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Performance and physiological consequences of completely replacing soy protein in rainbow trout (*Oncorhynchus mykiss*) diets with semi-defatted black soldier fly (*Hermetia illucens*) larval meal

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ABSTRACT

In recent years, the aquaculture industry has seen an increasing substitution of fishmeal (FM) with plant-based ingredients. However, challenges inherent in the use of such ingredients drive an ongoing search for sustainable and cost-effective alternatives. To study the potential of high insect protein content in rainbow trout diets, a dose-response study was set up to assess the effects of replacing up to 100 % of soy protein concentrate (SPC) with a meal of semi-defatted black soldier fly (*Hermetia illucens*) larvae. In a 10-week feeding experiment, a homogeneous group of 1100 rainbow trout (initial body weight: 135.8 ± 15.3 g) were supplied with 10 different feeds: a commercial soy-based control and diets in which the SPC content was replaced with increasing proportions of black soldier fly larval meal (BSF). All diets also incorporated the same low fishmeal content (7.5 %) and were tested with (Control+, 25BSF+, 50BSF+, 75BSF+ and 100BSF+) and without (Control, 25BSF, 50BSF, 75BSF and 100BSF) a faecal binder treatment. At the end of the experiment, growth performance, feed utilisation, organosomatic indices, and fillet yields were determined alongside histological and transcriptomic analysis of the liver and intestine. Results indicated that substitution with BSF was associated with increased feed intake and a significant, although non-linear, improvement in growth, hinting at nutritional deficiencies in the commercial SPC controls. However, fish fed with insect-free diets exhibited improved protein retention and feed conversion ratio (FCR). No significant differences were apparent in intestinal or liver histology, gene expression or fillet processing yield between treatments, indicating that even a complete replacement of SPC with BSF can take place without compromising rainbow trout health or productivity. This study is a pioneer in demonstrating that a complete substitution of SPC with BSF is possible without adversely affecting the performance of rainbow trout in aquaculture, highlighting once more the potential of these insects as an alternative for feeding salmonids in the near future, as long as regulations worldwide enable more sustainable production of insects for feed purposes.

1. Introduction

Feeding the world's growing population without exceeding the planet's environmental limits is one of the greatest challenges of the 21st century. Aquaculture plays a crucial role in meeting global demand for food, contributing with over 50 % of the globally consumed fish (FAO, 2024). However, as the sector continues to expand and become more integrated into the global food system, concerns about its environmental

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impact will increase accordingly (Newton et al., 2022). Sourcing high quality, cost-efficient feed ingredients in an intensifying sector, while meeting sustainability standards has become a significant challenge. Over the years, efforts have been made to maintain nutritional efficiency while reducing the weight of dependency on marine ingredients for aquafeeds, particularly for high-valued carnivorous species (Newton et al., 2022). Several vegetable protein alternatives are available to replace fishmeal (FM) in commercial aquaculture feeds, with soy-based variants being best established (van Riel et al., 2023). In 2020, approximately 40.5 % of salmonid diets were constituted of plant-based protein sources, with soy protein concentrates (SPCs) constituting approximately 21 % of feeds formulations (Aas et al., 2022a; 2022b). The production of SPCs comes at a relatively high cost, due to the processing required to maximise digestibility and reduce levels of anti-nutrients (Li et al., 2015). SPCs are used to mitigate the nutritional disadvantages of soybean meal (SBM), such as poor palatability, insufficiencies of essential amino acids (EAAs), minerals and vitamins (Zhu et al., 2020) and high levels of anti-nutritional factors (ANFs) linked to inflammatory responses in the distal intestine of fish (Merrifield et al., 2011). Although SPCs can successfully substitute FM in aquafeeds, as earlier suggested (Brinker and Reiter, 2010; Drew et al., 2007; Olli and Krogdahi, 1994), soy production is associated with numerous factors that render it a less desirable choice for aquaculture feeds. Its use impacts feed-food competition directly, since it could be used as a human food source and indirectly, by increasing the area of agricultural land used for feed production rather than human food production (van Riel et al., 2023). Soy production is also associated with environmentally problematic practices such as deforestation and the use of fertilisers and pesticides (Newton and Little, 2017; Pelletier et al., 2018). Nowadays, aquafeeds are the biggest contributor to the environmental impacts of aquaculture (Wind et al., 2022), amounting to approximately 57 % of the sector's total emissions, mostly through crop production and marine resource extraction for ingredients (MacLeod et al., 2020).

Incorporating insects into animal nutrition is not a novel concept (DeFoliart, 1989), but it has received considerable renewed attention in recent years. Scientific evaluations of their potential in alternative aquafeeds have significantly increased, pointing to a significant contribution in the future of the aquafeed industry (Hua et al., 2019; Tran et al., 2022; van Huis, 2019). As part of a natural diet, insects provide a sound balance of amino acids, lipids, vitamins, and minerals for most farmed salmonids (Nogales-Mérida et al., 2018), such as rainbow trout (*Oncorhynchus mykiss*). In Europe, products from seven different insect species are currently approved for use in aquaculture diets under an amendment to Commission Regulation (EU) 2017/893. Similar regulatory developments are also underway in other regions, reflecting a growing global interest in the use of insects in aquaculture. Of the approved species, the best studied are black soldier fly (*Hermetia illucens*, BSF), yellow mealworm (*Tenebrio molitor*) and common housefly (*Musca domestica*) (Gasco et al., 2020). Recent reviews identify insect meal as one of the most promising and sustainable alternatives for fish feeds in the forthcoming years (Hua, 2020; Hua et al., 2019; van Huis, 2019).

In the context of animal feed BSF larval meal (BSF) is credited with several advantages over other insect ingredients (Gasco et al., 2020; Tran et al., 2022). Besides being a polyphagous and a particularly protein rich organisms, the intestinal tract of BSF contains considerable amounts of amylase, lipase, and protease activity, rendering it a high-quality bioconverter (Barragan-Fonseca et al., 2017). BSF larvae are fast-growing, able to perform rapid conversion of feed to body mass (van Huis, 2012). For salmonids, besides presenting lower in methionine and cysteine, a BSF-based diet can offer a suitable and well-balanced amino acid profile including EAAs, and a potentially valuable lipid fraction—rich in both EPA and DHA when the insects are properly provided with diets enriched in these fatty acids (English et al., 2021). In addition, a plastic development trajectory means BSF can be easily manipulated by diet (Oonincx and Finke, 2020), to express an optimal nutrient profile in its larval form. BSF larvae can contain between 40 % and 45 % protein and 26–35 % lipids as a proportion of a dry weight, making it a specially promising alternative for carnivorous fish, which typically require 45–55 % crude protein and 16–30 % crude lipid in their diets (Barragan-Fonseca et al., 2017; Jobling, 2011; Nogales-Mérida et al., 2018). BSF larvae are also rich in bioactive compounds, including some with antibiotic properties, reducing the presence of bacterial pathogens and enhancing gut health (Xia et al., 2021). Furthermore, BSF can be reared on organic waste streams, requiring less land and causing relatively lower of greenhouse gas emissions compared to conventional sources. However, this method is currently not permitted in the EU and remains restricted in several other countries (Makkar et al., 2014; Oonincx et al., 2015; van Huis, 2012).

The applicability of BSF as a feed ingredient in the salmonid industry has already shown promising, if controversial, results (Weththasinghe et al., 2021a). The majority of published studies focus on partial replacement of FM with BSF (Belghit et al., 2018; Couto et al., 2022; Melenchón et al., 2022; Stadtlander et al., 2017), while fewer publications reported its potential for substituting plant-derived ingredients (Dietz and Liebert, 2018; Hossain et al., 2021; Randazzo et al., 2021a). Furthermore, although considerable debate continues regarding optimal replacement levels, the potential of BSF as a complete substitute for SPC in salmonid diets has yet to be investigated (Weththasinghe et al., 2021a).

This study hypothesises that a replacement of up to 100 % of SPC with BSF, with and without an inclusion of 0.3 % guar gum (GG) as a faecal binder, will maintain or even improve growth performance and feed utilisation of rainbow trout, without inducing adverse physiological effects on fish. To test this hypothesis a 10-week dose-response experiment was conducted using 10 balanced experimental diets which SPC was progressively replaced with BSF at 0 %, 25 %, 50 %, 75 %, and 100 %, with and without the addition of GG. The study aimed to evaluate the effects of these dietary modifications on growth performance, feed efficiency, and fish health, and to investigate possible physiological consequences of using BSF in fish diets through histological analysis and gene expression profiling. An account of the consequences of dietary treatments on faecal rheology will form part of a follow up publication.

