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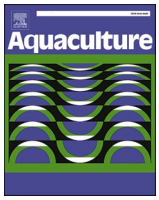

Black soldier fly larvae (*Hermetia illucens*) meal is a viable protein source for Atlantic salmon (*Salmo salar*) during a large-scale controlled field trial under commercial-like conditions

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ABSTRACT

Black soldier fly larvae meal (BSFL) from *Hermetia illucens* is a promising alternative protein source in diets for farmed fish. The larvae can efficiently convert low-value organic material into high-value protein in a production cycle with low arable land and freshwater inputs. A few recent studies have shown that BSFL is a suitable protein source for Atlantic salmon (*Salmo salar*) in smaller controlled experiments. However, industry-relevant field trials conducted under large scale near-commercial conditions over a longer period are lacking. In this study, a feeding trial was performed to evaluate the impact of BSFL on growth performance and health of Atlantic salmon during the grow-out phase in seawater, in a commercial site in Vestland county, Norway. A total of 181,046 post-smolt Atlantic salmon were distributed into six duplicate sea cages and fed one of three diets (commercial-like control diet and two test diets partially replacing the protein content of the control diet with 4% and 8% defatted BSFL meal) for 21 weeks, until a relevant commercial slaughter size of 4.5–5.0 kg was reached. Health parameters were assessed, including histology of the distal intestine (DI), DI microbiota identification (by 16 s rRNA-seq) and salmon RNA-seq of DI and head kidney (HK). The results showed that the inclusion of BSFL meal supported growth performance and had no adverse effect on gut health. The beta diversity of the DI microbiota and the relative abundance of families *Lactobacillaceae* and the chitinolytic *Bacillaceae* increased in the fish fed the BSFL diets. Additionally, no histopathological changes were attributable to BSFL meal intake. Results from RNA-seq in DI revealed that BSFL inclusion modulates metabolic processes associated with lipids, response to estrogens, activity of immune receptors (to chemokines), phagocytosis and extracellular vesicles. Based on these results, black soldier fly larvae meal was shown to be a suitable alternative protein ingredient in inclusions of up to at least 8% for Atlantic salmon under industrial fish farming conditions.

1. Introduction

Global salmon production reached nearly 2.9 million tons in 2021 (Kontali, 2022), and it is estimated to exceed 4 million tons in 2030 (Norwegian Seafood Council, 2022). To support further growth in the sector, there is an emerging need to develop high quality feed ingredients with a low environmental footprint. Since the 1990s, the content of fishmeal in commercial salmon feeds has decreased because of limited supply (Tacon et al., 2011) and high market prices, while the content of plant ingredients such as soy protein concentrate, corn gluten meal, rapeseed meal, sunflower meal and legumes has increased (Aas et al., 2022) because they are abundant and cheap. Increased use of protein sources of plant origin has, however, several shortcomings (Barroso et al., 2014; Bruni et al., 2018; Makkar et al., 2014). This includes a high fiber content, imbalanced amino acid profile and their content of several anti-nutritional factors (ANFs), including saponins, lectins, phytases and protease inhibitors, which can negatively impact fish growth performance and health. Some plant-based ingredients have led to low-grade chronic intestinal inflammation, which over time may lead to (pre)neoplastic changes in Atlantic salmon (Bjørgen et al., 2019;

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Dale et al., 2009). In addition, increased use of plant ingredients is questionable from a sustainability point of view. The production of plant-based raw materials puts a large demand on natural resources and increases feed-food competition (van Hal et al., 2019). Increased demand for plant-based raw materials due to the expansion of the agricultural sector is a potential driver of tropical deforestation and consequently greenhouse gas emissions, as well as loss of biodiversity and degradation of ecosystems (Pendrill et al., 2022).

To overcome these challenges, alternative protein ingredients produced by upcycling organic waste streams into circular protein sources can be an alternative. In this scenario, insects represent a promising alternative (Sogari et al., 2019). Black soldier fly larvae (BSFL) have shown potential as a suitable insect species for aquafeeds, as they have a rapid growth rate, are easy to rear and have a high nutritional value. They are sustainable due to their ability to efficiently convert organic waste into larval biomass rich in high quality protein and lipids (Kinasih et al., 2018; Shumo et al., 2019; Spranghers et al., 2017; Katayama et al., 2008). BSFL can grow on a wide range of substrates including fiber-rich plant by-products, fruits and vegetables and a few animal by-products. The main challenge when using BSFL meal in salmonid diets are small production volumes, which are currently too low to compete with commodity protein sources such as fish meal and soy proteins and consequential high cost. Currently, insect production is under strict regulation with respect to the feed substrates which limit the possibility for upscaling. A change in the EU regulations to allow use of alternative substrates would facilitate a more rapid upscaling, but use of such alternatives would require documentation with respect to food safety.

BSFL has a high nutritional value; they can contain about 40% protein and 30% fat, depending on the stage of development, composition of the rearing substrate and how they are processed after harvest (Newton et al., 2005). In general, BSFL meal meets the amino acid requirements for Atlantic salmon, except for methionine and lysine (Caligiani et al., 2018; NRC, N, 2011), which suggests that BSFL meal is more suitable than soy protein concentrate as a fishmeal replacement (English et al., 2021). BSFL also contain about 3–10% chitin. The chitin content of BSFL meal can vary depending on the stage of development of the larvae (Xiao et al., 2018) and how the meal is processed (Weththasinghe et al., 2021). Salmonids have a poor ability to digest chitin (Olsen et al., 2006) and high inclusions of chitin have shown to reduce digestibility of crude protein and lipid (Karlsen et al., 2017; Hansen et al., 2010). Despite these limitations, BSFL meal is still considered a valuable feed ingredient due to its high protein content and low environmental footprint. Additionally, BSFL meal contains several bioactive components with potential health beneficial effects, including modulation of gut microbiota, increasing microbial diversity and facilitating the growth of chitinolytic bacteria that helps the digestion of chitin (Huyben et al., 2019; Weththasinghe et al., 2022).

Several scientific publications exist describing the potential of various insect species as alternative feed ingredients for fish feed in smaller short-term controlled experiments (Barroso et al., 2014; Nesic and Zagon, 2019; Al-Qazzaz et al., 2016; Fisher et al., 2020; Belghit et al., 2019; Cardinaletti et al., 2019a), but long-term trials under commercial conditions with relevance for the industry are lacking. To our knowledge, this is the first published field experiment of commercial-like scale evaluating the effects of BSFL meal as a protein source for Atlantic salmon. Therefore, the overall goal of this study was to investigate the impacts of partially replacing conventional protein ingredients with insect meal in the diets for 181,046 Atlantic salmon on growth performance and health during the grow-out phase in seawater. Health was assessed by histology, 16 s rRNA sequencing of the distal intestine (DI) microbiota and RNA sequencing of DI, and head kidney (HK).

2. Materials and methods

2.1. BSFL meal

BSFL meal was produced by HiProMine S.A., Poznań, Poland. The larvae were fed vegetables and cereal based diets. The chemical composition and total amino acids are presented in Table 1. The insect meal was manufactured in accordance with HACCP and quality management system ISO 9001:15, GMP+ B2, GMP+ BA1 to ensure the consistency, safety and traceability of the product.

2.2. Experimental diets

Three experimental diets containing either 0% BSFL meal (control diet: IM0), 4% BSFL meal (IM4) and 8% BSFL meal (IM8) meal were produced at Aller Aqua A/S (Christiansfeld, Denmark). The diets were formulated to meet or exceed requirements for all indispensable amino acids and other nutrients for Atlantic salmon (NRC, N, 2011). In IM0, the remaining protein ingredients evenly replaced the insect meal. The pellet size was 9 mm throughout the experimental period. Composition, chemical content and amino acids are presented in Table 2. The ingredients were ground with a 0.8 mm screen high-speed hammer mill. The coarseness was 90% $<$ 315 μm. The diets were extruded with a single screw extruder (model EX920, Andritz Feed & Biofuel Technologies, Esbjerg, Denmark). In the extrusion process, the temperature in the conditioner was 98 °C and in the extruder it was 115–125 °C. The specific mechanical energy (SME) was around 30–35 kW ton^{-1}. The moisture level was 8% in the raw materials, and 6% in the final feed. The feed was vacuum coated by a vertical vacuum coater (Andritz Feed & Biofuel Technologies, Esbjerg, Denmark) with vertical mixing, adding

Table 1
Analyzed composition (% of dry matter) of black soldier fly larvae meal.

