



**T.R.
İSTANBUL UNIVERSITY
INSTITUTE OF SCIENCES**



Ph.D. THESIS

**INVESTIGATION OF AMINO ACID METABOLISM OF
JUVENILE GILTHEAD SEA BREAM (*Sparus aurata*)
FED WITH ALTERNATIVE PROTEINS**

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PREFACE

This doctoral thesis evaluated amino acid metabolism in juvenile seabream feed diets in which fish meal was partially replaced with hydrolyzed feather meal or poultry by-product meal. In addition, the necessary histological examinations were done to understand how the digestive system was affected by the diets used in the study.

The diets used in the research and the feeding experiments were prepared at the Mediterranean Fisheries Research, Production, and Training Institute in Beymelek, Antalya, affiliated to the Ministry of Agriculture. Proximate analyzes of diets and whole-body fish as well as histological analyzes were carried out in the Department of Aquaculture and Fish Diseases, Faculty of Aquatic Sciences, Istanbul University. Amino acid analysis in diets, whole-body and fish feces were carried out in GTS Test Laboratories-Department of Chemistry.

I am grateful to the Scientific Research Council of Istanbul University (İÜ BAP) for funding this doctoral thesis under grant number 33014. This work would not have been possible without the contribution of many people whom I sincerely thank for their help and support.

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May all the people whom I have not named and who have contributed in one way or another to the realization of this work forgive me, and may they find here the expression of my perfect gratitude. |

December, 2022

|Naoual DAMIR|

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols	Explanations
FBW	: Final body weight
IBW	: Initial body weight
TL	: Total length
t	: Duration of the trial in days

Abbreviations	Explanations
AAs	: Amino Acids
ADC	: Apparent protein digestibility coefficient
ALAT	: Alanine aminotransferase
ASAT	: Aspartate aminotransferase
CAAs	: Crystalline amino acids
CAC	: Crude ash content
CF	: Condition Factor
CFC	: Crude fiber content
CLC	: Crude lipid content
CPC	: Crude protein content
CS	: Calculation of Survival
DE	: Digestible energy
DHA	: Docosahexaenoic acid
DMC	: Dry matter content
EAA s	: Essential amino acids
EPA	: Eicosapentaenoic acid
FCR	: Feed conversion ratio
FE	: Feed efficiency
FEAP	: Federation of european aquaculture producers
FM	: Fish meal
GPT	: Glutamo-pyruvic transaminase
GOT	: Glutamo-oxalo-acetic acid transaminase
HFM	: Hydrolyzed feather meal

HSI	: Hepatosomatic indices
LCMS	: Liquid Chromatography mass spectrometer
ME	: Metabolisable energy
NEAAs	: Non-essential amino acids
NNE	: Non-nitrogen extracts
PAP	: Processed animal proteins
PBM	: Poultry by-product meal
PEF	: protein efficiency ratio
SGR	: Specific Growth Rate
TCA	: Acid three phosphate
TSAA	: Total sulfur amino acid
TME	: Total Metabolizable Energy
VSI	: Viserosomatic indices
WG	: Weight gain

ABSTRACT

[THESIS Ph.D.]

[INVESTIGATION OF AMINO ACID METABOLISM OF JUVENILE GILTHEAD SEA BREAM (*Sparus aurata*) FED WITH ALTERNATIVE PROTEINS]

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[ADVISOR

Prof. Dr. Mustafa YILDIZ]

This study evaluated the effects of partially replacing (55%, 65%, 75%) fishmeal (FM) with poultry by-product meal (55PBM, 65PBM, 75PBM) or hydrolyzed feather meal (55HFM, 65HFM, 75HFM) on growth performance and amino acid metabolism of juvenile gilthead seabream (*Sparus aurata*, 35.01 ± 0.11 g). Seven experimental iso-nitrogen (49% crude protein) and iso-lipid (19% crude lipid) experimental diets were prepared based on the three meals (FM, PBM, and HFM) and fed to juvenile seabream for 120 days.

Proximate diet and whole-body analyzes were performed to determine the effects of animal protein sources (PBM and HFM) on the growth performance, quality of diet, and feed intake of gilthead seabream. The protein efficiency ratio (PER) and amino acid profile of the dietary treatments as well as the whole body amino acid profile of the fish at the start and end of the experiment were assessed to determine the effectiveness and quality of proteins and whether there are deficiencies in essential amino acids (EAAs), which may limit the growth and normal

development of gilthead seabream. The digestibility of proteins and amino acids was studied to determine whether the proteins affect seabream's protein metabolism. The amino acid profile of fish feces was assessed to determine the levels of amino acids eliminated in indigestible form. Histological examination of the liver and digestive system was performed to assess the effects of dietary treatments on the welfare of gilthead seabream.

The results of this thesis show that growth performance, feed intake, and digestibility were negatively affected when juvenile seabream were fed with HFM-based diets (55HFM, 65HFM, 75HFM) ($P < 0.05$). The partial substitution of fish meal (FM) with poultry by-product meal (PBM) in the juvenile gilthead seabream diet is feasible without compromising their growth performance. Similarly, condition factor, hepatosomatic index (HSI) and viscerosomatic index (VSI) did not differ between the experimental groups fed PBM (55PBM, 65PBM, and 75PBM) and the group fed with fishmeal (FM) ($P > 0.05$). In addition, these groups of fish had similar feed consumption and feed conversion rates to the control group ($P > 0.05$).

There was no significant difference between the whole-body protein content in the fish-fed PBM diets and the control group ($P > 0.05$). Protein Efficiency Ratio (PER) values showed that PBM contains good quality and efficient protein.

This result was confirmed by the digestibility coefficients which were similar to the control group and showed no significant difference with the control group ($P > 0.05$).

At the end of the experiment, the amino acid profile of whole-body of juvenile seabream fed with PBM-based diets was not found to be deficient in EAAs. Similarly, dietary PBM content did not negatively affect protein and amino acid digestibility in juvenile seabream. In addition, the level of EAAs and non-essential amino acids (NEAAs) eliminated in indigestible form is low and similar to the control group.

Except for a slight accumulation of a few lipid droplets in the liver of the group fed with PBM-based diets, histological examination revealed no sign of pathology of the liver or digestive tract among these dietary treatments (55PBM, 65PBM, and 75PBM). Histology of the hindgut revealed no lipid deposit accumulation in fish fed PBM-based diets.

Keywords: gilthead seabream (*Sparus aurata*), terrestrial animal proteins, hydrolyzed feather meal, poultry by-product meal, amino acid metabolism



SUMMARY

[Ph.D. THESIS]

**[INVESTIGATION OF AMINO ACID METABOLISM OF
JUVENILE GILTHEAD SEA BREAM (*Sparus aurata*) FED WITH
ALTERNATIVE PROTEINS]**

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Istanbul University

Institute of Sciences

[Department of Aquaculture and Fish Diseases]

Supervisor: [Prof. Dr.] [Mustafa YILDIZ]

[It is seen that there has been a stagnation in the amount of fish obtained through capture fisheries in the last 20 years. As known, fish protein is important for human nutrition. Due to the increasing human population, natural fish production does not meet the nutritional needs. For this reason, fish production through aquaculture has gained great importance to meet the nutritional needs of people. Aquaculture is one of the fastest growing food production sectors in the world and Turkey.

In spite of the increasing aquaculture production, it is seen that there is a gradual decrease in fish meal production. From an economic point of view, the cost of feed, which ranges from 40% to 70% of the total operating cost, has become one of the main constraints to the growth of aquaculture.

From a nutritional point of view, protein is quantitatively the most important nutrient for all fish species. Fish feeds typically contain between 20% and 60% crude protein, depending on the fish species, and fish meal is the traditional source of protein and therefore essential amino acids in aquafeeds. However, the supply of fish meal has become limited and its price has also increased. Rising costs and dwindling fishmeal supplies have prompted scientists to search for cheaper but more consistent and sustainable alternative protein sources for fish feed. Alternative vegetable and animal protein sources that can be used instead of fishmeal are being investigated. Studies have shown that many vegetable and animal proteins are used in fish feeds.

Most of the studies conducted until recent years have shown that protein sources are limited by the growth performance of fish, feed use, and body amino acid composition. In this research, we evaluated the effects of partially (55, 65 and 75%) replacing dietary fish meal (FM) with Hydrolyzed Feather Meal (HFM) or Poultry by-Product Meal (PBM) on the growth performance, feed intake, amino acid levels in the body, proteins and amino acid digestibility, and digestive system histology, in juveniles *Sparus aurata*. In particular, this thesis determined how the protein sources used in the research affected the amino acid metabolism in juvenile gilthead seabream. |

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Keywords: gilthead seabream (*Sparus aurata*), terrestrial animal proteins, hydrolyzed feather meal, poultry by-product meal, amino acid metabolism. |

1. INTRODUCTION

1.1. WORLD FISHERIES AND AQUACULTURE PRODUCTION

The fisheries and aquaculture sectors have been increasingly recognized for their essential contribution to global food security and nutrition in the twenty-first century (FAO, 2022). According to the latest United nation's (Food and Agriculture Organization) report 2022, the total fisheries and aquaculture production (not including marine plants) has significantly increased in the past seven decades going from 19 million tonnes in 1950 to a record of about 179 million tonnes in 2018, with an annual growth rate of 3.3% (Figure.1.1). However, a slight decrease from this was recorded in 2020. The decrease is most likely due to the COVID-19 pandemic, causing both social and economic damage to various sectors, including fisheries and aquaculture. This led to lockdowns and closures of markets, ports, and borders resulting in a significant slowdown of trade, and disrupting seafood production and distribution. Fishing was disrupted and aquaculture struggled to maintain its planned production cycles (FAO, 2022).

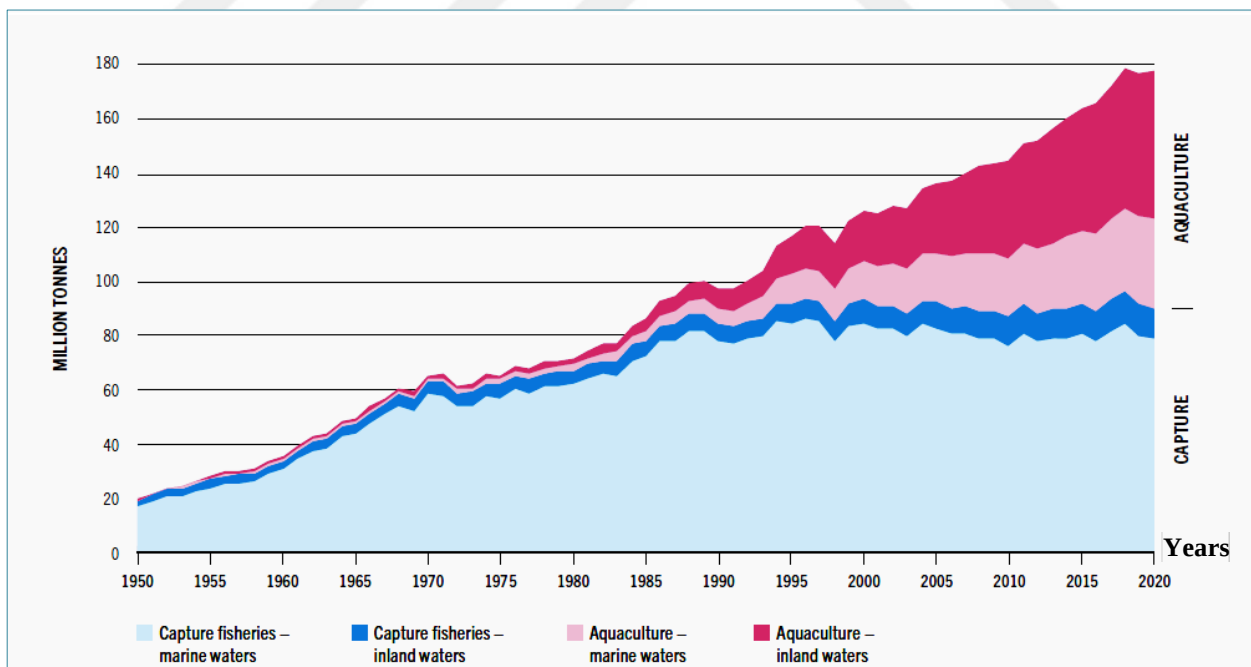


Figure 1.1: World capture fisheries and aquaculture production (FAO, 2022).

In 2020, capture fisheries and aquaculture contributed 90 million tonnes (51%) and 88 million tonnes (49%) respectively of the total fish production globally. The total production, 112 million tonnes (63%) were in marine water (70% from capture fisheries and 30% from aquaculture) and 66 million tonnes (37%) in inland waters (83% from aquaculture and 17% from capture fisheries) (FAO, 2022).

According to the FAO (2022) data, over 157 million tonnes (89%) of the total aquaculture animal products, in the world were used for human consumption and 20 million tonnes of the remaining part were destined for non-food uses, to produce mainly fishmeal and fish oil (Figure 1.2).

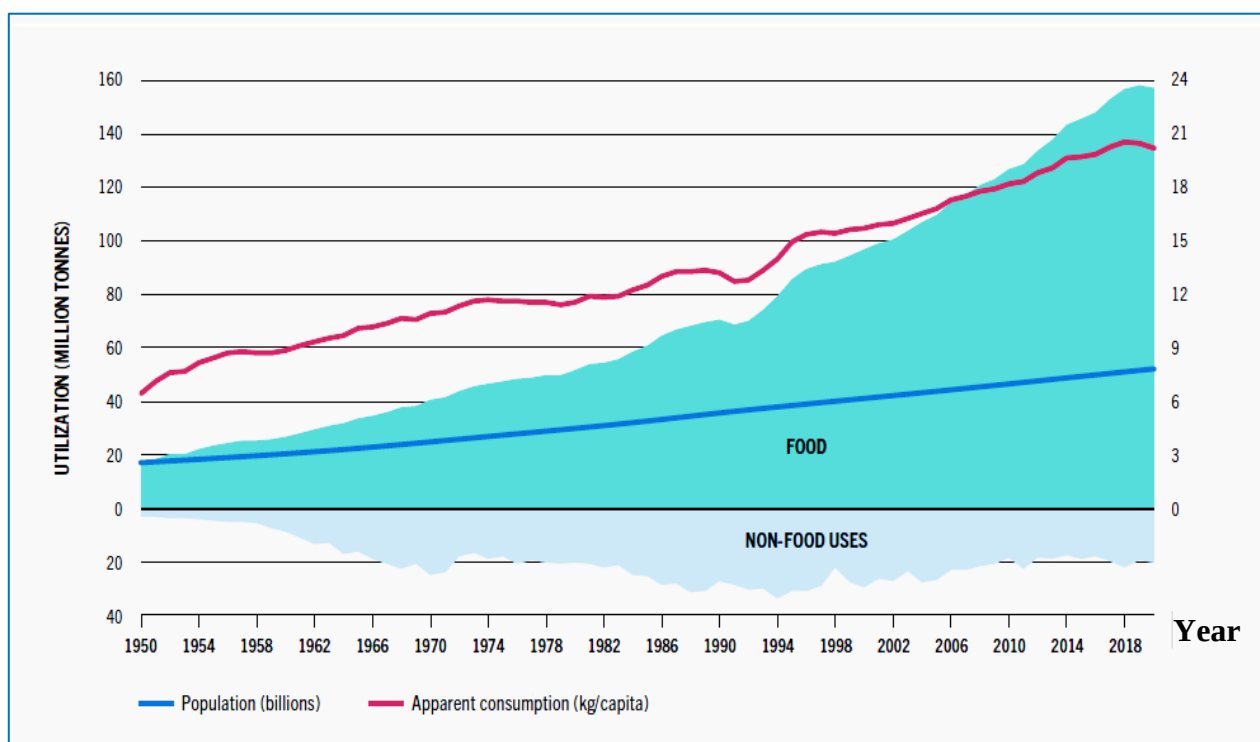


Figure 1.2: World fisheries and aquaculture production utilization and consumption (FAO, 2022).

1.2. WORLD AQUACULTURE PRODUCTION

World aquaculture production is expected to continue to grow, as part of the solution to provide food for more than nine billion people in 2050 (FAO, 2018). Aquaculture production is expected to increase to 106 million tonnes in 2030, with an overall growth of 22% or nearly 19 million tonnes compared with 2020. The share of farmed species in global fisheries and aquaculture

production (for food and non-food uses) is projected to grow from 49 percent in 2020 to 53% in 2030 (Figure 1.3) (FAO, 2022).

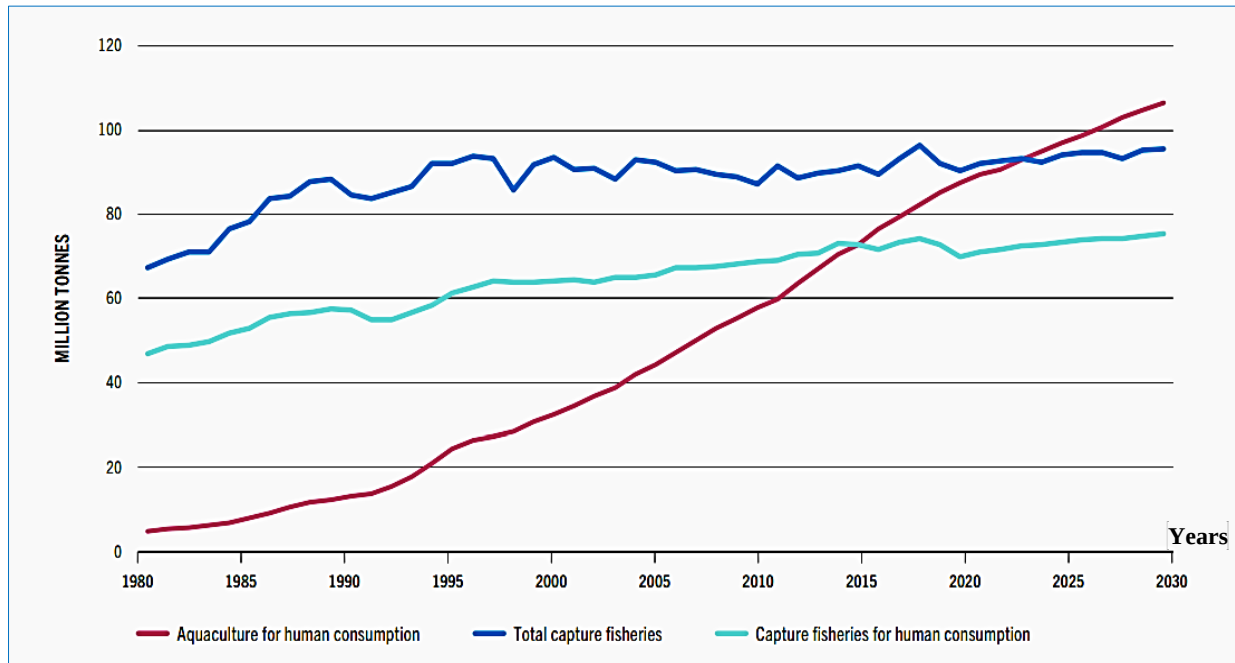


Figure 1.3: World capture fisheries and aquaculture production, 1980–2030 (FAO, 2022).

The data of FAO published in 2022, reported that in 2020, the finfish represented 76% of the total production of aquatic animals, with marine fishes representing 51% of the total finfish and 39% of the total aquatic animal production, followed by freshwater fishes, representing 43% of the total finfish and 33% of the total aquatic animal production (FAO, 2022). World marine and coastal aquaculture production of finfish is dominated by Asian countries with China being the biggest producer, with 1.7 million tonnes, followed by Norway (1.5 million tonnes), Chile (1.1 million tonnes), Indonesia (901 Tt), Philippines (412 Tt), Egypt (350 Tt), VietNam (305 Tt) and in the eighth position Turkiye with a production of 289 Thousand tonnes (Figure 1.4) (FAO, 2022).

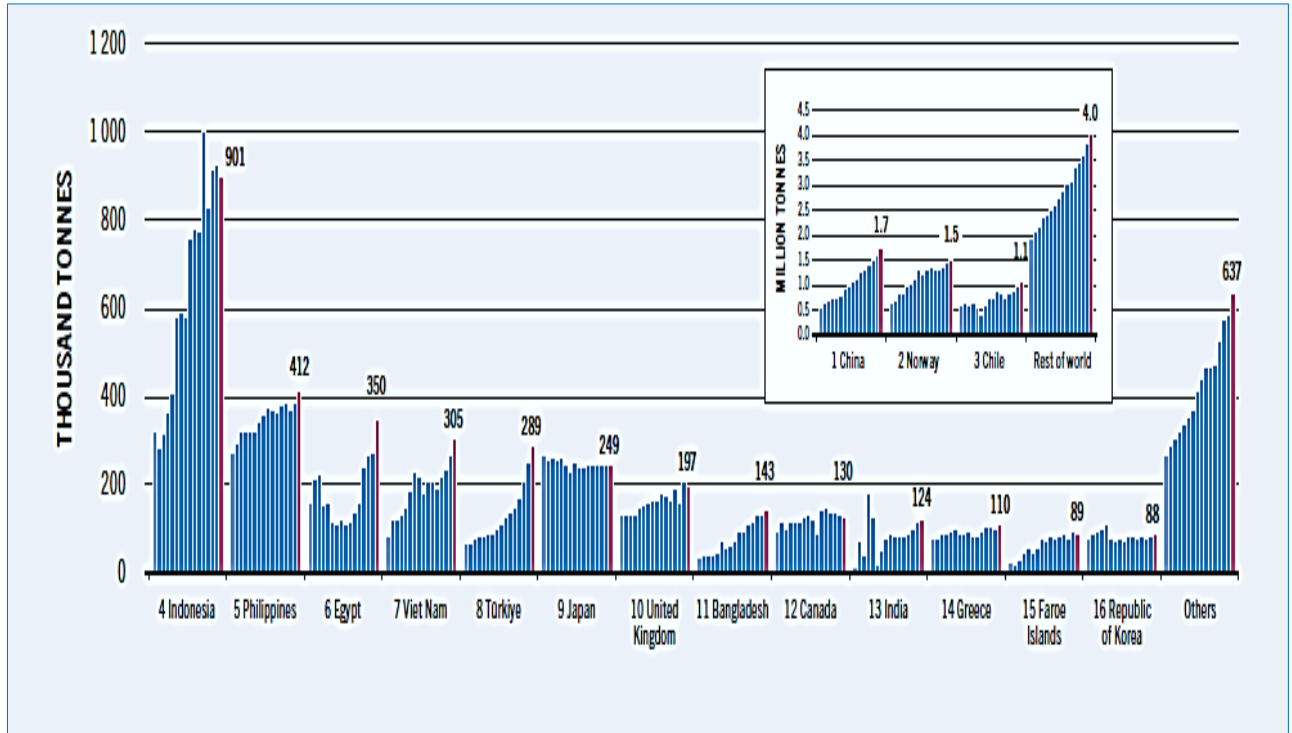


Figure 1.4: World marine and coastal aquaculture production of finfish by major producers (FAO, 2022).

1.3. AQUACULTURE PRODUCTION IN TURKEY

Turkey has a coastline of almost 8300 km along the Mediterranean sea in the South, the Aegean sea in the west, and the Black sea in the North, with three major varied biogeographical and climate, which give the country the characteristics necessary for the development of marine aquaculture. This country has also fluvial, lakes, and reservoirs, which provide the ideal condition for developing inland aquaculture. With many areas sheltered in bays, Turkey has an ideal position to become a global aquaculture producer. Aquaculture in Turkey started in the 1960s with the culture of rainbow trout and progressed along the Aegean and Mediterranean seas with seabream /seabass in the mid-1980s, which was followed by the cage culture of trout in the Black Sea during the 1990s (Uğur *et al.*, 2020). According to the Turkish Statistical Institute (TURKSTAT) 2022, the Turkish aquaculture sector has been growing steadily over the years. Inland water aquaculture is overwhelmingly dominated by the cultivation of salmonids, mainly rainbow trout, which accounts for 30% of the total (both marine and inland) farmed fish production.

According to TURKSTAT (2022), in 2021, aquaculture production increased by 11.9%, occurring as 472 Tt, 295 Tt at the marine fish, and 33 Tt at the inland waters (Figure 1.5, TURKSTAT, 2022).

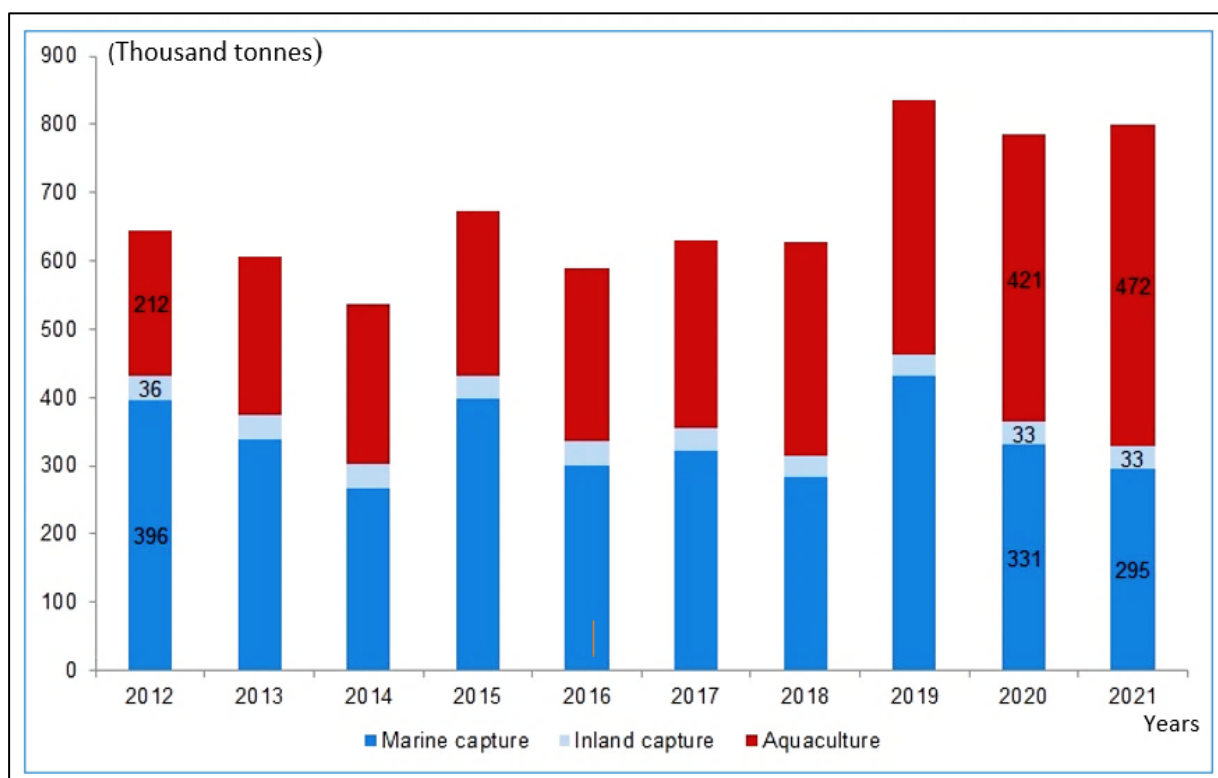


Figure 1.5: Fishery and aquaculture production in Turkey (2012-2021)
(TURKSTAT, 2022).

Inland water aquaculture was dominated by the cultivation of salmonids, mainly rainbow trout, which accounted for 30% of the total (both marine and inland) farmed fish production. Marine aquaculture was represented by sea bass (*Dicentrarchus labrax*) 155 Tt and seabream (*Sparus aurata*) 133 Tt (Figure 1.6) (TURKSTAT,2022). Yıldız and Ofori-Mensah (2017) reported that sea bass and seabream produced from culture and capture fisheries are ideal dietary sources of nutritional protein and AAs as they meet the human dietary requirements of protein and/or AAs.

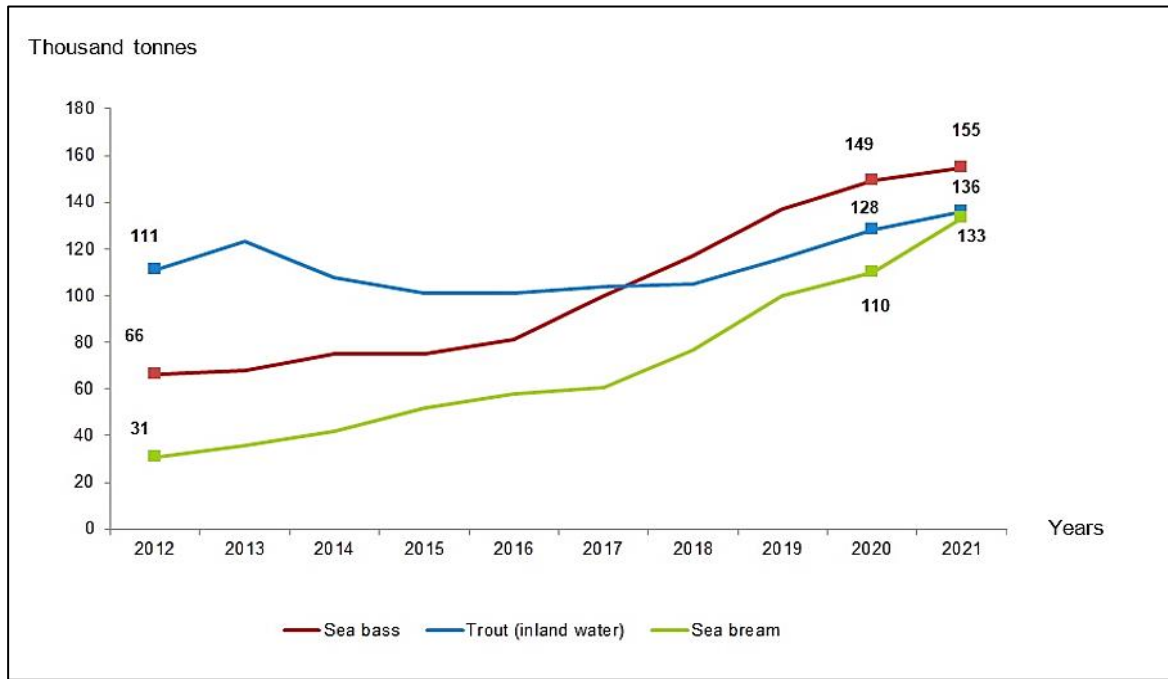


Figure 1.6: Major marine fish species production in Turkey (2012-2021)
(TURKSTAT, 2022).

1.4. WORLD AQUACULTURE AND AQUAFEED USE

World aquaculture production continues to grow each year. This growth rate needs a similar sustainable supply of feed input to meet the demand (Tacon and Metian, 2008). Figure 1.7 shows that marine fish production was estimated at 3.10 million tonnes and increased to production of 5.89 million tonnes. Similarly, total commercial feed usage was estimated at 4.32 million tonnes in 2017 and is estimated to increase to 5.89 million tonnes by 2025. Thus, a parallel relationship between world marine fish production and aquafeed use exists.

