

Sluttrapport for the FHF project:

# HoloFish: use of microbiome-genome co-optimisation to improve gut-health and growth in farmed salmon

Authors:

Jaelle C Brealey, Miyako Kodama, Jacob A Rasmussen, Søren B Hansen, Luisa Nielsen, Even Fjære, Martin Hansen, Lene M Secher, Lise Madsen, Karsten Kristiansen, Harald Sveier, Marcus Thomas Pius Gilbert, Michael D Martin, Morten T Limborg

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# Summary / Sammendrag

## Sammendrag (Norsk)

Laksenæringen har en nøkkelposisjon for å bidra til en grønn omstilling mot økonomisk og miljømessig bærekraftig matproduksjon. Likevel, som med mange typer oppdrettsdyr, står lakseoppdrettsnæringen overfor utfordringer som regelmessige sykdomsutbrudd, ineffektiv fôrkonvertering og tap på grunn av uforutsigbar variasjon i fiskestørrelse ved slakt. Mens forskere konvensjonelt har undersøkt om slik variasjon kan forklares under den tradisjonelle 'fenotype = genotype\*miljø'-modellen, har oppmerksomheten nylig vendt mot en alternativ forklaring, fiskens tarm-mikrobiom.

HoloFish-prosjektet har for første gang utviklet og brukt denne såkalte hologenomiske tilnærmingen for å bedre forstå og forbedre fôrkonvertering i en kommersiell kohort av oppdrettslaks. Dermed ble vertslaksen og dens mikrobiom studert som en enkelt enhet ved å generere molekylære data fra både vertslaksen og dens tilhørende mikrobiom. Ved å ta prøver av 463 laks som er slakteklare, hadde vi som mål å forstå antatte nøkkelvert-mikrobiota-interaksjoner i laks som ble oppdrettet på to forskjellige kommersielle dietter, samt representerte tre vilkårlig definerte størrelsesklasser fra små, middels eller store. Molekylære data for vertsgenomene, epigenomene, transkriptomene ble generert ved bruk av sekvensering med high throughput sequencing. Tilsvarende ble det mikrobielle metagenomet så vel som tarmmetabolomet generert for hver laks som ble tatt ut sammen med nøkkelegenskaper for KPI.

Samlet sett avslørte resultatene en rekke nye funn samt demonstrasjonen av en ny hologenomisk tilnærming for å studere de underliggende biologiske mekanismene for vekst og helse hos laks. Kort fortalt, mens det ikke var bemerkelsesverdige forskjeller mellom de to forskjellige diettene, avslørte multi omics-dataene et slående mønster med lav biomasse og lavt mangfold av tarm-mikrobiomet til oppdrettslaks, i sterk kontrast til landlevende husdyr. Videre, til tross for begrenset genomisk variasjon blant fisk, observerte vi en konsistent forskjell i sammensetningen av mikroorganismer i laks av forskjellig størrelse til tross for at all laks var av identisk alder, stamfisk og miljømessig opprinnelse. Det ble også observert at infeksjon med intestinale bendelormer har en effekt på vertslaksens mikrobiota, noe som peker på en sekundær effekt av bendelorm som også påvirker mikrobiotabalansen. Videre avslørte denne første storskalascreeningen av tarm-metabolomet hvordan fôr metabolsk påvirkes av både verts- og mikrobiom avledede funksjoner til fiskevekst og biomasse. Avslutningsvis demonstrerte HoloFish verdien av en ny hologenomisk tilnærming for å bedre forstå hvordan komplekse vert-mikrobiota-interaksjoner former vekst og helseytelse hos oppdrettslaks.

## Summary (English)

The salmon industry holds a key position to help steer a green transition towards economically and environmentally sustainable food production. Yet, as with many types of farmed animals, salmon aquaculture faces challenges such as regular disease outbreaks, inefficient feed conversion and losses due to unpredictable variation in fish size at harvest. While researchers have conventionally explored whether such variation might be explicable under the traditional 'phenotype = genotype\*environment' model, recent attention has turned towards an alternate explanation, the fishes' gut microbiome.

The HoloFish project has for the first time developed and applied this so-called hologenomic approach to better understand and improve feed conversion in a commercial cohort of farmed salmon. Thus, the host salmon and its microbiome was studied as a single unit by generating molecular data from both the host salmon as well as its associated microbiome. By sampling 463 ready to harvest salmon we aimed to understand putative key host - microbiota interactions in salmon that were reared on two distinct commercial diets, as well as representing three arbitrarily-defined size classes of small, medium or large. Molecular data for the host genomes, epigenomes and transcriptomes were generated using high throughput sequencing. Similarly, the microbial metagenome as well as the intestinal metabolome were generated for each sampled salmon, together with key KPI traits.

Overall, results revealed a range of novel findings, as well as the demonstration of a new hologenomic approach to study the underlying biological mechanisms of growth and health in salmon. Briefly, while there were no noteworthy differences between the two different diets, the multi-omics data revealed a striking pattern of a low biomass and low diversity of the intestinal microbiome of farmed salmon, in stark contrast to terrestrial livestock. Further, although there was limited genomic variation among fish, we observed a consistent difference in the composition of microorganisms in salmon of different sizes, despite all salmon being of identical age, broodstock and environmental origin. It was also observed that infection with intestinal tapeworms had an effect on the host salmon's microbiota, pointing at a secondary effect of tapeworm in also affecting the microbiota balance. Further, the inclusion of the first large-scale screening of the intestinal metabolome revealed how feed is metabolically translated by both host- and microbiome-derived functions into fish growth and biomass. In conclusion, HoloFish demonstrated the value of a new hologenomic approach to more fully understand how complex host - microbiota interactions shape growth and health performance in farmed salmon.

# Table of contents

<b>Summary / Sammendrag</b>	<b>2</b>
Sammendrag (Norsk)	2
Summary (English)	3
<b>Table of contents</b>	<b>4</b>
<b>Introduction</b>	<b>5</b>
Scientific background	5
The HoloFish project	7
<b>Research questions and objectives</b>	<b>9</b>
<b>Results and discussion</b>	<b>10</b>
WP 1: Sampling	10
WP 2 & 3: Independent analyses	11
Microbial metagenome	11
Microbiome composition	11
Microbiome phenotypes	12
Microbial 16S profiling	15
Host genome	18
Size class	18
Microbiome phenotypes	19
Host epigenome	21
Host transcriptome	22
Size class	22
Microbiome phenotypes	23
Untargeted meta-metabolomics	24
Size class	24
Microbiome phenotypes	26
Host fatty acid profiling	27
WP 3: Holo'omic analysis	29
Multi-omics analysis	29
Summary	33
WP 4: Validation experiment	34
<b>Conclusions and main findings</b>	<b>35</b>
<b>References</b>	<b>38</b>
<b>Deliverables</b>	<b>39</b>
Outreach and implementation of results	39
<b>Appendices</b>	<b>42</b>

# Introduction

## Scientific background

Aquaculture provides >50% of all consumed fish, is the fastest growing food-producing sector worldwide and is predicted to grow by  $\geq 5\%$  annually in years to come. Atlantic salmon is one of the most important aquaculture species with an annual production >2 million tonnes, worth >90 billion NOK, and its demand has been growing steadily in recent decades. Norway is both the world's leading producer of Atlantic salmon and the second largest seafood exporter.

The salmon industry's rapid growth presents an urgent need for a greener transition towards economically and environmentally sustainable production. Yet, as with many types of farmed animals, salmon aquaculture faces challenges such as regular disease outbreaks, inefficient feed conversion and losses due to unpredictable variation in fish size at harvest. Improving feed utilisation and efficiency presents a major potential for salmon farmers; as nearly 60% of salmon production costs come from fish feed, there is a demand for new solutions to reduce such costs. In recent years, the aquaculture industry has endeavoured to develop more sustainable and higher quality fish products. These efforts included (1) promoting the use of plant-based, cost-effective fish feed, (2) focusing on functions provided by the gut-microbiota, and (3) selective breeding to achieve higher yield and improved fish health.

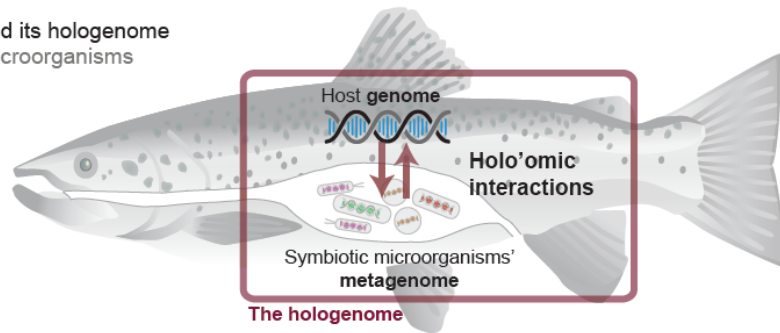
Researchers have conventionally explored whether such variation might be explicable under the traditional 'phenotype = genotype\*environment' model, with limited success. As such, attention has recently turned towards an alternate explanation, the fishes' gut microbiome. Rapidly accumulating evidence suggests that many host-associated microorganisms are not passive passengers, but active crew, who can affect and even condition phenotype (e.g. health, immune and growth profiles) in complex organisms like plants and animals. Indeed, their relevance for health and growth has now been recognised in salmon aquaculture: There is documented evidence that salmon gut microbiota affect growth, reproduction and vulnerability to disease. Numerous studies have attempted to understand the relationships between gut microbes and salmon phenotypes, but it is becoming clear that growth or disease resistance cannot simply be explained by the effect of the microbes alone. Might there be a solution to these challenges? We propose the answer is yes, and that it lies within their 'hologenome'.

Hologenomic theory offers potential to better understand and improve feed conversion in farmed fish. Specifically, hologenomic theory argues that the genomes of host organisms and their associated microbial communities are subjected to co-evolutionary

forces and constitute a larger super-organism, the 'holobiont'. Thus, the host organism and its microbiome should be studied as a single unit, the hologenome (Figure 1). Intriguingly, recent scientific work indicates that changes in phenotypes of farmed fish result from the interplay among the host genotypes, host physiology, host gut microbiota, as well as feed and environmental factors. This is particularly true for traits heavily affected by gut microbiota (e.g. growth metabolism, immune function, feed conversion rate). Therefore, to best manipulate such traits, one must consider the genome and microbiome together. Such an attempt has never been made in an aquaculture context.

Issues addressed in this project target the prioritised area 'Reduction of losses and robust fish' according to FHF's action plan, and specifically relates to research for improving and maintaining proper gut health in salmonids.

A) The holobiont and its hologenome  
Host + symbiotic microorganisms



B) Holo'omic interactions

Biomolecular interactions between hosts and symbiotic microorganisms triggered by environmental factors yield different holobiont phenotypes

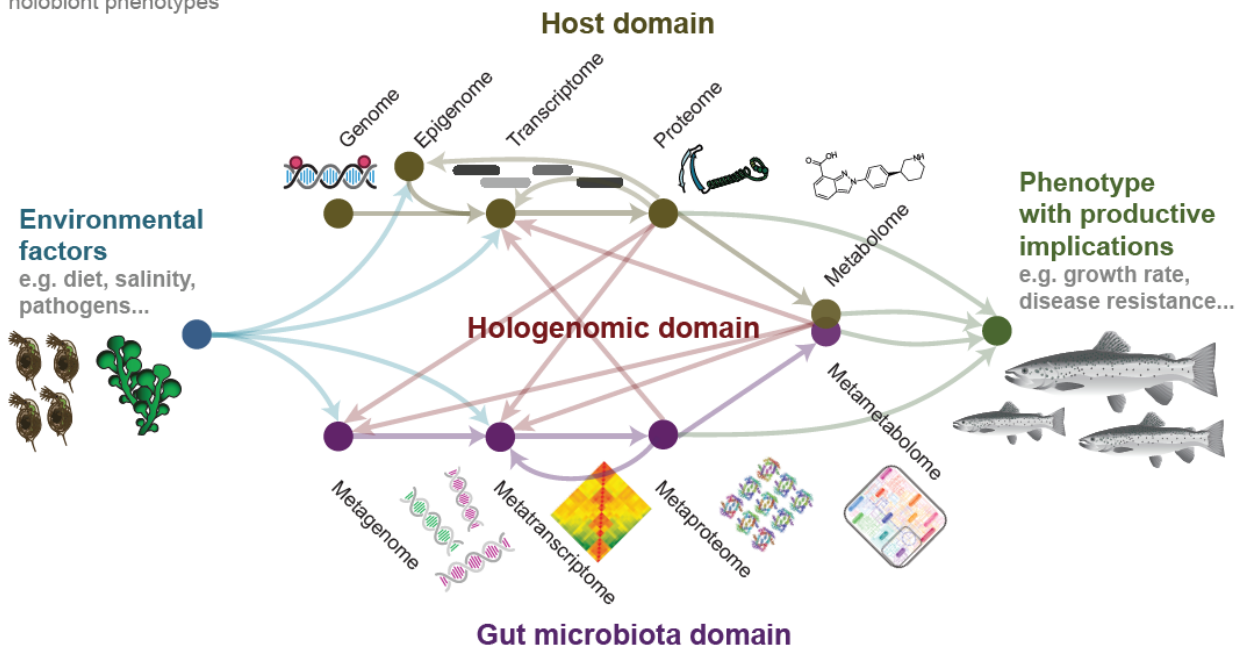


Figure 1. Conceptual framework of HoloFish: the holobiont and the hologenome (A), and the holo-omic interactions between the host and its gut microbiota (B). Arrows indicate the directionality of the effect. Blue arrows indicate environmental effects, which might affect metagenomic composition, gene expression of both the host and its intestinal microorganisms, and also introduce epigenomic variation. Brown arrows show molecular interactions within the host domain, while purple arrows show molecular interactions within the gut microbiota domain. Red arrows highlight holo-omic interactions, in other words potential reciprocal effects between the host and its intestinal microorganisms at different omic levels. Finally, green arrows link all these interactions with host phenotypes. Note the overlapping circles of the host metabolome and the microbiota metagenome; this indicates that the source of metabolites often cannot be assigned either to the host or the microbiota, but are the result of the combination of both domains. Figure based on (Limborg et al. 2018).

## The HoloFish project

The HoloFish project was set up to develop and apply the above-mentioned hologenomic framework to compare host genomes and microbiome data to better understand observed size differences in a commercial salmon cohort. HoloFish was originally planned to run for 3 years from 2018–2020, but was extended by one year based on unforeseen challenges, including COVID-19, but without affecting the budget.

