

## Full length article

# Insect (black soldier fly, *Hermetia illucens*) meal supplementation prevents the soybean meal-induced intestinal enteritis in rainbow trout and health benefits of using insect oil

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## ABSTRACT

Black soldier fly larvae (BSFL) and their oils (BSFLO) are receiving increasing attention as sustainable ingredients in fish feeds, but mostly as replacements to marine sources. There were two aims to this study; in exp. 1, soybean meal (SBM)-based diets were formulated to contain BSFL as supplements at 0 (SBM), 8 (SBM + BSFL<sub>low</sub>) or 16% (SBM + BSFL<sub>high</sub>) with a control diet being fishmeal-based (FM). In exp. 2, diets included only fish oil (FO), soybean oil (SBO), BSFLO or BSFLO + bile acid (BA), and all lipid sources were added at 16%. Both experiments were run at the same time and fed to rainbow trout (32 g) with each treatment being triplicated. After 10 weeks the fish were sampled for liver and distal intestine histology, expression of genes responsible for inflammation in the intestine and kidneys, and serum peroxidase and lysozyme activities. In exp. 1, supplementations of BSFL effectively prevented SBM-induced intestinal enteritis, down-regulated intestinal prostaglandin and interferon regulatory factor 1 (*IRF-1*), while the SBM + BSFL<sub>high</sub> diet significantly increased serum lysozyme activity. In exp. 2, BSFLO caused no histomorphological change to the liver or intestine, but kidney interleukin-8, tumor necrosis factor and *IRF-1* were significantly upregulated along with significantly higher serum peroxidase activity. The inclusion of BA in the BSFLO diets significantly upregulated intestinal prostaglandin gene expression. Overall, BSFL supplementations of 8 or 16% prevented SBM-induced intestinal enteritis based on histological observations, which was supported by a down-regulation in pro-inflammatory genes and enhanced innate immunity. Meanwhile, the use of BSFLO showed some immunological benefits. Therefore, these sustainable resources are recommended in the diets of rainbow trout, especially when using elevated levels of plant-based proteins.

## 1. Introduction

The aquaculture industry continues to expand globally, and this has led to an upward rise in the demand for protein and lipid sources. Traditionally, this demand was satisfied by fishmeal (FM) and fish oil (FO), however this is no longer possible due to a substantial reduction in availability and increased cost. Currently, plant-based proteins and oils are the dominant ingredients in formulated fish feeds, with the majority being sourced from soybeans. Soybean meal (SBM) has a relatively well-balanced amino acid profile, but high inclusion levels can cause

deleterious effects in fish performance, causing intestinal inflammation and damage to the epithelial barrier, and reducing the absorptive function of the enterocytes [1]. For instance, higher replacement of FM with SBM in rainbow trout diets have been reported to cause negative impacts on performance, gut health and membrane integrity [2]. Also, high inclusion of SBM was noted to induce inflammatory processes in the distal intestine of normal strains of rainbow trout [2]. Other authors have also reported similar effects such as inflammatory cell infiltration, intestinal structure damage, changes in gut microbial diversity, mucosal fold shortening, and suppression of immune system and growth in

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**Table 1a**

Ingredients (%) and proximate composition (% as is basis) of the experimental diets.

| Ingredients                     | Fishmeal | Soybean meal (SBM) | SBM + 8% BSFL | SBM + 16% BSFL |
|---------------------------------|----------|--------------------|---------------|----------------|
| Fish meal                       | 24       | 9.5                | 9.5           | 9.5            |
| Soybean meal                    | 0        | 21                 | 21            | 21             |
| Black soldier fly larvae (BSFL) | 0        | 0                  | 8.0           | 16             |
| Poultry meal                    | 10.2     | 11.8               | 7.8           | 5.5            |
| Soy protein concentrate         | 6.0      | 5.5                | 5.0           | 4.0            |
| Wheat gluten                    | 5.0      | 5.0                | 5.0           | 5.0            |
| Corn Protein Conc               | 6.0      | 6.5                | 6.0           | 5.0            |
| Blood meal                      | 5.0      | 5.0                | 5.0           | 4.5            |
| Wheat flour                     | 24.9     | 15.6               | 13.3          | 11.1           |
| Fish oil                        | 16.0     | 17.0               | 16.4          | 15.5           |
| Dicalcium phosphate             | 1.2      | 1.2                | 1.2           | 1.2            |
| Choline chloride (60%)          | 0.6      | 0.6                | 0.6           | 0.6            |
| Vitamin premix                  | 0.8      | 0.8                | 0.8           | 0.8            |
| Trace mineral mixture           | 0.1      | 0.1                | 0.1           | 0.1            |
| Vitamin C, Stay C-35)           | 0.2      | 0.2                | 0.2           | 0.2            |
| Lysine                          | 0        | 0.1                | 0.06          | 0.02           |
| Methionine                      | 0        | 0.1                | 0.08          | 0.05           |
| Total                           | 100      | 100                | 100           | 100            |
| Proximate composition           |          |                    |               |                |
| Moisture (%)                    | 6.0      | 5.8                | 6.0           | 5.9            |
| Crude protein (%)               | 44.8     | 44.5               | 43.9          | 43.8           |
| Crude lipid (%)                 | 21.0     | 21.0               | 21.1          | 20.8           |
| Ash (%)                         | 7.6      | 7.1                | 7.0           | 7.3            |

different fish species [1,3–7]. This is likely due to a combination of antinutritional factors within SBM that cause intestinal enteritis, which is a major obstacle to the use of higher SBM inclusions.

As the aquafeed industry embraces higher inclusion rates of plant proteins in fish diets, there is an urgent need to devise strategies to protect intestinal membrane integrity, especially in carnivorous fish. This is important to ensure adequate nutrient absorption, immune response, gut health and growth when fed diets high in SBM. Some strategies to improve intestinal health in trout subjected to high SBM diet have included exploring the potential of different feed additives in attenuating inflammatory processes in fish. For instance, dietary tributyrin supplementations in a high-SBM based diet was reported to enhance growth performance, reduce oxidative damage, and improve the intestinal morphology structure in yellow drum, *Nibea albiflora* and black sea bream, *Acanthopagrus schlegelii* [7,8]. Rimoldi et al. [1] reported that 0.2% butyrate and taurine supplementations mitigated inflammation in European sea bass, *Dicentrarchus labrax*, fed a high SBM-based diet. In spite of the beneficial impact of some of these additives, the direct use in fish diets are often hampered either due to their offensive odour or fast absorption before reaching the intestine [8]. Recent findings indicate that insect meal contains bioactive peptides, which have been reported to play an important role in boosting the innate immunity of teleost fish, improve survival, and reduce intestinal inflammation [9–11]. The presence of these bioactive peptides are reported to have originated from the abilities of insects to grow and survive in harsh environments thus prompting them to produce compounds with antimicrobial functions [12,13].

