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A review on fish *in vitro* digestion studies

Nazly Y. Martin-Culma[1,2,3] – Otilia Antal – Krisztina Takács[4*] – Péter Bársony[3]

[1]Faculty of Agricultural, Food Sciences, and Environmental Management. University of Debrecen, 4032, Debrecen, Hungary.
[2]Doctoral School of Animal Science, University of Debrecen, 4032, Debrecen, Hungary.
[3]Institute of Animal Science, Biotechnology and Nature Conservation, University of Debrecen, 4032, Debrecen, Hungary.
[4]Hungarian University of Agriculture and Life Sciences, Institute of Food Science and Technology, Department of Nutrition Science, 1118, Budapest, Hungary.
*Correspondence: takacs.krisztina@uni-mate.hu

SUMMARY

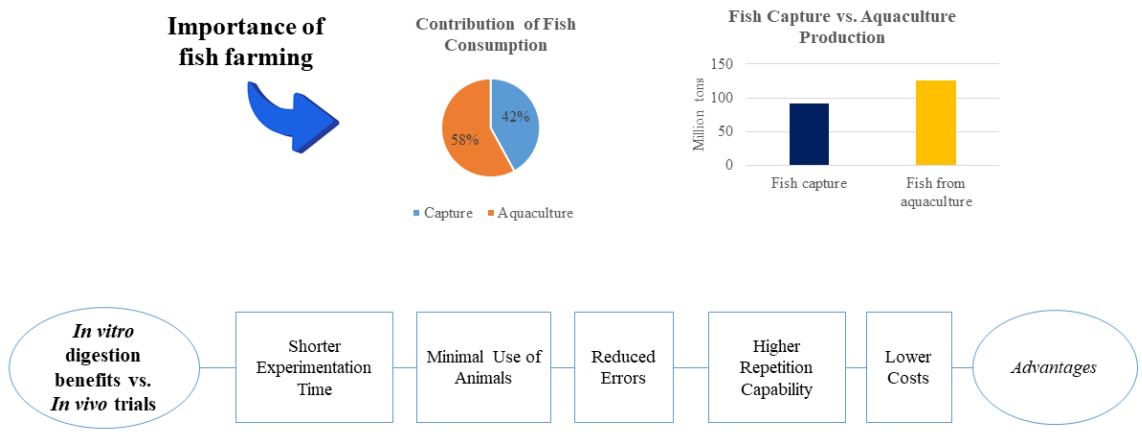
(1) Background: In vitro experiments in fish have been developed to search for dietary substitutes of fish meal in aquafeeds by measuring the digestibility of various feedstuffs by simply simulating the conditions of the stomach or portions of the digestive tract. In vitro digestion studies involving mainly commercial fish species have been conducted to determine the digestibility of conventional and alternative ingredients in fish diets using diverse digestion models. However, there remains a significant knowledge gap, particularly regarding the enzyme functionality, digestion mechanisms in many fish species, and the factors influencing enzymatic activity. This review article is focused on the importance of the use of different enzyme sources in the in vitro digestion model to predict protein hydrolysis.;(2) Methods: For this review, a comprehensive analysis of articles was conducted to gain insights into diverse enzyme interactions and feed evaluation assessments, from the nutritional science and biotechnology sector.; (3) Results: A total of 36 peer-review papers, which include original, and review articles were selected.; (4) Conclusions: In vitro digestion studies offer a valuable tool for evaluating the digestibility of conventional and alternative feed ingredients, helping to identify sustainable dietary substitutes for fish meal while reducing dependence on live animal trials.

