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Review Article

The application of protease in aquaculture: Prospects for enhancing the aquafeed industry

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ABSTRACT

Low-fishmeal and protein-saving diets are two prominent nutritional strategies utilized to address challenges related to the scarcity and sustainability of protein sources in aquaculture. However, these diets have been associated with adverse effects on the growth performance, feed utilization, and disease resistance of aquatic animals. To mitigate these challenges, exogenous protease has been applied to enhance the quality of diets with lower protein contents or fishmeal alternatives, thereby improving the bioavailability of nutritional ingredients. Additionally, protease preparations were also used to enzymatically hydrolyze fishmeal alternatives, thus enhancing their nutritional utilization. The present review aims to consolidate recent research progress on the use of protease in aquaculture and conclude the benefits and limitations of its application, thereby providing a comprehensive understanding of the subject and identifying opportunities for future research.
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1. Introduction

Aquaculture is a burgeoning sector within the food production industry and occupies a critical position in the provision of nutrition (FAO, 2020). The consumption of aquatic foods has been increasing at a rate of 3.0% annually between 1961 and 2019, outpacing the corresponding growth rate of the global population and nearly doubling it within the same timeframe (FAO, 2020). However, despite this growth, the application of fishmeal obtained from wild forage fish as aquatic feed remains a significant challenge. The global fishmeal production consumed by the aquaculture sector jumped from 33% to 66% between 2000 and 2016 (Naylor et al., 2021) and grew to 78% after 2019 (European Commission, 2021). Harvesting forage fish always brings about overexploitation that adversely impacts aquatic ecosystems because these fish play a vital role in the conversion of plankton into sustenance for species at higher trophic levels (Cury et al., 2000). Furthermore, fishmeal production is heavily influenced by weather patterns, such as the El Niño-Southern Oscillation phenomena (Maulu et al., 2021; Naylor et al., 2009). The long-term global fishmeal production is expected to stabilize at around 5 million metric tons (Bachis, 2022; FAO, 2020), which is insufficient to meet the projected expansion of aquaculture (Hua et al., 2019; Shepherd and Jackson, 2013). Additionally, the price of fishmeal seems to be rising much faster than its production, varying from 657 USD per metric ton in Jul 2002 to 1,610 USD per metric ton in Jul 2022 (Index Mundi, 2022). Therefore, minimizing the use of fishmeal in aquafeeds is imperative (Tacon and Metian, 2008). Two main nutrition strategies, i.e., low-fishmeal diets (LFD) and protein-saving diets (PSD), could be conducted to cope with the above problem. LFD involves substituting fishmeal with alternative proteins including fishery and terrestrial animal by-products, plant-based meals, single-cell proteins, and insect meals in the formulated feed to satisfy fish's standard protein

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requirements (Agboola et al., 2020; Galkanda-Arachchige et al., 2020; Kaiser et al., 2022; Wang et al., 2022; Yan et al., 2023). A growing amount of fishmeal from fishery by-products is reportedly being used and was estimated at over 27% of the global fishmeal used in aquaculture in 2020 (FAO, 2020). Insects and single-cell proteins are a promising alternative to conventional feedstuffs because they have a short life cycle, their production does not require huge arable land, and they have high digestible protein with amino acids profile similar to fishmeal (Li et al., 2021; Maulu et al., 2022; Wang et al., 2022). Plant-based proteins, such as cottonseed protein concentrate, soybean meal, and rapeseed meal, have been widely studied as a substitute for fishmeal recently (Kaiser et al., 2022; Wang et al., 2020; Xie et al., 2023). The protein-saving diet is a nutritional regimen characterized by a lower content of dietary protein, which is complemented with essential nutrients supplementation, including amino acids, high lipids, and high sugar (Dong et al., 2013; Lee et al., 2019; Li and Robinson, 1998; Shiau et al., 1990; Yu et al., 2022).

While LFD and PSD have been recognized to offer cost-saving advantages and reduce dependence on fishmeal, studies have also linked them to adverse effects on fish and shrimp (Panigrahi et al., 2019; Willora et al., 2022). Currently, plant-based proteins dominate fishmeal substitution in aquafeed (Chen et al., 2022a, 2022b); however, these proteins have several drawbacks, including anti-nutritional factors (ANFs), low digestibility, poor bioavailability, and palatability issues (Shomorin et al., 2019). Previous research has indicated that diets incorporating plant proteins may impede the activity of digestive enzymes. This hindrance is attributed to the presence of protease inhibitors in plant proteins, which bind to the active sites of endogenous proteases (Francis et al., 2001; Gatlin et al., 2007; Kamel et al., 2015; Segobola, 2016; Xu et al., 2022). Adding exogenous enzymes to aquatic feed is a proven nutritional approach that can improve the quality of diets that include plant-based proteins or other undesirable proteins (Dalsgaard et al., 2012; Li et al., 2016; Liang et al., 2022; Zheng et al., 2020). Proteases, as a dietary enzyme additive, can improve protein utilization by addressing endogenous enzyme deficiencies and hydrolyzing macromolecular proteins (Saleh et al., 2022). Additionally, they can enhance other nutrient absorption (Cowieson and Roos, 2016; Zaworska-Zakrzewska et al., 2022). However, a significant challenge in the commercial application of exogenous protease additives exists; proteases are heat-sensitive additives that undergo a reduction in efficacy during feed processing, and the extrusion process is primarily responsible for the degradation of thermosensitive nutrients (Espinosa et al., 2020). The feed industry relies on this process to gelatinize the starch, inactivate ANFs, and destroy pathogenic microorganisms (Drulyte and Orlien, 2019; Glencross et al., 2012; Shi et al., 2016); consequently, the optimal method of feed protease supplementation necessitates careful consideration. Presently, protease preparations are also creatively applied to the enzymatic hydrolysis of protein sources (Boyd et al., 2020). Protease-treated proteins always show higher crude protein digestibility and more bioactive substances, while the ANFs present in the enzymatically hydrolyzed proteins can be reduced (Caine et al., 1998; Rooke et al., 1998; Wu et al., 2020). The present review concludes the application of protease in aquafeed, encompassing its advantageous attributes as well as inherent limitations, aiming to facilitate a thorough comprehension of the topic and suggest prospective avenues for further investigation in order to fully exploit all potential outcomes.

2. Feed proteases

Proteases, which were initially identified in 1903, are enzymes responsible for breaking down complex proteins into smaller units through hydrolysis (Rawlings, 2013; Vines, 1903). Despite this, exogenous proteases have only been used as a mono-component commercial enzyme additive for the past 10 to 15 years (Cowieson and Adeola, 2005; Fru-Nji et al., 2011; Li et al., 2012). As an emerging product, its global market share is multiplying, valued at $3,454.3 million in 2020, and current projections indicate that it will reach $5,762.7 million by 2030 (Allied Market Research, 2022). Commercial feed proteases, such as alcalase, papain, flavourzyme, neutrase, and trypsin, can be classified into three categories based on their source: animal, plant, and microorganisms (Islam et al., 2022a). Also, they can be divided into acid, neutral, and alkaline proteases based on working pH values (Flores et al., 2019). pH and temperature always influence protease activity. Certain studies propose that these variables may alter protease structure, consequently impacting their activity (Awad et al., 2020; Buchholz et al., 2020). Hence, the outcomes may fluctuate based on the pH of the animal's gastrointestinal tract and the cultivation temperature. Considering the variability in pH ranges within aquatic animals' gastrointestinal tracts, it is imperative to account for these factors when employing proteases in aquafeed. Nevertheless, it appears that current applications in aquafeed do not take this into consideration. Information on some commercial feed proteases used in aquaculture is provided in Table S1. Most of them are derived from microorganisms' fermentation, making up nearly two-thirds of the market. The primary reason for the prevalence of microbial proteases is their characteristic as extracellular enzymes, which makes them easy to extract and cost-effective to produce without sacrificing their high catalytic activity (Beg and Gupta, 2003). Additionally, various microorganisms can produce industrial proteases, which can be modified using advanced technologies (Ali et al., 2016; Beg and Gupta, 2003). Proteolytic enzymes can break down proteins into peptide fragments composed of 2 to 20 amino acids or free amino acids (Islam et al., 2022b). Of note, the protease activities are highly specific in their cleavage. For instance, trypsin and alcalase are two different proteases with distinct substrate specificities. Trypsin targets peptide bonds that are located at the C-terminal side of lysine and arginine residues, whereas alcalase exhibits a broader specificity, preferring to hydrolyze peptide bonds located at the C-terminal side of hydrophobic residues, as shown in Fig. 1 (Vogelsang-O'dwyer et al., 2022). Peptides consist of a particular sequence of amino acids released from bulk protein and show different activities such as antioxidative, antibacterial, immunoregulation, and anti-inflammatory profiles (Jia et al., 2021). In aquaculture, the use of proteases can be categorized into two distinct applications. Firstly, as a feed additive, and secondly, for pre-treating fishmeal alternatives. The effects of feed protease preparation in aquaculture can be summarized as follows (Fig. 2): supplementing the deficiency of endogenous proteases and hydrolyzing complex proteins into simpler units. Supplementing endogenous digestive enzymes benefits the digestibility of nutrients by farmed animals, with a particular emphasis on proteins. Consequently, it effectively reduces nitrogen (N) emissions. Moreover, the breakdown of large protein molecules promotes the formation of bioactive peptides and the degradation of certain proteinaceous antinutrients. These effects not only positively influence the health and growth of farmed fish but also yield economic benefits (Mohammadigheisar and Kim, 2018; Schneider and Lazzari, 2022; Zheng et al., 2020).

