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P u b l i s h e r s

Feeding black soldier fly larvae (*Hermetia illucens*) reared on organic rest streams alters gut characteristics of Atlantic salmon (*Salmo salar*)

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RESEARCH ARTICLE

Abstract

The Atlantic salmon (*Salmo salar*) aquaculture industry is growing, and with it, the need to source and optimise sustainable ingredients for aquafeeds. Black soldier fly (BSF) larvae (*Hermetia illucens*) have received increasing research attention since they are a good source of protein that can efficiently convert a wide range of low-value organic material into valuable resources. This study investigated the impact of three differently processed BSF meals, an untreated BSF diet (BSFC+), a dechitinated BSF diet (BSFC-) and a fermented BSF diet (BSFC+P+) at a 10% inclusion level replacing fish meal in a fish meal control (FM) and a marine and soy protein concentrate based control diet (SPC). Growth performance, gut microbiome and gut histology of salmon fry was assessed. The inclusion and processing methods of BSF showed no adverse impacts on either growth performance or gut histology. However, the gut microbiome of fish was significantly altered by both the protein source and the processing method of the BSF larvae. Fish fed BSFC+, had an increased diversity and evenness of the community compared with conventional protein sources alone, and compared with the other BSF processing methods. However, control diets had a greater presence of lactic acid bacteria and genera associated with faster growing hosts. Fish fed BSF had a high relative abundance of the genus, *Exiguobacterium*, a chitin-degrading bacterium and in BSFC+P+ fed fish this bacterium completely dominated the community, indicating the presence of dysbiosis. Future studies should determine, why *Exiguobacterium* has dominated the community for the BSFC+P+ diet, and if it provides a digestive function to the host and identify bacteria that are indicators of optimal host performance and resilience. The results confirmed that BSF is a promising fish meal replacement for salmon, and it demonstrated that BSFC+ has a potential prebiotic impact on the gut microbiome of Atlantic salmon.

Keywords: microbiome, salmonids, insects, processing, chitin

1. Introduction

Atlantic salmon (*Salmo salar*) aquaculture has grown rapidly in recent decades in Europe and with it, the demand for high quality feed ingredients (Naylor *et al.*, 2009). Salmon are carnivorous and require high levels of dietary protein, particularly during early developmental phases (NRC, 2011). Traditionally, salmonid feeds relied heavily on fish meal, but as demand has grown and exerted increasing pressure on already exploited wild capture fisheries, sustainable and

scalable alternatives were needed by the industry (Tacon and Metian, 2009; Tacon *et al.*, 2011; Ytrestøyl *et al.*, 2015). In recent decades soy protein concentrate has replaced a large proportion of the fish meal in salmonid diets, yet challenges and bottlenecks remain for the use of plant ingredients (Aas *et al.*, 2019). Hence, there is a need for alternative sources of protein for Atlantic salmon feeds that can reduce the pressure on marine protein sources and which simultaneously complements plant protein meals in

commercial formulations (Randazzo *et al.*, 2021; Turchini *et al.*, 2019).

Insect meals are a promising alternative in aquafeeds and as a fish meal or plant protein replacement (Barroso *et al.*, 2014; Henry *et al.*, 2015; Weththasinghe *et al.*, 2021b). They have a suitable nutritional profile and are part of the natural diet of many fish species (Lock *et al.*, 2018). They can be produced in high density with low water use and methane emissions, and production is not location dependent (Rumpold and Schlüter, 2013; Van Huis and Dunkel, 2017). Additionally, insect meals can be reared on a wide range of organic substrates, efficiently converting low-value biomasses such as vegetable rest raw materials from the food industry, to valuable proteins for use in feed applications (Ojha *et al.*, 2020). This provides benefits for the development of a circular economy which reduces the impact of feed and food production and as such is prioritised in European legislation (EC Directive No. 2008/98) (Barragan-Fonseca *et al.*, 2017; Zarantoniello, *et al.*, 2020a). Insect meals derived from black soldier fly (BSF) larvae (*Hermetia illucens*) have received increasing attention in the salmon aquafeed literature (Askarian *et al.*, 2012; Belghit *et al.*, 2018; Fisher *et al.*, 2020; Weththasinghe *et al.*, 2021a,b). The reduced production cost has led to increased interest and availability of BSF for feed producers (English *et al.*, 2021; Henry *et al.*, 2015; Tomberlin and Van Huis, 2020). Existing research indicates that BSF is a promising alternative to fish meal without adverse effect on the physio-chemical qualities of the fillet (Bruni *et al.*, 2020). Yet, the processing of BSF for use in aquafeeds is not fully optimised, and there is thus a call to optimise the application of this novel protein source to maximise growth performance, as well as health and welfare of fish (Henry *et al.*, 2015).

Both the type of protein (Gajardo *et al.*, 2017), and processing methods can alter the gut microbiome of Atlantic salmon (Catalán *et al.*, 2018). During early developmental stages of salmonids the gut microbiome is particularly malleable and strongly influenced by the diet (Michl *et al.*, 2017, 2019) and during the first-feeding period, intestinal microbiomes are established (Dehler *et al.*, 2017). The gut microbial community of fish has important consequences for health resilience, disease resistance (López Nadal *et al.*, 2020; Yukgehnaish *et al.*, 2020), growth performance (Perry *et al.*, 2020), metabolism (Dvergedal *et al.*, 2020), digestion and nutrient uptake (Ghanbari *et al.*, 2015; Llewellyn *et al.*, 2014). The existing research indicates that the presence of certain bacteria such as lactic acid bacteria (LAB) can have functional benefits for digestive function and immune development of the fish, which are highly desirable for commercial aquaculture (Ringø *et al.*, 2018). Few studies exist on the impact of insect meals on the gut microbiome of salmonids, but existing research demonstrates feeding BSF larvae to juvenile Rainbow trout (*Oncorhynchus mykiss*)

increased both alpha diversity and presence of LABs in the distal gut (Huyben *et al.*, 2019), increased Firmicutes in the digesta (Bruni *et al.*, 2018) and may improve alpha diversity measures when combined at 10% with 50% poultry meal compared to vegetable meal diets (Gaudioso *et al.*, 2021). In Atlantic salmon, BSF altered the gut digesta microbial community and increased richness and diversity during the seawater phase (Li *et al.*, 2021). The impact of BSF on the gut microbiome in the early feeding stages of Atlantic salmon during the freshwater stage is lacking from the existing literature. Evidence from zebrafish (*Danio rerio*) fed BSF indicates that high inclusion levels (75-100%) modified the gut microbiota, reducing the number of species detected and the chao1 diversity (Zarantoniello *et al.,* 2020b) and that different rearing substrates can influence the resulting fish gut microbiome (Osimani *et al.*, 2019). Dietary changes that alter the gut microbiome, can have consequences for fish behaviour, for example shoaling or swimming behaviours, due to the bi-directional signalling between the gut and the brain, termed the gut-brain-axis (Borrelli *et al.*, 2016). While this field is in it´s infancy, emerging evidence from zebrafish (*D. rerio*), indicates that BSF in fish diets does not alter locomotive behaviour compared to conventional proteins (Zarantoniello *et al.*, 2020b).

Novel processing techniques provide a promising strategy for optimised BSF meal inclusion in aquafeed which is currently limited to only partial inclusion, thus, further research is needed to improve its nutritive value (Barragan-Fonseca *et al.*, 2017; Kroeckel *et al.*, 2012). Fermented feed ingredients have improved application in aquaculture feeds with positive effect on nutritional quality and promoting health and resilience in farmed fish (Catalán *et al.*, 2018; Dawood and Koshio, 2020; Ringø *et al.*, 2020). Fermentation of insects with LABs could lengthen their shelf-life, thus increasing their microbial safety (Klunder *et al.*, 2012). Fermenting feed ingredients can enhance their nutritional value for aquaculture by improving digestibility (Refstie *et al.*, 2005) and driving potentially beneficial changes to the gut microbiome (Catalán *et al.*, 2018). Probiotic LABs have been added to BSF to enhance diets for freshwater crayfish (*Chera cainii*) (Foysal *et al.*, 2021) but the impact of bacterial fermentation of BSF for Atlantic salmon fry diets has yet to be investigated. A limitation to the use of insects such as BSF in aquafeeds is the chitin content in their exoskeleton, which is a highly abundant mucopolysaccharide polymer of N-acetyl-d-glucosamine, (1-4)-linked 2-acetamido-2-deoxy-β-d-glucan (Henry *et al.*, 2015; Li *et al.*, 2019; Park and Kim, 2010; Zarantoniello *et al.*, 2020a). Chitin has been considered a potentially problematic component of BSF and has been suggested to cause reduced feed utilisation in Atlantic salmon (Olsen *et al.*, 2006) and that dechitination could be beneficial (Weththasinghe *et al.*, 2021a). Contrary to this, other research indicates that chitin may have beneficial prebiotic effects for fish (Ringø *et al.*, 2006), including increased alpha diversity in Rainbow trout

gut microbiomes (Huyben *et al.*, 2019), and altered the gut microbiome in Atlantic salmon (Askarian *et al.*, 2012). There is a need to determine the impact of chitin on the gut bacteria of farmed fish (Zhou *et al.*, 2013) and to use molecular methods to elucidate the impact to community composition and complement existing research for Atlantic salmon (Askarian *et al.*, 2012).