Keywords: *nutrient digestibility; fish diet; in vitro digestion; novel ingredients*

INTRODUCTION

Fish meat and its byproducts have a high concentration of omega 3 fatty acids, a good amino acid profile and high meat digestibility, with an average consumption of 20 kg per capita. Fish can usually be obtained from capture and aquaculture systems. Annually, fish capture is estimated at 92 million tons. Moreover, the global aquaculture production reached 126 million tons in 2021 (FAO, 2021). This large difference in fish harvesting is due to the limited availability of fish in open water. One of the primary objectives of aquaculture is the proper nutrition of fish, and digestibility techniques are used to identify the most advantageous and sustainable diet. However, fish farming in aquaculture systems is facing a crisis due to the scarcity and high cost of fish meal (FM) and fish oil (FO). It is crucial to find nutritional alternatives to feed ingredients and perform faster digestibility tests to decrease the cost of production and reduce the selling price (Brander, 2007) (*Figure 1*). Fish nutrition evaluates the absorption and utilization of nutrients from various diet sources. At the end of the last century, the development of *in vitro* digestive models began due to the replication of the digestive tract and the use of enzymes. Additionally, the pH, temperature, bowel movements, retention rates, and absorption of specific ingredients should be controlled. Currently, *in vitro* digestion models have advanced and integrated molecular biology, computational modeling, and analytical chemistry techniques.

Figure 1. **The importance of *in vitro* digestion methods in modern fish aquaculture production**



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Compared with *in vivo* digestibility experiments, *in vitro* methods have shorter experimentation times, minimal use of animals to promote animal welfare, and lower costs (Bohn et al., 2018; Bryan and Classen, 2020; Wang et al., 2021). *In vitro* fish digestion is commonly employed to accelerate trials, reducing the average duration of traditional experiments, which are usually longer, at six to sixteen weeks (Bell et al., 2003; Nguyen et al., 2009; Randazzo et al., 2021; Valente et al., 2006). *In vitro* assays can usually be performed in a few days, depending on the design of the experiment. In addition, they reduce the use of resources and can solve the problem of the limited capacity of fish facilities to perform experiments, allowing more repetitions and diet inclusions. In addition, there is no need for constant feeding. For example, in a 16-week salmon digestibility experiment, feed intake reached 6 tons (Bell et al., 2003), representing a significant cost for the investigation. These long experimental times can also lead to possible errors from humans or machinery within fish systems. These mistakes can affect the veracity of the data collected or, in critical scenarios, can lead to large animal losses. *In vitro* fish digestion is a feasible alternative to traditional experiments. The main objective of this review article is to present a comprehensive overview of the utilization of *in vitro* models during fish digestibility studies and to evaluate bioactive compounds of interest from fish by-products.

MATERIALS AND METHODS

The article presents the results of a systematic search from 1993–2024 using PRISMA review protocol, with a total of 1167 articles found through. Both original and review pieces were presented. It was identified 415 duplicates. After removing duplicates, the records screened were 752. From which were removed 594 scientific papers not related with fish *in vitro* digestion. A total of 158 articles were assessed for eligibility from this group of articles 116 articles were excluded because they were mainly focus on in vitro fish hydrolysates. Finally, a total of 36 articles were included in this review. Additionally, the review did not include encyclopaedias, book chapters, abstract conferences, or study areas unrelated to fish farming, animal science, or the medical or pharmaceutical industries. The ScienceDirect, JSTOR, Springerlink, SciELO, and Scopus databases were searched for relevant articles. The search was delimited via the following search terms: *in vitro* digestion AND/OR, INFOGEST AND/OR, static model AND/OR, *in vitro* antioxidant activity AND/OR, and fish AND/OR.

The information was analyzed in terms of data-based discrimination through the date of the article, relevance to the topic, *in vitro* digestion studies on fish. In *in vitro* studies of fish, the focus has been on the inclusion of different feed alternatives tested under different gastric conditions. The positive impact of *in vitro* digestion methods in the aquaculture industry aimed at reducing assay costs and procuring the well-being of the experimental animals involved was also of interest.

