Norwegian University of Life Sciences
Faculty of Biosciences
Department of Animal and Aquacultural Sciences

Philosophiae Doctor (PhD)
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Nutritional, health, and technical properties of black soldier fly (*Hermetia illucens*) in Atlantic salmon (*Salmo salar*) feeds

Næringsverdi, helse effekter og fysiske egenskaper ved bruk av svarte soldatfluer (*Hermetia illucens*) i fôr til Atlantisk laks (*Salmo salar*)

Pabodha Weththasinghe

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With love,
Pabodha

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1 Abbreviations and definitions

AA	Amino acids
ALT	Alanine aminotransferase
AMP	Antimicrobial peptides
AST	Aspartate aminotransferase
ASVs	Amplicon sequence variants
BSF	Black soldier fly
CK	Creatine kinase
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
FA	Fatty acids
FCR	Feed conversion ratio
FRAP	Ferric reducing antioxidant power
GSMMs	Genome-scale metabolic models
LAB	Lactic acid bacteria
LDA	Linear discriminant analysis
LER	Lipid efficiency ratio
N	Nitrogen
P	Phosphorous
PD	Phylogenetic diversity
PER	Protein efficiency ratio
SFA	Saturated fatty acids
SGR	Specific growth rate

2 List of papers

Paper I

Weththasinghe, P., Hansen, J.Ø., Nøkland, D., Lagos, L., Rawski, M., Øverland, M., 2021. Full-fat black soldier fly larvae (*Hermetia illucens*) meal and paste in extruded diets for Atlantic salmon (*Salmo salar*): Effect on physical pellet quality, nutrient digestibility, nutrient utilization and growth performances. *Aquaculture*. 530, 735785. https://doi.org/10.1016/j.aquaculture.2020.735785.

Paper II

Weththasinghe, P., Lagos, L., Cortés, M., Hansen, J.Ø., Øverland, M., 2021. Dietary inclusion of black soldier fly (*Hermetia Illucens*) larvae meal and paste improved gut health but had minor effects on skin mucus proteome and immune response in Atlantic Salmon (*Salmo Salar*). *Frontiers in Immunology*. 12, 599530. https://doi.org/10.3389/fimmu.2021.599530.

Paper III

Weththasinghe, P., Hansen, J.Ø., Rawski, M., Józefiak, D., Ghimire, S., Øverland, M. Insects in Atlantic salmon (*Salmo salar*) diets – Comparison between full-fat, de-fatted, and de-chitinized meals, and oil and exoskeleton fractions. Manuscript submitted to *Journal of Insects as Food and Feed*.

Paper IV

Weththasinghe, P., Rocha, S.D.C., Øyås, O., Lagos, L., Hansen, J.Ø., Mydland, L.T., Øverland M. Modulation of Atlantic salmon (*Salmo salar*) gut microbiota composition and predicted metabolic capacity by feeding diets with processed black soldier fly (*Hermetia illucens*) larvae meals and fractions. Manuscript submitted to *Animal Microbiome*.

Paper V

Weththasinghe, P., Hansen, J.Ø., Mydland, L.T., Øverland, M. A systematic meta-analysis based review on black soldier fly (*Hermetia illucens*) as a novel protein source for salmonids. Manuscript submitted to *Reviews in Aquaculture*.

3 Summary

Black soldier fly (BSF) (*Hermetia illucens*) has been identified as a promising novel ingredient in fish feeds. The knowledge on optimal downstream processing of BSF is, however, lacking in the literature. The main objective of this thesis was to contribute to increased knowledge on this topic. This thesis presents five papers.

Paper I investigated the dose-dependent effects of two differently processed full-fat BSF larvae products in diets for Atlantic salmon (*Salmo salar*) on technical pellet quality, nutrient utilization, and growth performance. Atlantic salmon pre-smolts were fed full-fat BSF meal or full-fat BSF paste, added to extruded diets, replacing increasing levels of protein from fishmeal and plant protein sources (6.25, 12.5 and 25% by meal and 3.7 and 6.7% by paste). The expansion and water stability of feed pellets numerically decreased with increasing inclusion of BSF meal and paste. Replacement of up to 12.5% and 6.7% of protein with full-fat BSF meal and paste, respectively, did not compromise fish growth performance, but growth rate decreased at 25% meal replacement. Protein digestibility decreased linearly with increasing dietary level of BSF meal or paste, but the protein retention was not affected by dietary BSF inclusion.

Subsequently, **Paper II** investigated the dose-dependent effects of dietary full-fat BSF larvae meal and paste on gut health and systemic immune responses in Atlantic salmon. Replacement of up to 12.5% protein with meal and 6.7% with paste improved gut health by improving distal intestine histology and reducing enterocyte steatosis in pyloric caeca. Replacing 25% protein with full-fat meal did not cause inflammatory changes in distal intestine histology but increased the level of pro-inflammatory cytokine IFNγ in the distal intestine and gave mild to moderate enterocyte steatosis in pyloric caeca. The BSF meal and paste in diets caused minor effects on protein expression in skin mucus and systemic immune responses in fish.

Paper III investigated the effect of inclusion of BSF larvae meals and fractions in Atlantic salmon diets on technical pellet quality, nutrient utilization, and growth performance. Atlantic salmon pre-smolts were fed extruded diets containing meals (full-fat, de-fatted or de-chitinized meals) or fractions (oil or exoskeleton) of BSF.

Meals replaced 15% protein from fishmeal and plant sources in a control diet. Lipid-rich full-fat and de-chitinized meals in the diets numerically reduced expansion and water stability of pellets. Full-fat and de-chitinized meals improved fish growth rate, while de-fatted meal, oil and exoskeleton diets supported similar growth performance as the control diet. Full-fat BSF meal also gave a higher growth rate and feed intake than other meals and fractions. Nevertheless, de-fatted meal and exoskeleton gave a better feed conversion than full-fat meal. Full-fat meal and oil did not compromise protein digestibility, but de-fatted and de-chitinized meals and exoskeleton reduced protein digestibility. Protein retention was not affected by BSF meals and fractions in the diets, except for the de-fatted meal, which increased the retention of digested protein.

Paper IV investigated how dietary BSF larvae meals and fractions affect the gut microbiota composition and their predicted metabolic capacity in Atlantic salmon. The diets that contained BSF chitin, i.e., BSF meals and exoskeleton diets, increased the abundance of chitinolytic *Lactobacillales* and *Actinomyces* in fish gut. The diets that contained BSF lipids, i.e., BSF meals and oil diets, increased the abundance of *Bacillaceae*. Fish fed full-fat meal had a phylogenetically diverse and unique gut microbial composition, dominated by beneficial lactic acid bacteria and *Actinomyces*, and showed a predicted increase in microbial mucin degradation.

Overall, differently processed BSF could partially replace protein and lipid in Atlantic salmon diets without compromising the growth performance, and fish response to dietary BSF varied with the protein replacement level and processing method. At 15% protein replacement, full-fat meal improved growth performance in salmon, and de-fatting and de-chitinization did not further improve fish growth.

Additionally, in **Paper V**, we conducted a meta-analysis to determine the effect of dietary BSF on nutrient utilization and growth performance in salmonids including Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*). This analysis showed that dietary BSF did not compromise the protein utilization and growth performance in salmonids. Nevertheless, replacement of fishmeal by BSF decreased growth rate and feed intake in salmonids, but replacement of non-fishmeal sources improved growth rate and feed conversion. This stresses the importance of the protein source(s) used in the control diet when evaluating nutritional value of BSF.

4 Norsk sammendrag

Svart soldatflue (SSF) (*Hermetia illucens*) er en lovende og bærekraftig ingrediens i fiskefôr. Men det er allikevel behov for å se nærmere på ulike prosesseringer av SSF. Hovedmålet med denne doktorgraden var å øke kunnskapen innenfor dette fagfeltet. Denne doktorgraden presenterer resultater fra fem artikler:

I **Artikkel I,** undersøkes effekten av å tilsette økende mengde av to ulike prosesserte SSF larveprodukter i fôr til Atlantisk laks (*Salmo salar*) på teknisk fôrkvalitet, utnyttelse av næringsstoff og tilvekst. Laks i ferskvann ble fôret med fullfett SSF mel eller pasta som ble tilsatt i økende mengde i fôr for å erstatte protein fra fiskemel og planteproteinkilder (6.25, 12.5 og 25% med mel og 3.7 og 6.7% med pasta). Ekspansjonen og vannstabiliteten av fôrpelleten ble numerisk redusert med økende innblanding av SSF-mel og pasta. Erstatning av 12.5% og 6.7% av proteinet med henholdsvis mel eller pasta, ga ingen negative effekter på vekst hos laksen, men veksten ble redusert med 25% erstatning med BSF mel. Proteinfordøyeligheten ble gradvis redusert ved økt innblanding av både SSF-mel og pasta i fôret, men proteinretensjonen ble ikke påvirket.

I **Artikkel II**, undersøkes effekten av økende mengde fullfett SSF larver-mel og pasta i fôret på tarmhelse og systemisk immunrespons i laks. Ved å erstatte opp til 12.5% av proteinet med mel og 6.7% med pasta, ble tarmhelsen bedret gjennom en forbedret histologi i baktarm og redusert forekomst av fettakkumulering i enterocyttene i blindsekkene (steatose). Erstatning av 25% av proteinet med fullfett SSF-mel førte ikke til forandringer i baktarm, men en mild til moderat steatose i enterocyttene i blindtarmen og økt nivå av den pro-inflammatoriske cytokinen IFNγ i baktarmen ble observert. SSF-mel og pasta i fôr til laks ga liten effekt på proteinproduksjon i slimlaget fra fiskeskinnet eller på det systemiske immunforsvaret.

Artikkel III undersøkte effekten av ulike mel og fraksjoner fra SSF larver i fôr til laks med fokus på teknisk fôrkvalitet, utnyttelse av næringsstoffer og vekst. Laks i ferskvann ble fôret med ekstruderte fôr som inneholdt mel (fullfett, avfettet, eller avkitinisert) eller fraksjoner (olje eller skall) fra SSF. Melene erstattet 15% av

proteinet fra fiskemel og planteproteiner i en kontrolldiett. Innblanding av de fettrike SSF melene, fullfett- og avkitinisert mel, ga en reduksjon i pelletekspansjon og vannstabilitet. Høyfettmelene økte også veksten hos laks, mens fôrene med avfettet mel, olje og skallfraksjonen fra SSF gav samme vekst som kontrollfiskene. Fullfettmelet gav også høyere vekst og fôropptak enn de andre SSF-fôrene. Likevel gav avfettet mel og skallfraksjonen en bedre fôrutnyttelse enn fullfettmelet. Fôrene med fullfettmel og SSF-olje gav ingen reduksjon i fordøyelse av protein, mens avfettet- og avkitinisert mel og SSF skallfraksjon reduserte proteinfordøyeligheten. Proteinretensjonen ble ikke påvirket med tilskudd av SSF i fôrene, bortsett fra en økt proteinretensjon med avfettet SSF-mel.

Artikkel IV undersøkte hvordan sammensetningen av tarmmikrobene og deres predikerte metabolske kapasitet i laks ble påvirket av å tilsette ulike mel og fraksjoner fra SSF larver i fôret. De kitinholdige SSF-melene og fôret med skallfraksjonen økte andelen av kitinnedbrytende melkesyrebakterier og *Actinomyces*. Diettene som inneholdt mye insektsfett økte andelen av *Bacillaceae* I laksetarmen. Fisken som ble fôret med fullfettmel hadde en større fylogenetisk diversitet og en unik sammensetning av tarmmikrobiota, hvor de gunstige melkesyrebakteriene og *Actinomyces* dominerte og viste en predikert økning i nedbrytning av mucin.

Disse resultatene viste at ulike prosesserte SSF-mel kan delvis erstatte protein og fett i fôret til laks uten å redusere tilveksten. De ulike responsene hos laksen varierer med mengde protein som blir erstattet med SSF og type prosessering av SSF. Ved å erstatte 15% av proteinet med fullfett SSF viste laksen økt vekst, men en videre prosessering som å fjerne fett- eller skall-fraksjonen fra SSF, førte ikke til bedre vekst hos fisken.

I Artikkel V ble det utført en metaanalyse for å se på den overordnede effekten av å inkludere SSF i fôret til laks og regnbueørret (*Oncorhynchus mykiss*) med fokus på fôrutnyttese og vekst parametre. Resultatet fra denne metaanalysen viste at fôring med SFF mel ga ingen reduksjon i vekst, fôrutnyttelse eller proteinutnyttelse hos laksefisk. Erstatning av fiskemel med SFF resulterte i redusert vekst, mens en erstatning av plante-baserte produkter gav en økning i både vekst og fôrutnyttelse. Dette demonstrer hvor viktig proteinkildene som brukes i kontrollfôret er, og hvordan de eventuelt er evaluert før en fôrformulering.

5 Synopsis

5.1 Introduction

Aquaculture production is increasingly contributing to the global food supply [1], and is projected to reach 109 million tonnes in 2030 [2]. It holds a significant potential to address malnutrition and diet-related disorders [1]. Feed is considered the most critical input in aquaculture production [3], and the aquaculture industry is challenged by a limited supply of sustainable protein and lipid sources for aquafeeds [4]. Fishmeal and fish oil are ideal feed resources for the aquaculture [5], however, the use of fishmeal and fish oil in aquafeeds is now limited due to the depletion of wild forage fish, high market prices, resource use conflicts and sustainability concerns [6]. Alternative plant sources have widely been used in aquafeeds [7], but their use is also limited by the presence of anti-nutritional factors [8], conflicts with human food consumption [9], and environmental issues [10]. Hence, the increase in global aquaculture production prompt the search for novel sustainable feed sources. Over the last few years, insects have been identified as an important future source of sustainable raw materials for aquafeeds, but the production volume of insects is still needed be increased to compete with conventional feed sources and increase price competitiveness [11]. The upscaling of insects as a novel feed ingredient was promoted with the approval of processed insects in aquafeeds by the European Commission (Regulation 2017/893/EC, 2017). Black soldier fly (*Hermetia illucens*; Diptera: Stratiomyidae) (BSF) is a promising insect species as a feed source due to the high nutritional value [12], efficiency in conversion of wide range of organic matter and suitability for large scale production [13]. BSF larvae consist of three major fractions; protein, lipid, and exoskeleton [14] and each of these contain unique composition that can cause differential responses in nutrient utilization, growth performance and health of fish.

The BSF can be processed by applying different heat treatments and/or separation of the protein, lipid, or exoskeleton fractions. The quality and nutritional composition of BSF vary with the processing method [15, 16]. When considering commercial production, less processed full-fat BSF is more cost-efficient, because the processing

of BSF, particularly drying and separation of fractions is an additional cost. Furthermore, the drying method and temperature may have a large impact on the nutritional quality of BSF as in other protein sources such as fishmeal and soybean meal [17-19]. In a recent review, Oonincx and Finke [20] showed that drying method and temperature has a large impact on protein digestibility of insects, and destruction of vitamins and denaturation of proteins can occur due to heat. The processing of BSF at low temperatures to produce a paste would be beneficial for aquaculture industry due to maintenance of the nutritional value of BSF and reduction of production cost. On the other hand, the partial separation of lipid and exoskeleton fractions of BSF was reported to improve the nutritional value of BSF. For instance, such processed BSF larvae increased maximum inclusion level (40-60%) in the diets without compromising growth performance in salmonids [21, 22] and improved nutrient digestibility in European sea bass (*Dicentrarchus labrax*) [23]. Nevertheless, so far, it is not certain how or which fractions would beneficially affect Atlantic salmon (*Salmo salar*) (hereafter referred to as salmon) and whether the separation of fractions is necessary. This makes it challenging to recommend optimum downstream processing conditions for BSF to be used in practical diets for salmon. Hence, further research is needed to compare the effects of the use of differently processed BSF in salmon feeds on nutrient utilization and growth performance.

When introducing a novel ingredient into fish feed, it is crucial to demonstrate that it will not compromise fish health. The BSF is known to contain bioactive compounds such as polysaccharides especially chitin, lauric acid and antimicrobial peptides (AMP) [24], which have antioxidant and immunostimulatory properties in fish [25-29]. The BSF might thus be considered as a functional feed ingredient, and the assessment of health effects beyond the nutritional value will, therefore, provide an added value to this novel ingredient. According to our knowledge, three studies evaluated the effect of processed BSF meals on gut health in salmon [30, 31], but they did not focus on systemic innate immune responses. The knowledge on the dose-dependent effect of full-fat BSF on gut health and innate systemic immune responses of salmon is scarce. Kumar et al. [32] reported BSF lipid fraction has health-promoting properties and suggested that it may not be necessary to de-fat BSF larvae. Lipid fraction could provide an important added value when full-fat BSF is used as a

protein source in fish feed. Therefore, the evaluation of beneficial health properties of full-fat BSF has a great importance.

Fish nutrition and immunity are heavily influenced by gut microbiota [33, 34]. Dietary inclusion of BSF was reported to modulate gut microbiota in salmon post-smolts [35] and rainbow trout (*Oncorhynchus mykiss*) [36-38], and suggested that the bioactive compounds in BSF might be responsible for its' effect towards modulating gut microbiota in fish. Nevertheless, the specific roles of these compounds in modulating gut microbiota are not yet certain. Each fraction of BSF has unique composition of bioactive compounds. Studying possible effects of different fractions of BSF on gut microbiota composition and their functions are thus worth of attention to identify potential roles of BSF fractions in fish performance and health. This provides important knowledge to determine optimal downstream processing of BSF for positive modulation of gut microbiota, and thereby to improve the performance and health status of fish.

The present thesis was based on the hypothesis that full-fat and processed BSF can partially replace the conventional feed resources in salmon diets and the degree of success of BSF in salmon diets is partially determined by the processing method of BSF. The general objective of the present thesis was to investigate how full-fat BSF, processed BSF meals and fractions of BSF affect the extrusion processing, technical feed quality, nutrient utilization, growth performance, gut microbiota, gut health, and immune responses of salmon. To achieve this objective, this thesis consists of five papers. In **Paper I**, we investigated the dose-dependent effects of two differently processed full-fat BSF larvae in salmon diets on extrusion processing, technical feed quality, nutrient utilization, and growth performance, while **Paper II** focused on the dose-dependent effects of full-fat BSF larvae on gut health, and systemic immune responses in salmon.

To get an in-depth understanding on the roles of protein, lipid, and exoskeleton fractions of BSF larvae and to determine if it is necessary to process BSF larvae by separation of these fractions, in **Paper III**, we investigated how dietary inclusion of meals (full-fat, de-fatted and de-chitinized) and fractions (oil and exoskeleton) affects the extrusion processing, pellet quality, nutrient utilization and growth performance

in salmon. In **Paper IV**, the effects of the dietary BSF meals and fractions on salmon gut microbiota were evaluated.

With the increasing number of studies regarding nutrient utilization and growth performance of fish fed BSF in literature, the determination of dietary effects of BSF in the salmonid population can provide a better estimation than individual studies. The diverse nature of BSF rearing, downstream processing, and study designs, makes it difficult to compare the reported results to draw a general conclusion. Therefore, a meta-analysis was conducted in **Paper V** to systematically review the results from different studies available in the literature. In this paper, we focused on the effect of the use of BSF in salmonid diets on nutrient utilization and growth performance and to identify the factors causing the variation in response of salmonids to dietary BSF.

5.2 Background

5.2.1 Insects as a novel ingredient in fish feeds: Benefits and limitations

In recent years, insects have received growing attention as a sustainable ingredient for fish feed production [11]. The production of insects has environmental benefits such as lower greenhouse gas and ammonia emissions [39], high land-use efficiency [40], low water requirement [41] and efficient nutrient conversion [13, 39]. The land, feed, and water required for the production of insects is lower than the requirements for livestock species (Fig. 1) [42]. Insects are found in aquatic environments and part of the natural diets of carnivorous and omnivorous fish [43], which makes insects an ideal feed alternative for fish. The use of processed insects in aquafeeds was approved by the European Commission (Regulation 2017/893/EC, 2017). The authorized seven insect species to be reared and used in aquafeeds include BSF, common housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), banded cricket (*Gryllodes sigillatus*) and field cricket (*Gryllus assimilis*). Amongst, BSF has attracted attention as one of the most promising insect species to be used in feeds [13].

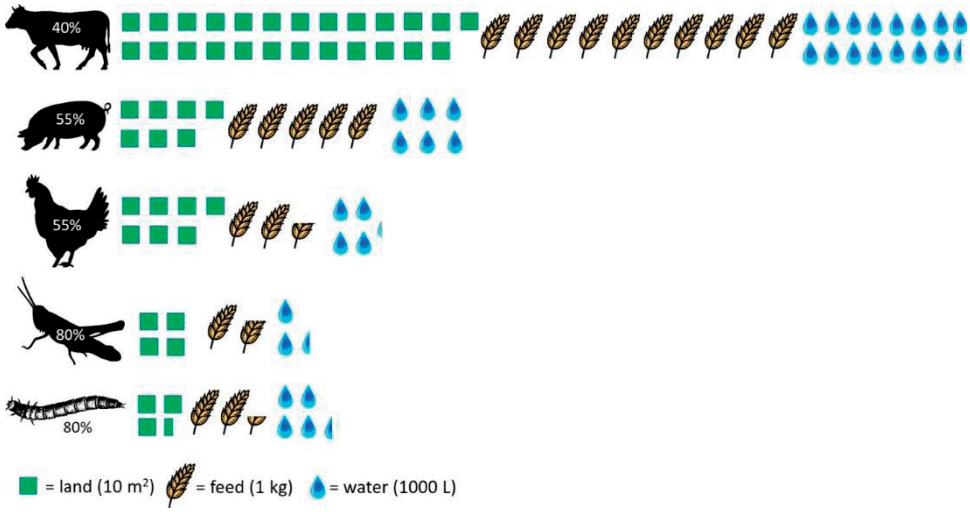

Fig. 1. Amount of land, feed, and water required to produce 1 kg of live animal weight and edible percentage of the live animal weight. This figure was taken from Dobermann et al. [42] with the permission of the authors.

Although a myriad of opportunities exists for using insects in fish feeds, there are some hurdles to overcome to reach its full potential. It is claimed that insects contain anti-nutritional compounds such as chitin [42], although the effect of insect chitin in fish has not been verified yet. A major consideration in the use or applicability of any novel feed ingredient is to demonstrate its safety. Regarding feed and food safety, it is reported that insects cannot accumulate mycotoxins, pesticides and antibiotics [44-46], but they can accumulate heavy metals when present in the rearing substrate [47, 48]. In a recent review, Lievens et al. [49] reported that accumulation of contaminants in BSF larvae do not exceed the European feed legislation limits, except for several metals such as cadmium, lead, and zinc. The monitoring of contaminants, especially for heavy metals in both rearing substrates and insects used as feed ingredients is required to ensure feed and food safety along the value chain [44]. The rearing substrates of BSF can be processed to effectively decontaminate and prevent exceeding of faecal indicator organisms and pathogen concentrations in fish feed pellets above microbiological quality standards for insect processed animal proteins [50]. The current trading price of insect meals is not yet competitive enough and the production of insects in sufficient volumes to compete with fishmeal and plant sources is yet to be achieved [11].

5.2.2 Production of insects: Role of rearing substrates

Insects can be fed on a wide range of organic materials, including feed, manure, faeces, slaughterhouse by-products, animal-based products and by-products, restaurant and food waste, fruit and vegetables, industrial by-products, cereal remnants, and algae. However, in the EU, the use of ruminant proteins, catering waste, meat-and-bone meal and manure is prohibited for insect rearing, in line with regulations on transmissible spongiform encephalopathies and bovine spongiform encephalopathy. Hence, the possibility of using low quality organic material as rearing substrates for growing insects is still limited by the regulatory framework in EU. The selection of substrates for insect rearing is based on the regulatory framework, cost, nutritional composition, biomass production, feed efficiency, the time needed to reach the harvesting stage, availability, steady year-round supply, absence of hazards, and ease of removal during harvesting [51].

Being poikilotherms with no investment of metabolic energy in maintaining a constant body temperature, insects demonstrate a higher feed conversion efficiency than livestock species [13]. Among the insect species, BSF can digest and convert organic matter into biomass more efficiently [52, 53]. The BSF was reported to reduce dry matter mass of rearing substrates by 33-58%, phosphorous (P) by 62-71% and nitrogen (N) by 30-50% [54]. Oonincx et al. [52] showed that the N conversion efficiency could reach up to 43–55% in BSF fed diets composed of food by-products. On the other hand, it is also reported that BSF could reduce harmful bacteria such as *Escherichia coli* counts in dairy manure [55] and *Salmonella enterica* serovar enteritidis [56] and house fly populations [57, 58] in chicken manure.

The rearing substrate has a significant influence on production parameters of BSF such as total yield, final body weight, biomass conversion ratio, and development time as well as substrate mass reduction by BSF [51, 59]. For instance, BSF reared on animal manure had poor performance compared to BSF reared on vegetable restaurant waste and poultry feed [60]. Oonincx et al. [52] and Lalander et al. [59] reported that BSF larvae developed faster on a high protein substrate because less substrate is required to attain a sufficient amount of protein for development. Excess carbohydrates caused a significant increase in the development time of BSF larvae to pre-pupae [61]. The high moisture content of the rearing substrate can negatively

affect the performance parameters and feed conversion efficiency of BSF [62]. Mixing high protein substrates with substrates containing easily available carbon can be a better approach to increase utilization of available nutrients by BSF [59]. The rearing substrate influenced the composition [20, 52, 63, 64] and *in vitro* protein digestibility of BSF for monogastrics [63]. Thus, it is possible to tailor BSF into a nutrient profile more suited for specific feed or food purposes by modulating the rearing substrate [65].

5.2.3 Black soldier fly: A promising insect species for fish feeds

The BSF is distributed worldwide [66]. The life cycle of BSF can be divided into five phases: eggs, larvae, pre-pupae, pupae, and adults (Fig. 2) [67]. The adult BSF do not possess disease transmission risks [68]. The larval, pre-pupal and pupal stages of BSF are considered as potential sources to be used in fish feeds. The BSF is a good source of protein, lipid, and minerals [12]. They also contain bioactive compounds such as chitin, lauric acid and AMP [24], which are known to possess modulatory effects on gut microbiota and antioxidant and immunostimulatory properties in fish [25-28, 69]. In addition, BSF can valorise a wide range of low-quality organic material efficiently into high-quality nutrients [70], thus, can contribute to sustain circular economy [71]. Furthermore, BSF is a good candidate for industrial-scale production, due to its high intrinsic growth rate, weight gain per day and feed conversion efficiency, the potential to rear on organic side streams and suitability for automation [13].

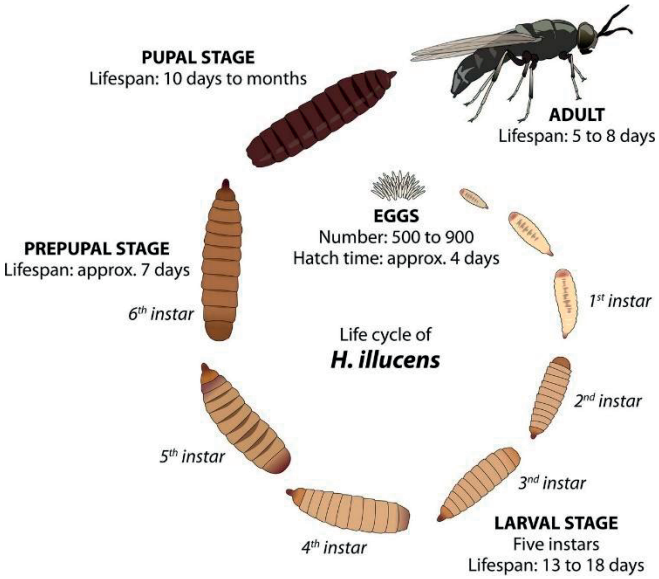

Fig. 2. Life cycle of black soldier fly (*Hermetia illucens*). This figure was taken from De Smet et al. [67] with the permission of authors.

5.2.4 Nutritional composition in black soldier fly

The BSF consist of three major fractions; protein, lipid, and exoskeleton [14]. The BSF, on average, contain a moderate level of protein content, ranging from 22 to 53% (dry matter basis) [64, 72]. The lipid fraction, ranging from 8 to 48% (dry matter basis) [65, 73] provides an added value to the BSF as an energy source in comparison to conventional protein resources [74]. The protein and lipid contents of BSF are variable, depending on the developmental stage [73], processing method [15], and rearing substrates [64]. Spranghers et al. [74] showed that rearing substrate had no substantial influence on the amino acids (AA) composition of BSF, whereas Lalander et al. [59] reported that there were some differences in the AA profile of BSF reared in different substrates. The radar chart of essential AA contents in Fig. 3, shows that BSF, in general, meets the AA requirements of salmon and rainbow trout, except methionine and lysine [75].

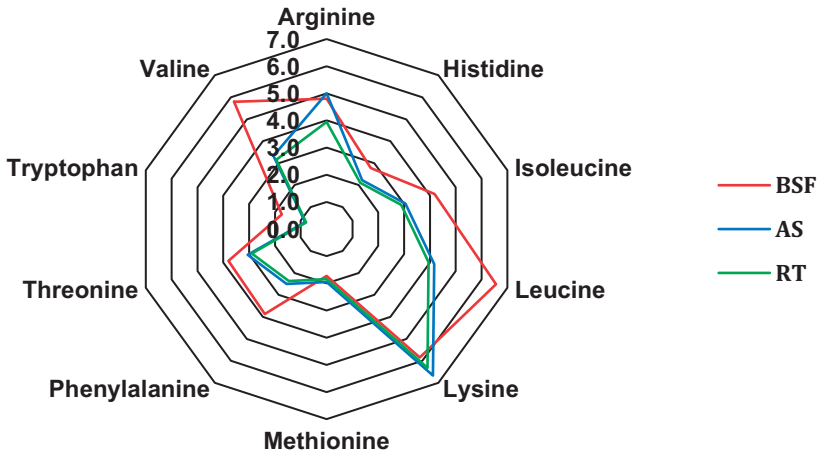

Fig. 3. Radar chart comparing amino acids (in g/100 g crude protein) in black soldier fly with the corresponding requirements in Atlantic salmon and rainbow trout [75]. BSF: black soldier fly (*Hermetia illucens*), AS: Atlantic salmon, RT: Rainbow trout. Sources: [14, 21, 22, 59, 65, 73, 74, 76-93].

As shown in Fig. 4, the fatty acids (FA) profiles of insects are species-specific. In BSF, the majority of the FA are saturated fatty acids (SFA), ranging from 43% [80] to 87% [72] of total FA. The most abundant FA in BSF is medium-chain 12:0 lauric acid, ranging from 13% [94] to 68% [95] of total FA. In contrast, FA in mealworm is dominated by oleic acid 18:1n9 and housefly by 16:0 palmitic acid. The lipid fraction of BSF is very poor in polyunsaturated FA, in particular omega-3 and omega-6 FA, compared to fish oil. According to the correlation matrix shown in Fig. 5, there are positive correlations between BSF larvae/pre-pupae and their substrates for the content of 16:1 palmitoleic acid, 18:1 oleic acid, 20:4n6 arachidonic acid, 20:5n3 eicosapentaenoic acid (EPA) and 22:6n3 docosahexaenoic acid (DHA). In contrast, 14:0 myristic acid showed a negative correlation. Hence, BSF seem to have the capacity to modify its FA profile according to their rearing substrate. Even though BSF contains very low level of desirable omega-3 fatty acid FA, it is possible to enrich BSF

with omega-3 fatty acids by feeding diets containing omega-3 FA, making BSF a suitable replacement for fishmeal/fish oil.

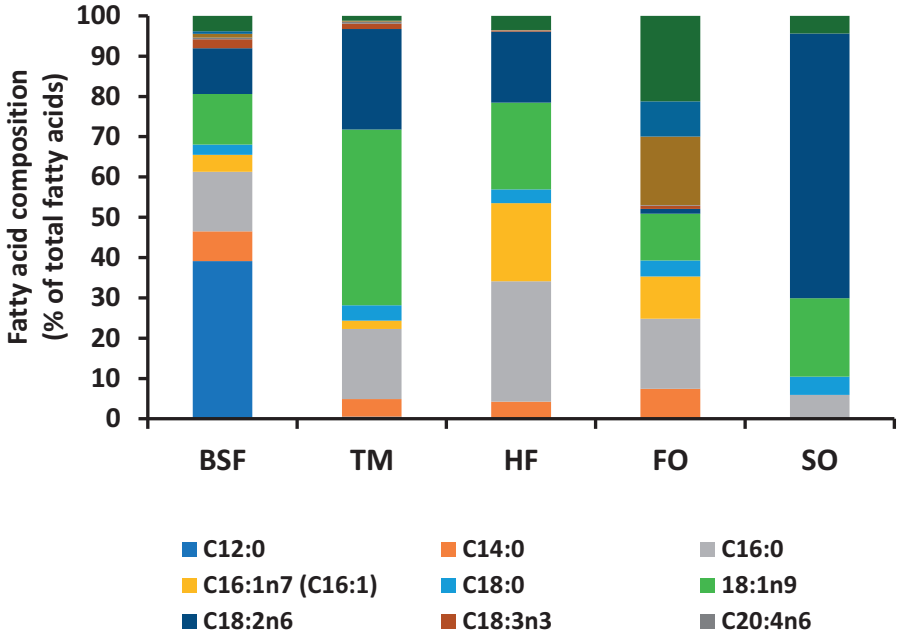

Fig. 4. Fatty acid (FA) composition in black soldier fly compared with the FA composition in two other insect species of interest for aquaculture, fish oil and soybean oil. BSF: black soldier fly (*Hermetia illucens*), TM: mealworm (*Tenebrio molitor*), HF: housefly (*Musca domestica*), FO: fish oil, SO: soybean oil. Sources: [21, 63-65, 72-75, 86, 90, 94-128].

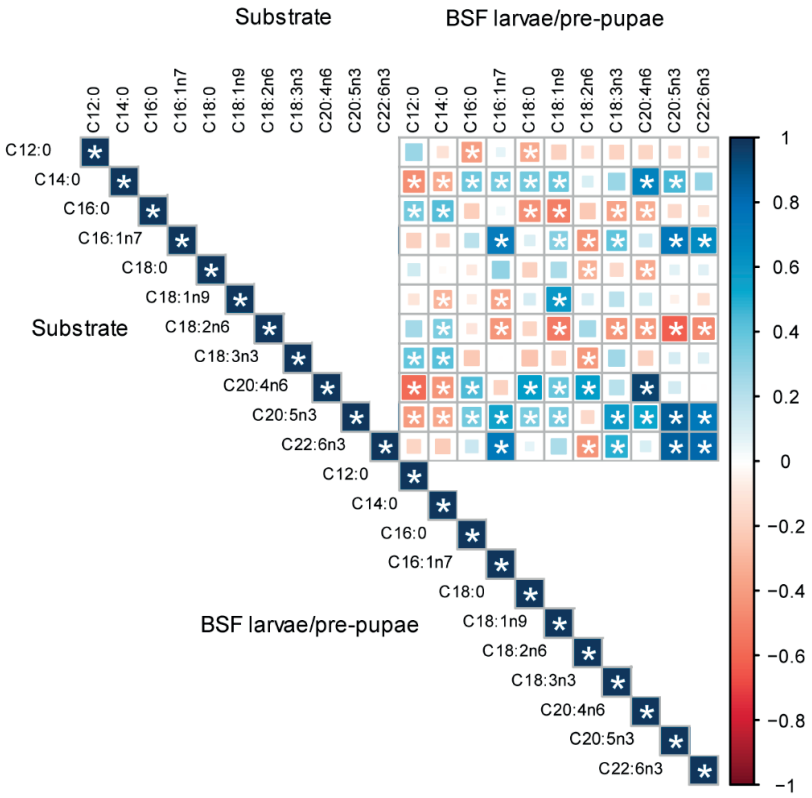

Fig. 5. Correlation matrix between the fatty acid (FA) composition (% of total FA) of black soldier fly (BSF) larvae/pre-pupae and the corresponding fatty acid composition (% of total FA) in their substrates. Sources [64, 65, 72, 74, 94, 97, 100].

Besides protein and lipid, BSF is also a rich source of minerals, particularly calcium [12, 82]. The mineral content in BSF vary with the rearing substrate [65, 74] and is higher than other insects [129] providing a substantial advantage of BSF over other insects nutritionally [70]. However, a high ash content could also be undesirable for the use of BSF as an ingredient in a feed formulation, but rearing on energy-rich substrates with a low content of ash and fibre, appeared to result in a reduced ash content, making BSF more suitable as a feed ingredient [74].

5.2.5 Bioactive compounds in black soldier fly and their effects in fish

Chitin

Chitin is a polymer of N-acetylglucosamine present in the exoskeleton of insects [130]. The chemical structure of chitin is shown in Fig. 6. The chitin content has been estimated to be 3-10% (dry matter basis) in BSF [64, 72]. The reported content of chitin in BSF in different studies depends on the analytical method [14] and the developmental stage of BSF [131]. Chitin is known to cause both negative and positive effects in fish, and the specific role of chitin in fish diets is still controversial and can be related to its dietary level of inclusion [132]. Chitin was reported to contain around 17.1 kJ/g of energy content [133]. Salmon and rainbow trout, however, have a poor capacity to digest chitin (13–40% and 3-5% respectively) [134, 135], meaning chitin can work as a filler with low digestible energy content [136]. Previous studies reported that feeding chitin containing krill meal or chitin from shrimp shells reduced growth in salmon [136, 137]. In contrast, Lellis and Barrows [138] reported that feeding 6% chitin-rich krill shell improved growth in steelhead trout (*Oncorhynchus mykiss*).

The protein content in feeds and feed ingredients is normally calculated based on the N content using the standard N-to-protein conversion factor of 6.25, which can overestimate protein content in BSF due to the presence of non-protein N from chitin. A conversion factor of 4.2 and 5 might be more appropriate for BSF to avoid this as reported by Janssen et al. [85] and Belghit et al. [139]. The overestimation of the protein content in the BSF meal can result in a low dietary protein level than the requirement of fish especially in high BSF-containing diets, which may negatively affect fish growth. Dietary chitin (5-10%) can also cause a reduction of nutrient digestibility as previously shown in salmon [136] and tilapia (*Oreochromis niloticus* × *O. aureus*) [140]. Insect exoskeleton comprises a chitin matrix with bound proteins, lipids and other compounds [141, 142], which may reduce the access of digestive enzymes to bound nutrients, and thereby the digestibility [143]. Chitin can further reduce protein digestibility due to its capacity to bind proteins [144] and immobilize [145] or reduce the activity of proteolytic enzymes such as the brush border enzyme,

leucine aminopeptidase that break down peptides into AA [21]. The exoskeleton of BSF is mineralized with calcium [82]. Alternate layers of protein and chitin impregnated with calcium carbonate are in the insect exoskeleton [146]. The ash content of BSF has been shown to affect its *in vitro* protein digestibility, hypothesizing that calcium carbonate can prevent the digestion of bound protein in the exoskeleton [63]. It has also been suggested that feeding chitin leads to decrease in lipid digestibility by decreasing bile acid levels in the pylorus, which is essential for activation of lipase and efficient FA absorption [137].

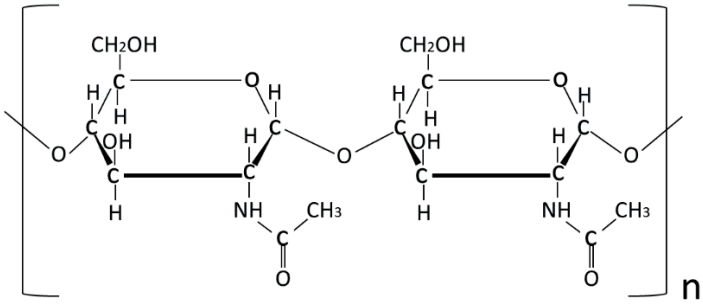

Fig. 6. Chemical structure of chitin. Modified from Berezina [147].

Chitin, on the other hand, can have immunomodulatory [25, 28] and antioxidant properties [29]. Chitin can act as pathogen-associated molecules [148], and has complex and size-dependent effects on innate and adaptive immune responses [149, 150]. The size of chitin particles determines whether it is biologically inert (large chitin polymers), pro-inflammatory (smaller fragments), or anti-inflammatory (even smaller fragments) [149]. Da Silva et al. [148] reported that chitin fragments (40–70 μm) could trigger inflammation and cytokine production via the pattern recognition receptors in mice. Several studies also showed immunomodulatory properties of chitin in fish, but such effects of chitin were dose [25] and time-dependent [25, 28]. For instance, feeding 1% chitin increased serum lysozyme activity in common carp (*Cyprinus carpio*) [28] and modulated the immune system and the disease resistance in Indian major carp (*Cirrhina mrigala*) [151]. Nevertheless, <1% inclusion of chitin did not affect immune responses in gilthead seabream (*Sparus aurata*) as shown by serum lysozyme activity and phagocytic activity of head kidney leukocytes [25].

Dietary chitin was also shown to modulate gut microbiota in fish including salmon [69] and Atlantic cod (*Gadus morhua* L.) [152]. In particular, gut microbiota of salmon fed 5% chitin was dominated by beneficial microbes such as *Lactobacilli, Bacillus, Staphylococcus and Acinetobacter* [69]. The positive modulation of fish gut microbiota by dietary chitin can be due to its prebiotic properties [37, 38] or antimicrobial and bacteriostatic activity against several pathogenic Gram-negative bacteria [153]. Chitin can also increase gut microbiota diversity in fish [152].

Fatty acids

The high SFA in BSF can reduce lipid digestibility. Lipid digestibility in salmonids decreases linearly with an increasing concentration of dietary SFA when dietary total FA are above 23% [154]. The major FA in BSF, medium-chain lauric acid is a good source of energy for salmonids compared to other FA and used less for tissue deposition [22, 155]. The increased energy production by FA such as lauric acid/medium-chain triglycerides can increase protein retention due to a protein-sparing effect [156, 157], as observed previously in salmon [158, 159]. In contrast, Liland et al. [160] showed in a recent meta-analysis that high dietary SFA (>39% of total FA), and increasing dietary level of lauric acid decreased normalized final body weight of fish fed BSF. The rapid oxidation of medium-chain FA, rather than deposited as fat, can reduce appetite and feed intake as shown in humans and rats [161, 162]. Even if some studies showed that feed intake in salmon decreased when fed diets containing medium-chain FA [158, 159], the inhibitory effect of dietary medium-chain FA on salmonid feed intake is more ambiguous [15] and seems to vary among fish species and source of medium-chain FA [155].

Lauric acid, on the other hand, can possess antimicrobial properties. For instance, monoglyceride of lauric acid or lauric acid in BSF oil have shown antimicrobial effects against *Clostridium perfringens* and D-streptococci, but demonstrated low impact on *E. coli*, *Salmonella* spp. and coliforms [163, 164]. For human gut microbes, lauric acid was shown to have low antimicrobial activity against commensal lactic acid bacteria (LAB), but higher activity against pathogenic *Bacteroides* and *Clostridium* [165]. Lauric acid can thus contribute to the modulation of gut microbiota in fish. Apart from the effects on gut microbiota, medium-chain FA could positively affect gut health by

improving the intestinal morphology and function, through their beneficial effects on crypt cell renewal [164]. Supplementation of medium-chain FA and triglycerides has also reported to reduce intestinal inflammation in pigs [166]. On the other hand, the SFA can have the potential to prevent or reduce enterocyte steatosis (abnormal lipid accumulation in enterocytes) in the proximal intestine, as observed in Arctic char (*Salvelinus alpinus*) [167]. The BSF are rich sources of choline [82, 168], which is important in lipid transport across the intestinal mucosa of salmon [169]. Choline can prevent enterocyte steatosis in the proximal intestine in salmon as previously shown when fed diets containing choline chloride (0.37–0.4%) [169, 170].

Antimicrobial peptides

The BSF is known to contain an expanded range of AMP with activity against many bacteria [171-174]. The antimicrobial activity of these AMP might facilitate the proliferation of specific bacteria(s) by eliminating certain bacteria in the fish gut. For instance, the dietary supplementation of AMP showed potential in suppression of harmful microbes, such as coliforms and *Clostridium* spp., by favouring beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* in broilers and pigs [175]. The AMP derived from BSF have great potential as alternatives to antibiotics and treatments of animal diseases due to their extensive antimicrobial properties and lower tendency to induce resistance [176]. In addition to antimicrobial effects, AMP have also shown antioxidant and immunostimulatory properties in fish [26].

5.2.6 Downstream processing of black soldier fly

Processing is important to optimize the use BSF in future fish feeds. The BSF can be processed by applying different heat treatments and/or separation of protein, lipid or exoskeleton fractions, to enhance nutritional value, palatability, digestibility [177], inclusion level in the diet and storage potential [178]. However, when considering commercial production, less processed BSF is more cost-efficient, because the processing of BSF, particularly drying, de-fatting, or de-chitinization is an additional cost and require high energy usage.

The processing of BSF into dried meal has been the standard practice. Drying can increase storage potential due to the low water activity. Once dried, BSF can be

ground into a meal for inclusion in fish feeds. Furthermore, like other protein sources such as fishmeal and soybean meal, the drying method and temperature may have a large impact on the nutritional quality of BSF [17-19]. In a recent review, Oonincx and Finke [20] showed that drying method and temperature has a large impact on protein digestibility of insects, and destruction of vitamins and denaturation of proteins can occur due to heat. Thus, processing BSF at low temperatures to produce a paste while maintaining the nutritional value and reducing the production cost and energy usage could be beneficial. The BSF larvae paste has previously been used successfully in mirror carp (*Cyprinus carpio*) [179] and California perch (*Micropterus salmoides*) [180]. The use of BSF paste as an ingredient in extruded feed may, however, be challenging and reduce storage potential due to higher moisture content [16].

The use of full-fat BSF can be a challenge to the feed industry due to its high lipid content that can interfere with the extrusion process [181] and reduce technical pellet quality [182]. This limits the maximum inclusion level of full-fat BSF in the diet. Thus, processing of BSF biomass into partially de-fatted protein-rich meal has become a practice, which allows high inclusion levels of BSF meal in fish diets without reducing the technical pellet quality of extruded feeds [178]. The separation of lipid fraction from BSF can produce high-protein meals (over 60%) [12, 74] and BSF oil, which is a good alternative lipid source in practical fish feed [74, 102]. Feed can be vacuum coated with BSF oils after pellet extrusion to prevent interference with the extrusion process [178].

Since it is suggested that the presence of chitin-rich exoskeleton can reduce the nutritional value of BSF, processing BSF into de-chitinized meal by separating exoskeleton might improve the nutritional value of BSF. Belghit et al. [21] reported that partially de-chitinized BSF protein meal in diets did not compromise the growth performance of salmon even at 60% inclusion level, but it still reduced nutrient digestibility. However, the anti-nutritive properties of BSF chitin are not confirmed yet, and being a bioactive compound, chitin might have positive effects on fish. In addition, the removal of the exoskeleton might increase the lipid content in the meal, which can further interfere with extrusion processing.

5.2.7 Extrusion processing: Role of lipid and moisture in the feed mash

Extrusion processing has become the primary technique used for commercial salmon feed production. Extrusion is a thermomechanical process of fish feed production where the technical pellet quality and expansion rate is controlled by steam and viscous dissipation of mechanical energy (heat), moisture level, and the physicochemical and rheological properties of the feed ingredients [183]. In a previous study, extrusion processing increased *in vitro* organic matter digestibility in BSF-containing feed blends, indicating extrusion may represent a valuable technology for processing BSF-based feed [184].

The lipid and moisture content in the feed ingredients directly affect extrusion processing and technical pellet quality (expansion, texture, pellet strength/integrity) of extruded pellets [185]. Lipids act as lubricants; therefore, a high lipid level in feed mash reduces the friction in the extruder [181, 185] and dough temperature [181]. Lower dough temperature, together with hydrophobic properties of high lipid in the extruder, can reduce the starch gelatinization [181, 186] and thereby the expansion [187] and technical pellet quality [186]. Being a lubricant, lipid can also decrease the extruder barrel fill and decrease the residence time of the dough in the extruder [185], thus the heat transfer between the extruder barrel and the dough becomes less efficient [188]. This can further reduce the degree of cooking, expansion and technical quality of pellets and produce a denser product [185]. The expansion can also be reduced by the lower pressure during extrusion caused by high lipid levels in the feed mash [184]. In agreement, a previous study also showed that extrudate expansion could decrease due to high lipid content in the mash [189]. However, if the lipid is bound, such as in a coarsely ground or whole oil seed, then higher lipid levels may be tolerated than unbound lipid [185].

The role of water in extrusion is to moisten the feed mash sufficiently to enable the starch and protein granules to rupture uniformly. The moisture within the extruder also permits the dough to pass through the die. The moisture level should be sufficiently high to retain its fluidity as the material discharges from the die and gelatinize starch, but low enough to ensure that the starch becomes stiff after passing through the die. Excessively low moisture limits the lubricating effect of dough in the

extruder and causes high energy consumption [185]. On the other hand, the higher moisture content in the extruder can act as a lubricant and decrease friction and dough temperature [181, 188]. This can lead to the production of feed pellets with poor technical quality as mentioned above.

5.2.8 Effects of black soldier fly in salmonid feeds

In recent years, an increasing number of studies have successfully used BSF as a novel feed ingredient for salmonids and have shown promising results. These studies mainly focused on BSFs' effects on nutrient utilization, growth performance, gut microbiota, gut health, and immune responses of salmon and rainbow trout. The research findings on the effect of BSF in salmonid feeds are summarized below.

Impact of black soldier fly on growth performance in salmonids

In the majority of previous studies, BSF meals did not compromise feed intake in salmon [21, 77] and rainbow trout [22, 178, 190], indicating no adverse effects of BSF on palatability. The BSF meal could also replace dietary protein sources at least partially without causing detrimental effects on salmonid growth performances [15, 21, 77, 83, 132, 190-192]. The response of fish to dietary BSF varied with inclusion level. Dietary inclusion of low to moderate levels of BSF meal (<20%) did not compromise growth performance in salmonids [77, 83, 178], whereas higher inclusion levels (>20%) reduced growth [83, 178]. In contrast, Belghit et al. [21] and Renna et al. [22] reported that partially de-chitinized or de-fatted meal in diets did not compromise the growth performance of salmon and rainbow trout, respectively, even at 40-60% inclusion level. Randazzo et al. [193] and Roques et al. [194], on the other hand, showed that replacing plant protein with 8-45% of de-fatted BSF meal and 10-15% BSF protein hydrolysate, respectively, improved rainbow trout growth. Few studies investigated and reported that BSF larvae oil (2.5-12% inclusion in the diet) could partially or totally replace fish and/or plant-based oil in diets for salmon [21] and rainbow trout [178] without compromising growth performance.

Impact of black soldier fly on nutrient utilization in salmonids

The protein digestibility of BSF larvae meal was reported to be 89% in salmon [83] and 85% in rainbow trout [178], which was lower than soybean meal and higher than corn protein concentrate [83]. In addition, Dumas et al. [178] reported that the digestibility of essential AA of BSF larvae meal varied from 84% to 96% in rainbow trout. The lipid digestibility of BSF larvae meal was reported to be 97% in salmon and not different from that of soybean meal and corn protein concentrate [83].

The digestibility of dry matter, protein, and lipid in BSF meals and oil containing diets were generally above 70% in salmonids despite the inclusion level [21, 38, 77, 83, 178]. Dietary inclusion of 5-40% of full-fat or de-fatted BSF larvae or pre-pupae meal did not compromise the digestibility of protein [22, 38, 77, 83, 86, 178, 192] and lipid [22, 38, 77, 83, 192] in salmonids. Additionally, Dumas et al. [178] showed that 20% BSF oil in the diet did not affect protein and lipid digestibility in rainbow trout. In contrast, de-fatted BSF meal reduced lipid digestibility at 20% inclusion in rainbow trout [178], while partially de-chitinized BSF larvae protein meal reduced digestibility of protein and lipid at 60% inclusion in salmon [21]. The protein and lipid digestibility of salmonids fed BSF varied in literature from 82 to 95% and 73–99%, respectively. The variation in digestibility results reported in the literature can be due to the faeces collection method, i.e., faeces collection from water vs stripping. For instance, the faeces collection for digestibility estimation from water might overestimate the protein digestibility compared to stripping, due to leaching of N depending on the type of feed as explained by Shomorin et al. [195]. Furthermore, in a recent review, English et al. [16] reported that nutrient digestibility in salmonids fed BSF could be dependent on the quality of the rearing substrates of the BSF.

In literature, the nutrient utilization of salmonids fed BSF shows a varying degree of success. The majority of studies showed that dietary inclusion of full-fat or processed BSF meal (5-60%) and/or oil (5-12%) in salmonid diets did not affect the protein and lipid utilization, as determined by efficiency ratio, apparent retention or whole-body composition [15, 21, 22, 77, 86, 91]. Nevertheless, BSF meal increased protein efficiency ratio (PER) in salmon at 30% inclusion, but did not affect at 10-20% [83]. Dietary BSF meal has also previously been shown to decrease PER and protein

retention, and increase lipid efficiency ratio (LER) and retention in rainbow trout at 28% inclusion [196].

Impact of black soldier fly on gut health in salmonids

When introducing a novel protein source into fish feed, assessing health effects beyond the nutritional value is essential. While the nutritional value of BSF has been extensively evaluated in salmonids, its influence on salmon health remains largely unexplored. Histological and gene expression analyses of fish have provided evidence regarding changes in gut health due to BSF inclusion in the diet.

In salmon, BSF inclusion in the diet reduced the severity and prevalence of enterocyte steatosis in the proximal intestine in both pre- [31] and post-smolts [30] compared to the fishmeal and plant protein-based control diets fed fish. The BSF larvae meal in the diet, however, increased submucosa cellularity, in the proximal intestine in salmon post-smolts [30]. Dietary inclusion of BSF larvae meal (5-60%) did not affect the histology of the mid and distal intestine in pre-smolt [31] and post-smolt [15]. Nevertheless, Li et al. [30] observed signs of enteritis in the histology of intestine of salmon post-smolts fed 15% de-fatted BSF-based diet similar to control diet fed fish. In addition to the histological analyses, BSF inclusion showed minor effects on the gene expression profile in the proximal and distal intestines of salmon pre- [31] and post-smolts [30].

In rainbow trout, the inclusion of full-fat or de-fatted (3-40%) BSF showed no to mild inflammatory signs and/or no changes in villus height in the histology of proximal [91, 192, 197], mid [132] and distal intestine [32, 132, 192]. The BSF oil also showed no to mild inflammation and/or no changes in villus height in proximal and/or distal intestine in rainbow trout [32, 178]. Nevertheless, mucous cells in the distal intestine increased at 21% full-fat meal inclusion and fold length in the mid intestine reduced at 10.5-21% inclusion in rainbow trout, indicating reduced capacity for nutrient absorption [132]. Previous studies also showed that the response of intestinal villi height of rainbow trout to BSF can be dose-dependent [178, 192]. Gaudioso et al. [198] showed that feeding BSF meal (8-45%) did not compromise the gut barrier function as indicated by plasma markers and gene expression profiles. Suggesting a specific anti-inflammatory role, dietary BSF could prevent the occurrence of

inflammatory signs of soybean meal-induced intestinal enteritis in the distal intestine histology and supported by downregulated genes involved in inflammation in rainbow trout [32, 193].

Impact of black soldier fly on immune responses, antioxidant capacity and tissue damage indices in salmonids

In literature, few studies reported immunomodulatory effects of BSF in salmonids, and the results vary with the level of BSF in the diet. Inclusion of 16% BSF meal in the diet increased serum lysozyme activity of rainbow trout [32], but did not affect serum/plasma lysozyme activity and/or total immunoglobulin at 6-11% inclusion levels [32, 86]. On the other hand, feeding 16% BSF oil increased serum lysozyme activity in rainbow trout compared to soybean oil, and upregulated the expression of IL-8, TNF-α1 and IRF-1 genes in the kidney compared to fish oil [32]. Further, Stenberg et al. [199] reported that head kidney leukocytes isolated from salmon fed BSF had indications of potential impacts on the cellular stress response when treated with lipopolysaccharides.

Oxidative stress within cells or tissue has adverse effects on fish health; thus antioxidants can have significant health-benefits [199]. The fish fed BSF meals or oil could maintain the antioxidant capacity in rainbow trout in most of the previous studies, but in a dose-dependent manner [32, 86, 190, 197]. The presence of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatine kinase (CK) in blood indicates liver [200], muscle or heart tissue [201] damages. Several studies demonstrated that the activities of these enzymes in serum/plasma were not altered or, in some cases, even decreased by dietary inclusion of de-fatted BSF larvae meal in salmon [77, 155] and rainbow trout [178]. Increasing levels of BSF in diet (5-40%) had no adverse effects on histological traits in liver and spleen in rainbow trout [197], and liver in salmon [15]. Hence the majority of previous studies indicated that BSF did not cause any adverse effects on tissue health. These results would also be in accordance with the maintenance of antioxidant capacity in salmonids fed with BSF diets, since an increased oxidative stress environment can be related to tissue damage [202, 203]. On the contrary, Cardinaletti et al. [132] reported an up-regulation of a gene involved in stress response, i.e., heat-shock protein-70, in the

liver of rainbow trout fed diets containing 21% BSF for 98 days, suggesting a physiological activation of stress/inflammation response when feeding BSF for a prolonged period.

Impact of black soldier fly on gut microbiota in salmonids

Previous studies reported that the dietary inclusion of full-fat and de-fatted meals of larval and pre-pupal stages of BSF modulated gut microbiota in salmon post-smolts [35] and rainbow trout [36-38, 198, 204]. Huyben et al. [36] further showed that the response of gut microbiota of salmonids to BSF depended on the development stage and lipid content of BSF meal. In general, previous studies showed that BSF meal in diets increased the abundance of beneficial microorganisms that contribute to the health of the host such as lactic acid [36-38] and butyrate [38] producing bacteria in the gut of rainbow trout. Recent research also showed that BSF increased abundance of bacteria that can produce chitinase, which helps to digest chitin that usually is difficult for salmonids to digest [36, 198].

High gut microbial diversity is in general considered a positive and desired feature associates with the health status of the host [38]. Species-rich communities are thought to out-compete pathogens for nutrients and colonization, consequently resisting pathogen invasion and intestinal infection [205, 206]. Feeding de-fatted BSF meal increased richness and diversity in rainbow trout [38, 204] and salmon post-smolts [35].

Gut microbes carry out many metabolic reactions, which play a critical role in host nutrition, physiological functions, and health [204, 207]. In a previous study, the metagenome data provided evidence that dietary BSF inclusion can shape the predicted metabolic activity of the gut microbiota in rainbow trout [204]. In this study, BSF meal showed a predicted enrichment of pathways involved in sugar and starch metabolism. In addition, Li et al. [35] carried out a multivariate association analysis between gut microbial clades and host responses in salmon post-smolts fed BSF, and showed that *Brevinema andersonii* and *Spirochaetaceae* were associated with the expression of genes related to pro- and anti-inflammatory responses and barrier function in the distal intestine, respectively.

5.2.9 Methods to evaluate dietary effects on fish health

To understand how dietary compounds affect fish health, a combined description of biochemical, physiological, histological, and multi-omics techniques can be used. The gut is the main site of the direct exposure to nutrients and antigens [208]. The histological analysis of the gut is considered a good indicator of the nutritional and health status of fish [209, 210]. Such analyses rely on tissue staining techniques to visualize intestinal morphology and specific cell markers [211]. The gut histological analyses can be supported with molecular biological approaches to evaluate gene expression profiles, which represent valuable tools to provide early information on inflammation processes, even in the absence of clear histopathological evidence [212]. In addition to the gut, the liver is an important organ involved in metabolic and immune functions and, consequently, is often considered a second target organ when testing novel fish feed ingredients. Histological and molecular analyses can also be applied to the liver, to assess dietary impacts in fish [211].

Fish possess both innate defence mechanisms such as phagocytosis, as well as specific humoral and cellular responses mediated by lymphocytes. Fish rely more on their innate defences, primarily the skin and mucus [213]. The skin mucus contains various proteins in the innate immune system such as complements, lysozyme, immunoglobulins, cytokines, protease and lectins that protect fish against pathogens [214]. The protein expression profile in skin mucus, thus, indicates modulations of systemic immune responses by the diet [213]. Further systemic immune responses such as phagocytic activity and population of immune cells in the head kidney and spleen can be assessed using *in vitro* cell culture techniques and flow cytometry. In addition, omics techniques (proteomics and transcriptomics) can be used to further identify the immunomodulatory effects of dietary components. Measurement of plasma indices also provides information regarding the health status of fish fed specific dietary ingredients. Among the various plasma components, the activities of alkaline phosphatase, AST, ALT, CK, C-reactive protein (CRP), lysozyme, immunoglobulins and cytokines seem to be useful indices for health assessment in fish [213]. On the other hand, the reactive oxygen species production and the response of the antioxidant markers in kidney and liver, as well as blood, can be used as indications of oxidative stress and antioxidant capacity in fish [215].

The use of different omics approaches is expected to give a better understanding of the fish immune mechanisms [216]. Protein can undergo post-translational modifications that markedly alter its function and activity. Thus, the evaluation of protein expression is advantageous compared to gene expression [217]. The validation of proteomics results can be done through antibody-based techniques or other complementary omics technologies. Antibody-based methods such as enzyme linked immunosorbent assays (ELISA) and western blotting are very commonly used for proteomic data validation. In addition, integrative studies combining proteomics with other omics platforms such as transcriptomics and metabolomics provide a broader vision of the effects of diets in fish [218] and have the potential to characterize phenotypes fully [216].

5.2.10 The interplay between diet, gut microbiota, and fish

The gut microbiota plays a crucial role in digestive function, nutrient metabolism, growth performance, fish physiology, barriers against pathogens, immune response, disease resistance, welfare, and health in fish [33, 34, 219-222]. Thus, the improvement of nutrient utilization, growth performance, and health in fish is possible by positive modulation of gut microbiota. Diet is one of the main drivers that can modulate the gut microbiota [223, 224]. Different dietary components can selectively induce compositional and functional alterations of the gut microbiota, which in turn could inflict important implications on nutrient utilization, host health and disease resistance [219, 225]. The characterization of the gut microbial communities modulated by different dietary compounds is an effective approach towards a better understanding of the nutritional effects in fish. Fish microbiota studies are advancing with rapid, low-cost and reliable DNA sequencing technologies, such as amplicon sequencing of bacterial marker genes to characterize gut microbiota in fish [34]. However, yet there is a narrow interpretation of the findings of studies in the context of gut microbiota-microbial metabolites-host interactions in fish.

5.2.11 Meta-analysis

Meta-analysis is a quantitative method to compile and statistically analyse results from a number of individual studies addressing similar research questions and produce integrated and broader interpretations. The meta-analyses can provide better estimates in the population. However, the meta-analysis has several limitations, including publication bias, small sample sizes, and the heterogeneity between studies included in the analysis [226]. However, some of these problems can be overcome, for instance, standardized effect size; Hedges' g can be used for the analysis as it corrects for bias with small sample sizes and produces a statistical standardization of the findings for each study [227]. The publication bias can be assessed using Graphical (funnel plots) and statistical (two-tailed Begg's rank correlation and Egger's linear regression asymmetry) tests and can be corrected using various methods [228]. In addition, the presence of heterogeneity and variables that can cause the heterogeneity between the studies can be identified through meta-regression (for both categorical and continuous variables) or sub-group analysis (for categorical variables) [229].

5.3 Status of knowledge

An increasing number of studies in literature have reported the effect of BSF as a protein or lipid source in diets for salmon on nutrient utilization and growth performance with a varying degree of success. The reasons for variation in response of salmon to BSF in diet have not yet been confirmed. It seems the processing method of BSF can have a larger impact on this. Differently processed BSF larvae meals such as full-fat, partially de-fatted or partially de-chitinized meals and BSF fractions such as BSF oil were used in previous studies. However, there is still a scarcity of information to make recommendations on optimal downstream processing of BSF to be used in practical diets for salmon, and there are many unknown factors for the effect of processing on the nutritional value in fish. In the case of full-fat BSF, all the studies used dried full-fat meal, but full-fat BSF paste processed at low temperatures has not been studied for salmon. Further, the differently processed BSF meals (full-fat, de-fatted and de-chitinized) and fractions (oil and exoskeleton) have still not been

evaluated and compared in a single study. It is not certain how, or which fractions would beneficially affect salmon and whether processing to separate BSF fractions is necessary. In addition, limited information exists on how differently processed BSF and its fractions affect the technical quality of extruded feed pellets.

While the nutritional value of BSF has been extensively evaluated in salmon, its influence on salmon health remains largely unexplored. Only three studies evaluated the effect of processed BSF meals on general gut health in salmon using histological and/or gene expression methods [15, 30, 31]. An additional study also evaluated *in vitro* bacterial and viral-induced gene response in head kidney leukocytes isolated from salmon fed BSF [199]. These studies did not focus on the protein expression in the intestine and systemic innate immune responses in the plasma, spleen, and skin mucus of salmon. The knowledge on the dose-dependent effect of full-fat BSF larvae on gut health and systemic immune responses of salmon is lacking in literature. The health-promoting properties of BSF lipid fraction have already been shown in rainbow trout [32], thus, the lipid fraction could provide important added value when full-fat BSF is used as a protein in salmon feed. Hence, it is worthwhile to evaluate the effect of full-fat BSF on salmon health when included in graded levels in the diet.

The dietary inclusion of BSF meal was previously reported to modulate gut microbiota in salmonids and it is possible that the bioactive compounds in BSF might be responsible for this. The gut microbiota modulatory roles of specific compounds in BSF are however not certain. To date, majority of studies on gut microbiota of fish fed BSF have been restricted to analysis of taxonomic composition. We are still far from understanding how BSF and its specific compounds affect the functional profile of gut microbiota in salmon, which is essential to identify potential fish-microbiota interactions. Investigation of the impacts of different dietary BSF fractions on gut microbiota composition and their metabolic capacity are thus worth of attention to identify how dietary BSF modulate fish performance and health and to make recommendations on optimal downstream processing methods for BSF.

The investigation of the effect of dietary BSF on nutrient utilization and growth performance in a population of salmonids can provide a better estimation than individual studies. Reviews with narrative and qualitative approaches are available summarizing scientific literature on the effect of BSF in salmonid diets [16, 230].

Nevertheless, the reported data on effect of BSF in salmonid diets have not yet been analysed using a quantitative approach; thus, there is a need to conduct a meta-analysis to investigate the effect of dietary BSF on nutrient utilization and growth performance in salmonids including salmon and rainbow trout and to identify the factors responsible for the variation in response of salmonids to the use of BSF in diet.

5.4 Hypotheses, objectives and aims

Overall hypothesis: Full-fat and processed BSF can partially replace the conventional feed resources in salmon diets without compromising technical pellet quality, nutrient utilization, growth performance, and health of fish, and the degree of success of BSF in salmon diets is partially determined by the processing method of BSF.

General objective: To investigate how the replacement of conventional feed resources with differently processed BSF affects the extrusion processing, technical pellet quality, nutrient utilization, growth performance, gut microbiota, gut health, and immune responses in salmon.

The present thesis consists of five papers. The hypotheses and aims of each paper are as follows:

Paper I

Hypothesis: Differently processed full-fat BSF larvae can partially replace conventional feed resources in salmon diets without compromising technical quality of extruded feed pellets, nutrient utilization, and growth performance.

Aim: To investigate the dose-dependent effect of two differently processed full-fat BSF larvae products (i.e., full-fat meal and paste) in diets on extrusion processing, technical feed pellet quality, nutrient utilization, and growth performance in salmon.

Paper II

Hypothesis: Full-fat BSF larvae contains bioactive compounds that can improve gut health and systemic immune responses in salmon.

Aim: To investigate the dose-dependent effect of full-fat BSF larvae meal and paste in diets on gut health and innate systemic immune responses in salmon.

Paper III

Hypothesis: Full-fat BSF larvae meal in diet can support similar nutrient utilization and growth performance as processed BSF larvae meals and fractions in salmon.

Aim: To determine whether it is necessary to apply costly processing of BSF larvae by separation of protein, lipid and exoskeleton fractions when included in salmon diets at moderate levels.

Paper IV

Hypothesis: Low processed full-fat BSF larvae meal in salmonid diets promotes a more favourable gut microbiota than processed meals and fractions.

Aim: To investigate the effect of BSF larvae meals (full-fat, de-fatted and de-chitinized) and fractions (oil and exoskeleton) in diets for salmon on gut microbiota composition and predicted microbial metabolic capacity.

Paper V

Hypothesis: The BSF can replace conventional protein sources in diets of salmonids without compromising nutrient utilization and growth performance, and the response of salmonids to dietary BSF depends on the salmonid species, the processing method of BSF, development stage of BSF, type of protein(s) replaced, feed production method, BSF inclusion level in the diet and fish body size.

Aim: To determine the effect of dietary BSF on nutrient utilization and growth performance in salmonids using a meta-analytic approach, and to identify which factors were responsible for variation in response of salmonids to dietary BSF between the studies.

5.5 Materials and Methods

Two fish experiments were conducted in the present thesis, and the results of the first experiment were documented in **Paper I** and **II**, whereas the results of the second experiment were documented in **Paper III** and **IV**. The overview of the two fish experiments is shown in Fig. 7. Additionally, a meta-analysis was conducted, and the results were documented in **Paper V**.

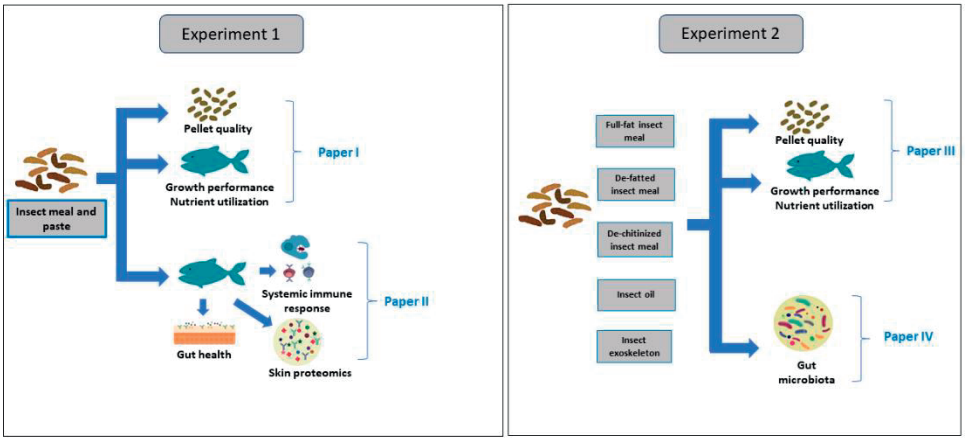

Fig. 7. The overview of two fish experiments (**Paper I** to **IV**) in the present thesis.

5.5.1 Paper I

A batch of BSF larvae was divided in to two parts and processed to a dried full-fat meal and a ground full-fat paste preserved with formic acid (2.5%). Seven isonitrogenous, isolipidic, and isoenergetic experimental diets were formulated: a control diet based on fishmeal, plant protein sources and fish oil (Control-1); three diets with increasing levels of full-fat BSF meal, replacing 6.25% (6.25IM), 12.5% (12.5IM), and 25% (25IM) of the protein content of Control-1, corresponding to 8%, 16% and 32% inclusion level respectively; two diets with increasing levels of full-fat BSF paste, replacing 3.7% (3.7IP) and 6.7% (6.7IP) of protein from Control-1, corresponding to 20% (wet weight) and 35% (wet weight) inclusion level respectively; and an extra control diet with 0.88% of formic acid (Control-2). The diets were produced using a five-section Bühler twin-screw extruder (BCTG 62/20 D, Uzwil, Switzerland) with reduced capacity, bypassing the conditioner. A screw configuration suitable for reduced extruder capacity was used. The screw speed was

increased when extruding BSF diets. In addition, the water addition to the extruder was reduced when extruding BSF paste diets. The impact of dietary inclusion of BSF on the extrusion processing and technical pellet quality were assessed.

