Digestibility and functionality of black soldier fly larvae meal in Atlantic salmon feeds

Gopika Radhakrishnan

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
2024

UNIVERSITY OF BERGEN

Digestibility and functionality of black soldier fly larvae meal in Atlantic salmon feeds

Gopika Radhakrishnan

Thesis for the degree of Philosophiae Doctor (PhD)
at the University of Bergen

Date of defense: 09.02.2024

Year: 2024

Title: Digestibility and functionality of black soldier fly larvae meal in Atlantic salmon feeds

Name: Gopika Radhakrishnan

Print: Skipnes Kommunikasjon / University of Bergen

Scientific environment

This Ph.D. project started in October 2020 as a collaboration between the Department of Biology at the University of Bergen (UiB) and the research group feed and nutrition at the Institute of Marine Research in Bergen (IMR). The work for this doctoral thesis was performed under the supervision of Dr. Ikram Belghit (IMR), Dr. Antony J. Prabhu Philip (IMR, current affiliation, NOFIMA) and Dr. Nina Liland (IMR, UiB) at the Institute of Marine Research, Bergen, Norway.

This Ph.D. has been part of the project SUSINCHAIN (SUStainable INsect CHAIN), funded by the European Union's Horizon 2020 research and innovation program, under grant agreement nº 861976.

Financial support in terms of fellowship was provided by the Indian Council of Agricultural Research, New Delhi, India, under Netaji Subhas-ICAR International Fellowship India for a period of 3 years. Financial support for an additional six months was provided by the Institute of Marine Research in Bergen.

Acknowledgements

I am extremely thankful to a lot of people for making my Ph.D. possible! First, I would like to express my gratitude to all my supervisors at Institute of Marine Research, Dr. Ikram Belghit, Dr. Antony J. Prabhu Philip, Dr. Nina Liland for all your excellent guidance, help and support throughout.

I am extremely thankful to Ikram, who has been there with me from the very beginning of my Ph.D. journey till the end. Thank you so much for bearing with me the entire time! You were always there helping me, guiding me, making me things understand, getting things done on time, picking me up when stressed, consoling…and lot more, there is no words to describe how much it meant for me. Thank you so much!

I am deeply indebted to Antony, without whom none of this have occurred at the first place. He has been a truly inspiring and a person to look up to whenever I face problems in research. “Something seems off? he would fix it!”- was being the motto! Thank you so much for clearing my doubts, answering my stupid questions, and putting me on right tract without being judgemental.

I am so thankful and mostly so happy that I had a chance to work with you Nina! You are so joyful and a much welcoming person. Thank you so much for your encouragement, advice, and enormous support for making my PhD successful. You were always easy to approach whenever I had a question or when I needed something. Thank you very much!

Thank you so much Erik-Jan, for your immense support and help that I received when I reached Norway and beyond. You were always ready to help. Thank you very much! A special thanks to Marta, you had always time for me, motivated me during my hard times. I am really happy to have you in IMR. To my ‘Pagal’, Pedro I am really grateful for giving me your time for helping me understand my enormous data that I used to cry upon. Thank you so much for everything!

I would like to express my gratitude to everyone in the fish nutrition group for an amazing working environment. A big thanks to Hui, Amalie, Sarah, Natalia, Joseph, Nawaraj, Kjersti, Ana for making my ‘Lab Life’ most cherishing! I am also thankful to all co-authors and people in Matre and Austevol who have helped me enormously with preparation and running fish experiments and samplings. In my entire Ph.D. journey, I have received a remarkable support from my dears…

Sahar, and Angie! Thanks for being there always and listening to all my ‘Ph.D. crisis’. You people made it easy throughout in the midst of tension!

I would like to thank all my friends, Chandru, Anne, Madhushri, Rita, Chavindi, Alice, Peng, Hoang, Lakshmi, Hari, Ashbin, Shafeek, Maria, Anandhu, Rithwik and many others…, for all the beautiful memories!! Never felt alone. You guys are the best!

My deepest gratitude to Dr. Ashutosh D. Deo, and Dr. Shamna N. for all their prayers and support for applying for Ph.D.

I would like to thank Yashwanth, Sahana, Jyothi, Kushala and Sowndarya for being there during my hard times!

Thank you bigL for being there for me always, supporting me and motivating me!!!! A big thanks to Rama akka, Preethi akka Dhamu sir and Shankar sir for making me feel home! It was more like having family here!!

I want to express my deepest gratitude to my family, Mom, Dad, my kunju sister Sree, for all their support, and prayers throughout. Without them nothing would have happened! A great and elephant part of my Ph.D. lies with them. I love you guys!!!!!

-Gopika-

Table of Contents

Abstract in English

The digestibility and functionality of partially defatted black soldier fly larvae (BSFL) meal has been studied in Atlantic salmon (*Salmo salar*) feeds. Insect meals are a promising alternative to mainstream feed ingredients due to their good protein and amino acid (AA) composition.

The first aim of this thesis (**Paper I**) primarily focused on evaluating the protein and amino acid digestibility in farmed Atlantic salmon using *in vivo* and *in vitro* methods. Salmon and mammalian enzyme sources were considered for studying the *in vitro* protein and AA solubility of BSFL meal. The BSFL meal had a crude protein digestible value >85% and protein solubility of 24%. The digestibility reported >80% for most of the AA and *in vitro* AA solubility values for BSFL meal ranged between 10-44%. A higher solubility for AA was observed with commercial enzymes than fish enzymes. Furthermore, the study attempted a comparison between the *in vivo* and *in vitro* methods to assess the suitability of *in vitro* methods. A high correlation (though not significant) between *in vitro* and *in vivo* protein digestibility in BSFL meal was observed using salmon enzyme but not at the AA level.

Subsequently, **Paper II** investigated the performance, survival, health and welfare of farmed Atlantic salmon under production relevant conditions. The trial was performed in open sea cages, exposed to natural stressors resembling a realistic commercial farm condition. The fish grew from ~ 0.2 kg to harvest size ~4.5-5 kg. Atlantic salmon fed BSFL meal at 5% reduced the plasma cortisol in salmon under chronic stress (pathogen infestation and environmental stressors) but without dietary effect during delousing stress. Furthermore, BSFL 5% diet also improved the blood profile, and altered the skin mucosal characteristics. Nevertheless, an increased response of pro-inflammatory genes was observed in the same dietary group, underlining an activation of innate immune response in salmon fed dietary BSFL meal. Moreover, the BSFL-based diet did not have negative effect on liver function, instead reduced the plasma aspartate aminotransferase, and alanine aminotransferase when fed BSFL 5%. Thus, inclusion of dietary BSFL at 5% had a positive immunomodulatory effect in Atlantic salmon.

Paper III investigated the dietary effects of BSFL meal on fillet quality of farmed Atlantic salmon from paper II. In general, inclusion of dietary BSFL meal up to 10% did not compromise the fillet physical, chemical, nutritional or sensory attributes. No dietary effect was observed on fillet color and texture. However, it was noticed that salmon fillet irrespective of dietary group had a higher yellowness index which was assumed to be due to fillet storage duration. Furthermore, BSFL 10% diet influenced the nutritional quality indices of salmon, though it was marginal. Also, a higher level of zinc and iron was observed in the salmon fillet fed BSFL 10%. The sensory analysis conducted on salmon fillet showed that dietary BSFL did not significantly affect the overall 'liking' of fillet.

This thesis also attempts to investigate the sustainability of insect-based salmon feeds based on carbon footprint. The analysis showed that BSFL-based diet had a lower carbon footprint (CO_2e per kg feed), when replaced plant protein sources (soy protein concentrate (SPC) and guar meal). Using the CO_2 equivalent values for SPC, guar protein and insect meal we could achieve a relative reduction of 0.15 to 0.35kg CO_2 equivalent per kg feed. This corresponds to a reduction of 6-15% in CO_2 emissions relative to 2.3kg CO_2 equivalent per kg feed in present Norwegian salmon farming. Thus, replacement up to 10% can reduce the carbon emission contributed from feed which can subsequently improve the overall sustainability of salmon feed in terms of carbon emissions.

Overall, dietary inclusion of BSFL meal at 5% inclusion improved the innate immune responses and up to 10% in Atlantic salmon feed is possible without significant negative effects on digestibility, health and fillet quality.

Abstract in Norwegian

Fordøyeligheten og funksjonaliteten til delvis avfettet mel av svarte soldatfluelarver (BSFL) er studert i fôret til atlantisk laks (*Salmo salar*). Insektmel er et lovende alternativ til konvensjonelle fôringredienser på grunn av deres gode protein- og aminosyre (AA)-sammensetning.

Det første målet i denne avhandlingen (Artikkel I) fokuserte primært på å evaluere protein- og aminosyrefordøyeligheten hos oppdrettet atlantisk laks ved hjelp av *in vivo*- og *in vitro*-metoder. Laks- og pattedyrenzymer ble vurdert for å studere *in vitro* løselighet av proteiner og AA i BSF-mel. BSFL-mel hadde en fordøyelig verdi for råprotein >85% og en protein løselighet på 24%. Fordøyeligheten ble rapportert som >80% for de fleste AA, og *in vitro*-løselighetsverdiene for AA i BSFL-mel varierte mellom 10-44%. Det ble observert høyere løselighet for AA med kommersielle enzymer enn med fiskeenzymer. Videre forsøkte studien å sammenligne *in vivo*- og *in vitro*-metodene for å vurdere egnetheten til *in vitro*-metodene. Det ble observert en høy korrelasjon (dog ikke signifikant) mellom *in vitro*- og *in vivo*-fordøyelighet av proteiner i BSFL-mel, men ikke på nivået av AA.

Deretter undersøkte Artikkel II prestasjon, overlevelse, helse og velferd hos oppdrettet atlantisk laks under produksjonsrelevante forhold. Forsøket ble utført i åpne merder i sjø, eksponert for naturlige stressfaktorer og dermed lignende realistiske forhold på en kommersiell gård. Fisken vokste fra ~ 0,2 kg til høstingsstørrelse ~4,5-5 kg. Atlantisk laks som ble fôret med BSFL-mel ved 5% hadde redusert plasmakortisol under kronisk stress (patogeninfeksjon og miljøstressfaktorer), men uten diettvirkning under avlusningsstress. Videre forbedret BSFL 5% dietten også blodprofilen og endret egenskapene til hudens mukosa. Imidlertid ble det observert økt respons av proinflammatoriske gener i den samme dietten, noe som understreker aktivering av den medfødte immunresponsen hos laks som ble fôret med BSFL-mel. Videre hadde BSFL-basert diett ingen negativ effekt på leverfunksjonen; i stedet reduserte den plasmakonsentrasjonen av aspartataminotransferase og alaninaminotransferase hos laks

fôret med BSFL 5%. Dermed hadde inkludering av BSFL-mel i kosten ved 5% en positiv immunmodulerende effekt på atlantisk laks.

Artikkel III undersøkte de diettrelaterte effektene av BSFL-mel på vurdering av filetkvalitet hos oppdrettet atlantisk laks under produksjonsrelevante forhold. Generelt sett kompromitterte inkluderingen av BSFL-mel i dietten opp til 10% ikke fysisk, kjemisk, næringsmessig eller sensorisk attributter for fileten. Det ble ikke observert effekter av diett på filetfarge eller -tekstur. Imidlertid ble det lagt merke til at uavhengig av dietten hadde laksefileten en høyere gulfargeindeks, antatt å skyldes lagringsvarighet av fileten. Videre påvirket BSFL 10% dietten de ernæringsmessige kvalitetsindikatorene til laks, selv om det var marginalt. Det ble også observert høyere nivå av sink og jern i laksfileten fôret med BSFL 10%. Sensorisk analyse utført på laksefileten viste at en diett med BSFL ikke påvirket betydelig den sensoriske kvaliteten.

Denne avhandlingen forsøkte også å undersøke bærekraften til insektbaserte lakseför basert på karbonavtrykk. Analysen viste at dietten basert på BSFL hadde et lavere karbonavtrykk (CO2e per kg fôr) sammenlignet med planteprotein kilder (soyaproteinkonsentrat (SPC) og guarmel). Ved å bruke CO2-ekvivalentverdiene for SPC, guarpotein og insektmel, kunne vi oppnå en relativ reduksjon på 0,15 til 0,35 kg CO2-ekvivalent per kg fôr. Dette tilsvarer en reduksjon på 6-15% i CO2-utslipp sammenlignet med 2,3 kg CO2-ekvivalent per kg fôr i dagens norske lakseoppdrett. Dermed kan erstatning opp til 10% redusere karbonutslippet fra fôr, noe som deretter kan forbedre den generelle bærekraften til laksefôr med hensyn til karbonutslipp.

Totalt sett forbedret diettinntak av BSFL-mel ved 5% inkludering de medfødte immunresponsene, og opp til 10% i fôret til atlantisk laks er mulig uten betydelige negative effekter på fordøyelighet, helse og filetkvalitet.

List of Publications

Paper I

Radhakrishnan, G., Silva, M.S., Lock, E.J., Belghit, I. and Philip, A.J.P., 2022. Assessing amino acid solubility of black soldier fly larvae meal in Atlantic salmon (*Salmo salar*) *in vivo* and *in vitro*. *Frontiers in Physiology*, 13, p.2439. https://doi.org/10.3389/fphys.2022.1028992

Paper II

Radhakrishnan, G., Liland, N.S., Koch, M.W., Lock, E.J., Philip, A.J.P. and Belghit, I., 2023. Evaluation of black soldier fly larvae meal as a functional feed ingredient in Atlantic salmon (*Salmo salar*) under farm-like conditions. *Frontiers in Aquaculture*, 2, p.1239402. https://doi.org/10.3389/faquc.2023.1239402

Paper III

Radhakrishnan, G., Philip, A.J.P., Caimi, C., Lock, E.J., Araujo, P., Liland, N.S., Rocha, C., Cunha, L.M., Gasco, L and Belghit, I. 2023. Evaluating the fillet quality and sensory characteristics of Atlantic salmon (*Salmo salar*) fed black soldier fly larvae meal in open sea-cages.

Submitted manuscript to aquaculture reports.

Abbreviations

AA	Amino acids
ADC	Apparent digestibility coefficient
ALT	Alanine transaminase
AMP	Antimicrobial peptides
AI	Atherogenicity index
AST	Aspartate aminotransferase
APD	Apparent protein digestibility
BSFL	Black soldier fly larvae
CP	Crude protein
CL	Crude lipid
DH	Degree of hydrolysis
DHA	Docosahexaenoic acid
DPA	Docosapentanoic acid
DM	Dry Matter
EAA	Essential amino acids
EPA	Eicosapentaenoic acid
ELISA	Enzyme-linked immunosorbent assay
FA	Fatty acids
FAA	Free amino acids
FCR	Feed conversion ratio
FFA	Free fatty acids
FM	Fish meal
FO	Fish oil
HCl	Hydrochloric acid
Hb	Hemoglobin
HPI	Hypothalamic-pituitary-interrenal axis
LC-PUFA	Long chain polyunsaturated fatty acids
il1β	Interleukin 1 *β*
NQC	Norwegian quality cut
n-3	Omega 3
n-6	Omega 6
MUFA	Monounsaturated fatty acids
MW	Molecular weight
NaOH	Sodium hydroxide
NQC	Norwegian Quality Cut
PUFA	Polyunsaturated fatty acids
RBC	Red blood cell
RPD	Relative protein digestibility
SBM	Soybean meal
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SFA	Saturated fatty acids
sMOS	Specific mannan-rich yeast parietal fraction

SPC	Soy protein concentrate
TI	Thrombogenicity index
WHC	Water holding capacity

1. Introduction

1.1 Aquaculture: significance and production

By 2050, the human population is expected to reach around ten billion people (FAO 2020). This growth will lead to a substantial increase in the demand for food, creating an estimated food gap of roughly 65% and a protein gap of 76% that we must address by then (Searchinger *et al*.,2019, UN 2020). Fish and other aquatic foods are valuable providers of essential amino acids (EAA), omega-3 fatty acids, minerals and vitamins (Hicks *et al*.,2019, Golden *et al.,* 2021, Zamborain-Mason *et al*., 2023). The aquaculture sector, which is growing at a rate of 5.7% per year, is one of the fastest growing food production sectors, contributing 56% to the global production of aquatic food in 2020 (FAO 2020). The production has been steadily increasing from 115.9 MT in 2018 to 122.6 MT (live weight) in 2020 and projected to reach 140 MT by 2050 (FAO 2022). Hence, capable of bridging approximately 1% of the projected "food gap" and 14% of the anticipated "animal protein gap" by 2050 (Searchinger *et al*., 2014, UN 2022a).
Aquaculture production can vary significantly among and within the regions and on the species cultured. Asia shares the highest contribution to global aquaculture (61.9 %) and Oceania being comparatively lowest (1%). In 2020, grass carp (*Ctenopharyngodon Idella*) emerged as one of the leading finfish species with a production volume of 5.8 MT, constituting 11.8% of global inland aquaculture. Similarly, Atlantic salmon (*Salmo salar*) stood out as another prominent species, producing 2.7 MT and accounting for a substantial 32.6% of all finfish species in marine and coastal aquaculture (FAO 2022).

1.1.1 Atlantic salmon aquaculture

Atlantic salmon is the dominant farmed species in Norwegian aquaculture, with an annual traded volume of close to 1.4 MT (data from 2020; Directory of Fisheries, 2021). Norway alone contributed more than half (51%) (Iversen *et al*., 2020) of global salmon production followed by Chile (25 %). Thus, such intensification of salmon aquaculture needs to be developed sustainably without harming existing ecosystems or the natural resources required for aquaculture production (Boyd *et al*., 2020). However, one of the major sustainability threats in salmon farming is in the utilization of feed and its

ingredients relative to the salmon produced (Aas *et al*., 2019, Cadillo-Benalcazar *et al*., 2020, Pelletier *et al*., 2009, Ytrestøyl *et al*., 2015). In 2020, a total of 1.98 MT of feed ingredients were used to produce 1.4 MT of salmon (Aas *et al.*, 2022), where ~92 % of the feed ingredients were imported from outside Norway (Statistics Norway, 2022), creating a high dependency on protein ingredients with higher footprint for salmon feed.

1.1.2 Ingredients used in salmon feeds

Salmon requires a high-quality diet containing ingredients sourced from targeted marine finfish species in the form of fish meal (FM) and fish oil (FO) into salmon feed. Levels of FM and FO in salmon feeds are now approximately 20% of total feed inclusion compared with 90% around 20 years ago (Ytrestøyl *et al*., 2015). These sources can provide high dietary protein, EAA and poly-unsaturated fatty acids (PUFA). However, over the years, the exclusive use of these ingredients in the feed made them expensive and unsustainable, thereby making aquaculture, including salmon production globally challenging (Lu, 2022). Thus, diversifying the protein sources was one of promising solution to address this challenge and improve the efficiency and sustainability of salmon farming (Norwegian Research Council 2008; Norwegian Scientific Committee for Food Safety 2009; Waagbø *et al*. 2001). In this regard, the composition of salmon feed faced gradual shift from these marine-based to plant-based ingredients, thereby reducing the dependency on FM from ~66 % in 1990 to as little as ~12 % in 2022 (Aas *et al*., 2022b) (Fig. 1). Soy proteins (~19 %), rapeseed and camelina oil (~20 %) and wheat gluten (~18 %) are the dominant plant sources used in salmon feed (Fig. 1, Aas *et al*., 2016). The advantage of including plant sources into the diet lies in their year-round availability and relatively lower cost compared to FM and FO, while still providing the necessary nutritional composition for a salmon diet. Regardless of these advantages, major limitations related to plant sources were the presence of antinutritional factors and high amount of plant matter such as starch (Gillund and Myhr, 2010). Again, some of the main problem identified with soy proteins is associated with the higher crop land use change emissions (arising from soybean production) (MacLeod *et al*., 2020).

Therefore, Norwegian government aims to increase the sustainability of the sector through the production of sustainable feed and that all feed for the aquaculture industry must be from sustainable sources by 2030 (Norwegian Ministry of Climate and Environment 2021). Hence further search for novel feed ingredients were undertaken by the scientific community and aquafeed industry for replacing or substituting the marine and plant-based ingredients in salmon feed. Selection of novel or alternative feed ingredients should fulfil certain criteria such as to meet the nutritional requirement of farmed species, better digestibility, growth and welfare of fish, cost effectiveness, sustainability and satisfy the basic principles of circularity (Barasa *et al*., 2022). Further, the composition of the feed used in salmon farming plays a significant role in contributing to the carbon footprint of salmon production (Winther et al., 2017). Hence, shifting ingredients from marine/terrestrial to novel ingredients can help in minimising carbon footprint of feed. Thus, some of the alternative ingredients identified were algae, fungi, seaweeds, molluscs and insects (Maulu *et al*., 2022). Moreover, the use of these ingredients should be approved by the regulations for its inclusion into the salmon feed.

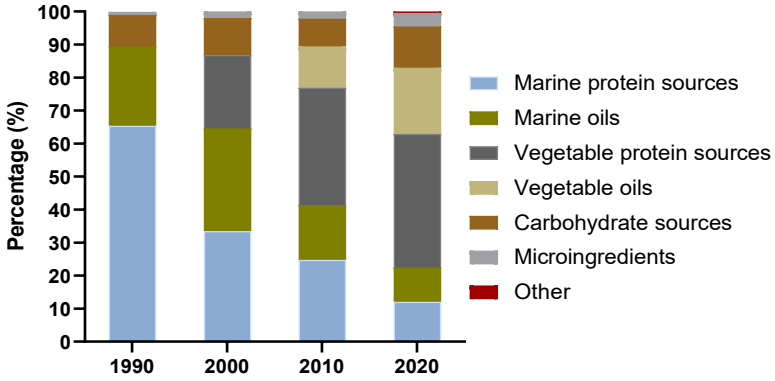


Figure 1: Sources of feed ingredients (% of feed) in Norwegian salmon feed from 1990 to 2020, adapted from Aas *et al*., 2022.

1.1.3 Insects in fish feed

Among the studied alternative ingredients, insects gained widespread attention in its use in fish feeds (Fig. 2) due to its high protein, good profile of amino acids (AA), vitamins and minerals (Mousavi *et al*., 2020). Moreover, insects have been a part of the natural diet of different carnivorous and omnivorous fish species (Whitley and Bollens, 2014). Insects can be used as a major source of protein as well as functional feed ingredient in fish diet due to the presence of various immunostimulants and bioactive compounds (e.g., chitin, lauric acid and antimicrobial peptides) (van Huis and Gasco, 2023). Several studies investigated the effect of replacement of the traditional protein sources with insect-based proteins in different fish species such as seabass, salmon, rainbow trout as shown in Fig. 2 (Abdel-Tawwab *et al*., 2020; Belforti *et al*., 2015; Belghit *et al*., 2019a; Belghit *et al*., 2018; Chemello *et al*., 2020; Gasco *et al*., 2020; Iaconisi *et al*., 2019; Sogari *et al*., 2019).

Insects and insect-derived products entered the European market since first being acknowledged as a valuable protein source for feed and food production at the end of the 2010s. In 2017, the European Union approved the use of several insect species such as black soldier fly (*Hermetia illucens*), common housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), banded cricket (G*ryllodes sigillatus)* and field cricket (*Gryllus assimilis*) (Commission Regulation (EU) 2017/893) for its use in aquafeed. Today, the aquafeed market consumes approximately 50% of European animal feed containing insects and this is expected to rise to 75% by 2030 (IPIFF, 2021). The commercial production of insect proteins in Europe is expected to reach 60,000 tonnes by 2025 and 200,000 tonnes by 2030. However, one of the key challenges faced by the insect sector to enter the aquaculture feed market is the small production volumes and unfavorable market economics in replacing traditional protein sources with an expensive insect protein (Gasco *et al*., 2023). In general, insects in fish feed can be beneficial to aquaculture industry as a potential alternative ingredient by improving growth and health of fish.

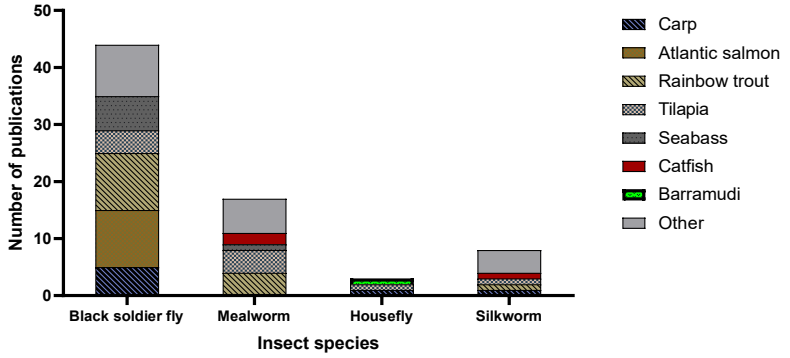


Figure 2: Number of publications in different fish species with different insect species (Adapted from Tran *et al*., 2022, from 1965 to 2021).

1.1.4 Insects in salmonid feed

In salmonids, such as in rainbow trout (*Oncorhynchus Mykiss*), dietary inclusion levels up to 50% of yellow mealworm larvae (Gasco *et al*., 2014) and silkworm pupae (up to 50% inclusion) (Dheke, 2013) have been successful. However, dietary inclusion of 9.2% of maggot meal in rainbow trout was not very successful as it affected fish growth and the fillet quality (St-Hilaire *et al.*, 2007). Dietary inclusion levels of black soldier fly (BSF) prepupae have shown a similar weight gain to that of fish fed FM when fed at 15% (St-Hilaire *et al.*, 2007) or 18–36% inclusion in rainbow trout (Sealey *et al*., 2011), and 5–25% inclusion in Atlantic salmon, where the BSF diets were supplemented with AAs (Lys and Met) (Lock *et al*., 2016). Thus, in general the dietary inclusion of insect meal up to 20% to 30% in salmonids diets is possible without any adverse effects in fish growth but a meta-analysis has shown that an inclusion level exceeding 25% can reduce the crude protein digestibility (Liland *et al*., 2021). Also, partial inclusion of dietary insect meal (silkworm pupae, BSF prepupae) at 10–50% in the diet of fish does not affect the fatty acids (FA) profiles, aroma or flavor enough to be detected by consumers (Nandeesha *et al*., 2000; Sealey *et al*., 2011). The feeding trials in Atlantic salmon and rainbow trout with BSF have shown promising results for maintaining similar growth performance, digestibility, health and fillet quality compared to the standard aquafeeds (Belghit *et al*., 2019a; Belghit *et al*., 2018; Dumas

et al., 2018; Fisher *et al*., 2020) (Table 1). Recent studies on growth performance have indicated that the optimal specific growth rate (SGR) for salmonids is achieved at a dietary inclusion of 100 g/kg BSFL meal in feed and has reported to reduce SGR above the optimal level (compared to FM-based control diets) (English *et al*., 2021). However, the dietary inclusion of defatted BSFL meal up to 300 g/kg (Terova *et al*., 2019) and full-fat BSFL meal up to 210 g/kg (replaced FM at 50%) is suitable in rainbow trout feeds (Cardinaletti *et al*., 2019). In a recent study performed on salmon fry and pre-smolts reported that the replacement of FM with full fat BSFL meal at 5 % and 10 % inclusion did not negatively impact salmon growth, feed utilization, digestibility, or health. However, the inclusion of BSFL at 15 % reduced the body weight, protein lipid efficiency ratio and increased the feed conversion ratio (FCR) of the fish (Mikołajczak *et al*., 2023) (Table 1).

Table 1: Dietary effects of black soldier fly larvae (BSFL) meal on digestibility, growth, health and fillet quality in Atlantic salmon

BSFL inclusion level (g/Kg)	Duration of experiment	Growth and digestibility	Health	Fillet	References
BSFM1 100-250* BSFM2 50-250*	Nd	No negative effects until 100 % inclusion of BSF2	No signs of intestinal hyper-vacuolisation with BSFL incorporation	No changes in the sensory analysis	Lock et al. 2016
BSFM 600 BSFO 120	56 days	No effects on growth performances, Reduced ADC of CP, CL and AA	Proteinase activity was similar, but leucine aminopeptidase activity was lower; Reduced enterocyte steatosis in the proximal intestine; Similar expression of genes indicative of stress response, immune	Nd	Belghit et al., 2018; Li et al., 2019a
BSFM 49-147	112 days	Similar ADC of protein, lipids, and AA	No effects on liver lipid accumulation; Similar digestive enzyme activities; Increase microbial richness and diversity	Small changes in the fillet sensory quality; did not impair the physico-chemical quality; increased n-3 polyunsaturated fatty acids; no effects on the volatile organic profile	Belghit et al., 2019; Li et al., 2020b; Bruni et al., 2020a
BSFM 100-300	112 days	Reduced specific growth rate, and increased protein efficiency ratio and feed conversion ratio (300 g/kg)	Nd	Nd	Fisher et al., 2020
BSFM 80-323 BSFP 198-351	49 days	Increased growth rate, feed conversion ratio, ADC of protein and lipid, protein efficiency ratio and lipid retention	Improved distal intestine histology and plasma lysozyme; Reduced enterocyte steatosis in pyloric caeca; Increased IFNg and reduced IgM in distal intestine; Increased plasma antioxidant capacity	Nd	Weththasinghe et al., 2021a and b
BSFM 100 BSFD 100 BSFF 100	75 days	No negative effects on growth performances	No adverse effects on gut histology; Gut microbiome altered by BSF inclusion and the processing	Nd	Leeper et al., 2022
BSFM 50-100	395 days	No negative effects on growth or mortality	Positive response on the general skin mucosal, hematological, and gene expression profiles	Nd	Radhakrishnan et al., 2023

*Different nutrient isolation and processing techniques were used to produce two types of insect meals (Lock et al., 2016). Nd= not determined; ADC= apparent digestibility coefficient; BSFM= black soldier fly meal; BSFO = black soldier fly oil; BSFP = black soldier fly paste; BSFD = black soldier fly dechitinated; BSFF = black soldier fly fermented; CP= Crude protein; CL = crude lipid; AA = amino acids; IFNg = interferon gamma; IgM = immunoglobulin M. Note that the Table represents some examples on the dietary effects of BSFL meal in Atlantic salmon, and further publications on this topic can be found.

1.1.5 Black soldier fly larvae

Black soldier fly (*Hermetia illucens L.*) is a dipteran species, native to American continent but now widely spread across the continents (Brammer and von Dohlen, 2007). The BSF has a short life cycle having 5 stages namely, egg (4 days), larvae (18 days), prepupae (14 days), pupae and adult (9 days) (De Smet *et al*., 2018). The Black soldier fly larvae (BSFL) are scavengers feeding on a wide range of organic substrates; however, adults do not feed and rely on the fat storage from the larval stage. These species can withstand extreme environmental conditions such as dehydration, food shortage and oxygen deficiency (Diener *et al*., 2011).

Nutritional composition of black soldier fly larvae: The black soldier fly larvae have high protein (30 - 63% on a DM basis) and good profile of EAA, vitamins and minerals, depending on the rearing substrate for the larvae (Liland *et al.*, 2017; St-Hilaire *et al.*, 2007) and protein quantification method (Eggink *et al*., 2022a). The AA profile of BSFL matches with the profile of FM (Fig. 3a) (Tschirner and Simon, 2015). Various minerals including iron, zinc, phosphorous, copper and magnesium have been reported from BSFL (Dierenfeld and King, 2008). Minerals such as calcium, potassium and sodium were found in lower concentrations in all insect species including BSFL (Rumpold and Schlüter, 2013), however exception is seen for house fly larvae (Hwangbo *et al*., 2009).

The lipid content in BSFL can vary between 11-47% (DM) and consists of 58-72% of saturated fatty acids (SFA) with lauric acid accounting for around 28-50% and 19-40% monounsaturated fatty acid (MUFA) and PUFA, respectively (Fig. 3b) (Kroeckel *et al*., 2012). The most stressed limitation in dietary inclusion of BSFL comes with the level of SFA which in higher amounts can affect the growth of carnivorous fish species. Also, a lack of n-3 PUFA in BSFL can modulate the FA content in fillet and may even affect the nutritional indices of salmonid fillet (St-Hilaire *et al.*, 2007). Nevertheless, this FA composition of BSFL can be modified based on their feeding substrate. Studies reported an increased omega-3 fatty acids (such as eicosapentaenoic acid; EPA) in BSFL when fish offal or seaweeds was included in the larval diet (St-Hilaire *et al*., 2007a; Liland *et al*., 2017).

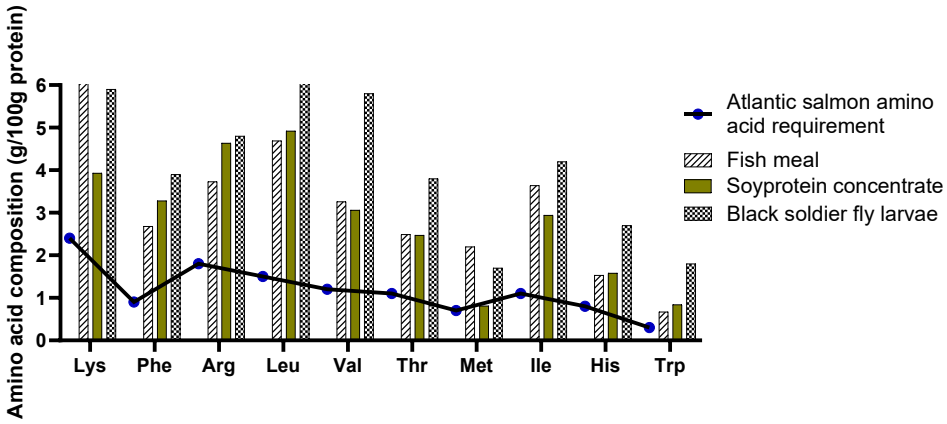


Figure 3a: Available essential amino acid composition (g/100 g protein) of fishmeal (herring, mechanically extracted; International feed number:5-02-000) (NRC, 2011), soy protein concentrate (NRC, 2011) and black soldier fly larvae in fish feed and salmon amino acid requirements (Weththasinghe *et al*., 2020; Fisher *et al*., 2020; Renna *et al*., 2017; Janssen *et al*., 2017; Melenchon *et al*., 2021; Liland *et al*., 2017; Lalander *et al*., 2018; Smets *et al*., 2020; Abdel-Tawwab *et al*., 2020; Belghit *et al*., 2019; Cummins *et al*., 2017; De Marco *et al*., 2015; Devic *et al*., 2018; Dietz *et al*., 2018; Veldkamp *et al*., 2015; Finke, 2013, NRC, 2011).

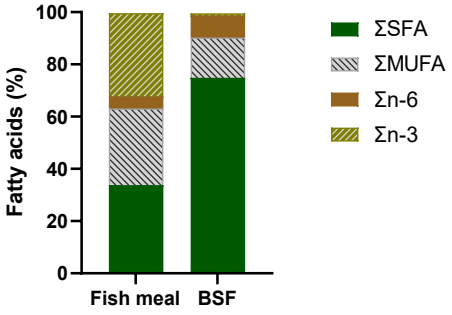


Figure 3b: Fatty acid profile (% of total fatty acids) of fishmeal and black soldier fly (Adapted from Tran *et al*., 2022).

1.2 Digestibility

1.2.1 Digestion and absorption of nutrients in salmon

Atlantic salmon is a carnivorous fish with a well-structured digestive system, divided into mouth, buccal cavity, esophagus, stomach, intestine, anus and associated organs (Sahlmann, 2013). After ingestion, the food enters a short muscular elastic tube called the esophagus. As the food leaves the esophagus, it enters a muscular bag like structure called stomach which secretes acids and enzymes. The stomach produces hydrochloric acid (HCL), which initiates digestion by denaturing the proteins, with the help of an enzyme called pepsin. The acidic environment (pH 1-5) created by HCL inside the stomach converts inactive pepsinogen to active pepsin. The stomach is followed by numerous blind tubules called pyloric caeca which increases the area of absorption and facilitates digestion (Krogdhal *et al*., 2015). The pyloric caeca are connected to the intestine, which can be divided into proximal, medial and distal. The proximal intestine possesses pseudo villi to increase the area of absorption by ~20 fold with the help of enterocytes. The medial intestine digests and absorbs the protein molecules through the process called endocytosis. The distal intestine is often differentiated as rectum with short microvilli and numerous mitochondria. Exocrine pancreas is an associated organ which produces and stores digestive enzymes such as trypsin, chymotrypsin, lipase, amylase, elastase, carboxypeptidase, DNAase and RNAase. The liver located in the peritoneal cavity secretes bile which is temporarily stored in the gall bladder. The bile is released into the intestinal lumen when the chyme enters the proximal intestine mainly for digesting the fat (Hardy and Kaushik, 2021).

1.2.2 Digestive proteases

The digestive proteases can be classified into endoproteases (breaks the peptide bond within) and exoproteases (breaks the peptide bond at the end of polypeptide chain). The acidic endoproteases such as pepsin is released from oxyntopeptic cells following the secretion of HCl (Fig.4). The gastric protease secretion is simulated by the distension of stomach wall as food enters the stomach or through external stimulus as reported in mammals. In salmon, pepsin release is rapid and peaks within one hour of feeding in pre-starved salmon (Einarsson *et al.* 1996). Followed by gastric digestion, as food enters

small intestine, the intestinal proteases along with sodium bicarbonate are released from pancreatic like tissues associated with fat surrounding the pyloric caeca (Einarsson & Davies 1996). The secretion is stimulated by the presence of digestion products of both fat and proteins (Liddle 2000). The major intestinal enzymes include trypsin and chymotrypsin belonging to class of serine proteases (Stryer 1988). Other enzymes include carboxypeptidase A and B and elastase. The presence of AA, such as tryptophan, phenylalanine (Liddle 2000), and lysine (Grendell & Rothman 1981) stimulates the secretion of trypsin.

1.2.3 Absorption of peptides and amino acids

The major site of AA and peptide absorption happens across the brush broader epithelium cells in the intestine of salmon. The digested proteins are absorbed as AA or peptides via different mechanisms. These include *i)* passive diffusion *ii)* carrier mediated independent of ion gradient *iii)* energy dependent carrier mediated transport coupled with ion gradients (Collie &Ferraris 1995) and *iv)* endocytosis of intact low molecular weight protein sources across distal parts of intestine (Sire & Vernier 1992). In salmon, passive diffusion accounts for a major uptake of AA and peptides via the brush border epithelium of intestine (Bakke-McKellep *et al.* 2000). Pyloric caeca occupy major part of the intestine, and it was reported that in salmon, 59% of AA in commercial salmon feed is digested and absorbed by the proximal intestine (Krogdahl *et al.* 1999). The absorbed AA and peptides are released into blood stream via the hepatic portal system (Ash 1985). A simple conceptual diagram of the whole process is summarised in Fig.4.

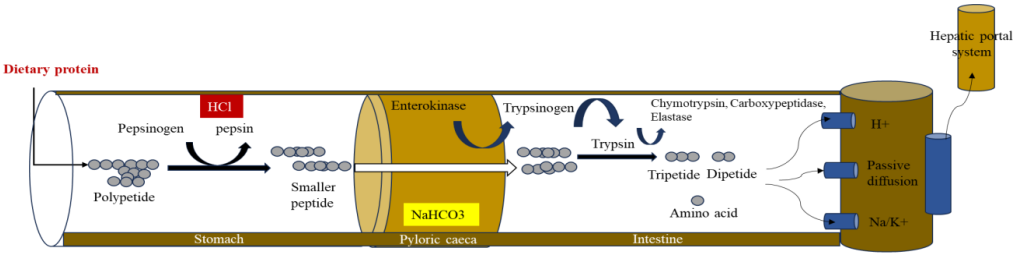


Figure 4: A conceptual diagram of digestion and absorption of dietary proteins in the gastrointestinal tract of fish. HCl = Hydrochloric acid; NaHCO3 = Sodium Bicarbonate; Na/K+ = Sodium-potassium pump.

1.2.4 Methods to study digestibility

The term digestibility is often defined as the amount or proportion of nutrients or categories of nutrients that disappear from the meal as it passes through the digestive system and is excreted in feces (NRC 2011). Bioavailability (biological availability) is a term used to describe the proportion of a nutrient in food that is utilized for normal body functions (Fairweather-Tait and Southon 2003).

In vivo digestibility. Digestibility can be determined by direct (Edin, 1918) and indirect methods (De la Noüe and Choubert, 1986). In a direct method, the digestibility is measured by feeding the fish with known quantity of feed and collecting the total amount of feces later (Cho, 1979; Hajen *et al.*, 1993). The in direct method uses an inert, non-toxic, indigestible marker such as chromic oxide (Cr_2O_3) or yttrium oxide (Y_2O_3) that are added to the feed and passes through the digestive tract along with the feed. This method assumes that the feces samples collected are representative of the feces collected over a period. The indirect method is the most widely used method to study digestibility (Hardy and Kaushik, 2021).

To determine digestibility of BSFL meal through indirect method, it involves a nutritionally balanced diet named as “reference diet” and a “test diet” which are formulated and extruded to study the digestibility of ingredients. The reference diet is the standard diet fed to the species of interest and test diet includes the ingredient to be tested combined with reference diet in 70:30 or 80:20 ratio respectively. This is because most of the feed ingredients cannot be fed alone, and it also ensures normal digestion and sufficient fecal materials for analysis. These *in vivo* feeding trials are the most ideal method used to determine ingredient digestibility. However, this demands a lot of time, labor and most importantly a large number of fish for experiments.

