Single Cell Protein in Fish Feed: Effects on Gut Microbiota

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Abstract

The microbiota has been shown to be important for nutrition and health. It provides the host with an extensive arsenal of dietary enzymes that can contribute to host metabolism by conversion of otherwise undigestible compounds to metabolites that the host can utilize. It can also provide benefits to the host by protecting against colonization of unwanted pathogens and by maintaining mucosal immunity. However, the composition of the microbiota have also been linked with diseases and susceptibility for infections, thus it is evident that the composition of the microbiota has an important role for health. The microbiota in fish is still a fairly unexplored ecosystem and it is not well described how different dietary components influence its composition.

This licentiate thesis focuses on the microbiota in fish and how partial replacement of fishmeal with microbe based protein sources or mussel meal influence the composition and diversity of the gut microbiota, in Arctic charr *(Salvelinus alpinus)* and rainbow trout (*Oncorhynchus mykiss*). The gut microbiota was characterized by using 16S rRNA gene amplicon sequencing using Illumina MiSeq. In paper I, five experimental diets were tested; intact (ISC), extracted (ESC) yeast cells of the species *Saccharomyces cerevisiae*, the micro fungi *Rhizobium oryzae* (RHO), mussel meal (MYE) and as reference, fishmeal (REF). Analyses showed that the gut microbiota was dominated by *Firmicutes* and *Proteobacteria*. Principal component analysis (PCA) of the data revealed that microbiota in proximal and distal regions of the intestine had similar composition. Replacement of fish meal with yeast and filamentous fungi also affected microbiota composition, primarily with higher relative proportions of *Photobacterium* and *Lactobacillus*.

In paper II, rainbow trout were fed diets with 3 different inclusion levels of either *S. cerevisiae* (SC20, SC40, SC60) or *Wickerhamomyces anomalus* (WA20, WA40, WA60) and as controls, rainbow trout fed fishmeal. Intestinal microbiota were dominated by *Leuconostocaceae*, *Lactobacillaceae* and *Photobacterium* and significant differences in composition of the microbiota were found between fish fed WA40 and WA60 compared with those fed the FM diet. In addition, a reduction in bacterial diversity in fish fed the diet WA40. These results showed that feeding diets with high inclusion of *W. anomalus* significantly changed the intestinal microbiota of rainbow trout while lower inclusion levels and diets of *S. cerevisiae* did not.

Keywords: Rainbow trout, Arctic charr, *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, blue mussel, bacteria, 16S rRNA

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Dedication

Till Astrid och Esmeralda

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Nyman A., Huyben D., Lundh T. and Dicksved J. (2016). Effects of microbe and mussel-based diets on the gut microbiota in Arctic charr (*Salvelinus alpinus*). *Aquaculture Reports* (submitted).

II Huyben D.*, Nyman A.*, Vidakovic A., Kiessling A., Dicksved J. and Lundh T. (2016). Effects of feeding the yeasts *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* on gut microbiota of rainbow trout. (manuscript).

* These authors contributed equally to the manuscript

The contribution of AN to the papers included in this thesis was as follows:

I Designed the microbiota study together with the supervisors, performed the laboratory analyses, analysed most of the microbiota samples and wrote the manuscript.

II Took part in sampling and participated in start-up of yeast culture, and wrote the manuscript together with DH.

Abbreviations

ESC	Diet containing extracted *Saccharomyces cerevisiae*
ISC	Diet containing intact *Saccharomyces cerevisiae*
RHO	Diet containing the filamentous fungus *Rhizopus oryzae*
MYE	Diet containing blue mussel, *Mytilus edulis*
REF	Fish meal-based reference diet
SCP	Single cell protein
SC	*Saccharomyces cerevisiae*
WA	*Wickerhamomyces anomalus*

1 Background

1.1 Fisheries and Aquaculture

The increasing demand for seafood has led to a dramatic upswing in the aquaculture sector. In recent years, the production of farmed fish has been almost as great as the amount produced in the capture industry (Figure 1). It is common to include fish meal or fish oil in fish feed and therefore aquaculture itself is a great consumer of fish products from the capture industry. Fish meal is produced from fish or fish waste that is pressed and dried. This waste was initially used in agriculture as fertiliser and later in pig and poultry feed, but it is currently used mainly in aquaculture, as fish feed. In 2010, 73% of the fish meal and 71% of the fish oil produced were used by aquaculture (Figure 2).