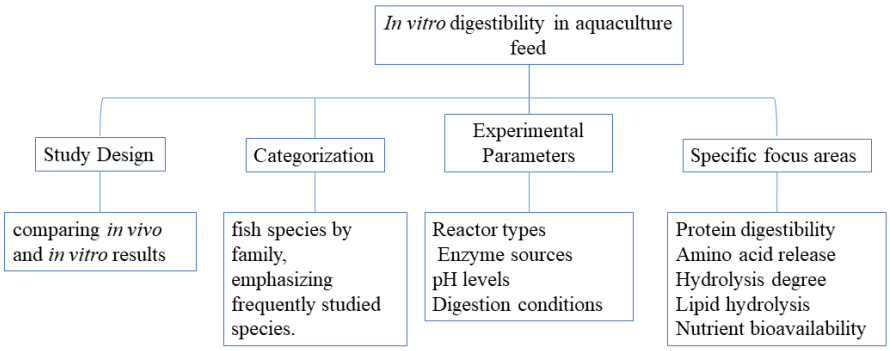
Qualitative and quantitative data analysis

A total of 58 relevant articles on fish *in vitro* digestion, published between 1985 and 2024, were identified, with a particular emphasis on those published after 2015. The selection focused on studies relevant to *in vitro* digestibility assessments for aquaculture feed evaluation. Articles with incomplete or missing information were excluded, while the final selection prioritized comprehensive studies, particularly those comparing in vivo and *in vitro* results.

Fish species were categorized by families, with frequently studied species highlighted. Experimental setups were analyzed, considering reactor types, enzyme sources, pH levels, and digestion conditions (e.g., alkaline, acidic, or combined environments). Key variables included digestion times, temperatures, and ingredient combinations, typically comprising four sources: vegetal, animal, mineral, or commercial meals.

The study was primarily focused on comparing fish meal digestion with alternative protein sources. Specific assessments included the degree of hydrolysis, amino acid release, and protein digestibility. Additional metrics evaluated were residual nitrogen, lipid hydrolysis, bioavailability, nutrient toxicity, and sugar release. Moreover, it was evaluated the use of extracts from the pyloric caeca and stomach to ensure accuracy in replicating fish digestive processes (*Figure 2*).

Figure 2. **Study design of the search of data. The information found in the scientific articles selected was classified according to study design, categorized by emphasis and experimental parameters, and main *in vitro* nutrient digestibility indices**



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RESULTS AND DISCUSSION

RESULTS

Advancements regarding *in vitro* digestibility studies for sustainable aquaculture feeds

Aquaculture has been a burgeoning industry since 1950; this practice significantly contributes to the provision of animal protein sources that offer high nutritional value and is a sustainable alternative that reduces the burden on wild fish populations. However, the availability of protein sources for fish farming has been decreasing due to the increasing human population and climate change.

This issue has led to the search for alternative nutritional ingredients that can be more sustainable and do not compete with human feed sources to encourage the consumption of fish. For example, the effect of the consumption of different types of fish in humans has been tested *in vivo* to determine the potential benefits of fish consumption, whereas in fish farming, the inclusion of different types of vegetables and insect meals and plant oils as partial replacers of fish meal and fish oil has been tested. Nonetheless, *in vivo* studies are costly and time-consuming, and they necessitate a suitable infrastructure to grow and maintain the fish, as well as human labor to regulate the tank's ideal settings and ensure the wellbeing of the experimental animals. As an alternative, *in vitro* experiments can help to determine the digestibility of a wide variety of feeds and are an optimal method of feed assessment (Yasumaru and Lemos, 2014; Rodríguez-Rodríguez et al., 2024). The main fish species used for *in vitro* digestion assays were: Rainbow trout (Yasumaru & Lemos, 2014; Sánchez-Muros et al., 2016; Márquez et al., 2013; Morales et al., 2011; Morales et al., 2013; Oldham et al., 2023), cobia (Yasumaru & Lemos, 2014; Sánchez-Muros et al., 2016), Nile tilapia (Yasumaru & Lemos, 2014; Sánchez-Muros et al., 2016; Rahmah et al., 2020). Pacific bluefin tuna (Castillo-Lopez et al., 2016; Román-Gavilanes et al., 2015), Atlantic salmon (Tibbetts et al., 2020; Tibbetts & Patelakis, 2022). sea bream (Rodrigañez et al., 2011), larger amberjack (Takakuwa et al., 2022b), white trevally (Takakuwa et al., 2022a). Common carp (Watanabe et al., 2016; Toledo-Solís et al., 2020), bagrid catfish (Arndt et al., 2015; Rahmah et al., 2020), zebrafish (Xiao et al., 2023; Yu et al., 2022), salmon (Silva et al., 2020), gilthead sea bream and flathead gray mullet (Martínez-Antequera et al., 2023).