Nutrient	%
Dry matter	94.1
Content in dry matter	
Crude protein	65.2
Crude lipid	5.7
Ash	2.1
Starch	NA[a]
Calcium	0.46
Sodium	0.09
Magnesium	0.04
Potassium	0.13
Phosphorous	0.31
Chitin	5.9
Amino acids[b]	
Essential amino acids	
Methionine	0.85
Asparagine	3.65
Threonine	2.53
Valine	2.27
Isoleucine	1.85
Leucine	4.23
Phenylalanine	2.38
Histidine	1.05
Lysine	3.01
Arginine	3.06
Non-essential amino acids	0.54
Cysteine	3.65
Aspartic acid	
Serine	2.49
Glutamic acid	50.4
Proline	2.3
Glycine	2.48
Alanine	3.27
Tyrosine	1.53
Total amino acids	42.5

[a] Under limit of detection.
[b] Water corrected values.

Table 2
Ingredients and analyzed chemical composition of experimental diets with increasing inclusion of black soldier fly larvae meal.[1]

	IM0	IM4	IM8
Ingredients (%)			
Fish meal LT[a]	23.22	21.97	20.73
Corn gluten[b]	9.20	8.71	8.21
Soy protein concentrate[c]	8.40	7.95	7.50
Wheat gluten[d]	7.64	7.23	6.82
Defatted insect meal[e]	0.00	4.00	8.00
Rapeseed oil[f]	19.28	19.04	18.81
Fish oil[g]	9.50	9.50	9.50
Wheat[h]	16.79	15.68	14.56
Starch[i]	1.00	1.00	1.00
Binder[j]	1.50	1.50	1.50
Monoammonium phosphate[k]	1.07	1.08	1.09
Amino acids[l]	1.65	1.59	1.53
Vitamin/mineral premix[m, n]	0.75	0.75	0.75
Chemical composition (%)			
Dry matter	92.4	92	94.3
Crude protein	38.1	36.3	37.5
Crude fat	27.6	27.4	29.0
Ash	7.1	7.1	7.2
Starch	12.8	12.6	11.8
Gross energy (MJ kg^{-1})	27.6	27.4	29.0
Macro mineral composition (%)			
Ca	1.3	1.2	1.2
Na	0.4	0.4	0.4
Mg	0.2	0.2	0.2
K	0.6	0.6	0.7
P	1.1	1.1	1.1
Amino acids[2] (%)			
Essential amino acids			
Methionine	0.80	1.39	1.08
Asparagine	2.35	2.32	2.31
Threonine	1.17	1.14	1.16
Valine	1.17	1.12	1.17
Isoleucine	1.33	1.27	1.29
Leucine	2.73	2.56	2.61
Phenylalanine	1.43	1.31	1.31
Histidine	0.87	0.80	0.78
Lysine	2.46	2.41	2.48
Arginine	1.78	1.67	1.69
Non-essential amino acids			
Cysteine	0.39	0.36	0.37
Aspartic acid	2.35	2.32	2.31
Serine	1.38	1.31	1.33
Glutamic acid	6.71	6.29	6.23
Proline	2.08	2.01	2.07
Glycine	1.38	1.33	1.36
Alanine	1.59	1.57	1.66
Tyrosine	0.78	0.98	0.99
Total amino acids	30.41	29.83	29.89

[1] 4% IM and 8% IM: Insect meal from black soldier fly larvae substituted 4% and 8% of the protein content of the control diet, respectively.
[2] Water corrected values.
[a] FF Skagen, Skagen, Denmark.
[b] Heinz & Co, Zurich, Switzerland.
[c] Nordic Soya, Uusikaupunki, Finland.
[d] Heinz & Co, Zurich, Switzerland.
[e] HiProMine, Robakowo, Poland.
[f] Emmelev, Otterup, Denmark.
[g] Triplenine, Esbjerg, Denmark.
[h] DLG, Fredericia, Denmark.
[i] Emsland Starch, Emsland, Germany.
[j] Imerys, Fur, Denmark.
[k] Timab, Murcia, Spain.
[l] L-lysine, DL-methionine, L-histidine, CJ Europe, Frankfurt, Germany.
[m] Vilofoss, Gråsten, Denmark.
[n] Choline chloride was added in the premix. Pellet diameter 6 and 9 mm.

both fish- and rapeseed oils.

2.3. Chemical analysis

The feed ingredients and insect meal were analyzed for dry matter by drying to constant weight at 104 °C, crude protein using Kjeldahl nitrogen (Commission dir. 152/2009/EC) × 6.25 (a conversion factor of 4.76 was used to evaluate crude protein in the insect meal, as suggested in (Janssen et al., 2017), instead of 6.25) with the Kjeltec™ 8400 (Foss, Denmark), crude lipid by The Sotex™ 8000 Extraction System (Foss, Denmark) (Commission dir 152/2009), ash by incineration at 550 °C (Commission dir. 152/2009/EC). Gross energy content was determined with a Parr 6400 bomb calorimeter (Parr Instrument Company, Illinois, USA), according to ISO 9831. Amino acids were analyzed according to Commission dir. 152/2009/EC on a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, UK). The chitin content of the insect meal was analyzed as described in (Finke, 2007).

2.4. Physical pellet quality

Length and width of 15 randomly selected pellets per diet was measured three times for each pellet with electronic calipers. Expansion was calculated as: ((pellet width − die diameter)/(die diameter)) × 100. Hardness was measured on 15 pellets per diet with a Kahl pellet hardness tester (Amandus Kahl, Hamburg, Germany). Sinking velocity was determined by measuring the mean value of the time required for 30 random pellets per diet to sink 1 m in room tempered tap water with a salinity of 35 ppt. Pellet durability was tested with a DORIS pellet tester (AKVAsmart, Bryne, Norway) using 100 g pellets. After the pellets passed through the pellet tester, the pellet collector was emptied on an 8 mm screen with a collector and sieved for 60 s at 0.5 amplitude with a Retsch AS 200 Control (Haan, Germany). The material remaining on the 8 mm screen after sieving was weighed. The durability was defined as the percentage of pellets remaining on the screen. The testing was conducted in triplicates for each diet. Water stability was determined by weighing 20 g of each diet into pre-weighed 20 cm circular wire netting baskets with 3 mm mesh size and a diameter of 8 cm. The bottom of each basket was flat and situated 2 cm above the lower end of the netting tube. Baskets with feed samples were placed in 600 mL beakers, to which were added 300 mL of MilliQ water. Three beakers per diet were then incubated in a water bath (SBK 25 D, Salvis AG, Reussbühl, Switzerland) at 23 °C and 120 rpm for 30, 60 and 120 min, respectively. After incubation, the baskets were gently drained and weighed before incubation in a heating cabinet at 105 °C for 20 h. Thereafter, the baskets were again weighed to determine the residual dietary dry matter in each basket. The testing was conducted in triplicates for each diet. Water stability was calculated as:

$$Water\ stability = \frac{\left(\frac{pellets\ starting\ weight}{pellets\ residual\ weight}\right) \times 100}{diet\ dry\ matter\%} \times 100$$

2.5. Fish husbandry and feeding

A total of 181,046 Atlantic salmon post-smolts with average weight 420 g (by Bremnes Seashore, Trovåg, Norway) were distributed into six 12,500 m^3 net pens in seawater close to Isane, Vestland county, Norway, in July 2020. The fish were fed a standard salmon feed until feeding with experimental diets started in October 2020. Each diet was administered to duplicate net pens over a period of 21 weeks. Feed was distributed with automatic feeders according to appetite monitored by cameras. The average sea temperature was 10.7 °C during the experimental period. All fish groups were deloused three times with Thermolicer treatment using a water bath holding 30–34 °C during the experimental period.

2.6. Sample collection

Ten fish from each diet group (30 in total) were collected after 21 weeks of feeding with experimental diets and euthanized with a sharp blow on the head. Weight and length of the fish was measured before the abdominal cavity was opened and the digestive organs were taken out and cleared of adipose tissue. Digesta from the distal intestine was collected and immediately frozen at −80 °C. Fish without distal intestine content were excluded from sampling. A tissue sample with 1 cm distal intestine and 10 intestinal folds were collected and stored in phosphate-buffered formaldehyde solution for histological examination. Head kidney and distal intestine tissue were collected from each fish in 2 × 3 mm pieces and fixed in RNAlater™ (Thermofisher Scientific, Waltham, MA, USA) at room temperature for 24 h, then stored at −80 °C.