Black soldier fly (*Hermetia illucens*) larvae (BSFL) is a highly sustainable candidate insect species due to their viable commercial production and have been shown to be suitable as an ingredient in aquaculture diets [14–16]. Notably, studies have indicated that the inclusion of insect meals in fish diets could help to improve the survival, antioxidant capacity, and immune response in fish and shrimp [9,18,19]. Recently, house fly larvae (HFL) meal was tested in swamp eel, *Monopterus albus*, which had positive effects on their immunity and gut microbial diversity [11].

**Table 1b**

Ingredients (%) and proximate composition (% as is basis) of the experimental diets.

| Ingredients                           | FO   | SBO  | BSFLO | BSFLO + BA |
|---------------------------------------|------|------|-------|------------|
| Fish meal, sardine <sup>a</sup>       | 24   | 24   | 24    | 24         |
| Poultry meal <sup>a</sup>             | 10.2 | 10.2 | 10.2  | 10.2       |
| Soy protein concentrate <sup>b</sup>  | 6    | 6    | 6     | 6          |
| Wheat gluten meal <sup>a</sup>        | 5    | 5    | 5     | 5          |
| Corn Protein Concentrate <sup>c</sup> | 6    | 6    | 6     | 6          |
| Blood meal <sup>a</sup>               | 5    | 5    | 5     | 5          |
| Wheat flour <sup>a</sup>              | 24.9 | 24.9 | 24.9  | 23.4       |
| Fish oil <sup>a</sup>                 | 16   | 0    | 0     | 0          |
| Plant oil <sup>d</sup>                | 0    | 16   | 0     | 0          |
| Insect oil <sup>d</sup>               | 0    | 0    | 16    | 16         |
| Bile salt (Ox-Bile) <sup>e</sup>      | 0    | 0    | 0     | 1.5        |
| Dicalcium phosphate <sup>a</sup>      | 1.2  | 1.2  | 1.2   | 1.2        |
| Choline chloride (60%) <sup>a</sup>   | 0.6  | 0.6  | 0.6   | 0.6        |
| Vitamin premix <sup>f</sup>           | 0.8  | 0.8  | 0.8   | 0.8        |
| Trace Mineral mixture <sup>g</sup>    | 0.1  | 0.1  | 0.1   | 0.1        |
| Vitamin C (Stay C-35%) <sup>h</sup>   | 0.2  | 0.2  | 0.2   | 0.2        |
| Total                                 | 100  | 100  | 100   | 100        |
| Proximate composition                 |      |      |       |            |
| Moisture (%)                          | 6.0  | 6.0  | 6.2   | 7.4        |
| Crude protein (%)                     | 44.8 | 44.5 | 44.1  | 44.4       |
| Crude lipid (%)                       | 21.0 | 21.7 | 21.4  | 20.9       |
| Ash (%)                               | 7.6  | 8.4  | 8.6   | 7.8        |

FO, fish oil; SO, soybean oil; BSLO, black soldier fly larvae oil; BA, bile acid.

<sup>a</sup> Rangen Inc., Buhl, ID, USA.

<sup>b</sup> Profine VF, The Solae Company, St. Louis, MO, USA.

<sup>c</sup> Empyrean® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.

<sup>d</sup> EnviroFlight™, USA.

<sup>e</sup> Oxbile extract powder (cholic acid: 51.2%), Lot No. 786269, Creative enzymes, USA.

<sup>f</sup> Vitamin premix supplied the following per kg diet: vitamin A, 2.4 mg; vitamin D, 0.15 mg; vitamin E, 267 mg; vitamin K as menadione sodium bisulfite, 20 µg; thiamin as thiamin mononitrate, 32 mg; riboflavin, 64 mg; pyridoxine as pyridoxine-HCl, 64 mg; pantothenic acid as Ca-D-pantothenate, 192 mg; niacin as nicotinic acid, 240 mg; biotin, 0.56 mg; folic acid, 12 mg; vitamin B<sub>12</sub>, 50 µg; and inositol as meso-inositol, 400 mg.

<sup>g</sup> US Fish and Wildlife Service Trace Mineral Premix #3. It supplied the following (mg/kg diet): Zn (as ZnSO<sub>4</sub>·7H<sub>2</sub>O), 75; Mn (as MnSO<sub>4</sub>), 20; Cu (as CuSO<sub>4</sub>·5H<sub>2</sub>O), 1.54; I (as KIO<sub>3</sub>), 10.

<sup>h</sup> Skretting USA, Tooele, UT, USA.

Although previous findings have reported the suitability of replacing dietary FM or SBM with BSFL in different fish species, little is known regarding BSFL as a functional feed ingredient in fish diets, especially when using diets high in plant-based proteins. Moreover, scarce attention has been given to BSFL oil (BSFLO) as a replacement to FO. This can provide important information because BSFL are relatively high in crude lipid, which makes BSFLO an abundant resource or by-product. In this two-part study, the aims were to: (i) evaluate the additive effect of BSFL in a SBM-based diet; (ii) examine the use of BSFL oil as a substitute for FO and soybean oil (SBO) in the diets of rainbow trout. The study emphasizes the dietary effects of BSFL by evaluating histological effects in the liver and distal intestine of rainbow trout as well as the immune response indices, and the expression of inflammatory genes.

## 2. Materials and methods

### 2.1. Diet preparation and feeding trial

Experiment I: Four iso-nitrogenous and iso-lipidic diets were formulated to contain a fishmeal (FM) based diet (control), and SBM-based diets (21% SBM that replaced 60% of FM) with increasing supplemental amounts of black soldier fly larvae (BSFL) at 0%, 8% or 16% (Table 1a). All ingredients were mixed together homogeneously in a Hobart mixer, water was added and then cold pelleted using a laboratory pellet mill (California Pellet Mill Company, San Francisco, CA, USA).

