NMR Metabolomics and Lipid Analyses in Salmonid Tissue - Response to Alternative Feeds

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Cover: The cover shows the main work of my thesis. Atlantic salmon and Arctic charr were fed with different diets and analysed chromatographically
(photo: graphic design by P. Sprenger (kampagner.de) and Arctic charr: A. Vidakovic

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Abstract

Salmonids are fatty fish and contain high amounts of omega-3 polyunsaturated fatty acids, beneficial in human nutrition. However, the aquaculture industry has expanded greatly in recent decades, increasing demand for fishmeal (FM) and fish oil (FO) for fish feed, while the supply of fish raw materials has remained static or declined. This has created a need for alternative sustainable raw materials for fish feeds.

This thesis examined the effect of replacing FM or FO with alternative plant (vegetable oils (VO), sesamin), microbial (zygomycete-, yeast fungi) and marine (krill, mussel) raw materials on growth performance, lipid content, fatty acid composition, lipid classes and fish metabolism in Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*).

There was a significant negative effect on growth performance when sesamin, zygomycete fungi and extracted yeast were included in fish diets. Replacement of FM or FO with alternative materials affected the lipid profile in fish tissues, *e.g*. compared with the control, docosahexaenoic acid (DHA) level was higher in fish fed krill meal and mussel meal and lower in fish fed VO, but unaffected in fish fed extracted and non-extracted yeast and sesamin. Zygomycete fungi meal inclusion decreased fish lipid content, thereby increasing DHA by 54% of total lipid content. Metabolomics analyses revealed an effect on several metabolites depending on the diet. Inclusion of sesamin increased the levels of some metabolites associated with energy metabolism, suggesting metabolic disturbance in the fish. Replacement of FM with non-extracted yeast affected liver metabolites, while extracted yeast mainly increased muscle metabolite levels. These increases suggested that the test ingredients produced a significant response in fish muscle metabolism. The altered metabolic fingerprint in fish fed krill and mussel meal diets compared with FM suggested differences in ingredient composition and physiological response to the diets.

These results provide new information on the effects of non-traditional ingredients on fish metabolism, *e.g.* lipid-, single-carbon metabolism and tricarboxylic acid cycle. More studies are needed to identify the biochemical mechanisms involved and commercialise alternative fish feed materials.

Keywords: Atlantic salmon, Arctic charr, sesamin, krill, mussel, microorganism, zygomycete fungi, yeast, fish metabolism, fatty acids, DHA

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Dedication

To my little family – Mathilda and Sven

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 Schiller Vestergren A.*, **Wagner, L.***, Pickova, J., Rosenlund, G., Kamal-Eldin, A. & Trattner, S. (2012). Sesamin modulates gene expression without corresponding effect on fatty acids in Atlantic salmon (*Salmo salar* L.). *Lipids* 47(9), 897-911. *Contributed equally.

II **Wagner, L.**, Trattner, S., Pickova, J., Goméz-Requeni, P. & Moazzami, A.A. (2014). ^{1}H NMR-based metabolomics studies on the effect of sesamin in Atlantic salmon (*Salmo salar*). *Food Chemistry* 147(0), 98-105.

III **Wagner, L.**, Gómez-Requeni, P., Moazzami, A.A., Pickova, J., Vidakovic, A., Langeland, M., Kiessling, A. & Lundh T. (2015). ^{1}H NMR-based metabolomics and lipid profile analysis of muscle and liver of Arctic charr (*Salvelinus alpinus*) fed microbial or mussel meal. (submitted to *British Journal of Nutrition*)

IV **Wagner, L.**, Pan, J., Didone, R., Müllner, E., Brännäs, E., Pickova, J. & Moazzami, A.A. (2015). Fatty acid analysis and ^{1}H NMR-based metabolomics study of Arctic charr (*Salvelinus alpinus*) fed with marine sources. (manuscript)

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

I Participated in planning the lipid analyses and experimental work together with the supervisors. Performed the analytical work, the evaluation and data analyses of the results. Was mainly responsible for preparing the lipid part of the manuscript.

