[2] Yue, G.H., Ma, K.Y., Xia, J.H. (2024). Status of conventional and molecular breeding of salinity-tolerant tilapia. Reviews in Aquaculture, 16(1): 271-286. https://doi.org/10.1111/raq.12838

[3] Metwaly, S., Nasr, H., Ahmed, K., Fathi, M. (2025). Multifaceted stress response in Nile tilapia (*Oreochromis niloticus*) fingerlings: Integrative analysis of salinity,

ammonia, and stocking density effects on growth, physiology, and gene expression. Fish Physiology and Biochemistry, 51(1): 48. https://doi.org/10.1007/s10695-025-01462-6

[4] Seale, A.P., Cao, K., Chang, R.J.A., et al. (2024). Salinity tolerance of fishes: Experimental approaches and implications for aquaculture production. Reviews in Aquaculture, 16(3): 1351-1373. https://doi.org/10.1111/raq.12900

[5] Dawood, M.A.O., Alkafafy, M., Sewilam, H. (2022). The antioxidant responses of gills, intestines and livers and blood immunity of common carp (*Cyprinus carpio*) exposed to salinity and temperature stressors. Fish Physiology and Biochemistry, 48(2): 397-408. https://doi.org/10.1007/s10695-022-01052-w

[6] Grosell, M. (2011). Intestinal anion exchange in marine teleosts is involved in osmoregulation and contributes to the oceanic inorganic carbon cycle. Acta Physiologica, 202(3): 421-434. https://doi.org/10.1111/j.1748-1716.2010.02241.x

[7] Whittamore, J.M. (2012). Osmoregulation and epithelial water transport: Lessons from the intestine of marine teleost fish. Journal of Comparative Physiology B, 182(1): 1-39. https://doi.org/10.1007/s00360-011-0601-3

[8] Tran-Ngoc, K.T., Schrama, J.W., Le, M.T.T., Nguyen, T.H., Roem, A.J., Verreth, J.A.J. (2017). Salinity and diet composition affect digestibility and intestinal morphology in Nile tilapia (*Oreochromis niloticus*). Aquaculture, 469: 36-43. https://doi.org/10.1016/j.aquaculture.2016.11.037

[9] Monier, M.N., Shady, S.H.H., Grana, Y.S., et al. (2025). Interactive impact of salinity and oxygen level on the growth performance, digestive enzymes, serum biochemistry, antioxidative, immunity, and histological status of Nile tilapia (*Oreochromis niloticus*). Fish Physiology and Biochemistry, 51(6): 200. https://doi.org/10.1007/s10695-025-01608-6

[10] Eldessouki, E.A.A., Elshopakey, G.E., Elbahnaswy, S., et al. (2024). Influence of astaxanthin-enriched *Haematococcus pluvialis* microalgae on the growth efficacy, immune response, antioxidant capacity, proinflammatory cytokines, and tissue histomorphology of hybrid red tilapia. Aquaculture International, 32(6): 7447-7468. https://doi.org/10.1007/s10499-024-01524-1

[11] Monier, M.N., Abd El-Naby, A.S., Fawzy, R.M., et al. (2025). Growth performance, antioxidant, and immune responses of Nile tilapia (*Oreochromis niloticus*) fed on low-fishmeal diets enriched with sodium chloride and its adaptability to different salinity levels. Fish Physiology and Biochemistry, 51(1): 6. https://doi.org/10.1007/s10695-024-01426-2

[12] Lu, J.F., Luo, S., Jin, T.C., et al. (2021). Betaine protects ayu (*Plecoglossus altivelis*) against Vibrio anguillarum infection in salinity by regulating the immunomodulatory activity of monocytes/macrophages. Aquaculture, 536: 736482. https://doi.org/10.1016/j.aquaculture.2021.736482

[13] Song, Y., Chen, R., Yang, M., Liu, Q., Zhou, Y., Zhuang, S. (2021). Dietary betaine supplementation improves growth performance, digestive function, intestinal integrity, immunity, and antioxidant capacity of yellow-feathered broilers. Italian Journal of Animal Science, 20(1): 1575-1586. https://doi.org/10.1080/1828051X.2021.1986681

[14] Yousefi, M., Molayemraftar, T., Hoseini, S.M., Ghafarifarsani, H. (2025). Dietary betaine improves immune function, antioxidant status, immune-related signaling molecules, and disease resistance in fish species. Aquaculture Nutrition, 2025(1): 4596572. https://doi.org/10.1155/anu/4596572

[15] Hersi, M.A., Genc, E., Pipilos, A., Keskin, E. (2023). Effects of dietary synbiotics and biofloc meal on the growth, tissue histomorphology, whole-body composition and intestinal microbiota profile of Nile tilapia (*Oreochromis niloticus*) cultured at different salinities. Aquaculture, 570: 739391. https://doi.org/10.1016/j.aquaculture.2023.739391

[16] Firdaus, M.Z.A., Bello, A.M., Arifin, N.B., Hariati, A.M., Fakhri, M. (2026). Dietary proline enhances physiological adaptation and oxidative stress resistance in Nile tilapia cultured under chronic salinity stress. Aquaculture Studies, 26(6): 642–652.

[17] Qiang, J., Tao, Y.F., Lu, S.Q., Ma, J.L., He, J., Xu, P. (2022). Role of astaxanthin as a stimulator of ovarian development in Nile tilapia (*Oreochromis niloticus*) and its potential regulatory mechanism: Ameliorating oxidative stress and apoptosis. Aquaculture Nutrition, 2022(1): 1245151. https://doi.org/10.1155/2022/1245151

[18] Luo, Z., Tan, X.Y., Liu, X.J., Wen, H. (2011). Effect of dietary betaine levels on growth performance and hepatic intermediary metabolism of GIFT strain of Nile tilapia *Oreochromis niloticus* reared in freshwater. Aquaculture Nutrition, 17(4): 361-367. https://doi.org/10.1111/j.1365-2095.2010.00805.x

[19] Takei, Y. (2021). The digestive tract as an essential organ for water acquisition in marine teleosts: Lessons from euryhaline eels. Zoological Letters, 7(1): 10. https://doi.org/10.1186/s40851-021-00175-x

[20] Elbahnaswy, S., Elshopakey, G.E. (2024). Recent progress in practical applications of a potential carotenoid astaxanthin in aquaculture industry: A review. Fish Physiology and Biochemistry, 50(1): 97-126. https://doi.org/10.1007/s10695-022-01167-0

[21] Ismail, T., Hegazi, E., Dawood, M.A.O., et al. (2020). Using of betaine to replace fish meal with soybean or/and corn gluten meal in Nile tilapia (*Oreochromis niloticus*) diets: Histomorphology, growth, fatty acid, and glucose-related gene expression traits. Aquaculture Reports, 17: 100376. https://doi.org/10.1016/j.aqrep.2020.100376