The HoloFish consortium is anchored at the Norwegian University of Science and Technology (NTNU) and made up of a total of five partners from Norway and Denmark, who are presented together with their scientific roles in the project in Table 1. See also the associated website at FHF<sup>1</sup>.

Table 1. Overview of the HoloFish consortium

Partner	Expertise	Role in HoloFish
Norwegian University of Science and Technology (NTNU)	Genomics and hologenomics	Project coordinator and leading hologenomics analyses
University of Copenhagen (UCPH)	Metagenomics and hologenomics	Analysing metagenome data and contributing to hologenomics analyses
Institute for Marine Research (IMR)	Fish and consumer health	Analysis of fatty acid profiles in salmon muscle
Aarhus University (AU)	Environmental metabolomics	Analyses of the intestinal metabolomics landscape in salmon
Lerøy Seafood Group (LSG)	Salmon producer	Providing samples from a commercial production. Interpretation of results and future implementations.

The HoloFish project has been organised around four work packages (WPs) designed around the different stages of the work. The work packages are summarised in Table 2.

<sup>1</sup> <https://www.fhf.no/prosjekter/prosjektbasen/901436/>



The project execution and methods are in detail described in Appendix 7. The results are summarised in the following sections.

Table 2. Overview of the HoloFish work packages

<b>Work Package (WP)</b>	<b>WP description</b>
<b><i>WP 1: Growth and feed experiments and sampling</i></b>	There will be sampled several tissues and gut contents from 360 adult Atlantic salmon that have been raised under three different diets in commercial sized cages (120 fish per diet).
<b><i>WP 2: Molecular analyses</i></b>	In this work package there will be generated multiple levels of molecular data for the salmon hosts: i) genome, ii) gene expression profiles, iii) epigenome profile, and iv) muscle fatty acid profile. Similarly, for the salmon gut microbiome there will be generated data for: i) microbial genomes, ii) microbial gene expression profile <sup>2</sup> , and iii) metabolome of the gut.
<b><i>WP 3: Data analyses</i></b>	Data from WP 2 will be analyzed using first existing protocols for describing molecular differences in host and gut-microbial composition/function among size groups and commercial diets. Second, we explored whether correlations exist between salmon genome and/or epigenome and/or transcriptome, with microbial genome and/or genes and/or transcriptome.
<b><i>WP 4: Validation experiments</i></b>	New biomarkers for monitoring fish health will be developed based on results in WP 3, and tested for application in commercial production by Lerøy. These new biomarkers will provide a tool for helping aquacultural firms improve fish health as well as their productivity.

<sup>2</sup> These data were not generated due to a re-prioritisation to increase sample sizes of each size group

# Research questions and objectives

We focus on new knowledge for **improving gut-health** in salmonids; our aim is to identify how the growth rate of farmed salmon is affected by the interacting effects of the feed composition, gut-microbiota and the salmon host genome, by using an innovative hologenomic approach (Figure 1). We will document how the host genotype co-interacts with the feed regime to influence the composition and activity of the gut microbiome communities in farmed salmon. These results will be verified at a commercial scale through participation of Lerøy and used directly to develop a new strategy for breeding and feeding regimes. These will focus on optimising the match between the salmon's genetic background and its diet, so as to optimise gut-health and growth output.

Our primary objective is to use a hologenomics approach to decipher if, and how, farmed Atlantic salmon genomic mechanisms regulate their gut microbial community composition and activity in response to two high-quality commercial diets.

## The specific **objectives** of HoloFish are:

- O1. To build an analytical framework to exploit these interactions for optimising salmon health, feed conversion and overall growth in aquaculture.
- O2. To use this framework to guide a validation trial in collaboration with Lerøy Seafood Group.
- O3. To disseminate the findings to not only the salmon aquaculture industry, but also the wider agri- and aquacultural industries.

In order to design the research activities towards reaching these objectives, we have further defined four specific project aims that are aligned with the four WPs described above. These aims are listed here and re-addressed at the end of this report.

## Our specific **aims** relate to four planned WPs and these are to:

- A1. Sample tissue, gut content and gut-mucosal samples from sea-farmed salmon that are fed on three commercial diets, and within each treatment, exhibit variable sizes at harvest age (**WP 1**).
- A2. Use this data to characterise the genome, epigenome and transcriptome for each individual salmon, as well as the metagenomes, transcriptomes and metabolomes of their gut contents (**WP 2**).
- A3. Apply association mapping to these parameters within a hologenomic framework in order to decipher the link between salmon genomes and their gut microbiota composition and activity, how this relates to dietary treatment and how these in turn affect growth, feed conversion, health and muscle fatty acid profile (**WP 3**).
- A4. Perform a validation growth trial to evaluate findings and commercial potential of the hologenomics approach (**WP 4**).

# Results and discussion

## WP 1: Sampling

A total of 463 ready to harvest Atlantic salmon were sampled from two commercial feeds (253 from Feed1 and 210 from Feed2). Gutted weight of the salmon varied from 0.78 to 7.8 kg and approximately 70 fish from each size class (small, medium and large) were sampled per commercial feed type (Figure 2A). Generally the fish were in good condition; however, we observed a high incidence of tapeworm, with 378 (81.6%) of the 436 individuals having at least 1 tapeworm present in their intestine. Individuals heavily parasitised with tapeworm were significantly smaller than non-parasitised fish (Figure 2B).

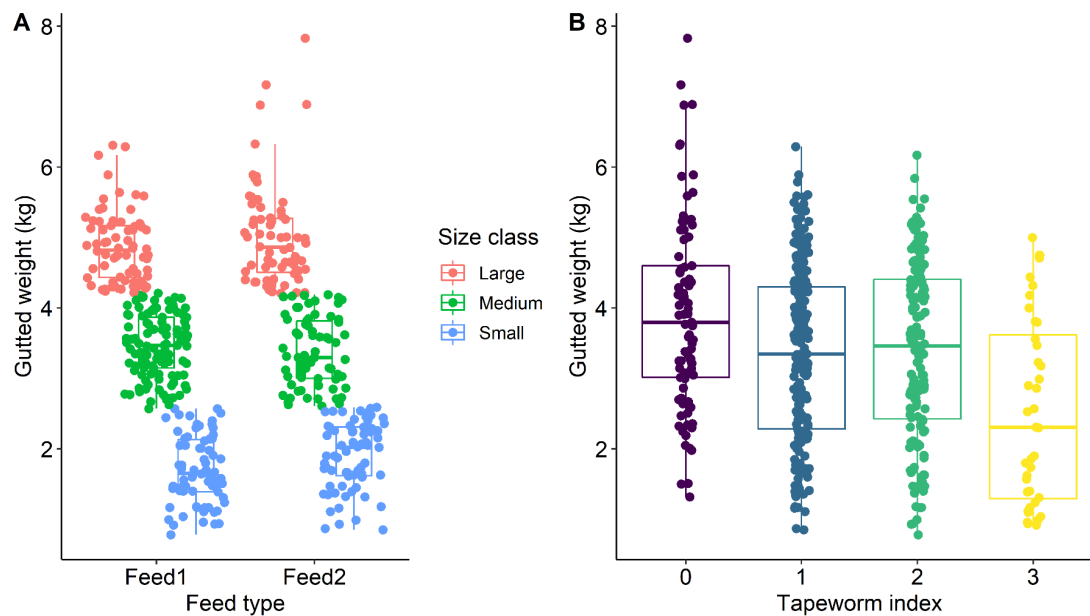


Figure 2. Gutted weight distribution between feed types (A) and tapeworm infection levels (B). Size classes in (A) were defined based on gutted weight and with the aim of including 70 fish per feed type in each class. In (B), fish heavily parasitised with tapeworm (index = 3) were significantly smaller than non-parasitised fish (index = 0).

## WP 2 & 3: Independent analyses

The following datasets were generated for each individual:

- microbial metagenome from the gut content
- microbial microbiota from 16S profiling of the gut epithelium
- host genome
- host epigenome
- host fatty acid profiles from muscle
- host transcriptome from the gut epithelium
- host + microbial metabolome (meta-metabolomics) from the gut content.

Each dataset was analysed independently before being integrated into an hologenomic framework. The microbial metagenome was used to define microbiome phenotypes for each fish, which were then used as additional variables, along with feed type and size class, in association tests within each of the other datasets.

### Microbial metagenome

#### Microbiome composition

The salmon gut microbiome consisted of 14 bacterial genomes. Of these, five were related to the mycoplasma family of bacteria (*Mycoplasma* and *Mycoplasmataceae*). Two of these mycoplasma genomes were also identified in the internal microbiome of tapeworm samples and have been named accordingly (*Mycoplasmataceae* CEseq1 & *Mycoplasma* CEseq7). This diversity is lower than what is commonly observed for the human gut microbiome (Qin et al. 2010) but is consistent with similar studies of salmonids (Rasmussen et al. 2022; Holben et al. 2002; Llewellyn et al. 2016).

The bacterial genome identified as *Mycoplasma salmoninae*, a known commensal of Atlantic salmon (Rasmussen et al. 2021), dominated the majority of samples, with an average relative abundance of 92% (Figure 3A). However, *M. salmoninae* abundance was significantly higher in larger individuals (Figure 3A), suggesting a role in promoting a healthy gut environment and increased growth in salmon. In contrast, smaller individuals instead had more varied microbiomes (as indicated by higher alpha diversity, Figure 3B) with higher relative abundances of two other mycoplasma genomes (an unknown *Mycoplasma* species and *Mycoplasmataceae* CEseq1) and *Photobacterium phosphoreum* (Figure 4). Bacteria of the genus *Photobacterium* can be pathogenic in fish (Ina-Salwany et al. 2019), while *Mycoplasmataceae* CEseq1 was first identified in the internal tapeworm microbiome. Furthermore, while *M. salmoninae* abundance tended to be lower in fish parasitised with tapeworm (Figure 3C), the presence of the other four mycoplasma species was higher in parasitised fish (72% in parasitised fish

vs. 19% in non-parasitised fish). Thus, these bacteria may be associated with a suboptimal salmon gut microbiome.

Feed type had little effect on the salmon gut microbiome, as only one bacterial genome, an unknown *Aliivibrio* species, was more abundant in fish fed Feed2 compared to Feed1 (Figure 3D).

The gene content of the bacterial genomes varied depending on taxonomy, as expected (Figure 5B). The *M. salmoninae*, *Mycoplasmataceae* CEseq1 and *Mycoplasma* CEseq7 genomes have been characterised in more detail elsewhere (Appendix 5, (Rasmussen et al. 2021)).

### Microbiome phenotypes

Based on these results, we defined the following microbiome phenotypes to include in association testing with the other 'omics datasets:

- High (>75%) relative abundance of *M. salmoninae* (binary True/False variable) and relative abundance (continuous variable)
- Detection (presence/absence, binary variable) and summed relative abundance of 'other' mycoplasma species (i.e. the four *Mycoplasmataceae* genomes, excluding *M. salmoninae*)
- Detection and summed relative abundance of *Vibrionaceae* species (four *Photobacterium* and *Aliivibrio* genomes)
- Detection and relative abundance of *Brevinema* species (one genome)
- Alpha diversity (Hill's Shannon index; continuous variable)
- Coordinates from the microbiome NMDS ordination (Figure 5A, dimensions 1 and 2; continuous variables)

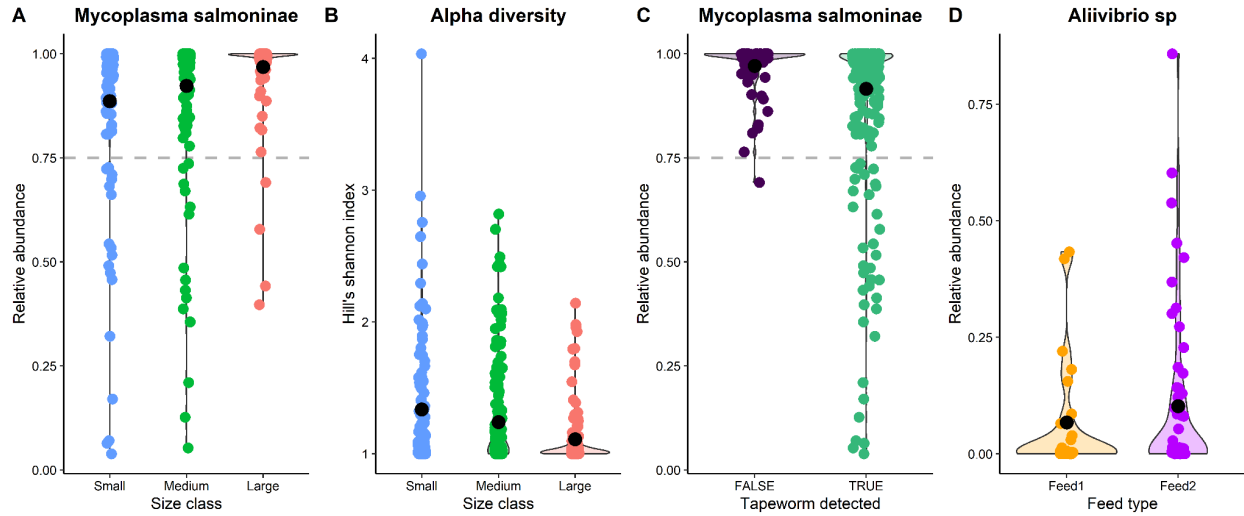


Figure 3. (A) Relative abundance of *M. salmoninae* by size class, which was higher in abundance in larger fish. (B) Alpha diversity of the salmon gut microbiome, as measured by Hill's Shannon, which was lower in smaller fish. (C) Relative abundance of *M. salmoninae* by tapeworm presence/absence, which was higher in fish where tapeworm was not detected. (D) Relative abundance of an unknown *Aliivibrio* sp. by feed type, which was higher in abundance in Feed2 compared to Feed1. Dashed line in (A) and (C) indicates cut-off threshold (75%) for "high" vs "low" abundance of *M. salmoninae*. Black dots indicate the mean value in each group.

