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Evaluation of black soldier fly larvae meal as a functional feed ingredient in Atlantic salmon (*Salmo salar*) under farm-like conditions

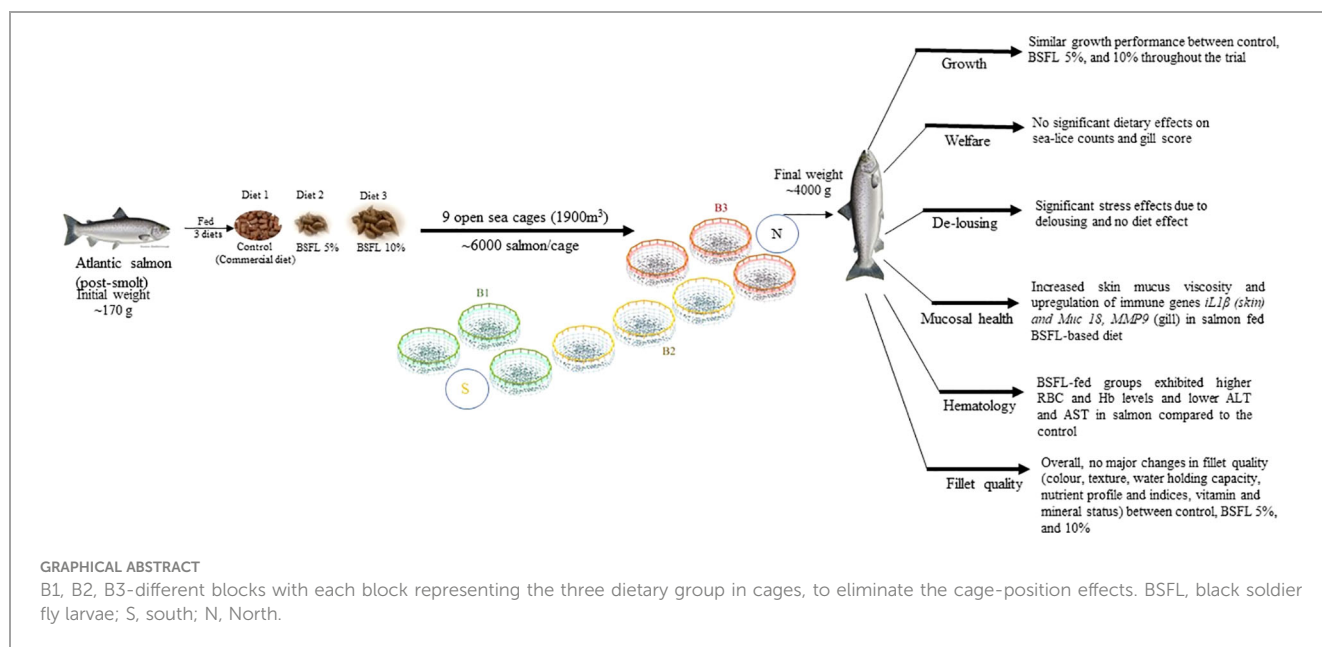
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Atlantic salmon (*Salmo salar*) were fed diets containing black soldier fly larvae (BSFL) meal at two inclusion levels for 13 months in open sea-cages. BSFL meal replaced plant-based ingredients and dietary insect meal inclusion levels were at 5% and 10%. A commercial salmon diet was fed as a control diet. Fish were reared from ~173 g to ~4 kg and were randomly distributed into nine open sea-cages with ~6000 salmon/cage (12×12 m²; 1900 m³). Fish from the sea-cages were sampled at two time points (mid and final samplings) to study the dietary effects of BSFL meal on the general health and welfare. Monthly assessments of sea-lice and gill score were conducted to evaluate the overall well-being of the salmon. The findings from the current study revealed that dietary inclusion of BSFL meal up to 10% did not have any significant effects in general growth, welfare or survival. However, significant positive response was observed in the general skin mucosal, hematological, and gene expression profiles of salmon. Notably, the group of salmon fed with 5% BSFL meal showed a significant decrease in plasma aspartate transaminase and alanine transaminase. Also, a significantly higher expression of *interleukin1β* in both skin and gill along with upregulation of *matrix metalloproteinase9* and *mucin18* in gill were observed in salmon fed BSFL at 5%, which aided in increased immune responses. Apart from that, this group had significantly higher mucus secretions, decreased cortisol response and increased number of erythrocytes. Furthermore, the delousing stress had a significant effect on the plasma cortisol, and these responses were independent of the dietary effect. Moreover, these immune responses behaved differently at different fish size and time points, acknowledging the influence of various factors in immune modulation. Overall, the findings from this study showed the effects of dietary BSFL meal to modulate the immune status of salmon. This study aims to fill the existing knowledge gaps regarding the impact of incorporating BSFL meal as a functional feed ingredient into the salmon diet on health and immune status replicating real farm conditions.

KEYWORDS

aquafeed, long term feeding, bioactive compounds, immunostimulants, health, insect meal, salmonids



1 Introduction

Aquaculture has been a rapidly developing industry during the past four decades and is expected to continue in the foreseeable future to feed the growing population (Garlock et al., 2022). Atlantic salmon (*Salmo salar*) is one of the most farmed species (2.7 MT), accounting for 32.6 % of marine and coastal aquaculture of all finfish species in 2020 (FAO, 2022). Norway is the world's leading producer of Atlantic salmon, producing around 1.4 MT in 2020. However, the downside of intensification of salmon industry has been the economic losses, due to mortality caused by salmon louse and treatments, infectious diseases, mucosal health, or multiple environmental stressors (Norwegian fish health report 2022). It has been shown that nutritional approach using functional feed ingredients (e.g., β -glucans or mannan-oligosaccharides, nucleotides, or plant extracts) can modulate the immune system of fish species (Ringø et al., 2012) and can protect fish from disease, parasites and various stressors (Hossain et al., 2023), thereby decreasing the mortality rate.

Recently, great attention has been given to the potential of using insect meal as sustainable feed ingredients due to its good nutritional profile (well-balanced content of amino acids, fatty acids, vitamins and minerals), presence of immunostimulant and bioactive compounds (Van Huis and Gasco, 2023). Among the approved insect species for its inclusion in aquafeed, black soldier fly larvae (BSFL) have gained widespread attention at research and commercial scale (Veldkamp et al., 2022b). This popularity of the black soldier fly might be due to its short life cycle, fast larval growth and ability to convert a variety of waste into valuable mass, thereby being an advantage for upscaling the insect production for use by the feed sector (Tran et al., 2022). Dietary BSFL meal can replace both marine and plant-based ingredients (such as fish meal (FM) and soy protein) and an inclusion up to 30% in aquafeed is possible

without affecting the growth performance of fish species (Liland et al., 2021). However, dietary inclusion of BSFL meal should not only ensure growth but also modulate the immune responses and thus improve the health of farmed fish species (Mouithys-Mickalad et al., 2020).

