A multi omics approach unravels metagenomic and metabolic alterations of a probiotic in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Background

Animal protein production is increasingly looking towards microbiome associated services such as the design of new and better probiotic solutions to further improve gut health and production sustainability. Here, we investigate the functional effects of bacteria based pro- and synbiotic feed additives on microbiome associated functions in relation to growth performance in the commercially important rainbow trout (*Oncorhynchus mykiss*). We combine complementary insights from multiple omics datasets from gut content samples, including 16S bacterial profiling, whole metagenomes, and untargeted metabolomics, to investigate bacterial metagenomic assembled genomes (MAGs) and their molecular interactions with host metabolism.

Results

Our findings reveal, that (I) feed additives changed the microbiome and that rainbow trout reared with feed additives had a significantly reduced relative abundance of the salmonid related *Candidatus* Mycoplasma salmoninae in both the mid and distal gut content, (II) genome resolved metagenomics revealed that alterations of microbial arginine biosynthesis and terpenoid backbone synthesis pathways were directly associated with presence of *Candidatus* Mycoplasma salmoninae, (III) differences in the composition of intestinal microbiota among feed types were directly associated with significant changes of the metabolomic landscape, including lipids and lipid-like metabolites, amino acids, bile acids, and steroid-related metabolites, (IV) performance data of pro- and synbiotic additives indicates alterations of protein and lipid efficiency ratios in rainbow trout.

Conclusion

Our results demonstrate how use of multi-omics to investigate complex host-microbiome interactions enable us to better evaluate the functional potential of probiotics compared to studies that only measure overall growth performance or that only characterise the microbial composition in intestinal environments.

Background

Understanding how feed types and different biotic additives shape the intestinal microbiota and the biological interactions between host and bacteria is of paramount importance to continually boost sustainability of animal production. Pro- and prebiotics have often been considered to promote gut health and growth by decreasing the prevalence of intestinal pathogens and changing the synthesis of bacterial exo-metabolites[1] related to health and growth[2–4]. Furthermore, application of biotic additives has also shown an increase of the absorptive surface for nutrient uptake, by increased density and length of host

villi and microvilli[5,6]. Bacteria able to increase production efficiency have been heavily investigated in terrestrial livestock, including both omnivorous species such as swine and poultry as well as herbivorous ruminants such as cattle and sheep. Highly investigated probiotics strains include *Lactobacillus*, *Pediococcus*, *Bifidobacterium*, and *Enterococcus* for ruminants[7–10], poultry[11,12], and swine[13–15]. Further, an investigation of autochthonous probiotics suggested that bacteria that are naturally adapted to the gastrointestinal tract of a respective host species is more likely to colonise when provisioned as probiotics in the feed[16]. In light of this, detailed characterisation of host-microbe interactions, using more holistic approaches, is needed to better understand how we can actively optimise beneficial services provided by the gut microbiota for livestock[17–19].

Despite the projected growth and importance of aquaculture in feeding a growing human population[20], there is a vast knowledge gap on how the gut microbiota support their host fish[21], especially when compared to terrestrial livestock where intestinal metagenomes have been increasingly investigated[19,22–25]. It is therefore of utmost importance to gain more specific knowledge of the functional potential of fish related gut microbiomes. Some feed types containing probiotics have already been tested in relation to their effects on growth efficiency and disease resilience[26–29]. The probiotic strains considered for aquacultural species so far are often derived from terrestrial species and thus not known to be related to the fish gut environment[16] and despite probiotic related lactic acid bacteria, including *Pediococcus, Leuconostoc*, and *Lactobacillus* previously has shown to be present in the gut microbiome of salmonids[5,30], very little is still known about their function in the fish gut environment regarding nutrient utilisation and immune response modulations[30,31]. Selection and testing strategies needs to be optimised further for the following reasons; first, fish are exotherms, which means that temperature conditions can vary a lot compared to the more stable body temperatures of terrestrial animals. Second, many farmed fish are carnivorous and known to have highly divergent gut microbiota communities compared to their herbivorous terrestrial counterparts[32]. Thus, there is need for more specific knowledge of the functional potential of the gut microbiota related to fish species. Nevertheless, the type of diet needed to produce fish in a sustainable manner is closer to that of terrestrial farmed animals than of wild fish, and as such probiotic bacteria that could help the host optimise digestion and utilisation of non-fishmeal-based diets would be of great interest.

Several studies based on 16S rRNA gene profiling of the gut microbiome of salmonids have demonstrated that the gut microbiome is highly variable and influence by a variety of external factors, but also that the environment is often characterised by low biodiversity of the gut microbiota, and domination by *Proteobacteria*, *Shewanella*, and *Mycoplasma* genera[33–35]. One study showed that an increase of insect-based proteins to rainbow trout (*Oncorhynchus mykiss*) increases the relative abundance of *Mycoplasma* in the gut microbiota[34]. While multiple studies have shown that abundance of *Mycoplasma* is positively associated with fish health and that it is an often dominant and species in the gut microbiota of both wild and farmed salmonids[35–42], very little is known on the function of this *Mycoplasma* and its metabolic interplay with its salmonid host. Furthermore, the underlying mechanisms of the discrepancy

of *Mycoplasma*, being highly dominant or totally absent still remains unknown, despite recent interests[42,43]. To overcome these unknowns, we advocate for more functionally oriented approaches to better understand the functional interactions between host fish and their associated microbiota species[2].

In this study we use a non-targeted multi omics approach to unravel the functional effects on the intestinal microbiota and metabolism when providing a probiotic to farmed rainbow trout. Specifically, we I) investigate microbial shifts in the gut environment caused by probiotic and synbiotic additives using both 16S rRNA gene profiling and whole genome metagenomics sequencing, II) Investigate the functional diversity of the gut microbiota, using metagenomics combined with high resolution untargeted metabolomics, including both UHPLC-MS/MS and IC HR-MS/MS, III) investigate differential abundance of key growth and health related metabolites in light of metagenomic profiles among fish reared on feed types with and without biotic additives, IV) use novel network based approaches for chemical structural annotation to break down unknown metabolite classes and improve knowledge of unknown microbial metabolites, which may be correlated with higher performance in rainbow trout.

Results

Pro- and synbiotic additives are associated with a reduction in relative abundance of Mycoplasma throughout the rainbow trout gut

During the experimental period the rainbow trout were fed control feed (CTRL), probiotic feed (PRO), and synbiotic feed (SYN), respectively (Table 1). Bacterial profiling of both the mid and distal intestinal content of 120 juvenile rainbow trout, using the V3-V4 region of the 16S rRNA gene, resulted in 382 amplicon sequence variants (ASVs). The five most abundant ASVs comprised 85.1% of the total number of microbial reads from rainbow trout in this trial, revealing a low intestinal microbiota diversity (mean effective ASV richness of 34.78 ± 15.8 Hill numbers). Diversity analysis, based on Hill numbers, revealed significantly higher diversity of the core microbiome in both the PRO and SYN groups compared to CTRL (Supplementary Fig. 1). Taxonomy assignment revealed that the five most abundant ASVs included genera of *Mycoplasma*, *Pediococcus*, *Pseudomonas*, *Massilla*, and *endosymbiont8* (genus of Enterobacteriaceae). Bacterial profiling throughout the gut revealed significant changes in the microbial composition among different diet groups with fish from the CTRL group being dominated by *Mycoplasma*, compared to fish from the other two groups that were largely characterised by a higher relative abundance of *Massilla* and *endosymbiont8*, though *Mycoplasma* were still present with individual variation. (Fig. 1a). Further, the recovered *Pediococcus* ASV revealed an exact match with the administered probiotic strain of *P. acidilactici* MA18/5M.

