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REVIEW

Nutritional implications of exogenous proteases in fish feeding

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Abstract - The increase in the demand for fish production drives the search for food strategies to maximize productivity. In this review, the nutritional implications of the use of exogenous proteases in fish growth are described. Exogenous proteases help in digestive processes, acting in the hydrolysis of macromolecules and in the release of smaller particles, such as amino acids and peptides. Dietary supplementation improves fish growth, mainly because of the availability and greater use of nutrients. The action of proteases is directly linked to better intake, feed efficiency and protein synthesis. On the other hand, enzyme activity depends on the substrate and dietary composition as there are limitations on digestibility. Furthermore, in feed processing, thermal stability and the form of inclusion of the protease influence its efficiency. However, the increase in fish weight gain can offset the cost of including the enzyme in the diet. However, there are still gaps regarding the effects of protease in fish feeding, for example, enzyme: specific substrate and enzyme: digestive system ratio; and, stabilization technologies (mainly extruded diets), so further studies are needed.

Keywords: Antinutrients. Growth. Enzyme. Nutrition. Fish farming.

Implicações nutricionais de proteases exógenas na alimentação de peixes

Resumo - O aumento na demanda da produção de peixes impulsiona a busca por estratégias alimentares a fim de maximizar a produtividade. Nesta revisão foram descritas as implicações nutricionais do uso de proteases exógenas sobre o crescimento dos peixes. As proteases exógenas auxiliam nos processos digestivos atuando na hidrólise de macromoléculas e na liberação de partículas menores, como aminoácidos e peptídeos. A suplementação dietética melhora o crescimento dos peixes, principalmente pela disponibilidade e maior aproveitamento dos nutrientes. A ação de proteases está ligada diretamente a melhor ingestão, eficiência alimentar e síntese de proteína. Por outro lado, a atividade da enzima depende do substrato e da composição dietética já que há limitações na digestibilidade. Além disso, no processamento de rações, a estabilidade térmica e a forma de inclusão da protease influenciam na sua eficiência. Contudo, o aumento no ganho em peso dos peixes pode compensar o custo de inclusão da enzima na dieta. No entanto, ainda existem lacunas quanto aos efeitos da protease na alimentação de peixes, como exemplos, relação enzima: substrato específico e enzima: sistema digestório; e, tecnologias de estabilização (principalmente, dietas extrusadas), por isso, são necessários estudos adicionais.

Palavras-chave: Antinutrientes. Crescimento. Enzima. Nutrição. Piscicultura.

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Introduction

Aquaculture is a significant activity for the world's food supply, being recognized as the fastest growing agribusiness sector. In 2020, this sector kept production in line with market demand, guaranteed supply and provided record exports (BRAZILIAN FISH FARMING ASSOCIATION - PEIXE BR, 2021). Worldwide, fish is the most consumed animal protein in human food. The increase in consumption is stimulated by increased production and improved distribution channels, associated with population growth, urbanization, and rising incomes (FAO, 2018; 2020).

In 2018, aquaculture grew by 3.2% over the previous year, with fish production accounting for 46% (82.1 million tons) of the world's aquaculture (FAO, 2020). The increase in production is accompanied by an increase in demand for ingredients and feed. The choice of ingredients for the acquisition of rations depends mainly on the feeding habit of the species (PORTZ; FURUYA, 2012). Carnivorous fish require diets with a higher amount of protein and use of animal sources in the diets when compared to herbivorous and omnivorous fish. This difference is directly related to the morphology of the digestive tract of each species (RODRIGUES *et al*., 2012).

The obstacles in the fish feeding indirectly stimulate the search for nutritional strategies that improve the use of these rations, notably, protein, which is one of the most important and most expensive nutritional fractions. The low availability and high cost of animal sources means that the amount of these sources in the rations is reduced and plant sources and/or by-products are used in fish feeding. Plant sources contain antinutrients that can impair fish growth (FRANCIS; MAKKAR; BECKER, 2001). On the other hand, the use of exogenous enzymes can improve the nutritional growth of animals, in addition to optimizing the cost/benefit ratio of feeding, survival and growth of animals (CASTILLO; GATLIN, 2015; KEMIGABO *et al*., 2019; KUMARI *et al*., 2013).

Enzymes are biological catalysts that accelerate biochemical reactions using alternative pathways, this allows for greater availability of nutrients for absorption by the body, which under normal conditions would not occur (GOMES *et al*., 2019). In animal production, commercial enzymes began to be used in the 1990s. Since then, animal production associated with biotechnology has advanced in levels, sources, efficacy and substrate specificity (BOYD *et al*., 2020). The presence of enzymes allows for changes in the plant base and greater inclusion of dietary ingredients (HASSAAN *et al*., 2019; SALEH *et al*., 2021). In fish feed, the most used enzymes are proteases, lipases, phytase and carbohydrates (CASTILLO; GATLIN, 2015; ZHENG *et al*., 2019).

Exogenous proteases can compensate for the deficiency of endogenous enzymes and help break down macromolecular proteins that are difficult to digest (SALEH *et al*., 2021; SHI *et al*., 2016). The use of exogenous proteases has allowed the reduction of protein and fish meal levels in diets for Nile tilapia (*Oreochromis niloticus*) (LIU *et al*., 2016; RAGAA *et al*., 2017; SALEH *et al*., 2021) and prussian carp (*Carassius auratus gibelio*) (SHI *et al*., 2016). Nutritional implications were also observed in rainbow trout (*Oncorhynchus mykiss*), prussian carp (*C. auratus* gibelio) and Nile tilapia (*O. niloticus*) as better feed efficiency, higher apparent protein digestibility coefficient and higher growth, respectively (DREW *et al*.,

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2005; GODA *et al*., 2020; LIU *et al*., 2016).

There are few studies on the use of exogenous proteases in fish diets compared to other enzymes such as phytase and carbohydrase (BOYD *et al*., 2020). Thus, the objective was to compile existing studies and describe the nutritional implications of exogenous proteases on fish growth responses. In addition to pointing out issues that have not yet been elucidated and the additional studies necessary for an adequate use of proteases in the fish diet.

Exogenous enzymes used as fish additives

Enzymes are proteins that accelerate reactions and break the bonds between molecules, providing nutrients and allowing greater action of digestive enzymes on a given substrate (GOMES *et al*., 2019). In animal production, the use of commercial enzymes started in the 1990s, consequently, this technology has advanced a lot in terms of levels, sources, efficacy, and substrate specificity (BOYD *et al*., 2020). The main categories of enzymes used in fish feed include proteases; lipases; phosphatases (phytase) and carbohydrases (xylanase, β-glucanases, etc). In addition to improving the availability of nutrients, they are also considered nutritional additives that improve the utilization of low-quality food in the diet of aquatic animals (HASSAAN *et al*., 2019).