2. Materials and methods

The experiment was conducted at the fisheries research station of Baden-Württemberg (Langenargen, Germenay), strictly following the recommendations of the German and European guidelines on the protection and welfare of animals used for scientific purposes (TierSchVersV, 1 August 2013 and Directive 2010/63/EU of the European Parliament of the European Union Council).

2.1. Experimental diets

This study deployed 10 iso-lipidic (29 % crude lipid, DM basis) and iso-nitrogenous (46.7 % crude protein, DM basis) extruded diets (pellet size 4.5 mm) whose ingredients and proximate composition are shown in Table 1. The diets were formulated to meet the specific nutritional requirements of rainbow trout (Jobling, 2011; NRC, 2011) and to test the effects of SPC, initially included at 30 % (on ingredient basis), substitution with increasing levels of BSF on fish performance and health, while maintaining a constant FM inclusion of 7.5 % in all diets. A soy-based commercial standard diet (control) was used as a reference, while treatments incorporated four increasing substitution levels of BSF at 25 (25BSF), 50 (50BSF), 75 (75BSF), and 100 % (100BSF). An additional 0.3 % GG was added to five of the resulting duplicate diets (control+, 25BSF+, 50BSF+, 75BSF+, and 100BSF+). To account for potential nutritional deficiencies, all diets were balanced with respect to EAAs and supplemented with selected additives, such as vitamin and mineral premixes. Fish oil (FO) and rapeseed oil were added to ensure adequate levels of long-chain unsaturated fatty acids (LC-PUFAs). The

Table 1
Formulation and proximate composition of the experimental diets.

Diets	Control	25 BSF	50 BSF	75 BSF	100 BSF	Control+	25 BSF+	50 BSF+	75 BSF+	100 BSF+
Ingredients, %										
Soy protein concentrate[a]	30.00	20.00	10.14	5.00	-	30.00	20.00	9.10	5.60	-
Black soldier fly meal[b]	-	10.00	20.00	30.00	40.00	-	10.00	20.00	30.00	40.00
Fish meal[c]	7.50	7.50	7.50	7.50	7.50	7.50	7.50	7.50	7.50	7.50
Soybean meal[d]	-	-	5.00	5.00	-	-	-	5.00	5.00	-
Wheat[e]	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Wheat gluten[f]	16.58	18.45	17.35	17.71	18.71	15.00	18.00	18.12	16.9	18.3
Guar meal[g]	5.00	5.00	5.00	1.48	-	6.91	5.65	5.00	0.72	-
Fish oil[h]	9.30	9.73	10.53	11.55	16.40	9.18	9.67	10.50	12.87	16.24
Rapeseed oil[i]	14.23	12.25	10.10	8.22	3.00	14.04	12.27	10.11	7.00	3.00
Additives n′ premixes	11.39	11.06	8.38	7.54	8.38	11.07	10.61	8.36	8.11	8.67
Guar gum[j]	-	-	-	-	-	0.30	0.30	0.30	0.30	0.30
Yttrium oxide	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Proximate composition (% DM)										
Dry matter (DM), %	92.70	92.90	93.20	92.40	93.40	93.60	93.00	93.30	93.20	92.70
Crude protein	46.17	46.50	46.78	46.86	47.00	46.26	46.24	46.95	46.67	47.14
Crude fat	28.91	28.85	28.86	29.00	29.76	28.63	28.71	28.51	28.54	29.77
Ash	4.85	4.84	5.36	5.95	6.96	4.91	4.84	5.36	6.22	6.80

Diets proximate composition was provided as estimated values for near-infrared spectroscopy (NIRS).
[a] European Commodity Company S. a., Kaliningrad Region.
[b] Black soldier fly (*H. illucens*, BSF) meal: 15% crude fat; 56% crude protein; chitin content not estimated. Protix Ingredients BV, Bergen op Zoom, Netherlands. S. a., Kaliningrad Region.
[c] Pelagia Egersund Sildoljefabrikk, Egersund, Norway.
[d] Felleskjøpet, Stavanger, Norway.
[e] Lantmännen, Mälmo, Sweden.
[f] Tereos Starch and Sweet BE (FR dep.), Lillebonne, France.
[g] Sunita Hydrocolloids PVT.LTD, Rajasthan, India.
[h] Pelagia Måløy Sildoljefabrikk, Norway.
[i] European Commodity Company S. a., Kaliningrad Region.
[j] Sa Seah International, Boulogne-sur-mer Cedex, France.

diets also included 0.2 % indigestible yttrium oxide (Y_2O_3) as an inert marker for digestibility measurements. All the manufacturing details are property of Skretting (Stavanger, Norway), and the proximate dietary compositions are provided as estimated values for near-infrared spectroscopy (NIRS). All feeds were kept in an aerated cooling chamber at a constant temperature of 5 °C to preserve their properties.

2.2. Feeding experiment

The feeding experiment followed a dose-response design and was conducted at the Fisheries Research Station of Baden-Württemberg (Langenargen, Germany) over a 10-week period, following the recommendations of the German and European guidelines on the protection and welfare of animals used for scientific purposes. During an acclimatisation of 3 weeks, a homogenous group of 1100 rainbow trout from a local strain (initial body weight (IBW): 135.8 ± 15.3 g), was fed with a commercial feed (EFICO Enviro 920 Advance, BioMar Group, Denmark). After acclimatisation, the fish were randomly distributed into 2 systems comprising a total of 20 green circular fiberglass tanks (55 fish per tank) with a volume of 0.33 m^3 . The 2 systems were operated in flow-through mode, using sand-filtered freshwater from Lake Constance (with a constant flow rate of 5 L min^{-1}). During the experiment, the photoperiod was fixed at 12 L:12D, *i.e.*, 12 h light and 12 h darkness (Lumilux® daylight lamps provided approximately 160 lux at the water surface between 07:30 a.m. and 07:30 p.m., with a 30 min sigmoidal transition period designed to simulate dawn and dusk. In order to maintain stable water parameters, the system was equipped with probes that continuously monitored temperature (Temperature Probes, Oxyguard, Denmark) and dissolved oxygen (Oxygen Probes, OxyGuard, Denmark), which were maintained automatically at 10.2 ± 1.72 °C and 11.0 ± 1.45 mg L^{-1} respectively. The fish were hand fed twice a day (8:30 a.m. and 3:30 p.m.), 6 days per week (Monday to Saturday), until apparent satiation. Thus excess feeding was kept to a minimum, resulting in a daily feed intake of 1.1 % of the body weight. All treatments were tested in duplicate, and the feed allocated to each tank was quantified daily. The animals were all-female rainbow trout, to eliminate the possibility of precocious maturation and minimise variability by sex-related effects.

2.3. Sampling

At the beginning and end of the experiment, all fish were individually weighed (g) and examined for external and internal macroscopic abnormalities. A pool of 20 fish from the initial stock and a pool of 5 fish from each tank at the end of the experiment were randomly sampled for transcriptome analysis of the proximal intestine. The samples were placed in cryotubes and stored at −80 °C until required for analysis. During the final sampling, 2 additional fish per tank were randomly sampled for histological analysis and tissues were collected from the liver and the proximal and distal intestine. Faecal material from all tanks was collected by dissection of distal intestine to perform the digestibility measurements. The faeces were immediately frozen at −20 °C until analysis. In addition, 10 fish per tank were sampled and filleted by an experienced person to assess processing yields. All fish were sampled by first stunning with a sharp blow to the head followed by a sacrificial gill cut.

2.4. Histological analysis

Histological evaluation of tissues from the liver, proximal, and distal intestines was performed at the University of Veterinary Medicine in Hannover, using standard methods (Miebach et al., 2023). After dissection, tissue samples were fixed using 4 % buffered formaldehyde (pH 7.2). Tissues were collected immediately after euthanasia; the liver was sampled from the mid to posterior part of the organ, while the proximal intestine, from the part immediately after the pyloric caeca, and the distal intestine in the final section of the tract, just before the anus. All tissues were dissected using sterile instruments under aseptic conditions on a cooled dissection tray. Before analysis, the tissues were dehydrated in series of graded ethanols, and embedded in paraffin to

form solid blocks. Posteriorly, histological cross-sections of 2 μm were cut, mounted, deparaffinised in butyl acetate and isopropanol and stained with haematoxylin eosin (HE) or alcian blue-PAS (AB-PAS). HE stained sections were used to measure the thickness of *stratum granulosum*, and *stratum compactum* (4 measurements/slide). Inflammatory cell infiltration into *lamina propria* was graded using a predetermined scoring system, (0: absent; 1: low; 2: moderate; and 3: high). AB-PAS stained sections were used to determine the density, as well as the goblet cells filling levels, also based on a score from 1 to 3, representing low to high respectively. The entire analysis was randomised, and blindly evaluated by light microscopy.

