

Nutrition and intestinal health in Atlantic salmon (*Salmo salar*): Involvement of antinutrients and microbiota

Ernæring og tarmhelse hos Atlantisk laks (*Salmo salar*):

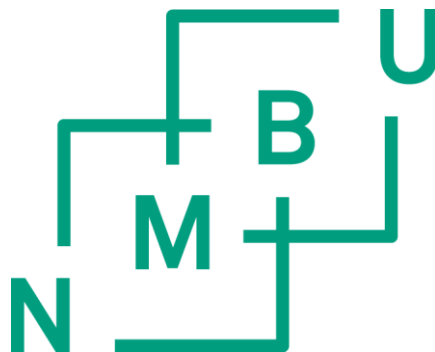
Betydning av antinæringsstoffer og mikrobiota

Philosophiae Doctor (PhD) Thesis

Karina Gajardo

Department of Basic Sciences and Aquatic Medicine
Faculty of Veterinary Medicine and Biosciences
Norwegian University of Life Sciences

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SUMMARY

The use of alternative protein ingredients to replace fishmeal in diets for Atlantic salmon (*Salmo salar*) can have negative consequences for the fish intestinal health. The most severe intestinal physiological and morphological effects were first observed to be induced in the distal intestine (DI) by soybean meal and called soybean meal induced enteritis (SMBIE). Other intestinal health disorders suggested to be related to the increasing use of plant ingredients in the diets include lipid malabsorption syndrome and intestinal tumors. SBMIE has been linked to the presence of antinutritional factors (ANFs) in soybean meal, which are also present in other legumes. Among the ANFs, saponins – alone or in combination with other ANFs – have been suggested as the most plausible cause of SBMIE and other legume-induced enteropathies. The syndrome seems to vary with stage of fish development, breed of soybeans and possibly other biotic, as well as abiotic conditions. Among other intestinal conditions that might influence the severity of SBMIE is the intestinal microbiota, for which present knowledge is very limited both in general and in their potential involvement in enteric pathologies in the salmon intestine.

The work presented in this thesis contributes to the basic knowledge regarding the effects of saponins and intestinal microbiota necessary for the understanding of the normal intestinal health of Atlantic salmon and the consequences of increasing use of alternative protein ingredients as replacement to fishmeal in the diet. Three aims were formulated;

Aim 1: to find the important mechanisms behind effects of soya saponins on the intestinal function and their role in SBMIE,

Aim 2: to characterize the digesta- and mucosa-associated microbiota along the intestine of Atlantic salmon fed a commercial diet, and

Aim 3: to characterize the effect on intestinal microbiota caused by the inclusion of alternative protein sources in the diet of Atlantic salmon and the microbiota's role for the function and health of the intestine.

The work conducted to reach *Aim 1*, to find mechanisms of saponins effects and saponins' role in SBMIE, is presented in **Paper I**. This dose-response study demonstrated that soya saponins

alone supplemented to a fishmeal based salmon diet caused SBMIE-like symptoms in the DI at doses as low as 2-4 g/kg. In this experiment, fish kept in a flow-through system with salt water, were fed one of two basal diets; one with fishmeal as the sole protein source and the other with a mix of lupin meal (20%), wheat gluten and fishmeal as protein sources. Batches of these two diets were supplemented with five doses of soya saponins: 0, 2, 4, 8, 10 g/kg. The feeding trial lasted 10 weeks. Feed delivery, but not wasted feed, was recorded.

Fish fed the fishmeal-based diets supplemented with 2-4 g/kg soya saponin and above showed all the typical signs described for SBMIE in the DI, i.e. loss of normal morphology and functionality, increased cell proliferation, along with reduced levels of cholesterol in plasma. Fish fed the lupin-containing diet showed similar SBMIE symptoms at the same soya saponin doses as those fed the fishmeal-based diet, despite apparently consuming less of the diets and therefore growing significantly less than fish fed the FM diet. These results suggest, as indicated in earlier studies, that presence of other ANFs in the lupin meal potentiate the effect of soya saponins. In conclusion, this study clearly showed that soya saponins alone may cause SBMIE-like symptoms in the DI of salmon. Consequently, when feed is formulated using more than one ingredient with saponins, care should be taken to avoid too high levels of saponins. The results also suggest that fishmeal diets supplemented with soya saponins may be used for induction of enteritis and thereby improving of the model earlier used for studies of mechanisms underlying enteritis. A model based on the use of saponin gives better opportunity to the study of factors involved in the enteritis development, as it would not depend on the highly variable composition of soybean meal. Thus, a model, which thereby becomes independent of soybean meal, will allow further investigation of interaction between saponins and other antinutrients, as well as a range of biotic and abiotic conditions, which may be of importance for the severity of the enteritis.

The work to reach *Aim 2*, to, characterize digesta- and mucosa-associated microbiota along the intestine of salmon, is presented in **Paper II**. The fish, kept in a recirculation unit with salt water, were fed for four weeks a commercial diet containing a combination of fishmeal and plant ingredients. Using high-throughput sequencing, the microbiota of five compartments of the intestine was characterized separately; proximal intestinal digesta (PID), mid intestinal digesta

(MID), distal intestinal digesta (DID), mid intestinal mucosa (MIM) and distal intestinal mucosa (DID).

The results showed clear significant differences between the bacterial communities in the digesta and the mucosa samples. The mucosa-associated microbiota presented lower richness than the digesta-associated microbiota. Moreover, there was a gradual change in the microbiota along the intestine in both the mucosa- and the digesta-associated microbiota: the difference between the MIM and DIM, as well as between the PID and MID, PID and DID were significant, but the differences between MID and DID did not show significance. The operational taxonomic units (OTUs) of all the digesta samples showed a high abundance of Proteobacteria (47%), Firmicutes (38%), Fusobacteria (7%) and Actinobacteria (6%). The mucosa compartments showed very high abundance of Proteobacteria (90%). A core microbiota of 22 OTUs was found in 80% of the samples of all compartments.

The work conducted to reach *Aim 3*, to characterize effects of alternative protein sources on intestinal microbiota and intestinal function and health, is presented in **Papers III** and **IV**. The fish, kept in a recirculating system with salt water, were fed one of five different diets for 48 days. A diet with fishmeal (FM) as sole protein source served as the control diet. The four substitution diets contained one of four alternative protein sources/mixes replacing a proportion of the fishmeal: one with 58% poultry meal (PM), a second with soybean meal (30% of the diet) mixed with wheat gluten (22% of the diet) (SBMWG), a third with soy protein concentrate (30%) mixed with poultry meal (6%) (SPCPM) and a fourth with guar meal (30%) mixed with wheat gluten (14.5%) (GMWG).

The results showed that the SBMWG diet compared to the FM diet, induced negative effects in the DI function and structure, which corresponded to what is described in fish in the chronic stage of SBMIE: histological signs of mild/moderate enteritis, high water content in the chyme, altered expression profile of water transport and cellular stress genes, and high number of PcnA immunopositive cells indicating higher cell proliferation. Concomitant to the functional and morphological changes in the DI, fish fed SBMWG also presented the highest modulation in the DI-associated microbiota compared to FM fed fish, especially in the digesta. In comparison with

FM fed fish, the fish fed SBMWG showed very high relative abundance of lactic acid bacteria (LAB) in both digesta and mucosa.

The fish fed the GMWG diet showed alteration in some of the genes related with water transport and cell proliferation. The latter was also confirmed by immunohistochemistry of the Pcn α . However, no clear signs of enteritis were observed in these fish, suggesting that the observed altered parameters could be the result of a normal adaptation to the diet. On the other hand, these fish also showed high abundance of LAB in the digesta.

The fish fed the other two substitution diets, PM and SPCPM, showed moderate modulation of the microbiota, but the observed indicators of intestinal health of these two experimental groups did not show alterations, which indicate no health related challenges.

Overall conclusions: The work presented in this thesis contributes with important knowledge regarding the responses of the salmon intestine to soya saponins and the role of this ANF as the causative agent of the SBMIE. The work also presents the most detailed characterization of the salmon intestinal microbiota to date revealing differences between the mucosa and digesta, as well as the between proximal and distal compartments. The influence of diet composition on the intestinal microbiota is clearly documented, and was found to be much more pronounced in digesta than the mucosa. The results do not give a solid basis for drawing conclusions regarding relationship between intestinal microbiota, function and health. Much more work need to be done combining several “omics” tools to reach a solid enough basis. The microbiota results presented in this thesis rise questions on whether the microbiota of specific compartments differ regarding their role for the intestinal function and health of salmon. In addition, the high abundance of LAB observed in fish undergoing SBMIE seems to be contradictory with the supposedly positive effect that this group of bacteria have been reported to have in homoeothermic species rising the question whether LAB in salmon may have other effects than in homoeothermic.

The work presented in this thesis supply important information strengthening the basis for the future work towards and understanding of the role of the intestinal microbiota for function and health of the intestine and of the fish overall.

SUMMARY IN NORWEGIAN (SAMMENDRAG)

Bruk av alternative proteinkilder som erstatning for fiskemel i fôr til atlantisk laks (*Salmo salar*) kan medføre redusert tarmhelse. De første tegn på fysiologiske og morfologisk helseeffekter i tarmen, ble observert i den distale delen av tarmen (DI), og var induert av innblanding av soyamel i fôret. Symptombildet ble kalt soyainduert enteritt (SBMIE). Laksens tarm er grundig studert når det gjelder slike negative effekter av proteinrike alternative fôrmidler. Blant øvrige effekter på tarmhelse som kan ha sammenheng med den økte broken av planteingredienser i fôret er en tilstand som er beskrevet som lipidmalabsorpsjonssyndrom, og en annen som fører til tarmsvulst. Soyainduert enteritt ser ut til å ha sammenheng med innholdet av antinæringsstoffer (ANFs) og er vist å kunne indueres også av frø fra andre belgvekster. Blant de aktuelle ANFs i soyamel ser saponinene, alene eller i samvirke med andre antinæringsstoffer, ut til å være de mest sentrale for utviklingen av SBMIE. Symptomene kan variere med fiskens utviklingsstadium, sorten soya som brukes, og muligens med andre biotiske og abiotiske forhold. Blant andre tarmforhold som kan være av betydning for alvorlighetsgraden av SBMIE, er sammensetningen av microbiota som vi har svært lite relevant kunnskap om både generelt og når det gjelder den rolle den spiller for patologiske tilstander hos laks.

Arbeidet som presenteres i denne avhandlingen bidrar med grunnleggende kunnskaper om virkninger av saponiner og om tarmens microbiota. Denne kunnskapen er nødvendig for forståelsen av normal tarmfunksjon hos laks og virkninger på tarmhelsen av økt bruk av alternative proteinfôrmidler som erstatning for fiskemel. Tre mål ble formulert for arbeidet;

Mål 1: å finne de viktige mekanismene bak effektene av soyasaponiner på tarmfunksjon og rollen de spiller for utviklingen av SBMIE,

Mål 2: å beskrive tarmens microbiota, både den som finnes i tarminnholdet og den som er forankret i mukosa langs hele tarmkanalen hos laks som spiser et kommersielt fôr,

Mål 3: å beskrive effekter på tarmens microbiota av ulike proteinkilder i fôret til laks og rollen microbiotaen spiller for tarmens funksjon og helse.

Arbeidet som ble gjennomført for å nå *Mål 1*, å finne mekanismer bak saponineffekter og betydning for SBMIE, er publisert i **Paper I**. Dette dose-responsstudiet viste at soyasaponiner

alene, tilsatt som supplement til et fiskemelbasert fôr, ga SBMIE-liknende symptomer i DI ved innblandingsnivåer på 2-4 g/kg og over. Forsøket ble gjennomført med laks i sjøvann i et gjennomstrømmingssystem. Fisken ble fôret ett av to basalfôr, det ene inneholdt fiskemel som den eneste proteinkilden, mens det andre inneholdt lupinmel (20%), der proteinet i lupinmelet erstattet protein i fiskemel. Batcher av disse basaldiettene ble tilsatt soyasaponin i dosene: 0, 2, 4, 8, 10 g/kg. Fôringsforsøket varte 10 uker. Utfôring, men ikke fôrspill, ble registrert.

Fisken som fikk det fiskemelbaserte basalfôret i doser på 2-4 g/kg eller høyere viste de fleste typiske tegn som er beskrevet for SBMIE, dvs unormal morfologi og endringer i en rekke vitale funksjoner i DI, og dessuten redusert kolesterolnivå i plasma. Fisk som spiste basalfôret med lupinmel viste de samme symptomene ved over 2 – 4 g/kg som fisken som fikk basalfôret basert på fiskemel, til tross for at de vokste vesentlig mindre og derfor etter all sannsynlighet spiste mye mindre. Det vil si at de fikk i seg mindre saponiner. Resultatene indikerer, som tidligere foreslått, at innholdet av andre antinæringsstoffer kan forsterke effekten av saponinene. Hovedkonklusjonen på dette arbeidet er at soyasaponiner alene kan forårsake SBMIE i DI hos laks. Dette medfører at man ved formulering av fôr med mer enn en ingrediens som inneholder saponiner, må unngå at innholdet av saponiner blir for høyt. Resultatene av dette arbeidet gir også grunnlag for bruke soyasaponiner til å framkalle fôrindusert enteritt og derved forbedre modellen som tidligere har vært brukt for å studere mekanismer for utvikling av enteritt. Modellen, som da blir uavhengig av soyamel, en fôringrediens som varierer betydelig i sammensetning, vil også gi betydelig bedre muligheter for å kunne studere interaksjoner mellom saponiner og andre antinæringsstoffer, positive og negative, og for en rekke andre biotiske og abiotiske forhold som kan ha betydning for alvorlighetsgraden av enteritt.

Arbeidet som ble gjennomført for å nå *Mål 2*, dvs å beskrive mikrobiota i tarminnhold og i mukosa langs tarmkanalen hos laks, er publisert i **Paper II**. Fisken ble holdt i et resirkuleringssystem med salt vann og fôret i fire uker på et kommersielt fôr basert på en blanding av fiskemel og flere planteingredienser som proteinkilder. Ved bruk av «high-throughput sequencing» ble mikrobiota i to prøver av tarmmukosa og tre prøver av tarminnhold, dvs tarminnhold fra framre (PID), midtre (MID) og bakre (DID) tarmavsnitt, og to prøver av mukosa, dvs fra midtre (MIM) og bakre (DID) tarmavsnitt.

Resultatene viste klare og signifikante forskjeller mellom bakteriepopulasjonene i prøvene av tarminnhold og mukosa, der mukosa viste mindre «richness» enn tarminnholdet. De viste også en klar gradvis endring langs tarmkanalen både i tarminnholdet og i mukosa: forskjellen var signifikant mellom MIM og DIM, mellom PID og MID og mellom PID og DID, men ikke mellom MID og DID. Alle prøvene av tarminnhold viste, basert på «Operational taxonomic units (OTUs), høy forekomst (abundance) av Proteobacteria (47%), Firmicutes (38%), Fusobacteria (7%) og Actinobacteria (6%) mens prøvene av mukosa viste svært høy forekomst av Proteobacteria (90%). En kjernemikrobiota (core microbiota) på 22 OTUs ble påvist i 80%, alle prøvene sett under ett.

Arbeidet som ble gjennomført for å nå *Mål 3*, å beskrive effekter av alternative proteinkilder på mikrobiota og helse i tarmen hos laks, er presentert i **Papers III** og **IV**. Fisken gikk i et resirkuleringsystem med saltvann og ble fôret ett av fem fôr i 48 dager. Et fôr (FM) var basert på fiskemel som eneste proteinkilde. Dette fungerte som kontrollfôr. De fire andre, kalt substitusjonsfôr, inneholdt ett av fire alternative proteinkilder/blandinger av proteinkilder som delvis erstattet fiskemel: ett inneholdt 58 % fjørfemel (PM), et annet en blanding av soyamel (30 % av fôret) og hvetegluten (22 % av fôret) (SBMWG), det tredje soyakonsentrat (30 %) og fjørfemel (6 %) (SPCPM), det fjerde guarmel (30 %) og hvetegluten (14,5 %) (GMWG).

Resultatene viste at SBMWG, sammenlignet med FM, forårsaket negative effekter i bakre tarmavsnitt (DI) både for funksjon og struktur, dvs endringer som tidligere er observert ved kronisk SBMIE, som høyt vanninnhold i tarminnholdet, endret ekspresjonsprofil for gener som koder for vanntransport, cellulær stress og celleproliferasjon, histologiske forandringer som viste mild til moderat enteritt og økt celleproliferasjon.

Samtidig med de funksjonelle og morfologiske forandringen i DI viste fisken som fikk SBMWG, sammenlignet med FM-fôret fisk, også endringer i mikrobiota, særlig for mikrobiota i tarminnholdet. Fiskene viste svært høyt innhold av melkesyrebakterier (LAB) både i tarminnholdet og i mukosa.

Fisken som ble føret GMWG viste, sammenlignet med den som fikk FM, endringer i noen gener som er relatert til vanntransport, cellulær stress og proliferasjon. Sistnevnte ble også bekreftet immunohistokjemisk. Ettersom fisken ikke viste tegn på enteritt, var disse endringene sannsynligvis indikasjoner på en normal tilpasning til endringer i førets sammensetning. Denne fisken viste imidlertid høy forekomst av LAB i tarminnholdet.

Fisken som fikk de to resterende substitusjonsdiettene, PM og SPCPM, viste moderate endringer i mikrobiota, men resultatene for tarmfunksjon og helse for disse to behandlingene viste ingen endringer som tilsier helserelaterte utfordringer for fisken.

Hovedkonklusjoner: Arbeidet som presenteres i denne avhandlingen, bidrar betydelig til kunnskapsgrunnlaget når det gjelder responsen i tarmen hos laks på soyasaponiner i føret og rollen dette antinæringsstoffet spiller for utviklingen av SBMIE. Arbeidet gir også den mest detaljerte beskrivelse av mikrobiota i tarmen hos laks som er publisert til dags dato, og viser viktige forskjeller mellom mikrobiota i tarminnhold og mukosa så vel som mellom fremre og bakre tarmavsnitt. Effekter av førets sammensetning er også klart dokumentert og viste at effektene var betydelig større tarminnholdet enn i mukosa. Resultatene gir ikke tilstrekkelig grunnlag for å trekke konklusjoner om sammenhengen mellom tarmens mikrobiota, funksjon og helse. Betydelig mer arbeid må gjøres ved bruk av flere «omics» verktøy for å nå en tilstrekkelig basis for slike konklusjoner. Resultatene gir imidlertid grunnlag for å reise spørsmål om hvorvidt mikrobiota i mukosa og tarminnhold, foran og bak i tarmen har ulik betydning for tarmens funksjon og helse. Funnet av høy forekomst av LAB hos fisk som hadde SBMIE kan og hevdes å ha hos varmblodige dyr og reiser spørsmålet om LAB har andre effekter hos laks enn hos varmblodige.

Arbeidet som presenteres i denne avhandlingen bidrar med basalkunnskap som er nødvendig for det framtidige arbeid mot å forstå betydningen av tarmens mikrobiota for tarmens og fiskens funksjon og helse.

SUMMARY IN SPANISH (RESUMEN)

El uso de fuentes de proteínas alternativas para reemplazar la harina de pescado en las dietas de salmón del Atlántico (*Salmo salar*) puede tener consecuencias negativas para la salud intestinal de los peces. Los efectos más severos en la morfología y fisiología intestinal fueron por primera vez observados después de la inducción de la llamada enteritis en el intestino distal (DI) inducida por soya (SMBIE). Entre otros desordenes intestinales supuestamente relacionados al incremento en el uso de ingredientes vegetales en la dieta también se incluyen el síndrome de mala absorción y tumores intestinales. La enteritis inducida por soya ha sido relacionada con la presencia de factores antinutricionales (ANFs), los cuales también están presentes en otras legumbres. Entre los ANFs sugeridos, la saponina sola o en combinación con otros ANFs han sido las causas más probables de SMBIE y otras enteropatías inducidas por otras legumbres. Este síndrome parece depender de la etapa de desarrollo del pez, variedad de la soya y posiblemente otras condiciones bióticas y abióticas. Otro factor que podría influenciar la severidad de SMBIE, es la microbiota intestinal, de la cual su conocimiento es muy limitado y más aún su rol en la patologías entéricas del salmón.

El trabajo presentado es esta tesis contribuye a el conocimiento básico con respecto a los efectos de la saponinas en la microbiota intestinal necesarios para el entendimiento de la salud de la salud intestinal del salmón del Atlántico y las consecuencias de aumentar el uso de proteínas alternativas para reemplazar la harina de soya en la dieta. Tres objetivos fueron formulados:

- 1 Objetivo: Encontrar los mecanismos importantes detrás del efecto de las saponinas de la soya en la función intestinal y sus rol en SBMIE,
- 2 Objetivo: Caracterizar la microbiota asociada a la mucosa y la digesta a lo largo del intestino de salmón del Atlántico alimentado con una dieta comercial, y
- 3 Objetivo: Caracterizar el efecto en la microbiota intestinal causado por la inclusión de fuentes de proteína alternativas en la dieta de salmón del Atlántico y el rol de la microbiota en la función y la salud del intestino.

El trabajo desarrollado para alcanzar el objetivo 1, encontrar los mecanismos del efecto de la saponina y su rol en la enteritis inducida por soya, es presentado en el artículo 1. Este estudio de

dosis respuesta demostró que solo con la suplementación de saponina de la soya en una dieta de salmón basada en harina de pescado causaron síntomas compatibles con SBMIE en el DI a las dosis tan bajas como 2-4g/kg. En este experimento, los peces fueron mantenidos en un sistema de flujo continuo en agua salada y alimentados con una de dos dietas basales; una con harina de soya como única fuente de proteína y la otra con una mezcla de harina de lupino (20%), gluten de trigo y harina de pescado como fuentes de proteínas. Lotes de estas dos dietas fueron suplementadas con cinco dosis de saponina de soya: 0, 2, 4, 8, 10 g/kg. El experimento de alimentación duró 10 semanas. Se registró el suministro de alimento pero no el alimento sin consumir.

Los peces alimentados con la dieta basada en harina de pescado y suplementada con una dosis de saponina de soya de 2-4 g/kg o superior mostraron todo los síntomas típicos de SBMIE en el DI, tales como pérdida de la morfología y funcionalidad normal, incremento de la proliferación celular, así como niveles reducidos de colesterol en plasma. Los peces alimentados con la dieta de lupino mostraron síntomas similares a SBMIE en las mismas dosis de saponina de soya que los peces alimentados con una dieta basada en harina de pescado, a pesar de un consumo aparentemente menor de las dietas y por lo tanto crecieron significativamente menos que los peces alimentados con FM. Estos resultados junto con estudios anteriores sugieren, que la presencia de otros ANFs en la harina de lupino potencian el efecto de la saponina de soya. En conclusión, este estudio claramente demostró que la saponina de soya sola puede causar síntomas semejantes a SBMIE en el DI del salmón. Basado en estos resultados, las dietas de harina de pescado suplementadas con saponina de soya pueden ser usados para la inducción de enteritis y de este modo mejorar el modelo usado anteriormente para estudiar los mecanismos fundamentales de la enteritis. Un modelo el cual sea independiente de harina de soya la cual es altamente variable en su composición, también daría mejores oportunidades para estudiar las interacciones entre las saponinas y otros antinutrientes, así como la relación con un rango de condiciones bióticas y abióticas las cuales pueden ser importantes en la severidad de la enteritis.

El trabajo para alcanzar el objetivo 2, caracterizar la microbiota asociada a la mucosa y la digesta a lo largo del intestino de salmón esta presentado en el artículo II. Los peces fueron mantenidos en un sistema de recirculación en agua salada y alimentados por cuatro semanas con una dieta

comercial que contenía una combinación de harina de pescado e ingredientes de origen vegetal. Usando secuenciación de alto rendimiento, se caracterizó separadamente la microbiota de cinco compartimientos del intestino; digesta del intestino proximal (PID), digesta intestino medial (MID), digesta intestino distal (DID), mucosa intestino medio (MIM) y mucosa intestino distal (DID).

Los resultados mostraron claras diferencias significativas entre las comunidades bacterianas de la mucosa y la digesta. La microbiota asociada a la mucosa presentó más baja riqueza de especies que la microbiota asociada a la digesta. Más aun, hubo un cambio gradual en la microbiota a lo largo del intestino tanto en la microbiota asociada a la mucosa como en la asociada a la digesta: la diferencia entre MIM y DIM y entre PID y MID, PID y DID fueron significativas al contrario que entre MID y DID donde no hubo diferencias significativas. Las unidades operacionales taxonómicas (OTUs) de todas las muestras de la digesta mostraron una alta abundancia de Proteobacterias (47%), Firmicutes (38%), Fusobacteria (7%) y Actinobacteria (6%). Los compartimientos de la mucosa mostraron una abundancia muy alta de Proteobacterias (90%). Una microbiota común de 22 OTUs fue encontrada en el 80% de las muestras de todos los compartimientos.

El trabajo para alcanzar el objetivo 3, caracterizar los efectos de diferentes fuentes alternativas de proteína en la microbiota intestinal y la función intestinal y salud, está presentado en el Artículo III y IV. Los peces fueron mantenidos en un sistema de recirculación de agua salada y alimentados durante 48 días con una de cinco dietas diferentes. Una dieta de harina de pescado (FM) como única fuente de proteína fue utilizada como dieta control. Las cuatro dietas restantes contenían una de las cuatro fuentes de proteínas puras o mezcladas reemplazando la harina de pescado; una con 58% de harina de pollo (PM), una segunda con harina de soya (30%) mezclada con gluten de trigo (22%) (SBMWG), una tercera con concentrado de proteico de soya (30%) mezclado con harina de pollo (6%) (SPCPM) y una cuarta con harina de guar (30%) mezclada con harina de trigo (14.5%) (GMWG).

Los resultados demostraron que la dieta SBMWG comparada con la dieta de FM, indujo efectos negativos en la función y estructura del DI, lo cual corresponde a lo descrito en peces durante la

fase crónica de SBMIE: signos histológicos compatibles con enteritis media a moderada, alto contenido de agua en el quimo, alteración del perfil de expresión de genes de transporte de agua y estrés celular y alto número de células inmuno-positivas a Pcná indicativas de alta proliferación celular. Concomitante a los cambios morfológicos y funcionales en el DI, los peces alimentados con SBMWG también presentaron la modulación más alta en la microbiota asociada al intestino distal comparado con los peces alimentados con FM, especialmente en la digesta. En comparación a los peces alimentado con FM, los peces alimentados con SBMWG mostraron un abundancia relativa de las bacterias ácido lácticas (LAB) más alta tanto en la digesta como en la mucosa.

Los peces alimentados con la dieta GMWG mostraron alteración en algunos genes relacionados con el transporte de agua, estrés celular y proliferación celular. Lo anterior fue también confirmado por inmunohistoquímica del Pcná. Sin embargo, en estos peces no se observaron signos claros de enteritis, sugiriendo que los parámetros alterados podrían ser el resultado de una adaptación normal a la dieta. Por otro lado, estos peces también mostraron alta abundancia de LAB en la digesta.

Los peces alimentados con las otras dietas PM y SPCPM, mostraron una modulación moderada de la microbiota, pero los indicadores observados de salud intestinal de estos dos grupos experimentales no mostraron alteraciones, lo cual indica que no hubo un deterioro de la salud.

Conclusiones generales: El trabajo presentado en esta tesis contribuye de forma importante al conocimiento respecto a las respuestas intestinales del salmón a la saponina de la soya y el rol de este ANF como agente causal de SBMIE. El trabajo también presenta la caracterización más detallada de la microbiota intestinal del salmón a la fecha, revelando las diferencias entre la mucosa y la digesta, coma también entre los compartimientos distal y proximal. La influencia de la composición de la dieta en la microbiota intestinal es claramente documentada, e indica que es mucho más pronunciada en la digesta que en la mucosa. Los resultados no dan una base sólida para concluir la relación entre la microbiota, la función intestinal e implicaciones en la salud. Es necesario realizar mayores esfuerzos en combinar diversas herramientas “omics” para una base lo suficientemente sólida. Los resultados de la microbiota presentados en esta tesis plantean la

pregunta si la microbiota de los diferentes compartimientos intestinales difieren en términos de sus roles en la función intestinal y la salud del salmón. Adicionalmente, la alta abundancia de LAB observada en los peces que están experimentando SBMIE parece ser contradictorio con los efectos positivos que se han reportado tener este grupo de bacterias en especies homotérmicas trayendo el interrogante si LAB en salmón pueden tener otros efectos que los vistos en animales homotérmicos.

El trabajo presentado en esta tesis aporta información importante para fortalecer la base del trabajo futuro hacia el entendimiento del rol de la microbiota intestinal en la función y salud del intestino y de los peces en general.

LIST OF ABBREVIATIONS

ANF	Antinutritional factor
cDNA	Complementary DNA
DI	Distal intestine
DID	Distal intestine digesta
DNA	Deoxyribonucleic acid
DIM	Distal intestine mucosa
FM	Fishmeal
HTS	High-throughput sequencing
IEL	Intraepithelial lymphocytes
LEfSe	Linear discriminant analysis effect size
MI	Mid intestine
MID	Mid intestine digesta
MIM	Mid intestine mucosa
NSTI	Nearest sequenced taxon index
OTU	Operational taxonomic unit
Pcna	Proliferation cell nuclear antigen
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PI	Proximal intestine
PICRUSt	Phylogenetic investigation of communities by reconstruction of unobserved states
PID	Proximal intestine digesta
PIM	Proximal intestine mucosa
QIIME	Quantitative insights into microbial ecology
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RFLP	Restriction Fragment Length Polymorphism
SBM	Soybean meal
SBMIE	Soybean meal induced enteritis

LIST OF ARTICLES

Paper I

Soya saponins induce enteritis in Atlantic salmon (*Salmo salar* L.)

Åshild Krogdahl, Karina Gajardo, Trond M. Kortner, Michael Penn, Min Gu, Gerd Marit Berge, Anne Marie Bakke. Journal of Agricultural and Food Chemistry 2015; 63(15), 3887-3902

Paper II

A high-resolution map of the gut microbiota in Atlantic salmon. A basis for comparative gut microbial research

Karina Gajardo, Ana Rodiles, Trond M. Kortner, Åshild Krogdahl, Anne Marie Bakke, Daniel L. Merrifield, Henning Sørum
Accepted by Scientific Reports

Paper III

Intestinal fluid permeability in Atlantic salmon (*Salmo salar* L.) is affected by dietary protein source

Haibin Hu, Trond M. Kortner, Karina Gajardo, Elvis Chikwati, John Tinsley, Åshild Krogdahl
Submitted to PLOS ONE

Paper IV

Alternative protein sources in the diet modulate microbiota and functionality in the distal intestine of Atlantic salmon (*Salmo salar*)

Karina Gajardo, Alexander Jaramillo-Torres, Trond M. Kortner, Daniel L. Merrifield, John Tinsley, Henning Sørum, Anne Marie Bakke, Åshild Krogdahl
Manuscript

INTRODUCTION

Fish health and growth performance are two of the main concerns when alternative sources of protein are included in Atlantic salmon (*Salmo salar*) diets in replacement of fishmeal. Several protein-rich plant ingredients commonly used in diets for land animals have been found also to be suitable alternatives for fishmeal in the diets of salmon. However, their inclusion is limited by the effects they seem to have on aspects of intestinal function and health. Disorders such as enteritis, lipid malabsorption syndrome, intestinal tumors and intestinal dysbiosis that may be related to changes in the diet composition have been reported with increasing frequency over the last two decades (Veterinærinstituttet, 2005). These symptoms may all be related to the continuous increase in the use of alternative resources such as plant ingredients in the diet, which not only affect health and normal functionality of the intestine, but the organism as a whole, as well as fish welfare. In the present context, some of the major research foci are the impacts of a group of molecules naturally present in plants, namely the antinutritional factors (ANFs) or antinutrients. Among them, saponins that are present at high levels in legumes, especially soybean, have gained particular attention and have been suspected to be the key agent responsible for the enteropathy caused by soybean meal. At the time of initiation of this thesis work, the question whether saponins alone would cause soybean meal induced enteritis (SBMIE) was not substantiated and so were many questions regarding the role of the intestinal microbiota for the enteritis development. The present work addresses these questions. Methodology well established in our laboratory, along with new sequencing techniques for characterization of the intestinal microbiota offered the possibilities of strengthening the basis for understanding the role of soya saponins and the microbiota for the enteritis development. The laboratory of Dr. Daniel Merrifield offered excellent cooperation for exchange of expertise allowing studies of the role of the microbiome for intestinal function in the fish.

The chapters below presents background information and knowledge of relevance for this thesis work.

Aquaculture production

There is no doubt about the key role that the aquaculture industry has and will continue to have as a source of food for people around the world. The world fish aquaculture industry has had an average annual growth of 6.2% in the last years (FAO, 2014), producing in the year 2012 66.6 million tons of food. By 2030, projections from the Food and Agricultural Organization of the United Nations (FAO) estimate that the aquaculture sector will contribute with over 60% of the fish for human consumption in the world, about 93.6 million tons, with Atlantic salmon contributing with at least 5 million tons (FAO, 2014) (Figure 1).

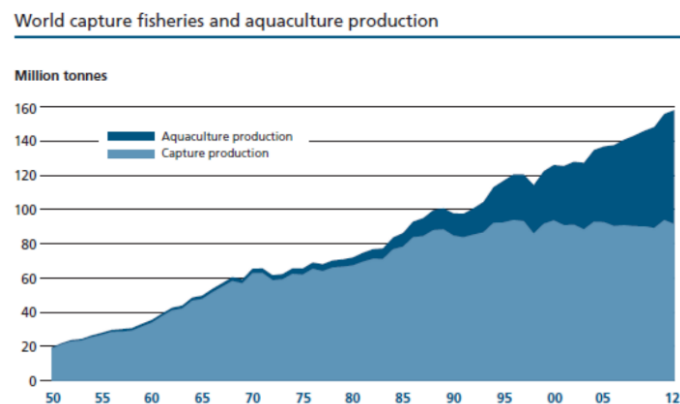


Figure 1. Global fisheries capture and aquaculture production (FAO, 2014).

As the aquaculture industry grows, the demand for ingredients for fish feed production also increases. In the past, the main ingredients in the diet of farmed carnivorous fish species have been fishmeal and fish oil obtained from wild catch. However, the supply of total wild catch has reached a plateau over the last decades (FAO, 2014). In order to reach the FAO projections of fish supply in coming decades, the aquaculture industry must base the production on alternative protein and lipid sources to produce sustainable and high quality feeds for farmed fish. Less expensive and more abundant sources, such as plant ingredients, are now used more and more as alternatives to fishmeal and fish oil in the diets of carnivorous fish. However, the use of alternative plant-based resources, especially those rich in protein, in the diets for salmonids have been limited by the presence of ANFs. These are naturally present in plants supposedly as

protection against disease-causing microorganisms and predation by higher animals. Most of them affect nutrient utilization negatively and many cause detrimental effect in the intestinal functionality and morphology (reviewed by (Krogdahl *et al.*, 2010).

In salmon as in other farmed fish species, the search for new sustainable alternative feed sources has demanded new knowledge and stimulated research, in particular regarding diet effects on intestinal physiology and health.

The alimentary tract of Atlantic salmon

The alimentary tract has as main function to digest and absorb the nutrients from the ingested food along with excreting the organism's waste and unused food components. Among other functions, the alimentary tract is responsible for hormone release and osmoregulation. Moreover, the alimentary tract acts as a physical, chemical and immunological barrier against pathogens and potentially harmful exogenous molecules. The alimentary tract also harbors a complex microbial community that influences various host functions.

Morphology and function

In general, the alimentary tract of Atlantic salmon, as most animals, can be divided in three main segments: the pre-gastric, gastric and post-gastric compartments. The pre-gastric segment comprises the mouth, where the ingestion of food occur, the pharynx, and the esophagus, the latter forming a tube connecting the mouth with the lower part of the alimentary tract. The gastric compartment or stomach is in charge of mechanical and initial enzymatic digestion of food particles. The stomach possesses oxynticopeptic cells that secrete hydrochloric acid and thus maintaining acidic conditions, as well as pepsinogen, the precursor of the gastric protease pepsin. The hydrochloric acid denatures proteins in the feed and converts the pepsinogen into its active form pepsin, which initiates hydrolysis of the feed proteins. The post-gastric compartment encompasses the intestine, also called the gut, and can be subdivided into three macroscopically distinguishable regions (Figure 2); the proximal intestine (PI) with the pyloric caeca (PC), the

mid intestine (MI) and the distal intestine (DI) (Nordrum *et al.*, 2000). After passing the stomach, the acidic semi-digested feed (chyme) enters the intestine. The PI is the location where most digestion and nutrient absorption occurs. In the PI, the pH of the chyme becomes more basic by the addition of bicarbonate from bile and pancreatic secretions. At this pH, nutrients are further digested with the help of digestive enzymes and bile salts secreted by the pancreas and liver, respectively, that function more optimally at a more neutral pH. The DI, as the rest of the intestine of salmon, and in contrast to the mammal's colon, presents enterocytes with microvilli forming a brush border. Consequently, digestion and absorption of nutrients may take place along most of the intestinal tract. Furthermore, macromolecules, such as large peptides, proteins and lipids, escaping previous digestion can be absorbed in the DI and may play a role in the development of the intestinal immune system.

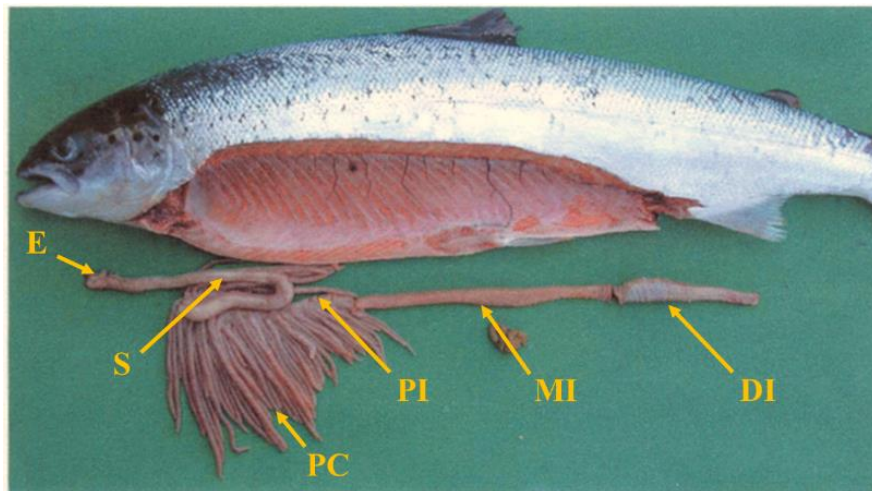


Figure 2. Anatomy of the alimentary tract of Atlantic salmon. Abbreviations: E, esophagus; S, stomach; PI, proximal intestine; PC, pyloric caeca; MI, mid intestine; DI, distal intestine. Image property of Åshild Krogdahl.

Along the alimentary tract, a mucus layer covers the mucosa. The mucus is produced by goblet cells, which are present in the mucosa between the enterocytes. The function of the mucus is varied; it creates a protective physical and chemical barrier; contains antimicrobial substances such as lysozymes, lectins and antimicrobial peptides and immunoglobulins, aid in the digestion and absorption of nutrients and in maintaining the intestinal homeostasis (Gomez *et al.*, 2013).

Histomorphology of the intestine

Figure 3 A-D shows the normal histological morphology of the intestine of Atlantic salmon. The intestine is a tube, which transversally comprises four main layers: *tunica serosa*, *tunica muscularis*, *tunica submucosa* and *tunica mucosa*.

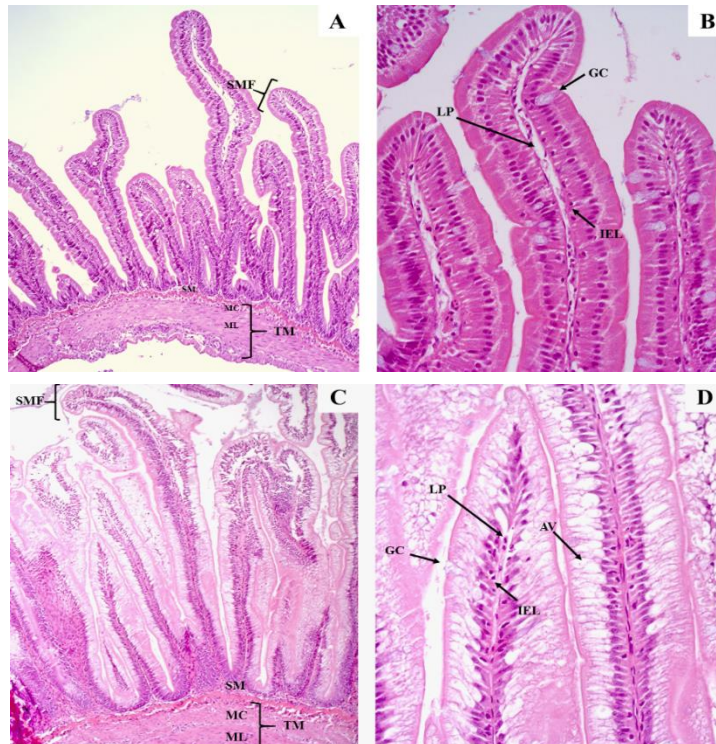


Figure 3. Normal histomorphological representation of sections of the intestine of Atlantic salmon, stained with hematoxylin and eosin. (A and B) Representative images of a longitudinal section of PI, PC and MI at lower (A) and higher (B) magnification; (C and D) Representative images of a longitudinal section of the DI at lower (C) and higher (D) magnification. Abbreviations: SMF; simple mucosal fold; SM, *tunica submucosa*; TM; *tunica muscularis*; MC, muscularis circularis; ML, muscularis longitudinalis; GC, goblet cell; LP, lamina propria; IEL, intraepithelial lymphocyte; AV, absorptive vacuoles.

The *tunica serosa* is the most external structure of the tube. It is made of mesothelial cells and connective tissue. The *tunica muscularis* consists of both a circular and a longitudinal layer of muscle in charge of the peristaltic movements of the intestine. The *tunica submucosa* with its stratum compactum is an extra layer of connective tissue that supports the innermost layer. The *tunica mucosa* or mucosa consists of a single layer of absorptive epithelial cells and lamina

propria, forming simple and complex mucosal folds. The enterocytes of the epithelial layer present a brush border membrane, which facilitate final digestion and absorption of nutrients. Alkaline phosphatase, leucine-aminopeptidase and some disaccharidases are produced in epithelial cells and subsequently located in the brush border membrane, aiding in the hydrolysis of peptides and low molecular carbohydrates, respectively ((Bakke-McKellep *et al.*, 2000) and reviewed by (Krogdahl *et al.*, 2005)). Moreover, the enterocytes may present absorptive vacuoles depending on the feeding state of the fish and the section of the intestine they belong. Goblet cells are mucus-producing cells. The function of rodlet cells is poorly understood but has been associated with immunological functions in the intestine of fish (Reite, 2005). Intraepithelial lymphocytes (IEL) are part of the defensive mechanism of the intestine and in accordance, their number grow when a possible threat is detected. The lamina propria, located in the middle of the mucosal folds in close contact with the enterocytes and submucosa, contains vascularized connective tissue, along with nerves and immune cells, including T- and B-lymphocytes (McMillan & Secombes, 1997, Bakke-McKellep *et al.*, 2007).

The immune system of the intestine

The immune system is the main defense system against potential harmful component and organisms of animals. As other animals, fish possesses an innate immune system formed by an in-born humoral and cellular defense system that include monocytes/macrophages, neutrophils, phagocytic cells and the mucus. The latter covers the mucosal layer in the intestine and contains various protective and antimicrobial substances, such as complement, mucins, enzymes, piscidins and defensins (Silphaduang *et al.*, 2006, Zou *et al.*, 2007). The presence of pattern recognition receptors (PPRs), such as Toll-like receptors in several phagocytic cells such as macrophages, neutrophils and dendritic cells, permits the recognition of external molecules as in the case of so-called pathogen-associated molecular patterns (PAMPs) of microbes (reviewed by (Tlaskalova-Hogenova *et al.*, 2005). PRRs help direct the immune response by activating signals that increase expression of soluble mediators, which are in charge of recruiting and regulating the immune and inflammatory cells that initiate or enhance immune responses. The complement system is composed by several protein with different functions such as elimination of pathogen, induction

of inflammatory responses, clearance of cell debris and modulation of adaptive immune response (reviewed by (Boshra *et al.*, 2006).

The other part of the immune system is the adaptive or specific immune system, which is also present in teleost fish. It is characterized by the humoral and cellular immune response mediated by B- and T-lymphocytes. Antibodies, also known as immunoglobulins, are produced by B cells against specific antigens and mediate the humoral response. Two immunoglobulins have been described in the intestinal immune system of teleost fish; IgM (Bengtén *et al.*, 1991) and IgT or Z (Hansen *et al.*, 2005, Savan *et al.*, 2005, Savan *et al.*, 2005). In fish, the specific cellular immune response is, as in mammals, also T lymphocyte-mediated. Regulatory T cells (Tregs) function in maintaining homeostasis. T cell-mediated immunity can include antigen-specific cytotoxic T lymphocytes, as well as various T helper (Th) cells such as Th1, Th2 and Th17 cells with various roles and specificities depending on the source of injury and type of immune response.

Common for both the innate and adaptive immune responses are the production and release of cytokines, both in maintaining homeostasis – by so-called anti-inflammatory cytokines – as well as in response to an injury or antigen – by so-called pro-inflammatory cytokines. Thus cytokines are signaling substances that orchestrate cross-talk between the different immune cells, e.g. as needed to mount an appropriate immune response.

The intestine's mucosal immune system can be divided in two sites; the induction sites namely the intestinal (gut) associated lymphoid system (GALT) and the effector sites, which include the mucosa presenting intraepithelial lymphocyte (IEL), dendritic cells and macrophages. The GALT in many teleost fish, including salmon, lacks specialized structures such as Peyer's patches or lymph nodes that are present in mammals. But the intestinal epithelium and the lamina propria of the mucosal folds still contain diffusely distributed lymphocytes, plasma cells, granulocytes, macrophages etc., all important players in the mucosal immune system.

Intestinal microbiota

The intestinal microbiota is a key element for the health and normal development of the host. It is defined as the total population of microorganisms inhabiting the host's intestine. It comprises mainly bacteria. In addition yeast, viruses, protozoa and archaea have been described as normal members of the intestinal microbiota of animals, including fish (reviewed by (Sekirov *et al.*, 2010) and (Nayak, 2010). Endogenous (health status, developmental stage) but also exogenous factors are known as important modulators of the intestinal microbiota. Among the latter, diet, temperature, salinity and microbiota of the environment exert an important influence on the microbial composition.

The intestinal microbiota can be divided in two groups according to their location in the intestine: the allochthonous and the autochthonous microbiota. The allochthonous microbiota comprise the microorganisms in transit, namely in the digesta, whereas the autochthonous microbiota refers to microorganisms living in close contact with the mucosal layer of the intestine (Savage, 1977, Savage, 1989, Ringø & Birkbeck, 1999). It has been demonstrated that the intestinal microbiota contribute with a number of host-related functions such as protection, development of the mucosal immune system, digestion and angiogenesis (Rawls *et al.*, 2004, Ringø *et al.*, 2007)(and reviewed by (Nayak, 2010, Sekirov *et al.*, 2010).

In mammals, intestinal dysbiosis (microbiota community imbalance) has been associated with a number of metabolic diseases revealing that the intestinal microbiota play key roles in several pathologies such as obesity, Crohn's disease and inflammatory bowel disease (IBD) (Bäckhed *et al.*, 2004, Sokol *et al.*, 2008, Nishikawa *et al.*, 2009, Ridaura *et al.*, 2013).