Anti-nutritional factors

Anti-nutritional or anti-nutrient factors are components present in plant ingredients, cereals and legumes, which can hinder the activity of digestive enzymes and the absorption of nutrients from food (FRANCIS; MAKKAR; BECKER, 2001; LAZZARI *et al*., 2010). Antinutrients include trypsin inhibitors, hemagglutinating agents, phytic acid, gossypol, alkaloids, thiaminase, among others. These antinutrients are present in plant ingredients commonly used in fish nutrition, such as soybean meal, canola meal and sunflower meal. When inactivated by heat treatment, antinutrients can reduce amino acid availability and protein digestibility by fish (NATIONAL RESEARCH COUNCIL - NRC, 2011; HARDY; BARROWS, 2002). However, there are other strategies to eliminate and/or minimize the effects of these components, such as selective breeding, genetic modification, or through supplementation (enzyme, mineral, etc.) (FRANCIS; MAKKAR; BECKER, 2001). As observed in recent studies, diets with a high inclusion of plant ingredients resulted in fish with better performance, immune system, digestibility, intestinal microbiological community and increased bioavailability of essential amino acids when supplemented with exogenous protease (GODA *et al*., 2020; HASSAAN *et al*., 2019; ZHENG *et al*., 2019). Thus, the hydrolysis of protein into individual amino acids and peptides during digestion is believed to be the main function of proteases (BOYD *et al*., 2020).

Exogenous proteases

Proteases perform various biological functions in homeostasis, apoptosis, signal transduction, reproduction and immunity. They account for more than 50% of global enzyme production, however, in animal production, they represent only 5% of the global feed enzyme market. Most studies include the protease within a cocktail, making it difficult to assess the enzyme's direct impacts on animal performance (BOYD *et al*., 2020).

Exogenous proteases are produced from bacteria (strains of *Bacillus* sp.), fungi (genus *Aspergillus* sp.) and yeasts (LI *et al*., 2013). Its stability in fish diets is mainly dependent on its pH specificity and thermolabile nature (KUMARI *et al*., 2013; ZHENG *et al*., 2019). Therefore, materials are used from mesophilic organisms and techniques such as amino acid modification and metal bonding to ensure enzymatic stability (LI *et al*., 2013; YEO; BAEK; PARK, 2001). However, the stability of the biomolecule in the bioconjugate material can perform the necessary functions efficiently (GOLE *et al*., 2001). Since the molecular recognition mechanism is characterized by the specificity of an enzyme in identifying and interacting with the exact substrate, through chemical affinity (ZHENG *et al*., 2019).

The food bolus substrate consists of the undigested fraction that serves as food for microbial fermentation in the intestine of animals, and its increased amount can cause digestive disorders. Exogenous protease in the diet can aid and compensate for the activity of digestive enzymes, so that macromolecular protein can be solubilized and hydrolyzed into low molecular peptides, peptones and various amino acids, available to be digested and/or absorbed, reducing the amount of substrate (SHI *et al*., 2016).

The level of protease inclusion in diets is influenced by many factors, mainly diet composition and digestive system. Few studies are related to the problems with the high inclusion of exogenous enzymes in the diet, but the unregulated use of these enzymes can cause damage to the intestinal mucosa and, consequently, induce negative effects on growth (KUMARI *et al*., 2013; LIU *et al*., 2016).

Nutritional implications of using exogenous proteases

The addition of exogenous protease to diets containing protein sources improves fish growth (GODA *et al*., 2019; HASSAAN *et al*., 2019; SALEH *et al*., 2021). Endogenous and exogenous enzymes can act in different ways on fish performance. Exogenous proteases can: (I) break down complex proteins and make amino acids and peptides available; (II) increase endogenous peptidase production; (III) be related to increased food intake; (IV) improve feed conversion; (V) reduce the effects caused by antinutrients present in plant protein sources; (VI) increase the protein efficiency rate, through protein consumption; (VII) decrease the use of fish meal.

Effects on growth

The growth of fish fed diets containing exogenous protease is related to higher feed intake and improved feed conversion and feed efficiency (Table 1) (GODA, *et al*., 2019; HASSAAN *et al*., 2019; SHI *et al*., 2016). Enzymes can directly infer the rate of absorption of nutrients in the gastrointestinal tract (DEBNATH *et al*., 2005; GODA *et al*., 2012). After digestion, amino acids are absorbed and transported through the hepatic portal vein from the intestine to the liver and rapidly metabolized (PORTZ; FURUYA, 2012). The rate of absorption and concentration of free plasma amino acids varies with dietary ingredients. For channel catfish (*Ictalurus punctatus*) fed diets containing soybean meal and fish meal, free amino acid concentrations peaked at 12 hours after feeding (AMBARDEKAR; REIGH; WILLIAMS, 2009). For rainbow trout (*O. mykiss*) fed soybean meal and malt protein meal, the peak amino acid concentration was 21 hours after feeding (YAMAMOTO; UNUMA; AKIYAMA, 1998). However, proteases can hydrolyze protein complexes from lower quality protein sources and promote the use of nutrients from the release of amino acids and peptides, to stimulate food intake and improve feed efficiency as observed in Nile tilapia (*O. niloticus*) (RAGAA *et al*., 2017; SALEH *et al*., 2021), sea bass (*Dicentrarchus labrax* L.) (GODA *et al*., 2019), black carp (*Mylopharyngodon piceus*) (CHEN *et al*., 2009) and rainbow trout (*O. mykiss*) (DREW *et al*., 2005).

The mechanism that improves food consumption in fish fed diets supplemented with enzymes still needs to be studied and explained. Diets containing distiller dry grains in place of soybean meal and exogenous protease (1000 mg kg^{-1}) showed lower food intake compared to fish fed the control diet (without distillers dry grains and exogenous protease) (GODA *et al*., 2019). In contrast, fish fed diets containing exogenous protease resulted in higher food intake (CHEN, 2009; KEMIGABO *et al*., 2019; RAGAA *et al*., 2017; SALEH *et al*., 2021). However, there are two possible explanations for the improvement in food consumption: (I) increased palatability of diets since diets containing exogenous proteases can make dietary amino acids available (HASSAAN *et al*., 2019) and release more free amino acids plasma levels and, consequently, stimulate food intake; (II) greater digestibility of nutrients, diets containing exogenous protease may result in a faster passage of ingested food through the digestive system, and accelerate the return of appetite, potentially promoting greater food intake (DEBNATH *et al*., 2005).