Replacing conventional proteins with BSF in salmon diets may reduce the presence of gut inflammation (Weththasinghe *et al.*, 2021b) which can have important consequences for health, welfare and nutrient uptake (Refstie *et al.*, 2000). This impact could be particularly important at very early developmental stages when the gut morphology is developing during and following first feeding (Sahlmann *et al.*, 2015). Existing studies indicate that BSF does not negatively alter Atlantic salmon gut health during the seawater phase even when all fish meal in the diet is substituted (Li *et al.*, 2020) or in the freshwater phase when 85% of protein was from BSF (Li *et al.*, 2019). In Rainbow trout, it has been indicated that the presence of BSF may even decrease inflammation of the gut associated with soybean meal intestinal enteritis (Kumar *et al.*, 2021). It will be important to understand how different processing methods effect juvenile Atlantic salmon gut histology to determine the optimal method and most suitable for combination with conventional aquafeed proteins.

Ensuring there is no adverse impact to growth performance, gut microbiome, and gut health, will help to determine that replacing conventional proteins with BSF, and methods selected to process BSF, do not have adverse impacts to fish welfare. Unsuitable dietary alteration can adversely impact fish welfare in aquaculture, compromising health or inducing suffering. Thus, these are key consideration for any candidate proteins for aquafeed inclusion (Ashley, 2007; Bonaldo *et al.*, 2015; Oliva-Teles, 2012).

The objective of this study was to optimise the use of BSF sourced from a circular bioeconomy model for salmonid aquafeeds. The impact of three differently processed BSF meals, an untreated BSF diet (BSFC+), a dechitinated BSF diet (BSFC-) and a fermented BSF diet (BSFC+P+) on growth performance, gut microbiome and gut histology of Atlantic salmon fry was assessed. This study answers a call in the literature to further elucidate the impact of insect meals for farmed Atlantic salmon (Lock *et al.*, 2018).

2. Materials and methods

Experimental animals and study design

Atlantic salmon (*S. salar*) hatched by Stofnfiskur Ltd. (Vogar, Iceland) and reared at 5.5 °C, was brought to first feeding using standard commercial techniques and commercial start-feed diet BioMar Inicio-plus (Grangemouth, UK) of

0.5 mm pellet size and at a water temperature of 10 °C. Fry were transferred to Matís Aquaculture Research Station (MARS) on March 1st 2018, where they were acclimated for one week to the study facilities. All fish within the experiment were individually weighed following a 12-hour fasting period under anaesthetic (2-phenoxyethanol of 300 mg/kg). Fish were split into twenty 20L-White circular PVC tanks, in quadruplicate for each feed treatment. Each tank contained 30 individual fish with similar initial weight (1.34±0.2 g). Fish were kept at 9.0±0.5 °C under 24-hour photoperiod of 40±10 lux, oxygen levels were maintained above 80% saturation. Fish were fed with the experimental feed treatments for 75 days. The experiment was performed following European and Icelandic guidelines and within the permits and licenses of the MARS facility. The licence number for these experiments was FE-1134 (Rekstrarleyfi) from MAST and UST201707 (Starfsleyfi) from the Icelandic Environment Agency. The authors complied with the ARRIVE guidelines to ensure ethical standards were met.

Production and processing of insects

Insect biomass was sourced from existing black soldier fly producers Better Origin (Cambridge, United Kingdom). Larvae were reared at 28 °C at 70% relative humidity in 80×90 cm trays. Larvae were fed on organic substrate consisting of 80% shredded potatoes and 20% spent brewer's yeast, sourced from Milton Brewery (Cambridgeshire, UK). Larvae were harvested at the 5th and 6th instar stages and were euthanised by exposure to cold. Larvae were washed in cold water to remove residual detritus and water-soluble dirt. The biomass was blanched to partially pasteurise the material. Three different treatments of the biomass were applied to produce three types of insect meal. An untreated black soldier fly meal (BSFC+) was sterilised by autoclaving (121 °C for 15 minutes). A chitin-negative black soldier fly meal (BSFC-) was processed with a proprietary dechitination apparatus which separated the solid chitin by-product fraction and a liquid protein and fat-rich product fraction. The liquid fraction was then sterilised (121 °C for 15 minutes). A processed black soldier fly meal (BSFC+P+) was sterilised and then subjected to bioprocessing (fermentation with *Pediococcus acidlactici* at 37 °C). All insect meals were then dried (80 °C for 24 hours) and milled to reduce the particle size for feed inclusion (IPHARMACHINE, Ruian City, Zhejiang, China P.R.). The nutritional profile of each insect meal is provided in (Table 1).

Experimental feeds and feeding

There were five dietary treatments formulated for this investigation. A fish meal-based control (FM) diet and a commercially comparable control with marine and soy protein concentrate based control (SPC) and three insect meal diets, BSFC+, BSFC-, BSFC+P+ Insect meal

Table 1. Chemical composition of the protein sources used in feed treatments for this study.

Composition (g/kg)	Protein raw materials[1]		Corn gluten meal	BSFC+	BSFC-	BSFC+P+
	Fish meal	Soy protein concentrate				
Dry matter	909	925	910	979	916	946
Crude protein	659	633	582	438	373	437
Crude lipid	107	2	10	292	308	262
Ash	139	90	23	124	101	114
Essential amino acids (g/kg)						
Arginine	42.3	42.9	18.6	24.4	21.2	21.5
Histidine	18.2	15.5	12.2	14.7	11.6	13.8
Isoleucine	30.2	27.5	23.2	19.8	16.8	19.3
Leucine	57.1	47.4	96.1	32.7	26.6	32.1
Lysine	58.8	37.0	9.1	31.0	28.6	31.0
Methionine	19.6	9.0	14.3	8.2	8.3	8.2
Phenylalanine	28.9	32.1	37.2	21.3	19.3	21.2
Threonine	33.3	25.1	20.3	19.8	16.9	19.8
Valine	37.8	28.5	28.0	28.3	20.0	28.0
Tryptophan	7.6	8.6	3.1	7.3	6.4	7.5
Non-essential amino acids (g/kg)						
Alanine	47.2	26.3	52.7	29.1	20.7	29.7
Aspartic acid	69.2	71.3	36.2	47.3	42.3	46.0
Glycine	50.3	25.6	17.4	27.0	19.0	27.1
Glutamic acid	105.0	114.0	130.0	58.4	50.7	53.9
Cysteine	5.8	8.6	11.4	3.5	3.5	3.5
Tyrosine	24.6	22.3	30.6	34.3	26.7	32.6
Proline	32.1	30.9	55.7	24.1	17.2	23.8
Serine	32.6	32.4	33.5	20.0	15.3	22.2

[1] BSFC- = dechitinated black soldier fly (BSF) diet; BSFC+ = untreated BSF diet; BSFC+P+ = fermented BSF diet.

was included at 10% in the feed formulation, primarily replacing fish meal and fish oil (Table 2). The diets were produced through cold pelletisation and drying followed by a crumbling process (<0.5 mm diameter crumbs) at Matís ohf., Iceland. All dry ingredients were milled to bring all materials to equal particle size (IPHARMACHINE). Dry ingredients were then homogenised in a standard food mixer (KitchenAid, Benton Harbour, MI, USA) and the mix was returned to the same mill to improve the homogeneity of the feed. The dry mix was returned to the food mixer and fish oil was added while simultaneously mixing. A small volume of water was added to produce the ideal consistency for the next stage (500 ml) The mix was then spread thinly onto a drying tray lined and oven dried at 30 °C (Convotherm, Eglfing, Germany) until moisture content was <10%. The dried material was then crumbled to 0.5 mm diameter crumbles. During the 75-day feeding trial, tanks were fed 15 times per day by electric belt-feeder between the hours of 09:00 and 01:00. All tanks were fed identical volumes, with 15% excess based on feed requirements at this developmental stage.