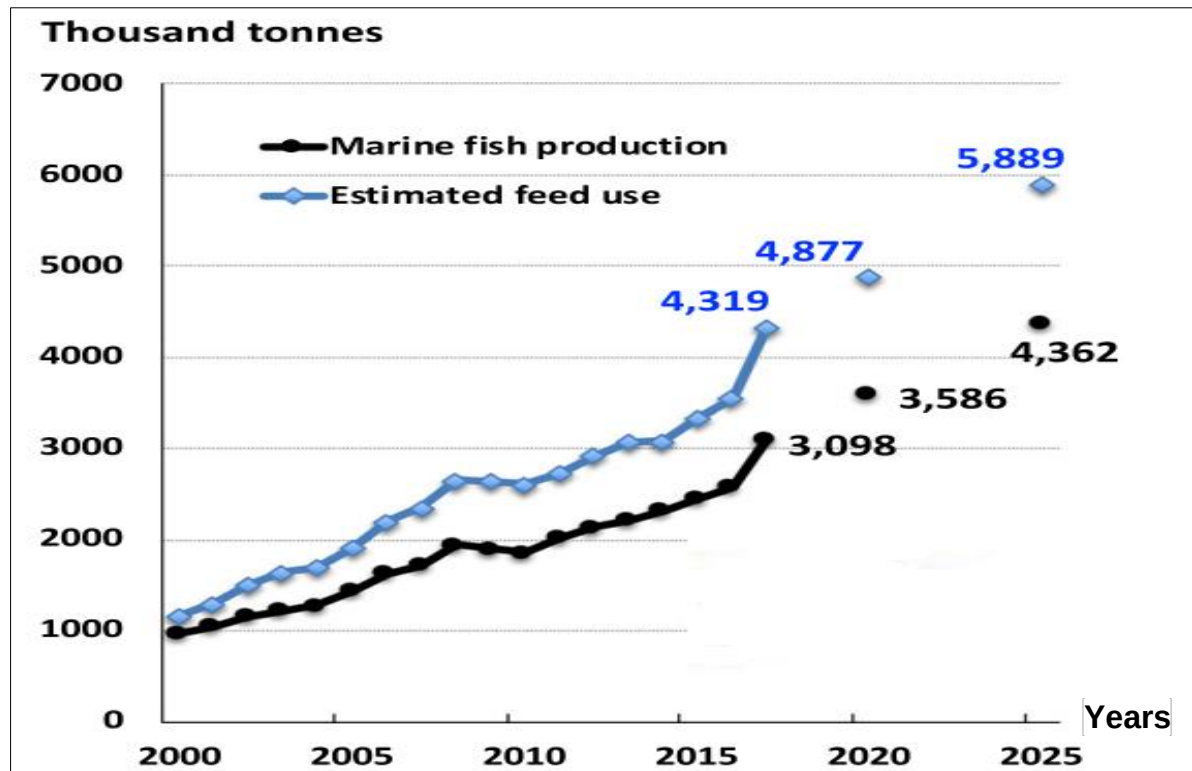


Figure 1.7: World marine fish production (FAO 2019) and estimated commercial feed usage between 2000 and 2025 (Albert G. J. Tacon, 2019).

One of the most important factors that will hinder the development of global aquaculture is feed (FAO, 2016). Along with the increasing demand for aquafeed, the prices of fish feed are also increasing because, in the aquaculture industry, more than 70% of aquaculture production is based on feed input, which typically accounts for 50%–60% of the total operating costs (Esmaeili *et al.*, 2016). This high price of aquafeed is often caused by the shortage and the rising cost of fishmeal and fish oil. According to the estimates of the Marine Ingredients Organization (IFFO), in 2020 about 86% of fishmeal was used in aquaculture, while 9% was destined for pig farming, 4% for other uses (mainly pet food), and 1% for poultry. In the same year, about 73% of fish oil was destined for aquaculture, 16% for human consumption, and 11% for other uses (including pet food and biofuel) (Figure 1.8) (FAO, 2022).

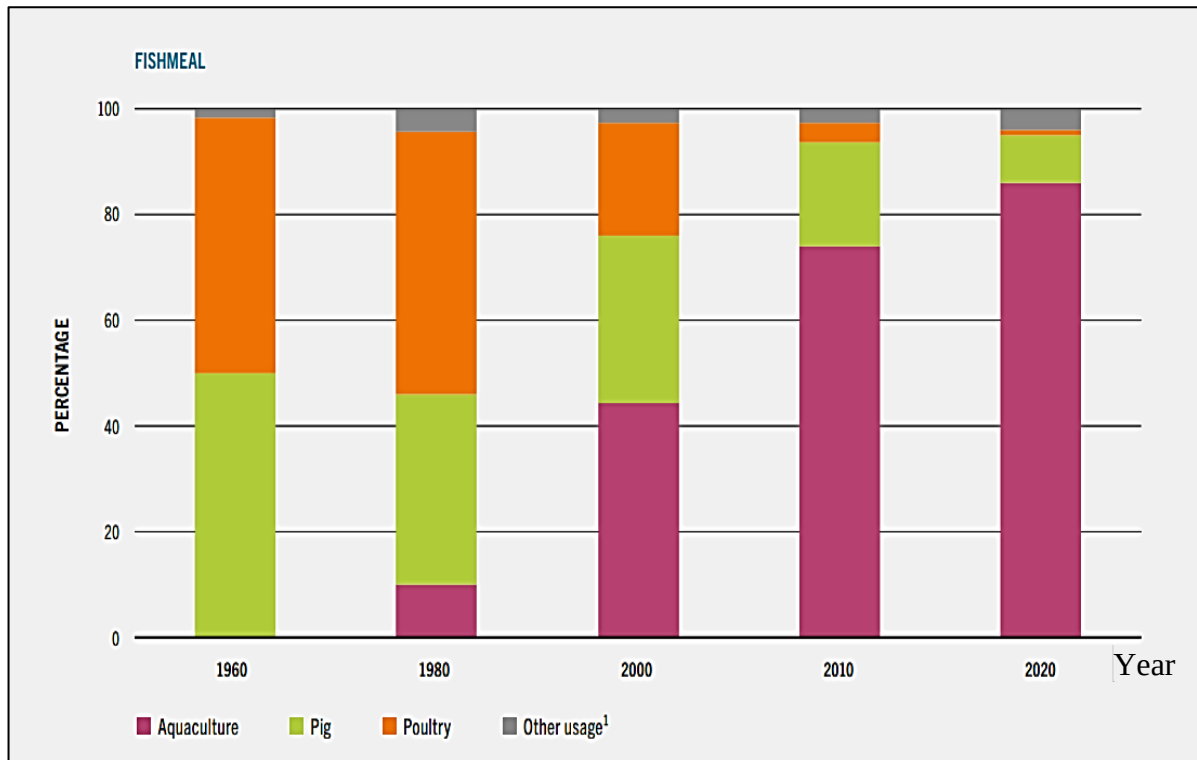


Figure 1.8: Global fishmeal utilization (FAO, 2022).

The increasing demand for fishmeal led to an increase in its price (Figure 1.9) (FAO, 2022), resulting from increasing demand and decreasing availability of forage fish.

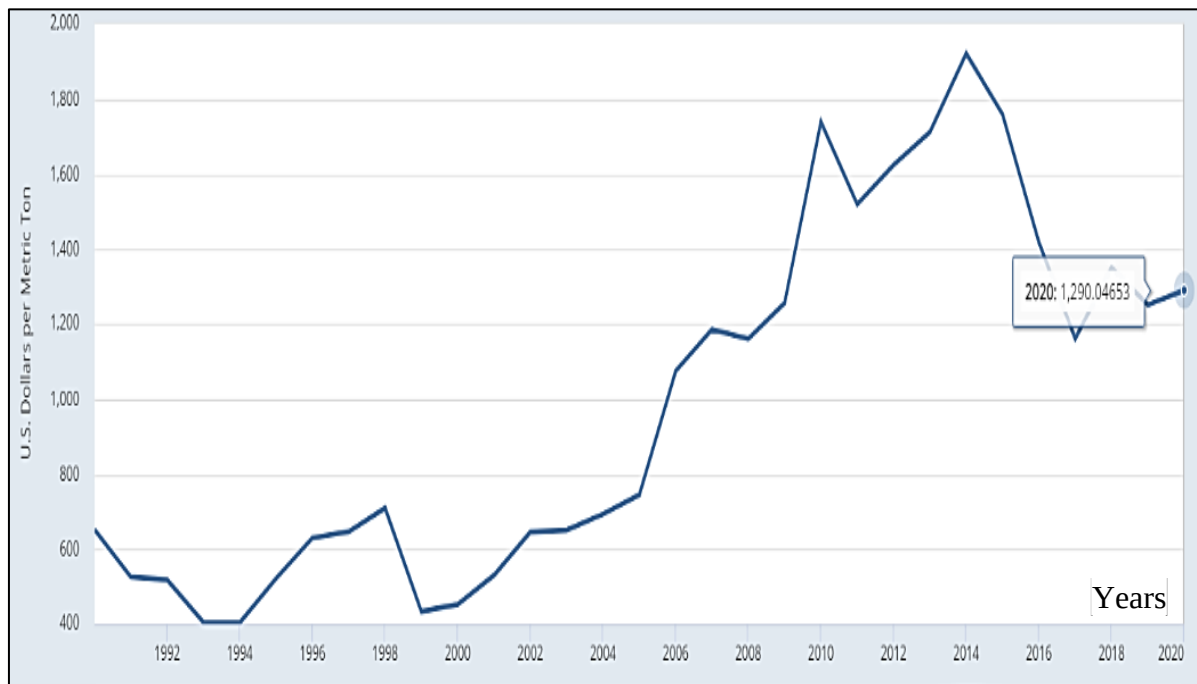


Figure 1.9: Global price of fish meal (International monetary fund, 2022).

However, due to high cost, fluctuating supply, unavailability, and shortage of quality, aquaculturists are continuously searching for alternative protein sources (plant and animal origin) as a replacement for fishmeal in aquafeed.

Aquaculture feed is a key sector for developing sustainable aquaculture. According to the FAO (2022) estimates both the production of fish and demand for aquaculture feed is expected to increase significantly till 2050. Therefore, emphasis on continued research to replace fishmeal with alternative and new ingredients, such as animal by-products, seaweed, and plant-based or insect sources is warranted.

1.5. SOME ALTERNATIVE PROTEIN SOURCES IN GILTHEAD SEABREAM DIET

1.5.1. Animal by-Products

The increase in aquaculture production lead to the rapid development of aquafeed production, with fish meal in the past decades being the preferred source of protein in aquafeeds, due to their high-quality (usually 60-75% protein content). It has an excellent amino acid profile (Gatlin *et al.*, 2007; Miles and Jacob, 2011; Hill *et al.*, 2019), high nutrient digestibility, and the absence of antinutrient factors. As aquaculture increases in production, it is only natural that the demand for fishmeal and fish oil will become even higher and according to the “The State of World Fisheries and Aquaculture” report (FAO, 2016), it is expected that during the 2010-2030 period, fishmeal prices are going to escalate up to 90%. If aquaculture wants to maintain the expansion verified in the previous years, the industry cannot keep relying on a protein source that is known to have such a high variable cost and that relies on the harvest of wild stocks. With this in mind, it is essential to look for more sustainable and less expensive alternatives that can partially or even completely replace fishmeal without negatively affecting fish performance.

Sustainable aquaculture will depend in part on a reduction in fishmeal use in aquafeed because of its increasing demand, unstable supply, and high price. There is a need, therefore, to look for sustainable alternatives. Alternative ingredients can usually be divided into those being derived from either terrestrial plant or animal origin (Nogueira *et al.*, 2012).

Finding an alternative source of protein to replace fishmeal to develop sustainable and cost-effective aquafeed is one of the major topics in aquafeed research.

Reducing the amount of fishmeal in diet formulations, without harming the fish performance, could have a positive impact on the profitability of commercial fish production. Several scientific researchers were interested in the effectiveness of different alternative proteins of both plant and animal origin in different farmed fish species *Salmo gairdneri* (Dabrowski *et al.*, 1989), *Sparus aurata* (Gomez-Requeni *et al.*, 2003; Gomez-Requeni *et al.*, 2004), *Pagrus major* (Biswas *et al.*, 2007), *Oncorhynchus mykiss* (Yürüten Özdemir and Yıldız., 2019). In general, animal protein sources do better than plant proteins in diets, especially for carnivorous species (Hardy, 1998). Compared to fish meal, plant proteins used in the aquafeed industry have progressively increased, although the use of these resources in fish diets has been limited by the presence of a variety of anti-nutritional factors (Francis *et al.*, 2001; Gatlin *et al.*, 2007; Kaushik, 1990), deficiency in some indispensable amino acids (Hardy, 2006) and reduction in protein digestibility (Santigosa *et al.*, 2008). However, terrestrial animal proteins categorized as Processed Animal Proteins (PAP) like poultry by-product meal (PBM), hydrolyzed feather meal (HFM), etc., were reintroduced in aquafeeds in European Union (EU) since July 1st, 2013 (European Commission, 2013).

Compared to plant proteins alternative animal proteins can substitute fishmeal in a farmed fish diet due to their abundance, higher protein contents, high digestibility, less expensive compared to fish meal, and being free from anti-nutritional factors (Bandara, 2018). Hertrampf *et al.*, (2000) mentioned in their book “*Handbook on Ingredients for Aquaculture Feeds*” that « poultry by-product meal is a good source for essential amino acids ». Fontinha *et al.* (2021) described that poultry by-product meal has a high protein content (58-65%) and essential amino acid profile similar to that of fishmeal, except for the lower levels of lysine and methionine. In this context, Davies *et al.* (2009) reported that fish as *Sparus aurata* do not have a definitive protein requirement as such, but instead require a balanced complement of the 10-essential amino acids (EAAs) to meet known requirements. Bandara (2018), showed that poultry by-product meal include a low level of some essential amino acids (lysine, methionine, and histidine), the effect on nutrient availability by processing technology variable level of digestibility and impaired growth rates at higher inclusion levels.

Hydrolysates of animal by-products are rich in proteins and their protein composition depends directly on the organs (feather, skin, etc.) used for hydrolysis. In the field of nutrition, hydrolyzed proteins have advantages because they have better digestibility (Robert, 2014).

Feather meal is rich in indispensable amino acids such as cysteine, threonine and arginine, and pepsin digestible protein (Nogueira *et al.*, 2012). Bureau *et al.* (1999) and Shapawi *et al.* (2007), reported that the higher inclusion rates of feather meal in fish diets are dependant upon production technology. Laporte (2007) indicated that the hydrolysis of feathers transforms the keratin which is not digested by mono-gastric animals and fish in its original structure into better-digested peptides. In aquafeed, the introduction of hydrolyzate in fish feed is known to improve growth in several species: *Oreochromis mossambicus* (Abwao Ocho, 2014), *Clarias gariepinus* (Absalom *et al.*, 2017), *Dicentrarchus labrax* (Campos *et al.*, 2017), *Sparus aurata* (Psofakis *et al.*, 2020). Several studies also show an increase in food intake (Refstie, 2004), improved feed efficiency (Aksnes *et al.*, 2006) and protein digestibility (Psofakis *et al.*, 2020). The use of hydrolysates also shows an improvement in the maturation of the digestive system in larvae (Zambonino-Infante *et al.*, 1997) and improves digestion and assimilation of nutrients.

Gilthead seabream (*Sparus aurata*) is one of the most important farmed fish species worldwide, particularly in the Mediterranean and Europe (FEAP, 2020). As this species is carnivorous, fishmeal is an excellent source of protein and its balanced amino acids profile (Gatlin *et al.*, 2007; Miles and Jacob, 2011). The increase in fishmeal demand will potentially increase the cost of aquaculture feed (Tacon and Metian, 2008). For sustainable aquaculture it is very interesting from an economic and environmental point of view to select new sources of protein that are nutritionally adequate for each species, available on the market at a competitive price and respectful of the environment. For this objective, several researchers involved in protein nutrition are studying various alternative protein sources to find good quality and economically sustainable alternative protein sources to replace fishmeal in the gilthead seabream (*Sparus aurata*) diet (Laporte, 2007; Hardy, 2010; Davies *et al.*, 2018).

1.6. OBJECTIVES OF THIS STUDY

Feeding the rapidly growing world population depends on increasing agricultural production and its fair distribution. The world population, which was 3 billion in the 1960s, is around 8 billion today, and if the population growth rate continues in this way, it will be on the threshold of 10-12 billion in the 2050s. Considering that the current world population has serious problems with eating a balanced and healthy diet, it is certain the situation tomorrow will not be any more trouble-

free than today. With the increase in aquaculture production in the world, feed consumption is also increasing. As it is known, fish oil is used as an energy source and fish meal is used as a protein source in fish feeds. However, the prices of these raw materials are increasing due to the limitations in fishmeal and fish oil production. Carnivorous fish species need 40-50% protein in their feed for normal development. For this reason, it has become a necessity to use a fish meal to provide essential amino acids from basic nutrients in feeds. Today, with the increase in the need for fish feed, the need for fish meal is also increasing.

However, fish meal produced today has become unable to meet the needs of aquaculture feeds. Therefore, it is of great importance to research alternative protein sources that can be used in feeds. Feeds for carnivorous finfish like gilthead seabream are usually composed of fish products, like fishmeal and fish oil, because farmed fish need to eat diets with similar nutritional profiles to those they would have in the wild. However, because fish ingredients are limited and expensive, they are often partially or totally replaced with other ingredients.

In order to develop sustainable aquaculture, it is therefore essential to be able to have feed guaranteeing both; maximum growth of raised species and sustainable management of wild fish stocks while having low production costs (for example; reducing the use of fishmeal in feeding by introducing alternative sources of protein efficient). This objective gives rise to numerous scientific studies on the nutrition of farmed fish and creates a constantly evolving field of research.

The aim of the present study was to evaluate the effects of replacing dietary fish meal with poultry by-product meal or hydrolyzed feather meal in the diet on growth performance, amino acid metabolism and histology of liver and digestive system in juvenile gilthead seabream.

The specific objectives of this doctoral thesis are to:

- i) Assess the growth performance and survival rates of juvenile seabream during and after feeding experiments,
- ii) Analyzed the whole-body amino acid composition,
- iii) Amino acid digestibility was analyzed,
- iv) Examine the histology of the liver and digestive system,

2. GENERAL PARTS

2.1. BIOLOGY OF GILTHEAD SEABREAM

Gilthead seabream (*Sparus aurata* Linnaeus, 1758), is systematically classified at the level of perciform fish which belongs to the family of Sparidae, genus *Sparus*, species *aurata*. According to Quero and Vayne (2008), the distinguishing features of this species are the presence of a golden stripe on the forehead, between the eyes, and the presence of a black spot at the beginning of the lateral line. In addition, it has from 4 to 6 strong canines in the front of each jaw, long and sharp teeth; and laterally 2 to 4 rows of molars (Figure 2.1.A). With regards to geographical distribution, it is common throughout the Mediterranean (Figure 2.1.B), present along the eastern Atlantic coast from Great Britain to Senegal, and rare in the Black Sea. Gilthead seabream is normally found on rocks and seagrass beds (*Posidonia oceanica*) but is also frequently caught on sandy bottoms. Young fish stay in relatively shallow areas (up to 30 m), while adults can reach deeper waters, usually no more than 50 m. (FAO, 2006).

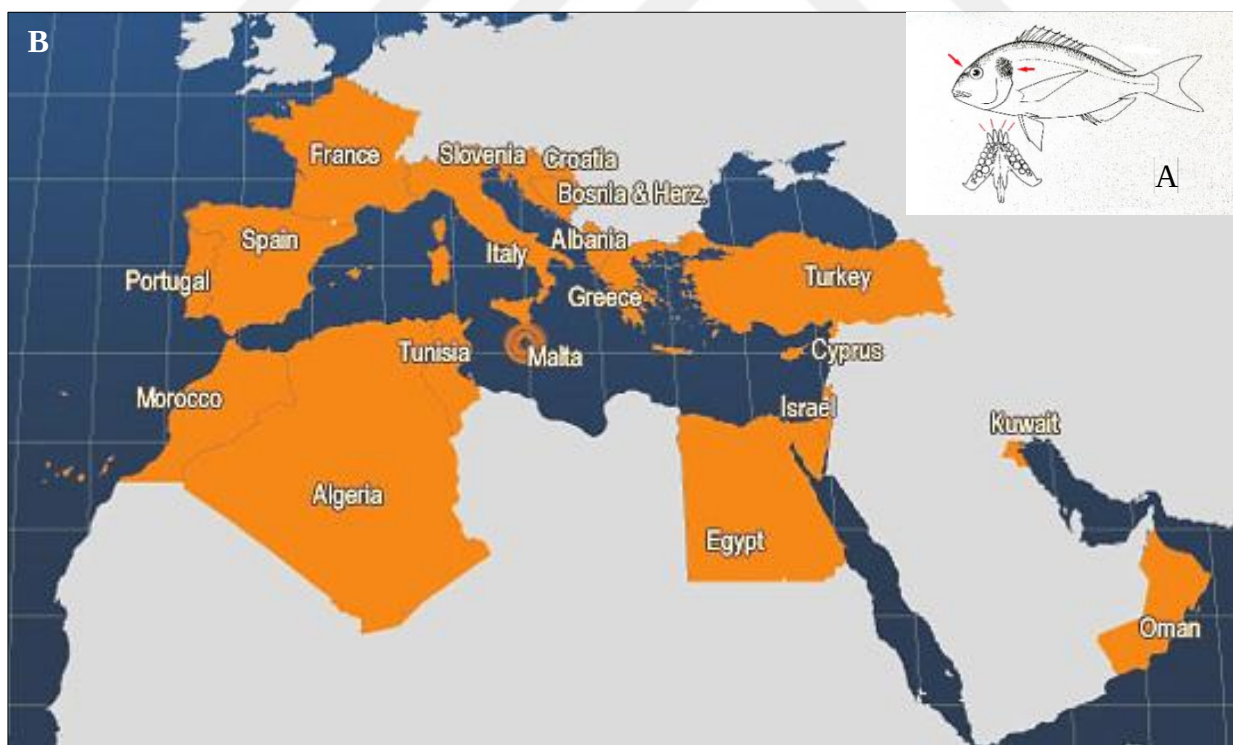


Figure 2.1: Major countries producing seabream (FAO Fishery Statistics, 2006).

Sparus aurata is generally a protandry hermaphrodite; it is the first male until the age of 2 years (20-30 cm) then it becomes female at the age of 2-3 years (33-40 cm). cm) (FAO, 2006). Generally,

in this specie, reproduction takes place from December to April at water temperatures of 13 to 17°C. Females can lay 20,000–80,000 eggs each day for up to 4 months. The production cycle of seabream is shown in Figure 2.2. At the start of the breeding season, the brood stock is transferred to the breeding tanks. For the rootstocks to produce eggs more easily they can be manipulated by the environment and/or by hormones. (FAO, 2006). Hatching begins about 48 hours after laying at 16/17°C. Newly hatched gilthead seabream larvae have their eyes and mouth closed and must rely on their egg sac for food (Laporte, 2007). They exhaust their yolk sac after 3–4 days of endogenous feeding. After this stage, the eyes are pigmented and the mouth is developed, allowing the larvae to feed on the larvae of other organisms. In the intensive rearing system, seabream larvae are fed first with rotifers (*Brachionus plicatilis*); which are chosen because they are easy to grow on a large scale.

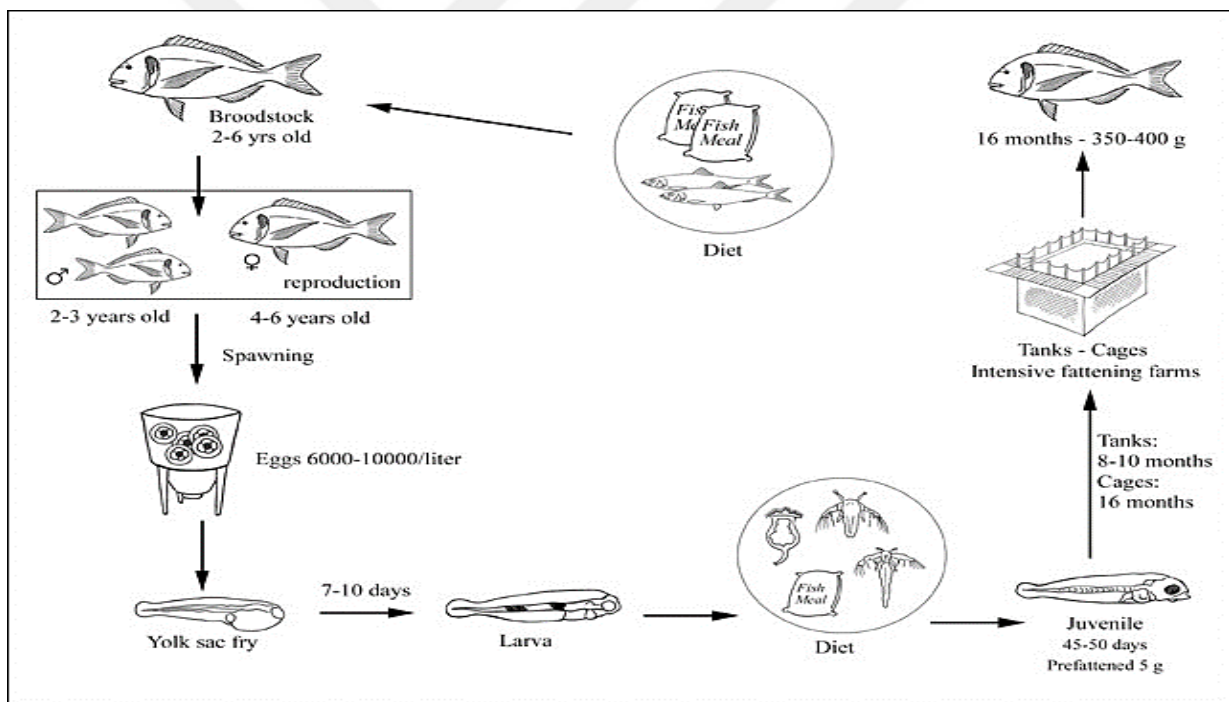


Figure 2.2: Production cycle of *Sparus aurata* in intensive breeding system (FAO, 2005-2021).

After 10–11 days, the rotifers are distributed with *Artemia salina* nauplii until the larvae complete their metamorphosis (32–35 days after hatching).

Before being distributed to the gilthead seabream larvae, rotifers and brine shrimp are enriched daily with commercial lipid preparations, to improve their levels of certain essential fatty acids

(EPA; DHA) and vitamins, which are critically important for good growth, development and survival. They continue to be fed brine shrimp until they reach a weight of around 5-10 mg. They are then fed with dry and formulated food rich in protein (50-60%) in large production tanks containing water at 18°C and 37‰ salinity.

After the larval stage, the juveniles of about 45 days are usually transferred to tanks or cages of different capacities, depending on their size and production requirements (10-20 (individuals/litre) at the temperature of 18°C and between 35-37‰ salinity. In this stage, feeding is usually done by automated systems or by hand. Typically, small individuals (1-3 g) are hand-fed using bait microparticles of 150 to 300 µm at intervals of 2 hours between 8 a.m. and 8 p.m. The food distributed is adjusted according to the size of the fish and the temperature of the water used. It is important to make a selection of individuals at least two or three times during the rearing season to avoid growth differences between reared fish. Juveniles of gilthead seabream reach commercial size (between 350 and 400g) after one and a half years on average (FAO, 2006).

2.2. WORLD AQUACULTURE PRODUCTION OF GILTHEAD SEABREAM

According to FAO (2021), seabream is the 33rd largest fish farmed in global aquaculture. The total world production of gilthead seabream in 2019 was estimated at 258,754 Ton (FAO, 2021). Turkey has overtaken Greece and Egypt has overtaken Spain since 2015. Figure 2.3 represents the top 9 world-producing countries of gilthead seabream in 2019. Turkey leads the world production with a share of 38.54% of production, followed by Greece with 21.43% and Egypt with 13.87%. (Food business, 2021).

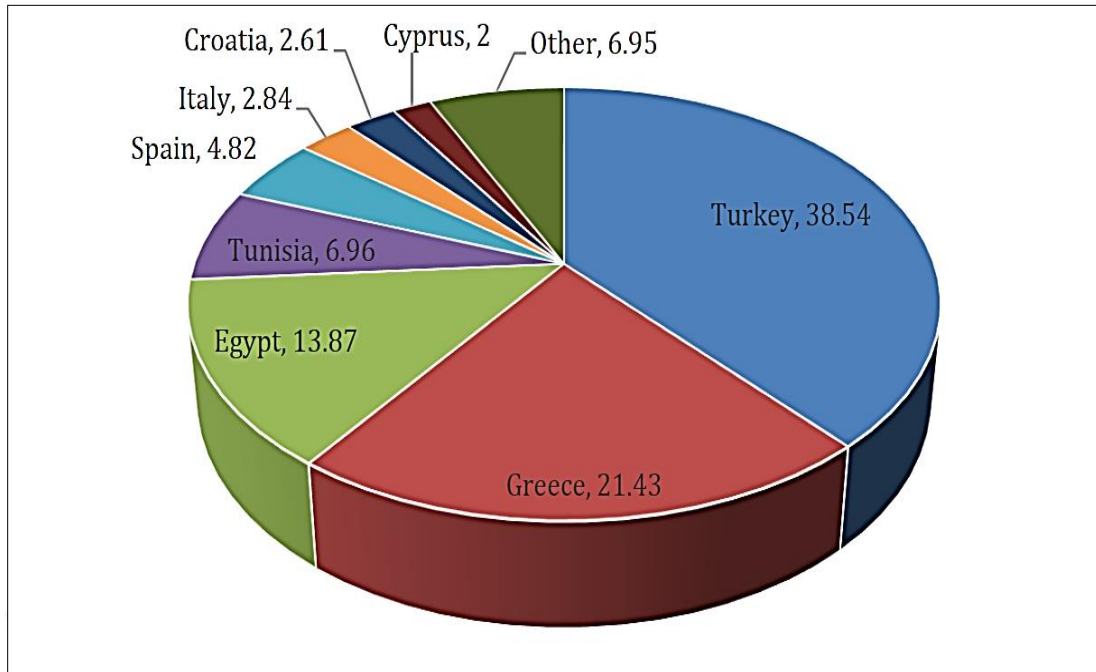


Figure 2.3: Top 9 countries production of gilthead seabream in 2019
(Food business, 2021).

2.3. PROTEIN REQUIREMENT IN DIET OF GILTHEAD SEABREAM

Proteins are polymers of amino acids arranged into polypeptides that are involved in the structure and many key physiological functions. In fish nutrition, protein is the most important and expensive ingredient. Proteins are large complex organic nitrogenous molecules. They are present in the cells of all animals and plants which are essential for the growth and maintenance of life. Compounds of amino acids linked by peptide bonds and cross-linked between the chains by sulfhydryl and hydrogen bonds. There are about 300 amino acids in natural sources, but only 20 form the basis of most proteins, each with different physical and chemical properties. For this reason, it is necessary to precisely determine the exact inclusion levels of the different protein sources in the feed formulation. The protein used in the formula must be balanced in amino acid composition with respect to the needs of the species. Nutrient balance and ingredient cost affect the profitability of feeds. The optimal level of protein in the fish diet is generally dependent on the fish species (salmonids, carnivorous...), its physiological state (age and size), protein quality, environmental parameters and feeding level. *Sparus aurata* is a carnivorous species, and has dietary protein requirements generally higher than that of salmonids (Laporte, 2007). The first dose-response survey conducted by Sabaut and Luquet (1973) showed that, juvenile gilthead

seabream require 40% protein in their diet. Dietary protein is used by fish in three different ways; in growth through the synthesis of new tissues, in maintenance (source of energy, synthesis of carbohydrates, lipids, proteins, hormones, enzymes, antibodies), and in the reconstitution of depleted tissues. These major tasks are, in fact, accomplished through the catabolism and anabolism of amino acids: although we often speak of a dietary need for protein, it is indeed essential to note that fish do not have a need for proteins as such but a need for a certain level of well-balanced essential amino acids that fish cannot synthesize (Laporte, 2007).

2.3.1. Amino Acids

Amino acids (AAs) are the building blocks of proteins. AAs constitute the monomers of proteins. In addition to the carbon, hydrogen and oxygen found in carbohydrates and lipids, they contain nitrogen. All AAs used for protein synthesis have the same general structure.

They have two functions; a carboxylic acid function ($-\text{COO}^-$) and an amino function ($-\text{NH}_3^+$). These two functions are carried by the same carbon atom called alpha carbon (the carbon atom next to the carboxylate group) in an L configuration, an atom of hydrogen and a chemical group called a. side chain ($-\text{R}$) characteristic of each different amino acid. AAs are found in two forms called the D and L forms, that is, they rotate the plane of polarized light as in the D and L forms of carbohydrates (mirror images). In nature, most AAs are L-form especially those in animal tissues such as in fish, unlike plants and microorganisms which can synthesize D-amino acids and fish have the ability to utilize D-amino acids to build tissues (Kim *et al.*, 1992a; Deng *et al.*, 2011). Each AAs has unique characteristics resulting from the size, shape, charge, hydrophobicity, and reactivity properties of its R group. Accordingly, the R group of AAs exerts a profound effect on the structure and biological activity of proteins.

therefore, amino acids are often classified according to the polarity of the side chain ($-\text{R}$) in to:

- 1) *neutral amino acids nonpolar aliphatic* (glycine, alanine, valine, leucine, and isoleucine);
- 2) *neutral amino acids polar aliphatic* (serine, threonine, and glutamine);
- 3) *neutral amino acids aromatic* (phenylalanine, tyrosine, and tryptophan);
- 4) *neutral amino acids sulfur-containing* (cysteine, methionine);
- 5) *neutral amino acids containing a secondary amino group* (proline);
- 6) *acidic amino acids* (aspartic acid, glutamic acid); and

7) *basic amino acids* (lysine, arginine, histidine)

Francesca *et al.* (2020) have summarized in Table 2.1 the major roles of amino acids in fish tissues.

