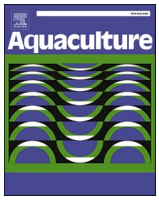
Aquaculture 576 (2023) 739861

Contents lists available at ScienceDirect

Aquaculture

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



Single cell protein from methanotrophic bacteria as an alternative healthy and functional protein source in aquafeeds, a holistic approach in rainbow trout (*Oncorhynchus mykiss*) juveniles

Alberto Ruiz [a], Ignasi Sanahuja [a], Nana W. Thorringer [b], Julie Lynegaard [b], Eleni Ntokou [b], Dolors Furones [a], Enric Gisbert [a,*]

[a] IRTA, Centre de la Ràpita, Aquaculture Program, Crta. Poble Nou, km 5.5, 43540 la Ràpita, Spain
[b] Unibio A/S, Langebjerg 1, 4000 Roskilde, Denmark

ARTICLE INFO

Keywords:
Alternative protein
Circularity
Disease resistance
Nutrition
Methylococcus capsulatus
Sustainability

ABSTRACT

Bacterial single cell protein (BSCP) obtained from methanotrophic bacteria was tested as an alternative protein source to fishmeal (FM) in compound diets for rainbow trout (*Oncorhynchus mykiss*) juveniles (initial body weight, BW = 11.3 ± 0.2 g, mean ± standard deviation). Different levels of FM replacement (0, 25, 50, 75 and 100%) by a BSCP (0, 3.75, 7.5, 11.25 and 15% of Uniprotein® Aqua) were tested in isoproteic (crude protein: 42%) and isolipidic (crude fat: 21%) extruded diets. The trial was divided in two different parts, a nutritional trial that lasted 83 days during which the impact of experimental diets was evaluated in terms of key performance indicators (KPIs) associated to fish growth performance and feed utilization, which was followed by a challenge with a pathogenic bacterium (*Aeromonas salmonicida* subsp. *salmonicida*) to evaluate the immunological competence of fish fed the experimental diets (15 days). At the end of the nutritional trial, somatic growth in terms of body weight (BW) responded quadratically ($P < 0.05$) to the dietary inclusion of BCSP with the lowest BW values (183.4 ± 4.5 g) recorded in fish fed 100% FM replacement with BSCP, whereas the rest of dietary groups showed similar BW values (191.3–201.2 g). Feed intake was not affected by the level of FM replacement by BSCP, whereas feed conversion ratio (FCR) slightly increased with increasing levels of BSCP ($P < 0.05$), even though the lowest FCR values were found in fish fed 50% FM replacement by BSCP (0.79 ± 0.01) in comparison to the control group (0.81 ± 0.01). When using quadratic linear regression, the optimal dietary FM replacement by BSCP in terms of BW values was estimated as 41.7%. The nutritional quality of the fillet was not altered with experimental diets; all dietary groups showed similar amino acid levels as well as their content in HUFA n-3 and n-6 that remained unaltered ($P > 0.05$) and lipid nutritional indexes ($P > 0.05$). No negative effects were observed in the histological organization of the liver nor the proximate intestine, neither in the lipid peroxidation levels nor activity of antioxidative stress enzymes in the liver from fish fed increased inclusion levels of BSCP ($P < 0.05$). The replacement of FM by the tested BSCP obtained from methanotrophic bacteria did not cause an imbalance or dysbiosis in the intestinal microbiota in rainbow trout regardless of the level of FM replaced. The replacement of FM by BSCP at 50% improved disease resistance in rainbow trout exposed to furunculosis when compared to the control diet (cumulative mortality = 83.3 ± 7.2% vs. 49.2 ± 15.6%; $P < 0.05$), which suggested that this ingredient may also have some immunomodulatory properties that would protect fish from infections, although their mode of action deserves further attention. Current results indicated that the tested BSCP from methanotrophic bacteria is a safe, functional, and sustainable alternative protein source for aquafeeds.

1. Introduction

The sustainability principle is widely used by practically all economic activities; thus, all sectors are committed to being sustainable and aspire to introduce sustainable practices in their business models; however, this goal is not easily put into practice. Sustainability is not a final condition nor a tangible goal; sustainability is a way ahead and a way of working. Under this scenario, sustainable strategies for

* Corresponding author.
E-mail address: enric.gisbert@irta.cat (E. Gisbert).

https://doi.org/10.1016/j.aquaculture.2023.739861
Received 25 March 2023; Received in revised form 29 June 2023; Accepted 1 July 2023
Available online 5 July 2023

improving blue food economies are essential to design a new approach for transitioning towards more responsible, comprehensive, exploitable production and consumption models with a positive impact for society and the environment (D'amato and Korhonen, 2021). Under this scenario, the aquafeed industry is not an exception, making considerable progress over the past 20 years in enhancing the efficiency of use of marine resources for feed formulation (Naylor et al., 2021). This was achieved by a combination of feed conversion ratio (FCR) improvement, reduction in fishmeal (FM) and fish oil (FO) inclusion levels, and increased use of FM from trimmings. However, the volume of wild-caught forage fish needed for supporting the increase in aquafeeds production (87.1 million tons in 2025) is unattainable based on current aquafeed formulations (Hua et al., 2019), and consequently, the dietary inclusion rates of FM and FO should diminish to guarantee and support the growth of this industry (Gephart and Golden, 2022). Among the vast variety of alternative FM sources, ranging from plant ingredients (e.g., soybean, corn gluten and rapeseed meals) to animal by-products (e.g., meat, bone and poultry meals, and trimmings of fish processing from both wild-caught and aquaculture sources) as reviewed by Hua et al. (2019) and Aragão et al. (2022) for their pros and cons, single cell proteins (SCP) have received special attention over the last years (Ritala et al., 2017; Glencross et al., 2020, 2023; Jones et al., 2020; Pereira et al., 2022).

Regardless of their origin (microalgae, yeast, fungi, or bacteria), SCP have important advantages over conventional protein ingredients as they have a good nutritional profile, require a shorter production time, less use of land, their production may not be affected by seasonal and climatic variations, and they may be produced from a wide array of cost-free substrates (Ritala et al., 2017; Sharif et al., 2021; Pereira et al., 2022). Among different SCP sources, bacterial SCP (BSCP) are one of the most popular and sustainable ones, since they are characterized by their high protein levels (up to 80% on a dry weight basis) in comparison to other sources of SCP, good amino acid profile that resembles that of FM, as well as for their content in vitamins, phospholipids, and other functional compounds (Pereira et al., 2022). In addition, bacteria may be produced using a wide range of substrates and nutrients ranging from industrial and agricultural residues to bioindustry by-products (Jones et al., 2020); thus, allowing the valorisation of waste streams and reducing the downstream costs related to industrial waste disposal (Ritala et al., 2017). As the former authors pointed out, selling residual biomass as feed is preferable to selling it as fertiliser. As reviewed by Jones et al. (2020), one of the main drawbacks of BSCP is their palatability which in some studies has been reported to compromise feed intake (FI), although this issue must be assessed case by case, since different sources of BSCP may differ in their nutritional properties (i.e., chemical composition from amino acids, nucleic acids, minerals, and vitamins) based on the bacterial strain used and their production technology (Sharif et al., 2021).

The objective of this study was to evaluate the suitability of BSCP from methanotrophic bacteria in compound diets for rainbow trout (*Oncorhynchus mykiss*) juveniles as an alternative ingredient to FM in order to test the hypothesis that this may be used as a safe and valuable alternative protein source for aquafeeds. For this purpose, we conducted a holistic approach on the use of this alternative protein ingredient based on the evaluation of classical key performance indicators (KPIs) related to growth and feed efficiency, as well as the impact of this ingredient on the nutritional quality of the fillet, the histological organization of the liver and intestine, and gut microbiota. The above-mentioned analyses were coupled to the assessment of whether the replacement of FM by BSCP might compromise the immune response of fish when challenged with a bacterial pathogen responsible of furunculosis in salmonids.

2. Material and methods

2.1. Ethics statement

All animal experimental procedures complied with the Guiding Principles for Biomedical Research Involving Animals (EU2010/63), the guidelines of the Spanish laws (law 32/2007 and RD 1201/2015) and were authorized by the Ethical Committee of the Institute for Research and Technology in Food and Agriculture (IRTA, Spain) for the use of laboratory animals (E-10/2020) and by the competent authority (Generalitat de Catalunya, Spain; FUE-2022-02675719).

2.2. Experimental diets

Five experimental diets with different levels of FM replacement (0, 25, 50, 75 and 100%) by a BSCP, corresponding to the nominal inclusion of 3.75, 7.5, 11.25 and 15% of BSCP in experimental diets, were formulated to evaluate the effect of this novel ingredient on rainbow trout juveniles (Table 1). The BSCP (Uniprotein® Aqua, Unibio, Roskilde, Denmark) was produced as described in the catalogue of feed materials (Commission Regulation (EU) 2022/1104 of 1 July 2022, 2023). Diets (crude protein: 42%, crude fat: 21%) were named as D1, D2, D3, D4 and D5 according to five increasing levels of FM replacement by the tested BSCP. The amino acid and fatty acid profiles of experimental diets are presented in Tables 2 and 3, respectively. The BSCP was produced by bacterial cultivation in a closed patented U-Loop® technology fermentation process (Unibio, Roskilde, Denmark). In this

Table 1
Formulation and proximate composition on dry matter basis of experimental diets replacing increased levels of fishmeal with bacterial single cell protein (BSCP, Uniprotein® Aqua, Unibio) tested in rainbow trout (*Oncorhynchus mykiss*) juveniles.

Ingredients, %	D1 (0% BSCP)	D2 (25% BSCP)	D3 (50% BSCP)	D4 (75% BSCP)	D5 (100% BSCP)
Fishmeal Super Prime	15.00	11.25	7.50	3.75	–
Uniprotein® Aqua*	–	3.75	7.50	11.25	15.00
Porcine blood meal	2.00	2.00	2.00	2.00	2.00
Poultry meal	5.00	5.00	5.00	5.00	5.00
Soy protein concentrate	10.00	10.00	10.00	10.00	10.00
Pea protein concentrate	4.00	4.00	4.00	4.00	4.00
Wheat gluten	8.00	8.20	8.40	8.60	8.80
Corn gluten meal	5.00	5.00	5.00	5.00	5.00
Soybean meal 48	5.00	5.00	5.00	5.00	5.00
Rapeseed meal	3.00	3.00	3.00	3.00	3.00
Sunflower meal	5.00	5.00	5.00	5.00	5.00
Wheat meal	11.00	10.60	10.30	9.90	9.50
Faba beans	5.00	5.00	5.00	5.00	5.00
Fish oil	5.50	5.50	5.50	5.50	5.50
Rapeseed oil	13.80	13.90	13.90	14.00	14.00
Vitamin & mineral premix	1.00	1.00	1.00	1.00	1.00
Betaine HCl	0.40	0.40	0.40	0.40	0.40
Antioxidant	0.20	0.20	0.20	0.20	0.20
Monocalcium phosphate	0.80	0.80	0.80	0.80	0.90
L-Lysine HCl 99%	0.30	0.40	0.50	0.60	0.70
Total	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Crude protein	42.1	41.9	42.0	42.1	41.9
Crude fat	21.1	21.0	21.1	21.0	21.0
Ash	6.5	6.2	5.9	5.5	5.3
Gross Energy (MJ/kg feed)	22.1	22.1	22.2	22.3	22.3

* Proximate composition of Uniprotein® Aqua (Unibio, Roskilde, Denmark): crude protein, 63.7%; crude fat, 8.8%; ash, 5.9%; water, 5.9%.

Table 2
Amino acid (% dry matter basis) content of experimental diets formulated with increased levels of fishmeal replaced with bacterial single cell protein (BSCP, Uniprotein® Aqua, Unibio).

Amino acids	D1 (0% BSCP)	D2 (25% BSCP)	D3 (50% BSCP)	D4 (75% BSCP)	D5 (100% BSCP)
Aspartic acid	4.10	4.01	4.16	4.05	3.91
Glutamic acid	9.70	9.49	9.80	9.57	9.41
Serine	2.44	2.39	2.49	2.40	2.35
Histidine	0.87	0.83	0.84	0.89	0.87
Glycine	2.45	2.32	2.45	2.30	2.21
Threonine	1.55	1.52	1.59	1.62	1.55
Arginine	2.82	2.74	2.89	2.91	2.87
Alanine	2.44	2.38	2.57	2.57	2.50
Taurine	<0.4	<0.4	<0.4	<0.4	<0.4
Tyrosine	1.58	1.57	1.69	1.77	1.73
Valine	1.79	1.77	1.89	1.97	1.89
Phenylalanine	2.10	2.07	2.18	2.21	2.08
Isoleucine	1.65	1.61	1.67	1.68	1.58
Leucine	3.68	3.59	3.74	3.69	3.53
Lysine	2.29	2.14	2.17	2.11	2.02
Proline	2.83	2.76	2.90	2.72	2.71
Cysteine	0.42	0.38	0.42	0.39	0.41
Methionine	1.13	1.14	1.15	1.10	1.11

process, methane derived from biogas (upgraded by carbon dioxide (CO_2) and impurities removal to obtain biomethane) was used as the sole carbon source and ammonia as the sole nitrogen source consumed by methanotrophic bacteria to produce new biomass.