1.2.5 Digestibility of black soldier fly larvae and diets containing BSFL meal

The apparent digestibility coefficient (ADC) of dry matter, crude protein and lipid of BSFL as an ingredient was 87%, 89% and 97% respectively when fed for salmon parr in freshwater phase (Fisher *et al*., 2020). In rainbow trout, the ADCs of dry matter, crude protein and lipid of BSFL as an ingredient was 69%, 85%, and 43% respectively fed for a period of 12 weeks. The ingredient ADCs of amino acid ranged between 57-

96% (Dumas *et al*., 2018). The ADC of protein was high with 88% and digestibility AA was above 80%, but the of lipid and dry matter digestibility in BSFL based diet was relatively low at 73% and 75%, respectively (Dumas *et al*., 2018). In pre-smolt Atlantic salmon, the ADC of protein, lipid and all AA in the diets were significantly reduced when 85% of the dietary FM and soy protein concentrate (SPC) were replaced with BSFL meal when fed for a period of 8 weeks (Belghit *et al*., 2018).

1.2.6 Use of *in vitro* methods to study nutrient digestibility in fish

In 2015, an estimated 192 million animals were reported to have been sacrificed in experiments worldwide (Taylor and Alvarez 2019). According to the data by Eur-Lex (2022), in the whole EU and Norway, fishes were the second most used in animal experiments (24.6%) after mice (52.5%). Given the large-scale commercial production of Atlantic salmon in Norway, the number of fishes in experiments is high (Knudsen *et al*. 2005) with 10.6 million salmon, in 2016. Thus, the sustainable use of animals and their welfare has become an increasing concern in general public and scientific community (Balcombe, 2016; Message and Greenhough, 2019). Therefore, the 3Rs principles (Replacement, Reduction and Refinement) were implemented to promote animal welfare and to find alternatives for live animals used in the research (Grimholt *et al.*, 2009). Hence, *in vitro* methods align well with the principles of the 3Rs, offering cost-effectiveness, and less time compared to *in vivo* approaches, making them suitable for preliminary screening of ingredients. Therefore, to perform *in vitro* studies in fish, it is important to understand how nutrients are digested and absorbed inside salmon gut.

Digestive systems in fish are biological reactors and membranes to digest food and extract nutrients. Thus, different static and dynamic *in vitro* models were developed under this principle to study the digestibility of nutrients by simulating the physiological conditions of fish gastrointestinal tract using laboratory equipment (Moyano *et al.*, 2015). Different analytical methods are used to evaluate the *in vitro* digestibility of ingredients in fish. These include pH-stat method (Dimes and Haard, 1994; Tibbetts *et al.*, 2011), pH drop (Ezquerra *et al.*, 1998) free amino group measurement (Church *et al.*, 1983), O-phtaldialdehyde (OPA) method (Saenzde *et al*., 2011), trichloroacetic

acid-soluble protein, and peptide distribution by either Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Alarcón *et al.*, 2002; Lemos *et al.*, 2004) or size-exclusion chromatography.

Source of enzyme. These *in vitro* studies are well known to use either a single or multiple mixtures of commercial enzymes like proteases, trypsin, chymotrypsin and peptidase originating from mammals or bacteria (Hsu *et al.*, 1977; Satterlee *et al.*, 1977). So far, there are no commercial fish digestive enzymes, thus these enzymes need to be extracted from fish under study to perform the *in vitro* analysis. These enzymes are extracted from the digestive tissues (stomach and pyloric ceca) (Yasumaru and Lemos, 2014) and the enzymes extracted from these tissues include gastric pepsin, pancreatic trypsin, chymotrypsin, amylase, and lipase. These enzyme secretions can vary with ingredient composition and progression of digestive processes (Bakke *et al.*, 2010) causing high variations between studies.

Simulation of gastrointestinal conditions. The gastrointestinal conditions are simulated by adding the feed ingredient (fixed protein concentration) into the reaction tube, followed by the digestive fluids and enzymes for each digestion phase. The enzyme:substrate (E:S) ratio is calculated based on the measured total enzymatic activity in fish digestive tissue, and the fish feed ingested (Morales and Moyano, 2010).The *in vitro* operating conditions include gastric and intestinal pH, temperature, duration and enzyme:substrate ratio (Gilannejad *et al.*, 2017). The pH values of the incubation mixtures for analyses of pepsin and intestinal proteases ranged between 1-3 and 7-9 (Krogdahl *et al.*, 2015). In general temperature of 37°C is chosen for the assays for the ease of comparison with homoeothermic animals (Krogdahl *et al.*, 2015). However, in salmonids lower temperatures are also used to set up the *in vitro* conditions due to the pronounced stability of salmon cold adapted enzymes (Ásgeirsson and Bjarnason, 1993). Different *in vitro* techniques used in fish digestibility studies are as given in Table 2 (Wang *et al.*, 2021). Some of the major limitations in *in vitro* models revolve around using enzymes extracted from fish as it involves significant variations and lack of reproducibility between the studies. This is because digestive enzymes secreted by fish are highly dependent on the environmental and nutritional factors (Lallès, 2020). Apart from this, the operating conditions or the digestion model can

itself affect the ingredient or feed digestibility. Thus, these limitations seek out the inability of *in vitro* methods to fully reflect the complexity of the digestion process that occurs *in vivo*, thereby making it less accurate compared to an *in vivo* method (Wang *et al.*, 2021).

Table 2: *In vitro* digestibility assay conditions and parameter measured on different fish species (modified and adapted from Wang *et al*., 2021).

Nd = not determined; DH = pH-stat degree of hydrolysis; FAA = free amino acids; APD = *in vivo* based apparent protein digestibility; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis; P = phosphorus; Fe = iron; Zn = zinc.

Fish species	Ingredient	pH	E:S ratio (U/mg protein)	T (°C)	Time (min)	Measured parameter	Note	References
O. mykiss, R.canadum , O. niloticus	Protein	2 & 8	Nd	25	60 & 60	DH	Two-stage pH stat, Compare caged and farm tilapia	Yasumaru and Lemos, 2014
T. orientalis	Fish and soybean meal	2 & 8	12.5 & 16.7	37	60 & 180	Peptide fraction, FAA, residual protein	Two-stage digestion, with commercial pepsin and fish crude intestinal extract	Román-Gavilanes *et al.*, 2015
T. orientalis	Fish and poultry meal	2 & 8	12.5 & 16.7	37	60 & 180	Peptide fraction, FAA, residual protein	Two-stage digestion, with commercial pepsin and fish crude intestinal extract	Castillo-Lopez *et al.*, 2016
M. nemurus	Soybean meal on different composition with fish meal	8	Nd	37	60	DH, pH drop, zymography, APD	One-stage intestinal digestion with 4 enzyme system: fish crude extract and 3 mammalian enzymes mixture	Rahmah *et al.*,2016
L. guttatus	13 protein ingredients	3 & 8	24.125 & 2.875	37	15 & 45	DH, FAA, Zymography	One and Two-stage pH stat, Comparison between two fish developmental stage	Peña *et al.*, 2017
T. magnodali	Soybean concentrate on 7 different level	3.5 & 8	6.25	37	15 & 45	DH, Nutrient retention (N, P)		Trejo-Escamilla *et al.*, 2017
O. mykiss, L. vannamei	*Nannochloropsis granulata*	8	Nd	25	60	DH	Evaluate different treatment by supercritical CO2 extraction	Tibbetts *et al.*,2017
S. aurata	Commercial feed	3.5–6.5 & 6.5–8.5	125–500 & 50–200	Nd	240–480 & 480–640	FAA, Reducing sugar	Two-stage membrane reactor, with fish crude extract	Gilannejad *et al.*, 2018

Fish species	Ingredient	pH	E:S ratio (U/mg protein)	T (°C)	Time (min)	Measured parameter	Note	References
P. mesopotamicus	*Three commercial diets*	2 & 7	Nd	37	120 & 120	*In vivo* assessment, residual protein, Minerals (P, Fe, Zn)	Two-step digestion with final product dialysis separation, Added bile extract	(Cian *et al.*, 2018)
A.trimaculatus	*16 protein ingredients*	3.5 & 8	Nd	Nd	15 & 45	DH, FAA	Intestinal extract	(Toledo-Solís *et al.*, 2020)
S. salar	*Two mixed diets*	2.1 & 8	Nd	Nd	60 & 240	Minerals	Intestinal extract, compared with and without inorganic supplementation	(Silva *et al.*, 2020)
C. idella	*Soybean β-conglycinin*	Nd	13.02 & 11.27	37	Nd	SDS page, FAA, qPCR	Stomach and pyloric extract	(Duan *et al.*, 2020)
M. nemurus	*Fish, soybean, and squid meal*	8	Nd	37	10	pH drop	One-stage intestinal digestion with 4 enzyme system: fish crude extract and 3 mammalian enzymes mixture	(Rahmah *et al.*, 2020)
-	*H. illucens*	2 & 7		38	1440 & 5760	Crude protein digestibility	Two-step enzymatic method (digestion with pepsin and trypsin-enriched pancreatin). Porcine enzyme	Marono et al., 2015
O. niloticus	*T. molitor* *Fish meal* *Soy meal*	7.8		37	10	Amino acid quantification using o-phthaldialdehyde	Intestinal proteases	Muros et al 2015
S. salar	*H. illucens*	2 & 8		37	120&240	Mineral and amino acid solubility	Two stage hydrolysis using commercial enzyme	Silva et al., 2021
S salar	*H. illucens*	3 & 8		22-24	60 & 60	Amino acid solubility	Two stage hydrolysis using commercial and salmon enzyme	Radhakrishnan et al. 2022

1.3 Functional properties of BSFL

Functional feeds are defined as feeds with growth, health, or other physiological benefits above and beyond the levels normally achieved when basal nutritional requirements are met (Jensen *et al.*, 2015a; Martin and Król, 2017). Apart from being used as source of protein, BSFL meal contains diverse bioactive and immunostimulant compounds such as antimicrobial peptides (AMPs) (e.g., α-helical peptides, cysteine-rich peptides, proline-rich peptides, glycine-rich peptides), fatty acids (lauric acid) and polysaccharides (chitin and chitosan) (Veldkamp *et al.*, 2022). The AMPs play a key part in the innate immune response and have been shown to improve fish resistance to diseases (Hu *et al.*, 2021; Rashidian *et al.*, 2021). Lauric acid is known for its antiviral and antibacterial activities (Lieberman *et al.*, 2006) and is also considered as a good source of energy for salmon (Belghit *et al.*, 2019b). The amount of chitin in BSFL is known to be found between 4-6% (Mohan *et al.*, 2022). Chitin with a caloric content of 17.1 kJ/g could be used as a carbon and nitrogen source in fish (Gutowska *et al*., 2004). However, the role of chitin and its derivates in fish is controversial as it is reported to exert positive (prebiotics, protection against infections) (Ringø *et al*., 2006; Terova *et al*., 2019; Esteban *et al*., 2001; Gopalakannan and Arul, 2006) and negative effects on fish (reduce nutrient digestibility and growth) (Shiau and Yu, 1999; Eggink *et al*., 2022b). For instance, in rainbow trout, administration of chitin stimulated macrophage activity and improved the disease resistance against *Vibrio anguillarum* (Sakai *et al*., 1992). Whereas the digestibility of protein decreased when BSFL meal with the highest dietary chitin contents (15.4% DM) were included in Nile tilapia and rainbow trout diets (Eggink *et al.*, 2022b). It was studied that the immune property of chitin is suggested to be a function of particle size of chitin (10^3-10^4 Da) (Alvarez *et al*., 2014).

1.3.1 Evaluating the immune responses of BSFL in salmonids

Immune system in salmon. The immune system in salmon consists of both innate (non-specific) and adaptive (specific) immune system. Innate immunity in general has phagocytic mechanism associated with macrophages and granular leukocytes which attack the microorganisms that invade the fish. Apart from these, other soluble factors such as lysozyme and complement factors also help in destroying the invading

pathogens (Hardy and Kaushik, 2021). Adaptive immunity is highly specific to a specific antigen and provides a long-lasting memory with the help of T cell receptors, immunoglobulins, and major histocompatibility complex. Compared to vertebrates, fish are mostly dependent on innate immune system (Mokhtar *et al.*, 2023), since adaptive immunity is less developed compared to mammals. The innate immune system in salmon is generally classified into physical barriers, cellular or humoral components (Magnadóttir, 2006).

The physical barrier includes scales, mucus (covering skin and gill) and the epidermis (Ellis, 2001; Ingram, 1980). Fish scales are plates of dermal bone that are embedded in the dermis and epidermis of fish (Wainwright, 2019), mainly for mechanical protection against predators, collisions with other fish or obstacles and from other mechanical threats (Khayer Dastjerdi and Barthelat, 2015). The mucus is considered the first line of defense and is secreted by goblet cells. Mucus contains immune parameters such as pentraxins, nucleotides, lysozyme, antimicrobial peptides, C-reactive protein, alkaline phosphatase, proteases and immunoglobulins which are responsible for deteriorating, inactivating, and controlling infections (Alexander and Ingram, 1992). Mucins and nucleotides are among the major molecules found in the mucus that are responsible for mucus viscosity, pathogen entrapment, and skin surface protection (Mokhtar *et al.*, 2022). The epidermis prevents the entry of foreign materials with the help of macrophages, eosinophilic granular cells, and lymphocytes found in the epithelial cells of the skin (Ángeles Esteban, 2012). Humoral immune parameters are molecules soluble in plasma and other body fluids (Uribe *et al.*, 2011). These include transferrin, lectins, lysozyme, antimicrobial peptides, cytokines, natural antibodies, and complement components, which can destroy microorganisms or suppress their growth (Magnadóttir, 2006).

Dietary modulation of immune factors in fish. The differences in the composition of diet and levels of each nutrient in feed are known to affect the overall immune responses and health in fish (Lim and Webster, 2001). Nutritional approaches can modulate the components of the immune system by acting on immune cells directly or indirectly through metabolic, neurological, or endocrine pathways (Reddy and Frey 1992). Dietary immunomodulation has a great potential in providing a good nutritional status

that can aid in improving the disease resistance in fish (Lim and Webster, 2001). Such dietary modulation included the use of functional or additive feed ingredients containing various immunostimulants and bioactive compounds such as nucleotides, organic acid, probiotics, prebiotics, extracts from plants, seaweeds and insects that may provide a health benefit beyond basic nutrition. For instance, feeding diets supplemented with probiotics such as β-1,3-1,6-glucans or mannan oligosaccharides (Refstie *et al.*, 2010; Sweetman *et al.*, 2010), commercial mixture of natural identical compounds (Jensen *et al.*, 2015b) and dietary nucleotides (Burrells *et al.*, 2001) has reported reduction in sea-lice infestations and mortalities in salmon. Previous research findings suggest that supplementing of nucleotides and yeast cell extracts in the diet of Atlantic salmon enhances growth performance, improves mucosal health and gut microbiota (Burrells *et al.*, 2001; Wang *et al.*, 2022). Furthermore, the supplementation of prebiotics such as *Pediococcus acidilactici* to the salmon diet could potentially stimulate the immune system and trigger the release of pro-inflammatory cytokines to fight possible pathogen attack (Jaramillo-Torres *et al.*, 2019).

Therefore, the modulation of immune responses due to dietary intervention such as manipulating the levels of various nutrients/ingredients, adding certain non-nutrient immunostimulatory compounds, and altering feeding strategies can be studied using various immune factors to monitor the health in salmon. Insect meals due to the presence of bioactive compounds can stimulate immune responses and can improve resistance towards diseases. For example, dietary inclusion of 15% of BSFL meal enhanced the gut microbial richness and diversity (Li *et al.*, 2020) and 12.5 % of BSFL meal reduced enterocyte steatosis in pyloric caeca and increased plasma lysozyme in salmon (Weththasinghe *et al.*, 2021). Dietary inclusion of BSFL meal up to 25% did not affect the overall liver and muscle health of salmon, as indicated by plasma alanine transaminase (ALT), aspartate aminotransferase (AST) and creatine kinase (Weththasinghe *et al.*, 2021b). In rainbow trout, dietary inclusion of BSFL at 20% significantly increased the gut mucin production and decreased the glutathione peroxidase activity (Elia *et al.*, 2018).

1.4 Evaluating the fillet quality of salmonids fed BSFL meal

The term 'quality' is an arbitrary word that involves all the attributes, and characteristics of the products and the extent to which the product satisfies the consumer requirements. The 'quality' related to seafood involves product nutritional content, safety, freshness, and physical attributes such as color and texture (Altintzoglou *et al.*, 2022; Botta, 1996; Bremner, 2000). One of the main goal of salmon farming industry is the production of high quality fillet meaning; high water holding capacity, firm texture, good amount of the fat and fatty acids especially n-3 PUFA, with a color intensity of pink to deep red color (Mørkøre *et al.*, 2020), and decent nutritional indices (Lutfi *et al.*, 2023; Sigurgisladottir *et al.*, 1997). Several studies were performed on salmonids to evaluate the effect of BSFL meal in fish fillet quality (Belghit *et al*., 2019a, Belghit *et al*., 2019b, Bruni *et al*., 2020a, Fisher *et al*., 2020, Li *et al*., 2020, Lock *et al*., 2016, Weththasinghe *et al*., 2021a, Weththasinghe *et al*., 2022, Li *et al*., 2021). In Atlantic salmon, complete replacement of BSFL meal can result in small changes in fillet sensory characteristics but did not impair the physico-chemical quality of fillet (Belghit *et al*., 2019a, Bruni *et al*., 2020a). In rainbow trout, dietary inclusion of 50% of BSFL meal did not impair the fillet quality (Bruni *et al*., 2020b).

1.4.1 Methods to evaluate fillet quality

Water holding capacity (WHC). is defined as the ability of salmon fillet to retain water. The water is often lost during storage, thawing and processing as drip loss thawing loss or cooking loss (Kaale *et al.*, 2014). These losses occur due to the extrusion of tissues juices due to the structural changes in the muscle (Huff-Lonergan and Sosnicki, 2002), implying nutrient loss. WHC is one of the useful tools to measure the quality and freshness of fillet. For instance, high WHC, low drip loss implies high protein functionality and increased net weight, which is better accepted by the consumers, thereby contributing to profitability of the fillet (Chan *et al.*, 2020).

Texture. The food texture is a collective term that covers several related physical properties such as form, viscosity and structural complexities (Stribiţcaia *et al.*, 2020). The salmon muscle texture is mostly characterized by proteins and fibers (Cheng *et al.*,

2014). Fillets that have small fibers were firmer than fillets with medium-sized or large muscle fibers (Mørkøre *et al*., 2009). A firm and elastic texture is an essential criterion for consumer appreciation and processing of salmon fillet (Østbye *et al.*, 2018). The texture of salmon fillet can be influenced by various environmental, biological and husbandry factors (Bahuaud *et al.*, 2010). Further, rate and extent of *postmortem* changes, fat content, fatty acid profile in the muscle can also influence the salmon fillet texture (Sigurgisladottir *et al.*, 1997). Bourne (1978) developed the General Foods Texture Profile Analysis method to evaluate the food texture. This method was based on the principle that texture is composed of diverse sensation parameters, such as hardness, brittleness, chewiness, gumminess, adhesiveness, cohesiveness, springiness, and viscosity, as perceived by sensory procedures. Borderias *et al*. (1983) applied this method to study these characteristics in fish. Recently, instrumental analysis using Instron texture test system is used commonly and sample behavior is registered when a force greater than gravity was applied mostly expressed in terms of shear force (N).

Color. The high market value and elite image of salmon is probably due to its characteristic 'red color' of the fillet (Grunenwald, 2018). This red color is due to the deposition of organic pigments, known as carotenoids (Sae-Lim *et al.*, 2022). Farmed salmon are dependent on the dietary supplementation of carotenoids such as astaxanthin due to the absence of de-nova synthesis of carotenoids in salmon (Lutfi *et al.*, 2023). Therefore, the salmon feed is fortified with dietary astaxanthin (recommended limit ≤ 80 mg kg^{-1} feed) (Wrolstad and Culver, 2012), either sourced from algae or from synthetic source (Elbahnaswy and Elshopakey, 2023). Furthermore, the salmon color can be affected by the fat content, or due to sea temperature. High degree of fat in the fillet can dilute the concentration of astaxanthin and also interfere with the perceived color (Sigurgisladottir *et al.*, 1997). Also, high sea temperature causes oxidative stress producing free radicals, and astaxanthin is metabolized to prevent the interaction of free radicals with cell membrane and DNA, thereby reducing the fillet color (Nordberg, 2018). The color of fresh fillet can be evaluated using color standards such as SalmoFan^{TM} (Roche, Switzerland) (Kono *et al.*, 2017) or by using instrument such as colorimeter, which measures light reflection from the flesh in comparison to a standard

calibration plate (Yeşilayer, 2020). The color measured in this instrument is generally in terms of lightness (L*), red-green chromaticity (a*) and yellow-blue chromaticity (b*) as recommended by the International Commission on Illumination (CIE, 1978). The chroma (C_{ab}*) is the expression of intensity and clarity of the color and is calculated using the a* and b* values (Hunt, 1977).

Proximate composition. Farmed salmon provides a high level of protein, and omega-3 long-chain polyunsaturated fatty acids (LC-PUFA), particularly eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) acids, compared to many other fish species (Sprague *et al.*, 2016). The EPA and DHA are widely acknowledged as beneficial for human health and are known to reduce cardiovascular diseases. The recommended intake is 1.75 g EPA + DHA per week (250 mg/day) as advocated by EFSA (2005). However, it should be noted that the reduced use of marine ingredients in aquafeeds had a significant effect on the EPA + DHA levels in farmed fish (Ytrestøyl *et al.*, 2015).

2. Objectives of the PhD work

The PhD thesis has two main objectives (Fig. 5):

(i) To study the digestibility of BSFL meal in Atlantic salmon *in vivo* and *in vitro*

(ii) To evaluate the effects of feeding salmon with diets containing BSFL meal in real farm like condition, for the entire production cycle in the sea-cages.

The PhD work was divided into four tasks as follows:

- Comparison between salmon crude enzyme extract and commercial (mammalian) enzymes for *in vitro* method.
- Evaluation and comparison of protein and amino acid solubility *in vitro* and *in vivo* in Atlantic salmon.
- Assessing the functionality of BSFL meal towards fish health and welfare in Atlantic salmon for entire production cycle in the sea-cages
- Evaluation of the nutritional and aesthetic quality of the fillet of Atlantic salmon fed BSFL meal for the entire production cycle in the sea-cages.

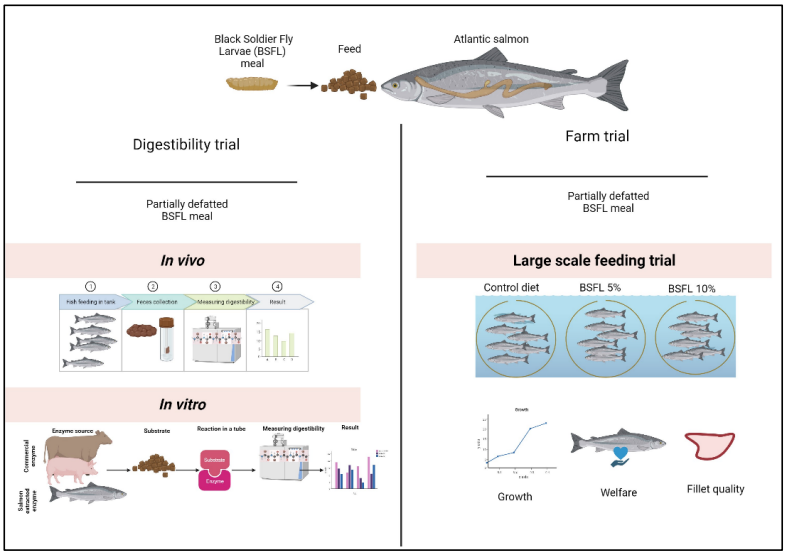


Figure 5: Summary of the PhD work, including the two main objectives of the thesis.

3. Methodological considerations

The introduction of novel feed ingredients into the diet of Atlantic salmon needs studies concerning its digestibility. Moreover, the impact of these ingredients on salmon health, welfare and fillet quality needs to be validated at conditions replicating real farm conditions unlike land-based experiments that are performed under controlled conditions.

3.1 Digestibility trials

3.1.1 Ingredients and experimental diets

The partially defatted BSFL meal required for the digestibility trial were procured from Protix Biosystems BV (Dongen, The Netherlands) and their experimental diets (test diet, and reference diet) were produced at Nofima's Aquaculture Technology Centre, Bergen, Norway. Reference diet represented a standard commercial diet containing FM and SPC as protein source and FO and rapeseed oil as lipid source was formulated. Tests diets were formulated by combining 80% of the reference diet with 20% of BSFL meal as test ingredient (**Paper I**).

In digestibility trials, ingredients are generally combined with basal diets in a 70:30 ratio as was first suggested by Cho & Slinger (1979), who applied this ratio to simulate feed inclusion levels in practical diets. However, any proportion can be used as long as the fish can consume the diet, and the proportions ensure the normal digestion process (NRC, 2011). Glencross *et al*. (2007), recommended inclusion levels from 20 to 40% for the test ingredients to simulate the levels used in practical diets. In the current study, the 80:20 ratio was considered to ensure that the experimental diets remained as close as possible to the nutritional composition of the reference diet. This approach aims to minimize differences in fish consumption due to the inclusion level of the test ingredient.

3.1.2 Digestibility trial – feces collection method

Atlantic salmon used in this trial had a mean initial body weight of 83.2±0.36g. Each diet was tested in triplicate tanks thus the salmon were randomly distributed into 9 tanks

(110 x 110 cm,465 L water volume) containing 140 fish each. To estimate the apparent nutrient digestibility of the BSFL meal and diets, feces were collected by manual stripping (**Paper I**).

The collection of feces for measuring the nutrient digestibility via indirect method can be through active and passive method. Active method of feces collection involves manual stripping (manually stripping the feces from the distal intestine by a gentle pressure on the abdominal cavity) (Windell *et al.*, 1978), dissection (killing the fish to remove the feces from distal intestine) (Austreng, 1978) and vacuum removal (insertion of a small tube into the anus and applying vacuum pressure to remove feces) (Windell *et al.*, 1978). Whereas passive method involves feces collection that are naturally egested by the fish. These feces are collected from the effluent water from the tank through filtration or screening. Different systems have been developed to collect feces, such as the still widely used Guelp (settling column) and Choubert systems.

The feces collection method plays a significant part in calculating the digestibility values since methods using columns for collecting feces may overestimate the protein digestibility compared to other faecal collection method such as stripping, due to leaching of nitrogen depending on the type of feed (Shomorin *et al*., 2019). Thus, the current study considered applying stripping as feces collection method to minimize the error in estimation of nutrient digestibility. However, caution must be taken while stripping as too much pressure on abdomen can result in blood and other fluid contamination into the feces.

3.2 *In vitro* methods

In vitro digestibility of BSFL meal was studied by simulating the gastrointestinal conditions similar to salmon observed *in vivo.* This approach involved the use of enzyme extracted from salmon and the enzymes commercially available from the market (pork/bovine). These two sources of enzymes were selected to compare the efficiency of each enzyme based on their ability to hydrolyse the proteins. This comparison was performed based on the principles of pH-stat method with a standard

protein source such as hemoglobin (for gastric enzyme) and casein (for intestinal enzymes). The pH-stat is an appropriate method to understand and standardise the efficiency of both enzymes based on their degree of protein hydrolysis (DH%). However, some of the limitations of using pH-stat method involves inconsistency in reproducing the result from earlier studies mainly due to the assumed α value which is dependent on temperature and the protein ingredient (peptide chain length and terminal amino acid) (Mat *et al.*, 2016). Moreover, the buffering capacity of the feed ingredient can affect the DH % (Márquez *et al.*, 2013). Additionally, titration with sodium hydroxide (NaOH) during the intestinal phase also measures the lipid hydrolysis of the ingredient (Wang *et al.*, 2021).

Once after having a standardised enzyme concentration, these enzymes were added into selected substrates having a fixed protein concentration for a fixed time interval under constant rotation at room temperature (21-24°C) for simulating the gastrointestinal conditions. This *in vitro* method involved two-stage hydrolysis involving both acidic and alkaline phases of digestion, where the pH (acidic- pH 3 and alkaline pH 8) for the entire experiment was maintained using pH-stat between the two phases. This experiment primarily measured the protein and amino acid solubilities using the non-soluble part in the digestion tube. It was found that salmon pepsin had a higher DH % on hemoglobin than the pepsin from commercial sources, being contrast to the DH % observed for the intestinal enzymes where commercial enzyme had higher DH % on casein than salmon enzymes (**Paper I**).

3.3 Large scale feeding trial

In the current study, two levels of dietary inclusion for BSFL meal were selected considering the current availability (5%) and a potential future standard for insect protein meals in animal diets (10%) (Veldkamp *et al*., 2022). As indicated by the meta-analysis by Weththasinghe *et al*. (2021), the type of protein source replaced plays a significant part in affecting the fish growth performance. This meta-analysis revealed that replacement of BSFL meal with non-fish meal protein sources increased the growth rate and reduced feed conversion ratio compared to the replacement of FM by BSFL

meal. Furthermore, as stated in the systematic review by Tran *et al*. (2022), inclusion of plant derived ingredients in salmonid feed can place a high pressure on the environment especially relating to land and water uses compared to fish meal. Thus, the current study considered replacing the plant-based protein sources in the feed for salmonids. The salmon in the present trial were reared from ~173 g to ~ 4 kg and were randomly distributed into nine open sea-cages with ~6000 salmon per cage (12×12 m^2; 1900 m^3), following a randomized block design to consider the cage position effects. This trial was conducted to study the dietary effects of BSFL meal on growth, body indices, general health, welfare **(Paper II)** and fillet quality **(Paper III)** when reared under conditions replicating real farm **(**Fig. 6).

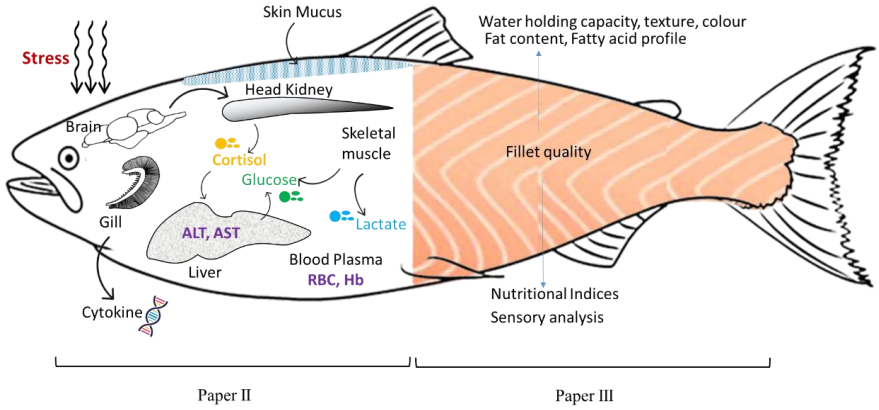


Figure 6: An overview of experimental design used in **Paper II** and **Paper III**

4. Results and Discussion

4.1 Digestibility of BSFL meal in Atlantic salmon *in vitro* and *in vivo*

4.1.1 *In vitro* solubility of BSFL meal in Altantic salmon

Dietary protein is a crucial nutrient in aquatic feeds as it provides essential amino acids for the growth and development of aquatic organisms. Therefore, it is important to evaluate the digestibility of raw protein sources used in the feeds (Sáenz de Rodrigáñez *et al*., 2011). Thus, one of the aims of this thesis was to assess the potential of *in vitro* method for rapid screening of protein quality for farmed Atlantic salmon, considering its relevance for feed digestibility studies. This chapter discussed about the protein and amino acid solubilities of partially defatted black soldier fly larvae (BSFL) meal and its diet by using commercial and salmon enzymes. This method employed two-stage hydrolysis involving a gastric followed by an alkaline phase for protein hydrolysis, with pH adjustment made between the phases (**Paper I**).

In the current thesis, salmon and mammalian enzyme sources were considered for studying the *in vitro* protein and AA solubility of BSF meal. The relative protein digestibility (RPD) obtained for BSFL meal was found to be 24% when used both salmon and commercial enzyme. One would expect a similar solubility value since these enzymes were standardized on similar degree of protein hydrolysis using pH-stat (**Paper I**). Carter *et al*. (1999), proposed that *in vitro* setups using commercial enzyme gave higher digestibility values on bluefin tuna (*Thunnus maccoyii*) feed than fish enzyme (Atlantic salmon and blue fin tuna) and that commercial enzymes are more suited to a wider range of feed ingredients. However, later in the study done by Moyano *et al*. (2001), the authors found that seabream (*Sparus aurata*) enzyme extracts produced a higher hydrolysis of proteins compared to purified mammalian enzymes due to the differences in their sensitivity to inhibitors, catalytic efficiency, and substrate affinity (Moyano *et al*., 2001). Also, within fish species, the extent of protein hydrolysis can be different. This was documented in the study by Hamadan *et al*. (2009), where enzyme extracts from two different species, meagre (*Argyrosomus regius)* and

European seabass (*Dicentrarchus labrax)*, were tested for their ability to hydrolyze protein in a commercial feed. The results showed that the enzymes from the two species had different extents of protein hydrolysis, even when operating under identical conditions. These differences could be due to variations in the proportions of digestive proteases or differences in their catalytic parameters (Hamadan *et al*., 2009). These studies emphasized the importance of also including tests with the digestive enzyme from the target species when working on setting up *in vitro* digestibility methods. Thus, salmon digestive proteases such as 'pepsin like' enzyme (acidic proteases) extracted from the stomach and 'proteases like enzyme' extracted from pyloric caeca + intestine (alkaline proteases) were used in this study. However, the alkaline proteases were not distinguished between trypsin and chymotrypsin as performed in other studies (Tibbetts *et al*., 2011b). The commercial proteases used in the study were the available products from porcine and bovine sources. Different sources of enzymes were considered to obtain more detailed information on differences in the action mechanisms of enzymes and the products resulting from digestion in Atlantic salmon. In the current study, commercial enzymes had higher solubility values for most of the AA on BSFL meal compared to salmon enzymes. Similar observations were made under the study conducted by Moyano *et al*. (2001), where the average AA solubility values were higher when using commercial proteases than enzyme extracts from seabream when performed on protein substrates such as casein, FM and soybean meal when used a digestion cell containing a semi-permeable membrane. Interestingly, in the current study but also in the study conducted by Moyano *et al*. (2001), similarly high solubilities for glycine and alanine have been observed compared to other AA when performing *in vitro* methods. These results could be related to the molecular weight (MW) of the AA. As can be seen in Fig. 7, a linear trend was observed between the AA solubility values (%) and the MW of AA (Dalton). The results showed that AA with the lower molecular weight especially alanine, (89 Dalton) had the highest solubility values. The lower molecular weight (MW) of AA enhances their hydration interactions with water molecules, contributing to increased solubility. Consequently, amino acids like glycine and alanine may experience higher solubilization due to their lower MW (Kramer et al., 2012). It is important to note that AA solubility can be influenced by their MW. Larger amino acids

can have increased hydrophobic interactions among protein molecules, potentially decreasing their solubility (Kramer et al., 2012). However, it is worth mentioning that higher MW amino acids do not consistently lead to lower solubility.

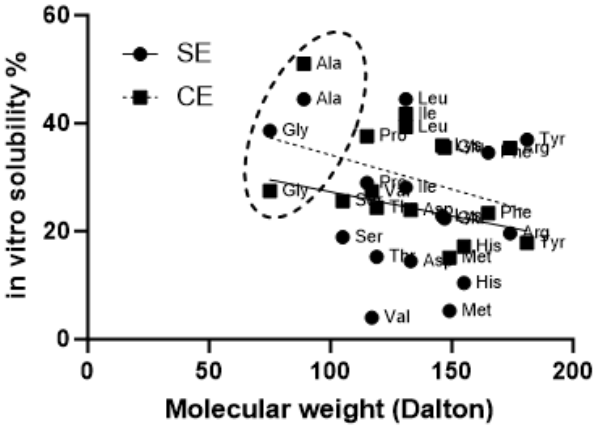


Figure 7: Relationship between molecular weight (Dalton) and *in vitro* solubility (%) of the amino acids. The regression equations are as follows, SE = Salmon enzyme (Y = -0.089*X + 36.2); CE = commercial enzyme (Y = -0.13*X + 46.9). The slopes and intercepts were not significantly different.

Comparison studies between fish and mammalian proteases were performed earlier on traditional protein sources such as FM and soybean meal. This was performed using pH-stat and a digestion cell for protein hydrolysis and these enzymes were standardized based on the enzyme activity (Moyano *et al*., 2001). The standardization of enzyme presented in the current thesis was based on degree of protein hydrolysis (**Paper I**), a method adopted from a study conducted by Yasumaru *et al*. (2014), where pH-stat method was used to standardize the digestive proteases extracted from the rainbow trout, cobia (*Rachycentron canadum*) and Nile tilapia (*Oreochromis niloticus*) based on their hydrolytic capacity (Yasumaru *et al*., 2014). This method of standardization based on their digestive capacity is proposed to be much better than considering a fixed volume of enzyme extract (Divakaran *et al*., 2004; Lan and Pan, 1993). Therefore, in

the current study, salmon and mammalian enzymes were standardized based on their hydrolytic capacity (**Paper I**). After the standardization of salmon and commercial enzymes, a two-stage hydrolysis of protein ingredients was performed. The results showed that *in vitro* AA solubility values for BSFL meal ranged between 10-44% (**Paper I**). In a study conducted by Moyano *et al*. (2001), the solubility of AA for FM ranged from 2-20% and soybean meal from 5-10%, when using seabream enzyme extracts in a digestion cell performing two-stage hydrolysis. Similarly, in a study conducted by Saenzde *et al*. (2011), *in vitro* analysis of animal based raw material using intestinal enzyme extracted from sole (*Senegalese sole*) showed that the total amino acid released from protein hydrolysis ranged between 2-22%, measured using o-phthaldialdehyde method (Saenzde *et al*., 2011). Our results imply that insect meal behaves in a similar range or even higher than animal protein sources. However, it must be noted that the differences in AA solubility between these studies are more likely due to the different *in vitro* methods employed. To summarize, different enzyme sources can be standardized based on degree of protein hydrolysis. Further, higher AA solubility was obtained with commercial enzymes when compared to salmon enzymes.

4.1.2 Apparent digestibility of protein and amino acid

In **Paper I**, BSFL meal was found to be highly digestible by Atlantic salmon with high crude protein ADC (90.6±5.42%), and true protein ADC (99.2±7.27). Similar results were obtained for the protein ADC of BSFL meal (89%) in salmon parr (Fisher *et al*., 2020) and around 85% in rainbow trout (Dumas *et al*., 2018). The AA digestibility of BSFL meal displayed >80% for almost all AA, except for aspartic acid (70%). Similar result was reported by Dumas *et al*. (2018), who reported the digestibility of essential AA of BSFL meal between 84% to 96% in rainbow trout. Thus, the higher digestibility of BSFL meal suggests its potential as an alternative protein ingredient in low fish meal and high plant-based protein diet.

The diets containing BSFL meal had similar ADC with control diet for crude protein (85.6±0.79), true protein (80.5±0.82) and crude lipid (93.0±0.69). Similarly, Belghit *et al*. (2019a), showed ADC of crude protein at 82%, when salmon were fed with defatted BSFL meal (15% BSFL inclusion; 100% FM replacement) for 16 weeks. The ADC

values obtained in the current study agrees with previously reported ADC value for salmonids for protein and lipid (Belghit *et al*., 2019a; Fisher *et al*., 2020; Terova *et al*., 2019). Although ADC of protein in BSFL meal diets were not different from control diet, the reported protein digestibility (80.5±0.82) (**Paper I**) was comparatively low to other reported study in salmonids (Dumas *et al*., 2018). This reduction may have occurred because of insect meal processing such as fractionation (separation of protein and lipid either by dry or wet fractionation, as shown in different studies (Eggink *et al*., 2022b). This highlights the importance of processing, which can influence the nutrient digestibility/solubility of insect meals and future studies should be testing the effect of different processing methods on the nutrient digestibility in fish species.

4.1.3 Comparison between *in vitro* and *in vivo* of protein and amino acids

In this thesis (**Paper I**), comparison between *in vivo* digestibility and *in vitro* solubility was performed to establish a physiological relevant study for digestibility assessments. In the current study, a strong positive correlation (r=0.989, p=0.09) was observed between BSFL meal measured *in vitro* to the *in vivo* apparent protein digestibility (APD), when using salmon enzyme. However, a relatively weaker correlation was observed when commercial proteases were used (r=0.74 p=47). With regard to AA solubility, the salmon enzyme provided better correlation (r=0.50; p=0.07) than commercial enzyme *in vitro*. This emphasizes the significance of using species specific enzymes for *in vitro* studies. Similar observations were made by Mirzakhani *et al*. (2018a), where the authors were able to fit linear regressions between *in vivo* APD and *in vitro* DH% with fish enzyme extracts in feed ingredients for Siberian sturgeon (r = 0.94–0.99). This kind of linear relation between *in vivo* APD and *in vitro* protein hydrolysis suggests a close relationship between peptide bond breakage by digestive proteases *in vivo* and *in vitro* (Mirzakhani *et al*., 2018a). Furthermore, a good correlation was not observed for the diets, but studies have reported a higher correlation (r = 0.962) on experimental diets *in vivo* to *in vitro* when using crude intestinal enzyme extract of bagrid catfish (*Mystus nemurus*) (Rahmah *et al*., 2016a). Thus, our results showed in general that protein digestibility *in vivo* can be better correlated to *in vitro* protein

hydrolysis data. As a result, *in vitro* method, by using species specific animal enzymes can be a useful tool to provide information on ingredient digestibility.