The aquafeed industry is constantly searching for partial replacements of fish meal; for this reason, the *in vitro* digestion technique has been employed to speed up the evaluation of diverse feed ingredients and interactions with fish metabolism and health, as shown in *Figure 1*.

Within the area of fish *in vitro* digestion, 58 relevant articles published from 1985–2023 were identified. In addition to articles published after 2015. Among these articles, a total of 13 provided a comparative evaluation of *in vivo* and *in vitro* results. The fish samples used were classified according to the fish families studied, revealing the following distributions: *Anabatidae* (1), *Carangidae* (2), *Cichlidae* (8), *Clariidae* (2), *Clupeidae* (1), *Cyprinidae* (5), *Elotridae* (1), *Gadidae (1), Lepisosteidae* (1), *Moronidae* (1), *Mugilidae* (1), *Osphronemidae* (1), *Palinuridae* (1), *Paralichthyidae* (1), *Rachycentridae* (1), *Salmonidae* (20), *Sciaenidae* (2), *Scombridaem* (4), *Scophthalidae* (1), *Soleidae* (1), *and Sparidae* (7). Notably, the most extensively studied fish species was rainbow trout (*Oncorhynchus mykiss*), with 17 publications. This finding can be attributed to the significant market importance of this species. With respect to the assessment of the degree of hydrolysis, all researchers have employed simple closed reactors. However, there were variations in terms of the enzyme sources and pH levels used. Some investigations mimic either an alkaline + acidic environment (21), whereas others have focused on alkaline (28) or acidic (2) conditions. On average, each experiment involved the use of a combination of four ingredients from vegetal, animal, mineral complex, or commercial meal sources, and the experiments involved the use of different temperatures, enzymes, and *in vitro* digestion times. The primary focus of most experiments was to compare the digestion of fish meals with that of other protein sources, such as soybean or alternative ingredients. Among these experiments, 16 specifically aimed to measure the release of amino acids, 19 examined the degree of hydrolysis, and three measured the relative protein digestibility. Additionally, certain studies have assessed residual nitrogen, lipid hydrolysis, tyrosine bioavailability, protein fractions, casein digestibility, nutrient bioaccessibility and toxicity, and sugar release. During these investigations, purified extracts derived from the pyloric caeca and stomach were commonly employed. After 2015, new studies were found. Articles with missing information were excluded from the compilation, and the best results are listed in *Table 1*.

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Table 1. ***In vitro*** **fish digestion studies**

