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ROLES OF DIETARY SUPPLEMENTATION OF EXOGENOUS PROTEASE IN LOW FISHMEAL AQUAFEED − A MINI REVIEW

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Abstract

The replacement of fishmeal (FM) with economical and nutritious FM alternatives including plant- and animal-based protein ingredients has become a global research priority. However, the presence of several anti-nutritional factors (ANFs) in these alternatives may impair nutrient utilization in fish. The supplementation of exogenous protease as feed additives could be an effective approach to improve the nutrient digestibility of these alternative proteins. Proteases are protein-digesting enzymes that catalyze the hydrolysis of complex protein macromolecules into simpler amino acids. Exogenous protease supplementation stimulates the activities of endogenous proteolytic enzymes for better nutrient digestion and absorption in fish. This review article summarizes the optimum protease supplementation levels in various fish species and its beneficial effects on growth performance, nutrient digestibility, intestinal morphology, digestive enzyme activities, whole-body composition, physiochemical parameters, immunity, and gene expression of fish. Moreover, the interactive effects of exogenous protease with organic acids and probiotics are also discussed. Overall, the supplementation of exogenous protease in fish feed is a viable solution for poor nutrient utilization of FM alternatives. Important gaps, however, exist in the current knowledge, particularly with regard to the effect of protease supplementation in animal-based proteins as most of the studies have focused on the effect of protease supplementation on plant-based proteins as FM alternatives. Moreover, additional studies focused on the effect of protease supplementation on the digestive enzyme activities, immunity, gut microbes and antioxidant parameters of fish will provide the basis for a better understanding of the mode of action of protease. The scarce knowledge in these areas limits the scope of protease supplementation as a feed additive in the aquaculture sector.

Key words: growth, nutrient digestibility, digestive enzyme activities, gut histology, antioxidant enzymes

Aquaculture is the most diverse food-producing sector in the world. It is playing a significant role in eradicating global famine, malnutrition, and food security issues by providing fish and other aquatic animals rich in essential amino acids, fatty acids, vitamins, and minerals (Subasinghe et al., 2009). This sector has been developing, expanding, and intensifying rapidly for the last four decades across the globe (Tacon, 2020). The intensive development of aquaculture has raised the demand for feed ingredients. (Gatlin III et al., 2007). Fishmeal (FM) is widely used in feed formulations due to its high palatability and digestibility (Hernandez et al., 2010). It has a well-balanced nutritional profile of essential amino acids, fatty acids, nucleotides, vitamins, and minerals (Tacon et al., 2009). However, the limited supply and high market price of FM resulted in the search for economical and nutritious FM alternatives for the maximum profitability and sustainability of the aquaculture sector (Shi et al., 2016).

Many animal-based proteins and plant-based proteins are being utilized as protein sources in feed formulations (Hekmatpour et al., 2019; Hlophe-Ginindza et al., 2016; Hosseini Shekarabi et al., 2021 a, b; Lee et al., 2020). The utilization of these FM alternatives has significantly reduced the cost of fish production (Gatlin III et al., 2007). However, the complete replacement of FM with these alternatives resulted in poor growth performance and low nutrient digestibility in fish (Sinha et al., 2011). The presence of some anti-nutritional factors (ANFs) including non-starch polysaccharides (NSPs) and proteinaceous anti-nutrients (e.g., glycinin and β-conglycinin) in FM alternatives induced stress on the gastrointestinal tract (GIT) of fish (NRC, 2011). These undesirable substances could reduce the villus length, and increase the crypt depth in the intestine, resulting in poor nutrient absorption (Gu et al., 2016; Palacios et al., 2004; Wu et al., 2020). Moreover, ANFs impair protein digestion and these partially digested proteins undergo fermentation (Cowieson and Roos, 2013; Hosseini Shekarabi et al., 2021 a) and yield ammonia, amines, phenols, and other toxic substances in the GIT and ultimately induce disease conditions in the body (Bikker et al., 2006; Williams et al., 2005). To resolve this problematic situation, researchers are supplementing exogenous protease in

feed to improve the intestinal health and growth performance of fish.

Proteases are the enzymes that catalyze the hydrolysis of complex proteins into simpler units for better digestion and absorption of nutrients. They significantly improve protein digestion and mitigate the emission of nitrogenous compounds by hydrolyzing the complex proteins and anti-nutrients into simpler units. Moreover, exogenous protease supplementation stimulates the release of endogenous digestive enzymes to break down proteins and lipids into simpler units (Hassaan et al., 2019). Proteases employed in fish feed formulations are commonly derived from yeast (*Saccharomyces cerevisiae*), bacteria (*Bacillus subtilis*), and fungi (*Aspergillus niger*, *Trichoderma reesei*) (Anadón et al., 2019). They are classified as exopeptidase or endopeptidase based on the cleavage site. They are also differentiated as chymosin-like, trypsin-like, or chymotrypsin-like for their similarity with endogenous proteases (García-Carreño, 1992). Based on sensitivity to surrounding pH, they are characterized as acidic, neutral, and alkaline proteases (Sriket, 2014). They are further categorized into different categories based on the active site (serine, aspartic, metalloprotease, cysteine, threonine, and glutamic) for catalysis (Rawlings et al., 2004; Sriket, 2014) (Figure 1). To date, the roles of dietary supplementation of exogenous protease in fish nutrition have not been reviewed. Hence, the main aim of this review article is to summarize the current research regarding exogenous protease supplementation in fish nutrition, with special attention to describing the mode of action in improving nutrient utilization in fish. Table 1 provides the information about the optimum protease supplementation levels in different fish species.