**Table 2**  
Primers and probes used for one-step quantitative real-time (RT-PCR).

| Protein                          | Gene            | Forward Primers (5'-3')   | Reverse Primers (5'-3')      | Probes                     | Accession numbers |
|----------------------------------|-----------------|---------------------------|------------------------------|----------------------------|-------------------|
| Interleukin 8                    | IL-8            | AGCCAGCCTTGTCTGTTGTG      | AGT TTA CCAATTCGTCTG CTT TCC | TCCTGGCCCTCCTGACCATTACTGAG | AJ279069          |
| Tumor necrosis factor $\alpha 1$ | TNF- $\alpha 1$ | TGGAGCCTCAGCTGGAGATATT    | CCGGCAATCTGCTTCAATGTATT      | CATTGGTGCAAAAGATAC         | AJ401377          |
| Interferon Regulatory Factor 1   | IRF-1           | GAAGACAGTCACCAAGAAACCCTTA | GCTCAGGAACCTCTTGTGCTTT       | ACACTGCCTTGCTCCC           | NM_001124293      |
| Prostaglandin D                  | ProstD          | GGCTCTTGCTGGAGGATGAC      | GAAGCGGCCTGGGATGT            | CTGGCCAAGAAGACTG           | AF281353          |
| $\beta$ -actin                   | bact            | CCCTCTTCCAGCCCTCCT        | AGTTGTAGGTGGTCTCGTGGATA      | CCGCAAGACTCCATACCGA        | AF254414          |

attached with a 2.4 mm die at the University of Idaho, Hagerman Fish Culture Experiment Station. The feed was dried in a forced-air dryer set at 40 °C until the moisture content was less than 10%. The diets were stored at ambient temperature (20–22 °C) until use. The proximate composition of the diets is shown in Table 1a.

Experiment II: Four iso-nitrogenous and iso-lipidic FM-based diets were formulated in which fish oil (FO) was completely replaced with either soybean oil (SBO) or black soldier fly larvae oil (BSFO). One of the BSFO diets were supplemented with bile acid (1.5% Ox-Bile) (IOB). The diets preparation was a described above with the exception of variance oil in the formulation (Table 1b).

Both of these experiments were run concurrently under identical laboratory conditions. Rainbow trout (525, average weight: 32 g) were stocked at 25 fish per tank (145-L) in triplicate. Each tank was supplied with 8 L min<sup>-1</sup> of constant temperature (15 °C), gravity-fed spring water. Fish were hand fed to apparent satiation twice daily for 10 weeks. The photoperiod was maintained at 14 h light: 10 h dark using an electric timer-controlled fluorescent light. During this time, water quality was monitored for ammonia-N, dissolved oxygen, pH, and temperature and were all within acceptable levels for rainbow trout.

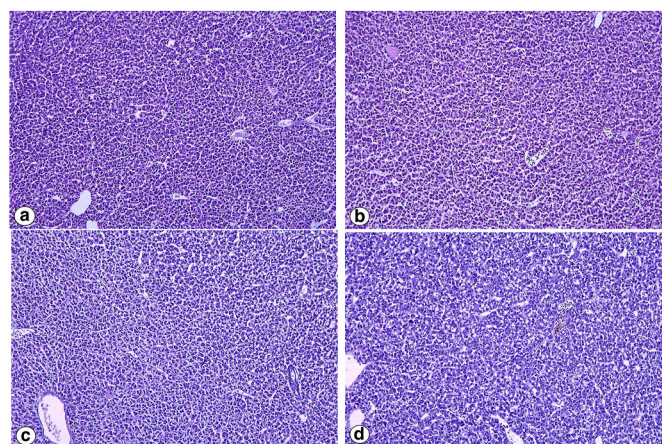
All fish handling and sampling, plus the experimental protocols used in the feeding trial were approved in advance by the University of Idaho's Institutional Animal Care and Use Committee.

## 2.2. Sampling

Upon completion of the feeding trial, three fish per tank (n = 9) were randomly selected and euthanized with tricaine methanesulfonate (MS-222, 300 mg L<sup>-1</sup>, buffered to pH 7.0) for blood collection. The blood was kept in ice for 4 h, and serum was collected after centrifugation at 2000×g for 10 min at 4 °C and used for peroxidase and lysozyme assay. The sacrificed fish were dissected the liver, kidney, and distal intestine were collected for analysis. The liver and distal intestinal samples were fixed in 10% formalin for 24 h, and then placed in isopropanol. They were soon after processed for histological study (section 2.3). The head kidney and distal intestinal samples were placed into 1.5 mL RNAase free tubes (AXYGEN™) for gene expression study, frozen in liquid N<sub>2</sub> and stored at –80 °C until processed (section 2.4).

## 2.3. Liver and intestinal histology

The liver and distal intestines were dehydrated using a graded series of alcohol baths and cleared of alcohol using xylene in a Tissue-Tek Vacuum Infiltration Processor (Sakura Finetek Inc., Torrance, CA). Tissues were then infiltrated with paraffin wax using a Tissue-Tek TEC Tissue Embedding Console System (Sakura Finetek USA Inc., Torrance, CA). Using a rotary microtome (ThermoHM 325, ThermoScientific), a total of four cross sections from each replicate were cut at 5-μm. All sections were then stained with haematoxylin and eosin and the slides were examined and representative pictures were taken under a light microscope (Leica DM 750) at 100 and 200 fold magnification.