A total of 1260 salmon pre-smolts with 34 g of mean initial weight were randomly distributed into 21 fiberglass tanks and fed with one of seven experimental diets *ad libitum* for seven weeks (n=3). The growth performance, nutrient digestibility and nutrient utilization were evaluated. The data were analysed using one-way ANOVA, followed by a Tukey's test for comparison of means. Linear and quadratic polynomial contrasts were used to determine the relationship between growth performance or nutrient utilization parameters and dietary BSF meal or paste levels.

5.5.2 Paper II

The details on the production of experimental diets and conduction of the fish experiment are reported in **Paper I**. Six fish from each tank were randomly sampled at the end of the experimental period to collect pyloric caeca and distal intestine sections, blood, and skin mucus. In addition, head kidney and spleen were collected from three fish from each tank of four dietary groups (i.e., two controls, 12.5% BSF meal and 6.7% BSF paste groups). The histological sections of the pyloric caeca were assessed for the increase in width and inflammatory cell infiltration of the submucosa and changes in the vacuolization of the enterocytes. For distal intestine, shortening of mucosal fold height, increase in width and inflammatory cell infiltration of the submucosa and lamina propria, and loss of enterocyte supranuclear vacuolization were evaluated. The levels of immunoglobulins (IgM and IgD) and pro-inflammatory cytokine (IFN_{γ} and IL-1β) in the distal intestine and skin mucus were measured using indirect ELISA. Besides, the protein expression profile in skin mucus was assessed using mass spectrometry. Plasma was analysed for biochemical and immune parameters including ferric reducing antioxidant power (FRAP), ALT, AST, CK, CRP and lysozyme. Head kidney and spleen (three fish per tank and pooled) leukocytes were isolated. The phagocytic capacity of head kidney macrophage-like cells against *Piscirickettsia salmonis* was measured *in vitro*, and IgD+, IgM+ and CD8+ splenocytes were counted using flow cytometry. The type of analyses used in this paper to assess gut health and immune responses are illustrated in Fig. 8.

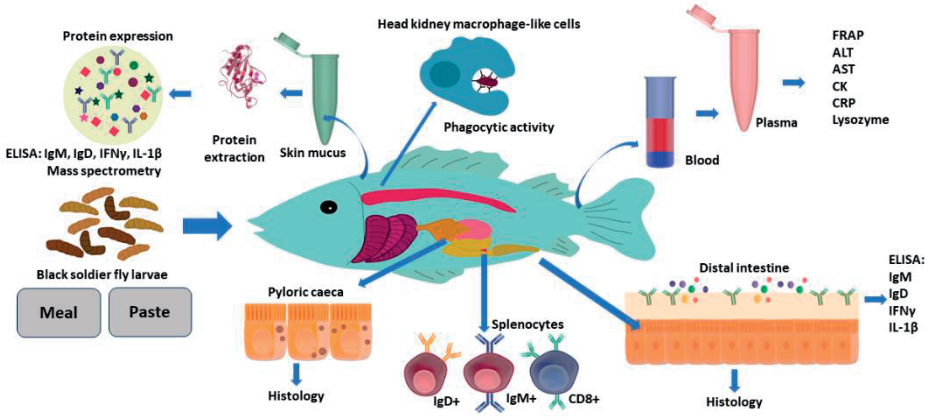

Fig. 8. The analyses used in **Paper II** to assess the gut health and systemic immune responses in fish fed experimental diets.

The significant differences in histological scores were analysed using ordinal logistic regression. Proteomics raw data were transferred to log normalization and then volcano plot analysis, multivariate statistical analysis, and data modelling were conducted. The other data were analysed using one-way ANOVA, followed by Tukey's multiple comparison test. In addition, polynomial contrast analysis was used to evaluate the relationship between plasma parameters and dietary BSF meal or paste levels.

5.5.3 Paper III

A batch of BSF larvae was processed into three meals (full-fat, de-fatted and de-chitinized) and two fractions (oil and exoskeleton). Six experimental diets with similar AA profiles and lipid contents were formulated: a control diet containing fishmeal, plant protein and fish oil (CD); full-fat BSF meal diet (IM); de-fatted BSF meal diet (DFIM); de-chitinized BSF meal diet (DCIM); BSF oil diet (IO) and BSF exoskeleton diet (EX). The full-fat, de-fatted and de-chitinized meals replaced 15% of the protein in the control diet. The BSF oil and exoskeleton were added to the diets to match the BSF oil and chitin contents in full-fat meal diet, respectively. The corresponding BSF inclusion levels in the diets were 20% full-fat meal, 15% de-fatted

meal, 25% de-chitinized meal, 6% oil and 7% exoskeleton. The diets were produced using a five-section Bühler twin-screw extruder (BCTG 62/20 D, Uzwil, Switzerland) with reduced capacity, bypassing the conditioner. A screw configuration suitable for reduced extruder capacity was used. The impact of dietary inclusion of meals and fractions of BSF larvae on the extrusion processing and technical pellet quality were evaluated.

A total of 900 salmon pre-smolts with 28 g of mean initial weight were randomly distributed into 18 fiberglass tanks and fed with one of seven experimental diets *ad libitum* for eight weeks (n=3). The effect of dietary inclusion of meals and fractions of BSF on growth performance, nutrient digestibility and nutrient utilization were evaluated. The data were analysed using one-way ANOVA, followed by a Tukey's multiple comparison test.

5.5.4 Paper IV

Details of the production of experimental diets and conduction of the fish experiment are reported in **Paper III**. Six fish from each tank were randomly sampled at the end of the experimental period to collect digesta from the distal intestine. Two tank water samples were also collected. Next, DNA was extracted from digesta, water as well as feed samples. The library preparation was conducted according to the Illumina 16S Metagenomic Sequencing Library Preparation protocol [231]. The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified. After the PCR amplification and cleaning of PCR products, they were examined by 1% agarose gel electrophoresis. The 13 digesta samples with the strongest bands of each dietary group were used for sequencing. The library was sequenced using the Miseq Reagent Kit v3 (600-cycle) (Illumina; catalog no., MS-102-3003) on the Miseq System (Illumina, San Diego, California, USA). The raw sequence data were processed by the DADA2 1.18.0 in R 4.0.4 [232] to infer amplicon sequence variants (ASVs) [233]. The Silva version 138.1 database [234, 235] was used as a reference database for the taxonomy assignment.

The differences in microbial abundances between the diets were determined using linear discriminant analysis (LDA) effect size (LEfSe) tool [236]. The statistical differences were evaluated using the factorial Kruskal-Wallis rank-sum test, followed

by a pairwise Wilcoxon test. A threshold between 3.5 and 4.0 was used for the LDA. The alpha (observed ASVs, Pielou's evenness, Shannon's index, and Faith's phylogenetic diversity (PD)) and beta (Jaccard distance, unweighted UniFrac distance, Aitchison distance and PHILR transformed Euclidean distance) diversity of gut microbiota of fish fed diets were assessed. The statistical differences in alpha diversity indices between the dietary groups were evaluated using Kruskal-Wallis test, followed by multiple comparisons using Wilcox pair-wise comparison test. The four beta-diversity distance matrices were visualized by the principal coordinates analysis. The permutation multivariate analysis of variance with 999 permutations (PERMANOVA) was performed to compare beta diversity. The ASVs were mapped to metabolic reactions via an available collection of genome-scale metabolic models (GSMMs) of gut microbes. The mean abundances of each reaction for each pair of diets were compared using two-sample t-test. The metabolic subsystem classification of reactions was obtained from the GSMMs, and Fisher's exact test was used to identify enriched subsystems among the significantly different reactions.

5.5.5 Paper V

A systematic literature search was conducted in ISI WEB OF KNOWLEDGE (1945-2021) and SCOPUS (1939-2021) to select studies. The growth performance data, i.e., specific growth rate (SGR), feed conversion ratio (FCR), and feed intake and nutrient utilization data, i.e., protein digestibility and PER of salmonids fed BSF diets were either extracted or calculated using available reported information. We included data from both salmon and rainbow trout in the present analysis to increase the number of comparisons. The standardized effect size; Hedges' g, was used to calculate differences in growth performance and nutrient utilization parameters between fish fed control diets and BSF diets within studies. The meta-analysis was performed in comprehensive meta-analysis version 3 software (Biostat Inc., Englewood, New Jersey, USA) using random effects models to account for the variation among the populations of studies. When significant heterogeneity was detected, meta-regression analysis was conducted to explore possible causes of heterogeneity in effect sizes of SGR, FCR and feed intake between the studies. The categorical variables including fish species (Atlantic salmon vs. rainbow trout), feed production method

(extrusion vs. pelleting), type of protein source(s) replaced (fishmeal vs. fishmeal + plant protein sources vs. non-fishmeal), BSF development stage (larvae vs. prepupae/pupae) and BSF processing method (full-fat vs. de-fatted) and two continuous variables including dietary inclusion level of BSF and fish body size were included in the meta-regression analysis. Sub-datasets were sorted from the full dataset according to the fish species, life stage of salmon, type of protein source(s) replaced, BSF development stage, BSF processing method and/or feed production method. The meta-analyses were conducted for growth performance and nutrient utilization parameters in these sub-datasets. Linear and quadratic regression analyses between dietary inclusion levels of BSF meal, fishmeal replacement level or dietary chitin level and effect sizes of SGR, FCR, feed intake, protein digestibility or PER were performed using IBM SPSS Statistics 27 software (IBM Corp., Armonk, NY, USA).

5.6 Results

5.6.1 Full-fat black soldier fly larvae in the diets for salmon: Impact on technical pellet quality, nutrient utilization, and growth performance (Paper I)

Full-fat black soldier fly larvae meal

Pellet durability and hardness were not adversely affected by the inclusion of full-fat BSF meal in the diet, but the expansion and water stability of pellets decreased numerically with increased inclusion of BSF meal. The BSF meal in the diet did not affect the feed intake of fish. Salmon SGR and FCR were not affected when up to 12.5% of protein was replaced by full-fat BSF meal, but SGR decreased at 25% replacement. Further, increasing BSF meal level in the diet linearly decreased SGR and increased FCR. Protein digestibility did not differ between fish fed BSF diets and the control diet, but linearly decreased with increasing level of BSF meal in the diet. Despite the reduced protein digestibility, total AA digestibility and protein retention were not compromised by the inclusion of BSF meal in the diet. The lipid digestibility and retention/accretion decreased linearly with increasing level of BSF meal in the diet.

Replacing 25% protein decreased PER, LER, lipid retention/accretion and energy retention. Dietary inclusion of full-fat BSF meal did not affect faecal P excretion and retention in salmon.

Full-fat black soldier fly larvae paste

Similar to the full-fat BSF meal, the inclusion of BSF paste in the diet did not adversely affect pellet durability and hardness, but the expansion and water stability of pellets decreased numerically with the increased inclusion of BSF paste. The SGR, FCR, and feed intake of salmon were not affected by replacing up to 6.7% of protein. Increasing dietary levels of BSF paste linearly reduced protein digestibility, but protein retention was not affected. Lipid digestibility was not affected by dietary inclusion of BSF paste, but lipid utilization increased at 3.7% replacement. The increased dietary level of BSF paste linearly increased faecal excretion of P accompanied by linearly decreased P retention.

5.6.2 Full-fat black soldier fly larvae in the diets for salmon: Impact on gut health and systemic immune responses (Paper II)

Full-fat black soldier fly larvae meal

The control diets containing fishmeal and plant protein sources caused mild to moderate enterocyte steatosis (abnormal lipid accumulation in enterocytes) in pyloric caeca and inflammatory changes in distal intestine characterized by mild to moderate mucosal fold shortening due to a loss in enterocyte vacuolization. Replacing up to 12.5% of dietary protein with full-fat BSF meal improved gut health by reducing enterocyte steatosis in pyloric caeca and improving distal intestine histology. At 25% protein replacement level, BSF meal prevented the occurrence of inflammatory changes in distal intestine histology but gave mild to moderate enterocyte steatosis in pyloric caeca and increased pro-inflammatory cytokine IFNγ in the distal intestine. The BSF meal did not increase IgM, IgD and IL-1β in the distal intestine. The BSF meal showed minor effects on protein expression in skin mucus and innate systemic responses in salmon. Plasma lysozyme content did not differ between control and BSF meal fed fish. The ELISA results showed no effect of BSF on immunoglobulin (IgM and IgD) and pro-inflammatory cytokines (IFNγ and IL-1β) in the skin mucus.

Proteomics results showed that fish fed the 12.5% BSF diet uniquely expressed skin mucus proteins that regulate phagocytosis and antimicrobial responses, i.e., calpains and histone, whereas the 25% BSF diet resulted in differentially expressed stress-related proteins in skin mucus, i.e., overexpressed apolipoprotein D, reduced expression of NAD(P)H dehydrogenase [quinone] 1-like and uniquely expressed superoxide dismutase. The 12.5% BSF diet tended to improve immune responses by numerically increasing lysozyme activity in plasma and phagocytic activity in head-kidney macrophages-like cells, but did not affect immune markers (IgD, IgM and CD8) in splenocytes. Feeding BSF meal seemed to have no negative effects on liver and muscle health as indicated by plasma ALT, AST and CK, and the plasma antioxidant capacity of fish as indicated by plasma FRAP.

Full-fat black soldier fly larvae paste

Full-fat BSF paste improved gut health by reducing enterocyte steatosis in pyloric caeca and preventing inflammatory changes in the distal intestine histology, except at 3.7% protein replacement, in which fish showed mild inflammatory changes in the distal intestine. Dietary inclusion of BSF paste did not increase immunoglobulin (IgM and IgD) and pro-inflammatory cytokine (IFN_{γ} and IL-1β) levels in the distal intestine. Feeding BSF paste caused minor changes in the expression profile of proteins in skin mucus, but no effects on the plasma lysozyme content. The 6.7% paste diet had no impact on phagocytic activity of head kidney macrophages-like cells and immune markers (IgD, IgM and CD8) in splenocytes. An increasing level of BSF paste linearly increased plasma antioxidant capacity as shown by FRAP. There was a quadratic relationship between plasma AST and dietary BSF paste level with the highest level at 3.7%, and the same trend was observed between plasma CK and dietary BSF paste level ($p = 0.07$). On the other hand, the presence of formic acid in diets seemed to have no effects on the gut, skin mucus and other general health parameters in plasma, spleen, and head kidney in salmon.

5.6.3 Meals and fractions of black soldier fly larvae in diets for salmon: Impact on technical pellet quality, nutrient utilization, and growth performance (Paper III)

All the diets showed a higher pellet durability, but the lipid-rich full-fat (30%) and de-chitinized (44%) meals in the diets numerically reduced pellet expansion and water stability. The de-fatted meal, oil and exoskeleton supported similar growth performance as the control. Feeding full-fat and de-chitinized meals improved SGR of salmon. Full-fat meal also gave a higher fish SGR than other BSF meals and fractions and accompanied by higher feed intake. However, feeding de-fatted meal and exoskeleton diets resulted a lower FCR in salmon than full-fat meal diet. Protein digestibility was not compromised by full-fat meal and BSF oil in the diets but decreased by de-fatted and de-chitinized meals and exoskeleton. De-fatted meal diet increased the retention of digested protein compared to the full-fat meal and control diets. Meals and fractions of BSF did not affect lipid digestibility. Full-fat meal diet gave lower lipid retention/accretion than the other insect diets, whereas de-chitinized meal fed fish showed the highest lipid retention/accretion. The diets containing full-fat and de-chitinized meals reduced faecal P excretion indicating improved P digestibility, while the fish fed de-fatted meal showed a higher P retention.

5.6.4 Meals and fractions of black soldier fly larvae in diets for salmon: Impact on gut microbiota (Paper IV)

A higher microbiota overlap between the gut and feed was observed compared to that between the gut and water. At the phylum level, feeding BSF larvae meals and fractions increased relative abundance of *Firmicutes* and decreased relative abundance of *Proteobacteria* in the salmon gut. The four diets that contained BSF chitin, i.e., BSF meals (1.4% in full-fat and de-fatted diets and 0.5% in de-chitinized diet) and exoskeleton (1.4%) diets enriched *Lactobacillales* and *Actinomyces* in fish gut. The full-fat meal diet fed fish showed the highest abundance of *Lactobacillales*, *Actinomyces* and *Enrerococcus* among all the dietary groups. The four diets that contained BSF lipids, i.e., BSF meals (6% in full-fat diet, 1.8% in de-fatted diets and 11% in de-chitinized diet) and oil diets (6%) increased the abundance of *Bacillaceae*. The de-chitinized meal fed fish had the highest abundance of *Corynebacterium* and

Brevibacterium in gut microbiota. The fish in this group also had a higher abundance of *Acinetobacter* compared to the control group. The exoskeleton diet enriched *Staphylococcus* in salmon gut.

As shown by the observed ASVs, the species richness did not differ between the gut microbiota in BSF and control groups, but the gut microbiota in fish fed full-fat meal diet had a numerically higher average. The lower Pielou's evenness in the gut microbiota of fish fed full-fat meal and BSF oil indicated the presence of dominated bacterial group(s) in the gut. Supporting this, the lower Shannon's index of full-fat meal group also suggested that the gut microbiota might be dominated by specific species. Faith's PD showed that phylogenetic diversity in full-fat meal fed fish was higher compared to the control diet fed fish, indicating the gut microbiota might consist of species from diverse clades in the phylogeny tree. The principal coordinates analysis of beta diversity showed that gut microbiota of BSF groups clustered together but separated from the control group. Moreover, statistical analyses showed that there were differences in beta-diversity between microbiota in the full-fat meal group and other BSF groups.

The meals and fractions of BSF also differently modulated the predicted metabolic capacity of gut microbiota. The gut microbiota in fish fed full-fat meal showed a predicted increase in mucin O-glycan degradation compared to all the other groups and reduction in lipopolysaccharide biosynthesis and FA synthesis compared to the control group. Feeding de-fatted meal caused predicted enrichment of mucin O-glycan degradation and starch and sucrose metabolism in gut microbiota compared to the control group. The de-chitinized meal and fractions of BSF led to a predicted increase in gut microbial FA synthesis and metabolism of several AA in comparison with the control group.

5.6.5 Meta-analysis: Impact of black soldier fly in diets on nutrient utilization and growth performance in salmonids (Paper V)

The meta-analysis showed that, on average, SGR, FCR and feed intake in salmonids fed BSF did not differ from those fed control diets. The analysis further showed a presence of heterogeneity in effect sizes of SGR, FCR and feed intake between studies. According to meta-regression, fish species, the protein source(s) replaced, and BSF

inclusion level partly caused the heterogeneity in SGR between the studies. Among the tested variables, only the protein source(s) replaced and BSF inclusion level partially explained the heterogeneity in FCR and feed intake, respectively.

Both salmon and rainbow trout sub-datasets showed that, on average, SGR, FCR and feed intake did not differ between the fish fed BSF diets and control diets, but salmon tended (p = 0.095) to increase FCR and salmon pre-smolts even increased FCR when fed BSF diets compared to fish fed control diets. Both full-fat and de-fatted BSF sub-datasets, on average, showed no differences in SGR, FCR and feed intake between BSF and control groups. The sub-datasets sorted based on the protein source(s) replaced showed that the replacement of fishmeal by BSF reduced SGR and feed intake in salmonids but did not affect the FCR. Replacement of both fishmeal and plant protein with BSF did not affect SGR and feed intake of salmonids, but it increased the FCR. Replacing non-fishmeal protein sources even increased the SGR and tended (p = 0.095) to increase feed intake, as well as reduced FCR in salmonids. The SGR and feed intake linearly decreased while FCR linearly increased with increasing level of fishmeal replacement by BSF. There were no linear or quadratic relationships between the dietary levels of BSF or chitin and effect sizes of SGR, FCR or feed intake. However, BSF larvae dataset showed a tendency (p = 0.088) to a linearly reduced SGR in salmonids, while salmon dataset tended (p = 0.05) to increase FCR linearly with increasing dietary BSF levels.

Similar to the growth performance data, the meta-analysis also showed that, on average, dietary inclusion of BSF did not compromise the PER in salmonids. However, feeding BSF tended to decrease protein digestibility in salmonids compared to the control diets. Several sub-datasets including salmon dataset also showed that the use of BSF in diets decreased protein digestibility compared to control diets. The influencing factors for these results were not considered in the present study, and the comparison across the studies is thus complicated.

5.7 Discussion

The BSF is considered a promising feed resource in fish diets. The knowledge on optimal downstream processing is however lacking in the literature. The present thesis aimed to investigate the impact of feeding differently processed BSF to salmon on nutrient utilization, growth performance, general gut health, gut microbiota, and immune responses. In summary, **Paper I** showed that the partial replacement of protein from fishmeal and plant protein sources with low to moderate levels of full-fat BSF larvae meal (6.25-12.5%) and paste (3-7-6.7%) did not compromise the growth performance of salmon. At 25% replacement level, fish SGR was, however, compromised by full-fat BSF larvae meal. The effects of full-fat BSF larvae on gut health and systemic immune responses are shown in **Paper II**. Replacing fishmeal and plant protein sources with moderate levels of full-fat BSF meal and paste (6.25-12.5%) improved gut health by reducing enterocyte steatosis in pyloric caeca and improving distal intestinal histology but replacing higher levels (25%) showed negative effects on gut health. Full-fat BSF meal and paste had minor impact on skin mucus proteome and innate systemic immune responses in salmon. The comparison of meals and fractions of BSF larvae in **Paper III** showed that replacing 15% of fishmeal and plant protein with de-fatted meal had no impact, but full-fat and de-chitinized meals increased SGR of the fish. The full-fat BSF meal also gave the highest SGR and feed intake among processed meals and fractions. As shown in **Paper IV**, the improved growth of fish fed full-fat meal was accompanied by phylogenetically diverse and unique gut microbiota, dominated with beneficial *Lactobacillales* and *Actinomyces*. The meta-analysis in **Paper V** showed that dietary inclusion of BSF in either full-fat or de-fatted forms did not compromise the SGR, FCR and feed intake in salmonids. However, according to **Paper I and III**, the use of high levels of lipid/moisture-rich full-fat meal/paste and de-chitinized meal of BSF larvae in diets can be a challenge due to the interference with the extrusion processing and reduced technical pellet quality.

5.7.1 Impact of processed black soldier fly larvae on extrusion processing and technical pellet quality

Extrusion processing has become the primary technique used for commercial salmon feed production. The ingredients in the feed mash have a direct impact on the extrusion processing and the technical quality of the extruded feed pellets [185]. Even though we hypothesized in **Paper I** that the inclusion of full-fat BSF would not result in poor quality pellets, increasing inclusion of full-fat meal and paste negatively affected the technical pellet quality. In addition, in **Paper III**, both full-fat and de-chitinized meals reduced the technical pellet quality. Full-fat and de-chitinized BSF contained a high lipid level (30-44%) (**Paper I** and **III**). The addition of full-fat and de-chitinized BSF thus increased the lipid level in the mash prior to extrusion. Concurrently, BSF paste also increased moisture content in the feed mash. High lipids or moisture contents in feed mash lead to a reduction in dough temperature [181, 188], which can reduce expansion [187] and technical pellet quality [186]. The expansion can further be reduced by the lower pressure during extrusion caused by high lipid levels in the feed mash [184]. Previous reports also showed that increased lipid content in feed mash decrease the extrudate expansion [189]. The fifth barrel of the extruder was cooled when extruding full-fat and de-chitinized BSF meals to obtain the desired bulk density in both **Paper I** and **III**, and this reduced temperature in the fifth barrel and the die, which might cause further reduction in pellet expansion [237-239]. The decreased expansion of BSF paste diets might also be associated with the high moisture content of the feed mash [239]. As shown by Hansen et al. [189], the high lipid levels in the feed mash followed with reduced pellet expansion is, most probably, explaining the reduced pellet water stability of full-fat and de-chitinized BSF diets. In accordance, a previous study has also showed reduced water stability of extruded fish feed pellets containing full-fat BSF meal [87].

The present results indicated that the use of lipid-rich full-fat or de-chitinized meals, and moisture-rich paste of BSF could be a challenge to the feed industry. This limits the maximum inclusion level of full-fat and de-chitinized BSF meals and paste in the diet. In **Paper I**, the screw speed was increased and/or the fifth barrel in the extruder was cooled down when the level of full-fat BSF meal and paste increased. In addition to this, the water addition to the extruder was reduced when extruding BSF paste

diets to obtain pellets with desirable technical quality. In **Paper III**, the throughput and water addition to the extruder were increased, and the fifth barrel in the extruder was cooled down to obtain pellets with desirable technical quality in the full-fat and de-chitinized BSF meals included diets. The present results, however, indicates that these modifications were not optimal to prevent negative effects caused by the increased lipid and moisture levels. Modification of the screw configuration during extrusion of the high BSF-containing diets could have improved the results, as this has a large effect on the extrudate characteristics [240]. It should be noted that, in the present thesis, we used an extruder with a reduced capacity and with by-passing of the pre-conditioner before extrusion, which is not the case in commercial feed production. Commercial extruders with pre-conditioners would tolerate high lipid and moisture levels without compromising technical pellet quality than what we used in this thesis.

5.7.2 Impact of processed black soldier fly larvae in salmon diets on nutrient utilization, performance, gut microbiota, and health

Interplay between black soldier fly larvae in diets, salmon performance and nutrient utilization, and gut microbiota

In agreement with our hypothesis, the present thesis showed that replacing conventional feed resources with BSF was possible without compromising growth performance, but the degree of success depended on BSF processing method and the dietary protein replacement level/BSF inclusion level. In **Paper I** and **III**, dietary inclusion of differently processed BSF meals, paste and fractions did not compromise feed intake of salmon, while 20% inclusion of full-fat meal (replacing 15% dietary protein) in **Paper III** even increased the feed intake. This indicates that BSF may even have a positive effect on the palatability in fish. In accordance, Rawski et al. [87] reported that the inclusion of full-fat BSF meal (10-30%) increased feed acceptance in Siberian sturgeon (*Acipenser baerii*). The observed increase in feed intake might also be due to the lower pellet water stability of the full-fat meal diet in **Paper III** as many water-soluble nutrients are known as chemo-attractants and feed stimulants [241].

Replacing low to moderate levels of protein from fishmeal and plant protein sources with full-fat meal (6.25-12.5%) and paste (3.7-6.7%) in **Paper I**, as well as with de-fatted meal (15%) in **Paper III** did not compromise the growth performance in salmon. **Paper III** reported further that fish fed diets with BSF fractions (6% oil and 7% exoskeleton) resulted in similar growth performance as those fed the control diet. In accordance, previous studies also showed that low to moderate dietary inclusion of BSF meals (<20%) or BSF oil (<10%) did not affect growth performance in salmonids [77, 83, 178]. Dietary BSF paste (<18%) did likewise not compromise growth performance in other fish species such as California Perch and mirror carp [179, 180].

The results of **Paper III** further showed that full-fat and de-chitinized meals can even increase SGR in salmon when replacing 15% dietary protein, while the use of full-fat BSF meal, rather than processing into de-fatted or de-chitinized meals or fractions supported higher SGR. Hence, it may not be necessary to de-fat or de-chitinize BSF at moderate inclusion levels in salmon diets, as this would be an additional cost. The increased feed intake can mainly be due to the improvement in SGR of salmon fed diets with full-fat BSF meal replacing 15% protein, but other processes may also involve in an increased growth response of salmon. For instance, the bioactive compounds in three fractions in full-fat meal, i.e., protein, lipid, and exoskeleton fractions, might act together to modulate gut microbiota positively, and thereby to improve the growth performance in fish.

According to the results of **Paper IV**, fish fed full-fat meal had phylogenetically diverse and unique gut microbiota composition, enriched and dominated by beneficial bacteria such as *Lactobacillales* and *Actinomyces* compared to other BSF groups. *Lactobacillales*, are commonly known as LAB, and generally considered as favourable microbes due to their abilities to enhance digestive function, mucosal tolerance, immune response, and disease resistance in host [242]. They also involve in production of lactic acid and bactericidal compounds that may prevent the colonization of pathogens to the intestinal surface [242-244]. *Actinomyces* belongs to the class *Actinobacteria* (**Paper IV**), which can produce antimicrobial compounds against fish pathogens and involve in the intestinal barrier function of the fish [245]. The enrichment and dominance of these beneficial bacteria might be the mode of

action for improved growth in fish fed full-fat meal in **Paper III**. This is further in accordance with our hypothesis that low processed full-fat BSF larvae meal promotes a more favourable gut microbiota than processed meals and fractions. **Paper IV** further showed that the full-fat meal fed fish had a predicted enrichment of mucin O-glycan degradation in gut microbiota compared to the fish fed control diet and other BSF meals and fractions. Mucin with a vast array of O-glycan structures is the major component in mucus layer covering intestinal epithelium [246]. Mucus nature can benefit certain mucin-degrading bacteria and thereby, shaping the gut microbiota composition at the mucosal surface. Concurrently, full-fat meal showed a predicted decrease in microbial lipopolysaccharide biosynthesis and FA synthesis pathways compared to fish fed control diet. According to the present results, it is likely that full-fat meal benefited the metabolic activity of salmon gut microbiota and consequently the fish growth.

In contrast to the results obtained for low and moderate BSF inclusion levels in **Paper I**, the diet with the highest full-fat BSF meal inclusion (32%) replacing 25% of dietary protein reduced SGR in salmon, accompanied by decreased PER, LER, and lipid digestibility and retention/accretion. The reduction of fish SGR at higher inclusion levels may be attributed to the presence of a higher level of chitin in the BSF (2.3%), although chitin seems to have no or even positive effects at low inclusion levels. Poorly digestible chitin can act as a filler [136] and might limit the growth of salmon. Confirming the negative effects of chitin, in a previous study, partial removal of exoskeleton allowed to include 60% BSF without compromising the growth performance of salmon [21]. The lower level of chitin in the diet, i.e., 0.4-1.2% in full-fat meal and paste diets in **Paper I** and, 1.4% in de-fatted meal and exoskeleton diets and 0.5% in de-chitinized meal diet in **Paper II**, may not be sufficient to cause adverse effects on fish performance. The presence of up to 2.1% of BSF chitin in diets did not reduce the SGR in rainbow trout in previous studies [22, 38]. Even though replacing 15% dietary protein with de-chitinized insect meal supported SGR of fish, it was lower than full-fat BSF meal in **Paper III**, indicating positive effects of BSF chitin at certain levels. Confirming beneficial effects of BSF exoskeleton fraction, inclusion of BSF exoskeleton (7%) in the diet increased feed utilization in fish compared to the

full-fat meal. Moreover, Lellis and Barrows [138] also reported that feeding 6% chitin-rich krill shell improved growth in steelhead trout (*Oncorhynchus mykiss*).

Protein digestibility in fish fed full-fat BSF meal and paste in **Paper I** and full-fat meal and BSF oil in **Paper III** did not differ from the control diets but decreased with increasing dietary levels of full-fat BSF meal and paste in the **Paper I**. **Paper III** further showed that dietary inclusion of de-fatted and de-chitinized meals and exoskeleton reduced protein digestibility in salmon. In accordance with these results, dietary inclusion of high levels of partially de-chitinized BSF meal (60%) adversely affected the protein/AA digestibility in salmon [21]. The presence of chitin in the diet can be responsible for the reduced protein digestibility in BSF-based diets, since chitin can reduce the activity [21, 145] or availability of protease enzymes [143]. In addition, calculating the protein content in the faeces based on N content can underestimate protein digestibility of the diet due to increased faecal excretion of non-protein N from the low-digestible chitin.

Despite reduced protein digestibility, total AA digestibility and/or protein retention in salmon were not affected by full-fat BSF meal and paste in **Paper I,** and BSF meals and fractions in salmon in **Paper III**. De-fatted BSF meal even increased retention of digested protein in salmon compared to both the control and full-fat meal diets, accompanying higher feed utilization than the fish fed full-fat meal (**Paper III**). Hence, de-fatting can be applied to improve the nutritional value of BSF larvae. According to the metabolic capacity prediction of gut microbiota in **Paper IV**, feeding de-fatted BSF meal showed a predicted increase in microbial starch and sucrose metabolism. The increased ability of gut microbiota to utilize dietary carbohydrates might partially be responsible for improved feed utilization in the fish fed de-fatted meal in the present study. Additionally, the fermentation of dietary carbohydrates by the gut microbiota can lead to the formation of a variety of beneficial substances, including short-chain fatty acids [204].

In **Paper I**, lipid digestibility was not affected by low levels of full-fat BSF larvae meal and paste in the diet (3.7-6.7% protein replacement), but moderate to high levels of full-fat BSF meal in the diet (12.5-25% protein replacement) reduced lipid digestibility. In accordance with these results, Belghit et al. [21] reported reduced lipid digestibility with the replacement of 85% of protein by BSF meal (60%

inclusion) in diets for salmon. Chitin can be responsible for this as they are suggested to decrease bile acid levels in the pylorus, which is essential for activation of lipase and efficient lipid absorption [137]. The SFA-rich full-fat BSF larvae (65% of total FA) can additionally increase the dietary SFA level. At higher levels, increasing dietary SFA can linearly decrease lipid digestibility as shown previously in salmonids [154]. The lower lipid digestibility in **Paper I** was accompanied by lower lipid retention/accretion at higher BSF meal inclusion levels. The most abundant FA in BSF, lauric acid (40% of total FA) is a good source of energy for salmonids, thus, fish use this FA largely for energy purposes and less for deposition in tissues [22, 155] and subsequently reduce lipid retention.

In contrast to **Paper I**, meals and fractions of BSF did not adversely affect lipid digestibility and retention in salmon in **Paper III**. The differences in BSF batches and the inclusion levels of BSF in the diet may explain these contrasting results. During the de-chitinization process, there was an increase of the relative lipid content of BSF meal (44%), making de-chitinized meal diet the one with the highest level of BSF lipids (**Paper III**). The BSF lipid rich-de-chitinized meal diet gave a higher lipid retention and LER, although de-chitinized meal diet contained the highest proportion of lauric acid. This indicates an occurrence of lipid deposition rather than oxidation, which is in contrast to the above explanation, but this is most likely due to the lower lipid content in this diet. The de-chitinized meal enriched *Corynebacterium* in gut microbiota of fish in **Paper IV**, which has been reported to produce lipase [247], indicating complementing effects of gut microbiota in lipid digestibility. Hence, the lower total lipid content with higher proportion of BSF lipids in this diet might influence enrichment of *Corynebacterium* although this should still be confirmed. On the other hand, the full-fat meal diet in **Paper III** gave lower lipid retention/accretion compared to other BSF meals and fractions diets, suggesting an increased utilization of dietary lipid for energy requirements. Higher amount of dietary protein is, thus, available for tissue synthesis due to protein sparing effect [156, 157] and consequently improve the fish growth [248].

Interplay between black soldier fly larvae in salmon diets, salmon health and gut microbiota

The gut is the first organ exposed to the diet and has a pivotal importance in feed utilization and growth. A healthy gut efficiently convert feed into fish biomass; thus, the impact of full-fat BSF larvae on gut health was assessed in **Paper II**. The two control diets containing fishmeal and plant protein in **Paper II** negatively affected gut health showing mild to moderate enterocyte steatosis in the pyloric caeca and mild inflammatory changes in the distal intestine, mainly shortening of the mucosal fold height due to the loss of enterocyte supranuclear vacuolization. The high inclusion of plant ingredients might lead to these observed changes as shown previously in pyloric caeca [249] and distal intestine [250, 251] of salmon. The soy protein concentrate that we used in **Paper II** was processed by water-extraction, thus, antinutritional factors such as saponin was only partly removed. Soy saponins are known to induce enteritis in the distal intestine salmon [252-254]. It is also possible that the inflammatory events in the gut of control fish were partially caused by the gut microbiota. Even though the gut microbiota of fish were not assessed in **Paper II**, the results of **Paper IV** showed that the BSF-free control diet containing fishmeal and plant meals, which had a similar formulation as the control diet in **Paper I**, increased the abundance of Gram-negative *Proteobacteria*. At higher abundances, *Proteobacteria* can facilitate inflammation in the gut [255]. **Paper IV** also showed that the gut microbiota in fish fed the control diet had a predicted enrichment of lipopolysaccharide biosynthesis compared to full-fat meal fed fish. Lipopolysaccharides are produced and presented on cell surface by most Gram-negative bacteria [256, 257]. Fish immune system recognizes bacterial lipopolysaccharides as pathogen-associated molecules, and can trigger an immune response in fish [258]. Hence, it is possible that the observed inflammatory changes in the intestine of fish fed control diets in **Paper II** can partially be caused by a similar modulation of gut microbiota and their metabolic reactions as in the control diet in **Paper IV**.

Paper II showed that the inclusion of full-fat meal and paste could improve gut health by preventing the development of enterocyte steatosis and inflammatory changes in the distal intestine in a dose-dependent manner. It should also be noted that the

reason for the observed improvements in gut health could be related to lower levels of plant ingredients in BSF diets in **Paper II**, in particular the saponin-containing soy protein concentrate compared to the control diet. On the other hand, in accordance with our hypothesis, these improvements in the gut might also be caused by the bioactive compounds in full-fat BSF that have beneficial health effects in fish. The replacement of dietary protein up to 12.5% with full-fat BSF reduced enterocyte steatosis in the pyloric caeca (**Paper II**). The presence of choline [82, 168] and SFA in BSF (65% of total FA) might prevent excessive lipid accumulation in the pyloric caeca as previously shown in salmon [169, 170] and Arctic char [167], respectively. Furthermore, full-fat BSF meal and paste in **Paper II** also prevented inflammatory changes in the distal intestine, except for the lowest BSF inclusion of 3.7%, which seems insufficient to mitigate inflammation in the intestine. Furthermore, replacing up to 12.5% protein with full-fat BSF did not increase pro-inflammatory cytokines IL-1β or IFNγ levels in the distal intestine. The full-fat BSF meal and paste did also not increase IgM and IgD levels in the distal intestine, indicating full-fat BSF is devoid of substances that can act as antigens to increase immunoglobulin levels in the distal intestine. Even though BSF contain compounds with immunostimulant properties in fish such as chitin and AMP [25, 26, 28], it seems like they may not be in sufficient amounts in the diet (at low to moderate BSF levels) to induce an immune response in the gut or may act as inducers of anti-inflammatory responses. Others also reported that the dietary inclusion of BSF could prevent the occurrence of inflammatory signs of soybean meal-induced intestinal enteritis in the distal intestine histology and supported by down-regulated expression of inflammatory markers/pro-inflammatory cytokine genes in rainbow trout, suggesting a specific anti-inflammatory role of BSF [32, 193]. The medium-chain lauric acid in the lipid fraction of full-fat BSF larvae (40% of total FA) might also contribute to the absence of intestine inflammation in **Paper II**. Medium-chain FA and triglycerides have also been suggested to improve gut health under inflammatory conditions in pigs [166]. In a previous study, the total replacement of fish or soybean oil with BSF oil did not induce any histopathological changes in the intestine of rainbow trout [32]. Hence, the lipid fraction of BSF can be beneficial for gut health in salmonid and may not be necessary to separate when including in salmon diets.

Another potential explanation for the positive effect of full-fat BSF on gut health may be related to its ability to modulate gut microbiota. The modulatory effects of full-fat BSF on gut microbiota were not investigated in **Paper II**. Nevertheless, the enriched LAB and predicted increase in microbial mucin degradation in gut microbiota of full-fat meal group in **Paper IV** may explain the beneficial effects of full-fat BSF observed in **Paper II**. The lactic acid produced by LAB can repair or prevent intestinal damage in fish, as shown previously with fish fed soybean meal [259]. On the other hand, the mucin-degrading bacteria play an important role in modulating gut inflammatory response at the mucosal surface [260] and host immune responses [261], and some may possess anti-inflammatory properties as shown for human microbes by Hansen et al. [262].

Accompanying the lower fish growth (**Paper I**), the higher inclusion of full-fat BSF meal, replacing 25% dietary protein showed a relatively poor gut health (**Paper II**). This diet gave mild to moderate enterocyte steatosis in the pyloric caeca. It is possible that the increased level of chitin might be causative factors for enterocyte steatosis, because BSF could previously reduce the prevalence of enterocyte steatosis in salmon even at 60% inclusion when chitin level in the meal was reduced [31]. Similarly, at 15% protein replacement level, de-chitinized meal in **Paper III** also reduced the prevalence and severity of enterocyte steatosis in pyloric caeca (Appendix-1 Fig. S1). Despite the absence of histological alterations in distal intestine, 25% full-fat meal diet increased the pro-inflammatory cytokine IFNγ level in distal intestine in **Paper II**. Thus, potential histological inflammatory changes can be expected in prolonged feeding with diets containing higher level of full-fat BSF. Furthermore, the BSF lipid rich-de-chitinized meal in **Paper III** also increased the expression of pro-inflammatory cytokine IL-1β in the distal intestine (Appendix-1 Fig. S2), meaning higher level of BSF lipid might trigger an inflammatory response in the gut, in addition to chitin.

Despite the major impacts on gut health, full-fat BSF had minor effects on systemic immune responses in salmon (**Paper II**). Antioxidant capacity is highly associated with fish health and immunity [263]. As shown in plasma FRAP in **Paper II**, feeding full-fat BSF meal did not compromise antioxidant capacity in fish, and BSF paste even improved antioxidant capacity. On the other hand, the two diets which gave higher

SGR compared to the control diet in **Paper III**, i.e., full-fat and de-chitinized meals diets also showed higher FRAP in plasma (Appendix-1 Fig. S3), indicating a positive role of plasma antioxidant defence on growth performance. This could be a result of BSF compounds with antioxidant properties, such as chitin and AMP [26, 29]. The results of **Paper II** further indicated that full-fat BSF meal or paste might not negatively affect liver and muscle health as indicated by plasma ALT, AST, CK, which are indicators of liver [200], muscle and heart tissue [201] damage. In general, feeding full-fat BSF meal and paste for seven weeks in **Paper II** had minor effects on the expression profile of proteins in the skin mucus and did not affect systemic innate immune responses in fish. In **Paper III**, feeding de-fatted and de-chitinized BSF meals (replacing 15% dietary protein) for eight weeks increased plasma lysozyme activity in salmon (Appendix-1 Fig. S4). The bioactive compounds in BSF, chitin and AMP, are known to possess immunomodulatory effects in fish [25, 26, 28, 166]. In addition, the immunomodulatory effects of the lipid fraction of BSF has recently been reported in rainbow trout by Kumar et al. [32]. According to the results of present thesis, the immunomodulatory effects of BSF seem to be dependent on the BSF processing method, dietary inclusion level and the feeding period. It is also possible that the immune system of fish might have a small window of activation that can be triggered by the dietary concentration of bioactive and other compounds in BSF. The minor effects of BSF meal and paste on the immune response and skin mucus proteome in **Paper II** indicate that the effect of full-fat BSF might be more local, as observed in the gut, than systemic. However, it is also possible that the skin mucus sampling method [264], lower mucus viscosity in freshwater fish [265] and the use of trypsinized peptides in the mass spectrometry analysis [266] could influence the observed minor effects of BSF on the proteome of salmon skin mucus.

Roles of black soldier fly larvae compounds in modulating salmon gut microbiota

In **Paper IV**, we investigated how the meals and fractions of BSF larvae influence the gut microbiota in salmon. The results showed that the gut microbiota composition of the fish fed BSF meals and fractions differ from the control fish. The diets that contained BSF chitin, i.e., BSF meals and exoskeleton diets (1.4% in full-fat meal, de-fatted meal, and exoskeleton diets and 0.5% in de-chitinized meal diet) enriched LAB

and *Actinomyces* compared to the control group, while this was not the case for chitin free BSF oil diet. Chitin has prebiotic properties [37, 38, 219]. Being chitinase producing bacteria, chitin can be a preferential growth substrate for LAB [219] and *Actinomyces* [267]. This selective promotion of the growth of certain chitinolytic bacteria by chitin is in agreement with previous observations in salmon [69] and Atlantic cod (*Gadus morhua* L.) [152]. Chitin itself has also shown antimicrobial and bacteriostatic activity against several pathogenic Gram-negative bacteria [153], which can explain the reduced abundance of *Gammaproteobacteria*, *Vibrionaceae* and *Photobacterium* in BSF groups in **Paper IV**.

The presence of chitin in full-fat meal diet can partially be the reason for the improved phylogenetic diversity in this group as shown previously [27]. This can be due to the fact that chitin increases the abundance of chitinolytic bacteria and a higher phylogenetic diversity exists within chitinolytic bacteria [267]. However, in the present study, other chitin-containing processed meals and exoskeleton diets did not increase the phylogenetic diversity in gut microbiota as full-fat meal, indicating other compounds in the full-fat meal apart from chitin may also be responsible for this.

Several BSF based diets in **Paper IV** enriched *Bacillaceae* (the family of *Bacillus*) and *Acinetobacter*. These two bacteria also produce chitinase [69, 268], but they were not enriched in all the chitin-containing diets and *Bacillaceae* was only enriched in diets that contained BSF lipids. This indicates the potential influence of other factors in BSF such as lauric acid, AMP, or other compounds. The FA composition of BSF, in particular, high medium-chain FA and lack of long-chain polyunsaturated omega-3 FA, can also be responsible for increased LAB as reported by Rimoldi et al. [244] and Huyben et al. [269], although the BSF oil diet did not enrich LAB in **Paper IV**. Medium-chain FA might be responsible for reducing the relative abundance of *Gammaproteobacteria* in fish fed BSF diets in **Paper IV** as also shown previously by Rimoldi et al. [244]. Furthermore, lauric acid has also shown antimicrobial activity against some *Staphylococcus* species [270], and this can be the reason for enrichment of *Staphylococcus* only in the exoskeleton diet which contained negligible amount of BSF lipids (0.8%) (**Paper IV**).

In a previous study, low omega-3 FA level in the diet elevated FA synthesis and AA metabolic pathways in the gut microbiota of salmon, suggesting that the gut

microbiota can compensate for a lack of omega-3 FA in the diet by increasing the abundance of FA producing bacteria [269]. Similarly, the lack of omega-3 FA in the lipid fraction of BSF larvae can be responsible for the observed predicted enrichment of FA synthesis and AA metabolic pathways in fish fed BSF lipid-rich de-chitinized meal and BSF oil diets in **Paper IV**. However, the other two diets that contained higher BSF lipids, i.e., full-fat and de-fatted meals diets, did not enrich these two metabolic pathways, thus, further research is needed to confirm this.

5.7.3 Black soldier fly as a protein source in salmonid feeds: A synthesis of previous research findings

The findings of studies investigated the effect of BSF on nutrient utilization and growth performance were systematically reviewed using a meta-analytic approach in **Paper V**. The present dataset consisted of studies with wide range of experimental conditions, and biological and dietary factors, that can influence the response of fish to the use of BSF in the diet. Based on our results, fish species, the protein source(s) replaced, and/or BSF inclusion level were partially responsible for the heterogeneity in growth performance of fish between studies. However, on average, SGR, FCR, feed intake and PER did not differ between the salmonids fed BSF diets and control diets. The use of BSF in salmonid diets is thus possible without compromising growth performance. Similar results were observed when the data only for salmon were included in the analysis, but salmon tended to increase FCR and salmon pre-smolts had even a higher FCR when fed with BSF diets.

In contrast to the results for growth performance and PER, on average, the dietary inclusion of BSF tended to decrease protein digestibility in salmonids (**Paper V**). Furthermore, feeding BSF, on average, decreased protein digestibility in salmon. However, the influencing factors for the reported results in literature which are related to BSF, fish experiments and method of digestibility estimation, were not considered in the present study and the comparison across the studies is thus complicated.

The processing methods of BSF used in **Papers I** and **III** were crucial points that directly affected the nutrient utilization and growth performance in salmon. On the contrary, the meta-analysis in **Paper V** showed that the processing method of BSF

(full-fat vs de-fatted) did not cause heterogeneity in growth performance of salmonids across the studies. Both full-fat and de-fatted BSF in the diet did not affect growth performances of salmonids. In agreement with this, Hua [271] also showed that full-fat and de-fatted BSF meals affected SGR of fish similarly when only the nutrient balanced diets were included in the dataset. However, these results should be interpreted with caution because the diets in the datasets contained BSF with varying degrees of de-fatting (partially or fully de-fatted) and processed by various drying methods/temperatures.

The inclusion level of insect meals can directly relate to the physiological response in fish [271]. The combined results in **Paper I** and **III**, as well as previous studies also showed dose-dependent responses of fish to increasing dietary BSF levels [83, 178]. In agreement with this, the dietary level of BSF could partially explain the heterogeneity of SGR and feed intake between the studies in **Paper V**. Furthermore, increasing dietary level of BSF also tended to increase FCR linearly in salmon.

The present results showed that the protein source(s) replaced had a significant impact on fish responses to the use of BSF in diets **(Paper V)**. The replacement of fishmeal by BSF negatively affected fish growth, whereas replacement of non-fishmeal positively affected fish growth. The growth reduction in salmonids when fishmeal was replaced by BSF can be due to decreased feed intake, while the tendency to increase in feed intake might be responsible for improved fish growth when replacing non-fishmeal sources. This points out the importance of the type of protein source(s) replaced by BSF when evaluating the nutritional values of BSF in salmonids. Supporting the results of **Paper I and III** in which salmon performance was dependent on protein replacement level, a dose-dependent responses between fishmeal replacement level and growth performances in salmonids were observed in **Paper V**. The present dataset consisted of AA balanced experimental diets except one study that did not report dietary AA profiles or information regarding supplementation of AA [93]. This indicates that the actual differences between the nutritional values of the different protein sources might cause differential responses of fish to dietary BSF, rather than an artifact of inconsistencies in the dietary AA profiles. For instance, BSF might limit in certain digestible AA compared to fishmeal and depress growth performance of salmonids when replacing fishmeal. This further

illustrates the importance of determining the digestible nutrient contents in the control diet and the BSF ingredients when evaluating nutritional value of BSF. The studies used in the present meta-analysis did not report any consideration of digestible AA in diet formulations. Beyond the digestible nutrient content, fishmeal may also contain nutritional components that are often overlooked and enhance growth, such as taurine and low molecular weight compounds [272-274]. The reported taurine content in BSF larvae (0.02%, dry matter basis) [275] was lower than that in fishmeal (0.9%, dry matter basis) [276], which might result in lower growth in comparison with fishmeal. The diets containing non-fishmeal protein sources might lack of these dietary components, thus replacing such protein sources by BSF may cause better growth performance [277].

5.8 Identified gaps for future study

The present results revealed that the use of cost-effective and resource-efficient low-processed full-fat BSF in salmon feeds is promising. Especially at moderate inclusion levels, full-fat BSF larvae meal in the diet even improved growth and gut microbiota in salmon than the processed BSF meals by separation of lipid or exoskeleton fractions (**Paper III** and **IV**). The full-fat BSF larvae meal and paste in the diet could also improve the gut health of salmon (**Paper II**). The use of full-fat BSF meal/paste in extruded fish feed is, however, limited due to higher lipid and moisture contents, even though commercial extruders with pre-conditioners can tolerate comparatively higher inclusion levels. Full-fat BSF paste can be a cheaper alternative. When produced locally, it can be an alternative to the dried meal, which requires a costly drying process and higher energy inputs. High-temperature processing can reduce the nutritional quality of protein and chemical conformation of immunomodulatory components, such as AMP. Thus, the paste processed using low temperatures can have a higher nutritional value and functional properties. In the present thesis, the maximum allowable protein replacement level for BSF paste to prevent poor technical pellet quality was 6.7%, and it is possible that fish might perform better when feeding higher inclusion levels of paste compared to the meal. Thus, future research into a modification of extruder parameters to increase the maximum inclusion level of lipid/moisture-rich BSF ingredients will facilitate the use of less-

processed BSF in commercial feed production. Nevertheless, a paste can still bring challenges with transportation and storage, in addition to its limitations in using in extrusion processing. Therefore, it is important to conduct a cost-benefit analysis of the use of BSF paste in fish feeds.

In **Paper I** and **II**, due to the practical implications, the number of dietary inclusion levels of each BSF products were not equal (4 levels for BSF meal diets and 3 levels for BSF paste diets). Further, the protein replacement levels in BSF meal and paste diets were different (6.25, 12.5 and 25% in BSF meal diets and 3.7 and 6.7% in BSF paste diets). Hence, our design was not suitable for a nested ANOVA which could have been beneficial to determine the main and interaction effects of the processing method and protein replacement level of BSF. In this case, we used one-way ANOVA, as this would be more powerful. Using one-way ANOVA and a post-hoc test, we could compare BSF meal and paste separately, but we did not compare BSF meal diets with paste diets as this cannot provide meaningful interpretations at unequal inclusion levels. If possible, it is advisable to consider this in future studies investigating the dose-dependent effects of different BSF ingredients.

The present thesis showed that the combined effect of all the three fractions in BSF; protein, lipid, and exoskeleton fractions, can be more effective than the individual effect of each fraction at 15% protein replacement in salmon diets (**Paper III**). This is, however, still needed to be tested at other inclusion/protein replacement levels. The roles of specific bioactive compounds in these fractions such as AMP, lauric acid and chitin are also yet to be verified. According to our results, it seems that the activation of fish immune system by bioactive compounds in BSF depends on their dietary concentration. Hence, it is important to determine the optimum inclusion levels and future studies should investigate the effect of graded levels of various fractions as well as extracted and purified bioactive components of BSF on growth performance, nutrient utilization, gut microbiota, gut health, and immune responses in fish. In addition, studies with more extended feeding periods or challenges tests merit further investigation to confirm the significance of immune responses and disease resistance *in vitro* as well as *in vivo* with fish fed BSF, their fractions or bioactive components. The effect of feed processing and digestion on AMP extracted

and purified from BSF is also worthy of attention, because the AMP must remain stable during these processes to cause beneficial effects in fish.

The present results showed that BSF meals and fractions could shape the metabolic activity of salmon gut microbiota, but these results are functional predictions. The use of genome databases for such predictions can be less reliable due to their bias towards human-related microbiota [278]. Therefore, predictive metabolic profiles should be interpreted with caution and determination of real functional profile of gut microbiota using other techniques such as metaproteomics or metabolomics would be preferred.

The dataset used in the present meta-analysis in **Paper V** covered a wide range of experimental conditions. The presence of a number of variable factors from study to study can cause controversial interpretation of effect sizes obtained in a meta-analysis [279]. Many of the variations that exist across studies were considered in the present meta-analysis of growth performance data. However, the present meta-analyses and meta-regressions also revealed that there was an unexplained heterogeneity still existed between the studies even after considering these influencing factors. This unexplained heterogeneity might be caused by the rearing substrates of BSF, nutrient composition of BSF, level of anti-nutritive as well as bioactive compounds in BSF, degree of de-fatting of BSF, drying method of BSF and temperatures, other processing methods such as hydrolysation, level of protein replaced by BSF, variations in diet formulations, rearing conditions and culture systems used in studies. On the other hand, none of the factors that can influence protein digestibility and PER were not considered in the present analysis due to insufficient data, although such factors might largely influence the fish response to dietary BSF. Therefore, this topic should be addressed again when more research has been carried out to elucidate various factors influencing the degree of success of use of BSF in fish diets, draw concrete conclusions and make recommendations.

5.9 Conclusions

The present thesis revealed that differently processed BSF larvae can partially replace fishmeal and plant proteins in Atlantic salmon pre-smolts diets without compromising the growth performance, but the response of salmon to BSF in the diet varied with the level of protein replaced by the BSF and the type of processing method. In **Paper I**, the replacement of low to moderate protein levels with full-fat BSF larvae meal (6.25-12.5%) and paste (3.7%-6.7%) did not compromise the growth performance. In **Paper III**, replacing 15% de-fatted larvae meal had no impact, but full-fat and de-chitinized meals increased SGR of fish. The full-fat BSF larvae meal also gave the highest SGR and feed intake among processed meals and fractions in **Paper II**. This was accompanied by phylogenetically diverse and unique gut microbial composition, dominated by beneficial bacteria such as LAB and *Actinomyces* (**Paper IV**). Hence, the combined effect of all the three fractions in BSF; protein, lipid, and exoskeleton fractions, seems to be more effective than the individual effect of each fraction at 15% protein replacement. Our results further showed that the inclusion of full-fat BSF larvae affects gut health in a dose-dependent manner, in particular moderate levels of full-fat meal and paste could mitigate the development of enterocyte steatosis in pyloric caeca and inflammatory responses in the distal intestine and thereby improved gut health (**Paper II**). These results appear to indicate that it may not be necessary to separate lipid or exoskeleton fractions from BSF, which would only be an additional cost when included in the diets of salmon. However, high levels of lipid/moisture-rich full-fat and de-chitinized BSF meals and paste in diets can be a challenge due to their high lipid and moisture contents that can interfere with the extrusion processing hence reducing the technical pellet quality (**Paper I** and **III**). At 25% protein replacement, full-fat BSF larvae meal compromised fish growth rate, accompanied by reduced nutrient utilization (**Paper I**), mild to moderate enterocyte steatosis in pyloric caeca and increased pro-inflammatory cytokine IFNγ in distal intestine (**Paper II**). The meta-analysis in **Paper V** showed that, on average, dietary inclusion of BSF in either full-fat or de-fatted forms did not compromise the protein utilization and growth performance in salmonids. When only the data for salmon were considered in analysis, fish decreased protein digestibility and tended to increase FCR. Further meta-analysis of sub datasets sorted according

to the protein source(s) replaced showed that replacement of fishmeal by BSF decreased SGR and feed intake in salmonids, but the replacement of non-fishmeal sources improved SGR and FCR. This stresses the importance of the protein source(s) used in the control diet when evaluating the nutritional value of BSF in salmonids.

6 References

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7 Appendices

7.1 Appendix -1: Supplementary figures

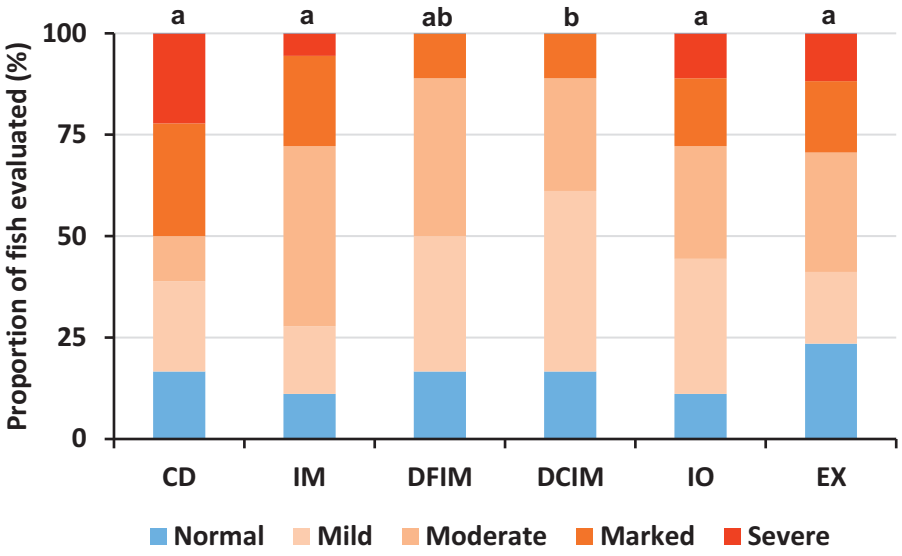

Fig. S1. Histological evaluation of pyloric caeca of fish fed experimental diets. (A) Proportion of pyloric caeca tissue sections that were scored "normal", "mild", moderate", "marked" or "severe" for enterocyte steatosis. Different superscript letters denote significant differences (p<0.05). CD: Control diet, IM: Full-fat black soldier fly (BSF) larvae meal diet, DFIM: De-fatted BSF larvae meal diet, DCIM: De-chitinized BSF larvae meal diet, IO: BSF larvae oil diet, EX: BSF larvae exoskeleton diet.

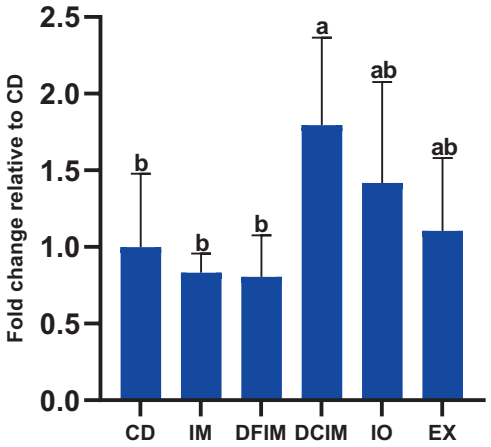

Fig. S2. Detection of IL-1β levels in distal intestine of fish fed experimental diets. Error bars indicate standard deviation. Error bars that are labelled with different superscript letters are significantly different (p<0.05). CD: Control diet, IM: Full-fat black soldier fly (BSF) larvae meal diet, DFIM: De-fatted BSF larvae meal diet, DCIM: De-chitinized BSF larvae meal diet, IO: BSF larvae oil diet, EX: BSF larvae exoskeleton diet.

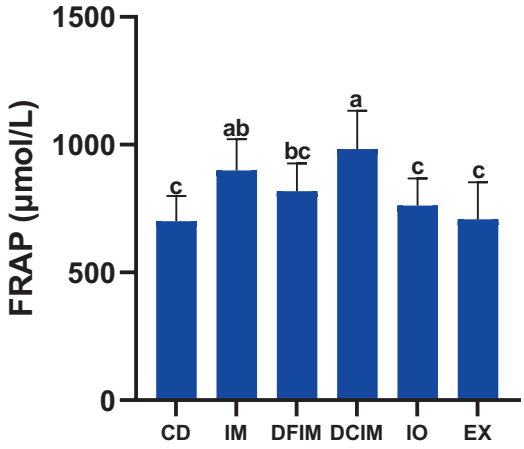

Fig. S3. Plasma ferric reducing antioxidant power (FRAP) (mmol/L) of fish fed experimental diets. Error bars indicate standard deviation. Error bars that are labelled with different superscript letters are significantly different (p<0.05). CD: Control diet, IM: Full-fat black soldier fly (BSF) larvae meal diet, DFIM: De-fatted BSF

larvae meal diet, DCIM: De-chitinized BSF larvae meal diet, IO: BSF larvae oil diet, EX: BSF larvae exoskeleton diet.

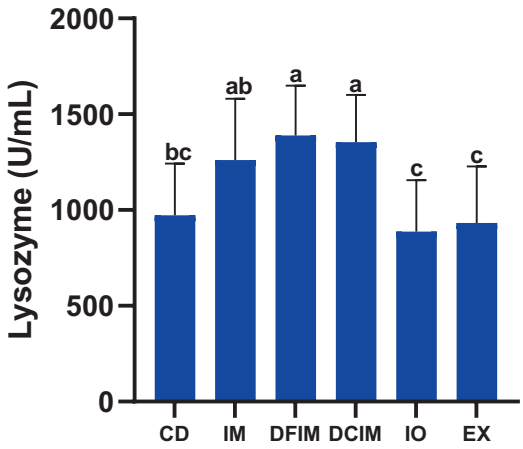

Fig. S4. Plasma lysozyme (U/mL) level of fish fed experimental diets. Error bars indicate standard deviation. Error bars that are labelled with different superscript letters are significantly different (p<0.05). CD: CD: Control diet, IM: Full-fat black soldier fly (BSF) larvae meal diet, DFIM: De-fatted BSF larvae meal diet, DCIM: De-chitinized BSF larvae meal diet, IO: BSF larvae oil diet, EX: BSF larvae exoskeleton diet.

7.2 Appendix -2: Papers I – V

Paper I

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Full-fat black soldier fly larvae (*Hermetia illucens*) meal and paste in extruded diets for Atlantic salmon (*Salmo salar*): Effect on physical pellet quality, nutrient digestibility, nutrient utilization and growth performances

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Nutrient utilization
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ABSTRACT

The present study investigated the effect of graded levels of black soldier fly larvae (BSFL) (*Hermetia illucens*) meal and paste on physical pellet quality, digestibility and utilization of nutrients and growth performances in extruded diets for Atlantic salmon (*Salmo salar*). A total of 1260 Atlantic salmon with 34 g of mean initial weight were randomly distributed into 21 fiberglass tanks and fed with one of seven isonitrogenous, isolipidic and isoenergetic diets for seven weeks. The experimental diets consisted of a control diet based on fishmeal, soy protein concentrate, corn gluten, faba bean and fish oil (Control-1); three diets with increased levels of full-fat BSFL meal, substituting 6.25% (6.25IM), 12.5% (12.5IM) and 25% (25IM) of the protein content of Control-1; two diets with increased levels of full-fat BSFL paste, substituting 3.7% (3.7IP) and 6.7% (6.7IP) of protein from Control-1 and an extra control diet with 0.88% of formic acid (Control-2). Pellet durability and hardness were overall high for all diets. However, the expansion, sinking velocity and water stability of feed pellets were lower with increased inclusion of BSFL meal and paste. Dietary inclusion of BSFL meal or paste did not affect the feed intake of fish. Further, replacing the protein content of the control diet with up to 12.5% and 6.7% of BSFL meal and paste, respectively, did not compromise fish growth rate or feed conversion ratio, although polynomial contrast analysis showed that increasing BSFL meal level in the diet linearly ($p < .05$) decreased these parameters. However, apparent digestibility coefficient (ADC) of protein and lipid, protein efficiency ratio and lipid retention were reduced linearly ($p < .05$) with increasing inclusion level of BSFL meal. Further, increasing dietary levels of BSFL paste linearly ($p < .05$) reduced ADC of protein, protein efficiency ratio and phosphorous retention. Despite the decreased ADC of protein, protein retention was not compromised by the inclusion of BSFL meal or paste. Replacement of 25% of dietary protein with BSFL meal decreased ($p < .05$) growth rate, accompanied by lower ($p < .05$) ADC and utilization of lipids and protein efficiency ratio. The present study showed that BSFL meal and paste could replace up to 12.5% and 6.7% of dietary protein, respectively, without compromising growth performance in Atlantic salmon.

1. Introduction

In recent years, insects have received growing attention as a sustainable ingredient for aquafeed production (Henry et al., 2015; Makkar et al., 2014; Nesic and Zagon, 2019) although the production of insects in sufficient volumes to compete with fishmeal and plant protein sources is yet to be achieved (Sogari et al., 2019). The production of insects has environmental benefits such as lower greenhouse gas and ammonia emissions (Oonincx et al., 2010), high land use efficiency (Alexander et al., 2017) and efficient nutrient conversion (Oonincx et al., 2010; van Huis, 2013). The feed conversion ratio (FCR) of insects fed food by-products ranged from 1.4 to 19.1 and nitrogen (N) conversion efficiency ranged from 22 to 87% depending on the insect species and growth media (Oonincx et al., 2015). The use of processed

Abbreviations: FCR, feed conversion ratio; N, nitrogen; BSFL, black soldier fly larvae; AA, amino acids; Ca, calcium; DM, dry matter; SPC, soy protein concentrate; ADC, apparent digestibility coefficient; WHC, water holding capacity; FA, fatty acids; Y, yttrium; Mg, magnesium; K, potassium; Na, sodium; P, phosphorous; SME, specific mechanical energy; SGR, specific growth rate; FBW, final body weight; PER, protein efficiency ratio; LER, lipid efficiency ratio; SFA, saturated fatty acids

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insects in feed for aquaculture animals was recently allowed by the European Commission (Regulation 2017/893/EC, 2017), which promotes upscaling of this novel feed ingredient.

One of the most promising insect species for feed purposes is black soldier fly larvae (BSFL) (*Hermetia illucens*) (van Huis, 2013). BSFL efficiently convert low-grade organic matter to high-quality nutrients (Diener et al., 2009). As shown by Oonincx et al. (2015), the N conversion efficiency can reach up to 43–55% in black soldier fly fed diets composed of food by-products. BSFL contain a moderate level of protein (31–59%) and has an amino acid (AA) profile closer to fishmeal and superior to soybean meal (Barroso et al., 2014; Makkar et al., 2014; Nogales-Mérida et al., 2019). In addition, BSFL is a good source of lipid (11–49%) (Makkar et al., 2014; Nogales-Mérida et al., 2019) and minerals, particularly calcium (Ca) (Finke, 2013; Makkar et al., 2014).

BSFL have successfully been used as a protein and lipid source in diets for Atlantic salmon (*Salmo salar*) reared in freshwater and seawater. Dietary inclusion of dried, defatted and chitin-reduced BSFL meal (60%) combined with BSFL oil (4.8%) (Belghit et al., 2018) and dried BSFL meal (10–20%) (Fisher et al., 2020) resulted in similar growth performance when compared with fishmeal and other protein sources in Atlantic salmon pre-smolts. Similar findings have also been shown in Atlantic salmon post-smolts when feeding partially defatted, dried BSFL meal (5–15%) (Belghit et al., 2019b; Lock et al., 2016). Hence, previous research demonstrated that dried or partially defatted BSFL meal, has potential as an alternative protein source in salmon feeds.

When considering commercial production, the processing of insect meal, particularly defatting is an additional cost. Thus, the use of whole BSFL meal is more cost-efficient. The use of full-fat insect meals can be a challenge to the feed industry due to its high lipid content that can interfere with the extrusion process (Lin et al., 1997), hence reducing the pellet quality (Sørensen et al., 2009). Thus, processing of BSFL biomass into partially defatted protein-rich meal has become a common practice, which allow high inclusion levels of insect meal in fish diets without reducing the technical quality of extruded diets (Dumas et al., 2018). Several studies have used full-fat dried BSFL meal in pelleted diets for rainbow trout (Sealey et al., 2011) and yellow catfish (Xiao et al., 2018), and extruded diets for rainbow trout (Józefiak et al., 2019b) and Siberian sturgeon (Józefiak et al., 2019a). However, none of them reported information on feed processing conditions or the impact of full-fat BSFL meal on extruder parameters and physical pellet quality.

High-temperature processing can reduce the nutritional quality of protein feed resources (Ljøkjel et al., 2000; Opstvedt et al., 1984; Opstvedt et al., 2003). Thus, processing BSFL at low temperatures to produce a paste, maintaining the nutritional value and reducing the production cost, could be beneficial for the aquaculture industry. As reported by Xu et al. (2020) feeding diets containing undried BSFL pulp (4.4–17.5%) to mirror carp did not affect growth rate and FCR. To our knowledge, there is no literature available regarding the use of undried full-fat BSFL ingredients in salmon diets.

Therefore, the present study used two types of BSFL; full-fat dried BSFL meal and BSFL paste (ground frozen BSFL preserved with formic acid) and investigated their effect on nutrient digestibility, nutrient utilization and growth performances when added in graded levels in extruded diets for Atlantic salmon pre-smolts. In addition, the effect of increasing levels of BSFL meal or paste on extruder parameters and physical pellet quality was also investigated.

2. Materials and methods

2.1. Experimental diets

BSFL meal and BSFL paste were produced at HiProMine S.A., Poznań, Poland. The BSFL feed was normalized in terms of dry matter (DM) content by the addition of wheat middlings (17%) to fresh vegetables and fruit mix, consisting of apples (15%), carrots (50%), potatoes (15%), and cabbage (20%) and established at the level of 22% DM. Fresh vegetable and fruit pre-consumer waste was ground (2000 rpm/1 min, (HPM milling system, 55 kw, Poland) to pass 2 mm screen and offered *ad libitum* to BSFL. Substrates were not contaminated by any animal products in accordance with EC regulation (no 1069/09).

Table 1
Analyzed chemical composition (%, dry matter) of black soldier fly larvae (BSFL) meal and paste.

Nutrient	BSFL meal	BSFL paste
Dry matter (%)	90.5	23
Crude protein	42	40.5[a]
Crude lipid	32	34.2[b]
Ash	9.25	
Formic acid	0	2.47
Total phosphorous	0.86	
Calcium	1.92	
Magnesium	0.27	
Chitin	8	
Amino acids[c]		
Essential amino acids		
Methionine	0.63	0.57
Threonine	1.34	1.26
Valine	1.9	1.62
Isoleucine	1.48	1.37
Leucine	2.9	2.17
Phenylalanine	1.48	1.43
Histidine	0.88	1.11
Lysine	2.12	2.28
Arginine	1.65	1.42
Tryptophan	0.77	0.12
Non-essential amino acids		
Cysteine	0.25	0.26
Aspartic acid	3.18	2.9
Serine	1.23	1.29
Glutamic acid	4.25	4.12
Proline	1.92	1.97
Glycine	1.53	1.53
Alanine	2.45	2.04
Tyrosine	1.84	2.77
Total amino acids	31.8	30.2

[a] Corresponds to 9.32% in BSFL paste.
[b] Corresponds to 7.87% in BSFL paste.
[c] Water corrected values.