Effect of protease supplementation on growth performance and feed utilization

The growth performance of fish reflects the efficacy of incorporated feed ingredients and feed additives. Hassan et al. (2019) reported that Nile tilapia (*Oreochromis niloticus*) fed diets containing different ratios of fishmeal and cottonseed meal supplemented with 2500 U/kg protease exhibited the best growth performance and feed utilization compared to those fed diets without protease supplementation. In another study, Saleh et al. (2021) observed a significant improvement in growth performance in Nile tilapia fed protease supplemented diets than fish fed unsupplemented diets. Furthermore, Li et al. (2015) reported that supplementation of 175 mg/kg protease in a compressed low fishmeal diet significantly improved the weight gain (613 without protease vs 744 with protease) and feed conversion ratio (1.62 without protease vs 1.49 with protease) of tilapia. A study conducted on genetically improved farmed tilapia by Wu et al. (2020) indicated that fish fed the plant-based diet (soybean meal, rapeseed meal, and cottonseed meal) containing 5.52 U/g protease had significantly higher final body weight, weight gain, specific growth rate and best feed conversion ratio to that fed other diets. Moreover, Ragaa et al. (2017) showed that the addition of 400 mg/kg serine-based protease significantly improved the growth performance of Nile tilapia when compared to control groups.

Furthermore, Drew et al. (2005) illustrated that exogenous protease supplementation of 250 mg/kg in coextruded canola and pea diet (1:1) significantly improved the feed conversion ratio of rainbow trout (*Oncorhynchus mykiss*). In rohu (*Labeo rohita*), an eight-week feeding trial conducted by Maryam et al. (2022) noted that fish fed the poultry by-product meal based (PBM) diet supplemented with 450 mg/kg protease had similar growth performance, feed utilization, and body indices to that fed positive control diet (fishmeal based diet). Similarly, Chen et al. (2009) reported that the addition of 01 per mil of neutral protease in the diet of black carp (*Mylopharyngodon piceus*) significantly improved the growth performance and feed utilization of fish. In another study, protease supplementation at the level of 1250 unit/kg and 1100 unit/kg significantly improved the growth performance of African catfish (*Clarias gariepinus*) larvae and fingerlings respectively (Kemigabo et al., 2019). In European seabass (*Dicentrarchus labrax*), a significant improvement in weight gain, final body weight, specific growth rate, and feed intake was reported for fish fed up to 50% high-protein distiller dried grain supplemented with 1 g/kg protease (Goda et al., 2020).