The examination of the immunomodulatory effects of BSFL meal as a functional feed ingredient in fish serves to offer insights into the mechanisms and behavior of immune parameters in salmon fed BSFL-based diets. The BSFL meal contains diverse bioactive and immunostimulant compounds such as antimicrobial peptides (AMPs) (e.g., α -helical peptides, cysteine-rich peptides, proline-rich peptides, glycine-rich peptides), fatty acids (lauric acid) and polysaccharides (chitin and chitosan) (Veldkamp et al., 2022a). Insect AMPs play a key part in the innate immune response and have been shown to improve fish resistance to diseases (Hu et al., 2021; Rashidian et al., 2021). Lauric acid is known for its antiviral and antibacterial activities (Lieberman et al., 2006) and is also considered as a good source of energy for salmon (Belghit et al., 2019b). Furthermore, it has been shown that BSFL meal contains chitin, a polysaccharide that can function as a potential prebiotic in animal feed (Song et al., 2014). Studies in juvenile Mozambique tilapia (*Oreochromis mossambicus*) showed that incorporating small doses (0.5 g/kg feed) of dried prepupae from BSF in their diet had enhanced the hematological parameters (Ushakova et al., 2018). Moreover, dietary inclusion of housefly (*Musca domestica*) at low levels (between, 0.75% and 7.5%) increased the innate immunity and disease resistance of red sea bream (*Pagrus major*) (Ido et al., 2015) and black carp (*Mylopharyngodon piceus*) (Ming et al., 2013). An inclusion of dietary insect meals at low levels might act as a functional feed ingredient with potential health beneficial effects for fish species.

Therefore, based on this knowledge, the current study aimed to investigate the effects of dietary inclusion of BSFL meal as a

functional feed ingredient in the Atlantic salmon diet (5% and 10%), reared in open sea-cages over the course of one year, aiming to simulate commercial farming conditions. The study aimed to assess the mucosal health and innate immune responses of salmon when exposed to chronic stress (pathogens and parasites, fluctuating temperature, salinity and water quality) and acute stress (delousing and handling).

2 Materials and methods

2.1 Experimental diets

Three experimental diets were produced by Skretting Norway (Averøy, Norway) and were formulated to be iso-nitrogenous and iso-lipidic diets, containing the same level of FM (100 g/kg, [Table 1](#)).

TABLE 1 Formulation (%) and proximate composition (% of dry matter) of experimental diets (4.5 mm, and 9.0 mm) fed to Atlantic salmon during 13 months in open sea-cages.

Ingredients	4.5 mm			9.0 mm		
	Control	BSFL 5%	BSFL 10%	Control	BSFL 5%	BSFL 10%
Fishmeal ¹	10.0	10.0	10.0	10.0	10.0	10.0
Guar Meal 58% CP roasted ²	10.0	8.10	5.00	12.0	8.4	4.8
Horse beans dehulled ³	8.58	7.00	6.75	5.5	4.8	4.8
Soy protein concentrate ⁴	19.0	17.7	16.1	7.0	6.0	4.0
Sunflower meal ⁵	–	–	–	7.0	7.0	7.0
BSF larvae meal ⁶	–	5.00	10.0	–	5.0	10.0
Fish oil crude high ⁷	4.52	4.45	4.37	5.10	5.04	4.96
Fish oil crude low ⁸	4.22	4.44	4.60	4.25	4.38	4.50
Rapeseed oil ⁹	14.2	13.7	13.2	22.80	22.53	22.17
Camelina oil ¹⁰	–	–	–	2.55	2.34	2.12
Wheat ¹¹	4.00	4.00	4.00	7.7	7.7	7.7
Wheat gluten vital pellets ¹²	20.0	20.0	20.0	11.76	12.42	13.67
Micro-nutrients ¹³	2.98	3.04	3.06	2.15	2.16	2.20
Vitamin- mineral mix ¹³	0.81	0.80	0.80	0.86	0.87	0.87
Water	1.68	1.77	2.15	1.32	1.33	1.28
Proximate composition						
Dry Matter %	92.8	92.3	93.5	94.2	93.9	93.4
Moisture %	7.2	7.8	6.5	5.76	6.11	6.63
Crude Protein %	49.0	48.0	49.0	41.0	42.0	41.0
Crude lipid%	24.0	27.0	25.0	31.0	33.0	33.0
Ash %	4.5	4.5	4.7	4.5	4.8	4.7
TBARS (nmol/g ww)	8.1	10.0	12.0	12.0	13.0	15.0
Gross energy (MJ/kg)	24.6	26.1	25.2	26.2	26.4	26.6

¹North Atlantic, min. 64% protein.

²Roasted, min. 53% protein.

³Dehulled, min. 25% protein.

⁴Non-GM, min. 58% protein.

⁵Min. 34% protein.

⁶Protix.

⁷North Atlantic.

⁸South American.

⁹Degummed.

¹⁰Degummed.

¹¹Min. 10% protein.

¹²Vital, min. 80% protein.

¹³Skretting.

¹³Skretting.

BSF, black soldier fly; TBARS, thiobarbituric acid reactive substances.

July 2021 to January 2022, 4.5 mm pellet.

February 2022 to August 2022, 9.0 mm pellet.

A control diet (Control) was formulated using a standard commercial recipe, containing FM and plant-based protein (guar meal, horse beans, sunflower meal and soy protein concentrate). The main lipid sources in all diets were fish oil and vegetable oils (rapeseed oil and camelina oil). The two experimental diets were formulated by using partially defatted BSFL meal (Protix, 53% crude protein, and 13% crude lipid). Plant-based ingredients were replaced with BSFL meal at 14% (BSFL 5%) and 26% (BSFL 10%) for 4.5 mm pellets. While for the 9.0 mm pellets, 19% (BSFL 5%) and 34% (BSFL 10%) of plant-based ingredients were replaced by the BSFL meal. These two levels of inclusion for BSFL meal were chosen: a low level (5%), which is currently feasible given the available quantities of insect meal, and a high level (10%), which could potentially represent the future standard for insect protein meals in animal diets, expected to be achievable in 10 to 15 years when the insect market can provide larger quantities of insect products (Veldkamp et al., 2022b).