Table 2.1: The major roles of amino acids in fish tissues (Francesca *et al.*, 2020).

Tissues	Amino acids	Physiological and/or metabolic role
Liver	Met	The main role of Met is the synthesis of taurine (for bile production)
	Val and Ile	Val and Ileu are used as glucogenic amino acids
	Leu	Leu could be metabolized to ketone bodies, providing energy for tissues such as brain and heart during starvation
	Ala	Ala is used as substrate for glycogen and/or glucose production in liver
	Met, Leu and Lys	In hepatocytes, Met, Leu and Lys could regulate glycolysis, gluconeogenesis and lipogenesis; particularly relevant their involvement in regulation of metabolism related gene expression
Blood system	Ala, Gln, and Arg	Concentration of Ala, Gln, and Arg in plasma may be increased during long-term starvation for gluconeogenesis
	Gln, Arg and Orn	Gln, Arg and Orn contribute to remove ammonia from blood during food deprivation
Nervous system	Glu	The main role of Glu is to provide energy for the enterocytes and precursors of biologically molecules like glutathione
	Gly	Inhibitory neurotransmitter in the central nervous system; co-agonist with glutamate for N-methyl-D-aspartate receptor receptors; antioxidant; anti-inflammation; one-carbon metabolism; conjugation with bile acids
Muscle	Ala, Asp, Glu, and Leu	Ala and Asp serve as glucogenic precursors during the rest time. Moreover Ala concentration in muscle is increased to support muscular activity Leu is oxidized during food deprivation in fish to produce ATP for swimming
Gut	Glu and Gln	Glu and Gln are major energy source, and used for glutathione synthesis. They play a crucial role intestinal health of fish, by modulating intestinal structure, protecting against oxidative damage and acting as energy substrate for the enterocytes.
	Arg	Arg has a role to improve intestinal health. Increased activity of several intestinal enzymes and changed composition of the intestinal microbiota.

All 20 amino acids were traditionally classified nutritionally into two groups. Essential (indispensable) amino acids (EAAs) and Non-essential (indispensable) amino acids (NEAAs)

2.3.1.1. Non-Essential Amino Acids (NEAAs)

Amino acids considered non-essential (NEAAs) for fish are: alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine and tyrosine.

These so-called non-essential amino acids (NEAAs) because they can be adequately synthesized by the fish's body at higher rates. Either from cellular metabolic intermediates or by transamination of other amino acids. Therefore, These non-essential amino acids are of nutritional importance because their presence in the diet helps conserve the energy needed for protein synthesis. Diets completely devoid of NEAAs have been shown to result in reduced growth rate of fish (Schuhmacher *et al.* 1995). Some dispensable amino acids can partially replace or spare essential amino acids. Phenylalanine can be partially replaced by tyrosine and methionine by cystine. Thus, phenylalanine and methionine requirements decrease when tyrosine and cystine, respectively, are present in the diet. The ratio of essential amino acids to non-essential amino acids (EAAs/NEAAs ratio) in dietary protein has important effects on protein utilization by fish.

2.3.1.2 Essential Amino Acids (EAAs)

There are ten AAs considered required by fish for growth and maintenance of life. These essential amino acids (EAAs) are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Millamena, 2002). These EAAs cannot be synthesized in the animal's body or are insufficiently synthesized *de novo* by the animals in an amount sufficient to maintain maximum animal development.

Thus, they must be provided by food under conditions where the utilization rates are higher than the synthesis rates (Sergio., 2016). Millamena (2002) reported that; essential amino acids in addition to being the building blocks of proteins, can also be precursors or provide part of the structure of other substances. Methionine is the precursor of cysteine and cystine. Methionine also provides methyl groups (CH₃) for creatine, choline and many other substances. When a hydroxyl (OH) group is added to phenylalanine, tyrosine is formed. tyrosine is needed to form the hormones thyroxine, epinephrine and norepinephrine, and the pigments melanin. Arginine gives ornithine when urea is formed in the urea cycle. Removal of a carboxyl (COOH) group from histidine forms histamine. Tryptophan is the precursor to serotonin and the vitamin nicotinic acid. All fish need the same ten essential amino acids.

2.4. AMINO ACIDS REQUIREMENTS OF JUVENILES GILTHEAD SEABREAM

In fish, a diet containing an optimal essential amino acid (EAAs) profile and sufficient protein content is a prerequisite for improving the utilization of AAs for growth and, therefore, for reducing the 'Nitrogen (N) excretion. This is particularly important for carnivorous species such as seabream, which use proteins rather than lipids or carbohydrates as an energy source (Peres and Oliva-Teles., 1999). Determination of quantitative EAAs requirements has generally been established in experiments where dose-response curves use growth as the response criterion (response measured is weight gain). The principle of this method is based on weight gain which is linearly related to increasing dietary levels of the essential amino acid at or below the required level. When the requirement is met, weight gains level off abruptly, then decline if the dietary concentration of the nutrient exceeds the animal's tolerance. (Nutritional Requirements of Fish, 1993). However, several problems are known to accurately determine the amino acid requirements of fish when based on growth studies: a) the growth rates that are commonly observed in amino acid test diets are often less or less than those observed with natural or intact proteins. b) some of the amino acids in the diets tested may be reduced during feeding. c) the interpretation of the breakpoint in the growth response curve could be subjective if the appropriate statistical tool is not used. There is a simple method that allows an approximation of the EAAs requirements, it is the analysis based on the EAAs profile of a muscle or a whole body. Wilson and Poe (1985) demonstrated a good correlation between the apparent requirements of catfish (*Ictalurus punctatus*) and EAAs levels in body tissues.

According to Peres and Oliva-Teles (2009), the optimal balance of essential amino acids in the diet of juvenile gilthead seabream is (g/16g N): Arginine, 5.55; Histidine, 1.89; Isoleucine, 2.55; Leucine, 4.75; Lysine 5.13; Methionine 2.60, Phenylalanine + tyrosine 5.76; threonine, 2.98; Tryptophan, 0.75 and Valine 3.21. More recently, Gaber *et al.*, (2016) showed that the quantitative EAAs requirements (%) of gilthead seabream (*Sparus aurata*) diets were as follows: Arginine 1.39, Histidine 1.6, Isoleucine 0.47, Leucine 2.4, Lysine 2.27, Methionine 1.09, Phenylalanine 1.43, Threonine 0.61, Tryptophan 0.42 and Valine 1.22. Essential amino acid requirements have generally been established in fish by the dose-response method. But few studies have reported on the maintenance requirements of EAAs (Richard *et al.*, 2010; He *et al.*, 2013). However, the factor modeling approach is increasingly used to estimate EAAs requirements.

This approach is based on information on maintenance requirements and AAs deposition for body protein and its utilization efficiency. Reliable estimates of AAs maintenance requirements are therefore important for developing feed formulation strategies to meet fish AAs requirements and to achieve maximum feed efficiency and minimum nitrogen (N) waste production. Hua (2013a) conducted a study using a mathematical modeling approach. Using this mathematical approach, he determined in gilthead seabream its EAAs requirements as a result (mg/kg PC0.75/day) for arginine (7.7 ± 6.6), histidine (9.8 ± 2.4), isoleucine (6.4 ± 24.8), leucine (9.1 ± 13.7), lysine (15.6 ± 8.2), methionine (18.4 ± 1.9), phenylalanine (14.6 ± 3.2), threonine (5.4 ± 4.4), tryptophan (0.5 ± 1.3) and valine (9.3 ± 26.6). Due to standard errors associated with a very limited number of studies, it was not possible to draw a conclusion for isoleucine, leucine, and valine.

2.5. AMINO ACIDS METABOLISM IN FISH

Amino acids (AAs) are molecules that have both an amine function and other carboxylic acid. The primary function of amino acids is their use as building blocks for protein synthesis. Additionally, amino acids serve as precursors to non-protein nitrogenous compounds such as nucleic acid, porphyrin, and creatine, and they also serve as energy substrates. Amino acid metabolism involves two complementary processes: 1) Catabolism (breakdown) which takes place in two reactions. First, removal of the amino group (removal of the amino nitrogen), and the remaining carbon skeleton, called alpha-keto acid (it is no longer an amino acid) is degraded into intermediates. 2) Anabolism using metabolic intermediates as substrates for AAs biosynthesis and as precursors of bioactive molecules.

2.6. MORPHOLOGY OF THE DIGESTIVE TRACT OF THE GILTHEAD SEABREAM

Digestion can be defined as the preparation of food by the animal for absorption. These processes can include mechanical particle size reduction, enzymatic solubilization of organic matter, and emulsification of lipids. Absorption includes the various processes that allow ions and molecules to cross the membranes of the intestinal tract into the blood or lymph to be metabolized by the animal.

The major parts of the alimentary canal are the mouth, esophagus, pharynx, stomach, intestine, rectum, and secretory glands, which include the liver and pancreas (Figure 2.4). The digestive tract

is a long tube that runs from the mouth to the rectum (Lovell., 1989). The digestive tract of carnivorous fish consists of an esophagus, the stomach which opens into the pyloric caeca, then a proximal intestine and a distal intestine. The liver and its gallbladder are connected to the proximal intestine, allowing the secretion of bile into the intestinal chyme. (Borey, 2017). Figure 2.4 shows the in situ digestive tract of juvenile gilthead seabream. The esophagus opens into the stomach in a Y-shape. The beginning of the intestine consists of four short pyloric caeca, at the base get the hepatic duct and the pancreatic duct discharge. The digestive tract forms two bends. After the second bend, there is a narrowing corresponding to a valve, visible from the outside. The relative length of the intestine is 5-6 cm. The liver consists of two lobes, not fully divided; the much larger left lobe protrudes posteriorly. The gallbladder is situated dorsally and appears as a sac as long as the entire abdominal cavity. Small masses of pancreatic tissue can be shown along the first intestinal tract (Cataldi *et al.*, 1987).

The stomach, pancreas, pyloric appendages, and intestinal mucosa are sources of protein-digesting enzymes in fish (Hoar and Randall, 1978). Some authors have wondered about the study of protein digestion in fish. Kenyon (1967) reported peptic activity of stomach tissues in carp, bluegill, pike, crappie, and also yellow perch. He found trypsin activity in extracts of carp pancreas and hepatopancreas, crappie pyloric caeca and pike. Protease activity was found in the intestinal mucosa of carp and pike. Some differences in protein digesting enzymes between species have been reported (Hoar and Randall., 1978). Kitamikado *et al.* (1964b) observed that protease activity is higher in the gut of rainbow trout than in carp. This is probably due to the higher consumption of protein feed by rainbow trout than by carp. However, Morishita *et al.* (1966) found no significant difference in the protease activity of the digestive tract in five species of fish. However, enzymes were more active at lower temperatures than any other species studied.

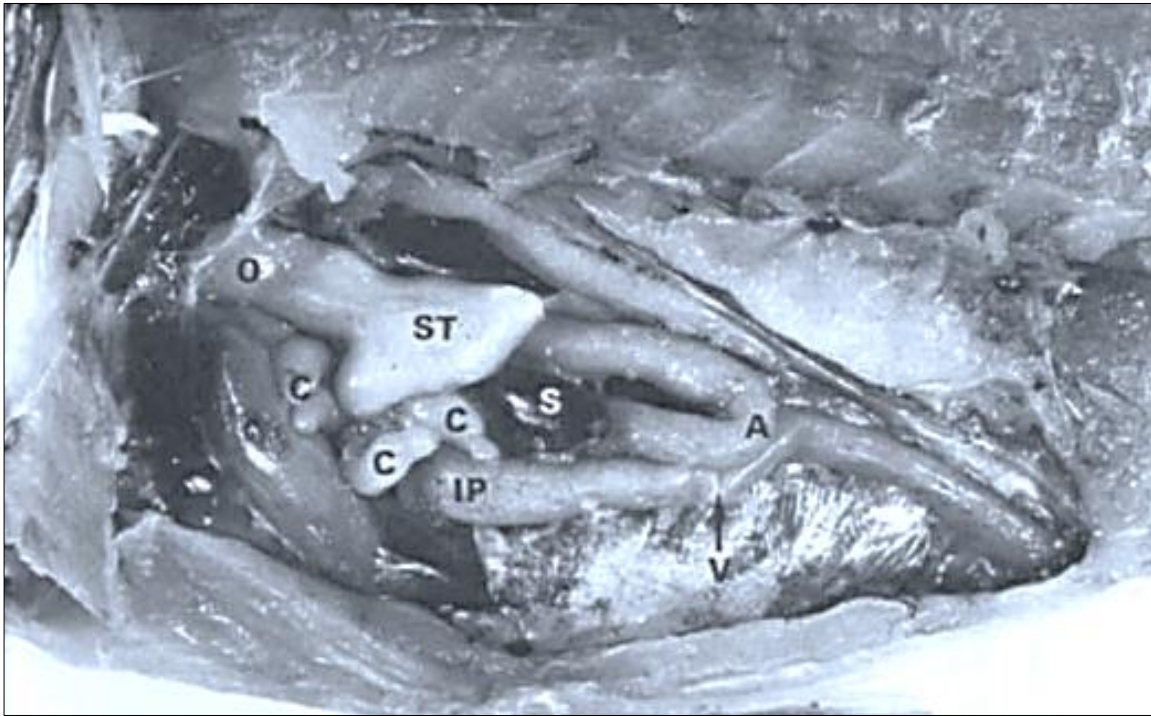


Figure 2.4: Morphology of digestive tract of *Sparus aurata*: O. oesophagus; ST. stomach; C, pyloric caeca; S. spleen; A. intestine anterior bend; IP, intestine posterior bend; V. intestine valve; (rectum is not clear in figure 2.4) (Cataldi *et al.*, 1987).

2.6.1. Protein Digestion, Amino Acids Absorption and Transportation

2.6.1.1. Protein Digestion

When food enters the stomach, digestive processes (digestive enzymes) transform food proteins, which are large molecules (which cannot be absorbed as such by the digestive epithelium), into molecules that can be absorbed by the stomach. Proteins will be broken down by a series of processes into free amino acids and di/tripeptides that could be taken up by intestinal enterocytes (Robert, 2014). For this reason, several enzymes are involved in the breakdown of food proteins (Figure 2.5) (Table 2.2). These enzymes are divided into two groups: the endopeptidases; (endo = internal) (pepsin, trypsin, and chymotrypsin) cleave internal peptide bonds, and exopeptidases (exo = external) carboxypeptidases and aminopeptidases hydrolyze terminal peptide bonds of amino acids (Figure 2.6).

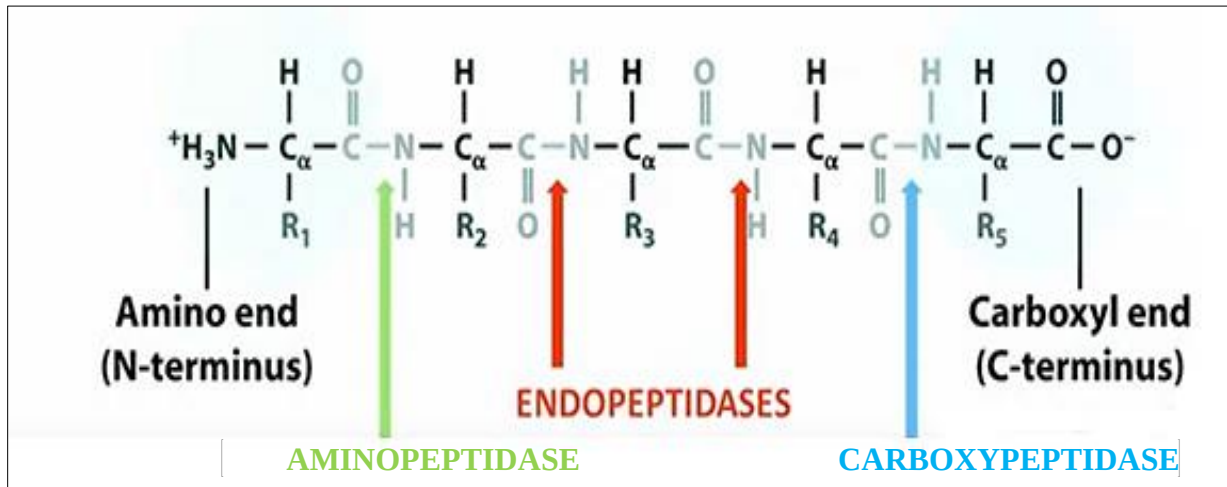


Figure 2.5: Diagram of protein digestion mechanisms.

Table 2.2: Proteolytic digestive enzyme cleavage sites (Robert, 2014).

Enzymes	Hydrolysez bonds
Pepsin	NH ₂ of aromatic amino acids and diacids
Trypsin	COOH from arginine and lysine
Chymotrypsin	COOH of aromatic amino acids
Elastase	Aliphatic amino acids
Carboxypeptidases	Amino acids Cter
Aminopeptidase	Nter amino acids
Di and tri peptidase	Peptide bonds of di and tri peptides

In gastric fish, the digestive phase begins in the stomach. Protein hydrolysis is initiated by the action of the enzyme pepsin, active in an acid medium (in fish at pH 1.5 to 3.0) to form polypeptides (Love1, 1989) (Pepsinogen, secreted by the main cells of the gastric epithelium, is an inactive form of pepsin, becomes active in an acid medium). After hydrolysis, the mixture of food with gastric juice turns into a mush called chyme. In mammals, as in fish, this chyme, when it has become completely and sufficiently liquid, is gradually secreted into the foregut. At this level, proteolysis continues under the action of pancreatic and intestinal enzymes (Lovell., 1989). The pancreas secretes many types of proteases, including trypsinogen and chymotrypsinogen

(inactive forms of trypsin and chymotrypsin), elastase, and carboxypeptidases A and B (exopeptidases) as an activated zymogen in the gut lumen.

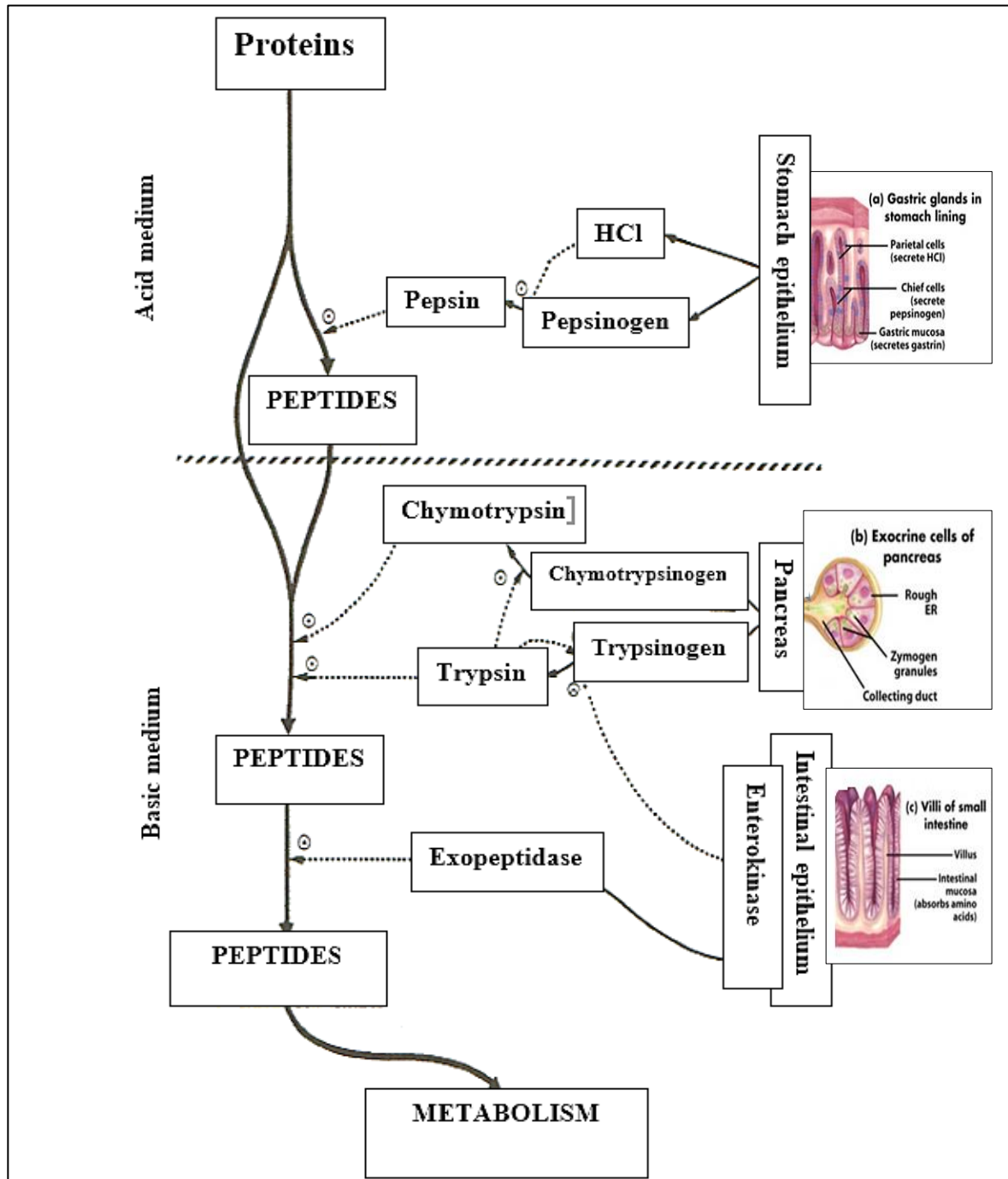


Figure 2.6: General scheme of proteolytic enzyme cleavage sites

Trypsinogen is activated by brush border enterokinase for old trypsinogen which in its active form acts on polypeptides. Trypsin, in turn, activates chymotrypsinogen, converting it to chymotrypsin. Other pancreatic enzymes: carboxypeptidases A and B, their action on polypeptides is supplemented by enterocyte brush border enzymes (aminopeptidase N), including dipeptidyl peptidase IV, leucine aminopeptidase, phenylalanine-glycine peptidase, γ -glutamyl transferase and other aminopeptidases. All these enzymes cleave peptide bonds at very specific sites (Table 1.1), the hydrolytic action of these gastric and pancreatic endoproteases results in the release of small peptides (di- and tripeptides) and free amino acids. Carboxypeptidases remove a single amino acid from the carboxy terminus of amino acids. The actions of pancreatic enzymes reduce proteins into a mixture of oligopeptides (2 to 6 amino acids) and free amino acids. Oligopeptides produced by gastric and pancreatic proteases are further cleaved by membrane brush border peptidases to release amino acids or di- and tripeptides before being absorbed. This step is the last step in protein digestion.

2.6.1.2. Absorption and Transport

The general anatomical structure of the intestinal wall of fish is similar to that of vertebrates. An absorptive epithelium faces the luminal contents and is responsible for the transport of nutrients (eg amino acids). However, unlike mammals, the intestinal epithelium of fish is not divided into regions of villi and crypts (Hochachka and Mommsen, 1989). According to Vernier in Hochachka and Mommsen (1989), regional differentiation of the gut in teleost fish gives rise to a foregut, in which amino acids and peptides are absorbed, and a hindgut, where proteins are absorbed as macromolecular.

Di/tri-peptides and free amino acids pass through the brush border membrane to reach the cytosol via transporters then exiting through the basolateral membrane in series and finally ending up in the blood (Figure 2.7). Basically, amino acids cross the basolateral and brush border membrane with the help of Na^+ -dependent transporters, Na^+ -independent transporters, or with the help of simple diffusion. However, other ions such as Cl^- have been reported in the uptake of nutrients by the marine teleost (Hochachka and Mommsen, 1989). Reshkin and co-workers from European eel gut identified three AA transport pathways: (1) Na^+ -dependent carriers for proline and glutamate; (2) independent Na^+ carriers; for alanine, lysine, and phenylalanine; and (3) a glycine transporter that required both an inward-directed Na^+ gradient (from blood to cell) and an outward-directed K^+ gradient.

Free amino acids were formally considered the only form in which hydrolyzed dietary proteins absorbed. However, the discovery of the specific transporter PepT1, by Fei *et al.* (1994) on the brush border of enterocytes, shows that peptides are also absorbed in the form of di/tri-peptides. The latter, when they enter the cytosol of the enterocytes are hydrolyzed into free amino acids by the cytosolic peptidase called Leucine-alanine peptidase or prolidase (Borey, 2017). This transporter is characterized by low specificity but by high capacity, it can transport all the di/tri-peptides with a lower energy cost than the transport of free amino acids (Robert, 2014). Recently, another transporter orthologous to the B0AT1 transporter which exists in mammals has been demonstrated in sea bass (Robert 2014).

A more recent study by Xu *et al.* (2016) revealed the existence of 12 transporters of peptides and amino acids in the intestinal wall of juvenile turbot (*Scophthalmus maximus*) named as follows: named; peptide transporters 1 and 2 (PepT1 and PepT2) mediating the uptake of all di- and tripeptides, neutral AA transporters including B0-type AA transporter 1 (B0AT1), L-type AA transporter 2 (LAT2), ASC system AA transporter 2 (ASCT2), sodium-coupled neutral AA transporter 2 (SNAT2), AA transporter T-type 1 (TAT1), proton coupling transporter AA 1 (PAT1), and AA cation transporters including AA transporter type b0,+ (b0,+AT), y+L system AA transporters 1 and 2 (y+LAT1 and y+LAT2) and cationic AA transporter 2 (CAT2) (Xu *et al.*, 2020).

Several works suggest the existence of transporters similar to those of mammals and figure 2.8, represents the mechanisms of absorption of amino acids and di/tri-peptides in enterocytes.

Currently, we have a detailed, albeit incomplete, description of AAs entry and exit pathways for a carnivorous juvenile turbot (*Scophthalmus maximus*) fish species. Information on the characteristics of nutrient transporters in other species would aid our understanding of nutrient uptake and metabolism. Regarding the mechanisms of AAs transport across the basolateral membrane, much less is known.

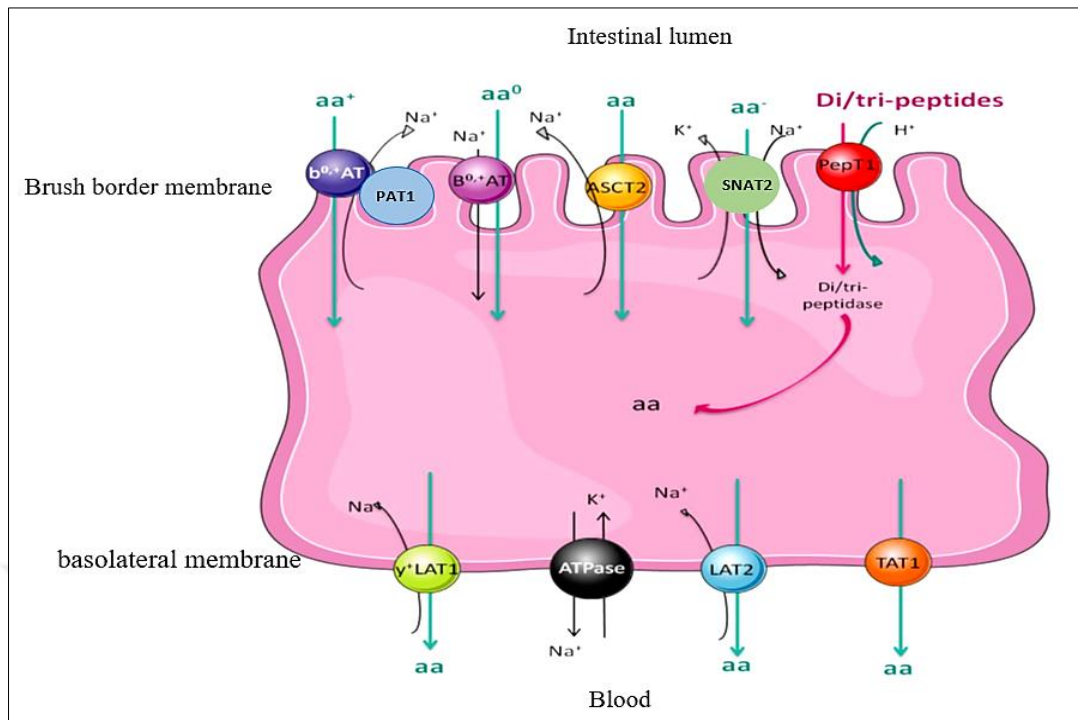


Figure 2.7: Schematic representation of the absorption mechanisms of amino acids and di/tri-peptides in enterocytes. ASCT2, system ASC amino acid transporter-2; b₀,+AT, b₀,+-type amino acid transporter; B₀AT1, B₀-type amino acid transporter 1; LAT2, L-type amino acid transporter 2; PAT1, proton-coupled amino acid transporter 1; PepT1, peptide transporter; SNAT2, sodium-coupled neutral amino acid transporter 2; TAT1, T-type amino acid transporter 1; y+LAT1, system y+L amino acid transporter (From Palacin et al., 2005 in Robert, 2014 modified by author).

2.6.1.3. Transport of Free Amino Acids From The Basolateral Membrane of Enterocytes into Blood

Di/tripeptides entering enterocytes are cleaved into free amino acids by cytoplasmic di- and tripeptidases. They are then released into the blood via amino acid transporters mediated by the basolateral membrane of enterocytes. For active uptake of AA, energy flows along the Na⁺ gradient and is supported by Na⁺ pumps (Na⁺ / K⁺ -ATPases), allowing it to penetrate the basolateral membrane and enter the blood (Figure 2.7) (Hochachka and Mommsen., 1989).

2.7. AMINO ACIDS METABOLISM IN FISH

2.7.1. Catabolism

Excess amino acids that exceed what is needed for their growth and development are then degraded because the animals cannot store the excess amino acids. In fish, amino acid catabolism occurs primarily in the liver, with the breakdown of amino acids leading to the release of ammonia (Ballantyne, 2001). In fish, amino acid catabolism occurs in two main ways; oxidative deamination (the ammonium ion is reduced and recycled to form another amino acid or eliminated) and by transamination (amino transfer), which is a general reaction of AAs metabolism, since it is involved both in their catabolism and in their synthesis.

Oxidative deamination is an energetic reaction catalyzed by dehydrogenase. Deamination of glutamate produces α -ketoglutarate and ammonia. Usually, ammonia is present in two forms: molecular NH_3 and cationic NH_4^+ (Figure 2.8.A). In liver cells, some amino acids can be catabolized by specific deaminases (histidase, asparaginase, serine dehydratase, and threonine dehydratase) to release NH_3 in the cytosol. Ammonia is usually released as NH_4^+ from the α -amino group of various amino acids by transamination, which requires a combination of cytosol aminotransferases and mitochondrial glutamate dehydrogenase in hepatocytes (Walton and Cowey, 1977; French *et al.*, 1981; Ballantyne, 2001). Deamination of glutamate by the enzyme glutamate dehydrogenase leads to the formation of NH_4^+ within the mitochondrial matrix. The mitochondrial matrix of some species also contains glutaminase, which releases NH_3 from glutamine amide-N (Ballantyne, 2001).

Ammonia, formed in hepatocytes, enters the blood after crossing the mitochondrial and plasma membranes. In plasma, ammonia is present mainly (> 95%) in the form of NH_4^+ . Ammonia circulates through the plasma to other parts of the body. Since ammonia is a toxic molecule, it must be excreted from the body to avoid accumulation (Ip and Chew, 2018). Since most teleost species are ammoniotelic, they have the ability to excrete nitrogen directly as ammonia (excrete more than 50% of nitrogen waste as NH_4^+ (Ip and Chew., 2018).

Lovell (1989) mentions that teleost fish emit 60 to 90% of nitrogen waste as ammonia through gills at lower energy costs. Unlike excretion in the form of urea, which requires more energy. The advantage of this form is that it is less toxic when accumulated in the animal.