2.5. Microarray hybridisation and quantitative PCR analysis

Samples from the proximal intestine (approximately 2 g) of fish fed diets containing 0 % or 100 % BSF, with or without an additional 0.3 % GG (control, control+, 100BSF, and 100BSF+), were added to 1 mL of TRIzol (Thermo Fisher Scientific, MA, USA) along with five 2.8-mm ceramic beads (Peqlab Biotechnologie, Germany) in reaction tubes. After incubation on ice for 5 min, the samples were homogenised using a Precellys 24 homogeniser (Bertin Technologies SAS, France). RNA was isolated by first adding chloroform to the TRIzol-tissue mixture, followed by isopropanol to precipitate the RNA. The resulting RNA pellet was dissolved in 100 μl of ultrapure water and further purified using the Isolate II RNA Mini Kit (Biocat/Meridian Bioscience, OH, USA) according to the manufacturer's instructions. Individual RNA samples were then converted to Cy3-labeled cRNA and hybridised with 8 × 60 K Agilent Salmon Oligo Microarrays (ID 020938, Agilent Technologies; GEO platform: GPL21057) following the Agilent 60-mer oligo microarray processing protocol of Martorell-Ribera et al. (2022). A G2505C Microarray Scanner System (Agilent Technologies) was used to scan the fluorescence signals of the hybridised Agilent microarrays at a resolution of 2 μm. The full complement of microarray data was deposited in the NCBI database Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/; accession: GSE243211). The reliability of the microarray-predicted expression difference was validated using reverse transcription-quantitative PCR (RT-qPCR). A dlgap4-specific oligonucleotide primer pair (exon-exon junction spanning sense primer: 5'-GGAGAACGGCAGGCCACTCA-3'; antisense primer: 5'-GAGTACGCAGTGGTGCGGCA-3') was designed using the Pyrosequencing Assay Design software (v.1.0.6; Biotage, Uppsala, Sweden) to amplify an 186-bp amplicon. Rps5 (ribosomal protein S5), rna18s (18S ribosomal RNA (Köbis et al., 2016)), actb (beta-actin (Rebl et al., 2008)), and eef1a1 (eukaryotic translation elongation factor 1, alpha 1 (Bowers et al., 2007)) were chosen as reference genes (with coefficients of variation, CV < 0.20). Individual RNA samples from rainbow trout (system A and system B, each n = 4) fed with 100BSF, 100BSF+, control, and control+ were reverse-transcribed into cDNA using the SensiFAST cDNA Synthesis Kit (Bioline/Meridian Bioscience). The qPCR analysis was conducted with the LightCycler-96 system (Roche, Mannheim, Germany) using the SensiFAST SYBR No-ROX Kit (Bioline/Meridian Bioscience, Luckenwalde, Germany), according to the following program: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s; primer annealing at 60 °C for 15 s; elongation at 72 °C for 15 s; and fluorescence measurement at 72 °C for 10 s. Melting curve analysis validated the amplification of the target fragment. In addition, amplicons were visualised on 3 % agarose gels to evaluate product size and quality.

2.6. Apparent dry matter digestibility

To determine dry matter (DM) and yttrium oxide (Y_2O_3) contents, faecal matter dissected from the distal intestines was first lyophilised and homogenised, following the methods presented by Schumann et al. (2016). After 100 mg of faecal material was transferred into pressure digestion vessels and processed in a microwave pressure digestion system (SPEEDWAVE Four, Berghof, Eningen, Germany). To accomplish the digestion, a digestion reagent solution was prepared by adding 2 mL nitric acid (HNO_3; 65 %), 1 mL hydrogen peroxide (H_2O_2; 30 %, stab. p. a.) and 5 mL distilled water and added to the vessels. The DM content of each sample was obtained from the ratio of dry to wet weight, before and after lyophilisation. After sample preparation, the Y_2O_3 levels were determined using inductively coupled plasma mass spectrometry (ICP-MS) at the federal chemical analysis service of Baden-Württemberg (Sigmaringen, Germany).

The apparent digestibility coefficient (ADC) of dry matter was calculated as follows:

$$ADC(\%, DM) = 100 - (100 \times Y_2O_{2(diet)} / Y_2O_{2(faeces)})$$

2.7. Analytical determinations

The collected data was used to determine the specific growth rate (SGR), thermal-unit growth coefficient (TGC), total feed intake (TFI), feed conversion ratio (FCR), protein efficiency ratio (PER), hepatosomatic index (HSI), viscerosomatic index (VSI), head-on gutted yield (HOG) and skin-on fillet yield (SK-ON FIL) obtained as follows:

$$SGR(\%/day) = (\ln FBW(g) - \ln IBW(g)/time(days) \times 100$$

$$TGC = (FBW^{1/3}(g) - IBW^{1/3}(g)) \times (\sum T(^{\circ}C) \times time(days))$$

$$TFI(g) = \sum daily\ consumed\ feed(g)$$

$$FCR = total\ feed\ intake(g)/weight\ gain(g)$$

$$PER(\%) = weight\ gain(g)/protein\ intake(g) \times 100$$

$$HSI(\%) = liver\ weight(g)/body\ weight(g) \times 100$$

$$VSI(\%) = viscera\ weight(g)/body\ weight(g) \times 100$$

$$HOG(\%) = head - on\ gutted\ weight(g)/body\ weight(g) \times 100$$

$$SK - ON\ FIL(\%) = filletw/skin\ weight(g)/body\ weight(g) \times 100$$

2.8. Data analysis

Growth performance, feed utilisation, organosomatic indices, histological analysis, and processing yields data were expressed as grand marginal means ± SD, considering each tank as an experimental unit. All data was tested for homogeneity and normality. Differences between treatments were detected by a generalised linear mixed model (GLMM), considering insect protein percentage and GG as independent variables, with tank as a random effect factor. When the results showed significance, means between treatments were compared using Tukey's post-hoc test to correct for multiple comparisons. The analysis described above was performed using JMP® Pro, Version 17.2.0 (2023) SAS Institute Inc., Cary, NC, 1989–2024. Differential gene expression between the control and treatment group (100BSF+) was evaluated using the comparative ΔΔCt method. Microarray-image files were read and background-corrected using the Agilent Feature Extraction Software (FES) 10.7.3.1. Features that passed this quality control were further analysed with the limma package (Smyth GK. "limma: Linear Models for Microarray Data" (Gentleman et al., 2005), in RStudio (2022.02.3). Following quantile normalisation, pairwise comparisons of the transcript abundances from the individual datasets from 100BSF/100BSF+ *vs*. control/control+ were employed. Hierarchical/K-means clustering and heatmap matrix analysis were performed using the R packages pheatmap, factoextra, and cluster. To control for false discovery rate, p-values were adjusted (Benjamini and Hochberg, 1995) returning only one feature (ID: A_05_P448532) with an adjusted *p-value* (*q-value*) of < 0.05. This feature was annotated using the Basic Local Alignment Search Tool (BLAST) (tax id: 8022; coverage and

sequence identity: > 98 % of > 90 %, E value: 1 ×10–22). The RT-qPCR data were extracted using the LightCycler-96 analysis software v. 1.1.0.1320 (Roche) and normalised against the geometric mean of individual reference-gene expression values. The GraphPad Prism software (v10.0.2) was used for the statistical analysis (Student's *t* test) of normalised RT-qPCR data.

The results were considered significantly different at $p < 0.05$ level.

3. Results

3.1. Fish performance and feed utilisation

All experimental diets were well accepted by rainbow trout and no differences in behaviour were observed during hand-feeding. Regardless of treatment, fish performed well in terms of growth and feed utilisation for all parameters measured, as shown in Table 2. At the end of the experiment, fish growth–expressed as FBW, WG, SGR, and TGC–exhibited no significant differences between treatments, except for SGR, which presented significantly lower values (between 1.34 ± 0.05 and 1.35 ± 0.02 % day^{-1}) for groups fed control diets ($F_{(4,10)} = 4.97$; $p = 0.018$), compared to the test groups (between 1.42 ± 0.06 and 1.48 ± 0.03 % day^{-1}). The control groups also exhibited significantly lower TFI values ($F_{(4,10)} = 5.10$; $p = 0.017$), compared to fish fed with increasing levels of BSF. In terms of feed utilisation, even though they were consumed less, the control treatments showed a better FCR, between 0.75 ± 0.01 and 0.76 ± 0.02 ($F_{(4,10)} = 6.79$; $p = 0.007$), and higher PER, between 3.07 ± 0.06 and 3.08 ± 0.05, differing significantly from the remaining groups ($F_{(4,10)} = 11.53$; $p = 0.001$). Increasing proportions of dietary BSF did appear to impact on the ADC_{DM}, with values between 72.0 ± 0.34 and 77.4 ± 0.48 % ($F_{(4,10)} = 3.12$; $p = 0.066$). The addition of 0.3 % GG did not appear to exert any significant effect on any of the analysed parameters. All reported mortality was attributable to occasional escapes from the tanks, with survival rates varying between 99.09 % and 100 %, with no significant differences between treatments.