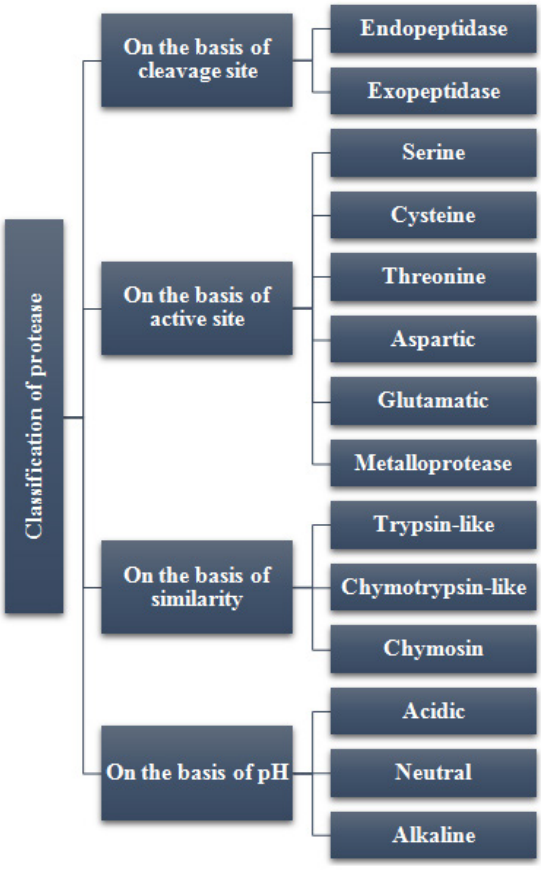


Figure 1. Classification of proteases

Table 1. Effects of protease supplementation on the performance of fish

Fish species	Source of protease	Optimum protease level	Synergy	Diet	Trial duration	Impact	Reference
1	2	3	4	5	6	7	8
Rainbow trout (*Oncorhynchus mykiss*)	Alkaline serine endopeptidase, optimal pH of 8.55	240 g/t	–	Coextruded canola and pea diet, coextruded flax and pea diet	56 days	Significant improvement in growth performance and nutrient digestibility for canola and pea diet	Drew et al., 2005
African catfish (*Clarias gariepinus*)	Neutral protease	1,100−1,250 unit/kg	–	Commercial dry feed	60 days	Significant improvement in feed utilization, protein retention, and growth performance	Kemigabo et al., 2019
Gibel carp (*Carassius auratus gibelio*)	Neutral protease	400 mg/kg	–	Fishmeal, soybean meal, peanut meal, rapeseed meal	84 days	Significant improvement in growth, nutrient digestibility, biochemical indices, and intestinal health	Liu et al., 2018
Gibel carp	Alkaline serine endopeptidase, with an optimal pH of 8.5	150−175 mg/kg	–	Fishmeal, soybean meal, cottonseed meal, rapeseed meal	84 days	Significant improvement in growth performance, feed utilization, nutrient digestibility, and nutrient retention	Shi et al., 2016
GIFT tilapia (*Oreochromis niloticus*)	Neutral protease (EC3.4.23.18) with enzyme activity 13,830 U/g, produced by *Humicola* sp. L8 fermentation	5.52 U/g	–	Soybean meal, cottonseed meal, rapeseed meal	60 days	Significant improvement in growth performance, intestinal health, immunity, and disease resistance	Wu et al., 2020
Nile tilapia (*Oreochromis niloticus*)	Commercial protease produced from *Bacillus licheniformis*, 600,000 U/g enzyme activity	500 g/ton	–	Fishmeal, soybean meal, yellow corn, corn gluten meal	98 days	Significant improvement in growth performance, nutrient digestibility, intestinal health, and blood parameters	Saleh et al., 2021
European seabass (*Dicentrarchus labrax*)	Commercial protease enzyme product, enzyme activity 2,000,000	1.0 g/kg	–	Fishmeal, soybean meal, corn gluten meal, high-protein distiller dried grain	70 days	Significant improvement in growth, intestinal health, immunity, and physiological indices	Goda et al., 2020
Tilapia	Serine-protease, 75000 PROT/g	200 mg/kg	–	Fishmeal, yellow corn, corn gluten, soybean meal	56 days	Significant improvement in growth performance and protein digestibility	Ragaa et al., 2017
Tilapia	Commercial protease, 5000 U/g enzyme activity, pH 7.5	2500 U/g	–	Fishmeal, soybean, cotton seed meal, yellow corn	84 days	Significant improvement in growth, nutrient assimilation, and hematology and gene expression	Hassan et al., 2019
Tilapia	Commercial protease	2500, 5000 U kg^{-1}	Malic acid	Soybean meal, corn gluten, yellow corn, and wheat bran	84 days	Significant improvement in growth performance; feed utilization efficiency, and digestive enzymes	Hassan et al., 2020
Tilapia	Neutral protease (EC3.4.23.18) produced from the fermentation of *Humicola* sp. L8, pH of 8.0, enzyme activity 13,830 U/g.	0.5 g/kg	*Bacillus pumilus*	Soybean meal, corn gluten, yellow corn, and wheat bran	84 days	Significant improvement in growth performance, intestinal histological morphometric, immune response, hematological, biochemical blood, metabolic gene expression, and intestinal bacterial flora	Hassan et al., 2021
Tilapia	Heat-stable serine protease (AG175TM) produced by a bacterial fermentation with an optimal pH of 8.5	175 mg/kg	Organic acid	Fishmeal, meat bone meal, soybean meal, rapeseed meal, cottonseed meal	63 days	Significant improvement in growth performance	Huan et al., 2018

Table 1. – contd.

1	2	3	4	5	6	7	8
Tilapia	Commercial protease, 5000 U/g enzyme activity, pH 7.5	500–750 U/g		Fishmeal, soybean meal, yellow corn, rice bran	84 days	Significant improvement in growth performance, biochemical indices, immunity, and intestinal health	Elnabi et al., 2020
Rohu (*Labeo rohita*)	Commercial protease produced from *Bacillus licheniformis*, 600,000 U/g enzyme activity	450 mg/kg		Fishmeal, poultry by-product meal, sunflower meal, rice polish	56 days	Significant improvement in growth performance, nutrient digestibility, digestive enzymes activities, and hematological parameters	Maryam et al., 2022
Siberian sturgeon (*Acipenser baerii*)	Exogenous multi-enzyme (Ronozyme®)	25 mg/kg xylanase, 30 mg/kg protease and 7.5 mg/kg phytase	0.2 g/kg multi-strain probiotics	Commercial feed	70 days	Significant improvement in growth performance, intestinal health, whole-body composition, and digestive enzyme activity	Ghodrati et al., 2021 a
Siberian sturgeon	Multi-enzyme complex	250 mg/kg exogenous multi-enzymes (beta-glucanase, cellulase, alpha-amylase, protease, xylanase and phytase)	0.2 g/kg probiotics mixture	Herring fishmeal, soybean meal, molasses, wheat flour	56 days	Significant improvement in growth performance, digestibility, intestinal health, and immune response	Shekarabi et al., 2022
Siberian sturgeon	Multi-enzyme complex	7.5 mg/kg phytase, 30 mg/kg protease, and 25 mg/kg xylanase	0.2 g/kg probiotics mixture	Commercial feed	70 days	Significant improvement in hemato-biochemical parameters and immunological responses	Ghodrati et al., 2021 b

Moreover, Liu et al. (2018) found that gibel carp fed the diet containing 300 mg/kg neutral protease had significantly higher specific growth rate (1.63 positive control diet vs 1.66 protease diet) and protein efficiency ratio (166.13 positive control diet vs 172 protease diet) than fish fed control diets. In another study on gibel carp (*Carassius auratus gibelio*) by Shi et al. (2016), it was observed that supplementation of 150−175 mg/kg alkaline serine protease in pelleted diet improved the growth performance of fish in comparison to negative control diet (low fishmeal diet) and showed the similar growth performance as positive control diet (high fishmeal diet). However, no significant difference in growth performance was recorded for protease supplementation in extruded diets.

Furthermore, Ghomi and colleagues (2012) reported a significant improvement in the final weight gain and feed conversion ratio of sturgeon (*Acipenser baerii*) in response to the supplementation of 250 mg/kg multi-enzyme complex. In addition, Soltan et al. (2009) reported that exogenous supplementation of 500 mg/kg multi-enzyme cocktail comprising protease (200000 U/kg), amylase (5500000 U/kg), gluconase (3000 U/kg), lipase (150000 U/kg), xylanase (500000 U/kg) and cellulase (1500 U/kg) had significant positive impacts on the growth, feed utilization, and nutrient digestibility in PBM based diet than fishmeal diet in Nile tilapia. They suggested that the presence of high oil content in fishmeal inhibited the enzyme activity in the control group (Soltan, 2009).

The improvement in growth performance may be attributed to better feed utilization, nutrient digestibility, and intestinal health of fish. This is due to the proteolytic action of protease in plant-based proteins as it disrupts the cell wall matrix and enhances the contact between digestive enzyme and cell content, resulting in enhanced protein digestibility. In addition, the degradation of ANFs and grain storage proteins by protease in a plant-based diet also improves the available energy in fish (Goda et al., 2020). Besides this, protease supplementation stimulates the production of endogenous peptidase that ultimately enhances the endogenous protease activity which consequently improved amino acid utilization and nutrient absorption (Lin et al., 2007).