The proximate composition of the diets was analyzed according to AOAC (2010) methods. Samples were freeze dried for 48 h (FreeZone 18 Liter Console, Labconco, USA) to obtain the dry matter. The dried feed samples were ground into a fine powder for the analysis of nitrogen (N), fat and ash content. Nitrogen content was analyzed using a CHNS elemental analyzer (Vario Macro Cube, Elementar Analysensysteme GmbH, Langensfeld, Germany) and quantified (AOAC, 2010). The instrument was calibrated with Ethylenediamine tetraacetic acid (Leco Corporation, Saint Joseph, MI, USA). Sulfanilamide (Alfa Aesar GmbH & Co, Karlsruhe, Germany) and a standard meat reference material (SMRD 2000, LGC Standards, Teddington, UK) were used as control sample. Crude protein was calculated as $N\% \times 6.25$. Crude lipid was measured gravimetrically after acid hydrolysis. Gross energy was measured by adiabatic bomb calorimetry using manufactures protocol (Parr Instrument Co., Moline, IL, USA). The feed formulation and analyzed nutrient composition of the feeds are provided in Table 1.

2.2 Experimental trial

The feeding trial was conducted at Austevoll research station, Institute of Marine Research, Norway, from July 2021 to August 2022 (13 months). On the 22nd of July 2021, a total of 56000 Atlantic salmon post smolts (Aquagen strain) were transported from Biofish AS (Tørvikbygd) to the Austevoll station by using a well boat. Approximately 6000 fish were distributed into each sea-cage using sensors, which detect and record the fish before releasing them into each cage. The fish were randomly distributed into nine open sea-cages ($12 \times 12 \text{ m}^2$; 1922 m^3). Diets were assigned to the sea-cages in triplicates using a randomized block design, containing three blocks (B1, B2, and B3) to account for cage position effects. The fish were acclimatized to the control diet one week prior to the start of the experiment. The feed was dispersed using automatic surface feeders with meals given every 3 min during seven hours a day (20 doses/h). The feeding volume were recorded and registered daily using the Mercatus Farmer software (Scale AQ, Norway) to estimate the biomass and feed conversion ratio. The pellet size (4.5- and 9.0-mm pellet) and size of meals were adjusted according to the

fish size, biomass and feeding behavior by visual observation from the surface. Lumpfish (*Cyclopterus lumpus*) and Ballan wrasse (*Labrus bergylta*) were added to each cage to keep lice levels low (Imsland et al., 2014; Imsland et al., 2018), according to standard routines at the station (Ratio of cleaner fish to salmon was 12% with the same ratio in all cages). The number of dead fish (salmon and cleaner fish) were calculated by counting them daily through visual observation. Towards the end of the trial (week 21-30), the dead fish were macroscopically examined. Microbiological and histological analyses were performed (Pharmaq Analytic, Bergen, Norway) on gill and heart of dead salmon to diagnose for potential bacterial or viral infections.

2.3 Rearing environment

Temperature, dissolved oxygen and salinity in the Austevoll research station were monitored throughout the year using Mercatus software (Scale AQ, Norway). The average temperature, salinity and oxygen at different depths (0.5, 5, 10, and 20 m) for the entire feeding period were recorded. The monthly average for temperature (°C), salinity (‰) and oxygen (% of saturation) (10 m depth) for an entire year from July 2021- August 2022, ranged between 6-15 °C, 17-30 ‰, and 94-108 (% of saturation), respectively. At 10 m depth, the temperature (°C) range was 14 ± 0.3 (June-August), 13 ± 0.1 (September-November), 8 ± 0.1 (December-February), and 14 ± 0.2 (March-May). Likewise, salinity (‰) was 32 ± 1.2 (June-August), 23 ± 2.0 (September-November), 30 ± 0.6 (December-February), and 32 ± 0.3 (March-May). Oxygen level (% of saturation) was 100 ± 0.6 (June-August), 97 ± 0.5 (September-November), 96 ± 0.5 (December-February), and 101 ± 0.6 (March-May).

2.4 Samplings

Fish were monitored for general growth, health and welfare parameters at multiple time points during the feeding trial:

- Initial sampling (July 2021): 50 fish/cage were removed for recording weight and length.
- Weekly samplings: 20 fish/cage/week were removed to monitor the sea-lice and gill score as a part of welfare assessment for the whole experiment. The gill scoring was qualitative and was evaluated per fish based on a scale of 0 to 5, where 0 represents no signs of infection and 5 represents severe infection (Taylor et al., 2009). The sea-lice (*Lepeophtheirus salmonis*) were counted manually and the permissible limit in Norwegian salmon farms is 0.2 mature females per salmon during spring and 0.5 for rest of the year (Norwegian Food Safety Authority).
- Mid (April 2022; fed for 9 months) and final samplings (August 2022; fed for 13 months) were conducted to obtain skin mucus (10 fish/cage), blood, plasma, gill and skin tissue (6 fish/cage). Additionally, 6 salmon were sampled from each sea-cage for fillet quality and sensory analysis (results described elsewhere).

-Delousing: During the entire experimental trial, a total of three thermal delousing was performed for different cages before exceeding the recommended limits by the Norwegian Food Safety Authority. In the current study, samples were taken from one of the delousing performed in May 2022 (fed for 10 months). In this sampling, six fish/cage were sacrificed to obtain the mucus and plasma samples. Fish were sampled before delousing (pre-stress) and after delousing (post-stress). Thermal delousing was performed using a thermolicer according to the approved guidelines of Norwegian Food Safety Authority. In short, the fish were pumped from each cage into the thermolicer where it passes through the processing loop for 30 seconds, with a water temperature of 28–32 °C. After the treatment, salmon was pumped back into the same sea-cage.

The initial body weight (IBW; 173 ± 2.7 g), final body weight (FBW), specific growth rate (SGR), weight gain (WG) and weight gain percentage (WG %) ($n=50$ per cage) and body indices such as hepatosomatic index (HSI), viscero-somatic index (VSI) and condition factor (K) ($n=6$ per cage) were measured at the termination of the trial. For each sampling, fish were collected from the sea-cage by using a large net hung from one side of the cage for fish to voluntarily jump into a smaller and separate section of the cage. Fish from this smaller and shallower section were taken out of the sea-cage using hand nets and transferred to large tubs. Fish were anesthetized with Finquel vet. (Tricaine Mesylate, 30 mg/L) to check the sea-lice and gill structure and were overdosed with anesthesia (60 mg/L, according to the guidelines of the Norwegian Regulation on Animal Experimentation and European Community Directive 86/609/EEC) and killed by cephalic concussion before collecting tissue samples.

