MASTER THESIS

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Influence of different inclusion levels of insect meal on the liver of Atlantic salmon

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Abbreviations

alpha-Linolenic acid an omega 3 fatty acid – ALA
Arachidonic acid – ARA
Black solider fly – BSF
Canola oil – CA
Dihomo-gamma-linolenic acid – DGLA
Docosahexaenoic acid an omega 3 fatty acid – DHA
Eicosapentaenoic acid an omega-3 fatty acid – EPA
Field peas – PP
Genetically modified organism – GMO
Heavy Black solider fly 10 % – HB
Heavy Mealworm 30 % – HM
Hepatosomatic index – HIS
Insect meal – IM
Light Black solider fly 5 % – LB
Light Meal worm 15 % – LM
Linseed oil – LI
Meal worm – MW
medium-chain fatty acids – MCFA
Melano-macrophage centres – MMC
n-3 polyunsaturated fatty acids – PUFA
n-6 fatty acids are a class of polyunsaturated fatty acids – PUFA's
Narrow leafed lupin – LP
Plant proteins – PP
Short-chain fatty acids – SCFA
Soybean concentrate – SBC
Soybean protein concentrate – SPC
Soybean meal – SB
Vegetable oils – VO

Abstract

In this study the liver of Atlantic salmon (*Salmo salar*) was assessed to determine the effects of replacing vegetable oils (VO) and soybean protein concentrate (SPC) with insect meal (IM) of black solider fly (BSF) and mealworm (MW). Post smolt juvenile Atlantic salmon were fed with a standardised control diet consisting of fish meal, fish oil, soy SPC, and vegetable oils. Modified standard diets contains BSF at 5 % and 10 % and MW at 15 % and 30 %, replacing rapeseed oil, SPC and wheat gluten. The trial lasted for 11 weeks. Histology, Immunohistochemistry, gas chromatography (GC) and qPCR was done on the liver samples to assess the lipid metabolism and overall conditions of the livers. Histology and Immunohistochemistry did not show significant deviation from control group. GC did not show any short fatty acid chains (SFAC) or C12:0 in the livers, but there was detected C12:0 in the feed derived from BSF. GC results indicated a significant difference CO to HM, LB to HM in the fatty acid chain C18:1n-7, HB to CO, HB to HM, HB to LM in the fatty acids C18:2n-6, C18:3n-3, C20:1n-9, C20:3n-6, C22:1n-11, C22:5n-3. HSI parameters showed to be between 1% - 2% indicating that the livers are healthy. Genes of interest are cd36, FATP1, LPL, APOA1, apoB100, SREBP1, they did not show any significant difference in up/down regulation in comparison to control. The study shows positive results in the replacement of VO and PP with BSF and MW in regard to liver welfare.

Acknowledgments

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1.0 Introduction

According to the Norwegian Directorate of fisheries (2022) in 2021 there was 1370 assigned licenses, with 32 locations on land and 990 locations at sea, which in total produces 1,467,655 tonnes of Atlantic salmon (*Salmo salar*). Norway has taken a huge leap in Atlantic salmon and Rainbow trout production since 1976 (Figure 1), and is one of the leading suppliers of salmon, producing over 50% of all salmon world-wide (Iversen et al., 2020)

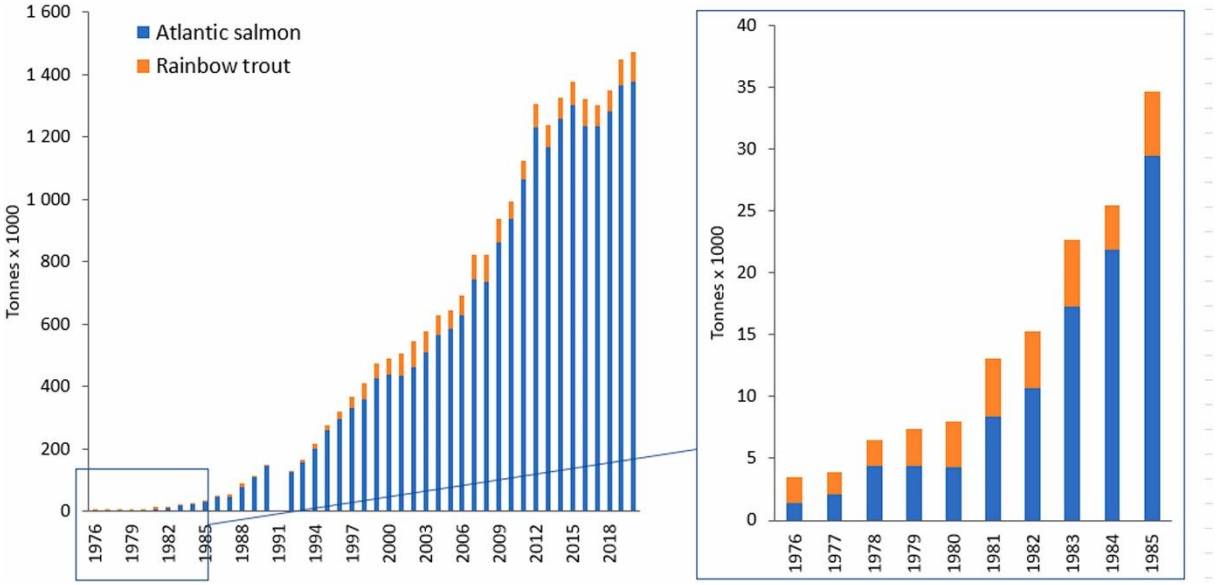

Figure 1: Annual sale of farmed Atlantic salmon and rainbow trout in Norway in 1976–2020 (Directory Of Fisheries, 2021; Statistics Norway, 2019).

To feed the vast number of salmon 1,976,709 tonnes of feed ingredients were used, comprising of 22.4% marine ingredients, 73.1% vegetable ingredients, and 4.1% micro ingredients such as vitamin and mineral premixes, astaxanthin, and crystalline amino acids. Additionally, only 0.4%, which is equivalent to 8126 tonnes of single cell protein, insect meal, fermented products, and microalgae were also utilized in salmon feeds (Figure 2). Norwegian marine protein and marine oil accounted for 8.3% of the ingredients used, while the remaining 91.7% of the ingredients were imported from all over the world (Figure 2) (Aas et al., 2022a)

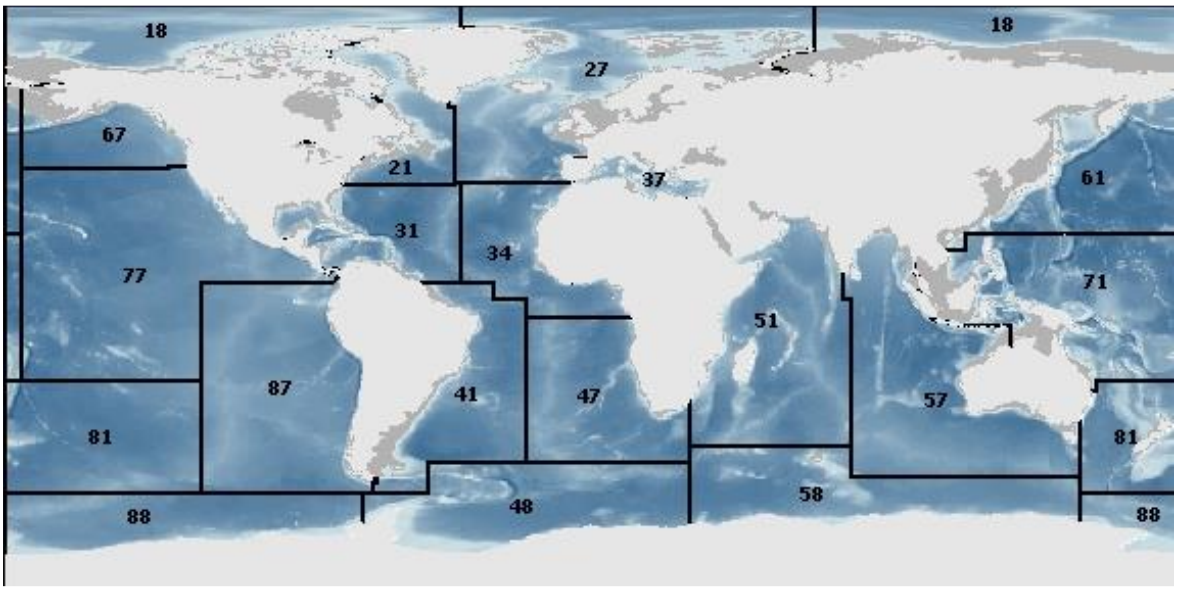

Figure 2: Represents the origin of marine resources according to FAO (FAO Fishing areas, https://www.fao.org/fishery/en/area/search)

Aquaculture struggles to meet the demand for sustainable high-quality feed. Fish meal and fish oil were one of the major protein and lipid sources applied in feed for aquaculture species around the world (Bandara, 2018). The issue with fish meal (FM) and fish oil (FO) is that it is a limited resource and therefore does not meet sustainability goals (Sørensen., 2011). Species like Peruvian anchoveta (*Engraulis ringens*), herring (*Clupea harengus*) and sprat (*Sprattus sprattus*) are used for FM and FO. Anchoveta is one of the most central species anchoveta have many uses as a food source but also as an ingredient in fish feed. The population of anchoveta has been declining over decades due to fishing overcapacity (Aranda, 2009). Although fisheries have been restricted the population has been struggling to recover. This can be caused by climate change as well as El Nino (Ferguson-Cradler, 2018). El Nino is an irregular weather pattern when, among other things, the surface waters rise in temperatures which results in migration of shallow swimming, schooling fish to the deeper parts of the ocean where fishing vessels cannot operate (Laws, 1997).The limited access to anchoveta put strain on feed production aquaculture resulting in rising prices and sca availability. It is unfeasible to rely on finite resources due to increasing production of Atlantic salmon (Figure 1). This lead to a considerable change of feed composition resulting in inclusion of plant-based ingredients such as plant proteins and vegetable oils (Aas, 2020). However, majority of these oils are not suitable or have deficiencies in n-3 highly unsaturated fatty acids (Caballero-Solares et al., 2018; Turchini et al., 2010; Yang et al., 2020).

In 1990, the salmon feed that was used in Norway, consisted of 90% marine originated ingredients but in 2013 it dropped down to 30% where the composition consisted of fish meal 18% and fish oil 11% (Ytrestøyl et al., 2015). In 2020, the average Norwegian salmon feed was produced with 12.1 % fish meal, 10.3 % fish oil, the remaining components of the feed are 40.5 % vegetable protein sources, 20.1 % vegetable oils, 12.5 % carbohydrate sources and 4.1 % micro ingredients. And a small amount of 0.4 % of ingredients such as insect meal, single cell protein, fermented products or micro algae (Figure 3) (Aas, 2022).