3. Effects of protease application on aquatic animals

3.1. Growth performance

Previous research has consistently demonstrated the growth-enhancing benefits of protease supplementation in diets, as

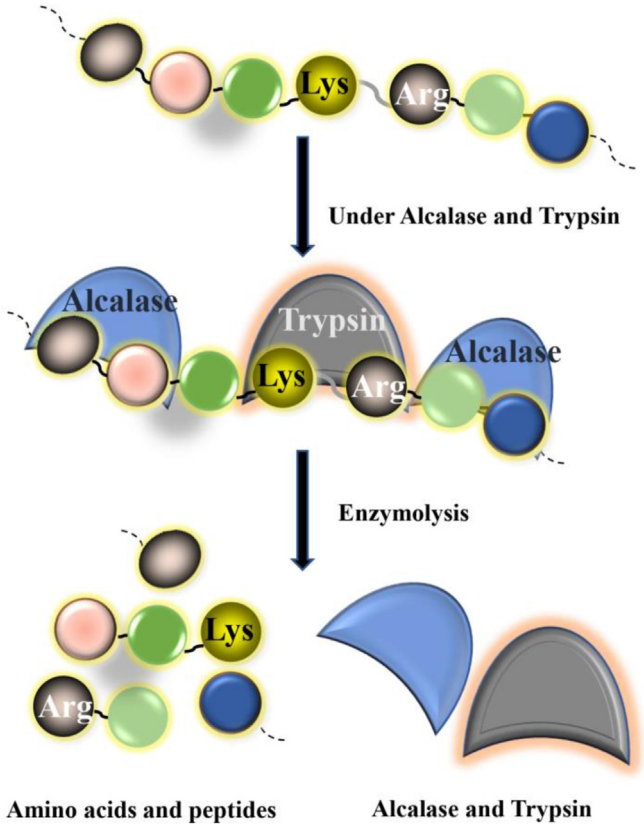

Fig. 1. Schematic representation of the enzymatic hydrolysis of proteins into peptides or free amino acids by alcalase and trypsin.

outlined in Table 1. In the case of omnivorous fish, a dosage of 500 mg/kg of protease supplementation in PSD diets has been found to significantly improve various growth performance indicators of Nile tilapia *Oreochromis niloticus*, including final body weight (FBW), weight gain (WG), specific growth rate (SGR), protein efficiency ratio (PER), and feed conversion ratio (FCR) (Saleh et al., 2022). Similarly, a study on blue tilapia *O. niloticus* × *Oreochromis aureus* reported that the supplementation of 175 mg/kg of protease in fishmeal-free diets could significantly improve FBW, WG, and FCR parameters (Li et al., 2019). Furthermore, a study conducted on the carnivorous fish, European seabass *Dicentrarchus labrax*, suggested that dietary supplementation of high levels of high-protein distiller's dried grains (HPDDG) with protease inclusion increased growth performance, as well as improved FCR (Goda et al., 2020). For crustacean species, multiple studies conducted on Pacific white shrimp (*Litopenaeus vannamei*) demonstrated that the addition of protease in LFD diets proved to be an effective nutritional strategy for improving shrimp growth (Yao et al., 2019; Li et al., 2016). Similar positive outcomes were observed in a study on the Chinese mitten crab *Eriocheir sinensis* (Chowdhury et al., 2018). However, Wu et al. (2020) reported that high-dose protease could inhibit growth performance in Nile tilapia. Other research also indicated that the excessive supplementation of exogenous protease always induced negative growth-promoting benefits (Liu et al., 2018; Wu et al., 2020). It is speculated that this may be due to the excessive addition of protease, leading to metabolic disorders of other nutrients in compound feed. According to Guan et al. (2021), supplementing with high levels of protease resulted in a 42.1% reduction in abdominal fat by regulating glucose and lipid metabolism. Meanwhile, this supplementation also led to a decrease in protein efficiency ratio (PER) and hindered the growth performance of largemouth bass *Micropterus salmoides*. Additionally, Song et al. (2017) proposed that excessive protease inclusion could cause damage to the intestines by hydrolyzing mucosal proteins when there are insufficient substrates available for hydrolysis. The effect of protease addition on aquatic animal growth is also influenced by various pelleting methods. Shi et al. (2016) supplemented LFD with exogenous protease at 125, 150, and 175 mg/kg levels, and processed them using either pelleting or extruding technology. The study findings indicated that gibel carp (*Carassius auratus gibelio*) showed significantly better growth performance when fed pelleted diets at all three supplementation levels, as opposed to extruded diets supplemented with protease. Additionally, the growth-promoting action of protease supplementation is significantly affected by the compound feed ingredients. For example, a study on common carp *Cyprinus carpio* found that supplementing 175 mg/kg protease in 20% fishmeal-based diets did not significantly impact fish growth (Leng et al., 2008). However, when the fishmeal inclusion level in diets was reduced to 10% or 6% with protein being replaced with soybean meal, the WG of fish increased significantly compared to groups without protease (Leng et al., 2008). In aquaculture production, diets are formulated with a nutrition strategy that enables an animal to achieve the best growth performance with minimum costs. Suppose the experimental diets' nutritional requirements for the animal being studied are not reduced to the minimum optimal level, any increase in nutrient utilization or fish growth caused by exogenous proteases cannot reflect the actual animal response (Son and Ravindran, 2011).

Enzymatic hydrolysis of protein under protease treatment to replace an appropriate proportion of untreated proteins or fishmeal is also beneficial for the growth of fish. According to Pfeuti et al. (2019), incorporating protease-treated feather meal into the diet of rainbow trout *Oncorhynchus mykiss* contributed to a growth rate increase of 10.5% to 11.5% compared to the untreated counterparts. Similarly, Cao et al. (2020) recorded that keratinase-treated feather meal (KFM) could promote an 81% higher growth rate of juvenile turbot *Scophthalmus maximus* than the steam-processed group. Replacing around three-quarters of un-hydrolyzed pre-mixed protein with pre-mixed protein hydrolysates in the diet of larval snakehead *Channa argus* led to a 38.1% increase in their FBW (Sheng et al., 2023). A study on juvenile barramundi *Lates calcarifer* showed that raw alga could only replace 20% fishmeal in diets based on the WG indicator, while the replacement level could be increased up to 40% when supplemented with protease-treated alga (Van Vo et al., 2020). For soy protein with protease treatment (SPP), Song et al. (2014) confirmed that replacing up to 85% of fishmeal protein with SPP negatively affected the growth of starry flounder *Platichthys stellatus*. However, the low to moderate levels of replacement (15% to 50%) could significantly improve growth parameters compared with full fishmeal protein diet groups. Moreover, manifold research investigating the effects of dietary protease-treated proteins on larval fish suggested that these enzymatically hydrolyzed products can facilitate rapid growth by serving as a highly digestible source of essential amino acids (EAA) and protein in diets (Delcroix et al., 2015; Kvale et al., 2009; Ovissipour et al., 2014; Sheng et al., 2023; Srichanun et al., 2014). Unlike adults, fish larvae show a weak ability to fully utilize conventional formulated feeds due to the immature digestive tract and lack of functional digestive systems. Compared to bulky proteins, protein hydrolysates (equal to be pre-digested) owning low molecular weight are more comfortably absorbed by the intestinal epithelial cells. However, overconsumption of protein hydrolysates in the diet also negatively influences larval fish growth (Kolkovski and Tandler, 2000). As feed additives, protease-based hydrolysis of proteins has successfully been used in PSD and LFD to mitigate growth inhibition caused