Blood was collected from the caudal vein using 38 mm blood collection needles and Ethylenediamine tetraacetic acid (EDTA) coated vacutainers. Samples were kept at 4°C for up to 24 h before measuring the total red blood cell (RBC) and hemoglobin (Hb) counts. The plasma samples were obtained by centrifuging the blood at 3500 rpm for 10 min in 1.5 mL Eppendorf tube. The plasma samples were aliquoted into small centrifuge tubes for analyzing plasma metabolites. All the samples were frozen immediately and stored at -80 °C until further analysis. For collecting the skin mucus, fish were taken out from the water by holding on the tail until the water dripped off. Later the fish were placed on one side on a clean surface without disturbing the dorsal surface. The skin mucus was collected by absorption according to the method described by [Tartor et al. \(2020\)](#) using sterile medical wipes (2.5 × 7 cm each; Kimberly-Clark, Kent, UK) to measure the total DNA in the skin mucus. The mucus was collected from the dorsal side above the lateral line excluding the head, fins and tail to avoid contamination of samples due to handling. For collecting the skin, and gill tissue for gene expression analysis, a small area of skin tissue from right below the dorsal fin was removed and the upper lamellae of the second gill arch was sampled. These tissues for gene expression studies were flash frozen in liquid nitrogen and later stored at -80 °C until further analysis.

2.5 Hematological analysis

Plasma metabolites such as alkaline phosphatase (ALP) (U/L), alanine transaminase (ALT) (U/L), aspartate transaminase (AST) (U/L), glucose (mmol/L), amylase (U/L), total protein (g/L), total cholesterol (mmol/L) and triglyceride (mmol/L) were analyzed using clinical bioanalyzer (Pentra C400 Horiba Medical, Montpellier, France). Plasma cortisol was analyzed using the cortisol ELISA kit (DEH 3388, Demeditec Diagnostics GmbH, Germany). This enzyme immunoassay was based on the principle of competitive binding and the samples were analyzed using a 96 well microplate based on the manufactures protocol. The absorbance was measured at 450 nm using a plate reader (Tecan Sunrise™, Tecan Trading AG, Switzerland). Plasma cortisol is expressed as ng/mL. The plasma osmolality was measured using Fiske® Micro-Osmometer Model 210 (Fiske® Associates, two Tech ways, Norwood, Massachusetts, USA). The osmolality values are expressed in mOsm/kg. Plasma ions such as K⁺, Na⁺, Ca⁺⁺, Cl⁻, along with glucose and lactate were measured using ABL 90 Flex Plus (Bergman Diagnostika). The concentration of ions is expressed as mmol/L.

The total RBC and Hb was measured using Diluter771 Swelab and Cell-Dyn 400 (Sequoia-Turner, Santa Clara, CA, USA) according to the manufacturer's instructions, using Para 12 control blood (Streck, MedMark, 218777) for calibration. To measure the RBC, 40 µL of blood was diluted with 10 mL of phosphate buffered saline (PBS, Merck, 4873) in a tube using the diluter and mixed thoroughly. The samples were diluted again with 10 ml of PBS in a Nunc cup and were placed in the Cell-Dyn 400. The RBC values are expressed as the value obtained ×10¹² cells/L. For the Hb, 40 µL of blood was diluted with 10 mL of PBS in a tube using the diluter followed by 6 droplets of Zap-o-globin lytic reagent (Beckman Coulter 7546138) to the solution. The resulting solution was thoroughly mixed by inverting the tubes. After 30 min the tubes were centrifuged for 10 min at room temperature. Later the solution was transferred into a Nunc cup and placed under the capillaries on the Cell-Dyn400 to measure Hb. The Hb measured is expressed as g/100mL.

2.6 Skin mucus analysis

The mucus DNA concentration was measured using Quant-iT™ PicoGreen™ dsDNA Assay Kit (P7589 Thermo Fisher Scientific, UK) according to manufactures protocol, based on fluorescence (480/520 nm excitation/emission, Ex/Em) using Quant-iT™ PicoGreen™ dye. The skin mucus DNA is expressed in ng/mL. The mucus lysozyme activity was measured using a Lysozyme Activity Assay Kit (ab211113) (Abcam, Cambridge, UK) according to manufactures protocol. The kit is based on the ability of lysozyme to cleave a synthetic substrate, 4-Methylumbelliferone and release a free fluorophore which can be easily quantified using a fluorescence microplate reader at fluorescence 360/445 nm (Ex/Em). Skin mucus lysozyme is expressed as pmol/min/mL. The mucus protein

concentration was measured using Pierce 660 assay according to manufactures protocol. The total protein concentration in the mucus sample was determined using bovine serum albumin as a standard using a 96 well plate at 660 nm. Total protein is expressed in ug/uL.

2.7 Gene expression analysis

Gene expression analysis was done on mucosal tissues such as skin and gill. The candidate genes selected for monitoring the general health and welfare of salmon were for stress response: *heat shock protein 70 (hsp70)*, *heat shock protein 90 (hsp90)*, immune response: *inducible nitric oxide synthase (inos)*, *lipoxigenase5 (lox5)*, *interleukin1β (il1β)* and *interleukin4/13a (il413a)*, mucus production: *mucin5 (muc5)*, *mucin18 (muc18)* and wound repair and healing: *matrix metalloproteinase9 (mmp9)*. The procedure for RNA extraction, reverse transcription and quantitative PCR (qPCR) followed were as described in [Hundal et al. \(2021\)](#). In brief, the total RNA was extracted from skin and gill tissue using EZ1 RNA Universal Tissue Kit (Qiagen) and the BioRobot EZ1 according to the manufacturer’s descriptions. Quality and integrity of RNA were assessed with the NanoDrop ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies) and the Agilent 2100 Bioanalyzer (Agilent Technologies). A two-step real-time PCR protocol was followed to assess the mRNA transcriptional levels of the selected target genes. The qPCR was run on a LightCycler® 480 Real-Time PCR System with the SYBR Green Mastermix (Roche Applied Sciences, Basel, Switzerland) and using the following temperature program: 5 min denaturisation and activation at 95°C, 45 cycles of 10 s denaturisation at 95°C, 10 s annealing at 60°C and 10 s synthesis at 72°C. A melting point analysis was performed before cooling to 4°C. The stability of the reference genes (geometric mean of both *βact* and *elf1α*) and mean normalized expression of the target genes were calculated using CFX Maestro software (Bio-Rad CFX maestro version 1.1, Bio-Rad laboratories). The list of primers and reference genes used are as given in [Table 2](#). The efficiency of all PCR runs was higher than 96% (ranging from 96 to 125%).