At the prepupal stage (10th day of rearing), larvae were harvested, sieved through 3 mm screen and washed with water on drum separator at 90 °C for 10 min (HPM cleaning system, Poland). A batch of BSFL was divided into two parts and frozen at −50 °C to produce BSFL paste or dried for meal. The BSFL were dried first at 130 °C for 1 h, and then at 80 °C for 23 h until a constant weight was reached, using a chamber air flow dryer (HiProMine S.A., Poznań, Poland) to produce BSFL meal. In the case of BSFL paste, BSFL were ground to pass 4 mm screen on continue flow homogenizer (HPM milling system, 25 kw, Poland) and preserved with formic acid (2.5%). The analyzed chemical composition of BSFL meal and paste is shown in Table 1.

Seven isonitrogenous, isolipidic and isoenergetic diets were formulated. The diets were formulated to meet or exceed NRC (2011) requirements for all indispensable AA and other nutrients for Atlantic salmon. The experimental diets consisted of a control diet based on fishmeal, soy protein concentrate (SPC), corn gluten, faba bean and fish oil (Control-1); three diets with increasing levels of full-fat BSFL meal, substituting 6.25% (6.25IM), 12.5% (12.5IM) and 25% (25IM) of the protein content of Control-1. In addition, two diets with increasing levels of full-fat BSFL paste, substituting 3.7% (3.7IP) and 6.7% (6.7IP) of the protein content Control-1 and an extra control with 0.88% of formic acid (Control-2) were evaluated. The diet Control-2 was included as a control for BSFL paste diets, since the BSFL paste was preserved with formic acid. Yttrium oxide was included in all the diets as an internal

Table 2
Ingredient and analyzed chemical composition of experimental diets with increased inclusion level of black soldier fly larvae (BSFL) meal and paste.[1]

	Control-1	6.25IM	12.5IM	25IM	Control-2	3.7IP	6.7IP
Ingredients (%)							
Fish meal[a]	25	23.24	21.48	17.69	25	20.27	16.62
SPC[b]	35.5	33.45	30.92	25.58	35.5	29.18	23.92
Corn gluten[c]	4	3.72	3.44	2.59	4	3.24	2.66
Faba bean[d]	1.85	1.72	1.59	1.03	1.85	1.5	1.23
BSFL meal[e]	0	8.07	16.13	32.27	0	0	0
BSFL paste[f]	0	0	0	0	0	19.8	35.12
Wheat flour[g]	13.64	13.64	13.64	13.64	13.64	11.91	10.56
Wheat bran[h]	4	2.47	1.28	0	3.12	2.16	0.98
Fish oil[i]	15	12.68	10.51	6.19	15	11.06	8.13
Formic acid[j]	0	0	0	0	0.88	0	0
Yttrium oxide[k]	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Vit/min premix[l]	0.65	0.65	0.65	0.65	0.65	0.57	0.5
Methionine[m]	0.2	0.2	0.2	0.2	0.2	0.17	0.15
Choline chloride[n]	0.15	0.15	0.15	0.15	0.15	0.13	0.12
Chemical composition (%, as is)							
Dry matter	92.9	92.5	91.5	90.8	91.1	91.9	91.9
Crude protein	46.8	47.4	46.4	45.7	45.8	47.2	47.9
Crude lipid	14.6	15.5	17.2	15.9	16.2	13	13.3
Starch	12.3	12.4	11.5	11.5	11.6	12	12.8
Ash	5.52	5.88	6.17	6.83	5.3	5.67	6.08
Formic acid	0	0	0	0	0.72	0.58	1.1
Gross energy (MJ kg^{-1})	21.9	21.7	21.7	21.5	21.6	21.4	21.1
Macro mineral composition (%, as is)							
Total phosphorous	0.91	0.89	0.87	0.88	0.91	0.9	0.89
Calcium	0.93	1.02	1.1	1.21	1.01	1.06	1.05
Magnesium	0.1	0.13	0.12	0.15	0.11	0.11	0.12
Potassium	0.52	0.62	0.66	0.66	0.46	0.58	0.61
Sodium	0.46	0.45	0.48	0.39	0.46	0.44	0.4
Amino acid composition[o] (%, as is)							
Essential amino acids							
Methionine	0.96	0.97	0.94	0.91	0.92	0.96	0.83
Threonine	1.54	1.56	1.53	1.5	1.52	1.55	1.52
Valine	1.58	1.61	1.61	1.62	1.57	1.63	1.67
Isoleucine	1.69	1.72	1.69	1.66	1.67	1.75	1.78
Leucine	3.19	3.14	3.07	2.92	3.12	3.19	3.2
Phenylalanine	1.9	1.89	1.85	1.81	1.91	1.93	1.93
Histidine	1.02	1.05	1.04	1.03	1	1.06	1.08
Lysine	2.48	2.46	2.43	2.36	2.4	2.5	2.58
Arginine	2.54	2.51	2.43	2.22	2.64	2.6	2.54
Non-essential amino acids							
Cysteine	0.44	0.44	0.43	0.39	0.43	0.45	0.46
Aspartic acid	3.98	3.97	3.84	3.71	3.85	3.99	4.12
Serine	1.77	1.78	1.72	1.66	1.76	1.79	1.82
Glutamic acid	7.45	7.34	7.11	6.6	7.2	7.42	7.49
Proline	2.15	2.08	1.96	2.06	2.07	2.16	2.19
Glycine	1.74	1.75	1.73	1.72	1.74	1.79	1.79
Alanine	1.89	1.94	1.97	2.04	1.86	1.94	1.99
Tyrosine	1.26	1.36	1.45	1.79	1.29	1.38	1.44
Total amino acids	37.58	37.57	36.8	36	36.95	38.09	38.43

[1] Control-1: Control diet. 6.25IM, 12.5IM and 25IM: BSFL meal substituted 6.25%, 12.5% and 25% of protein content of Control-1. Control-2: Control diet with 0.88% of formic acid. 3.7IP and 6.7IP: BSFL paste substituted 3.7% and 6.7% of protein content of Control-1.

[a] LT fishmeal, Norsildmel AS, Bergen, Norway

[b] Soy protein concentrate AX3®, Triple A AS, Hornsyld, Denmark.

[c] Corn gluten meal, Baolingbao Biology, Shangdong Yucheng, China.

[d] Faba beans, Norgesfôr, Oslo, Norway.

[e] Black soldier fly larvae meal, HiProMine S.A., Poznań, Poland.

[f] Black soldier fly larvae paste, HiProMine S.A., Poznań, Poland.

[g] Wheat flour 78%, batch number: 5093060546, Norgesmøllene, Bergen, Norway.

[h] Wheat bran, Norgesmøllene, Bergen, Norway.

[i] Fish oil, Norsildmel AS, Bergen, Norway.

[j] Formic acid, Ensil Maursyre 85%, Felleskjøpet, Norway.

[k] Yttrium oxide (Y_2O_3), Metal Rare Earth Limited, Shenzhen, China.

[l] Vit/min premix, Farmix, Trouw Nutrition, LA Putten, The Netherlands. Per kg of feed; retinol 2500.0 IU, cholecalciferol 32,400.0 IU, α-tocopherol SD 0.2 IU, menadione 40.000 mg, thiamine 15.0 mg, riboflavin 25.0 mg, d-Ca-pantothenate 40.002 mg, niacin 150.003 mg, biotin 3000.0 mg, cyanocobalamin 20.0 mg, folic acid 5.0 mg, pyridoxine 15.0 mg, ascorbate polyphosphate 0.098 g, Cu: Cu sulphate 5H2O 11.998 mg, Zn: Zn sulphate 89.992 mg, Mn: Mn(II) sulphate 34.993 mg, I: K-iodine 1.999 mg, Se: Na-selenite 0.200 mg, Cd Max. 0.0003 mg, Pd max. 0.028 mg, Ca 0.915 g, K 1.380 g, Na 0.001 g, Cl 1.252 g.

[m] L-methionine, Bestamino™ Cj Cheiljedang, Seoul, Korea.

[n] Choline chloride 70%, $C_5H_{14}ClNO$, 139.6 g/mol, Vilomix, Hønefoss, Norway.

[o] Water corrected values.

marker for the determination of apparent digestibility coefficient (ADC) of nutrients. Crystalline methionine was added to all the diets to ensure that the diets met or exceeded the methionine requirement of Atlantic salmon pre-smolts (0.7%, DM basis (NRC, 2011)). The ingredient and analyzed chemical composition of the experimental diets are shown in Table 2.

2.2. Production of experimental diets

The experimental diets were produced at the Norwegian University of Life Sciences (NMBU) Centre for Feed Technology (Fôrtek), Ås, Norway. The BSFL paste was ground frozen using a meat grinder (Tripas-Wexiö, RK-82, Sweden). After thawing overnight at room temperature, water was added to the paste (1 kg of water per 6 kg of paste) and ground using a pump grinder (Pedrollo TR 1.1, San Bonifacio (VR), Italy). The macro ingredients including SPC, fishmeal, corn gluten meal, wheat bran, faba beans, wheat flour, and BSFL meal were weighed and mixed with an ISDECA mixer (60-l paddle-mixer, prototype, Fôrtek, Forberg, Norway) for 2.5 min. The material mix was then ground in a small Hammer mill (Bill bliss, horizontal, 18.5 kW, USA) with a 1 mm sieve. The ground macro ingredients mixture was mixed with the micro-ingredients. BSFL paste was also added using the ISDECA mixer when producing the 3.7IP and 6.7IP diets. Formic acid was added to the Control-2 diet using the ISDECA mixer equipped with spray nozzles (nozzle type: 11004, Spraying Systems Co., Norway).

The diets were extruded in a five-section Bühler twin-screw extruder (BCTG 62/20 D, Uzwil, Switzerland) with reduced capacity, bypassing the conditioner, fitted with four 2.5 mm die holes. A small K-tron feeder was used to feed the material directly into the first extruder section. A screw configuration suitable for reduced extruder capacity was used for all the diets (Fig. 1). The screw speed was increased when the BSFL meal or paste content was increased in the diet. The pellets were dried at 60 °C using fan heaters (15KW, Inelco heaters, Dania-heater 15 kW, Fjerritslev, Denmark) for 1 h and cooled at room temperature. The dried uncoated pellets were sieved (1.6 mm screen) and the percentage of dust/broken pieces was calculated. Dried extruded pellets were vacuum coated with fish oil in Gentle Vacuum Coater (GVC) – 80 prototype (Fôrtek, Amandus-Kahl).

2.3. Fish experiment and rearing facilities

The fish experiment was conducted at the Center for Fish Research, NMBU, Ås, Norway. The experimental procedures were performed in accordance with the national guidelines for the care and use of animals (The Norwegian Animal Welfare Act and the Norwegian Regulation and Animal Experimentation). A total of 1260 Atlantic salmon (Aqua Gen Atlantic QLT-innOva SHIELD) with 34 g of mean initial weight were distributed into 21 fiberglass tanks (300 l capacity) with 60 fish per tank. The fish were kept under continuous light in recirculated freshwater with a water supply of 8.5 l min^{-1}. The average water temperature was 14.8 °C during the experimental period. Dissolved oxygen levels were kept above 7.0 mg l^{-1} in the outlet water. Triplicate tanks of salmon were fed one of seven experimental diets over a period of seven weeks. Fish were fed *ad libitum* (i.e. 10% excess) with electrically driven belt feeders twice a day for 2.5 h. Daily feed intake in each tank was quantified according to Helland et al. (1996), by collection of uneaten feed using wedge wire screens as explained by Shomorin et al. (2019). Fish weight was measured at the start and end of seven-week experimental period. Fifteen fish at the start of experiment and five fish from each tank at the end of the seven-week experimental period were randomly sampled and euthanized by a sharp blow to the head and stored at −20 °C. All sampled fish at the start of experiment and all sampled fish per tank at the end of the seven-week experimental period were pooled, homogenized and freeze dried prior to analysis of the chemical composition. After the seven-week experimental period, fish were fed the experimental diets for another two weeks for fecal collection. Fish were carefully stripped three times with seven days interval (i.e. 0, 7 and 14 days after whole body sampling) for fecal collection from the posterior intestine according to Austreng (1978). The feces were immediately weighed and stored at −20 °C prior to freeze drying. Prior to weighing, sampling and stripping, fish were anesthetized with tricaine methanesulfonate (MS-222) (80 mg l^{-1}) in small aerated tanks.

2.4. Physical pellet quality analysis

Physical quality parameters of oil-coated pellets were measured. Water stability of pellets during 30, 60 and 120 min were measured according to Baeverfjord et al. (2006). The durability of the pellets was estimated in triplicates using a Doris pellet tester (AKVAsmart, Bryne, Norway) according to Hansen et al. (2010). Hardness was measured using 15 randomly picked pellets from each diet with a Texture analyzer equipped with a 5 kg load cell (Tinius Olsen, H5KT, Salfords, England) according to Øverland et al. (2009). The width of 30 randomly selected pellets per diet was recorded using the Texture analyzer (Tinius Olsen, H5KT, Salfords, England) to determine expansion. The expansion (%) was calculated as ((Pellet width-die diameter) × die $diameter^{-1}$) × 100. The sinking velocity was determined by measuring the mean value of the time required for 10 randomly picked pellets to sink 1 m in 17 °C tap water. In addition, the water holding capacity (WHC) was measured according to Nguyen et al. (2015). The degree of starch gelatinization of feed was analyzed by Nofima AS, Ås, Norway, according to Kraugerud and Svihus (2011).

2.5. Chemical analysis

The feed and freeze-dried feces and fish were ground. DM content was measured by oven drying at 104 °C until a constant weight was reached. Ash content was determined by combustion at 550 °C. The N content of feed, feces and fish was analyzed by CHNS Elemental Analyzer (Vario El Cube elemental analyzer system GmbH, Hanau, Germany) and crude protein content was determined as N × 6.25. The crude protein content of BSFL meal and paste was estimated by Kjeldahl N × 6.25 according to Commission Regulation (EC) No 152/ 2009. Samples were extracted with petroleum ether and acetone (70/30) and crude lipid content was determined using an Accelerated Solvent Extractor (ASE200; Dionex Corp., Sunnyvale, CA, USA). The starch content was determined as described by McCleary et al. (1994) with some modifications. Briefly, the samples were treated with heat-stable α-amylase and amyl glucosidase-enzymes to degrade starch into glucose and glucose content was measured by a spectrometer (RX4041 Randox Daytona+, England). Gross energy was measured with PARR 1281 Adiabatic Bomb calorimeter (Parr Instruments, Moline, IL, USA) according to ISO 9831. AA except tryptophan contents were analyzed

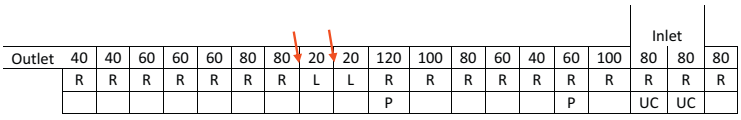

Fig. 1. The screw configuration used during the extrusion of experimental diets. 20; 40; 60; 80; 100; 120: Length in cm of each screw element. R: Right. L: Left (Flow direction of each screw element). P: Polygon. UC: Undercut conveying screw element (larger channel depth than the other conveying screw elements). The arrows indicate 5 mm spacer ring and 90^0 offset between the screw elements.

according to Commission Regulation (EC) No 152/2009 on a Biochrom 30 AA Analyzer (Biochrom Ltd., Cambridge, UK). Tryptophan content was analyzed using a Dionex Ultimate 3000 HPLC system (Dionex Softron GmbH, Germering, Germany) equipped with a Shimadzu RF-535 fluorescence detector (Shimadzu Corporation, Kyoto, Japan) according to Commission Regulation (EC) No 152/2009. The fatty acid (FA) content was determined using Trace GC Ultra gas chromatograph (Thermo Fisher Scientific, US) according to O'fallon et al. (2007) by synthesizing the FA to FA methyl esters (FAME). Yttrium (Y), Ca, magnesium (Mg), potassium (K) and sodium (Na) contents were measured using a microwave plasma atomic emission spectrometer (MP-AES 4200, Agilent Technologies, USA) after acid decomposition in a microwave digestion system (Start D, Milestone Srl, Italy). Total phosphorous (P) content was analyzed using a commercial spectrophotometric kit (PH8328, Randox laboratories, County Antrim, UK) after combustion and acid digestion according to Commission Regulation (EC) No 152/2009. The chitin content of BSFL meal was measured according to Finke (2007). The formic acid content in BSFL paste, Control-2 diet and diets containing BSFL paste was determined using HPLC-UV at Eurofins Agro Testing Norway AS.

2.6. Calculations

Specific mechanical energy (SME) (Wh kg^{-1}) was calculated as $(2 \times \pi \times 60^{-1}) \times (S_{rpm} \times T_{knm} \times T_{t/h}{}^{-1})$, where S_{rpm} is screw speed, T_{knm} is Torque and $T_{t/h}{}^{-1}$ is throughput. Specific growth rate (SGR) (% body weight day^{-1}) was calculated as [(ln (Final body weight (FBW) (g $fish^{-1}$)) – ln (Initial body weight (g $fish^{-1}$)))/ Experimental period (days)] × 100%. Feed intake (g DM $fish^{-1}$) was calculated as Total feed intake (g DM $tank^{-1}$)/Number of fish per tank. FCR (g g^{-1}) was calculated as Feed intake (g DM $fish^{-1}$)/ (FBW (g $fish^{-1}$) - Initial body weight (g $fish^{-1}$)). ADC of nutrients (%) was calculated as (1- [(Y concentration in diet/Y concentration in feces) × (Nutrient concentration in feces/Nutrient concentration in diet)]) × 100. Fecal excretion of minerals and N (%) was calculated as (100 – ADC of minerals or N). The dissolved N fraction (g kg^{-1} fish body weight gain) was calculated as (([Feed intake (g $fish^{-1}$) × N content in feed (%)/100] × ADC of N/100) – ([(FBW (g) × Final N content in fish (g g^{-1})) – (Initial body weight (g) × Initial N content in fish (g g^{-1}))])/ ([FBW (g $fish^{-1}$) - Initial body weight (g $fish^{-1}$)]/1000). Protein and lipid efficiency ratios (g g^{-1}) were calculated as (FBW (g $fish^{-1}$) - Initial body weight (g $fish^{-1}$))/ [Feed intake (g $fish^{-1}$) × Protein or lipid content in feed (%)/100]. Apparent nutrient retention (% intake) was calculated as [(FBW (g) × Final nutrient content in fish (g g^{-1})) – (Initial body weight (g) × Initial nutrient content in fish (g g^{-1}))]/ [Feed intake (g $fish^{-1}$) × Nutrient content in feed (%)/100] × 100.

2.7. Statistical analysis

The data were analyzed using one-way ANOVA, followed by a Tukey's test for comparison of means. Differences at $p < .05$ were considered as significant. Linear and quadratic polynomial contrasts were used to evaluate the relationship between different parameters and dietary BSFL meal or BSFL paste levels as indicated in tables. The chosen level of significance was $p < .05$ and threshold level of tendency was $p < .1$. All the statistical analyses were performed using IBM SPSS Statistics 26 software.

3. Results

3.1. Feed production and pellet quality

The extruder parameters used during the production of experimental diets are shown in Table 3. The full-fat BSFL meal increased the lipid content and the BSFL paste increased the moisture content of the feed mash prior to extrusion. To compensate this, the screw speed of the extruder was increased to obtain pellet with desirable expansion and physical quality. Despite the increased screw speed, decreased die pressure and torque were observed, which resulted in a decreased SME. In addition to increased screw speed, the water added to the BSFL paste diets in the extruder was reduced.

Inclusion of BSFL meal and paste in the diets numerically increased the amount of fines before coating. Pellet durability and hardness were overall high for all diets, but with a numerical reduction in durability for the diet with 12.5% BSFL meal replacement (Table 4). The expansion, sinking velocity and water stability of pellets after 30, 60 and 120 min were numerically lower with increased inclusion of both BSFL meal and paste (Table 4 and Fig. 2). Starch gelatinization varied among the experimental diets, ranging from 54.4 to 95.2%, but with high analytical variation within the treatments.

3.2. Fish performance

Only one fish died throughout the experimental period. Fish fed the BSFL meal diets had similar feed intake compared to Control-1. According to the linear polynomial contrasts, FBW and SGR reduced ($p < .05$) with increasing dietary BSFL meal level. According to ANOVA, there were no differences in FBW and SGR between the fish fed 6.25IM and 12.5IM diets and the fish fed Control-1 diet, while FBW and SGR were lower ($p < .05$) in fish fed 25IM diet than fish fed Control-1 diet. FCR of fish fed the BSFL meal diets were not different from the fish fed Control-1, though it was lower ($p < .05$) in 12.5IM than 25IM diet. In addition, a linear relationship ($p < .05$) was observed between FCR and BSFL meal level in the diet (Table 5).

In contrast, 3.7IP and 6.7IP diets did not affect the growth performances of fish compared to Control-1 or Control-2. A positive linear tendency ($p = .08$) was, however, observed between dietary BSFL paste level and FCR of fish. Growth performances did not differ between the two controls (Table 5).

3.3. Digestibility of nutrients, fecal excretion of minerals and dissolved fraction of N

Although ADC of protein and energy in BSFL meal diets did not differ from Control-1, ADC of protein decreased linearly ($p < .05$) with increasing dietary BSFL meal level. ADC of lipid was lower ($p < .01$) for the 12.5IM and 25IM diets compared to the Control-1, whereas ADC of starch was highest ($p < .01$) for the 25IM diet. Furthermore, a negative liner relationship ($p < .001$) was found between dietary BSFL meal level and ADC of lipid, and a positive linear relationship ($p < .01$) was found in ADC of starch (Table 6).

ADC of AA, except tyrosine in the BSFL meal diets did not differ from the Control-1. Tyrosine digestibility was lower ($p < .01$) in 12.5IM and 25IM diets when compared with the Control-1. It was also observed linear reductions ($p < .05$) of the ADC of phenylalanine, histidine, lysine and tyrosine with increasing level of BSFL meal in the diet. The same trend was observed for ADC of arginine ($p = .05$) and cysteine ($p = .07$). However, the ADC of total AA was unaltered by the dietary inclusion of BSFL meal (Table 7).

The ADCs of nutrients in salmon fed the BSFL paste diets did not differ from fish fed the Control-1 or the Control-2 diets. However, negative linear effects ($p < .05$) were observed between dietary BSFL paste level and ADCs of protein and energy (Table 6).

Fecal excretion of P and Mg were not affected by dietary inclusion of BSFL meal. Fecal Ca and K excretions were lower ($p < .05$) in 25IM diet and 12.5IM diet respectively, compared with Control-1. Further, linear relationships ($p < .05$) were observed between fecal Ca and K excretions and dietary BSFL meal level (Table 6).

Fecal P excretion increased linearly ($p < .001$) with increasing level of BSFL paste in the diet and BSFL paste diets showed higher ($p < .01$) P excretion than Control-2. Linear ($p < .05$) and quadratic ($p < .05$) relationships were observed between fecal Ca and Mg

Table 3
Extruder parameters during the production of experimental diets with increased inclusion level of black soldier fly larvae (BSFL) meal and paste.[1]

Extruder parameter	Control-1	6.25IM	12.5IM	25IM	Control-2	3.7IP	6.7IP
Throughput (kg h^{-1})	54.3	54.3	54.3	54.7	54.3	46.8	43.0
Barrel 1 (°C)	38.9	31.4	29.9	34.3	40.3	35.0	34.2
Barrel 2[a] (°C)	61.2	47.1	47.9	68.9	63.6	62.3	56.5
Barrel 3[a] (°C)	108	96.6	92.5	96.1	97.2	112	109
Barrel 4[a] (°C)	117	118	115	104	112	114	112
Barrel 5[b] (°C)	75.1	64.2	58.7	53.8	79.9	84.7	85.2
Die temperature (°C)	96.5	92.5	90.5	78.5	92.0	92.5	94.0
Die pressure (Bar)	29.5	20.2	16.2	1.35	22.8	6.80	3.95
Screw speed (rpm)	270	290	345	475	275	400	400
Torque (Nm)	339	270	226	103	277	180	156
Drive power (kW)	9.30	8.25	8.10	5.10	7.75	7.50	6.50
SME[c] (Wh kg^{-1})	177	151	150	93.6	147	161	152
Water addition[d] (kg h^{-1})	14.0	14.0	14.0	15.5	14.0	6.50	0
Lipid[e] (%)	2.67	5.04	7.64	12.6	2.67	4.32	6.14

[1] Control-1: Control diet. 6.25IM, 12.5IM and 25IM: BSFL meal substituted 6.25%, 12.5% and 25% of protein content of Control-1. Control-2: Control with 0.88% of formic acid. 3.7IP and 6.7IP: BSFL paste substituted 3.7% and 6.7% of protein content of Control-1.
[a] Heating around these sections of the extruder barrel.
[b] Cooling around this section of the extruder barrel.
[c] Specific mechanical energy.
[d] Water added into the extruder.
[e] Percentage of lipid in the feed mash prior to extrusion, on dry matter basis.

Table 4
Physical pellet quality of experimental diets with increased inclusion level of black soldier fly larvae (BSFL) meal and paste.[1]

Pellet quality parameter	Control-1	6.25IM	12.5IM	25IM	Control-2	3.7IP	6.7IP	SEM[2]
Fines before coating (%)	0.25	1.31	4.08	1.93	0.25	0.62	0.73	
Durability (%)	99.1	98.9	96.3	98.8	99.6	99.0	98.6	0.28
Hardness (N)	35.5	37.0	35.5	31.3	46.4	32.7	34.4	0.66
Expansion (%)	13.8	6.9	3.8	−9.0	12.2	3.6	−2.9	0.66
Sinking velocity (m S^{-1})	0.09	0.10	0.08	0.06	0.09	0.07	0.07	0.00
Water holding capacity (g wet/g dry)	2.59	2.48	2.53	2.36	2.58	2.10	1.84	0.06
Gelatinization (%)	72.7	95.2	75.8	84.2	83.0	54.4	67.9	3.75

[1] Control-1: Control diet. 6.25IM, 12.5IM and 25IM: BSFL meal substituted 6.25%, 12.5% and 25% of protein content of Control-1. Control-2: Control diet with 0.88% of formic acid. 3.7IP and 6.7IP: BSFL paste substituted 3.7% and 6.7% of protein content of Control-1.
[2] Standard error mean.

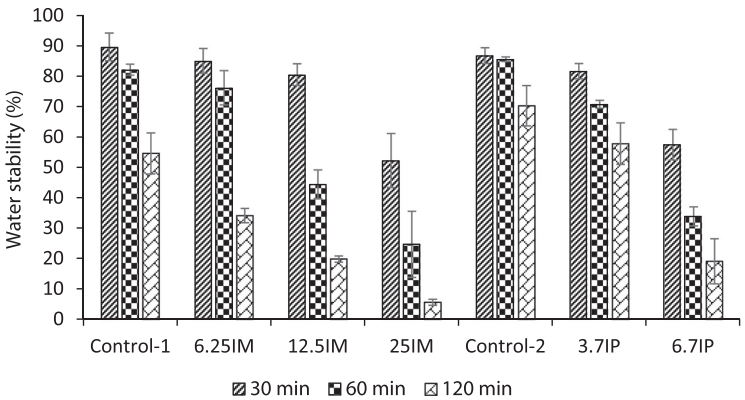

Fig. 2. Water stability (dry matter retention %) of pellets with increased inclusion of black soldier fly larvae (BSFL) meal and paste after 30, 60 and 120 min. Error bars indicate standard deviation. Control-1: Control diet. 6.25IM, 12.5IM and 25IM: BSFL meal substituted 6.25%, 12.5% and 25% of protein content of Control-1. Control-2: Control diet with 0.88% of formic acid. 3.7IP and 6.7IP: BSFL paste substituted 3.7% and 6.7% of protein content of Control-1.

excretions and dietary BSFL paste level. 6.7IP diet had higher ($p < .01$) Ca excretion compared to Control-2 (Table 6).

The dietary inclusion of BSFL meal or paste did not affect the dissolved N discharges, whereas fecal N excretion increased linearly ($p < .05$) when increasing the level of BSFL meal and paste in the diet (Table 6).

3.4. Nutrient utilization

The protein efficiency ratio (PER) linearly decreased ($p < .05$) with increasing BSFL meal level and 25IM diet showed a lower ($p < .05$) PER than Control-1, but dietary inclusion of BSFL meal did not affect the apparent protein retention in fish. Lipid efficiency ratio (LER) was lower ($p < .01$) in all BSFL meal diets compared to Control-1. Further, linear ($p < .001$) and quadratic ($p < .001$) relationships were

Table 5
Performance of fish fed experimental diets with increased inclusion level of black soldier fly larvae (BSFL) meal and paste.[1]

									Comparison 1 – BSFL meal diets[2]			Comparison 2 – BSFL paste diets[2]		
Performance indicator	Control-1	6.25IM	12.5IM	25IM	Control-2	3.7IP	6.7IP	SEM[3]	p_{ANOVA}[4]	p_{linear}[4]	p_{quad}[4]	p_{ANOVA}[5]	p_{linear}[5]	p_{quad}[5]
Initial body weight (g)	34.4	34.3	34.3	34.3	34.3	34.4	34.3	0.03	0.79	0.36	0.89	0.75	0.60	0.59
Final body weight (g)	94.4^{a}	92.4^{ab}	93.9^{a}	89.1^{b}	85.2	94.8	89.3	1.13	0.03	0.01	0.3	0.23	0.43	0.18
Specific growth rate (%)	2.25^{a}	2.20^{ab}	2.24^{ab}	2.12^{b}	2.02	2.25	2.12	0.03	0.04	0.014	0.33	0.28	0.47	0.22
Feed intake (g)	46.4	45.4	45.5	44.3	37.5	46.1	44.1	0.87	0.65	0.25	0.99	0.069	0.10	0.16
Feed conversion ratio	0.77^{ab}	0.78^{ab}	0.76^{b}	0.81^{a}	0.74	0.76	0.81	0.01	0.028	0.023	0.06	0.18	0.08	0.64

p_{ANOVA}: p value for one-way ANOVA. Values in the same row of Control-1, 6.25IM, 12.5IM and 25IM diets (Comparison 1) with different superscripts (a-b) are statistically different ($p < .05$) according to Tukey's multiple comparison test. p_{linear} and p_{quad} are the p values of linear and quadratic components of the polynomial contrast analysis between each performance indicator and BSFL meal/paste protein level in the diet: Control-1 was excluded in the polynomial contrast analysis of BSFL paste diets (Comparison 2).

[1] Control-1: Control diet. 6.25IM, 12.5IM and 25IM: BSFL meal substituted 6.25%, 12.5% and 25% of protein content of Control-1. Control-2: Control diet with 0.88% of formic acid. 3.7IP and 6.7IP: BSFL paste substituted 3.7% and 6.7% of protein content of Control-1.

[2] Two group comparisons were conducted: Comparison 1, between Control-1, 6.25IM, 12.5IM and 25IM diets; Comparison 2, between Control-1, Control-2, 3.7IP, and 6.7IP.

[3] Standard error mean.

[4] p values for Comparison 1.

[5] p values for Comparison 2.

observed between the LER and dietary BSFL meal level. Apparent lipid retention and apparent energy retention decreased linearly ($p < .01$) with increasing dietary BSFL meal level, where 25IM diet showed the lowest ($p < .05$) retentions (Table 8).

PER decreased linearly ($p < .05$) with increasing level of BSFL paste in the diet, and PER was lower ($p < .05$) in the 6.7IP diet than in the Control-2 diet. However, apparent protein retention was not affected by dietary inclusion of BSFL paste. The 3.7IP diet showed higher LER ($p < .05$) compared to the two controls and higher apparent lipid retention ($p < .01$) compared to Control-2. Linear ($p < .05$) and quadratic ($p < .01$) relationships were also observed between LER and apparent lipid retention and the level of BSFL paste in the diet. Further, apparent P retention decreased linearly ($p < .05$) when increasing dietary BSFL paste level (Table 8).

4. Discussion

4.1. Feed production and physical pellet quality

Adding the BSFL meal and paste to the diets led to an increased lipid and moisture content in the mash prior to extrusion, respectively. Lipids act as lubricants, therefore a high lipid level in extrusion increases the lubrication and reduces the friction in the extruder (De Pilli et al., 2015; Ilo et al., 2000; Lin et al., 1997), resulting in a decreased dough

Table 6
Apparent digestibility coefficient (ADC) of nutrients (%), fecal excretion of minerals and nitrogen (%) and dissolved fraction of nitrogen (g kg^{-1} fish body weight gain) of fish fed experimental diets with increased inclusion level of black soldier fly larvae (BSFL) meal and paste.[1]

									Comparison 1 – BSFL meal diets[2]			Comparison 2 – BSFL paste diets[2]		
	Control-1	6.25IM	12.5IM	25IM	Control-2	3.7IP	6.7IP	SEM[3]	p_{ANOVA}[4]	p_{linear}[4]	p_{quad}[4]	p_{ANOVA}[5]	p_{linear}[5]	p_{quad}[5]
Apparent digestibility coefficients														
Crude protein	87.2	86.9	84.7	83.3	89.6	88.9	86.7	0.55	0.12	0.027	0.92	0.33	0.03	0.36
Crude lipid	95.2^{a}	94.6^{a}	91.3^{b}	88.0^{c}	96.0	95.2	94.5	0.62	< 0.001	< 0.001	0.76	0.54	0.12	0.91
Starch	64.3^{b}	65.1^{b}	66.5^{b}	70.4^{a}	63.2	66.3	62.7	0.60	0.004	0.001	0.39	0.07	0.81	0.018
Energy	77.1	77.1	75.8	75.4	80.0	79.6	77.1	0.51	0.717	0.31	0.93	0.30	0.045	0.27
Fecal excretion of minerals and nitrogen														
Phosphorous	49.1^{X}	48.0	51.2	44.6	39.8^{Y}	44.6^{XY}	50.6^{X}	1.28	0.68	0.46	0.49	0.004	< 0.001	0.4
Calcium	105$^{a, X}$	98.1^{ab}	95.9^{ab}	92.7^{b}	80.8^{Y}	81.1^{Y}	99.3^{X}	2.06	0.017	0.004	0.17	< 0.001	0.004	0.025
Magnesium	53.7	43.4	52.9	47.5	46.2	43.1	53.2	1.24	0.11	0.47	0.66	0.08	0.038	0.016
Potassium	13.7^{a}	9.77^{ab}	8.80^{b}	8.82^{ab}	10.9	9.03	9.95	0.47	0.036	0.02	0.052	0.09	0.31	0.14
Nitrogen	12.8	13.1	15.3	16.7	10.4	11.1	13.3	0.55	0.12	0.027	0.92	0.33	0.03	0.36
Dissolved fractions														
Nitrogen	22.2	22.7	19.9	22.2	20.0	23.2	24.2	0.54	0.26	0.78	0.22	0.34	0.14	0.70

p_{ANOVA}: p value for one-way ANOVA. Values in the same row that share same superscripts are not statistically different ($p > .05$) according to Tukey's multiple comparison test. The letters a-c denote significant differences among Control-1, 6.25IM, 12.5IM and 25IM diets (Comparison 1), whereas the letters X-Y denote significant differences among Control-1, Control-2, 3.7IP, and 6.7IP diets (Comparison 2). p_{linear} and p_{quad} are the p values of linear and quadratic components of the polynomial contrast analysis between each parameter and BSFL meal/paste protein level in the diet: Control-1 was excluded in the polynomial contrast analysis of BSFL paste diets (Comparison 2).

[1] Control-1: Control diet. 6.25IM, 12.5IM and 25IM: BSFL meal substituted 6.25%, 12.5% and 25% of protein content of Control-1. Control-2: Control diet with 0.88% of formic acid. 3.7IP and 6.7IP: BSFL paste substituted 3.7% and 6.7% of protein content of Control-1.

[2] Two group comparisons were conducted: Comparison 1, between Control-1, 6.25IM, 12.5IM and 25IM diets; Comparison 2, between Control-1, Control-2, 3.7IP, and 6.7IP.

[3] Standard error mean.

[4] p values for Comparison 1.

[5] p values for Comparison 2.

Table 7
Apparent digestibility coefficient (ADC) of amino acids (%) of fish fed experimental diets with increased inclusion level of black soldier fly larvae (BSFL) meal.[1]

Amino acid	Control-1	6.25IM	12.5IM	25IM	SEM[2]	p_{ANOVA}[3]	p_{linear}[4]
Essential amino acids							
Methionine	93.0	92.8	92.0	91.6	0.34	0.46	0.15
Threonine	85.7	86.1	84.6	84.6	0.68	0.86	0.53
Valine	89.2	89.1	87.9	87.5	0.51	0.60	0.22
Isoleucine	90.5	90.4	89.2	88.8	0.49	0.56	0.21
Leucine	91.4	91.3	90.4	89.7	0.42	0.50	0.16
Phenylalanine	91.5	90.7	89.6	88.7	0.46	0.14	0.029
Histidine	88.2	87.9	86.3	84.7	0.63	0.19	0.042
Lysine	91.3	90.6	89.4	88.4	0.51	0.17	0.036
Arginine	95.0	94.8	94.1	93.3	0.30	0.22	0.05
Non-essential amino acids							
Cysteine	75.8	77.3	73.1	70.4	1.25	0.23	0.07
Aspartic acid	81.5	82.5	81.6	82.8	0.74	0.92	0.65
Serine	87.9	88.3	87.0	86.9	0.59	0.84	0.49
Glutamic acid	91.9	92.2	91.1	90.4	0.45	0.56	0.21
Proline	89.1	89.1	87.0	87.4	0.54	0.39	0.2
Glycine	83.9	84.1	82.2	82.4	0.65	0.69	0.36
Alanine	90.0	89.9	89.0	88.8	0.44	0.75	0.34
Tyrosine	87.4[a]	83.1[ab]	78.8[bc]	75.6[c]	1.44	0.001	< 0.001
Total amino acids	89.2	89.2	87.8	87.2	0.53	0.49	0.16

[1] Control-1: Control diet. 6.25IM, 12.5IM and 25IM: BSFL meal substituted 6.25%, 12.5% and 25% of protein content of Control-1.
[2] Standard error mean.
[3] p value for one-way ANOVA. Values in the same row with different superscripts are statistically different ($p < .05$) according to Tukey's multiple comparison test.
[4] p values of linear component of the polynomial contrast analysis between each ADC and BSFL meal protein level in the diet. Only the linear component of the polynomial contrasts is shown because all quadratic contrasts were not significant.

temperature (Hansen et al., 2011; Lin et al., 1997). Similarly, increased water content in the extruder can also act as a lubricant and decrease friction, leading to reduced dough temperature (Huang et al., 1995; Lin et al., 1997). Lower dough temperature can reduce starch gelatinization (Garber et al., 1997; Lin et al., 1997; Morken et al., 2012), which results in reduced expansion (Garber et al., 1997) and physical pellet quality (Morken et al., 2012). Hence, to reduce the adverse effect of high lipid and moisture contents in the mash during extrusion, the screw speed was increased with increasing BSFL meal and paste levels in the diet.

In general, a higher screw speed creates higher SME and leads to a higher dough temperature during extrusion (Morken et al., 2012; Rolfe et al., 2001). A decreased dough temperature with increased screw speed can also occur due to a decreased filling rate of the extruder (Huang et al., 1995) or decreased residence time of the dough in the extruder, resulting in a less efficient heat transfer between the extruder barrel and the dough (Della Valle et al., 1987; Huang et al., 1995; Lin et al., 1997). A similar effect might have occurred in the present study, as indicated by the reduced SME, die pressure and torque, because the resistance to screw rotation was proportional to the filling rate (Akdogan, 1996). In addition, the low barrel and die temperature, torque and SME in the high BSFL diets indicates that increased screw speed was not optimal in compensating for the increased lipid level in the present study. Further, modification of the screw configuration during extrusion of the high BSFL containing diets could have given better results, as this has large effect on the extrusion parameters (Gogoi et al., 1996).

Starch gelatinization was reported to be reduced at high lipid (Hansen et al., 2010; Hansen et al., 2011; Lin et al., 1997) and increased screw speed (Lin et al., 1997) due to low dough temperature and hydrophobic properties of high lipid in the extruder. However, no clear relationship was found between starch gelatinization and increased inclusion of dietary BSFL meal in the present study. The decreased starch gelatinization observed with increased inclusion of BSFL paste was probably due to reduced dough temperature from the higher moisture content in feed as shown by Lin et al. (1997) and this is associated with reduction of WHC (Artz et al., 1990).

The reduced pellet expansion with increased dietary BSFL meal in the present study is in line with previous reports indicating that a decreased extrudate expansion was due to high lipid content in mash (Hansen et al., 2011; Ilo et al., 2000; Navale et al., 2015). The decreased pellet expansion in high BSFL meal diets might also be related to lower barrel temperature, in particular the fifth barrel temperature (Bandyopadhyay and Rout, 2001; Kothakota et al., 2013; Pathania et al., 2013). The decreased expansion of BSFL paste diets might be associated with the higher screw speed and moisture content of the feed mash (Bandyopadhyay and Rout, 2001). This increased level of lipid content in the feed mash followed with reduced level of SME and pellet expansion is also, most probably, explaining the reduced pellet water stability in the present study as shown by Hansen et al. (2011).

Although Sørensen et al. (2009) showed that even a small increase in lipid content might adversely affect the physical quality of extruded diets, in the present study, pellet durability and hardness were not notably reduced with the inclusion of BSFL meal or paste in the diet. Similarly, BSFL and cricket meal did not affect the pellet durability in the extruded fish feed (Irungu et al., 2018). The presence of formic acid might also contribute to this in BSFL paste diets. As reported by the others, dietary supplementation of sodium diformate (Morken et al., 2011) and potassium diformate (Morken et al., 2012) increased durability/hardness of salmonid feed pellets.

4.2. Fish performance and nutrient digestibility and utilization

Dietary inclusion of both BSFL meal and paste did not affect the palatability of Atlantic salmon diets, as indicated by the similar feed intake among diets. Similar results were shown in Atlantic salmon pre-smolts (Belghit et al., 2018) and post-smolts (Belghit et al., 2019b) and rainbow trout (Dumas et al., 2018; Renna et al., 2017) fed BSFL meal diets.

As observed for both BSFL meal and paste in the present study, low dietary inclusion of BSFL meal, did not affect growth performance in Atlantic salmon pre-smolts (10–20%) (Fisher et al., 2020) or post-smolts (5–15%) (Belghit et al., 2019b) and rainbow trout (20%) (Józefiak et al., 2019b) compared to control diets based on fishmeal and other protein sources. Xu et al. (2020) also observed that dietary

Table 8
Nutrient retention parameters in fish fed experimental diets with increased inclusion level of black soldier fly larvae (BSFL) meal and paste.[1]

								Comparison 1 – BSFL meal diets[2]				Comparison 2 – BSFL paste diets[2]		
Nutrient retention parameter	Control-1	6.25IM	12.5IM	25IM	Control-2	3.7IP	6.7IP	SEM[3]	p_{ANOVA}[4]	p_{linear}[4]	p_{quad}[4]	p_{ANOVA}[5]	p_{linear}[5]	p_{quad}[5]
Protein efficiency ratio	$2.57^{a,XY}$	2.50^{ab}	2.58^{a}	2.46^{b}	2.70^{X}	2.56^{XY}	2.39^{Y}	0.03	0.02	0.031	0.28	0.03	0.016	0.75
Lipid efficiency ratio	$8.21^{a,\ YZ}$	7.65^{b}	6.98^{c}	7.07^{c}	7.62^{cZ}	9.28^{X}	8.57^{XY}	0.18	< 0.001	< 0.001	< 0.001	0.003	0.022	0.008
Apparent protein retention (%)	51.5	51.6	52.5	49.0	55.9	51.9	51.0	0.64	0.29	0.17	0.22	0.20	0.10	0.58
Apparent lipid retention (%)	$114^{a,X}$	103^{ab}	97.4^{ab}	93.3^{b}	103^{Y}	123^{X}	113^{XY}	2.45	0.03	0.007	0.16	0.003	0.031	0.005
Apparent energy retention (%)	56.4^{a}	56.1^{a}	56.1^{a}	51.5^{b}	57.7	55.8	55.3	0.58	0.02	0.006	0.13	0.65	0.31	0.78
Apparent phosphorous retention (%)	55.3	51.5	56.3	57.6	62.4	57.3	53.3	0.94	0.10	0.11	0.36	0.06	0.032	0.97

p_{ANOVA}: p value for one-way ANOVA. Values in the same row that share same superscripts are not statistically different ($p > .05$) according to Tukey's multiple comparison test. The letters a-c denote significant differences among Control-1, 6.25IM, 12.5IM and 25IM diets (Comparison 1), whereas the letters X-Z denote significant differences among Control-2, 3.7IP, and 6.7IP diets (Comparison 2). p_{linear} and p_{quad} are the p values of linear and quadratic components of the polynomial contrast analysis between each nutrient retention parameter and BSFL meal/paste protein level in the diet: Control-1 was excluded in the polynomial contrast analysis of BSFL paste diets (Comparison 2).

[1] Control-1: Control diet. 6.25IM, 12.5IM and 25IM: BSFL meal substituted 6.25%, 12.5% and 25% of protein content of Control-1. Control-2: Control diet with 0.88% of formic acid. 3.7IP and 6.7IP: BSFL paste substituted 3.7% and 6.7% of protein content of Control-1.

[2] Two group comparisons were conducted: Comparison 1, between Control-1, 6.25IM, 12.5IM and 25IM diets; Comparison 2, between Control-1, Control-2, 3.7IP, and 6.7IP.

[3] Standard error mean.

[4] p values for comparison 1.

[5] p values for Comparison 2.

fishmeal replaced with BSFL pulp (4.4–17.5%), which contained crushed fresh larvae, did not influence the SGR or FCR of juvenile mirror carp. Yet, the highest BSFL meal inclusion level which replaced 25% of dietary protein showed lower FBW and SGR in the present study. Similarly, FBW and/or SGR were also reported to be reduced in other studies where high levels of BSFL meal were included in the diets of Atlantic salmon pre-smolts (30%) (Fisher et al., 2020) and post-smolts (25%) (Lock et al., 2016) and rainbow trout (26.4%) (Dumas et al., 2018). In contrast, dietary inclusion of high levels of BSFL meal caused no adverse effect on growth performance in Atlantic salmon (60%) (Belghit et al., 2018) and rainbow trout (20–40%) (Renna et al., 2017).

The reduction of SGR in the present and previous studies may be attributed to the presence of chitin in the BSFL. Chitin is a major component of insect cuticle (Chapman, 1998; Tharanathan and Kittur, 2003). In the present study, whole BSFL meal including the cuticle was used. The chitin content in the BSFL meal (i.e. 8% in DM basis) corresponded to a chitin level of 0.6,1.2 and 2.3% for the meal diets and 0.4 and 0.6% for the paste diets. Previous studies reported reduced SGR in juvenile turbot fed BSFL meal containing chitin (1.6–7.3%) (Kroeckel et al., 2012), Atlantic salmon fed chitin from prawn shells (1–5%) (Karlsen et al., 2017) or krill meal containing chitin (2%) (Hansen et al., 2010) and reduced weight gain in tilapia fed chitin (2–10%) (Shiau and Yu, 1999). Furthermore, feeding high levels of chitin-reduced BSFL meal had no adverse effect on growth performance of Atlantic salmon (Belghit et al., 2018).

Chitin was reported to contain around 17.1 kJ/g of energy content, which could constitute a substantial percentage of total energy intake (Gutowska et al., 2004), but, Atlantic salmon have been reported to have a poor capacity to digest chitin (13–40%) (Olsen et al., 2006). This indicates that chitin function as a filler with low digestible energy content (Karlsen et al., 2017) which might limit growth rate at high inclusion levels. In addition, the reduction in growth rate could also be a result of the reduced ADC of nutrients. In accordance with the decreased ADC of protein with increasing dietary levels of BSFL meal and paste in the present study, dietary inclusion of high levels of BSFL meal adversely affected ADC of protein/AA in salmon pre-smolt (60%) (Belghit et al., 2018) and rainbow trout (40%) (Renna et al., 2017). On the contrary, some research showed that ADC of protein and most of the AA were not affected by dietary BSFL meal inclusion in Atlantic salmon post-smolts (5–25%) (Belghit et al., 2019b; Lock et al., 2016) and rainbow trout (20%) (Dumas et al., 2018). In agreement with present results for BSFL meal diets, Belghit et al. (2018) and Belghit et al. (2019a) reported reduced ADC of lipid and most fatty acids with the inclusion of BSFL meal and oil in diets for Atlantic salmon. The lower ADC of nutrients might also be attributed to chitin, because previous studies showed that feeding diets containing chitin reduced ADC of nutrients in Atlantic salmon (Hansen et al., 2010; Karlsen et al., 2017) and tilapia (Shiau and Yu, 1999). The chitin in insect cuticle exists in a matrix with proteins, lipids and other compounds (Chapman, 1998; Kramer et al., 1995), which may reduce the access of digestive enzymes, thus reducing ADCs of nutrients (Henry et al., 2015). In addition, chitin might further reduce ADC of protein due to its capacity to bind proteins (Piccolo et al., 2017) and immobilize (Muzzarelli, 1980) or reduce the activity of proteolytic enzymes such as the brush border enzyme, leucine aminopeptidase that break down peptides into AA (Belghit et al., 2018). It has also been suggested that feeding chitin leads to decreased bile acid levels in the pylorus, and thereby reduce ADC of lipid as bile acid is essential for activation of lipase and efficient lipid absorption (Hansen et al., 2010). In addition, the FA composition of BSFL meal is presented in the supplementary table (Table A.1) showing that the majority of the FA in BSFL meal were saturated fatty acids (SFA) (65% of total FA), which might increase the SFA content in BSFL diets. High SFA dietary concentrations may also partially explain the decrease in ADC of lipid in the present study, as the ADC of lipid decreases linearly with an increasing concentration of dietary SFA. This has previously been demonstrated in salmonids (Hua and Bureau,

2009).

Based on the present results, acid detergent fiber fraction in BSFL meal contained 12% of AA, which was bound to chitin, and probably not available for digestion. The observed reduction of ADCs of several AA in the present study might be because these AA were trapped in chitin that is concealed for enzymatic digestion. Furthermore, in the analysis of AA, it appeared that the peak for tyrosine in the HPLC chromatogram was overlapped with glucosamine, which is the building block of chitin (Ng et al., 2001). Therefore, the reduced ADC of tyrosine was most likely linked to an overestimation of the tyrosine content in the feces. However, the dietary inclusion of BSFL meal did not affect the ADC of total AA although the ADC of protein was reduced. Thus, the observed reduction of ADC of protein might partially be explained by the poorly digestible chitin.

Despite the decreased ADC of protein, apparent protein retention was not compromised by the inclusion of BSFL meal and paste, probably indicating an increased utilization of digested proteins in the fish fed the BSFL diets. The similar ADC of total AA and dissolved N level among the diets might partially explain this. In addition, this might also partially be due to the content of nucleic acid in BSFL, which may have an N-sparing effect in salmon. As shown by other protein sources such as bacterial meal, nucleic acids were suggested to have an N-sparing effect and increased N retention in salmon, although the nutrient digestibility was slightly lower (Øverland et al., 2010). In line with the present results, dietary replacement of fishmeal with BSFL meal did not affect protein retention in Atlantic salmon post-smolts (Belghit et al., 2019b; Lock et al., 2016), gilthead seabream (Karapanagiotidis et al., 2014) and yellow catfish (Xiao et al., 2018). However, dietary inclusion of BSFL meal negatively affected PER and replacement of 25% of dietary protein with BSFL meal reduced PER in the present study. In contrast, replacement of fishmeal and/or plant protein with BSFL meal did not affect the PER in Atlantic salmon pre-smolts (Belghit et al., 2018) and rainbow trout (Józefiak et al., 2019b; Renna et al., 2017). Further, Fisher et al. (2020) reported even an increased PER at 30% inclusion level in Atlantic salmon pre-smolts.

The apparent lipid retention values above 100% observed in several treatments of the present study indicated lipid synthesis outweighed lipid catabolism. Both LER and apparent lipid retention were negatively affected by the dietary inclusion of BSFL meal and the effect was worse with increasing level of BSFL meal in the diet. In agreement with this, lipid retention decreased at dietary inclusion of 33% BSFL meal and higher in juvenile turbot (Kroeckel et al., 2012). In addition, two studies showed that dietary BSFL meal negatively affected the whole-body lipid composition in rainbow trout (St-Hilaire et al., 2007) and juvenile turbot (Kroeckel et al., 2012). The observed lower lipid utilization in salmon fed diets containing BSFL meal was accompanied by low ADC of lipid in these diets and can be attributed to the presence of chitin as discussed above. In addition, the most abundant SFA in BSFL was medium-chain lauric acid (40% of the total FA). Lauric acid is considered to be a good source of energy for salmonids as it seems to be oxidized to a larger extent and used less for lipid deposition, resulting in low tissue deposition (Belghit et al., 2019a; Renna et al., 2017) and subsequently reduce lipid retention and LER. The increased energy production by lauric acid might also explain the observed comparable protein retention of BSFL diets despite reduced ADC of protein due to a protein-sparing effect (Karalazos et al., 2011). Teo et al. (1989) also reported the potential protein-sparing effect of medium-chain triglycerides. In agreement with this, previous studies have also shown that dietary inclusion of medium-chain triglycerides improved N/protein retention in Atlantic salmon (Nordrum et al., 2000; Nordrum et al., 2003). In addition, protein synthesis is a highly energy requiring process (Nordrum et al., 2000) and the high energy contribution by lauric acid might, therefore, have a positive effect on protein retention. Nevertheless, the chitin and BSFL oil content in BSFL paste diets did not seem to be sufficient to cause a negative impact on ADC of lipid. However, in contrast to BSFL meal, it was observed that 3.7% replacement of dietary protein with BSFL paste improved both LER and apparent lipid retention. This might be due to improved utilization of digested nutrients when the BSFL were subjected to low temperature processing and preserved with formic acid or included in the diet at lower levels.

According to the results of present and previous studies (Finke, 2013; Fisher et al., 2020), BSFL meal is more abundant in micronutrients (P, Ca, Mg, K) and BSFL have a mineralized cuticle in which Ca is incorporated into the cuticle (Finke, 2013). In general P content in BSFL meal is lower than fishmeal (Liland et al., 2017), which was reflected by a slight reduction of P level in BSFL meal diets. But the P in insects is likely to be readily available, unlike plant-based phytate P (Finke, 2002). This might be the reason for unaltered fecal P excretion and P retention of BSFL meal diets in the present study. Similarly, whole fish P content was not altered by BSFL meal diets in Atlantic salmon pre-smolts (Belghit et al., 2018). The observed fecal Ca excretion values closer to or above 100% in the present study is most likely due to Ca uptake by fish from water. The decreased fecal Ca excretion when increasing BSFL meal level in the diet indicated that higher dietary inclusion of BSFL meal improved ADC of Ca. It has been reported that the supplementation of diets with formic acid affects the intestinal pH of rainbow trout and improves the ADC of P, Ca and Mg (Vielma and Lall, 1997). However, an opposite result was observed for BSFL paste containing formic acid, where the increased dietary level of BSFL paste increased fecal excretion of P indicated decreased ADC of P and accompanied by decreased P retention. Similarly, increased fecal Ca excretion was observed with increasing inclusion level of BSFL paste in the diet, indicating decreased ADC of Ca. The increased fecal N excretion of BSFL meal and paste diets and increased fecal P excretion of BSFL paste diets indicate an increased environment impact of low processed insect products as alternative protein sources, although the fecal P excretion of BSFL meal diets and dissolved N fraction of the BSFL meal and paste diets were similar to the control diet. Future work on further processing such as defatting and dechitinization can help alleviate potential adverse environmental effects of such insect ingredients.

5. Conclusions

The present study showed that BSFL meal and paste could replace up to 12.5% and 6.7% of dietary fishmeal and plant proteins, respectively, without compromising the growth performance or protein retention in Atlantic salmon. Nevertheless, protein and lipid digestibility, protein efficiency ratio and lipid retention decreased linearly with increasing dietary BSFL meal level, whereas increasing dietary BSFL paste level linearly decreased protein digestibility, protein efficiency ratio and phosphorous retention. At higher replacement level of 25% BSFL meal, the growth rate was reduced, accompanied by a reduction in digestibility and utilization of lipids and protein efficiency ratio.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735785.

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Paper II

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Dietary Inclusion of Black Soldier Fly (*Hermetia Illucens*) Larvae Meal and Paste Improved Gut Health but Had Minor Effects on Skin Mucus Proteome and Immune Response in Atlantic Salmon (*Salmo Salar*)

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The present study investigated effects of dietary inclusion of black soldier fly larvae (BSFL) (*Hermetia illucens*) meal and paste on gut health, plasma biochemical parameters, immune response and skin mucus proteome in pre-smolt Atlantic salmon (*Salmo salar*). The seven-week experiment consisted of seven experimental diets: a control diet based on fishmeal and plant protein (Control-1); three BSFL meal diets, substituting 6.25% (6.25IM), 12.5% (12.5IM) and 25% (25IM) of protein; two BSFL paste diets, substituting 3.7% (3.7IP) and 6.7% (6.7IP) of protein and an extra control diet with 0.88% of formic acid (Control-2). The 6.25IM diet reduced enterocyte steatosis in pyloric caeca, improved distal intestine histology, and reduced IgM in distal intestine. The fish fed 12.5IM diet reduced enterocyte steatosis in pyloric caeca, improved distal intestine histology, had a higher plasma lysozyme content compared to 6.25IM, and tend to increase phagocytic activity in head-kidney macrophages-like cells. On the other hand, 25IM diet improved distal intestine histology, but showed mild-moderate enterocyte steatosis in pyloric caeca, increased IFNγ and reduced IgM in distal intestine. In the case of BSFL paste diets, 3.7IP diet caused mild inflammatory changes in distal intestine, although it reduced enterocyte steatosis in pyloric caeca. The 6.7IP diet reduced enterocyte steatosis in pyloric caeca and improved distal intestine histology. Increasing level of BSFL meal in the diet linearly decreased plasma C-reactive protein, whereas increasing level of BSFL paste linearly increased plasma antioxidant capacity. Dietary inclusion of BSFL meal and paste had minor effects on the expression profile of proteins in skin mucus and no effects on immune markers in splenocytes. BSFL meal showed no negative effect on liver and muscle health as indicated by plasma alanine aminotranseferase, asparate aminotransferase and creatine kinase. The present study showed that replacing conventional protein sources with low to moderate levels of BSFL meal (6.25% and 12.5%) or paste (3.7% and 6.7%)

reduced enterocyte steatosis in pyloric caeca, while replacing up to 25% with BSFL meal or 6.7% with paste improved distal intestine histology. Further, dietary inclusion of BSFL meal and paste had minor effects on skin mucus proteome and immune response in Atlantic salmon.

Keywords: black soldier fly larvae, meal, paste, Atlantic salmon, gut health, plasma biochemical parameters, immune response, skin mucus proteome

INTRODUCTION

Insects represent great potential as a sustainable alternative to conventional protein sources in aquafeeds (1–3). Black soldier fly larvae (BSFL) (*Hermetia illucens*) has attracted attention as one of the most promising insect species to be used in feeds (4). This is mainly due to its high nutritional value with 31-59% protein, 11–49% lipid (3, 5, 6) and its ability to valorize low-quality organic material (7) and ensure sustainable industrial-scale production (4).

The effect of BSFL as a replacer of conventional protein sources such as fishmeal and plant protein on growth performance has been studied in several aquaculture fish species, including salmonids. Previous studies reported that BSFL can partially replace dietary protein sources without adverse effects on salmonid growth performance (8–14). However, when introducing a novel protein source into fish feed, assessment of health effects beyond the nutritional value is important. BSFL are known to contain bioactive compounds such as chitin (15, 16) and antimicrobial peptides (AMP) (17, 18) which have antioxidant and immunostimulatory properties in fish (19–22). Furthermore, BSFL contain high amounts of medium-chain fatty acid, lauric acid (C12:0) (14, 23), which has antimicrobial effects against gram-positive bacteria (24, 25). Others have reported that dietary inclusion of BSFL meal increased the abundance of beneficial microorganisms that contribute to the health of the host such as lactic acid (26–28) and butyrate (27) producing bacteria in the gut of rainbow trout (*Oncorhynchus mykiss*). Several studies have evaluated the effect of BSFL on salmonid health. Dietary inclusion of defatted BSFL meal (60%) or partially defatted BSFL meal (15%) did not compromise gut health in pre-smolt (29) and post-smolt Atlantic salmon (*Salmo salar*) (30), respectively. Further, partially defatted BSFL meal in diets (20–40%) caused no adverse effects on the histology of liver, spleen and gut in rainbow trout (31). In addition, defatted BSFL meal did not cause negative effect on liver health in salmon as indicated by decreased or unaltered activities of plasma markers of liver damage such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (9, 32), and unaffected expression of genes related to stress response in the liver (32). On the contrary, Cardinaletti, Randazzo (13) reported an up-regulation of a stress-related gene in the liver of rainbow trout fed diets containing 21% full-fat BSFL meal for 98 days, suggesting a physiological activation of stress/inflammation response.

Previous studies focused on the health effects of dietary inclusion of dried BSFL meal for salmonids, in particular dried defatted BSFL meal for salmon. The present study investigated the nutritional value and health effects of two differently processed BSFL types in diets for pre-smolt Atlantic salmon. The two types of BSFL were low-processed and included full-fat dried BSFL meal and undried BSFL paste preserved with formic acid. The results on digestibility and utilization of nutrients and growth performance were reported by Weththasinghe, Hansen (14). In brief, BSFL meal and paste could replace low to moderate levels of dietary protein without compromising growth performance in Atlantic salmon (14). In addition, the fish in this study were sampled to investigate the effects on gut health, plasma biochemical parameters and immune response in pre-smolt salmon fed graded levels of BSFL meal and paste. Furthermore, according to our knowledge, none of the previous studies focused on the effect of dietary insects on the protein expression in skin mucus and distal intestine (DI) of fish. Therefore, we also analyzed the protein expression in skin mucus and DI using mass spectrometry and indirect enzyme-linked immunosorbent assay (ELISA). To further investigate the effect of dietary BSFL meal and paste on the immune system of fish, the phagocytic activity of head kidney (HK) macrophages-like cells isolated from salmon fed BSFL meal and paste was investigated in an *in vitro* challenge study with *Piscirickettsia salmonis* (*P. salmonis*).

MATERIALS AND METHODS

Experimental Diets and Fish Rearing

Full-fat BSFL meal and paste were produced at HiProMine S.A., Poznan, Poland. BSFL from a same batch were used to produce dried BSFL meal and undried BSFL paste preserved with formic acid (2.5%). Seven isonitrogenous, isolipidic and isoenergetic diets were prepared according to the nutrient requirements of pre-smolt Atlantic salmon (33). The experimental diets consisted of a control diet based on fishmeal, plant protein sources (i.e. water-extracted soy protein concentrate, corn gluten, faba bean) and fish oil (Control-1); three diets with increasing levels of BSFL meal, substituting 6.25% (6.25IM), 12.5% (12.5IM) and 25% (25IM) of the protein content of Control-1. In addition, two diets with increasing levels of BSFL paste, substituting 3.7% (3.7IP) and 6.7% (6.7IP) of the protein of Control-1 and an extra control with 0.88% of formic acid (Control-2) were evaluated. Considering BSFL paste was preserved with formic acid, the Control-2 diet was included as a control for BSFL paste diets. The ingredient and chemical composition of the experimental diets are shown in **Table 1**. Further details on BSFL meal and paste

TABLE 1 | Ingredient and analyzed chemical composition of experimental diets[1].

	Control-1	6.25IM	12.5IM	25IM	Control-2	3.7IP	6.7IP
Ingredients (%)							
Fishmeal	25	23.24	21.48	17.69	25	20.27	16.62
Soy protein concentrate	35.5	33.45	30.92	25.58	35.5	29.18	23.92
Corn gluten	4	3.72	3.44	2.59	4	3.24	2.66
Faba bean	1.85	1.72	1.59	1.03	1.85	1.5	1.23
BSFL meal	0	8.07	16.13	32.27	0	0	0
BSFL paste	0	0	0	0	0	19.8	35.12
Wheat flour	13.64	13.64	13.64	13.64	13.64	11.91	10.56
Wheat bran	4	2.47	1.28	0	3.12	2.16	0.98
Fish oil	15	12.68	10.51	6.19	15	11.06	8.13
Formic acid	0	0	0	0	0.88	0	0
Yttrium oxide	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Vit/min premix	0.65	0.65	0.65	0.65	0.65	0.57	0.5
Methionine	0.2	0.2	0.2	0.2	0.2	0.17	0.15
Choline chloride	0.15	0.15	0.15	0.15	0.15	0.13	0.12
Chemical composition (%, as is)							
Dry matter	92.9	92.5	91.5	90.8	91.1	91.9	91.9
Crude protein	46.8	47.4	46.4	45.7	45.8	47.2	47.9
Crude lipid	14.6	15.5	17.2	15.9	16.2	13	13.3
Starch	12.3	12.4	11.5	11.5	11.6	12	12.8
Ash	5.52	5.88	6.17	6.83	5.3	5.67	6.08
Formic acid	0	0	0	0	0.72	0.58	1.1
Gross energy (MJ kg^{-1})	21.9	21.7	21.7	21.5	21.6	21.4	21.1

[1]*Control-1: Control diet. 6.25IM, 12.5IM and 25IM: black soldier fly larvae (BSFL) meal substituted 6.25%, 12.5% and 25% of protein content of Control-1. Control-2: Control diet with 0.88% of formic acid. 3.7IP and 6.7IP: BSFL paste substituted 3.7% and 6.7% of protein content of Control-1.*

and chemical composition and production of diets were reported by Weththasinghe, Hansen (14).

The fish experiment was conducted at the Center for fish research, Norwegian University of Life Sciences (NMBU). The experimental procedures were performed in accordance with the national guidelines for the care and use of animals (The Norwegian Animal Welfare Act and the Norwegian Regulation on Animal Experimentation). The details of the fish experiment were reported by Weththasinghe, Hansen (14). In brief, a total of 1260 Atlantic salmon (Aqua Gen Atlantic QLT-innOva SHIELD) with 34 g of mean initial weight were distributed into 21 fiberglass tanks (300 L capacity) with 60 fish per tank. Three replicate tanks were randomly assigned to each of the seven experimental diets. Fish were fed *ad libitum* (i.e. 10% excess) with experimental diets over a period of seven weeks. The fish were kept under continuous light in recirculated freshwater and the average water temperature was 14.8 °C during the experimental period.

Sample Collection

At the end of the feeding period, six fish from each tank were randomly sampled, anesthetized with tricaine methanesulfonate (MS-222) (80 mg L^{-1}). The fish in all tanks were fed up to three to four hours before sampling. The skin mucus was immediately collected from the skin using sterile plastic spatulas, avoiding bleeding and fecal contamination. The collected mucus was immediately frozen in liquid nitrogen and stored at -80 °C until analysis. After mucus collection, fish were euthanized by a sharp blow to the head. Blood was collected from the caudal vein and centrifuged. Plasma samples were kept on dry ice until transferred to -20 °C and stored at -80 °C.

The DI was defined as a darker color, larger diameter section of the intestine where annular rings were visible (34). The DI was opened longitudinally and, the content was removed carefully. For histology, a piece of DI and a piece of pyloric caeca (PC) were fixed in 4% phosphate-buffered formalin for 24 h at room temperature before storage in 70% ethanol until further processing. For protein extraction, a piece of DI was rinsed in phosphate-buffered saline (PBS) and placed in cryotubes, frozen in liquid nitrogen and stored at −80 °C. For flow cytometry analysis, three fish per tank were sampled, HK and spleen were removed under aseptic conditions into tubes containing L-15 medium (Sigma-Aldrich) and used immediately for extraction of macrophages-like cells and splenocytes, respectively.

Histology

Histological sections of PC and DI (18 samples per dietary group) were processed by Aquamedic and at the Veterinary Institute Laboratory in Oslo, Norway according to their respective standard operating procedures. Briefly, formalin-fixed tissue samples were dehydrated in ethanol, equilibrated in xylene and embedded in paraffin. Sections of 3 µm thickness from each intestinal segment were prepared and stained with hematoxylin and eosin. The sections of PC and DI were then examined blindly by light microscopy with a focus on the morphological changes observed in soybean meal-induced enteritis as previously described for Atlantic salmon DI mucosa. The criteria included shortening of mucosal fold

height, increase in width and cellularity of the submucosa and lamina propria, and loss of enterocyte supranuclear vacuolization (35). Additionally, for the PC, changes in the vacuolization of the intestinal enterocytes were evaluated. The degree of change for the different morphological characteristics evaluated for the PC and DI, was graded using a scoring system with a scale of 0–4 where 0 represented normal; 1, mild changes; 2, moderate changes; 3, marked changes and 4, severe changes.

Detection of Immunological Markers by Indirect ELISA

The number of DI and skin mucus samples used for the detection of immunological markers were nine per dietary group, except for DI IgM and IgD, where 18 per dietary group were included. Samples were thawed on ice and homogenized using beads and ice-cold lysis buffer (Tris 20 mM, NaCl 100 mM, Triton X-100 0.05%, EDTA 5 mM, and protease inhibitor cocktail 1x) in a bead mill homogenizer (Qiagen RETSCH tissuelyser). Then, the homogenate was centrifuged at 12000 x g for 25 min at 4 °C. The supernatant, containing soluble proteins, was then transferred to new tubes on ice and stored at -20 °C until use. All protein samples were quantified by a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions. The extracted soluble proteins from DI and skin mucus were used for the detection of IgM, IgD, IFNγ and IL-1β using indirect ELISA. Briefly, each sample was diluted in carbonate buffer (60 mM NaHCO3 pH 9.6) to 45 ng μL^{-1} and 100 µL of diluted samples were seeded (in duplicate) in a 96-well plate (Nunc) for overnight incubation at 4 °C. Next, 200 µL of blocking solution (5% Blotting-Grade Block (BioRad) diluted in PBS was added to each well and incubated for 2 h at 37 °C. Next, 50 µL of the primary antibody was added to each well and plates were incubated for 90 min at 37 °C. The primary antibodies used were as follows: monoclonal anti-IgD, monoclonal anti-IgM, polyclonal anti-IFNγ or polyclonal anti-IL-1β at 1:200 dilution as reported by Sahlmann, Djordjevic (36) and kindly donated by Dr. Luis Mercado. Next, 50 µL of a secondary antibody diluted to 1:7000 (goat anti-mouse IgG-HRP or mouse anti-rabbit IgG-HRP) was added and incubated for 60 min at 37°C. Finally, 100 µL of chromagen substrate 3,3′,5,5′-tetramethylbenzidine single solution (TMB, Thermofisher) was added and incubated for 30 min at room temperature. The reaction was stopped with 50 µL of 1 N sulfuric acid and read at 450 nm on a Spectra Max microplate reader (Spectra Max M2; Molecular Devices).

Plasma Analysis for Biochemical and Immune Parameters

The ferric reducing ability of plasma (FRAP), ALT, AST, creatine kinase (CK), C-reactive protein (CRP) and lysozyme in plasma were analyzed (18 samples per dietary group) at Skretting Aquaculture Research Centre (ARC), Stavanger, Norway. The ALT, AST, CK and CRP of plasma samples were analyzed using kits from Thermo Fisher Scientific (article numbers: ALT 981361, AST 981363, CK 981828, CRP 981934) on a Konelab 30i Chemistry Analyzer (Thermo Fisher Scientific). Plasma FRAP and lysozyme content were analyzed using in-house protocols adapted from Benzie and Strain (37) and Parry, Chandan (38), respectively.

