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Growth performance of gilthead sea bream (*Sparus aurata*) fed a mixture of single cell ingredients for organic diets

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ABSTRACT

The aim of this study was to investigate the growth performance of gilthead sea bream (*Sparus aurata*) fed with a mixture of a single cell ingredient (SCI) (bacterial protein, yeast protein and algae) for organic aquaculture. Sea bream with an initial mean body weight of 6.87±0.07 g were randomly allocated to 250-litre tanks. Four isonitrogenous, isolipidic and isoenergetic diets were formulated by replacing organic fishmeal trimmings with 0% (control), 12% (SCI12), 15% (SCI15) and 18% (SCI18) of the SCI mixture. Each diet was distributed to the groups in triplicate. The fish were fed three times daily until satiation and the experimental period was 60 days. Survival rates and voluntary feed intake were similar in the fish with the different feed treatments ($p > 0.05$). Similar ($p > 0.05$) growth performance (final weight, SGR) and feed conversion ratio (FCR) were observed in fish fed all SCI-based diets, which was superior to the control diet treatment. Apparent protein digestibility was similar among treatments, but lipid digestibility was significantly reduced in the SCI15 and SCI18 groups compared to the SCI12 group. The specific activities of trypsin, total alkaline protease and lipase were increased in fish fed the SCI15 diet ($p < 0.05$). The protein content in the fillet was significantly increased and the lipid content was decreased in the SCI12 and SCI18 groups compared to the other groups. No adverse effects on liver and intestinal histology were observed in the different groups. The results suggest that organic fishmeal trimmings can be successfully replaced by innovative SCIs based on microbial protein, yeast meal and algae, and that this SCI mixture could be a candidate for organic aqua feed for gilthead sea bream.

1. Introduction

In recent decades, consumer preferences have changed and the demand for formed seafood has increased; as a result, aquaculture production reached 88 million tons in 2020 (FAO, 2022). Organic aquaculture can be a promising sector in the global ecology and economy (FiBL & IFOAM – Organics International, 2020); this area reflects a specific production approach driven by the growing public interest in the sustainable use of resources (Mente et al., 2019; Lembo and Mente, 2019). Compared to conventional fish, organic fish has a high growth performance and fillet quality without a high environmental impact (Di Marco et al., 2017). In the context of sustainability, the search for innovative and sustainable ingredients is of increasing interest. The increasing demand for certified organic feed ingredients for organic aquaculture leads to a supply storage which drives up costs and represents a bottleneck for the growth of organic aquaculture (EUMOFA, 2022). In addition, novel ingredients tailored to individual fish species to produce organic feed are a major obstacle to organic aquaculture production.

Fish processing trimmings accounts for 45–60% of fish raw materials and includes skin, scales, viscera, head, fins, muscles and fillet frames (Elavarasan, 2019; Gasco et al., 2020; Siddik et al., 2021), which could be used as animal feed (Rajeh et al., 2021) and organic fertiliser (Ahuja et al., 2020), which is beneficial in terms of sustainability,

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environmental friendliness and recycling economy. Hydrolyzed fish waste is a good source of proteins, lipids (Vázquez et al., 2020; Araujo et al., 2021; Coppola et al., 2021), amino acids, bioactive peptides, and antioxidants, which play an important role in fish health and growth (Siddik et al., 2021). Salmon by-products can contain up to 16% fish oil/kg, which is rich in DHA and EPA (Fouda, 2020). Replacing fishmeal (FM) with fish waste meal contributes to a more sustainable range of ingredients for fish feed; however, fish waste meal does not have the same nutritional value as whole fish body meal (Mo et al., 2018). According to EU Regulation 2018/848 (EU 2018/848 European Parliament and Council Regulation of 30 May, 2018), fish trimmings approved for carnivorous organic aquafeeds should come from sustainable fisheries for human consumption or from whole fish, crustaceans or molluscs from sustainable fisheries for non-human consumption. As we move into the 2030 s the clear focus for the marine ingredients sector is to consolidate sustainability and increase its their diversification (Glencross, 2023).

Single-cell proteins and oils have recently gained scientific and commercial interest due to their nutritional value, which rivals that of fishmeal (FM) and fish oil (FO). Regardless of their origin (microalgae, yeast, fungi, or bacteria), single-cell proteins have important advantages over conventional protein ingredients, as they have a good nutritional profile, require a shorter production time, and use less land, their production is not affected by seasonal and climatic variations, and they can be produced from a variety of cost-free substrates (Sharif et al., 2021). Single cell ingredients include bacteria, fungi and yeasts, microalgae and the combination of all or some of them, such as Biofloc (Glencross et al., 2020). The cultivation medium of the microorganisms plays a decisive role in the nutritional quality of the unicellular proteins (Glencross et al., 2020). Research is constantly being conducted in this area to find new substrates that utilize industrial and agricultural by-products and stream waste (Glencross et al., 2020; Jones et al., 2020; Bouras et al., 2020). However, the production costs for these types of protein and lipid sources remain high (Spalvins and Blumberga, 2018).

Bacteria are a promising source of proteins that can be produced sustainably with renewable energy sources, CO_2 and a minimum of water and land (Sillman et al., 2019). Single-cell protein derived from bacteria has a comparable amino acid profile to FM (Sharif et al., 2021). Methanotrophic bacteria such as *Methylophilus* spp. and *Methylomonas* spp. contain up to 41% protein and essential amino acids, and these bacteria can be cultured on sewage sludge by-products (gaseous and liquid); therefore, the bacteria are suitable candidates to replace FM in fish feed (Zha et al., 2021). In addition, methane-oxidising bacteria cultured in nitrogen-rich waste streams are rich in essential amino acids (Khoshnevisan et al., 2020). In terms of functional feed, *Bacillus subtilis* is an ingredient that can improve the digestibility and assimilation of fish and shrimp feed, improve water quality in rearing, prevent the development of pathogens and increase the immune status of animals (Olmos et al., 2020).

Yeasts are common by products of the brewing industry that are high in protein and other nutrients and, when hydrolyzed, have shown high protein digestibility in carnivorous fish (San Martin et al., 2020). Yeast meal, such as *Saccharomyces cerevisiae* meal, has a nutrient composition compatible with FM and has been used as a nutraceutical ingredient in aquafeed (Shurson, 2018; Agboola et al., 2021). Furthermore, selenized yeast has been shown to be an effective method to increase selenium accumulation in fish (Ferreira et al., 2022). In addition, microbial oil extracted from *Yarrowia lipolytica* cultured in fish oil waste from the smoking process contained high levels of fatty acids, including long-chain fatty acids such as DHA and EPA (Fabiszewska et al., 2021). Protists are also unicellular components, and *Schizochytriym limacinum.* is a promising source of n-3 fatty acids (Jones et al., 2020). In terms of aquaculture sustainability, the results of the (FutureEUAqua project), have shown that the substitution of fish oil with heterotrophically produced microalgae, a low trophic level organism, in salmon feed has a positive effect on fish feed in aquaculture (Kousoulaki et al., 2022).