Our analysis revealed a clear alteration of the microbiota as a result of feed type. A Principal Coordinates analysis (PCoA) revealed that 96.4% of the variance of the microbiota was explained by two principal components, and that the microbiota of CTRL clustered alone, whereas the microbiota of PRO and SYN clustered together (Fig. 1b). This pattern was repeated between both the mid and distal gut sections with no significant differences between the gut sections (Fig. 1b). Differential abundance analysis of the top

50 most abundant ASVs confirmed the significantly higher abundance of *Mycoplasma* in the CTRL group (Fig. 1c), indicating that feed additives may have a suppressing effect on the presence of *Mycoplasma* and *Bifidobacterium* (Fig. 1c). On the other hand, our data reveals an increase of the phylum Proteobacteria, including *Pseudomonas*, Enterobacteriaceae, *Massilia.* Also, an increase of the phylum Firmicutes, including Clostridiales, *Weissella*, *Staphylococcus.* The abundance of the probiotic *Pediococcus* ASV was significantly higher in the SYN group, compared to both the CTRL and the PRO groups, indicating that the usage of galacto oligosaccharides (GOS) as a supplemental prebiotic in the SYN group did increase the abundance of *P. acidilactici* MA18/5M (Fig. 1c).

Mycoplasma abundance is associated with microbial pathways of known relevance for salmonid metabolism

A random subset of individuals from each feeding group were selected to investigate inherent microbes in the rainbow trout intestinal content. Deep sequencing of each individual was required to get a decent coverage, since biomass of the microbes in intestinal samples was shown to be low from qPCR quantification of the V3-V4 region of the 16S rRNA gene (Supplementary Table S2.1). To cope with the high level of host DNA in the gut content, we generated more than 1.5 Tb of raw sequence data to obtain a metagenome from this low biomass microbiome. Raw reads were host filtered, assembled, binned, and a MAG database was curated, resulting in a metagenome of 5.01 Mb, consisting of no more than 5,574 genes from two MAGs and one bin (Fig. 2a). The metagenome for Candidatus *Mycoplasma salmoninae* mykiss (referred as *Mycoplasma* in this study) has previously been reported[42], but here we present the whole intestinal metagenome data retrieved from six rainbow trout, including both the mid and distal gut sections. *Mycoplasma* had an identical match with our previously found *Mycoplasma* ASV from the 16S rRNA gene profiling. Further, a MAG of an unknown genus of Enterobacteriaceae corresponded to the presence of the *endosymbiont8* ASV, which we hypothesise to be the corresponding MAG for the *endosymbiont8* ASV. Our analysis also revealed a bin of an unknown *Lactobacillus*. Lastly, short read mapping of the metagenome revealed low levels of *Pediococcus acidilactici* MA18/5M genes present in rainbow trout from the PRO and SYN groups, indicating that the probiotic strain seems to be abundant at a low level in the intestinal content.

Our metagenomic analysis confirmed the bacterial composition found by 16S rRNA gene metabarcoding, where *Mycoplasma* was found to be highly dominant in CTRL and especially in the midgut, corresponding to 76.8–84.5 % of all microbial reads in the midgut and between 56.4–68.7 % of all microbial reads in the distal gut. This *Mycoplasma* dominance resulted in a Q2-Q3 mean coverage of 3,667-5,939 X in the midgut samples and 317–847 X in distal gut samples for CTRL, whereas the coverage of *Mycoplasma* in PRO and SYN was extremely low, except for one sample in SYN (Supplementary Table S3.1). Both *Lactobacillus* and Enterobacteriaceae were found at higher relative abundance in fish from the PRO and SYN groups, a reflection of a reduced *Mycoplasma* biomass (Fig. 2a). Interestingly, the coverage of *Lactobacillus* and Enterobacteriaceae were in general very low and ranged from 0.00 to 8.43 X Q2-Q3 mean coverage across all samples for *Lactobacillus* and 0.00 to 5.16 X

Q2-Q3 mean coverage for Enterobacteriaceae, clearly indicating a low bacterial load even when *Mycoplasma* was reduced.

The functional potential of metagenomes also varied significantly among the feeding groups. Differential abundance analysis of the metagenome data revealed that 670 out of a total of 5,574 non-redundant genes were significantly more abundant in CTRL (adjusted p-value < 0.05) (Fig. 2b, Supplementary Table S3.2), including genes encoding for Arginine biosynthesis pathway, such as *arcA*, *arcC*, and *otc* (Fig. 2b), genes associated with the Cellobiose PTS system, referred to as cellulosome. Interestingly, we found Terpenoid Backbone Synthesis encoding genes, from the non-mevalonate (MEP) pathway, including *ispE*, *ispF*, *ispG*, and *ispH* to be enriched in the CTRL group (Fig. 2a-b). These MEP related genes were all present in the *Mycoplasma* MAG, clearly indicating that alterations of *Mycoplasma* abundance are the main driver of the observed metagenomic variation among feed groups. Surprisingly, we found that genes related to Terpenoid Backbone Synthesis had a dramatically higher log fold change than the rest of the *Mycoplasma* MAG related genes, which we hypothesise could reflect a higher gene copy number.

Diet and Mycoplasma abundance are associated with the intestinal metabolism of rainbow trout

To increase comprehensiveness of ionic properties and polarity in our metabolic analysis, we included UHPLC-MS/MS and IC HR-MS/MS data generation[44,45] resulting in a total of 22,222 mass spectral features with associated tandem mass spectrometric data, which we here use as a proxy for metabolites. Out of the 22,222 metabolites, 12,706 metabolites were generated from UHPLC-MS/MS and 9,516 metabolites were generated from IC HR-MS/MS.

Using the molecular networks, we retrieved *in silico* annotated chemical classes for 7,190 metabolites (56.59%) of UHPLC-MS/MS. Out of the 12,706 metabolites, 741 metabolites were included in the study after filtering for false positives and zero elimination. Overall metabolic variations revealed a clear differentiation among the CTRL, PRO, and SYN (Fig. 3a). Specifically, we investigated metabolite classes putatively synthesised by enzymes encoded by genes found to be differentially abundant in the metagenomes (Fig. 2b). We found clear differentiations in compositions of metabolite subclasses, including amino acids, peptides, and analogues, terpenoids, bile Acids, alcohols, and derivatives (Fig. 3b-d). Composition of especially terpenoids did not only cluster samples based on feed type alone, but also revealed some clustering of samples with a high relative abundance of *Mycoplasma* irrespective of feeding types (Fig. 3c).

For UHPLC-MS/MS, 419 (3.29%) could be matched to known compounds in the GNPS library. For IC HR-MS/MS, 282 (2.96%) could be matched to known compounds in the mzCloud database, which in total resulted in 240 known compounds after deduplication of isoforms of compounds and filtering (Supplementary Table S3.4). Differential intensity analysis of the 240 known metabolites resulted in 25 differentially abundant metabolites, whereas 19 of these metabolites were more abundant in CTRL (Fig. 3e). These included pantothenic acid, indole-3-carboxylic acid, 5-methoxyindole, and 5-hydroxyindole-3-acetic acid, indicating a higher amount of vitamin B_5 and degradation of tryptophan[46] in

the gut of rainbow trout from the CTRL group. These differences indicate alterations of important immune related metabolites among fish reared on the different feed types[46,47]. Furthermore, we found an increase of succinic semialdehyde in CTRL, indicating butyrate related short chain fatty acid (SCFA) metabolism occurring in the gut of rainbow trout[48], which corresponds to previous findings that *Mycoplasma* dominates the microbiome of both wild and farmed Atlantic salmon[36]. SCFAs are known to be the end-products of dietary fibre fermentation by gut microbiota and have been suggested to be an essential nexus between microbiota and different host organ systems[49]. We found an increase of lauroyl-carnitine in PRO and SYN indicating fatty acid oxidation and thereby an increase in lipid metabolism. Furthermore, gluconic acid lactone were found increased in PRO and SYN, indicating induced sugar degradation, which we hypothesise is due to sugar formation by present *Lactobacillus* or *P. acidilactici* MA18/5M, which would make sense for SYN, where galacto oligosaccharides were added to the feed (Fig. 2a and Fig. 3e).