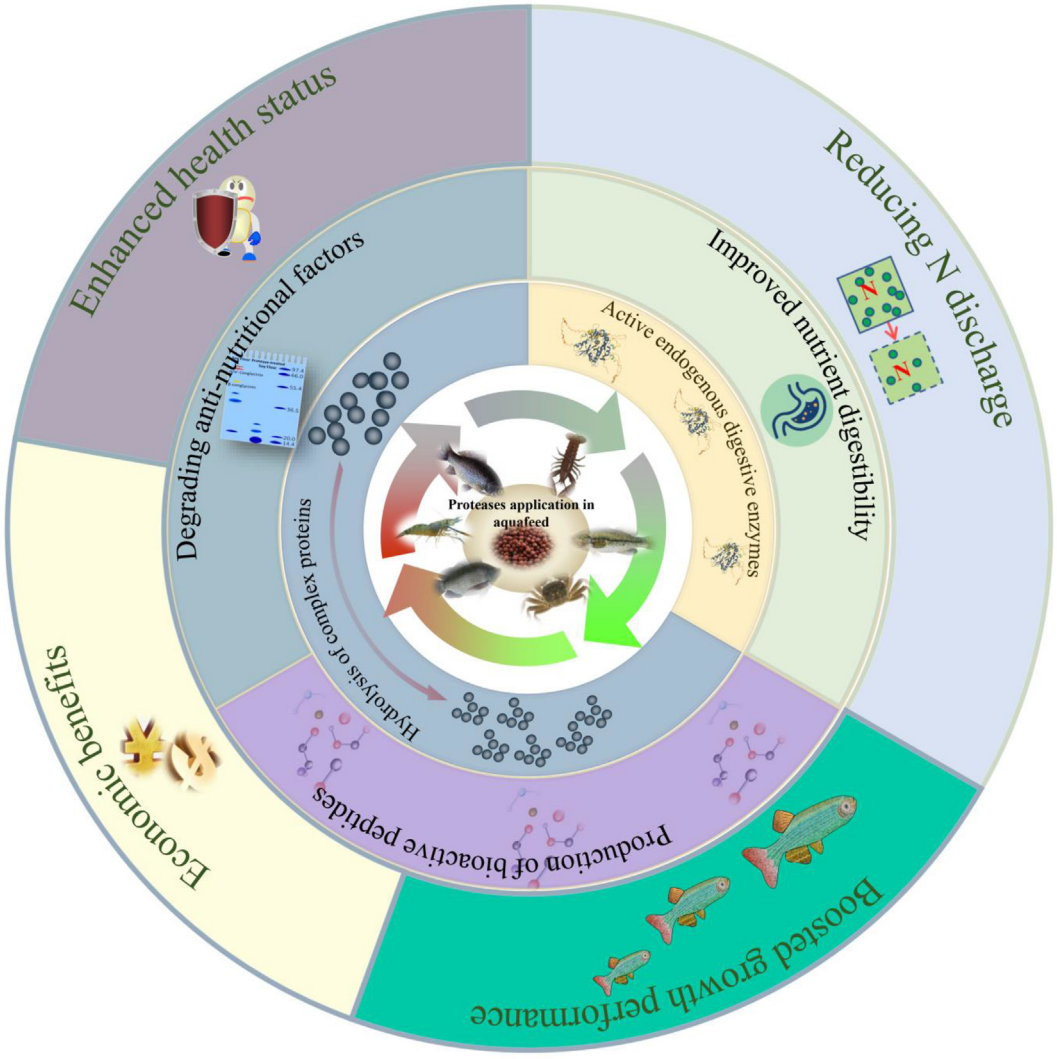

Fig. 2. An illustration of the benefits of exogenous protease in fish diets. N = nitrogen.

when high levels of fishmeal are replaced with plant-based proteins in aquafeeds. In herbivorous fish, Xiao et al. (2017) found that supplementing grass carp *Ctenopharyngodon idella* diet with 10 g/kg of protease-treated soybean protein increased their growth performance significantly. The difference in growth performance between the fish under the high-protein diet (34% CP) and those receiving the protease-treated soybean protein supplement (added to a 32% CP diet) was not statistically significant. In carnivorous fish, European seabass, the addition of 30 g/kg of anchovy and jumbo squid hydrolysates demonstrated the ability to reduce the negative effects of LFD (Costa et al., 2020). Also, 33.4 g/kg shrimp hydrolysate, 28.8 g/kg tilapia hydrolysate, and 31.2 g/kg krill hydrolysate supplemented in LFD of juvenile olive flounder *Paralichthys olivaceus* yielded better results on the fish's growth performance (Khosravi et al., 2018). Supplementation of protein enzymatic hydrolysate partially alleviated the amino acid deficiency caused by the PSD or LFD, leading to improved growth by meeting the animals' requirements (Wang et al., 2021). Moreover, some protein hydrolysates have shown superior attractiveness to aquatic animals (Barroso et al., 2013; Kolkovski et al., 2000a; Leal et al., 2010). For example, Cheng et al. (2019a) suggested that the diets containing cottonseed meal protein hydrolysate (CPH) were more attractive compared to those including squid extract, yeast nucleotides, betaine, and allicin in Chinese mitten carb *Eriocher sinensis*; consequently, 0.6% of CPH was recommended to be added in the diets as attractants. In a further study, dietary supplementation of CPH at 6 g/kg was proved to stimulate the appetite and increase the feeding rate of carp via the target of rapamycin (TOR) signaling pathway, which finally contributed to enhanced growth performance (Cheng et al., 2019b).

3.2. Degradation of ANFs and hydrolysis of complex proteins into simpler units

The promotion of growth in aquatic animals by protease is also partly attributed to its positive effects on the hydrolysis of proteinaceous antinutrients which are known to be harmful (Han et al., 2020; Hart et al., 2010; Zhu et al., 2021). Studies have demonstrated that protease pretreatment could significantly degrade ANFs (Table 2). For example, Yu et al. (2018) suggested that keratinase treatment could effectively degrade nearly 60% β-soybean globulin and 37% soybean globulin in soybean meal. Tan and Sun (2017) investigated the hydrolysis ability of different proteases from the Chinese market on degrading ANFs of soybean meal in vitro and found that protease DP100 could significantly eliminate 73.3% and 52.1% of glycinin and β-conglycinin, respectively. Moreover, it was

Table 1
The effects of dietary protease as a mono-component enzyme additive on growth and physiological parameters of aquatic animals.

Aquaculture species	Inclusion level(s)	Effects on the growth and physiological parameters	References
Omnivorous fish			
Nile tilapia or GIFT (*Oreochromis niloticus*)	Four diets with two controls: high protein (30%) and low protein (29%). The third diet contained 500 mg/kg protease, the fourth contained 250 mg/kg protease	Dietary supplementation of protease improved the productive performance of the fish besides sparing the protein inclusion and producing economical diets	Saleh et al. (2022)
	2,500 U/kg diet protease supplementation	Protease supplementation can improve growth, nutrient assimilation, and hematology and alter gene expression of growth hormone and insulin-like growth factor I of Nile tilapia	Hassaan et al. (2019)
	500 mg/kg of protease inclusion in the diets	Growth performance and feed utilization, including highest goblet cells, the thickness of muscularis, mucosal folds, and enterocytes. Furthermore, the immune parameters of the fish were improved	Hassaan et al. (2020)
	Protease supplemented in plant-based diets at the levels of 0, 1.38, 2.76, 5.52, and 11.04 U/g diet	5.52 U/g protease supplementation could promote growth performance, intestinal physical barrier function, innate immunity, and the fish's resistance against *Streptococcus agalactiae*	Wu et al. (2020)
Blue Tilapia (*Oreochromis niloticus* × *O. aureus*)	Compressed (CD) or extruded (ED) diets containing 30 g/kg or 90 g/kg fishmeal were supplemented with or without protease	Weight gain was improved and the feed conversion ratio decreased significantly with the supplementation of protease in 30 g/kg fishmeal CD	Li et al. (2016)
	Protease supplemented 0 (control), 1,000, and 1,500 mg/kg in diets	Significantly improved growth performance and feed utilization of the fish	Lin et al. (2007)
	Supplementation of protease to the diets at 175 mg/kg	Improved growth performance with no significant effect was observed on the whole-body composition and protein retention	Huan et al. (2018)
	Protease supplemented in fishmeal-based diets at 175 mg/kg diet	Improved the growth and nutrient utilization, higher intestinal villus length, and promoted the retention of crude protein and phosphorous	Li et al. (2019)
Gibel carp (*Carassius auratus gibelio*)	Four diets: 75, 150, 300, and 600 mg/kg protease in the diet	Improved growth performance. 150 or 600 mg/kg of protease led to foregut muscular thickness thinner, and protease activities in hepatopancreas and foregut were higher in the fish fed 150 or 300 mg/kg protease	Liu et al. (2018)
	500 mg/kg protease added in low fishmeal diets (LFD)	Improved the growth and immune response of the fish	Xu et al. (2022)
	Pelleted LFD containing 30 g/kg fishmeal (60 g/kg fishmeal replaced by soybean meal) supplemented with 125, 150, and 175 mg/kg exogenous protease	150 to 175 mg/kg protease supplementation in a pelleted LFD improved the growth performance, crude protein, and retention of protein and lipid of the fish	Shi et al. (2016)
Common carp (*Cyprinus carpio*)	The fish diets were supplemented with 0.0%, 0.1%, 0.2%, 0.3%, and 0.4% of exogenous protease papain	Enhanced growth performance in terms of length gain, weight gain, and specific growth rate	Patil et al. (2019)
Rohu (*Labeo rohita*)	Poultry by-product meal-based diets supplemented with exogenous protease at the levels of 150, 300, 450, 600, and 750 mg/kg diet	Growth performance, whole-body composition, and blood biochemistry were enhanced in the fish fed with protease-supplemented diets	Maryam et al. (2022)
Carnivorous fish			
African catfish (*Clarias gariepinus* B.)	Four artificial diets formulated and enriched with protease enzyme at levels of 0 (control), 750, 1,000, and 1,250 U/kg diet	Improved larval growth and survival compared with control	Kemigabo et al. (2019)
European seabass (*Dicentrarchus labrax*)	Protease supplemented at 30%, 40%, and 50% of high protein distiller's dried grains supplemented with 1,000 mg/kg of protease as a replacement for soybean meal	Protease supplementation at 50% significantly increased growth performance, feed utilization, and better feed conversion ratio. Further, hematology and serum biochemistry, humeral immune parameters (total protein, globulin, cholesterol, lysozyme activity), and total antioxidant capacity significantly increased	Goda et al. (2020)
Crustacean species			
Pacific white shrimp (*Litopenaeus vannamei*)	A high fish meal diet (HFD) with 250 g/kg fishmeal and an LFD with 225 g/kg fishmeal. 175 mg/kg protease supplemented to LFD	Enhanced growth, including higher weight gain and lower feed conversion ratio than the fish fed with diets without protease addition	Li et al. (2016)
	Protease supplemented in LFD at 125, 150, and 175 mg/kg	Enhanced total superoxide dismutase and polyphenol oxidase contents in both serum and hepatopancreas were higher and serum malondialdehyde content and the cumulative mortality during disease challenge tests were significantly reduced	Song et al. (2017)
	Protease complex (175 mg/kg) in LFD (10% fishmeal)	Improved the growth performance and nutrient utilization of the shrimp. However, no differences in whole-body proximate composition, intestinal villi width, and hepatopancreatic lipase activity	Yao et al. (2019)
Chinese mitten crab (*Eriocheir sinensis*)	Diets supplemented with 125, 150, and 175 mg/kg of a dietary protease	175 dietary proteases enhanced the fish's protein and lipid retention efficiencies	Chowdhury et al. (2018)

CD = compressed diets; ED = extruded diets; LFD = low fishmeal diets; HFD = high fishmeal diets.