In fish, transamination is the main route of deamination. It involves the transfer of an amine function at the α -position of amino acid 1 to a ketone function at the α -position of α -keto acid 2. This transfer of amino groups will allow the formation of a new amino acid 2 and a new α -keto acid 1. Aminotransferase enzymes are specific to amino acids. In mammals, as in teleost fish, alanine aminotransferase (ALAT) or glutamo-pyruvic transaminase (GPT) and aspartate aminotransferase (ASAT) or glutamo-oxalo-acetic acid transaminase (GOT) are quantitatively the most important aminotransferases in transamination (Fig. 2.8.B) (Cowey and Walton, 1989). A study conducted by Lupianez *et al.* (1989) showed an increase in ALAT activity in the liver of rainbow trout (*Oncorhynchus mykiss*), which received a high protein/carbohydrate-free diet. Similarly, Meton *et al.* (1999) observed increased ALAT activity in gilthead seabream (*Sparus aurata*) fed a high protein diet. On the other hand, Kim *et al.* (1992) did not observe the effects of dietary protein on ALAT activity in the liver of rainbow trout. All amino acids can be transaminated except for lysine and threonine.

Catabolism of the carbon skeleton of amino acids leads to the formation of pyruvate or oxaloacetate, α -ketoglutarate, succinyl-CoA, fumarate, acetyl-CoA and acetoacetyl-CoA. The amino group released from oxidized amino acids can be used to synthesize new non-essential AAs by transamination (α -ketoglutaric acid + ALA = GLU + pyruvate) and can be converted into ammonia by deamination ($\text{GLU} + \text{NAD} + \text{H}_2\text{O} = \alpha\text{-ketoglutaric acid} + \text{NH}_4^+$). AAs can also be classified based on the metabolic fate of the carbon skeleton (ketoacids) following removal of ammonia from ketogenic, glucogenic, or both glucogenic and ketogenic amino acids. Ketogenic amino acids whose carbon skeletal cleavage yields acetyl-CoA or acetoacetyl-CoA but no metabolites that can be converted to glucose (Cowey and Walton, 1989). Lysine and leucine are the only exclusively ketogenic amino acids (fig. 2.8.C). These amino acids increase blood levels of acetoacetate (ketone body) when consumed in large amounts of glucogenic amino acids, the catabolism of which leads to the formation of pyruvate or metabolites of the citric acid cycle (α -ketoglutarate, oxalacetate, fumarate, succinyl-CoA), can be converted into glucose by gluconeogenesis (Kaushik, 2001), which increases blood sugar when consumed in large quantities. Glucogenic amino acids are alanine, arginine, asparagine, aspartate, cysteine, glutamate, glycine,

histidine, methionine, proline, serine, and valine (Cowey and Walton, 1989). Some AAs can be both glucogenic and ketogenic because they can produce certain precursors that can become glucose and FAs. This type of AAs includes Isoleucine, phenylalanine, tryptophan, tyrosine, and threonine.

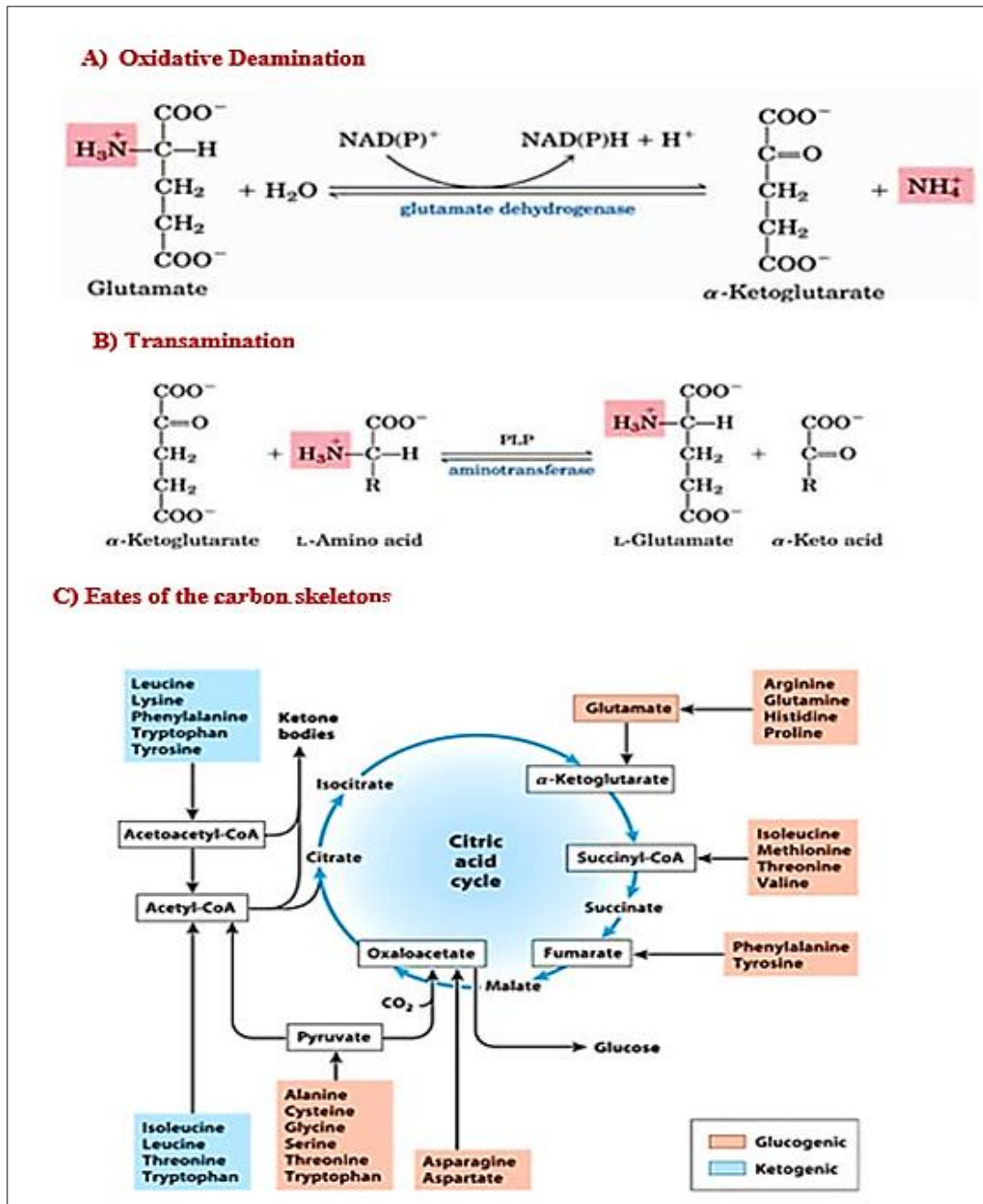


Figure 2.8: Degradation pathways of amino acids. A: Some amino acids (e.g glutamic acid/glutamate) can undergo oxidative deamination to their corresponding keto acids (e.g acetoglutaric acid/ α -ketoglutarate). B: Most amino acids undergo transamination, with the amino group being transferred to α -

ketoglutarate to form the keto acid and glutamate, which can then be directly deaminated as above. C: The keto acids produced from the above reactions are converted into small amounts of metabolites that fuel the citric acid cycle. In addition, some amino acids are glucogenic (pink boxes) producing glucose, while others are ketogenic (blue boxes) and produce acetoacetyl-CoA and therefore ketone bodies. Note that some amino acids may be either glucogenic or ketogenic. alanine, arginine, asparagine, aspartate, cysteine, glycine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine.

2.7.2. Anabolism

Amino acids transported via the basolateral membranes of enterocytes pass along the portal vein to the liver of the fish. The essential amino acids are those amino acids the body must obtain from the diet. The non-essential amino acids are synthesized from glycolytic intermediates; pyruvate, oxaloacetate, alpha-ketoglutarate, and 3-phosphoglycerate. Aspartate and asparagines are synthesized by transamination of oxaloacetate through transfer of glutamate and amide from glutamine, respectively. Glutamate, glutamine, proline and arginine are formed from the intermediate α -ketoglutarate of the acid three phosphate cycle (TCA) ring. The three alpha-keto acids pyruvate, oxaloacetate, and alpha-ketoglutarate can be transaminated in a single step to alanine, aspartate, and glutamate, respectively. The synthesis of glutamate can take place through the action of glutamate dehydrogenase in the presence of alphaketoglutarate acid and NH_4^+ and NADPH, H^+ or according to the reaction of an aminotransferase (transaminase). For glutamine it is synthesized under the action of glutamine synthetase. Proline is synthesized in two steps from glutamate which is transformed into glutamate semi-aldehyde, the latter giving proline in the presence of NADPH, H^+ . Serine, cysteine, and glycine are synthesized from 3-phosphoglycerate precursors of acid three phosphate cycle (TCA).

2.8. THE CONCEPT OF PROTEIN DIGESTIBILITY

Digestibility is a measure of the degree of net absorption of protein broken down into absorbable units (amino acids) in the digestive tract of fish. It describes the fraction of the nutrient or energy in the ingested food that is not excreted in the feces. The nutritional value of protein depends on its chemical composition, but also on the amount of amino acids or energy that the fish can absorb

and use. The bioavailability of nutrients or energy in fish feed can be defined primarily in terms of digestibility or, in the case of energy, metabolizability.

Metabolizability describes the fraction of digested energy that is not eliminated in the urine and through the gills. Digestible Energy (DE) and Metabolizable Energy (ME) have been used to express fish feed values, but many researchers only use and report Digestible Energy (DE) values due to difficulties in obtaining Metabolizable Energy (ME) values for fish. The digestibility of a nutrient is not a constant value, like the chemical analyzes of a nutrient in a particular sample of feed. Certain factors, such as diet and conditions of the fish (species, age, gut health, physiological state), influence digestibility. For example; the digestibility of proteins with excessive heat treatment, which leads to the formation of inter or intramolecular covalent bonds resistant to enzymatic digestion. Digestibility is measured directly from the differences between food consumption and fecal production. Total collection of feces in water is not possible for fish, so an inert indicator is added to the diet which accumulates in the feces (Allen Davis, 2015). Methods for determining digestibility coefficients involve direct or indirect measurement of the amount of nutrient (e.g. protein) ingested and then excreted.

The percentage of apparent protein digestibility coefficient (ADC) can be estimated using the following formula:

$$\text{ADC (\%)} = 100 - [100 \times (\text{Cr}_2\text{O}_3\text{Food} / \text{Cr}_2\text{O}_3\text{Faeces}) \times (\text{Nutrient}_{\text{Faeces}} / \text{Nutrient}_{\text{Food}})]$$

2.9. HISTOLOGY OF THE DIGESTIVE TRACT OF THE GILTHEAD SEABREAM

To ensure the sustainability of the aquaculture production of carnivorous fish species such as gilthead seabream (*Sparus aurata*), a species of interest for its particular dietary adaptations throughout its life cycle and for its economic importance in aquaculture, Mokhtar (2017) showed that some differences in the histological constitution of the digestive tract were observed. They are related to certain factors such as eating habits, food, age, body shape and weight. Similarly, the gastrointestinal tract of herbivorous fish showed a very significant increase in length compared to that of carnivorous species such as seabream. The digestive tract of this species has been the subject of numerous studies (Cataldi *et al.*, 1987; Cerezuela *et al.*, 2012; Madeira *et al.*, 2014; Mirabet *et al.*, 2017; Tina *et al.*, 2018) for determining the functions of its structures that have a relationship with the different dietary adaptations.

According to Cataldi *et al.* (1986) the gastrointestinal tract of *Sparus aurata* begins at the esophagus, extends to the stomach, intestine and ends at the anus (rectum). Its histological structure consists of mucosa, submucosa, muscularis and serosa. The mucosa (m) (Figure 2.9), is the most internal tunic, it is composed of a lining epithelium bordering the intestinal lumen. It can be united or pluristratified and of a connective tissue called Lamina propia. The submucosa (sm) is formed by connective tissue where many glands can be found. The muscle tunic is composed, as its name suggests, of smooth muscle cells (mi) which form a circular layer and a longitudinal layer (me). This tunic allows the contraction of the digestive tract. Finally, the serosa(s), the outermost tunic, is a layer of very dense connective tissue. These 4 tunics are constant along the digestive tract from the esophagus to the rectum in many species of fish including those of *Sparus aurata*. However, a specific cellular organization of each organ is observed.

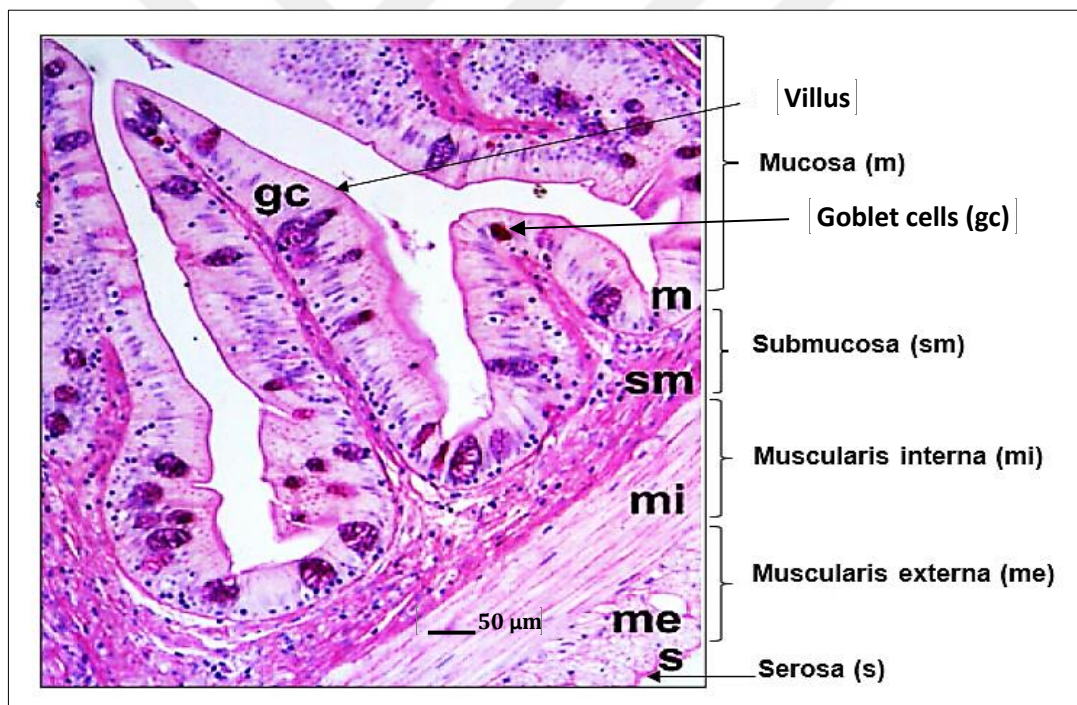


Figure 2.9: Histological section of a gilthead seabream intestine showing the organization of the tunics of the digestive tract (Cerezuela and Fumanal., 2012 modified by the author).

The tunica organization of the digestive tract of juvenile gilthead seabream was determined by Cataldi *et al.* (1987) as follows:

Esophagus

The muscular tunica is very thick in the first part and consists of striated muscle fibers which form a continuous circular layer on the outside and large longitudinal bundles on the inside. The submucosa and the lamina propria are very thin in juveniles and with age become thicker in adults. Among epithelial cells, there are mucus-secreting cells, which are globule-shaped, and other cells, similar in shape to mucus-secreting ones, are full of highly acidophilic granules. Taste buds are present on the ridges of the folds.

Stomach

Mokhtar, (2017) reported that in fish, the stomach is divided into 3 regions; cardiac, fundic and pyloric. Mokhtar, (2017) described the structure of the catfish stomach as follows: “The cardiac mucosa is characterized by a large number of long, well-defined folds, occupied by simple, tight branching tubular gastric glands which are lined by a type of cells with granular cytoplasm. The fundic region is characterized by a large number of simple branching tubular gastric glands consisting of oxyntico-peptic cells which contain a tubulovesicular network and a large number of large mitochondria. The most important feature of the fundic region is the presence of a large number of pits of different sizes”. However, Robert, (2014) distinguished two regions in the stomach; 1) the anterior region called the cardiac or fundic region and 2) the posterior region called the pyloric region. Similarly Cataldi *et al.*, 1987 observed that juvenile *Sparus aurata* has two stomach parts; the cardiac part presents a muscular tunic, composed of striated fibers which preserve in the first part the same arrangement as in the oesophagus. Smooth fibers gradually replace striated fibers and the longitudinal musculature appears on the outer wall and persists along the digestive tract. In the pyloric part, the circular muscularis is strongly thickened. The submucosa is much more developed in adults. In the stomach, the mucosa forms folds and cavities, and is composed of a columnar epithelium, with nuclei located at the base. The free surface of epithelial cells is smooth, without micro-ridges, sometimes with small spheroidal or cylindrical bubbles. Simple tubular gastric glands are present in the cardiac region, but they are completely absent in the pyloric region.

Intestine

The muscularis and submucosa are very thin in juvenile *Sparus aurata* but developed in adults. The typically wrinkled intestinal mucosa is composed of monolayer columnar cells whose free surfaces are entirely covered with microvilli. Numerous mucus-secreting cells are present in the epithelium. These goblet cells increase in number in the lower part of the intestine. The pyloric appendages have a simple columnar epithelium, with more abundant and longer microvilli than in the intestine. At the ring-shaped narrowing seen in the lower bowel region, the mucosa forms a funnel-shaped internal valve. After this valve, the epithelium has a different appearance: above the nucleus, the cells contain numerous vacuoles rich in large refractile eosinophilic granules.

Histology of other organs involved in protein digestion in gilthead seabream

Hepato-Pancreas

The liver is made up of a tissue of epithelial cells, through which a network of vessels and the sinusoids flow. The hepatic duct enters the first part of the intestine, after running parallel to a short pancreatic duct. Masses of glandular tissue surround the intestine and vessels, and in adults also infiltrate the liver. Small islets of Langerhans are included in the exocrine masses.

2.10. ALTERNATIVE PROTEIN SOURCES USED IN THIS STUDY

In 2001, the use of terrestrial animal by-products in animal feed was banned in European Union countries, due to problems with BSE in the late 20th century. But in 2013, in European Commission Regulation No. 56/2013, the safety of terrestrial animal by-products (Class 3 processed animal protein) was declared safe and allowed to be included in aquaculture feed. To this end, research (within EU countries) on the use and inclusion of animal by-products in aquaculture feed has been stagnant for several years. Replacing fishmeal with ground animal protein is one of the main sources of reducing the amount of fishmeal in fish feed. These animal proteins, such as poultry meal, blood meal, feather meal, can be used at relatively high levels in aquaculture feeds (Laporte, 2007; Amirkolaie *et al.*, 2014; Daniel., 2017; Hekmatpour *et al.*, 2018; Bandara, 2018; Karapanagiotidis *et al.*, 2018; Sabbagh *et al.*, 2019; Fontinha *et al.*, 2021).

These protein sources have high levels of protein; and relatively balanced amino acid profiles; compared to that fishmeal (Nogueira *et al.*, 2012). Poultry by-product meal, and hydrolyzed feather meal have been extensively tested in diets for *Sparus aurata* (Nogueira *et al.*, 2012; Karapanagiotidis *et al.*, 2019; Aragao *et al.*, 2020; Psfakis *et al.*, 2020; Fontinha *et al.*, 2021).

2.10.1. Poultry by-Product Meal (PBM)

A study conducted by Karapanagiotidis *et al.* (2019) showed that the total and partial replacement of fishmeal with PBM without and with the supplementation of lysine and methionine amino acids showed that PBM protein can successfully replace 50% of FM in the diet of juvenile *Sparus aurata* without adverse effects on survival, feed intake, growth performance, the proximal composition of body and muscle. Another study conducted by Hekmatpour (2018) showed that the overall performance of Sparidentex hasta juveniles improved when fishmeal was used in combination with poultry by-product meal. Fishmeal protein could be replaced up to 55% by PBM in the formulated diets of sobaity seabream juveniles without any negative effects on growth performance, feed utilization, and whole-body amino acid profile. Sabbagh *et al.* (2019) demonstrated the eventual possibility of partial or total fishmeal (FM) replacement by PBM in the diet of farmed juvenile gilthead seabream (*Sparus aurata*), without any adverse effects on growth performance, amino acids profile, and protein pattern in the fillet, digestive enzyme activities and liver histology.

2.10.2. Hydrolyzed Feather Meal (HFM)

Terrestrial animal by-product hydrolysates are rich in protein and their protein composition directly depends on the organs used. Poultry feather hydrolyzes proteins in the field of aquaculture nutrition, have advantages because they have better digestibility. The introduction of hydrolyzate in fish feed is known to improve growth in several species such as *Sparus aurata*. However, compared to fishmeal, hydrolyzed feather meal is low in some amino acids like lysine and methionine but high in cysteine content (Compos *et al.*, 2017). Psfakis *et al.* (2020) reported that compared to fishmeal, hydrolyzed feather meal is poorer in methionine, lysine, and histidine, but it is high in cysteine and in water-soluble amino acids such as arginine, proline, glycine and aspartic acid.

However, according to Wilson and Halver (2002), fish have a total sulfur amino acid (TSAA) requirement rather than a specific methionine requirement because cystine can spare a portion of methionine when this is not adequate in the diet for maximum growth. Further, supplementing the diet with crystalline amino acids (CAAs) like L-methionine or L-lysine is a commonly adopted practice to meet dietary EAAs requirements at a lower cost. However, in the aquaculture industry, there is still controversy over biological efficacy and gastrointestinal absorption rates in the literature. Some studies have found CAA use to be as effective as dietary protein bound AAs (Kim *et al.*, 1991; Kim *et al.*, 1992; Kim *et al.*, 1992a,c; Rollin *et al.*, 2003), while others studies have demonstrated lower biological efficacy of crystalline amino acids (CAAs) compared to intact protein (Zarate *et al.*, 1999; Dabrowski *et al.*, 2007; Dabrowski *et al.*, 2010). During digestion, digestive enzymes break down long-chain polypeptides into short peptides which are absorbed more efficiently. When CAAs are delivered in food, this process does not take place and therefore free AAs are eliminated from the gastrointestinal tract and their absorption is faster than protein-bound AAs, thus resulting in metabolic dyssynchrony with AAs released from digestion and thus affecting protein synthesis.

In this context, Laporte (2007) indicated that the hydrolysis of feathers transforms the keratin (which is not digested by mono-gastric animals including fish) in its original structure into better-digested peptides. Additionally, according to previous studies, the crude protein digestibility of hydrolyzed feather meal varies between fish species and studies such as at high levels in freshwater species but reduced in marine species such *Sparus aurata* 22% (Davies *et al.*, 2009). However, a recent study authored by Psafakis *et al.* (2020) indicated that 50% of fishmeal replacement by hydrolyzed feather meal in juvenile gilthead seabream *Sparus aurata* diet had no adverse effects on digestive protease activities. The same authors reported that the nutrient availability of hydrolyzed feather meal can vary according to the processing method involved in the feathers. Similar results were reported by Meeker and Hamilton, 2006. These authors suggested that any differences in the quality of poultry by-products may be due to the influence of processing methods.

Up to now, there is very little information available on the use of hydrolyzed feather meal and poultry by-product meal in the gilthead seabream *sparus aurata* diet.

2.11. FORMULATION OF DIET FOR GILTHEAD SEABREAM

Various constraints must be taken into account when formulating an aquaculture feed. Indeed, it must be adapted to the target animals. It must take into account the species, the physiological stage, the nutritional needs as well must. Aquafeeds must not only be adapted in terms of nutritional composition but also in terms of texture and size. To meet these constraints, the choice of raw materials is an essential step.

Generally, the feed prepared must contain a source of protein (fishmeal and/or of vegetable origin and/or of animal origin), a source of lipids (fish oil and/or of vegetable origin and/or of animal origin) a source of carbohydrates as well as mixtures of vitamins and minerals. Taking these constraints into account, a feed formula suitable for the diet of *Sparus aurata* was prepared for this experiment. |

3. MATERIAL AND METHODS

3.1. MATERIALS

3.1.1. Experimental Location and Period

The experimental diets of this study were prepared at the Mediterranean Fisheries Research, Production, and Training Institute in Beymelek, Antalya, affiliated with the Ministry of Agriculture. Feeding experiments were completed in 8 periods of 15 days and a total of 120 days between 31st July and 28th November 2019.

3.1.2. Fish Used in the Experiments

Juvenile gilthead seabream (*Sparus aurata*) with initial average weight of 35.01 ± 0.11 g and average length of 13.34 ± 0.02 cm belonging to the 2019 production period were used in the feeding experiments. The fish came from the production unit of the Mediterranean Fisheries Research, Production, and Training Institute in Beymelek, Antalya, affiliated to the Ministry of Agriculture. In the study, a total of 1050 fish were randomly placed in 21 tanks, with 50 in the experimental tank (Figure 3.1)



Figure 3.1: *Sparus aurata* (Linnaeus, 1758) gilthead seabream used in feeding experiments.

3.1.3. Experimental Condition

A total of 21 cylindrical fiberglass tanks (500 L capacity) were used for evaluating growth performance where each dietary group was assigned to triplicate tanks (Figure 3.2). For the digestibility tests, a total of 14 cylindroconical experimental tanks (150 L capacity) were used in duplicates. A separate fecal collection system was attached to each tank for collection of fish feces (Figure 3.3). Filtered seawater was used in the test tanks.

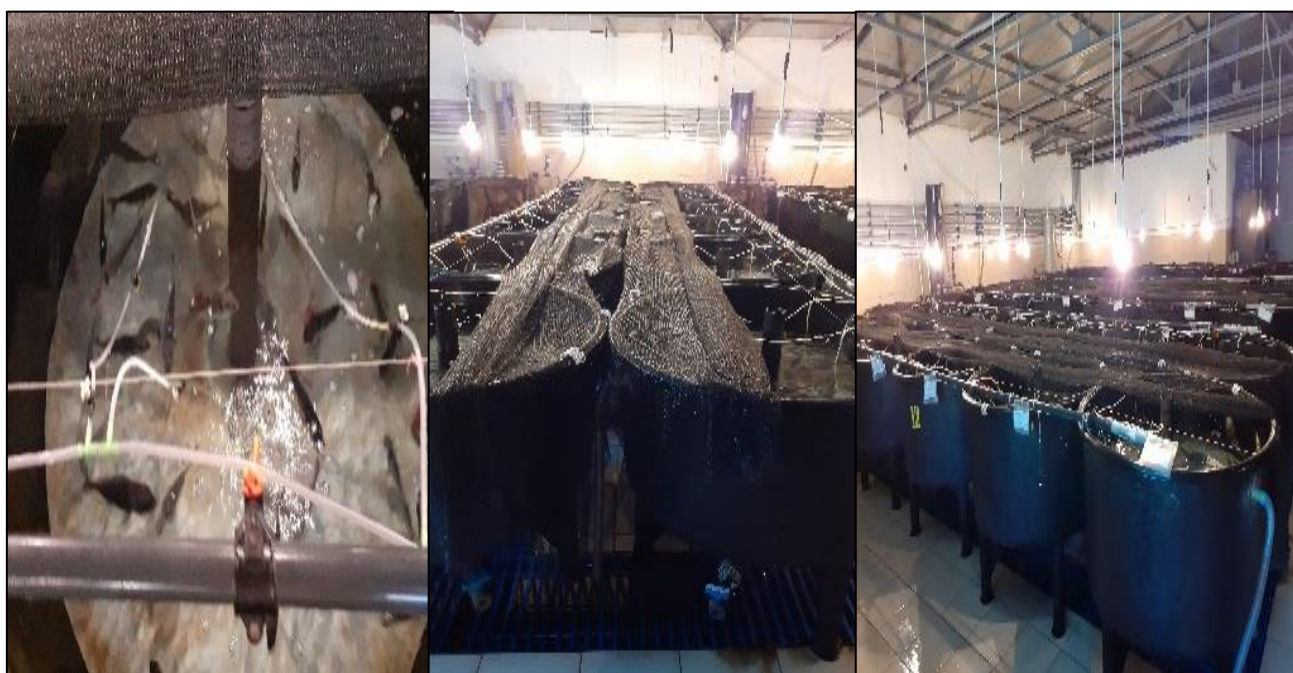


Figure 3.2: Juveniles gilthead seabream and the experimental system.



Figure 3.3: Cylinder-conical tanks used in the collection of fish feces during the experiment.
(A) cylindro-conical experimental tanks (B) fecal collection system.

3.1.4. Trial Fish Feed

The poultry by-product meal and the hydrolyzed feather meal used in the experimental diets were imported from feed factories that produce fish feed in SOLEVAL France. The remaining raw materials were purchased from the feed factories which produce fish feed in Turkey. Diets were formulated to meet the nutritional requirements of seabream juveniles (figure 3.4). Fish meal (FM), poultry by-product meal (PBM), and hydrolyzed feather meal (HFM) constituted the protein sources, where FM diet was the control. The other six dietary groups were prepared by substituting 55%, 65% ,and 75% of fish meal with either poultry by-product meal (corresponding to 55PBM, 65PBM, and 75PBM, respectively) or hydrolyzed feather meal (corresponding to 55HFM, 65HFM, and 75HFM, respectively). The formulation and proximate composition of the feed are shown in Table 3.1 where as the amino acids profiles of the meals used and diets are shown in Tables 3.2 and 3.3 respectively. Prepared diets were stored at a temperature of 4°C prior to feeding. For digestibility experiments, diets were prepared by adding 1% Cr_2O_3 provided it was tied to the same feed formulation as previously indicated in Table 3.1. These diets were used in tanks prepared for digestibility experiments.



Figure 3.4: An example of the diets used in the study.

Table 3.1: Formulation and proximate composition of experimental diets¹.

Diets	Feeding trial ²						
	FM	PBM55	PBM65	PBM75	HFM55	HFM65	HFM75
Formulation (g/kg⁻¹ diet)							
Fish meal	500	225	175	125	225	175	125
Poultry by-product meal	0	275	325	375	0	0	0
Hydrolyzed feather meal	0	0	0	0	275	325	375
Wheat gluten meal	80	110	120	125	60	55	50
Corn gluten meal	90	110	110	110	60	55	50
Dextrin	70	20	10	5	110	120	130
Fish oil	140	140	140	140	150	150	150
Gelatine	50	50	50	50	50	50	50
Mineral premix ^a	30	30	30	30	30	30	30
Vitamin premix ^a	30	30	30	30	30	30	30
Cr ₂ O ₃	10	10	10	10	10	10	10
Analyzed Proximate Composition (%) energy							
Dry matter	8.13	7.87	7.16	7.04	7.76	7.96	7.78
Crude Protein	48.94	48.26	48.44	48.86	48.78	48.54	48.95
Crude Lipid	18.47	19.43	19.41	19.28	19.84	19.62	19.69
Ash Content	7.94	9.15	9.71	9.63	5.48	5.13	5.18
Crude Cellulose	0.88	0.75	0.77	0.79	0.93	0.98	0.96
AEM ^c	15.64	14.54	14.51	14.40	17.21	17.77	17.44
MOE ^d	15.22	15.36	15.38	15.40	15.76	15.68	15.75
Total Energy	21.57	21.60	21.62	21.65	22.34	22.29	22.36

¹ Values (n = 3) are presented as the mean and the standard error of the mean (Mean±SD.). ²FM: Fish meal; PBM: Poultry by-product meal; 55PBM: 55% PBM+35%FM, 65PBM: 65%PBM+35%FM; 75PBM: 75%PBM+25%FM; HTU: Hydrolysis feather meal; 55HTU: 55%HTU+45%FM; 65HTU: 65%HTU+35%FM, 75HTU: 75%HTU+25%FM, ^aMineral mixture: Calcium carbonate 2.1g; Calcium phosphatedibasic 73.5g; Citric acid 0.227g; Cupric citrate 0.046g; Ferric citrate (16 to 17% Fe) 0.558g; Magnesium oxide 2.5g; Manganese citrate 0.835g; Potassium iodide 0.001g; Potassium phosphatedibasic 8.1g; potassium oxide 6.8g; Sodium chloride 3.06g; Sodium phosphate 2.14g and Zinc citrate 0.133g. ^bVitamin blend: Vitamin A 10,582.00 IU; Vitamin D3 881.84 IU; Vitamin E 176.36mg; Vitamin B12 48.52 µg; Riboflavin 52.92mg; Niacin 246.92mg; D-pantothenic acid 88.20mg; Menadione 5.28mg; Folic acid 7.04mg; Pyridoxine 17.68mg; Thiamine 31.80mg; D-biotin 1.24mg. ^cAEM: Nitrogen-Free Extracts. ^dMOE: Metabolizable Energy.

Table 3.2: Amino acid composition (g 100 g⁻¹ crude protein) of proteins used in experimental diets¹.