2.8 Statistical analysis and calculations

Statistical analyses were done using Statistica 13.4 (Statsoft Inc.) and GraphPad Prism version 9.0 (Graphpad Software Inc.). Data were tested for homogeneity of variance and normality using a Kolomogorov–Smirnov test and Shapiro–wilk test, respectively for normal distribution, and non-parametric test were used for non-normal distribution. A randomized block design (RBD) with three blocks (B1, B2, and B3) was used to account for cage-position effects. Data from gene expression analysis were log-transformed before statistical analysis. Data from blood, mucus, plasma ions, osmolality, metabolites, and cortisol and gene expression analysis were subjected to a factorial ANOVA, with diet, block and time points (mid and final sampling) as the main factors and cage as random factor, followed by Tukey multiple comparison tests. Data

TABLE 2 Primers used for quantitative PCR.

Primers	Forward	Tm	Reverse	Tm	Accession number	Primer length	Ta	Product size
<i>hsp70</i>	CCTGCCTACTTCAACGATTCACAGAGACA	56	CCAGCGATCACTCCAGCGTCCTTA	56	XM_045720591.1	25N	60 °C	59 bp
<i>hsp90</i>	GTGTGAACAAATGGAAATGGAACA	49	CAGCGTGCATGTTATGTTGCA	47	BT125328.1	25N	60 °C	81 bp
<i>inos</i>	ACAGACATTGGCCAGAGAC	49	CTCCATTCCCAAAGGTGCTA	47	AF088999.1	25N	60 °C	140 bp
<i>mmp9</i>	CTGGCCAGATAATTTGGAT	45	CATGGCTTTTGAGCCAGTTC	47	NM_001140457.1	25N	60 °C	133 bp
<i>il1β</i>	GCTGGAGAGTGCTGGAAAGAAC	54	CGTAGACAGGTTCAAATGCACCTTTGTG	53	AY617117	25N	60 °C	220 bp
<i>il413a</i>	GCATCGTTGTGAAGAGCCCAAGA	50	GAACTCTCTCAGCTCCACCT	51	AB574339	25N	60 °C	63 bp
<i>muc5</i>	CCGTGCTGGGAGACATTATGAAAT	52	TGCTGGAGAGGGAAGGGTAAC	52	XM_045690381.1	25N	60 °C	81 bp
<i>muc18</i>	AAGACAGCGAGGTGGTG	47	TCCGTTGACTTGGCAGATGA	47	XM_045724352.1	25N	60 °C	78 bp
<i>βact</i>	CCAAAGCCAAACAGGGAGAA	46	AGGGACAACACTGCCTGGAT	49	BC933897	25N	60 °C	91 bp
<i>elf1α</i>	TGCCCTCCAGGATGTCTAC	51	CACGGCCACAGGTACTG	50	AF321836	25N	60 °C	57 bp

Tm, melting temperature; Ta, annealing temperature.

from mortality, gill score and sea-lice were subjected to Kruskal–Wallis non-parametric analysis, using the median test and multiple pair wise comparisons by ranks. Data from delousing were subjected to a two-way ANOVA, with diet and delousing stress as the two main factors. Only in those cases where a significant effect was observed within a factor, one-way ANOVA followed by tukey’s multiple comparisons were performed for each factor separately. For all statistical tests, P values < 0.05 were considered significant. All results are expressed as mean ± standard error. All the graphs were made using GraphPad Prism version 9.0 (Graphpad Software Inc.).

Specific growth rate (SGR, % per day) =

$$\left[\frac{\ln(\text{Final Body Weight (g)}) - \ln(\text{Initial Body Weight (g)})}{\text{Number of feeding days}} \right] \times 100$$

Weight gain (WG, g) = (Final Body Weight (g) – Initial Body Weight (g))

$$\text{Weight gain (WG, \%)} = \left[\frac{(\text{Final Body Weight (g)} - \text{Initial Body Weight (g)})}{\text{Initial Body Weight (g)}} \right] \times 100$$

Fulton’s condition factor (K) =

$$(\text{Final Body Weight (g)}/\text{Final Body Length(mm)}^3) \times 100$$

Hepatosomatic index (HSI, %) = 100 × Liver weight (g)/Fish weight (g)

Viscerosomatic index (VSI, %) = 100 × Viscera weight (g)/Fish weight (g)

3 Results

3.1 Fish growth and survival

The growth performance indices such as, IBW and FBW were not significantly affected ($P > 0.05$) by the experimental diets (Table 3). Salmon were fed for 13 months with one of three experimental diets in triplicate seawater cages, the fish had grown to individual weights of 3394 ± 74 g with an SGR value similar in all dietary groups. The WG and WG % were not significantly different among the dietary groups (Table 3). The body indices such as HSI, VSI and K measured at the

TABLE 3 Growth and body indices of Atlantic salmon fed diets containing BSFL meal during 13 months in open sea-cages.

Growth parameter	Control	BSFL 5%	BSFL 10%	P value
IBW (g)	176±2.70	169±2.56	174 ±2.74	ns
FBW (g)	3407±76.89	3466±70.30	3318±70.68	ns
SGR (%)	1.4±0.03	1.4±0.03	1.4±0.03	ns
WG (g)	3257±126.9	3286±195.0	3186±90.2	ns
WG (%)	1838±112.9	1963±171.5	1804±42.1	ns
SR%	64±1.50	66±3.50	61±5.70	ns
Body indices				
HSI	1.1±0.14	1.1±0.07	1.0±0.06	ns
VSI	9.3±0.83	10.4±0.40	9.6±0.37	ns
K	1.4±0.05	1.4±0.02	1.4±0.06	ns

BSFL, black soldier fly larvae meal; IBW, initial body weight; FBW, final body weight; SGR, specific growth rate; WG, Weight gain; WG (%), Weight gain percentage; SR, survival rate; HSI, hepatosomatic index; VSI, viscerosomatic index; ns, not significant; Values are expressed as mean ± standard error. Growth parameters (IBW and FBW) and body indices were calculated using 50 and 6 fish per cage (triplicate cages per diet), respectively.