Table 2
Beneficial effects of protease pretreatment on the nutrient profiles of proteins.

Proteins	Incubation condition	Nutritional profiles	References
Defatted soy flour	Flavourzyme, novozym and alcalase; material-water ratio 1:20; pH 7.0	Each enzyme degraded both β-conglycinin and glycinin	Hrčková et al. (2018)
Soybean protein isolate	Alkaline protease and papain mixture; material-water ratio 1:20; 50 °C; pH 8.5; for 4 h	Decreasing glycinin and β-conglycinin	Cao et al. (2022)
Soybean meal	1.0 mg/g of *Bacillus subtilis* subtilisin-protease; 50 °C; pH 4.5; for 16 h	Increasing soluble matter and soluble crude protein, and decreasing the level of soybean trypsin inhibitors	Caine et al. (1998)
Soybean meal	10 mg/kg protease DP100	Decreasing 73.3% glycinin and 52.1% β-conglycinin	Tan and Sun (2017)
Soybean meal	6 mg/g keratinase; material-water ratio 5:4; for 24 h	Decreasing 37% glycinin and 60% β-conglycinin	Yu et al. (2018)
Cottonseed meal	Subtilisin; material-water ratio 1:25; 45 °C; pH 7.0; for 5 h	Increasing soluble crude protein and amino acids, and increasing peptide contents	Liu et al. (2005)
Cottonseed meal	AS1.398 protease; 45 °C; pH 70.0; for 5 h	Increasing the soluble protein by 125%, amino acids (74 to 180 Da) by 59.4%, and the small peptides (180 to 1,983 Da) by 605.3%	Gui et al. (2010a)
Soybean meal and cottonseed meal mixture (1:1)	A multiple enzyme complex including neutral protease; material-water ratio 1:5; 50 °C; pH 7.5; for 6 h	Increasing water-soluble nitrogen and decreasing molecular weight	Song et al. (2018)
Perilla meal protein	7% alcalase; material-water ratio 1:22; 61.4 °C; for 4 h	Increasing the soluble peptide or protein concentration, and increasing the DPPH scavenging capacity	Zhang et al. (2022)
Antarctic krill	8% trypsin; 45 °C; pH 7.9; for 8.5 h	Increasing amino nitrogen content, decreasing molecular weight	Liu et al. (2019)

Da = Dalton; DPPH = 2,2-diphenyl-1-picrylhydrazyl.

reported that the addition of protease in diets could also reduce the ANFs during feed production. For instance, Wu et al. (2020) investigated the effect of four graded levels of protease supplementation in plant-based diets containing soybean globulin (16.65 g/kg) and β-conglycinin (15.85 g/kg) on Nile tilapia. The study findings revealed a significant dose-dependent reduction in the concentration of these two ANFs.

Another main function of protease is believed to be the enzymatic hydrolysis of protein into individual amino acids and peptides (Table 2). Song et al. (2018) used neutral protease to break down a blend of soybean meal and cottonseed meals (1:1) and found that water-soluble nitrogen contents increased from 8.3% to 42.7%. In a more detailed analysis of molecular mass distribution, results showed that the contents of four different ranges (<1,000 Da, 1,000 to 3,000 Da, 3,000 to 5,000 Da, and >5,000 Da) all increased significantly (Song et al., 2016, 2018). Small peptides are known to be more easily absorbed than high molecular weight proteins, partly explaining the growth-promoting effects of protease. It is well known that the small peptides and free amino acids show distinct mechanisms for absorption, which reduces the antagonism caused by the competition for common absorption sites of free amino acids (Gilbert et al., 2008). Additionally, small peptides can be fully absorbed into the circulatory system and utilized by the liver to directly create proteins, yielding a higher rate of protein synthesis compared to the utilization of amino acids alone (Gilbert et al., 2008). However, a study involving spotted seabass *Lateolabrax maculatus* found that replacing 50% of the fishmeal in their diet with protease-based hydrolyzed soybean protein isolates resulted in a decrease in both WG and SGR (Cao et al., 2022). Another study on totoaba *Totoaba macdonaldi*, replacing dietary fishmeal at 40% with SPP also indicated that the fish was significantly affected negatively (Villanueva-Gutiérrez et al., 2022). This outcome can be largely attributed to the factor that the resulting increased levels of luminal peptides and free amino acids may saturate intestinal peptides and amino acid transporters. This could cause rapid peptide influx, which may in turn accelerate amino acid oxidation and endogenous excretion (Zhang et al., 2002). Consequently, the excessive amino acids in diets containing protease-treated proteins may be excreted as intact molecules through urine or gills, which could partially account for the observed impairment of growth (Berge et al., 1994). As shown in the work of Yuan et al. (2019a, 2019b), substituting high levels of cottonseed meal protein hydrolysate for fishmeal resulted in a decrease in fish growth performance. Specifically, the replacement caused a decrease in amino acid metabolism and activated the *AMPK/SIRT1* pathway while inhibiting the *TOR* signaling pathway.

3.3. Apparent digestibility coefficient (ADCs) of nutrients

The growth improvement of aquatic animals by protease is also mainly accredited to the enhancement of nutrient digestibility. The ideal amount of exogenous protease added to feed shows a considerable impact on nutrient digestibility, particularly crude protein (ADC_{CP}), as evidenced by Table 3. Hassaan et al. (2019) confirmed that feeding Nile tilapia diets including protease led to an improvement in ADCs for essential amino acids (ADC_{EAA}). Lee et al. (2020) examined the impact of dietary protease inclusion on the digestibility of amino acids in rainbow trout that were fed 17 different feed ingredients. The findings validated a boost in ADC_{EAA} and non-essential amino acids (ADC_{NEAA}). Apart from crude protein and amino acids, an optimal dose of exogenous protease inclusion could also effectively improve the digestibility of other nutrients: crude ash (ADC_{Ash}), crude lipid (ADC_{CL}), gross energy (ADC_{GE}), and dry matter (ADC_{DM}) (Table 3). For example, Maryam et al. (2022) found that 150 to 750 mg/kg protease supplementation in a diet based on poultry by-products significantly enhanced ADC_{Ash}, ADC_{CL}, and ADC_{DM} in rohu *Labeo rohita*. Parallelly, Drew et al. (2005) reported that supplementing canola: pea mixtures-based diets with moderate protease led to significant increases in ADC_{CL}, ADC_{GE}, and ADC_{DM}. In terms of trace elements and macroelements, one investigation conducted on tilapia demonstrated that dietary protease supplementation in LFD did not yield statistically significant improvements in ADCs for calcium (ADC_{Ca}), phosphorus (ADC_{P}), iron (ADC_{Fe}), and copper (ADC_{Cu}) (Huan et al., 2018). However, other studies indicated that ADC_{P} levels increased as a result of protease supplementation (Dalsgaard et al., 2012; Li et al., 2019). Furthermore, Ayhan et al. (2008) and Cho and Bureau (2001) reported that supplementing gilthead sea bream *Sparus aurata* diets with protease contributed to a significant increase in the ADCs of nitrogen (ADC_{N}), a crucial factor affecting water quality.

Studies have partly attributed the improved nutrient digestibility by protease to the improvement of the intestinal

Table 3
The effects of dietary protease as a mono-component enzyme additive on aquatic animals' nutrient digestibility and digestive enzyme activity.

