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Effects on growth and welfare of Atlantic salmon parr, feed diets with 10% BSFL meal, with different inclusions of BSFL stickwater.

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Abstract

To achieve sustainable growth in aquaculture, it is important to develop feed from more sustainable sources. Black soldier fly larvae meal (BSFL) is a novel ingredient approved by the EU legislation to be used in aquaculture feed. In the process of creating BSFL meal, the BSFL are pressed by a hydraulic press, and becomes a presscake and liquid is removed, the liquid is known as stickwater (SW). SW has a high content of free amino acids and water-soluble proteins, though there is still insufficient knowledge about the properties of SW in diets for salmon. BSFL meal has several challenges, including high levels of chitin and manganese (Mn). BSFL SW has a very low content of Mn and adding BSFL SW in diets for salmon could be beneficial. Would diets with high content of Mn, have a negative effect on fish welfare?

In this study, the BSFL SW was recovered back in the BSFL meal. Four experimental feeds were made, containing 10% BSFL meal with different inclusions of SW, and one control formulated to be similar to commercial diets for Atlantic salmon. There were three tanks per diet, each tank containing 100 Atlantic salmon parr. The feeds were formulated to have similar nutritional value and apparent digestibility coefficient (ADC).

We saw no significant differences between the different dietary treatments, and all fish preformed similarly. BSFL meal contains high amounts of dietary Mn, and the diets with most Mn contained 120mg/kg of this essential trace mineral. There were seen no sign of Mn oversaturation in the fish, nor reduced welfare. Fish feed all experimental diets, preformed as well as those fed control diet. There was no sign of increased growth or welfare from inclusions of BSFL SW, nor any sign of negative impact of high levels of dietary Mn.

Abbreviations

BSF: Black soldier fly

BSFL: Black soldier fly larvae

EU: European Union

NQC: Norwegian quality cut

SW: Stick water

FM: Fish meal

AA: Amino acids

ADC: apparent digestibility coefficient

Work performed by the candidate and the host institute's personnel

The experiment described in this thesis was performed in the facilities of Nofima by me (the candidate) with help of professional technicians. During my research stay at Nofima for the completion of my study I learned and performed myself fish feeding, oxygen quality measurements, fish dissection, tissue sampling, fish welfare indicator scoring, use of x-ray machine and analysis of x-ray images, freeze drying and sample preservation, different methods of homogenising, ICP analysis

Prior to joining this study, the feeds used in this study, were formulated and made by the staff at Nofima. Start up of the study, raising fish and distributing them to different tanks, feeding and water quality measurements were done by institute staff. Before termination and end sampling, I was taught by the staff how to calibrate feeders and measure water quality. The lipid and protein analysis were conducted by Biolab.

1. Introduction

For the Norwegian aquaculture industry to become more sustainable, it will be necessary to reduce the carbon footprint. Feed manufacturing is an energy demanding process, and has a high carbon footprint, the Norwegian statistical central bureau (SSB) estimates that salmon feed production stands for as much as 83% of the carbon footprint of Norwegian salmon farming (SSB, Oppdrettslaks til heile verda 18.05.2020). In order to make the Norwegian aquaculture more sustainable, it will be necessary to produce more sustainable feed ingredients and reduce carbon footprint from transportation of feed ingredients and finished feed (Aas et al. 2020, Boyd et al.2020, Eidem & Melås 2021). One step towards sustainable aquaculture, is to exchange soy products with an alternative more sustainable protein resource. Black soldier fly larvae (BSFL) has a high potential, as it can convert waste, to high quality protein (Belghit et al 2019). BSF do not require large land areas to grow, and they have short life cycles, and can be harvested often and year-round (Belghit et al 2019). BSFL has successfully been tried for salmon and have replaced up to 100% fish meal with good results (Belghit et al. 2019).

In fish meal (FM) production, stickwater (SW) is removed from the presscake. It was discovered that FM SW has a high content of water-soluble proteins and free amino acids (AA). Today SW is added back to the FM (Mahdabi & Shekarabi 2018, Kousoulaki et al. 2018). BSFL meal can be produced similarly to FM. To support circular economy, it is important to investigate the potential benefits of adding BSFL SW to the BSFL meal in diets for salmon.

There are some challenges in feeding BSFL meal to salmon, it contains high levels of dietary Mn (Zulkifli et al. 2022, Kousoulaki et al. 2022). If dietary Mn levels are too high the fish will adapt by reducing intestinal absorption, enhance liver metabolism, and increase excretion through the faeces (Prabhu et al. 2019). Over saturation of dietary Mn could reduce welfare and performance of the fish. If BSFL is to replace soy in salmon diets, it will be necessary to gain a better understanding of how the fish react to diets with high content of Mn, and where in the tissues Mn is deposited.

1.1 Aim and project hypotheses

The aim of this study is to gain better understanding of dietary BSFL meal effects on farmed salmon growth and welfare.

One aim of this study is to determine if BSFL SW in diets to Atlantc salmon *(Salmo salar)* has positive effects. BSFL SW is currently not used commercially, and BSFL SW could be an unutilized resource that could be beneficial to include in diets for Atlantic salmon.

BSFL meal contains high amounts of dietary Mn. It is important to determine what are the effects of Mn and weather and where it is deposited in the body of the fish.

1.2 Main hypothesis

Can the dietary incorporation of water-soluble proteins and free amino acids from BSFL SW can increase growth and welfare of Atlantic salmon?

1.3 Other project hypotheses

Can high concentrations of dietary Mn induce up-concentration of Mn in the body and tissues of Atlantic salmon parr?

Can high concentrations of dietary Mn have negative effects on fish performance and welfare?

2. Theoretical background

In the fall of 2015, the United Nations (UN) announced 17 sustainability goals, that was to be achieved by 2030. Two of these goals was zero hunger, and sustainable life at sea (UN, Sustainable Development, 2015). In response to these goals, the Norwegian government created a plan called “The aquaculture strategy - An ocean of possibilities” to achieve sustainable growth in Norwegian aquaculture, contributing to sustaining life at sea, by reducing capture fisheries, and sustainable development of aquaculture. (Regjeringen, Havbruksstrategien - Et hav av muligheter, 2021). In the “The aquaculture strategy - An ocean of possibilities” the Norwegian government wished to create sustainable growth in aquaculture by 2030. Fish raised in aquaculture farms have a considerably lower carbon footprint than any other domesticated animals, making farmed fish among the most sustainable animal protein available (Hundal et al. 2022). By developing sustainable aquaculture, it is possible to produce a more sustainable protein to feed a growing population, and thereby reduce world hunger, and contributing to the UN sustainability goals. Producing more seafood through aquaculture, causes less reliance on capture fisheries, and creates an economic interest in protecting the seas, thereby contributing to two of the UN sustainability goals.

Though aquaculture has considerably less carbon footprint than other animal protein, it is currently considered not sustainable (Boyd et al.2020). The Norwegian statistical central bureau (SSB) estimates that 83% of the carbon footprint in Norwegian salmon production, is from feed production (SSB, Oppdrettslaks til heile verda, 18.05.2020), This is due to feed production being a high energy demanding process. Feed has a very high transportation carbon footprint, ingredients transported long distances to feed factories, and finished feed is transported long distances. (Aas et al. 2020, Eidem & Melås 2021).

Salmon are a carnivorous fish, in the wild their diets mainly consist of protein and lipid. Over the past 30 years, feed ingredients used in farmed salmon feed have changed significantly. In the 1990s, salmon feed contained 65% fish meal, and 24% fish oil. In 2020 salmon feed only contain 12% fish meal, and 10% fish (oil figure 1) (Aas et al. 2020). Today the main protein source in salmon feed is soy protein concentrate (SPC), and the main lipid source is rapeseed oil (Aas et al. 2020).