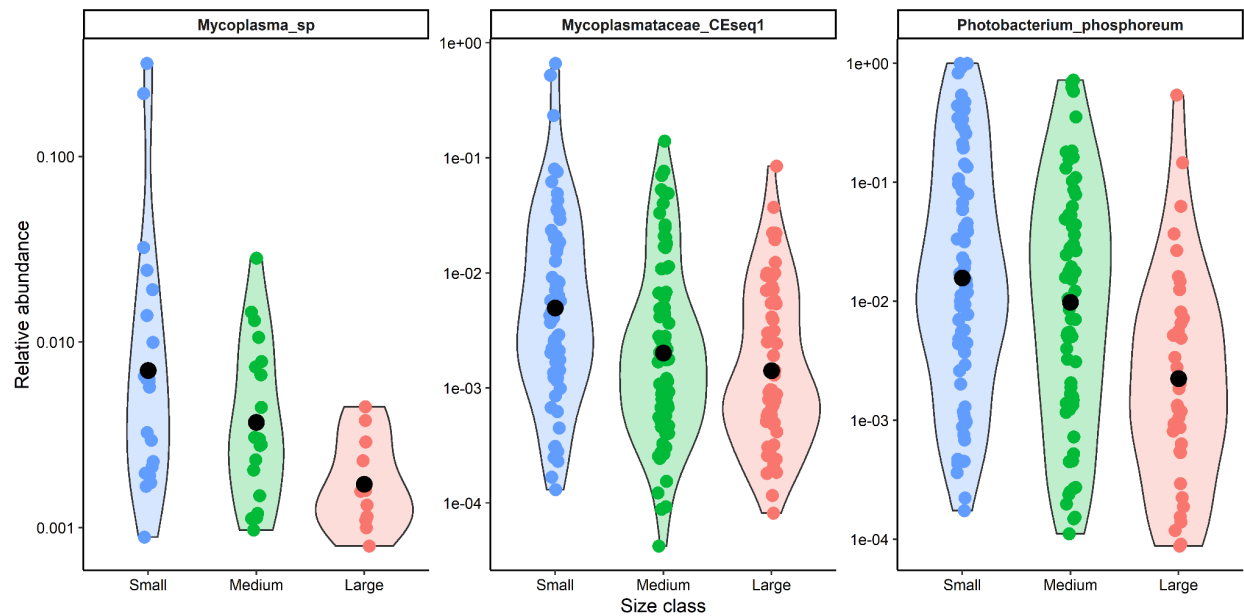


Figure 4. Relative abundance of an unknown *Mycoplasma* sp., *Mycoplasmataceae* CEseq1 and *P. phosphoreum* by size class. All three bacteria decreased in abundance in large fish. Samples in which these bacteria were not detected are not shown. Black dots indicate the mean value in each group.

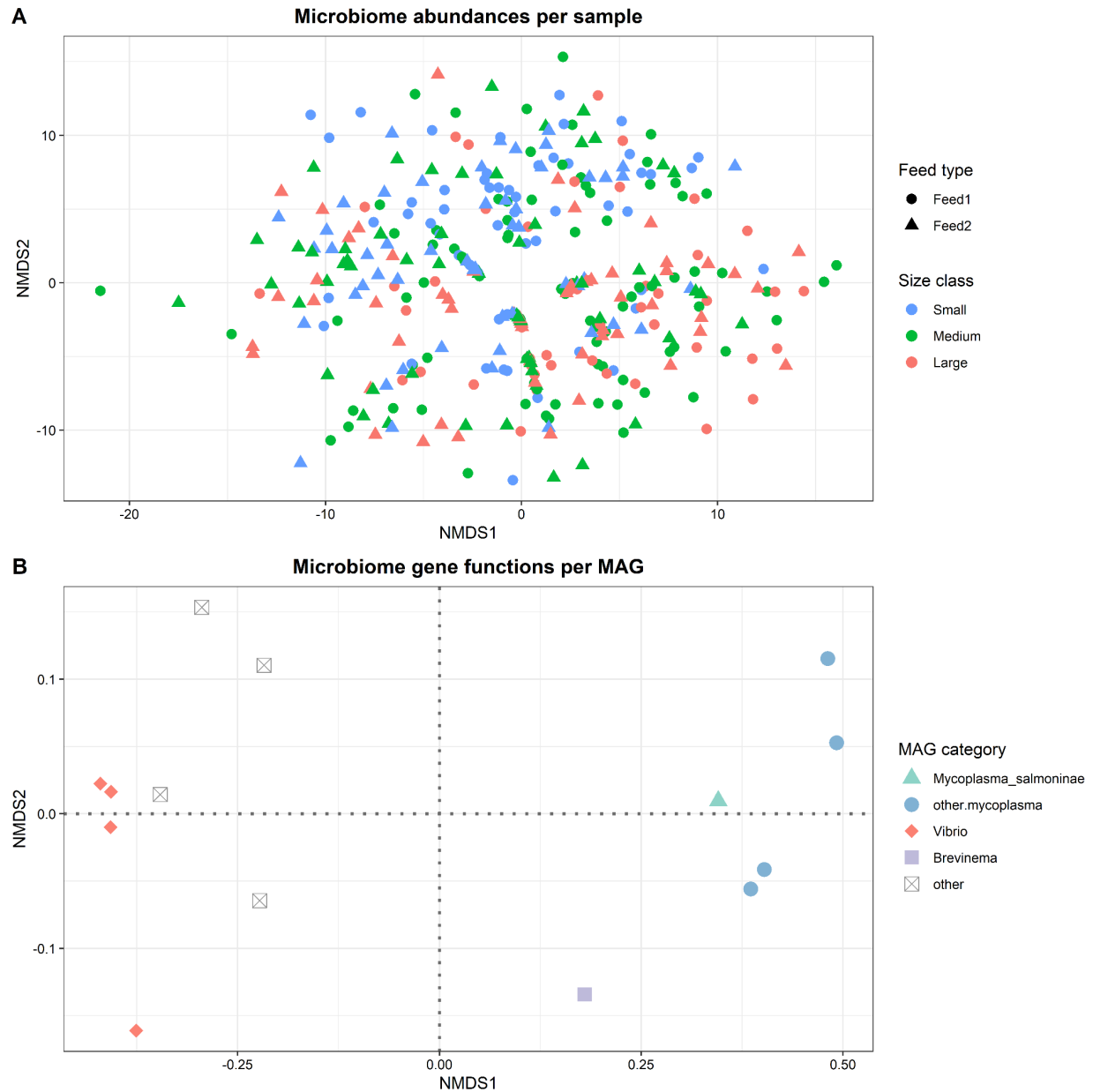


Figure 5. (A) Ordination by NMDS of microbiome composition (bacteria relative abundances) per sample. Each point represents a sample, colored by size class and shaped by feed type. Samples located more closely together have more similar microbiome compositions. Samples do not tend to cluster by size or feed type, indicating that generally, the microbiome composition of the salmon are quite similar. (B) Ordination by NMDS of microbiome gene functions (KEGG pathway presence/absence) per bacterial genome (MAG). Each point represents a bacterial genome, colored and shaped by taxonomy ('MAG category'). Bacterial genomes located more closely together contain more similar gene functions. The genomes tend to cluster by taxonomy.

## Microbial 16S profiling

The main aim of the 16S dataset was to compare gut content (from which both 16S and metagenomic data were generated) and the resident microbial community present in the gut mucosa (16S data generated from gut epithelium scrapes). 16S data was also generated for investigation of the effect of tapeworm infection on the gut microbiota of the salmon and characterisation of the internal tapeworm microbiota.

A core microbiota was observed between the salmon gut content and gut mucosa samples, dominated by *Mycoplasma*, *Photobacterium*, *Brevinema* and *Aliivibrio* (Figure 6). However, the gut content samples were more varied, containing additional bacteria, such as *Carnobacterium* and *Lactobacillus* (Figure 6). *Lactobacillus* was dominant in feed pellets from both commercial feeds, suggesting that this bacteria is introduced by the diet and is transient in the gut.

Comparison of gut mucosa samples between different levels of tapeworm infection revealed decreases in the abundance of mycoplasma related to *M. salmoninae* as the level of tapeworm infection increased, with corresponding increases in other, potentially pathogenic bacterial genera, like *Photobacterium* (Figure 7), consistent with results from the metagenomic data (Figure 3C). In contrast to the salmon gut microbiota, the internal microbiota of the tapeworm was dominated by mycoplasma related to *Mycoplasmataceae* CEseq1 & *Mycoplasma* CEseq7 (Figure 7). Our results suggest that the tapeworm may act as a Trojan horse, introducing new species of bacteria to the salmon gut microbiome and potentially contributing to a “less healthy” salmon gut microbiome composition.



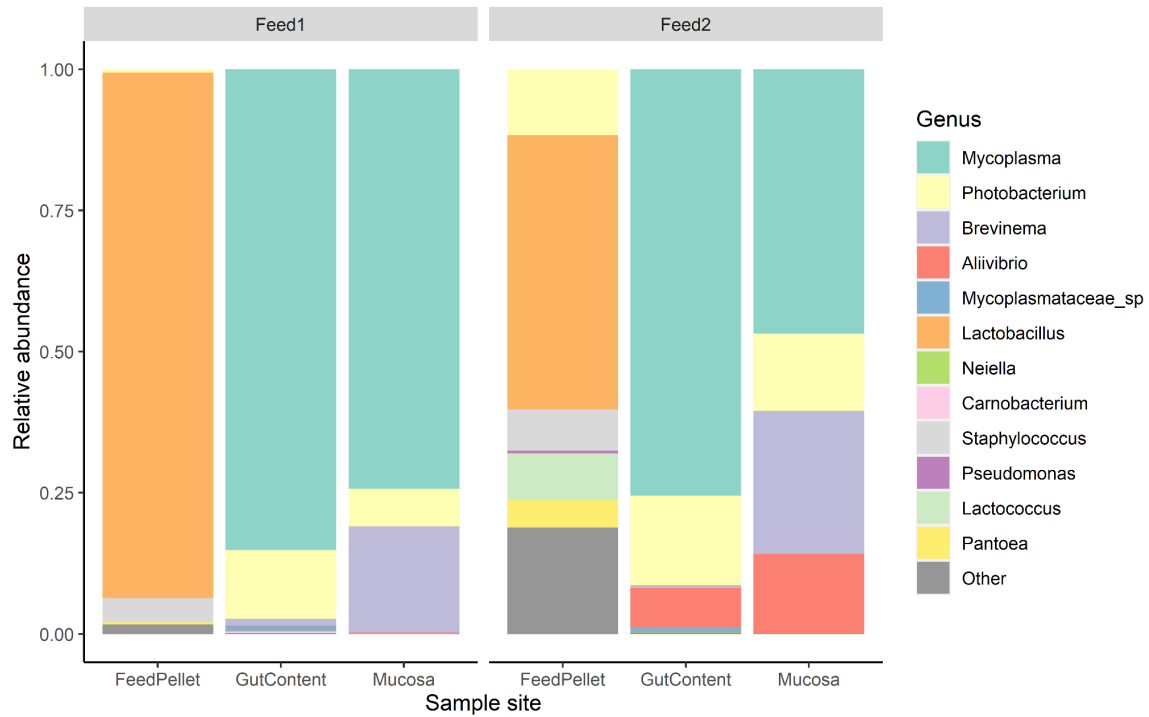


Figure 6. Relative abundance of bacterial genera identified by 16S profiling in the feed pellets, gut content and gut mucosa microbiota samples. In both feed types, *Mycoplasma* dominated salmon gut content and mucosa samples. However, gut content samples contained additional bacterial genera at low abundances. Feed pellets from both feed types were dominated by *Lactobacillus*. Only the 10 most abundant genera are shown, with the rest grouped as “Other”.

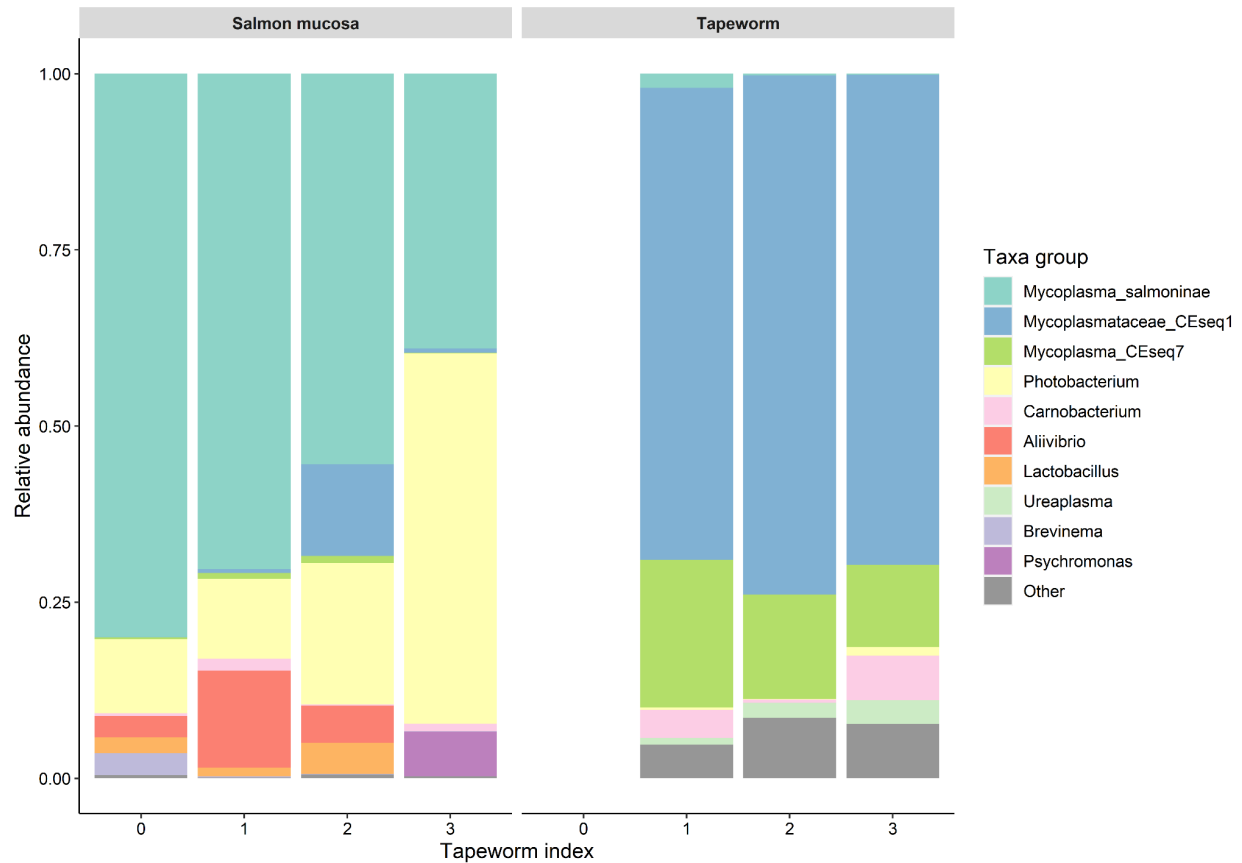


Figure 7. Relative abundance of bacterial taxa identified by 16S profiling in the salmon mucosa and internal tapeworm samples. Individuals are pooled by tapeworm index (0 = no tapeworm present, 3= heavily parasitised; no internal tapeworm samples exist for tapeworm index = 0). The mycoplasma group of bacteria have been separated into taxa genetically related to *Mycoplasma salmoninae*, *Mycoplasmataceae* CEseq1 and *Mycoplasma* CEseq7. Otherwise, the 7 most abundant genera are shown, with the rest grouped as “Other”. Generally, taxa related to *M. salmoninae* dominated the salmon gut mucosa, though the abundance decreased as tapeworm infection level increased. Taxa related to *Mycoplasmataceae* CEseq1 and *Mycoplasma* CEseq7 dominated the tapeworm internal microbiota.

