Replacing fishmeal with new alternative protein sources in the diet of European sea bass (*Dicentrarchus labrax*): effects on fish growth and disease resistance

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ABSTRACT

Due to the natural limit found in overexploitation of fishing, and the increased demand of aquatic products for different industries, aquaculture is presented as the solution to the high demand of aquatic products exerted by the world-wide increasing human population. But intensive aquaculture production needs to face different challenges to fulfill the demand increase in a context of sustainability, and covering the requirements of the "OneHealth", "Green Deal" and "Zero Waste" concepts. Two of the barriers that must be overcome are, i) the necessity of new ingredients in order to replace fishmeal and fish oil in the aquafeeds to decrease the high pressure and environmental impact of the fisheries and avoiding negative effects on fish health. ii) the impact on the health due to emergent pathogens and thermal-stress. Climate change is inducing an increase of infections by the genus *Vibrio*, an opportunistic group of bacteria that can cause economic losses by mortality or affecting the health of our individuals, and therefore, their animal welfare. The control of infectious diseases is necessary through prophylactic strategies to avoid the use of antibiotics in aquaculture and immunonutrition is one of the prophylactic tools available.

Processing animal proteins (PAPs) are among the different ingredients available for aquafeeds, but also the insect meals (IM) and the single cell proteins (SCP) are nowadays promising ingredients for aquafeeds, not only by their nutritional values, but also because of their immunonutritional properties. Therefore, this study aims to evaluate the use of PAPs or the combined use of insect meal and SCP in aquafeeds on fish growth and disease resistance in European sea bass (*Dicentrarchus labrax*). For this purpose, five experimental diets with two levels of fishmeal replacement (50 or 100%) by either PAPs or insect + SCP were tested for 12 weeks to evaluate growth performance, and the resistance to *Vibrio anguillarum* infection in sea bass juveniles. Overall, the results show that the diets did not compromise growth performance, except the diet with total replacement of FM by IM and SCP, that also is the diet that compromises survival after *Vibrio* infection.

1.- INTRODUCTION

Aquaculture, according to FAO, is defined as the cultivation under controlled conditions of species that develop in the aquatic environment and that are useful for humanity (APROMAR, 2023). Specifically, at the global level, fishing and aquaculture production reached a new maximum in 2022, increasing the aquaculture production up to 130.9 million tons, valued at 312.8 billion USD, 59% of the world fishing and aquaculture production (FAO, 2024). However, fisheries production is stagnated whereas aquaculture production continues increasing all around the world (APROMAR, 2023). At a national level, the aquaculture harvest in Spain in 2022 was 326,520 tons and a first sale value of 760.7 million euros (APROMAR, 2023). This increasing production continues to be the subject of discussion and concern due to the estimated increase in demand for products of aquatic origin by the increase of the world population, estimated by the United Nations to exceed 9.7 billion people in 2050, on a planet in which the carrying capacity is limited and in which resources are depleted at an increasing rate (U.N., 2024). For this, aquaculture is presented as a solution, being one of the objectives included in the FAO report: "Tracking progress on food- and agriculture-related SDG indicators" (Agenda point 23). If we look back and compare consumption data regarding products of aquatic origin between 1961 and 2022, we can observe marked differences. FAO (2024) estimated that the aquatic production growth rate (165 million tons), has doubled the production of 1961 and the world annual consumption of aquatic foods of animal origin per capita has increased from 9.1 kg in 1961 to 20.7 kg in 2022 (FAO, 2024). To face this increasing demand, with a large number of problems and challenges to solve, it is necessary to search for sustainable and ecological aquaculture (Wang *et al.*, 2021), and to avoid the spread of diseases and pathogens from a food health from the animal point of view (Maldonado Miranda *et al.*, 2022; Freitag *et al.*, 2022), being respectful of current legislation regarding animal welfare (APROMAR, 2024).

Therefore, one of the pillars of aquaculture through which we can address the vast majority or at least be a part of their solution, is nutrition. Aquafeed is one of the main costs of production in aquaculture and aquafeed formulas are highly dynamic, changing ingredients depending on the availability of ingredients. In turn, it must be sustainable with the environment, and must cover the objectives of the European Green Deal and the Zero waste policy. Without doubt, those objectives must be covered through the progressive reduction of the dependence of fishmeal (FM) and fish oil (FO) by the aquafeeds, by

replying those ingredients by alternative ingredients, that provide good health and digestibility to aquaculture animals, and be economically viable from a business perspective for its production.

Why are aquafeeds still dependent on a certain amount of fishmeal and fish oils? Retrospectively and with the parallel objective of giving a vision of the problems that aquaculture has already overcome in this area and the current lines of research, a general overview of its historical evolution can be analyzed: Aquatic fish, and specifically marine fish, depend on fish oil to obtain long-chain polyunsaturated fatty acids (LC-PUFAs) as these organisms are unable to elongate and desaturate 18:C fatty acids to 20:C or 22:C fatty acids. Besides, fishmeal has the best amino acid profile for aquatic organisms. Thus, traditionally fishmeal and fish oils were used as ingredients for aquafeeds. However, increased overfishing and increasing aquaculture production during the nineties reduced the availability of these ingredients. In 1998, a strong climate event called “el Niño” dramatically reduced the production of fishmeal and fish oil and aquafeeds needed to challenge this drastic reduction of ingredients while the product demand continued growing with the increase of human population and the demand of fishmeal and oil by the aquafeeds itself and other industries such as other animal feeds (Tacon and Metian, 2008).

This scenario was further complicated by the prohibition of use of ingredients of animal origin due to the incidence of the Bovine Spongiform Encephalopathy (BSE), that in turn eliminated all the animal ingredients from aquafeeds. The strategy was then to find ingredients to minimize the use of fishmeal and fish oil through the replacement by other sources of protein in aquafeed formulation (Hardy, 2010). The best candidates to be used as protein and lipidic sources were the meals and oils coming from agriculture, vegetable meals (VM) and oils (VO), but different limitations were found to use those ingredients: a) the lack of LC-PUFAs content in vegetable oils, that are rich in alpha linolenic acid (18:3n-3 - LNA) and linoleic acid (18:2n-6 - LA) and b) the relatively poor amino acid profile and the low digestibility of vegetable meals by marine fish. LNA and LA are the basis of the essential fatty acids docosahexaenoic acid (22:6n-3 - DHA) and eicosapentaenoic acid (20: 5n-3-EPA), two fatty acids that are the most valuable product in fish with high nutritional value from the human nutrition point of view (Watters *et al.*, 2012). Freshwater fish are able to elongate and desaturate LNA to EPA and DHA, but marine fish are not able to metabolize LNA into EPA and DHA. Those LC-PUFA have direct metabolic roles in growth, development of the central nervous system and vision, and a vital role in the immune system. In a large number of research studies, the conclusion was the possible substitution of

up to a certain percentage of fishmeal and oil with vegetable counterparts, such as soybean, linseed (Pianesso *et al*., 2020), rapeseed or olive (Mourente *et al*., 2005). However, this is not possible in marine carnivorous fish since they do not transform well the Alpha-linolenic acid (ALA), omega 3 of plant origin incorporated into the diet (Oliva-Teles *et al*., 2015). In addition partial substitution of fishmeal by vegetable meals and oils, in long-term periods, could compromise final nutritional quality of the fish filet and also compromise fish health, even when growth is not affected (Izquierdo *et al.*, 2005; González-Félix *et al*., 2016). In addition to these two essential fatty acids, there is arachidonic acid (AA), a polyunsaturated fatty acid of the omega 6 series that, in addition to being part of membrane phospholipids, is the precursor in the biosynthesis of eicosanoids, essential in the growth and survival of fish (Izquierdo *et al.*, 1996; Bessonart *et al.*, 1999). Therefore we can summarize that, to a large extent, until 2013, a large part of the lines of research in terms of nutrition in aquaculture were based on the replacement of FM/FO by VM/VO, with supplements of certain amino acids and fatty acids (Naylor *et al*., 2021).