In the current work no strong correlation was observed for AA, neither at the ingredient level nor in the diets, between *in vitro* and *in vivo*. A similar lack of correlation between individual AA was observed by Márquez *et al*. (2013), where the authors used a gastrointestinal model to assess potential AA bioavailability in diets for rainbow trout. The study presented a poor correlation between total masses of amino acids dialyzed *in vitro* and ADC of protein (per kg diet, r=0.037) (Márquez *et al*., 2013). The possible reason for lacking correlation between *in vivo* and *in vitro* amino acid solubility in the current study, might be due to the following reasons; firstly, DH% that was used to standardize the enzymes, quantified the number of peptide bonds hydrolyzed in a given protein source. This eliminates the detection of pre-hydrolyzed peptides already present in the reaction mixture. Thus, these undetected amino acids, when analyzed, will interfere with the final solubility values. Secondly, the differences might be because of the site of hydrolysis by the proteases. This can be related to the specificity of enzymes in cleaving the peptide bond within (endoproteases) or at the end (exoproteases) of protein molecule (Alarcón *et al*., 2002). Hydrolysis by endo- or exo- proteases by different enzyme sources also depends on the assay conditions. These include factors like the assay temperature, biological age of fish, salinity of the rearing water, particle size of ingredients and diets (Lu *et al*., 2011). Furthermore, the method cannot reproduce the complex digestion process which includes physical contraction, fluid mechanics of mixing and gradual emptying of food particles, as well as the contribution by the gut microbiota. However, these factors were not considered in this study, and might possibly explain the differences in the relative amino acid solubility values between *in vitro* and *in vivo*. Though the chapter discusses a better correlation of protein digestibility between the two systems (*in vivo* and *in vitro*), the same does not hold true for their respective amino acid digestibility (**Paper I**).

To conclude, *in vitro* assays can be highly useful in predicting the digestibility of protein and amino acids in fish feed ingredients. Though commercial enzymes displayed higher AA solubility than salmon enzyme, it could be observed that the latter had a strong

complementarity with *in vivo* studies. Thus, when choosing the enzymes for *in vitro* studies, the use of species-specific enzymes is proposed for a better correlation with *in vivo* digestibility values. Thus, considering the 3R principle of reduction, refinement and replacement in animal experiments, a refined method for digestibility studies could help to reduce animals in experiments.

4.2 Functional property of dietary BSFL meal in modulating the fish health for its better welfare

Expanding aquaculture has marked question on the fish health and welfare. It was recognized that 'stress' is a key issue in fish welfare, which challenges the state of homeostasis, and thus threatens the health of fish (Fletcher, 1997). Therefore, it is in the commercial interest of aquaculture industry to try to improve the welfare by minimizing stress for better fish growth and production. This part of discussion deals with the dietary effect of BSFL meal on welfare of fish, focusing on the health, immune responses, and survival when replacing plant-based protein sources in commercial salmon feed in the marine phase of salmon production. In the present thesis, the stress encountered by salmon included handling stress such as netting; environmental stress (temperature, salinity, oxygen, pathogens); and stress due to treatment such as thermal delousing (**Paper II**).

Plasma cortisol (further referred to as 'cortisol') is the most frequently used indicator of stress in fish and is part of the primary stress response. This hormone is released into the fish bloodstream from the interrenal cells of the head kidney through the activation of the hypothalamic-pituitary interrenal (HPI) axis (Ellis *et al*., 2012). The cortisol response is species specific and depends on the magnitude of duration (acute/chronic) of stress. For instance, in a study by Fanouraki *et al*. (2011), it was observed that European seabass had a cortisol response reaching 900 ng/mL, while grouper had a response level of 40 ng/mL and seabream had an intermediate response with 300 ng/mL when exposed to acute stress such as netting (Fanouraki *et al*., 2011). In the present thesis (**Paper II**), salmon had a cortisol response between 200-400ng/mL when exposed to chronic stress from different factors including pathogenic infestations

(*Pasteurellosis*, and *Branchiomonas Cysticola*) and/or environmental stressors. However, it was interesting to observe a dietary effect of BSFL meal (5% and 10% inclusion) in reducing the cortisol response (~200ng/mL) compared to control group (~350ng/mL) in salmon. Similar dietary effects were obtained in rainbow trout fed diets containing spirulina (*Spirulina platensis*), where 10% inclusion led to lower the plasma cortisol levels compared to control group, which might be due to the presence of bioactive compounds contained in spirulina (Yeganeh *et al*., 2015). This idea agrees with Cain *et al*. (2003), who stated that tilapia fed the diet containing immunostimulants, i.e., β-glucan produced by the bacterium (*Agrobacterium biovar* 1) had lower cortisol values. Thus, it can be hypothesized that the presence of bioactive compounds in insect meal might have reduced the cortisol level in salmon fed BSFL-based diets.

Another major welfare issue associated with high cortisol release in salmon farms is salmon lice management in marine salmonid aquaculture (Walde *et al*., 2022). Both farmers and fish health personnel widely acknowledge that delousing operations have detrimental effects on salmon, and that non-medicinal treatments exert a more pronounced negative impact on fish compared to chemotherapeutic methods (Overton *et al*. 2019, Grefsrud *et al*. 2021). In the current rial (**Paper II**), thermal delousing (using high water temperature) was applied on salmon for lice removal, which resulted in a cortisol response ranging between 300-400 ng/mL, one hour after delousing. According to the report by Bui *et al*. (2023), delousing treatments that included a 34°C thermal bath caused poorer welfare outcomes. Studies reported that dietary modulation via functional feeds was successful in reducing sea lice in salmon, thereby improving the welfare of salmon (Jensen *et al*., 2015b). As an example, feeding salmon glucosinolate-enriched feed resulted in 25% reduction in lice counts (Jodaa Holm *et al*., 2016). However, dietary BSFL meal was unable to provide any such better resistance to delousing process as evident from the cortisol response after delousing (**Paper II**). Similarly in a study by Selvam *et al*. (2021), dietary changes in EPA/DHA levels were not able to reduce the delousing stress in salmon. Thus, the current study was not

successful in alleviating the delousing stress via dietary inclusion of BSFL meal in salmon (**Paper II**).

Secondary responses result in immediate actions at blood/plasma or tissue levels due to effects of released stress hormones. Thus, blood/plasma analysis (**Paper II**) were performed to study the dietary effect on secondary immune responses as a result of cortisol release due to inflicted stress in salmon. It was observed that red blood cell (RBC) and hemoglobin (Hb) were lower in control group that witnessed higher cortisol level. Whereas salmon fed dietary BSFL meal had higher levels of RBC and Hb as a response to lower cortisol response in BSFL fed group (**Paper II**). Hoseini *et al*. (2021), reported such an inverse proportionality between erythrocytes and cortisol in rainbow trout (RBT) when fed with roselle (*Hibiscus sabdariffa*) meal, underlining the reason being the antioxidant capacity of the roselle meal (Hoseini *et al*., 2021). Therefore, the blood/plasma analysis of salmon in the feeding trial revealed a positive modulation of immune responses in salmon fed dietary BSFL meal compared to control diet (**Paper II**). Similar increases in the levels of RBC, white blood cells, hemoglobin, total protein, and albumin results were observed when *Spirulina platensis* was fed at 10% in the diet of rainbow trout compared to a control diet (Yeganeh *et al*., 2015). This property of increasing RBC was attributed to the presence of iron, as iron is known to have a significant effect on erythropoiesis, the production of red blood cells.

Serum alanine transaminase (ALT) and aspartate aminotransferase (AST) are commonly used as an indicator of liver dysfunction. The normal range for serum ALT for healthy adult salmon is reported to be 4-8 U/L (Sandnes *et al*., 1988), while the ALT values in this study ranged between 10-23 U/ L, with lowest value obtained for salmon fed dietary BSFL meal at 5%. Similar results were obtained by Ward *et al*. (2016), where 5–20% spirulina decreased the serum levels of AST and ALT in tilapia (Abo El-Ward *et al*., 2016). Whereas study on krill meal at 12% inclusion on post smolt Atlantic salmon had no effect on ALT levels or other serum components (Mørkøre *et al*., 2020). However, studies have reported hepatic disturbances in fish fed diets containing rendered animal proteins. For instance, in juvenile barramundi, feeding diets that completely replaced fishmeal with poultry by-product meal or a combination of poultry

by-product meal and insect meal led to increased serum levels of AST and glutamate dehydrogenase, which were associated with hepatic multifocal necrosis (Chaklader *et al*., 2020; Chaklader *et al*., 2019). Incidences of steatosis in fish hepatocytes were attributed to the high lipid level and low levels of EPA and DHA (Zhou *et al*., 2020). Thus, it can be hypothesized that decreased AST and ALT levels in salmon fed dietary BSFL meal at 5% indicate no negative health effects related to the liver dysfunction with salmon fed insect.

In the current thesis, skin epithelial health was monitored in terms of quantitative/qualitative evaluation of concentration of mucus compounds such as the DNA concentration and lysozyme activity. Mucus, which contains mucins and DNA play important roles in displaying the viscoelastic nature of mucus (Hill *et al*., 2014). A study by Kaitlyn *et al*. (2022), reported that DNA in mucus is associated with greater mucus viscoelasticity. Thus, in the current study (**Paper II**), the mucus DNA concentration in salmon skin was measured and the results displayed an increased DNA concentration in salmon fed BSFL meal at 5%. However, DNA concentration alone may not be a reliable indirect measure of mucus secretion. Furthermore, the exact relationship between mucus composition, DNA concentration, and viscoelastic properties is still not fully understood and needs further study (Kaitlyn *et al*., 2022). Studies have used different methods to monitor the skin epithelial health. For instance, Weththasinghe *et al*. (2021b), used the expression profile of proteins in the skin mucus to investigate the effects of dietary inclusion of BSFL meal and paste and revealed minor effects on the skin mucus in salmon fed BSFL-based diets (Weththasinghe *et al*., 2021b). Furthermore, a study conducted by Leclercq *et al*. (2020), used crude skin mucus weight (mg of mucus / cm of fish) for comparison of relative skin mucus level between experimental groups. The study displayed increased skin mucus (+46%) and goblet cell density (+25%) when basal diet was supplemented with a specific mannan-rich yeast parietal fraction (sMOS) (4 kg/T feed) after 6 weeks in Atlantic salmon (Leclercq *et al*., 2020). Thus, it can be concluded that functional ingredients have diet-induced proliferation of goblet cells in gut and skin highlighting the potential contribution of functional ingredients beyond their effects on the intestine (Leclercq *et*

al., 2020). Thus, the observed effect seen in the current study on skin mucus DNA concentration might be due to the functional properties of dietary BSFL meal.

However, the skin mucus lysozyme activity reported (**Paper II**) was not increased due to dietary inclusion of BSFL meal compared to the control group. The result is quite contradictory to what has been previously reported on fish where the skin lysozyme levels were higher than the control diets underlining the immunostimulatory effects of dietary BSFL meal (Tippayadara *et al*., 2021). In seabass, dietary BSFL meal showed no effect on serum lysozyme levels when FM was replaced by BSFL meal at 30% inclusion (Hender *et al*., 2021). The process of lysing intact bacteria by lysozyme is complex and involves breaking down the surface structures of the cell, such as the cell wall and protoplasmic membrane (Fleming, 1922; Fleming and Allison, 1922). Some authors have demonstrated a linear proportionality between plasma cortisol and skin mucus lysozymes in Atlantic salmon when exposed to crowding stress (Djordjevic *et al*., 2021, Demers and Bayne, 1997). Fevolden *et al*. (1994), discussed that the elevated levels of plasma cortisol and rise in lysozyme could indicate higher susceptibility of organism to pathogens and this proportional relationship might be a compensatory adaptation to enhance innate immunity. Thus, lower levels of lysozyme in BSFL fed diets might indicate that salmon fed BSFL diet were comparatively less stressed due to immunostimulant property of BSFL. Overall, there exists limited studies exploring the effect of insect meal on skin mucus lysozyme activity in salmon and so far, the existing studies on fish along with the current study reports no negative effect on skin epithelial health due to dietary BSFL meal in fish. Further, the pro-inflammatory cytokines such as interleukin1 *β (il1β)* in gill were upregulated in salmon fed BSFL-based diets at 5% compared to other experimental diets (**Paper II**). Similar upregulation of *il1β* in gut was observed in pre-smolt salmon when fed spruce tree extracts with conidendrin or isorhapontin, added at 0.2% and 0.02% respectively (Rocha *et al*., 2023). The inflammatory cytokine marker such as *il1β* can activate lymphocytes and phagocytic cells, which can increase fish resistance to bacterial infections and reduce mortalities (Sakai *et al*., 2021). Thus, the upregulation of inflammatory genes due to dietary BSFL meal indicates the immunostimulatory effect in salmon. Furthermore, substitution of

alternative ingredients such as plant protein-based and insect-based diets can have negative effects on the health of the intestinal epithelium, leading to disturbances in its absorptive function (Aragão *et al*., 2020; Vidakovic *et al*., 2016). Therefore, it is also equally important to assess the integrity of the intestinal epithelium for evaluating proper absorption of nutrients in the digestive system. However, the current study lacks an assessment on intestinal health, the result of which would have expanded the information on the effect of dietary BSFL meal on intestinal health.

Thus, it can be hypothesized that reduction in stress, better hematological profile, and mucus concentration and cytokine production observed in salmon fed BSFL-based could be attributed to different bioactive compounds present in insect meal. For instance, it was reported that proteins such as Hemopexin-like proteins and Calreticulin from salmon skin mucus are involved in protection to cells from oxidative damage and in the synthesis of mucins (Micallef *et al*., 2017). Similar proteins/peptides such as defensins or proline-rich antimicrobial peptides are present in BSFL meal which can evoke potential immune responses (Veldkamp *et al*., 2022a; Mattiuzzo *et al*., 2007). Furthermore, insects are reported to contain immunostimulants such as chitin that can have positive effects on animal health (Gasco *et al*. 2018b). Low levels of chitin are known to have immunostimulatory effects in seabream by activating the innate immune response (Esteban *et al*., 2001). This is because chitin is considered as a potential prebiotic that could help to maintain a balanced and healthy gut microbiota (Ringø *et al*., 2006; Terova *et al*., 2019). The gut microbiota is involved in intestinal mucosa development and maturation, immunity, and disease resistance (Schroeder et al., 2018; Llewellyn *et al*. 2014). Thus, the improved immune responses and reduced stress observed in salmon fed BSFL meal (**Paper II**) can be linked to the presence of bioactive compounds such as antimicrobial peptides, and chitin. However, quantification of AMPs and chitin was not performed in the current study (**Paper II**). To conclude, dietary BSFL meal in the diet of salmon positively modulates the hematological parameters, improves the skin mucus matrix, and cytokine production in salmon.

If the stress encountered by fish is severe and is prolonged for a longer duration, the extent of such a high stress response could result in poor growth and survival (tertiary

responses). The salmon in the current study grew from ~170g to ~3.5kg reporting no negative effects on growth and thus validates its dietary inclusion up to 10% in salmon feed on a commercial scale. Our study along with the Eide *et al*. (2024) are among the firsts to take previous knowledge on BSFL meal to the industrial scale salmon farming level. The current study agrees with the trial results obtained by Eide *et al*. (2024), detecting no significant differences in weight gain and growth rate. The present study (**Paper II**), however lacks a concrete supporting data for the production parameters such as feed conversion ratio, specific growth rate, feed intake, as the study posed limitations regard to the sampling size and documenting the amount of feed and feces in open sea cages.

Mortality data provides an overview of a fish welfare state and low mortality in a fish population is said to be a minimum requirement for good welfare. Though no difference was observed in the mortality data among the different dietary groups, the current study witnessed a high mortality rate of ~34% (**Paper II**) towards the end of the trial, making the overall mortality higher than the industry average in Norway (~15%) in sea water production phase (Oliveira *et al*., 2021). This high mortality in the current study was due to infectious diseases and regular handling at the research location. It could also be due to the location of farming zone as the southwest coast is known for a higher mortality compared to the northern part of Norway (Oliveira *et al*., 2021). Another cause of mortality in Norwegian salmon farms is the treatment for lice and related handling involved in the processes. Numerous studies have reported increased mortalities related to delousing which could be a consequence of skin and gill injuries that initiated inflammation and high stress responses marked by elevated plasma cortisol levels in salmon (Helgesen *et al.*, 2021; Overton *et al*., 2019). However, in the current study, dietary BSFL meal was not able to reduce the mortality, though some interesting trend was observed in the immune responses in BSFL fed salmon as discussed. Thus, according to the results obtained in this study, it can be concluded that functional property of dietary BSFL meal can positively modulate the fish health for its better welfare and does not have detrimental effects on growth in commercial farm like conditions.

4.3 Effects of dietary BSFL meal on fillet quality

In the current thesis (**Paper III**), the dietary effect of BSFL meal on fillet quality traits was studied for possible effect on fillet physical, chemical, nutritional and sensory characteristics. Thus, assessing the fillet quality is important since dietary treatment (Thomassen 2007), and environmental factors (Chen, 2020) can have positive, negative or neutral effects on the fillet quality. The data in the current work provides evidence that dietary inclusion of BSFL meal up to 10%, did not affect the fillet physical, chemical and sensory qualities when fed BSFL-based diet in real farm conditions compared to control diet. However minor changes were observed in the nutritional indices in salmon fillet when fed with insect meal. The results are in agreement with previous study conducted using BSFL meal in rainbow trout (Borgogno *et al*., 2017; Bruni *et al*., 2020; Melenchón *et al*., 2021; St-Hilaire *et al*., 2007b). Moreover, studies on dietary inclusion of other novel ingredients such as Antarctic krill meal (Mørkøre, *et al*. 2020), microalgae and organic-mineral meal (Kousoulaki *et al*., 2016) and glutamate supplemented meal (Larsson *et al*., 2014) are known to enhance fillet quality (water holding capacity, texture, color, nutritional indices, and taste) in Atlantic salmon.

4.3.1 Dietary effect on water holding capacity, texture and color

Liquid losses/water holding capacity play an important role in the seafood industry since loss of water in terms of drip loss can reduce the weight and yield of fillet affecting the financial and consumer aspects. Incorporating a protein source such as insect meal up to 10% did not affect the liquid losses (drip loss% = 1.6±0.2) in salmon fillet compared to the control diet farmed in open sea cages (**Paper III**). However, in a study on salmon reared in similar environment, showed a higher drip loss when fed with a low protein-to-lipid (P/L) diet (drip loss% = 1.9±0.1) compared to fish fed with a high P/L diet (drip loss% = 1.6±0.1) (Chen, 2020). Softer texture and gaping (separation of muscle fibers) in the fish are considered quality challenges because they can negatively impact the overall quality of the fish and the consumer's experience. Collagen is the responsible protein for fillet firmness. Thus, soft texture of fillet is associated with an increase in soluble collagen and a decrease in insoluble collagen (Espe *et al*., 2004).

In the current study, dietary inclusion of BSFL meal up to 10% had neutral effect on salmon firmness (25.3±1.23 N) when performed texture analysis. Study by Larsson *et al*. (2014), reported that glutamate supplementation can result in firmer fillets in salmon after ice storage (10.1 N) and after frozen storage (8.7 N) (Larsson *et al*., 2014). Whereas studies conducted on krill meal demonstrated a reduction in gaping and increased firmness (~9 N) by supplementing salmon finishing diets composed of 12% krill meal, 5% FM, and 8% FO compared to dietary treatment with 15% FM and 10% FO (Mørkøre *et al*., 2020). Though dietary BSFL meal had no effect on fillet firmness, it is worth noting here that the value obtained in the current trial for fillet texture (**Paper III**) had a relatively higher value (24-25 N) than the above-mentioned studies. According to Torgersen *et al*. (2014), fillet firmness (breaking force, N) range of salmon were categorized as soft (6.6–7.5 N), high (13.1– 16.7 N) and hard (17.7–20.9 N) firmness. The harder texture of the fillet can be attributed to factors such as collagen content, aerobic metabolism, protein degradation (Torgersen *et al*., 2014) which may have occurred during storage.

Apart from the drip loss, and texture, color have received the most attention in the salmon market. As pale or unevenly distributed color is regarded as a downgrading factor because they can reduce the overall quality and market value of the salmon fillets. Salmon fed marine-based ingredients are known to have more astaxanthin pigmentation thereby characteristics red/pink color (Berntzen, 2016). In this work (**Paper III**), no difference in the fillet color was observed when fed BSFL-based diet in salmon. Similar results were reported by Gouveia *et al*. (2002), where fish fillets color was unaffected by the dietary intervention despite the astaxanthin dietary source provided by *Chlorella vulgaris* (Chlorophyta, Volvocales) in the gilthead sea bream fillets (Gouveia *et al*., 2002). However, dietary inclusion of ingredients such as krill meal increased the overall color (SalmoFan score ≥25), compared to FM group (Mørkøre *et al*., 2020). It should be noted that though there was no difference in fillet colorimetry, the value for yellowness index in the current study was different than the one reported by Bruni *et al* (2020a) irrespective of the experimental group. This could be attributed to factors such as storage duration and packaging (Chan *et al*.,2021).

4.3.2 Dietary effect on total fat, PUFA and nutritional indices

A strong relationship exists between the composition and properties of the diet and the nutritional traits in fish fillet (García *et al*., 2010; Randazzo *et al*., 2021; Tibaldi *et al*., 2015). In this study (**Paper III**), the fat content of Norwegian Quality Cut (NQC) fillet from salmon was 104±0.55 g/kg when fed BSFL-based diets. Atlantic salmon is regarded as a fatty fish usually having 60g/kg to 220g/kg fat content and an average of 150g/kg to 160g/kg standard fat level in NQC (Rørå, Kvåle *et al*. 1998). Such variations in the chemical composition of fish are closely related to the fish species and size, sexual variation, nutrition, living circumstances, catching season, and other environmental conditions (Shearer, 1994). For instance, the plant ingredients (particularly soybean meals) can reduce lipid absorption and retention in fish tissues (Hua and Bureau, 2009; Martínez-Llorens *et al*., 2007; Romarheim *et al*., 2008) whereas their partial replacement with BSFL meal and poultry by-product meals in graded levels (20% and 40%) in the feed for gilthead bream can improve the lipid absorption (Randazzo *et al*., 2021).

In monogastric animals such as fish, broilers and rabbit, the FA profile of the fillet/meat tends to have more saturated FA profile when fed BSFL meal diets (Zotte, 2021). Similar observation was made in the current study (**Paper III**), where dietary inclusion of BSFL meal had an increased level of SFA, such as lauric acid in the fillet fed BSFL meal at 10% (**Paper III**). A similar increase in SFA was observed in rainbow trout when fed full-fat mealworms at 25 and 50% inclusion replacing FM (Iaconisi *et al*., 2018). Further, no changes were observed in levels of PUFA in the fillet except for docosapentanoic acid (DPA), the level of which was reduced on the dietary inclusion of insect meal. This is because BSFL meal is not recognized as a good source of PUFA, and hence will not be reflected in the fish fillet. However, in a study conducted by Gard. (2023), salmon when fed diet supplemented with microalgae (*Schizochytrium sp*.) the chemical composition of the fillets had an increased level of oleic acid, linoleic acid and DHA in the fillet (Gard, 2023) as the algae is known for a rich source of PUFA. Thus, the limitation of high amounts of SFA in insect meal can be modulated by rearing them on nutrient rich substrate rich in PUFA. Such modulation of the nutrient composition

of BSFL meal has been reported by Liland *et al*. (2017) where, n-3 PUFA such as EPA concentrations were increased in the larvae when grown on media containing up to 50% *Ascophyllum nodosum* enriched with EPA (Liland *et al*., 2017).

The content of EPA and DHA were not affected by the diets, but the inclusion of partially defatted BSFL meal up to 10% inclusion had a significant effect on the unsaturation ratio due to the higher content of total SFA in insect meal-based diets (**Paper III**). A similar trend was observed when yellow meal worm was used in the diet of rainbow trout (Iaconisi *et al*., 2018). Moreover, other indices such as atherogenicity (AI) and thrombogenicity (TI) were higher with higher dietary inclusion of BSFL meal. It was studied that reduction in fish oil in the diet can reduce the n-3 PUFA levels in fillet and can affect the overall nutritional indices (Zarantoniello *et al*., 2022). Thus, the reasons for the observed changes in the nutritional indices in the current study (**Paper III**) is due to the reduction of fish oil in the diet while using BSFL meal to make the diet iso-lipidic, and iso-energetic. Similar increase in these indices were observed in rainbow trout when replaced up to 50% fish meal with partially defatted BSFL meal (Bruni *et al*., 2020b). The substitution of marine materials with alternate ingredients does not necessarily have a negative effect on salmon fillet quality but there may be subtle changes in salmon product quality due to ingredient substitution. For example, a study found that the levels of EPA and DHA in farmed Scottish salmon declined over time due to the substitution of fish oil with terrestrial oils (Sprague *et al*., 2016). It is important to assess the quality of fillets resulting from newly developed diets. However, given the small magnitude of this difference in the estimated nutritional indices (**Paper III**), which were within the limits for salmon, states no negative effect of the dietary BSFL meal on salmon nutritional indices.

4.3.3 Dietary effect on sensory attributes

The sensory testing conducted on the salmon fillet (**Paper III**) was unable to detect any difference between the control and BSFL-based diets. In case of fish, similar results were obtained in studies involving trained and non-trained panelists in Atlantic salmon where partial or total replacement of dietary FM with BSFL did not impair the physico-chemical quality of the fillet (Bruni *et al*., 2020a; Lock *et al*., 2016). Odor is an

important parameter in sensory evaluation of a food product. A notable rancid odor and metallic taste were found associated with BSFL-based diet (**Paper III**). The 'typical' aroma of fish is known to be associated with volatile compounds that are formed as a result of the oxidation of n-3 PUFA by specific lipoxygenases (German *et al*., 1985; Lindsay, 2007; Milo and Grosch, 1995). Thus, any change from 'typical' to bitter or sour taste was suggested as an indication of protein/lipid degradation of food due to storage period.

The chewiness of the fillet was not significantly different between BSFL-based diet and control diet (**Paper III**). Similarly, in other feeding trials, the fillet sensory attributes of salmonids were not affected by dietary inclusion of BSFL meal in the diets (Lock *et al*., 2016; Sealey *et al*., 2011). However, in a study by Kotzamanis *et al*. (2020), the hardness and chewiness of the fillets from seabass were higher in fish supplemented with taurine at 10 or 20g/kg when compared to low FM diet (150g/kg). Further in the same study a blind panel test showed that seabass fillets had no significant differences in taste, elasticity, cohesiveness, or likeness among the dietary treatments supplemented with taurine (Kotzamanis *et al*., 2020). Apart from aquatic species, the use of BSFL meal has been used as a feed ingredient for terrestrial animal such as broiler and observed no difference in meat physical traits, proximate composition, and sensory evaluation from that obtained from control diets. While the studies conducted on rabbit fed the defatted BSFL meal had difference in meat color (increased redness) and oxidative stability (lower thiobarbituric acid reactive substances). Altogether, in the present thesis, the aesthetic quality of fillet was not affected by the dietary inclusion of BSFL meal up to 10% in Atlantic salmon when reared in real farm.

4.4 BSFL meal in salmon feed and sustainability

The Norwegian aquaculture sector aims for a sustainable feed production in coming years. Feed is the most contributing factor to carbon footprint for Norwegian salmon production. According to SINTEF report, 1kg of salmon feed has a CO_2 equivalent of 2.3kg and this accounts for 75-80% of farmed gate of CO_2 emissions in Atlantic salmon production. It has been suggested that major gains in terms of reducing CO_2 footprints

of farmed salmon can be achieved through improved feed efficiency and use of novel ingredients. Insect meal and algae oil as novel alternatives can reduce CO_2 footprint by 2% (SINTEF report). In this regard, the current thesis (**Paper II**), evaluated inclusion of BSFL meal at 5 and 10% by replacing SPC, and guar protein. The biological performance of the insect meal diets was on par with the control (**Paper II**). In addition, the quality of the product was also not compromised in the insect fed diet group (**Paper III**). Further, the inclusion of BSFL meal replacing plant proteins such as SPC, and guar proteins resulted in appreciable reduction in CO_2 footprint of the feed.

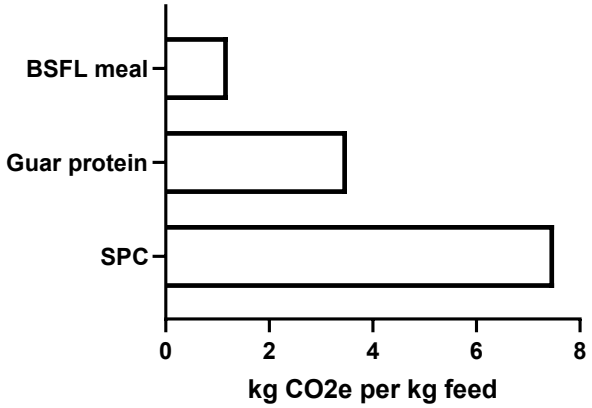


Figure 8: CO_2e (kg /kg feed) of black soldier fly larvae (BSFL) meal, guar protein and soy protein concentrate (SPC)

Although it was not possible to calculate the CO_2 equivalent of the control feed, using the CO_2 equivalent values for SPC, guar protein and BSFL meal, we could achieve a relative reduction of 0.15 to 0.35kg CO_2 equivalent per kg feed with BSFL5% and BSFL10%, respectively. This corresponds to a reduction of 6-15% in CO_2 emissions relative to 2.3kg CO_2 equivalent per kg feed in present Norwegian salmon farming (Figs. 8 and 9).

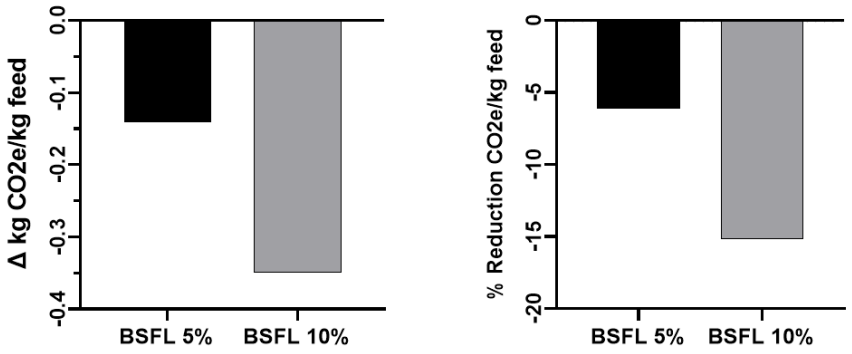


Figure 9: Reduction in CO_2e per kg feed when included BSFL meal (BSFL5% and BSFL10%) compared to the control diet (Control).

Typically, commercial feed formulations are computed considering least-cost calculations, without compromising the nutrient content, and optimal inclusion levels affecting performance of the species. Since different feed ingredients, their composition, and inclusion levels can significantly contribute to the carbon footprint of the feed, considering the CO2e per kg feed in the diet formulations is required. As observed from the current study, inclusion of insect meal can help reduce CO2e from the feed. However, key challenges faced by the insect sector lie in the small production volumes and high cost (2,500 and 5,500 €/t) and not competitive when compared to fishmeal (1,200-1,600 €/t) or soybean meal (450-700 €/t) (Mancuso *et al*., 2019; Pippinato *et al*., 2020). Considering that Norwegian salmon farming consumed 1.89 MT of feed, the inclusion up to 10% of insect meal would require 189000 tonnes which are levels far above the current insect production, when considering the current European production (Gasco *et al*., 2023). The gap between requirement and availability of novel ingredients thus challenge in reducing carbon footprint of salmon feed.

5. Concluding Remarks

The present work investigated *(i)* the digestibility/solubility of black soldier fly larvae meals in Atlantic salmon *in vivo* and *in vitro* and *(ii)* demonstrated the functionality of this ingredient in salmon reared for the entire sea-water production phase in real farm like conditions.

The main findings are highlighted as follows:

- BSFL meal and its diets had high protein and amino acid digestibility *in vivo* (>80%).
- *In vitro* analysis using salmon enzyme and commercial enzyme shows that they can be standardized based on their protein hydrolysis capacity (similar DH%) but deviates from the AA solubility values.
- In general, commercial enzymes had a higher AA solubility than salmon for BSFL meal and diets. Also, AA with lower molecular weights had higher solubility than aromatic or higher carbon AA.
- A comparison between *in vivo* digestibility and *in vitro* solubility of BSFL meal and diets tested was demonstrated. *In vitro* methodology using salmon enzyme in the current thesis showed a good correlation for protein solubility with *in vivo*. However, no correlation was found between amino acid solubility *in vivo* and *in vitro*.
- The functional properties of BSFL meal could positively influence the welfare of farmed salmon based on effects observed in skin mucus, and pro-inflammatory cytokines in skin and gill tissues, as well as related to stress handling.
- The dietary inclusion of BSFL meal does not negatively affect the fillet quality of salmon. Nutritional quality indices, and sensory attributes such as taste and odor could be affected by increasing BSFL meal in the diet.

6. Future Perspectives

The main objective of this work was to demonstrate the suitability and the functionality of BSFL meal as feed ingredient for Atlantic salmon reared in real farm-like conditions. The results of this work demonstrated the importance of using insect meals as the future sustainable feed ingredients for aquacultured fish species. In the recently devised community mission strategy on sustainable feed, it has been proposed to reduce the climate footprint of the amount of feed raw materials imported for farmed fish and livestock in Norway by 55 percent points by 2034, with an estimated annual reduction of at least 5 percentage points from 2026. Further, all feed for livestock and farmed fish in Norway should be from sustainable sources by 2034. However, now only 0.4% of the ingredients used in Norwegian aquafeed come from sustainable feed ingredients, which also suffer from significant cost and sustainability challenges. Therefore, upscaling the insect production for aquafeed is necessary in coming years.

More specifically, the work done during this Ph.D. aimed at adopting *in vitro* methods based on the 3 Rs to measure the protein digestibility of fish feed ingredients. *In vitro* methods can be used as rapid screening tool to test the digestibility of feed ingredients based on their protein hydrolysis data. However, the method holds limitations regarding its reproducibility and better correlation with *in vivo*. There exists a large variation in protein hydrolysis data obtained for the same ingredients using different methods. Therefore, the utilization of a standardized protocol or models capable of generating data that complements *in vivo* studies is essential. Implementation of this approach requires the compilation of extensive datasets incorporating real values from *in vivo* experiments, biological factors, and the fish species used, among other considerations.

Furthermore, it would be interesting to know how fish enzyme and commercial enzyme are different in cleaving the same protein/peptide chain when subjected to species specific gastrointestinal model. Since studies have been suggesting the differences in catalytic efficiency and sensitivity to inhibitors as some of the reasons underlying the differences. Thus, future research on enzyme properties and its catalysis efficiency in gut simulated models can help in improving standardization of fish enzymes *in vitro*.

Extensive research exists on the health promoting effect of BSFL meal in different species of fish. However, the exact mechanism behind the health promoting effect of chitin, lauric acid or antimicrobial peptides on improving health still lacks clarity. Though the blame on chitin exists for exerting its antinutrient property on digestibility, it has also been addressed as an immunostimulant in modulating health and immune response. Thus, knowledge on how exactly these bioactive compounds modulate different innate and specific responses in fish is required to provide information on better mechanism of action of these compounds.

Furthermore, it is reported that salmon fillet fed BSFL meal is associated with metallic/rancid odor. This property is associated with 'external (storage)/internal (nutrient interaction) factors. Thus, future studies on investigating why these sensory traits are affected by insect meal and if it is possible to improve the fillet property with the help of available research in reducing rancidity or oxidation such as use of chitosan-based nanoparticles which are reported to reduce lipid oxidation in fish fillet.

7. References

Abdel-Tawwab, M., Khalil, R.H., Metwally, A.A., Shakweer, M.S., Khallaf, M.A. and Abdel-Latif, H.M.R., 2020. Effects of black soldier fly (*Hermetia illucens* L.) larvae meal on growth performance, organs-somatic indices, body composition, and hemato-biochemical variables of European sea bass, *Dicentrarchus labrax*. Aquaculture 522: 735136. https://doi.org/10.1016/j.aquaculture.2020.735136

Abo El-Ward, A., Eid, A.E., Mohamed, K.A., Abd-elfattah, B. and Hasan, M.A., 2016. Growth performance of Nile tilapia (*Oreochromis niloticus*) fingerlings fed diet supplemented with different of *Spirulina platensis* levels. Egyptian Journal of Animal Production 53: 181-190.

Alarcón, F.J., Moyano, F.J. and Díaz, M., 2002. Evaluation of different protein sources for aquafeeds by an optimised pH-stat system. Journal of the Science of Food and Agriculture 82: 697-704. https://doi.org/10.1002/jsfa.1100

Alexander, J.B. and Ingram, G.A., 1992. Noncellular nonspecific defence mechanisms of fish. Annual Review of Fish Diseases 2: 249-279.

Altintzoglou, T., Cordeiro, C.M., Honkanen, P. and Onozaka, Y., 2022. "It gives me peace of mind". A new perspective on the identification of quality cues on salmon fillet products in Japan and the USA. Aquaculture 554: 738112. https://doi.org/10.1016/j.aquaculture.2022.738112

Alvarez, F.J., 2014. The effect of chitin size, shape, source and purification method on immune recognition. Molecules 19: 4433-4451. 10.3390/molecules19044433

Ángeles Esteban, M., 2012. An overview of the immunological defenses in fish skin. International scholarly research notices 2012.

Aragão, C., Cabano, M., Colen, R., Fuentes, J. and Dias, J., 2020. Alternative formulations for gilthead seabream diets: Towards a more sustainable production. Aquaculture Nutrition 26: 444-455.

Ásgeirsson, B. and Bjarnason, J.B., 1993. Properties of elastase from Atlantic cod a cold-adapted proteinase. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology 1164: 91-100. https://doi.org/10.1016/0167-4838(93)90116-9

Ash, R., 1985. Protein digestion and absorption. Nutrition and feeding in fish.: 69-93.

Austreng, E., 1978. Digestibility determination in fish using chromic oxide marking and analysis of contents from different segments of the gastrointestinal tract. Aquaculture 13: 265-272. https://doi.org/10.1016/0044-8486(78)90008-X

Bahuaud, D., Gaarder, M., Veiseth-Kent, E. and Thomassen, M., 2010. Fillet texture and protease activities in different families of farmed Atlantic salmon (*Salmo salar* L.). Aquaculture 310: 213-220. https://doi.org/10.1016/j.aquaculture.2010.10.008

Bakke, A.M., Glover, C. and Krogdahl, Å., 2010. 2 - Feeding, digestion and absorption of nutrients. In: M. Grosell, A.P. Farrell and C.J. Brauner (Eds.), Fish Physiology. Academic Press, pp. 57-110. https://doi.org/10.1016/S1546-5098(10)03002-5

Bakke-McKellep, A.M., Nordrum, S., Krogdahl, Å. and Buddington, R.K., 2000. Absorption of glucose, amino acids, and dipeptides by the intestines of Atlantic salmon (*Salmo salar* L.). Fish Physiol Biochem 22: 33-44.

Balcombe, J., 2016. What a fish knows: The inner lives of our underwater cousins. Scientific American/Farrar, Straus and Giroux.

Barasa, J.E., Mukhongo, P.N. and Ngetich, C.C., 2022. Perspectives on Salmon Aquaculture: Current Status, Challenges and Genetic Improvement for Future Growth. Salmon Aquaculture.

Belforti, M., Gai, F., Lussiana, C., Renna, M., Malfatto, V., Rotolo, L., De Marco, M., Dabbou, S., Schiavone, A., Zoccarato, I. and Gasco, L., 2015. *Tenebrio molitor* Meal in Rainbow Trout (*Oncorhynchus Mykiss*) Diets: Effects on Animal Performance, Nutrient Digestibility and Chemical Composition of Fillets. Italian Journal of Animal Science 14: 4170. 10.4081/ijas.2015.4170

Belghit, I., Liland, N.S., Gjesdal, P., Biancarosa, I., Menchetti, E., Li, Y., Waagbø, R., Krogdahl, Å. and Lock, E.-J., 2019a. Black soldier fly larvae meal can replace fish meal in diets of sea-water phase Atlantic salmon (*Salmo salar*). Aquaculture 503: 609-619. https://doi.org/10.1016/j.aquaculture.2018.12.032

Belghit, I., Liland, N.S., Waagbø, R., Biancarosa, I., Pelusio, N., Li, Y., Krogdahl, Å. and Lock, E.-J., 2018. Potential of insect-based diets for Atlantic salmon (*Salmo salar*). Aquaculture 491: 72-81. https://doi.org/10.1016/j.aquaculture.2018.03.016

Belghit, I., Waagbø, R., Lock, E.-J. and Liland, N.S., 2019b. Insect-based diets high in lauric acid reduce liver lipids in freshwater Atlantic salmon. Aquaculture Nutrition 25: 343-357. https://doi.org/10.1111/anu.12860

Berntzen, J., 2016. Feeding Strategy and Effect on Fillet Quality of Atlantic Salmon (*Salmo salar*).

Borderías, A.J., Lamua, M. and Tejada, M., 1983. Texture analysis of fish fillets and minced fish by both sensory and instrumental methods. International Journal of Food Science & Technology 18: 85-95.