## Host genome

### Size class

In the independent analyses, the host genomic dataset was used to determine whether certain genotypes or single nucleotide polymorphisms (SNPs) in the genome were associated with size class or the microbiome variables, while accounting for feed type. Some population structure was observed (Figure 8), with individuals from one sub-population tending to contain more large individuals. No strong differences were observed by feed type (Figure 8). In contrast, in the genome wide analysis study (GWAS), many SNPs were associated with feed type (Figure 9A). However, these SNPs appear to be stochastic outliers that were not consistently detected in other tests, thus they are unlikely to be biologically significant in our data and we do not consider them further. No SNPs were associated with gutted weight (after controlling for feed type) at the strict significance threshold of  $p < 5 \times 10^{-8}$ , although there was weak evidence of an association for several SNPs in multiple chromosomes ( $p < 1 \times 10^{-5}$ ; Figure 9B).

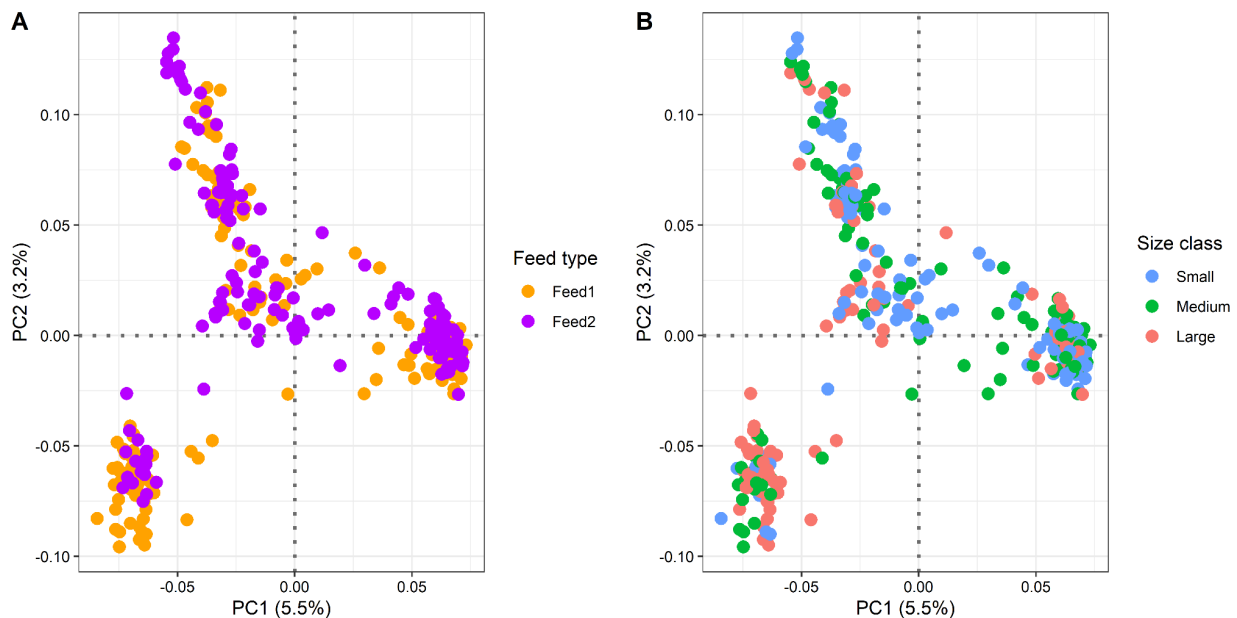


Figure 8. PCA showing individuals clustering into sub-populations in PC1 and PC2, based on genomic variation. Individuals are coloured by feed type (A) and size class (B). While individuals from both feed types are fairly evenly spread between subpopulations in (A), more large individuals are present in the sub-population in the bottom left corner of the plot in (A).

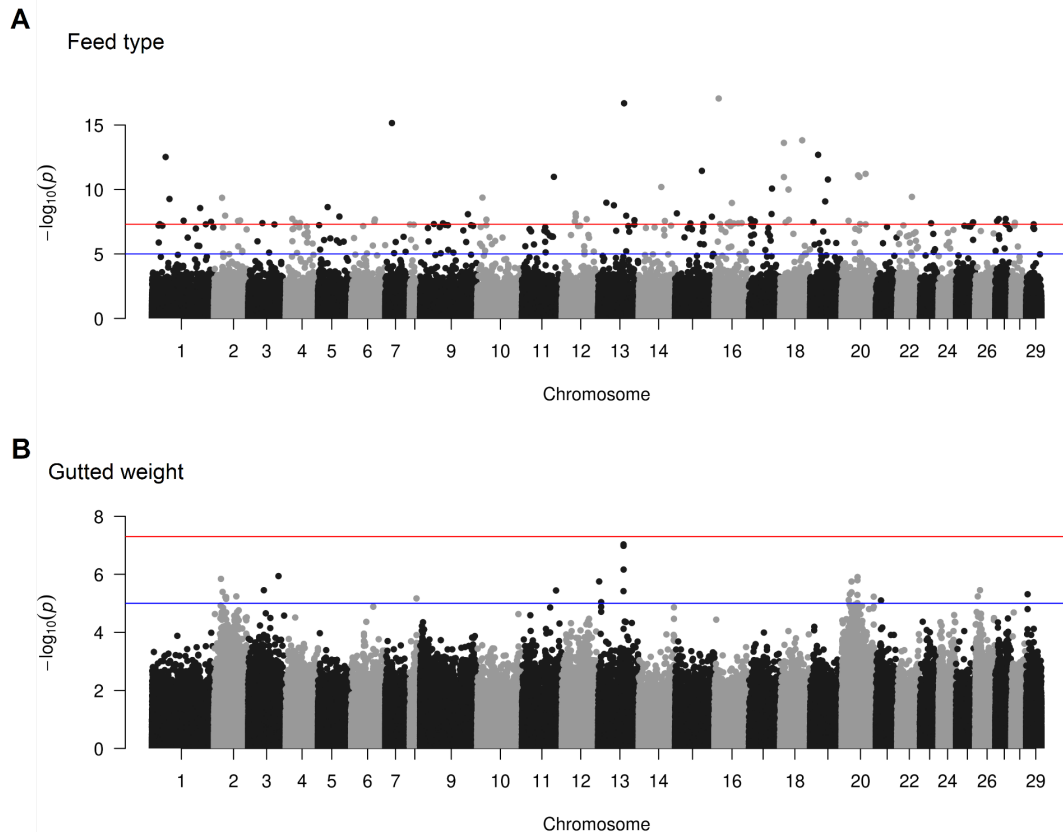


Figure 9. Manhattan plot showing (A) feed type and (B) size (gutted weight) GWAS results of p values (shown on a  $-\log$  axis) of SNPs across the genome. Several SNPs were significantly associated with feed type at  $p < 5 \times 10^{-8}$  (red line). No SNPs were significantly associated with salmon gutted weight; however, several SNPs in multiple chromosomes tended to correlate with gutted weight ( $p < 1 \times 10^{-5}$ ; blue line).

### Microbiome phenotypes

We used microbiome GWAS (mGWAS) to identify SNPs in the salmon host genome associated with the microbiome phenotypes defined above, while correcting for feed type, size class and tapeworm effects. Overall, we observed very few associations between SNPs and microbiome composition. However, a clear SNP peak in chromosome 5 was associated with the presence of an unknown *Mycoplasma* sp. (Figure 10A). Three genes were located in this region, coding for proteins likely involved in signalling pathways and innate immune responses (specifically, ankyrin repeat and KH domain-containing protein 1-like, involved in RNA binding and innate immune responses; Teneurin-2-like, involved in neural development and signal transduction; glutamate receptor 1-like, a neurotransmitter). Two additional SNPs were significantly ( $p < 5 \times 10^{-8}$ ) associated with the presence of an unknown *Mycoplasmataceae* sp. (Figure 10B) and the presence of *Photobacterium iliopiscarium* (Figure 10C). The *Mycoplasmataceae* associated SNP was located in a gene which encodes for a transcriptional coactivator (zinc finger MIZ domain-containing protein 1-like), while the *P.*

*iliopiscarium* associated SNP was located in a gene coding for an enzyme which controls the activation of important signalling pathways (diacylglycerol kinase delta).

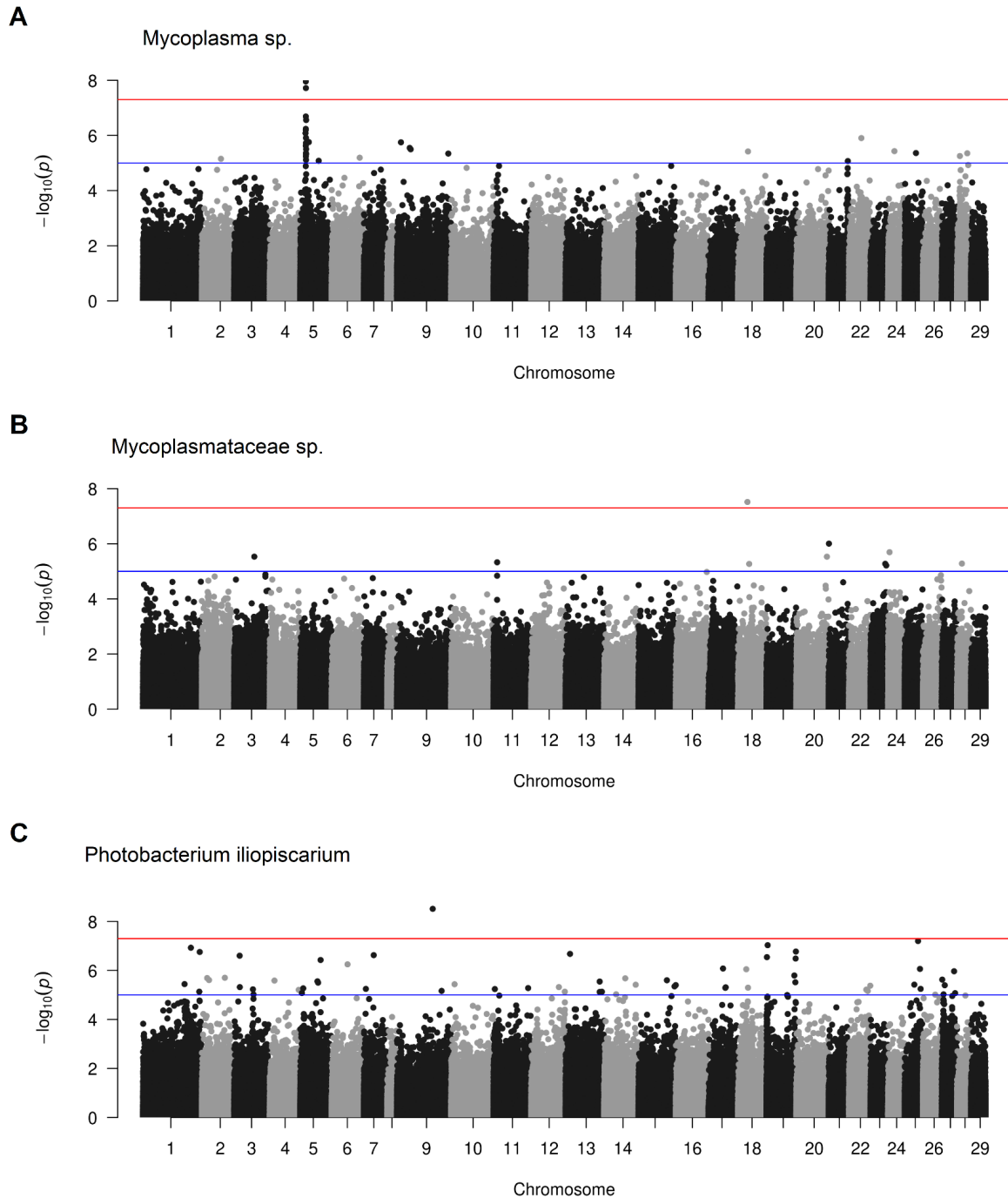


Figure 10. Manhattan plots from the mGWAS, identifying SNPs across the salmon genome associated ( $p < 5 \times 10^{-8}$ ; red line) with presence/absence of 3 bacterial genomes generated from the metagenomics data: (A) unknown *Mycoplasma* sp., (B) unknown *Mycoplasmataceae* sp. and (C) *Photobacterium iliopiscarium*.

## Host epigenome

The host epigenomic dataset was intended to be used to identify DNA methylation in the salmon genome associated with size class and gut microbiome composition. However, due to unexpected technical problems, the generated data were of low sequencing depth and thus of low coverage across the genome per individual. This low coverage prevented accurate identification of methylation differences between size classes with single site resolution. However, the estimated methylation levels in the individual samples can still be valuable for estimating and comparing the general CpG-methylation level between groups.

Furthermore, we are currently analysing newly generated epigenomic data from 20 individuals (10 small, 10 large, 5 from each feed type) with different microbiomes (based on the microbial metagenomic analysis), to find candidate regions with different methylation patterns correlated with microbiome composition. Data generation was outsourced to a reputable company we have had previous good experience with. The data was not available until a few weeks before the official end of the project; however, our quality control shows the data is of higher quality and unaffected by the previous technical issues. Preliminary results indicate that the epigenetic variation based on genome-wide DNA methylation patterns is generally not explaining the differences in size class and microbiome composition (Figure 11). This is however a method based on exploring large scale general differences. There may still be specific regions differentially methylated connected to microbiome composition or size, which further analysis will reveal.