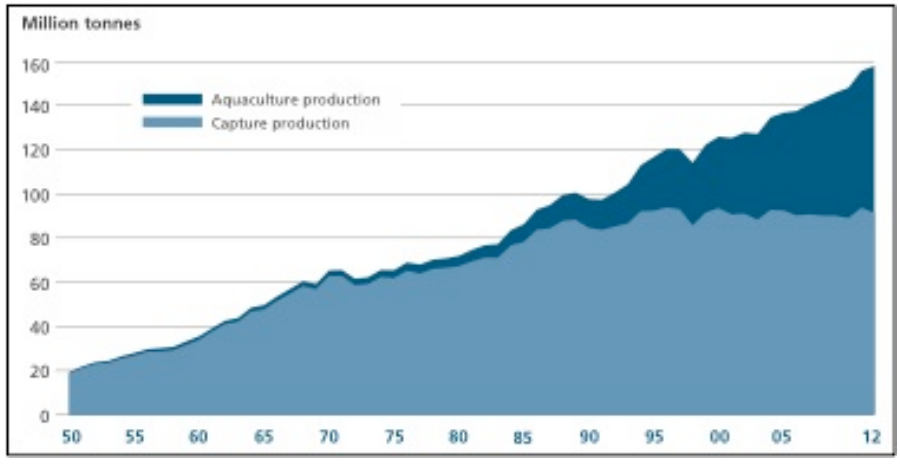

Figure 1. Total world fish production 1950-2012, showing the growing proportion contributed by aquaculture (FAO, 2014).

With this growing demand for resources, aquaculture may come to compete with humans for proteins and therefore it is vital to find a way to increase the overall available protein (Tacon *et al*., 2011). At the present time, fish are being caught for use as fish oil and fish meal in the feed industry and at each trophic level there is a substantial loss of net protein. With a growing global human population, it is important to formulate a feed that utilises resources which are unsuitable for human consumption and converts these to proteins suitable for human consumption.

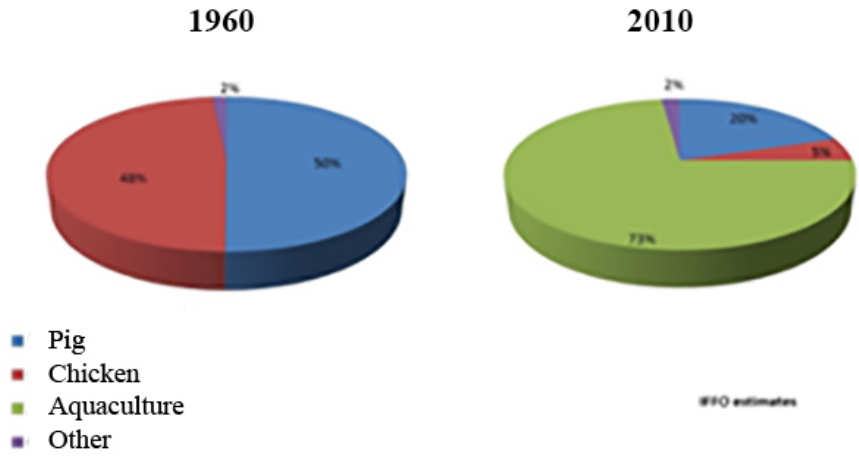

Figure 2. Changing uses of fish meal over time. In 1960, the usage was divided fairly equally between the poultry and pig industries. Now (2010), aquaculture uses around three-quarters of the fish meal produced world-wide.

1.2 Alternative feedstuffs

As fish meal is not a sustainable protein source, the aquaculture industry is looking for alternatives that are cheaper and more environmentally friendly. Among those alternative sources are plant-based and microbe-based feedstuffs.

1.2.1 Plant feedstuffs

Inclusion of plant feedstuffs in fish feed has increased markedly and, consequently, so has exposure to anti-nutritive factors that are novel to most farmed fish species, particularly carnivorous species (Krogdahl *et al*., 2010). Much research has been carried out to find alternative feed sources of protein, with plant materials such as oilseeds, legumes and cereal grains often being used (Gatlin *et al*., 2007). Plant feedstuffs such as soybean meal have the advantage of being much cheaper than fish meal and are considered to be a high quality source of protein (FAO, 2014). However, soybean meal has also been shown to be problematic as a feedstuff for Atlantic salmon (*Salmo salar*) due to its high content of anti-nutritive factors that can increase intestinal permeability and induce enteritis (Knudsen *et al*., 2008).

1.2.2 Single cell protein

Single cell protein (SCP) has become a very interesting alternative protein source for inclusion in fish feed (Øverland *et al*., 2013; Vidakovic *et al*., 2015; Langeland *et al*., 2016). Single cell protein has many positive traits, as it is fast growing, renewable and could be grown on substrate from industrial waste products. The nutritional value of its constituents, such as proteins, B-vitamins, pigments and β-glucans, has been suggested to be sufficiently high to make it a good replacement for fish protein (Sanderson & Jolly, 1994; Tacon, 1995). Anupama *et al.* (2000) listed the following characteristics of organisms suitable as single cell proteins:

- Non-pathogenic to plants, humans and animals
- Usable as food and feed
- Have good nutritional values
- Do not contain toxic compounds
- Have low production costs.

Bacterial biomass

Bacteria are fast growing, with a life cycle of hours rather than days, and there have therefore been attempts to harvest specific bacteria as a source of protein. For example, *Methylophilus methylotrophus*, an obligate methane-utilising organism, has been used to produce a commercially available product called PRUTEEN (Stringer, 1982). However, this product was never a major commercial success, since the biomass produced contains too high levels of nucleic acids to be used for human consumption and removal of these in a subsequent step would have made the production process more expensive than that of conventional protein However, other studies examining bacterial biomass in animal feed have found some types to be a good alternative protein source (Rumsey *et al*., 1992).