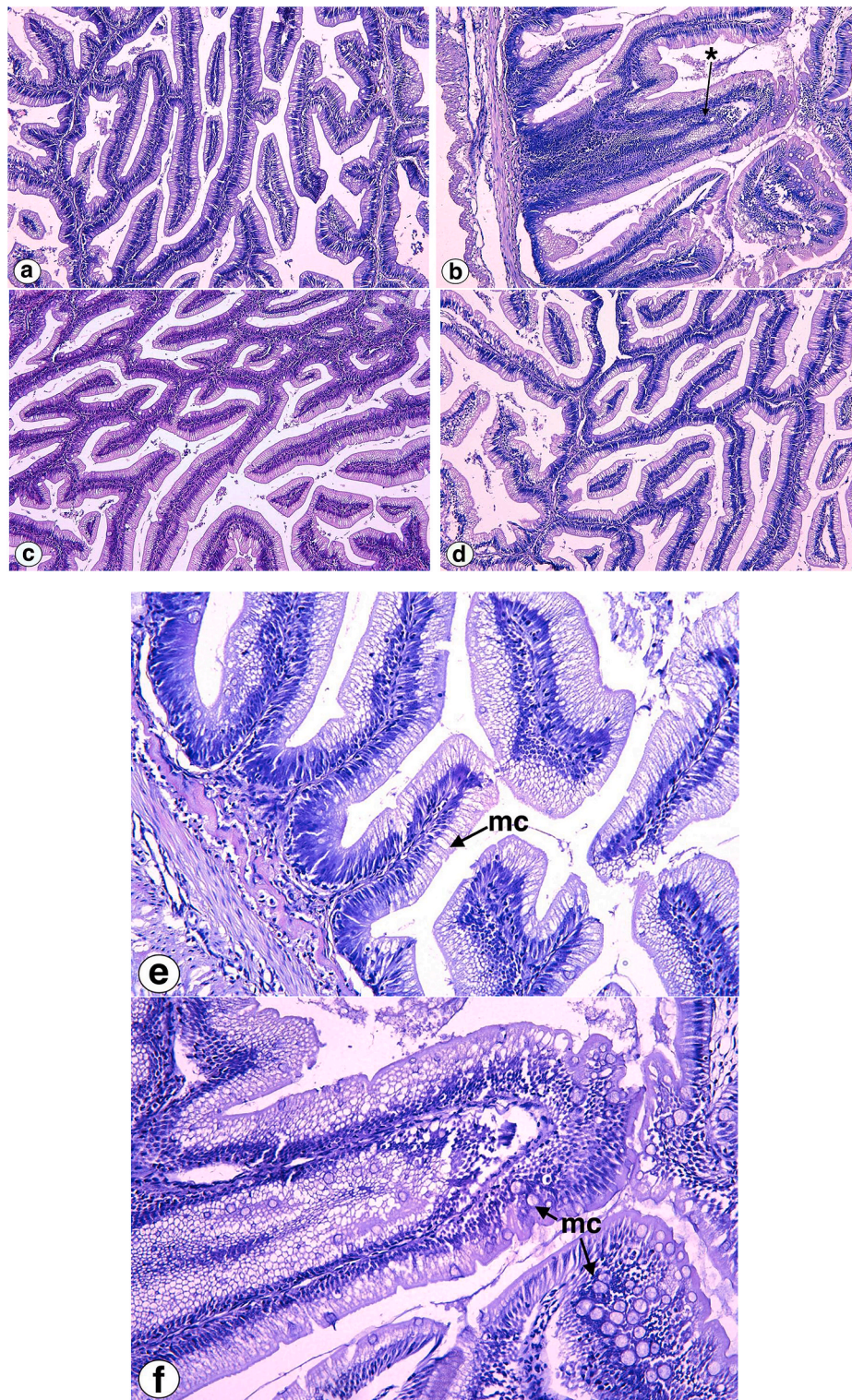
**Fig. 1.** Liver histological sections of rainbow trout fed fishmeal-based diet (a), soybean meal (SBM)-based diet (b) SBM-based diets with 8% BSFL (c) or 16% BSFL (d), with all livers appearing normal with no evidence of inflammation or damage. Haematoxylin & eosin staining; × 200 magnification.

## 2.4. Gene expression

Total RNA was isolated from collected liver samples (n = 9 fish per diet) using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The extraction was done in a biosafety cabinet. The RNA purity and quantity of samples were determined by measuring absorbance at 260 and 280 nm (NanoDrop 2000 spectrophotometer, Thermo Scientific, USA). To ascertain the level of inflammatory-related gene expression, a quantitative real-time RT-PCR was performed with an ABI QuantStudio Real-Time PCR system using the TaqMan One-Step RT-PCR Master Mix Reagents kit (Foster City, CA, USA). The concentration of each reaction was: Master Mix, 1 × (contains AmpliTaq Gold enzyme, dNTPs including dUTP, a passive reference, and buffer components); MultiScribe reverse transcriptase, 0.25 U μl<sup>-1</sup>; RNase inhibitor mix, 0.4 U μl<sup>-1</sup>; forward primer 600 nM; reverse primer 600 nM; probe, 250 nM; total RNA, 75 ng. The primer sequences of each gene, accession numbers, and probes are depicted in Table 2 β-actin was used as the reference housekeeping gene. The thermocycler conditions used for the tested genes consisted of 30 min at 48 °C, 10 min at 95 °C, then 40 PC R cycles at 95 °C for 15 s followed by 60 °C for 1 min. After carrying out the melting curve analysis to confirm a single PCR product in each reaction, the comparative CT method was used to determine the relative expression of the target genes (Interleukin-8 (IL-8), tumor necrosis factor α1 (TNF-α1), interferon gamma (IFN-γ) and prostaglandin), and the values were normalized by 2-ΔΔCT method with β-actin as the reference gene. Each sample assay was run in duplicate.

## 2.5. Data analysis

Data were tested for normality and homogeneity of variance prior to one-way Analysis of Variance (ANOVA). If significant differences were found, data were subjected to Duncan's multiple range test to separate



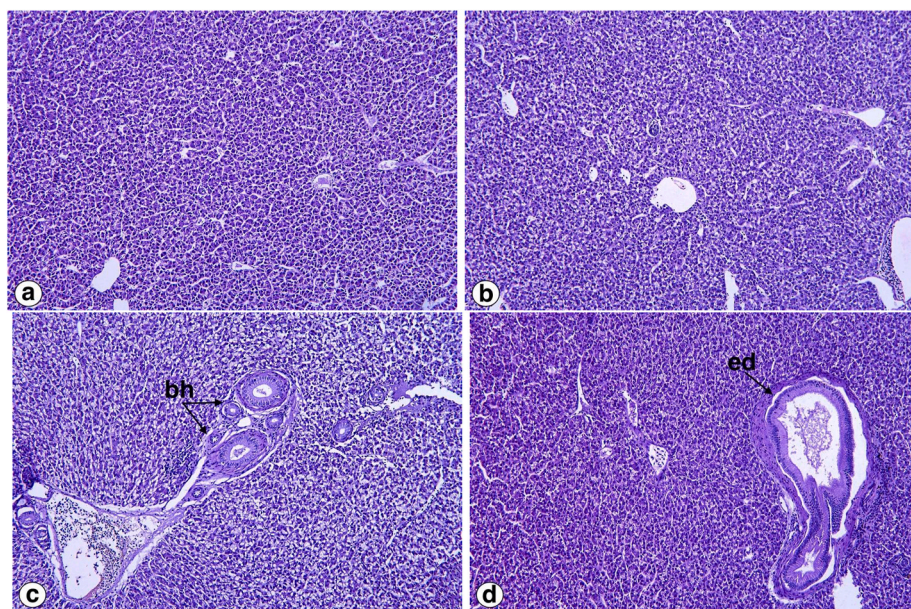
**Fig. 2.** Distal intestinal histological sections of rainbow trout fed fishmeal-based diet (a), soybean meal (SBM)-based diet (b) SBM-based diets with 8% BSFL (c) or 16% BSFL (d). Inflammation of the intestinal villi and lamina propria (asterisk) were observed in rainbow trout was observed in SBM group (b). Haematoxylin & eosin staining;  $\times 100$  magnification. Magnified view of distal intestinal histological sections of rainbow trout fed fishmeal-based diet (e) and soybean meal (SBM)-based diet (f). More mucus cells (mc, goblet cells) observed in the SBM treatment. Haematoxylin & eosin staining;  $\times 200$  magnification.

the means at a significance level of  $p < 0.05$ .