Characterization of the microbiota has in the past been performed by several different methodologies. In general, these methods can be divided in two major groups: culture dependent and culture independent. The first group has been classically applied for the study of microorganisms. It is relatively inexpensive but presents obvious limitations since only a low number of the intestinal bacterial species of fish grow under the laboratory conditions used in previous studies. In salmon, the reported cultivable bacteria have been estimated to not exceed

1% of the total population (Navarrete *et al.*, 2009). In the last 10 years, however, culture-independent methods have become the preferred choice due to their potential for identification and quantification of far more bacteria. Nowadays, there are several different culture-independent methods available (Figure 4) and their suitability depends on the aim of the investigation. The most commonly used culture-independent methods use the bacterial 16S rRNA gene for the characterization and quantification of the microbial communities in the intestine. Fingerprint analysis such as denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TTGE) were commonly chosen for microbial diversity analyses (Muyzer *et al.*, 1993, Merrifield *et al.*, 2009, Navarrete *et al.*, 2012, Grammes *et al.*, 2013, Reveco *et al.*, 2014).

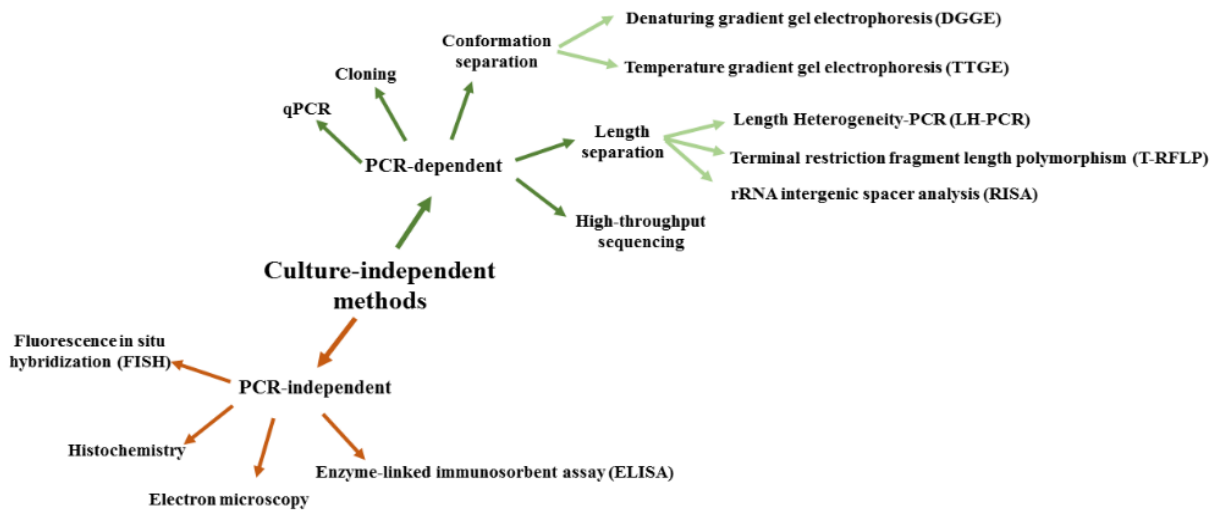


Figure 4. Culture-independent methods applied for study of bacterial communities in the intestine of fish (Based on Merrifield & Ringo (2014)).

The implementation of high-throughput sequencing (HTS) now permits characterization of millions of microorganisms simultaneously. The implementation of this technique has led to a more complete knowledge about the bacterial communities residing in the intestine of several animal species (Qin *et al.*, 2010, Hong *et al.*, 2011, Mao *et al.*, 2012, Ridaura *et al.*, 2013, Bolnick *et al.*, 2014, Daniel *et al.*, 2014, Looft *et al.*, 2014).

In fish, the knowledge about the intestinal microbiota's role for the host lags behind that regarding humans and other mammals. The wide spectrum of characterization techniques used, the different sections of the intestine studied and the pooling of digesta and mucosal samples together among other methodological differences have made comparisons across fish studies and even among studies in the same fish species a challenge.

The studies conducted so far, including culture dependent as well as independent methods, have identified several phyla present in the intestine of fish. Among them Proteobacteria and Firmicutes, Fusobacteria, Bacteroidetes and Actinobacteria have been the most commonly reported (reviewed by (Ringø *et al.*, 1995, Nayak, 2010, Llewellyn *et al.*, 2014). The same regards the salmonid intestinal microbiota (Hovda *et al.*, 2007, Navarrete *et al.*, 2009, Mansfield *et al.*, 2010, Desai *et al.*, 2012, Navarrete *et al.*, 2013, Hartviksen *et al.*, 2014, Reveco *et al.*, 2014, Zarkasi *et al.*, 2014, Llewellyn *et al.*, 2015, Lowrey *et al.*, 2015, Schmidt *et al.*, 2016, Zarkasi *et al.*, 2016). A brief summary of the published studies characterizing the intestinal microbiota of Atlantic salmon and the techniques applied for the characterization is given in Table 1.

Among the bacterial communities reported in salmon, the presence of different bacterial groups such as lactic acid bacteria (LAB) and also some genera such as *Bacillus*, *Pseudomonas* and the family Enterobacteriaceae have been commonly reported across studies using DGGE and quantitative polymerase chain reaction (qPCR) analyses (Hovda *et al.*, 2007, Navarrete *et al.*, 2009, Hartviksen *et al.*, 2014, Reveco *et al.*, 2014). In the last two years, a few studies characterizing the digesta associated microbiota of salmon using HTS of the 16S rRNA gene have confirmed and extended some of the previous knowledge about digesta associated microbiota of salmon (Zarkasi *et al.*, 2014, Llewellyn *et al.*, 2015, Schmidt *et al.*, 2016, Zarkasi *et al.*, 2016). The same phyla described in studies using older methods and named above, also appear as the most abundant in the latest studies, but the novelty in these studies reside in the high resolution of the characterization. These last studies have added a body of detailed information about bacterial communities in digesta and their modulation by external factors such as water temperature, location and diet, which has contributed to the understanding of the dynamics of the intestinal microbial communities at a level exceeding by far the level reached in earlier studies of the intestinal microbiota of salmon.

Table 1. Summary of studies characterizing the Atlantic salmon intestinal microbiota

Article title	Compartments studied/ Water type	Methods used	Most important findings
<i>Carnobacterium inihbens</i> sp. nov., isolated from the intestine of Atlantic salmon (<i>Salmo salar</i>) (1999) (Jöborn <i>et al.</i> , 1999)	Intestine	Culture + 16S rRNA gene amplification and sequencing	Strain K1 ^T (closely related to <i>Carnobacterium alterfunditum</i>) has the capacity to inhibit the growth of <i>Vibrio anguillarum</i> and <i>Aeromonas salmonicida</i>
Lactic acid bacteria associated with the digestive tract of Atlantic salmon (2000) (Ringø <i>et al.</i> , 2000)	Fore-, mid and distal intestinal mucosa/ Seawater	Culture + PCR amplification and sequencing	29.9% LAB of which 28.8% were classified as <i>Carnobacterium</i> spp
Phylogenetic analysis of intestinal microflora indicates a novel <i>Mycoplasma</i> phylotype in farmed and wild salmon (2002) (Holben <i>et al.</i> , 2002)	Digesta distal intestine (wild and farmed salmon)/ Seawater	16S rRNA gene PCR amplification, cloning and sequencing	<i>Mycoplasma</i> phylotype
The effect of dietary krill supplementation on epithelium-associated bacteria in the hindgut of Atlantic salmon (<i>Salmo salar</i> L.): a microbial and electron microscopical study (2006) (Ringø <i>et al.</i> , 2006)	Mucosa distal intestine/ Seawater	Culture + 16S rRNA gene PCR amplification and sequencing + Electron microscopy	Krill meal influences bacterial composition
Effects of dietary soyabean meal, inulin and oxytetracycline on intestinal microbiota and epithelial cell stress, apoptosis and proliferation in the teleost Atlantic salmon (<i>Salmo salar</i> L.) (2007) (Bakke-McKellep <i>et al.</i> , 2007)	Digesta and mucosa mid and distal intestine/ Seawater	Culture + 16S rRNA gene PCR amplification and sequencing	Soybean meal modulates bacterial communities in the intestine
Molecular characterization of the intestinal microbiota of farmed Atlantic salmon (<i>Salmo salar</i> L.) (2007) (Hovda <i>et al.</i> , 2007)	Mucosa fore, mid and hind intestine and digesta hind intestine/ Seawater	Culture + 16S rRNA gene PCR amplification, DGGE and sequencing	<i>Lactobacillus</i> spp., <i>Lactococcus</i> sp., <i>Bacillus</i> sp., <i>Photobacterium phosphoreum</i> , <i>Acinetobacter</i> sp., <i>Pseudomonas</i> sp. and <i>Vibrio</i> sp dominated the intestinal microbiota
Sequencing of variable regions of the 16S rRNA gene for identification of lactic acid bacteria isolated from the intestinal microbiota of healthy salmonids (2007) (Balcázar <i>et al.</i> , 2007)	Intestine	Culture + 16S rRNA gene PCR amplification and sequencing	<i>Carnobacterium maltaromaticum</i> , <i>Lactobacillus curvatus</i> , <i>Lactobacillus sakei</i> , <i>Lactobacillus plantarum</i> , <i>Lactococcus lactis</i> subsp. <i>cremoris</i> , <i>Lactococcus lactis</i> subsp. <i>lactis</i> , and <i>Leuconostoc mesenteroides</i>
Effect of intraperitoneal injection of immunostimulatory substances on allochthonous gut microbiota of Atlantic salmon (<i>Salmo salar</i> L.) determined using denaturing gradient gel electrophoresis (2008)(Liu <i>et al.</i> , 2008)	Digesta proximal and distal intestine/ Fresh water	16S rRNA gene PCR amplification, DGGE and sequencing	Intraperitoneal injection of lipopolysaccharide from <i>A. salmonicida</i> affects to some extent the digesta intestinal microbiota of Atlantic salmon

Oxytetracycline treatment reduces bacterial diversity of intestinal microbiota of Atlantic salmon (2008) (Navarrete <i>et al.</i> , 2008)	Entire intestine/ Fresh water	Culture + 16S rRNA gene PCR amplification, RFLP and sequencing	Microbiota of oxytetracycline treated fish presented lower diversity compared to non-treated fish
Use of 16S rRNA gene sequencing analysis to characterize culturable intestinal bacteria in Atlantic salmon (<i>Salmo salar</i>) fed diets with cellulose or non-starch polysaccharides from soy (2008) (Ringø <i>et al.</i> , 2008)	Digesta and mucosa mid and distal intestine/ Seawater	Culture + 16S rRNA gene PCR amplification and sequencing + Electron microscopy	No significant effects of diet composition were observed on total population of culturable bacteria. The composition of the intestinal microbiota was sensitive to dietary changes
Phylogenetic analysis of intestinal bacteria of freshwater salmon <i>Salmo salar</i> and sea trout <i>Salmo trutta trutta</i> and diet (2008) (Skrodenytė-Arbačiauskienė <i>et al.</i> , 2008)	Digesta distal intestine(wild salmon)/ Fresh water	Culture + 16S rRNA gene PCR amplification and sequencing	Enterobacteriaceae family (23%), <i>Plesiomonas</i> (19.2%) and <i>Carnobacterium</i> (15.3%) dominated the intestinal microbiota
The culturable intestinal microbiota of triploid and diploid juvenile Atlantic salmon (<i>Salmo salar</i>) a comparison of composition and drug resistance (2011) (Cantas <i>et al.</i> , 2011)	Digesta proximal, mid and distal intestine	Culture + 16S rRNA gene PCR amplification and sequencing	No ploidy effect observed in the bacterial species isolated. Triploids had a significant increase in total intestinal microbiota levels
Seasonal variations in the intestinal microbiota of farmed Atlantic salmon (<i>Salmo salar</i> L.) (2012) (Hovda <i>et al.</i> , 2012)	Digesta and mucosa mid and hind intestine/ Seawater	16S rRNA gene PCR amplification, DGGE and sequencing	No seasonal variations in the intestinal microbiota. Lactic acid bacteria (LAB) was present all year
Culturable autochthonous gut bacteria in Atlantic salmon (<i>Salmo salar</i> L.) fed diets with or without chitin. Characterization by 16S rRNA gene sequencing, ability to produce enzymes and in vitro growth inhibition of four fish pathogen (2012) (Askarian <i>et al.</i> , 2012)	Mucosa proximal and distal intestine/ Seawater	Culture + 16S rRNA gene PCR amplification and sequencing	<i>Pseudomonas</i> spp. in PI and DI and <i>Psychrobacter pulmonis</i> <i>Aeromonas</i> spp in PI
<i>Candida utilis</i> and <i>Chlorella vulgaris</i> counteract intestinal inflammation in Atlantic salmon (<i>Salmo salar</i> L.) (2013) (Grammes <i>et al.</i> , 2013)	Digesta distal intestine/ Seawater	16S rRNA gene PCR amplification, DGGE and sequencing	<i>Candida utilis</i> and <i>Chlorella vulgaris</i> were effective to counteract SBMIE
Short-term effects of dietary soybean meal and lactic acid bacteria on the intestinal morphology and microbiota of Atlantic salmon (<i>Salmo salar</i>) (2013) (Navarrete <i>et al.</i> , 2013)	Digesta distal intestine/ Fresh water	Culture + 16S rRNA gene PCR amplification, RFLP and sequencing	Specific bacterial groups were not correlated with the development of enteritis, but there was a correlation between diet and bacterial communities present in intestine
Dietary synbiotic application modulates Atlantic salmon (<i>Salmo salar</i>) intestinal microbial communities and intestinal immunity (2013) (Abid <i>et al.</i> , 2013)	Digesta and mucosa anterior and posterior intestine	qPCR + 16S rRNA PCR amplification, DGGE and sequencing	Lower total bacteria levels in anterior and posterior mucosa and posterior digesta in synbiotic treated fish compared to control fish
Dietary soybean protein concentrate-induced intestinal disorder in marine farmed Atlantic salmon, <i>Salmo salar</i> is associated with alterations in gut microbiota (2013) (Green <i>et al.</i> , 2013)	Faecal material and mucosa proximal and distal intestine/ Seawater	PCR amplification, T-RFLP + 16S rRNA PCR amplification and sequencing	Mucosa intestinal microbiota is complex at the population level, but simple and highly variable at individual level. Feeding soy protein concentrate to salmon increased the bacterial diversity in the intestine
Alternative dietary protein sources for Atlantic salmon (<i>Salmo salar</i> L.) effect on intestinal microbiota,	Faecal material and mucosa proximal and distal intestine/	qPCR	Diet altered digesta and mucosa-associated intestinal microbiota of DI. Mucosa-associated

intestinal and liver histology and growth (2014) (Hartviksen <i>et al.</i> , 2014)	Seawater		microbiota of PI remained unchanged by the change in diet
The effect of diet and environmental temperature on the faecal microbiota of farmed Tasmanian Atlantic Salmon (<i>Salmo salar</i> L.) (2014) (Neuman <i>et al.</i> , 2014)	Faecal material/ Seawater	Culture + 16S rRNA PCR amplification and sequencing	The number of different bacterial groups between the two diets varied and increased as water temperature increased. In summer, <i>Vibrio</i> sp increased and LAB decreased
Intestinal bacterial community structure differs between healthy and inflamed intestines in Atlantic salmon (<i>Salmo salar</i> L.) (2014) (Reveco <i>et al.</i> , 2014)	Digesta mid and distal intestine/ Seawater	16S rRNA PCR amplification, DGGE and sequencing	Higher abundance of Firmicutes in fish fed SBM (compared to fishmeal fed fish). Fish fed SBM presented reduced richness and evenness compared to fishmeal fed fish
Pyrosequencing-based characterization of gastrointestinal bacteria of Atlantic salmon (<i>Salmo salar</i> L.) within a commercial mariculture system (2014) (Zarkasi <i>et al.</i> , 2014)	Faecal material/ Seawater	16S rRNA gene PCR amplification + HTS	Dynamic fecal bacterial communities were less influenced by diet than by season and fish growth phases
High variability of levels of <i>Allivibrio</i> and lactic acid bacteria in the intestinal microbiota of farmed Atlantic salmon (2014) (Godoy <i>et al.</i> , 2015)	Mucosa (and digesta by rinsing) proximal (mid) and distal intestine/ Seawater	Culture + 16S rRNA PCR amplification and sequencing + cloning and sequencing	<i>Allivibrio</i> sp dominated in one fish and LAB represented over 50% of identified clones in other two fish
The biogeography of the Atlantic salmon (<i>Salmo salar</i>) gut microbiome (2015) (Llewellyn <i>et al.</i> , 2015)	Digesta mid and distal intestine/ Fresh water and seawater	16S rRNA gene PCR amplification + HTS	Differences between environmental and intestinal bacterial communities. Intestine microbial composition was not significantly impacted by geography. The lifecycle stage strongly defined both the diversity and identity of microbiota in intestinal microbiota
Probiotic and pathogen ex-vivo exposure of Atlantic salmon (<i>Salmo Salar</i> L.) Intestine from fish fed four different protein sources (2015) (Hartviksen <i>et al.</i> , 2015)	Entire intestine and mucosa distal intestine/ Seawater	qPCR	No dietary effect on bacterial adherence. Both <i>Carnobacterium divergens</i> and <i>Aeromonas salmonicida</i> adhered to mucosa
Atlantic Salmon (<i>Salmo salar</i> L.) Gastrointestinal microbial community dynamics in relation to digesta properties and diet (2016) (Zarkasi <i>et al.</i> , 2016)	Faecal material/ Seawater	16S rRNA gene PCR amplification + HTS	Bacterial communities in faecal material were highly dynamic over time in fish fed low protein diet. Low energy diet associated with a most divergent community structure
The influence of fishmeal-free diets on microbial communities in Atlantic salmon <i>Salmo salar</i> recirculation aquaculture systems (Schmidt <i>et al.</i> , 2016)	Mid intestine mucosa and digesta combined	16S rRNA gene PCR amplification + HTS	Diet influenced the microbiome structure of the intestine. More abundance of Lactobacillales in fish fed fishmeal free diets

Feeding alternative sources to Atlantic salmon

Alternative protein sources

As a result of the shortage of wild catch, at present, less than 30% of the ingredients in Norwegian salmon feed come from marine resources (Ytrestøyl *et al.*, 2015). Protein-rich plant ingredients substitute the fishmeal used previously in fish diets, in particular for carnivorous fish. Examples are processed products from soybeans, peas, lupins, sunflower, linseed and rape seeds for which nutritional value in Atlantic salmon has been thoroughly investigated (Rumsey *et al.*, 1993, Olli *et al.*, 1994, Gomes *et al.*, 1995, Aslaksen *et al.*, 2007, Øverland *et al.*, 2009) and are included in salmonid diets. However, utilization of some of these plant-based products has been limited by the presence of ANFs also called antinutrients (Table 2). The ANFs are natural constituents of plants, including the majority of ingredients mentioned above (reviewed by (Krogdahl *et al.*, 2010)). As the term indicates, they negatively affect animal nutrition, causing inferior nutrient utilization, altered nutrient balances of diets, inhibition of growth, intestinal dysfunction and altered immune functions. Removal or inactivation, complete or partial, of ANFs can be performed by processes such as heat or enzyme treatments, alcohol extraction, and fermentation. However, the degree of difficulty and success of the removal or inactivation, and thus the cost of the processing, depend on the ANF.

Also certain animal products may serve as nutritious alternative ingredients for use in in aquafeeds, e.g. rendered animal protein ingredients including blood meal, feather meal and various slaughter byproduct meals. These were, however, forbidden until recently in Europe. The re-authorization of non-ruminant processed animal proteins in aquafeeds (Regulation 56/2013), namely from poultry and swine, open an economical animal alternative for the replacement of FM in fish feed. They generally have a more complete amino acid profile compared to plant ingredients and low level of ANFs (Naylor *et al.*, 2009). Although the quality varies depending on the origin, processing and storage, animal byproducts have improved in quality in the later years and new further processed products are underway. At present, the main limiting factor for

these products is the consumers' reluctance to their inclusion in feed for fish destined for human consumption (Bureau *et al.*, 1999, Naylor *et al.*, 2009).

Table 2. Antinutritional factors found in alternative feed sources for Atlantic salmon. Detrimental effects and processing necessary for removal or reduction (From (Krogdahl *et al.*, 2010) with modifications

Antinutritional factor	Sources	Effect	Processing
Proteinase inhibitors	Legumes	Inhibition of trypsin, chymotrypsin, elastases and carboxypeptidases	Heat
Amylase inhibitors	Peas	Inhibition of amylase	Heat
Lipase inhibitors	Beans	Inhibition of lipase	Heat
Lectins	Plant seeds	Carbohydrate binding. Agglutination of cells such as blood cells and enterocytes	Heat
Phytic acid	Plants	Reduction of trypsin stability	Enzyme
Fiber	Plants	Reduce nutrient absorption	Dehulling
Tannins	Rape seeds	Binding digestive enzymes Astringent or bitter flavor	Dehulling
Saponins	Legumes	Form micelles that intercalate into cholesterol-containing membranes forming holes	Alcohol extraction
Sterols	Legumes	Reproductive negative effects	Alcohol/non-polar extraction
Oestrogens	Beans	Induce estrogenic activity	Alcohol/non-polar extraction
Gossypol	Cotton seeds	Toxic. Decrease feed consumption, growth, hematocrit, hemoglobin and reproductive capacity	Non-polar extraction
Oligosaccharides	Legumes	Osmotic active compounds (may cause diarrhea)	Alcohol/aqueous extraction
Quinolozidine alkaloids	Lupins	Decrease feed intake due to bitter taste	Aqueous extraction
Goitrogens	Rape seed	Thyroid hormone production disruptor	Enzyme

Soybean meal-induced enteritis; a model for the study of intestinal enteropathies

Soybean meal (SBM) is widely used as a protein source for land-based production animals. Several characteristics make SBM an attractive potential replacement of FM in the diets of salmon; it has an amino acid profile quite suitable for the salmon, is widely available and economically favorable. However, the inclusion of SBM even at levels below 10% has resulted in pathological changes in the DI, particularly in salmon (Krogdahl *et al.*, 2003, Uran *et al.*, 2009), but also in other fish species (Romarheim *et al.*, 2008, Urán *et al.*, 2008, Merrifield *et al.*,

2009). This condition is commonly referred to as soybean meal induced enteritis (SBMIE) (Baeverfjord & Krogdahl, 1996). SBMIE is highly reproducible in salmon, making this pathological condition an excellent model for the study of intestinal inflammation and associated dysfunction and immunological response.

The condition has been widely studied and found to imply disturbance of digestive functionality (Bakke-McKellep *et al.*, 2000, Venold *et al.*, 2012, Venold *et al.*, 2013), morphology (Van den Ingh *et al.*, 1991, Baeverfjord & Krogdahl, 1996, Van den Ingh *et al.*, 1996), the immune apparatus (Bakke-McKellep *et al.*, 2007, Marjara *et al.*, 2012, Sahlmann *et al.*, 2013) and microbial populations (Bakke-McKellep *et al.*, 2007, Reveco *et al.*, 2014) of the DI of salmon. Previous studies points to saponins as a key ANF in the development of the SBMIE, but whether also other ANFs are necessary for development of the symptoms has been unclear (Bureau *et al.*, 1998, Knudsen *et al.*, 2007, Knudsen *et al.*, 2008, Chikwati *et al.*, 2012, Kortner *et al.*, 2012, Grammes *et al.*, 2013). Saponins occur naturally in legumes such as soy, pea, sunflower, lupin and guar. They are heat-stabile triterpenoid or steroid amphipathic glycosides. Some studies indicate beneficial characteristics of saponins when included in the diet at low levels, e.g. on growth performance (reviewed by (Francis *et al.*, 2002) and they have a potential as adjuvants in vaccines (as reviewed by (Kensil, 1996). They have showed anticancer and antifungal activity (reviewed by (Sparg *et al.*, 2004), and have a clear hypocholesterolemic effect in several animal species (Olli *et al.*, 1994, Kaushik *et al.*, 1995, Krogdahl *et al.*, 2003, Sørensen *et al.*, 2011). On the other hand, saponins may alter the permeability of cell membranes, forming holes due to the ability to bind cholesterol and they interact with micelle formation.

The work of Knudsen *et al.* (Knudsen *et al.*, 2008), demonstrated that when salmon were fed a FM diet supplemented with a soya saponin extract (65% purified) the fish presented SBMIE-like symptoms. Based on these results, the authors suggested that soya saponins were the most plausible causative agent of the negative effect of SBM in salmon. However, this study did not exclude effects of other ANFs that might have been present in the saponin preparation. The study of Chikwati *et al.* (Chikwati *et al.*, 2012) using a >95% pure preparation supplemented to diets with various plant ingredients reported similar effects when combined with pea protein concentrate. This study therefore supported the conclusions of Knudsen *et al.* (Knudsen *et al.*,

2008) by clearly showing that saponins are key elements in development of enteritis. However, the question whether saponins alone would cause SBMIE was still unanswered, which was the basis for the work presented in **Paper I**.

Characteristics of SBMIE; histomorphology and immune response

The work of Baeverfjord & Krogdahl (1996) (Baeverfjord & Krogdahl, 1996) characterized the morphological changes in the intestine of Atlantic salmon fed SBM and described the pathology as a non-infectious subacute enteritis. Inflammation is the body response to an injurious agent, which include elimination of the harmful stimuli and reparation of the damaged tissue. The five cardinal signs of inflammation are redness, swelling, heat, pain and loss of function. In the case of the DI of fish undergoing SBMIE the presentation of the signs for inflammation also occur. The DI of salmon presenting signs of SBMIE (Figure 5) shows infiltration of the lamina propria by inflammatory cells, goblet cells in addition to edema (swelling), increased blood flow to the intestine (redness) and shortening of the mucosal folds with a decreased number of absorptive vacuoles in the enterocytes (loss of function). Heat and pain are difficult to confirm and are not been described in salmon undergoing SBMIE.

Yet the exact mechanism behind the development of SBMIE is still not well understood (Knudsen *et al.*, 2008). Increased expression profile of inflammatory genes has been reported during the first five days after start feeding salmon on a diet with 20% SBM (Marjara *et al.*, 2012, Sahlmann *et al.*, 2013). Moreover, elevated trypsin gene expression and enzymatic activity (Krogdahl *et al.*, 2003, Lilleeng *et al.*, 2007) in the DI of salmon, and elevated gene expression of proteinase-activated receptors (PARs) (Thorsen *et al.*, 2008) have been observed in DI during the development of SBMIE. In mammals, Protease-activated receptors (PARs) are present in several tissues, including the intestine, and are described as initiators of inflammatory processes (Cenac *et al.*, 2002). Thus, results in salmon, suggest the involvement of both trypsin and PARs in the early development of the pathology.

Recent studies in Atlantic salmon have described the immune response at the transcriptional level. Induction of genes for acute pro-inflammatory cytokines and chemokines, NFkB and

TNF α -, in addition to regulators of B- and T-cell function, tissue proliferation and remodeling have been observed (Skugor *et al.*, 2011, Kortner *et al.*, 2012, Marjara *et al.*, 2012, Grammes *et al.*, 2013, Sahlmann *et al.*, 2013, De Santis *et al.*, 2015, Król *et al.*, 2016). Concomitant to the latter, studies have also shown the presence of a number of different immune cells in the lamina propria and submucosa of the DI. Macrophages, granulocytes and especially a large number of T-cells have been reported in several studies in the DI of fish undergoing SBMIE (Bakke-McKellep *et al.*, 2007, Lilleeng *et al.*, 2009, Marjara *et al.*, 2012, Sahlmann *et al.*, 2013). The cytokine responses and the presence of CD3, CD4 and CD8 effector T-cells may suggest that this pathology is the result of a T-cell mediated hypersensitivity reaction similar to the one observed in human celiac disease or inflammatory bowel disease (Bakke-McKellep *et al.*, 2007).

In the work of Marjara *et al.* (Marjara *et al.*, 2012) evaluating the gene regulation of inflammatory markers during SBMIE in salmon in comparison with fish fed FM, it was observed that clusters of differentiation CD4 α and CD8 β seemed to increase their expression before any sign of inflammation was demonstrated by histology. Their expression continued to increase for 3 weeks. The same study observed marked upregulation of interleukin 17A (IL-17A), which indicates involvement of Th17 cells, supporting the hypothesis that SBMIE is a T-cell mediated DI pathology.

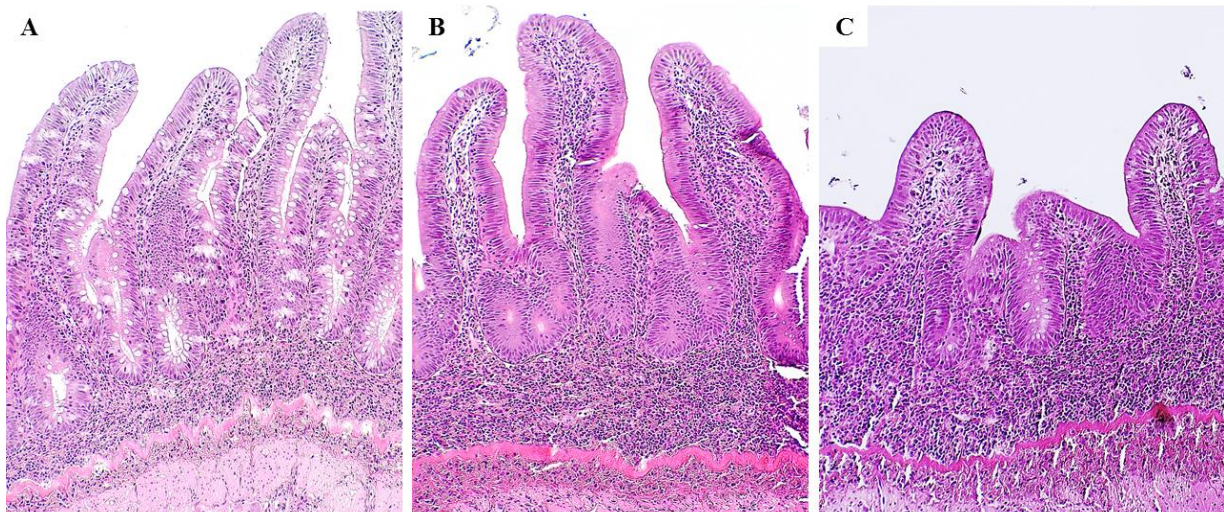


Figure 5. Representative light microscopy images of SBMIE in the DI of Atlantic salmon with different scores of severity. Picture A shows moderate changes. Picture B shows marked to severe changes and picture C shows severe changes in the morphology of the DI. Hematoxylin and eosin staining. Images taken by E. Chikwati. Readers are referred to Figure 3 for images of the healthy DI.

Characteristics of SBMIE; cell turnover

One of the characteristic features of the DI of fish presenting signs of SBMIE is an increased presence of immature cells along the mucosal fold, concomitant with an increment in apoptotic cells in the apices of the mucosal folds as a result of the chronic inflammatory process (Bakke-McKellep *et al.*, 2007). The immature cells migrate from the basal proliferative zones to the apices of the folds, apparently replacing the dead cells in the inflamed tissue causing hyperplasia of immature cells along the mucosal fold (Sanden *et al.*, 2005, Bakke-McKellep *et al.*, 2007, Romarheim *et al.*, 2011, Chikwati *et al.*, 2013, Venold *et al.*, 2013). By the use of immunohistochemistry (Bakke-McKellep *et al.*, 2007) and gene expression profiling of the cell proliferation marker proliferative cell nuclear antigen (*pcna*) (Kortner *et al.*, 2013), a semi-quantitative evaluation of the cell turnover can be performed (Figure 6A-B).

Moreover, the expression profile of metabolic and water channel genes such as fatty acid binding protein (*fabp2*) (Grammes *et al.*, 2013, Venold *et al.*, 2013) and aquaporin 8ab (*aqp8ab*) (Kortner *et al.*, 2012, Grammes *et al.*, 2013) have also been found to be decreased in the DI of fish with SBMIE. From studies in mammals, it has been suggested that a decrease in expression of *Aqp8* could be a marker of loss of functionality and inflammation (Hardin *et al.*, 2004) as the result of the increased cell turnover occurring in the inflamed intestine, since, as demonstrated in human studies, these proteins are only present in mature functional cells (Fisher *et al.*, 2001).

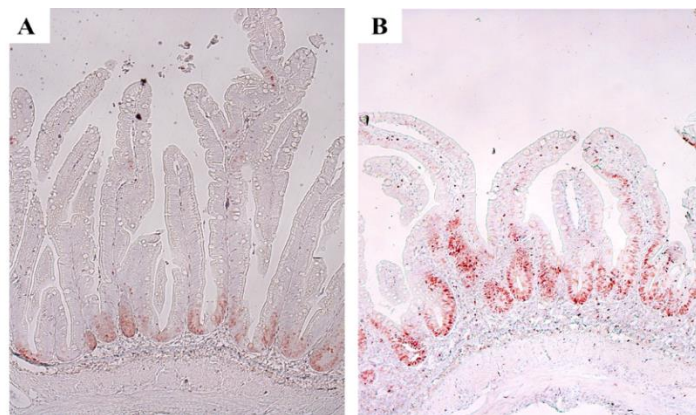


Figure 6. Representative images of the localization and distribution of immunohistochemically stained PcnA protein in the DI of A. salmon. PcnA distribution and localization in (A) normal DI and (B) inflamed DI with SBMIE. Images taken by M. Gu.

SBM apparently causes increased permeability of the distal intestinal epithelium in salmon (Nordrum *et al.*, 2000), which likely disrupts water and ion balance and often causes diarrhea (Baeverfjord & Krogdahl, 1996, Refstie *et al.*, 2000, Refstie *et al.*, 2001, Krogdahl *et al.*, 2003). Alteration in expression of genes related to regulation of water transport across the intestinal mucosa (such as *aqp8*) and tight junction proteins could assist in explaining the diarrhea observed in fish with SBMIE. An investigation of these underlying mechanisms formed the basis for the work presented in **Paper III**.

Characteristics of SBMIE; intestinal microbiota

From earlier studies, employing classical techniques, it is quite clear that inclusion of SBM in salmon diets modulates the intestinal microbiota of the fish. Whether this alteration plays an important role in development of or is a result of SBMIE is not known. The study of Heikkinen *et al.* (Heikkinen *et al.*, 2006) on rainbow trout (*Oncorhynchus mykiss*) was the first describing the effect of SBM on the intestinal microbiota in salmonids using both culture dependent and independent methods (PCR amplification). Heikkinen *et al.* (Heikkinen *et al.*, 2006) characterized the microbiota of the DI when 45% SBM was included in the diet in comparison with a FM diet fed the diets for 8 or 18 weeks. The authors reported that both diets decreased the total cultivable bacteria in the digesta during the first 4 weeks. During the next 4 weeks, the level of cultivable bacteria increased in FM fed fish but remained low in the SBM fed fish. Among the detectable bacteria, the study reported that *Aeromonas*, *Shingomonas*, *Chryseomonas*, *Lactococcus* and *Lactobacillus* appeared to be most abundant for both diets with some differences in the bacterial abundances between diets. On the other hand, Merrifield *et al.* (Merrifield *et al.*, 2009) did not find any difference in number of viable bacterial populations in either digesta or mucosa samples from rainbow trout fed either FM or SBM (50% of protein replacement) diets for 16 weeks. The authors found that FM-fed fish presented higher number of isolates classified as *Aeromonas* than SBM-fed fish, whereas the latter group presented higher abundance of *Psychrobacter* and yeast. In agreement with the latter study, Ringø *et al.* (Ringø *et al.*, 2008) reported no differences in the number of viable bacteria in mucosa or digesta between salmon fed FM and SBM (46% inclusion). In contrast with Ringø *et al.* (Ringø *et al.*, 2008), the study of

Bakke-McKellep *et al.* (Bakke-McKellep *et al.*, 2007) reported higher number of culture-viable bacteria in the mucosa of MI and DI and in digesta DI of fish fed SBM diet. In the last years, culture-independent techniques employed in several studies have reported that the inclusion of SBM modulate the intestinal microbial composition (Mansfield *et al.*, 2010, Desai *et al.*, 2012, Reveco *et al.*, 2014). The studies of Mansfield *et al.* (Mansfield *et al.*, 2010) and Desai *et al.* (Desai *et al.*, 2012) with rainbow trout, as well as the studies of Reveco *et al.* (Reveco *et al.*, 2014) with Atlantic salmon, have shown that fish fed SBM presented higher relative abundance of Firmicutes in comparison with fish fed FM diets. Moreover, higher abundance of LAB in fish fed SBM than fish fed FM was also observed.

Lactic acid bacteria are supposed to exert a positive effect in the intestine of animals, including fish (reviewed by (Ringø & Gatesoupe, 1998). Therefore, the reported high abundance of LAB in the DI of fish presenting signs of SBMIE may appear contradictory to the health promoting effect LAB have been associated with. Thus, the increased abundance of LAB in fish undergoing SBMIE is not well understood. Reveco *et al.* (Reveco *et al.*, 2014) have suggested that the increment in the relative abundance of a LAB member, namely *Lactococcus lactis* in fish undergoing SBMIE, could be the result of bacterial antagonisms through antimicrobial peptides present in this species of bacteria. Anyhow, intestinal microbiota profiling has now become a valuable endpoint measurement in order to assess and understand fish intestinal health status and effects of diet. A detailed investigation of the Atlantic salmon intestinal microbiota and effects of diet formed the basis for the work presented in **Paper II** and **Paper IV**.

Hypotheses

The following hypotheses were formulated based on the information presented in the introductory chapter. These hypotheses were the basis for the aims of the current thesis work:

- Soya saponins alone are capable of inducing SBMIE in the Atlantic salmon's DI
- The Atlantic salmon's intestinal microbiota present different bacterial communities along the intestinal sections
- Plant- and animal-based alternative proteins have a modulatory effect in the Atlantic salmon's intestinal microbiota that can affect the normal functionality of the intestine

AIMS OF THE STUDY

The overall goal of the present work was to secure that intestinal functions and therefore the health of Atlantic salmon are not compromised by increasing level of alternative protein ingredients in the diet.

In order to achieve the main goal of this project three aims were formulated:

1. To find the important the mechanisms behind effects of soya saponins on the intestinal function and their role in SBMIE (work presented in **Paper I**).
2. To increase the knowledge of the intestinal microbiota by characterization of the digesta- and mucosa-associated microbiota along the intestine of Atlantic salmon fed a commercial diet (work presented in **Paper II**).
3. To characterize the effect on intestinal microbiota caused by the inclusion of alternative protein sources in the diet of Atlantic salmon and the microbiota's role for the function and health of the intestine (work presented in **Paper III and IV**).

MATERIALS AND METHODS

Materials

Fish

All experiments were conducted in compliance with law and regulations for the experimentation with live animals in Norway and Denmark as overseen by the Norwegian Animal Research Authority and the Danish Animal Experiments Inspectorate respectively. The experiments were performed using samples from mix gender Atlantic salmon post smolt allocated in seawater – containing tanks.

Diets

Table 3-5 show the diets formulations used in the work presented in **Papers I to IV**.

Table 3. Diet formulation for the 10-week feeding trial presented in **Paper I***

	FM-0	FM-2	FM-4	FM-6	FM-10	LP-0	LP-2	LP-4	LP-6	LP-10
Ingredients (g/kg)										
Fishmeal**	587	587	587	587	587	235	235	235	235	235
Lupin meal†						200	200	200	200	200
Wheat gluten‡						190	190	190	190	190
Wheat meal	156	156	156	156	156	67	67	67	67	67
Soya saponinsΔ	0	2	4	6	10	0	2	4	6	10
Wheat starch	10	8	6	4	0	10	8	6	4	0
Fish oil¶	221	221	221	221	221	242	242	242	242	242
Vitamin mix□	20	20	20	20	20	20	20	20	20	20
Mineral mix□	5	5	5	5	5	5	5	5	5	5
Ca(H ₂ PO ₄) 2H ₂ O						10	10	10	10	10
Carophyll pink	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Yttrium oxide	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Lysine HCl						17	17	17	17	17
DL-Methionine						3.6	3.6	3.6	3.6	3.6
Chemical composition (%)										
Crude protein	42.3	42.3	42.3	42.3	42.3	44.1	44.1	44.1	44.1	44.1
Crude lipid	29.3	29.3	29.3	29.3	29.3	30.3	30.3	30.3	30.3	30.3
Starch	10.3	10.3	10.3	10.3	10.3	7.5	7.5	7.5	7.5	7.5
Gross energy (MJ/kg)	23.9	23.9	23.9	23.9	23.9	24.2	24.2	24.2	24.2	24.2

*FM, Fishmeal diet; LP, lupin meal diet; -0, -2, -4, -6, -10, levels of soya saponins inclusion in diets (g/kg).

**Fishmeal 122/11, LTQ fish meal, produced by Welcon AS; †Supplied by Holterman AS; ‡Wheat gluten 159/10, Amytex 100, produced by Syral, Belgium; Δ 95% Concentrate, supplied by Organic Technologies, USA; ¶ Fishoil O5/10, NorSalmOil, produced by Norsildmel.

□ Supplemented to meet requirements

Table 4. Diet formulation for the 4-week feeding presented in **Paper II***

Ingredients	Diet (% of total energy)
Fishmeal	45
Soy protein concentrate	15
Wheat gluten	6
Sunflower meal	4
Wheat	13
Fish oil	12
Rape seed oil	5
Chemical composition	
Crude protein	50
Crude lipid	23
Crude fibre	1
Gross energy (MJ/kg)	23

* Commercial diet. Astaxanthin added as pigment to the diet

Table 5. Formulation of the experimental diets for the 48-day feeding trial presented in **Papers**

III and IV*	FM	PM	SBMWG	SPCPM	GMWG
Ingredients (g/kg)					
Fish meal ^β	723.5	143	202.4	362.2	276.6
Wheat gluten [€]			220		145
Poultry meal ^Ω		580		60	
HiPro Soya [¥]			300		
SPC				300	
Guar meal					300
Tapioca ^α	110	110	110	110	110
Rape oil [□]	80	80	80	80	80
Fish oil [⊖]	80	80	80	80	80
Premix N [£]	6	6.5	7.1	7.3	7.9
Yttrium	0.5	0.5	0.5	0.5	0.5
Chemical Composition (%)					
Crude protein	51.4	49.4	47	46.4	49.9
Crude lipid	23.3	25.7	20.5	22.3	22
Starch	10.6	11.4	12	11.8	13.3
Ash	9.3	9.6	5	7.3	6

Abbreviations: FM, Fishmeal diet; PM, Poultry meal diet; SBMWG, Soybean meal wheat gluten diet; SPCPM, Soy protein concentrate poultry meal diet; GMWG, Guar meal wheat gluten diet. ^β Fish meal (Norsild, NOR); [€] Wheat gluten (Lantmännen, SW); ^Ω Poultry meal (Gepro, D); [¥] HiPro Soya Scan Mills (Germany); ^α Tapioca (KMC, DK); [□] Rape oil (Emmelev-Scanola, DK); [⊖] Fish oil (FF Skagen, DK); [£] Premix N (BioMar premix). Minerals, vitamins and synthetic amino acids to cover the nutrient requirements of the species.

Methods

The following chapter presents methods high-throughput sequencing (HTS), used for the characterizing of microbiota, and quantitative polymerase chain reaction (qPCR), used for

intestinal gene expression analysis. As other methods used in the papers are well established, they are not described here and readers are referred to the individual papers for brief descriptions.

Sampling the intestine for microbiota studies

For the intestinal microbiota experiments (work presented in **Paper II** and **IV**), the digesta and mucosa sampling was performed following two different protocols. Differences in the sampling protocol in the work presented in **Paper IV** were the result of sampling streamlining and use of a DNA extraction kit, which demanded a bigger mucosal sample size (details in discussion chapter). Despite differences in the sampling protocols, no differences were expected in the results as a product of this sampling modification since enough material for the sequencing of the intestinal bacterial community was obtained in both samplings.

In both experiments, euthanized fish were cleaned ventrally with 70% ethanol, the abdominal cavity was opened at the mid line and the whole intestine was aseptically removed and divided into segments PI, MI, and DI. Thereafter, for the work presented in **Paper II**, the three segments of the intestine were opened longitudinally and the digesta of each segment was collected separately. The tissues of PI, MI and DI were washed three times in PBS to remove possible remnants of intestinal digesta and samples to evaluate the mucosa-associated microbiota were obtained by scraping two centimeters lengths of mucosal tissue from the mid-section of each region with a sterile scalpel,. For the experiment presented in **Paper IV**, the digesta of the DI was collected by carefully squeezing it out of the intestine and into sterile tubes. The mucosal tissue of the DI was then washed with PBS to ensure removal of any remaining digesta. To evaluate the mucosa-associated microbiota, whole tissue segments of 1 cm were sampled from the middle of the DI.

For both experiments, all samples were frozen immediately in liquid N₂, and thereafter stored at -80°C until further processing.

Extraction of DNA for high-throughput sequencing

For characterization of the digesta and intestinal mucosa-associated microbiota, DNA was extracted from frozen (-80°C) samples. The following compartments of the intestine were characterized:

- Work presented in **Paper II**: digesta from PI, MI and DI and mucosa from MI and DI separately, i.e., five compartments.
- Work presented in **Paper IV**: digesta and mucosa of DI separately, i.e., two compartments.

Phenol/Chloroform-buffer DNA extraction protocol

The DNA extraction protocol used for the work presented in **Paper II** was a combination between the classical phenol/chloroform DNA extraction method and the use of buffers from the QIAamp Stool Mini Kit (Qiagen, Crawley, UK) extraction kit. Firstly, the method included the use of lysozyme to lyse bacterial cells, by hydrolyzing the peptidoglycan in the wall. Gram-positive bacteria are particularly susceptible to lysozyme since their cell wall contain higher amount of peptidoglycan in comparison with Gram-negative bacteria. This step was followed by the use of the QIAamp Stool Mini Kit (Qiagen, Crawley, UK) according to manufacturer's instructions. Modifications in the cleanup steps were added to the protocol in order to recover larger amount of DNA (Waines *et al.*, 2011) and included the use of ice-cold Tris-buffered phenol and chloroform and overnight precipitation of DNA in ice-cold isopropanol.

To assess adequate DNA concentration and purity, the extracted DNA was evaluated using NanoDrop™ 1000 spectrophotometer (Thermo Scientific Ltd, DE, USA).

Glass beads/QIAamp DNA extraction protocol

The DNA extraction for the characterization of the DI microbiota for the work presented in **Paper IV** was performed using the QIAamp Stool Mini Kit (Qiagen, Crawley, UK) according to

manufacturer's specification with some modifications in the lysis process. The QIAamp Stool Mini Kit has been widely applied for the extraction of bacterial DNA from the intestine. This protocol performs well for digesta as well as mucosa samples in mammals (Li *et al.*, 2003). Each sample was mixed with 150 mg of Glass beads (Merck, Darmstadt, Germany) and the ASL buffer (first buffer in the QIAamp Stool Mini Kit protocol) and this solution was homogenized using the FastPrep®-24 instrument (MP Biomedicals, France) at 6.0 m/s two times for 25 sec with a pause of 25 sec between the runs. Other modifications such as increased temperature during heating incubation (from 70 to 90°C) and increased treatment with proteinase K (from 10 to 15 min) were also implemented in order to improve the DNA extraction. NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific Ltd, DE, U.S.A.) was used to quantify the concentration and purity of the obtained DNA.

For the work presented in **Paper II** and **IV**, the final DNA yield varied among samples, mainly between samples from digesta compared to samples from mucosal origin (higher DNA yield from mucosa samples, as a result of significant amounts of host DNA present in the samples). The concentrations were not normalized at this stage of the pipeline but normalization was instead performed as a pre-step before high-throughput sequencing.

Polymerase chain reaction amplification and amplicon purification

In order to characterize the salmon intestinal microbiota, amplification of the hypervariable V1-V2 region of the 16S ribosomal RNA (rRNA) gene (Figure 7A) was performed. The 16S is part of the 30S subunit of the 70S prokaryotic ribosome (Figure 7B). The 16S rRNA gene contains several species-specific regions and is widely applied for the identification of bacterial organisms at genus and species level (Kim *et al.*, 2011).