2.7. DNA extraction of distal intestine microbiota

DNA from 200 mg distal intestine digesta and ground feed was isolated with the QIAamp Fast DNA Stool Mini Kit, cat. no. 51604 (Qiagen, Hilden, Germany) according to the instructions of the manufacturer with some modifications: for cell lysis, samples were put in 300 μL ASL stool lysis buffer (Qiagen, cat. no. 19082) with 100 μL 0.1 mm glass beads (Qiagen, cat. no. 13118–400); digesta samples were homogenized with a Qiagen TissueLyser bead mill (Retsch, Haan, Germany) at 20 Hz for 3 min, put on ice for 2 min, then homogenized again for 3 min; samples were heat incubated at 95 °C for 5 min, followed by a second incubation at 90 °C for 15 min after adding 15 μL proteinase K and 200 μL AL buffer; at the end of the procedure, the DNA was eluted in 50 μL ATE buffer. DNA was also isolated with the same procedure from three 500 mL water samples collected at three different spots in the salmon farm, filtered through a 0.2 μm pore size MF-Millipore membrane filter (Sigma-Aldrich, Saint-Louis USA, cat. no. GSWP04700). To ensure the quality of DNA extraction and sequencing, negative controls and positive controls were included. A negative control without sample was included during the DNA extraction from digesta, a blank 0.22 μm pore membrane filter, together with a positive control (in duplicate) containing a known microbial community of two yeasts and eight bacteria (Zymo- BIOMOICS ™, Zymo research, California, SA, cat. no. D6300). DNA concentration was measured in duplicates with the Invitrogen™ dsDNA BR Assay Kit (Thermo Fisher Scientific, cat. no. Q32863) using a Qubit 4 Fluorometer (Invitrogen, Waltham, MA, USA).

PCR amplification of the V3-V4 variable region of the 16 s rRNA gene and cleaning of the PCR product, was performed as described in the Illumina 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, 2013), with one modification: all digesta samples were amplified with 30 PCR cycles instead of 25 due to lower DNA concentrations compared to the non-digesta samples (feed samples, controls). PCR products were evaluated with 1% agarose gel electrophoresis to ensure the amplification of the variable region.

Library preparation was performed as described in the Illumina 16S Metagenomic Sequencing Library Preparation protocol (Illumina, 2013). PCR products were quantified using the Invitrogen Quant-iT™ Qubit™ dsDNA BR (Broad Range) assay kit (Thermo Fisher Scientific, Cat no: Q32853) with the Qubit 4 Fluorometer (Invitrogen). The combined library and PhiX control were then loaded in a concentration of 8 pM and sequenced using the Miseq System (Illumina, San Diego, CA, USA) with the Miseq Reagent Kit v3 (600-cycle) (Illumina, catalog no. MS-102-3003). The clustering density was 1338 K mm^2 and 83% clusters passing filter. The data output was demultiplexed FASTQ format files.

2.8. Histology

Histologic examination was performed on 30 distal intestine samples, 10 from each dietary group. The tissue sections covered the transition from mid to distal intestine with approximately one centimeter of mid intestine and 10 folds of the distal intestine. Standard procedures were followed including tissue fixation in 10% phosphate-buffered formalin, embedding in paraffin, and staining of sections from all blocks with H&E, periodic acid-Schiff and Alcian blue for detection of mucins.

The severity of inflammation, epithelial vacuolization and ectopic goblet cells were scored as normal, minimal, mild, modest, marked and severe by one operator, where each category was assigned a number from 0 to 4 with increasing severity. The number of single ectopic goblet cells, ectopic goblet cell clusters containing less than five cells and ectopic goblet cell clusters containing at least five cells were counted manually by one operator.

2.9. Salmon RNA-seq

RNA was isolated from distal intestine and head kidney tissue from 6 fish per dietary group. The tissue samples stored in RNAlater™ solution (Invitrogen, Waltham, USA) were thawed on ice, and 50 mg tissue was transferred to a 2 mL microtube (Sarstedt, Nümbrecht, Germany), containing 900 μL QIAzol lysis reagent (Qiagen, Hilden, Germany, cat. No. 79306) and a 5 mm stainless steel bead (Qiagen, cat. No. 69989). The tissues were lysed with a Qiagen TissueLyser bead mill (Retsch, Haan, Germany, cat. no. 85220) and run at 20 Hz for 2 min (twice). The lysate was transferred to 2 mL tubes, and 180 μL chloroform was added before vortexing. The tubes were centrifuged at 12000 rpm (4 °C) in a MicroCL21 microcentrifuge (Thermo Scientific, Waltham USA) for 15 min. Next centrifugations were run at 1200 rpm at 4 °C. The upper phase containing RNA and water was transferred to new tubes, and 450 μL isopropanol was added. Tubes were shaken by hand and incubated for 10 min at room temperature, before centrifugation for 15 min. Excess liquid was removed and 200 μL 70% ethanol was added before centrifugation at 10 min. The supernatant was removed, and the samples were centrifugated for 3 min. With the lids open, the pellets were left to dry for 5–10 min. The RNA was then dissolved in 40 μL RNAse-free water, followed by a 10 min incubation at 55 °C. RNA concentration was measured with a NanoDrop™ 8000 spectrophotometer (Thermo Scientific) according to instructions from the manufacturer. RNA integrity (RIN) values were measured with the Agilent 4150 TapeStation (Agilent, Santa Cara, CA, USA) according to the instructions from the manufacturer. For all samples RIN ≥ 7. Library preparation and RNA-seq were performed by Novogene (Cambridge, United Kingdom) using Illumina NovaSeq 6000 System (150 bp paired-end RNA sequencing with 30 million reads sequencing depth).

2.10. Calculations

Feed conversion ratio (FCR):

$$FCR = \frac{kg\ diet\ consumed}{(kg\ final\ biomass - kg\ initial\ biomass + kg\ dead\ fish)}$$

Specific growth rate (SGR):

$$\mathrm{SGR} = 100 \times \frac{ln(\mathrm{W}_2) - ln(\mathrm{W}_1)}{(\mathrm{t})}$$

Where W_2 = final biomass (kg), W_1 = initial biomass (kg), t = time (days).

2.11. Data analysis and statistics

Production results, physical pellet quality and the histological ectopic goblet cell counts (total ectopic goblet cells, clusters of ectopic goblet cells, clusters of ≥5 ectopic goblet cells) were analyzed using one-way ANOVA and Tukey's post hoc test in IBM SPSS Statistics version 29.0.0.0. Histology severity scores were analyzed in R, version 4.1.0 by ordinal logistic regression using the polr command (MASS R package

version 7.3–58.1), followed by the Fisher's exact test for count data (Fifer package version 1.0). The level of significance was $p < 0.05$.

For microbiota, sequence data was processed in R version 4.1.0 as described in (Weththasinghe et al., 2022), where the DADA2 1.18.0 pipeline processed raw sequence data and generated amplicon sequence variants (ASVs) (Callahan et al., 2016; Callahan, 2023). One sample from the control diet was excluded because of a very low ASV count compared to all the other samples. Taxonomic analysis showed that 76.9% of ASVs were assigned at the genus level and 14.2% of ASVs were assigned at species level. The ASVs, alpha diversity indices (observed ASVs, Pielou's evenness, Shannon's index and Faith's phylogenetic diversity (PD)) and beta diversity indices (Jaccard distance, unweighted UniFrac distance, Aitchison distance and PHILR transformed Euclidean distance) were computed as in Li et al. (Li et al., 2021). The ASV table was rarefied based on minimum sequence size (14,084) in the samples to compute Jaccard distance and unweighted UniFrac distance.

RNA-seq raw data from the distal intestine were analyzed following Morales-Lange et al. 2021 (Morales-Lange et al., 2021). Raw reads were cleaned by BBDuk (v34.56) to trim/remove low quality reads, adapter sequences and PhiX (Illumina spike-in). Then, cleaned reads were aligned to *Salmo salar* genome SSAL_v3.1 (GenBank assembly accession: GCA_905237065.2). Fragments were counted using featureCounts (v1.4.6-p1) and differentially expressed genes (DEGs) were estimated between diets using SARTools R package (v1.7.3). Significant DEGs were determined when the adjusted *p* value (padj) was <0.05. ShinyGO v0.76.3 was used (with default parameters) for the enrichment analysis and functional classification of DEGs, as described in (34). Term categories (FDR < 0.05) were displayed and sorted by fold enrichment (minGSSize = 2%).

3. Results

3.1. Overall health status and growth performance

Fish were of good condition without notable clinical signs (external and internal), except for a few individuals with tapeworms in the intestinal tract, which was observed in all dietary groups. Routine inspections by an external fish veterinarian were conducted throughout the experimental period. Fish welfare was defined as satisfactory the fish showed normal swimming behavior and no disease outbreaks were detected. Growth performance results after 21 weeks of feeding with experimental diets are given in Table 3. There were no statistically significant differences between the groups related to fish growth performance.