The next turning point in terms of nutrition and feed formulation was in 2013 with the new legislation at the European level of regulation (EU) No. 56/2013, which lifted the repeal of the use of terrestrial animal proteins. in the formulation of feed in aquaculture. This was a great revolution since it opened the door to new sources of formulas that could be used to prepare meals, especially chicken and its derivatives (Bowyer *et al*., 2012).

The next notable milestone in this evolutionary line of research was in 2017 the approval of insect meals as a formulation ingredient for aquaculture feed, with 15 insect species approved in EPPO (European and Mediterranean Plant Protection Organization). Taking into account that at this historical moment the later feed formulation ingredients increased in cost between 50% and 75% (Sanchez-Muros *et al.*, 2014), including those recently approved as proteins of terrestrial animal origin (Gasco *et al.*, 2016). The nutritional needs of monogastric species, especially fish, include high quality and quantity of protein in the diet. The amino acids of several insect species are compared to the composition of soybeans and fishmeal (Van Huis, 2013). As a source of protein, insects, depending on the species, have an adequate amino acid profile. The most common limiting amino acids are histidine, lysine and tryptophan, which could be incorporated into the diet. Insects appear to be a sustainable source of proteins with attractive quantity and quality and acceptable nutritional properties and from the point of view of nutritional value, the use of insects as a protein-rich and sustainable feed ingredient in diets is technically feasible and opens new perspectives in animal feeding (Basto *et al.*, 2020). However, the use of insect meal is not

free of problems and detractors. Taking into account that we must not lose sight of the objective that the purpose of aquaculture is human consumption as food, we must therefore take into account the wishes and preferences of the consumer when producing a good, attractive and competent product. A study on the consumer acceptance of insect meal in aquaculture in the Spanish market showed that consumer even accept to pay an extra cost for the fish produced from these meals, since at the same time they know that they are contributing to an improvement in environmental sustainability, although consumer also have some concern on the organoleptic attributes of this product (Ferrer Llagostera *et al.*, 2019).

Other interesting ingredients are the proteins coming from single-cell microorganisms, including bacteria, microalgae, fungi or yeast, that are defined as single cell- proteins (SCP) and can be considered as a protein source. Single cell protein meals are generally rich in amino acids and vitamins. These sources can be grown on waste substrates, making them highly profitable and sustainable raw materials, and are optimal for the approaches of the green deal and the zero-waste policies. In particular, meals derived from single bacterial cells stand out for having a very low environmental footprint and for growing rapidly in various conditions. In addition, these meals have a very high protein content (50-80% in dry matter), with a balanced profile of essential amino acids, and can be rich in vitamins, phospholipids and other functional compounds. One of the SCPs with high promising results is *Corynebacterium glutamicum* (Carvalho *et al.*, 2023), that has been included up to 20% in replacement of vegetable proteins without any negative effect in gilthead sea bream growth (Marchi *et al*., 2023). Those ingredients are covering the amino-acid side of the diets, but the essentiality of LC-PUFA must be covered by another ingredient that contains high amounts of EPA and DHA. Microalgae oils have gained vital importance nowadays as an ingredient rich in LC-PUFAs. As pointed out by Carvalho *et al*. (2023) "*This is because microalgal oils are possibly the most promising source to replace fish oil (FO) in aquafeeds in the future, due to their very rich content of n-3 long chain polyunsaturated fatty acids (LC-PUFA), particularly DHA".*

To summarize; from the fishmeal/fish oil times (the 90s) through the vegetable oils (the 2000s), the re-utilization of ingredients (the 2010s), to the new and emergent ingredients (the 2020s), once the entire chronological line of evolution in terms of the nutrition of aquaculture feeds has been explained, and both its strengths and weaknesses and current challenges of science have been pointed out, currently, the following questions remains unsolved to open different lines of ingredients utilization: i) the use of blends of

those ingredients to totally replace fishmeal and fish oil and ii) to explain how these new alternative ingredients, in addition to alleviating the problem of sustainability can also be used as a functional additive or immunonutrient, enhancing fish immune defenses or at least not affecting negatively the fish health. Those types of ingredients can be used also as additives with a role of stimulating and strengthening the immune system of fish against the possible stress generated by the intensive aquaculture practices and the changing environment that cause increases of the incidence of certain pathogens such as vibriosis.

The term vibriosis describes primary systemic infections caused by a pathogenic *Vibrio* species and does not include those nonspecific infections in which large numbers of *Vibrio* spp. may be involved as secondary opportunistic agents. Regarding the causal agent of the infection, it can develop from different species belonging to the genus *Vibrio*, such as *V. harvey, V. tubiashii, V. ordalii, V. vulnificus, V. alginolyticus* biotype 2, *V. parahaemolyticus, V. salmonicida* and *V. anguillarum* (Bekaert *et al.*, 2021). Without a doubt, vibriosis is the most serious bacterial infection that marine fish can suffer from, both in the wild and bred in captivity, of vital importance, positioning itself as the disease of aquaculture production that generates the most economic losses annually in the world (Maldonado-Miranda *et al*., 2022). Focusing exclusively on *V. anguillarum*, we define it as a Gram-negative oxidase-positive bacillus and facultative anaerobes widely distributed in nature, being responsible for causing characteristic hemorrhagic septicemia in a wide variety of fish species of great economic importance, such as Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), turbot (*Scophthalmus maximus*), sea bass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*), striped bass (*Morone saxatilis*), cod (*Gadus morhua*) and Japanese and European eel (*Anguilla japonica* and *Anguilla anguilla*) (Ina-Salwany *et al*., 2019; Manchanayake *et al.*, 2023).

The symptoms of fish affected by vibriosis are somewhat diffuse and generalized. This pathogen affects the initial stages of larvae and fry, directly causing death, without previously observing symptoms or any perceptible physical sign (Kyeong-Jun *et al*., 2024; Dubert *et al.*, 2016). However, in adult individuals it does have a much more characteristic clinical form, presenting a variety of symptoms that can vary depending on the species of *Vibrio*. In particular, *V. anguillarum* induces external injuries, including hemorrhages on the skin, especially around the fins and base of the tail, ulcers and necrosis on the skin, reddened and swollen areas. Internal symptoms can be pointed out internal bleeding, especially in the liver, kidney and muscle, inflammation and necrosis of internal organs, pointing out with special interest their digestive tract. Early symptoms of vibriosis are abnormal behavior,

with erratic swimming, lethargy or inactivity together with a loss of appetite. In addition, other manifestations can also be observed such as exophthalmia, general darkening of the skin and ascites (M.Y.Ina-Salvany *et al.*, 2019).

