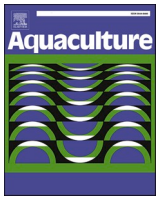
Aquaculture 592 (2024) 741257

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



Feeding of rainbow trout (*Oncorhynchus mykiss*) with organic ingredients replacing fish meal

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ARTICLE INFO

Keywords:
Organic trout
Fishmeal substitution
Organic farm
Rainbow trout
Microbiota
Health status

ABSTRACT

Demand for organic aquaculture is rising, but its viability will depend on the availability of economically viable raw materials to formulate organic diets. In the current work, organic diets were formulated based on different alternative protein sources distinguished by their ecological origin, insect meal (IN), seabass by-products (SB) and Iberian pig by-products (IB) in rainbow trout (*Oncorhynchus mykiss*) and their effects on growth, efficiency, productivity, and intestinal health. Fish with an initial weight of 67.2 g were fed two times a day until apparent satiation for 150 days. The control diet containing fish meal (FM) originated the highest final weight (298 g). Results obtained in the final body weight and the specific growth rate feed conversion ratio average daily gain indicate that the SB-FM, SB-IB, and SB-IN-IB diets presented a lower performance (272 g, 257 g, and 258 g final weight respectively) and FM-IN and IN-IB diets had the lowest final weight (215 g and 183 g respectively). An improvement in growth performance and nutrient utilization was observed in the SB-FM, SB-IB, and SB-IN-IB diets concerning the FM-IN and IN-IB diets. The lowest retention efficiencies of protein, fat, and essential amino acids were found in the IN-IB diet. The highest apparent digestibility coefficients (ADCs) of protein, energy, calcium, and phosphorus are found in Control and FM-IN diets. Results of enzymes showed that both trypsin and chymotrypsin are relatively low in IN-IB and SB-IN-IB. Fish-fed FM-IN and IN-IB diets showed histological changes in the liver and intestine. Considering the intestinal microbiota composition, the three dominant phyla were *Firmicutes* (59–89%), *Spirochaetota* (5–35%), and *Proteobacteria* (3–16%), but no differences between diets were obtained. No significant differences were observed on the Alpha diversity Shannon index. Therefore, although differences in growth were observed, the high substitution of fishmeal did not imply an alteration of the intestinal microbiota, possibly due to the high dominance of *Firmicutes*. Nevertheless, from an economic point of view, SB-IB diets gave the lowest economic conversion index and the highest economic profit index. In conclusion, the substitution of fishmeal affected the growth of the animal, registering the best results in the control followed by diets containing fishmeal of marine origin, but the lowest price of animal by-products originated the best economic results.

1. Introduction

Ecological production, also called biological or organic, is an agri-food management and production system that combines the best environmental practices, a high level of biodiversity, preservation of natural resources, and the application of demanding standards on animal welfare (FAO, 2020). Mainly, organic aquaculture has grown significantly in recent years, achieving in 2020 6.4% of total EU aquaculture production, around 74.032 t (EUMOFA, 2022). Variations in regulatory frameworks pertaining to organic certification exist globally. In this document, the term “organic” conforms to the regulations stipulated by the European Union, delineating the criteria necessary for certifying raw

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https://doi.org/10.1016/j.aquaculture.2024.741257
Received 16 January 2024; Received in revised form 22 May 2024; Accepted 18 June 2024
Available online 20 June 2024

materials as such. Therefore, organic aquaculture is a modest but rising part of the worldwide food production industry (Willer et al., 2021), based on four guiding principles: (1) health, (2) ecological, (3) equity, and (4) welfare (Gould et al., 2019). Most aquaculture production, 82%, is centered in Asia, primarily in China, with the remaining 18% in Europe (Willer et al., 2023).

Trout is the EU's third most abundant organic aquaculture species, accounting for 4.590 t, mainly distributed as follows: France with 2.346 t, Spain with 917 t, and Denmark with 642 t (EUMOFA, 2022). Trout is the main species of Spanish continental production, reaching its maximum production peak in 2001 (36,000 tons), but its production was drastically reduced to around 19,400 t in 2020 (APROMAR, 2020). Consequently, organic production may be an excellent option to reinforce trout consumption by providing a differentiated product with added value.

Few studies have been performed about fishmeal substitution for organic diets, being current work the first in rainbow trout. The findings from the research conducted on seabream and seabass indicate that completely substituting fishmeal with organic ingredients, including rainbow trout by-products, Iberian pig viscera, and insects, brings several benefits in terms of digestibility, histology, and growth performance (Tefal et al., 2023b; Tefal et al., 2023a). According to (Lunger et al., 2007; Lunger et al., 2006), up to 40% of fishmeal protein can be substituted by NuPro (an organically certified yeast-derived protein source) in juvenile cobia (*Rachycentron canadum*) without harming growth performance. Other studies compared commercial and organic feeds with organic soybean cake and wheat in organic farming of seabass and sea bream, obtaining a similar performance in both diets (Di Marco et al., 2017) without evidence that nutrition affected stress and immunological response.

One of the possible reasons for the few studies performed on organic diet formulation is the need for organic raw ingredients, in addition to reducing the inclusion of raw material from fisheries through fishmeal or oil substitution by alternative sources to achieve sustainable production. This is a general problem in the aquaculture sector, but organic production is more critical due to the low availability of high-quality alternative sources with an organic origin.

Under organic European Council Regulation, the diet may contain up to 60% organic plant ingredients and synthetic amino acid addition is prohibited in organic aquaculture diets. Furthermore, the transitional period for using 30% non-organic fish offal ended in 2014, making the organic diet formulation more complicated. A possible alternative may be the inclusion of Transformed Animal Proteins (TAPs) from non-ruminant animals, whose use is allowed in conventional aquaculture (RD 578/2014), and insects (Reg. EU 893/2017) but currently not explicitly authorized in organic aquaculture. The use of TAPs with organic origin does not imply any contradiction to the regulations, allowing the formulation of organic aquaculture feed without capturing fishmeal, only using the recovery of by-products resulting from the transformation of organic aquaculture itself or even TAPs from terrestrial organic farms. Organic plant sources are scarce, except soybean, and their amino acid profiles are insufficiently matched to produce an optimal fishmeal. Therefore, considering previous studies of fishmeal substitution in carnivorous species, different alternative protein sources have been investigated: plant-based protein sources, insect meal, TAPs, or organic fish by-products from the industry. Fishmeal replacement with plant-based protein sources has been widely studied, with the most promising the inclusion of soybean meal (SBM) (Heikkinen et al., 2006) or canola protein concentrate (Drew et al., 2007), since the addition of soybean meal to the diet, which replaced 45% of fishmeal (FM), led to an increase in feed conversion rate (FCR) and histopathological alterations. The differences in microbiology or inflammation did not impact the animals' performance in trials that lasted up to 18 weeks (Heikkinen et al., 2006). Substituting fish oil and fishmeal with vegetable oils and proteins can lower the concentration of polychlorinated dibenzodioxins and polychlorinated dibenzofurans (PCDD/F) and dioxin-like polychlorinated biphenyls (DL-PCB) in rainbow trout and influence their growth rate (Drew et al., 2007). Alternative animal proteins have also been used, such as krill, feather meal, meat, bone meal, and bacterial protein; however, mixes of plant-based proteins have produced the best development outcomes (Albrektsen et al., 2022; Lee et al., 2010). In a study on Atlantic cod, Toppe et al. (2006) utilized fish bone meal (FBM) as a dietary ingredient and study's findings were encouraging, indicating that it may be possible to substitute up to 45% of dietary FM protein with the FBM, which has a crude % protein content of 56%.

Insect meal has been used as a protein source for fish and crustaceans (Mousavi et al., 2020; Quang et al., 2022; Rumbos et al., 2021). St-Hilaire et al. (2007) reported that a rainbow trout diet containing 15% black soldier fly prepupae or housefly pupae had no adverse effect on feed conversion efficiency throughout a 9-week feeding period; the average initial weight was 22.6 g.

Aquaculture protein by-products are a sustainable and cost-effective alternative to conventional fishmeal-based feeds, which can help mitigate the environmental impact of fish processing and reduce the dependence on wild fish stocks for fishmeal production.

Several studies have investigated the use of aquaculture protein by-products in aquaculture feed. For instance, (Li et al., 2021) evaluated a combination of shrimp hydrolysate and plant proteins in the diets of largemouth bass, demonstrating that up to 30% of fishmeal can be replaced without any negative effect on growth performance. Similarly, (Gunathilaka et al., 2021) studied the use of shrimp protein hydrolysate and krill meal in the diets of red seabream. They found that incorporating shrimp protein hydrolysate can reduce fishmeal usage by up to 20%. Another study by Khieokhajonkhet and Surapon (2020) assessed the use of fish protein hydrolysate in the diets of Nile tilapia, showed that incorporating 10% resulted in the highest growth performance, feed, and protein utilization. These findings highlight the potential of aquaculture protein by-products as a valuable ingredient in aquaculture feed formulations.

Summing up, the objective of the current work was to develop for the first time a 100% organic protein diet for the most relevant European freshwater aquaculture species, rainbow trout, using alternative organic raw materials and evaluate its possible effect on health status through intestinal microbiome composition.

2. Materials and methods

2.1. Ethics approval

The Committee of Ethics and Animal Welfare of the Universitat Politècnica de València (UPV) reviewed and approved the experimental protocol following the Spanish Royal Decree 53/2013 and the Eu Directive 2010/63/UE on the protection of animals used for scientific purposes.

2.2. Experimental setup

The growth assay was conducted at Naturix S.L. (Valderrebollo, Guadalaraja, Spain), a certified organic fish farm for rainbow trout, using 24 cylindrical swimming pools (4 m^3) in an open freshwater system. During the experiment, average water parameters were as follows: temperature of 11 $\pm$ 3.3 °C and oxygen levels of 7.2 $\pm$ 1.06 mg/L. The nitrite, nitrate, and ammonia levels were not detectable during the experiment. All tanks had similar lighting conditions, with a natural photoperiod from July to December.

2.3. Fish and acclimatization

Rainbow trout were provided by Naturix S.L. (Valderrebollo, Guadalaraja, Spain), and acclimatization to pool conditions was necessary. This process lasted two weeks, during which the fish were fed daily to apparent satiation by hand, three times per day (8:00, 13:00, and 18:00)

with a control diet (Table 1). A total of 4560 fish were weighed before starting the growth assay (initial weight, 67.2 ± 3.82 g) and then randomly distributed into the 24 experimental pools (190 animals/tank).

2.4. Diets

Six extruded diets were formulated using organic plant and animal ingredients. The first diet contained fishmeal (FM) as the protein source (Control), while the second diet (FM-IN) used organic insect meal (IN), concretely Hermetia, and fishmeal as the protein source. The third diet (IN-IB) used organic insect meal and Iberian pig by-product meal (IB). The fourth diet (SB-FM) used by-products of organic seabass by-product meal and fishmeal as the protein source. The fifth diet used by-products of organic seabass by-product meal and Iberian pig by-product meal (SB-IB) as the protein source, and the sixth diet (SB-IN-IB) contained by-products of both organic seabass and Iberian pig, and insect meal (Table 1). The diets were supplemented with calcium phosphate and vegetable amino acids (lysine and methionine).

Table 1
Ingredients and proximal composition of diets tested in the growth assay.