Microalgae

Microalgae, for example Spirulina, are unicellular, filamentous blue-green algae. Like bacteria, they are fast growing organisms that have gained popularity in the health and food industry and increasingly as a protein and vitamin supplement to aquaculture diets (Usharani *et al*., 2012). Spirulina has long been used as a dietary supplement by people living close to alkaline lakes, where it is naturally occurring. Some Spirulina species, like the blue-green alga *Spirulina platensis*, have received more attention because they have a high nutritional content (Usharani *et al*., 2012). Spirulina can play an important role not only in

human and animal nutrition, but also in environmental protection through wastewater recycling.

Yeast

Yeast has been suggested as an alternative protein source for inclusion in aquaculture feeds *(*Øverland *et al*., 2013; Vidakovic *et al.*, 2015; Langeland *et al*., 2016). The nutritional value of yeast is high, with a high crude protein content. Yeast is also rich in B-vitamins, pigments and β-glucans, which increases its potential to replace fish meal in aquaculture feeds (Sanderson & Jolly, 1994; Tacon, 1995). Moreover, yeast has a similar amino acid profile to fish meal (Vidakovic *et al*., 2015). The β-glucans in the yeast cell walls have been suggested to stimulate immunological maturation in fish (Gatesoupe, 2007). Atlantic salmon has been reported to show better resistance to sea lice infection and also improved feed uptake and ability to counteract soybean meal-induced enteritis when β-glucans and mannan oligosaccharides derived from cell walls of SC are added to the feed (Refstie *et al*., 2010).

Paper I of this thesis examined the effect of microbial protein on the intestinal microbiota in Arctic charr (*Salvelinus alpinus*). To the best of my knowledge, that was the first study focusing on how different forms of single cell protein affect the microbial community in Arctic charr. Paper II examined how two different species of yeast at different levels of inclusion in the diet affected the gut microbiota and also examined the effects of feed extrusion on yeast viability.

Filamentous fungal biomass

Some filamentous fungi are fast growing, and in times of famine people have made efforts to produce *Fusarium* spp. and *Rhizopus* spp. cultures as a protein supplement. Fungal biomass production can vary widely depending on type of fungus and the substrate on which it is grown. *Rhizopus oryzae* is a protein-rich filamentous fungus with an amino acid profile similar to that of fish meal and has been used in a few previous studies to replace fish meal protein (Bankefors *et al.*, 2011; Abro *et al*., 2014). Abro *et al*. (2014) found that trans-epithelial resistance (TER) was higher in the distal intestine of Arctic charr fed *R. oryzae* than in fish fed fish-meal and that they had a reversed corresponding mannitol transport.. This indicates that dietary filamentous fungi or their digested metabolites affect the gut integrity in fish.

1.3 The fish intestine

In carnivorous fish, the length of the intestine varies from one-half to two-thirds of body length. Omnivores, on the other hand, have a gut of intermediate length, while it is often elongated and folded in herbivorous fish, where the gut length may be five to six times body length. The proximal region begins immediately after the pyloric caecum and has distinct epithelial, absorptive and secretory cells (Einar Ringø & Gatesoupe, 1998). The distal region is an extension of the proximal region (Sundell & Sundh, 2012)) (Figure 3), with gradually diminishing digestive and absorptive functions, near neutral pH of between 7.8 and 8.4 and increased levels of mucus production. The gut epithelium of both the proximal and distal region has extended foldings (Einar Ringø & Gatesoupe, 1998).

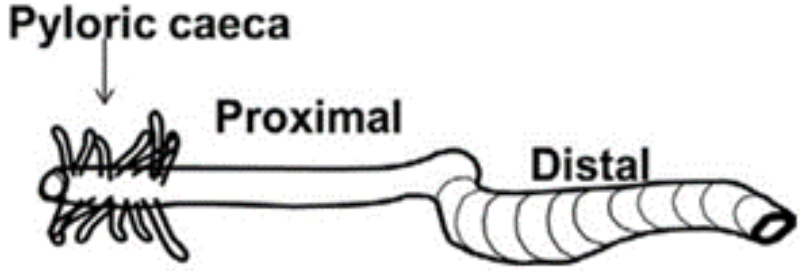

Figure 3. Schematic drawing of the intestine of salmonid fish.

1.4 Gut microbiota in fish

The gut microbiota in fish is involved in digestion, amino acid production and secretion of inhibitory compounds that protect against bacterial pathogens in the intestine (Austin, 2006; Nayak, 2010). The microbiota also has a role as an immuno-stimulant (Einar Ringø & Gatesoupe, 1998). The distal part of the gastrointestinal tract is a favourable ecological niche for microorganisms, and is colonised by a wide range of microbes (Skrodenytė-Arbaĉiauskienė, 2007). It is well known that the intestinal microbiota of freshwater and marine fish harbour different microorganisms, *e.g*. Sakata *et al*. (1980) showed that the intestinal microbiota of freshwater fish is composed of different species of bacteria than the intestinal microbiota of marine fish, while Ringø & Strøm (1994) found that *Lactobacillus* spp. constituted a large percentage of the intestinal microbiota of Arctic charr.