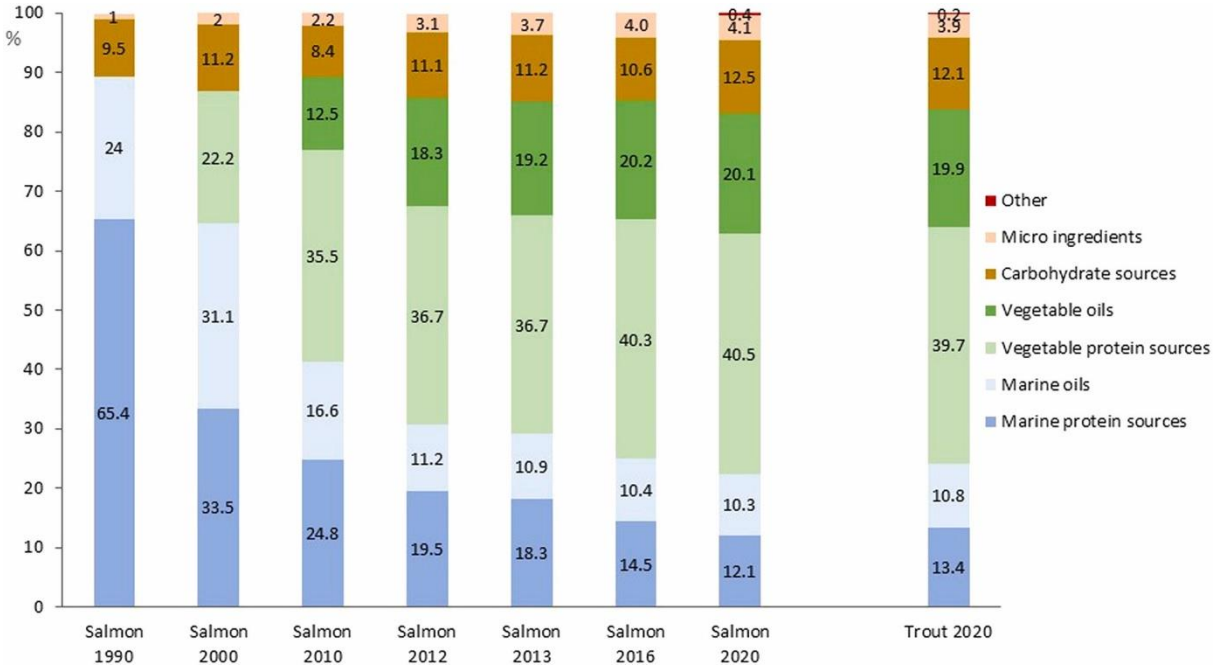

Figure 3: Sources of feed ingredients (% of feed) in Norwegian trout feed in 2020 (at right), compared to data for salmon feed from 1990 to 2020 (Aas et al., 2022b).

Incorporation of plant-based ingredients is driven by the availability and lower prices of these raw materials as compared to marine ones. Ingredients such as grain (wheat and corn), oilseeds (soybean, sunflower, rapeseeds, cottonseed), and pulses (beans, lupins, and peas) are used as a replacement. Vegetable ingredients are selected by nutrient content that they are able to provide. Parameters that are highly favoured are carbohydrates and indigestible antinutrients, high protein levels, good amino acid profile, high digestibility, low levels of fibres, and good palatability (Raskovic et al., 2011). By replacing 33% of fish meal protein with plant-based proteins such as, soybean concentrate (SBC), Narrow leafed lupin (LP), or field peas (PP) has shown to have no negative effect on the growth (C.G Carter, 2000). Other replacements that are worth mentioning are plant derived oils. Some of the plant-based oils are a good

replacement. Examples of these are linseed oil (LI) and canola oil (CA) (Ruyter, 2022). However, vegetable ingredients are insufficient in meeting the nutritional requirements, implementation of plant-based proteins into a diet of a carnivorous fish, cause negative effects on the intestine because of antinutritional factors (Bandara, 2018; Booman et al., 2018). Central organs in digestion and metabolism of incoming nutrients through digestive tract are intestine and liver, as they are responsible for absorption and metabolization of the incoming nutrients from fishes' diet. Therefore, monitoring these organs is crucial. Studies show that Atlantic salmon (*Salmo salar)* is susceptible to soybean meal-induced enteritis (SBMIE) (Kiron et al., 2020). Enteritis can be assessed as visible changes on the digestive system of the fish described as "non-infectious subacute enteritis", this includes shortening of the intestinal villi, loss of supranuclear vacuolization of the enterocytes, widening of lamina propria of villi, and infiltration of inflammatory cells (Raskovic et al., 2011). Other welfare indicators that shouldn't be overlooked is gene expression. In comparison between aquafeed that contains marine ingredients with terrestrial plant-based feed, at the transcript level, it has been shown that plant-based feeds perform more poorly (Caballero-Solares et al., 2020). PCR data show that ingredients of terrestrial origin modulated expression of genes related to inflammation, metabolism, growth-related mechanisms, and oxidative stress in the liver of Atlantic salmon (Caballero-Solares et al., 2020). In addition, correlations between transcript abundance and response parameters such as hepatosomatic index (HSI) and liver Arachidonic acid (ARA), DHA, and EPA levels indicated physiological impacts of nutrient-gene interactions induced by the terrestrial feed ingredients (Caballero-Solares et al., 2020). Physiological responses in the liver of Atlantic salmon (*Salmo salar*) show that gene expression profiles were more divergent between fish fed the marine and terrestrial diets. The analysis of hepatic biomarker gene expression via multiplex PCR revealed potential physiological impacts and nutrient-gene interactions in the fish with lower levels of marine source nutrients (Caballero-Solares et al., 2020). Complicating factor like welfare, cost and sustainability contributed to an increased interest in insects as an ingredient that might improve the feed with VO and plant protein (PP). Additionally, there have been efforts to use more sustainable and locally sourced ingredients in feed, such as insects, algae, marine by-products as well as agricultural waste (Belghit et al., 2019; Belghit et al., 2018; Liland et al., 2017; Sheppard, 1994). To be able to substitute the preferred ingredients such as FO it must optimally resemble the composition of which is rich in unsaturated fatty acids (n-3 HUFA), as well as the main energy source must come from monounsaturated fatty acids (MUFA) and saturated fatty acids (Henderson, 1984). Insects are a great source of nutrients as well as non-food ingredient, meaning that it is an ingredient that

is not primarily consumed by humans, in contrast to fish (fish meal, fish oil). Insects contain all macronutrients proteins, carbohydrates and oil as well as have a low environmental footprint during production, particularly when grown on industrial side streams that otherwise should have been waste (Melenchón et al., 2020). Species that are commonly used in feed are Black solider fly (*Hermetia illucens*) and Mealworm (*Tenebrio molitor*). Insect meal and insect oil is therefore a more sustainable solution in contrast to plant-based diet as well as FM and FO (Mikolajczak & Rawski, 2022). Insect meal and especially black soldier fly (BSF) are high in protein containing around (40% - 45%) and lipids (26% - 35%) thus it has been recognized suitable for aquaculture feed production (Sheppard, 1994; Tran, 2015). BSF can provide a large variety of nutrients including omega-3 fatty acid eicosapentaenoic acid (20:5n-3) if they are provided with adequate substrate that includes seaweed brown algae (Ascophyllum nodosum) (Liland et al., 2017). Atlantic salmon can utilize insects as a protein source and that their growth and health are not negatively affected when they are included in the diet (C. Caimi et al., 2021). Complete dietary replacement of fishmeal with Black solider fly do not compromise the physiochemical quality of Atlantic salmon filets. In addition, it increased neutral n-3 polyunsaturated fatty acids (PUFA) (Bruni et al., 2020) in filets. When using insect derived oils in feed it is important to note that standardised feeds of today do not contain medium-chain fatty acids (MCFA) or short-chain fatty acids (SCFAs) (Fawole et al., 2021; Ruyter, 2022). Nutrient rich deoxygenated blood in liver is supplied to the hepatocytes through the hepatic portal vein from the digestive tract (Peate & Nair, 2016). Hepatocyte's task is to detoxify, filter, store and process the nutrients from the digestive tract. The nutrients that hepatocytes receive are either converted to energy, stored, or used as building blocks for new molecules. Morphological changes in the liver may occur if the feed is not adequately formulated. If the lipid levels in the feed are high or the lipids are unable to be metabolized, hepatocytes vacuolization (micro vacuolization), fat accumulation in the liver (macro vacuolization) can occur (Mota et al., 2016). Furthermore changes in metabolic activity, changes in liver parenchyma and necrosis (Raskovic et al., 2011). Hepatocytes are the most abundant type of cells in liver they are polygonal and have a clear central core with strongly coloured chromatin alongside of nuclear membrane and a clear nucleolus. Hepatocytes have an eosinophilic cytoplasm which consists of glycogen and fat as well as other cellular components. Although the most central lymphoid organs (immune system) in fish are the kidney (mid, and head kidney), spleen and thymus, the liver is proven in some species to aid in immune system defence especially inflammation (Wolf & Wolfe, 2005; Wolke, 1992). Immune system is usually divided into two groups although they work hand in hand to defend organisms from outside

threats, and they are termed innate and adaptive response. The innate immune system represents the microbiological, physical and chemical barrier responses, but it also includes cellular elements of the immune system (neutrophiles, monocytes, macrophages, complement, cytokines, and phase proteins) and is often called the first line of defence (Hynes et al., 2011; Parkin & Cohen, 2001). The second line of defence is the adaptive immune system, which is much more specific and has memory of previous pathogens. It is specific due to antibody-antigen interactions/reactions. This means that cells such as T lymphocytes and B lymphocytes are involved when this occurs (Taylor & Ruiz Daniels, 2022). The specific characteristic of the adaptive immune system is used in several analytical methods. The usual morphological diagnosis routine consists of more basic stain types such as haematoxylin and eosin (Fischer et al., 2008), but IHC can locate the cells that represent the targeted antigen in the specific tissue, such as liver. Immunohistochemistry is a methodology that specifically involves localization and identification of antibody-antigen interaction where the specific antibody has been tagged with a visible label (Speilberg et al., 1994). Qualitative immunohistochemistry (IHC) analysis is a method used in histology to evaluate the presence or absence of a specific protein in a tissue sample. In qualitative IHC analysis, the objective is to determine whether the protein of interest is present in the sample. This type of analysis can be useful for identifying the specific cells or tissue types that express the protein. Liver is incessantly exposed to dietary and commensal bacterial products that pose inflammatory risks. Thus, the liver is a crucial indicator of inflammation in our case for *(Salmo salar)*. Antibodies are responsible for marking pathogens that can enter the organism through the digestive tract, with improper feed the risk of the pathogens entering the organisms is higher. Therefore, marking antibodies such as MHC II & CD8 is important. MHC II major histocompatibility complex is a heterodimer protein molecule that consists of two homologous peptides, with an α and β chain. These molecules have both extracellular regions and transmembrane sequences and a cytoplasmic tail. Each MHC molecule present a peptide sequence termed epitope that are recognised by the immune system. When such a complex presents a self-antigen, it will prevent the organism's immune system from targeting the cell. But when MHC molecule present foreign and/or pathogen-derived proteins, the cell will be eliminated. MHC II can be presented in cells such as dendric cells, B cells and MMC's (Speilberg et al., 1994; Torstensen et al., 2009), which in return can present foreign antigens to cells like T cell (Rock et al., 2016). Such cells can represent inflammation. CD8 is a protein found on the surface of certain immune cells. It is a transmembrane protein that plays a key role in the immune response to viral infections. CD8 is part of a larger family of proteins called the cluster of differentiation (CD) proteins, which are used to identify and

classify different types of immune cells. CD8 is expressed on cytotoxic T cells, which are a type of T cell that can recognize and discard infected cells. CD8 functions as a co-receptor, working with the T cell receptor (TCR) to bind to and recognize specific antigens that are displayed on the surface of infected cells. When CD8 binds to the antigen, it helps to activate the cytotoxic T cell, which then releases chemical signals that trigger the death of the infected cell (Rock et al., 2016).

To determine what type of lipids are retained or metabolised in the liver a fatty acid analysis is needed. Fatty acid analysis is a laboratory technique used to determine the types and proportions of fatty acids present in a sample of fat or oil. This analysis is typically used in the aquaculture industry to characterize the fat content of fatty acid composition of biological samples such as liver tissue. One of the methods used to perform fatty acid analysis is GC. Fatty acid analysis involves extracting the fatty acids from the sample, alternating them into a form that can be measured, and then using an analytical technique to determine the types and proportions of the different fatty acids present. The result of the analysis provides valuable information about the composition of the sample that are being analysed.