Fish Species	Source	Enzyme source	Type of hydrolysis (pH)	Time (min)-Temperature (°C)	Enzyme: Substrate ratio	Measured parameter	References
Oreochromis niloticus; Rachycentron canadum; Oncorhynchus mykiss	Animal (fish meals, blood meals, feather meals, and poultry byproduct meals). Vegetable (corn grain, corn gluten meal, cotton seed meal, rapeseed meal, soybean meals, soy protein concentrate, wheat bran, wheat flour, and wheat gluten).	Stomach and pyloric caeca/intestine enzymes.	Acidic: 2 Alcaline: 8	300–25 ℃	50, 200, 600, 1000 µL :80 mg protein	Degree of protein hydrolisis	Yasumaru & Lemos, 2014
Thunnus orientalis	Animal (Fish Meal, Poultry By-Product Meal)	Pancreatic crude extract from bluefin tuna.	Acidic: 2 Alcaline: 8	270–37 ℃	16. 7 U: 0.02 g/ml	Degree of protein hydrolisis	Castillo-Lopez et al., 2016
Cichlasoma trimaculatum	Animal (hemoglobin, premium chicken meal, shrimp head meals, squid meal, beef meat meal, chicken guts and meat meal, fish meal, blood beef meal. Hydrolyzed feather meal) Vegetal (maize gluten, sorghum meal, soy meal, wheat meal, soy paste). Lipid ingredients (salmon oil, fish oil, beef tallow oil, olive oil, maize oil, soy oil, sunflower oil).	Stomach and Intestinal enzymes.	Acidic: 3.5 Alcaline: 8	60–37 ℃	stomach extract 87 U/ml: 8 mg/ml of protein Intestine extract: 30.5 U/ml of activity: 8 mg/ml of protein	Degree and speed of hydrolysis	Toledo-Solís et al., 2020
Danio rerio	Vegetal (Microalgae)	Pepsin from porcine gastric mucosa and trypsin from porcine pancreas	Acidic: 2 Alcaline: 7.5	110–37 ℃	3000 units mg−1 protein) and trypsin from porcine pancreas (250 units mg−1 protein)	Protein fractions	Yu et al., 2022
Salmo salar L	Vegetal (Pavlova sp.459 Microalgae)	Commercial porcine pepsin and pancreatin	Acidic: 4.5 Alcaline: 9	320–39 ℃	300 µL trypsin enzyme solution 250 mg of test sample: ~25 g	Apparent digestibility coefficients	Tibbetts & Patelakis, 2022
Oreochromis niloticus	Vegetal (Peanut Meal, Rice Husk)	Intestinal enzymes	Alcaline: 8	180–25 ℃ 300–25 ℃ 360–25 ℃	6.0 to 7.3 mg l−1	Degree of protein hydrolisis	Nogueira et al., 2022
Oncorhynchus mykiss	Enzyme (Phytase)	Stomach enzymes	Acidic: 2 Alcaline: 9	240–37 ℃	31 and 19 U mg-1	Nitrogen and phosphorus bioaccessibility	Morales et al. 2013
Oncorhynchus mykiss	Mineral (ZnSO4, Zn-Bioplex)	Mammaliam enzymes	Acidic: 2.2 Alcaline: 8.9	240–37 ℃	2 mg/mL	Protein quantification	Oldham et al., 2023

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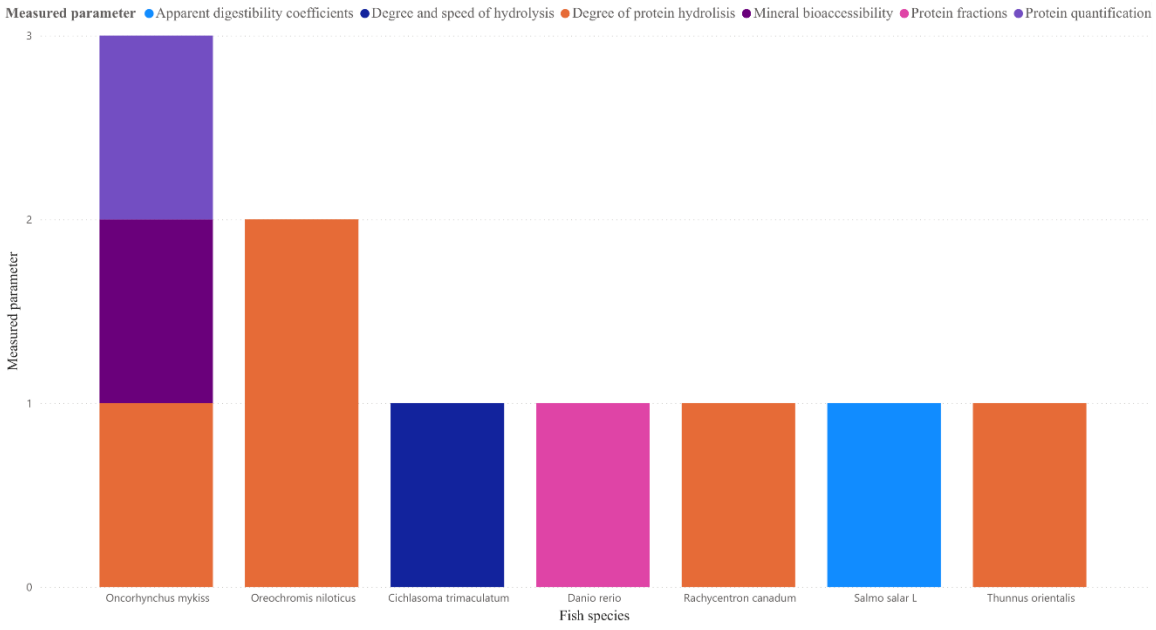
DISCUSSION