Growth performance

After 75 days of feeding, all individual fish from each treatment and replicate tanks were weighed (wet weight (g)) and measured (total length (cm)), following a 12-hour fast. From this data the Fulton´s condition factor (K) and specific growth rate (%) (SGR) over the study period could be calculated: K = (Weight / Total Length^3) × 100 and SGR = ((Ln(Final Weight) – Ln(Initial Weight)) × 100) / t, where t is the number of days over which the trial was run. Mortality was monitored daily throughout the feeding trial.

Gut sampling

After the assessment of growth performance, all fish were left for one week to recover from handling and fed the same experimental diets. At the end of this recovery

Table 2. Feed formulation and chemical composition for feed treatments of study.[1]

	FM	SPC	BSFC+	BSFC-	BSFC+P+
Formulation (g/kg)					
Fish meal[2]	630.0	467.6	405.9	417.0	406.8
Pre-gelatinised wheat[3]	180.8	133.1	117.0	108.6	113.1
Vitamin-mineral premix[4]	10.0	10.0	10.0	10.0	10.0
Fish oil[2]	101.2	119.7	97.4	94.7	100.5
Soy protein concentrate[2]	0.0	191.6	191.6	191.6	191.6
Corn gluten meal[3]	78.0	78.0	78.0	78.0	78.0
BSFC+	0.0	0.0	100.0	0.0	0.0
BSFC-	0.0	0.0	0.0	100.0	0.0
BSFC+P+	0.0	0.0	0.0	0.0	100.0
Composition (g/kg)					
Dry matter	867	884	909	922	894
Crude protein	467	484	492	503	495
Crude lipid	219	202	199	202	217
Ash	75	88	96	97	96

[1] BSFC- = dechitinated black soldier fly (BSF) diet; BSFC+ = untreated BSF diet; BSFC+P+ = fermented BSF diet; FM = fish meal; SPC = soy protein concentrate.
[2] Laxá hf., Akureyri, Iceland.
[3] Emmelev A/S, Otterup, Denmark.
[4] Laxa salmon premix 2006 1%, Trouw Nutrition, the Netherlands.

period, samples were taken for gut microbiome analysis and gut histology analysis. For both sample types, fish were fed just two hours prior to sampling to ensure high volumes of gut content. For the gut microbiome analysis, twelve fish per feed treatment three fish per tank were randomly selected. Selected fish were euthanised with a lethal dose of anaesthetic (phenoxyethanol) 600 mg/kg and the outside of the fish washed in 90% ethanol followed by sterile distilled water. The digesta from the distal part of the gastro-intestinal tract was extracted under sterile conditions. Triplicate samples of each dietary treatment were collected. All samples were stored at -80 °C prior to downstream processing. A further twelve fish per feed treatment (three fish per tank) were randomly selected for gut histology sampling. Selected fish were euthanised with a lethal dose of anaesthetic (phenoxyethanol) 600 mg/kg. Fish were dissected to remove the distal gut, and gut content was washed out of the gut section with phosphate buffered solution. Samples were stored in 10% buffered formalin for 48 h and then transferred to 70% ethanol and stored at 4 °C prior to downstream processing.

DNA extraction, PCR amplification and sequencing

Distal gut digesta samples and dietary treatment samples for gut microbiome analysis were transferred to a sterile 2 ml Eppendorf tube with 300 μl of sterile 1 mm diameter sterile silica beads (BioSpec Products, Bartlesville, OK, USA). 800 μl of CD1 from the QIAamp PowerFecal Pro DNA kit (QIAGEN, Hilden, Germany) was added to the Eppendorf tube. Samples were vortexed for 5 seconds and shaken at maximum speed (30 Hz) in a laboratory mixer mill (Retsch MM400, Haan, Germany) for 1 minute. The supernatant (~800 μl) was transferred to the PowerBead Pro Tube from the QIAGEN QIAamp PowerFecal Pro DNA kit. The protocol for this DNA extraction kit was then followed according to manufacturer instructions, and finally eluted with 80 μl of solution C6. A negative control with no material was also run to ensure no contamination occurred during the DNA extraction protocol. DNA concentrations were measured in 2 μl of sample using the Invitrogen Qubit dsDNA BR Assay kit (Invitrogen, Carlsbad, CA, USA). DNA were diluted to 4 ng/μl in a 50 μl aliquot. Samples were then subjected to PCR of a region covering the V3-V4 regions of the 16S rRNA gene with a universal bacterial primer pair S-D-Bact-0341-b-S-17 (5'–CCTACGGGNGGCWGCAG-3')/S-D-Bact-0785-a-A-21(5'-GACT-ACHVGGGTATCTAATCC-3') (Klindworth *et al.*, 2013). The PCR master mix included the diluted DNA, nuclease-free water, Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA), Q5 GC Enhancer, 0.5 μM of each primer containing Illumina overhang adapters, and 1× Q5 Reaction buffer, 200 μM dNTPs (New England Biolabs). Included in the PCR were both positive and negative samples to monitor for successful amplification and absence of contamination of the target region only. The thermocycling protocol had an initial denaturation step (98 °C for 30 s), followed by 35 cycles of, denaturation (98 °C for 10 s), annealing (52 °C for 30 s), and extension step (72 °C for 30 s), with a final extension (72 °C

for 2 min). Libraries were multiplexed with Nextera XT v2 barcodes (Illumina, San Diego, CA, USA), normalised using *Sequel-Prep* Normalisation Plates (ThermoFisher Scientific, Waltham, MA, USA) and sequenced on a MiSeq desktop sequencer (Illumina) using v3 chemistry and 2×300 cycles.

Histological sample processing and analysis

Distal gut tissues were equilibrated in xylene and embedded in paraffin wax following standard histological procedures. Sections of 4 μm thickness were cut and stained with haematoxylin and eosin. The sections were cut longitudinally (perpendicular to the folds). Processing was carried out at the Veterinary department of The Norwegian University of Life Sciences (NMBU). Blind histological examination was performed on images taken using a light microscope with camera connected (Olympus BX51, Tokyo, Japan) with a magnification of (×100). Tissue morphology was evaluated using a semi-quantitative scoring system with scores from 1-3 for lamina propria thickness and submucosal connective tissue width (Barnes *et al.*, 2014; Colburn *et al.*, 2012; Knudsen *et al.*, 2007). The scoring criteria used is described in Table 3.

Statistical methods

Statistical analyses were performed in R version 3.6.1 (2019-07-05). All tests were two-tailed with a significance level set to α=0.05. To assess growth performance, two dependent variables were statistically assessed, the average tank SGR% and K. A linear model with the package *nmle* (Pinheiro, 2020) was selected where the feed treatment was a fixed factor. To support the statistical robustness of this test and to account for potential variation at the tank level further

Table 3. Histological scoring system used on Atlantic salmon fed experiment feed treatments (modified from Barnes *et al.*, 2014; Colburn *et al.*, 2012; Knudsen *et al.*, 2007).

Score	Appearance
	Lamina propria of simple folds
1	Thin and delicate core of connective tissue in all simple folds.
2	Lamina propria slightly more distinct and robust in some of the folds.
3	Clear increase in lamina propria in most of the simple folds
	Connective tissue between base of folds and stratum compactum
1	Very thin layer of connective tissue between base of folds and stratum compactum.
2	Slightly increased amount of connective tissue beneath some of the mucosal folds.
3	Clear increase of connective tissue beneath most of the mucosal folds.

statistical analysis was run. Firstly, a generalised linear mixed model (GLMM)f with the package *lme4* (Bates *et al.*, 2015) on weight of all fish at the start of the trial, where feed treatment was a fixed factor and tank was a random nested factor of feed treatment to confirm there was no difference across fish body weight between tanks at the start of the trial. Secondly, another GLMM on the weight of all fish at the end of the trial where feed treatment was a fixed factor and tank was a random nested factor of feed treatment.