3.2. Organosomatic indices and processing yields

At a macroscopic level, all trout appeared to be in good health, with no significant lesions or deformities observed during visual inspections of external body condition. Livers and intestines appeared normal in shape and colour, and no signs of apparent necrosis were observed. HSI (between 1.45 ± 0.01 and 1.87 ± 0.09 %) and VSI (between 14.45 ± 2.05 and 16.84 ± 0.77 %) did not differ within treatments. The determination of HOG carcass and SK-ON FIL yields showed values between 83.16 ± 0.77 and 85.55 ± 2.05 % for HOG and between 49.99 ± 0.75 and 51.70 ± 0.89 % for SK-ON FIL and were not influenced by the different diets (Table 3).

3.3. Histological investigations

Histological examinations of the liver structure were performed to check for signs of hepatic inflammation and lipid accumulation by the presence of hepatocyte steatosis, as well as other disorders and inflammatory responses to the experimental diets (Figs. 1 and 2; Table 4). No influence of increasing dietary levels of BSF was observed on the structural parenchyma of liver tissues (Fig. 1). However, liver sections from all treatments presented a low to moderate degree of lipid accumulation, as evident from the levels of hepatocyte vacuolisation with a mean score from 0.6 to 1.5 (Fig. 1; Fig. 2A). No significant inflammatory responses were observed on liver tissue, with mean score values from 0.1 to 1 for cell infiltration (Fig. 2B). No significant effect of increasing dietary levels of BSF was observed on the degree of vacuolisation ($F_{(1,17)} = 2.682e\text{-}6$; $p = 0.999$) or inflammation ($F_{(1,20)} = 0.31$; $p = 0.582$) between experimental treatments.

The intestinal structure was found to be normal for farmed rainbow trout, with no apparent histopathological alterations of the proximal or distal tissues (Fig. 3). Proximal and distal cross-sections revealed mean diameter values of 3176 – 4399 μm and 5052 – 6587 μm respectively, with no statistical significance between treatments ($F_{(4,9)} = 0.50$; $p = 0.738$ and $F_{(4,5)} = 5.00$; $p = 0.06$, respectively), nor were mucosal layer thicknesses affected by the different feeds (Table 4). Observed rates of inflammatory cell infiltration of the *lamina propria* of the proximal intestine ranged from 0.25 to 0.88 (Fig. 4A and B). This represents a low density of inflammatory cells, and the difference between treatments was not significant ($F_{(4,11)} = 0.17$; $p = 0.947$). The same scenario was observed in the distal intestine with infiltration rates ranging from 0 to 0.75 ($F_{(4,30)} = 0.003$; $p = 1.000$) (Fig. 4A and B). In both intestinal segments, enterocyte vacuolisation was observed in the *lamina epithelialis* but not influenced by dietary treatment. Low to moderate vacuolisation was reported in the proximal intestine, scoring from 0.94 to 2.06 ($F_{(4,30)} = 0.003$; $p = 1.000$), and low values from 0.19 to 0.94 ($F_{(4,30)} = 0.003$; $p = 1.000$) were observed in the distal intestinal epithelium (Fig. 4C and D). Superficial observations of epithelium

Table 2
Growth performance, feed utilisation and apparent dry matter digestibility of rainbow trout fed with experimental diets.

Diet	IBW (g)	FBW (g)	Survival (%)	WG (g/fish)	SGR (%/day)	TGC	TFI (g/fish)	FCR	PER (%)	ADC_{DM} (%)
Control	138.4 ± 5.47	339.0 ± 3.42	100.0 ± 0.00	200.7 ± 8.89	1.34 ± 0.03^{a}	2.72 ± 0.19	152.6 ± 4.63^{a}	0.76 ± 0.01^{a}	3.07 ± 0.04^{a}	73.7 ± 1.98
Control+	135.5 ± 1.85	327.3 ± 5.19	100.0 ± 0.00	191.9 ± 3.34	1.35 ± 0.01^{a}	2.72 ± 0.29	143.8 ± 4.17^{a}	0.75 ± 0.01^{a}	3.08 ± 0.04^{a}	72.0 ± 0.24
25BSF	134.4 ± 0.57	347.0 ± 1.18	100.0 ± 0.00	212.6 ± 1.74	1.43 ± 0.04^{b}	2.91 ± 0.35	163.1 ± 1.36^{b}	0.77 ± 0.00^{b}	3.02 ± 0.00^{b}	75.3 ± 0.79
25BSF+	136.9 ± 3.18	352.0 ± 4.11	99.1 ± 0.91	277.6 ± 55.30	1.44 ± 0.04^{b}	2.94 ± 0.18	168.5 ± 3.96^{b}	0.78 ± 0.01^{b}	2.96 ± 0.03^{b}	74.4 ± 1.42
50BSF	134.8 ± 1.05	351.8 ± 1.99	99.1 ± 0.91	316.7 ± 98.82	1.43 ± 0.05^{b}	2.93 ± 0.38	168.4 ± 0.33^{b}	0.77 ± 0.00^{b}	2.96 ± 0.01^{b}	76.3 ± 0.87
50BSF+	139.3 ± 0.40	351.9 ± 11.52	100.0 ± 0.00	213.1 ± 11.91	1.42 ± 0.04^{b}	2.90 ± 0.16	166.1 ± 10.94^{b}	0.78 ± 0.01^{b}	2.93 ± 0.03^{b}	77.4 ± 0.34
75BSF	135.5 ± 1.89	351.7 ± 6.91	100.0 ± 0.00	216.2 ± 8.80	1.47 ± 0.03^{b}	2.99 ± 0.21	173.7 ± 9.36^{b}	0.80 ± 0.01^{b}	2.89 ± 0.04^{b}	75.5 ± 2.29
75BSF+	135.1 ± 0.32	352.1 ± 11.09	99.1 ± 0.91	228.5 ± 22.14	1.44 ± 0.01^{b}	2.94 ± 0.27	169.0 ± 6.69^{b}	0.78 ± 0.00^{b}	2.94 ± 0.02^{b}	76.2 ± 0.11
100BSF	135.7 ± 1.17	354.6 ± 2.80	100.0 ± 0.00	219.0 ± 1.63	1.48 ± 0.02^{b}	3.03 ± 0.33	170.5 ± 3.29^{b}	0.78 ± 0.01^{b}	2.93 ± 0.03^{b}	76.8 ± 1.56
100BSF+	133.0 ± 0.81	354.7 ± 8.40	100.0 ± 0.00	221.7 ± 9.21	1.47 ± 0.01^{b}	3.01 ± 0.28	173.8 ± 7.37^{b}	0.78 ± 0.00^{b}	2.92 ± 0.00^{b}	75.6 ± 0.72
Model effects (*p-value*)										
P	0.765	0.055	0.737	0.453	0.018	0.834	0.017	0.007	0.001	0.066
GG	0.386	0.243	1000	0.312	0.843	0.995	0.333	0.295	0.785	0.358
P*GG	0.487	0.782	0.382	0.870	0.971	0.999	0.770	0.155	0.362	0.754

Values are presented on tank basis as grand marginal means ± SEM (n = 2), superscript letters indicate significant differences among treatments ($p < 0.05$). Insect protein (P): 0 (control), 25, 50, 75 and 100 % substitution level; Guar Gum (G): 3 % inclusion vs.0 % inclusion; P*GG: Interaction between insect protein and guar gum. IBW: Initial body weight; FBW: Final body weight; WG: Weight gain; SGR: Specific growth rate; TGC: Thermal-unit growth coefficient; TFI: Total feed intake; FCR: Feed conversion ratio; PER: Protein efficiency ratio; ADC_{DM}: Apparentdigestibility coefficient of dry matter.

Table 3
Organosomatic indices and processing yields of rainbow trout fed with the experimental diets.