Furthermore, differential intensity analysis of metabolites with no spectral hits revealed a total of 168 metabolites from UHPLC-MS/MS with a significantly different abundance between CTRL and the two other groups after FDR correction for multiple tests (adjusted p-value < 0.05) (Supplementary Table S3.4). Furthermore, we found that 89 of the 168 metabolites were higher abundant in the CTRL group, whereas 79 of the metabolites were higher abundant in PRO or SYN. Metabolites higher abundant in the CTRL group included the metabolite classes: Prenol lipids, Steroids and Steroid Derivatives, Carboxylic Acids and Derivatives, and Benzenes and Substituted Derivatives (Supplementary Fig. 3, Supplementary Table S3.5). These metabolite classes clearly indicate a differentiation in steroid and terpenoid production in the intestinal environment. Especially prenol lipids, which include classes of terpenoids, were found to be highly affected by feed type and more abundant in the CTRL group thereby mimicking the differential abundance of *Mycoplasma* among feeding groups (Supplementary Fig. 3). Further investigation of differentially abundant metabolites observed across feeding types confirmed our previous finding of the CTRL group having a distinct metabolomic landscape compared to the PRO and SYN groups (Fig. 3a, Supplementary Fig. 4).

Deciphering unknown metabolites associated with gut microbiota

To investigate association between specific metabolites and presence of microbes, we computed the correlation between the relative abundance of the ASVs per sample to the concentrations of a subset of the metabolites. We restricted our analysis to the 26 samples, which included both 16S rRNA gene profiling and metabolomics. The 26 samples included 10 fish from the CTRL group, nine from the PRO group, and seven SYN samples. Filtering out rare ASVs resulted in a total of six ASVs (Fig. 1a), while zero inflation of metabolites validated a total of 569 metabolites for this association analysis. Association tests between metabolite intensities and the relative abundances of ASVs revealed four metabolites that are significantly associated with the ASV abundances after Bonferroni correction (Supplementary Table S3.6). We investigated the top 25 most significantly bacterial associated metabolites (BAMs) post Bonferroni correction, using an enhanced molecular network[50–54] to infer these unknown metabolites in the intestinal metabolomic landscape of rainbow trout (Supplementary Table S3.7).

Network analysis of 350 metabolites, including the top 25 BAMs and their related molecular families, were used to decipher molecular structures of unknown metabolites (Fig. 4). GNPS successfully classified 92.5% of the metabolites, where 28.2% of the classifications were confirmed by SIRIUS + CSI:FingerID. Of the 25 BAMs and their related molecular families, we were able to classify 11 BAMs and their related molecular families. The molecular families included prenol lipids from terpenoid backbone synthesis, which were associated with intestinal bacteria. Furthermore, we found a molecular family of unknown lipids, with indications of water loss, indicating formation of steroids, suggesting formation of bacterial related steroids in the intestinal environment (Fig. 5). Interestingly, we found BAMs related to networks of benzenoids, including putative stilbenes and phenylpropanones, indicating production of antibacterial BAMs in the intestinal environment of rainbow trout, which could target bacterial cell walls[55,56].

Furthermore, we found BAMs in molecular families of fatty acyls with a relatively low mass-charge, indicating conjugation of SCFAs. A molecular family of peptide structures, containing substructural motifs of SCFA related aminobutyrate, indicating degradation, biosynthesis or conjugation of an aminobutyrate-like peptide by bacteria in the intestinal environment. A network of putative peptides with BAMs revealed three shared substructural motifs between metabolites, including traces of histidine, alkylamine, and creatinine, indicating incorporation of ammonia derivatives into peptides by intestinal bacteria in rainbow trout (Fig. 4). These findings could confirm our metagenomic observation of arginine biosynthesis, which includes metabolism of ammonia rich peptides.

Feed additives and nutrient utilisation

Feed performance was evaluated based on bulk weights and counts of rainbow trout from five replicate tanks for each of the three feeding groups tested. Registration of total fed feed for each tank, as well as near infrared spectroscopically determined content of protein and lipid in each feed group was recorded (Supplementary Fig. 5a-e).

Our findings indicate that nutrient related phenotypes of juvenile rainbow trout can be affected by feed additives. Overall, our analyses revealed no significant differences in percent weight gain (WG), Feed Conversion Ratio (FCR), and the inverse of FCR, Feed Efficiency Ratio (FER), among feeding groups (Supplementary Fig. 5a-c). It should be noted that nutritional analysis of feed revealed a lower number of calories (MJ/Kg) and fat content (%) in PRO feed, and a lower amount of protein in the CTRL diet (Table 1). This may explain some of the observed differences in the performance data and complicates the further interpretations of feed efficiency indices (Supplementary Fig. 5).

However, the Lipid Efficiency Ratio (LER) suggest a significantly more effective conversion of feed lipids into biomass in the PRO group ($F_{(2,12)} = 9.84$, $p = 0.0029$) (Supplementary Fig. 5d), indicating usage of *Pediococcus acidilactici* MA18/5M could improve efficiency of lipid utilisation in rainbow trout. Analysis of Protein Efficiency Ratio (PER) showed that the CTRL group had a significantly higher efficiency than the other feeding types ($F_{(2,12)} = 9.88$, $p = 0.0029$). This could indicate that use of feed with pro- or synbiotic additives may decrease protein utilisation as a response to higher lipid efficiency ratio

(Supplementary Fig. 5e). An accurate LER and PER determination would require isoenergetic/proteinic diets and analysis of whole-body fat and protein rather than bulk weight of the fish.

Discussion

We combine a rigorous, comparative feed trial with highly robust multi omic data analysis, including deeply sequenced metagenomics, untargeted metabolomics, and bacterial 16S rRNA gene profiling of the intestinal compartment of rainbow trout. This multi-omics data set enabled us to first characterise rainbow trout associated MAGs, including a recently described candidate *Mycoplasma* species, in relation to estimates of nutrient utilisation in rainbow trout. We then complemented the metagenome data with metabolomics insights on the variation in intestinal metabolites of rainbow trout. Together, we show how probiotic feed changes are associated with distinct microbiota functions and metabolic profiles in rainbow trout and how this could be associated with nutrient uptake between feeding types.

Our multi-omic investigation revealed significant effects of feed additives on the intestinal environment in rainbow trout. The growth trial showed an alteration of protein efficiency ratio and lipid efficiency ratio, when using probiotic or synbiotic feed additives, though we acknowledge that a stricter performance experimental setup is needed with identical nutritional content, and we therefore suggest that the nutrient utilisation data indicate associations rather than firm conclusions. Our analysis revealed that the abundance of the dominant gut bacterial genus *Mycoplasma* was significantly reduced in fish that had been feeding on diets with pro- or synbiotic additives. On the other hand, our data revealed an increase of *Pseudomonas*, Enterobacteriaceae, and *Massilia.* Based on these observations we hypothesise that this probiotic causes a change in the microbiota, which can be mirrored in the meta-metabolism, potentially affecting both PER and LER. While such changes may be beneficial, it is interesting to note that previous studies have shown that *Mycoplasma* is consistently present in wild salmonid populations[36,39,42]. Further, neutral modelling comparing environmental and intestinal frequency distributions of *Mycoplasma* have previously suggested that Atlantic salmon associated *Mycoplasma* are adapted to colonisation of their hosts[57]. Together, our results add to previous findings and support the hypothesis that this salmonid associated *Mycoplasma* genus might be a native symbiont to salmonid species.