Amino acids	Proteins used ²		
	FM	PBM	HFM
<i>Essential amino acids (EAA)</i>			
Arginine	6.48±0.04 ^b	6.70±0.13 ^a	7.02±0.29 ^a
Histidine	3.25±0.02 ^a	2.82±0.62 ^{ab}	0.81±0.06 ^c
Isoleucine	4.24±0.16 ^{ab}	4.14±0.38 ^b	4.91±0.25 ^a
Leucine	7.64±0.13 ^a	6.86±0.59 ^b	7.78±0.13 ^a
Lysine	6.65±0.35 ^a	6.55±0.21 ^a	2.26±0.02 ^b
Methionine	3.06±0.03 ^a	2.98±0.27 ^a	0.65±0.01 ^b
Phenylalanine	4.02±0.08 ^b	3.60±0.20 ^c	4.97±0.21 ^a
Threonine	7.37±0.01 ^b	7.45±0.02 ^a	5.05±0.15 ^c
Tryptophan	0.89±0.03 ^a	0.50±0.02 ^c	0.61±0.02 ^b
Valine	5.15±0.24 ^b	4.16±0.04 ^c	7.18±0.22 ^a
<i>Non-essential amino acids (NEAA)</i>			
Alanine	4.52±0.06 ^a	3.50±0.40 ^b	3.95±0.15 ^{ab}
Aspartic acid	11.26±0.05 ^a	10.62±0.45 ^{ab}	6.99±0.09 ^c
Cysteine	1.14±0.01 ^b	0.94±0.01 ^c	3.60±0.08 ^a
Glutamic acid	12.28±0.27 ^b	13.53±0.29 ^a	9.97±0.17 ^c
Glycine	6.26±0.08 ^b	7.69±1.05 ^a	7.92±0.30 ^a
Proline	4.03±0.05 ^c	6.82±0.16 ^b	11.03±1.12 ^a
Serine	4.37±0.03 ^b	4.70±0.52 ^b	9.20±0.19 ^a
Tyrosine	2.70±0.09 ^b	3.86±0.40 ^a	2.72±0.32 ^b
ΣEAA	51.76±0.98^a	45.76±0.59^b	40.93±0.76^c
ΣNEAA	46.56±1.32^c	51.66±1.34^b	55.75±1.09^a
ΣAA	98.32±0.98^a	97.42±1.93^a	96.67±1.85^{ab}

¹Values (n = 3) are presented as mean and standard error of the mean (Mean±SD). The statistical difference between the values shown with different upper letters on the same line was found to be significant at the 95% accuracy level according to the Tukey multiple comparison test. ²FM: Fish meal, PBM: Poultry by-product meal, HFM: Hydrolyzed feather meal

Table 3.3: Amino acid composition (g 100 g⁻¹ crude protein) of the experimental diets¹.

Amino acids	Diets of feeding trial ²						
	FM	55PBM	65PBM	75PBM	55HFM	65HFM	75HFM
Essential amino acids (EAA)							
Arginine	7.72±0.56 ^a	7.51±0.11 ^a	7.46±0.05 ^a	7.42±0.18 ^a	5.46±0.07 ^c	6.02±0.23 ^{bc}	5.78±0.05 ^c
Histidine	2.37±0.04 ^a	2.43±0.37 ^a	2.17±0.07 ^b	2.10±0.02 ^b	1.58±0.13 ^c	1.36±0.01 ^c	1.15±0.04 ^c
Isoleucine	6.03±0.66 ^a	3.60±0.22 ^c	3.51±0.13 ^c	3.35±0.31 ^c	4.92±0.13 ^b	4.98±0.24 ^b	4.94±0.04 ^b
Leucine	7.45±1.84 ^b	7.47±0.12 ^b	7.40±0.07 ^b	6.56±1.00 ^{bc}	8.10±0.07 ^a	8.54±0.15 ^a	8.35±0.01 ^a
Lysine	6.76±1.75 ^a	6.31±0.18 ^a	6.10±0.26 ^a	5.12±0.15 ^b	3.72±0.11 ^c	3.31±0.09 ^{cd}	2.83±0.08 ^d
Methionine	3.70±0.10 ^a	2.83±0.21 ^b	2.75±0.56 ^b	2.53±0.18 ^b	1.82±0.04 ^c	1.56±0.01 ^d	1.33±0.04 ^d
Phen.	4.89±0.57 ^a	3.67±0.05 ^b	3.69±0.02 ^b	3.89±0.07 ^b	3.80±0.04 ^b	3.88±0.12 ^b	3.92±0.05 ^b
Trypt.	0.91±0.03 ^a	0.56±0.01 ^c	0.41±0.01 ^d	0.39±0.27 ^d	0.63±0.01 ^b	0.62±0.01 ^b	0.57±0.01 ^b
Threonine	4.36±0.17 ^c	5.66±0.18 ^a	5.36±0.16 ^{ab}	5.29±0.10 ^b	5.96±0.59 ^a	5.47±0.25 ^a	5.32±0.14 ^b
Valine	5.21±0.61 ^b	3.92±0.09 ^c	3.73±0.08 ^d	3.99±0.06 ^c	5.43±0.13 ^b	5.63±0.24 ^a	5.57±0.05 ^a
Non-essential amino acids (NEAA)							
Alanine	5.25±0.14 ^c	5.68±0.08 ^b	5.71±0.20 ^b	5.95±0.05 ^a	3.53±0.06 ^d	3.35±0.03 ^e	3.33±0.01 ^e
Asp. Acid	8.64±0.10 ^c	9.03±0.10 ^b	9.15±0.01 ^{ab}	8.98±0.09 ^b	9.59±0.59 ^a	8.43±0.16 ^c	8.27±0.32 ^c
Glycine	5.69±0.01 ^d	5.80±0.11 ^d	6.17±0.11 ^c	6.29±0.02 ^c	7.40±0.14 ^b	8.22±0.57 ^{ab}	8.72±0.48 ^a
Glut. acid	15.19±0.42 ^c	19.03±0.47 ^b	19.28±0.14 ^b	20.22±0.30 ^a	15.31±0.03 ^c	14.36±0.90 ^d	14.26±0.12 ^d
Serine	3.89±0.40 ^e	4.51±0.21 ^{de}	4.84±0.06 ^d	4.75±0.15 ^d	7.98±0.26 ^c	8.66±0.17 ^b	9.79±0.10 ^a
Cysteine	0.94±0.03 ^c	0.88±0.03 ^d	0.88±0.01 ^d	0.76±0.11 ^e	1.86±0.07 ^b	1.95±0.07 ^a	2.19±0.03 ^a
Tyrosine	3.38±0.32 ^a	2.73±0.14 ^{bc}	2.88±0.10 ^b	2.83±0.09 ^b	1.65±0.01 ^c	1.63±0.04 ^c	1.40±0.01 ^d
Proline	6.00±0.20 ^d	5.82±0.38 ^d	5.91±0.01 ^d	6.42±0.17 ^c	8.72±0.43 ^b	9.63±0.09 ^a	9.79±0.07 ^a
ΣEAA	49.41±0.25 ^a	43.97±0.02 ^b	42.18±1.10 ^b	40.61±1.37 ^{bc}	42.75±0.91 ^b	41.36±1.10 ^{bc}	39.76±0.27 ^c
ΣNEAA	48.99±0.34 ^c	53.48±1.36 ^b	54.82±0.04 ^b	56.20±0.33 ^a	56.02±0.56 ^a	56.23±1.16 ^a	57.75±1.08 ^a
ΣAA	98.40±0.08 ^a	97.45±1.34 ^a	97.00±1.05 ^{ab}	96.81±1.71 ^{ab}	98.25±0.73 ^a	97.59±2.16 ^a	97.52±1.35 ^a

¹Values (n = 3) are presented as mean and standard error of mean (Mean±SD). The statistical difference between the values shown with different upper letters on the same line was found to be significant at the 95% accuracy level according to the Tukey multiple comparison test. ²FM: Fish meal; PBM: Poultry by-product meal; 55PBM:55% PBM+35%FM, 65PBM: 65%PBM+35%FM; 75PBM: 75%PBM+25%FM; HTU: Hydrolysis feather meal; 55HTU: 55%HTU+45%FM; 65HTU: 65%HTU+35%FM, 75HTU:75%HTU+25%FM.

3.2. METHODS

3.2.1. Preparation of Diets

For this study, 7 different iso-nitrogenous (48 g/kg crude protein) and iso-lipidic (20 g/kg crude fat) feeds were prepared. The diets were prepared at the Mediterranean Institute for Fisheries Research, Production, and Training Institute in Beymelek, Antalya. Ingredients used in the preparation of the diets were purchased ready-to-use with a diameter varying between 400 and 500 μm . Initially, these ingredients were weighed as indicated in the feed formulation (Table 3.1.) and mixed until they became homogeneous. These diets containing various proteins (fishmeal, poultry by-product meal, and hydrolyzed feather meal) were produced in 4 mm pellets using an industrial-type meat grinder. For digestibility experiments, similar diets were produced by adding 1% Cr_2O_3 to the dietary formulation. At the end, the prepared diets were dried at an ambient temperature of 30°C for 3 days.

3.2.2. Experimental Conditions

All the main water quality parameters (dissolved oxygen, temperature, salinity, and pH) were monitored regularly to ensure that they remained within acceptable levels throughout the experimental period. The water temperature in the test tanks, dissolved oxygen in the water, pH, and salinity were measured once a week (Table 4.8) using a YSI Pro DSS Digital automatic device. Maintaining good water quality involved regular cleaning of tanks and filters in the morning at 11 am and in the evening at 7 pm. The flow rate of seawater into the tank was 1 litre/min. The photoperiod was set at 12 hours of light and 12 hours of darkness. A total of 1050 juvenile gilthead seabream used in this feeding experiment were randomly assigned to the growth tanks at 50 fish per each. Juvenile gilthead seabream were fed commercial diets for adaptation in experimental tanks for two weeks before being fed the prepared diets. During the experiment, the fish were hand-fed to satiation twice a day, at 9:30 am and at 5 pm.

In evaluating the digestibility of test diets, 9 fish/tanks were randomly assigned to 14 cylindroconical tanks (150 litres each) in duplicates per dietary group and fed diets containing Cr_2O_3 . Fish feces were collected by attaching fecal collecting devices to the outlet pipes of each tank to be used in the calculation of amino acid digestibility by fish.

The feces were collected 2 hours after feeding the fish. These feces were then stored in plastic containers in a freezer at a temperature of -80°C for further analysis. Spectrophotometric measurements were made in the analysis of Cr_2O_3 concentration in feed and fish feces according to Petry and Rapp (1970).

1.2.3. Measurement of Length and Weight of Fish

During the experimental period (research), the fish were not fed the day before weighing. At the beginning of the feeding experiments and every 15 days, the individual weight (g) of the fish in the experimental tanks were taken using a balance sensitive to 0.1 g. The final weight of individual fish was recorded at the end of the experiment. At the start and at the end of the experiment, the individual lengths (cm) of the fish in the experimental tanks were also determined using a measuring board on stable ground. The fish were anesthetized (1.6 g of Benzaquin + 10 ml of acetate + 10 l of water) before recording the individual length and weight. Fish growth performance parameters were assessed according to Ricker (1979).

3.2.4. Calculation Of Growth Indices

3.2.4.1. Weight Gain

The individual body weight gain (WG, g/fish) of the experimental fish was calculated by subtracting the first live weight (IBW) from the (FBW). The WG is calculated with the following formula:

$$\text{WG} = \text{FBW} - \text{IBW}$$

Where:

G: weight gain **FBW:** final body weight **IBW:** initial body weight

3.2.4.2. Condition Factor

The relationship between length and weight was defined using the condition factor. The condition factor is the parameter in which morphological structure is used in determining the nutritional status and developmental criteria in farmed fish. It is reported that a gilthead seabream with good nutritional conditions should have a CF of 1.2 (optimum 1.37). It is affected by the degree of

development of the structure. Condition factor is the ratio of the fish's Final Body Weight (FBW)(g) to the cube of the Total Length (TL)(cm).

The condition factor was calculated with the following formula:

$$CF = (FBW \text{ (g)} / TL^3 \text{ (cm}^3)) \times 100$$

Where:

CF: condition factor, **FBW**: Final body weight of fish, and **TL**: indicates the total length of the fish.

3.2.4.3. Specific Growth Rate

To calculate the specific growth rate (SGR, %/day), average weight measurements were taken every 15 days from the start of the trial. The specific growth rate, which indicates the rate of daily live weight gain, is obtained by multiplying by 100 the ratio of the difference between the natural logarithm of the final and initial values of the specific weight during the period of the experiment.

$$SGR \text{ (%/day)} = [(Lnwf - Lnwi) / t] \times 100$$

Where:

Lnwf is the natural logarithm of final weight and **Lnwi** is the natural logarithm of initial weight. **t** is the duration of the trial in days.

3.2.4.4. Calculation of Survival

The percentage of survival (%) was calculated as the number of individuals surviving at the end of the experiment compared to the number of individuals at the start of the experiment.

$$\text{Survival (\%)} = 100 - ((\text{Final Nb} / \text{Initial Nb}) \times 100)$$

Where:

Initial Nb is the number of fish at the beginning of the trial and **Final Nb** is the number of fish at the end of the trial

3.2.4.5. Calculation of Hepatosomatic (HSI) and Viserosomatic (VSI) Indices

Somatic hepatic and visceral indices were calculated by taking the total weight of the liver and internal organs, respectively of 15 randomly selected fish. At the beginning of the experiment, 10 fish were taken from each group and at the end of the experiment, 5 fish from each group were taken. The calculation of these indices was carried out according to the following formulas

$$\text{HSI} = [\text{Fish liver weight (g)} / \text{Fish live weight (g)}] \times 100$$

$$\text{VSI} = [\text{Weight of the internal organs of the fish (g)} / \text{Live weight of the fish (g)}] \times 100$$

3.2.4.6. Calculation of Feed Conversion Rate

The quantity of feed consumed during the entire feeding period was recorded. The feed utilization rate, or feed conversion rate, is one of the most widely used indicators for measuring fish growth (Aquamedia, 2006). Feed Conversion Ratio (FCR) = 2 is the average value for marine fish. The value of FCR varies depending on different factors; sizes of fish species, different growth conditions, feeds and raw materials used. The feed utilization rate, in addition to being a measure of weight gain, ensures the production of healthy, high-quality fish that can enter the market in the short term.

The feed utilization rate was calculated by dividing the average amount of feed eaten by the fish by the mass value gained by the fish during the experiment.

Feed Conversion Ratio (FCR) was Calculated according to the formula:

$$\text{FCR} = \frac{\text{Amount of feed consumed during study (g)}}{\text{Individual body weight increase (g)}}$$

3.2.4.7. Calculation of Feed efficiency

$$\text{FE} = \text{wet weight gain/feed intake.}$$

3.2.4.8. Calculation of Protein efficiency ratio PEF

$$\text{PER} = \text{weight gain/protein intake.}$$

3.2.5. Sample Collection

Before sampling, fish fasted for 24 h. Gastrointestinal tissue were collected from 10 fish and preserved in 10% formaldehyde solution for use in gut histological analysis. Another sample consisting of 10 fish for proximate analysis, 10 fish for amino acid profile analysis, and 10 other fish for HS and VS indices were also taken.



Figure 3.5: General view of the internal organs of experimental fish.

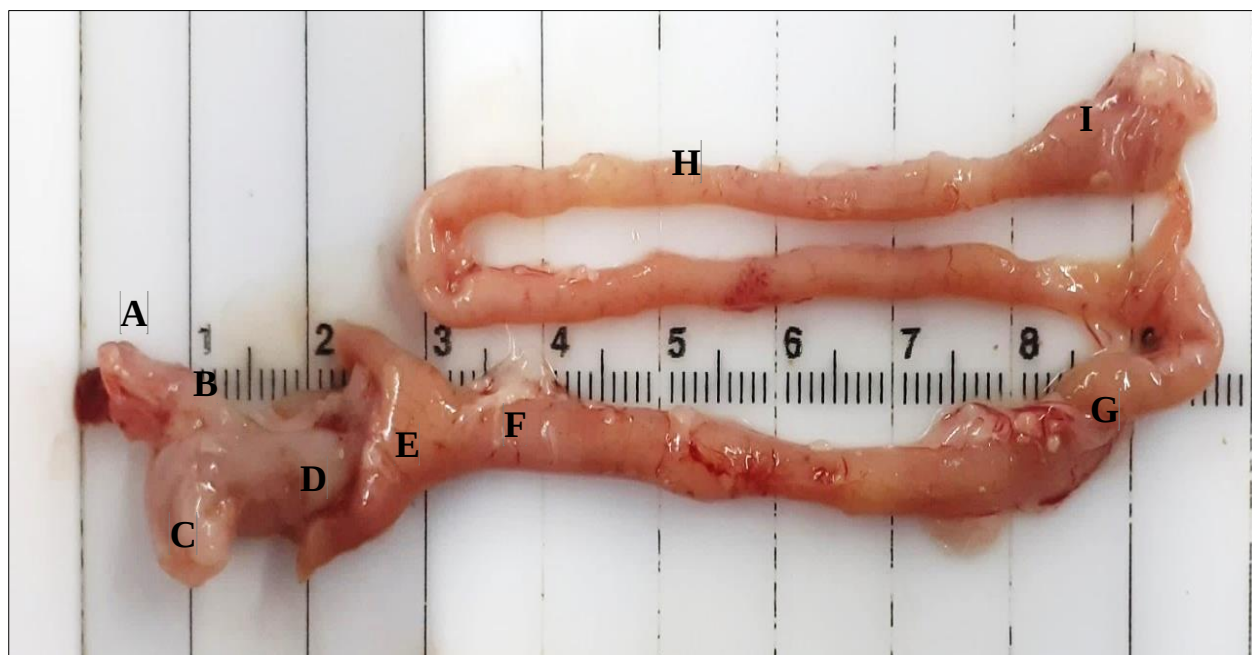


Figure 3.6: Structure of the digestive tract of *Sparus aurata*. (A) Esophagus, (B) cardiac stomach, (C) fundic stomach, (D) pyloric stomach, (E) pyloric caeca, (F) anterior intestine, (G) mid intestine, (H) posterior intestine, and (I) rectum.

At the end of the feeding experiments, a group of 105 fish was taken, i.e. from each tank, 5 fish were taken, making a total of 15 fish from each dietary group for analyses later. Thus, 5 fish were used for the proximate analysis and for the amino acid analyses. 5 to calculate HSI and VSI indices and 5 for histological analyzes of the digestive tract and liver. The samples taken for the histological analysis were stored in a 10% formaldehyde solution as mentioned above, while the rest of the samples taken for the other analyzes were stored in the refrigerator at -80°C . At the end of the experiments, all samples were transferred to the Fish Nutrition Laboratory of Istanbul University in polystyrene containers.

3.2.6. Chemical Analysis

The nutrient composition [dry matter (DM), crude ash (CA), crude protein (CP) (nitrogen $\times 6.25$), crude lipid (CL) (ether extracts), and crude fiber (CF)] and amino acid analyses of the feed and whole body were determined according to AOAC (2006) in triplicate. The analyses were carried out in the Fish Nutrition Laboratory of the Faculty of Aquatic Sciences of Istanbul University. Nitrogen-free feed extracts, total energy, and metabolized energy were calculated from the analysis results.

The total amount of energy and metabolized energy (ME kJ/g) in the feed was calculated according to Arthur and Phillips (1972). The Liquid Chromatography Mass Spectrometry (LCMS) method was used to determine the amino acid contents of meals, diets, fish, and fecal samples. Fish and dietary samples were prepared for analysis using a laboratory homogenizer and then stored in plastic containers at -20°C for until analyses.

3.2.6.1. Dry Matter Content

To balance the ratio, it is important to know the water content of each component; in addition, the humidity of prepared feeds should be monitored as high levels of humidity pose a risk of contamination with fungi and bacteria. Feeds that contain less than 10% moisture are the most commonly used feeds in intensive aquaculture because they are easy to store, and handle and are of more consistent quality. The method is based on drying a sample and determining the moisture content by the difference in weight between the dry and wet material.

The prepared samples were kept in an oven at 105°C for 4-6 hours. After this step, the samples were transferred to a desiccator and brought to room temperature. The amount of DM in the sample was found from the ratio of the difference between the first and final weighing to the mass of the sample.

The weight loss was recorded as the moisture content of the studied samples. The moisture content was calculated using the formula below.

$$\text{DMC (\%)} = \frac{|\text{Initial sample weight} - \text{Final sample weight}|}{|\text{Initial Sample weight (g)}|} * 100$$

3.2.6.2. Crude Ash Content

Ash is the total mineral or inorganic content of animal feed. It is determined after the incineration of a sample. Feed and whole fish samples were weighed to approximately 1.5g, placed in porcelain crucibles, and fired in a muffle furnace at a temperature of 450- 550°C for 5-6 hours.

At the end of this period, the samples were removed from the muffle furnace and the porcelain crucibles were cooled to room temperature in the desiccator and then weighed using a precision balance. The weight of the porcelain crucibles was subtracted from this result and the percentage raw ash results were calculated by dividing the sample weight and multiplying by 100 using the formula below.

$$\text{CAC (\%)} = \frac{(\text{Weight of porcelain crucible (g)} + \text{residue(g)}) - (\text{weight of porcelain crucible})}{\text{Initial sample weight (g)}} \times 100$$

3.2.6.3. Crude Protein Content

Homogenized samples (ingredients, feed, and whole body) were used in crude protein analysis. About 0.2-0.5g were weighed on precision balances and then transferred to Kjeldahl flasks. About 2 g of catalyst and 20 ml of sulfuric acid (H₂SO₄) were added to the samples. The wet bake process was carried out at 450°C for approximately 1.5 hours or until the liquid in the tube turned bright yellow. After the combustion process, the tubes were allowed to cool to room temperature. After cooling, the tubes were attached to the protein analyzer (Kjeldahl, Gerhardt Vapodest, 45 S) and continued with the distillation and titration processes. Using the values obtained from the titration process, the percentage of crude protein in the sample was calculated. The crude protein ratio was calculated using the formula below.

$$\text{CPC (\%)} = \frac{1.4007 \times 0.001 \times [\text{Acid used (ml)}] \times 6.25}{\text{Sample weight (g)}} \times 100$$



Figure 3.7: Digestion unit (A) and distillation unit (B) of the Kjeldahl system used for crude protein analysis in the Fish Nutrition Laboratory at Istanbul University.

3.2.6.4. Crude Lipid Content

Total lipids are generally defined as food components soluble in organic solvents and insoluble in water (Laporte, 2007). To measure the amount of total lipids in our biological samples (ingredients, feed, and whole body), we used the Soxhlet automatic extraction method. The samples (ingredients, feed, and whole body) which were homogenized, about 5g were weighed and transferred to the oil cartridges, and the upper parts of the cartridges were covered with cotton. The cartridges were placed in the Soxhelat device, (Velp Scientifica Ser,148). The weights of the oil crucibles, which were cleaned and dried in an oven at 105°C, were determined and placed in the oil analyzer (Soxhelat, Velp Scientifica Ser, 148). Lipid extraction was carried out for about 1 hour with diethyl ether (Figure 3.8). Once the extraction was complete, the oil crucibles were removed and stored in an oven at 105°C. For 1 hour after drying and cooling in the desiccator. The ratios of the crucible and crude oil were determined as a result of the weighing performed after this process. The percentage of total lipids in the samples was calculated by subtracting the weight of the empty crucible from the weight

of the full oil crucible and subtracting the weight of the sample, dividing by the initial weight of the sample, and multiplying by 100.



Figure 3.8: Soxhlet system used for crude lipid analysis in the Fish Nutrition Laboratory at Istanbul University.

3.2.6.5. Crude Fiber Content

The feeds were boiled for 30 minutes with different concentrations of H_2SO_4 and potassium hydroxide (KOH) to dissolve organic substances in the feeds except for crude fiber. All organic residues were washed with dilute H_2SO_4 , sodium hydroxide (NaOH), then with distilled water, and finally with acetone. Finally, the resulting residues were dried in an oven at $130^\circ C$ for 1 hour and cooled to room temperature in a desiccator, and weighed. The percentage of crude fiber content in the sample was calculated by dividing the difference between the second weighing and the weighing by the amount of sample and multiplying by 100. The ratio of crude fiber was calculated using the formula below.

$$\text{CFC (\%)} = \frac{[\text{Final weighing} - \text{First weighing}]}{[\text{Sample weight (g)}]} * 100$$

3.2.6.6. *Non-Nitrogen Extracts (NNE)*

In this study, the percentages of nitrogen-free extracts in fish feed were not determined by analyzes, but by subtracting the values of the previous analyzes (DMC, AC, PC, LC, and FC) from 100. The percentage of the nitrogen-free extract was calculated using the following formula.

$$\text{NNE (\%)} = 100 - (\% \text{DMC} + \% \text{CAC} + \% \text{CPC} + \% \text{CLC} + \% \text{CFC})$$

3.2.6.7. *Total Metabolizable Energy Value*

The percentage of a total energy value was calculated from the amount of energy crude protein content, crude lipid content, and carbohydrate content, which are nutrients obtained from analysis in diet samples.

$$\text{Total energy (\%)} = \frac{5.65 (\% \text{CPC}) + 9.45 (\% \text{CLC}) + 4.10 (\% \text{NNE}) * 10}{1000 * 4.186}$$

Metabolizable energy (ME) was calculated based on the digestibility values of energy-containing nutrients in carnivorous species.

$$\text{ME (\%)} = \frac{3.9 (\% \text{CPC}) + 8.0 (\% \text{CLC}) + 1.6 (\% \text{NNE}) * 10}{1000 * 4.186}$$

$$\left. \begin{array}{l} \text{For proteins: 3.9 k kal/g} \\ \text{For lipids: 8.0 k kal/g} \\ \text{For carbohydrates: 1.6 k kal/g} \end{array} \right\} \quad \left[1 \text{ kal} = 4.186 \text{ joules} \right]$$

3.2.7. Amino Acids Digestibility

In order to determine the digestibility of amino acids in feed samples and feces, 1% Cr₂O₃ indicator was added to the feeds. After being fed to the fish in the digestibility tanks, after fish feeding, approximately after 1.5 – 2 hours, the digestibility tanks and the feces collection system were cleaned up, making sure that there is no feed residue in the tanks and feces collection system, afterwards the feces have been collected for 5-6 hours approximately. The collected feces were stored in a deep freezer at -80°C until analyzed by placing them in lidded and sterile containers.

The digestibility ratios of amino acids were calculated according to the following formula (Maynard *et al*, 1979).

Amino acids digestibility rate (%) =

$$100 \left[100 \left(* \frac{\% \text{Cr}_2\text{O}_3 \text{ in diets}}{\% \text{Cr}_2\text{O}_3 \text{ in faeces}} \right) * \left(\frac{\% \text{Amino-acids in faeces}}{\% \text{Amino-acids in diets}} \right) \right]$$

3.2.8. Amino-Acids Analysis

Amino acid composition and digestibility are critical factors in determining the nutritional value of test ingredients and prepared foods as a source of protein. For this study, the amino acids were

analyzed by a private external laboratory. Analyzes were performed using a high-sensitivity, high-speed triple quadrupole mass spectrometer method for the Liquid Chromatography Mass Spectrometer system (LCMS/8050). This method allowed a faster analysis of several components (ultra-fast speed) as well as allowing a qualitative and quantitative analysis simultaneously. This method has made it possible to obtain results of unparalleled quality.

First, a 1g sample (raw materials, feed, fish feces, and fish whole body) was weighed with a 0.0001g precision balance and poured into a Schott bottle. 10 ml of petroleum ether was added and the oil was vortexed for 2 minutes. Then the samples were centrifuged for 3 minutes and the upper petroleum ether phase was removed with a pipette. This step was repeated once more. Then the oil was removed in a 50 ml tube, 25 ml of 6NHCl solution was added to the sample, then poured into a 250 ml Schott bottle. In order to be sure that no sample remains in the falcon 50 ml tube. Again, 25 ml afterward the open 250 ml Schott bottles were placed into an oven preheated to 110°C. The bottles were left open for an hour in the oven. After 1 hour, the bottles were closed and sealed to continue hydrolysis for 23 hours.

After 24 hours, the 250 ml Schott bottle was removed from the oven and stored until they reached room temperature, and subsequently transferred into 200 ml bottles. In order to prevent any sample from remaining in the Schott bottle, it was washed with distilled water and emptied into the balloon pot. Distilled water was then added to reach the mark. The samples were filtered using a filter (45 micron). 15 ml of the liquid was transferred to a 15 ml falcon tube. The samples were diluted 1500 times before sending them to the LCMS-MS device (Figure 3.9). The reason for this high dilution rate is that the LC-MS device measures with high precision.



Figure 3.9: Liquid Chromatography Mass Spectrometer system (LCMS 8050) used for the analysis of amino acids in the GTS Test Laboratories-Department of Chemistry.

3.2.9. Chromic Oxide Analysis

To assess the digestibility of formulated feeds, chromic oxide (Cr_2O_3), a substance more commonly used as a marker, was added to the feeds. Tanks were siphoned two hours after feeding fish to clean the tanks and remove uneaten feed. The fish fecal matter was then collected 5 hours after this by opening the outlet pipes of the fecal collection system (of the cylindroconical experimental tanks).

The feces were then transferred to small covered bottles and stored at -20°C until further analysis. To determine the amount of Cr_2O_3 in feed and feces the method of Kimura and Miller (1957) was applied. First, a standard curve was prepared. For this, quantities of Cr_2O_3 at 16 different points varying between 0.0003 and 0.0005 were weighed using a precision balance of 0.0001g, wrapped in filter paper, and placed in 250 ml Kjeldahl tubes. Two other empty tubes (without sample or

filter paper) and 2 other tubes as controls (containing only filter paper) have been used. Once the standard curve was determined, freeze-dried samples of 0.3g of feeds containing 1% Cr_2O_3 and between 0.003 and 0.005g feces were weighed, wrapped in filter paper, and placed in Kjeldahl tubes of 250 ml. 3 ml of concentrated nitric acid (HNO_3 , Sigma, CAS 7697-37-2) was added to all samples in the tubes for a period of time to allow homogeneous contact. Then these tubes were kept in the combustion unit for 30 minutes at 150 °C and one hour at 170°C. After combustion, the tubes were cooled to room temperature. Then 1.5 ml of 70% perchloric acid (HClO_4) was added to the tubes and heated again at 220°C for 15 minutes. During this combustion process, the tubes were left open until a color transformation occurred.

After the liquid part in the tubes cooled, it was sublimated into 50 ml vials and made up to 50 ml by adding distilled water to samples less than 50 ml. Finally, the absorbance value was defined in a spectrophotometer at a wavelength of 346.5 nm. The Cr_2O_3 concentrations of the samples were defined using the standard curve created at the beginning.

3.2.10. Histological Examination

For the histological evaluation under the optical microscope, 14 fish at the beginning of the experiment and 3 fish per tank at the end of the test were used. All fish were sacrificed with anesthesia prior to dissection. The liver as well as the digestive tract were quickly fixed in 10% formalin buffer and stored until further processing. Dehydration (in a graded series of alcohol), clarification (in xylene) and coating (in fibrowax) were the next steps.

After fixation, manually processed tissue samples were covered with embedding material and embedded in paraffin blocks. Tissue samples cut in a Leica microtome 4–5 μm thick were stained with the hematoxylin-eosin staining method and examined under a light microscope (Culing, 1963; Drury and Walington, 1980).

3.2.11. Statistical Analysis

The Windows SPSS 22.0 was used to statistically evaluate data related to growth measures throughout the experiment, and the analysis used one-way ANOVA (single-way ANOVA) and assessed the difference between the means with Tukey's multiple comparison test at a significance level $P < 0.05$ |

4. RESULTS

4.1. AMINO ACIDS COMPOSITION OF EXPERIMENTAL DIETS

The amino acid profile of the experimental diets is shown in Table 3.3. The amino acid composition of diets directly reflects the amino acid composition of the respective meals used. Total amino acids in experimental diets ranged from 96.81 to 98 g/100g of protein. EAAs values in FM and PBM-based diets were found to be higher than that of HFM. However, the sum of NEAAs are higher in HFM-based diets (varies from 56.02 to 57.75 g/100g of protein) than FM (48.99 g/100g of protein).