To assess the microbiome of digesta of the distal gut and the dietary treatments, demultiplexed FASTQ files from Illumina were processed to produce amplicon sequence variants (ASVs) using the DADA2 package version 1.16.0 (Callahan *et al.*, 2016) in Rstudio version 4.0.2 (Team, 2020). The function filterAndTrim set variables as, truncLen=c(280,250), trimLeft= 21, maxN=0, maxEE=c(2,2), truncQ=2. The SILVA database version 138 was used to assign Taxonomy to the ASVs (Quast *et al.*, 2013). The microbial community was analysed using R packages phyloseq (McMurdie and Holmes, 2013), microbiome (Lahti and Shetty, 2017) and vegan (Oksanen *et al.*, 2020), and visualised with ggplot2 (Wickham, 2016). The average number of reads per sample output from the DADA2 pipeline were 16,213±7,515 for all samples except for a single digesta sample from the SPC diet tank 3 that did not produce reads and was removed from downstream processing. Three PCR negative samples were also sequenced to control for any contamination during the sample processing steps, and were used as controls to remove suspected contamination from the samples using the package *decontam* (Davis *et al.*, 2017). The prevalence method and a threshold of 0.5. For all subsequent analysis, the read depth was normalised across samples with the function *rarefy_even_depth* to the sample with the lowest read depth. Raw 16S rRNA gene amplicon reads are deposited in the Sequence Read Archive under BioProject ID PRJNA733893 available at http://www.ncbi.nlm.nih.gov/bioproject/733893. The gut microbial community of fish fed the study diets, and the microbial community of the diets were quantitatively analysed using alpha and beta diversity measures. The selected alpha diversity measures where the observed richness of ASVs, Shannon diversity, Chao1 diversity and Pielou´s Evenness. For the gut samples, a GLMM was used to assess if there was a significant difference in these alpha diversity measures between the fish gut digesta fed different dietary treatments. In this model feed treatment was a fixed factor and tank was a nested random factor of feed treatment. The random nested factor of tank was tested by a likelihood ratio test (Fox *et al.*, 2011). For the diet microbiome, a linear model was selected to assess if the alpha diversity measures between the diet samples were significantly different, in this model the feed treatment was a fixed factor. Post-hoc testing was carried out using a Tukey test. The gut microbiome community assemblage for fish fed each of the dietary treatment and

the microbiome community of the diets respectively were transformed using a *Bray-Curtis* dissimilarity matrix and non-metric multidimensional scaling was applied. An Analysis of similarity (ANOSIM) test was conducted to assess for significant difference between and within fish fed different feed treatments. To further investigate the microbiome community assemblage the relative abundance of taxa as a proportion was visualised at the phylum level in stacked bar plots for direct comparison. The genus level was then visualised, with all genera present at less than 1% of the relative abundance grouped into a category called 'Other' and the genera present at greater than 1% relative abundance were visually displayed using boxplots for each feed treatment. To assess the gut histology, a non-parametric Kruskal-Wallis test was run on the two gut histology variables, lamina propria of simple folds, and connective tissue at base of folds. In this model, feed treatment was a fixed factor.

3. Results

Growth performance

There was no significant difference in SGR% among the dietary treatments with similar average values (Figure1A). There was also no significant difference in K between feed treatments with similar average values (Figure 1B). There was no significant difference in fish weight between dietary

treatments or between tanks at either the start or the trial or the end of the trial.

Gut microbiome

There was a significant difference in all alpha diversity measures among dietary treatments. The observed richness of ASVs (Figure 2A) was highest in the BSFC+ diet, followed by BFC- and SPC, which all had higher values than the FM control diet, and BSFC+P+ had the lowest observed richness of all the dietary treatments tested. The Shannon diversity index (Figure 2B) was higher is BSFC+ than all other treatments expect for SPC, BSFC+P+ had the lowest values compared to all other dietary treatments and FM had the second lowest values. The Chao1 diversity (Figure 2C) followed an identical pattern to the Shannon diversity index, and BSFC- showed a larger range of diversity values. Pielou´s evenness (Figure 2D) was significantly higher in BFC+ and SPC, followed by FM and BSFC-. The lowest community evenness was found in BSFC+P+. The alpha diversity indices in the diet samples (Supplementary Figure S1) differ slightly from the digesta. For the observed richness of ASVs (Supplementary Figure S1A), and the Chao1 diversity (Supplementary Figure S1C), BSFC- diet values are closer to the BSFC+ values than for the digesta. Whereas, for the Shannon diversity (Supplementary Figure S1B), and Pielou´s evenness (Supplementary Figure S1D), BSFC- had the lowest values, and BSFC+P+ values for
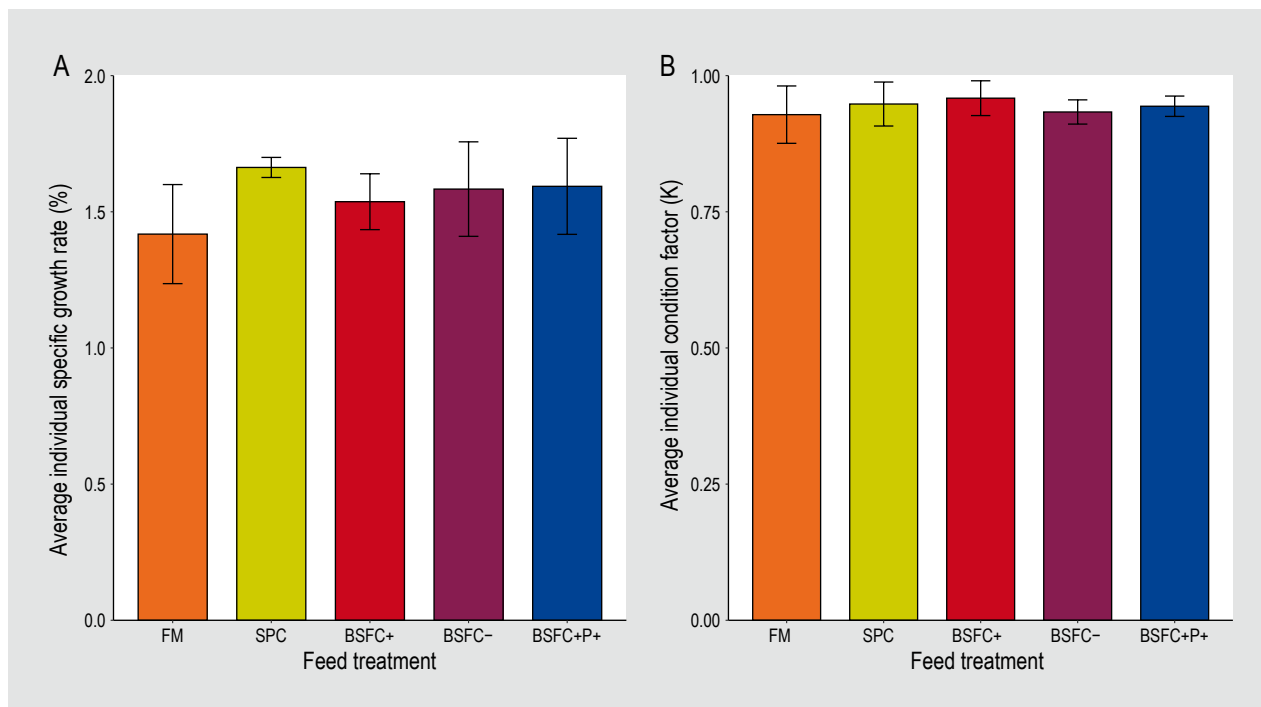

Figure 1. Bar plot of (A) the average individual specific growth rate (SGR) (%) and (B) the average individual condition factor (K) of fish fed the experimental feed treatments. Bars represent the standard deviation of the data. BSFC- = dechitinated black soldier fly (BSF) diet; BSFC+ = untreated BSF diet; BSFC+P+ = fermented BSF diet; FM = fish meal; SPC = soy protein concentrate.

Pielou´s evenness was closer to BSFC+ and the FM, and SPC diet samples.