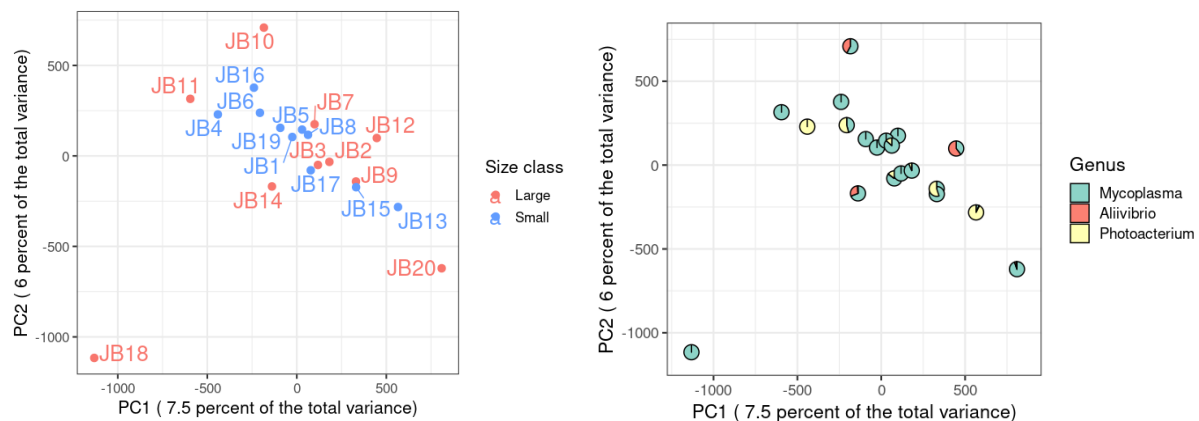


Figure 11. PCA showing the epigenomic variation of the 20 samples colored by size class (left) and microbiome composition (right). No strong clustering of samples by feed type or microbiome composition is evident.

## Host transcriptome

### Size class

The host transcriptome dataset was used to identify differences in host gut epithelium gene expression between size classes, while taking into account feed type. Widespread changes in gene expression were clear by feed type (Figure 12A) and to a lesser extent by size class (Figure 12B). These changes were confirmed by the differential expression analysis, with a large set of differentially expressed genes identified between feed types (>8000 genes) and a smaller set of differentially expressed genes identified between size classes (>1000 genes). Genes upregulated in large fish tended to have functions involving metabolism, such as lipid and fatty acid biosynthesis and generation of energy (examples in Figure 13).

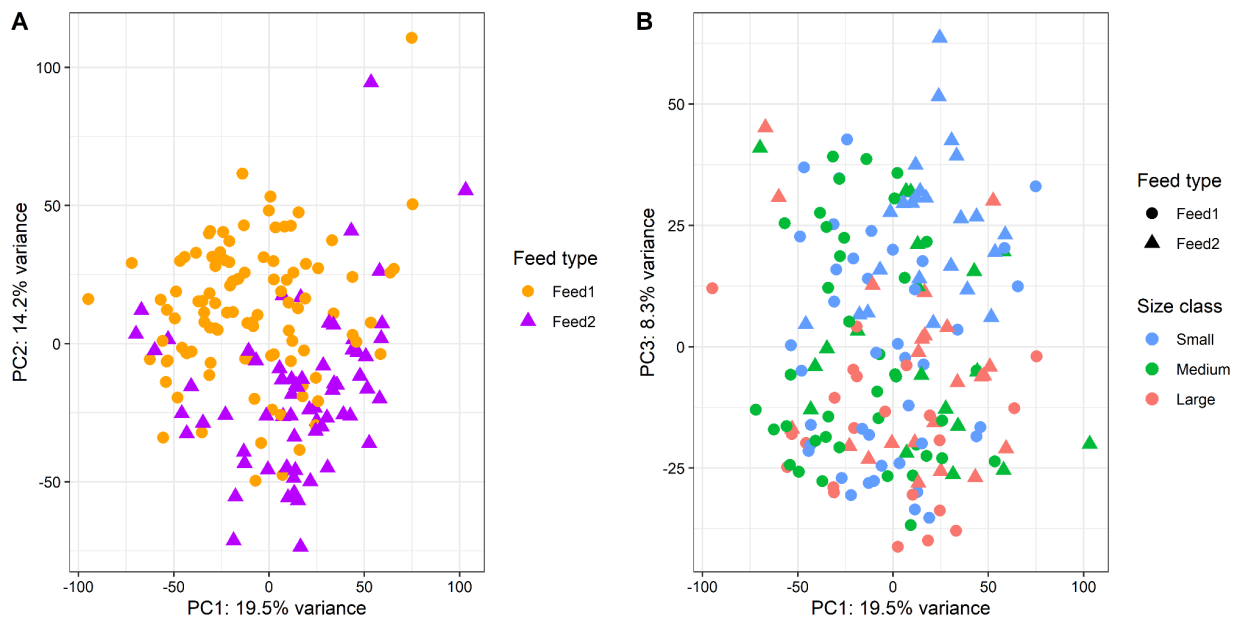


Figure 12. (A) PCA of gene expression by feed type in the first two principal components (PC1 & PC2). (B) PCA by size class in PC1 and PC3. Samples cluster by feed type in (A) and to a lesser extent by size class in (B).

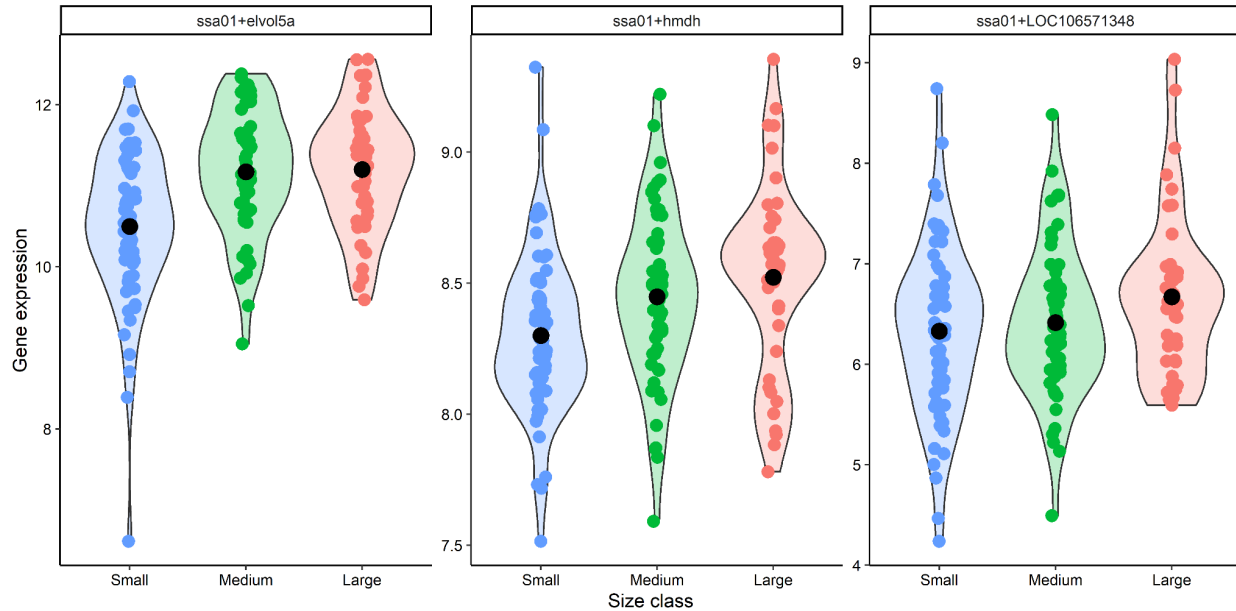


Figure 13. Examples of genes with functions related to lipid biosynthesis with increased expression in large fish. Genes are labelled by chromosome (*ssa*) and gene ID: **elvol5a**: polyunsaturated fatty acid elongase; **hmdh**: 3-hydroxy-3-methylglutaryl-coenzyme A reductase; **LOC106571348**: elongation of very long chain fatty acids protein 7-like. Black dots indicate the mean value in each group.

### Microbiome phenotypes

After controlling for size class, feed type and tapeworm index, 75 differentially expressed genes were associated with high abundance of *M. salmoninae*. Genes associated with *M. salmoninae* tended to have functions involving fatty acid and lipid metabolism and isoprenoid biosynthesis. For example, two genes coding for key enzymes in isoprenoid biosynthesis were downregulated in individuals with high *M. salmoninae* abundances (Figure 14). Eleven differentially expressed genes were associated with the presence of other mycoplasma species; however, no biologically meaningful patterns could be discerned from their functions. No other microbiome phenotype was associated with changes in host gut epithelial gene expression.



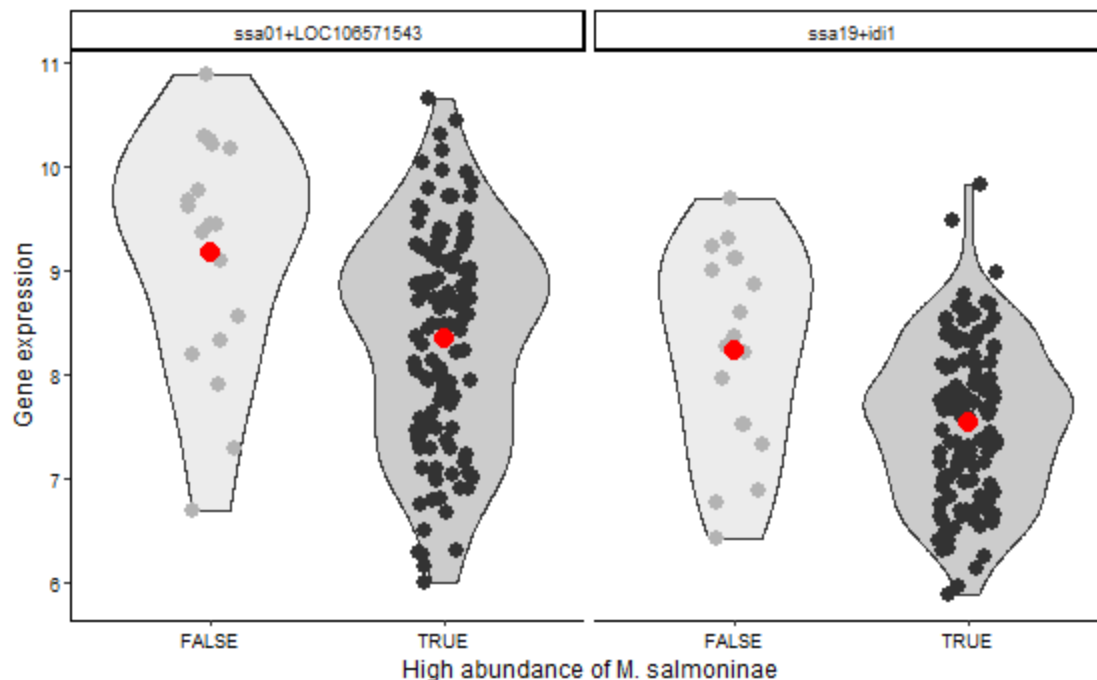


Figure 14. Examples of genes with functions related to isoprenoid biosynthesis with decreased expression in fish with high abundances of *M. salmoninae*. Genes are labelled by chromosome (ssa) and gene ID: **LOC106571543**: 3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble); **idi1**: isopentenyl-diphosphate delta isomerase 1. Red dots indicate the mean value in each group.

## Untargeted meta-metabolomics

The metabolomics dataset was used to explore interactions between the gut microbes and the host metabolism. Of the 971 metabolites identified passing quality control, 765 (78.78%) could be annotated at the superclass level using MS2 spectra, while 456 (46.96%) could be annotated at the most specific level. With this level of annotation, it is currently difficult to distinguish between host-derived and microbiome derived metabolites, thus in this analysis, we focus on broad changes in overall gut metabolism.

### Size class

No strong clustering by feed type or size class was observed in overall metabolite abundances (Figure 15B-C), although strong batch effects could be observed (Figure 15A), a known problem in metabolomics data (Viant et al. 2019) which we accounted for in subsequent analyses (details in Appendix 7). After controlling for batch effects, almost 400 metabolites were associated with size class, while almost 250 metabolites were associated with feed type. Network analysis revealed that many of the metabolites associated with size class were related to fatty acids and amino acids. For example, metabolites related to linoleic acids and prostaglandins were at higher abundance in

large fish (Figure 16). These results are consistent with the host transcriptomics results of increased abundance of genes related to lipid and fatty acid biosynthesis in large fish.

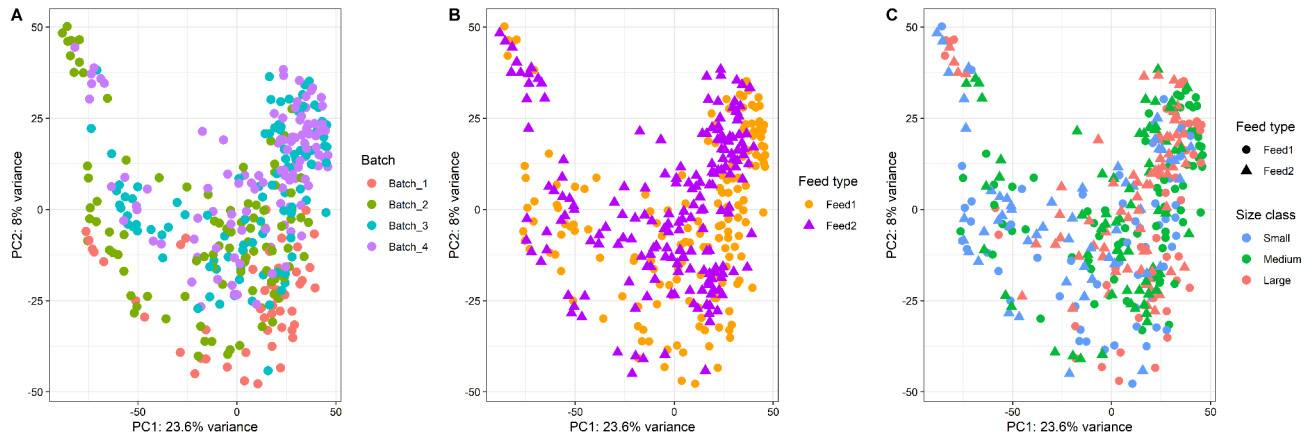


Figure 15. PCA of metabolite abundances, coloured by metabolomics processing batch (A), feed type (B) and size class (C). Samples tend to cluster by batch, rather than by feed time or size class, indicating that batch effects do occur. Thus, we controlled for these batch effects in all subsequent analyses (details in Appendix 7).