4.2. GROWTH PERFORMANCE VALUES

The individual starting weights of the fish placed in the experimental tanks and the individual body weights obtained during the experiment are shown in Table 4.1. The initial weights of fish in the various dietary groups were similar ($P > 0.05$) at stocking into the experimental tanks. The individual body weights of fish-fed FM-containing diets and fish-fed 65BPM and 75PBM diets at the end of the first period (15 days) were found to be similar ($P < 0.05$). However, the individual body weights of these three groups were higher than those fed HFM-based diets ($P < 0.05$). After 30 days of feeding, individual body weights of fish-fed HFM-based diets were the lowest ($P < 0.05$). At the end of the feeding experiments, the individual body weights of HFM-fed fish were at the lowest level ($P < 0.05$). At the end of the experiment, individual body weights of fish fed PBM and HFM were 3.77% and 69.42%, respectively lower, than FM-fed fish.

Table 4.1: Individual weights of fish (g)¹.

Period (Days)	Trial groups ²						
	FM	55PBM	65PBM	75PBM	55HFM	65HFM	75HFM
Survival %	100	100	100	100	76	66	46
Start	35.10±0.10 ^a	34.94±0.04 ^a	34.95±0.05 ^a	35.04±0.22 ^a	34.96±0.12 ^a	35.10±0.07 ^a	34.99±0.10 ^a
15	45.86±0.22 ^a	44.37±0.08 ^a	45.33±1.92 ^a	46.23±1.04 ^a	37.14±0.80 ^b	37.17±0.12 ^b	36.96±0.18 ^b
30	57.48±0.11 ^a	55.91±0.51 ^a	56.43±0.93 ^a	57.83±2.20 ^a	37.95±0.83 ^b	38.23±0.56 ^b	37.54±0.24 ^b
45	71.97±0.42 ^a	67.06 ±0.59 ^b	69.92±0.78 ^{ab}	69.83±2.61 ^{ab}	39.77±0.36 ^c	39.73±0.55 ^c	37.82±0.08 ^c
60	85.32±0.50 ^a	79.45±0.37 ^b	82.93 ±0.98 ^a	82.82±2.04 ^a	40.96±0.35 ^c	41.03±0.59 ^c	39.09±0.12 ^c
75	98.06±0.21 ^a	92.56±0.53 ^b	96.09±1.37 ^a	95.95±1.93 ^a	41.64±0.27 ^c	41.67±0.34 ^c	39.36±0.35 ^c
90	112.84±0.85 ^a	106.22±1.50 ^b	109.87±0.51 ^{ab}	108.08±2.36 ^{ab}	42.26±0.19 ^c	41.92±0.38 ^c	40.57±0.31 ^c
105	125.77±0.55 ^a	120.77±1.19 ^b	124.24±0.67 ^a	121.62±1.18 ^b	42.66±0.21 ^c	42.12±0.42 ^c	41.04±0.03 ^c
120	140.78±0.45 ^a	135.20±0.29 ^b	139.30±0.92 ^a	135.46±0.50 ^b	43.05±0.10 ^c	42.38±0.38 ^{cd}	41.56±0.10 ^d

¹Values (n = 3) are presented as mean and standard error of the mean (Mean±SD). The statistical difference between the values shown with different upper letters on the same line was found to be significant at the 95% accuracy level according to the Tukey multiple comparison test.

²FM: Fish meal; PBM: Poultry by-product meal; 55PBM: 55% PBM+35%FM, 65PBM: 65%PBM+35%FM; 75PBM: 75%PBM+25%FM; HTU: Hydrolysis feather meal; 55HTU: 55%HTU+45%FM; 65HTU: 65%HTU+35%FM, 75HTU: 75%HTU+25%FM.

The results obtained concerning the live weight gains of the fish during the experiment are presented in Table 4.2. Individual body weight gains of fish fed the FM diet were not statistically different from those of all groups fed PBM-based diets ($P > 0.05$). After the 30-day feeding period until the end of the experiment, individual body weight gains of fish-fed HFM diets were found to be the lowest ($P < 0.05$). Throughout the feeding period, the individual body weight gains of fish from the PBM groups were similar to the FM values. At the end of the 120 days of experimental feeding, the individual body weight gains of the HFM-fed fish were found to be the lowest ($P < 0.05$).

Table 4.2: Individual weight gains of fish (g)¹.

Period (Days)	Trial groups ²						
	FM	55PBM	65PBM	75PBM	55HFM	65HFM	75HFM
15	10.76±0.18 ^a	9.43±0.09 ^a	10.37±1.91 ^a	11.19±1.08 ^a	2.18±0.91 ^b	2.07±0.19 ^b	1.97±0.27 ^b
30	11.62±0.29 ^a	11.53±0.55 ^a	11.11±2.38 ^a	11.59±3.15 ^a	0.81±0.48 ^b	1.05±0.66 ^b	0.58±0.35 ^b
45	14.49±0.38 ^a	11.16±1.09 ^a	13.48±0.88 ^a	12.01±4.81 ^a	1.81±0.58 ^b	1.51±0.13 ^b	0.28±0.17 ^b
60	13.35±0.33 ^a	12.38±0.23 ^a	13.02±1.68 ^a	12.99±1.17 ^a	1.19±0.65 ^b	1.30±0.38 ^b	1.26±0.19 ^b
75	12.74±0.29 ^a	13.11±0.90 ^a	13.16±1.83 ^a	13.12±2.24 ^a	0.68±0.28 ^b	0.64±0.54 ^b	0.28±0.22 ^b
90	14.78±0.67 ^a	13.66±1.89 ^a	13.78±1.88 ^a	12.14±2.78 ^a	0.62±0.44 ^b	0.25±0.09 ^b	1.21±0.04 ^b
105	12.93±0.64 ^a	14.55±2.53 ^a	14.37±0.82 ^a	13.54±2.49 ^a	0.40±0.12 ^b	0.20±0.09 ^b	0.47±0.33 ^b
120	15.01±0.49 ^a	14.43±1.17 ^a	15.06±1.58 ^a	13.84±1.64 ^a	0.39±0.25 ^b	0.26±0.15 ^b	0.52±0.13 ^b
TWG³	105.68±0.44 ^a	100.26±0.32 ^b	104.34±0.96 ^a	100.42±0.38 ^b	8.09±0.21 ^c	7.28±0.33 ^{cd}	6.57±0.16 ^d

¹Values (n = 3) are presented as mean and standard error of the mean (Mean±SD). The statistical difference between the values shown with different upper letters on the same line was found to be significant at the 95% accuracy level according to the Tukey multiple comparison test.

²FM: Fish meal; PBM: Poultry by-product meal; 55PBM: 55% PBM+35%FM, 65PBM: 65%PBM+35%FM; 75PBM: 75%PBM+25%FM; HTU: Hydrolysis feather meal; 55HTU: 55%HTU+45%FM; 65HTU: 65%HTU+35%FM, 75HTU: 75%HTU+25%FM.

³ Total Weight gains

Fish specific growth rates were calculated using the periodic weight results. The results obtained are presented in Table 4.3. Examination of the results presented in the table shows that the specific growth rates of the fish fed with HFM-based diets were lower at the end of the first period. Similar results were obtained at the end of the second period ($P < 0.05$) and until the end of the experiment. The reverse of these results was obtained in fish fed with PBM, with a growth rate similar to that of FM. At the end of the feeding experiment, the mean specific growth rate of fish 55HFM, 65HFM, and, 75HFM were the lowest than FM ($P < 0.05$).

Table 4.3: Specific growth rates of fish¹.

Period (Days)	Trial groups ²						
	FM	55PBM	65PBM	75PBM	55HFM	65HFM	75HFM
15	1.78±0.03 ^a	1.59±0.01 ^a	1.73±0.28 ^a	1.85±0.16 ^a	0.40±0.16 ^b	0.38±0.03 ^b	0.37±0.05 ^b
30	1.51±0.04 ^a	1.54±0.07 ^a	1.46±0.33 ^a	1.49±0.39 ^a	0.14±0.08 ^b	0.19±0.12 ^b	0.10±0.06 ^b
45	1.50±0.03 ^a	1.19±0.06 ^a	1.43±0.10 ^a	1.26±0.50 ^a	0.31±0.10 ^b	0.26±0.02 ^b	0.05±0.03 ^c
60	1.13±0.03 ^a	1.15±0.03 ^a	1.14±0.15 ^a	1.14±0.12 ^a	0.20±0.11 ^b	0.21±0.06 ^b	0.22±0.03 ^b
75	0.93±0.02 ^a	1.02±0.07 ^a	0.98±0.13 ^a	0.98±0.17 ^a	0.11±0.05 ^b	0.10±0.09 ^b	0.05±0.04 ^b
90	0.94±0.04 ^a	0.92±0.12 ^a	0.89±0.13 ^a	0.79±0.18 ^a	0.10±0.07 ^b	0.04±0.01 ^c	0.20±0.01 ^b
105	0.72±0.04 ^a	0.86±0.15 ^a	0.82±0.05 ^a	0.79±0.15 ^a	0.06±0.02 ^c	0.03±0.01 ^c	0.08±0.05 ^c
120	0.75±0.03 ^a	0.75±0.06 ^a	0.76±0.08 ^a	0.72±0.09 ^a	0.06±0.04 ^c	0.04±0.02 ^c	0.08±0.02 ^c
FSGR³	1.16±0.01 ^a	1.13±0.01 ^a	1.15±0.01 ^a	1.13±0.01 ^a	0.17±0.01 ^c	0.16±0.01 ^c	0.14±0.01 ^c

¹Values (n = 3) are presented as mean and standard error of the mean (Mean±SD). The statistical difference between the values shown with different upper letters on the same line was found to be significant at the 95% accuracy level according to the Tukey multiple comparison test. ²FM: Fish meal; PBM: Poultry by-product meal; 55PBM: 55% PBM+35%FM, 65PBM: 65%PBM+35%FM; 75PBM: 75%PBM+25%FM; HTU: Hydrolysis feather meal; 55HTU: 55%HTU+45%FM; 65HTU: 65%HTU+35%FM, 75HTU: 75%HTU+25%FM. ³Final Specific growth rates

During the experiment, individual feed consumption of fish was calculated periodically and presented in Table 4.4. At the end of the first period of the feeding, the highest individual feed consumption amount was observed in the 75PBM group ($P < 0.05$). As shown in the table, a clear statistical difference was observed between the groups fed with PBM and HFM compared to the control group fed with FM as a source of dietary protein. Naturally, the quantity of diet consumed by fish increased with the increase in the weight of the fish as the feeding trial progressed. At the end feeding trial, feed consumption of the fish-fed HFM-based diets was significantly lower than

that of FM and PBM-based diets ($P < 0.05$). Feed consumption was similar ($P > 0.05$) between FM and 75PBM-fed group.

Table 4.4: Average individual feed consumption rates (g)¹.

Period (Days)	Trial groups ²						
	FM	55PBM	65PBM	75PBM	55HFM	65HFM	75HFM
15	11.21±0.08 ^a	9.05±0.03 ^b	10.77±0.18 ^a	11.01±0.59 ^a	5.16±0.59 ^c	4.99±0.50 ^c	5.67±0.25 ^c
30	13.15±0.99 ^a	11.10±0.06 ^a	11.23±0.19 ^a	12.93±0.65 ^a	2.65±0.36 ^b	2.82±0.70 ^b	2.87±1.38 ^b
45	16.30±0.51 ^a	12.21±0.11 ^b	13.44±0.33 ^b	10.57±0.55 ^c	4.54±0.52 ^d	4.61±0.33 ^d	1.28±0.62 ^e
60	15.04±0.16 ^a	13.34±0.21 ^b	14.93±0.64 ^a	15.39±0.14 ^a	3.50±0.36 ^c	2.20±0.07 ^d	2.69±0.16 ^{cd}
75	14.31±1.33 ^a	14.06±0.03 ^a	15.37±0.25 ^a	14.74±0.28 ^a	3.05±0.04 ^b	2.09±0.51 ^{bc}	1.06±0.41 ^c
90	16.44±0.50 ^b	15.83±0.35 ^b	19.17±0.83 ^a	16.47±0.36 ^b	2.42±0.46 ^{cd}	1.72±0.59 ^d	4.46±1.96 ^c
105	14.22±1.56 ^b	18.07±0.06 ^a	14.08±0.63 ^b	19.22±0.26 ^a	2.05±0.68 ^c	1.47±0.25 ^c	2.07±1.07 ^c
120	16.30±0.30 ^b	19.70±1.22 ^a	15.36±0.07 ^b	20.21±0.78 ^a	1.16±0.50 ^c	0.85±0.32 ^c	1.88±0.12 ^c
TFI³	116.96±1.35 ^{ab}	113.36±1.24 ^b	114.36±0.96 ^b	120.54±0.85 ^a	24.32±0.39 ^c	20.35±2.37 ^c	21.96±2.38 ^c

¹Values ($n = 3$) are presented as mean and standard error of the mean (Mean±SD). The statistical difference between the values shown with different upper letters on the same line was found to be significant at the 95% accuracy level according to the Tukey multiple comparison test.

²FM: Fish meal; PBM: Poultry by-product meal; 55PBM: 55% PBM+35%FM, 65PBM: 65%PBM+35%FM; 75PBM: 75%PBM+25%FM; HTU: Hydrolysis feather meal; 55HTU: 55%HTU+45%FM; 65HTU: 65%HTU+35%FM, 75HTU: 75%HTU+25%FM. ³TFI: Total Feed intake

During the feeding trials, feed conversion ratios of juvenile gilthead seabream were calculated periodically, and also feed efficiency and protein efficiency ratio were calculated and presented in Table 4.5. FM and PBM groups had lower feed conversion ratios ($P < 0.05$) at the end of the first period. Examination of the results shows that although there were statistically significant differences between the experimental groups during the experiment, it was found that the average

feed efficiency of PBM-fed fish at the end of the experiment was similar to that of FM-fed fish ($P < 0.05$). The average feed conversion ratio of these groups was found to be less than 1.23. FM and PBM groups had lower values in feed efficiency. Protein efficiency ratio of the groups fed with PBM and the control group fed with FM as a good source of dietary protein efficiency.

Table 4.5: Feed conversion rate of fish¹.

Period (Days)	Trial groups ²						
	FM	55PBM	65PBM	75PBM	55HFM	65HFM	75HFM
15	1.04±0.02 ^b	0.96±0.01 ^b	1.06±0.18 ^b	0.99±0.06 ^b	2.68±1.13 ^a	2.41±0.25 ^a	2.90±0.33 ^a
30	1.13±0.07 ^c	0.96±0.04 ^c	1.05±0.24 ^c	1.19±0.39 ^c	3.87±1.57 ^b	3.74±2.57 ^b	5.31±0.84 ^a
45	1.12±0.02 ^c	1.12±0.04 ^c	1.00±0.05 ^c	0.98±0.37 ^c	2.72±1.02 ^b	3.08±0.35 ^{ab}	5.35±2.26 ^a
60	1.13±0.04 ^d	1.06±0.05 ^d	1.16±0.19 ^d	1.19±0.11 ^d	3.45±1.52 ^a	1.82±0.66 ^c	2.16±0.31 ^b
75	1.12±0.09 ^b	1.08±0.08 ^b	1.19±0.18 ^b	1.15±0.23 ^b	4.96±1.86 ^a	5.07±3.20 ^a	4.75±1.74 ^a
90	1.11±0.06 ^{de}	1.17±0.18 ^{de}	1.40±0.14 ^d	1.40±0.28 ^d	5.14±2.81 ^b	6.92±1.41 ^a	3.67±1.55 ^c
105	1.10±0.17 ^d	1.27±0.25 ^d	0.98±0.06 ^d	1.45±0.28 ^c	5.01±2.18 ^b	5.15±1.03 ^{ab}	5.67±2.78 ^a
120	1.09±0.04 ^c	1.37±0.16 ^b	1.03±0.12 ^c	1.47±0.13 ^b	3.58±1.59 ^a	3.56±0.91 ^a	3.83±1.18 ^a
FFCR ³	1.11±0.01 ^c	1.12±0.02 ^c	1.11±0.01 ^c	1.23±0.04 ^b	3.93±0.41 ^a	3.97±0.29 ^a	4.20±0.35 ^a
FFE ⁴	0.90±0.01 ^a	0.88±0.02 ^{ab}	0.91±0.01 ^a	0.83±0.01 ^b	0.33±0.01 ^c	0.36±0.04 ^c	0.30±0.04 ^c
FPER ⁵	1.84±0.02 ^a	1.83±0.02 ^a	1.88±0.02 ^a	1.70±0.01 ^b	0.68±0.01 ^{cd}	0.74±0.07 ^c	0.61±0.06 ^d

¹Values (n = 3) are presented as the mean and standard error of the mean (Mean±SD). The statistical difference between the values shown with different upper letters on the same line was found to be significant at the 95% accuracy level according to the Tukey multiple comparison test.

²FM: Fish meal; PBM: Poultry by-product meal; 55PBM: 55% PBM+35%FM, 65PBM: 65%PBM+35%FM; 75PBM: 75%PBM+25%FM; HFM: Hydrolysis feather meal; 55HFM: 55%HFM+45%FM; 65HFM: 65%HFM+35%FM, 75HFM: 75%HFM+25%FM.

³FFCR: Final Feed conversion ratio = feed intake/wet weight gain ⁴FFE: Final Feed efficiency = wet weight gain/feed intake.

⁵FPER: Final Protein efficiency ratio = weight gain/protein intake.

In order to calculate the condition factor, the lengths and weights of individual fish were measured at the beginning of the experiment and periodically until the end of the experiment. The individual lengths obtained during the experiment are shown in Table 4.6. The statistical values obtained, there was no significant difference between the lengths of individual fish periodically of fish feeding with PBM and FM ($P > 0.05$). Their lengths also increased systematically with the growth of fish.

Table 4.6: Average individual length of fish (cm)¹.

Trial groups²	Start of the trial	End of trial
FM	13.37±0.22	18.82±0.28 ^a
55PBM	13.32±0.26	18.73±0.55 ^a
65PBM	13.31±0.27	18.62±0.98 ^a
75PBM	13.33±0.24	18.11±0.56 ^{ab}
55HFM	13.34±0.21	14.28±0.15 ^c
65HFM	13.36±0.23	14.43±0.06 ^c
75HFM	13.35±0.24	13.92±0.11 ^{cd}

¹Values ($n = 3$) are presented as the mean and standard error of the mean (Mean±SD). The statistical difference between the values shown with different upper letters on the same line was found to be significant at the 95% accuracy level according to the Tukey multiple comparison test.

²FM: Fish meal; PBM: Poultry by-product meal; 55PBM: 55% PBM+35%FM, 65PBM: 65%PBM+35%FM; 75PBM: 75%PBM+25%FM; HFM: Hydrolysis feather meal; 55HFM: 55%HFM+45%FM; 65HFM: 65%HFM+35%FM, 75HFM: 75%HFM+25%FM.

Fish condition factors were calculated at the start of the experiment and periodically throughout the experimental period. The results obtained are shown in Table 4.7. The results show that the fish condition factors at the start of the experiment were approximately 1.53. The condition factor values at the end of the experiment were higher than at the beginning of the experiment. When the condition factors of HFM groups were compared at the end of the trial, there was a significant lower between the PBM groups and the control group ($P < 0.05$).

Table 4.7: Condition factors of fish¹.

Trial groups²	Start of the trial	End of trial
FM	1.53±0.08	1.60±0.14 ^b
55PBM	1.50±0.06	1.70±0.16 ^{ab}
65PBM	1.52±0.09	1.72±0.10 ^{ab}
75PBM	1.53±0.08	1.74±0.98 ^a
55HFM	1.51±0.09	1.37±0.12 ^c
65HFM	1.56±0.07	1.28±0.11 ^c
75HFM	1.54±0.05	1.29±0.09 ^c

¹Values (n = 3) are presented as the mean and standard error of the mean (Mean±SD). The statistical difference between the values shown with different upper letters on the same line was found to be significant at the 95% accuracy level according to the Tukey multiple comparison test.

²FM: Fish meal; PBM: Poultry by-product meal; 55PBM: 55% PBM+35%FM, 65PBM: 65%PBM+35%FM; 75PBM: 75%PBM+25%FM; HFM: Hydrolysis feather meal; 55HFM: 55%HFM+45%FM; 65HFM: 65%HFM+35%FM, 75HFM: 75%HFM+25%FM.

The results of the hepatosomatic and viscerosomatic indices were calculated at the beginning and at the end of the experiment. The results obtained are presented in Table 4.8. At the end of the experiment, the difference between the groups was statistically significant ($P < 0.05$). However, the values of the hepatosomatic index and the viscerosomatic index at the end of the experiment were lower than the values at the beginning of the experiment ($P < 0.05$).

Table 4.8: Hepatosomatic and viscerosomatic index values¹.

Trial groups ²	Hepatosomatic index (HSI)		Viserosomatic index (VSI)	
	Start of the experiment	End of experiment	Start of the experiment	End of experiment
FM	1.47±0.25	1.08±0.23 ^a	9.10±1.29	7.35±1.17 ^a
55PBM	1.47±0.25	0.95±0.20 ^{ab}	9.10±1.29	6.76±1.22 ^{ab}
65PBM	1.47±0.25	1.04±0.24 ^a	9.10±1.29	6.92±1.32 ^{ab}
75PBM	1.47±0.25	1.10±0.18 ^a	9.10±1.29	7.05±1.18 ^a
55HFM	1.47±0.25	0.76±0.17 ^c	9.10±1.29	5.78±1.02 ^c
65HFM	1.47±0.25	0.77±0.18 ^c	9.10±1.29	5.09±0.98 ^d
75HFM	1.47±0.25	0.70±0.14 ^c	9.10±1.29	5.27±1.33 ^{cd}

¹Values (n = 3) are presented as the mean and standard error of the mean (Mean±SD). The statistical difference between the values shown with different upper letters on the same line was found to be significant at the 95% accuracy level according to the Tukey multiple comparison test.

²FM: Fish meal; PBM: Poultry by-product meal; 55PBM: 55% PBM+35%FM, 65PBM: 65%PBM+35%FM; 75PBM: 75%PBM+25%FM; HFM: Hydrolysis feather meal; 55HFM: 55%HFM+45%FM; 65HFM: 65%HFM+35%FM, 75HFM: 75%HFM+25%FM.

4.3. WHOLE-BODY COMPOSITION

The results of body composition at the end of the feeding trial are shown in Table 4.9. Overall, body compositions such as dry matter, crude ash, crude lipid, and crude protein were comparatively higher at the end than at the beginning of the test in fish fed with FM and PBM. However, the final proximate compositions of fish-fed HFM-based diets were lower than the values at the start of the trial. At the end of the experiment, crude fat levels in the livers of fish belonging to the HFM-based diets fish groups were about twice as high as at the start of the experiment. The FM and 55PBM groups had the lowest level of liver fat ($P < 0.05$).

Table 4.9: Whole-body composition and liver fat percentages of fish (%)¹.

Body Comp.	Trial groups ²							
	Start	FM	55PBM	65PBM	75PBM	55HFM	65HFM	75HFM
Dry Matter	33.06±0.04 ^{ab}	34.89±0.52 ^a	33.40±0.04 ^{ab}	33.45±0.21 ^{ab}	34.75±0.14 ^a	26.80±0.02 ^c	27.57±0.04 ^c	23.95±0.04 ^d
Crude protein	15.04±0.24 ^c	16.14±0.19 ^{ab}	16.92±0.02 ^{ab}	17.25±0.70 ^a	16.06±0.19 ^{ab}	13.78±0.03 ^{de}	14.26±0.03 ^d	13.43±0.03 ^e
Crude Fat	10.86±0.05 ^b	12.40±0.09 ^{ab}	12.25±0.96 ^{ab}	12.97±0.52 ^a	12.87±1.09 ^a	7.60±0.04 ^c	6.58±0.04 ^c	4.69±0.04 ^d
Ash	3.81±0.03 ^c	3.89±0.03 ^c	3.71±0.05 ^c	3.24±0.04 ^d	4.61±0.09 ^a	4.20±0.04 ^b	4.64±0.06 ^a	4.88±0.03 ^a
Liver fat	11.77±0.53 ^d	15.38±0.34 ^c	15.29±0.57 ^c	16.57±0.23 ^{bc}	16.44±2.47 ^{bc}	17.46±1.82 ^{ab}	18.94±0.67 ^{ab}	19.74±0.58 ^a

¹Values (n = 3) are presented as the mean and standard error of the mean (Mean±SD). The statistical difference between the values shown with different upper letters on the same line was found to be significant at the 95% accuracy level according to the Tukey multiple comparison test. ²FM: Fish meal; PBM: Poultry by-product meal; 55PBM:55% PBM+35%FM, 65PBM: 65%PBM+35%FM; 75PBM: 75%PBM+25%FM; HFM: Hydrolysis feather meal; 55HFM:55%HFM+45%FM; 65HFM: 65%HFM+35%FM, 75HFM:75%HFM+25%FM.

3.24±0.04^f

4.4. PHYSIOCHEMICAL PROPERTIES OF WATER USED IN EXPERIMENTAL TANKS

The water quality parameters were measured during the feeding experiments and are given in Table 4.10. According to the statistical calculations performed for all the measurements obtained from the beginning to the end of the experiment, the difference between the data turned out to be insignificant. During the research, the water temperature was 23.6-28.3 °C, dissolved oxygen 7.1-7.80 mg/l, salinity 38.1-38.5 mg/l, and pH from 7.8 to 7.9.

Table 4.10: Quality parameters of the water used in the research.

Period (Days)	Temperature (°C)	Dissolved oxygen (mg/l)	Salinity (mg/l)	pH
Start	27.20±0.01 ^{ab}	7.20±0.40	38.10±0.01	7.80±0.01
15	27.40±0.02 ^{ab}	7.30±0.50	38.20±0.02	7.80±0.01
30	27.80±0.02 ^{ab}	7.10±0.30	38.30±0.02	7.80±0.03
45	28.30±0.01 ^a	7.20±0.60	38.40±0.01	7.80±0.02
60	28.00±0.03 ^a	7.30±0.50	38.50±0.02	7.90±0.02
70	26.00±0.01 ^b	7.40±0.40	38.40±0.01	7.90±0.01
90	25.90±0.02 ^b	7.50±0.80	38.50±0.01	7.90±0.01
105	25.80±0.04 ^b	7.03±0.20	38.44±0.11	7.90±0.06
120	23.60±0.02 ^c	7.80±0.20	38.45±0.12	7.90±0.03

4.5. WHOLE-BODY AMINO ACID PROFILE

The whole-body amino acid profiles of the experimental fish are shown in Table 4.11. It can be seen from the results that the amino acids in the body of the fish reflect the amino acids in the feed. Fish fed the control diet and the groups of fish fed 55% and 65% poultry by-product meal showed significantly higher levels in total EAAs. Fish fed 75HFM diet had significantly lower body concentrations in histidine, leucine, lysine, methionine, threonine, and valine than the control group (FM), 55PBM, and 65PBM respectively. At the end of the 120-day feeding experiment, total EAAs of fish-fed diets containing hydrolyzed feather meal (55HFM, 65HFM, and 75HFM) had low EAAs concentrations than the control group but relatively high compared to the level at beginning of the experiment.

Table 4.11: Whole-body amino acid profile of fish (g/100 g protein)¹

		Diets of feeding trial ²						
Amino-acids	Start	FM	55PBM	65PBM	75PBM	55HFM	65HFM	75HFM
Essential amino acids (EAA)								
Arginine	5.56±0.52 ^{ab}	5.78±0.16 ^{ab}	5.75±0.12 ^{ab}	6.04±0.46 ^a	5.11±0.83 ^b	5.40±0.25 ^b	5.02±0.23 ^b	6.07±0.55 ^a
Histidine	2.75±0.10 ^{ab}	3.09±0.13 ^a	3.06±0.06 ^a	3.07±0.18 ^a	2.72±0.33 ^{ab}	2.68±0.06 ^{ab}	2.54±0.29 ^c	2.51±0.12 ^c
Isoleucine	3.99±0.70 ^c	5.27±0.45 ^a	5.08±0.42 ^a	4.89±0.41 ^a	5.11±0.33 ^a	4.56±0.24 ^b	4.91±0.34 ^a	4.25±0.54 ^b
Leucine	8.51±0.07 ^b	9.77±0.36 ^a	9.73±0.18 ^a	9.45±0.07 ^a	9.22±0.49 ^{ab}	9.20±0.09 ^{ab}	9.10±0.48 ^{ab}	8.47±0.26 ^b
Lysine	7.27±0.10 ^c	8.37±0.04 ^{ab}	8.33±0.13 ^{ab}	8.63±0.36 ^a	7.82±0.68 ^b	7.89±0.15 ^b	7.35±0.43 ^{bc}	7.10±0.45 ^c
Methionine	2.33±0.20 ^{ab}	2.46±0.08 ^a	2.52±0.16 ^a	2.,60±0.09 ^a	2.39±0.33 ^{ab}	2.62±0.40 ^a	2.19±0.13 ^b	2.40±0.29 ^{ab}
Phe-alanine	3.72±0.14 ^c	4.25±0.13 ^a	4.28±0.08 ^a	4.21±0.13 ^a	4.00±0.14 ^{ab}	3.99±0.11 ^{ab}	3.90±0.24 ^{ab}	3.84±0.10 ^b
Threonine	4.67±0.28 ^{ab}	4.93±0.24 ^a	4.83±0.44 ^{ab}	5.05±0.44 ^a	4.39±0.81 ^b	5.19±0.10 ^a	4.41±0.10 ^b	4.59±0.22 ^{ab}
Valine	4.63±0.38 ^b	5.34±0.13 ^a	5.34±0.15 ^a	5.26±0.29 ^a	5.06±0.43 ^{ab}	5.09±0.12 ^b	4.88±0.17 ^b	4.73±0.20 ^b
Non-essential amino acids (NEAA)								
Alanine	6.85±0.65 ^a	6.80±0.77 ^a	6.70±0.74 ^a	6.30±0.67 ^{ab}	6.66±0.46 ^a	6.41±0.54 ^{ab}	6.63±0.33 ^a	6.03±0.50 ^b
Asp. Acid	9.17±0.33 ^{ab}	9.20±0.73 ^{ab}	9.40±0.52 ^a	9.00±0.83 ^{ab}	9.57±0.89 ^a	8.79±1.53 ^b	8.73±0.15 ^b	7.98±0.76 ^c
Cysteine	1.29±0.21 ^a	0.61±0.31 ^c	0.61±0.28 ^c	0.84±0.31 ^b	0.61±0.29 ^c	1.15±0.14 ^a	0.51±0.23 ^{cd}	0.66±0.20 ^c
Glut. Acid	14.21±0.33 ^{ab}	13.99±0.27 ^{ab}	13.81±0.12 ^{ab}	13.67±0.33 ^{bc}	15.00±0.03 ^a	12.05±1.59 ^d	13.41±0.10 ^{bc}	12.87±0.60 ^c
Glycine	7.74±0.10 ^{ab}	6.58±0.13 ^b	7.02±0.38 ^{ab}	7.07±0.69 ^{ab}	6.73±0.24 ^{ab}	6.81±0.52 ^{ab}	7.14±0.21 ^{ab}	8.14±0.99 ^a
Serine	4.32±0.20 ^{ab}	4.22±0.38 ^{ab}	4.22±0.23 ^{ab}	4.04±0.12 ^b	4.15±0.30 ^{ab}	4.30±0.26 ^{ab}	4.43±0.25 ^a	4.61±0.05 ^a
Tyrosine	4.99±0.18 ^a	3.35±1.05 ^{bc}	3.41±1.11 ^{bc}	3.89±0.98 ^b	3.48±1.22 ^{bc}	5.16±0.66 ^a	3.08±0.49 ^c	3.88±0.87 ^b
Proline	3.57±0.33 ^a	3.30±0.18 ^{ab}	3.30±0.19 ^{ab}	3.13±0.09 ^{ab}	3.43±0.10 ^a	3.51±0.35 ^a	2.96±0.12 ^b	2.79±0.25 ^b
ΣEAA	43.22±0.31 ^b	49.26±0.76 ^a	48.92±0.05 ^a	49.20±0.07 ^a	45.82±3.84 ^{ab}	46.61±0.15 ^{ab}	44.32±0.25 ^{ab}	43.96±0.08 ^b
ΣNEAA	52.13±1.36 ^a	48.04±0.85 ^b	48.47±0.04 ^b	47.94±0.02 ^c	49.64±0.03 ^b	48.18±2.87 ^c	46.89±0.00 ^d	46.97±0.01 ^d
ΣAA	95.34±1.63 ^{ab}	97.30±1.60 ^a	97.39±0.09 ^a	97.13±0.06 ^a	95.46±3.85 ^b	94.79±2.85 ^b	91.21±0.25 ^c	90.92±0.09 ^c

¹Values (n = 3) are presented as the mean and standard error of the mean (Mean±SD). The statistical difference between the values shown with different upper letters on the same line was found to be significant at the 95% accuracy level according to the Tukey multiple comparison test.²FM: Fish meal; PBM: Poultry by-product meal; 55PBM:55% PBM+35%FM, 65PBM: 65%PBM+35%FM; 75PBM: 75%PBM+25%FM; HFM: Hydrolysis feather meal; 55HFM:55%HFM+45%FM; 65HFM: 65%HFM+35%FM, 75HFM:75%HFM+25%FM.