The gut microbial composition of the fish fed different diets was significantly different. There was larger difference in the microbial communities of fish fed different diets, than in fish within the same dietary treatment group (ANOSIM *P*=0.001, R0.8949) the clustering of the NMDS (stress=0.14) (Figure 3) is quite distinct. This suggests the dietary treatments in this study created fish with distinct gut microbial communities in the digesta of the distal gut. The FM and SPC control diet clusters were close together, and that the BSFC+ and BSFC- diet clusters were close together, but BSFC+P+ was clustered away from all other dietary treatments. At the phylum taxonomic level (Figure 4), the gut communities were dominated by Firmicutes in all dietary treatments (FM=0.55±0.31, SPC=0.87±0.15, BSFC+=0.76±0.15, BSFC-=0.79±0.09, BSFC+P+=0.97±0.03). The second most dominant phyla were Actinobacteriota (FM=0.16±0.26, SPC=0.05±0.06, BSFC+=0.12±0.04, BSFC-=0.15±0.07, BSFC+P+=0.03±0.02) and Proteobacteria (FM=0.27±0.32, SPC=0.07±0.08, BSFC+=0.12±0.12, BSFC-=0.06±0.07, BSFC+P+=0.00±0.00). For BSFC+P+ fed fish, Proteobacteria was absent. There was also some

tank effect present, which was most noticeable for fish in tank 4 fed the FM control which had much higher abundance of Proteobacteria than any other fish within that treatment. The phyla present in the diet microbiota samples (Supplementary Figure S2) follow similar trends to the phyla in the digesta, with a few exceptions, across all diet samples. There was a greater relative abundance of Actinobacteriota, and the diet microbiota sample for BSFC+P+ has a greater proportion of Proteobacteria than in the fish digesta. For the BSFC- diet samples, there was a greater presence of Bacteroidota compared to the fish digesta samples.

At the genus taxonomic level (Figure 5) there were seven genera that with relative abundance above 1% of the community assemblage. All other genera present showed a low abundance. For the genus *Bacillus* there were high levels in both fish fed the BSFC+ and BSFC- diets, low levels in those fed the SPC diets, but none present in either FM or BSFC+P+ diets. For *Clostridium_sensu_stricto_1*, *Enterococcus*, *Lactobacillus* and *Peptostreptococcus*, the same pattern was observed, with moderate levels for the FM control, the highest levels for SPC control, and decreasing levels for the BSFC+ and BSFC- diets and none present
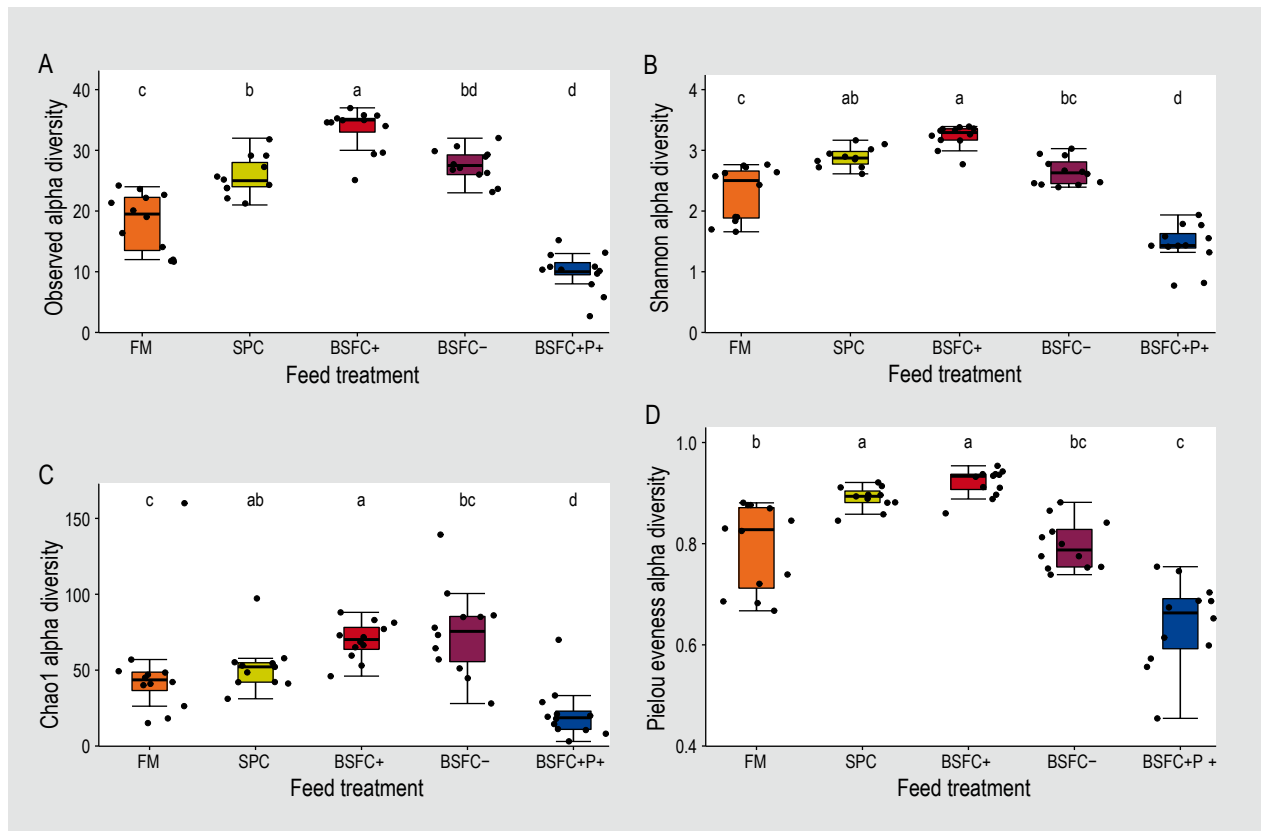
Figure 2. Box plots of alpha diversity measures for the gut digest of fish fed each dietary treatment: (A) the observed richness of ASVs, (B) Shannon diversity, (C) Chao1 diversity, (D) Pielou´s evenness. The *P*-value for each variable is displayed on its respective graph. BSFC- = dechitinated black soldier fly (BSF) diet; BSFC+ = untreated BSF diet; BSFC+P+ = fermented BSF diet; FM = fish meal; SPC = soy protein concentrate.

for the BSFC+P+ diet. A similar pattern was observed for *Clostridium_sensu_stricto_7,* except none were present in the FM control. The only genera present above 1% in BSFC+P+ was *Exiguobacterium,* which was detected in high relative abundance for the BSFC- diet, with low levels for the BSFC+ diet and none present in either FM or SPC control diets. The genera detected in the diet microbiota samples (Supplementary Figure S3) have several bacteria present at greater than 1% relative abundance that were not detected at greater than 1% in the digesta. The diet samples for BSFC+P+ were more diverse than for the resulting digesta samples. There were no LABs greater than 1% relative abundance in the feeds. The relative abundance of the genera, *Clostridium_sensu_stricto_1* and *Clostridium_sensu_stricto_7* was higher in the FM feeds than in the resulting FM digesta.

Gut histology

There was no significant difference in lamina propria of simple folds between the dietary treatments with similar average values observed (FM: 1.18±0.40, SPC: 1.25±0.45, BSFC+: 1.18±0.40, BSFC-: 1.33±0.49, BSFC+P+:1.17±0.39). There was also no significant difference in connective tissue at base of folds between the dietary treatments with similar average values observed (FM: 1.00±0.00, SPC: 1.00±0.00, BSFC+: 1.00±0.00, BSFC-: 1.08±0.29, BSFC+P+:1.08±0.29). An example image from the gut histology samples in provided for reference (Figure 6).

4. Discussion

The present study reports the impact of BSF reared on organic rest streams from the food industry, on the growth performance, gut microbiome, and gut histology of Atlantic salmon fry. Three different processing treatments of BSF (an untreated BSF diet (BSFC+), a dechitinated BSF diet (BSFC-) and a fermented BSF diet (BSFC+P+)) were compared to a fish meal control diet (FM) and a mixed fish meal and soy protein concentrate control diet (SPC) to optimise the application of BSF in formulated aquafeeds.