On the contrary, no significant improvement in growth performance and feed conversion ratio was documented for protease supplementation in rainbow trout fed soybean meal-based diet (Yigit et al., 2018). This may be ascribed to the usage of dehulled soybean meal in the experiment. Dehulling significantly reduces the anti-nutritional substances of plant-based proteins (Pal et al., 2017). Therefore, no remarkable effects of protease were observed in this experiment. Further, Drew et al. (2005) observed no significant effects of protease supplementation on the growth performance of rainbow trout fed coextruded flax and pea product (Figure 2).

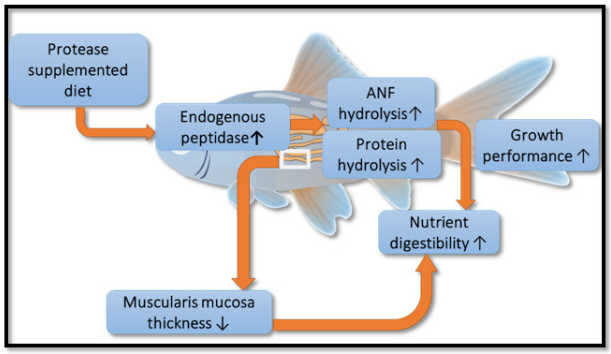


Figure 2. Effect of exogenous protease supplementation on the growth and nutrient digestibility of fish

Effect of exogenous protease supplementation on nutrient digestibility

Previous studies showed that the presence of some ANFs and fiber in plant feedstuff may impair nutrient digestibility in fish (Luo et al., 2008; Zhou and Yue, 2012). Hence, protease supplementation could be an effective strategy to mitigate the adverse effects of these ANFs on fish health (Hassaan et al., 2020; Lin et al., 2007; Shi et al., 2016). A study conducted by Drew et al. (2005) reported that supplementation of 250 g/t alkaline serine-protease obtained from bacterial fermentation in coextruded canola and pea diet significantly improved apparent digestibility coefficient (ADCs) of crude protein (0.760 without protease vs 0.836 with protease), crude lipid (0.899 without protease vs 0.968 with protease), dry matter (0.625 without protease vs 0.813 with protease) and gross energy (0.681 without protease vs 0.831 with protease) of rainbow trout. However, protease supplementation in coextruded flax and pea diet did not significantly improve the nutrient digestibility.

In another study, exogenous supplementation of 175 mg/kg protease in the diet of rainbow trout significantly improved the ADCs of 12 out of 17 feed ingredients (Lee et al., 2020). They observed that fish fed the tested ingredients supplemented with protease had significantly higher ADCs of digestible energy than fish fed ingredients without protease addition (66.6% without protease vs 69.2% with protease). Moreover, Lee et al. (2020) noted a significant improvement in the ADCs of amino acids of most tested ingredients by the addition of protease supplementation. They reported that soybean meal supplemented with protease showed the highest improvement in the ADC of 13 out of 18 amino acids. They concluded that protease supplementation could reduce the dietary requirement of essential amino acids in fish diet. In rohu, supplementation of protease up to 450 mg/kg significantly improved ADCs of dry matter, crude protein, crude lipid, and crude ash of PBM and reached the similar level as fishmeal based diet (Maryam et al., 2022).

A study conducted by Liu et al. (2018) on gibel carp showed that fish fed the diet receiving 150 mg/kg protease

had significantly higher ADCs of crude protein (82.29% positive control vs 87.9% protease diet) and crude lipid (73.9% positive control vs 90.91% protease diet) when compared to the positive control diet. Moreover, another study conducted by Shi et al. (2016) on gibel carp demonstrated that protease supplementation of 150−175 mg/kg in low fishmeal pelleted diet significantly improved the nutrient digestibility when compared to un-supplemented low fishmeal diet and showed similar ADCs values of dry matter (0.616 high fishmeal diet vs 0.605 protease diet) and crude protein (0.872 high fishmeal diet vs 0.868 protease diet) as high fishmeal pelleted diet. Furthermore, a 12-week feeding trial conducted on tilapia showed that diets supplemented with 2500 U/kg protease had significantly higher ADCs of crude protein, crude lipid, and gross energy when compared to un-supplemented diets (Hassaan et al., 2019). Another study on tilapia showed that supplementation of 175 mg/kg protease in 30 g/kg compressed fishmeal based diet significantly improved ADCs of crude protein compared to un-supplemented diet (80.5% without protease vs 83.9% with protease) (Li et al., 2015). Ragaa et al. (2017) reported that Nile tilapia fed diets containing 28% crude protein and 400 mg/kg serine-protease had the highest crude protein digestibility compared to un-supplemented diet (84.73% without protease vs 90.68% with protease). The improvement in nutrient digestibility might be ascribed to the hydrolysis of complex proteins and ANFs by protease addition in the fish diet (Ao et al., 2010). Moreover, protease supplementation resulted in the thinning of muscularis mucosa, which in turn slows down the peristalsis rate and increases the time for nutrient digestion and absorption in the small intestine in fish (Liu et al., 2018).

On the other hand, Yigit et al. (2018) showed that the addition of protease obtained from *Peniophora lycii* in soybean meal-based diet had no significant impact on the digestibility of crude protein and crude lipids of rainbow trout. Moreover, supplementation of 100 unit/kg protease in the extruded diet failed to improve the nutrient digestibility of Nile tilapia (Putnuan and Jintasataporn Srinoy, 2020). The authors of this study attributed this low nutrient digestibility to the low dosage, quality of protease, and loss of enzyme activity during the extrusion processing (Putnuan and Jintasataporn Srinoy, 2020). Similarly, Huan et al. (2018) showed that protease supplementation in meat and the bone meal had no significant effect on nutrient digestibility in tilapia. Huan et al. (2018) concluded that exogenous protease supplementation acts more remarkably on plant proteins than animal proteins. This may imply that animal-based proteins have relatively lower ANFs than plant-based proteins and protease enzyme finds less substrates in these proteins sources for its proteolytic activity. To date, very few studies have undertaken the effect of protease supplementation on animal-based proteins. Therefore, more concerning efforts should be devoted to this regard.