Ingredients (g kg^{-1})	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB
Raw materials (g kg^{-1})						
Fishmeal[a]	310	100		100		
Insect meal[b]		335	275			160
Seabass by-product[c]				284	293	163
Iberian pork viscera[d]			80		80	50
Wheat[e]	151	112	136	149	144	149
Soybean meal[f]	312	289	309	323	336	309
Wheat gluten[g]	60	60	60	60	60	60
Fish oil	127	59	60	49	32	44
Calcium phosphate	25	15	20	15	25	15
Vegetable methionine[h]	5	10	25	5	10	20
Vegetable lysine[i]		10	25	5	10	20
Vitamins and mineral mix[j]	10	10	10	10	10	10
Nutritional composition[k]						
DM (%)	86.3	85.0	85.2	89.6	89.2	88.5
CP (% DM)	43.1	43.6	42.5	42.2	41.4	41.7
CF (% DM.)	18.3	18.3	18.1	18.6	19.8	19.1
Ashes (%)	10.2	8.9	8.3	10.2	10.0	8.8
CHO (%)[l]	28.4	29.2	31.1	29	28.8	30.4
Gross energy (kJ/g)[m]	26.2	26.8	26.8	25.2	25.6	25.9

FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

[a] Fishmeal (91.9% DM, 73.9% CP, 11.2% CL, 14.3% Ash); CORPESCA S.A.

[b] Insect meal (92.6% DM, 37.6% CP, 28.5% CL, 20% CHO, 13.9% Ash) (Entomoch, Spain).

[c] Seabass by-product (97.5% DM, 34.5% CP, 41.7% CL, 3% CHO, 20.80% Ash) (Andrómeda, Spain).

[d] Iberian pig by-products (92.6% DM, 53.0% CP, 28.6% CL, 14.6% CHO, 3.8% Ash) (Jamón y Salud, Spain)).

[e] Wheat (92.3% DM, 12.7% CP, 1.3% CL, 1.7% Ash, 19.7 kJ^{-1} Energy); (PIENSOS ecoLUCAT, Barrax, Albacete, Spain).

[f] Soybean meal (94.6% DM, 43.1% CP, 9.3% CL, 6.3% Ash, 19.7 kJ^{-1} Energy); (PIENSOS ecoLUCAT, Barrax, Albacete, Spain).

[g] Wheat gluten (93.7% DM, 87.6% CP, 2.2% CL, 10.2% CHO, 0.05% Ash); (PIENSOS ecoLUCAT, Barrax, Albacete, Spain).

[h] Vegetable methionine (Adibio S.L.| Edificio Galileo, C/ Enebros 74, 2ª planta | 44,002 Teruel (Spain).

[i] Vegetable lysine (Adibio S.L.| Edificio Galileo, C/ Enebros 74, 2ª planta | 44,002 Teruel (Spain).

[j] Vitamin and mineral mix (g kg − 1): retinol acetate, 1,000,000 IU kg − 1; calcipherol, 500 IU kg − 1; DL-a-tocopherol, 10;menadione sodium bisulphite, 0.8; thiamine hydrochloride, 2.3; riboflavin, 2.3; pyridoxine hydrochloride, 15; cyanocobalamin, 25; nicotinamide, 15; pantothenic acid, 6; folic acid, 0.65; biotin, 0.07; ascorbic acid, 75; inositol, 15; betaine, 100; polypeptides 12. Soy concentrate as excipient.

[k] The nutritional composition results of the feeds are the average of the six analyses carried out throughout the growth test each time the diets were manufactured.

[l] Carbohydrates (g kg^{-1}) = 100 − CP (g kg^{-1}) − CL (g kg^{-1}) − Ash (g kg^{-1}).

[m] Gross energy (kJ/g) = ((23,9*CP/DM) + (39,8*CF/DM) + (17,6*CHO/DM)).

Experimental diets were manufactured as pellets using a semi-industrial twin-screw extruder (CLEXTRAL BC-45, Firminy, St Etienne, France) at the Universitat Politècnica de València. The processing conditions included 100 rpm screw speed, 110 °C temperature, 20 atm pressure, and 2–4 mm diameter pellets. The formulation and manufacture of diets were carried out using organic raw materials approved and labelled by Regulation (EU) 2018/848. The ingredients were prepared using organic seabass filleted to obtain sea bass by-products. Additionally, the Iberian by-products were fresh, and we were dried, preparing the meal accordingly. Once manufactured, the diets were packaged and stored in a thermally insulated tank. The inclusion levels of IN, SB, and IB and their combination in the experimental dietary formulations were tailored to fulfill the specific nutritional demands of rainbow trout. Our objective was to achieve the protein and fat levels of existing commercial trout feeds with as few raw materials as possible. The selection of these ingredients was guided by their nutritional profiles and suitability for organic aquaculture practices.

2.5. Growth assay: Nutritional and biometric parameters

The trial lasted for 150 days, during which trout were weighed monthly to evaluate their growth and determine nutritional parameters. Throughout the experiment, the fish were fed by hand to apparent satiation three times per day during the first 60 days (at 8:00, 13:00, and 18:00) and twice per day (at 9:00 and 14:00) from then up to the end. The feeding workers distributed the feed slowly, allowing all fish to eat in a weekly regime of feeding days and one fasting day. Every 30 days, all fish were weighed after being previously anesthetized with 10 mg/L clove oil (Guinama®) containing 87% eugenol. Before weighting, the fish were starved for 24 h. Ten fish were sampled initially at the beginning of the growth trial and stored at −30 °C for further whole-body composition analysis. At the end of the experiment, 15 fish per tank were sampled (60 per diet) to assess biometric parameters. Three fish per tank were randomly sampled and pooled to determine proximate composition, fatty acids, and amino acids. Final weight (FW), specific growth rate (SGR), survival, feed intake (FI), and feed conversion ratio (FCR) were determined using the tank as an experimental unit. Condition factor (CF), viscerosomatic index (VSI), hepatosomatic index (HSI), and meat index (MI) were also measured at the beginning and end of the growth trial. Additionally, the protein and fat retention indexes were calculated to determine their efficiency using the following Eqs. 1 and 2:

$$\text{Protein Productive Value}[\%], \text{PPV} = 100 \times \text{Protein fish gain } [g] / \text{Protein intake}[g] \quad (1)$$

$$\text{Fat Productive Value } [\%], \text{FPV} = 100 \times \text{Fat fish gain } [g] / \text{Fat intake } [g] \quad (2)$$

2.6. Digestibility assay

The digestibility assay for the experimental diets was conducted at the aquaculture laboratory of Universitat Politècnica de Valencia from February to July 2021 using 300 g rainbow trout specimens from Naturix. The trial was performed in four experimental tanks (190 L fiberglass tanks, 88 cm high, 62 cm wide, and 188 cm deep) set in an open freshwater system based on the Guelph system to collect the faecal material in the settling column. Five fish were placed in each tank. At 10:00 AM, the fish were given one meal daily to prevent waste when the fish were actively feeding. To avoid the pollution of faeces with the

diets, the drainpipe and settling column were brushed off an hour after the meal. The following morning at 8:00 AM, faeces were gravity-collected from the settling column's base into a plastic container. After collecting faeces, the fish were fed again at 10:00 am, allowing two hours between the activities to reduce stress.

The experiments lasted for 30 days for each diet and each replicate. Prior to analysis, the collected faeces were dried to a consistent weight in a 60 °C oven for 48 h and then stored in airtight plastic containers pending nutrient component and inert marker examination. The apparent digestibility of the diets was estimated indirectly using chromic oxide (Cr_2O_3) (5 g kg^{-1}) as an inert and indigestible marker and measuring its concentration in the diets and faeces. Additionally, dry matter, crude protein, energy, calcium, and phosphorus in both diets and faeces were analyzed using the same method. After acid digestion, the amount of chromium oxide in the diets and faeces was measured using an atomic absorption spectrometer (Perkin Elmer 3300, Perkin Elmer, Boston, MA, USA). Analyses were performed twice. The diets' apparent digestibility coefficients (ADC) were calculated according to (Cho et al., 1982). The ADCs (ADC*dm*, %) dry matter of the diets were calculated using the following Eq. 3:

$$\mathrm{ADCdm\%} = 1 - (\%Cr_2O_3 \text{ in diet} / \%Cr_2O_3 \text{ in faeces}) \quad (3)$$

The following formulas were used to determine the ADCs% of each specific nutritional variable (protein, Energy, calcium, and phosphorus) in the diets:

$$\mathrm{ADCnut} = 1 - ((\text{marker diet}/\text{marker faeces}) \times (\text{nutrient faeces}/\text{nutrient diet})) \quad (4)$$

In this equation, the terms nutrient diet (g kg^{-1}) and nutrient faeces (g kg^{-1}) refer to the nutritional parameters of concern (e.g., protein or energy) in the diet and the faeces, respectively. The terms marker diet (g kg^{-1}) and marker faeces (g kg^{-1}) refer to the marker content of the diet and the faeces, respectively.

2.7. Macronutrients and amino acids analysis

Diets and their ingredients, as well as the whole fish, were analyzed according to AOAC (1990) procedures: dry matter official method 934.01 (105 °C to constant weight); ash official method 942.05 (incinerated at 550 °C for 5 h); crude protein official method 990.03 (determined by direct combustion method DUMAS using LECO CN628, Geleen, Netherlands), and crude lipid official method 920.39 (extracted with methyl-ether using ANKOMXT10 Extractor, Macedon, NY, USA). All analyses were performed in triplicate. Diets and whole-body fish amino acids composition (Tables 2 and 3) were analyzed using a Waters HPLC system that included two pumps (Model 515; Waters), an autosampler (Model 717; Waters), a fluorescence detector (Model 474; Waters), and a temperature control module, as described by Bosch et al. (2006). After hydrolysis, an internal standard of aminobutyric acid was introduced. AQC (6aminoquinolylNhydroxysuccinimidyl carbamate) was used to derivatize amino acids. After oxidation with performic acid, methionine and cysteine were identified as methionine sulphone and cysteine acid, respectively. Waters AcQ isolated amino acids using a C18 reverse-phase column. 150 mm × 3.9 mm tag. Three fish per tank were randomly sampled and pooled to determine amino acids. All the analyses were carried out in duplicate.

2.8. Fatty acids analyses

The preparation of fatty acid methyl esters (FAME) from total lipids followed the method described by O'Fallon et al. (2007). The analysis of FAME was conducted using a Focus Gas Chromatograph (Thermo, Milan, Italy) equipped with a split/splitless injector and a flame ionization detector. The methyl esters were separated on a fused silica capillary column SPTM 2560 (Supelco, PA, USA) with 100 m × 0.25 mm × 0.2 μm film thickness dimensions. Helium was used as carrier gas with a 20 cm/s linear velocity. The samples were injected with a split ratio of 1/100. The initial oven temperature was set at 140 °C for five minutes, then increased to 240 °C at a rate of 4 °C/min and held at that temperature for 30 min. The detector and injector temperatures were both set at 260 °C. They identified individual fatty acids involved and compared their retention times with standards of fatty acid methyl esters provided by Supelco. Only fatty acids present at a minimum level of 0.1% were considered. To quantify the fatty acids, the sample weight data obtained from the analysis was used to calculate the grams of fatty acids per 100 g of sample, using C13:0 as the internal standard. The fatty acid composition of the experimental diets is presented in Table 3. Three fish per tank were randomly sampled and pooled to determine fatty acids. All the analyses were carried out in duplicate.

Table 2
Composition of essential and non-essential amino acids in experimental diets.