1.1 OBJECTIVES

- To evaluate the effectiveness of the partial or total replacement of fishmeal and oil with diets that include blends of alternative ingredients (PAPs or insect + SCP meals) on the growth of European sea bass (*Dicentrarchus labrax*).
- To evaluate the effects of those blends of ingredients on sea bass disease resistance through the use of a challenge test against *V. anguillarum*

2. MATERIALS AND METHODS

This section includes both the type of facilities and organisms used for the experiment, as well as all the techniques used to obtain the results and their subsequent analysis. The present experiment was approved by the bioethical committee of Las Palmas de Gran Canaria University (OEBA-ULPGC 26/2023) and approved by the authority (Consejería de Ganadería, Gobierno de Canarias).

2.1. Facilities

The experiment was carried out in the Aquaculture facilities of the Taliarte marine technological science park (PCTM) (Cod.REGA: ES350260026567). It was conducted in three different units of research within the facilities
(https://www.ecoaqua.eu/es/taliarte.html)

- The feeding and rearing plant for fry.
- The bioassay laboratory where the challenge was carried out.
- The highly specialized analysis service for aquaculture and biotechnology.

2.2. Experimental design: On-growing phase

Four hundred and fifty healthy European sea bass (*Dicentrarchus labrax*) were used in their juvenile stage. These were raised and supplied by a local farm (Aquanaria, Castillo del Romeral, Gran Canaria, Canary Islands), and were acclimated to the facility during 15 days. After the acclimation period, fish were randomly distributed in 15 fiberglass tanks (triplicate tanks per each dietary treatment, see details below), with a capacity of 500 L. The tanks were equipped with a continuous water (flow-through system) and were well aerated. Oxygen and temperature were monitored in each tank continuously. Each tank contained 30 individuals with an average weight range of 38.3 ± 2.1 g.

Regarding water conditions and parameters, dissolved oxygen levels ranged between 6.6 and 6.1 ppm and the water temperature ranged between 18.2 and 20.2°C. The fish were held under a natural photoperiod of approximately 12 hours of light and 12 hours of darkness (12L:12D).

2.3 Experimental diets

Based on fishmeal substitution, the experimental diets were formulated by the company SPAROS (Sparos LTD, Olhao, Portugal). Five isoprotein and isoenergetic experimental diets with partial (50%) or total (100%) replacement of the dietary FM were formulated. The Control diet, with 20 % marine proteins, was formulated to mimic a current commercial diet used for this species, including also 20% PAPs (from poultry meal, poultry blood meal and feather meal) and 2% of a bacterial SCP from *Corynebacterium glutamicum*. The other experimental diets were based on partial (50%) or total (100%) substitution of the fishmeal by increasing PAPs in the diet (PAP50 and PAP 100, respectively) or by the inclusion of an insect meal from black soldier fly (*Hermetia illucens*) and meal from of *C. glutamicum* (ALT50 and ALT100). The experimental diets and the proximal composition are described in Table 1 and summarized in Table 2.

Table 1. Dietary ingredients and proximal composition of the experimental diets.

Ingredients (%)	CTRL	PAP50	PAP100	ALT50	ALT100
	%	%	%	%	%
Fishmeal Super Prime	12.5				
Fishmeal 60	5.0	7.5		7.5	
Krill meal	2.5	2.5		2.5	
Poultry meal	10.00	12.00	20.25	10.00	10.00
Poultry blood meal	5.00	9.75	9.75	5.00	5.00
Feather Meal hydrolysate	5.0	5.0	5.0	5.0	5.0
Insect meal (BSF - PROTIX)				4.00	6.25
Aminopro NT70 - C. glutamicum	2.0	2.0	2.0	6.8	12.1
Corn gluten meal	12.0	12.0	12.0	12.0	12.0
Guar korma	6.0	6.0	6.0	6.0	6.0
Rapeseed meal	7.0	7.0	7.0	7.0	7.0
Wheat meal	10.38	11.98	12.80	10.18	10.58
Wheat bran	3.0	3.0	3.0	3.0	3.0
Whole peas	5.0	5.0	5.0	5.0	5.0
Fish oil	6.0	6.0	6.0	6.0	6.0
Algae oil (Veramaris)		0.25	0.55	0.25	0.55
Poultry fat	5.7	5.7	5.7	5.7	5.7
Vitamin and mineral premix	1.0	1.0	1.0	1.0	1.0
Choline chloride 50%	0.20	0.20	0.20	0.20	0.20
Antioxidant	0.20	0.20	0.20	0.20	0.20
Sodium propionate	0.10	0.10	0.10	0.10	0.10

Monoammonium phosphate	**0.20**	**1.30**	**1.80**	**1.30**	**2.10**
L-Lysine HCl 99%	**0.20**	**0.35**	**0.45**	**0.60**	**0.85**
DL-Methionine		**0.15**	**0.18**	**0.15**	**0.25**
Yttrium oxide	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**
Rapeseed lecithin (Liquid)	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**
TOTAL	**100**	**100**	**100**	**100**	**100**
Proximate composition (%wet weight)					
Crude protein	**45.33**	**45.78**	**46.27**	**45.46**	**44.66**
Crude lipid	**17.58**	**17.89**	**17.71**	**18.14**	**19.41**
Ash	**6.07**	**5.97**	**5.96**	**6.32**	**5.80**
Moisture	**6.21**	**7.79**	**7.27**	**7.75**	**7.23**

Table 2. Summary of the main characteristics of the diets used in the experiment.

CTRL	Mimicking a practical formula with: • 20% marine proteins (from fishmeal and krill meal) • 20% land animal proteins (from poultry meal, poultry blood meal and feather meal) • 2% single cell protein (*Corynebacterium glutamicum*)
PAP50	• 50% reduction of marine proteins by PAPs • Increase of land animal proteins without altering the rest of the formula • The reduction of EPA+DHA due to FM reduction was corrected with algae oil (Veramaris) • It implied some adjustments on monoammonium phosphate, Lys and Met to avoid deficiencies

PAP100	• 100% reduction of marine proteins by PAPs • Further increase of land animal proteins without altering the rest of the formula • The reduction of EPA+DHA due to FM reduction was corrected with algae oil (Veramaris) • It implied some adjustments on monoammonium phosphate, Lys and Met to avoid deficiencies
ALT50	• 50% reduction of marine proteins by Insect meal & SCP • Land animal proteins were kept identical to CTRL • Increase of alternative protein sources (BSF meal and single cell protein) without altering the rest of the formula • The reduction of EPA+DHA due to FM reduction was corrected with algae oil (Veramaris) • It implied some adjustments on monoammonium phosphate, Lys and Met to avoid deficiencies • Increase in formulation cost was <5%
ALT100	• 100% reduction of marine proteins by Insect meal & SCP • Land animal proteins were kept identical to CTRL • Increase of alternative protein sources (BSF meal and single cell protein) without altering the rest of the formula • The reduction of EPA+DHA due to FM reduction was corrected with algae oil (Veramaris) • It implied some adjustments on monoammonium phosphate, Lys and Met to avoid deficiencies • Increase in formulation cost was <5%

The composition of the diets can basically be differentiated into two of them formulated with protein of terrestrial animal origin and the other two with protein of alternative origin. By always taking into account the economic cost of the diets, only a maximum of 6% of insect meal could be used, which is why the amount of protein of single-cell origin (Aminopro NT70) (Mazzoleni, Italy) was increased.