Borgogno, M., Dinnella, C., Iaconisi, V., Fusi, R., Scarpaleggia, C., Schiavone, A., Monteleone, E., Gasco, L. and Parisi, G., 2017. Inclusion of *Hermetia illucens* larvae meal on rainbow trout (*Oncorhynchus Mykiss*) feed: effect on sensory profile according to static and dynamic evaluations. Journal of the Science of Food and Agriculture 97: 3402-3411. https://doi.org/10.1002/jsfa.8191

Botta, J.R., 1996. Evaluation of seafood freshness quality. John Wiley & Sons.

Bourne, M.C., Kenny, J.F. and Barnard, J., 1978. Computer-assisted readout of data from texture profile analysis curves 1. Journal of Texture Studies 9: 481-494.

Boyd, C.E., D'Abramo, L.R., Glencross, B.D., Huyben, D.C., Juarez, L.M., Lockwood, G.S., McNevin, A.A., Tacon, A.G.J., Teletchea, F., Tomasso Jr, J.R., Tucker, C.S. and Valenti, W.C., 2020. Achieving sustainable aquaculture: Historical and current perspectives and future needs and challenges. Journal of the World Aquaculture Society 51: 578-633. https://doi.org/10.1111/jwas.12714

Brammer, C.A. and von Dohlen, C.D., 2007. Evolutionary history of Stratiomyidae (Insecta: Diptera): The molecular phylogeny of a diverse family of flies. Molecular Phylogenetics and Evolution 43: 660-673. https://doi.org/10.1016/j.ympev.2006.09.006

Bremner, H.A., 2000. Toward Practical Definitions of Quality for Food Science. Critical Reviews in Food Science and Nutrition 40: 83-90. 10.1080/10408690091189284

Bruni, L., 2020. Innovative protein sources in feed for salmonids: Effects on lipid metabolism, gut microbiota and fillet quality.

Bruni, L., Belghit, I., Lock, E.-J., Secci, G., Taiti, C. and Parisi, G., 2020a. Total replacement of dietary fish meal with black soldier fly (*Hermetia illucens*) larvae does not impair physical, chemical or volatile composition of farmed Atlantic salmon (*Salmo salar* L.). Journal of the Science of Food and Agriculture 100: 1038-1047. https://doi.org/10.1002/jsfa.10108

Bruni, L., Randazzo, B., Cardinaletti, G., Zarantoniello, M., Mina, F., Secci, G., Tulli, F., Olivotto, I. and Parisi, G., 2020b. Dietary inclusion of full-fat *Hermetia illucens* prepupae meal in practical diets for rainbow trout (*Oncorhynchus Mykiss*): Lipid metabolism and fillet quality investigations. Aquaculture 529: 735678. https://doi.org/10.1016/j.aquaculture.2020.735678

Bui, S., Skern-Mauritzen, R., Saito, T. and Thompson, C., 2023. Optimising delousing strategies: developing best practice recommendations for maximal efficacy and positive welfare-Final Report. Rapport fra havforskningen.

Burrells, C., Williams, P.D., Southgate, P.J. and Wadsworth, S.L., 2001. Dietary nucleotides: a novel supplement in fish feeds: 2. Effects on vaccination, salt water transfer, growth rates and physiology of Atlantic salmon (*Salmo salar* L.). Aquaculture 199: 171-184. https://doi.org/10.1016/S0044-8486(01)00576-2

Cadillo-Benalcazar, J.J., Giampietro, M., Bukkens, S.G.F. and Strand, R., 2020. Multi-scale integrated evaluation of the sustainability of large-scale use of alternative feeds in salmon aquaculture. Journal of Cleaner Production 248: 119210. https://doi.org/10.1016/j.jclepro.2019.119210

Cain, K.D., Grabowski, L., Reilly, J. and Lytwyn, M., 2003. Immunomodulatory effects of a bacterial-derived β-1, 3 glucan administered to tilapia (*Oreochromis nilotocus* L.) in a Spirulina-based diet. Aquaculture Research 34: 1241-1244.

Carter, C.G., Houlihan, D.F. and McCarthy, I.D., 1992. Feed utilization efficiencies of Atlantic salmon (*Salmo salar* L.) parr: effect of a single supplementary enzyme. Comparative Biochemistry and Physiology Part A: Physiology 101: 369-374.

Castillo-Lopez, E., Espinoza-Villegas, R.E. and Viana, M.T., 2016. In vitro digestion comparison from fish and poultry by-product meals from simulated digestive process at different times of the Pacific Bluefin tuna, *Thunnus orientalis*. Aquaculture 458: 187-194.

Chaklader, M.R., Siddik, M.A.B. and Fotedar, R., 2020. Total replacement of fishmeal with poultry by-product meal affected the growth, muscle quality, histological structure, antioxidant capacity and immune response of juvenile barramundi, *Lates calcarifer*. PloS one 15: e0242079.

Chaklader, M.R., Siddik, M.A.B., Fotedar, R. and Howieson, J., 2019. Insect larvae, *Hermetia illucens* in poultry by-product meal for barramundi, *Lates calcarifer* modulates histomorphology, immunity and resistance to *Vibrio harveyi*. Scientific Reports 9: 1-15.

Chan, S.S., Roth, B., Skare, M., Hernar, M., Jessen, F., Løvdal, T., Jakobsen, A.N. and Lerfall, J., 2020. Effect of chilling technologies on water holding properties and other quality parameters throughout the whole value chain: From whole fish to cold-smoked fillets of Atlantic salmon (*Salmo salar*). Aquaculture 526: 735381. https://doi.org/10.1016/j.aquaculture.2020.735381

Chemello, G., Renna, M., Caimi, C., Guerreiro, I., Oliva-Teles, A., Enes, P., Biasato, I., Schiavone, A., Gai, F. and Gasco, L., 2020. Partially Defatted *Tenebrio molitor* Larva Meal in Diets for Grow-Out Rainbow Trout, *Oncorhynchus Mykiss* (Walbaum): Effects on Growth Performance, Diet Digestibility and Metabolic Responses. Animals (Basel) 10. 10.3390/ani10020229

Chen, X., 2020. Fillet quality of Atlantic salmon (*Salmo salar*) as affected by dietary and environmental treatments.

Cheng, J.-H., Sun, D.-W., Han, Z. and Zeng, X.-A., 2014. Texture and Structure Measurements and Analyses for Evaluation of Fish and Fillet Freshness Quality: A Review. Comprehensive Reviews in Food Science and Food Safety 13: 52-61. https://doi.org/10.1111/1541-4337.12043

Cho, C.Y., 1979. Apparent digestibility measurement in feedstuffs for rainbow trout. Finfish nutrition and fishfeed technology.

Church, F.C., Swaisgood, H.E., Porter, D.H. and Catignani, G.L., 1983. Spectrophotometric Assay Using o-Phthaldialdehyde for Determination of Proteolysis in Milk and Isolated Milk Proteins1. Journal of Dairy Science 66: 1219-1227. https://doi.org/10.3168/jds.S0022-0302(83)81926-2

Cian, R.E., Bacchetta, C., Cazenave, J. and Drago, S.R., 2018. In vitro assays predicts mineral retention and apparent protein digestibility of different fish feed

measured using a juvenile *P. mesopotamicus* model. Aquaculture Research 49: 2267-2277.

Collie, N.L. and Ferraris, R.P., 1995. Nutrient fluxes and regulation in fish intestine, Biochemistry and molecular biology of fishes. Elsevier, pp. 221-239.

Committee, E.S., 2015. Statement on the benefits of fish/seafood consumption compared to the risks of methylmercury in fish/seafood. EFSA journal 13: 3982. https://doi.org/10.2903/j.efsa.2015.3982

Cummins, V.C., Rawles, S.D., Thompson, K.R., Velasquez, A., Kobayashi, Y., Hager, J. and Webster, C.D., 2017. Evaluation of black soldier fly (*Hermetia illucens*) larvae meal as partial or total replacement of marine fish meal in practical diets for Pacific white shrimp (*Litopenaeus vannamei*). Aquaculture 473: 337-344. https://doi.org/10.1016/j.aquaculture.2017.02.022

De la Noüe, J. and Choubert, G., 1986. Digestibility in rainbow trout: comparison of the direct and indirect methods of measurement. The Progressive Fish-Culturist 48: 190-195.

De Marco, M., Martínez, S., Hernandez, F., Madrid, J., Gai, F., Rotolo, L., Belforti, M., Bergero, D., Katz, H. and Dabbou, S., 2015. Nutritional value of two insect larval meals (*Tenebrio molitor* and *Hermetia illucens*) for broiler chickens: Apparent nutrient digestibility, apparent ileal amino acid digestibility and apparent metabolizable energy. Animal Feed Science and Technology 209: 211-218.

De Smet, J., Wynants, E., Cos, P. and Van Campenhout, L., 2018. Microbial Community Dynamics during Rearing of Black Soldier Fly Larvae (*Hermetia illucens*) and Impact on Exploitation Potential. Applied and Environmental Microbiology 84: e02722-02717. 10.1128/AEM.02722-17

Demers, N.E. and Bayne, C.J., 1997. The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. Developmental & Comparative Immunology 21: 363-373. https://doi.org/10.1016/S0145-305X(97)00009-8

Devic, E., Leschen, W., Murray, F. and Little, D.C., 2018. Growth performance, feed utilization and body composition of advanced nursing Nile tilapia (*Oreochromis niloticus*) fed diets containing Black Soldier Fly (*Hermetia illucens*) larvae meal. Aquaculture Nutrition 24: 416-423.

Dheke, S., 2013. Growth Response of Rainbow Trout (*Oncorhynchus Mykiss*) on Substitution of Shrimp Meal by Different Protien Sources.

Diener, S., Zurbrügg, C., Gutiérrez, F.R., Nguyen, D.H., Morel, A., Koottatep, T. and Tockner, K., 2011. Black soldier fly larvae for organic waste treatment-prospects and constraints. Proceedings of the WasteSafe 2: 13-15.

Dierenfeld, E.S. and King, J., 2008. Digestibility and Mineral Availability of Phoenix Worms, *Hermetia illucens* , Ingested by Mountain Chicken Frogs, Leptodactylus fallax. Journal of Herpetological Medicine and Surgery 18: 100-105. 10.5818/1529-9651.18.3-4.100

Dietz, C. and Liebert, F., 2018. Does graded substitution of soy protein concentrate by an insect meal respond on growth and N-utilization in Nile tilapia (*Oreochromis niloticus*)? Aquaculture Reports 12: 43-48.

Dimes, L.E. and Haard, N.F., 1994. Estimation of protein digestibility—I. Development of an in vitro method for estimating protein digestibility in salmonids (*Salmo gairdneri*). Comparative Biochemistry and Physiology Part A: Physiology 108: 349-362. https://doi.org/10.1016/0300-9629(94)90106-6

Divakaran, S., Forster, I.P. and Velasco, M., 2004. Limitations on the use of shrimp Litopenaeus vannamei midgut gland extract for the measurement of in vitro protein digestibility. Aquaculture 239: 323-329. https://doi.org/10.1016/j.aquaculture.2004.06.004

Djordjevic, B., Morales-Lange, B., McLean Press, C., Olson, J., Lagos, L., Mercado, L. and Øverland, M., 2021. Comparison of Circulating Markers and Mucosal Immune Parameters from Skin and Distal Intestine of Atlantic Salmon in Two Models of Acute Stress, International Journal of Molecular Sciences. 10.3390/ijms22031028

Duan, X.-D., Feng, L., Jiang, W.-D., Wu, P., Liu, Y., Jiang, J., Tan, B.-P., Yang, Q.-H., Kuang, S.-Y. and Tang, L., 2020. The dynamic process of dietary soybean β-conglycinin in digestion, absorption, and metabolism among different intestinal segments in grass carp (*Ctenopharyngodon idella*). Fish Physiol Biochem 46: 1361-1374.

Dumas, A., Raggi, T., Barkhouse, J., Lewis, E. and Weltzien, E., 2018. The oil fraction and partially defatted meal of black soldier fly larvae (*Hermetia illucens*) affect differently growth performance, feed efficiency, nutrient deposition, blood glucose and lipid digestibility of rainbow trout (*Oncorhynchus Mykiss*). Aquaculture 492: 24-34. https://doi.org/10.1016/j.aquaculture.2018.03.038

Eggink, K.M., Lund, I., Pedersen, P.B., Hansen, B.W. and Dalsgaard, J., 2022a. Biowaste and by-products as rearing substrates for black soldier fly (*Hermetia illucens*) larvae: Effects on larval body composition and performance. PloS one 17: e0275213. 10.1371/journal.pone.0275213

Eggink, K.M., Pedersen, P.B., Lund, I. and Dalsgaard, J., 2022b. Chitin digestibility and intestinal exochitinase activity in Nile tilapia and rainbow trout fed different black soldier fly larvae meal size fractions. Aquaculture Research 53: 5536-5546. https://doi.org/10.1111/are.16035

Eide, L.H., Rocha, S.D.C., Morales-Lange, B., Kuiper, R.V., Dale, O.B., Djordjevic, B., Hooft, J.M. and Øverland, M., 2023. Black soldier fly larvae (*Hermetia illucens*) meal is a viable protein source for Atlantic salmon (*Salmo salar*) during a large-scale controlled field trial under commercial-like conditions. Aquaculture: 740194. https://doi.org/10.1016/j.aquaculture.2023.740194

Einarsson, S. and Davies, P.S., 1996. On the localisation and ultrastructure of pepsinogen, trypsinogen and chymotrypsinogen secreting cells in the Atlantic salmon, *Salmo salar* L. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 114: 295-301.

Einarsson, S., Davies, P.S. and Talbot, C., 1996. The effect of feeding on the secretion of pepsin, trypsin and chymotrypsin in the Atlantic salmon, *Salmo salar* L. Fish Physiol Biochem 15: 439-446.

Elbahnaswy, S. and Elshopakey, G.E., 2023. Recent progress in practical applications of a potential carotenoid astaxanthin in aquaculture industry: a review. Fish Physiol Biochem. 10.1007/s10695-022-01167-0

Elia, A.C., Capucchio, M.T., Caldaroni, B., Magara, G., Dörr, A.J.M., Biasato, I., Biasibetti, E., Righetti, M., Pastorino, P., Prearo, M., Gai, F., Schiavone, A. and Gasco, L., 2018. Influence of *Hermetia illucens* meal dietary inclusion on the histological traits, gut mucin composition and the oxidative stress biomarkers in rainbow trout (*Oncorhynchus Mykiss*). Aquaculture 496: 50-57. https://doi.org/10.1016/j.aquaculture.2018.07.009

Ellis, A.E., 2001. Innate host defense mechanisms of fish against viruses and bacteria. Developmental & Comparative Immunology 25: 827-839.

Ellis, T., Yildiz, H.Y., López-Olmeda, J., Spedicato, M.T., Tort, L., Øverli, Ø. and Martins, C.I.M., 2012. Cortisol and finfish welfare. Fish Physiol Biochem 38: 163-188. 10.1007/s10695-011-9568-y

English, G., Wanger, G. and Colombo, S.M., 2021. A review of advancements in black soldier fly (*Hermetia illucens*) production for dietary inclusion in salmonid feeds. Journal of Agriculture and Food Research 5: 100164. https://doi.org/10.1016/j.jafr.2021.100164

Espe, M., Ruohonen, K., Bjørnevik, M., Frøyland, L., Nortvedt, R. and Kiessling, A., 2004. Interactions between ice storage time, collagen composition, gaping and textural properties in farmed salmon muscle harvested at different times of the year. Aquaculture 240: 489-504.

Esteban, M.A., Cuesta, A., Ortuño, J. and Meseguer, J., 2001. Immunomodulatory effects of dietary intake of chitin on gilthead seabream (*Sparus aurata* L.) innate immune system. Fish Shellfish Immunol 11: 303-315. https://doi.org/10.1006/fsim.2000.0315

Eur-Lex, European Commission 2022 (Access on 2022, November 17) Available online: https:// ec. europa. eu/ envir onment/ chemi cals/ lab_ anima ls/ pdf/ SWD20 19_ Part_A_ and_B. pdf

European Union (EU), 2017. Commission Regulation (EU) 2017/893 of 24 May 2017 amending Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council and Annexes X, XIV and XV to Commission Regulation (EU) No 142/2011 as regards the provisions on processed animal

protein. Official Journal of the European Union L 138: 92. Available at: https://tinyurl.com/uzt956aw

Ezquerra, J.M., García-Carreño, F.L. and Carrillo, O., 1998. In vitro digestibility of dietary protein sources for white shrimp (*Penaeus vannamei*). Aquaculture 163: 123-136. https://doi.org/10.1016/S0044-8486(98)00217-8

Fairweather-Tait, S.J. and Southon, S., 2003. BIOAVAILABILITY OF NUTRIENTS. In: B. Caballero (Ed.), Encyclopedia of Food Sciences and Nutrition (Second Edition). Academic Press, Oxford, pp. 478-484. https://doi.org/10.1016/B0-12-227055-X/00096-1

Fanouraki, E., Mylonas, C.C., Papandroulakis, N. and Pavlidis, M., 2011. Species specificity in the magnitude and duration of the acute stress response in Mediterranean marine fish in culture. General and comparative endocrinology 173: 313-322.

FAO (2022) Fishery and aquaculture statistics global aquaculture production 1950–2020 (FishStatJ) In: FAO fisheries and aquaculture division online. rome updated 2022.wwWfaOorg/fishery/statistics/software/fishstatj/en

FAO, F., 2020. Agriculture Organization of the United Nations The state of world fisheries and aquaculture 2020: Sustainability in action. Rome: Food and Agriculture Organization of the United Nations: 1-244.

FAO-UN. 2020. The state of world fisheries and aquaculture 2020: Sustainability in action. Food and 305 Agriculture Organization of the United Nations.Feed 7: 743-759.

Fevolden, S.E., Røed, K.H. and Gjerde, B., 1994. Genetic components of post-stress cortisol and lysozyme activity in Atlantic salmon; correlations to disease resistance. Fish Shellfish Immunol 4: 507-519.

Finke, M.D., 2013. Complete nutrient content of four species of feeder insects. Zoo biology 32: 27-36.

Fisher, H.J., Collins, S.A., Hanson, C., Mason, B., Colombo, S.M. and Anderson, D.M., 2020. Black soldier fly larvae meal as a protein source in low fish meal diets for Atlantic salmon (*Salmo salar*). Aquaculture 521: 734978. https://doi.org/10.1016/j.aquaculture.2020.734978

Fleming, A. and Allison, V.D., 1922. Observations on a bacteriolytic substance ("lysozyme") found in secretions and tissues. British journal of experimental pathology 3: 252.

Fleming, A., 1922. On a remarkable bacteriolytic element found in tissues and secretions. Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character 93: 306-317.

Fletcher, T.C., 1997. Dietary effects on stress and health. Fish stress and health in aquaculture 62: 223-246.

García, J.R., Kalinowski, C.T.H., Izquierdo, M.S.L. and Robaina, L.E.R., 2010. Marine and freshwater crab meals in diets for red porgy (*Pagrus pagrus*): Effect on growth, fish composition and skin colour. Aquaculture Research 41: 1759-1769.

Gard, S.E., 2023. Skin health and product quality of Atlantic Salmon (*Salmo salar* L.) fed increased LC-PUFAs from micro algae (*Schizochytrium sp*.).

Gasco, L., Acuti, G., Bani, P., Dalle Zotte, A., Danieli, P.P., De Angelis, A., Fortina, R., Marino, R., Parisi, G., Piccolo, G., Pinotti, L., Prandini, A., Schiavone, A., Terova, G., Tulli, F. and Roncarati, A., 2020. Insect and fish by-products as sustainable alternatives to conventional animal proteins in animal nutrition. Italian Journal of Animal Science 19: 360-372. 10.1080/1828051X.2020.1743209

Gasco, L., Belforti, M., Rotolo, L., Lussiana, C., Parisi, G., Terova, G., Roncarati, A. and Gai, F., 2014. Mealworm (*Tenebrio molitor*) as a potential ingredient in practical diets for rainbow trout (*Oncorhynchus Mykiss*), Insect to feed the world. Wageningen UR, pp. 69-69.

Gasco, L., Biasato, I., Dabbou, S., Schiavone, A. and Gai, F., 2019. Animals Fed Insect-Based Diets: State-of-the-Art on Digestibility, Performance and Product Quality, Animals. 10.3390/ani9040170

Gasco, L., Oddon, S.B., Vandenberg, G.W., Veldkamp, T. and Biasato, I., 2023. Factors affecting the decision-making process of using insect-based products in animal feed formulations. Journal of Insects as Food and Feed: 1-12. 10.3920/JIFF2022.0164

German, J.B., Chen, S.E. and Kinsella, J.E., 1985. Lipid oxidation in fish tissue. Enzymic initiation via lipoxygenase. Journal of Agricultural and Food Chemistry 33: 680-683.

Gilannejad, N., Martínez-Rodríguez, G., Yúfera, M. and Moyano, F.J., 2017. Estimating the effect of different factors on the digestive bioaccessibility of protein by the Senegalese sole (*Solea senegalensis*); combination of response surface methodology and in vitro assays. Aquaculture 477: 28-34. https://doi.org/10.1016/j.aquaculture.2017.04.037

Gilannejad, N., Martínez-Rodríguez, G., Yúfera, M. and Moyano, F.J., 2018. Modelling digestive hydrolysis of nutrients in fish using factorial designs and desirability function. PloS one 13: e0206556. 10.1371/journal.pone.0206556

Gillund, F. and Myhr, A.I., 2010. Perspectives on Salmon Feed: A Deliberative Assessment of Several Alternative Feed Resources. Journal of Agricultural and Environmental Ethics 23: 527-550. 10.1007/s10806-010-9237-7

Glencross, B.D., Booth, M. and Allan, G.L., 2007. A feed is only as good as its ingredients–a review of ingredient evaluation strategies for aquaculture feeds. Aquaculture Nutrition 13: 17-34.

Golden, C.D., Koehn, J.Z., Shepon, A., Passarelli, S., Free, C.M., Viana, D.F., Matthey, H., Eurich, J.G., Gephart, J.A., Fluet-Chouinard, E., Nyboer, E.A., Lynch, A.J., Kjellevold, M., Bromage, S., Charlebois, P., Barange, M., Vannuccini, S., Cao, L., Kleisner, K.M., Rimm, E.B., Danaei, G., DeSisto, C., Kelahan, H., Fiorella, K.J., Little, D.C., Allison, E.H., Fanzo, J. and Thilsted, S.H., 2021. Aquatic foods to nourish nations. Nature 598: 315-320. 10.1038/s41586-021-03917-1

Gopalakannan, A. and Arul, V., 2006. Immunomodulatory effects of dietary intake of chitin, chitosan and levamisole on the immune system of *Cyprinus carpio* and control of *Aeromonas hydrophila* infection in ponds. Aquaculture 255: 179-187.

Gouveia, L., Choubert, G., Pereira, N., Santinha, J., Empis, J. and Gomes, E., 2002. Pigmentation of gilthead seabream, *Sparus aurata* (L. 1875), using *Chlorella vulgaris* (Chlorophyta, Volvocales) microalga. Aquaculture Research 33: 987-993. https://doi.org/10.1046/j.1365-2109.2002.00751.x

Grefsrud, E.S., Karlsen, Ø., Kvamme, B.O., Glover, K.A., Husa, V., Hansen, P.K., Grøsvik, B.E., Samuelsen, O.B., Sandlund, N. and Stien, L.H., 2021. Risikorapport norsk fiskeoppdrett 2021-kunnskapsstatus-Kunnskapsstatus effekter av norsk fiskeoppdrett. Rapport fra havforskningen.

Grendell, J.H. and Rothman, S.S., 1981. Digestive end products mobilize secretory proteins from subcellular stores in the pancreas. American Journal of Physiology-Gastrointestinal and Liver Physiology 241: G67-G73.

Grimholt, U., Johansen, R. and Smith, A.J., 2009. A review of the need and possible uses for genetically standardized Atlantic salmon (*Salmo salar*) in research. Laboratory Animals 43: 121-126. 10.1258/la.2008.008013

Grunenwald, M., 2018. Factors affecting pigmentation quality in Atlantic salmon (*Salmo salar* L.) at elevated temperature.

Gutowska, M.A., Drazen, J.C. and Robison, B.H., 2004. Digestive chitinolytic activity in marine fishes of Monterey Bay, California. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 139: 351-358. https://doi.org/10.1016/j.cbpb.2004.09.020

Hajen, W.E., Higgs, D.A., Beames, R.M. and Dosanjh, B.S., 1993. Digestibility of various feedstuffs by post-juvenile chinook salmon (*Oncorhynchus tshawytscha*) in sea water. 2. Measurement of digestibility. Aquaculture 112: 333-348.

Hamdan, M., Moyano, F.J. and Schuhardt, D., 2009. Optimization of a gastrointestinal model applicable to the evaluation of bioaccessibility in fish feeds. Journal of the Science of Food and Agriculture 89: 1195-1201. https://doi.org/10.1002/jsfa.3574

Hardy, R.W. and Kaushik, S.J., 2021. Fish nutrition. Academic press.

Helgesen, K.O., Horsberg, T.E., Stige, L.C. and Tarpai, A., 2021. The surveillance programme for resistance in salmon lice (*Lepeophtheirus salmonis*) in Norway 2020. Surveillance Program Report.

Hender, A., Siddik, M.A.B., Howieson, J. and Fotedar, R., 2021. Black Soldier Fly, *Hermetia illucens* as an Alternative to Fishmeal Protein and Fish Oil: Impact on Growth, Immune Response, Mucosal Barrier Status, and Flesh Quality of Juvenile Barramundi, *Lates calcarifer* (Bloch, 1790), Biology. 10.3390/biology10060505

Hicks, C.C., Cohen, P.J., Graham, N.A.J., Nash, K.L., Allison, E.H., D'Lima, C., Mills, D.J., Roscher, M., Thilsted, S.H., Thorne-Lyman, A.L. and MacNeil, M.A., 2019. Harnessing global fisheries to tackle micronutrient deficiencies. Nature 574: 95-98. 10.1038/s41586-019-1592-6

Hill, D.B., Vasquez, P.A., Mellnik, J., McKinley, S.A., Vose, A., Mu, F., Henderson, A.G., Donaldson, S.H., Alexis, N.E. and Boucher, R.C., 2014. A biophysical basis for mucus solids concentration as a candidate biomarker for airways disease. PloS one 9: e87681.

Hoseini, S.M., Hoseinifar, S.H. and Van Doan, H., 2021. Growth performance and hematological and antioxidant characteristics of rainbow trout, *Oncorhynchus Mykiss*, fed diets supplemented with Roselle, Hibiscus sabdariffa. Aquaculture 530: 735827. https://doi.org/10.1016/j.aquaculture.2020.735827

Hsu, H., Vavak, D., Satterlee, L. and Miller, G.J.J.o.F.S., 1977. A multienzyme technique for estimating protein digestibility. 42: 1269-1273.

Hu, Q.-Y., Wu, P., Feng, L., Jiang, W.-D., Liu, Y., Kuang, S.-Y., Tang, L., Li, J. and Zhou, X.-Q., 2021. Antimicrobial peptide Isalo scorpion cytotoxic peptide (IsCT) enhanced growth performance and improved intestinal immune function associated with janus kinases (JAKs)/signal transducers and activators of transcription (STATs) signalling pathways in on-growing grass carp (*Ctenopharyngodon idella*). Aquaculture 539: 736585. https://doi.org/10.1016/j.aquaculture.2021.736585

Hua, K. and Bureau, D.P., 2009. Development of a model to estimate digestible lipid content of salmonid fish feeds. Aquaculture 286: 271-276.

Huff-Lonergan, E. and Sosnicki, A., 2002. Water-holding capacity of fresh meat. Fact Sheet 4669: 1-8.

Hunt, R.W.G., 1977. The specification of colour appearance. I. Concepts and terms. Color Research & Application 2: 55-68.

Hwangbo, J., Hong, E.C., Jang, A., Kang, H.K., Oh, J.S., Kim, B.W. and Park, B.S., 2009. Utilization of house fly-maggots, a feed supplement in the production of broiler chickens. Journal of Environmental Biology 30.

Iaconisi, V., Bonelli, A., Pupino, R., Gai, F. and Parisi, G., 2018. Mealworm as dietary protein source for rainbow trout: Body and fillet quality traits. Aquaculture 484: 197-204. https://doi.org/10.1016/j.aquaculture.2017.11.034

Iaconisi, V., Secci, G., Sabatino, G., Piccolo, G., Gasco, L., Papini, A.M. and Parisi, G., 2019. Effect of mealworm (*Tenebrio molitor* L.) larvae meal on amino acid

composition of gilthead sea bream (*Sparus aurata* L.) and rainbow trout (*Oncorhynchus Mykiss* W.) fillets. Aquaculture 513: 734403.

Ingram, G.A., 1980. Substances involved in the natural resistance of fish to infection–A review. Journal of Fish Biology 16: 23-60. https://doi.org/10.1111/j.1095-8649.1980.tb03685.x

IPIFF, 2021. An overview of the European market of insects as feed. International Platform of Insects for Food and Feed, Brussels. International Platform of Insects for Food and Feed, Brussels, Belgium. Available at: https://ipiff.org/wp-content/uploads/2021/04/Apr-27-2021-IPIFF_The-European-market-of-insects-as-feed.pdf.

Iversen, A., Asche, F., Hermansen, Ø. and Nystøyl, R., 2020. Production cost and competitiveness in major salmon farming countries 2003–2018. Aquaculture 522: 735089.

Janssen, R.H., Vincken, J.-P., Arts, N.J.G., Fogliano, V. and Lakemond, C.M.M., 2019. Effect of endogenous phenoloxidase on protein solubility and digestibility after processing of *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens* . Food Research International 121: 684-690. https://doi.org/10.1016/j.foodres.2018.12.038

Jaramillo-Torres, A., Rawling, M.D., Rodiles, A., Mikalsen, H.E., Johansen, L.-H., Tinsley, J., Forberg, T., Aasum, E., Castex, M. and Merrifield, D.L., 2019. Influence of Dietary Supplementation of Probiotic Pediococcus acidilactici MA18/5M During the Transition From Freshwater to Seawater on Intestinal Health and Microbiota of Atlantic Salmon (*Salmo salar* L.). Frontiers in Microbiology 10.

Jensen, L.B., Provan, F., Larssen, E., Bron, J.E. and Obach, A., 2015b. Reducing sea lice (Lepeophtheirus salmonis) infestation of farmed Atlantic salmon (*Salmo salar* L.) through functional feeds. Aquaculture Nutrition 21: 983-993. https://doi.org/10.1111/anu.12222

Jodaa Holm, H., Wadsworth, S., Bjelland, A.-K., Krasnov, A., Evensen, Ø. and Skugor, S., 2016. Dietary phytochemicals modulate skin gene expression profiles and result in reduced lice counts after experimental infection in Atlantic salmon. Parasites & Vectors 9: 271. 10.1186/s13071-016-1537-y

Kaitlyn, R.R., William, J.K., Matthew, R.M. and David, B.H., 2022. Effects of mucin and DNA concentrations in airway mucus on *Pseudomonas aeruginosa* biofilm recalcitrance. bioRxiv: 2022.2003.2024.485733. 10.1101/2022.03.24.485733

Khayer Dastjerdi, A. and Barthelat, F., 2015. Teleost fish scales amongst the toughest collagenous materials. Journal of the Mechanical Behavior of Biomedical Materials 52: 95-107. https://doi.org/10.1016/j.jmbbm.2014.09.025

Knudsen G, Johansen R, Smith AJ (2005), (access: 2022, November 23) An analysis of the fish used in research in Norway in 2003: Numbers, species and use. Oslo:

Laboratory Animal Unit, Norwegian School of Veterinary Science http:// oslov et. veths. no/ analy sisfi sh2003. Pdf

Kono, S., Kon, M., Araki, T. and Sagara, Y., 2017. Effects of relationships among freezing rate, ice crystal size and color on surface color of frozen salmon fillet. Journal of Food Engineering 214: 158-165. https://doi.org/10.1016/j.jfoodeng.2017.06.023

Kotzamanis, Y., Tsironi, T., Brezas, A., Grigorakis, K., Ilia, V., Vatsos, I., Romano, N., van Eys, J. and Kumar, V., 2020. High taurine supplementation in plant protein-based diets improves growth and organoleptic characteristics of European seabass (*Dicentrarchus labrax*). Scientific Reports 10: 12294. 10.1038/s41598-020-69014-x

Kousoulaki, K., Mørkøre, T., Nengas, I., Berge, R.K. and Sweetman, J., 2016. Microalgae and organic minerals enhance lipid retention efficiency and fillet quality in Atlantic salmon (*Salmo salar* L.). Aquaculture 451: 47-57. https://doi.org/10.1016/j.aquaculture.2015.08.027

Kramer, R.M., Shende, V.R., Motl, N., Pace, C.N. and Scholtz, J.M., 2012. Toward a molecular understanding of protein solubility: increased negative surface charge correlates with increased solubility. Biophys J 102: 1907-1915. 10.1016/j.bpj.2012.01.060

Kroeckel, S., Harjes, A.G.E., Roth, I., Katz, H., Wuertz, S., Susenbeth, A. and Schulz, C., 2012. When a turbot catches a fly: Evaluation of a pre-pupae meal of the Black Soldier Fly (*Hermetia illucens*) as fish meal substitute — Growth performance and chitin degradation in juvenile turbot (*Psetta maxima*). Aquaculture 364-365: 345-352. https://doi.org/10.1016/j.aquaculture.2012.08.041

Krogdahl, Nordrum, SØRensen, Brudeseth and RØSjØ, 1999. Effects of diet composition on apparent nutrient absorption along the intestinal tract and of subsequent fasting on mucosal disaccharidase activities and plasma nutrient concentration in Atlantic salmon *Salmo salar* L. Aquaculture Nutrition 5: 121-133.

Krogdahl, Å., Sundby, A. and Holm, H., 2015. Characteristics of digestive processes in Atlantic salmon (*Salmo salar*). Enzyme pH optima, chyme pH, and enzyme activities. Aquaculture 449: 27-36. https://doi.org/10.1016/j.aquaculture.2015.02.032

Kaale, L.D., Eikevik, T.M., Rustad, T. and Nordtvedt, T.S., 2014. Changes in water holding capacity and drip loss of Atlantic salmon (*Salmo salar*) muscle during superchilled storage. LWT-Food Science and technology 55: 528-535.

Lalander, C., Diener, S., Zurbrügg, C. and Vinnerås, B., 2019. Effects of feedstock on larval development and process efficiency in waste treatment with black soldier fly (*Hermetia illucens*). Journal of Cleaner Production 208: 211-219.

Lallès, J.-P., 2020. Intestinal alkaline phosphatase in the gastrointestinal tract of fish: biology, ontogeny, and environmental and nutritional modulation. Reviews in Aquaculture 12: 555-581. https://doi.org/10.1111/raq.12340

Lan, C.C. and Pan, B.S., 1993. In-vitro digestibility simulating the proteolysis of feed protein in the midgut gland of grass shrimp (*Penaeus monodon*). Aquaculture 109: 59-70. https://doi.org/10.1016/0044-8486(93)90486-I

Larsson, T., Koppang, E.O., Espe, M., Terjesen, B.F., Krasnov, A., Moreno, H.M., Rørvik, K.-A., Thomassen, M. and Mørkøre, T., 2014. Fillet quality and health of Atlantic salmon (*Salmo salar* L.) fed a diet supplemented with glutamate. Aquaculture 426-427: 288-295. https://doi.org/10.1016/j.aquaculture.2014.01.034

Leclercq, E., Pontefract, N., Rawling, M., Valdenegro, V., Aasum, E., Andujar, L.V., Migaud, H., Castex, M. and Merrifield, D., 2020. Dietary supplementation with a specific mannan-rich yeast parietal fraction enhances the gut and skin mucosal barriers of Atlantic salmon (*Salmo salar*) and reduces its susceptibility to sea lice (*Lepeophtheirus salmonis*). Aquaculture 529: 735701. https://doi.org/10.1016/j.aquaculture.2020.735701

Leeper, A., Benhaïm, D., Smárason, B.Ö., Knobloch, S., Òmarsson, K.L., Bonnafoux, T., Pipan, M., Koppe, W., Björnsdóttir, R. and Øverland, M., 2022. Feeding black soldier fly larvae (*Hermetia illucens*) reared on organic rest streams alters gut characteristics of Atlantic salmon (*Salmo salar*). Journal of Insects as Food and Feed 8: 1355-1372. 10.3920/JIFF2021.0105

Lemos, D., Navarrete del Toro, A., Córdova-Murueta, J.H. and Garcia-Carreño, F., 2004. Testing feeds and feed ingredients for juvenile pink shrimp *Farfantepenaeus paulensis*: in vitro determination of protein digestibility and proteinase inhibition. Aquaculture 239: 307-321. https://doi.org/10.1016/j.aquaculture.2004.05.032

Li, Y., Bruni, L., Jaramillo-Torres, A., Gajardo, K., Kortner, T.M. and Krogdahl, Å., 2021a. Differential response of digesta- and mucosa-associated intestinal microbiota to dietary insect meal during the seawater phase of Atlantic salmon. Animal Microbiome 3: 8. 10.1186/s42523-020-00071-3

Li, Y., Gajardo, K., Jaramillo-Torres, A., Belghit, I., Lock, E.-J., Kortner, T.M. and Krogdahl, Å., 2021b. Reproducible Changes In The Intestinal Microbiota Of Atlantic Salmon Fed Diets Containing Black Soldier Fly Larvae Meal. Aquaculture 2021.

Li, Y., Kortner, T.M., Chikwati, E.M., Belghit, I., Lock, E.-J. and Krogdahl, Å., 2020. Total replacement of fish meal with black soldier fly (*Hermetia illucens*) larvae meal does not compromise the gut health of Atlantic salmon (*Salmo salar*). Aquaculture 520: 734967. https://doi.org/10.1016/j.aquaculture.2020.734967

Liddle, R.A., 2000. Regulation of cholecystokinin secretion in humans. Journal of gastroenterology 35: 181-187.

Lieberman, S., Enig, M.G. and Preuss, H.G., 2006. A Review of Monolaurin and Lauric Acid: Natural Virucidal and Bactericidal Agents. Alternative and Complementary Therapies 12: 310-314. 10.1089/act.2006.12.310

Liland, N.S., Araujo, P., Xu, X., Lock, E.-J., Radhakrishnan, G., Prabhu, A. and Belghit, I., 2021. A meta-analysis on the nutritional value of insects in aquafeeds. Journal of Insects as Food

Liland, N.S., Biancarosa, I., Araujo, P., Biemans, D., Bruckner, C.G., Waagbø, R., Torstensen, B.E. and Lock, E.-J., 2017. Modulation of nutrient composition of black soldier fly (*Hermetia illucens*) larvae by feeding seaweed-enriched media. PloS one 12: e0183188. 10.1371/journal.pone.0183188

Lim, C. and Webster, C.D., 2001. Nutrition and fish health. Food Products Press New York.

Lindsay, R.C., 2007. Flavors, Fennema's food chemistry. CRC Press, pp. 651-700.

Llewellyn, M.S., Boutin, S., Hoseinifar, S.H. and Derome, N., 2014. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Frontiers in Microbiology 5: 207.

Lock, E.R., Arsiwalla, T. and Waagbø, R., 2016. Insect larvae meal as an alternative source of nutrients in the diet of Atlantic salmon (*Salmo salar*) postsmolt. Aquaculture Nutrition 22: 1202-1213. https://doi.org/10.1111/anu.12343

Lu, Q., 2022. Salmon Aquaculture. BoD–Books on Demand.

Lu, Y., Kim, S. and Park, K., 2011. In vitro-in vivo correlation: perspectives on model development. Int J Pharm 418: 142-148. 10.1016/j.ijpharm.2011.01.010

Lutfi, E., Berge, G.M., Bæverfjord, G., Sigholt, T., Bou, M., Larsson, T., Mørkøre, T., Evensen, Ø., Sissener, N.H., Rosenlund, G., Sveen, L., Østbye, T.-K. and Ruyter, B., 2023. Increasing dietary levels of the n-3 long-chain PUFA, EPA and DHA, improves the growth, welfare, robustness and fillet quality of Atlantic salmon in sea cages. British Journal of Nutrition 129: 10-28. 10.1017/S0007114522000642

MacLeod, M.J., Hasan, M.R., Robb, D.H.F. and Mamun-Ur-Rashid, M., 2020. Quantifying greenhouse gas emissions from global aquaculture. Scientific Reports 10: 11679.

Magnadóttir, B., 2006. Innate immunity of fish (overview). Fish Shellfish Immunol 20: 137-151. https://doi.org/10.1016/j.fsi.2004.09.006

Mancuso, T., Pippinato, L. and Gasco L., 2019. The European insects sector and its role in the provision of green proteins in feed supply. Quality – Access to Success 20: 374-381

Marono, S., Piccolo, G., Loponte, R., Di Meo, C., Attia, Y.A., Nizza, A. and Bovera, F., 2015. In Vitro Crude Protein Digestibility of *Tenebrio molitor* and *Hermetia*

illucens Insect Meals and its Correlation with Chemical Composition Traits. Italian Journal of Animal Science 14: 3889. 10.4081/ijas.2015.3889

Márquez, L., Øverland, M., Martínez-Llorens, S., Morken, T. and Moyano, F.J., 2013. Use of a gastrointestinal model to assess potential amino acid bioavailability in diets for rainbow trout (*Oncorrhynchus mykiss*). Aquaculture 384-387: 46-55. https://doi.org/10.1016/j.aquaculture.2012.12.008

Martin, S.A.M. and Król, E., 2017. Nutrigenomics and immune function in fish: new insights from omics technologies. Developmental & Comparative Immunology 75: 86-98.