Growth performance of Atlantic salmon fry in this study was comparable across all dietary treatments for both the controls and the 10% inclusion of BSF, and all values were within normal range for the freshwater (FW) phase and experiment temperature (Nathanailides *et al.*, 1995; Ørnsrud *et al.*, 2002). The type of processing applied to the BSF did not have any measurable impact on either SGR (%) or K. Existing studies reported similar findings for low to moderate protein substitution levels of BSF, where adding up to 12.5% BSF meal did not adversely affect growth performance for FW phase Atlantic salmon (Weththasinghe *et al.*, 2021a). The same study reported that higher substitution levels of 25% reduced growth performance. Whereas, another study on Atlantic salmon fry showed no adverse impacts on growth performance with inclusion up to 20%, but reduced performance at 30% inclusion (Fisher *et al.*, 2020). A further study, showed that 85% of protein replacement with BSF had no adverse impact on growth performance (Belghit *et al.*, 2018). In post-smolt
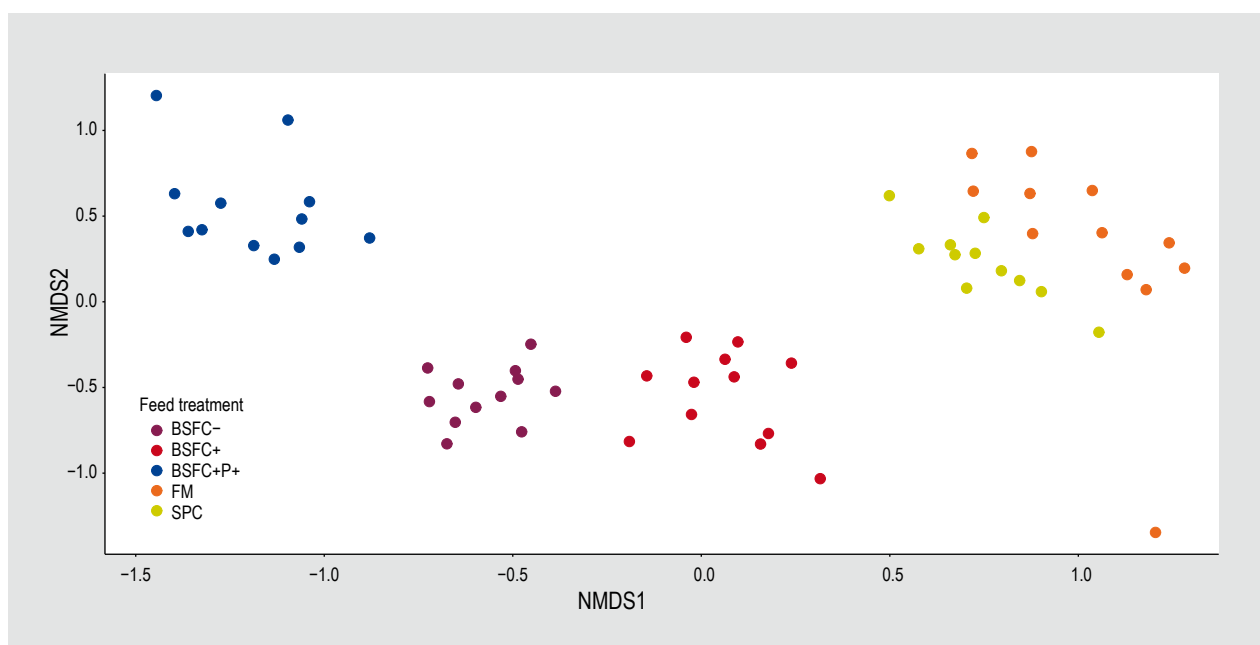

Figure 3. Non-metric multidimension scaling (NMDS) of study fish from each dietary treatment. Each point represents a single fish and colour indicates the feed treatment inclusion level. BSFC- = dechitinated black soldier fly (BSF) diet; BSFC+ = untreated BSF diet; BSFC+P+ = fermented BSF diet; FM = fish meal; SPC = soy protein concentrate.
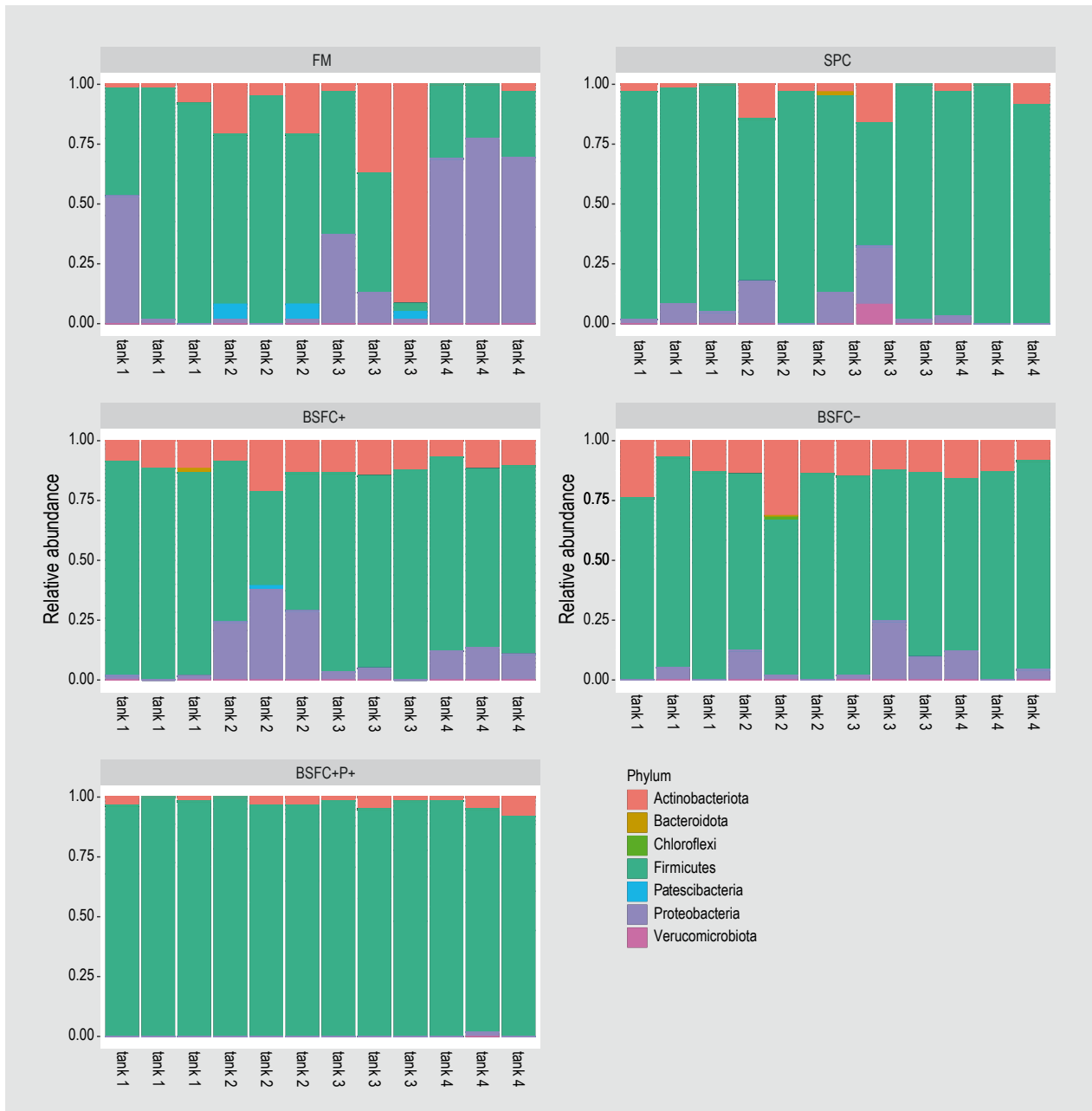
Figure 4. Stacked bar plot of gut bacterial composition using relative abundance of the most common phyla (above 1% relative abundance) for the study fish from each dietary treatment. BSFC- = dechitinated black soldier fly (BSF) diet; BSFC+ = untreated BSF diet; BSFC+P+ = fermented BSF diet; FM = fish meal; SPC = soy protein concentrate.