Previous investigations of *P. acidilactici* MA18/5M in bigger rainbow trouts have shown an increase of *Mycoplasma* in the gut microbiome, when using *P. acidilactici* MA18/5M as feed additive, suggesting that these microbial alterations, induced by feed additives, are complex and further dependent on age of host, water salinity, diet composition of protein and fat, and dose of *P. acidilactici* MA18/5M[31,58].

Functional insights from metagenomic analyses provided information on molecular pathways associated with the *Mycoplasma* MAG. The *Mycoplasma* MAG contains genes encoding enzymes involved in arginine biosynthesis, cellulosome, and terpenoid backbone synthesis, which is rather uncommon in *Mycoplasma*[1]. Induction of genes involved in terpenoid backbone synthesis in the transcriptome of salmonids has previously been reported to be positively correlated with FER[59], suggesting the importance of terpenoid backbone synthesis for growth metabolism. Therefore, we hypothesise that the terpenoid

backbone synthesis encoded by the *Mycoplasma* genome might add a supplemental boost to the metabolism of their salmonid hosts. Subsequently, a recent study of *Mycoplasma* in Atlantic salmon correlated terpenoid production to increased pigmentation of salmon flesh, suggesting *Mycoplasma* is affecting the metabolism in Atlantic salmon[60]. Barring any unknown adverse effects of this dominant component of the rainbow trout intestinal tract, such metabolic contributions and apparent degree of host-adaptation observed for the *Candidatus* Mycoplasma salmoninae, could suggest a fine-tuning of the performance of the trout from a hologenomics perspective[18].

Microbial contributions to the arginine biosynthesis pathway can affect host gut health. Microbial functions are likely to either i) increase arginine analogous like ornithine and citrulline or, ii) decrease the amount of toxic ammonia in the intestine of ammonotelic teleosts such as salmonids through anabolic carbamate kinase activity during feeding[61–64]. Citrulline and ornithine have previously been found more efficient for amino acid uptake in rainbow trout[65,66]. Ammonia reduction could have an impact for farmed fish, since they are often fed excessively, which suggests that *Mycoplasma* might serve as an advantageous gut symbiont that increases the tolerance of the host fish towards accumulated ammonia or suggests that *Mycoplasma* increase the availability of essential amino acids in the gut. Furthermore, we also found BAMs related to incorporation of ammonia rich moieties into putative peptides, further suggesting that bacteria in the intestinal environment are potentially detoxifying ammonia levels using several strategies[64]. The potential of using probiotics to improve growth and health in production animals has received immense attention across life sciences over the past decades. While there are successful examples of biotic additives, including decrease of vertebral column compression syndrome[67], increased innate immune response in rainbow trout fingerlings[68], modulations of antiviral response[69], and an increase of absorptive surface in the host[5,6] the underlying metabolic functions causing improved performance, or lack thereof, often remains unknown and especially so in the aquaculture field.

Conclusions

Indeed, the microbiome of salmonids, and other commercially important fish species, have mainly been described using 16S rRNA gene profiling. These studies have led to several interesting and important hypotheses of host-microbiome interactions, but these hypotheses remain largely speculative as they are solely based on compositional data. Our results demonstrate how use of multi-omics to investigate complex host-microbiome interactions enable us to better evaluate and explore the functional potential of probiotics compared to studies that only measure overall growth performance or that only characterise the microbial composition in intestinal environments. Furthermore, we demonstrate a more hologenomic approach to better understand complex host-microbe interactions in production animals based on a better functional understanding of intestinal microbiomes.

Methods And Materials

Fish and trial design

The sourcing of fish and rearing procedures used in this study have previously been described in a separate study[70]. Briefly, we obtained rainbow trout eggs from the AquaSearch FRESH strain (all-female, AquaSearch OVA, Billund, Denmark). Eggs were hatched and reared at the Bornholm Salmon Hatchery (Nexø, Denmark) that has a disease-free record and upon arrival, the eggs were disinfected using Desamar K30. Prior to experimental feeding, the fish were transported to the BioMar A/S research facilities (Hirtshals, Denmark).

An experimental feeding trial was carried out over an eight-week period. Three experimental, proprietary feed formulations were selected (Table 1): I) a control feed without any pre- or probiotic additives, II) control feed plus the commercial probiotic BACTOCELL with *Pediococcus acidilactici* MA18/5M, and III) control feed with a synbiotic additive, consisting of BACTOCELL and galacto oligosaccharides.

Table 1. Overview of the feed types included and the main differences.

Ingredient	Control Samples **(CTRL)**	Probiotic Samples **(PRO)**	Synbiotic Samples **(SYN)**	
Core feed composition	Fat content (%) (SD ± 1.21)	20.0	17.1	19.7
Protein content (%) (SD ± 1.46)	52.8	57.4	55.1	
Calories (MJ/Kg) (SD ± 0.406)	22.37	21.55	22.45	
Additives	*Pediococcus acidilactici* MA18/5M (1 x 10^6 CFU/gram)	**-**	**+**	**+**
Galacto Oligosaccharides (GOS)	**-**	**-**	**+**	

In order to minimise sampling bias, all feeding types were blinded before sampling. This was upheld throughout the feed trials, sample processing and analysis, and finally unblinded post analysis.

Feeding and Sample collection

For metabolomic investigation, 50 mg of gut content were sampled from the same region of the distal gut in each individual. Samples were immediately frozen on dry ice and subsequently transferred to a -80 °C freezer within hours. For microbial profiling, samples were taken from mid- and distal gut sections at both time points by dissecting gut content from both sections, using sterile scalpels and tweezers. Inoculation loops were used to ensure a normalised amount of gut content from each sample. All samples were preserved in SHIELD™, provided by Zymo Research, following the Zymo Research standard procedure. Weight, fork length, and qualitative comments regarding wounds were recorded for each individual. All

individuals were euthanised, according to the approved experimental guidelines, using Benzocaine in water prior to dissection, as detailed in a previous study[70].

Profiling the V3-V4 region of the bacterial 16S rRNA gene

DNA extractions for 16S profiling were carried out using Zymo Research Quick-DNA/RNA (Cat. D2131) following suppliers' recommendation. Prior to analysis, all samples were randomised. Extracts were quality controlled for inhibitors and optimal PCR settings prior to metabarcoding. Two extraction blanks were included for each plate. Metabarcoding was carried out by amplifying the V3-V4 region of the bacterial 16S gene, using the primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3)[71] combined with unique forward and reverse 8bp tags (Supplementary Table S2.2). All amplifications were carried out in triplicates to minimise procedural false positives[72]. Library preparation was carried out using Illumina NEBNext® Ultra™ IIDNA Library Prep Kit. Amplicons were sequenced on an Illumina NovaSeq 6000 PE250bp to obtain 250bp paired-end reads aiming for a minimum 10,000 reads per PCR replica.

Metagenomic data generation

Prior to analysis, all samples were randomised. Extraction of DNA for metagenomics was carried out using ZymoBiomics DNA miniPrep for a total of six rainbow trout, where two intestinal sections, including midgut and distal gut, were included, resulting in 12 samples (Supplementary Table S2.3) for further details. Fragmentation of DNA to 400 bp was carried out, using Covaris M220 with microTUBE-50 AFA Fiber Screw-Cap. Samples were normalised to 400 ng input for library preparation. Library preparation was based on Single-tube library preparation for degraded DNA[73], see Supplementary information 2.2. Prior to the indexing of libraries, all libraries were analyzed with quantitative PCR (qPCR) to estimate optimal cycle settings on a Mx3005P qPCR System (Agilent Technologies), see (Supplementary Table S2.3).