Extruded diets were manufactured by Sparos Lda. (Portugal) as follows: all powder ingredients were mixed accordingly to the target formulation in a double-helix mixer (TGC Extrusion model 500 l, France) and ground (<400 mm) in a micropulverizer hammer mill (Hosokawa-Alpine model SH1, Germany). Diets (pellet size: 3.0 mm) were manufactured with a twin-screw extruder (Clextral model BC45, France) with a screw diameter of 55.5 mm. Extrusion conditions were: feeder rate (80–85 kg/h), screw speed (247–266 rpm), water addition in barrel 1 (345 ml/min), temperature in barrel 1 (32–34 °C), temperature in barrel 2 (59–62 °C) and temperature in barrel 3 (111–114 °C). Extruded pellets were dried in a vibrating fluid bed dryer (TGC Extrusion model DR100, France). After cooling, oils were added by vacuum coating (Dinnissen model PG-10VCLAB, Netherlands). Coating conditions were: pressure (700 mbar), spraying time under vacuum (approximately 90 s), and return to atmospheric pressure (120 s). Immediately after coating, diets (20 kg each) were packed and shipped to IRTA research facilities (la Ràpita, Spain) where the nutritional trial was conducted.

2.3. Fish and experimental design

Unvaccinated rainbow trout juveniles were purchased from Truchas de Leiza SL (Leiza, Navarra, Spain), and transported to IRTA research facilities in la Ràpita (Tarragona, Spain). Trouts were acclimated during two weeks in a 6 m^3 tank connected to a water recirculation system (IRTAmar™). After the end of the acclimation period, all fish ($N = 720$) were individually measured and randomly distributed in 20,500-l tanks ($n = 30$ fish per tank; 4 replicate tanks per diet) connected to an IRTAmar™ unit with mechanical, biological filtration and UV water treatment. At the beginning of the trial, rainbow trout juveniles weighted 11.3 ± 0.2 g (mean ± standard deviation) body weight (BW) and measured 9.0 ± 0.1 cm in standard length (SL).

The trial was divided in two different parts, a nutritional trial that lasted 83 days during which the impact of experimental diets was evaluated in terms of KPIs associated to growth and feed performance. This was followed by a challenge trial with a pathogenic bacterium (*Aeromonas salmonicida* subsp. *salmonicida*), the causative agent of furunculosis, to evaluate the immunological competence of fish fed the experimental diets (15 days).

Table 3
Fatty acid composition (μg fatty acid/mg lipid) of experimental diets formulated with increased levels of fishmeal replaced with bacterial single cell protein (BSCP, Uniprotein® Aqua, Unibio).

Fatty acids	D1 (0% BSCP)	D2 (25% BSCP)	D3 (50% BSCP)	D4 (75% BSCP)	D5 (100% BSCP)
14:0	10.57 ± 0.16	9.53 ± 1.80	6.50 ± 0.43	7.24 ± 0.39	6.68 ± 0.93
15:0	1.05 ± 0.07	1.20 ± 0.39	0.97 ± 0.02	0.98 ± 0.20	1.04 ± 0.10
16:0	74.00 ± 1.48	75.59 ± 3.59	73.56 ± 1.52	74.96 ± 2.49	80.63 ± 4.75
18:0	18.84 ± 0.03	18.46 ± 0.22	18.46 ± 0.81	17.68 ± 0.67	18.21 ± 0.78
22:0	3.43 ± 0.07	3.38 ± 0.04	3.29 ± 0.11	3.30 ± 0.01	3.39 ± 0.11
24:0	1.90 ± 0.12	2.02 ± 0.11	2.14 ± 0.02	2.11 ± 0.08	2.17 ± 0.16
Σ SFA	1.03 ± 0.05	1.00 ± 0.12	0.85 ± 0.09	0.87 ± 0.06	0.74 ± 0.08
16:1	19.41 ± 0.44	19.86 ± 0.98	19.70 ± 1.21	23.12 ± 2.33	26.7 ± 2.14
18:1n-9	338.13 ± 6.93	330.02 ± 9.78	334.70 ± 8.11	346.24 ± 3.98	329.83 ± 7.87
24:1	1.90 ± 0.04	1.76 ± 0.04	1.64 ± 0.09	1.47 ± 0.12	1.41 ± 0.20
Σ MUFA	329.83 ± 8.72	385.73 ± 11.57	392.97 ± 6.92	397.45 ± 8.31	389.89 ± 9.31
18:2n-6	139.33 ± 2.25	137.77 ± 1.49	135.01 ± 0.94	144.61 ± 1.35	144.32 ± 3.86
18:3n-6	1.30 ± 0.01	1.23 ± 0.11	1.23 ± 0.04	1.24 ± 0.03	1.19 ± 0.09
20:4n-6	3.65 ± 0.27	3.35 ± 0.01	2.61 ± 0.01	2.31 ± 0.01	2.26 ± 0.09
Σ n-6 PUFA	144.29 ± 2.53	142.36 ± 1.59	138.85 ± 0.92	148.16 ± 1.32	147.77 ± 3.95
18:3n-3	47.90 ± 1.18	47.58 ± 1.60	48.11 ± 2.99	50.71 ± 3.63	47.32 ± 1.97
18:4n-3	6.43 ± 0.10	6.10 ± 0.37	5.75 ± 0.52	5.97 ± 0.69	5.22 ± 0.09
20:4n-3	1.68 ± 0.07	1.81 ± 0.19	1.87 ± 0.10	1.78 ± 0.12	1.42 ± 0.09
22:5n-3	4.86 ± 0.18	4.50 ± 0.19	4.66 ± 0.71	4.85 ± 1.01	4.76 ± 1.13
22:6n-3	29.67 ± 0.80	27.19 ± 1.09	24.96 ± 1.95	22.28 ± 1.89	19.44 ± 1.32
Σ n-3 PUFA	130.63 ± 3.53	126.57 ± 4.88	124.42 ± 6.73	104.21 ± 7.48	114.36 ± 5.74
Σ PUFA	274.92 ± 6.06	268.93 ± 6.47	263.27 ± 6.82	272.78 ± 3.52	254.25 ± 9.99

During the nutritional and bacterial challenge trials, water quality parameters were daily monitored with following results: water temperature (15.2 ± 1.2 °C), dissolved oxygen (8.6 ± 0.4 mg/l) (OXI330, Crison Instruments, Spain) and pH (8.2 ± 0.1) (pHmeter 507, Crison Instruments). Ammonia (0.17 ± 0.14 mg NH_4^+/l) and nitrite (0.22 ± 0.2 mg NO_2^-/l) levels (HACH DR 900 Colorimeter, Hach Company, Spain) were weekly controlled. Photoperiod followed natural changes according to the season of the year (November to February). Diets were distributed using automatic feeders (ARVO-TEC T Drum 2000; Arvotec, Huutokosk, Finland) at a feeding rate of 3.0% of the stocked biomass, which approached apparent satiation. The daily feed ratio was evenly distributed in 2 meals at 08:00 and 13:00 h (at each meal, the corresponding feed ration was distributed during 1 h). Two hours after each meal, uneaten pellets were collected, dried overnight (120 °C) and weighted (g) for calculating daily feed intake values, while feed ration was adjusted to guarantee 10–15% of uneaten pellets; thus, confirming that fish were fed ad libitum.

2.4. Fish sampling, growth and feed efficiency KPIs

At the end of the nutritional trial, fish were fasted overnight and all fish in each tank were anesthetized with MS-222 (100 mg/l) and their BW_f and SL_f were individually measured. The following equations were used for calculating common KPIs associated to growth, body condition, and feed efficiency performance of fish fed experimental diets:

$$\text{Specific growth rate in body weight (SGR, \%BW/day)} = 100 \text{ x } \left[\left(ln\ BW_f – ln\ BW_i\right)/\text{days}\right];$$

$$\text{Fulton's condition factor (K)} = 100 \text{ x } \left(BW_f/SL_f^3\right);$$

$$\text{Hepatosomatic index (HSI, \%)} = 100 \text{ x } \left[\text{Liver weight (g)}/BW_f\ (g)\right];$$

$$\text{Perivisceral fat index (PVFI, \%)} = 100 \text{ x } \left[\text{Perivisceral fat weight (g)}/BW_f\ (g)\right];$$

$$\text{Feed conversion ratio (FCR)} = \text{Feed ingesta per tank (g)} / \text{Increase in fish biomass per tank (g)}.$$

In addition, eight fish per tank ($n = 32$ per diet) were euthanized with an overdose of anaesthetic (350 mg/l) for biochemical, histological, and gut microbiota analyses. In particular, the liver, right-side fillet and intestine of each sampled fish were dissected. Liver and anterior-mid intestinal samples (ca. 1 cm^2; $n = 4$ fish per tank) were fixed in 4% buffered formaldehyde (pH = 7.4) and stored at 4 °C until further processing for histological analyses. This region of the intestine was chosen because most of dietary lipids and amino acids are absorbed there (Krogdahl et al., 1999). The fillet and the rest of the dissected liver (n = 4 fish per tank) were frozen at −80 °C for biochemical analyses, whereas the intestine of four supplementary fish was frozen at −80 °C for microbiota analyses.

2.5. Proximate composition, fatty acid, and amino acid profiles

Experimental diets and fillets from rainbow trout juveniles were analysed for proximate composition and, fatty acid and amino acid profiles. For each tank, the fillet of each five sampled fish were grinded and pooled for biochemical analyses. In particular, crude protein was determined as total nitrogen (N x 6.25) using the Kjeldahl's method (AOAC, 2011). Total lipids were determined according to the Folch's method (Folch et al., 1957). Dry matter was obtained by weight loss after drying samples in a stove at 105 °C overnight. Ash content was estimated by incineration in a muffle oven at 450 °C for 6 h. In order to evaluate the fatty acid profile of feeds and rainbow trout fillets, methyl esters were extracted twice using isohexane: diethyl ether (1:1, v:v), and analysed by gas-liquid chromatography as described in Skalli et al. (2020). Results of fatty acid content were expressed as μg fatty acid/mg lipid. Amino acid analyses were conducted following AOAC (1984) guidelines.

To estimate the nutritional quality of the lipid fraction from trout fillets, three separate indexes were calculated from the percentages of saturated fatty acids (SFA; lauric C12:0, myristic C14:0, palmitic C16:0, and stearic C18:0 acids), monounsaturated fatty acids (MUFA; oleic C18:1 n-9 acid), and polyunsaturated fatty acids (PUFA; γ-linolenic C18:3 n-6, α-linolenic C18:3 n-3, eicosapentaenoic C20:5 n-3, and docosahexaenoic C22:6 n-3 acids) as described in Chen and Liu (2020). The above-mentioned indexes were calculated as follows:

$$\text{Atherogenicity index (AI)} = [(C12:0 + (4 \text{ x } C14:0) + C16:0)]/(\Sigma MUFA + \Sigma n-6 + \Sigma n-3);$$

$$\text{Thrombogenicity index (TI)} = (C14:0 + C16:0 + C18:0)/[(0.5 \text{ x } \Sigma MUFA) + (0.5 \text{ x } \Sigma n-6) + (3 \text{ x } \Sigma n-3) + (\Sigma n-3/\Sigma n-6)];$$

$$\text{Hypocholesterolemic/hypercholesterolemic fatty acids ratio (h/H)} = (C18:1n-9 + C18:3n-6 + C18:3n-3 + C20:5n-3 + C22:6n-3)/(C12:0 + C14:0 + C16:0).$$

2.6. Hepatic lipid peroxidation and activity of antioxidant stress enzymes

To evaluate the oxidative stress condition of the liver, a pool of four liver pieces (ca. 60 mg per liver) per tank were homogenized (IKA T25 digital ULTRA-TURRAX, IKA Works) in 5 volumes *v/w* of buffer (150 mM KCl, 1 mM EDTA, pH 7.4), centrifuged (9000 xg, 30 min, 4 °C), and the supernatant was collected for hepatic antioxidative status analysis. Glutathione reductase (GR) activity was measured at λ = 340 nm using NADPH (0.09 mM) as cofactor and disulphide glutathione (GSSG, 0.9 mM) as substrate (Carlberg and Mannervik, 1975). Catalase (CAT) activity was measured at λ = 240 nm, with H_2O_2 (50 mM) as substrate (Aebi, 1974). Glutathione peroxidase (GPX) activity was measured at λ = 340 nm using NADPH (0.292 mM) as cofactor, and glutathione reduced (GSH, 2.5 mM) and cumene hydroperoxide (CHP, 0.625 mM) as substrates (Günzler and Flohé, 1985). Lipid peroxidation (LPO) levels were measured using 1-methyl-2-phenylindole (10.3 mM) diluted in acetonitrile and methanol (1:3 *v/v*), using 1,1,3,3-tetramethoxypropane as malondialdehyde (MDA) precursor (Solé et al., 2004). Enzyme activities and lipid peroxidation levels were normalized to soluble protein (Bradford, 1976). All assays were read in triplicate at 25 °C using 96-well microplates (ref. 467,340, Thermo Scientific, Denmark) by UV/Vis spectrophotometer (Tecan Infinite M200 Plate Reader, Tecan, Switzerland) and analysed with the specific software Magellan™ (v6, Tecan).