As fishmeal replacers, single-cell ingredients have been proved to be beneficial for fish. Bacterial protein enhances growth performance in several fish species (Estévez et al., 2021; Li et al., 2021; Xu et al., 2021; Guo et al., 2022; Ma et al., 2022; Zhang et al., 2022; Zheng et al., 2023). In addition, when yeast protein was used as fishmeal replacement, it improved the fish growth (Fronte et al., 2019; Rimoldi et al., 2020; Hines et al., 2021; Nazzaro et al., 2021; Reis et al., 2021; Ferreira et al., 2022). Furthermore, *Schizochytrium* sp. proved to have positive effects on fish growth (Ganuza et al., 2008; Kousoulaki et al., 2017; Santigosa et al., 2021; Kousoulaki et al., 2022). Nevertheless, although considerable research has been performed on the evaluation of the single-cell ingredients as fishmeal replacers, only a few studies have assessed their fish growth performance as a mixture (Vasilaki et al., 2023).

The aim of this study was to investigate the growth performance and digestibility of gilthead sea bream (*Sparus aurata*) fed with a mixture of single cell ingredients consisting of bacterial protein, yeast meal and algae for organic aquaculture. To the best of the authors' knowledge, this is the first study to investigate a "cocktail" of innovative protein ingredients as a replacement for fishmeal trimmings and plant-based ingredients for the diet of gilthead sea bream in organic aquaculture.

2. Material and methods

2.1. Ethics approval

All experimental procedures were performed in accordance with the guidelines of EU Directive 2010/63/EU on the protection of animals used for scientific purposes and were applied by FELASA- accredited scientists (Functions A-D). The experimental protocol was approved by the Ethics Committee of the Region of Thessaly, Veterinary Directorate, Department of Animal Welfare Medicine Veterinary Applications (No 18403/05–09–2019). The experiment was conducted in the registered experimental facility (EL-43BIO/exp-01) of the Laboratory of Aquaculture, Department of Ichthyology and Aquatic Environment, University of Thessaly.

2.2. Diet formulation

Four experimental diets were formulated to be isonitrogenous (48.3%), isolipidic (16.2%) and isoenergetic (21.3 kJ/g). The feed was thermically extruded using a laboratory scale twin-screw extruder (model EV025A10FAA, CLEXTRAL) and the oil was added by vacuum coating (DINISSEN). The control feed was designed to simulate the composition of a commercial feed containing organic FM trimmings, organic fish oil and soybean meal (organic and concentrate). A mixture of single cell ingredients(SCI) was incorporated into the diets at three different levels (12, 15 and 18%), replacing the FM trimming and vegetable ingredients, resulting in three experimental diets (SCI12, SCI15 and SCI18). The SCI mixture consisted of 66.7% bacterial protein *Methylococcus capsulatus* (FeedKind® from Calysta Inc) and 33.3% yeast protein *Saccharomyces cerevisiae* (NuPro® from Alltech). A further one percent (1%) of algae meal *Schizochytrium* (AlgaPrime™ DHA from Corbion) was added to the feedsSCI12, SCI15 and SCI18. The addition of krill meal was probably necessary to counteract palatability problems resulting from the use of unicellular ingredients. *Schizochytrium* was added to increase the DHA content of the diet, and rapeseed oil replaced fish oil in an amount that balanced the essential fatty acid profile between the diets. Yttrium oxide (Y_2O_3) was added at a level of 0.05% as an inert marker for digestibility. The formulation and immediate composition of the experimental diets and the individual cell components are shown in Table 1. The amino acid and fatty acid profiles of the experimental feeds are listed in Tables 2 and 3 respectively.

2.3. Experimental trial

The experimental trial was conducted in 12 cylindrical 250 L fibre

Table 1
Formulation and proximate composition of experimental diets and the single cell ingredients.

Ingredients (%)	Control	SCI12	SCI15	SCI18
Organic fish meal from fish trimmings	20	19	16	13
Krill meal	0	5	5	5
SCI mixture	0	11	14	17
AlgaPrime™ DHA	0	1	1	1
Wheat meal	10.20	17.43	16.83	16.33
Wheat gluten	6	11	10	11
Corn gluten	15	12.50	11	11
Organic soybean meal	8	0	0	0
Soybean concentrate	24	8	11	10
Organic oil from fish trimmings	11	5	5	5
Rapeseed oil	3	7	7	7
Monocalcium Phosphate	1.50	1.80	1.80	2.10
Mineral premix[a]	0.15	0.15	0.15	0.15
Vitamin premix[b]	0.15	0.15	0.15	0.15
Lysine	0.30	0.80	0.90	1.10
Methionine	0.20	0.12	0.12	0.12
Yttrium oxide	0.05	0.05	0.05	0.05
Moisture (%)	6.33	5.92	6.33	6.03
Dry Weight (%)	93.66	94.07	93.66	93.96
Proteins (%)	48.75	48.15	48.15	48.10
Lipids (%)	16.33	16.08	16.16	16.43
Ash (%)	7.39	7.19	6.98	6.77
Energy (Kj/g)	21.22	21.35	21.34	21.40
Single Cell Ingredients (SCI)	Bacterial protein	Yeast meal	Algae meal	
Proteins (%)	69.5	46.2	12.2	
Lipids (%)	1.0	0.3	55.7	
Moisture (%)	5.8	4.7	0.1	
Ash (%)	7	5.6	5.7	

[a] Mineral premix consisted of Se, Cu, Mn, Zn in organic form.
[b] Vitamin premix consisted of biotin, folic acid, niacin, pantothenic acid, pyridoxine, riboflavin, thiamin, vitamin B12, ascorbic acid, tocopherol acetate and vitamin K. Chemical composition of diets and SCI ingredients is expressed on a dry weight basis.

Table 2
Amino acid ratio (A/E) profile of experimental diets.