In vitro digestion method

During *in vitro* digestion assessments, different methodologies have been applied. The pH-stat method was the first approach used for *in vitro* digestion. This method measures the extent of peptide bond hydrolysis by protease enzymes during the incubation period (Dong et al., 1993). A key component of this method is the incubation of feed with digestive enzymes and the measurement of the protons that are liberated during the cleavage of peptide bonds to calculate digestibility (Moyano et al., 2015). With this methodology, many varieties have been developed to adapt the model to a specific feed compound and the human or animal gastric system. Another common methodology is the INFOGEST protocol (Minekus et al., 2014; Brodkorb et al., 2019), which reproduces the most important aspects of the human digestive system by applying standardized digestive enzyme activities. However, some studies do not show a significant difference between the results of these methodologies, as both are optimal for use during *in vitro* assessments. In general, the *in vitro* digestion technique uses different types of enzymes. During the oral (α-amylase), gastric (pepsin), and intestinal (pancreatic enzymes) phases.

Every phase has a specific pH and temperature, which imitate the general conditions of the digestive system. Some more complex, dynamic *in vitro* digestion systems (e.g., the dynamic gastric model (DGM), engineered stomach and small intestinal model (ESIN), human gastric simulator (HGS), and TNO *in vitro* (gastro-) intestinal model (TIM)). Also, reproduce the complex motility patterns of the stomach and intestine, which can significantly influence the degradation of macromolecules and the absorption of hydrolysis products (DuPont et al., 2019; Antal et al., 2020). As shown in *Figure 3* the most studied parameters were degree and/or speed of protein hydrolysis, it was also found studies that evaluated protein fractions, trypsin hydrolysis, mineral bioaccessibility, and apparent digestibility coefficients to evaluate the digestion in fish diets. Usually, an enzyme inhibitor is added to complete the digestive process (Brodkorb et al., 2019), and the digesta is analyzed via a variety of analytical techniques, such as high-performance liquid chromatography (HPLC), gas chromatography (GC), or spectrophotometry.

Figure 3. **Main parameters measured during *in vitro* digestion studies**



Alternative aquafeeds and digestibility in fish via *in vitro* digestion

The development of an appropriate *in vitro* digestion protocol requires not only a thorough understanding of physiological digestive processes but also a meticulous evaluation of the parameters that must be replicated to achieve the desired research outcomes. A device with four chambers represents the digestive system, the stomach (chamber 1), which has acidified gastric fluid. Chamber 2: Proximal intestine and pyloric ceca, which have alkaline digestive fluid. Chamber 3: Medium intestine that has alkaline digestive fluid and absorption; finally, chamber 4: represented by the distal intestine, where fermentation and absorption occur (*Figure 4*). However, the number of chambers can differ according to the experimental conditions. During the whole process, the pH, temperature, incubation time during each digestion phase, imitation of bowel movement and consequent stopping process significantly influence the results of the digestion. After the process finishes, the products