To further analyse the interactions of the fatty acids qPCR analysis is usually done. Polymerase chain reaction (PCR) is a laboratory technique used to amplify specific DNA sequences. It is not directly related to metabolism, but it can be used to study fatty acid transportation and metabolism by amplifying and analysing small amounts of DNA that may be present in samples. Measurements of up/down regulation of different genes can give an overview of the internal interactions. Metabolism is the chemical processes that occur within an organism to maintain life. These processes involve the breakdown of molecules to produce energy and the synthesis of molecules needed for growth and repair. PCR can be used to amplify and analyse the DNA of specific enzymes involved in metabolic pathways. The selected genes play a key role in transporting and metabolizing lipids. cd36 is a gene that encodes for a protein called cluster of differentiation 36, which is a membrane receptor involved in the uptake of fatty acids from the bloodstream into cells. cd36 plays a crucial role in the regulation of fatty acid metabolism and transport (Torstensen et al., 2009), and it is highly expressed in the liver and adipose tissue of Atlantic salmon. The cd36 facilitates the uptake of long-chain fatty acids into cells, where they can be used for energy production or stored as lipids. FATP1, which stands for fatty acid transport protein 1, is another gene involved in fatty acid metabolism. FATP1 is responsible for the transport of long-chain fatty acids across the cell membrane, facilitating their uptake. FATP1 is highly expressed in the liver and muscle tissues of Atlantic salmon

(Torstensen et al., 2009) and plays a crucial role in regulating fatty acid metabolism and lipid homeostasis. The transport of the fatty acids related to these genes has been described in hepatocytes. The expression of the genes has been regulated when the diet has been induced with vegetable oils (Sanchez-Gurmaches et al., 2011). LPL, or lipoprotein lipase, is an enzyme encoded by the LPL gene that plays a crucial role in the metabolism of lipoproteins, which are involved in transporting lipids, including triglycerides, in the bloodstream. LPL is expressed in various tissues, including the liver, muscle, and adipose tissue, and it hydrolyses triglycerides from lipoproteins, releasing fatty acids that can be taken up by cells for energy production or lipid synthesis. It has been suggested that LPL is linked to lipid accumulation in species such as rainbow trout and gilthead seabream (Kaneko et al., 2013). APOA1 is a gene that encodes for apolipoprotein A1, a major component of high-density lipoprotein (HDL). APOA1 plays a crucial role in the metabolism of lipids. APOA1 is expressed in various tissues, including the liver and intestine. Apolipoprotein B100 *(apoB100* gene) encodes for a large glycoprotein that is an essential component of very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) particles. In Atlantic salmon, apoB100 is mainly synthesized in the liver and is involved in the transport of lipids from the liver to peripheral tissues (Geay & Ferraresso, 2011). The expression of apoB100 in Atlantic salmon is affected by dietary lipid composition, with high-fat diets resulting in increased expression. SREBP1 gene plays a role in the regulation of lipid metabolism and homeostasis in Atlantic salmon. SREBP1 is involved in the synthesis and uptake of fatty acids. It is expressed in various tissues, including the liver, adipose tissue, and muscle, and it regulates the expression of genes involved in lipid metabolism (Minghetti et al., 2011), including those encoding for enzymes involved in fatty acid synthesis. Main aim of the study is to investigate the effects of partial replacement VO, with two types of insect meal at two inclusion levels, with two different insect species, black soldier fly (*Hermetia illucens*) and mealworm (*Tenebrio molitor*) on salmon liver. Planed experiments are immunohistochemistry, fatty acid composition and gene expression analysis this will give a good indication on what consequences this might have on the liver physiology. As the liver is a central organ that plays a key role in the breakdown, storage, and use of lipids in the body.

2.0 Materials & Methods

The NON-Fôr «Non-Food Organic Resources-based feeds optimised for salmon until post smolt stages» project is funded by NordForsk. The project is led by the Professor Mette Sørensen, Nord University, Bodø, Norway. The feeding trial was performed at Mørkvedbukta research station in Nord University. The trial was conducted within the guidelines of the National Animal Research Authority (FDU, ID-5887 in Norway), and the experiment was approved by Mattilsynet, complying with the guidelines under the Norwegian animal welfare act (LOV-2009-06-19-97) and the European Union act (EU/2010/63).

2.1 Experimental design

The fish used, was from Salten smolt in Breivika with a batch size of 193300 fish. The eggs hatched on 04/03/21, with a mortality of 223 (0.12%). Temperature for incubation was 6.9 °C on average. Juvenile fish were vaccinated at an average weight of ± 55g with Alphaject 6-2 from Pharmaq with a dosage of 0.05 ml. Fish went through two treatments with formaldehyde on 17/05/21 and 24/11/21, with a temperature of 13,2 °C. They were fed a diet of standardised feed (Table 1).

Table 3: Represents the feed that was used before the trial and the pellet size. The feed was aquired by Salten smolt Breivika

Ewos HARMONY start	0.6 mm	Used till 05/10/21
Ewos HARMONY start	0.9 mm	Used till 27/10/21
Ewos Harmony	1.3 mm	Used till 13/12/21
Harmony 15P	1.3 mm	Used till 26/02/22
Harmony 5P	1.7 mm	Used till 10/03/22
Ewos Harmony RAS 5P	1.7 mm	Used till 10/03/22
Harmony RAS 15P 500	2.1 mm	Used till 24/03/22
Ewos Micro 40	2.1 mm	Used from 30/03/22 till 02/05/22
Ewos Micro 80	2.1 mm	Used from 02/05/22 till transfer to experiment in Hall 1

The fish was transferred to the experimental Hall 1 (08.06.22), under continuous light conditions with no mortalities (Figure 1). The initial weight ranged from 143 ± 12.89g. The fish

were divided in 4 replicates tanks per diet group (4*5 = 20 tanks) (Figure 2). Total number of fish are 520 which resulted in 26 fish per tank. The fish are kept in hall 1 with a flow-through system holding filtered (200 µm), which is maintained at 1000 L flow rate and is supplied seawater from a depth of 250 m in Saltenfjorden (67°11'28" N 14°00'01" E).

The different feeding groups were CO, LB, LM, HB, HM. Duration of the experiment was 11 weeks. Average weight of the fish after 11 weeks on the day of sampling (25.08.22) was 438.1 ± 53,57 g. Fork length average of 30.25 ± 1,07 cm and the average weight of the livers were 4.72 ± 0,76 g. The second day of sampling (26.08.22) the average weight of the fish was 457 ± 46,30 fork length average of 30.64 ± 0,94 cm and average liver weight of 5.27 ± 1.00 g.

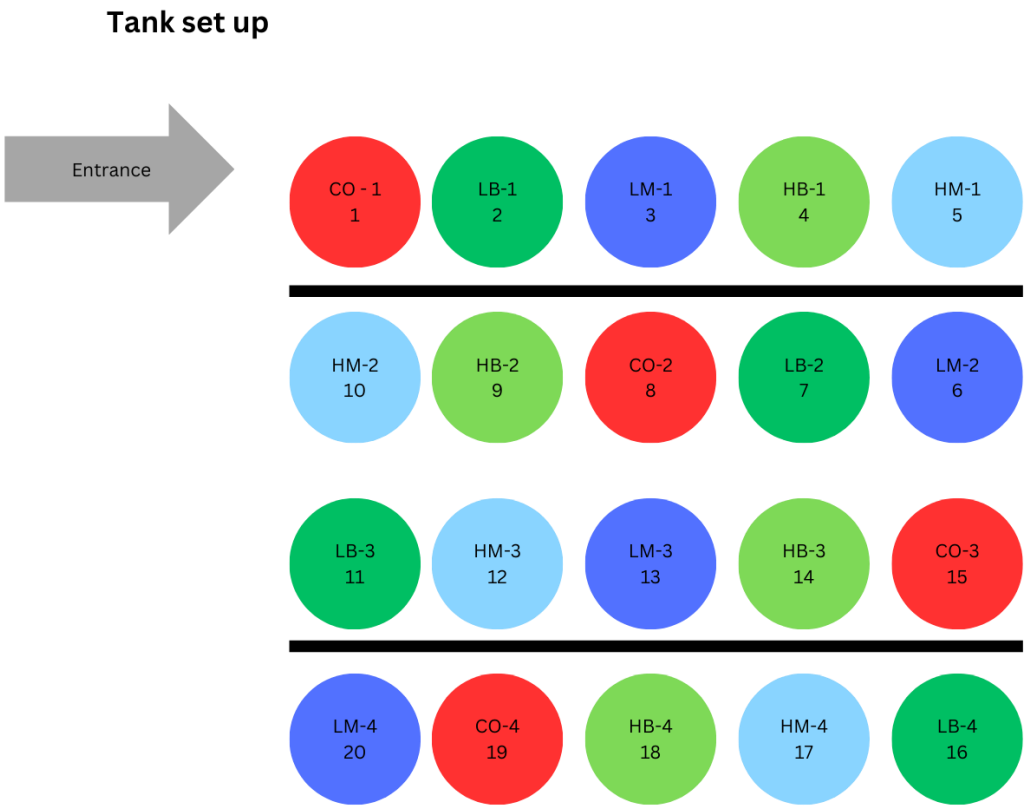

Figure 4: Is the experimental setup of the different feeds. CO is control, LB is 5% solider fly, HB is 10% solider fly, LM 15% meal worm, HM 30% meal worm. Each group has 4 replicates, meaning there are 4 fish per group. The total number of fish 80 individuals

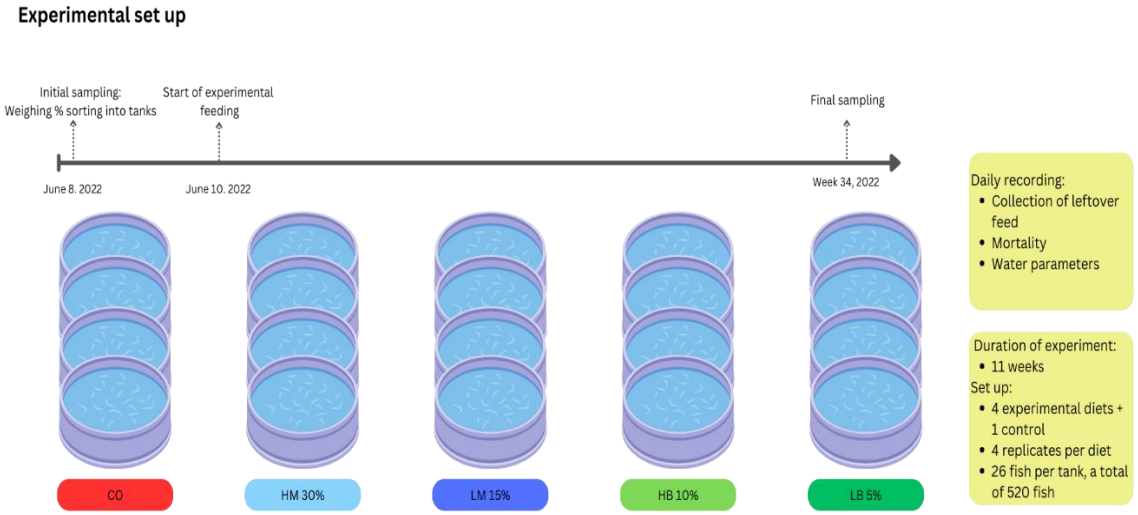

Figure 5: Is an overview of the experiment with different feeding trials and their groups. Atlantic salmon initial weight approx. 141-144 g. Four replicate tanks per diet group (4x5 = 20 tanks) 26 fish per tank, total 520 fish (0.8m3). Duration: 12 weeks Place: Hall 1, Research station @Nord Light: 24h continuous. Feeding Rate: 1.2% ,1.4% and changed to 1.6%.

2.2 Feed ingredients in experimental feed

The black solider fly larvae (BSFL) were made by Urbanmat AS Gjøvik, Norway. Mealworm (MW) was made by Invertapro AS Voss, Norway, and they were fed on cereal products with fruit and vegetables respectively. The feed was formulated and manufactured by BioMar's Technology Centre in Brande. They were fed composed diet induced with insect meal (Table 2), for quality of the pellets (Table 3). All of the different groups were fed twice a day with their corresponding feed and, where the feeding rate ranged from 1.2% to 1.6% due to good appetite. The different feeding groups were CO. LB with levels of (5%, 10%) and MW with levels of (15%, 30%). All diets contain constant levels of fish meal, 20%. And the replacement of soy protein concentrate (SPC), wheat gluten and rapeseed oil were replaced by insect meals in all four tested diets.

