ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

DOTTORATO DI RICERCA IN SCIENZE VETERINARIE

Ciclo XXXVI

Settore Concorsuale: 07/G1 SCIENZE E TECNOLOGIE ANIMALI

Settore Scientifico Disciplinare: AGR/20 ZOOCOLTURE

MEDITERRANEAN SPECIES AS MAIN CHARACTERS IN FEEDING STRATEGY FOR THE DEVELOPMENT OF SUSTAINABLE SYSTEMS AND RESILIENT FARMING. EFFECTS EVALUATION OF FOUR EXPERIMENTAL DIETS ADMINISTERED TO LARVAE, JUVENILES AND ADULTS OF EUROPEAN SEABASS AND TO MEAGRE, ON GROWTH PERFORMANCE, GUT HEALTH AND ANIMAL WELFARE.

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Esame finale anno 2024

ABSTRACT

Aquaculture is a growing wide industry, that until now and in the future represents a sector with enormous potential. Despite that, it is still correlated with environmental issues and ambiental impacts. For several years, both the scientific community and the industry have been working together to make this sector increasingly sustainable and resilient. This doctoral thesis deals with the study and valorisation of alternative ingredients chosen for their nutritional characteristics and potential, with the final aim of giving new inputs to aquaculture to become more sustainable. In particular, this study was focused on:

-Evaluation of green technologies applied to production of microalgae biomass. Renewable energy sources, such as geothermal, can reduce the environmental impact of aquafeed production.

- The use of by-products from fishery and aquaculture in order to replace fish meal fish oil and soybean meal with microalgae. By-products have a well-known great potential, considering both economic and environmental aspects, especially if is considered the huge amount of waste produced throughout the entire fish supply chain. Soy is considered an excellent ingredient for aquafeed as an alternative source of protein. However, it can have an impact on sustainability as competitor with human nutrition, and it's also involved in soil degradation and other ambiental issues. Microalgae, on the other hand, have a recognised role in fish nutrition due to their nutritional characteristics and the richness and high-quality fatty acids profile. Are, in fact, a recognized source of Omega-3 in aquafeed, for every single life stage of finfish and several species.

-Evaluate the effect of experimental diets on different life stages of a commercial species widely distributed in the Mediterranean Sea, European Seabass.

- Among two Mediterranean species considered, Meagre is an emerging species, considered a valid substitute for the most widespread commercial species such as Gilthead Seabream and European Seabass. Emerging species are a very powerful tool to reduce pressure on those species already heavily bred.

- Organic diets were tested on an emerging species, relating both the sustainability of organic diets and the sustainability of emerging species.

SUMMARY

1. Sustainable Aquaculture

Environmental concerns are already recognised by the aquaculture industry, which has made great progress in recent years and continues to improve in that sense. Aquaculture is a diverse industry, and environmental impacts vary with species, farming methods, management techniques, location, and local environmental conditions and wildlife (European Commission et al., 2015).

Many of challenges facing aquaculture regarding their impact on wildlife, can be mitigated with a proactive approach. In order to create sustainable aquaculture, it is crucial to balance the development of aquaculture with the conservation of natural resources (Frankic and Hershner, 2003).

“Sustainable development is the management and conservation of the natural resource base and the orientation of technological and institutional change in such a manner as to ensure the attainment and continued satisfaction of human needs for present and future generations. Such sustainable development (in the agriculture, forestry and fisheries sectors) conserves land, water, plant and animal genetic resources, is environmentally non-degrading, technically appropriate, economically viable and socially acceptable”. This is the definition elaborated by FAO in 1995 which demonstrates how complex is this topic and why has been debated for over 30 years. Sustainability could be considered as the result of the interaction of various forces from different fields (Figure 1).

Figure 1 The dimension of Sustainability (Fezzardi et al., 2013).

In fact, there are a lot of topics and factors to consider in sustainable aquaculture. On one hand, this can be overwhelming and make it difficult to find a solution. On the other hand, it also provides a lot of opportunities to improve different aspects of aquaculture by making them work together.

As the result of interactions of many forces, sustainability can be applied to the aquaculture sector in all aspects. Follow, are major voices:

1. Water: mainly distinct in marine and freshwater aquaculture (marine and brackish waters are interchangeable, where the sea meets freshwaters). Can also distinguished by other two categories: closed and open systems. In a closed system, such as a tank or enclosed pond, water is contained, controlled and recirculated. In an open system, such as sea cages, water from the natural environment can't be contained and ambiental parameters can't be settled or fixed.
2. Farming system type: intensive, semi-intensive, extensive. Intensive aquaculture managers provide all the feed for cultured species, while in extensive aquaculture, feed comes from the natural environment. In semi-intensive systems, managers supply natural sources of feed (European Commission et al., 2015).
3. Species: can be classified as 'finfish' (salmon, carp, Seabass), shellfish (which includes bivalves, mussels and crustaceans) or plants (such as seaweed or watercress).

Working on the sustainability of one or all of these voices must be the aim of both research and industry fields if the aquaculture sector wants to continue to grow and improve customer acceptance.

1.1 Organic aquaculture

Organic aquaculture is a farming method that is based on ecosystem management, intending to achieve environmental sustainability. The use of synthetic chemicals is prohibited in order to minimize environmental impacts. Organic fish production is an alternative approach to farming that is becoming increasingly popular due to the growing interest in sustainable resource management. Aquaculture under organic management, aims to produce fish that are socially responsible, economically viable, and ecologically sustainable (Ahmed et al., 2020). Organic aquaculture has recently been studied by both researchers and industry. Research priorities in fish farming include organic feeds, fish nutrition, food safety, environmental concerns, and trade issues. When it comes to organically cultured fish, the quality of flesh differs from conventional systems due to differences in feeds and nutrition. This difference is a significant factor that affects consumer choice (Mente et al., 2011). At the same time, Organic agriculture in Europe benefits from financial support and premium prices, but labour is expensive. A comprehensive analysis of European farm economics, which considers labour usage, yields, prices, costs, and support payments, reveals that the profits on organic farms are similar to those on conventional farms on average (FAO, 2002). From an economic point of view, in many conventional

aquaculture operations, feed accounts for over 50% of the variable operating cost. Organic operations assume a 50% surcharge for organic certified feeds, although lowering feed conversion ratios can compensate for their costs (Mente et al., 2011). The European Union's initial legislative framework for organic aquaculture was the Directive (EEC) 2092/91. This has now been replaced by Directives (EC) 834/07 and (EC) 889/08, introduced in 2007 and 2008 respectively. The maximum level of plant products inclusion in organic aquaculture is regulated by the EC Regulation 710/2009.

It's therefore necessary to find a good compromise between economic and environmental aspects.

1.2 RAS-Recirculated Aquaculture System

Among all intensive breeding systems, the recirculated aquaculture system (RAS) shown in Figure 2 is recognized as the most sustainable farming system (Fudge et al., 2023). Common systems, as sea cages and land farms, are spread worldwide because of their minor initial costs and easy management. On the other hand, are often correlated with high pollution rates and the risk of escape and foul-up of the wild genetic pool is an important issue debated. On the contrary, RAS has higher initial and management costs, but environmental impact is valuable reduced. In RAS, the initial amount of water introduced into the cycle is never completely exchanged and the quality of water is guaranteed by filters and mechanical methods. Subsequent additions of water are mainly due to special maintenance reasons such as evaporation, leaks or once-off manutention service. Recirculation aquaculture is essentially a technology based on the use of mechanical and biological filters which allow farming aquatic organisms by reusing water in the cycle and avoiding water waste (Bregnballe et al., 2015).

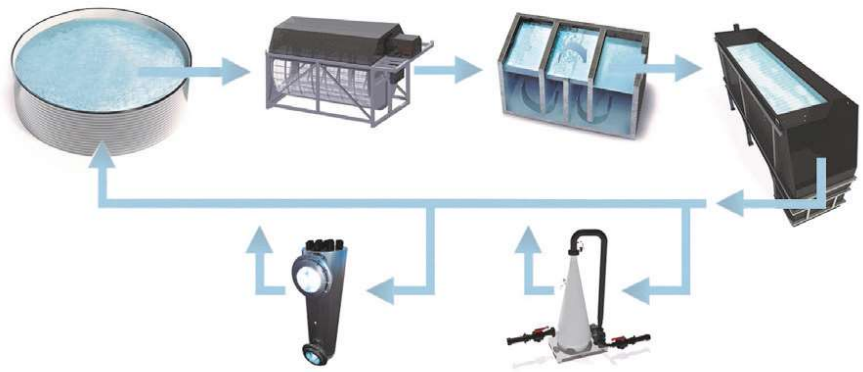

Figure 2 Simplified scheme of RAS (Recirculated Aquaculture System) (Bregnballe et al., 2015)

1.3 Alternative ingredients

The growth of farmed fish and crustaceans has been aided by the availability of feed inputs in major aquaculture producing countries over the past two decades. Recent statistical data from the FAO indicates that 55.2% of the total global aquaculture production in 2017 (FAO, 2019), rely on external nutrient inputs. These inputs are usually in the form of commercially manufactured feeds, with only a small portion coming from fresh feed inputs.

Commercial aquaculture feeds consist of a combination of vegetable and animal feed ingredients, mixed balancing micro and macronutrients, depending on the target species. Fish meal and fish oil have traditionally been used as primary ingredients in aquafeeds. However, global production of these products is not enough to support the growth of aquaculture at current feed formulation usage levels.

The availability of feed ingredients still relies on conventional sources such as plant oilseed meals, protein concentrates and oils, as well as captured and aquaculture fishery by-products meals and oils, terrestrial animal by-product meals and fats, cereal by-product meals, protein concentrates and oils. Furthermore, the feed industry has made substantial strides toward using lower-cost, locally sourced feed ingredients that typically have lower nutrient content and digestibility, and these include microbiologically produced single-cell proteins (SCP) like algal, bacterial, and yeast SCP (Boyd et al., 2020).

Nowadays, there is a growing recognition of the importance of using fishery and aquaculture by-products to improve economic and environmental efficiency, as well as food security. Finding innovative ways to utilize by-products has become a key factor in remaining competitive and profitable in the aquaculture industry, as well as in other food production sectors. Due to slim processing margins, it is essential to explore and fully utilize by-products to their maximum potential (Ytrestøyl et al., 2015). By-product can be defined as all the raw material, left over by food processing. For finfish, by-products typically include trimmings, skins, heads, frames (bones with attached flesh), viscera (guts) and blood (Stevens et al., 2018).

Microalgae are aquatic small photosynthetic organisms known as primary producers in aquatic ecosystems. In aquaculture, microalgae have multiple roles, from improving the quality of the water to serving as a food source. Microalgae play a crucial role as a direct or indirect source of food for wild fish, which are essential for fishmeal and fish oil production. In larval aquatic animals, microalgae are mainly used as live prey due to their appropriate size and nutritional value. They also serve as important food sources and nutrient supplements

for other live prey such as rotifers, *Artemia*, and copepods (Chen et al., 2021). Microalgae are an important source of food for farmed aquatic animals. They can also be used as a potential source of protein, lipids, and additives for aquafeeds. Furthermore, microalgae in fish culture systems are capable of reproducing by utilizing excess nutrients and converting CO2 into O2, which is essential for aquatic animal life (Ma and Hu, 2023).

2. Mediterranean species under consideration

2.1 European Seabass as commercial species

European Seabass is the common name of *Dicentrarchus labrax* a marine bonefish belonging to the Serranidae family (Figure 3). In the wild, its geographical distribution covers all Mediterranean costs, also reaching the North Sea and Baltic Sea. Its incredible spread is mainly due to euroalinic and eurothermical characteristics. It is a voracious predator and its diet is composed of small shoaling fish and a wide range of invertebrates including shrimps, prawns, crabs, squids and molluscs. The average size of the Seabass is about 50 cm, with a maximum value of 100 cm (FAO, 2005).

Figure 3 European Seabass adult

Consumers largely appreciate Seabass for its lean but tasty meat. *Dicentrarchus labrax* was the first marine non-salmonid species to be commercially and nowadays is the most important commercial fish widely farmed in Mediterranean areas from Greece to Turkey, Italy, Spain, Croatia and Egypt. In European aquaculture, Seabass is bred mainly intensively, with a strong differentiation for each vital phase of the animal, from larvae to adult (FAO, 2010).

The reproduction of Seabass is controlled from start to hatcheries, using brood stock selected in fish farms. The eggs fertilised by males, are collected on the surface of the tank and placed in an incubator, where they

hatch. The larvae are transferred to rearing tanks. Once the yolk sac is absorbed, larvae have a very specific diet: at first time enriched with micro-algae and zooplankton, and then, as they grow, with small crustaceans, typically *Artemia salina*. After one or two months, the larvae are fed with an artificial diet, obtaining the fry, which are transferred to the juvenile unit, where they feed by pellets. After two months, they can be moved to the grow-out farm. In most cases, fish are farmed in floating cages but animals are also raised in land-based tanks, generally using a recirculation system. Farmed Seabass are generally harvested when they 18 weigh 300 g to 500 g, which takes from a year and a half to two years, depending on water temperature (European Commission, 2012).

2.2 An emerging species: Meagre

Argyrosomus regius (Asso, 1801), commonly named Meagre (Figure 4), is a carnivorous species belonging to Sciaenidae. It's widespread all over the Mediterranean Sea and along the cost of West Africa. They present an anadromous migration behaviour and usually live in warm temperate climate environments (FAO, 2005). During their reproductive migration, adult meagre approach the coastline in mid-April and then move into estuaries at the end of May to spawn.

Figure 4 An adult of Meagre

This finfish can reach a huge size, into the wild (up to 2 m and reach >50 kg) and in rearing condition, and for this reason and fast growth capacity, is now widely evaluated. Its history in aquaculture is recent, and the first commercial product was documented in France in 1997 (FAO, 2005). Meagres are mainly farmed in France and Italy with similar farming techniques used with other commercial species as European Seabass or Gilthead Seabream. Aquafeed is supplied by all major producers. The feed is similar to that used for other mediterranean species and contains 45-48% protein and up to 20-24% lipid. In land-based farms, meagre is fed 2-3 times per day, while in sea cages they are typically fed once daily. The feed conversion rate (FCR) is about 1.7:1 but some trials have shown even better FCRs, especially in large sea cages with a stocking density below 50 m^3

(FAO, 2005). Due to its fast growth and excellent fillet quality (Costa et al., 2013) Meagre is spreading among Mediterranean farmers with excellent results. It is therefore considered an emerging species, which can fit into the European fishing sector.

3. Aim

The purpose of this study was to provide technologically advanced applications research on aquafeed and fish nutrition to make European aquaculture more productive, sustainable and resilient. Innovative aquafeeds were formulated, tested and validated, each targeting specific issues and under the principles of climate change mitigation, circular economy and sustainability.

The sustainability spectrum was extended to the production process of the ingredients, to different life phases and to different commercial species, trying to test more sustainable aquafeed. Replacing wild Fish Meal and Fish Oil with by-products, as well as organic ingredients are just some of sustainable practices that can be applied to fish nutrition, but which can give a new face to aquaculture. During trials was possible to formulate an enriched feed produced from sustainable energy sources tested on Seabass larvae. Was also possible to find new protein sources in by-product from fishing and aquaculture. The success of the organic sector in agriculture led the way to organic aquaculture as sustainable alternative, to be tested on new emerging species that may support commercial species.

Chapter 4.

A blend of microalgae products for the enrichments of *Artemia salina*: effects on growth performance, antioxidant status and anomalies rate of European Seabass (*Dicentrarchus labrax*) larva.

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Published on: Aquaculture Reports, (2024)

DOI: https://doi.org/10.1016/j.aqrep.2024.102070

4.1 Abstract

The recent fast development of the microalgae-related biotechnologies has enabled the availability of sustainable and nutrients-rich raw materials to be used as substitution of conventional sources in aquafeed formulation. However the cost of energy of the photobioreactors used to produce these microorganisms still constrained their wider inclusion into aquafeed compounds. By exploiting industrial waste outputs, *Nannochloropsis* sp. and *Spirulina* sp. were produced and utilised to formulate two experimental enrichments (LM1 and LM2). During a 60 days trial, their efficacy have been tested as *Artemia* metanauplii enrichments in the diet of European seabass (*Dicentrarchus labrax*) larvae, in comparison to a commercial control. Larvae were fed with isonitrogenous and isolipidic dietary treatments. Each dietary treatment presented a specific profile for the long chain polyunsaturated fatty acids ARA, DHA and EPA. Ratio of DHA/EPA in enriched *Artemia* of 4:1 as in the case of LM1 and LM2 in the presence of 1.4% of ARA (as a % on total fatty acid) guaranteed equal results to the control which displayed a ratio of DHA/EPA of 2:1 with 2.2% of ARA. No significant effects of the dietary treatments were detected in final survival, growth performance and incidence of skeletal anomalies. Regarding oxidative status, larvae fed with LM2 enrichment presented lower catalase activity than control larvae with no signs of oxidative damage, suggesting a potential antioxidant effect of LM2. The present study contributes to expanding the existing literature on successful utilisation of microalgae and cyanobacteria, used to produce valuable nutrients, in a perspective of circular nutrients economy.

Keywords

Microalgae, Cyanobacteria, Photobioreactors, Live feed enrichment, European seabass larvae, Skeletal anomalies.

4.2 Introduction

Aquaculture production of marine fish, such as European seabass (*Dicentrarchus labrax*), relies on the availability of a large number of healthy and fully weaned juveniles for the on-growing phase. The standard hatchery protocol involves feeding newly hatched larvae with live prey for a period before weaning them onto formulated dry feed completely. This initial reliance on live prey is necessary to provide essential nutrients and properly sized food for the small mouths of the altricial marine fish larvae. *Artemia* metanauplii (*Artemia salina*) are extensively used as live prey due to being cost-effective, easy-to-handle and polyvalent (Sorgeloos et al., 2001), despite their natural deficiency in long chain polyunsaturated fatty acids (LC-PUFAs) (Monroig et al., 2006; Ramos-Llorens et al., 2023). To overcome this bottleneck, enrichments are customarily performed to load up *Artemia* metanauplii with the adequate nutritional value for fish larvae (Boglino et al., 2012).

In fact, marine fish have a limited capacity to synthesise LC-PUFAs, such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA) (Izquierdo, 2005), although these molecules are important for the synthesis of new cellular structures (Gisbert et al., 2005) and for the primary role on larval survival, growth and immune system modulation (Tocher et al., 2019; Roo et al., 2019; Betancor et al., 2021; Pham et al., 2023). On the other hand, due to their highly peroxidable nature, it has been suggested that an excess of dietary LC-PUFAs, without a proper balance of antioxidant nutrients, can lead to controversial effects on fish oxidative stress (Izquierdo et al., 2013; Betancor et al., 2015; Wischhusen et al., 2022; Pham et al., 2023). Oxidative stress has been linked to morphogenic and skeletogenic alterations during early development stages, which may result in various degrees of skeletal anomalies in the larval development of hatchery-produced fish (Conceição et al., 2007; Lall and Lewis-McCrea, 2007; Izquierdo et al., 2013). Standard LC-PUFAs rich ingredients used by aquaculture industry are derived either from wild marine fish, in the form of fish oil, or from a number of microorganisms including microalgae and cyanobacteria, which are the primary producers of EPA and DHA (Mutale-Joan et al., 2023). On one hand, the utilisation of marine fish oil is posing several environmental and economic concerns as this contributes to the over-exploitation of pelagic fish stocks (Egerton et al., 2020; Glencross et al., 2020) along with presenting very high and unstable prices, due to the competition with human nutrition and other livestock production. In addition, this finite commodity is susceptible to seasonal fluctuation in the fatty acids profile (Gámez-Meza et al., 1999; Zlatanos and Laskaridis, 2007) and to contamination with dioxin-like polychlorinated biphenyls (PCBs), persistent organic pollutants

(POPs) and heavy metals, which bioaccumulate in animals at a higher trophic level (Sun et al., 2018; Santigosa et al., 2021; Zatti et al., 2023) and may interfere with normal larval physiological functions (Horri et al., 2018; Bogevik et al., 2023). On the other hand, the exploitation of microalgae and cyanobacteria can address some of the concerns related to fish oil, despite their production is often constrained by the high costs associated with cultivation. These microorganisms can be produced by farming on non-arable land, reduce water demands and convert CO2 and other waste products into valuable ingredients (Shah et al., 2018; Yarnold et al., 2019; Chen et al., 2021; Tocher et al., 2019; Conde et al., 2021). They are oxygenic photoautotrophs capable of producing LC-PUFAs together with a diverse array of unique and potent substances (Ma et al., 2020; Nagappan et al., 2021) such as some antioxidant molecules produced by Spirulina *spp* (Fernandes et al., 2023). Their incorporation into hatchery feeding regimens has been found to exhibit probiotic and antibacterial properties (Vadstein et al., 2018; Ognistaia et al., 2022), resulting in a beneficial effect on larval gut microbiota (Huang et al., 2023), positively influencing the production key performance indicators (Boglino et al., 2012; Paulo et al., 2020; Gui et al., 2022; Pan et al., 2022). From a production perspective, industrial-scale cultivation of microorganisms can be achieved through outdoor systems, utilising ponds or tanks, or by employing controlled environments such as photo bioreactors (Mutale-Joan et al., 2023). While outdoor systems can partially reduce energy costs by using sunlight as an energy input, seasonal environmental variations may affect the composition and quality of microorganism cells, potentially resulting in lower yields (Xu et al., 2020). On the other hand, photobioreactors, offering precise control over environmental conditions, require substantial energy inputs for lighting, temperature regulation, agitation, and nutrient supplementation. In the context of the circular economy, the reutilisation of industrial by-products as a source of energy in more advanced transformation processes is becoming increasingly important. In this context, European Union is funding several projects aimed at bridging the gap between research and industrial application. VAXA (VAXA Technologies Ltd.; Reykjavík, Iceland) has developed a new technology platform perfecting the cultivation of LC-PUFAs rich microorganisms by exploiting geothermal energy produced by a geothermal plant in Iceland. This allows to transform the waste outputs of a geothermal plant, hot and cold water and natural CO2 emissions, to produce a number of auto trophic microorganisms such as *Nannochloropsis* sp. and *Spirulina* sp. This system results in a year-round microorganisms production, resilient to the environmental variation, free from chemical contamination and with 80% reduction in production cost due to geothermal energy utilisation.

The aim of this study was to examine the effects of two products at development stage (LM1 and LM2) contained different proportions of *Nannochloropsis* sp. and *Spirulina* sp. produced with this more sustainable technology compared to a commercial lipid emulsion control (C), as *Artemia* metanauplii enrichments in the diet of European sea bass larvae. Besides their effects on larval survival, growth performance and fatty acid profile, the antioxidant status and the incidence of skeletal anomalies were also evaluated.

4.3 Materials and Methods

4.3.1 Larval origin and rearing methods

European Seabass larvae at 7 days post-hatching (dph) coming from one spawning batch were obtained from a commercial marine hatchery (Ca Zuliani Soc Agricola Srl., Italy). Larvae were transferred to the Laboratory of Aquaculture at the University of Bologna (Cesenatico, Italy) into a 50 L liquid storage bag filled with 1/3 seawater and 2/3 compressed oxygen, placed inside a polystyrene box for thermal isolation. Once at the laboratory, larvae were randomly allocated into nine 50 L up-welling tanks (2200 larvae tank-1) by using volumetric allocation (Parma et al., 2013). Tanks were supplied with seawater and connected to a closed recirculation system consisted of a mechanical sand filter (PTK 1200; Astralpool, Barcelona, Spain), ultraviolet lights (UV PE 45; Sita Srl, Barcelona, Spain) and a biofilter (PTK 1200; Astralpool, Barcelona, Spain). Water exchange rate in the tanks was initially of 0.5 L min-1, increased to 2.5 L min-1 following larval development. Photoperiod was set at 12 h light/12 h darkness regime (with light period from 8 am to 8 pm), through artificial light at < 50 lux at the water surface. Temperature was maintained at 18 ± 2 °C. Dissolved oxygen in the tanks was maintained at > 9 mg/L through an automatic liquid oxygen system connected to a software controller (B&G Sinergia snc, Chioggia, Italy). Sodium bicarbonate was added to keep the pH constant at 7.8–8.0 and avoid alkalinity fluctuation. Salinity ranged between 25 and 30 g L-1.

4.3.2 Experimental feeding regimes and live prey enrichments

Three different enrichment products were used to enrich *Artemia* metanauplii. Two experimental products, LM1 and LM2, consisting of microalgae blends with different proportions of *Nannochloropsis* sp. and *Spirulina* sp. produced by using geothermal resources (VAXA Farm, Reykjavík, Iceland) and a commercial control C (Red Pepper©, Bernaqua, Olen, Belgium). Both LM1 and LM2 were produced without addition of fish oil, LM2 contained a higher proportion of Spirulina compare to LM1. The feeding protocol was set, with

some modifications, according to those used in commercial hatcheries for European Seabass and based on previous published works (Villamizar et al., 2009; El-Dahhar et al., 2022). Wild caught *Artemia* cysts of EG strain coming from Great Salt lake in USA (Catvis B.V., 's-Hertogenbosch, The Netherlands) were incubated and hatched in seawater (salinity 30 g L-1) at 28 °C for 18 hours. After this, *Artemia* metanauplii were harvested and enriched for 12 hours at 26 °C by transferring metanauplii at a concentration of 300 individuals mL -1 to new container filled with seawater and 750 mg L-1 of the enriching products. Triplicate groups of larvae were fed with non-enriched *Artemia* nauplii from 7 to 26 dph (5 nauplii mL-1). Enriched *Artemia* metanauplii were then incorporated from 27 dph to 60 dph (7.5 metanauplii mL-1 from 27 to 50 dph and 10 metanauplii mL-1 from 51 to 60 dph). The feeding protocol with *Artemia* metanauplii without including formulated dry feed was maintained until advanced larvae development to avoid any possible bias on the outcomes of this research. The proximate composition and the fatty acids profile of the enriched *Artemia* metanauplii are shown in Table 1.

4.3.3 Samplings and growth calculations

Before each sampling procedure, larvae were euthanised with 300 mg L 1 of MS222. At 7, 19, 26, 32, 39, 46 and 54 dph, 20 larvae tank 1 were randomly collected in order to determine wet weight (WW) and dry weight (DW). At the end of the trial (60 dph), 100 larvae tank 1 were collected to determine WW and DW. WW was measured after rinsing larvae with de-ionised water on a 400μm sieve and removing the excess of moisture away from behind the sieve using lint-free paper towel (Bonaldo et al., 2011). DW was determined by oven-drying the larvae at 70 ◦C for 12 hours. At the end of the trial, all larvae were removed from each tank and counted to determine survival rate. Larvae were then rinsed in distilled water, and frozen (80 ◦C) until biochemical analyses or fixed in 10% formalin buffered with phosphate buffer saline (PBS, pH 7.2) for 48 h at 4 ◦C for skeletal anomalies analysis. Fixed samples were then washed in PBS (pH 7.5) for 48 h at 4 ◦C and then stored in 70% ethanol until further processing. Growth rate was calculated as specific growth rate (SGR, % day 1) according to the equation: SGR = 100 * (ln FBW - ln IBW)/days (where FBW and IBW represent the final and the initial body weights, respectively). Survival rate was calculated per tank as a percentage of the initial number of larvae. All experimental procedures were evaluated and approved by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna, in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes.

4.3.4 Lipid and fatty acids profiles

Lipids in both *Artemia* nauplii and metanaupli and European Seabass larvae were extracted according to (Bligh and Dyer, 1959) by using methanol and chloroform as solvents. Fatty acids were subjected to a methylated ester method (FAMEs) following the procedures as described in (Lepage and Roy, 1986). Briefly, methylated-FAs (FAMEs) were extracted into toluene. The FAMEs were analysed by gas chromatography (GC/FID, Clarus 500, Perkin-Elmer) using an SP-2330 fused silica capillary column (30 m × 0.25 mm i.d., 0.20 μm; Supelco Inc., Bellefonte, USA).

4.3.5 Evaluation of the antioxidant status

The antioxidant status of the larvae was assessed by measuring catalase activity (CAT), protein carbonylation (PC), lipid peroxidation (LPO) and mitochondrial reactive oxygen species production (mtROS). For the analyses of CAT, PC and LPO, three pools of 3 larvae per replicate tank (n = 9 pools per treatment) were sampled at the end of the growth trial (60 dph) and snap-frozen in liquid nitrogen. Samples were homogenised in 500 µl ultra-pure water using an Ultra-Turrax® Homogeniser (IKA®-Werke, Germany). One aliquot containing 4 % butylated hydroxytoluene (BHT) in methanol was used for the determination of LPO. The remaining homogenate was diluted (1:1) in 0.2 M K-phosphate buffer, pH 7.4, and centrifuged for 10 min at 10,000 g (4 ºC). The post-mitochondrial supernatant (PMS) was kept in –80 ºC for the analysis of catalase activity (CAT) and protein carbonylation (PC). For mtROS determination 3 larvae per replicate tank (n = 9 pools per treatment) were sampled and snap-frozen in liquid nitrogen. Samples were homogenised in 200 µl ice-cold mitochondria isolation buffer (225 mM mannitol, 75 mM sucrose, 1 mM EGTA and 4 mM HEPES, pH 7.2). Then, the homogenate was centrifuged for 10 min at 600 g and 4 °C. The supernatant was picked off and centrifuged again for 10 min, at 11,000 g and 4 ºC. The pellet was resuspended in buffer containing 250 mM sucrose and 5 mM HEPES (pH 7.2). Catalase activity (CAT) was determined by measuring decomposition of the substrate H2O2 at 240 nm (Clairborne, 1985). Protein carbonylation (PC) was measured by the reaction of 2,4-dinitrophenylhydrazine (DNPH) with carbonyl groups, according to the DNPH alkaline method (Mesquita et al., 2014). Endogenous lipid peroxidation (LPO) was determined by measuring thiobarbituric acid-reactive substances (TBARS) (Bird and Draper, 1984). Mitochondrial reactive oxygen species (mtROS) production was assessed by the dihydrodichloro-fluorescein diacetate method, H (2) DCF-DA (van der Toorn et al., 2009). The protein content of PMS (CAT, LPO and PC determinations) and mtROS samples was

determined according to the Bradford method (Bradford, 1976) using bovine serum albumin as standard. All biomarkers were determined in 96 well flat bottom microplates using a temperature-controlled microplate reader (Synergy H1, BioTek Instrument, Inc., USA).

4.3.6 Evaluation of severe skeletal anomalies

A total of 859 European Seabass larvae at 60 dph were stained with alcian blue and Alizarin red according to Pousis et al. (2022). Briefly, the specimens were immersed in a solution of 0.5% KOH and 3% H2O2 and subjected to prolonged exposure (5-6 hours) to sunlight. Then all the samples were stained in 200 ml cartilage staining solution (120 ml 95% ethanol, 80 ml glacial acetic acid, 50 mg Alcian blue) and in 200 ml of bone staining solution (60 mg Alizarin red in 200 ml KOH) in the dark for 90 min and 2 hours respectively. After each staining step the samples were washed three times in 0.5% KOH for 5 minutes. Finally, the samples were subjected to the process of diaphanization by consecutive immersion in containing 0.5% KOH and increasing glycerol concentrations (0.5% KOH: glycerol 3:1; 0.5% KOH: glycerolm1:1; 0.5% KOH: glycerol 1:3; 100% glycerol). The duration of the first two steps was about 48 hours while the while the third step was repeated several times until the 0.5% KOH: glycerol 1:3 solution remained colourless after 24 h of immersion.

Double-stained specimens were observed twice by the same operator, under a stereomicroscope (Leica WILD M3C, Wetzlar, DE), in order to reduce possible errors during the analysis of the samples and all the malformed fish were photographed by a digital camera (DFC 420; Leica Microsystems, Cambridge, UK). For each group, severe skeletal anomalies were recorded according to Prestinicola et al. (2013), and the following variables were calculated: 1) relative frequency (%) of individuals with at least one severe anomaly. 2) severe anomalies load (number of severe anomalies/number of individuals with severe anomalies). 3) relative frequencies (%) of severe anomalies in the in the four regions of the vertebral column and in the skull.

4.3.7 Statistical analysis

Significant differences in growth performance, fatty acid profile, antioxidant status and frequencies of severe skeletal anomalies between the groups were determined using one-way ANOVA and, whenever significant differences were identified, means were compared by the Post hoc multiple comparisons Tukey's test ($P < 0.05$). Prior to ANOVA, skeletal malformation data were arcsine transform as appropriate for proportion (Sokal and Rohlf, 1969). The assumptions related to data normal distribution and homogeneity of variances were explored through Anderson Darling's test and Levene 's test, respectively. In the case data presented a non-

normal distribution, significant differences between the groups were determined using Kruskal-Wallis non-parametric test. Analyses were performed using Minitab software (Minitab 19.0.1; Pennsylvania State University, USA). Results are presented as mean ± standard deviation (SD), unless otherwise mentioned.

4.4 Results

4.4.1 Growth performance

Wet weight (WW) and dry weight (DW) measured during the trial of European Seabass larvae fed *Artemia* metanauplii enriched with the different products are shown in Figure 5. At the end of the trial no significant differences were detected in specific growth rate (SGR) (7.95 ± 0.31, 7.92 ± 0.14 and 8.33 ± 0.56, respectively in LM1, LM2 and C) and survival rate (65.0 ± 6.9, 68.5 ± 7.1 and 61.5 ± 1.3, respectively in LM1, LM2 and C), among the different groups.