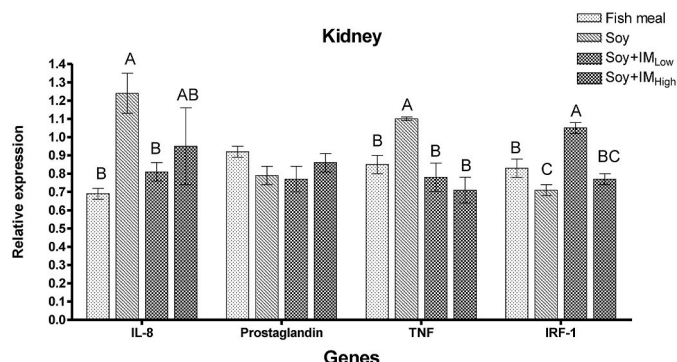
### 3. Results

#### 3.1. Histological examination

Experiment I: Histological appraisal of the livers in fish fed all the diets had normal sinusoid organization with no congestion,



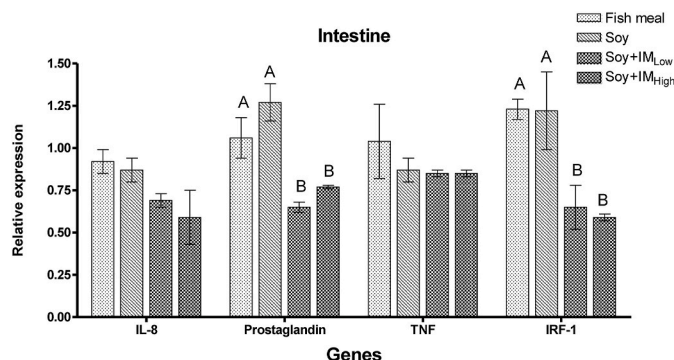
**Fig. 3.** Liver histological sections of rainbow trout fed the fish oil (FO)-based diet (a), soybean oil (SBO)-based diet (b), black soldier fly larvae oil (BSFLO)-based diet (c) or BSFLO-based diet + bile acid (BA) supplementation (d). Livers appeared normal in the FO (a) and SBO groups (b), with no evidence of excessive lipid storage, but there were instances of biliary hyperplasia (bh) in the BSFLO group (c) and enlarged bile ducts (ed) in the BSFLO + BA groups. Haematoxylin & eosin staining;  $\times 200$  magnification.



**Fig. 4.** Relative gene expression (mean  $\pm$  SE) of interleukin-8 (*IL-8*), prostaglandin D (*ProstD*), tumor-necrosis factor (*TNF*) and interferon regulatory factor 1 (*IRF-1*) in the kidneys of rainbow trout fed the fishmeal-based diet, soybean meal (SBM)-based diet (b) SBM-based diets with 8% BSFL (SBM + BSFL<sub>low</sub>) or 16% BSFL (SBM + BSFL<sub>high</sub>). Different letters within each gene indicate significant differences ( $p < 0.05$ ).

vacuolization or evidence of inflammation (Fig. 1a–d). In the distal intestine, the fish fed the FM diet (a), SBM + BSFL<sub>Low</sub> (c) or SBM + BSFL<sub>High</sub> diet (d) showed no inflammation in the villi or mucosa, and the microvilli were well-shaped (Fig. 2a, c & d). However, fish fed the with SBM (and no BSFL) (Fig. 2a, b) showed inflammation of the villi and lamina propria in the majority of sections due to infiltrations of leucocytes. Additionally, there were occasional instances of structural disintegration of the villi architecture (Fig. 2b). Magnified view of distal intestinal histological shown that SBM fed group exhibited the presence of mucus cells (goblet cells) (Fig. 2f) compared to the FM control (Fig. 2e).

**Experiment II:** The livers of fish fed the FO-based diet (Fig. 3a) and SBO-based diet (Fig. 3b) showed normal sinusoid organization with no congestion and no excessive hepatic vacuolization of lipid. In the livers of fish fed the BSFLO (Fig. 3c) and BSFLO + BA diet (Fig. 3d), there were occasional biliary hyperplasia (BH) and enlarged bile ducts (ED), respectively.

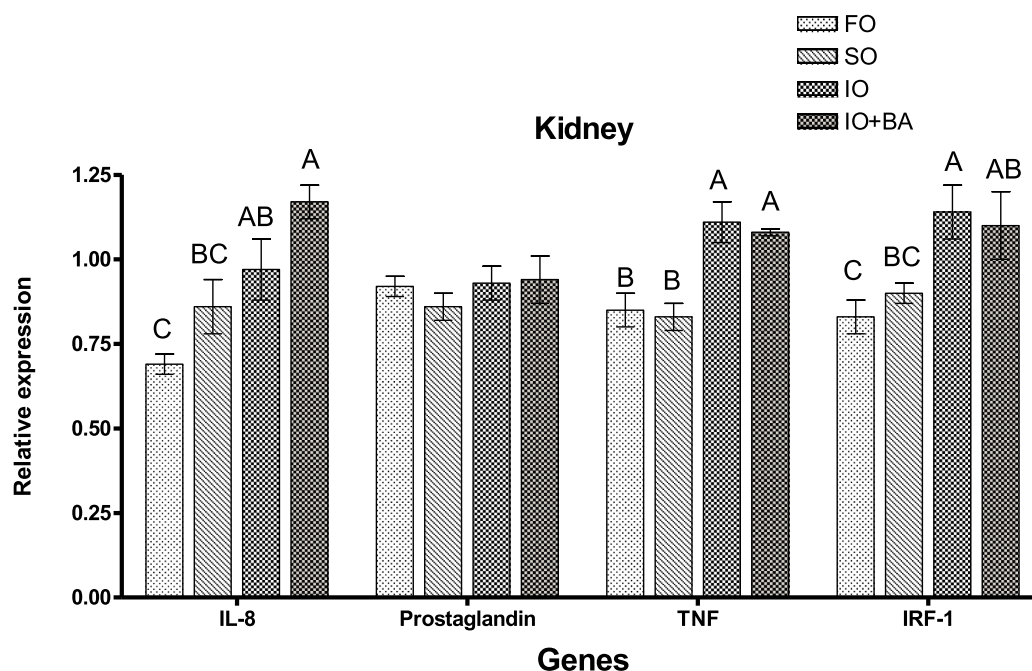


**Fig. 5.** Relative gene expression (mean  $\pm$  SE) of interleukin-8 (*IL-8*), prostaglandin D (*ProstD*), tumor-necrosis factor (*TNF*) and interferon regulatory factor 1 (*IRF-1*) in the intestines of rainbow trout fed the fishmeal-based diet, soybean meal (SBM)-based diet (b) SBM-based diets with 8% BSFL (SBM + BSFL<sub>low</sub>) or 16% BSFL (SBM + BSFL<sub>high</sub>). Different letters within each gene indicate significant differences ( $p < 0.05$ ).