2.7. Histological analyses

Fixed samples of the liver and anterior-mid intestine ($n = 4$ samples per tank, $n = 16$ per diet) were dehydrated in increasing-concentration of ethanol solutions, cleared with xylene (MYR STP 120, Especialidades Médicas Myr, Spain) and embedded in paraffin (MYR EC-350, Especialidades Médicas Myr). Serial sections (4 μm thickness) were obtained with a microtome (Leica RM2155, Leica Microsystems, Germany) and stained with haematoxylin and eosin with an automatic stainer (MYR MYREVA SS-30, Especialidades Médicas Myr). Sections were examined under a light microscope (Leica DM LB, Leica Microsystems) and photographed at 600 dpi (Olympus DP70 Digital Camera, Olympus Europa, Germany). Gut inflammation and accumulation of fat deposits in the hepatic parenchyma and intestinal samples were also evaluated semi-quantatively in both tissues as described by Ruiz et al. (2023) by means of a blinded and order-randomized histological examination by two different observers (Meyerholz and Beck, 2018).

2.8. Intestinal microbiota

Before fish sampling for gut microbial analyses, fish were fasted overnight to avoid allochthonous microbiota (Hao and Lee, 2004). Then, the intestinal tract just after the pyloric caeca of four fish per tank ($n = 16$ per diet) was dissected and aseptically opened lengthwise. The internal walls of the intestine were gently scraped with a round edge spatula and the collected content of each intestine was separately frozen at −80 °C until DNA extraction. Posteriorly, samples of ca. 150 mg of the intestinal scraped product were taken for DNA extraction following the manufacturer procedures for the DNeasy PowerSoil Pro Kit (ref. 47,016, QIAGEN, Germany). DNA concentration and purity were measured by

means of a Nanodrop-2000® spectrophotometer (Thermo Fisher Scientific, USA). The A_{260}/A_{280} absorbance ratios were higher than 1.80 and DNA concentrations were higher than 100 ng/μL.

The V3-V4 region of the 16S rRNA gene was amplified with the primers 341F (5'-CCTACGGGNGGCWGCAG-3′) and 805R (5'-GACTACHVGGGTATCTAATCC-3′) according to the 16S Metagenomic Sequencing Library Preparation guide (Illumina, 2013). First PCR was performed with Q5® High-Fidelity DNA Polymerase (ref. M0491L, New England BioLabs, USA) following the manufacturer's instructions: a polymerase activation and initial denaturation step of 30 s at 98 °C, followed by 25 cycles of 10 s at 98 °C, 30 s at 55 °C, 30 s at 72 °C, and a final extension of 2 min at 72 °C. Enough quantity and integrity of the amplified product was checked by means of a gel electrophoresis. After that, a second 8-cycle amplification was run to add the specific barcodes to the templates. The amplified region was sequenced on an Illumina MiSeq Platform (2 × 300 bp paired-end). Forward and reverse primers were removed from the raw reads with Cutadapt in QIIME2 Software (version 2021.11). The paired-ended reads were trimmed at a Phred score quality of 30 and merged in the R library *Dada2* (Callahan et al., 2016). The merged reads with an overlap length < 12 nucleotides or with >0 mismatches in the overlap region were discarded from the analysis. After identification and removal of chimeras (3.33% of the sequences), the taxonomic classification into amplicon sequence variants (ASVs) was carried out against SILVA database (v138.1) as a reference library. The ASVs with a bootstrapping confidence <80% were classified as unassigned (Smith et al., 2020). A total of 7,578,818 sequences clustering into 765 ASVs from 80 samples were generated. According to rarefaction curves (*vegan* library; Supplementary Fig. S1), sample depths were rarefied to the lowest number of total reads from the sample with the least number of reads (52,170) and normalized by total sum scaling (TSS), as recommended by McKnight et al. (2019).

2.9. Pathogenic bacterial challenge

To investigate whether the replacement of FM by increased levels of BSCP compromised the immune response of rainbow trout juveniles fed experimental diets, an in vivo bacterial challenge with the strain IRTA-17-44 of *A. salmonicida* subsp. *salmonicida* (courtesy of HIPRA culture collection, code: AS8074) was performed at the end of the nutritional period. For this purpose, bacterial suspensions were prepared from a stock stored in glycerol at −80 °C, defrosted and grown in TSA (23.0 ± 1.0 °C for 48 h) and prepared to an optical density ($OD_{550\ nm}$) of 1.23, corresponding to a density of 10^8 CFU/ml that was previously established by serial dilutions and plate counting. The bacterial suspension was 10-fold serially diluted in sterile PBS, to prepare the desired inoculum, which was confirmed by CFU's plate counting. Prior to the challenge trial, the lethal dose of 50% (LD50) for *A. salmonicida* subsp. *salmonicida* was determined using fish fed the control diet (D1). For this purpose, 30 rainbow trouts were injected intraperitoneally (IP) with 0.2 ml of three concentrations of the pathogenic bacterial 10^6, 10^7, and 10^8 CFU/ml (10 fish injected with each dose). Additionally, ten fish were injected with PBS as methodological control. The concentration of 10^7 CFU/ml was established as the nearest LD50 (data not shown).

For the challenge trial, twenty-four trout per dietary group weighting between 185 and 205 g were selected and transferred to forty 100 l tanks connected to an IRTAmar™ unit (8 fish per tank; stocking density = 20 kg/m^3; three tank replicates per diet) at IRTA's biosafety challenge room (Salomón et al., 2021). During the acclimation period (7 days) and during the bacterial challenge (15 days), fish were fed ad libitum with the same experimental diets used in the nutritional assay. After acclimation, fish were anesthetized, and IP injected with 0.2 ml of 2.3 × 10^7 CFU/ml of *A. salmonicida* subsp. *salmonicida*. Fish mortality occurring 12 h post-injection (hpi) was considered to be induced by the pathogen infection rather than handling stress, since no casualties were found in the control group injected with PBS. During the bacterial challenge, fish were supervised every 2 h, six times per day, including weekends. For ethical issues and to avoid unnecessary suffering, when the animals became moribund (i.e., loss of equilibrium, swollen abdomens, haemorrhaging in the anal area, and erratic swimming), they were euthanized with an overdose of MS-222. At the end of the trial, alive fish were sacrificed following the same procedure. Cause of death confirmation was determined by the recovery of the bacteria from all moribund animals (sampled head kidney platted on TSA), followed by specific PCR using *A. salmonicida* molecular tools as described in Salomón et al. (2021).

2.10. Statistical analyses

Data are presented as mean ± standard deviation (SD). Following confirmation of normality and homogeneity of variance, differences in key performance indicators among dietary groups differing in the level of BSCP were analysed with one-way analysis of variance (ANOVA). Data expressed as percentages were arcsine-transformed prior to the ANOVA analysis. Significant differences among experimental groups were decomposed in a linear relationship with the level of BSCP of the diet and a residual component (deviation) with polynomial orthogonal contrasts. The broken-line regression method was used to determine the breakpoint that represents the optimal replacement of FM by BSCP in diets for rainbow trout based on BW_f values. Regarding microbiota studies, statistical analyses and figures were performed with the R library *Microeco* as follows (Liu et al., 2021). The following alpha diversity indices were calculated: estimated richness was measured by means of the Chao1 and ACE indices, the latter considering ASVs with <10 reads per sample; and diversity, by means of the Shannon and Simpson indices, both based on species richness and evenness (Kim et al., 2017). Significant differences in alpha diversity ($P < 0.05$) were determined by the Kruskal-Wallis test, followed by Dunn's *post-hoc* test adjusted with false discovery rate (FDR) for multiple comparisons. Beta diversity was estimated based on the Bray-Curtis dissimilarity (Bray and Curtis, 1957), which was plotted by a principal coordinate analysis (PCoA) and the significant differences among experimental groups were checked with a permutational multivariate analysis of variance (PERMANOVA, $P < 0.05$). The test of Kruskal-Wallis and Dunn's post-test ($P \leq 0.05$) were used for assessing significant differences on the relative abundance of phyla ≥0.2% and genera ≥0.02% among groups.

3. Results

3.1. Survival, growth, somatic condition, and feed KPIs

Survival rates were not affected by dietary treatments at the end of the nutritional trial, with average survival values ranging between 92.5 and 97.5% (Table 4; $P > 0.05$). The replacement of FM by different levels of BSCP affected BW_f and SGR values that responded quadratically to the level of dietary BSCP levels (Table 4; $P < 0.05$). No significant differences in somatic growth (BW_f and SGR) were found between rainbow trout fed the control diet (D1) and their congeners fed the rest of experimental diets with different levels of FM replacement by BSCP (D2-D5) ($P > 0.05$); however, fish fed D3 (201.2 ± 6.1 g) grew better than those fed D5 (183.4 ± 4.5 g) ($P < 0.05$). When using quadratic linear regression, the optimal dietary FM replacement by the tested BSCP in terms of BW_f values was estimated as 41.7% as depicted in Fig. 1.

No differences in SL_f values were found among dietary groups, neither on selected somatic indexes like K, HSI and PVFI (Table 4; $P > 0.05$). When considering feed performance variables, no differences in FI were detected among dietary groups ($P > 0.05$), whereas the replacement of FM by the tested BSCP affected FCR values that linearly increased with increasing levels of dietary BSCP (Table 4; $P < 0.05$).

3.2. Proximate composition, fatty acid, and amino acid profiles

Results in terms of fillet proximal composition are shown in Table 5.

Table 4
Survival, growth performance (final body weight (BW_f), final standard length (SL_f) and specific growth rate (SGR), somatic condition indexes (Fulton's condition factor (K), hepatosomatic index (HIS), and perivisceral fat index (PVFI)), and feed efficiency parameters [feed intake (FI) and feed conversion ratio (FCR)] in rainbow trout (*Oncorhynchus mykiss*) fed experimental diets replacing increasing levels of fishmeal with bacterial single cell protein (BSCP, Uniprotein® Aqua, Unibio). Values are expressed as mean ± SD (n = 4 tanks).

	D1	D2	D3	D4	D5	Polynomial orthogonal contrast[a]	
	(0% BSCP)	(25% BSCP)	(50% BSCP)	(75% BSCP)	(100% BSCP)	Linear	Quadratic
Survival (%)	95.8 ± 3.2	93.3 ± 2.7	95.0 ± 1.9	92.5 ± 6.9	97.5 ± 5.0	–	–
BW_f (f)	191.3 ± 3.2	197.9 ± 2.01	201.2 ± 6.1	192.1 ± 9.7	183.4 ± 4.5	$P > 0.05$	$R^2 = 0.50$; $P < 0.05$
SL_f (cm)	22.5 ± 0.5	22.9 ± 0.2	23.2 ± 0.2	23.0 ± 0.3	22.6 ± 0.2	–	–
SGR (% BW/day)	3.41 ± 0.04	3.45 ± 0.02	3.47 ± 0.04	3.41 ± 0.06	3.36 ± 0.01	$P > 0.05$	$R^2 = 0.51$; $P < 0.05$
K	1.63 ± 0.04	1.64 ± 0.04	1.57 ± 0.06	1.56 ± 0.07	1.57 ± 0.05	–	–
HSI (%)	0.75 ± 0.07	0.85 ± 0.15	0.74 ± 0.13	0.79 ± 0.19	0.87 ± 0.08	–	–
PVFI (%)	1.38 ± 0.28	1.39 ± 0.25	1.42 ± 0.29	1.37 ± 0.23	1.32 ± 0.31	–	–
FI (g/tank)	4171 ± 125.1	4231 ± 135.2	4264 ± 93.4	4176 ± 117.6	4166 ± 119.6	–	–
FCR	0.81 ± 0.01	0.81 ± 0.02	0.79 ± 0.02	0.84 ± 0.02	0.83 ± 0.02	$R^2 = 0.22$; $P < 0.05$	$P > 0.05$

[a] If statistically significant differences were found among groups (ANOVA, $P < 0.05$), the polynomial orthogonal contrast was applied and the regression model that better fitted data was selected and the R-squared value indicated.