	Control	SCI12	SCI15	SCI18
EAA (% of total EAA)				
Arginine	12.33	11.23	11.69	11.11
Histidine	4.65	4.54	4.47	4.55
Isoleucine	9.49	8.68	8.64	9.09
Leucine	20.40	20.42	19.82	19.70
Lysine	11.39	12.76	13.21	13.64
Methionine	5.22	5.62	5.59	5.56
Phenylalanine	10.91	10.72	10.67	10.61
Threonine	8.06	8.17	8.13	8.08
Valine	10.44	10.21	10.16	10.10
Tyrosine	7.12	7.66	7.62	7.58
NEAA (% of total NEAA)				
Alanine	10.87	10.85	11.00	11.08
Asparagine	16.11	13.77	14.39	14.49
Glycine	10.07	10.02	10.16	9.80
Serine	9.26	9.18	9.31	8.95
Glutamine	37.86	39.65	38.93	39.63
Hydroxyproline	1.73	1.50	1.40	1.15
Proline	14.10	15.03	14.81	14.91
EAA/NEAA	0.84	0.81	0.83	0.84

tanks of three autonomous recirculating aquaculture systems. Each tank contained individual faecal sedimentation traps in each outlet. The gilthead sea bream juveniles were acclimated to the laboratory conditions for 15 days. During the acclimatization period, the fish were hand-fed a commercial diet until apparent satiation. Gilthead sea bream with an initial mean body weight of 6.87±0.07 g were randomly allocated to the tanks (40 fish per tank in triplicate groups). During the growth phase of the experiment, the fish were hand-fed three times a day (8:30, 14:00 and 17:00) 7 days a week for 60 days and twice a day (8:30 and 17:00) for the following 30 days to determine the digestibility of the feed. The water quality (21.4±0.02 °C, dissolved oxygen (DO) 7.22±0.0 mg/l, pH 8.43±0.0, salinity (S) 30.63±0.01 ppt, L:D 12:12) was similar in all three systems and remained stable during the experiment. The experiment lasted a total of 90 days, during which the behaviour and condition of the fish were observed before, during and after each meal. The fish were fasted 24 hours before sampling, and weighed individually during sampling to determine growth performance.

Table 3
Fatty acid profile of experimental diets.

Fatty acids (% of total)	Control	SCI12	SCI15	SCI18
14:0	2.95	2.04	2.00	1.83
14:1	0.10	0.09	0.09	0.09
15:0	0.21	0.16	0.16	0.15
16:0	13.44	14.04	14.30	14.42
16:1n9	0.17	0.11	0.10	0.09
16:1n7	3.47	3.67	3.99	4.17
17:0	0.21	0.29	0.29	0.28
18:0	2.80	2.46	2.37	2.25
18:1n9	36.06	42.10	42.35	43.29
18:2n6	16.47	17.64	17.61	17.79
18:3n3	3.51	3.71	3.71	3.61
20:1n9	5.67	3.49	3.23	3.17
20:2n9	0.33	0.20	0.18	0.17
20:5n3 (EPA)	3.32	2.22	2.23	2.14
22:1n9	6.62	3.53	3.33	3.15
24:0	0.55	0.38	0.31	0.25
22:6n3(DHA)	3.84	3.68	3.57	3.04
24:1n9	0.28	0.19	0.17	0.11
Total n3	10.67	9.61	9.51	8.79
Total n6	16.47	17.64	17.61	17.79
n3/n6	0.65	0.54	0.54	0.49

At the end of the experiment, eighteen gilthead sea bream (six per dietary treatment) were euthanized with an overdose of tricaine methanesulfonate (MS 222, 300+ mg/l) according to Directive 2010/63/EU and FELASA guidelines. The wet weight of the fish was measured, white muscle, stomach and pyloric caecum tissues were quickly dissected out, and the samples were weighed and stored at −80 °C for digestive enzyme and proximal composition analysis. Liver, posterior and anterior intestinal samples were also collected from each fish for histologic analysis.

2.4. Faecal collection and analysis

During the last 30 days of the experiment, the tanks and settling ponds were cleaned 1 hour after the second feeding of the day to remove all uneaten feed and faeces. Isothermal bags filled with ice were attached to each sedimentation column to ensure that faecal samples remained at a low temperature during the night. The faeces were collected from the sedimentation columns of each tank at 8:15 am before the first feeding of the day, centrifuged (3000 rpm, 4 °C) and stored at −20 °C. The faecal samples from the individual tanks were pooled and freeze-dried at the end of the experiment until further analysis.

2.5. Analysis of digestive enzymes

Digestive enzyme analysis of the stomach and pyloric caeca was performed to evaluate the effects of the experimental diets on digestive enzyme activities. Samples (a pooled sample of six fish tissues per dietary treatment) were freeze-dried and prepared for enzyme analysis in two replicates as described by Mente et al. (2017). Stomach and pyloric caeca samples were homogenized in 5 volumes v/w distilled water (4 °C) using an Ultra Turrax® homogenizer (IKA T25 digital ULTRA-TURRAX, IKA, USA) and centrifuged (3.300 x g, 3 min at 4 °C) to remove cell debris, and the supernatant was collected, aliquoted and frozen at −80 °C to determine the gastric and pancreatic enzymes

(Gisbert et al., 2009). To prevent the samples from degrading during storage and treated as described by Solovyev and Gisbert (2016). The enzyme activities of trypsin, total alkaline protease, α-amylase and bile salt-activated lipase were measured in the pyloric caeca tissue, while pepsin was measured in the stomach. Trypsin was measured with BAPNA as substrate in 50 mM Tris-HCl and 20 mM $CaCl_2$ buffer (pH 8.2). One unit of trypsin per mL (U) was defined as 1 μmol of BAPNA hydrolyzed per min per mLof extract at λ = 407 nm (Holm et al., 1988). Total alkaline proteases were determined using 0.5% (*w/v*) azo-casein as substrate in 50 mM Tris-HCl buffer (pH 8.0). One unit of total alkaline proteases per mL (U) was defined as 1 μmol of azo-casein hydrolyzed per min per mL of extract at λ = 366 nm (García-Carreño and Haard, 1993). α-amylase was measured using 0.3% soluble starch dissolved in Na_2HPO_4 buffer (pH 7.4) as substrate, and its activity (U) was defined as mg of hydrolyzed starch per min per mL ofextract (λ = 580 nm) (Métais and Bieth, 1968). Lipase activity was determined according to Iijima et al. (1998) using *p*-nitrophenyl myristate as a substrate in 0.25 mM Tris-HCl (pH 7.9), 0.25 mM 2-methoxyethanol and 5 mM sodium cholate buffer. Lipase activity (U) was defined as the μmol substrate hydrolyzed per min and mL extract (λ = 405 nm). Enzyme activities were expressed as total specific acidity (mU/mg protein), and the soluble protein in the enzyme extracts was quantified by the Bradford method (using bovine serum albumin as a standard (Bradford, 1976)). The activity of all enzymes was determined at 25 °C, and the activities were determined using a spectrophotometer (Tecan™ Infinite M200, Maennedorf, Switzerland) in duplicate per sample (methodical replicates).

2.6. Proximate composition analysis

The proximate composition of the feed, the ingredients and the white fish muscle (fillet) was determined. Crude protein of feed, white muscle and faeces was determined in dry samples by Kjeldahl analysis (N × 6.25; Behr Labour-Technik GmbH, Germany) and crude fat by exhaustive Soxhlet extraction with petroleum ether (40–60 °C, BP) in a Soxtherm Multistat/SX PC (Sox-416 Macro, Gerhard, Germany). The Y_2O_3 content was determined as described by Reis et al. (2008).