The biological and dietary characteristics of the species influence the growth response from a diet containing exogenous protease. The comfort temperature of the species affects the speed of nutrient digestion. Cold water fish such as rainbow trout (*O. mykiss*) result in longer digestion time compared to warm water fish such as channel catfish (*I. punctatus*) (AMBARDEKAR; REIGH; WILLIAMS, 2009). In addition, the slower digestion rate and absorption of plant ingredients may be related to the digestive processes and the delay in the evacuation of the system since omnivorous fish fed diets containing plant protein sources showed better protein utilization (LARSEN; DALSGAARD; PEDERSEN, 2012). On the other hand, carnivorous fish tend to evacuate the digestive system more quickly, thus having a lower ability to adapt the digestive system to dietary changes compared to omnivorous fish (PORTZ; FURUYA, 2012). Although exogenous proteases act on the use of nutrients and on performance parameters, the specific characteristics of the species must be considered during the formulation of diets.

Carnivorous fish demand diets with higher protein content and ingredients of high biological value compared to omnivorous and herbivorous fish (PORTZ; FURUYA, 2012). Fish feed represents more than half of the total production costs, and, among nutrients, protein is one of the most expensive (FAO, 2018). Studies focused on the addition of exogenous proteases in the diet of carnivores exert direct action on the appeals of sustainability. The use of the enzyme in the diet of carnivorous species results in improved performance parameters and greater digestibility of nutrients (DREW *et al*., 2005; FARHANGI; CARTER, 2007; GASCO *et al*., 2016; SOARES *et al*., 2008; YIGIT *et al*., 2018; LEE *et al*., 2020), greater retention of nitrogen and phosphorus (OGUNKOYA *et al*., 2006) and positive effects on health status (GODA, *et al*., 2019), in addition to allowing the use of lower cost vegetable protein sources compared to fish meal (DALSGAARD *et al*., 2012, 2016; FARHANGI; CARTER, 2007).

Table 1. Effects on growth of the addition of exogenous protease in diets with plant-based ingredients in fish feed.

Specie	Protease inclusion (mg kg^{-1})	Results	Reference
Prussian carp (*C. auratus gibelio*)	400[a]	↑ SGR ↓ FC ↑ PER	LIU *et al.* (2016)
Nile tilapia (*O. niloticus*)	250[b]	↑ WG ↑ SGR ↓ FC ↑ PER ↑ FI	SALEH *et al.* (2021)
Black carp (*M. piceus*)	1000, 2000 e 3000[c]	↑ WG ↓ FC ↑ FI	CHEN *et al.* (2009)
Peacock bass (*Cichla* sp.)	100[d]	↑ WP ↑ SGR ↓ FC	SOARES *et al.* (2008)
Nile tilapia (*O. niloticus*)	500[e]	↑ WG ↓ FC ↑ PER	HASSAAN *et al.* (2019)
Prussian carp (*C. auratus gibelio*)	150 e 175[f]	↑ WG ↓ FC ↑ PER	SHI *et al.* (2016)
Tilapia (*O. niloticus* × *O. aureus*)	175[g]	↑ WG ↓ FC	LI *et al.* (2015)
Nile tilapia (*O. niloticus*)	200 e 400[h]	↑ WG ↑ SGR ↓ FC ↑ PER ↑ FI	RAGAA *et al.* (2017)
Rainbow trout (*O. mykiss*)	250[i]	↓ FC	DREW *et al.* (2005)
Sea bass (*D. labrax* L.)	1000[j]	↑ WG ↑ SGR ↓ FC ↑ PER ↑ FI	GODA *et al.* (2019)
African catfish (*Clarias gariepinus*)	1100[k]	↑ WG ↑ FI ↓ FC	(KEMIGABO *et al.*, 2019)

[a]coated neutral protease (Kemin Industries Zhuhai Co., Ltd.); [b]protease (600.000 U g^{-1}, Novus Company, USA); [c]neutral protease (8000 U g^{-1}, Zhiwei); [d]fungal protease (Alltech Brazil); [e]protease (5000 U g^{-1}, Huvepharma, Antuérpia, Belgium); [f]alkaline protease (AG175™, JEFO Nutrition, Inc. Saint-Hyacinthe, Quebec, Canada); [g]alkaline protease (JEFO Nutrition, Inc. Saint-Hyacinthe, QC, Canada); [h]protease (Ronozyme ProAct™, DSM Nutrition Products, SP, Poland); [i]protease (Domestic poultry-250™; JEFO Nutrition, Inc. St.-Hyacinthe, QC); [j]protease (PROXYM ULTRA5®, Gloray Vet COMPANY); [k]protease (Kemin Industries (Zhuhai) Co. Ltd., China). Legend: Weight gain (WG); Specific growth rate (SGR); Feed conversion (FC); Protein efficiency rate (PER); Food intake (FI); Higher (↑); Lower (↓).

Diet composition influences the performance responses of fish fed exogenous proteases. A level of dietary protein that exceeds the need for maintenance and growth, results in negative effects on growth in fish fed diets containing exogenous protease (ADEOYE *et al.*, 2016a; GODA, *et al.*, 2019; SHI *et al.*, 2016). On the other hand, the 2% reduction of crude protein in the diet containing fish meal, soybean meal and exogenous protease (200 and 400 mg kg^{-1}) in Nile tilapia (*O. niloticus*) feed maintained the performance parameters and improved the use of protein (RAGAA *et al*., 2017). Also, positive effects on the performance of the species in question were observed when fed with a diet containing 1% reduction of crude protein and exogenous protease (500 mg kg^{-1}) (SALEH *et al.*, 2021). The prussian carp (*C. auratus gibelio*) showed a higher specific growth rate and lower feed conversion when fed a diet containing 2% reduction in crude protein and exogenous protease (300 mg kg^{-1}) (LIU *et al*., 2016). In summary, exogenous proteases work best in diets with less protein and when the composition contains lower quality ingredients.

Exogenous proteases may not result in effects on fish performance parameters (DALSGAARD *et al*., 2012; DREW *et al*., 2005; FARHANGI; CARTER, 2007; YIGIT *et al*., 2018). Part of these results is directly related to the formulation, composition, characteristics, and level of inclusion of the enzyme, quality of protein sources and processing of diets (GODA *et al*., 2019; LI, *et al.*, 2015; SHI *et al*., 2016). Ingredients containing antinutrients interfere with protease activity (DREW *et al.*, 2005; HASSAAN *et al.*, 2019). Thus, an alternative is the removal of antinutrients and the inclusion of exogenous protease in the diet to assess the effect on fish growth.