II Participated in planning the metabolomics analyses and experimental work together with the supervisors. Performed the analytical work, the evaluation and data analyses of the results. Interpreted the results together with the supervisors. Prepared the first draft of the manuscript.

III Was mainly responsible for planning the lipid and metabolomics analyses and experimental work. Participated in collecting the samples. Performed the laboratory work, the evaluation and data analyses of the results. Was mainly responsible for writing the first draft of the manuscript.

IV Participated in planning the study and was mainly responsible for planning the lipid and metabolomics analyses and experimental work. Participated in collecting the samples. Was responsible for the analytical part, evaluation and data analysis of the results and prepared the manuscript.

Abbreviations

ALA	Alpha-linolenic acid (18:3n-3)
ANOVA	Analysis of variance
ARA	Arachidonic acid (20:4n-6)
DHA	Docosahexaenoic acid (22:6n-3)
EPA	Eicosapentaenoic acid (20:5n-3)
EY	Extracted yeast (*Saccharomyces cerevisiae*) meal
FA	Fatty acids
FAME	Fatty acid methyl esters
FM	Fish meal
FO	Fish oil
GC	Gas chromatography
KM	Krill meal
LA	Linoleic acid (18:2n-6)
LCPUFA	Long-chain polyunsaturated fatty acids
MM	Mussel meal
MUFA	Monounsaturated fatty acids
n-3	Omega-3
n-6	Omega-6
NMR	Nuclear magnetic resonance spectroscopy
NY	Non-extracted yeast (*S. cerevisiae*) meal
PL	Phospholipids
PUFA	Polyunsaturated fatty acids
SFA	Saturated fatty acid
SGR	Specific growth rate
TAG	Triacylglycerol
TCA	Tricarboxylic acid
TL	Total lipids
TLC	Thin layer chromatography
TMAO	Trimethylamine n-oxide

TSP-d_4	Sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate
TTA	Tetradecylthioacetic acid
VIP	Variable influences in projection
VO	Vegetable oil
ZYG	Zygomycete fungi meal

1 Background

1.1 Aquaculture

The global population is expected to increase from 7 to 9.6 billion people by 2050. Providing fish, which will be a major component of the food supply for this growing population, will be a challenge (FAO, 2014). At present, total fish production in the world is approximately 158 Mt, including capture and aquaculture production (FAO, 2014) (Figure 1). The catch from wild fisheries stabilised in the 1990s at around 90 Mt and is not expected to increase any further, since the stocks are overexploited and some of them even depleted (Turchini *et al.*, 2009). The rest of the fish supply (approx. 68 Mt) comes from aquaculture production.

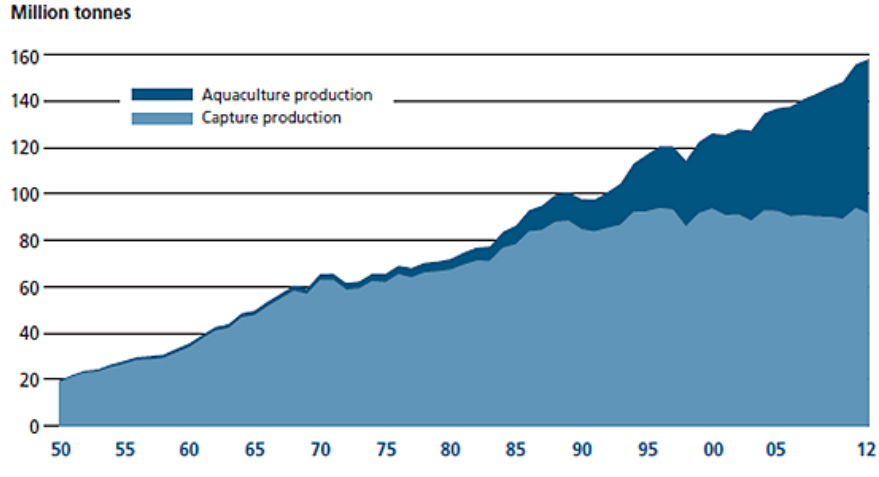

Figure 1. World capture fisheries and aquaculture production (1950-2012) (source: FAO (2014)).