Polymerase chain reactions (PCR) were carried out using the bacterial universal primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 338R-I (5' GCW GCC TCC CGT AGG AGT 3'), 338R-II (5' GCW GCC ACC CGT AGG TGT 3') pooled together in equal amounts and used at the same concentration as the forward primer. The PCR was performed with or without a touch down approach. Digesta samples from the work presented in **Paper II** were the only ones

amplified without the touch down approach. The obtained amplicons were evaluated in a 1.5% agarose gel.

Thereafter, the PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Crawley, UK), following the manufacturer's instructions.

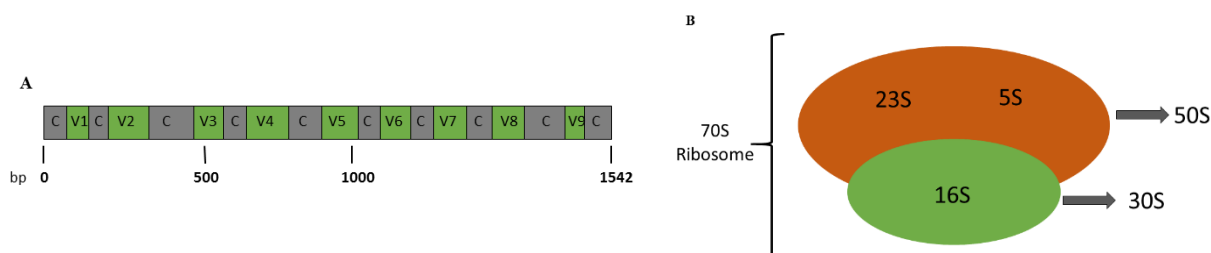


Figure 7. Schematic image of the (A) 16S rRNA gene and the (B) 70S ribosome RNA from prokaryotic organisms. Abbreviations: bp, base pair; C, conserved region (grey); V, hypervariable region (green).

The desirable values of purity obtained from Nanodrop of the purified PCR amplicons for further use in high-throughput sequencing should be as following:

- A260/280 ratio: between 1.8 and 2.0
- A260/230 ratio: above 1.5, close to 1.8

High-throughput sequencing

High-throughput sequencing (HTS), also known as next generation sequencing, is a relatively new method of massive parallel sequencing, which has permitted the sequencing of millions of short fragments of DNA simultaneously. The characterization of the microbiota for the work presented in **Paper II** and **IV** was performed using the Ion Torrent Proton/personal genome machine (PGM) platform. This platform sequences up to 400 base pairs (bp) with an accuracy of about 98%. The technology is based on the detection of hydrogen ion released during the polymerization of DNA. Figure 8 represents the workflow for the sequencing process. In summary, library preparation was performed by assessing amplicon fragment concentration and adjustment of the concentrations to 26 pM for each sample (normalization of samples). Library

preparation, attachment of the amplified clones to the Ion sphere particles, sequencing and finally removal of low quality sequences were performed according to manufacturer's instruction following the Ion Torrent sequencing workflow.

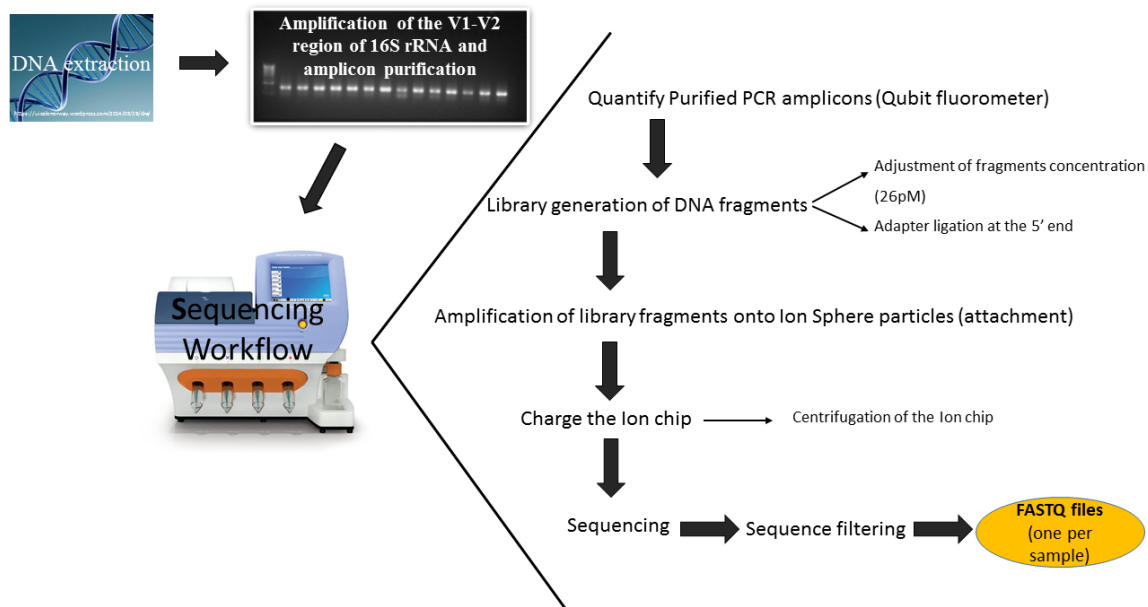


Figure 8. Workflow for the sequencing of bacterial amplicons from DNA samples of digesta and mucosa using the Ion Torrent PGM platform.

Bioinformatics

Bioinformatic work is needed to filter and further analyse the large amount of data produced by HTS platforms. There are several ways to analyse the sequence data resulting from HTS. The open-source software, Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso *et al.*, 2010) was used for the analysis of the sequencing data presented in **Paper II** and **IV**. This software is not the only open-source bioinformatic pipeline for microbiome analysis, although similar results should be expected by the use of other pipelines. The election of the pipeline was mainly based in bioinformatic knowledge and experience.

For the work presented in **Paper II** and **IV**, the bioinformatic workflow (Figure 9) started with an evaluation of the obtained sequences using the FastQC software (Andrews, 2010) to explore

the quality of the reads. Then the sequences were filtered by quality with FASTX-Toolkit (Hannon Lab, USA) using a minimum quality score of 20 in 80% of samples. Samples were then concatenated into one file using seqtk-master (available at <https://github.com/lh3/seqtk>).

The bioinformatic analyses performed thereafter uses the operational taxonomic unit (OTU) as the term to refer to the bacteria found in the samples. The OTU(s) can be defined as a species or group of species when DNA sequenced data has been used to identify individuals or group of individuals in a dataset using a defined sequence similarity threshold (Blaxter *et al.*, 2005). Analyses of the quality-filtered data were performed with QIIME to obtain the list showing the OTU abundances. QIIME performs other analyses of data that are useful for the understanding of microbial ecology, such as alpha and beta diversity and the computation of the core microbiota of the samples.

Alpha diversity is a term expressing the diversity (in this case OTU diversity) found within site, or in this case intestinal compartment or dietary treatment (Whittaker, 1972). Several indices can be calculated in QIIME for the estimation of the alpha diversity. To calculate alpha diversity indexes, samples were rarefied (random subsampling) based on information obtained at the lowest number of reads found in the dataset (sample with lowest reads) and rarefaction curves were constructed. Rarefaction curves represent the number of observations as function of sequencing depth. The curve will increase as long as there are still observations to be considered and plateaus when the majority of the observations in the sample are taken into consideration.

Alpha diversity indexes calculated by QIIME and presented in our studies are the following:

- Chao 1 index (Chao, 1984); estimates the richness of the sample. This index correct the number of species by the number of singletons and doubletons (OTUs represented by only one or two reads, respectively).
- Observed species; number of OTUs in the samples.
- Shannon's diversity index (Shannon, 2001): estimates the diversity of a sample taking into account both richness and abundance of the bacteria (OTUs) present in the samples.

- Good's coverage estimator; percentage of the total OTUs of the sample that are represented (sample coverage).

Beta diversity is defined as “the extent of species replacement or biotic change along environmental gradients” (Whittaker, 1972) i.e. a term expressing the differences in species, in this case OTUs, between different sites, or as in this case different intestinal compartment or diets. The calculation of the beta diversity was performed using weighted and unweighted UniFrac measures (Lozupone & Knight, 2005, Lozupone *et al.*, 2007). UniFrac measures the distance (beta diversity) between sets of taxa based on phylogenetic distance, using the phylogenetic tree of the taxa found in the different sites (Lozupone & Knight, 2005). When calculated, weighted UniFrac take into account OTU abundances (quantitative), whereas unweighted UniFrac does not (qualitative). Principal coordinates analysis (PCoA) plots are commonly used to compare groups in a graph based on measurements such as UniFrac distances as presented in the work of **Paper II** and **IV**. In these plots, each dot represents one sample and the distance between dots represents the similarity or dissimilarity between bacterial communities in the sample. The closer the dots the more similarity among samples bacterial communities.

The core microbiota can be defined as the OTUs shared among the different study groups (e.g. diets groups or intestinal compartments). A core or shared OTUs has been described in several fish studies (Roeselers *et al.*, 2011, Wong *et al.*, 2013) and can aid in the identification of the most commonly observed OTUs in the intestine.

Finally, statistical analyses of the alpha and beta diversity metrics were also performed. Alpha diversity metrics can be analysed using classical parametric or non-parametric statistical analyses. As for beta diversity, the use of permutational multivariate analysis of variance (PERMANOVA) has been demonstrated to be a useful choice for analyses of the differences in bacterial communities between groups. This non-parametric analysis has as only condition the assumption that the observations are exchangeable under a true null hypothesis (independence between observation and similar distributions).

Another statistical method performed to improve our understanding about the modulation of the microbial populations in our studies was the linear discriminant analysis (LDA) effect size

(LEfSe) analysis (Segata *et al.*, 2011). This algorithm can be used to characterize the differences between two or more conditions. The algorithm uses a non-parametric factorial Kruskal-Wallis sum-rank test to detect features with significant differential abundance with respect to the class of interest. The significance is then assessed using a set of pairwise tests among subclasses using Wilcoxon rank-sum test. Finally, LEfSe is used to estimate the effect size of the abundant feature(s) (Segata *et al.*, 2011).

Further analyses of microbiota data focused on gaining knowledge about functional properties of the bacterial communities and statistical analyses of the data obtained. Functional profiling (metabolomics or metatranscriptomics) are currently expensive and excessively time consuming. Therefore, prediction of the functionality of microbial communities was considered a valid alternative. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) is an open-source, bioinformatic tool designed to predict metagenome functional content from marker gene datasets (Langille *et al.*, 2013). PICRUSt uses an algorithm and a reference genome database for microorganisms to predict bacterial functionality of data resulting from 16S rRNA gene sequencing. As the method relies on annotation/presence in the database of the OTUs identified in the samples (or some phylogenetically close related microorganism), some limitations to the approach have to be considered, especially in new environments and/or environments with a large number of microorganisms that are poorly annotated in functional databases (Xu *et al.*, 2014). PICRUSt was performed for the work presented in **Paper II** using a close reference OTU table (demanded by PICRUSt) extracted from the OTUs original table, based on the Greengenes database (version 13.5.). The resulting table contained only about 10% of the OTU table generated by the original open reference strategy.

The PICRUSt analysis was performed using default settings for OTU normalization by copy number, predicted gene family abundances and finally metagenome inference using KEGG orthology (KOs) (Kanehisa *et al.*, 2011). Nearest sequenced taxon index (NSTI) was calculated as an estimate of the phylogenetic distance between each assigned OTU and the closest relative with a sequence reference genome.

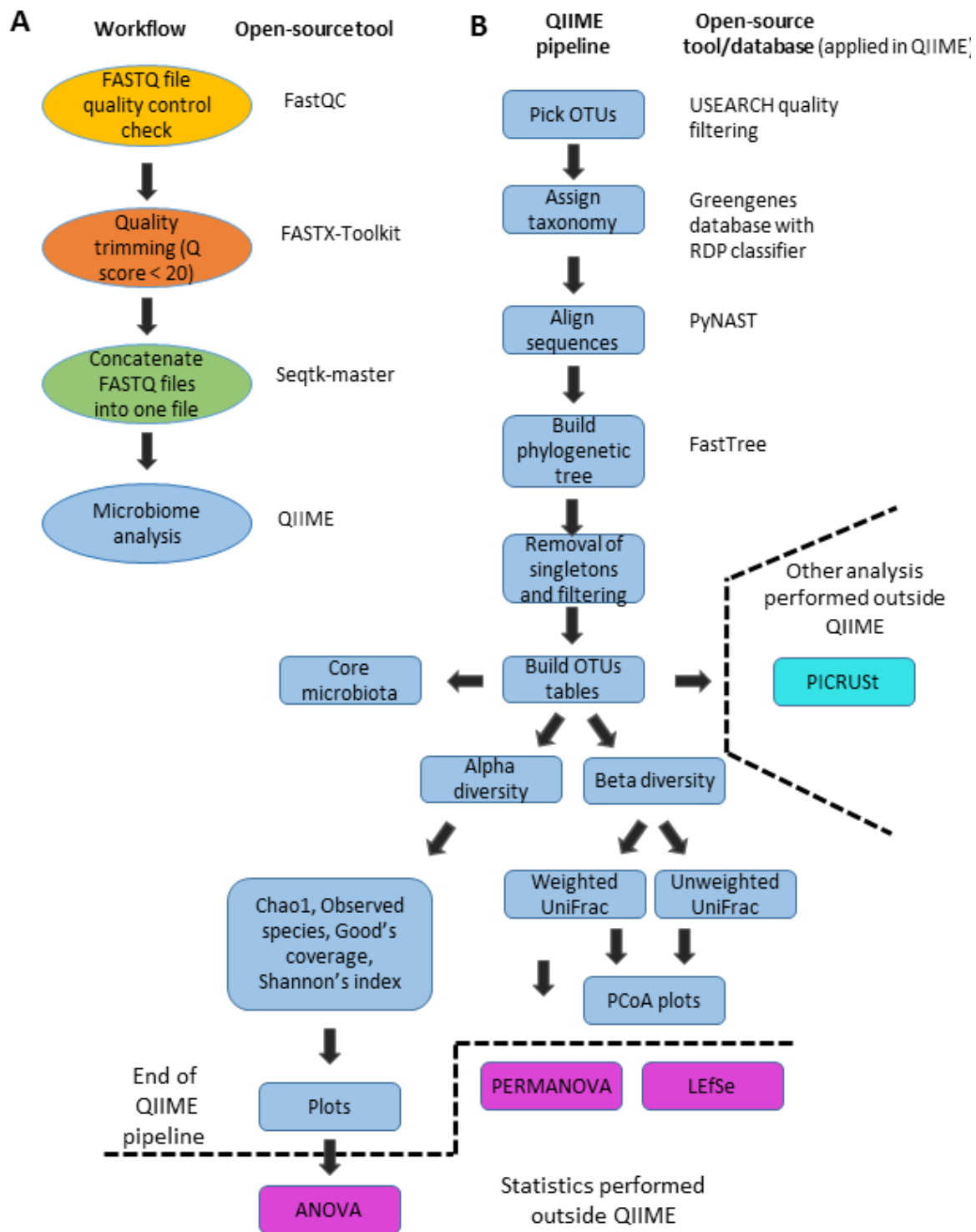


Figure 9. Workflow for the bioinformatic work for the microbiota characterization. A) General workflow for data analysis. B) QIIME pipeline as performed for the work presented in **Paper II** and **IV**.

Quantitative polymerase chain reaction (qPCR) for host gene expression profiling

Total RNA purification from DI samples, quality control, DNase treatment, and cDNA synthesis was performed as described by Kortner *et al.* (Kortner *et al.*, 2013). Primers for the work presented in **Paper I, III** and **IV** were obtained from previously published studies or designed using Primer3web software version 4.0.0 (<http://primer3.ut.ee/>). Annealing temperatures were tested for all new primers and melting curve analysis and agarose gel visualization were performed for all new primer pairs to confirm amplification specificity.

Gene expression profiling was performed for the following groups of relevant, health-related target genes:

- Immunity: for the work presented in **Paper I** and **IV**
- Metabolism: for the work presented in **Paper I, III** and **IV**
- Mucus production: for the work presented in **Paper I** and **IV**
- Water channel: for the work presented in **Paper I** and **III**
- Stress related: for the work presented in **Paper I** and **IV**
- Cell integrity: for the work presented in **Paper III**

In order to determine stable reference genes, several candidates were evaluated (Kortner *et al.*, 2011). The geometric average expression of *gapdh* and *rnapolIII* (work presented in **Paper I**) and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), RNA polymerase 2 (*rnapolii*) and hypoxanthine phosphoribosyltransferase 1 (*hprt1*) (work presented in **Paper III** and **IV**) were used for target gene normalization. The mean normalized expression of the target genes was calculated from raw C_q values by relative quantification (Muller *et al.*, 2002).

DISCUSSION: Material and Methods

Material

Post-smolt Atlantic salmon in seawater tanks were used in all experiments included in this thesis. However, not all experiments were performed in the same research facility. The experimental trials for the work presented in **Paper I** and **II** were performed at the Nofima's aquaculture research station at Sunndalsøra, Norway, while the experimental trial for the work presented in **Paper III** and **IV** was performed at BioMar's research station in Hirtshals, Denmark.

Previous studies have described that the microbiota can be modulated by external factors and that factors such as geographical location, water temperature, salinity and management practices in the research facilities can influence the composition of the intestinal microbiota in fish (Bakke-McKellep *et al.*, 2007, Merrifield *et al.*, 2009, Wong *et al.*, 2013, Zarkasi *et al.*, 2014). This is especially relevant when results from different studies are compared. The experimental trials for **Paper II** and **IV** were performed in two different facilities and even when both were located in Scandinavia, factors such as water temperature and salinity may differ in both experiments, having a modulatory effect in the intestinal microbiota. In the feeding trials reported in **Paper II** and **IV**, the fish were fed different diets. Moreover, the length of the feeding trials differed: four weeks in the trial presented in **Paper II** against 48 days for the trial presented in **Paper IV**. All these factors have modulatory effects on the intestinal microbiota (Merrifield *et al.*, 2009, Desai *et al.*, 2012, Hartviksen *et al.*, 2014) and should be considered when comparing the intestinal microbiota results.

Methods

Intestinal sampling procedures and extraction of DNA

For the work presented in **Papers I, II and III and IV**, sampling for the study of different parameters of the intestine was performed following methods well established in our laboratory.

The exceptions were the methods for the collection of samples for the characterization of the intestinal microbiota for the work presented in **Papers II** and **III**. Despite the differences in sampling procedures, mucosa scraping vs intact intestine sampling, both protocols demonstrated to be effective in providing high quantity and quality material to extract bacterial DNA from both digesta and mucosal samples. However, the mucosa sampling method used in the work presented in **Paper II** resulted in less material (approximately 50 mg of mucosal tissue per sample) compared with the method used in the work presented in **Paper IV** (over 200 mg of intestine per sample). The lower amount of mucosal tissue produced by the method used in the work presented in **Paper II** resulted in a concomitant lower amount of total DNA. However, the latter did not appear to be important since the difference in DNA yield obtained between the studies was not a difference in mucosa-associated bacterial DNA, but host DNA from the outer layers of the intestine, which should not influence the intestinal microbiota results.

Some possible challenges regarding the sampling methodology used in the work presented in **Paper II** resulted in changes implemented when sampling for the work presented in **Paper IV**. The first method was time consuming and the resulting mucosa sample size was much lower than the amount recommended for the use of the QIAamp Stool Mini Kit (Qiagen, Crawley, UK). The use of the kit was preferred against the classical phenol/chloroform method (work presented in **Paper II**) as it has been demonstrated that the kit method is efficient and faster (Li *et al.*, 2003). Variation in DNA extraction protocols has been found to be an important source of variation in studies of microbial communities, favouring the DNA extraction of one or more bacterial groups against others (Mackenzie *et al.*, 2015). Nevertheless, the study of Mackenzie *et al.* 2015 (Mackenzie *et al.*, 2015) comparing different DNA extraction methods for the study of the human intestinal microbiota found no significant differences between the phenol/chloroform method and the QIAamp Stool Mini Kit DNA extraction method in terms of OTU relative abundances. In the work for this thesis, the comparison between DNA extraction methods was assessed by extracting DNA from one sample with both methods (phenol/chloroform and QIAamp Stool Mini Kit) and performing the downstream work, including HTS, processing and analysis of the resulting data (data not shown). In agreement with the previously mentioned study, the results from this assessment did not show any significant differences between the two DNA extraction methods.

An additional aspect of importance when discussing the DNA extraction method and the use of highly sensitive sequencing approaches such as HTS is the chance of obtaining bias in the results due to bacterial contamination in reagents as described by Salter *et al.* (Salter *et al.*, 2014). These authors found that this type of misrepresentative results is particularly important in low bacterial biomass samples. This is important to consider when analyzing results such as shared OTUs among samples (as in the case of the core microbiota) as low abundance OTUs may originate from contaminated reagents or other kinds of contamination, even when the origin of the sample is an environment with high bacterial biomass (as the intestine). To avoid misleading conclusions, negative controls are required during all steps of sample processing. Besides negative controls, measures of safety to avoid external DNA contamination were taken when handling samples for the work presented in **Paper II** and **IV**, including use of flame, use of 70% ethanol solution to clean instruments, DNA away (Molecular Bio-products) on surfaces and gloves, and the sterilization of tubes and water.

Polymerase chain reaction (PCR) amplification

As explained in the Material and methods chapter, the amplification of one or more hypervariable regions of the 16S rRNA gene for the bacterial characterization is widely applied. One methodological challenge when performing HTS of the 16S rRNA gene for the characterization of the microbiota is the introduction of bias in the PCR amplification of bacterial DNA. This may be a result of the specific hypervariable 16S rRNA gene region selected for PCR amplification. The choice of the hypervariable region to be amplified was performed according to the following criteria:

- Amplicon length (preferably around 300 bp, matching the read length of the HTS platform)
- High quality amplification of bacterial DNA from intestinal fish samples
- Preferably, amplicon should have more than one hypervariable region

Studies comparing the performance of several hypervariable regions of the 16S rRNA gene for the characterization of the intestinal microbiota in humans and other terrestrial animals have suggested that several hypervariable regions performed well in terms of accuracy in

characterizing the bacterial communities in the samples (Huse *et al.*, 2008, Kim *et al.*, 2011). However, similar studies comparing different regions from the 16S rRNA gene for the study of microbial communities in the intestine of fish have not been conducted so far. Therefore, several primer sets targeting different 16S rRNA gene hypervariable regions were tested in this thesis (Table 6, Figure 10 A-D).

For our studies, we selected primers targeting the V1-V2 region (Roeselers *et al.*, 2011). Primers 27F and 338R-I and 338R-II (pooled together) were the ones resulting in adequate amplification of our intestinal samples in terms of brightness of the bands in the agarose gel (Figure 10 A).

Table 6. Primers tested for the amplification of the 16s rRNA gene.

Variable region	Primers	Length	Sequence	Observations ¹	Reference
V1-V2	27F 338R-I 338R-II	311	5' AGA GTT TGA TCM TGG CTC AG 3' 5' GCW GCC TCC CGT AGG AGT 3' 5' GCW GCC ACC CGT AGG TGT 3'	Good amplification	(Roeselers <i>et al.</i> , 2011)
V3	P2 P3	193	5'-CCT ACG GGA GGC AGC AG-3' 5'-ATT ACC GCG GCT GCT GG-3'	Good amplification	(Muyzer <i>et al.</i> , 1993)
V4-V5	Com1 Com2	407	5' CAG CAG CCG CGG TAA TAC 3' 5 'CCG TCA ATT CCT TTG AGT TT 3'	Poor amplification	(Schwieger & Tebbe, 2000)
V4	577F 926R	349	5' AYT GGG TDT AAA GNG 3' 5' CCG TCA ATT CMT TTR AGT 3'	Poor amplification	(Rodrigues <i>et al.</i> , 2013)

¹ Observation based in terms of brightness of the band for all samples in the agarose gel.

Another challenge when using the 16S rRNA gene of bacteria for the microbiota characterization is its similarity with the 16S rRNA gene of chloroplast. It has been suggested that chloroplasts evolved from cyanobacteria, therefore bacteria and chloroplasts share a highly related 16S rRNA gene. For this reason, a high number of reads obtained from the HTS – 78% in the work presented in **Paper II** and 61% in the work presented in **Paper IV** – were subsequently classified as Cyanobacteria/Chloroplast by QIIME. Since these reads represented eukaryote plant sequences, possibly from water, biofilm or dietary origin that were ingested by the fish, these reads were removed from all the analyses (Baldo *et al.*, 2015).

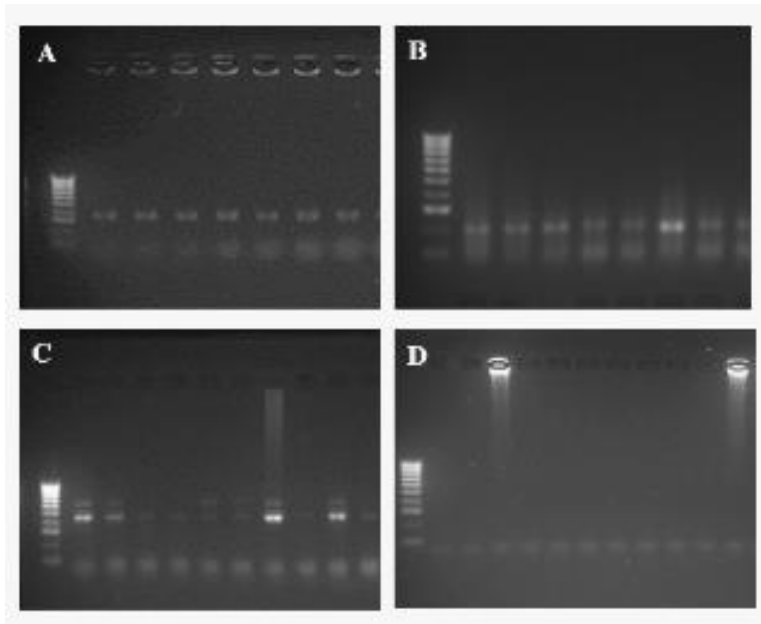


Figure 10. Representative images of 16S rRNA gene PCR amplification from DNA samples testing different hypervariable regions of the 16S rRNA gene using different primers set. A) Hypervariable region V1-V2, primers 27F and 338R I-II (pooled together); B) Hypervariable region V3, primers P2 and P3; C) Hypervariable region V4-V5, primers COM1 and COM2; D) Hypervariable region V4, primers 577F and 926R.

New DNA extraction kits that remove the CG-methylated DNA are an effective tool to remove the host DNA, but their efficiency against plant DNA present in samples remains unclear. Another strategy to avoid the chloroplast amplification is to use primers targeting a different 16S region presenting differences between bacteria and chloroplasts, as in the case of the position 783–799, which have four base pair mismatches (Chelius & Triplett, 2001) that could potentially be useful to avoid chloroplast amplification (Hanshew *et al.*, 2013). Primers tested for targeting the V4-V5, which included the previously named base pair position, did not showed good amplification and were not used in this thesis work.

In order to work more efficiently and take full advantage of the HTS technology, future work in salmon should be performed using DNA extraction kits that exclude eukaryotic DNA and primer sets that amplify only bacterial DNA, thus avoiding chloroplast amplification.

Bioinformatics

The use of HTS for the study of the microbiota depends on reliable bioinformatics tools capable of processing the data resulting from massive sequencing. Nowadays, open-source bioinformatic tools are still improving and new software versions and pipelines are regularly being released with improved, more accurate and effective options for the analysis of microbial communities. For the work presented in **Paper II** and **IV**, the pipeline QIIME version 1.8 was used for the processing and analysis of quality-trimmed sequences. QIIME has been widely applied in the study of the microbiota in samples from different environments including several studies of the intestinal microbiota of fish (Wong *et al.*, 2013, Falcinelli *et al.*, 2015, Standen *et al.*, 2015). Studies comparing different pipelines have demonstrated possible bias in the results depending on the different cluster algorithms for OTUs picking (open or closed reference approach), as well as other choices selected in the pipelines such as the databases used, chimera filtering software, and identity level assigned (Edgar, 2013, Pylro *et al.*, 2014). In our studies, we performed open reference (pick_otus.py script with USEARCH) as clustering method and the Greengenes database has been used in QIIME to perform clustering, chimera checking and quality filtering. Currently, there is no standardized bioinformatic protocol for the characterization of the microbiota in fish. Therefore, when comparing results across studies, it is important to consider the pipeline and the stringency of the parameters used in the bioinformatic pipeline, since changes in the stringency of bioinformatic approaches could result in over or underestimation of OTUs present in the samples (reviewed by (Hiergeist *et al.*, 2015).

Bacterial characterization: the past, the present and the future

Classical culture methods have been used for more than 100 years for the characterization of bacteria. With the development of the culture-independent methods, some of the limitations of the bacterial culture approach have been eliminated or are diminished. The new methods based mainly on the sequencing of the 16S rRNA gene allow scientists to assess not only culturable bacteria, but also non-culturable bacterial communities present in different environments. The use of DGGE for the characterization of the intestinal microbiota has been widely used in several animals (Simpson *et al.*, 1999, Noor *et al.*, 2010), including fish (Hovda *et al.*, 2007, Grammes

et al., 2013, Reveco *et al.*, 2014). The DGGE approach gives a “picture” (fingerprinting) of the microbial community (usually PCR amplicons of hypervariable regions of the 16S rRNA gene) represented in bands in a polyacrylamide gel. These bands could subsequently be sequenced in order to identify the bacterial species to which they represent. Although useful, this technique presents several limitations. One is microheterogeneity of bands, i.e. PCR amplicons representing different OTUs migrating to the same position in the gel (Sekiguchi *et al.*, 2001). On the other hand, bands with different migration patterns can originate from the same bacteria species. Nowadays, the use of HTS of the 16S rRNA gene for the characterization of bacterial communities is being widely spread. This technique is currently replacing other culture-independent techniques as a result of superior resolution of bacterial community profiles in samples in an efficient and affordable manner. Nonetheless, HTS is not error free, i.e., the sequencing may contain some minor errors, and the possibilities of introduction of bias in the results due to technical and methodological procedures has to be minimized by producing good quality samples to be sequenced, as discussed previously. One of the main disadvantages of the microbiota characterization by the use of HTS of the 16S rRNA gene or another conserved marker is that this method only permits the study of community structures without the possibility for functional microbiota profiling. Metabolomics, metatranscriptomics or shotgun metagenomic sequencing, i.e. sequencing of all genetic material in a sample allowing both characterization and functional profiling of samples, have not yet been applied to a large extent due to excessive cost and time constraints. As an alternative, the bioinformatic tool for prediction of the functional profile of the microbiota, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), has been developed (Langille *et al.*, 2013) and used in several studies in animals, including fish (Mao *et al.*, 2012, Jiménez *et al.*, 2014, Sullam *et al.*, 2015). The PICRUSt analysis was performed for the results presented in **Paper II**. The outcome was not considered to be useful as about 90% of the original OTUs were excluded from the analysis and the NSTI value was high at 0.27 ± 0.01 (mean \pm SEM). Improvements in database annotations by expanding the number of bacterial annotations are necessary in order to benefit from tools predicting functional bacterial profile in salmon.

Host gene expression profiling: qPCR

Molecular processes are of key importance in maintaining the optimal functionality of the intestine and are known to be a measurable intestinal response to dietary-related challenges, such as SBMIE (Kortner *et al.*, 2012, Marjara *et al.*, 2012). The study of gene expression by qPCR is performed in order to evaluate the intestinal response, mainly functional and immune responses, to different external factors such as alternative protein in the diet that may affect intestinal health. The technique is easy, cost efficient and a viable alternative in lieu of a shortage of salmon-specific antibodies for protein expression analyses. The qPCR approach is an established method, although like many other molecular methods, if not performed correctly, bias can be introduced to the results. One of the main concerns when applying qPCR to measure gene expression is the correct normalization of the data. The most common normalization method is the use of reference genes. These reference genes should have a constant expression through all analysed samples. Evaluation of the reference genes should be performed for each experiment, since there is no universally applicable reference gene. The study of Kortner *et al.* (Kortner *et al.*, 2011) gives a detailed explanation of evaluation procedure for the possible genes of reference and the importance of effective normalization of gene expression data in the study of dysfunctionality in the intestine of Atlantic salmon.

At present, microarrays and RNA-sequencing are two relatively new methods that have been introduced and successfully applied for the study of the intestinal responses to different environmental factors such as diet, at a gene expression level. The microarray technique is able to measure the expression of thousands of specific genes at the same time (transcript-specific probe dependent). This technique has been applied in a number of studies of Atlantic salmon undergoing SBMIE (Kortner *et al.*, 2012, Grammes *et al.*, 2013, Sahlmann *et al.*, 2013, Król *et al.*, 2016). Although useful, microarrays are expensive and only able to quantify the specific genes that the array is designed for. In contrast, RNA-sequencing (RNA-seq), which is another application of the HTS technology, does not depend on transcript-specific probes, which means that this technique is able to detect and quantify with high sensitivity and specificity all transcripts present in the sample. The major drawback of the RNA-seq technology, besides its cost, is the

demand for bioinformatics expertise that is needed to process and analyse the enormous amount of data resulting from the high-throughput sequencing.

It is expected that in the next years the RNA-seq technology becomes more available with lower cost and user-friendly analysis tools. These tools could make easily possible the integration of data resulting from different HTS approaches, such as the case of RNA-seq and microbiota characterization, leading to a better understanding of the health modulation, functionality and interactions of complex organic systems like the intestine.

SUMMARY OF RESULTS

Paper I presents work assessing whether soya saponins alone, i.e. without the presence of other plant antinutrients, may cause SBMIE in Atlantic salmon. The fish, kept in seawater flow-through tanks, were fed ten diets based on two basal diets, one containing fishmeal as the main protein source (FM) and the other contained 20% lupin meal (LP) and 19% wheat gluten as sources of protein as partial replacement of FM. The experimental diets were made by supplementing batches of each of the basal diets with a highly purified (95%) soya saponin preparation at 0, 2, 4, 6 or 10 g/kg. The feeding trial lasted 10 weeks.

Soya saponin supplementation caused dose-dependent effects on most of the selected endpoints in fish fed both basal diets. However, fish fed LP diet presented similar negative effects, despite eating less than the FM-fed fish. The histological analysis showed that the soysaponins elicited signs of inflammation in the DI of salmon when supplemented to either of the diets in doses at and above 2-4 g/kg. These inflammatory changes were similar to the ones described when SBM is included in diets at a level above 8% (Baeverfjord & Krogdahl, 1996). The saponin-induced inflammation was also associated with alterations in digestive functions such as reduction of the total bile acid concentration along the intestine, increased trypsin activity in DI content, and reduction of the activity and capacity of the brush border enzyme leucine aminopeptidase (LAP) in fish fed both saponin-supplemented basal diets. In addition, plasma cholesterol and total bile acids decreased with increasing soya saponins supplementation for both the FM and LP-fed fish. Plasma cholesterol level was, however, about 50% lower in fish fed the LP diets compared to the fish fed the FM diet.

Moreover, supplementation with soya saponins triggered modulation of the expression of several genes in the DI. The expression of immune-related genes such as *interleukin 17a (il-17a)*, *interferon γ (ifn γ)* and *myeloid differentiation factor 88 (myd88)* were found significantly increased, and *interleukin1 β (il1 β)* and *matrix metalloproteinase 13 (mmp13)* displayed a similar trend when soya saponins were supplemented. On the other hand, functionally related genes such as *Aquaporin 8ab (aqp8ab)* and *fatty acid binding protein 2a1 (fabp2a1)* were markedly and dose

dependently decreased with no significant difference between the basal diets. Moreover, elevated expression profile of the *pcna* gene, supported by the immunohistochemical detection of PcnA, showed elevated number of cells presenting this protein in fish fed the saponin-supplemented diets.

The main conclusions from this study were: Saponins induce SBMIE when present as the only antinutrient in diets without other protein sources than fishmeal for Atlantic salmon above a level between 2 to 4 g/kg. The same range of saponin-supplementation in the diet with 20% lupin meal replacing fishmeal caused similar changes. As the effects for some of the endpoints, however, in spite of lower exposure due to lower feed intake, were similar in fish fed the LP basal diet, the results seem to indicate that some LP component(s) may potentiate the saponin effects. Consequently, when feed is formulated using more than one ingredient with saponins, care should be taken to avoid too high levels of saponins. The results also suggest that fishmeal diets supplemented with soya saponins may be used for induction of enteritis and thereby improving of the model earlier used for studies of mechanisms underlying enteritis.

A model based on the use of saponin gives better opportunity to the study of factors involved in the enteritis development, as it would not depend on the highly variable composition of soybean meal. Thus, a model, which thereby becomes independent of soybean meal will allow to investigate further interaction between saponins and other antinutrients, as well as a range of biotic and abiotic conditions which may be of importance for the severity of the enteritis.

Paper II presents work supplying basic information regarding a key element of the function and health of the salmon intestine, namely the compartmental distribution of the intestinal microbiota, hitherto mostly unknown for fish and most other animals. In this study, we performed an in-depth characterization of the digesta and mucosa-associated microbiota along the intestine of farmed Atlantic salmon post-smolt kept in a salt water recirculating system. The fish were fed a commercial diet containing 38% plant-meals (soy protein concentrate, wheat, wheat gluten and sunflower meal) and 45% fishmeal for four weeks. This diet was considered moderate in terms of plant-meal inclusion levels and was expected to maintain satisfactory intestinal health. By employing high-throughput sequencing of the V1-V2 region of the 16S rRNA gene, the characterization of the digesta-associated microbiota in the proximal (PID), mid (MID) and distal

(DID) intestine and mucosa-associated microbiota of the mid (MIM) and distal (DIM) intestine, i.e. in five compartments, was performed.

This study revealed significant differences between the microbial communities within and between the digesta and mucosa compartments. The digesta presented higher microbial richness (higher number of OTUs) and higher Shannon's diversity index than the mucosal compartments (alpha diversity). Moreover, the statistical analysis revealed significant differences between most of the intestinal compartments (beta diversity). The exception was a lack of significance for the differences between MID and DID.

At phylum level, all the digesta compartments showed high relative abundance of OTUs for Proteobacteria followed by Firmicutes, Fusobacteria and Actinobacteria. At genus level, the digesta compartments differed as follows: in PID *Photobacterium*, *Delftia*, *Weissella* and *Leuconostoc* dominated followed by *Janthinobacterium*; in MID *Photobacterium*, *Leuconostoc* and *Weissella* dominated followed by *Peptostreptococcus* and *Janthinobacterium*; for DID *Photobacterium*, *Leuconostoc* dominated followed by *Janthinobacterium*, *Weissella* and *Peptostreptococcus*. The results for the mucosa compartments were as follows: both MIM and DIM showed a dominance of the phylum Proteobacteria with a high abundance of the genera *Janthinobacterium*, *Delftia* and *Variovorax*. In addition, MIM presented high relative abundance of the genus *Phyllobacterium* and DIM showed high relative abundance of the family Brevinemataceae and the genus *Stenotrophomonas*.

The results of the LEfSe analysis showed that the class Fusobacteriia and the order Vibrionales were significantly different in PID compared to the other compartments, while the class Clostridia was significantly higher in MID. The DID showed significantly higher abundance of the class Bacilli and Actinobacteria. As for the mucosal compartments, LEfSe results indicated that alpha- and betaproteobacteria were more abundant in MIM than the other compartments and *Delftia* were most abundant in DIM. Finally, the computed core microbiota, i.e. the shared OTU found in 80% of the samples, comprised 22 OTUs, with the highest number of OTUs belonging to the phylum Proteobacteria.

In this study, the microbiota differed substantially regarding all observed characteristics of the microbial population between the mucosa and digesta, as well as between the regions along the intestine. The differences clearly suggest that when sampling for investigation of relationship between intestinal microbiota and intestinal health or other host functions, it is necessary to differentiate between mucosa and digesta and to sample from several intestinal regions.

Paper III and IV present results of an experiment conducted to study effects in Atlantic salmon fed commercially relevant diets containing high levels of alternative protein sources on health indicators of the DI including microbiota. The results presented in **Paper III** report characteristics of the diets regarding production performance, nutritional quality, histomorphological changes, water-content of the DI digesta, plasma osmolality and aquaporin, ion transporter, tight junction and adherence junction expression in the DI tissue. The results presented in **Paper IV** comprise effects of the alternative diets on immune, cellular stress and cell proliferation responses in the DI tissue, as well as mucosa and digesta-associated DI microbiota. The fish used were Atlantic salmon post-smolt kept in a seawater recirculating system and fed one of five experimental diets for 48 days. The control diet (FM) contained fishmeal as the sole source of protein, while the fishmeal was partially replaced by the following alternative ingredients in four substitution diets: poultry meal diet (PM); a mix of soybean meal and wheat gluten (SBMWG); a mix of soy protein concentrate and poultry meal diet (SPCPM); or a mix of guar meal and wheat gluten diet (GMWG).

General results

Specific growth rate presented no significant differences (**Paper III**) between the fish fed FM and the fish fed the substitution diets. However, the substitution diets presented significantly higher feed conversion ratio, possibly reflecting the higher fiber content of these diets.

Regarding the general characterization of the microbiota present in the DI (**Paper IV**), the general results showed differences between the fish fed the FM and the substitution diets and between digesta and mucosa compartments. The alpha diversity index observed species were significantly different between digesta and mucosa, showing lower richness for the mucosa compared to the

digesta samples. Alternative ingredients caused differences in DI digesta and mucosa-associated microbiota compared to FM fed fish (beta diversity). The results from LEfSe indicated significant differences in DI microbiota between the fish fed FM diet and the fish fed the diets containing alternative proteins in both digesta and mucosa. A higher number of OTUs showed significant differences between the dietary groups in digesta compared to mucosa. Regardless of the diet, the DI mucosa showed lower abundance of Firmicutes and Fusobacteria and higher abundance of Proteobacteria, Bacteroidetes and OD1 in comparison with digesta. For all samples, the core microbiota consisted in 19 OTUs.

Diet specific results

SBMWG diet

Compared to the fish fed FM, the fish fed SBMWG showed the largest number of differences in all the evaluated histomorphological and physiological parameters analyzed in plasma and the DI tissue and chyme. These included increased fecal water content of DI chyme and higher plasma osmolality (**Paper III**). The histomorphological evaluation (**Paper III**) showed alteration in the normal morphology of the intestine indicating moderate enteritis (SBMIE), although gene expression of various immune-markers that have previously been differentially expressed in salmon with SBMIE were not differentially expressed in the current study (**Paper IV**). The latter could be the result of low levels of saponins in the soybean meal used in the diet or a chronic stage of the inflammatory process with an attempt to repair the damage produced by the diet. In line with these findings, the gene expression profile suggested that the diarrhea and high plasma osmolality could be at least partially explained by the alterations observed in several genes related to water regulation, transport and intestinal permeability, i.e. genes coding for aquaporins, ion transporters, tight junction and adherence junction proteins in the DI tissue (**Paper III**). Among the studied aquaporins, aquaporin 8ab (*aqp-8*), which has been suggested as a marked gene for the study of SBMIE (Kortner *et al.*, 2012), was markedly reduced. Together, the gene expression profile suggested reduced transcellular transport of water. Moreover, the expression of a gene related with cellular stress, i.e. shock protein 70 (*hsp70*), was increased in fish fed the SBMWG diet. In addition, fish fed the SBMWG diet presented high cell proliferation as seen by high

expression of the *pcna* gene and confirmed by immunohistochemical detection of Pcn protein tissue expression and distribution (**Paper IV**).

Fish fed SBMWG diet showed differences in the abundances of the DI bacterial communities. These fish also showed a different mucosal-associated microbiota compared to that of FM fed fish. Weighted and unweighted UniFrac PCoA plots supported the previous results, showing a clear separation between the digesta and mucosa compartments, as well as between diets. Results from the LEfSe analysis indicated that in digesta, the fish fed SBMWG diet presented significantly higher abundance of several members of the LAB group compared to the FM-fed fish. In the mucosa, SBMWG fed fish presented higher abundance of one LAB member, namely *Weissella* (**Paper IV**).

GMWG diet

Similarly to the SBMWG-fed fish, the fish fed GMWG diet also presented certain alterations in the expression profile of genes related to water transport such as *aqp-8ab* and *aqp-10* (**Paper III**), *frim*, *pcna* and *cat*, as well as an increased expression of Pcn-positive cells in the immunohistochemical detection (**Paper IV**). However, fish fed GMWG diet not showed concomitant alterations in the chyme water content or histomorphological signs of inflammation (enteritis) in the DI tissue. Guar meal is known to contain antinutrients such as saponins, which may explain the alteration in the water transport genes, as well as the cellular stress and increased number of Pcn positive cells. Fish fed the GMWG also presented high abundance of LAB in digesta and mucosa compared to FM fed fish. Together these results for fish fed GMWG diet may indicate that even when antinutrients may be altering the gene functionality and other functional components of the intestine, other mechanisms or mucosal elements may need to be altered in order to cause diarrhea and inflammation. Guar saponins, for example, may not be as concentrated in guar meal and/or as potent in their biological/antinutritional effects as soya saponins.

SPCPM and PM diets

Fish fed the SPCPM and the PM diet presented the least changes in the evaluated characteristics compared to the FM-fed salmon. The fish fed SPCPM diet showed elevated water content of the DI chyme and a trend towards lower gene expression in *aqp-8* and *aqp-10*, but otherwise no signs of inflammation. On the other hand, fish fed PM diet showed a decrease in the chyme water content, which did not appear to be related to alteration in gene expression or histomorphological disruption of the DI. This decrease in chyme water content was most likely an indirect result of high chyme lipid content in the chyme, as a result of low lipid digestibility.

In conclusion, the study presented in **Paper III** and **IV** clearly showed that the diet containing soybean meal negatively affected the gut health of salmon, causing diarrhea and inflammation with the concomitant changes in gene expression and morphology of the DI. In addition, fish fed SBMWG presented the largest modulation in the microbial-associated community of the intestine with high relative abundance of LAB in both digesta and mucosa compared to fish fed FM diet. The GMWG diet also presented some modifications in gene expression, cell proliferation and microbial modulation in the DI. However, as the health of the gut appeared unaffected, as indicated by the histological appearance, the effects observed in fish fed the GMWG diet most likely were a result of normal physiological adaptations to the dietary composition. The other two substitute diets produced only minor changes in the DI functionality, but otherwise were apparently not harmful for the health and functionality of Atlantic salmon.

Based on the summary above regarding the results of this thesis work, the following overarching topics appear to deserve discussion beyond what is already presented in **Papers I-IV**:

- Proof of saponins as inducers of enteritis allow further applications of the enteritis model
- Importance and implications for the host of digesta and mucosa-associated microbiota
- Mucosa-associated microbiota is less affected by diet than the digesta-associated - implications for intestinal function
- Soybean affect functional characteristics of the intestine but may not always significantly affect the immunological response as assessed by gene expression analysis
- Lactic acid bacteria dominate digesta-associated microbiota of fish fed SBM and GM

DISCUSSION: Results

Saponins as inducers of enteritis allow further applications of the enteritis model

Results from the work presented in **Paper I** showed that soya saponins alone are able to induce SBMIE in the DI of salmon when supplemented to a fishmeal based diet at levels above 2-4 g/kg. This knowledge found the basis for improvement of the frequently used model for the study of diet-induced enteritis. The model has until now depended on commercially available soybean meal and the results have varied unpredictably regarding the characteristic of the enteritis (Uran *et al.*, 2009). One cause for the differences in the results is the variation in genetic background of the soybeans (Uran *et al.*, 2009). Soybeans on the world market are in general not traceable and differ greatly in genetic background. Differences in soybean processing and refinement technologies enlarge the variation. The difference in soybean quality may greatly affect the outcome of the model, e.g. feed intake of the fish may vary and thereby antinutrient exposure (Uran *et al.*, 2009). An improved model using a diet based on high quality fishmeal supplemented with purified soya saponins would supposedly give a much more predictable induction of enteritis than the SBM-dependent model used until now. Full control would not be possible, however, as also the genetic background (Venold *et al.*, 2012) of the fish and developmental stage (Sahlmann *et al.*, 2015) may cause variation in enteritis symptoms. In addition, environmental conditions may also be a source of variation in the development of enteritis, e.g. water temperature has been shown to affect the feed intake in fish (Handeland *et al.*, 2008). The latter sources of variation are easier to control than the variation caused by soybean quality.