Purified libraries were indexed and amplified for sequencing, using customized index primers for MGI-2000. Sequencing was carried out, using 150 PE chemistry on a MGI-2000 at BGI Europe. Data for five of the 12 samples were generated for a previous study[42]. The remaining 7 samples were processed, and the generated data was analysed for this study and has not been published prior to this study., see (Supplementary Table S2.4) for further details.

Metabolomic extraction and preparation

A subset of ten samples from each of the three feeding types were selected according to Fulton's condition factor (five random samples below K=2, and five samples above K=2), resulting in a total sample size of 30 samples. In order to minimise batch effects all samples were randomised prior to any

laboratory processing. Samples were homogenised in 100 % methanol (MeOH) in a 1:10 sample:solvent ratio. Homogenisation was carried out in an OMNI Bead Ruptor 24, using dry ice in order to keep homogenised samples around 0°C to minimise degradation of metabolites during homogenisation. Six procedural blanks were included in homogenisation. A volume of 100 µl of all samples were collected into Quality Control samples (QC samples) used for normalization to enhance detection of metabolites, all samples were purified after homogenisation, using solid phase extraction (SPE), 2 mg/HRP-microSPE. The SPE was carried out conditioning with 200 µL 100 % MeOH and washing with 200 µL 0.1 % formic acid. Samples were eluted with 2 x 100 µL MeOH. Samples were concentrated using SpeedVac (ThermoFisher Scientific) and resuspended in 100 µL 5% MeOH. In order to correct for biases related to injection order, samples were divided into two replicates and ordered in an antiparallel order prior to nano-flow ultra-high pressure liquid chromatography–tandem high-resolution mass spectrometry analysis. Metabolites were detected and quantified using a Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (ThermoFisher Scientific) operated in positive ion data-dependent acquisition mode (Supplementary Information 2.3). Sample extracts were also analysed for more polar metabolites with ion exchange chromatography hyphenated to a Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (ThermoFisher Scientific) operated in negative ion data-dependent acquisition mode. A Dionex IonPac AS19-4 µm (2 x 250 mm) column was fitted with a Dionex AG19-4 µm (2 x 50 mm) Guard and connected to an ADRS 600 (2 mm) suppressor. Potassium hydroxide was used as an eluent.

Bacterial 16S rRNA gene profiling of Taxonomy and compositional analysis

Raw sequence data were quality controlled, using FastQC/v0.11.8[74] to remove low quality reads. Demultiplexing and removal of adaptors and low-quality reads were done with AdapterRemoval/v2.2.4[75], with a base quality threshold of 30 and a minimum read length of 50bp. Microbial 16S data were further filtered with a Maximum EE score or 2 for forwards and reverse reads. Reads were trimmed according to the error rate algorithm applied in DADA2[76]. ASVs were clustered, using the clustering algorithm implemented in DADA2. Taxonomy was assigned through DADA2 using Silva/v138. Post clustering algorithms were applied to minimise false positives using LULU[77] and subsequently contaminations were removed from samples, using decontam[78]. Composition analysis were carried out using phyloseq[79] and differential abundance analysis across feeding groups were carried out using metacoder using wilcoxon rank sum test and FDR correction for multiple comparisons[80]. Diversity analysis of the gut microbiome across feeding types was carried out applying Hill numbers, using hilldiv[81,82].

Metagenomic bioinformatics; Filtering, Assembly, Binning, Refinement

A subset of the data generated for this study were used for a separate, comparative study. Processing of data is presented in[42]. Raw sequence reads were quality controlled, using FastQC/v0.11.8[74] to assess filtering and quality steps. Removal of adapters and low-quality reads were done with AdapterRemoval/v2.2.4[75], with a quality base of 30 and a minimum length of 50bp. Duplicates were removed, and reads were re-paired to remove singletons, using bbmap/v.38.35[83]. In order to increase assembly efficiency by reducing eukaryotic contaminants, data were filtered for the phiX174 genome, Human (HG19) genome, and the rainbow trout (Omyk_1.0), using minimap[84]. Filtered data were both single assembled and co-assembled, using MegaHIT/v.1.1.1[85] with a minimal length of 1000 bp per scaffold, using meta-sensitive flag for metagenomic purpose and assembled contigs were quality controlled with Quast/v.5.02[86]. To increase effective binning, we used the anvi'o pipeline[87], as described in a previous study[42]. Relative abundance of each MAG was calculated based on percentage read recruitment across all samples from the specific host. We annotated functions, using Pfam[88], COG[89], and KEGG[90]. Differential abundance of all genes was carried out, using generalised linear models (GLMs), using DESeq2[91] in R. False Detection Rate correction to p-values to account for multiple tests were applied.

Metabolomic annotation and metabolite substructural analysis

ThermoFisher Scientific UHPLC-Orbitrap-MS/MS RAW files were converted into mzML files using Proteo Wizard[92]. A molecular network was created using the classical molecular networking workflow https://ccms-ucsd.github.io/GNPSDocumentation on the Global Natural Product Social Molecular Networking (GNPS) platform http://gnps.ucsd.edu[54,93].

In order to enhance identification of unknown metabolites, unsupervised substructures were discovered using MS2LDA[53,94], and MS2 spectra were annotated in silico using Network Annotation Propagation (NAP)[51]. Furthermore, peptidic natural products (PNPs) were annotated in silico, using DEREPLICATOR[95]. Chemical classes were retrieved for all GNPS library hits and in silico structures using ClassyFire[52]. Finally, all structural annotations were combined within one network using MolNetEnhancer[50]. Further annotation of metabolites was carried out, using MetDNA[96].

The obtained IC HRMS/MS data processed using Compound Discoverer 3.2.0.421 (Thermo Scientific). The optimized workflow performed retention time alignment, compound identification (detailed in Supplementary Table S3.3).

Metabolomic compositional analysis of UHPLC-Orbitrap-MS and differential intensity analysis of UHPLC-Orbitrap-MS/MS

and IC HR-MS/MS known metabolites

Quality assessment of initial data from UHPLC-Orbitrap-MS/MS was carried through PCoA plotting to ensure proper quality of quality pools, samples, and procedural blanks (Supplementary Fig. 2). To minimise false positive metabolites, we removed high abundant (metabolites above 5 x 10^6 Summed Precursor Ion Intensities) metabolites present in procedural blanks, which resulted in 9,863 metabolites. Metabolites present in >50 of the 60 samples were kept minimising zero inflation in metabolomic data, resulting in 741 metabolites. Procedural replicates were average between individuals for group comparisons, as the replicates did not seem to affect the variation between groups (Supplementary Fig. 2). Principal Coordinate Analysis (PCoA) of metabolites were carried out, using phyloseq[79]. We carried out a PCoA based on jaccard distances to minimise biases related to relative abundance of precursor intensities of unknown metabolites generated from UHPLC-Orbitrap-MS/MS.

Detected compounds from UHPLC-Orbitrap-MS/MS and IC HR-MS/MS were imputed to minimise zero inflation and normalised with variance stabilising normalisation, using MetaboDiff[97]. Technical replicates were averaged. Precursor intensities of isomers and conjugated compounds were summed (detailed in Supplementary information 3.3). Differential intensity analysis was carried using MetaboDiff (Supplementary Table 3.4). To increase biological inference of compounds we analysed compounds, using MetaCyc[98] (detailed in Supplementary information 3.3).

Association between bacterial ASVs abundances and metabolites

We restricted our analysis to the 26 samples that had their microbiome profiled using the 16S amplicon sequencing and had metabolites measured. Of these 26 samples, including 10 from CTRL, 9 from PRO, and the remaining 7 from the SYN group.