There is a growing interest in fish farming to control diseases using alternative dietary components, such as prebiotics and probiotics, in order to reduce the need for antibiotics in aquaculture (Pérez *et al*., 2010). It has been shown that certain lactic acid bacteria can counteract the effects of pathogenic microorganisms, possibly by competition for nutrients and adhesion receptors, and act as probiotics in rainbow trout (*Oncorhynchus mykiss*) (Balcázar, De Blas, Ruiz-Zarzuela, Vendrell, Gironés, et al., 2007; Vendrell et al., 2008). In aquaculture, previous studies have sought to determine dietary effects on changes in microbial composition, especially focusing on soybean meal as a replacement for fish meal in salmonid fish such as rainbow trout (Heikkinen *et al*., 2006) and Atlantic salmon (Bakke-McKellep *et al*., 2007).

1.4.1 Lactic acid bacteria in fish

Lactic acid bacteria have high nutritional demands, requiring carbohydrates, amino acids, peptides, nucleic acid derivatives and vitamins (Einar Ringø & Gatesoupe, 1998). Lactic acid bacteria include members of the genera *Streptococcus, Enterococcus, Lactobacillus, Aerococcus, Carnobacterium, Leuconostoc, Lactococcus* and *Pediococcus*. Different species of lactic acid bacteria have adapted to grow under varying environmental conditions and they are widespread in nature.

Lactic acid bacteria are commonly found in the gastrointestinal tract of many endothermic animals (Tannock *et al*., 1982), in dairy products (Coeuret *et al*., 2003), meat products (Schillinger & Lücke, 1987) and seafood products (Najjari *et al*., 2008) and on some plants (Keddie, 1951). Lactic acid bacteria are often used in the production and preservation of food products, *e.g*. dairy products including cheese and yoghurt, and also in lacto-fermentation of vegetables and in silage making (Keddie, 1951; Coeuret *et al*., 2003; Najjari *et al*., 2008).

The function of lactic acid bacteria in the gastrointestinal tract of endothermic animals has been thoroughly studied and reviewed (Tannock *et al*., 1982; Conway *et al*., 1987; Fernandez *et al*., 2003). In fish, however, only a few studies have reported that lactic acid bacteria are part of the normal intestinal microbiota. Some studies on the presence of lactic acid bacteria in the digestive tract of salmonids have been reviewed by Ringø *et al.* (1998) and Salinas *et al.* (2008), who concluded that pretreatment of the salmonid intestine with probiotic lactobacilli can prevent the damaging effect of *Aeromonas*. However, Ringø *et al.* (1998) also found that some species of lactic acid bacteria present in the intestines of salmonids were pathogenic.

The fry of salmonids have no indigenous lactic acid bacteria, since they come with ingested food. Since sources of lactic acid bacteria are mainly of freshwater and terrestrial origin, one can speculate that they originate from consumption of insects by fish.

A better understanding of the many factors affecting lactic acid bacteria in the digestive tract may be of interest in aquaculture.

2 Aims of the thesis

The overall aim of this thesis was to investigate how replacement of fish meal with alternative protein sources, as microbes and blue mussel, affects the composition and diversity of the gastrointestinal microbiota.

Specific aims in Papers I-II were to:

- Examine differences between the proximal and distal part of the intestine regarding composition of the microbiota (Paper I).
- Compare the effects on the gut microbiota of diets with different sources of microbial protein (Paper I)
- Investigate how different levels of yeast inclusion affect the microbiota (Paper II).
- Investigate how two different yeast species affect the microbiota (Paper II).

3 Materials and Methods

3.1 Sampling

In Paper I, Arctic charr were fed five experimental diets containing: intact *Saccharomyces cerevisiae* (ISC), extracted *S.* cerevisiae (ESC), *Rhizopus oryzae* (RHO), *Mytilus edulis* (MYE) or a fish meal-based reference diet (REF) and the effects on fish growth were investigated. The fish were dissected and the intestines were sectioned with strings at the anus, ileorectal valve and below the pyloric caecum, in order to avoid flow of digesta between the distal and proximal intestine. After sectioning, the whole intestine of each fish was wrapped in aluminium foil and flash-frozen in liquid nitrogen. Thereafter, the samples were stored at -80 °C until they were examined for effects on intestinal microbiota. The intestinal samples were thawed, dissected and scraped, one region at a time, under sterile conditions.

In Paper II, rainbow trout were fed two different yeasts, *S. cerevisiae* (SC) and *Wickerhamomyces anomalus* (WA), at different inclusion levels (20%, 40% and 60%) to examine effects on growth and intestinal microbiota. In this experiment all intestinal samples were taken on-site by aseptically opening the ventral side of the fish. The distal intestine was cut between the sphincter at the start of the distal intestine and the anus. The intestine was cut open with sterile scissors and a scalpel was used to scrape the intestinal contents and mucosal layer into tubes, which were stored at 4°C until they were transported to the laboratory. There, the intestinal contents and mucosa were mixed with peptone water and frozen at -20 °C until further analysis.