Diet	VSI (%)	HSI (%)	HOG (%)	SK-ON FIL (%)
Control	15.3 ± 0.34	1.45 ± 0.01	84.7 ± 0.34	50.9 ± 0.34
Control+	16.4 ± 0.43	1.45 ± 0.03	83.6 ± 0.43	50.4 ± 0.45
25BSF	15.3 ± 0.34	1.85 ± 0.04	84.7 ± 0.34	50.9 ± 0.27
25BSF+	16.8 ± 0.38	1.57 ± 0.08	83.2 ± 0.38	50.4 ± 0.43
50BSF	16.2 ± 0.34	1.79 ± 0.14	83.8 ± 0.34	50.0 ± 0.73
50BSF+	15.8 ± 0.36	1.78 ± 0.31	84.2 ± 0.36	50.4 ± 0.57
75BSF	15.9 ± 0.66	1.68 ± 0.08	84.1 ± 0.66	51.5 ± 0.78
75BSF+	14.5 ± 0.68	1.87 ± 0.06	85.6 ± 0.68	51.6 ± 0.98
100BSF	15.1 ± 0.64	1.76 ± 0.17	84.9 ± 0.64	51.8 ± 0.55
100BSF+	15.1 ± 0.45	1.74 ± 0.19	84.9 ± 0.45	50.0 ± 0.32
Model effects (*p-value*)				
P	0.351	0.312	0.351	0.415
GG	0.221	0.576	0.221	0.634
P*GG	0.165	0.540	0.165	0.613

VSI: Viscerosomatic index; HSI: Hepatosomatic index; HOG: Head-on, gutted; SK-ON FIL: Skin-on fillet. Values for VSI, HOG and SK-ON FIL are presented grand marginal means ± SEM (n = 10). Values for HSI are presented grand marginal means ± SEM (n = 2), the absence of superscript letters indicates no significant differences among treatments ($p > 0.05$). Insect protein (P): 0 (control), 25, 50, 75 and 100 % substitution level; Guar Gum (G): 3 % inclusion vs. 0 % inclusion; P*GG: Interaction between insect protein and guar gum.

suggested both intestinal segments exhibited normal goblet cell counts, which were also similar between treatments and between different parts of the intestine (Table 4). Also, the goblet cell filling did not vary between groups in each analysed section, with moderate values observed for both proximal (from 1.9 to 2.4 ($F_{(4,30)} = 0.005$; $p = 1.000$)) and distal (from 1.4 to 2.1 ($F_{(4,13)} = 0.18$; $p = 0.944$)) tissues (Fig. 4E and F). Regardless of the percentage of dietary BSF, all intestinal sections contained goblet cells stained purple by PAS-staining and vivid blue by AB-staining, indicating the presence of both neutral and acidic mucins. No differences were evident between treatments in either tissue type (scored at 2.4–3 ($F_{(4,30)} = 0.21$; $p = 0.932$) for proximal intestine and 2.6–3 ($F_{(4,18)} = 0.06$; $p = 0.934$) for distal intestine) (Fig. 4G and H).

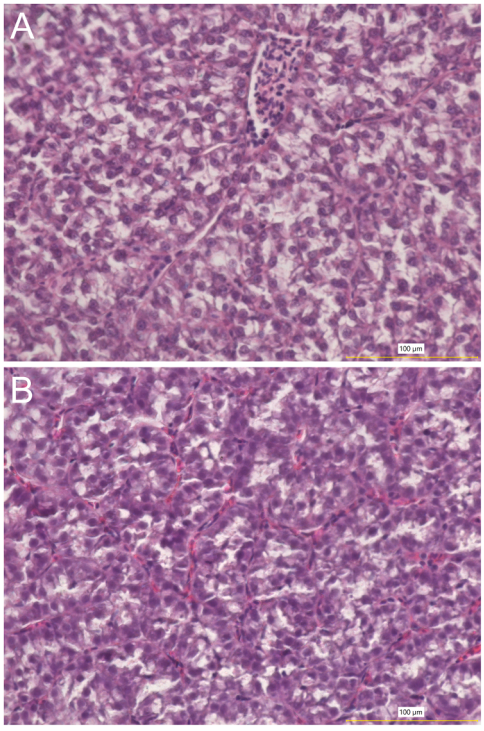

Fig. 1. Representative light micrographs of liver tissue from rainbow trout fed the reference control diet (A) and the 100BSF diet (B). Images captured at 400 × magnification.

No level of dietary BSF appeared to induce any significant alteration in rainbow trout intestine when compared with trout fed SPCs, and not did the inclusion of 0.3 % GG influence any of the evaluated histological parameters.

3.4. Expression profiling of the intestine

The complete substitution of dietary SPC with BSF plus 0.3 % GG yielded only a small effect on the transcriptome of proximal intestine in experimental fish (Fig. 5A), with a 1.5-fold upregulation of Agilent feature A_05_P448532 predicted in the 100BSF+ group compared to the controls (with $q = 0.036$). This feature was identified as *Oncorhynchus mykiss* gene LOC110491086 (NCBI gene ID: 110491086), which corresponds with a human orthologue encoding disks large-associated protein 4 (DLGAP4). The elevated transcript concentration in the proximal intestine of trout fed 100BSF+ was confirmed via RT-qPCR, revealing a 1.96-fold greater expression ($p = 0.047$) compared with fish fed the control diet (Fig. 5B).

4. Discussion

This study investigates the effects of replacing SPCs with graded levels of BSF, focusing on the growth performance, feed utilisation, and health of rainbow trout. To our knowledge, no other study has yet investigated the complete replacement of SPCs, which still is the main protein source in low FM diets for rainbow trout.

From the beginning of the experiment, all fish, regardless of treatment, actively foraged for the supplied feed, suggesting that the insect content had no adverse effects on diet palatability or acceptance. This was further evidenced by the increased feed intake in BSF-fed fish. Similar behaviour was previously observed for both rainbow trout and Atlantic salmon (*Salmo salar*) fed dietary BSF (Belghit et al., 2018; Cardinaletti et al., 2022). Replacing up to 100 % of SPC content with BSF did not result in any notable effect on overall fish performance. In fact, fish on all treatments demonstrated robust growth and feed utilisation. Additionally, the presence of 0.3 % GG did not impact any of the measured parameters, confirming the findings of Schumann et al. (2022).

After 10 weeks, there were no observable negative effects on fish growth, of even total replacement of dietary SPC with BSF. On the contrary, fish fed diets including some proportion of BSF exhibited enhanced growth compared to those on the commercial reference diets, which mainly contained SPC as a protein source. This suggests that even

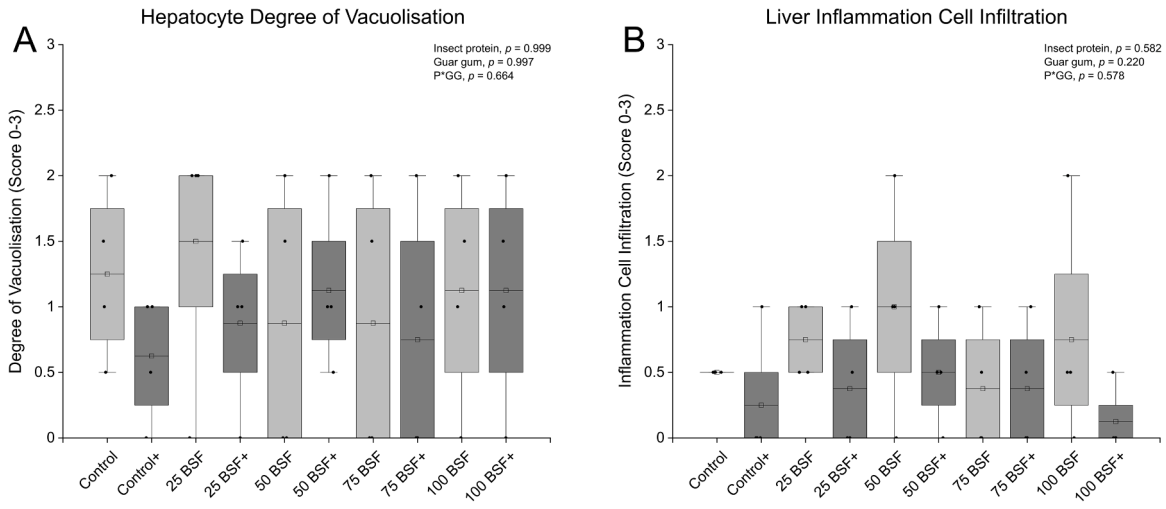

Fig. 2. Examination of the liver of trout for hepatocyte vacuole formation (A), and signs of inflammatory reactions (B) of rainbow trout fed diets composed with increasing levels of *H. illucens* larval meal.

at just 25 % replacement BSF can have a positive effect on rainbow trout growth rates, though this effect was not further improved by higher levels of BSF inclusion, results that are aligned with previous studies showing no adverse effects on fish performance when some level of BSF was included in their diets (Bordignon et al., 2021; Drosdowech et al., 2024b). This suggests that insect content might provide mitigation of some SPC nutritional deficiencies, possibly related to soybean remain ANFs, which appears can be fully compensated even by the lowest BSF level (Dietz and Liebert, 2018). Studies have already demonstrated the potential of BSF as an FM replacement for salmonid diets (up to 50 %) not only without adversely affecting growth performance (Caimi et al., 2021; Renna et al., 2017), but actually improving SGR (Weththasinghe et al., 2021b). Our results are not directly comparable with the previous literature due to differences in the used feed materials, principally the substitution of high-concentrated SBM ingredients in low FM formulations. Comparable growth was previously reported by Hossain et al. (2021) for rainbow trout fed on FM- and soy-based diets with low levels of BSF. However, contrary to our results, fish fed with higher levels of BSF exhibited weaker performance. Compared to SBM, SPC is characterised by optimal digestibility and low ANFs content (Kaushik et al.,

Table 4
Effects of dietary BSF inclusion in rainbow trout's proximal and distal intestine morphology and mucin-producing goblet cells.