Table 4: Represents ingredient composition and analysed chemical composition of the experimental diets.

Ingredient (%) Diets	CO	LB	HB	LM	HM
Black soldier fly meal		5.00	10.00		
Yellow mealworm meal		0.0	0.0	15.00	30.00
Fish meal	20.00	20.00	20.00	20.00	20.00
Soy protein concentrate	20.00	20.00	19.77	15.30	2.00
Wheat gluten	14.46	12.88	11.46	9.49	9.59
Vegetable RM*	18.76	18.76	18.76	18.76	18.76
Fish oil	13.30	13.31	13.32	13.38	14.19
Rapeseed oil	6.23	4.40	2.57	3.63	0.0
Other (incl. Pigment)	7.18	5.57	4.06	4.37	5.40
Yttrium	0.05	0.05	0.05	0.05	0.05
Analysed chemical composition					
Dry matter	93.68	93.97	93.48	94.32	93.25
Energy (kJ/g-100)	22.00	22.13	22.46	22.75	22.90
Crude protein	44.63	46.00	45.30	45.70	47.00
Ash	7.30	7.60	7.40	7.20	6.60
Crude lipid	23.17	22.53	22.51	24.10	24.50

Table 3: Pellet Quality, BioMar.

Pellet Quality, BioMar	CO	LB	HB	LM	HM
Floatability, SW %	0.5	0	0	0	0
Dust, %	0.05	0.01	0.02	0.02	0.95
Oxypress, hours	>150	>150	>150	>150	>150
Centrifuge test (free oil) %	0.03	0.01	0.01	0.02	0.02

2.3 Sample collection and other parameters

The fish was manually netted from each tank and placed into a sedative tank. Sedation used Tricaine Pharmaq 140mg/g, dosage 30g/L. Then the fish was weighed, length was measured and the appropriate samples for several different analysis was included. The data for HSI was acquired by liver weight and weight of the fish. The liver samples were divided into 4 pieces and distributed with a scalpel and tweezers (Figure 3). The samples for histology and IHC were

immediately fixated in a solution of Formaldehyde 4% stabilised, buffered (Art.no 9713.9010, VWR Chemical). The amount of formaldehyde that the samples were fixated in is 10 times the volume of the samples. For the gene expression the samples were taken from the middle part on the left side of the liver (Figure 3). Samples were chopped into smaller pieces and placed into a small tube and snap frozen in liquid nitrogen, later placed in – 80℃ until further processing. The remaining parts of the liver were placed on an aluminium foil sheet and wrapped snap frozen in liquid nitrogen until they were moved into – 80℃. Histology and IHC samples were stored for fixation for 24hrs at room temperature (RT). After incubation time they were stored at +4 ℃ until processing.

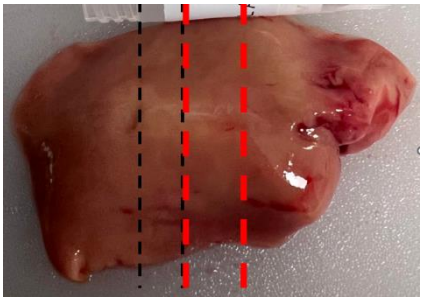

Figure 6: Represents the taken samples, histology (red lines) and gene expression (black lines). In addition, the remaining pieces of liver were used for liver fat analysis.

2.4 Preparation of the slides

The samples are dehydrated, which is followed by clearing and infiltration of the liver tissue. This was done with Thermo Fisher Scientific - Shandon Citadel 2000 by following a standard protocol (Suvarna et al., 2018). Individual liver samples were grouped together to form a group of 4 individuals from their respective treatment and placed in Microstar II cassettes. Next step is embedding to create a formalin-fixed, paraffin-embedded (FFPE) block of tissue following a standard protocol. Instrument used is Leica EG1150H at 60 ℃. With paraffin Tissue-Tek paraffin wax TEK III (polymer) with a melting point of 55-56 ℃. When FFPEs are poured they are cooled and stored at -20℃ until sectioning. Fixed tissue was embedded with paraffin and sliced into continuous sections on Cool-cut Thermo scientific, cooling device microm Cool-Cut. The blocks are then trimmed with thickness of 15 µm and cut into thin tissue sections with a thickness of 4 µm using a Microm HM355S rotary microtome with low-profile blades from Sakura Art.no 4689. The sliced sections were transferred by Thermo scientific section transfer

system microm STS for rotary microtomes. The tissue sections are placed on the glass slides polysine vw international. Slides are then air dried 24 hrs and stored at RT.

2.5 Haematoxylin & eosin staining

Haematoxylin–eosin (HE) staining was used to evaluate tissue morphology a standard protocol was followed with a dip and dunk technique. The steps that are involved are removing the wax, hydration of the section, haematoxylin nuclear stain, counterstaining, rinse, dehydrate and cover slip mounting.

2.6 CD8 and MHC II staining

For IHC staining a standard procedure was followed, antiserum used is CD8 and MCH II with a concentration of 1 µl/1000 µg ml^{-1} the stock solution was stored at -80°C. Stock solutions were diluted with glycerol at 1:2 ratio and stored at -20°C until further processing. Processing of the slides started with rehydration and followed by heat induced epitope retrieval (HIER) procedure with Grant CBB28 2V0923001. During HIER slides are submerged into citric buffer and placed into boiling water for 30 minutes. To block endogenous peroxidase activity a solution of 30 ml Methanol 70 ml tris-buffered saline and Polysorbate 20 (TBST) and 1 ml of 30 % H_2O_2 hydrogen peroxide UN2014 is used. To prevent the slides from drying out during incubations, the slides are placed into an incubation box with wet paper towels for 30 minutes. Slides are rinsed in TBST 3 times with a period of 3 min. Next step is to block the unspecific binding sites. Mixed solution of 20 ml TBST, 0,6 g BSA, 1 ml of Normal goat serum and 1 ml Normal donkey serum. 100 µL is then added to the slides per tissue section with an incubation time of 60 min. Excess solution is removed, and the slides are prepared for incubation with the primary antibody for MHC II or CD8 1:200, flowed by incubation with secondary antibody conjugated horse radish. One of the slides was chosen to become a negative control and this slide was not incubated with the primary antibody. Only secondary antibody was used here. The primary antibody is diluted in blocking solution, and 200 µL is then added to the slides per tissue section except the one slide that is a negative control. The slides are incubated for 24hrs at + 4 ℃, 200 µL of secondary anti-body is added to the slides per tissue section also for the negative control. 30 min in advance before the incubation is complete, Streptavidin conjugated horse radish is diluted in PBST 1:1000. The 200 µL of diluted Streptavidin conjugated horse radish is then added to the slides per tissue section for all.

Next step is to follow standard protocol for *Vector® NovaRED™ SUBSTRATE KIT FOR PEROXIDASE Catalog Number SK-4800 and stain the tissue. After the tissue is stained it is dehydrated and the samples are fixated with mounting media and a glass cover.

2.7 Total fat analysis of liver

The method that is used is a modification of Bligh and Dyer procedure (1959). Stored liver samples are gathered from the freezer with a set temperature of – 80 ℃, and are moved into a freeze drier, Sentry 2.0, VirTis, model # 2KBTXL -75 ℃ at -80 ℃, with vacuum 91mT. With a separately attached pump Trivac D2,5E serial #190293122, Leybold, made in Czech Republic. Moisture is removed from the samples and each liver sample is manually crushed. Individual liver samples from their respective dietary group were pooled. Each respective group had an average weight of 0.0534 g. The replicates of each Feeding group are repeated twice.

Samples are hydrated with 0.8 ml dH_2O. Then 2 ml of methanol HiPerSolv chromanorm HPLC-gradient grade 20864.320 is added followed by 1 ml chloroform SupraSolv for GC ECD and FID 1.02432.2500. Concentration of 19:0 was 1.56 mg/ml CHCl3 where the average 0.54 mg ± 0.07 internal standard was added to each sample. When the mixture is cooled homogenization is done with Fisher Scientific FB120 with a frequency of 20 kHz with intervals of 10 sec and resting time of 5 sec, which is done 3 times in total. Next step is to add 1 ml of chloroform and homogenize for 10 sec, then 1 ml of dH_2O is added and the sample is homogenized again for 10 sec. The samples are then placed on ice to cool before further processing. After that samples are Centrifuged with Thermo Scientfic Sorvall Legend X1R centrifuge at 3900 rpm for 20 min. The chloroform phase is pipetted and placed into another vial, which is then flushed with N gas to prevent oxidation, and then stored at + 4 ℃. From each stored vial, 0.3 ml is taken out and placed in a tiny cup. The chloroform is then set to evaporate on a heating board with at temperature of 40 ℃. After solvent evaporation the beakers are placed into an exicator at RT for 24 hrs to ensure that there is no more moisture left. Results were determined gravimetrically.

2.8 Total fat analysis of feed

The method that is used is a modification of Bligh and Dyer procedure (1959). The exact same protocol was used as mentioned in 3.1.2 materials and methods paragraph for the extraction of total lipids from livers as for feed. The feed was tested for total fat analysis. Results were determined gravimetrically. Feed was also tested for fatty acid analysis with GC and the protocol used is the same as 3.1.4 isolation of fatty acid methyl esters mentioned in the

paragraph below. The data was collected from the Compass CDS 4.1.0.329 and transferred to excel.

2.9 Isolation of fatty acid methyl esters

Procedure used is based on that described by Metcalfe et al. (1966). Chloroform phase that was stored for total fat analysis is used for further processing. The steps involved are saponification and methylation. Evaporation of 0,5 ml of chloroform phase is complete. Lipids then undergo saponification with 1 ml of 0,5 M NaOH is added with 1 ml of methanol. The sample is flushed with N_2 gas and incubated for 15 min at 100°C, and then cooled on ice for 5 minutes until further processing. Methylation step is done by adding 2 ml of BF3 methanol and flushed with N_2. and heating at 100 ℃ for duration of 5 min. The tube in then chilled on ice and 1 ml of hexane is added and the mixture incubated for 1 min at 100°C and cooled again on ice.

Then 3 ml of saturated NaCl solution are added, and thoroughly mixed by shaking. The hexane phase is then extracted and placed in a vial, followed by another extraction 0,5 ml of hexane which is pooled with the first extraction. After dilution with hexane to obtain concentration of about 0,25 mg FAMES pr ml, the fatty acids were analysed with GC Bruker, model CP-8400 with CP-8400 Autosampler, with wax embedded column, part number CP7713, CP-Wax 52CB 25m x 0,25 mm x 0,20 μm, Agilent Techonlogies. The cycles were done with split 1:50 and the column was initiated at 90°C for 1 min, then 45°C/min until 150°C, and then 4°C/min until 225°C hold 2 min = which in total results in 23.08 min pr sample. The results are transferred to a program called Compass CDS 4.1.0.329. where the data can be collected and moved into an excel file.

2.10 RNA extraction and cDNA synthesis

RNA from Atlantic salmon livers were extracted using reagent E.Z.N.A Total RNA kit (R6834-02, Omega Bio-tek, USA) standard protocol was followed which was advised by manufacturer. When the RNA extraction was complete and any contaminating DNA was eliminated, the RNA is then tested for purity, quantity, and quality. For purity NanoDrop One^c (ThermoFisher scientific Verona Rd. Madison, WI 53711 USA) was used, for quantity Qubit 3.0 Fluorometer Life Technologies) was used with Qubit RNA BR Assay Kit (Invitrogen, Life Technologies USA). For quality gel electrophoresis 1.2 % (w/v) was performed and image was captured with Bio-Rad ChemiDoc MP Imagining system, USA (Figure 10).

QuantiTect Reverse Transcription Kit (QIAGEN Hilden) was used for cDNA synthesis. Briefly, 1 µg of RNA sample was used, and standard protocol was followed as mentioned from manufacturer.

2.11 Electrophoresis

One of the quality procedures is electrophoresis. All the samples showed adequate quality with both 28S rRNA and 18S rRNA are visible.