Aquaculture is the fastest growing food industry sector worldwide and, in the coming decade, total production from aquaculture and wild fisheries is

expected to exceed that of beef, pork and poultry. However, a total of 136.2 Mt of fish and fish products is used for human consumption and aquaculture covers roughly half the global demand. The annual expansion in the world fish aquaculture industry from 2000-2012 was 6.2%, which was a slower growth rate than in the preceding decade (9.5% in 1990-2000) (FAO, 2014).

Aquaculture production differs between continents. Asia is currently producing 89% of total aquaculture production, with China being the main producer (62%) and carp the main fish species, while Europe produces 4.3% of total production. Aquaculture production in Norway, the largest producer in Europe, and in Sweden is dominated by salmonids, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), respectively, but production of Arctic charr (*Salvelinus alpinus*) has also increased in Sweden in recent years. Most fish species farmed in European aquaculture are traditionally fed smaller fish, since they are predatory species. Therefore the natural feed ingredients contain fish raw material (fishmeal and fish oil).

1.2 Aquafeed

With the increase in aquaculture production, the requirements for fishmeal (FM) and fish oil (FO), two traditional components and key sources of feedstuff for aquaculture, are also steadily growing. Fishmeal and FO can be produced from whole fish, especially small pelagic fish species, or fish by-products. The main fish species used for production are menhaden (*Brevoortia tyrannus*), sandeel (*Ammodytes tobianus*), blue whiting (*Micromesistius poutassou*), capelin (*Mallotus villosus*), herring (*Clupea harengus*), sardine (*Sardina pilchardus*), mackerel (*Scomber scombrus*). However, a small part of catches of this species are also used for direct human consumption (Wijkström, 2012).

In aquafeeds, FM and FO are generally used at higher levels in feeds for carnivorous species, such as salmon and trout, and at lower levels in feeds for omnivorous species and crustaceans (Hardy, 2010).

The production of FM and FO had been relatively stable over the decades but has shown a decline in recent years, of 4-6 Mt and 0.8-1.0 Mt, respectively, whereas the use has dramatically increased. For example, the proportion of FM used by aquaculture was 73% in 2010, while it was only 33% in the year 2000 (Shepherd & Jackson, 2013). Fishmeal is the main dietary protein source in aquafeeds and typically contains 60-72% protein, 10-20% ash and 5-12% lipids (Shepherd & Jackson, 2013). Fish meal represents the optimal protein source for aquaculture because of its good digestibility and suitable amino acid profile for maximal protein utilisation and growth (Cho & Kim, 2011; Trushenski *et*

al., 2006). During FM production, FO is obtained as a by-product. The proportion of FO used by aquaculture in 2010 was 71%, with estimates showing that salmonid production uses 70% of total FO production (Shepherd & Jackson, 2013). High energy diets for salmonids contribute to better growth performance of the fish by allowing them to exploit the lipids in the feed to the maximum and convert dietary protein into muscle protein (Sargent *et al.*, 2002).

Interest in FO for human consumption is currently increasing (24% of crude oil supply in 2010), mainly to treat hearth diseases, diabetes and other (FAO, 2014; Shepherd & Jackson, 2013).

However, the production of FM and FO for aquafeeds is becoming non-sustainable due to the increasing demand from the growing aquaculture industry and high production costs (Naylor *et al.*, 2009; Tacon & Metian, 2008). Therefore, in the past decade most research efforts have been directed towards obtaining suitable raw materials that can act as alternatives to FM and FO for the aquaculture industry.

1.3 Alternative dietary sources

1.3.1 Animal protein sources

Vegetable protein sources are low in price and the global trade in wheat, coarse grains (maize, barley, sorghum, rye, oats, millet and mixed grains), soybeans and soybean products is increasing (Figure 2).