3.2. Technical pellet quality analysis

All diets had high physical pellet quality, but insect meal inclusion significantly reduced expansion, durability, sinking velocity and water stability after 60 min (Table 4). Pellet length and hardness were not significantly affected, but a numerical reduction with increasing BSFL meal inclusion was observed.

Table 3
Growth performance after 21 weeks.

Growth parameter	IM0	IM4	IM8	*p-value*[1]	SEM[2]
Initial weight (g)	1340	1380	1240	0.50	0.28
Final weight (g)	4556	4747	4651	0.74	232.4
Specific growth rate (%)	0.87	0.86	0.91	0.75	0.17
Feed conversion ratio	1.27	1.35	1.35	0.39	0.06
Feed consumption (kg)	134,740	128,929	126,473	0.89	8612
Mortality (%)	3.7	7.7	6.5	0.55	3.34

IM0: 0% insect meal; IM4: 4% insect meal; IM8: 8% insect meal.
[1] Significance of the one-way ANOVA.
[2] Standard error of mean.

Table 4
Physical pellet quality of the experimental diets containing increasing inclusion level of black soldier fly larvae meal.

Pellet quality parameter	Insect meal (% diet)			p_{ANOVA}[1]	SEM[2]
	0	4	8		
Pellet length (mm)	9.23	9.04	8.87	0.2475	0.15
Hardness (kg)	8.27	7.77	7.60	0.0814	0.21
Durability (%)	96.3^{a}	98.5^{b}	90.3^{c}	<0.0001	0.49
Expansion (%)	41.2^{a}	38.0^{ab}	36.9^{b}	0.0196	1.07
Sinking velocity (mS^{-1})	0.123^{a}	0.117^{a}	0.097^{b}	<0.0001	0.00
Water stability 30 min	95.2	94.4	94.1	0.2413	0.43
Water stability 60 min	96.2^{a}	93.5^{b}	94.5^{ab}	0.0134	0.44

[1] Significance of the one-way ANOVA. Values in the same row with different superscripts are significantly different according to Tukey's multiple comparison test ($P \leq 0.05$).
[2] Standard error mean.

3.3. 16 s rRNA sequencing of distal intestine microbiota

The most abundant phyla in the distal intestine microbiota were *Proteobacteria* and *Firmicutes*, which represented >90% of the bacterial abundance for all three diets (Fig. 1a). *Psycrobacter* and *Carnobacterium* were the most abundant genera for all the diets (Fig. 1b). *Psycrobacter* was the most abundant geneus in diets IM0 and IM8, accounting for >40% and 30% of the average abundance, respectively. In IM4, *Carnobacter* was the most abundant genus, accounting for approximately 25% of the abundance, and *Psycrobacter* approximately 20%. Of the fifteen most abundant taxa, *Bacillaceae* ($p = 0.008$), *Lactobaccillaceae* ($p = 0.042$) *and Oceanobacillus* ($p = 0.0034$) were significantly more abundant in the insect meal diet groups compared to the control diet (IM0) group (Fig. 2).

The overlap of amplicon sequence variants between microbiota from digesta, feed and water (Fig. 3) revealed that the intestinal microbial composition overlaps with both feed and water. The ASV overlap between digesta and feed was, however, much larger than the overlap between digesta and water.

None of the four alpha diversity indices (observed ASVs, Shannon's index, Pielou's evenness, Faith's PD) showed significant differences between the three diets. Four indices were used to evaluate beta diversity and there was a separation between the control cluster and insect meal clusters in all indices (Fig. 4). Significant differences between control diet and insect meal diets were found for the Jaccard ($p = 0.001$), Aitchison ($p = 0.001$) and Unweighted Unifrac ($p = 0.023$) distance matrix (Table 5). Test for homogeneity of multivariate dispersions revealed no significant differences between the diets (Table 6).

3.4. Histology

No significant differences between diets were found for frequency of ectopic goblet cells (Fig. 5A), extent of epithelial vacuolization (Fig. 5B), and inflammation (Fig. 5C). There was a tendency of more clusters of ectopic goblet cells in the IM4 diet ($p = 0.058$, Table 7). The variability between samples within the same diet was apparent, as high and low severity scores were found within all dietary groups for the three analyzed parameters. Evaluation of the distal intestine revealed normal and healthy macroscopic morphology for sampled fish in this study.

3.5. RNAseq

DEGs number and term enrichment per diet comparison showed different patterns among groups for each organ (Table 8). In distal intestine, IM4 compared to IM0 (Fig. 6A) showed only downregulated terms (e.g., isoprenoid biosynthetic processes, terpenoid backbone biosynthesis, unsaturated fatty acid metabolic processes and alpha-Linolenic acid metabolism). In IM8 compared to IM0 (Fig. 6B), two terms were upregulated (response to estrogen and positive regulation of

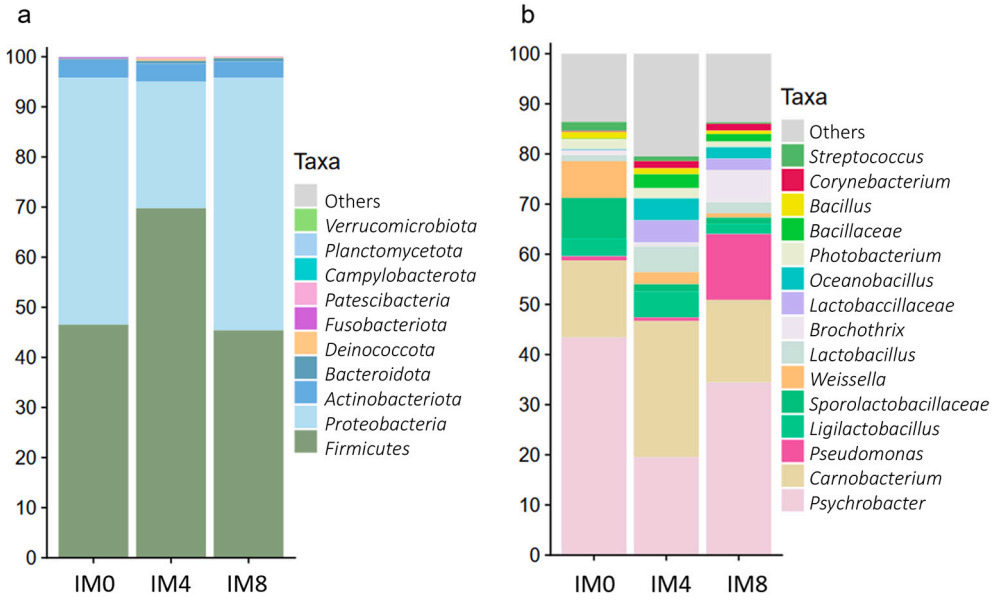

Fig. 1. Relative abundance of bacteria in distal intestine microbiota. (**a**): The ten most abundant phyla in the distal intestine microbiota. (**b**): The fifteen most abundant genera in the distal intestine microbiota. IM0: control diet containing 0% insect meal; IM4: 4% insect meal; IM8: 8% insect meal.