Martínez-Llorens, S., Moñino, A.V., Tomás Vidal, A., Salvador, V.J.M., Pla Torres, M. and Jover Cerdá, M., 2007. Soybean meal as a protein source in gilthead sea bream (*Sparus aurata* L.) diets: effects on growth and nutrient utilization. Aquaculture Research 38: 82-90.

Mat, D.J.L., Le Feunteun, S., Michon, C. and Souchon, I., 2016. In vitro digestion of foods using pH-stat and the INFOGEST protocol: Impact of matrix structure on digestion kinetics of macronutrients, proteins and lipids. Food Research International 88: 226-233. https://doi.org/10.1016/j.foodres.2015.12.002

Mattiuzzo, M., Bandiera, A., Gennaro, R., Benincasa, M., Pacor, S., Antcheva, N. and Scocchi, M., 2007. Role of the *Escherichia coli* SbmA in the antimicrobial activity of proline-rich peptides. Molecular Microbiology 66: 151-163.

Maulu, S., Langi, S., Hasimuna, O.J., Missinhoun, D., Munganga, B.P., Hampuwo, B.M., Gabriel, N.N., Elsabagh, M., Van Doan, H., Abdul Kari, Z. and Dawood, M.A.O., 2022. Recent advances in the utilization of insects as an ingredient in aquafeeds: A review. Animal Nutrition 11: 334-349. https://doi.org/10.1016/j.aninu.2022.07.013

Melenchón, F., Larrán, A.M., de Mercado, E., Hidalgo, M.C., Cardenete, G., Barroso, F.G., Fabrikov, D., Lourenço, H.M., Pessoa, M.F. and Tomás-Almenar, C., 2021. Potential use of black soldier fly (*Hermetia illucens*) and mealworm (*Tenebrio molitor*) insectmeals in diets for rainbow trout (*Oncorhynchus Mykiss*). Aquaculture Nutrition 27: 491-505. https://doi.org/10.1111/anu.13201

Message, R. and Greenhough, B., 2019. “But It’s Just a Fish”: Understanding the Challenges of Applying the 3Rs in Laboratory Aquariums in the UK, Animals. 10.3390/ani9121075

Micallef, G., Cash, P., Fernandes, J.M.O., Rajan, B., Tinsley, J.W., Bickerdike, R., Martin, S.A.M. and Bowman, A.S., 2017. Dietary Yeast Cell Wall Extract Alters the Proteome of the Skin Mucous Barrier in Atlantic Salmon (*Salmo salar*): Increased Abundance and Expression of a Calreticulin-Like Protein. PloS one 12: e0169075. 10.1371/journal.pone.0169075

Mikołajczak, Z., Mazurkiewicz, J., Rawski, M., Kierończyk, B., Józefiak, A., Świątkiewicz, S. and Józefiak, D., 2023. Black Soldier Fly Full-Fat Meal in

Atlantic Salmon Nutrition – Part B: Effects on Growth Performance, Feed Utilization, Selected Nutriphysiological Traits and Production Sustainability in Pre-Smolts. Annals of Animal Science 23: 239-251. doi:10.2478/aoas-2022-0071

Milo, C. and Grosch, W., 1995. Detection of odor defects in boiled cod and trout by gas chromatography-olfactometry of headspace samples. Journal of Agricultural and Food Chemistry 43: 459-462.

Mirzakhani, M.K., Abedian Kenari, A. and Motamedzadegan, A., 2018. Prediction of apparent protein digestibility by in vitro pH-stat degree of protein hydrolysis with species-specific enzymes for Siberian sturgeon (*Acipenser baeri*, Brandt 1869). Aquaculture 496: 73-78. https://doi.org/10.1016/j.aquaculture.2018.07.014

Mohan, K., Rajan, D.K., Muralisankar, T., Ganesan, A.R., Sathishkumar, P. and Revathi, N., 2022. Use of black soldier fly (*Hermetia illucens* L.) larvae meal in aquafeeds for a sustainable aquaculture industry: A review of past and future needs. Aquaculture 553: 738095. https://doi.org/10.1016/j.aquaculture.2022.738095

Mokhtar, D.M., Hussein, M.M. and Sayed, R.K.A., 2022. Novel Identification and Microscopy of the Intestinal Bulb of Molly Fish (*Poecilia sphenops*) with a Focus on Its Role in Immunity. Microscopy and Microanalysis 28: 1827-1839. 10.1017/S1431927622012089

Mokhtar, D.M., Zaccone, G., Alesci, A., Kuciel, M., Hussein, M.T. and Sayed, R.K.A., 2023. Main Components of Fish Immunity: An Overview of the Fish Immune System, Fishes. 10.3390/fishes8020093

Morales, G.A. and Moyano, F.J., 2010. Application of an in vitro gastrointestinal model to evaluate nitrogen and phosphorus bioaccessibility and bioavailability in fish feed ingredients. Aquaculture 306: 244-251. https://doi.org/10.1016/j.aquaculture.2010.05.014

Mousavi, S., Zahedinezhad, S. and Loh, J.Y., 2020. A review on insect meals in aquaculture: The immunomodulatory and physiological effects. International Aquatic Research 12: 100-115.

Moyano, F.J. and Savoie, L., 2001. Comparison of in vitro systems of protein digestion using either mammal or fish proteolytic enzymes. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 128: 359-368. https://doi.org/10.1016/S1095-6433(00)00315-9

Moyano, F.J., Saénz de Rodrigáñez, M.A., Díaz, M. and Tacon, A.G.J., 2015. Application of in vitro digestibility methods in aquaculture: constraints and perspectives. Reviews in Aquaculture 7: 223-242. https://doi.org/10.1111/raq.12065

Mørkøre, T., Moreno, H.M., Borderías, J., Larsson, T., Hellberg, H., Hatlen, B., Romarheim, O.H., Ruyter, B., Lazado, C.C., Jiménez-Guerrero, R., Bjerke, M.T., Benitez-Santana, T. and Krasnov, A., 2020. Dietary inclusion of Antarctic krill meal during the finishing feed period improves health and fillet quality of Atlantic salmon (*Salmo salar* L.). British Journal of Nutrition 124: 418-431. 10.1017/S0007114520001282

Mørkøre, T., Ruohonen, K. and Kiessling, A., 2009. Variation In Texture Of Farmed Atlantic Salmon (*Salmo salar* L). Relevance Of Muscle Fiber Cross-Sectional Area. Journal of Texture Studies 40: 1-15. https://doi.org/10.1111/j.1745-4603.2008.00166.x

Nandeesha, M.C., Gangadhara, B., Varghese, T.J. and Keshavanath, P., 2000. Growth response and flesh quality of common carp, *Cyprinus carpio* fed with high levels of nondefatted silkworm pupae. Asian Fisheries Science 13: 235-242.

National Research, C., 2011. Nutrient requirements of fish and shrimp.

Nordberg, M., 2018. Seasonal variation in fillet quality of Atlantic salmon (*Salmo salar*).

Oliveira, V.H.S., Dean, K.R., Qviller, L., Kirkeby, C. and Bang Jensen, B., 2021. Factors associated with baseline mortality in Norwegian Atlantic salmon farming. Scientific Reports 11: 14702. 10.1038/s41598-021-93874-6

Overton, K., Dempster, T., Oppedal, F., Kristiansen, T.S., Gismervik, K. and Stien, L.H., 2019. Salmon lice treatments and salmon mortality in Norwegian aquaculture: a review. Reviews in Aquaculture 11: 1398-1417. https://doi.org/10.1111/raq.12299

Pelletier, N., Tyedmers, P., Sonesson, U., Scholz, A., Ziegler, F., Flysjo, A., Kruse, S., Cancino, B. and Silverman, H., 2009. Not all salmon are created equal: life cycle assessment (LCA) of global salmon farming systems. ACS Publications.

Peña, E., Hernández, C., Ibarra-Castro, L. and Álvarez-González, C.A., 2017. In vitro protein digestibility of different grow-out stages of spotted rose snapper (*Lutjanus guttatus*, Steindachner, 1869). Aquaculture Nutrition 23: 1204-1215. https://doi.org/10.1111/anu.12489

Pippinato, L., Gasco, L., Di Vita, G. and Mancuso, T., 2020. Current scenario in the European edible-insect industry: a preliminary study. Journal of Insects as Food and Feed 6: 371-381. https://doi.org/10.3920/JIFF2020.0008

Radhakrishnan, G., Liland, N.S., Koch, M.W., Lock, E.-J., Philip, A.J.P. and Belghit, I., Evaluation of black soldier fly larvae meal as a functional feed ingredient in Atlantic salmon (*Salmo salar*) under farm-like conditions. Frontiers in Aquaculture 2: 1239402.

Rahmah, S., Aliyu-Paiko, M. and Hashim, R., 2016. In vivo and in vitro protein digestibility in juvenile bagrid catfish *Mystus nemurus* (Cuvier and Valenciennes 1840) fed soybean meal-based diets. Aquaculture Research 47: 1392-1401.

Rahmah, S., Hashim, R. and El-Sayed, A.F.M., 2020. Digestive proteases and in vitro protein digestibility in bagrid catfish *Mystus nemurus* (Cuvier and Valenciennes 1840). Aquaculture Research 51: 4613-4622.

Randazzo, B., Zarantoniello, M., Cardinaletti, G., Cerri, R., Giorgini, E., Belloni, A., Contò, M., Tibaldi, E. and Olivotto, I., 2021. *Hermetia illucens* and poultry by-product meals as alternatives to plant protein sources in gilthead seabream (*Sparus aurata*) diet: A multidisciplinary study on fish gut status. Animals 11: 677.

Rashidian, G., Moosazadeh Moghaddam, M., Mirnejad, R. and Mohammadi Azad, Z., 2021. Supplementation of zebrafish (*Danio rerio*) diet using a short antimicrobial peptide: Evaluation of growth performance, immunomodulatory function, antioxidant activity, and disease resistance. Fish Shellfish Immunol 119: 42-50. https://doi.org/10.1016/j.fsi.2021.09.035

Reddy, P.G. and Frey, R.A., 1990. Nutritional modulation of immunity in domestic food animals. Advances in Veterinary Science and comparative medicine 35: 255-281.

Refstie, S., Baeverfjord, G., Seim, R.R. and Elvebø, O., 2010. Effects of dietary yeast cell wall β-glucans and MOS on performance, gut health, and salmon lice resistance in Atlantic salmon (*Salmo salar*) fed sunflower and soybean meal. Aquaculture 305: 109-116.

Renna, M., Schiavone, A., Gai, F., Dabbou, S., Lussiana, C., Malfatto, V., Prearo, M., Capucchio, M.T., Biasato, I., Biasibetti, E., De Marco, M., Brugiapaglia, A., Zoccarato, I. and Gasco, L., 2017. Evaluation of the suitability of a partially defatted black soldier fly (*Hermetia illucens* L.) larvae meal as ingredient for rainbow trout (*Oncorhynchus Mykiss* Walbaum) diets. Journal of Animal Science and Biotechnology 8: 57. 10.1186/s40104-017-0191-3

Ringø, E., Olsen, R.E., Vecino, J.L.G., Wadsworth, S. and Song, S.K., 2012. Use of immunostimulants and nucleotides in aquaculture: a review. J Mar Sci Res Dev 2: 104.

Rocha, S.D.C., Morales-Lange, B., Montero, R., Teklay Okbayohanese, D., Kathiresan, P., McLean Press, C., Torunn Mydland, L. and Øverland, M., 2023. Norway spruce extracts (NSEs) as bioactive compounds in novel feeds: Effect on intestinal immune-related biomarkers, morphometry and microbiota in Atlantic salmon pre-smolts. Journal of Functional Foods 111: 105888. https://doi.org/10.1016/j.jff.2023.105888

Román-Gavilanes, A.I., Martínez-Montaño, E. and Viana, M.T., 2015. Comparative Characterization of Enzymatic Digestion from Fish and Soybean Meal from Simulated Digestive Process of Pacific Bluefin Tuna, Thunnus orientalis. Journal of the World Aquaculture Society 46: 409-420. https://doi.org/10.1111/jwas.12204

Romarheim, O.H., Skrede, A., Penn, M., Mydland, L.T., Krogdahl, Å. and Storebakken, T., 2008. Lipid digestibility, bile drainage and development of morphological intestinal changes in rainbow trout (*Oncorhynchus Mykiss*) fed diets containing defatted soybean meal. Aquaculture 274: 329-338.

Rumpold, B.A. and Schlüter, O.K., 2013. Nutritional composition and safety aspects of edible insects. Molecular nutrition & food research 57: 802-823.

Rørå, A. M. B., et al. (1998). "Process yield, colour and sensory quality of smoked Atlantic salmon (*Salmo salar*) in relation to raw material characteristics." Food Research International 31(8): 601-609.

Sae-Lim, P., Boison, S. and Baranski, M., 2022. 566. Multi-trait genome wide association study in correlated traits: fillet colour and body weight in Atlantic salmon, Proceedings of 12th World Congress on Genetics Applied to Livestock Production (WCGALP). Wageningen Academic Publishers, pp. 2347-2350. doi:10.3920/978-90-8686-940-4_566

Sáenz de Rodrigáñez, M.Á., Medina, E., Moyano, F.J. and Alarcón, F.J., 2011. Evaluation of protein hydrolysis in raw sources by digestive proteases of Senegalese sole (*Solea senegalensis*, Kaup 1858) using a combination of an in vitro assay and sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis of products. Aquaculture Research 42: 1639-1652. https://doi.org/10.1111/j.1365-2109.2010.02758.x

Sahlmann, C., 2013. The digestive system of Atlantic salmon (*Salmo salar* L.)-Ontogeny and response to soybean meal rich diets–. Norwegian School of Veterinary Science, Olso.

Sakai, M., Hikima, J.-i. and Kono, T., 2021. Fish cytokines: current research and applications. Fisheries Science 87: 1-9.

Sandnes, K., Lie, Ø. and Waagbø, R., 1988. Normal ranges of some blood chemistry parameters in adult farmed Atlantic salmon, *Salmo salar*. Journal of Fish Biology 32: 129-136.

Satterlee, L., Kendrick, J. and Miller, G.J.N.R.I., 1977. Rapid in vitro assays for estimating protein quality [of food].

Schroeder, B.O., Birchenough, G.M.H., Ståhlman, M., Arike, L., Johansson, M.E.V., Hansson, G.C. and Bäckhed, F., 2018. Bifidobacteria or fiber protects against diet-induced microbiota-mediated colonic mucus deterioration. Cell host & microbe 23: 27-40.

Sealey, W.M., Gaylord, T.G., Barrows, F.T., Tomberlin, J.K., McGuire, M.A., Ross, C. and St-Hilaire, S., 2011. Sensory Analysis of Rainbow Trout, *Oncorhynchus Mykiss*, Fed Enriched Black Soldier Fly Prepupae, *Hermetia illucens* . 42: 34-45. 10.1111/j.1749-7345.2010.00441.x

Searchinger, T., Waite, R., Hanson, C., Ranganathan, J., Dumas, P. and Matthews, E., 2019. World resources report: Creating a sustainable food future. World Resources Institute.

Selvam, C., Philip, A.J.P., Lutfi, E., Sigholt, T., Norberg, B., Bæverfjord, G., Rosenlund, G., Ruyter, B. and Sissener, N.H., 2022. Long-term feeding of Atlantic salmon with varying levels of dietary EPA + DHA alters the mineral status but does not affect the stress responses after mechanical delousing stress. British Journal of Nutrition 128: 2291-2307. 10.1017/S0007114522000514

Shearer, K. D. (1994). "Factors affecting the proximate composition of cultured fishes with emphasis on salmonids." Aquaculture 119(1): 63-88.

Shi, Z., Li, X.-Q., Chowdhury, M.A.K., Chen, J.-N. and Leng, X.-J., 2016. Effects of protease supplementation in low fish meal pelleted and extruded diets on growth, nutrient retention and digestibility of gibel carp, *Carassius auratus gibelio*. Aquaculture 460: 37-44. https://doi.org/10.1016/j.aquaculture.2016.03.049

Shomorin, G.O., Storebakken, T., Kraugerud, O.F., Øverland, M., Hansen, B.R. and Hansen, J.Ø., 2019. Evaluation of wedge wire scree as a new tool for faeces collection in digestibility assessment in fish: The impact of nutrient leaching on apparent digestibility of nitrogen, carbon and sulphur from fishmeal, soybean meal and rapeseed meal-based diets in rainbow trout (*Oncorhynchus Mykiss*). Aquaculture 504: 81-87.

Sigurgisladottir, S., ØTorrissen, O., Lie, Ø., Thomassen, M. and Hafsteinsson, H., 1997. Salmon quality: Methods to determine the quality parameters. Reviews in Fisheries Science 5: 223-252. 10.1080/10641269709388599

Silva, M.S., Matos, R., Araujo, P., Lock, E.J., Gopika, R., Prabhu, P.A.J. and Belghit, I., 2022. In vitro assessment of protein digestibility and mineral solubility of black soldier fly larvae meals for monogastric animals. Journal of Insects as Food and Feed 8: 953-966. 10.3920/JIFF2021.0197

Silva, M.S., Prabhu, P.A.J., Ørnsrud, R., Sele, V., Kröckel, S., Sloth, J.J. and Amlund, H., 2020. In vitro digestion method to evaluate solubility of dietary zinc, selenium and manganese in salmonid diets. Journal of Trace Elements in Medicine and Biology 57: 126418. https://doi.org/10.1016/j.jtemb.2019.126418

Sire, M.F. and Vernier, J.-M., 1992. Intestinal absorption of protein in teleost fish. Comparative Biochemistry and Physiology Part A: Physiology 103: 771-781.

Smets, R., Verbinnen, B., Van De Voorde, I., Aerts, G., Claes, J. and Van Der Borght, M., 2020. Sequential extraction and characterisation of lipids, proteins, and chitin from black soldier fly (*Hermetia illucens*) larvae, prepupae, and pupae. Waste and Biomass Valorization 11: 6455-6466.

Sogari, G., Amato, M., Biasato, I., Chiesa, S. and Gasco, L., 2019. The Potential Role of Insects as Feed: A Multi-Perspective Review, Animals. 10.3390/ani9040119

Sprague, M., Dick, J.R. and Tocher, D.R., 2016. Impact of sustainable feeds on omega-3 long-chain fatty acid levels in farmed Atlantic salmon, 2006–2015. Scientific Reports 6: 21892. 10.1038/srep21892

Statistics Norway, 2022. 09283: Exports of fish, by country/trade region/continent 2007–2021. https://www.ssb.no/en/statbank/table/09283/

St-Hilaire, S., Sheppard, C., Tomberlin, J.K., Irving, S., Newton, L., McGuire, M.A., Mosley, E.E., Hardy, R.W. and Sealey, W., 2007a. Fly Prepupae as a Feedstuff for Rainbow Trout, *Oncorhynchus Mykiss*. 38: 59-67. 10.1111/j.1749-7345.2006.00073.x

St-Hilaire, S., Sheppard, C., Tomberlin, J.K., Irving, S., Newton, L., McGuire, M.A., Mosley, E.E., Hardy, R.W. and Sealey, W., 2007b. Fly Prepupae as a Feedstuff for Rainbow Trout, *Oncorhynchus Mykiss*. Journal of the World Aquaculture Society 38: 59-67. https://doi.org/10.1111/j.1749-7345.2006.00073.x

Stribiţcaia, E., Evans, C.E.L., Gibbons, C., Blundell, J. and Sarkar, A., 2020. Food texture influences on satiety: systematic review and meta-analysis. Scientific Reports 10: 12929. 10.1038/s41598-020-69504-y

Stryer, L., 1988. Biosynthesis of amino acids and heme. Biochemistry: 575-600.

Sweetman, J.W., Torrecillas, S., Dimitroglou, A., Rider, S., Davies, S.J. and Izquierdo, M.S., 2010. Enhancing the natural defences and barrier protection of aquaculture species. Aquaculture Research 41: 345-355. https://doi.org/10.1111/j.1365-2109.2009.02196.x

Taylor, K. and Alvarez, L.R., 2019. An estimate of the number of animals used for scientific purposes worldwide in 2015. Alternatives to Laboratory Animals 47: 196-213.

Terova, G., Rimoldi, S., Ascione, C., Gini, E., Ceccotti, C. and Gasco, L., 2019. Rainbow trout (*Oncorhynchus Mykiss*) gut microbiota is modulated by insect meal from *Hermetia illucens* prepupae in the diet. Reviews in Fish Biology and Fisheries 29: 465-486. 10.1007/s11160-019-09558-y

Thomassen, M., Gudding, R., Norberg, B., Jørgensen, L., Bjerkeng, B., Ytrestøyl, T., & Olsen, R. E. (2007). Aquaculture Research: From Cage to Consumption. Oslo: The Research Council of Norway.

Thomassen, M.S., 2007. Aquaculture research: from cage to consumption.

Tibaldi, E., Chini Zittelli, G., Parisi, G., Bruno, M., Giorgi, G., Tulli, F., Venturini, S., Tredici, M.R. and Poli, B.M., 2015. Growth performance and quality traits of European sea bass (*D. labrax*) fed diets including increasing levels of freeze-dried Isochrysis sp. (T-ISO) biomass as a source of protein and n-3 long chain PUFA in partial substitution of fish derivatives. Aquaculture 440: 60-68. https://doi.org/10.1016/j.aquaculture.2015.02.002

Tibbetts, S.M., Verreth, J.A.J. and Lall, S.P., 2011. In vitro pH-Stat protein hydrolysis of feed ingredients for Atlantic cod, *Gadus morhua*. 2. In vitro protein

digestibility of common and alternative feed ingredients. Aquaculture 319: 407-416. https://doi.org/10.1016/j.aquaculture.2011.07.020

Tibbetts, S.M., Yasumaru, F. and Lemos, D., 2017. In vitro prediction of digestible protein content of marine microalgae (*Nannochloropsis granulata*) meals for Pacific white shrimp (*Litopenaeus vannamei*) and rainbow trout (*Oncorhynchus Mykiss*). Algal Research 21: 76-80. https://doi.org/10.1016/j.algal.2016.11.010

Tippayadara, N., Dawood, M.A.O., Krutmuang, P., Hoseinifar, S.H., Doan, H.V. and Paolucci, M., 2021. Replacement of Fish Meal by Black Soldier Fly (*Hermetia illucens*) Larvae Meal: Effects on Growth, Haematology, and Skin Mucus Immunity of Nile Tilapia, *Oreochromis niloticus*, Animals. 10.3390/ani11010193

Toledo-Solís, F.J., Martínez-García, R., Díaz, M., Pena Marin, E.S., Di Yorio, M.P., Vissio, P.G., Álvarez-González, C.A. and Saenz de Rodriganez, M., 2020. Potential bioavailability of protein and lipids in feed ingredients for the three-spot cichlid *Amphilophus trimaculatus*: An in vitro assessment. Aquaculture Research 51: 2913-2925.

Torgersen, J.S., Koppang, E.O., Stien, L.H., Kohler, A., Pedersen, M.E. and Mørkøre, T., 2014. Soft texture of Atlantic salmon fillets is associated with glycogen accumulation. PloS one 9: e85551.

Tran, H.Q., Nguyen, T.T., Prokešová, M., Gebauer, T., Doan, H.V. and Stejskal, V., 2022. Systematic review and meta-analysis of production performance of aquaculture species fed dietary insect meals. Reviews in Aquaculture 14: 1637-1655. https://doi.org/10.1111/raq.12666

Tran, H.Q., Nguyen, T.T., Prokešová, M.D., Matoušek, J., Tomčala, A., Van Doan, H., Kiljunen, M. and Stejskal, V., 2023. Insight into bioavailability of various insect meals for European perch (*Perca fluviatilis*): A nutritional and stable isotopic evaluation. Aquaculture 563: 738912. https://doi.org/10.1016/j.aquaculture.2022.738912

Trejo-Escamilla, I., Galaviz, M.A., Flores-Ibarra, M., Alvarez Gonzalez, C.A. and López, L.M., 2017. Replacement of fishmeal by soya protein concentrate in the diets of *Totoaba macdonaldi* (Gilbert, 1890) juveniles: effect on the growth performance, in vitro digestibility, digestive enzymes and the haematological and biochemistry parameters. Aquaculture Research 48: 4038-4057.

Tschirner, M. and Simon, A., 2015. Influence of different growing substrates and processing on the nutrient composition of black soldier fly larvae destined for animal feed. Journal of Insects as Food and Feed 1: 249-259. 10.3920/JIFF2014.0008

Uribe, C., Folch, H., Enriquez, R. and Moran, G., 2011. Innate and adaptive immunity in teleost fish: a review. Veterinární medicína 56: 486-503.

van Huis, A. and Gasco, L., 2023. Insects as feed for livestock production. Science 379: 138-139. 10.1126/science.adc9165

Veldkamp, T. and Bosch, G., 2015. Insects: a protein-rich feed ingredient in pig and poultry diets. Animal Frontiers 5: 45-50.

Veldkamp, T., Dong, L., Paul, A. and Govers, C., 2022a. Bioactive properties of insect products for monogastric animals – a review. Journal of Insects as Food and Feed 8: 1027-1040. 10.3920/JIFF2021.0031

Veldkamp, T., Meijer, N., Alleweldt, F., Deruytter, D., Van Campenhout, L., Gasco, L., Roos, N., Smetana, S., Fernandes, A. and van der Fels-Klerx, H.J., 2022b. Overcoming Technical and Market Barriers to Enable Sustainable Large-Scale Production and Consumption of Insect Proteins in Europe: A SUSINCHAIN Perspective, Insects. 10.3390/insects13030281

Vidakovic, A., Langeland, M., Sundh, H., Sundell, K., Olstorpe, M., Vielma, J., Kiessling, A. and Lundh, T., 2016. Evaluation of growth performance and intestinal barrier function in Arctic Charr (*Salvelinus alpinus*) fed yeast (*Saccharomyces cerevisiae*), fungi (*Rhizopus oryzae*) and blue mussel (*Mytilus edulis*). Aquaculture Nutrition 22: 1348-1360.

Wainwright, D.K., 2019. Fish scales: morphology, evolution, and function.

Walde, C.S., Stormoen, M., Pettersen, J.M., Persson, D., Røsæg, M.V. and Bang Jensen, B., 2022. How delousing affects the short-term growth of Atlantic salmon (*Salmo salar*). Aquaculture 561: 738720. https://doi.org/10.1016/j.aquaculture.2022.738720

Wang, J., Jaramillo-Torres, A., Li, Y., Brevik, Ø.J., Jakobsen, J.V., Kortner, T.M. and Krogdahl, Å., 2022. Gut Health and Microbiota in Out-of-Season Atlantic Salmon (*Salmo salar* L.) Smolts Before and After Seawater Transfer Under Commercial Arctic Conditions: Modulation by Functional Feed Ingredients. Frontiers in Marine Science 9.

Wang, R., Mohammadi, M., Mahboubi, A. and Taherzadeh, M.J., 2021. In-vitro digestion models: a critical review for human and fish and a protocol for in-vitro digestion in fish. Bioengineered 12: 3040-3064. 10.1080/21655979.2021.1940769

Weththasinghe, P., 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.

Weththasinghe, P., Hansen, J.Ø., Nøkland, D., Lagos, L., Rawski, M. and Øverland, M., 2021a. 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

Weththasinghe, P., Lagos, L., Cortés, M., Hansen, J.Ø. and Øverland, M., 2021b. 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.

Whitley, S.N. and Bollens, S.M., 2014. Fish assemblages across a vegetation gradient in a restoring tidal freshwater wetland: diets and potential for resource competition. Environmental Biology of Fishes 97: 659-674. 10.1007/s10641-013-0168-9

Windell, J.T., Foltz, J.W. and Sarokon, J.A., 1978. Methods of fecal collection and nutrient leaching in digestibility studies. The Progressive fish-culturist 40: 51-55.

Winther, U., Hognes, E.S., Jafarzadeh, S. and Ziegler, F., 2020. Greenhouse gas emissions of Norwegian seafood products in 2017. SINTEF Ocean.

Wrolstad, R.E. and Culver, C.A., 2012. Alternatives to Those Artificial FD&C Food Colorants. Annual Review of Food Science and Technology 3: 59-77. 10.1146/annurev-food-022811-101118

Waagbø, R., Torrissen, O. and Austreng, E., 2001. Fôr og fôrmidler-den største utfordringen for vekst i norsk havbruk.

Yasumaru, F. and Lemos, D., 2014. Species specific in vitro protein digestion (pH-stat) for fish: method development and application for juvenile rainbow trout (*Oncorhynchus Mykiss*), cobia (*Rachycentron canadum*), and Nile tilapia (*Oreochromis niloticus*). Aquaculture 426-427: 74-84. https://doi.org/10.1016/j.aquaculture.2014.01.012

Yeganeh, S., Teimouri, M. and Amirkolaie, A.K., 2015. Dietary effects of Spirulina platensis on hematological and serum biochemical parameters of rainbow trout (*Oncorhynchus Mykiss*). Research in Veterinary Science 101: 84-88. https://doi.org/10.1016/j.rvsc.2015.06.002

Yeşilayer, N., 2020. Comparison of Flesh Colour Assessment Methods for Wild Brown Trout (*Salmo trutta macrostigma*), Farmed Rainbow Trout (*Oncorhynchus Mykiss*) and Farmed Atlantic Salmon (*Salmo salar*). Pakistan Journal of Zoology 52.

Ytrestøyl, T., Aas, T.S. and Åsgård, T., 2015. Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway. Aquaculture 448: 365-374. https://doi.org/10.1016/j.aquaculture.2015.06.023

Zamborain-Mason, J., Viana, D., Nicholas, K., Jackson, E.D., Koehn, J.Z., Passarelli, S., Yoo, S.-H., Zhang, A.W., Davin, H.C., Duggan, C.P., Schmidhuber, J. and Golden, C.D., 2023. A Decision Framework for Selecting Critically Important Nutrients from Aquatic Foods. Current Environmental Health Reports 10: 172-183. 10.1007/s40572-023-00397-5

Zarantoniello, M., Pulido Rodriguez, L.F., Randazzo, B., Cardinaletti, G., Giorgini, E., Belloni, A., Secci, G., Faccenda, F., Pulcini, D., Parisi, G., Capoccioni, F., Tibaldi, E. and Olivotto, I., 2022. Conventional feed additives or red claw crayfish meal and dried microbial biomass as feed supplement in fish meal-free diets for rainbow trout (*Oncorhynchus Mykiss*): Possible ameliorative effects on growth and gut health status. Aquaculture 554: 738137. https://doi.org/10.1016/j.aquaculture.2022.738137

Zhou, Z., Yao, W., Ye, B., Wu, X., Li, X. and Dong, Y., 2020. Effects of replacing fishmeal protein with poultry by-product meal protein and soybean meal protein on growth, feed intake, feed utilization, gut and liver histology of hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) juveniles. Aquaculture 516: 734503. https://doi.org/10.1016/j.aquaculture.2019.734503

Zotte, A.D., 2021. Do insects as feed ingredient affect meat quality? Теория и практика переработки мяса 6: 200-209.

Østbye, T.K.K., Ruyter, B., Standal, I.B., Stien, L.H., Bahuaud, D., Dessen, J.E., Latif, M.S., Fyhn-Terjesen, B., Rørvik, K.A. and Mørkøre, T., 2018. Functional amino acids stimulate muscle development and improve fillet texture of Atlantic salmon. Aquaculture Nutrition 24: 14-26. https://doi.org/10.1111/anu.12528

Aas, T.S., Ytrestoyl, T. and Asgard, T., 2016. Utilization of feed resources in the production of Atlantic salmon *Salmo salar*. Norway: An update for.

Aas, T.S., Ytrestøyl, T. and Åsgård, T., 2019. Utilization of feed resources in the production of Atlantic salmon (*Salmo salar*) in Norway: An update for 2016. Aquaculture Reports 15: 100216.

Aas, T.S., Åsgård, T. and Ytrestøyl, T., 2022. Utilization of feed resources in the production of Atlantic salmon (*Salmo salar*) in Norway: An update for 2020. Aquaculture Reports 26: 101316. https://doi.org/10.1016/j.aqrep.2022.101316

I

Paper I

Gopika Radhakrishnan, Marta S. Silva, Erik-Jan Lock, Ikram Belghit, Antony Jesu Prabhu Philip (2022).

Assessing amino acid solubility of black soldier fly larvae meal in Atlantic salmon (*Salmo salar*) *in vivo* and *in vitro*. **Frontiers in Physiology**, 13, 1028992.

DOI: 10.3389/fphys.2022.1028992

frontiers | Frontiers in Physiology

TYPE Original Research
PUBLISHED 23 November 2022
DOI 10.3389/fphys.2022.1028992

OPEN ACCESS

EDITED BY
Francisco Javier Moyano,
University of Almeria, Spain

REVIEWED BY
Houguo Xu,
Yellow Sea Fisheries Research Institute (CAFS), China
Carlos Alfonso Alvarez-González,
Universidad Juárez Autónoma de Tabasco, Mexico

*CORRESPONDENCE
Ikram Belghit,
antony.philip@hi.no

SPECIALTY SECTION
This article was submitted to Aquatic Physiology, a section of the journal Frontiers in Physiology

RECEIVED 26 August 2022
ACCEPTED 07 November 2022
PUBLISHED 23 November 2022

CITATION
Radhakrishnan G, Silva MS, Lock E-J, Belghit I and Philip AJP (2022), Assessing amino acid solubility of black soldier fly larvae meal in Atlantic salmon (*Salmo salar*) *in vivo* and *in vitro*. *Front. Physiol.* 13:1028992. doi: 10.3389/fphys.2022.1028992

Assessing amino acid solubility of black soldier fly larvae meal in Atlantic salmon (*Salmo salar*) *in vivo* and *in vitro*

Gopika Radhakrishnan[1,2], Marta S. Silva[1], Erik-Jan Lock[1,2], Ikram Belghit[1]* and Antony Jesu Prabhu Philip[1]

[1]Institute of Marine Research, Bergen, Norway, [2]Department of Biological Sciences, University of Bergen, Bergen, Norway

In vitro and *in vivo* methods were used to evaluate amino acids solubility of black soldier fly (BSF) larvae meal and two experimental diets (reference and test diets) for Atlantic salmon. The current study used *in vitro* method such as pH stat to compare and standardise the salmon extracted enzyme (SE), and commercial enzyme (CE) based on their hydrolytic capacity on a purified protein substrate. Further, an *in vitro* amino acid solubility of feed ingredients and diets were measured using the standardised enzyme volume from SE and CE. Results showed that SE and CE exhibit similar protein hydrolytic capacity upon standardisation on purified substrates. However, when using the two-stage hydrolysis (acidic and alkaline steps), significantly higher amino acid solubility was observed with CE except for glycine, and proline which were equally solubilised by both SE, and CE. No significant difference was observed between reference and test diet using the SE except for tyrosine, valine, leucine, and phenylalanine, which were significantly higher solubilised in reference diet than test diet. Whereas higher solubility of valine, isoleucine, aspartic acid, and glutamic acid was observed in test diet using CE than SE. Similarly, the solubility of valine, isoleucine, and glutamic acid were higher in BSF larvae meal when CE was used. The *in vivo* true protein digestibility of BSF larvae meal was 99%, and 81% for the test diet containing BSF larvae meal. The results demonstrated a positive correlation ($r = 0.91$; $p < 0.01$) between salmon and commercial enzymes but overall, no significant correlation was observed for amino acid solubility between *in vivo* and *in vitro*. However, there was a strong positive correlation for protein solubility using SE ($r = 0.98$) than CE ($r = 0.74$) with the *in vivo* true protein digestibility. The efficiency of SE, and CE can be compared, and standardised based on DH%, and hence correlates better with the *in vivo* protein digestibility but not with amino acid solubilities.

KEYWORDS

aquafeed, protein digestibility, *in vitro* assay, pH Stat, insect meal, Atlantic salmon

Introduction

Marine-based ingredients are a major protein source in the diet of farmed Atlantic salmon. However, due to the growing concerns about economic and environmental sustainability of marine-based ingredients, plant-based ingredients have gradually replaced them in Atlantic salmon feeds, decreasing from ~90% in 1990 to ~25% in 2016 (Aas et al., 2019). However, plant-based ingredients require large amount of land and water, and have significant amount of antinutritional factors, and indigestible fibres (Kokou and Fountoulaki, 2018). Because protein is the most expensive, and a significant nutrient to be replaced, numerous research on alternative protein sources were undertaken (Glencross, 2020; Wang et al., 2021). As a result, novel sources of protein such as algae, yeast, and insects were introduced in aquafeed (Albrektsen et al., 2022). Among these, insect meal has received widespread interest due to its low carbon footprint (Huis, 2013), favourable nutritional profile, and high protein digestibility (Rodríguez-Rodríguez et al., 2022).

The European Union (EU) has approved the use of insects in animal feed, including crickets, yellow meal worms, black soldier flies, and maggots [Commission Regulation (EU) 2017/893]. Among these, black soldier fly (BSF) larvae are considered as one of the potential species because of its high growth rate, short rearing period, and well-balanced amino acid profile (Nogales-Mérida et al., 2019). Thus, BSF larvae meal has been used as a protein source in the salmonid diets (Belghit et al., 2019abib_belghit_et_al_2019a; English et al., 2021; Fisher et al., 2020; Weththasinghe et al., 2021) exhibiting positive (Randazzo et al., 2021), negative (St-Hilaire et al., 2007; Kroeckel et al., 2012; Gasco et al., 2016), or no effect on growth performance and nutrient digestibility (Lock et al., 2016; Dumas et al., 2018; Belghit et al., 2019b; Weththasinghe et al., 2021). However, these findings display an inconsistent result as discussed by English et al. (2021) mainly due to inter-study variations and could be linked to their digestibility. Hence, more research is required to investigate the digestibility of BSF larvae meal in the fish diet.

Nutrient digestibility is typically done *in vivo* by conducting a feeding trial. However, the feeding trial requires the use of many experimental fish, demands time, and labour (Tibbetts, 2012; Lewis et al., 2019). The EU has implemented legal obligations to put the 3Rs concepts of replacement, reduction, and refinement (Russell and Burch, 1959) into practice (Directive 2010/63/EU). In this scenario, *in vitro* approaches can be regarded as complementary research methods to study nutrient digestibility. The *in vitro* solubility method is in line with the 3Rs principles for assessing the digestibility of ingredients and diets in the laboratory employing simulated gut conditions and enzymes (Wang et al., 2021). A significant number of *in vitro* studies with varied methodologies were used for the nutritional evaluation of different feed ingredients using purified commercial proteases and enzymes extracted from fish (Hsu et al., 1977; Grabner, 1985; Grabner and Hofer, 1985; Eggum et al., 1989; Rahmah et al., 2016; Mcdonough et al., 2020; Rahmah et al., 2020). The current study used commercial proteases and enzyme extracted from Atlantic salmon with equal concentration. In previous studies, these proteases are standardised based on their specific enzymatic activity (CHONG et al., 2002). However, it was observed in the study by Yasumaru *et al.* (2014) that standardisation of enzymes for *in vitro* studies is more accurate by measuring the hydrolytic capacity (DH%) of enzyme. Considering these research background, a comparison between *in vitro* and *in vivo* nutrient digestibility sheds a light on the reliability of *in vitro* in predicting the nutrient digestibility. Also, emphasis on using the enzyme extracted from salmon is recommended to have a more realistic data on salmon physiology. However, commercial enzymes are most widely used for a rapid analysis. Hence, its comparison with the commercial enzyme source will enable to understand the differences in the enzyme properties. Therefore, the aim of the current study was 1) to standardise salmon extracted enzyme (SE) and commercial enzyme (CE) based on DH% and to compare the digestibility pattern and catalytic efficiency between them. 2) to carry out *in vitro* solubility using two stage hydrolysis where after the alkaline hydrolysis, the digested products are recovered for evaluating the amino acid solubility. 3) comparison between *in vivo* and *in vitro* protein digestibility and amino acid solubility to understand the complementarity of *in vitro* methods in predicting the solubility of aquafeed ingredients.

Materials and methods

Chemicals and reagents

Analytical reagent grade chemicals and Milli-Q® water (18.2 MΩ cm) (EMD Millipore Corporation, Billerica, MA, United States) were used unless otherwise stated. Tris-HCl (GE Health care), Trichloroacetic acid ($C_2HCl_3O_2$, Merck Life Science AS), Hemoglobin from bovine blood (95% crude protein), and casein from bovine milk (90% crude protein) from Sigma Aldrich, St. Louis, MO, United States. Sodium hydroxide (NaOH, Emsure® ACS, ISO), hydrochloric acid (HCl, Emsure® ACS, ISO, 37% w/w) and hydrogen peroxide (H_2O_2, Emsure® ACS, ISO, 30% w/w) were purchased from Merck (Darmstadt, Germany). Pepsin (2500U, P7012, from porcine gastric mucosa), trypsin (5000U, T0303, from

TABLE 1 Formulation, and chemical composition of experimental diets fed to Atlantic salmon.