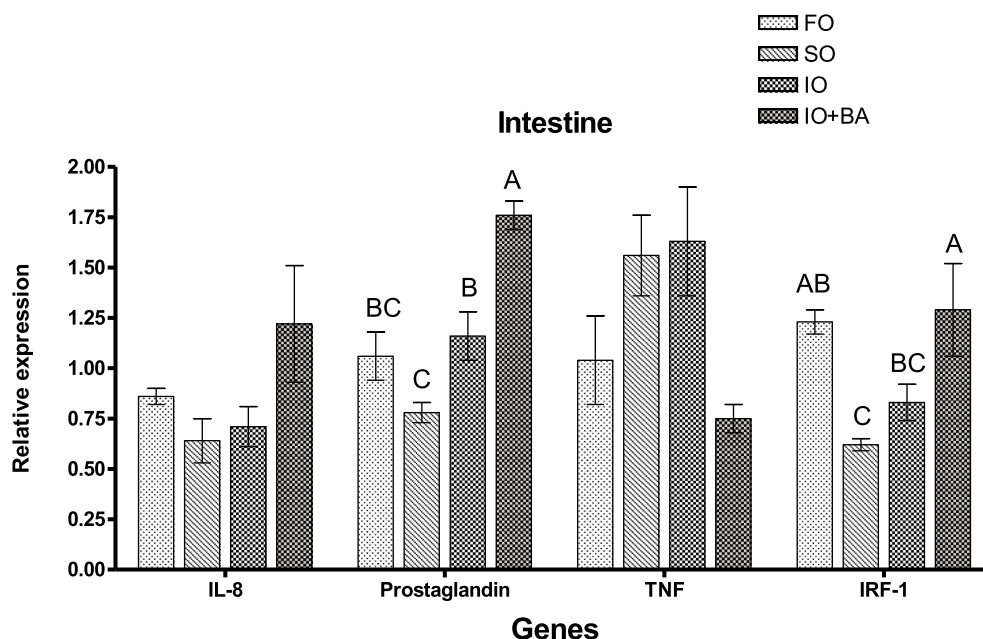
### 3.2. Immune-related inflammatory gene

**Experiment I:** The relative expression of proinflammatory cytokine (*IL-8*), tumor necrosis factor  $\alpha 1$  (*TNF- $\alpha 1$* ) and Interferon regulatory factor 1 (*IRF-1*) genes in the kidney of rainbow trout were influenced by the diet type (Fig. 4). The fish fed SBM diet showed a significantly higher expression of *IL-8* as well as *TNF- $\alpha 1$*  in the kidneys compared to other dietary groups ( $p < 0.05$ ). The expression of *IRF-1* was found to be up-regulated in SBM + BSFL<sub>Low</sub> fed fish but significantly down-regulated in SBM group. However, no dietary effect on prostaglandin D (*ProstD*) expression in the kidneys was observed among the groups ( $p > 0.05$ ) (Fig. 4). The intestinal gene expression of *IL-8* and *TNF- $\alpha$*  were unaffected by the diet (Fig. 5), however *ProstD* and *IRF-1* were significantly ( $p < 0.05$ ) downregulated in the SBM + BSFL<sub>low</sub> and SBM + BSFL<sub>high</sub> groups compared to those fed the FM and SBM diets (Fig. 5).

**Fig. 6 Experiment II:** In the kidney, trout fed BSFLO + BA had a significantly higher ( $p < 0.05$ ) expression of *IL-8* compared to the FO fed fish, however, no effect was observed on the *ProstD* among the dietary groups ( $p > 0.05$ ; Fig. 6). The kidney of trout fed BSFLO-based diet had an up-regulated expression of *TNF- $\alpha$*  and *IRF-1* compared to FO and SBO groups, although the *IRF-1* expression in BSFLO + BA was similar to the



**Fig. 6.** Relative gene expression (mean  $\pm$  SE) of interleukin-8 (*IL-8*), prostaglandin D (*ProstD*), tumor-necrosis factor (*TNF*) and interferon regulatory factor 1 (*IRF-1*) in the kidneys of rainbow trout fed the fish oil (FO)-based diet, soybean oil (SBO)-based diet, black soldier fly larvae oil (BSFLO)-based diet, or BSFLO-based diet + bile acid (BA) supplementation (BSFLO + BA). Different letters within each gene indicate significant differences ( $p < 0.05$ ).



**Fig. 7.** Relative gene expression (mean  $\pm$  SE) of interleukin-8 (*IL-8*), prostaglandin D (*ProstD*), tumor-necrosis factor (*TNF*) and interferon regulatory factor 1 (*IRF-1*) in the intestines of rainbow trout fed the fish oil (FO)-based diet, soybean oil (SBO)-based diet, black soldier fly larvae oil (BSFLO)-based diet, or BSFLO-based diet + bile acid (BA) supplementation (BSFLO + BA). Different letters within each gene indicate significant differences ( $p < 0.05$ ).

SBO fed fish (Fig. 6). The intestinal expression of *ProstD* and *IRF-1* were found higher in BSFLO + BA whereas SBO fed trout recorded a significantly lower expression ( $p < 0.05$ ; Fig. 7). No significant differences were observed in the *IL-8* and *TNF- $\alpha$*  among the dietary groups ( $p > 0.05$ ).

### 3.3. Effect of insect meal and oil on the immune response of rainbow trout

Experiment I: The supplemental BSFL at higher inclusion level (SBM

+ BSFL<sub>High</sub>) significantly increased the serum lysozyme activity ( $p < 0.05$ ; Fig. 8), but the activity of peroxidase among the various dietary groups were similar ( $p > 0.05$ ).

Experiment II: Trout fed BSFLO + BA recorded the highest serum lysozyme activity which is similar to the FO but significantly higher than the BSFLO and SBO fed groups ( $p < 0.05$ ; Fig. 9). The peroxidase activity in trout fed BSFLO-based diets registered higher value compared to the SBO and FO fed fish ( $p < 0.05$ ; Fig. 9).