Diet	Diameter (μm)	Goblet cells amount (10 ×100 μm)	Thichness *str. granulosum* (μm)	Thichness *str. compactum* (μm)
Proximal intestine				
Control	3175.6 ± 414.00	4.81 ± 0.52	31.0 ± 11.04	23.5 ± 9.28
Control+	4144.0 ± 534.43	3.93 ± 1.13	28.8 ± 8 08	20.5 ± 3.41
25BSF	4398.6 ± 917.01	4.94 ± 0.52	42.1 ± 14.50	22.4 ± 9.09
25BSF+	3781.8 ± 234.51	4.69 ± 0.63	38.7 ± 13.91	26.0 ± 12.36
50BSF	3546.7 ± 695.95	5.63 ± 1.30	38.0 ± 11.64	21.8 ± 7.00
50BSF+	3897.3 ± 569.79	4.19 ± 0.94	38.5 ± 13.06	21.2 ± 8.85
75BSF	3466.7 ± 465.04	5.19 ± 1.55	29.5 ± 6.54	16.4 ± 3.58
75BSF+	4264.2 ± 361.64	4.56 ± 1.07	35.1 ± 20.02	18.4 ± 5.23
100BSF	3619.8 ± 308.93	4.50 ± 0.35	35.4 ± 8.17	18.9 ± 2.22
100BSF+	3563.7 ± 414.00	5.50 ± 1.06	40.9 ± 18.03	16.7 ± 5.65
Model effects (*p-value*)				
P	0.738	0.812	0.492	0.142
GG	0.0795	0.289	0.820	0.462
P*GG	0.311	0.315	0.928	0.626
Distal intestine				
Control	4544.5 ± 260.22	4.86 ± 1.16	36.8 ± 10.78	16.7 ± 3.52
Control+	5251.3 ± 1125.36	4.94 ± 1.13	27.6 ± 10 12	18.0 ± 3.15
25BSF	6145.1 ± 503.88	4.00 ± 0.74	29.8 ± 10.99	16.0 ± 9.26
25BSF+	6587.0 ± 84.15	4.25 ± 1.32	32.4 ± 10.94	17.0 ± 5.70
50BSF	5413.6 ± 826.85	5.31 ± 1.30	31.1 ± 6.84	18.7 ± 2.42
50BSF+	5587.2 ± 238.86	5.00 ± 1.40	40.0 ± 17.75	16.1 ± 4.50
75BSF	4541.5 ± 2368.10	4.31 ± 1.14	43.4 ± 14.21	18.9 ± 7.64
75BSF+	5347.0 ± 187.26	5.06 ± 1.42	34.0 ± 12.40	14.4 ± 2.87
100BSF	4327.3 ± 1226.35	3.88 ± 0.95	39.7 ± 14.60	23.1 ± 7.52
100BSF+	5052.3 ± 235.11	4.88 ± 1.16	26.9 ± 2.71	19.0 ± 7.59
Model effects (*p-value*)				
P	0.061	0.435	0.645	0.609
GG	0.392	0.940	0.260	0.762
P*GG	0.919	0.786	0.311	0.794

Values are presented grand marginal means ± SD (n = 4), the absence of superscript letters indicate no significant differences among treatments ($p > 0.05$). Insect protein (P): 0 (control), 25, 50, 75 and 100 % substitution level; Guar Gum (G): 3 % inclusion vs.0 % inclusion; P*GG: Interaction between insect protein and guar gum.

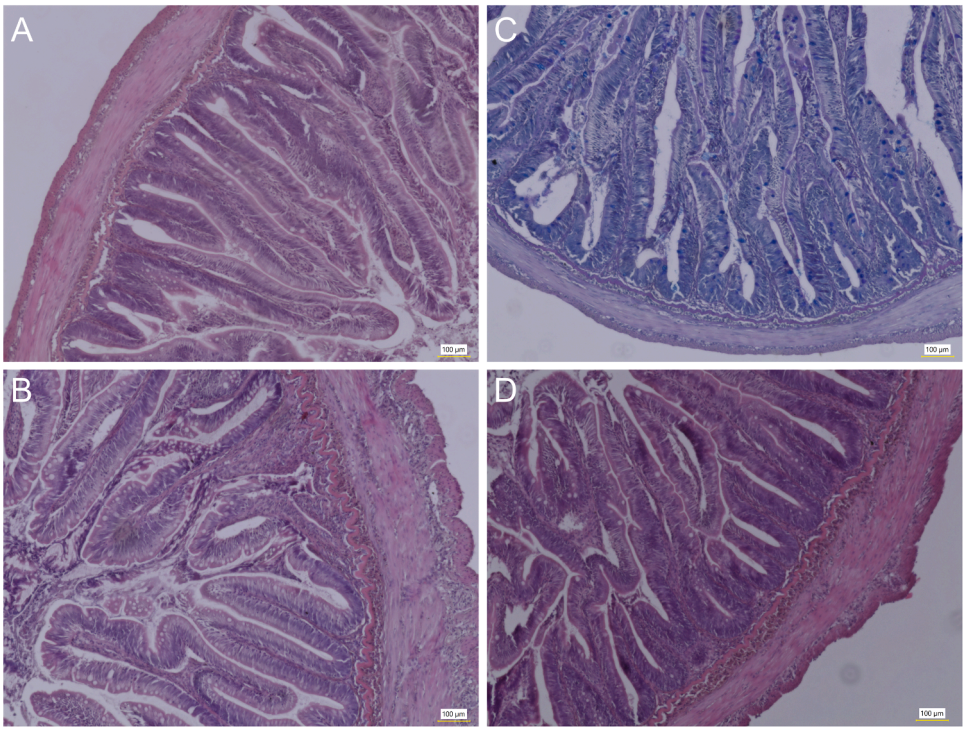

Fig. 3. Representative light micrographs of intestinal tissue from rainbow trout fed the reference control diet and the 100BSF diet. (A) Proximal intestine of fish fed the control diet; (B) Distal intestine of fish fed the control diet; (C) Proximal intestine of fish fed the 100BSF diet; (D) Distal intestine of fish fed the 100BSF diet. Images captured at 100 × magnification.

1995; Li et al., 2015), possibly explaining the discrepant outcomes of both studies, through the reduction in dietary SPC in Hossain's study, while substantial levels of SBM were maintained. Furthermore, variances in BSF quality related to insect life stage, diet, and processing methods could contribute to such discrepancies, as they can distinctly influence the fatty acid, vitamin, and mineral content of the resulting product (Oonincx and Finke, 2020).

Previous studies have reported improved SGRs in fish fed with insects, often linked to higher feed intakes (Weththasinghe et al., 2021b). In our study, all experimental groups exhibited good feed uptake, although fish consumed significantly more feed containing BSF compared to both insect-free groups. This may be related to the levels of FO in the BSF diets, which increased proportionally with the inclusion of insects. The use of FO in aquafeeds is known to significantly enhance palatability to rainbow trout, which as a carnivorous species have a preference for diets rich in *n*-3 long-chain polyunsaturated fatty acids (ω-3 LC-PUFAs) (Huyben et al., 2021; Roy et al., 2019). Despite their increased feed intake, fish fed with BSF exhibited reduced feed efficiency, indicating a less efficient digestion (Davis and Hardy, 2021). Recently, Randazzo et al. (2021b) reported that rainbow trout fed 45 % BSF (60 % replacement level) in a diet completely deprived of FM, exhibited better growth performance and feed utilisation than those given a plant-based diet, and similar or slightly better than those on an FM-based diet. These results coincided with increased feed intake as the proportion of insects increased. Comparable trends in growth and feed efficiency were observed in our study when SPC was replaced by BSF, although an opposite effect on FCR was observed for the plant-based reference diet, as mentioned before. These discrepancies can again be attributed to the use of SPC as the main reference protein source and the very low to non-existent levels of SBM in the present diets, supported by the significantly higher PER in groups fed the control diets.