The feed was dispensed by specialized aquaculture technicians at 3 feedings per day until apparent satiation for 90 days. The experiment was conducted in blind, technicians knowing the abbreviation of the diets, but not the ingredients and the whole formulae. After

the last meal of the day, the uneaten pellets were collected and weighed to precisely estimate the daily feed intake.

2.4. Sampling and anesthetic management

All individuals in the 15 tanks were individually weighed (whole fish weight) and measured (total length) monthly to obtain the growth curve of the respective diets. The specific growth rate was calculated as SGR (%/day): (Ln final mean weight – Ln initial mean weight)/nº of days x 100.

This was carried out through anesthesia management in accordance with the regulations of the European Union Directive (2010/63/EU) and Spanish legislation (RD 53/2013) for experiments with animals. The manipulation of the fish was carried out under natural anesthesia with clove oil (0.2 ml/L; Guinama S.L; Spain, Ref. Mg83168), and discomfort, stress and pain of the animals was avoided as much as possible.

2.5. *Vibrio anguillarum* bacteria preparation

2.5.1. Preparation of the culture medium for the bacteria

To work with the bacteria, we must first sow it and to do this, prepare a culture medium where they develop their metabolism and replicate. This culture medium will be the BHI (brain heart infusion)(Condalab, Spain). We prepared 250 mL of both the solid medium (agar) and the liquid medium (broth), for this we used the proportion of powder and water that is provided in the container by the manufacturer of the medium. 9.25 grams of medium were required for the broth and 13 grams of medium for the agar, both in 250 mL of distilled water. In our case, the medium was prepared at 1.5% so we added 0.375 grams of salt.

Both mixtures were placed in a magnetic stirrer and we activated both the temperature (about 55ºC) and the stirring, until it was enough for the entire liquid to move. We boiled it for one minute and sterilized it in an autoclave at 121ºC for 15 minutes. We placed the samples in the refrigerator at 3ºC. As for the liquid medium, we poured it into 50 mL plastic falcon tubes.

2.5.2. Seeding of the bacteria

The *Vibrio anguillarum* sample for seeding belongs to the collection of bacteria strains from ECOAQUA universitary institute (ULPGC), and is identified as strain 507 and is kept at -80ºC. The bacteria seeding process was carried out in two stages: initially on solid medium and subsequently on liquid medium.

2.5.2.1 Sowing in solid medium

Plating on agar was carried out using the multiple streak technique (with three streaks) under a Bunsen burner to maintain sterile conditions (Fig. 1). The seeded plates were incubated at 26ºC, which is the average body temperature of the fish (simulating their natural environment), for 48 days.

Figure 1. Sowing in solid medium.

2.5.2.2. Liquid Culture Preparation

Two 50 mL Falcon tubes were prepared; one was filled with 20 mL of liquid medium and the other with 5 mL of liquid medium (the latter acted as a control to verify the absence of external contamination). After two days of incubation, a colony of significant size was picked from the plate. To ensure complete collection of the colony, a square was cut around it (this included the medium) and transferred to the tube with 20 mL of liquid medium. Finally, the tube with 5 mL of liquid medium did not receive any colonies and served as a negative control.

2.5.3.3. Liquid Culture Incubation

The Falcon tubes (one with the colony and the other as a control) were incubated at 26ºC under constant shaking to promote the growth of the bacteria. The start time of incubation was recorded for accurate monitoring of bacterial growth. This method ensures the controlled and sterile growth of *Vibrio anguillarum.*

After the initial preparation, periodic measurements were made to determine the moment at which the bacteria reached the desired concentration (10^7 CFU/mL). These measurements were carried out at specific intervals over several days: at half an hour, one hour, two hours and three hours. Two different methods were used to measure bacterial growth:

- Optical density: A 96-well plate was prepared in which 200 microliters of the sample and control were placed, in duplicate, at different times. The plate with the samples was introduced into the densitometer, which measured the absorbance at 595 nanometers, providing an immediate result.

- Culture in Serial Dilutions Plate: Six 1.5 mL Eppendorf tubes were prepared with 900 microliters of PBS each. A sample was removed from the incubator and 100 microliters were placed in the first Eppendorf, homogenizing the mixture (1/10 dilution). The process was repeated successively until the sixth dilution (1/1,000,000). A plate with half agar was taken and three circles were drawn at the base for each dilution, from the third to the sixth. Sowing 10 microliters of each

dilution in their respective circles in triplicate to avoid errors. The plates were allowed to dry and incubate for one day at 26ºC.

2.6. Challenge test against *Vibrio anguillarum*

Ninety European sea bass were randomly selected, 6 from each tank, making a total of 18 per dietary treatment. Fish body weight was around 80 ± 10g.

Each 6 fish from the same tank were confined using PVC buckets connected to an oxygen supply. At the same time, a batch of the seeded sample of *Vibrio anguillarum* corresponding to 10^6 CFU was loaded into 1 mL syringes. Under anesthesia using clove oil diluted in water, 0.1 mL of activated bacteria was inoculated intraperitoneal into each of the specimens. Therefore, the final load of *Vibrio anguillarum* in each of the European sea bass was 10^5 CFU.

2.6.1. Microbial diagnosis: Verification of *Vibrio anguillarum* presence in fish tissues

To verify that the inoculated bacteria is causing infection in fish, an extraction of bacterial DNA from different tissues of the fish (liver, craneal kidney and spleen) was carried out and subsequently a PCR was carried out to confirm and identify our bacteria.

It was done in four steps to purify the genetic material:

- Tissue Preparation: we cut 40 mg of tissue and transferred it to a 1.5 mL Eppendorf. We added 400 µL of Tissue lysis(TL)(E.Z.N.A, Omega Biotek) buffer and 25 µL of proteinase K, mixed the solution using a vortex and incubated at 55ºC while moving for 3 hours, mixing every 20 minutes.

- Cell Lysis and DNA Purification: after incubation, we centrifuged the sample at maximum speed 14.100rcs/min for 5 minutes. We transferred the supernatant to a new 1.5 mL Eppendorf without disturbing the pellet. We added 420 µL of BL buffer, mixed again with a vortex and incubated at 70ºC for 10 minutes. We added 420 µL of 100% ethanol again and mixed again with a vortex.

- DNA Filtration and Washing: we inserted an extraction column into a 2 mL collection tube and transferred the entire sample to it, including any precipitate. We centrifuged at 14.100rcs/min for 1 minute, discarded the filtrate and reused the collection tube. This step had to be repeated due to the volume of the sample. Finally, we added 500 µL of HBC buffer, for the third time we centrifuged again at maximum speed (14.100rcs/min) for 30 seconds and discarded the filtrate along with the collection tube.