The gross energy content of feed and faeces was determined adiabatically in dry samples using an IKA oxygen bomb calorimeter (C5000; IKA Werke GmbH, Staufen, Germany). Total lipids in dry faeces were determined using a micromethod as described by Nengas et al. (1995). The amino acid composition of the tissue samples and feed was determined using the method described in Lyndon et al. (1993) and Mente et al. (2002). Amino acid results were expressed in g/100 g of sample. The ratio of essential amino acids (A/E) of each essential amino acid (EAA) was calculated as a percentage of the total EAA and the ratio of non-essential amino acids (NEAA) (Arai, 1981). Fatty acid analysis of the feed samples was performed as described by Fountoulaki et al. (2003).

2.7. Growth performance and digestibility parameters

The estimation of growth performance and nutritional parameters was done using the following formulae:

Weight gain (WG, g/fish) = Final body weight (g) – Initial body weight (g)

Specific growth rate (SGR, %/day) = 100 × [ln(final body weight) – ln(initial body weight)] × $days^{-1}$

Feed conversion ratio (FCR) = feed intake (g) × $wet\ weight\ gain^{-1}$ (g)

Protein efficiency ratio (PER) = wet weight gain (g) × $protein\ intake^{-1}$ (g)

Voluntary feed intake (VFI, %body weight/day) = 100 × total dry feed intake (g) × [(initial + final body weight (g)) × 0.5 × days]$^{-1}$

Survival (%) = 100 × number of fish at the end of the experiment × $number\ of\ fish\ stocked^{-1}$

Hepatosomatic index (%) = 100 × liver weight (g) × $body\ weight^{-1}$ (g)

Apparent Digestibility Coefficient (ADC, %) = 100 × [1 – (dietary Y_2O_3 level × faeces Y_2O_3 $level^{-1}$)]

$ADC_{protein/energy/lipid/starch}$ (%) = 100 × [1 – (dietary Y_2O_3 level × faeces Y_2O_3 $level^{-1}$) × (faeces protein/energy/lipid/starch level × dietary protein/energy/lipid level)]

2.8. Histological examination of liver and gut

For the histological examination, liver, foregut, and hindgut samples were taken from six fish per feed treatment. The tissue samples were fixed in Davidson fixative for 24 hours. They were then dehydrated in a graded series of ethanol, immersed in xylene, and embedded in paraffin. Sections 4–7 μm thick were taken and stained with hematoxylin-eosin. The histological slides were examined under a microscope (Bresser Science TRM 301, Bresser GmbH, Rhede, Germany), and digital images (600 dpi) were captured with a camera (Bresser MikroCam 5.0 MP, Bresser GmbH, Rhede, Germany). The width of the brush border of the foregut and the diameter of the lipid droplets were measured from the microscope images according to Berillis et al. (2017).

2.9. Statistical analysis

The values are given as means ± standard error of the means (S.E.M). Growth and nutrient indices, biochemical parameters and ADC were tested for normality using the Shapiro—Wilk test and for homogeneity using the Levene test. A one-way ANOVA was used to analyze the effects of feed treatments on fish growth performance, followed by a Tukey comparison test. Pearson correlation coefficients were calculated between the composition of amino acids in feed and tissue. Significance was accepted at 5%. All statistical analyses were performed using SPSS Statistics, version 26 (SPSS, Chicago, IL, USA).

3. Results

3.1. Growth performance feed conversion and digestive enzyme activity

The fish fed with the experimental diets (SCI12, SCI15 and SCI18) showed similar values for weight gain, SGR and PER ($p > 0.05$), which were significantly increased compared to fish fed the control diet (Table 4; $p < 0.05$). Fish fed SCI12, SCI15 and SCI18 also had similar FCR values ($p > 0.05$) which were significantly lower compared to fish fed the control diet ($p < 0.05$). Voluntary feed intake and survival rate

Table 4
Growth performance, nutrient utilization and somatic indexes of *Sparus aurata* fed the experimental diets.

Indexes	Control	SCI12	SCI15	SCI18
Final Weight (g)	14.65 ±0.46^{a}	19.44 ±0.48^{b}	19.86 ±0.49^{b}	19.37 ±0.45^{b}
Weight gain (g)	7.85 ±0.11^{a}	12.37 ±0.57^{b}	12.75 ±0.35^{b}	12.14 ±0.97^{b}
SGR[1] (%/day)	1.21 ±0.02^{a}	1.68 ±0.07^{b}	1.69 ±0.04^{b}	1.63 ±0.08^{b}
FCR[2]	1.28 ±0.07^{b}	1.05 ±0.02^{a}	0.99 ±0.03^{a}	0.99 ±0.04^{a}
PER[3]	1.60 ±0.08^{a}	1.98 ±0.05^{a,b}	2.08 ±0.08^{b}	2.09 ±0.10^{b}
Voluntary feed intake (% BW/day)	1.49 ±0.10^{a}	1.63 ±0.09^{a}	1.56 ±0.09^{a}	1.51 ±0.11^{a}
Survival (%)	96.6 ±1.66^{a}	100 ±0.00^{a}	89.76 ±7.73^{a}	96.42 ±1.79^{a}
Hepatosomatic index	1.16 ±0.21^{a}	1.78 ±0.05^{b}	1.74 ±0.13^{b}	1.8±0.05^{b}

Values are presented as means ± standard error of means. Means sharing the same superscript are not significantly different from each other ($p > 0.05$). 1Specific growth rate, 2Feed conversion ratio, 3Protein efficiency ratio.

were not affected by the feed treatments ($p > 0.05$). In addition, the HSI value was reduced in the control group compared to the SCI-fed fish ($p < 0.05$).

The digestibility (ADC) of dry matter was significantly higher in all three SCI diets than in the control diet, but the ADC of protein was similar in all diets (Table 5; $p > 0.05$). The ADCs of lipids, energy and starch were significantly increased in all three SCI diet treatments, with the SCI12 group had the highest ADC lipid and ADC energy values. The activities of the digestive enzymes trypsin, alkaline protease, and lipase in the pyloric caeca of gilthead sea bream were significantly increased in fish fed SCI15 compared to fish fed the control diet ($p < 0.05$) (Table 6). α-amylase activity in the pyloric caeca and pepsin activity in the stomach of fish fed the control and SCI15 diets were similar ($p > 0.05$).

3.2. Proximal composition of the fillet

The moisture of the white muscle of the gilthead sea bream was not affected by the diet ($p > 0.05$) (Table 7). The protein content in the fillet of SCI12 and SCI18 fish was significantly reduced compared the other groups, and the highest value was observed in fish fed the control diet ($p < 0.05$). In contrast, the lipid contents of the SCI12 and SCI18 fish were significantly increased, and the highest value was observed in fish fed the SCI18 diet. The energy content of fish fillets increased ($p < 0.05$) with the increase of SCI mixture in the diet, while the ash content of SCI15 was significantly increased compared to that of SCI18.