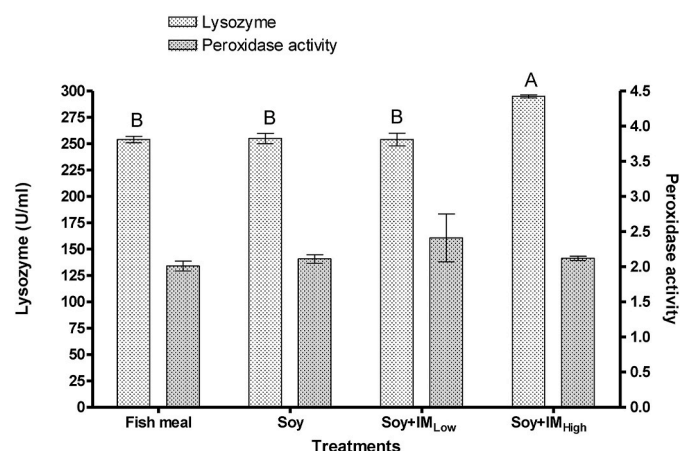


Fig. 8. Serum lysozyme and peroxidase activity (mean  $\pm$  SE) in rainbow trout fed the fishmeal-based diet, soybean meal (SBM)-based diet (b) SBM-based diets with 8% BSFL (SBM + BSFL<sub>low</sub>) or 16% BSFL (SBM + BSFL<sub>high</sub>). Different letters indicate significant differences ( $p < 0.05$ ) among the treatments.

## 4. Discussion

### 4.1. Experiment I

It is known that high inclusions SBM in the diets of rainbow trout often elicit inflammatory responses and damage to the intestinal barrier thereby reducing the absorptive functions of the enterocytes [2]. This similarly occurred in the present study whereby rainbow trout fed the SBM had highly inflamed distal intestines due to infiltration of leucocytes in the villi and lamina propria as well as structural disintegration of the architecture in some cases. These deleterious effects are attributed to the presence of various antinutritional factors (ANFs) found in leguminous plant, including SBM [19]. However, recent findings have shown that insect meals contain bioactive peptides that can prevent or reduce the induction of inflammatory processes in fish [11]. In this study, it was similarly observed that rainbow trout fed SBM-based diets, but with BSFL supplementation, effectively prevented the occurrence of

inflammation. This dramatic improvement may be attributed to bioactive peptides inherent in BSFL meal and further research should explore these compounds in order to potentially increase these levels in meals containing BSFL.

The effective mitigation of intestinal enteritis in rainbow trout fed BSFL was supported by gene expression results. Various genes responsible for inflammation were measured that included, interleukins, tumor necrosis factor and interferon regulatory factors [20]. In particular, interferon regulatory factor 1 (*IRF-1*) has been reported to play a key role in the genesis of proinflammatory cytokines, prostaglandins, and chemokines mediated by inflammation as well as immune cell activation [21,22]. Therefore, the observed up-regulation of *IRF-1* and prostaglandin D (*ProstD*) in the intestine of trout fed the SBM-based diet further reinforces the histological evidence of the damaging effects of high dietary SBM on the intestine. A similar result has been reported in northern snakehead, *Channa argus*, in which the expression of *IL-1 $\beta$*  (pro-inflammatory cytokine) was up-regulated after being fed diets with high SBM inclusions [20]. Remarkably, there was a decrease in the proinflammatory cytokines *IL-8*, *TNF- $\alpha$ 1* in the kidney and *IRF-1/ProstD* in the intestine in BSFL-fed groups (corresponding with the FM group), which indicates the potency of BSFL in attenuating the onset of inflammation in trout fed high SBM diets. Our result is congruent with an earlier study where dietary HFL supplementations significantly decreased the hepatic expression of proinflammatory interleukin (*IL-1 $\beta$* ) and *hepcidin* in *M. albus* [11,17].

The SBM diet also significantly upregulated the expression of *TNF- $\alpha$ 1* and *IL-8* in trout head kidney, although no differences were noticed in the intestine. Potentially, upregulation would be found if evaluated after feeding longer than ten weeks. The kidney is an important lymphohematopoiesis organ which plays a pivotal role in the clearing of circulating cytokines from the blood and the removal of these cytokines can limit inflammation [23,24]. Therefore, the observed up-regulated expression in the SBM fed group could be a result of increased lymphocyte production in the head kidney, and this has been reported to be associated with increased production of proinflammatory cytokines [22]. Additionally, this could also be in response to intestinal damage to the enterocytes and subsequent removal of cytokines released into the blood.

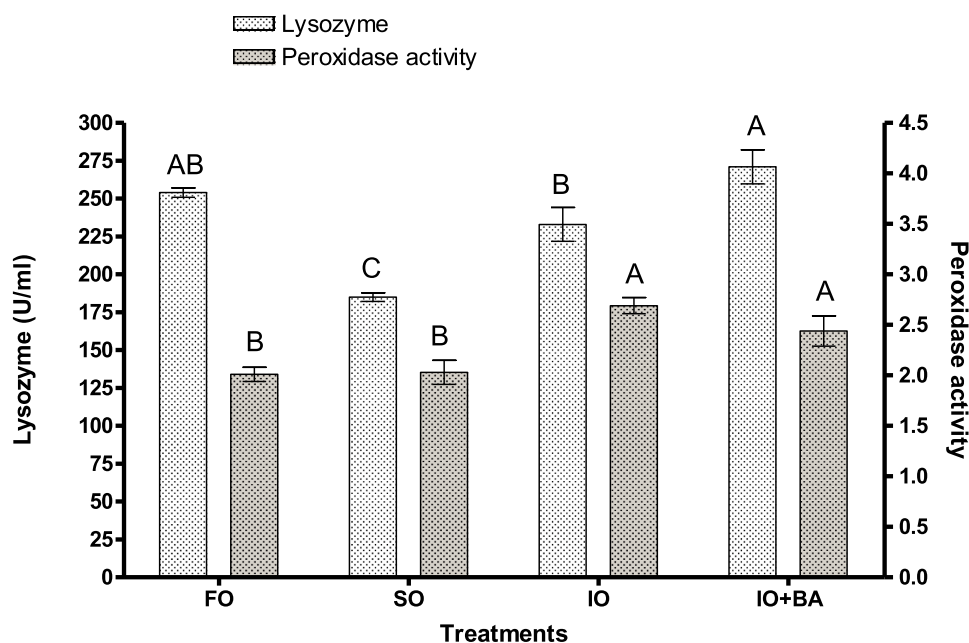


Fig. 9. Serum lysozyme and peroxidase activity (mean  $\pm$  SE) in rainbow trout fed the fish oil (FO)-based diet, soybean oil (SBO)-based diet, black soldier fly larvae oil (BSFLO)-based diet, or BSFLO-based diet + bile acid (BA) supplementation (BSFLO + BA). Different letters indicate significant differences ( $p < 0.05$ ) among the treatments.