Species	Fish size, g	Experimental diets and protease information [1]	Nutrient digestibility and digestive enzymes activities [2]	Reference
Omnivorous fish				
Genetically improved farmed tilapia GIFT (*Oreochromis niloticus*)	18.5	Protease (EC3.4.23.18 with 13,830 U/g) supplementation at 0, 100,200, 400, and 800 mg/kg in the basal diets	↑ADC_{CP} at 200 and 400 mg/kg, but ↓ADC_{DM} and ADC_{CP} at 800 mg/kg; ↑Protease at all doses in all distal, mid, and proximal intestine	Wu et al. (2020)
	7.56	500 and 1,000 mg/kg protease (CAS No. 9001-927) addition in low fishmeal diets (LFD) with different levels of malic acid	↑Chymotrypsin, trypsin, and lipase at both protease supplementation levels with malic acid supplementation	Hassaan et al. (2020)
	8.76	Exp. 1) Measurement of in vitro digestibility of several raw materials with or without protease (pineapple waste extract) supplementation; Exp. 2) Adding 1%, 2%, and 3% of pineapple waste extract in basal diets	Exp. 1) ↑ADC_{CP} of fishmeal (65%, 60%, and 53% CP) and soybean meal (51% and 48% CP) ingredients in vitro; Exp. 2) →ADC_{CP} at all levels in vivo, but ↑ADC_{CP} in all supplementation levels of protease in vitro	Yuangsoi et al. (2018)
	11.6	Addition of 500 mg/kg of protease (5,000 U/g) in LFD with partial dietary fishmeal (FM) replacement with cottonseed meal (CSM; FM:CSM = 2:1, 1:1, and 1:2)	↑ADC_{CP}, ADC_{CL}, ADC_{DE}, and ADC_{DM} in all diets: except for →ADC_{DM} in FM:CSM = 1:2 diets; furthermore, ↑ADC_{EAA} in all diets: except for →ADC_{Thr} and ADC_{Val} in FM: CSM = 1:2 diets	Hassaan et al. (2019)
Blue tilapia (*Oreochromis niloticus* × *O. aureus*)	1.70	Inclusion of 175 mg/kg AG175™ (35,000 U/g) in either 30 g/kg or 90 g/kg FM-based diets, exposed to compressing or extruding processing	↑ADC_{CP} and ADC_{DM} at 30 g/kg FM diets but not 90 g/kg FM diets	Li et al. (2016)
	7.70	175 mg/kg AG175™ addition in LFD	↑ADC_{CP}, ADC_{DM}, and AD_{CP}, while →ADC_{Ca}	Li et al. (2019)
	15.0	175 mg/kg PT125™ (25,000 U/g) added in diets containing 40 g/kg cork	↑ADC_{DM} and →ADC_{CP}; ↑Protease and amylase in the intestine	Yang et al. (2019)
	7.00	Supplementation of 175 mg/kg AG175™ in LFD (FM was replaced with meat and bone meal)	→ADC_{CP}, ADC_{DM}, ADC_{Ca}, ADC_{P}, ADC_{Fe}, and ADC_{Cu}	Huan et al. (2018)
Gibel carp (*Carassius auratus gibelio*)	8.08	75, 150, 300, and 600 mg/kg protease (neutral protease) supplementation in protein-saving diets (PSD)	↑ADC_{CP} at 150 mg/kg supplementation diets, and ↑ADC_{CL} by 75 to 300 mg/kg protease addition, while →ADC_{DM} by all the inclusion levels of protease; ↑Protease at 600 mg/kg diets both in hepatopancreatic and foregut tissues	Liu et al. (2018)
	17.2	500 mg/kg protease (20,000 U/g) added in LFD	→ADC_{DM}, ADC_{CP}, and ADC_{P}; ↑Chymotrypsin and trypsin in the liver, meanwhile, ↑Trypsin and amylase in the intestine	Xu et al. (2022)
	35.0	125, 150, and 175 mg/kg AG175™ supplemented in the LFD (60 g/kg FM was isonitrogenous replaced by soybean meal) subjected to either pelleting or extruding processing	↑ADC_{CP} at all the inclusion levels and ADC_{DM} at 150 and 175 mg/kg in pelleting process diets, however, →ADC_{CP} and ADC_{DM} at all inclusion levels in extruding processing diets	Shi et al. (2016)
Common carp (*Cyprinus carpio* L.)	11.9; 48.7	Exp. 1: 175 mg/kg Aquagrow (alkaline protease) added in three diets with different FM contents (10%, 15%, and 20%); Exp. 2: 175 mg/kg of the same protease added in an LFD containing only 6% of FM	Exp. 1) ↑Protease at 10% FM diets but not 15% and 20% FM diets; Exp. 2) ↑Protease at the PSD diets	Leng et al. (2008)
Jian carp (*Cyprinus carpio* var. Jian)	52.5	175 mg/kg AG175™ supplemented in LFD	↑ADC_{CP} and ADC_{Ca}, and →ADC_{P}	Zhang et al. (2017)
Rohu (*Labeo rohita*)	11.3	150, 300, 450, 600, and 750 mg/kg CIBENZA DP® (600,000 U/g) included in LFD (FM was replaced with poultry by-product meal in all diets)	↑ADC_{Ash}, ADC_{CP}, ADC_{CL}, and ADC_{DM} at all levels; except for ADC_{CP} at 750 mg/kg; ↑Amylase, protease, and lipase are both in the hepatopancreas and intestine at all supplementation levels, except for protease at 750 in the intestine	Maryam et al. (2022)
Sterlet (*Acipenser ruthenus*)	37.0	10 and 20 g/kg of papain added to the basal diets	↑Amylase at 20 g/kg in the posterior intestine, ↑Lipase at 20 g/kg in the anterior intestine, ↓Trypsin at both inclusion levels in the anterior intestine, and ↑ LAP at 20 g/kg in the posterior intestine	Wiszniewski et al. (2022)
	56.0	1,000 and 2,000 mg/kg of bromelain (Sigma-Aldrich with 900 U/g) added to a commercial diet	↑Pepsin in the stomach and lipase in the gastrointestinal tract at both inclusion levels, however, ↓Trypsin and LAP in the gastrointestinal tract at both levels, and ↓Amylase at 2,000 mg/kg in the gastrointestinal tract	Wiszniewski et al. (2019)

(*continued on next page*)

Table 3 (*continued*)

Species	Fish size, g	Experimental diets and protease information [1]	Nutrient digestibility and digestive enzymes activities [2]	Reference
Carnivorous fish				
Black carp (*Mylopharyngodon piceus*)	3.03	0.05%, 0.1%, 0.2%, and 0.3% of protease (neutral protease with 8,000 U/g) included in basal diets	↑ADC_{CP}, and →ADC_{DM} ↑Protease in the hepatopancreas but not in the intestine improved by 1%, 2%, and 3% inclusion of protease in the diets, however, →Amylase in the hepatopancreas and intestine by all the inclusion levels	Chen et al. (2009)
African catfish (*Clarias gariepinus* B.)	10.1	Larvae and fingerlings diets contained 0, 750, 1,000, and 1,250 U/kg protease	↑ADC_{CP} at all supplementation levels	Kemigabo et al. (2019)
Catfish (*Pangasius hypothalamus*)	2.23	Basal diets were supplemented with 0, 2,000, 4,000, 6,000, and 8,000 mg/kg of crude papain	↑ADC_{CP} at all supplementation levels	Rachmawati and Prihartono (2019)
Rainbow trout (*Oncorhynchus mykiss*)	190	Exp. 1: 250 mg/kg Poultrygrow-250 (5,840 U/g) added in coextruded flax:pea mixtures- and canola:pea mixtures-based diets. Exp. 2: 250 mg/kg of the same protease added in dehulled flax-based diets	Exp. 1) ↑ADC_{CP}, ADC_{GE}, and ADC_{DM} in canola:pea-based diets but not in flax:pea diets; Exp. 2) →ADC_{CP}, ADC_{GE}, and ADC_{DM} in dehulled flax-based diets	Drew et al. (2005)
	88.0	1,000 and 2,000 mg/kg of RONOZYME ProAct (75,000 U/g) added to basal diets	→ADC_{CP} and ADC_{CL} at all inclusion levels	Yigit et al. (2016)
	110; 106; 73.0	228 mg/kg protease was added in three kinds of LFD; soybean meal-based, sunflower meal-based, and rapeseed meal-based	↑ADC_{CP}, ADC_{Ash}, ADC_{CL}, ADC_{DM}, and AD_{CP} in soybean meal-based diets, but →ADC_{CP}, ADC_{Ash}, ADC_{CL}, ADC_{DM}, and AD_{CP} in sunflower meal-based and rapeseed meal-based diets;	Dalsgaard et al. (2012)
	110	237 mg/kg protease (a bacterial mono-component) added in LFD	↑Xylose, mannose, and uronic acids	Dalsgaard et al. (2016)
	250	175 mg/kg protease (a protease extracted from bacteria) added in 17 feed ingredients to evaluate the digestibility;	↑ADC_{DM} in feather meal-2 and soybean meal, ↑ADC_{GE} in feather meal-2, single cell protein, and poultry by-product-2, and ↑ADC_{AA} (at least one) among these 17 ingredients	Lee et al. (2020)
Largemouth bass (*Micropterus salmoides*)	31.9	300 and 500 mL/t neutral proteases included in LFD (FM was replaced with cottonseed protein concentrate)	↑Protease at both levels of inclusion in the foregut and hindgut	Guan et al. (2021)
Gilthead sea bream (*Sparus aurata*)	89.5	2,000 mg/kg protease added in LFD (FM was replaced with soybean meal)	→ADC_{CP}, ADC_{DM}, and ADC_{P}, while ↑ADC_{N}	Ayhan et al. (2008)
Olive flounder (*Paralichthys olivaceus*)	5.26	175 mg/kg protease supplemented in LFD	↑Trypsin in the intestine	Bae et al. (2020)
Yellow perch (*Perca flavescens*)	0.59	0.1% pancreatin in basal diets	→Trypsin, chymotrypsin, and pepsin	Kolkovski et al. (2000b)
Crustacean species				
Red swamp crayfish (*Procambarus clarkii*)	8.18	0, 100, 200, 400, 800, and 1,600 mg/kg of protease (20,000 U/g) added to LFD (FM were replaced by plant protein)	↑Protease both in the intestine and hepatopancreas at 200 and 400 mg/kg protease inclusion levels, ↑Amylase in hepatopancreas at all levels and in the intestine at 200 mg/kg, and ↑Lipase in both intestine and hepatopancreas at all levels, except for at 1,600 mg/kg in the intestine	Yang et al. (2022)
Chinese mitten crab (*Eriocheir sinensis*)	0.73	0, 125, 150, and 175 mg/kg AG175™ added in LFD (40 g/kg FM replaced with 20 g/kg soybean meal and 40 g/kg CSM)	↑Trypsin in hepatopancreatic tissues at 150 and 175 mg/kg	Chowdhury et al. (2018)
Pacific white shrimp (*Litopenaeus vannamei*)	2.12	175 mg/kg AG175™ included in LFD (10% FM was replaced with a combination of soybean meal and meat-and-bone meal)	↑ADC_{CP} and ADC_{DM}; ↑Protease in hepatopancreatic tissues, and →Amy and Lip	Yao et al. (2019)
	3.30	175 mg/kg AG175™ included in PSD (reducing FM contents)	↑Protease in the hepatopancreas but not in the intestine	Li et al. (2016)
	1.71	1,000 mg/kg of four kinds of protease (Cenzyme; Enzeco protease 180; Multizyme AK; and Fungal protease) supplemented in basal diets	↑ADC_{CP} in four kinds of protease supplementation diets (in vitro)	Divakaran and Velasco (1999)
	0.33	125, 150, and 175 mg/kg of a commercial protease in LFD (FM was replaced with soybean meal and peanut meal)	↑Trypsin at all inclusion levels, ↑Lipase at 150 and 175 mg/kg inclusion levels, as well as ↑Amylase at 175 mg/kg level of protease	Song et al. (2017)
	2.96	175 mg/kg of AG175™ added to LFD (FM was replaced with soybean meal)	→ ADC_{CP} and ADC_{DM}; ↑Protease in the hepatopancreas, while →Amylase and Lipase in the hepatopancreas	Yao et al. (2017)
	4.55	175 mg/kg of protease (alkaline serine protease) supplemented in basal diets	↑Protease in the intestine and hepatopancreas, and ↑Lipase in the hepatopancreas, meanwhile, →Amylase in the hepatopancreas	Tan et al. (2013)