3.2 Pellet making and extrusion

The production of pelleted fish feed starts with the mixing of dry ingredients with boiling water and part of the fish oil for 10 minutes. The feed is then left to cool down. Thereafter, the pellets go through an extruder, where the feed is subjected to 120-130 °C for 30 seconds. The feed is then pressed through a sieve and cut to appropriate lengths within the extruder, to make it suitable for different sizes and species of fish. The pellets are air-dried over night at 40 °C and then passed through a vacuum coater where fatty acids such as fish oil are added (Figure 4). The effects of extrusion on yeast viability were examined in Paper II.

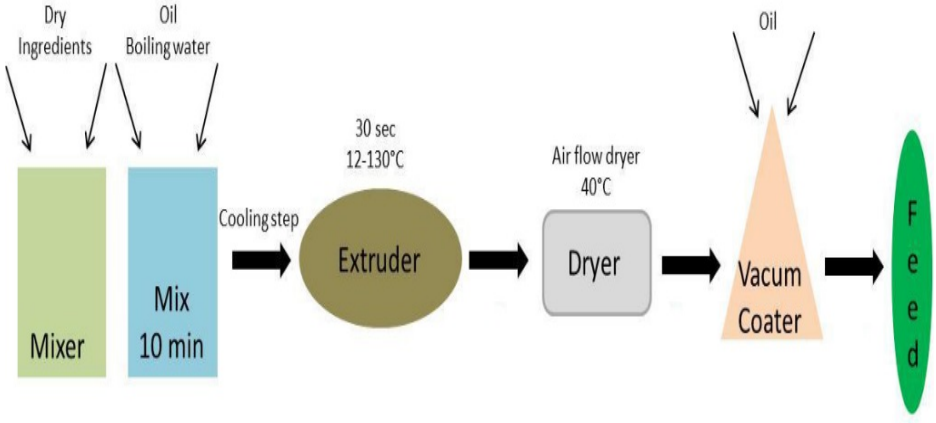

Figure 4. Schematic flowchart of extruded pellet fish feed production.

3.3 Analysis of the composition of the microbiota

The knowledge about the intestinal microbiota in fish has expanded along with the development of new techniques. Previously, characterization of the microbiota relied on culture based approaches to study the structure of the microbial community. Samples were taken from digesta or homogenates of the intestine, which were then grown on agar plates either on selective or non-selective media. This process is both expensive and time-consuming as the intestinal bacteria of cold water species often is slow-growing and thus need long incubation times of up to 4 weeks. Another disadvantage is that they would only show the species suitable for cultivation.

However, since the last 20 years, molecular methods have been developed and used to study the microbiota, which now enables a good picture of the total bacterial representation in the intestine.

3.3.1 Fingerprinting techniques

Microbial community fingerprinting is a term for a number of molecular methods which have been developed for studying the composition of microbial communities. These methods usually relies on separation of multi-template PCR products, which generate a molecular fingerprint of the microbial community.

One of the most common approaches is denaturing, or temperature, gradient gel electrophoresis (DGGE and TGGE, respectively) which separates PCR fragments based on their GC content. Another commonly used fingerprinting approach is terminal-restriction fragment length polymorphism (T-RFLP) which generate a fingerprint based on polymorphisms in restriction sites in the PRC fragments (Dicksved, 2008). The fingerprinting techniques are generally quite cheap, but suffers of a quite poos resolution and inability to characterize the species composition.

3.3.2 Next generation sequencing

Next generation sequencing (NGS) the collective term for a number of sequencing methods developed after the traditional Sanger sequencing method. The NGS platforms generates millions of sequences in each run and has provided an important analytical platform to study microbial ecology. Using NGS to sequence the 16S rRNA gene have revealed that it is possible to get a detailed picture of the structure of gut microbial communities. This technique has been used both to determine microbial diversity in Arctic charr (Lyons et al., 2015) and to quantify shifts in intestinal microbiota in response to infection in rainbow trout (Ingerslev et al., 2014).

3.3.3 Generation of 16S amplicon libraries

In both papers, amplicons were generated from the V4 region of the 16S rRNA gene using Illumina MiSeq. An initial PCR was used to target and amplify the V4 region of the 16S rRNA genes from the isolated DNA using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3' (Hugerth et al., 2014) and 805R (5'-GACTACHVGGGTATCTAATCC-3' (Herlemann et al., 2011).

4 Results and Discussion

4.1 Community composition

In paper I, the microbiota was characterized in Arctic charr and we found that two phyla covered 85% of the total relative abundance in the data set, with *Firmicutes* (65.7%) and *Proteobacteria* (19.3%) being the most dominant. Among the *Firmicutes*, the largest proportion was classified as lactic acid bacteria (LAB) and had an average relative abundance of 36.2% in the data set. The most dominant OTU was *Leuconostocaceae* which had an average relative abundance of 14.2%. The major representative of the Proteobacteria phylum was *Photobacterium*.

In paper II, the microbiota was characterized in rainbow trout, and was dominated by two phyla: with 75 % of the sequences classified as *Firmicutes* and 19 % as *Proteobacteria*. Among the *Firmicutes*, LAB represented the largest fraction of the OTUs, with *Leuconostocaceae*, *Lactobacillaceae* and *Streptococcus* being most abundant. *Proteobacteria* was dominated by the taxa *Photobacterium* and *Enterobacteriaceae*.