Effects on nutrient digestibility

The presence of exogenous proteases in fish feed provides greater digestibility of nutrients, especially protein (Table 2) (FARHANGI; CARTER, 2007; RAGAA *et al.*, 2017; SHI *et al*., 2016; KEMIGABO *et al.*, 2019). Greater nutrient use is directly related to the release of amino acids and peptides in the digestive system. On the other hand, nutrient use can be impaired by the presence of antinutrients, as observed in sea bass (*D. labrax* L.) fed diets containing insect meal and exogenous protease (200 mg kg^{-1}) (GASCO *et al*., 2016) and in rainbow trout (*O. mykiss*) fed diets containing canola and pea seeds and exogenous protease (250 mg kg^{-1}).

Nutrient digestibility depends on the activity of endogenous and exogenous proteases in the digestive system. The pH of the gastrointestinal tract of fish has a direct influence on the activity of proteases (DABROWSKI; GLOGOWSKI, 1977). Acid proteases, such as pepsin, act according to their name, in an acidic environment, in the stomach of fish, while neutral and/or alkaline proteases, trypsin and chymotrypsin, act in neutral and/or alkaline portions of the intestine. In fish without a stomach, initial digestion is carried out by pancreatic alkaline trypsin (BALDISSEROTTO, 2013). Nile tilapia (*O. niloticus*) fed plant diets with the addition of exogenous enzymes (protease, β-glucanase and xylanase) increased the secretion of endogenous protease (trypsin) in the digestive system and greater protein retention (LIN; MAI; TAN, 2007). According to Francis; Makkar; Becker (2001), the presence of antinutrients (trypsin inhibitors) in the diet may not cause negative effects on performance, as these effects can be offset by the synthesis of endogenous proteases in the

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fish digestive system. In addition, the greater activity of endogenous enzymes in the gastrointestinal tract is related to the quality of the protein of the ingredients, since the silver catfish (*Rhamdia quelen*) fed with animal meal showed greater activity of endogenous proteases and greater weight gain, when compared to fish fed diets based on soybean meal and yeast (LAZZARI *et al*., 2010). However, it is observed that more concentrated ingredients that present proteins of high biological value and better nutritional quality require greater activity of endogenous proteases and tend to provide growth, consequently, the activity of exogenous proteases is lower, this is because these ingredients are highly digestible by fish.

Table 2. Effects on nutrient digestibility by the addition of exogenous protease in diets with plant ingredients in fish feed

Specie	Protease inclusion (mg kg^{-1})	Results	Reference
Nile tilapia (*O. niloticus*)	200 e 400[a]	↑ ADC CP	RAGAA *et al.* (2017)
Sea bass (*D. labrax* L.)	200[b]	↓ ADC DM; PC and ADF	GASCO *et al.* (2016)
Rainbow trout (*O. mykiss*)	250[c]	↑ ADC DM; GE; CP and fat	DREW *et al.* (2005)
Rainbow trout (*O. mykiss*)	300[d]	↓ ADC DM; GE and fat ↑ ACD CP	FARHANGI; CARTER (2007)
Black carp (*M. piceus*)	1000, 2000 e 3000[e]	↑ ADC CP	CHEN *et al.* (2009)
Nile tilapia (*O. niloticus*)	500[f]	↑ ADC CP, DE; and fat	HASSAAN *et al.* (2019)
Prussian carp (*C. auratus gibelio*)	150 e 175[g]	↑ ADC DM and CP	SHI *et al.* (2016)
Tilapia (*O. niloticus* × *O. aureus*)	175[h]	↑ ADC DM and CP	LI *et al.* (2015)
Truta arco íris (*O. mykiss*)	175[i]	↑ ADC DM and AA	LEE *et al.* (2020)
African catfish (*C. gariepinus*),	1100[j]	↑ ADC CP	(KEMIGABO *et al*., 2019)

[a]protease (Ronozyme ProAct ™, DSM Nutrition Products, SP, Poland); [b]protease (Ronozyme ProAct™, DSM, Heerlen, Netherlands); [c]protease (Domestic poultry-250™; JEFO Nutrition, Inc., St. Hyacinthe, QC); [d]protease (Bio-Feed™ Pro, Novo Nordisk, Bagsvaerd, Denmark); [e]neutral protease (8000 U g^{-1}, Zhiwei); [f]protease (5000 U g^{-1}, Huvepharma, Antwerp, Belgium); [g]alkaline protease (AG175™, JEFO Nutrition, Inc. Saint-Hyacinthe, Quebec, Canada); [h]alkaline protease (JEFO Nutrition, Inc. Saint-Hyacinthe, QC, Canada); [i]protease (JEFO Nutrition, Inc., Quebec, Canada); [j]protease (Kemin Industries (Zhuhai) Co. Ltd., China). Apparent digestibility coefficient (ADC); Crude protein (CP); Dry matter (DM); Acid detergent fiber (ADF); Gross energy (GE); Digestible energy (DE); Amino acids (AA); Higher (↑); Lower (↓).

The efficiency of exogenous protease activity is related to the development of the mucosa and intestinal structures of fish, as the increase in villi allows a greater capacity for nutrient absorption and, indirectly, stimulates the activity of endogenous enzymes and, consequently, promotes growth (ADEOYE *et al*., 2016a; GODA *et al*., 2019; KUMARI *et al*., 2013; ZHANG *et al*., 2012). Fish fed diets containing exogenous protease presented villi with greater height and surface area and goblet cells distributed along the villi,

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indicating a better morphological state of the intestine (ABD ELNABI *et al*., 2020; KUMARI *et al*., 2013; SALEH *et al*., 2021; WU *et al*., 2020). However, the hormone cholecystokinin inhibits stomach acid secretion and stimulates the secretion of endogenous enzymes by the pancreas into the intestinal lumen (BALDISSEROTTO, 2013). While the secretin hormone increases the release of pancreatic alkaline secretion. Possibly, the availability of nutrients and the improvement of the morphological state of the intestine induce the hormone cholecystokinin to secrete endogenous enzymes, since most of the digestion of food takes place in the intestine (CAHU *et al*., 2004; BALDISSEROTTO, 2013; HLOPHE-GININDZA *et al*., 2015).

Diet processing can influence nutrient digestibility and fish body composition, as observed in Prussian carp (*C. auratus gibelio*) (SHI *et al*., 2016) and tilapia (*O. niloticus* × *O. aureus*) (LI *et al*., 2015). In contrast, diets containing exogenous enzymes did not influence the body composition of tilapia (*O. niloticus*) (ADEOYE *et al*., 2016a), prussian carp *(C. auratus gibelio*) (LIU *et al*., 2016), black carp (*M. piceus*) (CHEN *et al*., 2009), pompano (*Trachinotus marginatus*) (SIMIÃO *et al*., 2018), rainbow trout (*O. mykiss*) (YIGIT *et al*., 2018) and sea bass (*D. labrax* L.) (GASCO *et al*., 2016). However, the digestibility of nutrients may be affected by both the processing of diets and exogenous proteases and interaction, as in the study by Shi *et al*. (2016). The authors observed greater protein and lipid retention in fish fed the extruded diet containing exogenous protease, suggesting better use of nutrients. Although, in the extrusion process, some proteins can be denatured and, consequently, facilitate the action of proteases during the digestion process (NRC, 2011), exogenous proteases must have thermal and pH stability to ensure enzymatic activity (LI *et al*., 2015).