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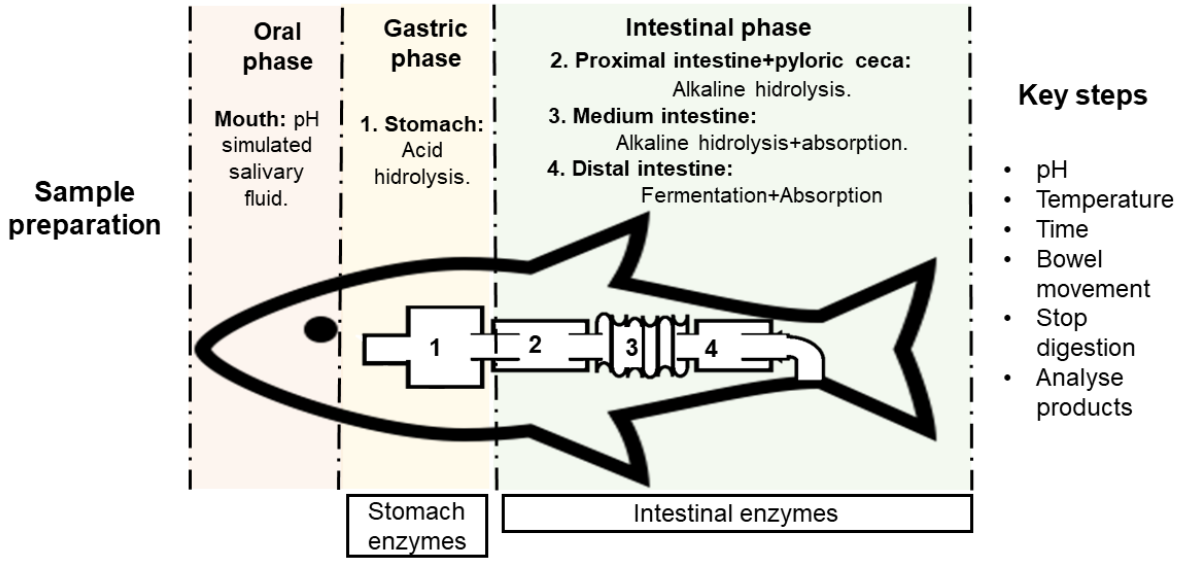
can be analyzed according to the goals of the study (*Table 1*).

Some studies overlooked the gastric phase of digestion in favor of concentrating only on the intestinal stage, which is crucial for simulating digestion in fish with functioning stomachs. Pepsin and an acidic pH are present during the gastric phase, which improves the digestion of proteins, deactivates trypsin inhibitors, and facilitates the solubility of iron (Fe) and phosphorus (P).

Figure 4. **The fish were subjected to *in vitro* digestion (based on Moyano et al., 2015)**



Most studies utilize the pH-stat method to assess the degree of hydrolysis. However, a significant limitation of this approach is its ability to simulate only the alkaline phase of digestion (pH > 8.0). Consequently, alternative methods such as OPA, TNBS, or HPLC are employed to quantify amino acid release (Moyano et al., 2015). Furthermore, SDS–PAGE is frequently used to assess protein digestibility. Since commercially available vertebrate enzymes have unique properties, such as optimal pH, sensitivity to inhibitors, and reaction speed, the use of fish enzyme extracts in research is essential for determining the bioaccessibility of nutrients. Hence, it is essential to standardize specific fish enzyme-to-substrate ratios. Furthermore, it is important to recognize that the digestibility of substances can be influenced by a variety of factors, including age, nutrition, temperature, feed, prior treatment, and environmental conditions. Furthermore, the size of the meal is an important factor since it has a significant effect on how well minerals and amino acids are absorbed. Unfortunately, many articles fail to mention the feed size, overlooking an important aspect. Considering the abovementioned points, there is a need to develop *in vitro* methods that generate results that correlate well with *in vivo* observations.

Studies conducted post-2008, as detailed in *Table 1*, have predominantly extracted enzymes from stomach, intestinal, and pancreatic sources, with limited use of bovine and porcine digestive enzymes noted in only three studies (Arndt et al., 2015; Yu et al., 2022; Rodríguez-Rodríguez et al., 2024). The choice of enzyme source aligns with the significance of pH in *in vitro* assays. However, several studies have employed various pH ranges that do not reflect the actual conditions in the *in vivo* stomach (pH 3.5–5) and intestines (pH 6.5–7.2). The pH conditions during experimentation varied, with alkaline reactions spanning a range of 6.2–9, whereas acidic reactions were observed within a range of 2–4. Such discrepancies may influence the accuracy of the results due to alterations in pepsin activity. Similarly, temperature also plays a significant role in these trials. *In vivo* body fish temperatures typically range from 5 to 15 °C from cold water sources and from hot water sources ranging from 6 to 30 °C, and deviating from these temperatures can significantly affect the reaction rates. However, some papers utilize temperatures as high as 39 °C (Tibbetts & Patelakis, 2022), which may not accurately represent physiological conditions. However, this decision was presumably made because the use of high temperatures increases the speed of the reactions.