Atlantic salmon, 100% replacement of fish meal has been reported without compromising growth performance (Belghit *et al.*, 2019; Lock *et al.*, 2016). The results of the present study further confirm the existing findings, that lower inclusion levels of BSF provide comparable SGR and K to conventional protein sources for Atlantic salmon during the FW phase. At this low inclusion level of 10%, the removal of chitin from BSF did not produce marked benefit to growth performance, suggesting that chitin is not a limiting factor when the inclusion of insect protein is low, as some studies have suggest (Kroeckel *et al.*, 2012;

Olsen *et al.*, 2006; Weththasinghe *et al.*, 2021a; Xiao *et al.*, 2018) although further clarification of impact of chitin at higher inclusion levels is required. This study suggests that fermentation of insect proteins did not provide any measurable benefits to growth performance in juvenile salmonid diets. There is lack of existing research that assesses the impact of fermenting insect protein sources for aquafeeds. Existing studies have observed improved growth performance in several carp species (*Catla catla*, *Cirrhinus mrigala, Labeo rohita, Hypophthalmychtyhys molitrix*) (Rangacharyulu *et al.*, 2003) and broiler chickens
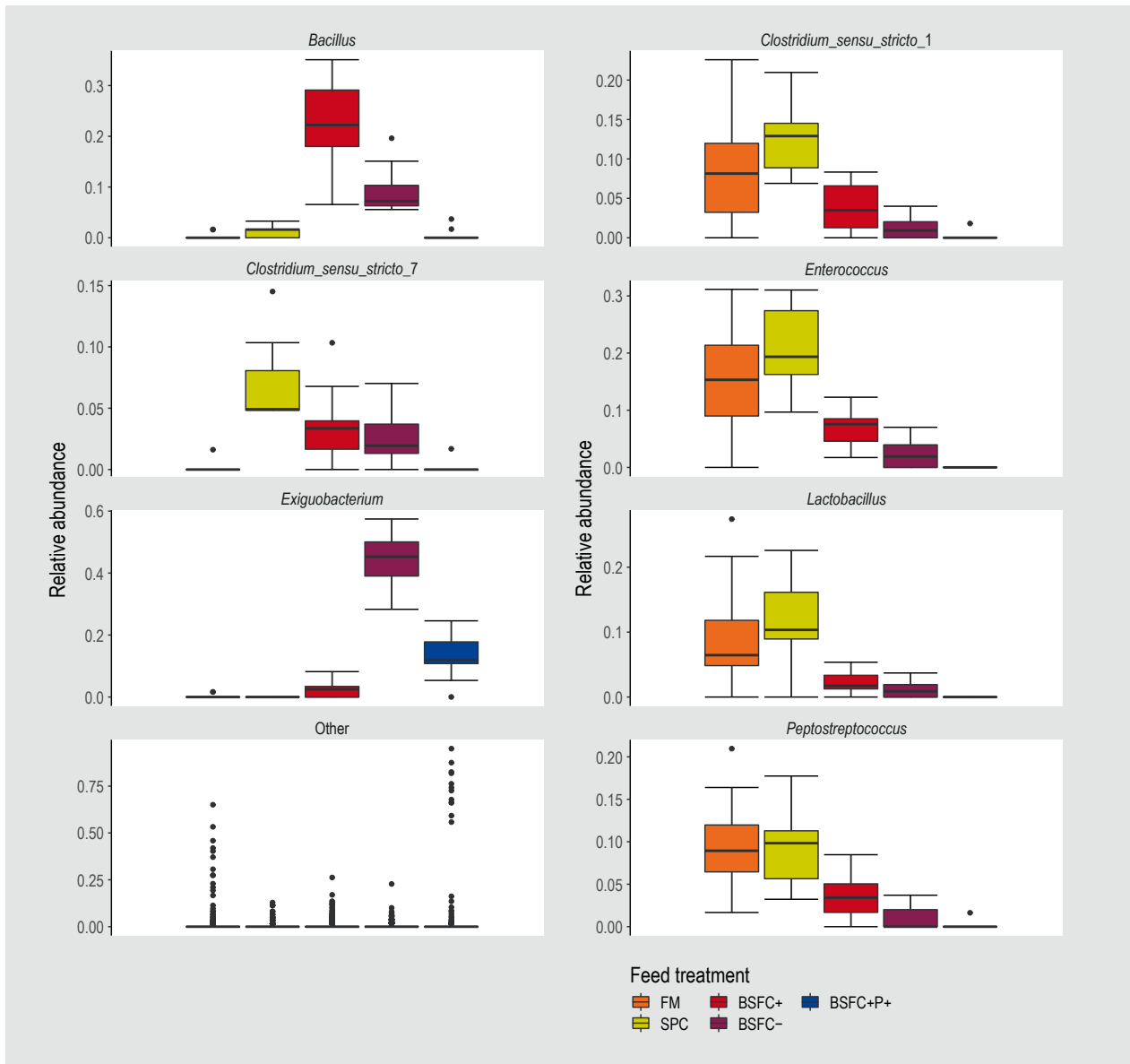
Figure 5. Boxplots of gut bacterial composition using relative abundance of the most common genera found in fish from each dietary treatment. BSFC- = dechitinated black soldier fly (BSF) diet; BSFC+ = untreated BSF diet; BSFC+P+ = fermented BSF diet; FM = fish meal; SPC = soy protein concentrate.

(Rao *et al.*, 2011) fed fermented silkworm pupae silage. Weight gains have been observed for broiler chickens fed up to 25% of fermented sago larvae replacing fish meal (Sjofjan and Adli, 2021). Future studies should elucidate the impact of processing to improve protein quality of BSF in salmonid diets. It would also be valuable to assess if dechitination has an impact on growth performance when BSF is fed at higher inclusion levels, since the upper limits of BSF in the diet of Atlantic salmon are unclear (English *et al.*, 2021), and dechitination may influence maximum inclusion levels. It will be important to optimise all aspects of BSF production to account for discrepancies between existing findings which may be driven by several factors, such as differences in rearing conditions (English *et al.*,
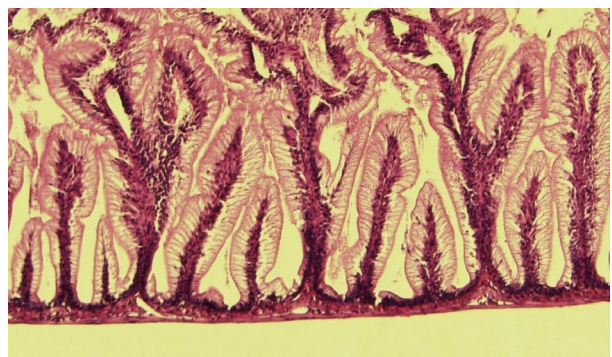
Figure 6. Gut histology image of FW Atlantic salmon assessed in this study under blind histological scoring, where no difference was found between feed treatments.

2021), variable substrates used during BSF production (Zarantoniello *et al.*, 2020a), and different processing of the BSF prior to dietary inclusion (Barragan-Fonseca *et al.*, 2017). Future studies should determine the optimal method to maximise inclusion of BSF as a substitute for conventional aquafeed ingredients (English *et al.*, 2021).

The alpha diversity measures in distal gut digesta of fish fed diets in the present study show that the untreated BSF (BSFC+) had higher diversity, richness, and evenness measures than all the processed BSF dietary treatments and the FM control. BSFC+ also had comparable values as the SPC control diet except for the ASV richness, which was higher in BSFC+. These results indicate that chitin in the BSF may have a prebiotic impact on the gut microbiome of these juvenile fish. This would be beneficial for aquaculture since higher microbial diversity levels and an even community could both be signs of a beneficial gut health and can support productivity (Infante-Villamil *et al.*, 2021). To the knowledge of these authors, the present study is the first to report the impact of BSF on the gut microbiome of juvenile Atlantic salmon during the FW phase. Existing studies in post-smolt Atlantic salmon in the seawater (SW) phase detected similar patterns for alpha diversity as found herein, reporting increased microbial diversity and richness in digesta of fish fed diets with a 15% inclusion of BSF replacing conventional protein sources in a mixed protein source diet (Li *et al.*, 2021). Similarly, in studies with Rainbow trout (*Oncorhynchus mykiss*) fed a diet with 30% BSF larvae and pre-pupae inclusion, replacing fish meal (Huyben *et al.*, 2019) and a 25 and 50% replacement of fish meal with BSF (Bruni *et al.*, 2018) respectively, alpha diversity increased which was attributed to the presence of chitin (Huyben *et al.*, 2019). Chitin supplementation has also been observed to alter gut microbiota of Atlantic salmon (Askarian *et al.*, 2012) and have a beneficial prebiotic potential for aquafeed inclusion (Bruni *et al.*, 2018; Ringø *et al.*, 2012). Removal of chitin in the BSFC- diet in the present study reduced the alpha diversity measures of gut microbiome compared with the untreated BSF (BSFC+), diet but the measures were still comparable to those fish fed the SPC diet, except for the community evenness which was lower for BSFC-, but comparable with the FM control fish. This suggests that dechitination does not provide potential benefits for the gut microbiome, and may even have adverse consequences associated with reduced microbial diversity on gut health and resilience (Apper *et al.*, 2016). Fish fed the fermented BSF diet (BSFC+P+) in the present study had the lowest diversity and the most uneven community in their distal gut digesta, indicating possible dysbiosis and limited bacterial competition to protect against pathogens entering the gut, conditions associated with poor health and resilience in fish (Apper *et al.*, 2016; Infante-Villamil *et al.*, 2021). This finding is contrary to indications from the existing literature, that fermenting insects increases the presence of

bioactive compounds (Castro-López *et al.*, 2020) and their nutritional value (Kewuyemi *et al.*, 2020) that might be expected to have benefits for the gut microbiome. Further investigation of the impact of insect meal fermentation on the fish gut microbiome will be necessary to determine the causes of the low microbial community diversity and unevenness shown by the present study.