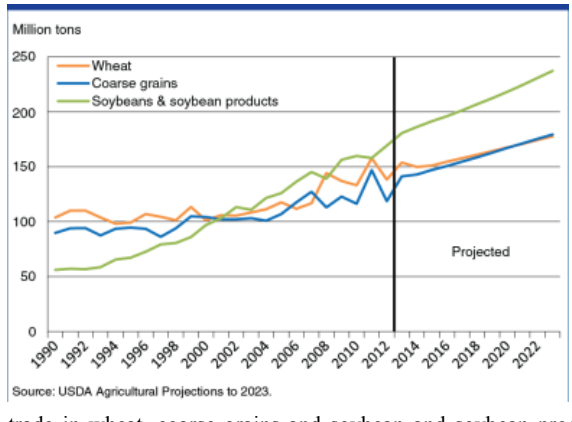

Figure 2. Global trade in wheat, coarse grains and soybean and soybean products, 1990-2022. (USDA Agricultural Projections to 2023).

Therefore, vegetable protein sources have been widely used as alternatives to FM in fish feeds. However, although these plant sources have a suitable protein content, the amino acid profile and non-soluble carbohydrate content are not optimal and the presence of anti-nutritional compounds, which might cause lower feed intake, reduced digestibility and inflammation, has to be taken into consideration (Hardy, 2010). A potential replacement for FM in aquafeeds is aquatic organisms that are further down in the food chain, such as krill or mussels, but microorganisms might also be suitable as an alternative source for aquafeeds.

Antarctic krill (*Euphausia superba*) belongs to the family of euphausiids, of which 85 species have been reported so far (Nicol & Endo, 1999). These marine pelagic crustaceans contain 11.9-15.4% protein and 0.4-3.6% lipids (Grantham, 1977). The lipid content and composition of krill range seasonally and depend on species, sex, maturity stage, age, diet and other biological factors (Kolakowska *et al.*, 1994; Kolakowska, 1991). Marine organisms including krill are rich in omega-3 polyunsaturated fatty acids (n-3 PUFA) such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), due to the presence of marine microalgae in their diet (Kolakowska *et al.*, 1994). The use of Antarctic krill as a potential FM replacer (partial or total) in the diet has been studied for a number of fish species, such as Atlantic salmon (Hansen *et al.*, 2011; Suontama *et al.*, 2007; Olsen *et al.*, 2006; Julshamn *et al.*, 2004), Atlantic halibut (*Hippoglossus hippoglossus*) (Tibbetts *et al.*, 2011; Suontama *et al.*, 2007), yellowtail (*Seriola quinqueradiata*) (Yoshitomi & Nagano, 2012), Atlantic cod (*Gadus morhua*) (Tibbetts *et al.*, 2011) and rainbow trout (Yoshitomi *et al.*, 2007).

Blue mussels (*Mytilus edulis*) are marine bivalve molluscs that feed on phytoplankton. They have a high protein content with a suitable amino acid composition to feed to fish (Langeland *et al.*, 2014). Even though the fat content is low, the lipid composition satisfies the requirements for essential FA (EPA and DHA) (Berge & Austreng, 1989). In addition, blue mussels are easy to cultivate and are able to decrease the concentrations of nitrogen and phosphorus dissolved in seawater by filtering microalgae (Jönsson & Elwinger, 2009). Mussels have two drawbacks, however. First, to obtain a suitable protein meal the bulk of shells have to be removed. Second, they absorb biotoxins, which might pose a risk for their use as a food ingredient for animal and human consumption (Grienke *et al.*, 2014). Nevertheless, it has been reported that some mussels-derived molecules can promote health, including providing protection against cardiovascular disease and inflammation (Grienke *et al.*, 2014). Krill and mussel meals have similarities to FM and can be used as potential replacements.