Common in both studies was that *Firmicutes* and *Proteobacteria* were the dominant phyla. In addition, in both studies LAB made up a large proportion of the *Firmicutes* and *Leuconostocaceae* was most abundant. This result is consistent with previous studies on rainbow trout reporting high presence of LAB among the *Firmicutes*, especially *Weissella*, *Streptococcus* (Desai et al., 2012) and *Leuconostoc* (Desai et al., 2012; Ingerslev et al., 2014). Ringø & Strøm (1994) found that *Lactobacillus* spp. constituted a large proportion (~20%) of the intestinal microbiota of Arctic charr. Proteobacteria were dominated by the taxon *Photobacterium*, which was more dominant in the fish fed microbial protein in paper I.

4.2 Diversity

In paper I, Shannon diversity and Chao-1 richness in the proximal and distal gut differed depending on diet. However, there were no significant differences in diversity between the regions. In the proximal region, the highest diversity was found in fish fed the ISC and RHO diets, both significantly higher than the REF diet. Shannon's diversity index showed a similar pattern to Chao-1, but with no significant differences between the diets. In the distal region, the highest values of Chao-1 richness were again in fish fed microbe-based diets and the lowest in those fed the MYE and REF diets. The fish fed ESC had a significantly higher

Chao-1 index than those fed REF. Shannon's index also differed between the diets, with a significantly higher value for the ISC diet compared with REF in pair-wise comparisons.

In paper II, the microbial diversity showed no effects on fish fed the SC or WA diets compared to the fishmeal diet, except for a significant decrease in Chao-1 richness for the WA40 diet. Although not significant, fish fed the WA20 diet had the highest number of taxa, Shannon diversity and Chao-1 richness.

In paper I, we found significant effects of diet on diversity in both proximal and distal regions of the intestine. However, there were no significant differences between the regions. In paper II, we only examined the distal part of the intestine and might thus have missed dietary effects in the proximal region. However, fish fed the WA40 diet had a lower bacterial diversity in the distal part than fish fed other diets.

One of the theories about diversity is that a higher diversity is desirable as it can contribute to ecosystem stability by "functional redundancy". This means that more organisms in a community can perform the same function, thus reducing the risk of damage if any of the organisms should disappear from the ecosystem.

4.3 Feed impact on the gut microbiota

In paper I, principal component analysis (PCA) was performed on the data set to determine if there was a correlation between test ingredients in the diets and the composition of the bacterial microbiota in the intestine. In the PCA plots, the first and second principal components together explained 64% of the variation in the dataset and showed that the different diets had an impact on the intestinal bacterial community composition. There were no separate clustering between samples from the proximal and distal gut illustrating that there was no apparent difference in microbiota com-position between the regions. In terms of diet effects, the microbial diets clustered separately compared to the REF diet, which indicated that there were differences in microbial composition related to the diet type. Also, the microbial based diets yielded similar compositions of the microbiota, contrary to what we saw in Paper II. The microbiota in fish fed the MYE diet was also different from the microbial-based diets when samples from both proximal and distal regions were pooled.

In paper II, the PCA showed an impact of diet with diverging groups of fish fed FM, SC and WA diets. Biplots from the PCA revealed that *Streptococcus*

luteciae, *Lactobacillus* and *Leuconostocaceae* were associated with diet FM, *Streptococcus sobrinus* and *Leuconostoc* with diet SC20, *Photobacterium* with diet WA20, *Lactobacillaceae* with diet WA40 and *Enterobacteriaceae* and *Pseudomonas veronii* with diet WA60. The different yeasts yielded different compositions of the microbiota. The SC diets had no major impact on the microbiota, perhaps such impact would be more evident if both proximal and distal region had been examined.

In order to see if there were any similarities or differences in microbiota composition between the studies, the data sets were merged and analyzed together. Analysis of data from both studies together using Principal Coordinate Analysis (PCoA) showed a difference in clustering according to PC2, between the studies. The rainbow trout clustered separately with no differences between the fishmeal, SC20, SC40, SC60 and WA20. WA40 and WA 60 were significantly different than all other treatments. The Arctic charr also cluster separately, with the REF and MYE significantly different from the other diets. The microbial based diets also formed a group significantly differed from the others (Figure 5).