4.6. AMINO ACID PROFILE IN FAECES

The data presented in Table 4.12 summarizes the analysis of the amino acid profile in the feces obtained from each of the diets fed juvenile gilthead seabream. This shows the concentrations of the 9 essential amino acids analyzed and the 8 non-essential amino acids in the feces of the fish tested. From the analysis of the data presented in Table 4.12, it was found that the amino acids profiles in the fecal matter of the experimental fish reflected the quantities of amino acids eliminated by the fish. The amount of EAA in the fecal matter of the groups fed with HFM was higher than that of the control group and those fed with PBM-based diets. Fish fed hydrolyzed feather meal had considerable losses of certain essential amino acids; arginine, isoleucine, leucine, phenylalanine, threonine, and valine compared to groups fed with FM and PBM ($P < 0.05$). However, the fecal amino acid profiles of the PBM-fed groups showed statistically nonsignificant ($P > 0.05$) differences from the FM-fed group. On the other hand, the content of NEAA in the fecal matter cysteine, glutamic acid, glycine, and serine, of fish of the HFM group increased by 9% compared to the control group ($P < 0.05$).

4.7. APPARENT DIGESTIBILITY COEFFICIENTS (%) OF AMINO ACIDS IN DIETS

Table 4.13 shows the calculated profiles of the apparent digestibility coefficient (ADC) of each experimental diet tested. According to the results, FM-fed fish recorded digestibility values of 86%-92% for essential amino acids, and between 82%-93% for non-essential amino acids. Similar, results were obtained in the groups fed with PBM-based diets ($P > 0.05$). However, all three groups of hydrolyzed feather meal (HFM) did not perform well in the digestibility coefficient in this assessment for juvenile gilthead seabream and the digestibility of essential amino acids was low with no indication that the treatment hydrolysis had any benefit in improving the digestibility of alternative proteins for this species. The coefficients were statistically different ($P < 0.05$). HFM had lower values in EAAs than the other groups, in particular histidine, isoleucine, lysine, alanine, and, tyrosine.

Table 4.12: Amino acid profile in experimental fish feces (g/100 g protein)¹

Amino-Acids	Diets of feeding trial ²						
	FM	55PBM	65PBM	75PBM	55HFM	65HFM	75HFM
Essential amino acids (EAA)							
Arginine	0.83±0.05 ^b	0.85±0.04 ^b	0.94±0.03 ^b	0.97±0.08 ^b	2.22±0.11 ^a	2.30±0.12 ^a	2.31±0.05 ^a
Histidine	0.46±0.05 ^{bc}	0.48±0.05 ^{bc}	0.38±0.03 ^c	0.24±0.03 ^d	0.63±0.01 ^a	0.69±0.04 ^a	0.70±0.10 ^a
Isoleucine	0.72±0.09 ^c	0.75±0.01 ^c	0.69±0.04 ^c	0.69±0.14 ^c	1.90±0.04 ^b	2.11±0.03 ^a	2.21±0.03 ^a
Leucine	0.93±0.05 ^b	1.03±0.09 ^b	1.02±0.03 ^b	1.16±0.02 ^b	3.16±0.08 ^a	3.20±0.09 ^a	3.28±0.03 ^a
Lysine	0.82±0.09 ^b	0.85±0.04 ^b	0.76±0.06 ^{bc}	0.61±0.06 ^c	1.12±0.06 ^a	1.27±0.04 ^a	1.26±0.02 ^a
Methionine	0.49±0.04 ^a	0.52±0.01 ^a	0.40±0.01 ^{ab}	0.24±0.06 ^b	0.52±0.05 ^a	0.54±0.08 ^a	0.48±0.04 ^a
Phe-lalanine	0.96±0.02 ^b	1.04±0.01 ^b	0.91±0.08 ^b	1.08±0.06 ^b	1.29±0.27 ^{ab}	1.37±0.11 ^{ab}	1.58±0.02 ^a
Threonine	0.79±0.10 ^b	0.43±0.02 ^c	0.84±0.03 ^b	0.97±0.07 ^b	2.13±0.13 ^a	2.15±0.11 ^a	2.21±0.04 ^a
Valine	0.84±0.08 ^{bc}	1.01±0.23 ^b	0.77±0.04 ^{bc}	0.98±0.07 ^b	2.24±0.04 ^a	2.20±0.08 ^a	2.16±0.02 ^a
Non-essential amino acids (NEAA)							
Alanine	1.23±0.02 ^{ab}	1.11±0.06 ^b	1.23±0.05 ^{ab}	1.30±0.04 ^{ab}	1.43±0.29 ^a	1.49±0.02 ^a	1.50±0.02 ^a
Asp. Acid	2.13±0.02 ^c	2.35±0.01 ^{bc}	2.05±0.07 ^c	2.07±0.08 ^c	2.79±0.35 ^{ab}	2.93±0.10 ^a	3.17±0.02 ^a
Cysteine	0.19±0.04 ^c	0.17±0.01 ^c	0.19±0.00 ^c	0.24±0.01 ^c	0.64±0.13 ^{ab}	0.80±0.06 ^a	0.87±0.03 ^a
Glut. Acid	2.80±0.08 ^{cd}	2.24±0.02 ^{de}	2.70±0.01 ^d	2.49±0.34 ^d	5.09±0.51 ^a	3.74±0.04 ^{bc}	3.82±0.08 ^b
Glycine	0.89±0.02 ^c	0.98±0.16 ^c	0.95±0.06 ^c	0.94±0.06 ^c	3.49±0.51 ^a	2.04±0.06 ^b	2.08±0.17 ^b
Serine	0.80±0.05 ^c	0.93±0.03 ^c	0.78±0.06 ^c	0.86±0.08 ^c	3.07±0.50 ^a	2.69±0.09 ^b	2.79±0.04 ^b
Tyrosine	0.79±0.18 ^{ab}	0.86±0.02 ^a	0.73±0.05 ^{ab}	0.85±0.08 ^a	0.90±0.08 ^a	0.97±0.07 ^a	0.37±0.03 ^c
Proline	0.55±0.03 ^c	0.72±0.03 ^b	0.56±0.03 ^c	0.57±0.01 ^c	2.77±0.50 ^a	2.80±0.06 ^a	2.87±0.02 ^a
ΣEAA	6.84±0.06 ^c	6.96±0.03 ^c	6.73±0.03 ^c	6.95±0.08 ^c	15.21±0.13 ^b	15.84±0.23 ^{ab}	16.19±0.06 ^a
ΣNEAA	9.37±0.14 ^c	9.35±0.20 ^c	9.19±0.02 ^c	9.31±0.19 ^c	20.21±0.61 ^a	17.46±0.38 ^b	17.46±0.22 ^b
ΣAA	16.20±0.20 ^c	16.31±0.10 ^c	15.92±0.01 ^c	16.27±0.27 ^c	35.40±0.40 ^a	33.29±0.06 ^b	33.64±0.16 ^b

¹Values (n = 3) are presented as mean and standard error of mean (Mean±SD.). The statistical difference between the values shown with different upper letters on the same line was found to be significant at the 95% accuracy level according to the Tukey multiple comparison test.²FM: Fish meal; PBM: Poultry by-product meal; 55PBM:55% PBM+35%FM, 65PBM: 65%PBM+35%FM; 75PBM: 75%PBM+25%FM; HFM: Hydrolysis feather meal; 55HFM:55%HFM+45%FM; 65HFM: 65%HFM+35%FM, 75HFM:75%HFM+25%FM.

Table 4.13: Apparent digestibility coefficients (%) of protein and amino acids in diets¹

Amino-acids	Diets of feeding trial ²						
	FM	55PBM	65PBM	75PBM	55HFM	65HFM	75HFM
Crude protein	75.38±0.68 ^a	74.25±0.15 ^a	74.93±0.54 ^a	72.15±0.78 ^b	32.21±2.70 ^c	35.56±2.48 ^c	36.60±2.54 ^c
Essential amino acids (EAA)							
Arginine	92.34±0.10 ^a	92.03±0.49 ^a	91.48±0.22 ^a	92.06±0.86 ^a	54.78±1.67 ^b	52.75±4.24 ^b	44.22±0.71 ^c
Histidine	86.04±1.30 ^a	85.80±3.67 ^a	88.10±0.46 ^a	83.03±0.85 ^{ab}	39.02±2.71 ^c	37.30±3.25 ^b	30.21±5.57 ^d
Isoleucine	91.29±1.98 ^a	85.24±1.07 ^b	86.71±1.32 ^b	87.51±1.44 ^b	57.06±0.23 ^c	47.69±3.31 ^d	37.73±0.30 ^e
Leucine	90.77±2.76 ^a	90.23±0.74 ^a	90.68±0.33 ^a	89.10±1.87 ^a	56.58±0.80 ^b	53.80±0.52 ^{bc}	45.23±0.52 ^c
Lysine	90.94±3.25 ^a	90.49±0.69 ^a	91.65±0.36 ^a	90.79±0.55 ^a	66.44±2.75 ^b	52.73±0.22 ^c	38.10±1.00 ^d
Methionine	90.59±1.00 ^a	86.89±0.62 ^{ab}	89.84±2.45 ^a	90.11±1.85 ^a	68.11±3.67 ^b	57.75±6.74 ^{bc}	49.34±5.59 ^c
Phenylalanine	85.77±1.94 ^a	79.95±0.54 ^a	83.35±1.49 ^a	83.24±1.22 ^a	53.82±8.18 ^b	45.43±1.22 ^{bc}	43.93±0.06 ^c
Threonine	86.91±2.14 ^b	94.68±0.38 ^a	89.46±0.76 ^{ab}	88.91±1.03 ^{ab}	60.22±1.52 ^c	51.38±4.84 ^{cd}	41.91±2.55 ^d
Valine	88.51±0.28 ^a	81.83±3.73 ^b	86.10±0.34 ^a	85.09±0.90 ^a	54.19±2.00 ^b	51.65±3.82 ^b	45.84±1.03 ^{bc}
Non-essential amino acids (NEAA)							
Alanine	83.23±0.20 ^{ab}	86.22±0.53 ^a	85.45±0.07 ^a	86.78±0.32 ^a	54.78±9.79 ^c	45.12±1.11 ^{cd}	37.37±1.24 ^d
Aspartic Acid	82.36±0.01 ^{ab}	81.62±0.10 ^{ab}	84.91±0.57 ^a	86.09±0.43 ^a	67.46±6.08 ^c	57.08±2.27 ^{cd}	46.60±1.66 ^d
Cysteine	85.59±2.34 ^a	86.17±0.32 ^a	85.44±0.04 ^a	80.36±2.26 ^{ab}	61.38±6.48 ^c	49.61±5.65 ^d	44.31±2.87 ^{de}
Glu. Acid	86.81±0.01 ^{ab}	91.64±0.35 ^a	90.56±0.11 ^a	92.56±1.24 ^a	62.99±4.07 ^{cd}	67.87±2.31 ^c	62.63±1.07 ^{cd}
Glycine	92.27±5.21 ^a	88.09±2.17 ^{abc}	89.65±0.44 ^{ab}	90.96±0.53 ^{ab}	63.23±2.40 ^d	69.41±1.20 ^{bcd}	66.64±4.55 ^{cd}
Serine	85.35±0.53 ^a	85.43±1.11 ^a	89.12±1.01 ^a	89.08±0.69 ^a	57.13±0.57 ^c	61.64±1.98 ^b	60.30±0.21 ^{bc}
Tyrosine	83.41±2.18 ^a	77.66±0.57 ^a	83.04±0.66 ^a	81.91±2.34 ^a	39.01±5.74 ^b	26.48±3.43 ^b	25.90±4.24 ^b
Proline	93.49±0.53 ^a	91.27±0.18 ^a	93.60±0.38 ^a	94.59±0.09 ^a	64.60±1.12 ^b	64.17±1.01 ^b	55.52±2.34 ^c

¹Values (n = 3) are presented as mean and standard error of mean (Mean±SD.). The statistical difference between the values shown with different upper letters on the same line was found to be significant at the 95% accuracy level according to the Tukey multiple comparison test. ²FM: Fish meal; PBM: Poultry by-product meal; 55PBM:55% PBM+35%FM, 65PBM: 65%PBM+35%FM;75PBM: 75%PBM+25%FM; HFM: Hydrolysis feather meal; 55HFM:55%HFM+45%FM; 65HFM: 65%HFM+35%FM,75HFM:75%HFM+25%FM. ³PER: Protein efficiency ratio= weight gain / crude protein intake.

In order to assess the impact of the experimental diets tested in this study on the liver, the stomach, and the intestinal absorption surface, histological sections of the liver and digestive tract of *Sparus aurata* were made.

4.8. Histology of Liver Gilthead Seabream

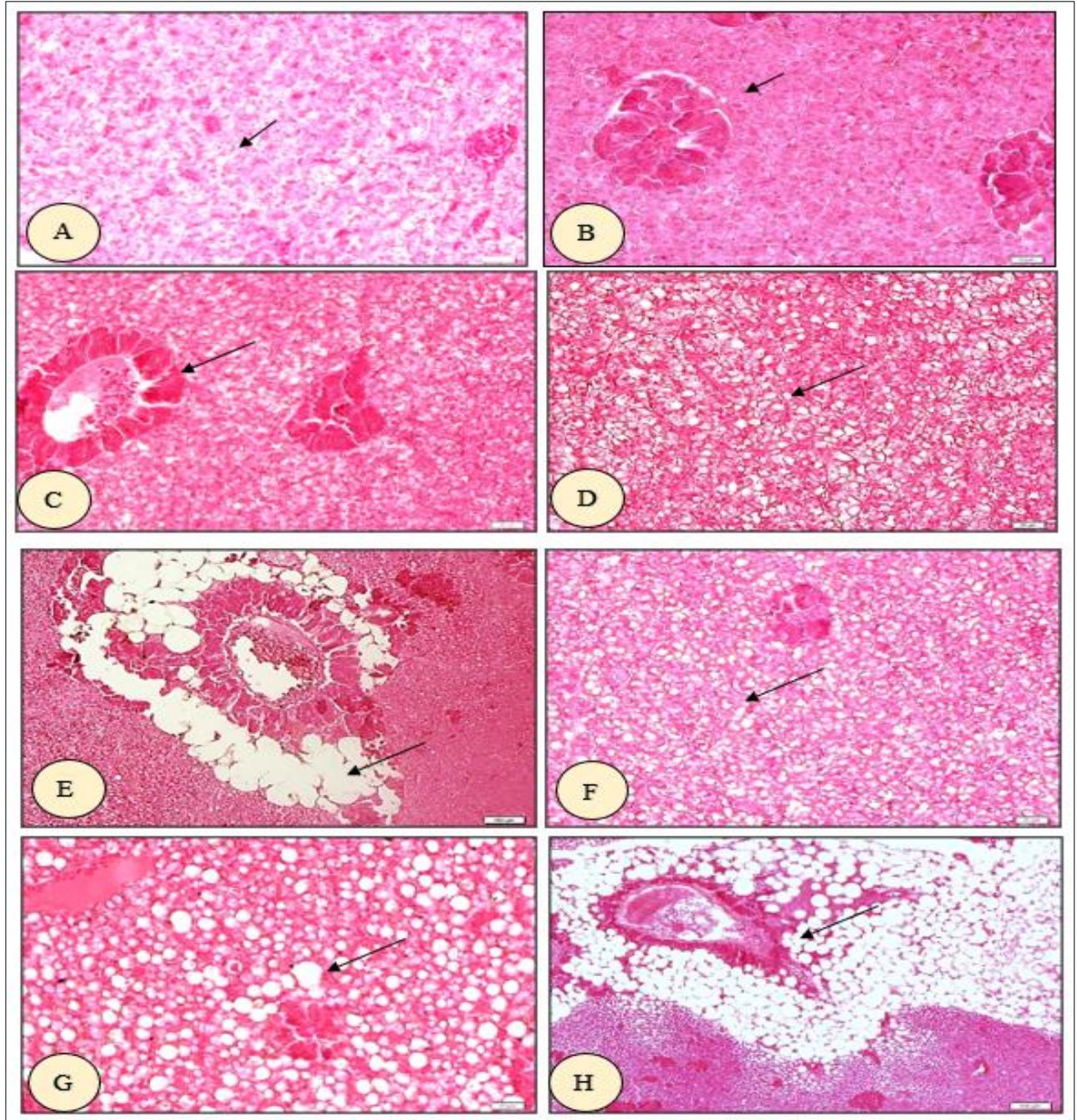


Figure 4.1: Histological sections of liver from gilthead seabream fed experimental diets. (A) Initial group: small amount of fatty accumulation and dense blood vessels (hyperemia) around hepatocyte cells. (B) FM: centrally located hepatocyte cells and pancreatic tissue. (C) 55PBM: hepatocyte cells and pancreatic tissue containing centrally located nuclei. (D) 65PBM: small amount of fat droplets and hepatocyte cells. (E) 75PBM: dense fat accumulation around pancreatic tissue. (F) 55HFM: necrosis and fat vacuoles in hepatocyte cells. (G) 65HFM: intense steatosis between hepatocyte cells. (H) 75HFM: necrotic pancreatic and hepatocyte cells, intense steatosis and congestion. |

Histological sections of livers from fish-fed experimental diets showed normal histological features of fish-fed (B) FM and (C) 55PBM. Fish fed with 65PBM showed hepatocytes (indicated by arrow) and pancreatic tissue containing nuclei and fat droplets located in the center (indicated by arrow). Seabream fed with a 75PBM diet showed dense fat accumulation around the pancreatic tissue compared to fish-fed FM, 55PBM, and 65PBM (Figure 4.1).

Liver histological sections of fish fed with a 55HFM diet showed the presence of necrosis and fatty vacuoles in the hepatocyte cells, whereas fish fed with 65HFM had intense steatosis between the pyknotic hepatocyte cells (indicated by arrow). The liver histology of seabream fed with 75HFM diet show necrosis of pancreatic and hepatocyte cells, intense steatosis, and congestion. Overall, intense steatosis was detected in hepatopancreas tissue when the amount of HFM used in the diet increased.

4.9. Gastric Histology of Gilthead Seabream

The histological sections of the stomachs of juvenile seabream fed with the different diets used in this experiment are presented below in figure 4.2. The internal wall of the stomach of the initial group is typically composed of five layers represented by the mucosal epithelium (ME) forming folds on the side of the intestinal lumen (IL), the lamina propria (LP), the submucosa (SM), the internal (IM) and external muscularis (EM) and serosis (S). Gastric histology sections of juvenile gilthead seabream fed with different experimental diets show that the groups fed with 55PBM and 65PBM had slightly longer and branched villi, in comparison with the other groups (indicated by the arrows) (Figure 4.2).

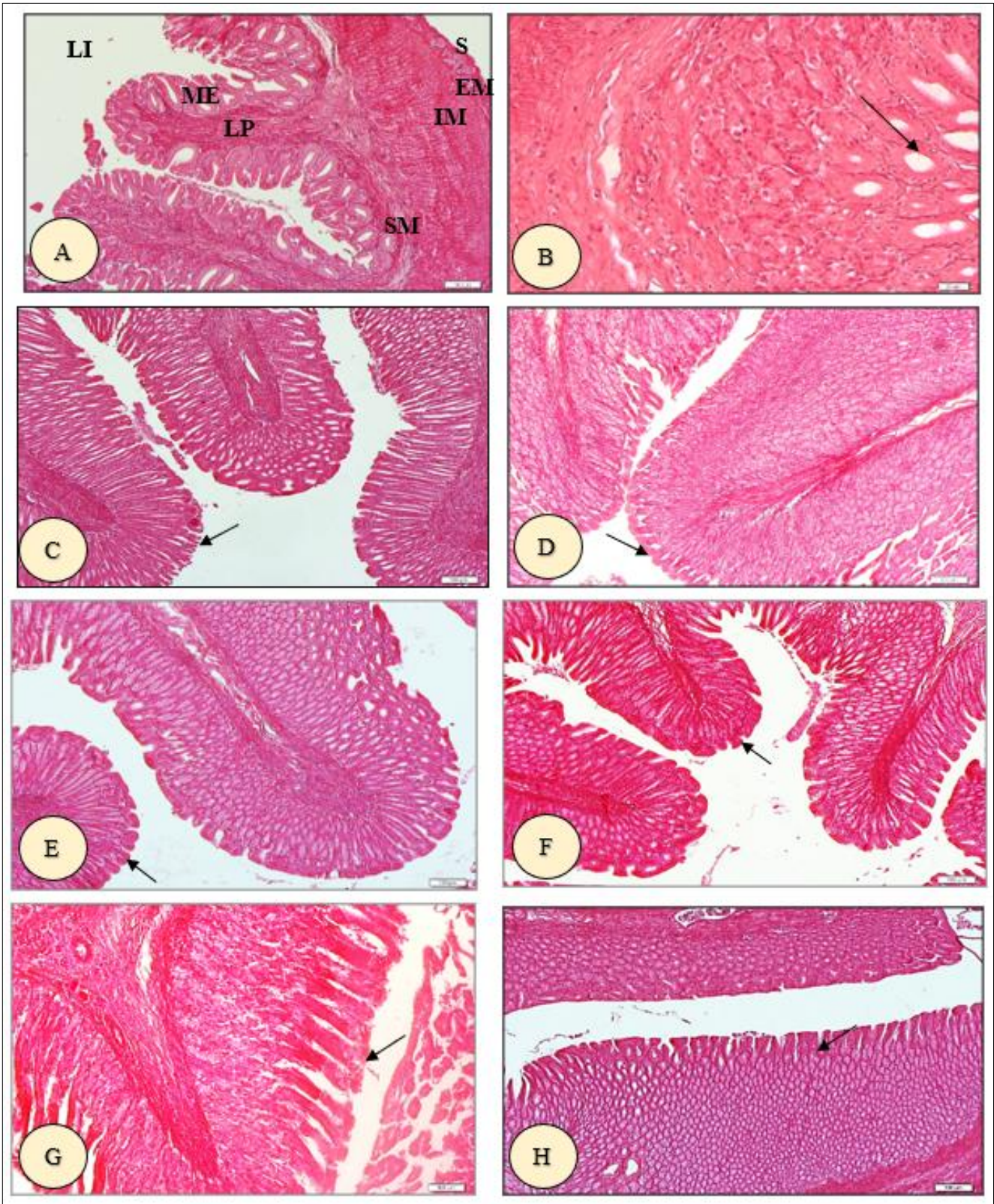


Figure 4.2: Histological sections of the stomach of *Sparus aurata* fed experimental diets. (A) initial group: short branching villi (B) FM: internal and external muscularis with presence of small amount of fat droplets. (C) 55PBM: long and uniform villi. (D) 65PBM: wide surface of epithelial tissue and short villi. (E) 75PBM: long and uniform villi. (F) 55HFM: altered short villi. (G) 65HFM: partially weathered short villi. (H) 75HFM: very short altered villi. (IL) intestinal lumen, (LP) lamina propria, (SM) submucosa, (IM) internal muscularis, (ME) external muscularis, (S) serosis.

4.10. Histology of Anterior Intestine of Gilthead Seabream

Anterior foregut sections showed, branching in the villi of fish-fed PBM-based diets, which increased with the increase in the amount of PBM in the diet. When the foregut tissues of HFM group fish were examined histologically, elongations and branching were detected in the villi due to the increased amount of HFM in the diet (Figure 4.3, F, G, H). Intense branching was observed in the 75HFM group. Furthermore, no fatty vacuoles were detected in the villi of the intestinal tissue of fish belonging to this group.

4.11. Histology of Mid Intestine of Gilthead Seabream

The results of histological sections of the midgut of the experimental groups fed with PBM and HFM-based diets showed intestinal villi which tend to be longer and more ramified as the portion of dietary PBM and HFM increased (Figure 4.4, F, G, H). The number of goblet cells in the midgut of fish fed partially with 55% and 65% PBM was slightly higher compared to the original group and the control group fed 100% fish meal (Figure 4.4, B, C, D, E). This trend suggests a beneficial effect of PBM and HFM on intestinal health in juvenile gilthead seabream.

4.12. Histology of Posterior Intestine of Gilthead Seabream

Experimental groups fed partially with PBM and HFM showed greater development of intestinal villi compared to the group fed with FM (Figure 4.5). The post-intestinal absorption surface became larger as the amount of PBM and HFM increased in the diet. In addition, the increase in the quantity of hydrolyzed feather meal in the feed led to a significant increase in the number of goblet cells in the intestine of *Sparus aurata*, both in the proximal and middle part (Figure 4.4 F, G, H, and Figure 4.5, F, G, H). A moderate number of lymphocytes (indicated by the arrows) in the mucosa of the proximal intestine of the 75PBM group (Figure 4.5) was observed (indicated by arrow).

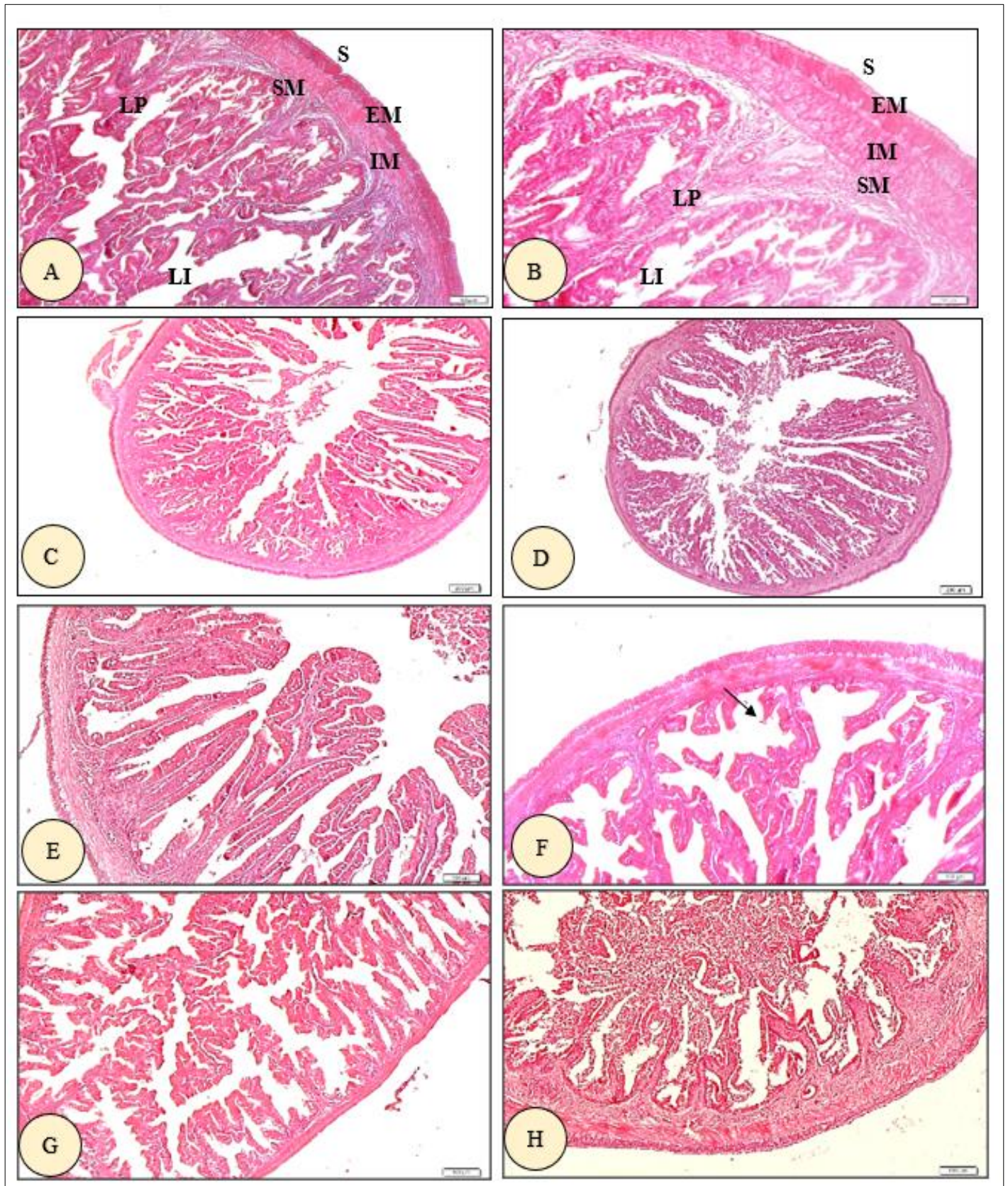


Figure 4.3: Histological sections of the anterior intestine of gilthead seabream fed experimental diets. (A) Initial group: long and densely branched villi and few goblet cells, (B) FM: few goblet cells and branched villi. (C) 55PBM: densely branched villi and few goblet cells. (D) 65PBM: goblet cells and densely branched villi. (E) 75PBM: intense branching at villus tips, few goblet cells, shedding in enterocyte cells surrounding villi. (F) 55HFM: numerous goblet cells and branching villi. (G) 65HFM: goblet cells and densely branched villi. (H) 75HFM: excretion of enterocyte cells surrounding the villi. (IL) intestinal lumen, (LP) lamina propria, (SM) submucosa, (IM) internal muscularis, (ME) external muscularis, (S) serosis.

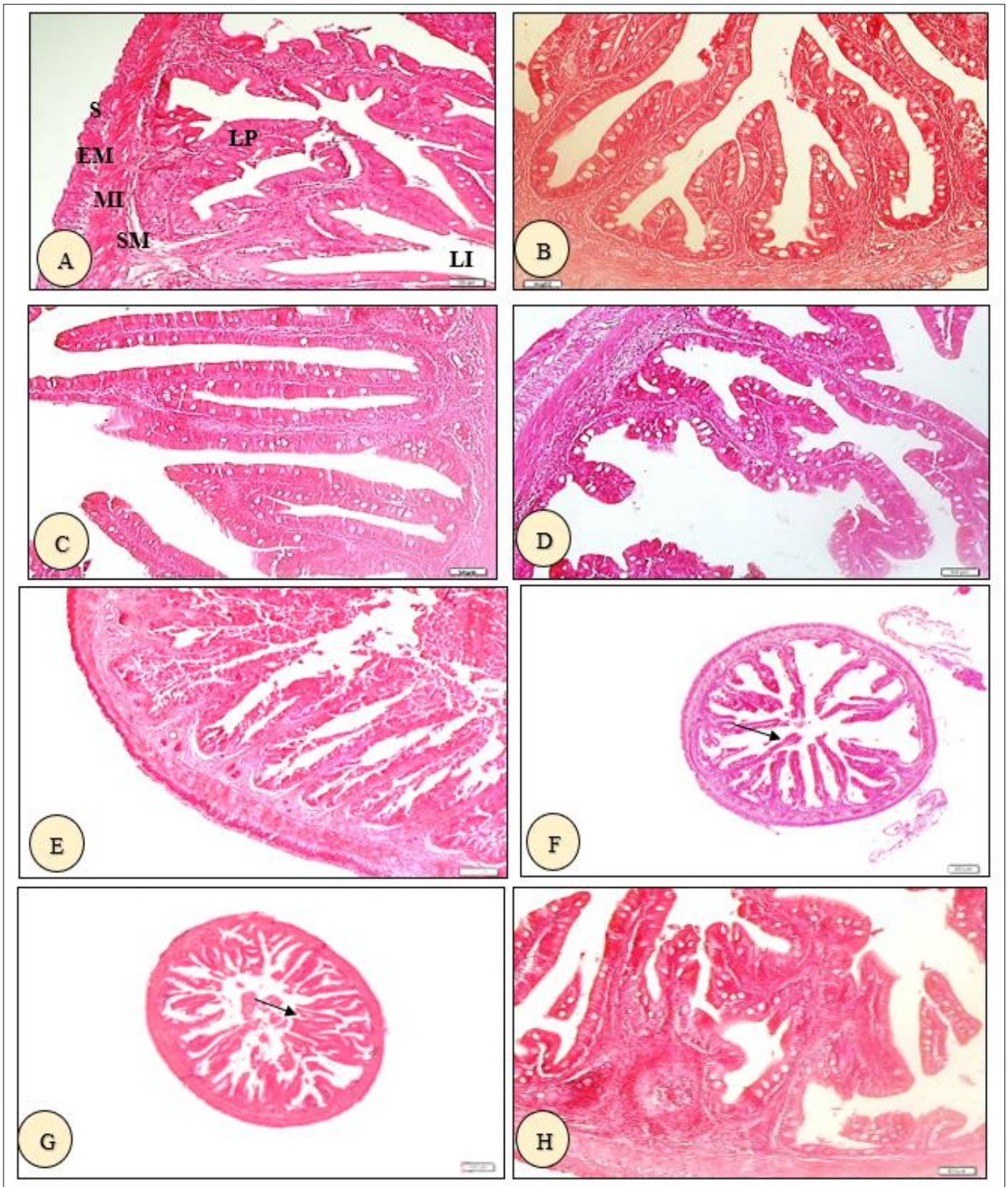


Figure 4.4: Histological sections of the mid gut of gilthead seabream fed experimental diets. (A) initial cluster: long, branching villi and dense goblet cells. (B) FM: long and branched villi. (C) 55PBM: long and branched villi. (D) 65PBM: long and branched villi. (E) 75PBM: goblet cells, long branching villi and enterocyte cells surrounding the villi (F) 55HFM: goblet cells and elongated villi (G) 65HFM: elongated and branching villi and some goblet cells (H) 75HFM: goblet cells and much branching villi. (IL) intestinal lumen, (LP) lamina propria, (SM) submucosa, (IM) internal muscularis, (ME) external muscularis, (S) serosis.