Diets	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB
Essential amino acids (g 100 g^{-1} DM)						
Arginine	3.48	2.40	2.62	2.80	2.56	3.13
Histidine	0.34	0.8	0.62	0.72	0.56	0.64
Isoleucine	1.12	1.40	1.39	1.48	1.36	1.39
Leucine	2.15	2.59	2.73	2.70	2.59	2.56
Lysine	2.81	2.04	2.12	2.52	2.17	2.74
Methionine	0.92	0.88	0.76	0.99	0.87	0.88
Phenylalanine	1.37	1.89	1.89	1.83	1.78	1.72
Threonine	1.57	1.47	1.59	1.60	1.44	1.67
Valine	1.90	2.11	2.13	2.15	2.00	2.12
Non-essential amino acids (g 100 g^{-1} DM)						
Alanine	2.77	1.63	1.77	1.80	1.64	2.07
Aspartic acid	4.51	3.57	4.25	3.85	4.22	4.98
Cysteine	0.40	0.44	0.45	0.40	0.48	0.69
Glutamic acid	5.69	10.09	9.48	8.39	9.18	6.62
Glycine	3.82	1.64	2.07	1.93	1.83	2.50
Proline	2.28	2.98	2.96	2.34	2.58	1.95
Serine	2.16	1.89	2.58	1.93	2.26	2.58
Tyrosine	1.10	1.42	1.23	1.36	1.19	1.21

FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic. DM: Dry Matter.

Table 3
Fatty acid composition in experimental diets (g 100 g^{-1} of sample dry matter).

Diets	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB
SFA						
(C14:0)	0.37	1.35	0.33	0.53	0.53	0.89
(C15:0)	0.04	0.07	0.04	0.06	0.05	0.06
(C16:0)	2.66	2.89	3.13	2.78	3.20	3.38
(C17:0)	0.05	0.05	0.05	0.05	0.06	0.06
(C18:0)	0.06	0.04	0.06	0.05	0.05	0.05
MUFA						
(C16:1)	0.42	0.53	0.42	0.60	0.62	0.62
(C17:1)	0.02	0.03	0.03	0.04	0.04	0.03
(C18:1n-9c)	4.04	2.86	4.95	4.10	4.91	4.28
(18:1(n-7))	0.38	0.23	0.43	0.43	0.47	0.38
(C20:1)	0.23	0.16	0.01	0.70	0.73	0.39
PUFA						
(C18:2n-6c) LA	6.31	2.62	5.76	4.05	4.29	4.26
(C18:3n-3) LNA	0.93	0.52	0.79	0.69	0.69	0.60
LC-PUFA						
(C20:4n-6) ARA	0.08	0.04	0.19	0.07	0.13	0.12
(20:5n-3) EPA	0.60	0.35	0.45	0.57	0.51	0.46
(22:6n-3) DHA	0.99	0.61	0.68	1.18	1.00	0.77

SFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids, LC-PUFA: Long chain-Polyunsaturated fatty acids LA: Linoleic acid, LNA: Linolenic acid, ARA: Arachidonic acid, EPA: Eicosapentaenoic acid, DHA: Docosahexaenoic acid. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

2.9. Blood parameters

Twelve fish per treatment were anesthetized at the end of the study, and blood samples were taken by puncturing the caudal vein with heparinized syringes laced with an anticoagulant. For later analysis, samples were promptly kept at −40 °C. The concentrations of glucose, lactate dehydrogenase (LDH), and total protein (TP) were measured using ultraviolet spectrophotometry (Ortho Clinical Diagnostics, Raritan, NJ, EUA). The ICTIOVET SCP laboratory (Barcelona, Spain) conducted all these analyses.

2.10. Enzymatic activity

Three fish's digestive tracts per tank were sampled at the experiment's end. Trout were fed the night before at 20:00 and the day of the sampling at 8:00 to guarantee the presence of content along the whole digestive tract. Fish were sedated with clove oil and slaughtered by cold shock before being dissected to extract the digestive tract. Caeca samples were considered and collected. To prepare for enzymatic extraction, they were kept at −20 °C. Manual disaggregation, dilution in distilled water (1 g of sample: 3 mL of distilled water), homogenization by T 25 digital ULTRA-TURRAX® (IKA®, Staufen, Germany), keeping tubes on ice, and centrifugation at 12,000 rpm and 4 °C for 15 min were used to prepare enzyme extracts for protease analysis. Until enzyme analysis, the supernatant was kept at −20 °C. According to the procedure created by Erlanger et al. (1961), trypsin and chymotrypsin activities were obtained by a kinetic test utilizing N-Benzoyl-DL-arginine p-nitroanilide (0.5 mM BAPNA) as a substrate in 50 mM Tris-HCl buffer containing 20 mM CaCl2. Every 30 s for five minutes, an increase in absorbance at 405 nm was observed. With an extinction value of 0.0637 mL μg^{-1} $cm^{-1}$of p-nitroanilide produced per minute, it was used as the unit of activity. The solubility of protein was determined by the Bradford method (Bradford, 1976).

The enzyme activity of trypsin, chymotrypsin, and the trypsin/chymotrypsin ratio (T/C) are expressed in activity per gram of fish (U g^{-1} trout), per gram of caeca tissue (U g^{-1} caeca) and mg of soluble protein (U mg^{-1} soluble protein).

2.11. Liver and intestinal histology

At the end of the experiment, intestine and liver samples from three fish from each tank were collected and dissected into small pieces and preserved in formalin 10%. All the formalin-fixed tissues were routinely dehydrated in ethanol, equilibrated in ultraclean, and embedded in paraffin according to standard histological techniques. Transverse sections were cut with a Microtome Shandon Hypercut to a thickness of 5 μm and stained with Alcian blue for gut and liver examination. A total of 400 sections of the liver and 100 of the intestines were examined under a light microscope (Eclipse E400 Nikon, Izasa S.A., Barcelona, Spain).

The measurements and observations of the intestine were performed using a combination of criteria used in previous studies (Adamidou et al., 2009; Nogales-Mérida et al., 2016) and the following parameters were measured: serous layer (SL), muscular layer (ML), submucous layer (SML), villi length (VL), villi thickness (VT), and lamina propria length (LP). All the images of samples were taken with an optical microscope Nikon JAPON 0.90. The images were analyzed using Photoshop software and converted into metric units.

2.12. Microbiome

At the end of the growth trial, three fish per tank (12 fish per diet) were slaughtered on ice and dissected to obtain the gastrointestinal tract. Fish were fasted for 24 h before sampling. After discarding the stomach and pyloric caeca, the first intestinal third of the gut (foregut) was dissected, sliced longitudinally, and washed with phosphate-buffered saline solution to remove digestion. Intestinal mucosa was scraped using sterilized large scalpel blades, stored in Eppendorf tubes, frozen in liquid nitrogen, and stored at −80 °C.

The microbiota of 48 samples collected from posterior rainbow trout intestine were characterized by 16S rRNA gene amplicon sequencing on the Illumina MiSeq platform.

2.12.1. Microbial DNA extraction

According to the manufacturer's instructions, DNA was extracted from 200 L of bacterial suspension using the DNeasy PowerSoil® Kit (Qiagen, Milan, Italy). The samples were lysed in PowerBead Tube using a TissueLyser II (Qiagen) for 2 min at 25 Hz. Like negative control of the extraction procedure, a sample with only lysis buffer was processed in parallel with the samples. The concentration of the extracted DNA was measured with the NanoDropTM 2000 spectrophotometer (Thermo Scientific, Milan, Italy) and stored at —20C until the PCR reaction was performed.

2.12.2. Preparation of 16S amplicon library and sequencing

Libraries of 16S ribosomal RNA gene amplicons were prepared using primer pair sequences for the V3-V4 region following the Illumina protocol "16S Metagenomic Sequencing Library Preparation" for the Illumina system. Bacterial 16S rRNA gene amplicons were generated from 50 ng of microbial genomic DNA in 25 L PCR using High Fidelity Platinum® Taq DNA Polymerase Kit (Thermo Fisher Scientific, Italy) and Pro341F (50-CCTACGGGNBGCASCAG −30) and Pro805R (50-GACTACNVGGGTATCTAATCC -30) selected by Takahashi et al. (2014).

The expected size in the Agilent 2100 bioanalyzer trace after the amplicon PCR step was ~550 bp. The complete procedure for preparing and sequencing the 16S rRNA gene library is described in Rimoldi et al. (2018). Briefly, Nextera XT unique reference Illumina paired-end adapters were ligated to 16S amplicons using the Nextera XT Index Kit (Illumina, San Diego, CA, USA). Next, qPCR quality controlled all libraries using KAPA Illumina® Platforms library quantification kits (Kapa Biosystems Ltd., London, UK) at equimolar concentrations and diluted to 6 picomolar. Pooled libraries were then multiplexed and sequenced on an Illumina HiSeq X Ten platform (Illumina, San Diego, CA, USA) at 2 × 300 bp paired sequences.

2.12.3. Metabarcoding raw data analysis

Raw FASTQ data from sequencing were processed using the open-source program QIIME 22021.4 (Bolyen et al., 2019). Raw data was quality filtered using the q2-demux plugin, followed by denoising with DADA2(Callahan et al., 2016) (via q2-dada2). All amplicon sequence variants (ASVs) were aligned with mafft (Katoh et al., 2002) (via q2-alignment) and used to construct the phylogeny with fasttree2 (Price et al., 2010) (via q2-phylogeny). Taxonomy was assigned to ASVs using the q2 feature classifier (Bokulich et al., 2018) to classify sklearn with its naive Bayes taxonomy V4 (Quast et al., 2013). The same analysis was performed after filtering ASV classified as mycoplasmas. In that case, the samples were thinned to 800 sequences per sample.

2.13. Statistical analysis

Growth, nutritive, and biometric indices, and all analyses were analyzed through an analysis of variance using the statistical package Statgraphics ® Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA), with a Newman-Keuls test for the comparison of the means and a level of significance of $p < 0.05$. Relative microbiota data were statistically analyzed by one-way analysis of variance using the Newman-Keuls test. Differences were considered statistically significant when $p < 0.05$. The data was expressed as the mean and the standard error of the mean.

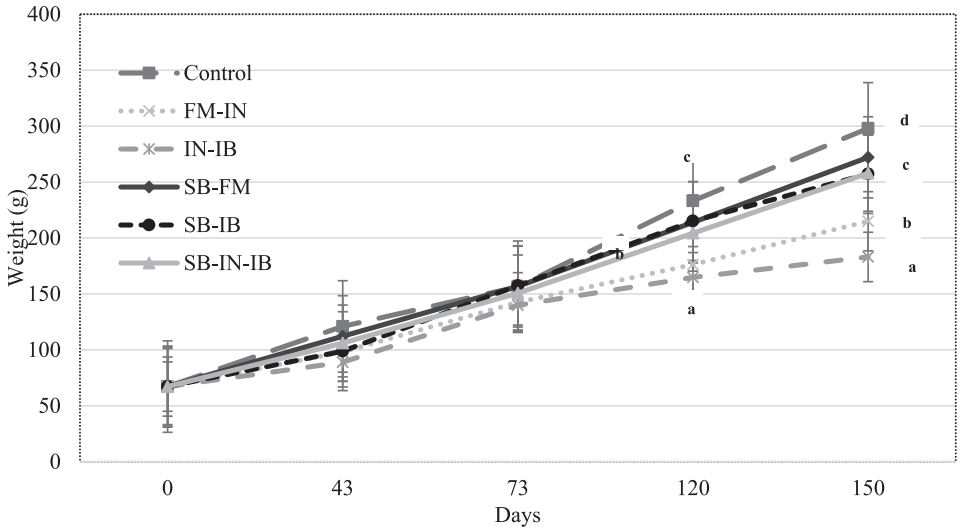

Fig. 1. Evolution of weight along the experiment. Values represented as mean ± standard deviation ($n = 4$). Different letters in each sampling means significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

3. Results

3.1. Growth, nutritive, and biometric assessment

The evolution of fish weight along the growth trial is shown in Fig. 1. During the first 73 days, no differences appeared, but significant differences among diets were observed in the last two samplings.

At the end of the experiment, fish fed the Control diet reached the highest final body weight, followed by fish fed SB-IN-IB and SB-FM diets, while fish fed IN-IB presented the lowest (Table 4).