The improved model would allow more detailed studies of the basic mechanisms underlying diet-induced enteritis, as well as the study of the interaction between different antinutrients during the development of the enteritis. Using this model, the questions arising from the present work (**Paper I**), as well as previous in-vivo and in-vitro studies (Knudsen *et al.*, 2008, Chikwati *et al.*, 2012, Bakke *et al.*, 2014), on whether other antinutrients enhance the saponin effects, could also be answered. In addition, a refined model may allow thorough investigation on the role of the intestinal microbiota in enteritis development.

Importance and implications for the host of digesta and mucosa-associated microbiota

The work presented in **Paper II** showed that the microbiota in the salmon's intestine varied from the proximal to the distal intestine. The differences in the intestinal internal milieu along the intestine are certainly important factors for the regional differences of the intestinal microbiota. Since the chyme pH is relatively constant from the proximal to the distal intestine (Krogdahl *et al.*, 2015), changes in the digesta-associated microbiota along the intestine would rather be due to variation in other intestinal characteristics than pH. On one hand, variation in the concentration of nutrients might be one important factor that may explain these changes. On the other hand, diminishing concentrations along the intestine of components such as bile salts, a wide range of digestive enzymes, along with the increment in the concentration of indigestible material such as fiber, may supply the correct environment and substrates for the growth of different bacteria species along the intestine. Accordingly, bacteria with preference for indigestible substrates would be found in higher abundance in the most distal regions of the intestine.

Even though the present work supplies new and important information on the differences along the intestine (**Paper II**) and the dietary modulation of the intestinal-associated microbiota (**Paper IV**), the importance for the host of these differences is quite unclear. Studies in humans describing the metagenomic bacterial profiles have showed that the magnitude of microbiota modulation do not necessarily correlate with the magnitude of changes in the functional gene profile of the microbiota, suggesting functional redundancy between bacterial species (Jones *et al.*, 2008, Mahowald *et al.*, 2009, Ferrer *et al.*, 2013). Whether the differences in intestinal bacterial community composition modulate the functional profile of the intestinal microbiota in salmon remains unclear.

The results of both our studies on intestinal microbiota (**Paper II** and **IV**) showed lower number of OTUs (observed species) in mucosa compared to digesta-associated microbiota. A number of factors could explain differences in abundances and number of OTUs between digesta and mucosa. On the host side, the pattern recognition receptors (PRRs), e.g. TLRs and NODs, located in several immune cells of the mucosal surface are in charge of the recognition of commensal bacteria, and thus, of maintaining intestinal homeostasis. At the same time, these receptors

activate responses against harmful bacteria that may come in contact with the intestinal mucosa (reviewed by (Tlaskalova-Hogenova *et al.*, 2005). On the bacterial side, the residence time in the intestine, the presence or absence on the bacterial surface of lectins, adhesins, fimbriae, adhesive pili and/or cell wall anchored mucus-binding proteins (MUBs), along with bacterial mechanisms to resist antimicrobial immune secretions by the host, are all important factors that determine the ability of bacteria species to attach and live in close contact to the host (reviewed by (Donaldson *et al.*, 2016). In addition, the presence or absence of dietary components, such as lectins that can compete with the bacteria lectins for attachment to the mucosal cell surface, and other bioactive components that may modulate the intestinal immune responses, have consequences for the attachment of the various bacteria and hence for the composition of the mucosa-associated microbiota (Lee *et al.*, 2006, Zinger-Yosovich & Gilboa-Garber, 2009, Clement & Venkatesh, 2010, Queipo-Ortuño *et al.*, 2012). It may be suggested that the binding or attachment of non-pathogenic bacteria, i.e. commensal microbiota, affect the function of the mucosa and possibly other physiological functions of the salmon, for better or for worse, depending on the receptors involved. However, knowledge on the effects of interactions between the commensal bacteria and systemic functionality in salmon are still a largely unexplored area.

The role of the digesta-associated microbiota, on the other hand, has had more attention among researcher. Studies on land animals including humans have shown that lack of attachment to the mucosa does not mean marginal importance for the host (Garrett *et al.*, 2010, Karlsson *et al.*, 2012). Digesta-associated microbiota interact with the host through their metabolites. In homoeothermic animals, the resident anaerobic, colonic bacteria ferment the ingested non-digestible carbohydrates and produce short chain fatty acids (SCFA) such as acetate, butyrate and propionate. These are of high relevance for the health of the host. They have anti-inflammatory effects (Park *et al.*, 2007, Cox *et al.*, 2009), influence motility in the colon (Ono *et al.*, 2004), fluid/electrolyte uptake (Vidyasagar & Ramakrishna, 2002) and ion transport (Yajima, 1988). Butyrate seems to be the most important source of energy for the enterocytes, necessary for functions such as cell growth and differentiation, ion absorption, motility, immune regulation, intestinal barrier functions etc. (reviewed by (Canani *et al.*, 2011). In addition, absorbed acetate and propionate serve as energy sources for peripheral tissues (Bergman, 1990). Atlantic salmon do not have a colon and whether the most distal regions of the intestine contain lower oxygen

levels than the proximal regions does not appear to have been investigated. In general, metabolite production, including SCFAs, by microbiota in various intestinal regions in salmon and implications for intestinal and systemic health deserves further attention.

Based on available information regarding other animals, it is highly likely that SCFAs play important roles for the optimal functionality and health of mucosal cells also in salmon. At present, little information seems to be available regarding characteristics of SCFA-producing bacteria in the salmon intestine. The only relevant study found in the scientific literature describes the effects of SCFA, i.e. sodium salts of acetic, propionic and butyric acid, supplemented in diets for salmon. No positive effects on specific growth rate and mortality were observed (Bjerkeng *et al.*, 1999). Attempts have been made to estimate production of SCFAs based on presence of bacteria species recognized as SCFA producers (Barcenilla *et al.*, 2000). However, SCFA production may vary greatly depending of the diet and the bacterial species preset in the intestine (Goodlad & Mathers, 1990), making prediction of SCFA production difficult. An alternative might be to focus only on butyrate production as suggested by the work of (Louis & Flint, 2007) describing a method for the detection of a gene specifically related to butyrate production present in most butyrate producing bacteria. Indirect information on butyrate production, indicated by the presence of this gene, might help us to understand the role of butyrate as health promotor and to identify the butyrate-producing bacteria in the intestine of salmon.

Many unanswered questions dominate the present status regarding the role of the microbiota for the function and health of the salmon intestine. The way forward should be based on a good in-depth characterization of the microbial communities present in various regions along the intestine in both digesta and mucosa compartments. Until the time the work of the present thesis was initiated, only three studies were published describing the intestinal microbiota of farmed salmon using HTS. One additional study described the intestinal microbiota of wild salmon. However, all these studies characterized the intestinal microbiota based on samples containing both mucosa and digesta (Schmidt *et al.*, 2016), DI digesta (Llewellyn *et al.*, 2015) or feces collected by stripping (Zarkasi *et al.*, 2014, Zarkasi *et al.*, 2016). It is our understanding that the present PhD work, describing the intestinal microbiota of five compartments (three digesta and two mucosa compartments), represents an important step forward in the characterization of the intestinal

microbiota of farmed salmon. The work strengthens the basis for acknowledging the diversity of the intestinal microbiota and calls for more diversified and thorough investigations taking into account the regional organization of the intestine and the related variation in microbiota. By combining characterization of the microbiota of different regions of the intestine with analysis of the transcriptome and metabolome of the same compartments, it may be possible to elucidate bacterial functions in the salmon intestine and their role in health and disease.

Mucosa-associated microbiota is less affected by diet than the digesta-associated microbiota - implications for intestinal function

The importance of the commensal intestinal microbiota in the development and the maintenance of the immune system homeostasis has been indicated in studies with several animal species (Kelly *et al.*, 2004, Rawls *et al.*, 2004, Vijay-Kumar *et al.*, 2010, Lee *et al.*, 2011, Olszak *et al.*, 2012). For example, the study of Olszak *et al.* (Olszak *et al.*, 2012) with gnotobiotic (germ-free) mice shown that germ-free animals have elevated number of invariant natural killer T-cells, leading to increased morbidity after experimental induction of IBD. Moreover, the mucosa-associated microbiota acts as a physical and chemical barrier against pathogenic bacteria by preventing their attachment to the mucosa of the intestine (Ramare *et al.*, 1993, Nicaise *et al.*, 1999, Portrait *et al.*, 1999). With this background, the question arises on whether modulation on the intestinal microbiota as a result of variation in diet composition, as shown by the present work (**Paper IV**), may affect the development of the immune system and the barrier function of the intestinal mucosa of salmon. The present work does not give the answer to this question, but provides basis for some considerations for the planning of future work. The results presented in **Paper IV** confirmed the results presented in **Paper II**, showing that mucosa-associated microbiota had lower richness than digesta-associated microbiota. Moreover, salmon fed the PM, as well as those fed the SBMWG diet, showed a mucosa-associated microbiota that differed significantly from fish fed the FM diet (unweighted UniFrac). The differences were small, however, compared to the diet-induced microbial variation seen in the digesta-associated microbiota. Together, the results from the studies presented in **Paper II** and **IV** indicate that the stability of the microbial communities in the intestine differs between digesta and mucosa and that mucosa-associated microbial communities are more resistant to dietary modulation than the

digesta-associated microbiota. As explained before, attachment, survival and growth of bacteria in close contact with the intestinal mucosa of the host may depend on bacterial properties, at the same time that it is highly monitored by the local immune cells. Thus, it can be suggested that through those interactions, the mucosa-associated microbiota could have a more important role, at least for the immune system of the host, than the digesta-associated microbiota. Therefore, the bacterial stability and the lower number of bacteria in the mucosa-associated microbiota could be the result of evolutionary host-microbiota selection and adaptation. These could explain the lower richness associated with the mucosa and also the resilience or microbiota stability observed in the mucosa of both our studies. This means that mucosa-associated microbiota may belong to a more select group of commensal bacteria that are both well adapted but also allowed to live in close contact with the host.

Soybean affect functional characteristics of the intestine but may not always significantly affect the immunological response as assessed by gene expression analysis

In our study presented in **Paper III** and **IV**, the inclusion of SBM in one of the diets induced most of the expected effects indicating SBMIE, i.e. effects on histology and molecular markers such as PcnA-immunohistochemistry and increased gene expression of cellular proliferation, typical for an inflammatory condition (Baeverfjord & Krogh, 1996, Bakke-McKellep *et al.*, 2007, Grammes *et al.*, 2013). The parallel repair and new damage induced by the constant ingestion of SBM was clearly represented by the high level of PcnA staining, the loss of functionality of the DI of salmon, and diarrhea. However, the profile expression of the analysed immune genes were not significantly modulated, in contrast to what has been observed most frequently in our earlier studies of the effects of SBM inclusion in salmon diets (Bakke-McKellep *et al.*, 2007, Lilleeng *et al.*, 2009, Marjara *et al.*, 2012, Sahlmann *et al.*, 2013). As discussed before, soybean meals may vary greatly in content of antinutrient due to variation in soybean genetic background as well as in processing conditions. Based on our results showing that soya saponins induce enteritis in a dose-dependent manner (**Paper I**), it may be suggested that the SBM used in the present diet was low in saponins, or that the diet might contain other components, which may have reduced the SBMIE symptoms. On the other hands, it may also be hypothesized that the apparent lack of responses in immune genes was that the DI of these fish

was undergoing a transition to a more chronic stage of the inflammatory process, attempting to repair the damage produced by the SBMWG diet.

Lactic acid bacteria dominate digesta-associated microbiota of fish fed SBM and GM

Studies in mammals have described the changes in the intestinal microbiota related with different enteric diseases, such as inflammatory bowel disease, obesity and colitis (Bäckhed *et al.*, 2004, Frank *et al.*, 2007, Nishikawa *et al.*, 2009). Even if the direct relationship between intestinal disease and modulation of the intestinal microbiota is not well understood, present information suggests that changes in bacterial communities are important factors in several intestinal disorders. The experimental diets in the work presented in **Paper IV**, containing different protein sources from animal and plant origin, caused a modulation in the bacterial communities in the DI digesta compared to that of fish fed the FM diet. Modulation of the microbiota in salmonid digesta caused by incorporation of plant proteins and processed animal proteins (PAP, e.g. poultry meal) other than from fish have been reported previously (Desai *et al.*, 2012, Hartviksen *et al.*, 2014, Revenco *et al.*, 2014). There is, however, no clear link regarding functional and health consequences of this modulation.

Among the fish fed the substitution diets, the group showing the highest effects for all studied parameters, was the fish fed SBMWG (**Paper III** and **IV**). Concomitant with changes in functionality and morphology of the DI, these fish presented the highest abundance of LAB in both the digesta and the mucosa of the DI. Our results are in line with earlier observations in fish fed SBM (Desai *et al.*, 2012, Revenco *et al.*, 2014). Also fish fed the GMWG diet presented increased relative abundance of LAB. The increment in LAB abundance in fish fed these two diets may be the result of their higher amount of non-digestible carbohydrates, several of which are suitable substrates for many LAB. The high abundances of LAB was puzzling, as LAB generally are considered to have positive gut health effects (reviewed by (Pérez-Sánchez *et al.*, 2014). These fish, and in particular those fed the SBMWG diet, showed signs indicating loss of function and cellular stress. Present knowledge does not provide a basis for conclusions regarding possible positive or detrimental effects of LAB on the intestinal health of salmon. The present picture may be interpreted as indicative of unfavorable effects, possibly related to high production

of lactic acid or other metabolites. The possibility exists, however, that without the increase in LAB, the SBMIE-symptoms might have been more severe. The improved SBMIE model, suggested above, would be useful for investigations aiming to clarify the role of LAB for development of SBMIE. Combined with analytical tools such as metagenomics, transcriptomics, metatranscriptomics, metaproteomics and metabolomics, deeper insight into relationships between diet, microbiota and function will be revealed. This kind of knowledge is needed for the understanding and prevention of dietary intestinal health challenges, e.g. those related to the use of new ingredients in fish diets.

MAIN CONCLUSIONS

In salmon:

- Supplementation of a fishmeal based diet with highly purified (95%) soya saponins at levels 2-4 g/kg and above caused SBMIE-like symptoms in the DI, confirming that soya saponins alone can cause enteropathy similar to SBMIE
- Despite eating less, the fish fed the diet with lupin and fishmeal as protein sources supplemented with highly purified (95% purified) soya saponins at levels 2-4 g/kg and above showed similar severity on the SBMIE-like symptoms in the DI than fish fed the fishmeal diets supplemented with soya saponins. The result indicated potentiation of the saponin effects by lupin antinutrients
- The bacterial communities of the intestinal microbiota in fish fed a current commercial diet with a mix of fishmeal and several plant ingredients as protein sources differed significantly between mucosa and digesta, as well as between regions along the intestinal tract. The digesta-associated microbiota was dominated by Firmicutes and Proteobacteria, followed by Fusobacteria, Actinobacteria and Bacteroidetes. Whereas the mucosa-associated microbiota were highly dominated by members of the Proteobacteria, Bacteroidetes and OD1 phyla
- Dietary partial replacement of fishmeal with a mix of SBM-wheat gluten caused significant shifts in the relative abundance in the DI digesta-associated microbiota. The most pronounced effect was a large increase in the relative abundance of LAB. These fish also showed disruption of the normal histomorphology, increment of immature cells, increment of water content in the chyme, alteration in the gene expression indicating cellular stress and decrease water transport, but no effects on immune related genes. Whether there was a link between the SBMIE-like symptoms and the high LAB abundance cannot be concluded based on the present work

- Dietary partial replacement of fishmeal with a mix of guar meal and wheat gluten caused significant shifts in the relative abundance in the DI digesta-associated microbiota. As for the SBM-wheat gluten fed fish, the most pronounced effect was an increase in the relative abundance of LAB in the DI digesta-associated microbiota, although this increment was smaller than for the SBM-wheat gluten fed fish. Minor alterations in expression of some functional genes most likely reflected normal adaptations to dietary changes
- Dietary partial replacement of fishmeal with a mix of SPC and poultry meal caused only minor modulations in abundance of bacterial communities in the DI digesta-associated microbiota. These fish showed increased water content in the DI chyme, but only small alteration in water transport related genes, underlining that also other antinutrients beside saponins, such as fiber and phytates, may affect water content of the chyme in DI
- Dietary partial replacement of fishmeal with poultry meal caused moderate modulations in abundance of bacterial communities in the DI digesta-associated microbiota. Again, the most pronounced effect was increased LAB abundance, although the effect was smaller than for the substitution diets containing SBM and guar meal. These fish showed decreased water content in the DI chyme, but no alteration in water transport-related genes or other analyzed genes. The decrease in chyme water content was most likely due to higher lipid content resulting from low lipid digestibility
- In contrast to the digesta-associated microbiota, the mucosa-associated microbial communities seemed less affected by the partial replacements of fishmeal with the alternative protein sources described above

About the methodology used in this thesis work:

- Despite efforts to predict the functional profile of the intestinal microbiota in the samples using a bioinformatic tool, i.e. PICRUSt, the method and outcome was not considered useful for either of the studies presented in this thesis characterizing the intestinal microbiota of Atlantic salmon

- Improvement in the number of bacterial annotations in current databases used by PICRUSt and other bioinformatic tools predicting the functional profile of bacterial communities are necessary in order to benefit from softwares predicting functional bacterial profile
- The combination of several analytical “omics” are necessary in order to acquire insights about the importance of the intestinal microbiota and its role health and disease in salmon

FUTURE PERSPECTIVES

The present thesis contributes with new knowledge regarding the effect of soya saponins on intestinal health, characterization of salmon microbiota along the intestine and the effect of alternative sources of protein in salmon diets on functionality and microbial community modulation. Nevertheless, the following questions remain unresolved and ought to be addressed in future studies to understand the intestinal host-microbe interaction, the effect of alternative resources in salmon diets and their role in the salmon health:

- Does intestinal microbiota modulation by dietary components affect the intestinal health? If so, which bacteria are responsible for possible negative effects of this modulation?
- What are the functional roles of the different bacterial populations inhabiting the intestine of salmon and which of those bacterial populations present beneficial properties that might help to improve the salmon health?
- What are the functional implications of the differences observed in microbial communities between digesta and mucosa and along the intestine?
- What is the role of digesta and mucosa-associated microbiota in SBMIE
- Is the severity of SBMIE modulated by the presence of LAB in the DI? If so, are LAB beneficial or detrimental for the health of the host?
- How can we modulate the intestinal microbiota of salmon in order to achieve better health and welfare?

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PAPERS I TO IV

Paper I

Soya Saponins Induce Enteritis in Atlantic Salmon (*Salmo salar* L.)

Åshild Krogdahl,^{*,†} Karina Gajardo,[†] Trond M. Kortner,[†] Michael Penn,[‡] Min Gu,[§] Gerd Marit Berge,^{||} and Anne Marie Bakke[†]

[†]Department of Basic Sciences and Aquatic Medicine, School of Veterinary Medicine, Norwegian University of Life Sciences (NMBU), N-0033 Oslo, Norway

[‡]U.S. Fish and Wildlife Service, Lamar, Pennsylvania 16848, United States

[§]Marine College, Shandong University, Weihai 264209, People's Republic of China

^{||}Sunnalsøra Aquaculture Research Station, Nofima, N-6600 Sunndalsøra, Norway

S Supporting Information

ABSTRACT: Soybean meal-induced enteritis (SBMIE) is a well-described condition in the distal intestine of salmonids, and saponins have been implicated as the causal agent. However, the question remains whether saponins alone cause SBMIE. Moreover, the dose–response relationship has not been described. In a 10 week feeding trial with Atlantic salmon, a highly purified (95%) soya saponin preparation was supplemented (0, 2, 4, 6, or 10 g/kg) to two basal diets, one containing fishmeal as the major protein source (FM) and the other 25% lupin meal (LP). Saponins caused dose-dependent increases in the severity of inflammation independent of the basal diet, with concomitant alterations in digestive functions and immunological marker expression. Thus, saponins induced inflammation whether the diet contained other legume components or not. However, responses were often the same or stronger in fish fed the corresponding saponin-supplemented LP diets despite lower saponin exposure, suggesting potentiation by other legume component(s).

KEYWORDS: inflammation, intestine, soybean, lupin meal, fishmeal

■ INTRODUCTION

To fulfill the increase in demand for cultivated fish and at the same time to improve sustainability of the industry, new nutrient sources are sought with increasing intensity to reduce reliance on fishmeal. In this context, several high-protein plant ingredients are considered suitable candidates for use in aquafeeds.¹ Among these are various legumes, often highly processed to produce high-protein concentrates and reduce the concentration of antinutritional factors (ANFs). Wheat and maize gluten, rapeseed, sunflower, and lupin meals are also used. Soybean meal (SBM), the most important high-protein source used in production of animal feeds on the world market, has also been investigated as a feed ingredient for salmonids. However, the use of full-fat and solvent-extracted SBM in salmonid feeds is limited due to negative effects on growth performance^{2–5} and intestinal health, causing severe inflammation in the distal intestine (DI).^{3,4,6–13} The condition, often called SBM-induced enteropathy (SBMIE), is described as a noninfectious, subacute enteritis, characterized by shortening of the mucosal folds, infiltration of the lamina propria by various inflammatory cells, and decreased numbers of absorptive vacuoles in the enterocytes.^{6,8,10,13}

The exact cause and mechanisms behind the inflammation and other negative effects are not fully understood. However, several investigations indicate that alcohol-soluble ANFs,^{3,7,14} especially soya saponins,^{15–20} are potential causatory factors. Present knowledge does not allow firm conclusions on whether saponins are solely responsible or if additional components also play a role.^{16,18,19} Saponins occur naturally in legumes and other seed crops such as soy, pea, sunflower, and lupin.^{18,21}

They are heat-stable, triterpenoid or steroid, amphipathic glycosides.²¹ The amphiphilic property provides saponins the ability to bind and form nonabsorbable complexes with cholesterol.^{22,23} In mammals, saponins apparently have the ability to bind to membrane cholesterol of intestinal epithelial cells and thus form holes and alter membrane permeability, possibly facilitating the uptake of molecules, including antigens and potential toxins, that normally are not absorbed by the enterocytes.²⁴ Saponins have also been suggested to interfere with digestion of lipids and proteins,²⁵ and have a hypocholesterolemic effect in several animal species.^{23,26,27} At low dietary levels, saponins have immune-stimulating effects, and their use as adjuvants in vaccines is well documented.^{28–32} They may also act as antifungal, antiviral, and anticancer agents (reviewed by Sparg et al.²⁸). In line with this, at least low levels of saponins appear to improve growth in some mammals²⁵ and fish.³³

Analyses of SBM have shown saponin levels in a range of 5–7 g/kg.^{34,35} In contrast, lupin seeds contain 0.4–0.7 g/kg,^{36,37} levels that are insufficient to induce pathological changes in the DI of salmon at inclusion levels up to 30% in the diet.³⁸ However, inaccuracy and imprecision of the available assay for saponin analysis in legumes, foods, and feeds is high,³⁹ which causes uncertainties regarding the levels and types of saponins present. As for SBM, lupin seeds can also contain other ANFs

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Table 1. Formulation of the Experimental Diets^a

	FM-0	FM-2	FM-4	FM-6	FM-10	LP-0	LP-2	LP-4	LP-6	LP-10
Ingredients (g/kg)										
fishmeal ^b	587	587	587	587	587	235	235	235	235	235
lupin meal ^c						200	200	200	200	200
wheat gluten ^d						190	190	190	190	190
wheat meal	156	156	156	156	156	67	67	67	67	67
soya saponins ^e	0	2	4	6	10	0	2	4	6	10
wheat starch	10	8	6	4	0	10	8	6	4	0
fish oil ^f	221	221	221	221	221	242	242	242	242	242
vitamin mix ^g	20	20	20	20	20	20	20	20	20	20
mineral mix ^g	5	5	5	5	5	5	5	5	5	5
Ca(H ₂ PO ₄) ₂ ·2H ₂ O						10	10	10	10	10
carophyll pink	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
yttrium oxide	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
lysine hydrochloride						17	17	17	17	17
DL-methionine						3.6	3.6	3.6	3.6	3.6
Chemical Composition (%)										
crude protein	42.3	42.3	42.3	42.3	42.3	44.1	44.1	44.1	44.1	44.1
crude lipid	29.3	29.3	29.3	29.3	29.3	30.3	30.3	30.3	30.3	30.3
starch	10.3	10.3	10.3	10.3	10.3	7.5	7.5	7.5	7.5	7.5
gross energy (MJ/kg)	23.9	23.9	23.9	23.9	23.9	24.2	24.2	24.2	24.2	24.2

^aAbbreviations: FM, fishmeal diet; LP, lupin meal diet; 0, 2, 4, 6, 10, levels of soya saponin inclusion in diets (g/kg). ^bFishmeal 122/11, LTQ fishmeal, produced by Welcon AS, Norway. ^cSupplied by Holterman AS, Norway. ^dWheat gluten 159/10, Amytex 100, produced by Syral, Belgium. ^eA 95% concentrate, supplied by Organic Technologies, United States. ^fFish oil OS/10, NorSalmOil, produced by Norsildmel, Norway. ^gSupplemented to meet requirements.

such as lectins, protease inhibitors, oligosaccharides, phytate, alkaloids, and cyanogenic glycosides.^{40,41} Despite the presence of these ANFs, lupin meals are considered highly digestible and good protein sources for salmonids.^{42,43}

The purpose of this study was to investigate whether soya saponins alone may affect performance and induce enteritis in Atlantic salmon in the absence of other legume components in the diet, and to disclose the dietary threshold level. The recent introduction of a more purified soya saponin product (95% purity) allows for more conclusive data regarding this line of query. To assess threshold levels when other legume components are present, saponins were also supplemented to a legume-meal-containing diet. To this end, a dose–response design was employed using two basal diets without and with the presence of lupin meal, a diet ingredient that has previously been suggested to potentiate the effect of soya saponins.¹⁶

MATERIALS AND METHODS

Animal Husbandry. The experiment was conducted in compliance with laws regulating the experimentation with live animals in Norway as overseen by the Norwegian Animal Research Authority (FDU). The feeding trial was performed at Nofima's Aquaculture Research Station at Sunndalsøra, Norway. For each diet, duplicate, mixed-gender groups of 22 Atlantic salmon postsmolts of the Sunndalsøra strain with an initial mean body weight of 442 g (33 g SEM) were randomly distributed into 20 1 m³ fiberglass tanks containing 250 L seawater replaced at a rate of 20 L/min. The temperature during the feeding trial decreased from 12 to 9 °C due to seasonal variation and affected all tanks equally. Oxygen saturation was above 85% throughout the experiment, whereas salinity varied between 32 and 33 g/L. Fish were fed continuously by automatic disk feeders. The economy and available facilities did not allow quantification of feed waste, but qualitative observations of appetite and waste accumulation in the tanks were made throughout the experiment. As the role of saponins in development of enteritis was the main goal of the study, this was considered an acceptable condition for

the purpose of our study. A regimen of 24 h lighting was employed during the experimental period.

Diets. Diet formulations and their chemical compositions are shown in Table 1. Two basal diets were formulated, differing regarding protein sources. One was made with fishmeal supplying about 95% of the protein (FM). In the other, lupin meal and wheat gluten supplied about 25% and 35% of the protein, respectively, with fishmeal the remaining source (LP). Batches of each basal diet were subsequently supplemented with 0, 2, 4, 6, and 10 g/kg purified soya saponins (certified to be 95% pure, obtained from Organic Technologies, Coshocton, OH), replacing wheat starch. Accordingly, the diets were termed FM-0 and LP-0, FM-2 and LP-2, FM-4 and LP-4, FM-6 and LP-6, and FM-10 and LP-10. The basal diets were formulated to be isoenergetic (24 MJ GE/kg; GE = gross energy), and all diets were supplemented to fulfill the fishes' requirements for lysine, methionine, vitamins, and minerals. Fish oil and wheat meal were added as lipid and carbohydrate sources, respectively, to balance the nutrient composition of the diets. The fish were fed the experimental diets for 10 weeks.

Sampling. At the termination of the experiment, 10 randomly selected fish from each tank were sampled. All fish were fully anesthetized with tricaine methanesulfonate (MS222, Argent Chemical Laboratories) and subsequently euthanized by cervical dislocation prior to tissue sampling. Only fish with digesta throughout the intestinal tract were sampled to ensure intestinal exposure to the diets. Body weights and lengths were recorded for all fish. From five anesthetized fish per tank, blood was collected in heparinized vacutainers for plasma preparation. The abdominal cavities of all 10 euthanized fish per tank were opened, and the intestine was removed and cleared of any mesenteric and adipose tissue. Content from the proximal and distal halves of the proximal intestine (PI1 and PI2, respectively), the midintestine (MI), and the proximal and distal halves of the DI (DI1 and DI2, respectively) was collected into separate, preweighed tubes, frozen in liquid N₂, and then stored at –80 °C until further preparation and analyses could be conducted. The empty stomach, intestinal segments (PI, MI, and DI), liver, spleen, head kidney, and gall bladder were weighed. DI tissue samples for the histological evaluation were taken from six of the ten sampled fish per

tank, placed in 4% phosphate-buffered formaldehyde solution for 24 h, and subsequently stored in 70% ethanol until further processing. For mRNA extraction, DI samples from three of the six fish sampled for histology were placed in RNAlater (Ambion, Carlsbad, CA) at 4 °C for 24 h and subsequently stored at -20 °C. DI samples from the same three fish were taken for Western blots. These were frozen in liquid N₂ and then stored at -80 °C. For brush border membrane enzyme activity assessment, PI, MI, and the remaining DI tissues from all 10 fish per tank were frozen in liquid N₂ and stored at -80 °C.

Diet and Digesta Analyses. Feed and feces samples (from DI2) were analyzed for dry matter (EU 71/393), Kjeldahl nitrogen (N) (EU 93/28), lipid (HCl hydrolysis and diethyl ether extraction (EU 98/64)), and starch (AOAC enzymatic method 996.11). Gross energy was measured by bomb calorimetry (Parr 1271 bomb calorimeter, Parr, Moline, IL). Amino acid (except tryptophan) analysis of all samples was performed with a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, U.K.) after hydrolysis, according to EC Commission Directive 98/64/EC. Tryptophan was analyzed on a Dionex Summit HPLC system, with a Shimadzu RF-535 fluorescence detector. Fatty acid composition of the feeds was determined by use of GC (gas chromatography) as previously described.⁴⁴ Yttrium oxide concentration in feed and feces was determined by inductively coupled plasma mass spectroscopy (ICPMS) as previously described by Refstie et al.⁴⁵

Plasma Cholesterol, Total Bile Acids, and Other Metabolites. Analyses of plasma levels of cholesterol, total bile acids (conjugated and nonconjugated bile acids), total protein, glucose, triglycerides, free fatty acids, sodium, and phosphorus were performed by the Central Laboratory of the NMBU School of Veterinary Medicine (Oslo, Norway) according to standard protocols (Advia 1800, Siemens Healthcare Diagnostics, Erlangen, Germany).

Trypsin Activity and Total Bile Acids in the Digesta. Trypsin activity was determined colorimetrically as described by Kakade et al.⁴⁶ using the substrate benzoylarginine *p*-nitroanilide (Sigma no. B-4875, Sigma Chemical Co., St. Louis, MO) and a curve derived from standardized bovine trypsin solution. Total bile acid concentrations were determined using the Enzabile test kit (catalog no. 550101, BioStat Diagnostic Systems, Cheshire, U.K.) and a curve derived from standardized taurocholic acid solution.

Brush Border Membrane Enzyme Activity. The activity of the brush border membrane enzyme leucine aminopeptidase (LAP) was measured in PI and DI tissue homogenates diluted in ice cold Tris-mannitol buffer (1:20, w/v) containing 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc SC, Pentapharm Ltd., Basel, Switzerland) as a serine proteinase inhibitor. The activity was determined colorimetrically using the substrate L-leucine β-naphthylamide hydrochloride as described by Kroghdal et al.⁹ LAP specific activity was calculated following tissue protein concentration analysis using the BioRad protein assay (BioRad Laboratories, Munich, Germany).

Histology. Fixed DI tissue samples were processed according to standard histological techniques and stained with hematoxylin and eosin (H&E). Examination was conducted blindly with a light microscope using a continuous scoring scale from 0 to 10 as described elsewhere.⁴⁷ The following histological characteristics were evaluated: length and fusion of mucosal folds, cellular infiltration and width of the lamina propria and submucosa, enterocyte vacuolization, nucleus position within the enterocytes, and the relative number of goblet cells.

Immunohistochemistry. Detection of proliferating cell nuclear antigen (Pcna) in fixed sections of DI tissue was performed by immunohistochemistry. Sections were deparaffinized using xylene and subsequently rehydrated. Nonspecific antibody binding was reduced by incubating the sections for 20 min with 5% bovine serum albumin in Tris-buffered saline (BSA/TBS) containing normal horse serum diluted 1:50. This was followed by overnight incubation at 4 °C with the primary antibody (mouse monoclonal anti-Pcna, M0879, Dako Norge, Oslo, Norway) diluted 1:200 in 1% BSA/TBS. After being rinsed in PBS, sections were incubated with biotinylated horse antimouse secondary antibody at 1:200 in 1% BSA/TBS for 30 min. A Vectastain ABC-PO (mouse IgG) kit was used to visualize

immunoreactivity. Sections were counterstained with Mayer's hematoxylin. Staining was evaluated by measuring the Pcna staining height as described elsewhere.¹¹

Western Blot. Quantification of Pcna and fatty acid binding protein 2 (Fabp2) levels was performed in DI tissue samples from three fish from the control groups (FM-0 and LP-0) and from the 10 g/kg saponin groups (FM-10 and LP-10). Samples of 40 μg of protein were prepared, separated by SDS-PAGE, and blotted onto nitrocellulose membranes as previously described.⁴⁸ Membranes were incubated overnight at 4 °C with primary antibodies for the two target proteins: mouse monoclonal anti-Pcna (1:2000) and goat polyclonal anti-Fabp2 (1:4000, NB100-59746, Novus Biologicals, Littleton, CO). Primary antibodies for candidate reference proteins included rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (Gapdh; 1:250, PA1-987, Thermo Scientific, Waltham, MA) and rabbit polyclonal anti-actin (1:500, A5060, Sigma-Aldrich, St. Louis, MO). All antibodies were previously validated for use in Atlantic salmon.^{48,49} After subsequent incubations with alkaline phosphatase-conjugated secondary antibodies, target and reference proteins were visualized with fluorescence by the ECF Western blotting reagent pack (GE Healthcare) and Typhoon 9200 imager system (Amersham Biosciences). ImageQuant software (Amersham Biosciences) was applied to quantify the band intensities. Both Gapdh and actin expression levels showed significant variation between diet groups and were therefore not suitable as reference proteins in the present study. Instead, total membrane protein content was visualized using a MemCode protein stain kit (Thermo Scientific) and used as a qualitative loading control.

Quantitative Real Time PCR (qPCR). RNA purification and quality control, DNase treatment, cDNA synthesis, and qPCR assays were performed as described by Kortner et al.⁵⁰ A selection of previously proposed marker genes for gut health and metabolism was profiled in DI tissue samples. Primer details are shown in the Supporting Information (Table SI 1). *gapdh*, *rnapolIII* (RNA polymerase II), and *hprt1* (hypoxanthine phosphoribosyltransferase 1) were evaluated for use as reference genes as described by Kortner et al.⁵³ The geometric average expression of *gapdh* and *rnapolIII* was used as the normalization factor. Mean normalized expression of the target genes was calculated from raw Cq values by relative quantification.⁵¹

Calculations. The specific growth rate (SGR) was calculated using the tank means for initial body weight (IBW) and final body weight (FBW) and calculated as follows:

$$\text{SGR} = [(\ln \text{FBW} - \ln \text{IBW}) / \text{number of days}] \times 100$$

The organosomatic index (OSI) was calculated as follows:

$$\text{OSI} (\%) = [\text{organ weight (g)} / \text{body weight (g)}] \times 100$$

The apparent digestibility (AD) was estimated indirectly using Y₂O₃ and calculated as follows:

$$\text{AD} = 100 - 100(M_{\text{feed}}/M_{\text{feces}})(N_{\text{feed}}/N_{\text{feces}})$$

where M_{feed} and M_{feces} are the concentrations (%) of marker (Y₂O₃) in the feed and feces and N_{feed} and N_{feces} are the concentrations (%) of a nutrient in the feed and feces.

The Pcna-positive proliferative compartment length (PCL) was corrected for body length (PCL_{BL}):

$$\text{PCL}_{\text{BL}} (\mu\text{m}/\text{m}) = [\text{PCL} (\mu\text{m}) / \text{body length (cm)}] \times 100$$

Statistical Analysis. Data were tested for normality and variance homogeneity using the Shapiro-Wilk *W* goodness of fit test and the Bartlett test, respectively. When necessary, data were transformed to achieve normal distribution (indicated by a superscript "†" in Table 10). Effects of the inclusion of levels of soya saponins were evaluated using regression analysis (GLM). The results were fit to polynomial models of first, second, and third degrees. The model considered to fit the results best on the basis of visual examination and the observed *r*² is reported. Comparison of basal diet effects was performed employing two-way analysis of variance (ANOVA) with the basal diet and saponin level as class variables. When interaction effects were

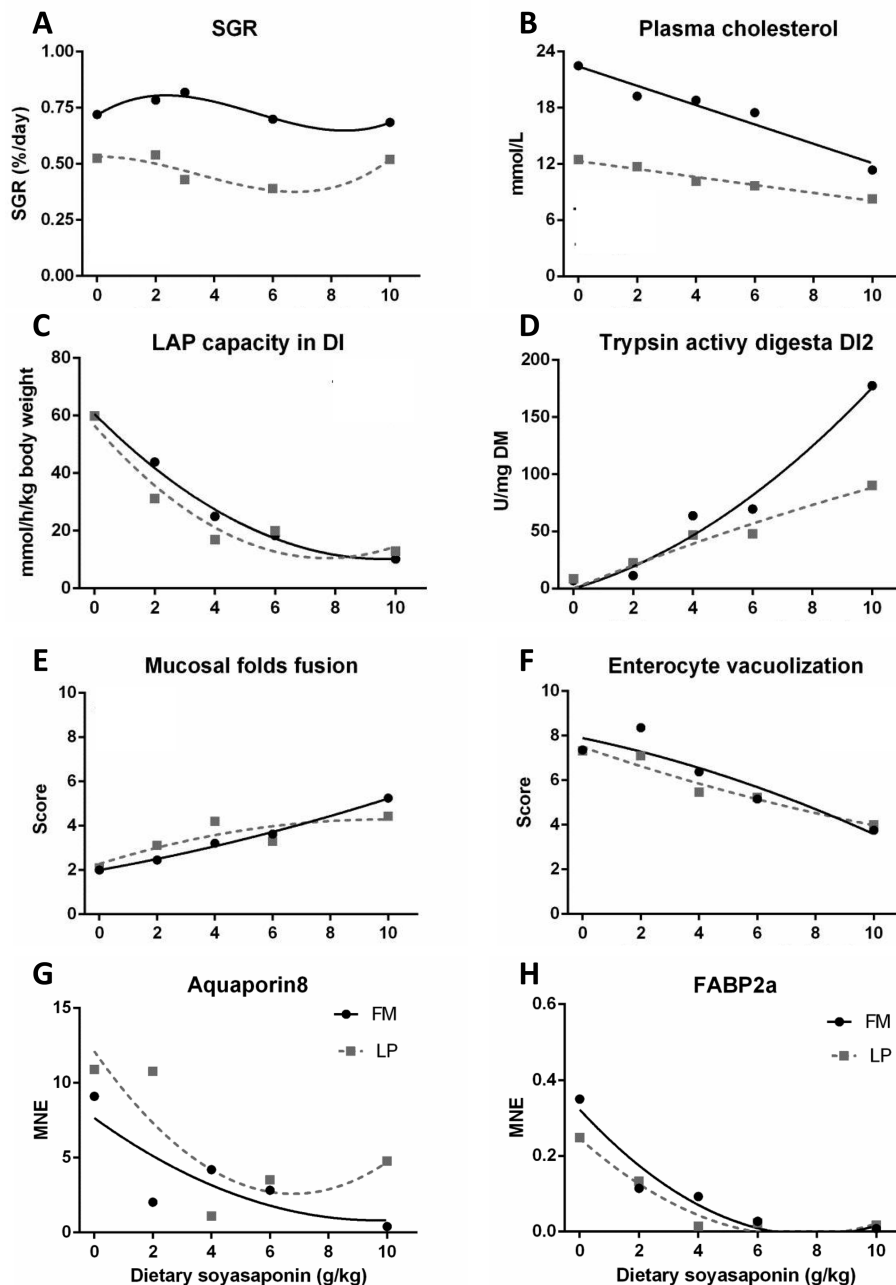


Figure 1. Illustration of relationships between level of dietary saponin supplementation (g/kg) and (A) specific growth rate (SGR; % per day), (B) plasma cholesterol level (mmol/L), (C) total capacity of leucine aminopeptidase (LAP) activity in the distal intestine (DI; mmol/h/kg of body weight), (D) trypsin activity of chyme from the DI (U/mg dry matter [DM]), (E) mucosal fold fusion in the distal intestine, (F) enterocyte vacuolization in the DI (score), (G) mean normalized expression (MNE) of *aqp8* in DI tissue, and (H) MNE of *fabp2a1* in DI tissue. The curves illustrate the regression that fits the results best for fish fed the fishmeal diet (FM; solid lines) and the diet with lupin meal (LP; dashed lines) according to the regressions shown in Tables 2, 4, and 7.

significant, one-way ANOVA and the posthoc Tukey–Kramer HSD test were performed to aid in the interpretation of interactions.

Since histology and some qPCR data did not fulfill the requirement of normal distribution, the analysis was performed using the Wilcoxon/Kruskal–Wallis test followed by the posthoc Steel–Dwass method to compare the means. Statistical analysis was performed using JMP statistical software (version 10, SAS Institute, United States), and graphs were made using GraphPad Prism, version 6.0 (GraphPad Software Inc., 2013). The level of significance for all analyses was set at $P < 0.05$. Results showing P values between 0.05 and 0.10 are discussed as results showing clear trends.

RESULTS

The presentation of the results is organized as follows: The response variables are grouped in subsections on the basis of their relationship regarding localization, function, and method of analysis. Within each subsection, the evaluation by regression analyses of saponin supplementation within the two basal diets is given first, followed by a comparison of the two basal diets based on the results of the two-way ANOVA. To allow interpretation of significant interaction effects, means and statistics resulting from a one-way ANOVA are also provided in the respective tables.

Table 2. Results of Regression Analysis of Increasing Doses of Soya Saponins on Specific Growth Rate (SGR), Nutrient Apparent Digestibility (AD), and Organosomatic Index (OSI) Data from Atlantic Salmon Fed the Experimental Diets^a

	FM						LP					
	P (model)	R ²	intercept	X	X ²	X ³	P (model)	R ²	intercept	X	X ²	X ³
SGR	0.0277	0.76	0.71	0.087	-0.027	0.001	0.1137	0.60	0.529	0.026	-0.017	0.001
crude protein AD	0.0098	0.73	84.91	0.3201	-0.0652		0.9048	0.03	91.20	0.0288	-0.0053	
lipid AD	0.0040	0.79	95.29	-0.6398	0.0297		0.5167	0.17	96.38	0.1492	-0.021	
starch AD	0.4718	0.32	69.182	-2.093	0.624	-0.041	0.1261	0.58	66.771	1.900	-0.325	0.017
OSI stomach	0.5043	0.30	0.456	0.011	-0.003	0.0002	0.7432	0.17	0.41	0.007	-0.001	0.00006
OSI PI	0.1616	0.55	1.570	0.0649	-0.009	0.0004	0.1037	0.62	2.05	-0.192	0.0576	-0.0038
OSI MI	0.9587	0.05	0.23	0.0017	-0.0006	0.00005	0.1869	0.52	0.21	-0.029	0.0079	-0.0005
OSI DI	0.0002	0.84	0.48	-0.013			0.0143	0.54	0.45	-0.010		
OSI liver	0.0124	0.56	1.14	-0.019			0.2309	0.17	1.26	-0.008		
OSI spleen	0.2813	0.44	0.04	0.003	-0.0013	0.0001	0.4809	0.31	0.060	-0.004	0.0009	-0.00005
OSI head kidney	0.708	0.19	0.10	-0.0067	0.0013	-0.00006	0.7918	0.14	0.11	0.00407	-0.0013	0.00009
OSI gall bladder	0.0054	0.77	0.02	-0.0018	0.0001		0.8408	0.04	0.02	-0.0003	0.00004	

^aAbbreviations: FM, fishmeal diet; LP, lupin meal diet.

Feed Acceptance and Growth Performance. During the course of the feeding trial, differences in feed acceptance of the basal diets regardless of saponin supplementation were observed visually. More feed waste was observed in the tanks of fish fed the LP diets compared to the FM diets, and this was reflected in differences in specific growth rates (Figure 1A). The regression analysis (Table 2) showed a significant cubic (third-degree) relationship between growth and soya saponin level for fish fed the FM basal diet, indicating a stimulating effect at the lower inclusion levels. Growth was similar in fish fed the FM-0 and FM-10 diets. The results did not indicate any negative effect of saponin inclusion within the investigated range of saponin inclusion. For LP-fed fish, the relationship between growth and saponin level was less clear and not significant, and no indication of a stimulating effect at low levels was observed. For fish fed the LP-0 diet, SGR was 0.53, which was 34% less than for fish fed the FM-0 diet with an SGR of 0.72 ($P < 0.0001$).

Apparent Nutrient Digestibilities (See Tables 2 and 3 and Supporting Information Tables SI 2–5). Regression analyses revealed a significant inverse relationship between the dietary saponin level and AD of crude protein and lipid, which followed a second-degree relationship; i.e., the effect became more pronounced at higher levels. The same picture was seen for amino acid ADs. For about half of the amino acids, the results fit a first-degree relationship best, while for the remainder a second-degree relationship was best. However, for crude protein and all amino acids, the effect depended on the basal diet and was clearly significant only for fish fed the FM diet. The LP diet showed significantly and substantially higher ADs for crude protein and all amino acids at all saponin supplementation levels compared to the respective FM diets. For the FM-0 and LP-0 diets the difference in AD of protein was 6%. The apparent digestibility of taurine also decreased with increasing saponin level, but again the effect was only seen in fish fed the FM basal diet. Significantly lower values were observed for fish fed the LP basal diet than those fed the FM diet. For crude lipid AD, an increasing saponin level generally decreased digestibility, but a clear effect was seen only for fish fed the FM diets. ADs of the individual fatty acids showed the same picture. In fish fed the LP diets, negative effects of saponins were observed for C16:0, with a similar trend for C18:0. Fish fed the LP diet generally showed the highest values

Table 3. Soya Saponin and Basal Diet Effects on the Apparent Digestibility (%) of Macronutrients in Atlantic Salmon Fed the Experimental Diets^a

	crude protein	lipid	starch
Two-Way ANOVA Model			
P (model)	<0.0001	<0.0001	0.205
pooled SEM	0.55	0.46	1.28
P Values from Two-Way ANOVA			
saponin level	0.015	0.001	0.061
basal diet	<0.0001	<0.0001	0.473
interaction	0.026	0.021	0.476
Means of One-Way ANOVA			
FM-0	84.9 b	94.9 c	69
FM-2	85.4 b	94.8 c	68
FM-4	84.3 b	93.0 d	67
FM-6	85.1 b	92.1 d	70
FM-10	81.4 c	91.9 d	69
LP-0	91.0 a	96.0 abc	66
LP-2	91.8 a	97.0 ab	69
LP-4	90.4 a	97.2 a	71
LP-6	91.5 a	95.6 bc	69
LP-10	90.9 a	95.9 abc	70

^aAbbreviations: FM, fishmeal diet; LP, lupin meal diet; 0, 2, 4, 6, 10, levels of soya saponin inclusion in diets (g/kg). Mean values with different online letters a, b, c, and d within a column are significantly different ($P < 0.05$).

for crude lipid and fatty acid ADs. ANOVA showed a significant interaction between basal diet and saponin level. For the FM-0 and LP-0 diets, the difference was 1%, whereas for FM-10 and LP-10 diets, the difference was 5%. No significant effect was observed for the AD of starch, either for saponin level or for basal diet. On the basis of the observed ADs of protein, lipid, and starch for diets FM-0 and LP-0, digestible energy values for the basal diets were estimated to be 20.7 and 21.8 MJ/kg, respectively. The values 23.6, 30.5, and 17.3 kJ/g were used, respectively, for the GE of protein ($N \times 6.25$), lipid, and starch.