- Washing and Elution of DNA: we inserted the column into a new 2 mL collection tube and added 700 µL of DNA Wash Buffer. We centrifuged at 14.000rcs/min for 30 seconds, discard the filtrate and reused the collection tube. We repeated this step. We centrifuged at 14.100rcs/min for 2 minutes to completely dry the column. We transferred the column to a 1.5 mL nuclease-free Eppendorf and added 100 µL of Elution Buffer heated to 70ºC. We let the mixture rest for 2 minutes at room temperature and centrifuged at maximum speed (14.100rcs/min) for 1 minute.

2.6.2 PCR

Immediately afterward we performed the PCR: The total reaction volume was 25 µL of reaction buffer containing deionized water, 1 × buffer A, 1.0 mM MgCl2, 0.2 µM of each forward and reverse primer, 0.1 mM dNTP, 1.5 U of Taq polymerase and 5.0 ng of genomic DNA as template. We put the volume of mixture and then the extracted DNA in each well of a 96-well plate. PCR was carried out with an initial pre denaturation at 94 ◦C for 5 min, followed by 35 cycles (1 min denaturation at 94 ◦C, 1 min primer annealing at 55 ◦C, and 1 min primer extension at 72 ◦C). then the final extension step of 7 min at 72 ◦C. Also prepared a positive control with a bacteria confirmed strain of our storage and a white that was water (Jaruboonyakorn *et al.,* 2022; Innis MA. 1990). prepared a 2% agarose gel (1.2 grams of agarose + 60 mL of 0.5X TBE) and dissolved it by heating it in the microwave until it was transparent. After removing it, we added 1.5 microliters of GelRed (Biotium). poured it into a suitable mold, removed the bubbles, placed the comb on it, and left it covered until it dried. Electrophoresis: made a mix of 1 microliter of gel loading buffer + 2 microliters of PCR product + 3 microliters of MQ water (for each sample and control). Made another mix for the ladder, replacing the 2 microliters of PCR product with the ladder. placed the gel in the electrophoresis tank so that the wells were at the location of the positive

node. loaded the mixes into each well of the gel, starting with the ladder, followed by the negative control, the samples, and the positive control. Finally, I covered the tank and ran it at 80V for 1 hour. The reading was carried out using a computer and UV light.

2.6.3. Microbiological Diagnosis

To certify that the cause of the deaths was our pathogen and not another, a post-mortem microbiological diagnosis was carried out on the first nine individuals who died in the challenge to check Koch's postulates.

The dissection was performed in a completely sterile area and always under the flame of the Bunsen burner. Previously sterilized samples of anterior kidney, liver and spleen were collected. These organs were selected since they are the main ones in terms of lymphatic tissue in which the bacteria *Vibrio anguillarum* acts. These samples were collected and seeded in a BHI culture medium and waited for 48 hours for the growth of bacterial colonies.

Subsequently, the three tests necessary to confirm vibriosis were performed on each of them following Koch's postulates test (Walker *et al.*, 2006):

Catalase: Catalase is an enzyme that catalyzes the breakdown of hydrogen peroxide into water and oxygen. The test is used to check for the presence of the enzyme catalase which is found in most aerobic and facultative anaerobic bacteria that contain cytochrome oxidase. The test was carried out using the slide procedure. We placed two drops of 3% hydrogen peroxide to which we added a colony previously collected with the sowing loop.

Oxidase: determines the presence of oxidase enzymes. The oxidase reaction is due to the presence of a cytochrome oxidase system. Cytochromes are enzymes that are part of the electron transport chain in aerobic respiration, transferring electrons to oxygen, with the formation of water. The cytochrome oxidase system is generally only found in aerobic organisms. We placed a piece of filter paper approximately 3x3cm in a Petri dish. We added 2-3 drops of 1% liquid solution of dihydrochloride tetramethyl-p-phenylenediamine Kovacs reagent (Condalab, Madrid, Spain) to the center of the filter paper. We spreaded a colony on the impregnated paper with the sowing loop.

Mobility test: Used to determine whether an organism is mobile or immobile. Bacteria move by means of their flagella, which are found mainly between the bacilli. We placed the sample on a slide and observed it under the microscope.

Gram stain: This staining requires four solutions, a basic dye, a mordant (a substance that increases the affinity between the cell and the dye), a decolorizer (removes the dye from a stained cell) and a second dye or contrast dye (color dye different from the initial one). After staining with the first dye (Crystal violet) we carried out the decolorization with ethanol that removes the dye only in the Gram negative cells, while in the Gram positive cells the dye is retained and the cells remain blue. The Gram-negative cells were then stained with the contrast dye (safranin) so they can be seen (Tobon and Hoyos, 2012).

2.6.4. Macroscopically clinical signs of infection

Throughout the challenge, the majority external injuries were analyzed and visually described like, identifying the typical signs of septicemic diseases include hemorrhagic patches on different areas of the fish surface and the base of the fins, ascites, hemorrhages at the base of the pectoral fin and around the operculum, Congestion of the pelvic, caudal and anal fins, as described by Bukha *et al.*, (2023)

2.7. Statistical analysis

Statistical treatment of the data was performed with IBM SPSS Statistics v26 software (IBM Corp. Armonk, NY, United States). The results of the experiment were analyzed using one and One-way ANOVA tests and the post hoc Tukey range test, considering significant differences when $p < 0.05$ for growth parameters. The Kaplan-Meier survival curve was used to analyze the survival rate. The normality of the experimental data was tested with the Shapiro-Wilk test and the homogeneity of the variances was tested with the Levene's test.

3. RESULTS

3.1. Growth parameters

No mortality was recorded in any of the specific dietary treatments during the experiment. Fish accepted all diets well, and no significant differences were found in the ingesta (data not shown).

After 90 days of feeding, the utilization of the different diets did not induce changes in fish growth, except for fish fed the diet ALT100. The fish fed PAP 50, PAP 100, and ALT 50 diets showed similar growth (final weight) compared to the control diet (Table 3). However, the fish fed with the ALT 100 diet presented a significantly ($p<0.05$) lower final weight and weight gain compared to the other experimental diets (Table 3). Moreover, although no significant ($P>0.05$) differences were observed, SGR tended to be worse in fish fed the ALT 100 diet compared to fish fed the other experimental diets.

Table 3. Growth performance of European sea bass (*Dicentrarchus labrax*) juveniles after a feeding trial of 90 days with the experimental diets.

	Control	PAP 50	PAP 100	ALT 50	ALT 100
Initial body weight (g)	36.85 ± 0.50	36.53 ± 0.45	37.14 ± 0.52	36.86 ± 0.26	36.54 ± 0.27
Final body weight (g)	125.75 ± 1.24^{a}	122.81 ± 5.24^{ab}	125.04 ± 4.53^{a}	122.97 ± 1.27^{ab}	112.12 ± 6.72^{b}
Initial body length (cm)	14.84 ± 0.12	14.87 ± 0.1	14.89 ± 0.03	14.92 ± 0.02	14.85 ± 0.03
Final body length (cm)	20.74 ± 0.12	20.78 ± 0.19	20.85 ± 0.34	20.83 ± 0.08	20.25 ± 0.39
SGR (%day^{-1})	1.36 ± 0.03	1.35 ± 0.04	1.35 ± 0.05	1.34 ± 0.01	1.25 ± 0.08

Different letters as superindex in values (mean ± SD) in the same row indicate significant differences ($p<0.05$). Control: control diet with 20% FM; PAP 50: 50% of FM replacement by PAPs; PAP 100: 100% replacement by PAPs; ALT 50: 50% replacement by the inclusion of Insec meal and SCP; ALT 100: total replacement FM by the inclusion of insect meal and SCP. PAP: Processed animal proteins; SCP: Single cell protein

The utilization of the different diets did not induce alterations in the proportion of the body shape, as no differences were observed in the ratio body weight/fish length within all the experimental period (Fig. 2). Each fish grew adequately in the length/weight ratio without any type of variations in the condition factor.