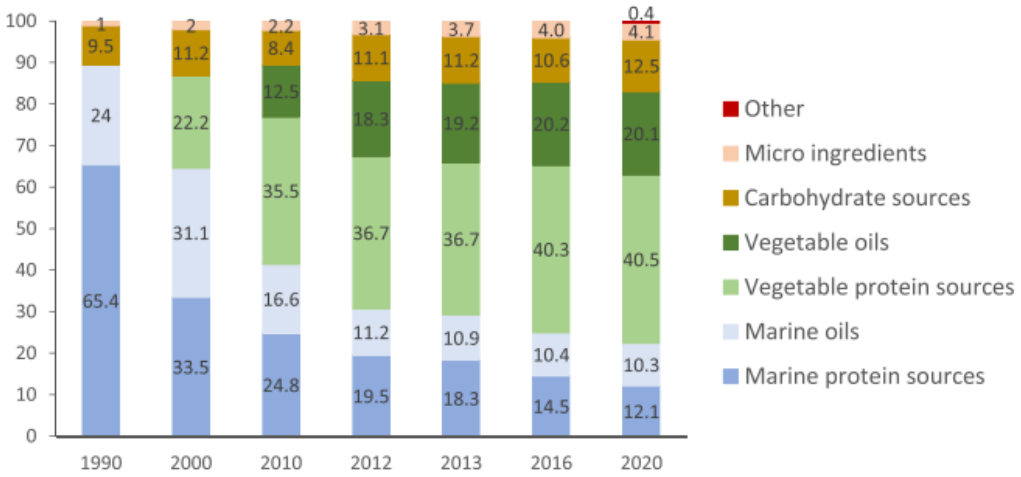


Figure 1. Sources of feed ingredients (% of feed) in Norwegian salmon feed in 2020 compared to previous years (Aas et al., 2019; Ytrestøyl et al., 2015). Micro ingredients include vitamin- and mineral premixes, phosphorus sources, astaxanthin, crystalline amino acids. 'Other' includes insect meal, single cell protein, fermented products, and microalgae.

The Norwegian salmon industry has increased rapidly the past three decades, in the 1990's Norway produced less than 200 000 tonnes of salmon, and in 2020 Norway produced 1 467 655 tonnes of salmon (Aas et al. 2020). In the 1990 there was an abundance of marine ingredients available to be used in salmon feed. Since the 1990's the salmon industry has grown, and the demand for marine feed ingredients has increased, the supply and availability of fish meal has not increased (figure 2) (Aas et al. 2020).

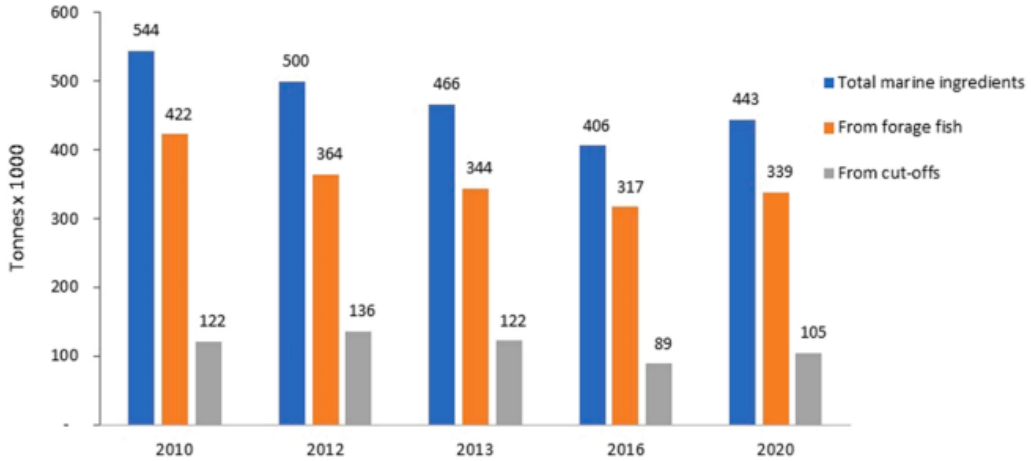


Figure 2. Marine ingredients (tonnes × 1000) from forage fish and cut-offs used in Norwegian salmon feed in 2010–2020. Data from 2010 to 1016 are from Ytrestøyl et al. (2015) and Aas et al. (2019).

As a result, farmed salmon today, are fed feed that is mainly produced of plant ingredients, grown through agriculture (Aas et al. 2020, Eidem & Melås 2021). Agricultural production has a high usage of freshwater, which is a limited resource in many parts of the world. Most agriculture production is based on monocultures, and use pesticides, which greatly limits biodiversity (Boyd et al.2020). Most crops grown in agriculture can go directly to human consumption.

To achieve sustainable growth in salmon farming, it is important to explore new potentially more sustainable novel feed ingredients, that can be produced in large quantities, all year round. Moreover, aquafeeds should not compete with human consumption, and should not decrease welfare for the fish. There are several different novel feed ingredients that have the potential to meet these demands. The novel feed ingredients that are in use, or most likely to be used in near future are macroalgae, microalgae, yeast and insect meal. (Belghit et al. 2019, Mitra 2021).

When investigating new feed ingredients, it could be beneficial to explore feed resources which are natural for the salmon to eat. Atlantic salmon start their lives in freshwater river systems. In and by these river systems there are an abundancy of aquatic and aerial insects, which the salmon parr will predate upon. In a study done in Louvenga river Kola Peninsula, Russia stomach content of wild Atlantic salmon parr was studied, and it was found 24% aerial insects and 68.2% aquatic insects. Insects are a natural part of salmon parr diets (Orlov et al. 2006).

The European commission has since May 2017 approved seven species of insects as novel feed ingredients in aquaculture feed. The following species are approved black soldier fly *(Hermetia illucens)*, common housefly *(Musca domestica)*, yellow mealworm *(Tenebrio molitor)*, lesser mealworm *(Alphitobius diaperinus)*, house cricket (*Acheta domesticus)*, banded cricket *(Gryllodes sigillatus)* and field cricket *(Gryllus assimilis)* (Bruni et al. 2019).

Though several of the named species are approved, black soldier fly (BSF) will be in focus in this study. BSF is a true fly (Diptera) of the family Stratiomyidae. BSF is originally native to the Americas. The BSF occurs worldwide in temperate regions, but it is sensitive to colder climates, and thereby cannot become an invasive species in colder regions (Wang & Shelomi

2017). Adult BSF consume only water, black soldier fly larvae (BSFL) can consume and convert vegetables, fruit, industrial food waste and manure to high quality protein, making them an exceptional resource for sustainable circular biomass production (Wang & Shelomi 2017, Belghit et al. 2019). BSF contain high amounts of protein 40%, and up to 30% lipid (in dry weight). BSF has an amino acid profile similar to that of fish meal, and studies have shown that BSFL can replace soybean meal and fish meal in the diets of poultry, pigs, ruminants, Atlantic salmon, Atlantic cod, Atlantic halibut and European sea bass (Makkar et al. 2014, Lock et al. 2016, Belghit et al. 2019, Bruni et al. 2019, Mitra 2021). Nevertheless, other studies convey less promising results, where dietary inclusions of BSFL meal has led to reduced fish body condition, or has reduced feed palatability (Kroeckel et al 2012, Gasco et al. 2016).

In the process of making fish meal, the raw materials are cooked, then pressed to make a presscake, and in this process the liquid is pressed out. The liquid that is removed is known as stickwater (SW). Fish meal SW was previously disposed of, though it was discovered that the SW contained many nutrients and water-soluble proteins, which can be highly beneficial for fish. SW and hydrolysate from krill contain more free amino acids and water-soluble proteins. Water soluble proteins can make the feed more palatable and can increase the appetite. The increased amount of free amino acids can help support growth, and welfare of the farmed fish (Aksnes et al. 2006, Kousoulaki et al. 2009, Oterhals & Samuelsen 2015, Mahdabi & Shekarabi 2018, Kousoulaki et al. 2018). Today the SW is recovered back to the presscake to make high quality fish meal (Mahdabi & Shekarabi 2018). BSFL are in some cases processed similarly to fish meal, however today the SW from production of BSFL meal are in some cases disposed of, instead of utilized (Zulkifli et al. 2022).