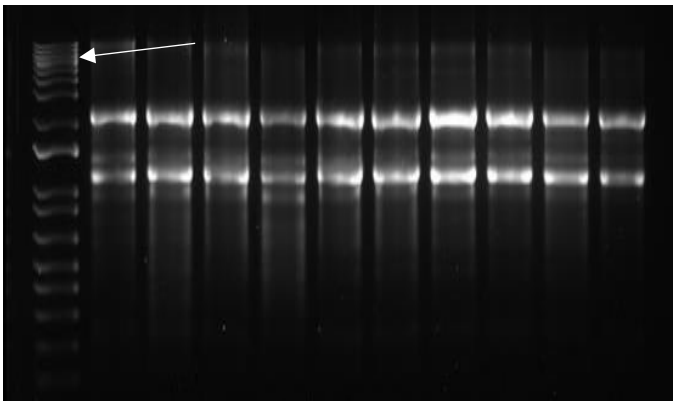

Figure 7. Represents a picture of an agarose gel plate ranging from the sample number 141-158. This was done to quality check the extracted RNA. The white arrow represents the ladder.

2.12 qPCR

All qPCR reactions were done in duplicates in 10 µL reactions consisting of 5 µL of All qPCR reactions were done in duplicates in 10 µL reactions consisting of 5 µL using FastStart Universal SYBR Green Master (ROX), 1 µL of gene-specific primer pair (5 µM each) and 4 µL of 2-fold diluted cDNA sample. Non-reverse transcription control was not included. Template controls were included for each primer pair at 500 µL of master mix and 100 µL of forward and reverse primers. Details of the primers used for target they are provided in (Table 4). Thermocycling parameters as follows: initial denaturation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 sec, optimized annealing temperature (60°C) for each gene (Table 4) for 30 s. The specificity of amplification was determined by melting curve analysis. The standard curve was obtained by running a 5-point series of 2-fold dilution (1:2, 1:4, 1:8, 1:16 and 1:32) pooled cDNA. The results from the Light Cycler were obtained with Light Cycler 96 1.1 and transferred to Excel.

2.13 Primers

Table 4: represents the chosen primers from published papers (Sanchez-Gurmaches et al., 2011; Torstensen et al., 2011) and previous lab work at Nord University lab. Primers, *actb* (Beta-actin), *eef1ab* (protein synthesis), *uba52* (protein comprising ubiquitin) which are house keeping genes. The targeted genes that relate to of lipid transport throughout the organism are *fatp1* (transporter in cellular FA uptake in tissues with rapid FA metabolism), *cd36* (linked to steatosis), *lpl* (Hydrolyse TGA to FA and make them available for cells), *apoa1*(HDL transports cholesterol back to liver), *apoB100* (LDL transports lipids from liver to organs) *srebp1* (Regulates genes related to lipid and cholesterol production, is regulated by sterols).

Gene	Forward Primer, 5' to 3'	Reverse Primer, 3' to 5'	Annealing Temperature C°
ACTB	CCAAAGCCAACAGGGAGAA	AGGGACAACACTGCCTGGA	60
EEF1AB	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG	60
UBA52	TCAAGGCCAAGATCCAGGAT	CGCAGCACAAGATGCAGAGT	60
SREBP1	CCCCAGTTTATCAAGGCTGA	TCCATCATCACTGGCACTGT	60
FATP1	TGGGAGCTTGTGGGTTCAA	ACTTTCATGAGGCGGATTGG	60
CD36	TTTCCTGCTGCGCACCTT	GGTGCGGGTCATGAAGATTT	60
LPL	GCCCGACCTTTGAGTTTGC	ACGTCCACAAAGAGAGCATCGT	60
APOA1	ACCCACCAGACCACCATCAT	CAGCTGAGAGGGAGCATCAG	60
ApoB100	TTGCAGAGACCTTTAAGTTCATTCA	TGTGCAGTGGTTGCCTTGAC	60

2.14 Procedures & statistical analysis

Qualitative analysis of the lives with H&E was done with a microscope and one observer. The scoring for this was set at 0 to 3 where 0 is the standard referring to the control slides.

Qualitative analysis of the livers with CD8 and MHC II was done with a microscope and one observer. The scoring list is from 2 to -3 where the standard is set at 0 respective of the control.

Hepato-somatic index % was calculated using the formula listed below. One way ANOVA was preformed and Levene's test was done to determine the homogeneity of variance.

$$Hepato\text{-}somatic\ index\ (\%) = \frac{Liver\ weight\ (g)}{Fish\ weight\ (g)} \times 100$$

All the statistical analyses were done with R-studio (Version 2022.12.0+353) except for GC fat analysis on livers, this was done with SPSS (Version 26). Microsoft® Excel (Version 2212) was used to create tables and data sheets. Significance threshold for all statistical analysis were 0,05. Plots were created in R-studio. Results are presented as mean ± standard deviation (SD). Difference between total lipids and fatty acids on liver in between the treatments were determined through one way ANOVA test. Levene's test were done to test for homogeneity of variances. Post hoc Tukey test was performed with a confidence level of 0.95 to determine which of the groups differed significantly. Shapiro-Wilk test was used to check for normality. Results are presented as mean of mg/g Significance was established at $p < 0.05$ and 95%. The graphs are presented as percentage of mg/g.

Difference between total lipids and fatty acids on feed between the treatments were determined through ANOVA. Levene's test was done to determine the homogeneity of variance. Results are presented as mean of mg/g Significance was established at $p < 0.05$ and 95%. The graphs are presented as percentage of mg/g.

To compare the different expression of genes (*cd*36, *fatp*1, *lpl*, *apoa*1, *apoB*100, *SREBP*1) within the different feeding groups (CO, LB, LM, HB, HM). Shapiro-Wilk test was used to check for normality. The non-parametric test Kruskal-Wallis was performed for the genes *cd36, fatp1, lpl, apoa1, srebp1*. For the gene *apoB100* One-way ANOVA was performed. A pairwise T-test was performed to compare the between the treatments with their respective gene of interest. Results are presented as fold change; significance was established at $p < 0.05$ and 95%. The graphs are presented with relative expression.

3.0 Results

3.1 HSI

Data were verified for homogeneity of variances ($p > 0.05$) for HSI, with Levene's test. Pr (>F) = 0.898, meaning that the assumption of homogeneity with equal variance was met. The test showed no significant difference (F=1.46, P-value = 0.22). The average HIS % is 1.11 ± 0.14 throughout all groups. CO groups average is 1.12 ± 0.12, LB groups average 1.14 ± 0.15, LM groups average 1.03 ± 0.16, HB groups average 1.12 ± 0.10, HM groups average 1.12 ± 1,12 (Figure 12).

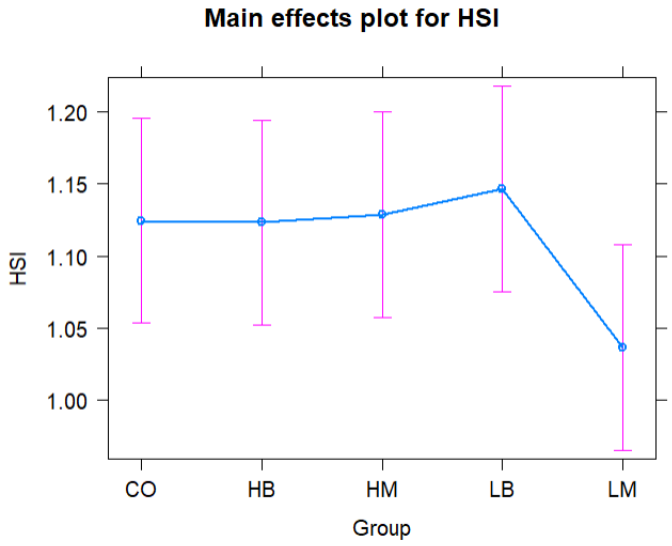

Figure 8: Represents HSI parameters on the livers in percent

3.2 Liver histomorphometry

The histologically prepared microscope slides with liver samples (n = 80). Where studied with qualitative analysis of Haematoxylin & eosin staining. The samples were studied under light microscopy using Olympus BX51 with an image taking attachment Olympus SC180 for identification of histopathological alternations in the tissue. Observation results did not show any significant derivation of the control (Table 5 & Figure 5). No vacuolization or displacement of nuclei was observed among the dietary treatments. Among of all groups only CO 8 showed some area specific vacuolization and displacement of nucleus in slide (Figure 5).

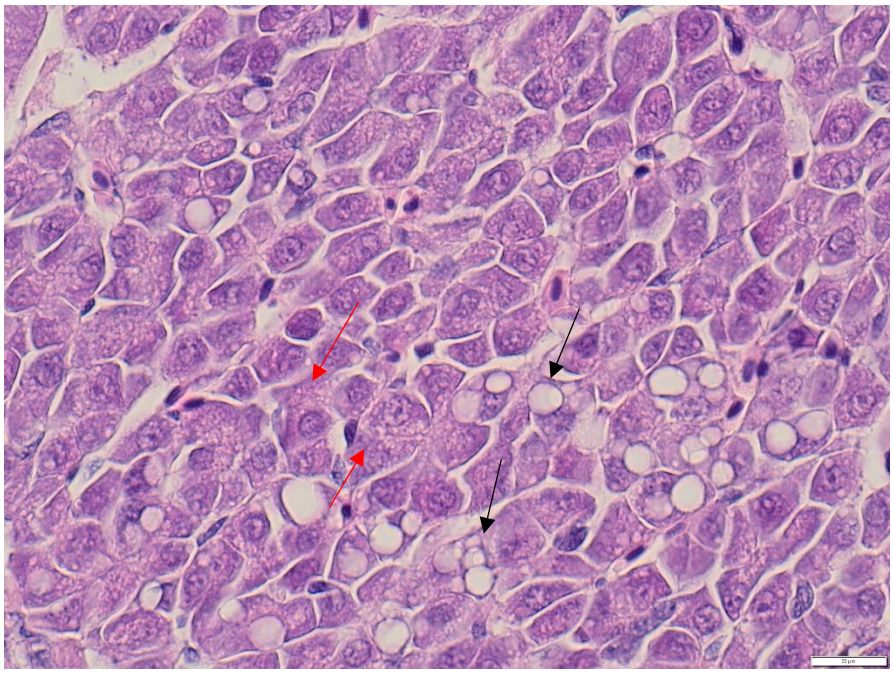

Figure 9: Represents micro and macro vacuolization in sample CO 8. The image was captured using magnification 60 x. The black arrows indicate macro vacuolization, whereas red arrows indicate micro vacuolization with displacement of nucleus.

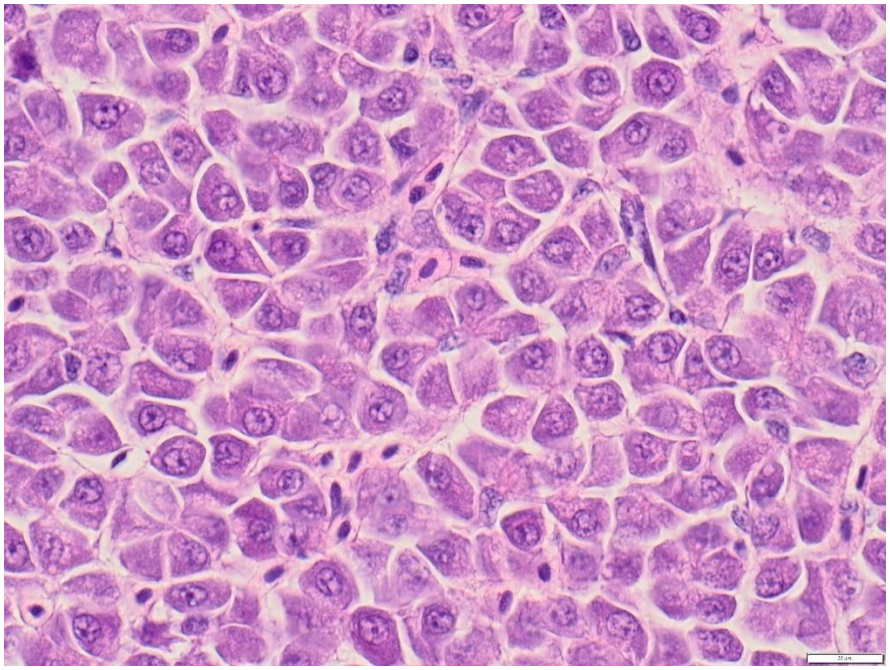

Figure 10: Represents hepatocytes with centred nucleus and no micro or macro vacuolization sample HM 5. The image was captured using magnification 60 x

Table 5: Represents the observed vacuolization and nucleus placement with a ranking system 0/3 where 0 is reference point from the control.