Flow Cytometry and Phagocytic Capacity

HK and spleen (three fish per tank and pooled) leukocytes were isolated, according to Iliev, Thim (39). To investigate the surface expression of IgD, IgM and CD8 in splenocytes, the samples were washed with ice-cold PBS and incubated with primary antibody (anti-IgD at 1:400 dilution, anti-IgM at 1: 400 dilution or anti-CD8 at 1:200 dilution) for 1 h in PBS, 5% FBS on ice. Then the cells were incubated with secondary Alexa546 coagulated antibody diluted to 1 µg/ml in PBS, 5% FBS for 30 min on ice. Thereafter, cells were washed twice in PBS and analyzed by flow cytometry using a Beckman Coulter Gallios flow cytometer.

The capacity of HK cells to uptake *P. salmonis in vitro* was measured according to methods described by Lagos, Tandberg (40). Briefly, 1 ml of isolated adherent HK leukocytes (i.e. HK macrophages-like cells) (1×10^6 cells/ml) per sample was incubated for 1 h at 15°C with inactivated CFSE labeled *P. salmonis* (1×10^7 CFU/ml), or PBS as a control, without any centrifugation step to enhance the infection. After the incubation, cells were centrifuged at 600 × g for 10 min and the pellet was washed three times with ice-cold PBS and analyzed by flow cytometry using a Beckman Coulter Gallios flow cytometer. The fluorescence of CFSE-conjugated *P. salmonis* was measured before and after the addition of trypan blue (0.025% final concentration) to quench extracellular fluorescence. Data were analyzed using Kaluza software v.1.2 (Beckman Coulter) and at least 10,000 events were collected for each sample.

For morphological characterization, HK macrophages-like cells co-cultured with CFSE-conjugated *P. salmonis* were seeded in an 8-chamber tissue cultured treated glass Falcon CultureSlide® (Corning, New York, USA) at a density of 150,000 cells per chamber. After 1 h, cells were washed with PBS and fixed with 3% paraformaldehyde (Sigma-Aldrich) for 20 min at 4°C. Then, cells were washed three times for 3 min with PBS and left to air dry. Once dried, plastic chambers were removed from the slides. Three drops of mounting medium, Fluoroshield (Sigma-Aldrich), containing DAPI were added to the slides and covered with a coverslip. Confocal laser microscopy (Zeiss LSM 800) was used for imaging and the images were analyzed by ImageJ software.

Skin Mucus Proteomics

The extracted and quantified soluble protein from skin mucus (three samples per dietary group) were used for mass spectrometry analysis. Briefly, 20 µg of total protein in PBS were pH adjusted to 8 by adding ammonium bicarbonate (Sigma-Aldrich, Darmstadt, Germany). The samples were then digested with 1 µg trypsin (Promega, sequencing grade) overnight at 37 °C. The tryptic peptides were analyzed using an Ultimate 3000 RSLCnano-UHPLC system connected to a Q Exactive liquid chromatography-mass spectrometer (LC-MS/MS) (Thermo Fisher Scientific, Bremen, Germany). LC-MS/MS was run at the Proteomics Core Facility (PCF) at the University of Oslo, Norway. The acquired raw data were analyzed using

MaxQuant (41) version 1.4.1.2. and Perseus version 1.6.0.7 based on MS1 intensity quantification. Proteins were quantified using the MaxLFQ algorithm (42). The data were searched against the salmon proteome (82390 sequences, June 2019). Peptide identifications were filtered to achieve a protein false discovery rate (FDR) of 1% using the target-decoy strategy. The analysis was restricted to proteins identified in at least two of the three replicates per dietary group. Protein raw data were transferred to log normalization and then volcano plot analysis, multivariate statistical analysis and data modeling were performed in R (R Core Team, 2019) using the package DEP (43) and vsn (44). In addition to differentially expressed proteins, the proteins uniquely expressed in each diet were identified. UniprotKB database was used for the functional annotation of the proteins. The potential functions of these proteins were inferred from the homologs for their UniprotKB sequence. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (45) partner repository with the dataset identifier PXD019125.

Statistical Analysis

Differences in histological scores for the morphological characteristics of the DI and PC tissue were analyzed for statistical significance using ordinal logistic regression run in the R statistical package (version 3.4.2; 2017) within the RStudio interphase (version 1.1.383; 2017). Three different group comparisons were conducted, and these comprised of: comparison 1, between Control-1, 6.25IM, 12.5IM and 25IM diets, comparison 2, between Control-1, Control-2, 3.7IP, and 6.7IP and comparison 3, between Control-2, 3.7IP, and 6.7IP. Differences were examined based on odds ratios of the different dietary groups having different histology scores compared to the reference diet. Reference diets were Control-1 for comparison 1 and 2 and Control-2 for comparison 3.

The biochemical and immune parameters of plasma, results of ELISA, surface expression of IgD, IgM and CD8 in splenocytes and HK macrophages-like cell phagocytic activity were analyzed using one-way ANOVA, followed by Tukey's multiple comparison test for comparison of means. Differences at $p<0.05$ were considered as significant. In addition, polynomial contrast analysis was used to evaluate the relationship between plasma parameters and dietary BSFL meal or paste levels. The results of ELISA were presented as fold changes relative to Control-1 and two different group comparisons were conducted, and these comprised of: comparison 1, between 6.25IM, 12.5IM and 25IM diets and comparison 2, between Control-2, 3.7IP, and 6.7IP diets. The means of each diet were also compared with Control-1 diet using Dunnett's multiple comparison test. The statistical analyses of plasma parameters were performed using IBM SPSS Statistics 26 software, whereas statistical analyses of ELISA results, surface expression of IgD, IgM and CD8 in splenocytes and HK macrophages-like cell phagocytic activity were performed using GraphPad Prism 8.3.1. Prior to ANOVA, these data were tested for homogeneity of variance by Levene's test and normal distribution of residuals was checked using Kolmogorov-Smirnov test. These two tests were performed in IBM SPSS Statistics 26 software. When the assumption of equal variance was violated, the data were tested using Brown-Forsythe ANOVA test and Welch's ANOVA test, followed by Dunnett's T3 multiple comparison test. Kruskal-Wallis test was used when the data were not normally distributed or the data showed both heterogeneity of variances and non-normal distribution, and followed by Dunn's multiple comparison test. These tests were performed in GraphPad Prism 8.3.1.

RESULTS

Gut Health Assessment

Pyloric Caeca

Mild to moderate accumulation of lipid in vacuoles in the epithelial cells, also called enterocyte steatosis was the main morphological change observed in the PC of all diet groups, as illustrated in **Supplementary Figure 1**. High occurrence and severity of the enterocyte steatosis was observed in Control-1, Control-2 and 25IM (**Figure 1A**). The degree of the steatosis was lower (less in number and severity of the steatosis) ($p<0.01$) in 6.25IM, 12.5IM, 3.7IP and 6.7IP diets fed fish compared to Control-1 (**Figure 1A**). The PC submucosa of fish fed all the diets were normal with regards to other morphological changes, i.e. increase in the width and inflammatory cell infiltration in the submucosa (**Figure 1B**).

Distal Intestine

Evaluation of the DI revealed normal and healthy morphology for most of the fish in the present study (**Figure 2**). Increased width and infiltration of the submucosa and lamina propria by inflammatory cells in DI were not observed in any of the groups except for focal lesions in two fish fed Control-2 diet (**Figures 2A, B**). The fish fed Control-1 showed mild inflammation changes (**Supplementary Figure 1**) characterized by a mild to moderate mucosal fold shortening (**Figure 2C**) due to a loss in enterocyte vacuolization (**Figure 2D**). Similar to Control-1, the Control-2 and 3.7IP diets also showed inflammatory changes in terms of mucosal fold shortening (**Figure 2C**) and loss of supranuclear vacuolization (**Figure 2D**). However, BSFL meal diets and 6.7IP diet fed fish showed no such inflammatory changes in the DI (**Figures 2C, D**).

Immune Parameters in Distal Intestine and Skin Mucus

Immunoglobulin and pro-inflammatory cytokine levels in the DI and skin mucus are shown in **Figure 3** as fold changes relative to Control-1. Considering BSFL paste was preserved in formic acid, we also compared the effect of BSFL paste diets relative to Control-2 (**Supplementary Figure 2**).

IgM level was lower ($p<0.001$) in DI of fish fed 6.25IM (0.4-fold change) and 25IM (0.3-fold change) diets, relative to Control-1, while fish fed 12.5IM diet showed higher IgM level ($p<0.01$) compared to 6.25IM and 25IM diets (**Figure 3A**). The 25IM diet showed a 9.2-fold increase ($p<0.01$) of DI IFNγ relative to Control-1. DI IFNγ in 25IM was also higher ($p<0.05$) than

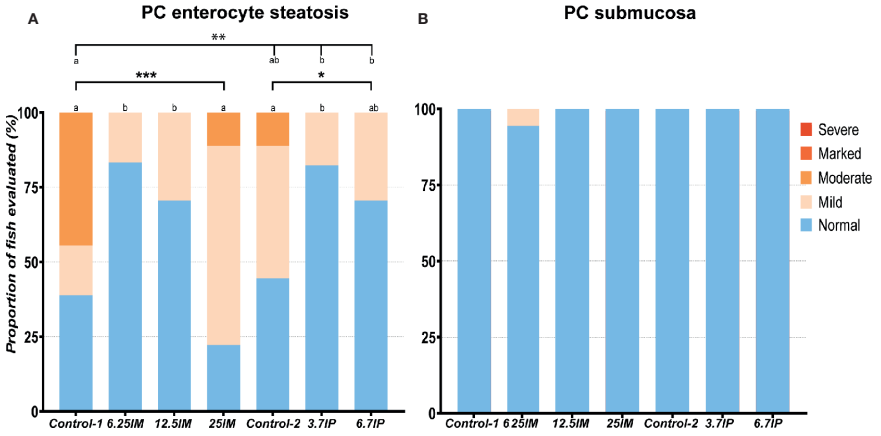

FIGURE 1 | Histological evaluation of pyloric caeca (PC). **(A)** Number of PC tissue sections that were scored "normal", "mild", moderate", "marked" or "severe" for enterocyte steatosis and **(B)** increase in the width and inflammatory cell infiltration in the submucosa. Column charts, within each of the demarcation bars that mark Comparison 1 (Control-1, 6.25IM, 12.5IM and 25IM), Comparison 2 (Control-1, Control-2, 3.7IP, and 6.7IP) and Comparison 3 (Control-2, 3.7IP and 6.7IP), that do not share superscript letters are statistically different ($p < 0.05$). Asterisks denote level of significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) following outcomes of an ordinal logistic regression for differences in distribution of histological scores between the dietary groups. Each measurement was performed in triplicate using 6 fish per tank. Control-1: Control diet. 6.25IM, 12.5IM and 25IM: BSFL meal substituted 6.25%, 12.5% and 25% of protein content of Control-1. Control-2: Control diet with 0.88% of formic acid. 3.7IP and 6.7IP: BSFL paste substituted 3.7% and 6.7% of protein content of Control-1.

other BSFL meal diets (**Figure 3C**). The IgD and IL-1β levels in the DI were not affected by the dietary inclusion of BSFL meal (**Figures 3B, D**). In BSFL paste fed salmon, the 3.7IP diet showed a 0.4-fold decrease (p<0.001) in DI IgM relative to Control-1 (**Figure 3A**). The DI IgM level in the 3.7IP diet was also lower (p<0.05) than Control-2 and 6.7IP. The IgD, IFNγ and IL-1β levels in the DI were not affected by the dietary inclusion of BSFL paste (**Figures 3B–D**).

In the skin mucus, the IgM, IgD, IFNγ and IL-1β levels were not affected by the dietary inclusion of BSFL meal (**Figures 3E–H**). Similarly, BSFL paste did not change the skin mucus IgM and IL-1β levels (**Figures 3E, H**). However, the fish fed 3.7IP diet showed a lower (p<0.05) IgD level than 6.7IP diet (**Figure 3F**). Further, 3.7IP diet also showed a 0.4-fold decrease (p<0.01) of IFNγ level relative to Control-1, which was also lower (p<0.05) than Control-2 and 6.7IP diets (**Figure 3G**). We observed high variations in immunoglobulin and cytokine levels in the DI and skin mucus among the fish within groups.

Plasma Immune and Biochemical Parameters

Plasma FRAP, ALT, AST, CK, CRP and lysozyme contents in BSFL meal and paste diets were not statistically different from Control-1 (**Table 2**). Plasma lysozyme content was higher (p <0.01) in 12.5IM fed fish compared to 6.25IM fed fish. Plasma FRAP content was higher (p <0.01) in 6.7IP compared to Control-2. According to polynomial contrast analysis, there was a negative linear relationship between plasma CRP and dietary BSFL meal level (p <0.05). Also, the plasma FRAP content increased linearly with increasing dietary BSFL paste level (p <0.01)). In addition, there was a quadratic relationship between plasma AST and dietary BSFL paste level (p <0.05) with the highest level at 3.7%, and the same trend was observed between plasma CK and dietary BSFL paste level (p = 0.07).

Immune Markers in Spleen and Phagocytic Activity in Head Kidney Macrophages-Like Cells

Considering the results presented above, the immune markers in spleen and phagocytic activity in HK macrophages-like cells were measured only in four diets, i.e. Control-1, 12.5IM, Control-2 and 6.7IP. The number of IgM+, IgD+ and CD8+ splenocytes was not affected by dietary treatments (**Figure 4A**). Further, dietary treatments did not affect the phagocytic activity in HK macrophages-like cells. However, macrophages-like cells isolated from HK of fish fed 12.5IM diet were more prone to incorporate labeled-*P. salmonis* compared to other diets, although it was not statistically significant (**Figure 4B**). The incorporation of *P. salmonis* was confirmed by confocal microscopy (**Figure 4C**).

Skin Mucus Proteomics

The proteins present in the skin mucus of fish fed BSFL meal or paste were analyzed by mass spectrometry. A total of 1636 salmon proteins were identified (**Supplementary Table 1**). After filtering for proteins presented in at least two of the three replicates per dietary group, 968 proteins were selected for further analyses. Volcano plots displaying normalized log2 of LFQ (label-free quantification) protein abundance ratio between Control-1 and other experimental diets, or between Control-2 and BSFL paste diets, against statistical

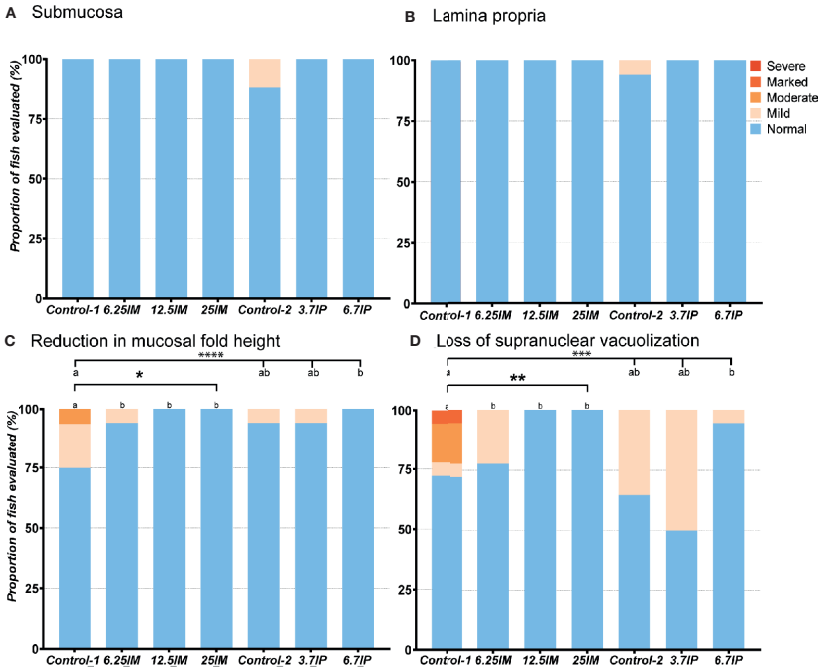

FIGURE 2 | Histological evaluation of distal intestine (DI). **(A)** Number of DI tissue sections that were scored "normal", "mild", "moderate", "marked or "severe" for the morphological characteristics of increase in width and inflammatory cell infiltration of the submucosa and **(B)** lamina propria, **(C)** reduction in mucosal fold height and **(D)** loss of enterocyte supranuclear vacuolization. Column charts, within each of the demarcation bars that mark Comparison 1 (Diets Control-1, 6.25IM, 12.5IM and 25IM) and Comparison 2 (Control-1, Control-2, 3.7IP, and 6.7IP), that do not share superscript letters are statistically different ($p<0.05$). Asterisks denote level of significance (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$) following outcomes of an ordinal logistic regression for differences in distribution of histological scores between the dietary groups. Each measurement was performed in triplicate using 6 fish per tank. Control-1: Control diet. 6.25IM, 12.5IM and 25IM: BSFL meal substituted 6.25%, 12.5% and 25% of protein content of Control-1. Control-2: Control diet with 0.88% of formic acid. 3.7IP and 6.7IP: BSFL paste substituted 3.7% and 6.7% of protein content of Control-1.

significance measurements (−log10 *p* value) are shown in **Supplementary Figure 3**. The dietary inclusion of BSFL meal and paste had minor effects on the expression profile of proteins. The exception was the expression of apolipoprotein D and NAD(P)H dehydrogenase [quinone] 1- like in 25IM diet compared to Control-1. In addition, 12.5IM diet over-expressed heterogeneous nuclear ribonucleoprotein A0-like and reduced the expression of RNA-binding protein cabeza-like and serum deprivation-response protein compared to Control-1. Further, compared to Control-2, the 3.7IP diet showed lower expression of beta-globin (**Table 3**).

The detected salmon proteins were checked for unique proteins. The criteria used for the identification of unique proteins was the presence of the particular protein in at least two of the three replicates in a dietary group. The unique proteins expressed in the skin mucus of fish fed experimental diets are shown in **Supplementary Table 2**. The BSFL meal diets and 6.7IP fed fish uniquely expressed several immune-related proteins, whereas 25IM also uniquely expressed superoxide dismutase.

DISCUSSION

The present study reports the effect of dietary inclusion of graded levels of BSFL meal and paste on gut health, plasma biochemical parameters, immune response and protein expression in skin mucus in pre-smolt salmon.

The gut is considered as the main site of exposure to nutrients and antigens (46). In a normal and healthy gut, almost no vacuolization is observed in the enterocytes of the proximal intestine (30). Increased vacuolization of the enterocytes in the PC is called enterocyte steatosis, which reflects an abnormal lipid droplet accumulation within the enterocytes due to impaired lipoprotein synthesis (47, 48) or lipid transport across the intestinal mucosa to the circulatory system (49). This condition is observed in fish affected by the so-called lipid malabsorption syndrome (50). The extensive accumulation of lipid droplets in enterocytes might cause damage to the epithelium affecting the integrity of the epithelial barrier. Such damage can translocate pathogenic or potentially pathogenic

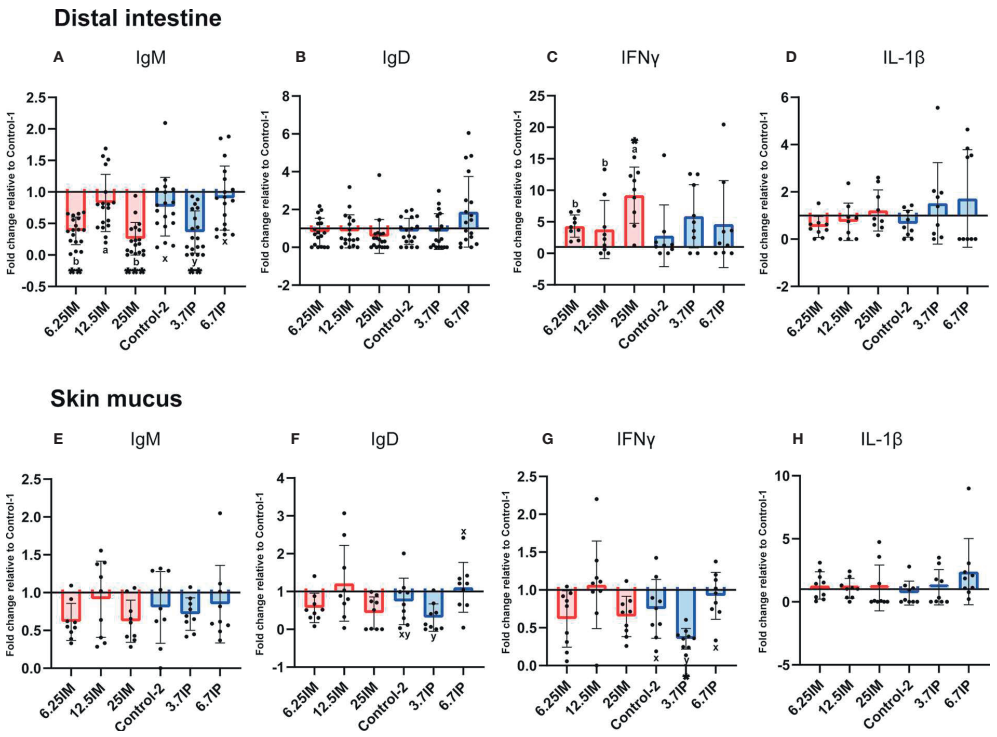

FIGURE 3 | Detection of immunoglobulin and pro-inflammatory cytokine levels in distal intestine and skin mucus of fish fed experimental diets containing black soldier fly larvae (BSFL) meal and paste. **(A)** IgM, **(B)** IgD, **(C)** IFNγ and **(D)** IL-1β levels in distal intestine and **(E)** IgM, **(F)** IgD, **(G)** IFNγ and **(H)** IL-1β levels in skin mucus. Results are expressed as fold change relative to Control-1. Error bars indicate standard deviation. Error bars that are labelled with different superscript letters are significantly ($p<0.05$) different according to Tukey's multiple comparison test or Dunn's multiple comparison test. The letters a-b denote significant differences among 6.25IM, 12.5IM and 25IM diets (Comparison 1), whereas x–y denote significant differences among Control-2, 3.7IP and 6.7IP (Comparison 2). Asterisks denote level of significant difference with Control-1 (*= $p<0.01$, ** = $p<0.001$, *** = $p<0.0001$) according to Dunnett's multiple comparison test or Dunn's multiple comparison test. Control-1: Control diet. 6.25IM, 12.5IM and 25IM: BSFL meal substituted 6.25%, 12.5% and 25% of protein content of Control-1. Control-2: Control diet with 0.88% of formic acid. 3.7IP and 6.7IP: BSFL paste substituted 3.7% and 6.7% of protein content of Control-1.

bacteria into the host and, having detrimental effects on fish health (47, 48). The observed mild to moderate enterocyte steatosis in the PC of fish fed the control diets in the present study might be due to the inclusion of high levels of plant ingredients (49–51) or lack of dietary choline (50, 52) as observed previously. However, a reduced enterocyte steatosis was observed in the PC of fish fed diets with low to moderate dietary BSFL meal and paste inclusion. Li, Kortner (30) and Li, Kortner (29) have also shown that post-smolt salmon fed diets containing 15% BSFL meal and pre-smolt salmon fed diets with 60% of de-chitinized BSFL meal presented a lower degree of enterocyte steatosis in the proximal intestine. The reason for the reduction in enterocyte steatosis in the BSFL meal and paste fed fish in the present study could be related to lower levels of plant ingredients or the presence of bioactive components in the BSFL such as choline. As reported by others, insects, including BSFL, are rich sources of choline (15, 53). Choline is important in lipid transport across the intestinal mucosa of salmon (50) and dietary choline chloride (0.37–0.4%) prevented excessive lipid accumulation in the proximal intestine in post-smolt Atlantic salmon (50, 52). In addition, the majority of fatty acids in BSFL meal were saturated (14) which might lead to reduced enterocyte steatosis, as observed in Arctic char (*Salvelinus alpinus*) by Olsen, Myklebust (48). On the contrary, we observed that 25% replacement of protein with BSFL meal caused mild-moderate enterocyte steatosis, similar to the control diets. This finding was associated with a lower lipid digestibility in fish fed diets with 25% replacement of protein with BSFL meal (14), suggesting that high level of chitin from BSFL could also be a causative factor for the enterocyte steatosis. The BSFL meal used in the present study contained 8% of chitin in dry matter basis, this corresponds to chitin levels of 0.6,1.2 and 2.3% for the meal diets and 0.4 and

TABLE 2 | Immune and biochemical parameters in plasma of fish fed black soldier fly larvae (BSFL) meal and paste[1].

									Comparison 1—BSFL meal diets[2]			*Comparison 2—BSFL paste diets*[2]		
	Control-1	**6.25IM**	**12.5IM**	**25IM**	**Control-2**	**3.7IP**	**6.7IP**	**SEM**[3]	p_{value}[4]	p_{linear}[4]	p_{quad}[4]	p_{value}[5]	p_{linear}[5]	p_{quad}[5]
Ferric reducing antioxidant power (FRAP) (μmol/L)	920.5^{XY}	961.4	961.4	979.1	889.9^{Y}	993.0^{XY}	1017.5^{X}	11.55	0.52	0.19	0.58	0.005	0.005	0.40
Alanine aminotransferase (ALT) (U/L)	14.3	13.1	18.2	14.8	14.2	13.7	11.8	0.78	0.09	0.68	0.40	0.55	0.35	0.69
Aspartate aminotransferase (AST) (U/L)	637.4	538.2	652.7	532.9	530.3	685.5	535.9	18.72	0.15	0.27	0.67	0.09	0.82	0.016
Creatine kinase activity (CK) (U/L)	19739.4	16880.3	23553.1	18441.1	17438.0	25792.3	21333.8	997.7	0.09	0.98	0.41	0.13	0.27	0.07
C-reactive protein (CRP) (mg/L)	2.7	2.3	1.1	0.5	0.8	1.6	0.9	0.23	0.07	0.018	0.75	0.04	0.67	0.11
Lysozyme (U/ml)	1199.9^{ab}	1040.5^{b}	1339.1^{a}	1198.3^{ab}	1236.9	1185.9	1266.5	20.84	0.003	0.35	0.47	0.63	0.73	0.29

[1]*Control-1: Control diet. 6.25IM, 12.5IM and 25IM: BSFL meal substituted 6.25%, 12.5% and 25% of protein content of Control-1. Control-2: Control diet with 0.88% of formic acid. 3.7IP and 6.7IP: BSFL paste substituted 3.7% and 6.7% of protein content of Control-1.*

[2]*Two group comparisons were conducted: Comparison 1, between Control-1, 6.25IM, 12.5IM and 25IM diets; Comparison 2, between Control-1, Control-2, 3.7IP, and 6.7IP.*

[3]*Standard error mean.*

[4]*p values for comparison 1.*

[5]*p values for Comparison 2.*

p_{value}*: p value for one-way ANOVA, Kruskal-Wallis test or Welch's ANOVA test. Different superscript letters of lysozyme values of Control-1, 6.25IM, 12.5IM and 25IM diets (Comparison 1) indicate significant (p<0.05) differences according to Tukey's multiple comparison test. Different superscript letters of FRAP values of Control-1, Control-2, 3.7IP and 6.7_IP diets (Comparison 2) indicate significant (p<0.05) differences according to Dunnett's T3 multiple comparison test.* p_{linear} *and* p_{quad} *are the p values of linear and quadratic components of the polynomial contrast analysis between each plasma parameter and BSFL meal/paste protein level in the diet: Control-1 was excluded in the polynomial contrast analysis of BSFL paste diets (Comparison 2).*

0.6% for the paste diets (14). The diet that replaced 25% of protein with BSFL meal also had the highest level of lauric acid and this could also affect this condition. Further studies are thus needed to investigate the effect of lauric acid on the enterocyte steatosis in Atlantic salmon when fed BSFL.

Mild inflammatory changes were observed in the DI of fish fed the two control and 3.7IP diets in the present study. These inflammatory changes comprised predominantly of the shortening of the mucosal fold height due to the loss in the enterocyte supranuclear vacuolization. Loss of the vacuolization is also known to indicate a block in the enterocytic activity in the DI (54). As for enterocyte steatosis, the high inclusion of plant ingredients might lead to these changes in the present study (55, 56). Further, the soy protein concentrate that we used was water-extracted thus antinutritional factors such as saponin were not fully removed. Soya saponin is known to induce enteritis in the DI of Atlantic salmon (57–59). In accordance with the present results of BSFL meal diets and 6.7IP diet, others also reported that dietary inclusion of BSFL meal showed normal and healthy histology in DI of pre-smolt salmon (29) and rainbow trout (60) and mid intestine of post-smolt salmon (10). The reason for the absence of DI inflammatory changes in the BSFL meal diets and 6.7IP diet fed fish in the present study could be related to inclusion of BSFL or lower levels of plant protein ingredients. The fatty acid composition of BSFL might contribute for the absence of intestine inflammation. BSFL contain high amounts of medium-chain lauric acid (C12:0) (14, 23), which has antimicrobial effects against gram-positive bacteria (24, 25, 61, 62) and viruses (61, 62). Medium-chain fatty acids and medium-chain triglycerides have also been suggested to improve gut health under inflammatory conditions (63), which might be associated with the induction of the expression of host defense peptides in the gut (64). Another possible explanation for the absence of intestine inflammation in fish fed BSFL meal and paste may be related to BSFLs' ability to modulate gut microbiota and increased microbial lactic acid and butyrate production. Although our study did not include microbiota analysis, others have reported that dietary inclusion of BSFL meal increased the abundance of lactic acid (26–28) and butyrate (27) producing bacteria in the gut of rainbow trout. It has been reported that lactate and butyrate could repair or prevent the intestinal damage caused by dietary soybean meal or oxidized soybean oil in fish (65, 66). The anti-inflammatory properties of microbe-derived butyrate in gut and its role in enhancing intestinal barrier function and mucosal immunity are well studied in human (67). It is also possible that the short-chain fatty acids including butyrate produced by gut microbiota might induce the expression of host defense peptides and prevent inflammation in the gut as observed in mammals and birds (64). Further experiments are needed to unravel the effect of the full-fat BSFL meal and paste on gut microbiota diversity of salmon.

Pro-inflammatory cytokines are known to be released as part of the innate immune response in fish (68). The dietary replacement of fishmeal and plant protein sources up to 12.5% with BSFL meal and 6.7% with BSFL paste, did not affect IL-1β or IFNγ levels in DI. This explains the absence of inflammatory changes in DI histology of fish fed these diets. Dietary inclusion of BSFL meal has been reported not to affect the intestinal expression of pro-inflammatory cytokine genes including IFNγ and IL-1β in pre-smolt (29) and post-smolt (30) salmon. Similarly, dietary inclusion of defatted BSFL meal did not affect

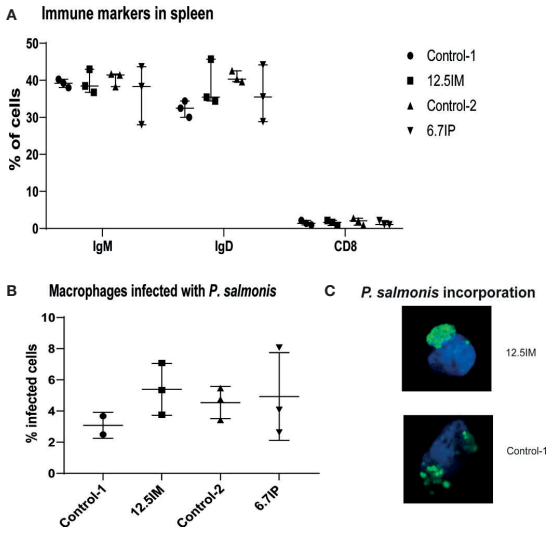

FIGURE 4 | Expression of immune markers of splenocytes and phagocytic capacity of head kidney macrophages-like cells isolated from salmon fed experimental diets containing black soldier fly larvae (BSFL) meal and paste. **(A)** Flow cytometry showing the number of splenocytes expressing IgM, IgD or CD8. **(B)** number of macrophages-like cells able to incorporate labeled-bacteria (*P. salmonis* CFSE labelled). **(C)** confocal microscopy confirming the incorporation of labelled *P. salmonis*. Each measurement was performed using 3 fish per tank. Control-1: Control diet; 12.5IM: BSFL meal substituted 12.5% of protein content of Control-1; Control-2: Control diet with 0.88% of formic acid; 6.7IP: BSFL paste substituted 6.7% of protein content of Control-1.

the intestinal inflammatory cytokines concentrations of TNF-α, IL-6 and IL-8 in juvenile Japanese seabass (*Lateolabrax japonicus*) (69). In contrast, high (25IM) inclusion level of BSFL meal induced high levels of IFNγ in DI. Thus, despite the absence of histological alterations in DI, a potential morphological effect might be expected in prolonged feeding with BSFL meal diets. Natural antibodies are crucial components of the innate humoral immune system, as they provide immediate, early and broad protection against pathogens (68). The IgD level in DI was not altered by the inclusion of BSFL meal and paste. However, the DI IgM presented low levels in 6.25IM, 25IM and 3.7IP diets fed fish compared to Control-1. The lower DI IgM might also be related to the absence of inflammatory changes in DI histology in BSFL meal containing diets, because elevated IgM level in intestinal mucosa might be a sign of inflammation (70). However, this effect was not observed in 3.7IP diet.

Oxidative stress within cells or tissue has adverse effects on fish health, thus antioxidants can have significant health-benefits (71). In the present study, the antioxidant capacity in the plasma was measured in terms of FRAP. The results of FRAP in the present study showed an increased plasma antioxidant capacity when increasing the level of dietary BSFL paste, whereas salmon fed BSFL meal showed unaltered plasma antioxidant defense capacity. Previous studies also reported that the activity of serum antioxidant enzymes did not alter or even increased with dietary inclusion of BSFL meal or pulp in Jian carp (*Cyprinus carpio* var. Jian) (72), yellow catfish (*Pelteobagrus fulvidraco*) (73) and mirror carp (*Cyprinus carpio* var. specularis) (74). However, Zhou, Liu (75) observed that serum antioxidant capacity was not affected by partial replacement of dietary fishmeal with BSFL meal in Jian carp, while complete replacement reduced serum antioxidant capacity.

ALT and AST are enzymes present in liver and spleen, and leak into the bloodstream upon liver cell damage; therefore, high levels of these enzymes in blood are indicators of the liver damage (76). On the other hand, CK is concentrated in muscle and heart tissue and CK in the blood indicates damage of these tissues (77). In the present study, plasma AST, ALT and CK levels were not affected by BSFL meal, suggesting that dietary BSFL meal might not affect liver and muscle health. In accordance with the present study, several studies demonstrated that the activities of serum ALT and AST were not altered or in some cases even decreased by dietary inclusion of BSFL meal or pulp in pre-smolt (32) and post-smolt salmon (9), Jian carp (72), Japanese seabass (69) and mirror carp (74). In addition, Belghit, Waagbø (32) and Vargas-Abúndez, Randazzo (78) observed that dietary inclusion of BSFL meal did not affect the expression of genes involved in stress response (heat-shock protein-70 and superoxide dismutase) in the liver of pre-smolt salmon and clownfish (*Amphiprion ocellaris*) respectively, suggesting no induction of stress response and further

TABLE 3 | Differentially expressed proteins in the skin mucus of fish fed black soldier fly larvae (BSFL) meal or paste[1].

Diet	SwissProt accession no	Protein name	Gene name	Fold change	Adjusted *p* value
Abundance compared to Control-1					
12.5IM	A0A1S3RTG2	Heterogeneous nuclear ribonucleoprotein A0-like	*LOC106604941*	2.27	0.024
12.5IM	A0A1S3S0N2	RNA-binding protein cabeza-like	*LOC106606097*	–2.65	0.024
12.5IM	A0A1S3PL20	Serum deprivation-response protein	*sdpr*	–0.64	0.024
25IM	B5XEY8	Apolipoprotein D	*APOD*	1.58	0.003
25IM	A0A1S3PWS4	NAD(P)H dehydrogenase [quinone] 1-like	*LOC106587985*	–1.61	0.004
Abundance compared to Control-2					
3.7IP	Q91470	Beta-globin	*HBB1*	–3.53	0.028

[1]*Control-1: Control diet. 12.5IM and 25IM: BSFL meal substituted 12.5% and 25% of protein content of Control-1. Control-2: Control diet with 0.88% of formic acid. 3.7IP: BSFL paste substituted 3.7% of protein content of Control-1.*

confirming that BSFL ingredients did not cause any negative effect on liver health. On the contrary, increased expression of heat-shock protein-70 gene was observed in the hepatopancreas of Jian carp fed BSFL meal diets, suggesting an induced stress response only when dietary substitution of fishmeal exceeded 75% (72). In agreement with the present results, dietary inclusion of BSFL meal did not affect the plasma CK level in rainbow trout (60) or intestinal CK in Japanese seabass (69). These results might be associated with the diminished CRP level in the plasma of fish fed BSFL meal. CRP is an acute-phase serum protein and a mediator of innate immunity (79). The blood CRP level is increased in response to acute infection, inflammation or tissue injury (80, 81). Further, the serum CRP level was increased in rainbow trout reared in unfavorable environment, i.e. high-water temperature (82). The diminished CRP level in the plasma of fish fed BSFL meal in the present study might also be associated with the absence of DI inflammatory changes in BSFL meal diets. However, according to the polynomial contrast analysis, plasma AST and CK levels tend to increase at 3.7% replacement of protein with BSFL paste.

Lysozyme is an important defense molecule of the innate immune system of fish (83, 84), which is important in mediating protection against microbial invasion. Lysozyme is distributed in mucus, lymphoid tissue, plasma and other body fluids of freshwater and marine fish (84). It has been reported that dietary defatted BSFL meal did not affect the serum lysozyme activity in Japanese seabass (69). However, we observed low lysozyme level in fish fed 6.25IM, but increased level in fish fed 12.5IM. A dose-response of BSFL meal on serum lysozyme activity was observed in yellow catfish, with higher activity at lower dietary levels (73). Like B cells, macrophages are considered the principal phagocytic cell population in fish (85, 86) and phagocytosis is one of the main effector mechanisms of innate immunity against pathogens in fish (87). The HK macrophages-like cells of fish fed 12.5IM diet showed a numerically higher phagocytic activity when challenged by *P. salmonis*, the pathogen that cause Piscirickettsiosis in salmonid fish (88). A previous study also reported increased phagocytic activity of peritoneal leukocytes in red sea bream (*Pagrus major*) fed housefly pupae homogenate (89). Xiao, Jin (73) reported that dietary inclusion of BSFL meal did not affect the phagocytic index (Intracellular total bacterial count/Number of cells involved in phagocytosis) of white blood cells in yellow catfish, whereas the percentage of phagocytic cells involved in phagocytosis was lower at high dietary levels of BSFL meal (46–59%).

The skin mucus contains different innate immune parameters such as complements, lysozyme, immunoglobulins, cytokines, protease and lectins that protect fish against pathogens (90). As indicated by Esteban (90), the levels of immunoglobulin and pro-inflammatory cytokines in skin mucus varied between individuals and were detected in small quantities in the present study. The dietary inclusion of BSFL meal and paste did not affect the immunoglobulin and pro-inflammatory cytokine levels in the skin mucus, except the 3.7IP diet reduced both IgD and IFNγ in skin mucus. In accordance, mass spectrometry results also showed that the dietary inclusion of BSFL meal and paste had minor effects on the expression profile of proteins in the skin mucus. The higher inclusion of BSFL meal, replacing 25% of dietary protein, increased expression of apolipoprotein D which is involved in lipid transport. It has been demonstrated that apolipoprotein D gene expression *in vitro* was associated with several pathological and stressful conditions and pro-inflammatory stimuli in human cell lines (91). Further, 25IM diet also reduced the expression of NAD(P)H dehydrogenase [quinone] 1-like. NAD(P)H dehydrogenase [quinone] 1 isoform 1 gene is a biomarker of hepatotoxicity (92). The replacement of 3.7% of dietary protein with BSFL paste reduced the expression of beta-globin compared to formic acid containing control, which is a part of the hemoglobin complex and involved in oxygen transport.

The two diets with low and moderate levels of BSFL meal, i.e. 6.25IM and 12.5IM, uniquely expressed calreticulin-like. In a recent study, dietary inclusion of yeast cell wall extract increased abundance and expression of a calreticulin-like protein in the skin mucus of salmon (93). Further, calreticulin was over-expressed in the proteome of the DI of Atlantic salmon fed a probiotic feed additive 24 h after inducing inflammation. This suggest that it has a key role in many cellular and immunoregulatory functions, which help to counteract the inflammation (94). In addition, the fish fed 12.5IM diet uniquely expressed calpain-9-like and calpain-2 catalytic subunit-like. Calpains are calcium-dependent proteases (95, 96), which regulate phagocytosis and bacterial killing in macrophages (97). Further, the fish fed 12.5IM diet uniquely expressed high mobility group protein B1, H1 histone family

member 0 like protein and galectin. High mobility group box 1 protein is known as an extra-cellular cytokine that triggers inflammatory and immune responses (98). Zhao, Hu (98) demonstrated that a high mobility group box 1 homolog from red drum (*Sciaenops ocellatus*) could function as a secreted cytokine in response to bacterial infection and promote innate defense through the activation of macrophages, and Xie, Hodgkinson (99) reported that in goldfish (*Carassius auratus*), high mobility group box 1 is a critical regulatory cytokine of inflammatory and antimicrobial response. Histone fragments or histone derived peptides from skin mucus of rainbow trout (100) and Atlantic salmon (101) were reported to possess antimicrobial properties. The 6.7IM diet fed fish uniquely expressed C-type lectin lectoxin-Thr1-like, which is a C-type lectin superfamily member and calpain small subunit 1. Fish lectins are reported to possess antimicrobial effects. In the presence of Ca^{2+}, C-type lectins initiate a broad range of biological processes such as adhesion, endocytosis, and pathogen neutralization (102). The skin mucus of fish fed 25IM diet uniquely expressed superoxide dismutase, which is an antioxidant enzyme (103, 104) and a marker of stress response (32).

The minor effects of BSFL meal and paste on the immune response and skin mucus proteome in the present study indicate that the effect of BSFL might be more local, as observed in the gut, than systemic. In addition, many other reasons can explain the minor effects of BSFL on skin mucus proteome, such as the sampling time, i.e. seven weeks after feeding or the sampling method, i.e. scraping with plastic spatulas, which could influence the type and amount of protein obtained as shown in Fæste, Tartor (105). Besides, the fish were in the freshwater phase, which has been shown to have lower mucus viscosity than in seawater, meaning a different protein and glycosylation pattern (106). It is important to note that in the present study, trypsinized peptides were used in the mass spectrometry analysis, that might affect the protein conformation (107). It is possible that BSFL might have an effect on the conformation or glycosylation of skin mucus protein, which was not assessed in the present study.

In summary, the present study showed that 6.25IM diet reduced enterocyte steatosis in PC, improved DI histology, and reduced IgM level in DI. The fish fed 12.5IM diet reduced enterocyte steatosis in PC, improved DI histology, had a higher plasma lysozyme activity compared to 6.25IM, and tend to increase phagocytic activity in HK macrophages-like cells against *P. salmonis*. In addition, this diet showed uniquely expressed skin mucus proteins that regulate phagocytosis and antimicrobial responses, i.e. calpains and histone. On the other hand, 25IM diet improved DI histology, but showed enterocyte steatosis in PC, increased pro-inflammatory cytokine IFNγ in DI and reduced IgM in DI. Concurrently, this diet differentially expressed stress related proteins, i.e. over-expressed apolipoprotein D, reduced expression of NAD(P)H dehydrogenase [quinone] 1-like and uniquely expressed superoxide dismutase. In the case of BSFL paste diets, 3.7IP diet caused mild inflammatory changes in DI, reduced DI IgM and skin mucus IgD and IFNγ, and tend to increase plasma AST and CK, although it reduced enterocyte steatosis in PC. The 6.7IP diet reduced enterocyte steatosis in PC and improved DI histology, accompanied by higher plasma FRAP. Further, this diet uniquely expressed proteins that regulate phagocytosis and antimicrobial responses such as calpain and lectin. These results suggest that 6.25% and 12.5% replacement of dietary protein with BSFL meal and 6.7% replacement with BSFL paste, were more prone to cause positive impacts on gut health and immune response in Atlantic salmon, in comparison to low (3.7IP) and high inclusion levels (25IM). Further, the presence of formic acid in diets seemed to have no or minor effects on the gut, skin mucus and other general health parameters in plasma, spleen and HK. However, further studies with a longer feeding period or exposing fish to pathogenic challenges, are needed to confirm the significance of these results. The results observed in the present study might be attributed to the presence of chitin in the BSFL containing diets: 0.6% (6.25IM);1.2% (12.5IM); 2.3% (25IM) in BSFL meal diets and 0.4% (3.7IP); 0.6% (6.7IP) in BSFL paste diets (14). Chitin has complex and size-dependent effects on innate and adaptive immune responses (108), as chitin act as pathogen-associated molecular patterns (109). Large chitin polymers are biologically inert, while smaller fragments are pro-inflammatory, and even smaller fragments stimulate the production of anti-inflammatory cytokine (108). Da Silva, Hartl (109) reported that 40–70 μm sized chitin fragments could trigger inflammation and cytokine production via the pattern recognition receptors in mice. Numerous studies regarding the effect of chitin on the fish immune system indicated that chitin could be used as an immunostimulant when supplemented in fish diets (1, 21). However, chitin's immunomodulating effect in fish has also been suggested to be dependent on the dietary inclusion level (13, 21). For instance, it has been reported that dietary inclusion of 1% chitin increased serum lysozyme activity in common carp (22), whereas <1% inclusion did not alter serum lysozyme activity and phagocytic activity of HK leukocytes in gilthead seabream (*Sparus aurata*) (19). However, Esteban, Cuesta (19) also reported that administration of a chitin diet (<1%) enhanced seabream immune activity through the non-specific modulation of haemolytic complement activity, leucocyte respiratory burst activity and cytotoxicity in a dose-dependent and a time-dependent manner. As discussed by Sánchez-Muros, Barroso (110) dietary incorporation of chitin stimulated macrophage activity in rainbow trout. Chitin or chitosan enriched diet (1%) could also modulate the immune system and the disease resistance in *Cirrhina mrigala* (111). Chitin and its derivatives such as chitosan, were also reported to have antioxidant properties (112–114). In addition to chitin, BSFL also contain AMP (17, 18) which could also contribute for the present results. A previous study also reported that dietary inclusion of AMP could improve immunity and oxidation resistance in common carp (*Cyprinus carpio*) (20). It seems that the stimulation of the immune system has a small window of activation that might be triggered by the concentration of chitin, AMP or other components present in the BSFL containing diet. Hence, future studies should investigate the effect of graded levels of various

bioactive components of BSFL on gut health and immune response in fish.

CONCLUSION

The present study showed that replacing conventional protein sources with low to moderate levels of BSFL meal (6.25% and 12.5%) or paste (3.7% and 6.7%) reduced enterocyte steatosis in pyloric caeca, while replacing up to 25% with BSFL meal or 6.7% with paste improved distal intestine histology. The increasing BSFL meal level in the diet linearly decreased plasma C-reactive protein, while increasing BSFL paste linearly increased plasma antioxidant capacity. The dietary inclusion of BSFL meal and paste had minor effects on skin mucus proteome and immune response in Atlantic salmon.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/pride/archive/, PXD019125.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the fish experiment was conducted at Center for Fish Research at Norwegian University of Life Sciences (NMBU), which is a research facility approved by Norwegian Animal Research Authority (permit no. 109) and operates in accordance with Norwegian Regulations of 17th of June 2008 No. 822: Regulations relating to Operation of Aquaculture Establishments (Aquaculture Operation Regulations). The experimental procedures were performed in accordance with the national guidelines for the care and use of animals (The Norwegian Animal Welfare Act and the Norwegian Regulation on Animal Experimentation). All the experimental diets were formulated to meet the known nutrient requirement of salmon (NRC, 2011); thus, the fish were not exposed to nutrient deficiencies during the experiment. Insects are found in aquatic environments and part of the natural diets of salmon. The other ingredients used in the experimental diets were commonly used in commercial fish feed production. Therefore, no apparent distress in fish was expected by feeding the experimental diets containing black soldier fly larvae. The water quality parameters were maintained at optimal levels and checked frequently during the experiment. In this study no invasive techniques were applied to the fish. No surgery, administration of test substance or physical treatments were performed in live fish. Fish were randomly sampled, anaesthetized, and killed by a sharp blow to the head, in accordance with the Norwegian Animal Welfare act. Skin mucus samples were only retrieved from euthanized fish and other samples were collected after killing the fish.

AUTHOR CONTRIBUTIONS

PW, LL, and MØ contributed to conception and design of the study. PW, LL, MS, and JH involved in methodology, investigation, and analysis of data. All the authors contributed to interpretation of data and discussion. LL and MØ acquired funding. PW wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.599530/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Paper III

Insects in Atlantic salmon (*Salmo salar*) diets – Comparison between full-fat, defatted, and de-chitinized meals, and oil and exoskeleton fractions

Running header: Black solider fly larvae meals and fractions for salmon

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Abstract

The present study investigated the effect of meals and fractions of black soldier fly larvae (BSFL; *Hermetia illucens*) in diets for Atlantic salmon (*Salmo salar*) on the physical quality of feed pellets, nutrient utilization, and growth performance. Six extruded diets were produced: control diet containing fishmeal and plant protein (CD); full-fat BSFL meal diet (IM); defatted BSFL meal diet (DFIM); de-chitinized BSFL meal diet (DCIM); BSFL oil diet (IO) and BSFL exoskeleton diet (EX). The full-fat, defatted and de-chitinized meals replaced 15% of protein in the control diet. An eight-week study was conducted using salmon with average 28 g initial weight. The pellet durability was high in all the diets, but the full-fat and de-chitinized meals in the diets numerically reduced pellet hardness, expansion, and water stability. The full-fat and de-chitinized meals improved growth rate of salmon, whilst defatted meal, oil and exoskeleton supported similar growth performance as the control. The improved growth rate of fish fed full-fat meal diet was accompanied by higher feed intake. Defatted and de-chitinized meals and insect fractions gave lower fish growth rate than full-fat meal, but defatted meal gave a better feed conversion ratio than full-fat meal. Defatted meal, de-chitinized meal and exoskeleton reduced protein digestibility in fish, however; defatted meal increased the digested protein retention. Neither insect meals nor fractions affected lipid digestibility, whereas full-fat meal diet fed fish had lower lipid retention than for the other insect diets fed fish. In conclusion, use of full-fat BSFL meal improved feed intake and growth rate of salmon when replacing 15% of dietary protein. Use of defatted meal gave similar growth performance as the control diet fed fish, but improved feed utilization compared to the full-fat meal diet. De-chitinization did not further improve the nutritional value of BSFL for salmon.

Key words: black soldier fly larvae, insect fractions, pellet quality, fish growth performance, nutrient utilization

Conflicts of interest statement

The authors declare no competing conflicts of interest.

1. Introduction

Black soldier fly larvae (BSFL) (*Hermetia illucens*) have a great potential as a sustainable novel feed ingredient in fish feed due to its high nutrient value (Barroso *et al.*, 2014; Makkar *et al.*, 2014; Nogales-Mérida *et al.*, 2019) and its ability to utilize wide variety of organic materials efficiently (Wang and Shelomi, 2017). Numerous studies reported the effect of dietary inclusion of BSFL on nutrient utilization, growth performance, and health of various fish species including Atlantic salmon (*Salmo salar*) (Belghit *et al.*, 2019a; Belghit *et al.*, 2018; Fisher *et al.*, 2020; Lock *et al.*, 2016; Weththasinghe *et al.*, 2021a; Weththasinghe *et al.*, 2021b).

BSFL contain the three major fractions protein, lipid and exoskeleton (Müller *et al.*, 2017; Ravi *et al.*, 2020). The results of previous studies suggest that these fractions might differently affect the nutrient utilization and growth performance of fish. In previous studies, dietary inclusion of moderate levels of BSFL meal (<20%) did not compromise growth performance in salmon (Belghit *et al.*, 2019a; Fisher *et al.*, 2020; Weththasinghe *et al.*, 2021a), whereas higher inclusion levels (>20%) reduced growth rate (Fisher *et al.*, 2020; Weththasinghe *et al.*, 2021a). In contrast, Belghit *et al.* (2018) reported that de-chitinized BSFL protein meal in diets did not compromise the growth performance of salmon even at 60% inclusion level. The reduction of growth rate at higher BSFL levels was thus suggested to be attributed to the presence of chitin in the exoskeleton of BSFL (Dumas *et al.*, 2018; Weththasinghe *et al.*, 2021a). Dietary inclusion of BSFL meal has also shown to reduce the nutrient digestibility in salmon (Belghit *et al.*, 2018; Weththasinghe *et al.*, 2021a). This might also be due to chitin (Hansen *et al.*, 2010; Karlsen *et al.*, 2017; Shiau and Yu, 1999) or high level of saturated fatty acids (Hua and Bureau, 2009) present in BSFL. Further, Weththasinghe *et al.* (2021a) reported that the lipid retention decreased at high dietary inclusion of BSFL meal in salmon. It was hypothesized that this was caused by high level of lauric acid (C12:0) in BSFL, which is preferred as a substrate for oxidation in salmon (Belghit *et al.*, 2019b; Renna *et al.*, 2017). On the other hand, bioactive compounds present in BSFL, such as antimicrobial peptides (Müller *et al.*, 2017; Park *et al.*, 2015; Park *et al.*, 2014), chitin, as well as lauric acid possess antimicrobial properties (Askarian *et al.*, 2012; Skřivanová *et al.*, 2006; Spranghers *et*

al., 2018), which could have a positive effect on gut microbiota, gut health and subsequently the growth performance in fish.

The quality and antimicrobial peptides in the protein fraction, fatty acid composition in the lipid fraction and the chitin in the exoskeleton fraction of BSFL might have contrasting effects on the nutrient utilization and growth performance in fish. However, to the best of our knowledge, such effects of different fractions of BSFL have still not been evaluated in a single study. Further, limited information exists on the BSFL fractions on technical quality of extruded feed pellets. Therefore, the present study investigated the effects of meals (full-fat, defatted and de-chitinized) and fractions (oil and exoskeleton) of BSFL in extruded diets on physical pellet quality, nutrient utilization, and growth performance in Atlantic salmon pre-smolts.

2. Materials and Methods

2.1 Rearing and processing of black soldier fly larvae

BSFL were reared and processed into meals or fractions at HiProMine S.A., Robakowo, Poland. The BSFL were fed *ad libitum*. The dry matter (DM) content of BSFL feed was normalized to 22% by adding wheat middlings (17%) to fresh vegetables and fruit pre-consumer waste mix, consisting of apples (15%), carrots (50%), potatoes (15%), and cabbage (20%). The feed mixture was ground (2000 rpm, 55 kW; HPM milling system, Robakowo, Poland) to sieve through a 2 mm screen. In accordance with EC regulation (no 1069/09), the BSFL feed did not contain any animal products.

The larvae were harvested on the seventh day of rearing, sieved using a 3 mm screen, and washed with water on drum separator at 90 ^{0}C for 10 min (HPM cleaning system, Robakowo, Poland). A batch of BSFL was divided into two parts for further processing. The first part was dried at 110 °C for 1 h and then at 80 °C for 23 h until a constant weight was reached using a chamber air flow dryer (HiProMine S.A., Robakowo, Poland) for full-fat BSFL meal production, then a part of it was defatted to obtain partially defatted meal and oil with use of oil press (Reinartz, model AP14/22, Neuss, Germany). The second part of BSFL was used to obtain partially de-chitinized meal and exoskeleton fraction. For the separation of partially de-chitinized BSFL and

exoskeleton fraction, the mechanical de-chitinization was applied using food press twin-screw processor with 0.3 mm screen diameter (Angel Juicer, model 7500, Busan, Korea). The de-chitinized BSFL and exoskeleton were dried at 110 °C for 1 h and then at 80 °C for 23 h until a constant weight was reached using a chamber air flow dryer (HiProMine S.A., Robakowo, Poland). The full-fat and de-chitinized meals and BSFL oil were tested for *Salmonella* at Eurofins Agro Testing Norway AS, Moss, Norway and *Salmonella* were not detected. All the products were stored at 4 °C before use for feed production. The chemical compositions of BSFL ingredients are shown in Table 1 and Table S1.

Table 1: Chemical composition (%, as is) of meals and fractions of black soldier fly larvae (BSFL)

Nutrient	Full-fat BSFL meal	Defatted BSFL meal	De-chitinized BSFL meal	BSFL oil	BSFL exoskeleton
Dry matter	88.1	86.8	90.7	99.0	91.7
Crude protein	37.6	51.4	31.2	2	59.9
Crude lipid	29.6	11.7	43.5	96.0[a]	11.1
Ash	5.82	8.03	6.97	1.03	5.59
Chitin	7.05	9.65	2.15		19.8
Amino acids[b]					
Essential amino acids					
Methionine	0.41	0.56	0.42	NA	0.29
Threonine	1.16	1.58	1.10	NA	1.71
Valine	1.46	1.99	1.33	NA	2.75
Isoleucine	1.23	1.68	1.17	NA	1.91
Leucine	2.02	2.75	1.91	NA	3.11
Phenylalanine	1.24	1.69	1.19	NA	1.73
Histidine	0.95	1.30	0.88	NA	1.70
Lysine	1.92	2.62	1.90	NA	2.14
Arginine	1.48	2.03	1.42	NA	2.09
Tryptophan	0.22	0.29	0.23	NA	0.10
Non-essential amino acids					
Cysteine	0.23	0.32	0.23	NA	0.24
Aspartic acid	2.48	3.39	2.40	NA	3.34
Serine	1.27	1.73	1.17	NA	2.30
Glutamic acid	3.97	5.42	3.78	NA	5.89
Proline	1.96	2.68	1.78	NA	3.87
Glycine	1.52	2.08	1.37	NA	3.07
Alanine	2.25	3.07	2.07	NA	4.01
Tyrosine	2.56	3.50	2.25	NA	5.74
Total amino acid	28.33	38.68	26.61	NA	45.97

[a] Calculated as Crude lipid = Dry matter – (Crude protein + Ash). [b] Water corrected values. The amino acids compositions of full-fat and de-chitinized BSFL meals are calculated values based on the analysed amino acid compositions of defatted BSFL meal and BSFL exoskeleton. NA: Not analysed.

2.2 Experimental diets

Six experimental diets were formulated to have similar amino acid profiles and lipid contents, and to meet or exceed NRC (2011) requirements for all essential amino acids and other nutrients for Atlantic salmon. The experimental diets comprised of a control diet (CD) containing fishmeal, soy protein concentrate (SPC) and corn gluten

as protein sources, and fish oil as the main lipid source; three diets with full-fat BSFL meal (IM), defatted BSFL meal (DFIM) or de-chitinized BSFL meal (DCIM), replacing 15% of protein from fishmeal, SPC and corn gluten in the control diet; a diet containing BSFL oil (IO) and a diet containing BSFL exoskeleton (EX). The BSFL oil and exoskeleton were added to the diets to match the BSFL oil and chitin contents in full-fat BSFL meal diet, respectively. The internal marker, Yttrium oxide was added in all the diets for the determination of apparent digestibility coefficient (ADC) of nutrients. Crystalline methionine and monocalcium phosphate were added to all the diets to ensure that the diets met or exceeded the methionine (0.7%, DM basis (NRC, 2011)) and phosphorous (P) (0.8%, DM basis (NRC, 2011)) requirements of Atlantic salmon, respectively. Table 2 shows the ingredient and chemical composition of the six experimental diets.

Table 2: Ingredient and chemical composition of experimental diets with meals or fractions of black soldier fly larvae (BSFL)[1]

	CD	IM	DFIM	DCIM	IO	EX
Ingredients (%)						
Fishmeal[a]	22.50	18.57	18.57	18.57	22.50	21.78
Soy protein concentrate[b]	34.50	28.48	28.48	28.48	34.50	33.39
Corn gluten[c]	5.50	4.54	4.54	4.54	5.50	5.32
Full-fat BSFL meal[d]	0.00	20.36	0.00	0.00	0.00	0.00
Defatted BSFL meal[e]	0.00	0.00	14.89	0.00	0.00	0.00
De-chitinized BSFL meal[f]	0.00	0.00	0.00	24.53	0.00	0.00
BSFL oil[g]	0.00	0.00	0.00	0.00	6.24	0.00
BSFL exoskeleton[h]	0.00	0.00	0.00	0.00	0.00	7.20
Wheat flour[i]	14.65	14.65	14.65	14.65	14.65	14.65
Fish oil[j]	16.00	10.47	14.75	5.82	10.05	15.36
Methionine[k]	0.20	0.20	0.20	0.20	0.20	0.20
Choline chloride[l]	0.15	0.15	0.15	0.15	0.15	0.15
Yttrium[m]	0.01	0.01	0.01	0.01	0.01	0.01
Vit/min premix[n]	0.65	0.65	0.65	0.65	0.65	0.65
Monocalcium Phosphate[o]	0.80	0.80	0.80	0.80	0.80	0.80
Wheat bran[p]	5.04	1.13	2.31	1.60	4.74	0.49
Chemical composition (%, as is)						
Dry matter	91.6	91.9	93.0	92.9	93.3	91.7
Crude protein	46.6	44.4	46.0	46.6	46.6	47.3
Crude lipid	19.6	20.3	17.8	12.9	18.3	17.0
Starch	13.1	12.2	12.4	12.4	12.6	11.7
Ash	6.70	6.60	6.77	7.23	6.70	6.61
Chitin[q]		1.44	1.44	0.53		1.43
Macro mineral composition (%, as is)						
Total phosphorous	1.13	1.10	1.10	1.24	1.11	1.10
Calcium	1.11	1.19	1.29	1.28	1.28	1.19
Magnesium	0.18	0.19	0.20	0.23	0.17	0.17
Amino acid composition[r] (%, as is)						
Essential amino acids						
Methionine	0.98	0.90	0.90	0.94	0.90	0.94
Threonine	1.59	1.48	1.51	1.56	1.58	1.59
Valine	1.55	1.52	1.54	1.51	1.56	1.64

Isoleucine	1.78	1.65	1.70	1.75	1.80	1.79
Leucine	3.40	3.05	3.12	3.23	3.40	3.36
Phenylalanine	1.99	1.76	1.81	1.90	1.97	1.88
Histidine	1.07	1.04	1.05	1.06	1.07	1.09
Lysine	2.62	2.47	2.49	2.59	2.60	2.58
Arginine	2.72	2.42	2.46	2.57	2.71	2.63
Non-essential amino acids						
Cysteine	0.45	0.41	0.41	0.43	0.44	0.43
Aspartic acid	4.13	3.68	3.82	3.99	4.12	4.05
Serine	1.84	1.66	1.71	1.71	1.80	1.85
Glutamic acid	8.05	7.08	7.32	7.55	8.00	7.90
Proline	2.12	2.05	2.17	2.07	2.16	2.32
Glycine	1.70	1.64	1.67	1.63	1.69	1.78
Alanine	1.96	1.99	2.02	1.91	1.96	2.11
Tyrosine	1.21	1.41	1.43	1.24	1.25	1.48
Total amino acid	39.2	36.2	37.1	37.6	39.0	39.4

[1] CD: Control diet. IM: Full-fat BSFL meal diet. DFIM: Defatted BSFL meal diet. DCIM: De-chitinized BSFL meal diet. IO: BSFL oil diet. EX: BSFL exoskeleton diet. [a] LT fishmeal, Norsildmel AS, Bergen, Norway. [b] Soy protein concentrate, Tradkon SPC HC-200, Sojaprotein, Becej, Serbia. [c] Corn gluten meal, Baolingbao Biology, Shangdong Yucheng, China. [d] Full-fat black soldier fly larvae meal, HiProMine S.A., Poznań, Poland. [e] Defatted black soldier fly larvae meal, HiProMine S.A., Poznań, Poland. [f] De-chitinized black soldier fly larvae meal, HiProMine S.A., Poznań, Poland. [g] Black soldier fly larvae oil, HiProMine S.A., Poznań, Poland. [h] Black soldier fly larvae exoskeleton, HiProMine S.A., Poznań, Poland. [i] Wheat flour 78%, batch number: 5093060546, Norgesmøllene, Bergen, Norway. [j] Fish oil, Norsildmel AS, Bergen, Norway. [k] L-methionine, Bestamino™ Cj Cheiljedang, Seoul, Korea. [l] Choline chloride 70%, $C_5H_{14}ClNO$, 139.6g/mol, Vilomix, Hønefoss, Norway. [m] Yttrium oxide (Y_2O_3) Metal Rare Earth Limited, Shenzhen, China. [n] Vit/min premix, Farmix, Trouw Nutrition, LA Putten, The Netherlands. Per kg of feed; Vitamin A 3250 IU, Vitamin D3 1950 IU, Vitamin E 260 IU, Vitamin K3 13 mg, Vitamin B1 20 mg, Vitamin B2 33 mg, d-Ca-pantothenate 52 mg, Niacinamide 98 mg, Vitamin B6 20 mg, Folic acid 6.5 mg, Vitamin B12 33 mcg, Vitamin C 163 mg, Biotin 358 mcg, Calcium iodate, anhydrous, Iodine 3.9 mg, Manganese (II) oxide, Manganese 20 mg, Zinc oxide, Zinc 137 mg. [o]

Monocalcium Phosphate, Monohydrate, BOLIFOR® MCP-F, Yara Phosphates Oy, Animal Nutrition, Sweden. [p] Wheat bran, Norgesmøllene, Bergen, Norway. [q] Calculated based on the chitin content of the respective BSFL ingredient and its inclusion level in the diet. [r] Water corrected values.

2.3 Production of experimental diets

The extruded experimental diets were produced at the Norwegian University of Life Sciences (NMBU) Centre for Feed Technology (Fôrtek), Ås, Norway. First, all the weighed ingredients (except micro ingredients, fish oil and BSFL oil) were mixed for 3 min in an ISDECA mixer (60-l paddle-mixer, prototype, Fôrtek, Forberg, Norway). The material mixture was then ground in a Hammer mill (Bill bliss, horizontal, 18.5 kW, USA) with a 1 mm sieve, and mixed with micro-ingredients. The feed mash of full-fat and de-chitinized meal diets were ground again in the Hammer mill (Bill bliss, horizontal, 18.5 kW, USA) with a 0.5 mm sieve to prevent the production of coarse particles and less integrated pellets after the extrusion. The diets were extruded in a five-section Bühler twin-screw extruder (BCTG 62/20 D, Uzwil, Switzerland) fitted with four 2.5 mm die holes. The extruder operated without a pre-conditioner and the screw configuration shown previously by Weththasinghe *et al.* (2021a) was used during the extrusion of diets. The feed mash was fed into the first section of the extruder using a small K-tron feeder. The screw speed was increased when the extrusion of insect ingredients containing diets. The extruded pellets were dried at 60 °C for 1 h using fan heaters (15KW, Inelco heaters, Dania-heater 15 kW, Fjerritslev, Denmark). The pellets were then cooled at room temperature and vacuum coated with fish oil and/or BSFL oil in Gentle Vacuum Coater (GVC) – 80 prototype (Fôrtek, Amandus-Kahl).

2.4 Fish study, rearing facilities, and sampling

The fish study was conducted at the Centre for Fish Research, NMBU, Ås, Norway. The study consisted of 900 Atlantic salmon (Aqua Gen Atlantic QLT-innOva SHIELD) with an average 28 g initial weight. The fish were randomly distributed into 18 fiberglass tanks (50 fish per tank) with recirculated freshwater (average temperature of 14.4±0.4 °C). The tanks were supplied water at 6 l/min and dissolved oxygen levels

were kept above 7.0 mg/l in the outlet water. The study lasted for eight weeks and triplicate tanks of salmon were fed one of the six experimental diets. The fish were kept under continuous light and fed *ad libitum* (i.e. 10% excess) with electrically driven belt feeders according to a six-hours feeding program per day. The uneaten feed was collected daily using the wedge wire screens fitted to the outlet of tanks as explained by Shomorin *et al.* (2019) and daily feed intake in each tank was quantified according to Helland *et al.* (1996). The fish mortality was checked daily. Initial and final body weights of fish were measured at the start and end of the eight-week study period. Fifteen fish at the start and five fish per tank at end of the study were sampled, pooled, homogenized and freeze dried for whole body composition analysis as explained by Weththasinghe *et al.* (2021a). In addition, at the end of the study, another six fish from each tank were randomly sampled, anesthetized, euthanized by a sharp blow to the head and weighed individually. The fish were dissected, and the liver was removed. The attached adipose tissue and fat around liver were removed and the liver weight was measured to calculate hepatosomatic index (HSI). After the eight-week study period, fish were fed with experimental diets for two additional weeks for faeces collection. Fish were carefully stripped three times with seven days interval for faecal collection from the posterior intestine according to Austreng (1978). The stripped faeces were stored immediately at −20 °C prior to freeze drying. Tricaine methanesulfonate (MS-222) (80 mg/l) was used to anesthetize fish during weighing, sampling, and stripping. All the experimental procedures were conducted adhering to the guidelines for the care and use of animals in Norway (The Norwegian Animal Welfare Act and the Norwegian Regulation and Animal Experimentation).

2.5 Physical pellet quality analysis

The bulk density of the uncoated pellets was measured after the extrusion. The other physical quality parameters were measured in oil-coated pellets. As explained by Hansen *et al.* (2010), Doris pellet tester (AKVAsmart, Bryne, Norway) was used to estimate pellet durability. The durability of the pellets was measured in triplicates using a 2 mm screen. Hardness was measured using 15 pellets with average length and diameter from each diet with a Texture analyser with a 5 kg load cell (Tinius Olsen, H5KT, Salfords, England) according to Øverland *et al.* (2009). The expansion

of the extruded pellets was determined by measuring the width of 30 randomly selected pellets per diet using the Texture analyser (Tinius Olsen, H5KT, Salfords, England). The mean value of the time required for 10 randomly picked pellets to sink 1 m in 17 °C tap water was recorded to determine the sinking velocity of the pellets. The method explained by Baeverfjord *et al.* (2006) was used to measure the water stability of pellets within 30 and 60 min.

2.6 Chemical analysis

The feed and freeze-dried faeces and fish were ground. The samples were oven dried at 104 °C until a constant weight was reached to measure DM content. Ash contents were determined by combustion at 550 °C. The nitrogen (N) contents of BSFL ingredients and fish were estimated by Kjeldahl method according to Commission Regulation (EC) No 152/ 2009. The N contents of faeces were analysed by CHNS Elemental Analyzer (Vario El Cube elemental analyser system GmbH, Hanau, Germany). The crude protein content was determined as N × 6.25. The N content in diets was measured by both methods and the values obtained by CHNS Elemental Analyzer were used for protein digestibility estimates, whereas the values obtained by Kjeldahl method were used for protein retention estimates. The chitin contents of BSFL ingredients were measured as explained by Finke (2007). The crude lipid contents of BSFL ingredients, faeces and fish were determined after extraction with petroleum ether and acetone (70/30) using an Accelerated Solvent Extractor (ASE200; Dionex Corp., Sunnyvale, CA, USA). The crude lipid contents of diets were measured by acid hydrolysis and ether extraction according to NMKL 160 (modified) at Eurofins Agro Testing Norway AS, Moss, Norway. The method explained by McCleary *et al.* (1994) was used with some modifications to measure starch content. Briefly, the starch in the samples were converted into glucose using heat-stable α-amylase and amyl glucosidase-enzymes, and glucose content was measured by a spectrometer (RX4041 Randox Daytona+, England). The Biochrom 30 Amino Acid Analyser (Biochrom Ltd., Cambridge, UK) was used to analyse amino acid contents according to Commission Regulation (EC) No 152/2009. The fatty acid content of BSFL oil was determined by synthesizing the fatty acid to fatty acid methyl esters (FAME) using Trace GC Ultra gas chromatograph (Thermo Fisher Scientific, US) as

explained by O'fallon *et al.* (2007). Yttrium (Y), calcium and magnesium contents were measured after acid decomposition in a microwave digestion system (Start D, Milestone Srl, Italy) using a microwave plasma atomic emission spectrometer (MP-AES 4200, Agilent Technologies, USA). Total P contents were analysed using a commercial spectrophotometric kit (PH8328, Randox laboratories, County Antrim, UK) after combustion and acid digestion according to Commission Regulation (EC) No 152/2009.