There is a high demand for sustainable feed ingredients, and BSFL meal could be a good candidate to partly replace soy protein concentrate (SPC) in aquacultural feeds. However, BSFL meal comes with several challenges, such as high content of chitin, requirement for processing optimization and the limited availability. There is still much that is unknown about the effects of dietary chitin, however some studies report that chitin can have a negative effect the digestibility and utilization of nutrients (Askarian et al. 2012, Kroeckel et al. 2012, Zulkifli et al. 2022). Another challenge with BSFL meal, is its high content of manganese (Zulkifli et al. 2022, Kousoulaki et al. 2022).

Manganese (Mn) is a micro mineral that is found in virtually all diets, though in small concentrations. Mn is an essential mineral, that supports growth, vertebral development, and

antioxidant metabolism. Mn deficiency can result in reduced growth, dwarfism, and poor bone mineralization (Prabhu et al. 2019, Aschner & Aschner 2005, Watanabe et al. 1997). EU legislation has set an upper limit of 100mg/kg of Mn (EU Regulation 2017/893/EC, Sele et al. 2020, Zulkifli et al. 2022). BSFL meal can contain as much as four times the legal amount of manganese (Kousoulaki et al. 2022). If BSFL meal is to replace large amounts of SPC, it will be necessary to study further the effects of high concentrations of Mn on the welfare of the fish in aquaculture.

To achieve sustainability in salmon farming, it will be necessary to use more sustainable feed resources in salmon feeds (Belghit et al. 2019, Mitra 2021). Including BSFL SW in diets for Atlantic salmon can stimulate an increase in growth. This is due to the BSFL SW containing more water-soluble proteins and free amino acids. Utilizing BSFL SW in feed, instead of disposing of it, supports more sustainable growth. BSFL presscake has a high content of dietary Mn and BSFL SW has a very low content of dietary Mn and utilizing BSFL SW in diets for salmon can reduce the dietary Mn levels. Utilizing the whole biomass of BSFL could both be beneficial for the salmon, and support more sustainable growth, by utilizing all available nutrients in BSFL meal processing.

3. Methods

3.1 Feed composition

The BSFL meal used in present study was produced non commercially by Innovafeed in Paris (France), under the scope of the Millennial salmon project (Grant number: 319987, funded by the Research Council of Norway and the industrial partners INNOVAFEED, Corbion, Cargill, Auchan, MOWI and Labyrie fine foods). The BSFL were produced small scale process and their diet mainly consisted of wheat bran. The BSFL, were pressed to a press cake, and SW was separated from the press cake by sieving. Following the, the oil was removed from the SW prior to spray drying. The BSFL meal and dried SW, was sent to Nofima's Aquaculture feed technology centre in Bergen, in May 2022. The BSFL presscake and SW had a protein content of 61.3 and 50.0 % (dry matter) respectively, while most fish meal have a protein content above 70%. All protein in the SW meal was water soluble protein. There was a higher ash content in the SW, but at the same time lower content of Ca and micro elements.

Particularly interesting that Mn was 10-times lower in the SW compared to the presscake meal (Table 3).

A control diet, which contained neither BSFL meal, nor SW, was formulated based on the dietary levels of ingredients used by Norwegian salmon feed producers in 2020 (Aas et al. 2020). In addition, four experimental diets containing BSFL meal, without (BSFL 10%) or with different levels of SW (BSFL9% +SW1%), (BSFL 8%+ SW 2%) and (BSFL 6%+ SW 4%). The diets were balanced with essential amino acids in crystalline form, vitamins, minerals and phospholipids to meet salmon parr nutrient requirements. The formulation and chemical composition of the trial diets are listed in (Table 4). The inert marker used in the feed was Yttrium oxide. The feeds were produced using a combined preconditioner and co-rotating twin-screw extruder system (TX-52, Wenger Manufacturing inc., Sabetha, KS, United States). The feed was dried in a dual layer carousel dryer (Model 200.2; Paul Klockner GmbH, Nistertal, Germany. The feeds was were coated with oil using Dinnissen vacuum coater (Sevenum, The Netherlands). The pellet produced had a diameter of 2.5 mm.

Table 3. Nutritional content of BSFL presscake and BSFL stickwater.

Analysis	Units	BSFL presscake	BSFL stickwater
Proximal			
Dry matter	%	97.8	95.7
Protein	%	61.3	50.0
Fat	g/100g	10.7	9.8
Ash	g/100g	5.9	11.1
Energy	Kcal/kg	5672.0	4782.0
Water soluble protein	g/100g	<5.0	50,3
Mineral			
P	mg/kg	10400.0	12200.0
Ca	mg/kg	13100.0	3120.0
Mg	mg/kg	4380.0	4610.0
Cu	mg/kg	15.0	4.5
Fe	mg/kg	207.0	62.7
Mn	mg/kg	584.0	48.6
Zn	mg/kg	240.0	53.0

Table 4. Formulation of the experimental diets.

Ingredients	Control diet	BSFL 10%	BSFL 9% + SW 1%	BSFL 8% + SW 2%	BSFL 6% + SW 4%
	%	%	%	%	%
Fish meal 1/22	15.00	15.00	15.00	15.00	15.00
SPC 3/21	15.00	15.00	15.00	15.00	15.00
Wheat gluten 12/21	14.00	14.00	14.00	14.00	14.00
Fava bean 8/21	8.00	8.00	8.00	8.00	8.00
Wheat 20/21	15.69	13.69	13.69	13.69	13.69
Fish oil 19/21 coating	7.00	7.00	7.00	7.00	7.00
BSFL cake P20/22		10.00	9.00	8.00	6.00
BSFL stick water P22/22		0.00	1.00	2.00	4.00
Rapeseed oil 17/21	3.00	4.00	4.00	3.90	3.80
Linseed oil 5/22	1.00			0.10	0.20
L-Threonine P3/20	0.49	0.49	0.49	0.49	0.49
L-Lys 79% T4/20	0.94	0.94	0.94	0.94	0.94
Lecithin from rapeseed 5/19	0.95				
Vitaminpremix T11/20	0.50	0.50	0.50	0.50	0.50
Monosodiumphosphate (26% P) T14/20	2.00	2.00	2.00	2.00	2.00
Carop. Pink (10% Astax) T16/19	0.05	0.05	0.05	0.05	0.05
Marin lecithin P41/21	0.05	1.00	1.00	1.00	1.00
Yttrium oksid T3/21	0.01	0.01	0.01	0.01	0.01
Vitamin C P18/20	0.05	0.05	0.05	0.05	0.05
Metionin T2/20	0.42	0.42	0.42	0.42	0.42
Histidin P14/19	0.15	0.15	0.15	0.15	0.15
Mineral premiks T10/18	0.50	0.50	0.50	0.50	0.50
Choline chloride (70%) 5/20	0.20	0.20	0.20	0.20	0.20

Table 5. Energy, lipid, protein, ash, dry matter, water and water-soluble protein content of the experimental diets.