Effect of exogenous protease supplementation on the gut health

GIT plays an important role in digestion, nutrient absorption, gas exchange, and osmoregulation. However, the GIT of farmed fish is exposed to high stress levels induced by ANFs in plant-based proteins. The presence of ANFs including non-starch polysaccharides (NSPs) and proteinaceous anti-nutrients in plant feedstuff could reduce the villus length and increase the crypt depth in the intestine resulting in low nutrient digestion and absorption in the gut (Sinha et al., 2011). These partially digested proteins may undergo bacterial fermentation and result in the production of toxic substances, amines, ammonia, and phenols which in turn impair the functioning of the intestinal barrier, reduce electrolyte absorption, and consequently induce inflammatory reactions in the body (Wu et al., 2020). Previous studies showed that exogenous protease supplementation in fish feed significantly improved the intestinal health of fish (Saleh et al., 2021; Goda et al., 2020; Abd Elnabi et al., 2020). However, Liu et al. (2018) found no significant differences in villi length, width, and length to width ratio of gibel carp fed control diets and protease supplemented diets. However, a significant enhancement in intestinal folds was recorded for protease supplemented diets. Moreover, fish fed the diet containing 300 mg/kg protease supplemented diet had thinner muscularis mucosa than fish fed the positive control diet (54.47 positive control vs 41.39 protease diet). The thinning of muscularis mucosa reduced the intestinal peristalsis and increased the rate of nutrient digestion and absorption which consequently improved the growth and nutrient digestibility of fish (Liu et al., 2018). Besides this, Liu et al. (2018) observed that excessive protease supplementation beyond the optimum level induced negative effects on the intestine's absorptive surface (villus) in gibel carp. The positive impacts of protease supplementation on intestinal health are illustrated in Figure 3.

Mucus barrier in GIT plays an essential role in immune defense and homeostasis in fish (Rombout Jan et al., 2011). The functional integrity of this barrier depends upon the integrity of tight junctions of intestinal epithelial cells, which in turn are associated with tight junction protein complex including occludin, claudin, and zonula occludens (Turner, 2006). Supplementation of 5.52 U/g protease (produced by *Humicola* sp. fermentation) improved the intestinal barrier by downregulating the mRNA of occludin, claudin, and zonula occludens in tilapia. It also downregulated the inflammation-causing cytokines, including tumor necrosis factor alpha (TNF-α) and cytokine interleukin-1β (IL-1β), which resulted in better disease resistance in tilapia (Wu et al., 2020). Moreover, the downregulation of heat shock protein 70 (HSP70), in this study, by protease addition, reduced the intestinal stress caused by the ANFs in FM alternatives (Wu et al., 2020).

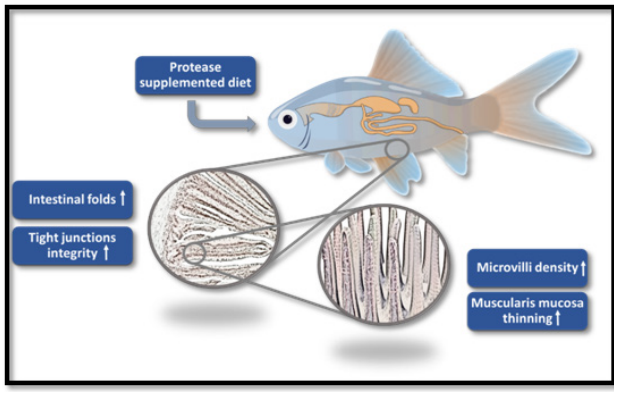


Figure 3. Effects of protease supplementation on the intestinal health of fish

Effect of exogenous protease supplementation on digestive enzymes activities

Digestive enzyme activity is an important index to determine the nutritional value and ingredients availability of feed (Haider et al., 2018). Digestive enzymes are present in the gastrointestinal lumen and linked with the intestinal epithelial cell brush-border membrane (Bakke et al., 2010). The activities of digestive enzymes are greatly influenced by the quantity and quality of incorporated protein in fish feed (Buddington and Krogdahl, 2004). A study conducted on rohu showed that protease supplementation up to 450 mg/kg, significantly enhanced the activities of proteases, amylases, and lipases in intestinal and hepatopancreatic tissues (Maryam et al., 2022). Further, Liu et al. (2018) also found significant improvement in the activities of endogenous proteases in the intestinal and hepatopancreatic tissues of gibel carp fed protease supplemented diets. Similar findings were reported in common carp (Ding-Yun and Yong-Hong, 2008) and gibel carp (Wang, 2003). These findings confirmed that exogenous protease supplementation stimulates the production of endogenous enzymes for better nutrient digestion. Further, the production of endogenous enzymes such as acid proteases, alkaline proteases, lipases, and amylases in the digestive tract depends upon the health status, feeding, age, genetics, and environment of an animal (Brito et al., 2000). Till now, the exact mechanism of exogenous protease supplementation in enhancing digestive enzymes activities is not found. Therefore, future studies in this context are recommended.

Effect of exogenous protease supplementation on biochemical indices

Serum proteins, mainly albumin and globulin proteins have been identified as valuable tools for monitoring fish physiology. Their levels in serum are associated with immune response (Goda et al., 2020). No significant changes were recorded in the level of glucose, albumin, and total protein for protease supplementation in gibel carp (Liu et al., 2018), rohu (Maryam et al., 2022), and tilapia (Saleh et al., 2021). These findings indicated that optimum protease supplementation did not induce any stress on fish physiology. However, a significant increase in serum globulin and albumin was noted in tilapia fed the diet having high inclusion level of cottonseed meal supplemented with protease (Hassaan et al., 2019). Their high serum levels correspond to the presence of huge free gossypol content in the diet.