Organosomatic Indices (See Table 2 and Supporting Information Table SI 6). Increasing soya saponin levels did not affect the OSI of the stomach, PI, or MI in fish fed either basal diet. The basal diet composition, on the other hand, affected these indices significantly. Fish fed the LP diet had a

Table 4. Results of Regression Analysis of Increasing Doses of Soya Saponins on Plasma Variables, Intestinal Mucosal Enzyme Activities, and Digesta Bile Acid Concentration and Trypsin Activity Data from Atlantic Salmon Fed the Experimental Diets^a

	FM						LP					
	P (model)	R ²	intercept	X	X ²	X ³	P (model)	R ²	intercept	X	X ²	X ³
plasma total protein	0.090	0.31	43.68	-0.21			0.056	0.38	43.14	-0.55		
plasma total bile acids	0.009	0.59	24.80	-1.82			0.026	0.47	11.72	-0.66		
plasma cholesterol	<0.0001	0.91	22.40	-1.03			0.003	0.68	12.31	-0.42		
plasma triacylglycerides	0.572	0.14	3.242	0.387	-0.034		0.646	0.11	4.25	-0.09	0.002	
plasma free fatty acids	0.705	0.01	0.36	0.005			0.460	0.06	0.37	-0.003		
plasma glucose	0.256	0.46	5.723	-0.728	0.168	-0.01	0.383	0.37	4.624	0.377	-0.105	0.007
plasma phosphorus	0.860	0.11	3.996	0.090	-0.017	0.001	0.705	0.19	4.031	-0.114	0.042	-0.003
plasma sodium	0.352	0.25	164.06	-0.541	0.060		0.478	0.19	162.31	0.163	-0.051	
LAP PI capacity	0.079	0.33	209.93	2.88			0.0105	0.57	194.12	4.803		
LAP DI capacity	<0.0001	0.95	60.53	-10.468	0.543		0.0002	0.91	56.524	-11.955	0.775	
LAP PI activity	0.3201	0.41	431.68	39.131	-12.396	0.89	0.7608	0.16	361.83	16.683	-2.929	0.152
LAP DI activity	0.0002	0.84	352.8	-26.44			0.0013	0.74	345.22	-25.86		
TBA digesta-PI1	0.4588	0.33	279.14	136.22	-25.99	1.230	0.0104	0.82	533.30	-14.37	-4.266	0.455
TBA digesta-PI2	0.0048	0.78	382.99	-18.63	0.127		0.0974	0.48	366.40	-16.350	1.06	
TBA digesta-MI	0.0152	0.69	491.98	-12.825	-0.092		0.0737	0.52	461.65	-23.32	1.626	
TBA digesta-DI1	0.0041	0.66	257.83	-9.41			0.0597	0.37	234.37	-7.433		
TBA digesta-DI2	0.4163	0.22	71.055	-0.870	0.303		0.9159	0.02	69.184	0.587	-0.106	
trypsin digesta-PI1	0.2109	0.36	258.48	3.936	-0.970		0.4617	0.20	285.47	-14.567	1.066	
trypsin digesta-PI2	0.4348	0.34	160.33	-30.56	8.409	-0.576	0.2166	0.50	165.08	9.472	-3.577	0.233
trypsin digesta-MI	0.1499	0.42	108.89	-4.410	0.692		0.1898	0.38	107.81	-3.171	0.119	
trypsin digesta-DI1	0.0001	0.85	51.526	12.54			0.0384	0.43	61.315	3.690		
trypsin digesta-DI2	0.0001	0.92	5.144	5.767	1.13		0.0014	0.84	9.409	6.977	0.096	

^aAbbreviations: FM, fishmeal diet; LP, lupin meal diet; LAP capacity, leucine aminopeptidase capacity in PI or DI; LAP activity, leucine aminopeptidase activity in PI or DI; PI, proximal intestine; DI, distal intestine; TBA digesta, total bile acid in the intestinal content in PI1, PI2, MI, DI1, or DI2; PI1, proximal section of proximal intestine; PI2, distal section of proximal intestine; MI, midintestine; DI1, proximal section of distal intestine; DI2, distal section of distal intestine; trypsin digesta, trypsin activity in the intestinal content in PI1, PI2, MI, DI1, or DI2; X, soya saponin level.

lower OSI of the stomach and MI and a higher OSI of the PI than fish fed the FM basal diet. For the DI, on the other hand, the basal diets did not affect the OSI differently, but for both, a clear negative effect of increasing soya saponin level was observed and the relationship fit a first-degree function best. The liver OSI decreased significantly for the FM fish with increasing soya saponin level, also fitting a first-degree function best. For the LP-fed fish, the relationship was not significant. The two-way ANOVA model did not show significant effects. The saponin level affected the gall bladder OSI significantly in fish fed the FM basal diet only, following a decreasing, second-degree relationship best, with a diminishing effect with increasing level. According to the results of the two-way ANOVA, there was no clear difference between the two basal diets in gall bladder OSI. Neither the spleen nor head kidney indices showed a significant relationship to soya saponin level or basal diet.

Plasma Values (see Table 4, Figure 1B, and Supporting Information Table SI 7). Increasing levels of soya saponins reduced plasma cholesterol following a first-order relationship. The magnitude of the effect, however, depended significantly on the basal diet. Fish fed the LP diet generally showed cholesterol levels 50% lower than those of fish fed the FM diet. Likewise and possibly related to the lower plasma values in general, the effect of saponin dose was less pronounced in fish fed the LP diet than fish fed the FM diet. Fish fed the FM-10 diet showed a reduction in plasma cholesterol of about 50% compared to those fed FM-0. For the LP fish the reduction was 23%. Plasma total bile acid

concentrations showed a trend similar to that of the cholesterol results, but with somewhat greater variation. The effect depended on the basal diet. For fish fed the LP-0 diet, the total bile acid concentration was 35% lower than that for fish fed the FM-0 diet. The difference between fish fed the FM-0 and FM-10 diets was about 65%, whereas the decrease was 61% in fish fed the respective LP diets. The other plasma variables showed no significant effects either of soya saponin level or of basal diet.

Brush Border Membrane Leucine Aminopeptidase Activity (See Tables 4 and 5 and Figure 1C). Activities are presented as total capacity (mM/kg body weight) and specific activity (mM/mg of protein). In the PI, increasing levels of soya saponin inclusion significantly increased the total LAP capacity. The regression showed a significant first-order relationship for fish fed the LP diets, with the same trend for the FM-fed fish. In DI, the relationship was opposite and significant for both basal diets, fitting a second-degree relationship best with diminishing reductions with increasing saponin level. The specific activity was also affected by the saponin level, but only in the DI, fitting a first-order function best. Basal diet effects were also only observed on specific activity in the DI, whereas LAP capacity was not affected in any of the intestinal segments.

Total Bile Acid Concentration and Trypsin Activity in Intestinal Content (See Tables 4 and 6 and Figure 1D). Results reveal that increasing saponin levels decreased the chyme total bile acid concentration significantly in PI2, MI, and DI1 significantly for fish fed the FM diet, while a similar trend

Table 5. Soya Saponin and Basal Diet Effects on Leucine Aminopeptidase Total Capacity and Specific Activity in Intestinal Tissue Homogenate from the Proximal (PI) and Distal (DI) Intestine of Atlantic Salmon Fed the Experimental Diets^a

	total capacity (mmol/h/kgfishweight)		specific activity (mmol/h/mg of tissue protein)	
	PI	DI	PI	DI
Two-Way ANOVA Model				
<i>P</i> (model)	0.009	<0.0001	0.005	<0.0001
pooled SEM	11.5	2.9	22.2	28.7
<i>P</i> Values from Two-Way ANOVA				
soya saponin level	0.001	<0.0001	0.354	<0.0001
basal diet	0.309	0.450	0.0007	0.828
interaction	0.361	0.493	0.999	0.931
Means of One-Way ANOVA				
FM-0	209	59 a	436	324 abc
FM-2	210	43 ab	449	338 ab
FM-4	236	24 cd	470	267 abcd
FM-6	217	18 cd	401	156 de
FM-10	239	10 d	477	95 e
LP-0	194	59 a	365	398 a
LP-2	200	31 bc	370	271 abcd
LP-4	224	16 cd	410	174 cde
LP-6	210	19 cd	379	201 bcde
LP-10	245	12 d	389	111 de

^aAbbreviations: FM, fishmeal diet; LP, lupin meal diet; 0, 2, 4, 6, 10, levels of soya saponin inclusion in diets (g/kg); BW, body weight. Mean values with different online letters a, b, c, d, and e within a column are significantly different ($P < 0.05$).

was observed for the LP-fed fish. In PI2, the difference between the two basal diets was significant (significant interaction), with a more pronounced effect for fish fed the FM diet. For trypsin

activity, significant effects of saponin level were observed only for the two most distal segments, DI1 and DI2, in which increasing dietary soya saponin levels increased the activity of trypsin markedly. The magnitude of the effect depended on the basal diet, with a greater effect for fish fed the FM diet. In DI1 the relationship followed a first-degree function, whereas in DI2 the effect seemed to accelerate with increasing saponin level; i.e., it fit a second-degree function better.

Morphology of the Distal Intestine (See Tables 7–9 and Figures 1E,F and 2A–C). The soya saponin level affected all the characteristics assessed and showed alterations typical for mucosal inflammation. The severity increased with increasing level of soya saponin inclusion. The effects seemed independent of the basal diet composition. The results showed decreased height and increased fusion of the mucosal folds, increased width and cellular (leucocyte) infiltration of the lamina propria and submucosa, reduced numbers of supra-nuclear absorptive vacuoles in enterocytes, displaced nuclei toward the apexes of the cells, and increased frequency of goblet cells in the mucosa. For all these characteristics, regression followed a second-degree relationship best, with increasing effect with increasing saponin level.

Immunohistochemical Examination of the Distal Intestine (See Table 9 and Figure 2D–F). Results of the PcnA staining indicate that increases in saponin levels significantly increased the height of the staining in the mucosal folds, regardless of the basal diet. The regression analyses showed that a second-degree function fit the results best. The height of PcnA staining did not differ for the two basal diets.

Western Blot (See Figure 3). PcnA protein levels in the DI were increased 2-fold by the 10 g/kg saponin dose, but the effect was only observed for fish fed the FM diet. In contrast, Fabp2a protein levels were significantly reduced and again only for fish fed the FM diet.

Table 6. Soya Saponin and Basal Diet Effects on Total Bile Acid Concentration and Trypsin Activity in the Digesta of Atlantic Salmon Fed the Experimental Diets^a

	total bile acids (mg/g DM)					trypsin (U/mg DM)				
	PI1	PI2	MI	DI1	DI2	PI1	PI2	MI	DI1	DI2
Two-Way ANOVA Model										
<i>P</i> (model)	0.445	<0.0001	0.001	0.006	0.341	0.281	0.214	0.038	<0.0001	<0.0001
pooled SEM	87.2	21.2	24.7	17.5	13.1	29.3	17.2	8.7	14.9	12.3
<i>P</i> Values from Two-Way ANOVA										
saponin level	0.303	<0.0001	0.0004	0.0009	0.429	0.072	0.054	0.720	<0.0001	<0.0001
basal diet	0.239	0.134	0.172	0.315	0.272	0.604	0.150	0.050	0.002	0.017
interaction	0.670	0.012	0.154	0.638	0.217	0.667	0.739	0.025	0.001	0.001
Means of One-Way ANOVA										
FM-0	264	403 a	493 a	252 a	70	258	161	99 ab	59 b	6 c
FM-2	511	310 ab	475 ab	256 a	70	265	122	121 ab	73 b	11 c
FM-4	414	301 ab	405 ab	203 ab	74	248	143	102 ab	97 b	63 bc
FM-6	462	308 ab	438 ab	203 ab	74	255	151	95 ab	118 ab	69 bc
FM-10	269	200 b	349 b	165 ab	93	199	119	137 a	184 a	177 a
LP-0	533	362 a	456 ab	255 a	69	280	167	108 ab	64 b	8 c
LP-2	490	344 a	438 ab	231 ab	73	258	163	101 ab	74 b	22 bc
LP-4	437	321 a	376 ab	145 b	56	275	171	97 ab	67 b	46 bc
LP-6	391	298 ab	386 ab	202 ab	79	206	138	93 ab	76 b	47 bc
LP-10	417	311 ab	391 ab	173 ab	62	252	136	88 b	104 ab	90 b

^aAbbreviations: FM, fishmeal diet; LP, lupin meal diet; 0, 2, 4, 6, 10, levels of soya saponin inclusion in diets (g/kg); DM, dry matter; PI1, proximal section of proximal intestine; PI2, distal section of proximal intestine; MI, midintestine; DI1, proximal section of distal intestine; DI2, distal section of distal intestine.

Table 7. Results of Increasing Doses of Soya Saponins on the Regression Analysis for Distal Intestine Histology and Gene Expression Data from Atlantic Salmon Fed the Experimental Diets^a

	FM				LP					
	P (model)	R ²	intercept	X	P (model)	R ²	intercept	X	X ²	X ³
MF height	0.0005	0.89	8.185	-0.212	0.0078	0.75	06.900	-0.337	0.015	
MF fusion	0.0003	0.92	2.001	0.233	0.027	0.64	2.275	0.408	-0.020	
LP width	0.0048	0.78	1.301	0.079	0.03	0.63	1.497	0.167	0.0006	
LP cellularity	0.006	0.76	1.244	0.031	0.01	0.67	1.398	0.214	-0.003	
SM width	0.007	0.76	1.092	0.072	0.026	0.65	1.368	0.184	-0.001	
SM cellularity	0.002	0.72	0.792	0.264	0.004	0.64	1.384	0.170		
ent vacuolization	0.001	0.84	7.893	-0.2711	0.004	0.78	7.492	-0.448	0.009	
ent nucleus position	0.0006	0.88	1.672	0.410	0.004	0.78	1.704	0.423	-0.015	
goblet cells	0.0009	0.87	1.582	0.372	0.0005	0.88	1.559	0.397	-0.008	
<i>cd4a</i>	0.08	0.51	0.0009	-0.0001	0.42	0.22	0.0008	6.978 × 10 ⁻⁶	-0.000007	
<i>cd8β</i>	0.46	0.20	0.0312	0.0001	0.37	0.24	0.0322	0.0017	-0.0001	
<i>tcy</i>	0.74	0.18	0.0072	-0.0004	0.06	0.67	0.0064	0.0013	-0.0003	-0.00002
<i>mhcI</i>	0.62	0.24	1.4474	0.0722	0.90	0.08	1.2925	0.1934	-0.0513	0.0033
<i>il-1β</i>	0.051	0.57	0.0023	-0.001	0.64	0.12	0.0019	4.984 × 10 ⁻⁵	5.96 × 10 ⁻⁶	
<i>il-17a</i>	0.071	0.53	0.0011	-0.0002	0.56	0.15	0.0017	0.0017	-0.0001	
<i>ifnγ</i>	0.046	0.71	0.0008	0.0005	0.24	0.48	0.0018	-0.0003	0.0002	-0.00002
<i>gilt</i>	0.005	0.78	25.556	1.7998	0.46	0.20	27.667	-0.023	-0.0362	
<i>par2a</i>	0.41	0.36	0.2464	0.0197	0.23	0.48	0.2609	0.0367	-0.0069	0.00034
<i>par2b</i>	0.58	0.14	0.0536	-0.0010	0.84	0.05	0.0570	0.0004	-8.113 × 10 ⁻⁵	
<i>try</i>	0.04	0.73	0.0002	0.0002	0.64	0.23	0.0005	-0.0001	0.00002	-0.000001
<i>myd88</i>	0.006	0.76	0.0550	0.0002	0.15	0.42	0.0603	0.0026	-0.0001	
<i>pcna</i>	0.32	0.42	0.2002	-0.0041	0.63	0.24	0.1934	-0.0074	0.0033	-0.0002
<i>mmp13</i>	0.02	0.68	0.0158	-0.0057	0.22	0.35	0.0101	0.0099	-0.0007	
<i>muc2</i>	0.08	0.52	8.1271	-0.7806	0.63	0.12	11.407	-0.560	0.0479	
<i>aqp8ab</i>	0.19	0.37	7.6597	-1.413	0.02	0.64	12.118	-2.805	0.2064	
<i>fabp2a1</i>	0.07	0.53	0.3218	-0.0843	0.04	0.58	0.2460	-0.0694	0.0046	
<i>fxr</i>	0.70	0.19	0.0130	0.0006	0.57	0.26	0.0144	0.0020	-0.0004	0.00002

Histology

Gene Expression

^aAbbreviations: FM, fishmeal diet; LP, lupin meal diet; MF, mucosal fold; LP, lamina propria; SM, submucosa; ent, enterocyte; *il-17A*, interleukin 17A; *ifnγ*, interferon γ ; *il-1β*, interleukin 1 β ; *muc2*, mucin-2; *aqp8ab*, aquaporin 8ab; *fabp2a1*, fatty acid binding protein 2a1; *try*, trypsin; *cd4a*, cluster of differentiation 4a; *gilt*, interferon γ -inducible lysosomal thiol reductase; *pcna*, proliferating cell nuclear antigen; *cd8β*, cluster of differentiation 8 β ; *par2a*, proteinase-activated receptor 2a; *par2b*, proteinase-activated receptor 2b; *mhcI*, mhc class I; *myd88*, myeloid differentiation factor 88; *tcy*, T-cell receptor γ ; *fxr*; farnesoid x receptor; *mmp13*, matrix metalloproteinase 13; X, soya saponin level.

Table 8. Soya Saponin and Basal Diet Effects on Histological Evaluation of the Distal Intestine of Atlantic Salmon Fed the Experimental Diets^a

	mucosal folds		lamina propria		submucosa		enterocytes		goblet cells
	height	fusion	width	cellular infiltration	width	cellular infiltration	vacuolization	nucleus position	relative nr
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
pooled SEM	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2
FM-0	8.0 a	2.0 de	1.3 e	1.3 e	1.2 d	1.1 d	7.4 ab	1.9 d	1.8 c
FM-2	8.1 a	2.5 cd	1.4 de	1.3 e	1.1 d	1.1 d	8.4 a	1.9 d	1.8 c
FM-4	7.2 a	3.2 bce	2.3 cd	2.0 bc	1.9 c	1.9 c	6.8 bc	3.7 bc	3.3 b
FM-6	5.8 c	3.6 abc	2.3 cd	2.1 cd	2.1 bc	2.0 bc	5.2 d	4.0 ab	3.6 b
FM-10	4.3 c	5.3 a	4.1 a	4.0 a	3.7 ab	3.7 ab	3.8 e	5.5 a	4.7 ab
LP-0	7.0 ab	2.1 d	1.4 de	1.3 de	1.3 d	1.3 d	7.3 ab	1.7 d	1.5 c
LP-2	7.0 ab	2.8 bcd	1.5 de	1.5 de	1.6 cd	1.6 cd	7.9 a	1.7 d	1.9 c
LP-4	5.3 cd	4.2 ab	2.7 abc	2.7 abc	2.7 abc	2.7 abc	5.5 cde	3.6 bc	3.4 b
LP-6	5.9 bc	3.3 abcd	1.9 cde	2.0 bcd	1.9 cd	1.9 bcd	5.2 cd	3.3 c	3.3 b
LP-10	4.9 cd	4.2 ab	3.4 ab	3.3 a	3.2 a	3.2 a	4.0 de	4.5 ab	4.8 a

^aAbbreviations: FM, fishmeal diet; LP, lupin meal diet; 0, 2, 4, 6, 10, levels of soya saponin inclusion in diets (g/kg). Data were analyzed using the Wilcoxon/Kruskal–Wallis test followed by the posthoc Steel–Dwass method. Mean values with different online letters a, b, c, d, and e within a column are significantly different ($P < 0.05$).

Table 9. Soya Saponin and Basal Diet Effects on PcnA Staining Height Analysis of the Distal Intestine of Atlantic Salmon Fed the Experimental Diets^a

	PcnA measurements	
	PCL (μm)	PCL _{BL} ($\mu\text{m}/\text{m}$)
Two-Way ANOVA Model		
P (model)	<0.0001	<0.0001
pooled SEM	14	31
P Values from Two-Way ANOVA		
saponin level	<0.0001	<0.0001
basal diet	0.136	0.106
interaction	0.612	0.31
Means of One-Way ANOVA		
FM-0	124 c	300 d
FM-2	137 c	336 cd
FM-4	133 c	336 cd
FM-6	186 abc	475 abc
FM-10	240 a	614 a
LP-0	126 c	320 d
LP-2	128 c	322 d
LP-4	132 c	334 cd
LP-6	162 bc	403 bcd
LP-10	205 ab	519 ab

^aAbbreviations: FM, fishmeal diet; LP, lupin meal diet; 0, 2, 4, 6, 10, levels of soya saponin inclusion in diets (g/kg); PCL, PcnA-positive proliferative compartment length; PCL_{BL}, PcnA-positive proliferative compartment length corrected for body length. Mean values with different online letters a, b, c, and d within a column are significantly different ($P < 0.05$).

Gene Expression (See Tables 7, 10, and 11 and Figure 1G,H). Among the immune-related transcripts, interleukin 17a (*il-17a*), interferon γ (*ifn γ*), and myeloid differentiation factor 88 (*myd88*) were significantly increased with increasing saponin levels, and interleukin1 β (*il-1 β*) and matrix metalloproteinase 13 (*mmp13*) displayed a similar trend. For *il-17a*, the effect was apparent only in fish fed the FM diet as indicated by the significant interaction. The posthoc test based on the results of the one-way ANOVA showed that the effect became pronounced only at the highest saponin inclusion level, i.e.,

for fish fed FM-10. The FM-10 group also showed the numerically highest values for the other above-mentioned transcripts. Increasing saponin levels down-regulated the expression of *aqp8ab* (Figure 1G), *fabp2a1* (Figure 1H), and interferon γ -inducible lysosomal thiol reductase (*gilt*). For *aqp8ab* this effect seemed to depend on the basal diet with somewhat different dose–response curves for the two. Comparison of the two basal diets showed that fish fed the LP diets presented higher expression of *ifn γ* and mucin 2 (*muc2*) than fish fed the FM diets. For the other expression variables, no significant differences were observed.

DISCUSSION

The data reveal that 2–4 g/kg soya saponins in diets from a 95% purified source elicited signs of inflammation in the DI of Atlantic salmon without the presence of other legume components. Supplementation with 2 g/kg modified some functional characteristics, while a level between 2 and 4 g/kg appeared to be needed to induce signs of inflammatory changes. However, inclusion levels as low as 8% SBM have previously caused inflammation with concomitant digestive dysfunction,⁹ which translates to a saponin level of ca. 0.4–0.6 g/kg in the diet. This is 70–80% lower than the lowest supplementation of 2 g/kg diet in the current study. Additionally, for several of the observed variables, such as enterocyte vacuolization, mucosal fold fusion, and *Fabp2a*, the dose–response effect did not clearly differ between the saponin-supplemented FM and LP diets, despite the apparent difference in feed intake and therefore saponin exposure. Thus, the saponin supplementation was more potent in the LP than the FM basal diet, most likely due to interactions with other lupin components. Therefore, the current study substantiates previous suggestions that the induction of inflammation by soya saponins is potentiated by other ANFs, antigens, or other components in SBM and other legumes such as peas and lupins.^{16,19,20}

Effects of Basal Diet Composition. The observed, although not quantified, differences in feed intake, which are most likely the major cause of the differences in growth observed between fish fed the two basal diets along with the implicit underlying differences in digestive and metabolic

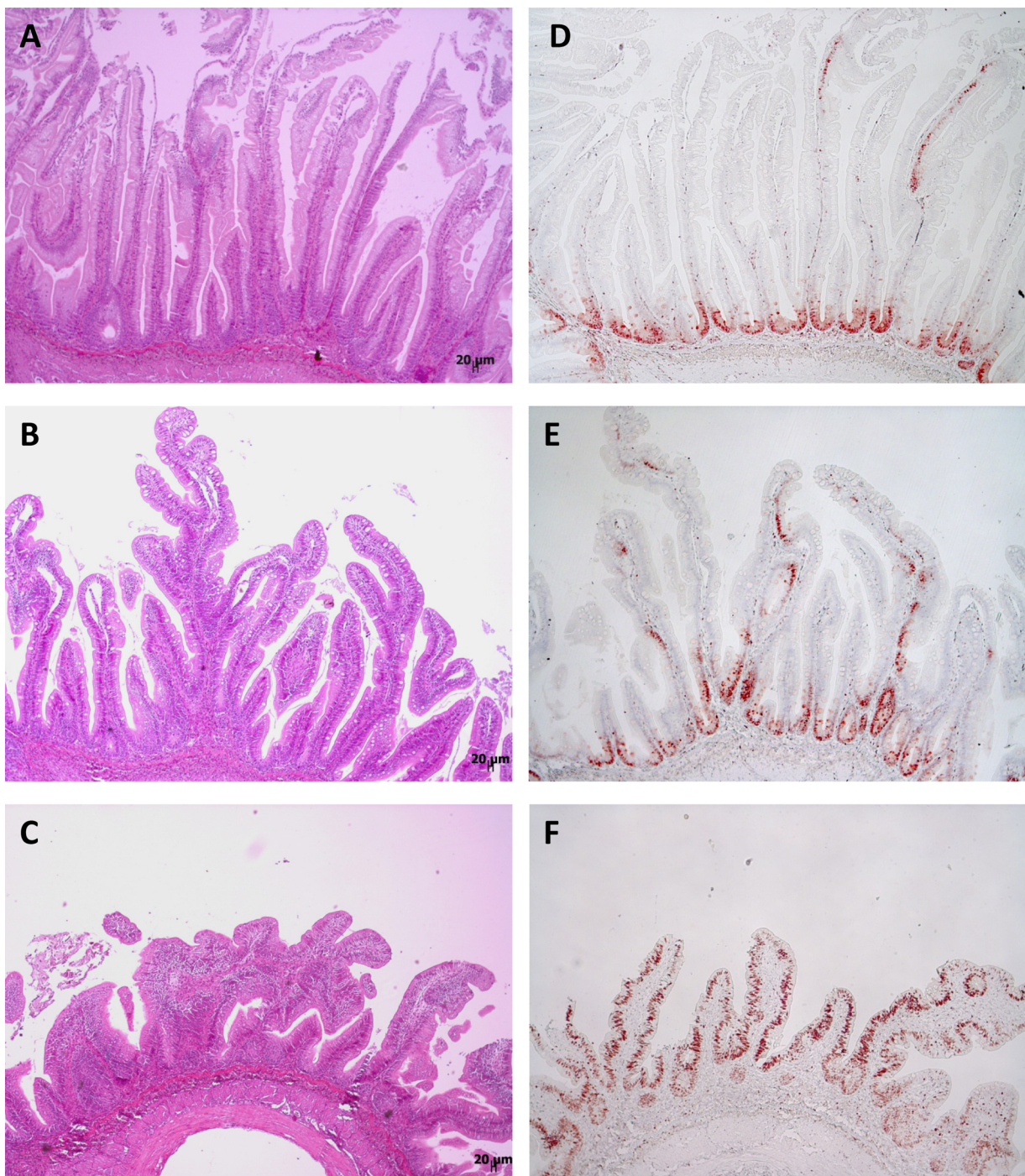


Figure 2. (A–C) Representative histomorphological images from hematoxylin and eosin-stained sections of the distal intestine of Atlantic salmon depicting the dose-dependent increase in the severity of the inflammatory changes with increasing soya saponin supplementation in fish fed the FM-0 (A), FM-6 (B), and FM-10 (C) diets. (D–F) Representative images of the localization and distribution of immunohistochemically labeled proliferating cell nuclear antigen (PcnA) protein in the epithelial cells of the distal intestine of Atlantic salmon depicting the dose-dependent increase in the relative number of proliferating cells with increasing soya saponin supplementation considered low for the FM-0 diet (D), moderately elevated for the FM-6 diet (E), and highly elevated for the FM-10 diet (F). FM = fishmeal diet, and 0, 6, and 10 indicate the levels of soya saponin inclusion in the diets (g/kg).

aspects, makes integration of results from the two groups challenging. The macronutrient digestibilities were generally higher for the LP than the FM diet. The estimates of digestible energy of the basal diets based on the ADs resulted in values of 20.7 and 21.8 MJ/kg for FM-0 and LP-0, respectively. Fish fed the LP-0 diet grew 32% less than those fed the FM-0 diet. Assuming their energy needs to be 17–19 MJ GE/kg of

growth,⁵⁶ feed consumption is estimated at 230–250 g for the LP diet and 360–400 g for the FM diet. Thus, the LP-fed fish ate ca. 36% less. Lupins contain bitter alkaloids, which may be the cause of the low feed intake in the LP fed fish.^{57,58} The lupin alkaloids, however, seem not to affect fish health importantly,^{57–60} as supported by the present study.

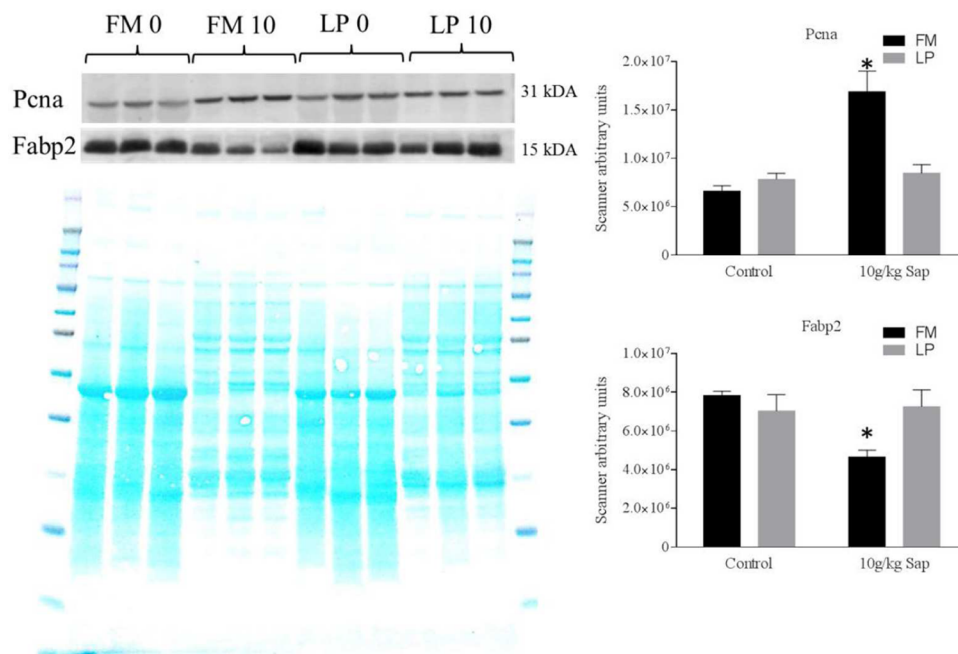


Figure 3. Western blot analysis of proliferating cell nuclear antigen (PcnA) and fatty acid binding protein 2 (Fabp2) proteins in the distal intestinal tissue of fish fed the four diets FM-0, FM-10, PM-0, and PM-10. Immunoblots are shown for $n = 3$ individual fish per diet group. The corresponding total protein stain is shown in blue below each individual blot. Relative staining intensity values in the columns of the graphs are means of the $n = 3$ fish per diet, with their standard errors represented by vertical bars. Asterisks denote saponin diet groups that are significantly different from their respective control diet groups.

The higher nutrient ADs for the LP-fed fish may at least partly be explained by the apparent difference in feed intake, which have been reported to decrease with increasing feed intake.^{61,62} However, reported effects of feed intake are in general rather small. The 6% difference in protein AD in the present study was therefore remarkably high, and can only be explained by a higher protein digestibility of the plant meals in the LP diet, i.e., lupin and wheat gluten, compared to the fishmeal in the FM diet. Experience has indicated that protein digestibility may vary greatly among fishmeals, most likely due to differences in the quality of the raw materials, ash content, and processing conditions such as heat treatment.⁶³

The lower relative weights of the stomach, MI, DI, and liver in the LP-fed fish were most likely also due to lower feed intake.⁶⁴ However, the higher PI weight, the section of the digestive tract that is responsible for the majority of nutrient absorption,⁶⁵ may be a result of (1) a higher concentration of digestible nutrients that can stimulate tissue growth and/or (2) a compensatory response to challenges to the digestive processes linked to fibers and possibly other ANFs in lupins. Fiber-rich diets have been shown to induce growth of intestinal tissues in Atlantic salmon⁶⁶ and cod,⁶⁷ possibly due to a need for a larger absorptive surface area when the volume of the chyme increases due to swelling of the fiber. Growth stimulation by fermentation products produced from the fibers in the digestive tract is another possible mechanism.^{68,69} In any case, the effects on PI weight were clearly greater than any diminishing effect of reduced feed intake could be expected to be.

Plasma cholesterol and bile salt levels also differed markedly between the fish fed the two basal diets, with lower values in the LP-fed fish, which can again be explained by lower feed intake and lower diet cholesterol levels in this diet. However, this is also a commonly observed response to plant feed

ingredients and foods, with their contents of fibers, phytosterols, phytoestrogens, and saponins all affecting cholesterol and bile salt absorption (reviewed by Krogdahl et al.¹⁸). In fish, low cholesterol and bile acid absorption and metabolism have been suggested to be a cause of the frequently observed reductions in lipid digestibility of plant ingredients, in particular standard SBM.⁷⁰ In the present experiment, however, lipid ADs did not significantly differ between the LP- and the FM-fed fish, although apparent lower feed intake in the LP-fed fish may have masked any negative effects.

Effects of Saponins. Under the condition that the difference in growth was due to a difference in feed intake, saponin exposure would have been lower in the LP fish. On the basis of feed intake estimates (see above), exposures of supplemented saponins were 0, 1.1, 1.7, 2.4, and 5.1 g/kg initial body weight for the fish fed the LP-0 to LP-10 diets and 0, 1.8, 3.8, 4.7, and 7.5 g/kg initial body weight for the FM-0 to FM-10 diets. For the LP diets the estimates are most likely lower than the actual values as the natural saponin contents of the lupins were not taken into account. However, the low levels reported in lupin seeds^{36,37} can be considered negligible in this context, adding less than 0.1 g/kg to the LP diet, i.e., less than 5% of the supplementation level of the LP-2 diet. Since current tools used for saponin analysis lack accuracy,³⁹ saponin analysis was not performed.

On the basis of the above considerations, it might be argued that saponin effects should be evaluated on the basis of exposure, e.g., expressed per unit of fish weight, rather than level in the diet. Similarly, estimates of acceptable dose should also be based on exposure per unit of fish weight. However, the apparent difference in feed intake most likely interacted several ways with saponin effects, making a discussion based on saponin intake very difficult and its value questionable. The discussion on the effects of saponin exposure below is therefore

Table 10. Soya Saponin and Basal Diet Effects on Gene Expression Analysis of the Distal Intestine of Atlantic Salmon Fed the Experimental Diets^a

	<i>cd4α</i>	<i>cd8β</i>	<i>mhcI</i>	<i>il-1β</i> [†]	<i>il-17a</i> [†]	<i>ifnγ</i> [†]	<i>gilt</i>	<i>par2a</i>	<i>par2b</i>	<i>try</i>	<i>pna</i>	<i>muc2</i>	<i>app8ab</i> [†]	<i>fabp2a1</i> [†]
<i>P</i> (model)	0.369	0.423	0.529	0.082	0.003	0.005	0.035	0.530	0.679	0.515	0.862	0.004	0.005	<0.0001
pooled SEM	0.0001	0.003	0.222	0.002	0.004	0.0007	2.57	0.022	0.005	8.11 × 10 ⁻⁵	0.018	0.916	2.100	0.091
saponin level	0.232	0.215	0.388	0.027	0.001	0.026	0.011	0.564	0.992	0.885	0.689	0.922	0.003	<0.0001
basal diet	0.788	0.459	0.653	0.963	0.909	0.007	0.343	0.182	0.388	0.240	0.802	0.0005	0.057	0.787
interaction	0.204	0.405	0.269	0.162	0.042	0.188	0.212	0.973	0.398	0.364	0.485	0.560	0.120	0.333
							Means of One-Way ANOVA							
FM-0	0.0009	0.031	1.4	0.0017	0.0005 b	0.0008 b	26	0.25	0.055	0.0002	0.20	8.1 bc	9.1 a	0.34 a
FM-2	0.0008	0.031	1.5	0.0019	0.001 ab	0.0016 ab	26	0.26	0.047	0.0005	0.18	6.9 ab	2.0 ab	0.11 abc
FM-4	0.0008	0.032	1.3	0.0019	0.006 ab	0.0018 ab	28	0.31	0.055	0.0003	0.18	5.8 b	4.2 ab	0.09 abcd
FM-6	0.0007	0.034	1.2	0.0015	0.004 ab	0.0018 ab	27	0.28	0.051	0.0002	0.17	6.8 ab	2.8 ab	0.02 abcd
FM-10	0.001	0.037	1.1	0.010	0.018 a	0.002 ab	15	0.26	0.057	0.0004	0.22	8.4 ab	0.4 b	0.008 d
LP-0	0.0008	0.032	1.2	0.0021	0.001 ab	0.0017 ab	27	0.26	0.056	0.0005	0.19	11.9 a	10.9 a	0.24 ab
LP-2	0.0008	0.033	1.7	0.0016	0.003 ab	0.0023 ab	28	0.30	0.060	0.0003	0.19	8.8 ab	10.7 a	0.13 abc
LP-4	0.001	0.039	1.1	0.0029	0.008 ab	0.0027 ab	26	0.32	0.054	0.0004	0.19	11.8 a	1.1 ab	0.01 cd
LP-6	0.0009	0.035	1.4	0.0021	0.001 ab	0.004 a	25	0.30	0.057	0.0004	0.21	8.9 ab	3.5 ab	0.02 bcd
LP-10	0.0008	0.033	1.4	0.0031	0.003 ab	0.0026 ab	24	0.28	0.053	0.0004	0.18	10.6 ab	4.7 a	0.01 bcd

^aAbbreviations: FM, fishmeal diet; LP, lupin meal diet; 0, 2, 4, 6, 10, levels of soya saponin inclusion in diets (g/kg); †, log-transformed data; *cd4α*, cluster of differentiation 4α; *cd8β*, cluster of differentiation 8β; *mhcI*, major histocompatibility class I; *il-1β*, interleukin 1β; *il-17a*, interleukin 17a; *ifnγ*, interferon γ; *gilt*, inducible lysosomal thiol reductase; *par2a*, proteinase-activated receptor 2a; *par2b*, proteinase-activated receptor 2b; *try*, trypsinogen; *pna*, proliferating cell nuclear antigen; *muc2*, mucin-2; *app8ab*, aquaporin 8ab; *fabp2a1*, fatty acid binding protein 2a. Mean values with different online letters a, b, c, d, and e within a column are significantly different ($P < 0.05$).

Table 11. Soya Saponin and Basal Diet Effects on Gene Expression Analysis of the Distal Intestine of Atlantic Salmon Fed the Experimental Diets^a

	<i>tcry</i>	<i>myd88</i>	<i>mmp13</i>	<i>fxr</i>
	Wilcoxon/Kruskal–Wallis Test			
<i>P</i> value	0.193	0.036	0.060	0.445
pooled SEM	0.0007	0.004	0.015	0.001
	Mean Values			
FM-0	0.0070	0.054	0.012	0.0129
FM-2	0.0072	0.055	0.015	0.0138
FM-4	0.0052	0.065	0.016	0.0127
FM-6	0.0064	0.062	0.019	0.0128
FM-10	0.0066	0.086	0.083	0.0154
LP-0	0.0064	0.061	0.015	0.0143
LP-2	0.0078	0.059	0.017	0.0170
LP-4	0.0079	0.072	0.036	0.0165
LP-6	0.0070	0.068	0.048	0.0157
LP-10	0.0089	0.068	0.028	0.0155

^aAbbreviations: FM, fishmeal diet; LP, lupin meal diet; 0, 2, 4, 6, 10, levels of soya saponin inclusion in diets (g/kg); *tcry*, T-cell receptor γ ; *myd88*, myeloid differentiation factor 88; *mmp13*, matrix metalloproteinase 13; *fxr*; farnesoid x receptor. Data were analyzed using the Wilcoxon/Kruskal–Wallis test followed by the posthoc Steel–Dwass method.

mainly based on the responses seen for the FM-fed fish. Comments regarding effects in the fish fed the LP diet are reserved for response variables of particular importance. Absence of saponin effects in LP fish, as compared to FM fish, is not discussed when they were most likely a result of low saponin exposure.

Low levels of saponin exposure stimulated growth in the FM group, an effect which may have been due to increasing feed intake, or some metabolic or physiological effects. As for other toxins and stressors generally considered detrimental for health at higher doses, the apparently beneficial effects observed at low levels of saponin exposure can be considered a hormetic response. This may also be related to the immune-stimulating effects of saponin's adjuvant characteristics.^{28–30,38} The apparent absence of such positive effects in LP-fed fish may be explained by antagonistic, possibly potentiating effects of other lupin components. The apparent lack of a significant effect of soya saponin exposure on growth for either basal diet group is in line with indications from other studies investigating similar saponin preparations in fish feed.^{16,19}

The observed negative effects of higher saponin exposure on nutrient digestibilities are in agreement with the results of the work of Chikwati et al.¹⁹ investigating the effect of adding 2 g/kg soya saponin to diets with different plant ingredients. However, in this earlier work, the saponin effects depended on the plant ingredient included in the diet and were significant for a diet containing pea protein concentrate, but not for the other diets with sunflower, horse beans, and rapeseed meal as alternative ingredients. The suggested explanation was differences in saponin level between the plant ingredients, as peas contain more saponins than the others, or other potentiating components in the pea meal.¹⁹

The mechanisms behind effects of saponin exposure on nutrient ADs may be related to reductions in cholesterol and bile salt levels in intestinal content and subsequently in plasma. Saponin binding to 3- β -hydroxysteroids such as cholesterol forms insoluble, nonabsorbable complexes and increases their

excretion,^{22,25,71} which alters the normal metabolic pathways of cholesterol and total bile acid production.⁵⁰ Thus, the current data support previous investigations suggesting that saponins are at least partially responsible for the drop in plasma cholesterol levels in several animal species fed plant meal diets.^{23,25–27,50} Moreover, bile salts have the ability to stabilize pancreatic proteolytic enzymes (reviewed by Kroghdal et al.¹⁸). Accordingly, a reduction in chyme bile salt concentration may cause reduced protein digestibility, as observed at the highest level of saponin supplementation in the present study. The marked reduction in LAP activity in the DI may also have played a role in the saponin effect on protein and amino acid ADs.

The present study clearly demonstrated that soya saponins alone, supplemented at levels of 2–4 g/kg of diet and above, caused a dose-dependent increase in the severity of inflammatory changes in the DI tissue as described for SBMIE in salmonids,^{6,8,9,13,19} strongly suggesting the involvement of saponins as a causatory agent. At the lowest saponin inclusion level, the effects were small, but trends were clearly observed for increased width and cellular infiltration in lamina propria and submucosa. The reductions in DI somatic index and digestive enzyme activities as well as marked reductions in gene expression for various functional proteins (see below) are signs of dysfunction caused by the inflammation and may be directly linked to the shortening of the mucosal folds, which has been suggested to be related to the increase in cell turnover with concomitant increases in proliferation, migration, and apoptosis observed in Atlantic salmon fed SBM.^{11,13,72} These phenomena were apparent in the present study as well, as supported by data regarding mucosal fold height and Pcn protein expression in the DI tissue.

At the gene expression level in the DI, alterations observed at the higher saponin inclusion levels in the present study correspond well with the more convincingly up- or down-regulated genes reported for salmon with SBMIE.^{52,54} Among these were genes related to recruitment and/or differentiation of inflammatory cells, i.e., *il-17a*, *ifn γ* , and *il-1 β* in the FM-10 group and remodeling of the extracellular matrix as suggested by induction of *mmp13*. The cytokine *il-17a* has been suggested as a sensitive molecular marker for SBMIE⁵² and may be a candidate marker for exposure to high levels of soya saponins as well, since transcript levels displayed a >30-fold induction in the FM-10 group. Other immune-related genes previously reported to be differentially regulated during SBMIE, but often less convincingly than those mentioned above,^{10,52,54} were not significantly or dose-dependently affected in the current study. These include *par2a*, *par2b*, *tcry*, *cd4a*, *cd8 β* , *gilt*, *mhcl*, *myd88*, *fxr*, and *muc2*. This may further support the theory that other SBM components may work synergistically with saponins to illicit a full inflammatory response.

Among functional transcript markers, *fabp2a1* for fatty acid binding protein and *aqp8ab* for one of the aquaporins were found to be strongly and dose-dependently down-regulated by soya saponin inclusion in both basal diets. A corresponding reduction in Fabp2 protein level was observed for the FM-10 group. These responses in Fabp2 protein and mRNA levels have also been observed during SBMIE^{49,73} and inflammation caused by the combination of pea protein concentrate and 2 g/kg soya saponins.²⁰ Reduced Fabp2 levels could explain the decreased lipid ADs typically observed in fish fed SBM.⁴⁹ The transcriptional expression of *aqp8ab* also displayed marked suppression with increasing soya saponin levels for both basal

diets. This response has previously been observed in salmon with DI inflammation^{20,73} and in mammals with intestinal inflammation.^{74,75} In these latter studies, down-regulation of *aqp8* was concomitant with decreases in the absorption of water, suggesting a possible role of aquaporins in the pathogenesis of diarrhea accompanying intestinal inflammatory disease.

The observation of progressively increasing trypsin activity of the intestinal content supports earlier findings in salmonids,^{9,55} as well as in other animals and humans,^{76,77} and suggests that this variable is a good marker candidate for inflammation in the DI. In mammals, such increases in chyme trypsin activity of the distal compartments of the intestine have been shown to activate PAR-2, which mediates pro-inflammatory effects in the colon of mammals.^{78,79} It may also be partly explained by decreased ability of the more immature or damaged epithelial cells to reabsorb pancreatic enzymes.^{66,72}

■ ASSOCIATED CONTENT

● Supporting Information

Primer pair sequence efficiency, amplicon size, and annealing temperature for the genes used for real-time PCR (Table SI 1), results of regression analysis of increasing doses of soya saponins on amino acid and fatty acid apparent digestibility (AD) data (Table SI 2), and soya saponin and basal diet effects on apparent digestibility (%) of essential amino acids (Table SI 3), nonessential amino acids and taurine (Table SI 4), and fatty acids (Table SI 5), on organosomatic indices (OSI) (Table SI 6), and on plasma parameters (Table SI 7). This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: ashild.krogdahl@nmbu.no. Phone: +47 2296 4534. Fax: +47 2259 7310.

Author Contributions

The contributions of the authors were as follows: Å.K., leadership, experimental design, data evaluation and interpretation, manuscript development; K.G., morphological evaluation, gene expression, statistical analysis, manuscript writing; M.P., experimental design, sampling, statistical analysis; T.M.K., gene expression analyses, Western blot; M.G., sampling, morphological evaluation, immunohistochemistry; G.M.B., feeding trial, sampling; A.M.B., experimental design, data evaluation and interpretation, manuscript development and review.

Notes

The authors declare no competing financial interest.