	Unit	Control diet	BSFL 10%	BSFL 9% + SW 1%	BSFL 8% + SW 2%	BSFL 6% + SW 4%
Energy	%	21.6	21.6	21.5	21.6	21.4
Lipid	%	22.4	23.0	22.9	22.1	22.1
Protein	%	43.5	44.0	43.6	43.9	44.2
Ash	%	6.7	5.9	6.0	6.0	6.2
Dry matter	%	92.3	91.7	91.4	91.7	91.6
Water	%	7.7	8.3	8.6	8.3	8.4
Water soluble proteins	mg/k	8.8	7.5	7.9	8.0	9.1

Table 6. Mineral content of experimental diets

		Control diet	BSFL 10%	BSFL 9% + SW 1%	BSFL 8% + SW 2%	BSFL 6% + SW 4%
P	%	1.14	1.08	1.07	1.06	1.11
Ca	mg/kg	8600.00	7000.00	6800.00	6800.00	6600.00
Mg	mg/kg	2300.00	2200.00	2100.00	2100.00	2100.00
Cu	mg/kg	17.00	17.00	15.00	15.00	15.00
Fe	mg/kg	250.00	240.00	240.00	250.00	250.00
Mn	mg/kg	60.00	120.00	110.00	110.00	94.00
Zn	mg/kg	180.00	180.00	170.00	170.00	170.00

Table 7. Peptide distribution of experimental diets

	Unit	Control diet	BSFL 10%	BSFL 9% + SW 1%	BSFL 8% + SW 2%	BSFL 6% + SW 4%
Water soluble proteins	mg/k	8.8 %	7.5 %	7.9 %	8.0 %	9.1 %
Mw-peptid >15000	g/100g	0.07	0.07	0.07	0.06	0.07
Mw-peptid 15000-10000	g/100g	1.21	1.26	1.31	1.16	1.26
Mw-peptid 10000-8000	g/100g	0.37	0.36	0.40	0.41	0.51
Mw-peptid 8000-6000	g/100g	0.46	0.39	0.44	0.47	0.59
Mw-peptid 6000-4000	g/100g	0.51	0.40	0.44	0.46	0.54
Mw-peptid 4000-2000	g/100g	0.58	0.44	0.48	0.51	0.60
Mw-peptid 2000-1000	g/100g	0.37	0.29	0.31	0.33	0.38
Mw-peptid 1000-500	g/100g	0.36	0.32	0.32	0.40	0.43
Mw-peptid 500-200	g/100g	0.51	0.43	0.47	0.50	0.60
Mw-peptid <200	g/100g	4.42	3.53	3.70	3.72	4.13

Table 8. Total amino acid composition in different diets, and amino acid content of BSFL presscake, BSFL SW and fish meal used in different diets.

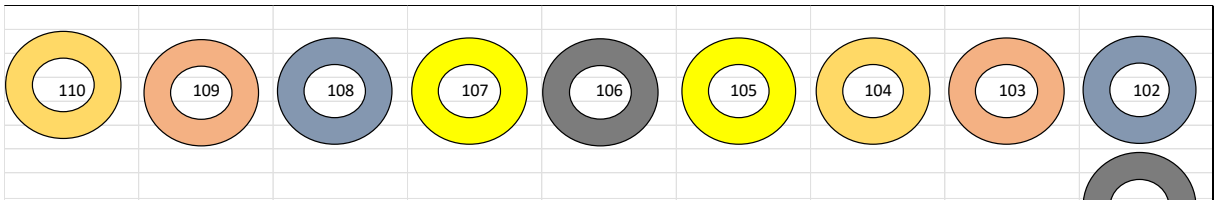
Total amino acids		Control diet	BSFL 10%	BSFL 9% + SW 1%	BSFL 8% + SW 2%	BSFL 6% + SW 4%	BSFL presscake	BSFL stickwater	Fish meal
Alanine	g/100g	1,90	1,90	1,80	1,80	1,90	4.1	3.1	4.3
Arginine	g/100g	2,30	2,20	2,20	2,20	2,20	3.0	1.3	4.3
Aspartic acid	g/100g	3,30	3,30	3,20	3,20	3,30	5.6	3.5	6.3
Glutamic acid	g/100g	8,70	8,90	8,80	8,80	9,10	5.5	7.3	9.2
Glycine	g/100g	2,00	1,90	1,90	1,80	1,90	3.2	2.0	4.3
Histidine	g/100g	1,10	1,10	1,10	1,10	1,10	1.7	2.1	1.6
Hydroxyproline	g/100g	0,16	0,11	0,11	0,11	0,11	<0.2	<0.2	0.6
Isoleucine	g/100g	1,70	1,80	1,70	1,70	1,80	2.7	1.1	3.0
Leucine	g/100g	3,00	3,00	2,90	2,90	3,00	4.7	1.3	5.3
Lysine	g/100g	3,00	2,90	2,80	2,80	2,80	4.1	1.6	5.4
Phenylalanine	g/100g	1,90	2,00	1,90	1,90	2,00	2.8	1.0	2.6
Proline	g/100g	2,70	2,80	2,80	2,8	2,90	4.7	1.6	3.0
Serine	g/100g	1,90	2,00	1,90	1,9	2,10	2.6	1.4	2.8
Threoine	g/100g	1,90	1,90	1,90	1,9	1,90	2.6	1.1	2.9
Tyrosine	g/100g	1,30	1,50	1,50	1,4	1,50	4.2	1.7	2.1
Valine	g/100g	1,90	2,00	1,90	1,9	1,90	4.0	1.6	3.4
Methionine	g/100g	1,20	1,10	1,10	1,1	1,10	1.3	0.2	2.2
Total sum		39,96	40,41	39,51	39,31	40,61			

Table 9. Free amino acids in diets

Free amino acids		Control diet	BSFL 10%	BSFL 9% + SW 1%	BSFL 8% + SW 2%	BSFL 6% + SW 4%
creatinine	g/100g	0.31	0.22	0.23	0.20	0.23
Aspartic acid	g/100g	0.03	0.03	0.03	0.03	0.03
Glutamic acid	g/100g	0.07	0.05	0.06	0.06	0.07
Hydroxyproline	g/100g	<0.01	<0.01	<0.01	<0.01	<0.01
Serine	g/100g	0.01	0.01	0.01	0.01	0.01
Asparagine	g/100g	0.02	0.02	0.02	0.02	0.02
Glycine	g/100g	0.03	0.02	0.02	0.02	0.03
Glutamine	g/100g	<0.01	<0.01	<0.01	0.01	0.01
Beta-alanine	g/100g	0.01	<0.01	0.01	<0.01	<0.01
Taurine	g/100g	0.16	0.1	0.11	0.10	0.11
Histidine	g/100g	0.14	0.13	0.14	0.14	0.15
Gamma-aminobutyric acid	g/100g	0.01	0.01	0.01	0.01	0.01
Citrulline	g/100g	<0.01	<0.01	<0.01	0.01	0.01
Threoine	g/100g	0.51	0.5	0.51	0.50	0.49
Alanine	g/100g	0.06	0.05	0.06	0.10	0.12
Carnosine	g/100g	<0.01	<0.01	<0.01	<0.01	<0.01
Arginine	g/100g	0.08	0.07	0.07	0.08	0.08
Proline	g/100g	0.02	0.02	0.02	0.04	0.06
Tyrosine	g/100g	0.02	0.02	0.02	0.02	0.03
Valine	g/100g	0.02	0.02	0.02	0.02	0.03
Methionine	g/100g	0.43	0.42	0.4	0.39	0.38
Cysteine	g/100g	<0.01	<0.01	<0.01	<0.01	<0.01
Isoleucine	g/100g	0.02	0.01	0.01	0.01	0.02
Leucine	g/100g	0.04	0.02	0.03	0.03	0.03
Phenylalanine	g/100g	0.02	0.02	0.02	0.02	0.02
Tryptophane	g/100g	0.01	0.01	0.01	0.01	0.01
Ornitine	g/100g	0.01	0.01	0.01	0.01	0.02
Lysine	g/100g	1.1	1.1	1.1	0.79	0.77
Total sum	g/100g	3.13	2.86	2.92	2.63	2.74

3.2 Trial

The feeding trial was conducted at Nofima's Research Station for Sustainable Aquaculture in Sunndalsøra, Norway. There were used 5 experimental diets in total, 4 containing different blends of BSFL and SW and 1 control diet. The diets were feed to Atlantic salmon (Salmo salar) parr, with starting body weight of approximately 25g, over a period of 8 weeks. Three tanks were used per dietary treatment, and a total of 15 tanks (Figure 10-13). in which the experimental diets were assigned randomly. In each tank there were 100 individuals, each tank was supplied with freshwater with temperature of 12 degrees Celsius average. At inlet, the water had an oxygen saturation degree of 106%. Feeding was performed continuously, using automatic feeders, set to release some feed every 15 minutes. The feeders stocked every three days, and water quality was measured daily. Uneaten feed was not collected, as pellet size was too small to be recovered.