Reptile				
Chinese pond turtle (*Chinemys reevesii*)	5.70	Adding 0, 150, and 200 mg/kg of protease in basal diets	→Pepsin, amylase, and lipase in the stomach; →Amylase and lipase in the liver and intestine; ↑Trypsin at all supplementation levels in the liver and 150 mg/kg in the intestine, but ↓Trypsin at 200 mg/kg in the intestine	Liang et al. (2019)

'↑' represents a significant rise ($P < 0.05$) compared to the group without protease supplementation; '↓' represents a significant decline ($P < 0.05$) compared to the group without protease supplementation; '→' represents no significant change ($P > 0.05$) compared to the group without protease supplementation.

[1] Abbreviations in the column of 'Experimental diets and protease information': LFD = low fishmeal diets; PSD = protein saving diets; Exp = experiment; FM = fishmeal; CSM = cottonseed meal. Any unmarked information about proteases refers to information that is not explicitly stated in the references.

[2] Abbreviations in the column of 'Nutrient digestibility and digestive enzymes activities': ADC_{CP} = apparent digestibility coefficients of crude protein; ADC_{DM} = apparent digestibility coefficients of dry matter; ADC_{CL} = apparent digestibility coefficients of crude lipid; ADC_{DE} = apparent digestibility coefficients of digestible energy; ADC_{EAA} = apparent digestibility coefficients of essential amino acid; ADC_{Ash} = apparent digestibility coefficients of crude ash; ADC_{Ca} = apparent digestibility coefficients of calcium; ADC_{P} = apparent digestibility coefficients of phosphorus; ADC_{Fe} = apparent digestibility coefficients of iron; ADC_{Cu} = apparent digestibility coefficients of copper; ADC_{GE} = apparent digestibility coefficients of gross energy; ADC_{AA} = apparent digestibility coefficients of amino acid; and ADC_{N} = apparent digestibility coefficients of nitrogen; CP = crude protein; Exp = experiment; FM = fishmeal; CSM = cottonseed meal; Thr = threonine; Val = valine; LAP = leucine aminopeptidase.

structure, which contributed to the thinning of intestinal muscular mucosa (Hassaan et al., 2020; Maryam et al., 2022). It was suggested that this thinning would dwindle the peristalsis speed and consequently prolong digestion and absorption time (Liu et al., 2018). Current research on terrestrial animals confirmed that incorporating moderate protease levels into their diets can enhance the morphology of intestinal mucosa (Cowieson et al., 2017; Ding et al., 2016; Peek et al., 2009; Xu et al., 2017). In aquatic animals, a study on grass carp reported that the height and width of folds in three intestinal segments increased when fed diets including protease (Feng et al., 2023). A study in tilapia elucidated that optimal levels of protease supplementation could enhance the development of villi and improve intestinal morphology (Wu et al., 2020). Similarly, Saleh et al. (2022) observed that 500 mg/kg protease in PSD diets and 250 mg/kg in a diet with optimal protein requirement both could significantly improve intestinal health in Nile tilapia. The health of the intestines could be influenced by the decrease in antinutrient factors (as mentioned in Chapter 3.2) and the release of bioactive peptides through protease hydrolysis of substrates (López-Barrios et al., 2014). The release of peptides or free amino acids facilitates absorption and provides energy for intestinal cells (Gilbert et al., 2008). Contrariwise, a previous study found that supplementing 75 to 600 mg/kg protease in gibel carp diets did not result in any significant changes in the villi length and width of the intestine besides not affecting the growth performance (Liu et al., 2018). The discrepancies in the reported outcomes may be partially explained by variations in the species, protease type, experimental settings, and dietary compositions.

Furthermore, nutrient digestion is aided by digestive enzymes (Wang et al., 2022). Numerous research studies supported the advantageous impact of incorporating dietary proteases in enhancing endogenous digestive enzyme secretion (Table 3). For example, Feng et al. (2023) stated that the inclusion of a moderate amount of protease K in the diets of grass carp can enhance the activities of intestinal brush border enzymes. Also, Maryam et al. (2022) discovered that administering a diet containing 450 mg/kg protease resulted in a significant improvement in the activities of amylase, lipase, and protease in rohu. Nevertheless, it should be emphasized that the incorporation of proteases is not invariably advantageous, as evidenced by several investigations documenting the restricted efficacy of this approach in enhancing certain nutrients' digestibility (Drew et al., 2005; Farhangi and Carter, 2007; Liu et al., 2018; Xu et al., 2022; Yigit et al., 2016). One possible explanation for these findings is that the inclusion level of the exogenous protease was not optimized in these experiments, and as a result, excessive dosages may have made a negative impact on the fish. In addition, feed ingredient composition could potentially affect the efficiency of dietary protease to some extent. Hassaan et al. (2019) revealed that replacing fishmeal diets with cottonseed meals resulted in a decrease in the improvement of ADC_{DM}, ADC_{CL}, ADC_{CP}, and ADC_{GE}, as the replacement ratios increased. Furthermore, Drew et al. (2005) found that protease supplementation could significantly increase ADC_{DM}, ADC_{CP}, ADC_{CL}, and ADC_{GE} of rainbow trout fed with canola-pea-based diets but not flax-pea diets. Interestingly, protease supplemented in the same formula diets with various granulation processes showed different effects on the digestibility of nutrients as well: pelleted diets showed higher ADC_{DM} and ADC_{CP} than the extruded diets (Shi et al., 2016). In the context of optimizing nutrient digestibility, the appropriate level of protease inclusion and diet processing method are critical factors that determine the beneficial results. Moreover, the selection of the appropriate protease type, in accordance with the properties of the protein substrate, is also necessary to achieve optimal outcomes. These considerations are of utmost importance for the development of effective and sustainable aquaculture practices.

3.4. Physiological function

Recently, some studies have confirmed the beneficial effects of proteases on the physiological function of aquatic animals, which may potentially promote growth performance (Table 1). In fish, reductions in blood aspartate aminotransferase (AST) and alanine aminotransferase (ALT) have been reported in Nile tilapia (Hassaan et al., 2019, 2020), gibel carp (Liu et al., 2018), and largemouth bass (Guan et al., 2021), fed with adequate protease supplementation levels. Also, Yang et al. (2022) reported that dietary 200 to 800 mg/kg protease supplementation could significantly decrease the contents of AST and ALT in red swamp crayfish *Procambarus clarkia* fed plant-based diets. Blood ALT and AST are crucial indicators of liver injury in aquatic animals, and their low levels always indicate liver health (Chen et al., 2021, 2022a, 2022b). The aforementioned findings substantiated the assertion that the incorporation of protease in LFD exerted a hepatoprotective effect on aquatic animals. The utilization of LFD, specifically dietary formulations substituting plant-based proteins for fishmeal, has been observed to considerably predispose fish or shrimp to oxidative stress-induced damage. The supplementation of protease has been verified to exert a positive protective effect against oxidative stress-induced damage. For example, a study on gibel carp showed that 500 mg/kg protease supplementation in LFD could significantly increase hepatic total antioxidant capacity (T-AOC), total superoxide dismutase activities (T-SOD), and glutathione peroxidase activities (GPX) as well as intestinal T-SOD, complement 4, and secretory immunoglobulin A contents (Xu et al., 2022). Yang et al. (2022) confirmed that protease supplementation significantly improved the hemolymph biochemical indices and decreased the contents of malondialdehyde (MDA) in red swamp crayfish. Similarly, supplementing the diet of Pacific white shrimp with 175 mg/kg of protease significantly improved the non-specific immune system. This was evident through the augmentation of polyphenol oxidase and T-SOD activities, both in the serum and hepatopancreas (Song et al., 2017). Furthermore, a reduction in the accumulation of hematological MDA of the shrimp challenged with *Vibrio parahaemolyticus* was observed in the study of Song et al. (2017). According to Wu et al. (2020), incorporating protease in diets can enhance serum antioxidant enzyme activities, scavenge free radicals, and regulate the mRNA expression of *tnf-α*, *il-1β*, and *hsp70*, which help protect endothelial cells against stress caused by plant-based diets.