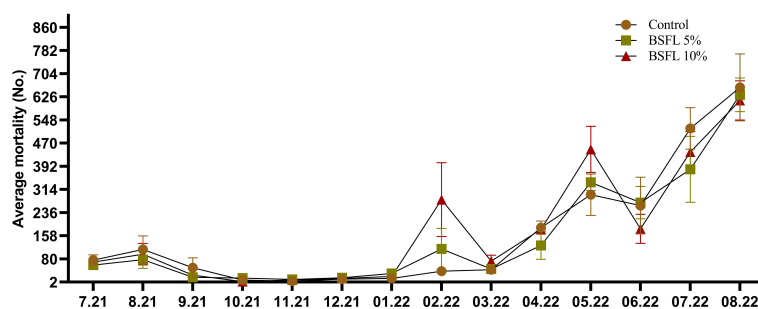


FIGURE 1

Total mortality (No.; number of dead salmon collected) during the 13 months experiment. The data are presented as the mean of dead fish/cage (± SE, triplicates cages per diet) collected during each month from July 2021–August 2022 (x-axis).

termination of the trial were not significantly different among the dietary groups (Table 3). The mortality data of salmon over the 13 months had no significant dietary effects ($P>0.05$) (Figure 1). The mortality was less until December 2021, but started to increase gradually, peaking in August 2022 (Figure 1). The autopsy result (Pharmaq Analytic, Bergen, Norway) verified the occurrence of *Pasteurellosis* and *Branchiomonas Cysticola* and reported heart inflammation and gill epitheliocysts.

3.2 Gill score and sea-lice

Figures 2A, B show the gill score and sea-lice count throughout the trial (August 2021–August 2022), respectively. No significant dietary effects on sea-lice counts and gill score were observed, except for in December 2021 where the 10% dietary inclusion of BSFL meal had a higher sea-lice compared with the fish fed control diet ($P<0.05$).

3.3 Hematological analysis

3.3.1 Cortisol

There was a significant ($P<0.05$) difference in the mean cortisol value from the two sampling points (mid and final) (Figure 3). The mean cortisol value from the final sampling (190–358 ng/mL) was almost double the mean value from the mid-sampling (113–144 ng/mL). Moreover, a significant dietary effect was observed among the

dietary groups from the final sampling, where the cortisol levels were significantly ($P<0.05$) lower in the 10% BSFL fed group than the control group.

3.3.2 Red blood cell and hemoglobin

The RBC count and Hb levels at the two sampling points are depicted in Figures 4A, B. No significant dietary effects ($P>0.05$) were noted during the mid-sampling. However, at the final sampling, the BSFL-fed groups exhibited significantly higher RBC and Hb levels ($P<0.05$) compared to the control.

3.3.3 Plasma metabolites

At the mid-sampling, the levels of ALT, AST and triglycerides were significantly ($P<0.05$) lower in salmon fed dietary BSFL meal at 5% compared to salmon fed control diet and BSFL 10% diet (Table 4). Cholesterol level was significantly higher in salmon fed BSFL 10% compared to salmon fed control and BSFL 5%. However, the levels of ALP, amylase, total protein, glucose, ions (K^+ , Na^+ , Ca^{++} , Cl^-) and Lac were not significantly affected ($P>0.05$) by diets at this time point. There were no significant differences in the plasma metabolites at the final sampling (Table 4).

3.4 Skin mucus analysis

Figures 5A, B show the mucus DNA concentration and lysozyme activity, at two sampling points (mid and final sampling), respectively.

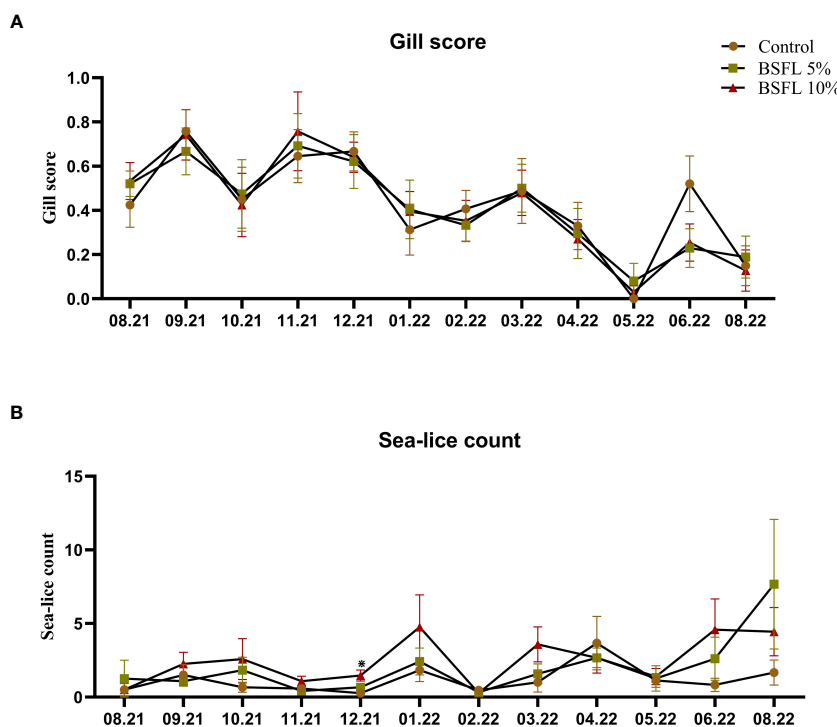
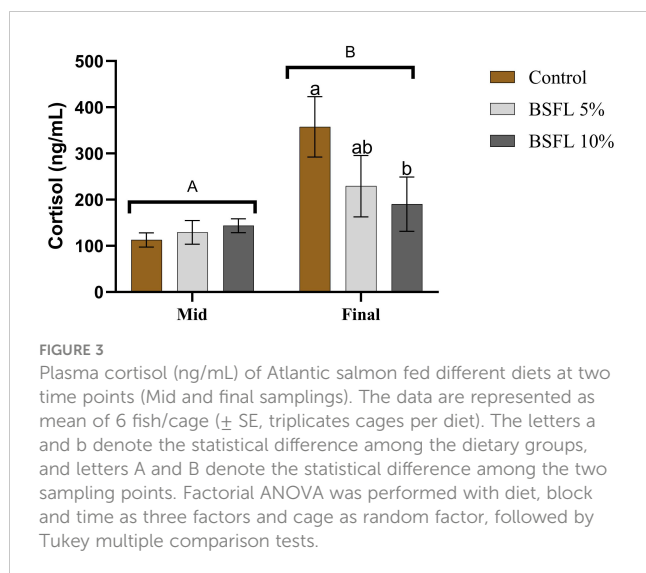


FIGURE 2

Gill score (A) and sea-lice count (B) of Atlantic salmon fed different diets during the 13 months experiment [from July 2021–August 2022 (x-axis)]. The data are presented as the mean of 20 fish/cage (\pm SE, triplicates cages per diet). The statistical difference among the dietary groups were detected with two-way ANOVA with diet and block as two factors and cage as random factor, followed by Tukey multiple comparison tests or multiple pair wise comparisons by ranks. *Denote the statistical difference between fish fed Control and BSFL10%.