Tank	Diet
105	Control
107	Control
118	Control
101	BSFL 10%
106	BSFL 10%
117	BSFL 10%
102	BSFL 9% SW 1%
108	BSFL 9% SW 1%
119	BSFL 9% SW 1%
103	BSFL 8% SW 2%
109	BSFL 8% SW 2%
116	BSFL 8% SW 2%
104	BSFL 6% SW 4%
110	BSFL 6% SW 4%
115	BSFL 6% SW 4%

Figure 10. Experimental tank and diet distribution.

Figure 11. Trial setup with tanks, as Nofima Research Station for Sustainable Aquaculture in Sunndalsøra. Each tank is numbered and assigned one experimental diet.

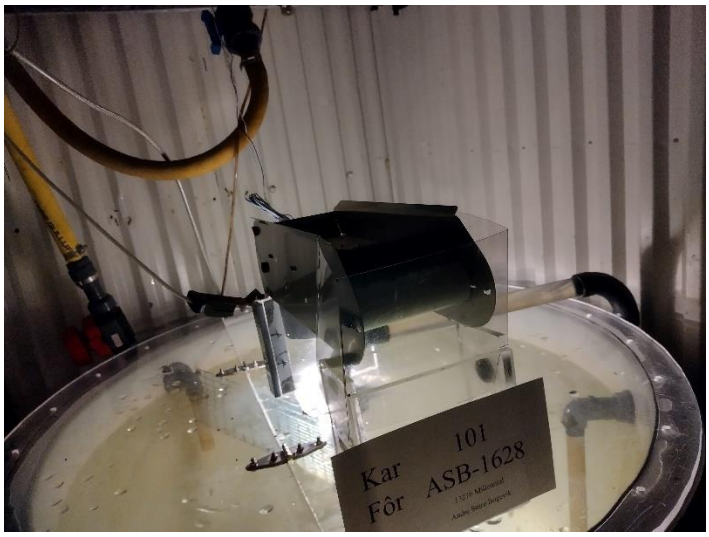


Figure 12. The fish were feed with a belt feeder. Each feeder was labelled, to reduce chances of tanks mistakenly being feed the wrong feed.

Figure 13. Image of the salmon parr during trial run.

3.3 Sampling

At the end of the 8-week trial the fish was euthanized. The fish was euthanized with Finquel 20g per litre of water, and sodium bicarbonate was used as buffer. From each tank 10 fish were collected. Weight, length, and welfare score was recorded (Figure 14). Hindgut content was collected and pooled in a container per tank. Liver, midgut and muscle tissue was pooled from 10 fish per tank and stored at -20˚C prior to freeze drying and chemical analysis. Gutted

weight, liver and viscera were weighed prior to sampling. The midgut was cut diagonally and rinsed with PBS- solution (Figure 15), and muscle was taken from the left NQC (Norwegian quality cut) area of the spine after fileting and removal of the skin. Five fish per tank were collected and cut in pieces, as a whole fish sample. In addition, 10 fish per tank were collected and stored in -20 freezer to be x-rayed (figure 16). The remaining fish in each tank was bulk weighed, and hind gut content was stripped for faeces according to Austreng et al. 1978.

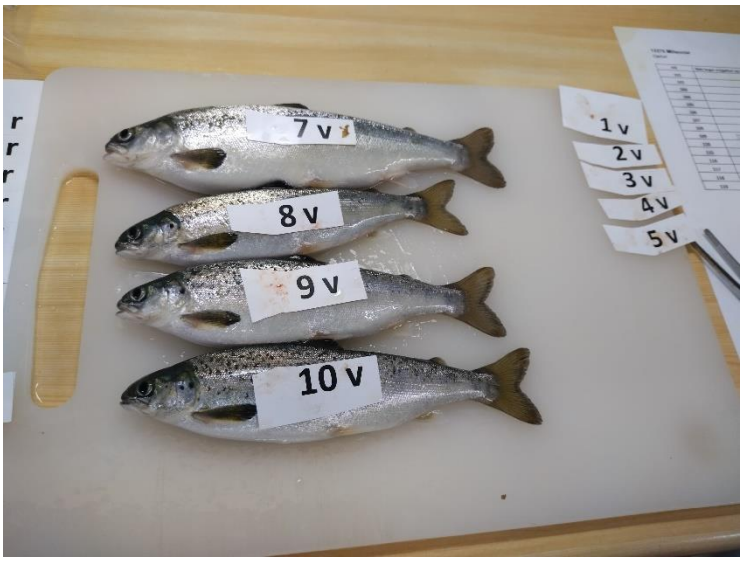


Figure 14. Each fish was labelled during sampling, to avoid human error.

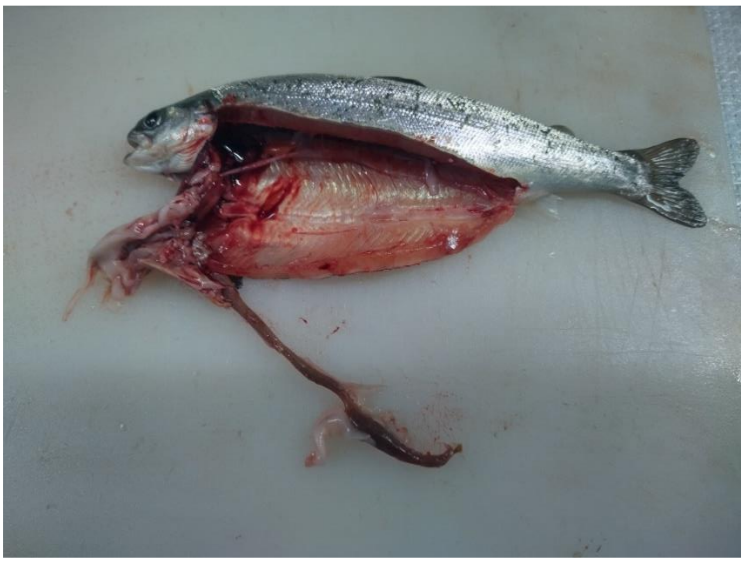


Figure 15. Image during tissue sampling. Here the gut has been pulled out, for sampling of mid gut, to be analysed for Mn content.

Figure 16. Ready samples, placed in freezer at -20°C. Fish flat for x-ray scanning.

The tissue samples were freeze dried at the laboratory at Nofima Research Station for Sustainable Aquaculture in Sunndalsøra. The whole fish samples where homogenized with a Bosch Mfw3520w meat grinder. Each sample contained an aliquot of homogenized 5 fish per tank and were also freeze dried prior to analysis. The muscle, liver and midgut samples, not homogenized prior to freeze drying. All samples were kept in containers marked with tank and tissue type, and all samples were pooled per tank.

10 fish from each tank were x-rayed to examine if there were any skeletal deformities (figure 17). Each fish was placed on a tray before x-ray analyses, using an IMS Giotto mammography equipment (Giotto, Pontecchio Marconi, BO, Italy) The image resolution was 20 pixels per mm2, with exposure at 22 kV and 100 mAs. X-ray images were recorded on coated photo-reactive phosphorous Fujifilm Computed Radiography (FCR) Imaging Plates (Fujifilm, Tokyo, Japan). Plates were read using FCR Profect Reader (Fujifilm, Tokyo, Japan). Each x-ray image was analysed for deformities. This was done by looking closely over each x-ray image and recording how many fish had different deformities. The most common deformity was spinal fusion. Each deformity was recorded and mean deformity per tank was calculated.

Figure 17. X-ray image of salmon. Fish nr 2 has fusion in vertebrate.

Following the sampling and freezing drying, the samples were sent to Nofima feed technology centre in Bergen where chemical analyses were performed by Nofima's accredited laboratory Biolab. Prior to each analytical procedure, the samples were ground to a fine homogenized powder, and stored in -20°C freezer, as a precaution to protect them from oxidation and degradation as if some humidity is left in the samples following freeze drying.