2.7 Calculations

The pellet expansion (%) was calculated as ((Width of pellet - Die diameter)/ Die diameter) × 100. Specific mechanical energy (Wh/kg) was calculated as ($2 \times \pi \times 60^{-1}$) × (Screw speed × Torque × Throughput). Specific growth rate (SGR) (%) was calculated as [(ln (Fish final body weight (g)) – ln (Fish initial body weight (g)))/ Study period (days)] × 100. Feed conversion ratio (FCR) was calculated as Feed intake of fish (g DM)/ Fish body weight gain (g). HSI (%) was calculated as Weight of liver (g)/Fish body weight (g) × 100. ADC of nutrients (%) was calculated as (1- [(Y concentration in diet/Y concentration in faeces) × (Nutrient concentration in faeces/Nutrient concentration in diet)]) ×100. Faecal excretion of nutrients (%) was calculated as (100 – ADC of nutrients). The dissolved nutrient fraction (g kg^{-1}) was calculated as [Nutrient digested (g) – (Final nutrient content in fish (g) – Initial nutrient content in fish (g))] / Fish body weight gain (kg). Protein and lipid efficiency ratios were calculated as Fish body weight gain (g)/ Protein or lipid intake (g). Apparent nutrient retention (% intake) was calculated as [(Final nutrient content in fish (g) – Initial nutrient content in fish (g))/ Nutrient intake (g)] × 100. Nutrient retention (% digested nutrient) was calculated as [(Final nutrient content in fish (g) – Initial nutrient content in fish (g))/ Nutrient digested (g)] × 100.

2.8 Statistical analysis

The data were analysed using one-way ANOVA and Tukey's multiple comparison test was used to compare the means. Differences at $p<0.05$ were regarded as significant. The analyses were performed using IBM SPSS Statistics 27 software.

3. Results

3.1 Feed production and physical pellet quality

The lipid-rich full-fat and de-chitinized insect meals increased the lipid content of the feed mash prior to extrusion. To compensate for this, the throughput and water addition to the extruder were increased to obtain pellets with desirable physical quality in the full-fat and de-chitinized insect meals included diets. In addition, the fifth barrel of the extruder was cooled to obtain the desired bulk density in these two diets, and this led to reduced temperature in the fifth barrel and the die. Further, decreased die pressure and torque were observed during the extrusion process of these two diets (Table 3).

Table 3: Extruder parameters during the production of experimental diets with meals or fractions of black soldier fly larvae (BSFL)[1]

Extruder parameter	CD	IM	DFIM	DCIM	IO	EX
Throughput (kg/h)	35	40	30	40	30	30
Barrel 1 (°C)	42	22	32	26	37	40
Barrel 2 (°C)	86	33	49	37	60	77
Barrel 3 (°C)	107	67	108	84	114	113
Barrel 4 (°C)	113	107	123	105	127	123
Barrel 5 (°C)	111	84	122	92	127	122
Die temperature (°C)	105	91	122	99	127	122
Die pressure (Bar)	23	4	12.3	2.8	25.2	22.4
Screw speed (rpm)	270	400	400	425	385	400
Torque (%)	53	43	48	38	60	49
Drive power (kW)	6.5	7.6	8.8	7.3	10.7	9
SME[a] (Wh/kg)	449	510	616	440	771	630
Water addition[b] (kg/h)	14	15.5	14	16.5	14	14
Lipid[c] (%)	2.50	7.97	3.75	12.64	2.48	3.01

[1] CD: Control diet. IM: Full-fat BSFL meal diet. DFIM: Defatted BSFL meal diet. DCIM: De-chitinized BSFL meal diet. IO: BSFL oil diet. EX: BSFL exoskeleton diet. [a] Specific mechanical energy. [b] Water added into the extruder. [c] Percentage of lipid in the feed mash prior to extrusion.

The pellet durability measured by the Doris pellet tester showed an overall high physical quality of the pellets. However, the full-fat and de-chitinized insect meal included diets showed numerically lower pellet hardness, expansion, and water

stability as well as numerically higher bulk density after the extrusion (Table 4 and Figure 1).

Table 4: Physical pellet quality of experimental diets with meals or fractions of black soldier fly larvae (BSFL)[1]

Pellet quality parameter	CD	IM	DFIM	DCIM	IO	EX	SEM[2]
Bulk density (g/l)	566.7	586.3	540.3	580	528.7	560.7	5.36
Durability (%)	97.7	96.4	98.0	93.7	98.6	98.3	0.42
Hardness (N)	18.2	12.3	13.4	11.1	23.6	14.0	0.82
Expansion (%)	18.4	1.67	17.4	-0.81	32.3	21.8	0.89
Sinking velocity (m/S)	0.10	0.08	0.09	0.08	0.08	0.09	0.002

[1] CD: Control diet. IM: Full-fat BSFL meal diet. DFIM: Defatted BSFL meal diet. DCIM: De-chitinized BSFL meal diet. IO: BSFL oil diet. EX: BSFL exoskeleton diet. [2] Standard error mean.

3.2 Growth performance

Only four fish died throughout the study period. The fish fed full-fat insect meal showed higher feed intake, accompanied by higher final body weight and SGR compared with the fish fed the control and the other insect diets. In addition, the inclusion of de-chitinized insect meal in the diet also increased final body weight and SGR of fish compared to the control diet but decreased compared to the full-fat meal diet. The defatted insect meal, oil and exoskeleton supported similar final body weight and SGR as the control diet fed fish. Although the FCR of the fish fed insect diets did not differ from the fish fed control diet, defatted meal and exoskeleton included diets gave lower FCR in fish than the full-fat meal diet. The HSI was not affected by the dietary treatments (Table 5).

Table 5: Performance of fish fed experimental diets with meals or fractions of black soldier fly larvae (BSFL)[1]

	CD	IM	DFIM	DCIM	IO	EX	SEM[2]	*p* value
Initial body weight (g)	28.3	28.3	28.3	28.3	28.3	28.3	0.007	0.14
Final body weight (g)	98.4^{c}	114.8^{a}	104.2^{bc}	107.1^{b}	99.8^{bc}	104.7^{bc}	1.41	<0.001
Specific growth rate (%)	2.01^{c}	2.26^{a}	2.10^{bc}	2.14^{b}	2.03^{bc}	2.11^{bc}	0.02	<0.001
Feed intake (g DM/fish)	53.1^{b}	68.8^{a}	55.9^{b}	59.4^{b}	55.7^{b}	57.1^{b}	1.31	<0.001
Feed conversion ratio	0.76^{ab}	0.80^{a}	0.74^{b}	0.76^{ab}	0.78^{ab}	0.75^{b}	0.006	0.013
Hepatosomatic index (%)	1.32	1.35	1.42	1.36	1.34	1.37	0.01	0.48

[1] CD: Control diet. IM: Full-fat BSFL meal diet. DFIM: Defatted BSFL meal diet. DCIM: De-chitinized BSFL meal diet. IO: BSFL oil diet. EX: BSFL exoskeleton diet. [2] Standard error mean. Values in the same row with different superscripts are significantly different at $p<0.05$.

3.3 Digestibility, faecal excretion, and dissolved fraction of nutrients

ADC of DM, crude lipid and starch were not affected by the dietary treatments. The full-fat insect meal and insect oil diets fed fish showed similar ADC of protein as the control diet fed fish. The defatted meal, de-chitinized meal and exoskeleton containing diets fed fish had lower ADC of protein, accompanied by increased faecal N excretion compared to the control diet fed fish. However, ADC of protein did not differ between full-fat insect meal diet and other insect diets (Table 6).

The fish fed full-fat and de-chitinized insect meals containing diets showed lower faecal excretion of P, whereas fish fed full-fat meal, defatted meal and insect oil containing diets had lower faecal excretion of calcium, than the fish fed control diet. The dissolved fractions of N and P in insect meals or fractions containing diets did not differ from the control diet, except defatted meal diet, in which the dissolved fraction of N was lower than the control and full-fat meal diets. Further, the defatted insect

meal fed fish showed a lower dissolved P fraction compared to de-chitinized insect meal diet fed fish (Table 6).

Table 6: Apparent digestibility coefficient (%), faecal excretion (%) and dissolved fraction of nutrients (g/kg of fish body weight gain) of fish fed experimental diets with meals or fractions of black soldier fly larvae (BSFL)[1]

	CD	IM	DFIM	DCIM	IO	EX	SEM[2]	*p* value
Apparent digestibility coefficients								
Dry matter	73.5	75.6	74.8	74.3	73.5	73.9	0.27	0.175
Crude protein	89.4^{a}	88.0^{abc}	87.4^{bc}	86.9^{c}	89.0^{ab}	86.7^{c}	0.28	0.001
Crude lipid	97.6	97.6	97.1	95.7	96.9	97.1	0.23	0.16
Starch	84.2	82.0	82.8	83.2	84.9	81.6	0.37	0.054
Ash	21.8^{b}	28.4^{ab}	31.0^{a}	28.0^{ab}	21.9^{b}	25.7^{ab}	1.02	0.016
Faecal excretions								
Phosphorous	49.0^{ab}	43.2^{c}	43.5^{bc}	42.2^{c}	52.9^{a}	47.8^{abc}	1.01	<0.001
Calcium	90.4^{a}	78.0^{b}	76.1^{b}	82.5^{ab}	79.2^{b}	83.3^{ab}	1.30	0.002
Magnesium	69.8	57.2	60.9	57.7	59.7	56.4	1.94	0.398
Nitrogen	10.6^{c}	12.0^{abc}	12.6^{ab}	13.2^{a}	11.0^{bc}	13.3^{a}	0.28	0.001
Dissolved fractions								
Nitrogen	25.3^{a}	25.0^{a}	21.5^{b}	23.2^{ab}	25.2^{a}	23.7^{ab}	0.42	0.020
Phosphorous	0.71^{ab}	0.91^{ab}	0.05^{b}	1.06^{a}	0.29^{ab}	0.57^{ab}	0.11	0.020

[1] CD: Control diet. IM: Full-fat BSFL meal diet. DFIM: Defatted BSFL meal diet. DCIM: De-chitinized BSFL meal diet. IO: BSFL oil diet. EX: BSFL exoskeleton diet. [2] Standard error mean. Values in the same row with different superscripts are significantly different at $p<0.05$.

3.4 Nutrient retention

The retention of ingested protein was not affected by dietary treatments. The protein efficiency ratio (PER) of fish fed insect diets did not differ from the fish fed control diet. Further, the retentions of digested protein of fish fed insect diets, except defatted insect meal diet, were also similar as the fish fed control diet. In defatted insect meal diet, the retention of digested protein was higher compared to both control and full-fat insect meal diets. Fish fed the insect diets except full-fat meal diet showed higher lipid efficiency ratio (LER) than the control diet fed fish, where the fish fed de-chitinized meal showed the highest. The retention of both ingested and digested lipid in fish fed the insect diets did not differ from the control diet, except for those fed the de-chitinized meal diet, which had a higher retention of lipid. Both LER and lipid

retentions were lower in the full-fat meal diet than the diets containing other insect meals or fractions. The apparent P retention was highest in defatted insect meal diet fed fish (Table 7).

Table 7: Nutrient retention parameters in fish fed experimental diets with meals or fractions black soldier fly larvae (BSFL)[1]

	CD	IM	DFIM	DCIM	IO	EX	SEM[2]	*p* value
Protein efficiency ratio	2.60^{ab}	2.61^{ab}	2.75^{a}	2.64^{ab}	2.57^{b}	2.59^{ab}	0.02	0.028
Lipid efficiency ratio	6.17^{d}	5.71^{e}	7.10^{b}	9.54^{a}	6.55^{c}	7.21^{b}	0.30	<0.001
Apparent protein retention (% intake)	48.4	47.4	50.5	48.6	48.6	48.3	0.32	0.087
Apparent protein retention (% digested protein)	54.1^{b}	53.8^{b}	57.8^{a}	56.0^{ab}	54.6^{ab}	55.7^{ab}	0.42	0.027
Apparent lipid retention (% intake)	88.3^{bc}	77.9^{c}	97.8^{b}	128.7^{a}	96.7^{b}	94.1^{b}	3.91	<0.001
Apparent lipid retention (% digested lipid)	90.4^{bc}	79.8^{c}	100.8^{b}	134.5^{a}	99.7^{b}	96.9^{b}	4.21	<0.001
Apparent phosphorous retention (% intake)	43.4^{b}	47.2^{b}	55.9^{a}	47.3^{b}	44.0^{b}	45.9^{b}	1.16	0.003

[1] CD: Control diet. IM: Full-fat BSFL meal diet. DFIM: Defatted BSFL meal diet. DCIM: De-chitinized BSFL meal diet. IO: BSFL oil diet. EX: BSFL exoskeleton diet. [2] Standard error mean. Values in the same row with different superscripts are significantly different at $p<0.05$.

4. Discussion

The present study investigated the effect of dietary inclusion of meals and fractions of BSFL on physical feed pellet quality, nutrient utilization, and growth performance in Atlantic salmon pre-smolts. The results provide important information to determine how the BSFL should be processed to optimize its use in extruded diets for salmon.

4.1 Feed production and physical pellet quality

The high level of lipid in full-fat and de-chitinized insect meals increased the lipid content in the feed mash during extrusion. As explained in Weththasinghe *et al.* (2021a) and Hansen *et al.* (2011), increase in lipid content in the mash can result in reduced dough temperature and starch gelatinization during extrusion, that further can lead to a lower physical quality of extruded pellets. This might explain the numerically lower hardness observed in full-fat and de-chitinized insect meals included diets in the present study. The higher bulk densities of full-fat and de-chitinized insect meal diets were accompanied by lower pellet expansion. The reduced pellet expansion in these two diets is in line with previous reports indicating that increased lipid content in feed mash decreased extrudate expansion (Hansen *et al.*, 2011; Ilo *et al.*, 2000; Weththasinghe *et al.*, 2021a). High lipid levels in the feed mash can reduce the pressure during the extrusion, resulting in poor expansion (Ottoboni *et al.*, 2018). In addition, the decreased pellet expansion in full-fat and de-chitinized insect meal diets might also be related to lower temperature in the fifth barrel of the extruder (Bandyopadhyay and Rout, 2001; Kothakota *et al.*, 2013; Pathania *et al.*, 2013). As shown by Hansen *et al.* (2011), the increased level of lipid content in the feed mash followed with reduced pellet expansion is also, most probably, explaining the reduced pellet water stability of full-fat and de-chitinized insect meal diets in the present study. Previous studies also showed reduced water stability of extruded fish feed pellets containing full-fat BSFL meal (Rawski *et al.*, 2020; Weththasinghe *et al.*, 2021a).

4.2 Nutrient utilization and growth performance in salmon

In the present study, the replacement of 15% of dietary protein with full-fat insect meal, rather than with processed BSFL meal by separation of lipid or exoskeleton fractions supported higher growth rate, and is, thus, more resource efficient and less costly. The improvement in growth rate of fish fed the full-fat meal diet can be a result of the higher feed intake of fish fed this diet. The increased feed intake indicates that moderate level of full-fat BSFL meal in diets (20%) might increase palatability for salmon. Rawski *et al.* (2020) also reported that dietary inclusion of full-fat BSFL meal (10-30%) increased feed acceptance in Siberian sturgeon (*Acipenser baerii*). In contrast, our previous study showed that 8-32% full-fat BSFL meal in diets had no effect on feed intake of salmon (Weththasinghe *et al.*, 2021a). In the present study, the improved feed intake might be due to lower pellet water stability of full-fat meal diet as many water-soluble nutrients are known chemo-attractants and feed stimulants (Simon *et al.*, 2021). In addition to the improved feed intake, other processes may also be involved in increase in growth rate of salmon fed full-fat insect meal in the present study. BSFL are rich in bioactive compounds such as chitin (Caligiani *et al.*, 2018; Finke, 2013), antimicrobial peptides (Müller *et al.*, 2017; Park *et al.*, 2015; Park *et al.*, 2014) and medium-chain fatty acid, lauric acid (C12:0) (40% of the total fatty acid) (Table S1). These compounds are shown to possess antimicrobial properties (Askarian *et al.*, 2012; Skřivanová *et al.*, 2006; Spranghers *et al.*, 2018), which could have a positive effect on gut health and growth performance in fish. Thus, it is possible that the improved growth of fish fed full-fat meal diet might partially be due to the functional properties of BSFL protein, lipid, and exoskeleton fractions. However, in contrast to the present results, replacing 6.25-12.5% of dietary protein with full-fat BSFL meal had no effect on salmon growth rate, whereas replacing 25% of dietary protein had adverse effects on growth rate in our previous study (Weththasinghe *et al.*, 2021a).

Previous studies reported that feeding krill meal containing chitin or chitin from shrimp shells reduced growth in salmon (Hansen *et al.*, 2010; Karlsen *et al.*, 2017). In the present study, feeding the full-fat insect meal, defatted insect meal and insect exoskeleton diets containing 1.4% of chitin did not compromise growth performance

in salmon, suggesting that this level of chitin may not be sufficient to cause negative effects on fish performance. Similarly, in previous studies, the presence of up to 1.2% and 2.1% of BSFL chitin in diets did not reduce growth rate in salmon (Weththasinghe *et al.*, 2021a) and rainbow trout (*Oncorhynchus mykiss*) (Renna *et al.*, 2017; Terova *et al.*, 2019), respectively. The replacement of dietary protein with de-chitinized insect meal supported higher growth rate of fish, but decreased compared to full-fat insect meal, indicating BSFL chitin might have a positive effect on growth rate of salmon. The present results also showed that dietary inclusion of 7.2% insect exoskeleton improved FCR in fish than full-fat meal, which further confirmed that the exoskeleton fraction of BSFL might not have negative impact in salmon.

Dietary inclusion of defatted insect meal, de-chitinized insect meal and insect exoskeleton reduced protein digestibility. This might be attributed to the presence of chitin in these diets; 1.4% in defatted meal and exoskeleton diets and 0.5% in de-chitinized meal diet. The exoskeleton of BSFL contains non-protein N from chitin and amino acids bound to a matrix (Finke, 2007), and thus probably have a low digestibility. The chitin digestibility capacity of salmon is low (13–40%) (Olsen *et al.*, 2006), leading to increased faecal N excretion. The protein content was calculated as total N × 6.25, which overestimate the protein content in the faeces and consequently underestimate the ADC of protein. In addition, chitin might reduce the availability of insect protein for protease enzymes (Henry *et al.*, 2015) or activity of protease enzymes (Belghit *et al.*, 2018; Muzzarelli, 1980). On the other hand, full-fat insect meal diet also contained similar level of chitin as defatted meal and exoskeleton diets, but the full-fat insect meal diet did not reduce the ADC of protein in the present study. The chitin may lead to a reduced protein digestibility in fish, but in accordance with the present study and other studies, limited effect on protein digestibility in salmon (Fisher *et al.*, 2020) and rainbow trout (Melenchón *et al.*, 2021) fed BSFL meal has been reported.

Despite the reduced ADC of protein, the inclusion of meals or fractions of insects did not compromise protein retention. In line with the present results, BSFL meal in diets for salmon (Weththasinghe *et al.*, 2021a) and rainbow trout (Melenchón *et al.*, 2021) did not affect protein retention. In the present study, dietary inclusion of defatted

insect meal even increased digested protein retention in salmon compared to both the control diet and the full-fat meal diet. In defatted insect meal fed fish, this was also accompanied by lower FCR compared to the fish fed full-fat meal. Thus, the replacement of 15% of dietary protein with defatted insect meal gave better feed utilization than the full-fat insect meal. This emphasized the importance of applying a defatting process as a strategy to improve the nutritional value of BSFL.

Neither the insect meals nor the fractions adversely affected ADC of lipid in the present study. In several treatments, the lipid retention values were above 100%. Similarly, Weththasinghe *et al.* (2021a) also reported lipid retention values above 100% in salmon, whereas Dumas *et al.* (2018) reported efficiency of lipid deposition values above 100% in rainbow trout, and indicated lipid synthesis outweighed lipid catabolism. The insect diets gave similar lipid retentions as the control diet, except for the de-chitinized meal diet which gave a higher lipid retention as well as LER in the present study. The lauric acid is shown a larger extent to be oxidized and a lesser extent to be deposited, resulting in reduced tissue lipid deposition (Belghit *et al.*, 2019b; Renna *et al.*, 2017) and subsequently reduced lipid retention. However, in the present study, de-chitinized meal diet with the highest proportion of lauric acid, had the highest lipid retention, indicating lipid deposited rather than oxidation. This is most likely due to the lower lipid content in this diet. On the other hand, the lower lipid retention in fish fed full-fat meal diet compared to other insect diets, might indicate a higher utilization of energy from lipid, thus increasing the amount of dietary protein used for tissue synthesis due to a protein sparing effect (Francis and Turchini, 2017; Karalazos *et al.*, 2011) and subsequently improved fish growth.

Corresponding to the higher retention of digested protein, defatted insect meal reduced the dissolved N discharges in salmon. Furthermore, the diets containing full-fat insect meal and de-chitinized insect meal reduced faecal P excretion indicating improved P digestibility, whereas the fish fed the diet containing defatted insect meal showed higher P retention. This indicates that the P in BSFL might be more bioavailable than the P in fishmeal and plant protein sources. Thus, the present results suggest that feeding fish with diets containing full-fat, defatted or de-chitinized insect meals reduced environmental impact of salmon production by

reducing either dissolved N or faecal P excretion. On the other hand, feeding diets with defatted meal, de-chitinized meal and exoskeleton also increased environmental impact by increasing faecal N excretion.

5. Conclusions

The full-fat insect meal improved feed intake and growth rate in salmon when replacing 15% of dietary protein from fishmeal and plant protein sources, however, defatted meal gave better feed utilization than full-fat meal. The present results suggest that BSFL might be optimal to use in less processed or defatted form in diets for salmon. The insect meals and fractions generally supported a high durability of the extruded pellets, however, the inclusion of lipid-rich full-fat and de-chitinized insect meals in the diet numerically reduced hardness, expansion, and water stability of the pellets. Future studies are needed to optimize the use of black soldier fly larvae in extruded fish diets.

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Figures

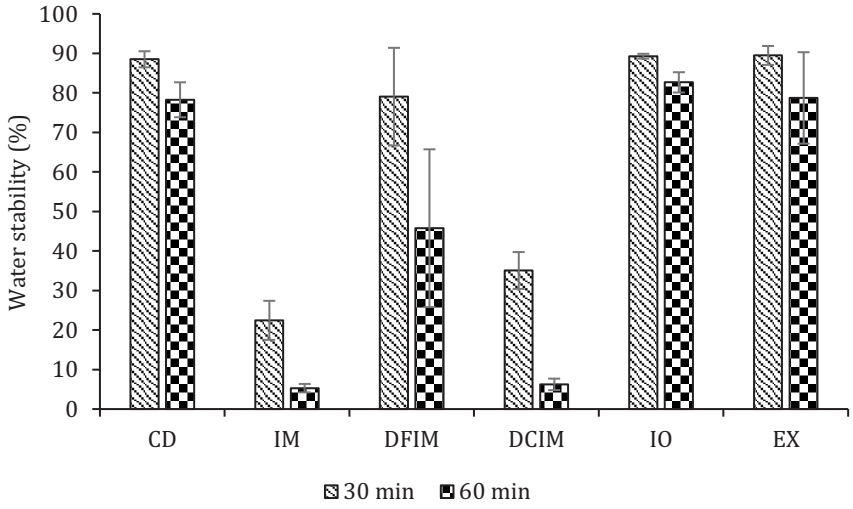

Figure 1: Water stability (dry matter retention %) of pellets of experimental diets with meals or fractions of black soldier fly larvae (BSFL) within 30 min and 60 min. Error bars indicate standard deviation. CD: Control diet. IM: Full-fat BSFL meal diet. DFIM: Defatted BSFL meal diet. DCIM: De-chitinized BSFL meal diet. IO: BSFL oil diet. EX: BSFL exoskeleton diet.

Supplementary table

Table S1: Fatty acid composition (% of total fatty acids) of black soldier fly larvae oil

Fatty acids	Value
C8:0	0.008
C10:0	0.68
C11:0	0.015
C12:0	39.7
C14:0	10.2
C14:1	0.21
C15:0	0.11
C16:0	16.4
C16:1n7	2.73
C17:0	0.14
C18:0	2.93
C18:1n9c	12.8
C18:2n6c	12.6
C20:0	0.11
C20:1	0.085
C18:3n3	0.87
C21:0	0.28
C20:2n6	0.016
C22:0	0.017
C20:3n6	0.03
C20:3n3	0.016
C20:4n6	0.007
C24:0	0.004
C20:5n3	0.006
C24:1	0.004

Paper IV

Modulation of Atlantic salmon (*Salmo salar*) gut microbiota composition and predicted metabolic capacity by feeding diets with processed black soldier fly (*Hermetia illucens*) larvae meals and fractions

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Abstract

Background: Black soldier fly (*Hermetia illucens*) is a promising insect species to use as a novel ingredient in fish feeds. Black soldier fly larvae consists of three major fractions, namely protein, lipid, and exoskeleton. These fractions contains bioactive compounds that can modulate the gut microbiota in fish such as antimicrobial peptides, lauric acid, and chitin. However, it is not certain how, or which fractions of black solider fly would affect gut microbiota in fish. In the present study, black soldier fly larvae were processed into three different meals (full-fat, defatted and de-chitinized) and two fractions (oil and exoskeleton), and included in diets for Atlantic salmon (*Salmo salar*). Atlantic salmon pre-smolts were fed with these diets in comparison with a commercial-like control diet for eight weeks to investigate the effects of insect meals and fractions on the composition and predicted metabolic capacity of gut microbiota. The gut microbiota was profiled by 16S rRNA gene sequencing, and the predicted metabolic capacities of gut microbiota were determined using genome-scale metabolic models.

Results: The inclusion of insect meals and fractions decreased abundance of *Proteobacteria* and increased abundance of *Firmicutes* in salmon gut. The diets that contained insect chitin, i.e., insect meals or exoskeleton diets, increased abundance of chitinolytic bacteria including lactic acid bacteria and *Actinomyces* in salmon gut, with fish fed full-fat meal diet showing the highest abundances. The diets that contained insect lipids, i.e., insect meals and oil diets enriched *Bacillaceae* in fish gut. The fish fed diets containing full-fat insect meal had phylogenetically diverse and unique gut microbiota dominated by beneficial lactic acid bacteria and *Actinomyces,* and showed a predicted increase in mucin degradation compared to the other diets.

Conclusions: The present results showed that the dietary inclusion of insect meals and fractions can differently modulate the composition and predicted metabolic capacity of gut microbiota in Atlantic salmon pre-smolts. The use of full-fat black soldier fly larvae meal in diets for salmon is more favorable for positive modulation of gut microbiota than larvae processed by separation of lipid or exoskeleton fractions.

Keywords: Black soldier fly, Atlantic salmon, gut microbiota, predicted microbial metabolic capacity, full-fat insect meal, defatted insect meal, de-chitinized insect meal, insect oil, insect exoskeleton

Background

Aquaculture has been the fastest growing food production sector over the last three decades and is expected to contribute significantly to the global animal-derived protein budget [1]. There is, however, a major constraint in supply of sustainable ingredients for fish feeds [2]. Fishmeal and fish oil have conventionally been used in fish feeds, but this practice is no longer sustainable due to depletion of wild forage fish, high market prices, conflicts about resource use, and environmental issues [3]. Alternative plant ingredients, such as soy products also raises serious ethical and sustainability concerns related with human food consumption [4], intensified crop production, deforestation, and other environmental issues [5, 6]. The presence of anti-nutritional factors further limits the use of plant ingredients [7]. Hence, aquaculture requires sustainable novel feed ingredients to remain economically and environmentally sustainable.

Over the last few years, there has been a growing interest in using insects as a sustainable novel fish feed ingredient [8]. Although the production volumes of insects cannot yet compete with conventional feed sources [8], the approval for use of processed insects in aqua feed by the European Commission (Regulation 2017/893/EC, 2017) promotes upscaling of insects as a fish feed ingredient. Due to the high nutritive value [9], low environmental impacts [10-12] and suitability for large scale production [13], black soldier fly (*Hermetia illucens*) (BSF) becomes a promising insect species to use for feed purposes. During the past decade, an increasing number of studies have successfully used BSF in diets for different fish species including Atlantic salmon (*Salmo salar*). The majority of studies showed that BSF did not compromise growth performance in salmon at low to moderate dietary inclusion levels [14-16], while other studies also showed positive effects of feeding BSF on gut health of salmon [17], confirming its potential as a novel ingredient in salmon feeds.

The gut microbiota plays a crucial role in digestive function, nutrient metabolism, growth performance, fish physiology, barriers against pathogens, immune response, disease resistance, welfare, and health in fish [18-23]. Thus, a positive modulation of

gut microbiota can be a key factor to improve nutrient utilization, growth performance, and health in fish. Diet is one of the main drivers in shaping the gut microbiota [24, 25]. Feeding diets containing BSF meal was previously reported to modulate gut microbiota in salmon [26] and rainbow trout (*Oncorhynchus mykiss*) [27-29]. The BSF consists of three major fractions namely protein, lipid, and exoskeleton [30]. Each fraction contains different bioactive compounds, such as antimicrobial peptides (AMP), lauric acid and chitin, respectively [31]. The expanded spectrum of AMP present in BSF have activity against many bacteria [32-35], while lauric acid has demonstrated antimicrobial effects against Gram-positive bacteria [36-38]. Dietary chitin has shown antimicrobial and bacteriostatic activity against several Gram-negative pathogens [39] but also to enrich beneficial microbiota in salmon gut due to its prebiotic properties [28, 29]. Hence, it is possible that the BSF might selectively modulate gut microbiota, which in turn could affect fish nutrient utilization, growth, and health. However, it is not certain how, or which specific compounds in BSF would affect gut microbiota in salmon. Characterizing the response of salmon gut microbiota to dietary full-fat BSF meal compared with different fractions of BSF and further processed BSF meals by separating lipid or exoskeleton fraction is, thus, worthy of attention. It is further important to determine how BSF should be processed to optimize its use in salmon diets. Although previous studies used full-fat and defatted BSF meals in salmonid diets, to the best of our knowledge, no studies evaluated the effects of different meals and fractions of BSF larvae on gut microbiota in a single study.

To date, majority of studies on gut microbiota of fish fed BSF have been restricted to analysis of taxonomic composition. Few previous studies showed that insect-based feeds could modulate the functional repertoire of gut microbiota in fish [40, 41] and the functional alterations of the gut microbiota to dietary insects varied with the fish species [40]. Nevertheless, we are still far from understanding how BSF and its specific compounds affect the functional profile of gut microbiota in Atlantic salmon, which is essential to identify potential fish-microbiota interactions. Therefore, the aims of the present study were to compare the composition, diversity and predicted metabolic capacities of gut microbiota in Atlantic salmon pre-smolts when fed with

BSF larvae meals (full-fat, defatted and de-chitinized meals) and fractions (oil and exoskeleton) by high-throughput sequencing technology.

Methods

Experimental diets, fish study and sampling

The BSF larvae were reared and processed into three meals (full-fat, defatted and de-chitinized) and two fractions (oil and exoskeleton) at HiProMine S.A., Robakowo, Poland. Six experimental diets were formulated to meet or exceed NRC [42] nutrient requirements of Atlantic salmon; a commercial-like control diet containing fishmeal, plant protein meals and fish oil (CD); three diets containing BSF meals and two diets containing BSF fractions. The three BSF meal diets contained either full-fat (IM), defatted (DFIM) or de-chitinized (DCIM) BSF meal replacing 15% of the protein content of CD. Two BSF fractions diets contained either BSF oil (IO) or exoskeleton (EX). The oil and exoskeleton of BSF were added to the diets to match the BSF oil and chitin contents in IM diet, respectively. Table 1 shows the ingredient and chemical compositions of the six experimental diets.

Table 1. Ingredient and chemical composition of experimental diets containing meals or fractions of black soldier fly (BSF) larvae[1]

	CD	IM	DFIM	DCIM	IO	EX
Ingredients (%)						
Fishmeal	22.50	18.57	18.57	18.57	22.50	21.78
Soy protein concentrate	34.50	28.48	28.48	28.48	34.50	33.39
Corn gluten	5.50	4.54	4.54	4.54	5.50	5.32
Full-fat BSF larvae meal	0.00	20.36	0.00	0.00	0.00	0.00
Defatted BSF larvae meal	0.00	0.00	14.89	0.00	0.00	0.00
De-chitinized BSF larvae meal	0.00	0.00	0.00	24.53	0.00	0.00
BSF larvae oil	0.00	0.00	0.00	0.00	6.24	0.00
BSF larvae exoskeleton	0.00	0.00	0.00	0.00	0.00	7.20
Wheat flour	14.65	14.65	14.65	14.65	14.65	14.65
Fish oil	16.00	10.47	14.75	5.82	10.05	15.36
Methionine	0.20	0.20	0.20	0.20	0.20	0.20
Choline chloride	0.15	0.15	0.15	0.15	0.15	0.15
Yttrium	0.01	0.01	0.01	0.01	0.01	0.01
Vit/min premix	0.65	0.65	0.65	0.65	0.65	0.65
Monocalcium Phosphate	0.80	0.80	0.80	0.80	0.80	0.80
Wheat bran	5.04	1.12	2.31	1.60	4.75	0.49
Chemical composition (%, wet-weight basis)						
Dry matter	91.6	91.9	93.0	92.9	93.3	91.7
Crude protein	46.6	44.4	46.0	46.6	46.6	47.3
Crude lipid	19.6	20.3	17.8	12.9	18.3	17.0
Starch	13.1	12.2	12.4	12.4	12.6	11.7
Ash	6.70	6.60	6.77	7.23	6.70	6.61
Chitin		1.44	1.44	0.53		1.43

[1] CD: Control diet; IM: Full-fat BSF larvae meal diet; DFIM: Defatted BSF larvae meal diet; DCIM: De-chitinized BSF larvae meal diet; IO: BSF larvae oil diet; EX: BSF larvae exoskeleton diet.

The fish study was conducted at the Center for Fish Research, NMBU, Ås, Norway. A total of 900 Atlantic salmon pre-smolts (Aqua Gen Atlantic QLT-innOva SHIELD) (28 g of average initial weight) were allocated into 18 fiberglass tanks, and fed *ad libitum* with one of the six experimental diets (n=3) for eight weeks. The fish were reared in

recirculated freshwater (14.4±0.4 °C) and kept under continuous light. At the end of the experiment, six fish from each tank were randomly sampled and anesthetized using tricaine methanesulfonate (MS-222) (80 mg/L). Fish were euthanized by a sharp blow to the head and recorded individual weight. The distal intestine was defined as the darker color section of the intestine with large diameter and annular rings [43]. The distal intestine was opened longitudinally and, the digesta was removed carefully using sterile plastic spatulas. The digesta was placed in cryotubes, snap-frozen in liquid nitrogen and stored at -80 ^{0}C. To maintain aseptic conditions, the digesta samples were collected near a gas burner and tools were cleaned and decontaminated using 70% ethanol spray and flaming between each fish. In addition, feed samples and water samples from the tank water source were collected into sterile plastic containers and stored at -80 ^{0}C.

Extraction of DNA from samples and controls

Total DNA from approximately 200 mg of digesta (18 samples per dietary group) and 100 mg of ground feed (2 samples per diet) were extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany, cat. no. 51604) according to the guidelines of the manufacturer with the following modifications: For the lysis of the samples, 300 µL (for digesta samples) or 500 µL (for feed samples) of Buffer ASL (Stool lysis buffer, Cat No./ID: 19082) was added to 2 mL prefilled bead tubes (Qiagen; Cat No., 13118-50) (100 µL of 0.1 mm glass beads). Then the samples were homogenized in a bead mill homogenizer (Qiagen RETSCH tissuelyser) at 20 Hz twice for 3 min, with a pause of 2 min (on ice) between the runs. The temperature for the heating incubation was 95 °C, and after adding proteinase K and buffer AL the incubation was 15 min at 90 °C. The extracted DNA was eluted with 50 µl of Buffer ATE and incubated 10 min at room temperature before centrifugation. In addition to digesta and feed, total DNA was extracted from two water samples. Water samples (500 mL) were filtered through a MF-Millipore membrane filter with 0.22 µm pore size (Sigma-Aldrich, Cat No. GSWP04700) and DNA was extracted using the same method as above but 600 µl of buffer ASL was added for the lysis.

To assess the reliability of the present workflow, two controls were added during DNA extraction: a blank negative control without a sample and a positive control containing a microbial community standard (mock), which consists of eight bacteria and two yeasts (Zymo- BIOMICS™, Zymo Research, California, USA; catalog no., D6300). The same DNA extraction procedure used for digesta samples was followed for both negative and positive (75 µL) controls. Further, the total DNA was extracted from a blank filter paper used for the filtration of water following the same procedure used for DNA extraction from water. After extraction, the DNA concentration was determined in duplicates using Invitrogen™ Quant-iT™ Qubit™ dsDNA HS (High sensitivity) assay kit (Thermo Fisher Scientific, California, USA, Cat No: Q32854) with the Qubit 4 Fluorometer (Invitrogen). The extracted DNA were stored at -20 ^{0}C until further analysis.

PCR amplification

A first PCR (in duplicates) was performed in 25 µL reactions to amplify the V3–V4 hypervariable regions of the bacterial 16S rRNA gene. The primers used were 341F (5'-CCTACGGGNGGCWGCAG-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3'). The reaction mix contained 2x KAPA HiFi HotStart Ready Mix (12.5 µL) (Roche Sequencing Solutions, Material No: 7958935001), DNA template (5 µL), and 1 µM primers (3.75 µL of each primer). The PCR thermal cycling began with an initial denaturation at 95 °C for 3 min and followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The duplicate amplified PCR products were pooled and purified using Agencourt AmPure XP beads (Beckman Coulter, Indiana, USA, Cat No: A63881). The cleaned PCR products were examined by 1% agarose gel electrophoresis. The 13 digesta samples with strongest bands of each dietary group were used for sequencing.

Library preparation and sequencing

The library preparation was conducted according to the Illumina 16S Metagenomic Sequencing Library Preparation protocol [44]. First, the cleaned PCR amplicons were indexed with the Nextera XT Index Kit v2 Set A (96 indexes, 384 samples) (Illumina,

California, USA, Cat. No: FC-131-2001). The index PCR products were purified using the AMPure beads and quantified using the Invitrogen™ Quant-iT™ Qubit™ dsDNA BR (Broad Range) assay kit (Thermo Fisher Scientific, California, USA, Cat no: Q32853) with the Qubit 4 Fluorometer (Invitrogen). The library size was determined using representative cleaned libraries with the Agilent DNA 1000 Kit (Agilent Technologies, California, USA; catalog no., 5067–1505). The libraries were diluted to 4 nM in 10mM Tris (pH 8.5). The libraries from negative control and blank filter paper had a concentration lower than 4 nM, and thus were not diluted. Equal volumes of diluted and undiluted libraries were pooled. The pooled library was denatured using 0.2 N NaOH. A standard Illumina generated PhiX control (Illumina, San Diego, Waltham, MA, USA, Cat No: FC-110-3001) was also denatured. The denatured library was combined with 5% Phix control (570 µL library + 30 µL PhiX control). The combined library and Phix control was then loaded at 8 pM and sequenced on the Miseq System (Illumina, San Diego, California, USA) using the Miseq Reagent Kit v3 (600-cycle) (Illumina; catalog no., MS-102-3003). The clustering density was 804 K/mm2 and 91% of clusters were passing filter. Data output from the sequencer were demultiplexed FASTQ format files.

Processing of sequence data

The processing of sequence data was done in R 4.0.4 [45]. The DADA2 1.18.0 was used to process the raw sequence data and generate amplicon sequence variants (ASVs) [46]. A total of 15.7 million raw reads were generated for digesta, feed, and water samples. The median of raw reads per sample were 165,648, while the minimum reads per sample was 46,075 and maximum was 815,888. The median Phred quality score of reads was crashed at position 298 bp in forward reads and at position 220 bp in reverse reads. The primer sequences and low-quality reads from where the median Phred quality score crashed were trimmed and filtered out from the demultiplexed paired ended reads. A model of error rates was developed, and error sequences were removed. The forward and reverse reads from each sample were merged, ASV table was constructed, and chimeric sequences were removed from the ASV table. A total number of 14,666 unique ASVs were generated after the sequence denoising and ASVs filtering. The resulted ASVs were assigned with

taxonomy using the reference database, Silva version 138.1 [47, 48]. The sequences obtained from the mock samples were matched with the expected reference sequences to evaluate the DADA2 performance. A phyloseq object was built using the R package phyloseq 1.34.0 for further analyses using the generated ASVs table, taxonomy table and sample metadata [49]. The undetermined sample in the sequence output was removed from the phyloseq object. The following ASVs were removed from the ASV table: ASVs identified as chloroplasts (4.7% of ASVs) or mitochondria (10% of ASVs), ASVs with no phylum-level taxonomic assignment and ASVs found in only one non-control sample. The contaminating ASVs due to reagent contamination and cross contamination were identified and removed from ASV table as explained by Li et al. [26]. Taxonomic analysis showed that 68.8% of ASVs were assigned at the genus level whereas only 10% of ASVs had a species-level annotation. The core ASVs, alpha diversity indices (observed ASVs, Pielou's evenness, Shannon's index and Faith's phylogenetic diversity (PD)) and beta diversity indices (Jaccard distance, unweighted UniFrac distance, Aitchison distance and PHILR transformed Euclidean distance) were computed according to Li et al. [26]. The ASV table was rarefied based on minimum sequence size (10,332) in the samples to compute Jaccard distance, unweighted UniFrac distance (Fig. S1).

Metabolic reaction level analysis

The reaction-level analysis of gut microbiota was performed as previously described by Yilmaz et al. [50]. The ASVs were mapped to metabolic reactions via an available collection of genome-scale metabolic models (GSMMs) of gut microbes [51], including only ASVs that could be mapped to a taxonomic rank of family or lower and to at least one GSMM. For each sample, we then calculated the normalized abundance of each reaction (i):

$$a_r(i) = \frac{\sum_{j=1}^{n} a_{\mathrm{ASV}}(j) E(i,j)}{\sum_{j=1}^{n} a_{\mathrm{ASV}}(j)}$$

where $a_{\mathrm{ASV}}(j)$ is the abundance of ASV *j* in the sample, *n* is the total number of ASVs, and $E(i,j)$ is the expected probability (frequency of occurrences) of reaction *i* in the GSMMs mapped to ASV *j*.

Statistical analysis

Linear discriminant analysis (LDA) effect size (LEfSe) tool [52] was used to characterize microbial differences of biological relevance between the dietary groups. The statistical differences were evaluated using factorial Kruskal-Wallis rank sum test, followed by pairwise Wilcoxon test and a threshold between 3.5 and 4.0 for the LDA. The strategy for multi-class analysis was one-against-all/all-against-all. The statistical analyses related to microbial diversity were run in R 4.0.4 [45]. The statistical difference between the dietary groups for the four alpha diversity indices were evaluated using Kruskal-Wallis test, followed by multiple comparisons using Wilcox pair-wise comparison test. The differences in beta-diversity were evaluated by performing permutation multivariate analysis of variance (PERMANOVA) [53] with 999 permutations using the R package vegan 2.5.7 [54], and followed by a pair-wise comparison. The four beta-diversity distance matrices were visualized by the principal coordinates analysis (PCoA). The homogeneity of multivariate dispersions among groups was evaluated by the permutation test, PERMDISP [55], using the R package vegan 2.5.7 [54]. The adjusted pair-wise comparisons by the Benjamini-Hochberg procedure were used where applicable [56]. Differences were regarded as significant when $p < 0.05$.

All analyses related to metabolic reactions were performed in Python 3.7.0. A two-sample *t*-test was used to compare the mean abundances of each metabolic reaction for each pair of diets. The Benjamini-Hochberg procedure [56] was used to correct for multiple testing, and reactions with adjusted $p \leq 0.05$ were considered to be significantly different between diets. The metabolic pathway classification of reactions was obtained from the GSMMs, and Fisher's exact test was used to identify enriched pathways among the significantly different reactions. The pathways with Benjamini-Hochberg-adjusted $p \leq 0.05$ were considered to be enriched. Further, principal component analysis (PCA) was performed separately on standardized ASVs (Fig. S2) and reaction abundances (z-scores) (Fig. S3).

Results

Microbiota associated with positive and negative controls

Confirming the reliability of the present workflow for assessing the gut microbiota, the eight expected bacterial genera in the mock were successfully identified. *Staphylococcus aureus* was identified at the species level as well (Fig. S4). The relative abundance of *Enterococcus*, *Listeria* and *Staphylococcus* were underestimated, whereas the relative abundance of *Bacillus*, *Lactobacillus* and *Pseudomonas* were overestimated. The Pearson correlation coefficient (Pearson's *r*) between the expected and observed taxonomic profile of the mock at genus level was 0.48, while it was 0.99 between the observed profiles. The dominant taxa identified in the blank filter paper were *Paracoccus* (21%) and *Corynebacterium* (22%). The contaminant taxa of the negative control was dominated by *Candidatus Nomurabacteria* (23%).

Microbiota associated with water and feed

The microbiota in tank water were dominated by phyla *Proteobacteria* (40%), *Bacteroidota* (29%), *Verrucomicrobiota* (7%), and *Patescibacteria* (7%) (Fig. 1a). At the genus or lowest taxonomy level, *Rudanella* (10%), *Sphaerotilus* (10%), *Rhodoferax* (6%), Hydrogenophaga (4%) and *Verrucomicrobiaceae* (3%) dominated the microbiota in tank water (Fig. 1b).

The taxonomic compositions of the feed samples were diet-dependent (Fig. 2). At the phylum level, the microbiota in the feed was dominated, regardless of the diet, by *Proteobacteria*, *Firmicutes* and *Actinobacteriota*. The CD feed had higher abundance of *Proteobacteria* (75%) compared to insect-based feed (28-47%). On the contrary, insect-based feed had higher abundance of *Firmicutes* (18% in CD and 39-55% in insect-based feed), and *Actinobacteriota* (3% in CD and 9-13% in insect-based feed) (Fig. 2a). At genus or lowest taxonomic level, microbiota associated with insect-based feed showed higher abundance of *Oceanobacillus*, *Actinomyces*, *Brevibacterium*, *Lactobacillales*, *Bacillaceae*, *Pseudogracilibacillus* and *RsaHf*231 compared to CD feed, while *Morganella* was solely found in insect-based feed pellets. The CD feed was dominated by *Photobacterium* (52%) (Fig. 2b).

Gut-associated microbiota

The taxonomic composition of the digesta samples were diet-dependent (Fig. 3). At the phylum level, *Firmicutes* was the dominant taxon of digesta samples of all the dietary groups, where the fish fed insect diets had higher abundance than the fish fed CD (CD, 49%; IM, 66%; DFIM, 61%; DCIM, 54%; IO, 64%; EX, 54%). The fish fed insect diets except IO also had higher abundance of *Actinobacteriota* (CD, 20%, IM, 30%; DFIM, 23%; DCIM, 30%, IO, 13%; EX, 23%), but all the insect groups had lower abundance of *Proteobacteria* (CD, 26%; IM, 2%; DFIM, 13%; DCIM, 15%; IO, 20%; EX, 15%) (Fig. 3a). At genus or lowest taxonomic level, *Lactobacillales* was the dominant taxon in IM (25%) and DFIM (15%) groups, and their abundances were followed by DCIM (11%), EX (9%), CD (6%) and IO (4%) groups. The abundance of *Actinomyces* was higher in IM (17%), DFIM (10%), DCIM (12%) and EX (9%) groups compared to the CD and IO groups (4%). The insect diets fed fish except EX showed higher abundance of *Bacillaceae* (IM, DFIM, 7%; DCIM 8%; IO, 15%, EX, 2%), compared to CD diet fed fish (2%). The DCIM group had the highest abundance of *Corynebacterium* (CD, 3%; IM, 4%; DFIM, 3%; DCIM, 8%; IO, 4%; EX, 4%). The IO and EX groups were dominated by *Oceanobacillus* (16%) and *Staphylococcus* (16%) respectively, whereas *Pantoea* (7%) and *Staphylococcus* (6%) were dominant in CD group (Fig. 3b).

To characterize the microbiota in fish gut with significant differences in abundances between the dietary groups, LEfSe was performed. The LEfSe results are presented in cladograms showing the phylogenetic distribution of the bacterial lineages and LDA column charts. Fig. 4 shows the significantly enriched taxa in all the dietary groups. At LDA score of 3.5, most of the significantly enriched taxa in CD group belonged to classes *Gammaproteobacteria* and *Clostridia*, while *Photobacterium* and *Vibrionaceae* were among the enriched taxa in *Gammaproteobacteria*. The significantly enriched taxa in IM group mainly belonged to phylum *Actinobacteriota* and class *Bacilli*, such as *Lactobacillales*, *Enterococcaceae*, *RsaHf*231, *Actinomyces* and *Enterococcus*. The DFIM significantly enriched family *Micrococcaceae* and genus *Pseudogracilibacillus*, whereas DCIM group had significantly higher abundance of genera *Corynebacterium* and *Brevibacterium*. The IO mainly enriched order *Bacillales*,

and genera *Oceanobacillus*, *Paenibacillus* and *Pseudomonas*. The EX mainly enriched phylum *Patescibacteria* and genera *Staphylococcus* and *Mycobacterium*.

Fig. 5 shows the LEfSe results for the comparison between CD and IM groups at LDA score of 4. LEfSe detected 52 bacterial clades (26 in each) showing statistically significant different abundances between the IM and CD groups. In comparison with CD group, IM diet enriched taxa belong to two main classes namely *Actinobacteria* and *Bacilli*. The enriched taxa in these classes included *RsaHf*231, *Lactobacillales*, *Bacillales*, *Bacillaceae*, *Actinomyces*, *Oceanobacillus*, and *Brevibacterium*. Most of these bacterial taxa were also significantly enriched in DFIM, DCIM and EX groups compared to CD group (Fig. S5-7). In addition, DCIM diet also enriched genera *Acinetobacter* and *Corynebacterium* (Fig. S6), and EX group enriched *Staphylococcus* compared to CD (Fig. S7). The EX group, however, did not enrich *Bacillaceae* and *Oceanobacillus* (Fig. S7). The IO diet mainly enriched taxa belonging to class *Bacilli* such as *Bacillaceae* and *Oceanobacillus* compared to the CD group (Fig. S8).

The gut microbial compositions in the gut partly resembled the microbiota in respective feed, but differed from the water microbiota. The ASVs overlap between the gut and feed was higher than that between the gut and water (Fig. 6).

At prevalence threshold of 80%, 210 ASVs were identified as core microbiota in fish gut. Two ASVs, classified as *Enterococcus* and *Lactobacillales* were identified as core ASVs in all the digesta sample types (Table S1). Additionally, fish fed insect diets shared 14 ASVs identified as *RsaHf*231, *Oceanobacillus*, *Actinomyces*, *Corynebacterium*, *Staphylococcaceae*, *Lactobacillales*, *Pseudogracilibacillus* and *Brevibacterium* (Fig. S9a; Table S1). The insect meal groups (IM, DFIM and DCIM) had 54 core ASVs and *Lactobacillales*, *Enterococcus*, *Corynebacterium*, *Brevibacterium*, *Oceanobacillus* and *Morganella* were among them (Fig. S9b; Table S1). The four diets that contained BSF lipid (IM, DFIM, DCIM and IO) shared 28 ASVs and most of them belonged to family *Bacillaceae* (Fig. S9c; Table S1). The four diets which contained BSF chitin (IM, DFIM, DCIM and EX) had 21 shared ASVs and *Enterococcus*, *Lactobacillales*, *Corynebacterium* and *Actinomyces* were among them (Fig. S9d; Table S1).

Alpha diversity

The diet significantly affected the alpha diversity indices of gut microbiota ($p < 0.001$) (Fig 7 and Table S2). The observed ASVs did not differ between the gut microbiota in insect groups and CD group, but IM group presented a numerically higher average (Fig. 7a). The IM and IO groups showed lower Pielou's evenness than the CD group, and the other groups did not differ from CD group (Fig. 7b). Further, IM diet also reduced Shannon's index compared to CD group (Fig. 7c). Following the trend for observed ASVs, IM group had higher Faith's PD compared to the CD group (Fig. 7d). The Shannon's index and Faith's PD in other groups did not differ from CD group (Fig. 7c-d). Additionally, the observed ASVs and Faith's PD were higher in IM group than IO and EX groups, but did not differ from DFIM and DCIM groups. Pielou's evenness was lower in IM compared to DFIM group, while Shannon's index in IM groups was lower than in DFIM and DCIM groups (Table S2).

Beta diversity

The PCoA plots for all four beta-diversity indices showed that insect groups clustered together, but separated from CD group (Fig. 8). Confirming the group separation in PCoA plots, PERMANOVA results also revealed differences between the gut microbiota of fish fed CD and insect diets. Although it was not clear in the PCoA plots, the statistical tests showed differences in beta-diversity between microbiota in the IM group and the other insect groups, regardless of the distance matrix used (Table S3). The box-plots and results of the tests for homogeneity of multivariate dispersions are shown in Fig. S10 and Table S4, respectively. For the four distances, IM and DCIM groups showed lower multivariate dispersions than the CD group, whereas no differences were observed between CD and insect fractions (IO and EX) groups. In addition, the IM diet had the lowest multivariate dispersions among the insect groups for all the four distances.

Metabolic capacity of gut microbiota

Of the 3590 ASVs, 1886 could be mapped to at least one genome-scale metabolic model (GSMM) from a published collection of GSMMs of gut microbes [51]. Among these, 1165 were matched to family with an average of 16 models per ASV, 665 were matched to genus with an average of 10 models per ASV, and 56 were matched to species with an average of 1 model per ASV (Fig. S11). In total, the models that were mapped to ASVs contained 4886 different reactions. Most of these reactions (78%) were present in all samples and all samples contained at least 82% of the reactions, but the abundances of many reactions differed significantly between samples and diets. Furthermore, PCA of reaction abundances allowed much more of the variability in the data to be explained in a few components than PCA of ASV abundances (Figs. S2 and S3).

Grouping reactions by metabolic pathways showed that 32 pathways were enriched reactions with significantly different mean abundances between dietary groups (Fig. 9). The mean differences in reaction abundances between groups are shown for enriched pathways in Fig. S12. The gut microbiota in fish fed IO enriched highest number of pathways (22) compared to CD group (Fig. 9; Fig. S12a). The first principal component of PCA on reaction abundances (z-scores) showed that IM and IO groups separated from the other groups in terms of their metabolic capacities (Fig. S3c). These two groups showed predicted enrichment of metabolic pathway mucin O-glycan degradation and fatty acid (FA) synthesis, respectively, compared to other groups (Fig. 9).

In comparison with CD group, IM group decreased lipopolysaccharide biosynthesis and FA synthesis (Fig. 9; Fig. S12d), whereas DFIM group enriched mucin O-glycan degradation, starch and sucrose metabolism and valine, leucine, and isoleucine metabolism (Fig. 9; Fig. S12i). The DCIM, IO and EX enriched predicted metabolic pathways amino acid/peptide metabolism and FA synthesis compared to the CD group (Fig. 9; Fig. S12a, c, h).

Discussion

Modulation of fish gut microbiota composition and diversity

The present study showed that inclusion of meals and fractions of insects in the diet can modify the gut microbiota of Atlantic salmon pre-smolts. Previous findings also showed that feeding BSF modulated gut microbiota in rainbow trout [27-29, 41, 57] and salmon post-smolts [26]. The phyla *Firmicutes*, *Proteobacteria*, *Actinobacteriota*, and *Bacteroidota* represented more than 94% of the gut microbiota in fish, regardless of the diet, which belong to the core gut microbiota in different fish species [22]. The observed increase of *Firmicutes*, *Actinobacteriota*, *Lactobacillales*, *Actinomyces*, *RSaHf231*, *Oceanobacillus*, *Bacillaceae*, *Brevibacterium, Acinetobacter*, *Staphylococcus* and/or *Corynebacterium* and decreased *Proteobacteria* abundances in comparison to the control fish, also observed previously in gut microbiota of salmon post-smolts [26] and rainbow trout [27, 29] when fed BSF meal.

The observed gut microbiota differed from water-associated microbiota. This can be related to the low water intake of fish in freshwater, and is in accordance with a previous study showing that gut microbiota of rainbow trout reared in freshwater did not reflect the microbiota of the surrounding environment [21]. The highly abundant taxa in feed containing insect meals and fractions were *Oceanobacillus*, *Actinomyces, Brevibacterium*, *Lactobacillales*, *Bacillaceae*, *RsaHF*231, and *Morganella*. Such taxa have also been identified in BSF whole larvae/prepupae or their gut [58-61]. The microbiota in all diets contained *Photobacterium*, but made up more than 50% of the microbiota in the control feed, similar to previous reports [57]. It is plausible that fishmeal was the main source of *Photobacterium* in the feed and its abundance is associated with the inclusion level because of the wide distribution of these bacteria in marine environment and fish [62, 63]. In future studies, analyzing the microbiota in the feed ingredients would provide useful information regarding the sources of microbes in the feed.

The modulation of gut microbiota in fish fed the insect-based diets can be explained by microbiota associated in feed and the composition of feed. There were overlaps between the microbes found in feed and fish gut, in particular the bacterial taxa,

Pantoea, *Oceanobacillus*, *Lactobacillales*, *Bacillaceae, Actinomyces* and *RsaHf*231. While high temperature during extrusion could eliminate microbiota in feed, dead bacteria and spores can still be profiled by the DNA sequencing technique. Hence, the observed microbial composition in the fish gut could reflect some dead or inactivated microbes in undigested feed. However, it is also possible that resistant bacterial spores could modulate microbial community in the gut, but the extent to which the observed feed microbiota contributed to shape gut microbiota cannot be identified using sequencing-based methods. Our results also suggested that fish gut microbiota was not merely originated from feed, but that the specific feed composition selectively modulated the microbiome.

Insect meals and exoskeleton fraction enriched *Lactobacillales*, which are commonly known as lactic acid bacteria (LAB). Among the dietary groups, the full-fat group had the highest abundance of *Lactobacillales* and *Enterococcus*, a genus belong to *Lactobacillales*. Previous studies also showed that dietary inclusion of BSF meal increased abundance of *Lactobacillales*, *Lactobacillus* and/or *Enterococcus* in salmon post-smolts [26] and rainbow trout [27, 29, 41]. The LAB are commonly observed microbes of the teleost fish gut in minor proportions of the overall community [64, 65]. In general, LAB are considered as beneficial gut microbes due to their abilities to enhance digestive function, mucosal tolerance, immune response, and disease resistance in host [66]. They are known to produce lactic acid and bactericidal compounds that may prevent colonization of pathogens on the intestinal surface [66-68] and even repair or prevent the intestinal damage caused by antinutritional factors present in plant-based ingredients such as soybean meal in fish [69].

The exoskeleton of BSF contains chitin [70], which can be associated with proliferation of LAB due to its prebiotic properties [18, 28, 29]. Our results strongly supported this, since only the diets containing insect chitin (1.4% in full-fat meal, defatted meal, and exoskeleton diets and 0.5% in de-chitinized meal diet) enriched abundance of *Lactobacillales* in the fish gut. In addition to LAB, these four chitin containing diets also increased abundance of *Actinomyces* in gut microbiota in fish, with the highest abundance observed from full-fat meal diet. The enrichment of *Actinomyces* has previously been shown when salmon post-smolts [26] and rainbow

trout [29, 57] fed BSF meal. *Actinomyces* are often identified as chitin degraders and might benefit from the presence of chitin [71]. The genus *Actinomyces* is within the class *Actinobacteria*, which is involved in the function of the intestinal barrier of the fish and playing an essential role in the synthesis of antimicrobial compounds against fish pathogens [72]. In addition, many bacterial species belonging to *Bacillus* of family *Bacillaceae* can produce chitinase [73, 74]. In the present study, the insect meals and oil diets enriched *Bacillaceae* in the fish gut. Huyben et al. [27] also showed similar results in rainbow trout fed full-fat or defatted BSF larvae meals. Hence, in the present study, chitin in the BSF larvae could have acted as a substrate and may have selectively promoted the growth of certain chitinolytic bacteria in the fish gut such as *Lactobacillales*, *Actinomyces* and *Bacillaceae* in agreement with previous observations in Atlantic salmon [73] and Atlantic cod (*Gadus morhua* L.) [75].

The lipid fraction of BSF larvae was rich with medium chain lauric acid (40% of total FAs), and did not contain long-chain polyunsaturated omega-3 FAs. This FA composition of BSF can also be responsible partially for increased LAB abundance as shown by Rimoldi et al. [68] and Huyben et al. [76], although the insect oil diet did not enrich LAB in the present study. Fish fed de-chitinized meal showed the highest abundance of *Corynebacterium* in gut microbiota as observed in rainbow trout fed BSF larvae or pre-pupae meal [27, 29]. During the de-chitinization process, there was an increase of the relative lipid content of insect meal (44%), making de-chitinized meal diet the one with the highest level of BSF lipids. Thus, BSF lipids might cause the increase in *Corynebacterium* in the gut of fish fed de-chitinized meal diet. Huyben et al. [27] also observed that the abundance in fish fed full-fat BSF meals were higher than in fish fed defatted meal. The *Corynebacterium* has been reported to produce lipase [77]. The de-chitinized meal decreased abnormal lipid accumulation in the pyloric caeca (P. Weththasinghe, J.Ø. Hansen, L.T. Mydland, L. Lagos, B. Morales-Lange, M. Øverland, unpublished observations), and it is possible that the enriched *Corynebacterium* might have played a role in preventing this condition. Moreover, despite of being chitinolytic bacteria, *Bacillaceae* were only enriched in fish fed BSF lipid containing diets and not in exoskeleton diet with insect chitin, indicating BSF lipid fraction was favorable for the proliferation of this bacteria. In the present study,

the exoskeleton diet gave the highest abundance of *Staphylococcus*, followed by the control diet. The other insect diets, which contained lauric acid, did not enrich *Staphylococcus*. Lauric acid has shown antimicrobial activity against some species of *Staphylococcus* [78]. This suggested that BSF lipid can also modulate salmon gut microbiota in addition to chitin. Altogether, BSF as a whole or its components, might explain the changes in the microbial community, and most importantly, the reduced abundance of Gram-negative *Gammaproteobacteria*, *Vibrionaceae* and *Photobacterium* in fish fed insect-based diets in comparison to control fish. Chitin was previously reported to have antimicrobial and bacteriostatic activity against several Gram-negative pathogens [39]. Furthermore, Rimoldi et al. [68] reported that lauric acid in the diet can also reduce the abundance of *Gammaproteobacteria*.

In the present study, fish fed full-fat meal diet presented a numerically higher average of species richness, as shown by observed ASVs. The decreased Pielou's evenness suggested that the fish fed full-fat meal and insect oil might have specific bacterial group(s) that dominated the gut microbiota, also supported by Shannon's index of full-fat meal group. Faith's PD measures the biodiversity, based on the phylogeny distance, showed that phylogenetic diversity in fish fed full-fat meal was higher than that in control fish, indicating presence of species from diverse clades in the phylogeny tree. Hence the alpha-diversity indices strongly indicated that fish fed full-fat meal might have a different species composition, but there might be a dominance of a specific group(s), i.e., LAB and *Actinomyces*. Higher abundance of these chitinolytic bacteria can be the reason for increased phylogenetic diversity in full-fat meal group, since a higher phylogenetic diversity exists within chitinolytic bacteria [71]. On the contrary to the results for fish fed full-fat insect meal, the species richness, evenness, and the diversity in gut microbiota in fish fed defatted and de-chitinized meals did not differ from the control fish. Previous studies, however, showed that feeding defatted BSF meal increased richness and diversity in rainbow trout [29, 41] and salmon post-smolts [26]. The beta diversity indices showed that gut microbiota of insect-based groups clustered closer than that of the control group, as previously observed in rainbow trout [27-29, 41]. Regardless of the distance matrix used, gut microbiota in full-fat meal group also differ from the other insect

groups. In general, high gut microbial diversity is commonly associated with positive health effects. Species-rich communities are thought to potentially provide further metabolic capabilities to the host [79] and out-compete pathogens for nutrients and colonization [80, 81], e.g., LAB can reduce pathogens adhesion by creating a biofilm in the gut [68, 82].

The fish fed full-fat meal showed higher growth performance compared to the fish fed control diet and other insect-based diets (P. Weththasinghe, J.Ø. Hansen, M. Rawski, D. Józefiak, S. Ghimire, M. Øverland, unpublished observations). The improved phylogenetic diversity and unique composition in gut microbiota in full-fat group, together with the enrichment and dominance of beneficial bacteria such as LAB and *Actinomyces*, may cause this improvement in growth. It is possible that chitin, lauric acid and other bioactive components such as AMP might have acted together in positively modulating gut microbiota in fish fed full-fat meal, and consequently improved fish growth performance. Altogether, this points to the use of full-fat BSF larvae meal in diets for Atlantic salmon as more efficient, than processed larvae by separation of lipid or exoskeleton fractions.

Modulation of metabolic capacity of gut microbiota

The gut microbiota carries out many metabolic reactions, which play a critical role in host nutrition, physiological functions, and health [41, 83]. In the present study, the predicted metabolic reaction profile of gut microbiota in fish fed full-fat BSF meal diet differed from other diets, as observed in PCA results. The full-fat insect meal enriched mucin O-glycan degradation in gut microbiota compared to the control as well as other insect-based diets. The mucus layer covering intestinal epithelium is mainly consisted of mucin with a vast array of O-glycan structures [84]. Mucus nature can benefit certain mucin-degrading bacteria and thereby, shaping the gut microbiota composition at the mucosal surface, gut inflammatory responses [85] and host immune responses [86]. At the same time, it was also observed lower levels of reactions in lipopolysaccharide biosynthesis pathway in fish fed full-fat meal diet compared to fish fed control and de-chitinized meal diets. Gram-negative bacteria produce and have lipopolysaccharides on cell surface [87, 88], which are recognized

as pathogen-associated molecules and can activate the innate immune response in fish [89]. This reduction in lipopolysaccharide biosynthesis is in accordance with drastic reduction in Gram-negative *Proteobacteria* in full-fat meal group. Considering the growth performance, it is likely that full-fat meal benefited the metabolic activity of Atlantic salmon gut microbiota and consequently the fish growth.

The predicted enrichment of FA synthesis in fish fed BSF lipid-rich de-chitinized meal and oil can mostly be due to the lack of omega-3 FAs in lipid fraction of BSF larvae. Gut microbiota can compensate for low levels of omega-3 FAs in the diet by increasing the abundance of FA producing bacteria [76]. Feeding defatted meal caused a predicted enrichment of starch and sucrose metabolism in gut microbiota, and similar results were previously shown by Rimoldi et al. [41] in rainbow trout fed BSF meal. This indicates gut microbiota of fish fed with defatted insect meal may have the capacity to improve dietary carbohydrates utilization by complementing the endogenous digestive enzymes [41]. However, predictive metabolic profiles should be interpreted with caution, because the reliability of metagenome prediction tools is questionable due to the biased databases towards human-related microbiota [90]. In particular, the GSMM collection used in the present study was originally created for microbes found in human gut microbiota. Therefore, metagenomic, metaproteomic or metabolomic analysis of digesta samples would be preferred to determine the real functional profile of gut microbiota.

Conclusions

The present results showed that feeding meals and fractions of BSF insect larvae differently modulated gut microbial composition, diversity, and predicted metabolic repertoire in Atlantic salmon pre-smolt. Both insect meals and fractions decreased *Proteobacteria* to *Firmicutes* ratio in the gut of fish. The diets containing BSF chitin, i.e., insect meals and exoskeleton diets, increased chitinolytic LAB and *Actinomyces*, while those containing BSF lipids, i.e., insect meals and oil diets, increased the abundance of *Bacillaceae*. Full-fat insect meal led to phylogenetically diverse and unique gut microbiota dominated by the beneficial LAB and *Actinomyces*, and showed a predicted increase in mucin degradation compared to the fish fed other diets.

Overall, the present results showed that full-fat BSF larvae meal was more favorable in positive modulation of gut microbiota than processed larvae by separation of lipid and exoskeleton fractions.

Abbreviations

ASVs: amplicon sequence variants; AMP: antimicrobial peptides; BSF: black soldier fly; FA: fatty acid; GSMMs: genome-scale metabolic models; LAB: lactic acid bacteria; LDA: linear discriminant analysis; LEfSe: linear discriminant analysis effect size; PD: phylogenetic diversity; PERMANOVA: permutation multivariate analysis of variance; PCA: principal component analysis; PCoA: principal coordinates analysis.

Declarations

Ethics approval and consent to participate

The fish experiment was conducted at Center for fish research at Norwegian University of Life Sciences (NMBU), which is a research facility approved by Norwegian Animal Research Authority (permit no. 109) and operates in accordance with Norwegian Regulations of 17th of June 2008 No. 822: Regulations relating to Operation of Aquaculture Establishments (Aquaculture Operation Regulations). The experimental procedures were in accordance with the national guidelines for the care and use of animals (The Norwegian Animal Welfare Act and the Norwegian Regulation on Animal Experimentation).

Consent for publication

Not applicable.

Availability of data and material

The raw 16S rRNA gene sequence files and metadata are deposited at the NCBI SRA database under the BioProject PRJNA762510. Other data and code for reproducing the results are available in the GitLab repository (https://gitlab.com/Pabodha/salmon_insects_microbiota_2021).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

PW, JØH, and MØ contributed to the conception. PW, JØH, MØ, LL, and LTM designed study. PW and JØH involved in feed production, fish experiment and sampling. PW and SDCR carried out laboratory works. PW, SDCR, and OØ performed bioinformatics, statistical analyses, and data visualization. MØ acquired funding. PW wrote the first draft of the manuscript. All the authors read, revised, and approved the final version of the manuscript.

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Figure legends

Fig. 1. Most abundant taxa in tank water (TW) samples. Top 10 most abundant taxa at phylum (**a**) and top 15 most abundant taxa at genus or lowest taxonomy (**b**) level.

Fig. 2. Most abundant taxa in feed samples. Top 10 most abundant taxa at phylum (**a**) and top 15 most abundant taxa at genus or lowest taxonomy (**b**) level. The plots in left side of the figure display the relative taxa abundances in all the feed samples. The samples are grouped by the diet. The plots in right side display the mean abundance of each taxon within the same diet. CD: control diet; IM: full-fat BSF (black soldier fly) larvae meal diet; DFIM: defatted BSF larvae meal diet; DCIM: de-chitinized BSF larvae meal diet; IO: BSF larvae oil diet; EX: BSF larvae exoskeleton diet.

Fig. 3. Most abundant taxa in distal intestine digesta samples from fish fed experimental diets. Top 10 most abundant taxa at phylum (**a**) and top 15 most abundant taxa at genus or lowest taxonomy (**b**) level. The plots in left side of the figure display the relative taxa abundances in all the samples. The samples are grouped by the diet. The plots in right side display the mean abundance of each taxon within the same dietary group. CD: control diet; IM: full-fat BSF (black soldier fly) larvae meal diet; DFIM: defatted BSF larvae meal diet; DCIM: de-chitinized BSF larvae meal diet; IO: BSF larvae oil diet; EX: BSF larvae exoskeleton diet.