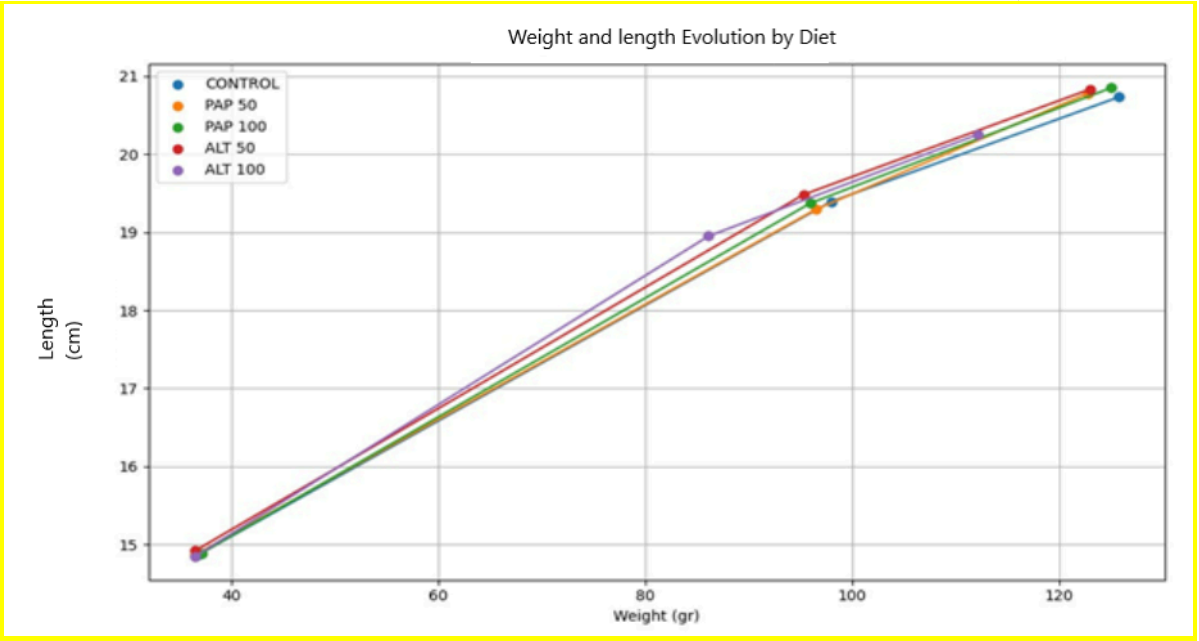

Figure 2. Correlation of length and weight of fish during the experiment. PAP: Processed animal proteins; ALT: Blend of Insect meal and Single cell protein (SCP)

3.2. Survival after challenge test with *Vibrio anguillarum*

Although there were no significant differences in survival among treatments (Log.rank (mantel-Cox) test: p value 0.050117), a clear tendency can be seen when PAP or ALT50 is used when compared to control or ALT100 diets. As observed in the Kaplan-Meier curve (Fig. 3), the fish fed the Control diet dropped quickly in survival to about 20% around 24-48 hours. Afterwards, it maintained a really low survival, achieving the worst results with the ALT100 diet (5.5%). PAP50 quickly dropped total survival to about 20% around 24-48 hours to end the challenge with a survival rate of 11.1%. PAP100 dropped rapidly total survival after 48 hours to a percentage of 30% but maintained a greater survival percentage (16.66%) when compared with the other experimental groups until 144 hours, presenting itself as the diet with the best results in our experiment. ALT50 began with high mortality, reaching 10% around 24-48 hours. However, afterwards it remained stable, achieving a survival of 11% of individuals, double that of the control diet (5.5%). ALT100 had the fastest drop in survival, with a peak at 48 hours of mortality of 84.44%. It remained stable for the next 72 hours and ended with a result identical to the Control diet (5.5%), positioning both as the two poorest diets in terms of results.

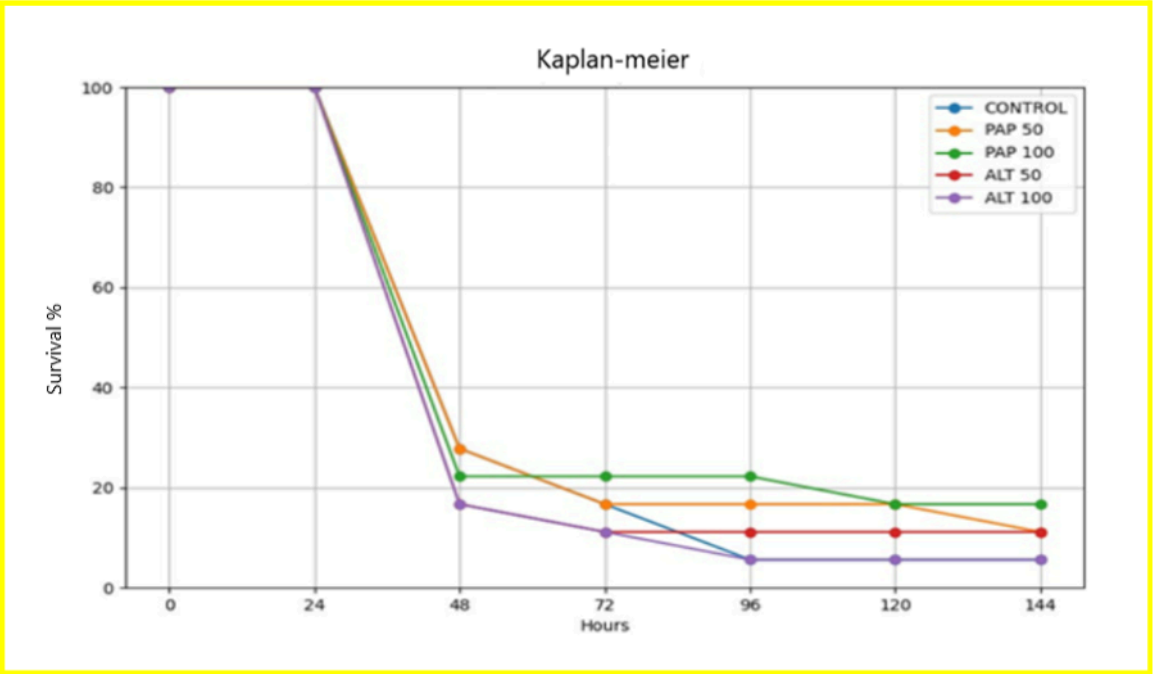

Figure 3. Kaplan Meier survival graph obtained after the resistance challenge against *Vibrio anguillarum* for 7 days in hours for sea bass fed the different experimental diets. PAP: Processed animal proteins; ALT: Blend of Insect meal and Single cell protein (SCP)

Two general observations can be pointed out: i) taking into account that they all started at the same time and no loss was observed in the following 24 hours, this justifies the correct procedure of all intraperitoneal injections, thus ruling out any death due to management error; ii) the utilization of ALT diets (50 or 100) seemed to induced a faster mortality, although ALT50 fish had better survival rate at the end of the period.