Moreover, studies have reported an improvement in antioxidant capacity and immunoreaction in diets composed of protease-treated proteins in fish and crabs. In grass carp fed PSD, Song et al. (2020) demonstrated that dietary enzymatically hydrolyzed soy protein can alleviate inflammatory responses by regulating the *NF-κB* and *TOR* signaling pathways. In Jian carp *C. carpio* var, Xiao et al. (2019) reported that protease-treated soy protein could decrease MDA and protein carbonyl contents, improve the activities of antioxidant enzymes and glutathione contents, and enhance mRNA expressions of antioxidant enzymes and *Nrf2*. Similarly, treating the fish with diets including soy protein hydrolysates as a replacement for fishmeal at 30% could significantly increase serum T-SOD and T-AOC activities while decreasing the MDA contents in juvenile starry flounder *P. stellatus* (Song et al., 2014). In Chinese mitten crab, dietary inclusion of 30 to 60 g/kg of cottonseed meal protein hydrolysate can significantly enhance the antioxidant capacity and immune response by activating immune-related genes such as *Tolls* and *MyD88* (Cheng et al., 2020).

Among the above indicators, MDA is a crucial marker for assessing oxidative damage in organisms (Chen et al., 2022a). The results consistently indicated that the addition of protease or the inclusion of protease-treated proteins has a reducing effect on MDA levels. The activity of antioxidant enzymes (such as T-SOD, T-AOC, and GPX) has been identified as a pivotal factor in impeding lipid peroxidation and ameliorating oxidative damage. The above passage partially explained the impact of proteases on oxidative damage and inflammatory responses from a molecular regulatory perspective, but it did not effectively elaborate on the specific mechanisms involved. Recent studies primarily focused on the effects of protease on fish growth and nutrient digestibility, with little attention given to its impact on physiological functions. The immune-enhancing function of proteases appears to be attributed mainly to the hydrolysis of protein. These released small molecule substances are believed to have some bioactive peptides possessing antioxidant and anti-inflammatory properties. These peptides exhibit the potential to function as an effective scavenging machine of reactive oxygen species. Certain peptides that are released show properties of metal ion chelation or the reduction of hydroperoxides to protect fish from oxidative stress (Jia et al., 2021). Therefore, there is a need for further studies to critically investigate this.

3.5. Disease resistance

Moreover, protease-treated protein inclusion is reported to improve fish survival rates after parasites or bacterial infections (Khosravi et al., 2018). Resende et al. (2022) reported that 30 g/kg of swine blood hydrolysates in LFD for European sea bass enhanced the fish's resistance against *Tenacibaculum maritimum* infection. Furthermore, feeding European sea bass larvae with protein hydrolysate additives significantly improved the fish's resistance to *Vibrio anguillarum* (Kotzamanis et al., 2007). Another study on the same adult species fed with shrimp protein hydrolysates showed a higher cumulative survival rate when the fish were challenged by an epizootic outbreak of *Vibrio Pelagius* (Gisbert et al., 2018). Similar results were also reported in the adult red sea bream *Pagrus major* fed with shrimp protein hydrolysates (Khosravi et al., 2015), and the juvenile fish fed with rill hydrolysate concentrate (Bui et al., 2014) both after being challenged by *Edwardsiella tarda*. Tuna viscera hydrolysate supplementation in the diets enhanced the resistance to *Streptococcus iniae* infection in pompano *Trachinotus blochii* (Pham et al., 2022) and juvenile barramundi *Lates calcarifer* (Siddik et al., 2018). Furthermore, a reduction in the accumulation of 96 h-cumulative mortality of the Pacific white shrimp challenged with *Vibrio parahaemolyticus* was observed in the study of Song et al. (2017). Therefore, protein hydrolysate incorporation in fish or shrimp diets could serve as a practical nutritional approach to prevent tenacibaculosis and reduce economic losses in aquaculture. Protease-treated proteins always contain some small peptides with biological activities, possessing potential physiological properties beyond normal and adequate nutrition (Fig. 3) (Hartmann and Meisel, 2007; López-Barrios et al., 2014; Udenigwe and Aluko, 2011). The bioactive peptides contained in proteins subjected to enzymes may be important components that enhance the disease resistance of animals. To date, numerous studies have explored the functional benefits of bioactive peptides derived from protein sources, such as soybean meal (Singh et al., 2014), cottonseed meal (Kumar et al., 2022), rapeseed meal (Ma et al., 2022), and marine or poultry by-product protein (Nirmal et al., 2022; Romero-Garay et al., 2022). In detail, Duan et al. (2021) confirmed that rapeseed protein was a good potential source of antimicrobial peptides (AMPs) using in silico approach. An article on multiple meta-analyses has demonstrated the beneficial effects of AMPs added to diets on aquatic animal health and body function (Wang et al., 2023). As gathered above, these bioactive peptides contribute to the health status of farmed animals, which also become the biggest selling point for the promotion of enzymatically hydrolyzed proteins in the feed industry (Singh et al., 2014).

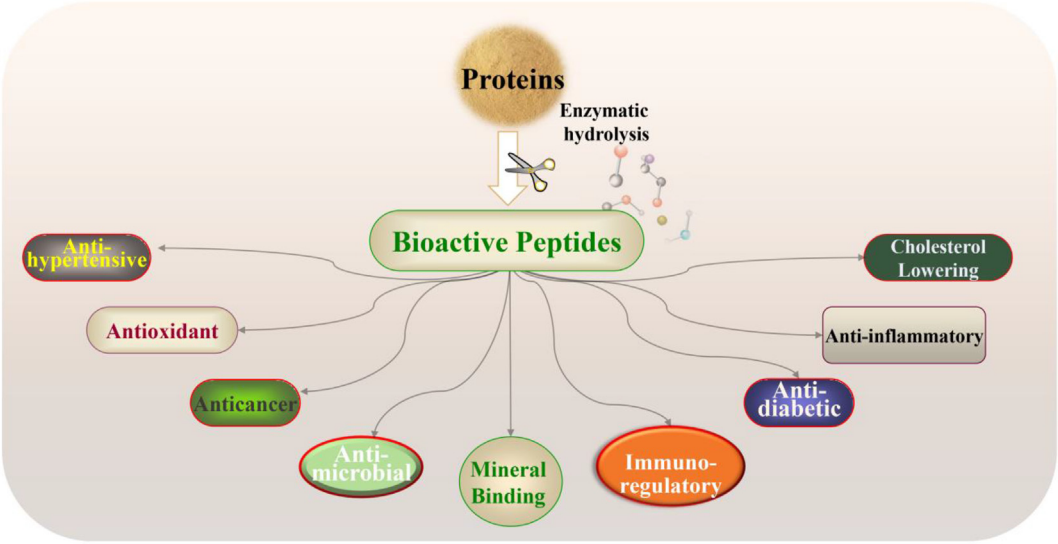

Fig. 3. The summary of functions related to bioactive peptides released from feed protein sources under protease treatment. Source: Singh et al. (2014).

3.6. Others

This section provides a primary exposition on the impact of proteases on the gut microbiota, behavioral response, water quality, flesh quality of aquatic animals, and economic effectiveness. Given the scarcity of available literature, the information presented in this section is condensed. The main objective is to incite further scholarly inquiry in this captivating domain.

Several studies have reported the positive impact of incorporating dietary protease on the intestinal microflora of various aquatic organisms (Adeoye et al., 2016; Dai et al., 2019; Hassaan et al., 2021; Zhu et al., 2022). For example, a study on Pacific white shrimp reported a significant increase in the abundance of dominant microflora such as Bacteroidetes, Proteobacteria, and Actinobacteria (Zhu et al., 2022). Gut microorganisms have been considered biosensors for the nutritional health of fish, by helping in absorption and improving the immune system (Hassaan et al., 2021). Proteases altered the gut microbiota and subsequently impacted metabolism and immunity, warranting further research.

An investigation into the behavioral response of common carp found that fish given dietary papain treatments had higher movement and activity, as well as a more intense feeding response (Tewari et al., 2018). In terms of water quality, Saleh et al. (2022) found that including 250 mg/kg of protease in Nile tilapia diets, with a typical protein requirement of 30% CP, contributed to a significant reduction in both ammonia and nitrite concentrations in aquaria water. Likewise, the addition of pineapple waste extract to the diets of tilapia, serving as a source of bromelain, resulted in a considerable reduction in the levels of free ammonia and total nitrogen in the culture water (Yuangsoi et al., 2018). Commercial exogenous enzymes composed of xylanase, alpha-amylase, acidic proteinase, and neutral-proteinase, have also been shown to present ecological benefits by reducing ammonia in aquaculture systems (Hassan et al., 2017). Furthermore, Islam et al. (2021) noted a decrease in the levels of phosphate, ammonia, nitrate, and nitrite in the environment of striped catfish (*Pangasianodon hypophthalmus*) that were fed a diet containing 500 mg/kg pepsin. The enhancement of water quality is largely credited to the improved utilization of nitrogen or phosphorus in feed, which can be linked to the addition of protease. Further evidence supporting this can be found in the previous section. However, Saleh et al. (2022) did not observe any significant improvement in water quality when protease was supplemented at 500 mg/kg in groups fed with PSD. A study by Tewari et al. (2018) also did not demonstrate a decrease in water NH_3 in which common carp fed with a conventional diet supplemented with papain was cultured. Hence, it can be inferred that the treatments of PSD or optimal protein requirement significantly curtail nitrogen emissions, thereby limiting the potential impact of protease on water quality improvement.