Fish weight (FW), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), Protein Efficiency ratio (PER), and average daily gain (ADG) were affected by the dietary composition (Table 4). At the end of the experiment, there were no differences among the treatments in fish survival. Trout fed the Control diet with FM presented the highest final weight, SGR, and ADG (298 g, 0.99% day^{-1}, 1.54 g day^{-1}, respectively) and trout fed the IN-IB diet had the lowest final weight, SGR, and ADG (183 g, 0.67% day^{-1}, 0.77 g day^{-1}, respectively). FI was lowest in the FM-IN diet (1.55 g 100 g fish^{-1} day^{-1}). FCR was highest in the IN-IB diet (3.37) and lower in the Control diet (2.15) without differences with the other diets (2.14–2.33). PER was highest in the SB-IB diet and lowest in the IN-IB diet (1.55).

3.2. Body composition and retention efficiency

The proximate composition of the whole body is shown in Table 5. Fish fed the IN-IB diet exhibited the lowest dry matter (22.9%) and fat content (24.8%) and consequently the highest body protein (66%) followed by fish fed FM-IN. In contrast, fish fed Control and SB-IN-IB diets showed the highest fat (29.9 and 30.0%) and lowest protein content (59 and 59.4%, respectively). No significant differences were found for ash content. Differences were observed in Productive Protein Value (PPV) and Productive Fat Value (PFV), which were the lowest values for the IN-IB diet for both (13.7 and 11.7%, respectively).

Regarding biometric parameters (Table 6), statistically significant differences were observed in condition factor (CF), Viscerosamatic Index (VSI) and Hepatosomatic index (HSI). Fish fed FM-IN presented the highest VSI (20.5%), and fish fed IN-IB diet obtained a lowest HIS and CF (2.0% and 1.0 g cm$_3^{-1}$ respectively). No differences were observed in the Meat index (MI).

3.3. Digestibility

The Apparent digestibility coefficients (ADCs) of dry matter for rainbow trout ranged from 78 to 90% without differences among diets (Table 7). ADC of Calcium was the lowest in the SB-IN-IB (23%) and SB-FM (27%) diets and highest in the Control diet (65%). The lowest ADC of phosphorus was for the SB-FM diet (53%), and highest for the Control

Table 4
Growth and Nutritive indices at the end of the experimental trial.

Diets	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB	SEM	*P-value*
Final weight	298.0^{d}	215.0^{b}	183.0^{a}	272.1^{c}	257.2^{c}	257.9^{c}	6.6	0.0000
Survival	78.3	80.5	75.2	69.2	69.7	82.8	5.2	0.3960
SGR [1]	0.99^{d}	0.77^{b}	0.67^{a}	0.93^{c}	0.89^{c}	0.89^{c}	0.02	0.0000
ADG[2]	1.54^{d}	0.98^{b}	0.77^{a}	1.37^{c}	1.27^{c}	1.27^{c}	0.04	0.0000
FI [3]	1.74^{ab}	1.55^{a}	1.91^{b}	1.71^{ab}	1.58^{ab}	1.59^{ab}	0.11	0.2644
FCR[4]	2.15^{a}	2.33^{a}	3.37^{b}	2.29^{a}	2.19^{a}	2.14^{a}	0.20	0.0043
PER[5]	1.14^{ab}	1.11^{ab}	0.84^{a}	1.08^{ab}	1.16^{ab}	1. 21^{b}	0.08	0.0560

Values represented as mean ± standard error ($n = 4$). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

[1] Specific growth rate (SGR) = 100 × ln (final weight/initial weight)/days.

[2] Average daily gain (ADG) = weight gain (g) / days.

[3] Feed intake (FI) (g 100 g fish^{-1} day^{-1}) = 100 × feed consumption (g)/average biomass (g) × days.

[4] Feed conversion ratio (FCR) = feed consumption (g) / weight gain (g).

[5] Protein Efficiency ratio (PER) = biomass gain (g) / protein intake (g).

Table 5
Body composition and retention efficiencies of trout at initial and after feeding with experimental diets.

	Initial	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB	SEM	*P-value*
Dry matter	24.13	25.63^{c}	23.45^{b}	22.87^{a}	25.12^{c}	25.18^{c}	24.78^{c}	0.23	0.0000
Protein	15.45	15.12^{a}	14.94^{b}	15.14^{c}	15.38^{ab}	15.41^{ab}	14.74^{a}	0.73	0.0004
Fat	6.49	7.66^{c}	6.12^{ab}	5.69^{a}	6.81^{ab}	7.10^{bc}	7.51^{c}	0.68	0.0140
Ash	2.04	2.43	2.36	2.29	2.35	2.39	2.37	0.44	0.9487
PPV [1]		19.9^{b}	18.2^{b}	13.7^{a}	18.7^{b}	20.4^{b}	19.9^{b}	1.30	0.0199
PFV [2]		25.1^{b}	17.8^{b}	11.7^{a}	19.5^{b}	20.6^{b}	23.8^{b}	1.79	0.0008

Values represented as mean ± standard error (n = 4). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

[1] Productive Protein Value (PPV %) = Protein retained (final fish protein × Final biomass (g)) × 100 - Initial fish protein x initial biomass (g) / Protein ingested (kg ingested food ×% crude protein).

[2] Productive Fat Value (PFV %) = Fat retained (final fish fat × Final biomass (g)) × 100 - Initial fish fat x initial biomass (g) / fat ingested (kg ingested food ×% crude fat).

Table 6
Biometric indices at the end of the experiment.

Diets	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB	SEM	*P-value*
VSI[1]	16.78^{a}	20.49^{b}	16.38^{a}	18.10^{a}	17.20^{a}	18.36^{a}	0.54	0.0000
HSI[2]	2.26^{b}	2.28^{b}	2.00^{a}	2.50^{b}	2.49^{b}	2.27^{b}	0.08	0.0000
CF[3]	1.20^{b}	1.19^{b}	1.00^{a}	1.31^{b}	1.18^{b}	1.20^{b}	0.03	0.0001
MI[4]	52.91	47.88	49.33	52.94	48.79	51.03	1.45	0.1230

Values represented as mean ± standard error ($n = 12$). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

[1] Viscerosomatic index (VSI) (%) = (visceral weight (g) / total fish weight. (g)) × 100.

[2] Hepatosomatic index (HSI) (%) = (liver weight (g) / total fish weight (g)) × 100.

[3] Condition factor (CF) (g cm_3^{-1}) = (total fish weight (g) / length3 (cm)) × 100.

[4] Meat index (MI) (%) = (meat weight (g) / total fish weight (g)) × 100.

and FM-IN diets (73% and 74%). The highest-protein ADCs were observed in fish-fed Control and FM-IN (95% both), with significant differences concerning the rest of the diets except the SB-IN-IB diet (87%). ADCs of energy were the highest in the Control and FM-IN diets (93% and 92%, respectively) and lowest in the SB-FM diet (82%).

3.4. Retention efficiency of essential amino acids

There were statistical differences in essential amino acid retention efficiency for histidine (His), Isoleucine (Iso), leucine (Leu), and phenylalanine (Phe) (Table 8). Fish fed the Control diet showed the highest retention for His, Iso, Leu, and Phe. Fish fed the IN-IB diet presented the lowest Iso, Leu, and Phe retention efficiency values. The retention efficiency of Arginine (Arg), lysine (Lys), methionine (Met), threonine (Thr), and valine (Val) did not show differences in all diets.

3.5. Enzymatic activity

Enzymatic activity of rainbow trout fed different experimental diets is shown in Fig. 2. Trypsin activity was lower in fish fed IN-IB and SB-IN-IB diets, but Chymotrypsin activity differs in function of units, lowest in SB-FM per g of trout, and lowest in IN-IB in the rest. The ratio Trypsin/Chymotrypsin showed that only was highest in fish fed IN-IB expressed respect to caeca tissue.

3.6. Blood parameters

No differences have been observed regarding parameters analyzed in the fish serum fed the experimental diets (Table 9).

3.7. Liver and intestinal histology

Regarding liver histology, some differences have been observed in the nucleus and hepatocytes diameter, as seen in Table 10, which were the highest in fish fed the SB-IB diet (11.16 and 27.58 μm, respectively), whereas they were the lowest (4.78 and 11.67 μm, respectively) in fish fed the FM-IN diet.

Differences were found in the qualitative aspect (Fig. 3) between the different diets. Regularly shaped nuclei were observed in the cell's center and peripheral areas. The liver of fish fed with the IN-IB diet showed the absence of white spaces, indicating the accumulation of lipids. The liver of fish fed with Control, FM-IN, and SB-IB diets had a

Table 7
Apparent digestibility coefficients of dry matter and different nutrients of rainbow trout fed experimental diets.

ADC (%) *	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB	SEM	*P-value*
Dry matter	89	90	82	79	84	78	7.12	0.1763
Calcium	65^{c}	58^{bc}	41^{ab}	27^{a}	41^{ab}	23^{a}	9.88	0.0001
Phosphorus	73^{c}	74^{c}	58^{ab}	53^{a}	68^{bc}	57^{ab}	6.36	0.0015
Protein	95^{c}	95^{c}	90^{ab}	87^{a}	90^{ab}	93^{bc}	2.18	0.0001
Energy	93^{d}	92^{d}	84^{b}	82^{a}	87^{c}	87^{c}	1.12	0.0000

Values represented as mean ± standard error (n = 4). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

$ADC_{dm} = 100 - (100 \times (\%\ Cr2O3\ in\ diet / \%\ Cr2O3\ in\ faeces))$.

$ADC_{nut} = 100 - (100 \times (\%\ feed\ marker / \%\ faeces\ marker) \times (\%\ nutrient.\ Energy.\ Amino\ acid.\ or\ fatty\ acid\ in\ faeces / \%\ nutrient.\ Energy.\ amino\ acid.\ or\ fatty\ acid\ in\ faeces)$.

* Apparent digestibility coefficients (ADC).

Table 8
Retention efficiency of essential amino acids of the rainbow trout fed with the experimental diets.

Diet	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB	SEM	*P-value*
Arginine	13.6	16.6	10.7	17.6	17.6	16.3	1.84	0.0992
Histidine	53.1^{b}	19.9^{a}	18.8^{a}	25.5^{a}	34.1^{a}	31.7^{a}	5.31	0.0026
Isoleucine	22.1^{b}	14.0^{ab}	12.1^{a}	16.5^{ab}	17.6^{ab}	17.91^{ab}	1.90	0.0270
Leucine	21.3^{b}	15.2^{ab}	11.4^{a}	16.9^{ab}	16.6^{ab}	18.6^{ab}	1.85	0.0300
Lysine	19.4	23.5	19.5	22.6	26.6	20.3	2.25	0.2178
Methionine	22.5	22.9	22.3	24.4	30.5	24.6	2.02	0.0838
Phenylalanine	19.2^{c}	12.1^{a}	10.0^{a}	14.4^{ab}	14.8^{ab}	16.3^{ab}	1.62	0.0141
Threonine	16.3	15.1	10.8	17.3	17.5	16.3	1.58	0.0679
Valine	18.7	14.9	12.1	16.8	17.8	17.6	1.63	0.0923

Values represented as mean ± standard error ($n = 4$). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