Metabolic effects

Monitoring the metabolic effects and health status of fish is an important tool for evaluating fish performance (Table 3). The evaluation of the activity of the aminotransferase enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in plasma reflects the health status of the liver and its functions (ZHAI; LU; CHEN, 2014). Diets containing low-quality ingredients can result in changes in the health status of fish. Ragaa *et al*. (2017) in Nile tilapia (*O. niloticus*) did not observe any influence of exogenous protease supplementation on plasma ALT and AST activity. Hassaan *et al*. (2019) observed that Nile tilapia (*O. niloticus*) fed diets containing cottonseed meal and exogenous protease had lower plasma ALT and AST activity, indicating lower activity of amino acid metabolism and greater immune response of fish, with increased total protein and albumin. The authors suggest that the immune response is due to the presence of gossypol, an anti-nutritional factor in cotton (FRANCIS; MAKKAR; BECKER, 2001). In addition, the lower activity of aminotransaminase enzymes may be due to the use of vegetables in diets and the absence of some essential amino acid, as suggested by Abd Elnabi *et al*. (2020).

Diet processing and the inclusion of exogenous protease did not influence the plasma concentration of total proteins and albumin in prussian carp (*C. auratus gibelio*) (SHI *et al*., 2016). The sea bass (*D. labrax*) when fed diets containing dry distiller grains and exogenous protease showed an increase in the plasma concentration of total proteins, in the number of red and white blood cells, in the concentration of hemoglobin and in the hematocrit rate, the authors attributed to these results the greater demand of oxygen for its

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dissociation (GODA *et al*., 2019). On the other hand, in the study by Adeoye *et al*. (2016b) no hematological changes were observed in fish fed a combination of exogenous enzymes (protease, carbohydrase and phytase). Currently, the use of diets enriched with plant protein sources and lower quality ingredients, supplemented with protease has not had a negative effect on fish health, however, studies related to the mechanism of action of exogenous proteases in relation to blood parameters need to be developed (GODA *et al*., 2020; HASSAAN *et al*., 2019; SHI *et al*., 2016).

Table 3. Metabolic effects of the addition of exogenous protease in diets with plant ingredients in fish feed.

Specie	Protease inclusion (mg kg^{-1})	Results	Reference
Nile tilapia (*O. niloticus*)	250[a]	Blood: ↑ number of white blood cells ↑ hematocrit rate ↓ cholesterol content	SALEH *et al*. (2021)
Nile tilápia (*O. niloticus*)	500[b]	Blood: ↑ concentration of hemoglobin ↑ hematocrit rate Plasma: ↑ concentration of total proteins ↑ concentration of albumin ↓ ALT and AST activity	HASSAAN *et al*. (2019)
Sea bass (*D. labrax* L.)	1000[c]	Blood: ↑ number of red blood cells ↑ number of white blood cells ↑ concentration of hemoglobin ↑ hematocrit rate Plasma: ↑ concentration of total proteins ↓ concentration of albumin ↑ cholesterol content Liver: ↓ ALT and AST activity ↓ concentration of AP	GODA *et al.* (2019)
Prussian carp (*C. auratus gibelio*)	400[d]	Serum: ↑ concentration of AP Hepatopancreas: ↓ AST activity	LIU *et al.* (2016)
Nile tilapia (*O. niloticus*)	500[e]	Blood: ↑ number of red blood cells ↑ number of white blood cells ↑ concentration of hemoglobin ↑ hematocrit rate Plasma: ↓ ALT and AST activity ↑ concentration of total proteins and albumin	HASSAAN *et al*., (2021)

[a]protease (600.000 U g^{-1}, Novus Company, USA); [b]protease (5000 U g^{-1}, Huvepharma, Antwerp, Belgium); [c]protease (PROXYM ULTRA5®, Gloray Vet COMPANY); [d]coated neutral protease (Kemin Industries Zhuhai Co., Ltd.); [e]SunHY Biology Co. Ltd., China. Legend: Aspartate aminotransferase (AST); Alanine aminotransferase (ALT); Alkaline phosphatase (AP); Higher (↑); Lower (↓).

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The aminotransferase enzymes, ALT and AST, are biomarkers of amino acid metabolism and their activity increases when there are deamination and transamination processes (NELSON; COX, 2004). The highest AST activity was observed in the hepatopancreas of prussian carp (*C. auratus gibelio*) fed with lower levels of protein and without exogenous protease in the diet, possibly due to the processes of amino acid deamination (LIU *et al*., 2016). Kumari *et al.* (2013) indicated a greater use of dietary protein with greater activity of aminotransferase enzymes in tissues, liver and muscle, of fish fed with nanoencapsulated trypsin. On the other hand, sea bass (*D. labrax* L.) fed diets containing the highest content of dry distillery grains (50%) and exogenous protease showed lower AST and ALT and alkaline phosphatase activity in the liver and greater growth (GODA, *et al*., 2019). However, it is observed that exogenous proteases do not have direct effects on protein metabolism, however, they can increase the availability of nutrients for the action of aminotransferase enzymes.

Synergistic effects

Exogenous proteases together with other exogenous enzymes result in improved growth (ALI ZAMINI *et al*., 2014; LIN; MAI; TAN, 2007; SIMIÃO *et al*., 2018), nutrient digestibility (HLOPHE-GININDZA *et al*., 2015) and alteration of the intestinal microbiota of fish (ADEOYE *et al*., 2016a, 2016b; HASSAAN *et al*., 2021). However, the addition of enzyme complexes or blends allows enzymes to act simultaneously, each one in its specific substrate, thus allowing greater availability of nutrients in the digestive system of fish (GOMES *et al*., 2019; LIN; MAI; TAN, 2007). Furthermore, the combination of acids and exogenous proteases in the diet showed positive results in growth, nutrient digestibility and villus height (HASSAAN *et al*., 2020; HUAN *et al*., 2018). On the other hand, to ensure beneficial effects, processing techniques and diet composition must be considered, as well as species and other factors that can inactivate and/or reduce enzymatic activity.