The DNA concentration varied between 10 000-15 000 ng/mL and remained consistent during the mid-sampling, without any dietary effects. However, during the final sampling, the group fed with 5% BSFL had a significantly ($P < 0.05$) higher DNA concentration than the control and BSFL 10% groups (Figure 5A). No significant dietary effect was observed on lysozyme activity during the mid-sampling, but at the final sampling the control group exhibited significantly higher lysozyme activity than the groups fed with BSFL (Figure 5B).

3.5 Gene expression analysis

Figures 6A-C display the results of mRNA expression analysis for selected genes such as *interleukin1 β* (*il1 β*), *mucin18* (*muc18*) and *matrix metalloproteinase 9* (*mmp9*) in skin and gill tissues from the two different sampling points. In the skin tissue, *il1 β* was significantly upregulated in salmon fed BSFL 5% at mid-sampling whereas in gill tissue *muc18* and *mmp9* expression were significantly upregulated in the BSFL 5% fed group compared to the two other dietary groups from the mid-sampling. Meanwhile, *il1 β* was significantly upregulated ($P < 0.05$) in gill of salmon fed BSFL 5% in both sampling points. The other genes *heat shock Protein* (*hsp70*, *hsp90*), *inducible nitric oxide synthase* (*inos*), *lipoxigenase* (*lox5*) and *interleukin413a* (*il413a*), *mucin* (*muc5*) that were investigated were not significantly affected due to dietary BSFL meal (data not presented). The average expression stability (M value) for the reference genes were 0.29 and 0.33 M value for gill and skin plates respectively.

3.6 Delousing

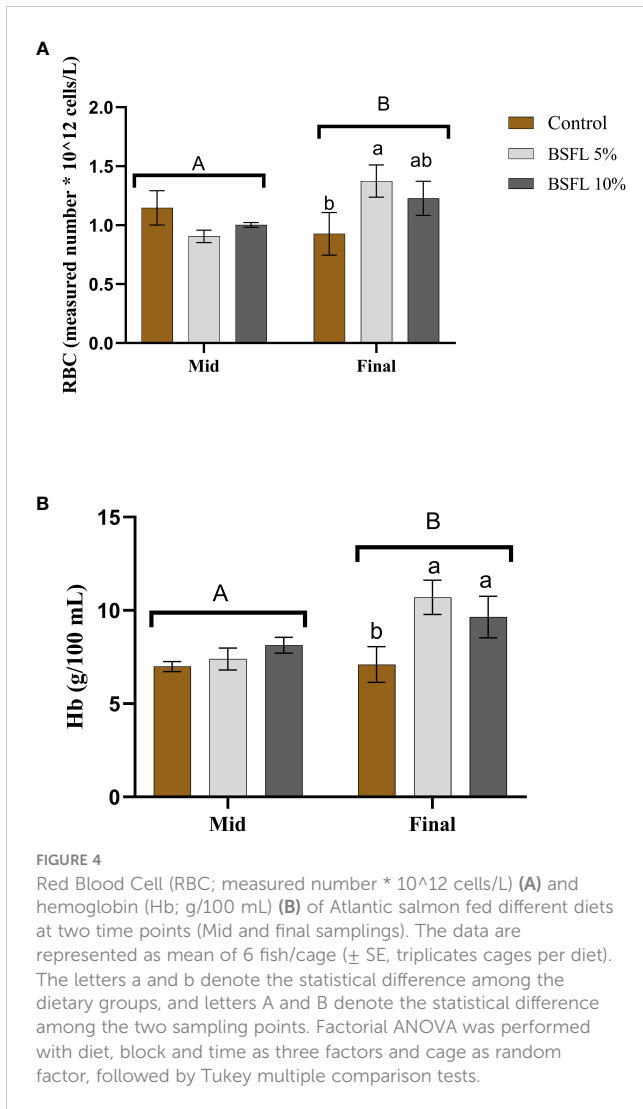
Figure 7 shows the skin mucus DNA concentration (A) and lysozyme activity (B) in skin mucus, as well as the plasma cortisol (C) and plasma osmolality levels (D) before and after delousing. No effects were detected in the mucus samples for either diet or cortisol-induced stress ($P > 0.05$). However, in the plasma samples,

a significant ($P < 0.05$) stress effect was observed in the cortisol level. Additionally, a significant interaction effect was observed between diet and stress in the plasma osmolality, with the BSFL 5% group exhibiting significantly higher levels ($P < 0.05$) than the control group (Figure 7D).

4 Discussion

The Atlantic salmon fed either 5% or 10% dietary BSFL meal showed a similar growth performance to the fish fed a diet with standard commercial protein sources. Earlier studies performed in laboratory scale in tanks on land, have also shown no effects on growth when using up to 12% full fat BSFL meal in pre smolts (Weththasinghe et al., 2021a) and up to 15% defatted BSFL meal in post smolt salmon (Belghit et al., 2019a) replacing the protein sources in the salmon feed (FM and plant-based protein). The current study thus confirms what has been seen earlier, but now verifying the lack of a negative impact on growth of Atlantic salmon fed BSFL meal in a situation much closer to a commercial aquaculture production.

The general welfare of salmon was monitored weekly (approximately 53 weeks) in terms of gill score and sea-lice count. These parameters were not affected by the experimental diet, except for December 2021, which is likely to be due to a random effect of sampling. Apart from that, the cumulative mortality rate was stable from the start of this trial (July-August 2021) until the mid-sampling (April 2022; 42 weeks post release into sea-cage) and was similar to the mortality pattern observed in Norwegian and Scottish salmon farms studied over the last 5 years (Soares et al., 2011; Bang Jensen et al., 2020), with an overall mortality rate of ~15% in Norwegian farms. However, after this time of the year till the end of the current trial, the mortality rate increased, which peaked in August 2022 (52 weeks post release into sea-cage), making the overall mortality rate of this trial ~34%. In fact, this rate was similar to the mortality rate (~23%) reported for salmon in a similar production zone in 2021 (Norwegian fish health report, 2022). This high mortality in the current study can be linked to various factors including multiple delousing, and diseases faced by salmon during that period. The salmon were exposed to thermal delousing to maintain the adult sea-lice population within the legal limits. Thermal delousing, a commonly used method in salmon farms to control and eliminate sea-lice, can contribute to increased mortality due to its exposure to higher water temperatures (up to 32 °C for ~30 sec), and repeated handling that create a highly stressful environment for the salmon (Moltumyr et al., 2021). During one of the delousing procedures, samples were taken before and after the process, indicating a notable increase in stress levels with higher plasma cortisol levels observed after one hour. However, the cortisol response was independent of the dietary BSFL meal in the salmon diet. Previous studies have also attempted different dietary modulation to reduce the delousing stress. For instance, Selvam et al. (2022) modulated the levels of dietary eicosapentaenoic acid and docosahexaenoic acid in Atlantic salmon but did not observe any significant dietary effect when exposed to mechanical delousing