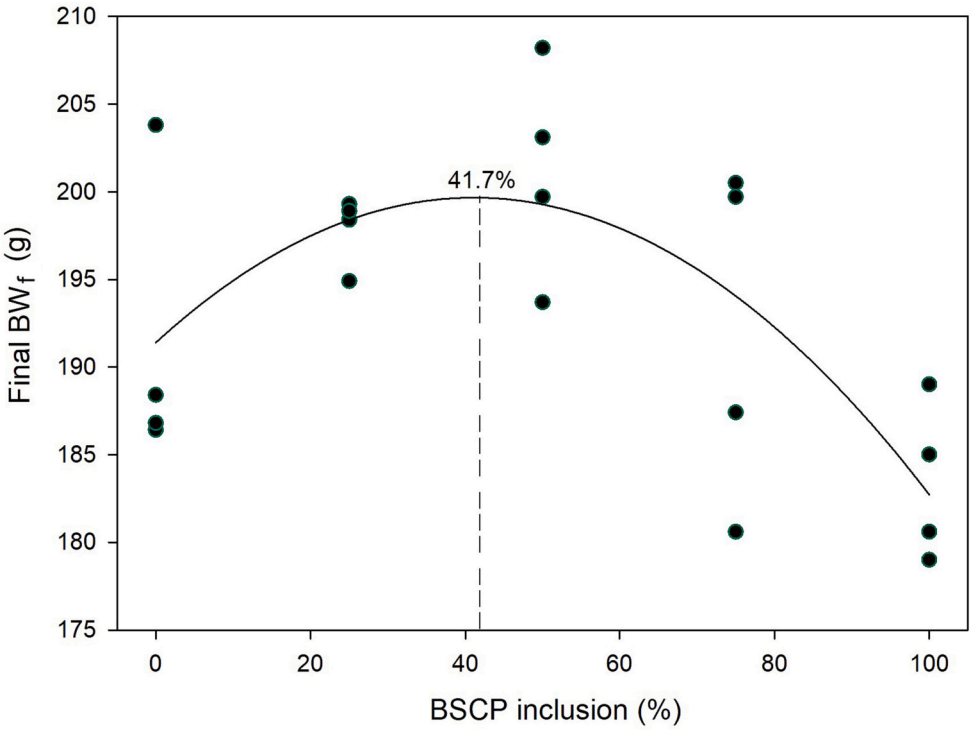


Fig. 1. Optimal fishmeal (FM) replacement by bacterial single cell protein (BSCP, Uniprotein® Aqua, Unibio) estimated according to final body weight (BW_f) of rainbow trout (*Oncorhynchus mykiss*) juveniles fed experimental diets ($n = 4$ per diet). The optimal dietary FM replacement by BSCP was estimated by quadratic regression ($y = y_0 + ax + bx^2$, where $y_0 = 191.4 \pm 3.04$, $a = 0.402 \pm 0.144$, $b = -0.0049 \pm 0.014$, $R = 0.71$, $P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Proximate composition (%) at dry matter basis of the rainbow trout (*Oncorhynchus mykiss*) fillet fed experimental diets replacing increased levels of fishmeal with bacterial single cell protein (BSCP, Uniprotein® Aqua, Unibio). Values are expressed as the mean ± SD ($n = 4$ tanks).

	D1	D2	D3	D4	D5	Polynomial orthogonal contrast[a]	
	(0% BSCP)	(25% BSCP)	(50% BSCP)	(75% BSCP)	(100% BSCP)	Linear	Quadratic
Crude protein	78.23 ± 1.02	75.54 ± 2.12	81.62 ± 1.69	82.42 ± 1.11	78.61 ± 2.04	$P < 0.05$, $R^2 = 0.66$	$P > 0.05$
Crude fat	17.55 ± 0.47	16.30 ± 2.75	14.80 ± 1.49	13.90 ± 1.39	17.32 ± 1.39	–	–
Carbohydrates	1.40 ± 0.10	1.47 ± 0.11	1.33 ± 0.12	1.29 ± 0.23	1.38 ± 0.11	–	–
Ash	1.71 ± 0.10	1.81 ± 0.10	2.01 ± 0.14	1.85 ± 0.33	1.83 ± 0.14	–	–

[a] If statistically significant differences were found among groups (ANOVA, $P < 0.05$), the polynomial orthogonal contrast was applied and the regression model that better fitted data was selected and the R-squared value indicated.

In particular, the replacement of increased levels of FM by the BSCP affected the crude protein in rainbow trout filet. The relationship between the content in crude protein of lipids and dietary inclusion of BSCP was linear (Table 5, $P < 0.05$). The highest crude protein content was measured in the fillets of fish fed D4 and the lowest values in those fillets from D1, D2 and D5, whereas fish from D3 had intermediate values. No differences were measured for crude lipid, carbohydrate, or ash contents in the fillets for any of the experimental diets ($P > 0.05$).

The fatty acid profile of the trout fillets fed different experimental diets was very conserved among dietary groups (Table 6). Fillets only

Table 6
Fatty acid composition (μg fatty acid/mg lipid) and health nutritional quality of the lipid fraction (atherogenicity and thrombogenicity indexes, and hypocholesterolemic to hypercholesterolemic fatty acids ratio) from rainbow trout (*Oncorhynchus mykiss*) fillets fed experimental diets replacing increased levels of fishmeal with bacterial single cell protein (BSCP, Uniprotein® Aqua, Unibio). Values are expressed as the mean ± SD (n = 4 tanks).

	D1	D2	D3	D4	D5	Polynomial orthogonal contrast[a]	
Fatty acids	(0% BSCP)	(25% BSCP)	(50% BSCP)	(75% BSCP)	(100% BSCP)	Linear	Quadratic
14:0	9.4 ± 1.9	8.8 ± 1.4	9.3 ± 0.7	9.6 ± 0.6	8.5 ± 1.2	–	–
15:0	3.6 ± 1.6	2.9 ± 1.3	2.2 ± 0.7	1.8 ± 0.2	2.1 ± 0.9	–	–
16:0	100.4 ± 3.8	99.7 ± 1.1	103.8 ± 1.3	105.3 ± 4.1	104.6 ± 2.7	–	–
18:0	25.9 ± 0.8	25.2 ± 1.6	26.3 ± 0.4	25.9 ± 0.7	25.4 ± 0.6	–	–
22:0	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.6	1.1 ± 0.1	–	–
24:0	1.3 ± 0.1	1.4 ± 0.3	1.5 ± 0.1	1.5 ± 0.1	1.4 ± 0.2	–	–
Σ SFA	141.8 ± 5.2	139.0 ± 0.8	145.2 ± 1.2	145.3 ± 0.2	143.1 ± 3.0	–	–
16:1	16.5 ± 1.4	18.2 ± 3.1	19.2 ± 0.5	19.4 ± 3.0	24.3 ± 2.5	$P < 0.05$; $R^2 = 0.50$	$P < 0.05$; $R^2 = 0.52$
18:1n-9	355.0 ± 9.4	341.5 ± 13.2	332.6 ± 11.9	326.9 ± 12.5	347.0 ± 10.6	–	–
24:1	1.7 ± 0.3	1.5 ± 0.2	1.3 ± 0.3	1.5 ± 0.1	1.3 ± 0.2	–	–
Σ MUFA	385.2 ± 10.0	372.5 ± 14.3	364.2 ± 11.8	358.8 ± 14.7	383.5 ± 12.0	$P > 0.05$	$P < 0.05$; $R^2 = 0.33$
18:2n-6	121.8 ± 3.5	120.5 ± 5.3	115.3 ± 1.8	114.4 ± 3.1	123.1 ± 4.3	–	–
18:3n-6	3.8 ± 0.8	3.2 ± 1.5	2.7 ± 0.7	3.3 ± 1.0	3.1 ± 0.9	–	–
20:4n-6	3.5 ± 0.1	3.4 ± 0.3	3.8 ± 0.4	4.0 ± 0.2	3.6 ± 0.3	–	–
Σ n-6 PUFA	129.1 ± 4.2	127.1 ± 5.9	121.8 ± 1.6	121.7 ± 3.8	129.8 ± 4.0	–	–
18:3n-3	26.0 ± 0.9	25.7 ± 2.1	25.7 ± 0.4	25.5 ± 0.6	26.7 ± 1.3	–	–
18:4n-3	2.2 ± 0.2	1.9 ± 0.3	1.8 ± 0.3	1.8 ± 0.2	1.8 ± 0.2	–	–
20:3n-3	2.1 ± 0.5	2.3 ± 0.1	2.3 ± 0.2	2.4 ± 0.3	2.1 ± 0.2	–	–
20:4n-3	2.0 ± 0.3	2.0 ± 0.2	1.9 ± 0.3	2.0 ± 0.1	1.8 ± 0.2	–	–
20:5n-3	23.7 ± 0.9	22.7 ± 1.8	23.0 ± 2.3	24.4 ± 0.6	21.8 ± 2.3	–	–
22:5n-3	5.5 ± 0.8	5.9 ± 0.7	5.9 ± 0.6	5.4 ± 1.3	5.5 ± 0.9	–	–
22:6n-3	78.4 ± 3.9	80.2 ± 9.3	84.6 ± 14.0	92.7 ± 9.6	77.4 ± 8.3	–	–
Σ n-3 PUFA	139.9 ± 5.6	140.6 ± 9.4	145.1 ± 17.0	154.2 ± 12.1	137.1 ± 8.4	–	–
Σ PUFA	269.0 ± 5.0	267.7 ± 8.6	267.0 ± 15.8	275.9 ± 8.4	266.8 ± 9.0	–	–
AI	0.21 ± 0.02	0.21 ± 0.01	0.23 ± 0.01	0.23 ± 0.01	0.21 ± 0.01	–	–
TI	0.20 ± 0.01	0.20 ± 0.01	0.21 ± 0.02	0.20 ± 0.01	0.21 ± 0.01	–	–
h/H	4.44 ± 0.21	4.36 ± 0.13	4.21 ± 0.11	4.12 ± 0.11	4.21 ± 0.15	–	–

Abbreviations: Σ SFA, total saturated fatty acids; Σ MSFA, total monounsaturated fatty acids; Σ n-6 PUFA, total polyunsaturated n-6 fatty acids; Σ n-3 PUFA, total polyunsaturated n-3 fatty acids; Σ PUFA, total polyunsaturated fatty acids; AI, atherogenicity index; TI, thrombogenicity index; h/H, hypocholesterolemic to hypercholesterolemic fatty acids ratio. [a] If statistically significant differences were found among groups (ANOVA, $P < 0.05$), the polynomial orthogonal contrast was applied and the regression model that better fitted data was selected and the R-squared value indicated.

differed in their content of palmitoleic (16:1), and in the content of total monounsaturated fatty acids (MSFA) ($P < 0.05$). In particular, the increase in FM replacement by BSCP increased the fillet content of 16:1, showing a quadratic relationship between both variables. Regarding total MUFA levels, the lowest levels were measured in the fillet fed the D4 in contrast to the D1 that showed the highest levels. The rest of dietary groups (D2, D3 and D5) showed intermediate levels. No differences were measured in terms of total n-6 and n-3 PUFA contents among experimental diets ($P > 0.05$). There were no differences in the AI, TI and H/H values of the fillet among experimental diets ($P > 0.05$). No differences were measured in the fillet's amino acid profile between the experimental diets (Table 7, $P > 0.05$).

3.3. Hepatic oxidative stress biomarkers

No differences were measured for total lipid peroxidation values in the liver of fish fed the different diets, nor in the activity of CAT and GR among experimental diets ($P > 0.05$). However, D5 had the lowest GPX activity level compared to D1 and D3 groups (Table 8; $P < 0.05$).

3.4. Histological organization of the liver and intestine

The histological organization of the hepatic parenchyma in rainbow trout juveniles was similar among the experimental diets. In general, the liver was composed of polyhedral hepatocytes with eosinophilic cytoplasm denoting glycogen and very low (5% of analysed livers) or absent (95% of analysed livers) accumulation of fat resulting in central nuclei (score of 1; Ruiz et al., 2023; Supplementary Fig. S1). Hepatocytes were arranged in packed anastomosed plates around the veins, and the hepatic parenchyma was surrounded by a thin capsule of fibroconnective tissue. No melanomacrophage centres nor infiltrations of lymphocytes were found among the experimental diets, which indicated the healthy hepatic condition of fish under different dietary conditions.

Regarding the intestine, there were no differences in the histological organization of the anterior-mid intestine among rainbow trout juveniles fed the experimental diets with increased levels of the tested BSCP replacing FM (score of 1; Ruiz et al., 2023; Supplementary Fig. S2). In all experimental groups, intestinal folds were lined by a simple columnar epithelium with basal nuclei, basophilic cytoplasm, and prominent microvilli. Scattered among enterocytes were abundant goblet cells producing and excreting into the gut lumen large amount of mucus (glycoproteins). Inflammatory disorders nor enteritis were not recorded in any of the experimental diets. The organization of the *lamina propria*, submucosa and tunica muscularis was normal. No lipid deposits were found either within enterocytes or in the vascular system.

3.5. Gut microbiota

The replacement of FM by BSCP did not modify the intestinal microbial community as no differences in the alpha diversity indices (Chao1, ACE, Shannon, and Simpson) were registered among dietary groups (Fig. 2a-d; $P > 0.05$). Furthermore, no differences in beta diversity (Bray-Curtis index) were measured in gut microbiota samples from different groups (Fig. 2e; PERMANOVA, $F = 0.448$, $R^2 = 0.023$, $P = 0.839$). Regarding the microbial composition at the phylum and genus levels, there were no differences among dietary groups (Fig. 3a and b, respectively), with Firmicutes, Desulfobacteria and Proteobacteria being the most abundant phyla. As previously stated, there was a large conservation in the microbial composition among dietary groups, being 96.9% of repeated sequences shared between experimental diets, as indicated by the Venn diagram (Supplementary Fig. S3).