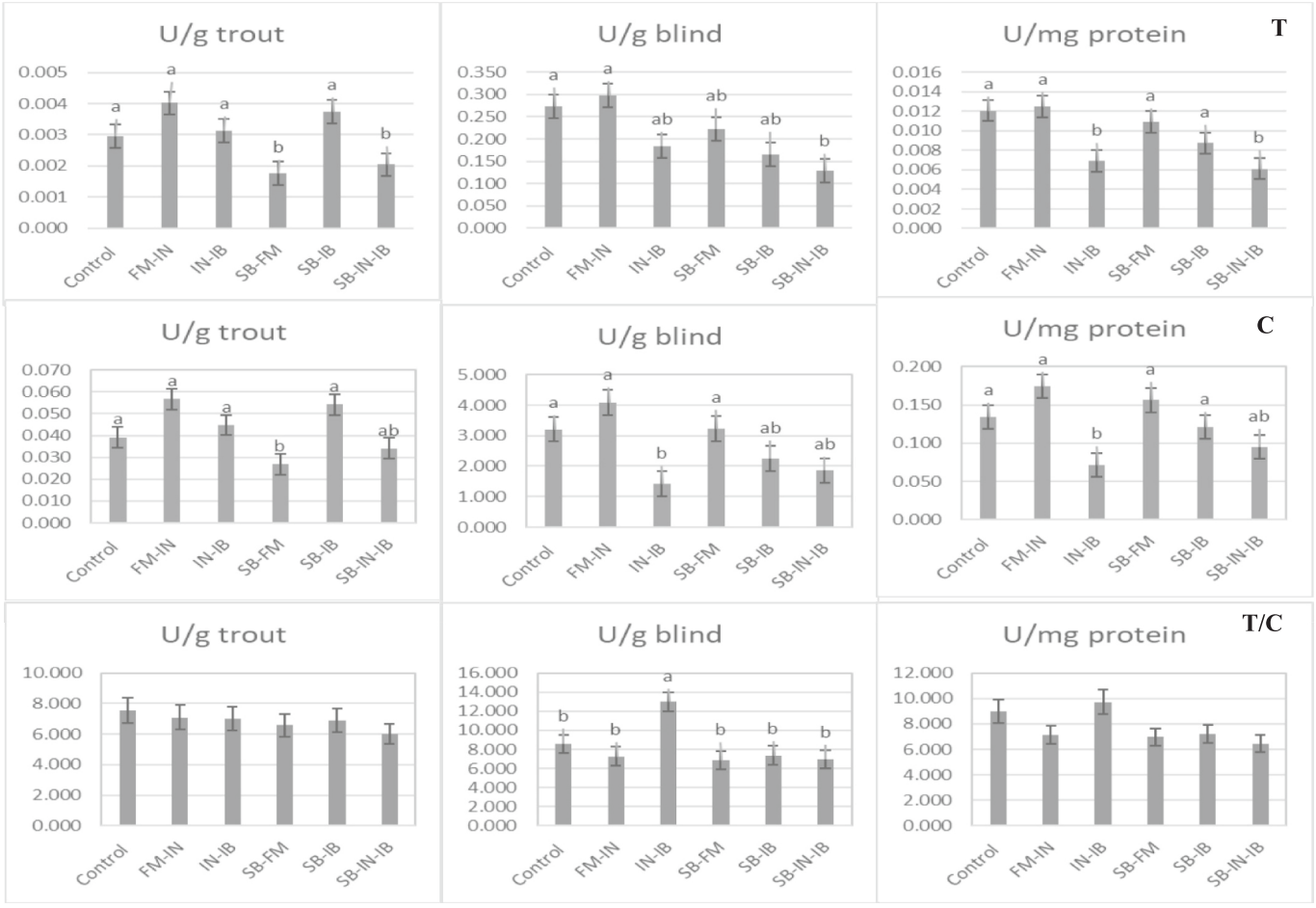

Fig. 2. Activity of digestive enzymes measured in the pyloric cecum of trout fed with experimental diets. Values represented as mean ± standard error ($n = 4$). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic. In the case of (T) trypsin, (C) Chymotrypsin, and the (T/C) trypsin/chymotrypsin ratio (T/C), the values for each enzyme are expressed in activity per gram of fish (U g trout^{-1}). Per gram of caeca tissue (U g caeca^{-1}) and mg of soluble protein (U mg soluble protein^{-1}).

slight fat accumulation. The SB-FM-IB and SB-FM diets showed micro and macro vacuoles with a defined border.

The results of anterior and posterior intestine measurements are reported in Table 11. Significant differences have been observed in all the measurements. The SB-IB diet had the lowest SL, ML, and SML parameters in the proximal and distal intestines (PI, DI), whereas the diet SB-IN-IB registered the highest at PI. LP was the highest for fish-fed SB-IB diet in PI and DI. VL was the lowest for the fish-fed SB-FM diet in PI and DI.

3.8. Gut microbiota composition

Considering the intestinal microbiota composition, 19 bacterial phyla were found in the sample set (Table 12). Regardless of diet, three dominant phyla were by far the most abundant, *Firmicutes* (64–81%). *Spirochaetota* (8–29%) and *Proteobacteria* (3−12), but no difference exists between experimental diets.

A number of 27 Genera were found in the microbiome. The dominant genera of microbiota were *Mycoplasma* (59–88%) within the *Firmicutes* phylum, followed by *Brevinema* (5–35%) (Table 13), but without statistical differences between diets. Clostridium and Xanthomonas were

Table 9
Effect of the experimental diets on blood parameters of the rainbow trout.

Diets	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB
Glucose (mg/dL)	120.3 ±15.4	147.4 ±9.4	172.9 ±34.7	131.7 ±8.2	130.9 ±17.0	132.0 ±9.3
LDH (U/L)[1]	3694.3 ±491.2	2354.9 ±188.6	2404.3 ±173.9	3826.7 ±687.4	4438.3 ±969.6	3172.8 ±466.0
TP (g/L)[2]	39.9 ±2.1	40.0 ±1.8	36.0 ±1.5	42.3 ±2.0	43.7 ±2.4	43.1 ±2.9

Values represented as mean ± standard error (n = 4). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.
[1] LDH: Lactate dehydrogenase.
[2] TP: Total protein.

highest in IN-IB.

Mycoplasma was genera predominant within the *Firmicutes* phylum without significant differences between groups, and due to the dominant character of the *Firmicutes* phylum and the *Mycoplasma* genera, the sequences were filtered for these genera since it could be camouflaging possible. After filtering, the dominant phylum (Table 14) was, *Spirochaetota* (27–54%) and *Proteobacteria* (18–37%).

After filtering, the most dominant genera in the *Spirochaetota* phylum was *Brevinema* as the most abundant genera in all the experimental diets, with a higher percentage in the SB-IB and FM-IN diets (54.2 ± 11.5% and 52.1 ± 12.5%, respectively (Table 15).

The *Proteobacteria* phylum was mainly represented by the classes *Alphaproteobacteria* and *Gammaproteobacteria*. The *Alphaproteobacteria* class had a differential representation according to the diet, with the genera *Sphingomonas, Roseobacter*, and *Brevundimonas* being more abundant in the "SB-IB" diet. On the other hand, the "IN-IB" diet had a higher representation of *Blastomonas* and the "Control" of *Bosea*. In the class *Gammaproteobacteria*, the principal genera found were *Dechloromonas* and *Thermomonas* in the "IN-IB" diet, *Hydrogenophaga* in the "SB-FM" diet, and *Shewanella* in the "FM-IN" diet. The third most abundant phylum was *Firmicutes*, with 8.98% of the total.

Despite genera differences in the different diets, when diversity was assessed using the Shannon index, there were no significant differences (Fig. 4).

Table 10
Histological measures of the liver of trout fed experimental diets.

Diets	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB	SEM	*P-value*
Nucleus diameter (μm)	5.77^{c}	4.78^{a}	5.28^{b}	5.92^{c}	11.1^{d}	5.71^{bc}	0.10	0.0001
Hepatocyte diameter (μm)	14.27^{c}	11.67^{a}	13.17^{b}	12.58^{b}	27.5^{d}	14.06^{c}	0.31	0.0000

Values represented as mean ± standard error ($n = 100$). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

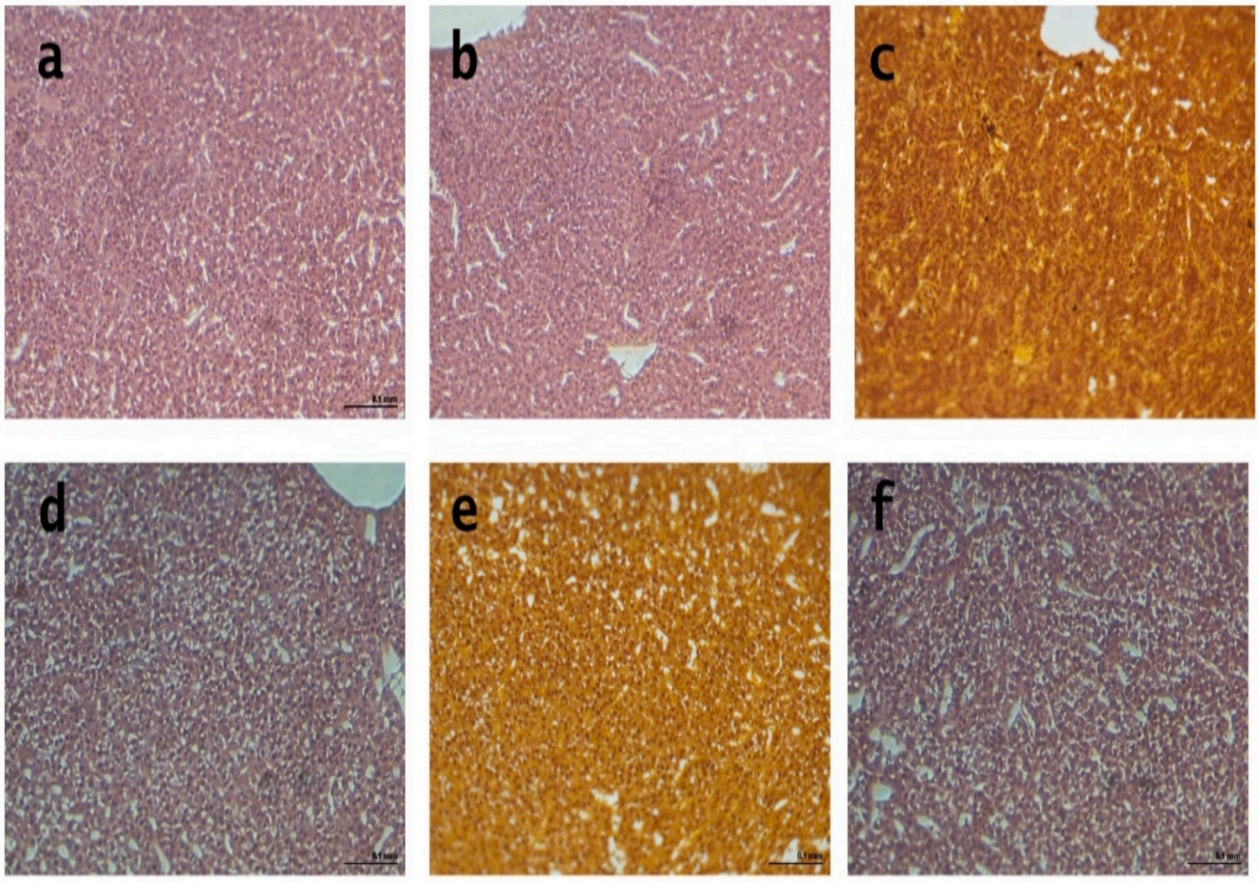

Fig. 3. Histological detail of the liver (20×) with Hematoxylin-Eosin staining of the trout fed with the experimental diet. (a: FM-IN: Fishmeal-Insect; b: IN-IB: Insect-Iberic; c: SB-FM: Seabass- Fishmeal; d: SB-IB: Seabass-Iberic; e: SB-f: IN-IB: Seabass-Insect-Iberic.

Table 11
Effect of the different diets on proximal and distal intestine measurements in rainbow trout.