3.4 Mineral analysis

For mineral analysis UltraWave and ICP-OES method was used. Midgut, liver, muscle, whole fish and faeces samples were analyzed. The ICP-OES analyses for 9 minerals, though in this study only the Mn and the yttrium marker content was needed. Two gram of sample was removed from each container, placed in a test tube, and weighed. Each sample was analysed in two parallels, making it a total of 150 samples. In each test tube with sample 1 ml of water was added, and 2 ml of nitric acid. All samples were run through an UltraWave apparatus.

Then, each sample was diluted with distilled water, to a final volume of 25 ml, which was run through the ICP-OES to get the content of the samples in different minerals. The faeces samples had very high mineral content, therefore these samples were diluted more, that is to a final volume of 50ml to achieve more accurate results.

3.5 Fat and protein analysis

Fat and protein analysis was conducted by the accredited laboratory BioLab, at Nofima in Bergen.

Crude protein in feeds, tissue samples and gastrointestinal (GI) content were analysed using the Kjeldahl method (Nx 6.25) (ISO 5983-1997).

Lipid content in diets and gut samples was quantified using the method previously described by Blight and Dyer (1959).

3.6 Data and statistics

Specific growth rate (SGR) is calculated using the following formula:

$$SGR = \left(\frac{(End\ weight - \ Start\ weight)}{Experimental\ period\ (days)}\right) x100$$

TGC was calculated by the following three equations.

Where (T) is temperature in ˚C, (t) is time in days W_0 is initial weight, and W_t is final weight after (t).

$$\sqrt[3]{W_t} = \sqrt[3]{W_0} + [(T/1000) \times t]$$

Under constant temperature TGC is calculated as:

$$TGC = [(\sqrt[3]{W_t} - \sqrt[3]{W_0})/(T \times t)] \times 1000$$

When growth predictions are made the formula becomes:

$$W_t = \{\sqrt[3]{W_0} + [(TGC/1000) \times (T \times t)]\}^3$$

Condition factor (CF) was calculated using the following formula:

$$\mathrm{CF} = \left(\frac{Body\ weight}{Length^3}\right) x100$$

Hepatosomatic index (HSI) was calculated using the following formula:

$$HSI = \left(\frac{Liver\ weight}{Body\ weight}\right) \times 100$$

Visceral somatic index (VSI) was calculated using the following formula:

$$VSI = \left(\frac{Visceral\ weight}{Body\ weight}\right) \times 100$$

Slaughter yield in % was calculated using the following formula:

$$Slaughter\ yield\% = \left(\frac{Head\ on\ gutted\ weight}{Body\ weight}\right) \times 100$$

Dry matter for each nutrient was calculated using following formula:

$$Nutrient\ dry\ matter = \left(\frac{100}{Dry\ matter}\right) \times nutrient$$

Fat and protein apparent digestibility coefficient (ADC) was calculated using the following formula:

$$ADC(\%) = 100 - \left(100 \times \frac{Marker\ concentration\ in\ feed}{Marker\ concentration\ in\ faeces}\right) \times \left(\frac{Nutrient\ concentration\ in\ feces}{Nutrient\ concentration\ in\ feed}\right)$$

For statistical analysis and graph plotting, the program IMB SPSS (version 28.0.0.0) for Windows was used.

one-way ANOVA was run to see statistical significance. For analysis of Mn a Tukey post hoc tests was run as well.

4. Results

4.1 Nutritional value of feeds

The feeds had similar nutritional values with crude protein content of approx 44%, total fat content of approx 22-23% and energy content of approx 21-22 kj/g (table 5). There were more water-soluble proteins in BSFL 6% SW4% as expected, as in this treatment there was included the highest dietary level of SW.

4.2 Mineral content of each feed

There were some variances in the mineral content of the experimental feeds. In this study we focused on manganese. As seen in table 4, Mn content was 60, 120, 110, 110 and 94 mg/k respectively for the Control, BSFL 10%, BSFL9+SW1%, BSFL8+SW2% and BSFL6+SW4%, diets, respectively. There was considerably more Mn in BSFL10% as compared to the diets containing BSFL SW and control diet.

4.3 Amino acids (AA)

There was a difference in the AA profile based on different ingredients as seen in table. 8. BSFL SW contains more glutamic acid and histidine than BSFL presscake. BSFL presscake contains more histidine, hydroxyproline, phenylalanine, proline, tyrosine and valine as compared to fish meal. Though fishmeal generally contains more amino acids than BSFL presscake and SW.

Amino acid composition in each diet, is well balanced, though there is some variance between diets. As seen in table. 8, there is more total amino acid Proline in experimental diets. Though more Lysin and Methionine in control diets due to a higher content of these essential amino acids in the fish meal compared to the BSFL meals.

In free amino acids, there is more Creatinine, Alanine and Proline in diets containing BSFL SW. Though considerably less Lysin in BSFL SW diets (table 9).

4.4 Growth and welfare

Performance results from the trial are summarized in Table 18. The salmon parr had a mean start weigh of 28g, and after 56 days ended up with the final average body weight of 80g as seen in Figure 20. Giving a specific growth ratio (SGR) between 1.9 and 2. There were seen no differences between dietary group in final weight or growth (one-way ANOVA; $p<0.05$). Similar condition factor, liver size and slaughter yield were also seen in fish from all dietary treatments. Welfare measures such as scale loss, dorsal fin damage and pectoral fin damage was recorded, again without observing any significant differences between dietary treatments (Figure 21). Skeletal deformities were recorded as well. There were few cases with skeletal deformities, and the deformities that were found were mainly vertebral fusions in the spine. There were more cases of skeletal deformities in control diet, though not enough differences to be significant. There was found no evidence of diet effecting deformities.

In SW there is often higher content of medium sized peptides at 500-10 000 daltons, which are known to stimulate appetite (Kousoulaki et al. 2012), as seen in table 7, there were more medium sized peptides in diets containing BSFL SW, though this is effect is not reflected in the results.

Table 18. Mean, standard deviation and p-value for Start weight in grams, end weight in grams, Specific growth rate (SGR), Thermal growth coefficient (TGC), condition factor (CF), Heposomatic index (HSI), Visceral somatic index (VSI), slaughter yield %, welfare issues such as scale loss, dorsal fin damage, pectoral fin damage, and skeletal deformities.

	Control diet	BSFL 10%	BSFL 9% + SW 1%	BSFL 8% + SW 2%	BSFL 6% + SW 4%	P-value
Start weight (g)	25.62 ± 0.19	25.76 ± 0.11	25.78 ± 0.08	25.77 ± 0.06	25.74 ± 0.05	0.103
End weight (g)	81.68 ± 3.79	78.77 ± 3.16	79.08 ± 5.87	79.33 ± 1.43	77.41 ± 4.64	0.267
SGR	2.03 ± 0.09	1.95 ± 0.06	1.96 ± 0.13	1.97 ± 0.04	1.92 ± 0.10	0.722
TGC	1.98 ± 0.10	1.89 ± 0.08	1.90 ± 0.15	1.91 ± 0.04	1.86 ± 0.12	0.741
CF	1.48 ± 0.06	1.49 ± 0.07	1.48 ± 0.07	1.49 ± 0.07	1.48 ± 0.07	0.591
HSI	0.11 ± 0.02	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.757
VSI	12.93 ± 1.81	12.87 ± 1.18	13.23 ± 0.99	13.06 ± 1.17	13.13 ± 1.06	0.821
Slaughter yield %	0.81 ± 0.22	0.87 ± 0.01	0.86 ± 0.01	0.87 ± 0.01	0.86 ± 0.01	0.458
Scale loss	10.66 ± 1.15	14.00 ± 4.35	10.33 ± 0.57	11.00 ± 1.00	9.66 ± 3.51	0.335
Dorsal fin damage	11.00 ± 4.58	12.66 ± 1.15	12.66 ± 5.85	14.66 ± 3.05	9.33 ± 5.68	0.663
Pectoral fin damage	6.00 ± 2.64	8.66 ± 1.52	8.33 ± 2.88	8.66 ± 1.52	6.66 ± 3.21	0.569
Vertebrate deformities	0.3 ± 0.1	0.13 ± 0.23	0.06 ± 0.11	0 ± 0	0.03 ± 0.05	0.226

Table 19. Apparent digestibility coefficient of lipid, protein and dry matter between diets.