Table 7
Amino acid composition (% of total amino acids) of rainbow trout (*Oncorhynchus mykiss*) fillets fed experimental diets replacing graded levels of fishmeal with bacterial single cell protein (BSCP, Uniprotein® Aqua, Unibio). Values are expressed as the mean ± SD (n = 4 tanks).

Amino acids	D1 (0% BSCP)	D2 (25% BSCP)	D3 (50% BSCP)	D4 (75% BSCP)	D5 (100% BSCP)
Aspartic acid	8.27 ± 0.29	8.59 ± 0.28	8.63 ± 0.13	8.75 ± 0.21	8.36 ± 0.11
Glutamic acid	11.12 ± 0.29	11.72 ± 0.53	11.73 ± 0.44	11.77 ± 0.34	11.49 ± 0.41
Serine	3.13 ± 0.03	3.29 ± 0.13	3.23 ± 0.09	3.29 ± 0.09	3.11 ± 0,15
Histidine	1.82 ± 0.07	1.83 ± 0.07	1.85 ± 0.07	1.92 ± 0.07	1.86 ± 0.06
Glycine	3.78 ± 0.10	3.80 ± 0.12	3.82 ± 0.16	3.84 ± 0.05	3.76 ± 0.08
Threonine	3.46 ± 0.06	3.51 ± 0.13	3.53 ± 0.15	3.61 ± 0.09	3.46 ± 0.09
Arginine	5.44 ± 0.11	5.43 ± 0.25	5.45 ± 0.19	5.59 ± 0.12	5.32 ± 0.22
Alanine	4.80 ± 0.08	4.89 ± 0.19	4.88 ± 0.09	4.98 ± 0.06	4.76 ± 0.14
Taurine	<0.4	<0.4	<0.4	<0.4	<0.4
Tyrosine	2.72 ± 0.06	2.80 ± 0.11	2.83 ± 0.06	2.88 ± 0.02	2.75 ± 0.08
Valine	3.77 ± 0.08	3.85 ± 0.16	3.90 ± 0.11	4.01 ± 0.07	3.82 ± 0.10
Phenylalanine	3.30 ± 0.05	3.33 ± 0.14	3.37 ± 0.13	3.43 ± 0.08	3.27 ± 0.08
Isoleucine	3.67 ± 0.10	3.75 ± 0.16	3.83 ± 0.09	3.91 ± 0.04	3.73 ± 0.11
Leucine	6.26 ± 0.12	6.44 ± 0.22	6.48 ± 0.17	6.60 ± 0.07	6.33 ± 0.18
Lysine	6.01 ± 0.23	5.85 ± 0.35	5.71 ± 0.41	6.11 ± 0.47	5.79 ± 0.43
Proline	2.04 ± 0.15	2.22 ± 0.12	2.24 ± 0.16	2.18 ± 0.11	2.08 ± 0.14
Cysteine	0.56 ± 0.01	0.54 ± 0,06	0.57 ± 0.04	0.55 ± 0.06	0.53 ± 0.04
Methionine	2.14 ± 0.12	2.16 ± 0.12	2.20 ± 0.09	2.29 ± 0.15	2.19 ± 0.13

3.6. Bacterial challenge

At the end of the nutritional study, a bacterial challenge with the causative agent of furunculosis was conducted to evaluate whether the replacement of FM by graded levels of tested BSCP might compromise the immune response of in fish. The highest cumulative survival rates were measured for D2 and D3 in comparison to D1, D4 and D5 groups (Fig. 4; $P < 0.05$). Cumulative survival was not linearly nor quadratically correlated to dietary BSCP levels as the polynomial orthogonal contrast indicated ($P > 0.05$).

4. Discussion

Testing and validating new aquafeed ingredients, especially those targeting the FM substitution, should not be conducted just by the assessment of conventional KPIs associated to growth and feed efficiency, since different studies have reported that FM replacement may result in adverse effects in fish physiology, metabolism, and health (Aragão et al., 2022). Thus, we run a holistic study in rainbow trout juveniles to evaluate the optimal FM replacement levels by a BSCP obtained by methanotrophic bacteria (Uniprotein® Aqua, Unibio) coupled with an integrative approach of several parameters related to i) tissue health and condition, ii) the nutritional profile of the fillet in terms of proximate, fatty acid and amino acid composition, iii) gut microbiota composition and diversity, and iv) disease resistance in front of a bacterial pathogen (*A. salmonicida* subsp. *salmonicida*) by means of an in vivo challenge. Under current experimental conditions, the tested BSCP could replace up to 75% of FM (11.25% BSCP) without compromising growth and feed efficiency KPI values. Remarkably, rainbow trout juveniles fed D3 (50% FM replacement by BSCP) grew better and had lower FCR values compared to the control group (D1). These results may be explained by the high content of nucleotides, nucleic acids and/or bioactive compounds in BSCP (Pereira et al., 2022; Sharif et al., 2021) that may promote fish growth. However, the effect of FM replacement by tested BSCP on growth performance was not linear; in fact, it displayed a quadratic response. Thus, considering this information, we estimated that the optimal level of FM replacement by Uniprotein® Aqua was ca. 42%. Results from our study when considering quadratic regression felt within the range of optimal FM replacement by BSCP values reported in the literature, although direct comparisons are not advisable since BSCP differing in their origin and production system do also differ in their nutritional profile (50–80% crude protein and variable amino acid profile) and palatable properties (Sharif et al., 2021; Pereira et al., 2022). Regarding salmonid species, in Atlantic salmon (*Salmo salar*), FM was replaced up to 52% with BSCP obtained from *Methylococcus capsulatus* without compromising growth performance, although the same BSCP was only successful to replace FM up to 38% when tested in rainbow trout (Overland et al., 2010). Another study in Atlantic salmon showed that BSCP (BioProtein®, Norferm AS, Stavanger, Norway) may replace up to 36% FM in compound diets considering growth and feed efficiency results, even though nutrient digestibility was reduced (Aas et al., 2006a). Similarly, a recent study by Zamani et al. (2020) found in rainbow trout fry that a BSCP (PL68®, Intraco Ltd., Antwerp, Belgium) obtained as a by-product of monosodium L-glutamic acid production by means of microbial fermentation of vegetal raw materials, could replace up to 52% of FM when growth data was considered. However, the former authors showed that this replacement could only be of 46.9% when muscle docosahexaenoic acid (DHA) content was used for calculating the optimal FM replacement levels. Regarding the use of BSCP in marine species, Chen et al. (2019) was able to replace FM with BSCP obtained from *Clostridium autoethanogenum* up to 58.2% without remarkable differences in growth performance in blackhead sea bream (*Acanthopagrus schlegelii*) fry, although

Table 8
Lipid peroxidation (μM MDA/mg protein) and activity of antioxidant enzymes (nmol/min/mg protein) in rainbow trout (*Oncorhynchus mykiss*) fed experimental diets replacing graded levels of fishmeal with bacterial single cell protein (BSCP, Uniprotein® Aqua, Unibio).

	D1 (0% BSCP)	D2 (25% BSCP)	D3 (50% BSCP)	D4 (75% BSCP)	D5 (100% BSCP)	Polynomial orthogonal contrast[a] Linear	Quadratic
Lipid peroxidation	0.15 ± 0.02	0.13 ± 0.02	0.15 ± 0.01	0.13 ± 0.02	0.13 ± 0.02	–	–
CAT	436.8 ± 73.0	330.8 ± 59.0	446.2 ± 117.7	465.5 ± 90.1	326.4 ± 72.5	–	–
GR	7.0 ± 1.4	7.46 ± 1.3	6.9 ± 1.5	6.4 ± 0.9	7.7 ± 0.6	–	–
GPX	48.8 ± 7.9	44.2 ± 4.8	49.0 ± 6.1	42.1 ± 3.6	35.7 ± 4.1	$P < 0.05$; $R^2 = 0.55$	$P > 0.05$

[a] If statistically significant differences were found among groups (ANOVA, $P < 0.05$), the polynomial orthogonal contrast was applied and the regression model that better fitted data was selected and the R-squared value indicated.

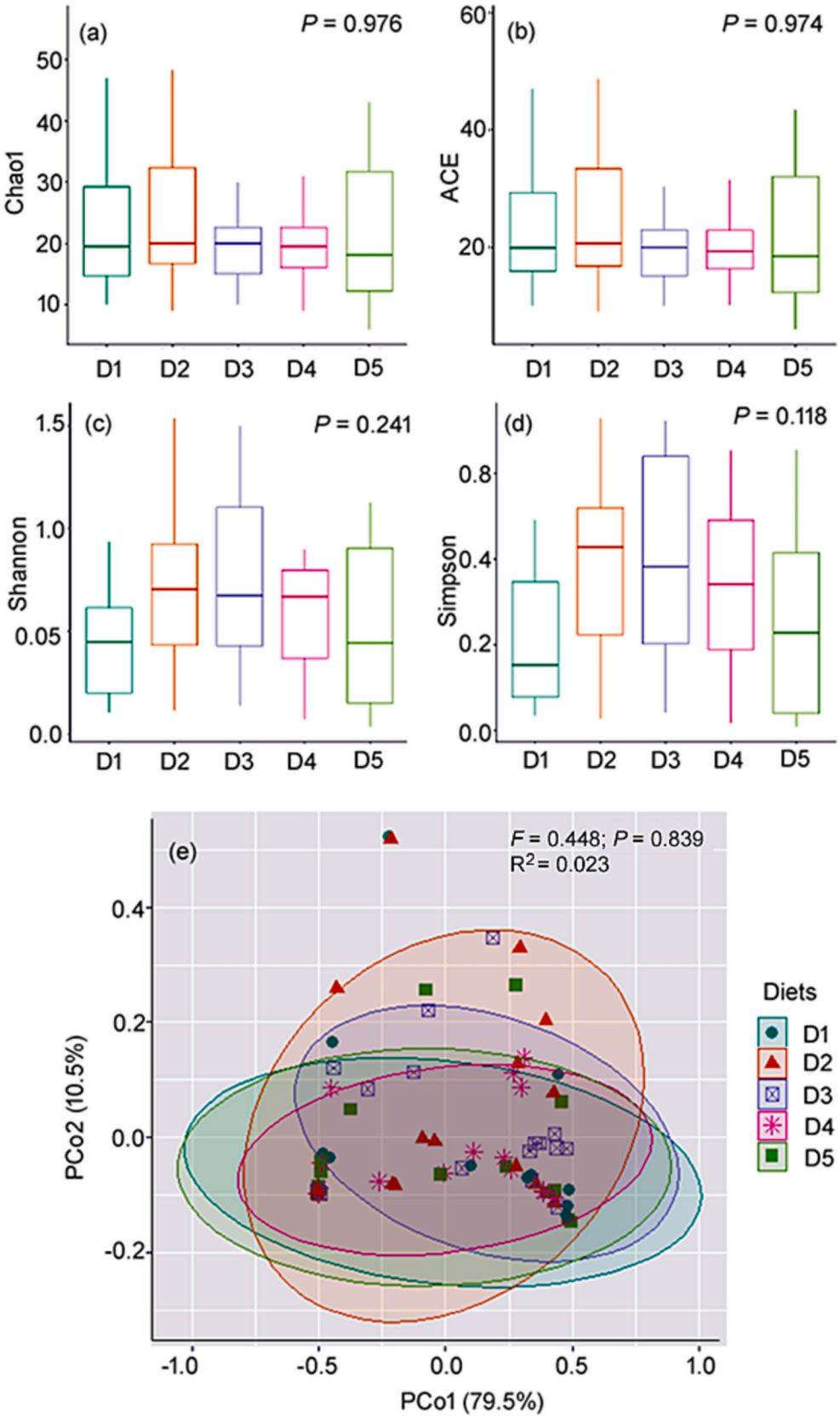


Fig. 2. Box-plots of alpha diversity metrics (a, Chao1; b, ACE; c, Shannon; d, Simpson indexes) and Principal Coordinate Analysis (PCoA) for beta diversity (Bray-Curtis index) of the intestinal microbiota composition from rainbow trout (*Oncorhynchus mykiss*) fillets fed experimental diets replacing increased levels of fishmeal with bacterial single cell protein (BSCP, Uniprotein® Aqua, Unibio) (D1: 0%, D2: 25%, D3: 50%, D4: 75% and D5: 100% BSCP; dietary contents of BSCP were 0, 3.75, 7.5, 11.25 and 15%, respectively). Values are expressed as the mean ± SD (n = 16 fish per experimental diet). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