Serum lipids, including triglycerides and total cholesterol levels, have been used to indicate cardiovascular and liver health (Peres et al., 2015). No significant difference in serum lipid was observed for protease supplementation in the findings of many researchers (Liu et al., 2018; Maryam et al., 2022; Ragaa et al., 2017; Shi et al., 2016). These results manifest the protective role of protease in the normal functioning of the cardiovascular system. Moreover, no significant difference in urea and creatinine was observed in tilapia, indicating that protease supplementation had no negative effects on the kidney (Saleh et al., 2021).

Aspartate aminotransferase (AST) and alanine transaminase (ALT) are non-plasma-specific enzymes involved in amino acid metabolism and gluconeogenesis (Hassaan et al., 2018). AST is localized in the mitochondria of hepatocytes while ALT is located in the liver and kidney (Yang et al., 2003). The enhanced activities of serum AST and ALT in fish may correspond to the leakage of enzymes across the damaged plasma membrane and the synthesis of enzymes by liver tissues (Yang et al., 2003). Thus, these enzymes are an important indicator to reflect the liver's health in fish (Zhai et al., 2016). Shi et al. (2016) observed no significant difference in the activities of alkaline phosphatase (ALP), AST and ALT for protease supplementation in fish diet. A similar finding was recorded in rohu where protease supplemented diets had no significant effect on ALP, ALT, and AST activities (Maryam et al., 2022). On the other hand, increased serum activities of these enzymes were reported at 600 mg/kg protease level in gibel carp (Liu et al., 2018). This implies that excessive protease supplementation beyond the optimum level could accelerate the activities of ALT, ALP, and AST which may induce negative effects on the liver health of fish.

Effect of protease supplementation on hematological parameters

Hematological parameters are often used as an important indicator of fish health. Protease supplementation significantly enhanced the red blood cells, hematocrit, and hemoglobin concentration in European seabass (Goda et al., 2020), rohu (Maryam et al., 2022), and tilapia (Hassaan et al., 2019). The increase in hemoglobin level could be ascribed to higher oxygen consumption due to high hemoglobin saturation and dissociation rate (Satheeshkumar et al., 2012). In addition, Saleh et al. (2021) also reported an increase in red blood cell concentration in tilapia fed protease supplemented diet (Saleh et al., 2021). The increase in red blood cells indicates better fish health (Satheeshkumar et al., 2012). On the other hand, no significant difference in hemoglobin and red blood cell was observed for protease supplemented diets

in tilapia (Adeoye et al., 2016; Saleh et al., 2021) and gibel carp (Liu et al., 2018). White blood cells (WBCs) play an important role in protecting the body against pathogens and infections. Evidence of non-significant effects of protease supplementation on WBCs was documented in tilapia (Hassan et al., 2019, 2020; Saleh et al., 2021) and rohu (Maryam et al., 2022). However, Goda et al. (2020) found a significant increase in WBCs of European seabass fed protease supplemented high-protein distiller's dried grains based diet. This indicates the stimulation of a strong immune response in fish due to the presence of high ANFs in the diet.

Effect of exogenous protease supplementation on antioxidant parameters

Antioxidants are substances that inhibit substrate oxidation by suppressing the production of reactive oxygen species (Neto et al., 2019). Exogenous protease supplementation improved the scavenging role of free radicals and serum antioxidant activity in farmed animals (Cowieson and Ravindran, 2008; Song et al., 2017). In gibel carp, protease supplementation accelerates the activities of intestinal superoxide dismutase, catalase, glutathione peroxidase, and total antioxidant capacity (Wu et al., 2020). The enhanced activities of these enzymes have an important role in the disposal of pathogens (Uzzau et al., 2002). Similarly, enhanced activity of phenol oxidase and reduced activity of malondialdehyde (a final product of lipid peroxidation) was reported in shrimp (*Litopenaeus vannamei*) fed protease supplemented diet (Song et al., 2017). These findings confirmed that exogenous protease supplementation significantly reduces oxidative stress and protects the body against the negative impacts of free radicals (Li et al., 2016; Song et al., 2017). However, attempts to analyze the effects of protease supplementation on the antioxidant properties of fish need more consideration.

Effect of exogenous protease supplementation on immunity

The spleen is an important component of the immune system and plays a significant role in eliciting an immune response against pathogens by promoting the maturation of lymphocytes in humoral and cellular defense (Elnabi et al., 2020). A study conducted by Elnabi et al. (2020) recorded a significant positive impact of protease supplementation on the spleen of tilapia. In another study, a significant increment in the level of serum lysozyme, and complement proteins (C3 and C4) was reported for protease supplementation in gibel carp (Wu et al., 2020). The better immune response of fish may owe to the hydrolysis of complex proteins and ANFs by protease supplementation, which resulted in enhanced protein availability which consequently increased the antibodies production in fish. Moreover, enhanced lysozyme activities due to proteolytic enzymes resulted in the breakdown of 1,4 glyosidic bonds in bacterial cell wall could also be the reason of better immune response in fish (Yano, 1996) (Figure 4). From above, the obvious evidence to show the relationship between immunity and protease supplementation is not found, thus more concerning efforts should be devoted.