Haematoxylin & eosin		**RANK 0/3**
CO 1	centred nucleus	0
CO 8	centred nucleus & vacuolization	0
CO 15	centred nucleus	0
CO 19	centred nucleus	0
HB 4	centred nucleus	0
HB 9	centred nucleus	0
HB 14	centred nucleus	0
HB 18	centred nucleus	0
HM12	centred nucleus	0
HM17	centred nucleus	0
HM5	centred nucleus	0
HM10	centred nucleus	0
LM2	centred nucleus	0
LM6	centred nucleus	0
LM13	micro/macro vacuolization centred nucleus	0
LM20	centred nucleus	
LB2	centred nucleus	0
LB 7	centred nucleus	0
LB 11	centred nucleus	0
LB 16	centred nucleus	0

3.3 IHC: MHC II & CD8

Qualitative analysis of MHC II staining showed no significant derivation from the control where appropriate cells were stained such as: Dendritic cell, Macrophages, Monocytes and B-cells. Cells that have foreign antigen on the exterior of the cell were marked with MHC II antibody. NovaRed was used for staining.

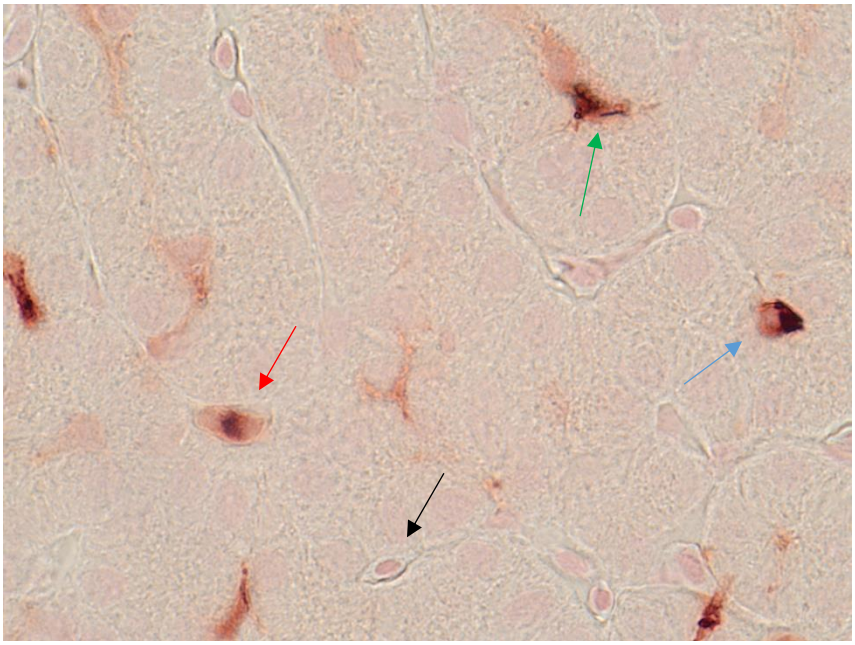

Figure 11: Shows Atlantic salmon liver. Red arrow points out macrophage cell, black arrow points out an erythrocyte, blue arrow shows a monocyte, and the green indicates a dendritic cell. The image was captured using magnification 60 x, group LB

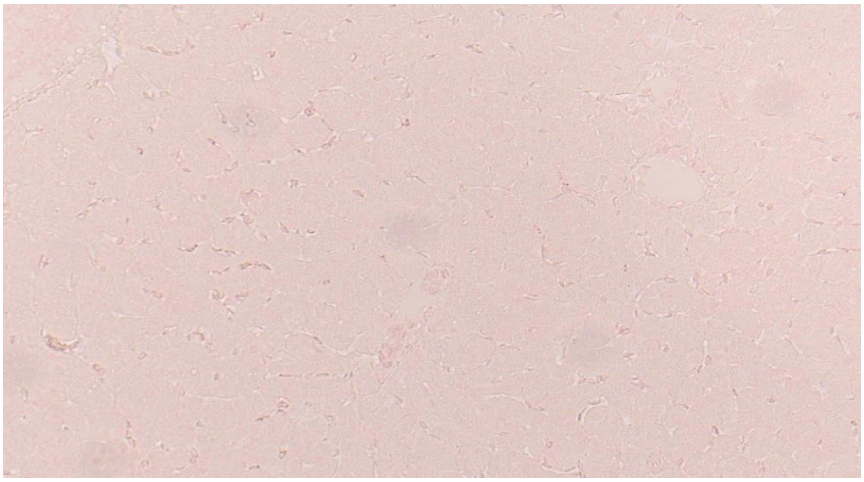

Figure 12: Represents the MHC II negative control, without primary antibody. The image was captured using magnification 10 x, group CO

The protocol was used on two different antibodies MHC II and CD8, where MHC II showed positive staining (Figure 6) and negative control did not show any staining (Figure 7). The qualitative analysis was performed by one observer and the results of MHC II showed a higher abundance of positively stained cells in livers in each dietary groups CO, LB, LM ranking closer to 0 (Table 6). There was a notable difference between groups. HM and HB groups showed lower amount of staining ranking closer to -3. There was a notable difference between groups. HM and HB groups showed lower amount of staining ranking closer to -3. Method used for qualitative analysis is a modification of the method used (Yousef & Matsumoto, 2021). The most common score was at -2 resulting in 35% the percentage of observations by their ranking is represented in (Table 7).

Table 6: Represents observations made by the observer ranging from -3/2 where the 0 is the reference point from the control.

MHC II	Observations	RANK -3/2
CO1	Adequate	0
CO8	Adequate	0
CO15	Adequate	-3
CO19	Adequate	-3
CO15	Adequate	-3
CO19	Adequate	-3
HB4	Adequate	-3
HB9	Adequate, with artifacts.	-2
HB14	Adequate	-2
HB18	Liver in some spots has more stained cells that in other parts. As well as overstaining close to the gull duct	-2
HM12	Artifact bubbles	-2
HM17	Artifact bubbles	-2
HM5	Adequate	-1
HM10	Adequate	-2
LM2	Adequate	-1
LM6	Adequate	-1
LM13	Adequate	0
LM20	Adequate	0
LB2	Adequate	-1
LB7	On this slide 2 of the livers had little to no staining, seems individual	-2
LB11	Adequate	0
LB16	Adequate	0

Table 7: Represents the percentage of the observations corresponding to the grading scale set of -3/2 where the -3 represents less staining than 2.

Scale	Percent
2	0%
1	0%
0	30%
-1	20%
-2	35%
-3	5%

The results from CD8 showed no positive staining (figure 8 & 9).

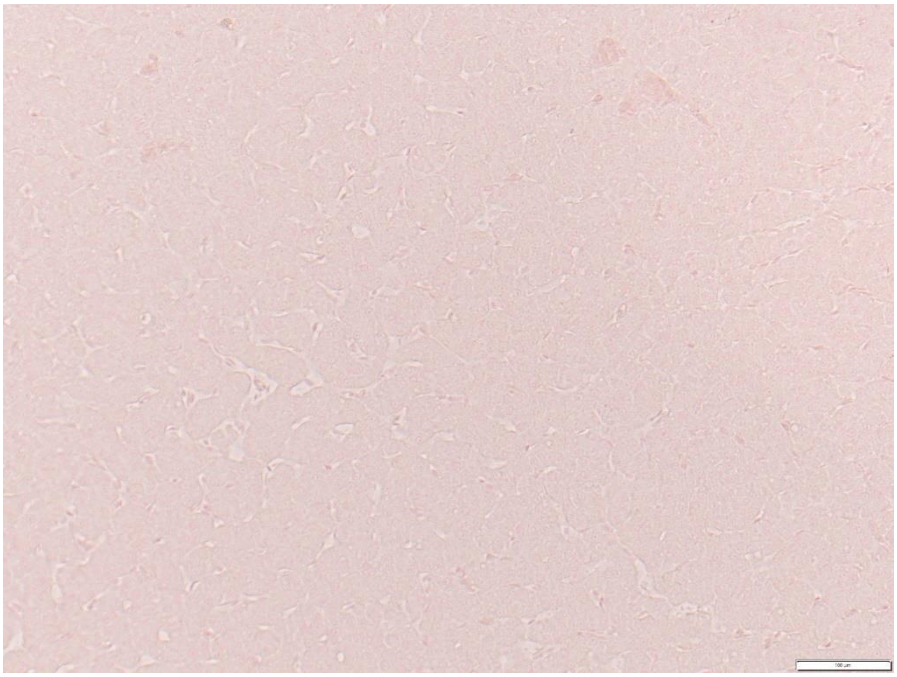

Figure 13: Represents CD8 staining LB (no colour). The image was captured using magnification 10 x

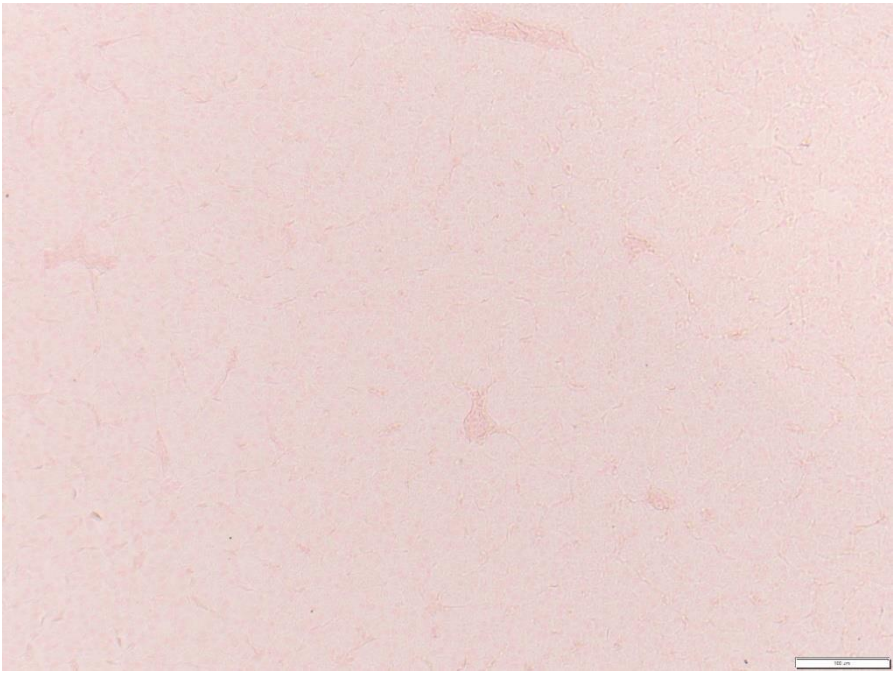

Figure 14: Represents CD8 staining CO (no colour). The image was captured using magnification 10 x

3.4 Total fat analysis for feed

CO groups average is 25.01 ± 0.36, LB groups average 23.04 ± 0.34, LM groups average 2.07 ± 0.41, HB groups average 22.79 ± 0.14, HM groups average 24.44 ± 0.40. For main fatty acids in the feed (Table 9). Data were verified for homogeneity of variances ($p > 0.05$) for the 5 feeds used with Levene's test. Pr (>F) = 2.2e-16, meaning that there is a significant difference between the variance. As the assumption of homogeneity of equal variance was not met, a Welch ANOVA was performed instead of one-way ANOVA. The test did not show any significant difference (F=10.3, P-value = 0.07).

Table 8: Represents fatty acid analysis on the feed in % of some fatty acids.