One study on grass carp reported that protease-treated soy protein supplementation in PSD could significantly improve the flesh tenderness, juiciness, and flavor of grass carp (Song et al., 2019). Good sensory quality always stimulates purchase intentions in consumers, and several studies were conducted to explore feasible strategies to improve flesh sensory quality presently (Tang et al., 2022; Yang et al., 2021). It appears that protease-treated protein supplementation in diets as a nutritional strategy to enhance the flavor of cultured fish is worthy of more research. In food science, food can be processed by enzymatic hydrolysis to improve its freshness and acceptability for human beings (Kang et al., 2019; Selamassakul et al., 2020). Providing fish with high-quality and appealing feed results in a dual advantage of yielding delectable fish fillets while promoting fish optimal growth and development.

Multiple studies have investigated the economic effectiveness of protease-enriched diets (Goda et al., 2020; Mo et al., 2020a, 2020b). For instance, one study conducted by Goda et al. (2020) explored the application of HPDDG as a replacement for soybean meal in European seabass diets. The finding indicated that supplementing the 50% HPDDG diet with protease resulted in the lowest total cost of feed per kilogram of fish gain. Similarly, Mo et al. (2020b) conducted a study on gold-lined seabream *Rhabdosargus sarba*, where they investigated two experimental diets with 30% and 60% fishmeal replaced by soybean dregs (SBD). The 30% and 60% fishmeal-replaced diets were supplemented with 0.5% papain (Exp. 1), while both a fishmeal-based diet and the 30% fishmeal-replaced diet were supplemented with 1.3% bromelain (Exp. 2). The results showed that the experimental diet with 60% SBD and papain (Exp. 1) reduced the cost of fish per kilogram by 31.8% compared to the 0% SBD diet. Furthermore, both groups with bromelain (Exp. 2) had a cost reduction of 27.0% and 46.7% compared to fishmeal-based diets. Mo et al. (2020a) stated that supplementing food waste-based

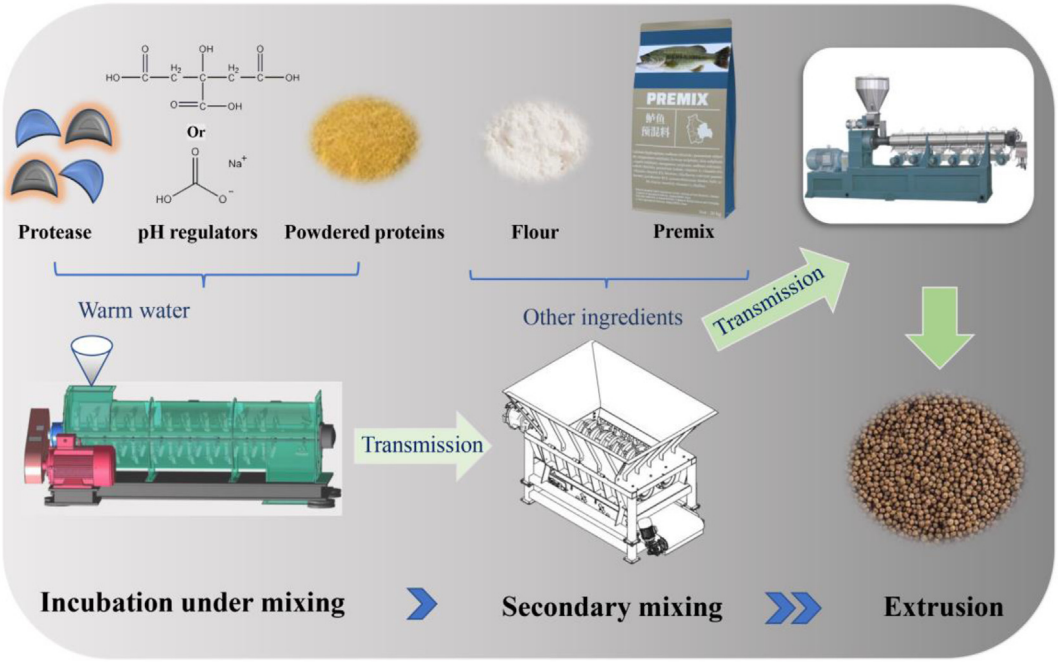

Fig. 4. Flow chart of incorporating the protease treatment into the real-time online processing of fish compound feed. The stages include: 1) Mixing proteins with water, protease, and pH regulators, followed by stirring and incubation of the mixture; 2) Direct transfer of the enzymatic hydrolysis products to the next mixer after incubation, along with other powdered and pre-mixed materials; 3) Conveying the mixed material into the flow buffering bin before transferring it to the puffing machine for feed production.

diets for grass carp with a combination of papain and bromelain is a viable nutritional strategy.

4. Conclusions and future perspective

Protease additives succeed in increasing the digestibility of feed nutrients, boosting fish growth performance, enhancing health status, and improving the aquaculture environment. Moreover, plenty of undesirable protein sources could be hydrolyzed by it to become high-quality protein raw materials with small molecular weight, high digestibility, low ANFs, and more bioactive peptides.

As feed additives, some critical bottlenecks may hinder its application in aquatic feed. One of them is their requisite incorporation into the feed components, in a manner that averts denaturation and inactivation during processing, such as extrusion, drying, and subsequent storage, or alternatively, facile solubilization and elimination via a straightforward top-coating process upon administration to the culture pond, particularly when the feed is not quickly consumed (Boyd et al., 2020). Another is fluctuant enzyme activities as the activities of endogenous digestive enzymes in fish are significantly affected by the temperature of the environment compared to homeothermic animals (Hashemi et al., 2018; Ma et al., 2019). It must be emphasized that accurate determination of the protease inclusion level is an important aspect of ensuring the efficiency of exogenous proteases in fish feeding. Low-dose supplementation consistently yielded no discernible effects, whereas high-dose supplementation could potentially exert adverse consequences on both the intestinal health and growth performance of cultivated fish (Liu et al., 2018; Wu et al., 2020). Therefore, more research on estimating the requirement for specific proteases of specific aquaculture species at different water temperatures should be carried out. Additionally, the pH of the digestive tract or stomach of fish is subject to variability among species and changes throughout development, which would also impact the effectiveness of protease additives. Nevertheless, advanced biotechnology holds promise in providing solutions to enhance the stability of proteases (Dotsenko et al., 2021; Navone et al., 2021). For instance, Su et al. (2022) successfully applied a combinatorial strategy via bioinformatics analysis and rational design to enhance keratinase thermostability for efficient biodegradation of feathers. Some polymers employed as protein or drug carriers can potentiate the efficiency of exogenous enzymes in medical and feed industries (Liang et al., 2022; Ye and Chi, 2018). One study has suggested that encapsulated shrimp enzymes with alginate–bentonite capsules contributed to a 27% higher enzyme activity when compared with the control diet (Rodriguez et al., 2018). Therefore, further research is needed on techniques that guarantee proper enzyme immobilization (Rodriguez et al., 2018).

For protease-treated proteins, pretreatment may negatively impact feed properties, including microbial contamination and final pellet characteristics such as firmness and texture (Liang et al., 2022). Moreover, the processing requirements of these commodities typically involve dehydration, comminution, packaging, and conveyance to feed manufacturers, inevitably leading to a surge in carbon footprint. Hence, optimizing the enzymatic hydrolysis conditions in a targeted manner is necessary. Also, future studies are required to adopt a more environmentally-friendly method, using real-time online enzymatic hydrolysis, so that the enzymatic hydrolysis products can pass directly into the subsequent granulation process. Prior research on phytase, another feed enzyme, showed that its inclusion in the real-time online processing of compound feed was effective in enhancing mineral availability for Atlantic salmon (*Salmo salar* L.) raised in low-temperature environments (Denstadli et al., 2006, 2007). Our recent work (Xue et al., 2022), published as a patent in China (CN 113005030 B), also confirmed that this method shows high efficiency and low consumption. The online processing operation can be broadly divided into three stages, which are illustrated in Fig. 4.

Protease preparations utilized in the feed industry are predominantly generated via microbial fermentation. Each stage of the enzymatic production process can substantially influence the safety of the resultant enzymes. As a result, the monitoring of microbial

strains is essential to ensure the effectiveness and safety of enzyme products. Additionally, harmful microorganisms and heavy metal pollutants can present significant risks to the safety of enzyme products. Therefore, it is crucial to strengthen the standardization of microbial and enzyme preparations.

Author contributions

Shiyou Chen: Conceptualization, data curation, investigation, methodology, validation, visualization, and writing-original draft. **Sahya Maulu:** Writing-original draft, and revising the manuscript critically for important intellectual content. **Jie Wang:** Review. **Xiaoze Xie:** Validation, visualization. **Xiaofang Liang:** Investigation. **Hao Wang:** Methodology. **Junjun Wang:** Editing. **Min Xue:** Conceptualization, supervision, writing-review and editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Data availability statement

All data generated or analyzed during this study has been cited in the article or included as supplementary information.

Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.11.001.

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