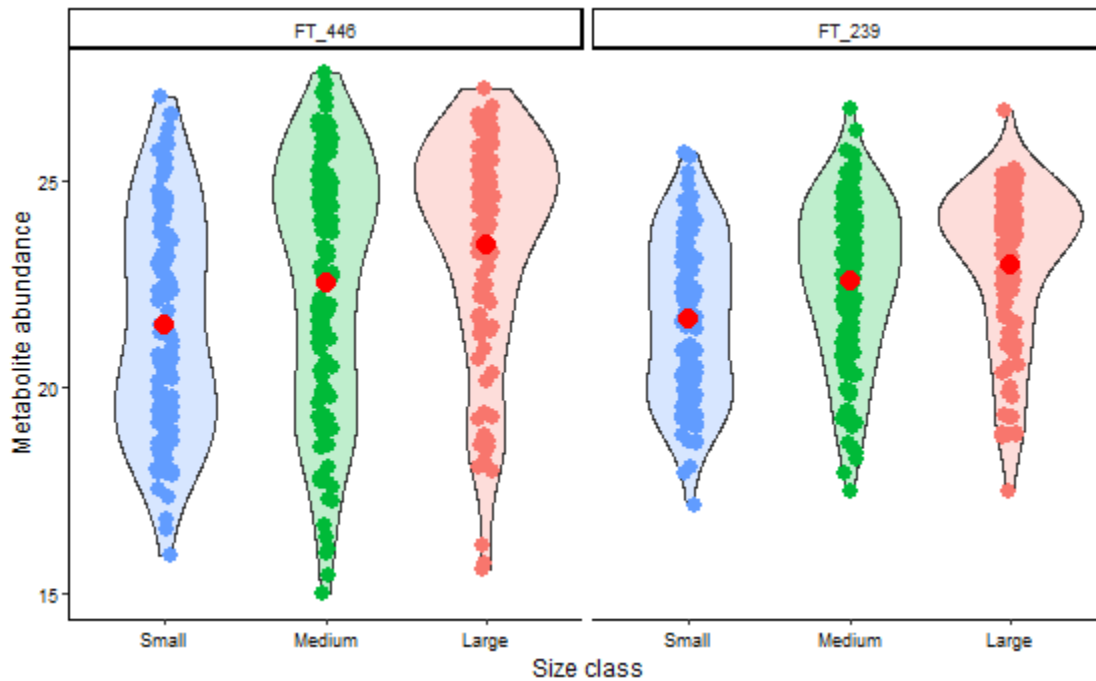


Figure 16. Examples of metabolites annotated as lipids with increased abundance in large fish . Metabolites are labelled by an arbitrary metabolite ID: **FT\_446**: prostaglandins and related compounds; **FT\_239**: linoleic acids and derivatives. Red dots indicate the mean value in each group.

## Microbiome phenotypes

Several microbiome phenotypes were associated with differential metabolite abundance, including high abundance of *M. salmoninae* (91 metabolites), detection of other mycoplasma species (42 metabolites) and detection of *Vibrionaceae* species (122 metabolites). In the network analysis, metabolites related to prenols were at decreased abundances in individuals with high abundance of *M. salmoninae* and at increased abundances in individuals where *Vibrionaceae* species were detected (examples in Figure 17). Prenols include isoprenoids, in concordance with the host transcriptomics finding of decreased expression of isoprenoid biosynthesis genes in salmon with high abundance of *M. salmoninae*. No metabolite networks were associated with detection of other mycoplasma species, although the differentially abundant metabolites included many annotations related to bile acids.

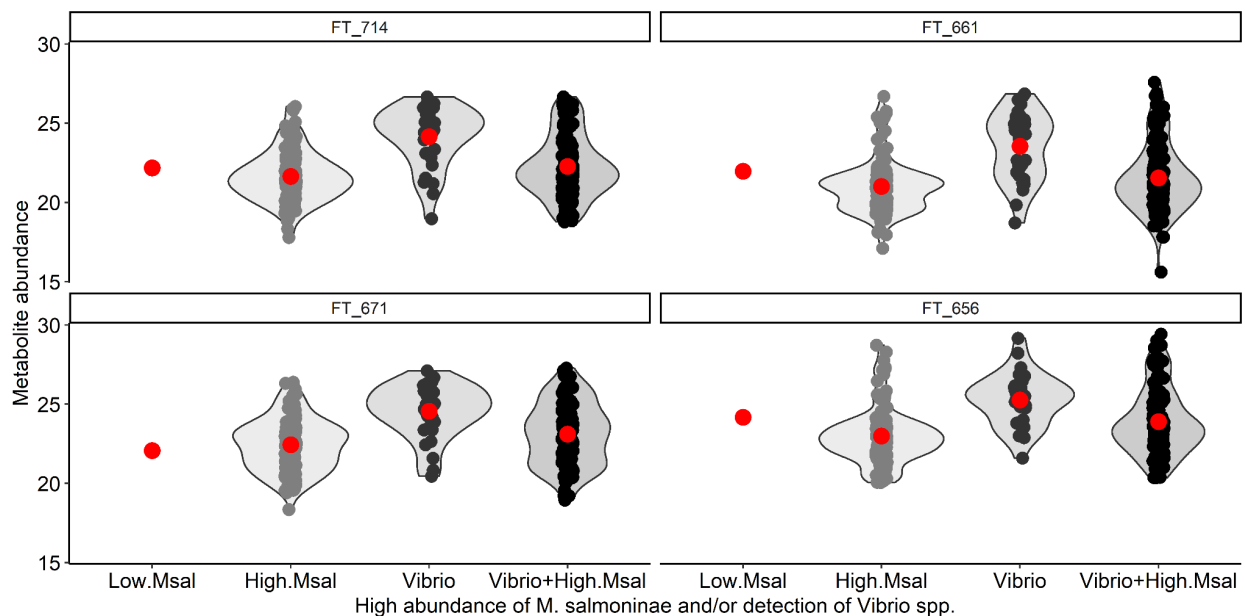


Figure 17. Examples of metabolites annotated as lipids related to prostaglandins and prenols with differing levels of abundance depending on microbiome phenotype. Samples have been categorised depending on their abundance of *M. salmoninae* and detection of *Vibrionaceae* spp.: **Low.Msal**: low abundance of *M. salmoninae* and no detection of *Vibrionaceae* spp. (n=1); **High.Msal**: high abundance of *M. salmoninae* and no detection of *Vibrionaceae* spp.; **Vibrio**: detection of *Vibrionaceae* spp. and low abundance of *M. salmoninae*; **Vibrio+High.Msal**: high abundance of *M. salmoninae* and detection of *Vibrionaceae* spp. Metabolites are labelled by an arbitrary metabolite ID: **FT\_714**: prenol lipids; **FT\_661**: prostaglandins and related compounds; **FT\_671**: prenol lipids; **FT\_656**: Fatty acids and conjugates. Red dots indicate the mean value in each group. Generally, samples with only *Vibrionaceae* spp. have the highest abundances of these metabolites, while samples with only high abundances of *M. salmoninae* have the lowest abundances of these metabolites.

## Host fatty acid profiling

The abundance of 45 fatty acids in the salmon muscle were profiled, along with the fatty acid composition of feed pellets from both feed types. The abundance of 17 fatty acids and 8 summed fatty acid classes were associated with size class after adjustment for feed type and batch effects. Many of these fatty acids, such as omega 3 fatty acids, EPA and DHA (Figure 18), are of interest to salmon aquaculture producers and consumers as being of higher nutritional value. These fatty acids were included as covariates in the multi-omics analysis detailed below. Consistent with the metabolomics results, alpha-linolenic acid (ALA) was also at higher abundance in the muscle of large fish, especially within Feed1 (Figure 18).

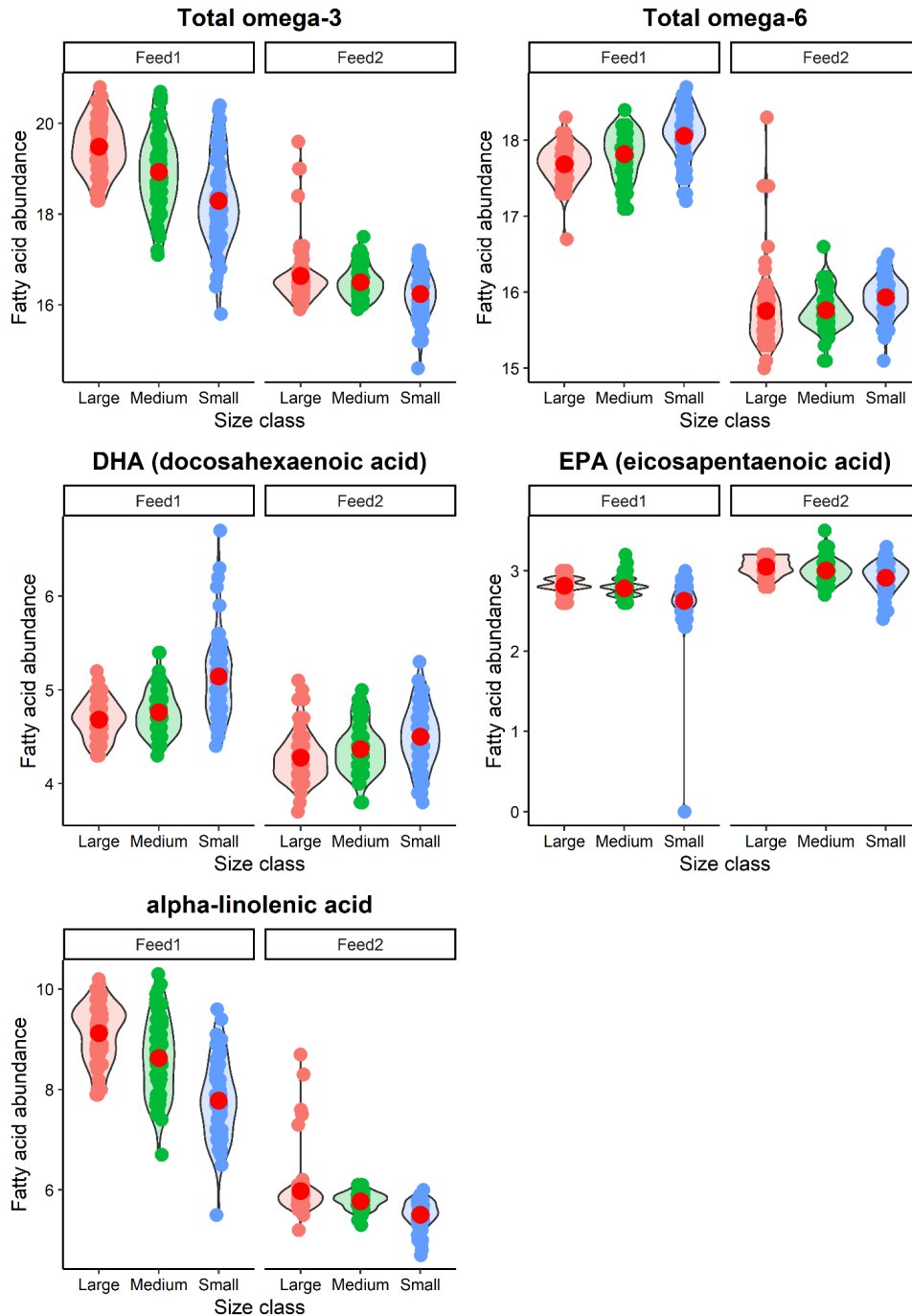


Figure 18. Examples of relative abundance of fatty acids in salmon muscle associated with size class after controlling for feed type. Red dots indicate the mean value in each group. EPA and the sum of all omega-3 fatty acids were more abundant in large fish, while DHA (another omega-3 fatty acid) and the sum of all omega-6 fatty acids were more abundant in small fish. Alpha-linolenic acid was more abundant in large fish, consistent with the metabolomics results.

## WP 3: Holo'omic analysis

### Multi-omics analysis

Unsupervised multi-omics factor analysis (MOFA) was used to identify hidden interactions among the host 'omics data layers. In MOFA, multiple multi-dimensional ('omics) datasets are reduced to a set of 'factors' that explain the variance in the datasets, with the first factor explaining the most variance, analogous to PCA in a single 'omics dataset (Argelaguet et al. 2018) (Figure 19A). These factors were then investigated for correlations with fish size class, feed type, tapeworm index, microbiome phenotypes and abundance of select fatty acids (DHA, EPA, total omega-3 and total omega-6) (Figure 19B). The top ~500 most variable host genome SNPs, expressed genes and metabolites were included as 'omics datasets (after correcting for batch effects). The first 6 factors together explained around 80% of the combined variance in the datasets, mostly from the transcriptomic and metabolomic layers (Figure 19A). Here we present the most interesting findings.

High abundance of *M. salmoninae* was associated with Factor 1 (host transcriptomics) (Figure 20A). Expressed genes contributing to this association were predominantly involved in host pathways related to important cellular functions, like adhesion, proliferation and inflammation, suggesting that the host cells do shape the presence of the intracellular *M. salmoninae* (Figure 20B).

High abundance of *M. salmoninae* and large fish were positively correlated with Factor 5 (metabolomics), whereas detection of *Vibrionaceae* spp. was negatively correlated with this factor (Figure 21). Many organic acids and some acyl carnitine-related lipids were at increased abundance in large fish with high *M. salmoninae* abundances (Figure 21A-D). Acyl carnitines are involved in cellular transport of long-chain fatty acids to the mitochondria for oxidation for energy generation. Metabolites at increased abundance in *Vibrionaceae* positive fish were mostly unannotated, but included some azoles like thiadiazoles (Figure 21E-F). Many thiadiazoles have activities against various bacteria, fungi and viruses, and are hence being explored as possible components for antimicrobial drugs. Thus, these results suggest a tentative link to increases in antimicrobial components by the host in response to increased *Vibrionaceae* abundances in the gut.

Size class was negatively correlated with Factor 4 (host genotypes), while detection of *Brevinema* tended to be positively correlated with this factor (Figure 22). *Brevinema* was not significantly associated with any SNPs in the mGWAS that tested for direct host genotype associations with microbiome composition, thus illustrating the potential power of MOFA to identify unexpected or weak associations missed by other methods.