Feed	**CO**	**LB 5%**	**LM 15%**	**HB 10%**	**HM 30%**
Fatty acids					
Saturated fatty acids (SFAs)					
C12:0	0.0	1.23	0.0	2.46	0.0
C14:0	6.13	6.57	6.17	7.03	6.77
C16:0	16.38	17.67	18.27	18.60	20.75
C18:0	3.80	4.03	4.08	4.06	4.53
∑SAFAs	27.64	30.81	29.79	33.47	33.19
Monounsaturated fatty acids (MUFAs)					
C16:1 n-9	0.0	0.0	0.0	0.15	0.14
C18:1 n-9	27.87	24.72	25.30	21.50	21.92
C20:1 n-9	1.45	1.42	1.26	1.31	1.11
∑MUFAs	38.91	36.10	35.68	32.85	32.35
Polyunsaturated fatty acids (PUFAs)					
C18:2 n-6 (LA)	11.28	10.94	12.90	10.90	13.57
C18:3 n-3 (ALA)	2.89	2.44	2.17	2.06	1.10
C20:5 n-3 (EPA)	9.63	9.73	9.45	10.23	10.02
C22:6 n-3 (DHA)	6.43	6.57	6.35	6.84	6.68
∑PUFA	30.23	29.68	30.87	30.03	31.37
∑n-3	21.89	21.81	20.86	21.62	20.85
∑n-6	11.97	11.69	13.60	12.49	14.49
n-3/n-6	1.83	1.87	1.53	1.73	1.44
DHA/EPA	0.67	0.68	0.67	0.67	0.67

Values are expressed as mean value of duplicate samples per diet.

3.5 Total fat analysis of livers in all groups

CO groups average is 15.89 ± 0.87, LB groups average 14.77 ± 0.81, LM groups average 14.77 ± 1.02, HB groups average 14.53 ± 0.94, HM groups average 14.22 ± 0.56 (Figure 11). Data were verified for homogeneity of variances ($p > 0.05$) for the 5 diets with Levene's test. P-value obtained was Pr (>F) = 0.29, meaning that the assumption of homogeneity of equal variance is met. One way ANOVA was performed to determine the significant difference in percentage between the total fat content of the different feed. The test showed a significant difference ($F_{4.889}$, P=0.003). The factor feeding group (CO, LB, LM, HB, HM) explained 29% of the variance. Post hoc Tukey test was performed with a confidence level of 0.95 to determine which

of the groups differed significantly. HM to Control adjusted p-value of 0.0019 and HB to Control adjusted p-value of 0.015, indicating that control group is significantly different to HM and HB. Other groups did not show the same trend.

Shapiro-Wilk test was used to check for normality P = 0.06 indicating that the data is normally distributed.

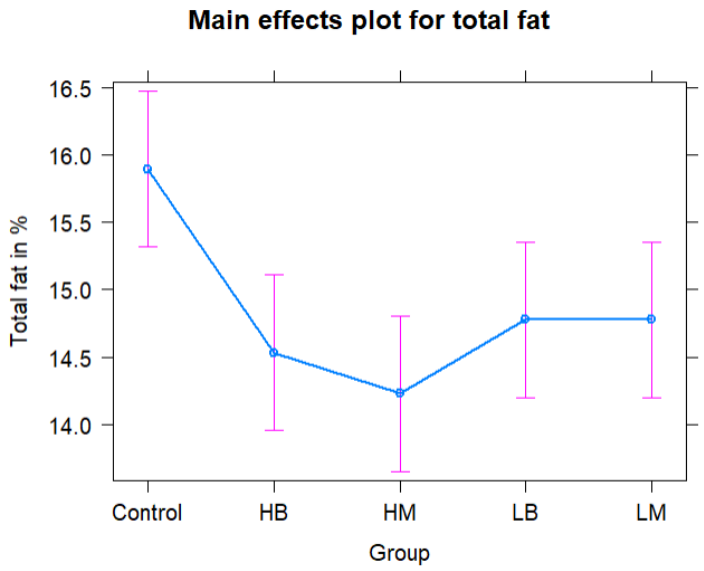

Figure 15: Represents total fat analysis on liver in percent

3.6 GC fat analysis on liver

Certain fatty acids had a rise without showing a significant difference such as C14:0 comparing CO 2.55 ± 2.34 to HM 4.04 ± 0.32, while it dropped when compared to HB 1.86 ± 1.11. Certain fatty acids did not show a significant difference but where higher compared to CO ∑SAFA in CO 23.90 ± 2.39 to HM 26.73 ± 0.54. Other fatty acids showed a decline in fatty acids when compared to CO ∑MUFA in CO 22.75 ± 2.34 to HM 19.07 ± 0.78. But some also showed very little variation such as ∑POLY in CO 53.36 ± 1.41 to HM 54.20 ± 0.63. ∑n-3 in CO 43.06 ± 1.62 to HM 43.90 ± 0,91as well as ∑n-6 in CO 10.30 ± 0.40 to HM 10.30 ± 1.09. Data were verified for homogeneity of variances ($p > 0.05$) for the 5 diets with Levene's test (Levene, 1960). Some of the groups did not meet the requirements of homogeneity of variance based on mean. These include fatty acids (C18:2n-6, C20:4n-6, C22:1n-11, C24:1n-9). The rest met the requirements of homogeneity. Shapiro wilk test was done to test for normality of variance, but the requirements of normality were not met. To test for difference between groups, non-parametric independent-samples Kruskal-Wallis's test was done. It showed that some of the fatty acids have as significant difference between groups. Fatty acids that showed significant

difference were CO 2.40 ± 0.17 to HM 2.04 ± 0.07, and LB 2.53 ± 0.19 to HM 2.04 ± 0.07 in the fatty acid chain C18:1n-7. HB to CO, HB to HM, HB to LM in the fatty acids C18:2n-6, C18:3n-3, C20:1n-9, C20:3n-6, C22:1n-11, C22:5n-3, they are marked by * (Table 10).

	CO		LB 5%		LM 15%		HB 10%		HM 30%	
Name	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**
C14:0	2.55	± 2.34	2.98	± 2.75	2.36	± 1.71	1.86	± 1.11	4.04	± 0.32
C16:0	15.37	± 0.43	15.43	± 0.78	16.14	± 0.26	16.20	± 0.54	16.15	± 0.40
C16:1n-9	0.49	± 0.06	0.56	± 0.05	0.55	± 0.06	0.56	± 0.06	0.49	± 0.05
C16:1n-7	1.78	± 0.18	1.90	± 0.05	2.00	± 0.21	1.84	± 0.11	1.76	± 0.10
C18:0	5.97	± 0.37	6.00	± 0.18	6.26	± 0.12	6.26	± 0.34	6.53	± 0.20
C18:1n-9	16.05	± 1.76	16.40	± 1.15	16.64	± 1.47	13.95	± 1.23	13.42	± 0.64
C18:1n-7*	2.40	± 0.17	2.53	± 0.19	2.37	± 0.14	2.33	± 0.08	2.04	± 0.07
C18:2n-6*	4.76	± 0.31	4.67	± 0.21	5.57	± 0.47	4.41	± 0.17	5.17	± 0.16
C18:3n-3*	0.94	± 0.20	0.86	± 0.03	0.94	± 0.10	0.77	± 0.06	0.52	± 0.02
C20:1n-9*	1.68	± 0.20	1.75	± 0.25	1.49	± 0.11	1.49	± 0.12	1.25	± 0.07
C20:3n-6*	1.26	± 0.09	1.28	± 0.09	1.37	± 0.06	1.19	± 0.06	1.40	± 0.10
C20:4n-6	3.35	± 0.21	3.08	± 0.15	1.94	± 0.88	3.83	± 0.41	2.76	± 0.96
C20:4n-3	0.55	± 0.04	0.52	± 0.02	0.57	± 0.04	0.53	± 0.03	0.49	± 0.03
C20:5n-3	7.75	± 0.54	7.67	± 0.35	7.60	± 0.27	8.29	± 0.86	8.13	± 0.63
C22:1n-11*	0.04	± 0.07	0.32	± 0.21	0.00	± 0.00	0.00	± 0.00	0.00	± 0.00
C22:3n-6	0.63	± 0.11	0.55	± 0.11	0.60	± 0.15	0.62	± 0.10	0.86	± 0.10
C22:4n-6	0.30	± 0.09	0.21	± 0.14	0.19	± 0.13	0.31	± 0.08	0.12	± 0.14
C22:5n-3*	2.91	± 0.12	2.88	± 0.05	3.07	± 0.07	2.99	± 0.12	3.24	± 0.14
C22:6n-3	30.91	± 1.14	30.23	± 0.73	30.16	± 1.13	32.27	± 0.76	31.51	± 0.49
C24:1n-9	0.30	± 0.09	0.18	± 0.19	0.19	± 0.20	0.28	± 0.08	0.11	± 0.12
Sum SAFA	23.90	± 2.39	24.41	± 2.15	24.66	± 1.24	24.32	± 0.88	26.73	± 0.54
Sum MUFA	22.75	± 2.34	23.65	± 1.67	22.83	± 2.63	20.46	± 1.44	19.07	± 0.78
Sum POLY	53.36	± 1.41	51.95	± 1.48	52.51	± 1.42	55.22	± 1.30	54.20	± 0.63
Sum n-3	43.06	± 1.62	42.15	± 1.08	42.55	± 1.61	44.86	± 0.88	43.90	± 0.91
Sum HUFA	42.11	± 1.56	41.30	± 1.05	41.68	± 1.80	44.09	± 0.86	43.38	± 0.91
DHA/EPA	4.01	± 0.27	3.95	± 0.14	3.82	± 0.25	3.94	± 0.43	3.90	± 0.29
EPA/ARA	2.33	± 0.25	2.50	± 0.16	4.33	± 1.39	2.17	± 0.06	3.44	± 1.39
Sum n-6	10.30	± 0.40	9.79	± 0.46	9.96	± 0.98	10.37	± 0.54	10.30	± 1.09
n-3/n-6	4.19	± 0.29	4.31	± 0.14	4.32	± 0.49	4.34	± 0.18	4.32	± 0.55

Table 9: Represents percentage mean and standard deviation on the fatty acids in the liver.

3.7 q-PCR

Primer efficiency *cd36* is 2.01 with mean fold change for CO is 0.078, for LB is 0,074, for LM is 0,073, for HB 0,058, for HM is 0,054. Primer efficiency *fatp1* is 2.1 with mean fold change

for CO is 1.15, for LB is 1.90, for LM is 1,91, for HB 1,55, for HM is 1,03. Primer efficiency Primer efficiency *lpl* is 2 with mean fold change for CO is 0,082, for LB is 0,67, for LM is 0,74, for HB 0,58, for HM is 0,52. Primer efficiency *apoa1* is 2.17 with mean fold change for CO is 0,75, for LB is 0,90, for LM is 0,72, for HB 0,50, for HM is 0,48. Primer efficiency *apoB100* is 1,95 with mean fold change for CO is 0,76, for LB is 0,71, for LM is 0,65, for HB 0,50, for HM is 0,48. Primer efficiency *serbp1* is 2.01 with mean fold change for CO is 0,81, for LB is 0,74, for LM is 0,75, for HB 0,78, for HM is 0.45. The statistics were done to compare the different expression of genes (*cd36, fatp1, lpl, apoa1, apoB100, srebp1*) within the different feeding groups (CO, LB, LM, HB, HM). Shapiro-Wilk test was used to check for normality, (p-value < 0.05) indicating that the data is not normally distributed. Except for *apoB100* were the p-value = 0.2771 meaning that the data is normally distributed. The assumption of homogeneity of variance was met for all the genes. The non-parametric test Kruskal-Wallis was performed for the genes (*cd36, fatp1, lpl, apoa1, srebp1*) where all of these had a (p-value > 0.05). For the gene *apoB100* One-way ANOVA was performed without showing any significant difference. Since there was no significant difference of gene expression within the feed groups, no post-hoc test was performed.

