Final Scientific Report - FHF Project 901435

GutMatters – Defining and improving intestinal health in farmed salmon

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Ås, Norway, September 2022

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1. Content

2. Sammendrag

Mål og strategier. Prosjektet hadde følgende mål: a) gjennomføre en feltundersøkelse (WP1) for å skaffe til veie informasjon om forekomsten av tarmhelseutfordringer og om produksjonsforhold som kan ha betydning for slike utfordringer hos laks i norske oppdrettsanlegg, b) klarlegge om forholdet mellom fiskemel og plantemel i fôret har betydning for forekomsten av tarmhelseutfordringer (WP2.1.), c) studere om funksjonelle ingredienser kan forebygge tarmproblemer (WP 2.1. og 2.3), og d) klarlegge kolins rolle for fettopphopning, steatose, i tarmens blindsekker og forhold som påvirker fiskens behov for kolin (WP2.1, 3 og 4). *Ex vivo* og *in vitro* studier ble gjennomført for å få innsikt i hvordan tarmbetennelse kan påvirke opptaket av et aktuelt pesticid og hvordan det kan påvirke funksjon i tarmceller (WP2.2).

Feltundersøkelsen (WP1) – Seks anlegg, tre tidspunkter. Prøver ble tatt og observasjoner ble gjort hos seks oppdrettere langs norskekysten, tre ganger i løpet av sjøfasen, dvs. like etter utsett om høsten, neste vår, og noen uker før slakting. Analysene av fisken omfattet makroskopiske og histologiske egenskaper, og måling av andre helsemarkører, som biokjemiske og molekylære markører relatert til fordøyelseskapasitet og immunfunksjoner langs tarmkanalen, sammensetningen i mikrobiota i tarminnholdet, og markører i blodet som indikerer fiskens helse og funksjon, basert på klassiske så vel som metabolomiske metoder. Også fôret som ble brukt i anleggene, ble grundig undersøkt for innhold av makronæringsstoffer, aminosyrer, fettsyrer, fibertyper og kolin. Dessuten ble opplysninger om fôringsregimer, miljøfaktorer og andre viktige produksjonsforhold innhentet så detaljert som mulig.

Undersøkelsen bekreftet at betennelser i baktarmen hos laks var hyppig forekommende og viste at forekomsten og alvorlighetsgraden økte med økende tid i sjøen. Steatose var også hyppig forekommende i alle oppdrettsanleggene, men forekomsten og alvorlighetsgraden var høyst variabel gjennom produksjonsfasen, med lavest forekomst i kalde perioder. Analysene av fôrets sammensetning viste at fôrprodusentene fulgte liknende strategier når det gjelder utviklingen i næringsstoffsammensetning gjennom produksjonsperioden. De viste også, basert på forskjeller i mønsteret av fiberkomponenter i fôret, at ingrediensene som ble brukt varierte mellom fôrprodusentene, og gjennom produksjonsperioden. Analysene viste stor variasjon i innhold av kolin i fôrene, men korrelasjon til forekomsten av steatose var uklar. Høyeste målte kolininnhold i fôrene var 3040 mg/kg. Noen klar sammenheng mellom fôrenes kjemiske sammensetning og tarmbetennelse ble ikke funnet.

Feltundersøkelsen omfattet også analyse av materiale fra et tidligere samarbeid mellom Mowi, Skretting og NMBU. Dette retrospektive studiet, omfattet grupper av fisk som hadde vært gitt fôr basert på kommersielle resepter. Målet med dette arbeidet var å påvise mulige betennelsesforandringer og gjøre et dypdykk ved bruk av nye histomorfologiske analysemetoder (inkl. *in situ* hybridisering) for å kunne generere ny kunnskap om inflammatoriske responser i laksetarmen. Et nytt scoringssystem for betennelse i laksetarm ble utviklet i dette arbeidet, som tok hensyn til variasjoner i respons på ulike steder i tarmveggen. Kalsifiseringer ble observert i både betente og ikke-betente tarmer, mens dislokaliserte celler kun ble funnet i betent vev. Viktigst var studiet av B-cellepopulasjoner i tarmen som påviste sterk IgD respons i forbindelse med betennelse. Slik respons kan skyldes hypersensitivitetsreaksjoner, og dette funnet åpner nye muligheter for å øke forståelsen for betennelsesprosesser i laksetarm i framtidige studier.

Virkninger av nivå av fiskemel i fôret og tilskudd av kolin og funksjonelle ingredienser på tarmfunksjon og -helse (WP2.1 og 2.3). To kontrollerte forsøk, WP2.1 og WP2.3, ble gjennomført for å øke kunnskapen om sammenhengen mellom fôrets sammensetning og tarmbetennelse og steatose, og for å finne muligheter for å forebygge slike helseutfordringer. Analysene som ble gjennomført på fisken, var de samme som de som ble brukt i feltundersøkelsen. Det første kontrollerte forsøket (WP2.1) omfattet åtte fôrblandinger med økende mengde fiskemel i fôret, fra 0 til 40 %. En konstant blanding av soyaproteinkonsentrat (42 %), hvetegluten (28 %), erteproteinkonsentrat (15 %) og solsikke (5 %) ble tatt ut av fôrblandingen i bytte for fiskemelet. To serier av disse fôrblandingene ble laget, en uten og en med en tilsetning i fôret av 0.3 % kolinklorid, 0.5 % Macrogard, og 0.5 % nukleotider, ingredienser som hevdes å kunne forebygge tarmbetennelse og steatose. Laksen i forsøket veide 186 g i snitt ved start av forsøket som varte 63 dager, og ble gjennomført i 1 m^2 kar med sjøvann (10.6 °C) med 35 fisker per kar. Ett kar ble brukt per fôr.

Resultatene viste, for fisken som fikk fôr uten funksjonelle ingredienser, at nivået av fiskemel i fôret ikke påvirket veksten eller fôrutnyttelsen, men økende innhold reduserte symptomene på steatose. Samtidig økte slakteutbyttet på grunn av redusert fettakkumulering i de indre organene. Det var ingen tegn til betennelse i baktarmen hos fisken, uavhengig av fiskemelnivå i fôret. Økt fiskemelnivå i fôret endret sammensetningen av bakterier i innholdet i baktarmen og økte antallet forskjellige bakterier.
Tilsettingen til fôret av blandingen av funksjonelle ingredienser påvirket ikke veksten hos fisken, men reduserte symptomene på steatose. Fisk som fikk fôr med 11 % fiskemel eller mer viste ingen symptomer på steatose, mest sannsynlig på grunn av økningen i innholdet av kolin i fôret. Blandingen endret uttrykket av enkelte gener som anses å være markører for akutt betennelse og stress, men ettersom det ikke ble påvist tarmbetennelse hos fisken, kan ikke den eventuelle forebyggende virkingen av blandingen på tarmbetennelsen vurderes. Blandingen ga, imidlertid, tydelig effekt på bakteriesammensetningen i tarmen og gjorde den ganske lik den hos fisken som fikk 40 % fiskemel i fôret. Resultatene indikerte et kolinbehov hos fisken i dette forsøket, på 3000 – 4000 mg/kg fôr.

I det andre kontrollerte forsøket (WP2.3) ble laks gitt to fôrblandinger som begge ble forventet å gi betennelse i baktarmen. I det ene (SBM) inngikk soyamel, i det andre (CoPea) inngikk en blanding av maisgluten- og erteproteinmel. Et referansefôr med høyt innhold av fiskemel var også med i forsøket. Virkninger av to blandinger av funksjonelle ingredienser, P1 (arginin og butyrat) og P2 (butyrat, β-glukan og nukleotider), som ble antatt å kunne forebygge betennelse ved å styrke immunfunksjoner og forbedre tarmmikrobiotaen, ble studert. Soyafôret ble testet med både P1 og P2, mens CoPea-fôret ble testet kun med P2. Forsøksbetingelsene i forsøket og analysene som ble gjennomført var svært like de i WP2.1. Hver behandling ble testet i duplikat. Resultatene viste at SBM-fôret induserte alvorlig betennelse i baktarmen, mens CoPea-fôret ga milde indikasjoner på betennelse. De to fôrblandingene ble derfor ansett som velegnet for å kunne samle informasjon om eventuelle gunstige effekter av P1 og P2 på tarmens immunfunksjon og tarmbetennelse. Resultatene viste at tilskudd av P1 til SBM fôret ikke endret de histologiske symptomene på tarmbetennelse, og heller ikke påvirket de fleste andre biomarkørene som ble målt, dvs. veksthastighet, fordøyelighet av næringsstoffer, forutnyttelse, vekten av baktarmen, proteasekapasiteten i tarmen, genekspresjon, konsentrasjonen av gallesalter og trypsinaktivitet i tarminnholdet i baktarmen, og metabolitter i plasma. Men, sammensetningen av mikrobiota viste markerte effekter. Tilsettingen gjorde

sammensetningen av mikrobiota ganske lik den i fisk som hadde fått kontrollfôr med høyt fiskemelinnhold. Som for P1, ga tilskudd av P2 ingen vesentlige effekter på de biomarkørene som ble analysert, og ga ingen effekt på forekomst av alvorlighetsgrad av tarmbetennelsen i baktarmen. Men, som for P1, ga P2 endring i sammensetningen av mikrobiota og gjorde den ganske lik sammensetningen hos fisk som fikk kontrollfôr.

Forhold som påvirker kolinbehov (WP3 og W4). I WP3 ble effekter på kolinbehov av fettnivået i fôret og vanntemperatur studert. Fôr med fire fettnivåer, fra 16 til 31 %, og vanntemperaturer på 8 og 15 ℃ ble brukt. Laksen (25 g startvekt) gikk i ferskvann i tanker med 100 fisker i hver tank. Prøvetaking og analyser ble gjennomført som i WP2.1. Resultatene når det gjelder relativ vekt (indeks) av blindsekkene, som anses som den beste indikatoren for estimering av kolinbehov, viste klar effekt av både fettnivå i fôret og vanntemperatur. Sammenholdt med tidligere studier av kolinbehov hos laks, indikerer resultatene fra WP3, at økningene i blindsekkindeks som følge av økningen i fettnivå i fôret tilsvarer en økning i kolinbehov på 1000 mg/kg fôr, og som følge av økningen i temperatur på 1500mg/kg fôr. Hvis kolinbehov til ulike funksjoner er additive, indikerer resultatene at fisk som får fôr med 31% fett, ved 15 ℃, har et kolinbehov som er 2500 mg/kg høyere enn for fisk som gis fôr med 15% fett ved 8 ℃.

I WP4 ble effekter på fiskens kolinbehov av fettsyresammensetning (rapsolje fra 0 til 23% i fôr med 35 % fett) og av fiskestørrelse (1,5 og 3,4 kg) undersøkt. Økningen i innhold av rapsolje endret i særlig grad innholdet i fôret av monoumettede fettsyrer, som gikk opp, mens innholdet av mettede fettsyrer og langkjedede flerumettede fettsyrer gikk ned. Fisken ble fôret i åtte uker i stålbur (100 fisker per bur, ett bur per fôr) i sjø ved LetSea's anlegg på Dønna. Prøvetakingen og analyser av prøvene ble gjort som i WP2.1. Resultatene som gjelder effekter av fettsyresammensetning og fiskestørrelse, ble vurdert som beskrevet for i WP3, basert på endringer i blindsekkindeks, og viste at økende innhold av rapsolje fra 0 til 23 % økte kolinbehovet, mens den store fisken hadde lavere kolinbehov enn den mindre. Effektene tilsvarte endringer i kolinbehov på omkring 600 mg/kg på grunn av økningen i innholdet av rapsolje, 800 mg/kg mellom de to fiskestørrelsene. Hvis kolinbehov er additive, indikerer resultatene at fisk på 1,5 kg som får fôr med 23 % rapsolje, trenger 1400 mg/kg mer kolin i fôret enn fisk på 3,5 kg som får fôr uten rapsolje. Imidlertid medførte økt innhold av rapsolje til økt fettfordøyelighet, og ettersom kolinbehovet i WP3 ble vist å øke med økende fettnivå i fôret (WP3), vil noe av økning i kolinbehov med økende rapsoljeinnhold trolig skyldes økingen tilførsel av fordøyelig fett, slik at anslaget for behovsendringen sannsynligvis er for høyt.

***Ex vivo* og *in vitro* undersøkelser (WP 2.2).** Disse arbeidene omfattet studier av tarmvev (*ex vivo*) og tarmceller (*in vitro*) og hadde som mål å svare på spørsmålet om hvorvidt en betennelsesreaksjon i tarmen, forårsaket av fôr som inneholder soyamel, kan øke permeabiliteten for pesticider, og hvorvidt pesticider kan forgifte tarmcellene. I *ex vivo*-studiet ble vev fra fisk som inngikk i WP2.3 studert. Chlorpyrifos (CPF) ble valgt som et relevant pesticid å studere. Resultatene indikerer at tarmbetennelsen i fisken fra WP2.3 ikke påvirket permeabiliteten for CPF. I cellestudiene ble den letale konsentrasjonen 50 % (LC50) ble målt til 89 µM CPF. Cellene viste imidlertid tegn på forstyrrelser av lipidmetabolismen ved 50 µM og brutt membranintegritet ved konsentrasjoner så lave som 13,5 µM CPF.

Praktisk betydning av resultatene.

Kolinbehov. Forsøkene i dette prosjektet som adresserte effekter på kolinbehov av variasjon i fôrsammensetning, omgivelsestemperatur og fiskestørrelse, var designet for å vise slike effekter så klart som mulig. Fôret var derfor formulert til å være svært mangelfullt for kolin og derfor ikke egnet for estimering av optimalt kolinbehov. Ettersom effektene på kolinbehov som resultatene indikerer ikke nødvendigvis er additive, vil en summering av estimatene av endring i behov kunne gi teoretiske totalbehov vesentlig høyere enn de biologiske. I feltundersøkelsen var, imidlertid, det høyeste målte kolinnivået 3040 ppm, i et anlegg der 8 av de 12 fiskene som ble undersøkt hadde alvorlig steatose, noe som indikerer at kolinbehovet hos stor fisk er godt over 3040 ppm. Arbeidet bør følges opp av et forsøk der kolinbehovet hos laks estimeres når det er på sitt høyeste, det vil si med fisk i rask vekst som gis fôr med høyt innhold av fett med høy fordøyelighet, og ved høy temperatur.

Økonomiske konsekvenser av kolinmangel. Et anslag for den økonomiske gevinsten av å gi fisken optimalt tilskudd av kolin kan baseres på resultatene i WP2.1. Forsøket i denne arbeidspakken viste at kolintilskudd til et fôr basert på planteingredienser opp til et nivå som dekker behovet, ga en reduksjon i vekten av blindsekkene tilsvarende ca 2% av kroppsvekten. Dette tilsvarer en økning i salgsverdi i størrelsesorden 10 mill. kr i året i et anlegg som produserer 10 000 tonn fisk til en salgspris av 50 kr/kg. I tillegg kommer bedringen i fôrutnyttelse som kommer som følge av redusert fettakkumulering i innvollene.

Bruk av funksjonelle ingredienser for å motvirke tarmbetennelse. Forsøkene med tilsetting av funksjonelle ingredienser for forebygging av tarmbetennelse viste ingen positive effekter hos fish med tarmbetennelse. Ettersom det foreligger lite annen tilsvarende dokumentasjon, det er behov for å styrke den vitenskapelige dokumentasjonen for helseeffekter av funksjonelle ingredienser, som basis for beslutninger om bruk av funksjonelle ingredienser i fiskefôr. Slik bruk øker fôrkostnadene og, ettersom det er energikrevende å stimulere immunforsvaret, reduserer fôrutnyttelsen.

3. Summary

Goals and strategies. The goals of the project were the following: a) through a field survey (WP1) to gather information on the occurrence of gut health challenges and on production conditions which may be of importance for the occurrence of such challenges, b) to clarify whether the ratio of fish meal to plant meal in the diet is of importance for the occurrence of the gut challenges (WP2.1), c) to find whether functional ingredients might prevent gut health challenges (WP2.1. and 2.3), and d) to identify the roles of choline supply for lipid accumulation in the pyloric ceaca and conditions which might affect choline requirement (WP2.1, WP3 and 4). Moreover, *ex vivo* and *in vitro* studies were carried out to get insight in how gut inflammation might affect uptake of a relevant pesticide and how it can affect function of gut cells.

Field survey (WP1) – Six farms, three timepoints. Samples were taken and observations made in six farms along the Norwegian coast, three times during the sea phase, in the autumn just after sea transfer, in the following spring, and some weeks before slaughter. The survey of the fish comprised macroscopic and histological characteristics, and observation of other gut health biomarkers such as biochemical and molecular indicators of digestive capacity, immune and barrier functions along the intestine, composition of the microbiota in gut content, blood biomarkers of health and function

based on classical and metabolome assays. Feed samples from the sites were characterized regarding macronutrients, amino acids, fatty acids, fibre composition, and choline level. Information on feeding regime and environmental conditions was also observed.

The survey confirmed that inflammation in the distal intestine was a frequent condition which increased in severity with time after sea transfer. Steatosis was also observed in all farms, but at variable severity throughout the production period, although lowest in the colder time periods. The information gathered on feed composition indicated that the feed producers delivering feed to the farms included in the survey followed similar strategies regarding development of nutrient composition throughout the production period. They also revealed, based on differences in pattern of fibre components in the diets, that the ingredients varied between the feed producers and throughout the production period. Moreover, the dietary content of choline, which in the other work packages was found to be an important factor for development of the steatosis, varied greatly, but not clearly with severity of the symptoms. The highest analysed level of dietary choline was 3040mg/kg. A clear relationship was not found between the chemical composition of the diets and the observed symptoms of steatosis.

This work package also included investigation of previously collected material from feed experiments in a collaboration between Mowi, Skretting and NMBU. This activity investigated groups of fish fed diets based on commercial receipts. The aim of this study was to detect possible changes with respect to inflammation and to perform a deep-dive with new histomorphological methods (including *in situ* hybridization) to reveal new traits in the inflammatory responses. In this work, a new scoring system for inflammation in the salmon gut was developed, taking into account variations in the response at different sites of the gut wall. Further, calcifications and dislocated epithelial cells and relation of these findings to the degree of inflammation were identified. Calcifications were observed in the tissues irrespective of symptoms of inflammation, whereas dislocated epithelial cells always were found only in connection with inflammation. Populations of B cells in the salmonid gut were also investigated and strong IgD responses in connection with inflammation were observed. Such responses may be attributed to hypersensitivity reactions, and this finding opens up new possibilities for our understanding of inflammatory conditions in the salmon intestine that should be pursued in future investigations.

Effects of level of fish meal in the diet and supplementation with choline and functional ingredients (WP2.1 and 2.3). Two controlled feeding experiments, WP2.1 and 2.3, were conducted, firstly to address the likely relationships between feed composition and gut inflammation and steatosis, and secondly to find possible means for remediation of these gut disorders. The same analytical tools as mentioned above for the field survey were used to characterize the fish regarding gut function and health. The first controlled experiment (WP2.1) comprised a dose-response trial with eight levels of fish meal in the diets from 0 to 40% at the expense of a constant mixture of protein concentrated meals of soybeans (42%), wheat (28%), peas (15%), corn (11%), and sunflower (5%). Two series of diets were made, one without and the other with a mixture in the diet of 0.3% choline chloride, 0.5% Macrogard and 0.5% nucleotides, ingredients which are suggested to prevent gut inflammation and steatosis. The salmon averaged 186g at start of the experiment, which was conducted in 1m^2 seawater tanks (10.6°C, 43 fish per tank) and lasted 63 days. One tank of fish was used for each treatment.

The results showed that in fish fed diets without functional ingredients, Increasing level of fish meal did not affect growth or feed conversion ratio, but clearly reduced the symptoms of steatosis. Concomitantly, the yield increased due to the reduction in lipid accumulation in the internal organs. No signs of inflammation were observed in the intestines. Increased fish meal level increased microbial richness, diversity, and composition in the digesta in the distal intestine. The supplement mix did not affect growth but eliminated all signs of steatosis in fish fed diets with fishmeal levels at 11% and above, supposedly due to the choline in the mix. The mix modulated expression of some genes which are generally regarded as markers of acute inflammation and stress. However, as no histological signs of inflammation were recorded, the preventive effect of the supplements on gut inflammation cannot be evaluated. However, the mix induced clear effects on the microbiota composition which became quite similar to that of the fish fed high fish meal diets without supplement. The results indicated a choline requirement of the fish in this study of 3000 – 4000mg/kg diet.

The second controlled experiment (WP2.3) comprised two feed receipts, both expected to induce gut inflammation. Soybean meal was used as the gut health challenger in one of the diets (SBM), and a mixture of corn gluten and pea protein concentrate in the other (CoPea). A high fish meal reference diet (Contr) was also included. Two mixes of functional ingredients suggested to improve gut health by strengthening the immune functions and improve microbiota composition was evaluated: P1 contained arginine and butyrate, P2 contained butyrate, β-glucan, and nucleotides. The SBM diet was tested with both P1 and P2, the CoPea only with P2. The conditions and analytical procedures in this experiment were very similar to those in WP2.1. The diets were tested in duplicate tanks of fish. The results showed that the SBM diet induced severe inflammation in the distal intestine, the CoPea diet a few, mild signs. Accordingly, the two diets would serve as useful models for gaining information on possible beneficial effects of P1 and P2. Addition of P1 to the SBM diet did not alter the histological signs of inflammation, neither did this mixture markedly alter other observed biomarkers considered to indicate gut health, such as growth rate, nutrient digestibility, feed conversion, gut tissue weight, and protease capacity of the intestine, gene expression, bile salt concentration and trypsin activity in the chyme from DI, or plasma metabolites. Even so, marked effects of the P1 supplementation on microbiota composition in the gut content of the distal intestine were observed, in a direction towards the microbiota composition of the fish fed the Contr feed. As for supplementation with the P1 to the SBM diet, supplementation with P2 caused no important effects on the observed biomarkers and did not reduce the prevalence or severity of the inflammation in the distal intestine. However, also for P2, marked effects, similar to those of P1, were observed on the composition of the microbiota in the content of the distal intestine. Similarly, supplementation of the CoPea diet with P2, did not alter the observed signs of inflammation in the distal intestine of these fish, but changed the composition of the microbiota which became similar to that of fish fed the Contr diet.

Conditions which affect choline requirement (WP3 and WP4). In WP3 effects on choline requirement of lipid level and water temperature were investigated. Salmon weighing 25g at start, and kept in fresh water at 8 and 15°C, were fed four diets varying in lipid level from 16 to 31%, in duplicate tanks of fish, with 100 fish per tank, for eight weeks. The sampling and analytical procedures were similar to those used in WP2.1, except that microbiota analyses were not conducted. The results for relative weight (index) of pyloric caeca, considered to be the best

biomarker for estimation of choline requirement, showed a clear, increasing effect of both lipid level and temperature. When compared to earlier studies estimating requirement of choline for prevention of steatosis, the increases corresponded to increases in dietary choline requirement of about 1000 and 1500mg/kg diet, respectively. If choline requirement for various functions are additive, the results indicate that fish fed diets with 31% fat, at 15 ℃, have a choline requirement which is 2500mg/kg higher that for fish given feed with 15% fat at 8℃.

In WP4 effects on choline requirement of varying fatty acid composition (0 – 23% rapeseed oil in diets with 35% fat) and fish size (1.5 and 3.5kg) were investigated. Increasing inclusion of rapeseed oil increased, in particular, level of monoene fatty acids and decreased level of saturated fatty acids and PUFAs in the diets. The fish were fed for eight weeks in steel cages (100 fish per cage) in sea water at LetSea's facility at Dønna. Sampling and analytical procedures were as described for WP3. The results regarding effects of fatty acid composition and fish size on choline requirement, evaluated as for WP3 based on alteration in pyloric caeca index, indicated that increasing content of rapeseed oil from 0 to 23% increased choline requirement, whereas the large fish required less choline than the smaller fish. If choline requirements are additive, the results indicate that fish weighing 1.5 kg which are fed high fat diets with 23 % rapeseed oil, need 1400mg/kg more choline in the diet than fish weighing 3.5 kg fed high fat diets without rapeseed oil. However, the increase in rapeseed level in the diet caused an increase in lipid digestibility, and, as choline requirement, according to the results of WP3, increase with lipid level in the diet, some of the indicated increase in choline requirement with increasing content of rapeseed oil, was most likely due to the concomitant increase in supply of digestible fat.

***Ex vivo* and *in vitro* studies (WP 2.2)**. WP2.2 comprised *ex vivo* and *in vitro* cell line studies with the aim to answer the question whether inflammation induced by feeding a diet with soybean meal might increase absorption of pesticides, and if pesticides may be toxic to enterocytes. For the *ex vivo* studies tissues were obtained from fish in WP2.1. Chlorpyrifos (CPF) was chosen as a relevant pesticide. Inflammation appeared not to affect intestinal uptake of CPF. For the cell studies, a cell line based on intestinal mucosal cells from rainbow trout were used. The lethal concentration 50% (LC50) were found to be 90µM CPF. However, cells displayed signs of disrupted lipid metabolism at 50µM CPF and disrupted membrane integrity at concentrations as low as 14µM CPF.

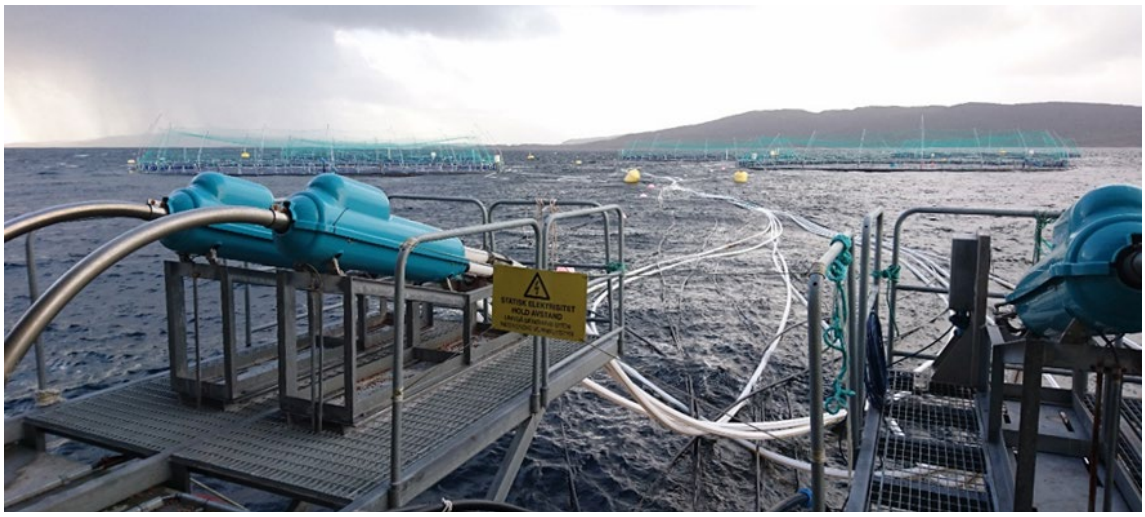
Practical implications of the results.

Choline requirement. The controlled experiments in this project addressing effects on choline requirement of variation in diet composition, environmental temperature, and fish size, were designed to reveal such effects as clear as possible. The diets were therefore designed to be severely choline deficient and did not supply information suitable for estimation of choline requirement. As the variation in choline requirement indicated in the present study, might not be additive, to sum them up, would indicate a theoretical total requirement which may be substantially higher than the biological. However, in the field survey, the feed sample showing the highest choline level, 3040mg/kg, was from a farm in which 8 out of 12 investigated fish, showed severe steatosis, indicating that choline requirement of large salmon is well above 3040mg/kg.
The work conducted in this project to clarify conditions which might affect choline requirement, should be followed up by an experiment in which choline requirement can be estimated at its

highest, which means with fish in rapid growth, given feed high in lipid of high digestibility, and at high water temperature.

Economic benefits of optimal choline supply. Excessive, abdominal lipid accumulation reduces fish yield and feed utilization, i.e., factors of great economic importance. The results of WP2.1 showed that choline supplementation of a plant-based diets reduced the weight of the pyloric intestine by about 2% of body weight, with a corresponding increase in slaughter weight. This increase in yield would increase sales value about 10 mill NOK per year in a farm producing 10 000 ton at a sales price of 50NOK/kg. In addition, the improvement in feed utilization as a result of the reduction in lipid accumulation in the belly, would also represent an economical benefit.

Use of functional ingredients for prevention of gut inflammation. The experiments with functional ingredients, evaluating possible preventive effects on gut inflammation, did not show clear, beneficial effect in fish suffering from gut inflammation. Very little similar documentation is available in the scientific literature. This situation calls for strengthening of present knowledge regarding health effects of functional ingredients, as basis for decisions regarding use of functional ingredients in feed for salmon, which are costly and most likely reduces feed efficiency of the diets as stimulation of the immune apparatus is energy demanding.



4. Introduction

Scientific background

Optimal intestinal health and digestive function are prerequisites for efficient feed utilization and for production of robust fish that will better cope with disease, stress, and farming conditions in general. However, at the time of the initiation of the GutMatters project, gut diseases were an underestimated challenge in farmed Norwegian salmon although gut inflammation and excessive lipid accumulation, steatosis, were frequently observed. Previous research suggested that the high levels of plant ingredients in the feeds, with their inherent antinutritional factors and fibres, can be important contributing factors. However, the significance of dietary contaminants, gut microbiota, nutritional and disease status, and developmental stage of the fish, as well as exogenous factors such as geographical location, other environmental conditions, and various management practices were not well understood. A more thorough understanding of the interplay between these factors was considered necessary to define causal relationships and subsequently prevent losses due to gut disease. The project aimed to generate information with relevance to these factors both at commercial fish farms and in controlled experiments, and hence to find means of improving gut health in farmed salmon.

Scope

The first part of the project was initiated in October 2017 with a program which was planned to finish in October 2020. The information gained from this program underlined and confirmed choline's role as essential nutrient in salmon diets for gut health. However, new questions raised regarding required level in the diet for efficient lipid transport at different life stages, varying lipid level in the diet, and varying environmental conditions. An application for an extension of scope and time was therefore submitted to FHF. The application was acknowledged with a deadline September 2022.

As planned the project was initiated with a field survey (WP1.1 and WP1.3) with focus on gut health, in which samples were taken of fish and feed in six farms along the Norwegian coast, at three times during the production period, i.e., shortly after sea transfer, in the middle of the growth period and close to termination of the sea water period. The samples were investigated with a wide range of analytical procedures to found the basis for understanding mechanisms underlying differences in gut health in the fish. Data on management and environmental conditions, and production parameters was also obtained. The aim was to collect as much information as possible to get an overview of the situation in Norwegian salmon production and increasing the chance to identify explanations for the development of gut health challenges. In parallel, a retrospective study was conducted investigating samples collected in a newly terminated study which was considered would be useful for the present project, and which could add useful information to the GutMatters project. In parallel to WP1, two controlled experiments were carried out (WP2.1 and 2.3) in order to strengthen understanding of the role of plant ingredients in the diets on the observed gut health challenges. WP2.1 comprised an experiment in which the importance of the balance between plant ingredients and fish meal in the diet were investigated in a dose-response trial with eight levels of plant protein to fish meal ratios. Possible modulating effects of current functional ingredients, i.e., nucleotides, β-glucan, and choline, were also included as main goals in the study. In WP2.3, effects of diets which were known or suspected to challenge the fish gut, were studied, i.e., soybean, pea, and corn gluten meals. Also in

this experiment, possible beneficial effects of functional ingredients were addressed, i.e., arginine, butyrate, and nucleotides. An in vitro study (WP2.2) was also included in this WP, to find whether compounds which might have polluted some of the plant ingredients present in the diets fed to the fish observed in the field trial, might affect the physiology and health of the gut.

The two WPs in the follow-up step of the project specifically addressed factors which might affect choline requirement. Choline's role in lipid transport is crucial. It is therefore likely that choline requirement depends on conditions such as environmental temperature, lipid level in the diet, lipid quality, and fish size. The two former sources of variation were addressed in WP3, the latter in WP4.

Project organization

The project has been anchored in the Nutrition and Health research unit (VM-Nutr) at the Department of Paraclinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences (NMBU), with professor Åshild Krogdahl as the leader, responsible for administration and coordination of the research activities. The project consortium comprised researchers from two research groups from NMBU, i.e., the units VM-Nutr and the Defense mechanisms and genetics (VM-Defense), and researchers from Aquamedic AS, NOFIMA AS, Institute of Marine Research (NIFES/IMR), and University of Copenhagen (UCPH). In the additional part of the project, one researcher from LetSea AS was also involved. The reference group comprised representatives from feed industry providing assistance regarding feed formulation and production, i.e., Skretting AS, Biomar AS, Cargill Inc., MOWI Feed Norway, and from the salmon producers, i.e., Lingalaks AS, Salmar AS, MOWI AS, Midtnorsk havbruk, and Cermaq AS, providing access to fish farms for the field survey.

Regular meetings have allowed good interdisciplinary communication and discussions between the scientific organizations involved and the feed and salmon production industries represented in the reference group.

Key researchers

The following researchers have been actively involved the project:

Professor Åshild Krogdahl, NMBU, VM-Nutr, project leader
Associate professor Trond Moxness Kortner, NMBU, VM-Nutr
Researcher Anusha Krishanthi Shyama Dhanasiri, NMBU, VM-Nutr
Post doc. Alexander Jaramillo-Torres, NMBU, VM-Nutr
PhD student Daphne Siciliani, NMBU, VM-Nutr
Professor Erling Olaf Koppang, NMBU, VM-Defense
Researcher Håvard Bjørgen, NMBU, VM-Defense
Chief Executive Officer Paul Midtlyng, Aquamedic AS
Senior researcher Elvis Chikwati, Aquamedic AS
Senior scientist Gerd Berge, NOFIMA AS
Senior scientist Aleksei Krasnov, NOFIMA AS
Senior scientist Svein Halvor Knutsen, NOFIMA AS
Researcher Øystein Sæle, NIFES/IMR
Professor Søren Balling Engelsen, UCPH

Associate professor Violetta Aru, UCPH
Associate professor Bekzod Khakimov, UCPH
Section leader, Henriette Hanssen, LetSea AS

Reference group (as of September 2022)

R&D Director Charles McGurk, Skretting AS
Senior scientist Anne Kristine Hansen, Biomar AS
Technical Application Lead, Synne Andersen, Chargill Inc.
Product Development Manager Guido Riesen, MOWI Norway
Biological coordinator, Anita Stevnebø, Lingalaks AS
Head of Feed and Fish Health at Cermaq Global Olai Einen, Cermaq AS
Feed and analyses lead, Arne Guttvik, Salmar AS

Challenges and result goals

Importance for the industry

Fish health and welfare were, and still are, among the issues in focus of the aquaculture industry as well as the general population. Research, which can help improve health and prevent diseases, will be beneficial for the industry and the society. In parallel with the great changes which have occurred during the last three decades regarding feed composition of the Atlantic salmon, with high substitution of marine ingredients with plant ingredients, reports of gut health problems have increased. However, until the initiation of the GutMatters project, no systematic collection of information on gut health had been conducted which could indicate occurrence, type and whether diet and other environmental conditions played a role in development of these health problems. Hence, the possibilities to take measures to counteract such problems, were limited. The GutMatters project was planned to give valuable information on the gut health status of salmon grown under commercial conditions in Norway at three time points over a period of one year. Together with observations and analyses regarding the management, environment, diet, production, health and function of the fish, the results were expected to be of importance for further development of salmon diets, management, and hence for salmon welfare, production, and the overall economy in the salmon industry.

Result goals

The overall goal of the GutMatters project has been to improve gut health in Atlantic salmon by:

1) *Establishing empirical knowledge on the current status of gut health in sea-farmed salmon*
2) *Assess the contribution of feeds and environmental and management factors to observed pathologies*
3) *Explore interactions between feed constituents and gut health*
4) *Explore avenues of remediation of gut disorders*

The research goals are explained in the following hypotheses:

1) *Interactions between endogenous and exogenous factors influence the manifestation and severity of gut disorders*
2) *Specific tissue responses can aid in diagnosing and discovering the causes of gut disorders*
3) *Biomarkers are present in faeces and blood and can be used as non-invasive diagnostic tools*
4) *Gut pathologies can be prevented*

5. Project design

The project comprised:

- ✓ A field survey to identify gut health problems encountered by the industry, visiting and sampling three times during the sea phase at six commercial production sites, along the Norwegian coast, from Hordaland in the south to Finnmark in the north. Thorough characterization of the diets offered to the fish, and details of the environmental conditions encountered by the fish were recorded to find possible relationships to the gut health.
- ✓ A retrospective study, utilizing samples from an earlier field study, for further investigations.
- ✓ Four controlled studies investigating how certain plant feedstuffs might challenge the intestine of salmon and the possibility of preventing gut disorders by dietary supplements
- ✓ In vitro studies employing intestinal tissues and cell cultures for generation of information on possible effects of contaminants.

6. Work packages, methodology, results, discussion, and conclusions

The project was structured with four main work packages (WP) as listed below:

WP1: Salmon Gut Health Survey 2017-2018 (responsible partners *Aquamedic* and *VM-Nutr*)

WP1.1 Prospective field survey (*Aquamedic*)

WP1.2 Retrospective survey (*VM-Defense*)

WP1.3 Sample analyses and data mining

Task 1: Gut histomorphology (*Aquamedic*)

Task 2: Gut immune status (*NOFIMA Ås*)

Task 3: Gut barrier function (*VM-Defense*)

Task 4: Gut digestive function (*VM-Nutr*)

Task 5: Gut microbiota (*VM-Nutr*)

Task 6: Metabolic profiling of faeces and blood (*UCPH*)

Task 7: Feed characterization (*VM-Nutr*)

Task 8: Multivariate data analysis (*UCPH*)

WP2: Controlled experiments (VM-Nutr)

WP2.1 Feeding trial I – Combination effects of legume level and functional ingredients (*VM-Nutr + NOFIMA*)

WP2.2 Ex vivo investigations (*NIFES*).

WP2.3 Controlled trial II – Remediation of gut inflammation (*VM-Nutr + NOFIMA*)

WP3: Effects of dietary lipid level and environmental temperature on the severity of steatosis in fish fed suboptimal level of choline (NOFIMA, VM Nutr, Aquamedic)

WP 4: Effects of fish size and lipid quality on the severity of steatosis in fish fed suboptimal level of choline (VM-Nutr, LetSea, Aquamedic)

WP1. Salmon Gut Health Survey 2017-2018

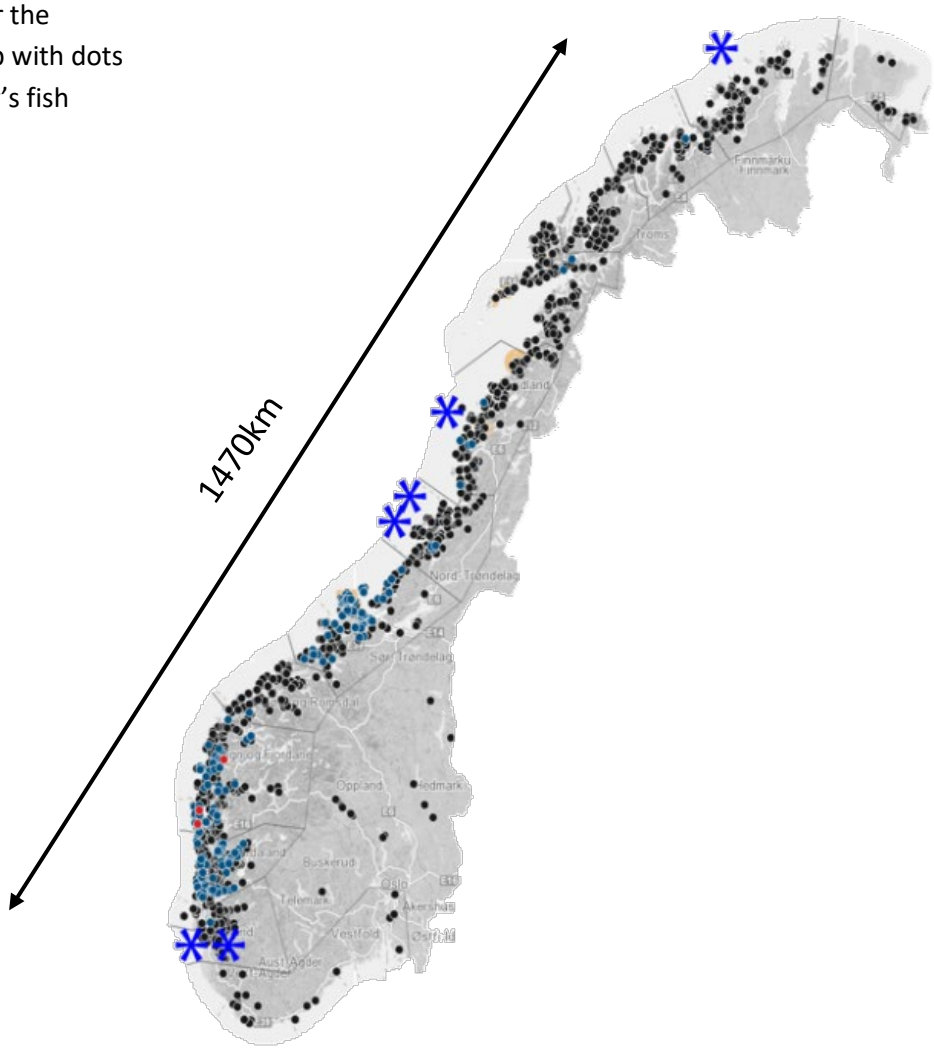
WP1.1 and 1.3 Prospective field survey

Responsible partner: Aquamedic AS

Background in brief

The prospective field survey (WP1) was conducted from early October 2017 to late November 2018. Sea farmed Atlantic salmon from two commercial sites in each of three major coastal farming regions (southwestern-, mid-, and northern Norway) were sampled. Sites belonging to reference group members from the farming industry, allowing return transport to the coordinator laboratory in Oslo within 12 hours of completing the sampling were selected for participation in the study: sites 1 and 2 in the south-west; site 3 and 4 two on the mid-Norway coast and sites 5 (inside) and 6 (near) the Arctic region (Figure 1.1).