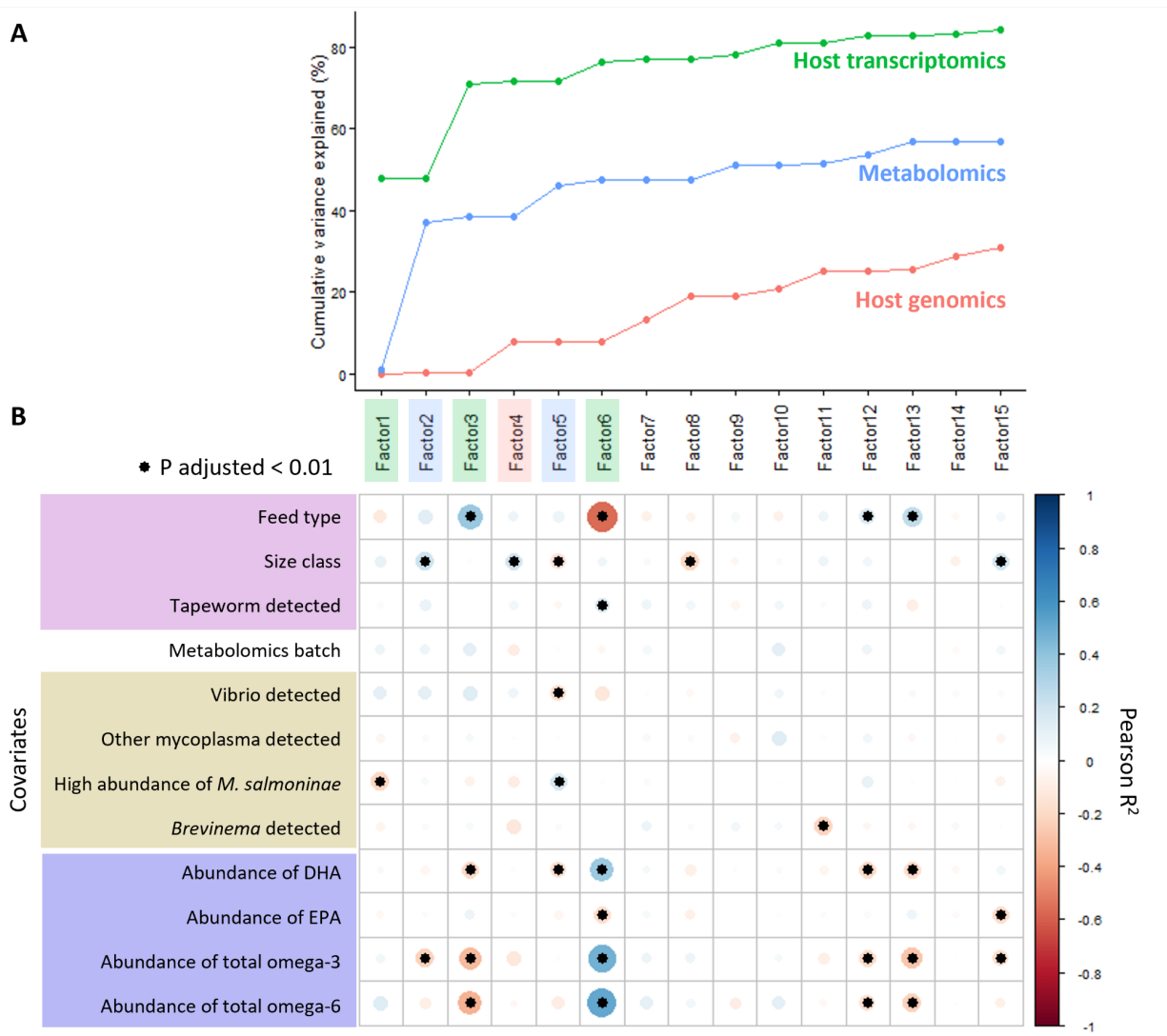


Figure 19. Summary of Multi-omics Factor Analysis results. (A) Cumulative amount of variation in the three 'omics datasets explained by each factor. Almost 80% of the variance is explained by the first 6 factors, in either the host transcriptomics (green), metabolomics (blue) and host genomics (red) datasets. (B) Correlations (Pearson R-squared values, circles) of covariates (rows) with each factor (columns). Covariates included fish traits (pink), technical batches (white), microbiome phenotypes (gold) and fatty acid abundances (purple). Positive correlations are shown in blue circles, negative correlations in red circles. Black asterisk indicates correlation was significant at  $p$ -adjusted < 0.01.

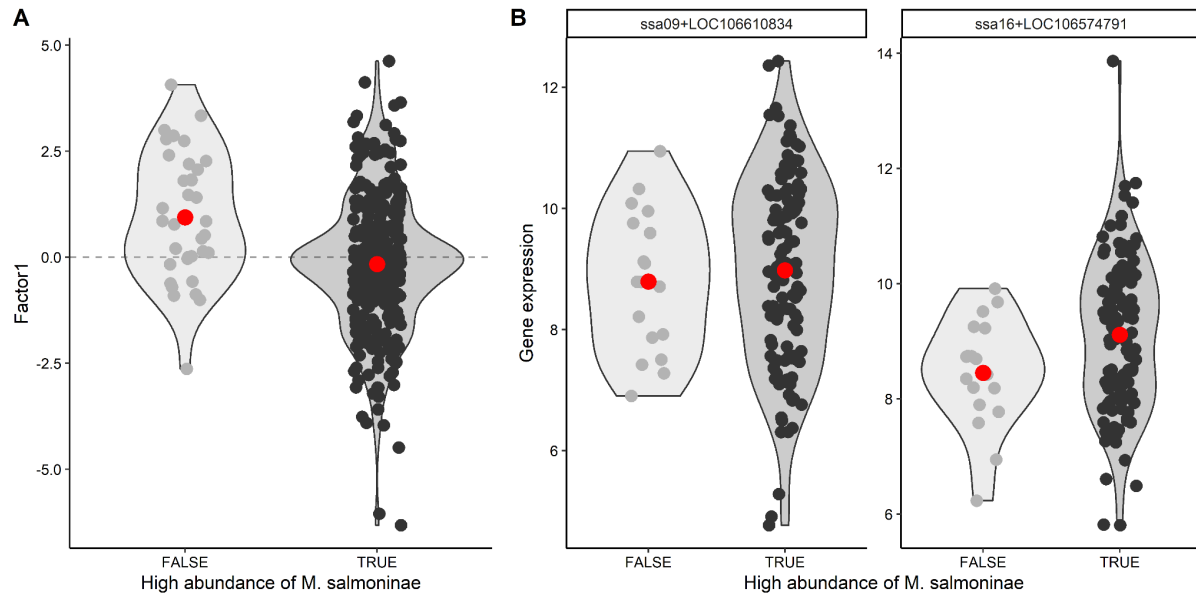


Figure 20. Factor 1 is negatively correlated with high abundance of *M. salmoninae* (A). Factor 1 is mostly capturing variance in the host transcriptomics data. Genes contributing to this variance include LOC106610834 (thrombospondin-1-like) on chromosome ssa09 and LOC106574791 (fibronectin-like) on chromosome ssa16, which both have decreased expression in salmon with high abundances of *M. salmoninae* (B). Both of these genes have functions related to host cell proliferation and adhesion, as well as actions in the inflammatory response.



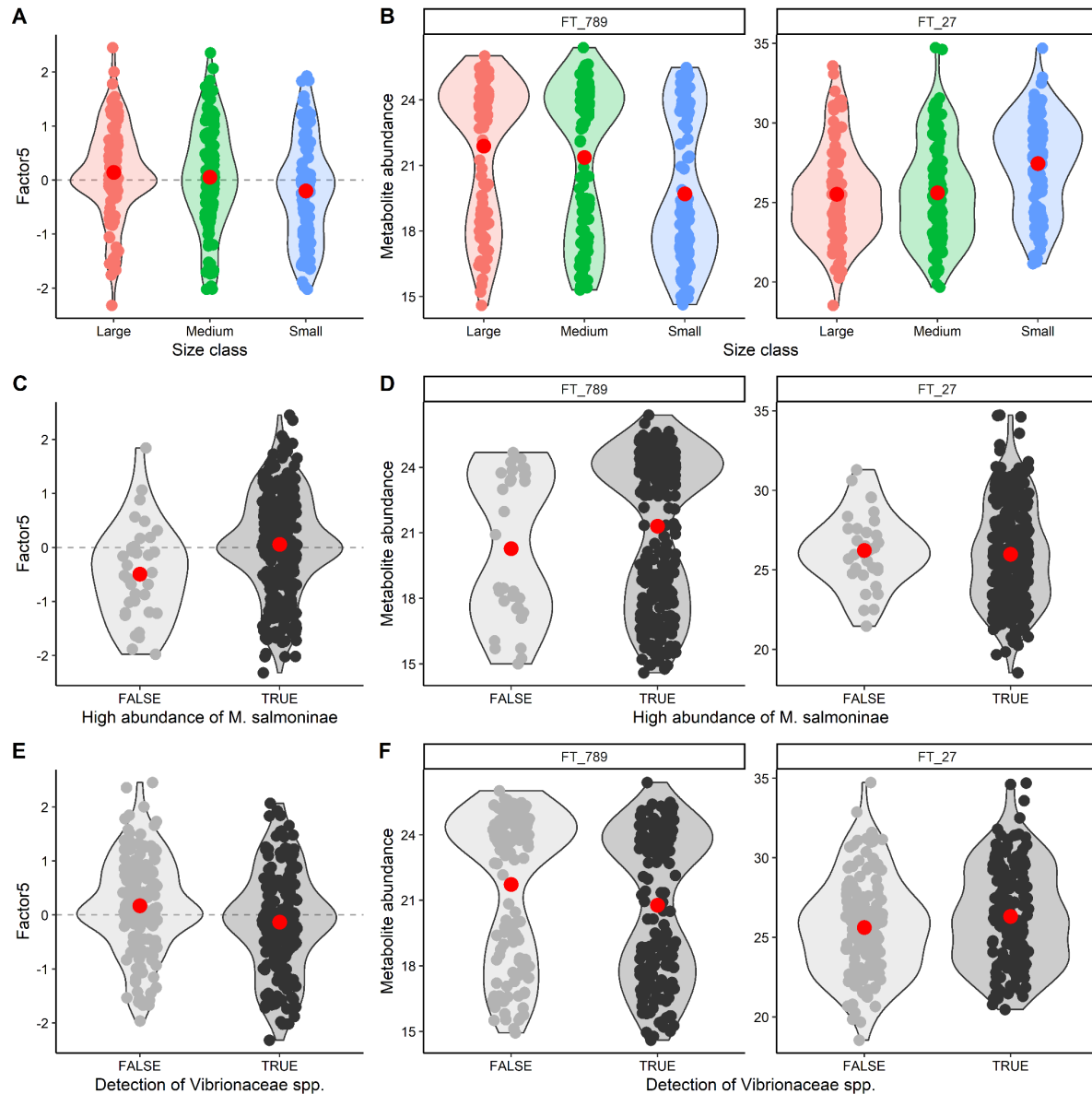


Figure 21. Factor 5 is positively correlated with large fish (A) and high abundance of *M. salmoninae* (C), and negatively correlated with detection of *Vibrionaceae* spp. (E). Factor 5 is mostly capturing variance in the metabolomics data. Metabolites contributing to this variance include FT\_789, an acyl carnitine lipid with increased abundance in large fish (B) and those with high abundance of *M. salmoninae* (D) and decreased abundance in fish where *Vibrionaceae* spp. were detected (F). In contrast, the metabolite FT\_27, a thiadiazole, has the opposite pattern, with increased abundance in small fish (B), those with low *M. salmoninae* abundances (D) and those positive for *Vibrionaceae* spp. (F).

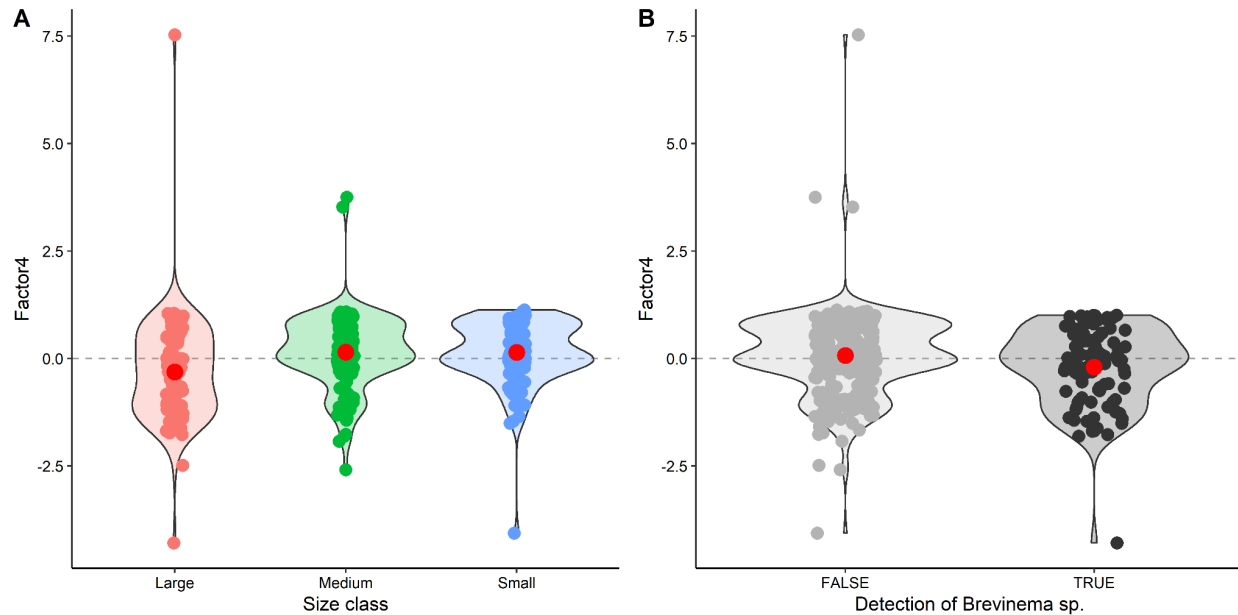


Figure 22. Factor 4 is negatively correlated with large fish (A) and detection of *Brevinema* sp. (B). Factor 4 is mostly capturing variance in the host genomics data.

## Summary

Overall, the holo'omics analysis provides only weak evidence for a host genetic link to the microbiome phenotype, at least at the low level of genetic variation observed in this cohort. In the pilot data, epigenomic associations with microbiome phenotype were also not observed, although this could be due to the small sample size.

However, we did observe that the microbiome is associated with the host phenotype - including growth, as measured by gutted weight, and health, as indicated by tapeworm infection levels. The bacteria *M. salmoninae* in particular seems to play an important role in the healthy salmon gut microbiome. The 16S profiling results confirm that this bacteria is a key resident of the salmon gut microbiome, as has been previously indicated (Rasmussen et al. 2021), and is not introduced solely by the feed. These larger, healthier fish with high *M. salmoninae* abundances are also characterised by shifts in gene expression and metabolic profiles, including changes in fatty acid and lipid metabolism, concordant with the higher levels of total omega-3 and specifically DHA fatty acids deposited in the muscle of larger fish. Furthermore, high abundance of *M. salmoninae* is associated with decreases in prenol-associated metabolism, such as isoprenoids. The genome of *M. salmoninae* contains an isoprenoid biosynthesis pathway that is not frequently observed in other *Mycoplasma* species (Rasmussen et al. 2021), suggesting a possible adaptation to decreased levels of available host isoprenoids in the gut.