1.3.2 Microbial protein sources

Microorganisms such as yeast and other fungi, bacteria and microalgae can become a suitable source of protein, vitamins, minerals, lipids and carbohydrates (Kuhad *et al.*, 1997) and might be candidates for use in aquafeeds. All types of microbial protein products contain high concentrations of nucleic acids, which can elevate the plasma level of uric acid and result in crystals of urate forming in tissue, causing kidney stones and gout in mammals (Kuhad *et al.*, 1997). In fish, including salmonids, high levels of nucleic acid are metabolised by their highly active liver uricase (Rumsey *et al.*, 1992). However, the present low-scale manufacturing and high production costs of microbial protein products limit their use in commercial aquafeeds (Kuhad *et al.*, 1997).

The zygomycete fungi *Rhizopus oryzea* is a filamentous microfungus which is cultivated on *e.g.* paper pulp wastewater fractions such as spent sulphite liquor. It is rich in protein (40-50% depending on harvesting, dewatering and drying method), with lipid, vitamin and amino acid concentrations similar to those in FM (Abro *et al.*, 2014; Edebo, 2008; Mydland *et al.*, 2007). Thus, zygomycete species have been tested as a potential FM replacer in feed for salmonids (Abro *et al.*, 2014; Bankefors *et al.*, 2011; Mydland *et al.*, 2007).

Another alternative protein source in aquafeeds could be baker's yeast (*Saccharomyces cerevisiae*). The protein content of baker's yeast is similar to that of FM (40-65%) (Nasseri *et al.*, 2011; Kuhad *et al.*, 1997). Essential amino acids are present in yeast, although the levels of sulphur-containing amino acids, such as methionine and cysteine, are lower and this can cause an amino acid imbalance in yeast-containing fish diets compared with FM (Nasseri *et al.*, 2011; Kuhad *et al.*, 1997). The inclusion of yeast as a protein source in the diets has been studied for a number of fish species, *e.g.* Atlantic salmon (Øverland *et al.*, 2013), sea bass (*Dicentrarchus labrax*) juveniles (Oliva-Teles & Goncalves, 2001), koi carp (*Cyprinus carpio*) fingerlings (Korkmaz & Cakirogullari, 2011), Nile tilapia (*Oreochromis niloticus*) fingerlings (Ebrahim & Abou-Seif, 2008), lake trout (*Salvelinus namuycush*) (Rumsey *et al.*, 1990) and rainbow trout (Rumsey *et al.*, 1991).

1.3.3 Vegetable oil sources

The production of vegetable oils (VO), such as palm, soybean, rapeseed, sunflower and peanut oil, has been increasing in recent decades (Figure 3) (Schmidt, 2015). The lower production costs, higher availability and sustainability of VO, compared with FO, make the use of VO as a feed ingredient in fish diets very attractive.

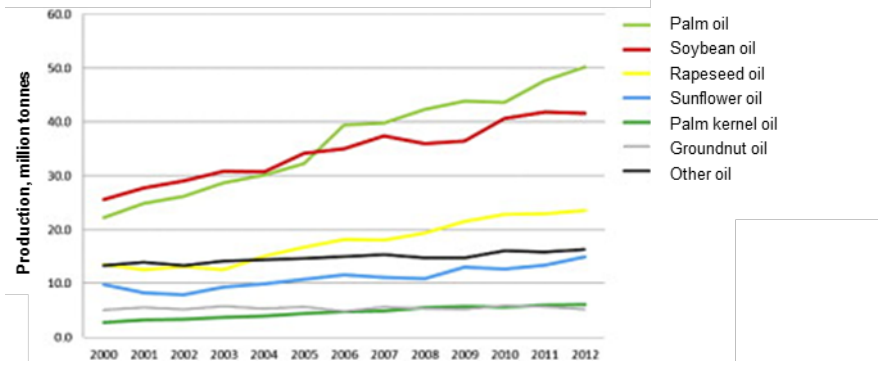

Figure 3. World production of vegetable oils (source: FAOSTAT (2013)).