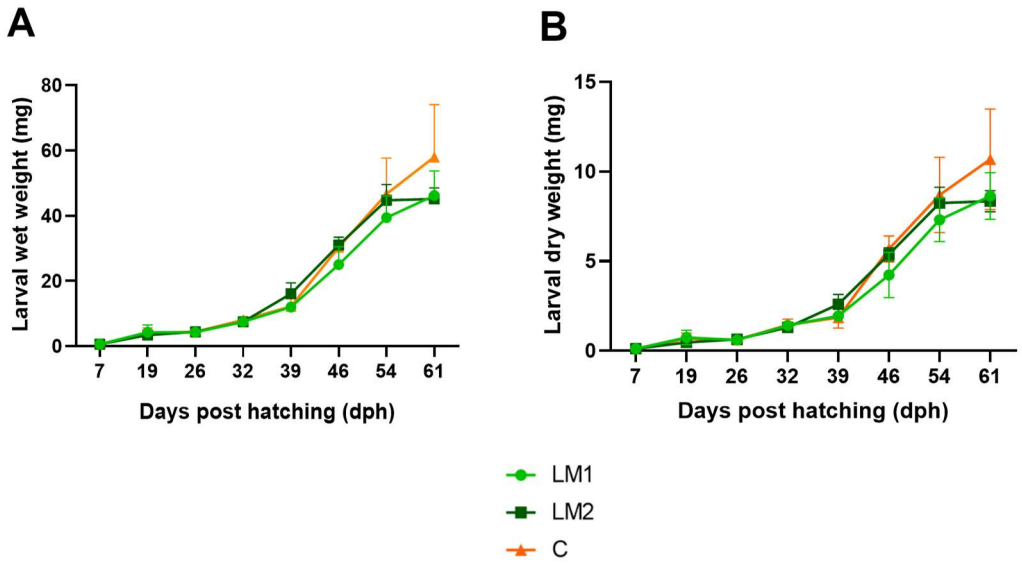

Figure 5 Larval wet weight (A) and dry weight (B) of European Seabass fed *Artemia* metanauplii enriched with the experimental and control enrichments from 7 to 60 dph.

4.4.2 Fatty acid profile in *Artemia* metanauplii and larvae

Proximate compositions and fatty acid profiles of *Artemia* metanauplii enriched with the different products are shown in Table 1. No significant differences were observed in the proximate composition of *Artemia* metanauplii (total protein, total lipids, total fatty acids and ash) among treatments ($P > 0.05$). For what concern the differences related to LC-PUFAs, significant differences were observed in the proportions of arachidonic (ARA 20:4 n-6) acid, which was found significantly higher in C compared to LM1 and LM2. Eicosapentaenoic acid (EPA 20:5 n-3) was found significantly higher in C compared to LM1 and LM2 and in LM2 compared to LM1. On the contrary, docosahexaenoic acid (DHA 22:6 n-3) was found significantly higher in LM2 compared to LM1 and C and in LM1 compared to C. According to these, the ratio n-3/n-6 PUFA was significantly higher in Artemia metanauplii enriched with LM1 and LM2, the amount of EPA + DHA was significantly higher in LM2, and the ratio EPA/DHA was significantly higher in the control Artemia metanauplii. Fatty acid profiles of European seabass larvae fed Artemia enriched with the different products are shown in Table 2. At the end of the trial (60 dph), no significant differences were detected in the total quantity of saturated, monounsaturated and total PUFA (n-3 + n-6) fatty acids among larvae from the different treatments groups. With regards to the differences related to LC-PUFAs, these follow the same pattern as the Artemia metanauplii treatments. Larvae fed Artemia metanauplii enriched with C presented significantly higher arachidonic (ARA 20:4 n6) end eicosapentaenoic (EPA 20:5 n-3 compared to LM1 and LM2. Docosahexaenoic acid (DHA 22:6 n-3) was found significantly higher in LM1 and LM2 compared to C. Concordantly, the ratio n-3/n-6PUFA was significantly higher in larvae fed Artemia metanauplii enriched with LM1 and LM2, the amount of EPA+DHA was significantly higher in LM2 compared to LM1 and C and in LM1 compared to C and the ratio EPA/ DHA was significantly lower in LM2 compared to LM1 and C and in LM1 compared to C.

Table 1 Proximate analysis (% of dry weight) and fatty acid composition (% of total fatty acids) of the non-enriched *Artemia* nauplii and enriched *Artemia* metanauplii with the experimental and control enriching products.

	Non-enriched	LM1	LM2	C	*P-value*
Total proteins	55.95 ± 0.47	55.18 ± 0.95	56.36 ± 0.62	55.71 ± 1.20	ns
Total lipids	20.07 ± 0.09	23.07 ± 0.32	23.19 ± 0.76	23.90 ± 0.41	ns
Total fatty acids	16.16 ± 0.53	18.24 ± 0.71	18.42 ± 0.25	18.94 ± 0.47	ns
Ash	8.54 ± 0.04	11.66 ± 1.38	10.33 ± 0.21	10.19 ± 0.06	ns
11:0	2.25 ± 0.00	2.29 ± 0.22	2.18 ± 0.08	2.03 ± 0.25	ns
14:0	0.81 ± 0.01	5.60 ± 0.18^{b}	5.68 ± 0.09^{b}	1.44 ± 0.08^{a}	0.000
15:0	0.22 ± 0.05	0.17 ± 0.02^{a}	0.18 ± 0.03^{a}	0.21 ± 0.01^{b}	0.001
16:0	12.04 ± 0.04	12.06 ± 0.11^{a}	12.07 ± 0.1^{a}	13.15 ± 0.12^{b}	0.000
17:0	0.83 ± 0.01	0.67 ± 0.05	0.68 ± 0.00	0.68 ± 0.02	ns
18:0	4.96 ± 0.00	4.46 ± 0.05	4.48 ± 0.03	4.43 ± 0.07	ns
20:0	0.25 ± 0.03	0.28 ± 0.02	0.25 ± 0.08	0.22 ± 0.06	ns
22:0	0.27 ± 0.04	0.40 ± 0.02^{a}	0.37 ± 0.04^{a}	0.54 ± 0.02^{b}	0.000
Σ saturated	21.63 ± 0.23	25.93 ± 0.28^{a}	25.89 ± 0.11^{a}	22.70 ± 0.41^{b}	0.000
14:1 n-5	0.58 ± 0.03	0.41 ± 0.04	0.40 ± 0.03	0.38 ± 0.01	ns
16:1 n-7	2.66 ± 0.00	2.08 ± 0.08^{a}	1.98 ± 0.02^{b}	2.16 ± 0.03^{a}	0.002
16:1 n-9	0.59 ± 0.00	0.46 ± 0.03	0.45 ± 0.01	0.47 ± 0.02	ns
18:1 n-7	7.46 ± 0.24	6.04 ± 0.21	5.94 ± 0.17	6.13 ± 0.11	ns
18:1 n-9	18.9 ± 0.05	15.31 ± 0.16	15.19 ± 0.03	15.35 ± 0.2	ns
20:1 n-9	0.59 ± 0.03	0.53 ± 0.06	0.55 ± 0.07	0.59 ± 0.06	ns
22:1 n-9	0.12 ± 0.01	0.16 ± 0.05	0.16 ± 0.04	0.19 ± 0.09	ns
24:1 n-9	0	0	0	0	ns
Σ monounsaturated	30.9 ± 0.31	24.99 ± 0.39	24.67 ± 0.25	25.27 ± 0.20	ns
16:2 n-6	0.28 ± 0.02	0.09 ± 0.05	0.07 ± 0.02	0.04 ± 0.01	ns
18:2 n-6 (LOA)	6.70 ± 0.04	5.10 ± 0.09^{a}	5.09 ± 0.06^{a}	5.75 ± 0.08^{b}	0.000
18:3 n-6	0.32 ± 0.01	0.29 ± 0.02	0.34 ± 0.05	0.30 ± 0.02	ns
20:2 n-6	0.26 ± 0.01	0.24 ± 0.03	0.24 ± 0.01	0.25 ± 0.02	ns
20:3 n-6 (DGLA)	0.07 ± 0.01	0.11 ± 0.05^{a}	0.10 ± 0.01^{a}	0.17 ± 0.02^{b}	0.002
20:4 n-6 (ARA)	1.35 ± 0.01	1.43 ± 0.03^{a}	1.42 ± 0.04^{a}	2.21 ± 0.05^{b}	0.000
22:4 n-6	0.07 ± 0.04	0.09 ± 0.03	0.07 ± 0.03	0.08 ± 0.01	ns
22:5 n-6 (DPA n-6)	0	1.98 ± 0.10^{a}	2.12 ± 0.06^{a}	3.22 ± 0.08^{b}	0.000
Σ n-6 PUFA	9.05 ± 0.09	9.33 ± 0.09^{a}	9.46 ± 0.04^{a}	12.02 ± 0.11^{b}	0.000
16:3 n-3 (HTA)	0.58 ± 0.02	0.42 ± 0.02	0.42 ± 0.03	0.40 ± 0.02	ns
18:3 n-3 (ALA)	30.58 ± 0.07	22.28 ± 0.24^{b}	21.53 ± 0.16^{a}	22.47 ± 0.34^{b}	0.001
18:4 n-3 (SDA)	4.14 ± 0.09	2.79 ± 0.02^{b}	2.64 ± 0.04^{a}	2.74 ± 0.04^{b}	0.001
20:4 n-3 (ETA)	0.66 ± 0.01	0.61 ± 0.03^{a}	0.58 ± 0.02^{a}	0.71 ± 0.00^{b}	0.000
20:5 n-3 (EPA)	1.70 ± 0.01	2.65 ± 0.04^{a}	2.75 ± 0.05^{b}	3.94 ± 0.03^{c}	0.000
22:5 n-3 (DPA n-3)	0.04 ± 0.05	0.07 ± 0.01^{a}	0.06 ± 0.01^{a}	0.20 ± 0.06^{b}	0.000
22:6 n-3 (DHA)	0.07 ± 0.02	10.43 ± 0.4^{b}	11.42 ± 0.15^{c}	9.09 ± 0.24^{a}	0.000
Σ n-3 PUFA	37.77 ± 0.02	39.25 ± 0.22	39.40 ± 0.12	39.55 ± 0.25	ns
Σ polyunsaturated	46.82 ± 0.04	48.58 ± 0.26^{a}	48.85 ± 0.18^{a}	51.57 ± 0.27^{b}	0.000
n-3/n-6	4.18 ± 0.05	4.21 ± 0.03^{b}	4.16 ± 0.02^{b}	3.29 ± 0.03^{a}	0.000
EPA+DHA	1.77 ± 0.02	13.08 ± 0.40^{a}	14.18 ± 0.13^{b}	13.03 ± 0.26^{a}	0.000
EPA/DHA	-	0.25 ± 0.01^{a}	0.24 ± 0.01^{a}	0.43 ± 0.01^{b}	0.000

Data are given as the mean (n=4) ± SD. Significant differences among treatments were detected $P \leq 0.05$ by using one way ANOVA + Tukey's test. Values from non-enriched *Artemia* nauplii were not included in the statistic.

Table 2 Fatty acid composition (% of total fatty acids) of European Seabass larvae fed *Artemia* metanauplii enriched with the experimental and control enriching products at the end of the trial.

	LM1	LM2	C	*P-value*
11:0	4.32 ± 0.22	3.79 ± 0.20	4.07 ± 0.27	ns
14:0	1.28 ± 0.03^{c}	1.05 ± 0.07^{b}	0.60 ± 0.06^{a}	0.001
15:0	0.25 ± 0.01^{a}	0.27 ± 0.02^{a}	0.29 ± 0.03^{b}	0.007
16:0	17.25 ± 0.24	17.09 ± 0.47	17.40 ± 0.49	ns
17:0	0.92 ± 0.05	0.91 ± 0.03	0.93 ± 0.02	ns
18:0	10.06 ± 0.09	10.30 ± 0.09	9.86 ± 0.16	ns
20:0	0.27 ± 0.03	0.28 ± 0.03	0.27 ± 0.02	ns
22:0	0.52 ± 0.10	0.47 ± 0.05	0.55 ± 0.03	ns
Σ saturated	34.86 ± 0.33	34.16 ± 0.65	33.98 ± 0.69	ns
14:1 n-5	0.14 ± 0.01	0.14 ± 0.02	0.15 ± 0.02	ns
16:1 n-7	0.84 ± 0.02^{ab}	0.78 ± 0.03^{a}	0.89 ± 0.04^{b}	0.031
16:1 n-9	0.53 ± 0.02	0.52 ± 0.02	0.53 ± 0.02	ns
18:1 n-7	5.12 ± 0.11	5.01 ± 0.10	5.25 ± 0.09	ns
18:1 n-9	12.49 ± 0.11	12.27 ± 0.08	12.28 ± 0.12	ns
20:1 n-9	0.81 ± 0.03	0.80 ± 0.03	0.81 ± 0.04	ns
22:1 n-9	0.23 ± 0.03	0.22 ± 0.02	0.25 ± 0.07	ns
24:1 n-9	0.58 ± 0.02	0.62 ± 0.09	0.56 ± 0.03	ns
Σ monounsaturated	20.75 ± 0.21	20.35 ± 0.16	20.72 ± 0.21	ns
16:2 n-6	0.06 ± 0.04	0.05 ± 0.01	0.06 ± 0.01	ns
18:2 n-6 (LOA)	2.62 ± 0.06^{a}	2.56 ± 0.08^{a}	2.94 ± 0.09^{b}	0.011
18:3 n-6	0.14 ± 0.01	0.16 ± 0.03	0.13 ± 0.02	ns
20:2 n-6	0.55 ± 0.07	0.56 ± 0.06	0.57 ± 0.03	ns
20:3 n-6 (DGLA)	0.14 ± 0.02^{a}	0.15 ± 0.03^{a}	0.19 ± 0.02^{b}	0.001
20:4 n-6 (ARA)	4.84 ± 0.08^{a}	4.86 ± 0.14^{a}	5.74 ± 0.11^{b}	0.000
22:4 n-6	0.21 ± 0.03	0.25 ± 0.12	0.24 ± 0.04	ns
22:5 n-6 (DPA n-6)	2.97 ± 0.07^{a}	3.09 ± 0.17^{a}	3.98 ± 0.12^{b}	0.000
Σ n-6 PUFA	11.54 ± 0.21^{a}	11.67 ± 0.22^{a}	13.85 ± 0.19^{b}	0.000
16:3 n-3 (HTA)	0.35 ± 0.08	0.35 ± 0.09	0.32 ± 0.09	ns
18:3 n-3 (ALA)	6.75 ± 0.15	6.22 ± 0.18	6.98 ± 0.22	ns
18:4 n-3 (SDA)	0.55 ± 0.02	0.53 ± 0.03	0.58 ± 0.04	ns
20:4 n-3 (ETA)	0.52 ± 0.14	0.47 ± 0.07	0.50 ± 0.04	ns
20:5 n-3 (EPA)	5.33 ± 0.08^{a}	5.30 ± 0.15^{a}	5.60 ± 0.13^{b}	0.010
22:5 n-3 (DPA n-3)	0.76 ± 0.03^{a}	0.72 ± 0.05^{a}	0.89 ± 0.06^{b}	0.011
22:6 n-3 (DHA)	17.99 ± 0.29^{b}	19.63 ± 0.62^{b}	15.94 ± 0.50^{a}	0.000
Σ n-3 PUFA	32.24 ± 0.29^{a}	33.21 ± 0.70^{a}	30.81 ± 0.63^{b}	0.004
Σ polyunsaturated	43.78 ± 0.38	44.88 ± 0.76	44.66 ± 0.77	ns
n-3/n-6	2.79 ± 0.06^{b}	2.85 ± 0.06^{b}	2.22 ± 0.03^{a}	0.000
EPA+DHA	23.31 ± 0.3^{b}	24.92 ± 0.72^{c}	21.55 ± 0.61^{a}	0.000
EPA/DHA	0.30 ± 0.01^{b}	0.27 ± 0.01^{a}	0.35 ± 0.01^{c}	0.000

Data are given as the mean (n=3) ± SD. Significant differences among treatments were detected $P \leq 0.05$ by using one way ANOVA + Tukey's test.

4.4.3 Oxidative status

Biomarkers related to oxidative status measured on European seabass larvae fed Artemia metanauplii enriched with the different products are shown in Figure 6. At the end of the trial (60 dph), CAT activity was found significantly lower in larvae fed Artemia metanauplii enriched with LM2 compared to C (P = 0.005). No significant differences were detected in mtROS, PC and LPO among experimental groups, although it was observed a trend to higher mtROS and LPO in larvae fed Artemia metanauplii enriched with LM2 and LM1, respectively.

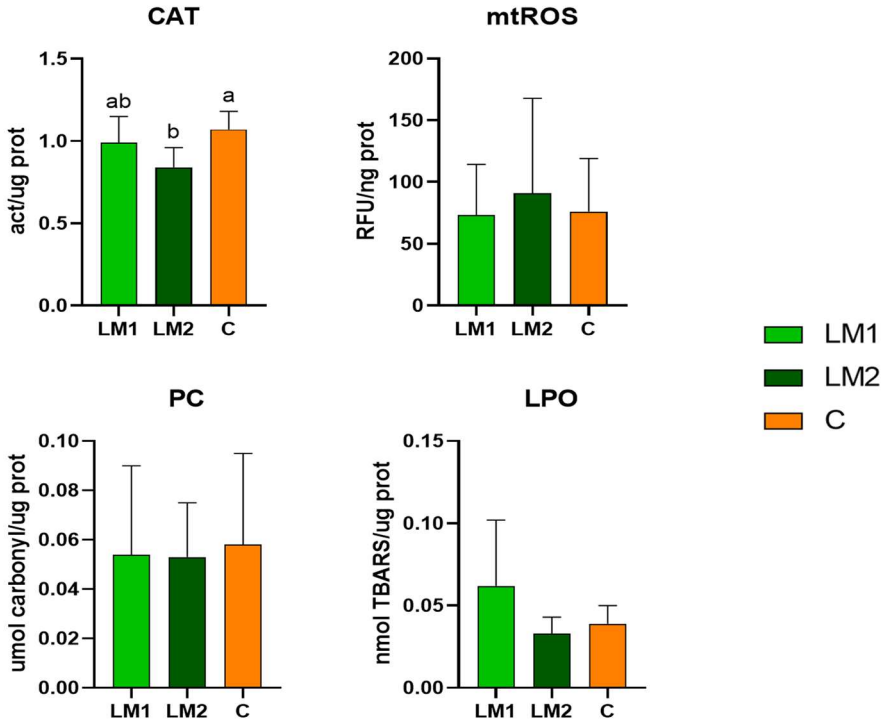

Figure 6 CAT (catalase activity), mROS (mitochondrial reactive oxygen species), PC (protein carbonylation) and LPO (lipid peroxidation) levels in European Seabass fed *Artemia* metanauplii enriched with the experimental and control enrichments at 60 dph. Data are given as the mean (n = 3) ± SD. Significant differences among treatments were detected $P \leq 0.05$ by using one way ANOVA + Tukey's test for CAT, mROS and PC and by Kruskal-Wallis non-parametric test for LPO.

4.4.4 Skeletal anomalies

All larvae with a non-inflated swim bladder (N = 166) were excluded from the analysis and the microscopic analysis to identify skeletal anomalies was performed on the remaining individuals (N = 693). Representative micrographs of the recorded anomalies in the three analysed groups are reported in Figure 7. No significant differences between the three experimental groups were found in the relative frequency (%) of individuals with at least one severe anomaly (LM1 = 47.5 ± 2.1; LM2 = 34.2 ± 10.7%; C = 37.5 ± 16.4%; $P > 0.05$) and in the severe anomalies load (LM1 = 2.5 ± 0.1; LM2 = 2.4 ± 0.5; C = 2.7 ± 0.2; $P > 0.05$) (Table 3). The relative frequencies of severe anomalies observed in each of the four regions of the vertebral column and in the skull are reported in Figure 8. Most of the severe anomalies affected the caudal vertebral region (LM1 = 58.8 ± 13.3; LM2 = 49.8 ± 10.9; C = 41.0 ± 2.7%), followed by skull anomalies in LM1 (14.7 ± 3.8%) and LM2 (17.4 ± 9.6%), and by anomalies of the cranial region of the vertebral column in C (29.1 ± 5.7%). No statistical differences were observed in the frequency of severe anomalies in any of the examined body regions ($P > 0.05$ for all comparisons).

Table 3 Skeletal anomalies of European Seabass larvae fed *Artemia* metanauplii enriched with the experimental and control enriching products at the end of the trial.

	LM1	LM2	C	*P-value*
Number of examined individuals	195	279	219	ns
Relative frequency of individuals with at least one severe anomaly (%)	47.5 ± 12.1	34.2 ± 10.7	37.5 ± 16.4	ns
Severe anomalies load	2.5 ± 0.1	2.4 ± 0.5	2.7 ± 0.2	ns

Data are mean of three subgroups (tanks) ± SD. No significant differences among LM1, LM2 and C groups were detected (ANOVA, $P > 0.05$).

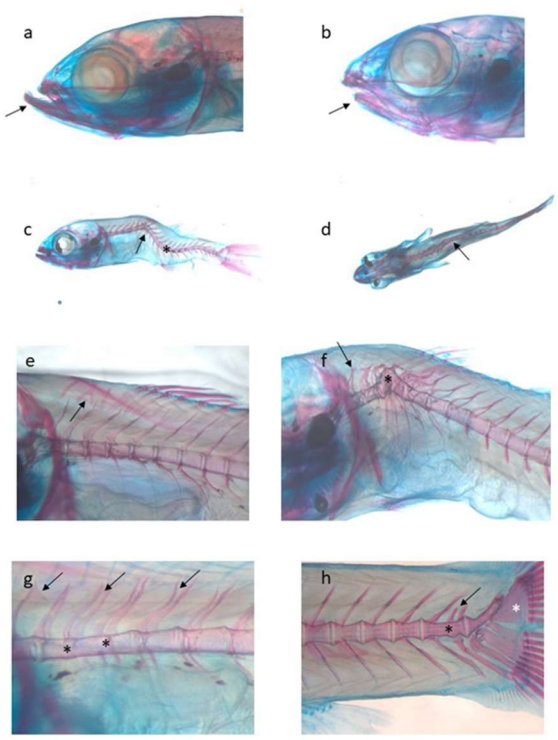

Figure 7 Skeletal anomalies in European Seabass assessed at 60 dph. (a) Anomalous dentary (prognathism) (arrow), (b) Anomalous dentary (lower jaw reduction) (arrow). (c) Pre haemal kyphosis (arrow) haemal lordosis (asterisk). (d) Scoliosis between pre haemal and haemal region (arrow). (e) Anomalous (forked) pre dorsal bone. (f) kyphosis, fused vertebrae and deformed bodies of vertebrae (asterisk) between cranial and pre haemal region; anomalous neural spines are also visible (arrow), (g) fused cranial vertebrae (asterisks); anomalous neural spines are also visible (arrows), (h) fused caudal vertebrae (black asterisk); anomalous neural spine (arrow); fused hypural (white asterisk).

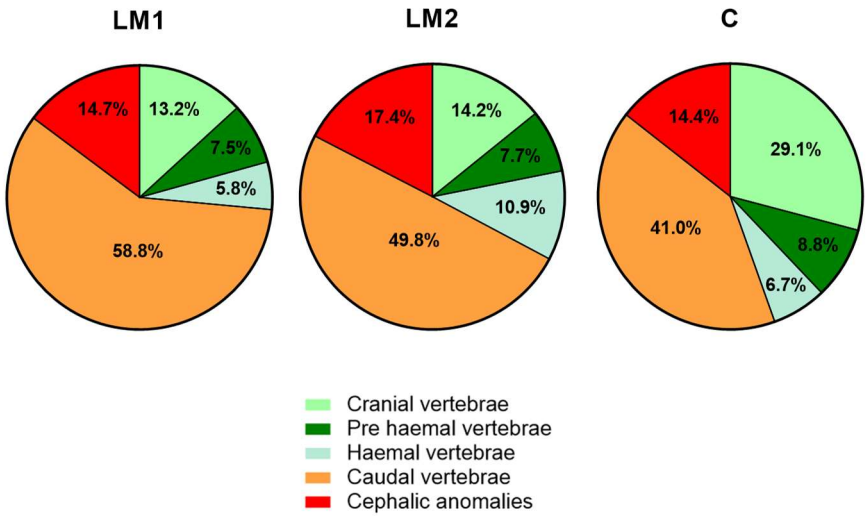

Figure 8 Relative frequencies (%) of severe anomalies in the four regions of the vertebral column and in the skull of European Seabass fed *Artemia* metanauplii enriched with the experimental and control enrichments at 60 dph. Data are given as the mean (n = 3) ± SD. No significant differences among LM1, LM2 and C were detected $P > 0.05$ by using one way ANOVA.

4.5 Discussion

One of the most fascinating aspects of phototrophic production of microalgae and cyanobacteria is the important remediation service offered to the environment (Chauton et al., 2015; Fern´ andez et al., 2020; Mishra et al., 2023). By performing photosynthesis, these microorganisms convert inorganic nutrients, carbon dioxide (CO2) and light radiation into valuable components, such as oils rich in long chain polyunsaturated fatty acids (LC-PUFAs), while releasing oxygen in the environment. In the case of the present study, the use of geothermic resources provides a constant source of heat and CO 2 contributing to reduce 80% of energy consumption, and at the same time, allowing to obtain a constant product quality all-year round. This is not a minor point because oils derived from wild marine fish suffer from seasonal composition variability which can affect the final fatty acid profile of the enrichment (Gámez-Meza et al., 1999; Zlatanos and Laskaridis, 2007). The present study contributes to expanding the existing literature on successful utilisation of microalgal based products, produced in a perspective of circular nutrients economy (Shah et al., 2018; Tocher et al., 2019; Yarnold et al., 2019; Santigosa et al., 2021; Chen et al., 2021; Harder et al., 2021; Carvalho et al., 2022; Mota et al., 2023). By feeding European seabass larvae with *Artemia* metanauplii enriched with two experimental products (LM1 and LM2) and a commercial control (C), the same results in terms of growth performance, survival rate and skeletal anomalies were obtained among the experimental groups. In terms of growth performance indicators, these outcomes are in line with previous studies conducted on European seabass larvae reared with a similar protocol (Sfakianakis et al., 2013; Yan et al., 2019; El-Dahhar et al., 2022; El Basuini et al., 2022). Like the majority of marine fish species, European seabass produce altricial larvae, which typically experience high mortality rates during the initial feeding phase. Compared to previous studies conducted by Vanderplancke et al. (2015) and Darias et al. (2010) reporting a survival rate at 45 dph of 45% and 68% respectively, the observed survival rates in the present study, ranging from 61% to 68% at 60 dph, were high in all experimental groups. Larvae mortality occurs both in nature and in captive conditions mostly during the critical phase of yolk sac reabsorption and mouth opening at the beginning of exogenous feeding (Yúfera and Darias, 2007). This is a challenge in fishery biology (Houde, 2008) and the underlying mechanism is not yet completely understood, although feed availability and quality are the main factors explaining mortality in experimental conditions (Cushing, 1973; Malzahn et al., 2022; Benini et al., 2022). Nutritional blunders or deficiency occurring during this delicate phase may impact the correct maturation of the digestive system, the

oxidative status and the skeletal ossification of adult fish (Izquierdo et al., 2013; Pham et al., 2023; Wischhusen et al., 2022), therefore it is important to provide larvae with an adequate level of nutrients. Among nutrients, dietary lipids are the main energy source for developing fish larvae. Lipids provide LC-PUFAs, which are needed for the synthesis of new cellular structures (Gisbert et al., 2005) and for the primary role on larval survival, growth and immune system modulation (Tocher et al., 2019; Roo et al., 2019; Betancor et al., 2021; Pham et al., 2023). In this trial, the three enriched *Artemia* metanauplii dietary treatments were isonitrogenous and isolipidic despite some differences in the fatty acids profiles. Due to the importance of bioactive LC-PUFAs such as DHA, EPA and ARA in larval nutrition (Tocher, 2015), this discussion is focused on the implications related to their contents, overlooking other differences observed in less bioactive fatty acids. With regard to the total amount of n-3 PUFAs evaluated in *Artemia* metanauplii in this trial, no difference among the different dietary treatments were observed. However, within n-3 PUFAs, DHA was more abundant in LM1 and LM2 enriched *Artemia* metanauplii while EPA in C. DHA plays an important role in the development of the visual and neural system of marine fish larvae and its deficiency may affect the development of the predatory behaviour resulting in a lower survival rate (Watanabe, 1993; Izquierdo et al., 2013; Roo et al., 2019; Pham et al., 2023). A functional evidence of DHA importance is represented by the very high content of DHA in marine fish eggs (Anderson et al., 1984; Watanabe, 1993; Xu et al., 2017). Still within n-3 PUFA fatty acid family, EPA plays an important role in larvae metabolism, since it regulates cell membranes integrity and function and enhances their fluidity (Izquierdo, 2005; Hashimoto and Hossain, 2018) much more than arachidonic acid (ARA) (Hagve et al., 1998), but less than DHA (Hashimoto et al., 1999). Moreover, it is considered an important regulator of eicosanoids production (Adam et al., 2017; Sissener et al., 2020), being a major substrate for both cyclooxygenases and lipoxygenase enzymes and the main precursor for leukotriene synthesis in many fish species (Izquierdo, 2005; Montero et al., 2015, 2019). Another metabolically active LC-PUFA from the n-6 family is ARA. In this trial, *Artemia* metanauplii enriched with C showed an increased content of ARA. This is not surprising since ARA is highly present in fish-derived products and C is an encapsulated fish-oil based enrichment specifically supplemented with ARA, as stated in the product label. ARA deficiency in aquafeed formulation become more of a problem with the reduction of fish meal and oil (Araújo et al., 2022). ARA is involved in important physiological and immunological functions in marine fish, such as the eicosanoids production, being the main precursors for prostaglandin synthesis (Izquierdo, 2005).