Figure 1.1: Location of the six marine farming sites enrolled for the project on a map with dots showing Norway's fish farming sites



Sampling methodology

In each of the participant sites, one net pen population deemed to be representative of the stocked groups was selected for the repeated samplings. The initial samplings were carried out 4-6 weeks post sea transfer, then 8-10 months into the final rearing, and finally 1-6 months prior to planned harvest, respectively. A minimum of 12, and a maximum of 20 individual Atlantic salmon (depending on weather and logistical limitations) were sampled at least 1 hour after the daily feeding had commenced, to ensure the sampled fish would have luminal content in the intestine.

Blood was collected from the caudal vein into heparin and EDTA anticoagulant vials and samples were spun down without delay. Plasma, tissue, and luminal contents of the pyloric and distal intestine for microbiota analysis were snap-frozen in liquid nitrogen. The pyloric ceca, mid and distal intestine, liver, heart, head kidney and spleen were all sampled into 10% neutral buffered formalin for fixation. Samples for gene expression analysis from the same tissues and organs were stored in RNAlater ®. The remaining intestinal tissue of the pyloric caeca, mid-, and distal region were separately weighed and stored in pre-tared vials before snap-freezing in liquid nitrogen and storage at -80 °C for digestive enzyme activity analyses. Intestinal content from the pyloric, mid, and distal intestine, that remained after the microbiota sample collection were separately sampled, snap frozen in liquid nitrogen, awaiting further analyses of bile salt content, pancreatic enzyme activities, as well as metabolomic assays. Upon return to the coordinator laboratory, all snap frozen samples were transferred to -80°C freezer storage.

At each sampling site visit, a 1-kg sample of the feed currently being fed to the sampled pen were collected by farm personnel directly from the feed silo. The feed sample was stored frozen at -20 °C awaiting further analysis. Stock and environmental data, and details of the sampled fish population were collected from the farm management software at each site visit, and after harvest of the population.

Fish population, production- and environmental data

The seawater temperature on the participant sites showed clear seasonal pattern. The lowest weekly averages were from 1.5 to 4.5℃ in winter, while the highest weekly averages were around 18℃ in both southern locations, but only 12-14.5℃ in both northern locations.
The average weight of the fish sampled few weeks after sea transfer varied between 128 and 772 g, the former having been transferred end of September to the northernmost site, and the latter having been transferred as 269 g, already seawater adapted post-smolts by mid-September.

The growth of the included populations, calculated after incorporation of harvest data were from 0.6 to 0.8% expressed as specific daily growth rate (SGR), and from 2.4 to 4.0 expressed as Thermal Growth Coefficient (TGC).

Except for 20 out of 308 fish (6,5%) showing some reddish discoloration, the gastric mucosa of the sampled fish was without any unhealthy signs. Discoloration of the pyloric caeca and of the mid-intestinal mucosa was seen in 69 out of 308 (22.4%) and 31 fish (10.1%), respectively. The distal intestinal mucosa showed visible reddish discoloration in 13 out of 308 sampled fish (4,2%). Sites 1 and 2 were infested with tapeworm at the second and third sampling, while no tapeworm was found in any of the sampling in the other sites.

Task 1. Gut histomorphology

Task methodology

From each sampling event, pyloric caeca, mid-, and distal intestine, and liver tissue from 12 individual fish were selected for histology evaluation. The assessment was performed using a standardized protocol employed at Aquamedic AS, adapted from scoring systems described by Bæverfjord and Krogdahl (1996) and Krogdahl et al. (2003) using classical light microscopy of haematoxylin-eosin (HE) stained sections. Enteritis (inflammation of the mucosa) and enterocyte vacuolization indicating lipid accumulation were scored using an ordinal scale from 0 (normal; no changes) to 4 (severe changes).

Results

In the first sampling, inflammatory changes of the distal intestine were only seen in samples from site1. In the second sampling, an increasing trend was observed with mild to moderate changes in less than half of the fishes from each site. A further increase in inflammatory scores were seen in the final sampling from site 3 and 4, and particularly in site 2 where all sampled intestines showed some degree on inflammatory change (Figure 1.2).

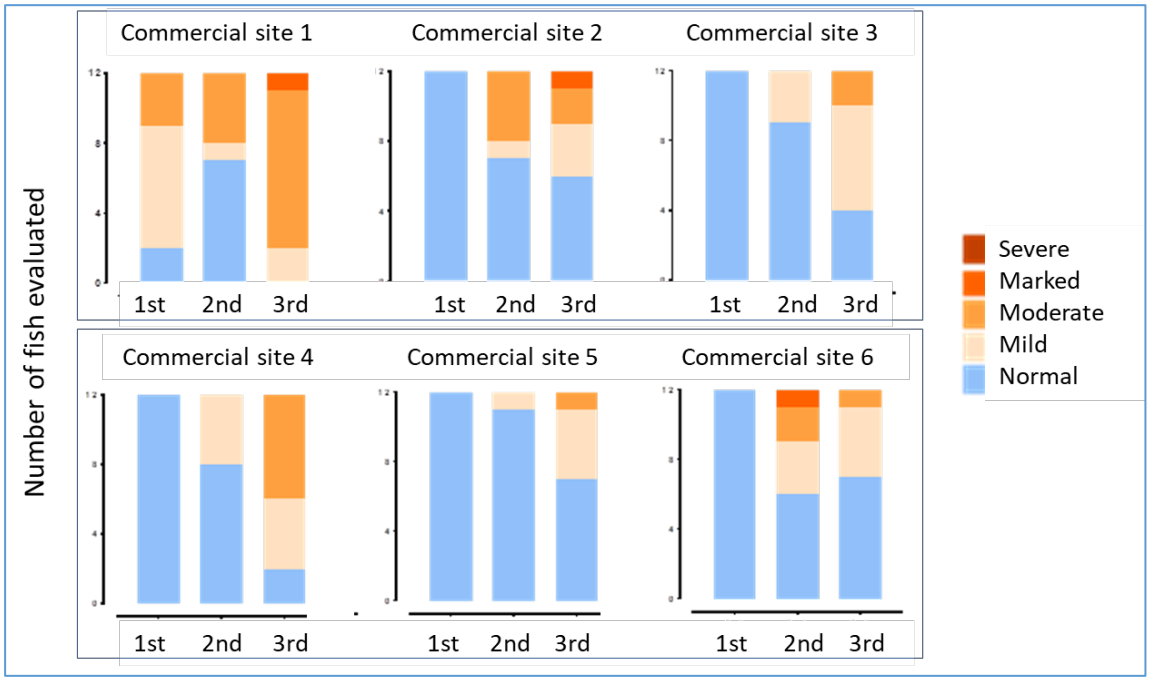


Figure 1.2: Degree of submucosal infiltration of mononuclear cells in the distal intestine of Atlantic salmon during 3 sampling events.

In the first sampling, all of the groups showed lipid vacuolisation of the pyloric mucosa; albeit highly variale between the sampled populations. Except for sites 2 and 4, from one to 8 fishes (out of 12) displayed marked or severe tissue changes. With the exception of site 5, the situation was largely normalized at the second sampling, while increasing again in the 3rd sampling, particlularly in the samples from sites 2 and 3 (Figure 1.3).

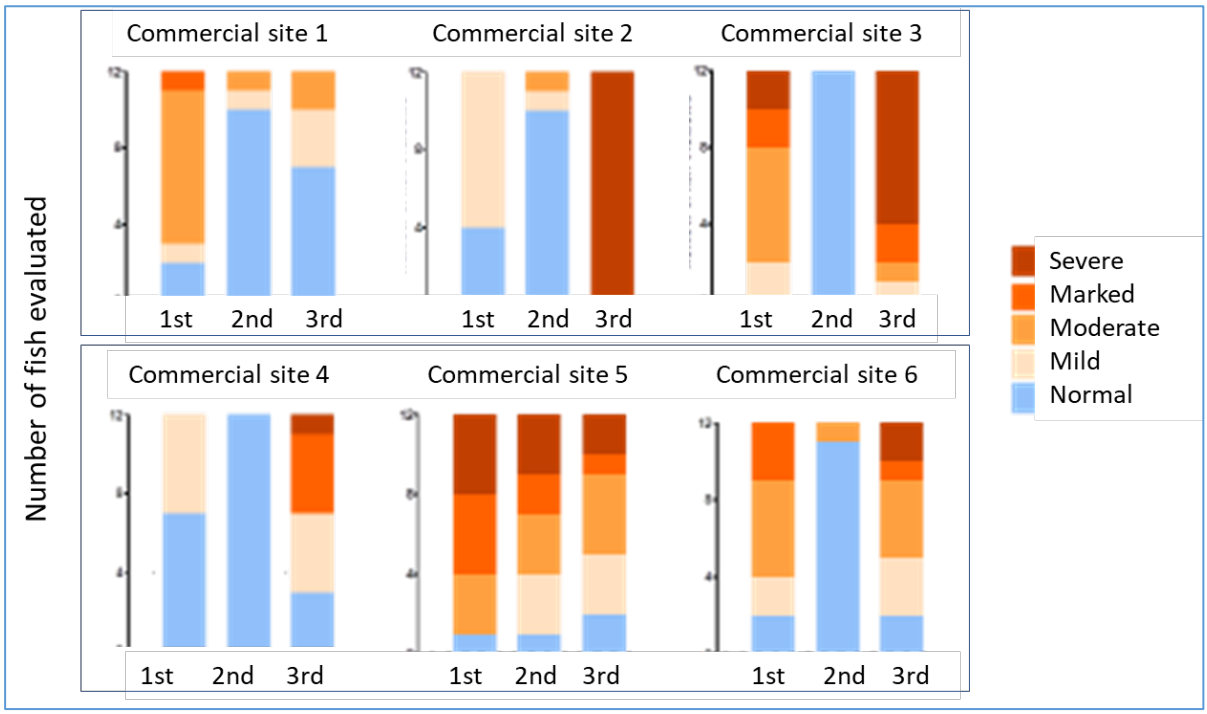


Figure 1.3: Prevalence and degree of lipid vacuolisation of the mucosa in pyloric caeca

Task 2. Gut immune status

Task methodology

Microarray analyses were performed using 15k microarray SIQ-6 at three time-points (n = 8 per site and time-point)

Results

The analyses found both common and site-specific expression changes. Comparison between samplings/time-points in combined data from all six sites found 322 differentially expressed genes (DEG). A search was conducted for functional groups (STARS annotation) with co-regulated genes (significant difference of mean $\log_2$- expression ratios from zero) and enriched terms of Gene Ontology – GO (Table 4.1). Both approaches suggested inflammation and structural changes at different levels as the main trends.

A hallmark of the inflammation was activation of chemokine signalling, antigen presentation and recruitment of immune cells (chemotaxis), while metalloproteinases (highly destructive inflammatory effectors) were down-regulated. Stimulation of innate antiviral immunity was observed mainly at second sampling. Intracellular (cytoskeleton and myofiber) and extracellular (collagens) proteins were downregulated together with genes involved in developmental and regeneration processes. Here, the results suggested preferential recruitment of lymphocytes.

Table 4.1: Co-ordinated temporal expression changes in the distal intestine of Atlantic salmon. Significant differences from the first time-point (t test) are indicated with underlined bold italics.

Functional group	DEG	E2-E1	E3-E1
Antigen presentation	9	0.63	0.84
Chemokines	12	0.31	0.71
Innate antiviral responses	54	1.16	0.16
Matrix metalloproteinases	5	-0.91	-0.89
Xenobiotic metabolism	5	-0.23	-0.85
Cytoskeleton	14	-0.57	-1.07
Myofiber proteins	9	-0.63	-0.99
Extracellular matrix (ECM)	10	-0.44	-0.91
Collagens	9	-0.66	-1.07
Regulators of differentiation	8	-0.27	-0.71
Growth factors	5	-0.61	-1.1

The expression changes in the field study showed results much in common with the transcription signature of enteritis induced with soy saponins and soybean meal. Many more genes showed site-specific changes between the time-points: the numbers of genes with differences between last and first samplings ranged from 315 at site S1 to 946 at site S6, totally 2716 DEG including 557 immune genes. Overall, expression of immune genes was lowest at sites 5 and 6, apparently in concordance with histoscores of inflammation.

Task 3. Gut barrier function

Task methodology

A representative number of samples showing different inflammatory scores, originating from the field material and from a previous experiment were subjected to analysis as described below. After microscopical assessment of HE stained sections, tissues were selected for follow-up using histological markers for structural changes in epithelial cells (cytokeratin), immune cell markers for T cells and B cells, and PAS stain to assess the quantity of mucin-producing cells. In addition, in situ hybridization for bacteria was performed (Løken, O.M. et al. 2020).

Results

Disruption of the basal membrane was not encountered in any of the investigated samples. In inflamed intestine, dislocated epithelial cells were occasionally observed beneath the basal membrane. This may indicate an increased permeability of this membrane. Detection of T cells and B cells in the inflamed tissue showed increased amounts particularly of IgD producing cells. This finding may indicate a hypersensitivity reaction in the intestine, previously not described with respect to intestinal inflammation in the teleost gastrointestinal tract. Importantly, no bacteria or any other antigens were detected beneath the basal membrane. Thus, the gut barrier seems intact in all samples examined. The findings from samples obtained in the prospective survey are similar to those as reported for the retrospective study of this project (WP1.2).

Task 4. Gut digestive function

Task methodology

Samples of gut content were analyzed regarding bile salt concentration and trypsin acitivity, and of gut tissues regarding activity of leucine aminopeptidase (LAP) in the mid (MI) and distal intestine (DI) as described in Wang et al. (2020).

Results

Our results regarding gut function, shown in Figure 1.4, revealed significant differences between sampling sites and sampling events. Moreover, there were significant interactions between site and event indicating that the effect of sampling event differed between the sites. The statistical significances for almost all of these indicators of gut function, for effect of site, event, and interaction, were high ($p<0.0001$). The intestinal functions are dynamically adapted to changes in diet composition and feed intake. Most of the observed effects of site and sampling event, may reflect such variations between the sites and sampling events. Multivariate analyses of the results from this survey, will indicate to what extent such variation can explain these differences. However, the increase in trypsin activity of the chyme and the decrease in LAP in DI with time, observed for most of the sampling sites, may reflect the increase in gut inflammation with time, in accordance with earlier studies of effects of gut inflammation on gut function in salmon (Krogdahl et al. 2003).

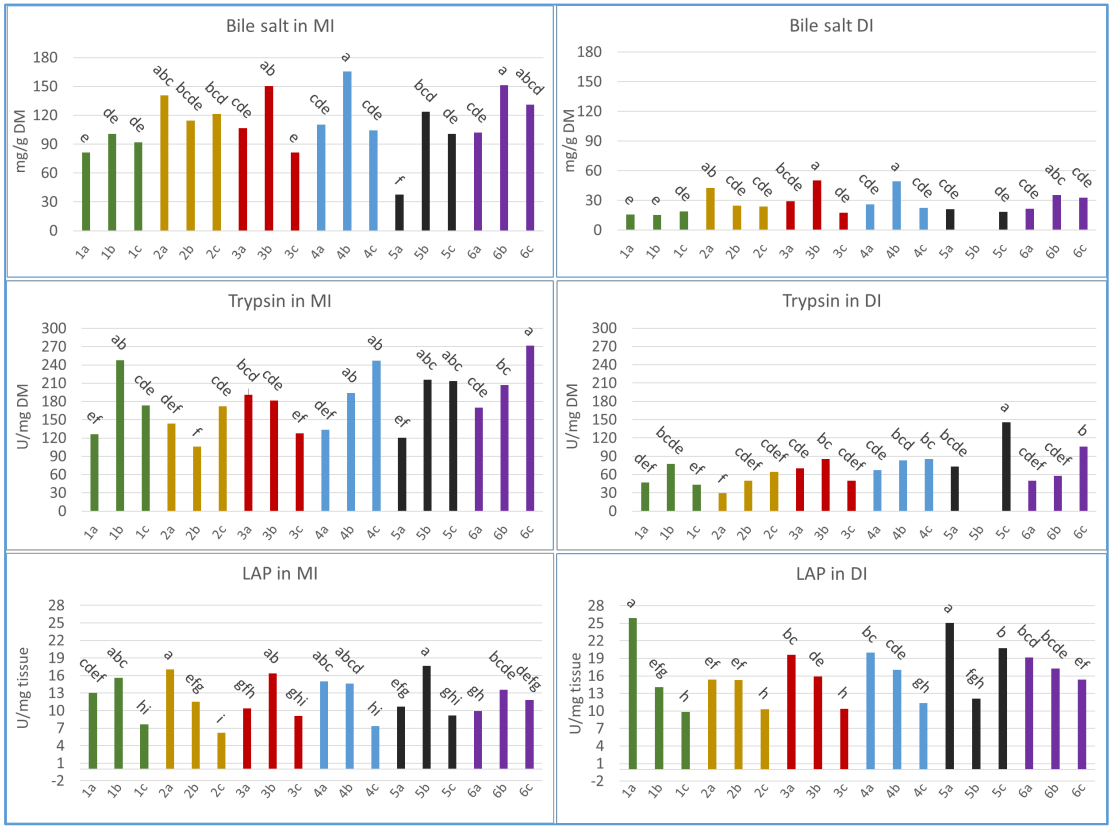


Figure 1.4. Indicators of gut function, i.e. concentration of bile salt and trypsin activity in content and activity of leucine aminopeptidase (LAP) in tissue from mid (MI) and distal intestine (DI) of fish sampled at the six sampling sites (1-6), three times (a-c) during the production period in the sea.

Task 5. Gut microbiota

Task methodology

Samples of gut content from the distal intestine (6 replicates, per site and sampling occasion); a total of 108 samples were analyzed and statistically processed as described in (Wang et al. 2021).

Results

The qPCR assay to indirectly quantify total bacterial DNA in the digesta samples indicated that there were significant differences in the total bacterial DNA between the time points of sampling. The Bacterial DNA was significantly higher in the second and third sampling point (b and c) compared to the first sampling point (a) (Figure 1.5).

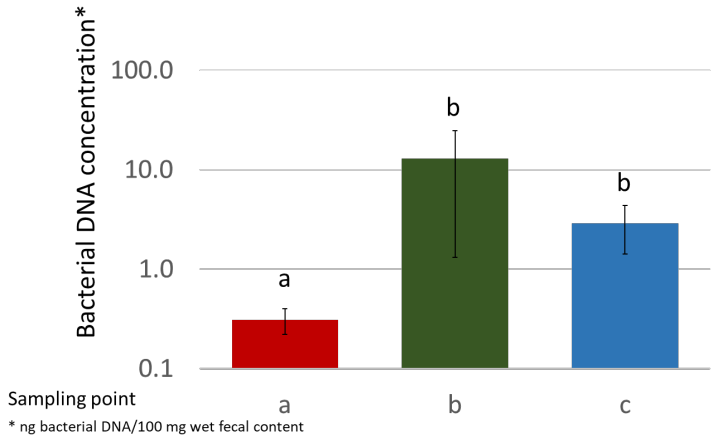


Figure 1.5. A bar plot graph showing bacterial DNA concentration (ng bacterial DNA per 100 mg of wet faecal content) by sampling point (n=6). Error bars indicate standard deviation.

The beta diversity (bacterial community structure) between sampling points and sites were driven by the sampling point. Samples from the first sampling point cluster together and were separated from the other two sampling points while there was no clear separation but an overlapping of samples between the second and third sampling points (Figure 1.6).

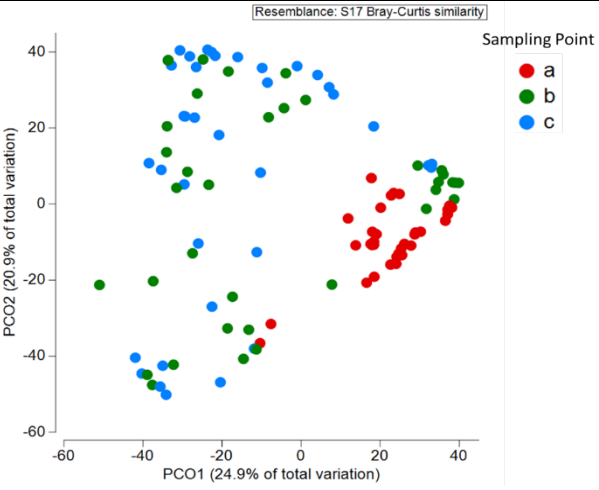


Figure 1.6. PCoA plots showing bacterial communities' structure (Beta diversity) in fish during the three sampling points (n=36).

Task 6. Metabolites of blood and faeces

Task methodology

Plasma metabolites were observed employing classical methods as well as metabolic profiling. The former, conducted at the central laboratory at NMBU, observed plasma glucose, cholesterol, triglycerides, free fatty acids, aspartate amino transferase (ASAT) and alanine amino transferase (ALAT) by standard methods. For the latter, profiled metabolites in plasma as well as faeces from the proximal (PI) and distal intestine (DI) by use of proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. ^{1}H NMR spectroscopy is an untargeted, unbiased, relative fast and non-destructive analytical technique, which gives an overview of the major chemical constituents (NMR LOD ~ 0.05mM) of the sample under investigation. A total of 296 individual plasma samples were measured by ^{1}H NMR. A total of 512 samples were measured while 111 samples (due to weight) were not sufficient for metabolite extraction and analysis. Plasma preparation for metabolomics analysis was performed as described by Aru et al. (2021) while sample preparation for the ^{1}H NMR analysis of faecal samples was performed as described by Cui et al. (2020). Further details on sample measurements can be found in Aru et al. (2021) and Cui et al. (2020).

Results

For all the observed plasma biomarkers observed by classical method, i.e., glucose, cholesterol, triglycerides, free fatty acids, aspartate amino transferase (ASAT) and alanine amino transferase (ALAT), the analyses showed statistically significant ($p<0.0001$) differences between sampling sites and sampling events, as well as significant interaction (Figure 1.4.1).

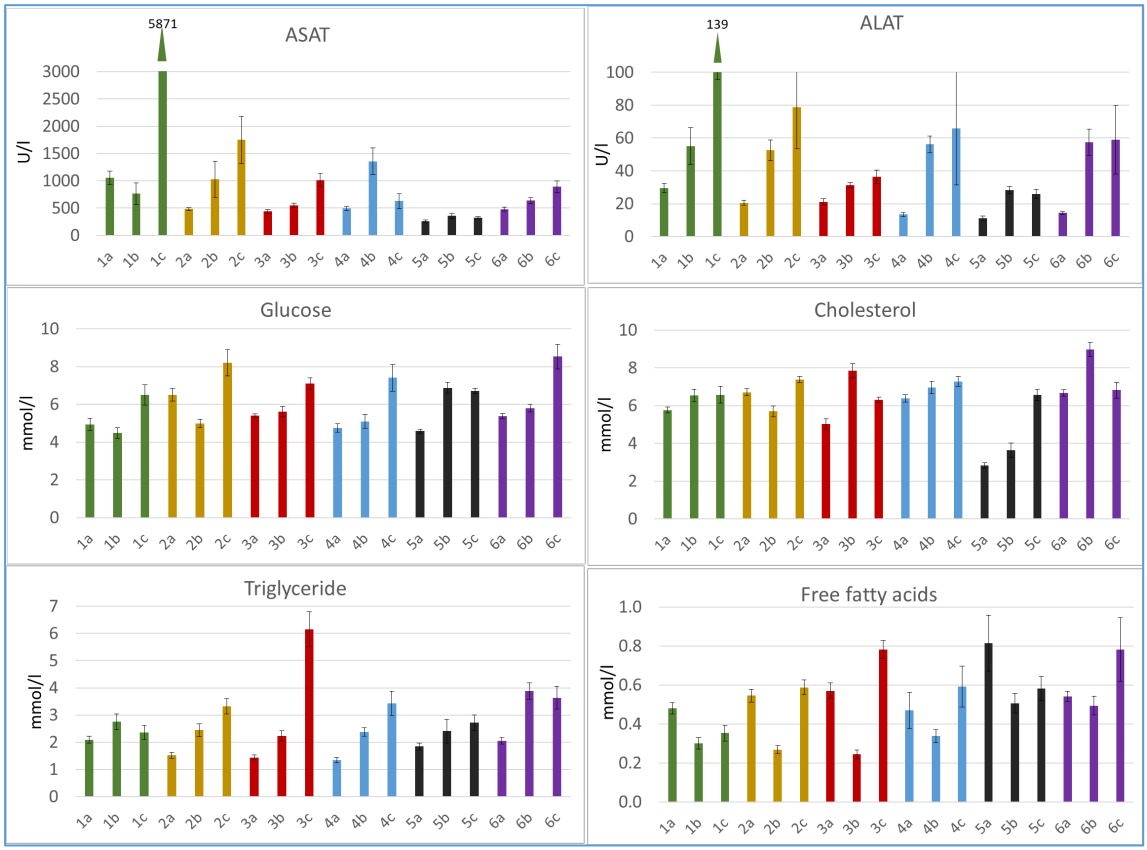


Figure 1.7. Results of classical, biochemical analyses of plasma samples from the fish, indicating nutrient supply and metabolism.

All these biomarkers, except free fatty acids increased from the first to the last sampling timepoint, in general reflecting the change in diet composition, i.e., increase in lipid and carbohydrate level. The results for free fatty acids indicated a lower value for the samples taken at the second sampling event, at which the results varied between 0.2 and 0.5 mmol/l, supposedly reflecting a lower water temperature and feed intake preceding the spring samplings. The plasma levels of alanine transaminase activity (ALT), an indicator of reduced liver function, showed increasing trend from the first to the last sampling timepoint. The values varied between 11 and 21 U/l at the first sampling time, and between 26 and 130 U/l at the last, seeming to indicate site differences with decreasing values towards the north.

The metabolome assay overall, identified 60 signals from metabolites and functional groups from lipids in lipoproteins in the ^{1}H NMR spectra of salmon plasma. In particular, the spectral landscape is dominated by the strong resonances stemming from lipids in lipoproteins (Figure 1.8.a and 1.8.b).

An overview of the metabolites identified in the ^{1}H NMR spectra of plasma and faecal PI and DI has been produced. Metabolite identification in complex spectral regions is ongoing – Exploratory metabolomics analysis is performed including SS (signature signals), SUS (signal of yet unknown spin systems) and unresolvable complex (BINS) regions.

Metabolic profiling of the faecal content provides an informative chemical overview of the faecal metabolome from both PI and DI, and it is suitable for assessing alterations in the digestive process occurring in the two different segments of the intestine. The delta metabolome was used to relate the fish metabolome to the histological profile of salmon gut. PI and DI represent different anatomical parts of the salmon intestine, hosting different steps of the digestive processes. The metabolomics analysis of the faecal content from PI and DI (delta metabolome) can help elucidating the biochemical alterations behind lipid malabsorption and inflammation.

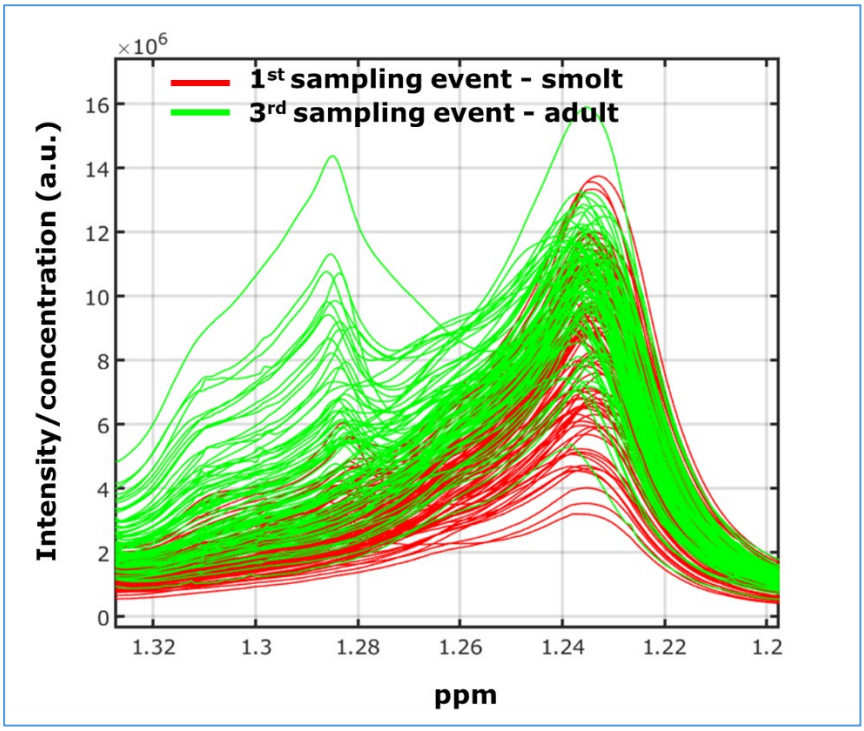


Figure 1.8.a. Expansion of methylene region of the ^{1}H NMR spectra of salmon plasma. Spectra are coloured according to the sampling event. The ^{1}H NMR signals from VLDL, HDL, and LDL are highlighted.

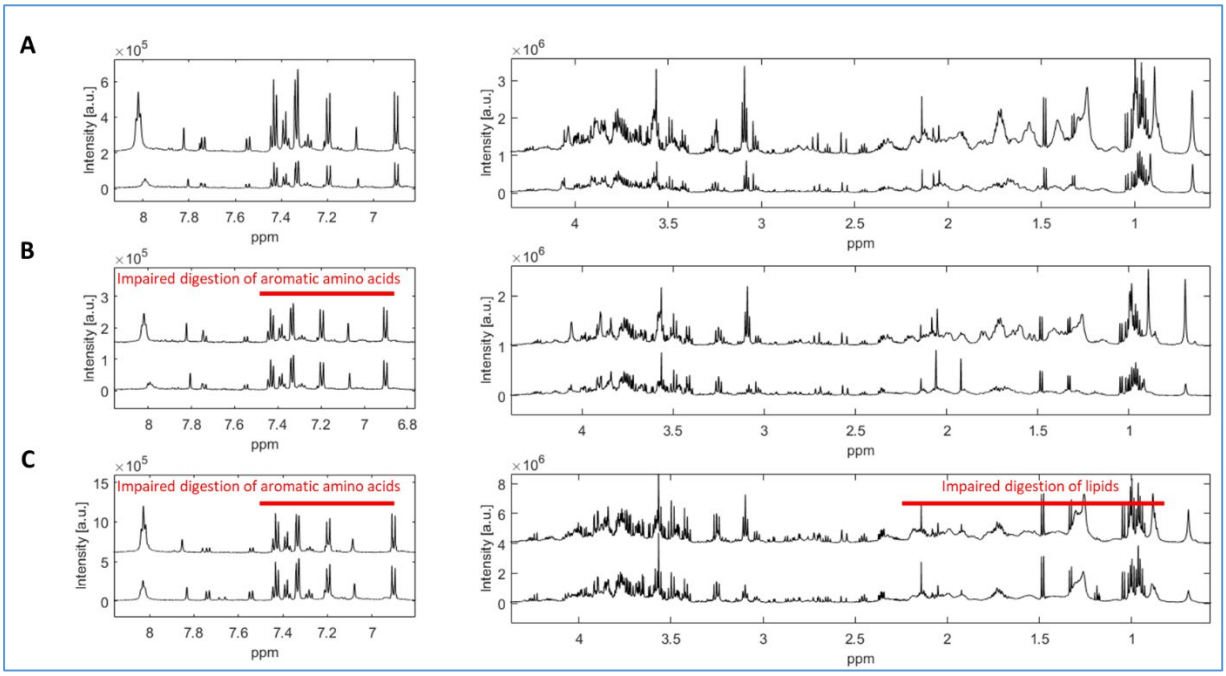


Figure 1.8.b. Representative ^{1}H NMR spectra of PI (top spectrum) and DI from three individual fishes with different histological profile. A: normal score. B: impaired digestion of aromatic amino acid tyrosine and phenylalanine. C: impaired digestion of lipids and aromatic amino acids. Spectra are normalized using a reference signal (ERETIC) before comparison.

Task 7. Feed characterization

Methodology

Nutritional analyses

The proximate composition of the feed samples was analyzed at LabTek, Norwegian University of Life Sciences, Ås, Norway, following international standard procedures. Choline content was analyzed by Eurofins according to the standard method as described in AOAC Official Method 2015.10. Contents of non-starch polysaccharides (NSP) and low-molecular saccharides were determined at NOFIMA's laboratory under the supervision of senior researcher Dr. Svein Halvor Knutsen. The procedures for NSP are based on methodologies described by Englyst et al. (1994). The chromatographic system was analyzed with HPAEC-PAD following a method adapted from Helgerud et al. (2016).

Toxicological analyses

Polycyclic aromatic hydrocarbons (PAHs), mycotoxins and pesticides analyses were performed at the Research Institute for Pesticides and Water, University Jaume I, 12071 Castellón, Spain. PAHs were analyzed by gas chromatography/tandem mass spectrometry (GC-MS/MS) with atmospheric pressure chemical ionization as described by Portolés et al. (2017). Mycotoxins were analyzed with Liquid chromatography/tandem mass spectrometer (LC-MS/MS) according to Beltrán et al., (2013). The pesticide analyses were done by chromatography coupled to triple quadrupole mass spectrometry (GC-APCI-MS/MS) and ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) according to Grimalt et al., (2010).

Results

Nutritional analyses

The general picture showed similar strategies among the feed companies and farmers regarding macronutrient composition of the diets throughout the production period, with decreasing protein level and increasing lipid level with increasing fish size. In the early stage after sea transfer, the protein level varied between 480 and 433 g/kg, at the latest sampling timepoint between 323 and 270 g/kg. For lipid, the level at first sampling varied between 230 and 258 g/kg, at the last sampling between 371 and 433 g/kg. There was no clear trend in the levels of starch between the samplings. Estimated digestible protein to digestible energy ratio varied between 18.5 and 20.8 g/MJ at the first sampling and decreased to levels between 9.8 and 12.3 g/MJ at the last sampling.
The results regarding amino acid composition of the diets showed that the levels of lysine, methionine + cystine and threonine varied around the required levels, without clear trends between samplings. For histidine however, the picture showed decreasing levels from the first to the last sampling, initially above and finally around the estimated requirement. Sum of essential amino acids, exclusive of tryptophan, in the protein was fairly stable throughout the production period, averaging 43%, varying between 41 and 46%. The level of long chain polyunsaturated fatty acids, i.e., C20:5 ω3 (EPA) and C22:6 ω3 DHA, did not vary systematically over the observation time. well above the requirement indicated by NRC, i.e., 10 g/kg (NRC, 2011). The content in the diets of C18:3 ω3, increased throughout the observation time, reflecting the increase in content of plant oil in the diets.

Choline level, both free and bound, varied greatly between sites and sampling timepoints, seemingly without any clear trend among the sites, nor with developmental stage of the fish. Total choline level varied between 1230 and 3040 mg/kg (Figure 1.9). Choline's essentiality was documented recently, preliminarily suggesting a requirement of at least 3400 mg/kg (Hansen et al. 2020a).

The feeds' contents of non-starch carbohydrates (soluble or insoluble) in the diets showed no clear trends regarding life stage of the fish or between sites. The levels of total non-starch polysaccharides (NSP) varied between 5 and 9 expressed as % of fat free diet. The soluble fraction of the NSP comprised about ¼ of the total NSP. Free glucose and fructose were low in all diets, while sucrose, raffinose, stachyose and verbascose, mostly stemming from bean products, were found in all feed samples, but in variable amounts. The sum of low-molecular carbohydrates did not show any clear trend with developmental stage of the salmon, nor with site, varying between 1.1 and 3.5% of fat free diet.

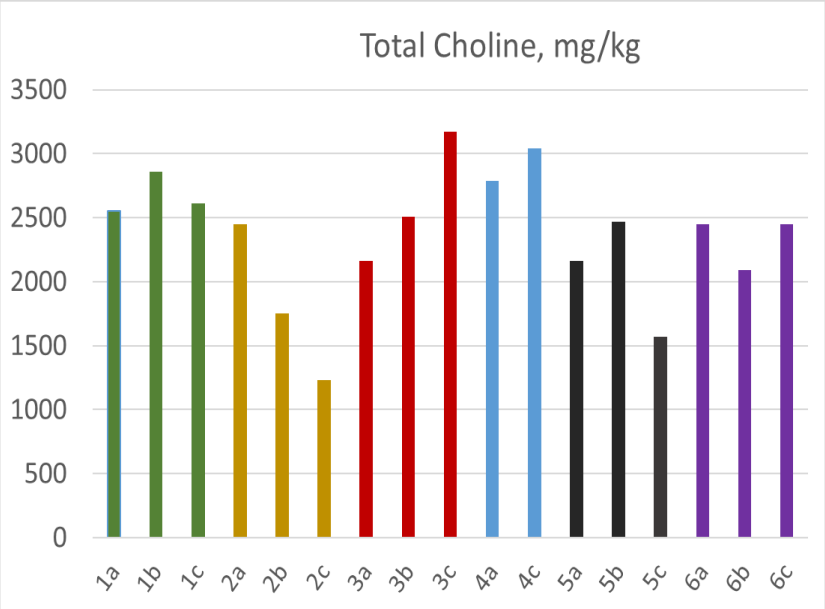


Figure 1.9. Results of analyses of total choline in diets sampled at the salmon farms three time during the production period in the sea. The numbers 1 – 6 indicate production site, b – c indicate sampling timepoint, i.e., just after sea transfer, in the spring next year, and some weeks before slaughter, respectively.

Toxicological analyses

Polycyclic aromatic hydrocarbons (PAHs) in the sampled feeds were non-detectable or low. The PAHs found in highest concentrations were fluorene, phenanthrene, anthracene, pyrene and fluoranthene, all of which are typically found in soybean grain which have been dried in a direct-fired furnace, a common practice in parts of Brazil (Silva et al., 2020). There are no maximum levels established for feed or feed ingredients for these individual PAHs. A maximum level for Sum PAH4 (anthracene, chrysene, fluoranthene, and pyrene) of 30.0 µg kg^{-1} is, however given for smoked fish according to amended Regulation (EC) No 1881/2006. The highest value of PAH4 found in this study was 0.6 µg kg^{-1}.

The mycotoxin aflatoxin (AFL) was detected in most feed samples. Information on the relative sensitivity to dietary aflatoxin exposure is lacking for Atlantic salmon, however the observed levels were under the general set MRL of 10 ug/kg, which does not include salmonid specific sensitivity. For other mycotoxin groups, fumonisin (FUM), OTA and ZEA the observed levels were lower than the estimated none observed effect level (NOEL) assessed by VKM for salmonids in 2013.

According to the National monitoring program for fish feed in Norway, the average ethoxyquin content in fish feed has decreased from 3000 ug/Kg in 2018 to 0.6 mg/Kg in 2019 and 19 ug/Kg in 2020 (Sele et al., 2021). The samples analysed in this study were collected from autumn of 2017 to the spring of 2018. Out of the 6 sites, 3 show a decrease in ethoxyquin in line with the results from the national monitoring program.

Task 8. Multivariate data analysis

Methodology

Metadata for multivariate analysis included fish biometrics, diets, environmental and geographical data, histology, microbiome, and gene expression (intestine), and NMR metabolomics data (plasma and faecal content from PI and DI). Data analysis algorithms used were principal component analysis (PCA) (Wold et al. 1987) and ANOVA simultaneous component analysis (ASCA) (Smilde et al. 2005).