The combination of exogenous proteases and probiotics has positive effects on fish performance (Table 4). However, when the level of enzyme inclusion in the diet is not enough, the functions performed by probiotics can be compromised and not achieve satisfactory results. Thus, the detection of alterations resulting from supplementation is not possible as observed in Channa argus (DAI *et al*., 2018). On the other hand, enzymes favor the action of probiotics, since they release substrate for probiotic activity, and the combination of both improves fish performance (ADEOYE *et al*., 2016a, 2016b; HASSAAN *et al*., 2021).

In diets developed for fish, soybean meal and corn ingredients supply most of the metabolic energy required (NRC, 2011). However, antinutrients can make it difficult to use this energy and other nutrients. On the other hand, the presence of probiotics in the digestive tract can produce metabolic products during fermentation, such as lipopeptide and peptidase. These metabolites can alter the activity of digestive enzymes and even the pH of the digestive tract (MURA; BAUER, 1978). It was possible to observe that the combination of exogenous enzymes and probiotics alters the intestinal microbiota without deleterious effects on the intestinal health of fish (ADEOYE *et al*., 2016b; HASSAAN *et al*., 2021; JIANG *et al*., 2014). The reason for such effects may be that exogenous enzymes alter the environment of the intestine, such alterations of pH or intestinal substrates caused by enzymatic decomposition since positive effects of the combination of exogenous enzymes and probiotics were not observed by Dai *et al*. (2018). However, the mechanism of action of this combination in terms of improving fish performance is not yet known.

Table 4. Synergistic effects of protease and probiotic mixture composition in fish diets

Specie	Composition	Results	Reference
Nile tilapia (*O. niloticus*)	1.85 × 10^5 *B. pumilus* (CFU kg^{-1}) + 500 mg protease kg^{-1}	Performance: ↑ WG; SGR; FI; PER ↓ FC Intestine histology: ↑ number of GC; mucosal thickness and enterocyte height Immunology: ↑ phagocytic activity ↑ concentration of IgM and Lys	(HASSAAN *et al*., 2021)
Snake head (*C. argus*)	1 × 10^6 *B. amyloliquefaciens* (CFU g^{-1}) + 300 mg protease kg^{-1}	Performance: = WG; SGR; FI; PER; FC Serum: = albumin, total proteins and Lys ↑ concentration of AP Digestive enzymes: ↑ pepsin in the stomach ↑ trypsin and amylase in the liver	(DAI *et al*., 2018)
Nile tilapia (*O. niloticus*)	20 mg kg^{-1} (*B. subtilis*, *B. licheniformis* e *B. pumilus*) + 30 mg protease kg^{-1}	Performance: ↑ SGR and PER ↓ FC Serum: = Lys Intestine histology: ↑ number of GC; MV and EAS	(ADEOYE *et al*., 2016a, 2016b)

Legend: Weight gain (WG); Specific growth rate (SGR); Feed conversion (FC); Protein efficiency rate (PER); Food intake (FI); Goblet cells (GC); Immunoglobulin (IgM); Alkaline phosphatase (AP); Lysozyme (Lys); Microvilli (MV); Enterocyte absorption surface (EAS). Same as control treatment (=); Higher (↑); Lower (↓).

Bottlenecks and opportunities for the use of exogenous proteases

The use of exogenous enzymes in animal production is an established nutritional strategy. Biotechnology companies tend to develop new enzymes and optimize existing methods to provide better rates of productivity and feed efficiency. Additionally, its use is increasingly recurrent because of the high cost of ingredients, variability in the composition and quality of animal flours, which increase the production cost and limit the profitability of fingerling production.

As previously described, exogenous proteases have many positive effects on growth, nutrient digestibility, health status, dietary quality, economics, and environment in fish production. However, knowledge of sources and characterization; protease inclusion levels, mechanisms of action and substrate: protease ratio are important aspects to ensure greater efficiency of exogenous proteases in fish feeding.

Sources and characteristics of proteases

The exogenous protease encapsulation technique can guarantee the stability and delivery of exogenous enzymes. Nanoencapsulation can prevent exogenous protease effects from being restricted in the digestive system by protease inhibitors and possible hydrolysis caused by digestive proteases (ZHENG *et al.*, 2019). Chitosan, as a thermostable material, can immobilize many enzymes and help protect biomolecules from adverse effects. Exogenous trypsin, when nano encapsulated in chitosan to strengthen its efficacy and mimic proteolytic activity in the gastrointestinal tract via controlled release, improved growth and nutrient digestibility in *Labeo rohita* fed for 45 days (KUMARI *et al.*, 2013). On the other hand, pepsin immobilized in colloidal gold nanoparticles had its activity investigated on 3D supports and its stability was observed compared to free enzyme, suggesting the authors that the enzyme has biocatalytic activity in material (GOLE *et al*., 2001). In another study, the protease was coated in a fluidized bed to increase its heat stability, it was possible to observe positive effects on the growth of prussian carp (*C. auratus gibelio*) (LIU *et al*., 2016).

Exogenous protease effects are responsive to gastric conditions that cause protein denaturation and degradation. Next, after being exposed to Nile tilapia (*O. niloticus*) gastrointestinal pH conditions, the protease activity of alginate capsules without and with bentonite at pH 3 did not undergo denaturation, but was affected at pH 2.5; 2 and 1.5 (RODRIGUEZ *et al.*, 2018). Bovine trypsin supplemented in the diet of common carp (*C. carpio*) resulted in increased proteolytic activity and this increase in activity was correlated with the proportion of exogenous trypsin (DABROWSKI; GLOGOWSKI, 1977).

Fish feed is processed by compression granulation (pelleting) and/or extrusion (NRC, 2011). During these processes, favorable conditions for enzymatic hydrolysis (temperature and humidity) may occur, in two ways: (I) inactivation of the exogenous enzyme, as they are thermosensitive; (II) enzymatic activation and, consequently, greater availability of nutrients from the diet (HASSAAN *et al.*, 2019). However, enzyme stability prevents enzymatic hydrolysis during these processes (LI *et al.*, 2015). In the study by Shi *et al.* (2016) protease inactivation was observed in the extrusion process, where only 37.65% of the proteolytic activity was maintained after this process; while during the pelleting of the feed the rate of retention of the protease activity was 77.98% and 79.30% (LI *et al.*, 2015; SHI *et al*., 2016), where both cases showed high thermal stability of the protease compared to the extruded feed. In Nile tilapia (*O. niloticus*) fed a diet containing shrimp enzyme microcapsules, alkaline protease activity was 27% higher than in fish fed a control diet without enzyme immobilization, indicating that alginate-bentonite capsules are good vehicles for enzyme delivery. On the other hand, the high temperature of extrusion processing causes loss of microencapsulated enzymes, making it difficult to include them in diets (RODRIGUEZ *et al*., 2018). Thus, additional studies involving growth bioassays are needed to demonstrate the action and efficiency of enzymes subjected to different compositions and feed processing techniques.