In our study, ADC_{DM} remained unaffected by dietary treatment, with a slight tendency for diets containing 50, 75, and 100 % BSF to be more digestible than those free of insects. Reduced digestibility in insect-based feeds is generally associated with the presence of chitin in insect meal. Chitin is a complex natural polymer—of β-1,4-linked subunits of N-acetyl-D-glucosamine—shown to lower dry matter digestibility of rainbow trout feeds when present in significant levels (*e.g.,* 15.4 %, DM basis) or larger particle sizes (> 400 μm), already recognised as having antinutritional effects (Eggink et al., 2022). However, BSF larvae present a relatively low chitin content, ranging from 1 % to 9 % (Meneguz et al., 2018; Eggink and Dalsgaard, 2023), and species such as rainbow trout exhibit chitinolytic activity, enabling chitin digestion and even upregulating exochitinase activity—depending on its concentration in their diet (Eggink et al., 2022). Moreover, fish fed diets including 1–5 % chitin also exhibited improvements in intestinal microbiota and a gut microflora richer in chitinase-producing bacteria (Askarian et al., 2011; Bruni et al., 2018; Drosdowech et al., 2024a; Huyben et al., 2018). Efficient digestion has already been observed in rainbow trout (Caimi et al., 2021), Atlantic salmon (Weththasinghe et al., 2021b), seabass (*Dicentrarchus labrax*) (Basto et al., 2020), gilthead seabream (*Sparus aurata*) (Moutinho et al., 2022) and barramundi (*Lates calcarifer*) (Le Boucher et al., 2024) fed non-dechitinised BSF with chitin levels ranging from 5 % to 7 %, compared to fish fed FM, with either neutral or positive effects on digestibility reported with increasing levels of insect content. Although not quantified, the chitin content in the studied diets likely remained below the threshold levels required to negatively impact digestibility. The observed tendency for a lower digestibility in the control groups could also be attributed to the presence of 30 % SPC in the control diets. Despite SPCs reduced levels of ANFs, they can still contain traces of soybean antigens like agglutinins, saponins, and phytic acid (Zhu et al., 2020). In addition, SPCs may also retain certain polysaccharides such as starch and insoluble fibre (Schumann et al., 2022), which could potentially reduce dry matter digestibility in our study. Similar ADCs (over 75 % ADC_{DM}) were reported in Atlantic salmon fed BSF as a replacement for SBM, although with significant improvements in the insect-based diet after a 16-week feeding experiment (Fisher et al., 2020).

The relative sizes of liver and viscera, together with the proportion of edible flesh obtained from farmed fish after processing, are key

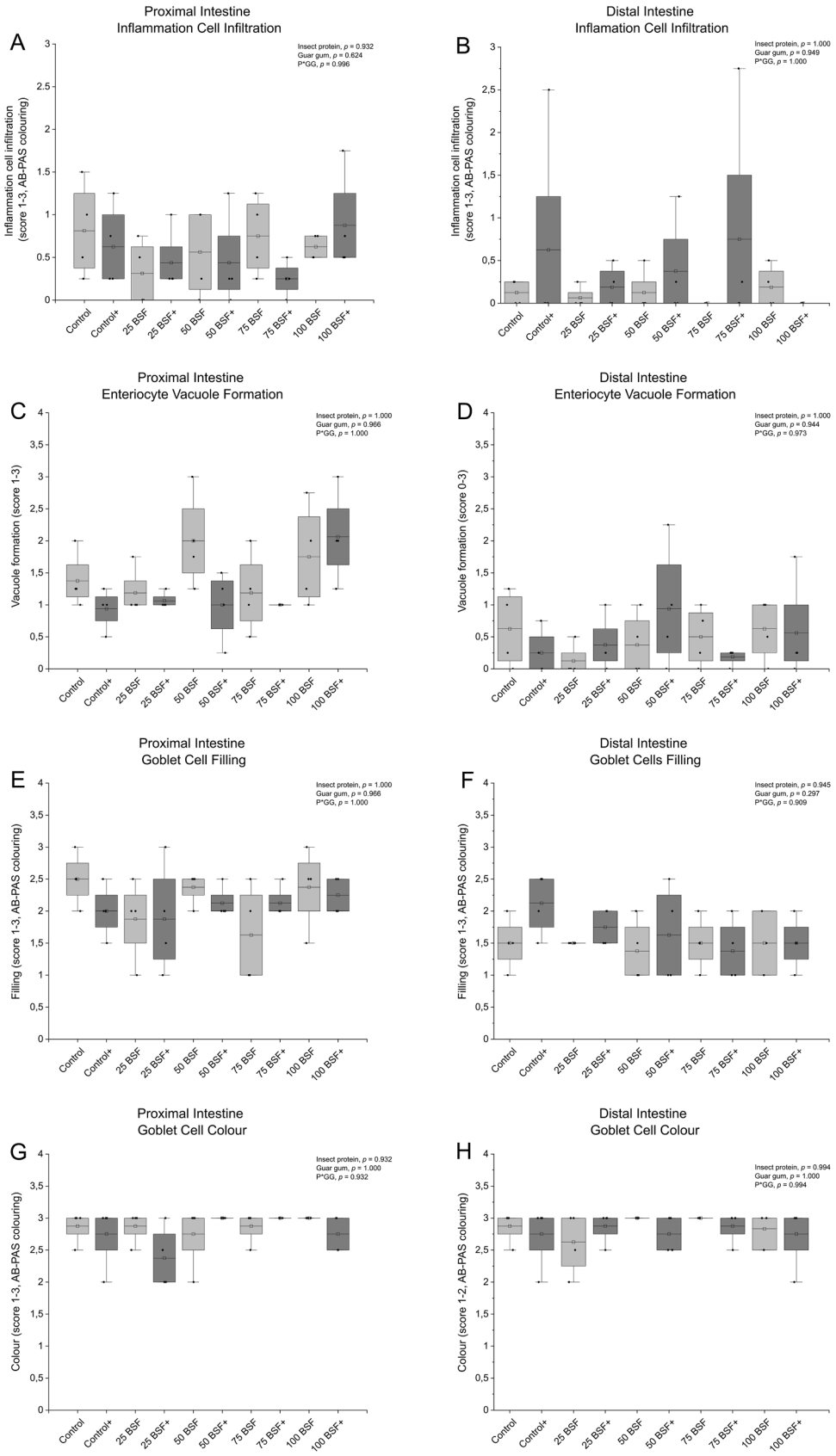

Fig. 4. Examination of the proximal and distal intestine of trout for signs of inflammatory reactions (A and B), enterocyte vacuole formation (C and D), goblet cells mucus filling (E and F), and goblet cells mucin colour (G and H) of rainbow trout fed diets composed with increasing levels of *H. illucens* larval meal.

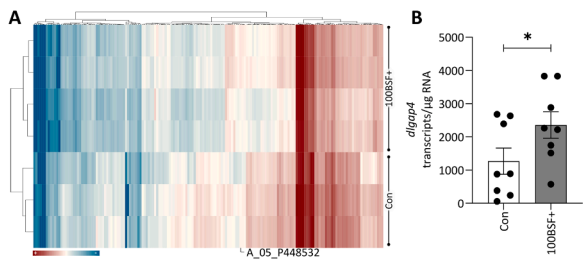

Fig. 5. Transcriptomic analysis of proximal-intestine samples from rainbow trout after feeding 100 % BSF+ or a control diet. (A) Hierarchical clustering of log10-transformed transcript abundance of differentially expressed genes (with $p < 0.001$) from the microarray-based comparison of 100 % BSF+ group (upper panel) versus control group (Control, lower panel). High and low gene expression intensities are represented by red and blue colours, respectively. Note that only one gene (dlgap4; Agilent ID: A_05_P448532) passed the threshold of $q < 0.05$. (B) Bar plot illustrating the dlgap4 transcript abundance in trout fed 100 % BSF+ group (grey bar) versus control trout (white bar) as assessed with RT-qPCR. Bars represent mean values (± standard error of the mean); dots represent individual transcript numbers; the asterisk indicates $p < 0.05$.

indicators of efficient feed utilisation leading to more profitable and sustainable production (Bugeon et al., 2010). The HSI is also an indicator of metabolic function and energy reserves, with proper liver function values ranging from 1 % to 2 % (Iaconisi et al., 2017), while the VSI reflects organoleptic health and visceral fat deposition, which are largely influenced by the lipid content of the feed (Jobling et al., 1998). In the current experiment, the insect protein treatments resulted in HSI and VSI values within the viable range for farmed rainbow trout, showing no dose-dependent effects. Even apparently small differences in processing yields can have a significant economic impact when long-term cumulative quantitative effects are considered (Bugeon et al., 2010; Davidson et al., 2014). Organosomatic indices calculated in the present study point to no difference in processing yields (HOG and SK-ON FIL) by the inclusion of insect protein in the diet, compared to the reference SPC diet, which represents normal values for farmed rainbow trout (Davidson et al., 2014). In this respect, BSF appears to be a viable SPC replacement in commercial diets without compromising production economics.