In addition, ARA is also involved in the stress response (Martins et al., 2013). The concern over n-6 PUFA derived eicosanoids is due to the dogma of n-6 PUFA derived eicosanoids being pro-inflammatory, as opposed to the n-3 PUFA derived being anti-inflammatory (Hundal et al., 2021; Dong et al., 2023). As a consequence, DHA, EPA and ARA are considered essential fatty acids for the majority of marine fish and are required to be supplied in the diet. Marine species do not have sufficient Δ12 (or n-6) and Δ15 (or n-3) desaturases and elongase activities to produce them from oleic (18:1 n-9) acid precursor (Furuita et al., 1996; Izquierdo, 2005; Tocher et al., 2019), hence their requirements have been extensively investigated in many marine species (Watanabe et al., 1989; Izquierdo, 2005; Izquierdo et al., 2013; Navarro-Guille´n et al., 2014; Roo et al., 2019; de Mello et al., 2022). An optimal level is set at 0.6 –2.3% for DHA, 0.7–2.3% EPA and up to 1% ARA considered as percentage on dry feed (Izquierdo, 2005), which are met in all experimental groups (data on % of fatty acid/dry feed not shown). What is more difficult is understanding the mechanisms of interaction between the LC-PUFAs. For instance, evidence of competition of LC-PUFAs on the same enzymatic pathways such as lipoxygenases, cyclooxygenases and phospholipases have been reported by several authors (Izquierdo, 2005; Norambuena et al., 2012; Kumar et al., 2016). As a results, when it comes to model the LC-PUFAs requirements, their ratio has to be carefully adjusted. Izquierdo, (2005) and Houston et al. (2017) proposed a ratio of 1:1 (EPA/DHA) with ARA values higher than 0.5% and a total 3% of LC-PUFAs, ideal for gilthead seabream growth. Betancor et al. (2016) found reduced growth when juveniles of gilthead seabream were fed diets with high EPA/DHA ratios, emphasising the importance of DHA. A similar superiority of DHA to EPA has been proposed also by Wu et al. (2002), reporting an increased growth in juveniles grouper (*Epinephelus malabaricus*) with lower ratio of EPA/DHA. The fatty acid profile of European seabass fingerling at 60 dph reflected the composition of their diets, as observed by several authors (Glencross, 2009; Boglino et al., 2012), with some differences, related to the metabolic transformation occurring in fish, resulting in a compensatory effect between the LC-PUFAs feed input and the final LC-PUFAs output deposited in larvae flesh. For instance, EPA content in *Artemia* metanauplii was 49% and 43% more in C compared to LM1 and LM2, respectively, while in larvae was smoothed over to an only 5% more in C compared to both LM1 and LM2. Likewise, ARA content in *Artemia* metanauplii was 55% more in C compared to both LM1 and LM2 while in larvae 18% more in C compared to both LM1 and LM2. Interestingly the ratio of difference in DHA content remained the same in *Artemia* metanauplii and larvae (approximately 14% and 25% lower in C compared to LM1 and LM2

respectively in both *Artemia* and larvae). Overall data on LC-PUFAs composition of larvae are in line with previous experiments conducted on gilthead seabream (Koven et al., 2001), European seabass (Gisbert et al., 2005), turbot (*Scophthalmus maximus*) and Senegale sole (*Solea senegalensis*) (Boglino et al., 2012), showing enough nutrients stored for the further development in all experimental groups. This statement is confirmed by the absence of differences, among the dietary treatments, in the n-9 PUFAs (particularly 18:1 n-9 and 20:1 n-9) contents in larvae, which are produced by elongation and increased values were proposed as indicator of LC-PUFAs deficiency in gilthead seabream (Rodriguez et al., 1994) and grouper (Wu et al., 2002). Excessive LC-PUFAs in diets may lead to increased risk of lipid peroxidation (Betancor et al., 2015; Qian et al., 2015; Adam et al., 2017), which is mediated by free radicals and reactive oxygen species (ROS) production (oxidative factors). Nevertheless, a recent study described the antioxidant protection capacity of EPA and DHA against cellular oxidative stress in humans (Aldhafiri, 2022). In line with this, dietary supplementation with a mixture of EPA + DHA has been reported to exert an antioxidant protective role in fish (Kumar et al., 2022). Balanced concentrations of anti- and pro-oxidant factors are continuously generated during regular cellular metabolism, which is known as ROS homeostasis (Lushchak, 2016). Between the metabolic antioxidant factors, the enzyme catalase catalyses the decomposition of hydrogen peroxide to water and oxygen, preventing the cascade of oxidation reactions and closing the lipid peroxidation catalytic cycle (Betancor et al., 2012). However, if ROS generation exceeds the antioxidant protection, the imbalance is called oxidative stress and may lead to reduced larval growth (Betancor et al., 2012; Roo et al., 2019), severe dystrophic lesions in the musculature (Betancor et al., 2012) and higher skeletal anomalies rate (Izquierdo et al., 2010). Lipid peroxidation and its by-products, such as protein carbonylation (Suzuki et al., 2010), have been commonly used as biomarkers of oxidative status in fish larvae (Monroig et al., 2006; Betancor et al., 2012; Guerreiro et al., 2022). In this trial, although not statistically significant, larvae from LM2 treatment group showed a trend to higher mitochondrial ROS production. By contrast, this group presented statistically lower catalase activity than larvae from C treatment and did not present any tendency to higher oxidative damage (measured as LPO, PC and larval growth performance). From a nutritional point of view, microalgae and cyanobacteria display diverse bioactive compound profiles, being those with antioxidant properties of particular interest. In that sense, both *Nannochloropsis* sp. and *Spirulina* sp. are rich in the pigmentsβ-carotene and zeaxanthin, although, on a dry weight basis, *Spirulina* sp. contains 2.5 and 15-fold higher β-carotene and zeaxanthin than

Nannochloropsis sp., respectively (Ghaeni et al., 2015; Bernaerts et al., 2020). In addition, Spirulina sp. is also rich in phycocyanin, a blue-coloured photosynthetic pigment with free radical scavenging capacity (Fernandes et al., 2023). Altogether, the higher EPA+DHA content found in Artemia enriched with LM2 and the higher content in antioxidant compounds of Spirulina sp., results might support a potential preventive effect against oxidative stress of LM2 enrichment (Coulombier et al., 2021; Vignaud et al., 2023). However, further studies are needed to conclusively unravel the antioxidant effects of LM2 blend. Several skeletal anomalies have been described in marine fish, representing a major problem in aquaculture for the economical, biological and ethical related concerns (Boglione et al., 2013). Anomalies in fish are often associated with a reduced growth and high mortality rate, being a major problem in a hatchery setting due to the derived economical losses. In this trial, the morphological analysis of European seabass did not show an effect of the microalgal enrichments LM1 and LM2 on the total number of severe anomalies and the relative frequencies in the different regions of vertebral column, compared to C. In a wild contest, it is fair to presume that individuals with physical anomalies will have a reduced capacity to swim properly, feed or escape a predator, thus reducing its likelihood to survive. In a survey conducted on wild populations by Boglione et al. (2001) a 4% of wild gilthead seabream presented some severe anomalies. On the contrary, in a hatchery setting, with controlled environmental conditions, food availability and absence of predation, the anomalies rate is expected to be higher. The findings from this study are in line with surveys conducted by Koumoundouros (2010) and Boglione et al. (2013), reporting an incidence of severe anomalies in Mediterranean marine hatcheries of 30–40% of the reared fish. A recent study conducted by (Viegas et al., 2023) on European seabass reported an incidence of severe anomalies of 33–37% at 64 dph. Even though anomalies in fish have been often associated with nutritional causes such as nutrient deficiency (Boglione et al., 2013) or nutrients unbalancing (Izquierdo et al., 2013; Roo et al., 2019), the severe anomalies observed in this trial are more likely to be attributed to general causes such as tank hydrodynamics, temperature and other abiotic and biotic factors.

4.6 Conclusion

Producing microorganisms such as microalgae and cyanobacteria exploiting industrial geothermal waste outputs could mitigate the overexploitation of marine fish stocks and reduce the costs associate with photobioreactors energy consumption. The results obtained in this study indicated that using blends of so produced products for the enrichments of *Artemia* salina produced equal results in terms of growth performance and larvae anomaly rate compared to a commercial control. Each dietary treatment presented a specific accumulation pattern for ARA, DHA and EPA long chain polyunsaturated fatty acids. Ratio of DHA/EPA of 4:1 as in the case of LM1 and LM2 in the presence of 1.4% of ARA (as a % on total fatty acid) guaranteed equal results to the control which display a ratio of DHA/EPA of 2:1 with 2.2% of ARA. The fatty acids profile of European seabass larvae sampled at 60 dph reflected the composition of the diet, showing no signs of LC-PUFAs deficiency in every dietary treatment. Results on the larvae oxidative status suggested that LM2 enrichment product may exert potential preventive effects against oxidative stress, which could be translated into enhanced fish larvae robustness and, in the long-term, improved health status of the fish. After the radiological survey, European seabass larvae at 60 dph presented a percentage of anomalies ranging from 34% to 47% in all dietary treatments. The relative frequency of anomaly mainly affected caudal vertebrae, followed by skull, cranial vertebrae, pre-haemal vertebrae and haemal vertebrae in all groups. These findings are in line with other research conducted on the species and on other Mediterranean marine fish.

4.7 Credit authorship contribution statement

Chrysovalentinos Pousis: Investigation, Methodology, Writing review & editing. **Manuel Yúfera**: Conceptualization, Investigation, Methodology, Writing – review & editing. **Isabel Medina**: Conceptualization, Investigation, Methodology, Writing – review & editing. **Maria Jesus González**: Investigation, Methodology. **Pier Paolo Gatta**: Conceptualization, Writing – review & editing. **Andrea Bertini**: Investigation, Methodology, Writing – original draft, Writing – review & editing. **Christel Nys**: Conceptualization, Methodology, Writing – review & editing. **Gianluca Ventriglia**: Investigation, Methodology, Writing – review & editing. Carmen Navarro-Guill´ en: Conceptualization, Investigation, Methodology, Writing – review & editing. **Luca Parma**: Conceptualization, Investigation, Methodology, Writing – review & editing. **Elisa Benini**: Methodology, Writing – review & editing. **Alessio Bonaldo**:

Conceptualization, Investigation, Methodology, Writing – review & editing. **Arianna Marchi**: Investigation, Methodology, Writing – review & editing.

4.8 Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests Author C.N is employed by VAXA technologies Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4.9 Acknowledgement

This research was funded by the project SUSTAINFEED (EIT-Food 21168, Co-funded by the European Union). The author Elisa Benini was supported by the Project code CN_00000033, Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, CUP J33C22001190001, Project title “National Biodiversity Future Centre—NBFC” under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4—Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of Italian Ministry of University and Research funded by the European Union—NextGenerationEU.

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Chapter 5.

Towards a free wild-caught fishmeal, fish oil and soy protein in European Seabass diet using by-products from fishery and aquaculture.

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Published on: Aquaculture, (2023)

DOI: https://doi.org/10.1016/j.aquaculture.2023.739571

5.1 Abstract

One of the main strategies to implement the sustainability of aquaculture is by reducing its dependence on feed raw materials derived from wild marine resources, unsustainable land and freshwater use. To totally replace wild-caught fishmeal (FM), fish oil (FO) and soy protein (SP) in European Seabass, five experimental diets were formulated including increasing levels of fishery and aquaculture by-products (control C, 0FM100FO, 0FM0FO, 0FM0FO-50SP, 0FM0FO-0SP). Diets were administered to triplicate fish groups of 50 individuals reared in a recirculation system for 119 days. No major differences ($p > 0.05$) between C and 0FM100FO were detected in growth (final body weight) and feed efficiency parameters (feed conversion rate, protein efficiency ratio, gross protein efficiency, lipid efficiency ratio, gross lipid efficiency) while they were reduced ($p < 0.05$) in 0FM0FO, 0FM0FO-50SP, 0FM0FO-0SP compared to C diet. At the end of the growth trial the overall metabolomic profiles of fish muscle was impacted by the diets. In particular, the increase in glycine and phosphocreatine in the muscle of fish fed with diets containing FM and FO by-products, may suggest changes or adaptation in metabolic pattern for energy production at muscular level; results which were also supported by the higher level in plasma creatinine, uric acid and lactate found in C diets. Concerning welfare indicators, overall plasma parameters were in line with the normal range for this species indicating a general optimal welfare condition under all dietary regimes. No effect of diets on overall gut microbiota layout was observed. However, the inclusion of the FM and FO by-product increase the relative abundance of several taxa such as Weissela, Enterococcus, Streptococcus and Bacillus which could potentially support immune system and disease resistance. Overall, this study highlighted the possibility of totally replacing wild-caught FM and FO using by-products from fisheries and aquaculture with an only marginal reduction of the overall performance.

Keywords

European Seabass, By-products, Growth Metabolomics, Gut microbiota

5.2 Introduction

The increase in world population and the improvement of living conditions have changed world food habits with a shift towards products of animal origin (FAO, 2017). Over the last two decades the aquaculture sector has been increasingly recognized for its essential contribution to global food security and nutrition (FAO, 2022). However, the rapid growth of aquaculture has led to an overexploitation of natural resources (wild fish populations, land and freshwater uses) with implication on ecological issues such as biodiversity concerns and biotic depletion (Ahmed and Thompson, 2019). The main challenge of aquaculture is to become more sustainable using nutritional resources that are produced through a circular bioeconomy approach (Colombo et al., 2022). In the last twenty years, several studies on soy derivates as alternative protein sources to fishmeal (FM) have reported positive results on the growth and health of several aquatic species (Zhou et al., 2018), such as salmonids (Collins et al., 2013) and Mediterranean marine species, including gilthead sea bream (Parma et al., 2016) and European Seabass (Bonvini et al., 2018a). However, plant ingredients may pose contrasts in sustainability and resource efficiency. In fact, several studies on life cycle assessment demonstrated that the substitution of marine ingredients with vegetable ingredients have shifted resource demand from the oceans onto the land with an overall environmental impacts of feed production expected to increase (Newton and Little, 2018; Malcorps et al., 2019). Besides being competing resources in the human food chain, vegetable ingredients when included at high dietary level may also inducing nutritional specification issues (nutrient limitation and antinutritional factor) (Glencross, 2009), may affect fish welfare (Saito et al., 2020) and could also alter the level of micro- and macro nutrient in the final fish product (Nichols et al., 2014; Sprague et al., 2016). Soy management, including growing, processing, transporting, and disposal is directly and indirectly associated with over exploitation of soil, deforestation, and high levels of carbon footprint (Eranki et al., 2019). Imported soybeans for aquafeed use are responsible for 75% of aquaculture greenhouse gas (GHG) emission estimated between 2.9 and 3.8 kg CO 2e kg 1 LW of fish at the farm gate (Ghosh et al., 2020). Moreover, the expansion of the aquaculture sector often requires inputs from wild fish stocks for feed formulation, exploiting a sector already overused both for animal and human consumption (Wang et al., 2015). In response to this, the marine ingredients industry is undergoing a period of change as increasing amounts of raw materials are sourced from the by-products of fisheries and aquaculture which now account for as much as 35% of FM worldwide (Newton and Little, 2018). Fishery and aquaculture by-products are now widespread as promising

alternative feed ingredients for fish farming due to their avail ability and nutritional quality (Stevens et al., 2018). The utilization of different fish and crustacean species with the inclusion of every kind of animal body part (from bone to skin) with a high content of micro and macronutrients such as protein, DHA and EPA, is what makes these ingredients highly attractive for the sector (Stevens et al., 2018; Malcorps et al., 2021). Fishery and aquaculture by-products can also be considered as sustainable ingredients because of their potential to reduce environmental impact, improve the efficiency of the industry, and reduce waste production (Stevens et al., 2018; Gasco et al., 2020; Munekata et al., 2020). The inclusion of low economic value fish by-products also showed the potential to lower the Fish in - Fish-out (FIFO) ratio (Kok et al., 2020) and the need for commonly used marine ingredient substitutes, such as plant ingredients, which affect EPA + DHA aquafeed content (Malcorps et al., 2021). Also, some vegetable by-products of the agriculture processing industry are a rich resource still greatly undervalued (Glencross et al., 2020). It was estimated that approximately 1.3 billion tons per year of food for human consumption is lost or wasted during and after pro cessing, and about half of this is represented by vegetable waste (Ran et al., 2019). However, most agri-food-derived by-products are a promising source of lipids, carbohydrates, proteins with nutritionally and functionally important compounds such as gluten, polyphenols, pectin and many others (Dhillon et al., 2016; Leonard et al., 2020). In recent years, due to emerging health concerns, gluten meal has become a significant cereal by-product of agriculture and is becoming widespread on account of its high protein content (Tapia-Hernandez et al., 2019). In aquaculture, it is used for its nutritional characteristics, safety, ease, cost-effectiveness of purification and, in comparison to soybean products, its production is less associated with unsustainable deforestation (Newton et al., 2023). Previous studies on the effect of this ingredient on rainbow trout (Tusche et al., 2012), salmon (Glencross et al., 2021), European Seabass (Fountoulaki et al., 2010) and gilthead sea bream (Aragao et al., 2020) have shown positive results on growth performance. Therefore, the present research aims to evaluate the effects of total replacement of wild-caught FM and FO by using fishery and aquaculture by-products and glutens as a valid alternative to soy protein (SP). Growth performance and gut health through gut microbiota investigation of European Seabass juveniles were evaluated. Furthermore, the effects of the different diets on muscle metabolome were evaluated by an NMR-based metabolomics approach and chemometrics analysis which proved to be an ideal tool for analysing complex samples such as fish muscle (Picone et al., 2011; Laghi et al., 2014; Ciampa et al., 2022).

5.3 Materials and Methods

5.3.1 Experimental diets

Five experimental diets were formulated to totally replace wild caught FM, FO and SP using fisheries and aquaculture by-products, and alternative vegetable protein. Wild-caught FM and krill meal (FM prime, krill meal) were totally replaced by adding a combination of FM trimming and shrimp meal (0%FM,100%FO, 0FM100FO). Wild-caught FO (FO Extra) was totally replaced with FO trimming (0 %FM, 0%FO, 0FM0FO). SP (soybean meal and soy protein concentrate) were half (0% FM, 0%FO, 50%SP, 0FM0FO-50SP) and totally replaced (0%FM, 0%FO, 0%SP, 0FM0FO-0SP) with a blend of corn gluten and wheat gluten. Diets were extruded at industrial level with a diameter of 3 mm. Feeds were produced by AIA – Agricola Italiana Alimentare S.p.A. (Verona, Italy). Ingredients, proximate composition and fatty acid composition of the experimental diets are shown in Table 4 and Table 5.

Table 4 Ingredients and proximate composition of the experimental diets.

	C	0FM100FO	0FMFO	0FMFO-50SP	0FMFO-0SP
Ingredients, % of the diet					
[1]Fish meal Prime	25.0	-	-	-	-
[2]Fish meal Trimming	-	25.0	25.0	25.0	25.0
[3]Krill meal	2.50	-	-	-	-
[4]Shrimps meal	-	2.50	2.50	8.50	8.50
[5]Fish oil Extra	14.6	14.25	-	-	-
[6]Fish oil Trimming	-	-	14.25	12.5	12.5
Wheat Meal	14.5	14.3	14.4	1.4	18.2
[7]Soybean Meal	19.2	19.2	19.2	9.6	-
[8]Soy protein Concentre	8.00	8.00	8.00	4.00	-
[9]Corn gluten	4.00	4.00	4.00	10.0	15.5
[10]Wheat gluten	-	2.60	2.50	2.75	9.50
Sunflower seed meal feed, dehulled	5.00	4.00	4.00	5.00	5.00
Guar germ meal	6.00	5.00	5.00	5.00	-
[11]Yeasts	-	-	-	-	2.00
[12]Microalgae	-	-	-	2.50	2.50
[13]Vitamin/mineral premix	1.20	1.20	1.20	1.20	1.20
Proximate composition, % on a wet weight basis					
Moisture	3.80	1.90	1.81	1.77	2.27
Protein	42.9	42.2	43.5	43.3	42.2
Lipid	18.1	17.5	16.5	17.0	17.7
Ash	7.02	8.91	9.08	9.84	9.02
PUFA, % of diet					
EPA	2.20	2.00	0.62	0.60	0.60
DHA	1.90	1.85	0.70	1.60	1.60
EPA+DHA	4.10	3.85	1.32	2.20	2.20

[1]Origin: Chile; Composition: protein 67%, lipid 10%, ash 14%. Antioxidant: butylated hydroxyanisole, BHA, 40 ppm; Butylated hydroxytoluene BHT, 200 ppm.
[2]Obtained from Atlantic mackerel (*Scomber scombrus)* and sardine (*Sardina pilchardus*). Origin: Morocco; Composition: protein 63%, lipid 10%, ash 20%. BHA, 80 ppm; BHT, 80 ppm.
[3]Composition: protein 54%, lipid, 18%, ash 13%.
[4] Obtained from whiteleg shrimp (*Litopenaeus vannamei*) heads; Composition: protein 52%, lipid 8%, ash 20%.
[5] Origin: Chile. Antioxidant: BHA 90 ppm, BHT 80 ppm, propylgallate, PG 40 ppm.
[6]Obtained from Atlantic salmon (*Salmo salar*). Origin: EU. Antioxidant: BHA 70 ppm, BHT 145 ppm, PG 40 ppm.
[7]Origin: Italy (gmo-free). Composition: protein 49%, lipid, 1%.
[8]Origin: Serbia. Composition: protein, 61%, lipid, 0.3%.
[9]Origin: Italy.
[10]Origin: Italy.
[11]*Saccaromices cerevisiae*.
[12] Obtained from processed *Schizochytrium* (Corbion, Brazil). 13 Vitamins/mineral premix (IU or mg kg 1 diet): vitamin A: 12000 IU; vitamin D: 2000 IU; vitamin E: 160 mg; vitamin C: 160 mg; Manganese: 40 mg; Zinc: 55 mg; Iron 20 mg; Copper: 8 mg; Iodine: 2 mg; selenium: 0.15 mg; BHA, 150 mg; PG, 75 mg.

Table 5 Fatty acid composition (% of total fatty acid methyl esters, FAME) of the experimental diets

	C	0FM100FO	0FM0FO	0FM0FO-50SP	0FM0FO-0SP
12:0	0.71 ± 0.18	0.92 ± 0.23	-	0.65 ± 0.17	-
14:0	6.37 ± 0.92	7.3 ± 1.0	3.25 ± 0.51	3.00 ± 0.48	2.78 ± 0.43
15:0	0.53 ± 0.13	0.66 ± 0.16	0.25 ± 0.06	0.25 ± 0.06	0.22 ± 0.05
16:0	21.0 ± 2.5	23.6 ± 2.7	15.4 ± 2.0	12.8 ± 1.8	13.8 ± 1.7
16:1n-7	7.00 ± 1.33	8.20 ± 1.62	3.59 ± 0.72	3.50 ± 0.65	3.19 ± 0.56
17:0	0.50 ± 0.12	0.58 ± 0.14	0.27 ± 0.06	0.24 ± 0.06	0.25 ± 0.06
17:1n-7	0.20 ± 0.05	0.22 ± 0.05	0.12 ± 0.0	0.11 ± 0.03	0.11 ± 0.03
18:0	4.32 ± 0.63	4.69 ± 0.68	3.41 ± 0.52	3.37 ± 0.52	3.38 ± 0.50
18:1n-9	15.9 ± 2.1	14.2 ± 1.9	30.6 ± 3.4	31.0 ± 3.4	29.6 ±. 3.2
18:1n-7	3.13 ± 0.49	3.16 ± 0.49	2.76 ± 0.44	2.82 ± 0.45	2.67 ± 0.42
18:1n-6	0.12 ± 0.03	0.13 ± 0.03	0.09 ± 0.02	0.09 ± 0.02	0.08 ± 0.02
18:2n-6	7.80 ± 1.2	7.60 ± 1.1	16.2 ± 2.0	16.1 ± 2.0	15.7 ± 1.9
18:3n-6	0.16 ± 0.04	0.17 ± 0.04	0.11 ± 0.03	0.11 ± 0.03	0.11 ± 0.03
18:3n-3	1.72 ± 0.35	1.32 ± 0.29	5.39 ± 0.80	5.71 ± 0.84	5.24 ± 0.72
18:4n-3	1.80 ± 0.35	1.77 ± 0.34	0.77 ± 0.18	0.82 ± 0.20	0.74 ± 0.17
20:0	0.31 ± 0.08	0.31 ± 0.07	0.29 ± 0.07	0.35 ± 0.09	0.36 ± 0.09
20:1n-9	1.66 ± 0.33	1.40 ± 0.30	2.22 ± 0.37	2.84 ± 0.44	2.54 ± 0.40
20:2n-6	0.28 ± 0.06	0.25 ± 0.06	0.50 ± 0.12	0.59 ± 0.14	0.55 ± 0.13
20:3n-6	0.12 ± 0.03	0.11 ± 0.03	0.15 ± 0.04	0.17 ± 0.04	0.17 ± 0.04
20:3n-3	0.13 ± 0.03	0.01 ± 0.02	0.28 ± 0.07	0.44 ± 0.10	0.34 ± 0.08
20:4n-6 (ARA)	0.97 ± 0.23	1.02 ± 0.24	0.38 ± 0.1	0.41 ± 0.10	0.42 ± 0.01
20:5n-3 (EPA)	11.3 ± 1.6	10.7 ± 1.5	3.46 ± 0.52	4.16 ± 0.61	3.85 ± 0.55
22:0	0.12 ± 0.03	0.09 ± 0.02	0.11 ± 0.03	0.15 ± 0.04	0.16 ± 0.04
22:1n-11	1.26 ± 0.30	0.94 ± 0.22	1.15 ± 0.27	1.91 ± 0.34	1.61 ± 0.29
22:1n-9	0.20 ± 0.05	0.12 ± 0.03	0.22 ± 0.05	0.42 ± 0.09	0.33 ± 0.08
22:2n-6	0.03 ± 0.00	-	0.05 ± 0.01	0.08 ± 0.02	0.07 ± 0.02
22:4n-6	0.09 ± 0.02	0.07 ± 0.02	0.04 ± 0.00	0.05 ± 0.01	0.05 ± 0.01
22:3n-3	0.01 ± 0.00	-	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
22:5n-6	0.38 ± 0.09	0.32 ± 0.08	0.84 ± 0.20	0.18 ± 0.04	1.13 ± 0.25
22:5n-3	1.26 ± 0.30	1.00 ± 0.24	0.75 ± 0.18	1.17 ± 0.27	1.03 ± 0.24
22:6n-3 (DHA)	7.30 ± 1.0	5.65 ± 0.83	5.46 ± 0.78	4.22 ± 0.62	7.40 ± 1.1
23:0	0.02 ± 0.00	0.09 ± 0.02	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
24:0	0.07 ± 0.02	0.05± 0.01	0.05 ± 0.01	0.01 ± 0.02	0.10 ± 0.02
24:1n-9	0.22 ± 0.05	0.19 ± 0.04	0.17 ± 0.04	0.33 ± 0.08	0.30 ± 0.07
Σ SFA	34.8 ± 2.8	39.3 ± 3.0	23.5 ± 2.2	21.5 ± 2.0	21.5 ± 1.8
Σ MUFA	31.2 ± 2.8	30.1 ± 3.7	41.7 ± 4.4	43.7 ± 4.5	41.2 ± 4.1
Σ PUFA	34.0 ± 2.4	30.6 ± 2.2	34.8 ± 2.4	34.8 ± 2.3	37.3 ± 2.4
Σ n-6	10.3 ± 1.2	10.1 ± 1.2	18.5 ± 2.0	17.9 ± 2.0	18.4 ± 1.9
Σ n-3	23.8 ± 2.0	20.7 ± 1.8	16.4 ± 1.3	16.9 ± 1.3	19.0 ± 1.5
n-3/n-6	2.31 ± 0.33	2.05 ± 0.30	0.89 ± 0.12	0.94 ± 0.13	1.03 ± 0.14
EPA/ARA	11.6	10.5	9.1	10.1	9.2
DHA/EPA	0.65	0.53	1.58	1.01	1.92

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: poly-unsaturated fatty acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; ARA: arachidonic acid.

5.3.2 Fish and rearing trial

The experiment was carried out at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. European Seabass juveniles were obtained from an Italian hatchery. At the beginning of the trial, 50 fish (initial average weight: 75.96 ± 6.99 g) per tank were randomly distributed into 15 square tanks with a capacity of 800 L. Each diet was administered to triplicate groups, with random assignment, over 119 days. Tanks were provided with natural seawater and connected to a closed recirculation system (overall water volume: 20 m3). The rearing system consisted of a mechanical sand filter (PTK 1200, Astralpool, Barcelona, Spain), ultraviolet lights (PE 25 mJ/cm2: 32m3 h 1, Blaufish, Barcelona, Spain) and a biofilter (PTK 1200, Astralpool, Barcelona, Spain). The water exchange rate was 100% every hour, while the overall water renewal amount in the system was 5% daily. During the trial, the temperature was kept at 24 ± 0.5 ◦C and the photoperiod was maintained at 12 h light and 12 h dark through artificial light. The oxygen level was kept constant (8.0 ± 1.0 mg L 1) by means of a liquid oxygen system regulated by a software programme (B&G Sinergia snc, Chioggia, Italy). Each day, ammonia (total ammonia nitrogen ≤0.1 mg L 1) and nitrite (≤ 0.2 mg L 1) were monitored by spectrophotometer (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany) and salinity (25 g L 1) was measured by a refractometer (106 ATC, Giorgio Bormac S.r.l., Carpi, Italy). Sodium bicarbonate was added if needed to keep pH constant at 7.8–8.0. Animals were fed to satiation with automatic feeders twice a day, set to release pellets gradually for one and a half hours. The uneaten pellets of each tank were collected, dried overnight at 105 ◦C, and weighed for feed intake (FI) calculation (Parma et al., 2019).

5.3.3 Sampling

At the beginning and at the end of the experiment, all animals in each tank were anaesthetised by MS222 at 100 mg L 1 and weighed. Specific growth rate (SGR) and feed conversion rate (FCR) were calculated. Moreover, wet body weight, length, viscera and liver were individually evaluated from five fish per tank to determine Viscerosomatic (VSI), Hepatosomatic indexes (HSI) and condition factor (CF). Proximate composition of the carcasses was determined on a pooled sample of 10 fish per tank at the beginning and on a pooled sample of 5 fish per tank at the end of the trial. For gut microbiota analysis, at the end of the trial, 12 h post meal, digesta content from the posterior intestine of five fish per tank was collected and immediately stored at 80 ◦C (Parma et al., 2020). For the assessment of plasma biochemistry, blood from 5 fish per tank

was collected from the caudal vein. Samples were then centrifuged (3000 xg, 10 min, 4 ◦C) and plasma aliquots were stored at 80 ◦C until analysis (Pelusio et al., 2021). At the same time 20 fish per treatment were sampled for metabolomic analyses of muscle. Overall experimental procedures were evaluated and approved by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna (ID 1136/2019), under European directive 2010/ 63/UE relating to the protection of animals used for scientific purposes.

5.3.4 Calculations

Following, the illustration of employed formulae:

Specific growth rate (SGR) (% day 1) = 100*(ln FBW- ln IBW) / days (where FBW represent the final body weight and IBW and the initial body weights).

Feed intake (FI, g kg ΔBW 1 day 1) = ((100*total feed ingestion)/ (ΔBW))/days.

Feed conversion ratio (FCR) = feed intake / weight gain.

Protein efficiency ratio (PER) = (FBW – IBW) / protein intake.

Gross protein efficiency (GPE) (%) = 100*[(% final body protein*FBW) - (% initial body protein*IBW)] / total protein intake fish.

Lipid efficiency ratio (LER) = (FBW - IBW)/lipid intake.

Gross lipid efficiency (GLE) (%) = 100*[(% final body lipid*FBW) (% initial body lipid*IBW)]/total lipid intake fish.

VSI = Viscerosomatic index (%) = 100*(viscera weight/FBW).

HSI = Hepatosomatic index (%) = 100*(liver weight/FBW). CF = condition factor = 100 × (body weight, g)/ (body length, cm)^3.

5.3.5 Proximate composition analysis

Diets and whole bodies of sampled fish were analysed for proximate composition. Moisture content was obtained by weight loss after drying samples in a stove at 105 ◦C until a constant weight. Crude protein was determined as total nitrogen (N) by using the Kjeldahl method, multiplying N by 6.25, according to AOAC International (AOAC, 2010). Total lipids were determined according to Bligh and Dyer's (1959) extraction method. Samples were incinerated to a constant weight in a muffle oven at 450 ◦C to estimate ash content (AOAC, 2010). Fatty acid analyses composition of diets was performed according to ISO16958:2015.

5.3.6 Metabolomic analysis

Samples were prepared according to Picone et al. (2011): for each diet named “DA”, “DB”, “DC”, “DD” and “DE”, 10 aliquots of 4 g of white dorsal muscle from 20 different Seabass specimens for each treatment were homogenized with 8 mL of 7% perchloric acid (1:2 w/v). The acidic mixtures were first centrifuged at 4 ◦C, 10000 rpm (Hermle z366 K) for 10 min and then neutralized to pH 7.80 ± 0.05 using 9 M KOH. Further centrifugation at 14000 rpm for 10 min at 4 ◦C (Scilogex D30243) was needed to remove potassium perchlorate precipitate. Supernatant (720 µL) was aliquoted and placed in Eppendorf microfuge tube adding 80 µL of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) 10 mM as inner standard and then centrifuged again at 14000 rpm for 10 min at 4 ◦C. Clear sample (690 µL) was placed into a standard 5 mm NMR tube with a TSP final concentration of 0.1 mM and measurements were performed. All the 300 1H NMR spectra were recorded at 300 K on a Bruker US+ Avance III spectrometer operating at 600 MHz, equipped with a BBI-z probe and a 24 SampleCase ™ cooled for sample storage and automation (Bruker BioSpin, Karlsruhe, Germany). Each spectrum was acquired using 32 K data points over a 7211.54 Hz spectral width and adding 256 transients. A recycle delay of 5 s and a 90◦ pulse of 11.4 µs were set up. Acquisition time (2.27 s) and recycle delay were adjusted to be 5 times longer than the T1 of the protons under investigation, which has been considered to be no longer than 1.4 s. The saturation of the residual water signal was achieved by irradiating it during the recycle delay at δ equal to 4.703 ppm. For each sample,128 scans were collected into 32 K data points covering a 12-ppm spectral width and requiring 22 min of measurement time. The phase correction and baseline were adjusted with TOPSPIN software version 3.5 pls (Bruker BioSpin, Karlsruhe, Germany) and successively the spectra were calibrated taking the chemical shift of the TSP signal to 0.000 ppm. For each sample, the analysis was performed in triplicate (Rocculi et al., 2019). Before statistical analysis, the NMR spectra underwent several preprocessing procedures, such as spectra alignment, removal of some peaks, normalization and a final binning. The alignment of all spectra is led on the internal standard (TSP) peak at 0.00 ppm, then some parts of spectra lacking metabolic information are removed: i) from 9.00 to 20.00 ppm and from 20.00 to 0.50 where there is only noise; ii) the part from 4.60 to 5.10 ppm where water peak may produce a high interference and iii) the part from 6.00 to 6.10 ppm where there is the TCA peak. The new dataset was normalized by the application of the Probabilistic Quotient Normalization (PQN) (Dieterle et al., 2006) which is based on the calculation of a most probable dilution factor by looking at the distribution of

quotients of amplitudes of a test spectrum by those of a reference one. Another further crucial data reduction is performed by using a binning also called bucketing algorithm (Craig et al., 2006). In this work, spectra were reduced to 431 bins of 100 points each which correspond to 0.0183 ppm of spectrum length. Bins with a loading value >1% of the overall standard deviation of all loading values were selected to determine the spectral regions encompassing most of the discriminative information (Picone et al., 2018).