Results

Multivariate data analysis is still ongoing and will comprise:

1) Exploratory analysis of the different metadata (separately)
2) Data fusion – distinct and common effects in the combined data matrices from various analytical platforms. Aim is to find possible biomarkers of gut health.

However, the available metadata from all analytical platforms is not fully balanced on the individual fish level (i.e., sample wise) limiting factors being microarray (144 samples) and microbiome (108 samples). The analysis of fully balanced data matrices thus requires downsizing the rest of the matrices to the microarray/microbiome sample number, which will result in an unbalanced experimental design (with very few samples per sampling event) for the faecal NMR-metabolome.
In order to preserve the statistical power of the data, data merging will be driven by the histology scores (221 samples).

Data analysis will be carried out as follows:

- Describe the baseline (healthy individuals)– correlations between different data matrices – we need a composite histology score (one for inflammation and one for dyslipidaemia)
- Describe the outliers (or sick individuals - few) – we can rely on microarray and microbiome.

Multiple confounding factors have been identified in the design of the prospective study and will have to carefully be considered before drawing any conclusion.

Discussion and conclusions

The histology investigation (Task 1) showed a low prevalence of mild to moderate inflammation of distal intestinal tissues; only one site (site 1) stood out with a higher general profile, especially in the final sampling when all fish were affected. The prevalence of lipid vacuolisation in pyloric caeca was generally higher, where all or the majority of sampled fishes were affected in the final sampling. The gene expression studies confirmed inflammatory transcription profiles (Task 2) like those earlier found in soybean meal- and saponin-induced enteritis of salmon. In task 3, the picture was supplemented with the discovery of IgD producing cell in the intestinal tissues, indicating hypersensitivity reactions. However, the findings indicated that the gut barrier functions were intact (Task 3).

The plasma biomarkers of digestive function varied between samplings, believed to reflect changes in feed composition, water temperature and feed intake as they were generally within the normal range of values (Task 4). The gut microbiota analyses showed highest bacterial DNA content in the spring sampling (Task 5), The diversity profiles from first sampling of the various sites clustered, while an overlapping pattern could be seen at the second and third sampling time points.
The metabolomics investigations revealed 60 distinct NMR spectroscopy signals in salmon plasma, predominantly from lipids and in particular from lipoproteins (Task 6). Work to differentiate the normal mid- and distal intestine metabolome vs. aberrant digestion profiles has commenced and yielded first results.

The feed analyses showed a normal pattern of decreasing protein content and increasing lipid content during the marine farming cycle (Task 7). All of the feeds showed choline levels below the available requirement estimates.

The multivariate data analysis (Task 8) is still ongoing and will comprise both exploratory analysis of the different separate metadata, and data fusion possible biomarkers of gut health.

In conclusion the survey and sample analyses are believed to yield observations and measured values which are representative of Norwegian commercial farming of Atlantic salmon during the seawater phase.

WP1.2 Retrospective survey

Responsible partner: VM-Defense
This part of the project was based on a material collected from a collaboration project between MOWI, Skretting and NMBU. Different diets (A to G) had been fed to triplicate groups of fish. Sampling of hindgut was performed in the start of the experiment (20 individuals), at mid-sampling (fifteen fish from each triplicate of groups), and similarly at end-sampling. The aim of the experiment was to assess the putative development of inflammation over time in the different groups and to characterize this progression by different methods. Samples from the experiment were therefore considered of relevance for further studies under the GutMatters project.

Methodology

The methods used included traditional histology, immunohistochemistry and in situ hybridization for the expression of selected molecules and genes. The material was first received in the laboratory fixed in formalin and processed routinely for histological analysis. Sections were made and stained with HE for evaluation. Following this evaluation, selected material was further processed for an in-depth analysis using immunohistochemistry, special staining methods and *in situ* hybridization. The methods used for immunohistochemistry and *in situ* hybridization have been described by Løken et al (2020)

Based on the histological findings in this material, we invented a new grading system for the description of inflammatory changes. This system is new in itself and will therefore be presented under “Results”.

Results

New scoring system

Previously, histological scoring has not taken into account variation of inflammatory changes within a sample. As we could detect this phenomenon to be prominent in the collected material, we developed a new scoring system based on the observations within the collected intestines.

- **0.0**: No identifiable changes
- **0.5**: No identifiable changes in most of the section + occasional focal regions with some inflammation.
- **1.0**: Some inflammation *or* no inflammation with occasional severe focal inflammation.
- **1.5**: Some to moderate inflammation *or* Some inflammation with occasional moderate or severe focal inflammation.
- **2.0**: Moderate inflammation
- **2.5**: Moderate to severe inflammation *or* Moderate inflammation with occasional severe inflammation
- **3.0**: Severe pan-inflammation

This system aims at grading the overall seriousness of the condition and not only attribute the scoring to a chosen part of the section. Previous systems have not to a sufficient grade taken into account variations within a sample. For instance, the intestine may appear un-affected apart from a focal or isolated area with severe inflammation. Such an area may display severe cellular changes (for instance dislocated epithelial cells). The novel grading system takes such changes into account. However, a grading system can never provide a precise description of the actual change, which should be presented in addition to a grade.

Scoring results based on HE-stained sections

The fifteen initial individuals (prior to the onset of the experiment) displayed a very low grade of detected inflammatory changes in their intestines. Nine individuals were without comments, the others only slight grade of inflammation. Highest individual score was 1.5, and the overall average score was 0.3.

In the mid-sampling, the variation was more pronounced, but in the entire sampling (all groups), there were just two individuals scoring as high as 2.5 and two individuals scored 2.0. The overall score was 0.6. This sampling was hampered with autolysis of some of the material and is best addressed as a collected sampling and not divided into different groups. This also serves the purpose of the material as the aim is to address the progression of inflammation over time and its characterization and not the feed effects on the different groups.

There was some group variation, but not spectacular, the outliers being Group D with a score of 0.4 and Group A with 0.8. As the diets for the different groups were not made known to the project, it is not the scope of this report to discuss the possible causes for this variation, however, it is more interesting to characterize the nature and the degree of inflammation observed over time. In this final sampling, the overall score was 0.9. A total of four individuals scored 3.0, and five individuals scored 2,5. These are both severe changes. So, of the 105 individuals investigated in this sampling, nine displayed severe changes, or roughly 9%.

Dislocated epithelial cells

The qualitative investigation of the individual sections included registration of possible dislocated epithelial cells, known from mammalian gastroenterology to be associated also with prolonged

inflammation. Here, HE sections were first validated for such cells by HE-staining. When suspicion was there, the samples were subjected to additional investigation by PAS staining, to detect mucus content. This method has proved to be efficient for the detection of dislocated epithelial cells (Dale et al. 2009). In the initial sampling, no dislocated epithelial cells were detected. In the mid-sampling, two individuals displayed this finding. Both these individuals scored 1.5. In the end-sampling, one sample, scoring 1.5 (low) (slight to moderate inflammation) was found to have dislocated epithelial cells (See examples in Figure 1.10). The same situation was found in one sample, scoring 2.0, one sample, scoring 2.5 and finally in two samples, scoring 3. Of note, the other two individuals scoring 3 in the end sampling showed no signs of dislocated epithelial cells. None un-affected intestines and intestines with scoring 1 were found to harbour dislocated epithelial cells.

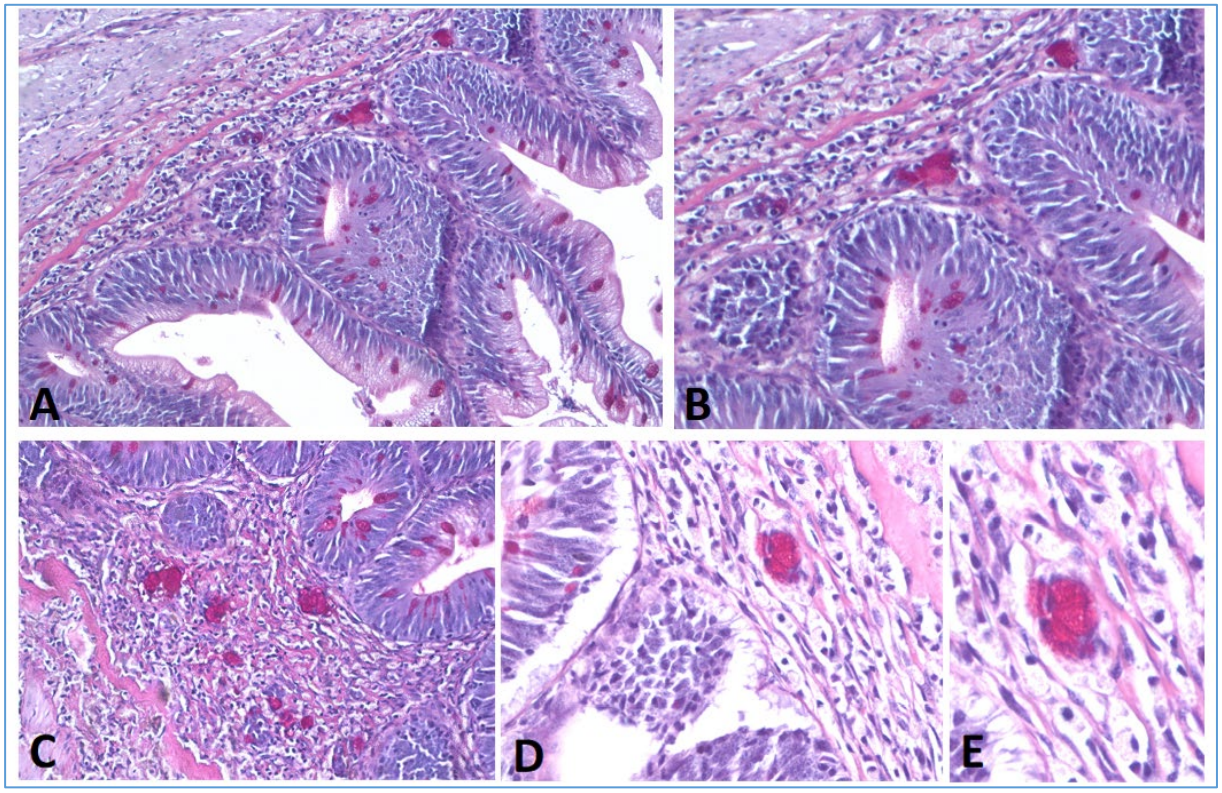


Figure 1.10. Examples of dislocated and mucus-containing epithelial cells in salmon intestine. In A to E, the mucus (red) is detected by PAS staining. A and B: Overview and magnified section of dislocated epithelial cells (red) in the submucosa. Inflammation score 2. C: Dislocated epithelial cells in submucosa of an individual with inflammation score 3. D: Dislocated epithelial cells in submucosa of an individual with inflammation score 1,5.

Calcifications

In HE-stain sections, calcifications were registered when observed. They were mostly found in the submucosa. There is just one former paper that have reported such changes previously, and was then termed “focal dystrophic calcinosis” (Mørkøre et al. 2020). The significance of this finding is un-known. The presence of the calcifications displayed no co-occurrence with inflammatory changes. Calcifications were found in the mid-sampling and in the final sampling, but not in the initial one. In

the *last sampling*, the results of these findings regarding number of individuals scoring 1 – 6 were as follows: Score 0: 6; Score 0.5: 0; Score 1.0: 11; Score 1.6: 6; Score 2.0: 5; Score 2.5; 2; Score 3: 3.

Immunohistochemistry for nitrotyrosine

Nitrotyrosine is an end-product of proteins subjected to oxidation. It is known to be detected in epithelial cells that may proceed into cancerous cells. We tested if this phenomenon may be detected in the salmon intestines, in particular those displaying dislocated epithelial cells (Figure 1.11). A number of individuals were tested, representing all grades of inflammatory changes. These experiments revealed no certain findings of nitrotyrosine in epithelial cells. Macrophage-like cells in the submucosa were frequently positive for cytokeratine, a sign of intracellular protein degradation. This is a normal finding in active macrophages.

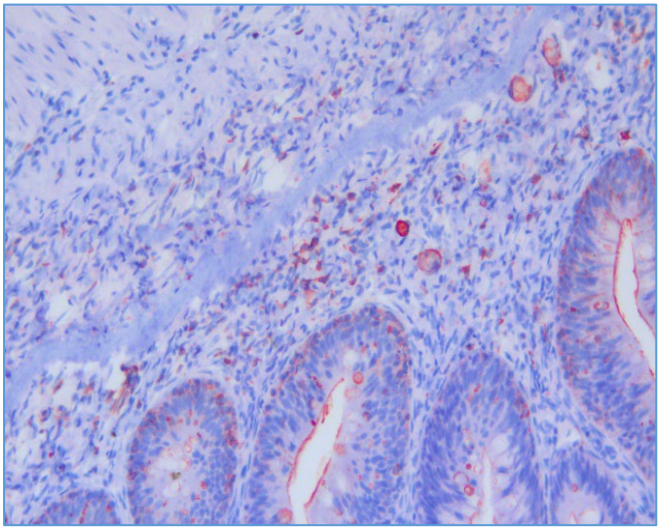


Figure 1.11. A salmon intestine with dislocated epithelial cells investigated for the presence of nitrotyrosine by immunohistochemistry. Macrophage-like cells and mucus-containing cells in the submucosa are positive (red). Positive Brush border of epithelial cells are interpreted at false positive. Macrophage-like cells within the epithelium are positive. No epithelial cells show uniform positive staining in the cytoplasm which would have been indicative for accumulation of nitrotyrosine and a pre-cancerous finding.

In situ hybridization for bacteria, IgM, IgT and IgD

Bacteria. Selected material was subjected to *in situ* hybridization for bacteria targeting 16S ribosomal RNA. The probe was provided by RNA Scope and should target all bacteria, Gram negative as well as Gram positive. The tested material included all grades of inflammatory changes from last sampling and also sections with dislocated epithelial cells. Bacteria were invariably detected in the intestinal lumen, frequently attached to the brush border of the epithelial cells but were not found within the epithelium or beneath the basal membrane. Thus, there were no variations in these findings regardless of the degree of inflammation or the presence of dislocated epithelial cells.

Immunoglobulins. The focus of feed-induced inflammatory changes with respect to leukocytes has been centred on T cells. No reports have so far addressed such changes with respect to the occurrence of IgM, IgT and IgD-expressing cells. Immunohistochemistry for IgM will also detect IgM bound to macrophages, and these are not B cells. But when targeting the mRNAs for the respective immunoglobulins, we are able to accurately determine the nature of positive cells. The procedure and probes have been presented previously (Løken, O.M. et al. 2020). Here, the tested material included all grades of inflammatory changes from last sampling and sections with dislocated

epithelial cells. In inflamed intestine, IgM mRNA positive cells were abundant in the inflamed regions (image below). However, the IgT mRNA positive cells were very low in numbers (Figure 1.12). In inflamed regions, the most prominent reactions were associated with in situ hybridization of IgD mRNAs (Figure 1.12). Here, positive cells were also detected in the epithelium. The images are representative for all investigated individuals with an inflammatory grade of 2,5. At lower grades, the presence of these cells gradually disappeared, and with an inflammation score at 0, almost no positive cells could be detected.

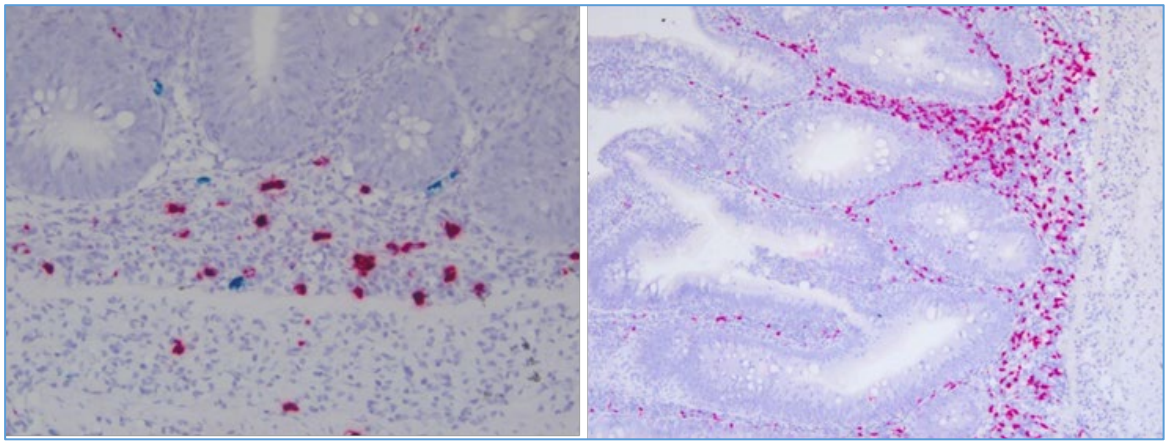


Figure 1.12. *In situ* Ig in inflamed hind-gut. **Left:** *In situ* hybridization for IgM (red) and IgT (blue). There are several IgM- positive cells (red) in the inflamed submucosa, but very few IgT-positive cells. **Right:** *In situ* hybridization for IgD. IgD-positive cells are found both in the epithelium and in inflamed tissue below the basal membrane.

Discussion

In this work package, we have followed fish which over time have received commercial feed. The changes in the investigated fish varied from not detectable to severe inflammation. At the end sampling, approximately 9% of the fishes showed serious inflammatory conditions in the intestine. A novel scoring system for assessing intestinal changes was developed. This system takes into account severity variation of changes within a sample.

Dislocated epithelial cells were registered in the intestines. This phenomenon has previously been shown to be a step in the tumour-neogenesis in salmon (Dale et al. 2009). However, it is not known if the presence of such dislocated epithelial may regress and turn back to a normal status over time or proceed into tumours. Either way may be thinkable, depending on so far unknown factors. Of note, such cells were only found in intestines displaying inflammation and never in no-inflamed intestine. We therefore cannot conclude what drives the process of loss of polarization of intestinal epithelial cells with subsequent displacement of these cells to the lamina propria.

With respect to calcifications, they were present both in inflamed and non-inflamed intestine. The reason for this phenomenon is obscure. This is hardly a normal condition and has only been reported once before (Mørkøre et al. 2020). Also, these authors had no explanation for this observation.

Inflammation is known to trigger the oxidation of proteins and their end product after such oxidative stress is nitrotyrosine. Detection of nitrotyrosine in epithelial cells is used in pathological investigations for investigating if such stress is present. Surprisingly, we did not detect nitrotyrosine in epithelial cells. If we had, this would have been an important finding for the explanation of the presence of dislocated epithelial cells, indicating cell damage. However, extensive testing for nitrotyrosine in the gut epithelium did not give any positive reactions.

Leaky gut syndrome is thought to occur in different intestinal conditions compromising the epithelial barrier. It has been speculated that such may occur in the salmon and that lumen ingredients might trigger and enhance inflammatory conditions. Bacteria have been suspected. Using a pan-bacterial probe, we investigated if bacteria were present in the submucosa of fish with varying degree of inflammation, from none to severe. In this material, we never encountered bacteria beneath the basal membrane including areas with severe inflammation. Bacteria were however invariably detected in the mucus layer covering the epithelium and in rare cases intraepithelially. This result seems to make it feasible to rule out leaky gut as a factor in the pathogenesis of feed-related inflammatory conditions in the salmon. Therefore, we conclude that intestinal leakage, at least when related to bacteria, is not a driver of the observed inflammatory response.

To the best of our knowledge, this is the first study to address the presence of IgM, IgT and IgD mRNA positive cells in feed-induced enteritis in fish. Previous studies have focused predominantly on T cells. Before these investigations, we believed IgT expression to be strongly reactive. IgT is assumed to be the IgA equivalent in fish and has shown responses with respect to mucosal infections (Zhang et al. 2021). However, the IgT responses seemed remarkably modest in inflamed intestine. On the other hand, IgM responses were strong, but most prominent was the IgD response. The inflamed portions were thus dominated by IgD mRNA positive cells. The findings were discussed with Dr. Carolina Tafalla in Madrid who pointed out that IgD-positive cells were known to be highly involved in mucosal hypersensitivity reactions. We believe this is a significant finding in this project. The observation opens up for a totally new concept of our understanding of feed-induced inflammation which should be pursued in future studies.

Conclusions

- The invention of a novel intestinal scoring system makes is more feasible to validate intestines displaying varying degrees of inflammation.
- Dislocated epithelial cells are a common finding in inflamed intestine. In un-affected intestine, they seem not to be present.
- Oxidative reactions seem not present in epithelium covering inflamed regions. This contrasts the situation of inflammatory bowel disease in mammals.
- Calcifications are common in intestines with and without inflammation.
- Leaky gut syndrome seems not to occur in feed-induced intestinal inflammation in salmon.
- The strong presence of IgD positive cells in inflamed intestine indicates hypersensitivity reactions. These may be triggering the inflammatory reactions observed.

WP2. Controlled experiments

WP2.1 Feeding trial I – Combination effects of legume level and functional ingredients

Responsible partner VM-Nutr and NOFIMA

The field survey of the project, presented above, confirmed that steatosis (lipid vacuolization) of the pyloric caeca (PC) and inflammation of the distal intestine (DI) were common findings in Norwegian Atlantic salmon. The aims of the present work package were to strengthen knowledge on the interaction between level of legumes and fishmeal in the diet and symptoms of steatosis in PC and inflammation in the DI, and to find whether supplementation with a mixture of choline and two functional ingredients, β-glucans (Macrogard®, Biorigin) and nucleotides (Lallemand®) might prevent the steatosis and reduce symptoms of inflammation in the DI of the salmon.

Methodology

Fish, fish facility, sampling, and diet formulation

The experiment was conducted at Nofima's Research Station at Sunndalsøra approved by Norwegian Animal Research Authority (NARA). Seawater tanks, $1m^3$, were used, for the study, each stocked with 43 fish with average weight 186g. Sixteen tanks were used. The temperature range averaged 10.6°C. At termination of the 63 days feeding period, 12 fish per tank were euthanized with an overdose of MS-222 (0.5–0.8 g/l) before tissue sampling. The fish were measured, and blood sampled taken from the caudal vein on heparinized vacutainers. Plasma was prepared and frozen in liquid N_2 and stored at -80°C. The fish were opened along the abdomen and the organs removed before the carcass was weighed. For 6 of the 12 fish sampled from each tank, this procedure was conducted under as clean conditions as possible for collection of samples of digesta and mucosa for microbiota analyses (Li et al. 2021). Liver weights and samples for histology were obtained. The intestine was cleaned of mesenteric fat and cut into sections, i.e., pyloric (PI), mid (MI) and distal (DI) intestine, which were weighed. The PI and the DI were divided in two halves, PI1 and PI2, and DI1 and DI2, respectively. The content of PI and DI sections were collected quantitatively and frozen in liquid N_2 for enzyme and bile salt analyses. From the mid sections of the PI and DI, samples were taken for histological examination, the remaining tissue was collected for enzymological, and gene expression analyses. The fish remaining in each tank were anaesthetized and stripped for collection of faeces in pools per tank, for analyses of nutrient composition and yttrium content, and calculation of nutrient digestibilities.

Two batches of eight diets, formulated to be similar regarding the content of digestible energy, digestible protein, and EPA+DHA, were made with fishmeal levels varying from 0 to 40% (Table 2.1.1). One of the batches was supplemented with a mixture of choline chloride (70%), Macrogard® (Biorigin) and nucleotides (Lallemand®). The regression design of this experiment made it appropriate to conduct this study without replicate tanks.

Analytical procedures

Due to limited resources, for some of the analytical procedures, only samples from fish fed low, two medium, and high FM inclusion levels, with and without supplements (i.e., diet 1, 4, 5, 8, 9, 12, 13, and 16) were analysed, as indicated in the figures and tables presenting the results.

Diet and faeces for digestibility measurement. Samples were analysed for dry matter, ash, crude protein, lipid, gross energy and yttrium as described by Refstie et al. (1997). Total lipids were extracted from homogenized samples of diets and faeces, following the method described by Folch et al. (1957).

Table 2.1.1. Ingredient and nutrient composition of the diets†

Fish meal level, %	0	6	11	17	23	29	34	40
Ingredients (g kg-1)								
Fishmeal LT	0	29	57	86	114	143	171	200
Fishmeal SA Superprime	0	29	57	86	114	143	171	200
Krill 56%††	20	20	20	20	20	20	20	20
Soya SPC >62	230	214	199	183	167	151	136	120
Sunflower Ext, low fibre	25	24	22	21	19	18	16	15
Wheat Gluten	152	137	123	108	94	79	65	50
Maize Gluten 60	62	55	49	42	35	28	22	15
Pea Protein 65	80	71	61	52	43	34	24	15
Wheat	100	101	103	104	105	106	108	109
Horse Beans, Dehulled	30	28	26	24	21	19	17	15
Fish Oil, EPA+DHA	111	105	98	92	85	79	73	66
Rapeseed Oil	128	132	136	139	143	147	151	155
Vitamin and mineral mix	10	10	10	10	10	10	10	10
$Ca(H_2PO_4)_2$ (MCP)	30.4	26.1	21.8	17.4	13.01	8.7	4.4	0
Amino acids	24	20.6	17.2	13.8	10.4	7	3.7	0.3
Yttrium	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Estimated nutrient content								
Digestible energy (MJ kg-1)	200	200	200	200	200	200	200	200
Crude protein (g kg-1)	423	425	427	429	432	434	436	438
Digestible protein (g kg-1)	385	385	385	385	385	385	385	385
Crude fat (g kg-1)	265	265	265	265	266	266	266	266
Ash (g kg-1)	59	63	68	72	76	80	84	89
EPA+DHA (g kg-1)	26	26	26	26	26	26	26	26
Analysed content								
Gross energy (MJ kg-1)	237	237	235	236	236	233	233	234
Crude protein (g kg-1)	413	413	422	418	430	432	434	446
Lipid (g kg-1)	298	289	292	297	296	289	291	289
Choline (Diet 1-8) (mg kg-1)	1050	1120	1240	1380	1530	1870	1770	2110
Choline (Diet 9-16) (mg kg-1)	2260	2410	2630	2750	3040	3100	3260	3470

† Two batches were made of each diet, one without and one with a mixture in the diet of 0.3% choline chloride (70%) supplied by Aker Biomarine, 0.5% Macrogard® produced by Biorigin and 0.5% nucleotides produced by Lallemand®

Plasma biomarkers

Classical methods: Plasma free (non-esterified) fatty acids, cholesterol, and total triacylglycerides were analysed according to standard procedures at the Central Laboratory of the Norwegian University of Life Sciences (NMBU).

Untargeted plasma metabolomics by 1H NMR: Plasma samples from six fish per tank were collected and analysed by ^{1}H NMR spectroscopy as previously described in the present document (page 23). Metabolite identification and quantification from the ^{1}H NMR spectra was performed using the

SigMa software (Aru et al. 2021; Khakimov et al. 2020). The resulting metabolite concentration table was imported into MATLAB 2020a (Mathworks Inc., Natick, MA, USA) where, for each metabolite, regression to dietary fishmeal (%) was calculated. Results are given as R^2, intercept, and p-value. Metabolite concentrations obtained from ^{1}H NMR and GC-MS measurements were tested for normal distribution and, prior to parametric statistics, variance analysis was performed to assess whether metabolite variability significantly differed between the two treatments (supplemented vs. non-supplemented diet). FDR-corrected p-values were used for the following analysis. Metabolites whose concentration significantly ($p<0.05$) differed between the two groups were selected for pathway analysis, which was performed using MetScape1 (Gao et al. 2010), a plugin for CytoScape2 (Shannon et al. 2003).

Histology

Formalin-fixed sections of the pyloric caeca (PC), distal intestine (DI), and liver (LI) were processed according to established internal histological methods to produce 4-5 µm thick H&E-stained sections. Periodic acid-Schiff (PAS)-stained sections were also prepared from liver tissue. Histological assessment, using light microscopy for PC and DI, focused on the characteristic morphological changes of soybean meal-induced enteritis (SBMIE) in Atlantic salmon DI, that consist of shortening of mucosal fold height, increase in width and cellularity of the submucosa and lamina propria compartments, and reduction in enterocyte supranuclear vacuolization. For the pyloric caeca, vacuolization of enterocytes, indicating lipid accumulation (enterocyte steatosis), was also assessed. Liver sections were evaluated for changes in hepatocyte morphology and the presence of specific pathological changes such as hepatocyte vacuolization, degeneration, haemorrhage, or inflammation. All morphological characteristics evaluated for PC, DI, and LI were graded on a scale of 0-4, where 0 represented normal; 1, mild change; 2, moderate change; 3, marked changes, and 4, severe changes.

Gene expression

Analyses were performed on tissues from the distal intestine of salmon fed four diets without supplements, containing 0, 11, 17, and 40% fishmeal, and from fish fed the corresponding diets with the supplement (six fish per treatment, totally, 48 samples) using Nofima's 15k Atlantic salmon oligonucleotide DNA microarray SIQ6 (GPL30031). Total RNA was extracted with Agencourt® RNAdvance Tissue kit (Qiagen, Hilden, Germany). Samples (~10 mg) were transferred into tubes with 400 µL lysis buffer containing 1 mg proteinase K (Qiagen, Hilden, Germany), and 3 mm magnetic beads, homogenised in a FastPrep-96 tissue lyser (MP Biomedicals, Eschwege, Germany) for 120 s at maximum speed and incubated for 25 min at 37°C. Lysed samples were processed with Biomek 4000 Automated Workstation (Beckman Coulter, Brea, CA, USA). RNA concentration and integrity were determined using a NanoDrop 8000 Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The microarrays were fabricated by Agilent Technologies (Santa Clara, CA, USA), and all reagents and equipment were purchased from the same provider. The Cy3-labelled RNA probes were produced with Low-Input Quick-Amp Labeling Kit (200 ng / reaction), fragmented with Gene Expression Hybridization Kit, and hybridized to microarrays in an oven for 17 h at 65°C, at rotation speed 10 rpm. The arrays were washed at room temperature with Gene Expression Wash Buffer I and II (1 min each) and scanned. Data were processed with Nofima's bioinformatics pipeline STARS (Krasnov et al. 2011). After equalizing the mean intensities of all microarrays, the individual values were divided by the mean of the features in all samples to calculate the expression ratios (ER). The log2-ER were normalized with lowess (locally weighted scatterplot smoothing). Relationship between the gene expression profiles

and FM levels was assessed with correlation (Pearson |r|>0.70) and linear regression (regression coefficient > 0.001). Feeds with and without supplements were compared, pairwise, differentially expressed genes were selected at a cut-off log_2-ER > 0.8 (1.75-fold) and <0.05 (t-test). Enrichment of Gene Ontology (GO) categories and KEGG pathways in the list of genes that responded to FM was assessed with Yates' corrected chi-square test ($p<0.05$), the minimum number of genes per term was set to five. Data were submitted to NCBI Geo Omnibus.

Microbiota

For intestinal microbiota analysis, gut contents were collected from the lumen of the pyloric (PI) and distal intestine (DI) from the same treatments as mentioned above for gene expression, i.e., containing 0, 11.4, 17.1, and 40% fishmeal. The DNA was extracted from the respective digesta samples from 6 fish from each dietary group following the protocol of QIAamp Fast DNA Stool Kit (Qiagen, Crawley, UK) with some modifications tested by our group (Li et al. 2021). For the quality control of the microbiota profiling protocol, along with each of the DNA extraction batch, two 'blanks' (without any sampling material) and two 'positive controls', i.e., mock (microbial community standard from Zymo-BIOMICS™, Zymo Research, California, USA) were included.
PCR amplification of the V1-V2 region of the 16S rRNA gene was carried out using 27F (5' AGAGTTTGATCMTGGCTCAG 3'), and 338R-I (5' GCWGCC TCCCGTAGGAGT 3') and 338R-II (5' GCWGCCACCCGTAGGTGT 3') to have about 300bp amplicons (Gajardo et al. 2017). PCRs were performed along with negative PCR controls following the protocol described previously (Li et al. 2021). Library preparations of the products form amplicon PCR (the Quick-16S™ NGS Library Prep Kit, Zymo Research) and subsequent quality control of the resulting libraries were performed as described previously (Li et al. 2021). The final pooled library was then denatured and diluted to 8 pM and sequenced on Illumina MiSeq platform with Miseq Reagent Kit v3 (600-cycle) (Illumina) to generate paired-end reads. 20% of 8 pM PhiX control was added as an internal control.

As an extra measure to identify contaminated sequences, qPCR was performed separately to quantity 16S rRNA gene in the diluted DNA templates (samples, blanks, and mock) used for the amplicon PCR as described in Li et al. 2021.

Bioinformatics analysis of microbiome sequencing data was performed using QIIME2 version 2 (Bolyen et al. 2019; Caporaso et al. 2010). The demultiplexed paired-ended reads were denoised, trimmed, and quality filtered using the DADA2 algorithm (Callahan et al. 2016) in QIIME2. The taxonomy was assigned to the resulting amplicon sequence variants (ASVs) tables by a Scikitlearn Naive Bayes machine-learning classifier (Bokulich et al. 2018), which was trained on the SILVA 132 99% ASVs (Quast et al. 2013) that were trimmed to exclusively include the regions of 16S rRNA gene amplified by the primers used in the current study. ASVs table was filtered for several criteria as detailed in the previous studies (Li et al. 2021). Contaminant sequences were detected as previously described by our group (Li et al. 2021). In total, 43 ASVs were removed from digesta samples. Among the removed ASVs were those belonging to the genera *Limnohabitans*, *Flavobacterium*, *Cutibacterium,* and uncultured bacteria from the order Oligoflexaceae. The ASVs filtered from the raw ASVs table were also removed from the representative sequences.

To compute the alpha and beta diversity indices, the ASVs table was rarified at 16,700 reads to have an even number of reads across all samples. Alpha diversity was calculated using the Observed species and Shannon`s diversity index with Kruskal-Wallis test. Beta diversity was evaluated using Bray-Curtis and weighted UniFrac distance metrics with PERMANOVA test. MicrobiomeAnalyst

package (Chong et al. 2020; Dhariwal et al. 2017) was used to analyse and graphical presentation of abundant taxa among the groups and visualization of alpha diversity, using ASV table at the feature level. Differentially abundant genera between the dietary groups were analyzed using DESeq2 (Anders and Huber, 2010) and the differences were considered statistically significant when the adjusted p-value (padj) with the Benjamini-Hochberg procedure ≤ 0.1.

Calculations

Growth of the fish was calculated as specific growth rate (SGR) = ((ln FBW/lnIBW)/D) X 100. IBW and FBW are the initial and final body weight (tank means) and D is number of feeding days; Condition factor (CF) = FBW * 100)/(Fork length cm)^3); Organ Indices= (organ weight/ body weight) X 100). Apparent digestibilities of the nutrients (ADn) of main nutrients was estimated by using Y_2O_3 as an inert marker (M) and calculated as follows: ADn = 100 – (100 X (M_{feed} / M_{faeces}) X (N_{feed} / N_{faeces})), where M represents the percentage of the inert marker in feed and faeces and N represents the percentage of a nutrient in feed and faeces.

Statistical evaluation

Regression analyses were used for evaluation of results regarding growth, feed utilization, organ indices, digestive functions, and processes. The models *Y=K + k*FM level* and *Y=K + k1*FM level + k2*Supplement* were used on results within diet series, and for the combined results, respectively. The aim of the use of the latter was to evaluate whether the effect of the supplementation caused a significant effect on the outcome of the feeding. For the latter analyses, only the p-value for the model and for the effect of the supplementation are given. The results were also analysed using a second-degree model, but no significant results were observed for the quadratic term.

Differences in histological scores for the various evaluated morphological characteristics of the PC, DI, and LI tissue were analysed for statistical significance using ordinal logistic regression run in the R statistical package (version 3.6.2; 2019) within the RStudio interphase (version 1.2.5033; 2019). Influence of supplementation and fishmeal level were examined based on odds ratios of the different feeding groups having different histology scores. For the histological features, the scores generated were categorical variables and the differences between the treatments were explored by contingency analysis using the chi-squared test.

For gene expression, microbiota and metabolome results, the data evaluation is described in the detailed procedures for these methods presented in the manuscript submitted for publication of these results.

Results

Growth, feed conversion, nutrient digestibility, and plasma metabolites

Neither growth nor feed conversion ratio (FCR) was significantly affected by fish meal level ($p>0.2$) (Figure 2.1.1). The same regarded effect of supplementation with the package of functional ingredients ($p>0.2$), which gave no significant effect.

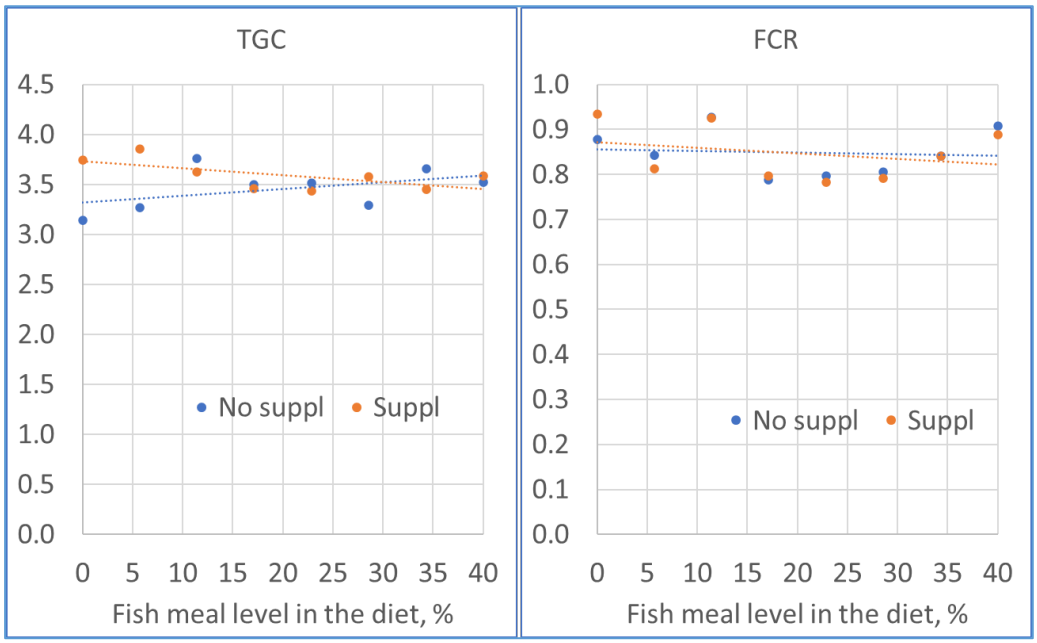


Figure 2.1.1. Results regarding growth (TGC) and feed conversion ratio (FCR). The statistical evaluation did not show significant relationship, neither with fish meal level ($p>0.2$) nor of the supplementation ($p>0.2$).

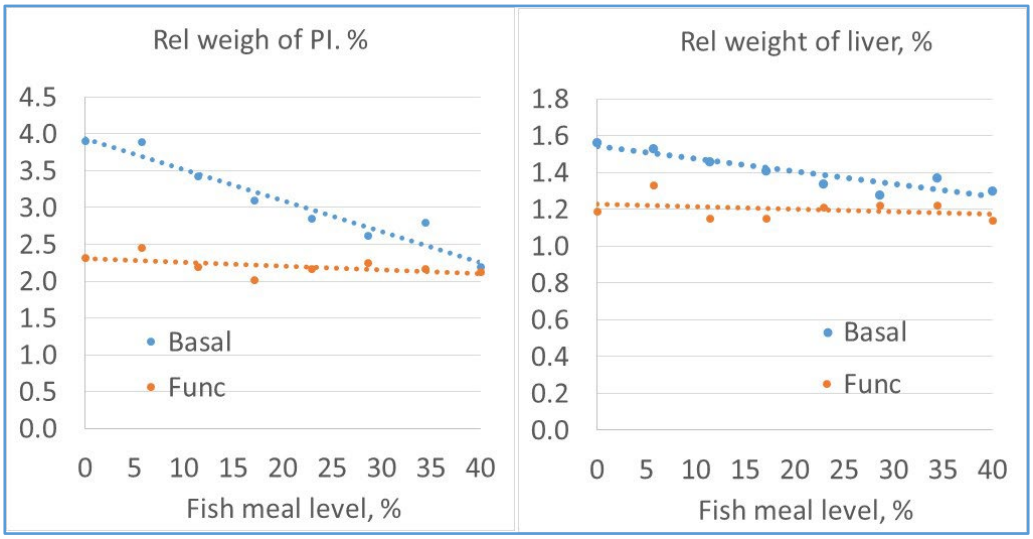


Figure 2.1.2. Pyloric intestine somatic index (PISI) and hepatosomatic index (HSI) in fish fed the two series of diets varying in fishmeal level, one series without supplements (No suppl) and the other supplemented (Suppl) with a mixture of choline chloride (0.3%), β-glucan (0.05%) and nucleotides (0.05%). For statistics see Table 2.1.2.