We filtered out the rare ASVs and retained only the six most abundant ASVs to restrict our analyses to only the most relevant ASVs, where we would have statistical power to detect associations. Metabolite data were filtered and normalised (detailed in Supplementary information 3.3). To measure the putative effect of ASVs on metabolite relative abundances, for each metabolite, we used a stepwise regression procedure, using R-package MASS, where we started with a linear model where the abundances of all six ASVs were used as explanatory variables (detailed in Supplementary information 3.3). The final model was selected when no ASVs could be removed without significant reduction in explanatory power of the model, and no ASVs could be added with significant improvement of fit.

Finally, note that we did not include feed type as an explanatory variable for the metabolite relative abundances, since the feed type was highly correlated with the ASV abundance information. Specifically, the abundance of *Mycoplasma* was indicative of control vs. non-control feed type. Thus, given the

modest sample sizes in this study, we decide to focus our test on the putative effects of ASV abundance on metabolite relative abundances.

For each metabolite, we tested the final model obtained from the stepwise procedure using a F-statistic to test the proportion of variance explained by the chosen ASVs, we used bonferroni correction for significance adjustment (detailed in Supplementary information 3.3).

Metabolomic network analysis of bacterial associated metabolites and substructural analysis

Networks for selected Bacterial associated metabolites (BAMs) were visualised using Cytoscape/v3.8.0[99]. Annotation of BAMs were carried out as detailed for UHPLC-MS/MS data and with a combination of GNPS network and SIRIUS4 with CSI:FingerID[100,101] (detailed Supplementary information 3.3). Information of nodes within the metabolic networks are detailed in supplementary (Supplementary Table S3.7).

Feed Performance Analysis

Feed performance parameters were analysed for each group based on recorded bulk fish weight, numbers of individuals and consumed feed during the experimental feeding period for each of the five replicate tanks in each experimental feed group. Furthermore, the specific fat and protein content for each experimental feed was determined using near-infrared spectroscopy as part of internal quality control at BioMar[102].

Calculations of parameters were calculated according to previous study[70] (Supplementary information 3.3.6).

Statistical analysis

All statistics were conducted using R (version 3.6.1) and Python (version 3.7.4).

Declarations

Ethical Approval and Consent to Participate

The Danish Animal Experiments Inspectorate, under license no. 2015-15-0201-00645 approved the protocols regarding experimental animals described for this study. The study is thus approved under the Danish law regarding experimental animals.

Consent for Publication

Not applicable

Availability of Data and Material

Summary of metagenome is located for review purpose in following share link: https://figshare.com/s/cdee30247b82787f953c and will be publicly available upon acceptance at: 10.6084/m9.figshare.13193846.

Anvio database for metagenomics analysis is located for review purpose in following share link: https://figshare.com/s/18715f789de6d13d624c and will be publicly available upon acceptance at: 10.6084/m9.figshare.13193861.

The data generated for 16S rRNA gene profiling of bacteria are available in ENA repository with project accession numbers PRJEXXXXX. The raw metagenomic dataset generated during the current study will be available in ENA repository with project accession numbers PRJEB40990.

The metabolomics datasets generated and analysed during the current study is available on the massive repository, using accession number MSV000084364, ftp://massive.ucsd.edu/MSV000084364/.

Any code used for the study used to generate results that are reported in the paper and central to its main claims are available at: https://github.com/JacobAgerbo/Multi_Omic_Rainbow_Trout.

Competing interests

Torunn Forberg is employed at Biomar Group, who produce, market and sell fish feed supplements with some of the ingredients tested in the current investigation. Furthermore, Biomar provided parts of the funding for this study. Jacob Agerbo Rasmussen, Kasper Rømer Villumsen, Madeleine Ernst, Martin Hansen, Shyam Gopalakrishnan, M. Thomas. P. Gilbert, Anders Miki Bojesen, Karsten Kristiansen, and Morten Tønsberg Limborg all declare no competing interests.

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Author’s Contributions

M.T.L. conceived the study with input from A.M.B., M.T.P.G, K.K. Trial and sampling was organised and performed by J.A.R., K.R.V, T.F., and M.T.L. J.A.R, K.R.V, and M.H. carried out laboratory work. J.A.R, M.E.,

S.G., and M.H. performed the computational analysis. J.A.R wrote the manuscript with input from all authors.

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Figures

Figure 1

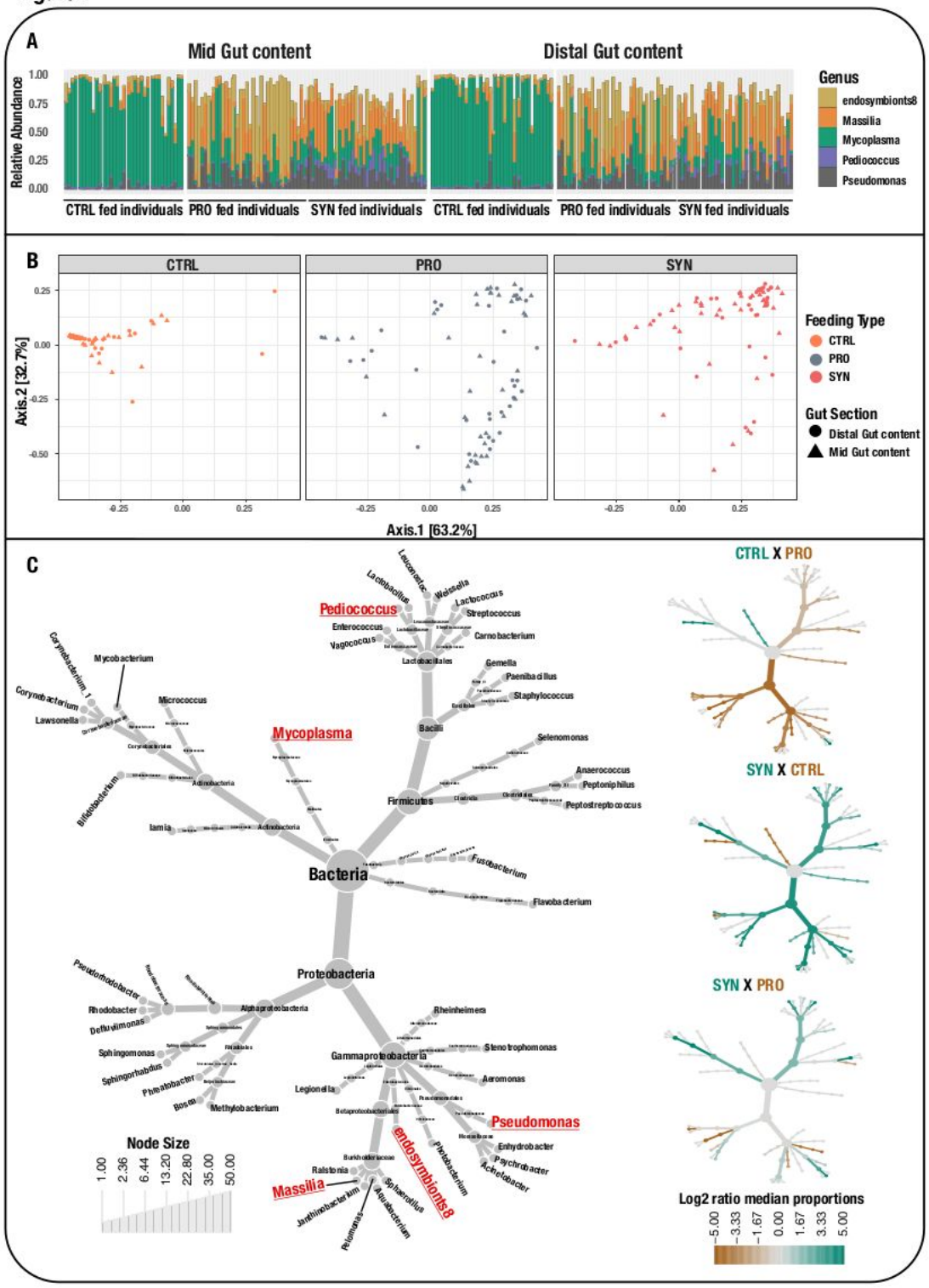

Figure 1

16S rRNA Gene profiling of the gut microbiome across feeding type and intestinal sections. A) For the mid and distal gut sections separately, the barplots show relative abundance of high abundant bacteria defined as being present in more than 2% of all samples. B) Principal Coordinates Analysis (PCoA) of the microbial composition in all samples and feeding groups using Unifrac distances. Distribution of individual samples for the three feeding groups are plotted for the same axes but shown in separate plots