5.3.7 Blood plasma analyses

The levels of glucose (GLU), urea, creatine, uric acid, total bilirubin, cholesterol (CHOL), triglycerides (TRIG), total protein (TP), albumin (ALB), aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), creatine kinase (CK), lactate dehydrogenase (LDH), calcium (Ca+2), phosphorus (P), potassium (K+) sodium (Na+), iron (Fe), chloride(Cl), magnesium (Mg) were determined in the plasma using samples of 500 µL on an automated analyser AU 480; 220 Olympus/Beckman Coulter, Brea, CA, United States) using specific methods (Olympus system 221 reagent, OSR) and according to Parma et al. (2023) . The Albumin/Globulin (ALB/GLO) ratio, lactate (LAC) and Current Calcium (Cur.Ca) were calculated.

5.3.8 Gut bacterial community DNA extraction, sequencing and analysis

Total DNA was extracted from individual distal gut content obtained from 5 fish per tank (300 mg per fish) at the end of the trial (for a total of 15 samples per experimental diet), as previously reported in Pelusio et al. (2021). DNA was then quantified with NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and stored at 20 ◦C until further processing. The amplification of the V3-V4 hypervariable regions of the 16S rRNA bacterial gene was carried out using the 341F and 785R primers with overhang sequencing adapters attached and 2 x KAPA HiFi HotStart ReadyMix (KAPA Byosystems). As already described by Pelusio et al. (2021), the thermal cycle consists of 30 amplification cycle, at the end the PCR products were purified, and indexed libraries were prepared following Illumina protocol “16S Metagenomic Sequencing Library Preparation”. Libraries were normalized to 4 nM and pooled, the resulting pool was denatured with 0.2 N NaOH and diluted to 6 pM with 20% Phix control. Sequencing was performed on Illumina MiSeq platform using 2 × 250 bp paired-end protocol according to the manufacturer's instructions (Illumina, San Diego, CA). At the end of the sequencing process, raw sequences were processed combining PANDAseq and QIIME2 pipelines (Bolyen et al., 2019; https://qiime2.org). High-quality reads, obtained after a filtering step for length (min/max = 350/550

bp) and quality with default parameters, were cleaned using DADA2 (Callahan et al., 2016) and clustered into amplicon sequence variants (ASVs) using VSEARCH algorithm (Rognes et al., 2016). Taxonomy was assigned using RDP classifier against SILVA database (Quast et al., 2013). Three different metrics were used to evaluate internal ecosystem diversity (alpha-diversity) – Faith's Phylogenetic Diversity (faith_pd), Shannon_entropy index, and number of observed ASVs (observed features). Unweighted UniFrac distances were computed to estimate inter-sample ecosystem diversity (beta-diversity) and used as input for Principal Coordinates Analysis (PCoA).

5.3.9 Statistical analysis

Growth and plasma biochemistry data are presented as mean ± standard deviation (SD). A single tank was used as the experimental unit for analysing growth performance and a pool of ten fish was considered the experimental unit for the analysis of carcass composition and nutritional indices. The homogeneity of variance assumptions was validated for all data preceding ANOVA. Tukey's post hoc test was performed. All statistical analyses for growth and plasma biochemistry were performed using GraphPad 8.0.1. The differences among treatments were considered significant at $P \leq 0.05$. NMR data underwent univariate and multivariate analyses according to Hatzakis (2019). In all pre-processing steps, univariate (ANOVA and T-Test) and multivariate data analyses (PCA) were implemented and conducted using the R free software environment for statistical computing (version 4.1.0). Essential bins responsible for group differentiation were chosen by applying the one-way analysis of variance (ANOVA) followed by Tukey's post hoc-test considering $P < 0.05$ as significant and by the Student's t-test. The chemical components from ANOVA were identified according to the literature, and by comparing their chemical shift and multiplicity with Chenomx software data bank, version 8.1 (Edmonton, AB, Canada). Microbiota analysis and respective plots were produced using R software (https://www.r-project.org/) with “vegan” (http://www.cran. r-project.org/package-vegan/), “Made4” (Culhane et al., 2005) and “stats” packages (https://stat.ethz.ch/R-manual/R-devel/library/stat s/html/00Index.html). Data separation was tested by a permutation test with pseudo-F ratios (function “Adonis” in “vegan” package). When required, Wilcoxon and Kruskal–Wallis tests were used to assess significant differences in alpha diversity and taxon relative abundance between groups. P-value ≤0.05 was considered statistically significant, while a P-value between 0.05 and 0.1 was considered as a trend.

5.4 Results

5.4.1 Growth

Results of growth performance and FI are summarized in Table 6. FBW was significantly higher in diet C and 0FM100FO compared to other treatments. SGR and WG were higher in C compared to other treatments, at the same time 0FM100FO was lower than C. FI values were significantly higher in 0FM0FO then 0FM0FO-0SP. FCR was statistically lower in C compared to 0FM0FO, 0FM0FO-50SP and 0FM0FO0SP.

Table 6 Growth performance and feed intake of Seabass fed experimental diets over 119 days.

	Experimental diets					
	C	0FM100FO	0FMFO	0FMFO-50SP	0FMFO-0SP	*P-value*
IBW	75.9 ± 1.06	75.8 ± 1.80	76.4 ± 0.79	75.8 ± 2.13	75.8 ± 1.45	*0.9875*
FBW	247 ± 4.75^{b}	239 ± 9.89^{b}	221 ± 5.89^{a}	222 ± 5.85^{a}	217 ± 9.24^{a}	***0.0021***
SGR	0.99 ± 0.01^{c}	0.97 ± 0.02^{b}	0.89 ± 0.04^{a}	0.90 ± 0.02^{a}	0.88 ± 0.02^{a}	***0.0013***
FI	1.19 ± 0.02^{ab}	1.22 ± 0.03^{ab}	1.24 ± 0.03^{b}	1.22 ± 0.02^{ab}	1.16 ± 0.04^{a}	***0.0294***
FCR	1.34 ± 0.02^{a}	1.40 ± 0.05^{ab}	1.50 ± 0.04^{b}	1.48 ± 0.05^{b}	1.43 ± 0.05a^{b}	***0.0038***
WG	171 ±3.97^{c}	164 ± 8.10^{b}	145 ± 5.57^{a}	146 ± 7.89^{a}	142 ± 7.84^{a}	***0.0012***
Survival	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	*1*

Data are given as the mean (n=3) ± SD. In each line, different superscript letters indicate significant differences among treatments (P ≤ 0.05).
IBW = Initial body weight.
FBW = Final body weight.
SGR = Specific growth rate (% day-1) = 100 * (ln FBW- ln IBW) / days.
Feed intake (FI, g kg ΔBW–1 day–1) = ((1000*total feed ingestion)/(ΔBW))/days.
FCR = Feed conversion rate = feed intake / weight gain.
WG = Weight gain (g)
Survival = Survival (%).

5.4.2 Proximate composition

Results of body proximate composition, nutritional indices and somatic indices are summarized in Table 7. No significant differences were observed for moisture, lipid and ash. Proteins in 0FM100FO were significantly higher compared to 0FM0FO. PER was higher in C than 0FM0FO, 0FM0FO-50SP and 0FM0FO-0SP, while 0FM0F0-0SP was higher than 0FM0FO and 0FM0FO-50SP. GPE was lower in 0FM0FO and 0FM0FO-50SP compared to C and 0FM100FO.Results of LER shown a P-value lower than 0.05 but non-specific differences among treatments were evaluated by multiple comparison Tukey's test. No effects were shown in GLE and CF. Results of VSI were statistically lower in diet C compared to 0FM0FO-0SP. 0FM0FO-0SP values of HSI were higher compared to 0FM0FO-50SP, 0FM0FO and C, at the same time 0FM0FO50SP was lower than 0FM0FO-0SP.

Table 7 Body composition, nutritional indices, somatic indices measured in Seabass.

	C	0FM100FO	0FMFO	0FMFO-50SP	0FMFO-0SP	*P-value*
			Whole body composition, %			
Moisture	60.9 ± 3.79	60.2 ± 8.55	60.3 ± 1.43	60.1 ± 0.48	60.8 ± 10.2	*0.4667*
Protein	17.0 ± 0.97^{ab}	17.2 ± 0.23^{b}	16.5 ± 0.52^{a}	16.8 ± 1.27^{ab}	16.9 ± 0.14^{ab}	***0.0253***
Lipid	18.6 ± 1.99	18.5 ± 5.6	19.4 ± 2.93	19.3 ± 0.47	18.7 ± 14.1	*0.1418*
Ash	3.67 ± 0.5	3.5 ± 1.99	3.25 ± 0.22	3.73 ± 0.57	3.57 ± 0.53	*0.0985*
			Nutritional indices			
PER	1.74 ± 0.02^{c}	1.69 ± 0.05^{bc}	1.51 ± 0.05^{a}	1.55 ± 0.05^{a}	1.66 ± 0.05^{b}	***<0.0001***
GPE	30.5 ± 1.51^{b}	29.9 ± 1.09^{b}	25.3 ± 0.54^{a}	26.6 ± 1.72^{a}	28.6 ± 1.09^{ab}	***0.0024***
LER	4.13 ± 0.05	4.07 ± 0.13	3.99 ± 0.02	3.97 ± 0.12	3.95 ± 0.13	***0.0376***
GLE	82.4 ± 3.52	80.6 ± 5.98	85.9 ± 5.26	85.2 ± 2.87	83.6 ± 4.56	*0.6476*
			*Somatic indice*s			
CF	1.31 ± 0.01	1.36 ± 0,02	1.24 ± 0.01	1.15 ± 0.00	1.24 ± 0.01	*0.1004*
VSI	11.9 ± 0.06^{a}	12.7 ± 0.18^{ab}	12.9 ± 0.12^{ab}	12.8 ± 0.01^{ab}	13.7 ± 0.22^{b}	***0.0226***
HSI	1.93 ±0.59^{a}	2.14 ± 0.47^{ab}	1.97 ± 0.74^{a}	2.35 ± 0.24^{b}	2.82 ± 0.42^{c}	***<0.0001***

Data are given as the mean (n=3 ± SD). In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
PER = Protein efficiency ratio = ((FBW-IBW)/protein intake).
GPE = Gross protein efficiency = 100*[(%final body protein*FBW) - (%initial body protein*IBW)]/total protein intake fish.
LER = Lipid efficiency rate = (FBW - IBW)/lipid intake.
GLE = Gross lipid efficiency = 100*[(% final body lipid ∗ FBW) - (% initial body lipid*IBW)]/total lipid intake fish.
CF = condition factor = 100 × (body weight, g)/ (body length, cm)3
VSI = Viscerosomatic index (%) = 100*(viscera weight/FBW).
HSI = Hepatosomatic index (%) = 100*(liver weight/FBW).
SD = Standard deviation.

5.4.3 Metabolomic analysis

The PCA obtained from the final data set together with the relative loadings plot for PC1 is shown in Figure 9. PC1 and PC2 explain, respectively, 60% and 15% of the total variance and the separation according to the diets occurs along PC1. The main metabolites which are involved along PC1 in the differentiation of samples, are shown by the loadings plot in Figure 9C. In Table 8 are listed the main bins contributing to the second principal component. Lactate was higher in C and 0FM0FO-0SP compared to the other treatments (Figure 9A). Creatine-p was higher in C compared to the other treatments while 0FM0FO-0SP was higher than 0FM100FO and 0FM0FO (Figure 9B). Glycine was lower in C compared to the other treatments except than 0FM0FO-0SP (Figure 9C). Taurine was higher in C compared to the other treatments while 0FM0FO-0SP was higher than 0FM100FO, 0FM0FO and 0FM0FO-50SP (Figure 10).

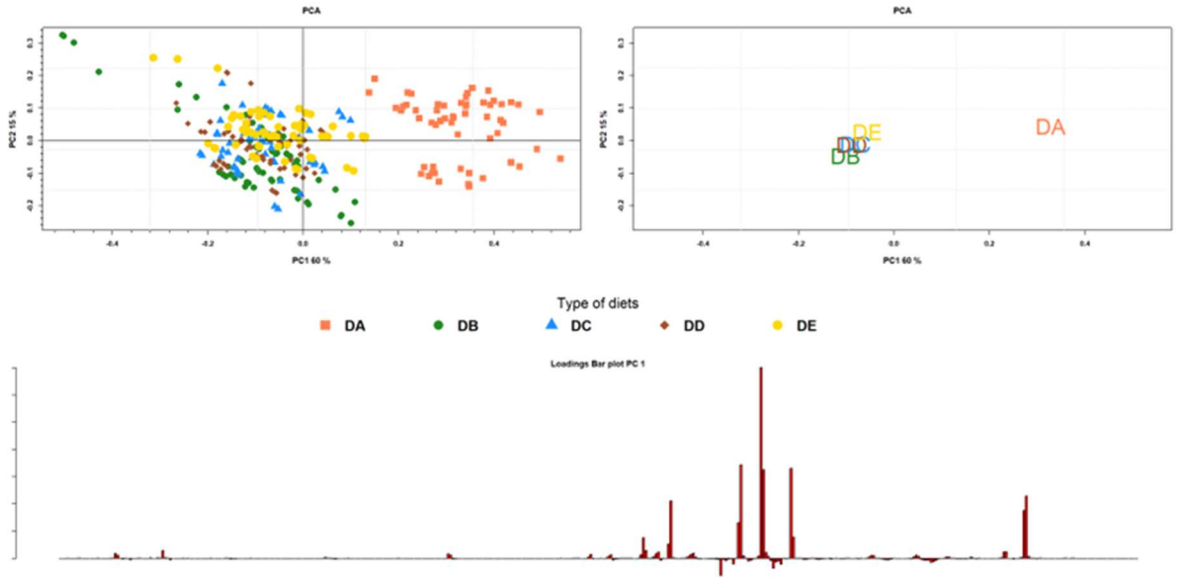

Figure 9 A) PCA and loading plot of Seabass samples reared with 5 different diets B) PCs centroids plot and C) Loandings plot from PC1.

Table 8 Significant bins and their corresponding metabolites from PC1 loadings plot.

Bin number	PC	ppm_s	ppm_f	Metabolite	Multiplicity*
234	1	4.146	4.110	Lactate	m
244-245	1	3.964	3.909	Creatine-p	s
265	1	3.579	3.543	Glycine	s
272-273	1	3.451	3.396	Taurine	t
281-282	1	3.287	3.232	Taurine	t
293-294	1	3.067	3.012	Creatine-p	s
386-387	1	1.366	1.311	Lactate	d

* s= singlet; d: doublet; dd: double doblet; m= multiplet; q= quartet

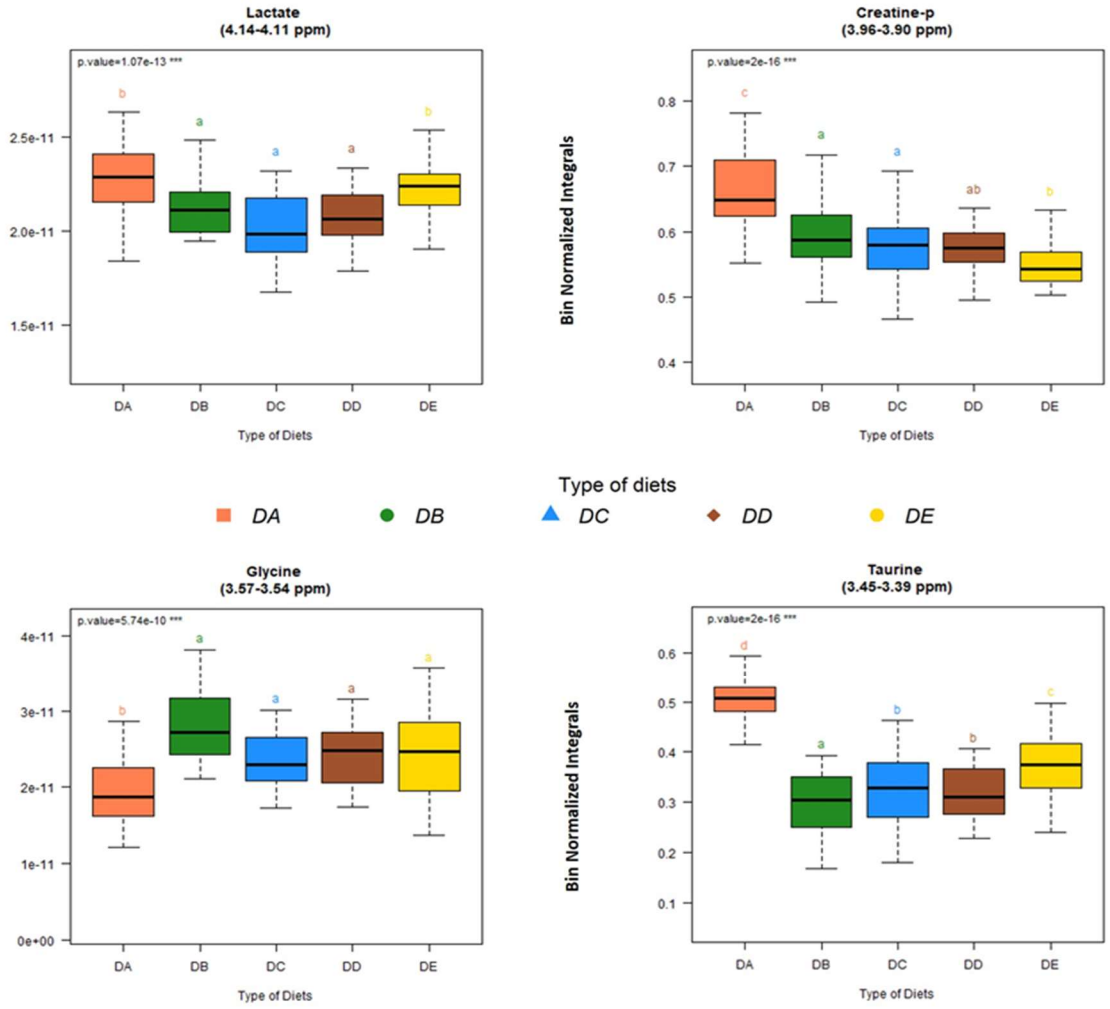

Figure 10 Boxplot of the main metabolites identified by PC analysis. Metabolites' concentrations were considered in area arbitrary units (normalized integrals). Statistical analysis was by one-way ANOVA, using Tukey's post-hoc test. Different letters in the same row indicate significant differences (at least $p < 0.05$).

5.4.4 Plasma biochemistry

Plasma parameter results are shown in Table 9. Among all data considered, statistical difference was highlighted in CREA, Uric Acid, ALP, P, Mg, HDL, TP, ALB/GLOB and LAC values. In animals fed with C diet, CREA value was higher compared to other treatments. Results of Uric Acid showed higher values in diet C compared to 0FM0FO, 0FM0FO-50SP and 0FM0FO-0SP. ALP and TP were both lower in diet 0FM100FO compared to 0FM0FO-0SP. P shown a P-value lower than 0.05 but non-specific difference among treatments was evaluated by multiple comparison Tukey's test. Diet C showed higher value of Mg compared to 0FM0FO-0SP. HDL value was lower in 0FM0FO compared to 0FM0FO-0SP. ALB/GLO was higher in 0FM0FO compared to 0FM0FO-0SP-values of LAC were lower in 0FM100FO, 0FM0FO-50SP and 0FM0FO-0SP compared to other treatments.

Table 9 Plasma biochemistry in Seabass fed with experimental diets.

	C	0FM100FO	0FMFO	0FMFO-50SP	0FMFO-0SP	*P - value*
GLUC	211 ± 86.5	194 ± 62.8	157 ± 37.4	196± 53.1	186 ± 55.2	*0.1591*
Urea	8.69 ± 2.35	8.98 ± 1.63	9.32 ± 2.05	8.77 ± 1.49	7.87 ± 1.43	*0.2596*
CREA	0.88 ± 0.15^{b}	0.41 ± 0.08^{a}	0.38 ± 0.08^{a}	0.41 ± 0.09^{a}	0.42 ± 0.09^{a}	***<0.0001***
Uric.Acid	0.54 ± 0.56^{b}	0.29 ± 0.26^{ab}	0.19 ± 0.18^{a}	0.19 ± 0.14^{a}	0.13 ± 0.1^{a}	***0.0018***
Tot Bil	0.03 ± 0.02	0.03 ± 0.03	0.04 ± 0.03	0.04 ± 0.03	0.04 ± 0.03	*0.7198*
Ast	109 ± 84.7	101 ± 106.9	116 ± 93.2	347 ± 837.0	157 ± 80.2	*0.3507*
Alt	18.6 ± 33.1	6.28 ± 4.73	8.18 ± 3.85	117.0 ± 417.7	17.3 ± 23.6	*0.4219*
Alp	65.4 ± 11.6^{ab}	61.7 ± 13.2^{a}	67.2 ± 8.04^{ab}	71.1 ± 14.3 ^{ab}	77.3 ± 13.2^{b}	***0.0093***
CK	1998 ± 1098	1755 ± 2515	2770 ± 2672	2649 ± 2096	2224 ± 1430	*0.6194*
LDH	148 ± 55.4	143 ± 75.3	185 ± 127	180 ± 142	136 ± 51.8	*0.5062*
Ca^{2+}	15.4 ± 1.51	14.1 ± 1.62	15.1 ± 1.49	15.2 ± 2.23	15.3 ± 1.3	*0.1937*
P	12.1 ± 1.26	11.3 ± 1.78	12.6 ± 1.48	12.7 ± 1.57	12.8 ± 1.25	***0.0483***
Mg	4.15 ± 0.76^{b}	3.51 ± 0.44^{ab}	3.72 ± 0.68^{ab}	3.77 ± 0.73^{ab}	3.45 ± 0.44^{a}	***0.0222***
CHOL	301 ± 64.4	265 ± 41.9	250 ± 42.8	262 ± 47.3	275 ± 45.4	*0.0569*
HDL	83.8 ± 20.3^{ab}	74.1 ± 19.3^{ab}	69.4 ± 18.1^{a}	76.2 ± 17.9^{ab}	88.5 ± 18.8^{b}	***0.0431***
TRIG	1370 ± 465	1184 ± 282	1378 ± 346	1505 ± 348	1215 ± 358	*0.1215*
TP	5.04 ± 0.72^{ab}	4.45 ± 0.58^{a}	4.82 ± 0.63^{ab}	4.73 ± 0.63^{ab}	5.22 ± 0.54^{b}	***0.0154***
Alb	1.43 ± 0.20	1.27 ± 0.16	1.39 ± 0.18	1.35 ± 0.18	1.41 ± 0.15	*0.0966*
Cur. Ca	17.5 ± 1.35	16.3 ± 1.50	17.2 ± 1.37	17.4 ± 2.06	17.4 ± 1.26	*0.2472*
ALB/GLO	0.39 ± 0.02^{ab}	0.39 ± 0.03^{ab}	0.41 ± 0.03^{b}	0.40 ± 0.03^{ab}	0.37 ± 0.03^{a}	***0.0477***
LAC	62.7 ± 19.5^{b}	47.9 ± 8.9^{a}	58.2 ± 8.44^{b}	49.0 ± 14.6^{a}	43.3 ± 9.30^{a}	***0.0004***
Fe	131 ± 42.3	112 ± 21.8	111 ± 26.8	108 ± 15.9	111 ± 20.5	*0.1330*
Na^{+}	181 ± 12.0	176 ± 13.8	177 ± 5.21	183 ± 16.6	179 ± 10.6	*0.5775*
K	2.18 ± 0.66	2.16 ± 0.86	2.20 ± 1.27	2.87 ± 1.21	2.67 ± 1.12	*0.1788*
Cl	145 ± 12.0	143 ± 11.2	143 ± 4.44	148 ± 13.6	145 ± 9.02	*0.6398*

Data are given as the mean (n=15 diet^{-1}) ± SD. Different letters indicate significant difference (One-way ANOVA $P \leq 0.05$) between treatments. GLU, glucose , (mg dL^{-1}); Urea , (mg dL^{-1}); CREA, creatinine , (mg dL^{-1}); Uric Ac, uric acid , (mg dL^{-1}); Tot Bil, total bilirubin , (mg dL^{-1}); Ast, aspartate aminotransferase , (U L^{-1}); Alt, alanine aminotransferase (U L^{-1}); Alp, alkaline phosphatase , (U L^{-1}); CK, creatine kinase , (U L^{-1}); LDH, lactate dehydrogenase , (U L^{-1}); Ca^{+2} , calcium , (mg dL^{-1}); P, inorganic phosphorus; , (mg dL^{-1}); Mg, magnesium , (mg dL^{-1}); CHOL, cholesterol , (mg dL^{-1}); HDL, high density lipoprotein; TRIG, triglycerides , (mg dL^{-1}); TP, total protein , (mg dL^{-1}); Alb, albumin , (g dL^{-1}); Cur Ca2+, current calcium (mg dL^{-1}); ALB/GLO, albumin/globulin; LAC, lactate (mmol L^{-1}); Fe, iron , (µg dL^{-1}); Na^{+}, sodium , (mEq L^{-1}); K^{+}, potassium , (mEq L^{-1}); Cl, chloride , (mEq L^{-1}); SD, standard deviation.

5.4.5 Gut microbiota

The 16S rRNA gene sequencing was performed on a total of 75 distal intestine content samples, yielding 593′045 high-quality reads (mean ± SD, 7′907 ± 2′938) and clustered into a total of 8′596 ASVs. In order to assess the effects of replacement of FM, FO and SP on the gut bacteria community during the growth process of Seabass, the gut microbiota (GM) was analysed for each dietary group at the end of the trial. Prin cipal Coordinates Analysis (PCoA) based on Unweighted UniFrac distances evaluated the GM variations between samples (beta-diversity). Moreover, the gut microbial community diversity, within each dietary group, was represented with faith-PD, Shannon_entropy and observed features. According to our results (Figure 11), in terms of overall GM composition, not all dietary groups showed a significant variation compared to the control group, regarding both alpha and beta diversity ($p > 0.05$). The overall GM composition at different phylogenetic levels was investigated, as reported in Figure 12, at phylum, family (Figure 13) and genus level (Figure 14). More specifically, at phylum level the most abundant taxa observed was Firmicutes (with an overall relative abundance mean of 71%), but no difference was evaluated among treatments. The most represented family was *Lactobacillaceae* (overall r.ab. mean 55%), followed by a much lower abundance of *Staphylococcaceae, Planctomycetaceae, Bacillaceae*, S*treptococcaceae* and *Enterococcaceae* (overall r.ab. mean 3%, 2%, 2%, 1% and 1%), all belonging to Firmicutes phylum except for *Planctomycetaceae*. Those last family groups all showed statistical differences among groups in terms of relative abundance, with lower values in Ctrl group compared to the others experimental diets (Wilcoxon rank-sum test $p < 0.05$) (Figure 13).

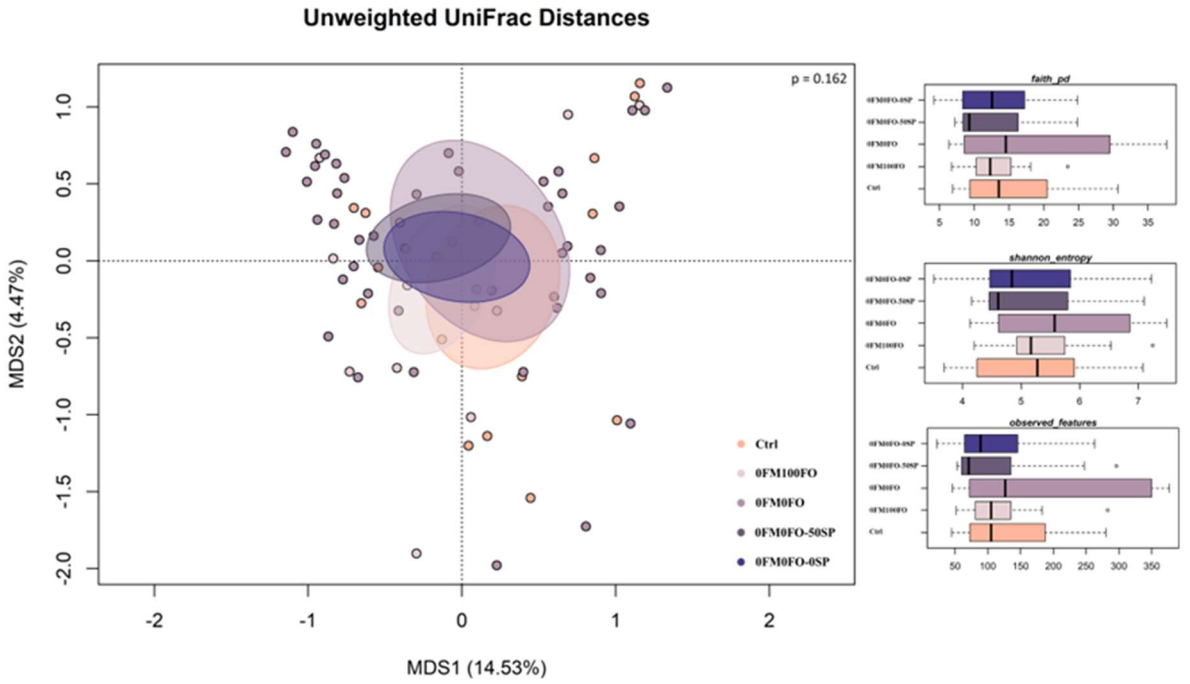

Figure 11 Beta diversity and alpha diversity of gut microbiota of Seabass fed with experimental diets ober 119 days. On the left, Principal Coordinates Analysis (PCoA) based on unweighted UniFrac distances between gut microbiota composition of animales fed with experimental diets. No significant separations were highlighted (permutation test with pseudo-F ratios Adonis; p= 0.162). On the right, Boxplots of alpha diversity values with 3 metrics, faith_pd, shannon_entropy and observed_features (ASVs). All metrics didn't higlight any significant variations (Kruskal-Wallis test $p > 0.05$) of alpha diversity among dietary groups.

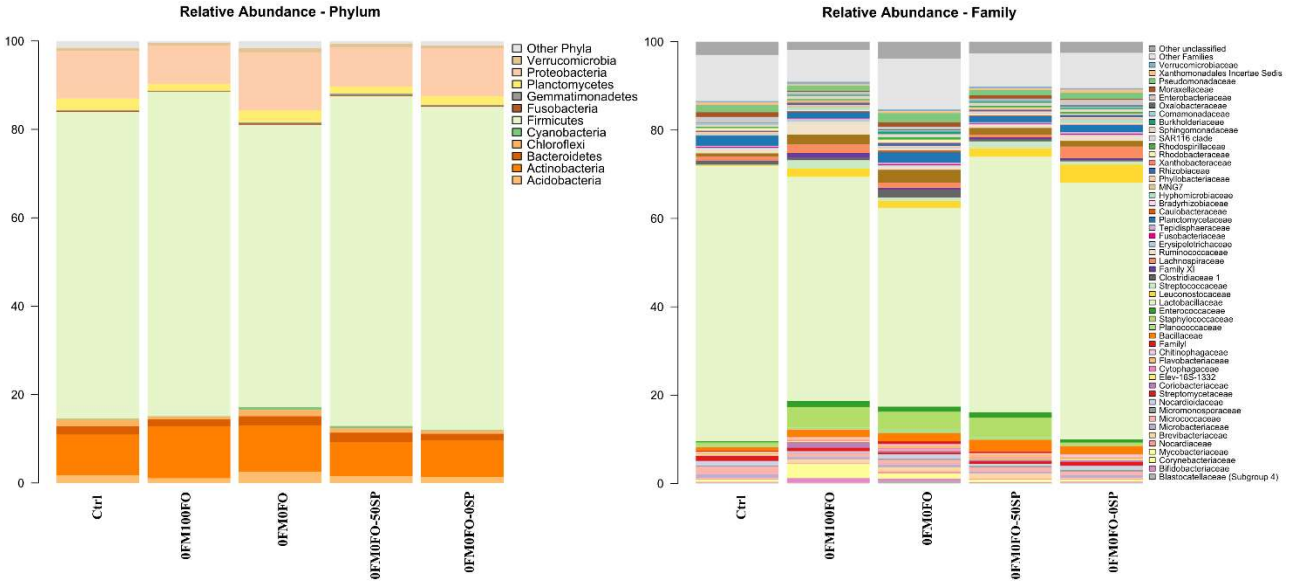

Figure 12 Microbiota composition of distal gut content of Seabass fed with experimental diets. Bar plot summarizing the microbiota composition at Phylum (left) and family (right) of fish intestinal content. Only phyla and families with a relative abundance ≥ 1.0% in at least 3 samples are showed.