Exogenous enzymes can change the physical quality of pellets, making them softer, and consequently, increase feed consumption and improve fish growth. In the same study, the forms of incorporation of enzymes in the feed did not differ (extruded feed without enzyme; feed with enzyme added before extrusion; feed with enzyme added in a vacuum coating machine after extrusion), however, the addition of enzyme after extrusion improved extrusion digestibility of nutrients in Atlantic salmon (*Salmo salar*) (JACOBSEN *et al*., 2018). The addition of exogenous protease in post-extrusion vaccum coater in diets containing soybean meal resulted in better digestibility of nutrients by rainbow trout (*O. mykiss*), and no positive effect was observed when supplementing the enzymes together (protease, β-glucanase and xylanase) in the diet containing soybean meal (DALSGAARD *et al*., 2012). For the same species and similar enzyme application, Dalsgaard *et al*. (2016) observed that exogenous enzymes can reduce the anti-nutritional effects of plant ingredients and improve nutrient digestibility and fish growth. The incorporation of post-extrusion enzymes with the use of vacuum coater is an important strategy in fish feeding, since in addition to improving pellet quality, it also increases the availability of nutrients and their use by the fish.

Protease levels in fish feed

Black carp (*M. piceus*) fed diets containing levels of 1, 2 and 3 g kg^{-1} of exogenous protease, low inclusion of fish meal (150 g kg^{-1}) and high inclusion of soybean meal (260 g kg^{-1}) resulted in increased weight gain and improved apparent protein digestibility coefficient (CHEN *et al*., 2009). However, fish showed no change in weight gain when fed with levels above 1 g kg^{-1} of protease. Goda *et al*. (2019) tested the inclusion of 1 g kg^{-1} of exogenous protease in the diet containing high fish meal inclusion (300 g kg^{-1}) and low distillery grain content (187.5 g kg^{-1}) and observed better growth and feed efficiency, higher plasma total protein concentration, lower alkaline phosphatase concentration, lower ALT and AST activity in the liver, greater villus length and height and increase in the number of goblet cells in the intestine of sea bass (*D. labrax* L.). The high content of distillery grains in substitution of soybean meal increase of 24% in the final weight of the fish, and in the economic evaluation, a lower cost of feed/kg of weight was observed with the replacement. On the other hand, in the study by Yigit *et al*. (2018) no greater growth, nutrient digestibility and change in body composition were observed in rainbow trout (*O. mykiss*) fed a diet containing low inclusion of fish meal (310 g kg^{-1}), high inclusion of soybean meal (440 g kg^{-1}) and two levels of protease (1 e 2 g kg^{-1}). Levels above 1 g kg^{-1} of exogenous protease in the diet did not result in increased weight gain or positive effects on apparent nutrient digestibility (CHEN *et al*., 2009; GODA *et al*., 2019; YIGIT *et al*., 2018).

The extruded diet (at 110±5ºC) containing low inclusion of fish meal (30 g kg^{-1}) and high inclusion of soybean meal (260 g kg^{-1}) showed lower exogenous protease activity when compared to a diet which contains 90 g kg^{-1} of fish meal and 160 g kg^{-1} of soybean meal. In the diet with the highest nutritional quality (90 g kg^{-1} of fish meal and 160 g kg^{-1} of soybean meal) supplementation with 125, 150 and 170 mg kg^{-1} of exogenous protease did not influence the performance of the prussian carp (*C. auratus gibelio*). However, fish fed a pelleted diet containing low inclusion of fish meal (30 g kg^{-1}) and high inclusion of soybean meal (260 g kg^{-1}) without supplementation showed lower growth; on the other hand, when supplemented with protease, this same diet resulted in an average increase of 11% in fish growth and better digestibility of nutrients (SHI *et al*., 2016). In tilapia (*O. niloticus* × *O. aureus*) fed a pelleted diet containing low inclusion of fish meal (30 g kg^{-1}) and high inclusion of soybean meal (300 g kg^{-1}) and 175 mg kg^{-1} of protease showed an average increase of 12% in final weight due to improved feed efficiency and apparent digestibility of nutrients when compared to the control group without exogenous protease in the diet. However, there was no difference in the final weight of fish fed the pelleted diet containing 90 g kg^{-1} of fish meal and 260 g kg^{-1} of soybean meal with enzyme supplementation in relation to the diet without protease. Furthermore, no greater growth was observed in tilapia fed the extruded diet containing 30 g kg^{-1} or 90 g kg^{-1} of fish meal and exogenous protease compared to fish fed the pelleted diet containing 90 g kg^{-1} of fish meal and protease (LI *et al*., 2015). Both studies showed negative effects of exogenous protease if added to extruded diets, but benefits were reported with feeding pelleted diets, and the effects can be attributed to the thermosensitive characteristic of the proteases.

Ragaa *et al*. (2017) observed in Nile tilapia (*O. niloticus*) an average increase of 13% in final weight, greater weight gain and apparent protein digestibility coefficient when fed a diet containing 400 mg kg^{-1} of exogenous protease, low inclusion of fish meal (80 g kg^{-1}) and high of soybean meal (361.1 g kg^{-1}). In addition, a 2% reduction in the protein level (70 g kg^{-1} of fish meal and 350.50 g kg^{-1} of soybean meal) with 200 and 400 mg kg^{-1} of protease in the fish diet, resulted in an improvement in apparent protein digestibility and a 7% increase in final weight compared to fish fed diets without enzyme. Nile tilapia (*O. niloticus*) that received diets containing high inclusion of fish meal (150 g kg^{-1}) and low inclusion of cottonseed meal (120 g kg^{-1}) and another containing low inclusion of fish meal (110 g kg^{-1}) and high cottonseed meal (160 g kg^{-1}), both containing 500 mg kg^{-1} of exogenous protease, resulted in an approximate 24% increase in final weight, better availability of amino acids and of the apparent digestibility coefficient of dry matter, protein and lipids (HASSAAN *et al*., 2019). Improvement in the apparent digestibility coefficient of protein and lipid was also observed in prussian carp (*C. auratus gibelio*) fed a diet containing low inclusion of fish meal (60 g kg^{-1}) and high inclusion of soybean meal (180 g kg-1) and 400 mg kg^{-1} of exogenous protease, promoting with this formulation an economy of 20 g kg^{-1} of protein in the diet (LIU *et al*., 2016).