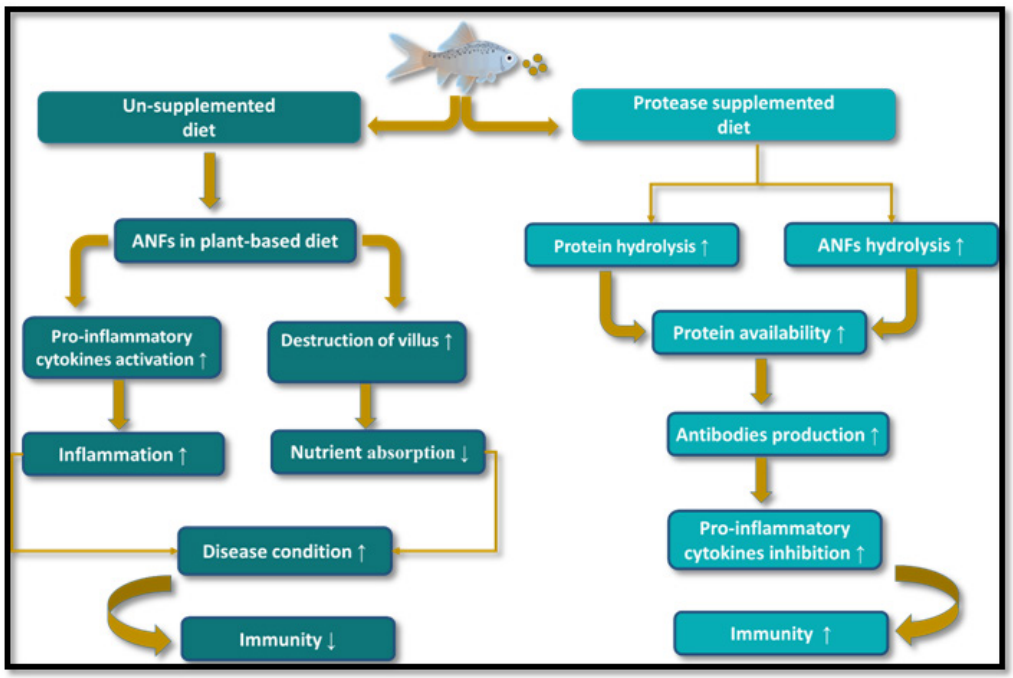


Figure 4. Effect of protease supplementation on the immunity of fish

Effect of exogenous protease supplementation on whole-body composition

The whole body proximate composition of fish is categorized into water, lipid, protein, and ash content. The difference in whole-body proximate composition corresponds to the difference in appetite, growth, survival, and reproduction of fish (Breck, 2014). It gives information about the energy density of fish calculated using standard energy values of lipids and protein (Haider et al., 2016). Previous studies showed that the exogenous protease supplementation in fish feed did not influence the whole body protein, lipid, moisture, and ash contents in fish (Adeoye et al., 2016; Hassaan et al., 2019; Lin et al., 2007; Shi et al., 2016). However, significant enhancement in body protein was reported for protease supplementation in African catfish larvae (Kemigabo et al., 2019), rohu (Maryam et al., 2022), tilapia (Li et al., 2016) and rainbow trout (Dalsgaard et al., 2012). This increased whole body protein content reflects the improved protein digestibility and feed utilization by protease supplementation. The discrepancy in these results may be due to the difference in fish species, diet composition, experimental conditions, and supplemented levels of protease.

Interactive effects of protease

Protease and malic acid

The supplementation of protease (2500 mg/kg) in combination with malic acid (5 g/kg) significantly improved the weight gain, feed conversion ratio, protein efficiency ratio, specific growth rate, feed intake, and final body weight gain in Nile tilapia (Hassaan et al., 2020). This significant improvement is attributed to the acidic environment in the stomach due to the addition of organic acids. The low pH by organic acid addition increased the activation of the pepsin enzyme which consequently improved nutrient absorption, feed utilization, and growth performance (Hassaan et al., 2020; Ng et al., 2009; Shah et al., 2021). This low pH may also facilitate the action of protease in degrading the complex proteins into a simpler form. In addition, significant enhancement in the activities of amylase, lipase, and chymotrypsin in the intestine was also recorded for protease and malic acid supplemented diet (Hassaan et al., 2020). The improvement in enzyme activities corresponded to improvement in the growth of intestinal epithelial cells and the height of villi, leading to better nutrient absorption in fish (Huan et al., 2018; Liu et al., 2014). This combination significantly enhanced the red blood cells, hematocrit, and hemoglobin (Hassaan et al., 2020). Moreover, the mentioned above combination significantly reduced the activities of serum ALT and AST activities and improved globulin, serum total protein, and albumin in tilapia (Hassaan et al., 2020). These findings confirmed that protease and malic acid improved the health status of Nile tilapia (Hassaan et al., 2020).

Besides this, protease in combination with organic salts is also reported as a useful combination for improving fish health. Supplementation of protease (175 mg/kg) in combination with an organic salt blend (922 mg/kg; calcium formate, calcium propionate and sodium acetate) in meat and bone meal based diet significantly improved the feed conversion ratio, weight gain, and protein retention and apparent digestibility coefficient of nutrients of tilapia (Huan et al., 2018). The improvement in growth performance and nutrient digestibility may be attributed to the enhanced catalytic activity of protease in response to the addition of organic salts in fish feed.