	Control diet	BSFL 10%	BSFL 9% + SW 1%	BSFL 8% + SW 2%	BSFL 6% + SW 4%	P-value
Dry matter	77 %	78 %	79 %	79 %	78 %	0,161
Protein	93 %	92 %	93 %	92 %	92 %	0,158
Lipid	96 %	96 %	97 %	97 %	97 %	0,191

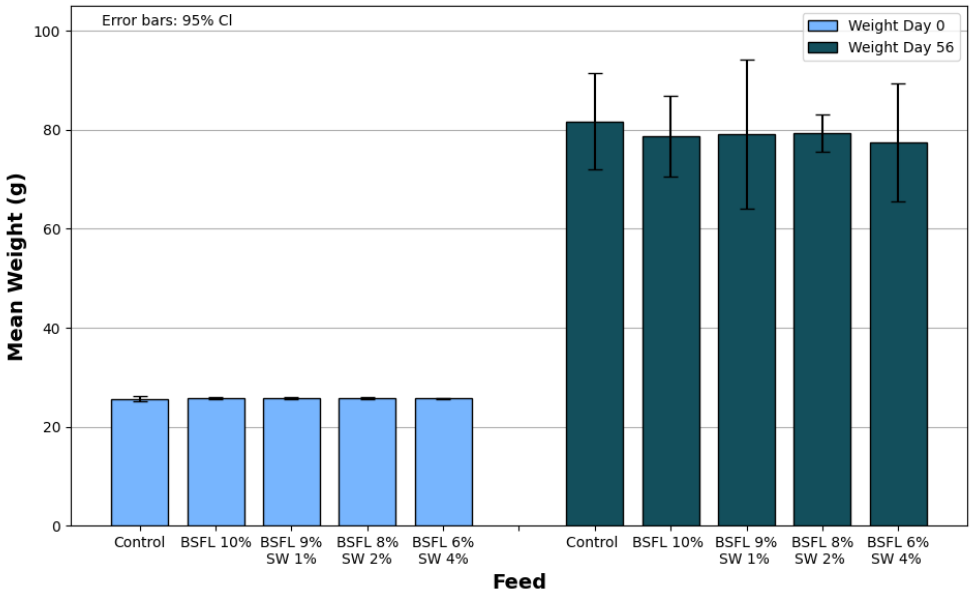


Figure 20. Fish start body weight, and end body weight after 56 days (N=3), compared to different dietary treatments.

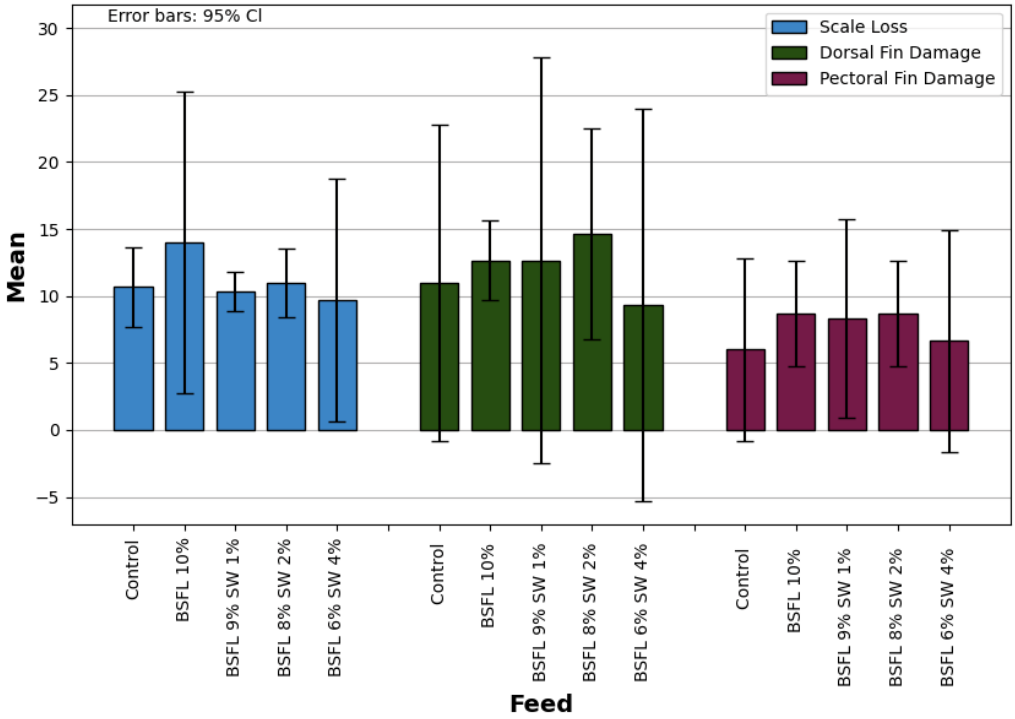


Figure 21. Scale loss, Dorsal fin damage and pectoral fin damage compared different feeds. (N=3, p>0.05) Non significant differences between diets. Welfare issues were recorded by severity and occurrence per 30 fish that were analysed per diet, these results were grouped together per tank, and error bars represent differences between tanks.

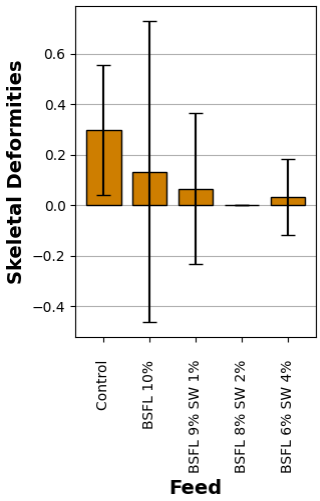


Figure 22. Skeletal deformities compared in fish feed different experimental feeds, (N=3, p>0.05) Non significant differences between diets.

4.5 Manganese

Manganese content of different salmon tissues varied. As seen in figure 23, there was very little Mn stored in muscles, and there was little variance between diets. The midgut contained most Mn among the tissues studied, though there were no significant differences between dietary groups. As seen in figure 24, there was significantly more Mn in fish faeces from experimental diets, compared to control diet (one-way ANOVA; $p<0.05$), followed by Tukey post hoc test showing differences between diets. The midgut tissues were scraped clean for faeces and rinsed in PBS solution prior to analysis. Midgut samples are in direct contact with faeces, we would expect it to contain more Mn than other tissue samples.

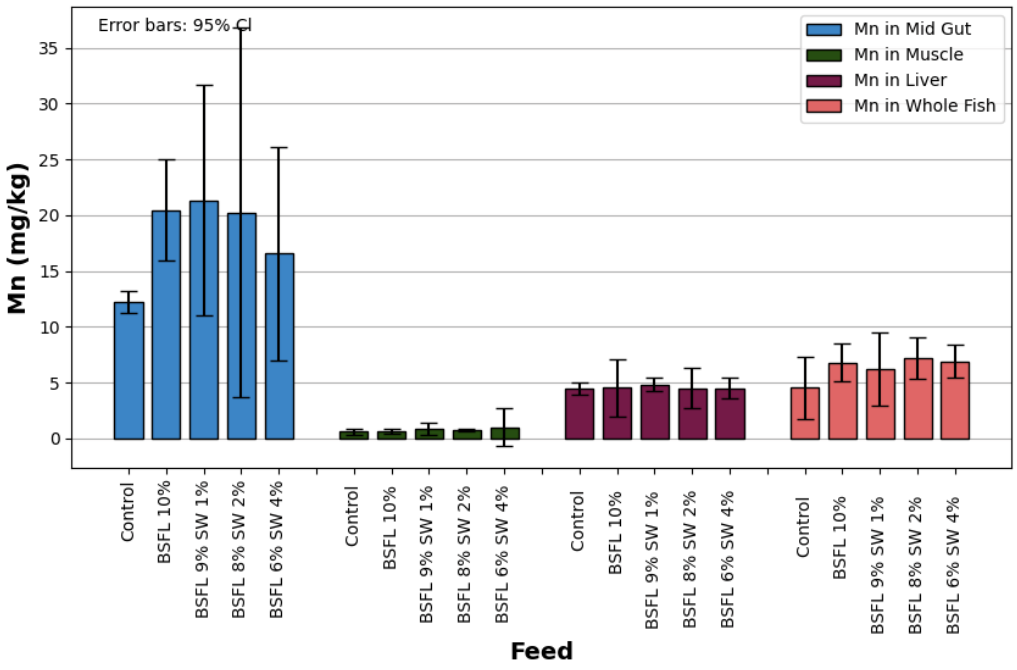


Figure 23. Manganese content in different tissue samples. (N=3, $p>0.05$) Non significant differences between diets.