	References diet	Test diet
Ingredients (g/100 g)		
BSF meal[1]	-	20.00
Fish meal[2]	30.00	24.00
SPC[3]	17.00	13.60
Wheat gluten[4]	15.00	12.00
Wheat[5]	10.15	8.12
Fish oil[2]	15.00	12.00
Rapeseed oil[6]	5.5	4.40
Lecithin. Rapseed[7]	1	0.80
Vitamin PMX[8]	0.7	0.56
Mineral PMX[8]	0.7	0.56
Monosodiumphosphate[8]	2.5	2.00
L-Lysine[8]	0.7	0.56
L-Threonine[8]	0.1	0.08
DL-Methionin[8]	0.3	0.24
L-Histidine[8]	0.3	0.24
Choline chloride[8]	0.5	0.40
Carop. Pink (10% Astax)[8]	0.05	0.04
Yttrium oxide[9]	0.05	0.04
Water adjustment	0.45	0.36
Chemical composition (%)		
Moisture	7.0	7.0
Crude Protein	55.0	55.0
True Protein[10]	41.0	42.9
Crude Lipid	11.9	11.5
Carbohydrate	5.7	5.0
Ash	9.0	9.0
Amino acid composition (mg/g)		
Hyp	2.4	2.0
His	11.8	12.9
Tau	1.49	1.19
Ser	24.4	24.3
Arg	26.6	26.2
Gly	25.5	25.6
Asp	43	47
Glu	113	104
Thr	19.9	20.7
Ala	24.0	26.7
Pro	35	34
Lys	36	37
Tyr	16.2	19.6
Met	14.2	13.1
Val	22.6	24.5
Ile	20.7	21.4
Leu	37	38
Phe	23.5	23.6

[1]Protix Biosystems BV (Dongen, Netherlands).
[2]Pelagia, Norway.
[3]Selecta, Brazil.
[4]Tereos Syral, Belgium.
[5]Norgesmøllene AS, norway.
[6]Emmelev, Denmark.
[7]Marvesa, Netherlands.
[8]Vilomix, Norway.
[9]VWR, norway.
[10]True protein = sum of anhydrous amino acids.
BSF , black soldier fly; SPC , soy protein concentrate.

TABLE 2 Chemical and amino acid composition of black soldier fly larvae meal (BSF), fish meal (FM) and soy protein concentrate (SPC).

	BSF	FM[a]	SPC[a]
Chemical composition (%)			
Crude Protein	53.0	65.9	63.3
True Protein[b]	39.0	-	-
Crude Lipid	13.4	10.7	2.0
Amino acid composition (mg/g)			
His	13.5	18.2	15.5
Ser	20.9	32.6	32.4
Arg	24.1	42.3	42.9
Gly	24.2	50.3	25.6
Asp	51.0	69.2	71.3
Glu	58.0	105	114
Thr	20.8	33.3	25.1
Ala	31.0	47.2	26.3
Pro	27.8	32.1	30.9
Lys	35.0	58.8	37.0
Tyr	28.0	24.6	22.3
Met	9.5	19.6	9.0
Val	29.7	37.8	28.5
Ile	23.4	30.2	27.5
Leu	36.0	57.1	47.4
Phe	22.1	28.9	32.1

[a]Data obtained from Leeper et al., 2022.
[b]True protein = sum of anhydrous amino acids.
BSF , black soldier fly; FM , fish meal; SPC , soy protein concentrate.

porcine pancreas) were obtained from Sigma Aldrich (St. Louis, MO, United States), and Chymotrypsin (≥40 U CAS Number 9004-07-3, from bovine pancreas) was purchased from Sigma Aldrich (St. Louis, MO, United States), and protease (11U, PT377-1G, from porcine pancreas) was purchased from Elastin Products Company (Owensville, MO, United States).

Test ingredient and experimental diets

The test ingredient, BSF larvae meal (53% crude protein, and 13% crude lipid) was procured from Protix Biosystems BV (Dongen, Netherlands) and the experimental diets (test diet, and reference diet) were produced at Nofima's Aquaculture Technology Center, Bergen, Norway. The fish trial was performed in Nofima's tank facilities in Sunndalsøra, Norway. Formulation and proximate composition of the experimental diets (3-mm pellet) are as given in Table 1. The chemical composition and amino acids profile of BSF larvae meal along with traditional protein sources used in aquafeed, such as fish meal and soy protein concentrate are provided in Table 2. Yttrium oxide (0.05%) was used as an inert digestibility marker and added to the experimental diets. The test diet was made by mixing the reference diet with BSF larvae meal at 80: 20 ratio. These ingredients (BSF larvae meal) and experimental diets (reference and test diets) were used for *in vitro* and *in vivo* studies.

Standardisation of pH stat method for *in vitro* solubility using salmon gut enzymes and commercial enzymes

Extraction of crude salmon enzymes (SE)

The extraction of crude salmon enzyme (SE) method was developed based on principles described elsewhere (Alarcón et al., 2002; Yasumaru and Lemos, 2014; Rahmah et al., 2016). Two Atlantic salmon, weighing around 600–700 g were taken from the laboratory facility at the Institute of Marine research, Norway. The fish were fed at 8:00 in the morning with a commercial feed (Supreme Plus15, Skretting) at *ad libitum*. After 4 h, the fish were sacrificed using overdose (100 mg/L) of MS222, followed by a quick cephalic concussion. The fish were dissected to remove the stomach, pyloric ceca, and intestine. The pH of the stomach (4.9–5) and intestines (7.8–7.9) were noted before the excision. The stomach and the intestine along with pyloric caeca were thoroughly washed with cold distilled water to remove the blood stains and fat. These tissues were chopped into smaller pieces and homogenised with cold distilled water in 1:1 ratio using a tissue homogeniser (Polytron PT 2100). The homogenisation was performed in several pulses of approximately 30 s to avoid overheating, and the entire process of homogenisation was done keeping a glass beaker on ice to avoid damage to the tissue protein and enzymes. The homogenised samples were then centrifuged at 3,220 × g for 30 min at 4°C (Fisher Scientific, Eppendorf™ 5810R Centrifuges with A-4-81 Model Rotor). The collected supernatant which constituted the crude enzyme extract (SE) were stored at −80°C until further use. The total protein concentration in the stomach and intestinal extract was determined using Pierce™ 660 nm Protein Assay Reagent (ThermoFisher Scientific, Waltham, MA, United States) using BSA as protein standard (ThermoFisher Scientific).

Preparation of commercial enzyme (CE) stock solution

The commercial enzyme (CE) stock solution required for performing the standardisation and *in vitro* solubility were prepared by mixing equal amount of pepsin for gastric hydrolysis, and trypsin, chymotrypsin, and protease for alkaline hydrolysis. The concentration of 5 mg/ml was considered as stock solution for pepsin and intestinal proteases after performing several attempts with different concentrations based on the previous studies (Hsu et al., 1977; Dimes and Haard, 1994).

Determination of enzyme activity

Total pepsin activity of SE and CE stock solution was assayed according to the method described by Anson and Mirsky (1932), using 2% hemoglobin solution as substrate. The assay was initiated by adding 5 ml of the substrate into the glass tubes named blank and test. All the tubes were placed at 37°C for approximately 10 min to equilibrate. This was followed by addition of 1 mL of enzyme solution into the test tubes and were placed at 37°C for 10 min to incubate. Later, the reaction was terminated by adding 10 ml of 5% trichloroacetic acid (TCA) to all tubes. One ml of respective enzyme solution was added into blank tube after adding TCA. All the tubes were mixed properly and were kept at 37°C for 5 min. The blank and test tubes were filtered using 0.45 µm syringe filter, and the absorbance were read at 280 nm (UV-VIS Spectrophotometer, Shimadzu, Model: UV-1800, United States). One unit of pepsin activity was defined as the change in absorbance of 0.001 per min at pH 2 at 37°C measured as TCA soluble products.

The total protease activity of SE and CE stock solution was measured according to Walter (1984). In this assay, the protease activity of the stock solution was measured using casein as the standard substrate. To begin with, 20 µL of enzyme solution was mixed with 0.5 ml of 0.1M Tris-HCl buffer (pH 8) at room temperature. The reaction was initiated by the addition of 0.5 ml of 1% casein and kept for 30 min. Later, the reaction was terminated by the addition of 0.5 ml of 20% TCA. The solution mix were allowed to stand for 10 min at room temperature, followed by centrifugation at 16,500 *g* for 5 min at 4°C. The absorbance of the reaction mixture was measured at 280 nm (UV-VIS Spectrophotometer, Shimadzu, Model: UV-1800). One unit of enzyme activity is defined as the 1 µg tyrosine released per min (Walter, 1984). All the measurements were carried out in duplicates.

Crude salmon and commercial enzymes standardization using pH stat method

The crude enzyme extract from SE and CE were standardised for their hydrolytic capacity using pH stat according to (Yasumaru and Lemos, 2014) technique using automated titrators (848 Titrino plus-Metrohm AG, Switzerland). The enzyme activity which exhibited a similar degree of hydrolysis (DH%) in the standard substrates for both SE and CE were selected for *in vitro* solubility using two stage hydrolysis. The values which showed similar DH (%) using hemoglobin were 2.33 (SE) and 1.94 (CE), whereas DH (%) obtained were 5.90 (SE) and 6.17 (CE) using casein as a substrate.

The standard substrates used were analytical grade hemoglobin, and casein. One mL of CE stock solution (both gastric and intestinal enzyme mix) and 1 ml of extracted salmon enzyme (stomach and intestine extract) were used. All these enzyme solutions were serially diluted in 1:2 ratio into 5 tubes. Protein suspension mixtures of the standard substrate was prepared by dissolving 80 mg of substrate protein in distilled water (10 ml total suspension volume). The pH of hemoglobin and gastric enzymes were adjusted to 3 and pH 8 for casein and intestinal enzymes using 0.01 N HCl or 0.01 N NaOH, respectively. The protein hydrolysis assay was initiated by the addition of respective enzyme into the protein suspension for 60 min under continuous stirring. The whole reaction was maintained at room temperature (20–23°C). The hydrolyses were carried out in duplicates.

In vitro solubility of black soldier fly larvae meal and diets

In vitro solubility of BSF larvae meal and diets were performed by two stage hydrolysis. The BSF larvae meal and the diets were sieved using a mesh to have fine particle size (˂1,000 µm) for measuring the DH% of the sample. The standardised enzyme activity was selected for gastric hydrolysis and alkaline hydrolysis. Two stage degree of hydrolysis was followed for determining the protein hydrolysis of the samples. Initially, the protein suspension (80 mg protein) of each test ingredient and pepsin were adjusted to the pH 3 with 0.01N HCl, and respective enzyme solutions were added into the mixture. The mixture was allowed to stand for 1 h at room temperature under continuous stirring. After 1 h, the pH of the solution was adjusted to pH 8 with 0.01 N NaOH, and intestinal enzymes were added. This mixture was again allowed to stand for 1 h at room temperature under continuous stirring. After the intestinal hydrolysis, soluble and insoluble fractions were separated. The insoluble fraction was immediately placed on ice to inactivate the enzyme activity. A blank was set up with same conditions without enzymes to determine the effect of autohydrolysis, and a control with only protein suspension in water. The amino acid solubility of the test samples, and blank were measured in relation to control values. All the tubes were stored at −20°C until further analysis. The non-soluble portion was analyzed for amino acid solubility using ultra performance liquid chromatography (UPLC, Waters Acquity UPLC system, Milford, MA, United States).

In vivo digestibility of black soldier fly larvae meal and diets

An *in vivo* digestibility study was carried out in Atlantic salmon to evaluate the amino acid digestibility of BSF larvae meal. The feeding trial was conducted at NOFIMA Research Station at Sunndalsøra, Norway according to Norwegian (FOR-2015-06-18-761) and European legislation (Directive 2010/63/EU). Atlantic salmon used in this trial had a mean initial body weight of 83.2 ± 0.36 g. Each diet was tested in triplicate tanks (flow-through; closed system) thus the salmon were randomly distributed to 6 tanks (110 × 110 cm, 465 L water volume) contained 140 fish each. The fish were fed with the experimental diets for 30 days at *ad libitum* using automatic feeders (72 feeding per day, 1 min per meal, at 20 min intervals). Salmon were reared in seawater (flow rate at 21 L/min) with continuous light (24 h) at 11°C and oxygen saturation was maintained above 80% during the whole experimental period. To estimate the apparent nutrient digestibility of the BSF larvae meal and diets, feces were collected by manual stripping and were stored in polypropylene containers for freeze-drying. During freeze-drying samples were placed without lids in a freeze dryer at −20oC and 0.2 mbar (FreeZone® 18 L, Kansas City, Labconco). The freeze-dried feces, diets, and BSF larvae meal were analysed for amino acids concentrations using ultra-performance liquid chromatography (UPLC, Waters Acquity UPLC system, Milford, MA, United States).

True protein calculation

True protein is calculated based on the sum of anhydrous amino acid residues, due to the presence of high concentrations of non-protein nitrogen (N) present in the BSF larvae. The protein content is often overestimated with 6.25 N-to-protein factor.

Amino acid analysis

The amino acid analysis of the salmon diet, BSF larvae meals, feces and residue after two stage hydrolysis were carried out by an ultra-performance liquid chromatography (UPLC, Waters Acquity UPLC system, Milford, MA, United States). The quantitative determination was based

on an accredited method by the Nordic Committee of Food Analysis (NMKL) and described in detail elsewhere (Espe et al., 2014; Belghit et al., 2019b). The results were integrated by Empower 3 (Waters, Milford, MA, United States). Amino acids were quantified using standards from Thermo Fisher Scientific (product number; 20,088 Rockford, IL 61105, United States).

Formulae and calculations

The degree of hydrolysis (DH) of protein with stomach extract during standardisation was calculated based on the formula (Diermayr and Dehne, 1990):

$$DH = [(V \times N) \div E] \times (1 \div P) \times FpH \times 100 \quad (1)$$

Where V, volume of acid consumed in the hydrolysis reaction (ml); N normality of the acid; E mass of substrate protein (g); P, number of peptide bonds cleaved (mol g protein^{-1}). For proteins which amino acid composition is not determined, P is generally suggested as 8.0. F_{pH} 1.08 (correction factor).

The DH with pyloric caeca/intestine extract during standardisation was calculated according to (Adler-Nissen, 1986):

$$DH = B \times Nb \times \left(\frac{1}{\alpha}\right) \times \left(\frac{1}{MP}\right) \times \left(\frac{1}{Htot}\right) \times 100 \quad (2)$$

Where B, is the volume of alkali consumed (ml); Nb, normality of the alkali, α average degree of dissociation of the α-NHgroups (1/α = 1.50 for pH 8.0 at 25°C); MP, mass of substrate protein (g); H_{tot}, total number of peptide bonds in the protein substrate [7.6–9.2 meqv g protein^{-1}, according to the source of protein (Adler-Nissen, 1986)].

Amino acid (AA) solubility of final products from two stage hydrolysis during the *in vitro* tests were calculated as follows:

$$AA\ (\%) = 100 - \left(\frac{AA\ present\ in\ residue\ after\ digestion}{AA\ present\ in\ residue\ in\ control} * 100\right) \quad (3)$$

Formulae used to determine apparent digestibility (AD) of nutrients in the diets and feed ingredients were previously described (Furukawa and Tsukahara, 1966). The apparent digestibility coefficient (ADC) of nutrients in the diets and apparent digestibility (AD) of the BSF larvae meal was calculated as follows:

$$ADC\ diets\ (\%) = 100 - 100\left(\frac{Y\ diet}{Y\ feces} * \frac{N\ feces}{N\ diet}\right) \quad (4)$$

where Y is concentration of the inert marker (i.e., yttrium oxide) and N is the concentration of the nutrient.

$$ADC\ of\ ingredients\ (\%) = \left(\frac{Nut\ TD * AD\ TD - 0.8 * Nut\ RD * AD\ RD}{0.2 * Nut\ Ing}\right) \quad (5)$$

Nut_{TD} is nutrient concentration in test diet, AD_{TD} is the apparent digestibility of nutrients in test diet, Nut_{RD} is nutrient concentration in the reference diet, AD_{RD} is apparent digestibility of nutrients in the reference diet and Nut_{Ing} is the nutrient concentration in test ingredient.

Statistical analysis

The software Statistica 13.4 (Statsoft Inc.) and GraphPad Prism (version 8.03, for Windows, GraphPad Software, La Jolla, CA, United States) were used for all statistical analysis. Data were tested for normality and homogeneity of variance using a Kolomogorov-Smirnov test and Shapiro-wilk test, respectively. Data from protein DH% from enzyme extract standardisation were transformed to arcsin ($x^{1/2}$) before statistical analysis. Enzymes dilution (DH%) data were subjected to two way full-factorial ANOVA followed by Duncan's Multiple Range Test at significance level of 95% using SAS 9.4 (SAS Institute Inc.) software for Windows (SAS, 2013, Institute, Cary NC). *In vitro* amino acid solubility data were subjected to two-way analysis of variance (ANOVA) with diet and enzyme effect as two factors. One-way ANOVA was performed to analyze any significant difference between diets for each enzyme, followed by Tukey's multiple comparison. The *in vivo* digestibility data were analysed using *t*-test to compare between test diet and reference diet. For all statistical tests, *p*-values < 0.05 were considered significant, and all the results are expressed as mean ± standard deviation. Figures and graphs were obtained by using GraphPad Prism (version 8.03, for Windows, GraphPad Software, La Jolla, CA, United States).

Results

Standardisation of pH stat method for *in vitro* solubility using salmon gut enzymes and commercial enzymes

In vitro solubility for enzyme standardisation using pH stat method exhibited decreased degree of hydrolysis (DH%) on purified substrates like hemoglobin and casein upon serial dilution as shown in Table 3. One mL of SE extract from the stock solution exhibited significantly higher ($p < 0.05$) hydrolytic capacity than the equivalent volume of CE stock solution on hemoglobin. Whereas hydrolytic capacity of 1 ml of CE was significantly higher ($p < 0.05$) than the equivalent volume of SE stock solution on casein. The standardised DH values for SE on haemoglobin and casein were 2.33% and 5.95%, and CE on haemoglobin and casein were 1.94% and 6.17%, respectively (Table 3). The CE and the SE extract were standardised based on the dilution that exhibited similar DH% on the purified substrates.

TABLE 3 The enzyme activity, and protein hydrolytic performance (DH%) of salmon enzyme and commercial enzyme on the standard substrate, hemoglobin, and casein.

Stomach enzyme				
Dilution tubes	**SE (U/ml)**	**SE (DH%)**	**CE (U/ml)**	**CE (DH%)**
1	166.00	6.28*	156.00	1.94*
2	83.00	5.39*	78.00	1.18*
3	42.00	4.58*	39.00	0.65*
4	21.00	3.99*	20.00	0.01*
5	10.00	3.01*	10.00	0.00*
6	5.00	2.33*	5.00	0.00*
Intestine enzyme				
1	5.10	5.95*	8.20	7.70*
2	2.50	4.21*	4.10	6.17*
3	1.25	4.09*	2.05	5.39*
4	0.63	3.03*	1.03	4.65*
5	0.31	2.70*	0.51	3.96*
6	0.16	1.98*	0.26	3.50*

In vitro pH stat protein hydrolysis (DH%) carried out for 60 min at room temperature, with 80 mg protein substrate and 6 tubes of serially diluted (1:2) enzyme solutions with specific activity (U/ml). '*' denotes statistical significance between the dilutions and were analysed through full-factorial ANOVA, followed by Duncan's Multiple Range Test at significance level of 95%.

Total pepsin activity in the SE and CE stock solution was 166 U/ml, and 156 U/mL, respectively. The gastric enzyme activity that provided similar DH for SE and CE were 5 U/ml, and 156 U/ml, respectively (Table 3). Total protease activity in SE, and CE stock solution was 5.1 U/ml, 8.2 U/mL respectively. The intestinal enzyme activity that provided similar DH for SE and CE were 5 U/ml, and CE 4 U/ml respectively (Table 3) for studying the *in vitro* amino acid solubility in diets and BSF larvae meals.

In vitro amino acid solubility of diets and black soldier fly larvae meal using standardised enzyme activity

The *in vitro* amino acid solubility of reference diet, and test diet using SE, and CE are as given in Figure 1 and Supplementary Table S1. The amino acid solubility between SE, and CE were significantly different ($p < 0.05$) despite of standardising with similar degree of protein hydrolysis (except for proline and glycine which were solubilised equally). In general, solubility of amino acids ranged between 70 and 80% using CE and 50–70% using SE, for reference diet and test diet clearly indicating the higher solubility using CE (Supplementary Table S1).

When compared between the diets using SE, there was no significant difference between test diet and reference diet except for tyrosine ($p = 0.003$), valine ($p = 0.001$), leucine ($p = 0.049$), and phenylalanine ($p = 0.008$), which exhibited significantly higher solubility in reference diet compared to test diet. Whereas significantly higher solubility was observed for aspartic acid ($p = 0.002$), glutamic acid ($p = 0.001$), valine ($p = 0.007$), and isoleucine ($p = 0.004$) in test diet when CE was used. There was a significant interaction effect between enzyme and diet on essential amino acid like valine, isoleucine, leucine, and phenylalanine, and non-essential amino acids like aspartic acid, glutamic acid, and tyrosine (Figure 1; Supplementary Table S1).

The *in vitro* amino acid solubility of BSF larvae meal is as given in Figure 2A and Supplementary Table S2. It was observed that the solubility (%) of essential amino acid such as arginine (35.42 ± 0.31), valine (27.35 ± 3.79), isoleucine (41.73 ± 9.87) and non-essential amino acid like glutamic acid (35.53 ± 0.39), and proline (37.60 ± 1.32) were significantly higher ($p < 0.05$) solubilised with CE, being contrast to leucine (44.46 ± 12.71), phenylalanine (34.56 ± 5.38), and tyrosine (36.98 ± 13.11), which were highly solubilised with SE ($p < 0.05$). Very low solubility was observed for histidine, threonine, methionine, and aspartic acid in the BSF larvae meal with both SE and CE (Figure 2A; Supplementary Table S2).

In vivo apparent digestibility of the diets and black soldier fly larvae meal

The *in vivo* apparent digestibility of true protein, crude protein, and crude lipid (%) of the diets were calculated and

10.3389/fphys.2022.1028992

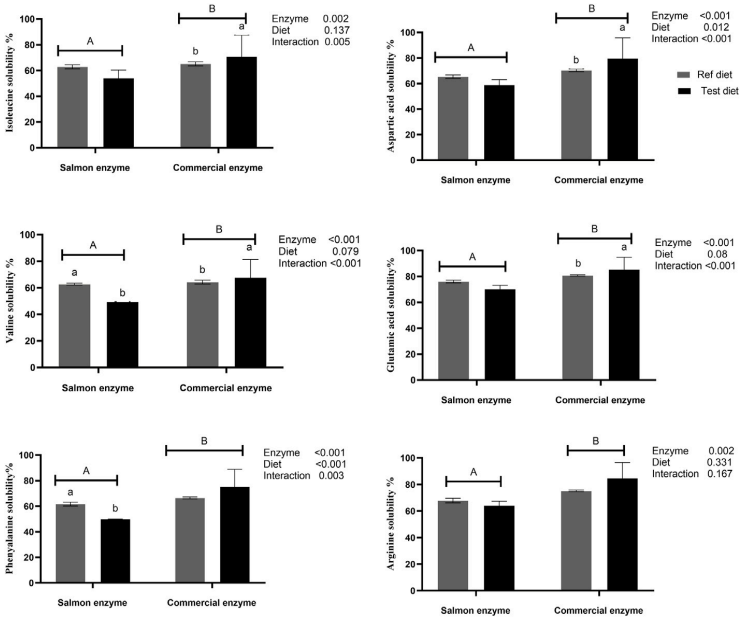


FIGURE 1
In vitro amino acids solubility of reference and test diets using salmon and commercial enzyme. Values are means of 2 values, with their standard deviation represented by vertical bars. Capital letters **(A,B)** indicate statistical difference between salmon enzyme and commercial enzyme detected with two-way ANOVA followed by Tukey's multiple comparisons. Small letters (a,b) indicate the statistical difference between diets detected with one-way ANOVA. *In vitro* solubility values of the reference and test diets for remaining amino acids are presented in Supplementary Table S1.

observed that there was no statistical difference between test diet and reference diet (Table 4). Furthermore, when compared among the amino acid digestibility, there was no significant difference between the reference diet and the test diet except for non-essential amino acids like aspartic acid (p = 0.009), and alanine (p = 0.031) which exhibited significantly higher digestibility in reference diet than test diet. The crude lipid, true protein, and crude protein digestibility (%) of the BSF larvae meal was observed to be 68.8 ± 4.4, 99.2 ± 7.3, and 89.9 ± 5.4, respectively. As given in Figure 2B, the apparent digestibility (%) of some of the amino acids such as arginine, serine, methionine, and isoleucine exhibited 92.0 ± 3.7%, 94.8 ± 6.9, 80.0 ± 6.7, and 87.98 ± 4.89, respectively.

Comparison between and within *in vitro* and *in vivo* digestibility methods

A comparison was made between *in vitro* and *in vivo* true protein digestibility of BSF larvae meal (Table 5). It was observed that a strong positive correlation (r = 0.98; p = 0.09) was obtained using SE with the *in vivo* than using CE (r = 0.74; p = 0.47). No significant difference was observed for protein hydrolysis using SE, and CE on BSF larvae meal. Likewise, a comparison of amino acid solubility of BSF larvae meal using SE, CE, with *in vivo* was performed. The BSF amino acid solubility was comparatively better correlated with SE (r = 0.46; p = 0.07) than CE (r = 0.19; p = 0.46). Also, a comparison of amino acid solubility was made between diets using SE, CE, with *in vivo*. A significant positive correlation (r = 0.91; $p < 0.01$) was found between SE, and CE, in reference diet. No correlation (r = 0.26; p = 0.32) was found between SE, and CE, in test diet. However, when compared between *in vitro* and *in vivo,* the correlation of reference diet was found to be much lesser with SE (r = 0.14; p = 0.61), and CE (r = 0.25 p = 0.34). Similarly, a comparison of amino acid solubility of diets within SE, CE, and *in vivo* was performed (Table 5). When correlated within *in vivo*, a significant positive correlation (r = 0.98; $p < 0.01$) was found between reference diet and test diet. Similar correlation (r = 0.81; $p <$ 0.01) was found between reference diet and test diet when SE was used. However, a weak but statistically significant

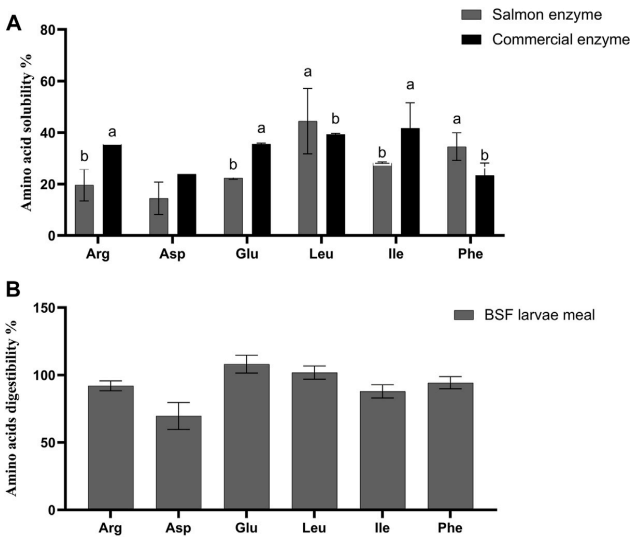


FIGURE 2
(A) *In vitro* amino acids solubility of black soldier fly larvae meal (BSF). Values are means of 3 values, with their standard deviation represented by vertical bars. Small letters **(A,B)** indicate the statistical difference between salmon enzyme, and commercial enzyme detected with one-way ANOVA followed by Tukey's multiple comparisons. *In vitro* solubility values of black soldier fly larvae meal (BSF) for remaining amino acids are presented in Supplementary Table S2 **(B)** *In vivo* amino acids digestibility of black soldier fly larvae meal (BSF). Values are means of 3 values, with their standard deviation represented by vertical bars. *In vivo* digestibility values of the black soldier fly larvae meal (BSF) for remaining amino acids are presented in Supplementary Table S3.

correlation ($r = 0.54$; $p = 0.03$) was observed between the two diets when CE was used.

Discussion

Standardisation of salmon extracted enzyme and commercial enzyme using pH stat method for *in vitro* solubility and comparison of enzyme efficiency between salmon extracted enzyme and commercial enzyme

The current study used enzyme extracted from salmon gut, and commercial proteases (bovine or porcine) to get an insight about the of degree of hydrolysis (DH%) upon dilution from a fixed concentration apart from the established enzyme concentration. The major goal of diluting the SE and CE was to determine the efficiency of enzyme required to have a comparable hydrolytic capability. According to the findings of this study, when standardised based on DH%, the SE, and CE exhibits similar protein hydrolytic performance. This is because, DH% quantifies the peptides that were cleaved by the amount of enzyme present in the reaction mixture. So, when the volume that exhibited similar DH% were selected, it indirectly quantifies the enzyme required to cleave the peptides. Hence, at this point, the volume selected will have a fixed quantity of enzyme to cleave the fixed substrate. This explains that SE, and CE could behave same once standardised based on DH%. This is different from the enzyme standardisation based on enzyme units which shows significant difference in the protein solubility even after being fixed to same enzyme units (Alarcón et al., 2002). This is primarily due to the differences in the enzyme kinetics, and catalytic properties shown between them. When the enzyme activity of the standardised volume was performed, the salmon pepsin exhibited higher enzyme activity than the equivalent volume of CE that was required to produce the same DH%. This agrees with the study conducted by Norris and Elam, (1940), where the pepsin activity of SE was higher than the porcine pepsin. This might be because cold adapted enzymes have higher catalytic efficiency due to a flexible tertiary structure that helps in lowering the activation energy during catalysis (Hochachka, 1984; Outzen et al., 1996). It can also be speculated that higher pepsin activity might be due to the feeding habit of salmon as pepsin activity is apparently related to predation and carnivorous have highest pepsin level than the herbivores

TABLE 4 Apparent digestibility coefficient (ADC%) of crude lipid, true protein, crude protein and amino acid of reference and test diets fed to Atlantic salmon.

Nutrients	References diet	Test diet	*p*-value
Crude lipid	92.99 ± 0.42	93.01 ± 0.69	Ns
True protein	80.37 ± 0.46	81.19 ± 1.03	Ns
Crude protein	85.34 ± 0.34	85.63 ± 0.79	Ns
Amino acids			
His	89.14 ± 0.71	88.85 ± 0.32	Ns
Ser	87.61 ± 0.67	86.69 ± 0.67	Ns
Arg	93.05 ± 0.50	92.51 ± 0.28	Ns
Gly	81.63 ± 1.03	82.25 ± 0.85	Ns
Asp	77.69 ± 0.57a	72.28 ± 1.89 b	0.01
Glu	93.16 ± 0.42	93.03 ± 0.38	Ns
Thr	85.51 ± 0.88	84.56 ± 0.74	Ns
Ala	88.86 ± 0.58a	87.04 ± 0.77 b	0.03
Pro	90.60 ± 0.59	91.04 ± 0.47	Ns
Lys	90.61 ± 0.32	90.24 ± 0.56	Ns
Tyr	90.47 ± 0.72	89.25 ± 0.32	Ns
Met	91.45 ± 0.65	90.91 ± 0.41	Ns
Val	88.57 ± 0.86	87.57 ± 0.64	Ns
Ile	89.42 ± 0.89	88.44 ± 0.41	Ns
Leu	90.63 ± 0.65	89.90 ± 0.43	Ns
Phe	90.55 ± 0.67	89.81 ± 0.31	Ns

The values are expressed as mean ± SD ($n = 3$). Ns, not statistically significant ($p > 0.05$). Statistical significance analysed through t test. Different superscript letters within an individual row denote statistically significant differences in ADC, values.

(Einarsson, 1993). Whereas salmon intestinal proteases exhibited similar enzyme activities with CE, as most of the studies suggested that the fish serine proteases are similar to the homeotherms in terms of their molecular size, amino acid composition, and sensitivity to protease inhibitors (Dimes and Haard, 1994; Shahidi and Janak Kamil, 2001).

Thus, it should be noted that even though the enzymes are standardised based on their hydrolytic capacity, the enzyme activity of SE and CE varies because of specific features shown by the enzymes from cold adapted species and carnivorous compared to their counterparts from homeotherms and herbivores/omnivores (Smalås et al., 1994). Therefore, on one hand species specific enzymes extracts are always considered better when performing *in vitro* solubility for feed ingredients, and diets as many other factors such as age, habitat, feeding status, and enzyme properties of the species can also alter the solubility values (Yasumaru and Lemos, 2014). On the other hand, the use of purified commercial enzymes is helpful since it allows the standardisation of *in vitro* digestion models and lab comparisons (Santos et al., 2019).

In vitro solubility of black soldier fly larvae meal and experimental diets using salmon extracted enzyme and commercial enzyme

The DH% is a quantitative measurement of the protein solubility and do not determine the solubility of individual amino acid. The standardised volume of enzyme was used further for determining the amino acid solubility of BSF larvae meal and diets. The two-stage hydrolysis helps in performing the *in vitro* digestion in a more realistic condition than single step hydrolysis (Yasumaru and Lemos, 2014). Moreover, gastric phase including HCl, and pepsin helps in breaking down the peptide bond and leads to higher protein hydrolysis and provides more homogenous solubility values

TABLE 5 Correlation between *In vivo*, and *in vitro* protein and amino acid solubility of black soldier fly (BSF) larvae meal, test diet and reference diet using salmon enzymes, and commercial enzymes.

	Correlation between *in vivo* and *in vitro* solubility		
	***In vivo*: *In vitro* SE**	***In vivo*: *In vitro* CE**	***In vitro* SE: *In vitro* CE**
Protein solubility			
Black soldier fly larvae meal	0.989 (0.09)	0.74 (0.47)	0.83 (0.37)
Amino acid solubility			
Black soldier fly larvae meal	0.50 (0.07)	0.19 (0.46)	0.44 (0.09)
Test diet	0.07 (0.79)	0.23 (0.4)	0.27 (0.32)
References diet	0.14 (0.61)	0.25 (0.34)	0.99 (<0.01)

Correlation values (r) are indicated before the parenthesis; p values are indicated inside the parenthesis; SE: salmon enzyme; CE: commercial enzyme.

(Alarcón et al., 2002) than single step hydrolysis. The conditions such as temperature, pH, and reaction time selected for the *in vitro* solubility in this study were based on the previous studies (Eggum et al., 1989; Rahmah et al., 2016; Silva et al., 2022). The products after second step of hydrolysis were recovered to evaluate the amino acid solubility of BSF larvae meal and diets. In general, *in vitro* solubility values range obtained for the BSF meal used in this study was similar to the solubility range obtained by Moyano & Savoie (2001) for ingredients like fish meal that showed 20–50% using purified commercial enzymes, and 5–20% using fish digestive extracts. However, low solubility of methionine in this study was observed probably due to the lesser availability of methionine in the BSF larvae meal (Makkar et al., 2014). The reduced solubility of amino acids in the BSF larvae meal using the SE might be associated with the chitin present in the BSF larvae meal which can negatively affect the protein digestibility (Marono et al., 2015; Eggink et al., 2022). Further, the level of lipids can affect the DH and amino acid solubility of BSF larvae meal. Lauric acid and linoleic acid are the most common saturated, and unsaturated lipid fraction present in the BSF larvae meal. Extraction of these lipids can concentrate the proteins and amino acids. This eventually increases the degree of hydrolysis and expose more ionizable amino and carboxylic groups for enzyme hydrolysis (Zayas et al., 1997).

In vitro solubility of the experimental diets used in this study was similar to the results obtained by Carter et al. (1999), where the digestibility values ranged from 74 to 89%, and 82–91% using salmon crude enzymes and commercial enzyme, respectively. Furthermore, the results from the study by Rahmah et al. (2020) showed that CE exhibited higher solubility in the experimental diets than the crude intestinal extract of bagrid catfish (*Mystus nemurus*). It can be speculated that this might be due to the combination ratio of various enzymes in the CE mixture or may be because of the higher thermostability of CE than SE (Smalås et al., 1994; Outzen et al., 1996). When compared between the two diets used in the study irrespective of the enzyme source, the amino acid solubility of reference diet and test diet presented similar values indicating that inclusion of insect meal does not affect the overall solubility as reflected *in vivo*. However, the lesser solubilities of lysine, phenylalanine, and tyrosine in test diet with SE could be possibly due to the limited accessibility of amino acid residues in the diet specific to serine proteases chymotrypsin, and trypsin (Alarcón et al., 2002). The other possible reason might be because of the interference of chitin present in the BSF that can prevent the protein break down from the non-soluble fraction. In this case, during hydrolysis, these amino acids might be less accessible for enzymatic breakdown due to aggregation (Slizyte et al., 2005). However, chemical treatment or enzymatic hydrolysis can be used to convert the chitin into more soluble products, and consequently increase the DH and solubility of amino acids.

In vivo digestibility of black soldier fly larvae meal and experimental diets and comparison between *in vitro* and *in vivo*

In the *in vivo* study, BSF larvae meal was found to be highly digestible by Atlantic salmon exhibiting the true protein digestibility around 99%. Similar results were obtained in digestibility studies where the ADC of protein was above 85% and almost 90% in rainbow trout, and Atlantic salmon, respectively using BSF larvae meals (Belghit et al., 2019a; Fisher et al., 2020). The *in vivo* amino acid digestibility values were also similar with the other digestibility trials performed on rainbow trout (Dumas et al., 2018). Similarly, the ADC of diets were comparable with other digestibility studies (Magalhães et al., 2017; Renna et al., 2017). The higher digestibility of BSF larvae meal suggests that it could be used as a potential ingredient in low fish meal and high plant-based protein diet. Furthermore, the main purpose of *in vitro* solubility was to find out the reliability of this method in predicting the digestibility values *in vivo*. A strong correlation between the reference diet and test diet within the *in vivo* and *in vitro* using SE suggests that the inclusion of BSF larvae meal in the diet of Atlantic salmon are equally digested. A strong positive correlation between SE, and *in vivo* was observed for protein solubility in BSF larvae meal suggesting that species specific enzyme extract gives a better reflection of the *in vivo* studies (Alarcón et al., 2002). The enzyme complex extracted from salmon cleaves the peptide bond at the specific sites as *in vivo*. This agrees with many studies that have established a significant correlation between the *in vitro* and *in vivo* methods using fish enzyme extract on protein solubility (Alarcón et al., 2002; Yasumaru and Lemos, 2014; Rahmah et al., 2016; Rahmah et al., 2020).

However, in the current study, an attempt was made to correlate amino acid solubility between *in vivo* and *in vitro*, but no strong correlation was found. The possible reason for lacking correlation between *in vivo* and *in vitro* amino acid solubility data might be due to the following reasons; firstly, DH% that was used to standardise the enzymes, quantified the number of peptide bonds hydrolysed in a given protein source. This eliminates the detection of pre-hydrolysed peptides already present in the reaction mixture. Thus, these undetected amino acids, when analysed will interfere with the final solubility values. Secondly, the differences might be because of the site of hydrolysis by the proteases. This can be related to the specificity of enzymes in cleaving the peptide bond within (endoproteases) or at the end (exoproteases) of protein molecules (Alarcón et al., 2002). Hydrolysis by endo- or exo-proteases by different enzyme sources also depends on the assay conditions. These include factors like the assay temperature, biological age of fish, salinity of the rearing water, particle size of ingredients and diets (Lu et al., 2011). Furthermore, the method cannot reproduce the complex digestion process which includes physical contraction, fluid mechanics of mixing and gradual emptying of food particles, as well as the contribution by the gut microbiota. However, these factors were not considered in this

study, and might possibly explain the differences in the relative amino acid solubility values between *in vitro* and *in vivo*.

Conclusion

Both *in vivo* and *in vitro* experiments revealed that black soldier fly larvae meal as a fish feed ingredient is highly digestible. Data from the present study showed that the enzyme standardised based on hydrolytic capacity using pH stat method can be used to compare and standardise between different source of enzymes. A strong correlation between *in vitro*, and *in vivo* protein solubilities might not necessarily reflect the amino acid solubilities mainly because of the specificity and catalytic properties of enzymes from varied sources. Therefore, species specific enzymes extracts are always better in correlating *in vitro* to *in vivo*. Since fish encompasses a vast number of species, it is difficult to follow the human approach to standardise the operating conditions. As a result, further research focusing on dynamic digestion models using purified fish enzymes will be more suitable in evaluating nutritional digestibility to ensure consistent and reproducible results.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the Norwegian (FOR-2015-06-18-761) and European legislation (Directive 2010/63/EU).

Author contributions

GR conducted the experiments, analysed, and interpreted the data, drafted, and revised the paper; MS supervised the study, interpreted the data, edited and revised the paper; EL edited and revised the paper; IB designed the study, supervised the study, interpreted the data, edited and revised the paper; AP designed the study, supervised the study, interpreted the data, edited and revised the paper.

Funding

This study was supported by the Institute of Marine Research and the European Union's Horizon 2020 (SUSINCHAIN, 861976).

Acknowledgments

The authors are thankful to Katerina Kousoulaki (Nofima AS, Department of Nutrition and Feed Technology, Bergen, Norway), Joachim Nordbø (Institute of Marine Research), and Anne Gulbrandsen Frøystein (University of Bergen) for their support and help.