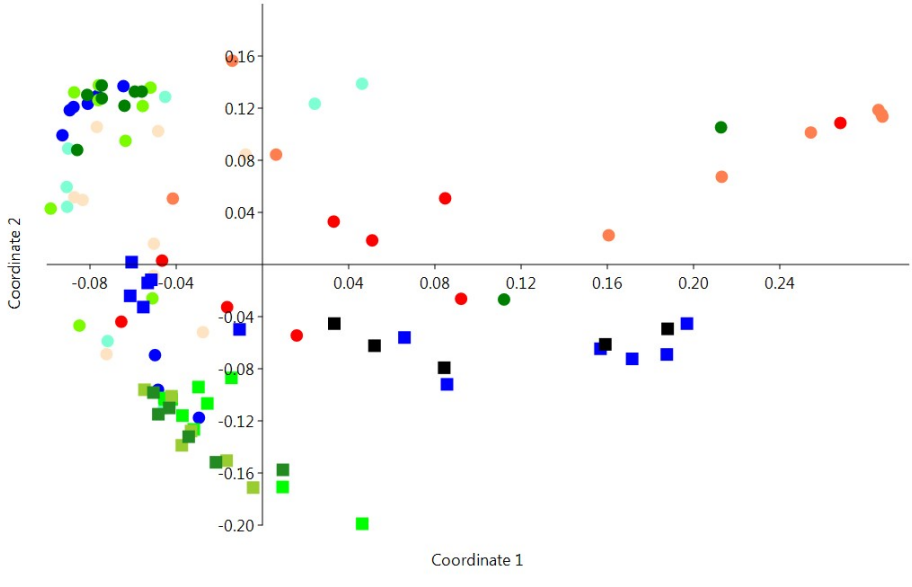

Figure 5. Scatterplot showing results of principal coordinate analysis (PCoA). Full circles represent rainbow trout (blue=fishmeal, turquoise=SC20, light green=SC40, dark green=SC60, pink=WA20, orange=WA40, red=WA60). Squares represent Arctic charr (blue=REF, black=MYE, green=ESC, moss green=ISC, dark green=RHO).

4.4 Dietary effects on specific OTUs

In Paper I, the OTUs with a mean relative abundance >1% in the dataset were analyzed with a univariate analysis in order to study the diet impact on specific OTUs. The OTUs that varied the most between diets was taxonomically classified as *Photobacterium*. This OTU had a significantly higher abundance in fish fed microbial based feeds compared with controls and the difference was evident in both proximal and distal regions. The diet also had an impact on an OTU classified as *Porphyromonas endodontalis* where fish fed the ESC or ISC diets had a lower abundance compared with fish fed the REF diet. Moreover, *Lactobacillus* sp. were significantly higher in the proximal intestine of fish fed microbial-based diets compared to the REF diet. However, this effect was limited to the proximal region. In addition, diet type also effected abundance of *Peptostreptococcus*, where there was no significant impact on the abundance of this OTU compared to controls fed the reference diet, there was a clear difference in abundance between fish fed the ESC and MYE diets in the distal region, whereas no such difference was found in the proximal region.

In paper II, the genera with a mean abundance of > 1 % in the data set was dominated by LAB (order Lactobacillales) and represented 70 % of the OTU abundance. The fish that were fed WA60 diet had a significantly lower abundance (47 %) compared with those fed the fishmeal diet. *Leuconostocaceae*, *Lactobacillaceae* and *Photobacterium* contributed between 37 and 60 % of the total OTU abundance but no significant differences existed between SC20, SC40, WA20 compared with the FM diet. However, fish fed the SC60 diet had a significantly lower abundance of *Photobacterium*. Moreover, fish fed the WA40 diet had differences in the abundance of *Leuconostocaceae*, *Lactobacillaceae*, *Photobacterium*, *Streptococcus sobrinus*, *Streptococcus luteciae*, *Carnobacterium* and *Leuconostoc*, while fish fed WA60 had

differences in *Leuconostocaceae*, *Enterobacteriaceae* and *Pseudomonas veronii*. For gut bacteria profiles, fish fed SC40 showed the lowest dissimilarity value compared with FM for the top four bacterial groups in fish fed all diets, whereas the highest dissimilarity value was found in fish fed diet WA40. Significant dissimilarities were only found for fish fed diet WA40 and diet WA60.

In paper I, we found significantly higher abundance of *Lactobacillus* in the proximal intestine of Arctic charr fed the microbe-based diets. We also found numerically higher abundance of *Lactobacillus* in the distal intestine of fish fed the microbe-based feeds, but the difference was not significant. The elevated abundance of lactobacilli in fish fed the microbe-based diets could be explained by the cell wall components in the microbe-based feeds having prebiotic properties. The yeast cell wall is made up of beta-glucans, mannan-oligosaccharides (MOS) and chitin. Beta-glucans have been shown in vitro to have a beneficial effect on the stress tolerance of intestinal *Lactobacillus* (Stack et al., 2010). It has also been shown that whole yeasts have similar effects to refined MOS when fed to pigs, resulting in elevated lactobacilli levels (White et al., 2002) and that MOS can function as prebiotics by providing favourable conditions for growth of *Lactobacillus* in monogastric animals (Flickinger & Fahey, 2002). However, we obtained similar results for the diets with intact and extracted yeast, where the cell wall had been removed, and the effect could possibly be due instead to some symbiotic relationship between yeast and lactobacilli. On the other hand, this is not very likely as most of the yeast had been inactivated during extrusion.
We observed an effect of diet on *Porphyromonas endodontalis* and *Peptostreptococcus*. *Porphyromonas* sp. has previously been found in the intestinal microbiota of Atlantic salmon (Zarkasi et al., 2016) and rainbow trout (Kim et al., 2007) and *Peptostreptococcus* has been found in the GI tract of freshwater fish (Romero et al., 2014). We could not find any evidence linking these two bacteria with microbe-based diets and further studies are needed to determine their importance.