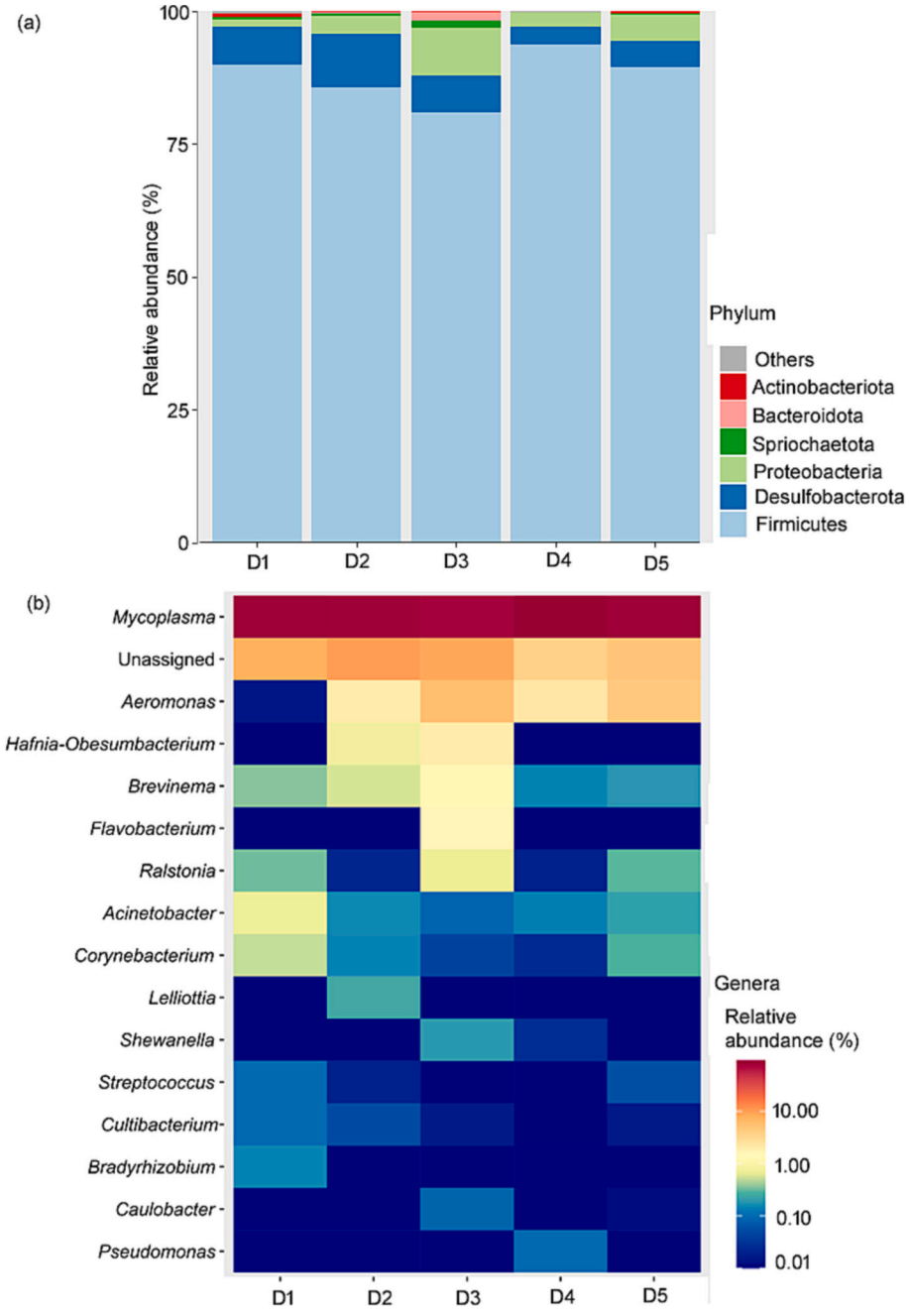


Fig. 3. Microbiota composition at phylum (a) and genus (b) levels of the intestine in rainbow trout (*Oncorhynchus mykiss*) fed experimental diets replacing increased levels of fishmeal with bacterial single cell protein (BSCP, Uniprotein® Aqua, Unibio) (D1: 0%, D2: 25%, D3: 50%, D4: 75% and D5: 100% BSCP; dietary contents of BSCP were 0, 3.75, 7.5, 11.25 and 15%, respectively). Those taxa with an average relative abundance of <0.2% were classified as "Others" at the level of phylum and not represented at the level of genus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

diets containing BSCP were supplemented with DL-methinionine and L-arginine to mimic the amino acid profile of the control diet. In yellowtail (*Seriola quinqueradiata*), FM might be replaced by BSCP obtained from *Methylococcus capsulatus* (FeedKind®, Calysta, Inc., CA, USA) at 30% without compromising growth performance and FCR values (Biswas et al., 2020). Furthermore, BSCPs have also been used as an ingredient for replacing vegetal protein sources (i.e., soybean meal, soy protein concentrate, and corn gluten). In particular, Marchi et al. (2023) reported that BSCP from *Corynebacterium glutamicum* might replace soy protein concentrate and corn gluten up to 20% in diets for gilthead sea bream (*Sparus aurata*) without negatively impacting fish growth and protein utilization. Similar results were described in Nile tilapia (*Oreochromis niloticus*) in which increasing levels of *C. autoethanogenum* meal could replace up to 20% of soybean meal (Maulu et al., 2021).Under current experimental conditions, the replacement of FM by the tested BSCP obtained from methanotrophic bacteria did not affect FI in rainbow trout, whereas it improved FCR in specimens fed D3 diet. Regarding FI, several authors have reported contradictory results depending on the source of BSCP tested. In particular, the content of free purines and other flavour compounds in BSCP might negatively affect feed palatability, especially in diets with high levels of FM replacement, and consequently, this may impair FI (Rumsey et al., 1992). In this sense, several authors have reported a FI reduction in fish fed diets containing a wide range of BSCP levels (10–58.2%) (Kiessling and Askbrandt, 1993; Hardy et al., 2018; Chen et al., 2019). However, similar to present results other studies have reported no significant effects of FM replacement by BSCP on FI (Berge et al., 2005; Aas et al., 2006a, 2006b; Zamani et al., 2020; Marchi et al., 2023; Glencross et al., 2023). In the present study, the improvement of FCR in rainbow trout fed D3 (50% FM replacement by BSCP) might be associated to a slight but not significant increase in FI values, even though the presence of bioactive compounds with growth-promoting effects (e.g., protein

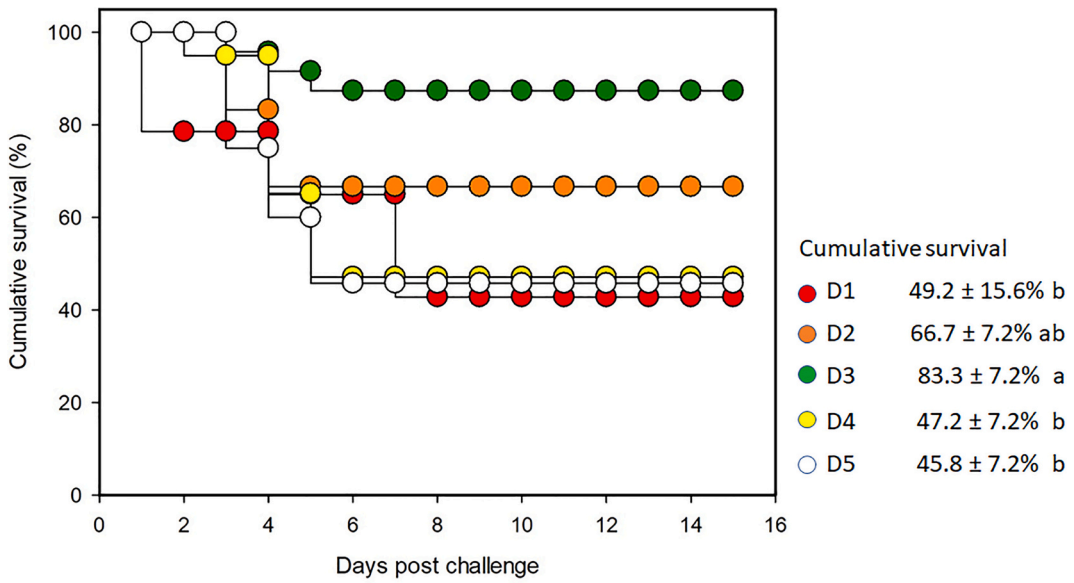


Fig. 4. Results of the bacterial challenge (*Aeromonas salmonicida* subsp. *salmonicida*; intraperitoneal injection dose of 2.3×10^7 CFU/ml) conducted in rainbow trout (*Oncorhynchus mykiss*) fed experimental diets replacing increased levels of fishmeal with bacterial single cell protein (BSCP, Uniprotein® Aqua, Unibio) (D1: 0%, D2: 25%, D3: 50%, D4: 75% and D5: 100% BSCP; dietary contents of BSCP were 0, 3.75, 7.5, 11.25 and 15%, respectively). Fish ($n = 8$ per tank) were intraperitoneally injected with 2 ml of 2.3×10^7 CFU/ml. Cumulative mortality corresponds to mean ± SD (three replicate tanks per experimental diet). Different letters indicate significant differences in cumulative survival among experiments diets (ANOVA, $P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hydrolysates, nucleotides, nucleic acids among others) in the tested BSCP might also be responsible for these results (Glencross et al., 2020; Hossain et al., 2020; Sharif et al., 2021).

Regarding somatic indices, no differences were found in terms of K, HSI and PVFI in rainbow trout fed increased levels of BSCP, which indicated no dietary effect on the assimilation and distribution of nutrients within the organism (Marchi et al., 2023). In particular, fish fed D3 and D4 (50 and 75% FM replacement by BSCP) showed slightly higher protein content than the rest of dietary groups, results that may be due to small differences in BW_f among them. Regardless of the above-mentioned small changes in fillet's protein content coupled to the absence in changes in their amino acid composition might indicated that protein synthesis and retention was not affected by dietary regimes (Carter et al., 2000). This is of special relevance since amino acid regulate key metabolic processes in the organism that are critical for the maintenance, growth, reproduction, and immune responses in vertebrates. In this sense, dietary amino acid imbalances may cause increased amino acid oxidation and lead to decreased feed conversion efficiencies as well as changes in feed intake (Li et al., 2008). Thus, present results showed that the amino acid profile of the tested BSCP and formulated feeds with graded replacement levels of FM met the nutritional requirements of rainbow trout juveniles, representing a good quality alternative in terms of both crude protein levels and amino acid profile.

Although no differences in fillet's crude fat content were found among dietary groups and the fatty acid profile of the fillet was quite conserved, increasing levels of BSCP resulted in higher content on palmitoleic (C16:1) acid while lower contents on oleic (C18:1n-9) acid and on total MSFA were found when increasing the levels of BSCP up to 75%. The positive linear regression between the amount in 16:1 in fillets and levels of FM replacement by BSCP ($r = 0.91$; $P < 0.05$) may indicate that this alternative ingredient might be rich in this MSFA. However, such pattern was not found for oleic acid and total monosaturated fatty acids. Remarkably, increasing levels of BSCP did not alter the n-3 and n-6 PUFA profiles of the fillet, which guarantees the fillets nutritional profile of rainbow trout fed with increased levels of BSCP. The nutritional indices for assessing fatty acids, FM replacement by BSCP did not change the atherogenicity, the thrombogenic nor the hypocholesterolemic to hypercholesterolemic fatty acids ratios, which under present experimental conditions were within the range of values reported for other fish species (Chen and Liu, 2020). These results indicated that the fatty acid profile from fillets of rainbow trout fed BSCP-based diets does not represent any potential harm for the consumer with regard to cardiovascular diseases (Chen and Liu, 2020).

The histological analysis of the liver and intestine provides a reliable assessment of the nutritional condition of fish under different dietary conditions, since both tissues accurately reflect any physiological disorder originated from unbalanced dietary conditions (Gisbert et al., 2008). Under present experimental conditions, no remarkable changes in the histological organization of the anterior-mid intestine and hepatic parenchyma were measured between the control diet and those with increased levels of BSCP, which indicated that the tested alternative protein source obtained from methanotrophic bacteria was innocuous when compared to FM. Good tissue condition evidenced by histological data were supported by biochemical analyses related to lipid peroxidation levels and activity of antioxidative stress enzymes like CAT, GR and GPX. In particular, no differences in lipid peroxidation values were observed in liver samples from fish fed BSCP diets in comparison to the control diet, which showed that BSCP did not alter the cellular redox state and the oxidative status of the liver. Considering the activity of oxidative stress enzymes, the increasing inclusion of BSCP in the tested diets did not show a hepatic response to oxidative stress nor toxicity in fish as CAT, one of the major antioxidant biomarkers, was similar among groups. In the same line, GR activity was not altered by the FM replacement, that altogether suggest a normal sustainability of the reactive oxygen species (ROS) concentration in hepatic cells (Harasgama et al., 2020) and metabolism (Wu et al., 2004). However, the enzymes tagged as antioxidant protectors also participate in important metabolic functions such as those involved in arachidonic acid, leukotriene, prostaglandin, and/or in estrogen metabolism (Garner et al., 1999; Wang et al., 2021). Thus, it was not surprising to observe that some of these enzyme activities varied among experimental diets even though others did not. In the case of GPX, values of this enzyme from D5 showed differences in its activity compared to specimens from D1 and D3, differences that may be attributed to changes in somatic growth

rather than liver health and condition (Lei et al., 2007), since this enzyme participates in the detoxification of ROS produced by metabolism (Wu et al., 2004).