for visual purposes. Grouping of rainbow trout reared on different feeding types were visualised as orange for CTRL, blue for PRO, and red for SYN. Shapes indicate different intestinal sections, where the circle indicates samples isolated from distal gut content and triangles indicate samples isolated from mid gut content. C) Heat Tree of species composition of the 50 most abundant ASVs throughout the gut combined with pairwise comparisons for the three feeding types, control, probiotics, and synbiotics. The grey tree on the lower left is a taxonomic reference for the smaller unlabelled trees. The most abundant genera from figure A are coloured in red and underlined. The colour of each taxon reflects differential abundance between the two groups being compared with colours determined by the log2 ratio of median proportions of reads observed in each feeding type. The Log2 changes were determined using a Wilcoxon rank-sum test followed by a Benjamini-Hochberg (FDR) correction for multiple comparisons. Size of nodes relates to the number of ASVs found within the given taxonomic group.

Figure 2

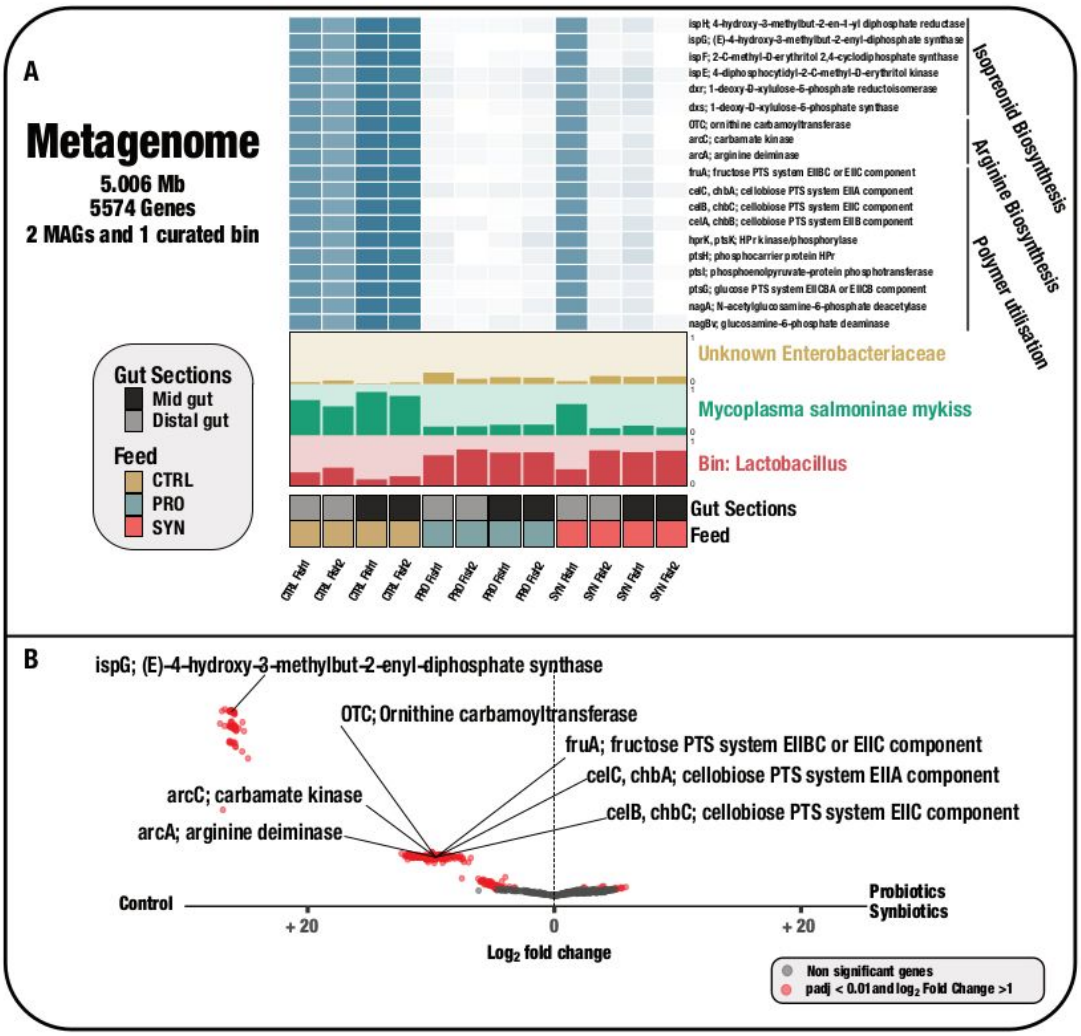

Figure 2

Genome-resolved metagenomics from gut microbiome across feeding type and intestinal sections. A) Two Metagenomic assembled genomes (MAGs) and one bin were found present in the metagenome, consisting of 5.006 megabases (Mb) and 5,574 non-redundant genes, which were visualised as yellow for Unknown Enterobacteriaceae, green for Candidatus Mycoplasma salmoninae mykiss, and red for Unknown Lactobacillus. Barplots indicate relative abundance of the two MAGs and the bin within each

sample. Grouping of rainbow trout reared on different feeding types were visualised as orange for CTRL, blue for PRO, and red for SYN. Intestinal sections (gut sections) were coloured as black for mid gut and grey for distal gut. Heatmap visualises a series of genes of interest, which are related to isoprenoid biosynthesis, arginine biosynthesis, and polymer utilisation. Intensity of blue colour indicates log10 of coverage of genes across samples. (B) Volcano plot of differentially abundant metagenomic genes between samples from CTRL vs. PRO and SYN. Colouration of nodes indicate significance, whereas red nodes are significant genes after correction and have a log2 fold change (FC) >1. Grey nodes are non-significant genes.