The amino acid ratio of white muscle of gilthead sea bream fed the experimental diets showed different patterns (Table 8). Arginine and isoleucine in the muscle tissue of the fish decreased in the groups fed SCI15 and SCI18 compared to the control group. On the other hand, the levels of leucine, lysine, methionine, phenylalnine, valine and tyrosine were higher in fish fed the SCI15 treatment were increased. In addition, histidine, isoleucine, methionine and threonine were increased in fish fed the SCI18diet. Leucine was decreased in fish fed the SCI18 diet and lysine was also decreased in fish in the control group. The arginine and isoleucine content in the white muscles was increased in fish fed the control diet, while the threonine content in the white muscles of the SCI15 diet-treated group decreased. In the white muscle of the SCI15 diet treatment, the amino acids aspartic acid and glycine were increased compared to the amino acid ratio of the fish fed the control and SCI18 diet treatments.

Pearson correlation showed a positive correlation between the amino acids of the experimental diets and white muscle tissue ($r = 0.762$, $p < 0.05$ for the control group, $r = 0.66$, $p < 0.05$ for the SCI15 group and $r = 0.69$, $p < 0.05$ for the SCI18 group). In addition, a strong correlation was found between the amino acids of the muscles of the control group and those of the SCI15 group ($r = 0.982$, $p < 0.05$) and between the control and SCI18 groups ($r = 0.987$, $p < 0.05$).

3.3. Histological examination

The liver in the control diet showed a normal structure and most hepatocytes contained central nuclei. The SCI-based diets showed a slight nuclear shift to the periphery of the hepatocytes due to increased lipid inclusions. Steatosis or haemorrhages were not detected in all samples (Fig. 1). The histological organisation of the anterior and posterior regions of the intestine appeared normal in all feeding groups, with normal distribution of goblet cells and distinct and well-organised villi (Fig. 2). There were no signs of inflammation in the anterior and posterior intestinal regions in all diets. Histological measurements showed that the brush border of the anterior intestine was significantly thinner in fish fed the control diet, while the lipid droplets in the liver of fish fed the SCI12 diet were larger (Table 9).

Table 5
Apparent Digestibility Coefficient (ADC) of dry matter, protein, lipid, energy, and starch of the experimental diets fed to *S. aurata*.

Indexes	Control	SCI12	SCI15	SCI18
ADC_{DM} (%)	65.59±0.53^{a}	82.77±0.02^{b}	82.49±0.21^{b}	82.04±1.35^{b}
$ADC_{Protein}$ (%)	91.53±0.03^{a}	94.16±0.24^{a}	93.00±0.33^{a}	92.95±0.91^{a}
ADC_{Lipid} (%)	90.06±0.82^{a}	94.18±0.09^{b}	93.56±0.42^{b}	92.59±1.10^{a,b}
ADC_{Energy} (%)	86.01±0.21^{a}	90.64±0.17^{b}	89.18±0.09^{b}	88.40±1.14^{a,b}
ADC_{Starch} (%)	91.62±0.74^{a}	94.11±0.19^{b}	94.19±0.20^{b}	93.87±0.32^{b}

Values are presented as means ± standard error of means. Means sharing the same superscript are not significantly different from each other ($p > 0.05$)

Table 6
Digestive enzyme specific activities (mU/mg protein) of pyloric caeca and stomach of *S. aurata* before (initial) *and after* fed the control and SCI15 diets.

Enzymes (mU/mg protein)	Initial	Control	SCI15
Pyloric caeca	4.87±0.02^{a}	76.86±3.00^{b}	97.26±2.31^{c}
Tryspin			
Total Alkaline Protease	91.52±4.64^{a,b}	79.48±1.93^{a}	116.56 ±6.17^{b}
α-Amylase	82.85±8.10^{a}	139.47 ±6.28^{b}	165.39 ±1.94^{b}
Lipase	32.41±0.49^{a}	33.11±3.69^{a}	57.54±6.05^{b}
Stomach	108.09	131.02	135.69
Pepsin	±12.72^{a}	±8.75^{a}	±6.54^{a}

Values are presented as means ± standard error of means. Means sharing the same superscript are not significantly different from each other ($p > 0.05$)

Table 7
Proximate composition of *S. aurata* white muscle fed the experimental diets.

	Control	SCI12	SCI15	SCI18
Moisture (%)	75.18±0.32^{a}	73.66±0.55^{a}	73.54±0.94^{a}	73.17±0.41^{a}
Dry matter (%)	24.81±0.32^{a}	26.33±0.55^{a}	26.45±0.94^{a}	26.82±0.41^{a}
Proteins (%)	75.53±0.15^{b}	71.28±0.13^{a}	73.83±0.47^{b}	70.17±0.58^{a}
Lipids (%)	17.63±0.50^{a}	22.37±0.35^{b}	18.89±0.68^{a}	24.27±0.44^{b}
Energy (Kj/g)	23.63±0.01^{a}	24.5±0.02^{c}	24.14±0.05^{b}	25.11±0.05^{d}
Ash (%)	5.80±0.09$^{a.b}$	6.04±0.30$^{a.b}$	6.14±0.09^{b}	5.35±0.07^{a}

Values are presented as means ± standard error of means. Means sharing the same superscript are not significantly different from each other ($p > 0.05$)

Table 8
Amino acid ratio (% of total amino acids) of *S. aurata* white muscle fed the experimental diets.

	Control	SCI15	SCI18
EAA (g/100 g sample)			
Arginine	4.61	4.47	4.27
Histidine	0.74	0.74	0.78
Isoleucine	3.49	3.42	3.45
Leucine	5.42	5.66	5.35
Lysine	7.46	8.36	8.02
Methionine	1.15	1.78	1.55
Phenylalanine	3.32	3.43	3.3
Threonine	3.11	2.7	3.22
Valine	3.21	3.52	3.36
Tyrosine	2.27	2.61	2.38
NEAA (g/100 g sample)			
Alanine	4.49	4.5	4.38
Aspartic acid	7.62	7.94	7.08
Glycine	3.13	3.92	3.87
Serine	2.36	2.35	2.47
Glutamic acid	8.84	8.02	7.91
Proline	2.69	2.5	2.5

4. Discussion

In the present study, an SCI mixture containing bacterial protein (66.7%), yeast protein (33.3%) and algae (1%) improved FCR and increased the growth performance of *S. aurata*. The results showed that replacing organic FM trimmings and plant ingredients with unicellular