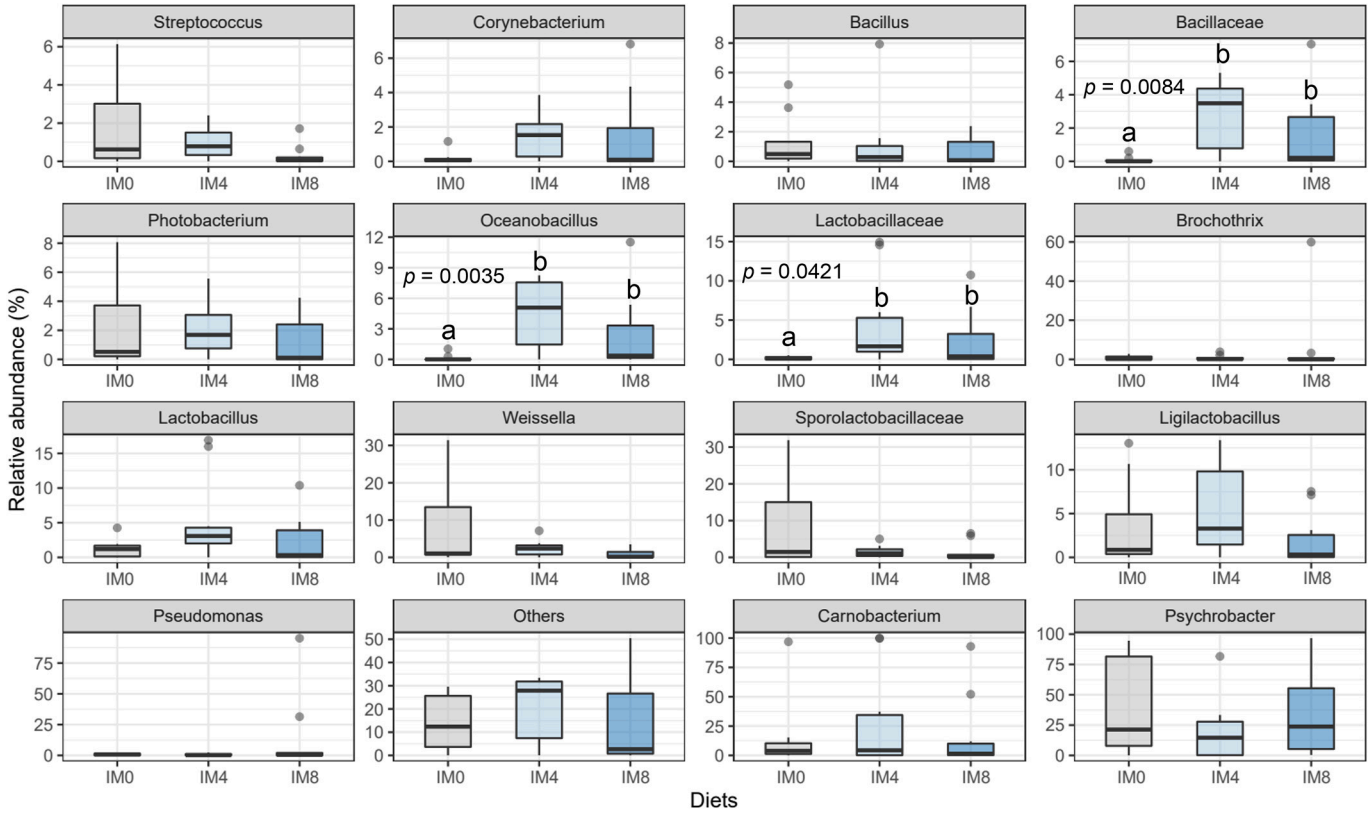

Fig. 2. Boxplots of relative abundance of the 15 most abundant taxa (genus or lowest taxonomic rank) in the digesta of fish fed experimental diets. The samples are grouped by diets. IM0: control diet containing 0% insect meal; IM4: diet containing 4% insect meal; IM8: diet containing 8% insect meal. Lower case letters a and b indicate taxa with significantly different relative abundance between the diets ($p < 0.05$).

gene expression) and one was downregulated (monocarboxylic acid biosynthetic processes). Moreover, the comparison between IM4 and IM8 (Fig. 6C) showed twenty-one terms upregulated (e.g., chemokine-mediated signaling pathway, extracellular vesicle, immune receptor activity, phagocytosis and positive regulation of cell population proliferation).

In head kidney, downregulated terms were detected in IM4 compared to IM0 (chromosome, telomeric region, Fig. 7A) and in IM8 compared to IM0 (e.g., indole-containing compound metabolic processes, aromatic amino acid family metabolic processes, stem cell differentiation and folate biosynthesis. Fig. 7B).

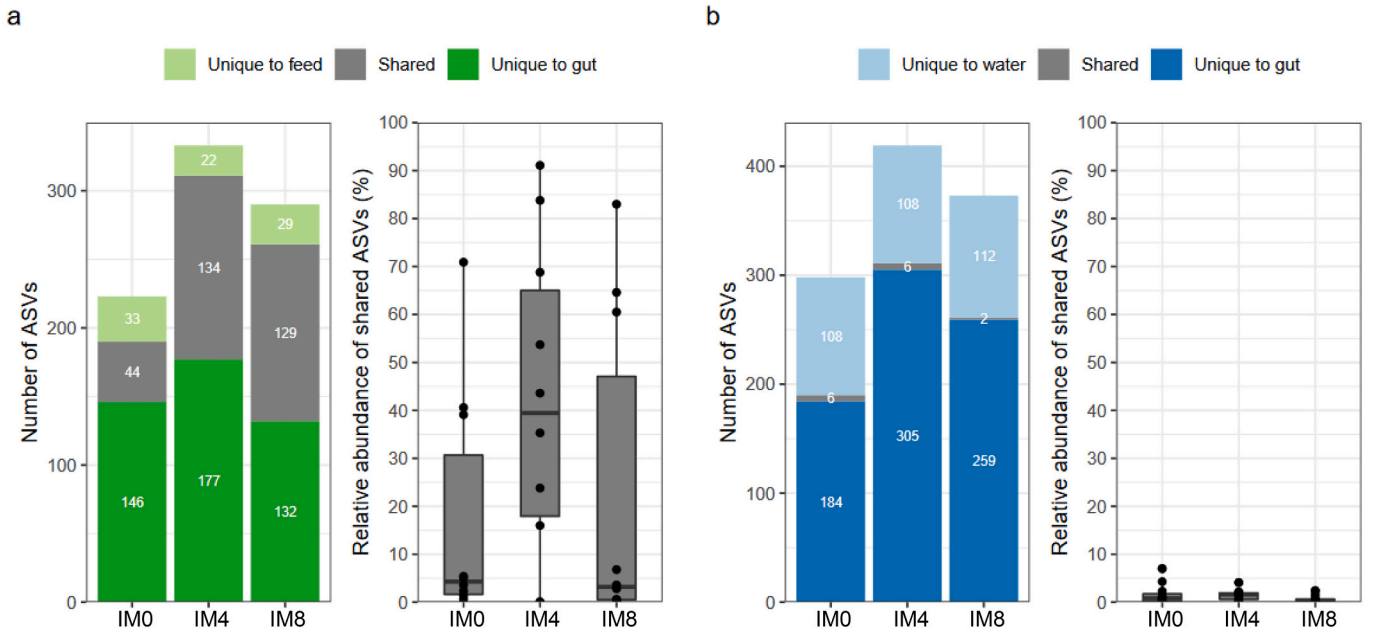

Fig. 3. Microbial overlap between the distal intestine digesta and feed (**a**) and the gut and seawater (**b**). The number of amplicon sequence variants is shown in the right figure of each panel. The minimum relative abundance of shared ASVs is shown on the right side of each panel. The minimum relative abundance of ASVs to be considered significant as present in a sample was 0.05%. Samples are grouped by diets: IM0: 0% insect meal; IM4: 4% insect meal; IM8: 8% insect meal.

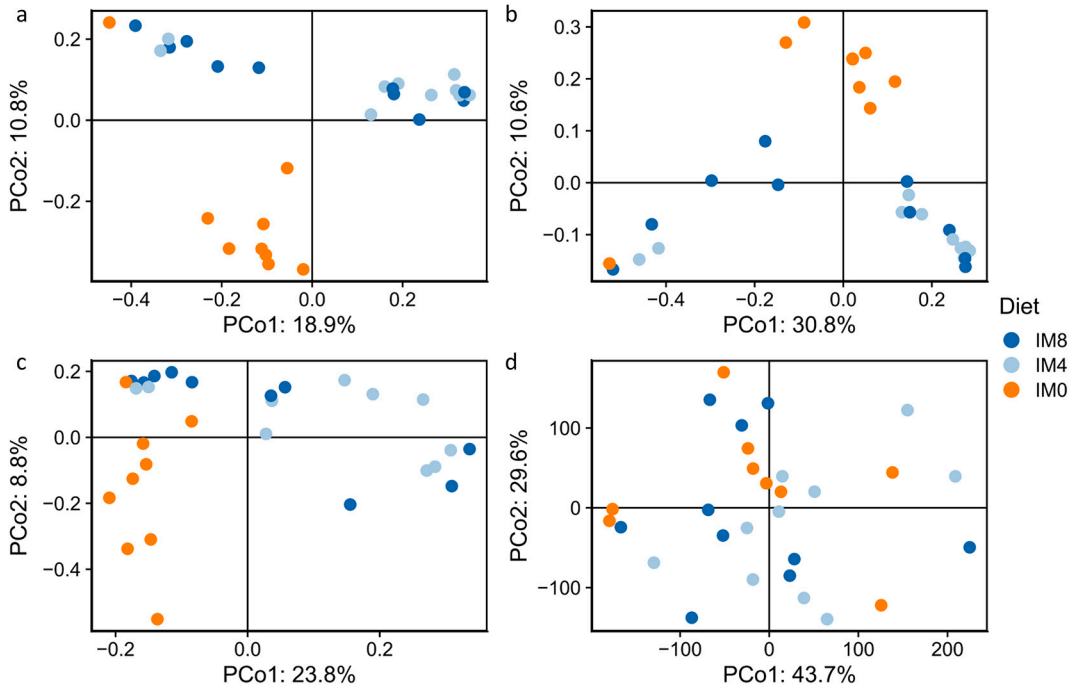

Fig. 4. Principal coordinate analysis (PCo) plots of beta diversity of gut microbiota in salmon fed experimental diets. The four different beta-diversity indices are (**a**) Jaccard distance (**b**) Unweighted Unifrac distance (**c**) Aitchison distance and (**d**) PhilR transformed Euclidean distance. The samples are grouped by diets: IM0: 0% insect meal; IM4: 4% insect meal; IM8: 8% insect meal.