As the digestive system represents the first direct interaction with novel diets, histological examination of liver and gut tissues is a routine component of nutritional trials, providing an integrative overview of organ integrity and functionality (Rašković et al., 2011; Wang et al., 2017). An inflammatory response is regarded as a primary indicator of negative effects (Miebach et al., 2023). In the current study liver histological examinations did not reveal severe inflammatory cell infiltration, and although low to moderate levels of cytoplasmic hepatocyte vacuolisation were observed, these were not linked to any dietary treatments. Previous studies have reported widely variable effects of insect diets on fish liver, with some reporting increased lipid accumulation to severe steatosis, with a linear increase of total saturated fatty acids (SFA) and associated decreases in PUFAs when insects were progressively incorporated into fish diets (Cardinaletti et al., 2019; Zarantoniello et al., 2020). Some, in line with our results, reported no effects when replacing FM or SBM with insect alternatives (Kumar et al., 2020; Melenchón et al., 2022), while others even report beneficial effects of BSF on lipid accumulation levels (Cardinaletti et al., 2022). In studies with Atlantic salmon (Belghit et al., 2018) and barramundi (Chaklader et al., 2021), authors also described a mitigating effect of BSF on liver lipid accumulation, hypothesising that the significant amount of lauric acid in insect meals might prevent lipid deposition by stimulating its rapid oxidation. Such differences are probably related to the different nutritional composition of the used experimental diets.

Histological parameters were also evaluated in both proximal and distal intestinal sections, revealing no evidence of inflammatory responses linked to increasing levels of BSF. This accords with several previous studies showing an absence of intestinal inflammatory signs when BSFs were applied in teleost diets (Caimi et al., 2020; Elia et al., 2018; Lock et al., 2015; Miebach et al., 2023). The majority of fish are planktivorous during their larval and juvenile life stages, and even species that become piscivores, herbivores or detritivores as adults may start their feeding activity as zooplanktivores, including insects as part of their natural diet. Carnivorous species like salmonids frequently prey on cladocerans, copepods and insect larvae as during their larval stage, while juvenile diets are dominated by larval, pupal or adult insects, before switching to fish-dominated prey (Nunn et al., 2011). In addition, insects contain certain bioactive peptides and polysaccharides that have already been shown to reduce intestinal inflammation in fish (Gasco et al., 2020). Particularly, the partial inclusion of BSF in aquafeeds has shown to be beneficial in preventing SBM-induced enteritis in rainbow trout (Kumar et al., 2020). Although the specific dietary agents involved in the upregulation and pro-inflammatory responses present in BSF were not analysed, these factors may have contributed to the intestinal health of the fish in our study (Gasco et al., 2020). No indications of enteritis were found, and dietary changes did not affect intestinal macro-morphology or goblet cell density. Besides no observable differences were found within the analysed intestinal sections, a more pronounced enterocyte steatosis with moderate level of cell vacuolisation was observed in the proximal intestine, whereas less evident in distal intestine. This phenomenon is known to be associated with high levels of plant inclusion in fish diets (Gu et al., 2013), and similar effects have previously been observed in both proximal and distal intestines when SPC was used as a replacement for FM in rainbow trout (Escaffre et al., 2007). In contrast to other studies, where BSF diets led to a reduction in enterocyte vacuole formation, no such effect in rainbow trout's intestine was found (Li et al., 2018; Weththasinghe et al., 2021b). An accumulation of lipids in the intestines of the control groups was to be expected, as plant ingredients are typically lower in phospholipids than FM, especially phosphatidylcholine, which plays a direct role in lipid transport across the intestinal mucosa (Krogdahl, et al., 2020). However, the lack of changes in enterocyte condition in fish fed the BSF diets compared to controls was unexpected. Insects are reported to contain significant amounts of choline–the essential nutrient of phosphatidylcholine–previously been shown to prevent lipid accumulation in the proximal intestine of Atlantic salmon (Hansen et al., 2020; Krogdahl et al., 2020; Oonincx and Finke, 2020). The reasons for this lack of differences remains unclear and a detailed comprehensive analysis of the used BSF should be considered in future investigations. Furthermore, no macroscopic signs of lipid malabsorption were observed among the analysed fish, nor any other indication of bad health. Our results point to considerable potential for BSF as a viable replacement for SPC in salmonid diets. In all experimental groups, goblet cells showed a considerable degree of filling and a diversified mucin composition, indicating good intestinal functionality, without excessive secretion of mucus into the intestinal lumen, which normally represents a defensive response to irritants, as part of the primary barrier against infection (Jung-Schroers et al., 2018). Variation in dietary levels of BSF did not influence the thickness of submucosal layers. These results suggest that rainbow trout can efficiently utilise BSF as a protein source without compromising liver and intestinal health.

Corroborating the histology results, the transcriptomic analysis of intestinal tissue samples from rainbow trout revealed that only the dlgap4 gene was significantly upregulated in trout fed with 100 % BSF and 0.3 % GG compared with controls. Although the precise function of the teleost dlgap4 is unknown, the human DLGAP4 ortholog links scaffold proteins and associated signalling molecules to membrane-bound ion channels and receptors, especially glutamate receptors, and indirectly controls their activity (Rasmussen et al., 2017). The glutamatergic receptor activity of the interaction between microbiota and intestine can, in turn, influence the visceral sensitivity and motility of

the gut, and alterations in glutamatergic transmission may contribute to localised pathogenesis (Baj et al., 2019). Previous studies consistently imply that partial substitution of FM with insect alternatives does not trigger the kind of inflammatory responses or physiological disorders seen in finfish fed on other substitute components with antinutritive properties, such as SBM (Seibel et al., 2022; Krogdahl et al., 2015). Moreover, the partial or complete substitution of FM by insect meal induces only low to modest changes in the expression of selected marker genes in defined gut segments of different aquaculture fishes, including gilthead sea bream (Carvalho et al., 2022; Piazzon et al., 2022) or Atlantic salmon (Li et al., 2018, 2020).

The demand for alternative feed ingredients to mitigate the aquaculture sector's demand for protein presents an opportunity for the insect industry (IPIFF, 2019). While currently contributing only a minimal part of salmonid diets (Aas et al., 2022a; 2022b), insects are already considered a reliable source for feed ingredients of aquafeeds. However, the question whether the insect industry can thrive in Europe remains unanswered. The production of insects still faces considerable challenges in Europe, making a large-scale European insect industry at best a long-term goal (Niyonsaba et al., 2023). The sustainable production of insect feeds, largely depends on to the possibility of using organic waste streams (Biteau et al., 2024; van Huis, 2019), which are still not allowed in several countries worldwide, making the sector economically expensive and environmentally unstable in the near term (Biteau et al., 2024; Veldkamp et al., 2022).

5. Conclusions

This study supports the viability of black soldier fly larvae as a complete replacement for soy protein concentrate in trout feeds at an inclusion level of 40 % without affecting fish growth, feed utilisation and dressing levels. High dietary incorporation BSF did not negatively affect palatability or dry matter digestibility and there were no adverse effects on liver and intestine histology compared to fish on the SPC-based control. These findings support the use of BSF larvae a possible nutritionally viable alternative to conventional plant-based protein sources in aquafeeds.

CRediT authorship contribution statement

Carsten Schulz: Writing – review & editing. **Alexander Brinker:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Dirk Koczan:** Resources. **Verena Jung-Schroers:** Resources, Methodology. **Sara de Sousa e Brito:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Mark Schumann:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Alexander Rebl:** Writing – review & editing, Visualization, Resources, Methodology, Formal analysis. **Tamara Wind:** Writing – original draft, Visualization, Investigation, Data curation, Conceptualization.

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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.

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Data Availability

Data will be made available on request.

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