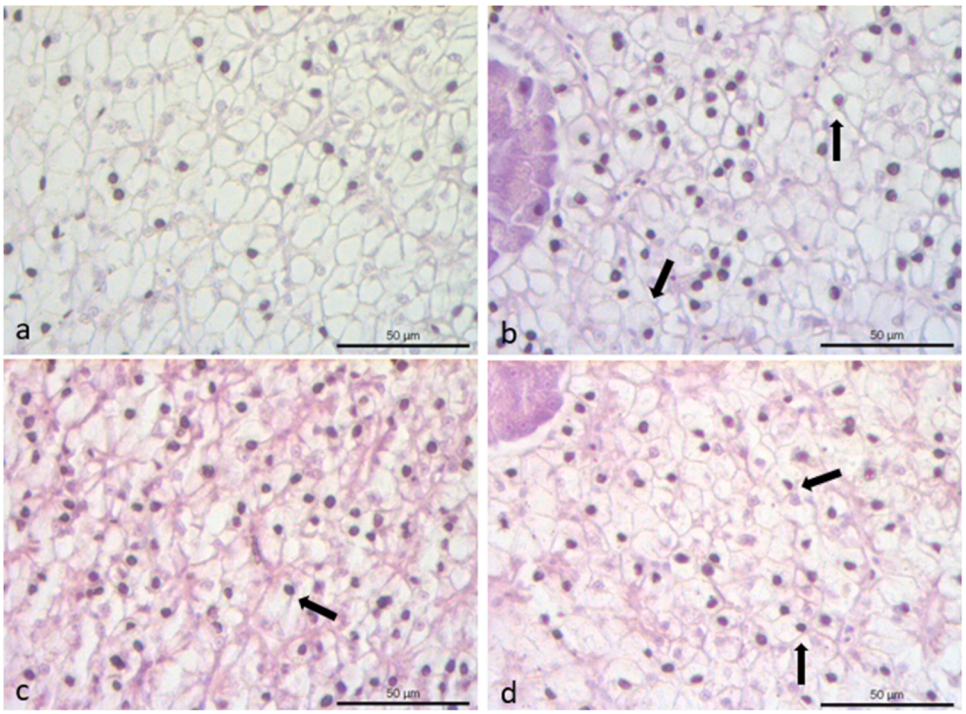

Fig. 1. Liver histological examination of *S. aurata* fed the experimental diets (a) control. (b) SCI12. (c) SCI15 and (d) SCI18. In SCI diets (b.c.d) nuclei displacement were detected (arrows) due to the presence of small lipid droplets. In general, fish fed the different diets had normal liver histology.

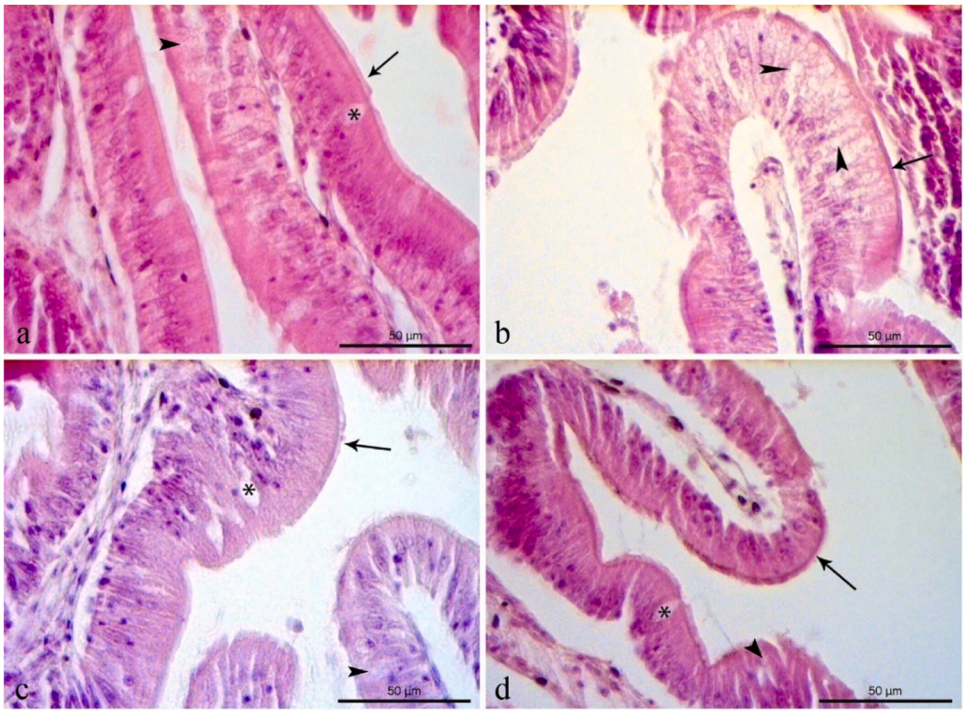

Fig. 2. Anterior gut histological examination of *S. aurata* fed the experimental diets (a) control. (b) SCI12. (c) SCI15 and (d) SCI18. All dietary treatments had normal structure with goblet cells (asterisks), thick brush border (arrows) and smaller or bigger supranuclear absorbing vascuoles in the enterocytes (arrowhead).

proteins up to 15–18% had a positive effect on the growth performance of gilthead sea bream, which accepted the SCI diets well. Since feed intake was not affected by the diet, the increased growth of the fish fed the SCI diets was due to the fact that the fish digested the SCI meal, especially the lipids, energy and starch, better than the organic FM trimmings. Single cell proteins have a good nutritional profile, they can be produced from a variety of cost-free substrates (Ritala et al., 2017) and could be candidates for the production of organic feeds. Mente et al. (2011) have investigated aspects of formulated feed use, feed composition and general nutritional principles and product quality in the context of organic aquaculture. However, there are few studies investigating the replacement of fishmeal in organic feed for sea bream.

Table 9
Histological measurements in anterior gut and liver of *S. aurata* fed the experimental diets.

Measurements	Control	SCI12	SCI15	SCI18
Anterior gut brush border width (μm)	1.77±0.07[a] (32)	2.39±0.05[b] (56)	2.50±0.04[b] (47)	2.38±0.04[b] (44)
Liver lipid droplets diameter (μm)	5.43±0.12[a] (81)	5.90±0.13[b] (76)	5.43±0.10[a] (84)	5.84±0.11[ab] (80)

Data represent means ± standard error of means. The number of measurements is represented within brackets. Different letters in the same line denote statistically significant differences ($p < 0.05$).

Estévez et al. (2021) have shown that fishmeal derived from trimmings can be replaced by up to 25% with organically certified pea proteins without compromising the growth, health and quality of the final fish product.

Microbial protein is an alternative source of high-quality protein that can replace animal protein, such as FM, in livestock and aquaculture diets (Matassa et al., 2016; Olmos et al., 2020). Ma et al. (2022) reported the same results on growth and feed parameters when FM was replaced up to 50% by *Clostridium autoethanogenum* protein in largemouth bass (*Micropterus salmoides*). Replacing 24.8% FM with *M. capsulatus* meal also did not change the growth performance of black sea bream (*Acanthopagrus schlegelii*), although optimal growth and feed conversion occurred at 8% FM replacement (Xu et al., 2021). Hines et al. (2021) found that growth and feed utilization parameters remained unchanged and gut bacteria were unaffected by a 20% replacement of FM with dry and lysed *S. cerevisiae* in the diet of *O. mykiss*. Reis et al. (2021) also found that the diet of gilthead sea bream supplemented with β-glucan from *S. cerevisiae* did not affect growth and feed conversion. In *Schizochytrium* sp., the inclusion of 2.5% as a lipid source and especially as a DHA source in microdiets of *S. aurata* had no negative effects on the larvae; however, the complete replacement of fish oil reduced growth and survival (Ganuza et al., 2008).