Fish fed the unsupplemented diets showed higher relative PI weights and more pronounced decreasing effect of increasing fishmeal level than those fed the supplemented diets (Figure 2.1.2). The liver (HSI) showed similar results but only for fish fed the unsupplemented diets. For the MI, the results indicated opposite effects of FM level and Supplement, but the effects were minor. The DI relative weight did not show significant effects of either FM level or Supplement.

Table 2.1.2. Results of regression analyses of body weight (BW), condition factor (CF), liver index (HSI), and intestinal section indices based on individual observations†‡

	BW	CF	HSI‡	PISI	MISI	DISI
No supplement						
p(model)	0.9663	0.1746	<.0001	<0.0001	0.0138	0.5669
R^2	0.000	0.020	0.180	0.7365	0.1248	0.0072
Intercept	532	1.443	1.471	3.933	0.3205	0.5222
Reg. coeff. FM level	0.0279	0.0008	-0.0051	-0.0419	-0.00173	0.0005
Supplemented						
p(model)	0.4601	0.2897	0.2052	0.0327	0.0184	0.7156
R^2	0.006	0.012	0.017	0.0954	0.1149	0.0029
Intercept	554	1.497	1.221	2.4076	0.3051	0.4925
Reg. coeff. FM level	-0.5120	-0.0007	-0.0010	-0.0056	-0.00323	0.00031
Combined						
p(model)	0.4877	0.5789	<0.0001	<0.0001	0.0005	0.0969
p(Supplement)	0.2434	0.3127	<0.0001	<0.0001	0.0022	0.0402

†Model Y=Intercept + k*FM level was used with the results for each diet series, i.e., *No supplementation* (No suppl) and *Supplemented* (Suppl). For the *combined* regression analyses, conducted to find whether the supplement affected the regression, the following model was used: Intercept + k_1*FM level + k_2*Supplement. Reg. Coeff=regression coefficient on fishmeal level; p(Supplement)=p-value for the effects of supplementation.
‡SI=Organosomatic index; PISI, MISI, and DISI=SI of pyloric region (PI), mid intestine (MI), and distal intestine (DI), respectively.

Digestibility of macronutrients and fatty acids showed significant effect of Supplement. The regression analyses showed small but significant effects on nutrient digestibility of FM level in unsupplemented diets, positive for dry matter (DM) (p=0.04), energy (p<0.01), lipid (sum of fatty acids) (p=0.05), saturated and mono-unsaturated fatty acids (Figure 2.1.3), negative for crude protein (p<0.01), EPA+DHA (p<0.001) and the sum of n-3 fatty acids (p=0.001). For fish fed the supplemented diet series, an effect of fishmeal level was observed only for EPA+DHA (p<0.001) and the sum of all n-3 fatty acids (p<0.001), and this relationship was negative. The statistical analyses of effect of supplementation showed significant results for MUFA, n-6, n-3, and EPA+DHA with p<0.001 for all three. Digestibility of choline, one of the components of the supplement, showed no effect of fishmeal level in the diet (Table 2.1.4). It was, however, significantly higher for the supplemented diet series.

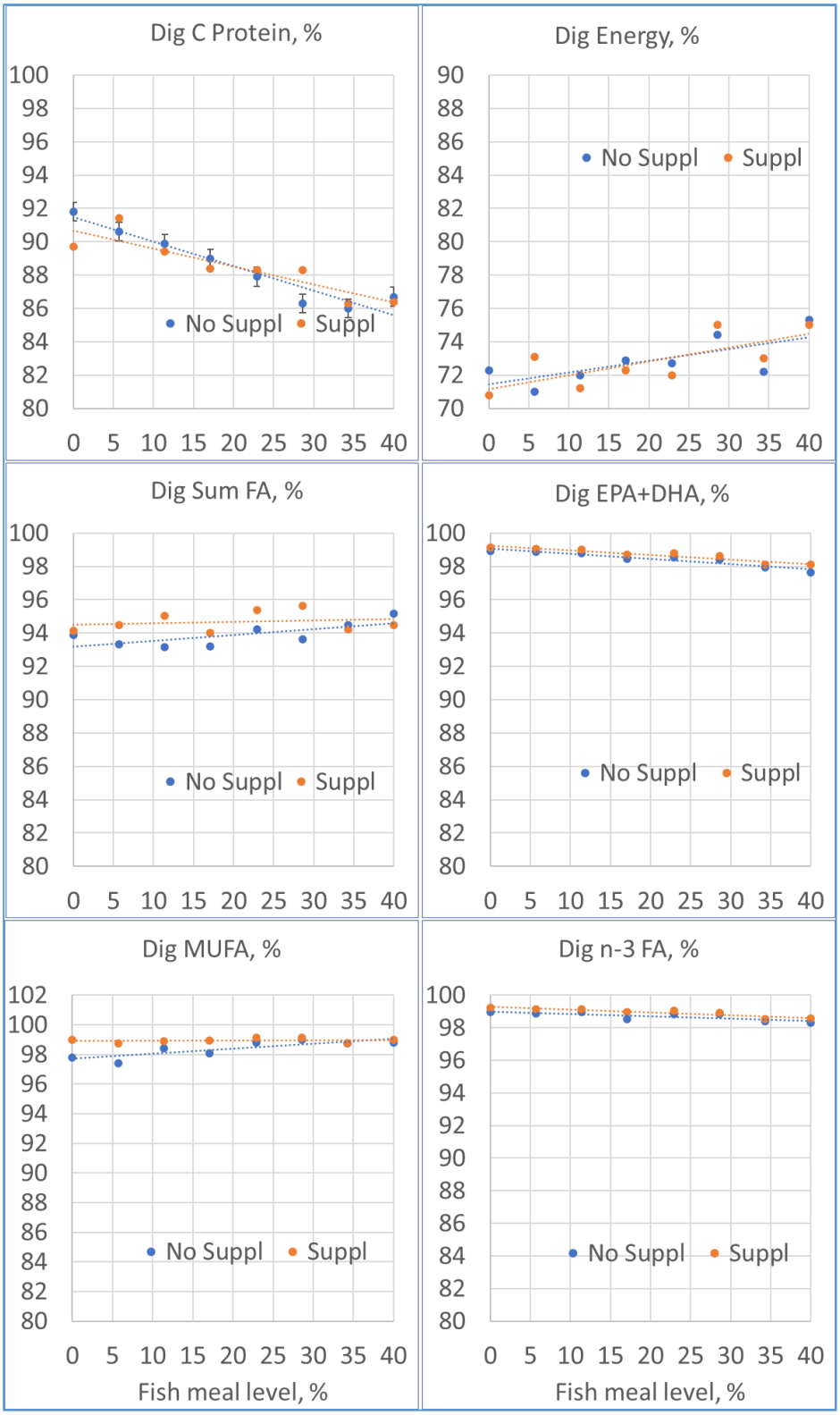


Figure 2.1.3. Digestibility of crude protein, energy, and fatty acids which showed significant effect of dietary fishmeal level as well as significant effect of the dietary supplement (No Suppl and Suppl), i.e., a mixture of 0.3% choline chloride, 0.05% β-glucan and 0.05% nucleotides. Note the expanded scales on the Y-axis. For statistics see Table 2.1.4.

Table 2.1.4. Results of regression analyses of nutrient and energy digestibilities on fishmeal level based on observations on tank level†

	DM	Energy	CP‡	Lipid	Sat	MUFA	n-6	n-3	EPA+ DHA	Choline
No supplement										
p(model)	0.0443	0.0027	0.0002	0.053	0.1046	0.0104	0.0115	0.023	0.0004	0.2359
R^2	0.52	0.80	0.92	0.49	0.38	0.69	0.68	0.61	0.89	0.22
Intercept	71.4	81.9	91.5	93.2	85.0	97.7	97.9	99.0	99.1	91.3
Reg.Coeff. FM level	0.07	0.07	-0.15	0.04	0.10	0.03	0.02	-0.01	-0.03	0.037
Supplemented										
p(model)	0.0347	0.0256	0.0025	0.6247	0.5352	0.7403	0.1641	0.0012	0.0005	0.118
R^2	0.55	0.59	0.80	0.04	0.07	0.02	0.30	0.85	0.88	0.36
Intercept	71.1	82.4	90.7	94.5	87.8	98.9	98.5	99.3	99.2	95.9
Reg.Coeff. FM level	0.08	0.06	-0.11	0.01	0.03	0.00	0.01	-0.02	-0.03	-0.012
Combined										
p(model)	0.007	0.0004	<.0001	0.0215	0.087	0.0031	0.0022	<.0001	<.0001	<0.0001
p(Supplement)	0.9254	0.6444	1.0000	0.0218	0.1301	0.0049	0.0127	0.0059	0.0064	<0.0001

† See explanation regarding statistics in Table 2.1.2.
‡CP=crude protein; DM=dry matter; Sat=sum saturated fatty acids; MUFA=mono-unsaturated fatty acids; n-3=sum ω-3 fatty acids; n-6=sum ω-6 fatty acids.

The results of the classical assays of plasma samples showed no significant effect on glucose of fishmeal level (p=0.09), neither of the supplementation (p=0.08). Regarding plasma cholesterol and triglyceride level clear effects of fishmeal level was seen for both unsupplemented and supplemented diets, and there was a significant effect of supplementation of the diets (Figure 2.1.4).

Also, by ^{1}H NMR spectroscopy diet effects on plasma cholesterol level were shown (Figure 2.1.5). However, the classical procedure seemed to separate the treatment effects more clearly, supposedly due to lower variation between observations. Analysis of the ^{1}H NMR data for plasma levels of the amino acid methionine, glutamine, methyl histidine, alanine and of the branched-chain amino acids isoleucine, leucine, and valine showed no effect of either fishmeal level or supplementation (data not shown). For tyrosine, a non-essential amino acid for which metabolism is closely linked to the metabolism of choline (Kall et al. 2019), a negative correlation to FM level was observed, which was strongest for the non-supplemented diets (R^2=0.60, p<0.001) (Figure 2.1.4).

Total choline in plasma showed a clear, increasing effect of fishmeal in fish fed the unsupplemented diet, whereas fish fed the supplemented diets showed no such effect. The supplementation elevated plasma choline level to a level as high as observed in fish fed the unsupplemented 40% fishmeal diet. Plasma dimethyl glycine, a metabolite in the metabolism of choline increased with increasing fish meal, similarly for the treatments without and with the supplementation.

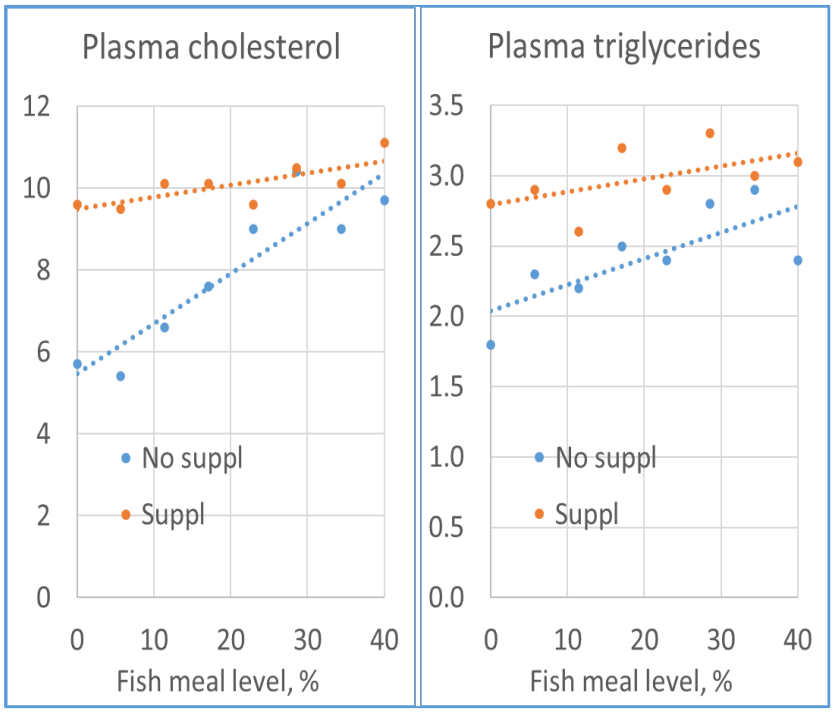


Figure 2.1.4. Results of analyses of plasma biomarkers which showed significant effects of dietary fishmeal level and of supplementation (No Suppl vs Suppl). Statistics: Effect of FM level in fish fed unsupplemented feed on plasma triglycerides: $p<0.001$, on plasma cholesterol: $p<0.001$; in fish fed supplemented diets: plasma triglycerides: $p=0.10$ and plasma cholesterol: $p<0.001$; Effects of supplementation: $p<0.001$.

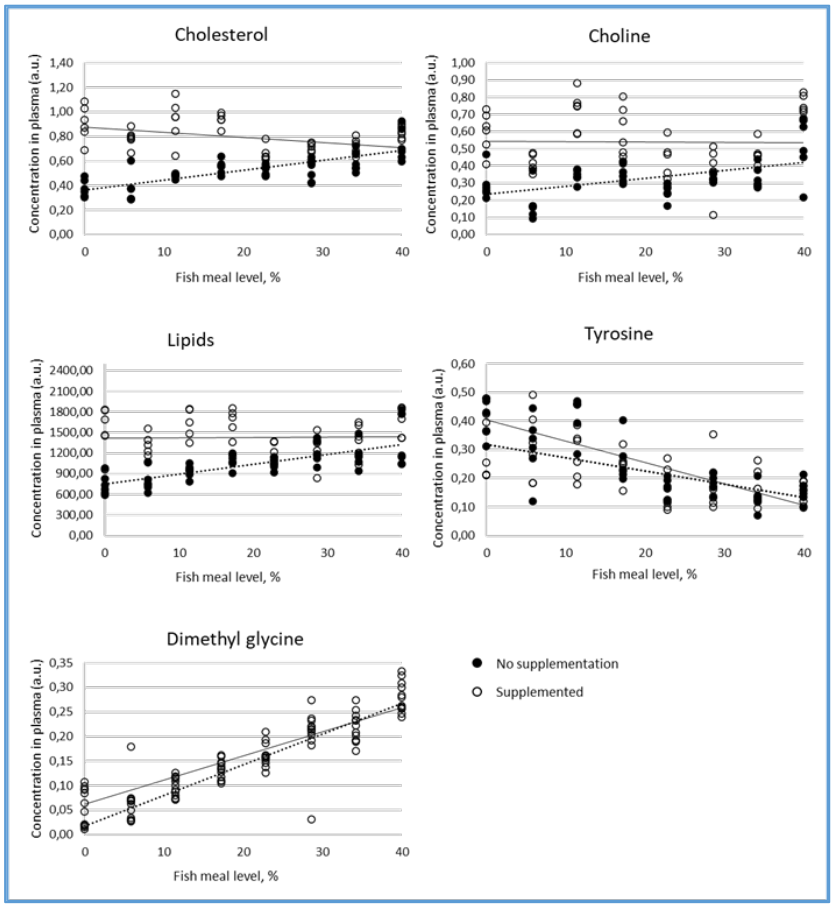


Figure 2.1.5. Scatter plots showing the results of the regression analysis between the metabolites concentrations as measured by ^{1}H NMR and the fishmeal content. Metabolite concentrations were scaled down by 10^{6} and are expressed as arbitrary units (a.u.).

Histology

As illustrated in Figure 2.1.6., a distinct effect of both fishmeal ($p<0.0001$) level and supplementation ($p<0.0001$) was observed regarding occurrence and severity of lipid accumulation (steatosis) in the enterocytes of the PI. A general trend for the fish fed the unsupplemented diet series was decreasing severity of the steatosis in proportion to increase in the FM level. For fish fed the supplemented diet series, steatosis was observed only for the two lowest FM levels. None of the fish fed diets with higher fishmeal levels showed signs of steatosis. No other abnormal morphological features were noted in the pyloric caeca.

In the distal intestine, histological examination regarding signs of inflammation (data not shown) indicated normal structure in most of the examined fish. However, fish fed the highest fish meal level, i.e., 5 of 36 individuals, regardless of supplementation showed mild and focal inflammatory cell infiltration into the submucosa and lamina propria compartments. Liver tissue was observed with no clear histopathological changes.

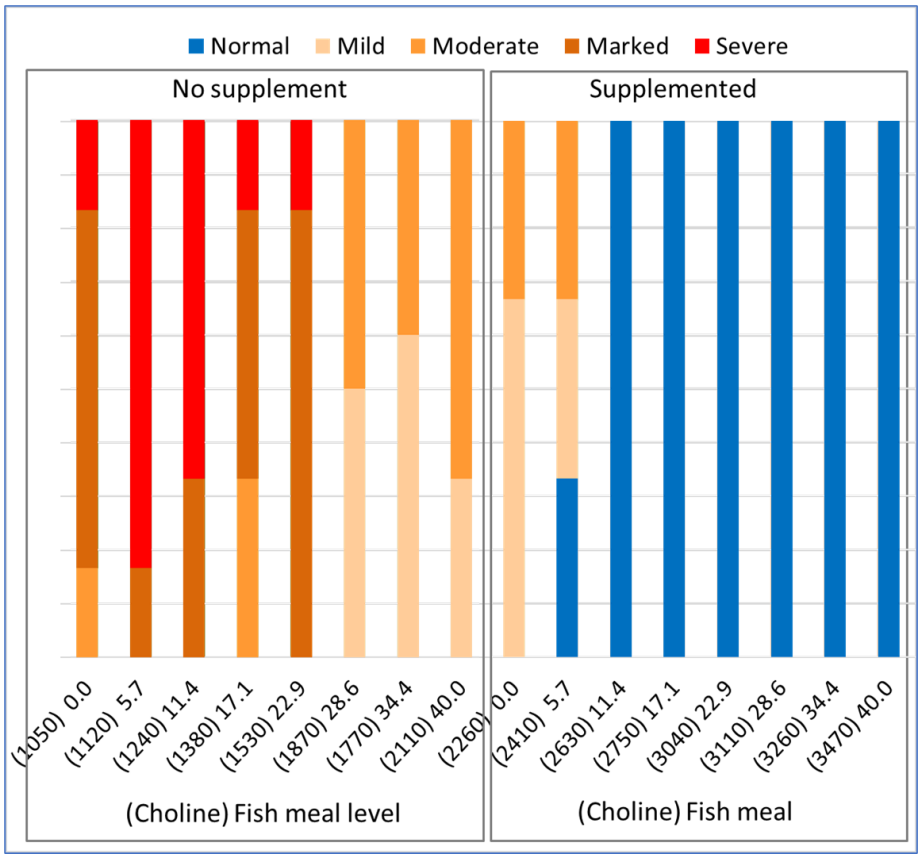


Figure 2.1.6. Results of histological examination of pyloric caeca regarding degree of enterocyte vacuolization, i.e., steatosis. Numbers at the bottom of the columns indicate (in parenthesis) choline level in mg/kg in the diet and (outside the parenthesis) fish meal level. Significant effects of fishmeal ($p<0.0001$) level as well as supplementation ($p<0.0001$) was observed.

Gene expression

The microarray analyses focused on the intestinal responses to fishmeal level and the supplement. Positive and negative relationship with FM levels was found in respectively 537 and 309 genes. Enrichment analysis suggests that fishmeal affected various metabolic pathways including

biotransformation, metabolism of amino acids, lipids, retinoids, steroids, iron and heme and genes encoding extracellular proteins, while immune changes were minor (Table 2.1.5, Figure 2.1.7).

Table 2.1.5. Enrichment of functional categories of Gene Ontology among genes that responded to the supplement.

Functional group	DEG[1]	Genes[2]	p value[3]
Upregulated			
Xenobiotic metabolic process	5	113	0.001
Arginine and proline metabolism	6	113	0.000
Heme binding	10	212	0.000
Iron ion binding	8	217	0.000
Lipid binding	5	189	0.039
Metallopeptidase activity	6	148	0.000
Monooxygenase activity	8	96	0.000
Antigen processing and presentation	8	88	0.000
Cell-matrix adhesion	5	157	0.012
Collagen	7	183	0.000
Downregulated			
Cytoskeleton	12	539	0.008
Muscle contraction	13	245	0.000
Cytokine-mediated signalling pathway	10	356	0.002
Defense response to virus	21	232	0.000
Innate immune response	18	624	0.000
Neutrophil chemotaxis	5	102	0.001
Response to bacterium	6	198	0.013
Extracellular matrix	7	288	0.033

[1]Nunber of differentially expressed genes per term. [2]Number of genes on the microarray platform. [3]Yates' corrected chi-test.

A

Function	Gene	Supp-	Supp+
Lipid	Acetyl-CoA acyltransferase 1	0.73	0.31
Lipid	Acid ceramidase	0.75	0.28
Lipid	Acyl-CoA synthetase short-chain f1	0.88	0.69
Lipid	Acyl-CoA synthetase short-chain f3m	0.82	0.49
Lipid	Acyl-CoA thioesterase 11	0.85	0.43
Lipid	Acyl-coenzyme A oxidase 3, peroxisomal	0.80	0.25
Lipid	Acyl-coenzyme A thioesterase 12-like	0.87	0.48
Lipid	Alpha-methylacyl-CoA racemase	0.78	0.20
Lipid	Apolipoprotein B-100	0.73	0.80
Lipid	ATP-binding cassette G5	0.90	0.72
Lipid	Carnitine O-acetyltransferase	0.89	0.40
Lipid	Diacylglycerol O-acyltransferase 2 *	0.82	0.56
Lipid	Elongation of long chain fatty acids	0.85	0.48
Lipid	Long-chain-fatty-acid--CoA ligase *	0.73	0.03
Lipid	PPAR delta b *	0.81	0.51
Lipid	Peroxisomal biogenesis factor 3	0.84	0.74
Lipid	Peroxisomal membrane protein 11C	0.83	0.54
Lipid	Peroxisome biogenesis factor 13	0.81	0.38
Retinol	Epidermal retinol dehydrogenase 2	0.82	0.37
Retinol	Retinol dehydrogenase 7-like	0.86	0.51
Retinol	Retinol dehydrogenase 8b *	0.76	0.21
Retinol	Retinol saturase	0.74	0.53
Steroid, bile	3-oxo-5-beta-steroid 4-dehydrogenase	0.85	0.43
Steroid, bile	7-dehydrocholesterol reductase	0.81	0.24
Steroid, bile	Cytochrome P450 3A27	0.78	0.74
Steroid, bile	Cytochrome P450 46A2	0.78	0.56
Steroid, bile	Fatty-acid amide hydrolase 1 *	0.73	0.32
Steroid, bile	Hydroxysteroid 11-beta dehydrogenase 2	0.77	0.48
Iron, heme	5-aminolevulinate synthase	0.46	0.70
Iron, heme	Ferritin, heavy polypeptide 1b	0.93	0.67
Iron, heme	Heme oxygenase-like	0.89	0.84
Iron, heme	Transferrin-a	0.91	0.54

B

Function	Gene	Supp-	Supp+
Redox	Catalase	0.74	0.32
Redox	Glutathione peroxidase 1-like	0.69	0.78
Redox	Selenoprotein H-like	0.76	0.73
Redox	Selenoprotein P, plasma, 1	0.75	0.57
Redox	Selenoprotein W_ 2a	0.78	0.50
Redox	Thioredoxin reductase 3	0.82	0.36
Xenobiotic	Aldose reductase-like	0.76	0.46
Xenobiotic	Aryl hydrocarbon receptor 2 gamma	0.73	0.24
Xenobiotic	Arylacetamide deacetylase	0.81	0.63
Xenobiotic	Arylamine N-acetyltransferase *	0.84	0.42
Xenobiotic	ATP-binding cassette G2b *	0.70	0.45
Xenobiotic	ATP-binding cassette, sub-family C	0.75	0.50
Xenobiotic	Cytochrome P450 1A1 *	0.86	0.87
Xenobiotic	Cytochrome P450 27C1 *	0.81	0.79
Xenobiotic	cytochrome P450 2D15-like *	0.70	0.34
Xenobiotic	cytochrome P450 2F3-like *	0.71	0.43
Xenobiotic	Glutathione S-transferase kappa 1	0.79	0.54
Xenobiotic	Glutathione S-transferase 3 *	0.75	0.39
Xenobiotic	Glutathione S-transferase P *	0.89	0.73
Xenobiotic	Glutathione S-transferase A	0.88	0.63
Xenobiotic	Glutathione S-transferase P	0.88	0.64
Xenobiotic	Glutathione S-transferase kappa 1 *	0.78	0.67
Xenobiotic	Glutathione S-transferase Mu 5 *	0.84	0.64
Xenobiotic	glutathione S-transferase A2-like	0.75	0.63
Xenobiotic	Glutathione S-transferase P *	0.77	0.42
Xenobiotic	UDP-glucuronosyltransferase 2A2 *	0.8	0.46
Xenobiotic	UDP glucuronosyltransferase 5a1	0.80	0.24
Xenobiotic	UDP glucuronosyltransferase 5b3	0.73	0.17
Xenobiotic	UDP-glucuronosyltransferase 2A2 *	0.74	0.33
Xenobiotic	D-amino-acid oxidase	0.89	0.47

Figure 2.1.7. Correlation between FM levels and expression of genes encoding proteins with metabolic functions in the distal intestine. Data are correlation (Pearson) with FM in feeds without and with supplements (Supp- and Supp+). Genes downregulated in the distal intestine with dietary enteritis (Kortner et al. 2012) are marked with *.

A: lipids, retinol, steroids, iron and heme.
B: metabolism of reactive oxygen species and biotransformation.

Adding the supplements to the diets markedly reduced the effects of fishmeal level on gene expression, as seen by the numbers of genes meeting the selection criteria – 34 and 12 genes with positive and negative correlations. The effects of supplementation were strongest at low fishmeal levels and negligible at the highest level: respectively 391 and 10 DEG. Though the number of genes that responded to both FM level and Supplement was relatively small, some expression changes indicated a certain similarity in their effects, such as stimulation of xenobiotic metabolism, metabolism of amino acids, lipids, heme and iron and proteases (Figure 2.1.8). The effects were similar for diets with 0 – 17% fishmeal and markedly decreased or disappeared at the highest level (FM40). Several DEG may affect intestinal performance. *Angiotensin-converting enzyme* plays a key part in the control of intestinal microbiota (Hashimoto et al., 2012). Downregulated *perilipin* promotes the formation of lipid droplets in various fish tissues including intestine (Wilson et al., 2021). The supplement decreased expression of several gene markers of acute inflammation and stress in Atlantic salmon including *neutrophil cytosolic factor 1* and *matrix metalloproteinases 9 and 13* (Krasnov et al., 2021).

Function	Gene	FM0	FM11	FM17	FM40
Antiviral	ISG15-like	***-3.57***	-0.83	***-12.88***	1.28
Antiviral	IFN-induced protein 44-1	***-3.34***	-0.71	***-6.76***	1.33
Antiviral	IFN-induced tetratricopeptide repeats 5-2	***-2.92***	-0.97	***-5.01***	1.55
Antiviral	Gig2	***-4.10***	-0.53	***-17.25***	1.45
Antiviral	Viperin (rsad)	***-2.80***	-1.00	***-5.00***	0.99
Immune	CD209 antigen-like protein C	***2.73***	***1.78***	***2.38***	1.31
Effector	Neutrophil cytosolic factor 1	***-3.77***	***-2.09***	***-2.29***	0.96
Effector	Matrix metalloproteinase-9	***-1.80***	***-2.57***	-1.53	1.16
Effector	Matrix metalloproteinase-13	-1.37	***-2.22***	-1.09	1.31
Amino acid	B(0,+)-type amino acid transporter 1	***2.89***	1.21	***3.08***	2.00
Lipid	Acyl-coenzyme A thioesterase 12-like	***2.26***	***2.01***	***2.06***	0.96
Lipid	Acyl-CoA synthetase short-chain family 1	***2.54***	***2.44***	***2.32***	1.04
Lipid	Fatty aldehyde dehydrogenase	***2.21***	***1.93***	1.32	1.29
Lipid	Fatty acid desaturase 6	***2.17***	1.50	***2.01***	1.30
Lipid	Apolipoprotein B-100	1.40	***3.05***	***2.65***	***3.66***
Metabolism L	Perilipin	***-1.84***	***-2.29***	***-2.31***	-1.01
Xenobiotic	D-amino-acid oxidase	***1.82***	1.64	***1.85***	1.00
Xenobiotic	Carboxylesterase	1.54	1.26	***1.89***	1.14
Xenobiotic	Cytochrome P450 7A1	***2.56***	***2.35***	***2.50***	1.27
Xenobiotic	Glutathione S-transferase A1	1.71	1.26	***1.87***	***0.94***
Xenobiotic	Glutathione S-transferase A2	1.68	1.20	***1.82***	***0.95***
Xenobiotic	Fatty aldehyde dehydrogenase	***2.14***	***1.82***	1.28	1.30
Xenobiotic	Cytochrome P450 27C1	1.63	1.48	***3.12***	1.52
Xenobiotic	Cytochrome P450 1A1	***2.01***	1.58	***2.28***	1.52
Digestion	Angiotensin-converting enzyme	***1.75***	1.53	***1.80***	0.95
Digestion	Duodenase-1	***2.66***	***4.02***	***2.05***	1.49
Digestion	Lactase - Likr	***2.05***	***2.06***	***2.79***	1.12

Figure 2.1.8. Effect of the supplement on gene expression in distal intestine. Data are expression ratios (folds) of diets with and without supplement, differential expression is marked with underlined bold italics.

FM might stimulate protection by increasing expression of ROS scavengers and genes encoding enzymes of biotransformation phase I (*cytochromes p450*) and phase II (*glutathione s-transferases* and *UDP-glucuronosyltransferase*). The up-regulated *d-amino acid oxidase* neutralizes potentially toxic products of intestinal microbiota (Sasabe et al., 2016). Previous studies revealed genes downregulated in distal intestine of Atlantic salmon with enteritis induced by soy saponins and peas

(Kortner et al. 2012) and 145 of these genes including sixteen genes of xenobiotic metabolism showed positive relationship with FM (underlined in Figure 2.1.8).

Microbiota

Digesta-associated microbiota measured by alpha or beta diversity metrices in PI and DI indicated no significant compartment effect for any of the dietary groups. Therefore, combined data from PI and DI were used for these analyses.

Alpha diversity (Figure 2.1.9). A diet effect on alpha diversity metrices including Shannon index (diversity) and Observed species (richness) was observed, showing significant increase in alpha diversity in high fishmeal treatment (FM40) compared to the zero and low fishmeal treatments (FM0 & FM11, Figure 2.1.9). However, diets supplemented with functional ingredients there were no significant changes in alpha diversity among the high and low fish meal. Supplementation significantly reduced alpha diversity in high fishmeal groups (FM17>FM17S & FM40>FM40S).

Beta diversity (Table 2.1.4) showed significant differences between fish fed low and high fishmeal levels, but only for fish fed diets without supplementation. However, beta diversity in fish fed with high fishmeal diets with supplements differed from that in fish fed with the respective diet without the supplements.

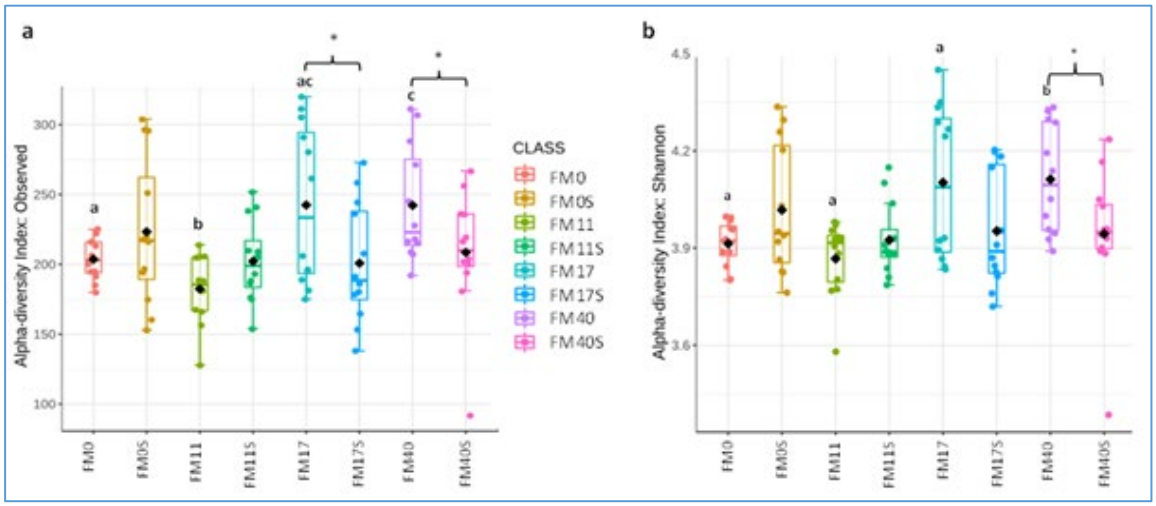


Figure 2.1.9. The alpha diversity indices for digesta-associated microbiota in the intestine of Atlantic salmon fed with diets containing diverse levels of fishmeal and their respective counterparts with functional ingredients. a) observed species and b) Shannon index. Different letters indicate significant differences among the dietary groups without supplementation. *Indicates significant difference between the dietary groups without and with supplementation.

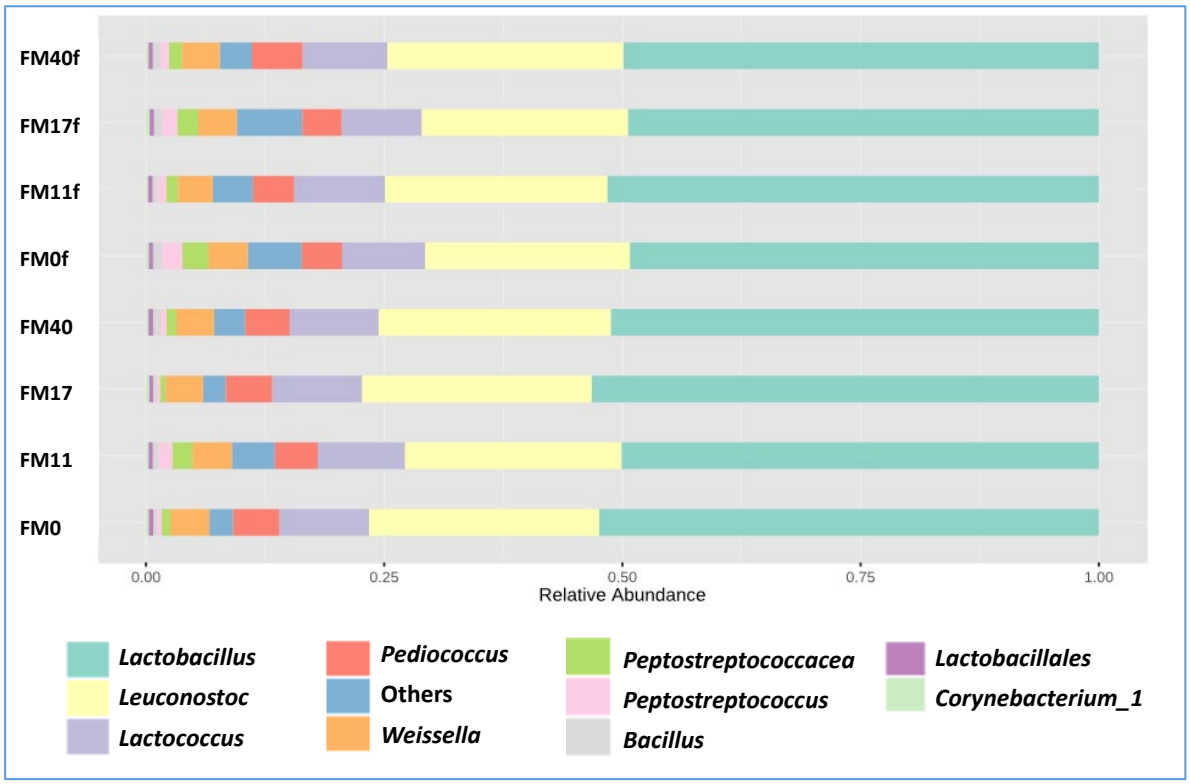


Figure 2.1.10. Results for the ten most abundant genera present in the treatments selected for these analyses, i.e., FM0, FM11, FM17, FM40, FM0S, FM11S, FM17S, and FM40S

Table 2.1.4. Beta diversity of gut microbiota in fish fed with diets containing diverse levels of fishmeal and their respective counterparts with functional ingredients

Pairwise comparisons	Bray–Curtis dissimilarity matric¤		Weighted UniFrac Distance¤	
	pseudo-F	p-value	pseudo-F	p-value
No supplement				
FM0*vs*FM11	1.1	0.339	0.1	0.393
FM0*vs*FM17	4.1	0.012*	6.99	0.018*
FM0*vs*FM40	4.1	0.001*	8.37	0.001*
FM11*vs*FM17	3.7	0.024*	7.84	0.006*
FM11*vs*FM40	3.7	0.001*	9.36	0.001*
FM17*vs*FM40	0.7	0.606	0.52	0.582
Supplemented				
FM0*vs*FM0f	2.3	0.066	3.49	0.081
FM11*vs*FM11S	1. 2	0.165	1.81	0.115
FM17*vs*FM17S	2.1	0.076	2.98	0.073
FM40*vs*FM40S	2.2	0.023*	4.44	0.021*

¤ PERMANOVA analysis with 999 permutations

* Pairs with statistically significant differences ($p < 0.05$) in beta diversity reported in the respective analysis

Dominating phyla (Figure 2.1.10). *Lactobacillus* (49-53%), *Leuconostoc* (21-25%), *Lactococcus* (8-9%), *Pediococcus* (4-5%) and *Weissella* (4%) were predominately detected in all the groups, irrespective of the dietary composition.

There was a significant increase in several bacterial genera with the addition of high fishmeal levels into the diets (Data to be published). For instance, fish fed diet with 17% (FM17) and 40% (FM40) fishmeal showed increased abundance in 14 and 21 genera ($q \leq 0.1$) respectively, compared to the diets without any fishmeal content (FM0). Eleven of the increased genera were common to both groups. Supplementation of functional ingredients to the respective diets tended to reduce abundances for most of the genera.

Discussion

Effects of varying fishmeal level

Although no significant effect of FM level on growth was observed, the relative weight of the internal organs decreased in fish fed the unsupplemented diets, the most for PI, but clear also for the liver. The sum of reduction in weight for the intestine and liver between fish fed the low and the high fish meal diet, accounted for about 2% of the body weight. As the weight of the PI with surrounding fat (not observed in this experiment) is substantially larger than the weight of the PI cleaned of fat, and in WP4 was 3 times higher, optimal choline supply, compared to deficient, can account for a substantial difference in yield, which economically would be of significant importance. Moreover, excessive lipid accumulation in the abdomen results in decreased feed utilization per unit of yield.

The histological appearance of tissue from the PI, showing that the severity of the steatosis decreased with increasing fish meal level, and the fact that steatosis symptoms were less severe or absent in the fish fed the supplemented diets, suggests that lipid transport was impaired in the low fish meal diets due to choline deficiency.

The many effects revealed by the microarray analyses on metabolism of amino acids, lipids, retinoids and steroids, iron and heme, and activity of proteases, growth, and differentiation, including DNA replication and repair, formation of tight junctions and extracellular matrix, epithelial cell differentiation, and immune functions, cannot be explained in detail, due to the use of practically relevant diets and a mixture of functional ingredients rather than single ingredients. The increasing level of fishmeal certainly altered the chemical composition of the diets greatly. Among the compounds which undoubtedly changed markedly were plant fibres and plant antinutrients for which the level was reduced, whereas the level of cholesterol, bile salts, iron, and choline increased. A great number of other components, present in lower amounts, were also changing with the change in FM level. They comprise minerals, vitamins, fatty acids, and soluble carbohydrate. It is well recognized that the animal body, for many functions, e.g., digestion, absorption, metabolism, and utilization, has the ability to adapt to changes in diet composition, seemingly in order to optimize feed utilization, growth and the ability to reproduce and tolerate stress. The observed alterations in gene expression with increasing fishmeal level in the diet, may be reflections of such, normal adaptations. They do not necessarily indicate that increasing FM level induced beneficial or detrimental responses, or that the plant ingredients were less efficient as nutrient sources. However, the changes in gene expression may be results of responses to deficient, excessive, or imbalanced composition of essential nutrients. One example which can indicate such effects, is the observed responses in *perilipin* (also named *plin2*), a highly preserved gene which codes for a protein with

functions at the surface of intracellular lipid droplets (Granneman et al. 2017). The decrease observed with increasing FM level was most likely related to decrease in lipid accumulation in the enterocytes of the PI, clearly indicated by the histology results and which correlated inversely with the content of choline in these diets.