Different studies have reported that the autochthonous microbiota of farmed rainbow trout is dominated a phylum level by Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Fusobacteria (Kononova et al., 2019; Rimoldi et al., 2021). In the present study, the remarkably high relative abundance of Firmicutes was due to the prevalent dominance of *Mycoplasma* in all dietary groups, which is a predominant genus generally found in the intestinal digesta and mucosa of salmonids, like Arctic char (*Salvelinus alpinus*; Knobloch et al., 2022), Atlantic salmon (Llewellyn et al., 2016) and rainbow trout (Huyben et al., 2018; Sibinga et al., 2022). Several of these studies have suggested that *Mycoplasma* has an important role in nutrient digestion and fermentation, producing metabolites that may benefit fish health. In particular, Rasmussen et al. (2021) have proposed a mutualistic relationship between *Mycoplasma* and salmonids, in which the host could take advantage of the potential of *Mycoplasma* to promote gut health and favour growth performance in the aquaculture industry. Thus, the high relative abundance of this genus reported in the current study may be interpreted as a positive aspect in terms of nutrient utilization and fish growth, which indicated that none of the tested experimental diets compromised the fish intestinal health status. On the other hand, fish gut microbiota might be modulated by multiple biotic factors, being the diet one of the most studied ones (see review in Ringø et al., 2016). In particular, several studies have shown that the replacement of FM by alternative protein sources like soybean meal or insect meal in rainbow trout resulted in changes in gut microbiota composition and diversity (Bruni et al., 2018; Merrifield et al., 2009; Kononova et al., 2019; Terova et al., 2019; Rimoldi et al., 2021). Similarly, a recent study from Marchi et al. (2023) evaluating different levels of FM replacement by BSCP obtained from *C. glutamicum* revealed that higher BSCP levels increased microbial diversity in gilthead sea bream. Contrary to other alternative protein sources used in rainbow trout aquafeeds and recent data provided by Marchi et al. (2023) in gilthead sea bream, under present experimental conditions, the replacement of FM by the tested BSCP did not modify the intestinal microbial community in rainbow trout. These findings are of relevance since diets formulated with moderate and/or high levels of FM are generally considered as reference ones in nutritional studies, as well as their effects on fish condition, physiology, and health, including the gut microbiota composition in fish fed those diets (Ringø et al., 2016; Vatsos, 2017). Thus, the replacement of FM by the tested BSCP obtained from methanotrophic bacteria did not cause an imbalance or dysbiosis in the intestinal microbiota in rainbow trout, which may be considered a good result due to its similarity to the control diet. These results can also be supported by our histological findings from the proximal intestine, since no inflammation or nutritional disorders (i.e., steatosis, epithelial desquamation) were found in the intestinal mucosa in fish fed all diets with different levels of BSCP inclusion.

One of the most interesting results from this study was the fact that replacing FM by the tested BSCP at 50% (D3) enhanced disease resistance in rainbow trout juveniles when challenged with *A. salmonicida* subsp. *salmonicida*, the causative agent of furunculosis. Although several studies have shown that some SCP obtained from yeast and/or microalgae may have some immunomodulatory benefits on the host, limited information is available on BSCP regarding this issue (Glencross et al., 2020). This unexpected result may be due to the presence of nucleotides and bioactive compounds in the tested BSCP (Pereira et al., 2022), although further research is needed for their proper characterization and quantification in this alternative protein source for aquafeeds, as well as its mode of action on host's immune system. Present results showed that using BSCP from methanotrophic bacteria may be a good and sustainable strategy against furunculosis in salmonids, as well as in marine species like European sea bass (*Dicentrarchus labrax*) suffering from this bacterial infectious disease (Fernández-Álvarez et al., 2016).

5. Conclusions

Results from the present study indicated that BSCP from methanotrophic bacteria produced in a closed patented U-Loop® technology fermentation process using methane derived from biogas and ammonia as the sole carbon and ammonia sources, can be incorporated into compound aquafeeds to reduce the use of traditional FM. Based on growth performance, FCR results, and cumulative survival after challenging rainbow trout juveniles with *A. salmonicida* subsp. *salmonicida*, we recommend to replace FM by the tested BSCP up to 50%. Furthermore, current results indicated that the tested BSCP from methanotrophic bacteria is a safe and functional alternative protein source with no negative effects on FI, liver and intestine condition, antioxidant enzymes and gut microbiota. The use of this BSCP promotes the conversion and reuse of industrial wastes, reducing the emission of methane, and has an enormous potential for its use in aquaculture within a sustainable and circular aquafeed industry.

CRediT authorship contribution statement

Alberto Ruiz: Methodology, Formal analysis, Visualization, Writing – review & editing. **Ignasi Sanahuja:** Methodology, Formal analysis, Visualization, Writing – review & editing. **Nana W. Thorringer:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Julie Lynegaard:** Writing – review & editing. **Eleni Ntokou:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Dolors Furones:** Methodology, Formal analysis, Writing – review & editing. **Enric Gisbert:** Conceptualization, Methodology, Formal analysis, Visualization, Supervision, Project administration, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

N.W. Thorringer, J. Lynegaard and E. Ntokou are current UNIBIO (Denmark) employers. The funders had no role in the design of the study; in the collection, statistical analyses, or interpretation of data.

Data availability

Data will be made available on request.

Acknowledgments

This work has been financed through the VALUEWASTE project that has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No. 818312. Alberto Ruiz was supported by a predoctoral grant (PRE2019-091259) and Ignasi Sanahuja was supported by a Juan de la Cierva postdoctoral contract (FJC2020-043933-I). Authors would like to express their gratitude to Magda Monllaó, Esteban Hernandez, Marta Sastre, Maria Curto, Olga Bellot and Edgar Bertomeu, staff from IRTA la Ràpita, for all their dedication and valuable assistance during the execution of the experiment.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.739861.

References

Aas, T.S., Grisdale-Helland, B., Terjesen, B.F., Helland, S.J., 2006a. Improved growth and nutrient utilisation in Atlantic salmon (*Salmo salar*) fed diets containing a bacterial protein meal. Aquaculture 259, 365–376. https://doi.org/10.1016/j.aquaculture.2006.05.032.

Aas, T.S., Hatlen, B., Grisdale-Helland, B., Terjesen, B.F., Bakke-McKellep, A.M., Helland, S.J., 2006b. Effects of diets containing a bacterial protein meal on growth and feed utilisation in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 261, 357–368. https://doi.org/10.1016/j.aquaculture.2006.07.033.

Aebi, H., 1974. Catalase. In: Bergmeyer, H.V. (Ed.), Methods in Enzymatic Analysis, 2. Academic Press, New York, NY, USA, pp. 674–684.

AOAC, 1984. Official methods of analysis. In: Amino Acid Analysis. Sample Hydrolysis, 43, p. 263.

AOAC, 2011. Official methods of analysis. In: Official Methods of Analysis of AOAC International, 18th ed. AOAC, Gaithersburg, MD (Revision 4).

Aragão, C., Gonçalves, A.T., Costas, B., Azeredo, R., Xavier, M.J., Engrola, S., 2022. Alternative proteins for fish diets: implications beyond growth. Animals 12, 1211. https://doi.org/10.3390/ani12091211.

Berge, G.M., Baeverfjord, G., Skrede, A., Storebakken, T., 2005. Bacterial protein grown on natural gas as protein source in diets for Atlantic salmon, *Salmo salar*, in saltwater. Aquaculture 244, 233–240. https://doi.org/10.1016/j.aquaculture.2004.11.017.

Biswas, A., Takakuwa, F., Yamada, S., Matsuda, A., Saville, R.M., LeBlanc, A., Silverman, J.A., Sato, N., Tanaka, H., 2020. Methanotroph (*Methylococcus capsulatus*, Bath) bacteria meal as an alternative protein source for Japanese yellowtail, *Seriola quinqueradiata*. Aquaculture 529, 735700. https://doi.org/10.1016/j.aquaculture.2020.735700.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bray, J.R., Curtis, J.T., 1957. An ordination of the upland forest communities of southern Wisconsin. Ecol. Monogr. 27, 326–349.

Bruni, L., Pastorelli, R., Viti, C., Gasco, L., Parisi, G., 2018. Characterisation of the intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) fed with *Hermetia illucens* (black soldier fly) partially defatted larva meal as partial dietary protein source. Aquaculture 487, 56–63. https://doi.org/10.1016/j.aquaculture.2018.01.006.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Carlberg, I., Mannervik, B., 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J. Biol. Chem. 250, 5475–5480.

Carter, C.G., Houlihan, D.F., He, Z.Y., 2000. Changes in tissue free amino acid concentrations in Atlantic salmon, *Salmo salar* L., after consumption of a low ration. Fish Physiol. Biochem. 23, 295–306. https://doi.org/10.1023/A:1011187026997.

Chen, J., Liu, H., 2020. Nutritional indices for assessing fatty acids; a mini review. Int. J. Mol. Sci. 21, 5695. https://doi.org/10.3390/ijms21165695.

Chen, Y., Sagada, G., Xu, B., Chao, W., Zou, F., Ng, W.-K., Sun, Y., Wang, L., Zhong, Z., Shao, Q., 2019. Partial replacement of fishmeal with *Clostridium autoethanogenum* single-cell protein in the diet for juvenile black sea bream (*Acanthopagrus schlegelii*). Aquac. Res. 51, 1000–1011. https://doi.org/10.1111/are.14446.

Commission Regulation (EU) 2022/1104 of 1 July 2022, 2023. Amending Regulation (EU) No 68/2013 on the Catalogue of Feed Materials (Text with EEA Relevance). C/2022/4474. OJ L 177, 4.7.2022, pp. 4–74. http://data.europa.eu/eli/reg/2022/1104/oj.

D'amato, D., Korhonen, J., 2021. Integrating the green economy, circular economy and bioeconomy in a strategic sustainability framework. Ecol. Econ. 188, 107143 https://doi.org/10.1016/j.ecolecon.2021.107143.

Fernández-Álvarez, C., Gijón, D., Álvarez, M., Santos, Y., 2016. First isolation of *Aeromonas salmonicida* subspecies *salmonicida* from diseased sea bass, *Dicentrarchus labrax* (L.), cultured in Spain. Aquac. Rep. 4, 36–41. https://doi.org/10.1016/j.aqrep.2016.05.006.

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol Chem. 226, 497–509.

Garner, A.P., Paine, M.J.I., Rodriguez-Crespo, I., Chinje, E.C., De Montellano, P.O., Stratford, I.J., Tew, D.G., Wolf, C.R., 1999. Nitric Oxide Synthases Catalyze the Activation of Redox Cycling and Bioreductive Anticancer Agents. Cancer Res. 59, 1929–1934.

Gephart, J.A., Golden, C.D., 2022. Environmental and nutritional double bottom lines in aquaculture. One Earth 5, 324–328. https://doi.org/10.1016/j.oneear.2022.03.018.

Gisbert, E., Ortiz-Delgado, J.B., Sarasquete, C., 2008. Nutritional cellular biomarkers in early life stages of fish. Histol. Histopathol. 23, 1525–1539.

Glencross, B.D., Huyben, D., Schrama, J.W., 2020. The application of single-cell ingredients in aquaculture feeds—a review. Fishes 5, 22. https://doi.org/10.3390/fishes5030022.

Glencross, B., Muñoz-Lopez, P., Matthew, C., MacKenzie, S., Powell, A., Longshaw, M., LeBlanc, A., 2023. Digestibility of bacterial protein by Atlantic salmon (*Salmo salar*) is affected by both inclusion level and acclimation time. Aquaculture 565, 739137. https://doi.org/10.1016/j.aquaculture.2022.739137.

Günzler, A., Flohé, L., 1985. Glutathione peroxidase. In: Greenwald, R.A. (Ed.), CRC Handbook of Methods for Oxygen Radical Research. CRC Press Inc., Boca Raton, FL, USA, pp. 285–290.

Hao, W.L., Lee, Y.K., 2004. Microflora of the gastrointestinal tract: a review. Methods Mol. Biol. 268, 491–502. https://doi.org/10.1385/1-59259-766-1:491.

Harasgama, J.C., Kasthuriarachchi, T.D.W., Kwon, H., Wan, Q., Lee, J., 2020. Molecular and functional characterization of a mitochondrial glutathione reductase homolog from redlip mullet (*Liza haematocheila*): disclosing its antioxidant properties in the fish immune response mechanism. Dev. Comp. Immunol. 113, 103785 https://doi.org/10.1016/j.dci.2020.103785.