	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB	SEM	*P-value*
Proximal								
SL (μm)	59.17^{bc}	56.87^{bc}	41.83^{ab}	73.63^{cd}	27.26^{a}	80.01^{d}	6.85	0.0000
ML (μm)	78.18^{a}	89.48^{b}	83.46^{ab}	68.39^{a}	67.95^{a}	141.15^{c}	5.06	0.0000
SML(μm)	46.31^{b}	65.09^{c}	65.82^{c}	47.86^{b}	35.13^{a}	80.99^{d}	2.98	0.0000
VL (μm)	740.68^{bc}	678.58^{ab}	670.48^{abc}	592.09^{a}	820.63^{c}	812.74^{c}	37.13	0.0026
VT (μm)	119.76^{a}	118.77^{a}	131.28^{ab}	121.90^{ab}	139.58^{b}	126.79^{ab}	5.92	0.0258
LP (μm)	$28,22^{b}$	$28,14^{b}$	$24,08^{ab}$	$30,44^{bc}$	$32,83^{c}$	$24,24^{a}$	1,64	0.1115
Distal								
SL (μm)	123.63^{c}	48.29^{a}	46.22^{a}	82.49^{b}	42.87^{a}	98.57^{b}	9.50	0.0000
ML (μm)	57.61^{c}	50.67^{ab}	63.57^{bc}	78.53^{c}	37.76^{a}	104.48^{d}	4.98	0.0000
SML(μm)	56.53^{a}	47.50^{a}	52.58^{a}	55.07^{a}	53.19^{a}	70.50^{b}	4.33	0.0251
VL (μm)	809.53^{b}	848.41^{ab}	563.01^{ab}	648.85^{a}	656.03^{ab}	802.08^{ab}	82.68	0.1267
VT 8(μm)	139.85^{b}	108.55^{a}	121.57^{ab}	120.83^{a}	126.30^{ab}	137.68^{b}	5.88	0.0074
LP (μm)	26.93^{a}	25.55^{a}	24.36^{a}	32.33^{bc}	36.04^{c}	28.02^{ab}	1.89	0.0026

Values represented as mean ± standard error ($n = 20$). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic. SL: Serous layer. ML: Muscular layer. SML: Submucous layer. VL: Villi length. VT: Villi thickness. LP: Lamina propria.

Table 12
Phyla found in microbiota sequencing.

Index (%)	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB	SEM	*P-value*
Firmicutes	72.57	79.47	77.19	81.07	64.25	78.31	10.87	0.5719
Spirochaetota	14.68	12.07	8.96	8.05	28.68	17.42	9.43	0.3032
Proteobacteria	8.43	5.48	12.03	7.34	4.36	2.64	5.50	0.5597
Actinobacteriota	2.10	1.56	0.51	1.12	1.11	0.97	0.74	0.5209
Bacteroidota	1.29	0.75	0.76	0.73	0.39	0.26	0.51	0.7444
Desulfobacterota	0.04	0.13	0.14	0.43	0.43	0.05	0.07	0.3623
Patescibacteria	0.02	0.01	0.08	0.02	0.00	0.02	0.04	0.5205
Bdellovibrionota	0.10	0.01	0.00	0.01	0.00	0.00	0.05	0.6724
Fusobacteriota	0.04	0.00	0.00	0.18	0.02	0.01	0.09	0.5159
Verrucomicrobiota	0.05	0.01	0.03	0.04	0.01	0.01	0.03	0.8322
Planctomycetota	0.08	0.03	0.02	0.02	0.01	0.01	0.02	0.2015
Acidobacteriota	0.01	0.00	0.00	0.08	0.01	0.07	0.03	0.1747
Deinococcota	0.00	0.00	0.00	0.18	0.02	0.02	0.08	0.3628
Deferribacterota	0.00	0.01	0.01	0.00	0.00	0.00	0.003	0.2482
Myxococcota	0.02	0.01	0.00	0.00	0.00	0.00	0.01	0.7288
Chlorolipids	0.02	0.01	0.00	0.08	0.01	0.00	0.04	0.4595
Gemmatimonadota	0.00	0.01	0.01	0.05	0.01	0.00	0.03	0.5074
Cyanobacteria	0.06	0.02	0.01	0.01	0.00	0.01	0.02	0.2674
Methylomirabilota	0.00	0.00	0.00	0.03	0.04	0.00	0.02	0.4633
Unassigned	0.50	0.41	0.24	0.57	0.65	0.18	0.06	0.5862

Values represented as mean ($n = 6$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

4. Discussion

Herbivorous and omnivorous species from organic production, as tilapia and catfish, seem to be easier than carnivorous species since organic feedstuff can cover their nutritional needs and thus easily replace conventional feedstuff (Craig and McLean, 2005).

Nevertheless, formulating organic diets for carnivores is much more difficult due to their high protein/essential amino acid requirement and the prohibition of organic terrestrial animal byproducts and synthetic amino acids in organic diets (Unión Europea, 2018). Nevertheless, the results of the present work are promising.

4.1. Fish performance and biometric parameters

No significant differences in mortality were observed among the experimental diets, ranging from 17% to 30%, with a concentrated increase between July 28th and August 2nd due to a flow-related issue.

Good results have been obtained in trout using diets with high substitutions of fishmeal for vegetable mixtures in conventional feeds (Burr et al., 2012; Watanabe et al., 1993), but there are no studies where growth is evaluated in trout fed with organic feed with high substitutions of fishmeal and fish oil. Some previous works with seabass compare conventional and organic diets. However, the results cannot be well compared since the organic diets had higher amounts of fishmeal (56%) than the conventional one (20%), resulting in better growth and feed conversion ratios in fish-fed organic feed. (Di Marco et al., 2017). In other similar studies carried out with sea bream, the organic feed also presented better growth than the conventional one, without significant differences, but the organic feed was also formulated with a higher percentage of fishmeal (63%) than the conventional one (50%) (Mente et al., 2012). Two studies evaluated the effect of organic raw materials such as organic insect meal, Iberian pig byproducts, and organic rainbow trout byproducts for gilthead seabream; total replacing fishmeal with organic raw materials provides numerous advantages in terms of digestibility, histology, and growth performance (Tefal et al., 2023b, 2023c). In another investigation with the same ingredients for seabass carried out by Tefal et al. (2023a), it was found that the complete substitution of fishmeal slightly impacted growth and certain efficiency parameters, although not significant enough to outweigh the economic benefits.

The control diet with fish meal as animal protein gave the best results, but the two experimental diets containing organic insect meal (IN-

Table 13
Taxonomy and percentage (%) of the bacterial genera detected in the microbiota of hindgut samples (for each experimental group).

Index (%)	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB	SEM	*P-value*
Mycoplasma (Firmicutes)	70,51	77,73	72,90	78,55	57,67	88,12	10.62	0.6323
Brevinema (Spirochaetota)	14,68	16,86	5,41	8,85	34,78	7,87	9.07	0.3614
Sphingomona (Proteobacteria)	0,58	0,76	0,51	1,55	0,69	0,34	0.32	0.2362
Clostridium (Firmicutes)	0,00	0,00	3,19	0,19	0,03	0,01	1.11	0.4441
Xanthomonas (Proteobacteria)	0,34	0,00	10,06	0,00	0,11	0,00	3.51	0.4403
Blastomonas (Proteobacteria)	1,74	0,17	1,23	0,00	0,01	0,00	0.75	0.4907
Crenobacter (Proteobacteria)	0,00	0,86	0,07	0,52	0,62	0,05	0.33	0.4816
Aeromonas (Proteobacteria)	0,26	0,08	0,05	0,76	0,10	0,48	0.29	0.6137
Corynebacterium (Actinobacteriota)	0,89	0,02	0,01	0,22	0,48	0,10	0.42	0.6075
Streptococcus (Firmicutes)	0,86	0,01	0,02	0,23	0,04	0,03	0.17	0.3534
Aquabacterium (Proteobacteria)	1,20	0,00	0,00	0,00	0,00	0,00	0.64	0.6298
Acinetobacter (Proteobacteria)	0,08	0,03	0,03	0,65	0,00	0,03	0.22	0.2571
Xanthobacteraceae (Proteobacteria)	0,18	0,00	0,01	0,24	0,07	0,29	0.14	0.6609
Amaricoccus (Proteobacteria)	0,00	0,00	0,00	0,34	0,00	0,36	0.19	0.5373
Bacteroides (Bacteroidota)	0,05	0,17	0,17	0,03	0,25	0,13	0.11	0.6789
Massilia (Proteobacteria)	0,03	0,00	0,01	0,69	0,00	0,02	0.27	0.3921
Mycobacterium (Actinobacteriota)	0,09	0,01	0,21	0,14	0,24	0,08	0.13	0.8515
Staphylococcus (Firmicutes)	0,12	0,10	0,03	0,01	0,50	0,02	0.17	0.3534
Bosea (Proteobacteria)	0,51	0,02	0,46	0,00	0,00	0,00	0.25	0.4276
Desulfovibrio (Desulfobacterota)	0,02	0,02	0,04	0,51	0,07	0,01	0.17	0.2960
Escherichia- Shigella (Proteobacteria)	0,01	0,00	0,42	0,07	0,05	0,05	0.15	0.4303
Clostridiaceae (Firmicutes)	0,25	0,00	0,00	0,02	0,13	0,00	0.12	0.5806
Hydrogenophaga (Proteobacteria)	0,27	0,03	0,45	0,02	0,00	0,00	0.15	0.2338
Brevundimonas (Proteobacteria)	0,15	0,00	0,00	0,06	0,01	0,23	0.11	0.6276
Roseococcus (Proteobacteria)	0,35	0,05	0,36	0,00	0,00	0,00	0.19	0.4824
Flavobacterium (Bacteroidota)	0,21	0,00	0,09	0,11	0,05	0,01	0.12	0.7961
Deefgea (Proteobacteria)	0,03	0,02	0,03	0,04	0,44	0,00	0.12	0.0814

Values represented as mean (n = 6). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

Table 14
Taxonomy and percentage (%) of the bacterial phyla detected in the microbiota of hindgut samples after Mycoplasma filtration.

Index (%)	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB	SEM	*P-value*
Spirochaetota	27,32	52,09	30,44	36,39	54,15	37,79	13.25	0.5787
Proteobacteria	43,74	18,09	37,48	26,98	21,37	28,35	8.24	0.2148
Firmicutes	7,15	13,12	15,06	8,47	8,95	10,87	4.84	0.8545
Actinobacteriota	7,43	2,90	4,93	9,36	6,36	9,09	3.77	0.8226
Bacteroidota	5,61	5,27	4,33	3,85	1,25	5,76	2.35	0.7134
Patescibacteria	0,04	0,05	0,33	0,04	0,00	0,27	0.13	0.2848
Desulfobacterota	0,07	2,12	1,32	3,29	1,18	0,45	1.52	0.7028
Fusobacteriota	0,00	0,16	0,02	0,64	0,11	0,13	0.26	0.5221
Planctomycetota	0,13	0,06	0,10	0,11	0,17	0,10	0.99	0.9861
Bdellovibrionota	0,17	0,00	0,00	0,05	0,00	0,03	0.07	0.5781
Verrucomicrobiota	0,08	0,14	0,44	0,10	0,01	0,00	0.17	0.4539
Methylomirabilota	0,00	0,00	0,00	0,29	0,00	0,00	0.12	0.4633
Deinococcota	0,00	0,01	0,00	0,84	0,04	0,14	0.25	0.1485
Chloroflexi	2,86	0,00	0,00	0,32	0,00	0,00	1.00	0.2909
Acidobacteriota	0,02	0,00	0,05	0,26	0,00	0,68	0.23	0.1836
Gemmatimonadota	0,00	0,19	0,02	0,18	0,00	0,00	0.11	0.5972
Cyanobacteria	0,05	0,13	0,03	0,02	0,00	0,14	0.08	0.6815
Myxococcota	0,03	0,09	0,25	0,00	0,00	0,00	0.10	0.4773
Deferribacterota	0,00	0,13	0,02	0,00	0,00	0,00	0.05	0.4633
No asignados	5,30	5,45	5,19	8,82	6,41	6,18	2.25	0.8692

Values represented as mean (n = 6). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

IB and FM-IN) obtained the lowest final weights, indicating a negative effect on the growth performance of rainbow trout. The poor growth observed is related with low dietary level of fish proteins (considering both fishmeal and seabass by-products) in these diets (10 and 0%, respectively, for IN-IB and FM-IN) when they are compared with the rest of the diets (38,4% in the SB-IB diet, 31% in the Control diet, and 16% in SB-IN-IB diet). Primarily, diminished growth may stem from factors such as reduced feed intake, diminished diet digestibility, or decreased retention efficiency. In the case of the IN-IB group, despite exhibiting heightened feed intake, their final weight was comparatively lower, potentially attributable to observed discrepancies in digestibility (refer to Table 7), thus elucidating the growth differentials. Furthermore, the disparities in retention efficiencies highlighted in Table 8 further elucidate the subdued growth associated with the IN-IB diet. Conversely, for the FM-IN group, the attenuated growth aligns with decreased retention efficiencies and feed intake, though not correlating with digestibility.