Plotting of the PI index results on the choline levels of the diets gave a regression line which indicated an average requirement for these fish in the range 2800 to 3200mg/kg. Taking into account an estimated tank variation (SEM) for such PI results of about 400mg/kg, as indicated in the study of Hansen et al. (2020a), a choline level in the range 3600 – 4000mg/kg would cover the needs of 95% of the fish in the present study. A similar consideration of the histological results indicates that a requirement of choline in the range 3200 – 3500mg/kg would cover the needs of 95% of the fish. Both estimates are close to the requirement of 3400mg/kg estimated recently for half kilo fish in salt water (Hansen et al. 2020a). In the work of Hansen et al. a lower choline requirement was estimated based on the histological results than the PI index results. As a histological picture describes the structure in a very limited are of the PI, whereas the PI index reflects the weight of the whole gut section, the requirement-estimate based on the PI index may be the most correct.

The gut microbiota profiling indicated significant elevation in alpha diversity and change in beta diversity in fish fed diets with increasing FM level. Alpha diversity of the fish gut microbiota can be increased or decreased based on the alternative protein source used to replace FM in the diet as indicated by several previous studies. For examples, alpha diversity was reduced in Atlantic salmon gut microbiota when the FM was partially replaced with soyabean meal and wheat gluten (Gajardo 2017) while alpha diversity was increased in the same species when the FM was fully replaced with insect meal (Li et al 2021). On the other hand, no significant difference was observed in alpha diversity in gut microbiota of rainbow trout fed with FM rich commercial diet or a full terrestrial plant-based diet devoid of fishmeal (Pérez-Pascual et al. 2021). Similar to the observation made in our study, diet induced beta diversity changes are commonly observed for digesta-associated microbiota in fish including Atlantic salmon (Gajardo 2017, Pérez-Pascual 2021, Li et al 2021).

The predominant genera *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus* and *Weissella* detected in the digesta of the fish irrespective of the dietary composition is in agreement with the results of earlier studies (Gajardo et al. 2016; Wang et al. 2021) showing that these genera are most often among the core digesta-associated microbiota in Atlantic salmon. This predominant occupancy of 88% (FM17and FM40) - 95% (FM0-FM11) of the gut microbiota by the members of lactic acid bacteria despite the varying level of FM in the diet, without and with the supplementation, may indicate their importance as core microbiota of the salmon during seawater stage. Lactic acid bacteria are presumed to have beneficial effects on salmon health and function through immune regulation, disease resistant and improvement of digestive process (Balcázar et al. 2007; Ringo & Gatesoupe 1998; Ringoe et al. 2010).

Effects of supplementation with a mixture of choline, β-glucans, and nucleotides

Some of earlier studies of effects of the functional ingredients investigated in the present study confirm the present results of absence of effects of the supplement mix on growth and feed conversion. Krogdahl et al (2020a) studied effects of choline, Refstie et al. (2010) β-glucan, and Wang et al. (2020) nucleotides. However, growth promoting effects of choline was documented in a publication (Hansen et al. 2020b), and for β-glucan and nucleotides, a number of review papers

present documentation for growth promoting effects (See reviews by Ringø et al (2012) and Fuchs et al. (2015)). The variation between studies may be related to variation in several aspects, e.g., in dosage, feeding regime, stocking density, developmental stage of the fish, and water parameters. Fuchs et al. (2015)) concludes for turbot at on-growing stages that, under optimal production conditions, diet additives probably do not have beneficial impacts. If supplementation with functional ingredients improves growth and immune function and increases resistance to stress and disease, it, most likely, is the diet composition which is not optimal. This consideration is supported by the gene expression results from the distal intestinal samples in the present study, which showed that the effect of supplementation was significant at low FM level and negligible at the highest Most fish nutrition researchers agree that present knowledge on requirement of many of the essential nutrients for optimal growth and disease resistance (NRC 2011) is insufficient. Until the knowledge is substantially strengthened, the mechanisms underlying beneficial effects of supplementation with functional ingredients cannot be understood. Moreover, use of such ingredients comes with a cost, not only in money, but seemingly and logically also as increased energy needs (Wang et al. 2020).

As no literature has been found indicating that either β-glucan or nucleotides may reduce lipid accumulation in the pyloric region and increase plasma levels of triglycerol and cholesterol, the choline in the supplement mix was most likely the cause these effects. This statement is supported by the fact that most of the genes which showed altered expression in the DI are involved in lipid transport and metabolism.

The fact that the distal intestine of the fish in the present study showed only vague signs of inflammation, most pronounced for the high FM diets, the possible preventing effects of β-glucan and nucleotides cannot be discussed in detail based on the observation given by this study. Supplementation increased, however, expression of two glutathione s-transferases, enzymes of phase II biotransformation even at the highest FM level, which might enhance protection.

The supplements significantly altered both the alpha and beta diversity, but only in the gut microbiota of the fish fed the high FM diet. This effect on microbiota could possibly be related to the high choline level in the diet, which increased continuously from the zero FM diet without supplement to the high FM diet with supplement.As we observed a clear difference in gut microbiota between the fish fed low and high FM diets, the supplement-induced effects exclusively in the microbiota of high FM diet fed fish may be due to the combined effect of FM level and supplements, possibly due to the additive effect of high choline levels from both sources. High choline levels might have reduced microbial richness and diversity and changed beta diversity. Observations made in weaned piglets (Qiu et al. 2020) of reduced richness and diversity of colonic microbiota upon choline supply, seem to be in line with this suggestion.

It seems that the supplements equalized the gut microbiota profiles in the fish fed the high FM diet to the profiles of the fish fed the low FM diets, i.e., no differences made for the alpha and beta diversity values between FM40S group and the FM0 group even though the latter group significantly differed from FM40.

Conclusions

Increasing level of fishmeal in the diet

- did not affect FCR, nor TGC, but increased yield due to reduction in lipid accumulation in the gut and liver weight
- improved digestibility of saturated and monounsaturated fatty acids as well as protein
- decreased somatic index of PI and MI, and symptoms of steatosis in the PI, supposedly related to increasing level of choline in the diets, a consideration supported by the gene expression results
- affected various metabolic processes and structural proteins with minor changes in the immune system
- indicated a choline requirement of 3600 – 4000mg/kg for the fish in this experiment
- increased microbial richness and diversity and changed composition of the gut microbiota in the digesta

Supplementation with a mixture of choline, β-glucan, and nucleotides

- did not affect FCR or growth
- greatly reduced relative weight of PI and symptoms of steatosis in the pyloric caeca
- gave clear effects on lipid transport and metabolism, indicating that the choline in the supplement was the main causing agent
- decreased gene markers of acute inflammation and stress
- stimulated genes involved in nutrient and xenobiotic metabolism in fish fed lower FM diets
- reduced richness and diversity and changed composition of gut microbiota in the fish fed diets with high FM levels

WP2.2 Ex vivo and in vitro investigations

Responsible partner HI

Even though chlorpyrifos (O,O-diethyl-O-3,5,6-trichlor-2-pyridyl phosphorothioate, CPF) was banned in the European union January 2020 due its harmful effects on brain development, it is still widely used in other parts of the world. Consequently, it finds its way into salmon feeds due to the use of imported plant ingredients. In this WP effects of CPF on permeability of the anterior intestine Atlantic salmon which had been exposed to a diet based on soy meal was investigated. Further, the effect of CPF on intestinal mucosal cells were studied using a cell line.

Methodology

Ex vivo gut sac analysis

The intestine of fish from experiment described in WP2.3 were analysed for permeability. Twelve fish from the control group and twelve from the soy meal (high in saponins) group were sacrificed with a blow to the head and the mid intestinal section were dissected. Gut sacs were prepared according to Mateer et al. (2016) with slight modifications. The intestinal segments of 5 -8 cm were immersed in Ringer's solution and the luminal content was gently flushed out. The posterior end of the intestine was then closed with a surgical suture before the segment was filled with 500 µM chlorpyrifos (CPF) in Ringer's with FITC-D or only Ringer's with FITC-D as a control.

The filled gut sacs were rapidly mounted in individual glass tubes with 45ml Ringer's solution and aerated with 97 % O_2 and 3 % CO_2. Tubes were immersed in a water bath regulated to 12°C, illustrated in Figure 2.2.1.

A sample of 100 µl Ringer's solution was collected from each tube every 20 min. and replaced by a fresh batch of 100 µl. Samples were taken for a total of 3 hours and measured at excitation wavelength 493 nm and emission wavelength of 518 nm on a multi label plate reader (PerkinElmer). At the end of the experimental period the intestinal sacs were cut open exposing the mucosal surface. The length and the width of each intestinal segment was measured. Tissue from the gut sac was also sampled for histological analysis on 4 % formaldehyde in phosphate buffered saline (PBS).

The average FITC-D counts for the standard were calculated and the concentration was found for each sample absorbance on the standard curve. The sample FITC-D concentration was determined by plotting the FITC-D counts of the standard versus the concentration of the FITC-D standards. For each time point the sample FITC-D concentration was converted to cumulative concentration (Qt) from the equation below.

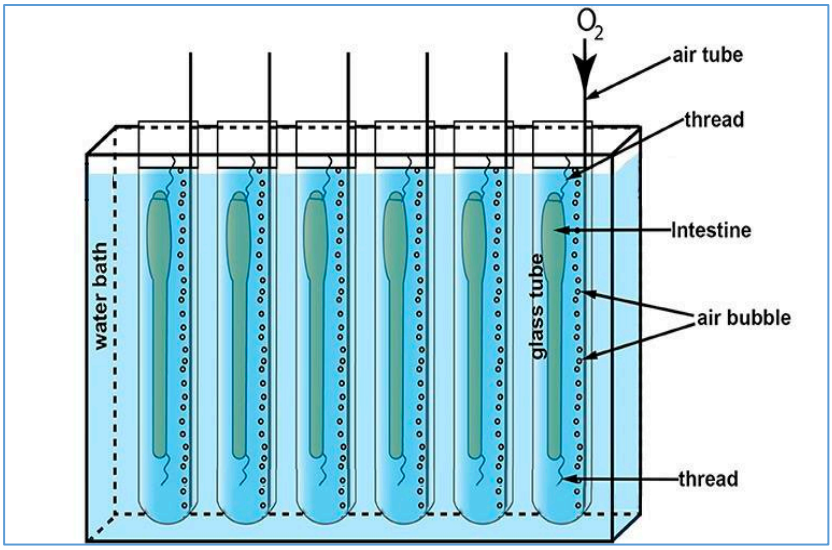


Figure 2.2.1. In vitro experimental setup. The prepared anterior intestinal sacs were placed in glass tubes containing Ringer's solution and immersed in a cold-water bath. Intestinal sacs were filled with Ringer's solution containing both FITC-D and CPF or just FITC-D (control). Oxygen was supplied to each glass tube with an individually mounted air tube.

$Qt=Ct \times Vr + \sum QtxVs$

(Qt= Cumulative concentration at time t; Ct= Concentration of the sample FITC-D at time t; Vr= Volume at the receiver side (external medium); Qt sum= Sum of all previous Qts; Vs= Volume sampled)

The slope of the dQ/dt, was calculated by plotting the cumulative concentration Qt versus time. At last, the apparent permeability was calculated for each intestinal sac from the following equation.

$Papp=\delta Q/\delta t \times 1/A \times C0$

(Papp= Apparent permeability; A = Area of the tissue; C0=Initial concentration of FITC-D inserted in sacs)

In vitro RTgutGC cell line

RTgutGC is a cell line established from a primary culture derived from the distal part of the gut of a female rainbow trout *Oncorhynchus mykiss* (Kawano et al. 2011*)*. These cells are heteroploid and possess an epithelial like morphology. It has also been reported that RTgutGC cells express alkaline

phosphatase activity which is an indication of enterocyte differentiation (Kawano et al. 2011). In previous studies, RTgutGC cells have been grown as monolayers on permeable supports, leading to a two-compartment intestinal barrier model consisting of a polarized epithelium (Kawano et al. 2011). The idea behind this is to mimic the *in vivo* intestinal lumen. RTgutGC is a physiologically adequate fish intestinal barrier model equivalent to the Caco-2 human intestinal epithelial cell line, which is used to study fish intestinal immune and barrier functions (Wang et al. 2019).

Culture. The RTgutGC cell line were aseptically cultured in L-15 medium containing 10% FBS and incubated at 19 °C. The cells were grown in 75 cm^2 cell culture flasks for 5-7 days, until they were harvested for experimental purposes. Cells were harvested by discarding the culture medium from the flask followed by rinsing the cell layer twice with 8-10 ml PBS. The cells were enzymatically detached from the flask by adding 1-2 ml of trypsin for 2 minutes in room temperature. The trypsination reaction was stopped by adding 10 ml of fresh culture medium and the detached cells were aspirated gently by pipetting. The resulting cell suspension was centrifuged at 1000 rpm, 19 °C for 3 minutes. Cells were resuspended in 1-2 ml growth medium, after discarding the supernatant. The density of the cells was determined by loading 10 µl of cell suspension on to a hemocytometer and the number of cells were counted manually. The cells were diluted to required volume in culture media and seeded in wells prior to experiments. Cells were split in 1:2 ratio for maintenance, after reaching confluency.

CPF effect on cell viability. xCELLigence Real Time Cell Analysis (RTCA) (Agilent) surveil physiological changes in cells. Cells were seeded at a density of 20,000 cells/well in 100 µl culture media in a E96 xCELLingence plate (Aligent). The plate was placed back in the xCELLigence and the adhesion, growth and proliferation of the cells were monitored every 15 min for 24 hours. 24 hours after seeding, the cells were exposed to a dose range of 0.05 - 500 µM CPF. Controls received medium with 0.2% DMSO and some wells were treated with 10% triton as a positive control. After exposure, the experiment was run for approximately 40 hours.

Validating epithelia formation and pesticide exposure. To evaluate the epithelial integrity of the RTgutGC barrier, transepithelial electrical resistance (TEER) was measured. Cells were seeded in trans well membrane inserts with 0.4 µm pore size at a density of 150,000 cells/ml L-15 medium per insert (1 ml added). Membrane inserts were plated in a 12-well plate filled with 1 ml of L-15 in each basolateral compartment. Cells were incubated at 19 °C, with a change of medium, allowing the cells to form a barrier. For the control (blank), empty (cell-free) membranes were filled with 1 ml of media in both apical and basolateral chamber. TEER levels were measured over time by using an EVOM voltmeter meter with STX2 chopstick electrodes according the EVOM instruction manual. The unit area resistance (Ω cm2) was calculated by subtracting the values obtained from the blank from membranes containing cells and by multiplying by the growth area of the insert. Cells were grown for at least 10 days at 19 °C before pesticide exposure experiments. Cells were exposed to CPF concentrations of 0.5, 5, 50, 100, 500 µM and DMSO 0.2% (control). The TEER was then measured 24 hours after exposure.

CPF exposure trial for mRNA quantification. Cells were seeded in six-well plates in 3 ml complete L-15 medium at a density of 600,000 cells/well and incubated at 19 °C for 3-4 days prior to exposure with a change of medium. The cells were then exposed to 0.5, 5, 50, 100, 500 µM CPF and 0.2% DMSO (control) in triplicates for 24 hours. After 24 hours of pesticide exposure, cells were washed twice with PBS. For lysing of cells 600 µl of RLT Plus buffer was added to each well and mixed well by

pipetting up and down a few times. The lysate was transferred to individual 1.5 ml tubes and stored at - 80 °C until further processing. See Fernando (2021) for details on RNA isolation, cDNA synthesis, primers and qPCR

Results

Ex vivo gut sac study of permeability of inflamed gut tissue for chlorpyrifos (CPF)

The apparent intestinal permeability for each intestinal segment for different experimental groups are plotted in Figure 2.2.2. The average apparent permeability of the mucosa for fish fed with SBM diet injected with 500 µM CPF and 0 µM CPF was 1.01 x 10-5 cms-1 and 1.31 x 10-5 cms-1 respectively. The average permeability for the FM fed group (control) injected with 500 µM CPF and 0 µM CPF was 1.50 x 10-5 cms-1 and 1.60 x10-5 cms-1 respectively.

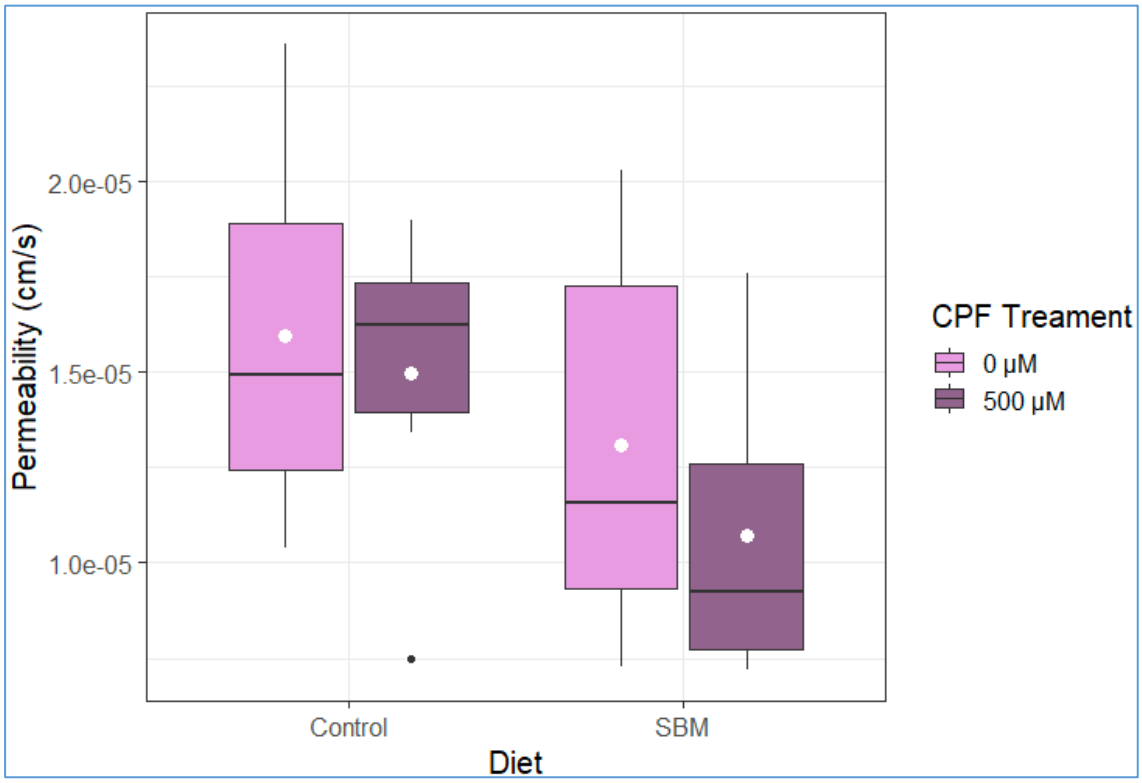


Figure 2.2.2. Apparent permeability of Atlantic salmon intestinal sacs of each experimental group and the effect of CPF treatment. Control 0 µM and Control 500 µM represents fish fed with fish meal later injected 0 and 500 µM CPF in the intestinal sac, respectively. SBM 0 µM and SBM 500 µM represents fish fed with soybean meal later injected with 0 and 500 µM CPF in the intestinal sac, respectively. The Lower box limit represents Q1 (lower quartile), middle – median and upper limit - Q3 (upper quartile). White circle - mean, black dots – outliers.

In vitro RTgutGC cell line

CPF effect on cell viability. The strength of cell adhesion is an expression of the physiological state of the cells and is represented as the Cell Index (CI) which is a unit-less measurement. The response to CPF exposure between 0.05 - 500 µM on the cells after 24 hours is shown in Figure 2.2.3 (linear fit), and Figure 2.2.4 (logistic regression). Controls (DMSO 0.2%) reached a mean cell index (CI) value of 2 ± 0.02. RTgutGC cells exposed to CPF up to 5 µM showed no significant difference compared with the

controls. Cells exposed to 10, 20, 50, 100 and 500 µM was significantly different compared with the controls (ANOVA). Cells exposed to 500 µM CPF died, giving a mean CI value around 0. CI of all positive controls (n=3) treated with 20% triton dropped down to negative values and a similar observation was made in one well of cells exposed to 500 µM CPF. Cells exposed to 5 µM

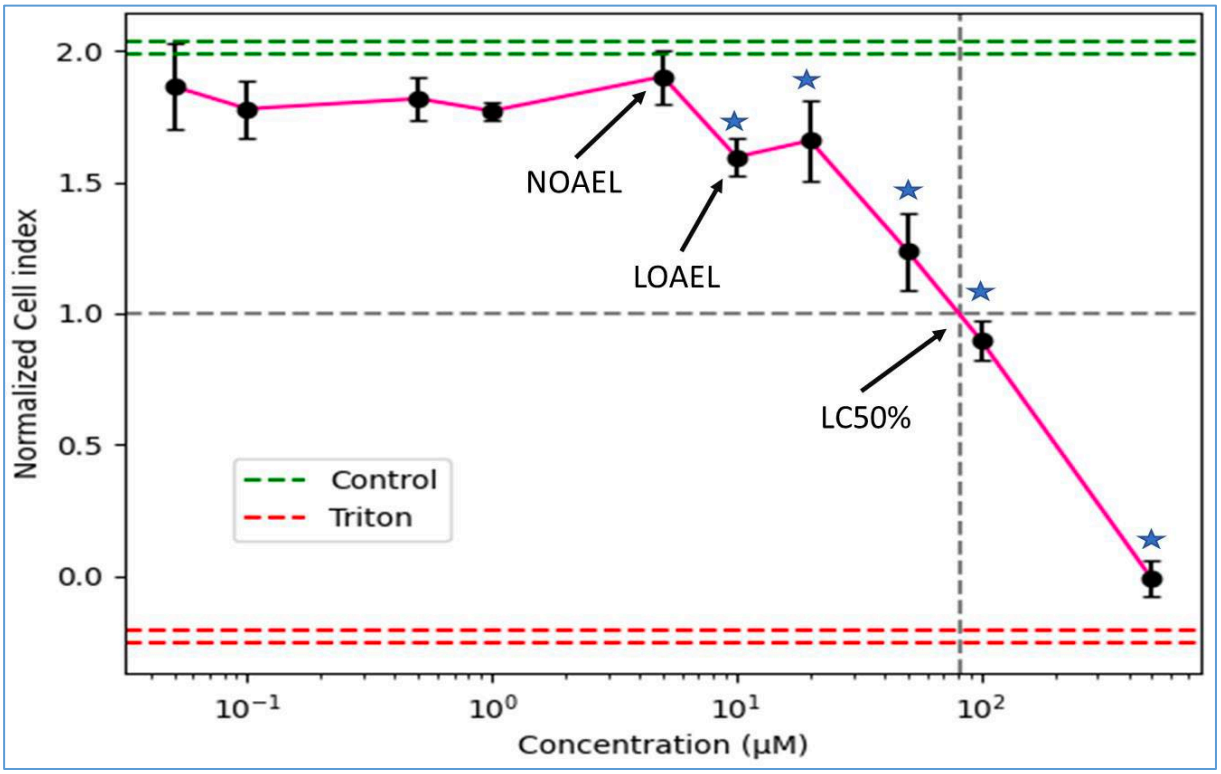


Fig. 2.2.3. Response curve of the cell index of RTgutGC cells upon CPF exposure. xCELLigence data shows cell adhesion as mean cell index ± SD of three replicates. Significant difference observed between the control and the exposed group by one-way ANOVA is indicated by star ($p < 0.05$). NOAEL = No observed adverse effect level, LOAEL = Lowest observed adverse effect level, Green lines = ± SD interval of the Control, Red lines = ± SD interval of the Triton 20%, Grey line = LC50% (Lethal concentration 50%).

CPF had a similar response to the control, indicating the NOAEL (No Observed Adverse Effect Level) value; and cells exposed to 10 µM CPF, being the lowest concentration significantly different from the control, indicating the LOAEL (Lowest Observed Adverse Effect Level) value. The LC50 of CPF in RTgutGC cells, as determined by linear interpolation, was 84 µM (Figure 2.2.4). According to the logistic regression plot there is little or no effect in the adhesion of cells at low concentrations of CPF. Beyond the concentration of 5 µM CPF the regression line starts to decline indicating a high adhesion of cells. From concentrations beyond 10 µM there is a clear decline in the adhesion of cells. At 500 µM CPF the adhesion of cell is a fraction of the adhesion at low concentrations. The measured BMR5 and LC50 for CPF in RTgutGC cell in this analysis are 13.5 ± 0.06 and 89 ± 0.12 µM at 95% confidence, respectively.

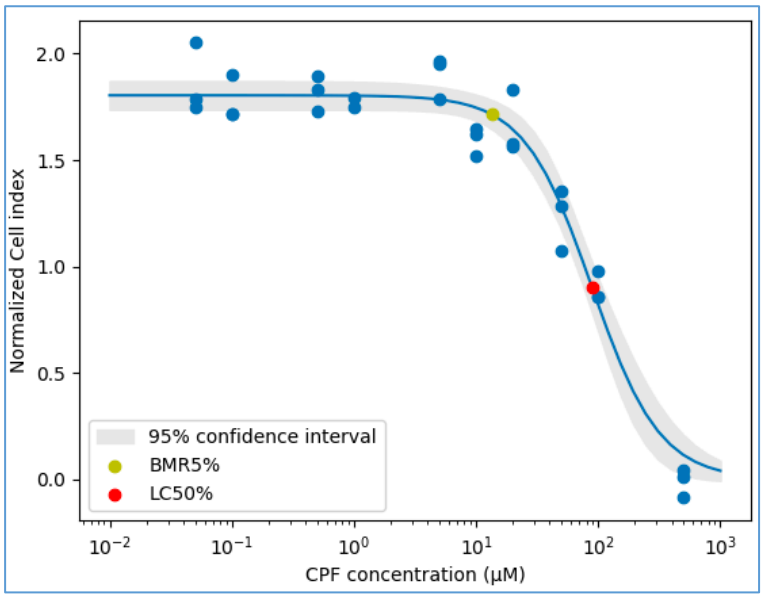


Fig.2.2.4. Sigmoid curve fit of the exposure response curve for CPF and Cell Index of RTgutGC cells. Cells were exposed to CPF in the dose range of 0.05 – 500 µM for 24 h using a xCELLigence system. Control and 20% Triton is not included in this analysis. Blue line = sigmoid curve, Blue dots = raw data, Yellow dot = Benchmark response 5% (BMR5), Red dot = Lethal concentration 50% (LC50), Grey line = 95% confidence interval.

Transepithelial electrical resistance. The effect of CPF on permeability of the RTgutGC cell monolayer after 24 hours is shown in Figure 2.2.5.

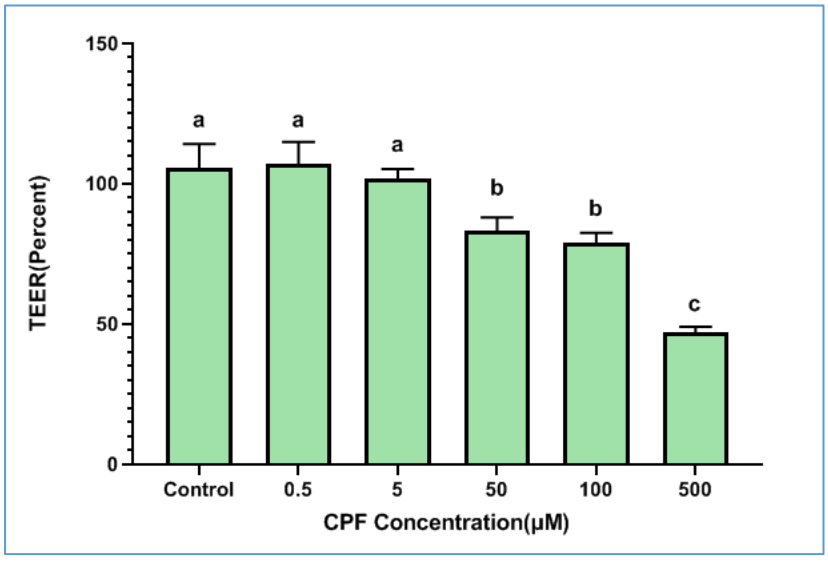


Fig 2.2.5. Transepithelial electrical resistance (TEER) values in RTgutGC cells after treatment with different concentrations of chlorpyrifos (CPF) for 24 h compared with the initial value. Results are expressed as the mean ± SD of three independent experiments. Different letters (a,b,c) indicate significant differences in TEER (One - way ANOVA, $p < 0.05$).

The TEER values are shown in percent, where the TEER values measured 24 hours after the addition of CPF divided by the TEER values measured before the addition of CPF multiplied by 100. The average baseline TEER values of the monolayers (on day 10) varied from 59 -76 Ω·cm2. Cell cultures in L-15 media containing 0.2% DMSO (control) did not significantly alter the initial TEER values after 24 hours. CPF caused a dose dependent decrease in TEER values. TEER levels of cells exposed to low concentrations of CPF were not affected, however TEER levels exposed to higher concentrations of

CPF (50 – 500 µM) were significantly lower than those of control cells. Cell death was induced in all wells exposed to 500 µM CPF, decreasing the TEER values close to background levels.

Gene expression. Figure 2.2.6 shows the induction of the cyp1a, cyp3a, gstr and ugt2a2 mRNA in RTgutGC cells 24 hours after exposed to 0.5 – 100 µM CPF. All four genes, cyp1a (p = 2.07e-12), cyp3a (p = 8.23e-07), gstr (p = 0.000113) and ugt2a2 (p = 2.16e-09) were significantly affected by the exposure. cyp1a was significantly upregulated at 0.5 and 5 µM CPF with a fold change of 8.8 and 6.9, respectively. cyp1a seemed to downregulate with the increasing CPF concentration. cyp3a expression was significantly upregulated at 50 µM CFP with a fold change of 1.86. Both gstr and ugt2a2 mRNA expression was significantly downregulated at 100 µM CPF but was not significantly altered in cells exposed to lower concentrations of CPF.

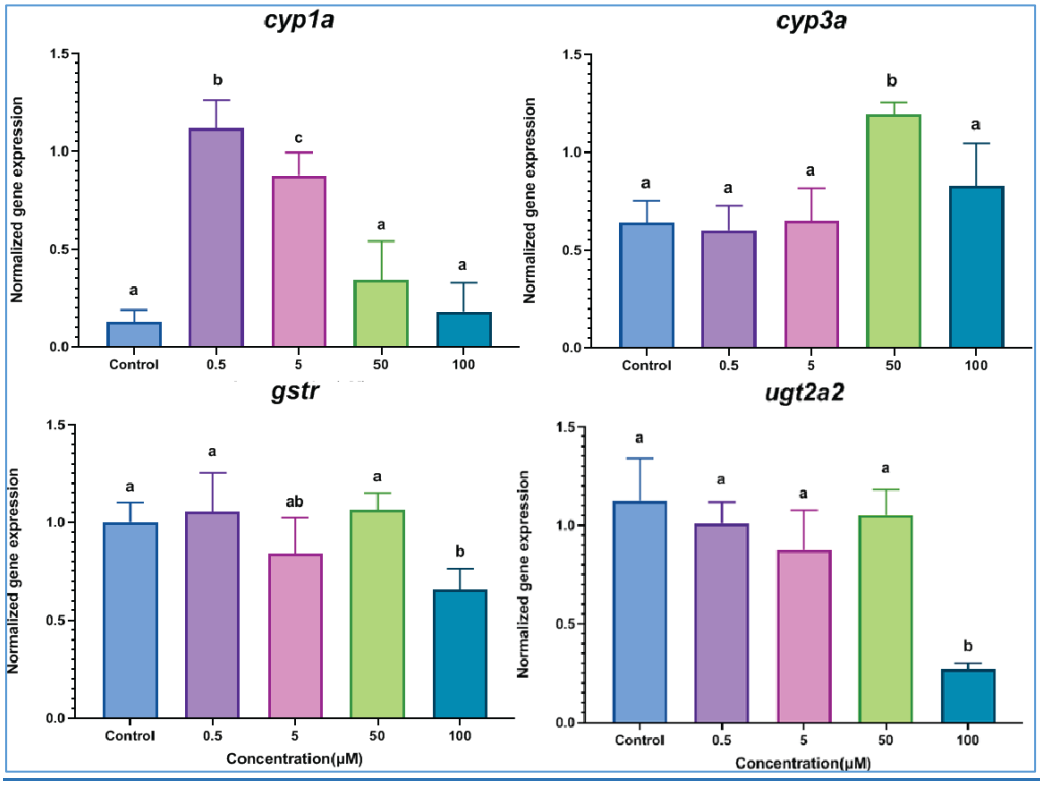


Figure 2.2.6. Expression of genes involved in detoxification, A) cyp1a, B) cyp3a, C) gstr, D) ugt2a2 in rainbow trout gut cells (RTgutGC) exposed to chlorpyrifos (CPF) and DMSO control (0.2%) for 24 hours. mRNA expressions are represented as mean normalized expression and the values represent mean±SD of six replicates (n=6). Different letters indicate statistical differences in mean values between treatments (One-way ANOVA, p<0.05).

The expression of genes involved in the lipid metabolism in RTgutGC cells 24 hours after exposed to 0.5 – 100 µM CPF are shown in Figure 2.2.7. *Lpcat2* (p = 0.00545) and *plin2* (p = 3.1e-13) were significantly affected by CPF exposure. The mRNA expression levels of *lpcat2* significantly decreased at 100 µM CPF. *lpcat2* expression slightly increased at 50 µM but was not significantly different compared to the control. The expression levels of *plin2* peaked at 50 µM CPF. *plin2* mRNA levels of

the cells exposed to 50 and 100 µM CPF had a fold change of 9.8 and 4.8 respectively compared to the control.

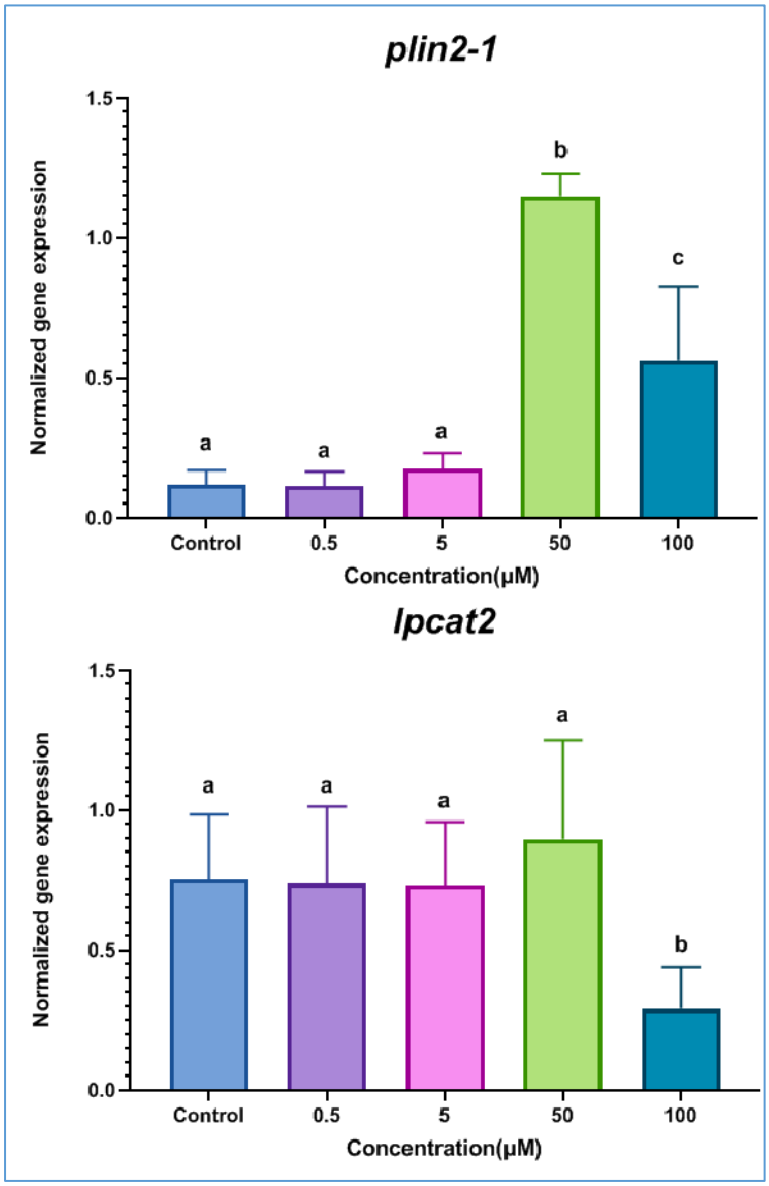


Figure 2.2.7. Expression of genes involved in lipid metabolism, lpcat2 and plin2-1 in rainbow trout gut cells (RTgutGC) exposed to chlorpyrifos (CPF) and DMSO control (0.2%) for 24 hours. mRNA expressions are represented as mean normalized expression and the values represent mean±SD of six replicates (n=6). Different letters indicate statistical differences in mean values between treatments (One-way ANOVA, $p<0.05$)

Discussion

Our first approach to study the impact on intestinal integrity of CPF was to use the well-established gut sac model (Mateer et al. 2016; Whittamore et al. 2016). We did preliminary tests to establish the concentration that showed increased permeability and found that 500 µM CPF significantly increased permeability (Fernando 2021). However, in the present study 500 µM CPF had no effect on permeability. The only hypothesis we have to offer is that the fish in this study was larger than the fish used for the preliminary studies.

Gut sacs from the group of fish that was fed soy meal, high in antinutrients such as saponins was believed to show increased leakage. However, this group did not differ from the controls. Nor did the gut sacs exposed to CPF. Chikwati et al. (2013) and van den Ingh et al. (1991) have demonstrated saponin induced inflammation in the hind gut of salmon, but not in the more anterior parts. This is in agreement with our results. The histology of the used gut sacs was investigated, and no indication of breached integrity was found. All groups displayed large amounts of mucosal cells, but no differences between treatments. Histology did not reveal any degree of inflammation either.

According to the international standard for in vitro cytotoxicity testing (ISO, 2009), the reduction of cell viability to contaminants by more than 30% is considered a cytotoxic effect. Based on the ANOVA analysis the xCelligence system showed 38%, 55% and 100% cell viability reduction, from the controls, in cells exposed to 50, 100, 500 µM CPF, respectively. The regression model showed 34%, 52% and 100% cell viability reduction from the baseline. Therefore, we can confirm that RTgutGC cells exposed to CPF concentrations of 50 µM and upwards induce cytotoxicity. Babín & Tarazona (2005) found CPF to be the most potent compound out of six pesticides tested in rainbow trout liver (RTL-W1) and rainbow trout gonadal (RTG 2) cell lines in a Neural red and FRAME KB protein assay and the inhibition of EROD activity was observed at the lowest tested concentration 0.02 mg/L for CPF. Søfteland et al. (2014) and Olsvik & Søfteland (2020) observed that Atlantic salmon hepatocytes exposed up to 1000 µM CPF and chlorpyrifos-methyl (CPM) were not cytotoxic, which is not in line with our results. These studies have used primary hepatocyte cell cultures and the reason for the differences in sensitivity between salmonid primary hepatocytes and cell lines to CPF is unknown (Søfteland et al. 2014).

Traditionally, NOAEL and LOAEL have been used to determine the point of departure (POD), which is a point on a dose/exposure response curve corresponding to estimated low effect or no effect level (EFSA 2016). Since this approach has limitations, the benchmark response (BMR) approach provides a promising method for calculating the POD (Crump 1984; EFSA 2016). NOAELs and LOAELs are based upon a hypothesis testing approach and provides a less detailed visualization and quantitative description of a toxic response than a regression model (Landis et al. 2017). Therefore, the BMR5 and LC50 determined from the regression model in this study would yield more accurate values.

As the xCelligence system allows for accurate measurements of the physiological state of the cells, the TEER measurements measure the integrity of the monolayer created by the cell line. In other words, the TEER analysis should also be able to pick up deteriorating tight junctions. The ANOVA analysis showed a decrease in TEER at 50 µM CPF. However, there was a decrease in TEER from 0.5 to 50 µM CPF, meaning that the cell layer became more permeable and therefore leaked more. We therefore conclude that the risk of increased leakage in intestinal epithelium starts at a much lower concentration than 50 µM.

CPF effect on gene regulation. CPF can undergo bioactivation by CYP1A to a more toxic metabolite called CPF-oxon, which is more potent and less stable than the parent compound (Sams et al. 2004). The sulphur ion released in CPF activation is highly reactive and is believed to bind immediately to the heme iron of the CYP protein inhibiting its activity (Neal 1980; Tang et al. 2002). It is also possible that CPF-oxon may inhibit the aryl hydrocarbon receptor (AhR), also called the xenobiotic receptor,

involved in gene regulation, or enzymes involved in the AhR pathway or increase the AhR repressor proteins, resulting in decreasing cyp1a expression levels.
Several studies have shown the upregulation of cyp1a mRNA expression after CPF exposure in different fish species (Jeon et al. 2016; Søfteland et al. 2014; Xing et al. 2014), which agrees with our results. This indicates that chlorpyrifos induces cyp1a expression, but that the expression may be inhibited at higher concentrations.