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Paper II

A high-resolution map of the gut microbiota in Atlantic salmon (*Salmo salar*). A basis for comparative gut microbial research

Karina Gajardo^{1*}, Ana Rodiles², Trond M Kortner¹, Åshild Krogdahl¹, Anne Marie Bakke¹, Daniel L. Merrifield², Henning Sørum³

¹ Department of Basic Sciences and Aquatic Medicine, Faculty of Veterinary Medicine and Biosciences, Norwegian University of Life Sciences (NMBU), Oslo, Norway

² Aquaculture and Fish Nutrition Research Group, School of Biological Sciences, Plymouth University, UK

³ Department of food Safety and Infection Biology, Faculty of Veterinary Medicine and Biosciences, Norwegian University of Life Sciences (NMBU), Oslo, Norway

***Corresponding author:** Karina Gajardo, PO Box 8146 Dep., 0033 Oslo, Norway; E-mail: karina.gajardo@nmbu.no; Tel. +47 67232024

Abstract

Gut health challenges possibly related to alterations in gut microbiota, caused by plant ingredients in the diets, cause losses in Atlantic salmon production. To investigate the role of the microbiota for gut function and health detailed characterization of the gut microbiota is needed. We present the first in-depth characterization of salmon gut microbiota based on high-throughput sequencing of the 16S rRNA gene's V1-V2 region. Samples were taken from five intestinal compartments: digesta from proximal, mid and distal intestine and of mucosa from mid and distal intestine of 67.3 g salmon kept in seawater (12-14°C) and fed a commercial diet for 4 weeks. Microbial richness and diversity differed significantly and were higher in the digesta than the mucosa. In mucosa, Proteobacteria dominated the microbiota (90%), whereas in digesta both Proteobacteria (47%) and Firmicutes (38%) showed high abundance. Future studies of diet and environmental impacts on gut microbiota should therefore differentiate between effects on mucosa and digesta in the proximal, mid and the distal intestine. A core microbiota, represented by 22 OTUs, was found in 80% of the samples. The gut microbiota of Atlantic salmon showed similarities with that of mammals.

Introduction

The recent development of new analytical tools and their use for detailed study of microbiota populating body surfaces have contributed to a new understanding of the vital importance of the microbiota for the health and welfare of the host ¹. The gut plays a pivotal role, harbouring the largest microbiota populations in the body and exposing these to the body's largest immunogenic organ, i.e. the gut wall. A rapidly increasing number of studies in man and other mammals have supplied fundamental information regarding characteristics of resident gut microbes. They are involved in modulating a variety of gut functions, including digestion and absorption of nutrients and signalling from the myriad of gut mucosal receptors to abdominal organs as well as the brain ^{2,3}. Gut dysbiosis seems to be implicated in a number of diseases, including but not limited to obesity, colitis and inflammatory bowel diseases ⁴.

The microbiota of the fish gut is also receiving increased attention, illustrated by the steady increase in the development and commercial application of “functional” fish feeds for species in production. These contain e.g. pre- and/or probiotics that purportedly exert modulating effects on the gut microbiota and thereby benefit fish growth or disease resistance. However, information and understanding regarding fish gut microbiota still lags behind that of man and other mammals, including evidence for cause-effect relationships between gut microbiota and host physiology. This is at least partially due to the wide range of both external and internal factors that can influence the diversity of microorganisms described in the fish gut ^{3,5-10}. Specifically, environmental factors such as water temperature, salinity and geographical location, as well as developmental stage, diet, farm management practices, medical interventions and stress have all been demonstrated to extensively modulate the gut microbiota ^{6,9,11-16}. The phyla Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Fusobacteria have been reported as residents of the gut in a variety of fish species ¹⁷. But the descriptions are rather incomplete regarding regional variation along the intestine ^{9-11,18-21} and distinction between the mucosa-associated autochthonous and the more transient or digesta-associated allochthonous microbial communities ^{6,8,13,22-24}. Additionally, attempts to compare salmonid and mammalian gut microbiota ²⁵ may jump-start attempts to link our increasing knowledge of salmonid intestinal microbiota with their possible functional properties.

As one of the most important cultured fish species, the composition of the gut microbiota of Atlantic salmon has been studied to some extent, mostly by applying culture-based techniques (reviewed by Cahill, ²⁶ and Ringø *et al.*, ¹⁴) and only recently semi-quantitative molecular techniques have been put to use ^{6,10,18,19,24} and now also high-throughput sequencing ^{11,16,27,28}. These recent studies have broadened our understanding of the microbiota populations in the salmon gut, but again, a detailed regional and spatial (digesta vs mucosa-associated microbial communities) characterization, as well as comparative aspects have to our knowledge not yet been reported.

The Atlantic salmon may serve as a model species for gut microbial research for a number of reasons. From an ecological perspective, its anadromous nature is of interest and its sequenced genome greatly facilitates investigations of interactions between gut microbiota and host genotype and phenotype. In addition, salmon are strictly piscivorous in nature, and yet Norwegian farmed salmon at later growth stages are now fed diets containing more than 70% plant materials ²⁹. No other production animal has experienced a comparable change in diet composition. A large body of literature has described the various effects of alternative plant-based nutrient sources in fish (reviewed by Gatlin *et al.* 2007 ³⁰). Atlantic salmon appear particularly susceptible to gut malfunctions caused by plant antinutrients ^{31,32} and therefore constitutes a model for studies of nutritional stress and diet-related gut disorders. Connections between shifts in microbiota caused by changes in diet composition and gut health challenges have been alluded to in some studies ¹⁰. Recent work has shown that lactic acid producing bacteria (LAB) are present in higher abundance in salmon fed a plant-based diet compared to those fed fishmeal-based diets ¹⁰. Also wild-caught salmon have been observed to have relatively low levels of LAB ¹⁶. Yet any substantiated conclusions rely on investigations of resident microbiota in all intestinal compartments using high-resolution methods as well as a functional characterization of the populations under various conditions.

The aim of the present study was therefore to conduct the first in-depth characterization of the digesta and mucosa-associated microbiota of various intestinal regions in healthy post-smolt farmed salmon. The fish were fed a current commercial diet with composition developed for the size of the experimental fish, containing 43% plant and 57% marine ingredients.

Results

Characteristics of the high-throughput sequence data

High-throughput sequencing of bacterial DNA resulted in a total of 2.3 million raw reads. After data quality filter processing, the number of effective reads (clean reads without Cyanobacteria and filtered at 0.005%) was 814 691. The total number of operational taxonomic units (OTUs) assigned for all of the studied compartments was 914 for those clustered at 97% sequence identity. For all samples, rarefaction curves (Supplementary Figure 1A-B) showed that samples from both the digesta and mucosa reached the saturation phase. Furthermore, the good coverage index was 0.9923 ± 0.0006 (mean \pm standard error of the mean (SEM)), indicating adequate depth of sequencing.

Gut microbiota in digesta and mucosa compartments along the intestine

Results from the analysis of the alpha diversity metrics showed significantly lower richness (Chao1 and observed species) and Shannon's diversity index for the gut mucosa-associated microbiota compartments compared to the digesta compartments (Table 1).

Tables

Table 1. Alpha diversity results of gut microbiota of Atlantic salmon fed a commercial diet^a.

	Chao1	Observed species	Shannon's index
<i>Statistics</i>			
P (model)	0.0011	0.001	0.0004
Pooled SEM	44	41	0.3
<i>Means values</i>			
PID	552 ^a	484 ^a	6.4 ^a
MID	629 ^a	562 ^a	7.4 ^{ac}
DID	622 ^a	561 ^a	7.4 ^{ac}
MIM	170 ^b	132 ^c	4.4 ^b
DIM	188 ^b	158 ^c	4.9 ^b

^aAbbreviations: PID, proximal intestine digesta; MID, mid intestinal digesta; DID, distal intestine digesta; MIM, mid intestine mucosa; DIM, distal intestine mucosa.

At phylum level, the bacterial taxonomic composition across the digesta compartments showed a high relative abundance of Proteobacteria (47%), followed by Firmicutes (38%), Fusobacteria (7%) and Actinobacteria (6%), while the mucosal compartments showed an almost complete dominance of Proteobacteria (90%; Figure 1 and Supplementary Table 1). A total of 88 OTUs were detected as core microbiota for the digesta compartments and 32 for the mucosal compartments (Figure 2). Across all compartments, 22 common OTUs were identified: 14 at genus level, *Janthinobacterium*, *Propionibacterium*, *Stenotrophomonas*, *Pseudomonas*, *Phyllobacterium*, *Delftia*, *Herbaspirillum*, *Burkholderia*, *Sphingomonas*, *Ochrobactrum*, *Variovorax*, *Microbacterium*, *Rhodococcus* and *Acinetobacter*; six at family level, Phyllobacteriaceae, Enterobacteriaceae, Rhizobiaceae, Comamonadaceae, Oxalobacteraceae and Caulobacteraceae; and one at order level, Rhizobiales and one at phylum level; Proteobacteria (Figure 2 and Supplementary Table 2).

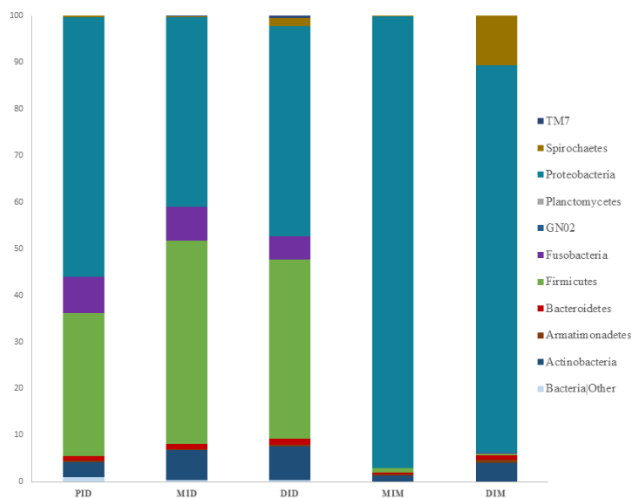


Figure 1. Gut microbiota composition (relative OTU composition) at phylum level. Composition of the five studied compartments: proximal intestinal digesta (PID), mid intestinal digesta (MID), distal intestinal digesta (DID), mid intestinal mucosa (MIM) and distal intestinal mucosa (DIM).

Results from the PERMANOVA analysis revealed significant differences between most of the intestinal compartments ($p=0.001$; Table 2). The exception was the difference between the mid intestinal digesta (MID) and the distal intestinal digesta (DID) ($p=0.795$). Accordingly, Principal

coordinates analysis (PCoA) plots (Figure 3) based on the weighted and unweighted UniFrac metrics showed that samples within each compartment clustered together for both the digesta and the mucosa with the exception of MID and DID, which clustered together without a clear separation between them.

Table 2. Result of the PERMANOVA analysis of the Weighted UniFrac for the different gut compartments studied of Atlantic salmon^a.

	P-value
PERMANOVA	0.001
PERMANOVA Pair-wise test	
PID. MID	0.034
PID. DID	0.004
PID. MIM	0.006
PID. DIM	0.01
MID. DID	0.795
MID. MIM	0.009
MID. DIM	0.012
DID. MIM	0.009
DID. DIM	0.013
MIM. DIM	0.008

^aAbbreviations: PID, proximal intestine digesta; MID, mid intestinal digesta; DID, distal intestine digesta; MIM, mid intestine mucosa; DIM, distal intestine mucosa.

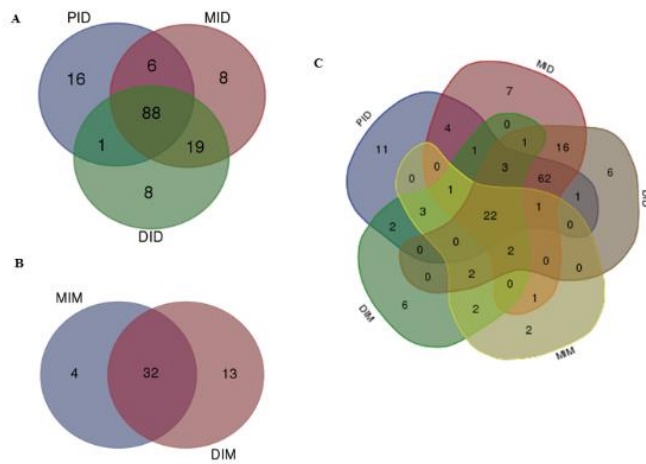


Figure 2. Venn diagrams showing compartmental core microbiota OTU distributions. (A) Digesta compartments: 88 OTUs were identified as core microbiota (80% of samples in each compartment) for the proximal intestinal digesta (PID), mid intestinal digesta (MID) and the distal intestinal digesta (DID). (B) Mucosa compartments: 32 OTUs were identified as core microbiota (80% of samples in each compartment) for the mid intestinal mucosa (MIM) and the distal intestinal mucosa (DIM). (C) Core microbiota (80% of samples in each compartment) for all studied compartments. Twenty two OTUs were found in all compartments.

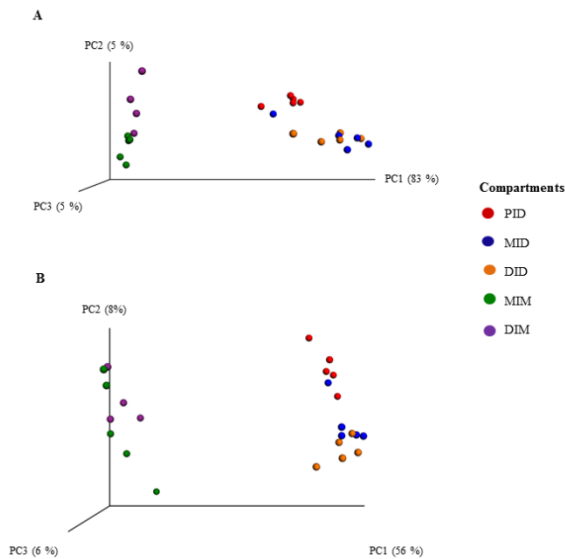


Figure 3. PCoA of Weighted (A) and Unweighted UniFrac (B) showing clustering of the compartments. Each dot represents one sample. Abbreviations: PID, proximal intestinal digesta; MID, mid intestinal digesta; DID, distal intestinal digesta; MIM, mid intestinal mucosa; DIM, distal intestinal mucosa.

Figure 4 shows the relative abundance of OTUs at the genus level in the various compartments. For the digesta, the genera *Photobacterium* (16%) *Delftia* (11%), *Weissella* (11%), *Leuconostoc* (8%) followed by *Janthinobacterium* (6%), showed the highest abundance in the proximal intestinal digesta (PID), whereas in MID and DID the genera *Photobacterium*, *Leuconostoc*, *Janthinobacterium*, *Weissella* and *Peptostreptococcus* were the most abundant. In the microbiota of the mucosa, the phylum Proteobacteria dominated and among these bacteria the genera *Janthinobacterium*, *Phyllobacterium*, *Variovorax* and *Delftia* showed the highest relative abundance mid intestinal mucosa (MIM), while *Delftia*, *Janthinobacterium*, *Variovorax* and *Stenotrophomonas* showed the highest relative abundance in the distal intestinal mucosa (DIM). In addition to genus from the Proteobacteria phylum, the family Brevinemataceae from the phylum Spirochaetes presented also high relative abundance in DIM (11%) (for a detailed list see Supplementary Table 1). In order to characterize the microbial communities that showed significant differences in abundances between the compartments, linear discriminant analysis (LDA) effect size (LEfSe) was performed. The results (Figure 5) showed that the class Fusobacteriia and the order Vibrionales were significantly different in PID, several OTUs from the class Clostridia were significantly different in MID compared to the other compartments, whereas class Bacilli and Actinobacteria (Actinomycetales) were significantly different in DID. Compared to the digesta compartments the mucosal compartments showed significantly higher abundance of the phylum Proteobacteria: Alphaproteobacteria and Betaproteobacteria for MIM and *Delftia* for DIM.

As the Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) analysis of the functional profile of the microbiota showed high Nearest sequenced taxon index (NSTI) values (0.22 ± 0.02 mean \pm SEM) and excluded of the majority of the OTUs, the results of the predicted functional profiling were considered not to be relevant for the interpretation of functional role of the microbiota in the gut of the fish in the present investigation. The results are therefore not presented.

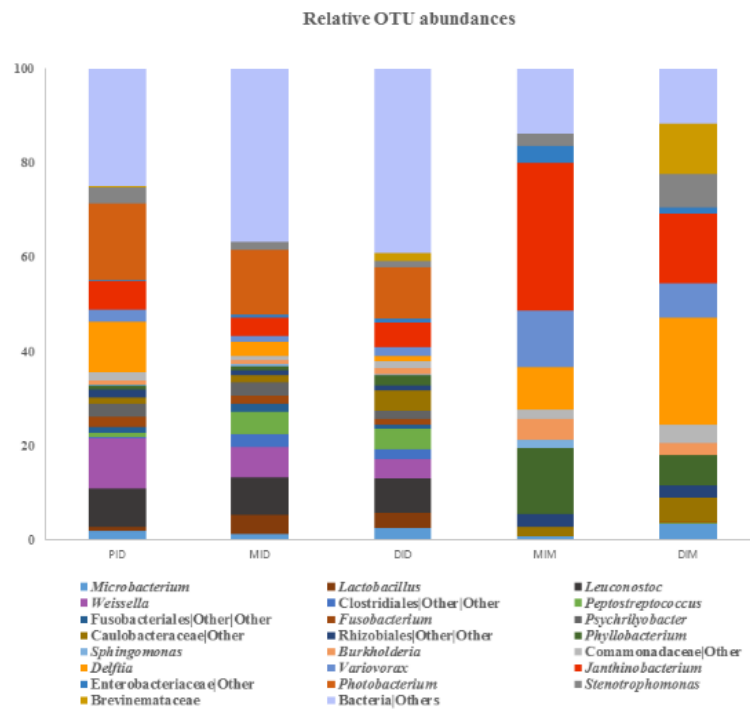


Figure 4. Gut microbiota composition (relative OTU composition) at genus level, or lowest taxonomic level determined by the analysis, in the gut compartments for the 12 genera showing the highest abundance. Abbreviations: PID, proximal intestine digesta; MID, mid intestinal digesta; DID, distal intestine digesta; MIM, mid intestine mucosa; DIM, distal intestine mucosa.

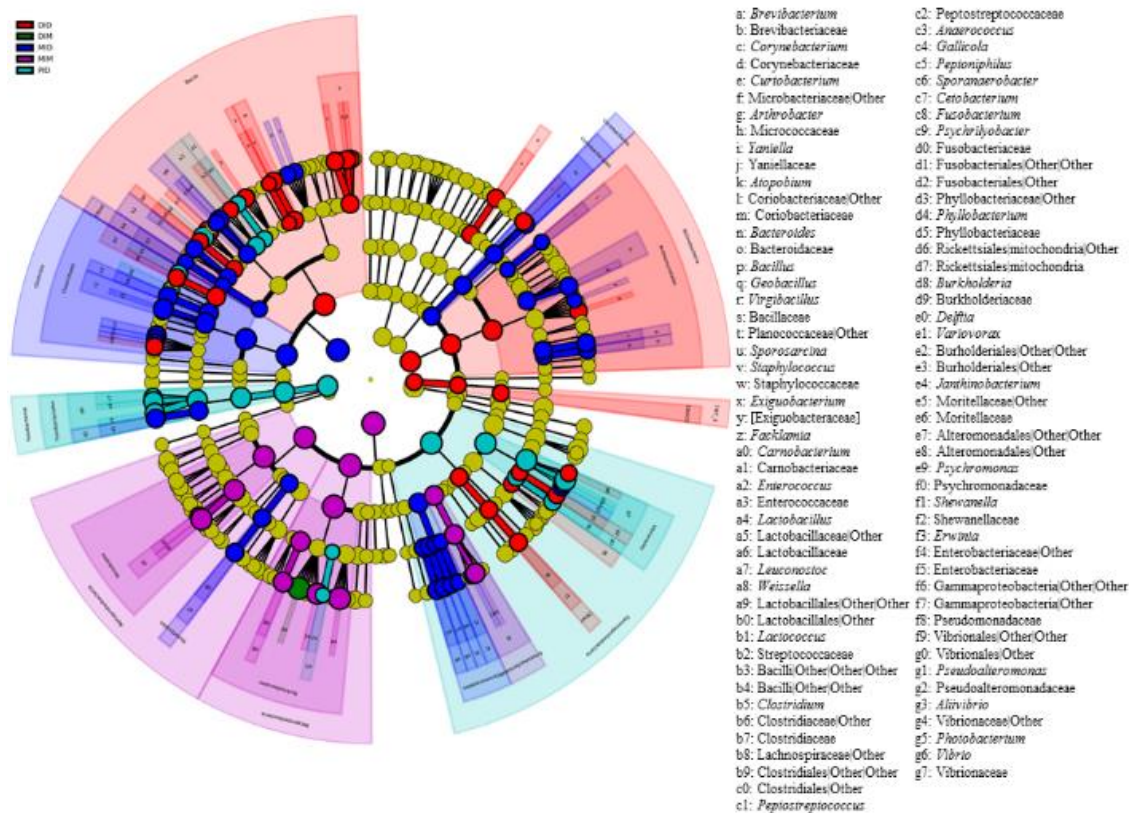


Figure 5. Circular cladogram reporting LEfSe results presenting the identified OTUs distributed according to phylogenetic characteristics around the circle. The dots in the centre present the OTUs at phylum level (name not given), whereas the outer circle of dots present the OTUs on genus level. The colour of the dots and sectors indicate the compartment in which the respective OTUs are most abundant. The colour explanation is given in the upper left corner. Yellow colour indicates OTUs that showed similar abundance in all compartments. The coloured sectors give information on class (full name in outermost circle, given only for classes showing significant difference between compartments), order (full name, next to the outer circle), family (indicated by letter, explanation given to the right in the figure) and genus (indicated by letter, explanation given to the right in the figure). Abbreviations: PID, proximal intestinal digesta (light blue); MID, mid intestinal digesta (blue); DID, distal intestinal digesta (red); MIM, mid intestinal mucosa (purple); DIM, distal intestinal mucosa (green).

Discussion

The present study provides the most detailed description of gut microbiota in Atlantic salmon to date. No studies of similar resolution regarding intestinal compartments and microbial population diversity for salmon are available in the scientific literature at present. The only other reports on

gut microbiota in farmed salmon using high-throughput sequencing are from the studies of Zarkasi *et al.*, one describing seasonal changes¹¹ and the other the bacterial community dynamic in relation to digesta and diet properties²⁷ and the study of Schmidt *et al.*,²⁸ describing the effect of fishmeal free diets in the microbial communities in a recirculation system. However, in those studies, only stripped faeces samples (for the first two studies) or a combination of mucosa and digesta of the mid-intestine (the last study) were investigated.

The observation that the dominating organisms in the digesta samples of our study belonged to the phyla Proteobacteria and Firmicutes is generally in agreement with previous studies of salmonids employing other methods of bacterial characterization than in the present^{9-11,14,18,21}. The observed high abundance of Firmicutes, especially genera belonging to the lactic acid bacteria (LAB) *Weissella* and *Leuconostoc* also agrees with previous observations from salmonids fed diets with high content of plant ingredients^{9,10,21}. However, an intermediate abundance of LAB was observed in the present study with salmon fed a diet with intermediate inclusion levels of both fishmeal and plant ingredients compared to more extreme diets containing either predominantly fishmeal or plant ingredients. In salmon fed high fishmeal diets the abundance of LAB seems to be lower^{9,10}. Likewise, adult wild salmon¹⁶ show low LAB abundance whereas the Proteobacteria and Firmicutes dominated in their intestinal contents. Variation in abundance of these Proteobacteria and Firmicutes between the wild salmon caught at different geographical locations was not significant, strengthening the many indications of a close relationship between diet and gut microbiota. The high abundance of LAB in the cultivated salmon fed plant-rich diets is likely to be the result of the presence of carbohydrates in the diet, digestible as well as indigestible, which may preferentially be used by these bacteria as substrate for growth. Their functional significance for systemic and gut health of salmon remain to be confirmed.

In their characterization of the microbiota of the distal intestinal digesta of farmed salmon, Zarkasi *et al.*,¹¹ reported higher abundance of the class Gammaproteobacteria and the phyla Firmicutes and Bacteroidetes, with variation in OTU dominance between the coldest and warmest months of the year. Greater abundance was observed during the cold season for the genera *Lactococcus*, *Weissella*, *Leuconostoc*, *Cloacibacterium*, *Carnobacterium* and *Diaphorobacter*, while in the warmest months (above 16°C) higher abundance was seen for the members of the Vibrionaceae family. Our findings from salmon held at 12 to 14°C confirm these results, as we detected a high

relative abundance of *Weissella* and *Leuconostoc* in the DID. On the other hand, and in contrast to the results of Zarkasi *et al.*,¹¹ the phylum Bacteroidetes and the genera *Carnobacterium* and *Lactococcus* were only found in minimal relative abundance, and, *Cloacibacterium* and *Diaphorobacter* were not detected at all in the present study. Geographical distance between Tasmania and Norway may help explain these differences between the studies. The results of Zarkasi *et al.*,¹¹ and those from studies of rainbow trout (*Oncorhynchus mykiss*) employing similar high-resolution tools⁹ show agreement with the current study regarding presence of *Leuconostoc*. Only a few of the earlier studies have associated *Leuconostoc* among the gut microbiota of salmon³³⁻³⁵, suggesting that the current approach give deeper insight into the characteristics of the gut microbiota.

Studies addressing composition of the microbiota associated with the gut mucosa in salmon are sparse^{6,36,37}, and the characterization methods, intestinal sections studied and the experimental conditions have differed. However, all report a high abundance of Proteobacteria, including the present study, although the quantitative aspects differ. While the previous studies report relative abundance of Proteobacteria of 30-40%, the present study indicated a far higher abundance of more than 90% of this mucosa-associated bacteria. The cause of this seemingly major difference may be related to methodological differences, but also differences in environmental conditions including dietary differences.

In both MIM and DIM, the genus *Janthinobacterium* was highly abundant. Interestingly, the species within this genus, especially *Janthinobacterium lividum*, are known to produce a pigment known as violacein, which has antiviral, antibacterial and antifungal properties³⁸. Of possible relevance are the findings indicating a mutualistic relationship between *Janthinobacterium lividum* and the red-backed salamander (*Plethodon cinereus*), preventing skin colonization and concomitant disease caused by the fungus *Batrachochytrium dendrobatidis*³⁸. It is not known whether Atlantic salmon might benefit from the high abundance of *Janthinobacterium* in the gut mucosa. Its presence, metabolism and physiological effects in salmon should be addressed in future studies.

The observation of lower richness and diversity indices for the gut mucosa-associated microbiota compared to the digesta indicates that only a fraction of the bacteria present in the intestinal digesta have the characteristics necessary for colonizing the mucosa of the host. On the other hand, it is

unlikely that microbes present in the mucosa are not present in the digesta. The observation in the present study of a core microbiota, represented by 22 OTUs, common for all compartments is partially in agreement with results of previous studies. The presence of *Pseudomonas*, *Acinetobacter*, *Microbacterium*, *Janthinobacterium*, *Burkholderia*, members of the Rhizobiales and Enterobacteriaceae has been described in several studies of the salmon gut microbiota^{6,11,16,23,24,34}. They may represent a group of well-adapted microorganisms able to colonize the gut of salmon in different environments around the world.

Our results from the PICRUSt analyses, conducted demanding clustering at 97% sequence identity, which produced a predicted functional profile, did not supply useful information. This is possibly due to qualitative and quantitative differences between our data and the databases used by PICRUSt. Similar challenges were experienced by Sullam *et al.*,³⁹ on samples from Trinidadian guppies. However, in the latter study, when using 94% sequence identity as criteria for OTU assignment, relevant results seemed to be produced for microbial populations. It has been suggested that results from PICRUSt should be applied with caution for new environments, especially when NSTI values are high⁴⁰. Improvements in the existing database annotation are necessary in order to improve the accuracy and benefit from tools predicting microbial functionality in a wider range of environments⁴¹.

When comparing the present results regarding gut microbiota of the salmon with comparable results from terrestrial mammals, similarities were found. The most common phyla found in the salmon, and also in other fish species, i.e. Proteobacteria, Firmicutes, Actinobacteria, Fusobacteria and Bacteroidetes, together with Verrucomicrobia and Spirochaetes, are also found to be the most common phyla colonizing the gut of terrestrial mammals^{17,42,43}. However, species-specific differences in the dominance of the phyla are apparent²⁵. In humans and other mammals, Firmicutes and Bacteroidetes are the most dominant phyla^{1,42} whereas in salmon, as discussed above, the Proteobacteria and Firmicutes dominate. As in mammals, the identified microbiota in salmon differed markedly between gut regions and whether the samples originated from digesta or mucosa^{42,44,45}. However, in contrast to mammals⁴² the mucosa-associated bacterial community in salmon was almost completely dominated by Proteobacteria.

Summary and concluding remarks

The present in-depth characterization of the bacterial microorganisms in five different compartments of the gut of Atlantic salmon showed that bacterial populations varied substantially between the regions of the intestine and especially between digesta and mucosa-associated compartments. Proteobacteria and Firmicutes were the most abundant phyla in the digesta while Proteobacteria almost completely dominated in the mucosa-associated microbiota. A core group of microbiota composed mainly of bacteria belonging to the phylum Proteobacteria was identified. Based on our data and previous reports on gut microbiota across species, we find similarities between salmon and mammalian gut microbial communities. As it is likely that the microbiota populating the mucosa interacts more closely, or at least differently, with the host than the microbiota populating the digesta, the present study strongly suggests that future work should observe and differentiate between microbiota in digesta and mucosa when effects of diet, environment and farm management practices are investigated. This will likely facilitate achieving much needed knowledge concerning truly functional interactions between the host, diet and the microbial communities of the gut.

Materials and Methods

Experimental fish and environmental conditions

The experiment was conducted in a recirculation system at Nofima's research station at Sunndalsøra, Norway in accordance with laws regulating experimentation with live animals in Norway and the experimental protocol was approved by the Norwegian Animal Research Authority (Forsøksdyrutvalget). Three groups of 40 PIT-tagged post-smolt Atlantic salmon weighing 67.3 ± 0.3 g (mean \pm SEM) were kept in 500 L seawater-containing tanks with a water renewal rate of 30 L/min. Water temperature during the feeding trial varied between 12 and 14°C, salinity between 32 and 33 g/L and oxygen saturation was above 85%. Fish were fed continuously by automatic disk feeders. A regimen of 24 h lighting was employed during the experimental period according to the routines of the facility.

Diet and feeding

The feeding trial lasted four weeks and feed was delivered at a rate of 120% of estimated requirement in an attempt to secure *ad libitum* feed intake. A commercial, extruded diet suitable for the size and fulfilling the nutritional requirements of the fish was used, containing 45% fishmeal, 15% soy protein concentrate, 6% wheat gluten, 4% sunflower meal, 13% wheat, 12% fish oil and 5% rape seed oil. The proximate composition was 50% crude protein, 23% crude lipid, 1% crude fibre, 13% nitrogen free extract, 9% ash and 4% water. The gross energy was calculated to be 23 MJ/kg.

Sampling procedure

At termination of the feeding period, five fish were randomly selected for sampling from the three tanks while in the fed state, two fish from two of the tanks and one from the third. Fish were anesthetized with tricaine methanesulfonate (MS222; Argent Chemical Laboratories, Redmond, WA, USA) and then euthanized by a sharp blow to the head. All sampled fish had digesta throughout the intestinal tract, ensuring intestinal exposure to the diet. The exterior of the fish was wiped clean with 70% ethanol, the abdomen opened at the ventral mid line and the whole intestine was aseptically removed from the abdominal cavity. The intestine was separated into the proximal, mid and distal intestinal regions as previously defined⁴⁶ and each was opened longitudinally. The digesta from each of the three regions; proximal, mid and distal intestine digesta (PID, MID, DID respectively) was collected separately. The mid- and distal sections of the intestine were then washed in PBS three times to remove remnants of the digesta and samples for investigation of the mucosa-associated bacteria collected by scraping the mucosal layer from a two centimetres length of the mid-section of each region (mid intestine mucosa; MIM and distal intestine mucosa; DIM) with a sterile scalpel. Mucosal samples from the proximal intestine were not collected due to difficulties in separating the mucosa from the digesta in this region. All samples were frozen immediately in liquid N₂, and thereafter stored at -80°C. The PID, MID, DID, MIM and DIM are hereafter defined as separate compartments.

DNA extraction

DNA was extracted from 100 mg of each digesta sample and 50 mg of each mucosal tissue sample. Following incubation with 50 mg mL⁻¹ of lysozyme at 37°C, DNA extraction was performed using

the QIAamp Stool Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's specification with modification as described elsewhere ⁴⁷ and summarized by Falcinelli *et al.* 2015 ⁴⁸. DNA concentrations were determined using NanoDropTM 1000 spectrophotometer (Thermo Scientific, DE, USA).

PCR amplification

To analyse the microbial populations, amplification of the variable region V1-V2 of the 16S rRNA gene was performed. The PCR was conducted using the bacterial universal primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 338R-I (5' GCW GCC TCC CGT AGG AGT 3') and 338R-II (5' GCW GCC ACC CGT AGG TGT 3') ⁴⁹. The reaction was carried out in 50 µl sample volume using 0.4 µl of DNA template for digesta samples and 2 µl of DNA template for mucosa samples, 25 µl Phusion[®] High-Fidelity PCR Master Mix (Thermo Scientific, CA, USA) and 1 µl of forward (27F) and reverse (pooled 338R-I and II) primers (50 pM). For digesta samples, the PCR was ran as follows: initial denaturation at 98°C for 2 min; 30 cycles of denaturation at 98°C for 10 s, annealing at 53°C for 30 s, and extension at 72°C for 60 s; followed by a final extension at 72°C for 10 min. For mucosa samples, a touch-down PCR strategy was used and run as follows: initial denaturation at 98°C for 3 min; 35 cycles of denaturation at 98°C for 15 s, annealing decreasing from 63°C to 53°C in 10 cycles for 30 s followed by 25 cycles at 53°C for 30 s, and extension at 72°C for 30 s; followed by a final extension at 72°C for 10 min. The resulting amplicons were then analysed in a 1.5% agarose gel. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Crawley, UK) following the manufacturer's instructions.

High-throughput sequencing

Qubit 2.0 Fluorometer (Invitrogen, CA, USA) was used to quantify the purified PCR products. The amplicons were then evaluated for fragment concentration using Ion Library Quantitation Kit (Life Technologies, CA, USA). Concentrations were adjusted to 26 pM for all samples. Amplicons were attached to Ion Sphere Particles (ISPs) using an Ion PGM Template OT2 400 kit (Life Technologies, CA, USA) according to the manufacturer's instructions. Multiplexed sequencing was conducted using 318 chip (Life Technologies, CA, USA) on the Ion Torrent Personal Genome platform (Life Technologies, CA, USA). Sequences were sorted by sample and filtered within the

PGM software to remove low quality reads. Finally, the data from each sample was exported as individual FastQ files.

High-throughput sequence data processing

Taxonomic analyses of sequence reads were performed after the removal of low quality scores (Q score < 20 in 80% of sequences) with FASTX-Toolkit (Hannon Lab). One sample of the DIM segment presented low quality reads and therefore this sample was excluded from the analysis. The rest of the sequences were concatenated and sorted by sequence similarity into a single fasta file. Sequences were then further analysed using QIIME pipeline⁵⁰. Greengenes database (version 13.8.) was used as reference database⁵¹. OTU picking was performed using the quality filter pipeline (USEARCH quality filter pipeline⁵²), with a 97% sequence identity. The taxonomic assignment was performed using RDP classified⁵³ with a confidence of 0.8. Multiple alignment was performed with PyNAST⁵⁴ with a minimum threshold length of 150 bp. The OTU table was made excluding the sequences that fail to align from the multiple alignment step. The resulting OTU table was filtered at 0.005% to reduce spurious OTUs⁵⁵ and sequences classified as Cyanobacteria were removed from the final data as they were considered to be diet associated (chloroplast sequences) and do not represent populations from the gut microbiota⁵⁶. QIIME was also used to calculate Phylogenetic tree⁵⁷, to identify the core microbiota of the compartments, defined in this study as the operational taxonomic units (OTUs) present in 80% of the samples per compartment, and to calculate the alpha and beta diversity metrics on rarefied OTU tables. Venn diagrams representing the results of the core microbiota were drawn using the web tool <http://bioinformatics.psb.ugent.be/webtools/Venn/>. Chao1, good coverage, Observed species and Shannon's diversity indices were calculated. EMPEROR⁵⁸ was used to visualize the PCoA plots from the weighted and unweighted UniFrac metrics⁵⁹. The results of the characterization of the microbiome are presented at the phylum and genus taxonomic levels.

Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt)⁴⁰ a tool predicting the functional profile of the microbiota was used. Since PICRUSt uses a closed reference OTU picking, based on the Greengenes database (version 13.5.), extraction of the OTUs from the original OTU table was performed using filter_otus_from_table.py script⁵⁰. After OTUs extraction, we observed that a large number of the original 97% OTUs were excluded and only about 10% retained for PICRUSt analysis. The analysis was performed using default settings for

OTU normalization by copy number, predicted gene family abundances and finally metagenome inference using KEGG orthology (KOs)⁶⁰. Nearest sequenced taxon index (NSTI) was calculated as an estimate of the phylogenetic distance between each assigned OTU and the closest relative with a sequence reference genome.

Statistical analysis of data

To assess the differences between the microbiota communities of the different compartments, the program PRIMER7 with PERMANOVA+⁶¹ was used. Permutation multivariate analysis of variance (PERMANOVA) was performed with 999 permutations to the weighted UniFrac distance matrix resulted from the beta diversity analysis of QIIME. Linear discriminant analysis (LDA) effect size (LEfSe)⁶² was used to characterize significant differences in OTUs among the compartments. The LEfSe analysis was performed using an alpha value of 0.01 for both the factorial Kruskal-Wallis rank sum test and pairwise Wilcoxon test and a threshold of 2.0 for the LDA. The approach used was an all-against-all multi-class analysis.

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Author Contributions

K.G. performed laboratory and bioinformatic work and wrote the manuscript. A.R. contributed with laboratory and bioinformatics support and manuscript corrections. T.M.K. planned the experiment and contributed with manuscript writing. Å.K. planned the experiment and contributed with manuscript writing and corrections. A.M.B. planned the experiment and contributed with manuscript corrections. D.L.M. contributed with experimental design and manuscript corrections. H.S. contributed with manuscript writing and corrections.

Additional Information: Sequence data have been deposited in the Sequence Read Archive of the National Centre for Biotechnology Information (SRA, NCBI) under the SRA accession SRP072036. **Competing financial interests:** The authors acknowledge that there are no conflicts of interests attached to the submitted manuscript.

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Paper III

Intestinal fluid permeability in Atlantic salmon (*Salmo salar* L.) is affected by dietary protein source

Haibin Hu^{1,2}, Trond M. Kortner¹, Karina Gajardo^{1*}, Elvis Chikwati¹, John Tinsley³, Åshild Krogdahl¹

¹ Department of Basic Sciences and Aquatic Medicine, Faculty of Veterinary Medicine and Biosciences, Norwegian University of Life Sciences, Oslo, Norway

² Key Laboratory of Aquaculture Nutrition and Feed (Ministry of Agriculture) & Key Laboratory of Mariculture (Ministry of Education), Ocean University of China, Qingdao, Shandong, P. R. China

³ BioMar Ltd., Grangemouth Docks, Grangemouth FK3 8UL, UK

* Corresponding author:

E-mail: karina.gajardo@nmbu.no (K.G).

Abstract

In Atlantic salmon (*Salmo salar* L.), and also in other fish species, certain plant protein ingredients can increase fecal water content creating a diarrhea-like condition which may impair gut function and reduce fish growth. The present study aimed to strengthen understanding of the underlying mechanisms by observing effects of various alternative plant protein sources when replacing fish meal on expression of genes encoding proteins playing key roles in regulation of water transport across the mucosa of the distal intestine (DI). A 48-days feeding trial was conducted with five diets: A reference diet (FM) in which fish meal (72%) was the only protein source; Diet SBMWG with a mix of soybean meal (30%) and wheat gluten (22%); Diet SPCPM with a mix of soy protein concentrate (30%) and poultry meal (6%); Diet GMWG with guar meal (30%) and wheat gluten (14.5%); Diet PM with 58% poultry meal. Compared to fish fed the FM reference diet, fish fed the soybean meal containing diet (SBMWG) showed signs of enteritis in the DI, increased fecal water content of DI chyme and higher plasma osmolality. Altered DI expression of a battery of genes encoding aquaporins, ion transporters, tight junction and adherens junction proteins suggested reduced transcellular transport of water as well as a tightening of the junction barrier in fish fed the SBMWG diet, which may explain the observed higher fecal water content and plasma osmolality. DI structure was not altered for fish fed the other experimental diets but alterations in target gene expression and fecal water content were observed, indicating that alterations in water transport components may take place without clear effects on intestinal structure.

1. Introduction

Feeding Atlantic salmon (*Salmo salar* L.) with diets containing high level of alternative protein sources, especially soybean meal and certain other legumes, may induce digestive disturbances including diarrhea-like conditions indicating impaired gut permeability of water. Altered permeability may lead to impaired digestive functions, reduced fish growth [1-7]. Diarrhea-like phenomenon is observed in particular in the distal compartment of the fish gut. Similar observations have been made in rainbow trout (*Oncorhynchus mykiss*) [8-10]. Modulation of digesta water content by certain plant ingredients has also been observed in pigs [11]. The effect may be a result of variation in osmotic conditions as well as level and function of the many proteins involved in regulation of intestinal fluid permeability [8, 12-15]. The latter involves both transcellular and paracellular routes. Figure 1 illustrates the many elements involved in intestinal fluid permeability. Symptoms of diarrhea, when induced by legume ingredients in the diet are frequently accompanied by altered permeability also for other components [12]. Altered permeability may be the key mechanisms in the often observed concomitant appearance of inflammation. Present knowledge on mechanisms underlying altered water permeability in diet induced diarrhea in fish is very limited. The present work aims to improve understanding of these mechanisms.

Water is absorbed from the intestinal lumen into enterocytes through Aqp-1a/1b, Aqp-8ab and Aqp-10 located at the brush border and in sub-apical regions. Water is further drawn along the osmotic gradient out of enterocytes and into the sub-mucosa through basolaterally located Aqp-8ab and finally arrives in the blood vessels. The basolaterally located Nka α -1c or α -1b, regulated by Fxyd-12, are the main and primary activated elements in ion transcellular transportation and will transport Na^+ out of enterocytes and K^+ into enterocytes. The apically located Nkcc-2 contributes to absorption of Na^+ , K^+ and Cl^- from the intestinal lumen and into enterocytes, whereas the basolaterally located Nkcc-1a secretes Na^+ , K^+ and Cl^- from enterocytes into submucosa and finally into the blood vessels. The apically and basolaterally located Cftr-II and Clc-3 transport chloride ions into and out of enterocytes. Guanylin, secreted by goblet cells into the intestinal lumen could affect the function of apically located Nkcc-2 and Cftr-II. Claudin-25b, Jam-1a/1b, Occludin, Tricellulin, E-cadherin and β -catenin could tighten the junction barrier between enterocytes and limit the paracellular flux of water, ions and macromolecules, whereas Claudin-

15 is a pore-forming protein promoting paracellular flux of cations, predominantly Na^+ and small molecules with radii $<4\text{\AA}$. These junction proteins are linked to the actin cytoskeleton via binding to Zo-1 and Cingulin. Black dotted arrows show the direction of water and ion transport, blue arrows show the function direction of junction barrier proteins. Abbreviations: Aqp, aquaporin; Nka, Na^+ , K^+ -ATPase; Nkcc, Na^+ , K^+ , 2Cl^- co-transporter; Clc, chloride channel; Cftr, cystic fibrosis transmembrane conductance regulator Cl^- channel; Jam, junctional adhesion molecule; Zo, zonula occludens.

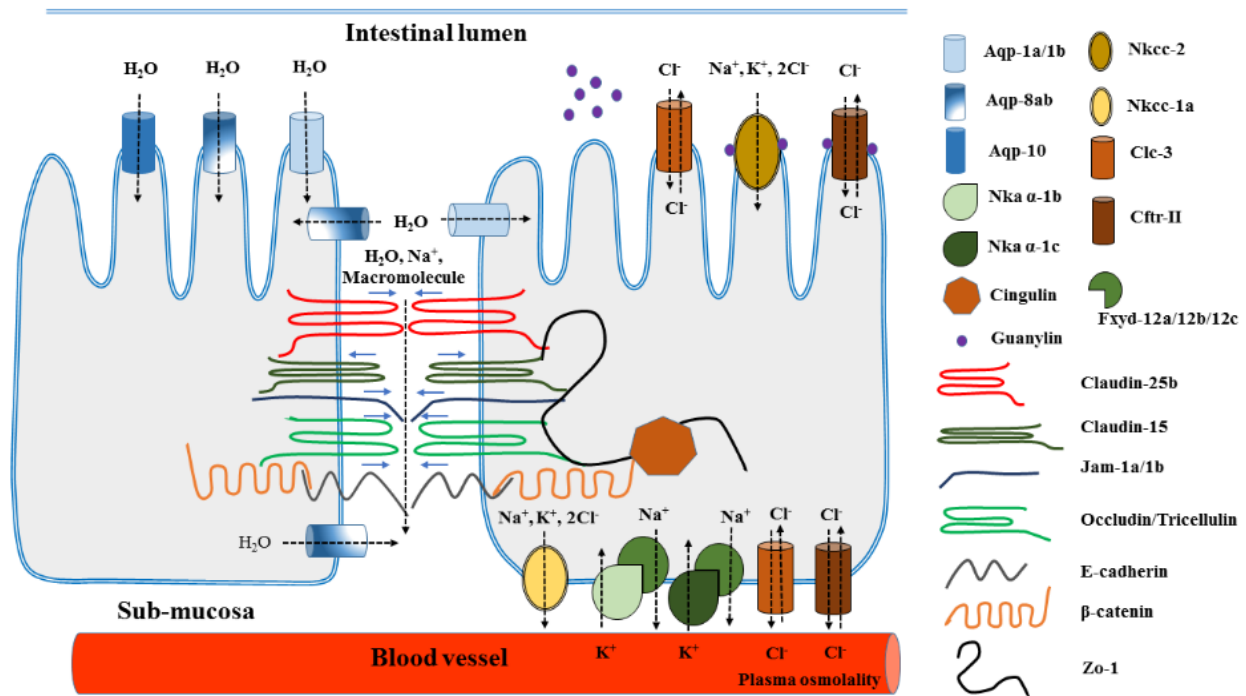


Figure 1. The elements involved in intestinal fluid permeability. Water is absorbed from the intestinal lumen into enterocytes through Aqp-1a/1b, Aqp-8ab and Aqp-10 located at the brush border and in sub-apical regions. Water is further drawn along the osmotic gradient out of enterocytes and into the sub-mucosa through basolaterally located Aqp-8ab and finally arrives in the blood vessels. The basolaterally located Nka α -1c or α -1b, regulated by Fxyd-12, are the main and primary activated elements in ion transcellular transportation and will transport Na^+ out of enterocytes and K^+ into enterocytes. The apically located Nkcc-2 contributes to absorption of Na^+ , K^+ and Cl^- from the intestinal lumen and into enterocytes, whereas the basolaterally located Nkcc-1a secretes Na^+ , K^+ and Cl^- from enterocytes into submucosa and finally into the blood vessels. The apically and basolaterally located Cftr-II and Clc-3 transport chloride ions into and out of enterocytes. Guanylin, secreted by goblet cells into the intestinal lumen could affect the function of apically located Nkcc-2 and Cftr-II. Claudin-25b, Jam-1a/1b, Occludin, Tricellulin, E-cadherin and β -catenin could tighten the junction barrier between enterocytes and limit the paracellular flux

of water, ions and macromolecules, whereas Claudin-15 is a pore-forming protein promoting paracellular flux of cations, predominantly Na^+ and small molecules with radii $<4\text{\AA}$. These junction proteins are linked to the actin cytoskeleton via binding to Zo-1 and Cingulin. Black dotted arrows show the direction of water and ion transport, blue arrows show the function direction of junction barrier proteins. Abbreviations: Aqp, aquaporin; Nka, Na^+ , K^- -ATPase; Nkcc, Na^+ , K^+ , 2Cl^- co-transporter; Clc, chloride channel; Cftr, cystic fibrosis transmembrane conductance regulator Cl^- channel; Jam, junctional adhesion molecule; Zo, zonula occludens.