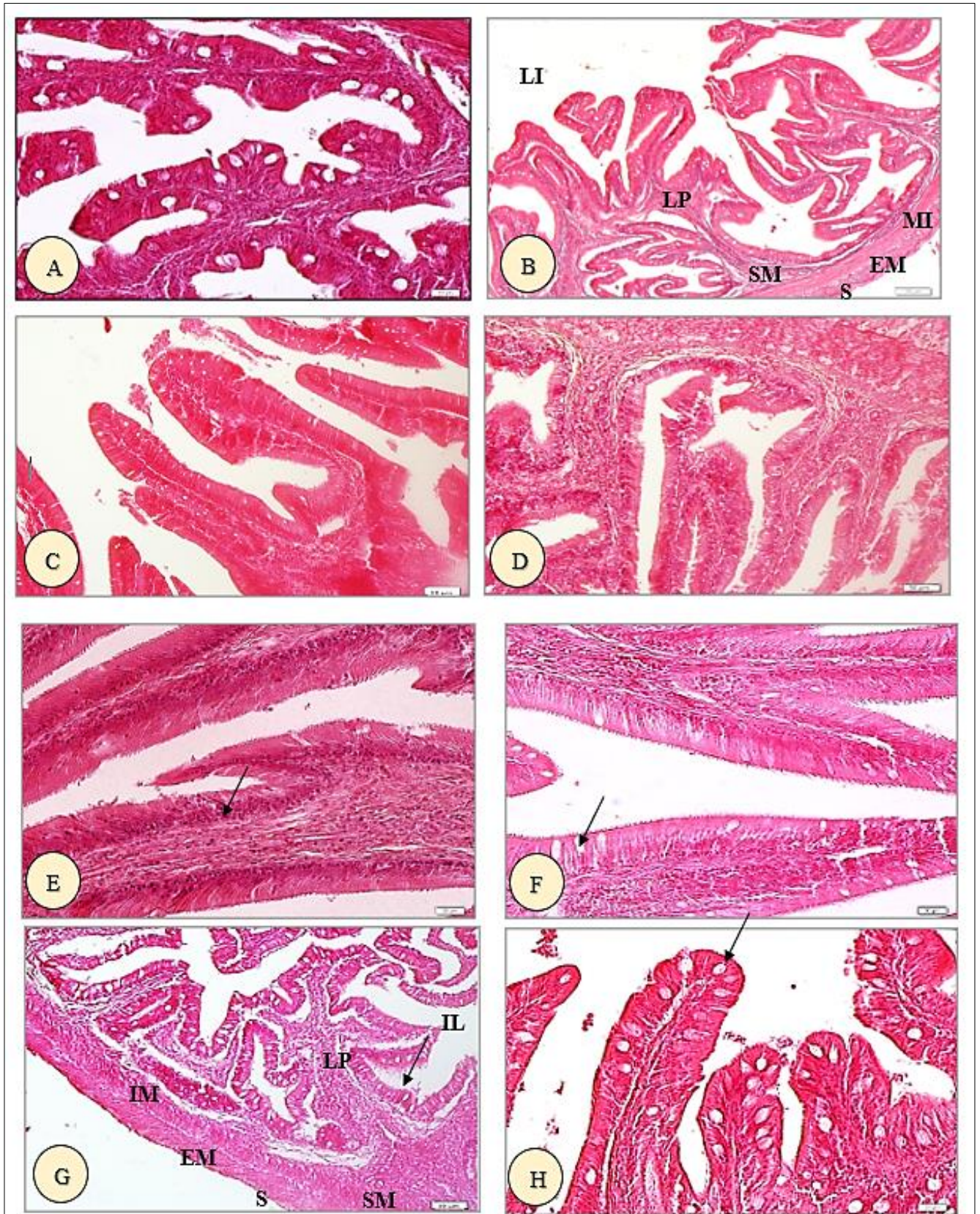


Figure 4.5: Histological sections of the posterior intestine of gilthead seabream fed experimental diets. (A) Initial group: long and branched villi and dense goblet cells. (B) FM: long and branched villi. (C) 55PBM: long and branched villi. (D) 65PBM: long and branched villi. (E) 75PBM: long villi. (F) 55HFM: long villi (G) 65HFM: elongated and branched villi and few goblet cells (H) 75HFM: many goblet cells, slightly long and sparsely branched villi. (IL) intestinal lumen, (LP) lamina propria, (SM) submucosa, (IM) internal muscularis, (ME) external muscularis, (S) serosis.

5. DISCUSSION AND CONCLUSION

Fishmeal is an excellent source of protein for carnivorous fish species such as gilthead seabream, a species of high economic value in the Mediterranean. The increase in demand for fishmeal has potentially increased its selling price. Therefore, alternative protein sources such as hydrolyzed feather meal (HFM) and poultry by-product meal (PBM) which are cheaper and of good quality, are needed to totally or partially replace dietary fishmeal. Studies based on HFM in *S. aurata* are extremely limited (Alexis, 1997; Nengas *et al.*, 1999; Nogueira *et al.*, 2012; Psafakis *et al.*, 2020). Psafakis in his study observed that total replacement of fishmeal with HFM resulted in reduced voluntary feed intake, but partial replacement HFM did not lead to values lower than those obtained in the FM-diet. In fact, fish-fed diets with 50% FM replacement, either supplemented or not with the essential amino acids lysine and methionine, had significantly higher feed consumption than the control group. It has been suggested that fish can increase their feed intake in order to obtain a constant supply of essential amino acids (Peres and Oliva-Teles, 2006). These results indicate that HFM does not exert a negative impact on fish feed intake when used at low levels of FM replacement in juvenile seabream diets. Interestingly, Campos *et al.* (2017) working with *Dicentrarchus labrax* suggested that even high levels of dietary HFM induce feed palatability that does not interfere with fish feed intake (% body weight/day). It has been suggested that HFM does not exert a negative impact on the food consumption of fish (Xue *et al.*, 2012; Wang *et al.*, 2010; Hu *et al.*, 2013), including seabream (Nogueira *et al.*, 2012) when used together with other animal-based protein.

In the diet of *S. aurata*, the low nutritional value of HFM compared to fishmeal was previously noted by Alexis (1997) and Nengas *et al.* (1999). These authors observed that the total replacement of fishmeal by a mixture of HFM with poultry meal, in a ratio of 1:3, led to a drop in feed consumption, mainly due to its low digestibility. (Nengas *et al.*, 1995). Although the hydrolysis process can improve the digestibility of HFM (Bureau *et al.*, 1999, Laporte, 2007; Guillaume, 2017), due to its high keratin content. Guillaume (2017) showed that hydrolyzed feather meal (HFM) is an economical feed ingredient with high protein content (75-85%) and is widely used in animal feed formulation.

Feathers are made up of over 90% a protein called keratin. Keratin is high in arginine, threonine, leucine, isoleucine, and valine, but relatively low in histidine, lysine, methionine, and tryptophan. Psfakis *et al.* (2020) reported that hydrolyzed feather meal is lower in methionine, lysine, and histidine, but is high in cysteine and water-soluble amino acids such as arginine, proline, glycine, and aspartic acid. Psfakis *et al.* (2020) also observed that the immediate whole body and muscle composition of juvenile seabream did not show considerable variability by the inclusion of HFM, except at the total replacement level, at which the fish had reduced lipid and energy contents.

Additionally, Psfakis *et al.* (2020) have shown that 50% of FM replacement by HFM had no adverse effects on digestive protease activities. This author studied the effect of HFM protein on the activities of the digestive enzymes, trypsin, and chymotrypsin in the pyloric caeca and pepsin in the stomach of juvenile *S. aurata*. The results showed that partial replacement (%50 FM replacement) of fish meal with HFM had no significant effect on the activities of any enzymes tested and indicates that HFM digestibility was not the main factor that reduced fish growth.

In our study, the inclusion of HFM at 55%, 65% and 75% in diets of juvenile *Sparus aurata* had negative impacts on growth performance, feed intake, feed utilization, condition factor, hepatosomatic, and viscera somatic index. Nutrient digestibility was also negatively affected. The digestibility coefficients of amino acids in these diets consumed by gilthead seabream showed low values. The analysis of the fecal material obtained from HFM diets and the calculated apparent digestibility coefficient (ADC) profiles of the diet shows high levels of undigested protein. Disappointingly, hydrolyzed feather meal used in this experiment did not perform well for gilthead seabream and protein digestibility was very poor with no indication that the hydrolysis treatment had any benefit in improving protein digestibility for this species.

According to a previous study, the crude protein digestibility of hydrolyzed feather meal varies among fish species (Campos *et al.*, 2017). The apparent protein digestibility coefficient is high in freshwater species but reduced in marine species such as *Sparus aurata*, where ADC was reported to be 22% (Campos *et al.*, 2017). However, a recent study by Psfakis *et al.* (2020) reported that 50% replacement of fishmeal with hydrolyzed feather meal in the juvenile gilthead seabream *Sparus aurata* diet had no adverse effect on digestive protease activities.

In this study, the lower levels of protein digestibility are likely to be explained by the lack of secretion of polypeptide digestive enzymes by pancreatic cells (chymotrypsinogen and trypsinogen, carboxypeptidase A and B) due to exposure to damage caused by feeding fish with chicken feathers (Figure 4.1, E, F, G).

So that the histological results of the liver and the digestive system showed the negative effect of HFM on the diet of seabream through the presence of necrosis and greasiness in the liver and inflammation of the short villi in the stomach. Histological findings did not show that the hindgut surface was affected, possibly because dietary amino acids and dipeptides/di-peptides were not absorbed into the enterocytes. They appear to be eliminated via the feces in the form of indigestible proteins, which may explain the higher amounts of amino acids in the feces of HFM-fed seabream.

These negative results could be attributed to the quality of hydrolyzed feather meal used in this experiment. Guillaume, (2017) showed three different processes of feather keratin hydrolysis (steam hydrolysis, bacterial fermentation and hydrolysis, and enzymatic hydrolysis) and that these processes influence the quality of the flour obtained. For steam hydrolysis (the treatment method most commonly used by the industry), it was shown that the hydrolysis conditions (temperature, pressure, and duration), widely vary according to the manufacturing facilities and change according to the type of industrial rendering equipment available. Another hydrolysis process; is fermentation and bacterial hydrolysis. Over the past decade, keratolytic microorganisms have been used to cause the hydrolysis of chemical bonds in keratin to occur by enzymes rather than high vapor pressure, which involves minimizing the destruction and the formation of cross-linked amino acids (CLAA). Degradation of keratin by microorganisms is possible due to their ability to produce both disulfide reductase and proteases (Yamamura *et al.* 2002; Ramnani *et al.* 2005). Secreted sulfide reductases disulfide bonds in keratin, allowing proteases to cleave the peptide in the protein, breaking it down into dipeptides and single amino acids (Bockle and Muller 1997, Yamamura *et al.* 2002). Protease enzymes can be used in the hydrolysis of feathers.

This type of hydrolysis can be achieved in minutes rather than days, which means faster production turnover. Several studies have assessed the nutritional value of enzymatically treated feather meal (Barbour *et al.* 2002, Laporte 2007, Davies *et al.* 2009).

Investigations revealed that the enzymatic hydrolysis of keratinocytes can be significantly improved by the addition of chemical reducers (Ramnani and Gupta 2007, Eslahi *et al.* 2013). As

such, the use of chemical reducers with proteases to convert raw feathers into food ingredients is an option worth further research.

Following the negative results obtained during the relative use of HFM in the diets of juvenile gilthead seabream and which is probably due to the poor quality of HFM, we will continue the discussion of the results of this study between the control group containing 100% fishmeal and groups containing PBM in diets of gilthead seabream.

The water temperature recorded during the growth period varied between 23 °C and 28.5 °C, while the salinity levels varied between 38.8 and 39.5 mg/l. During the research, the amount of dissolved oxygen in the water was recorded at 7.31 ± 0.3 mg/l and the average pH at 7.86 ± 0.05 . Except for water Temperature (which was slightly higher at the start of the experiment), the physical and chemical water parameters recorded in our study were similar to values considered suitable for rearing juvenile seabream (Mozes *et al.*, 2011).

Nengas *et al.* (1999) reported that replacing 50% of FM with PBM did not negatively affect growth performance in *Sparus aurata*, but the group-fed diets containing 75% PBM had very low growth values. Hekmatpour *et al.* (2018), reported that up to 55% fishmeal protein could be replaced by PBM in formulated diets of juvenile sparidentex seabream without any negative effect on growth performance. Fontinha *et al.* (2021), suggested that the partial replacement (37.5%) of fish meal with poultry by-product meal does not negatively affect the growth performance of *Sparus aurata*. Other similar studies by Karapanagiotidis *et al.* (2018) suggested that (PBM) can successfully replace up to 50% of FM in the diet of *S. aurata* without adverse effects on survival, feed intake and, growth performance but total replacement affects growth performance. However, the work of Sabbagh *et al.* (2019) shows that it is possible to partially (50%) or totally (100%) replace FM with PBM in the diet of gilthead seabream, without adverse effects on the growth performance of the fish.

In our study, the results showed that 75% FM could be replaced by PBM in the diets of juvenile seabream with no significant decrease in growth performance. This appears to be due to the balanced EAAs in particular lysine and methionine contents of the diet containing PBM, which were similar to the profile of the diet containing 100% fishmeal.

In comparison to the control diet, presented optimal levels of EAAs necessary for the growth and development of juvenile gilthead seabream. This implies that the PBM meal used in the feed

formulation in our feeding experiment was of good quality. The results of the profile of AAs in the whole body of *Sparus aurata* fed with PBM-based diets were similar to FM as well as to the profile required in the whole body for *Sparus aurata* (Nengas *et al.*, 1999; Marcouli *et al.*, 2006; Kaushik and Seilliez., 2010; Gaber *et al.*, 2016; Torfi Mozanzadeh *et al.*, 2018; Fantinha *et al.*, 2021; Hekmatpour *et al.*, 2018, 2019; Moutinho *et al.*, 2017). Yıldız and Ofori-Mensah (2017) reported that the EAA composition in fillets of *Sparus aurata* was affected by feeds and seasons ($P < 0.05$).

In addition, Fontinha *et al.* (2021) reported that the optimal level of substitution of FM with PBM strongly depends on the species of fish (carnivores, herbivores, or omnivores), diet formulation, and the quality of PBMs used. Recently, Davis *et al.* (2019) observed in an experiment with 22g of *Sparus aurata* that up to 75% of FM could be replaced by PBM. However, in a second experiment, with 10g of *Sparus aurata*, they observed that growth performance was significantly reduced when diets included 75% of PBM. Similarly, Karapanagiotidis *et al.* (2019) in an experiment observed that partial (50%) or total replacement of FM by PBM resulted in a decrease in growth performance and feeding efficiency in juveniles by 2.5g. Young individuals of *Sparus aurata* tolerate lower inclusion rates of PBM in contrast to large individuals. This indicates a positive relationship between the weight of juvenile seabream and the maximum acceptable PBM in their diets. In fact, young juveniles need a higher level of protein (stocks in EAAs, particularly lysine and methionine, which limit the growth of *Sparus aurata*) in the ration for good development, unlike the big juveniles of gilthead seabream.

This is consistent with the results obtained by Karapanagiotidis *et al.* (2019) in another experiment with third generation juveniles who observed no difference in growth performance when fed diets containing 50% PBM supplemented with the essential amino acids lysine and methionine.

Palatability is an important issue when fishmeal is partially or completely replaced by an alternative ingredient in the fish diet because poor palatability leads to a decrease in voluntary feed intake, thus negatively affecting fish growth (Gomez-Requeni *et al.*, 2003; Hu *et al.*, 2013; Hill *et al.*, 2019; Fontinha *et al.*, 2021).

According to Oliva-Teles *et al.* (2015), animal by-products including PBM are highly palatable. In this study, diets containing up to 75% PBM were found to be as palatable as a control diet containing 100% FM.

The feed intake (FI), feed conversion ratio (FCR), feed efficiency (FE), and protein efficiency ratio (PER) values obtained in this study were representative of diet quality, as juvenile seabream fed high levels of PBM (55PBM, 65PBM, and 75PBM) were effective in converting feed to body weight. The FCR values determined for 55PBM, 65PBM, and 75PBM were 1.12, 1.11, and 1.23, respectively and the PEF values recorded for the same feed groups were 1.83, 1.88, and 1.70, respectively. These results were effective and similar to the control group (FCR = 1.11 and PER = 1.84). Similar results were reported by other authors in other fish species *Oncorhynchus mykiss* (Yürüten Özdemir and Yıldız, 2019), *Sparus aurata* (Fantinha *et al.*, 2021; Karapanagiotidis *et al.*, 2018; Hekmetpour *et al.*, 2018; Hekmetpour *et al.*, 2019). Sabbagh *et al.* (2019) who completely replaced fishmeal with PBM without any essential amino acid supplement in the diet of young seabream with an initial body weight of 73.57 g, obtained an FCR value of 1.32.

Our PEF results confirmed that gilthead seabream had excellent potential for protein utilization of poultry by-product (PBM) meal. The best performing diet had an FCR value of 1.11 and PER of 1.88 similar to the control group. No deficiency in essential amino acids was observed between the groups of juvenile *Sparus aurata* fed with diets containing PBM and the FM group. The AA profiles of the prepared diets were reflected in the AAs profile of the whole body of the fish used in the present study. According to the results of our study, it would therefore be reasonable to say that PBM could replace up to 75% of fishmeal in the diet of juvenile gilthead seabream without any detriment to growth performance and food intake.

At the end of this experiment, when the results of the fish whole-body nutrient analysis were evaluated, no major differences were recorded between the PBM-fed fish and the FM-fed fish group. The crude protein contents were at the level of 16.92, 17.25, and 16.06 respectively in the whole body of fish fed with 55PBM, 65PBM, and 75PBM.

No significant difference was observed between the group's fed diets containing PBM and the control group (FM), whose protein content was around 16.14. Yıldız *et al.* (2006) reported that the balance between the amount of energy and protein in the diet is important because a deficiency in non-protein (fats and carbohydrates) energy means that part of the protein will be broken down for energy.

This implies that whole-body amino acid contents of gilthead seabream were not affected by replacing fishmeal with high levels of PBM. Similar results were obtained in the whole-body of

Sparus aurata by Nengas *et al.*, (1999); Mozanzadeh *et al.*, (2018), and by Yürüten Özdemir and Yıldız (2019) in the whole-body of *Oncorhynchus mykiss*. Similarly, at the end of the experiment, the lipid level in the whole body was recorded at around 12.87%, and no significant difference was observed between PBM and FM feeds. Similar results were obtained by Yürüten Özdemir and Yıldız (2019) in the whole body of *Oncorhynchus mykiss*. However, Nengas *et al.* (1999), Hekmatpour *et al.* (2018) Mozanzadeh *et al.*, 2018, obtained low lipid levels in the whole body of juvenile gilthead seabream. This is due to the low level of lipids contained in the formulated diets. The study conducted by Yıldız *et al.* (2006), show that, in seabream when the fat percentage in the diets increased the lipid percentage in the fish body increased.

Whole-body ash levels increased linearly with increasing PBM portion in the diet. This probably reflects the greater availability of minerals such as calcium and phosphorus in PBM meal than in FM. Similar results were reported by Karapanagiotidis *et al.* (2019) and Fontinha *et al.* (2021) who obtained ash values that varied from 3.7 to 4.3 and 5 and 5.3% respectively. According to the literature, several authors recommend a high level of protein is necessary for the diet of gilthead seabream, which is a carnivorous fish. Therefore, for optimal growth and development, gilthead seabream requires diets containing 40-55% protein (Sabant and Luquert, 1973; Santinha *et al.*, 1996; Vergara *et al.*, 1996 a,b) and 15-22% lipids (Vergara and Jancey, 1993; Santinha *et al.*, 1999). The formulated diets for juvenile seabream used in this thesis study contained 47.44% protein and 19.25%lipids, which are within the range of dietary protein and lipid contents of juvenile *Sparus aurata* specified in NRC (2011).

Measurement of total length and organ characteristics revealed that condition factor (CF) and hepatosomatic (HSI) and vesiculosomatic (VSI) indices of juvenile gilthead seabream showed a positive correlation with increasing levels of PBM in the diet.

No significant difference was recorded between the groups of fish fed with PBM-based diets and the group fed with FM. This means that the level of stored energy reserves in juvenile gilthead seabream is stored primarily as fat in the viscera without affecting liver function. In addition, our result may suggest that juvenile gilthead seabream tolerate poultry fat well and that organ health would not be threatened by the inclusion of up to 75% PBM in diets for juvenile gilthead seabream. These results were in agreement with those reported by Karapanagiotidis *et al.* (2018) and Fontinha *et al.* (2021). However, slightly elevated contents were recorded by Sabbagh *et al.* (2019). This is probably due to the use of two sources of oil; fish oil and soybean oil in the formulation of juvenile

gilthead seabream feed. Yıldız *et al.* (2006) reported that HSI was related to the body's metabolism which is affected by the seasons. The study conducted by Yıldız *et al.* (2007) show that for Sea Bass (*Dicentrarchus labrax* L.) the visceral lipid content of cultured and wild fish significantly increased in summer, while the liver lipid content significantly increased in winter ($P < 0.05$). Yıldız *et al.* (2007) reported that the percentage of the VSI and visceral lipid in the sea bass steadily increased with dietary fat content.

The structural adaptation of the digestive tract of each species of fish is distinct. This structure depends not only on body shape, sex, and body weight but also on its feeding behavior (Barlow *et al.*, 1993; Davis, 1985). Knowledge of the morphology and functioning of the digestive tract would be useful to establish a diet for gilthead seabream, a carnivorous species of increasing importance for aquaculture, especially in Turkey, and in the Mediterranean (Purushothaman *et al.*, 2016).

In our experiment, macroscopic observations of the digestive tract of juvenile seabreams showed a clearly recognizable Y-shaped stomach and a relatively short intestine composed of three parts anterior, middle, and posterior, as commonly seen in carnivorous species. Our results were similar to the literature (Cataldi *et al.*, 1987; Laporte, 2007; Tina *et al.*, 2018).

Nevertheless, the morphological examination carried out in our study tends to confirm the general ultrastructural characteristics of enterocytes in relation to their location and their functional specificities. Microscopic observations of histological sections of the stomachs of juvenile gilthead seabream fed 55PBM, 65PBM and 75PBM, showed branched and relatively short villi with the presence of mucosal epithelium, lamina propria, muscularis, and serosa. These results were similar to the literature (Cataldi *et al.*, 1987; Laporte, 2007; Purushothaman *et al.*, 2016; Tina *et al.*, 2018).

From our observations, PBM-based diets had no obvious effect on the gut morpho-histology of juvenile gilthead seabream. Indeed, the anterior, median, and posterior parts of the intestine were distinguished by the shape of their respective mucosal folds. Foregut histology of PBM-fed juveniles showed densely branched microvilli and few goblet cells. However, the median and posterior sections showed many longer (than the anterior section of the intestine) and branching microvilli adorning the intestinal epithelium, which includes numerous goblet and absorptive cells to fulfill the function of lubrication as well as to facilitate digestion and absorption of feed. In addition, the number of goblet cells increased from the anterior to the middle parts as well as the posterior part of the intestine.

It is generally recognized that the anterior part of the intestine is functionally specialized for the absorption of small molecules (Laporte., 2007). The intestine is where the majority of feed absorption takes place due to the action of various digestive enzymes during the digestion process. Absorption is greatly facilitated by the villi (folds) which multiply the absorption surface of the tube (Laporte, 2007).

The density of goblet cells is another criterion that generally makes it possible to distinguish the different parts of the intestine (Murray *et al.*, 1996). A higher number of globular cells in the distal part of the intestine may allow better lubrication for fecal evacuation (Laporte, 2007). Similarly, Machado *et al.* (2013) reported that the higher number of goblet cells in the hindgut appears to be a universal feature in fish and is likely helpful in increasing mucus production to protect the intestinal mucosa and facilitate fecal expulsion.

In this experiment, the results obtained by histological assessment of the liver demonstrated that juvenile gilthead seabream can adapt relatively well to feeds with a high content of poultry by-product meal (up to 75%). Except for the accumulation of a few lipid droplets around the pancreatic cells in the 75PBM-fed fish group, no signs of hepatic inflammation (tissue breakdown) were observed. Our results show that the high levels of PBM in the diet of juvenile *Sparus aurata* do not have negative effects on the normal functioning of the liver (absence of hepatic steatosis and necrosis in the groups of fish fed with PBM, in comparison with the control group fed with FM).

Juvenile gilthead seabream, therefore, seem more tolerant to feed rich in PBM as evidenced by the absence of inflammatory signs after 120 days of feeding.

Thus, the results obtained in this study seem to demonstrate that the growth performance of juvenile *Sparus aurata* fed with PBM comes from a phenomenon of good absorption of nutrients by the presence of optimal levels of proteolytic enzymes and an increase in the intestinal absorbent surface. The use of poultry by-products in the diet of gilthead seabream did not result in severe signs of intestinal steatosis.

Additionally, there was no evidence of serious impairment resulting from the 120-day feeding trial with PBM-based diets. No histopathological changes in the digestive tract and liver of gilthead seabream were reported in relation to the PBM-containing diet.

Digestibility is a very important aspect to consider when assessing protein quality in fish nutrition (Gauthier *et al.*, 1982). Information on the digestibility of specific nutrients will provide crucial information in order to formulate efficient fish diets (Bureau and Cho, 1999; Davies *et al.*, 2009). In the present study, the protein and amino acid digestibility of the experimental diets was evaluated after 120 days of testing to determine whether the fish adapt and accept the new diets containing increasing concentrations of PBM.

In our experiment, fishmeal provided the best results in terms of coefficients for protein (75.38%) EAAs (85.77%-92.34%), and NEAAs (82.36%-93.49%) digestibility. It was also observed that the digestibility coefficients for PBM-based diets were consistently high for all essential (81.83%-92.06%) and non-essential (77.66%-94.59%) amino acids. Indeed, the digestion of amino acids reflects the overall digestibility of proteins. However, these profiles may highlight fundamental variations with respect to specific amino acids. These results mean that juvenile gilthead seabream that consumed the diets containing PBM highly digested the proteins and amino acids contained in the PBM meal. In addition, the low values of AAs obtained in the feces could be explained by the higher dietary consumption of PBMs and which was similar to the diet containing 100% FM.

Our experiment demonstrated that the different concentrations (55%, 65%, and 75%) of PBM were highly digested by juvenile seabream in a limited feeding trial for the evaluation of the digestibility profiles of key nutrient components.

The ADC for proteins obtained with poultry by-product meal 55PBM, 65PBM, and 75PBM in our trial 74.25%, 74.93%, and 72.15% respectively were slightly lower than the value previously reported by Nengas *et al.* (1995) (89.9%), and Hekmatpour *et al.*, (2018) (79.02%). These authors have shown the effective use of a PBM for gilthead seabream, showing excellent performance in digestibility and growth (Nengas *et al.*, 1999; Hill *et al.*, 2019).

This result is however comparable to the value found by Nazzaro *et al.* (2021) who determined the ADC content between 71% and 88% in poultry by-product (PBM) meal fed to seabream. Using the same concept of fixed ingredient levels in mixed diets for their digestibility trials, different authors have revealed the excellent characteristics and potential of poultry by-products for a number of important fish species.

In terms of digestibility performance, poultry material has been reported favorably for a few marine fish species with ADC values ranging from 78.4% to 90.9% (Zhou *et al.*, 2004; Booth *et*

al., 2005; Tibbets et al., 2006), and the present study supports the potential of PBM in aquaculture feeds for juvenile gilthead seabream with very promising results. Indeed, PBM meal is probably the most effective animal protein alternative to fishmeal currently available both (Good protein quality and less expensive than fish meal) from a nutritional point of view in aquafeed for carnivorous fish such as gilthead seabream.

Conclusion

According to the results of this study, it is clear that PBM did not have any negative effect on growth performance, the condition factor, hepatosomatic and viscerosomatic indices, and feed conversion ratio in the juvenile seabream. The growth performance of fish fed with PBM-based diets were found to be similar to that of the control group.

There was no significant difference between the whole-body protein content of fish-fed diets containing PBM and those in the fish meal group. Likewise, the whole-body amino acid composition of fish was not significantly affected by PBM. However, the levels of essential amino acids in the whole body of fish-fed HFM-based diets were very low. Unlike fish-fed PPBM-based diets, fish-fed diets containing HFM had significantly reduced growth performance, and feed conversion rate.

Histological analyses of the liver and digestive system of fish-fed diets containing PBM showed no serious pathological disturbances.

However, a few lipid droplets were observed in the liver of fish feed with 75% PBM. In contrast, no lipid droplets were detected on histological examination of the hindgut.

In general, PBM used in the diet of juvenile *Sparus aurata* had excellent potential to significantly replace fish meal in the diet of juvenile gilthead seabream with respect to digestibility and nutritional profile. However, the low digestibility and EAAs deficiencies in hydrolyzed feather meal should limit its inclusion in the diet of juvenile gilthead seabream. The processing method applied to the feather meal (steam hydrolyzed method) did not significantly influence the nutrient digestibility of this feed.

For years, hydrolyzed products have been a subject of interest to the scientific community from the point of view of their nutritional qualities. However, hydrolyzate by-products remain complex mixtures whose composition are poorly known until now. For this reason, the fine qualitative and quantitative analyses of the hydrolyzates will make it possible to establish predictions on the nutritional potential of a hydrolyzate. In addition, to deepen the knowledge of the composition of hydrolyzate, it would be very interesting to develop a strategy to address the quantification of hydrolytic peptides.

Unlike Psoufakis *et al.*, 2020, who achieved positive results, dietary inclusion of HFM resulted in poor growth performance in juvenile gilthead seabream in our study. We are of the view that the method used in the hydrolysis of the feather was probably less efficient.

Future research should consider including crystalline EAAs in the diets of juvenile *Sparus aurata* containing HFM as a protein source. It will also be interesting, to try other methods for the hydrolysis of feathers to be certain whether these methods will yield products with better amino acid profiles.

In this study, the replacement of 75% of fishmeal with PBM in the diet of *Sparus aurata* showed growth performance similar to the control (FM) group. Positive results were observed in feed intake, whole-body amino acid profile, and digestive system in comparison to the control group.

Based on the results of the present study, it is possible to replace up to 75% of fishmeal with PBM in the diet of juvenile *Sparus aurata* without any adverse effects on growth performance and health. Proteins from poultry by-products currently constitute ingredients with high nutritional potential for the diet of intensively farmed fish. They also have the advantage of having a positive impact on the environment by promoting by-products and reducing the pressure and dependence of the aquafeed industry on fish meal. |

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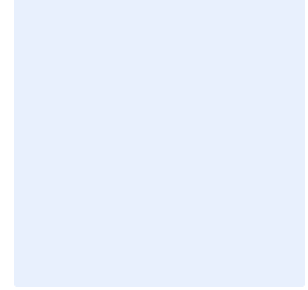
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APPENDICES

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