CYP3A is an important phase I enzyme mainly expressed in the intestine (Lee & Buhler 2003). Atlantic salmon hepatocytes exposed to CPF showed no significant effects on cyp3a expression (Olsvik et al. 2019). Jeon et al. (2016) showed upregulation and Ma et al. (2015) showed downregulation of cyp3a in zebrafish and goldfish exposed to CPF, respectively. These conflicting results demonstrate that regulation of cyp3a genes after CPF exposure is dependent on the species analysed.

Glutathione S-transferase (*gst*) and UDP-glucuronosyl-transferase (*ugt*) are genes coding for enzymes involved in the phase II of xenobiotic detoxification. In the present study *gst* and *ugt* did not respond to the treatment of CPF, except in the cells exposed to 100 µM CPF, which was significantly downregulated compared to the control. Although no significant difference was observed in the *gst* and *ugt* expression at low exposure concentrations in our study, these enzymes may still play an important role in the detoxification of CPF. However, the response of *gst* and *ugt* to toxicants is most likely dependent on the type of xenobiotics, concentration and time of exposure and the species involved (Botté et al. 2012).

To investigate if CPF may intervene with lipid metabolism, we analysed *perilipin 2* which is a controlling factor in cytosolic lipid droplet formation and *lpcat2*, a limiting factor in phospholipid remodelling. The increase in *plin2* at 50 µM CPF demonstrate that the cells are storing more lipid. Storage of lipid is a way for a cell to protect itself during stress (Jarc & Petan 2019). *Lpcat* was not affected by the lower concentrations of CPF indicating that phospholipid remodelling, vital for membrane maintenance was not initiated by CPF.

Conclusion

The integrity of the anterior intestine of salmon seems to be resilient to both saponin and CPF. This is in agreement with earlier histological analysis that only found inflammations in the hind gut post saponin exposure. Cell studies show a lethal concentration 50% (LC50) of 89 µM CPF. However, cells display signs of disrupted lipid metabolism at 50 µM CPF and membrane integrity at concentrations as low as 13.5 µM CPF.

WP2.3 Controlled trial II – Remediation of gut inflammation

Responsible partner VM-Nutr + NOFIMA

The aim of WP2.3 was to find whether supplementation with functional ingredients could remediate diet induced inflammation in the distal intestine of Atlantic salmon. Two diets with potentially inflammation-inducing ingredients which were studied without and with supplementation with mixtures of functional ingredients. The strategy was to observe differences between the basal diets and effects of the supplements on growth and feed utilization, structure, and function of the digestive tract, and on the microbiota of the distal compartment in which diet induced inflammation most often occur. Dysbiosis in the gut, which include loss of overall microbial diversity, loss of

beneficial microbial organisms, and expansion of pathogens or potentially harmful microorganisms, is recognized as key factors for immune function and disease resistance in an animal (Petersen & Round 2014).

Methodology

Fish, management and feeding

The feeding part of the experiment was conducted at Nofima's research facility at Sunndalsøra and lasted for 69 days. The fish used were Atlantic salmon with an initial weight of 177g. Eighteen flow-through tanks with seawater, 1.5m^2 surface area were used. The ambient water temperature averaged at 11.2C° over the feeding period. Oxygen level in the water was kept between 80 and 100%. Each tank was stocked with 57 fish which were fed in 15 – 20% surplus of expected requirement. The amount of uneaten feed was recorded allowing estimation of feed intake (Helland et al. 1996).

Feed composition

Six diets were made (Table 2.3.1): one control diet (Contr) with high fish meal level and five experimental diets low in fish meal. Three of the latter contained soybean meal an inducer of severe inflammation, the remaining two contained corn gluten and peameal, both considered to induce mild inflammation. Two packages (P1 and P2) of functional ingredients were made. The composition of the functional ingredient packages was chosen based on recommendations from the producers of the functional ingredients and supported by the feed industry partners involved in the project. P1 contained 0.01% butyrate and 1.5% arginine and P2 contained 0.1% β-glucan, 0.01% butyrate and 0.05% nucleotides. The soybean diet (SBM) was tested with both packages, the corn-pea diet (CoPea) only with P2. The six diets were produced by Skretting AS at their experimental feed production unit.

Sampling at termination of the feeding period

At termination of the feeding period, six fish per tank were euthanized by an overdose of MS-222 (0.05–0.08 g/l). The protocols used for sampling procedures were similar to those described by Wang et al (2021).

Sample analyses

The analytical procedures employed in the present WP were similar to those described above for WP2.1, and presented in more detail in earlier papers of ours (See (Li et al. 2020) and Wang et al. (2021). This regards analyses of diet and faecal macronutrient composition; biochemical biomarkers in plasma; histology of samples from the distal intestine (DI); microbiota composition in gut content and mucosa from the DI region. Expression of genes in tissue from the DI of salmon was conducted using Nofima's 15k Atlantic salmon oligonucleotide DNA microarray SIQ6 (GPL30031) as described by Krasnov et al. (2021), and plasma metabolome according to (Aru et al. 2021).

Table 2.3.1. Feed composition*

Diets	Contr	SBM	SBM+P1	SBM+P2	CoPea	CoPea+P2
Feed ingredients, %						
Fish meal	35.0	5.0	5.0	5.0	5.0	5.0
Soybean meal (HP)		30.0	30.0	30.0		
Corn gluten					25.0	25.0
Pea meal					7.5	7.5
Wheat gluten	20.0	21.0	21.0	21.0	20.0	20.0
Faba bean	5.0	5.0	5.0	5.0	5.0	5.0
Soy protein concentrate (SPC)	12.2	12.5	12.5	12.5	12.4	12.5
Wheat	10.6	6.8	5.3	6.6	4.0	4.1
Fish oil	8.5	9.4	9.3	9.4	10.6	10.4
Rapeseed oil	8.1	8.9	8.9	9.0	7.3	7.2
Astaxanthin	0.01	0.01	0.01	0.01	0.01	0.01
Vitamin Mix	0.11	0.11	0.11	0.11	0.11	0.11
Mineral Mix	0.1	0.1	0.1	0.1	0.1	0.1
Yttrium premix	0.1	0.1	0.1	0.1	0.1	0.1
Inositol		0.124	0.124	0.124	0.124	0.124
Inorganic phosphate	0.602	1.529	1.505	1.529	1.794	1.781
Methionine		0.195	0.188	0.195	0.088	0.083
Lysine		0.551	0.523	0.551	1.246	1.228
Choline	0.229	0.443	0.443	0.443	0.443	0.443
Functional ingredients, %						
β-Glucans				0.1		0.1
Butyrate			0.01	0.01		0.01
Nucleotides				0.05		0.05
Arginine			1.5			
Analysed nutrient composition, %						
Dry matter	92.3	92.2	92.6	92.0	93.0	92.4
Lipid	19.4	18.1	18.3	19.1	21.2	19.7
Choline, mg/kg	0.152	0.150	0.143	0.168	0.152	0.149
Nitrogen	8.4	7.3	7.6	7.4	7.7	7.8
Ash	6.1	4.5	4.4	4.5	4.1	4.1
Yttrium	0.008	0.007	0.008	0.007	0.007	0.007
Estimated values						
Gross Energy, MJ/kg	22	20	20	20	22	22
DP/DE, g/kg	22	20	20	20	22	0.22

*P1 and P2: functional package 1 and 2. Package 1 (P1) contained 0.01% butyrate and 1.5% arginine and package 2 (P2) contained 0.1% β-glucan, 0.01% butyrate and 0.05% nucleotides.); DP/DE: estimated ratio digestible protein/digestible energy.

Statistical evaluation

For the results of histology, gene expression, microbiota, metabolome and data evaluation see earlier detailed description (Wang et al. 2021). One-way ANOVA was used for the remainder of the variables employing the SAS 9.3 computer software (SAS 2017).

Results and discussion

Effects on growth, nutrient digestibility, digestive functions, and gut histology

The results regarding growth, estimated as TGC, and feed conversion ratio (FCR) based on all fish in the tanks are shown in Figure 2.3.1, whereas body measures taken for the fish sampled for further analyses are shown in Table 2.3.2. The fish fed the Contr diet showed the highest growth rate, with significantly higher values than for fish fed the SBM diet and the SBM+P2 diet. Fish in the SBM+P1, the CoPea and the CoPea+P2 diets showed intermediate values not differing significantly from the former diets. The body measures observed for the fish sampled for further analyses showed a similar picture as the means of all fish and are considered representative. The result for FCR showed a corresponding picture, i.e., lowest values for the Contr fed fish, highest for those fed the SBM diet.

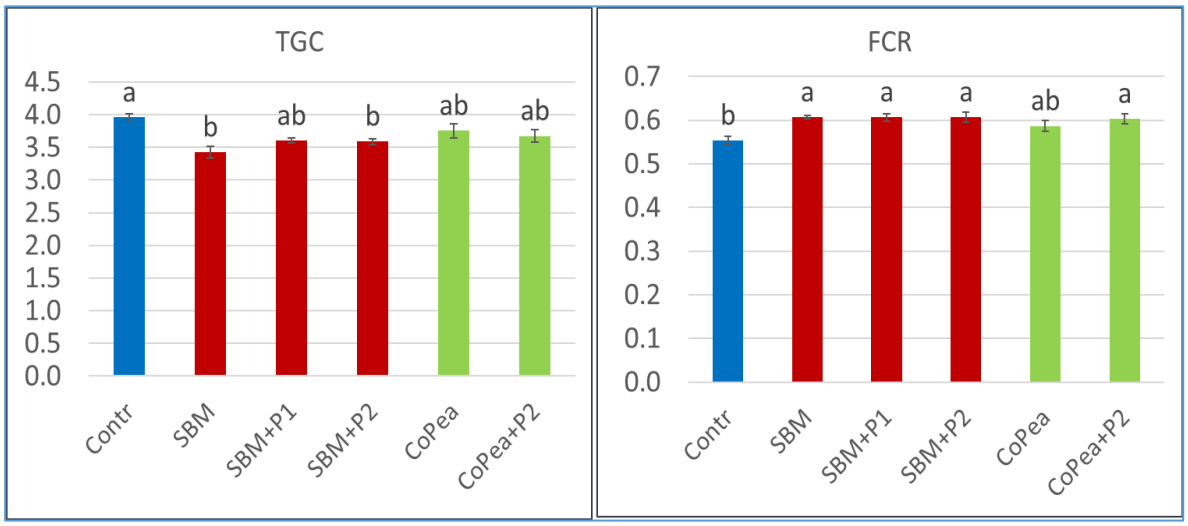


Figure 2.3.1. Results regarding growth estimate as thermal growth coefficient (TGC) and feed conversion ratio (FCR).

The figures 2.3.2-4 illustrate results regarding gut function such as relative weights of the proximal and distal intestine, capacity of leucine aminopeptidase (LAP) in PI and DI, chyme bile salt concentration the PI2 and DI1, and trypsin activity in the chyme of DI. They are all biomarkers which have shown clear responses upon feeding diets with various legume ingredients, such as soybean and pea meal, which induce inflammation in the intestine. Tables 2.3.3.-5. show dry matter of the chyme along the intestine, digestibility results for the evaluated diets and plasma biochemical markers, respectively.

Table 2.3.2. Results of observations made on sampled fish

	Control	SBM	SBM+PC1	SBM+PC2	CoPea	CoPea+PC2	P(model)
Body weight, g	644[a]	598[ab]	582[b]	599[ab]	630[ab]	645[a]	0.0453
Body length, cm	35.1	34.4	34.6	34.3	34.7	35.1	0.4370
Condition factor	1.50[a]	1.43[b]	1.40[b]	1.50[a]	1.50[a]	1.50[a]	0.0004
Carcass weight, g	574[a]	527[b]	511[b]	525[b]	552[b]	567[b]	0.0367
Rel. liver weight, %	1.13	1.2	1.17	1.17	1.27	1.2	0.0668

The relative weight of the proximal section of the intestine (PI) (Figure 2.3.2) was higher for fish fed the SBM diets and the CoPea diet than for fish fed the Contr diet, and intermediate for fish fed the CoPea+P2 diet. The higher values for the fish fed the plant rich diets are suggested to be due to the high content of plant ingredients with high fibre content of these diets. The high supply of fibre, which may challenge digestive functions, might have triggered an increase digestive capacity with increased tissue weight as a result. Also, the presence of antinutrients in these ingredients might have caused such effects. The results are in line with previous studies and corresponding observations of stimulated digestive capacities seen in other animals (Liener 1994; Nitsan et al. 1983). The reduction in plasma cholesterol level in fish fed the soybean containing diets, demonstrates the presence of soybean antinutrients , among which saponins have the most pronounced effect (Krogdahl et al. 2015).

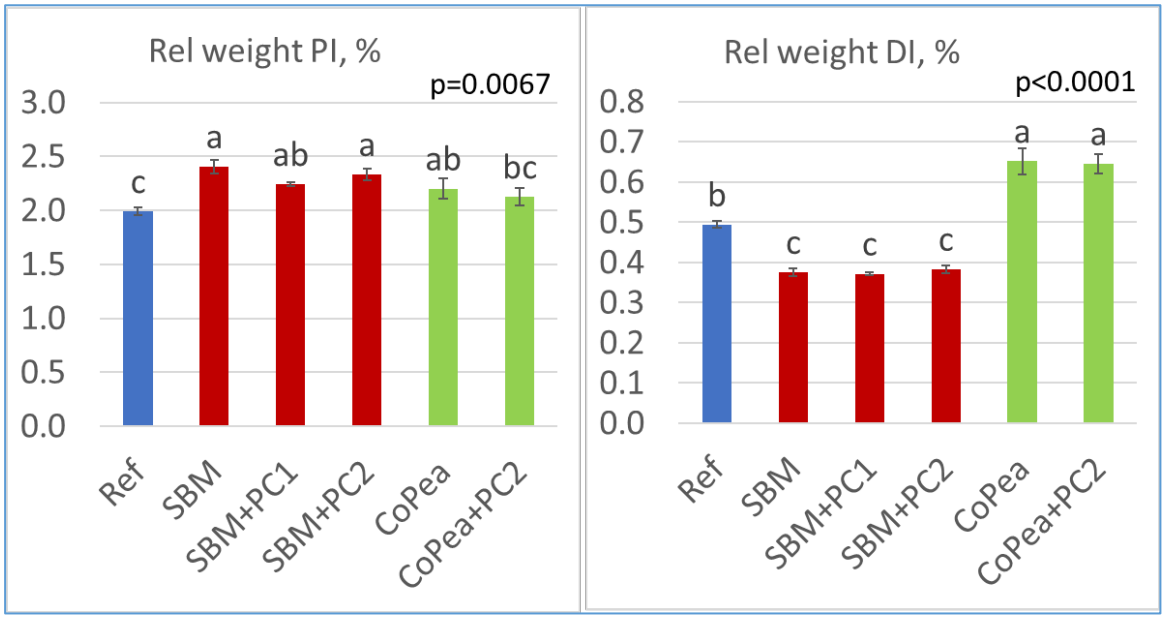


Figure 2.3.2. Results regarding relative weight (% of body weight) of the proximal (PI) and distal (DI) intestinal tissue. The results for the mid intestine showed no significant differences between treatments and averaged 0.17%, ranging from 0.17 – 0.18.

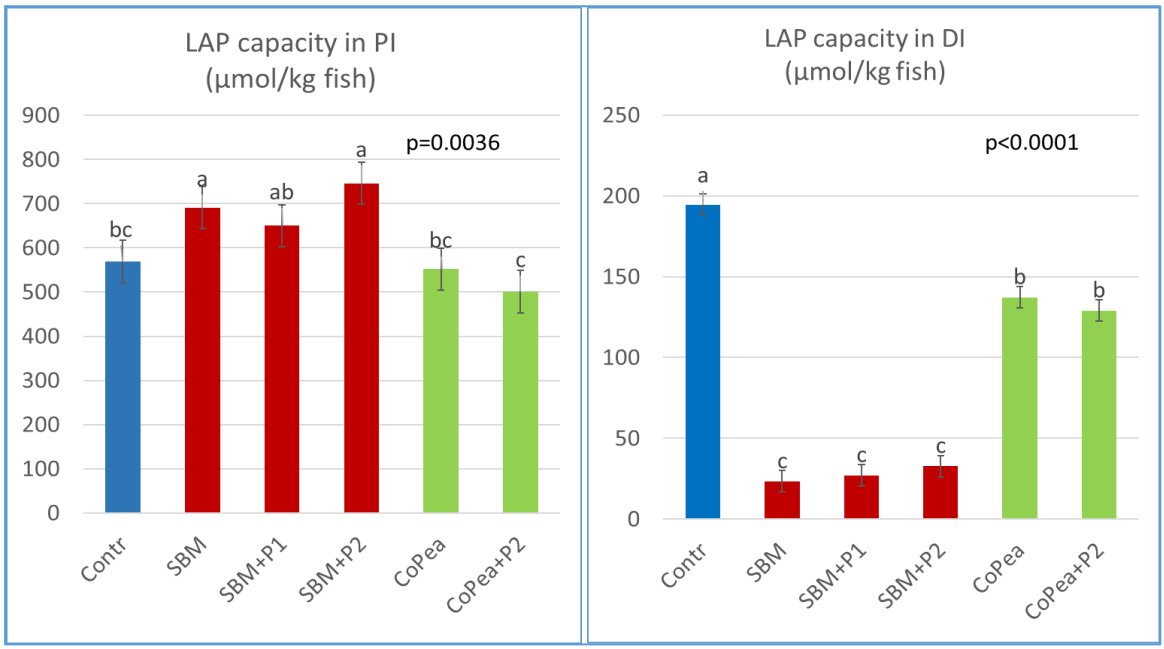


Figure2.3.3. Results regarding capacity of leucine aminopeptidase in the pyloric intestine (PI) and distal intestine (DI) expressed as µmol substrate produced per kg fish.

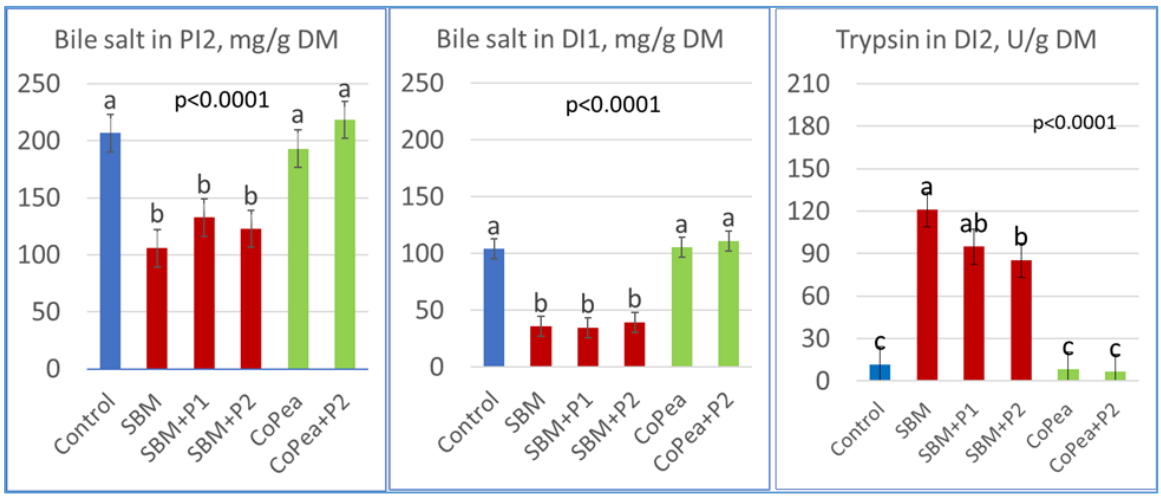


Figure 2.3.4. Results regarding concentration of bile salt (mg/g dry matter) in chyme from the distal half of the pyloric intestine (PI2) and activity of trypsin (U/g dry matter) in chyme from the distal intestine (DI).

The DI showed a different picture with greatly reduced relative tissue weight for the fish fed the SBM diets, with no significant difference between the three SBM diets. The effects in the SBM fed fish are in accordance with observations in several earlier studies addressing gut effects of standard qualities of soybean meal (Krogdahl et al. 2015; Krogdahl et al. 2020b) and correspond to the many signs of inflammation in this part of the intestine. They comprise reduction in LAP capacity, increase in chyme trypsin activity, a dry matter content of the chyme indicating diarrhoea, and not least the histological signs which all showed signs of inflammation (Figure 2.3.5). Supplementation of the SBM diets with the functional ingredients did not diminish the effects induced by the SBM on growth, nutrient

digestibility, digestive functions, and gut histology, neither for P1 or P2. The only exception was the diminishing effect of the P2 on trypsin activity in the chyme of DI (Figure 2.3.4) which might indicate a slight reducing effect on the tissue stress.

Table 2.3.3. Dry matter in chyme along the intestine, %

Sample	Contr	SBM	SBM+P1	SBM+P2	CoPea	CoPea+P2	*p(model)*
PI1	19.2^{b}	16.3^{c}	15.8^{c}	17.9^{bc}	22.1^{a}	18.2^{bc}	*0.0017*
PI2	20.6	19.6	16.6	19.0	21.0	19.1	*0.2964*
MI	17.9^{ab}	16.5^{c}	16.7^{bc}	16.9^{bc}	18.7^{a}	17.9^{ab}	*0.0175*
DI1	16.3^{a}	16.3^{a}	12.2^{b}	13.2^{ab}	16.4^{a}	15.0^{ab}	*0.0458*
DI2	15.9^{a}	10.6^{b}	10.2^{b}	10.9^{b}	15.5^{a}	14.4^{a}	*<0.0001*
Faeces	14.1^{ab}	10.3^{ab}	10.2^{bc}	10.0^{c}	16.6^{a}	13.6^{abc}	*<0.0001*

Table 2.3.4. Digestibility of crude protein, lipid and ash*, %

	Contr	SBM	SBM+P1	SBM+P2	CoPea	CoPea+P2	*p(model)*
Crude protein	89.6^{c}	90.1^{bc}	91.2^{ab}	90.9^{ab}	91.5^{a}	91.5^{a}	*0.0006*
Lipid	96.9	97.0	97.2	96.7	96.4	96.4	*0.3500*
Ash	1.8^{a}	-21.3^{b}	-20.3^{b}	-22.0^{b}	-1.4^{a}	-6.4^{a}	*0.0001*

*For ash the numbers are not indicating minerals absorbed from the diet, as minerals in drinking water is not accounted for in the input. The data indicate malfunction in the reabsorption of the minerals for the fish fed the SBM diets.

Table 2.3.5. Plasma biomarkers

Diet	Contr	SBM	SBM+P1	SBM+P2	CoPea	CoPea+P2	p(model)
Alanine transferase	13.6	18.3	12.6	13.9	15.3	15.3	0.8963
Cholesterol	9.7^{a}	8.4^{b}	8.3^{b}	8.5^{b}	9.5^{a}	9.0^{ab}	0.0093
Triglycerides	3.6	3.1	3.7	4.0	4.0	3.7	0.5270
Glucose	4.6	5.1	4.7	4.7	4.6	4.9	0.0912

Signs of DI inflammation in fish fed the CoPea diet were few, comprising clear cell infiltration in the submucosa and reduced LAP capacity in the tissue. No preventive effects of the supplementation were observed.

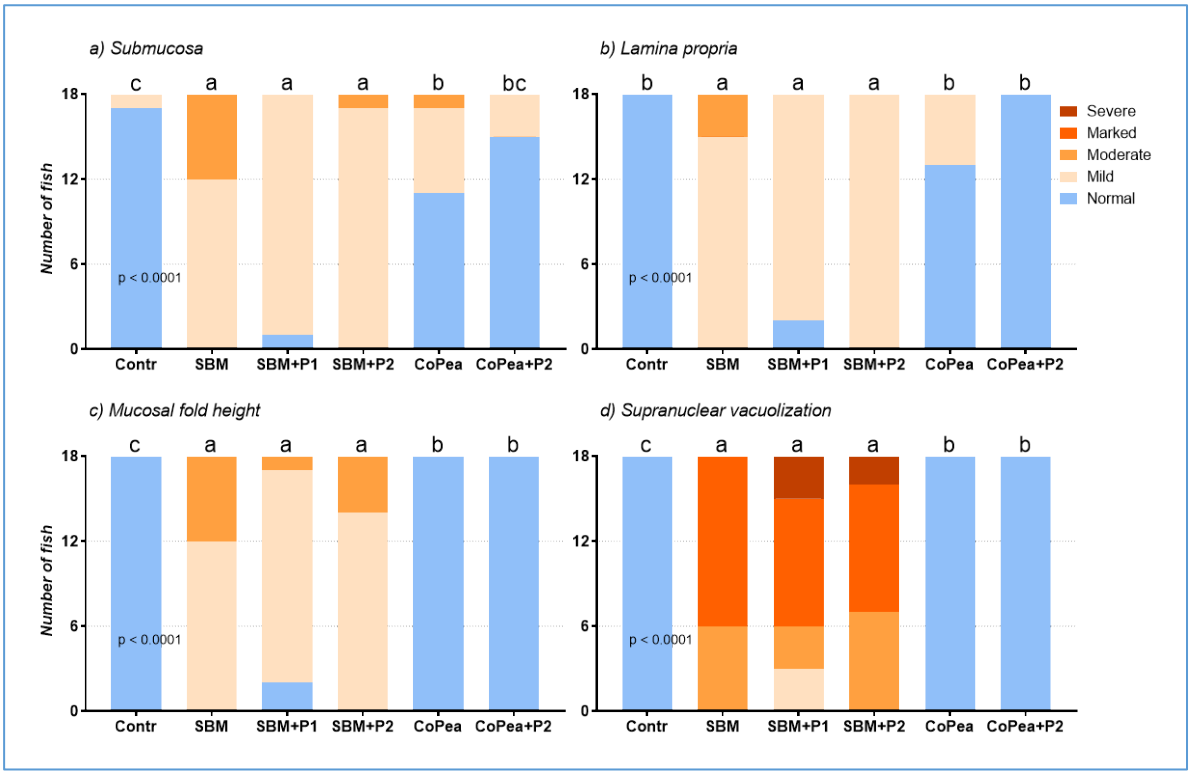


Figure 2.3.5. Results of histological evaluation of tissue from the DI regarding width and cellularity of submucosa (a) and lamina propria (b), mucosal fold height (c) and supranuclear vacuolization (d) graded as normal, mild, moderate, marked, and severe.

Gene expression

The analysis of gene expression in the DI tissue (Table 2.3.6 and Figure 2.3.6-8) generally confirmed the results presented and discussed above regarding effects on growth, nutrient digestibility, digestive functions, and gut histology. The number of genes showing different expression (DEG) between the Contr and the SBM fed fish was high, 849. The DEGs showing the greatest differences (fold change >1.5) between these treatments belonged to functional groups (Figure 2.3.6) such as protein folding, transport, mitochondria, proteases, lysozymes, vitamin metabolism, lipid metabolism, xenobiotic metabolism, lectin, immune regulator, and TNF-related. Details regarding specific genes affected among genes involved in stress and immune responses, and in metabolism are shown in figure 2.3.7 and 8. The results illustrates in detail the great impact of including soybean meal in salmon diets on the function of the DI.

Likewise, the gene expression analyses confirm the marginality of the difference between the Contr and CoPea fed fish, showing 26 DEGs, with significant effect only for the functional group proteases, vitamin metabolism, lipid metabolism, and more specifically only for the gene transcobalamine-2 which protects B12 from degradation.

The gene expression analyses also confirm the results discussed above regarding minor effects of P1 and P2 when supplemented to the SBM diet (81 and 121 DEGs, respectively). The effects of P1 regarded the functional groups chemokine, lectin, and immune regulator. Specific immune genes significantly affected were CC-motif chemokine 19-4, lectin putative proteoglycan and matrix metalloproteinase 9. Supplementation of the SBM diet with P2 affected more functional groups than

P1 and showed significant effects on the group protein folding and modification, amino acid metabolism, transport, mitochondria, antigen presentation, chemokine, lectin, and immune regulator. Regarding specific genes, significant effects were observed on the stress and immune genes DNA damage-inducible 4 protein, thioredoxin, cytochrome b-245 light chain, and arginase type II. Regarding genes classified as involved in metabolism, significant effects were observed for cysteine dioxygenase type 1, sulfontransferase, long-chain-fatty acid CoA ligase ACBG1, intestinal fatty acid binding protein, fatty acid desaturase 6, cytochrome P450 2D15, and carboxylesterase.

Table 2.3.6. Numbers of differentially expressed genes (DEGs) in tissue from the DI

Comparison	No of DEGs
SBM vs Contr	849
CoPea vs Contr	26
SBM+P1 vs SBM	81
SBM+P2 vs SBM	121
CoPea+P2 vs CoPea	49
SBM vs CoPea	698
SBM+P1 vs SBM+P2	133

Functional group	No	SBM-CL	CoPea vs control	SBM+P1 vs SBM	SBM+P2 vs SBM	CoPea+P2 vs CoPea	SBM vs CoPea	SBM+P1 vs SBM+P2
Protein folding & modification	24	***1.5***	1.0	1.0	***-1.2***	1.0	***1.5***	***1.2***
Oxidative stress response	20	***-1.7***	1.2	1.0	1.2	-1.1	***-2.1***	-1.2
Amino acid metabolism	34	-1.6	1.0	1.1	***1.2***	1.0	***-1.6***	0.9
Transport	26	***-2.3***	-1.1	1.0	***1.2***	1.1	***-2.1***	***-1.3***
Mitochondria	32	***-1.8***	-1.1	1.1	***1.2***	1.0	***-1.7***	-1.1
Proteases	46	***-1.5***	***-1.2***	-1.1	***1.1***	1.0	-1.3	***-1.3***
Lysosomes	9	***-2.2***	1.0	1.0	***1.2***	-1.2	***-2.1***	***-1.2***
Retinoid metabolism	10	-1.4	1.0	1.0	***1.3***	1.0	-1.4	***-1.3***
Vitamin metabolism	5	***-2.5***	***-1.1***	1.0	***1.3***	1.0	***-2.4***	***-1.3***
Cytochromes P450	10	-1.7	1.0	1.1	***1.5***	1.3	-1.7	***-1.4***
Lipid metabolism	60	***-2.0***	***-1.1***	1.0	***1.3***	***1.1***	***-1.8***	***-1.4***
Xenobiotic metabolism	45	***-1.9***	-1.1	1.1	***1.3***	1.1	***-1.8***	***-1.3***
Antigen presentation	18	***-1.5***	***1.8***	***-1.7***	1.2	-1.2	***-2.7***	***-2.0***
Chemokine	14	1.8	1.3	***-1.9***	***-1.6***	-1.2	1.4	-1.1
Lectin	13	***1.7***	-1.2	***-1.8***	***-1.6***	1.2	***2.0***	-1.2
Immune regulator	21	***1.9***	1.1	***-1.4***	-1.2	1.0	***1.7***	***-1.2***
TNF-related	9	***1.8***	1.1	-1.3	-1.1	1.1	***1.6***	-1.2

Figure 2.3.6. Effect of feeds on gene expression in distal intestine: functional groups. Data are folds; numbers of DEG are indicated. Significant expression differences ($p < 0.05$) are highlighted with underlined bold italics.

Gene	SBM vs Contr	CoPea vs Contr	SBM+P1 vs SBM	SBM+P2 vs SBM	CoPea+P2 vs CoPea	SBM vs CoPea	SBM+P1 vs SBM+P2
Heat shock cognate 70	***4.0***	-1.0	-1.2	-1.5	1.1	***4.1***	1.2
Heat shock protein 90, alpha	***2.1***	1.0	-1.4	-1.7	-1.1	***2.0***	1.2
Jun B-1	***4.0***	1.8	***-2.3***	-1.9	1.4	2.2	-1.2
Immediate early response 2-2	***2.0***	1.1	-1.5	1.0	1.3	***1.8***	-1.5
Immediate early response 5-1	***2.6***	1.2	-1.1	-1.0	1.1	***2.2***	-1.1
DNA damage-inducible 4 protein	***3.0***	2.1	-1.2	***2.0***	-1.2	1.4	***-2.4***
Thioredoxin	***-2.8***	1.1	1.4	***3.0***	-1.6	***-3.1***	***-2.1***
Superoxide dismutase [Mn]	***-1.8***	-1.2	1.3	1.4	-1.0	-1.5	-1.1
Selenoprotein P, plasma, 1	***-3.8***	-1.4	1.2	1.6	-1.2	***-2.7***	-1.3
Beta-2 microglobulin	***-1.8***	1.2	-1.3	1.2	-1.1	***-2.2***	-1.5
H-2 class I HC antigen, Q10 alpha chain	-7.7	2.9	-1.3	1.9	-1.7	***-22.2***	-2.4
MHC class I antigen	***-3.6***	1.2	-1.7	-1.1	1.1	***-4.3***	-1.6
C-C motif chemokine 19-4	***11.7***	2.4	***-3.8***	***-4.8***	-1.6	***4.8***	1.3
Chemokine CK-1 precursor	***4.1***	1.1	-1.6	-1.3	1.0	***3.7***	-1.3
Arachidonate 5-lipoxygenase-activating	***2.0***	-1.0	-1.6	-1.2	1.1	***2.1***	-1.3
Interleukin-6	***-2.7***	1.1	-1.3	1.2	-1.1	***-3.1***	-1.6
Interleukin-11	***4.2***	1.2	-1.4	1.3	1.1	***3.4***	-1.8
Interleukin-18	***2.3***	1.0	-1.1	-1.2	1.1	***2.2***	1.1
TNF decoy receptor	***3.6***	1.3	-1.7	-1.7	1.1	***2.7***	1.0
C1q and TNF domains	***4.2***	1.1	-1.3	-2.5	-1.0	***3.7***	1.9
Complement C1q protein 2	***3.3***	1.2	-2.0	-1.7	-1.6	***2.9***	-1.2
Cathelicidin (cath)	***2.8***	1.0	-1.9	-2.1	1.1	***2.7***	1.1
E-selectin	***4.2***	1.5	***-2.8***	-2.9	-1.1	***2.8***	1.0
Lectin, putative proteoglycan	2.6	-1.3	***-3.9***	-2.5	-1.2	***3.4***	-1.6
Cytochrome b-245 light chain	***4.8***	1.6	***-2.2***	***-2.1***	-1.1	***3.0***	-1.1
Arginase, type II	***13.4***	-1.0	-1.4	***-3.0***	1.0	***14.0***	2.1
Matrix metalloproteinase-9	1.5	-1.9	***-2.1***	-1.6	1.7	***2.8***	-1.3
MMP 13 or Collagenase 3	***4.9***	-1.2	1.1	-1.7	1.0	***5.7***	1.9

Figure 2.3.7. Effect of feeds on gene expression in distal intestine: genes involved in stress and immune responses. Data are folds; differential expression is highlighted with underlined bold italics. Genes – markers of Atlantic salmon responses to stress, inflammation and bacterial infections (Krasnov, A. et al. 2021) are in bold.

Gene	SBM vs Contr	CoPea vs Contr	SBM+P1 vs SBM	SBM+P2 vs SBM	CoPea+P2 vs CoPea	SBM vs CoPea	SBM+P1 vs SBM+P2
Solute carrier family 13	***-1.9***	-1.1	1.3	1.7	-1.3	***-1.8***	-1.4
Aquaporin 8b	***-8.1***	1.2	-1.8	1.1	1.2	***-9.8***	-2.1
Transcobalamin-2	***-33.8***	***-1.8***	1.7	2.1	1.1	***-19.1***	-1.2
Solute carrier family 6	***-3.1***	-1.1	-1.0	1.4	1.1	***-2.8***	-1.3
4-aminobutyrate aminotransferase	***-12.8***	-1.1	-1.1	2.3	1.3	***-11.3***	-2.7
Cysteine dioxygenase type 1	***-4.1***	-1.2	-1.0	***1.8***	1.2	***-3.5***	***-1.8***
Heme-binding protein 2	***-97.0***	-1.7	1.5	1.2	1.6	***-57.0***	1.3
Heme oxygenase	***-3.5***	-1.4	-1.1	1.2	1.3	***-2.4***	-1.3
Tissue inhibitor of metalloproteinase 2a	***-4.7***	1.3	1.1	-1.0	1.2	***-6.1***	1.1
Meprin A subunit alpha	***-5.6***	1.1	-1.4	1.3	1.1	***-6.0***	***-1.9***
Serine carboxypeptidase CPVL	***-3.4***	-1.1	-1.1	1.5	1.1	***-3.1***	-1.6
Angiotensin-converting enzyme	***-3.0***	-1.2	1.1	1.2	-1.2	***-2.6***	-1.1
7-dehydrocholesterol reductase (DHCR7)	***-10.4***	-1.4	-1.3	1.8	-1.0	***-7.7***	-2.4
Vitamin D3 hydroxylase-associated protein	***-2.6***	1.0	-1.0	1.3	-1.2	***-2.6***	-1.3
3-oxo-5-beta-steroid 4-dehydrogenase	***-2.3***	-1.1	1.1	1.2	-1.2	***-2.1***	-1.1
Solute carrier family 26 member 6	***-2.4***	-1.2	1.3	1.6	-1.0	***-2.0***	-1.2
Sulfotransferase	***-12.4***	-1.1	1.5	***1.9***	1.2	***-11.8***	-1.3
Apolipoprotein A-II	***-2.8***	1.0	1.4	1.8	-1.1	***-2.9***	-1.3
Acyl-coenzyme A thioesterase 11	***-11.5***	-1.4	-2.2	1.6	***2.6***	***-8.1***	-3.5
Long-chain-fatty-acid--CoA ligase ACSBG1	***-6.7***	-1.3	1.1	***2.4***	1.7	***-5.1***	***-2.2***
Fatty acid-binding protein, intestinal	***-3.7***	-1.2	-1.5	***2.0***	1.1	***-3.2***	***-2.9***
Fatty acid desaturase 6	***-11.5***	-1.5	1.3	***2.7***	1.4	***-7.9***	-2.0
Cytochrome P450 2F3	***-3.2***	-1.2	1.1	1.5	-1.0	***-2.8***	-1.4
Cytochrome P450 2D15	***-2.7***	1.1	1.3	***2.0***	-1.1	***-3.0***	-1.6
Cytochrome P450 XXVIA1	***-3.5***	1.0	1.2	1.7	-1.0	***-3.4***	-1.4
Cytochrome P450 1A1	-2.0	1.1	-1.0	1.2	1.3	***-2.1***	-1.2
Carboxylesterase	***-3.4***	-1.2	1.6	***1.9***	1.4	***-2.9***	-1.2
UDP-glucuronosyltransferase 2A2	***-3.4***	-1.2	-1.2	1.5	1.7	***-2.9***	***-1.8***
Guanylin precursor	***-4.0***	-1.4	1.1	1.5	-1.3	***-2.9***	-1.4

Figure 2.3.8. Effect of feeds on gene expression in distal intestine: genes involved in metabolism. Data are folds; differential expression is highlighted with underlined bold italics (Krasnov, A. et al. 2021) .

Metabolome

The metabolome results confirmed the modesty of the diet effects observed for biochemical markers. Exploratory principal component analysis (PCA) performed on the plasma metabolite concentrations showed no clear separation between plasma samples from fish fed the basal diets: Control, Corn/Pea, and SBM (Figure 2.3.9). Overall, the individual fish distribution along PC1 and PC2 (explaining about 33% and 13% of variation in the data, respectively, Figure 2.3.9) mostly reflected the high interindividual variability.

PCA was performed separately to analyse the Corn/Pea and SBM diets. As before, no clear separation was revealed between the CoPea diets, and sample distribution in the PC1 vs PC2 biplot (approx. 47% of the explained variance) was dominated by the great interindividual differences (Figure 2.3.10a). The same picture can be observed in Figure 2.3.10b, in which the comparison amongst the SBM diets is presented (approx. 38% of the explained variance).