stress. Nonetheless, previous studies reported reducing the sea-lice infestations and mortalities in salmon through functional feed ingredients (Jensen et al., 2015) and dietary nucleotides (Burrells et al., 2001a). Apart from the delousing and handling stress, increased mortality might be also due to bacterial infection with *Pasteurellosis*, and *Branchiomonas Cysticola*, observed in salmon during the regular farm monitoring (diagnosed in May-June 2022, analyzed by Pharmaq Analytic, Bergen, Norway). These bacteria are known to affect the heart and gills in salmon, if occurred in heavy load, leading to high mortality (Gjessing et al., 2021; Sandlund et al., 2021). However, in the current study, very high gill infection was not observed in the three dietary groups. Hence, a direct correlation between gill score and bacterial occurrences was not observed. Nevertheless, it is plausible that diseases alone might not induce mortalities, but the combined stress from handling, delousing, and pathogenic pressure could contribute to increased mortality among salmon (Segner et al., 2012). There were, however, no effects of diets on growth or survival, demonstrating that diets containing up to 10% BSFL meal can give a long-term performance equal to fish fed a commercial diet in open sea-cages.

Studying hematological indices and mucosal health can be an easy and an important diagnostic tool for observing stress, physical anomalies, diseases symptoms and monitoring health (Assefa and Abunna, 2018; Seibel et al., 2021). The most used indicator for assessing stress levels in fish is plasma cortisol, which was measured at the mid and final sampling of this study to assess potential differences in basal stress level due to diet. At the final sampling, the plasma cortisol levels were higher in the salmon fed the control diet than the fish fed diets containing BSFL meal. The observed higher cortisol in the final sampling is hypothesized to be due to the exposure of salmon to multiple stressors mentioned in this study towards the end of the trial. The series of stressful events eventually resulted in elevated stress in salmon resulting in higher cortisol response in plasma. Furthermore, parallel to the above findings, the control group had a lower RBC and Hb count than the fish fed BSFL

TABLE 4 Plasma metabolites, ions and osmolality of Atlantic salmon fed experimental diets during 8 and 13 months (mid and final samplings, respectively) in open sea-cages.

Parameter	Mid				Final			
	Control	BSFL 5%	BSFL 10%	P value	Control	BSFL 5%	BSFL 10%	P value
ALP (U/L)	164.1±13.6	199.7±25.9	206.2±15.8	ns	376.2±122.1	165.6±17.4	135.2±18.9	ns
ALT (U/L)	19.5±1.1 ^a	10.1±1.5 ^b	22.6±4.5 ^a	<0.05	23.2±3.0	20.2±1.7	16.4±1.5	ns
Amylase (U/L)	611.1±58.1	735.7±55.3	632.2±37.8	ns	1286.2±184.6	1192.6±89.8	1055.4±104.6	ns
AST (U/L)	1180.4±431.1 ^a	347.1±54.3 ^b	1496.3±359.3 ^a	<0.05	1128.3±212.9	1120.3±170.8	692.9±115.1	ns
Cholesterol (mmol/L)	5.5±0.19 ^b	5.7±0.41 ^b	6.5±0.30 ^a	<0.05	6.9±0.6	7.1±0.5	6.5±0.5	ns
Glucose HK (mmol/L)	6.4±0.18	6.5±0.26	6.9±0.16	ns	7.7±0.7	7.3±0.7	6.9±0.3	ns
Total protein (g/L)	34.5±1.08	36.0±1.88	37.4±0.96	ns	41.2±2.6	39.2±2.0	37.3±1.8	ns
Triglyceride (mmol/L)	3.0±0.29 ^a	1.9±0.27 ^b	3.3±0.52 ^a	<0.05	2.7±0.5	2.5±0.3	2.3±0.2	ns

(Continued)

meal into the diet may have a positive effect on the liver health of salmon. In the current study a reduction in plasma cholesterol and triglycerides was observed in the group fed BSFL which were similar to the studies reported in Japanese seabass (*Lateolabrax japonicus*) and African catfish (*Clarias gariepinus*) when FM was replaced by dietary BSFL meal (Wang et al., 2019; Fawole et al., 2020). This effect is likely due to the presence of chitin in BSFL, which has been documented to lower triglyceride and cholesterol levels (Wang et al., 2019). However, it is also possible that dietary cholesterol level or feeding status of salmon may have contributed to the observed effect, as the fish were sampled at a fed state.

Dietary inclusion of BSFL meal up to 10% in Atlantic salmon did not have any significant effects in general growth and survival. The salmon fed BSFL 5% showed an increase in the number of erythrocytes, and a reduced cortisol response when exposed to various stressors during the end of the trial and could indicate the antioxidant capacity of salmon fed BSFL diet. Moreover, this group of salmon exhibited a higher skin mucus DNA concentration which suggested higher mucus secretions enabling better protection against pathogens and stress conditions. Also, the inflammatory gene such as *il1 β* , was upregulated in skin and gill tissue (mid and final samplings), while the plasma ALT and AST levels were lower in salmon fed dietary BSFL meal at 5% (mid sampling). It is worth mentioning that the health indicators observed in salmon behave differently across different fish sizes, time points and environmental conditions. In conclusion, the results of this study showed that dietary BSFL meal can modulate the immune responses in Atlantic salmon reared at large scale under farm-like conditions.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

This trial is exempt from application to the Norwegian Food Safety Authority, according to the regulation “FOR-2015-06-18-761 Regulation concerning the use of animals for scientific purposes, §6. Godkjenning av forsøk”. The approval requirement does not apply to experiments involving only the killing of animals to use organs or tissues from them. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

GR: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources Data Curation, Writing -

Original Draft Writing - Review & Editing, Visualization. AP: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources Data Curation, Review & Editing, Visualization, Supervision. MK: Conceptualization, Methodology, Investigation, Review & Editing, Project administration. NL: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Review & Editing, Visualization. E-JL: Conceptualization, Methodology, Validation, Investigation, Review & Editing. IB: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources Data Curation, Writing - Original Draft Writing - Review & Editing, Visualization, Supervision, Project administration. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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