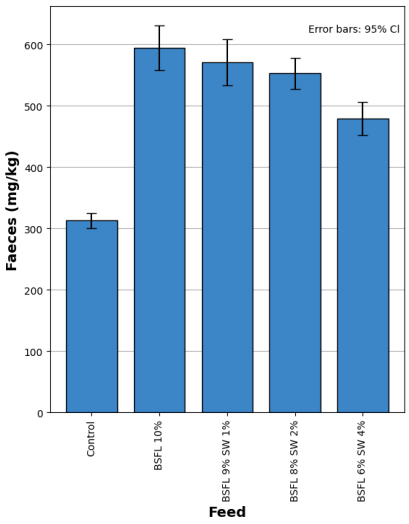


Figure 24. Manganese content in faeces. There was significantly less manganese in the control sample compared to the different experimental feeds determines by one-way ANOVA (N=3, $p<0.05$), followed by Tukey post hoc showing differences between diets.

5. Discussion

Atlantic salmon grew successfully from 28g to 80g feed on 10% BSFL meal with different inclusions of SW. The feeds used in this trial had similar apparent digestibility coefficient (ADC), all the fish grew similarly with no significant differences between diets.

In a similar study, where 6.25, 12.15 and 25% of the protein was replaced with BSFL meal in diets for salmon fry, start weight 34g. The fish feed diets containing 6.25 and 12.5 preformed similarly. Though fish feed diets containing 25% BSFL meal experienced decreased growth rate and ADC. (Weththasinghe et al. 2021). Compared to the present study, we experienced similar ADC results to that of Weththasinghe et al. 2021, though we did not see any negative effects on growth rate or ADC, which is likely due to low inclusions of BSFL meal.

SW from fish meal production contain high concentrations of water-soluble proteins and free amino acids. More water-soluble proteins can increase palatability, and more free amino acids can increase growth (Aksnes et al. 2012, Kousoulaki et al. 2009. Oterhals & Samuelsen 2015). In study conducted by Kousoulaki et al. 2009, there was found considerably more amino acids and water-soluble protein in fish SW compared to fish meal. Moreover, in

Kousoulaki et al. (2009) salmon feed diets with increasing levels fish meal SW showed increased bodyweight, specific growth rate (SGR) and thermal growth coefficient (TGC). In the present study, TGC values were lower than expected. Which could be due to lower water temperatures, than was expected.

In a study done by Kousoulaki et al. 2022, Atlantic salmon smolts were fed diets containing 20% BSFL meal with BSFL SW. The salmon performed well on their experimental diets. The BSFL meal contained the highest Histidine levels, similar levels to that found in fish meal. The BSFL meal in this study did contain high levels of dietary Mn, and there was not seen any negative effect on this (Kousoulaki et al. 2022).

In the present study, there were similar histidine levels in all diets. Although there is more histidine in both BSFL presscake and BSFL SW compared to FM.

Increased levels of free amino acid and other water-soluble nitrogenous compounds can increase diet palatability growth (Aksnes et al. 2012, Kousoulaki et al. 2009. Oterhals & Samuelsen 2015). Based on previously studies, it was hypothesized that in our trial also, the fish fed diets containing higher levels of BSFL SW would achieve greater growth rate, or higher feed intake. Feed intake was unfortunately not possible to measure in trials with so small fish as the pellet size is too small to be recovered.

The BSFL SW diets contained some more water-soluble proteins, with diet BSFL 6% SW 4% containing most water-soluble proteins of the experimental diets, close to similar content of water-soluble proteins, as the control diet (9.12 vs 8.86 g/100g, respectively). The diets contained similar amino acid profiles, though there were some variations in the amino acid composition of the diets. As expected, the diets added BSFL SW contained more water-soluble proteins and free amino acids than the diet added BSFL meal alone, though not near as much as is seen in fish SW. The reason why all the fish in this study preformed similarly, could be that the feeds were well formulated, with similar protein and lipid digestibility, and similar content of amino acids and water-soluble protein, with too small variations to induce significant performance effects.

As mentioned before, BSFL meal has some challenges, as for instance its high content in dietary Mn. European union (Eu) regulations have set an upper limit of 100 mg/kg of manganese in feed (EU Regulation 2017/893/EC, Sele et al. 2020, Zulkifli et al. 2022). In the feeds used in this study, the inclusion was only 10% BSFL meal and the feed that contained most Mn was BSFL 10%, with a Mn content of 120 mg/kg. The majority of the dietary Mn comes from the BSFL presscake. BSFL SW contains less dietary Mn, and by including more BSFL SW the levels of dietary Mn in diets are lowered. In extreme cases, if dietary intake of Mn is too high, the fish will adapt by reducing intestinal absorption, enhance liver metabolism, and increase excretion through the faeces. Once Mn is absorbed through the intestine, it gets transported through the blood to the liver, where it gets stored in the bones. Mn is also excreted through bile and is removed with the faeces when in excess. (Prabhu et al. 2019, Aschner & Aschner 2005). In the control diet, there was analysed 60 mg/kg Mn whereas the experimental diets contained 120, 110, 110 and 94 mg/kg (see figure 6). Accordingly, the faeces in the control treatment had significantly less Mn as compared to the faeces from fish in the BSFL experimental treatments ($p<0.05$), and little variation in the Mn levels of fish tissues was observed. This shows that salmon parr can effectively excrete excess dietary Mn through the faeces and do not accumulate higher amounts in the tissues when fed diets that contain Mn in excess. All the feeds had very high nutrient apparent digestibility coefficients, and all fish grew similarly. There were no significant differences in welfare indicators, such as scale loss, pectoral fin damage, dorsal fin damage or skeletal deformities.

6. Conclusion

Atlantic salmon parr grows well with no signs for reduced performance and welfare or changes in whole body and tissue composition when fed diets containing 10% BSFL with or without added SW. BSFL meal contains high levels of the essential trace mineral Mn leading at times to diets containing Mn levels above the legal limits. Nevertheless, we saw no evidence of over saturation of dietary Mn in the analysed salmon parr body or tissues (muscle, liver and midgut) when fed diets with increasing levels of Mn. On the contrary, the fish fed diets with excess Mn levels could effectively excrete those in their faeces. Thus, from this study, we do not see any negative effects in salmon performance fed diets with up to 120 mg/kg dietary Mn.

6.1 Future studies

Where in the BSFL body is Mn deposited? Is it possible to process this further to lower the BSFL meal Mn levels?

It is recommended to run a similar study to the present one, with larger fish using larger pellet size, in order to collect uneaten feed and determine if dietary BSFL SW inclusions may have any effects on feed intake rates in salmon.

More physiological responses in salmon fed BSFL SW or excess dietary Mn levels can be studied as for instance the occurrence inflammation in the gut studied by gut histology.

What effects has chitin on nutrient digestibility in salmon parr and smolt? At what levels would we se negative effects?

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