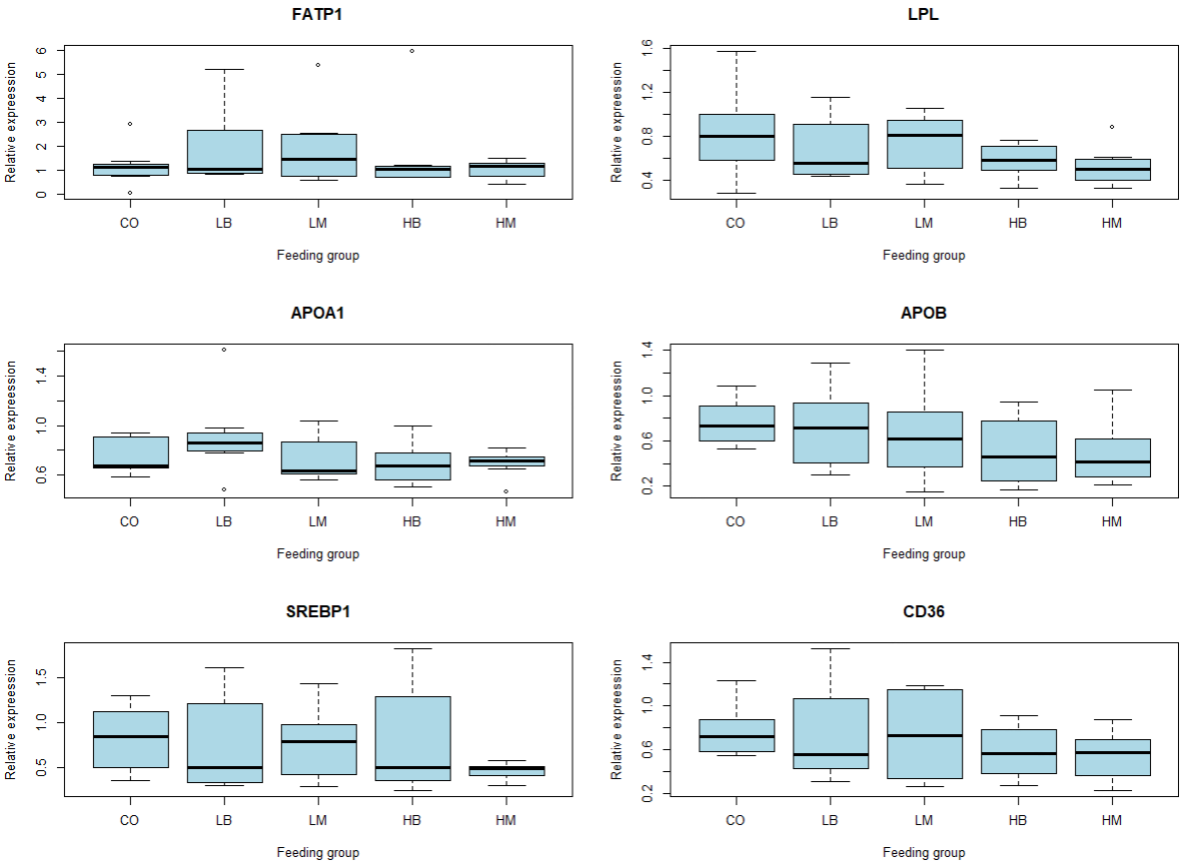

Figure 16: Relative expression of target genes in different feed groups. The box plots show the median and 25th and 75th percentiles, based on six biological replicates. No significant differences were observed.

4.0 Discussion

The purpose of this study is to assess the use of black soldier fly (BSF) and mealworm (MW) as a component in the feed for salmon in aquaculture, with particular emphasis on their effects on the lipid accumulation in the liver. The primary inquiry was the fate of the C12:0 medium-chain fatty acids and potential short-chain fatty acids (SCFAs) in the feed containing BSF (Table 8). These fatty acids are not a common occurrence in todays standardized feed for salmon species (Hundal, 2022). However, in the present study the results of HSI showed between 1%-2% (Figure 8). The results are similar with the other studies where inclusion of insect meal levels is slightly higher ranging from 33%-100% replacement of FM with a HIS result of 1.1 %. This study also has concluded that the statistical analysis did not show a significant difference (Belghit et al., 2019). Indicating the livers of the salmon seems healthy (C. Caimi et al., 2021) In the present study, histological analysis of liver with haematoxylin & eosin staining did not indicate any deviations from control (Table 5 & Figure 8). Some macro & micro vacuoles were observed in some of the control group but did not show a consistent vacuolization throughout the whole liver of the affected individual. These results show that inclusion of mealworm or black solider fly larvae does not affect the morphology of salmon liver in a negative manner. This indicates that the developed feed does not promote steatosis of the liver (Fountoulaki et al., 2017). Similar results were also observed in earlier studies that showed 8 %-16 % inclusion levels of BSF. The study did not vid evidence of inflammation or damage (Christian Caimi et al., 2021; Kumar et al., 2021). These results support the results of qPCR. In the present trial, IHC analysis in the dietary group did not indicate any large deviations from the CO group (Table 6 & Table 7). The IHC indicated positive staining of MCH II in the liver, stained cells consisted of dendritic cell, monocyte, and melano-macrophages (Figure 11). Cells that are stained with MHC II take part in innate immune system defense and maintenance against pathogens such as bacteria, viruses, fungi, and parasites, foreign substances such as toxins, drugs, and chemicals as well as cancer cells and abnormal cells in the body (Liaskou et al., 2012). Furthermore, blood circulating from the intestines of the liver is plentiful in bacterial products and nutrients (Gao et al., 2008). Staining shows that the immune cells in most cases are evenly spread throughout the liver samples. There are no signs of inflammation as it is expected to have immune cells present in the liver as part of maintaining homeostasis (Liaskou et al., 2012). The innate response cells are present in the fish even during early development, in zebrafish macrophages are present in the liver already at 12 h stage (Cheng et al., 2020). However, CD8 staining revealed absence of MCH I (Figure 13 & Figure 14). CD8 glycoprotein

that is present on cytotoxic T-cells as a co-receptor and will bind to the MHC I class (Russell, 1998). Regarding to the similar studies there is no little inflammation on the liver with insect meal induced feeds. These results support the results of Histology with haematoxylin &eosin staining. For the total fat analysis of the feed statistical analysis did not show a significant difference between the dietary groups showing consistent parameters. The formulated feed is deviating from the commercialized feed when looking into certain fatty acids (Hundal, 2022). Although C12:0 was found in the feed, research has revealed that the majority of lauric acid consumed is sent directly to the liver, where it is transformed into energy and other metabolites instead of being kept as fat (Dayrit, 2014). When looking into the total fat analysis of the livers a significant difference between the groups was noticed (Table 9 & Figure 15). GC analysis of different lipids showed that the essential fatty acids (EFAs) C18:2n-6 and C18:3n-3 were noted to be significantly different. C18:2n-6 is an omega-6 polyunsaturated fatty acids (PUFAs) also known as linoleic acid. C18:3n-3 is an omega-3 PUFA also known as alpha-linolenic acid (ALA). Additionally, C18:3n-3 is a precursor for the longer-chain omega-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). C20:1n-9: this is a monounsaturated fatty acid (MUFA) also known as eicosanoid acid. C20:3n-6: this is an omega-6 PUFA also known as dihomo-gamma-linolenic acid (DGLA). Like other omega-6 PUFAs is involved in the synthesis of eicosanoids, which can have both pro- and anti-inflammatory effects depending on the specific molecule produced. C22:1n-11 is a MUFA also known as erucic acid. C22:5n-3 is known as omega-3 fatty acid. It is also known as docosapentaenoic acid (DPA), which is an intermediate between eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the biosynthesis of long-chain polyunsaturated fatty acids (LC-PUFAs) in fish. However, the values show that there are quite high amounts of ∑HUFA and ∑PUFA in contrast to other fatty acid that are present (Table 9). Such high and consistent results of those fatty acids could be explained by the inclusion levels of FM that stayed consistent, as well as the consistent inclusion of fish oil with little deviation from each treatment (Table 2). The significant difference with inclusion levels is focused on soya SPS and VO. The significant difference of 18:2n-6 and 18:3n-3 fatty acids match the results of other studies performed on rapeseed oil, this would explain the results according to the formulated feed and exclusion levels of rapeseed oil (Bell et al., 2003).Other fatty acids such a C20:1n-9, C20:3n-6, C22:5n-3 could be explained by presence of the FM and FO, as well as the differences between the groups of HB and CO, HB to HM and HB to LM. Earlier studies showed that those fatty acids are present in fish derived ingredients as well as in small amounts in BSF (Fawole et al., 2021). C22:1n-11 and C18:1n-7 the isomer of oleic acid are harder to explain due to them

normally not being present in BSF or MW (Xu et al., 2020), but the trend is the same as with others where they are all groups are significantly different to HB. qPCR results showed to be consistent and did meet all the parameters such as quality (figure 7), purity, and quantity. As in other studies done on some of the same genes, chosen primers were working well (Sanchez-Gurmaches et al., 2011), and the liver is a good tissue to work with due to its high concentrations of genetic material. The qPCR analysis did not show any statistically significant difference in up/down regulation of the tested genes between different feeding groups. This could be explained by low inclusion levels and longer duration in comparison to other studies (Torstensen et al., 2011). These results are also supported by the histological analysis of this study as no steatosis was detected.

5.0 Conclusion

The result does not deviate from previous studies that have been done on inclusion of insects in feed for Atlantic salmon. There have not been any significant difference changes on the liver of different inclusion levels of insect meal. This indicates that with proper substrate it is possible to replace the vegetable derived nutrients with insects without causing severe negative effects on the liver, lipid uptake and metabolism. Results from qPCR indicates that the feed does not put stress on the livers homeostasis in contrast to vegetable feed (Morais, 2011). This is promising results that could indicate that the insect ingredients are more suitable for salmon than vegetable derived ingredients. From the aspect of sustainability this could be an option for aquaculture to change its course towards this solution and stop importing ingredients such as soy. If the production of the insects would take place in Norway this would make the industry less reliable on foreign ingredients and maybe focus on production locally. This could open doors for more opportunities in local communities of Norway. There has been some controversy around the production of the pellets that prove it to be difficult to commercially produce a good quality pellet with high inclusion of insects that has not been defatted (Weththasinghe, 2022). As of the project in total there has been no indication of negative results. The growth factors seem to be the same as with usage of the control feed, only thing that has been noted by other students is the colour of the filets, at high inclusion levels of MW, where the filets become more yellow in colour.

Future studies should include higher inclusion levels as well as prolonged experimental trials to assess the long-term effects of the formulated feed. Immunology studies could also be included to further assess the effects of insect meal as an ingredient.

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7.0 Appendix

Solutions

Note that all the solutions are stock solutions and must be diluted 1:10 before use.

Buffers

10X PBS (0.1M PBS, pH 7.2):

Na 2 HPO 4 (anhydrous) ---------- 10.9 g

NaH 2 PO 4 (anhydrous) ---------- 3.2 g

NaCl ---------- 90 g

Distilled water ---------- 1000 ml

Mix to dissolve and adjust pH to 7.2 using 0.1N HCl (typically pH ≈ 6.0).

Store this solution at room temperature. Dilute 1:10 with distilled water before use and adjust pH if necessary.

10X TBS (0.5M Tris, 9% NaCl, pH 7.6):

Trizma HCl (Sigma T3253) ---------- 60,6 g

Trizma base (Sigma T1503) ---------- 13,9 g

NaCl ---------- 90 g

Distilled water ---------- 1000 ml

Mix to dissolve and adjust pH to 7.6 using 0.1N HCl (typically pH ≈ 6.0).

Citrate Buffer (0.1 M, pH 6.0)

Sodium Citrate dihydrate ---------- 24.269 g

Citric acid ---------- 3.358 g

Distilled water ---------- 800 ml

Mix to dissolve and Adjust solution to desired pH using 0.1N HCl (typically pH ≈ 6.0).

Store this solution at room temperature. Dilute 1:10 with distilled water before use and adjust pH if necessary.