Protein digestibility was not affected by the feed treatments in the present study, but the digestibility of dry matter, lipids, energy and starch was significantly higher in all three SCI diets compared to the control diet. These results indicate a more efficient nutrient catabolism of the SCI mixture compared to FM trimmings in combination with soybean products, which in turn improved the growth performance of the fish, as previously mentioned. The SCI mixture consisted of microbial protein from *M. capsulatus* and *S. cerevisiae*. The suitability of these alternative proteins for replacing FM was previously investigated as individual feed ingredients, but not simultaneously in a mixture, as in the present study. Biswas et al. (2020), working with Japanese amberjack (*Seriola quinqueradiata*), reported that the growth performance, feed efficiency and digestibility of the fish were not affected by a 30% replacement of FM with *M. capsulatus*. Similar results were reported in turbot (*Scophthalmus maximus*), in which *M. capsulatus* replaced FM by 30%, and the authors found reduced dry matter digestibility in the tested diets (Zheng et al., 2023). *A. schlegelii* fed *M. capsulatus* as a replacement for FM also showed reduced dry matter digestibility and lipid digestibility, although fish growth, feed efficiency and protein digestibility were not affected by the diet (Xu et al., 2021). In contrast largemouth bass (*Micropterus salmoides*) fed diets containing up to 6% *M. capsulatus* meal as FM replacer showed better growth, unaffected feed utilization and higher digestibility of dry matter, protein and energy compared to fish fed the control diet (Guo et al., 2022). In addition, the higher the FM replacement, the lower the digestibility.

Yeast meal contains a considerable amount of crude protein (about 45–55%) and other bioactive components that are beneficial for the growth and development of fish (Agboola et al., 2021). According to Nazzaro et al. (2021), the inclusion of 20%-30% brewers spent germ and spent grain is a viable protein source for carnivores (*O. mykiss*, *S. aurata*) with similar growth and protein, lipid and amino acid digestibility as the FM feed. More specifically, the authors indicated that hydrolyzed beer by-products helped to improve protein digestibility in gilthead sea bream, but lipid digestibility appeared to be reduced in the experimental diets. On the other hand, *O. mykiss* fed dry spent grain or dry yeast showed better protein digestibility than the hydrolyzed specimen. In addition, better growth and protein efficiency were observed, and feed conversion of *S. aurata* was not affected by the inclusion of 20% brewers spent yeast compared to FM protein (Estévez et al., 2021). The protein and lipid digestibility coefficients showed that feeding 20% dry or hydrolyzed brewer's yeast had no adverse effects on protein and lipid digestibility of *S. aurata*.

In the present study, the digestibility of the feed was closely related to the activity of the digestive enzymes. The increase in bile salt-activated lipase-specific activity in fish fed the SCI15 diet resulted in an increase in lipid ADC compared to fish fed the control diet. A non-significant increase in α-amylase activity in fish fed the SCI15 diet resulted in higher apparent starch digestibility. On the other hand, gilthead sea bream fed the SCI15 diet showed increased trypsin and alkaline protease activity compared to fish in the control group and similar pepsin activity; however, no significant differences were observed for ADC protein. Therefore, similar proteolytic enzyme activity in the fish may be required for the SCI diets to achieve similar protein digestibility to the control diet. A similar pattern in pepsin activity was found in *A.* schlegelii fed *M. capsulatus* diets, although lipase and amylase activities were unaffected in the stomach and increased in the foregut (Xu et al., 2021). In contrast to the results of the present study, Zheng et al. (2023) observed that lipase and trypsin activities in the foregut decreased and pepsin activity increased in *S. maximus* fed *M. capsulatus* diets, which can be considered as a possible effect on the bacterial cell wall, diet formulation and/or buffering properties of the diet formulated with this ingredient. The protein content of the gilthead sea bream fillet increased but the lipid content decreased in the control and SCI15 dietary treatments, despite the high protein digestibility in all dietary treatments and the higher lipid digestibility in the FM replacement treatments. However, the protein content of *D. labrax* did not change when FM was replaced with fillet waste from *O. niloticus*, but the lipid content increased when the fish were fed the 45% replacement diet (Saleh et al., 2020). The same study reported that essential amino acids were not affected in the body of European sea bass, with the exception of tryptophan, which was found in the lowest amount in the reference diet. Furthermore, the composition of red drum remained unchanged when FM was replaced with a mixture of plant protein and FM trimmings (Yamamoto et al., 2021). The study by Jimoh et al., (2021) agrees with these results, while the replacement of FM with visceral meal protein in the African catfish diet did not result in changes in the protein and lipid content of the fish, although the highest levels were found in fish fed a diet with 15% FM replacement. Similarly, no changed in proximate composition were observed in Nile tilapia when the fish were fed salmon testis meal, although lipid levels increased in rainbow trout (Lee et al., 2015). Whole body protein content of European sea bass was not affected by feeding, but replacing FM with apple pomace fish silage resulted in reduced lipid levels (Davies et al., 2020).

The inclusion of SCI led to an increase in the lysine A/E ratio at all inclusion levels (SCI12, SCI15 & SCI18) compared to the A/E ratio of the control diet. A strong correlation was found between the white muscle amino acids of the control group and those of the SCI15 and SCI18 diet groups ($r = 0.982, 0.987$, respectively $p < 0.05$). According to hypothesis that an efficient diet should result in an amino acid profile largely similar to that of the experimental animal (Mente et al., 2003), the results of this study showed that a high intake of single-cell ingredients resulted in higher lysine, leucine, methionine, phenylalanine,