Fig. 4. Linear discriminant analysis (LDA) effect size (LEfSe) results on gut microbiota of fish. Circular cladogram reporting LEfSe results presents the identified amplicon sequence variants (ASVs) distributed according to phylogenetic characteristics around the circle (**a**). The dots closest to the center represent ASVs at the phylum level, whereas the outer circle of dots represent ASVs at the genus level. The color of the dots and sectors indicate the dietary group in which the respective ASVs are most abundant. The color explanation is given above the cladogram. Yellow color indicates ASVs that showed similar abundance in all dietary groups. The colored sectors give information on phylum, class (full name in outermost circles, given only for phylum or class showing significant difference between groups), order, family, and genus (indicated by letter). The explanation is given below the cladogram. Indicator taxa with LDA scores of 3.5 or greater in the microbial communities (**b**). p: phylum; c:

class; o: order; f: family; g: genus; CD: control diet; IM: full-fat BSF (black soldier fly) larvae meal diet; DFIM: defatted BSF larvae meal diet; DCIM: de-chitinized BSF larvae meal diet; IO: BSF larvae oil diet; EX: BSF larvae exoskeleton diet.

Fig. 5. Linear discriminant analysis (LDA) effect size (LEfSe) results on gut microbiota of fish fed CD and IM diets. Circular cladogram reporting LEfSe results presents the identified amplicon sequence variants (ASVs) distributed according to phylogenetic characteristics around the circle (**a**). The dots closest to the center represent ASVs at the phylum level, whereas the outer circle of dots represent ASVs at the genus level. The color of the dots and sectors indicate the dietary group in which the respective ASVs are most abundant. The color explanation is given in the upper left corner. Yellow color indicates ASVs that showed similar abundance in all dietary groups. The colored sectors give information on phylum, class (full name in outermost circles, given only for phylum or class showing significant difference between groups), order, family, and genus (indicated by letter). The explanation is given below the cladogram. Indicator taxa with LDA scores of 4 or greater in the microbial communities (**b**). p: phylum; c: class; o: order; f: family; g: genus; CD: control diet; IM: full-fat black soldier fly larvae meal diet.

Fig. 6. The microbial overlap between the gut and feeds (**a**) and between gut and water (**b**). The number of shared amplicon sequence variants (ASVs) is shown on the left side in each panel. The relative abundance of shared ASVs is shown on the right side in each panel. The minimum relative abundance of ASVs to be considered as present in a sample was 0.05%. CD: control diet; IM: full-fat BSF (black soldier fly) larvae meal diet; DFIM: defatted BSF larvae meal diet; DCIM: de-chitinized BSF larvae meal diet; IO: BSF larvae oil diet; EX: BSF larvae exoskeleton diet.

Fig. 7. The alpha-diversity of gut microbiota in salmon fed experimental diets. Asterisks denote statistically significant differences between control group and insect groups (*, $p < 0.05$; ***, $p < 0.001$). PD: phylogenetic diversity; CD: control diet; IM: full-fat BSF larvae meal diet; DFIM: defatted BSF larvae meal diet; DCIM: de-chitinized BSF (black soldier fly) larvae meal diet; IO: BSF larvae oil diet; EX: BSF larvae exoskeleton diet.

Fig. 8. The beta-diversity of gut microbiota in fish fed experimental diets. PCo: principal coordinate; CD: control diet; IM: full-fat BSF (black soldier fly) larvae meal diet; DFIM: defatted BSF larvae meal diet; DCIM: de-chitinized BSF larvae meal diet; IO: BSF larvae oil diet; EX: BSF larvae exoskeleton diet.

Fig. 9. Hierarchical clustering of the significantly enriched metabolic subsystems between each pair of dietary groups. Columns are diet pairs, rows are metabolic subsystem, and the color of each cell indicates whether the metabolic subsystem was enriched in diet 1 (blue) or diet 2 (red). CD: control diet; IM: full-fat BSF (black soldier fly) larvae meal diet; DFIM: defatted BSF larvae meal diet; DCIM: de-chitinized BSF larvae meal diet; IO: BSF larvae oil diet; EX: BSF larvae exoskeleton diet.

Supplementary figure legends

Fig. S1. Rarefaction curves based on observed amplicon sequence variants (ASVs) for the different sample types. The ASV table was rarefied based on minimum sequence size (10,332) in the sample for normalization of the sequence for computation of two of the beta-diversity indices (Jaccard distance and unweighted UniFrac distance). CD: control diet; IM: full-fat BSF (black soldier fly) larvae meal diet; DFIM: defatted BSF larvae meal diet; DCIM: de-chitinized BSF larvae meal diet; IO: BSF larvae oil diet; EX: BSF larvae exoskeleton diet.

Fig. S2. Principal component analysis (PCA) on standardized amplicon sequence variants (ASVs). Score plots for PC1 and PC2 (**a**) and PC1 and PC3 (**b**), mean scores (dark) with 95% confidence intervals for PC1 (**c**), PC2 (**d**), and PC3 (**e**), and percentage of variance explained by PCs (**f**). PC: principal component, CD: control diet; IM: full-fat BSF (black soldier fly) larvae meal diet; DFIM: defatted BSF larvae meal diet; DCIM: de-chitinized BSF larvae meal diet; IO: BSF larvae oil diet; EX: BSF larvae exoskeleton diet.

Fig. S3. Principal component analysis (PCA) on metabolic reaction abundances (z-scores). Score plots for PC1 and PC2 (**a**) and PC1 and PC3 (**b**), mean scores (dark) with 95% confidence intervals for PC1 (**c**), PC2 (**d**), and PC3 (**e**), and percentage of variance explained by PCs (**f**). PC: principal component, CD: control diet; IM: full-fat BSF (black soldier fly) larvae meal diet; DFIM: defatted BSF larvae meal diet; DCIM: de-chitinized BSF larvae meal diet; IO: BSF larvae oil diet; EX: BSF larvae exoskeleton diet.

Fig. S4. Expected and observed taxonomic profiles of the mock microbial community standard. Mock_1, Mock_2: observed taxonomic profiles of the mock. Mock_Exp: expected taxonomic profile of the mock.

Fig. S5. Linear discriminant analysis (LDA) effect size (LEfSe) results on gut microbiota of fish fed CD and DFIM diets. Circular cladogram reporting LEfSe results presents the identified amplicon sequence variants (ASVs) distributed according to phylogenetic characteristics around the circle (**a**). The dots closest to the center

represent ASVs at the phylum level, whereas the outer circle of dots represent ASVs at the genus level. The color of the dots and sectors indicate the dietary group in which the respective ASVs are most abundant. The color explanation is given in the upper left corner. Yellow color indicates ASVs that showed similar abundance in all dietary groups. The colored sectors give information on phylum, class (full name in outermost circles, given only for phylum or class showing significant difference between groups), order, family, and genus (indicated by letter). The explanation is given below the cladogram. Indicator taxa with LDA scores of 4 or greater in the microbial communities (**b**). p: phylum; c: class; o: order; f: family; g: genus; CD: control diet; DFIM: defatted black soldier fly larvae meal diet.

Fig. S6. Linear discriminant analysis (LDA) effect size (LEfSe) results on gut microbiota of fish fed CD and DCIM diets. Circular cladogram reporting LEfSe results presents the identified amplicon sequence variants (ASVs) distributed according to phylogenetic characteristics around the circle (**a**). The dots closest to the center represent ASVs at the phylum level, whereas the outer circle of dots represent ASVs at the genus level. The color of the dots and sectors indicate the dietary group in which the respective ASVs are most abundant. The color explanation is given in the upper left corner. Yellow color indicates ASVs that showed similar abundance in all dietary groups. The colored sectors give information on phylum, class (full name in outermost circles, given only for phylum or class showing significant difference between groups), order, family, and genus (indicated by letter). The explanation is given below the cladogram. Indicator taxa with LDA scores of 4 or greater in the microbial communities (**b**). p: phylum; c: class; o: order; f: family; g: genus; CD: control diet; DCIM: de-chitinized black soldier fly larvae meal diet.

Fig. S7. Linear discriminant analysis (LDA) effect size (LEfSe) results on gut microbiota of fish fed CD and EX diets. Circular cladogram reporting LEfSe results presents the identified amplicon sequence variants (ASVs) distributed according to phylogenetic characteristics around the circle (**a**). The identified ASVs are distributed according to phylogenetic characteristics around the circle. The dots closest to the center represent ASVs at the phylum level, whereas the outer circle of dots represent ASVs at the genus level. The color of the dots and sectors indicate the dietary group

in which the respective ASVs are most abundant. The color explanation is given in the upper left corner. Yellow color indicates ASVs that showed similar abundance in all dietary groups. The colored sectors give information on class (full name in outermost circles, given only for phylum or class showing significant difference between groups), order, family, and genus (indicated by letter). The explanation is given below the cladogram. Indicator taxa with LDA scores of 4 or greater in the microbial communities (**b**). c: class; o: order; f: family; g: genus; CD: control diet; EX: black soldier fly larvae exoskeleton diet.

Fig. S8: Linear discriminant analysis (LDA) effect size (LEfSe) results on gut microbiota of fish fed CD and IO diets. Circular cladogram reporting LEfSe results presents the identified amplicon sequence variants (ASVs) distributed according to phylogenetic characteristics around the circle (**a**). The dots closest to the center represent ASVs at the phylum level, whereas the outer circle of dots represent ASVs at the genus level. The color of the dots and sectors indicate the dietary group in which the respective ASVs are most abundant. The color explanation is given in the upper left corner. Yellow color indicates ASVs that showed similar abundance in all dietary groups. The colored sectors give information on phylum, class (full name in outermost circles, given only for phylum or class showing significant difference between groups), order, family, and genus (indicated by letter). The explanation is given below the cladogram. Indicator taxa with LDA scores of 4 or greater in the microbial communities (**b**). p: phylum; c: class; o: order; f: family; g: genus; CD: control diet; IO: black soldier fly larvae oil diet.

Fig. S9. Venn's diagram showing the shared and unique core ASVs in digesta samples belong to insect-based groups (**a**), insect meal groups (**b**), insect lipid containing groups (**c**) and insect chitin containing groups (**d**). The core ASVs were computed using a prevalence threshold of 80%. CD: control diet; IM: full-fat BSF (black soldier fly) larvae meal diet; DFIM: defatted BSF larvae meal diet; DCIM: de-chitinized BSF larvae meal diet; IO: BSF larvae oil diet; EX: BSF larvae exoskeleton diet.

Fig. S10. The boxplots for homogeneity of multivariate dispersions in gut microbiota of fish fed experimental diets. CD: control diet; IM: Full-fat BSF (black soldier fly)

larvae meal diet; DFIM: Defatted BSF larvae meal diet; DCIM: De-chitinized BSF larvae meal diet; IO: BSF larvae oil diet; EX: BSF larvae exoskeleton diet.

Fig. S11. Number of ASVs mapped to genome-scale metabolic models. Number of samples matched to models at different taxonomic levels (**a**) and the number of models mapped to each sample by taxonomic level (**b**).

Fig. S12. Results from t-tests comparing reaction abundances between pairs of diets. The t-statistic for each reaction is shown along with the mean across all reactions with 95% confidence interval for all significantly enriched subsystems. IO and CD (**a**), EX and IO (**b**), DCIM and CD (**c**), IM and CD (**d**), IO and DFIM (**e**), IO and DCIM (**f**), DFIM and IM (**g**), EX and CD (**h**), EX and DCIM (**i**), IO and IM (**j**), DCIM and IM (**k**), DFIM and CD (**l**), EX and DFIM (**m**), EX and IM (**n**) and DCIM and DFIM (**o**) groups. CD: control diet; IM: full-fat BSF (black soldier fly) larvae meal diet; DFIM: defatted BSF larvae meal diet; DCIM: de-chitinized BSF larvae meal diet; IO: BSF larvae oil diet; EX: BSF larvae exoskeleton diet.

Figures

Figure 1

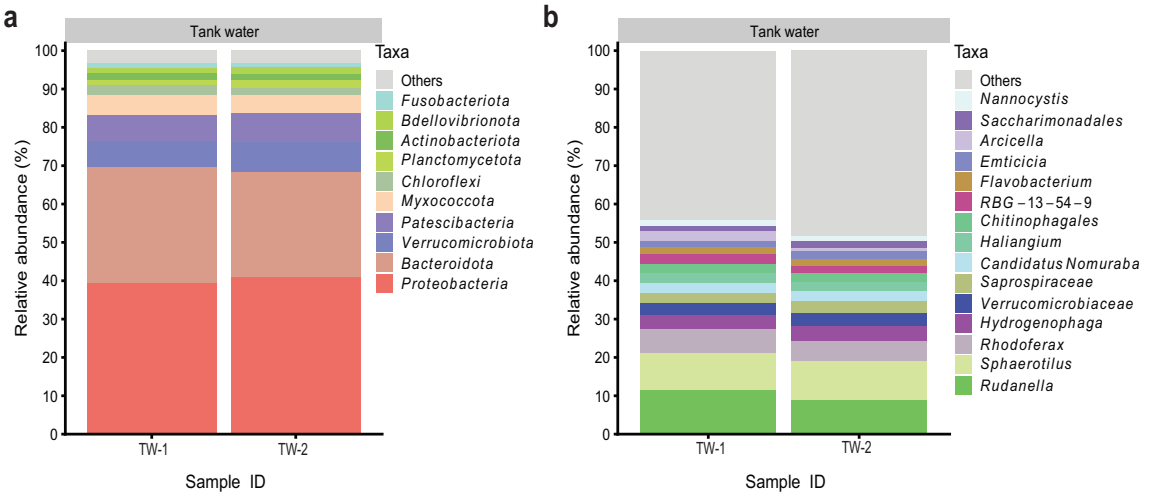


Figure 2

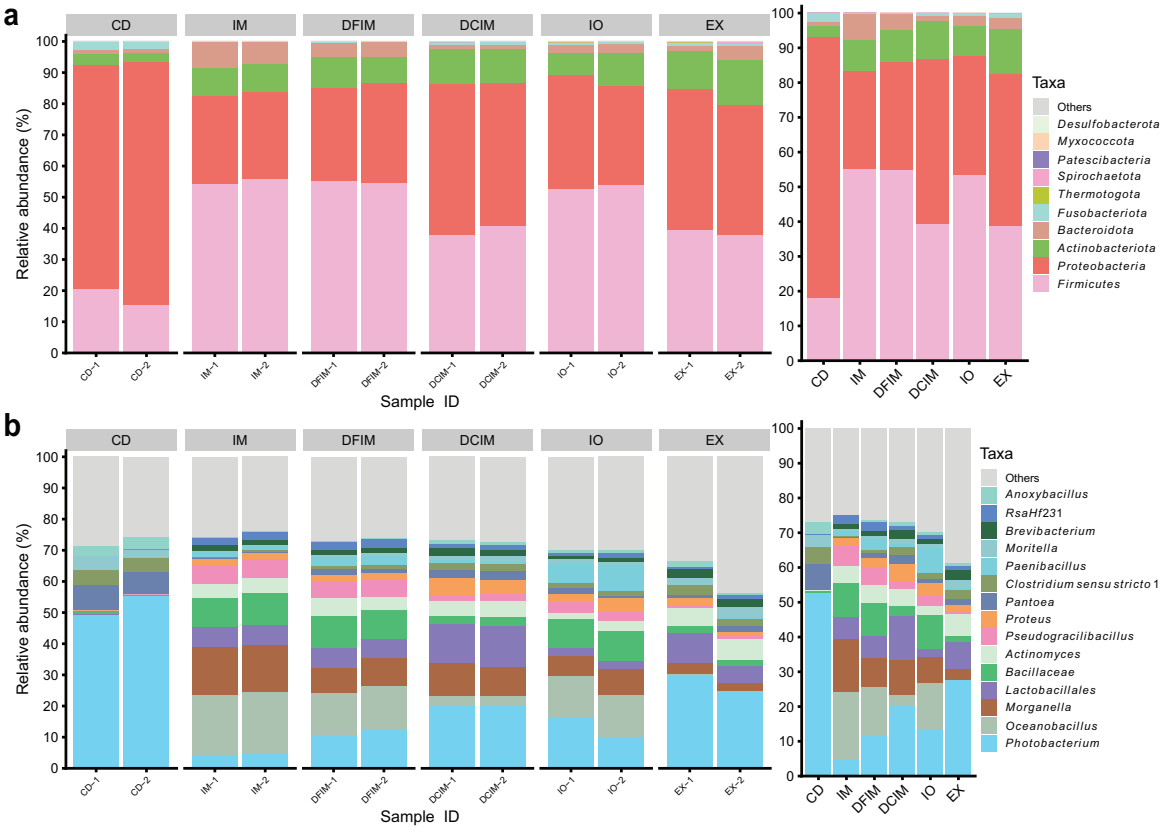
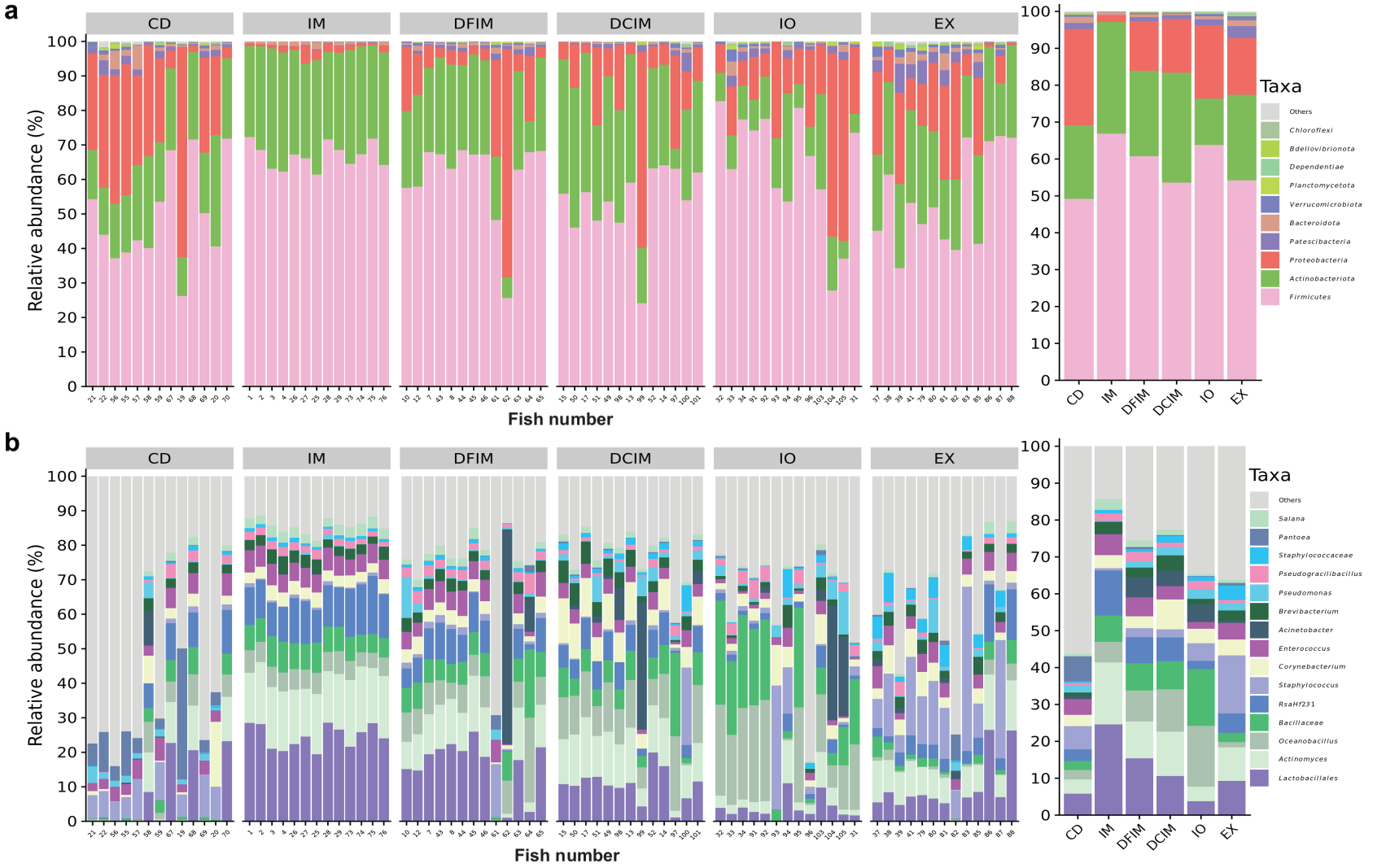


Figure 3


a
CD
IM
DFIM
DCIM
IO
EX
Relative abundance (%)
Fish number
Taxa
Others
Chloroflexi
Bdellovibrionota
Dependentiae
Planctomycetota
Verrucomicrobiota
Bacteroidota
Patescibacteria
Proteobacteria
Actinobacteriota
Firmicutes
b
Salana
Pantoea
Staphylococcaceae
Pseudogracilibacillus
Pseudomonas
Brevibacterium
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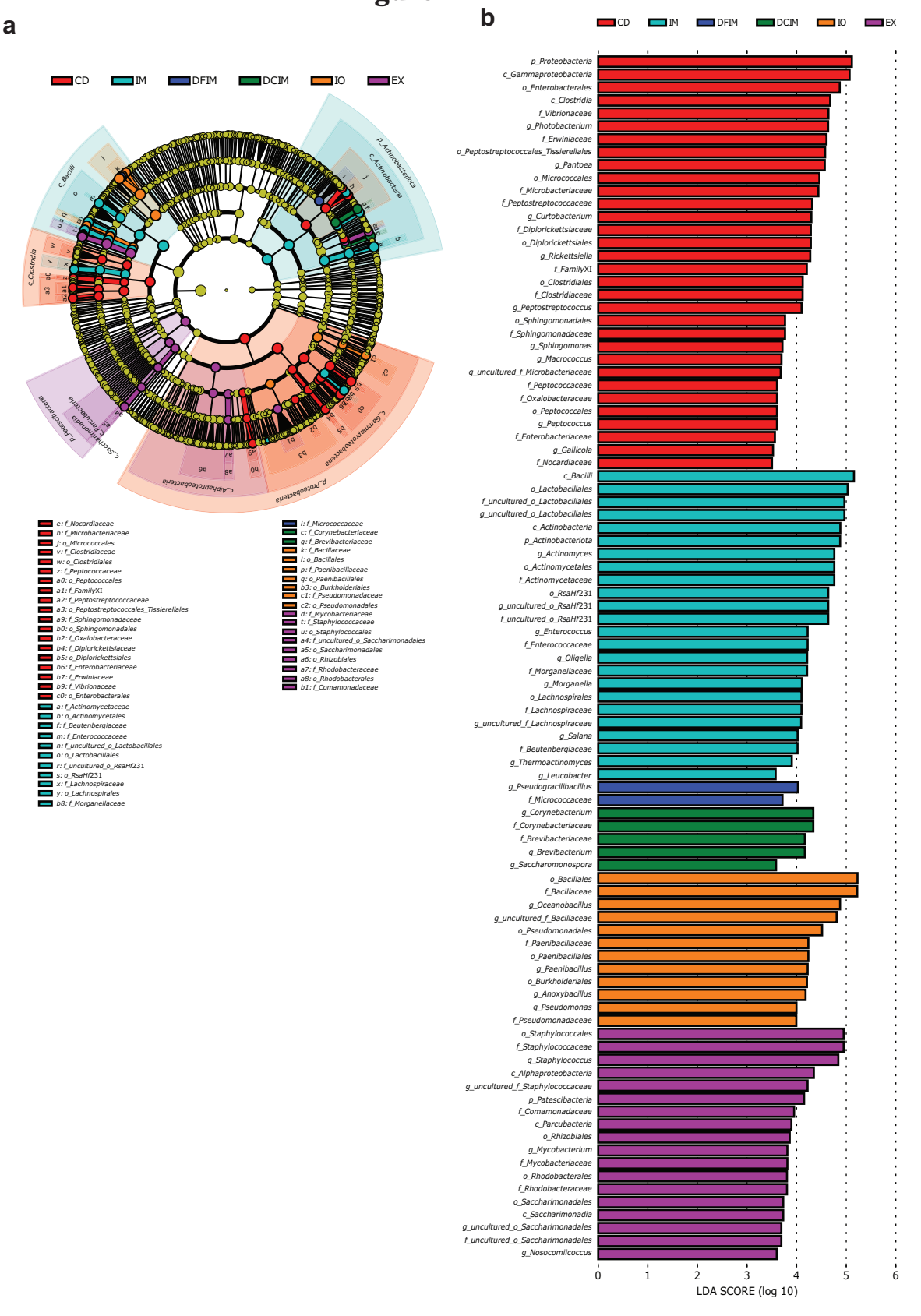
Enterococcus
Corynebacterium
Staphylococcus
RsaHf231
Bacillaceae
Oceanobacillus
Actinomyces
Lactobacillales

Figure 4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b
CD
IM
DFIM
DCIM
IO
EX
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c_Gammaproteobacteria
c_Alphaproteobacteria
p_Actinobacteriota
c_Actinobacteria
c_Bacilli
c_Clostridia
p_Bacteroidetes
c_Saccharimonadia
p_Patescibacteria
e: f_Nocardiaceae
h: f_Microbacteriaceae
j: o_Micrococcales
v: f_Clostridiaceae
w: o_Clostridiales
z: f_Peptococcaceae
a0: o_Peptococcales
a1: f_FamilyXI
a2: f_Peptostreptococcaceae
a3: o_Peptostreptococcales_Tissierellales
a9: f_Sphingomonadaceae
b0: o_Sphingomonadales
b2: f_Oxalobacteraceae
b4: f_Diplorickettsiaceae
b5: o_Diplorickettsiales
b6: f_Enterobacteriaceae
b7: f_Erwiniaceae
b9: f_Vibrionaceae
c0: o_Enterobacterales
a: f_Actinomycetaceae
b: o_Actinomycetales
f: f_Beutenbergiaceae
m: f_Enterococcaceae
n: f_uncultured_o_Lactobacillales
o: o_Lactobacillales
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s: o_RsaHf231
x: f_Lachnospiraceae
y: o_Lachnospirales
b8: f_Morganellaceae
i: f_Micrococcaceae
c: f_Corynebacteriaceae
g: f_Brevibacteriaceae
k: f_Bacillaceae
l: o_Bacillales
p: f_Paenibacillaceae
q: o_Paenibacillales
b3: o_Burkholderiales
c1: f_Pseudomonadaceae
c2: o_Pseudomonadales
d: f_Mycobacteriaceae
t: f_Staphylococcaceae
u: o_Staphylococcales
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a5: o_Saccharimonadales
a6: o_Rhizobiales
a7: f_Rhodobacteraceae
a8: o_Rhodobacterales
b1: f_Comamonadaceae
p_Proteobacteria
c_Gammaproteobacteria
o_Enterobacterales
c_Clostridia
f_Vibrionaceae
g_Photobacterium
f_Erwiniaceae
o_Peptostreptococcales_Tissierellales
g_Pantoea
o_Micrococcales
f_Microbacteriaceae
f_Peptostreptococcaceae
g_Curtobacterium
f_Diplorickettsiaceae
o_Diplorickettsiales
g_Rickettsiella
f_FamilyXI
o_Clostridiales
f_Clostridiaceae
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f_Sphingomonadaceae
g_Sphingomonas
g_Macrococcus
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g_Gallicola
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o_Lactobacillales
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g_uncultured_o_Lactobacillales
c_Actinobacteria
p_Actinobacteriota
g_Actinomyces
o_Actinomycetales
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o_RsaHf231
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g_Morganella
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g_Brevibacterium
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g_Paenibacillus
o_Burkholderiales
g_Anoxybacillus
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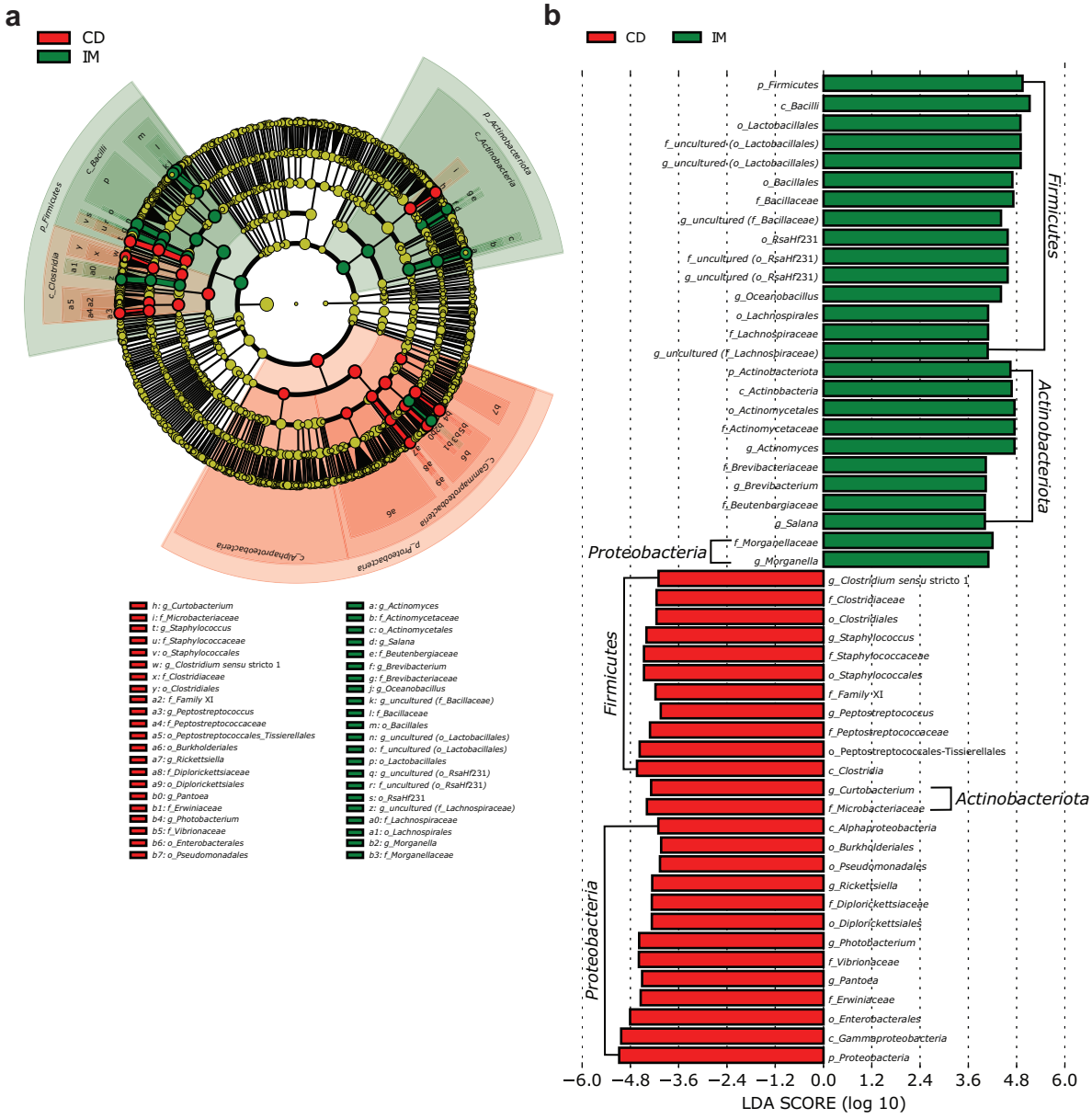
o_Rhodobacterales
f_Rhodobacteraceae
o_Saccharimonadales
c_Saccharimonadia
g_uncultured_o_Saccharimonadales
f_uncultured_o_Saccharimonadales
g_Nosocomiicoccus
0
1
2
3
4
5
6
LDA SCORE (log 10)

Figure 5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c_Alphaproteobacteria
h: g_Curtobacterium
i: f_Microbacteriaceae
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u: f_Staphylococcaceae
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y: o_Clostridiales
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a4: f_Peptostreptococcaceae
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a6: o_Burkholderiales
a7: g_Rickettsiella
a8: f_Diplorickettsiaceae
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b0: g_Pantoea
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d: g_Salana
e: f_Beutenbergiaceae
f: g_Brevibacterium
g: f_Brevibacteriaceae
j: g_Oceanobacillus
k: g_uncultured (f_Bacillaceae)
l: f_Bacillaceae
m: o_Bacillales
n: g_uncultured (o_Lactobacillales)
o: f_uncultured (o_Lactobacillales)
p: o_Lactobacillales
q: g_uncultured (o_RsaHf231)
r: f_uncultured (o_RsaHf231)
s: o_RsaHf231
z: g_uncultured (f_Lachnospiraceae)
a0: f_Lachnospiraceae
a1: o_Lachnospirales
b2: g_Morganella
b3: f_Morganellaceae
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CD
IM
p_Firmicutes
c_Bacilli
o_Lactobacillales
f_uncultured (o_Lactobacillales)
g_uncultured (o_Lactobacillales)
o_Bacillales
f_Bacillaceae
g_uncultured (f_Bacillaceae)
o_RsaHf231
f_uncultured (o_RsaHf231)
g_uncultured (o_RsaHf231)
g_Oceanobacillus
o_Lachnospirales
f_Lachnospiraceae
g_uncultured (f_Lachnospiraceae)
p_Actinobacteriota
c_Actinobacteria
o_Actinomycetales
f_Actinomycetaceae
g_Actinomyces
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g_Brevibacterium
f_Beutenbergiaceae
g_Salana
f_Morganellaceae
g_Morganella
Proteobacteria
g_Clostridium sensu stricto 1
f_Clostridiaceae
o_Clostridiales
g_Staphylococcus
f_Staphylococcaceae
o_Staphylococcales
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g_Peptostreptococcus
f_Peptostreptococcaceae
o_Peptostreptococcales-Tissierellales
c_Clostridia
g_Curtobacterium
f_Microbacteriaceae
c_Alphaproteobacteria
o_Burkholderiales
o_Pseudomonadales
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f_Diplorickettsiaceae
o_Diplorickettsiales
g_Photobacterium
f_Vibrionaceae
g_Pantoea
f_Erwiniaceae
o_Enterobacterales
c_Gammaproteobacteria
p_Proteobacteria
Firmicutes
Actinobacteriota
Firmicutes
Actinobacteriota
Proteobacteria
−6.0 −4.8 −3.6 −2.4 −1.2 0.0 1.2 2.4 3.6 4.8 6.0
LDA SCORE (log 10)

Figure 6

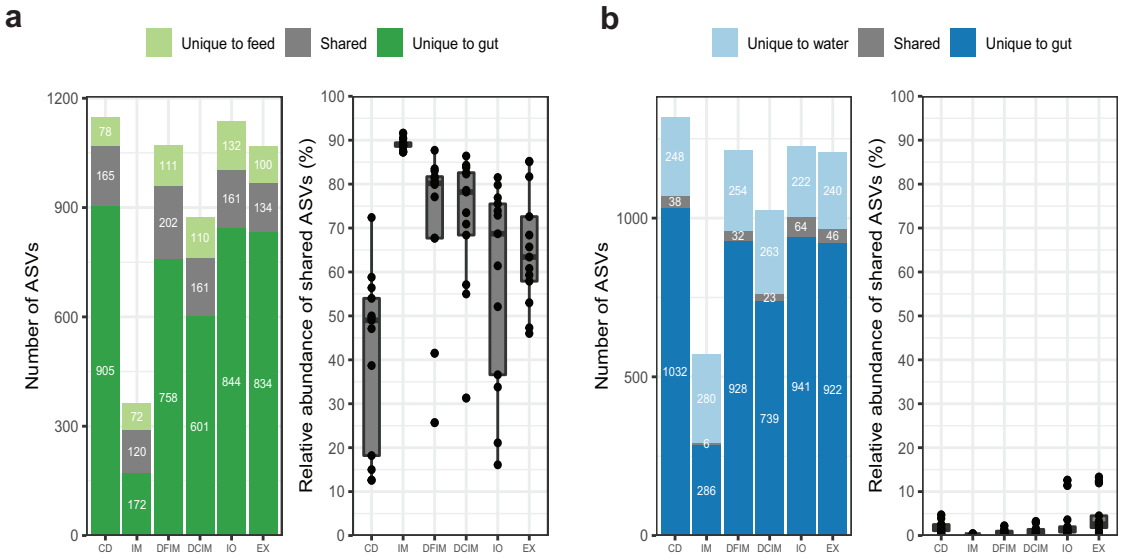

a
Unique to feed
Shared
Unique to gut
Number of ASVs
Relative abundance of shared ASVs (%)
CD
IM
DFIM
DCIM
IO
EX
b
Unique to water
Shared
Unique to gut
Number of ASVs
Relative abundance of shared ASVs (%)

Figure 7

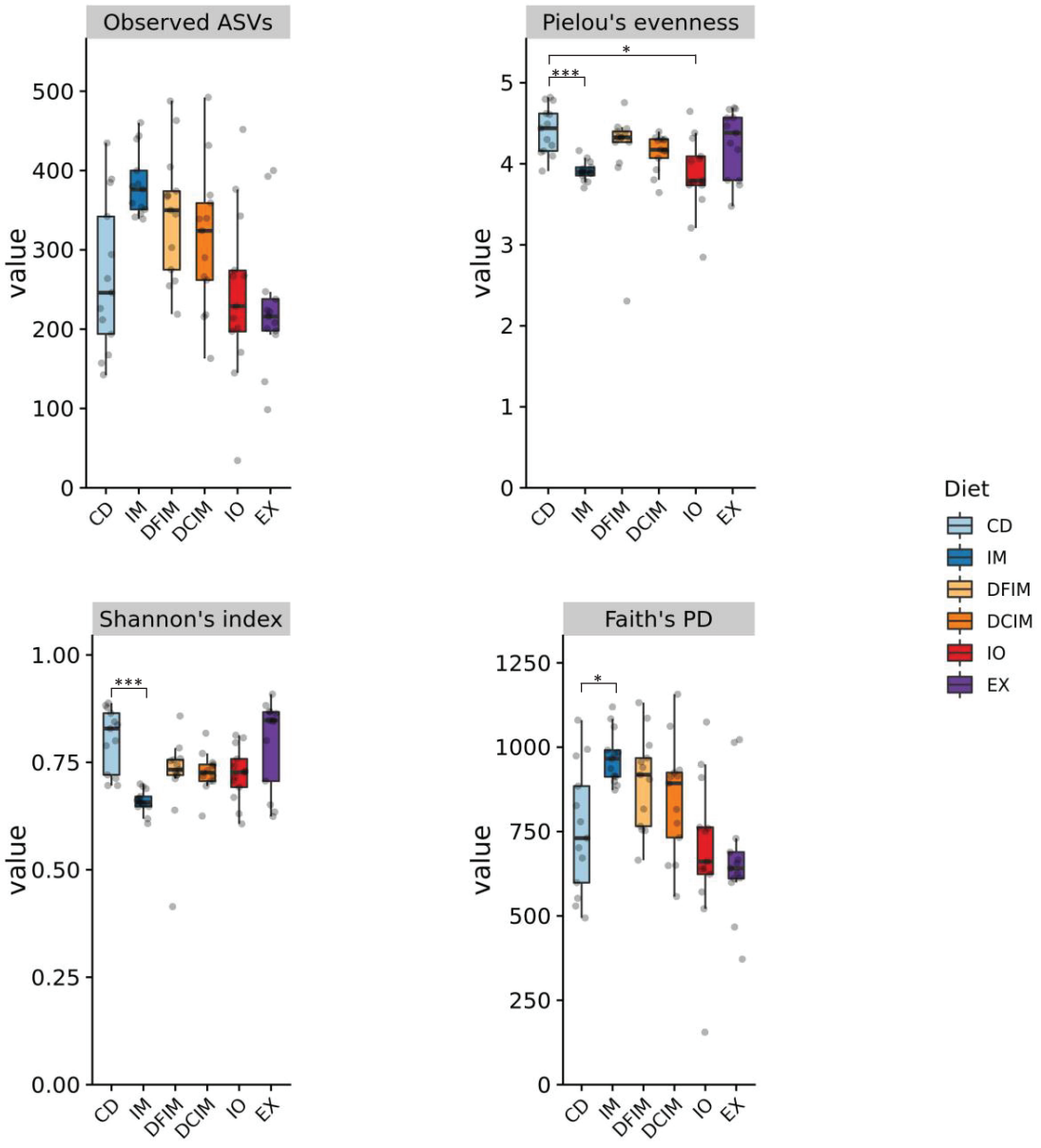

Observed ASVs
Pielou's evenness
Shannon's index
Faith's PD
value
CD
IM
DFIM
DCIM
IO
EX
***
*
Diet

Figure 8

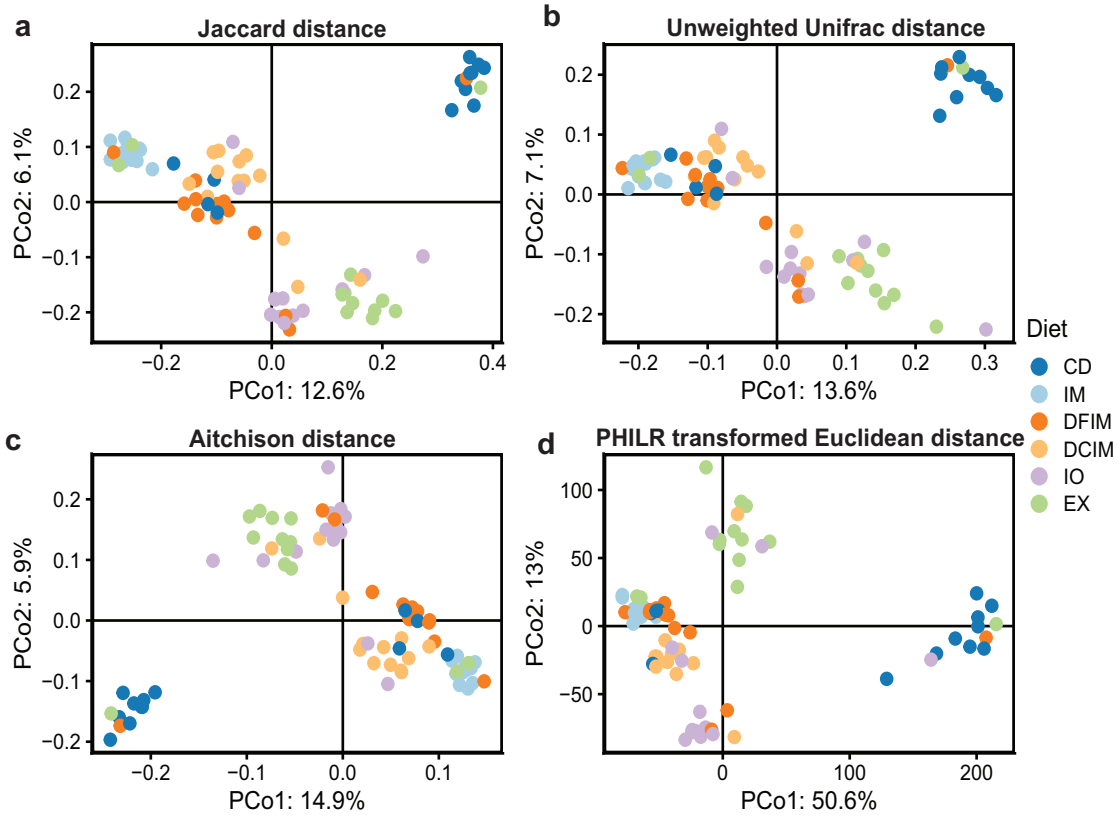
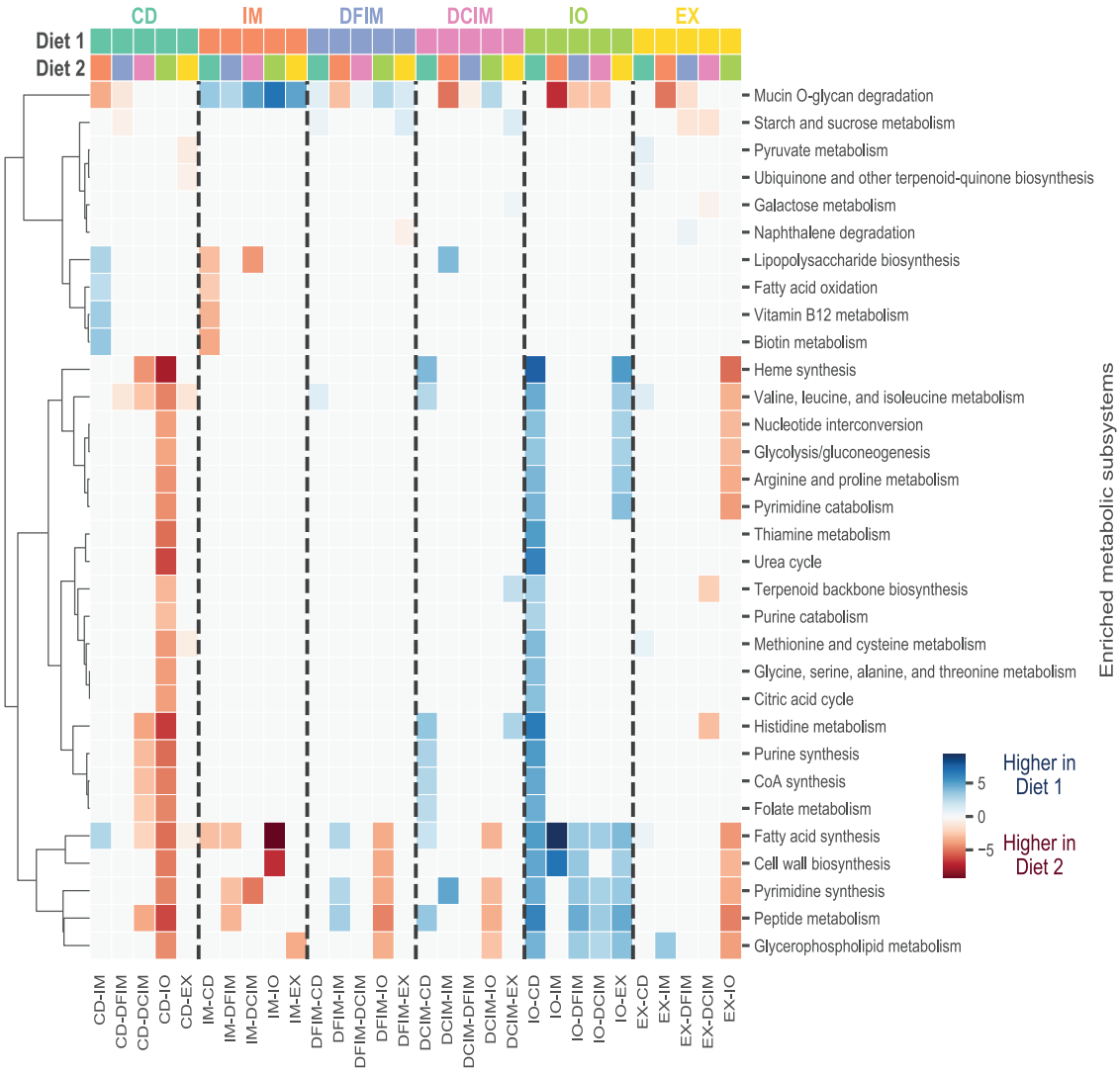

a
Jaccard distance
PCo2: 6.1%
PCo1: 12.6%
b
Unweighted Unifrac distance
PCo2: 7.1%
PCo1: 13.6%
c
Aitchison distance
PCo2: 5.9%
PCo1: 14.9%
d
PHILR transformed Euclidean distance
PCo2: 13%
PCo1: 50.6%
Diet
CD
IM
DFIM
DCIM
IO
EX

Figure 9


CD
IM
DFIM
DCIM
IO
EX
Diet 1
Diet 2
Mucin O-glycan degradation
Starch and sucrose metabolism
Pyruvate metabolism
Ubiquinone and other terpenoid-quinone biosynthesis
Galactose metabolism
Naphthalene degradation
Lipopolysaccharide biosynthesis
Fatty acid oxidation
Vitamin B12 metabolism
Biotin metabolism
Heme synthesis
Valine, leucine, and isoleucine metabolism
Nucleotide interconversion
Glycolysis/gluconeogenesis
Arginine and proline metabolism
Pyrimidine catabolism
Thiamine metabolism
Urea cycle
Terpenoid backbone biosynthesis
Purine catabolism
Methionine and cysteine metabolism
Glycine, serine, alanine, and threonine metabolism
Citric acid cycle
Histidine metabolism
Purine synthesis
CoA synthesis
Folate metabolism
Fatty acid synthesis
Cell wall biosynthesis
Pyrimidine synthesis
Peptide metabolism
Glycerophospholipid metabolism
Enriched metabolic subsystems
Higher in Diet 1
5
0
−5
Higher in Diet 2
CD-IM
CD-DFIM
CD-DCIM
CD-IO
CD-EX
IM-CD
IM-DFIM
IM-DCIM
IM-IO
IM-EX
DFIM-CD
DFIM-IM
DFIM-DCIM
DFIM-IO
DFIM-EX
DCIM-CD
DCIM-IM
DCIM-DFIM
DCIM-IO
DCIM-EX
IO-CD
IO-IM
IO-DFIM
IO-DCIM
IO-EX
EX-CD
EX-IM
EX-DFIM
EX-DCIM
EX-IO

Supplementary figures

Figure S1

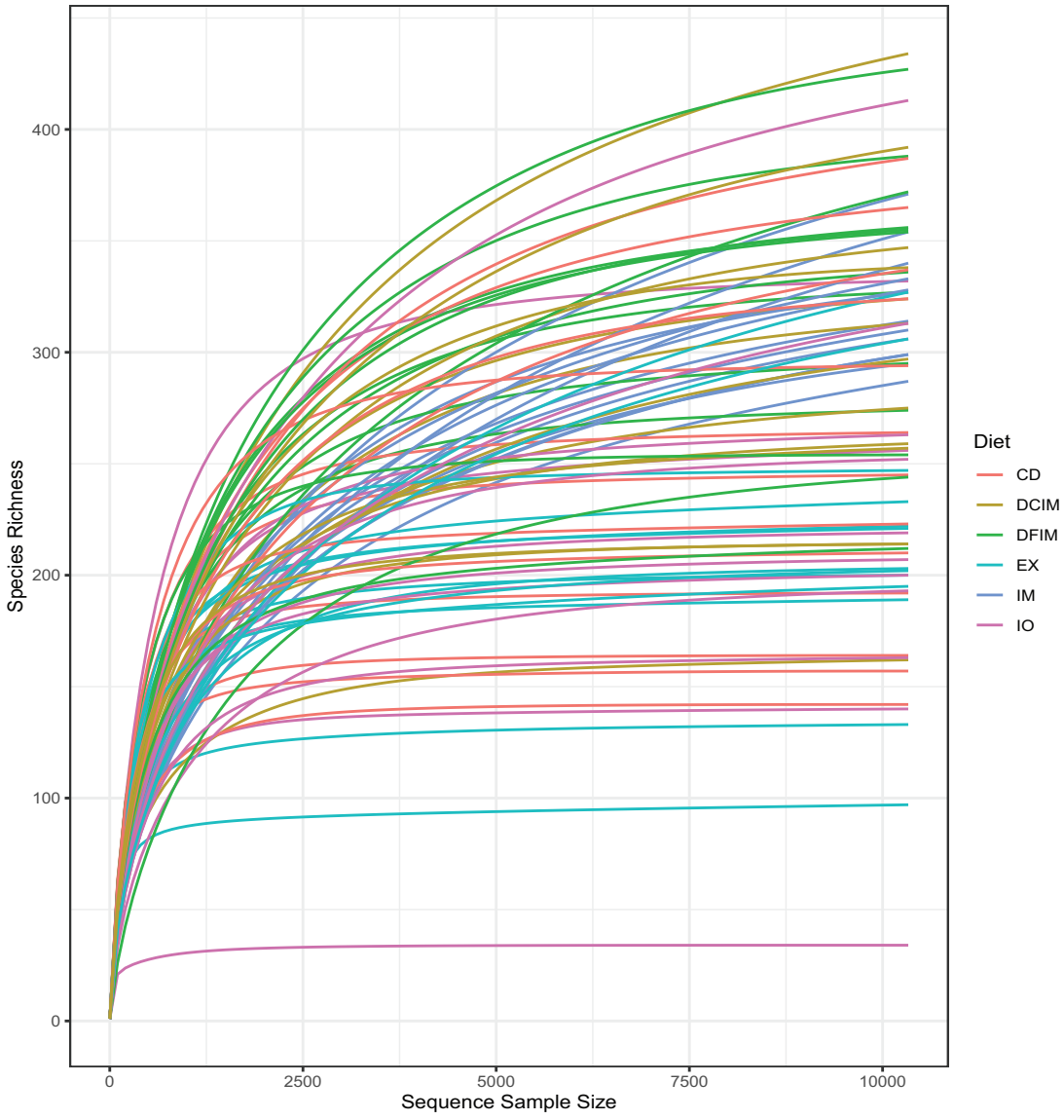


Figure S2

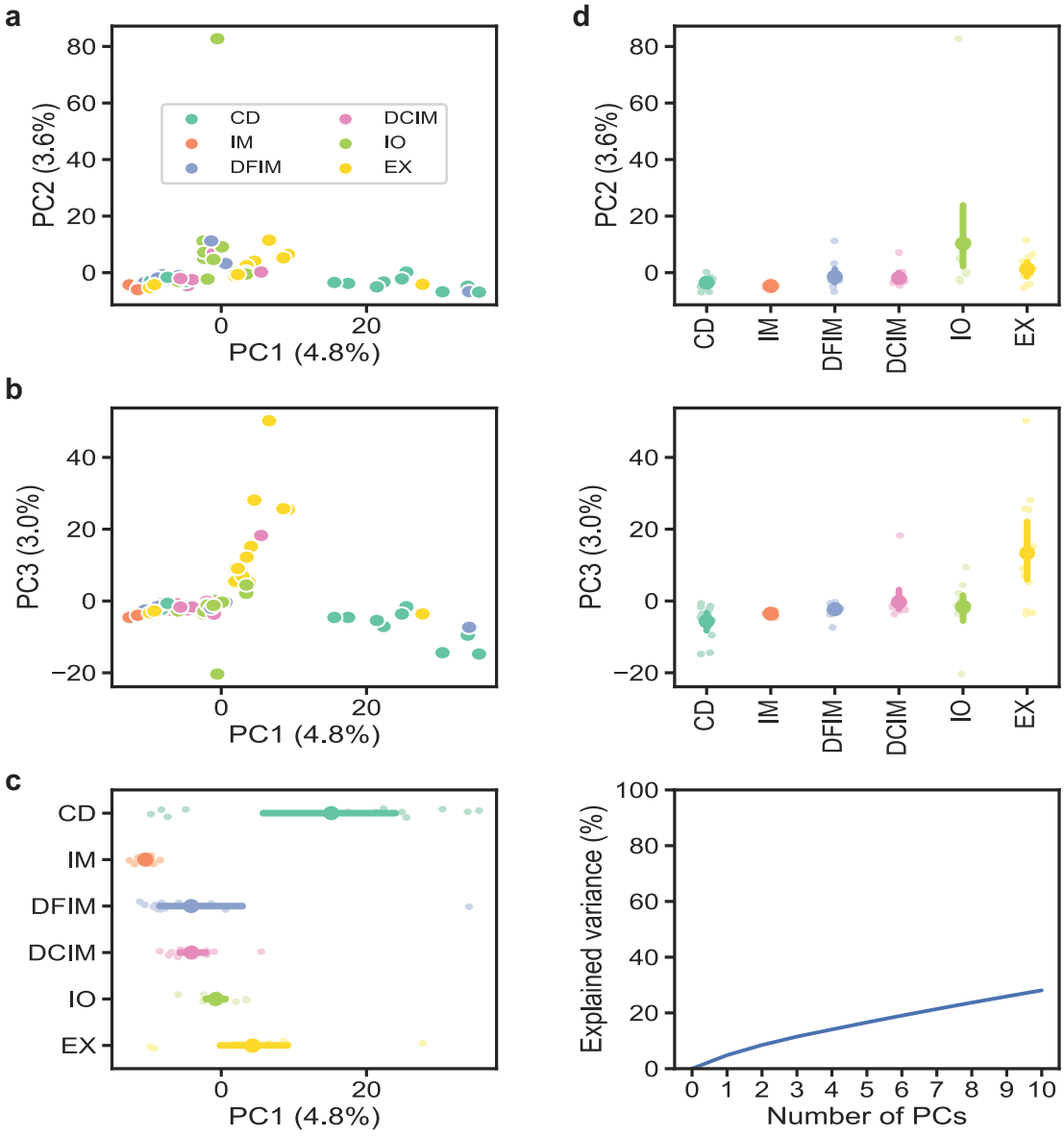

a
PC2 (3.6%)
PC1 (4.8%)
CD
IM
DFIM
DCIM
IO
EX
d
PC2 (3.6%)
CD
IM
DFIM
DCIM
IO
EX
b
PC3 (3.0%)
PC1 (4.8%)
PC3 (3.0%)
c
CD
IM
DFIM
DCIM
IO
EX
PC1 (4.8%)
Explained variance (%)
Number of PCs

Figure S3

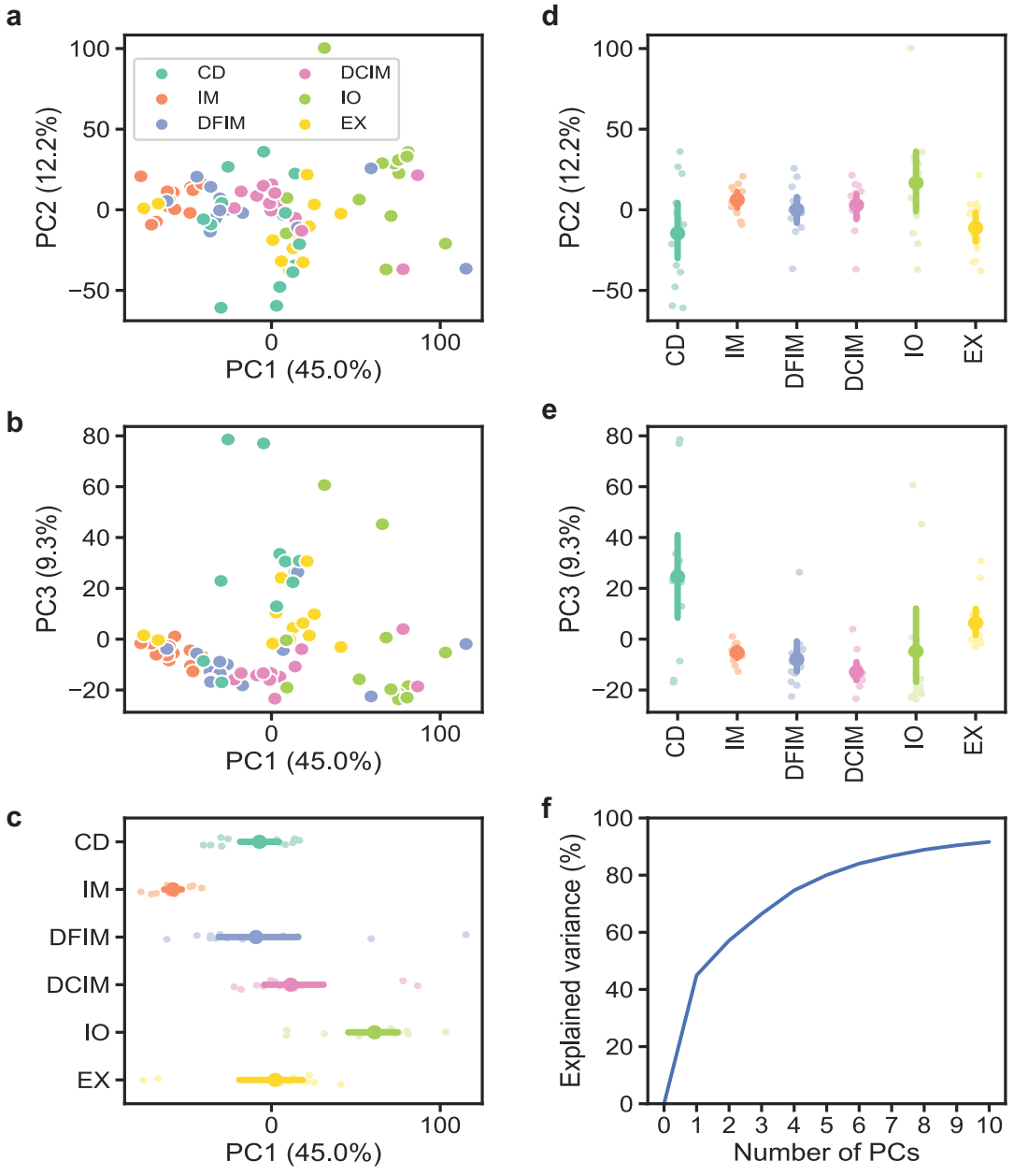

a
b
c
d
e
f
CD
IM
DFIM
DCIM
IO
EX
PC1 (45.0%)
PC2 (12.2%)
PC3 (9.3%)
Explained variance (%)
Number of PCs

Figure S4

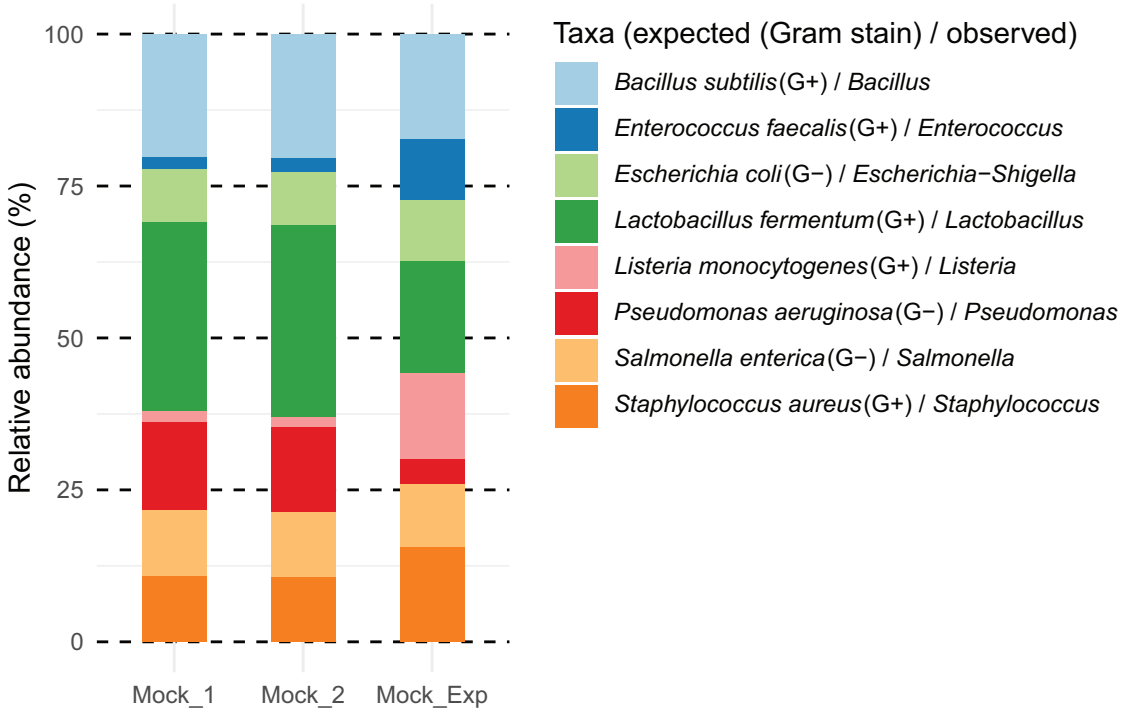

Relative abundance (%)
100
75
50
25
0
Mock_1
Mock_2
Mock_Exp
Taxa (expected (Gram stain) / observed)
Bacillus subtilis(G+) / Bacillus
Enterococcus faecalis(G+) / Enterococcus
Escherichia coli(G−) / Escherichia−Shigella
Lactobacillus fermentum(G+) / Lactobacillus
Listeria monocytogenes(G+) / Listeria
Pseudomonas aeruginosa(G−) / Pseudomonas
Salmonella enterica(G−) / Salmonella
Staphylococcus aureus(G+) / Staphylococcus

Figure S5

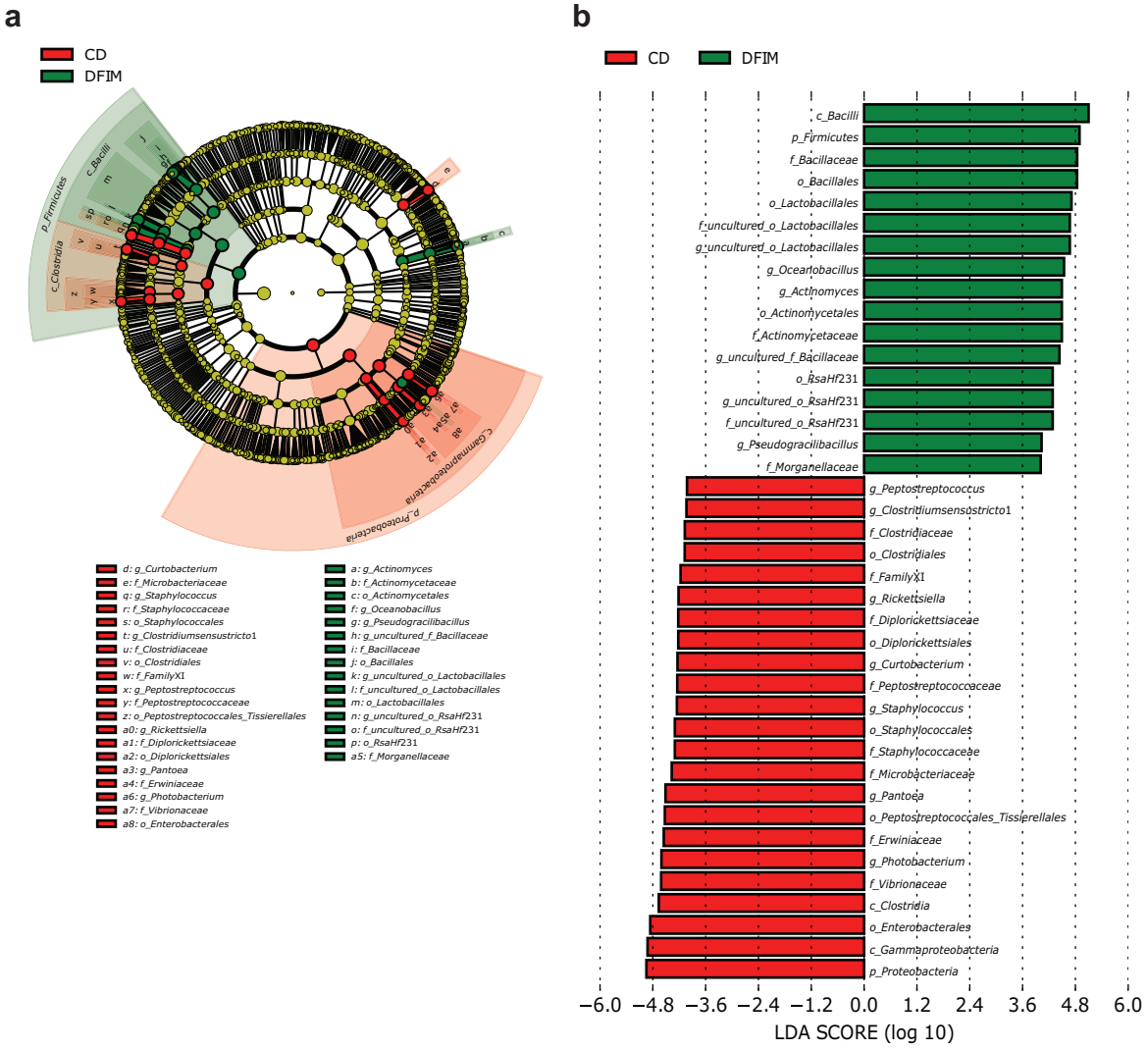

a
CD
DFIM
p_Firmicutes
c_Bacilli
c_Clostridia
p_Proteobacteria
c_Gammaproteobacteria
d: g_Curtobacterium
e: f_Microbacteriaceae
q: g_Staphylococcus
r: f_Staphylococcaceae
s: o_Staphylococcales
t: g_Clostridiumsensustricto1
u: f_Clostridiaceae
v: o_Clostridiales
w: f_FamilyX1
x: g_Peptostreptococcus
y: f_Peptostreptococcaceae
z: o_Peptostreptococcales_Tissierellales
a0: g_Rickettsiella
a1: f_Diplorickettsiaceae
a2: o_Diplorickettsiales
a3: g_Pantoea
a4: f_Erwiniaceae
a6: g_Photobacterium
a7: f_Vibrionaceae
a8: o_Enterobacterales
a: g_Actinomyces
b: f_Actinomycetaceae
c: o_Actinomycetales
f: g_Oceanobacillus
g: g_Pseudogracilibacillus
h: g_uncultured_f_Bacillaceae
i: f_Bacillaceae
j: o_Bacillales
k: g_uncultured_o_Lactobacillales
l: f_uncultured_o_Lactobacillales
m: o_Lactobacillales
n: g_uncultured_o_RsaHf231
o: f_uncultured_o_RsaHf231
p: o_RsaHf231
a5: f_Morganellaceae
b
CD
DFIM
c_Bacilli
p_Firmicutes
f_Bacillaceae
o_Bacillales
o_Lactobacillales
f_uncultured_o_Lactobacillales
g_uncultured_o_Lactobacillales
g_Oceanobacillus
g_Actinomyces
o_Actinomycetales
f_Actinomycetaceae
g_uncultured_f_Bacillaceae
o_RsaHf231
g_uncultured_o_RsaHf231
f_uncultured_o_RsaHf231
g_Pseudogracilibacillus
f_Morganellaceae
g_Peptostreptococcus
g_Clostridiumsensustricto1
f_Clostridiaceae
o_Clostridiales
f_FamilyX1
g_Rickettsiella
f_Diplorickettsiaceae
o_Diplorickettsiales
g_Curtobacterium
f_Peptostreptococcaceae
g_Staphylococcus
o_Staphylococcales
f_Staphylococcaceae
f_Microbacteriaceae
g_Pantoea
o_Peptostreptococcales_Tissierellales
f_Erwiniaceae
g_Photobacterium
f_Vibrionaceae
c_Clostridia
o_Enterobacterales
c_Gammaproteobacteria
p_Proteobacteria
−6.0 −4.8 −3.6 −2.4 −1.2 0.0 1.2 2.4 3.6 4.8 6.0
LDA SCORE (log 10)