4. Discussion

The present study reports the effects of replacing conventional protein ingredients with BSFL meal on growth performance, health, and intestinal microbiota in Atlantic salmon under industrial conditions in the grow-out phase in seawater. Published studies on how insect meal impacts Atlantic salmon have been performed in short-term smaller scale studies with considerably smaller sample sizes (Weththasinghe et al., 2021; Fisher et al., 2020; Belghit et al., 2019; Leeper et al., 2022; Radhakrishnan et al., 2022). Nevertheless, together with Radhakrishnan et al. (Radhakrishnan et al., n.d.), we are the first to take previous knowledge on BSFL meal to the industrial scale salmon farming level, which provides important knowledge on how these novel ingredients work under field conditions. Interestingly, both studies detected no differences in weight gain, growth rate, feed conversion ratio and mortality when replacing protein ingredients with BSFL meal, suggests

Table 5
PERMANOVA analysis for beta diversity of gut microbiota in Atlantic salmon fed experimental diets containing 0%, 4% and 8% black soldier fly larvae meal, adjusted *p*-value.

	Aitchison distance	Jaccard distance	Phylogenetic isometric log-ratio (PHILR) distance	Unweighted UniFrac distance
F value	2.3496	2.197	1.604	1.955
p value	0.001	0.001	0.141	0.023
Pair-wise comparisons				
IM0 vs IM4	0.003	0.003	0.183	0.012
IM0 vs IM8	0.003	0.003	0.726	0.078
IM4 vs IM8	0.744	0.633	1	0.822

IM0: 0% insect meal; IM4: 4% insect meal; IM8: 8% insect meal.

Table 6
Test of homogeneity of multivariate dispersions between diets, adjusted *p*-value.

	Aitchison distance	Jaccard distance	Phylogenetic isometric log-ratio (PHILR) distance	Unweighted UniFrac distance
F value	1.902	0.501	0.204	0.447
p value	0.169	0.604	0.816	0.664
Pairwise comparisons				
IM0 vs IM4	0.010	0.960	0.781	0.958
IM0 vs IM8	0.637	0.274	0.766	0.926
IM4 vs IM8	0.221	0.424	0.518	0.427

IM0: 0% insect meal; IM4: 4% insect meal; IM8: 8% insect meal.

that fish fed BSFL meal performed as well as those fed the conventional protein ingredients in the control diet.

The moderate inclusion levels of 4% and 8% BSFL were chosen due to limited bulk availability, relatively high prices, and limited knowledge on how higher inclusions levels of BSFL would affect technical feed quality, fish growth performance and health of Atlantic salmon during the grow-out phase. Challenges associated with BSFL as a feed ingredient are the adverse effects of the high lipid content and chitin on fish health. Chitin might influence growth performance by reducing feed intake and availability and digestibility of nutrients (Kroeckel et al., 2012). However, sustained growth performance is reported in other smaller scale studies where fishmeal was substituted with increasing amounts of BSFL meal in the diets for Atlantic salmon in seawater (Belghit et al., 2019; Lock et al., 2016). The upper limit for BSFL meal inclusion varies between studies, and it is difficult to compare as control diets differ from full fishmeal replacements to low BSFL inclusions replaced by plant protein sources. However, it has been suggested that Atlantic salmon show optimal growth up to 200 g kg^{-1} BSFL meal, as reviewed in (English et al., 2021).

The high lipid content in full-fat BSFL meal is challenging for the feed industry as it interferes with the extrusion process and reduces pellet quality (Weththasinghe et al., 2021; Sørensen et al., 2009; Lin et al., 1998). Even though pellet expansion, durability and sinking velocity decreased with insect meal inclusion, it did not cause any detectable effects on fish growth performance, since the pellets most likely had high quality in the relevant time frame between release of pellets until being eaten by the fish. Reduced pellet expansion with increasing BSFL inclusion has been reported in previous studies (Weththasinghe et al., 2021; Irungu et al., 2018). Even though the BSFL meal in the present study was fat-reduced, it contained 5.7% crude lipid. It is difficult to determine if the lipid content contributed to the measured reduction in physical pellet quality, but previous studies indicate that decreased expansion is due to high lipid content in the mash during extrusion (Hansen et al., 2011; Ilo et al., 2000; Navale et al., 2016). A high lipid level in extrusion increases lubrication and reduces friction in the extruder, resulting in a decreased dough temperature (Hansen et al., 2011; Lin et al., 1997). Lower dough temperature can reduce starch gelatinization (Lin et al., 1997; Garber et al., 1997; Morken et al., 2012), resulting in reduced expansion (Garber et al., 1997) and physical pellet quality (Morken et al., 2012). The results of the present study suggest

Table 7
Overview of ectopic goblet cells of the distal intestine.

Parameter	Diet	Avg.	Min.	Max.	Std. Dev.	*p* – value[1]
Single ectopic goblet cells	IM0	62.5	32	145	11.7	0.746
	IM4	73.4	19	187	16.6	
	IM8	57.9	3	120	15.1	
Ectopic goblet cell clusters	IM0	11.7	0	51	15.0	0.058
	IM4	18.6	6	52	16.5	
	IM8	3.5	0	21	6.6	
Clusters of ≥5 ectopic goblet cells	IM0	5.7	0	25	8.1	0.207
	IM4	12.3	0	63	21.4	
	IM8	1.5	0	9	2.8	

[1] *p*-value for one-way ANOVA. Statistical significance according to Tukey's post hoc test ($p < 0.05$). IM0: 0% insect meal; IM4: 4% insect meal; IM8: 8% insect meal.

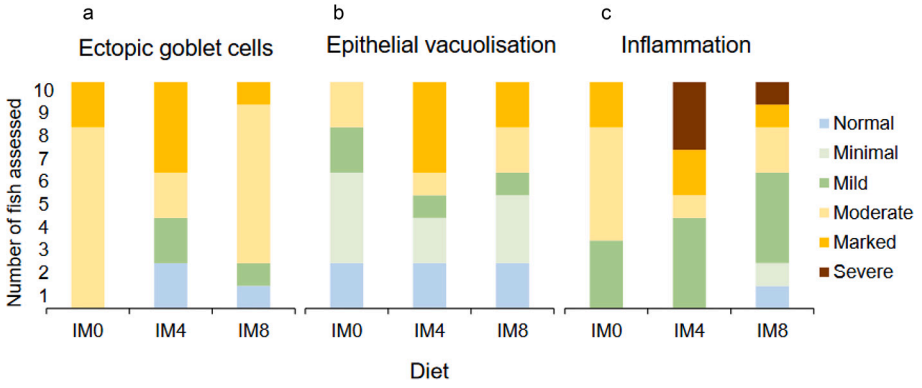

Fig. 5. Histopathological changes in the distal intestine of Atlantic salmon fed diets with black soldier fly larvae meal. IM0: 0% insect meal; IM4: 4% insect meal; IM8: 8% insect meal. Histological parameters assessed: (**a**) ectopic goblet cells, (**b**) epithelial vacuolization, (**c**) inflammation. Each parameter (**a-c**) was given a score of 0–5, where 0 represents normal morphology and 5 denotes severe morphology.

Table 8
Significant differentially expressed genes (DEGs) and term enrichment per comparison.

Sample	Comparison	DEGs		Term Enrichment	
		Upregulated	Downregulated	Upregulated	Downregulated
DI	IM4 | IM0	9	16	0	5
	IM8 | IM0	22	54	2	1
	IM4 | IM8	91	6	21	0
HK	IM4 | IM0	9	4	0	1
	IM8 | IM0	10	3	0	6
	IM4 | IM8	0	0	0	0

DI: Distal intestine. HK: Head kidney. IM0: 0% insect meal; IM4: 4% insect meal; IM8: 8% insect meal.

that high BSFL meal inclusions may compromise some physical pellet quality parameters, but a moderate inclusion of 8% did not cause any biologically relevant effects.