In the present study, the beta diversity of the distal gut digesta microbiota reveals a distinct microbial community composition across dietary treatments. The greatest differences were observed between the community composition of the FM and SPC control diets compared with the BSFC+ and BSFC- diets, with the most distinctly different community composition found in the fermented BSFC+P+ fish. There is strong consensus in the existing literature that the dietary protein source alters the community composition of salmonids (Gajardo *et al.*, 2017; Michl *et al.*, 2017). This has been reported when conventional proteins are replaced with BSF (Li *et al.*, 2021). The dominant phyla in the distal gufirmict digesta of fish across all diet treatments in the present study is comparable to other freshwater stage salmonids, which are commonly dominated by Firmicutes, Proteobacteria and Actinobacteria (Gajardo *et al.*, 2016; Huyben *et al.*, 2019). At the phyla level the strong dominance of Firmicutes in the digesta of fish fed the fermented BSF diet (BSFC+P+) explains the unevenness present in this community. At the genus level, the driver of community unevenness and the dominance of Firmicutes in fish fed the fermented diet, BSFC+P+ can be identified as the genera *Exiguobacterium*. *Exiguobacterium* is a bacterium that produces chitinase that degrades chitin (Anuradha and Revathi, 2013). In the present study, fish fed any diet containing BSF have *Exiguobacterium* present in this distal gut digesta in an abundance higher than 1%. The same bacteria has been detected in gut microbiome samples from both farmed Atlantic salmon fed commercial feed (Webster *et al.*, 2020), in wild flounder (*Paralichthys adspersus)* (Salas-Leiva *et al.*, 2017) but was not found in the gut microbiome of BSF fed rainbow trout (*O. mykiss*) using high-throughput sequencing (Huyben *et al.*, 2019). Chitinase activity has immune benefits for fish and presence in the gut microbiome community can be desirable (Zhang *et al.*, 2012). *Exiguobacterium acetylicum S01* isolated from soil was reported to inhibit the growth of fish pathogens and have a desirable probiotic role (Jinendiran *et al.*, 2019a), and as a dietary supplement, improved growth performance and immune responses in goldfish (*Carassius auratus*) (Jinendiran *et al.*, 2019b). Future work should isolate *Exiguobacterium* present in fish species fed BSF and assess its probiotic potential and should confirm that the bacterium is active in the fish gut environment. In the case of the fermented BSF diet in the present study, this bacterium dominates the community, and is driving the observed dysbiosis which may reduce the resilience of these fish and is undesirable (Apper *et al.*,

2016; Lozupone *et al.*, 2012). This suggests that fermented BSF may not be suitable for Atlantic salmon at such as early developmental stage when the gut microbiome is still becoming established (Rodríguez *et al.*, 2015) and this phenomenon requires further investigation. The strong presence of *Exiguobacterium* in fish fed the dechitinated diet (BSFC-) in the present study could be driven by the dechitination process which may have removed bulk chitin but left glycosidic bonds intact that this bacterium was able to efficiently exploit. It will be important for future studies to consider the indirect impacts of BSF processing methods. The present study also observed increased relative abundance of the genus *Bacillus* in both the untreated BSF diet (BSFC+) and the dechitinated BSF diet (BSFC-) compared with both controls. This was also observed in Rainbow Trout (*O. mykiss*) fed BSF diets (Huyben *et al.*, 2019) and in Atlantic salmon fed diets with 5% chitin supplementation, where the chitin may have supported the growth of *Bacillus* species by providing additional substrate for growth (Askarian *et al.*, 2012). Two genera of bacteria, *Clostridium_senus_stricto_1* and *Peptostreptococcus* were found in higher relative abundance in the two control diets of the present study and were slightly lower in the untreated BSFC+ diet and even lower in the dechitinated BSFC- diet. These bacteria have been associated with faster growing individuals in Rainbow trout (*O. mykiss*) (Chapagain *et al.*, 2019). No growth benefit was observed in the present study, and it will be important to extend this trial to monitor growth up to harvest size and to determine if the same bacteria are also growth rate indicators in Atlantic salmon. Two lactic acid bacteria (LAB) were detected above 1% relative abundance in fish in the present study, *Enterococcus* and *Lactobacillus*, these were both also in highest abundance in the two control diets, and decreased from BSFC+ to BSFC- diets, to absent in BSFC+P+ fed fish. This is contrary to existing studies that reported an increase in LABs within presence of BSF (Bruni *et al.*, 2018; Huyben *et al.*, 2019). It will be important to determine if this reduced LAB presence has any impact to health and resilience of Atlantic salmon and to establish a cause-effect relationship to elucidate their roles for the host (Gajardo *et al.*, 2017). The microbiota detected in the diet samples appears to influence the resulting microbial community in the fish gut digesta, which has been established in existing studies for salmonids (Gajardo *et al.*, 2017; Li *et al.*, 2021). However, there were notable differences between the microbial communities in both sample types, and the alpha diversity trends and genera present. This is particularly notable for the BSFC+P+ diet microbial community which is not dominated by *Exiguobacterium* in the same manner as the digesta samples for fish fed this diet.

The gut histology of Atlantic salmon fry in the present study did not differ among the dietary treatments and the low scores for histological characteristics suggest there was no adverse impact on gut health and no inflammation was detected for any of the diets fed. The controls of this study were FM and SPC protein sources which are minimally impactful to gut health in salmonids at the inclusion levels applied (Booman *et al.*, 2018; Krogdahl *et al.*, 2020). This finding confirms existing reports that BSF at low inclusion levels does not negatively impact gut health in salmonids compared with control diets (Elia *et al.*, 2018; Li *et al.*, 2019; Weththasinghe *et al.*, 2021b). One study reported no adverse effect to gut health when BSF replaced 100% of fish meal in Atlantic salmon diets (Li *et al.*, 2020). Furthermore, in a study where soybean meal was fed to Rainbow trout, intestinal enteritis was present and a supplementation of 16% BSF was found to prevent enteritis (Kumar *et al.*, 2021). Future studies should combine BSF with and without chitin with feed ingredients that are linked to poor gut health to investigate possible benefits for Atlantic salmon.

5. Conclusions

Our results support the existing literature that a 10% inclusion of BSF as a replacement for fish meal in formulated feeds, does not adversely impact growth performance or gut histology of Atlantic salmon during the freshwater phase. Furthermore, the BSF processing methods used in this study did not alter the growth performance or gut histology. The gut microbiome of Atlantic salmon in this trial was significantly altered by both the protein source and the processing method of the BSF larvae used. This study revealed that the untreated BSF fed fish, had a potentially prebiotic impact on the gut microbiome community alpha diversity and evenness compared with conventional protein sources alone. Compared with the other processing methods, dechitinated BSF or fermented BSF. Both control diets had a greater presence of both LABs and potential bacterial indicators of faster growing salmonid host than any of the diets containing BSF. All fish fed a diet containing BSF had a high relative abundance of the genus, *Exiguobacterium*, a chitin-degrading bacterium. The BSFC- diet fed fish had the highest relative abundance of this bacterium, but in digesta of the fish fed the fermented BSF diet (BSFC+P+) this bacterium completely dominated the community, and indicated the presence of dysbiosis, an undesirable state for the gut microbiome. It will be important in future studies to determine, why *Exiguobacterium* has dominated the community for the BSFC+P+ diet, if this bacterium is active and if it provides a digestive function to the host. It will also be very valuable for growing Atlantic salmon aquaculture industry to identify gut bacteria associated with characteristics of optimal production.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/JIFF2021.0105

Figure S1. Box plots of alpha diversity measures for the dietary microbial.

Figure S2. Stacked bar plot of bacterial composition using relative abundance of the most common phyla for the study dietary treatments.

Figure S3. Boxplots of bacterial composition using relative abundance of the most common genera found in each dietary treatment.

Acknowledgements

Conflict of interest

The authors declare a competing interest, M. Pipan, one of the co-authors is employed by Better Origins, a company that developed and markets black soldier fly larvae. All other authors declare that they have not competing interests.

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