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.1028992/full#supplementary-material

References

Aas, T. S., Ytrestøyl, T., and Asgård, T. (2019). Utilization of feed resources in the production of Atlantic salmon (*Salmo salar*) in Norway: An update for 2016. *Aquac. Rep.* 15, 100216. doi:10.1016/j.aqrep.2019.100216

Adler-Nissen, J. (1986). *Enzymic hydrolysis of food proteins*. Elsevier applied science publishers.Amsterdam, Netherlands

Alarcón, F. J., Moyano, F. J., and Díaz, M. (2002). Evaluation of different protein sources for aquafeeds by an optimised pH-stat system. *J. Sci. Food Agric.* 82, 697–704. doi:10.1002/jsfa.1100

Albrektsen, S., Kortet, R., Skov, P. V., Ytteborg, E., Gitlesen, S., Kleinegris, D., et al. (2022). *Reviews in aquaculture*. 14.Future feed resources in sustainable salmonid production: A review doi:10.1111/raq.12673

Anson, M. L., and Mirsky, A. E. (1932). The estimation of pepsin with hemoglobin. *J. Gen. Physiol.* 16, 59–63. doi:10.1085/jgp.16.1.59

Belghit, I., Liland, N. S., Gjesdal, P., Biancarosa, I., Menchetti, E., Li, Y., et al. (2019a). Black soldier fly larvae meal can replace fish meal in diets of sea-water phase Atlantic salmon (*Salmo salar*). *Aquaculture* 503, 609–619. doi:10.1016/j.aquaculture.2018.12.032

Belghit, I., Lock, E.-J., Fumière, O., Lecrenier, M.-C., Renard, P., Dieu, M., et al. (2019b). Species-specific discrimination of insect meals for aquafeeds by direct comparison of tandem mass spectra. *Animals.* 9, 222. doi:10.3390/ani9050222

Carter, C. G., Bransden, M. P., Van Barneveld, R. J., and Clarke, S. M. (1999). Alternative methods for nutrition research on the southern bluefin tuna, *Thunnus maccoyii*: *In vitro* digestibility. *Aquaculture* 179, 57–70. doi:10.1016/s0044-8486(99)00152-0

Chong, A. S. C., Hashim, R., and Ali, A. B. (2002). Assessment of dry matter and protein digestibilities of selected raw ingredients by discus fish (*Symphysodon aequifasciata*) using *in vivo* and *in vitro* methods. *Aquac. Nutr.* 8, 229–238. doi:10.1046/j.1365-2095.2002.00214.x

Diermayr, P., and Dehne, L. J. Z. F. L.-U. U.-F. (1990). [Controlled enzymatic protein hydrolysis in low pH areas. 1. Studies with bovine serum albumin]. *Z. Leb. Unters. Forsch.* 190, 516–520. doi:10.1007/BF01214073

Dimes, L. E., and Haard, N. F. (1994). Estimation of protein digestibility—I. Development of an *in vitro* method for estimating protein digestibility in salmonids (*Salmo gairdneri*). *Comp. Biochem. Physiology Part A Physiology* 108, 349–362. doi:10.1016/0300-9629(94)90106-6

Dumas, A., Raggi, T., Barkhouse, J., Lewis, E., and Weltzien, E. (2018). The oil fraction and partially defatted meal of black soldier fly larvae (*Hermetia illucens*) affect differently growth performance, feed efficiency, nutrient deposition, blood glucose and lipid digestibility of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 492, 24–34. doi:10.1016/j.aquaculture.2018.03.038

Eggink, K. M., Pedersen, P. B., Lund, I., and Dalsgaard, J. (2022). Chitin digestibility and intestinal exochitinase activity in Nile tilapia and rainbow trout fed different black soldier fly larvae meal size fractions. *Aquaculture Research* 53 (16), 5536–5546. doi:10.1111/are.16035

Eggum, B. O., Hansen, I., and Larsen, T. J. P. F. F. H. N. (1989). Protein quality and digestible energy of selected foods determined in balance trials with rats. *Plant Foods Hum. Nutr.* 39, 13–21. doi:10.1007/BF01092397

Einarsson, S. (1993). *Physiological and biological aspects*. Glasgow, Scotland, UK): University of Glasgow.Proteases in the atlantic salmon, *Salmo salar* L

English, G., Wanger, G., and Colombo, S. M. (2021). A review of advancements in black soldier fly (*Hermetia illucens*) production for dietary inclusion in salmonid feeds. *J. Agric. Food Res.* 5, 100164. doi:10.1016/j.jafr.2021.100164

Espe, M., Andersen, S. M., Holen, E., Rønnestad, I., Veiseth-Kent, E., Zerrahn, J. E., et al. (2014). Methionine deficiency does not increase polyamine turnover through depletion of hepatic S-adenosylmethionine in juvenile Atlantic salmon. *Br. J. Nutr.* 112, 1274–1285. doi:10.1017/S0007114514002062

European Union (Eu) (2017). 92. Commission Regulation (EU) 2017/893 of 24 May 2017 amending Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament Available at: https://tinyurl.com/uzt956aw.

Fisher, H. J., Collins, S. A., Hanson, C., Mason, B., Colombo, S. M., and Anderson, D. M. (2020). Black soldier fly larvae meal as a protein source in low fish meal diets for Atlantic salmon (*Salmo salar*). *Aquaculture* 521, 734978. doi:10.1016/j.aquaculture.2020.734978

Furukawa, A., and Tsukahara, H. (1966). On the acid digestion method for the determination of chromic oxide as an index substance in the study of digestibility of fish feed. *Bull. Jpn. Soc. Sci. Fish.* 32, 502–506. doi:10.2331/suisan.32.502

Gasco, L., Henry, M., Piccolo, G., Marono, S., Gai, F., Renna, M., et al. (2016). *Tenebrio molitor* meal in diets for European sea bass (*Dicentrarchus labrax* L.) juveniles: Growth performance, whole body composition and *in vivo* apparent digestibility. *Animal Feed Sci. Technol.* 220, 34–45. doi:10.1016/j.anifeedsci.2016.07.003

Glencross, B. D. (2020). A feed is still only as good as its ingredients: An update on the nutritional research strategies for the optimal evaluation of ingredients for aquaculture feeds. *Aquac. Nutr.* 6, 1871–1883. doi:10.1111/anu.13138

Grabner, M. (1985). An *in vitro* method for measuring protein digestibility of fish feed components. *Aquaculture* 48, 97–110. doi:10.1016/0044-8486(85)90097-3

Grabner, M., and Hofer, R. (1985). The digestibility of the proteins of broad bean (*Vicia faba*) and soya bean (*Glycine max*) under *in vitro* conditions simulating the alimentary tracts of rainbow trout (*Salmo gairdneri*) and carp (*Cyprinus carpio*). *Aquaculture* 48, 111–122. doi:10.1016/0044-8486(85)90098-5

Hochachka, P. W. (1984). Temperature adaptation. *Biochemical adaptations*, 355–449.

Hsu, H., Vavak, D., Satterlee, L., and Miller, G. J. J. O. F. S. (1977). A multienzyme technique for estimating protein digestibility. *J. Food Sci.* 42, 1269–1273. doi:10.1111/j.1365-2621.1977.tb14476.x

Huis, A. V. (2013). Potential of Insects as Food and Feed in Assuring Food Security, Annu Rev Entomol. 58, 563–583.doi:10.1146/annurev-ento-120811-153704

Kokou, F., and Fountoulaki, E. (2018). Aquaculture waste production associated with antinutrient presence in common fish feed plant ingredients. *Aquaculture* 495, 295–310. doi:10.1016/j.aquaculture.2018.06.003

Kroeckel, S., Harjes, A. G. E., Roth, I., Katz, H., Wuertz, S., Susenbeth, A., et al. (2012). When a turbot catches a fly: Evaluation of a pre-pupae meal of the Black Soldier Fly (*Hermetia illucens*) as fish meal substitute — growth performance and chitin degradation in juvenile turbot (*Psetta maxima*). *Aquaculture* 364-365, 345–352. doi:10.1016/j.aquaculture.2012.08.041

Leeper, A., Benhaïm, D., Smárason, B. Ö., Knobloch, S., Ömarsson, K. L., Bonnafoux, T., et al. (2022). Feeding black soldier fly larvae (*Hermetia illucens*) reared on organic rest streams alters gut characteristics of Atlantic salmon (*Salmo salar*). *J. Insects as Food Feed* 8, 1355–1372. doi:10.3920/jiff2021.0105

Lewis, M. J., Francis, D. S., Blyth, D., Moyano, F. J., Smullen, R. P., Turchini, G. M., et al. (2019). A comparison of *in-vivo* and *in-vitro* methods for assessing the digestibility of poultry by-product meals using barramundi (*lates calcarifer*); impacts of cooking temperature and raw material freshness. *Aquaculture* 498, 187–200. doi:10.1016/j.aquaculture.2018.08.032

Lock, E. R., Arsiwalla, T., and Waagbø, R. (2016). Insect larvae meal as an alternative source of nutrients in the diet of Atlantic salmon (*Salmo salar*) postsmolt. *Aquac. Nutr.* 22, 1202–1213. doi:10.1111/anu.12343

Lu, Y., Kim, S., and Park, K. (2011). *In vitro-in vivo* correlation: Perspectives on model development. *Int. J. Pharm.* 418, 142–148. doi:10.1016/j.ijpharm.2011.01.010

Magalhães, R., Sánchez-López, A., Leal, R. S., Martínez-Llorens, S., Oliva-Teles, A., and Peres, H. (2017). Black soldier fly (*Hermetia illucens*) pre-pupae meal as a fish meal replacement in diets for European seabass (*Dicentrarchus labrax*). *Aquaculture* 476, 79–85. doi:10.1016/j.aquaculture.2017.04.021

Makkar, H. P. S., Tran, G., Heuzé, V., and Ankers, P. (2014). State-of-the-art on use of insects as animal feed. *Animal Feed Sci. Technol.* 197, 1–33. doi:10.1016/j.anifeedsci.2014.07.008

Marono, S., Piccolo, G., Loponte, R., Di Meo, C., Attia, Y. A., Nizza, A., et al. (2015). *In vitro* crude protein digestibility of *Tenebrio molitor* and *Hermetia illucens* insect meals and its correlation with chemical composition traits. *Italian J. Animal Sci.* 14, 3889. doi:10.4081/ijas.2015.3889

Mcdonough, F. E., Sarwar, G., Steinke, F. H., Slump, P., Garcia, S., and Boisen, S. (2020). *In vitro* assay for protein digestibility: Interlaboratory study. *J. AOAC Int.* 73, 622–625. doi:10.1093/jaoac/73.4.622

Moyano, F. J., and Savoie, L. (2001). Comparison of *in vitro* systems of protein digestion using either mammal or fish proteolytic enzymes. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 128, 359–368. doi:10.1016/s1095-6433(00)00315-9

Nogales-Mérida, S., Gobbi, P., Józefiak, D., Mazurkiewicz, J., Dudek, K., Rawski, M., et al. (2019).Insect meals in fish nutrition. *Reviews in Aquaculture* 11, 1080–1103. doi:10.1111/raq.12281

Norris, E. R., and Elam, D. W. (1940). Preparation and properties of crystalline salmon pepsin. *J. Biol. Chem.* 134, 443–454. doi:10.1016/s0021-9258(18)73285-7

Outzen, H., Berglund, G. I., Smalås, A. O., and Willassen, N. P. (1996). Temperature and pH sensitivity of trypsins from atlantic salmon (*Salmo salar*) in comparison with bovine and porcine trypsin. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 115, 33–45. doi:10.1016/0305-0491(96)00081-8

Rahmah, S., Aliyu-Paiko, M., and Hashim, R. (2016). *In vivo* and *in vitro* protein digestibility in juvenile bagrid catfish *Mystus nemurus* (Cuvier and Valenciennes 1840) fed soybean meal-based diets. *Aquac. Res.* 47, 1392–1401. doi:10.1111/are.12595

Rahmah, S., Hashim, R., and El-Sayed, A.-F. M. (2020). Digestive proteases and *in vitro* protein digestibility in bagrid catfish *Mystus nemurus* (Cuvier and Valenciennes 1840). *Aquac. Res.* 51, 4613–4622. doi:10.1111/are.14807

Randazzo, B., Zarantoniello, M., Gioacchini, G., Cardinaletti, G., Belloni, A., Giorgini, E., et al. (2021). Physiological response of rainbow trout (*Oncorhynchus mykiss*) to graded levels of *Hermetia illucens* or poultry by-product meals as single or combined substitute ingredients to dietary plant proteins. *Aquaculture* 538, 736550. doi:10.1016/j.aquaculture.2021.736550

Renna, M., Schiavone, A., Gai, F., Dabbou, S., Lussiana, C., Malfatto, V., et al. (2017). Evaluation of the suitability of a partially defatted black soldier fly (*Hermetia illucens* L.) larvae meal as ingredient for rainbow trout (*Oncorhynchus mykiss* Walbaum) diets. *J. Anim. Sci. Biotechnol.* 8, 57. doi:10.1186/s40104-017-0191-3

Rodríguez-Rodríguez, M., Barroso, F. G., Fabrikov, D., and Sánchez-Muros, M. J. (2022). Vitro Crude Protein Digestibility of Insects: A Review, 13.*Insects* doi:10.3390/insects13080682

Russell, W. M. S., and Burch, R. L. (1959). *The principles of humane experimental technique*. Methuen.Massachusetts, USA

Santos, D. I., Saraiva, J. M. A., Vicente, A. A., and Moldão-Martins, M. (2019). "2 - methods for determining bioavailability and bioaccessibility of bioactive compounds and nutrients," in *Innovative thermal and non-thermal processing, bioaccessibility and bioavailability of nutrients and bioactive compounds*. Editors F. J. BARBA, J. M. A. SARAIVA, G. CRAVOTTO, and J. M. LORENZO (Elsevier Inc).United Kingdom

Shahidi, F., and Janak Kamil, Y. V. A. (2001). Enzymes from fish and aquatic invertebrates and their application in the food industry. *Trends Food Sci. Technol.* 12, 435–464. doi:10.1016/s0924-2244(02)00021-3

Silva, M. S., Matos, R., Araujo, P., Lock, E. J., Gopika, R., Prabhu, P. A. J., et al. (2022). *In vitro* assessment of protein digestibility and mineral solubility of black soldier fly larvae meals for monogastric animals. *J. Insects as Food Feed* 8, 953–966. doi:10.3920/jiff2021.0197

Šližytė, R., Daukšas, E., Falch, E., Storrø, I., and Rustad, T. (2005). Characteristics of protein fractions generated from hydrolysed cod (*Gadus morhua*) by-products. *Process Biochem.* 40, 2021–2033. doi:10.1016/j.procbio.2004.07.016

Smalås, A. O., Heimstad, E. S., Hordvik, A., Willassen, N. P., and Male, R. (1994). Cold adaption of enzymes: Structural comparison between salmon and bovine trypsins. *Proteins* 20, 149–166. doi:10.1002/prot.340200205

St-Hilaire, S., Sheppard, C., Tomberlin, J. K., Irving, S., Newton, L., Mcguire, M. A., et al. (2007). Fly prepupae as a feedstuff for rainbow trout, *Oncorhynchus mykiss*. *J. World Aquac. Soc.* 38, 59–67. doi:10.1111/j.1749-7345.2006.00073.x

Tibbetts, S. M. (2012). *Protein and energy nutrition of marine gadoids, Atlantic cod (*Gadus morhua *L.) and haddock (Melanogrammus aeglefinus L.)*. Wageningen University and Research.Wageningen, Netherlands

Walter, H., Bergmeyer, H. U., and Bermeyer, J. (1984). *Methods of Enzymatic Analysis*, 5. New York, NY, USA: John Wiley & Sons.Proteinases: Methods with hemoglobin, casein and azocoll as substrates

Wang, R., Mohammadi, M., Mahboubi, A., and Taherzadeh, M. J. (2021). *In-vitro* digestion models: A critical review for human and fish and a protocol for *in-vitro* digestion in fish. *Bioengineered* 12, 3040–3064. doi:10.1080/21655979.2021.1940769

Weththasinghe, P., Hansen, J. Ø., Nøkland, D., Lagos, L., Rawski, M., and 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. doi:10.1016/j.aquaculture.2020.735785

Yasumaru, F., and Lemos, D. (2014). Species specific *in vitro* protein digestion (pH-stat) for fish: Method development and application for juvenile rainbow trout (*Oncorhynchus mykiss*), cobia (*Rachycentron canadum*), and nile tilapia (*Oreochromis niloticus*). *Aquaculture* 426-427, 74–84. doi:10.1016/j.aquaculture.2014.01.012

Zayas, J. F. (1997). "Solubility of proteins," in *Functionality of proteins in food*. Editor J. F. ZAYAS (Berlin, Heidelberg: Springer).

frontiers

Supplementary Material

1 Supplementary Tables

Table 1: *In vitro* amino acid solubility of reference diet and test diet using salmon enzymes, and commercial enzymes after two stage hydrolysis

	***In vitro* amino acid solubility**						
	Salmon enzyme		**Commercial enzyme**		**Two-way ANOVA P-value**		
Amino acids	**Reference diet**	**Test diet**	**Reference diet**	**Test diet**	**Enzyme**	**Diet**	**Diet*Enzyme**
His	63.9±1.5	58.7±3.5	70.2±0.3	69.9±0.09	0.003	ns	ns
Ser	65.4±0.0	61.6±3.4	66.3±1.4	68.4±0.4	0.04	ns	ns
Arg	67.9±1.7	64.1±3.3	75.2±0.6	75.1±0.7	0.002	ns	ns
Gly	68.7±0.9	65.1±3.3	69.1±1.4	70.0±0.9	Ns	ns	ns
Asp	65.4±1.3	58.9±4.1	70.4±1.0^{b}	90.6±0.7^{a}	<0.001	0.012	<0.001
Glu	76.1±1.0	70.3±2.9	80.9±0.5 ^{b}	91.9±0.3 ^{a}	<0.001	ns	<0.001
Thr	63.8±2.1	59.1±0.4	66.0±1.2	66.6±1.1	0.04	ns	ns
Ala	66.0±1.4	62.9±3.7	67.9±1.4	70.5±0.1	0.03	ns	ns
Pro	73.0±1.3	69.4±3.0	75.4±1.0	73.6±2.8	Ns	ns	ns
Lys	67.6±0.9	54.8±0.3	73.3±1.4	80.9± 12.0	0.02	ns	ns
Tyr	63.0±1.9^{a}	48.4±5.9^{b}	66.4±0.9	64.6±0.0	0.01	0.021	0.05
Met	61.1±2.1	58.2±4.5	64.2±1.4	69.4±1.7	0.02	ns	ns
Val	62.8±0.7^{a}	49.5±0.05^{b}	64.3±1.4^{b}	77.9±0.8^{a}	<0.001	ns	<0.001
Ile	62.9±1.6	54.2±6.2	65.2±1.6^{b}	82.5±0.1^{a}	0.002	ns	0.005
Leu	65.7±1.3^{a}	61.2±0.6^{b}	69.2±0.9	69.9±1.5	0.001	ns	0.031
Phe	61.7±1.5^{a}	49.9±0.06^{b}	66.5±0.8	64.8±1.3	<0.001	<0.001	0.003

All data are shown as mean±SD (n=2). P values of two-way ANOVA are presented for factors 'diet', 'enzyme' and interaction between diet and enzyme. ns, not statistically significant ($p > 0.05$). Different superscript letters within an individual row denote statistically significant differences in amino acid solubility according to Tukey's multiple comparison test

Table 2: *In vitro* amino acid solubility of black soldier fly (BSF) larvae meal using salmon enzyme, and commercial enzyme after two stage hydrolysis

Amino acid	Salmon enzyme	Commercial enzyme	P value
His	10.5±5.6	17.2±1.3	ns
Ser	18.9±7.5	25.6±0.6	ns
Arg	19.6±6.2[b]	35.4±0.3[a]	0.046
Gly	38.6±15.3	27.4±0.6	ns
Asp	14.5±6.3	23.9±0.02	ns
Glu	22.4±5.3[b]	35.5±0.4[a]	0.038
Thr	15.3±6.2	24.4±0.2	ns
Ala	44.5±17.8	51.0±24.1	ns
Pro	28.9±8.5[b]	37.6±1.3[a]	0.023
Lys	22.7±5.9	35.9±1.7	ns
Tyr	36.9±13.1[a]	17.9±2.6[b]	0.043
Met	5.3±7.9	15.1±0.06	ns
Val	4.0±4.0[b]	27.4±3.8[a]	0.046
Ile	28.2±0.5[b]	41.7±9.9 [a]	0.015
Leu	44.5±12.7[b]	39.3±0.4 [a]	0.014
Phe	34.6±5.4[b]	23.4±4.8 [a]	0.009

The values are expressed as mean±SD (n=3). Small letters (a, and b) indicate the statistical difference between salmon enzyme, and commercial enzyme detected with one-way ANOVA followed by Tukey's multiple comparisons

Table 3. Apparent digestibility coefficient (ADC%) of crude lipid, true protein, crude protein, and amino acid of black soldier fly larvae meal (BSF)

Nutrients	**ADC% of BSF larvae meal**
Crude lipid	68.9±4.4
True protein	99.18±7.3
Crude protein	89.9±5.4
Amino acids	
His	112.8±3.9
Ser	94.8±6.9
Arg	92.0±3.7
Gly	90.9±8.6
Asp	69.7±10.0
Glu	108.1±6.6
Thr	93.5±6.8
Ala	99.6±5.1
Pro	100.4±5.7
Lys	104.2±4.2
Tyr	102.9±2.7
Met	80.0±6.7

Val	91.6±5.1
Ile	87.9±4.9
Leu	101.8±4.9
Phe	94.3±4.5

The values are expressed as mean±SD (n=3).

II

Paper II

Gopika Radhakrishnan, Nina S. Liland, Marianne Wethe Koch, Erik-Jan Lock, Antony Jesu Prabhu Philip, Ikram Belghit, (2023).

Evaluation of black soldier fly larvae meal as a functional feed ingredient in Atlantic salmon (*Salmo salar*) under farm-like conditions. **Frontiers in Aquaculture**, 2, 1239402.

DOI: 10.3389/faquc.2023.1239402

frontiers | Frontiers in Aquaculture

TYPE Original Research
PUBLISHED 10 October 2023
DOI 10.3389/faquc.2023.1239402

OPEN ACCESS

EDITED BY
Byron Morales-Lange,
Norwegian University of Life Sciences, Norway

REVIEWED BY
Leidy Lagos,
AKVA Group, Norway
Pabodha Weththasinghe,
University of Peradeniya, Sri Lanka
Hany M. R. Abdel-Latif,
Alexandria University, Egypt

*CORRESPONDENCE
Ikram Belghit
ikram.belghit@hi.no

†PRESENT ADDRESS
Erik-Jan Lock,
Nutrition and feed technology, NOFIMA, Bergen, Norway

RECEIVED 13 June 2023
ACCEPTED 21 September 2023
PUBLISHED 10 October 2023

CITATION
Radhakrishnan G, Liland NS, Koch MW, Lock E-J, Philip AJP and Belghit I (2023) Evaluation of black soldier fly larvae meal as a functional feed ingredient in Atlantic salmon (*Salmo salar*) under farm-like conditions. *Front. Aquac.* 2:1239402. doi: 10.3389/faquc.2023.1239402

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

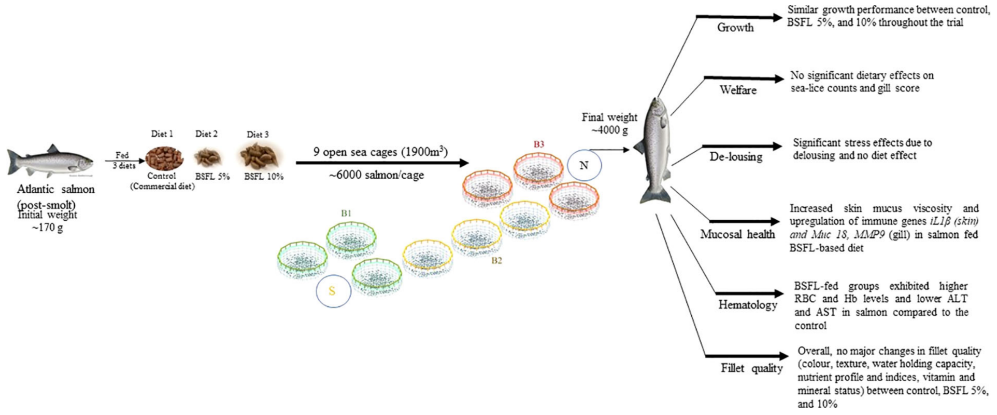
Gopika Radhakrishnan[1,2], Nina S. Liland[1], Marianne Wethe Koch[1], Erik-Jan Lock[1†], Antony Jesu Prabhu Philip[1] and Ikram Belghit[1*]

[1]Feed and Nutrition Group, Institute of Marine Research, Bergen, Norway, [2]Faculty of Mathematics and Natural Sciences, University of Bergen, Bergen, Norway

Atlantic salmon (*Salmo salar*) were fed diets containing black soldier fly larvae (BSFL) meal at two inclusion levels for 13 months in open sea-cages. BSFL meal replaced plant-based ingredients and dietary insect meal inclusion levels were at 5% and 10%. A commercial salmon diet was fed as a control diet. Fish were reared from ~173 g to ~ 4 kg and were randomly distributed into nine open sea-cages with ~6000 salmon/cage (12×12 m^2; 1900 m^3). Fish from the sea-cages were sampled at two time points (mid and final samplings) to study the dietary effects of BSFL meal on the general health and welfare. Monthly assessments of sea-lice and gill score were conducted to evaluate the overall well-being of the salmon. The findings from the current study revealed that dietary inclusion of BSFL meal up to 10% did not have any significant effects in general growth, welfare or survival. However, significant positive response was observed in the general skin mucosal, hematological, and gene expression profiles of salmon. Notably, the group of salmon fed with 5% BSFL meal showed a significant decrease in plasma aspartate transaminase and alanine transaminase. Also, a significantly higher expression of *interleukin1β* in both skin and gill along with upregulation of *matrix metallopeptidase9* and *mucin18* in gill were observed in salmon fed BSFL at 5%, which aided in increased immune responses. Apart from that, this group had significantly higher mucus secretions, decreased cortisol response and increased number of erythrocytes. Furthermore, the delousing stress had a significant effect on the plasma cortisol, and these responses were independent of the dietary effect. Moreover, these immune responses behaved differently at different fish size and time points, acknowledging the influence of various factors in immune modulation. Overall, the findings from this study showed the effects of dietary BSFL meal to modulate the immune status of salmon. This study aims to fill the existing knowledge gaps regarding the impact of incorporating BSFL meal as a functional feed ingredient into the salmon diet on health and immune status replicating real farm conditions.

KEYWORDS

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



GRAPHICAL ABSTRACT
B1, B2, B3-different blocks with each block representing the three dietary group in cages, to eliminate the cage-position effects. BSFL, black soldier fly larvae; S, south; N, North.

1 Introduction

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

Recently, great attention has been given to the potential of using insect meal as sustainable feed ingredients due to its good nutritional profile (well-balanced content of amino acids, fatty acids, vitamins and minerals), presence of immunostimulant and bioactive compounds (Van Huis and Gasco, 2023). Among the approved insect species for its inclusion in aquafeed, black soldier fly larvae (BSFL) have gained widespread attention at research and commercial scale (Veldkamp et al., 2022b). This popularity of the black soldier fly might be due to its short life cycle, fast larval growth and ability to convert a variety of waste into valuable mass, thereby being an advantage for upscaling the insect production for use by the feed sector (Tran et al., 2022). Dietary BSFL meal can replace both marine and plant-based ingredients (such as fish meal (FM) and soy protein) and an inclusion up to 30% in aquafeed is possible without affecting the growth performance of fish species (Liland et al., 2021). However, dietary inclusion of BSFL meal should not only ensure growth but also modulate the immune responses and thus improve the health of farmed fish species (Mouithys-Mickalad et al., 2020).

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

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

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

2 Materials and methods

2.1 Experimental diets

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

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

Ingredients	4.5 mm			9.0 mm		
	Control	BSFL 5%	BSFL 10%	Control	BSFL 5%	BSFL 10%
Fishmeal[1]	10.0	10.0	10.0	10.0	10.0	10.0
Guar Meal 58% CP roasted[2]	10.0	8.10	5.00	12.0	8.4	4.8
Horse beans dehulled[3]	8.58	7.00	6.75	5.5	4.8	4.8
Soy protein concentrate[4]	19.0	17.7	16.1	7.0	6.0	4.0
Sunflower meal[5]	–	–	–	7.0	7.0	7.0
BSF larvae meal[6]	–	5.00	10.0	–	5.0	10.0
Fish oil crude high[7]	4.52	4.45	4.37	5.10	5.04	4.96
Fish oil crude low[8]	4.22	4.44	4.60	4.25	4.38	4.50
Rapeseed oil[9]	14.2	13.7	13.2	22.80	22.53	22.17
Camelina oil[10]	–	–	–	2.55	2,34	2,12
Wheat[11]	4.00	4.00	4.00	7.7	7.7	7.7
Wheat gluten vital pellets[12]	20.0	20.0	20.0	11.76	12.42	13.67
Micro-nutrients[13]	2.98	3.04	3.06	2.15	2.16	2.20
Vitamin- mineral mix[13]	0.81	0.80	0.80	0.86	0.87	0.87
Water	1.68	1.77	2.15	1.32	1.33	1.28
Proximate composition						
Dry Matter %	92.8	92.3	93.5	94.2	93. 9	93.4
Moisture %	7.2	7.8	6.5	5.76	6.11	6.63
Crude Protein %	49.0	48.0	49.0	41.0	42.0	41.0
Crude lipid%	24.0	27.0	25.0	31.0	33.0	33.0
Ash %	4.5	4.5	4.7	4.5	4.8	4.7
TBARS (nmol/g ww)	8.1	10.0	12.0	12.0	13.0	15.0
Gross energy (MJ/kg)	24.6	26.1	25.2	26.2	26.4	26.6

[1]North Atlantic, min. 64% protein.
[2]Roasted, min. 53% protein.
[3]Dehulled, min. 25% protein.
[4]Non-GM, min. 58% protein.
[5]Min. 34% protein.
[6]Protix.
[7]North Atlantic.
[8]South American.
[9]Degummed.
[10]Degummed.
[11]Min. 10% protein.
[12]Vital, min. 80% protein.
[13]Skretting.
[13]Skretting.
BSF, black soldier fly; TBARS, thiobarbituric acid reactive substances.
July 2021to January 2022, 4.5 mm pellet.
February 2022 to August 2022, 9.0 mm pellet.

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

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

2.2 Experimental trial

The feeding trial was conducted at Austevoll research station, Institute of Marine Research, Norway, from July 2021 to August 2022 (13 months). On the 22nd of July 2021, a total of 56000 Atlantic salmon post smolts (Aquagen strain) were transported from Biofish AS (Tørvikbygd) to the Austevoll station by using a well boat. Approximatively 6000 fish were distributed into each sea-cage using sensors, which detect and record the fish before releasing them into each cage. The fish were randomly distributed into nine open sea-cages (12×12 m$^{2;}$1922 m^{3}). Diets were assigned to the sea-cages in triplicates using a randomized block design, containing three blocks (B1, B2, and B3) to account for cage position effects. The fish were acclimatized to the control diet one week prior to the start of the experiment. The feed was dispersed using automatic surface feeders with meals given every 3 min during seven hours a day (20 doses/h). The feeding volume were recorded and registered daily using the Mercatus Farmer software (Scale AQ, Norway) to estimate the biomass and feed conversion ratio. The pellet size (4.5- and 9.0-mm pellet) and size of meals were adjusted according to the fish size, biomass and feeding behavior by visual observation from the surface. Lumpfish (*Cyclopterus lumpus*) and Ballan wrasse (*Labrus bergylta*) were added to each cage to keep lice levels low (Imsland et al., 2014; Imsland et al., 2018), according to standard routines at the station (Ratio of cleaner fish to salmon was 12% with the same ratio in all cages). The number of dead fish (salmon and cleaner fish) were calculated by counting them daily through visual observation. Towards the end of the trial (week 21-30), the dead fish were macroscopically examined. Microbiological and histological analyses were performed (Pharmaq Analytic, Bergen, Norway) on gill and heart of dead salmon to diagnose for potential bacterial or viral infections.

2.3 Rearing environment

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

2.4 Samplings

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

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

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

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

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

2.5 Hematological analysis

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

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

2.6 Skin mucus analysis

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

10.3389/faquc.2023.1239402

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

2.7 Gene expression analysis

Gene expression analysis was done on mucosal tissues such as skin and gill. The candidate genes selected for monitoring the general health and welfare of salmon were for stress response: *heat shock protein 70* (*hsp70*), *heat shock protein 90* (*hsp90*), immune response: *inducible nitric oxide synthase* (*inos*), *lipoxygenase5* (*lox5*), *interleukin1β* (*il1β*) and *interleukin4/13a* (*il413a*), mucus production: *mucin5* (*muc5*), *mucin18* (*muc18*) and wound repair and healing: *matrix metallopeptidase9* (*mmp9*). The procedure for RNA extraction, reverse transcription and quantitative PCR (qPCR) followed were as described in Hundal et al. (2021). In brief, the total RNA was extracted from skin and gill tissue using EZ1 RNA Universal Tissue Kit (Qiagen) and the BioRobot EZ1 according to the manufacturer's descriptions. Quality and integrity of RNA were assessed with the NanoDrop ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies) and the Agilent 2100 Bioanalyzer (Agilent Technologies). A two-step real-time PCR protocol was followed to assess the mRNA transcriptional levels of the selected target genes. The qPCR was run on a LightCycler® 480 Real-Time PCR System with the SYBR Green Mastermix (Roche Applied Sciences, Basel, Switzerland) and using the following temperature program: 5 min denaturisation and activation at 95°C, 45 cycles of 10 s denaturisation at 95°C, 10 s annealing at 60°C and 10 s synthesis at 72°C. A melting point analysis was performed before cooling to 4°C. The stability of the reference genes (geometric mean of both *βact* and *elf1α*) and mean normalized expression of the target genes were calculated using CFX Maestro software (Bio-Rad CFX maestro version 1.1, Bio-Rad laboratories). The list of primers and reference genes used are as given in Table 2. The efficiency of all PCR runs was higher than 96% (ranging from 96 to 125%).

2.8 Statistical analysis and calculations

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

TABLE 2 Primers used for quantitative PCR.

Primers	Forward	Tm	Reverse	Tm	Accession number	Primer length	Ta	Product size
hsp70	CCTGCCTACTTCAACGATTCACAGAGACA	56	CCAGCGATCACTCCAGCGTCCTTA	56	XM_045720591.1	25N	60 °C	59 bp
hsp90	GTGTGAACAATGGGAAATGGAACA	49	CAGGGTGCATGTTATGTTGCA	47	BT125328.1	25N	60 °C	81 bp
inos	ACAGACATTGGCCCAGAGAC	49	CTCCATTCCCAAAGGTGCTA	47	AF088999.1	25N	60 °C	140 bp
mmp9	CTGGCGCAGATATTTTGGAT	45	CATGGCTTTTGAGCCAGTTC	47	NM_001140457.1	25N	60 °C	133 bp
il1β	GCTGGAGAGTGCTGTGGAAGAAC	54	CGTAGACAGGTTCAAATGCACTTTGTG	53	AY617117	25N	60 °C	220 bp
il413a	GCATCGTTGTGAAGAGCCAAGA	50	GAAGTCTCCTCAGCTCCACCT	51	AB574339	25N	60 °C	63 bp
muc5	CCGTGCTGGGAGACATTATGAAGT	52	TGCTGGAGAGGGAAAGGGTAAC	52	XM_045690381.1	25N	60 °C	81 bp
muc18	AAGAGCAGCCGAGGTGGTG	47	TCCGTTGACTTGGCAGATGA	47	XM_045724352.1	25N	60 °C	78 bp
βact	CCAAAGCCAACAGGGAGAA	46	AGGGACAACACTGCCTGGAT	49	BG933897	25N	60 °C	91 bp
elf1α	TGCCCCTCCAGGATGTCTAC	51	CACGGCCCACAGGTACTG	50	AF321836	25N	60 °C	57 bp

Tm, melting temperature; Ta, annealing temperature.

10.3389/faquc.2023.1239402

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

$$\text{Specific growth rate (SGR, \% per day)} = \left[\frac{\ln(\text{Final Body Weight (g)}) - \ln(\text{Initial Body Weight (g)})}{\text{Number of feeding days}}\right] \times 100$$

$$\text{Weight gain (WG, g)} = (\text{Final Body Weight (g)} - \text{Initial Body Weight (g)}$$

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

$$\text{Fulton's condition factor (K)} = (\text{Final Body Weight (g)}/\text{Final Body Length(mm)}\hat{}3) \times 100$$

$$\text{Hepatosomatic index (HSI, \%)} = 100 \times \text{Liver weight (g)}/\text{Fish weight (g)}$$

$$\text{Viscerosomatic index (VSI, \%)} = 100 \times \text{Viscera weight (g)}/\text{Fish weight (g)}$$

3 Results

3.1 Fish growth and survival

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

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

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

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

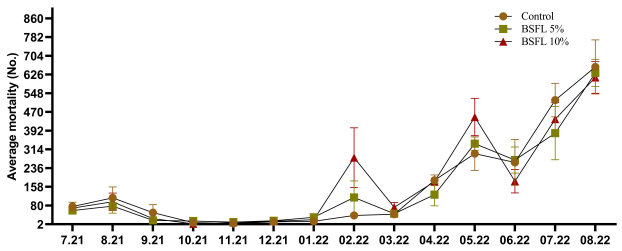


FIGURE 1
Total mortality (No.; number of dead salmon collected) during the 13 months experiment. The data are presented as the mean of dead fish/cage (± SE, triplicates cages per diet) collected during each month from July 2021-August 2022 (x-axis).

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

3.2 Gill score and sea-lice

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

3.3 Hematological analysis

3.3.1 Cortisol

There was a significant (P<0.05) difference in the mean cortisol value from the two sampling points (mid and final) (Figure 3). The mean cortisol value from the final sampling (190-358 ng/mL) was almost double the mean value from the mid-sampling (113-144 ng/mL). Moreover, a significant dietary effect was observed among the dietary groups from the final sampling, where the cortisol levels were significantly (P<0.05) lower in the 10% BSFL fed group than the control group.

3.3.2 Red blood cell and hemoglobin

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

3.3.3 Plasma metabolites

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

3.4 Skin mucus analysis

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

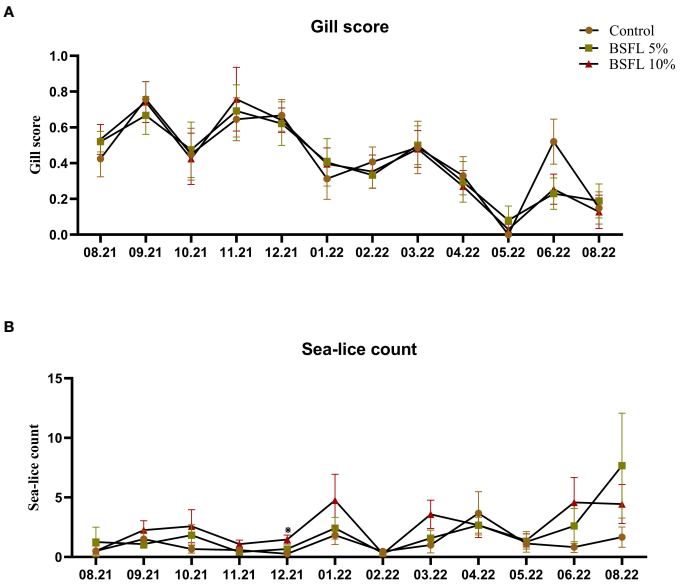


FIGURE 2
Gill score **(A)** and sea-lice count **(B)** of Atlantic salmon fed different diets during the 13 months experiment [from July 2021-August 2022 (x-axis)]. The data are presented as the mean of 20 fish/cage (± SE, triplicates cages per diet). The statistical difference among the dietary groups were detected with two-way ANOVA with diet and block as two factors and cage as random factor, followed by Tukey multiple comparison tests or multiple pair wise comparisons by ranks. *Denote the statistical difference between fish fed Control and BSFL10%.

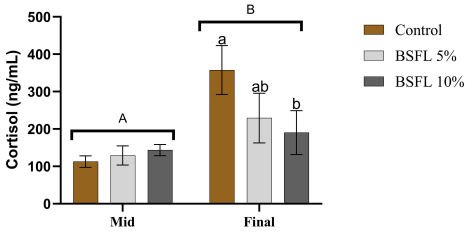


FIGURE 3
Plasma cortisol (ng/mL) of Atlantic salmon fed different diets at two time points (Mid and final samplings). The data are represented as mean of 6 fish/cage (± SE, triplicates cages per diet). The letters a and b denote the statistical difference among the dietary groups, and letters A and B denote the statistical difference among the two sampling points. Factorial ANOVA was performed with diet, block and time as three factors and cage as random factor, followed by Tukey multiple comparison tests.

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

3.5 Gene expression analysis

Figures 6A-C display the results of mRNA expression analysis for selected genes such as *interleukin1β* (*il1β*), *mucin18* (*muc18*) and *matrix metallopeptidase 9* (*mmp9*) in skin and gill tissues from the two different sampling points. In the skin tissue, *il1β* was significantly upregulated in salmon fed BSFL 5% at mid-sampling whereas in gill tissue *muc18* and *mmp9* expression were significantly upregulated in the BSFL 5% fed group compared to the two other dietary groups from the mid-sampling. Meanwhile, *il1β* was significantly upregulated ($P<0.05$) in gill of salmon fed BSFL 5% in both sampling points. The other genes *heat shock Protein* (*hsp70*, *hsp90*), *inducible nitric oxide synthase* (*inos*), *lipoxygenase* (*lox5*) and *interleukin413a* (*il413a*), *mucin* (*muc5*) that were investigated were not significantly affected due to dietary BSFL meal (data not presented). The average expression stability (M value) for the reference genes were 0.29 and 0.33 M value for gill and skin plates respectively.