Rainbow trout (*O. mykiss*) fed a diet containing low (280 g kg^{-1}) and high (350 g kg^{-1}) inclusion of fish meal and 175 mg kg^{-1} of protease showed better performance (ZHANG *et al*., 2012). In the study by Dalsgaard *et al*. (2012), in the extruded diet with 228 mg kg^{-1} of exogenous liquid protease, containing low inclusion of fish meal (201.7 g kg^{-1}) and high inclusion of soybean meal (343.8 g kg^{-1}), there was an improvement in the apparent digestibility of nutrients and in the growth of rainbow trout (*O. mykiss*), but there was no improvement in the digestibility of nutrients in the diets with sunflower meal (246 g kg^{-1}) and canola meal (263.5 g kg^{-1}) supplemented with protease. Dalsgaard *et al*. (2016) observed that exogenous protease supplemented under the same conditions as the previous study resulted in higher apparent digestibility of nutrients in rainbow trout (*O. mykiss*) diets. Although the supplementation of 250 mg kg^{-1} of exogenous protease to the canola diet (240 g kg^{-1}) resulted in improved feed efficiency, the flaxseed diet (240 g kg^{-1}) did not affect the digestibility of nutrients in rainbow trout (*O. mykiss*) (DREW *et al*., 2005). Thus, it is observed that the activity of exogenous enzymes is influenced by alternative sources of protein in fish feed and, consequently, can cause adverse effects on the use of nutrients and growth.

Positive effects on intestinal villi growth and health were observed in *L. rohita* fed chitosan-encapsulated trypsin in the diet than unencapsulated trypsin (KUMARI *et al*., 2013). The increase in the specific growth rate and in the protein efficiency rate and lower feed conversion showed that even after feed processing, trypsin nanoencapsulated in chitosan remained stable and active, indicating a positive correlation between feed intake and digestive enzyme activity. In tilapia (*O. mossambicus*) it was observed greater growth, improvement in apparent protein digestibility and intestinal enzyme activities when fed with a diet containing 500 mg kg^{-1} of exogenous protease, 77.1 g kg^{-1} of fish meal and 75 g kg^{-1} of kikuyu leaf bran (HLOPHE-GININDZA *et al*., 2015). Atlantic salmon (*Salmo salar* L.) fed a diet containing 339 g kg^{-1} of soybean meal and a mixture of proteolytic enzymes and carbohydrates (1 mg kg^{-1}) showed greater growth compared to fish fed the diet based on fish meal and/or with soybean meal without the addition of enzymes (CARTER *et al*., 1994). Common carp (*Cyprinus carpio* L.) fed a diet containing supplemental protease and 100 g kg^{-1} of fish meal showed better weight gain compared to the group that received diets containing 150 g kg^{-1} and 200 g kg^{-1} of fish meal (LENG *et al*., 2008). In contrast, the use of exogenous enzymes (300 mg kg^{-1} protease, 75 mg kg^{-1} phytase and 250 mg kg^{-1} xylanase) and probiotics as individual supplements did not show positive effects on Nile tilapia (*O. niloticus*) performance parameters (ADEOYE *et al*., 2016b), and the antinutrients, non-starch polysaccharides and trypsin inhibitors, are substrates for the xylanase and protease enzymes, respectively. Possibly, the differences between the results of growth and nutrient digestibility are related to other factors, such as the interaction of enzymes with the composition of diets and their substrates, digestive system, species and the stability of proteases in feed processing.

Protease: substrate interaction

Enzymes have degrees of specificity in relation to their different substrates, for example, digestive trypsin can hydrolyze a peptide bond in which the amino group is made up of basic amino acids such as lysine, arginine and histidine. In the study by Dalsgaard *et al*. (2012) it was observed that the addition of protease in diets containing soybean meal, sunflower meal and canola meal showed better nutrient digestibility with soybean meal than in other sources for rainbow trout (*O. mykiss*). Possibly, the presence of phytic acid in these ingredients prevented the enzyme's action in improving the use of nutrients (FRANCIS; MAKKAR; BECKER, 2001). Thus, an alternative is to evaluate the activity of exogenous proteases used in fish diets according to the enzyme-substrate specificity of each ingredient.

Sea bass (*D. labrax* L.) fed a pelleted diet containing 200 mg kg^{-1} of protease, high inclusion of fish meal (445.5 g kg^{-1}) and low inclusion of *Tenebio molitor* larvae meal (247.5 g kg^{-1}), showed no improvement in nutrient digestibility (GASCO *et al*., 2016). In the study by Henry *et al*. (2018) no change was observed in the immunological parameters of fish fed diets containing exogenous enzymes and *T. molitor* larvae meal (247.5 g kg^{-1}). However, the trypsin inhibition activity was reduced in the presence of proteases, leading the authors to believe that the enzymes are not recommended in diets containing *T. molitor* larvae meal, since they could have inhibited some protein responsible for immune functions and reduce the apparent digestibility of dietary protein.

In the study by Chen *et al*. (2009) there was an increase in the apparent digestibility of the protein, but not in the dry matter, so that the authors believed that the exogenous protease reached its substrate due to the increased availability of protein. However, not all enzymes are equally effective in digesting their substrates

(LIN; MAI; TAN, 2007) and the effects of enzymes can be diminished in diets that contain highly digestible, high-density ingredients (SHI *et al.*, 2016). Therefore, the enzyme: substrate relationship demands information more focused on the bioavailability of amino acids, on the interaction with endogenous enzymes during digestion and on the prior quality of the protein sources that make up the diets.

Final considerations

Supplementation of exogenous proteases in plant-based diet results in positive effects on fish growth, nutrient digestibility, and metabolism, and indirectly brings social, economic and environmental benefits. Although there are many studies with carnivorous species, due to the need for diets concentrated in protein, the use of exogenous proteases for omnivorous species also improves the growth and digestibility of nutrients from plant sources. Based on the results indicated, it is clear the importance of using exogenous proteases in fish feeding, either individually or in combination. Feeding fish represents a high cost in the production system, and the increase in fish weight gain provided by the use of protease may offset its additional cost in the diet.

There are contradictory results attributed to the levels of enzyme inclusion, the composition of the diets and the cultivation conditions and, consequently, it compares studies difficult. In addition, some issues related to the use of protease have not yet been elucidated: (I) protease and its effect on improving food consumption; (II) protease effect on hematological parameters; (III) mechanism of action of the protease to the specific substrate; (IV) dynamics involved between protease: probiotic: intestinal environment.

Future research is needed to understand the mechanism of action of proteases in plant protein sources, in addition to evaluations regarding the thermal stability of proteases, new enzymatic coating techniques and forms of incorporation into the feed, to optimize and guarantee economic production and sustainable

Conflict of interest

The authors declare that the research was conducted in the absence of any potential conflicts of interest.

Ethical statements

The authors confirm that the ethical guidelines adopted by the journal were followed by this work, and all authors agree with the submission, content and transfer of the publication rights of the article to the journal. They also declare that the work has not been previously published nor is it being considered for publication in another journal.

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