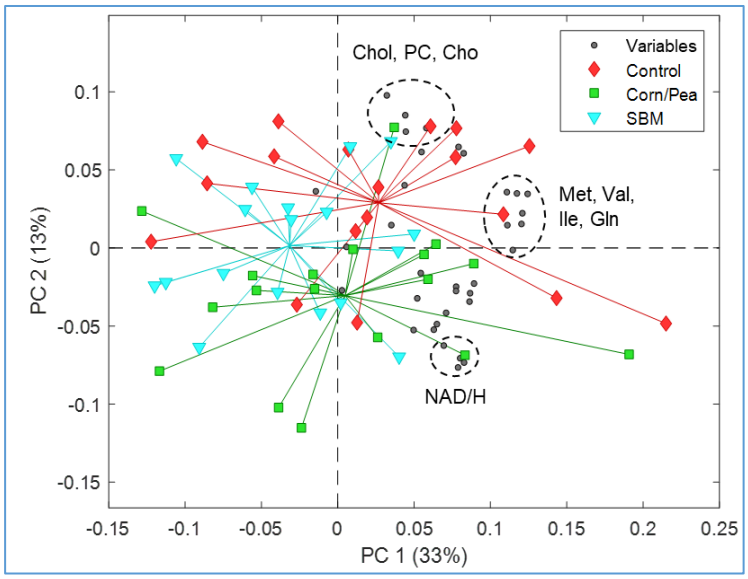


Figure 2.3.9. Biplots of the PCA performed on the metabolite tables of plasma samples from salmon (N=54) fed the basal diets: Control, Corn/Pea, and SBM. Keys: Cho: cholesterol; Chol: choline; PC: phosphocholine; Met: methionine; Val: valine; Ile: isoleucine; Gln: glutamine; NAD/H: NAD^+ and NADH. How to read the plot: different colours and symbols stand for different classes (see legend). Metabolites (variables, black dots) depicted close to a specific class are most abundant in that specific class/sample(s).

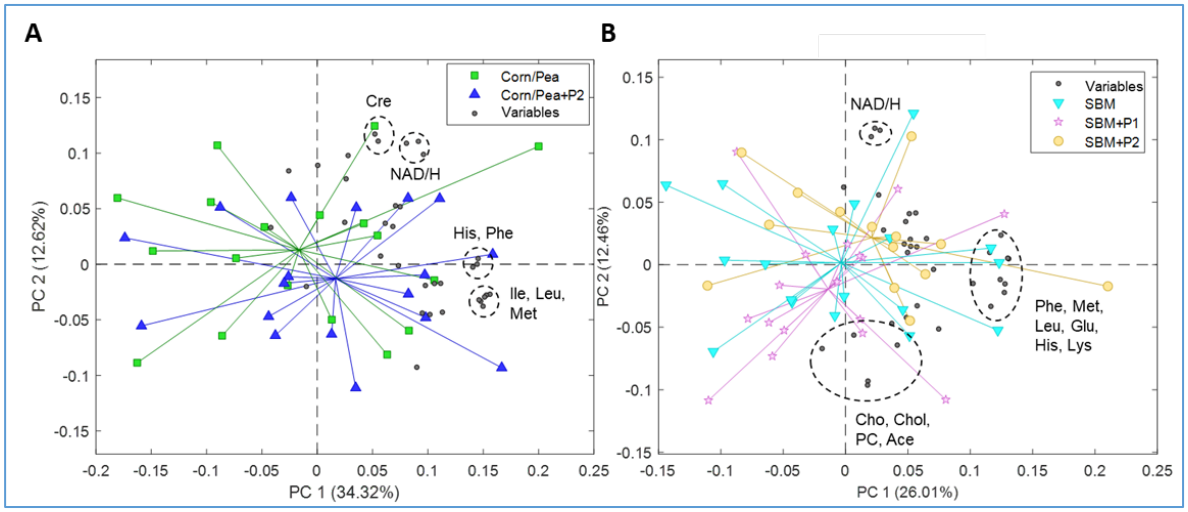


Figure 2.3.10. PCA biplots showing the comparison between Corn/Pea vs Corn/Pea+P2 (N=36) (A) and between SBM vs SBM+P1 vs SBM+P2 (N=52) (B). 2 outliers removed - 109 and 161. Keys: Ace: acetate; Cho: cholesterol; Chol: choline; PC: phosphocholine; Met: methionine; Val: valine; Ile: isoleucine; Gln: glutamine; His: histidine; Phe: phenylalanine; Leu: leucine; Cre: creatine. See the caption to Figure 2.3.9 for how to interpret the plot.

PCA was finally performed to compare the diets supplemented with the P2 package (Figure 2.3.11). A weak diet-related separation could be observed along PC1, with the Corn/Pea+P2 samples being characterized by a higher amino acid content in their plasma.

Overall, the results of the metabolomics analysis suggest a high degree of interindividual variability which can be partially ascribed to the fact that the diet composition (mainly in terms of amino acid composition), already in the basal diets, was not similar. As for the experimental diets, metabolites

whose concentration changed as a result of diet intake were mainly amino acids, which were highest in the all the experimental diets. Differently, choline and glycine plasma concentration decreased in fish fed all the experimental diets.

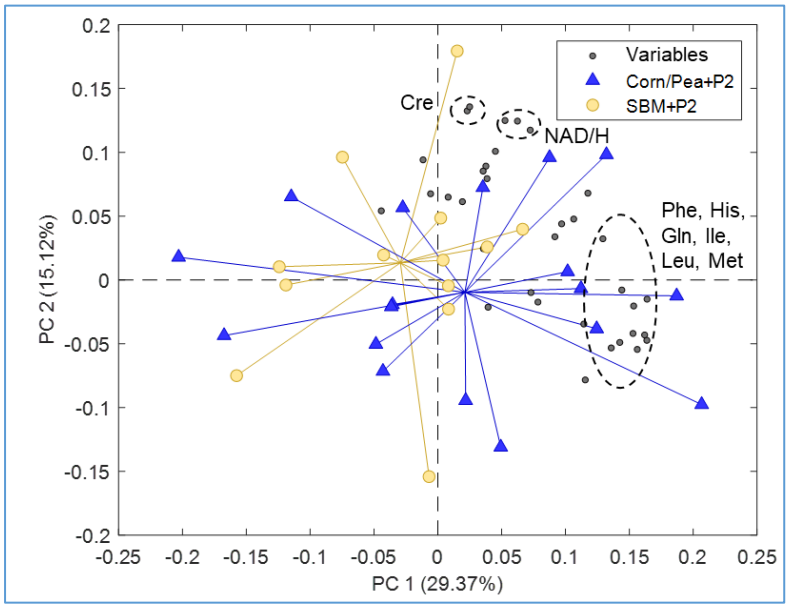


Figure 2.3.11. PCA biplots showing the comparison between plasma samples from fish fed the Corn/Pea+P2 and SBM+P2 diets (N=36). Keys: Met: methionine; Ile: isoleucine; Gln: glutamine; His: histidine; Phe: phenylalanine; Leu: leucine; Cre: creatine; NAD/H: NAD^+ and NADH. See caption to Figure 2.3.9 for how to interpret the plot.

Microbiota

Alpha diversity (Figure 2.3.12) in the digesta from DI showed that the CoPea fed fish, but not the SBM fed, differed significantly for both alpha diversity matrices. Comparison of the SBM and CoPea fed fish showed no significant difference. Supplementation with P1 and P2 to the SBM diet did not change the alpha diversity significantly, neither did supplementation with P2 to the CoPea diet. In the mucosa, significant difference was observed for the Shannon index in SBM fed compared to Contr fed fish, and Shannon in the SBM+P1 and observed taxa in the SBM+P2 treatments compared to the SBM diet. On the other hand, P2 supplementation to CoPea diet did not significantly change the alpha diversity indices.

Beta diversity (Figure 2.3.13). In the digesta, microbiota from 6 treatments grouped into different clusters (Figure 2.3.13a). Pairwise PERMANOVA analysis indicated significant differences in microbial composition between SBM and Contr, CoPea and Contr, and between SBM and CoPea. Supplementation of P1 and P2 to SBM diet caused a significant shift in microbial composition, whereas supplementation of P2 to CoPea diet showed significantly changed microbial composition only when assessed by Bray-Curtis dissimilarity index, but not by the unweighted Unifrac distance. In the mucosa, beta diversity of the microbiota did not show clear clustering (Figure 2.3.13b) for any of the treatments. The exception was the SBM treatment. In the mucosa, however, the significant diet effects found only between SBM and CoPea and when SBM diet supplemented with P1.

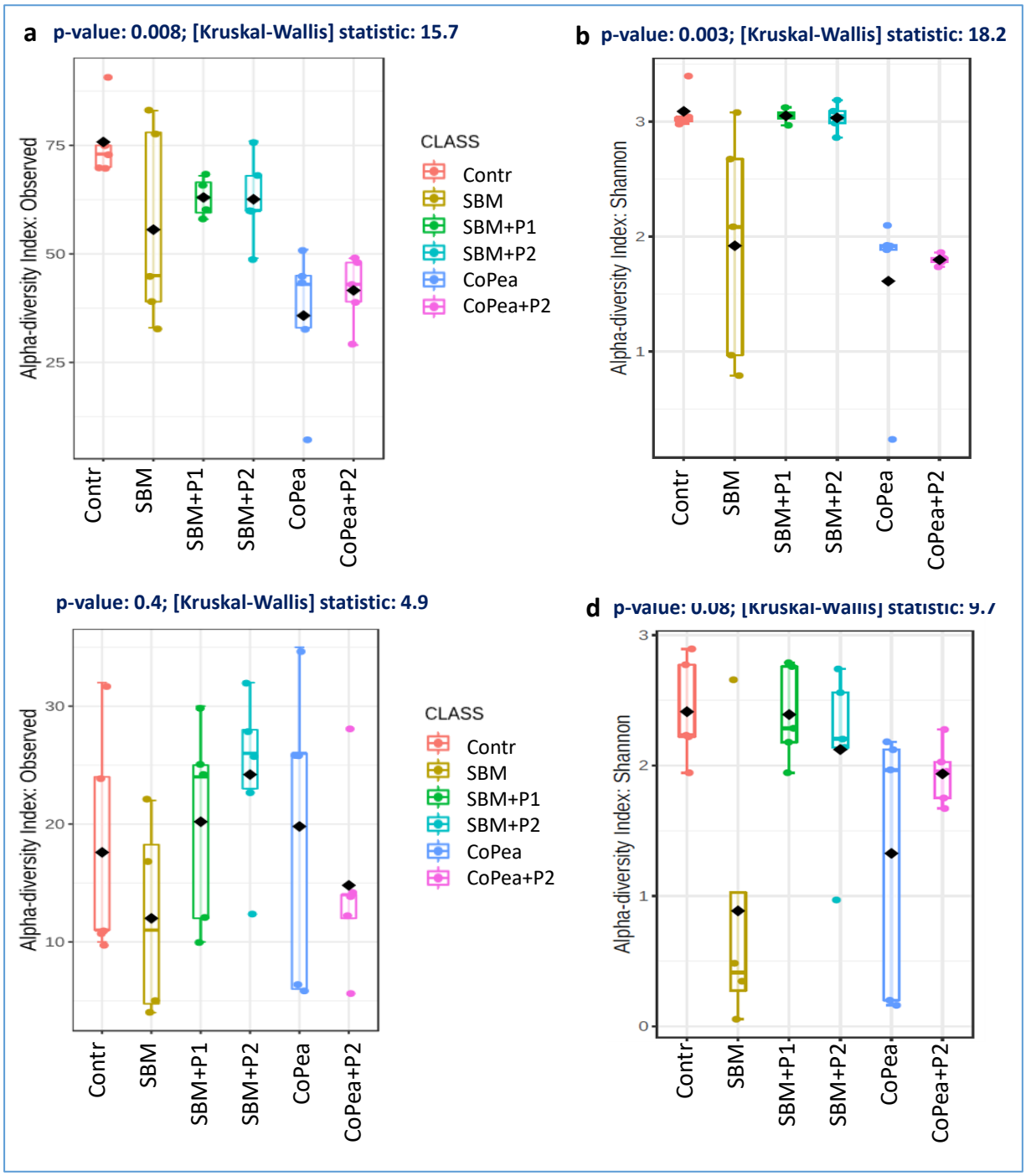


Figure 2.3.12. The alpha diversity indices for microbiota in digesta and mucosa of the distal intestine of Atlantic salmon fed six diets. a) observed species and b) Shannon indices for digesta; c) observed species and d) Shannon indices for mucosa. p-values and Kruskal-Wallis statistics (H values) are presented above each graph.

Dominating phyla (Figure 2.3.14). In the digesta the taxonomic analysis revealed that the phylum *Firmicutes* was predominant of five of the six treatments (79%- 98%). The exception was the SBM treatment (46%) (Figure 2.3.13). In the SBM fed fish *Proteobacteria* dominated comprising 52% of the total abundance. Supplementation with P1 and P2 reduced its abundance to 5% and 7%, respectively and increased abundance of *Firmicutes*, P1 to 92% and P2 to 89%. Contr and CoPea diet fed fish

contained 12 and 21% of *Proteobacteria* of the total abundance, respectively. Supplementation of P2 to CoPea diet reduced *Proteobacteria* abundance to 1%.

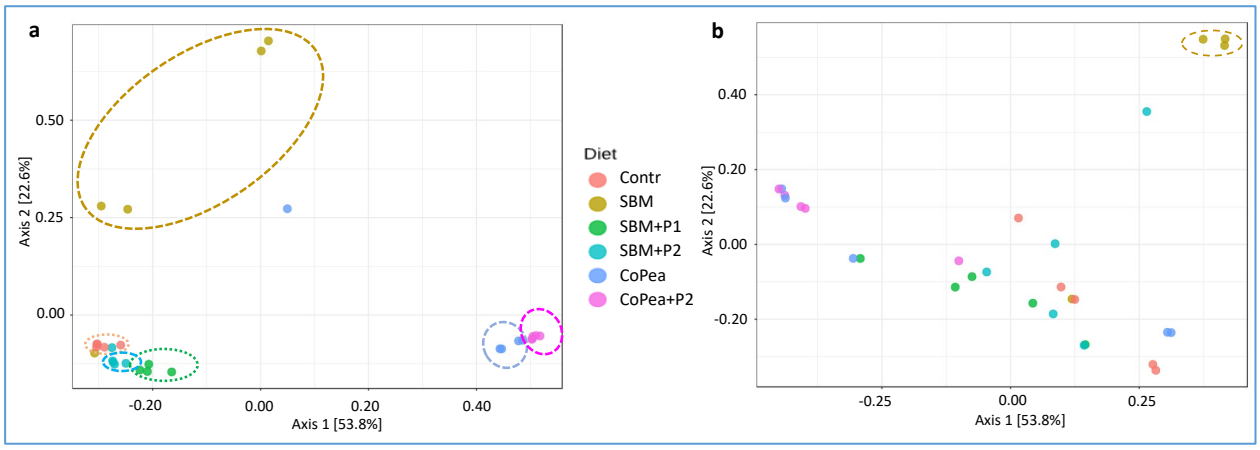


Figure 2.3.13. PCoA plots based on Bray-Curtis dissimilarity matrix showing bacterial community structures (beta diversity) in the distal intestine (a) digesta and (b) mucosa of the Atlantic salmon fed with six diets. The whole bacterial community of each sample is represented by a dot on the figure. Samples with similar bacterial compositions are closer to each other.

In mucosa, as observed in the digesta, the phylum *Firmicutes* was predominant in five of the six (Contr, 48%, CoPea, 57%, SBM+P1, 75%, SBM+P2,56.4%, CoPea+P2, 82%). The exception was SBM treatment (14.5%). In SBM group, *Proteobacteria* predominated with 80 % of total abundance, while in fish fed Contr and CoPea diets the *Proteobacteria* comprised 29% and 42% of total abundance. Supplementation to the SBM diet reduced *Proteobacteria,* for P1, 11% and P2, 34%, and increased *Firmicutes*. Supplementation with P2 to CoPea diet also reduced mucosal *Proteobacteria* abundance to 3.4%.

Dominating genera (Figure 2.3.15). In the digesta the most predominant genera differed clearly among the six treatments (Figure 2.3.14). In Contr fed fish *Leuconostoc* (28.6%) and *Weissella* (21%) were predominant genera followed by *Lactobacillus* (13.6%). In SBM fed fish the genera *Aliivibrio* (49.6%), *Leuconostoc* (16.6%) and *Weissella* (13%) predominated, while in CoPea fed fish *Lactobacillus* (65%) dominated. Supplementation with P1 and P2 to SBM changed the predominant genera to become more similar to those in the Contr group. Both SBM+P1 and SBM +P2 groups had *Leuconostoc* (25.5% and 28%), *Lactobacillus* (21.6% and 16.6%) and *Weissella* (17.5% and 22.4%) as predominant genera. In fish fed the CoPea-P2 diet a further increase in the presence of *Lactobacillus* to 86% of total abundance was observed while *Photobacterium* diminished to 0.06%.

In mucosa (Figure 2.3.16), as in the digesta, the treatments varied regarding predominating genera. In the Contr fed fish *Photobacterium* (12.4%) and Staphylococcus (11%) dominated, in SBM fed fish, *Aliivibrio* (71.7%), and in CoPea, *Lactobacillus* (40%), *Photobacterium* (40%) and *Staphylococcus*

(10%). Supplementation with P1 and P2 to the SBM diet changed the same genera in mucosa of both groups but in different proportions. Supplementation P1 greatly decreased *Aliivibrio* (1.6%) and

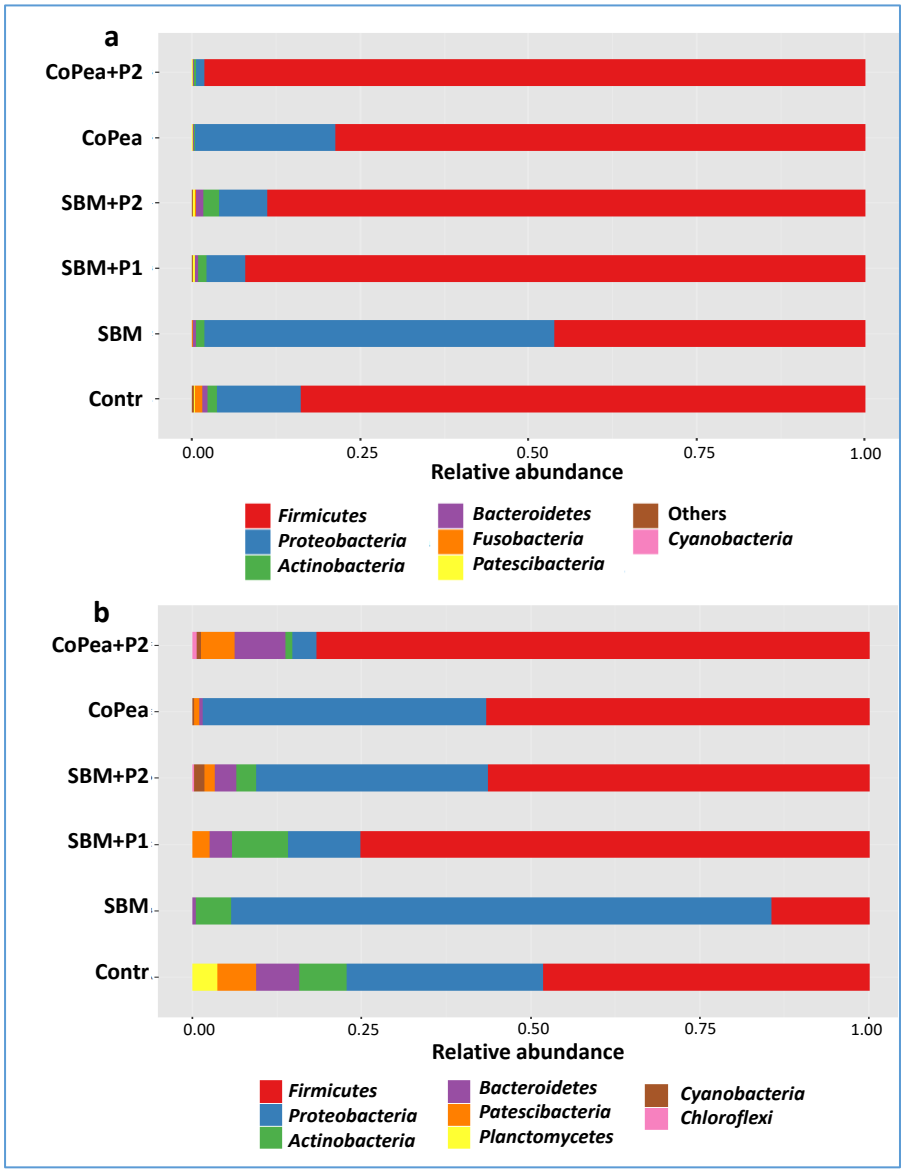


Figure 2.3.14. Most abundant phyla of digesta (a) and mucosa (b) from distal intestine of the Atlantic salmon fed with one out of six diets.

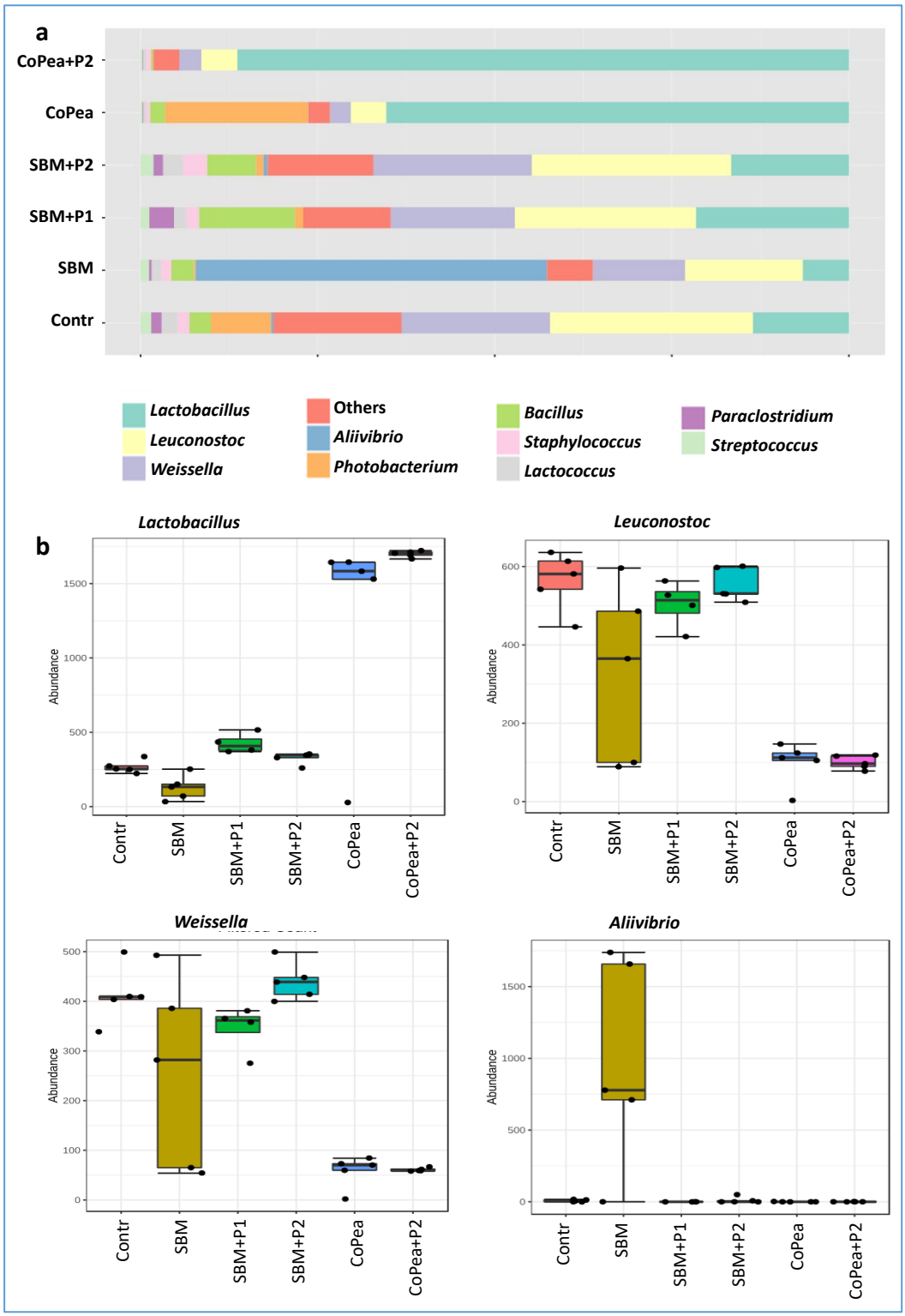


Figure 2.3.15. Ten most abundant genera of digesta from distal intestine of the Atlantic salmon fed with six diets (a). Box plots showing filtered absolute counts of some selected genera (b).

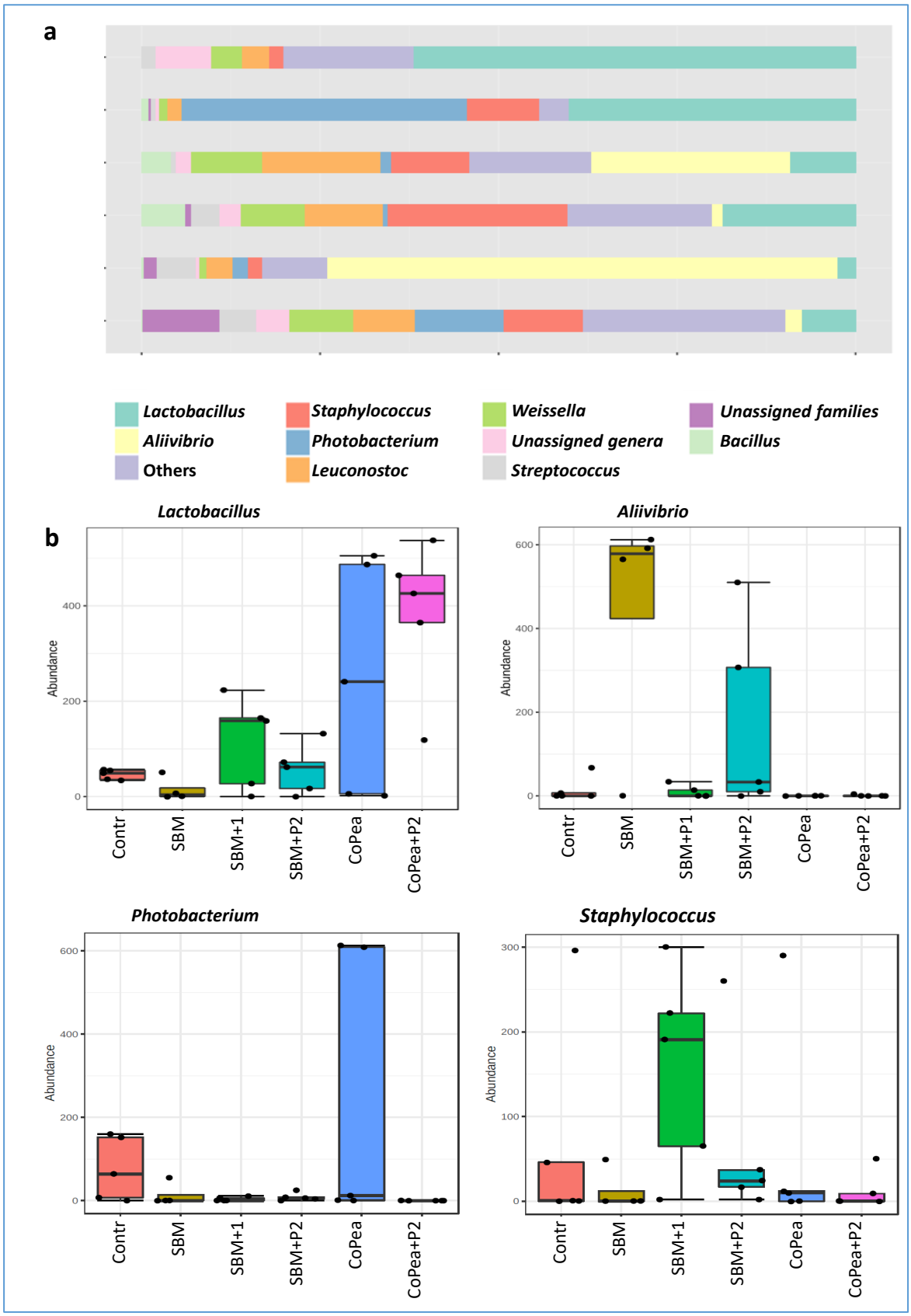


Figure 2.3.16. Ten most abundant genera of mucosa from distal intestine of the Atlantic salmon fed the six experimental diets (a). Box plots showing filtered absolute counts of some selected genera (b).

increased the abundance of *Staphylococcus* (25%), *Lactobacillus* (18.6%), *Leuconostoc* (11%) and *Weissella* (9%). P2 also decreased *Aliivibrio* (28 %) to some extent and mostly increased *Leuconostoc* (17%), *Staphylococcus* (11%), *Weissella* (10%) and *Lactobacillus* (9%). In mucosa, similar to the digesta, supplementation with P2 to CoPea increased abundance of *Lactobacillus* (62%) and diminished *Photobacterium. Staphylococcus* also decreased in fish fed theCoPea+P2 diet (2%) compared to CoPea group.

In general, SBM as well as CoPea diet fed fish showed reduced alpha diversity. In the DI digesta, the number of different microbial taxa in CoPea fed fish was significantly lower than in Contr fed fish, whereas SBM fed fish did not differ from the Contr fed fish in this aspect. Supplementation with P1 an P2 did not significantly affect this picture. In the DI mucosa, the number of different microbial taxa and their distribution (Shannon index only) in SBM fed fish showed significant reduction compared to the Contr fed fish, whereas the CoPea fed fish did not differ from the control fish in this aspect. Supplementation with P1 and P2 increased the number of different microbial taxa in SBM fed fish, but not in those fed the CoPea diet. Humans with inflammatory bowel diseases have been reported to have lower alpha diversity compared to the healthy individuals. In line with those findings, reduced alpha diversity of both digesta and mucosa -associated microbiota in SBM as well as CoPea diet fed fish could be associated with the distal intestinal inflammations. Even though supplementation increased the alpha diversity in SBM fed fish to the level of Contr fed fish, it did not seem to lessen the inflammation.

Even though fish fed the SBM diet showed distinct digesta and mucosa microbiota profiles having higher abundance of phylum *Proteobacteria* and genus *Aliivibrio*, it is very difficult to link this deference for the gut inflammation. *Proteobacteria* and *Aliivibrio* are previously reported to be among the core gut microbiota of Atlantic salmon. Therefore, the clear difference in microbiota in the digesta and mucosa might mainly have been reflections of the difference in substrates for the microbiota among the diets, as shown in earlier studies of fish, other animal species, experimental animals, and humans. Further, supplementation of P1 and P2 packages to SBM significantly changed the microbial composition with decreased in phylum *Proteobacteria* and genus *Aliivibrio* making it similar to Contr group, without any effect on the inflammation status. Reduction in aerobic bacteria, *Aliivibrio* and increase of facultative anaerobic/anaerobic bacteria *Leuconostoc*, *Lactobacillus* and *Weissella* may be due to the enforcement of anaerobic environment by butyrate as shown in the mammalian studies. These findings indicate that dietary changes may exert large changes in the gut microbiota without major impact on the gut health or other physiological and production related aspects.

When considering microbial taxa in CoPea fed fish, high fibre content in CoPea diet may have resulted in higher abundance of lactic acid bacteria, *Lactobacillus*. Lactic acid bacteria including Lactobacillus are known to produce SCFAs and mammalian studies indicate that intestinal epithelial cells obtain most of the energy requirements from the SCFAs. Therefore, increased energy supply from increased *Lactobacillus* levels could have enhanced the DI growth in CoPea group. Supplementation of P2 further increased the abundance of *Lactobacillus* probably due to the anaerobic environment, facilitated by butyrate as well as influence of beta glucan.

Conclusions regarding WP2.3 - remediating effects of functional ingredients.

The clear signs of inflammation induced by feeding the fish the SBM basal and the mild signs observed upon feeding with the CoPea diet indicate that our intention to create two inflammation models, was reached. They therefore are considered to provide conditions which make them suitable for elucidating whether the chosen functional ingredients might reduce signs of inflammation under such conditions and improve the gut health.

Supplementation with the mixture of arginine and butyrate (P1) to the SBM diet did not alter the histological signs of inflammation caused by the SBM diet, neither did this mixture markedly alter other of the observed biomarkers considered to indicate gut health, such as growth rate, nutrient digestibility, feed conversion, tissue weight, and protease capacity of the intestine, gene expression, bile salt concentration and trypsin activity in the chyme from DI, and plasma metabolites. Even so, marked effects on gut microbiota were observed, mostly in a direction towards the microbiota composition of the fish fed the Contr feed. As for supplementation of the SBM diet with P1, supplementation of this basal diet with the mixture of butyrate, β-glucan, and nucleotides (P2) caused no important effects on the observed biomarkers. However, also for P2, marked effects, similar to those of P1, were observed on the composition of the microbiota in the DI. Supplementation of the CoPea diet with P2, did not alter the observed signs of inflammation in the distal intestine of these fish.

The observed effects of inclusion of P1 and P2 in the SBM feed and of P2 in the CoPea feed, which changed the microbiota to become similar to that of the fish fed the control diet, high in fish meal, but without beneficial effect on the symptoms of inflammation, show that major changes in the gut microbiota may take place without clear effects on gut health.

WP3: Effects of dietary lipid level and environmental temperature on the severity of steatosis in fish fed suboptimal level of choline

Responsible partner: VM Nutr, NOFIMA

The aim of WP3 was to follow up the clear results from WP2.1 regarding the role of choline for development of steatosis in the pyloric caeca. Steatosis was frequently observed with varying severity during the salmon production cycle in the field survey in WP1. WP2.1 confirmed the essentiality (Hansen et al. 2020a) of choline for elimination of this condition and that diets with high plant content, unless supplemented with a source of choline, contain insufficient choline for efficient lipid transport across the intestine. Choline's role in lipid transport suggests that choline requirement may depend on level of lipid and fatty acid composition of the diet, environmental temperature, as well as developmental stage of the fish. In the present work package, the role of lipid level and temperature was in focus, whereas lipid quality and fish size were addressed in the following. Both were screening experiments addressing possible relationships. In both work packages, diets severely, but similarly, deficient in choline were used, to optimize the change of revealing possible effects on choline requirement.

Methodology

Diets

Four high plant diets with low level of choline were made with lipid level of 16%, 21%, 26%, 31% (Table 3.1). The experimental diets were produced by extrusion (feed pellet size 6 mm) at BioMar Feed Technology Centre (Brande, Denmark) using a BC 45 twin screw extruder (Clextral, France).

Table 3.1. Composition of experimental diets*

Raw material	**D16**	**D21**	**D26**	**D31**
Fish Meal NA Con-Kix 72%	20	20	20	20
Soya SPC >62%, non gmo	24.21	16.14	8.07	0
Wheat Gluten 80		5.80	11.61	20
Maize Gluten 60 (min. 58%)	10	9.64	9.28	3.41
Pea Protein 65%	13.28	13.4	13.6	15.06
Wheat Milling quality	14.81	11.6	8.39	0.51
Tapioca Starch		0.67	1.33	7.64
Fish Oil, 18 EPA+DHA Eth.free	3.16	4.94	6.73	8.14
Rapeseed Oil, Crude	7.37	10.34	13.30	14.86
Premix	0.48	0.48	1.08	1.07
Lecithin Soy, Liquid	0.8	0.8	0.8	2.0
Calcium carbonat (limestone)	2.52	1.68	0.84	0
Mono-sodium Phosphate (MSP)	3.06	3.1	3.1	2.98
L-Lysine HCl (78%)	0.19	0.49	0.80	1.4
DL-Methionine (99%)	0.2	0.2	0.2	1
L-Threonine (98%)		0.06	0.13	0.26
L-Histidine (74%)	0.3	0.35	0.4	0.53
Water change	-0.39	0.1	0.59	1.13

* The diets were supplemented with standard vitamin and mineral premixes in accordance with NRC guidelines (2011)[15]. Yttrium oxide (0.50 g/kg) was added as an inert marker for estimation of apparent nutrient digestibility.

Experimental fish and conditions, sampling, and analytical procedures.

The feeding trial was conducted at Nofima's Research Station in Sunndalsøra (NO). The fish had an initial weight of 25g, all coming from the same population, were randomly assigned to 200L flow through tanks, 100 fish per tank, with two water temperatures, 8 and 15℃. Two tanks were used per treatment, i.e., a total of 16 tanks. A 24-hour light regime was employed. The fish were fed using disc feeders, 10% above estimated requirement. After 8 weeks of feeding, six fish from each tank were sampled randomly, and samples taken as described above for WP2.1, except that blood samples and samples for microbiota analyses were not taken. Standard procedures for analyses of nutrient and yttrium composition in feed and faeces were employed. See Li et al. for further details (2020). The histological examination followed the procedure described by Hansen et al. (2020a). The same regarded the procedure for analyses of gene expression.

Statistical analyses

Data were subjected to two-way analyses of variance (ANOVA) followed by Shapiro–Wilk test to assess the normality of the variance. The level of significance for all analyses was set at $P < 0.05$, and P values between 0.05 and 0.1 were considered as indications of effects and mentioned as trends. All data are means ± SEM.

Results and discussion

Results regarding growth rate and nutrient digestibilities are shown in Table 3.2, regarding yield and relative weights of liver and pyloric intestine of sampled fish in Figure 3.1. The growth rate was, as expected, significantly affected by temperature, and higher at 15 than at 8℃, the same was carcass percentage, i.e., yield. Lipid level appeared not to affect growth significantly, but yield decreased with increasing lipid level.

Table 3.2. Mean results of growth and digestibility observations

		SGR	DLip	DCProt	DEnergy	DDM	FaecDM
Temp, ℃	Lipid, %						
	16	1.7	95.8[a]	90.0	81.9	75.1[b]	12.5[a]
	21	1.7	96.2[a]	90.9	86.1	79.0[b]	12.3[a]
	26	1.6	93.9[b]	90.4	83.5	78.7[b]	11.9[a]
	31	1.7	94.4[b]	91.8	88.1	83.0[a]	10.9[b]
8		0.99[b]	95.2	90.9	**	80.0	12.0
15		2.36[a]	94.9	90.6	84.9	77.9	11.8
Statistics							
p(model)		<0.0001	0.0148	0.3366	0.0101	0.2515	0.0028
p(Temp)		<0.0001	0.4323	0.6981		0.1271	0.2827
p(Lipid)		0.3285	0.0086	0.2346	0.0080	0.2615	0.0016

*As no significant interaction between temperature and dietary lipid level was observed the results shown in the table are based on analyses without the interaction term. Abbreviations: DLip=digestibility of lipid; DCProt=digestibility of crude protein; Dash=digestibility of ash; DEnergy=digestibility of energy; DDM=digestibility of dry matter; FaecDM=dry matter in faeces. Means with different letters were significantly different.

** Insufficient sample for analysis. One-way ANOVA.

Regarding macronutrient digestibility no significant temperature effects were seen. Lipid level in the diet affected lipid digestibility significantly and decreased with increasing lipid level. The cause of this decrease is not apparent. However, increasing lipid level increased dry matter digestibility, supposedly due to slowing of the passage rate, allowing more time for digestion of starch (not analysed), for which digestive capacity is low in Atlantic salmon (Krogdahl et al 2004).

Results for relative weights of liver and pyloric intestine (Figure 3.1) showed significant increase with both lipid level in the diet and temperature. This observation explains the decrease in yield with increasing dietary lipid level as well as with temperature, i.e., that increased lipid level in a choline deficient diet reduces yield of production.

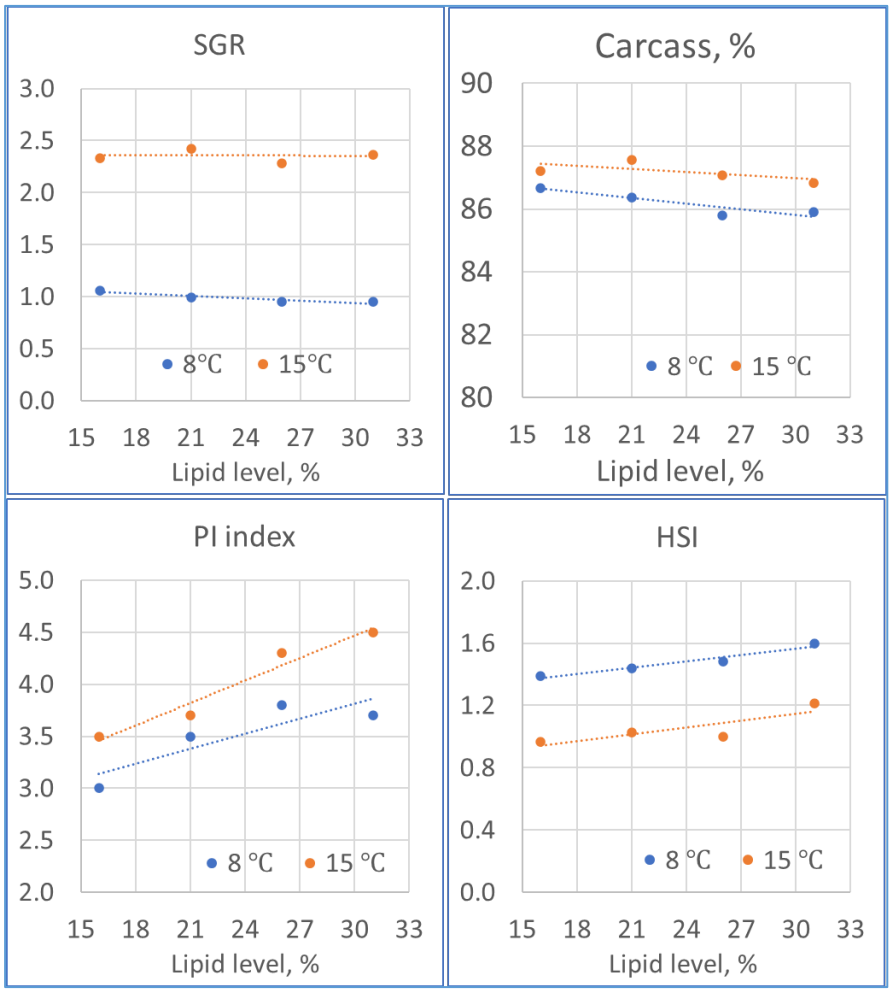


Figure 3.1. Results for growth rate (SGR) carcass percentage, liver index (HIS), and index of pyloric intestine (PI) in fish fed diets with four levels of lipid at two temperatures.