3.6 Delousing

Figure 7 shows the skin mucus DNA concentration (A) and lysozyme activity (B) in skin mucus, as well as the plasma cortisol (C) and plasma osmolality levels (D) before and after delousing. No effects were detected in the mucus samples for either diet or cortisol-induced stress ($P>0.05$). However, in the plasma samples, a significant ($P<0.05$) stress effect was observed in the cortisol level. Additionally, a significant interaction effect was observed between diet and stress in the plasma osmolality, with the BSFL 5% group exhibiting significantly higher levels ($P<0.05$) than the control group (Figure 7D).

4 Discussion

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

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

10.3389/faquc.2023.1239402

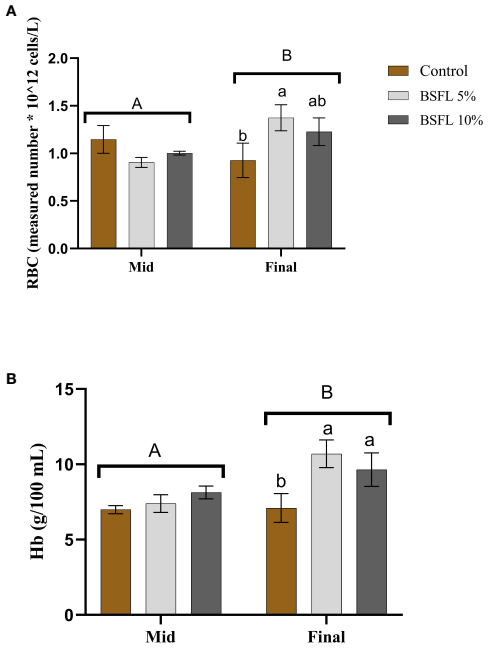


FIGURE 4
Red Blood Cell (RBC; measured number * 10^12 cells/L) **(A)** and hemoglobin (Hb; g/100 mL) **(B)** of Atlantic salmon fed different diets at two time points (Mid and final samplings). The data are represented as mean of 6 fish/cage (± SE, triplicates cages per diet). The letters a and b denote the statistical difference among the dietary groups, and letters A and B denote the statistical difference among the two sampling points. Factorial ANOVA was performed with diet, block and time as three factors and cage as random factor, followed by Tukey multiple comparison tests.

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

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

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

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

(Continued)

TABLE 4 Continued

Parameter	Mid				Final			
Metabolites	Control	BSFL 5%	BSFL 10%	P value	Control	BSFL 5%	BSFL 10%	P value
Ions (mmol/L)								
K+	3.7±0.28	4.1±0.33	3.8±0.22	ns	3.1±0.26	3.1±0.25	3.1±0.24	ns
Na+	173.2±0.88	172.2±2.63	176.8±1.15	ns	183.3±3.82	177.3±1.79	174.9±2.13	ns
Ca++	1.68±0.02	1.65±0.03	1.73±0.02	ns	1.64±0.04	1.68±0.02	1.61±0.03	ns
Cl-	149.0±0.55	149.5±2.30	149.4±0.75	ns	160.1±3.59	155.8±1.06	154.2±1.84	ns
Lac	8.8±0.34	10.3±1.24	10.5±0.60	ns	10.0±1.49	10.8±1.18	7.7±0.32	ns
Osmolality (mOsm/kg)	355.2±2.11	352.0±5.53	363.9±2.42	ns	380.3±9.03	367.2±4.21	359.8±3.94	ns

BSFL, black soldier fly larvae meal; ns, not significant; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; K, potassium; Na, sodium; Ca, calcium; Cl, chloride; Lac, lactate. The letters a and b denote the statistical difference among the dietary groups and were detected using two-way ANOVA, with diet and block as two main factors or Kruskal–Wallis non-parametric analysis, using the median test and multiple pair wise comparisons by ranks.

5% at the same time point. This suggests that the elevated stress condition may have reduced the RBC's oxygen-carrying capacity in control group compared to salmon fed BSFL meal, meaning that this group of salmon faced comparatively less stressful conditions in this experimental trial. A similar pattern of increased erythrocytes and decreased cortisol levels were observed in a study conducted in rainbow trout when fed with roselle (*Hibiscus sabdariffa*) meal, underlining the reason being the antioxidant capacity of the roselle meal (Hoseini et al., 2021). It has been shown that different insect species, including BSFL contain bioactive compounds with antioxidant properties (Priyadarshana et al., 2022; Veldkamp et al., 2022a) and is also known to modulate the oxidative stress in cellular and animal models (D'Antonio et al., 2021). Stress can affect the general health and welfare of fish. The results obtained in this study showed that salmon fed BSFL diets were able to better cope with the stressful events, probably due to the presence of bioactive compounds (chitin, AMPs) and antioxidant capacity, which might have reduced the oxidative stress in Atlantic salmon. The antioxidant capacity could also be confirmed by analyzing other potential stress biomarkers such as catalase, superoxide dismutase or respiratory burst activity. However, these analyses were not performed in the current study.

Lysozyme is one of the innate immune parameters studied in skin mucus (Pelusio et al., 2022). Differences in the mucus lysozyme activities mostly relate to the mucus composition (e.g., mucins, nucleotides, proteases, AMPs) (Fast et al., 2002) and an increase in lysozyme activity generally is triggered due to high microbial load (Ghafoori et al., 2014). Different studies have shown that plasma lysozyme activity increased in fish species fed diets containing insect meal, particularly after a challenge with pathogens (Su et al., 2017; Ido et al., 2019), triggering the innate immune responses. The evidence so far indicates a dose-dependent relationship between insect meal and plasma lysozyme activity in Atlantic salmon (6.25% vs 12.5% inclusion), as presented by Weththasinghe et al. (2021b). However, in the current study, the level of mucus lysozyme at the end of the feeding trial was lower in salmon fed BSF-based diets compared to the fish fed control diet. Apart from bacterial infection, it has been shown that higher lysozyme activity can also be due to high stress conditions (Dash et al., 2018). As discussed earlier, the control group exhibited high levels of cortisol, an indicator of stress condition, which might have increased the level of mucus lysozyme. A similar proportionality between plasma cortisol and skin mucus lysozymes was observed in Atlantic salmon when exposed to crowding stress (Demers and Bayne, 1997; Djordjevic et al., 2021). Fevolden et al. (1994), discussed that the elevated levels of plasma cortisol and rise in lysozyme could indicate higher susceptibility of organism to pathogens and this proportional relationship might be a compensatory adaptation to enhance

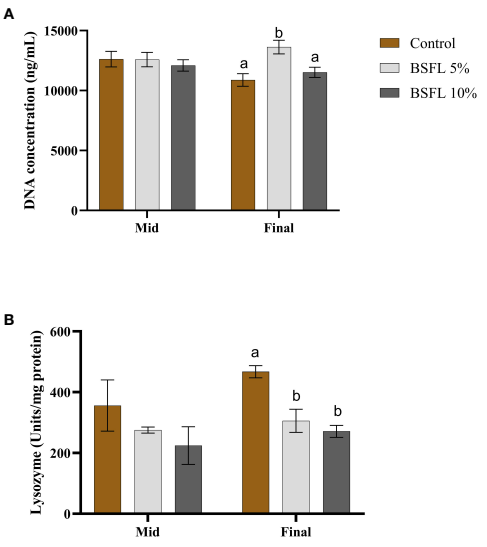


FIGURE 5
Mucus DNA concentration (ng/mL) **(A)** and mucus lysozyme (U/mg protein) **(B)** of Atlantic salmon fed different diets at two time points (Mid and final samplings). The data are represented as mean of 10 fish/cage (± SE, triplicates cages per diet). The letters a and b denote the statistical difference among the dietary groups, and letters A and B denote the statistical difference among the two sampling points. Factorial ANOVA was performed with diet, block and time as three factors and cage as random factor, followed by Tukey multiple comparison tests.

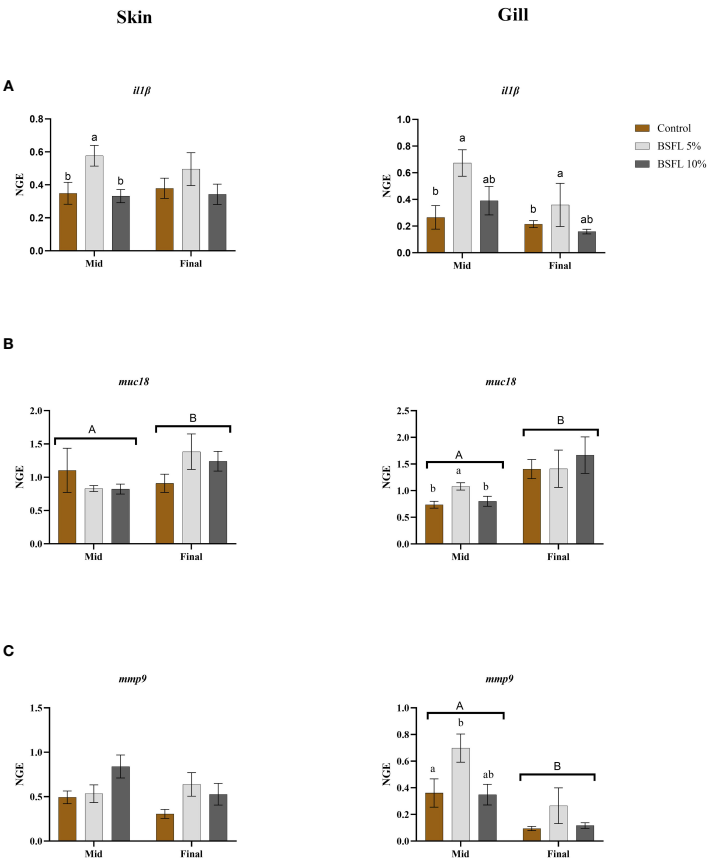


FIGURE 6
Skin and Gill mRNA expression of *interleukin1β* (*il1β*) **(A)**, *mucin18* (*muc18*) **(B)**, and *matrix metallopeptidase9* (*mmp9*) **(C)** of Atlantic salmon fed different diets at two time points (Mid and final samplings). The data are represented as mean of 6 fish/cage (± SE, triplicates cages per diet). The letters a and b denote the statistical difference among the dietary groups, and letters A and B denote the statistical difference among the two sampling points. Factorial ANOVA was performed with diet, block and time as three factors and cage as random factor, followed by Tukey multiple comparison tests (NGE, normalized gene expression).

innate immunity. Thus, lower levels of lysozyme in BSFL fed diets might indicate that salmon fed BSFL diet were comparatively less stressed due to immunostimulant property of BSFL.

The viscoelasticity of mucus is determined by the presence of various biopolymers such as DNA and mucins, responsible for trapping the pathogens and initiating the inflammatory responses (Kaitlyn et al., 2022). In humans, neutrophils are observed to actively release DNA, and this extracellular DNA plays a significant role in capturing harmful microorganisms (Brinkmann et al., 2004). This implies that DNA might have an immune function in mucus. In fish, the skin mucus contains DNA originating from host cells, as well as from beneficial or harmful bacteria (Brinchmann, 2016). Thus, higher secretions of DNA from neutrophils or other cell types into the skin's surface can potentially increase the thickness of the mucus layer and thereby protecting the skin by increasing its viscoelasticity (Cameron and Endean, 1973; Al-Hassan et al., 1985; Caballero et al., 2020). In the present study, the DNA concentration in the skin mucus was comparatively lower in the control diet than BSFL fed at 5% (final sampling) but lacked any significant differences at the mid-sampling. Earlier work has shown that gill mucus viscosity was significantly lower in Atlantic salmon and brown trout (*Salmo trutta* L.) affected with amoebic gill disease compared to healthy fish (Roberts and Powell, 2005). Hender et al. (2021) reported an increase in the number of acidic mucins in Asian seabass (*Lates calcarifer*), that enhanced the gut and skin mucosal immunity when 30% FM and fish oil were replaced with partially defatted BSFL meal. Previous research findings suggest that supplementing of nucleotides and yeast cell extracts in the diet of Atlantic salmon enhances improved mucosal health and gut microbiota (Burrells et al., 2001b; Wang et al., 2022). Our results are therefore in line with earlier reports. Research has indicated that the low molecular weight antimicrobial peptides

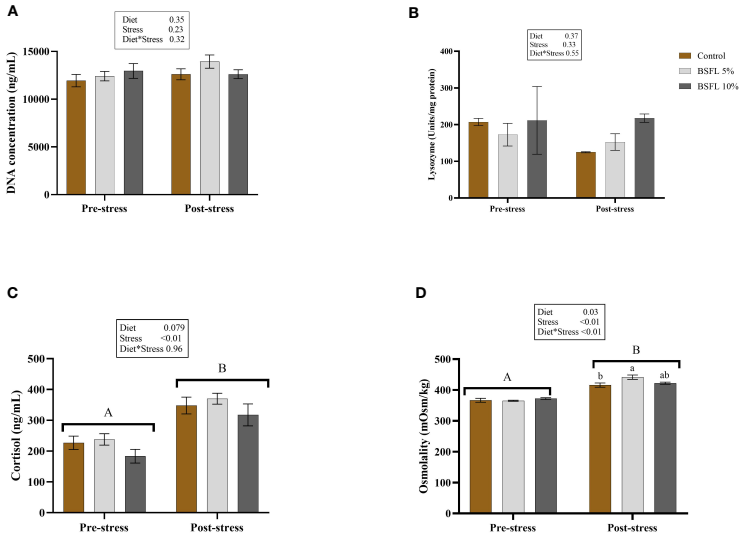


FIGURE 7
Mucus DNA concentration **(A)**, mucus lysozyme **(B)**, plasma cortisol **(C)** and plasma osmolality **(D)** before and after delousing in Atlantic salmon fed different diets. Salmon were sampled before delousing stress (pre-stress) and after one hour (post stress). The data are represented as mean of 6 fish/cage (± SE, triplicates cages per diet). The letters a and b denote the statistical difference among the dietary groups, and letters A and B denote the statistical difference before and after delousing. Two-way ANOVA was performed with diet and block as two factors and cage as random factor, followed by Tukey multiple comparison tests.

found in BSFL meal, specifically defensins, exhibit antibacterial properties. Defensins serve as active agents of the innate immune system, offering a first line of defense against infectious pathogens (Veldkamp et al., 2022a). Furthermore, proline-rich AMPs can interfere with DNA and RNA synthesis by binding to nucleic acids (Kragol et al., 2002) and exhibit antimicrobial properties against various bacteria (Mattiuzzo et al., 2007). Hence, there is a possibility that the presence of these antimicrobial peptides in the diet of salmon might have increased the mucus secretions in the form of DNA and mucins from neutrophils or by modulating the cellular process and producing more viscous mucus. However, further investigation is needed to comprehensively understand the link between diet and mucus defense systems, as well as more knowledge on how DNA concentrations can be used as a measure of mucus viscosity.

In the current study, higher mRNA expression of *muc18* and *mmp9* in gill (mid-sampling) were observed in salmon fed dietary BSFL at 5% compared to salmon fed control diet. These genes are mainly associated with mucus production and inflammatory processes such as remodeling and wound healing (Swain et al., 2006). An increased transcription of *il1β* in skin and gill (mid and final sampling) were also observed in salmon fed dietary BSFL meal. This observed transcription of *il1β* might be due to the bacterium *Branchiomonas cisticola* which is known to affect inflammatory genes (such as *mmp25, mmp13, il1β, il8*) (Gjessing et al., 2021). Thus, higher expression of *il1β* in salmon fed BSFL meal may indicate that they were able to comparatively resist better against pathogens. Furthermore, previous studies reported an upregulation of stress and immune related genes in gut of salmon fed with BSFL meal *in vivo* (Li et al., 2019; Li et al., 2020) and *in vitro* using isolated head kidney leucocytes from salmon challenged with bacterial and viral pathogens (Stenberg, 2018). Similar results were obtained in different tissues in Asian seabass, and European seabass when partially defatted BSFL meal and full fat BSFL meal was substituting 22% and 15% of the FM (Abdel-Latif et al., 2021; Hender et al., 2021). Furthermore, studies have also reported that supplementation of prebiotics such as *Pediococcus acidilactici* to the salmon diet could potentially stimulate the immune system and trigger the release of pro-inflammatory cytokines activation to fight possible pathogen attack (Jaramillo-Torres et al., 2019). In the current study, these observed effects might be linked to the immunostimulant properties of chitin and chitosan (Ghotloo et al., 2015). The chitin or chitosan from the BSFL meal is known to have the potential to stimulate the innate immune response (Veldkamp et al., 2022a). In the current study, however, the content of these components has not been measured. Therefore, the higher expression of these genes involved in the inflammatory process might be due to the content of chitin/chitosan as an immunomodulant in the BSFL meal or due to other compounds in the BSFL meal.

Inflammation triggers the innate immune system, which activates responses causing tissue damage and the release of AST and ALT into the body fluids (Samim and Vaseem, 2021). Compared to the other experimental diets, the diet with BSFL meal fed at 5% showed significantly lower levels of plasma ALT and AST at the mid-sampling. This suggests that incorporating BSFL

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

Dietary inclusion of BSFL meal up to 10% in Atlantic salmon did not have any significant effects in general growth and survival. The salmon fed BSFL 5% showed an increase in the number of erythrocytes, and a reduced cortisol response when exposed to various stressors during the end of the trial and could indicate the antioxidant capacity of salmon fed BSFL diet. Moreover, this group of salmon exhibited a higher skin mucus DNA concentration which suggested higher mucus secretions enabling better protection against pathogens and stress conditions. Also, the inflammatory gene such as *il1β*, was upregulated in skin and gill tissue (mid and final samplings), while the plasma ALT and AST levels were lower in salmon fed dietary BSFL meal at 5% (mid sampling). It is worth mentioning that the health indicators observed in salmon behave differently across different fish sizes, time points and environmental conditions. In conclusion, the results of this study showed that dietary BSFL meal can modulate the immune responses in Atlantic salmon reared at large scale under farm-like conditions.

Data availability statement

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

Ethics statement

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

Author contributions

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

Funding

This study was supported by the Institute of Marine Research and the European Union's Horizon 2020 (SUSINCHAIN, 861976). The authors thank the fellowship provided by the ICAR Netaji Subash International Fellowship.

Acknowledgments

The authors are thankful to Kjetil Stensland, Bjørn Magne Hufthammer, Ida Berger Waage and Gokstad Svanhild Lohne at Austevoll station and all the people worked under the project for the different samplings (Marta S Silva, David Dominguez, Charles Reffay, Paul Gottar, Chandrasekar Selvam, Angela Etayo, Sahar Yarahmadi, and Nawaraj Gautam). The authors thank the fellowship provided by the Indian Council of Agricultural Research (ICAR) through the ICAR Netaji Subash International Fellowship.

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.

Author IB declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

References

Abdel-Latif, H. M. R., Abdel-Tawwab, M., Khalil, R. H., Metwally, A. A., Shakweer, M. S., Ghetas, H. A., et al. (2021). Black soldier fly (*Hermetia illucens*) larvae meal in diets of European seabass: Effects on antioxidative capacity, non-specific immunity, transcriptomic responses, and resistance to the challenge with *Vibrio alginolyticus*. *Fish Shellfish Immunol.* 111, 111–118. doi: 10.1016/j.fsi.2021.01.013

Al-Hassan, J. M., Thomson, M., Criddle, K. R., Summers, B., and Criddle, R. S. (1985). Catfish epidermal secretions in response to threat or injury. *Mar. Biol.* 88, 117–123. doi: 10.1007/BF00397158

AOAC, I. (2010). *AOAC official methods of analysis*. Association of Official Analytical Chemists Arlington, Virginia.

Assefa, A., and Abunna, F. (2018). Maintenance of fish health in aquaculture: review of epidemiological approaches for prevention and control of infectious disease of fish. *Vet. Med. Int.* 2018, 10. doi: 10.1155/2018/5432497

Bang Jensen, B., Qviller, L., and Toft, N. (2020). Spatio-temporal variations in mortality during the seawater production phase of Atlantic salmon (*Salmo salar*) in Norway. *J. Fish Dis.* 43, 445–457. doi: 10.1111/jfd.13142

Belghit, I., Liland, N. S., Gjesdal, P., Biancarosa, I., Menchetti, E., Li, Y., et al. (2019a). Black soldier fly larvae meal can replace fish meal in diets of sea-water phase Atlantic salmon (*Salmo salar*). *Aquaculture* 503, 609–619. doi: 10.1016/j.aquaculture.2018.12.032

Belghit, I., Waagbø, R., Lock, E.-J., and Liland, N. S. (2019b). Insect-based diets high in lauric acid reduce liver lipids in freshwater Atlantic salmon. *Aquac. Nutr.* 25, 343–357. doi: 10.1111/anu.12860

Brinchmann, M. F. (2016). Immune relevant molecules identified in the skin mucus of fish using -omics technologies. *Mol. Biosyst.* 12, 2056–2063. doi: 10.1039/C5MB00890E

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., et al. (2004). Neutrophil extracellular traps kill bacteria. *Science* 303, 1532–1535. doi: 10.1126/science.1092385

Burrells, C., Williams, P. D., and Forno, P. F. (2001a). Dietary nucleotides: a novel supplement in fish feeds: 1. Effects on resistance to disease in salmonids. *Aquaculture* 199, 159–169. doi: 10.1016/S0044-8486(01)00577-4

Burrells, C., Williams, P. D., Southgate, P. J., and Wadsworth, S. L. (2001b). Dietary nucleotides: a novel supplement in fish feeds: 2. Effects on vaccination, salt water transfer, growth rates and physiology of Atlantic salmon (*Salmo salar* L.). *Aquaculture* 199, 171–184. doi: 10.1016/S0044-8486(01)00576-2

Caballero, S., Galeano, A. M., Lozano, J. D., and Vives, M. (2020). Description of the microbiota in epidermal mucus and skin of sharks (*Ginglymostoma cirratum* and *Negaprion brevirostris*) and one stingray (*Hypanus americanus*). *PeerJ* 8, e10240. doi: 10.7717/peerj.10240

Cameron, A. M., and Endean, R. (1973). Epidermal secretions and the evolution of venom glands in fishes. *Toxicon* 11, 401–410. doi: 10.1016/0041-0101(73)90115-3

D'Antonio, V., Serafini, M., and Battista, N. (2021). Dietary modulation of oxidative stress from edible insects: A mini-review. *Front. Nutr.* 8. doi: 10.3389/fnut.2021.642551

Dash, S., Das, S. K., Samal, J., and Thatoi, H. N. (2018). Epidermal mucus, a major determinant in fish health: a review. *Iranian J. Vet. Res.* 19, 72–81. doi: 10.1016/S1095-6433(02)00109-5.

Demers, N. E., and Bayne, C. J. (1997). The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. *Dev. Comp. Immunol.* 21, 363–373. doi: 10.1016/S0145-305X(97)00009-8

Djordjevic, B., Morales-Lange, B., Mclean Press, C., Olson, J., Lagos, L., Mercado, L., et al. (2021). Comparison of circulating markers and mucosal immune parameters from skin and distal intestine of Atlantic salmon in two models of acute stress. *Int. J. Mol. Sci.* 22(3), 1028. doi: 10.3390/ijms22031028

FAO. (2022). The State of World Fisheries and Aquaculture 2022. *Towards Blue Transformation*. FAO. doi: 10.4060/cc0461en

Fast, M. D., Sims, D. E., Burka, J. F., Mustafa, A., and Ross, N. W. (2002). Skin morphology and humoral non-specific defence parameters of mucus and plasma in rainbow trout, coho and Atlantic salmon. *Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol.* 132, 645–657. doi: 10.1016/S1095-6433(02)00109-5

Fawole, F. J., Adeoye, A. A., Tiamiyu, L. O., Ajala, K. I., Obadara, S. O., and Ganiyu, I. O. (2020). Substituting fishmeal with *Hermetia illucens* in the diets of African catfish (*Clarias gariepinus*): Effects on growth, nutrient utilization, haemato-physiological response, and oxidative stress biomarker. *Aquaculture* 518, 734849. doi: 10.1016/j.aquaculture.2019.734849

Fevolden, S. E., Reed, K. H., and Gjerde, B. (1994). Genetic components of post-stress cortisol and lysozyme activity in Atlantic salmon; correlations to disease and resistance. *Fish Shellfish Immunol.* 4(7), 507–519. doi: 10.1006/fsim.1994.1045

Garlock, T., Asche, F., Anderson, J., Ceballos-Concha, A., Love, D. C., Osmundsen, T. C., et al. (2022). Aquaculture: The missing contributor in the food security agenda. *Global Food Secur.* 32, 100620. doi: 10.1016/j.gfs.2022.100620

Ghafoori, Z., Heidari, B., Farzadfar, F., and Aghamaali, M. (2014). Variations of serum and mucus lysozyme activity and total protein content in the male and female Caspian kutum (*Rutilus frisii kutum*, Kamensky 1901) during reproductive period. *Fish Shellfish Immunol.* 37, 139–146. doi: 10.1016/j.fsi.2014.01.016

Ghotloo, S., Hoseini, M. H. M., Alimohammadian, M. H., Khaze, V., Memarnejadian, A., and Rostami, A. (2015). Immunomodulatory effects of chitin microparticles on Leishmania major-infected BALB/c mice. *Parasitol. Int.* 64, 219–221. doi: 10.1016/j.parint.2014.12.007

Gjessing, M. C., Spilsberg, B., Steinum, T. M., Amundsen, M., Austbø, L., Hansen, H., et al. (2021). Multi-agent in *situ* hybridization confirms Ca. *Branchiomonas cysticola* as a major contributor in complex gill disease in Atlantic salmon. *Fish Shellfish Immunol. Rep.* 2, 100026. doi: 10.1016/j.fsirep.2021.100026

Hender, A., Siddik, M. A. B., Howieson, J., and Fotedar, R. (2021). Black Soldier Fly, *Hermetia illucens* as an Alternative to Fishmeal Protein and Fish Oil: Impact on Growth, Immune Response, Mucosal Barrier Status, and Flesh Quality of Juvenile Barramundi, *Lates calcarifer* (Bloch 1790). *Biology* 10(6), 505. doi: 10.3390/biology10060505

Hoseini, S. M., Hoseinifar, S. H., and Van Doan, H. (2021). Growth performance and hematological and antioxidant characteristics of rainbow trout, Oncorhynchus mykiss, fed diets supplemented with Roselle, *Hibiscus sabdariffa*. *Aquaculture* 530, 735827. doi: 10.1016/j.aquaculture.2020.735827

Hossain, M. S., Small, B. C., Kumar, V., and Hardy, R. (2023). Utilization of functional feed additives to produce cost-effective, ecofriendly aquafeeds high in plant-based ingredients. *Rev. Aquac.*, 1–33. doi: 10.1111/raq.12824

Hu, Q.-Y., Wu, P., Feng, L., Jiang, W.-D., Liu, Y., Kuang, S.-Y., et al. (2021). Antimicrobial peptide Isalo scorpion cytotoxic peptide (IsCT) enhanced growth performance and improved intestinal immune function associated with janus kinases (JAKs)/signal transducers and activators of transcription (STATs) signalling pathways in on-growing grass carp (*Ctenopharyngodon idella*). *Aquaculture* 539, 736585. doi: 10.1016/j.aquaculture.2021.736585

Hundal, B. K., Liland, N. S., Rosenlund, G., Höglund, E., Araujo, P., Stubhaug, I., et al. (2021). Increasing the dietary n-6/n-3 ratio alters the hepatic eicosanoid production after acute stress in Atlantic salmon (*Salmo salar*). *Aquaculture* 534, 736272. doi: 10.1016/j.aquaculture.2020.736272

Ido, A., Iwai, T., Ito, K., Ohta, T., Mizushige, T., Kishida, T., et al. (2015). Dietary effects of housefly (Musca domestica) (Diptera: Muscidae) pupae on the growth performance and the resistance against bacterial pathogen in red sea bream (Pagrus major) (Perciformes: Sparidae). *Applied Entomology and Zoology* 50, 213–221. doi: 10.1007/s13355-015-0325-z

Ido, A., Hashizume, A., Ohta, T., Takahashi, T., Miura, C., and Miura, T. (2019). Replacement of Fish Meal by Defatted Yellow Mealworm (*Tenebrio molitor*) Larvae in Diet Improves Growth Performance and Disease Resistance in Red Seabream (*Pargus major*). *Animals* 9 (3), 100. doi: 10.3390/ani9030100

Imsland, A. K. D., Hanssen, A., Nytrø, A. V., Reynolds, P., Jonassen, T. M., Hangstad, T. A., et al. (2018). It works! Lumpfish can significantly lower sea lice infestation in large-scale salmon farming. *Biol. Open* 7 (9), bio036301. doi: 10.1242/bio.036301

Imsland, A. K., Reynolds, P., Eliassen, G., Hangstad, T. A., Foss, A., Vikingstad, E., et al. (2014). The use of lumpfish (*Cyclopterus lumpus* L.) to control sea lice (*Lepeophtheirus salmonis* Krøyer) infestations in intensively farmed Atlantic salmon (*Salmo salar* L.). *Aquaculture* 424, 18–23. doi: 10.1016/j.aquaculture.2013.12.033

Jaramillo-Torres, A., Rawling, M. D., Rodiles, A., Mikalsen, H. E., Johansen, L.-H., Tinsley, J., et al. (2019). Influence of Dietary Supplementation of Probiotic *Pediococcus acidilactici* MA18/5M During the Transition From Freshwater to Seawater on Intestinal Health and Microbiota of Atlantic Salmon (*Salmo salar* L.). *Front. Microbiol.* 10. doi: 10.3389/fmicb.2019.02243

Jensen, L. B., Provan, F., Larssen, E., Bron, J. E., and Obach, A. (2015). Reducing sea-lice (Lepeophtheirus salmonis) infestation of farmed Atlantic salmon (*Salmo salar* L.) through functional feeds. *Aquac. Nutr.* 21, 983–993. doi: 10.1111/anu.12222

Kaitlyn, R. R., William, J. K., Matthew, R. M., and David, B. H. (2022). Effects of mucin and DNA concentrations in airway mucus on *Pseudomonas aeruginosa* biofilm recalcitrance. *bioRxiv.* 7 (4), e00291-22. doi: 10.1128/msphere

Kragol, G., Hoffmann, R., Chattergoon, M. A., Lovas, S., Cudic, M., Bulet, P., et al. (2002). Identification of crucial residues for the antibacterial activity of the proline-rich peptide, pyrrhocoricin. *Eur. J. Biochem.* 269, 4226–4237. doi: 10.1046/j.1432-1033.2002.03119.x

Li, Y., Kortner, T. M., Chikwati, E. M., Belghit, I., Lock, E.-J., and Krogdahl, Å. (2020). Total replacement of fish meal with black soldier fly (*Hermetia illucens*) larvae meal does not compromise the gut health of Atlantic salmon (*Salmo salar*). *Aquaculture* 520, 734967. doi: 10.1016/j.aquaculture.2020.734967

Li, Y., Kortner, T. M., Chikwati, E. M., Munang'andu, H. M., Lock, E.-J., and Krogdahl, Å. (2019). Gut health and vaccination response in pre-smolt Atlantic salmon (*Salmo salar*) fed black soldier fly (*Hermetia illucens*) larvae meal. *Fish Shellfish Immunol.* 86, 1106–1113. doi: 10.1016/j.fsi.2018.12.057

Lieberman, S., Enig, M. G., and Preuss, H. G. (2006). A review of monolaurin and lauric acid: natural virucidal and bactericidal agents. *Altern. Complement. Therapies* 12, 310–314. doi: 10.1089/act.2006.12.310

Liland, N. S., Araujo, P., Xu, X. X., Lock, E. J., Radhakrishnan, G., Prabhu, A. J. P., et al. (2021). A meta-analysis on the nutritional value of insects in aquafeeds. *J. Insects as Food Feed* 7, 743–759. doi: 10.3920/JIFF2020.0147

Mattiuzzo, M., Bandiera, A., Gennaro, R., Benincasa, M., Pacor, S., Antcheva, N., et al. (2007). Role of the Escherichia coli SbmA in the antimicrobial activity of proline-rich peptides. *Mol. Microbiol.* 66, 151–163. doi: 10.1111/j.1365-2958.2007.05903.x

Ming, J., Ye, J., Zhang, Y., Yang, X., Wu, C., Shao, X., et al. (2013). The influence of maggot meal and l-carnitine on growth, immunity, antioxidant indices and disease resistance of black carp (*Mylopharyngodon piceus*). *J. Chin. Cereals Oils Assoc.* 28, 80–86.

Moltumyr, L., Gismervik, K., Gu, J., Gåsnes, S. K., Kristiansen, T. S., Rønnestad, I., et al. (2021). Does the thermal component of warm water treatment inflict acute lesions on Atlantic salmon (*Salmo salar*)? *Aquaculture* 532, 736048. doi: 10.1016/j.aquaculture.2020.736048

Mouithys-Mickalad, A., Schmitt, E., Dalim, M., Franck, T., Tome, N. M., Van Spankeren, M., et al. (2020). Black soldier fly (*Hermetia illucens*) larvae protein derivatives: potential to promote animal health. *Animals* 10 (6), 941. doi: 10.3390/ani10060941

Pelusio, N. F., Bonaldo, A., Gisbert, E., Andree, K. B., Esteban, M. A., Dondi, F., et al. (2022). Different fish meal and fish oil dietary levels in European sea bass: welfare implications after acute confinement stress. *Front. Mar. Sci.* 8. doi: 10.3389/fmars.2021.779053

Priyadarshana, M. K. C., Walpita, C. N., Ruwandeepika, H. A. D., and Magamage, M. P. S. (2022). Effects of black soldier fly, *Hermetia illucens* (Linnaeus 1758), larvae incorporated feed on histomorphology, gut microbiota and blood chemistry of cultured. *Asian Fisheries Sci.* 35, 269–281. doi: 10.33997/j.afs.2022.35.3.005

Rashidian, G., Moosazadeh Moghaddam, M., Mirnejad, R., and Mohammadi Azad, Z. (2021). Supplementation of zebrafish (*Danio rerio*) diet using a short antimicrobial peptide: Evaluation of growth performance, immunomodulatory function, antioxidant activity, and disease resistance. *Fish Shellfish Immunol.* 119, 42–50. doi: 10.1016/j.fsi.2021.09.035

Ringø, E., Olsen, R. E., Vecino, J. G., Wadsworth, S., and Song, S. K. (2012). Use of immunostimulants and nucleotides in aquaculture: a review. *J. Mar. Sci.: Res. Dev.* 2, 104.

Roberts, S. D., and Powell, M. D. (2005). The viscosity and glycoprotein biochemistry of salmonid mucus varies with species, salinity and the presence of amoebic gill disease. *J. Comp. Physiol. B* 175, 1–11. doi: 10.1007/s00360-005-0473-5

Samim, A. R., and Vaseem, H. (2021). Assessment of the potential threat of nickel(II) oxide nanoparticles to fish *Heteropneustes fossilis* associated with the changes in haematological, biochemical and enzymological parameters. *Environ. Sci. pollut. Res.* 28, 54630–54646. doi: 10.1007/s11356-021-14451-y

Sandlund, N., Rønneseth, A., Ellul, R. M., Nylund, S., and Sandlund, L. (2021). *Pasteurella* spp. Infections in Atlantic salmon and lumpsucker. *J. Fish Dis.* 44, 1201–1214. doi: 10.1111/jfd.13381

Segner, H., Sundh, H., Buchmann, K., Douxfils, J., Sundell, K. S., Mathieu, C., et al. (2012). Health of farmed fish: its relation to fish welfare and its utility as welfare indicator. *Fish Physiol. Biochem.* 38, 85–105. doi: 10.1007/s10695-011-9517-9

Seibel, H., Baßmann, B., and Rebl, A. (2021). Blood will tell: what hematological analyses can reveal about fish welfare. *Front. Vet. Sci.* 8. doi: 10.3389/fvets.2021.616955

Selvam, C., Philip, A. J. P., Lutfi, E., Sigholt, T., Norberg, B., Bæverfjord, G., et al. (2022). Long-term feeding of Atlantic salmon with varying levels of dietary EPA + DHA alters the mineral status but does not affect the stress responses after mechanical delousing stress. *Br. J. Nutr.* 128, 2291–2307. doi: 10.1017/S0007114522000514

Soares, S., Green, D. M., Turnbull, J. F., Crumlish, M., and Murray, A. G. (2011). A baseline method for benchmarking mortality losses in Atlantic salmon (*Salmo salar*) production. *Aquaculture* 314, 7–12. doi: 10.1016/j.aquaculture.2011.01.029

Song, S. K., Beck, B. R., Kim, D., Park, J., Kim, J., Kim, H. D., et al. (2014). Prebiotics as immunostimulants in aquaculture: a review. *Fish Shellfish Immunol.* 40, 40–48. doi: 10.1016/j.fsi.2014.06.016

Stenberg, O. K. (2018). *In vitro* bacterial and viral response in head kidney leukocytes of Atlantic salmon (*Salmo salar*) fed dietary insects meal (The University of Bergen). Available at: https://hdl.handle.net/1956/17767.

Su, J., Gong, Y., Cao, S., Lu, F., Han, D., Liu, H., et al. (2017). Effects of dietary Tenebrio molitor meal on the growth performance, immune response and disease resistance of yellow catfish (Pelteobagrus fulvidraco). *Fish Shellfish Immunol.* 69, 59–66. doi: 10.1016/j.fsi.2017.08.008

Swain, P., Sahoo, P. K., and Ayyappan, S. (2006). *Fish and shellfish immunology: An introduction* (India: Narendra Publishing House).

Tartor, H., Luis Monjane, A., and Grove, S. (2020). Quantification of defensive proteins in skin mucus of Atlantic salmon using minimally invasive sampling and high-sensitivity ELISA. *Anim. (Basel)* 10 (8), 1374. doi: 10.3390/ani10081374

Taylor, R. S., Muller, W. J., Cook, M. T., Kube, P. D., and Elliott, N. G. (2009). Gill observations in Atlantic salmon (*Salmo salar*, L.) during repeated amoebic gill disease (AGD) field exposure and survival challenge. *Aquaculture* 290, 1–8. doi: 10.1016/j.aquaculture.2009.01.030

Tran, H. Q., Nguyen, T. T., Prokešová, M., Gebauer, T., Doan, H. V., and Stejskal, V. (2022). Systematic review and meta-analysis of production performance of aquaculture species fed dietary insect meals. *Rev. Aquac.* 14, 1637–1655. doi: 10.1111/raq.12666

Ushakova, N. A., Ponomarev, S. V., Bakaneva, Y. M., Fedorovykh, Y. V., Levina, O. A., Kotel'nikov, A. V., et al. (2018). Biological Efficiency of the Prepupae *Hermetia illucens* in the Diet of the Young Mozambique Tilapia *Oreochromis mossambicus*. *Biol. Bull.* 45, 382–387. doi: 10.1134/S1062359018040143

Van Huis, A., and Gasco, L. (2023). Insects as feed for livestock production. *Science* 379, 138–139. doi: 10.1126/science.adc9165

Veldkamp, T., Dong, L., Paul, A., and Govers, C. (2022a). Bioactive properties of insect products for monogastric animals – a review. *J. Insects as Food Feed* 8, 1027–1040. doi: 10.3920/JIFF2021.0031

Veldkamp, T., Meijer, N., Alleweldt, F., Deruytter, D., Van Campenhout, L., Gasco, L., et al. (2022b). Overcoming technical and market barriers to enable sustainable large-scale production and consumption of insect proteins in Europe: A SUSINCHAIN perspective. *Insects* 13 (3), 281. doi: 10.3390/insects13030281

Wang, J., Jaramillo-Torres, A., Li, Y., Brevik, Ø.J., Jakobsen, J. V., Kortner, T. M., et al. (2022). Gut health and microbiota in out-of-season Atlantic salmon (*Salmo salar* L.) smolts before and after seawater transfer under commercial arctic conditions: modulation by functional feed ingredients. *Front. Mar. Sci.* 9, 860081. doi: 10.3389/fmars.2022.860081

Wang, G., Peng, K., Hu, J., Yi, C., Chen, X., Wu, H., et al. (2019). Evaluation of defatted black soldier fly (*Hermetia illucens* L.) larvae meal as an alternative protein ingredient for juvenile Japanese seabass (*Lateolabrax japonicus*) diets. *Aquaculture* 507, 144–154. doi: 10.1016/j.aquaculture.2019.04.023

Weththasinghe, P., Hansen, J. Ø., Nøkland, D., Lagos, L., Rawski, M., and Øverland, M. (2021a). 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. doi: 10.1016/j.aquaculture.2020.735785

Weththasinghe, P., Lagos, L., Cortés, M., Hansen, J. Ø., and Øverland, M. (2021b). 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*). *Front. Immunol.* 12. doi: 10.3389/fimmu.2021.599530

III

Paper III

Gopika Radhakrishnan, Antony Jesu Prabhu Philip, Christian Caimi, Erik-Jan Lock, Pedro Araujo, Nina S. Liland, Celia Rocha, Luís Miguel Cunha, Laura Gasco, Ikram Belghit (2023).

Evaluating the fillet quality and sensory characteristics of Atlantic salmon (*Salmo salar*) fed black soldier fly larvae meal for whole production cycle in sea cages.

Submitted manuscript.

Graphic design: Communication Division, UiB / Print: Skipnes Kommunikasjon AS

uib.no

ISBN: 9788230860786 (print)
9788230853351 (PDF)