valine, tyrosine, alanine and glutamine enrichment and lower leucine and aspartic amino acid enrichment in white fish muscle compared to the control diet. The SCI ingredients thus ensure a balanced amino acid profile in the fish fillet. Arginine and isoleucine in the muscle tissue of the fish decreased in the SCI15 and SCI18 dietary treatments compared to the control group, whereas methionine and tyrosine increased in fish fed the SCI15 dietary treatment. A balanced ratio of amino acids in the diet (including essential and non-essential amino acids) is important for optimal utilisation of dietary protein. An ideal protein is one that provides the exact balance of amino acids required for maximum growth and protein deposition. A feed formulated based on the ideal protein concept is an effective way to meet amino acid requirements with less dietary protein and achieve better protein efficiency. The amino acids arginine and threonine contained in the muscle of blackhead seabream (*Acanthopagrus schlegelii*) were positively affected by replacing FM with soluble FM in the diet (Irm et al., 2020). Mente et al. (2021) confirmed that a 16% replacement of marine protein with vegetable protein meets the amino acid requirements of sea bream. Dietary lysine influences the utilisation of arginine. If the body composition of wild sea bass is used as the ideal profile of essential amino acids, it can be seen that the requirement for lysine, leucine and arginine is higher than the requirement for the other essential amino acids (Mente et al., 2012). Berge et al. (1998) showed that the argine content in muscle and plasma tended decrease when Atlantic salmon were fed high levels of lysine. In this study, an increased amount of lysine in the diet may have minimised lysine–arginine antagonism. When bacterial protein from *M. capsulatus* replaced FM in the diet of *S. quinqueradiata*, it had no effect on the proximate composition of the fish, but the complete replacement of FM contributed to lower protein and lipid levels in the fish body (Biswas et al., 2020). According to Zheng et al. (2023), the replacement of FM with *M. capsulatus* meal affected the immediate composition of turbot, and indeed the ash content of the body increased; however, PUFAs in the muscle decreased when the replacement level was 100%. On the other hand, Nazzaro et al. (2021) reported that the inclusion of 10% dried or hydrolyzed commercial yeast in the diet and 7.5% brewers spent grain led to an increase in protein content in the gilthead sea bream muscle. In addition, the inclusion of 20% hydrolyzed commercial yeast and 10% hydrolyzed spent yeast in the feed led to an increased lipid content in the fish muscle.

In *S. aurata* fed with dried *Schizochytrium* sp. as a substitute for fish oil, an increase in DHA was observed, although the substitute had a negative effect on the growth performance of the fish (Eryalçin and Yildiz, 2015). On the other hand, according to Santigosa et al. (2021), the growth performance of sea bream fed with 3.5% *Schizochytrium* sp. oil as a substitute for fish oil was unaffected and the fatty acid composition of the fillet was consistent with the fatty acid composition of the diet, suggesting that feeding microalgae could result in an environmentally friendly final product rich in n-3 and n-6 fatty acids. In contrast, Zaihurin et al. (2021) found that the body protein of climbing perch (*Anabas testudineus*) was higher when fed fish waste, but the fat content was lower, although the replacement of 25% FM resulted in a similar growth rate to the control diet. Regarding the fatty acid composition of *A. testudineus,* fatty acid 17:1 n was decreased in fish fed fish waste meal, but 18:2n6, 20:5n3, and 22:6n3 were increased in fish fed replacement diets. According to Estévez et al. (2021), *S. aurata* fed diets in which FM was replaced with brewers' grain (7.5% and 15%) showed an increase in monounsaturated and polyunsaturated n-6 fatty acids in muscle, while sea bream fed brewers' grains showed an increase in saturated fatty acids in muscle and a decrease in n-3 polyunsaturated fatty acids compared to the control diet.

Replacing FM with the SCI mixture in gilthead sea bream did not adversely affect the histological organization of the liver and intestine. The liver appeared normal with small lipid droplets in some cases. The anterior and posterior intestine also appeared normal, indicating that the feeds were well metabolized. However, when the proportion of FM replaced by fermented tilapia waste was above 45%, a negative effect on the gut health of European sea bass was observed (Saleh et al., 2020). According to Li et al. (2021), the inclusion of up to 200 g/kg of dried *C. autoethanogenum* in the diet of Jian carp (*Cyprinus carpio* var. *Jian*) resulted in normal liver and intestinal structure without signs of pathological damage. Furthermore, replacing 150 g/kg FM with 129 g/kg *M. capsulatous* in largemouth bass (*M. salmoides*) resulted in intestinal damage (thickened intestinal wall and reduced villus height), suggesting that the optimal level of bacterial protein is 86 g/kg (Zhang et al., 2022). According to Zheng et al. (2023), juvenile turbot (*S. maximus*) fed diets replacing 45–100% FM with *M. capsulatus* gradually developed fatty liver with hepatocyte vacuolation, dislocation and disappearance of nuclei. Bacterial protein obtained from fermented agricultural waste had no effect of the histological organization of liver and intestine (Adeoye et al., 2021). In addition, the use of autolyzed yeast as a substitute for FM at a level of 5% resulted in a higher absorption area in the intestinal track (Fronte et al., 2019). Our histological analysis revealed that the brush border of the anterior intestine was thinner in fish fed the control diet, likely indicating lower nutrient absorption. This fact may reflect the lower SGR and weight gain observed in fish fed the control diet. Vasilaki et al. (2023) also concluded that thicker brush border may indicate higher nutrient intake. In fish fed SCI12 and SCI18, the lipid droplets in the liver appeared to be larger. Since no steatosis was observed, larger lipid droplets in the control diet do not correspond to lower lipid digestibility or higher fat content in the fish body.

Inflation, the rising cost of raw materials and the rising cost of producing aqua feed due to energy costs have caused the aqua feed industry to stagnate. Therefore, functional nutrition and alternative novel feed ingredients that deviate from current raw materials will play a key role in improving the resilience of aquaculture. However, the availability of 500 novel ingredients and their expansion will require high investments and lower prices to be economically feasible. Future sustainable aquaculture and organic aquaculture will depend on the supply chain of novel ingredients, their cost, safety, quality, eco-label, certification and knowledge of species-specific nutritional physiology to improve their metabolic strategies. The results suggest that organic FM waste and wild-collected plant ingredients can be successfully replaced by innovative SCI based on microbial protein, yeast meal and algae. A proportion of up to 18% of the SCI mixture had no adverse effects on growth performance, feed conversion, fillet composition and fish health, while liver and gut histopathology remained unaffected. This study improved our knowledge of the effects of novel aquafeed ingredients on growth performance of sea bream in organic aquaculture. The inclusion of bacterial protein, yeast meal and algae in fish feed could be used in organic feed formulations as an alternative to fish meal waste and plant-based ingredients and promote the sustainability of organic aquaculture, which could lead to environmentally friendly production.

CRediT authorship contribution statement

Styliani Andreopoulou: Formal analysis, Data curation. **Anna Tampou:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Ioannis T Karapanagiotidis:** Writing – review & editing, Validation, Methodology, Investigation. **Enric Gisbert:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Eleni Mente:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Efthimia Antonopoulou:** Writing – review & editing, Validation, Methodology, Investigation. **Ioannis Nengas:** Writing – review & editing, Validation, Methodology, Investigation. **Antigoni Vasilaki:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Panagiotis Berillis:** Writing – review & editing, Validation, Methodology, Investigation, Data curation.

Declaration of Competing Interest

Corresponding authors, on behalf of all the authors of a submission, must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. All authors, including those *without* competing interests to declare, should provide the relevant information to the corresponding author (which, where relevant, may specify they have nothing to declare). Corresponding authors should then use this tool to create a shared statement and upload to the submission system at the Attach Files step.

Data availability

Data will be made available on request.

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