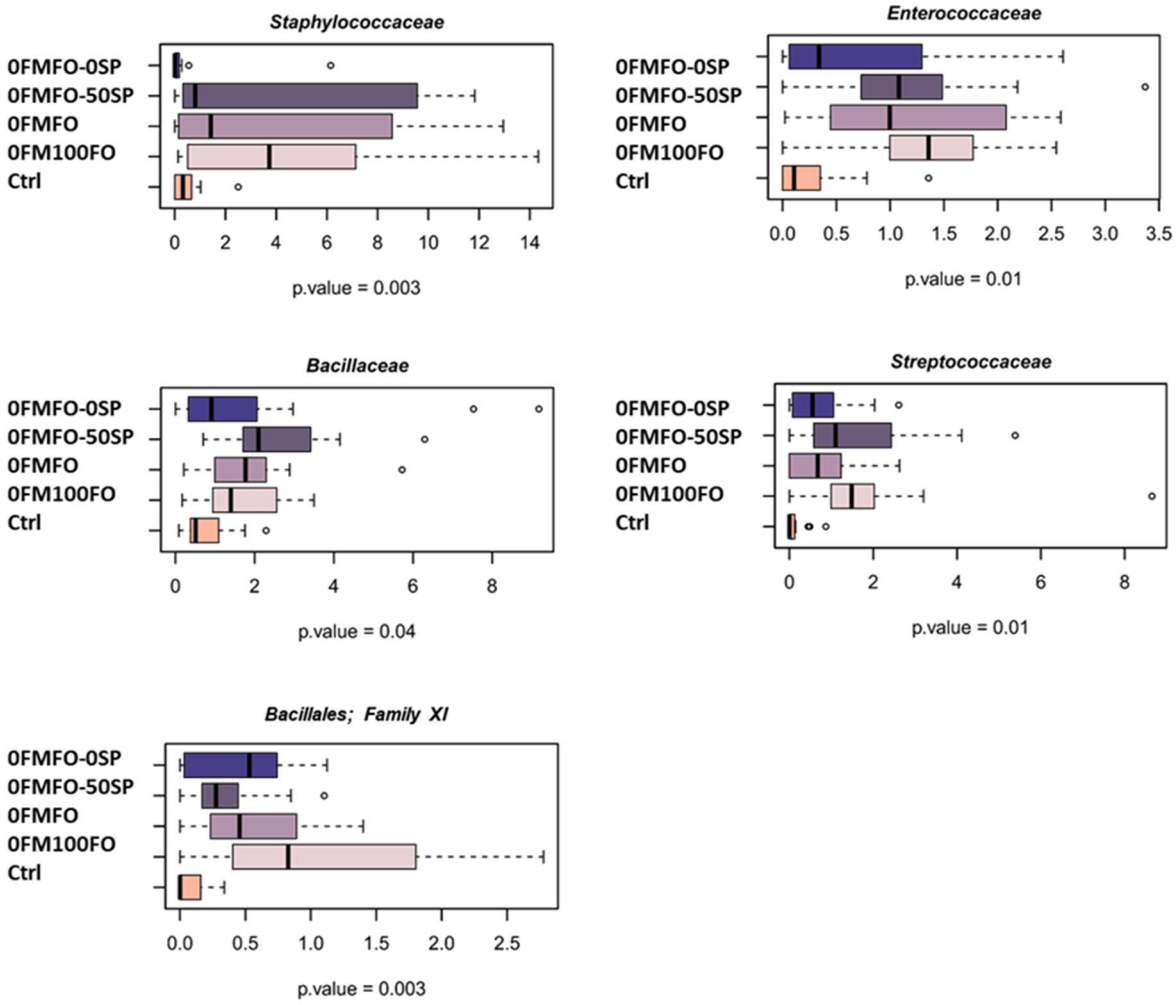

Figure 13 Taxonomic composition of bacterial communities of distal gut content of Seabass fed experimental diets. Distributions of relative abundance of families that showed a significant variation between groups fed with different diets (Wilcoxon rank-sum test, *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$). Only families with a mean relative abundance ≥ 1.0% in at least 3 samples were represented. The central box of each dataset represents the distance between the 25th and the 75th percentiles. The median between them is marked with a black line.

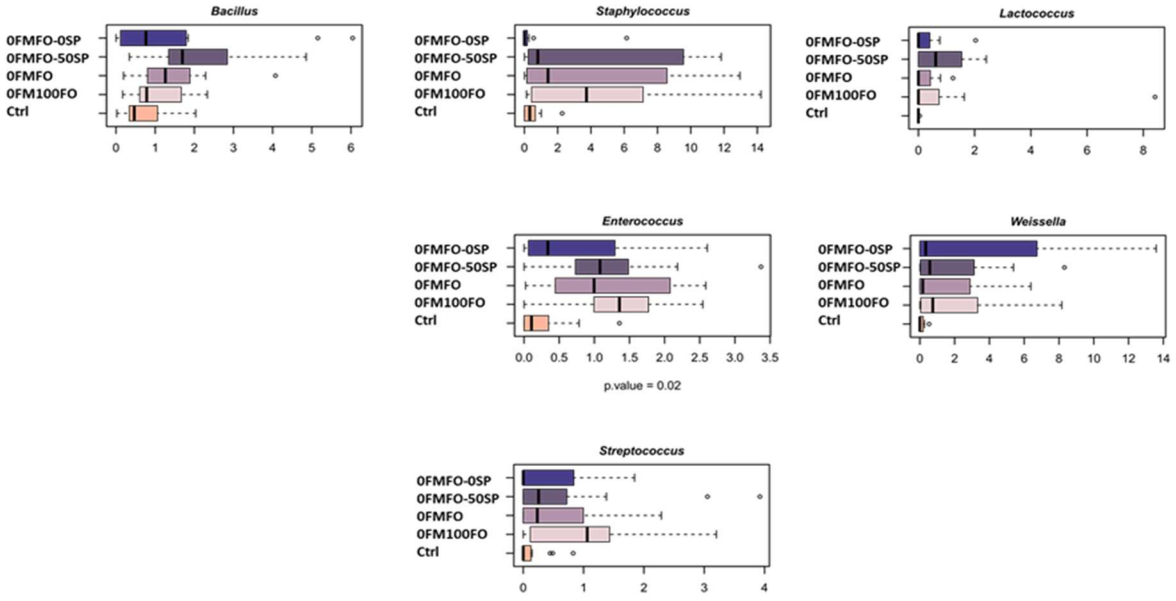

Figure 14 Taxonomic composition of bacterial communities of distal gut content of Seabass fed experimental diets. Distributions of relative abundance of genera that showed a significant variation between groups fed with different diets (Wilcoxon rank-sum test, *** $p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$). Only genera with a mean relative abundance $\geq 1.0\%$ in at least 3 samples were represented. The central box of each dataset represents the distance between the 25th and the 75th percentiles. The median between them is marked with a black line.

5.5 Discussion

Several studies have investigated the inclusion of plant derivates as alternative ingredients for FM and FO replacement in Mediterranean species, while less attention was paid to the application of fish byproducts. The use of discarded fish by-products reduces the demand for FM from whole fish, may reduce pressure on fish stocks and is associated with sustainable farming of aquatic animals (Colombo et al., 2022). According to the present study the total replacement of FM with FM by-products did not affect the overall growth performance, feed utilization and FI. Previous studies showed good potential to replace wild-caught FM using FM by-products in several carnivorous marine fish species (Li et al., 2004; Uyan et al., 2006; Benitez-Hernandez et al., 2018; Kim et al., 2022) even if most of these studies did not exceed 50% of the FM replacement. If by-products can satisfy fish nutrient requirements in terms of protein, or the amino acids profile of the byproduct is similar to standard FM, no negative effect can be expected. However, FM by-products may contain considerably high levels of bones with lower availability of nutrients such as phosphorus and calcium which can affect overall performance (Albrektsen et al., 2022). Further replacement of wild FO with FO from by-products resulted in reduced growth as observed in 0FM0FO. In fact, the total replacement of both wild FMFO leads to a ~ 10% reduction in SGR mainly due to a reduced FCR and specifically a reduced protein efficiency. Several studies have found reduction in performance of this species with the decreasing of marine FO in favour of vegetable oils. The reduction of LC-PUFA could affect growth performance as previously described on this species (Torrecillas et al., 2017a, 2018; Pelusio et al., 2022). However, a recent finding denotes that 0.9% dietary LC-PUFA was effective to cover the requirements for European Seabass from juveniles to adults (Castro et al., 2022). This finding is in line with the 0.7% LC-PUFA requirement established by Skalli and Robin (2004) for this species. Dietary Σn-3/Σn6 are also known to affect growth performance and stress response in fish species (Montero and Izquierdo, 2010). At this regard, the dietary Σn-3/Σn-6 reported in the present study are in line with previous studies on this species which provided a successfully nearly complete replacement of FO by vegetable oil when a suitable source of LC-PUFA was present (Torrecillas et al., 2017b; Castro et al., 2022). In the light of the above-mentioned studies, we may exclude a dietary deficiency in LCPUFA or altered Σn-3/Σn-6 able to affect the growth performance. However, it should be mentioned that different raw materials could have different lipid and energy availability for fish growth, which could have been responsible for the growth impairments observed. The replacement of soy products (SBM and SPC) with

more sustainable vegetable protein ingredients (wheat gluten and corn gluten, 0FM0FO-50SP and 0FM0FO0SP) in terms of land and water uses (Newton and Little, 2018, Newton et al., 2023) did not further alter growth except for a slight reduction in FI in comparison to 0FM0FO. Despite dietary SBM being well known to possibly induce inflammation processes in the distal intestine of some fish species (Baeverfjord and Krogdahl, 1996; Knudsen et al., 2008), the SBM level employed in the present study is within the tolerance range for European Seabass (Bonvini et al., 2018b). Previous studies on this and other Mediterranean species, have shown that 70% FM protein can be successfully replaced by wheat glutens in diets supplemented with the most limiting amino acids (Messina et al., 2013). However, reduction in palatability was also observed in gilthead sea bream fed 42% glutens in comparison to a blend of vegetable protein ingredients also including soybean meal (SBM, Parma et al., 2016). The application of plant protein mixture in aquafeed formulation is known to reduce the inhibition effect of feed intake given by a specific effect of a single ingredient (Bonaldo et al., 2015). On the other hand, the inclusion of yeast in 0FM0FO-0SP was probably responsible for restoring the FCR to that of the control group as also indicated by an increase in protein efficiency. In addition to an optimal protein profile (equivalent to SBM), yeasts are known to promote feed efficiency via the action of bioactive and prebiotic compounds such as vitamins B, β-glucans, mannoprotein and nucleotides (Agboola et al., 2021). The application of metabolomics tools to fish nutrition is in its early stages, but recently some studies have demonstrated that it can provide a global insight into muscle metabolism by the identification of multiple metabolites involved in the biological responses of individuals exposed to different dietary treatments (Roques et al., 2020). In the present study, the overall metabolomic profiles of fish muscle were clearly impacted by the diets. The PCA reveals a cluster of the control diet separated by the other diets which otherwise showed complete overlap. This finding agrees with previous studies where the substitution of FM with alternative protein sources such as soybean meal, insect meal, and feather meal (Jasour et al., 2017; Casu et al., 2017; Roqueset al., 2020b) impacted the metabolic profiles of fish muscle. In the present study, a deep investigation reveals the main metabolites which were responsible for the separation in the PC. Specifically, phosphocreatine and lactate tended to be lower in the muscle of fish fed with FM by-products compared to those fed wild FM diet. These metabolites have a key role on muscle energy metabolism both in energy store and energy production; thus, the changes of these metabolites could indicate a disturbance of energy metabolism or (as in the case of creatine) can also be attributed to a reduced supply due to plant-based diets or

other alternative ingredients to FM (Roques et al., 2020b). Interestingly, the higher level of glycine found at increasing FM replacement may also explain changes in metabolic pattern for energy production. In fact, increasing muscle level of glycine was previously found in fish fed a plant-based diet and may indicate an adaptation of energy metabolism towards an increased protein catabolism for the production of glucogenic amino acids into energy to counterbalance an energy-deficient state (Casu et al., 2017; Wei et al., 2017). Another explanation of the increase in the glycine could be in relation to the creatine synthesis since glycine together with arginine, is transformed into its precursor, the guanidinoacetate. Creatine seems to be synthesized in the fish muscle but its uptake can also occur from the feed (Wuertz and Reiser, 2022; Borchel et al., 2014). Therefore, exogenous creatine seems to regulate the endogenous creatine synthesis. The presence of both metabolites in the muscle (glycine and p-creatine) may then suggest a difference in creatine metabolism when wild FM was replaced, probably due to a lower creatine content in the FM by-product. Although it has not been measured, a lower creatine content in fishmeal by-products is expected since 95% of the creatine is stored in fish muscle which is usually a minor component of FM derived from trimming. This is also supported by the higher creatinine level found in the blood of fish fed wild FMFO indicating the higher creatine metabolism of fish fed this diet since creatinine formation occurs spontaneously during the conversion of creatine to phosphocreatine. Creatine metabolism and requirements in fish species are still poorly investigated; however, creatine is a key factor in the development of skeletal muscle and its inclusion seems advisable in carnivorous fish fed plant-based diets (Wuertz and Reiser, 2022). The higher level of taurine in the muscle of fish fed with the control diet could also explain the differences in growth performance. In turbot, a metabolomic study revealed that a high plant protein diet reduced taurine level in muscle in comparison to a FM control diet indicating that dietary taurine level may affect taurine metabolism in the muscle (Wei et al., 2017). Among its several physiological functions, dietary taurine promotes proliferation of muscle fibrils and elevates quantities of myosin and actin myofilaments to increase muscle growth of European Seabass (Saleh et al., 2020; Wassef et al., 2021). To evaluate welfare indicators, plasma biochemistry and gut microbiota were performed. Most of the plasma parameters analysed were in line with the normal range for this species, indicating a general optimal welfare condition for all the treatments (Pelusio et al., 2022; Bonvini et al., 2018b; Peres et al., 2014). The most significant differences occurred in the level of creatinine, uric acid and lactate with higher values in C diet compared to the other treatments. Interestingly, this value seems to be strictly correlated with the higher

phosphocreatine and lactate found in the muscle of the fish of the same treatment, supporting higher energy muscle metabolism in specimens fed C diet. In gilthead sea bream, a high level of plasma creatinine was previously associated to high FMFO diet in combination to a single meal during a feeding frequency trial or during different rearing density, indicating higher protein metabolism in comparison to a low FMFO diet (Busti et al., 2020a; Parma et al., 2020). On the other hand, in European Seabass the observed higher plasma lactate after stress exposure only in fish fed high FM, FO level (30% FM, 15% FO) indicates a stronger metabolic muscle response to crowding in comparison to a low FMFO level (10% FM, 3% FO) (Pelusio et al., 2022). It should also be noted that plasma Mg tended to be higher under this treatment. It is well known that Mg participates in muscle energy metabolism and Mg plasma level is positively associated with muscle performance in both human and animals (Zhang et al., 2017). Gut microbiota is a valid method to assess digestive condition and gut health especially concerning dietary formulation, which can drive gut microbiome towards potential consequences on metabolism and host immune response. Up to now several studies have addressed the effect of novel ingredients/additives including insect meal (Rangel et al., 2022; Perez-Pascual et al., 2020), oligosaccharides (Rimoldi et al., 2020), organic acid (Busti et al., 2020b), glycerol (Louvado et al., 2020); and vegetable ingredients (Parma et al., 2019; Serra et al., 2021). However, no studies have assessed the gut microbiota layout in response to the total replacement of wild marine feedstuff using by-products from fisheries and aquaculture. GM was dominated by Firmicutes phylum, while *Lactobacillaceae, Staphylococcaceae, Bacillaceae, Streptococcaceae* and *Enterococceae* were the most represented bacterial families. These data are in accordance with previous studies on this species, recognizing bacteria within Firmicutes phylum as promoters of a healthy intestinal epithelium and generally of a good fish health status in European Seabass and other Mediterranean species (Parma et al., 2019; Parma et al., 2020; Busti et al., 2020b; P´erez-Pascual et al., 2020). No effect of diets on overall gut microbiota composition were observed as highlighted by Alpha and Beta diversities indices. Data that agreed with previous observations where slight differences in the growth performance followed by FMFO replacement with alternative non-marine ingredients did not result in gut microbiome alteration (Pe´rez-Pascual et al., 2020). Indeed, in the present study a greater abundance of some specific taxa was observed at the increase of fish by-product inclusion (i.e. *Bacillus, Staphylococcus, Streptococcus, Enterococcus, Weissela, Lactococcus, Leuconostocacea*). Specifically, it was observed that the inclusion of the FM by trimming (0FM100FO) was mainly responsible for promoting taxa

such as *Streptococcus, Weissela* and *Enterococcus* while changes in *Bacillus* abundance were enhanced only after the FO by-product inclusion. These data support a general gut health status of fish fed under FM and FO byproduct; in fact, most of these families and genera belonging to Firmicutes phylum, such as *Bacillus, Weissela, Leuconostaceae, Streptococcus, Lactococcus, Enterococcus* are considered beneficial taxa for fish species with several positive contributions to nutrition, the immune system, and disease resistance (Ringø et al., 2020a, 2020b).

5.6 Conclusion

This study highlighted the potential of totally replacing wild-caught FM and FO and soy protein using by-products from fisheries and aquaculture and glutens, with only a marginal reduction of overall performance. In particular, when only wild-caught FM was totally replaced by FM by-products no differences were recorded, while the combined replacement of wild-caught FM and FO resulted in a performance reduction. The replacement of soy products (SBM and SPC) with more sustainable vegetable protein ingredients (wheat and corn gluten) in terms of land and water uses did not further alter growth. On the other hand, the inclusion of 2% of yeast in zero wild caught FMFO and soy protein was probably responsible for restoring the FCR and the protein efficiency to that of the control group. Muscle metabolomic profiles suggest changes or adaptation in the metabolic pattern for energy production at the muscular level when fish were fed FM and FO by-products. These findings deserve further investigation in regard to creatine and taurine metabolism or dietary supplementation for this species. The absence of differences in gut microbiota layout suggests no major implication of FM and FO byproducts on gut health. Rather, the inclusion of FM and FO byproducts promoted several taxa such as *Weissela, Enterococcus, Streptococcus* and *Bacillus* which can potentially support the immune system and disease resistance.

5.7 Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

5.8 Acknowledgement

This research was undertaken under the NewTechAqua (New technologies Tools and Strategies for a Sustainable, Resilient and Innovative European Aquaculture) project, which has received funding from the European Union's Horizon 2020 Programme under grant agreement No 862658 (https://www.newtechaqua.eu/). This study represents partial fulfilment of the requirements for the Ph.D. thesis of Daniel Scicchitano at the Ph.D. course of Innovative Technologies and Sustainable Use of Mediterranean Sea Fishery and Biological Resources (FishMed – University of Bologna, Italy). The authors would like to thank Gillian Forlivesi Heywood for English.

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Chapter 6

The use of fishery and aquaculture by-products with *Nannochloropsis* sp. allows total dietary replacement of wild-caught fishmeal, fish oil and soy protein in European sea bass juveniles.

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Published on: Aquaculture, (2024)

DOI: https://doi.org/10.1016/j.aquaculture.2024.741015

6.1 Abstract

Five experimental diets (CTRL, 50FMFO, 50FMFO-50MIC, 0FMFO-50MIC, 0FMFO-100MIC) were formulated to replace wild fishmeal (FM), wild fish oil (FO) and soy protein using fisheries, aquaculture by-product (BP) and microalgae (MIC). Fifty European Seabass juveniles were distributed in 15 tanks (initial body weight 46.66 ± 0.04 g) and reared in recirculating system for 88 days. Temperature, salinity, oxygen and photoperiod were kept constant throughout the experiment (22 ± 0.5°C, 25 g L^{-1} and 8.0 ± 1.0 mg L^{-1}, 12:12 light/dark, respectively). At the end, growth indexes, feed intake (FI), proximal composition, nutritional index, apparent digestibility, somatometric indexes, blood plasma biochemistry and digestive enzymes activity were evaluated. Also, gut microbiota composition was assessed through next-generation sequencing. Results showed that growth, digestibility, and nutritional performance were not affected by FM and FO and soy replacement using BP and MIC. Less than 50% of MIC and total replacement of FM and FO with BP have positive effects on enzyme activity. Moreover, inclusion of BP and MIC had positive effects on the microbiota richness and abundance. In conclusion, the utilization of BP and MIC is a valuable alternative to FM and FO as well as soy protein in feed for European Seabass juveniles.

Keywords

Fish meal, Fish oil, microalgae, gut microbiome, nutrition, growth.

6.2 Introduction

Fish by -products are playing a major role in reducing food losses and waste, enhancing food security and nutrition, promoting environmental sustainability and climate change mitigation across various food systems (FAO, 2019; Olesen et al., 2023). Recently, in the aquaculture sector, there has been a growing interest in utilizing fisheries and aquaculture by -products as valuable alternative raw materials to fish meal (FM) and fish oil (FO) derived from wild stocks. This interest comes from the aim to reduce the carbon footprint of finfish production and to preserve wild fish stocks (Newton et al., 2023). In this regard, fish processing procedures can generate a huge amount of discharged material (up to 70%), consisting of head, skin, bones and viscera (Kandyliari et al., 2020). However, the discharge material is still rich in micro and macronutrients such as protein s, lipid s and essential fatty acids, mineral s and vitamins (Mutalipassi et al., 2021). This material can be processed at an industrial scale with advanced technologies to extract highly nutritious fish meal and oil, ensuring that no part of the fish goes to waste (Coppola et al., 2021). Thus, fish by -products have been already integrated into 30% and 50% of the world FM and FO production, respectively and represent an extremely promising ingredient to be used by aquaculture feed industries (IFFO, 2022) . Microalgae are renowned in fish nutrition for their high content of EPA and DHA, essential fatty acids crucial for various life stages, from larvae to adults. Consequently, microalgae are frequently incorporated into feed formulations (Ansari et al., 2021). They also provide varying protein percentages, ranging from 18% to 46%, with some species reaching up to 69%, making them a significant source of essential amino acids (Nagarajan et al., 2021). Due to their nutritional profile and the growing demand for alternative aquafeed ingredients, the global market for microalgae is expected to surge from $32.6 billion annually in 2017 to $53.43 billion by 2026 (Nagarajan et al., 2021). Moreover, microalgae are highly sustainable, capable of thriving on diverse substrates and in various conditions, including waste materials such as wastewater (Gamboa - Delgado et al., 2018). Positive results with the addition of microalgae as protein sources in aquafeed have been reported in salmon (Gong et al., 2019) and many Mediterranean species, such as meagre (Estevez et al., 2022), gilthead sea bream (Carvalho et al., 2021) and European sea bass (Pascon et al., 2021). In this latest species, different microalgae species such as Tetraselmis sp. (Tulli et al., 2012), Isochrysis sp. (Tibaldi et al., 2015) and Pavlova viridis (Haas et al., 2016) have been tested as a replacement of fish meal and fish oil, with the percentage ranging from 20% to 100% of microalgae biomass. Recently, *Nannochloropsis* sp. showed promising results on growth

performances (Ayala et al., 2023) and intestinal health in gilthead sea bream (Saez et al., 2022) and European sea bass (Haas et al., 2016; Castro et al., 2016). Considering these various factors, it is important to note that despite numerous studies examining the effects of a diet based solely on fish oil and fish meal trimmings or microalgae on European sea bass, there is a scarcity of literature addressing the combined impact of these ingredients on both growth and health. Further research is needed to understand the potential distinct effects of incorporating both these components in the diet of European sea bass, providing valuable insights into optimizing nutrition and enhancing the well -being of this species. In this scenario, we postulate d that blending FM and FO obtained from trimming with microalgae in the European sea bass diet could serve as valuable alternatives to wild -caught fish and soy meal. This combination holds the promise of enhancing growth performance and gut health, leveraging the nutritional attributes of these ingredients while advancing aquaculture sustainability. Following a methodology akin to Marchi et al. (2023) for European sea bass, this study focused on earlier juvenile specimens. This choice was prompted by the acknowledgment of potentially heightened nutritional requirements, particularly concerning protein and fatty acid composition. Although this approach may entail increased feed costs, the potential to enhance animal quality justifies such investment.

6.3 Materials and Methods

6.3.1 Experimental diets

Five experimental diets were developed to sequentially substitute wild -caught FM and FO with fisheries and aquaculture by -products, alongside the replacement of soy protein concentrate with microalgae (MIC). Specifically, wild -caught FM (FM Prime) and FO (FO Extra) were first half and then totally replaced by FM and FO trimming (50% FM, 50% FO), (50FMFO), while soy protein concentrate was partially replaced by microalgae (50% FM, 50% FO, 50% MIC), (50FMFO -50MIC). Then wild -caught FM and FO were totally replaced by FM and FO trimming with a concomitant partial (0%FM, 0%FO, 50%MIC), (0FMFO -50MIC) or total (0% FM, 0%FO, 100%MIC), (0FMFO -100MIC) replacement of soy protein concentrate by the microalgae *Nannochloropsis* sp. Diets were produced with a diameter of 3 mm by the University of Almeria, Spain. The formulation was designed by Agricola Italiana Alimentare S.p.a – AIA, Verona, Italy; in accordance with the University of Almeria and the University of Bologna. Briefly, all ingredients were mixed in a 120 L mixer, grounded with a hammer mill (UPZ 100, Hosokawa -Alpine, Augsburg, Germany) to 0.5 mm. The diets were extruded in a twin -screw extruder (Evolum 25, Clextral, Firminy, France), fitted with 3 mm die holes.

The extruder barrel consisted of four sections and the temperature profile in each segment (from inlet to outlet) was 90, 95, 95, and 105 °C, respectively. The pellets were dried after extrusion at 27 °C using a drying chamber (Airfrio, Almería, Spain), and cooled at ambient temperature. Vacuum fat coating was done on the following day in a Pegasus PG -10VC LAB vacuum coater (Dinnissen, Sevenum, The Netherlands). Ingredients, proximate and fatty acids composition of the experimental diets are shown in Table 10 and Table 11.

Table 10 Ingredients and proximate composition of the experimental diets

Composition (%)	**C**	**50FMFO**	**50FMFO-50MIC**	**0FMFO-50MIC**	**0FMFO-100MIC**
Ingredients, % of the diet					
[1]Fish meal Prime	30	15	15	-	-
[2]Fish meal trimming	-	15	15	30	30
[3]Fish oil EXTRA	11.5	5.75	5.75	-	-
[4]Fish oil trimming	-	5.75	5.75	11.5	11.5
[5]Soy Protein Concentrate	15	15	10	10	-
[6]Microalgae	-	-	7.5	7.5	15
[7]Wheat meal	9.66	9.30	7.77	6.77	5
[8]Wheat gluten	15	15	16.5	16.5	-
[9]Pea Protein	12	12	12.5	12	12.5
[10]Rapeseed oil	6	5.75	5.3	5	5
Vit C 35%	0.05	0.05	0.05	0.05	0.05
Vit E 50%	0.02	0.02	0.02	0.02	0.02
Dl-Methionin*e*	0.06	0.075	0.055	0.35	0.38
L-Lysine 50%	-	0.60	0.60	0.80	0.67
L-Valine	-	-	-	0.40	0.89
L-Threonine	-	-	-	0.70	0.87
L-Tryptophan	-	-	-	0.20	0.92
[11]Premix	0.71	0.71	0.71	0.71	0.71
Proximate composition, % on a wet weight basis					
Moisture	6.88	5.80	6.85	6.95	6.57
Protein	50.19	50.78	50.25	48.72	48.22
Lipid	20.94	21.40	21.60	22.99	23.48
Ash	6.79	7.72	8.95	9.80	11.24

[1]Origin: Chile; Composition: protein 67%, lipid 10%, ash 14%. Antioxidant: butylated hydroxyanisole, BHA, 40 ppm; Butylated hydroxytoluene BHT, 200 ppm.
[2] Origin: Morocco; Obtained from Atlantic mackerel (*Scomber scombrus*) and sardine (*Sardina pilchardus*). Composition: protein 63%, lipid 10%, ash 20%. BHA, 80 ppm; BHT, 80 ppm.
[3]Origin: Chile. Antioxidant: BHA 90 ppm, BHT 80 ppm, propylgallate, PG 40 ppm.
[4] Origin: EU. Obtained from Atlantic salmon (*Salmo salar*). Antioxidant: BHA 70 ppm, BHT 145 ppm, PG 40 ppm.
[5]Origin: Serbia. Composition: protein, 61%, lipid, 0.3%.
[6]Origin: Spain; Obtained from processed *Nannocloropsis* (Almeria, Spain).
[7]Origin: Spain; Composition: 12% protein.
[9]Origin: Spain; Composition: 78% crude protein.
[9]Origin: Spain; Obtain from Pisane, Emilio Peña; Composition: 85% crude protein, 1.5% crude lipid.
[10]Origin: Spain; Obtain from Roviroli SL.
[11] Origin: Italy; Composition: BHA, 150 mg; PG, 75 mg.

Table 11 Fatty acid profile (% of total fatty acid methyl esters, FAME) of the experimental diets.

	C	50FMFO	50FMFO-50MIC	0FMFO-50MIC	0FMFO-100MIC
14:00	2.20 ± 0.36	2.29 ± 0.37	2.33 ± 0.37	2.33 ± 0.37	2.24 ± 0.37
16:00	14.1 ± 1.70	14.6 ± 1.80	13.3 ± 1.60	13.9 ± 1.70	15.6 ± 1.90
16:1n7	2.73 ± 0.00	3.75 ± 0.00	3.72 ± 0.00	4.49 ± 0.00	2.82 ± 0.00
18:00	3.94 ± 0.56	3.86 ± 0.56	3.55 ± 0.51	3.54 ± 0.51	4.26 ± 0.65
18:1n9	28.5 ± 3.10	27.0 ± 3.00	28.1 ± 3.00	27.1 ± 2.90	26.8 ± 3.00
18:1n7	2.53 ± 0.39	2.44 ± 0.39	2.56 ± 0.39	2.46 ± 0.38	2.42 ± 0.38
18:2n6	12.2 ± 1.60	11.9 ± 1.60	11.5 ± 1.40	11.3 ± 1.40	11.9 ± 1.60
18:3n3	3.67 ± 0.53	3.44 ± 0.51	3.56 ± 0.51	3.42 ± 0.50	3.42 ± 0.50
18:4n3	0.64 ± 0.16	0.63 ± 0.15	0.79 ± 0.19	0.69 ± 0.17	0.52 ± 0.12
20:1n9	2.01 ± 0.33	1.96 ± 0.33	2.82 ± 0.42	2.48 ± 0.38	1.25 ± 0.26
20:4n6	0.93 ± 0.22	1.01 ± 0.24	0.92 ± 0.21	1.06 ± 0.22	1.22 ± 0.26
20:4n3	< 0.0050	< 0.0050	< 0.0050	< 0.0050	< 0.0050
20:5n3	4.38 ± 0.62	5.58 ± 0.80	5.35 ± 0.72	6.15 ± 0.86	4.60 ± 0.65
22:5n3	1.18 ± 0.25	1.16 ± 0.25	1.36 ± 0.25	1.26 ± 0.24	1.03 ± 0.24
22:6n3	12.2 ± 1.60	12.0 ± 1.50	10.5 ± 1.40	10.7 ± 1.40	14.1 ± 1.80
Σ SFA	23.1 ± 1.9	23.7 ± 2.00	21.7 ± 1.70	22.4 ± 1.80	25.3 ± 2.10
Σ MUFA	39.2 ± 4.00	38.6 ± 3.90	41.7 ± 3.80	40.5 ± 3.70	35.6 ± 3.90
Σ PUFA	37.5 ± 2.50	37.7 ± 2.40	36.4 ± 2.30	37.2 ± 2.30	39.1 ± 2.60
Σ n-3	22.6 ± 1.80	23.3 ± 1.80	22.2 ± 1.70	22.9 ± 1.80	24.1 ± 2.00
Σ n-6	15.0 ± 1.70	14.4 ± 1.70	14.2 ± 1.50	14.4 ± 1.50	15.1 ± 1.70
Σ n-9	31.6 ± 3.10	30.0 ± 3.00	32.1 ± 3.10	30.7 ± 2.90	28.9 ± 3.00
n-3/n-6	1.51 ± 0.21	1.62 ± 0.23	1.56 ± 0.21	1.59 ± 0.21	1.60 ± 0.23
n-3 PUFA	17.8	18.7	17.2	18.1	19.7
EPA/DHA	0.36	0.46	0.51	0.57	0.33

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid.

6.3.2 Fish and rearing trial

The trial took place at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. European Seabass juveniles were obtained from an Italian hatchery, located in Adriatic coast of Mediterranean Sea. At the beginning of the trial, 50 fish per tank with initial body weight of about 46.66 ± 0.04 g, were randomly distributed into 15 square tanks with a capacity of 800 L. Each diet was randomly assigned and administered to triplicate groups, over 88 days. Tanks were provided with natural seawater and connected to a closed recirculation system (overall water volume: 20 m^3). The rearing system consisted of a mechanical sand filter (PTK 1200, Astralpool, Barcelona, Spain), ultraviolet lights (PE 25mJ/cm^2: 32m^3 h^{-1}, Blaufish, Barcelona, Spain) and a biofilter (PTK 1200, Astralpool, Barcelona, Spain). The overall water renewal amount in the system was 5% daily, while water exchange rate was 100% every hour. During the experiment, temperature was kept at 22 ± 0.5°C and the photoperiod was maintained at 12 h light and 12 h dark through artificial light. The oxygen level was kept constant (8.0 ± 1.0 mg L^{-1}) thanks to the connection with a liquid oxygen system regulated by a software programme (B&G Sinergia snc, Chioggia, Italy). Each day, ammonia (total ammonia nitrogen ≤ 0.1 mg L^{-1}) and nitrite (≤ 0.2 mg L^{-1}) were monitored by spectrophotometer (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany) and salinity (25 g L^{-1}) was measured by a refractometer (106 ATC, Giorgio Bormac S.r.l., Carpi, Italy).