Results from 16 s rRNA sequencing showed that insect meal modulated the distal intestine microbiota (Fig. 1). The gut microbiota in fish has a vital role in maintaining health, nutrient metabolism, digestion, disease resistance, growth, immune response and welfare (Ghanbari et al., 2015; Wang et al., 2018; Hoseinifar et al., 2015). Improved health, nutrient utilization and growth could therefore be achieved by positive modulation of the microbiota. The phyla *Firmicutes, Proteobacteria* and *Actinobacteria* accounted for over 95% of the intestinal microbiota, which is as expected for fish microbiota in general, shown in previous literature as reviewed in (Ghanbari et al., 2015). *Psycrobacter, Carnobacterium, Pseudomonas and Ligilactobacillus* were the most abundant genera in this study. *Psycrobacter, Carnobacterium, Pseudomonas* are commonly found in the salmonid intestine in seawater (Kristiansen et al., 2011; Askarian et al., 2012). The most abundant genera in the present study differ from a study on Atlantic salmon fed insect meal in seawater published in 2021 by Li et al., where the most abundant genera were *Aliivibrio, Brevinema and Mycoplasma* (Li et al., 2021). The relative abundance of the taxa *Bacillaceae, Lactobacillaceae and Oceanobacillus* was significantly higher in the insect diets compared to the control (Fig. 2). An increase of these taxa was also found in Atlantic salmon smolts by Weththasinghe et al. (Weththasinghe et al., 2022), in post-smolts by Li et al. (Li et al., 2021) and in rainbow trout fed BSFL meal (Huyben et al., 2019; Terova et al., 2019). A chemical analysis revealed that the insect meal in this study contained 5.9% (g/100 g) chitin. Several studies have suggested that chitin acts as a substrate for chitinase producing bacteria in the fish gut (Bruni et al., 2018; Huyben et al., 2019; Askarian et al., 2012). The significant increase in abundance of *Bacillaceae* (Fig. 2) in fish fed insect meal diets could be explained by chitin acting as a substrate to increase the proliferation of chitinolytic bacteria represented by several *Bacillus* species (Huyben et al., 2019; Weththasinghe et al., 2022; Cody, 1989). High inclusion of chitin has been suggested to reduce nutrient digestibility in fish (Cardinaletti et al., 2019a; Kroeckel et al., 2012; Magalhães et al., 2017), but th moderate inclusion of 4 to 8% insect meal in the present experiment was not expected to decrease nutrient digestibility. Feed intake and feed conversion ratio were not affected by insect meal inclusion in this study. Chitin has been suggested as a preferred substrate for lactic acid bacteria in salmon gut (Bruni et al., 2018; Askarian et al., 2012). The present study showed that *Lactobacillaceae,* a family of lactic acid producing bacteria, increased with BSFL meal inclusion, as in previous reports on salmon and rainbow trout (Huyben et al., 2019; Weththasinghe et al., 2022; Li et al., 2021; Terova et al., 2019; Rimoldi et al., 2021). Lactic acid bacteria have been characterized as a positive indicator of gut health in fish, because of their ability to stimulate digestive function, mucosal tolerance, immune response and disease resistance as reviewed in (Ringø et al., 2018).

Alpha diversity was not affected by insect meal inclusion in the present study, while beta-diversity significantly increased. These findings suggest that insect meal inclusion changed the microbial composition (beta diversity) without increasing microbial richness (alpha diversity). In previous studies both alpha and beta diversity were changed by BSFL meal inclusion with more pronounced differences in Atlantic salmon and Rainbow trout, compared to the findings in the present study (Huyben et al., 2019; Weththasinghe et al., 2022; Li et al., 2021; Terova et al., 2019). This can be explained by a higher insect meal inclusion (15% - 50%) in these studies and considerably smaller sample size. Additionally, Leeper et al. also found that alpha and beta diversity was significantly impacted by 10% insect meal inclusion in salmon fry (Leeper et al., 2022). Hence, the impact of microbiota modulation by insect meal inclusion seems to be impacted by life stage and inclusion level.

Previous studies have shown that the microbiota composition of the water has a lower impact on shaping the intestinal microbiota compared to that of the diets in fish (Agboola et al., 2022; Lyons et al., 2017). This is in line with the present study, where the gut and feed microbiota overlapped to a much larger extent than the gut and water microbiota (Fig. 3). The insect meal inclusion of 4% and 8% had 129 and 134 shared ASVs between feed and gut, compared to 44 between IM0 diet and gut (Fig. 3). This is likely a contributor to the increased beta diversity observed for the insect meal diets.

Regarding morphometric parameters of the distal intestine, findings by Dale et al. in 2009 and 2018 indicated that ectopic epithelial cell clusters in salmonid intestine are associated with inflammation, and that the presence of ectopic epithelial cells in submucosal inflammatory foci indicated early stages in tumorigenesis in salmon fed commercially available feeds. They suggested that inflammation and carcinogenesis should be a focus of investigation in future feed trials (Dale et al., 2009; Bjørgen et al., 2018). In the present study, histological sections of the distal intestine were analyzed to investigate the impact of insect meal on inflammation and ectopic goblet cells when feeding diets containing BSFL meal over several months. The variation in histological score of ectopic goblet cells, epithelial vacuolization and inflammation observed in fish within the same diet, indicates that these parameters were likely caused by other factors in the feed or in the environment rather than the inclusion of BSFL meal. The histological results did not suggest that there is an impact of insect meal inclusion up to the levels used in this study on the distal intestine.

RNAseq of the distal intestine revealed that several immune-related gene ontology terms regarding chemokine and cytokine receptors, signaling pathways and responses were upregulated when comparing IM4 to IM8 (Fig. 6C). A broad range of studies have investigated the effects of chitin in fish, and generally dietary chitin has been suggested to activate the innate immune system in fish (Ringø et al., 2012). Previous studies have shown both positive and negative effects in fish. Chitin might act as an immune-stimulant and anti-inflammatory molecule in fish at low levels but might reduce fish growth and intestinal inflammation at high levels (Cardinaletti et al., 2019b). Chitin inclusion is suggested to decrease lipid digestibility by decreasing bile acid in the pyloric intestine, which is essential for activation of lipase and fatty acid absorption (Hansen et al., 2010). However, upregulation of immune genes was not observed when comparing IM8 to IM0, which might indicate that the upregulation of immune genes in Fig. 6C was not induced by chitin. The upregulation of immune genes in IM4 likely has a