Statistics:
BW: p(Temp)<.0001
p(Lipid)=0.7969
Yield: p(Temp)<.0001
p(Lipid)=0.0.0248
PI: p(Temp)=.00016
p(Lipid)=0.0012
HSI: p(Temp)<.0001
p(Lipid)=0.0031

Figure 3.2 shows how dietary lipid level and temperature affect lipid content in the PI and liver. The PI lipid content increased with increasing lipid level and more than doubled when dietary lipid level increased from 16 to 25%. The increase seemed to peak at a dietary level around 24%. As the cause of the indicated peak was difficult to explain, the diets were reanalysed for content of choline. The result showed the following levels: D16: 1930 mg/kg, D21:1830mg/kg, D26:1940mg/kg, and D31: 2310mg/kg. The reanalysis showed that our intention to keep the choline level constant in the four diets was not reached for the D31 diet which showed higher level than planned for, 2300 instead of 1900mg/kg. This elevation turned out to be due to a higher inclusion of soybean lecithin in this diet, an error which occurred when this diet was remade in a hurry because the first batch was leaking fat during storage. The remediation of the leakage was an increase in soya lecithin. This can explain the observation of lower lipid accumulation in the PI of fish fed the D31 diet. The results for relative weight of the PI in fish fed at 8°C may indicate a levelling of the effects of lipid around 31% lipid in

the diet, whereas the results for fish fed at 15°C seemed to continue to increase with increasing dietary lipid level, even though the lipid concentration decreased.

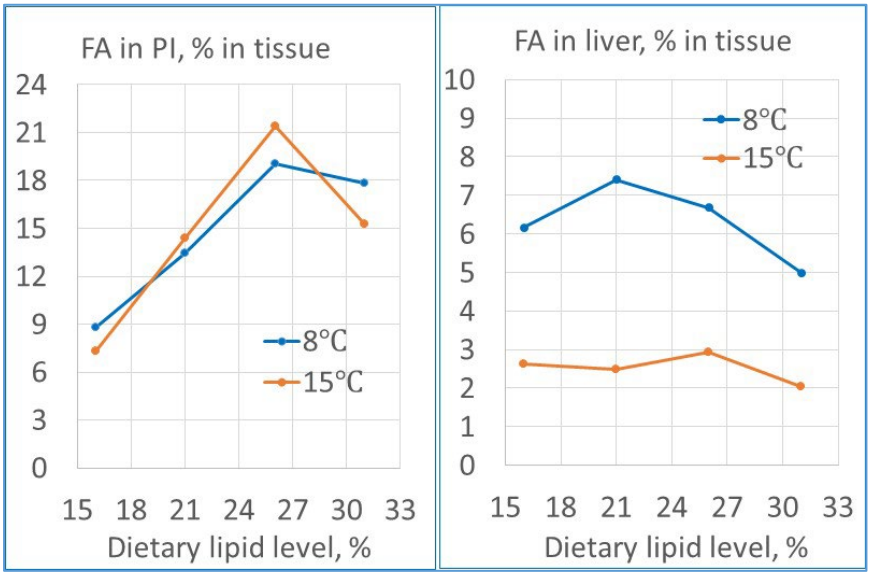


Figure 3.2. Sum of fatty acids (% in the tissue) in the pyloric intestine and liver

Figure 3.3 shows the results of the histological examination of the PI. They confirm the PI results of Figure 3.1. and 3.2, showing increasing steatosis with increasing dietary lipid level, and a tendency to more severe symptoms for fish fed at 15 compared to 8°C.

The results regarding the liver, both the HSI and the lipid content, indicate that the liver was affected in a different way than the PI. In contrast to the PI the index was highest for fish fed at 8°C. As observed for the PI, the index increased with increasing dietary lipid level, but the lipid content did not increase clearly with dietary lipid content. The histology examination of the liver (not shown) confirmed this picture, showing no clear differences in vacuolation, which would indicate lipid accumulation. The differences in responses of the PI and the liver, are difficult to explain. One clear limitation for the discussion is lack of knowledge regarding transport of lipid from the intestine to the organs and tissues in fish, which is generally not known. Dedicated studies of lipid transport are needed for understanding and discussion of these results and also for future studies involving transport and metabolism of lipid and lipid soluble compounds from the intestine to the tissues and organs of the body.

Taking into consideration the effect of the increase in dietary lipid level from 16 to 26%, the PI index increased 0.7 %-units in fish kept at 8°C, 1.0 %-units at 15°C. Evaluating these results in light of the results of Hansen et al. (2020a), estimating choline requirement in Atlantic salmon with diets containing 29% lipid, these shifts in PI index correspond to an increase in choline requirement of 1100 mg/kg. The consideration behind this estimate is illustrated in Figure 3.4. The corresponding difference in PI index in fish fed at 8 and 15°C was about 1.0 units, corresponding to an increase in the requirement by 1500mg/kg. As the conditions in the present study and that of Hansen et al. (2020a) were quite different, the shifts of 1100 – 1500mg/kg estimated above, are only indicative of

the real changes in choline requirement in the present study. Whether these shifts are additive or not, cannot be concluded upon based on the present study. However, what should be clear is that choline requirement depends on both lipid level and environmental temperature at a magnitude of importance for diet formulation and supplementation if prevention of steatosis is a goal.

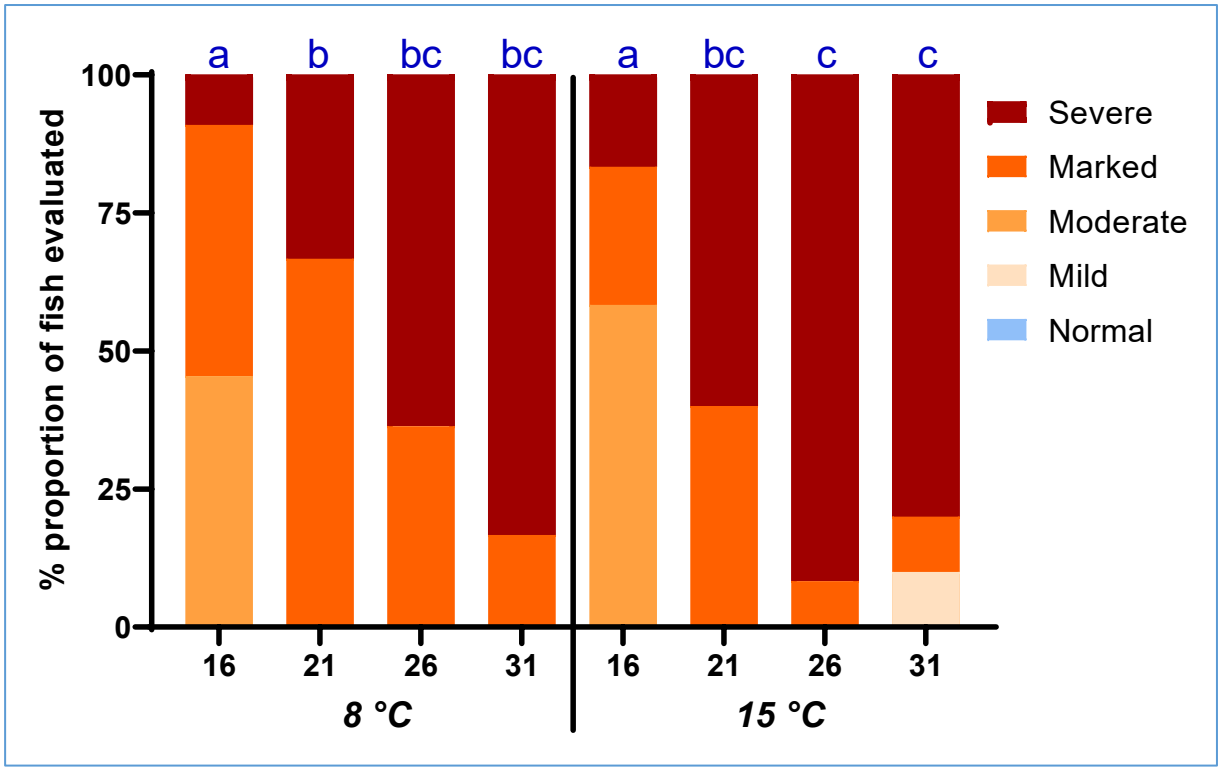


Figure 3.3. Results of histological examination, i.e., proportion of pyloric caeca tissue scored for enterocyte steatosis for the GM-9 feeding trial. X-axis presents dietary lipid level at two rearing water temperatures of 8 and 15℃. Superscript letters not shared by two columns represent a significant statistical difference following a Fisher exact test and a follow-up pairwise comparison of all groups using the ChiSquarePostHoc test in R. An ordinal logistic regression of impact of lipid level and water temperature level on the distribution of the histological scores among the diet groups for pyloric caecal enterocyte steatosis showed significant effect of lipid level (p=0.003, but not for temperature (p=0.317).

It can be discussed whether absence of steatosis is necessary, or if some signs are acceptable and normal. In a situation with marked signs of steatosis, lipid digestibility is often reduced, and followed by symptoms of lipid malabsorption. In severe cases the environment is polluted with floating faeces (Penn 2011).

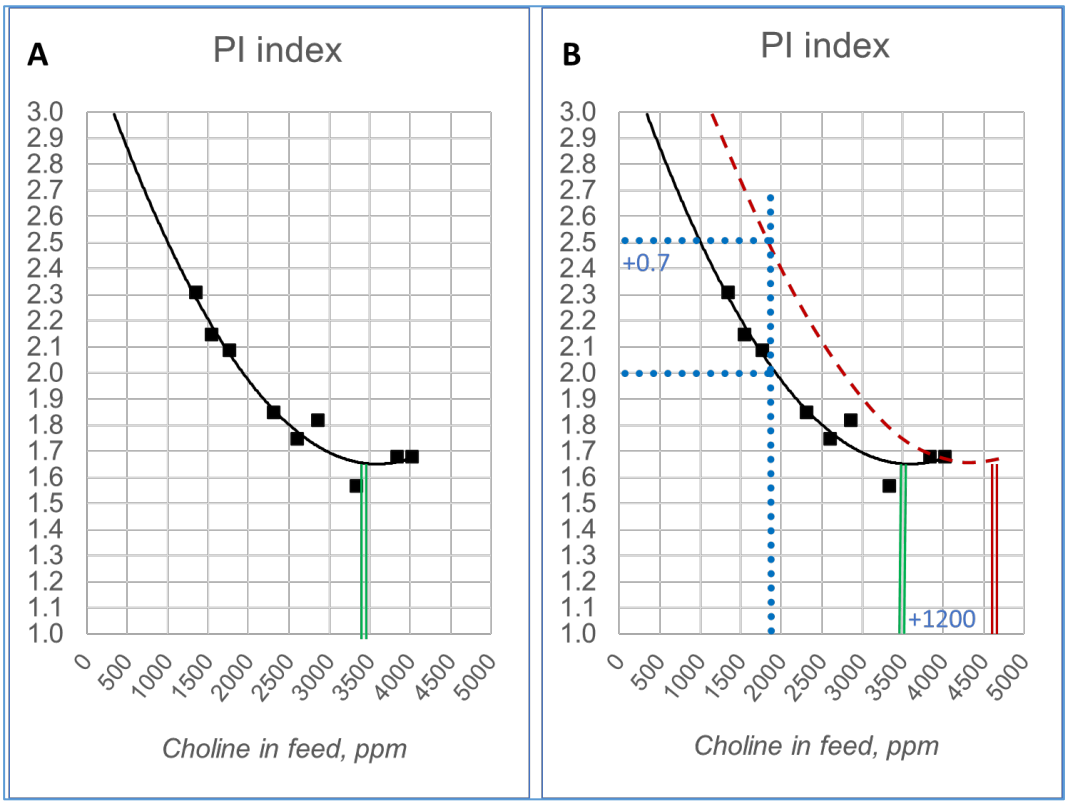


Figure 3.4. The left side of the figure (A) illustrates the results regarding function of PI index on choline supply in the study of Hansen et al. (2020a). Based on these results choline requirement was estimated, for fish weighing 450g and fed a diet with 29% lipid, to be 3400mg/kg (red line). The dose-response curve allows estimation of the effect of lipid level in the present study, which in the fish fed at 8℃ caused an increase in Pi index of 0.7 in fish fed a diet with 1900mg/kg choline. A diet with 1900mg/kg of choline in Hansen et al.'s study would correspond to a PI index of 2.0. Adding 0.7 to this for a diet with the same choline content (blue dotted lines) indicates a rightward shift in the dose-response curve (red line). The curve indicates that an increase in dietary lipid content in the present experiment from 15 to 26% increased the choline requirement to 4600, i.e., an increase of 1200mg/kg.

Conclusions

- Increasing lipid level in a choline deficient diet from 16 to 31% did not affect growth rate, increased relative weight of the pyloric caeca and histological symptoms of steatosis in the enterocytes, increased relative weight of the liver, and, accordingly, decreased fish yield.
- Elevation of the water temperature from 8 to 15℃, increased growth rate, relative weight of the pyloric caeca, and the histological symptoms of steatosis seemed to become more severe, decreased relative weight of the liver, and increased carcass weight.
- Estimation of the effects of lipid level and temperature on choline requirement based on an earlier dose-response study conducted to estimate choline requirement, indicate that an increase in lipid level in the diet increased from 16 to 26%, increased choline requirement by 1100mg/kg diet, the increase in temperature by 1500mg/kg.
- Dietary lipid level, as well as environmental temperature affect choline requirement to a magnitude of importance for fish biology and health, and for fish yield.

WP 4: Effects of fish size and lipid quality on the severity of steatosis in fish fed suboptimal level of choline

Responsible partner: VM-Nutr, LetSea

The aims of WP4 were to find to what degree lipid quality, i.e., fatty acid profile, and fish size might affect choline requirement.

Methodology

A regression design with six rape oil to fish oil ratios, from 0/35 to 23.5/7.7, and total lipid level of 35%. Otherwise, the diets were similar (Table 4.1.). As the diets used in WP3, these diets were severely deficient in choline. The diets were made at BioMar's experimental feed facility in Brande, Denmark.

Table 4.1. Feed ingredients and nutrient composition*

	RO_0	**RO_5**	**RO_9**	**RO_14**	**RP_19**	**RO_24**
Ingredients						
Fish Meal NA LT	13.0	13.0	13.0	13.0	13.0	13.0
Krill 56	2.0	2.0	2.0	2.0	2.0	2.0
Soya SPC	23.8	23.8	23.8	23.8	23.8	23.8
Pea Protein 65	7.5	7.5	7.5	7.5	7.5	7.5
Guar Meal	8.0	8.0	8.0	8.0	8.0	8.0
Wheat Milling quality	13.5	13.5	13.5	13.5	13.5	13.5
Fish Oil	31.2	26.5	21.8	17.1	12.4	7.7
Rapeseed Oil	0.0	4.7	9.4	14.1	18.8	23.5
Choline chloride, 70 %	0.03	0.03	0.03	0.03	0.03	0.03
Vitamin and mineral mix*	2.1	2.1	2.1	2.1	2.1	2.1
Lucantin Pink CWD 10%, BASF	0.1	0.1	0.1	0.1	0.1	0.1
Yttrium oxide	0.1	0.1	0.1	0.1	0.1	0.1
Analyzed content						
Protein (NIR), %	36.5					
Fat (NIR), %	35.0					
Choline, mg/kg	1523					
Estimated digestible energy, MJ/kg	21.5					

*The diets were made based on one mix of dry ingredients which was split in six for addition of the different fat mixtures. Analysed content of protein, fat and choline are averages of the six diets.
**The vitamin and mineral supplements secure coverage of the requirements as described by NRC (2011).

The experiment was carried out at LetSea's experimental farm at Dønna, Norway. The diets were fed to fish of two sizes, 1.5kg and 3.5kg at start of the experiment. The regression design allowed use of only one net pen (100 fish per pen) for each diet and fish size. The feeding period lasted 57 days.

At termination of the feeding period 12 fish were sampled from each net pen. The sampling procedure was the same as described for WP2.1, except that microbiota analyses were not conducted. The analyses of the collected samples were done mainly as described for WP2.1. In addition fatty acid profile of feed, digesta and tissues were analysed (O`fallon 2007).

The results were evaluated employing regression analyses regarding effects of rapeseed oil level within fish size. For comparison between the two sizes of fish two-way ANOVA was employed. For the histological features, the scores generated were categorical variables and the differences between the treatments were explored by contingency analysis using the chi-squared test.

Results and discussion

The fish grew well with an average thermal growth coefficient (TGC) for the small fish of 4.1, which was significantly different ($p<0.0001$) from that of the large fish: which was 2.9. Yield of sampled fish varied between 83 and 85%, but did not show significant effect, neither of rapeseed oil level nor fish size. Mortality was very low. In total 50 of the 1200 fish died, evenly distributed among the net pens. Feed conversion was high and varied between 1.0 and 1.2 with no significant effect of either rapeseed oil level or fish size.

Figure 4.1 presents the results for effects of lipid level and fish size on index of the PI and average histological scores.

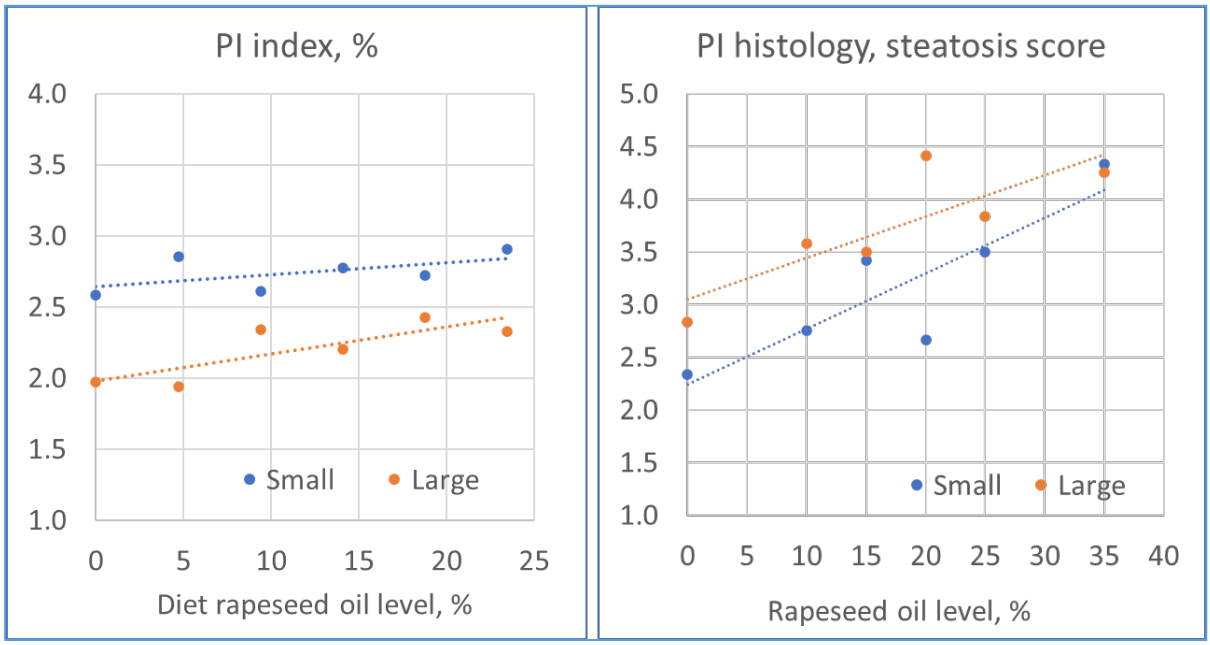


Figure 4.1. Results regarding indicators of steatosis in the pyloric intestine, i.e., index and average histological scores (number of fish characterized to show normal (1), mild (2), moderate (3), marked (4) and severe (5) symptoms.

The PI index showed a significant positive effect of rapeseed oil level in the diet ($p=0.0314$) as well as of fish size ($p<0.0001$) for which lower values were observed for the larger fish. Also the histological observations showed significant effects, with symptoms of steatosis increasing with increasing level of rapeseed oil ($p=0.035$). The same regarded fish size ($p=0.008$), i.e., the opposite effect indicated by

the PI results. The explanation for the seemingly opposite effect of fish size on PI index and histological symptoms of steatosis might be that the small fish rather than filling up the enterocytes with lipid, expanded the size of the organ allowing lower filling of each of the enterocytes. The difference in PI index between fish fed the low and the high rapeseed oil diets was 0.3 units for the small fish, 0.4 units for the larger fish.

Considering the PI index as the most appropriate biomarker for estimation of choline requirement (Hansen et al. 2020a), and employing a similar consideration as above for WP3, the shift in PI index of 0.3 and 0.4 with increasing rapeseed oil in the diet represents a difference in choline requirement of about 600mg/kg, and the average difference between the small and the large fish of 0.5, represents a change in requirement of about 800mg/kg.

The mechanisms underlying the increase in choline requirement with increasing dietary level of rapeseed oil may be found in the results shown in Figure 4.2. The figure shows the relationship between the level of rapeseed oil in the diet and digestibility of fat, estimated based on levels of fatty acids observed in the diet and in content from the distal half of the distal intestine. The difference was greater than expected, from about 80 to 94, i.e., about 15%. The results reveal that the fish oil had very low digestibility and that inclusion of rapeseed oil greatly improved digestibility, indicating a very high digestibility of most fatty acids in the rapeseed oil. A closer look at the digestibility of the individual fatty acids, represented by C16:0, reveals that the difference in digestibility was due to the differences in digestibility of the saturated fatty acids. Saturated fatty acids comprised a larger part of the fish oil, 40%, than of the rapeseed oil with about 15%. Digestibility of C16:0, which comprised 5% of the diet with only fish oil, increased from 55 to 85% in the large fish, and about the same in the small, with the increase in rapeseed oil from 0 to 35%. The increase in rapeseed oil decreased content of C16:0 in the diet to 2.7%. It may be suggested that the indicated increase in choline requirement with increasing level of rapeseed in the diet, was due increase in lipid digestibility and thereby an increase in need for choline for lipid transport across the gut mucosa.

Further details regarding fatty acid digestibilities and accumulation of fatty acids in the pyloric tissue, liver and in abdominal fat, and a discussion of fatty acid metabolism and transport between tissues and organs and metabolism in the will be published in the near future by Daphne Siciliani, a PhD student at NMBU, who has done part of her scientific work associated to this project.

LetSea's station at Dønna

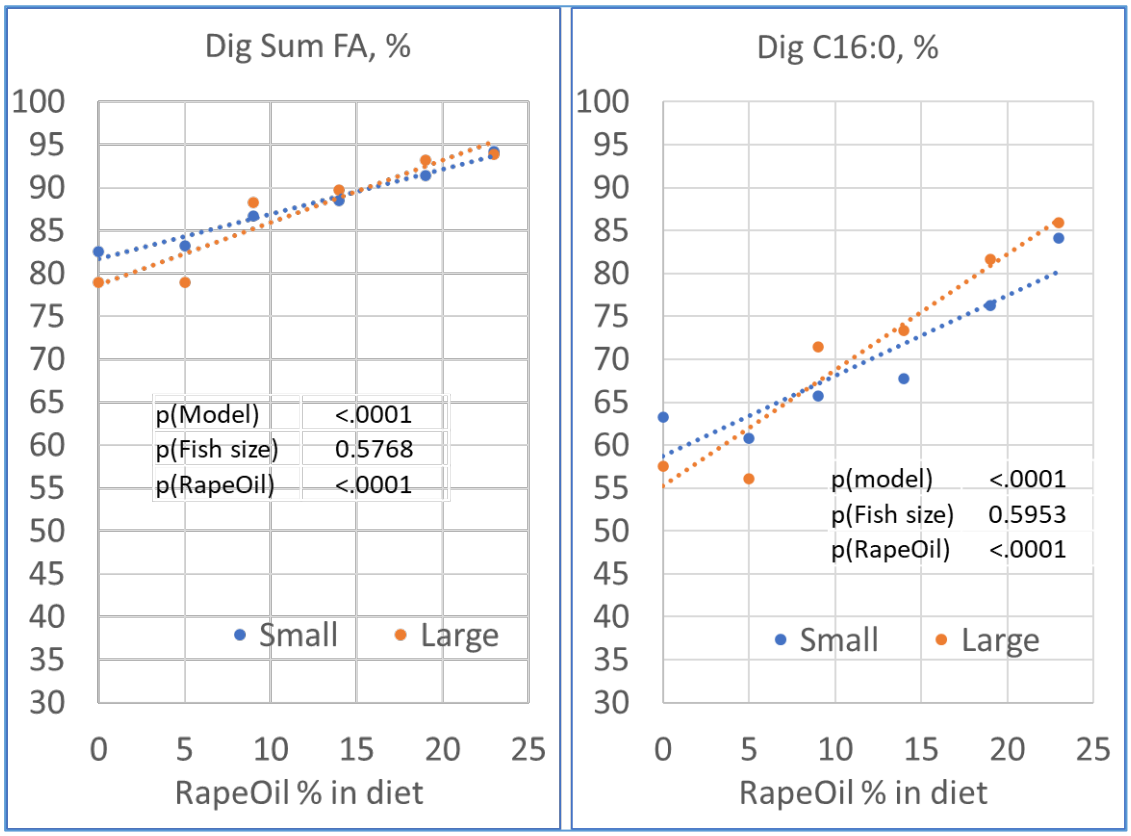


Figure 4.2. Digestibility of sum of fatty acid as indicated by level of fatty acids in the diet and in chyme collected from the distal half of the distal intestine.

Conclusions

- Increasing level of rapeseed oil in the diet containing 36% fat, from 0 to 23%, replacing fish oil, increased growth, relative weight of the pyloric ceaca, histological signs of steatosis, and digestibility of lipid, in particular of saturated fatty acids. Fish yield was not significantly affected, possibly due to high individual variation.
- The large fish showed lower growth rates than the smaller, lower relative weight of the pyloric caeca, more severe histological signs of steatosis in the enterocytes, similar lipid digestibility and yield.
- Estimation of the effects of content of rapeseed oil and fish size on choline requirement based on an earlier dose-response study conducted to estimate choline requirement, indicate that an increase in rapeseed oil from 0 to 23% in diets containing 36% fat, increased choline requirement of about 600mg/kg, and was 800mg/kg higher in fish weighing 1.5kg than fish weighing 3.5kg.
- The numbers should be taken only as indicative, as the reference experiment, used to quantify the shift in choline requirement, was conducted with fish a start weight of about 500 g and under other environmental conditions.
- The overall conclusion is therefore from this study that both lipid quality and fish size affect choline requirement, and that further studies are needed to define more accurately the requirement under varying conditions, i.e., employing dose-response studies with varying choline level in the diet.
- Economical losses due to reduced yield and feed utilization should also be taken into account.

Overall considerations of the results of WP1-4 in light of the hypothesis (H) which were described before start of the project:

H1: Interactions between endogenous and exogenous factors influence the manifestation and severity of gut disorders

The results of the field survey indicated that the severity of the steatosis in the pyloric caeca depend on seasonal variation more than on the feed composition. However, according to the results gained in the project regarding choline requirement, all the diet samples collected contained less choline than necessary for elimination of steatosis symptoms under conditions which require high supply, i.e., high feed intake, and high lipid level in the diets and high temperature.

Regarding the symptoms of inflammation, which increased throughout the seawater period, none of the many observed endogenous and exogenous factors were found to be closely related to the severity of these symptoms

H2: Specific tissue responses can aid in diagnosing and discovering the causes of gut disorders

Regarding the steatosis, variation within a population of fish in relative weight of the pyloric caeca and histological appearance of the tissue can indicate severity of the challenge. With today's feed composition, it is highly likely that the cause is deficient supply of choline, although deficient supply of essential long-chain fatty acids is another possible cause.

Regarding feed-induced inflammation in the distal intestine which compromises digestive functions of the tissue, variation in relative tissue weight and in activity of digestive enzymes in the tissue indicate severity of the inflammation. Lower values indicate more severe inflammation.

H3: Biomarkers are present in faeces and blood and can be used as non-invasive diagnostic tools

Regarding inflammation in the distal intestine, larger variation in trypsin activity in faeces within a population, can indicate severity of the inflammation. Variation in microbiota composition was considerable in the field survey as well as in the WP2.1 and 2.3 but did not reflect variation in gut disorders.

H4: Gut pathologies can be prevented

The steatosis observed in the field survey and the controlled experiments can be prevented by securing sufficient choline in the diets. However, choline requirement is not yet defined for salmon under all conditions. Further studies are needed.

Altogether, the scientific achievements from GutMatters have:

Generated the first systematic collection of information on gut health status of salmon grown under commercial conditions in Norway and linked the gut health status to observations and analyses regarding the management, environment, diet, production, health, and function of the fish. Steatosis, which varied with season, and gut inflammation, which increased in severity with time in the sea phase of the production, occurred frequently in the farms involved in the survey.

Contributed with new knowledge regarding how diet composition can affect gut health in salmon, and how dietary supplements to today's low marine salmon diets can improve gut health and function. The symptoms of steatosis increased with increasing content of plant ingredients in the diet but were eliminated by supplementation of the diets with choline, confirming that choline is an essential vitamin for Atlantic salmon. Choline requirement was further found to increase with increasing lipid level in the diet and environmental temperature, depend on fatty acid composition of the lipid, and decrease towards the end of the production period in seawater. On the other hand, supplementation of salmon diets with mixtures of functional ingredients, i.e., arginine, butyrate, β-glucan, and nucleotides did not reduce symptoms of intestinal inflammation induced by plant ingredients.

Developed and applied new approaches and analytical tools for the studies of gut health and function in salmon, including histomorphological, microbial and in vitro methods.

7. Main results

- Steatosis, which varied with season, and gut inflammation, which increased in severity with time in the sea phase of the production, occurred frequently in the farms involved in the field survey.
- Steatosis symptoms increased with decreasing fishmeal level in the diet, i.e., in parallel with the decrease in choline level, and were eliminated by supplementation of the diets with choline, confirming that choline is an essential vitamin for Atlantic salmon.
- Choline requirement was found to increase with increasing lipid level in the diet and with environmental temperature, to depend on fatty acid composition of the lipid. Steatosis may reduce fish yield and, accordingly, economical return.
- Symptoms of inflammation, severe induced by feeding diets with soybean meal, or mild induced by a mixture of corn gluten and pea protein concentrate, were not prevented by supplementation of the diets with mixtures of functional ingredients, i.e., arginine, butyrate, β-glucan, and nucleotides.
- Gut microbiota composition was affected by inclusion of plant ingredients in the diets. Supplementation of the diets with the functional ingredients diminished these effects, without improving gut inflammation.

8. Deliverables

Conference presentations – Orals and posters

1. Chikwati, E., Storsul, T., Midtlyng, P.J. 2017. Histological gut health monitoring of Norwegian sea-farmed Atlantic salmon. Abstract and Oral presentation of the project, by Elvis Chikwati, at European Association of Fish Pathologists conference, Sept 4. – 8., 2017, Belfast, Ireland.
2. Krogdahl, Å., Kortner, T., Koppang, E.O., Bjørgen, H., Midtlyng, P., Chikwati, E., Berge, G.M., Krasnov, A., Sæle, Ø., Engelsen, S.B., Abstract and Poster presentation of the project, by Åshild Krogdahl, at NFR's Havbrukskonferansen, April 18. – 20. 2018, Oslo.
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6. Aru, V., Khakimov, B., Chikwati, E., Torres, A.J., Krasnov, A., Kortner, T., Krogdahl, Å., Engelsen, S.B.. Oral presentation at MRFOOD2018 - 14th International Conference on the Applications of Magnetic Resonance in Food Science, September 17 - 21, 2018 - Rennes (France). Poster presentation at the Metabolomics conference, June 24 - 28, 2018, Seattle (U.S.A). For this presentation, Violetta was given the Early Career Award from the Metabolomics Society.
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8. Elvis Chikwati, Midtlyng P.J., Li Y., Wang J., Zhou W., Hage E., Præsteng K., Kortner T.M., Løkka G., Torres A.J., Bjørgen H., Koppang E.O., Berge G.M., Krasnov A., Sæle Ø., Aru V., Khakimov B., Engelsen S.B., Åshild Krogdahl. 2019. Gut Matters- prosjektet; kartlegging av tarmforandringer hos Atlantisk laks gjennom produksjonssyklus i sjø. Oral presentation at Tekna's Frisk fisk meeting 6. – 7. February 2019, in Tromsø.
9. Elvis Chikwati, Paul Midtlyng, Trond M. Kortner, Jie Wang, Yanxian Li, Weiwen Zhou, Guro Løkka, Alexander Jaramillo-Torres, Åshild Krogdahl. 2019. Lipid malabsorption in six Norwegian Atlantic salmon (Salmo salar L.) farms. Oral presentation at the EAP Conference in Berlin, Oct 7th – 10th
10. Kortner T.M., Chikwati E., Midtlyng P.J. Li Y. Wang J. Zhou W. Hage E. Præsteng K. Løkka G. Torres A.J. Bjørgen H. Koppang E.O., Berge G.M. Krasnov A., Sæle Ø., Aru V. Khakimov B. Engelsen S.B. Åshild Krogdahl. 2019. GutMatters prosjektet: Tarmhelsesituasjonen i oppdrettsanlegg langs kysten, i sør og nord, sommer som vinter. Muntlig innlegg, Det 14. norske fiskeernæringsseminaret, Solstrand, 13.-14. november 2019.
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12. Krogdahl, Å., Berge, G.M., Engelsen, S.E., Koppang, E.O., Midtlyng, P.J., Sæle, Ø., Aru, V., Bjørgen, H., Chikwati, E., Khakimov, B., Kortner, T., Krasnov, A., Torres. A.J. 2020. Tarmhelsen hos laks i norske oppdrettsanlegg kan forbedres med bedre fôrsammensetning. Abstract for oral presentation at Havbrukskonferansen 2020, in Bergen. Video conference June 9th, 2020.
13. Fernando, I.C., Krogdahl, Å., Berge, G.M., Le, H., Kortner, T.M., Sæle, Ø. 2020. Effekten av plantevernmidler i laksefôr på tarmhelse. Abstract for oral presentation at Havbrukskonferansen 2020, in Bergen, which was cancelled.

14. Siciliani, D., Berge, G.M., Kortner, T.M., Valen, E.C., Chikwati, E.M., Krogdahl, Å. 2021. Effects of dietary lipid level and environmental temperature on the severity of steatosis in Atlantic salmon fed suboptimal level of choline. Oral presentation at EAS conference, Madeira, October 5. – 7., 2021.
15. Krogdahl, Å., Chikwati, E.M., Krasnov, A., Torres, A.J., Berge, G.M., Midtlyng, P., Hillestad, M., Koppang, E.O., Sæle, Ø., Engelsen, S.B., Kortner, T.M. 2021. Gut health effects in Atlantic salmon (salmo salar l) of low dietary fish meal level can be limited by dietary supplements. Oral presentation at EAS conference, Madeira, October 5. – 7., 2021.
16. Thunes, V., Siciliani, D., Kortner, T.M. Hanssen, H., Chikwati, E.M., Krogdahl, Å. 2022. Effects of dietary lipid quality and fish size on choline requirement in Atlantic salmon (Salmo salar L). ISFNF, Sorrento, June 6. – 9., 2022.
17. Krogdahl, Å., Siciliani, D., Chikwati, E.M., Berge, G.M., Kortner, T.M. 2022. Choline requirement of Atlantic salmon (*Salmon salar* L) depends on dietary lipid level and environmental temperature. ISFNF, Sorrento, June 6. – 9., 2022.
18. Aru, V., Khakimov, B., Kortner, T.M, Krogdahl, Å., Engelsen, S.B. 2022.^{1}H-NMR metabolomics investigation of plasma and fecal samples from farmed salmons fed with reduced fishmeal and plant-based feed. Oral presentation at MRFood2022 - 15th International Conference on the Applications of Magnetic Resonance in Food Science, June 7-10, 2022 - Aarhus, Denmark.

Publications in trade journals

19. Krogdahl, Å., Chikwati, E., Kortner, T.M., Engelsen, S.B., Koppang, E.O., Berge, G. M., Sæle, Ø., Krasnov, A., Midtlyng, P. J. 2019. Tarmproblemer hos oppdrettslaks, i sør og nord, sommer og vinter. Norsk fiskeoppdrett 8, 114-117.
20. Krogdahl, Å., Chikwati, E., Engelsen, S.B., Koppang, E.O., Berge, G. M., Sæle, Ø., Krasnov, A., Midtlyng, P. J., Kortner, T.M. Laksen trenger mye kolin for effektivt å kunne transportere fett fra tarmen til blod, organer og vev. Manus under utvikling. Sendes til Norsk fiskeoppdrett.

Publications in scientific journals

21. Aru, V., Khakimov, B., Sørensen, K.M., Chikwati, E.M., Kortner, T.M., Midtlyng, P., Krogdahl, Å., Engelsen, S.B. 2021. The plasma metabolome of Atlantic salmon as studied by 1H NMR spectroscopy using standard operating procedures. Effect of aquaculture location and growth stage. Metabolomics 17(6) DOI: 10.1007/s11306-021-01797-0.
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23. Bjørgen, H., Oaland, Ø., Midtlyng, P., Tafalla, C., Krogdahl, Å., Koppang, E.O. IgD-transcript positive cells suggest hypersensitivity in feed-related intestinal inflammation in the Atlantic salmon. Submitted to Fish and Shellfish Immunology
24. Krogdahl, Å., Chikwati, E.M., Krasnov, A., Dhanasiri, A., Berge, G.M., Aru, V., Khakimov, B., Engelsen, S.B., Kortner, T.M. Gut function, microbiota, and health in Atlantic salmon (Salmo salar, L.) are modulated by dietary fishmeal level and functional ingredients. Submitted to Aquaculture Nutrition.
25. Krogdahl, Å., Dhanasiri, A., Krasnov, A., Aru, V., Khakimov, B., Chikwati, E.M., Berge, G.M., Engelsen, S.B., Kortner, T.M. Effects of functional ingredients on gut inflammation in Atlantic salmon (Salmo salar L). (In manuscript, to be submitted shortly).

Manuscripts under development

1. Siciliani, D., Chikwati, E.M., Berge, G.M., Kortner, T.M., Krogdahl, Å. 2022. Effects of dietary lipid level and environmental temperature on choline requirement in Atlantic salmon (*Salmo salar* L) parr.
2. Thunes, V., Siciliani, D., Kortner, T.M. Hanssen, H., Chikwati, E.M., Krogdahl, Å. 2022. Effects of dietary lipid quality and fish size on choline requirement in Atlantic salmon (Salmo salar L).
3. Aru, V., Khakimov, B., Sørensen, K.M., Chikwati, E.M., Kortner, T.M., Midtlyng, P., Krogdahl, Å., Engelsen, S.B. Using the salmon faecal delta metabolome to evaluate new plant-based feeding strategies.
4. Aru, V., Khakimov, B., Sørensen, K.M., Chikwati, E.M., Kortner, T.M., Midtlyng, P., Krogdahl, Å., Engelsen, S.B. Metabolomics investigation of salmon feeding trials targeted for reducing fishmeal.

Booklet under development

Midtlyng, P., Chikwati, E., Aru, V., Khakimov, B., Sørensen, K.M., Kortner, T.M., Krogdahl, Å., Engelsen, S.B.

Educational activities

Master degree (UiB, completed). Iresha Chavindi Fernando. Topic: Effects of chlorpyrifos, an organophosphate pesticide on the intestinal barrier of Atlantic salmon (Salmo salar): Finished (UiB/HI)

PhD (NMBU, ongoing). Daphne Siciliani. Topic: Effects of dietary choline supplementation to Atlantic salmon: Production conditions, gut health, and epigenetic regulation.

Research education program for vet students (ongoing). Vebjørn Thunes. Topic: Steatosis in Atlantic salmon. Effects of fat quality and fish size.

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