Therefore, the inclusion of alternative marine sources (organic seabass by-products) reversed the negative effect on fish growth, and it may be a more economical and environmentally sustainable option than only fishmeal or plant-based diets.

Likewise, the fish fed with the IN-IB diet had the worst FCR and poorest protein and lipid retention, which may indicate a nutrient imbalance (Goff and Gatlin, 2004). On the other hand, the SB-IN-IB diet

Table 15
Taxonomy and percentage (%) of the bacterial genera detected in the microbiota of hindgut samples after Mycoplasma filtration (for each experimental group).

Index (%)	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB	SEM	*P-value*
Brevinema (Spirochaetota)	28.9	52.1	27.3	54.2	35.5	28.9	13.25	0.5787
Sphingomonas (Proteobacteria)	17.6	2.8	5.7	5.2	35.5	17.6	3.03	0.0898
Clostridium (Firmicutes)	0	0	5	0.2	0.1	0	1.82	0.4004
Xanthomonas (Proteobacteria)	1	0	10.1	0.8	0	1	3.68	0.4031
Blastomonas (Proteobacteria)	3.2	1.7	1.2	0.1	0	3.2	1.46	0.4017
Crenobacter (Proteobacteria)	0	4.1	0.1	2.4	1.1	0	1.81	0.6105
Aeromonas (Proteobacteria)	0.4	1.2	0.1	0.1	3.8	0.4	2.12	0.5353
Corynebacterium (Actinobacteriota)	3.3	0.6	0.1	2.8	3.2	3.3	1.9	0.7483
Acinetobacter (Proteobacteria)	0	0.2	1.4	0	0.3	0	0.77	0.471
Xanthobacteraceae (Proteobacteria)	1.1	0	0.3	0.3	2.4	1.1	0.96	0.563
Bacteroides (Bacteroidota)	0.8	1.6	2.8	0.3	1.1	0.8	1.26	0.0826
Massilia (Proteobacteria)	0	0	0.1	0	0.4	0	0.73	0.2858
Mycobacterium (Actinobacteriota)	1.7	0.1	2.1	0.9	0.8	1.7	0.99	0.8177
Sthapylococcus (Firmicutes)	0.4	0.7	0.5	3	0.6	0.4	1.55	0.5775
Bosea (Proteobacteria)	0.9	0.2	0.5	0	0	0.9	0.39	0.307
Desulfovibrio (Desulfobacterota)	1.2	0	0	0	0.2	1.2	0.84	0.4716
Escherichia-Shigella (Proteobacteria)	1.9	0	6.7	0.4	1.3	1.9	2.41	0.4417
Other genera	37.5	31	35.3	29.1	9.7	37.5	11.23	0.5036
Lactobacillus (Firmicutes)	0	0.28	2.1	0.34	0.93	0	0.83	0.6015
Flavobacterium (Bacteroidota)	0.56	0	0.13	0.33	0.07	0.56	0.28	0.2475

Values represented as mean (n = 6). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

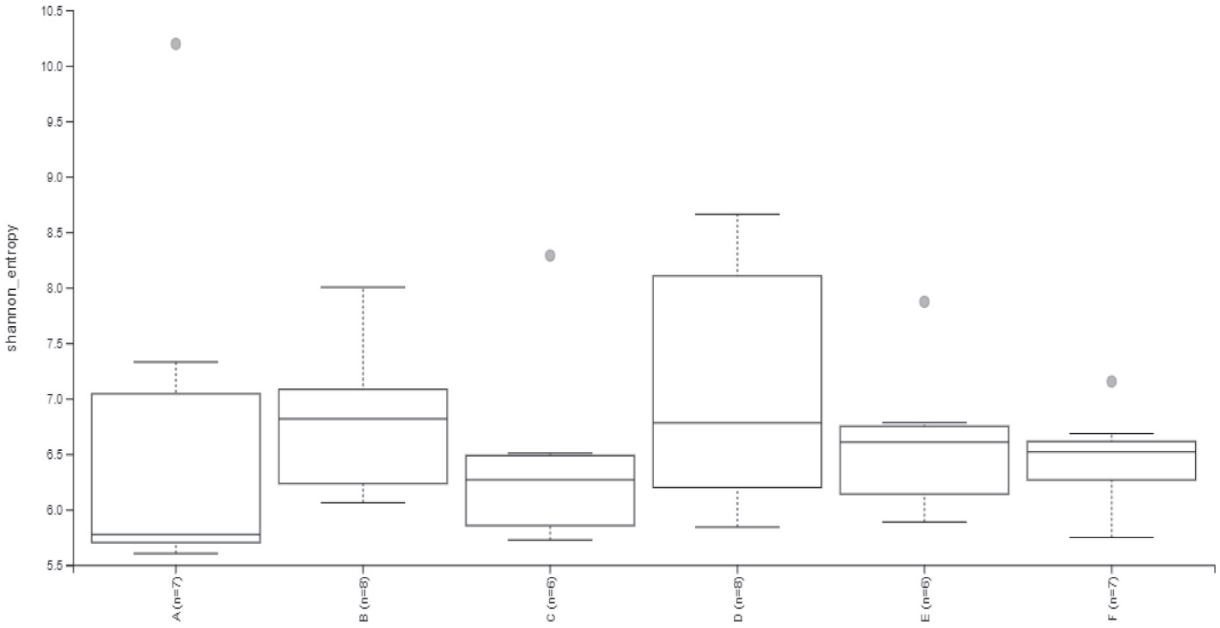

Fig. 4. Representation of diversity through the Shannon index between the different experimental groups.
(A) Control, (B) FM-IN: Fishmeal-Insect; (C) IN-IB: Insect-Iberic;(D) SB-FM: Seabass- Fishmeal; (E) SB-IB: Seabass-Iberic; (F) SB-f: IN-IB: Seabass-Insect-Iberic.

containing a lower percentage of insect meal (16% insect meal) was closer to the results obtained with the Control diet in nutritive parameters, particularly FCR. In previous studies, insect meal has partially or totally replaced fishmeal (50% and 45%) without affecting fish growth performance, feed utilization, digestibility, microbiota, and fillet quality (Iaconisi et al., 2017; Magalhães et al., 2017; Rimoldi et al., 2021; Terova et al., 2021). However, as in the current work, growth was affected when this substitution was 50% (Melenchón et al., 2022). Insects were generally high in fat (20%) compared to fishmeal (Domínguez, 2015); the fatty acid profile of the diets with insects (IN-IB and FM-IN) had a higher ratio of saturated fatty acids, which differs from that of fishmeal (Control), which is rich in n-3, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), lowest in insect diets (Table 3). Fish oil in insect diets had to be reduced to maintain the lipid level, but diets containing organic seabass byproducts (42% lipid) did not need fish oil. EPA and DHA were higher than in insect diets, and growth was higher, although lower than Control diet.

The differences in CF and HSI in this study were caused by the smaller size of the IN-IB fish and poor efficiency, which resulted in small size fish.

4.2. Body composition, retention efficiency, and amino acids retention

Concerning body composition, dry matter, and fat composition showed significant decreases when the fish were fed FM-IN and IN-IB diet, probably due to poor growth. Nevertheless, diet SB-IN-IB, containing a lower-level insect meal, showed a similar body profile to the control diet.

The current study's findings show that substituting fishmeal affects the apparent retention values of many essential amino acids (EAA),

which is one of the most severe issues with FM substitution with alternative ingredients is an essential amino acid deficiency (Kaushik and Seiliez, 2010), and unbalanced EAA levels in diets have been identified as a significant cause of low growth in the fish-fed animal by-products -based diets (Moutinho et al., 2017).

Control diets had high percentages of some EAA retention efficiency, and their values are comparable to previous works, but no differences appeared in arginine, lysine, and methionine retention (Moutinho et al., 2017). According to the growth results obtained with this diet, the fish fed with the IN-IB diet had the lowest retention efficiency for some EAA, histidine, isoleucine, leucine, and phenylalanine.

4.3. Enzymatic activity and digestibility

In the present study, the results of the enzymatic activity showed, in general, that the rainbow trout fed Control, IN-FM, and SB-FM diets increased the trypsin and chymotrypsin activity of the pyloric caeca (Fig. 3). On the contrary, fish fed with IN-IB diet showed a general decrease in trypsin and chymotrypsin activity. Since digestive enzymes are a useful indicator of feed digestion in fish, the characterization provides information on the digestive ability of fish to hydrolyse proteins in feed ingredients (Lemieux et al., 1999). Therefore, the low digestive capacity observed in the IN-IB diet is directly correlated with low growth and low protein and lipid retention efficiency, and poor digestibility obtained for this treatment, as shown in Table 3, Table 4, and Table 6, respectively.

In the present study, diets with a high fishmeal content demonstrated higher digestibility. The increased digestibility of dry matter, energy, nitrogen, and amino acid availability reaffirms the preference for fishmeal as the primary protein source in formulated aquaculture feeds. Previous research on salmonids has also supported the high digestibility coefficients observed (Smith and Guerin, 1995; Sugiura et al., 1998). Similarly, other ingredients, such as animal meals and protein extracts, such as gluten from corn and wheat, exhibit comparable digestibility to fishmeal in silver perch (Geoff et al., 2001). Therefore, it is evident from the current study that the Control diet, with the highest fishmeal content, resulted in enhanced digestibility. Indeed, the apparent digestibility coefficients (ADC) of Crude protein, Energy, Calcium, and Phosphorus significantly decreased when the fish were fed a diet of SB-FM.

4.4. Blood parameters

All the blood parameters observed are within the normal range of those established for this species (Carthy et al., 1971). No differences have been observed in the parameters analyzed in the serum of 12 fish per treatment.

4.5. Histological analysis

4.5.1. Liver

Significant differences were found in the liver histology of fish fed the experimental diets. Fish fed the SB-IB diet exhibited the highest nucleus and hepatocyte diameter measurements, whereas those fed the FM-IN diet demonstrated the lowest measurements for both parameters. This work indicates that the FM-IN experimental diet had a lower content of highly unsaturated fatty acids (HUFAs) than the Control diet. This finding is significant because it suggests that the substitution of certain ingredients in the experimental diets resulted in a decrease in the levels of essential fatty acids. According to numerous studies, these observations are consistent with the fact that high levels of substitution cause an increase in fat in the liver, increasing the hepatosomatic index, and the content of lipid vesicles in carnivorous fish (Jerusalén, 2017). Some authors have related that the reduction of the content of essential fatty acids in the diet tended to cause deposition of lipids in the liver since it is known that low levels of n3 HUFAs in the diets produce a decrease in the synthesis of lipoproteins, preventing the transport of lipids from the liver to other tissues (Cansino, 2002). This suggests that the altered fatty acid composition in the experimental diets may have contributed to the observed changes in liver histology. After analysing the measurements obtained in each treatment, a relationship was observed between the final weight, the diameter of hepatocytes, and nuclei in the liver Despite the established differences, no significant pathological alterations of the liver tissue were observed because of replacing the fishmeal.