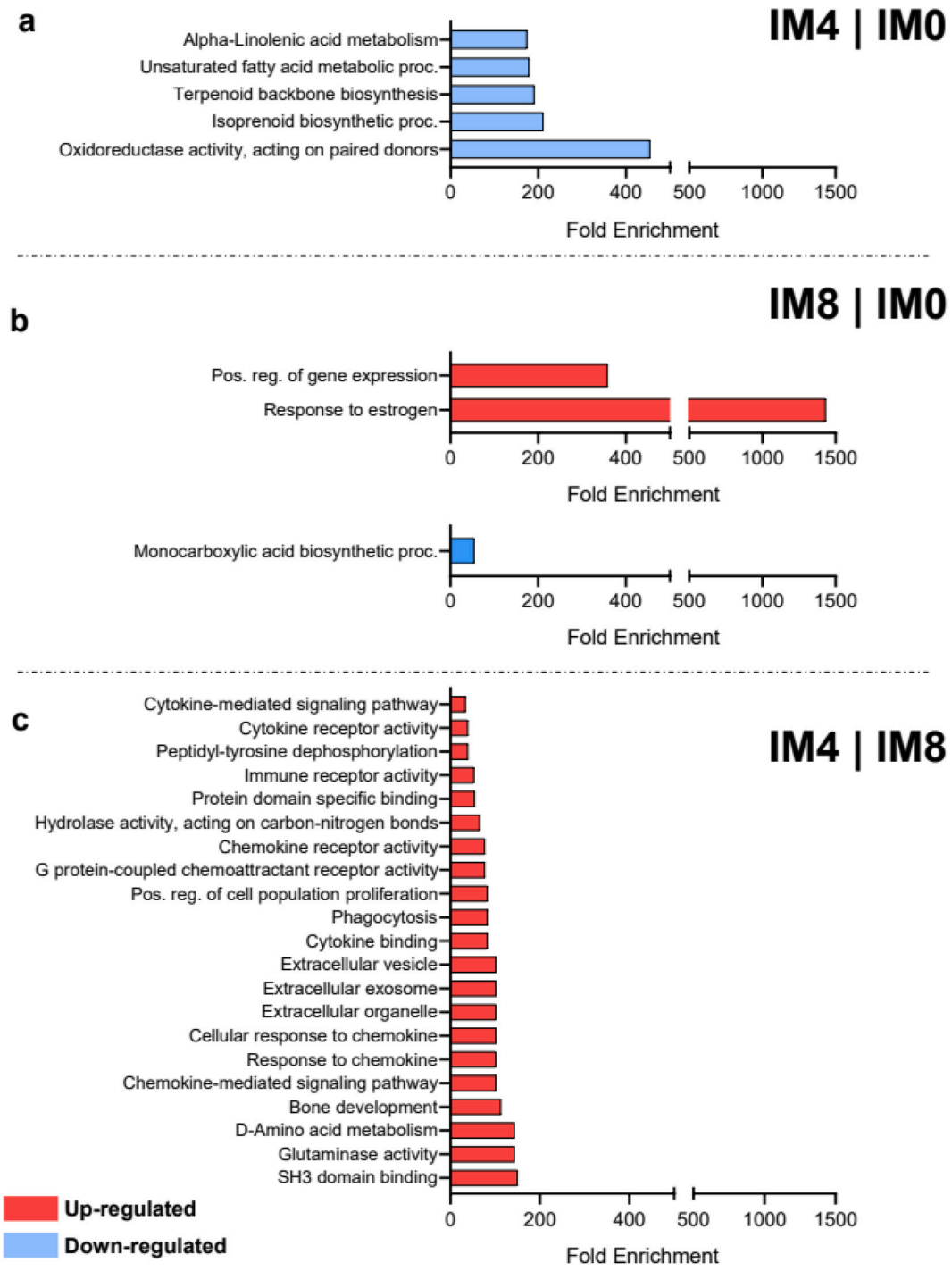

Fig. 6. Significantly enriched gene ontology (GO) terms in the distal intestine of fish fed 4% insect meal (IM4), 8% insect meal (IM8) and 0% insect meal (IM0). (**a**): GO terms in IM4 compared to IM0. (**b**): GO terms in IM8 compared to IM0. (**c**): GO terms in IM4 compared to IM8.

link to the frequency of severe inflammation found in some fish in the histology analyses. This is also in line with the 16 s rRNA sequencing results which showed that IM4 stood out from the two other diets. Alpha-linoleic acid metabolism and unsaturated fatty acid metabolic processes were downregulated when comparing IM4 to IM0 in the distal intestine, however the same effect was not seen in the comparison between IM0 and IM8, indicating that the effects were not enhanced by an increase in BSFL inclusion. As the insect meal in this study was defatted and contained 5.7% lipid, it is not a major contributor of fatty acids in the diets. The fish oil content was constant for the three diets. Weththasinghe et al. found that lipid digestibility and lipid retention decreased linearly by BSFL inclusion for Atlantic salmon in freshwater (Weththasinghe et al., 2021), which suggests that BSFL meal inclusion may also impact lipid profile of the fish.

The response to estrogen was upregulated in the IM8 group compared to control. Estrogen is known to modulate the gastrointestinal tract in fish (Buddington and Krogdahl, 2004). In the human gastrointestinal tract, estrogen and estrogen receptors have been shown to modulate the activity of epithelial ion secretion of HCO_3^- and Cl^-, which is important in maintaining body fluid homeostasis. The regulation of estrogen in ion channels and transporters are suggested to have beneficial impact (Yang et al., 2017). The gastrointestinal tract is recognized

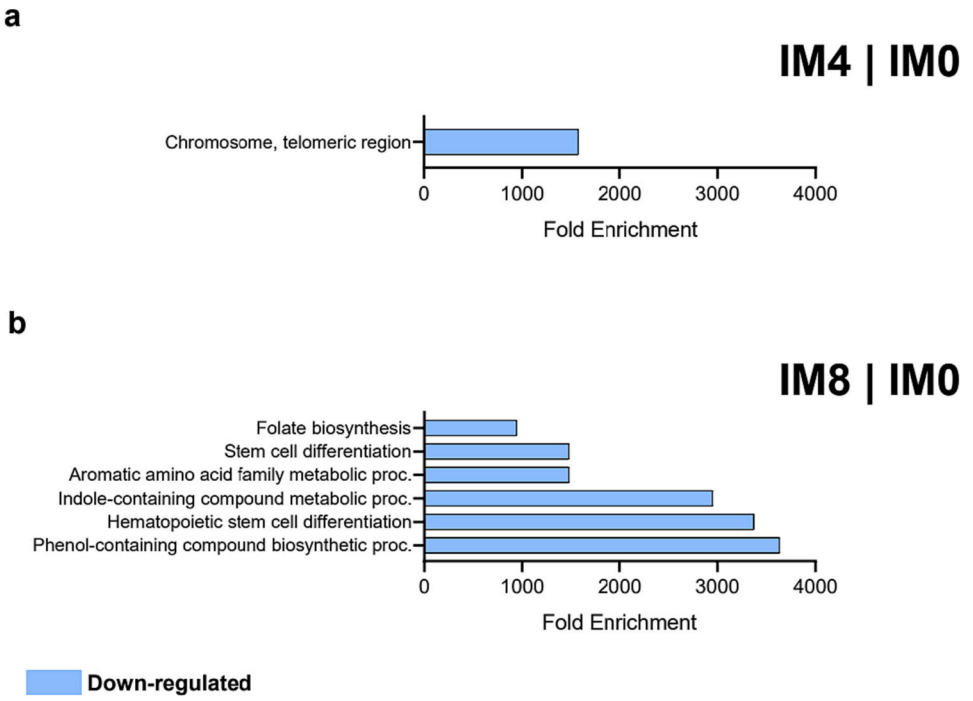

Fig. 7. Significantly enriched gene ontology (GO) terms in the head kidney of fish fed 4% insect meal (IM4), 8% insect meal (IM8) and 0% insect meal (IM0). (**a**): GO terms in IM4 compared to IM0. (**b**): GO terms in IM8 compared to IM0.

to be the largest endocrine organ in the vertebrate body (Holst et al., 1996), and secreted amounts of hormones change the characteristics of the gastrointestinal tract. The types of hormones released in the gastrointestinal tract of fish are partly determined by the conditions in the intestine, such as nutrients and pH (Buddington and Krogdahl, 2004). Altered microbiota and thereby metabolite composition in the intestine could be a driver of the regulation of the response to estrogen. The presence of estrogen receptors on fish leukocytes suggests that there is an estrogen-mediated immunoregulation and that estrogen modulates the innate immune response in fish (Szwejser et al., 2017).

The head kidney in fish recruits specific cell types during disease conditions like inflammation, in addition to several other immunological functions (Parra et al., 2015). Chitin acts as pathogen-associated molecular patterns (PAMPs) by binding to pattern recognition receptors (PRRs) and, thus, stimulating the production of cytokines and immune mediators (Stenberg et al., 2019). Previous studies have suggested that chitin has potential to stimulate the innate immune response in various fish species (Ringø et al., 2012; Cuesta et al., 2003; Esteban et al., 2001). However, no immune-related gene ontology terms were significantly enriched. In a study with post-smolt Atlantic salmon head kidney leukocytes, Stenberg et al. found that insect meal did not influence the transcription of inflammatory-related genes (Stenberg et al., 2019). The low number of significantly regulated terms in head kidney suggests that the inclusion of insect meal did not induce systemic inflammation. This is in line with the present study, where immune-related genes in the head kidney did not seem to be significantly affected by the 8% insect meal inclusion. On the other hand, the downregulation of gene ontology terms related to stem cell differentiation, metabolic and biosynthetic processes were observed (Fig. 7B). The downregulation of folate biosynthesis in IM8 could be caused by the lower inclusion of fishmeal (20.73% vs. 23.22% in IM0), which might have reduced the content of folate in IM8. It is difficult to draw conclusions based on the RNAseq results because of a small sample size and variability among fish from the same dietary group.

5. Conclusion

To conclude, replacing conventional protein sources with low to moderate levels of defatted BSFL meal (4 and 8%) did not compromise growth performance or health in Atlantic salmon under field conditions. These findings suggest that black soldier fly larvae meal when used at moderate inclusion levels is suitable as an alternative protein source in diets for Atlantic salmon in the grow-out seawater phase.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Acknowledgement

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