Figure 3

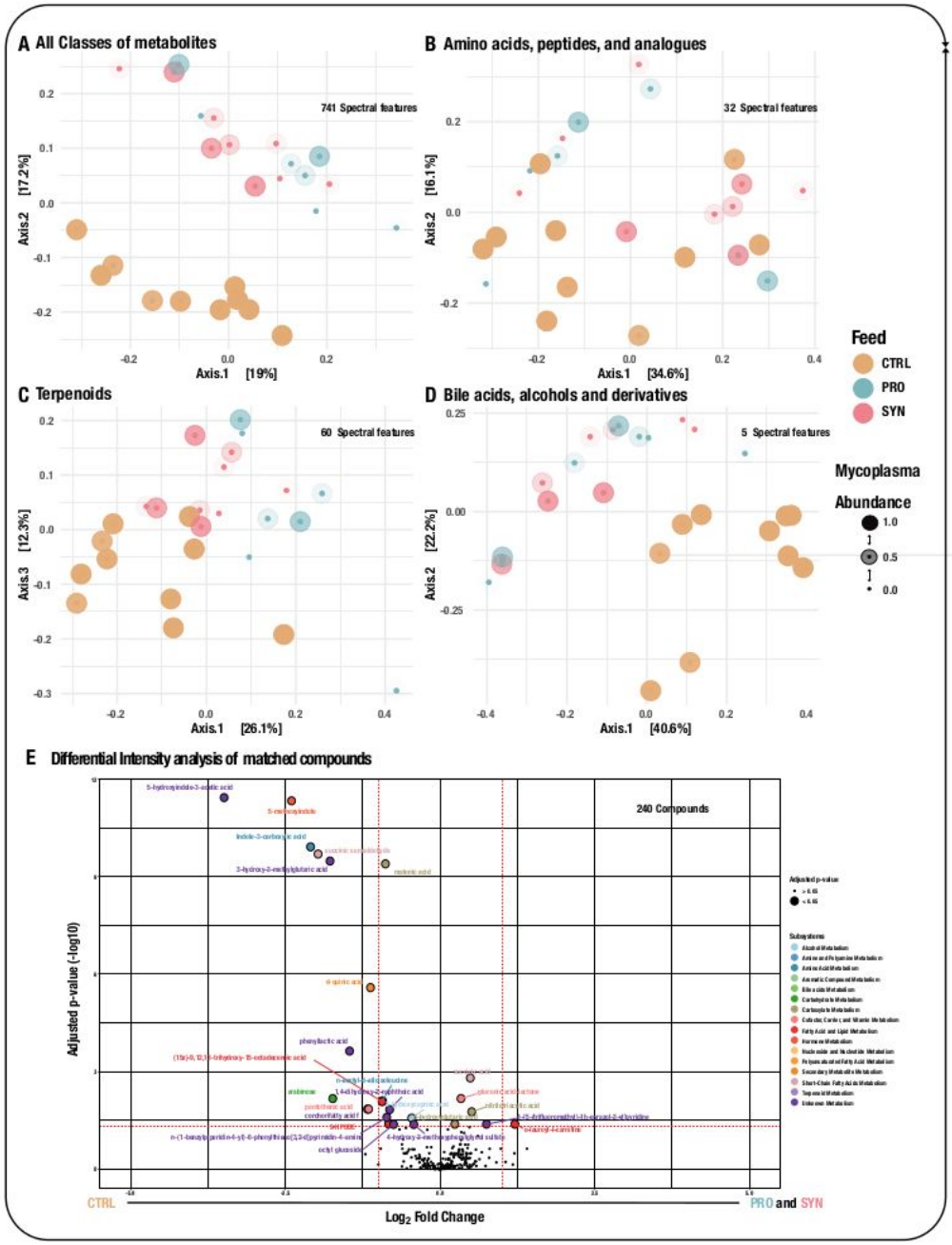

Figure 3

Composition of metabolic putative classes across feeding types and differentiation of known compounds. A-D) Principal Coordinate Analysis (PCoA) of metabolites across all three feeding types based on 741 of the 9,723 metabolites, which is occurring in more than 50 of the 60 samples. Opacity of nodes is related to relative abundance of Mycoplasma. A) All classes of metabolites, B) Amino acids, peptides, and analogues, C) Terpenoids, including monoterpenoid, diterpenoids, triterpenoids, and

sesquiterpenoids, D) Bile acids, alcohols, and derivatives. Grouping of rainbow trout reared on different feeding types were visualised as orange for CTRL, blue for PRO, and red for SYN. E) Volcano plot of a differential intensity test performed between the CTRL feed against the two feed types with biotics additives. The test was based on 240 VSN normalised metabolites with a spectral match to known compounds. Metabolites with an adjusted p-value below 0.05 were considered to be significant. Size of nodes are dependent on adjusted p-value, where big nodes are significantly different between feeding types ($p.adj < 0.05$) and small nodes are not significant ($p.adj > 0.05$). Colour of nodes are dependent on related metabolic subsystems, which are specified in legend.

Figure 4

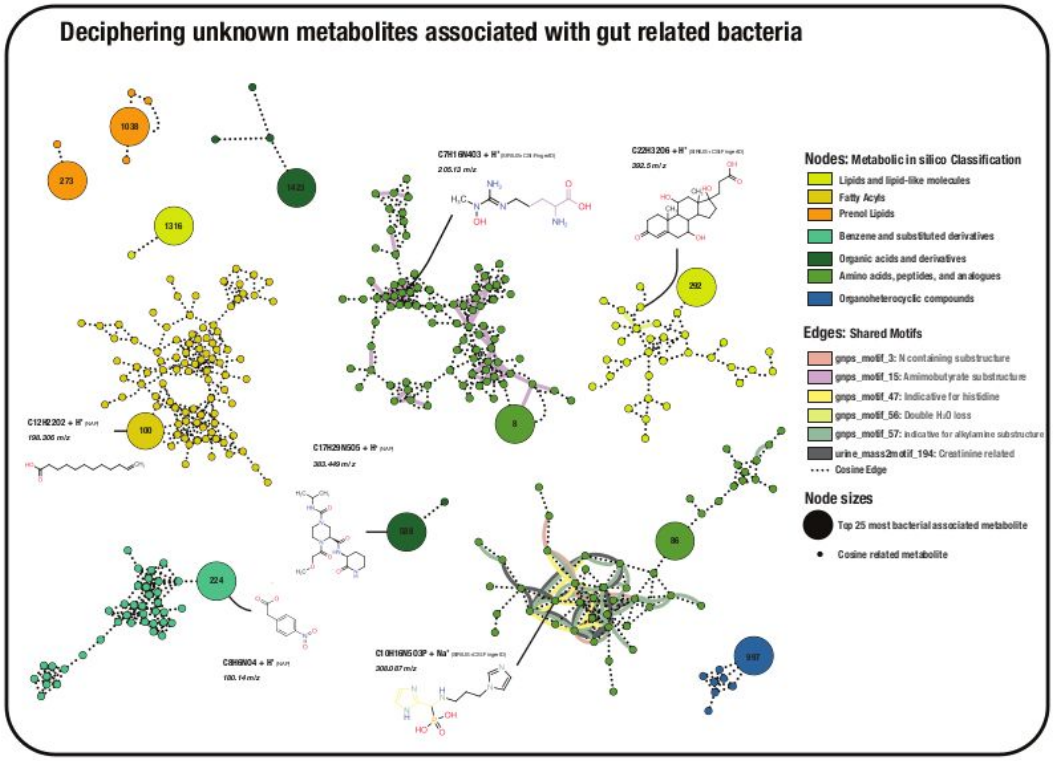

Figure 4

Network analysis of bacterial associated metabolites and their molecular families with in silico annotation and hypothetical structure Metabolomics based molecular network of 11 bacterial associated metabolites (BAMs) and their molecular related network coloured by chemical classes as indicated in the legend. A total of 350 metabolites were selected based on the bacterial association test and molecular relation to BAMs. Neighbour nodes of the significant nodes were co-selected in order to increase

knowledge of BAMs. Small nodes indicate metabolites in a network with bacterial associated metabolites. Edges are coloured according to shared motifs from MS2LDA and MotifDB databases or the cosine edge as indicated in the legend. Unclassified molecular families were removed from the figure to increase clarity. Hypothetical structures for molecular families are shown if identified. Shared motifs in molecular networks were visualised in hypothetical structures with corresponding edge colours.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplTableS21.xlsx
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- SupplTableS24.xlsx
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- SupplTableS34.xlsx
- SupplTableS35.xlsx
- SupplTableS36.xlsx
- SupplTableS37.xlsx
- Supplementary.docx