3.3. Microbiological diagnosis and macroscopical observations

The results obtained by PCR analysis were as expected (Figure 4), thus confirming our strain of *Vibrio anguillarum*.

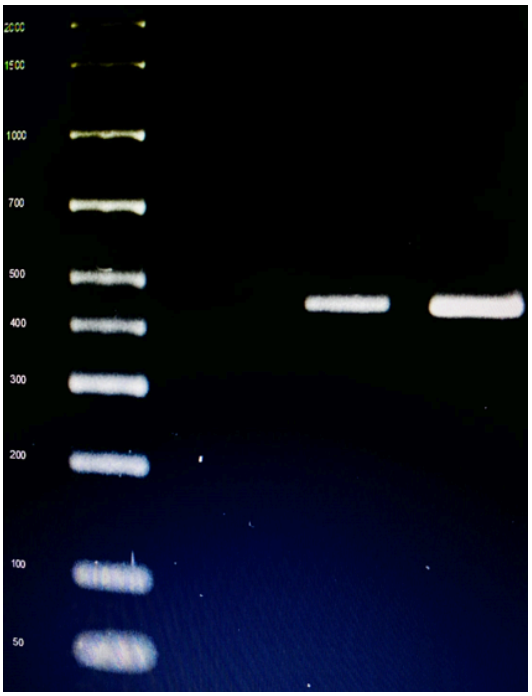

Figure 4. Result of PCR to confirm *V. anguillarum*

The Koch's postulates confirmed in all the tissues analyzed (Spleen, liver and head kidney) that the isolated pathogen was the inoculated pathogen (Table 4)

Table 4. Kosch' postulates test to confirm Vibriosis in samples of tissues from seabass fed the experimental diets

analyzed sample	Catalase	Oxidase	Mobility test	Gram stain
Spleen	+	+	+	−
Liver	+	+	+	−
Head kidney	+	+	+	−

Macroscopic analysis of the fish confirmed also the presence of vibriosis, with fish showing the typical external symptoms of vibriosis, including hemorrhages at the base of the pectoral fins and around the operculum (Fig. 5)

Figure 5. External symptoms and lesions of *Vibrio anguillarum***.**

Taking into account the different test and diagnosis followed, it can be concluded that the cause of mortality was *Vibrio anguillarum.*

4. DISCUSSION

The different strategies of FM supplementation in sea bass diets used in the present study are modulating fish growth in different ways, and the results obtained in the present study are in accordance with previous studies reporting supplementation of FM by alternative ingredients in sea bass or in other fish species. The utilization of PAPs, both partial (PAP50) or total (PAP100) substitution of FM, induced a similar sea bass growth when compared to control (commercial-like) diet, which in turns indicate that it is possible to replace FM by PAPs (poultry origin) in sea bass diets. Galkanda-Arachchige and co-authors (2020) summarized the use of poultry by-products in aquafeeds through a meta-analysis of the different published studies and concluded that the success of FM replacement depends on the species for both freshwater or marine fish, but depends also on the level of FM replacement for marine fish. This limitation in FM replacement is, in part, due to the decrease of essential fatty acids when reducing the fishmeal, which also contains a certain amount of fish oil. Carvalho *et al*. (2020) demonstrated for sea bream that the success of fishmeal and fish oil replacement depends on the supplementation of an oil that covers the requirements of essential fatty acids. Those authors supplemented the alternative

diets with a microalgal oil rich in EPA and DHA, obtaining thus similar growth with the low FM/FO diets than the control (FM/FO based) diet. However, in that study, the replacement without microalgal oil supplementation reduced fish growth. In our experiment, the success of our PAP50 and PAP100 diets is possible through the addition of a microalgae oil (Veramaris®), that provides both DHA and EPA. This strategy also permits n-3 LCPUFA sources allowing the complete replacement of marine ingredients through the use of a cheaper and sustainable lipid source.

Regarding the utilization of insect meal, Tran and co-authors (2022) recently reviewed the utilization of insect meals on performance of different aquaculture species through a systematic and meta-analysis of the different published papers and concluded that some insect meals could support the growth performance of aquaculture species depending on the inclusion levels, and the content of chitin level in the ingredient is a limiting factor when using marine species, specially those with limited capacity to digest chitin.

The results from the present study are in agreement with other studies, which use both our same protein source of insect origin (*Hermetia illucens*) that obtained an improvement in growth only by adding 1% of black soldier fly (*Hermetia illucens*) (Xu *et al*., 2021) or other authors who used other sources such as the hydrolyzed mealworm (*Tenebrio molitor*) and superworm (*Zophobas morio*) meals as a partial replacement for fishmeal in sea trout (*Salmo trutta*) (Mikolajczak *et al.*, 2020) and also obtained these positive results in the development of fish when using a limited amount of insect meal in the diet. Indeed, the utilization of the ALT100 diet with 100% FM substitution induced lower growth in sea bass, suggesting that it is still necessary to clearly define the limit of adding this type of ingredient to diets without causing an unfavorable effect. Those results are in agreement with the results for Largemouth bass (*Micropterus salmoides*) in which a 5% amount of black soldier fly (*Hermetia illucens*) led to a significant decrease of SGR (Xu *et al.,* 2021).

On the other hand, the use of Single cell proteins for aquafeeds has been also reviewed recently (Sharif *et al.*, 2021). Those authors pointed out that these ingredients can be produced at any time during a year and could be produced from various cost free substrates, in concordance with the "zero-waste" and "green deal" approaches. However, one of the main limitations on the use of SCP meals is the digestibility of this ingredient, that can have a negative impact on the protein digestibility of the diet, and thus must be included in a limited amount and depending on the species (Glencross *et al.*, 2022), in agreement with the results of the present experiment.

Both ingredients must be supplied in combination with other ingredients, as suggested by Sharif *et al.* (2021) and Tran *et al.* (2022), but little is known on the combined use of both ingredients in marine species such as sea bass. Thus our results contribute to the knowledge for sea bass diet formulation with new ingredients for aquaculture.

Although the use of alternative proteins to fishmeal allows a relative well-performance of aquaculture fish under a certain level of FM substitution and blend of different ingredients, the implications of the use of alternative proteins must go "beyond growth", as pointed out by Aragao and co-authors (2022). Those authors pointed out that alternative protein sources, including PAPs, insect meals, micro- and macroalgae, and single cell proteins may negatively impact gut microbiota and health, thus affecting fish health and disease resistance.