Incorporations of Aquaporins (Aqp) and ion transporters in the intestinal epithelial membrane are key elements in regulation of animal intestinal transcellular fluid transport [13]. Previous studies have shown that the water channel proteins Aqp-8ab, Aqp-10, Aqp-1a (also named Aqp-1aa) and Aqp-1b (also named Aqp-1 or -1ab) are the main aquaporins in the Atlantic salmon intestine [13, 16]. Of these, Aqp-1a and Aqp-1b are located in the brush border and sub-apical region of enterocytes. Aqp-8ab is found in the same position as Aqp-1a/1b as well as in basolateral regions of enterocytes [13, 16]. The basolaterally located Na^+ , K^- -ATPase (Nka), the main Na^+ , Cl^- transporter, has been suggested as the most important and first activated element in regulation of ion-coupled transcellular fluid transport in the fish intestine [13, 17, 18]. Studies on salmon intestinal tissue have indicated that Nka α -1c and α -1b isoforms are the functional isoforms, with Fxyd-12 acting as a main modulating protein [18, 19]. In addition, Na^+ , K^- , 2Cl^- co-transporters (Nkcc), especially the apically located absorptive isoform: Nkcc-2 and the basolaterally located secretory isoform: Nkcc-1a are playing key roles. The same seems to be the case for the chloride channels (Clc), such as cystic fibrosis transmembrane conductance regulator Cl^- channel II (Cftr-II) and Clc-3 in apical and basolateral membrane of enterocytes [18, 20]. Furthermore, Guanylin, secreted by intestinal goblet cells into the gut lumen, seems to be correlated with diarrhea in fish intestine [6, 20, 21].

Paracellular permeability in the fish intestine appears to be regulated mainly via the network of tight junction proteins (TJP) and adherens junction proteins (AJP) between intestinal epithelial cells [13, 14]. Of tight junction (TJ) transmembrane proteins, Claudins, especially Claudin-15 and -25b, play key roles in intestinal fluid permeability in Atlantic salmon [13, 14, 19, 22]. Among these Claudin-25b is barrier tightening whereas Claudin-15 act as a pore forming protein allowing paracellular flux of cations, predominantly Na^+ and small molecules with radii $<4\text{\AA}$. Moreover, occludin and Tricellulin, regulating water and large macromolecule flux presumably through the

non-restrictive leak pathway, as well as junctional adhesion molecule (Jam)-1 have been reported as important barrier tightening TJPs [13, 14, 22]. These TJ transmembrane proteins bind to TJ cytoplasmic plaque proteins, mainly Zonula occludens-1 (Zo-1), and then to Cingulin [13, 14]. In addition, AJs, predominantly composed by cadherin-catenin interactions especially E-cadherins - β -catenin interaction, also regulate animal intestinal paracellular pathway [14].

The purpose of this study was to find whether modulation of the various elements involved in water transport may explain the diarrhea-like symptoms observed in salmonids fed diets with high content of alternative protein sources, i.e. soybean meal and soybean concentrate. Poultry meal and guar meal were also included in the present study as relevant alternative feed ingredients. Whereas poultry meal is quite well described as fish feed ingredient, the opposite is the situation for the guar meal, a protein rich byproduct from the guar gum industry with protein content in the range 35-50%. No information has been found in the literature regarding its nutritional value and effects of guar meal on digestive physiology in fish. The presence of antinutrients such as trypsin inhibitors and saponins make the product interesting in comparison with soybean products among which some contain many antinutrients [23]. The following endpoints were selected to get a broad perspective on mechanisms that might be involved: expressions of genes coding for aquaporins, ion transporters, tight junction and adherens junction proteins in distal intestine, plasma osmolality, fecal dry matter and intestinal histology.

2. Materials and methods

2.1. Fish management

The experiment was conducted in strict compliance with laws regulating the experimentation with live animals in Denmark as overseen by the Danish Animal Experiments Inspectorate and since the present study was a feeding trial performed with Atlantic salmon, no further approval was required. The feeding trial was performed at BioMar's RAS research facilities in Hirtshals, Denmark. For each diet, mixed-gender groups of 22 or 23 Atlantic salmon post smolt with an initial mean body weight of 314 g (SEM=2 g) were randomly distributed into duplicate 0.8 m³ fiberglass tanks, containing 1000 L seawater. The temperature was 15°C during the feeding trial. Oxygen saturation was above 85% throughout the duration of the trial, and salinity was maintained

at 33g/L \pm 1 g/L. Fish were fed continuously by automatic Belt feeders (18 hours feeding period from 1 PM to 7 AM, and feed consumption was recorded daily. The uneaten pellets were removed and dried to estimate the feed intake. A regimen of 24 h lighting was employed during the experimental period.

2.2. Diets and feeding

Diets were produced at BioMar's Tech Centre in Brande, Denmark with a pellet size of 4.5mm. Diet formulations and their chemical compositions are shown in Table 1. The control diet (Diet FM) was formulated with fishmeal as the sole protein source. In the other four experimental diets fish meal was replaced with soybean meal mixed with wheat gluten (Diet SBMWG), soy protein concentrate mixed with poultry meal (Diet SPCPM), guar meal mixed with wheat gluten (Diet GMWG) or poultry meal (Diet PM). All diets were supplemented with vitamins and minerals to fulfill the fishes' requirements for all nutrients. The diets varied somewhat in ingredient composition to be practical according to BioMar's considerations. In the presentation and discussion of the results comparisons are therefore mainly based on the difference between the control diet and each of the other experimental diets, not the differences among the other diets. The fish were fed for 48 days.

Table 1. Formulation of the experimental diets^a

	FM	SBMWG	SPCPM	GMWG	PM
		<i>Ingredients (g/kg)</i>			
Fish meal ^b	723.5	202.4	362.2	276.6	143.0
HiPro Soya		300.0			
Wheat gluten ^c		220.0		145.0	
SPC			300.0		
Guar meal				300.0	
Poultry meal ^d			60.0		580.0
Tapioca	110.0	110.0	110.0	110.0	110.0
Rape oil ^e	80.0	80.0	80.0	80.0	80.0
Fish oil ^f	80.0	80.0	80.0	80.0	80.0
Premix N ^g	6.0	7.1	7.3	7.9	6.5
Yttrium	0.5	0.5	0.5	0.5	0.5
		<i>Chemical Composition (%)</i>			
Crude protein	51.4	47.0	46.4	49.9	49.4
Crude lipid	23.3	20.5	22.3	22.0	25.7
Starch	10.6	12.0	11.8	13.3	11.4
Ash	9.3	5.0	7.3	6.0	9.6
Phosphorus	1.4	0.7	1.0	0.8	1.6
Digestible energy (MJ/kg)	21.1	18.3	18.4	18.9	19.6

^a The diets were assigned abbreviations as indicated above the columns. ^b Fish meal (Norsild, NOR): ^c Wheat gluten (Lantmännen, SW): ^d Poultry meal (Gepro, D): ^e Rape oil (Emmelev-Scanola, DK): ^f Fish oil (FF Skagen, DK): ^g Premix N: BioMar premix[®], footnote: minerals, vitamins and synthetic amino acids to cover the nutrient requirements of the species.

2.3. Sampling

At the termination of the experiment, the weight and length of all fish were recorded for calculation of growth performance. Samples were taken from six randomly selected fish from each duplicate tank. Samples were taken only from fish with digesta throughout the intestinal tract, to ensure intestinal exposure to the diets until sampling. All fish were fully anesthetized with benzocaine (20ml/100L) (Kalmagin 20%, Centrovat) and subsequently euthanized by cervical dislocation prior to tissue sampling. From all euthanized fish, blood was collected in heparinized vacutainers for plasma preparation, then frozen in liquid N₂ and stored at -40 °C for plasma osmolality measurement. The fish were manually stripped for feces by gently applying pressure to the lower abdominal region. Initial urine expression was removed and discarded before feces could

be collected. Fecal samples were immediately frozen in liquid N₂, freeze-dried and ground prior to analysis. Thereafter, the abdominal cavity was opened, and the intestine was removed and cleared of mesenteric and adipose tissue. Content from the distal intestine (DI) was collected quantitatively in separate, pre-weighed tubes, and then frozen in liquid N₂ and stored at -80 °C pending analyses. Distal intestinal tissue samples from the same six fish were collected for histological evaluation, placed in 4% phosphate-buffered formaldehyde solution for 24 h, and subsequently stored in 70% ethanol until further processing. For RNA extraction, DI tissue samples (~100 mg) from the same six fish were taken and placed in RNAlater (Ambion, Carlsbad, CA) at 4 °C for 24 h, and were subsequently stored at -20 °C.

2.4. Diet and feces analyses

Feed and feces samples were analyzed for dry matter (EU 71/393), Kjeldahl nitrogen (N) (EU 93/28), lipid (HCl hydrolysis and diethyl ether extraction (EU 98/64)), and starch (AOAC enzymatic method 996.11). Gross energy was measured by bomb calorimetry (Parr 1271 bomb calorimeter, Parr, Moline, IL). Amino acids (except tryptophan) analysis of all samples were performed with a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, U.K.) after hydrolysis, according to EC Commission Directive 98/64/EC. Tryptophan was analyzed on a Dionex Summit HPLC system, with a Shimadzu RF-535 fluorescence detector. Yttrium oxide concentrations in feed and feces were determined by inductively coupled plasma mass spectroscopy (ICPMS) as previously described [24].

2.5. Histology

Histology slides with DI tissue sections from each fish stained with haematoxylin and eosin were evaluated using a semi-quantitative technique. All slides were evaluated using light microscopy. The evaluation procedure focused on the characteristic morphological changes of soybean meal induced DI enteritis in Atlantic salmon, that consist of changes in mucosal fold length, width and cellularity of the submucosa and lamina propria, enterocyte supranuclear vacuolization, and frequency of goblet cells, intra-epithelial lymphocytes, mitotic figures and apoptotic bodies within the epithelial layer. For each of the morphological characteristics, the degree of change was graded using a scoring system with a scale of 0-10 where 0-2 represented normal, >2 to 4 mild changes, >4 to 6 moderate changes, >6-8 marked changes, and >8-10 severe

changes. Scoring was done using a visual analogue scale to generate the scores as continuous variables that allowed conducting one way analysis of variance (ANOVA) statistical analyses on the data.

2.6. Plasma osmolality

Blood plasma osmolality from each fish was determined by freezing point depression by a cryoscopic method using a Knauer osmometer (semi-micro-osmometer type ML. no. A0299, Berlin, Germany).

2.7. Quantitative Real Time PCR (qPCR)

Total RNA was extracted from DI tissue samples (~30 mg) from the first five fish in the first duplicate tank and the first four fish in the second duplicate tank per treatment by using Trizol reagent (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA) and purified with Pure Link (Invitrogen™) including an on-column DNase treatment according to the manufacturer's protocol. The integrity of the RNA samples was verified by the 2100 Bioanalyzer in combination with RNA Nano Chip (Agilent Technologies, Santa Clara, CA, USA). RNA integrity numbers (RIN) were >8 for all samples, with an average RIN of 8.9. RNA purity and concentrations were measured using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). Total RNA was stored at -80°C until use. First-strand complementary DNA was synthesized from 0.8 µg total RNA from all samples using SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen™). Negative controls were performed in parallel by omitting RNA or enzyme. The qPCR primers for amplification of gene-specific PCR products were designed using Primer3web software version 4.0.0 (<http://primer3.ut.ee/>) or obtained from the literature. The primer details are shown in Table 2. All primer pairs were first used in gradient reactions in order to determine optimal annealing temperatures. To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analysis and visual inspection of the PCR products by agarose gel electrophoresis. PCR efficiency for each gene assay was determined using 2-fold serial dilutions of randomly pooled complementary DNA. The expressions of individual gene targets (9 fish per group, 4 or 5 individuals from each tank duplicate) were analyzed using the LightCycler 96 (Roche Diagnostics, Basel, Switzerland). Each 10 µl DNA amplification reaction contained 2

µl PCR grade water, 2 µl of 1:15 diluted complementary DNA template, 5 µl LightCycler 480 SYBR Green I Master (Roche Diagnostics) and 0.5 µl (10mM) of each forward and reverse primer.

Each sample was assayed in duplicates, including a no-template control. The three-step qPCR run included an enzyme activation step at 95°C (5 min), forty to forty-five cycles at 95 °C (10 s), 55–62 °C (depending on the primers used, 10 s; see Table 2) and 72°C (15 s) and a melting curve step. Distal intestinal gene expression was normalized to the geometric average of glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), RNA polymerase 2 (*rnapolii*) and hypoxanthine phosphoribosyltransferase 1 (*hpri1*) expression as evaluated elsewhere [25]. Mean normalized expression of the target genes was calculated from raw Cq values by relative quantification [26].

Table 2. Details of primer pairs used for real-time PCR assays*

Gene name	5'-3' primer sequence		Amplicon size (bp)	Annealing temperature (°C)	Primer efficiency	GenBank accession no.	Primer reference
	Forward	Reverse					
<i>aqp-1a</i>	CTACCTTCCAGCTGGTCCTG	TGATACCGCAGCCTGTGTAG	141	62	2.05	BT046625	[16]
<i>aqp-1b</i>	CTGTGGGTCTGGGACATCTT	TAAGGGCTGCTGCTACACCT	153	62	2.00	NM_001140000.1	[16]
<i>aqp-8ab</i>	GGAGCTGCCATGTCAAAGAT	CGCCCTAGCAATACTACCA	159	60	2.00	KC626879.1	[6]
<i>aqp-10</i>	GGTGTGGTGATCGGAGTCT	CGCCCTAAACACCTCATCC	121	60	1.68	DW569041	[16]
<i>nka α-1c</i>	GAGAGGGAGACGTACTACTAGAAAGCA	CAGCAAGACAACCATGCAAGA	69	60	2.01	NM_001124459	[47]
<i>nka α-1b</i>	CTGCTACATCTCAACCAACAACATT	CACCATCACAGTGTTCAATTGGAT	81	60	2.00	NM_001124460	[47]
<i>fxyd-12a</i>	ATCCCTCCGTCATTGGTCAA	TGCTTCATAACTGCTTCCCTG	96	62	2.01	NM_001123730.1	
<i>fxyd-12b</i>	AGGGCAGCTGACTACAGAGA	TGCATCAGGGTCTGACTCAG	139	55	1.96	NM_001123730.1	
<i>fxyd-12c</i>	GACCTTCGTTGCAGTCATCA	GGCTTCTCTCAACCTTCTTTC	102	62	2.00	NM_001123730.1	
<i>nkcc-1a</i>	GATGATCTGCGGCCATGTTC	CTCGTTCTTCATCAGCCAGC	100	60	1.97	NM_001123683.1	
<i>nkcc-2</i>	CCGCGTGCCCAACATC	GCACGGTTACCGCTCACACT	57	60	2.03	NM_001141520	[18]
<i>clc-3</i>	CGAGGGCATCTACGAATCAC	CTCCTTGGCGTCGAGGAA	61	60	1.95	NM_001173586	[18]
<i>cfr-11</i>	AGAATGGGACCGAGAAGTGG	CTCCCCAAAGTACAGCAGGA	115	62	1.91	NM_001123534.1	
<i>guanylin</i>	ATCTGGTCTCTGAAGCCAC	CCGTTTACACTGCTGCTCTC	115	60	2.02	BT047912.2	
<i>claudin-15</i>	GGCACGCTCTGAGAAACAACA	TAGGAAGTGGCAGCCTGACT	92	60	2.02	BK006395	[19]
<i>claudin-25b</i>	CCTGTAAGAGGGGTCCATCA	TGACACATGTTCTGCCCTGT	101	60	1.98	BK006399	[19]
<i>occludin</i>	GACAGTGAGTCCCCACCAT	ATCTCTCCCTGCAGGTCTT	101	60	2.12	NM_001173656.1	[22]
<i>tricellulin</i>	GGATGCCATGATGGGTAAC	AGGAAGGCTGGGTCACTCTT	111	60	2.03	DW548339	[22]
<i>jam-1a</i>	TCGGCAGGCTATTCAAGAGT	AGTAGGTTCTCTGGCACTG	110	60	2.00	GBR01043957.1	
<i>jam-1b</i>	CGTTGCGGAAGGGCGTAG	CCAGCATGTGTCGGATTTC	146	60	2.00	GBR01043958.1	
<i>zo-1</i>	CAAAGCCAGTGTATGCCAG	CAGTTCATACTCGGCTGA	119	60	1.93	>gnl SRA SRR403078.506834.2	
<i>cingulin</i>	AAAAGCACCTGGAGAGACA	TTTGTTCTCTCCTCCACCC	147	60	2.02	JT824570	
<i>e-cadherin</i>	ACTATGACGAGGAGGGAGGT	TGGAGCGATGTCATTACGGA	107	60	1.98	BT058864.1	
<i>β-catenin</i>	ATGAGGACGACAGATACCAA	CTGGGGTGTCTGGGAACTTT	126	62	2.07	NM_001140567.1	
<i>gapdh</i>	AAGTGAAGCAGGAGGGTGGAA	CAGCCTCACCCCATTTGATG	96	60	1.85	BT050045	[25]
<i>rnapii</i>	CCAATACATGACCAATATGAAAGG	ATGATGATGGGATCTTCTGCTC	157	60	1.80	BG936649	[25]
<i>hpri1</i>	CCGCCTCAAGAGTACTGTAAAT	GTCGGAACTCAAACCTATG	255	60	1.99	BT043501	[25]

*Abbreviations: *aqp*, aquaporin; *nka*, Na⁺, K⁻-ATPase; *nkcc*, Na⁺, K⁻, 2Cl⁻ co-transporter; *clc*, chloride channel; *cfr-11*, cystic fibrosis transmembrane conductance regulator Cl⁻ channel II; *jam*, junctional adhesion molecules; *zo*, zonula occludens; *gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *rnapii*, RNA polymerase 2; *hpri1*, Hypoxanthine phosphoribosyltransferase 1.

2.8. Calculation

The growth performances including specific growth rate (SGR), feed conversion ratio (FCR) and feed intake (FI), and apparent digestibility (AD) were calculated according to the previous studies [27, 28].

2.9. Statistical analysis

Data were tested for normality and variance homogeneity using the Shapiro–Wilk W goodness of fit test and the Bartlett test, respectively. When necessary, data were Log-transformed to achieve normal distribution (indicated by a superscript “†”). These data were subjected to one-way ANOVA using SPSS 20.0.0. When overall differences were significant ($P<0.05$), Tukey’s test was used to compare the means among individual treatments. Since some qPCR data did not fulfill the requirement of normal distribution after Log-transformation (indicated by a superscript “§”), the analysis was performed using the Wilcoxon/Kruskal–Wallis test followed by the Post.-hoc Steel-Dwass method to compare the means. Statistical analysis of these qPCR data was performed using JMP statistical software (version 10, SAS Institute, United States). The level of significance for all analyses was set at $P<0.05$. Mean values with different superscript letters within a column or a line indicated significant differences between groups ($P<0.05$). Two-tailed Pearson correlation coefficients were calculated to determine possible relations between plasma osmolality and intestinal gene expressions.

3. Results

3.1. General fish performance and nutrient digestibilities

Neither SGR nor FI was significantly affected by replacement of the fish meal with any of the alternative protein sources. However, for all replacement diets, significantly higher FCR was observed than that of the FM diet. The SBMWG diet increased ADs of protein and the amino acids Trp, Ser, Glu, Pro, Gly and Cys, whereas ADs of starch and energy decreased compared to the FM diet (Table 3 and 4; Data shown only for essential amino acids and Cys). Lipid digestibility was not significantly affected by SBMWG diet. Fish in SPCPM group showed significantly decreased ADs of protein and the amino acids Val, Thr, Ile, Leu, Lys, Met, Ser, Glu, Pro, Gly, Ala and Asp

(Table 3 and 4). Also AD of energy in SPCPM treatment was significantly decreased compared to fish in the FM treatment. Fish fed the GMWG diet showed significantly decreased ADs of starch, energy, and the amino acids Val, Arg, Thr, Ile, Leu, Phe, Lys, Ala, Asp and Tyr (Table 3 and 4), and the same trend was seen for AD of lipid, while significantly increased AD of amino acid Cys. The diet with PM reduced ADs of crude protein, all amino acids as well as energy (Table 3 and 4), and the same trend was seen for AD of lipid.

Table 3. Fish growth performance, chyme water content and macronutrients apparent digestibility, and plasma osmolality*

	SGR (%/day)	FI (%/day)	FCR [†]	Dig. Lipid, %	Dig. Protein, %	Dig. Starch, %	Dig. Energy, %	Chyme water, %	Plasma osmolality, mOsmol/kg
<i>One-way analysis of variance (ANOVA)</i>									
<i>P</i> value	0.06	0.30	<0.001	0.02	<0.001	<0.001	<0.001	<0.001	<0.01
Pooled SEM	0.04	0.02	0.02	0.5	1.2	1.9	0.8	0.45	1.3
<i>Mean values</i>									
FM	1.43	1.16	0.82 ^c	97.5 ^{ab}	89.3 ^b	85.5 ^a	91.0 ^a	86.69 ^b	295 ^b
SBMWG	1.20	1.20	1.00 ^a	98.7 ^a	92.7 ^a	70.8 ^{bc}	86.3 ^b	89.15 ^a	305 ^a
SPCPM	1.37	1.28	0.93 ^b	97.8 ^{ab}	86.9 ^c	82.6 ^a	84.4 ^{bc}	88.58 ^a	302 ^{ab}
GMWG	1.28	1.24	0.96 ^{ab}	95.5 ^{ab}	88.4 ^{bc}	66.0 ^c	83.0 ^c	86.38 ^b	300 ^{ab}
PM	1.15	1.14	0.99 ^{ab}	95.1 ^b	77.9 ^d	78.4 ^{ab}	82.3 ^c	83.64 ^c	293 ^b

*Abbreviations: SGR, specific growth rate; FCR, feed conversion ratio; FI, feed intake. For explanation of diet abbreviations see Table 1. Log-transformed data are indicated by †. Mean values with different superscript letters within a column are significantly different ($P < 0.05$).

Table 4. Apparent digestibility (%) of essential amino acids and Cys

	Val	Arg	Thr	Ile	Leu	Phe	Lys	His	Trp	Met	Cys
	<i>One-way analysis of variance (ANOVA)</i>										
<i>P</i> value	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00
Pooled	1	1	1	1	1	1	1	1	1	1	01
SEM	1.3	1.1	1.4	1.2	1.3	1.1	1.0	1.3	1.7	1.2	2.2
	<i>Mean values</i>										
FM	91.8 ^a	94.7 ^{ab}	91.1 ^a	92.2 ^a	93.5 ^a	93.7 ^{ab}	94.4 ^a	91.7 ^{ab}	86.9 ^b	93.4 ^a	79.8 ^b
SBMWG	93.9 ^a	96.1 ^a	91.8 ^a	94.3 ^a	94.8 ^a	95.9 ^a	94.5 ^a	93.6 ^a	91.6 ^a	95.0 ^a	88.1 ^a
SPCPM	88.7 ^b	93.4 ^{bc}	86.5 ^b	89.7 ^b	90.2 ^b	91.3 ^{bc}	92.4 ^b	89.5 ^b	86.1 ^b	90.4 ^b	74.5 ^b
GMWG	88.5 ^b	92.1 ^c	87.2 ^b	89.0 ^b	89.1 ^b	90.6 ^c	92.4 ^b	89.8 ^b	87.1 ^b	93.0 ^{ab}	86.3 ^a
PM	77.5 ^c	82.3 ^d	75.2 ^c	79.0 ^c	79.0 ^c	82.6 ^d	82.5 ^c	78.2 ^c	70.3 ^c	81.1 ^c	62.7 ^c

For explanation of diet abbreviations see Table 1. Mean values with different superscript letters within a column are significantly different ($P < 0.05$)

3.2. Chyme water content, plasma osmolality and distal intestinal morphology

Among the diets, SBMWG and SPCPM diets significantly increased chyme water content in the distal intestine (Table 3). The SBMWG also elevated plasma osmolality significantly whereas fish fed the SPCPM showed a clear trend in the same direction (Table 3). Alteration of DI morphology was seen only for the SBMWG diet with significant reductions in mucosal fold height. The observed alterations regarded the supranuclear vacuolization which was graded to be mild to moderate (Figures 2 and 3). The GMWG diet did not cause clear effects either on chyme water content or plasma osmolality, whereas the PM diet decreased DI chyme water content without clearly affecting plasma osmolality (Table 3). Neither of the two latter diets altered DI histology.

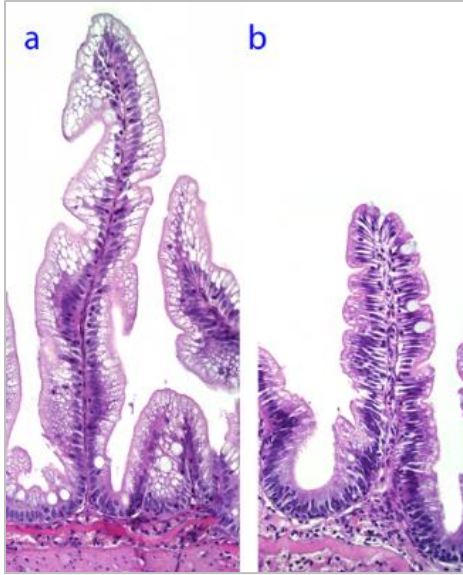


Figure 2. Representative histological appearances of distal intestine tissue scored as normal (a) and moderately altered (b).

3.3 Aquaporins, ion transporters and regulatory proteins in mucosa of the distal intestine

Fish fed the SBMWG diet showed the clearest effects on the observed gene expressions i.e. on aquaporins, ion transporters and their regulating proteins (Table 5 and 6). Significantly lower expression was observed for *aqp-8ab* and *aqp-10*, and *aqp-1a* and *aqp-1b* showed the same trend. Regarding the apically located ion transporters *nkcc-2* and cystic fibrosis transmembrane conductance regulator Cl⁻ channel II (*cftr-II*) gene expressions were not significantly affected, but expression of the gene coding for *guanylin*, a regulator of the Nkcc and Cftr transporters, was significantly reduced. Among the observed genes coding for laterally located transporters, *nka α-1c* showed significantly lower expression. Significantly lower gene expression was observed also for the protein *fxyd-12c*, serving as a regulatory subunit of Nka and/or modulator of ion transporters. Expression of the other investigated ion transporters and modulators, *nka α-1b*, *nkcc-1a*, chloride channels (*clc-3*), *fxyd-12a* and *fxyd-12b* were not significantly altered by SBMWG feeding. Similar directions of alterations as seen in fish fed SBMWG diet were observed in fish fed SPCPM diet, but the effects were not significant except for the effect on expression of the *fxyd-12c*. Fish fed the GMWG diet showed significant reduction in *aqp-8ab* and *aqp-10*. In these fish neither *nkcc-2*, *cftr-II* nor *guanylin* expression showed significant effect, whereas *nka α-1c*, *fxyd-12a*, *fxyd-12b* as well as *fxyd-12c* showed significantly reduced expression. Fish fed the PM diet

did not show significant alteration in expression of any of the aquaporins, ion transporters or their regulatory proteins.

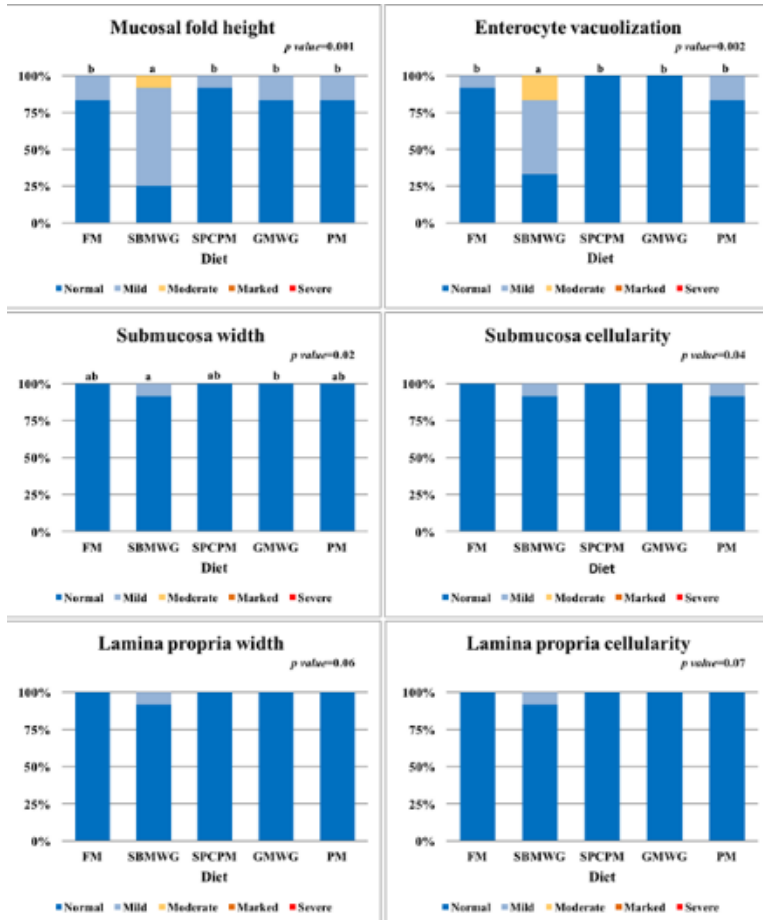


Figure 3. Contingency charts of the distal intestine morphology results For explanation of diet abbreviations see Table 1. Contingency charts showing proportions of sampled individuals that scored “normal”, “mild”, or “moderate” (none scored above moderate) for selected distal intestine morphological characteristics. P values for the one-way ANOVA analyses are given.

Table 5. Gene expression levels of aquaporins in the distal intestine

	<i>aqp-1a</i> [†]	<i>aqp-1b</i> [†]	<i>aqp-8ab</i>	<i>aqp-10</i>
<i>One-way analysis of variance (ANOVA)</i>				
<i>P</i> value	0.33	0.11	<0.001	0.001
Pooled SEM	0.002	0.0003	0.07	0.008
<i>Mean values</i>				
FM	0.013	0.0025	1.6 ^a	0.20 ^a
SBMWG	0.012	0.0021	0.7 ^b	0.11 ^b
SPCPM	0.015	0.0029	1.2 ^{ab}	0.15 ^{ab}
GMWG	0.013	0.0038	0.8 ^b	0.12 ^b
PM	0.022	0.0031	1.4 ^a	0.17 ^{ab}

For explanation of diet and gene abbreviations see Table 1 and 2. Log-transformed data are indicated by †. Mean values with different superscript letter within a column are significantly different ($P < 0.05$).

Table 6. Gene expression levels of ion transporters in the distal intestine

	<i>nkcc-2</i> [†]	<i>cfr-II</i>	guanylin [†]	<i>nka-1c</i>	<i>nka-1b</i> [†]	<i>fxyd-12a</i> [†]	<i>fxyd-12b</i> [§]	<i>fxyd-12c</i> [§]	<i>nkcc-1a</i>	<i>clc-3</i> [†]
<i>One-way analysis of variance (ANOVA)</i>										
				<0.0						
<i>P</i> value	0.44	0.77	0.03	1	0.44	<0.001	0.02	<0.001	0.22	0.43
Pooled SEM	0.006	0.001	0.03	0.08	0.0003	0.003	0.005	0.13	0.0005	0.001
<i>Mean values</i>										
FM	0.09	0.026	0.69 ^a	4.6 ^a	0.0037	0.125 ^a	0.174 ^a	5.8 ^a	0.0119	0.027
SBMWG	0.07	0.024	0.36 ^b	4.0 ^b	0.0047	0.108 ^{ab}	0.159 ^{ab}	4.2 ^c	0.0102	0.034
SPCPM	0.05	0.024	0.54 ^{ab}	4.2 ^{ab}	0.0040	0.110 ^{ab}	0.147 ^{ab}	4.4 ^{bc}	0.0103	0.029
GMWG	0.05	0.029	0.46 ^{ab}	3.7 ^b	0.005	0.094 ^b	0.135 ^b	4.3 ^c	0.0109	0.027
PM	0.06	0.026	0.52 ^{ab}	4.3 ^{ab}	0.0045	0.131 ^a	0.172 ^{ab}	5.2 ^{ab}	0.0134	0.028

For explanation of diet and gene abbreviations see Table 1 and 2. The † indicates log-transformed data, § indicates data analyzed by Wilcoxon/Kruskal–Wallis test and Post.-hoc Steel-Dwass method. Mean values with different superscript letters within a column are significantly different ($P < 0.05$).

3.4 Tight junction and adherens junction proteins in mucosa of the distal intestine

Also regarding gene expression of the tight junction proteins the clearest effects were seen in fish fed the SBMWG diet (Table 7). Significantly decreased expression was observed for *claudin-15* and *cingulin* whereas expression of *occludin*, *jam-1b*, *e-cadherin* and β -*catenin* were significantly elevated. The same direction of alteration was seen in fish fed the SPCPM diet, but none of these alterations were significant. Fish fed the GMWG diet showed significant diminishing effect on expression of *claudin-15*. For the other tight junction endpoints the effects were not significant. In fish fed the PM diet, one significant effect was observed: elevation of expression of *occludin*.

Table 7. Gene expression levels of junction proteins in the distal intestine

	<i>claudin-15</i> [†]	<i>claudin-25b</i> [§]	<i>occludin</i> [†]	<i>tricellulin</i> [†]	<i>jam-1a</i> [§]	<i>jam-1b</i>	<i>zo-1</i>	<i>cingulin</i> [†]	<i>e-cadherin</i> [§]	β - <i>catenin</i>
<i>One-way analysis of variance (ANOVA)</i>										
<i>P</i> value	<0.01	0.04	<0.001	0.05	0.03	0.02	<0.01	0.001	<0.01	0.04
Pooled SEM	0.06	0.04	0.006	0.007	0.002	0.006	0.004	0.0007	0.073	0.002
<i>Mean values</i>										
FM	1.7 ^a	0.59	0.06 ^b	0.14 ^b	0.113 ^{ab}	0.15 ^b	0.089 ^{ab}	0.012 ^a	1.940 ^{bc}	0.052 ^b
SBMWG	1.1 ^b	0.55	0.11 ^a	0.14 ^b	0.125 ^a	0.20 ^a	0.066 ^b	0.005 ^b	2.746 ^a	0.066 ^a
SPCPM	1.4 ^{ab}	0.67	0.07 ^b	0.16 ^{ab}	0.111 ^{ab}	0.17 ^{ab}	0.079 ^{ab}	0.008 ^{ab}	2.143 ^b	0.060 ^{ab}
GMWG	1.2 ^b	0.58	0.10 ^{ab}	0.16 ^{ab}	0.104 ^b	0.15 ^b	0.080 ^{ab}	0.007 ^{ab}	1.939 ^c	0.063 ^{ab}
PM	1.6 ^{ab}	0.94	0.13 ^a	0.20 ^a	0.111 ^{ab}	0.17 ^{ab}	0.107 ^a	0.011 ^a	2.156 ^b	0.053 ^b

For explanation of diet and gene abbreviations see Table 1 and 2. The † indicates log-transformed data, § indicates data analyzed by the Wilcoxon/Kruskal–Wallis test and post-hoc Steel-Dwass method. Mean values with different superscript letters within a column are significantly different ($P < 0.05$).

3.5. Correlation between plasma osmolality and gene expression

The mRNA level of *aqp-1a*, *nka a-1c*, *fxyd-12a*, *fxyd-12b*, *fxyd -12c*, *nkcc-2*, *claudin-15* and *cingulin* in Atlantic salmon DI showed significantly negative correlation with fish plasma osmolality ($P < 0.05$, Table 8).

Table 8. Two-tailed Pearson correlation coefficients between plasma osmolality and intestinal gene expressions.

	<i>aqp-1a</i>	<i>nka α-1c</i>	<i>fxyd-12a</i>	<i>fxyd-12b</i>	<i>fxyd-12c</i>	<i>nkcc-2</i>	<i>claudin-15</i>	<i>cingulin</i>
<i>r</i>	-0.30	-0.39	-0.38	-0.32	-0.43	-0.39	-0.37	-0.33
<i>P</i> value	<0.05	<0.05	<0.05	<0.05	<0.01	<0.01	<0.05	<0.05

For explanation of gene abbreviations see Table 2. Correlation was considered significant when $P < 0.05$. Only significantly correlated genes are shown.

Discussion

The results shown by fish fed the SBMWG diet gave the clearest basis for answering of the question of the present work: what are the physiological alterations underlying the elevated water content of chyme of the distal intestine? As the wheat gluten in the SBMWG diet has no identified antinutrients and has been found to have high nutritional value in Atlantic salmon, the causing agent was, in all likelihood, a soybean component [5, 29]. The observed effect of the SBMWG diet on chyme water content is in line with the results of numerous studies involving standard soybean meals, which when included in salmonid feed induce diarrhea-like conditions as well as enteritis [2-7]. The morphological alterations seen in the present study were less severe than often observed in Atlantic salmon fed diets with 30% soybean meal. The explanation may be related to level of antinutrients in the specific soybean variety used, processing conditions of the product, ingredient of the diets interacting with the antinutrient effects, feed intake, genetic characteristics of the fish, etc.

The results indicate that the elevation in chyme water content in fish fed the SBMWG was a result of reduced transport of water both into and out of the enterocytes via the aquaporins and ion transporters on both the apical and the basolateral side of the cell, i.e. as indicated by the observed decrease in expression of the genes *aqp-8ab* and *aqp-10*, *nka α-1c*, and the Nka associated, modulating gene *fxyd-12c*. The effect of the decrease in expression of *guanylin* is unclear as its product is known as an inhibitor of Na⁺-K⁺-transporters in the intestinal mucosa and to cause diarrhea, i.e. reduced expression would counteract the observed effects on the aquaporins and ion transporters [30]. However, Guanylin has also been suggested to exert the opposite effect, to increase intestinal ion and ion-coupled fluid absorption in fish in seawater by mobilizing Nkcc-2 activity via stimulating Cl⁻ supply into intestinal lumen through apical membrane Cftr-like channels [20, 21]. The effects observed on expression of tight junction and adherens junction proteins, i.e. decrease for *claudin-15* expressions and increase for *occludin*, *jam-1b*, *e-cadherin* and *β-catenin* expressions, all are expected to tighten the junction barrier and limit water and ion paracellular permeability [13, 14, 19, 22]. These results of the present study complement results of previous studies investigating effects of diet-induced distal intestinal inflammation on TJ function and fluid permeability in salmon. Similar to the observations in the present work, aquaporins, in particular *aqp-8*, were observed to be markedly reduced during distal intestinal inflammation in salmon, whereas *occludin* was found to be induced [6, 27, 31, 32]. Additionally, gene expression was reduced for several barrier tightening TJPs, including *claudin* and *cadherin* isoforms [6, 31, 32]. The study by Grammes et al. [31], as the present, also showed decreased expression of pore-forming protein *claudin-15*.

The saponins in the soybean meal were, most likely, the main trigger of the observed effects as they in pure form have been found to be able to induce enteritis in Atlantic salmon [6, 27]. Also other soybean antinutrients may be involved aggravating the symptoms as indicated in studies with other animal species and cell models. Dietary inclusion of soya fiber (10%) was found to increase *occludin* and/or *zo-1* expressions in ileum and colon of weaning piglets [33] and soya oligosaccharides (0.5%) also have similar effects on weaning piglets [46]. Moreover, phytoestrogens such as soya genistein have been observed to increase *occludin*, *zo-1*, *e-cadherin* and *β-catenin* mRNA and protein expressions in the *Apc^{Min/+}* mouse model of colorectal cancer and in Caco-2 cells [34, 35]. Also dietary phytate (4.4 g/kg) may decrease Nka activity as shown in chicken duodenum and jejunum [36, 37]. Dietary β-conglycinin (8%) in fishmeal diet was found

to decrease Nka activity in all intestinal segments of Jian carp [38], and β -conglycinin hydrolysates (0.5 g/L) could increase tight junction barrier formation in Caco-2 cells [39]. Accordingly, several of the antinutrients in soya, alone or in combination, may be of the triggers of effects of the SBMWG diet on expression of genes coding for proteins involved in intestine fluid permeability in the present study.

Atlantic salmon when in salt water, as well as other marine fish, are constantly losing water to the external hyperosmotic seawater across their body surfaces and via the kidney and need to drink salt water to maintain body osmolality [40]. Any factor reducing water absorption from the intestine would be expected to affect body osmolality as seen in the present study in fish fed the SBMWG diet. The higher plasma osmolality might further trigger thirst and stimulate fish drinking, which may aggravate the condition leading to diarrhea [41].

The moderate effects of the SPCPM diet observed on the elements involved in water transport across the intestinal mucosa indicate that processing of soybean meal into soy protein concentrate removes most but not all the antinutrients involved in gut effects of soybean meal. These results are consistent with the previous studies on Atlantic salmon showing decreased chyme dry matter as well as disturbances of nutrient digestibility when diets with high level of soybean concentrate were fed [8, 42, 43]. Intestinal paracellular fluid permeability alternation caused by soy protein concentrate in the diet has also been found in mice colon as indicated by increased expression of barrier-tightening TJPs *claudin-1* and *occludin* mRNA [44].

Among the antinutrients mentioned above as possible triggers of the observed effects saponins, phytoestrogens and oligosaccharides are removed during the alcohol water extraction in the production of the SPC, and the potentially antinutritional effects of β -conglycinin and other protein antinutrients such as protease inhibitors and lectins are inactivated to a large extent by the denaturing effects of the solvents involved in the processing. Left among the antinutrients are fiber and phytate [45]. These might be the partial reasons why the SPCPM diet changed ion-transporter expression of *fxyd-12c* in this study, as discussed above. The fact that fish fed the diets with the two soybean products, i.e. the SMBWG and SPCPM diet, both showed increased and very similar DI chyme water content indicates that the mechanisms underlying this response may occur without symptoms of enteritis and even if the effects on expression of the proteins involved in

water transport are minor. The components which the two products have in common, such as fiber (non-starch polysaccharides) and phytic acid are the most likely causing agent(s).

In the present study the GMWG diet decreased *aqp-8ab*, *aqp-10*, *nka α -1c*, *fxyd-12a*, *fxyd-12b*, *fxyd-12c* as well as *claudin-15* gene expression supposedly due to antinutrients in the guar meal. However, fish chyme water content was not affected, in contrast to what would have been expected from the effects of the SBMWG diet on expression of genes coding for aquaporin, ion transporter and tight junction proteins. The observed difference in effect of the SBMWG and GMWG diet on chyme water content may indicate that also other mucosal components involved in control of water permeability components may play a role for the resulting chyme water content.

The decrease in chyme water content observed in the fish fed the PM diet did not seem to be related to major alterations in water transport across the intestinal mucosa of the distal intestine as only expression of one of the observed water transport variables was significantly affected. Occludin which is considered as a tight junction tightening protein, was significantly increased, an effect which would be expected to reduce water transport and rather increase chyme water content. The decrease in chyme water content was most likely a result of the low lipid digestibility and a resulting higher lipid content.

As the recipes of the diets used in the present work were not designed for evaluation of nutritional value of specific ingredients, no comparison of nutritional quality is presented.

Conclusions

Increased water content in chyme of the distal intestine of Atlantic salmon with corresponding increase in plasma osmolality was observed in fish fed a diet containing standard soybean meal. These fish also showed alterations in expression of aquaporins, ion transporters, associated proteins, tight junction and adherens junction proteins, which can explain the chyme and plasma alterations. The work also indicated that alterations in the water transport components may take place without alterations in chyme water content and plasma osmolality.

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The authors' contributions were as follows: H. H. performed the gene expression and plasma osmolality work and wrote the manuscript; T. M. K. guided the gene expression work and revised the manuscript; E. C. and K. G. performed the histopathology work and revised the manuscript. Å. K., J. T. planned and supervised the study and reviewed the manuscript.

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Paper IV

Alternative protein sources in the diet modulate microbiota and functionality in the distal intestine of Atlantic salmon (*Salmo salar*)

Karina Gajardo^{1#}, Alexander Jaramillo-Torres², Trond M. Kortner¹, Daniel L. Merrifield², John Tinsley³, Henning Sørum⁴, Anne Marie Bakke¹, Åshild Krogdahl¹

¹ Department of Basic Sciences and Aquatic Medicine, Faculty of Veterinary Medicine and Biosciences, Norwegian University of Life Sciences (NMBU), Oslo, Norway

² Aquaculture and Fish Nutrition Research Group, School of Biological Sciences, Plymouth University, UK

³ BioMar Ltd., Grangemouth Docks, Grangemouth, UK

⁴ Department of Food Safety and Infection Biology, Faculty of Veterinary Medicine and Biosciences, Norwegian University of Life Sciences (NMBU), Oslo, Norway

Address correspondence to Karina Gajardo, karina.gajardo@nmbu.no.

Running Head: Salmon gut is affected by different diets

Abstract

The present study aimed to gain information whether alternative dietary protein sources modulate the microbial communities in the distal intestine (DI) in Atlantic salmon, and if alterations in microbiota profiles are reflected in modifications to host intestinal function and health status. A 48-days feeding trial was conducted feeding the fish either of five diets: A reference diet (Diet FM) in which fish meal was the only protein source and four diets with commercially relevant composition in which alternative ingredients replaced fish meal, i.e. Diet PM with poultry meal; Diet SBMWG with a mix of soybean meal and wheat gluten; Diet SPCPM with a mix of soy protein concentrate and poultry meal; Diet GMWG with guar meal and wheat gluten. Samples were taken of DI digesta and mucosa for microbial profiling and from DI whole tissue for immunohistochemistry and expression profiling of marker genes for gut health. Regardless of diet, there were significant differences between the microbial populations in the digesta and the mucosa in the salmon DI. Microbial richness were higher in the digesta than the mucosa. The digesta-associated bacterial communities were more affected by the diet than the mucosa-associated microbiota. Interestingly, both legume-based diets (SBMWG and GMWG) presented high relative abundance of lactic acid bacteria (LAB) in addition to alteration in the expression of a gene related cell proliferation. It was however not possible to conclude whether changes in bacterial communities were related to or contributed to the host responses to the diets.

Introduction

The use of alternative plant-based protein sources to partially replace fishmeal in diets for farmed Atlantic salmon (*Salmo salar*) is currently common practice (1). The use of certain feed ingredients of plant origin in salmonid fish diets has however been restricted as a consequence of the presence of antinutrients challenging function and health of the gut of fish (2). A number of studies describe a range of functional effects of plant based alternative protein ingredients on salmonid gut health and function, especially when soybean meal and other legumes are included in the diets (3-10). Some studies have also investigated modulatory effects of different protein sources on the gut microbiota in salmonids (11-16), but general knowledge regarding interactions between diets, gut microbiota and fish gut function and health is fragmentary and incomplete. There is no controversy any more regarding the critical importance of the gut microbiota for the host (reviewed by (17)). For example, studies in mammals have linked gut microbial dysbiosis closely to a number of health disorders such as obesity and inflammatory bowel disease (18, 19). In fish, investigation of functional aspects of the gut microbiota has so far had focus on the modulatory effect of dietary supplements such as pre- and probiotics. Possible links to variation in gut immune functions and growth performance have also been addressed (20-24). Based on experience from studies with mammals, gut microbiota profiles is expected to be a valuable endpoint measurement in order to assess and understand fish gut health status and effects of diet.

The work presented herein was part of a larger study evaluating effects of practical Atlantic salmon diets with high content of alternative protein sources, i.e. soybean meal and concentrate, guar meal and poultry meal. Results regarding growth performance, nutrient digestibilities and intestinal histomorphology are reported elsewhere (25). The main purposes of the work on cultivated Atlantic salmon presented in this paper were to *i*) strengthen knowledge on digesta and mucosa associated microbial composition in the distal intestine, *ii*) evaluate if high levels of current alternative protein sources modulate the microbial communities in the distal intestine, *iii*) evaluate if high levels of alternative protein sources modulate distal intestine health status and *iv*) evaluate if alterations in intestinal microbiota profiles may be reflected in alterations in host intestinal health status and functionality.