The digestion time is one of the most different aspects of fish digestion, which is attributed mainly to the fish size, metabolism, and diet. On average, the digestion time can vary from 12 hours to days (Rust, 2002). These variations are linked to fish size, type of eating habits (carnivorous (12–24 hours), herbivorous (12–48 hours), and omnivorous (12–36 hours)), metabolism and feed size. In general, small fish species digest faster than large fish do. The digestion time is also crucial during *in vitro* digestion assays, in which higher temperatures can speed up enzymatic reactions. However, the digestion times used in different studies vary greatly from those used for normal fish conditions.

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The digestion times ranged from 10 minutes (Rahmah et al., 2020) to 6 hours (Nogueira et al., 2022). These large differences in digestion time might lead to incomplete digestion due to the shorter time needed to break down proteins, affecting the measurements related to amino acid release, degree of hydrolysis, and protein digestibility. Shortening the digestion time may not allow the enzymes sufficient time to catalyze the proteins, influencing the rate and extent of protein hydrolysis. Increasing the temperature to speed up digestion could alter the kinetics of the digestion process. While higher temperatures might accelerate enzyme activity, they can also potentially denature enzymes or proteins, leading to inaccurate results of actual digestion. Shorter digestion times and alterations in temperature could produce results that do not reflect real-life digestion.

Additionally, numerous plant-based fish meal alternatives may contain protease inhibitors, and it may also be essential to determine whether the investigated food/feed has an inhibitory effect on digestive enzymes. Furthermore, it is important to recognize that the digestibility of substances can be influenced by a variety of factors, including age, nutrition, temperature, feed, prior treatment, and environmental conditions. Furthermore, the size of the meal is an important factor since it has a significant effect on how well minerals and amino acids are absorbed. Unfortunately, many articles fail to mention the feed size, overlooking an important aspect. As mentioned above, there is a need for feed size and inhibitors as crucial factors when performing *in vitro* digestion assays.

The enzyme concentration varies depending on the source, fish species and diet. The amount and precedence of enzymes differ according to the fish species and economic funding of the experiment. Enzymes from fish species are the most expensive because of the need for enzyme standardization; hence, many studies have opted to use mammalian enzymes. Despite the precedence, the amount should be enough to produce a saturation effect and a higher enzyme concentration that can improve the completeness of the digestion.

CONCLUSIONS

The continuous advancement of nutritional science and biotechnology sectors using *in vitro* digestion techniques can lead to assess the bioaccessibility of different foods. Moreover, *in vitro* digestion does not require live fish in long trials, so it can be an alternative to reduce cost and speed up research and adapt to the increasing demographic population demand of protein of the world. *In vitro* digestion provides an alternative to the more traditional long-duration trials with live fish, thereby reducing cost and hastening research to meet the needs of the growing global population. Sustainable solutions must be found for the supply of adequate food for human consumption as well as fish, without competing between the two resources. In addition, finding new uses for the growing volume of by-products from the fish farming industry can further help achieve better sustainability in production, reduce animal disease, and promote fish health and welfare in aquaculture.

The digestion protocols serve as a means of ascertaining the level of digestibility associated with substitute sources, including insect meals, sources of vegetal protein, and feed additives. Fish nutrition methods are designed to determine the degree of protein and lipid hydrolysis and the solubility of minerals. The digestibility of various vegetable and animal protein sources, as well as the impact of enzyme supplementation on the absorption of amino acids, are other interesting topics. However, these methods need to be improved, and fish *in vitro* digestion protocols must be adapted to different fish digestive tracts and functionalities, which can significantly differ among fish species, rearing methods, eating methods, and environmental conditions, as well to explore the use of nutraceuticals in fish diets. Depending on the objectives of the research, *in vitro* digestion is a topic that calls for increased comprehension of physiological mechanisms and the creation of sophisticated, standardized protocols. To confirm that *in vitro* digestive models are effective for each nutrient (e.g., proteins, lipids, minerals) or toxin under investigation, it is also advisable to compare the *in vivo and in vitro* results.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors wrote the manuscript, revised, and approved the final version of the manuscript.

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