4.5.2. Intestine

In the present study, the histological sections of the foregut showed typical morphologies of a rainbow trout were observed under normal conditions, except in the IN-IB diet that presented thickening, and a reduction of the intestinal villi height may be due to the accumulation of fluid and infiltration of inflammatory cells (Estruch et al., 2018). The SB-IN-IB diet was found with higher measurements of ML and SML than the rest, but without morphological alterations and with optimal growth. The Control, SB-FM, and SB-IB diets coincide with the most efficient diets regarding growth. The values obtained in the hindgut of rainbow trout did not show differences as evident as in the case of the foregut between the different treatments. However, the SB-IN-IB diet followed by the Control diet have been the diets that have recorded the greatest lengths and thicknesses. As in the foregut, in villi lengths the influence of fishmeal on intestinal morphology has once again been detected, where treatments without fishmeal tended to shorten villi length. In a previous study, the FM0 experimental group had higher VT and LP values at PI; however, the opposite trend appears to be observed at DI, with lower VT and LP values but no significant differences (Vélez-Calabria et al., 2021). The IN-IB diet's observed morphological alterations, such as smaller measurements and decreased absorption surface, could have contributed to its lower growth efficiency. Similar findings have been reported in previous studies. (Santigosa et al., 2008) investigated the replacement of fishmeal with vegetable raw materials in rainbow trout and found that as the fishmeal content decreased, there was a decreasing trend in villi length and smaller goblet cells, indicating potential negative effects on intestinal morphology. Furthermore, another study focused on replacing fishmeal with insect meal and found that the control diet with fishmeal had greater thicknesses, suggesting a positive impact on intestinal morphology (Melenchón et al., 2022). These findings support the idea that alterations in diet composition, particularly the substitution of fishmeal with other ingredients, can influence the morphological characteristics of the intestine. The observed smaller measures and alterations in villi length and goblet cells in the IN-IB diet may have contributed to the lower growth efficiency observed in the study.

4.6. Microbiome analysis

The microbial communities that inhabit the gastrointestinal tract of vertebrates are closely connected to their digestive physiology and gut health (Lyons et al., 2017). We can conclude that regardless of diet, the most dominant phylum and genera by far in the present study was *Firmicutes* and *Mycoplasma*, with a mean of 76.02 and 74.25%, respectively. These data largely agree with those shown by Lyons et al., 2017; Terova et al., 2019, where the phylum *Firmicutes*, *Proteobacteria*, and *Tenericutes* were dominant in the intestine of rainbow trout regardless the diet

Table 16
cost and economic performance metrics for experimental diets in rainbow trout.

	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB
Price of diet (€/kg)	1.86	3.34	2.95	1.38	1.26	2.21
Economic Conversion *(€/kg)*	4.00	7.78	9.96	3.15	2.77	4.73
Profit Index *(€/kg)*	4.00	0.22	−1.96	4.85	5.23	3.27

(Lyons et al., 2017; Terova et al., 2019).

Studies of the gut microbiome in salmonids showed *Mycoplasma* as the predominant genera. These salmonid-related *Mycoplasma* species are highly dominant in the gastrointestinal microbiota of all salmonids investigated, including rainbow trout (*Oncorhynchus mykiss*), Chinook salmon (*Oncorhynchus tshawytscha*), and Atlantic salmon (*Salmo salar*). Furthermore, phenotypic evidence points towards a beneficial role of *Mycoplasma*, such as disease resistance, given the inverse correlation between the abundance of *Mycoplasma* and *Vibrio* sp. (Brown et al., 2019; Lyons et al., 2017; Rasmussen et al., 2021).

Although the genera *Mycoplasma* are often exposed as an obligate parasite, studies have revealed that *Mycoplasma* species, as a natural host in salmonids, could be adapted explicitly for ammonotelic hosts as most teleosts, due to the ability to utilize ammonia. Previous studies have shown the importance of arginine and its derivatives, citrulline and ornithine, in the gastrointestinal tract of farmed fish. There is genetic evidence that *Mycoplasma* can use ammonia as a substrate for ornithine and citrulline biosynthesis due to the presence of genes encoding carbamate kinase (arcC) and ornithine transcarbamylase (otc), becoming an important power source (Andersen et al., 2013; Berge et al., 2002; Nguyen et al., 2018; Wang et al., 2020). This characteristic benefits salmonids since they cannot synthesize arginine de novo. In addition, it could increase the detoxification of ammonia in the intestine, which is usually found in high concentrations. On the other hand, ornithine absorption from the intestine may lead to increased growth in Atlantic salmon (Li et al., 2009; Rubino et al., 2014). Threfore, it is hypothesized that this could have facilitated a beneficial evolutionary relationship between *Mycoplasma* and its salmonid hosts (Rasmussen et al., 2021).

Finally, *Mycoplasma* in salmonids harbor genes capable of degrading long-chain polymers, such as chitin, which is usually abundant in insects and crustaceans, which constitute a significant proportion of the natural diet of juvenile salmonids. This could be beneficial for its host, as degradation of long-chain polymers increases the nutritional value of a chitin-rich diet and thus could be a coevolutionary driver between salmonid and *Mycoplasma* hosts. This hypothesis may also explain the increase in *Mycoplasma* in aquaculture cohorts, where an increase in *Mycoplasma* was shown in the intestinal region of rainbow trout reared on an insect-based diet, which has subsequently been shown to be beneficial (Orlov et al., 2006; Rimoldi et al., 2021; Rimoldi et al., 2019). Furthermore, chitin and its deacetylate derivative, chitosan, have antimicrobial properties and a bacteriostatic effect against various harmful gram-negative bacteria (Nawaz et al., 2018).

Once *Mycoplasma* filtered the data, *Firmicutes* decreased from high percentages to significantly lower values. The phylum that increased the most was *Spirochaetota*, with the genera *Brevinema* being the only representative, specially important in trout fed SB-IB diet, a genus associated with more excellent resistance to diseases (Mora-Sánchez et al., 2020). *Brevinema* is part of the central microbiota of Atlantic salmon (*Salmo salar*). It is associated with the expression of genes related to pro-inflammatory and anti-inflammatory responses. The *Spirochaetota* phylum has been associated with the expression of genes related to intestinal barrier function. Nevertheless, the *Proteobacteria* Phylum was more abundant than *Spirochaetota* in the control diet. Other genera, such as *Clostridium* and *Xanthomonas*, were highest in the trout-fed diet IN-IB, which had the worst growth results, and *Blastomonas* and *Aquabacterium* were the highest in the Control diet.

On the other hand, it is well documented that rainbow trout misuse dietary carbohydrates (Geurden et al., 2014; Guillaume et al., 2001), but the cause remains unclear (Lyons et al., 2017). Members of the phylum *Firmicutes* and *Spirochaetota* are known to play essential roles in the fermentation of dietary carbohydrates, transporting indigestible sugars across their cell membranes (Corrigan et al., 2015; Lyons et al., 2017). For most microbial fermentations, glucose dissimilation occurs via the glycolytic pathway. The molecule most frequently produced from this process is pyruvate. Therefore, the elevation of the glycolysis/gluconeogenesis and pyruvate metabolism pathways represents a further indication of the fermentative potential of the trout gut microbiome. This may be correlated with *Firmicutes* as one of the significant microbial phyla observed in the intestine of rainbow trout. Carbohydrate fermentation results in the formation of short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, which can be used in energy metabolism and have also been shown to promote enterocyte health (Hamer et al., 2008; Louis and Flint, 2009). Furthermore, high SCFA concentrations have previously been reported in various fish species, including rainbow trout (Clements et al., 2014; Lyons et al., 2017; Smith and Guerin, 1995).

The elevation of the genetic pathways responsible for the fermentation of amino acids and the production of peptidases could be related to the protein richness of the food. Rainbow trout require high levels of dietary protein, >35% of dietary dry matter, most likely associated with persistent amino acid catabolism for use as an energy source (Geurden et al., 2014; Kaushik and Seiliez, 2010).

Dietary proteins not digested by endogenous digestive enzymes are made available to bacteria for fermentation. Thus, microbiome fermentative activity may be significant in the distal intestinal region, where such enzymes are likely to have less influence (Lyons et al., 2017). On the other hand, in the *Firmicutes* phylum, there are *clostridia* with proteolytic and amino acid fermenting capacity (Neis et al., 2015). This could be an advantage for the group fed with the "FM-IN" diet since *Clostridium* was represented in a higher proportion.

4.7. Economic analysis

Although trout growth using organic ingredients was reduced, this work has demonstrated that trout can grow and allow commercial weight with organic byproducts from seabass and Iberian pork, with a similar feed conversion ratio. On the other hand, the growth and conversion ratio of experimental diets SB-FM, SB-IB, and SB-IN-IB was the same than using the commercial diet of the fish farm, (286 g and 1.9, data not showed), which opens an opportunity for cheaper organic diets, because the lower growth could be compensated by lower cost of diets (Table 16).

The price and economic conversion were lowest with diets SB-FM and SB-IB, and consequently, the Profit Index, expressed in terms of euros per kg of fish, was higher with these diets, particularly with SB-IB, without fish meal, which also improves the sustainability of trout feeding.

5. Conclusions

The study investigated the impact of substituting fishmeal with organic byproducts, such as seabass, Iberian pig byproducts, and insects, in the development of 100% organic protein diets for rainbow trout. The results revealed that, while the growth performance of trout was affected by the substitution of fishmeal, the best outcomes were observed in the control diet, followed by diets containing fishmeal of marine origin. Interestingly, the lowest price of animal byproducts, specifically in SB-IB diets, led to the best economic results, as indicated by the lowest economic conversion and profit indexes.

Despite a reduction in trout growth using organic ingredients, the study demonstrated that trout could reach commercial weight with organic byproducts from seabass and Iberian pork while maintaining a comparable feed conversion ratio, suggesting an opportunity for cost-effective organic diets.

The economic advantages of SB-FM and SB-IB diets, particularly the latter without fish meal, contribute to the overall sustainability of trout feeding. The findings highlight the potential for cheaper organic diets, where lower growth can be compensated by the reduced cost of diets. Therefore, these diets' profit index was higher, emphasizing the economic viability and sustainability of utilizing seabass and Iberian pig byproducts in rainbow trout aquaculture.

Funding

This project had been developed with the collaboration of the Biodiversity Foundation (Spanish Ministry for Ecological Transition and the Demographic Challenge), through the Pleamar Program, co-financed by the European Maritime and Fisheries Fund (EMFF). A full scholarship from the Ministry of Higher Education of the Arab Republic of Egypt funds the researcher Eslam Tefal.

CRediT authorship contribution statement

Eslam Tefal: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **David S. Peñaranda:** Writing – review & editing, Validation, Supervision, Investigation. **Silvia Martínez-Llorens:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Ana Tomás-Vidal:** Validation, Supervision, Data curation. **Ignacio Jauralde:** Investigation, Formal analysis. **Luis Lagos:** Methodology, Investigation. **Francisco Javier Moyano:** Investigation, Formal analysis. **Miguel Jover-Cerdá:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition.

Declaration of competing interest

The authors have no financial or personal conflict of interest to declare. The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgments

The first author was supported by a complete grant from the Ministry of Higher Education of the Arab Republic of Egypt, and the research project was developed with the collaboration of the Biodiversity Foundation (Spanish Ministry for Ecological Transition and the Demographic Challenge), through the Pleamar Program, co-financed by the European Maritime and Fisheries Fund (EMFF).

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