Interestingly, there was a higher expression of *IRF-1* in the kidney of fish fed SBM + BSFL<sub>Low</sub>, which may be related to an immunostimulatory effect of BSFL based on significantly elevated serum lysozyme activity in trout fed the SBM + BSFL<sub>High</sub> diet. Similarly, dietary BSFL or maggot meal significantly enhanced the lysozyme activity in yellow catfish, *Pelteobagrus fulvidraco*, marron, *Cherax cainii*, and black carp, *Mylopharyngodon piceus*, respectively [25–27]. The immunostimulatory results of using relatively low levels of dietary BSFL could have implications to disease resistance. In fact, Chaklader et al. [13] found that the inclusion of 10% BSFL improved various genes in the head kidney responsible for immunity in barramundi, *Lates calcarifer*, as well as their resistance to *Vibrio harveyi* challenge.

It should be noted that dietary BSFL has also caused deleterious effects in growth, liver histopathology and/or immunity in fish, which is often ascribed to excessive chitin found in BSFL. For example, when replacing FM with BSFL at 75% and above, intestinal histopathological damage was noted as well as some necrotic hepatocytes in the livers of Jian carp, *Cyprinus carpio* [29]. Lower inclusion of 26.4% of BSFL shortened the villi in the distal intestine of rainbow trout, which was believed as the cause for a worsening of the feed conversion ratio [30]. On the other hand, BSFL at 25% in the diets of rainbow trout caused lipid vacuolization in the liver, but there was no damage to either the liver or intestine while there were positive and negative effects to the oxidative status [30]. Dumas et al. [29] suggested that BSFL should not be included above 13%, while Elia et al. [30] suggested the maximum inclusion could be as great as 20% in the diets of rainbow trout. In this study, it can be conjectured that 16% is not only appropriate, but beneficial to the immunity and gut histomorphology of rainbow trout.

#### 4.2. Experiment II

The crude lipid content of BSFL can be upwards of 45%, and thus BSFL is often defatted mechanically or with solvents [31]. The resulting BSFL oil (BSFLO) by-product is likely to be a more readily available resource as BSFL production is expected to increase over time. Few studies, however, have examined the feasibility of replacing FO or plant-based oils with BSFLO in the diets of fish [28,29]. Li et al. [28] replaced SBO with BSFLO from 0 to 100% in the diets of *C. carpio*, and found no difference in growth, but did find an increase in their intraperitoneal fat, likely due to a significant upregulation of peroxisome proliferator-activated receptor alpha expression. Dumas et al. [29] found no negative effect on the use of dietary BSFLO when included at 2.5, 5.0 or 10.0% on the growth, feeding efficiency or gut histomorphology in rainbow trout and believed even higher inclusion levels were possible.

One of the features of BSFLO is the high content of lauric acid (C12:0) [32]. When consumed by animals, lauric acid is quickly oxidized rather than being stored in the liver, leading to reduced feed intake [16], and controls obesity in mammals [33,34]. In this study, the BSFLO had no significant effect on the expression of genes responsible for inflammation in the intestine or any evidence of excessive lipid storage in the liver. Liver histopathology did reveal biliary duct hyperplasia, which may indicate a response to release more bile to facilitate lipid digestion. For example, dietary BSFLO increased the intraperitoneal fat content of *C. carpio* when replacing SBO [28]. Interestingly, the inclusion of BA at 1.5% in the BSFLO diet (BSFLO + BA), led to some instances of enlarged bile ducts, which may indicate the BA inclusion was excessive. Indeed, the use of dietary BA can cause abnormalities to the internal organs of some fish species [35]. Intestinal histopathological examinations, however, showed no adverse effects here, but there was a significant upregulation to prostaglandin in the intestine. Compared to the FO treatment, there was an upregulation of *IL-8*, *TNF-α1* and *IRF-1* in the kidneys, which could be due to an immunostimulatory effect of the BSFLO. This is based on the BSFLO diets significantly increasing lysozyme activity compared to rainbow trout fed the SBO diet. Moreover, compared to rainbow trout fed the FO or SBO diets, there was

significantly higher peroxidase activity. These findings indicate that BSFLO can replace FO or SBO in the diets of rainbow trout as well as providing some health benefits. Recently, it was shown that BSFLO improved the oxidative status and reduced the expression of pro-inflammatory genes in *C. carpio* and was superior compared to those fed diets with oils from silkworm pupae or yellow mealworm [36].

#### 5. Conclusions

The occurrence of SBM-induced intestinal enteritis in rainbow trout is a major obstacle to its use at higher inclusions; however, BSFL supplementations effectively prevented this pathology and these results are highly encouraging for its use in feeds containing high levels of SBM. The total replacement of FO or SBO with BSFLO led to no histopathological changes in the liver or intestine, decreased inflammation and improved immunity based on the gene expression and some innate immunity parameters. These findings will likely increase the demand for BSFL and BSFLO as sustainable products to become a standard ingredient in the diets of rainbow trout. Moreover, these results appear to indicate that it may not be necessary to defat BSFL, which would only be an additional cost when included in the diets of rainbow trout.

#### CRediT authorship contribution statement

**Vikas Kumar:** designed the research project, diet formulation, interpretation of results and wrote the manuscript. **Femi J. Fawole:** contributed to diet formulation and conducted the feeding trial contributed to the design and planning of the experiment, fish sampling, histological slides preparation, and gene expression analyses. **Nicholas Romano:** has done the histological analysis and, contributed significantly to draft the manuscript. **Md Sakhawat Hossain:** contributed to the design and planning of the experiment, fish sampling, histological slides preparation, and gene expression analyses. **Shyam N. Labh:** contributed to the design and planning of the experiment, fish sampling, histological slides preparation, and gene expression analyses. **Ken Overturf:** contributed to planning of the experiment and interpretation of results and reviewed the manuscript. **Brian C. Small:** contributed to planning of the experiment and interpretation of results and reviewed the manuscript. All authors read the draft, corrected and approved the final manuscript.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2020.12.008>.

## Data availability statement

The data that support the findings of this study are available upon the request.

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