Animals were fed to satiation with automatic feeders twice a day, set to release gradually pellet for one hour. The uneaten pellets of each tank were collected, dried overnight at 105°C, and weighed for feed intake (FI) calculation (Parma et al., 2019).

6.3.3 Sampling

At the outset and upon conclusion of the experiment, all animals within each tank were anesthetized with MS222 at a concentration of 100 mg L^{-1} and weighed. Specific growth rate (SGR) and feed conversion rate (FCR) were calculated. The proximate composition of the carcasses was determined using a pooled sample of 10 fish initially and a pooled sample of 5 fish per tank at the end of the trial. For gut microbiota analysis, 12 hours post -meal at the conclusion of the trial, digesta content from posterior intestine of five fish per tank was collected and immediately stored at −80°C (Parma et al., 2020). At the same time 10 fish per tank were euthanized to collect feces to determine the apparent digestibility coefficient (ADC) of dry matter and protein using the indirect method with diets containing yttrium oxide (Busti et al., 2020). Blood

was also collected from 5 fish per tank for the assessment of plasma biochemistry. Blood samples were centrifuged (3000 x g, 10 min, 4°C) and plasma aliquots were stored at - 80°C until analysis (Pelusio et al., 2021). To assess enzymatic activity, 4 fish per tank were euthanized and the entire intestine was collected from each fish. Sample s were stored at -80°C until analyses. The experimental procedures were evaluated and approved by the Ethical -Scientific Committee for Animal Experimentation of the University of Bologna (ID 1136/2019), in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes.

6.3.4 Calculations

Following, the employed formulae:

Specific growth rate (SGR) (% day^{-1}) = 100*(ln FBW- ln IBW) / days (where FBW represent the final body weight and IBW and the initial body weights).

Feed intake (FI, g kg ΔBW^{-1} day^{-1}) = ((100*total feed ingestion)/(ΔBW))/days.

Feed conversion ratio (FCR) = feed intake / weight gain.

Protein efficiency rate (PER) = (FBW – IBW) / protein intake.

Apparent Digestibility (ADC (%)) = 100 – [100 *(Y in feed/Y in faeces) * (nutrient in faeces/nutrient in feed)].

Gross protein efficiency (GPE) (%) = 100*[(% final body protein*FBW) - (% initial body protein*IBW)] / total protein intake fish.

Lipid efficiency rate (LER) = (FBW - IBW)/lipid intake.

Gross lipid efficiency (GLE) (%) = 100*[(% final body lipid*FBW) - (% initial body lipid*IBW)]/total lipid intake fish.

6.3.5 Proximate composition analysis

To determine moisture content, samples were dried in a n oven at 105°C until a constant weight was achieved. Crude protein was determined as total nitrogen (N) by using the Kjeldahl method, multiplying N by 6.25. Total lipids were determined according to Bligh and Dyer's (1959) extraction method. Samples were incinerated to a constant weight in a muffle oven at 450 °C to estimate ash content (AOAC, 2010). The concentrations of yttrium oxide in both diets and feces were measured through Inductively Coupled Plasma -Atomic Emission Spectrometry (ICP -AES) using equipment from Perkin Elmer, MA, United States, following the method described by Busti et al. (2020).

6.3.6 Metabolic parameters in plasma

To determine plasma parameters was used 500 µL of sample on an automated analyser (AU 480; Olympus/Beckman Coulter, Brea, CA, United State) according to the manufacturer's instructions (Parma et al., 2023). The levels of glucose (GLU), urea, creatine, uric acid, total bilirubin, aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), creatine kinase (CK), lactate dehydrogenase (LDH), cholesterol (CHOL), triglycerides (TRIG), total protein (TP), albumin (ALB), calcium (Ca^{+2}), phosphorus (P), potassium (K^{+}) sodium (Na^{+}), iron (Fe), chloride (Cl), magnesium (Mg) were determined. The Albumin/Globulin (ALB/GLO) ratio, lactate (LAC) and Current Calcium (Cur.Ca) were calculated.

6.3.7 Determination of digestive enzyme activities

From 4 fish per tank, the entire digestive tract was extracted, and segments of the intestines were pooled. All assays were conducted in triplicate, respecting both proximal and distal regions of the intestine. To determine digestive enzyme activities, intestinal segments were manually homogenized in distilled water at 4°C to achieve a final concentration of 0.5 g mL^{-1}. Subsequently, the homogenized material was centrifuged (16,000×g for 12 min at 4°C), and supernatants obtained after centrifugation were immediately stored at −20°C until further analysis. Total soluble protein was quantified according to Bradford (1976) using bovine serum albumin as a standard. The activities of some of the most important digestive enzymes were measured spectrophotometrically, and specific enzymatic activity was expressed as unit per gram of tissue (Alarcon et al., 1998). Total alkaline protease (TAP) activity was assessed using 5 g L − 1 casein in 50 mM Tris HCl (pH 9.0) as substrate, with one unit of TAP defined as the amount of enzyme that released 1 µg of tyrosine per minute. This calculation considered an extinction coefficient for tyrosine of 0.008 $\mu g^{-1} mL^{-1} cm^{-1}$. The activity of TAP was measured spectrophotometrically at 280 nm. To determine the activities of trypsin and chymotrypsin, 0.5 mM BAPNA (N - a -benzoyl -DL -arginine - 4nitroanilide) (Erlanger et al., 1961) and 0.2 mM SAPNA (N -succinyl -(Ala)2 -Pro -Phe - Pnitroanilide) (DelMar et al., 1979) respectively, were prepared as substrate in 50 mM Tris -HCl, 10 mM CaCl 2 buffer (pH 8.5). Leucine aminopeptidase activity was assayed using 2 mM L -leucine -pnitroanilide (LpNa) in 100 mM Tris -HCl buffer, pH 8.8 while alkaline phosphatase was assessed with p -nitrophenyl phosphate in 1 M diethanolamine buffer, pH 9.5, containing 1 mM MgCl2 as substrates (Vizcaíno et al., 2014). Trypsin, chymotrypsin, and leucine aminopeptidase activities were measured spectrophotometrically at 405 nm and one unit of activity (U) was defined as the amount of enzyme

that releases 1 μmol of p -nitroanilide (pNA) per minute, considering the extinction coefficient 8800 M cm^{-1}. Moreover, one unit of alkaline phosphatase activity was defined as the amount of enzyme that 1 μg of nitrophenyl released per minute considering a coefficient molar extinction of p -nitrophenol, 17, 800 M cm^{-1}, measured at 405 nm (Galafat et al., 2022).

6.3.8 Gut bacterial community DNA extraction, sequencing and analysis

At the end of the feeding trial, total DNA was extracted from individual distal gut content (300 mg per fish) obtained from a total of 75 fish (15 fish per tank), as previously reported by Parma et al. (2016). Total DNA was then quantified with NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and stored at -20 °C until further processing. The amplification of the V3-V4 hypervariable regions of the 16S rRNA bacterial gene was carried out using the 341F and 785R primers with overhang sequencing adapters attached and 2 x KAPA HiFi HotStart ReadyMix (KAPA Byosystems). As already described by Parma et al., (2020) the thermal cycle consists of 30 amplification cycles and at the end PCR products were purified, and the indexed libraries were prepared following Illumina protocol “16S Metagenomic Sequencing Library Preparation”. Libraries were normalized to 4 nM and pooled together, the resulting pool were denatured with 0.2 N NaOH and diluted to 6 pM with 20% Phix control. Sequencing was performed on Illumina MiSeq platform using 2 x 250 bp paired-end protocol according to the manufacturer's instructions (Illumina, San Diego, CA). At the end of the sequencing process, raw sequences were processed by combining PANDAseq and QIIME2 pipelines (Bolyen et al., 2019; https://qiime2.org). High-quality reads, obtained after a filtering step for length (min/max = 350/550 bp) and quality step using USEARCH with a max error rate of 3% (Edgar, 2010), were cleaned and clustered into amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016). Taxonomy was assigned using a hybrid method combining VSEARCH and q2 classifier trained on the SILVA database release 138.1(Bokulich et al., 2018). Three different metrics were used to evaluate internal ecosystem diversity (alpha-diversity) – Faith’s Phylogenetic Diversity (faith_pd), Shannon_entropy index, and number of observed ASVs (observed features). Unweighted UniFrac distances were computed to estimate inter-sample ecosystem diversity (beta-diversity) and used as input for Principal Coordinates Analysis (PCoA).

6.3.9 Statistical analysis

All data are presented as mean ± standard deviation (SD). A s experimental unit single tank was used to evaluate growth performance and a pool of t en fish was considered the experimental unit for the analysis of carcass composition, nutritional indices and digestibility evaluation. Five individual fish per tank were used for analysing somatic indices, blood biochemistry and gut microbiota community profiles. Preceding ANOVA, normality and homogeneity of variance were checked using Shapiro -Wilk and Brown -Forsythe tests, respectively. Tukey's post hoc test was performed. All statistical analyses were performed using GraphPad 8.0.1. The differences among treatments were considered significant at $P \leq 0.05$. Microbiota analysis and respective plots were produced using R software (https://www.r - project.org/) with "vegan" (http://www.cran.r -project.org/package -vegan/), "Made4" (Culhane et al., 2005) and "stats" packages (https://stat.ethz.ch/R - manual/R - devel/library/stats/html/00Index.html). Data separation was tested by a permutation test with pseudo -F ratios (function "Adonis" in the "vegan" package). When required, Wilcoxon and Kruskal –Wallis test s were used to assess significant differences in alpha diversity and taxon relative abundance between groups. When necessary, p -values were corrected for multiple testing with Benjamini -Hochberg method, with a false discovery rate (FDR) ≤ 0.05 considered as statistically significant.

6.4 Results

6.4.1 Growth

Results of growth performance and FI are summarized in Table 12. No statistical differences were evaluated for all parameters considered (IBW, FBW, WG, FCR and SGR).

Table 12 Growth performance and feed intake of Seabass juveniles fed experimental diets over 88 days.

	Experimental diets					
	C	**50FMFO**	**50FMFO-50MIC**	**0FMFO-50MIC**	**0FMFO-100MIC**	***P-value***
IBW	46.6 ± 0.18	46.6 ± 0.10	46.7 ± 0.17	46.7 ± 0.11	46.7 ± 0.62	0.9942
FBW	169 ± 2.37	164 ± 2.44	163 ± 2.72	159 ± 4.69	176 ± 26.5	0.5653
WG	119 ± 2.32	118 ± 2.38	117 ± 2.57	112 ± 4.63	129 ± 26.8	0.5704
SGR	1.45 ± 0.02	1.43 ± 0.02	1.43 ± 0.02	1.39 ± 0.03	1.50 ± 0.17	0.5818
FI	1.67 ± 0.01	1.73 ± 0.01	1.66 ± 0.01	1.73 ± 0.06	1.63 ± 0.16	0.4371
FCR	1.32 ± 0.03	1.36 ± 0.01	1.31 ± 0.01	1.42 ± 0.09	1.29 ± 0.23	0.6317
Survival	100 ± 0.00	100 ± 0.00	99.30 ± 1.20	100 ± 0.00	100 ± 0.00	0.4516

Data are given as the mean (n=3) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
IBW = Initial body weight.
FBW = Final body weight.
WG = Weight gain (g)
SGR = Specific growth rate (% day-1) = 100 * (ln FBW- ln IBW) / days.
Feed intake (FI, g kg ΔBW–1 day–1) = ((1000*total feed ingestion)/(ΔBW))/days.
FCR = Feed conversion rate = feed intake / weight gain.
Survival = Survival (%).

6.4.2 Proximate Composition

Results of body composition, nutritional indices and somatic indices are summarized in Table 13. Value of moisture was significantly higher in control diet C compared to 50FMFO, 50FMFO-50MIC and 0FMFO-50MIC. Protein body content presented lower value in diet 50FMFO-50MIC than in diet 50FMFO and 0FMFO-100MIC. Lipid body content was significantly higher in 50FMFO, 50FMFO-50MIC and 0FMFO-50MIC compared to control diet. No statistical differences were detected for ash, apparent digestibility and all nutritional indexes considered. Table 14 presented results of fatty acid composition collected from the flesh of animals. No statistical differences were found.

Table 13 Body composition and nutritional indices measured in European Seabass

	C	**50FMFO**	**50FMFO-50MIC**	**0FMFO-50MIC**	**0FMFO-100MIC**	***P-value***
			Whole body composition, %			
Moisture	65.0 ± 1.80^{b}	62.2 ± 0.4^{a}	62 ± 1.3^{a}	62.1 ± 1.6^{a}	63.9 ± 1.2^{ab}	**0.0023**
Protein	16.8 ± 0.4^{ab}	17.3 ± 0.5^{b}	16.0 ± 1.08^{a}	17 ± 0.3^{ab}	17.1 ± 0.1^{b}	**0.0126**
Lipid	14.5 ± 1.9^{a}	17.6 ± 0.7^{b}	18.1 ± 0.5^{b}	17.8 ± 1.6^{b}	16 ± 1.1^{ab}	**0.0003**
Ash	2.90 ± 0.36	2.57 ± 0.17	3.16 ± 0.92	2.57 ± 0.92	2.84 ± 0.10	0.1632
			Apparent digestibility			
Protein	72.5 ± 4.4	76.8 ± 5.64	74.2 ± 6.58	76 ± 2.8	75.3 ± 3.88	0.6128
Dry Matter	95.3 ± 1.25	96.2 ± 0.78	95.4 ± 1.06	94.7 ± 0.69	93.9 ± 1.89	0.2078
			Nutritional indices			
PER	2.49 ± 0.05	2.35 ± 0.05	2.31 ± 0.05	2.30 ± 0.02	2.66 ± 0.55	0.4022
GPE	42.3 ± 0.61	41.5 ± 1.36	36.4 ± 3.86	39.8 ± 1.01	46.0 ± 9.77	0.2445
LER	5.73 ± 0.11	5.64 ± 0.11	5.47 ± 0.12	5.03 ± 0.05	5.51 ± 1.14	0.5475
GLE	87.1 ± 16.9	107 ± 6.54	110 ± 5.78	99.9 ± 12.6	94.5 ± 14.9	0.2137

Data are given as the mean (n=3 ± SD). In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).

PER = Protein efficiency ratio = ((FBW-IBW)/protein intake).

GPE = Gross protein efficiency = 100*[(%final body protein*FBW) - (%initial body protein*IBW)]/total protein intake fish.

LER = Lipid efficiency rate = (FBW - IBW)/lipid intake.

GLE = Gross lipid efficiency = 100*[(% final body lipid ∗ FBW) - (% initial body lipid*IBW)]/total lipid intake fish.

Table 14 Fatty acid composition (% of total fatty acid methyl esters, FAME) of European Seabass.

	C	50FMFO	50FMFO-50MIC	0FMFO-50MIC	0FMFO-100MIC	*P-value*
14:00	2.17 ± 0.01	2.15 ± 0.02	2.37 ± 0.42	1.82 ± 0.60	2.12 ± 0.07	0.4302
16:00	15.4 ± 0.29	15.5 ± 0.24	16.1 ± 1.11	15.6 ± 0.73	15.7 ± 0.50	0.6964
16:1n7	4.34 ± 0.08	4.22 ± 0.11	4.38 ± 0.41	4.19 ± 0.30	4.16 ± 0.13	0.7783
18:00	3.8 ± 0.10	3.83 ± 0.04	3.85 ± 0.23	3.94 ± 0.38	3.93 ± 0.15	0.9152
18:1n9	35.7 ± 0.22	34.8 ± 1.05	34.8 ± 1.37	36.0 ± 0.65	36.0 ± 0.8	0.3444
18:1n7	0.34 ± 0.01	0.29 ± 0.05	0.29 ± 0.02	0.31 ± 0.02	0.31 ± 0.00	0.2491
18:2n6	11.1 ± 0.02	11.1 ± 0.25	11.4 ± 0.38	11.1 ± 0.12	11.1 ± 0.10	0.4136
18:3n3	2.59 ± 0.01	2.62 ± 0.04	2.52 ± 0.12	2.6 ± 0.08	2.58 ± 0.06	0.5477
18:4n3	0.55 ± 0.03	0.52 ± 0.08	0.62 ± 0.07	0.58 ± 0.09	0.55 ± 0.12	0.7013
20:1n9	3.29 ± 0.26	3.04 ± 0.51	3.18 ± 0.35	3.16 ± 0.40	3.13 ± 0.35	0.9545
20:4n6	0.65 ± 0.01	0.73 ± 0.10	0.68 ± 0.15	0.67 ± 0.02	0.68 ± 0.04	0.8492
20:4n3	0.33 ± 0.02	0.31 ± 0.03	0.32 ± 0.03	0.78 ± 0.8	0.30 ± 0.04	0.4633
20:5n3	3.36 ± 0.05	3.4 ± 0.12	3.32 ± 0.04	2.99 ± 0.44	3.18 ± 0.30	0.3344
22:5n3	0.97 ± 0.19	1.04 ± 0.04	1.02 ± 0.02	1.02 ± 0.06	1.00 ± 0.09	0.9228
22:6n3	8.53 ± 0.18	9.55 ± 1.24	8.4 ± 1.44	8.59 ± 0.29	8.66 ± 0.32	0.5438
Σ SFA	21.4 ± 0.37	21.5 ± 0.20	22.4 ± 1.47	21.4 ± 1.22	21.7 ± 0.58	0.6737
Σ MUFA	43.7 ± 0.44	42.4 ± 1.73	42.7 ± 1.50	43.7 ± 0.28	43.4 ± 0.37	0.4526
Σ PUFA	28.1 ± 0.26	29.3 ± 1.58	28.3 ± 1.18	28.3 ± 0.23	28.1 ± 0.69	0.5534
Σ n-3	16.3 ± 0.27	17.5 ± 1.24	16.2 ± 1.34	16.6 ± 0.26	16.3 ± 0.78	0.4762
Σ n-6	11.7 ± 0.03	11.8 ± 0.36	12.1 ± 0.29	11.8 ± 0.13	11.8 ± 0.09	0.3834
Σ n-9	3.64 ± 0.25	3.34 ± 0.57	3.47 ± 0.36	3.48 ± 0.37	3.45 ± 0.35	0.9178
n-3/n-6	1.39 ± 0.02	1.47 ± 0.06	1.34 ± 0.13	1.40 ± 0.03	1.37 ± 0.07	0.3674
n-3 PUFA	13.2	14.3	13.1	13.4	13.2	0.4805
EPA/DHA	0.39	0.35	0.4	0.34	0.36	0.5764

SFA: saturated fatty acid; MUFA: SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid.

6.4.3 Plasma results

Plasma parameter results are shown in Table 15. Creatinine value was higher in CTRL than other treatments. ALP values in 0FMF O -50MIC and 0FMFO -100MIC were higher compared to 50FMFO -50MIC. Value s of CHOL were higher in CTRL compared to 50FMFO -50MIC and 0FMFO -100MIC diets. CTRL presented the highest value s of HDL while 50FMFO was higher than 0FMFO -50MIC and 0FMFO -100MIC diets. TP values were statistically higher in CTRL, and lowest in 0FMFO -100MIC diet. Fe values were lower in 50FMFO compared to CTRL. Diet 0FMFO -100MIC presented higher value s of Na than 50FMFO -50MIC and CTRL diets. Cl was lower in CTRL compared to 0FMFO -100MIC.

Table 15 Plasma biochemistry in European Seabass fed with experimental diets.

	C	50FMFO	50FMFO-50MIC	0FMFO-50MIC	0FMFO-100MIC	*P-value*
GLUC	91.0 ± 19.1	86.4 ± 18.5	76.2 ± 13.4	81.4 ± 17.6	81.6 ± 20.4	0.2229
Urea	10.2 ± 1.95	9.29 ± 2.66	9.17 ± 1.24	8.99 ± 2.11	8.96 ± 1.3	0.3233
CREA	0.43 ± 0.09^{b}	0.26 ± 0.07^{a}	0.26 ± 0.05^{a}	0.20 ± 0.04^{a}	0.21 ± 0.04^{a}	**<0.0001**
Uric Ac	0.12 ± 0.1	0.13 ± 0.1	0.08 ± 0.05	0.10 ± 0.04	0.13 ± 0.08	0.3803
Tot Bil	0.08 ± 0.04	0.07 ± 0.03	0.07 ± 0.02	0.08 ± 0.03	0.07 ± 0.02	0.7749
Ast	77.4 ± 40.2	78.0 ± 61.8	68.1 ± 60.3	84.4 ± 87.5	37.5 ± 21.3	0.2084
Alt	5.46 ± 2.23	4.20 ± 1.97	4.26 ± 2.91	5.14 ± 4.22	2.85 ± 1.35	0.1026
Alp	59.9 ± 11.8^{ab}	62.1 ± 16^{ab}	55.1 ± 6.21^{a}	69.5 ± 12.84^{b}	68.1 ± 12.9^{b}	**0.0132**
Ck	6436 ± 4445	4081 ± 4551	3064 ± 2891	5078 ± 5856	2220 ± 1659	0.0677
LDH	77.2 ± 22.6	72.5 ± 35.7	72.9 ± 60.7	75.5 ± 60.1	55.4 ± 30.4	0.6806
Ca^{2+}	15.8 ± 1.26	15.2 ± 1.05	14.8 ± 1.05	15.1 ± 1.3	15.3 ± 1.33	0.2166
P	14.3 ± 1.53	13.2 ± 1.51	13.6 ± 0.84	13.2 ± 1.32	13.9 ± 1.30	0.1266
Mg	3.67 ± 0.50	3.45 ± 0.53	3.22 ± 0.63	3.48 ± 0.66	3.43 ± 0.66	0.3887
CHOL	280 ± 100^{b}	234 ± 69.3^{ab}	202 ± 65.4^{a}	218 ± 51.2^{ab}	189 ± 51.6^{a}	**0.0084**
HDL	78.6 ± 25.4^{c}	59.1 ± 10.2^{b}	52.5 ± 11.9^{ab}	42.1 ± 15.3^{a}	42.2 ± 8.89^{a}	**<0.0001**
TRIG	1758 ± 385	1717 ± 345	1668 ± 251	1778 ± 392	1682 ± 411	0.9075
TP	4.87 ± 0.45^{b}	4.77 ± 0.5^{ab}	4.48 ± 0.53^{ab}	4.56 ± 0.58^{ab}	4.32 ± 0.45^{a}	**0.0277**
Alb	1.38 ± 0.16	1.33 ± 0.15	1.27 ± 0.14	1.34 ± 0.20	1.26 ± 0.12	0.2234
Alb/Glo	0.39 ± 0.02	0.38 ± 0.02	0.39 ± 0.03	0.41 ± 0.04	0.41 ± 0.03	0.0779
LAC	42.5 ± 9.7	44.3 ± 5.17	37.8 ± 7.42	42.1 ± 9.13	45.4 ± 9.96	0.1457
$CurCa^{2+}$	228 ± 37.8	202 ± 32.1	202 ± 22.1	200 ± 29.9	214 ± 34.1	0.0974
Na/K	94.7 ± 14.0	88.8 ± 14.8	89.4 ± 19.9	89.5 ± 28.5	102 ± 23.3	0.3101
Fe	137 ± 24.3^{b}	110 ± 28.2^{a}	116 ± 21.6^{ab}	127 ± 22.5^{ab}	130 ± 21.3^{ab}	**0.0184**
Na^{+}	174 ± 5.35^{a}	178 ± 3.39^{ab}	176 ± 3.39^{a}	177 ± 3.73^{ab}	181 ± 5.68^{b}	**0.0006**
K^{+}	1.88 ± 0.3	2.06 ± 0.39	2.06 ± 0.45	2.15 ± 0.59	1.86 ± 0.44	0.3155
Cl	138 ± 5.51^{a}	143 ± 3.05^{ab}	143 ± 3.50^{ab}	142 ± 4.38^{ab}	146 ± 5.64^{b}	**0.0010**

Data are given as the mean (n=15 diet^{-1}) ± SD. Different letters indicate significant difference (One-way ANOVA $P \leq 0.05$) between treatments. GLU, glucose , (mg dL^{-1}); Urea , (mg dL^{-1}); CREA, creatinine , (mg dL^{-1}); Uric Ac, uric acid , (mg dL^{-1}); Tot Bil, total bilirubin , (mg dL^{-1}); Ast, aspartate aminotransferase , (U L^{-1}); Alt, alanine aminotransferase (U L^{-1}); Alp, alkaline phosphatase , (U L^{-1}); Ck, creatine kinase , (U L^{-1}); LDH, lactate dehydrogenase , (U L^{-1}); Ca^{+2} , calcium , (mg dL^{-1}); P, inorganic phosphorus; , (mg dL^{-1}); Mg, magnesium , (mg dL^{-1}); CHOL, cholesterol , (mg dL^{-1}); HDL, high density lipoprotein; TRIG, triglycerides , (mg dL^{-1}); TP, total protein , (mg dL^{-1}); Alb, albumin , (g dL^{-1}); Alb/Glo, albumin/globulin; LAC, lactate (mmol L^{-1}); $CurCa^{2+}$, current calcium (mg dL^{-1}); Na/K, sodium/potassium; Fe, iron , (µg dL^{-1}); Na^{+}, sodium , (mEq L^{-1}); K^{+}, potassium , (mEq L^{-1}); Cl, chloride , (mEq L^{-1}); SD, standard deviation.

6.4.4 Digestive enzymes activities

The enzyme activity results assessed at the end of the trial were summarized in Table 16. LANP, TAP, and trypsin activity did not exhibit any significant differences among treatments, considering both the proximal and distal intestinal regions. However, AP and chymotrypsin showed significant differences among treatments and between segments. Alkaline phosphatase activity in proximal segment presented lower value s in CTRL and 0FMFO -100MIC diets compared to 50FMFO and 50FMFO -50MIC diets. In the distal tract of the intestine, alkaline phosphatase activity was lower in 50FMFO and 0FMFO -100MIC groups compared to the 50FMFO -50MIC group. In the proximal region, chymotrypsin exhibited higher values in the 50FMFO and 50FMFO -50MIC diets compared to the 0FMFO -50MIC diet. Conversely, in the distal segment, the 50FMFO -50MIC diet displayed the highest value compared to other diets, with 50FMFO, 50FMFO -50MIC and 0FMFO -50MIC showing higher values compared to CTRL and 50FMFO -100MIC.

Table 16 Digestive enzymes activities (U g tissue^{-1}) measured in European Seabass.

	C	**50FMFO**	**50FMFO-50MIC**	**0FMFO-50MIC**	**0FMFO-100MIC**	***P-value***
			Proximal Intestine			
AP	5.94±0.88^{a}	8.58±0.84^{b}	9.24±1.51^{b}	13.3±0.84^{c}	4.82±0.97^{a}	**<0.0001**
LANP	0.76±0.1	0.74±0.08	0.71±0.09	0.77±0.1	0.63±0.21	0.2761
Trypsin	0.15±0.08	0.24±0.12	0.23±0.14	0.32±0.16	0.39±0.19	0.1546
CT	1.74±1.84^{a}	8.20±1.94^{c}	11.0±1.97^{c}	3.57±1.61 ^{b}	1.46±1.92^{a}	**<0.0001**
TAP	1008±668	830±429	1431±381	917±325	1515±684	0.0217
			Distal Intestine			
AP	4.91±2.57^{ab}	4.58±4.61^{a}	5.88±4.42^{b}	8.19±3.59^{c}	4.07±3.65^{a}	**<0.0001**
LANP	0.78±0.08	0.60±0.1	0.76±0.09	0.80±0.1	0.67±0.21	0.0611
Trypsin	0.54±0.18	0.51±0.26	0.58±0.27	0.82±0.46	0.34±0.34	0.0742
CT	3.92±1.87^{a}	8.29±1.29^{b}	14.3±1.68^{c}	6.73±0.98^{b}	1.39±0.62^{a}	**<0.0001**
TAP	2096±943	2607±868	2449±1694	2665±756	3318±1616	0.3435

Data are given as the mean (n=3 ± SD). In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
AP: Alkaline phosphatase
LANP: Leucine aminopeptidase
CT: Chymotrypsin
TAP: Total alkaline protease.

6.4.5 Gut Microbiota

The 16S rRNA gene sequencing was performed on a total of 75 distal intestine content samples, yielding 306'385 high-quality reads (mean ± SD, 4'085 ± 2'618) and clustered into a total of 1'155 ASVs. In order to assess the effects of replacement of FM, FO and SP, on the gut bacteria community during the growth process of Seabass, the gut microbiota (GM) was analysed for each dietary group at the end of the trial. Principal Coordinates Analysis (PCoA) based on Unweighted UniFrac distances was used to evaluate the GM variations between samples (beta-diversity). Moreover, the gut microbial community diversity, within each dietary group, was represented with faith-PD, Shannon_entropy and observed-features.

According to our results (Figure 15), in terms of overall GM composition, a significant separation between groups was observed. Specifically, C and 50FMFO groups segregated compared to the other groups (50FMFO-50MIC, 0FMFO-50MIC, 0FMFO-100MIC) (Adonis, $p < 0.001$). At the same time, focusing on the internal ecosystem diversity of the fish gut microbiota, we observed a significant increase of both faith_pd and Shannon indices of 0FMFO-50MIC and 0FMFO-100MIC groups compared to the control group (Wilcoxon rank-sum test, $p < 0.05$). On the other hand, within the treatment groups we observed a significant increase of Shannon index of 50FMFO-50MIC, 0FMFO-50MIC and 0FMFO-100MIC groups compared to 50FMFO group ($p < 0.05$), while regarding faith_pd index only 0FMFO-100MIC group shown a significant higher value compared to 50FMFO group ($p < 0.05$). While only 50FMFO group showed a significant reduction of Shannon index compared to the CTRL group ($p < 0.05$).

The overall GM composition at different phylogenetic levels was investigated, as reported at phylum and family level in Figure 16, while specific genera significant variations were highlighted in Figure 17. More specifically, at phylum level the most abundant taxa observed was Firmicutes (with an overall relative abundance mean of 97%). The most represented families were *Streptococcaceae* (r.ab. mean ± SEM 60.0 ± 3.5 % CTRL; 28.4 ± 1.8 % 50FMFO; 20.3 ± 1.6 % 50FMFO-50MIC; 35.1 ± 4.0 % 0FMFO-50MIC; 34.6 ± 3.2 % 0FMFO-100MIC), and *Lactobacillaceae* (34.4 ± 3.7 % CTRL; 68.5 ± 1.9 % 50FMFO; 55.0 ± 3.8 % 50FMFO-50MIC; 38.0 ± 4.6 % 0FMFO-50MIC; 27.2 ± 6.2 % 0FMFO-100MIC) all belonging to Firmicutes phylum. Focusing on the specific variation between each group at genera level, we observed a significant decrease in the relative abundance of *Lactococcus*, *Bifidobacterium*, *Granulicatella*, *Lacteicaseibacillus* and *Streptococcus* genera in treatment groups compared to the CTRL group (Wilcoxon rank-sum test, $p < 0.05$),

as shown in Figure 17. On the other hand, we observed a significant relative abundance increase of *Pediococcus*, *Clostridium sensu stricto 1*, *Leuconostoc* and *Turicibacter* genera in treatment groups compared to the CTRL group (Wilcoxon rank-sum test, $p < 0.05$; Figure 17). Focusing the attention on the pinpoint variations within the treatment groups, we observed a significant decrease of *Pediococcus* genus in 0FMFO-100MIC group compared to 50FMFO ($p < 0.05$), a significant decrease of *Bifidobacterium* genus in 50FMFO-50MIC and 0FMFO-100MIC groups compared to 50FMFO group ($p < 0.05$) and a significant decrease in the relative abundance of *Leuconostoc* genus in the 0FMFO-50MIC and 0FMFO-100MIC groups compared to 50FMFO ($p < 0.05$; Figure 17). Furthermore, always compared to 50FMFO group, we observed a significant increase in the relative abundance of *Clostridium sensu stricto 1* and *Turicibacter* genera in all the other treated groups ($p < 0.01$; Figure 17).