Figure S6

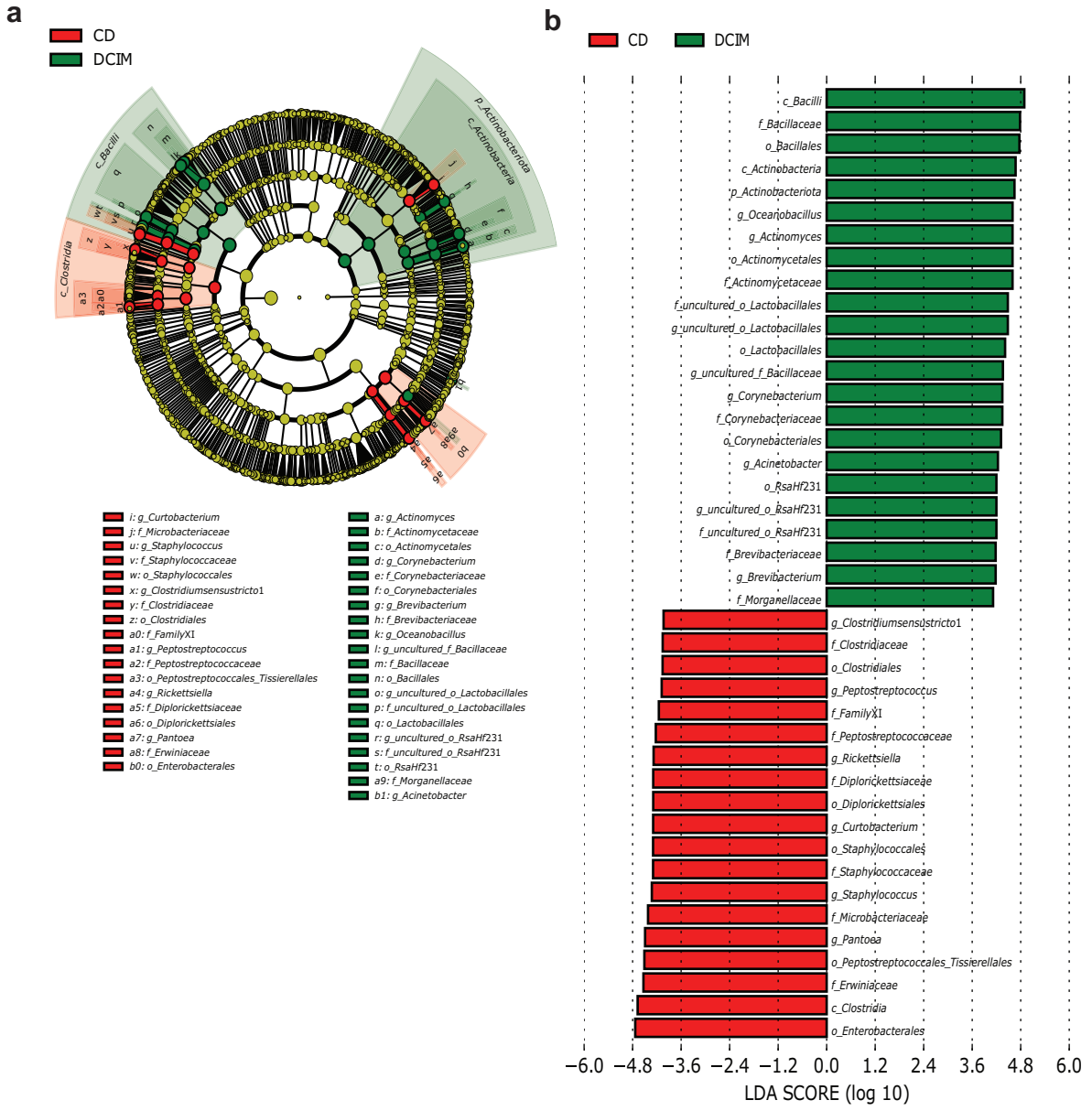

a
CD
DCIM
c_Bacili
p_Actinobacteriota
c_Actinobacteria
c_Clostridia
i: g_Curtobacterium
j: f_Microbacteriaceae
u: g_Staphylococcus
v: f_Staphylococcaceae
w: o_Staphylococcales
x: g_Clostridiumsensustricto1
y: f_Clostridiaceae
z: o_Clostridiales
a0: f_FamilyX1
a1: g_Peptostreptococcus
a2: f_Peptostreptococcaceae
a3: o_Peptostreptococcales_Tissierellales
a4: g_Rickettsiella
a5: f_Diplorickettsiaceae
a6: o_Diplorickettsiales
a7: g_Pantoea
a8: f_Erwiniaceae
b0: o_Enterobacterales
a: g_Actinomyces
b: f_Actinomycetaceae
c: o_Actinomycetales
d: g_Corynebacterium
e: f_Corynebacteriales
f: o_Corynebacteriales
g: g_Brevibacterium
h: f_Brevibacteriaceae
k: g_Oceanobacillus
l: g_uncultured_f_Bacillaceae
m: f_Bacillaceae
n: o_Bacillales
o: g_uncultured_o_Lactobacillales
p: f_uncultured_o_Lactobacillales
q: o_Lactobacillales
r: g_uncultured_o_RsaHf231
s: f_uncultured_o_RsaHf231
t: o_RsaHf231
a9: f_Morganellaceae
b1: g_Acinetobacter
b
CD
DCIM
c_Bacilli
f_Bacillaceae
o_Bacillales
c_Actinobacteria
p_Actinobacteriota
g_Oceanobacillus
g_Actinomyces
o_Actinomycetales
f_Actinomycetaceae
f_uncultured_o_Lactobacillales
g_uncultured_o_Lactobacillales
o_Lactobacillales
g_uncultured_f_Bacillaceae
g_Corynebacterium
f_Corynebacteriaceae
o_Corynebacteriales
g_Acinetobacter
o_RsaHf231
g_uncultured_o_RsaHf231
f_uncultured_o_RsaHf231
f_Brevibacteriaceae
g_Brevibacterium
f_Morganellaceae
g_Clostridiumsensustricto1
f_Clostridiaceae
o_Clostridiales
g_Peptostreptococcus
f_FamilyX1
f_Peptostreptococcaceae
g_Rickettsiella
f_Diplorickettsiaceae
o_Diplorickettsiales
g_Curtobacterium
o_Staphylococcales
f_Staphylococcaceae
g_Staphylococcus
f_Microbacteriaceae
g_Pantoea
o_Peptostreptococcales_Tissierellales
f_Erwiniaceae
c_Clostridia
o_Enterobacterales
−6.0 −4.8 −3.6 −2.4 −1.2 0.0 1.2 2.4 3.6 4.8 6.0
LDA SCORE (log 10)

Figure S7

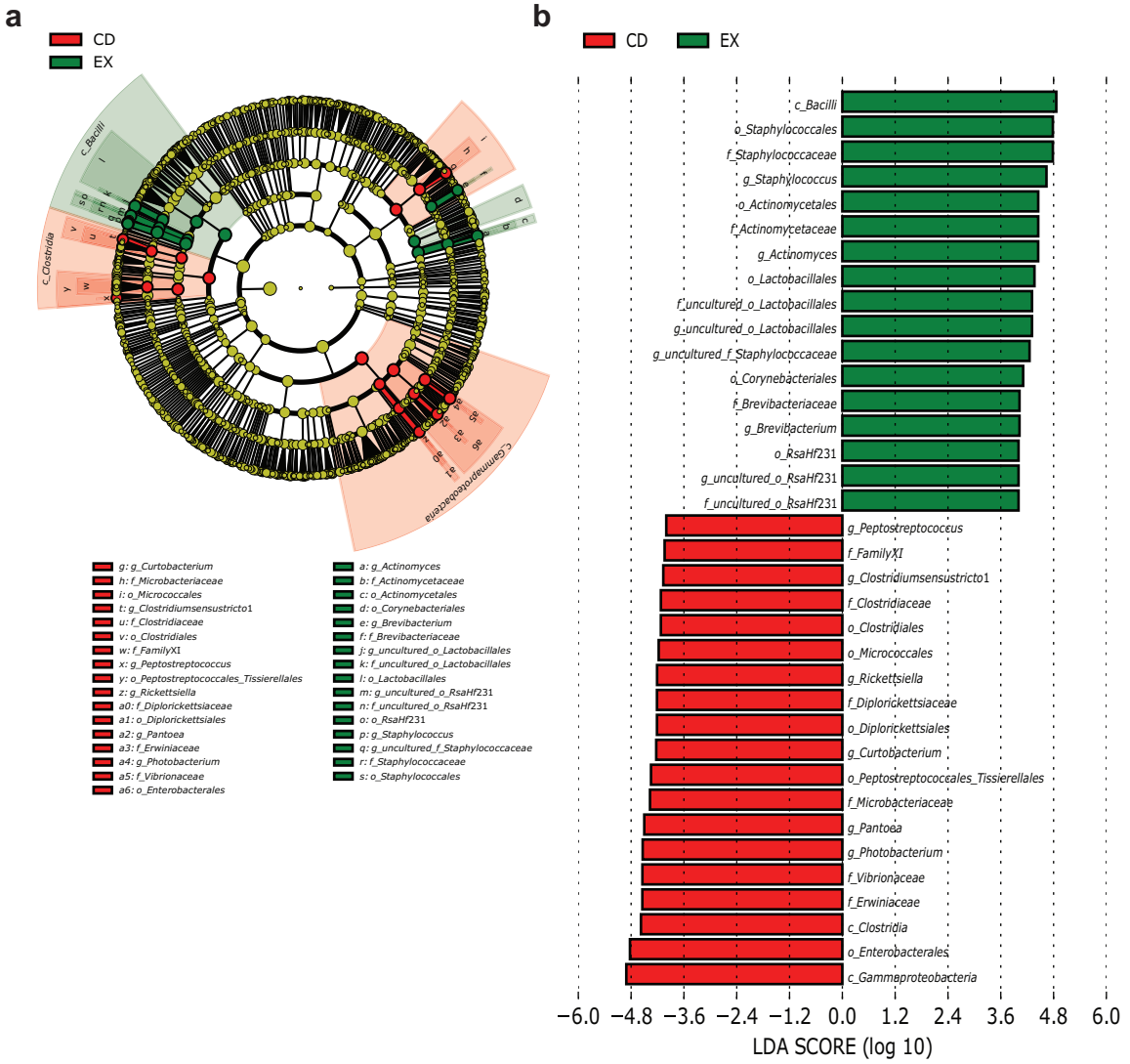

a
CD
EX
c_Bacilli
c_Clostridia
c_Gammaproteobacteria
g: g_Curtobacterium
h: f_Microbacteriaceae
i: o_Micrococcales
t: g_Clostridiumsensustricto1
u: f_Clostridiaceae
v: o_Clostridiales
w: f_FamilyXI
x: g_Peptostreptococcus
y: o_Peptostreptococcales_Tissierellales
z: g_Rickettsiella
a0: f_Diplorickettsiaceae
a1: o_Diplorickettsiales
a2: g_Pantoea
a3: f_Erwiniaceae
a4: g_Photobacterium
a5: f_Vibrionaceae
a6: o_Enterobacterales
a: g_Actinomyces
b: f_Actinomycetaceae
c: o_Actinomycetales
d: o_Corynebacteriales
e: g_Brevibacterium
f: f_Brevibacteriaceae
j: g_uncultured_o_Lactobacillales
k: f_uncultured_o_Lactobacillales
l: o_Lactobacillales
m: g_uncultured_o_RsaHf231
n: f_uncultured_o_RsaHf231
o: o_RsaHf231
p: g_Staphylococcus
q: g_uncultured_f_Staphylococcaceae
r: f_Staphylococcaceae
s: o_Staphylococcales
b
CD
EX
c_Bacilli
o_Staphylococcales
f_Staphylococcaceae
g_Staphylococcus
o_Actinomycetales
f_Actinomycetaceae
g_Actinomyces
o_Lactobacillales
f_uncultured_o_Lactobacillales
g_uncultured_o_Lactobacillales
g_uncultured_f_Staphylococcaceae
o_Corynebacteriales
f_Brevibacteriaceae
g_Brevibacterium
o_RsaHf231
g_uncultured_o_RsaHf231
f_uncultured_o_RsaHf231
g_Peptostreptococcus
f_FamilyXI
g_Clostridiumsensustricto1
f_Clostridiaceae
o_Clostridiales
o_Micrococcales
g_Rickettsiella
f_Diplorickettsiaceae
o_Diplorickettsiales
g_Curtobacterium
o_Peptostreptococcales_Tissierellales
f_Microbacteriaceae
g_Pantoea
g_Photobacterium
f_Vibrionaceae
f_Erwiniaceae
c_Clostridia
o_Enterobacterales
c_Gammaproteobacteria
−6.0 −4.8 −3.6 −2.4 −1.2 0.0 1.2 2.4 3.6 4.8 6.0
LDA SCORE (log 10)

Figure S8

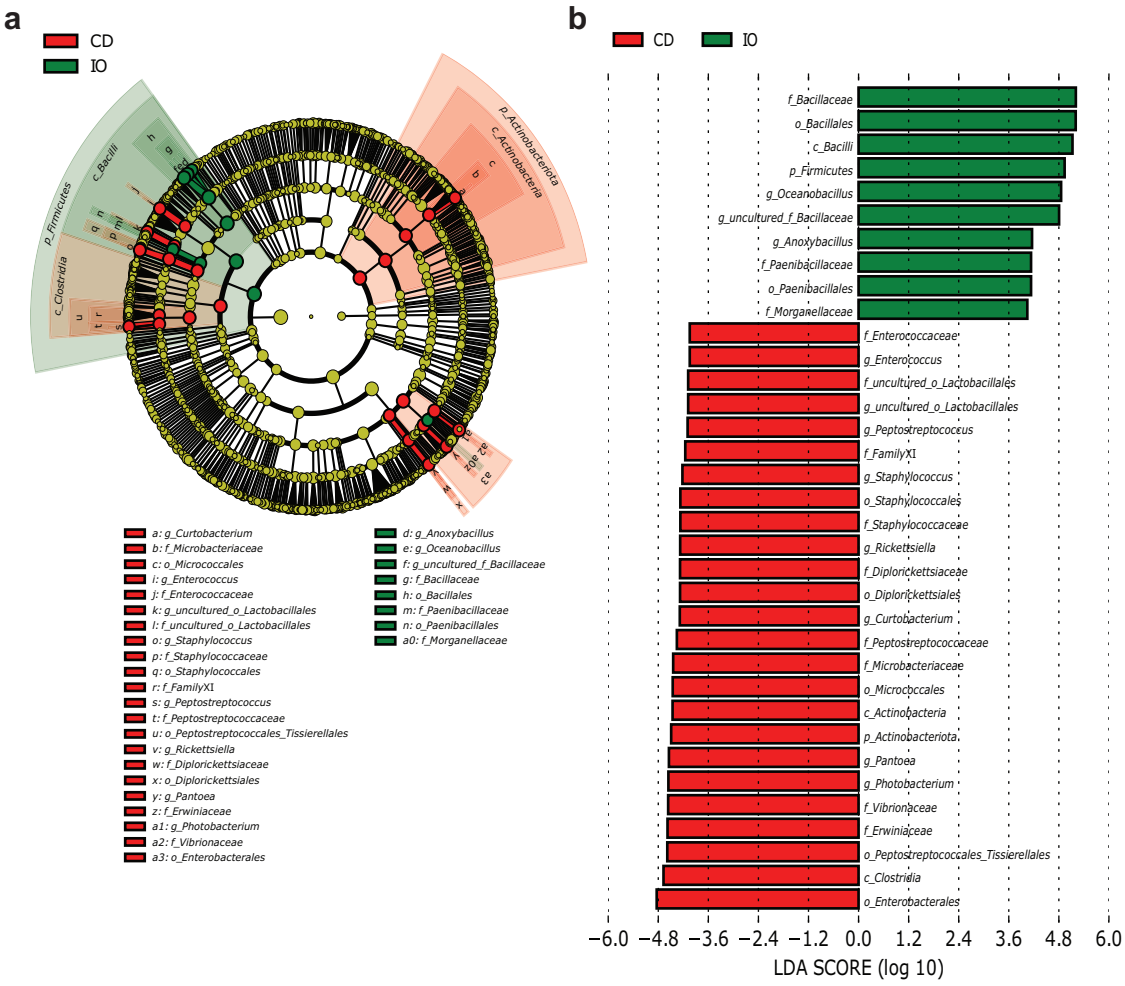

a
CD
IO
p_Firmicutes
c_Bacilli
c_Clostridia
p_Actinobacteriota
c_Actinobacteria
a: g_Curtobacterium
b: f_Microbacteriaceae
c: o_Micrococcales
i: g_Enterococcus
j: f_Enterococcaceae
k: g_uncultured_o_Lactobacillales
l: f_uncultured_o_Lactobacillales
o: g_Staphylococcus
p: f_Staphylococcaceae
q: o_Staphylococcales
r: f_FamilyX1
s: g_Peptostreptococcus
t: f_Peptostreptococcaceae
u: o_Peptostreptococcales_Tissierellales
v: g_Rickettsiella
w: f_Diplorickettsiaceae
x: o_Diplorickettsiales
y: g_Pantoea
z: f_Erwiniaceae
a1: g_Photobacterium
a2: f_Vibrionaceae
a3: o_Enterobacterales
d: g_Anoxybacillus
e: g_Oceanobacillus
f: g_uncultured_f_Bacillaceae
g: f_Bacillaceae
h: o_Bacillales
m: f_Paenibacillaceae
n: o_Paenibacillales
a0: f_Morganellaceae
b
CD
IO
f_Bacillaceae
o_Bacillales
c_Bacilli
p_Firmicutes
g_Oceanobacillus
g_uncultured_f_Bacillaceae
g_Anoxybacillus
f_Paenibacillaceae
o_Paenibacillales
f_Morganellaceae
f_Enterococcaceae
g_Enterococcus
f_uncultured_o_Lactobacillales
g_uncultured_o_Lactobacillales
g_Peptostreptococcus
f_FamilyX1
g_Staphylococcus
o_Staphylococcales
f_Staphylococcaceae
g_Rickettsiella
f_Diplorickettsiaceae
o_Diplorickettsiales
g_Curtobacterium
f_Peptostreptococcaceae
f_Microbacteriaceae
o_Micrococcales
c_Actinobacteria
p_Actinobacteriota
g_Pantoea
g_Photobacterium
f_Vibrionaceae
f_Erwiniaceae
o_Peptostreptococcales_Tissierellales
c_Clostridia
o_Enterobacterales
−6.0 −4.8 −3.6 −2.4 −1.2 0.0 1.2 2.4 3.6 4.8 6.0
LDA SCORE (log 10)

Figure S9

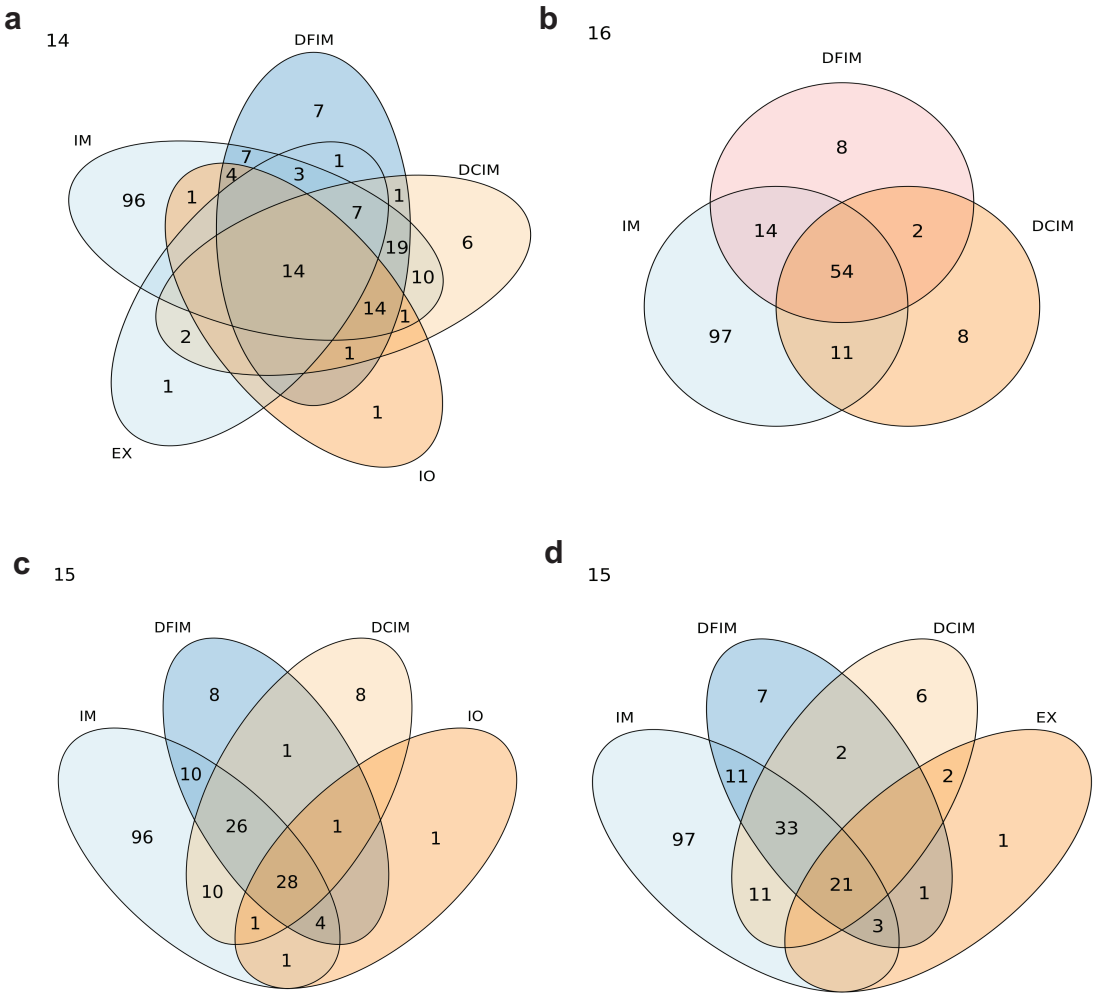

a
14
DFIM
IM
DCIM
EX
IO
7
7
4
3
1
96
1
1
7
19
6
14
10
14
1
2
1
1
1
1
b
16
DFIM
IM
DCIM
8
14
2
54
97
11
8
c
15
DFIM
DCIM
IM
IO
8
8
1
10
96
26
1
1
28
10
1
4
1
d
15
DFIM
DCIM
IM
EX
7
6
2
11
2
97
33
1
21
11
1
3

Figure S10

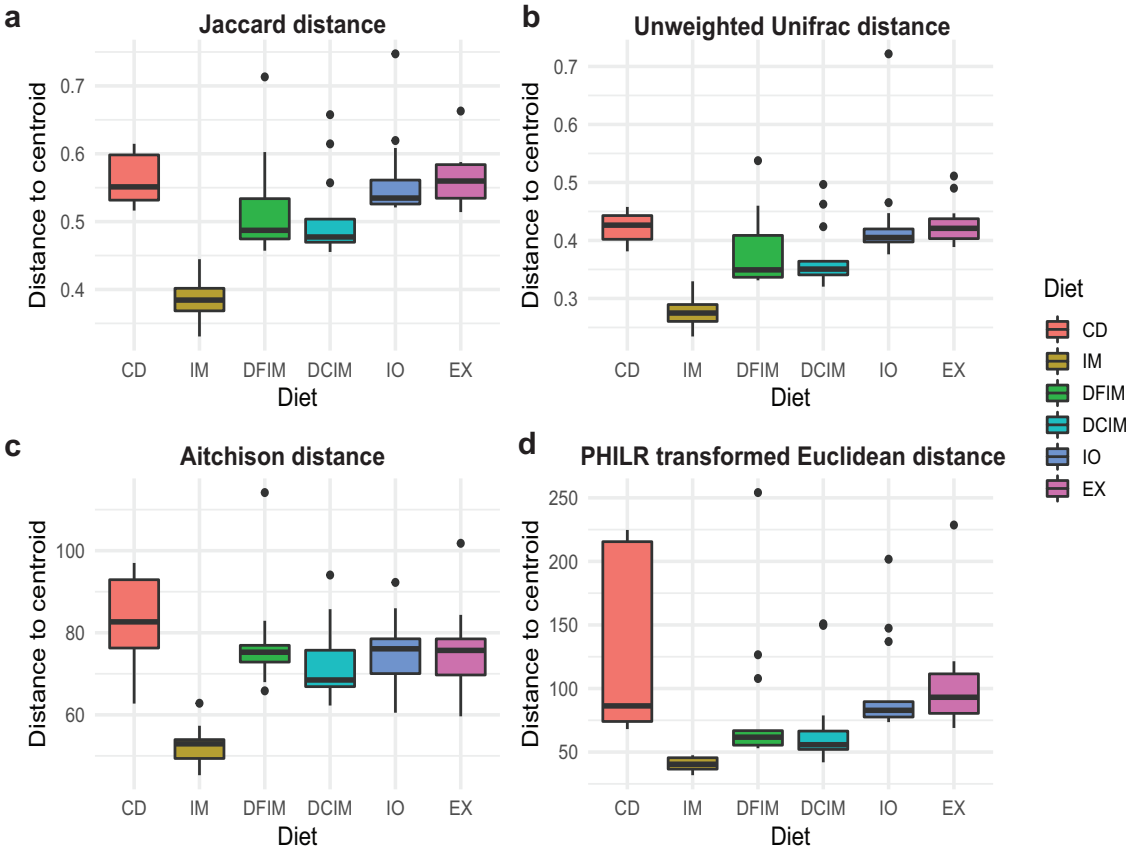

a
Jaccard distance
Distance to centroid
0.7
0.6
0.5
0.4
CD
IM
DFIM
DCIM
IO
EX
Diet
b
Unweighted Unifrac distance
Distance to centroid
0.7
0.6
0.5
0.4
0.3
CD
IM
DFIM
DCIM
IO
EX
Diet
c
Aitchison distance
Distance to centroid
100
80
60
CD
IM
DFIM
DCIM
IO
EX
Diet
d
PHILR transformed Euclidean distance
Distance to centroid
250
200
150
100
50
CD
IM
DFIM
DCIM
IO
EX
Diet
Diet
CD
IM
DFIM
DCIM
IO
EX

Figure S11

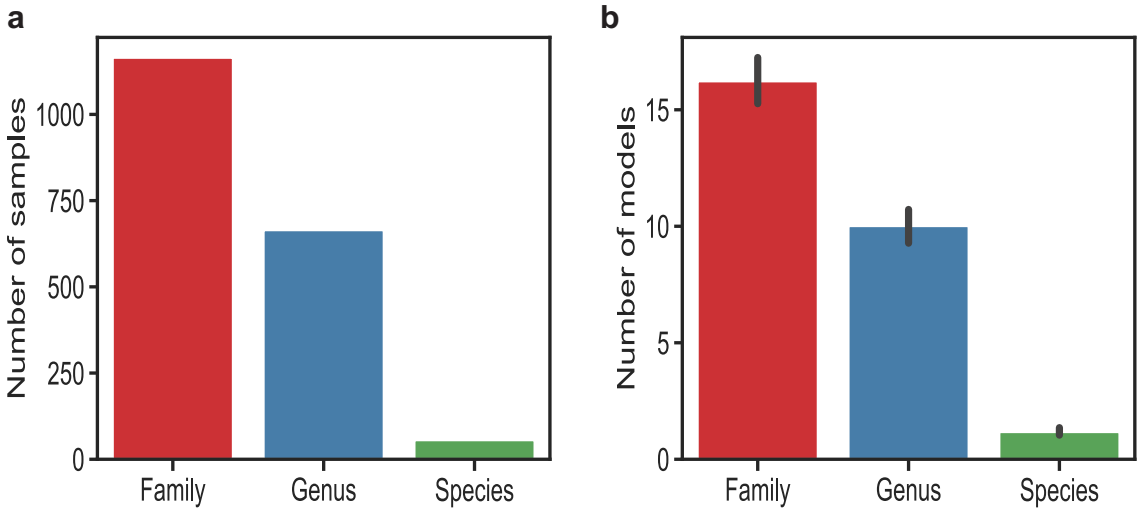
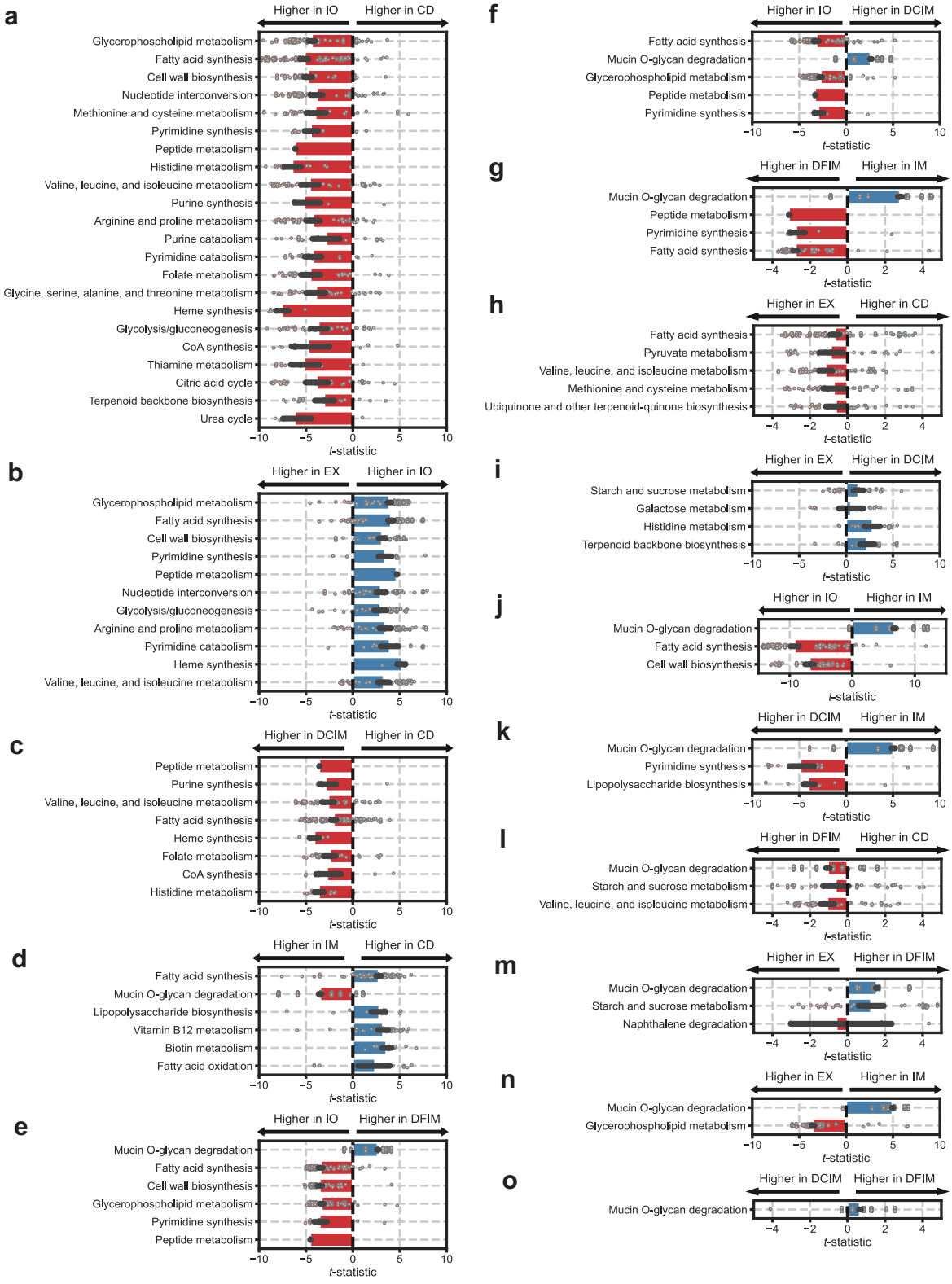

a
Number of samples
1000
750
500
250
0
Family
Genus
Species
b
Number of models
15
10
5
0
Family
Genus
Species

Figure S12


a
Higher in IO
Higher in CD
Glycerophospholipid metabolism
Fatty acid synthesis
Cell wall biosynthesis
Nucleotide interconversion
Methionine and cysteine metabolism
Pyrimidine synthesis
Peptide metabolism
Histidine metabolism
Valine, leucine, and isoleucine metabolism
Purine synthesis
Arginine and proline metabolism
Purine catabolism
Pyrimidine catabolism
Folate metabolism
Glycine, serine, alanine, and threonine metabolism
Heme synthesis
Glycolysis/gluconeogenesis
CoA synthesis
Thiamine metabolism
Citric acid cycle
Terpenoid backbone biosynthesis
Urea cycle
−10 −5 0 5 10
t-statistic
b
Higher in EX
Higher in IO
Glycerophospholipid metabolism
Fatty acid synthesis
Cell wall biosynthesis
Pyrimidine synthesis
Peptide metabolism
Nucleotide interconversion
Glycolysis/gluconeogenesis
Arginine and proline metabolism
Pyrimidine catabolism
Heme synthesis
Valine, leucine, and isoleucine metabolism
−10 −5 0 5 10
t-statistic
c
Higher in DCIM
Higher in CD
Peptide metabolism
Purine synthesis
Valine, leucine, and isoleucine metabolism
Fatty acid synthesis
Heme synthesis
Folate metabolism
CoA synthesis
Histidine metabolism
−10 −5 0 5 10
t-statistic
d
Higher in IM
Higher in CD
Fatty acid synthesis
Mucin O-glycan degradation
Lipopolysaccharide biosynthesis
Vitamin B12 metabolism
Biotin metabolism
Fatty acid oxidation
−10 −5 0 5 10
t-statistic
e
Higher in IO
Higher in DFIM
Mucin O-glycan degradation
Fatty acid synthesis
Cell wall biosynthesis
Glycerophospholipid metabolism
Pyrimidine synthesis
Peptide metabolism
−10 −5 0 5 10
t-statistic
f
Higher in IO
Higher in DCIM
Fatty acid synthesis
Mucin O-glycan degradation
Glycerophospholipid metabolism
Peptide metabolism
Pyrimidine synthesis
−10 −5 0 5 10
t-statistic
g
Higher in DFIM
Higher in IM
Mucin O-glycan degradation
Peptide metabolism
Pyrimidine synthesis
Fatty acid synthesis
−4 −2 0 2 4
t-statistic
h
Higher in EX
Higher in CD
Fatty acid synthesis
Pyruvate metabolism
Valine, leucine, and isoleucine metabolism
Methionine and cysteine metabolism
Ubiquinone and other terpenoid-quinone biosynthesis
−4 −2 0 2 4
t-statistic
i
Higher in EX
Higher in DCIM
Starch and sucrose metabolism
Galactose metabolism
Histidine metabolism
Terpenoid backbone biosynthesis
−10 −5 0 5 10
t-statistic
j
Higher in IO
Higher in IM
Mucin O-glycan degradation
Fatty acid synthesis
Cell wall biosynthesis
−10 0 10
t-statistic
k
Higher in DCIM
Higher in IM
Mucin O-glycan degradation
Pyrimidine synthesis
Lipopolysaccharide biosynthesis
−10 −5 0 5 10
t-statistic
l
Higher in DFIM
Higher in CD
Mucin O-glycan degradation
Starch and sucrose metabolism
Valine, leucine, and isoleucine metabolism
−4 −2 0 2 4
t-statistic
m
Higher in EX
Higher in DFIM
Mucin O-glycan degradation
Starch and sucrose metabolism
Naphthalene degradation
−4 −2 0 2 4
t-statistic
n
Higher in EX
Higher in IM
Mucin O-glycan degradation
Glycerophospholipid metabolism
−10 −5 0 5 10
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o
Higher in DCIM
Higher in DFIM
Mucin O-glycan degradation
−4 −2 0 2 4
t-statistic

Supplementary tables

Table S1. The prevalence of core ASVs in different sample types. (Since this table is big, it was not included in this thesis. This is available upon request)

Table S2. Pair-wise comparison of alpha diversity indices of gut microbiota in fish fed experimental diets containing meals and fractions of black soldier fly (BSF) larvae – adjusted *p* values

Pair-wise comparisons	**Observed ASVs**	**Pielou's evenness**	**Shannon's index**	**Faith's PD**
IM vs DFIM	1	0.007	0.016	1
IM vs DCIM	0.15	0.07	<0.001	0.35
IM vs IO	0.016	1	0.051	0.007
IM vs EX	0.012	0.45	0.051	0.007
DFIM vs DCIM	1	0.57	1	1
DFIM vs IO	0.15	0.32	1	0.07
DFIM vs EX	0.016	1	0.41	0.02
DCIM vs IO	0.87	0.57	1	0.51
DCIM vs EX	0.2	1	0.35	0.19
IO vs EX	1	0.32	0.35	1

IM: Full-fat BSF larvae meal diet; DFIM: Defatted BSF larvae meal diet; DCIM: De-chitinized BSF larvae meal diet; IO: BSF larvae oil diet; EX: BSF larvae exoskeleton diet.

Table S3. PERMANOVA analysis for beta-diversity of gut microbiota in fish fed experimental diets containing meals and fractions of black soldier fly (BSF) larvae – adjusted *p* value

	Jaccard distance	**Unweighted UniFrac distance**	**Aitchison distance**	**Phylogenetic isometric log-ratio (PHILR) transformed Euclidean distance**
F value	2.94	3.02	3.10	7.31
p value	0.001	0.001	0.001	0.001
Pair-wise comparisons				
CD vs IM	0.015	0.015	0.015	0.015
CD vs DFIM	0.03	0.06	0.06	0.03
CD vs DCIM	0.015	0.015	0.015	0.015
CD vs IO	0.015	0.015	0.015	0.03
CD vs EX	0.03	0.03	0.06	0.12
IM vs DFIM	0.015	0.015	0.015	0.015
IM vs DCIM	0.015	0.015	0.015	0.015
IM vs IO	0.015	0.015	0.015	0.015
IM vs EX	0.015	0.015	0.015	0.015
DFIM vs DCIM	0.03	0.09	0.045	0.38
DFIM vs IO	0.03	0.015	0.03	0.51
DFIM vs EX	0.045	0.015	0.03	0.045
DCIM vs IO	0.015	0.015	0.06	0.09
DCIM vs EX	0.015	0.03	0.015	0.015
IO vs EX	0.045	0.045	0.015	0.015

[1] CD: Control diet; IM: Full-fat BSF larvae meal diet; DFIM: Defatted BSF larvae meal diet; DCIM: De-chitinized BSF larvae meal diet; IO: BSF larvae oil diet; EX: BSF larvae exoskeleton diet.

Table S4. Test of homogeneity of multivariate dispersions among dietary groups

	Jaccard distance	**Unweighted UniFrac distance**	**Aitchison distance**	**Phylogenetic isometric log-ratio (PHILR) transformed Euclidean distance**
F value	21.6	15.5	15.3	5.52
p value	0.001	0.001	0.001	0.002
Pair-wise comparisons				
CD vs IM	0.001	0.001	0.001	0.001
CD vs DFIM	0.07	0.043	0.30	0.10
CD vs DCIM	0.013	0.01	0.019	0.013
CD vs IO	0.91	0.65	0.11	0.24
CD vs EX	0.98	0.49	0.14	0.40
IM vs DFIM	0.001	0.001	0.001	0.010
IM vs DCIM	0.001	0.001	0.001	0.004
IM vs IO	0.001	0.001	0.001	0.001
IM vs EX	0.001	0.001	0.001	0.001
DFIM vs DCIM	0.67	0.70	0.21	0.53
DFIM vs IO	0.10	0.08	0.62	0.40
DFIM vs EX	0.08	0.019	0.71	0.27
DCIM vs IO	0.041	0.036	0.32	0.06
DCIM vs EX	0.020	0.005	0.35	0.037
IO vs EX	0.91	0.90	0.95	0.78

CD: Control diet; IM: Full-fat BSF larvae meal diet; DFIM: Defatted BSF larvae meal diet; DCIM: De-chitinized BSF larvae meal diet; IO: BSF larvae oil diet; EX: BSF larvae exoskeleton diet.

Paper V

A systematic meta-analysis based review on black soldier fly (*Hermetia illucens*) as a novel protein source for salmonids

Short running title: Black soldier fly in salmonid diets

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict of interest disclosure

The authors declare no competing conflicts of interest.

Abstract

Black soldier fly (*Hermetia illucens*) have gained attention as a sustainable novel protein source in fish feed due to its high nutritional value and low environmental impacts. In the past decade, effects of the use of black soldier fly in aquafeeds have widely been studied in salmonids. A meta-analysis was conducted to compile and systematically quantify the effect of black soldier fly in diets for salmonids on growth performance and nutrient utilization. The meta-analysis showed that dietary inclusion of black soldier fly did not compromise the specific growth rate, feed conversion ratio, feed intake, protein digestibility and protein efficiency ratio in salmonids. A meta-regression was conducted to explore possible causes of variation in growth rate, feed conversion ratio and feed intake between the studies. Fish species, protein source(s) replaced, and black soldier fly inclusion level were partially responsible for the variation in growth rate between the studies. On the other hand, the protein source(s) replaced and black soldier fly inclusion level partially explained the variation in feed conversion ratio and feed intake, respectively. Replacement of fishmeal by black soldier fly decreased growth rate and feed intake in salmonids, but the replacement of non-fishmeal sources improved growth rate and feed conversion. This strengthened the importance of the type of replaced protein source(s) when evaluating nutritional values of black soldier fly for salmonids. In conclusion, the present meta-analysis showed that black soldier fly is a promising protein source for salmonid feeds.

Key words: Fishmeal replacement, insect meal, meta-analysis, meta-regression, nutrient utilization, salmonids

Introduction

Insect meals have received an increasing attention in recent years as a sustainable protein source for aquafeeds,[1] because insects are able to utilize organic side streams, and the production does not require any agricultural land, has low water usage and contributes to lower greenhouse gas emissions. [2] The approval of use of processed insects in aquafeeds by the European Commission (Regulation 2017/893/EC, 2017) further promotes upscaling of insects as a novel protein source. One of the most favourable insect species to be used in feed is black soldier fly (*Hermetia illucens*) (BSF).[3] BSF is a good source of protein, lipid, and minerals.[4] Further, BSF are capable of converting low-quality organic material efficiently into high-quality nutrients,[5] although the possibility of using low quality organic material as a substrate for growing insects is still limited by the regulatory framework in Europe. BSF is a good candidate for large-scale production due to its high growth rate and feed conversion efficiency, potential to be reared on organic side streams and suitability for automation.[3] In addition to nutritive value, BSF also contain bioactive compounds such as chitin, lauric acid and antimicrobial peptides, which can have health beneficial effects in animals.[6]

With the identification of great potential of BSF as a sustainable novel protein source in fish feed, the effects of the use of BSF in diets for salmonids such as Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) have widely been studied, focusing on growth performance, nutrient utilization, gut microbiota,[7,8] gut health and immune responses.[9,10] In literature, the studies on growth performance and nutrient utilization reported high variation in success of fish responses to BSF in diets. The use of BSF in diets was shown to have no effects,[11-14] negative effects[11,12,14] or even positive effects[15] on growth performance and nutrient utilization in salmonids. Furthermore, previous studies showed dose-dependent responses in fish to increasing dietary BSF levels or protein replacement levels.[11,14] In a recent review, English et al.[16] also discussed the inconsistency of results obtained in different studies investigating the effects of the use of BSF in diets on salmonid growth performance and nutrient utilization. The diverse nature of BSF rearing, downstream processing, and study designs, makes it difficult to directly compare the reported results to draw

a general conclusion and to determine the dose dependent responses across the studies.

Meta-analysis is a method to compile and statistically analyse results from a number of individual studies addressing similar research questions and produce integrated and broader interpretations. Recently, this approach was used to examine the effect of replacing fishmeal with insect meals on specific growth rate (SGR) of fish[17] and to determine the nutritional value of insects in aquafeeds.[18] These meta-analyses included data for various insect species as well as various aquatic species. The results of these two studies further emphasized that the analysis of individual insect and aquatic species can be more meaningful than the generalized results across different insect and aquatic species. The previous reviews on the topic of the effects of the use of BSF in salmonid diets concentrated on summarizing scientific literature in a narrative and qualitative approach.[16,19] The effect of BSF in salmonid diets has not yet been evaluated using a quantitative meta-analysis based approach according to our knowledge. In addition, the reasons of the inconsistency in success associated with the use of BSF in diets is important to identify, to optimize the use of BSF in salmonid diets and experimental designs. According to our knowledge, none of the previous meta-analyses took into consideration factors such as fish species, feed processing techniques, type of protein source(s) replaced by insects and developmental stage of insects, that can influence the fish response to dietary insects. Therefore, in the present study, a meta-analysis was conducted to 1) systematically review and summarize data from previous studies to determine the effect of dietary BSF on SGR, feed conversion ratio (FCR), feed intake, apparent digestibility coefficient (ADC) of protein and protein efficiency ratio (PER) in salmonids and 2) identify the factors causing the variation in response of salmonids to the use of BSF in diet.

Materials and methods

Literature search and dataset

The present meta-analysis was conducted adhering to the principles in the Cochrane Handbook for Systematic Reviews of Interventions[20] and the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement.[21] A systematic

literature search was conducted in ISI WEB OF KNOWLEDGE (1945-2021) and SCOPUS (1939-2021) on 11-15 March 2021, using the following search terms in combination with Boolean operators: insect; insects; black soldier fly; *Hermetia illucens*; salmon; Atlantic salmon; *Salmo salar*; Coho salmon; *Oncorhynchus kisutch*; Chinook salmon; *Oncorhynchus tshawytscha*; trout; rainbow trout and *Oncorhynchus mykiss*. The terms were used to search Topic in ISI WEB OF KNOWLEDGE and Title-Abstract-Keywords in SCOPUS. The literature search strategy was supplemented with manual searches.

The selection process of studies to be included in the meta-analysis dataset is shown in Fig. 1. To prevent selection bias, following pre-specified inclusion criteria were used: 1) Presence of a control group that did not include BSF; 2) Protein source(s) in the control diet replaced by BSF; 3) Studies investigated the effects of BSF on the growth performance (SGR, FCR and/or feed intake) or nutrient utilization (ADC of protein and PER) in salmonids. A study was considered as a growth study if the fish were fed for minimum seven weeks or the fish at least doubled in weight during the feeding period; 4) Reported standard deviation or standard error mean and 5) Written in English. In addition, the dataset included studies with nutrient balanced (major nutrients and/or amino acids) experimental diets to avoid the nutrient imbalances interference with the results. Duplicate reports, reviews and conference proceedings were not included. If a study contained more than one control diet, relevant BSF diets were compared separately with each individual control. When a study contained more than two treatments providing more than one comparison to the meta-analysis, the comparisons were individually coded.

Relevant data were extracted from each study using a standardized proforma. Data extracted included: growth performance and/or nutrient utilization parameters including SGR, FCR, feed intake, ADC of protein and PER, (calculated based on Equations (1), (2), (3) and (4) respectively), number of experimental units per treatment, salmonid species, life stage of salmon, final body weight of fish, feed production method, type of protein source(s) replaced by BSF, developmental stage of BSF, processing method of BSF, dietary inclusion level of BSF, dietary chitin level and fishmeal replacement level. The fishmeal replacement level of BSF diets was

calculated as [(Fish meal in the control diet (%)-Fishmeal in the BSF diet (%))/Fishmeal in the control diet (%)] × 100. In addition, the standard deviation or standardized error mean of SGR, FCR, feed intake, ADC of protein or PER from control and BSF diets-fed groups were extracted. Feed intake values were reported in numerous ways in the studies. Therefore, the feed intake values per fish per day was calculated using available information in reported studies, and the standard deviation of calculated feed intake values were determined using the prognostic method described by Ma et al.[22]

$$SGR = \frac{\ln FBW - \ln IBW}{t} \text{ x } 100 \quad (1)$$

where FBW = final body weight, IBW = initial body weight, t = number of days.

$$FCR = \frac{FI}{BWG} \quad (2)$$

where FI = feed intake, BWG = body weight gain.

$$ADC\ of\ protein = \left(1 - \frac{M_D}{M_F} \times \frac{P_F}{P_D}\right) \times 100 \quad (3)$$

where M_D = marker concentration in diet, M_F = marker concentration in faeces, P_F = protein concentration in faeces, P_D = protein concentration in diet.

$$PER = \frac{BWG}{PI} \quad (4)$$

where BWG = body weight gain, PI = protein intake.

Statistical analysis

The differences in growth performance and nutrient utilization parameters between control diets and BSF diets-fed fish within studies were calculated using a standardized effect size; Hedges' g (calculated based on Equations (5), (6) and (7)). The Hedges' g corrects for bias with small sample sizes and produces a statistical standardization of the findings for each study.[23]

The Hedges' g was calculated as:

$$\frac{\bar{X}_B - \bar{X}_C}{S_p} J \quad (5)$$

where $\bar{X}$ is the mean for the BSF (B) and control (C) groups, S_p is the pooled sample standard deviation, and J is a correction factor for bias with small sample sizes.

The pooled standard deviation (S_p) was calculated as:

$$S_P = \sqrt{\frac{(n_B - 1)SD_B^2 + (n_C - 1)SD_C^2}{n_B + n_C - 2}} \quad (6)$$

The correction factor (J) was calculated as:

$$J = 1 - \frac{3}{4(n_B + n_C - 2) - 1} \quad (7)$$

where n is the sample size and SD is the standard deviation of the BSF (B) and control (C) groups.

The meta-analysis was performed in comprehensive meta-analysis version 3 software (Biostat Inc., Englewood, New Jersey, USA) using random effects models to account for the variation among the populations of studies. The presence of true heterogeneity between the studies was identified with Cochran's Q -test[24] and the proportion of observed heterogeneity caused by true effects was quantified using I^2 statistics.[25] When significant heterogeneity was detected, meta-regression analysis was conducted to explore possible causes of heterogeneity. The categorical variables including fish species (Atlantic salmon vs. rainbow trout), feed production method (extrusion vs. pelleting), type of protein source(s) replaced (fishmeal vs. fishmeal + plant protein sources vs. non-fishmeal), BSF development stage (larvae vs. prepupae/pupae) and BSF processing method (full-fat vs. defatted) and two continuous variables including dietary inclusion level of BSF and fish body size were included in the meta-regression analysis. Further differentiations of other variables were not possible due to the limited number or lack of data for carrying out a meaningful analysis. Meta-regression was not conducted with ADC of protein and PER data due to lack of data points in at least one group of each categorical variable.

In addition, meta-analysis was conducted in sub-datasets sorted from the full dataset according to the fish species, life stage of salmon, type of protein source(s) replaced, BSF development stage, BSF processing method and feed production method. Estimated effect sizes were visually displayed in forest plots created with Comprehensive meta-analysis version 3 software (Biostat, Englewood, New Jersey, USA).

Linear and quadratic regression analyses between dietary inclusion level of BSF, dietary chitin level or fishmeal replacement level and effect sizes of SGR, FCR, feed intake, ADC of protein or PER were performed using IBM SPSS Statistics 27 software (IBM Corp., Armonk, NY, USA). The graphs were created using GraphPad Prism 9.0.0 software (GraphPad Software, San Diego, CA, USA).

The chosen level of significance was $p < 0.05$ and threshold level of tendency was $p < 0.1$. The possible publication bias was not conducted in the present study due to the occurrence of substantial heterogeneity with all outcomes, which may lead to false-positive claims for publication bias.[26]

Results

The nutritional compositions of BSF larvae, prepupae and pupae are shown in Table 1. The BSF on average contain 36-39% protein and 28-34% lipid. The amino acid profiles of BSF differ from mealworm (*Tenebrio Molitor*) and house fly (*Musca domestica*) (Table S1). The BSF contain lower content of most of the essential amino acids than fishmeal, especially lysine (5.6-6.4 g/ 100 g protein) and methionine (1.7 g/ 100 g protein) (7.8 and 3 g/ 100 g protein respectively in fishmeal) (Table S2). The methionine content in BSF is superior than in soy protein (1.4 g/ 100 g protein) (Table S2). The essential amino acid profile shows that BSF in general meet the amino acids requirements of Atlantic salmon and rainbow trout[27] (Table S2), except for lysine and methionine. However, the values in NRC[27] are more than a decade old and advances in genetic and breeding programs over the years has changed the nutritional requirements of salmonids.

The meta-analysis dataset consisted of 16 publications in peer-reviewed journals and one unpublished in-house study (Table S3). Amongst these studies, 13 studies reported the nutritional composition of used BSF ingredients (Table S4). The studies were reported between 2007 and 2021. Either BSF larvae, prepupae or pupae were used in these studies in full-fat or defatted form. The BSF were included in the experimental diets by replacing traditional protein sources such as fishmeal, plant protein sources and/or animal protein sources, and the BSF inclusion levels in diets ranged from 5 to 60%. The sample sizes of studies ranged from two to four experimental units/treatments.

Amongst the selected studies, 13 studies were used to conduct the meta-analysis for SGR (36 comparisons) and all the 17 studies were used for FCR (49 comparisons) and feed intake (50 comparisons). The most studied salmonid species was rainbow trout (i.e., 11 studies) accounting for 50%, 53% and 54% of the SGR, FCR, and feed intake data, respectively, whereas Atlantic salmon accounted for 50%, 47% and 46% of the SGR, FCR and feed intake data, respectively. Amongst Atlantic salmon, four studies used pre-smolts and two studies used post-smolts. Seven studies (19 comparisons) were used to conduct the meta-analysis for ADC of protein. Atlantic salmon accounted for 68% of the ADC of protein data, whereas rainbow trout accounted for 32% of the data. Although two additional studies reported ADC of protein, one study was excluded from the analysis being an outlier as it gave extreme effect sizes,[28] and the other one was excluded because ADC of protein was reported as a graphical presentation.[13] Eight studies (21 comparisons) were used to conduct the meta-analysis for PER. Atlantic salmon accounted for 71% of the PER data, whereas rainbow trout accounted for 29% of the data.

Specific growth rate

The forest plot in Fig. 2 shows the pooled effect of the use of BSF in diets on SGR in salmonids. In the full dataset of SGR, the Hedges' g between BSF diets and control diets ranged from -5.71 to 8, with 78% of the comparisons showing an increase or no change in SGR in fish fed BSF diets compared to control diets. The meta-analysis of SGR showed a mean effect size of -0.014 (Confidence interval: -0.615 to 0.586). On

average, SGR in salmonids fed BSF diets did not differ from those fed the control diets. The test of heterogeneity, Q-value was 168.0 with a corresponding *p* value of <0.001, showing a significant heterogeneity in true effect sizes of SGR between the studies. Further, the I^2 statistic showed that 79.2% of the observed heterogeneity was caused by the true effects rather than the sampling error. The variance of true effects (T^2) was 2.5 and the standard deviation of true effects (T) was 1.6.

According to the meta-regression, fish species, protein source(s) replaced, and dietary BSF level partially caused the heterogeneity in SGR between the studies in the full dataset. However, the other variables in the model including feed production method, BSF development stage, BSF processing method and fish body size did not explain heterogeneity at any significant level. The variables included in the meta-regression model could explain only 19% of the heterogeneity in SGR between studies (Table 2).

The meta-analyses of sub-datasets including salmon, salmon pre-smolts, rainbow trout, full-fat BSF and defatted BSF showed that, on average, the SGR of fish fed BSF diets did not differ from that of fish fed control diets. Three groups were identified according to the source(s) of protein replaced: 1) fishmeal, 2) fishmeal and plant protein sources and 3) non-fishmeal protein sources. The meta-analyses within these three groups showed that replacing fishmeal with BSF decreased SGR of salmonids, whereas replacement of both fishmeal and plant protein sources did not change SGR. The replacement of non-fishmeal protein sources with BSF even increased the SGR. The analyses further showed presence of unexplained heterogeneity between the studies in all the sub-datasets. Nevertheless, there were no sufficient data to conduct further subgroup analyses or meta regression in these datasets (Table 3).

Linear and quadratic regressions in the full dataset and sub-datasets revealed no linear or quadratic relationships between the dietary inclusion level of BSF (Table S5) or dietary chitin level (Tables S6) and effect sizes of SGR. In the BSF larvae dataset, however, SGR in salmonids tended (p = 0.088) to decrease linearly with increasing level of BSF in the diet. On the other hand, the fishmeal replacement level had negative linear relationships and/or quadratic relationships with effect sizes of SGR

in the full dataset, as well as in sub-datasets including salmon, rainbow trout, BSF larvae, BSF prepupae/pupae and defatted BSF (Fig. 3 and Table S7).

Feed conversion ratio

The forest plot in Fig. 4 shows the pooled effect of BSF inclusion in diets on FCR in salmonids. In the full dataset of FCR, the Hedges' g, between BSF diets and control diets ranged from -8 to 4.8, with 80% of the comparisons showing a decrease or no change in FCR in fish fed BSF diets compared to control diets. The meta-analysis of FCR showed a mean effect size of 0.094 (Confidence interval: -0.341 to 0.529). On average, FCR in salmonids fed BSF diets did not differ from those fed the control diets. The test of heterogeneity, Q-value was 185.2 with a corresponding *p* value of <0.001, showing a significant heterogeneity in true effect sizes of FCR between the studies. Further, the I^2 statistic showed that 74.1% of the observed heterogeneity was caused by the true effects rather than the sampling error. The variance of true effects (T^2) was 1.7 and the standard deviation of true effects (T) was 1.3.

According to the meta-regression, the protein source(s) replaced partially caused the heterogeneity in FCR between the studies in the full dataset. However, the other variables in the model including fish species, feed production method, BSF development stage, BSF processing method, BSF inclusion level and fish body size did not explain heterogeneity at any significant level. The variables included in the meta-regression model could explain only 2% of the heterogeneity in FCR between studies (Table 2).

The salmon dataset showed that, on average, dietary inclusion of BSF tended (p = 0.095) to increase FCR in salmon compared to control diets. The mean effect size for salmon pre-smolts showed an increased FCR in fish fed BSF diets compared to fish fed control diets. The meta-analysis of the FCR in rainbow trout group showed no statistically significant effect of BSF in the diet. The meta-analysis of the three groups categorized according to the type of protein source(s) replaced showed that replacing fishmeal with BSF did not change FCR of salmonids, whereas replacement of both fishmeal and plant protein sources increased FCR and replacement of non-fishmeal protein sources decreased FCR. The two sub-datasets sorted according to the

processing method of BSF showed that feeding either full-fat or defatted BSF had no impact on FCR in salmonids. Although all the sub-datasets revealed presence of unexplained heterogeneity between the studies, potential factors responsible for this could not be identified due to insufficient availability of data (Table 3).

Linear and quadratic regressions in the full dataset and sub-datasets revealed that there were no linear or quadratic relationships between the inclusion level of BSF (Table S5) or chitin level in the diet (Table S6) and effect sizes of FCR. In the salmon dataset, however, FCR tended (p = 0.05) to increase linearly with increasing level of BSF in the diet. Fishmeal replacement level on other hand had both positive linear relationships and quadratic relationships with the effect size of FCR in the full dataset, and the sub datasets including rainbow trout, BSF prepupae/pupae and defatted BSF (Fig. 5 and Table S7).

Feed intake

The forest plot in Fig. 6 shows the pooled effect of BSF inclusion in diets on feed intake in salmonids. In the full dataset of feed intake, the Hedges' g, between BSF diets and control diets ranged from -27 to 4.5, with 78% of the comparisons showing an increase or no change in feed intake in fish fed BSF diets compared to control diets. The meta-analysis of feed intake showed a mean effect size of -0.099 (Confidence interval: -0.476 to 0.277). On average, the feed intake in salmonids fed BSF diets did not differ from those fed the control diets. The test of heterogeneity, Q-value was 146.4 with a corresponding p value of <0.001, showing a significant heterogeneity in true effect sizes of feed intake between the studies. Further, the I^2 statistic showed that 66.5% of the observed heterogeneity was caused by the true effects rather than the sampling error. The variance of true effects (T^2) was 1.1 and the standard deviation of true effects (T) was 1.1.

According to the meta-regression, BSF inclusion level partially caused the heterogeneity in feed intake between the studies in the full data set. However, the other variables in the model did not explain heterogeneity at any significant level (Table 2). The sub-datasets showed that, on average, feed intake did not differ between fish fed BSF diets and control diets, except replacing fishmeal with BSF

decreased feed intake and replacing non-fishmeal protein sources tended (p = 0.095) to increase feed intake of salmonids (Table 3).

Linear and quadratic regressions in the full dataset and sub-datasets revealed that there were no linear or quadratic relationships between the inclusion level of BSF (Table S5) or chitin level in the diet (Table S6) and effect sizes of feed intake. Fishmeal replacement level on other hand had negative linear relationships and/or quadratic relationships with the effect size of feed intake in the full dataset, and the sub-datasets including salmon, BSF larvae, full-fat BSF and defatted BSF (Fig. 7 and Table S7).

Protein digestibility

The forest plot in Fig. 8 shows the pooled effect of BSF inclusion in diets on ADC of protein in salmonids. In the full dataset, the Hedges' g, between BSF diets and control diets ranged from -3.30 to 1.43, with 74% of the comparisons showing no change in ADC of protein in fish fed BSF diets compared to control diet. The meta-analysis of ADC of protein in the full dataset showed a mean effect size of -0.540 (Confidence interval: -1.096 to 0.017). On average, the dietary inclusion of BSF tended (p = 0.057) to decrease ADC of protein in salmonids compared to control diets. The test of heterogeneity, Q-value was 49.3 with a corresponding p value of <0.001, showing a significant heterogeneity in true effect sizes of ADC of protein between the studies. Further, the I^2 statistic indicates that 63.5% of the observed heterogeneity was caused by the true effects rather than the sampling error. The variance of true effects (T^2) was 0.9 and the standard deviation of true effects (T) was 1.0.

For salmon, BSF larvae and extruded feed datasets, the mean effect sizes of ADC of protein showed that dietary inclusion of BSF decreased ADC of protein compared to control diets. The full-fat BSF dataset showed no difference in ADC of protein between BSF and control groups (Table 3). There were no sufficient data available for other sub-groups to conduct meta-analysis.

Linear and quadratic regressions in the full dataset revealed that there were no significant linear or quadratic relationships between the dietary inclusion level of

BSF (Tables S5), dietary chitin level (Tables S6) or fishmeal replacement level (Table S7) and effect sizes of ADC of protein.

Protein efficiency ratio

The forest plot in Fig. 9 shows the pooled effect of BSF inclusion in diets on PER in salmonids. In the full dataset of PER, the Hedges' g, between BSF diets and control diets ranged from -4.8 to 3.4, with 76% of the comparisons showing an increase or no change in PER in fish fed BSF diets compared to control diet. The meta-analysis of PER in the full dataset showed a mean effect size of -0.064 (Confidence interval: -0.655 to 0.526). On average, the PER in salmonids fed BSF diets did not differ from those fed the control diets. The test of heterogeneity, Q-value was 68.9 with a corresponding *p* value of <0.001, showing a significant heterogeneity in true effect sizes of PER between the studies. Further, the I^2 statistic showed that 71% of the observed heterogeneity was caused by the true effects rather than the sampling error. The variance of true effects (T^2) was 1.3 and the standard deviation of true effects (T) was 1.1.

For salmon and full-fat BSF datasets, the mean effect sizes of PER showed that dietary inclusion of BSF had no impact on PER compared to control diets (Table 3). There were no sufficient data available for other groups to conduct meta-analysis.

Linear and quadratic regressions in the full dataset revealed that there were no significant linear or quadratic relationships between the dietary inclusion level of BSF (Tables S5), dietary chitin level (Tables S6) or fishmeal replacement level (Table S7) and effect sizes of PER.

Discussion

The present meta-analysis provided an overall insight into the direction of effects obtained across studies that used BSF in diets for Atlantic salmon and rainbow trout. The control diets in the present dataset contained fishmeal, plant, and land animal protein sources. More than 75% of data in the meta-analysis dataset showed that majority of the experimental diets containing BSF supported similar or superior growth rate, feed utilization and feed intake in salmonids compared to control diets.

The mean effect sizes for the full dataset in the present meta-analysis further revealed no differences in growth rate and feed conversion between the fish fed BSF diets and control diets. This was accompanied by the mean effect size for feed intake, showing no difference between BSF and control diets-fed fish. Hence, the use of 5-60% BSF in salmonid diets replacing fishmeal, plant and animal protein is possible without compromising growth performance. On the other hand, it is possible that the mean effect sizes in the meta-analysis averaged out the possible factors influencing the effectiveness of dietary BSF in salmonids.[17] The wide range of effect sizes of SGR, FCR and feed intake in the present analysis indicated the variation in the effectiveness of the use of BSF meal in diets for salmonids. The heterogeneity test also confirmed the possible effect of influencing factors and meta-regression was used to identify these factors in the present study.

Previous reviews showed that different fish species responded differently to dietary BSF.[19,29] In accordance, the present study also showed that the type of salmonid species was partially responsible for the heterogeneity of growth rate in salmonids between the studies. This confirmed the importance of conducting meta-analysis for salmon and rainbow trout separately. Separate meta-analyses for each fish species also showed that the use of BSF in diets in the form of either full-fat or defatted had no impact on growth rate, feed utilization and feed intake. However, further analysis revealed that the dietary inclusion of BSF depressed the feed utilization in salmon pre-smolts, indicating low utilization of BSF for salmon reared in freshwater.

As shown in the Table S8, the nutrient composition of BSF vary with the processing methods. In a recent review, English et al.[16] also showed the quality and nutritional composition of BSF can change dramatically based on processing. The processing method of insect meals is a crucial point that can have a direct effect on the growth performance and feed efficiency in fish.[30] In a previous study, growth performance of salmon differed by how the BSF was processed.[31] In addition, Basto et al.[32] reported that defatted BSF meal improved the digestibility of protein and amino acids compared to full-fat meal in European sea bass (*Dicentrarchus labrax*) juveniles. On the contrary, present results showed that processing method of BSF (full-fat vs defatted) did not cause heterogeneity in growth performance of salmonids between

the studies. The growth performances of salmonids fed BSF, on average, were similar to those fed control diets despite the BSF was in full-fat or defatted form. The meta-analysis conducted by Hua[17] showed that full-fat and defatted BSF meals affects SGR of fish similarly when only the nutrient balanced diets were included in the dataset. Although present and previous meta-analyses showed no differences in fish responses to the use of defatted and full-fat BSF in the diet, these results should be interpreted with caution because the diets in the datasets contained BSF with varying degrees of defatting (partially or fully defatted).

Feed production technologies such as extrusion might affect the nutritional values of feeds containing insect meals.[17] The processing method of feed (pelleting vs extrusion) was reported to affect the responses of fish to dietary changes.[33-35] For instance, fish fed extruded diets had higher weight gain at low dietary protein levels[34] and higher nutrient utilization[35] than pelleted feeds. When the low-fishmeal diets were supplemented with enzymes, pelleted feed, but not extruded feed, improved fish growth and nutrient utilization.[33] On the contrary, present results showed that the feed production method did not explain the heterogeneity of SGR, FCR and feed intake in fish fed BSF across the studies. Additionally, the developmental stage of BSF did not contribute to the heterogeneity between the studies, even though the nutrient composition varied with the developmental stage of BSF. This indicates that differences in nutrient composition during BSF stages may not be sufficiently large enough to have an impact on fish growth performance.

The meta-analysis conducted by Hua[17] demonstrated that the use of up to 29% BSF meals in diets had no adverse effect on fish growth rate in comparison with control diets with similar nutrient content but decreased at higher levels. Liland et al. [18] observed a linear reduction in SGR of fish and shellfish species used in aquaculture with increasing BSF level in the diet. In the present study, the growth performance parameters in salmonids did not show any linear or quadratic relationships with BSF inclusion level. Further, there was no clear breaking point detected for both SGR and FCR with the increasing level of BSF in the diet of salmonids (data not shown). Nevertheless, dietary level of BSF could partially explain the heterogeneity of SGR and feed intake in salmonids existing across the studies used. In addition, BSF larvae

dataset showed a tendency to reduce SGR linearly with increasing dietary BSF level. When only salmon was considered in the analysis, increasing dietary level of BSF also tended to increase FCR linearly (R^2 = 0.14 and p = 0.05).

As indicated by the meta-regression for growth performance parameters, the effects of the types of protein source(s) replaced by BSF is worth further investigation. Thus, meta-analysis was conducted in the sub datasets sorted according to the protein source(s) replaced by BSF. The replacement of fishmeal by BSF negatively affected the growth rate and feed intake in salmonids but did not affect the FCR. Although the replacement of fishmeal and plant protein sources with BSF did not affect SGR and feed intake of salmonids, it increased FCR. On the other hand, replacement of non-fishmeal protein sources with BSF even increased the growth rate, as well as reduced FCR in salmonids. Hence, the present results strengthened the importance of the type of protein source(s) replaced by BSF when evaluating the nutritional values of BSF in salmonids. The fishmeal replacement levels in the studies included in the present meta-analysis ranged from 0 to 100%. The linear regression analysis showed that the increasing fishmeal replacement by BSF negatively affected the SGR, FCR and feed intake in salmonids. All the sub datasets, except the full-fat BSF dataset, also showed linear decrease in SGR with increasing level of fishmeal replacement. Similarly, FCR in fish increased linearly with increasing level of fishmeal replacement in rainbow trout, defatted BSF and BSF prepupae/pupae datasets. Such linear reductions were also observed for feed intake in salmon, BSF larvae, full-fat and defatted BSF datasets. As stressed by Hua and Bureau[36], fishmeal replacement level might not be an objective parameter in evaluating nutritive values of alternative ingredients such as insect meals because the composition and nutritional value of fishmeal can vary widely. However, it can still provide a good indication on the dose response of fish for the replacement level of fishmeal in the diet.

All studies used in the present meta-analysis had balanced essential amino acid composition between the control and BSF diets, except one study that did not report any information regarding the amino acid profiles of diets or supplementation of amino acids.[37] Hence, the differences in fish responses according to the replaced protein source(s) were likely a reflection of the true differences between the

nutritional values of the BSF and other protein sources rather than an artefact of discrepancies in the dietary amino acid profiles. It is possible that the depressed growth performance of salmonids fed diets replacing fishmeal might be due to limiting digestible amino acids in BSF compared to fishmeal. This illustrates the importance of determining the digestible protein, amino acids and energy levels in both the control diet and the test ingredient. The studies used in the present meta-analysis did not report any consideration of digestible amino acids in diet formulations. In literature, limited information is available on protein and amino acid digestibility of BSF in salmonids. The protein digestibility coefficient of BSF larvae meal was reported as 89% in Atlantic salmon[12] and 85% in rainbow trout.[14] Fisher et al.[12] further showed that the protein digestibility of BSF was lower than soybean meal (96%) and higher than corn protein concentrate (85%). In addition, Dumas et al.[14] reported that the digestibility of essential amino acids in BSF larvae meal varied from 84% to 96% in rainbow trout, while the digestibility of conditionally essential amino acid-like taurine was 57%. In these two studies, faeces were collected for digestibility estimation using faecal collection columns attached to the tanks. This might overestimate the protein digestibility compared to other faecal collection methods such as stripping, due to leaching of nitrogen (N) depending on the type of feed as explained by Shomorin et al.[38] On the other hand, fishmeal may contain nutritional components that promote fish growth beyond the digestible nutrient content alone, such as taurine and low molecular weight compounds.[39-41] These components may be lacking in diets containing other protein sources and lead to better growth performance when such protein sources were replaced by BSF as explained in the review by Collins et al.[42] The growth reduction when fishmeal was replaced by BSF can also be due to decreased feed intake. However, the fishmeal replaced dataset contained two studies which gave comparatively lower effect sizes for feed intake than the other studies.[31,43] It is, thus, possible that these two studies might influence the overall results for feed intake in this group. The improved growth in non-fishmeal replaced group can also be related to feed intake, as there was a tendency to increase in feed intake in this group.

As observed for growth performance data, the present study also showed that majority of the experimental diets containing BSF in literature gave similar protein digestibility and PER in salmonids as the control diets. Further, the meta-analysis showed that, on average, the dietary inclusion of BSF did not affect PER, but tended to decrease protein digestibility. Several sub-datasets also showed that the use of BSF decreased protein digestibility in salmonids compared to control diets, indicating negative effects of BSF on protein digestibility. The datasets consisted of different variables that can influence these results. In a review, English et al.[16] showed that the rearing substrate of BSF can be responsible for the inconsistency of nutrient digestibility of salmonids fed BSF between the studies. Moreover, similar to other animal protein sources such as fishmeal,[44] the drying method and temperature may have a large impact on the nutritional quality and protein digestibility of insect meals.[45] These influencing factors were not considered in the present study, and the comparison across the studies is thus complicated.

The exoskeleton of insects characteristically contains chitin.[46] The chitin content of the BSF ingredients in the present dataset ranged from 3 to 17% (Table S4), and dietary chitin levels varied from 0.2 to 3% (dry matter basis) (Table S3). As previous studies suggested,[11,47,48] chitin can be the reason for observed negative effects (at least tend to) of BSF on growth performance and protein digestibility in several datasets in the present study. The dietary protein contents in the dataset mostly covered the requirement of salmon and rainbow trout[27] but the protein contents were calculated using the nitrogen-to-protein conversion factor of 6.25. Since BSF contain non-protein N from chitin, Janssen et al.[49] and Belghit et al.[50] have recently suggested that a factor between 4.2 and 5 might be more appropriate for BSF to avoid overestimation of the protein content. . Nevertheless, chitin is poorly digestible in salmon and rainbow trout (13–40% and 2-5% respectively)[51,52] and can thus act as a filler in the diet.[48] Poorly digestible chitin can also increase faecal N excretion, and lead to overestimate the protein content in the faeces and underestimate the protein digestibility. This shows the importance of correcting the protein digestibility for chitin excreted as non-protein N with faeces, but none of the studies in the present meta-analysis reported a such correction. In addition, the chitin matrix in the

exoskeleton of insects contains bound amino acids.[53] This might reduce the availability of protein in BSF for protease enzymes[29] or the activity of protease enzymes.[13] However, Basto et al.[32] reported that chitin alone cannot explain the lower nutrient digestibility in insect meals. Even though chitin can compromise protein digestibility, several studies reported no effect of the use of BSF meal on protein digestibility in both salmon[12] and rainbow trout.[54] The present regression analysis also showed no relationships between dietary chitin level and growth or nutrient utilization parameters. Nevertheless, this should be viewed with caution because only the data from six studies that reported chitin contents were used for the regression analyses.