Protease and probiotics

Probiotics are defined as the live microbial culture added into the feed to optimize the colonization of beneficial gut microflora for better growth performance of living organisms (Borch et al., 2015). Supplementation of protease in combination with probiotics has positive effects on the health and physiology of fish. A study conducted by Hassan et al. (2021) showed that combination of protease (0.5 g) with probiotics (1.85×10^5 CFU kg^{-1} diet) significantly improved the growth performance, feed utilization, innate immunity, and *S. agalactiae* resistance in Nile tilapia (Hassan et al., 2021). In addition, Ghodrati et al. (2021 a) noted the highest values of final body weight, final body length, and metabolic growth rate in Siberian sturgeon fed with the combination of 2 g/kg multi-strain probiotics (*P. pentosaceus* 1×10^9 and *L. lactis* 1×10^8 CFU/g) and exogenous multi-enzyme cocktail including xylanase (25 mg/kg), protease (30 mg/kg) and phytase (7.5 mg/kg). The improvement in growth performance and feed utilization owes to the cumulative effect of protease and probiotics in providing a suitable substrate for the biota of the intestine (Hassan et al., 2021). Moreover, this combination stimulates the appetite, production of useful metabolites, and flourishment of beneficial gut bacteria for better fish growth. Supplementation of protease in combination with probiotics also enhanced the rate of digestion and absorption of nutrients in fish by thinning down the muscularis mucosa and improving the intestinal structures, including enterocytes, goblet cells, microvillus density, and mucosal folds (Hassaan et al., 2020). Another study conducted by Shekarabi et al. (2022) showed that supplementation of 250 mg/kg multi-enzyme complex and 2 g/kg probiotics mixture markedly enhanced the nutrient digestibility and intestinal health in Siberian sturgeon when compared to the control group. Moreover, this combination significantly improved the hematological and biochemical parameters in fish (Hassaan et al., 2021; Ghodrati et al., 2021 b). Besides this, increased serum immunoglobulin, lysozymes, and phagocytic enzymes were also reported for this combination (Hassaan et al., 2021; Shekarabi et al., 2022). This enhanced immune response may be ascribed to the inhibitory effects against pathogens in the gastrointestinal tract due to the probiotics and protease combination (Kristina Borch et al., 2015). Moreover, the production of cytokines through macrophages and monocytes in

response to this combination is also regarded as a reason for enhanced immunity in fish (Ehsani et al., 2021). A significant improvement in gene expression of insulin growth factor (IGF-I0) was also recorded for protease and probiotic supplemented diet in Nile tilapia (Hassaan et al., 2021). However, the interaction of these mentioned additives is not well understood. Hence, additional studies are recommended to improve our understanding in this regard.

Conclusion and perspectives

The attempt to replace the fishmeal with plant and animal-based proteins in fish feed has been facing the challenges of low nutrient digestibility, poor palatability, imbalanced amino acid profile, and several anti-nutritional substances. Thus, dietary protease supplementation could be an effective strategy to improve nutrient digestibility and utilization in fish. The addition of protease in fish feed counteracts the scarcity of endogenous digestive enzymes and improved the nutrient utilization in fish. However, the use of proteases in fish feed has not been investigated as broadly as other enzyme classes. Therefore, more studies are needed to scrutinize the efficacy of protease in fish nutrition.

This review verified the beneficial effects of optimum protease supplementation on the growth performance of fish. The beneficial effects are owing to the hydrolysis of anti-nutritional factors, enhanced nutrient absorption, and improved feed utilization by protease addition. However, the underlying mechanisms through which exogenous protease causes these positive changes still require further scrutiny. Therefore, more studies are required that can help in exploring the pathways that might be involved in the changes caused by protease supplementation. Moreover, protease supplementation has more pronounced effects on plant proteins than animal based proteins. Thus, more collaborative efforts should be devoted to conducting studies with animal based proteins so that it provides the basis for comparing the effects of these two alternative protein sources in fish feed. This would help the feed manufacturers to choose the best feed ingredients for their aquaculture practices.

Detailed literature is available regarding the effects of protease on growth performance and nutrient digestibility. However, very few studies have investigated the effects of protease supplementation on the anti-oxidant system, immunity, digestive enzyme activities, gut microbes, hematological parameters and gene expression in fish. The scarce knowledge in these areas limits the scope of protease supplementation as a feed additive in the aquaculture sector. Therefore, future investigations are needed to clarify the influence of protease supplementation on these parameters. Finally, information gathered in this review will provide insight to feed manufacturers to formulate a well-balanced and cost effective feed for fish species. Moreover, the gaps identified in the review will provide an opportunity to researchers to explore a new arena of protease supplementation with their mode of action in fish farming.

Authors' contributions

Maryam: bibliography review, manuscript writing, content design; Syed Zakir Hussain Shah: supervising author, conceptualization of ideas and structure of the article; Mahroze Fatima: conceptualization of the information and revision; Hafsa Nadeem: content design and revision; Shahzad Ashraf and Majid Hussain: content design and revision.

Competing interests

The authors declare that there are no conflicts of interest.

Consent to participate

All authors contributed to the study conception and collections. All authors read and approved the final manuscript.

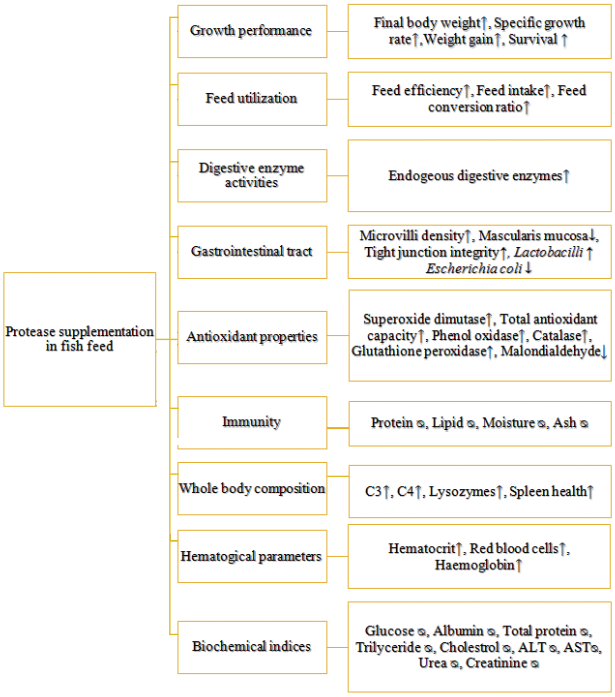
Consent for publication

All authors gave their consent for research publication.

Availability of supporting data

All the data supporting this study is available online as mentioned in the cited references.

Summary of protease supplementation on fish performance where ↑ means increase; ↓ decrease; ⊖ non-significant



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