Hardy, R.W., Patro, B., Pujol-Baxley, C., Marx, C.J., Feinberg, L., 2018. Partial replacement of soybean meal with *Methylobacterium extorquens* single-cell protein in feeds for rainbow trout (*Oncorhynchus mykiss* Walbaum). Aquac. Res. 49, 2218–2224. https://doi.org/10.1111/are.13678.

Hossain, M.S., Koshio, S., Kestemont, P., 2020. Recent advances of nucleotide nutrition research in aquaculture: a review. Rev. Aquac. 12, 1028–1053. https://doi.org/10.1111/raq.12370.

Hua, K., Cobcroft, J.M., Cole, A., Condon, K., Jerry, D.R., Mangott, A., Praeger, C., Vucko, M.J., Zeng, C., Zenger, K., Strugnell, J.M., 2019. The future of aquatic protein: implications for protein sources in aquaculture diets. One Earth 1, 316–329. https://doi.org/10.1016/j.oneear.2019.10.018.

Huyben, D., Sun, L., Moccia, R., Kiessling, A., Dicksved, J., Lundh, T., 2018. Dietary live yeast and increased water temperature influence the gut microbiota of rainbow trout. J. Appl. Microbiol. 124, 1377–1392. https://doi.org/10.1111/jam.13738.

Illumina, 2013. 16S Metagenomic Sequencing Library Preparation: Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System. Illumina, USA.

Jones, S.W., Karpol, A., Friedman, S., Maru, B.T., Tracy, B.P., 2020. Recent advances in single cell protein use as a feed ingredient in aquaculture. Curr. Opin. Biotechnol. 61, 189–197. https://doi.org/10.1016/j.copbio.2019.12.026.

Kiessling, A., Askbrandt, S., 1993. Nutritive value of two bacterial strains of single-cell protein for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 109, 119–130. https://doi.org/10.1016/0044-8486(93)90209-H.

Kim, B.R., Shin, J., Guevarra, R.B., Lee, J.H., Kim, D.W., Seol, K.H., Lee, J.H., Kim, H.B., Isaacson, R.E., 2017. Deciphering diversity indices for a better understanding of microbial communities. J. Microbiol. Biotechnol. 27, 2089–2093. https://doi.org/10.4014/jmb.1709.09027.

Knobloch, S., Skírnisdóttir, S., Dubois, M., Kolypczuk, L., Leroi, F., Leeper, A., Passerini, D., Marteinsson, V.T., 2022. Impact of putative probiotics on growth, behaviour and the gut microbiome of farmed Arctic char (*Salvelinus alpinus*). Front. Microbiol. 13, 912473 https://doi.org/10.3389/fmicb.2022.912473.

Kononova, S.V., Zinchenko, D.V., Muranova, T.A., Belova, N.A., Miroshnikov, A.I., 2019. Intestinal microbiota of salmonids and its changes upon introduction of soy proteins to fish feed. Aquac. Int. 27, 475–496. https://doi.org/10.1007/s10499-019-00341-1.

Krogdahl, Å., Nordrum, S., Sorensen, M., Brudeseth, L., Rosjo, C., 1999. Effects of diet composition on apparent nutrient absorption along the intestinal tract and of subsequent fasting on mucosal disaccharidase activities and plasma nutrient concentration in Atlantic salmon *Salmo salar* L. Aquac. Nutr. 5, 121–133. https://doi.org/10.1046/j.1365-2095.1999.00095.x.

Lei, X.G., Cheng, W.H., McClung, J.P., 2007. Metabolic regulation and function of glutathione peroxidase-1. Annu. Rev. Nutr. 27, 41–61. https://doi.org/10.1146/annurev.nutr.27.061406.093716.

Li, P., Mai, K., Trushenski, J., Wu, G., 2008. New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds. Amino Acids 37, 43–53. https://doi.org/10.1007/s00726-008-0171-1.

Liu, C., Cui, Y., Li, X., Yao, M., 2021. Microeco: an R package for data mining in microbial community ecology. FEMS Microbiol. Ecol. 97 https://doi.org/10.1093/femsec/fiaa255 fiaa255.

Llewellyn, M.S., McGinnity, P., Dionne, M., Letourneau, J., Thonier, F., Carvalho, G.R., Creer, S., Derome, N., 2016. The biogeography of the Atlantic salmon (*Salmo salar*) gut microbiome. ISME J. 10, 1280–1284. https://doi.org/10.1038/ismej.2015.189.

Marchi, A., Bonaldo, A., Scicchitano, D., Candela, M., De Marco, A., Falciglia, S., Mazzoni, M., Lattanzio, G., Clavenzani, P., Dondi, F., Gatta, P.P., Parma, L., 2023. Feeding gilthead sea bream with increasing dietary bacterial single cell protein level: implication on growth, plasma biochemistry, gut histology, and gut microbiota. Aquaculture 565, 739132. https://doi.org/10.1016/j.aquaculture.2022.739132.

Maulu, S., Liang, H., Ge, X., Yu, H., Huang, D., Ke, J., Ren, M., Mi, H., 2021. Effect of dietary *Clostridium autoethanogenum* protein on growth, body composition, plasma parameters and hepatic genes expression related to growth and AMPK/TOR/PI3K signaling pathway of the genetically improved farmed tilapia (GIFT: *Oreochromis niloticus*) juveniles. Anim. Feed Sci. Technol. 276, 114914 https://doi.org/10.1016/j.anifeedsci.2021.114914.

McKnight, D.T., Huerlimann, R., Bower, D.S., Schwarzkopf, L., Alford, R.A., Zenger, K.R., 2019. Methods for normalizing microbiome data: an ecological perspective. Methods Ecol. Evol. 10, 389–400. https://doi.org/10.1111/2041-210X.13115.

Merrifield, D.L., Dimitroglou, A., Bradley, G., Baker, R.T.M., Davies, S.J., 2009. Soybean meal alters autochthonous microbial populations, microvilli morphology and compromises intestinal enterocyte integrity of rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis. 32, 755–766. https://doi.org/10.1111/j.1365-2761.2009.01052.

Meyerholz, D.K., Beck, A.P., 2018. Fundamental concepts for semiquantitative tissue scoring in translational research. ILAR J. 59, 13–17. https://doi.org/10.1093/ilar/ily025.

Naylor, R.L., Hardy, R.W., Buschmann, A.H., Bush, S.R., Cao, L., Klinger, D.H., Little, D. C., Lubchenco, J., Shumway, S.E., Troell, M., 2021. A 20-year retrospective review of global aquaculture. Nature 591, 551–563. https://doi.org/10.1038/s41586-021-03308-6.

Overland, M., Tauson, A.H., Shearer, K., Skrede, A., 2010. Evaluation of methane-utlising bacteria products as feed ingredients for monogastric animals. Arch. Anim. Nutr. 64, 171–189. https://doi.org/10.1080/17450391003691534.

Pereira, A.G., Garcia-Oliveira, P., Otero, P., Soria-Lopez, A., Cassani, L., Cao, H., Xiao, J., Prieto, M.A., Simal-Gandara, J., 2022. Single-cell proteins obtained by circular economy intended as a feed ingredient in aquaculture. Foods 11, 2831. https://doi.org/10.3390/foods11182831.

Rasmussen, J.A., Villumsen, K.R., Duchêne, D.A., Puetz, L.C., Delmont, T.O., Sveier, H., von Gersdorff, J.L., Præbel, K., Martin, M.D., Bojesen, A.M., Gilbert, M.T.P., Kristiansen, K., Limborg, M.T., 2021. Genome-resolved metagenomics suggests a mutualistic relationship between *Mycoplasma* and salmonid *hosts*. Commun. Biol. 4, 1–10. https://doi.org/10.1038/s42003-021-02105-1.

Rimoldi, A., Antonini, M., Gasco, L., Moroni, F., Terova, G., 2021. Intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) may be improved by feeding a *Hermetia illucens* meal/low-fishmeal diet. Fish Physiol. Biochem. 47, 365–380. https://doi.org/10.1007/s10695-020-00918-1.

Ringø, E., Zhou, Z., Vecino, J.L.G., Wadsworth, S., Romero, J., Krogdahl, Å., Olsen, R.E., Dimitroglou, A., Foey, A., Davies, S., Owen, M., Lauzon, H.L., Martinsen, L.L., De Schryver, P., Bossier, P., Sperstad, S., Merrifield, D.L., 2016. Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? Aquac. Nutr. 22, 219–282. https://doi.org/10.1111/anu.12346.

Ritala, A., Häkkinen, S.T., Toivari, M., Wiebe, M.G., 2017. Single cell protein - state-of-the-art, industrial landscape and patents 2001–2016. Front. Microbiol. 8, 2009. https://doi.org/10.3389/fmicb.2017.02009.

Ruiz, A., Andree, K.B., Sanahuja, I., Holhorea, P.G., Calduch-Giner, J.À., Morais, S., Pastor, J.J., Pérez-Sánchez, J., Gisbert, E., 2023. Bile salt dietary supplementation promotes growth and reduces body adiposity in gilthead seabream *(Sparus aurata*). Aquaculture 566, 739203. https://doi.org/10.1016/j.aquaculture.2022.739203.

Rumsey, G.L., Winfree, R.A., Hughes, S.G., 1992. Nutritional value of dietary nucleic acids and purine bases to rainbow trout (*Oncorhynchus mykiss*). Aquaculture 108, 97–110. https://doi.org/10.1016/0044-8486(92)90321-B.

Salomón, R., Furones, M.D., Reyes-López, F.E., Tort, L., Firmino, J.P., Esteban, M.A., Espinosa Ruíz, C., Quintela, J.C., Pinilla-Rosas, J.M., Vallejos-Vidal, E., Gisbert, E., 2021. A bioactive extract rich in triterpenic acid and polyphenols from *Olea europaea* promotes systemic immunity and protects Atlantic salmon smolts against furunculosis. Front. Immunol. 12, 737601 https://doi.org/10.3389/fimmu.2021.737601.

Sharif, M., Zafar, M.H., Aqib, A.I., Saeed, M., Farag, M.R., Alagawany, M., 2021. Single cell protein: sources, mechanism of production, nutritional value and its uses in aquaculture nutrition. Aquaculture 531, 735885. https://doi.org/10.1016/j.aquaculture.2020.735885.

Sibinga, N.A., Lee, M.T., Johnson, E.L., Selvaraj, V., Marquis, H., 2022. Longitudinal sampling of the rainbow trout (*Oncorhynchus mykiss*) microbiome reveals effects of dietary cecropin A and *Yersinia ruckeri* infection. Front. Mar. Sci. 9, 901389 https://doi.org/10.3389/fmars.2022.901389.

Skalli, A., Firmino, J.P., Andree, K.B., Salomón, R., Estévez, A., Puig, P., Sabater-Martíne, M., Hechavarria, T., Gisbert, E., 2020. The inclusion of the microalga *Scenedesmus* sp. in diets for rainbow trout, *Onchorhynchus mykiss*, juveniles. Animals 10, 1656. https://doi.org/10.3390/ani10091656.

Smith, P.E., Waters, S.M., Gómez Expósito, R., Smidt, H., Carberry, C.A., McCabe, M.S., 2020. Synthetic sequencing standards: a guide to database choice for rumen microbiota amplicon sequencing analysis. Front. Microbiol. 11, 606825 https://doi.org/10.3389/fmicb.2020.606825.

Solé, M., Potrykus, J., Fernández-Díaz, C., Blasco, J., 2004. Variations on stress defences and metallothionein levels in the Senegal sole, *Solea senegalensis*, during early larval stages. Fish Physiol. Biochem. 30, 57–66. https://doi.org/10.1007/s10695-004-6786-6.

Terova, G., Rimoldi, S., Ascione, C., Gini, E., Ceccotti, C., Gasco, L., 2019. Rainbow trout (*Oncorhynchus mykiss*) gut microbiota is modulated by insect meal from *Hermetia illucens* prepupae in the diet. Rev. Fish Biol. Fisheries 29, 465–486. https://doi.org/10.1007/s11160-019-09558-y.

Vatsos, I.N., 2017. Standardizing the microbiota of fish used in research. Lab. Anim. 51, 353–364. https://doi.org/10.1177/0023677216678825.

Wang, B., Wu, L., Chen, J., Dong, L., Chen, C., Wen, Z., Hu, J., Fleming, I., Wang, D.W., 2021. Metabolism pathways of arachidonic acids: mechanisms and potential therapeutic targets. Signal Transduct. Target. Ther. 6, 94. https://doi.org/10.1038/s41392-020-00443-w.

Wu, G., Fang, Y.-Z., Yang, S., Lupton, J.R., Turner, N.D., 2004. Glutathione metabolism and its implications for health. J. Nutr. 134, 489–492. https://doi.org/10.1093/jn/134.3.489.

Zamani, A., Khajavi, M., Nazarpak, M.H., Gisbert, E., 2020. Evaluation of a bacterial single-cell protein in compound diets for rainbow trout (*Oncorhynchus mykiss*) fry as an alternative protein source. Animals 10, 1676. https://doi.org/10.3390/ani10091676.