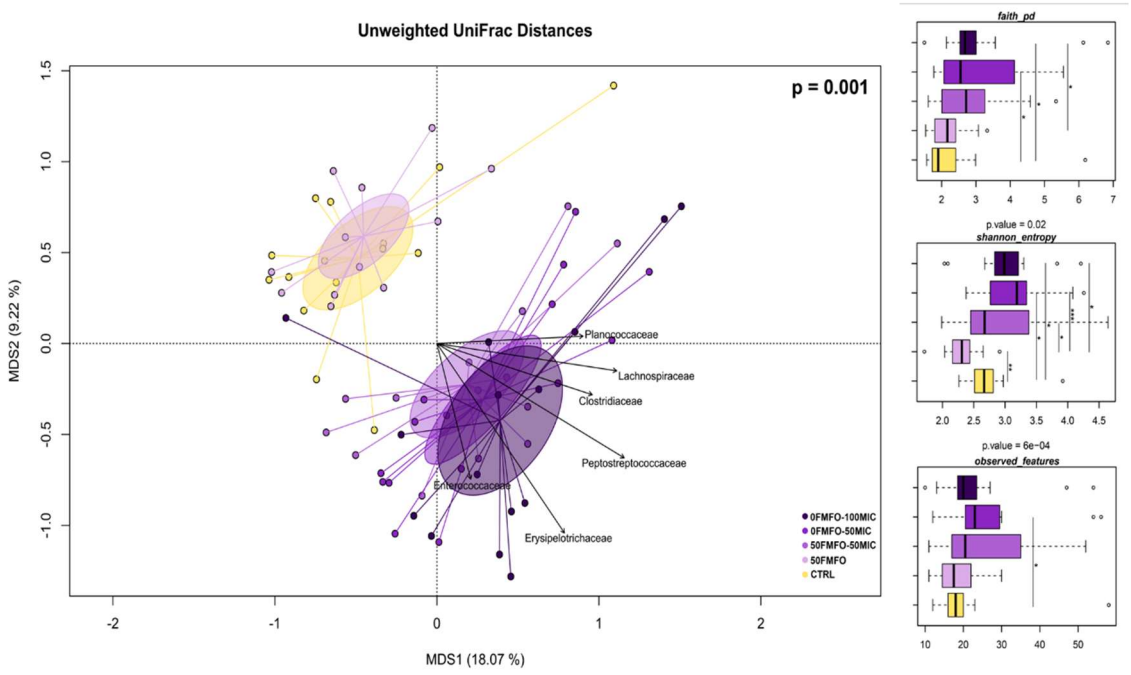

Figure 15 Beta diversity and alpha diversity of gut microbiota of Seabass fed with experimental diets over 88 days. On the left, Principal Coordinates Analysis (PCoA) based on unweighted UniFrac distances between gut microbiota composition of animals fed with experimental diets. Significant separations were highlighted (permutation test with pseudo-F ratios Adonis; $p = 0.001$). On the right, Boxplots of alpha diversity values with 3 metrics, faith_pd, shannon_entropy and observed_features (ASVs). Faith_pd and Shannon indices shown significant general variations (Kruskal-Wallis test $p < 0.05$) of alpha diversity among dietary groups, with specific significant variations between groups highlighted by a line and a different number of * based on the P-value (Wilcoxon rank-sum test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

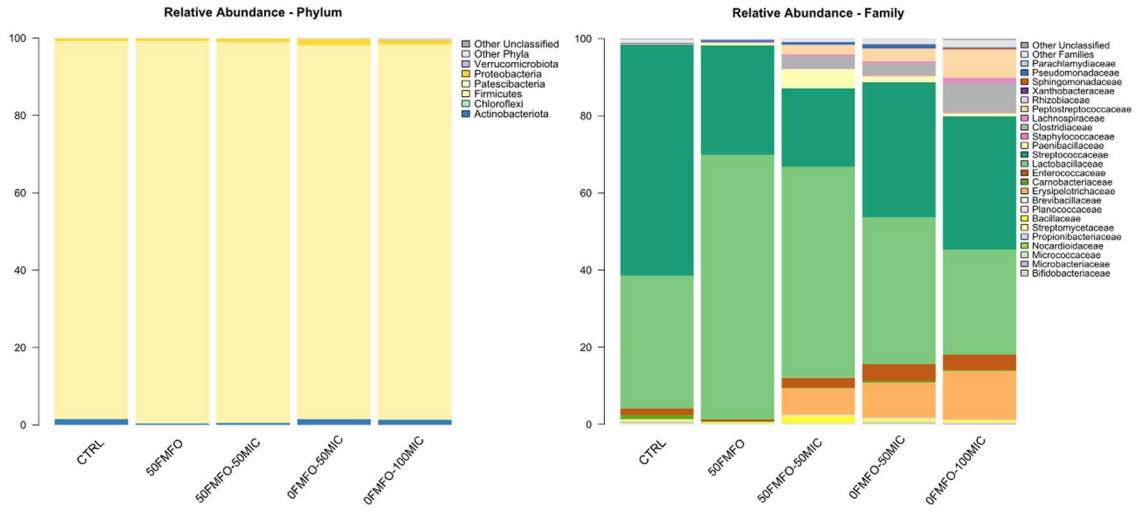

Figure 16 Microbiota composition of distal gut content of Seabass fed experimental diets. Bar plot summarizing the microbiota composition at phylum (left) and family (right) of fish intestinal content. Only phyla and families with a relative abundance ≥ 0.5% in at least 2 samples are shown.

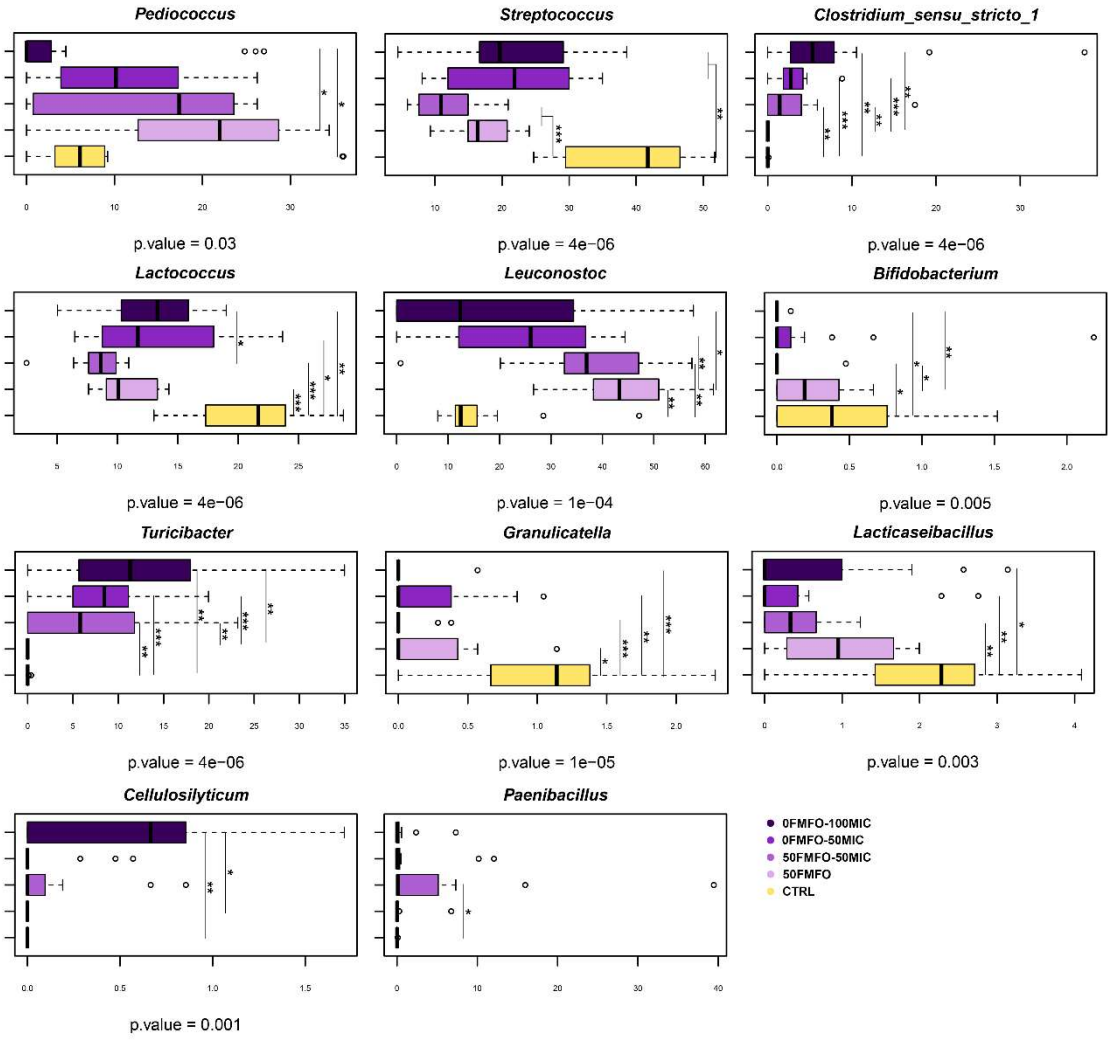

Figure 17 Taxonomic composition of bacterial communities of distal gut content of Seabass fed experimental diets. Distributions of relative abundance of genera that showed a significant variation between groups fed with different diets (Wilcoxon rank-sum test, *** $p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$). Only genera with a mean relative abundance ≥ 0.5% in

at least 2 samples were represented. The central box of each dataset represents the distance between the 25th and the 75th percentiles. The median between them is marked with a black line.

6.5 Discussion

In aquaculture, sustainable feed production and circularity are crucial for long-term viability and environmental conservation. Responsible ingredient sourcing and waste reduction support marine ecosystem health, while circular models prioritize resource efficiency and waste reduction, enhancing economic sustainability and minimizing environmental impact. The focus of the current study aligns perfectly with these principles as circular ingredients are tested for European Seabass aquaculture. Utilizing bycatch and trimmings as feed ingredients reduces waste and minimizes the environmental impact of fishing activities, contributing to more responsible and sustainable fishing practices. This approach maximizes the utilization of harvested fish and alleviates pressure on wild fish populations, promoting more efficient resource use within the aquaculture industry. Additionally, substituting soy meal with microalgae, such as *Nannochloropsis*, offers a sustainable alternative with potential nutritional and health benefits, including a blend of essential amino acids, healthy fatty acids as well as vitamins and pigments (Nagappan et al., 2021). Marchi et al. (2023) showed that the use of fishery and aquaculture by-products from mackerel and sardines is a valid strategy to totally replace wild-caught FM and FO in European Seabass at on-growing stage. Similarly, the results of growth, FI and feed utilization reported in the present study confirm also for juveniles the possibility to totally replace wild-caught FM and FO using trimmings without any negative effect on overall growth and feed efficiency indicators. In some cases, the substitution of wild caught FM with by-product meal or with microalgae led to an improvement of growth and feed utilization as for the olive flounder (*Paralichthys olivaceus*) fed with tuna by-product (Kim et al., 2014) or for Nile tilapia (*Oreochromis niloticus*) fed with a microalgae-blend (Sarker et al., 2020). For Pangasius catfish, the utilization of by-products from the filleting industry in feed has been implemented in several farms (Paripatananont, 2002). However, the quality of FM and FO from aquatic by-products may vary from the wild-catch one where FM produced from by-products and trimmings contains a lower protein content (as low as 50%–55%) and high ash levels (up to 20%–30%). Similar to the findings of this study, multiple prior research efforts have demonstrated that integrating dietary microalgae into fish feed typically does not lead to significant deviations in growth performance compared to control diets. For example, *Nannochloropsis oceanica* in Atlantic salmon, *Isochrysis* sp. and *Tetraselmis suecica* in European sea bass, *Arthrospira maxima* in red tilapia and *Scenedesmus almeriensis* in gilthead seabream, have all been investigated (Gong et al., 2020;

Tibaldi et al., 2015; Vizcaino et al., 2015, Tulli et al., 2012). Specifically, recent studies on European sea bass have shown that incorporating dietary microalgae levels of up to 15 -20% has resulted in growth and feed efficiency parameters similar to those observed in the present study. These parameters include specific growth rates (SGR) ranging from 1.00 to 1.70 and feed conversion ratios (FCR) from 1.15 to 1.64 (Pascon et al., 202 1; Valente et al., 2019). Concerning body composition, protein content was similar in fish fed 50% FM and FO from by -products compared to control diet. However, when SBM was substituted by 50% of MIC and FM and FO were replaced by trimming derivates, body protein content was lower compared to 50FMFO. Moreover, lipid composition was higher when FM and FO were replaced with by - product but also with partial substitution with MIC compared to the control diet. This increment is in accordance with a previous study conducted on European sea bass (Mota et al., 2023) and gilthead sea bream (Valente et al., 2019) fed with *Nannochloropsis* enriched diet, caused by high proportion of LC -PUFA in microalgae.

In contrast with previous study by Randazzo et al. (2023), where a blend of two microalgae whole cell dry biomass was utilized, in this study values of digestibility and nutritional indices were not affected by microalgae partial or total replacement of wild FM and FO. Additionally, fatty acid composition values are often used to evaluate the efficiency of microalgae-enriched diets to replace FO. In this study, the inclusion of microalgae allowed the maintenance of essential FA levels, such as DHA, EPA and ARA. This is in accordance with a previous study conducted on European seabass juveniles fed with 5 enriched diets with two different species of microalgae (*Pavlova viridis* and *Nannochloropsis* sp.) at two percentages as replacement of FO. Here it was shown how the percentages of the microalgae inclusion can influence the fatty acid composition of animal fillets (Haas et al., 2016).

Analyses of plasma biochemistry reveal that the experimental diets had minor effects on the health of the organism. However, only a few plasma parameters showed significant differences between treatment groups. Among those, creatinine (CREA), a metabolic waste product of the creatine, produced at kidney level (He et al., 2020) emerged as notable. Creatine, a nitrogenous organic acid naturally present in metabolically active tissue of all vertebrates, primarily serves to assess muscle condition (Fazial et al. 2018). Elevated levels of creatinine were observed in the blood of fish fed wild fishmeal-fish oil (FMFO), indicating heightened creatine metabolism in these fish. This is likely due to the spontaneous formation of creatinine during the conversion of creatine to phosphocreatine, as proposed by Marchi et al. (2023). Additionally, the experimental diet

influenced plasma cholesterol levels, showing a decrease when microalgae were incorporated. This reduction occurred alongside a decrease in soy protein concentrate, which is recognized for its hypercholesterolemic effects in European sea bass (Bonvini et al., 2018). In contrast, earlier studies did not observe any change in plasma cholesterol levels in European sea bass and Nile tilapia fed with *Nannochloropsis oceanica* (Batista et al., 2020) and *Nannochloropsis oculate* (Zahran et al., 2023), respectively. Nevertheless, microalgae, including Nannochloropsis species, are acknowledged as rich sources of phytosterols that could support lowering blood cholesterol levels (Randhir et al., 2020). Plasma proteins serve as reliable indicators of the well-being of well-nourished animals (Peres et al., 2014). Even if result of TP decreased, they remained the standard range. Plasma electrolytes are valuable indicators of cells health. In this study all values tended to increase, particularly with the higher level of 0FMFO-100MIC compared to control diet, which exhibited lower values, with a difference of 4%. Therefore, the partial and total replacement of wild-caught fish meal and fish oil with soy derivates and microalgae did not adversely affect animal health.

Enzymatic activity is often used as an indicator of digestibility and assimilation in fish feed enriched with microalgae. In this study, we observed no difference in trypsin activity between treatments excluding the antitrypsin action of the vegetable ingredient employed (Biswas et al., 2022). Moreover, the activity of AP reached its peak in fish fed diet with total replacement of wild-caught FM and FO and with half replacement of soy derivates with microalgae (50FMFO-50MIC). Alkaline phosphatase is a key enzyme of the intestinal brush borders, serving as an indicator of intestinal integrity and a general marker of nutrient absorption. The increase of these activities may enhance the overall efficiency of digestive and absorptive processes (Silva et al., 2010; Vizcaíno et al., 2014).

A clear impact of diets has been shown also through the analyses of the gut microbiome. In particular, the inclusion of different percentage of microalgae drives the shifting of the gut microbiome community, clearly segregating from the gut bacteria communities of fish fed diet without microalgae. In particular, *Clostridiaceae*, *Planococcaceae, Peptostreptococcaceae, Enterococcaceae* and *Erysipelotrichaceae*, were the mostly significant family responsible to drive the segregation between diets. These six families are founded extremely abundant in carnivorous fish species gut and well known as promoters of healthy intestinal epithelium (Egerton et al., 2018). At genera level, most promising results are represented by *Pediococcus, Leuconostoc* and *Turicibacter*, belonging to Firmicutes phylum, with a relative increasing in abundance from

control diet to diet with higher replacement of FM, FO and SP with microalgae. *Pediococcus* is a genus of bacterium recognized and widely used as a probiotic in aquaculture and is spready use as supplement in fish feed for marine species as Atlantic salmon (Jaramillo-Torres et al., 2019) as well as in European eel larval stages (Politis et al., 2023). Additionally, positive effect of this probiotic on fish health, increasing growth, influencing body composition and promoting intestinal histology health has been shown also for European Seabass (Eissa et al. in 2022). Moreover, probiotic *P. acidilactici* supplementation works positively in combination with β-glucans or fructooligosaccharides (FOS), also called prebiotics, in a synergic effect to promote fish growth performance (Torrecillas et al., 2018). Few studies have investigated the use of microalgae as potential prebiotic (Oviedo-Olvera et al., 2023) however recent studies aimed to show the positive effect of different microalgae strains on probiotics development and efficacy (Patel et al., 2021). *Turicibacter* is a genus of Firmicutes, largely present in European Seabass gut microbiota (Ofek et al., 2021) and other commercial species such as Tilapia, *Turicibacter* genus have a recognized role into the modulation of bacterial colonization, regulation of host energy metabolism, and host immunity (Bereded et al., 2022). Moreover, *Turicibacter* could produce short-chain fatty acids, and it was positively correlated with the content of butyric acid which is a functional fatty acid that inhibits enteritis and repairs the intestine (Hao et al., 2022). The positive effects of *Nannochloropsis* inclusion in feed on the intestinal microbiota of European Seabass has been recently demonstrate by Ferreira et al. (2022), marking the importance of integrating microalgae in fish feed, not only for their sustainability as aquafeed but also for their positive effect on the fish health.

6.6 Conclusion

This study highlighted the potential of totally replacing wild-caught FM and FO and soy protein using by-products from fisheries and aquaculture of mackerel and sardine as well as salmon trimming and the microalgae *Nannochloropsis* sp., without affecting growth performance. Growth parameters considered in this study do not seem to be affected by substitution of wild-caught FM and FO and soy protein with more sustainable alternatives. Moreover, the study showed that the experimental diets influenced the activity of the main digestive enzymes in the proximal and distal part of the intestine. Specifically, dietary replacement of 100% FM and FO with circular substitutes and 50% replacement of soymeal with microalgae increase the activity of alkaline phosphatase and chymotrypsin.

Based on the data collected in this study, we emphasize the multiple advantages connected with the use of microalgae as a protein source not only having no negative effects on the growth and health of the animal but, on the contrary, promoting important functions such as the absorption of nutrients at intestinal level, avoiding antinutritional factors. Moreover, we observed that the diets with by-products from fishery and aquaculture and microalgae had a positive effect on the richness and abundance of the microbiota, favouring those strains with a demonstrated beneficial effect on the animal's health. Hence, the integration of trimmed fish meal, oil, and microalgae presents a promising alternative to the conventional ingredients extensively employed in aquafeed production. Embracing the utilization of these components on a broader scale is imperative, not solely due to their benign impact on the organism's health and physiology but also owing to their circularity and reduced environmental footprint. By promoting the adoption of such sustainable practices, the aquaculture industry can make significant steps towards promoting ecological balance and mitigating its ecological footprint. This shift not only reflects a commitment to responsible resource management but also paves the way for a more resilient and environmentally conscious approach to aquaculture production.

6.7 Acknowledgement

This research was undertaken under the NewTechAqua (New technologies Tools and Strategies for a Sustainable, Resilient and Innovative European Aquaculture) project, which has received funding from the European Union's Horizon 2020 Programme under grant agreement No 862658 (https://www.newtechaqua.eu/). This partnership was a result of a previous collaboration within another Horizon 2020 European project, SABANA (Sustainable Algae Biorefinery for Agriculture and Aquaculture). Authors acknowledge the support of the University of Almeria (Experimental feeds Service, grant EQC2019-006380-P) on aquafeed elaboration.

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Chapter 7

Organic aquafeed for sustainable aquaculture: Meagre (*Argyrosomus regius*) as an emerging specie. Effect on growth, gut microbiota and plasma biochemistry.

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Unpublished

7.1 Abstract

Organic aquaculture is a growing industry that can help the industry become more sustainable and reduce its impact on the environment. Four experimental diets (C, PEA 7%, PEA 18%, PEA27%) were formulated to replace fishmeal (FM) and fish oil (FO) from trimming using increasing levels of organic pea protein. Each diet was administered to triplicate groups of 40 Meagre juveniles, assigned in a completely random manner, over 85 days. Temperature, salinity, oxygen and photoperiod were kept constant throughout the experiment (24 ± 0.5°C, 25 g L^{-1} and 8.0 ± 1.0 mg L^{-1}, 12:12 light/dark, respectively). At the end, growth indexes, feed intake (FI), proximal composition, somatometric indexes and blood plasma biochemistry were evaluated. Also, gut microbiota composition was assessed through next-generation sequencing. The replacement of Trimming FM and FO with organic ingredients didn't affect Growth performance and Feed Intake. Somatic index, VSI and HIS, highlighted significant differences in diet with replacement of 7% and 18%, respectively. Among Plasma parameters considered, Albumin and Albumin /Globulin ratio presented statistical differences among treatments, but values are in line with previous studies on Meagre. Moreover, inclusion of Pea Protein positive effects microbiota enhancing families with recognized good effects on gut health. In this article, an additional sustainable factor has been brought by replacing the Trimming Fish Meal (FM) and Fish Oil (FO), with organic ingredients. The innovative diets were then administered to Meagre, considered an emerging species, influencing animal health and gut richness.

Keywords

Organic ingredients, emerging specie, Meagre, gut microbiota

7.2 Introduction

Aquaculture is still a growing sector for animal protein production destined for human diets. This spread of aquaculture production put increasing strain on environmental challenges, leading to eutrophication, ecosystem alteration and habitat destruction. Because of those and many other reasons, consumers often associate this sector with an unhealthy and low-quality final product. The global rise in demand for organic products is the consequence of a conscious consumer who searches for healthy organic food produced in healthy ecosystems, respecting environmental and animal life (Gould et al., 2019). The aquaculture sector is still seeking new ways to become more sustainable without renouncing production growth and high-quality final products. In the last decade, several measures have been taken to mitigate the effect of this sector on the environment and one of some approaches to sustainable aquaculture is organic aquaculture (Ahmed and Thompson, 2019). Organic principles in aquaculture are the same as in agriculture system, underlining same rights and duties. Those points could be summered in four principles issues: health, ecology, fairness and care (FAO, 2021). Organic aquaculture is a method of cultivating aquatic species without the use of synthetic fertilizers, pesticides, herbicides, and GMOs. Instead, it focuses on using vegetable-based feed ingredients and additives such as soybean, corn, fish oil, and fish meal. This method of farming offers many benefits to the farmed aquatic animals, including improved nutrition, growth-promotion, and immunity. As well as organic farming has been growing in the last decades, organic aquaculture is spreading in global food production (Ahmed et al., 2020). According to the European Market Observatory for Fisheries and Aquaculture (EUMOFA) report, EU's organic aquaculture production has increased by 60% in five years (2015-2020), mainly due to organic mussel production EU organic aquaculture production, 2022.). Mente et al., (2011) describe organic aquaculture as a fast-growing sector, in terms of diversity of produce, production volumes and values, probably correlated to increasing demand for organic products by consumers. At the beginning of 2000, total production was only about 5000 metric tonnes, primarily from European countries and especially regarding few commercial species, such as salmon, trout and some species of mussels (FAO, 2002). In 2016, Asia was the highest producer, with China as dominant, responsible for more than 74% of the total organic aquaculture products. Europe followed, with a share of about 20% (Gambelli et al., 2019). In European Mediterranean regions, especially Greece (Perdikaris and Paschos, 2010) and Italy (Sicuro, 2019), organic aquaculture is focused on commercial species such as Seabass and sea bream. Those species are the only finfish

groups on an increasing trend: 2,750 tonnes in 2020 (2,000 tonnes in 2015), mostly from Greece. In North Europe, salmon (Lerfall et al., 2016) and trout (Pulcini et al., 2014), were well involved in organic aquaculture with good results on growth and health.

Emerging species are another tool to make the aquaculture sector more sustainable, promoting the conservation of biodiversity with all benefits linked with a healthy environment (Nguyen et al., 2009). Meagre (*Argyrosomus regius*) (Asso, 1801) is a carnivorous species distributed in the Mediterranean Sea, Black Sea and Atlantic coast of Europe. Meagre is considered a promising species for aquaculture diversification in the Mediterranean region, due to its high growth rate, rearing ability and appreciated flesh quality (Quéméner et al., 2002). As an emerging species, many studies were focused on nutrition and growth (Estevez et al., 2022). In a study conducted on Meagre larvae, amino acid profiles of carcasses were evaluated, in order to estimate the AA requirements of fish to find the formulation of an amino acid-balanced diet (Saavedra et al., 2015). Protein requirement for Meagre was set around 40–50%, and plant proteins can replace fish meal up to 76.2% of total dietary protein, without affecting the growth of animals (Estévez et al., 2011). Lipids requirement was established at around 17% and the essential fatty acids (EFA) requirement in practical diets for Meagre fingerlings was fixed at 2.1% of n-3 long-chain polyunsaturated fatty acids (LC-PUFA) in dry weight of the diet (DW) (Carvalho et al., 2019). In order to give a relevant contribution to sustainable aquaculture, this study aims to evaluate the effects of fish meal, (FM) and fish oil (FO) from trimming and organic ingredients as a valid protein alternative source on growth performance and gut health of Meagre juveniles.

7.3 Materials and Methods

7.3.1 Experimental diets

Four experimental (protein 46%, fat 16%) were formulated to contain increasing level of organic pea protein and seaweeds (PEA15.5%, PEA18%, PEA27%) in order to replace Fish meal (FM) and Fish oil (FO) from trimming. Diets were formulated by Irida S.A. (Grece) and produced via industrial extrusion with a diameter of 3 mm. Ingredients and proximate composition of experimental diets are shown in Table 17.

Table 17 Ingredients and proximate composition of the experimental diets.

Composition (%)	**C**	**PEA 7%**	**PEA 18%**	**PEA 27%**
Ingredients, % of the diet				
Fish meal Trimmings	58.5	50.0	41.0	32.0
Fish oil Trimmings	9.30	9.70	10.0	11.2
Organic Soybean meal	15.5	15.5	15.3	16.0
Organic wheat	16.0	10.2	8.00	6.00
Organic pea protein	-	7.00	18.0	27.6
Organic yeast	-	5.00	5.00	5.00
Seaweed	-	2.00	2.00	2.00
Organic premix (vitamins-minerals)	0.60	0.60	0.60	0.60
Natural antioxidant	0.03	0.03	0.03	0.03
Proximate composition, % on a wet weight basis				
Moisture	5.85	5.46	6.20	3.22
Protein	42.8	43.1	42.6	44.6
Lipid	14.0	15.5	15.9	15.9
Ash	17.6	16.1	14.8	13.8

Formulated according to the EU Reg. 2018/848, suitable for organic fish farming. Nutritional request for this species have been respected in order to guarantee the health of animals.

7.3.2 Fish and rearing trial

Meagre (*Argyrosomus regius*) trial was conducted at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. Meagre fries were obtained from Cromaris (Croatia) with approximate weight of around 10 g. Before the beginning of the trial, 400 animals were acclimated to the facilities for three weeks and fed with commercial feed in order to reach the optimal weight. At the beginning of the trial, 40 fish (initial average weight: 33.25 ± 0.14 g) were randomly distributed into twelve 800 L square tanks with a conical base. connected to a closed recirculation unit. Each diet was administered to triplicate groups, assigned in a completely random manner, over 85 days. Tanks were provided with natural seawater and connected to a closed recirculation system. The water exchange rate was kept at 100%/h in each tank and the overall water renewal in the system was at 5% daily. The rearing system consisted

of a mechanical sand filter (PTK 1200, Astralpool, Barcelona, Spain), ultraviolet lights (UV PE 45; Sita Srl, Barcelona, Spain) and a biofilter (PTK 1200, Astralpool, Barcelona, Spain). During the trial, the temperature was kept at 24 ± 1.0 °C and the photoperiod was maintained at 12 h light and 12 h dark through artificial light. Ammonia (total ammonia nitrogen ≤0.1 mg L−1), nitrite (≤0.2 mg L−1) and salinity (25 g L−1) were daily monitored spectrophotometrically (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany). Sodium bicarbonate was added daily to keep pH constant at 7.8–8.0. Feed was provided to satiation by oversupplying the feed via automatic feeders by approximately 10% of the daily ingested ration, twice a day (8:30, 16:30) for six days a week. Each meal lasted 1 h and the uneaten pellets of each tank were gathered, dried overnight at 105 °C, and their weight was deducted for overall calculation (Parma et al. 2020).

7.3.3 Sampling

At the beginning and at the end of the experiment, all animals in each tank were anaesthetised and weighed. Specific growth rate (SGR), feed intake (FI) and feed conversion rate (FCR) were calculated. Proximate composition of the carcasses was determined of the trial on a pooled sample of 10 fish at the beginning and on a pooled sample of 5 fish per tank at the end of the trial.

For the assessment of plasma biochemistry, from 5 fish per tank blood was collected from the caudal vein. Samples were then centrifuged (3000 x g, 10 min, 4°C) and plasma aliquots were stored at −80°C until analysis. Still at the end of the trial, five fish per tank were dissected sampled at 12 hours post meal to gut contents for microbiota analysis.

7.3.4 Calculations

Following, the illustration of employed formulae:

Specific growth rate (SGR) (% day-1) = 100*(ln FBW- ln IBW) / days (where FBW represent the final body weight and IBW and the initial body weights).

Feed intake (FI, g kg ΔBW–1 day–1) = ((100*total feed ingestion)/(ΔBW))/days.

Feed conversion ratio (FCR) = feed intake / weight gain.

Protein efficiency rate (PER) = (FBW – IBW) / protein intake.

Gross protein efficiency (GPE) (%) = 100*[(% final body protein*FBW) - (% initial body protein*IBW)] / total protein intake fish.

Lipid efficiency rate (LER) = (FBW - IBW)/lipid intake.

Gross lipid efficiency (GLE) (%) = 100*[(% final body lipid*FBW) - (% initial body lipid*IBW)]/total lipid intake fish.

Viscerosomatic index (VSI) (%) = 100*(viscera weight/FBW).

Hepatosomatic index (HIS) (%) = 100*(liver weight/FBW).

7.3.5 Proximate composition analysis

Diets and whole body of sampled fish were analysed for proximate composition. Moisture content was obtained by weight loss after drying samples in a stove at 105°C until a constant weight was achieved. Crude protein was determined as total nitrogen (N) by using the Kjeldahl method and multiplying N by 6.25. Total lipids were determined according to Bligh and Dyer's (1959) extraction method. Ash content was estimated by incineration to a constant weight in a muffle oven at 450°C (AOAC, 2010).

7.3.6 Metabolic parameters in plasma

To the assessment of plasma biochemistry were considered 25 indicators, chosen as perfect parameters to detect fish health. Levels of glucose (GLU), urea, creatinine, uric acid, total bilirubin, bile acid, cholesterol (CHOL), triglycerides (TRIG), total protein (TP), albumin (ALB), aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), creatine kinase (CK), lactate dehydrogenase (LDH), calcium (Ca ± 2), phosphorus (P), potassium (K ±) sodium (Na ±), iron (Fe), chloride (Cl), magnesium (Mg), were detected using samples of 500 μL on an automated analyser (AU 400; Beckman Coulter) according to the manufacturer's instructions. Moreover, Albumin/globulin (GLOB), Na/K ratio and Ca x P were calculated.

7.3.7 Gut microbiota

At the end of the feeding trial, total DNA was extracted from individual distal gut content (300 mg per fish) obtained from a total of 75 fish (15 fish per tank), as previously reported in Parma et al. (2016). Total DNA was then quantified with NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and stored at -20 °C until further processing. The amplification of the V3-V4 hypervariable regions of the 16S rRNA bacterial gene was carried out using the 341F and 785R primers with overhang sequencing adapters attached and 2 x

KAPA HiFi HotStart ReadyMix (KAPA Byosystems). As already described by Parma et al. (2020) the thermal cycle consists of 30 amplification cycle and at the end PCR products were purified, and the indexed libraries were prepared following Illumina protocol "16S Metagenomic Sequencing Library Preparation". Libraries were normalized to 4 nM and pooled together, the resulting pool were denatured with 0.2 N NaOH and diluted to 6 pM with 20% Phix control. Sequencing was performed on Illumina MiSeq platform using 2 x 250 bp paired-end protocol according to the manufacturer's instructions (Illumina, San Diego, CA). At the end of the sequencing process, raw sequences were processed combining PANDAseq and QIIME2 pipelines (Bolyen et al., 2019; https://qiime2.org). High-quality reads, obtained after a filtering step for length (min/max = 350/550 bp) and quality step using USEARCH with a max error rate of 3% (Edgar, 2010), were cleaned and clustered into amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016). Taxonomy was assigned using a hybrid method combining VSEARCH and q2 classifier trained on the SILVA database release 138.1(Bokulich et al., 2018). Three different metrics were used to evaluate internal ecosystem diversity (alpha-diversity) – Faith's Phylogenetic Diversity (faith_pd), Shannon_entropy index, and number of observed ASVs (observed features). Unweighted UniFrac distances were computed to estimate inter-sample ecosystem diversity (beta-diversity) and used as input for Principal Coordinates Analysis (PCoA).