We also observed large changes in host gene expression, metabolite abundance and muscle fatty acid levels between the two feed types, although no significant changes were observed in gut microbiome composition or in growth or health outcomes for the host. There were also some suggestions in the GWAS that host genetic variation could be observed between the two feed groups. These strong associations could be masking subtler changes in the 'omics data associated with size or microbiome phenotypes.

## WP 4: Validation experiment

Based on the results from the independent datasets and the integrative holo'omic analyses, a validation trial is currently underway to test whether using seaweed as a dietary additive can promote growth of the gut bacteria *M. salmoninae* and thereby boost salmon health and growth traits (details in Appendix 7; sampling ends in May 2022). As a macroalgae biomass, seaweed harbours a dynamic and diverse microbial biofilm community on its surface. Furthermore, seaweed is high in essential omega 3 fatty acids (Wells et al. 2017). It is therefore expected that using seaweed as a feed additive will promote the growth of beneficial gut microorganisms, including *M. salmoninae*, that will exert a positive effect on the salmon host's immune capacity (Wells et al. 2017). In extension, *M. salmoninae* has also been associated with increased disease resiliency in salmon (Bozzi et al. 2021). Given this general trend of *M. salmoninae* being positively associated with fish performance, we are also using our on-going trial as a validation of its use as a general biomarker for salmon health and performance.

## Conclusions and main findings

The main findings of HoloFish are summarised for each objective and project aim together with consideration of their future applicability for the aquaculture industry.

Table 3. HoloFish objectives and the associated outcome from the HoloFish project

Objective	HoloFish Objective	HoloFish outcome	Future applications
O1	To build an analytical framework to exploit these interactions for optimising salmon health, feed conversion and overall growth in aquaculture.	A true analytical framework to jointly analyse host and microbiota data did not exist at the launch of HoloFish. HoloFish activities and staff have contributed and led three key contributions to this effort through three published peer reviewed articles.	The three papers we have published will all help guide other researchers adopt a more holo'omics approach to better understand phenotypes including key production traits in farmed salmon and other species.
O2	To use this framework to guide a validation trial in collaboration with Lerøy Seafood Group.	In HoloFish we applied our holo'omic framework by generating multiple omics data sets from more than 400 farmed salmon. Preliminary insights have shown a convincing correlation with the relative abundance of a <i>Mycoplasma</i> species and fish performance as measured by the other data. For the validation we have aimed towards solutions that actively help boost abundance of this putative beneficial microbe. HoloFish joined forces with the related ongoing EU H2020 project HoloFood and is currently running a trial testing if the abundance of <i>Mycoplasma</i> can be modified by adding seaweed to the diet.	HoloFish should inspire future work that will add knowledge about the biological dynamics that shape the presence, abundance and activity of specific beneficial microbes and how novel solutions that foster the growth of a healthy gut can be developed and implemented into commercial production of salmon.
O3	To disseminate the findings to not only the salmon aquaculture industry, but also the wider agri- and aquacultural industries.	HoloFish partners have presented results at a wide range of events, including an invited talk at the Animal Microbiome conference in March 2021 that targeted a general food production audience with representatives from terrestrial livestock producing industries.	Inspire other areas of animal production systems to adopt the holo'omics framework towards a more sustainable food production.

Table 4. HoloFish objectives and the associated outcome from the HoloFish project

#	HoloFish Aim	HoloFish outcome	Future applications
A1	Sample tissue, gut content and gut-mucosal samples from sea-farmed salmon that are fed on two commercial diets, and within each treatment, exhibit variable sizes at harvest age ( <b>Work package 1</b> ).	Successful sampling of multiple different tissues and intestinal sites for a total of 463 salmon. Protocols for consistent sampling of both host and microbiota related molecular samples were developed and optimised during the project. Including choice of preservation buffers and transportation from field to lab.	Sampling protocols developed in the HoloFish project have already been used in more recently established holo'omics projects. Together, all these projects have contributed to a continued optimisation of holo'omics sampling protocols in similar and other animal systems.
A2	Use this data to characterise the genome, epigenome and transcriptome for each individual salmon, as well as the metagenomes, transcriptomes and metabolomes of their gut contents ( <b>Work package 2</b> ).	In WP2 we have generated the listed data sets as outlined in this report. In particular in relation to the epigenome, metagenome and metabolome HoloFish has pioneered the application of these methods to salmon, as similar data types at this size have not been generated for farmed salmon before.	HoloFish uncovered valuable experience from each of these data sets of great value to guide future omics studies in salmon. These include: i) the uncovering of a very low diversity metagenome, ii) shortcomings of bisulfite sequencing for high throughput methylation profiling, iii) the large potential of metabolomics data as well as the need for better methods to annotate metabolites.
A3	Apply association mapping to these parameters within a hologenomic framework in order to decipher the link between salmon genomes and their gut microbiota composition and activity, how this relates to dietary treatment and how these in turn affect growth, feed conversion, health and muscle fatty acid profile ( <b>Work package 3</b> ).	Overall, HoloFish results point to a pattern where, while feed showed correlations with gene expression, metabolites and some fatty acid profiles, the type of commercial diet seems to only have little effect on the observed health and growth phenotypes, as well on the microbiota. Alternatively, fish size (i.e. lifetime growth) is correlated with the metagenome composition suggesting some growth related relationship between the host and its gut microbiota. Lastly, association analysis points to a negligible effect of the host genotype in explaining observed differences in gut microbiota.	The lack of clear host genetic effects explaining the gut microbiota variation suggest that the observed associations between some bacteria and high growth performance should be followed up with a focus on how environmental and feed treatments may be used to actively boost gut health in farmed salmon.

A4	Perform a validation growth trial to evaluate findings and commercial potential of the hologenomics approach ( <b>Work package 4</b> ).	Based on results from WP3 we designed a validation study aiming at boosting the abundance of the identified <i>Mycoplasma</i> species immediately after transfer to sea, as this could serve to establish healthy gut microbiota in salmon during the sea phase. <i>Mycoplasma</i> is here considered as a general biomarker for high gut health. For this trial we decided to focus on the putative effect of adding seaweed as a natural prebiotic feed additive to the feed and then observe what effect this solution has on the abundance of <i>Mycoplasma</i> in the gut of salmon fed the seaweed diet.	For this trial we have joined forces with the related and ongoing EU H2020 project HoloFood to optimise use of resources for running the trial. The trial was started in October 2021 and will end in May 2022, whereafter results will be available during 2022, and reported as partly derived from the HoloFish project in peer reviewed publications crediting the FHF HoloFish grant.
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# Deliverables

## Outreach and implementation of results

Throughout the project we have engaged in a number of different outreach activities ranging from conference and workshop presentations, popular media and peer reviewed publications. All outreach activities are summarised in Table 5 and 6.

Table 5 Overview of planned outreach and dissemination deliverables

Deliverable	Status	URL / Appendix
Establishment of project blog (website) hosted by NTNU	A facebook profile was initially launched, but we have mainly used the FHF website as our online project home	<a href="https://www.fhf.no/prosjekter/prosjektbasen/901436/">https://www.fhf.no/prosjekter/prosjektbasen/901436/</a>
Outreach: popular articles/blog posts/presentation	Podcast on DR P1 (DK) --- Popular article published in the Aquaculture Europe Magazine March 2022 issue. --- Podcast on Evolutionary Hologenomics Podcast	<a href="https://www.dr.dk/lyd/p1/vildt-naturligt/vildt-naturligt-30">https://www.dr.dk/lyd/p1/vildt-naturligt/vildt-naturligt-30</a> --- Appendix 1; <a href="https://www.aquaeas.eu/publications-new/eas-magazine">https://www.aquaeas.eu/publications-new/eas-magazine</a> --- <a href="https://youtu.be/AWxqTksTxxk">https://youtu.be/AWxqTksTxxk</a>
Manuscript submitted: Review on hologenomic applications in aquaculture	Published in Trends In Biotechnology	Appendix 2 <a href="https://pubmed.ncbi.nlm.nih.gov/29395346/">https://pubmed.ncbi.nlm.nih.gov/29395346/</a>
Manuscript submitted: Methodological framework for fish hologenomics	Published in iScience	Appendix 3 <a href="https://www.sciencedirect.com/science/article/pii/S2589004220306040">https://www.sciencedirect.com/science/article/pii/S2589004220306040</a>
Manuscript submitted: Hologenomic analytical framework	Published in Nature Reviews Genetics	Appendix 4 <a href="https://www.nature.com/articles/s41576-021-00421-0#Ack1">https://www.nature.com/articles/s41576-021-00421-0#Ack1</a>
Manuscript submitted: Microbiomic basis of salmon phenotype	Accepted in mBio	Appendix 5
Manuscript submitted: Hologenomic basis of salmon microbiome composition	<i>In prep</i>	See this report for status on results. Manuscript intended for submission during 2022.



*Oral presentation on 'The hologenomic analytical framework' at: AQUA 2018	One keynote talk and one poster at <b>Bioengineering in Food and Feed Production</b> (one-day symposium hosted by DTU Bioengineering, DK)	<a href="https://www.tilmeld.dk/dtubioengineeringsymposium/programme.html">https://www.tilmeld.dk/dtubioengineeringsymposium/programme.html</a>
*Oral presentation on 'Hologenomic basis of salmon phenotype' at: Aquaculture Europe 2021	Invited oral presentation about Applied Hologenomics at the <b>ANIMAL MICROBIOME CONGRESS 2021</b>	<a href="https://www.kisacoresearch.com/events/animal-microbiome-congress-2021#speakers">https://www.kisacoresearch.com/events/animal-microbiome-congress-2021#speakers</a>
*Oral presentation at a relevant international conference in 2020	Two oral presentations and one Eposter on Hologenomics and the HoloFish project at EAS2021 --- Invited oral presentation on the HoloFish project at EMOP2021	<a href="https://www.aquaeas.org/">https://www.aquaeas.org/</a>  --- <a href="https://emop2020.org/">https://emop2020.org/</a>
*Presentations at annual FHF conferences	Oral presentation at the FHF workshop <b>R&amp;D on bacterial communities and microbiota in aquaculture – from lab to tank</b> (October 15th 2020)	<a href="https://www.fhf.no/arrangementer/arrangementer/rd-on-bacterial-communities-and-microbiota-in-aquaculture-from-lab-to-tank/">https://www.fhf.no/arrangementer/arrangementer/rd-on-bacterial-communities-and-microbiota-in-aquaculture-from-lab-to-tank/</a>
*Presentation of results to the farming industry at the Aqua-Nor 2020 fair	Oral presentation at GIA2020	<a href="https://www.gia2020.es/scientific-programme">https://www.gia2020.es/scientific-programme</a>

\* The concrete listed presentations were ones anticipated at the beginning of the project. Due to delays, COVID and other unforeseen events, our actual presentations and outlets have deviated from the originally planned ones. Here, we list the oral and poster presentations that we have performed and believe they at least make a 1:1 compensation of the promised activities listed in the first column.

Table 6 Overview of other outreach activities and collaborations

Type of activity	Date	Content	Topic	URL / Appendix
Peer reviewed article	May 2021	Published article in <i>Communications Biology</i>	Mycoplasma pangenome	<a href="https://www.nature.com/articles/s42003-021-02105-1#Sec9">https://www.nature.com/articles/s42003-021-02105-1#Sec9</a>
Education	Nov. 2021	MSc thesis report by Søren B Hansen from UCPH	Epigenome analysis of HoloFish samples	Appendix 6
Collaboration with the UCPH coordinated <b>HoloFood</b> project	2019 - 2021	Complementary trials and hologenomics analysis have been performed by the same consortium as HoloFish resulting in added value for both projects incl. development and publications of the hologenomic framework.	Salmon hologenomics	<a href="https://www.holofood.eu/">https://www.holofood.eu/</a>
Collaboration with the NMBU coordinated <b>ImprovAFish</b> project	2020 - 2021	Applying HoloFish developed protocols to screen gut microbiomes in salmon while testing a novel betamannan prebiotic candidate.	Salmon hologenomics	<a href="https://www.nmbu.no/en/research/groups/memo/research/node/39727">https://www.nmbu.no/en/research/groups/memo/research/node/39727</a>
Collaboration with the UCPH coordinated <b>FindingPheno</b> project	2021	Contributing already published data and experience to help the FindingPheno project develop novel machine learning methods to better analyse hologenomics data in the future	Data and analyses	<a href="https://www.findingpheno.eu/">https://www.findingpheno.eu/</a>
Collaboration with the UCPH coordinated <b>GP3</b> project also funded by FHF	2021 -	Applying protocols that were developed in HoloFish to generate microbiome data for phage detection	Data generation	<a href="https://www.fhf.no/prosjekter/prosjektbasen/901707/">https://www.fhf.no/prosjekter/prosjektbasen/901707/</a> & <a href="https://www.pasteur-ella.dk/">https://www.pasteur-ella.dk/</a>

# Appendices

Appendix 1: HoloFish popular science article: Pedersen (2022). Can the gut microbiota explain large size differences in farmed salmon? *Aquaculture Europe* magazine, **47**(1): 20–21.

Appendix 2: Review article: Limborg *et al.* (2018). Applied Hologenomics: Feasibility and Potential in Aquaculture. *Trends in Biotechnology*, **36**(3): 252–64.

Appendix 3: Hologenomics methodological framework: Nyholm *et al.* (2020). Holo-Omics: Integrated Host-Microbiota Multi-omics for Basic and Applied Biological Research. *iScience*, **23**: 101414.

Appendix 4: Hologenomics analytical framework: Alberdi *et al.* (2021). Disentangling host–microbiota complexity through hologenomics. *Nature Reviews Genetics*.  
<https://doi.org/10.1038/s41576-021-00421-0>.

Appendix 5: Submitted manuscript: Brealey *et al.* (2022). Microbiome ‘inception’: an intestinal cestode shapes a hierarchy of microbial communities nested within the host. Accepted at *mBio*.

Appendix 6: MSc thesis report: Hansen (2021). Analysis of epigenetic variation associated with tenacibaculosis and microbiome composition in Atlantic salmon (*Salmo salar*).

Appendix 7: HoloFish project execution and methods.