In the present study, the use of PAP50 or PAP100 did not affect fish survival after a challenge test against *Vibrio anguillarum*. In agreement with our results, the use of poultry byproducts as fishmeal replacers did not induce effects in cobia (*Rachycentron canadum*) (Zhou *et al.*, 2011) or rainbow trout (Gaudioso *et al.*, 2021) health and immune parameters. In the present study, fish fed the total substitution (PAP100) presented equal survival even when high substitution of FM by alternative ingredients can induce lower resistance to pathogens, as found in the study conducted by Torrecillas *et al.,* (2017) with sea bass in which mortality was significantly increased when substituting FM up to only 95% by vegetable meal. Therefore, it could be assumed that if the replacement of FM is made by processed animal proteins (poultry) and supplemented with microalgal oil, the survival against *Vibrio anguillarum* is not affected compared to almost total substitution by vegetable meals.

The utilization of the ALT50 diet, with partial substitution of FM by insect meal + SCP, caused a 10% increase in survival against the *Vibrio anguillarum* compared to the control diet (commercial-like formulated). The ALT50 diet included a 50% reduction in fishmeal and the addition of 40% insect meal, which resulted in improved fish survival against bacterial infection, in agreement with data from other studies in *Cirrhina mrigala*, in which mortality was 20% and 25% in infected fish fed with chitin or chitosan (Su *et al.*, 2017; Lourthu Samy Shanthi Mari *et al.*, 2014). Furthermore, in certain cases, fish are affected with different pathogens against which a favorable immune response is generated, this shows that not only a specific immune response is generated with respect to *V. anguillarum*. On the other hand, the use of SCP can also have positive effects on fish health,

including immunological and inflammatory responses and modulation of gut microbiome (Glencross *et al*., 2022). Bacterial SCP from *Methylococcus* has been proved to benefit the amelioration of soybean-induced distal enteritis in Atlantic salmon (*Salmo salar*) (Romarheim *et al*., 2013).

The possible reasons that we evaluated that explain these resistances are several, the protective effect of insects in the diet was hypothesized to be direct through the secretion of antimicrobial peptides by the insect (Hou *et al*., 2007). This occurs by increasing the population number of *Cetobacterium* in the intestinal microbiota of fish, which in turn, after multiple metabolic processes, generates multiple macromolecules, such as hyaluronic acid and chondroitin sulfate, which stimulate the primary immunity of the individual (Hou *et al*., 2007). Another possibility is the indirect way in which chitin and chitosan, as well as their derivatives, form the exoskeletons of arthropods including insects; they are substances known to activate the immune response in vertebrates. There are studies that demonstrate the benefits of chitin in this sense, such as the one suggested that the species *Cirrhina mrigala* fed with a diet enriched with 1% chitin improved the immune system and resistance against *Aphanomyces invadans* (Lourthu Samy Shanthi Mari *et al*., 2014). These chitosan derivatives incorporated into the diet have been shown to increase the total number of proteins, globulins and albumins in fish. These first ones (gamma globulin) are of vital importance in the formation of all the proteins necessary for the function of immunological activity, while albumin is essential to maintain osmotic pressure and acts as a plasma carrier and non-specific ligand (Ashish Kumar Jha *et al.,* 2007).

Finally, regarding chitin as a beneficial immune stimulant in our insect meal, its potential cytotoxic action could be highlighted. The leukocyte activity can be increased as a consequence of the incorporation of chitin and its derivatives into the diet in small amounts. This is supported by a study that the injection of this polymer induced the mobilization of phagocytes against *Vibrio anguillarum*. Similarly, the addition of low doses of housefly pupae (0.75 and 7.5%) in the diet of red sea bream (*Pagrus major*) during 10 days, significantly increased the phagocytic activity of peritoneal macrophages (Ido *et al.*, 2015). In parallel, an inclusion of 5% of housefly pupae in the diet for 2 months totally protected (100% survival) the fish against the bacterial pathogen *Edwardsiella tarda*, while all the fish in the control group died 12 days after the bacterial challenge (Esteban *et al.,* 2000; Ido *et al.*, 2015). Finally, although with a different ingredient for its formulation in the feed (*Tenebrio molitor*), a study argues that feeding early juveniles of European sea bass for 6 weeks with *Tenebrio molitor* larvae meal induced significant anti-inflammatory responses

(ceruloplasmin, myeloperoxidase and nitric oxide) (Henry *et al*., 2018). Although the bacteriolytic function of serum against Gram-negative bacteria was not affected by the Tenebrio diet, while both the antibacterial activity of lysozyme and the inhibition of trypsin in serum, generally linked to the antiparasitic activity of the fish, were increased notably (Henry *et al.*, 2018). The latter may be due to similarities in the composition of the exoskeleton of parasites and insects that can act as immunostimulants, potentially increasing antiparasitic activity.

As for the other line of our experiment, we can affirm that the inclusion of 6.8% single cell protein (*Corynebacterium glutamicum*) and at the same time a 50% reduction of FM used compared to our control diet, has provided resistance against *V. anguillarum*. This coincides with previous studies in which positive results are already estimated on SCP, such as the experimental study that adding 10% (25% FM replacement) of SCP (*Methylococcus capsulatus*) shows that sea bass had better growth compared to those not fed with these novel ingredients (Moroni *et al.*, 2024). The utilization of SCP improved the immune system of Nile tilapia by increasing Immunoglobulin M and the expression of pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin 8, interleukin 10 and interleukin 16 (Hamunjo Chama *et al.,* 2021). Vasilaki *et al.* (2023) found that the use of a blend of different SCP, including bacterial protein (Feedkind®), yeast protein (Nupro®) and algae meal (Algaprime™) in different levels of inclusion induced no inflammatory response in European sea bass juveniles and even found positive immunostimulation effects on lysozyme antibacterial activity and anti-protease activity), that could suggest a better potential of those fish to fight pathogenic infection, in agreement with the results from the present study.

Even when some studies are nowadays focusing on the use of blends of different ingredients on fish health (Aragao *et al*., 2022; Vasilakis *et al*., 2023, Carvalho *et al.*, 2023), the combination of both insect meals and SCP and their role on fish disease resistance and immunity is still poorly understood, and indirect effects through the action of these ingredients in the gut microbiome cannot be discarded (Aragao *et al.*, 2022). The results of the present study give new insights on how the combination of the new ingredients could contribute not only to fish performance but also to the improvement of fish health, and new studies must be done to understand how the blend of ingredients are modulating the fish immunity and disease resistance.

5. CONCLUSION

In conclusion, it is feasible to substitute (at least partially or even totally) the fishmeal by a blend of different ingredients with no effects on fish growth. The use of PAPs from poultry by-products supplemented with microalgae oil had no negative effects on fish growth and allows the utilization of more sustainable ingredients with lower costs than fishmeal. The use of a blend of insect meal and SCP is limited by the level of substitution, since total substitution of FM by the new ingredients induced a retardation of growth.

The use of new ingredients in aquafeeds must focus not only on fish performance but also in fish health and welfare. In this way, the use of PAPs + microalgae oils, or the partial replacement by an adequate percentage of insect meal/SCP, is improving fish survival after challenge test against *Vibrio anguillarum*, that could be related with a stimulation of the immune system in *Dicentrarchus labrax*. The present study gives new insights on how the strategy of fishmeal replacement in marine fish diets can contribute to the sustainable development (Zero Waste), the European green deal and the approaches of the OneHealth with a more sustainable blend of ingredients in aquafeeds.

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