7.3.8 Statistical analysis

All data are presented as mean ± standard deviation (SD). Tank was used as experimental unit to evaluate growth performance. Furthermore, a pool of ten fish was considered the experimental unit for the analysis of proximate composition and nutritional indices. Five individual fish were used for analyzing somatic indices, blood biochemistry and gut microbiota community profiles. The homogeneity of variance assumptions was validated for all data preceding ANOVA. Tukey's post hoc test was performed. All statistical analyses were performed using GraphPad 8.0.1. The differences among treatments were considered significant at $P \leq 0.05$. Microbiota analysis and respective plots were produced using R software (https://www.r-project.org/) with "vegan" (http://www.cran.r-project.org/package-vegan/), "Made4" (Culhane et al., 2005) and "stats" packages (https://stat.ethz.ch/R-manual/R-devel/library/stats/html/00Index.html). Data separation was assessed by a permutation test with pseudo-F ratios (function "Adonis" in "vegan" package). When required, Wilcoxon and Kruskal–Wallis test were used to assess significant differences in alpha diversity and taxon relative abundance

between groups. When necessary, P-values were corrected for multiple testing with Benjamini-Hochberg method, with a false discovery rate (FDR) ≤ 0.05 considered as statistically significant.

7.4 Results

7.4.1 Growth

Summary of the results growth index are presented in Table 18. No statistical difference was evaluated among treatments.

Table 18 Growth performance and feed intake of Meagre fed experimental diets over 85 days.

	C	PEA 7%	PEA 18%	PEA 27%	*P- value*
IBW	33.7 ± 0.06	33.3 ± 0.20	33.2 ± 0.06	33.2 ± 0.15	0.4058
FBW	171 ± 10.0	173 ± 22.5	189 ± 11.7	178 ± 23.7	0.5360
WG	139 ± 9.99	140 ± 22.5	156 ± 11.7	145 ± 23.9	0.6713
SGR	1.93 ± 0.07	1.93 ± 0.15	2.04 ± 0.07	1.97 ± 0.17	0.6754
FI	1.82 ± 0.04	1.82 ± 0.16	1.76 ± 0.03	1.77 ± 0.06	0.7828
FCR	1.21 ± 0.09	1.17 ± 0.20	1.09 ± 0.06	1.13 ± 0.07	0.6399
Survival	81.7 ± 13.8	90.0 ± 15.2	93.3 ± 1.40	87.5 ± 5.00	0.5962

Data are given as the mean (n=3) ± SD. In each line, different superscript letters indicate significant differences among treatments (P ≤ 0.05).
IBW = Initial body weight.
FBW = Final body weight.
SGR = Specific growth rate (% day-1) = 100 * (ln FBW- ln IBW) / days.
FI = Feed Intake (g feed/fish) = g feed ingested / number of fish
FCR = Feed conversion rate = feed intake / weight gain.
WG = Weight gain (g)
Survival = Survival (%)

7.4.2 Proximate Composition

Body composition, nutritional indices and somatic indices measured in Meagre are shown in Table 19. No statistical differences were evaluated for all parameters considered, except for GLE, VSI and HSI index. GLE was statistically higher in PEA 18% diet compared to PEA 7% and PEA 27%. Results of VSI shown a P-value lower than 0.05 but not specific difference among treatments was evaluated by multiple comparison Tukey's test. HIS is statistically higher in PEA 7% compared to PEA 18%.

Table 19 Body composition, nutritional indices and somatic indices measured in Meagre.

	C	PEA 7%	PEA 18%	PEA 27%	*P-value*
Whole body composition, %					
Moisture	70.9 ± 1.80	70.8 ± 0.80	68.8 ± 0.99	71.7 ± 0.57	0.1795
Protein	17.9 ± 0.11	18.3 ± 0.27	18.2 ± 0.6	17.8 ± 0.5	0.5180
Lipid	7.66 ± 1.89	7.44 ± 0.55	9.82 ± 0.72	7.33 ± 0.12	0.0599
Ash	2.78 ± 0.13	2.9 ± 0.03	3.07 ± 0.05	2.93 ± 0.2	0.1062
Nutritional indices					
PER	3.24 ± 0.23	3.25 ± 0.52	3.65 ± 0.27	3.25 ± 0.53	0.5712
GPE	58.1 ± 4.18	59.8 ± 9.57	66.6 ± 4.98	57.9 ± 9.54	0.4863
LER	9.90 ± 0.71	9.04 ± 1.45	9.78 ± 0.73	9.12 ± 1.5	0.7322
GLE	85.3 ± 5.47^{ab}	75.4 ± 10.8^{a}	109 ± 7.21^{b}	78.2 ± 11.5^{a}	**0.0074**
Somatic index					
CF	1.11 ± 0.04	1.12 ± 0.08	1.08 ± 0.04	1.07 ± 0.05	0.1946
VSI	3.95 ± 1.19	5.31 ± 1.75	3.78 ± 1.14	4.51 ± 0.86	**0.0486**
HSI	1.85 ± 0.67^{ab}	2.61 ± 0.78^{b}	1.74 ± 0.59^{a}	2.09 ± 0.42^{ab}	**0.0200**

Data are given as the mean (n=3 ± SD). In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
Data are given as the mean (n=3 ± SD). In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
PER = Protein efficiency ratio = ((FBW-IBW)/protein intake).
GPE = Gross protein efficiency = 100*[(%final body protein*FBW) - (%initial body protein*IBW)]/total protein intake fish.
LER = Lipid efficiency rate = (FBW - IBW)/lipid intake.
GLE = Gross lipid efficiency = 100*[(% final body lipid ∗ FBW) - (% initial body lipid*IBW)]/total lipid intake fish.
CF = condition factor = 100 × (body weight, g)/ (body length, cm)3
VSI = Viscerosomatic index (%) = 100*(viscera weight/FBW).
HSI = Hepatosomatic index (%) = 100*(liver weight/FBW).
SD = Standard deviation.

7.4.3 Plasma results

Results of plasma biochemistry are showed in Table 20. Statistical differences were evaluated in glucose, total protein, albumin, albumin/globulin, current calcium and sodium parameters. Glucose was higher in animals fed with control diets and lower in PEA27% diet. Total protein was lower in control diet and PEA 27% compared to PEA 18% diet. Albumin showed higher values in animal fed with PEA18% diet than control diet. Albumin/globulin values were higher in animals fed with PEA 27% diet than C and PEA 7% diets. Current calcium was lower in PEA 18% and PEA 27% diets compared to PEA 7% and Na was higher in C and PEA 7% than PEA 27%.

Table 20 Plasma biochemistry in Meagre fed with experimental diets.

	C	PEA 7%	PEA 18%	PEA 27%	P - value
GLU	164 ± 33.2^{b}	164 ± 28.1^{b}	123 ± 36.4^{ab}	104 ± 42.0^{a}	**0.0013**
Urea	6.74 ± 1.24	6.90 ± 1.49	7.53 ± 18	8.58 ± 1.80	0.0811
CREA	0.45 ± 0.12	0.37 ± 0.08	0.33 ± 0.1	0.51 ± 0.23	0.0519
Uric. Acid	0.08 ± 0.14	0.04 ± 0.03	0.05 ± 0.06	0.04 ± 0.04	0.5108
Tot Bil	0.12 ± 0.02	0.11 ± 0.02	0.10 ± 0.04	0.10 ± 0.04	0.5819
Ast	92.0 ± 92.0	93.0 ± 61.1	104 ± 95.0	131 ± 158	0.8578
Alt	17.0 ± 26.1	10.4 ± 44.4	12.4 ± 6.98	15.5 ± 15.8	0.8124
Alp	33.1 ± 4.86	38.3 ± 9.19	48.9 ± 30.9	53.8 ± 26.5	0.1615
CK	1077 ± 612	1707 ± 955	2284 ± 2624	1971 ± 2310	0.5591
LDH	445 ± 289	494 ± 322	518 ± 472	555 ± 607	0.9602
Ca^{2+}	13.5 ± 0.74	13.9 ± 0.49	13.7 ± 0.91	12.8 ± 1.79	0.0617
P	10.84 ± 0.89	11.4 ± 1.08	12.03 ± 1.99	11.7 ± 1.61	0.3780
Mg	2.59 ± 0.26	2.79 ± 0.12	2.70 ± 0.27	2.59 ± 0.28	0.2841
CHOL	181 ± 21.2	201 ± 22.6	187 ± 32.7	189 ± 39.4	0.5534
HDL	40.6 ± 5.17	42.6 ± 7.49	38.6 ± 5.41	39.9 ± 7.40	0.6203
TRIG	1488 ± 234	1614 ± 434	1457 ± 611	1515 ± 755	0.9349
TP	2.94 ± 0.22^{a}	3.09 ± 0.15^{ab}	3.28 ± 0.26^{b}	3.00 ± 0.16^{a}	**0.0090**
Alb	0.62 ± 0.05^{a}	0.64 ± 0.05^{ab}	0.71 ± 0.07^{b}	0.68 ± 0.06^{ab}	**0.0081**
Cur. Ca	16.3 ± 0.67^{ab}	16.7 ± 0.46^{b}	15.9 ± 0.94^{a}	15.6 ± 1.11^{a}	**0.0467**
ALB/GLO	0.27 ± 0.01^{a}	0.26 ± 0.02^{a}	0.27 ± 0.03^{ab}	0.29 ± 0.03^{b}	**0.0068**
LAC	29.4 ± 11.0	28.3 ± 7.53	26.2 ± 7.01	26.7 ± 7.52	0.8413
Fe	60.3 ± 16.2	60.6 ± 11.3	57.9 ± 20.9	56.7 ± 22.9	0.9612
Na	173 ± 4.46^{b}	175 ± 3.90^{b}	170 ± 2.49^{ab}	167 ± 3.82^{a}	**0.0010**
K	3.04 ± 0.42	2.76 ± 0.44	3.06 ± 0.83	3.18 ± 1.16	0.6873
Cl	147 ± 2.82	148 ± 3.27	145 ± 2.12	143 ± 4.89	0.0558

Data are given as the mean (n=15 $diet^{-1}$) ± SD. Different letters indicate significant difference (One-way ANOVA $P \leq 0.05$) between treatments. GLU, glucose , (mg dL^{-1}); Urea , (mg dL^{-1}); CREA, creatinine , (mg dL^{-1}); Uric Ac, uric acid , (mg dL^{-1}); Tot Bil, total bilirubin , (mg dL^{-1}); Ast, aspartate aminotransferase , (U L^{-1}); Alt, alanine aminotransferase (U L^{-1}); Alp, alkaline phosphatase , (U L^{-1}); CK, creatine kinase , (U L^{-1}); LDH, lactate dehydrogenase , (U L^{-1}); Ca^{+2} , calcium , (mg dL^{-1}); P, inorganic phosphorus; , (mg dL^{-1}); Mg, magnesium , (mg dL^{-1}); CHOL, cholesterol , (mg dL^{-1}); HDL, high density lipoprotein; TRIG, triglycerides , (mg dL^{-1}); TP, total protein , (mg dL^{-1}); Alb, albumin , (g dL^{-1}); Cur Ca2+, current calcium (mg dL^{-1}); ALB/GLO, albumin/globulin; LAC, lactate (mmol L^{-1}); Fe, iron , (µg dL^{-1}); Na^{+}, sodium , (mEq L^{-1}); K^{+}, potassium , (mEq L^{-1}); Cl, chloride , (mEq L^{-1}); SD, standard deviation.

7.4.4 Gut Microbiota

The 16S rRNA gene sequencing was performed on a total of 75 distal intestine content samples. The gut microbiota (GM) was analysed for each dietary group at the end of the trial, to assess the effect of organic pea protein. To evaluate the GM variations between samples (beta-diversity), Principal Coordinates Analysis (PCoA) based on Unweighted UniFrac distances was used. Moreover, the gut microbial community diversity, within each dietary group, was represented with faith-PD, Shannon_entropy and observed-features. In terms of overall GM composition, according to our results (Figure 18), not all dietary groups showed a significant variation compared to the control group, regarding both alpha and beta diversity ($p > 0.05$). The overall GM composition at different phylogenetic levels was investigated, as reported at phylum and family level in Figure 19, while specific genera significant variations were highlighted in Figure 20. More specifically, at phylum level the most abundant taxon observed was Proteobacteria (with an overall relative abundance mean of 74%), followed by Firmicutes (with an overall relative abundance mean of 15%). The most represented families were *Pseudomonadaceae* (r.ab. mean ± SEM 73.7 ± 4.83% C; 60.9 ± 9.21% PEA7%; 67.7 ± 6.72% PEA 18%; 71.5 ± 7.77% PEA27%), and *Lactobacillaceae* (4.92 ± 3.96 C; 2.81 ± 0.94 % PEA7%; 8.83 ± 5.11% PEA 18%; 8.74 ± 3.30% PEA27%) this last one belonging to Firmicutes phylum. The most abundant genus was *Pseudomonas* (with an overall relative abundance mean of 69%). Focusing on the specific variation between each group at family level, we observed a significant increase in the relative abundance of *Rhodobacteracea* and Marinomonadaceae genera in PEA7% groups compared to the C group (Wilcoxon rank-sum test, $p < 0.05$) and then a slight decrease in other groups as shown in Figure 20. Still in Figure 20 figurate, at genera level, the significant increase of *Lactobacillus* and *Levilactobacillus* groups, especially in PEA27% group compared to C group. On the other hand, we observed a significant decrease of *Nitrosomonadaceae*, in treatment groups compared to the C group while *Marinomonas* increased in PEA7% group compared to C and then decreased in other treated groups (Wilcoxon rank-sum test, $p < 0.05$; Figure 20).

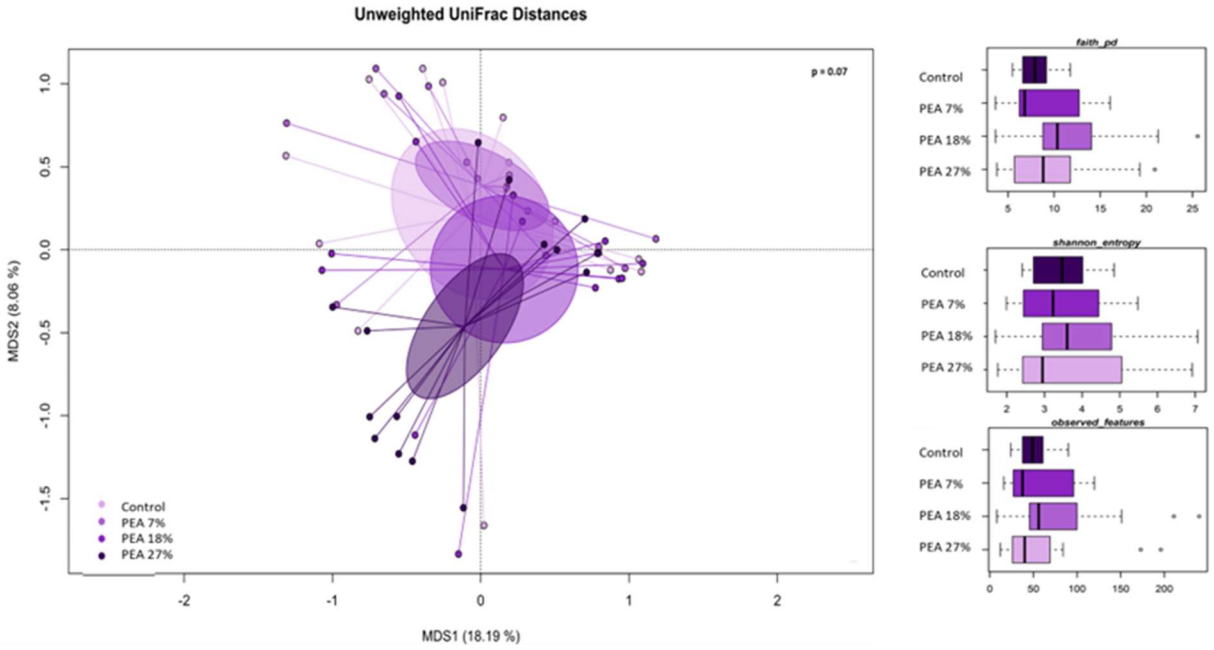

Figure 18 Beta diversity and alpha diversity of gut microbiota of Meagre fed with experimental diets over 85 days. On the left, Principal Coordinates Analysis (PCoA) based on unweighted UniFrac distances between gut microbiota composition of animals fed with experimental diets. No significant separations were highlighted (permutation test with pseudo-F ratios Adonis; p = 0.05).

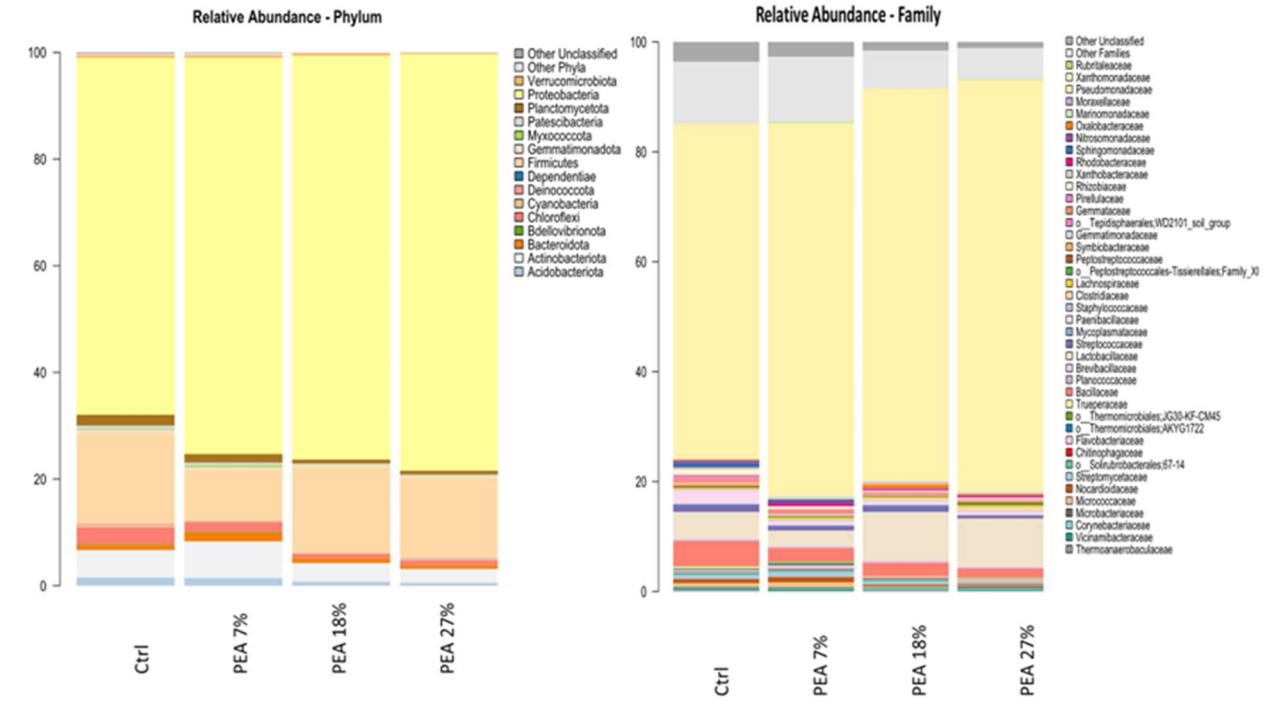

Figure 19 Microbiota composition of distal gut content of Seabass fed with experimental diets. Bar plot summarizing the microbiota composition at Phylum (left) and family (right) of fish intestinal content. Only phyla and families with a relative abundance ≥1.0% in at least 3 samples are shown.

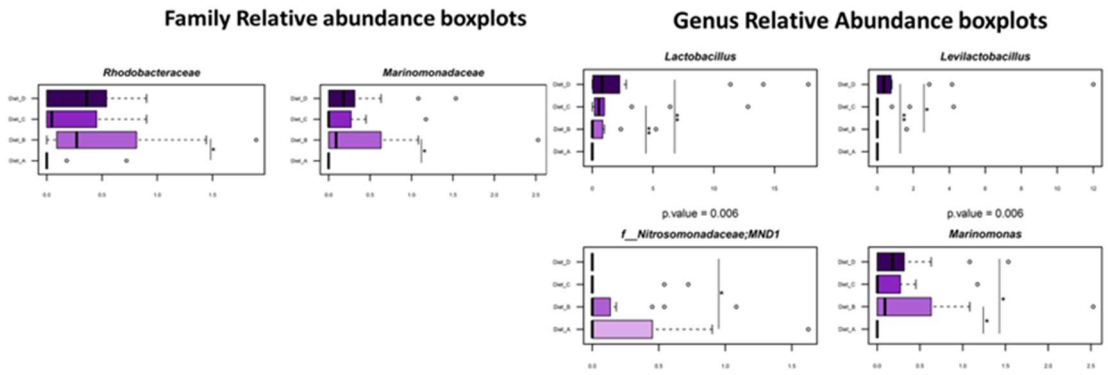

Figure 20 Taxonomic composition of bacterial communities of distal gut content of Meagre fed experimental diets. Distributions of relative abundance of families and genera that showed a significant variation between groups fed with different diets (Wilcoxon rank-sum test, *** $p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$). Only families with a mean relative abundance ≥1.0% in at least 3 samples were represented. The central box of each dataset represents the distance between the 25th and the 75th percentiles. The median between them is marked with a black line.

7.5 Discussion

This study highlighted the possibility of replacing FM and FO from trimming using organic ingredients and at the same time, investigating the effect of an organic experimental diet on Meagre, an emerging species, correlating the effect of organic ingredients with animal health and gut microbiota status. Growth parameters and Feed Intake are main indicators to detect the efficiency of a feed formulation. As shown in this study, no statistical differences were evaluated on growth index, and Feed Intake calculation. Same results were obtained in a previous study on Meagre, fed with an organic selenium supplement (Mansour et al., 2017). Comparing values from both studies, was possible to declare results obtained in this article as standard values. Forward, biometric parameters, body composition and nutritional index didn't present any statistical difference, except for GLE. In a study conducted on Meagre, with the aim to obtain lipid evaluation on farmed and wild Meagre fish (Sinanoglou et al., 2014), lipids values were found to be consistent with those observed in this study. As there were no statistical differences between treatments and values of both studies were the same, the authors could define these values as standards. Somatic indices are used in fish nutritional trials, in order to evaluate the effect of experimental diets on health of animals. These indices perfectly reflect health status of animals, and their interpretation is fundamental in nutrition field. For this reason, literature is abundant in this sense and is easy to find papers on freshwater species as salmon (Hart et al., 2021) and marine species, like European Seabass (Busti et al., 2020) and Gilthead Seabream (Busti et al., 2020) and also on emerging species like Meagre (Guroy et al., 2019; Lozano et al., 2017). In this study, statistical differences were shown in VSI and HIS indices. VSI, presented a P-value slightly lower than 0.05, while HIS presented differences between treatments, with the highest and lowest values in PEA7% and PEA18% diets, respectively. In a study conducted on Meagre by Chatzifotis et al., in 2018 on the effect of feed deprivation, HSI was influenced by starving conditions, decreasing during the trial. In this study, the lowest value of HSI, represented by PEA 18% diet, decreased by about 5% to Control. This is in accordance with Piccolo et al., (2008) who in a study conducted on Meagre, in order to evaluate the effect of two different proteins/fat reported similar values of HSI obtained in this trial. Other indicator to detect animal health and how an experimental diet can influence it, is plasma biochemistry. In Meagre, few studies used plasma as health indicator and most of them only consider stress markers (Herrera et al., 2021). No data was detected on the effect of partial replacement of FM Trimming with organic Pea Protein on health of this species. Generally, cortisol, lactate glucose and plasma

protein are used as indicators of stress conditions in animals. In this study, values of lactate and cortisol did not present any statistical difference, but glucose and Total protein did. Plasma proteins are good indicators of well-nourished animals as shown in previous studies on other marine species, like European Seabass (Marchi et al., 2023; Pelusio et al., 2021). In this case study, all values tend to increase than control diet, with higher value in animals fed with PEA 18% diet. At least, one of the main tools to investigate the effect of an experimental diet on animal health is the analysis of gut microbiota. As an emerging species, literature lacks gut microbiota analysis on Meagre, and only a few, deep investigated the diversity and richness of microbiota. In this study, there was a shift of fish gut microbiota towards an increase in Proteobacteria at the expense of beneficial bacteria from the Firmicutes. Despite this, the most represented families were *Pseudomonadaceae* and *Lactobacillaceae;* this last well recognized as probiotics. Same phyla were found in gut microbiota of Meagre in a study conducted on the effect of Black soldier fly meal on health conditions (Couto et al., 2022), but Firmicutes as dominant phylum. In that case study was attributed a beneficial effect of chitin against several Proteobacteria, thanks to its potential prebiotic properties and as a major substrate for Lactic acid bacteria (Askarian et al., 2012). The correlation between organic ingredients and gut microbiota composition is different for each species, but the authors didn't find any authoritative study to justify results obtained in this trial. In a study conducted on the effects of increasing dietary levels of organic acids and nature-identical compounds on growth, intestinal cytokine gene expression and gut microbiota of rainbow trout (*Oncorhynchus mykiss*) most abundant phyla were still Firmicutes and Proteobacteria, in this order. In conclusion, it could be an indirect action of organic ingredients to promote beneficial phyla with positive effect on gut health.

7.6 Conclusion

In conclusion, organic ingredients could be used in feed formulation without affecting growth and health, with a positive effect on sustainable aquaculture. In this study, was highlighted the use of organic raw materials, even in percentages just under 30%, does not affect the health and growth of the animal and can replace traditional protein sources without any complaints. This means that organic formulations can give breathing space to the aquaculture sector, already highly stressed by the search for alternative protein sources. Moreover, this study contributes to the characterization of the gut microbiota of this incredibly promising species, trying to fill some gaps in this topic. This is only the beginning in nutritional field to broaden the knowledge about the organic ingredients as well as in commercial species and also in emerging species and how an organic diet can interact with animals' health.

7.7 Acknowledgement

This research was undertaken under the NewTechAqua (New technologies Tools and Strategies for a Sustainable, Resilient and Innovative European Aquaculture) project, which has received funding from the European Union's Horizon 2020 Programme under grant agreement No 862658 (https://www.newtechaqua.eu/).

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8 Discussion and Conclusion

Sustainability is a current topic intensely studied and debated. The approach to this theme can be addressed from many points of view; social, economic, environmental and biological factors, connected to each other, all of them led the way to a balanced interaction between ecological and anthropological systems.

This PhD project was mainly focused on reducing the environmental impact of commercial aquafeed, maintaining high quality nutritional standards and promoting fish health. To achieve this result, this project was focused on fish meal and fish oil replacement, looking for alternative ingredients that could equate or exceed performance of commercial aquafeed, especially in terms of growth and health.

In the first study, feed formulation did not involve the use of sustainable alternative ingredients but the production of the ingredients in a sustainable way. This mainly depends on the life stage of the animals evaluated. In fact, in the early stage of European Seabass, mouth apparatus is not yet fully formed and after the absorption of the yolk sac, it is necessary to administer a food that is easy to ingest and digest. Not being able to operate on formulation, in this study, the sustainable production of microalgae was the main topic to investigate. Microalgae are commonly produced in photobioreactors, where ambient parameters are settled and controlled in order to avoid quality fluctuation or even quality loss. To standardize those parameters, a huge amount of energy and materials are used during the production, with a consequent huge amount of waste. In that way, such a noble product then becomes an impact factor on the environmental cycle. In this study, using geothermal energy as clean source, production impact on ambient was drastically reduced.

In the second and third case study, instead, sustainability was applied to FM, FO and SBM, three main ingredients in feed formulation, replacing them with alternative ingredients originating from by-products of fishery and aquaculture. In the third case, microalgae were also involved in feed formulation and administration, but as alternative protein source of SBM.

If in the first trial, differences in growth performance were evaluated, in the second none of growth parameters considered showed statistically different results between treatments. Those differences may be justified by different life stages of animals. Every single life stage has different needs, which reflect health status. Nutritional requirements must be considered during feed formulation and production. For example, the optimum protein level for Seabass juvenile feed was estimated around 50%, with a minimum of 45% up to

60% (Oliva-Teles, 2000). And since nutritional needs are different in adults, the sense organs can also be considered an influencing factor. In an interesting study conducted by Abbate et al., in 2012 on the morphology of the tongue of Seabass, the authors demonstrated how the structure of this organ had a fundamental role in the ingestion of aliments. Furthermore, on the surface of tongue were found numerous taste buds and fungiform and conical papillae which also may influence palatability. We can conclude that fishmeal, fish oil, and also soy, can be replaced with alternative protein sources, as long as biological and nutritional needs are respected.

Regarding blood parameters, we can notice that different values of Creatinine, HDL and Total Protein were reported in both studies. In both studies, highest values of all three parameters were shown in Control Diets compared to the other treatments. In this case, therefore, the replacement of fish meal and oil with by-products, as well as soybean meal with alternative protein sources, is not dependent on the life stage or physical characteristics. Or at least, it's easy to assume, that despite these differences, they respond the same way.

In both case studies, where microalgae were involved, no statistical differences were evaluated on growth performance. This is because the use of microalgae during larval fish rearing has many benefits on health, increasing nutritional quality profile of *Artemia*, but administration remains indirect and not strictly connected to animal growth (Qin, 2013). However, in Seabass adults, the administration of microalgae positively influences health, in particular at intestinal level, shifting and promoting those families of bacteria with a recognized beneficial effect on the animal health. The effects of dietary inclusion of some marine microalgae as *Nannochloropsis* sp, *Tisochrysis lutea* and *Tetraselmis suecica* demonstrated a positive effect on enhancing the innate immune response without affecting intestinal morphology and functionality (Picchietti et al., 2021). In the end, it’s possible to state that the inclusion of microalgae in fish feed, even as a substitute for vegetable protein sources, positively influences animal health. A healthy animal is less susceptible to disease, responds better to physiological and environmental stressors and generally has a higher performance.

In the last project, a further step toward sustainable aquaculture was made by using organic ingredients and selecting an emerging species. Is known that organic aquaculture excludes some products if not comply with organic processes. Final products obtained from organic processing presented higher quality and better nutritional profiles, because not afflicted by industrial food processing and respecting original nutritional characteristics. In this study, organic ingredients did not affect growth performance and health of animals, but

there is a lack of bibliography on organic aquaculture for this species, data were therefore compared with other two Mediterranean commercial species. Organic means, under definition, sustainable and the possibility of being able to breed distinct species becomes a sustainable choice allowing to lighten the pressure on those heavily bred species. Moreover, the introduction of new fish species and products can reduce the pressure on over-exploited fisheries and explore new segments for the EU market (Mylonas et al., 2019). Organic ingredients and emerging species became a combo that can boost aquaculture sector to sustainability.

This thesis highlights the potential of totally replacing wild-caught FM and FO and soy protein using by-products from fisheries and aquaculture, with only a marginal reduction of overall performance. The inclusion of microalgae enhances animal health, mainly at intestinal level, promoting beneficial bacteria. Besides, organic ingredients can be included in aquafeed for emerging species without any affection on growth and animal health. This PhD study also confirms that reaching sustainability is a set of factors that interact with each other's. To meet environmental issues, aquaculture may work not only on ingredient or feed formulation but also on feed production, target species, life stage of animal, or all of them. Not only a tool can be used, but several and all of these are important to reach the final goal, sustainability. Despite the scientific community investigated this field for almost 20 years, further studies need to be done, making sustainability a very current topic.

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Acknowledgement

Questa esperienza di Dottorato è stata per me un percorso estremamente formativo. Mi ha impartito tantissime lezioni che non solo, non credo avrei potuto imparare senza, ma che hanno riguardato tantissime sfaccettature della quotidianità e ad anche del lavoro che presumo andrò a svolgere.

Ringrazio i miei colleghi, che mi hanno supportata in questi tre anni, fra risate e chiacchiere, alleggerendo anche i momenti più duri. Chi fa da sé fa per tre, ma insieme si fa meglio e prima.

Doy gracias a España por ser tan bonita y acogedora y agradezco a los españoles por hacerlo así. Un abrazo enorme a la gente que conocí en Almería, que sigo llevando en el corazón y a la que espero volver a ver.

Ringrazio la mia migliore amica, che ancora ed ancora ha ascoltato tutti i miei problemi, spesso impotente, e spesso con una pietra sul cuore. Gli amici sono la famiglia che scegliamo per noi stessi.

Ringrazio Sandra ed Ettore ed il loro buon cuore. Vi ringrazio per avermi accolta nel vostro nido e nella vostra casa come se fossi stata lì da sempre e guardandomi come se non me ne dovessi mai andare. La mia promessa è di rimanere, per provare a sdebitarmi ancora ed ancora e ripagare quel debito di amore che devo sanare.

Questo risultato è mio quanto vostro e lo dedico a voi con tutto il mio cuore. Alzo la coppa al cielo pensando a voi e aspetto il momento in cui si stappa lo champagne e si festeggia per poter esultare insieme a voi. Durante il mio cammino, quando ho sentito di essere in difficoltà, mi sono guardata indietro e vi ho sempre trovati lì. Ma il mio percorso è pieno delle vostre tracce; siete di fianco a me quando ho bisogno di un sostegno e davanti a me quando ho bisogno dell'esempio. Nelle vostre orme, ci riconosco anche le mie e continuerò a seguirle sapendo di non perdermi mai.

Noi due andiamo oltre al tempo ed allo spazio, la nostra storia è cominciata in un attimo e dopo mesi, in un luogo ed in tante città. Vorrei avere il potere di congelare il tempo e lasciare tutto così, ma trepido in attesa di scoprire cosa ci riserverà il crescere insieme. Per noi voglio tutto e non voglio niente. Per me, però, io chiedo te!