In addition to chitin, other factors in the BSF, such as saturated fatty acids or other compounds can also cause negative effects on fish growth. A previous meta-analysis showed that high saturated fatty acids (>39% of total fatty acids), and increasing level of lauric acid in the diet decreased normalized final body weight of fish fed BSF.[18] Nevertheless, two previous studies showed that dietary inclusion of BSF larvae oil (2.5-12%) did not affect the growth performance of salmon[55] and rainbow trout[14], although the BSF oil diets in Belghit et al.[55] contained high levels of saturated fatty acids and lauric acid (48-51% and 22-29% of total fatty acids respectively). Hence, more research is needed to confirm the impact of fatty acid profile of BSF on salmonid performance.

A meta-analysis implies limitations associated with diverse nature of studies, and interpretation of effect sizes obtained in a meta-analysis may be controversial, especially if number of relevant studies are limited.[56] The comparison across studies should ideally consider all biological and dietary factors.[36] Many of the variables that exist across studies were considered in the present study, but unexplained heterogeneity still existed among the studies even after considering these influencing factors. Other factors causing discrepancy among studies might be related to quality of BSF, rearing substrates of BSF, nutrient composition of BSF, level of anti-nutritive as well as bioactive compounds in BSF, degree of defatting of BSF, drying method of BSF and temperatures, level of protein replaced by BSF, diet formulations, method of digestibility measurement, fish size, rearing conditions and culture systems used in studies. Nevertheless, insufficient data prevented us from including these factors in

the present analysis, although they might influence greatly the fish response to BSF. Therefore, this topic should be revisited when more research findings are available to identify the various factors affecting the response of salmonids to BSF in diets, which is important for drawing concrete conclusions and making recommendations.

Conclusions

The present meta-analysis showed that, on average, the growth rate, feed conversion, feed intake, protein digestibility and utilization in salmonids fed BSF diets did not differ from those fed control diets. Variations in these parameters, however, existed between the studies. The fish species, type of protein source(s) replaced, and BSF inclusion level were partially responsible for variation in fish SGR, whereas only the type of replaced protein source(s) and BSF inclusion level were detected as factors explaining the variations in FCR and feed intake, respectively. Replacement of fishmeal by BSF decreased SGR and feed intake of salmonids. On the other hand, the replacement of non-fishmeal sources improved SGR and feed conversion. This stressed the importance of type of replaced protein source(s) when evaluating the nutritional value of BSF for salmonids. Overall, the present meta-analysis showed that BSF is a promising protein source for salmonid feeds.

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Tables

Table 1. Proximate (% dry matter), mineral (g/ kg dry matter), amino acid (g/100 g crude protein) and fatty acid (% of total fatty acids) compositions of different developmental stages of black soldier fly (BSF).

Nutrient	Larvae		Prepupae		Pupae	
	Mean	SD	Mean	SD	Mean	SD
Dry matter (%)	32	5.3 (27)	35	5.9 (11)	40 (1)	
Crude protein	39	6.1 (58)	37	6.0 (16)	36	6.7 (2)
Crude lipid	28	8.7 (14)	34	9.2 (11)	28	17 (2)
Chitin	4.8	1.5 (9)	7.2	1.8 (9)	6.3 (1)	
Ash	10	4.5 (29)	10	4.6 (10)	14	7.4 (2)
Minerals						
Calcium	22	7.8 (12)	34	24 (5)	44 (1)	
Phosphorous	8.4	1.7 (17)	4.7	0.8 (5)	6.3 (1)	
Potassium	17	4.2 (12)	5.8	0.9 (5)	6.1 (1)	
Sodium	5	3.9 (12)	0.9	0.4 (5)	1.7 (1)	
Magnesium	3.8	1.3 (12)	2.8	0.6 (5)	3.7 (1)	
Manganese	0.2	0.05 (12)	0.2	0.1 (5)	0.4 (1)	
Iron	0.3	0.07 (12)	0.2	0.2 (5)	0.07 (1)	
Iodine	0.1	0.08 (10)				
Zinc	0.1	0.03 (11)	0.09	0.05 (4)	0.07 (1)	
Essential amino acids						
Arginine	4.8	0.6 (40)	4.8	1.1 (9)	5.5 (1)	
Histidine	2.7	0.5 (40)	3.0	0.7 (10)	3.4 (1)	
Isoleucine	4.2	0.7 (40)	4.2	0.9 (10)	4.7 (1)	
Leucine	6.6	0.8 (40)	6.6	1.4 (10)	7.8 (1)	
Lysine	5.9	1.0 (40)	5.6	1.3 (10)	6.4 (1)	
Methionine	1.7	0.3 (40)	1.7	0.4 (10)	1.7 (1)	
Phenylalanine	3.9	0.8 (40)	3.8	0.9 (10)	4.1 (1)	
Threonine	3.8	0.4 (40)	3.8	0.9 (10)	4.3 (1)	
Tryptophan	1.8	0.9 (21)	1.4	0.3 (6)	1.6 (1)	
Valine	5.8	0.7 (40)	5.8	1.3 (10)	6.8 (1)	
Non-essential amino acids						
Alanine	6.4	0.9 (35)	6.0	1.5 (8)	6.8 (1)	
Aspartic acid	8.6	0.9 (35)	8.3	2.2 (8)	11 (1)	
Glycine	5.1	0.6 (35)	5.1	1.3 (8)	6.5 (1)	
Glutamic acid	11	1.4 (34)	9.9	2.5 (8)	11 (1)	
Cysteine	0.8	0.7 (23)	1.1	1.0 (8)	0.8 (1)	
Tyrosine	5.7	1.4 (36)	5.9	2.0 (5)	6.8 (1)	
Proline	5.6	1.0 (34)	5.2	1.3 (8)	6.2 (1)	
Serine	4.2	0.4 (34)	4.1	1.5 (8)	4.7 (1)	
Fatty acids						
C12:0	37	9.9 (58)	43	11.9 (27)	65 (1)	
C14:0	7.5	1.4 (58)	6.9	2.0 (27)	9.7 (1)	
C16:0	16	3.1 (58)	13	3.6 (27)	8.6 (1)	

C16:1n7 (C16:1)	3.5	2.2 (55)	5.9	3.8 (27)	2.8 (1)
C18:0	2.9	0.9 (58)	1.8	0.8 (27)	1.2 (1)
18:1n9	13	4.3 (58)	12	5.0 (23)	6.8 (1)
C18:2n6	14	6.7 (58)	6.7	3.4 (27)	5.2 (1)
C18:3n3	1.5	0.8 (58)	3.9	6.9 (26)	0.7 (1)
C20:4n6	0.4	0.5 (39)			
C20:5n3	1.1	1.5 (41)	0.1	0.1 (5)	
C22:6n3	0.7	0.9 (30)	0.1	0.2 (5)	

SD: Standard deviation.

Values in parentheses are the number of datapoints used for calculating the mean.

Sources for proximate and mineral compositions: BSF larvae,[13,57-65] BSF prepupae[61,65-69] and BSF pupae.[60,65] Sources for amino acid compositions: BSF larvae,[11-13,28,49,54,57,64,65,70-80] BSF pre-pupae[37,65,66,69,80-82] and BSF pupae.[65] Sources for fatty acid compositions: BSF larvae,[11,13,54,57,58,62,65,83-89] BSF pre-pupae[65,67-69,90-93] and BSF pupae.[65]

Table 2. Heterogeneity in effect sizes (Hedges' g) and significance level (*p* value) of different categorical and continuous variables determined by meta-regression analysis.

Parameter	Heterogeneity explained by the model (%)	Test of the model	Heterogeneity unexplained by the model	*p* value						
				Fish species	Feed production	Type of protein source replaced	BSF development stage	BSF processing method	BSF inclusion level	Fish body size
SGR	0.19	***	***	**	NS	***	NS	NS	*	NS
FCR	0.02	**	***	NS	NS	**	NS	NS	NS	NS
Feed intake	-0.09	*	***	NS	NS	NS	NS	NS	*	NS

BSF: Black soldier fly (*Hermetia illucens*), SGR: Specific growth rate, FCR: Feed conversion ratio.

Asterisks denote level of significance (NS: not significant, $^{**}p < 0.01$, $^{***}p < 0.001$).

Table 3. Effect sizes (Hedges' g) of growth performance and nutrient utilization data in salmonids between experimental diets containing black soldier fly (BSF) and control diets (sub-datasets).

Parameter	Data subset	Number of studies	Number of comparisons	Random effect model			Heterogeneity		
				Hedges' g	95% CI	*p*	Q	*p*	I^2
SGR	**Species**								
	Atlantic salmon	5	18	-0.72	-1.7 to 0.2	NS	91.9	***	81.5
	Rainbow trout	8	18	0.60	-0.2 to 1.4	NS	72.5	***	76.5
	Salmon life stage								
	Pre-smolt	4	15	-0.36	-1.3 to 0.6	NS	73.4	***	80.9
	Post-smolt	No sufficient data							
	Protein source replaced								
	Fishmeal	7	14	-0.76	-1.4 to -0.2	*	29.7	**	56.2
	Fishmeal + Plant protein	3	12	-0.25	-1.4 to 0.9	NS	64.1	***	82.8
	Non-fishmeal	3	10	1.98	0.5 to 3.4	**	51.2	***	82.4
	BSF processing method								
	Full-fat	6	16	-0.37	-1.1 to 0.3	NS	50.3	***	70.2
	Defatted	8	20	0.26	-0.7 to 1.2	NS	113	***	83.1
FCR	**Species**								
	Atlantic salmon	6	23	0.51	-0.1 to 1.1	NS	77.4	***	71.6
	Rainbow trout	11	26	-0.31	-0.9 to 0.3	NS	104	***	76.1
	Salmon life stage								
	Pre-smolt	4	15	1.01	0.3 to 1.7	**	47.6	***	70.6
	Post-smolt	No sufficient data							
	Protein source replaced								
	Fishmeal	11	27	0.18	-0.3 to 0.6	NS	71.4	***	63.6
	Fishmeal + Plant protein	3	12	1.11	0.3 to 1.9	**	36.8	***	70.1
	Non-fishmeal	3	10	-1.89	-3.3 to -0.5	**	49.2	***	81.7
	BSF processing method								

	Full-fat	9	25	0.37	-0.1 to 0.9	NS	74.2	***	67.7
	Defatted	10	24	-0.30	-1.0 to 0.4	NS	109	***	78.9
Feed intake	**Species**								
	Atlantic salmon	6	23	-0.01	-0.5 to 0.5	NS	55.7	***	60.5
	Rainbow trout	11	27	-0.18	-0.7 to 0.4	NS	90.6	***	71.3
	Salmon life stage								
	Pre-smolt	4	15	0.08	-0.3 to 0.5	NS	21.2	NS	33.9
	Post-smolt	No sufficient data							
	Protein source replaced								
	Fishmeal	11	28	-0.70	-1.3 to -0.1	*	103	***	73.9
	Fishmeal + Plant protein	3	12	0.12	-0.4 to 0.7	NS	21.1	*	48.0
	Non-fishmeal	3	10	0.51	-0.1 to 1.1	NS	16.4	NS	45.0
	BSF processing method								
	Full-fat	9	25	-0.49	-1.1 to 0.1	NS	82.2	***	70.8
	Defatted	10	25	0.21	-0.3 to 0.7	NS	62.1	***	61.4
ADC of protein	**Species**								
	Atlantic salmon	4	13	-1.03	-1.7 to -0.4	**	28.7	**	58.2
	Rainbow trout	No sufficient data							
	BSF development stage								
	Larvae	6	16	-0.73	-1.4 to -0.1	*	45.2	***	66.8
	Prepupae/pupae	No sufficient data							
	BSF processing method								
	Full-fat	3	11	-0.52	-1.4 to 0.3	NS	36.7	***	72.2
	Defatted	No sufficient data							
	Feed production								
	Extrusion	5	15	-0.79	-1.5 to -0.1	*	44.4	***	68.5

	Pelleting	No sufficient data							
PER	**Species**								
	Atlantic salmon	4	15	-0.19	-1.0 to 0.7	NS	63.1	***	77.8
	Rainbow trout	No sufficient data							
	BSF processing method								
	Full-fat	5	14	-0.33	-1.0 to 0.4	NS	41.2	***	68.5
	Defatted	No sufficient data							

CI: Confidence interval, SGR: Specific growth rate, FCR: Feed conversion ratio, ADC of protein: Apparent digestibility of protein, PER: Protein efficiency ratio. Asterisks denote level of significance (NS: not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Figure legends

Fig. 1. Selection of studies to be included in the meta-analysis dataset. The selected 16 studies.[11-15,28,31,37,43,54,71,81,94-97]

Fig. 2. Forest plot of effect sizes (Hedges' g) of specific growth rate in salmonids between experimental diets containing black soldier fly (BSF) and control diets (full dataset). The mean effect size, calculated according to a random effects model, is indicated by the red diamond at the bottom. The size of the blue squares illustrates the weight of each study relatively to the mean effect size. Smaller squares represent less weight. CI: Confidence interval.

Fig. 3. The relationship between the fishmeal replacement level by black soldier fly and the effect sizes (Hedges' g) of specific growth rate for the full dataset **(A)**, salmon dataset **(B)**, rainbow trout dataset **(C)**, black soldier fly larvae dataset **(D)**, black soldier fly prepupae/pupae dataset **(E)** and defatted black soldier fly dataset **(F)**. Red lines represent linear relationships and blue lines represent quadratic relationships.

Fig. 4. Forest plot of effect sizes (Hedges' g) of feed conversion ratio in salmonids between experimental diets containing black soldier fly (BSF) and control diets (full dataset). The mean effect size, calculated according to a random effects model, is indicated by the red diamond at the bottom. The size of the blue squares illustrates the weight of each study relatively to the mean effect size. Smaller squares represent less weight. CI: Confidence interval.

Fig. 5. The relationship between the fishmeal replacement level by black soldier fly and the effect sizes (Hedges' g) of feed conversion ratio for the full dataset **(A)**, rainbow trout dataset **(B)**, black soldier fly prepupae/pupae dataset **(C)** and defatted black soldier fly dataset **(D)**. Red lines represent linear relationships and blue lines represent quadratic relationships.

Fig. 6. Forest plot of effect sizes (Hedges' g) of feed intake in salmonids between experimental diets containing black soldier fly (BSF) and control diets (full dataset). The mean effect size, calculated according to a random effects model, is indicated by the red diamond at the bottom. The size of the blue squares illustrates the weight of each study relatively to the mean effect size. Smaller squares represent less weight. CI: Confidence interval.

Fig. 7. The relationship between the fishmeal replacement level by black soldier fly and the effect sizes (Hedges' g) of feed intake for the full dataset **(A)**, salmon dataset **(B)**, black soldier fly larvae dataset **(C)**, full-fat black soldier fly dataset **(D)** and defatted black soldier fly dataset **(E)**. Red lines represent linear relationships and blue lines represent quadratic relationships.

Fig. 8. Forest plot of effect sizes (Hedges' g) of apparent digestibility coefficient of protein in salmonids between experimental diets containing black soldier fly (BSF) and control diets (full dataset). The mean effect size, calculated according to a random effects model, is indicated by the red diamond at the bottom. The size of the blue squares illustrates the weight of each study relatively to the mean effect size. Smaller squares represent less weight. CI: Confidence interval.

Fig. 9. Forest plot of effect sizes (Hedges' g) of protein efficiency ratio in salmonids between experimental diets containing black soldier fly (BSF) and control diets (full dataset). The mean effect size, calculated according to a random effects model, is indicated by the red diamond at the bottom. The size of the blue squares illustrates the weight of each study relatively to the mean effect size. Smaller squares represent less weight. CI: Confidence interval.

Figures

Figure 1

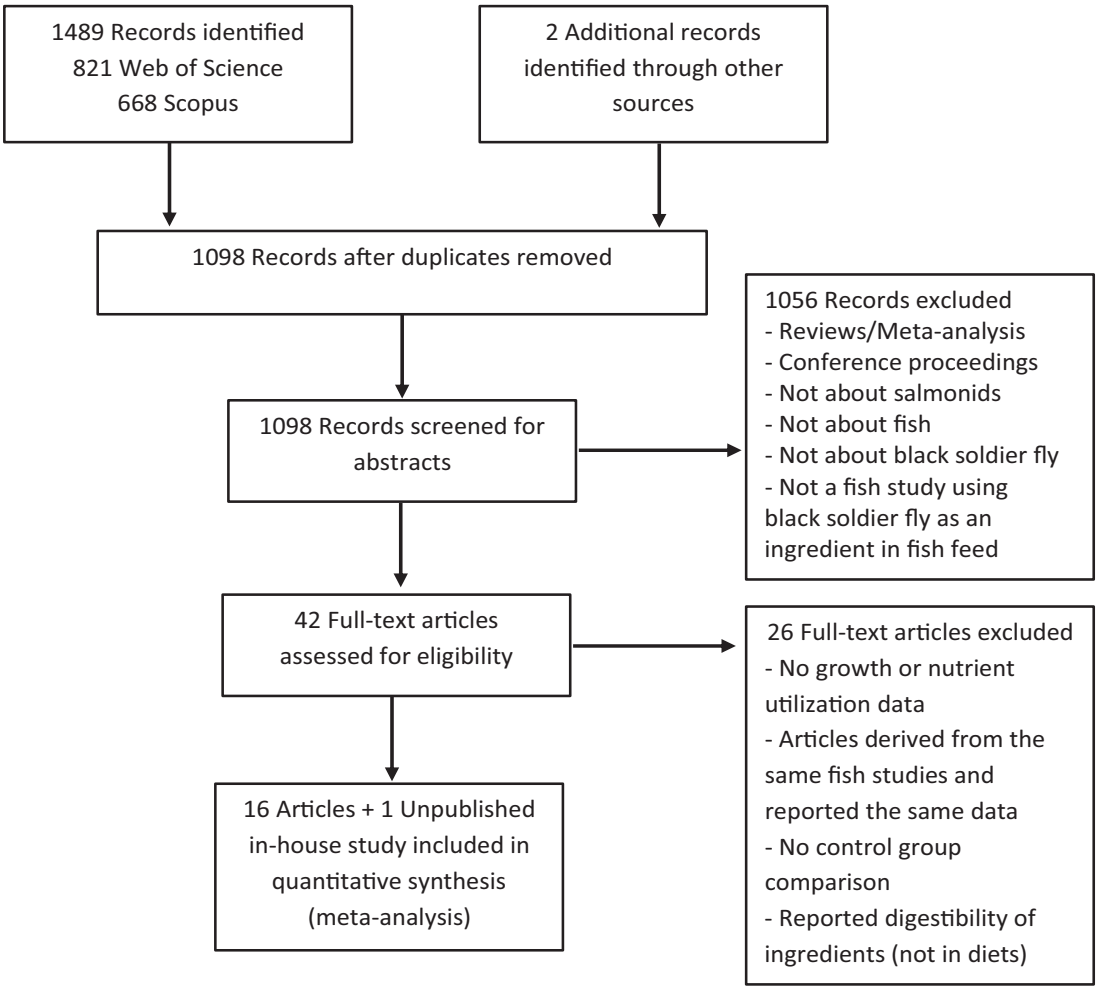


Figure 2

Fish species	Study name	Hedges' g	p-Value	Fishmeal replacement level (%)	Inclusion level (%)
Atlantic salmon	Weththasinghe et al. (2021a)_1	0.000	1.000	19	5
Atlantic salmon	Weththasinghe et al. (2021a)_2	3.538	0.004	19	5
Atlantic salmon	Belghit et al. (2019a)_1	-5.714	0.001	33	5
Rainbow trout	Roques et al. (2020)_1	0.632	0.351	0	5
Rainbow trout	Melenchón et al. (2021)_1	-0.870	0.182	14	6
Rainbow trout	Randazzo et al. (2021)_1	4.000	0.003	0	8
Rainbow trout	Randazzo et al. (2021)_2	8.000	0.001	0	8
Atlantic salmon	Weththasinghe et al. (2021a)_3	-0.769	0.265	7	8
Atlantic salmon	Weththasinghe et al. (2021a)_4	-2.000	0.022	34	8
Atlantic salmon	Weththasinghe et al. (2021a)_5	1.538	0.051	34	8
Atlantic salmon	Belghit et al. (2019a)_2	0.000	1.000	67	10
Atlantic salmon	Fisher et al. (2020)_1	0.483	0.441	0	10
Rainbow trout	Roques et al. (2020)_2	2.807	0.007	0	10
Rainbow trout	Terova et al. (2019)_1	-0.372	0.574	10	10
Rainbow trout	Cardinaletti et al. (2019)_1	-0.706	0.302	25	11
Rainbow trout	Melenchón et al. (2021)_2	-0.870	0.182	30	11
Atlantic salmon	Belghit et al. (2019a)_3	-5.714	0.001	100	15
Atlantic salmon	Unpublished_1	2.057	0.020	17	15
Rainbow trout	Roques et al. (2020)_3	3.293	0.004	0	15
Atlantic salmon	Weththasinghe et al. (2021a)_6	-0.154	0.814	14	16
Atlantic salmon	Fisher et al. (2020)_2	0.000	1.000	0	20
Rainbow trout	Józefiak et al. (2019)_1	-1.000	0.161	30	20
Rainbow trout	Renna et al. (2017)_1	0.669	0.294	25	20
Rainbow trout	Terova et al. (2019)_2	-1.098	0.131	20	20
Atlantic salmon	Unpublished_2	5.714	0.001	17	20
Rainbow trout	Cardinaletti et al. (2019)_2	-1.900	0.026	50	21
Rainbow trout	Randazzo et al. (2021)_3	4.000	0.003	0	23
Rainbow trout	Stadtlander et al. (2017)_1	0.320	0.628	46	28
Atlantic salmon	Fisher et al. (2020)_3	-2.899	0.002	0	30
Rainbow trout	Terova et al. (2019)_3	-1.098	0.131	30	30
Atlantic salmon	Weththasinghe et al. (2021a)_7	-2.000	0.022	29	32
Rainbow trout	Renna et al. (2017)_2	0.334	0.590	50	40
Rainbow trout	Randazzo et al. (2021)_4	4.000	0.003	0	45
Atlantic salmon	Belghit et al. (2018)_1	-0.870	0.182	83	60
Atlantic salmon	Belghit et al. (2018)_2	-5.652	0.000	83	60
Atlantic salmon	Belghit et al. (2018)_3	-3.478	0.001	83	60
		-0.014	0.963		

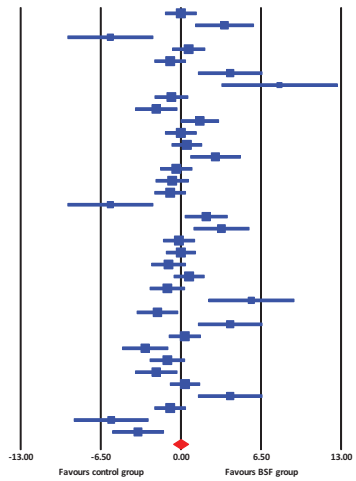


Figure 3

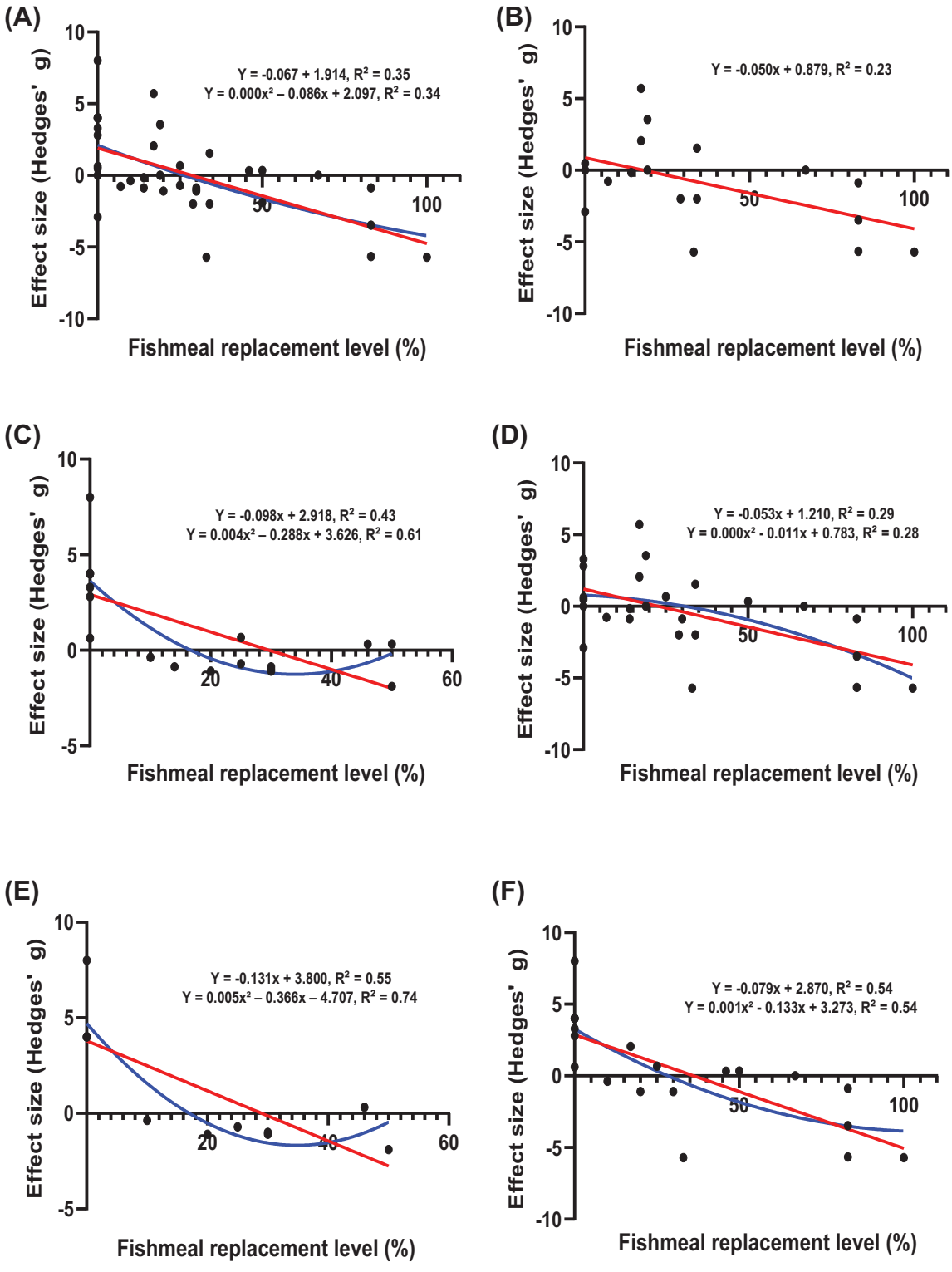

(A)
Y = -0.067 + 1.914, R² = 0.35
Y = 0.000x² – 0.086x + 2.097, R² = 0.34
Effect size (Hedges' g)
Fishmeal replacement level (%)
(B)
Y = -0.050x + 0.879, R² = 0.23
Effect size (Hedges' g)
Fishmeal replacement level (%)
(C)
Y = -0.098x + 2.918, R² = 0.43
Y = 0.004x² – 0.288x + 3.626, R² = 0.61
Effect size (Hedges' g)
Fishmeal replacement level (%)
(D)
Y = -0.053x + 1.210, R² = 0.29
Y = 0.000x² - 0.011x + 0.783, R² = 0.28
Effect size (Hedges' g)
Fishmeal replacement level (%)
(E)
Y = -0.131x + 3.800, R² = 0.55
Y = 0.005x² – 0.366x – 4.707, R² = 0.74
Effect size (Hedges' g)
Fishmeal replacement level (%)
(F)
Y = -0.079x + 2.870, R² = 0.54
Y = 0.001x² - 0.133x + 3.273, R² = 0.54
Effect size (Hedges' g)
Fishmeal replacement level (%)

Figure 4

Fish species	Study name	Hedges' g	p-Value	Fishmeal replacement level (%)	Inclusion level (%)
Atlantic salmon	Weththasinghe et al. (2021a)_1	-0.471	0.481	19	5
Atlantic salmon	Weththasinghe et al. (2021a)_2	0.941	0.183	19	5
Atlantic salmon	Belghit et al. (2019a)_1	0.000	1.000	33	5
Atlantic salmon	Lock et al. (2016)_1	0.000	1.000	25	5
Atlantic salmon	Lock et al. (2016)_2	-3.603	0.010	25	5
Rainbow trout	Roques et al. (2020)_1	-1.600	0.046	0	5
Rainbow trout	Melenchón et al. (2021)_1	1.087	0.106	14	6
Rainbow trout	Dumas et al. (2018)_1	1.518	0.054	25	7
Rainbow trout	Randazzo et al. (2021)_1	-1.143	0.118	0	8
Rainbow trout	Randazzo et al. (2021)_2	-8.000	0.001	0	8
Atlantic salmon	Weththasinghe et al. (2021a)_3	0.471	0.481	7	8
Atlantic salmon	Weththasinghe et al. (2021a)_4	1.882	0.027	34	8
Atlantic salmon	Weththasinghe et al. (2021a)_5	3.294	0.004	34	8
Atlantic salmon	Belghit et al. (2019a)_2	0.000	1.000	67	10
Atlantic salmon	Fisher et al. (2020)_1	-0.435	0.486	0	10
Atlantic salmon	Lock et al. (2016)_3	-1.524	0.052	50	10
Rainbow trout	Roques et al. (2020)_2	-2.824	0.007	0	10
Rainbow trout	Terova et al. (2019)_1	0.759	0.271	10	10
Rainbow trout	Cardinaletti et al. (2019)_1	0.582	0.389	25	11
Rainbow trout	Melenchón et al. (2021)_2	1.087	0.106	30	11
Rainbow trout	Dumas et al. (2018)_2	4.800	0.002	50	13
Atlantic salmon	Belghit et al. (2019a)_3	0.000	1.000	100	15
Atlantic salmon	Unpublished_1	-1.600	0.046	17	15
Rainbow trout	St-Hilaire et al. (2007)_1	0.248	0.688	25	15
Rainbow trout	Roques et al. (2020)_3	-3.765	0.003	0	15
Atlantic salmon	Weththasinghe et al. (2021a)_6	-0.471	0.481	14	16
Rainbow trout	Sealey et al. (2011)_1	-1.538	0.051	25	18
Rainbow trout	Sealey et al. (2011)_2	-1.538	0.051	25	20
Atlantic salmon	Fisher et al. (2020)_2	0.000	1.000	0	20
Rainbow trout	Józefiak et al. (2019)_1	0.800	0.248	30	20
Rainbow trout	Renna et al. (2017)_1	-0.966	0.144	25	20
Rainbow trout	Terova et al. (2019)_2	1.569	0.048	20	20
Atlantic salmon	Unpublished_2	3.200	0.005	17	20
Rainbow trout	Cardinaletti et al. (2019)_2	1.554	0.050	50	21
Rainbow trout	Randazzo et al. (2021)_3	-4.571	0.002	0	23
Atlantic salmon	Lock et al. (2016)_4	-2.241	0.022	100	25
Atlantic salmon	Lock et al. (2016)_5	2.530	0.017	100	25
Rainbow trout	Stadtlander et al. (2017)_1	0.140	0.830	46	28
Rainbow trout	St-Hilaire et al. (2007)_2	1.801	0.018	50	30
Atlantic salmon	Fisher et al. (2020)_3	3.043	0.002	0	30
Rainbow trout	Terova et al. (2019)_3	0.759	0.271	30	30
Atlantic salmon	Weththasinghe et al. (2021a)_7	1.882	0.027	29	32
Rainbow trout	Sealey et al. (2011)_3	-1.538	0.051	50	36
Rainbow trout	Renna et al. (2017)_2	0.000	1.000	50	40
Rainbow trout	Sealey et al. (2011)_4	-0.769	0.265	50	40
Rainbow trout	Randazzo et al. (2021)_4	-4.571	0.002	0	45
Atlantic salmon	Belghit et al. (2018)_1	0.870	0.182	83	60
Atlantic salmon	Belghit et al. (2018)_2	3.478	0.001	83	60
Atlantic salmon	Belghit et al. (2018)_3	1.739	0.021	83	60
		0.094	0.671		

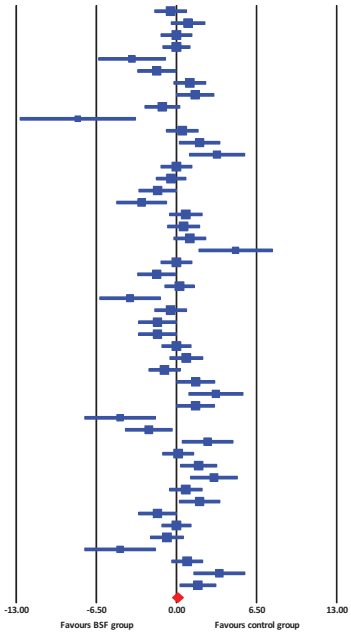


Figure 5

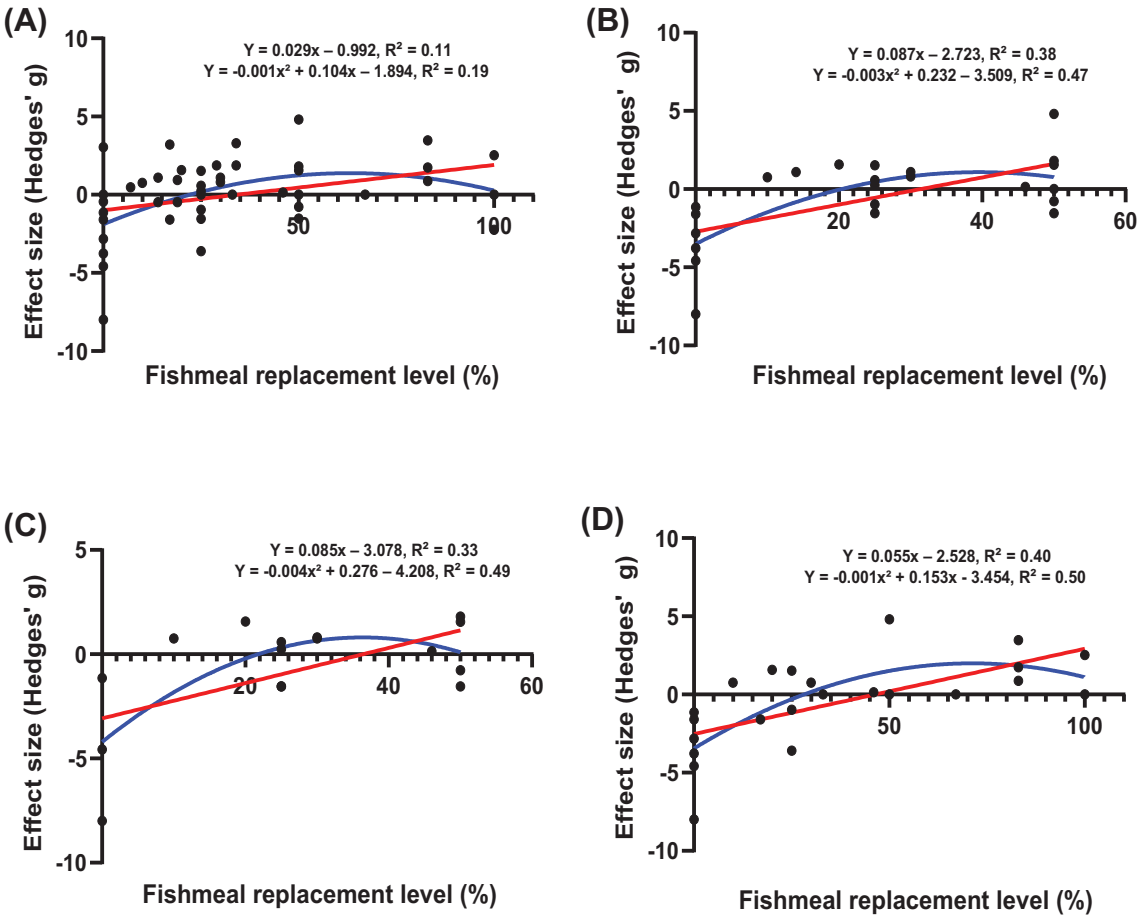

(A)
Y = 0.029x – 0.992, R² = 0.11
Y = -0.001x² + 0.104x – 1.894, R² = 0.19
Effect size (Hedges' g)
Fishmeal replacement level (%)
(B)
Y = 0.087x – 2.723, R² = 0.38
Y = -0.003x² + 0.232 – 3.509, R² = 0.47
Effect size (Hedges' g)
Fishmeal replacement level (%)
(C)
Y = 0.085x – 3.078, R² = 0.33
Y = -0.004x² + 0.276 – 4.208, R² = 0.49
Effect size (Hedges' g)
Fishmeal replacement level (%)
(D)
Y = 0.055x – 2.528, R² = 0.40
Y = -0.001x² + 0.153x - 3.454, R² = 0.50
Effect size (Hedges' g)
Fishmeal replacement level (%)

Figure 6

Fish species	Study name	Fishmeal replacement level (%)	Inclusion level (%)	Hedges's g	p-Value
Atlantic salmon	Belghit et al. (2019)_1	33	5	0.000	1.000
Atlantic salmon	Lock et al. (2016)_1	25	5	1.600	0.047
Atlantic salmon	Lock et al. (2016)_2	25	5	-13.143	0.005
Rainbow trout	Roques et al. (2020)_1	0	5	-0.267	0.685
Atlantic salmon	Weththasinghe et al. (2021b)_1	19	5	0.000	1.000
Atlantic salmon	Weththasinghe et al. (2021b)_2	19	5	1.350	0.076
Rainbow trout	Melenchón et al. (2020)_1	14	6	0.000	1.000
Rainbow trout	Dumas et al. (2018)_1	25	7	-1.067	0.140
Rainbow trout	Randazzo et al. (2021)_1	0	8	0.400	0.546
Rainbow trout	Randazzo et al. (2021)_2	0	8	0.800	0.248
Atlantic salmon	Weththasinghe et al. (2021b)_3	7	8	-0.388	0.558
Atlantic salmon	Weththasinghe et al. (2021b)_4	34	8	-0.530	0.430
Atlantic salmon	Weththasinghe et al. (2021b)_5	34	8	1.012	0.157
Atlantic salmon	Belghit et al. (2019)_2	67	10	0.000	1.000
Atlantic salmon	Fisher et al. (2020)_1	0	10	0.145	0.814
Atlantic salmon	Lock et al. (2016)_3	50	10	-3.886	0.009
Rainbow trout	Roques et al. (2020)_2	0	10	3.600	0.003
Rainbow trout	Terova et al. (2019)_1	10	10	0.533	0.427
Rainbow trout	Cardinaletti et al. (2019)_1	25	11	0.686	0.315
Rainbow trout	Melenchón et al. (2020)_2	30	11	-0.373	0.549
Rainbow trout	Dumas et al. (2018)_2	50	13	-0.533	0.427
Atlantic salmon	Belghit et al. (2019)_3	100	15	0.000	1.000
Rainbow trout	Roques et al. (2020)_3	0	15	3.733	0.003
Rainbow trout	St?Hilaire et al. (2007)_1	25	15	-2.733	0.003
Atlantic salmon	Unpublished_1	17	15	4.480	0.002
Atlantic salmon	Weththasinghe et al. (2021b)_6	14	16	0.000	1.000
Rainbow trout	Sealey et al. (2011)_1	25	18	-15.840	0.001
Atlantic salmon	Fisher et al. (2020)_2	0	20	0.000	1.000
Rainbow trout	Józefiak et al. (2019)_1	30	20	0.500	0.455
Rainbow trout	Renna et al. (2017)_1	25	20	0.373	0.549
Rainbow trout	Sealey et al. (2011)_2	25	20	-11.680	0.001
Rainbow trout	Terova et al. (2019)_2	20	20	0.933	0.187
Atlantic salmon	Unpublished_2	17	20	0.707	0.302
Rainbow trout	Cardinaletti et al. (2019)_2	50	21	0.000	1.000
Rainbow trout	Randazzo et al. (2021)_3	0	23	0.200	0.760
Atlantic salmon	Lock et al. (2016)_4	100	25	-12.229	0.005
Atlantic salmon	Lock et al. (2016)_5	100	25	-26.743	0.005
Rainbow trout	Dumas et al. (2018)_3	100	26	-2.000	0.022
Rainbow trout	Stadtlander et al. (2017)_1	46	28	0.300	0.649
Atlantic salmon	Fisher et al. (2020)_3	0	30	0.000	1.000
Rainbow trout	St?Hilaire et al. (2007)_2	50	30	-1.863	0.016
Rainbow trout	Terova et al. (2019)_3	30	30	1.333	0.079
Atlantic salmon	Weththasinghe et al. (2021b)_7	29	32	-0.446	0.503
Rainbow trout	Sealey et al. (2011)_3	50	36	-6.880	0.001
Rainbow trout	Renna et al. (2017)_2	50	40	0.000	1.000
Rainbow trout	Sealey et al. (2011)_4	50	40	-2.400	0.012
Rainbow trout	Randazzo et al. (2021)_4	0	45	0.200	0.760
Atlantic salmon	Belghit et al. (2018)_1	83	60	0.000	1.000
Atlantic salmon	Belghit et al. (2018)_2	83	60	-0.435	0.486
Atlantic salmon	Belghit et al. (2018)_3	83	60	-1.304	0.061
				-0.099	0.605

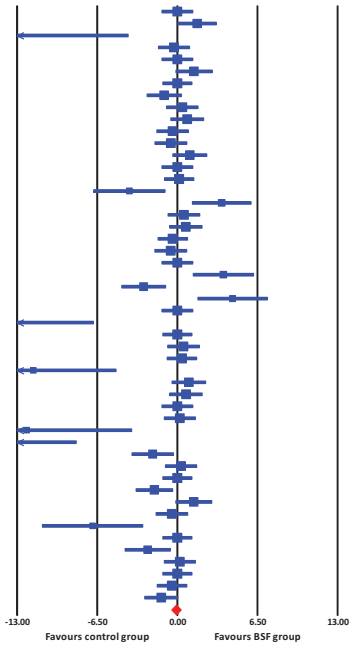


Figure 7

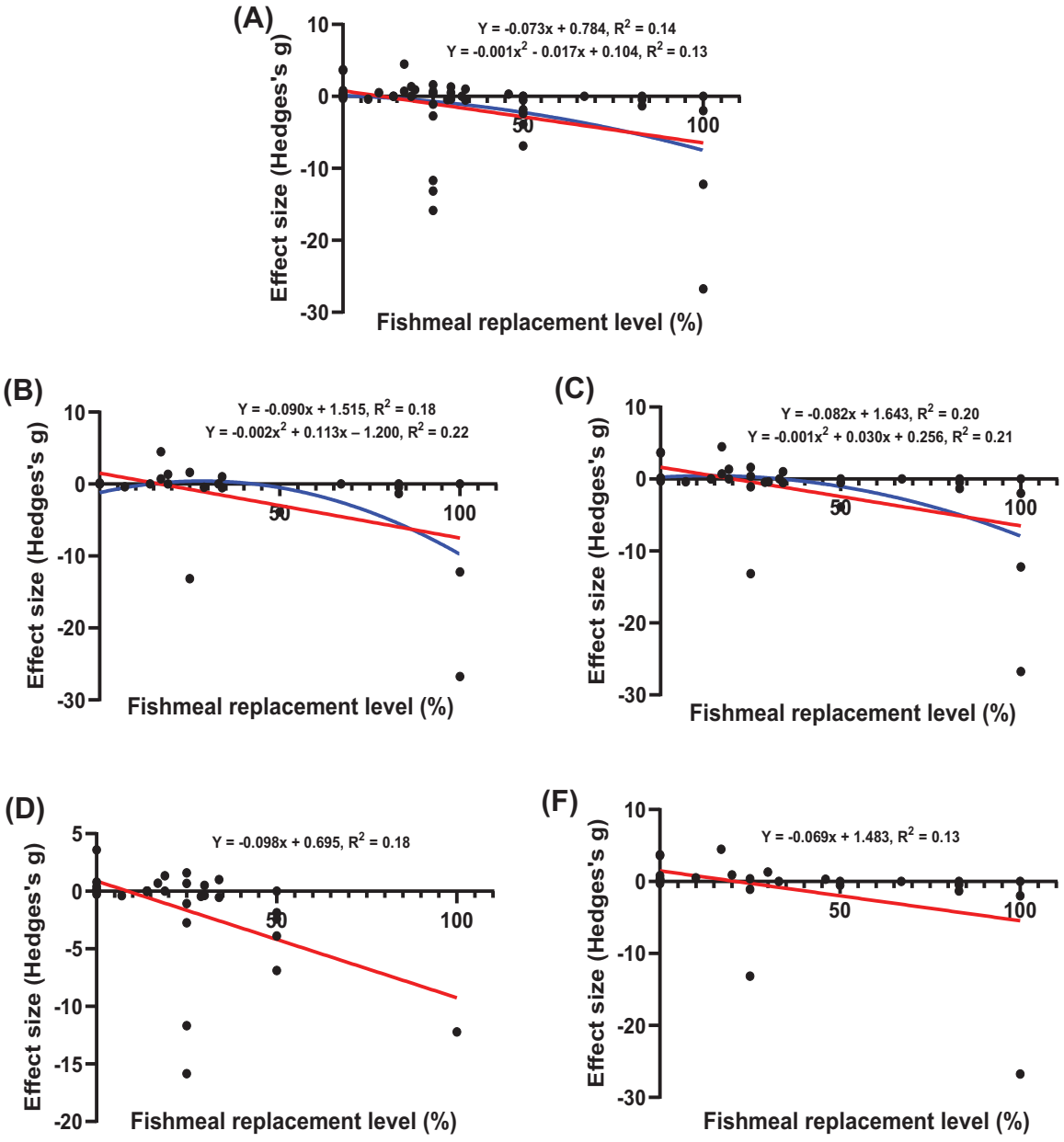

(A)
Y = -0.073x + 0.784, R2 = 0.14
Y = -0.001x2 - 0.017x + 0.104, R2 = 0.13
Effect size (Hedges's g)
Fishmeal replacement level (%)
(B)
Y = -0.090x + 1.515, R2 = 0.18
Y = -0.002x2 + 0.113x – 1.200, R2 = 0.22
Effect size (Hedges's g)
Fishmeal replacement level (%)
(C)
Y = -0.082x + 1.643, R2 = 0.20
Y = -0.001x2 + 0.030x + 0.256, R2 = 0.21
Effect size (Hedges's g)
Fishmeal replacement level (%)
(D)
Y = -0.098x + 0.695, R2 = 0.18
Effect size (Hedges's g)
Fishmeal replacement level (%)
(F)
Y = -0.069x + 1.483, R2 = 0.13
Effect size (Hedges's g)
Fishmeal replacement level (%)

Figure 8

Fish species	Study name	Hedges' g	p-Value	Fishmeal replacement level (%)	Inclusion level (%)
Atlantic salmon	Weththasinghe et al. (2021a)_1	1.427	0.065	19	5
Atlantic salmon	Weththasinghe et al. (2021a)_2	-0.588	0.384	19	5
Atlantic salmon	Belghit et al. (2019a)_1	-0.700	0.306	33	5
Rainbow trout	Melenchón et al. (2021)_1	1.401	0.048	14	6
Atlantic salmon	Weththasinghe et al. (2021a)_3	-0.252	0.702	7	8
Atlantic salmon	Weththasinghe et al. (2021a)_4	-0.420	0.528	34	8
Atlantic salmon	Weththasinghe et al. (2021a)_5	-2.434	0.011	34	8
Atlantic salmon	Belghit et al. (2019a)_2	-0.700	0.306	67	10
Rainbow trout	Terova et al. (2019)_1	0.205	0.754	10	10
Rainbow trout	Melenchón et al. (2021)_2	1.256	0.069	30	11
Atlantic salmon	Belghit et al. (2019a)_3	-1.400	0.068	100	15
Atlantic salmon	Unpublished_1	-3.299	0.004	18	15
Atlantic salmon	Weththasinghe et al. (2021a)_6	-2.099	0.018	14	16
Rainbow trout	Dumas et al. (2018)_1	-0.800	0.248	20	20
Rainbow trout	Terova et al. (2019)_2	-0.133	0.839	20	20
Atlantic salmon	Unpublished_2	-2.309	0.013	18	20
Atlantic salmon	Fisher et al. (2020)_1	0.000	1.000	30	30
Rainbow trout	Terova et al. (2019)_3	0.722	0.292	30	30
Atlantic salmon	Weththasinghe et al. (2021a)_7	-3.274	0.004	29	32
		-0.540	0.057		

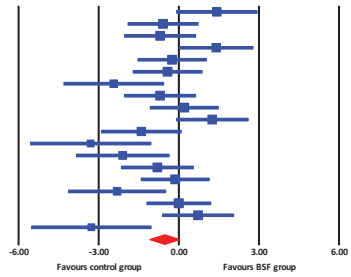
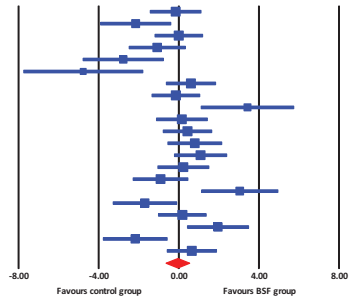


Figure 9

Fish species	Study name	Hedges' g	p-Value	Fishmeal replacement level (%)	Inclusion level (%)
Atlantic salmon	Weththasinghe et al. (2021a)_1	-0.154	0.814	19	5
Atlantic salmon	Weththasinghe et al. (2021a)_2	-2.154	0.017	19	5
Rainbow trout	Melenchón et al. (2021)_1	0.000	1.000	14	6
Atlantic salmon	Weththasinghe et al. (2021a)_3	-1.077	0.137	7	8
Atlantic salmon	Weththasinghe et al. (2021a)_4	-2.769	0.007	34	8
Atlantic salmon	Weththasinghe et al. (2021a)_5	-4.769	0.002	34	8
Atlantic salmon	Fisher et al. (2020)_1	0.609	0.337	0	10
Rainbow trout	Melenchón et al. (2021)_2	-0.145	0.814	30	11
Atlantic salmon	Unpublished_1	3.429	0.004	18	15
Atlantic salmon	Weththasinghe et al. (2021a)_6	0.154	0.814	14	16
Atlantic salmon	Fisher et al. (2020)_2	0.435	0.486	0	20
Rainbow trout	Józefiak et al. (2019)_1	0.800	0.248	31	20
Rainbow trout	Renna et al. (2017)_1	1.087	0.106	25	20
Atlantic salmon	Unpublished_2	0.229	0.728	18	20
Rainbow trout	Stadtlander et al. (2017)_1	-0.910	0.196	46	28
Atlantic salmon	Fisher et al. (2020)_3	3.043	0.002	0	30
Atlantic salmon	Weththasinghe et al. (2021a)_7	-1.692	0.038	29	32
Rainbow trout	Renna et al. (2017)_2	0.181	0.769	50	40
Atlantic salmon	Belghit et al. (2018)_1	1.957	0.013	83	60
Atlantic salmon	Belghit et al. (2018)_2	-2.174	0.008	83	60
Atlantic salmon	Belghit et al. (2018)_3	0.652	0.305	83	60
		-0.064	0.831		



Supplementary tables

Table S1. Average amino acid composition (g/100 g crude protein) of mealworm (*Tenebrio Molitor*) and house fly (*Musca domestica*).

	Mealworm larvae		**House fly larvae/pre-pupae**	
	Mean	SD	Mean	SD
Essential amino acids				
Arginine	5.1	1.0 (22)	4.9	0.7 (14)
Histidine	4.4	3.0 (22)	2.7	1.0 (14)
Isoleucine	4.0	0.9 (22)	3.1	0.7 (14)
Leucine	7.1	1.5 (22)	5.6	0.7 (14)
Lysine	5.3	1.2 (22)	6.2	1.4 (14)
Methionine	1.4	0.4 (22)	2.5	1.5 (14)
Phenylalanine	3.7	0.7 (22)	5.3	2.0 (14)
Threonine	3.8	0.7 (22)	4.0	1.4 (14)
Tryptophan	1.1	0.4 (6)	2.7	2.3 (9)
Valine	5.7	1.1 (22)	4.1	1.1 (14)
Non-essential amino acids				
Alanine	7.4	1.2 (17)	5.2	1.3 (11)
Aspartic acid	8.2	1.5 (17)	7.2	2.7 (11)
Glycine	5.3	1.0 (17)	3.9	1.2 (11)
Glutamic acid	12	1.6 (17)	10	3.8 (11)
Cysteine	1.0	0.5 (16)	1.5	0.9 (6)
Tyrosine	6.4	1.7 (17)	4.8	2.1 (11)
Proline	6.3	1.7 (17)	3.6	1.2 (7)
Serine	5.1	1.2 (17)	4.2	1.7 (11)

SD: Standard deviation.

Values in parentheses are the number of datapoints used for calculating the mean of each amino acid for each insect species.

Sources: Mealworm larvae[1-18] and housefly larvae/pre-pupae.[3,19-28]

Table S2. Essential amino acid (g/100 g of crude protein) compositions of fishmeal protein and soy protein, and their corresponding requirements in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*).

	Fishmeal protein†	**Soy protein‡**	**Atlantic salmon¶**	**Rainbow trout¶**
Arginine	5.6	7.3	5.0	3.9
Histidine	2.4	2.7	2.2	2.1
Isoleucine	4.7	4.5	3.1	2.9
Leucine	7.6	7.8	4.2	3.9
Lysine	7.8	6.4	6.7	6.3
Methionine	3.0	1.4	1.9	1.8
Phenylalanine	4.1	5.0	2.5	2.4
Threonine	4.3	3.9	3.1	2.9
Tryptophan	1.2	1.4	0.8	0.8
Valine	5.4	5.5	3.3	3.2

† Fishmeal (anchovy).[29]

‡ Soybean meal (solvent extracted 44% crude protein).[29]

¶ Amino acid requirements according to NRC 2011.[29]

Table S3. Source of data in the meta-analysis datasets.

Study name	Fish species	BSF type	BSF inclusion level in diet (%)	Replacement	Dietary chitin level (% dry matter basis)	Feed production	Available data
Unpublished in-house trial	Atlantic salmon (pre-smolt)	Full-fat BSF larvae meal	20	Fishmeal + Plant protein sources (Partial)	1.6	Extrusion	SGR, FCR, ADCP, PER, FI
		Defatted BSF larvae meal	15	Fishmeal + Plant protein sources (Partial)	1.6	Extrusion	SGR, FCR, ADCP, PER, FI
Randazzo et al. 2021[30]	Rainbow trout	Defatted BSF pupae meal	8-45	Plant protein sources (Partial)	NA	Extrusion	SGR, FCR, FI
Weththasinghe et al. 2021[31]	Atlantic salmon (pre-smolt)	Full-fat BSF larvae meal	8-32	Fishmeal + Plant protein sources (Partial)	0.6-2.6	Extrusion	SGR, FCR, ADCP, PER, FI
		Full-fat BSF larvae paste	5-8 (Dry matter basis)	Fishmeal + Plant protein sources (Partial)	0.4-0.7	Extrusion	SGR, FCR, ADCP, PER, FI
Fisher et al. 2020[32]	Atlantic salmon (pre-smolt)	BSF larvae meal	10-30	Plant protein sources + Animal protein sources (Partial)	NA	Pelleting	SGR, FCR, PER, FI[§]
	Atlantic salmon (reared in freshwater)	BSF larvae meal	30		NA	Pelleting	ADCP
Roques et al. 2020[33]	Rainbow trout	Defatted BSF larvae protein hydrolysate	5-15	Plant protein sources (Partial)	NA	Extrusion	SGR[†], FCR, FI[§]

Melenchón et al. 2021[5]	Rainbow trout	BSF larvae meal	6-11	Fishmeal (Partial)	0.9-1.8‡	Extrusion	SGR, FCR, ADCP, PER, FI§
Józefiak et al. 2019[4]	Rainbow trout	Full-fat BSF prepupae meal	20	Fishmeal (Partial)	NA	Extrusion	SGR, FCR, PER, FI§
Terova et al. 2019[34]	Rainbow trout	Partially defatted BSF prepupae meal	10-30	Fishmeal (Partial)	0.5-1.5	Pelleting	SGR, FCR, ADCP, FI§
Cardinaletti et al. 2019[35]	Rainbow trout	Full-fat BSF prepupae meal	11-21	Fishmeal (Partial)	NA	Pelleting	SGR, FCR, FI§
Belghit et al. 2019[36]	Atlantic salmon (post-smolt)	Partially defatted BSF larvae meal	5-15	Fishmeal (Partial to total)	NA	Extrusion	SGR, FCR, ADCP, FI
Dumas et al. 2018[37]	Rainbow trout	Partially defatted BSF larvae meal	7-26	Fishmeal (Partial to total)	0.2-0.7	Extrusion	FCR, ADCP, FI§
Belghit et al. 2018[38]	Atlantic salmon (pre-smolt)	De-chitinized BSF larvae protein meal	60	Fishmeal + Plant protein sources (Partial)	NA	Extrusion	SGR, FCR, PER, FI
Renna et al. 2017[39]	Rainbow trout	Partially defatted BSF larvae meal	20-40	Fishmeal (Partial)	1.1-2.1	Pelleting	SGR, FCR, ADCP, PER, FI§
Stadtlander et al. 2017[40]	Rainbow trout	Defatted BSF prepupae meal	28	Fishmeal (Partial)	NA	Extrusion	SGR, FCR, PER, FI§

Lock et al. 2016[41]	Atlantic salmon (post-smolt)	BSF larvae meal	5-25	Fishmeal (Partial to total)	NA	Extrusion	SGR, FCR, FI§
Sealey et al. 2011[42]	Rainbow trout	BSF prepupae meal	18-40	Fishmeal (Partial)	NA	Pelleting	FCR, FI§
St-Hilaire et al. 2007[27]	Rainbow trout	BSF prepupae meal	15-30	Fishmeal (Partial)	NA	Pelleting	FCR, FI§

† Not reported. Calculated based on the raw data received from the authors upon request.

‡ Not in dry matter basis.

§ Calculated feed intake.

NA: Not available.

BSF: Black soldier fly (*Hermetia illucens*), SGR: Specific growth rate, FCR: Feed conversion ratio, FI: Feed intake, ADCP: Apparent digestibility coefficient of protein, PER: Protein efficiency ratio.

Table S4. Average chemical composition of black soldier fly (BSF) ingredients included in the meta-analysis.

Study	BSF type	Gross energy (MJ/kg)	Dry matter (%)	Crude protein (%)	Crude lipid (%)	Chitin (%)	Ash (%)
Unpublished in-house trial	Full-fat BSF larvae meal		88	38	30	7.1	5.8
	Defatted BSF larvae meal		87	51	12	9.7	8.0
Weththasinghe et al. 2021[31]	Full-fat BSF larvae meal†		91	42	32	8	9.3
	Full-fat BSF larvae paste†		23	41	34		2.5
Fisher et al. 2020[32]	BSF larvae meal	20	96	56	12		
Melenchón et al. 2021[5]	BSF larvae meal		98	30	34	17	11
Józefiak et al. 2019[4]	Full-fat BSF prepupae meal†		98	40	34		7.1
Terova et al. 2019[34]	Partially defatted BSF prepupae meal†	26	90	49	21	5	8.7
Cardinaletti et al. 2019[35]	Full-fat BSF prepupae meal		79	31	33		10
Belghit et al. 2019[36]	Partially defatted BSF larvae meal			52	18		
Dumas et al. 2018[37]	Partially defatted BSF larvae meal†		92	47	20	2.7	13
Belghit et al. 2018[38]	BSF larvae†		31	34	22		11
Renna et al. 2017[39]	Partially defatted BSF larvae meal†	24	94	55	18	5	7.1
Lock et al. 2016[41]	BSF larvae meal 1	18	97	52	26		10
	BSF larvae meal 2	16	96	58	17		10
St-Hilaire et al. 2007[27]	BSF prepupae meal†		92	44	33		16

† Values are expressed as % dry matter (except dry matter values)

Table S5. Linear and quadratic regressions of effect sizes (Hedges' g) with *p* and adjusted R^2 values for the specific growth rate (SGR), feed conversion ratio (FCR), feed intake, apparent digestibility coefficient (ADC) of protein and protein efficiency ratio (PER) in salmonids fed increasing dietary levels of black soldier fly.

Parameter	Dataset	Regression type	Equation	*p*	R^2
SGR	Full	Linear	$Y = -0.052x + 1.149$	NS	0.05
		Quadratic	$Y = -0.002x^2 + 0.050x + 0.173$	NS	0.04
	Atlantic salmon	Linear	$Y = -0.058x + 0.355$	NS	0.07
		Quadratic	$Y = -0.002x^2 + 0.145x - 0.591$	NS	0.03
	Rainbow trout	Linear	$Y = -0.012x + 1.341$	NS	-0.06
		Quadratic	$Y = 0.006x^2 - 0.314x + 3.915$	NS	-0.001
	Larvae	Linear	$Y = -0.057x + 0.721$	NS	0.08
		Quadratic	$Y = -0.002x^2 + 0.087x - 0.577$	NS	0.09
	Prepupae/pupae	Linear	$Y = -0.030x + 1.887$	NS	-0.10
		Quadratic	$Y = 0.013x^2 - 0.675x + 8.069$	NS	0.22
	Full-fat	Linear	$Y = -0.074x + 0.947$	NS	0.02
		Quadratic	$Y = -0.006x^2 + 0.150x - 0.509$	NS	-0.01
	Defatted	Linear	$Y = -0.060x + 1.777$	NS	0.05
		Quadratic	$Y = -0.003x^2 + 0.135x - 0.210$	NS	0.05
FCR	Full	Linear	$Y = 0.025x - 0.557$	NS	0.004
		Quadratic	$Y = 4.7\times10^{-4}x^2 - 0.003x - 0.292$	NS	-0.02
	Atlantic salmon	Linear	$Y = 0.044x - 0.309$	NS	0.14
		Quadratic	$Y = -0.001x^2 + 0.090x - 0.722$	NS	0.11
	Rainbow trout	Linear	$Y = -0.017x - 0.296$	NS	-0.04
		Quadratic	$Y = -0.005x^2 + 0.219x - 2.364$	NS	-0.02
	Larvae	Linear	$Y = 0.039x - 0.332$	NS	0.07
		Quadratic	$Y = -3.5\times10^{-5}x^2 + 0.041x - 0.351$	NS	0.03
	Prepupae/pupae	Linear	$Y = -0.001x - 0.887$	NS	-0.07
		Quadratic	$Y = -0.011x^2 + 0.528x - 6.245$	NS	0.14

	Full-fat	Linear	$Y = -0.011x + 0.648$	NS	-0.04
		Quadratic	$Y = -0.001x^2 + 0.014x + 0.459$	NS	-0.08
	Defatted	Linear	$Y = 0.047x - 1.665$	NS	0.05
		Quadratic	$Y = 3.9\times10^{-4}x^2 + 0.022x - 1.419$	NS	0.003
Feed intake	Full	Linear	$Y = -0.028x - 1.077$	NS	-0.02
		Quadratic	$Y = 0.004x^2 - 0.272x + 1.268$	NS	0.01
	Atlantic salmon	Linear	$Y = -0.016x - 1.856$	NS	-0.05
		Quadratic	$Y = 0.006x^2 - 0.432x + 1.888$	NS	-0.01
	Rainbow trout	Linear	$Y = -0.055x - 0.101$	NS	-0.02
		Quadratic	$Y = 0.007x^2 - 0.367x + 2.657$	NS	-0.01
	Larvae	Linear	$Y = -0.026x - 0.921$	NS	-0.03
		Quadratic	$Y = 0.004x^2 - 0.293x + 1.465$	NS	-0.02
	Prepupae/pupae	Linear	$Y = -0.024x - 1.554$	NS	-0.07
		Quadratic	$Y = 0.009x^2 - 0.480x + 3.068$	NS	-0.08
	Full-fat	Linear	$Y = -0.141x + 0.293$	NS	0.06
		Quadratic	$Y = 0.012x^2 - 0.627x + 3.915$	NS	0.09
	Defatted	Linear	$Y = 1.1\times10^{-5}x - 1.144$	NS	-0.04
		Quadratic	$Y = 0.002x^2 - 0.102x - 0.126$	NS	-0.09
ADC of protein	Full	Linear	$Y = -0.048x - 0.018$	NS	0.03
		Quadratic	$Y = 0.005x^2 - 0.238x + 1.194$	NS	0.05
PER	Full	Linear	$Y = 0.022x - 0.666$	NS	-0.004
		Quadratic	$Y = -0.002x^2 + 0.152x - 1.954$	NS	0.05

NS: not significant.

Table S6. Linear and quadratic regressions of effect sizes (Hedges' g) with *p* and adjusted R^2 values for the specific growth rate (SGR), feed conversion ratio (FCR), feed intake, apparent digestibility coefficient (ADC) of protein and protein efficiency ratio (PER) in salmonids fed increasing dietary levels of black soldier fly chitin (full dataset).

Parameter	Regression type	Equation	*p*	R^2
SGR	Linear	$Y = -0.307x + 0.647$	NS	-0.06
	Quadratic	$Y = -0.924x^2 + 2.211x - 0.680$	NS	-0.10
FCR	Linear	$Y = -0.424x + 1.549$	NS	-0.03
	Quadratic	$Y = 0.943x^2 - 2.827x + 2.640$	NS	-0.01
Feed intake	Linear	$Y = 0.347x - 0.084$	NS	-0.02
	Quadratic	$Y = -0.908x^2 + 2.808x - 1.267$	NS	0.07
ADC of protein	Linear	$Y = -1.101x + 0.472$	NS	0.12
	Quadratic	$Y = -0.393x^2 - 0.050x - 0.058$	NS	0.06
PER	Linear	$Y = 0.938x - 1.722$	NS	0.02
	Quadratic	$Y = -2.223x^2 + 7.112x - 4.994$	NS	0.20

NS: not significant.

Table S7. Linear and quadratic regressions of effect sizes (Hedges' g) with *p* and adjusted R^2 values for the specific growth rate (SGR), feed conversion ratio (FCR), feed intake, apparent digestibility coefficient (ADC) of protein and protein efficiency ratio (PER) in salmonids fed diets with increasing fishmeal replacement level by black soldier fly.

Parameter	Dataset	Regression type	Equation	*p*	R^2
SGR	Full	Linear	$Y = -0.067x + 1.914$	***	0.35
		Quadratic	$Y = 2.3\times10^{-4}x^2 - 0.086x + 2.097$	***	0.34
	Atlantic salmon	Linear	$Y = -0.050x + 0.879$	*	0.23
		Quadratic	$Y = -0.001x^2 + 0.029x - 0.020$	NS	0.23
	Rainbow trout	Linear	$Y = -0.098x + 2.918$	**	0.43
		Quadratic	$Y = 0.004x^2 - 0.288x + 3.626$	***	0.61
	Full-fat	Linear	$Y = -0.020x + 0.276$	NS	-0.05
		Quadratic	$Y = -0.003x^2 + 0.122x - 0.600$	NS	0.01
	Defatted	Linear	$Y = -0.079x + 2.870$	***	0.54
		Quadratic	$Y = 0.001x^2 - 0.133x + 3.273$	**	0.54
	Larvae	Linear	$Y = -0.053x + 1.210$	**	0.29
		Quadratic	$Y = -4.7\times10^{-4}x^2 - 0.011x + 0.783$	*	0.28
	Pre-pupae	Linear	$Y = -0.131x + 3.800$	**	0.55
		Quadratic	$Y = 0.005x^2 - 0.366x + 4.707$	**	0.74
FCR	Full	Linear	$Y = 0.029x - 0.992$	*	0.11
		Quadratic	$Y = -0.001x^2 + 0.104x - 1.894$	**	0.19
	Atlantic salmon	Linear	$Y = 0.004x + 0.408$	NS	-0.04
		Quadratic	$Y = 1.2\times10^{-6}x^2 + 0.004x + 0.410$	NS	-0.09
	Rainbow trout	Linear	$Y = 0.087x - 2.723$	**	0.38
		Quadratic	$Y = -0.003x^2 + 0.232x - 3.509$	***	0.47
	Full-fat	Linear	$Y = -0.024x + 1.150$	NS	0.07
		Quadratic	$Y = 3.9\times10^{-4}x^2 + 0.009x + 0.684$	NS	0.07
	Defatted	Linear	$Y = 0.055x - 2.528$	**	0.40
		Quadratic	$Y = -0.001x^2 + 0.153x - 3.454$	***	0.50
	Larvae	Linear	$Y = 0.016x - 0.166$	NS	0.03

		Quadratic	$Y = -0.001x^2 + 0.074x - 0.877$	NS	0.07
	Pre-pupae	Linear	$Y = 0.085x - 3.078$	*	0.33
		Quadratic	$Y = -0.004x^2 + 0.276x - 4.208$	**	0.49
Feed intake	Full	Linear	$Y = -0.073x + 0.784$	**	0.14
		Quadratic	$Y = -0.001x^2 - 0.017x + 0.104$	*	0.13
	Atlantic salmon	Linear	$Y = -0.090x + 1.515$	*	0.18
		Quadratic	$Y = -0.002x^2 + 0.113x - 1.200$	*	0.22
	Rainbow trout	Linear	$Y = -0.041x - 0.084$	NS	0.02
		Quadratic	$Y = 0.001x^2 - 0.121x + 0.753$	NS	0.03
	Larvae	Linear	$Y = -0.082x + 1.643$	**	0.20
		Quadratic	$Y = -0.001x^2 + 0.030x + 0.256$	*	0.21
	Prepupae/pupae	Linear	$Y = -0.051x - 0.779$	NS	-0.03
		Quadratic	$Y = 0.005x^2 - 0.279x + 0.564$	NS	-0.01
	Full-fat	Linear	$Y = -0.098x + 0.695$	*	0.18
		Quadratic	$Y = -0.001x^2 - 0.017x - 0.429$	NS	0.17
	Defatted	Linear	$Y = -0.069x + 1.483$	*	0.13
		Quadratic	$Y = -0.001x^2 + 0.031x + 0.503$	NS	0.12
ADC of protein	Full	Linear	$Y = -0.008x - 0.471$	NS	-0.04
		Quadratic	$Y = 4.2\times10^{-6}x^2 - 0.009x - 0.463$	NS	-0.11
PER	Full	Linear	$Y = -0.010x + 0.156$	NS	-0.03
		Quadratic	$Y = 0.001x^2 - 0.105x + 1.302$	NS	0.08

Asterisks denote level of significance (NS: not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

Table S8. Average proximate composition (% dry matter) of differently processed black soldier fly (BSF) larvae and pre-pupae meals.

BSF product	Dry matter (%)		Crude protein		Crude lipid		Ash		Chitin		References
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
(Full-fat) BSF larvae meal	93	2.9 (8)	42	5.5 (9)	27	8.6 (9)	14	3.3 (8)	11	4.4 (4)	[5,31,32,43-47] and unpublished data from an in-house study
Defatted BSF larvae meal	93	2.9 (8)	56	6.8 (10)	14	6.9 (10)	9.3	5.2 (9)	6.8	3.1 (8)	[37,39,48-54] and unpublished data from an in-house study
(Full-fat) BSF pre-pupae meal	89	9.4 (3)	41	2.4 (3)	36	4.9 (3)	12	4.3 (3)			[4,27,35]
Defatted BSF pre-pupae meal	93	4.1 (2)	48	0.7 (2)	12	6.2 (2)	12	5.1 (2)	7.3	3.3 (2)	[34,55]

SD: Standard deviation

Values in parentheses are the number of datapoints used for calculating the mean.

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