Materials and Methods

Experimental fish

The experiment was conducted in accordance with laws regulating the experimentation with live animals in Denmark as overseen by the Danish Animal Experiments Inspectorate. The feeding trial was performed at the RAS research facilities of BioMar in Hirtshals, Denmark. For each diet, duplicate, mixed-gender groups of 22 or 23 post-smolt Atlantic salmon with an initial mean body weight of 314 ± 2 g (mean \pm SEM) were randomly distributed into ten 0.8 m³ fiberglass tanks containing 1000 L seawater. The temperature during the feeding trial was 15°C. Oxygen saturation was above 85% throughout the experiment, whereas salinity varied between 32-33 g/L. A regimen of 24 h lighting was employed during the experimental period.

Diets

The feeding trial lasted 48 days. Five diets were formulated, comprising one reference diet and four diets with commercially relevant ingredient composition. Supplementary Table 1 shows the formulations and chemical compositions of the diets. The reference diet (FM) was formulated with fishmeal as the only protein source (72%). The other experimental diets contained one of four different alternative protein sources/mixes replacing a proportion of the fishmeal; one experimental diet with 58% poultry meal (PM), a second with soybean meal (30%) mixed with wheat gluten (22%) (SBMWG), a third with soy protein concentrate (30%) mixed with poultry meal (6%) (SPCPM) and a fourth with guar meal (30%) mixed with wheat gluten (14.5%) (GMWG). Fish oil, rape oil and tapioca were added as lipid and carbohydrate sources, to balance the nutrient composition of the diets. The diets were supplemented to fulfil the fishes' requirements for lysine, methionine, vitamins and minerals. Fish were fed continuously by automatic Belt feeders, 18 hours feeding period from 1 PM to 7 AM, until termination of the trial. Feed consumption was recorded daily. The uneaten pellets were registered to estimate feed intake.

Sampling

At termination of the feeding period, fish were randomly selected for sampling, anesthetized with benzocaine (20ml/100L) (Kalmagin 20% Centrovat, Santiago, Chile) and then euthanized by cervical dislocation. All sampled fish presented digesta throughout the intestinal tract, which ensured intestinal exposure to the diets at the time of sampling. For analysis of microbiota five fish per diet (2 and 3 fish from each of the replicate tanks) were cleaned ventrally with 70%

ethanol. The abdominal cavity was then opened by the ventral mid line and the whole intestine was aseptically removed. Samples for investigation of the digesta-associated bacteria of the distal intestine (DI) of salmon were collected individually by carefully squeezing the digesta from the intestine into 1.5 ml sterile tubes. Samples for investigation of the mucosa-associated microbiota were collected from DI sections after they were opened and washed with sterile phosphate-buffered saline (PBS). Tissue segments of approximately 1 cm were sampled from the middle of the DI and subsequently transferred into 1.5 ml sterile tubes. All samples for microbiota analysis were frozen immediately in liquid N₂, and thereafter stored at -80°C. For RNA extraction, DI tissue samples from nine fish (4 and 5 fish from each replicate tank, including the fish sampled for microbiota analysis) per diet were taken and placed in RNAlater (Ambion®, Thermo Fisher Scientific, Waltham, MA, USA) at 4°C for 24 h, and subsequently stored at -20 °C. For immunohistochemical analyses, DI tissue samples from six fish per tank were collected, placed in 4% phosphate-buffered formaldehyde solution for 24 h, and subsequently stored in 70% ethanol until further processing.

DNA extraction

DNA was extracted from 200 mg of DI digesta samples and 200 mg of DI mucosa samples. The extraction was performed using the QIAamp Stool Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's specification with the following modifications; 1.4 ml Buffer ASL was added to the tubes containing the samples along with 150 mg of glass beads (Merck, Darmstadt, Germany). Then, samples were homogenized using the FastPrep®-24 instrument (MP Biomedicals, France) at 6.0 m/s two times for 25 sec with a pause of 25 sec between the runs. The temperature for the heating incubation was increased from 70 to 90°C and the incubation time after the addition of proteinase K and Buffer AL was increased from 10 to 15 min. DNA concentrations were determined using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, U.S.A.).

PCR amplification and high-throughput sequencing

To analyse the microbial population of the distal intestinal digesta and mucosa, amplification of the variable regions V1-V2 of the 16S rRNA was performed. The PCR was conducted using the bacterial universal primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 338R-I: (5' GCW GCC TCC CGT AGG AGT 3') and 338R-II (5' GCW GCC ACC CGT AGG TGT 3'). The reactions were carried out in 50µl volumes using 1 µl of DNA template, 25µl Phusion® High-Fidelity PCR Master Mix (Thermo Scientific, CA, USA) and 1µl of forward and reverse

(pooled 338R-I and II) primers (50pM). The PCR was run as following: initial denaturation at 98°C for 3 min, 35 cycles of denaturation at 98 °C for 15 sec, annealing decreasing from 63°C to 53°C in 10 cycles for 30 sec followed by 25 cycles at 53°C for 30 sec, and extension at 72°C for 30 sec; followed by a final extension at 72°C for 10 min. PCR products were then analysed in a 1.5% agarose gel and purified using the QIAquick PCR Purification Kit (Qiagen, Crawley, UK). High-throughput sequencing of the purified PCR products was carried out using the Ion Torrent Personal Genome Machine system (Life Technologies, CA, USA) as described elsewhere (26). Obtained sequences were grouped by sample and filtered within the PGM software to remove low quality reads.

High-throughput sequence data processing

Bioinformatic analyses of sequence reads were performed after the removal of low quality scores (Q score < 20 in 80% of sequences) with FASTX-Toolkit (Hannon Lab, USA). Sequences were concatenated and sorted by sequence similarity into a single fasta file. Sequences were further analysed using QIIME pipeline (27) as described elsewhere (26) using a length threshold for the multiple alignments of 250 bp. The following softwares were used in the data processing pipeline: USEARCH quality filter pipeline (28), PyNAST (29), RDP reference databases (30). Singletons and operational taxonomic units (OTUs) with less than 0.005% abundance were filtered out in order to reduce spurious OTUs (31). OTUs assigned as Cyanobacteria were excluded from the final data since they were considered to be of ingested content origin (chloroplast) and not part of the microbiota of the gut (32). QIIME was also used to identify the core microbiota (defined for this study as the OTUs present in 80% of the samples of each diet) and to rarefy the OTU tables to calculate alpha diversity metrics (Good's coverage, Observed species and Shannon index) and beta diversity metrics (unweighted and weighted UniFrac). The results are presented at phylum and genus taxonomic levels (or the lower taxonomic level assigned for OTUs).

RNA extraction and quantitative real time PCR (qPCR)

RNA purification and quality control, DNase treatment, cDNA synthesis and qPCR assays were performed as described elsewhere (25). RNA integrity numbers (RIN) were >8 for all samples, with an average RIN of 8.9. A selection of previously proposed marker genes for gut health and metabolism was profiled in DI tissue samples. Primer details are shown in Supplementary Table 2. *Gapdh*, *rna polymerase II (rnapolII)* and *hypoxanthine*

phosphoribosyltransferase 1 (hprt1) were evaluated for use as reference genes as described by Kortner *et al.* (33), and were found to be stably expressed based on their total variation and inter- and intraspecific variance. Thus, the geometric average expression of *gapdh* and *rnapolIII* and *hprt1* was used as the normalization factor. Mean normalized expression of the target genes was calculated from raw Cq values by relative quantification (34).

Immunohistochemistry

Immunohistochemistry was performed in fixed DI sections to detect the proliferating cell nuclear antigen (Pcna). Intestinal tissues were dehydrated and embedded in paraffin. Paraffin-embedded sections (5 µm) were transferred onto glass slides (Super-frost®, Thermo Scientific, CA, USA), dried overnight at room temperature, incubated for 1 hour at 58 °C, deparaffinized in xylene and rehydrated in graded alcohol baths (100%, 96% and 70%), then placed in dH₂O. Antigen retrieval was undertaken by heat-treatment in 10 mM citrate buffer, pH 6.0, at 120°C, for 15 min. Endogenous peroxidases were blocked by incubating the sections for 40 mins at 58°C in 0.05% phenylhydrazine (Sigma-Aldri-Aldrich, St. Louis, MO, USA).

Non-specific antibody binding was reduced by incubating the sections for 20 min at ambient temperature with 5% bovine serum albumin in TRIS-buffered saline (BSA/TBS) containing normal horse serum diluted 1:50 followed by overnight incubation at 4°C with the primary antibody (mouse monoclonal anti-Pcna; M0879, Dako Norge, Oslo, Norway) diluted 1:200 in 1% BSA/TBS (35, 36). The sections were then rinsed in PBS and incubated with biotinylated horse anti-mouse secondary antibody at 1:200 in 1% BSA/TBS for 20 min at ambient temperature. Vectastain ABC-PO (mouse IgG) kit was used to visualize immunoreactivity following the according to the manufacturer's instructions. Negative controls were prepared with 1% BSA/TBS instead of the primary antibody. Mayer's haematoxylin was used as counter-stain.

Pcna staining was evaluated blindly in two different intestinal sections for each fish using a light microscope. Sections evaluation was performed semi-quantitatively, scoring separately the immunopositive cells of two different regions of mucosal fold, the base and the apex. A total score was calculated by averaging the results in both regions based on the following staining patterns: Score 1, Very low/absent of positive cells; score 2, low positive score; score 3, medium positive score; score 4, high positive cells; score 5, very high positive cells.

Statistical analysis of data

For sequencing data, the unweighted and weighted UniFrac distance matrices were analysed by Permutation multivariate analysis of variance (PERMANOVA) with 999 permutations. For this propose the matrix was exported to the software PRIMER7 with PERMANOVA+ (37). Linear discriminant analysis (LDA) effect size (LEfSe) (38) was used to characterize microbial differences of biological relevance between the diets in mucosa within the two different compartments. The LEfSe analysis was performed using an alpha value of 0.01 for both the factorial Kruskal-Wallis rank sum test and pairwise Wilcoxon test and a threshold of 2.0 for the LDA. The approach used was an all-against-all multi-class analysis. The alpha diversity metric Observed species was subjected to a two-way ANOVA analysis with diet and compartment (digesta, mucosa) as class variables. Data were also analysed by one-way ANOVA with Tukey's Multiple Comparison test as post hoc test to aid in the interpretation of the two-way ANOVA results. For gene expression and immunohistochemistry, data was tested for normality and variance homogeneity using the Shapiro-Wilk W test and the Bartlett's test, respectively. Then the data were subjected to one-way ANOVA followed by Tukey's Multiple Comparison test using JMP statistical software (version 10; SAS Institute, USA). When necessary, the data was transformed to achieve normal distribution (indicated by "*" in tables). Since some qPCR data and the Shannon's diversity index did not fulfill the requirement of normal distribution, the analysis was performed using the Wilcoxon/Kruskal-Wallis test followed by post-hoc Steel-Dwass method to compare the means. The level of significance for all analyses was set at $P < 0.05$.

Results

General comments and characteristics of the high-throughput sequence data

The presentation and discussion of the results given below focus on differences observed between the reference diet and each of the diets with alternative ingredients. Differences between the experimental diets are not discussed, as their composition was not balanced for direct comparison.

After sequence quality filtering, trimming, filtering of the OTUs and discarding of cyanobacteria reads 1,191,799 sequences were retained for downstream analyses. The total

number of observations was 1660. The alpha diversity metric Good's coverage estimator was 0.9818 ± 0.0007 (mean \pm SEM), indicating adequate sequencing depth.

Gut microbiota in DI digesta

The statistical analysis (PERMANOVA) of the unweighted and weighted UniFrac matrices (Table 1) showed that all diets affected the unweighted UniFrac indicating that the microbial communities in the digesta of fish fed the experimental diets differed from those in the fish fed the FM diet. On the other hand, the weighted UniFrac showed that only fish fed PM and SBMWG differed significantly from that of fish fed the FM diet. The PCoA plots of the unweighted and weighted UniFrac (Figure 1 A-B) reflect the statistical analysis by showing clustering of samples by diet, especially in the PCoA plot showing the results of the unweighted UniFrac.

Results from the linear discriminant analysis (LDA) effect size (LEfSe) analysis further support the statistical results showing significant differences in microbial abundances between the experimental diets fed fish and the fish fed the FM diet (Figure 2 A). The experimental diets resulted in enrichment of several OTUs from different phyla. Compared to FM fed fish, fish fed SBMWG diet showed higher abundance of OTUs belonging to class Bacilli, genus *Bacillus*, and the genera *Weissella*, *Leuconostoc*, *Lactobacillus*, *Pediococcus*, *Erwinia* and *Sphingomonas*. Fish fed PM presented significant high abundance of the genera *Sporosarcina*, *Pseudomonadales*, *Jeotgalicoccus*, *Arthrobacter* and *Brevibacterium*. Fish fed SPCPM diet presented significant high abundance of *Streptococcus*, *Carnobacterium*, *Lactococcus*, *Shewanella*, *Ureibacillus*, *Geobacillus* while fish fed GMWG diet presented high abundance of *Anaerococcus* and the order Rickettsiales.

Table 1. Result of the statistical analysis of the Weighted UniFrac of the DI gut microbiota of Atlantic salmon fed diet with different protein sources^a.

	Weighted UniFrac	Unweighted UniFrac
	P-value	P-value
<i>Two-way PERMANOVA</i>		
Compartments	0.03	0.007
Diet	0.001	0.001
Interaction	0.582	0.213
<i>PERMANOVA Pair-wise test DIGESTA</i>		
FM, PM	0.046	0.008
FM, SBMWG	0.01	0.007
FM, SPCPM	0.254	0.013
FM, GMWG	0.086	0.008
<i>PERMANOVA Pair-wise test MUCOSA</i>		
FM, PM	0.085	0.013
FM, SBMWG	0.07	0.025
FM, SPCPM	0.13	0.275
FM, GMWG	0.396	0.232

^aAbbreviations: FM, Fishmeal diet; PM, Poultry meal diet; SBMWG, Soybean meal wheat gluten diet; SPCPM, Soy protein concentrate poultry meal diet; GMWG, Guar meal wheat gluten diet.

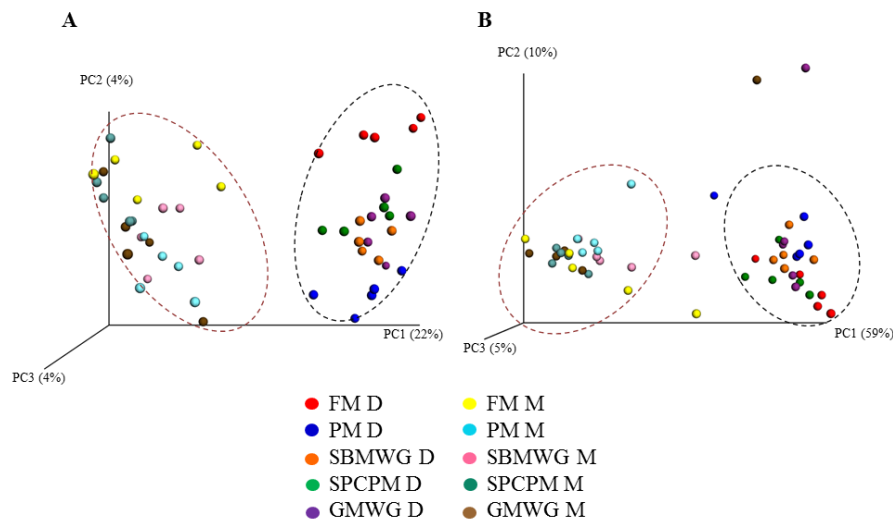


Figure 1. PCoA of unweighted (A) and weighted UniFrac (B) showing clustering samples. Each dot represents one sample. Abbreviations: FM, Fishmeal diet; PM, Poultry meal diet; SBMWG, Soybean meal with wheat gluten diet; SPCPM, Soy protein concentrate with poultry meal diet; GMWG, Guar meal with wheat gluten diet; D, digesta; M, mucosa.

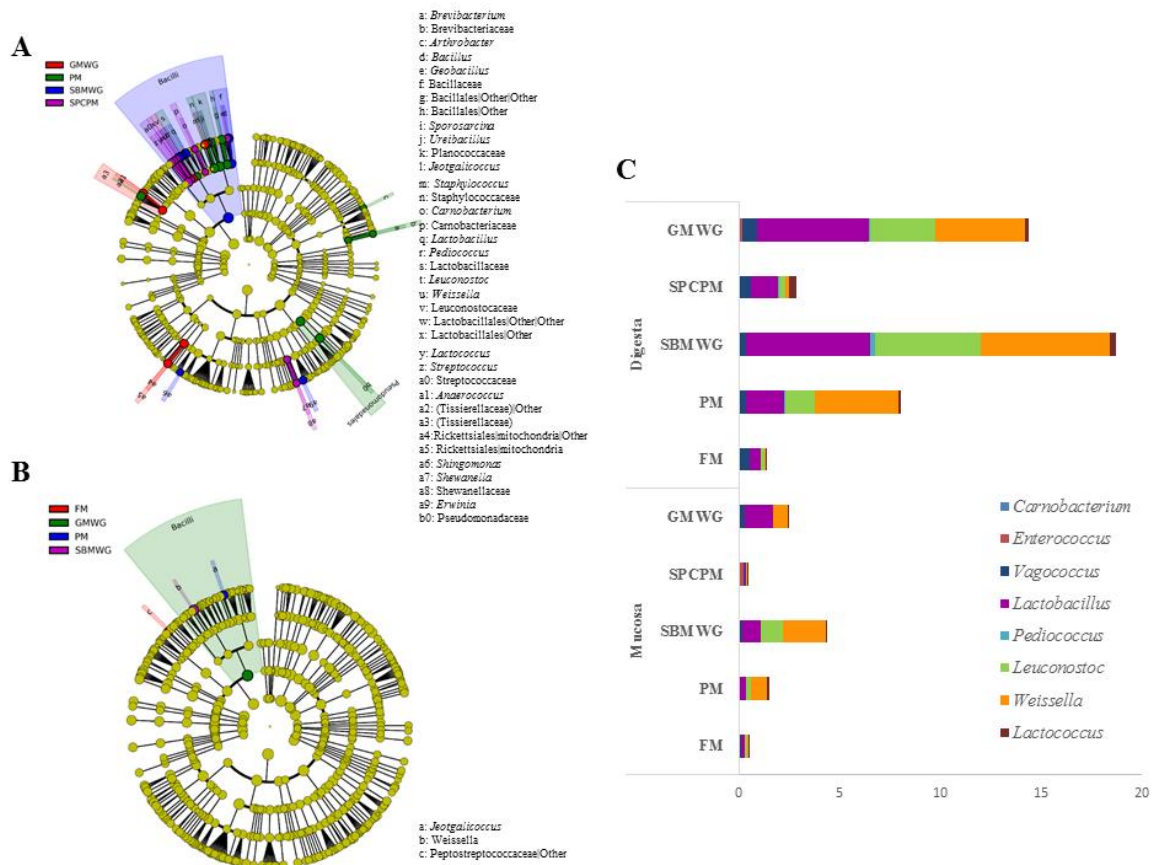


Figure 2. Results from the LEfSe analysis for the OTUs for (A) digesta and (B) mucosa and the relative abundance of LAB as percentage of the total OTUs found for each diet in digesta and mucosa (C). In figure A and B, the identified OTUs are distributed according to phylogenetic characteristics around the circle. The dots closest to the center represent the OTUs on phylum level, whereas the outer circle of dots present the OTUs on genus level. The color of the dots and sectors indicate the compartment in which the respective OTUs are most abundant. The color explanation is given in the upper left corner. Yellow color indicates OTUs that showed similar abundance in all compartments. The colored sectors give information on class (full name in outermost circle, given only for class showing significant difference between compartments), family, and genera are indicated by letter (explanation given to the right of the figure). Abbreviations: FM, Fishmeal diet; PM, Poultry meal diet; SBMWG, Soybean meal with wheat gluten diet; SPCPM, Soy protein concentrate with poultry meal diet; GMWG, Guar meal with wheat gluten diet.

Supplementary Figure 1 shows the relative abundance of the main OTUs at phylum level. Irrespective of diet, digesta OTUs belong mainly to the phyla Firmicutes, Proteobacteria, Fusobacteria, Bacteroidetes, OD1 and Actinobacteria. The digesta of fish fed the FM diet showed a high abundance of Firmicutes (39%), Proteobacteria (31%) and Fusobacteria (20%). Compared to fish fed the FM diet, fish fed the experimental diets presented higher abundance of Firmicutes (from 43% in fish fed PM, SBMWG and SPCPM diets to 54% in fish fed GMWG diet), and lower abundance of Fusobacteria (from 12% in fish fed GMWG to 16% in fish fed

SPCPM) and Proteobacteria (from 20% in fish fed GMWG diet to 29% in fish fed SBMWG diet).

Figure 3 shows the relative abundances of the main OTUs at genus taxonomic level in the digesta. Fish fed the FM diet showed a high abundance of *Photobacterium* (26%), *Peptostreptococcus* (15%), Clostridiales (14%) and *Cetobacterium* (4%). Compared to FM fed fish, fish fed the PM diet had lower relative abundance of *Photobacterium* (16%), *Peptostreptococcus* (8%) and Clostridiales (8%). Also the fish fed SBMWG diet showed lower relative abundance of *Photobacterium* (19%), *Peptostreptococcus* (9%) and Clostridiales (7%), but higher abundance of lactic acid bacteria (Figure 2 C) such as *Weissella* (6%), *Leuconostoc* (5%) and *Lactobacillus* (6%) compared to fish fed the FM diet. Fish fed the SPCPM diet showed abundance of *Peptostreptococcus* (16%) and Clostridiales (11%) quite similar to that of FM fed fish, whereas the relative abundance of both *Photobacterium* (20%) and Clostridiales (11%) were lower than in FM fed fish. Fish fed the GMWG diet as well as the fish fed the SBMWG diet showed high abundance of *Weissella* (4%), *Leuconostoc* (3%), *Lactobacillus* (6%), and lower levels of *Photobacterium* (15%) and Clostridiales (7%) compared to FM fed fish.

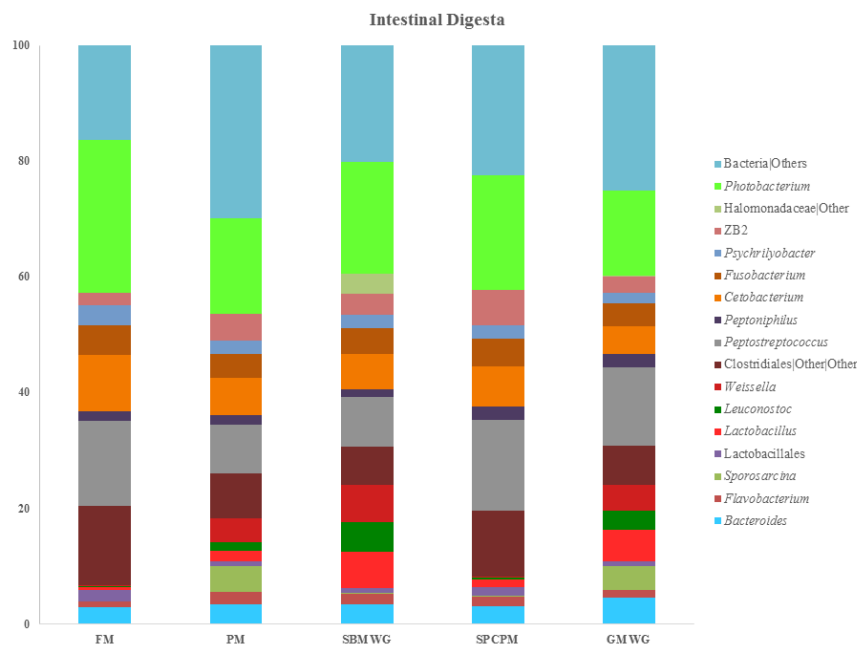


Figure 3. Gut microbiota composition (relative OTUs composition) at genus level (or the lower taxonomic level reached by the analysis) for digesta samples. Abbreviations: FM, Fishmeal diet; PM, Poultry meal diet; SBMWG, Soybean meal with wheat gluten diet; SPCPM, Soy protein concentrate with poultry meal diet; GMWG, Guar meal with wheat gluten diet

Gut microbiota in DI mucosa

Results of the PERMANOVA analysis showed significant differences (Table 1) in the unweighted UniFrac between the mucosa-associated microbial communities of fish fed PM and SBMWG diets compared to the FM diet.

Regarding the DI mucosa, the LEfSe analysis (Figure 2 B) showed significantly higher abundance of *Jeotgalicoccus* for fish fed PM compared to FM fed fish. The class Bacilli showed significant higher abundance in the GMWG fed fish compared to FM fed fish. *Weissella* presented higher abundance in fish fed SBMWG and an unidentified OTU from the family Peptostreptococcaceae showed lower abundances in fish fed the experimental diets compared to FM fed fish.

The dominant phyla in the mucosa-associated microbiota were Proteobacteria followed by Bacteroidetes, OD1 and Firmicutes. Proteobacteria represented 33% of the OTUs in FM diet fed fish and between 25 - 34% for the experimental diets (Supplementary Figure 1). The abundances of the other main phyla varied between the FM and the experimental diets with an increase of Bacteroidetes (from 15% in the FM diet to 20 - 24% in the experimental diets), and a decrease of Fusobacteria (from 12% in FM diet to 0.4 – 5% in the experimental diets). Compared to the FM fed fish, the relative abundance of the phyla OD1 and Firmicutes increased or decreased depending of the experimental diet. Higher relative abundance was observed for OD1 in fish fed SPCPM (24%) and GMWG (25%) and lower relative abundance in fish fed SBMWG (13%) and PM (19%) compared with FM fed fish. Firmicutes presented a higher relative abundance in SBMWG (8%), GMWG (9%) and PM (10%) fed fish compared with FM fed fish. The OTUs assigned to the mucosal samples (Figure 4) also showed differences at genus level when the experimental diets were compared to the FM diet, but the differences seemed to be smaller than those observed for the digesta samples. The fish fed the FM diet showed high relative abundance of ZB2 (class, no lower taxonomic classification possible) (16%), *Cetobacterium* (5%) and *Flavobacterium* (5%). Similar to fish fed FM diet, fish fed the experimental diets showed a high relative abundance of ZB2 (from 10% in SBMWG to 19% in GMWG), and higher relative abundance of *Flavobacterium* (from 8% in fish fed SBMWG to 10% in fish fed GMWG) compared to FM fed fish. The fish fed SBMWG

diet also showed high relative abundance of Halomonadaceae (10%) and Brevinemataceae (6%) compared to FM fed fish. For a detailed list of OTUs see Supplementary Table 3.

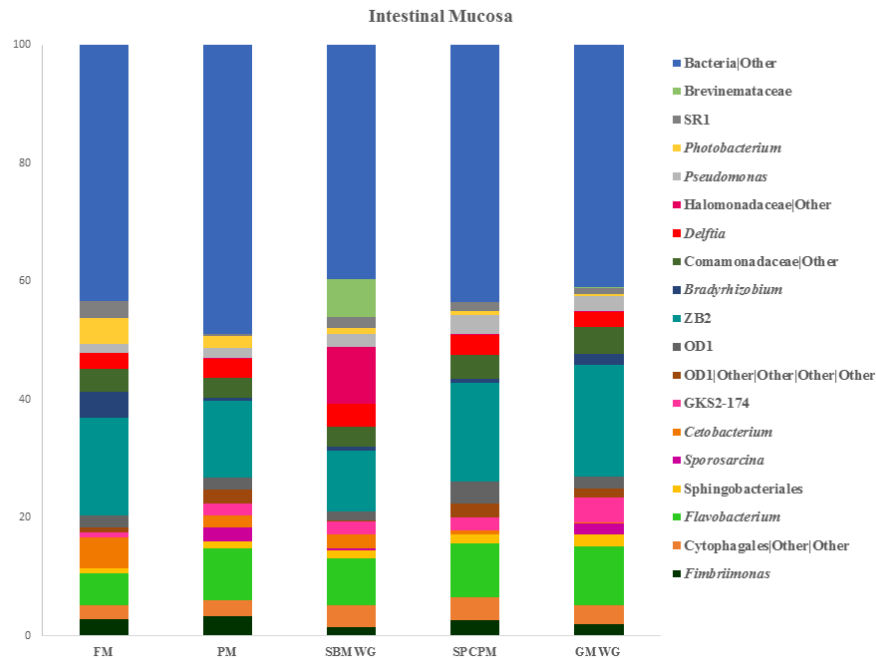


Figure 4. Gut microbiota composition (relative OTUs composition) at genus level (or the lower taxonomic level reached by the analysis) for mucosa samples. Abbreviations: FM, Fishmeal diet; PM, Poultry meal diet; SBMWG, Soybean meal with wheat gluten diet; SPCPM, Soy protein concentrate with poultry meal diet; GMWG, Guar meal with wheat gluten diet.

Digesta vs mucosa and core microbiota

The alpha diversity metrics for richness, observed species, showed significant differences between the two investigated compartments (Table 2) with lower number of observed species (OTUs) in samples of mucosa compared with digesta. The Observed species parameter of the alpha diversity metric presented higher values for fish fed PM and SBMWG compared to FM fed fish. On the other hand, the Shannon's diversity index, combining richness and abundance of the different OTUs, did not show significant differences between fish fed the experimental diets and the fish fed fishmeal (FM) diet (Table 2). Moreover, the statistical analysis of the unweighted and weighted UniFrac showed that microbial communities differed significantly between the digesta and mucosa compartments (Table 1). Supporting the previous statistical analysis, the PCoA plots of the unweighted and weighted UniFrac (Figure 1 A-B) presented clear separation between samples of digesta and mucosa origin.

Table 2. Alpha diversity results of DI gut microbiota of Atlantic salmon^a.

Richness		Diversity	
Observed Species		Shannon's index*	
Two-way ANOVA model			
P (model)	0.0002	P (model)	<0.0001
Pooled SEM	13	Pooled SEM	0.2
<i>P values from two-way ANOVA</i>		<i>Means for each diet/section studied</i>	
Segment	<0.0001	DIGESTA	
Diet	0.004	FM	5.4
Interaction	0.283	PM	6.6
<i>Means of the significant observations</i>		SBMWG	6.3
FM	269 ^b	SPMPM	6.0
PM	348 ^a	GMWG	6.0
SBMWG	329 ^a	MUCOSA	
SPCPM	320 ^{ab}	FM	6.7
GMWG	307 ^{ab}	PM	7.0
		SBMWG	6.3
Digesta	344 ^a	SPCPM	6.9
Mucosa	285 ^b	GMWG	6.5

^aAbbreviations: FM, Fishmeal diet; PM, Poultry meal diet; SBMWG, Soybean meal wheat gluten diet; SPCPM, Soy protein concentrate poultry meal diet; GMWG, Guar meal wheat gluten diet. * Non-parametric test.

The list with the OTUs representing the core microbiota, i.e. present in 80% of the samples irrespective of diet, is shown in Supplementary Table 4. In the digesta, 60 OTUs (Figure 5 A) were observed in all diets groups, showing a dominance of Firmicutes (24 OTUs) and Proteobacteria (19 OTUs). The core microbiota present in the mucosa was smaller than the one from the digesta with 37 OTUs shared by all diets (Figure 5 B). In mucosa, the core was dominated by OTUs belonging to Proteobacteria (15 OTUs) and Bacteroidetes (nine OTUs). The core microbiota for both digesta and mucosa across all samples comprised 19 shared OTUs (6 Bacteroidetes, 5 Proteobacteria, 3 Firmicutes, 3 Fusobacteria, 1 OD1 and 1 Armatimonadetes).

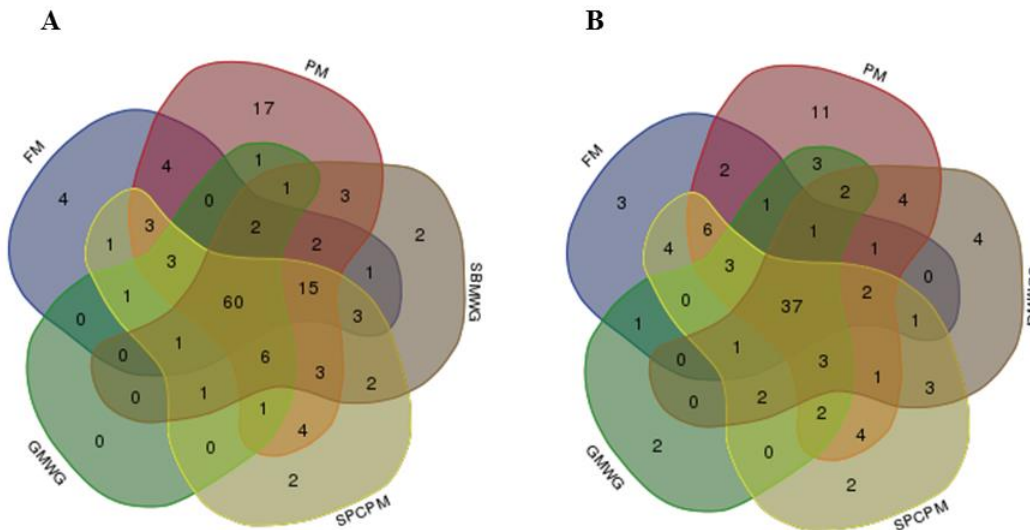


Figure 5. Venn diagrams showing compartmental OTU distributions. (A) OTUs distribution in the digesta samples. Sixty OTUs were identified as core microbiota (80% of samples) for all diets in digesta. (B) OTUs distribution in the mucosal samples. Thirty-seven OTUs were identified as core microbiota (80% of samples) for all diets in mucosa. Abbreviations: FM, Fishmeal diet; PM, Poultry meal diet; SBMWG, Soybean meal with wheat gluten diet; SPCPM, Soy protein concentrate with poultry meal diet; GMWG, Guar meal with wheat gluten diet.

Gene expression

Results regarding gene expression in tissue of the DI are presented in Table 3. Fish fed the SBMWG and GMWG diets showed the highest modulation in expression levels compared to the FM diet fed fish, A clear induction of *pcna* (proliferating cell nuclear antigen) was seen for fish fed SBMWG and GMWG, whereas *frim* (ferritin) and *cat* (catalase) expression was significantly reduced. The SBMWG diet also increased expression of *hsp70* (heat shock protein 70), and a similar trend was seen for fish fed the GMWG diet. In fish fed the SPCPM diet, the only significant finding was reduced levels of *cat*, whereas no significant changes were found for fish fed the PM diet. No significant changes in expression of immune-related gene were found for any of the experimental diets as compared to the FM diet fed fish.

Table 3. Effect of diets with different protein sources on gene expression analysis of the distal intestine of Atlantic salmon^α.

	<i>il-1β</i> *	<i>cd4a</i> *	<i>cd8β</i> *	<i>gilt</i>	<i>ifn-γ</i> *	<i>mmp13</i> *	<i>muc2</i> *	<i>frim</i>	<i>pcna</i> *	<i>cat</i>	<i>hsp70</i> *	<i>myd88</i> *	<i>mhc1</i> *	<i>tcr-γ</i> *	<i>il-6</i> ^Ω	<i>il-17a</i> ^Ω	<i>fabp2a1</i> ^Ω
<i>One-way ANOVA model</i>																	
P-value	0.17	0.12	0.40	0.09	0.40	0.20	0.26	0.0007	0.0001	<0.0001	0.006	0.02	0.06	0.62	0.32	0.21	0.12
Pooled SEM	0.0006	0.0005	0.0005	1.2	0.0005	0.004	0.97	3	0.03	0.0007	0.4	0.003	0.14	0.0002	0.0002	0.003	0.001
<i>Means values</i>																	
FM	0.0036	0.0030	0.0011	31.0	0.0011	0.029	10.3	51 ^a	0.16 ^b	0.0078 ^a	7.1 ^b	0.045 ^{ab}	0.78	0.0023	0.0012	0.004	0.013
PM	0.0030	0.0037	0.0014	30.8	0.0015	0.024	9.3	47 ^{ab}	0.18 ^b	0.0063 ^{ab}	7.2 ^b	0.046 ^{ab}	0.90	0.0021	0.0009	0.009	0.013
SBMWG	0.0021	0.0040	0.0012	27.6	0.0012	0.037	6.9	35 ^{bc}	0.31 ^a	0.0031 ^c	8.8 ^a	0.052 ^a	0.57	0.0018	0.0007	0.011	0.015
SPCPM	0.0016	0.0044	0.0024	31.1	0.0024	0.020	8.2	45 ^{abc}	0.24 ^{ab}	0.0042 ^{bc}	7.9 ^{ab}	0.047 ^{ab}	0.99	0.0021	0.0005	0.010	0.015
GMWG	0.0022	0.0028	0.0018	27.7	0.0018	0.024	7.3	32 ^c	0.32 ^a	0.004 ^{bc}	8.4 ^{ab}	0.037 ^b	0.56	0.0021	0.0006	0.003	0.015

^αAbbreviations: FM, Fishmeal diet; PM, Poultry meal diet; SBMWG, Soybean meal wheat gluten diet; SPCPM, Soy protein concentrate poultry meal diet; GMWG, Guar meal wheat gluten diet. * Log-transformed data. *il-1β*, interleukin 1β; *cd4a*, cluster of differentiation 4α; *cd8β*, cluster of differentiation 8β; *gilt*, interferon γ- inducible lysosomal thiol reductase; *ifnγ*, interferon γ; *mmp13*, matrix metalloproteinase 13; *muc2*, mucin-2; *frim*; *frim*, ferritin, middle subunit; *pcna*, proliferating cell nuclear antigen; *cat*, catalase; *hsp70*, heat shock protein 70; *myd88*, myeloid differentiation factor 88; *mhc1*, major histocompatibility class 1; *tcrγ*, T-cell receptor γ. Mean values with different superscript letters a, b, c within a column are significantly different (P<0.05). ^Ω Non-parametric test.

Pcna immunohistochemistry

Results of the Pcna staining analysis are shown in Table 4 and Figures 6 A-B. The results of the one-way ANOVA showed that the all the experimental diets, with exception of PM diet increased the Pcna staining intensity in the DI. The SPCPM diet fed fish presented moderate increment of the staining intensity whereas SBMWG and GMWG diets presented high intensity of the staining in comparison with the FM diet fed fish.

Table 4. Effects of diet on Pcna staining height analysis of the distal intestine of Atlantic salmon^a.

Pcna measurements	
<i>One-way ANOVA model</i>	
P-value	<0.0001
Pooled SEM	0.18
<i>Means values</i>	
FM	1.81 ^c
PM	2.00 ^c
SBMWG	3.56 ^a
SPCPM	2.81 ^b
GMWG	3.09 ^{ab}

^aAbbreviations: FM, Fishmeal diet; PM, Poultry meal diet; SBMWG, Soybean meal wheat gluten diet; SPCPM, Soy protein concentrate poultry meal diet; GMWG, Guar meal wheat gluten diet. Mean values with different superscript letters a, b, c within a column are significantly different (P<0.05).

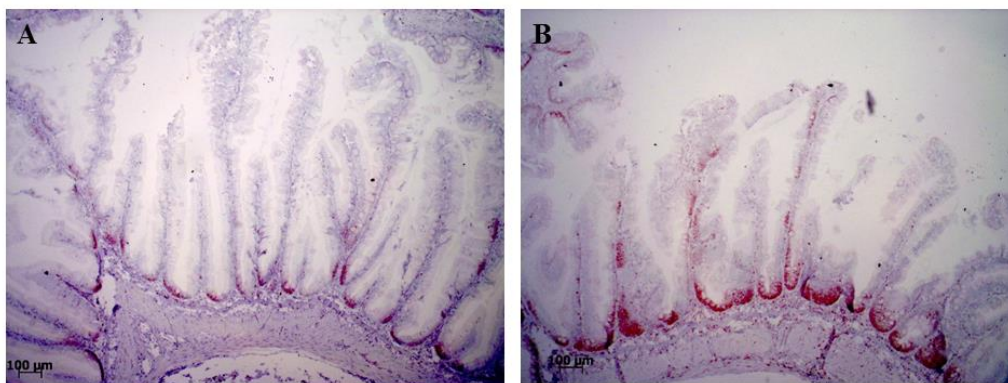


Figure 6. Representative images of the localization and distribution of immunohistochemically-labelled proliferating cell nuclear antigen (Pcna) protein in the epithelial cells of the distal intestine of Atlantic salmon. (A) Represents normal score. (B) Represents the highest score in the study.

Discussion

High resolution microbiota sequencing has been previously used to evaluate the diet's role in shaping the gut microbiota in fish (39, 40) including salmonids such as rainbow trout (*Oncorhynchus mykiss*) (14, 41, 42) and Atlantic salmon (43, 44). However, only a few of the studies conducted with salmonids, separated between the mucosa-associated autochthonous and the more transient or digesta-associated allochthonous microbial communities. Our recent study of characteristics of the microbiota in digesta and mucosa along the intestine of Atlantic salmon demonstrated important differences between the microbial communities of these two gut compartments (26), implying that dietary modulatory effects may be masked and therefore overlooked if only digesta-associated microbiota is characterized or if a homogenate of digesta and mucosal tissue is evaluated. The current study contribute with knowledge on such aspects aiming to characterize effects of alternative protein sources on microbiota-associated within the two compartments of the DI, i.e. digesta and mucosa, supposedly with a higher chance of finding possible correlations with functional changes in the mucosa of this gut region.

The results from the present study which demonstrated that, regardless of diet, there were significant differences between the microbial populations in the digesta and the mucosa in the salmon DI are in agreement with other studies characterizing the gut microbiota in fish (16, 26, 35, 39, 45, 46) . Furthermore, in agreement with Gajardo *et al.* 2016 (26) the alpha diversity metric observed species in the present study, showed lower values in mucosa compared with digesta samples, suggesting that not all bacteria present in the digesta are able to colonize the mucosa of the gut of salmon.

In addition, the apparently lower relative abundance of Firmicutes in mucosal samples compared to the digesta samples was in agreement with observations made in our previous study (26). In line with these results, the OTUs reported as core microbiota for all samples belonged mainly to three phyla; Proteobacteria, Firmicutes and Bacteroidetes. Several of the reported shared OTUs, such as *Fusobacterium*, *Microbacterium*, *Peptostreptococcus*, *Psychrilyobacter*, *Pseudomonas*, *Weissella*, *Photobacterium*, *Delftia*, *Psychrilyobacter* and *Bradyrhizobium* have been previously reported as members of the gut microbial communities of salmon (16, 26, 46-50). Together, these results suggest that certain bacterial species of some phyla may be more capable of inhabiting the gut of salmon despite changes in external environmental factors such as location and diet.

Regarding effects of diet composition on gut microbiota the present results showed the clearest difference between the SBMWG fed fish and fish fed the FM diet for all studied variables. The differences regarding the LAB, being 18 times higher in the digesta of fish fed SBMWG than the FM fed fish, were possibly the most interesting as these bacteria are generally supposed to be beneficial for gut health. Lactic acid bacteria comprise genera such as *Weissella*, *Leuconostoc*, *Lactobacillus*, *Pediococcus*, and *Carnobacteria* (51). The high abundance observed for *Leuconostoc* in digesta in the present study is in line with results of our previous work (26) as well as the work of Zarkasi *et al.* (47). Previous studies, employing classical methods, have reported *Carnobacterium*, another LAB and member of the order Lactobacillales, as highly abundant in the gut of farmed salmon (35, 46, 52-54). In the present study, in agreement with Zarkasi *et al.* (47) and (26), *Carnobacterium* was only present in minimal abundance. The apparent difference between studies in abundance of these two related bacteria might be due to differences in fish breeds, diet composition, environmental conditions or differences in sensitivity of the methodologies. A similar trend as found in the digesta were observed regarding the relative abundance of LAB in the mucosa-associated microbiota. LAB accounted for about 4% of the observed OTUs in the mucosa in fish fed SBMWG, whereas fish fed FM diet presented a LAB abundance of about 1%.

These results from the digesta samples are in agreement with previous studies reporting high abundance of LAB in DI digesta when soybean meal was added to the diet of salmonids (14, 48). The higher amount of low molecular indigestible carbohydrates, such as raffinose and stachyose, present in soybean diets and other plant-based diets may explain the higher abundance of LAB. The LAB is known to utilize such substrates for their metabolism and growth. In line with these considerations, fish fed the SPCPM diet, which do not contain low molecular carbohydrates, had low relative abundance of LAB. Also fish fed GMWG presented high relative abundance of LAB (14%) in the digesta compared to FM fed fish. The substrate for these LAB may be the highly water soluble galactomannan which is present in guar meal (55). Whether ingredient processing or other diet components such as antinutrients play a role in the way the diet modulates the gut microbiota requires further investigations.

Results from the present study which are reported elsewhere (25) showed that the histomorphology of fish DI was altered significantly by the SBMWG diet showing alterations typical for soybean

meal induced enteritis (SBMIE), whereas the histomorphology appeared normal for fish fed the other alternative diets. The degree of change in fish fed the SBMWG diet was milder than typically observed when salmon are fed SBM at the level used in the present experiment and no significant responses in variables indicating immunological status were observed. The explanation for differences in response to soybean meal between experiments may be variation in levels of antinutrients in the batch of soybeans used, in meal processing, diet composition and processing, in breeds of experimental fish and not at least feed intake, which vary with temperature and several other environmental conditions (10, 56, 57). The observed alterations in molecular variables in fish fed the SBMWG diet, all known to be associated with SBMIE, indicate a certain impairment of DI health status. Key indicators in this respect are increased cell proliferation, as indicated by the increased Pcn staining and increased *pcna* gene expression, and increased cellular stress as indicated by induction of *hsp70* and suppression of *frim* and *cat*. The direction and magnitude of change of these markers were in accordance with most previous reports on SBMIE in salmon (35, 58, 59). Fish fed GMWG and SPCPM showed alterations in some of the functional indicators. However, as the effects were small and overall morphology structure was not altered, it is likely that these changes were indicators of normal, physiological adaptations to diet composition.

As fish fed the SBMWG diet showed high LAB abundance and also showed signs of impaired gut health, the present work might appear to challenge the general understanding that certain bacteria among LAB have positive effects on gut health in fish (reviewed by (60) and (61)). Despite this, the results of the present cannot conclude on whether the increase in LAB was beneficial, detrimental or without importance for the gut health. Studies combining different omics techniques such as metagenomics, transcriptomics, metatranscriptomics, metaproteomics and metabolomics as well as the use of gnotobiotic animals would be expected to supply useful information in this regard. Such techniques have been used successfully in mammalian models and has thrown light on the role of the microbiota and their modulation and their role in disease and health (62-65).

Conclusions

The present work confirms our previous work showing clear differences between the digesta and mucosa in presence and abundance of bacteria. The OTUs found in both digesta and mucosa-

associated microbiota belonged mainly to the phyla Firmicutes, Proteobacteria, Fusobacteria, Bacteroidetes, and Actinobacteria. In addition, high relative abundance of the phylum OD1 was found in the mucosa-associated microbiota. The diet and the segment studied had an effect on the richness of the gut microbiota. Moreover, the digesta-associated microbiota showed clear dependency on the diet composition whereas mucosa-associated microbiota appeared less affected by diet composition. Fish fed the diet containing soybean meal showed mild distal intestinal enteritis and at the same time high relative abundance of LAB. Future research should improve understanding of the functional role of LAB and whether LAB or other bacterial groups may be of importance for the health status of the salmon gut.

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Author Contributions

K.G., performed laboratory and bioinformatic work and wrote the manuscript. A.J., performed and analysed immunohistochemistry and wrote in the manuscript, T.M.K., contributed with qPCR data interpretation and manuscript writing. D.L.M., contributed with manuscript corrections. J.T., contributed with planning the experiment and manuscript corrections. H.S. contributed with manuscript corrections. A.M.B., contributed with manuscript corrections. Å.K., contributed with manuscript writing and corrections.

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