We also observed dietary effects on *Photobacterium*, which belong to the phylum *Proteobacteria*. *Photobacterium* is common in marine environments, with some luminous species occurring as symbionts in the light-generating organs of several fish species (Dalgaard et al., 1997). To our knowledge, *Photobacterium* has not been found in freshwater environments, only in the GI of migrating fish (Budsberg et al., 2003). The high abundance of

Photobacterium in Arctic charr fed the microbe-based diets was unexpected, since this taxa is primarily of marine origin (Baumann & Baumann, 1977), although our microbe-based diets did contain some fish meal.

In paper II, *Lactobacillales* represented a large proportion of the intestinal microbiota in the examined rainbow trout. Although the biggest difference was between the different species of yeast, fish fed the WA40 had most impact on specific bacteria. The present study found high abundance of *Leuconostoc* and *Streptococcus*, which is similar to the findings of Ingerslev et al. (2014). These bacteria have been suggested to have a beneficial role for fish health (Lara-Flores et al., 2003; Vendrell et al., 2008). In this study, fish fed the WA40 diet had a significantly higher abundance of *Carnobacteria*. Lyons et al. (2015) also showed high abundance of *Carnobacteria*, which have previously been reported to be part of the healthy rainbow trout intestinal microbiota (Einar Ringø & Gatesoupe, 1998; Balcázar, De Blas, Ruiz-Zarzuela, Vendrell, Girones, et al., 2007). *Leuconostocaceae* and *Lactobacillaceae* where dominating taxa in most diets and both these bacteria families belong to the order *Lactobacillales*. There was also a high abundance of *Photobacterium*, which is common in marine environments (Dalgaard et al., 1997) and thus could originate from the fish meal component in the diets. The WA60 diet had a significantly lower abundance of *Leuconostocaceae* than the other diets, but significantly higher proportions of *Enterobacteriaceae* and *Pseudomonas veronii*. In Paper I, microbial based feeds increased the abundance of *Photobacterium*, while in Paper II *Photobacterium* was only affected in one of the treatments, and there the abundance decreased.

4.5 Yeast levels before and after extrusion

In Paper II, we examined what effect extrusion had on yeast viability. The yeast ingredients, *S. cerevisiae* or *W. anomalus* and *S. cerevisiae* mix, contained 9.1 and 10.0 log CFU g-1 and the SC and WA diets contained between 8.1-9.5 log CFU g-1 before extrusion. Diets of WA had significantly higher levels of live yeast than SC diets, while both SC and WA had significantly higher levels than the FM diet. After extrusion, levels of live yeast in the FM diet were similar as before while both SC and WA diets had log-reductions between 4.8-7.1 CFU g-1. No significant differences in levels of live yeast measured by CFU g-1 between diets were found, while yeast levels measured by cells g-1 were significantly higher for SC and WA diets (between 9.2-10.2 log cells g-1)

compared with FM (7.6 log cells g-1). Yeast cell viability was significantly higher for SC and WA diets (95.1 ± 0.5 %; mean ± SE) compared with FM (64.1 ± 3.5 %).

The low live count and high viability of yeasts in both SC and WA diets suggested that high temperature from extrusion inactivated yeasts, but did not disrupt the cells. Inactivated, non-disrupted yeast is not ideal because the yeast cannot cultivate the intestinal microbiota or release nutrients for metabolic uptake. Therefore, reducing extrusion temperature for yeast-based diets may be one solution for increasing the level of live yeast in diets and consequent ingestion and colonization in the gut of fish. However, reducing extrusion temperature may affect other factors, such as pellet expansion and pore size that could have negative effects on digestibility of carbohydrates. Thus, further research is necessary to optimize extrusion parameters of yeast-based feeds and ongoing research is being performed by our research group. It is notable that also the FM diet had considerable levels of yeast, which could be due to the diets having a plant based component from which the yeasts could originate.

5 Conclusions

Lactic acid bacteria represented a dominant fraction of the microbiota of farmed Arctic charr as well as in rainbow trout.

In paper I, the microbe-based feeds were associated with similar changes in microbiota composition, primarily characterized by an elevated abundance of *Photobacterium* and *Lactobacillus* when compared to reference fish meal-based diet. Microbiota composition was also similar in the proximal and distal intestine of Arctic charr, but dietary responses were specific to gut segment.

In paper II, these results show that feed extrusion inactivated high levels of yeast and feeding diets containing 40 and 60 % replacement of fish meal with *W. anomalus* and *S. cerevisiae* mix significantly changed the gut microbiota of rainbow trout. This favoured bacteria associated with the *Lactobacillales* and *Streptococcaceae* families, while lower levels and diets of *S. cerevisiae* had lesser effects.

Better knowledge on the normal gut microbiota in farmed fish can contribute to improved options for designing and applying pre and probiotic products for salmonids.

SCP can be a useful feedstuff as long as the inclusions are not too high, as they then could result in decreased growth. High inclusions could also affect the microbiota negatively. In one of the studies *Photobacterium* benefitted from yeast inclusions and because they are related to the often pathogenic *Vibrio*, this is not desirable.

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