Previous studies have found that using VO instead of FO in the diet of salmonids does not affect growth and taste, although the fish may have less rancid and marine characteristics (Torstensen *et al.*, 2005; Bell *et al.*, 2003; Caballero *et al.*, 2002; Bell *et al.*, 2001). Vegetable oils generally have higher levels of n-6 and n-9, mainly linoleic acid (LA, 18:2n-6) and oleic acid (18:1n-9), and lower levels of n-3 (except linseed oil) such as alpha-linolenic acid (ALA, 18:3n-3) (Regost *et al.*, 2004). Depending on the species, fish have the capacity to convert (elongate and desaturate) shorter polyunsaturated 18-carbon essential FA, such as ALA and LA, to long-chain polyunsaturated fatty acids (LCPUFA), including EPA, DHA and arachidonic acid (ARA, 20:4n-6). However, marine carnivorous species (containing in general higher n-3 LCPUFA levels) have a limited ability to produce EPA and DHA *in vivo* to support growth and fish health (Turchini *et al.*, 2009). Salmonids are able to synthesise ALA to n-3 LCPUFA. Therefore, to find the most suitable alternative to maintain the health benefits from salmon in the human diet, it is important to use VO that are: i) rich in saturated (SFA) and monounsaturated FA (MUFA) as an energy source for maintenance of fish growth and protein saving; ii) generally high in ALA, to maximise the conversion to EPA and DHA; and iii) low in LA, because it is poorly beta-oxidised by the fish metabolism and difficult to remove even when a finishing diet is used (Turchini *et al.*, 2009; Bell *et al.*, 2003). Therefore, linseed (high in ALA), rapeseed (high in MUFA) and palm oil (high in SFA) might be suitable FO replacers in combination. However, a natural drawback for consumers is that VO lack LCPUFA, *e.g.* EPA and DHA, which can therefore decrease the amount of these FA in the fish muscle (Torstensen *et al.*, 2005; Sargent *et al.*, 2002; Bell *et al.*, 2001). As a consequence of this VO replacement the n-3/n-6 ratio will decrease in fish tissue. This change will cause a higher n-6/n-3 dietary ratio in human diet (Simopoulos, 2002). Several studies suggest that

use of FO in the finishing diet could be a strategy to maintain a high n-3 LCPUFA content in farmed fish muscle, even though it requires an extended feeding time and higher production costs (Mraz *et al.*, 2012; Bell *et al.*, 2003; Robin *et al.*, 2003).

1.3.4 Studies on bioactive effects of sesamin

To counteract the negative effects when using VO in fish diets, several studies have examined the effects of improving conversion of ALA to EPA and DHA by adding a lipid modulator, such as bioactive compounds, to the fish feed (Mraz *et al.*, 2010; Trattner *et al.*, 2008a; Trattner *et al.*, 2008b).

Bioactive compounds are defined as naturally occurring non-nutritive compounds that typically occur in small quantities in plant products and lipid-rich food (Kitts, 1994). Numerous bioactive compounds appear to have beneficial effects on human health, *e.g*. by acting as antioxidants and anti-carcinogens, lowering blood pressure, exerting protective effects against liver damage by alcohol and decreasing the occurrence of cardiovascular disease, coronary heart diseases and stroke (Kris-Etherton *et al.*, 2002). Minor bioactive compounds, such as the lignin sesamin, have also been shown to affect lipid metabolism and/or FA composition in rodents (Ide *et al.*, 2001; Ashakumary *et al.*, 1999), humans (Hirata *et al.*, 1996) and fish (Alhazzaa *et al.*, 2012; Trattner *et al.*, 2008a).

Sesamin (Figure 4) is a minor bioactive lipid compound commonly found in refined sesame seed oil. The effect of sesamin on lipid metabolism in mammals is well documented. It has been shown to increase beta-oxidation in rodents (Ide *et al.*, 2009b; Ashakumary *et al.*, 1999) and has an effect on several lipid-related genes, such as *Δ*-5 and *Δ*-6 desaturase. In rats, sesamin inhibits *in vivo* the *Δ*-5 desaturase activity of n-6 FA, but not that of n-3 FA (Fujiyama-Fujiwara *et al.*, 1995) and it affects cholesterol levels (Kamal-Eldin *et al.*, 2000). In salmonids, this bioactive compound increases the DHA level of white muscle in the freshwater stage (Trattner *et al.*, 2008a).

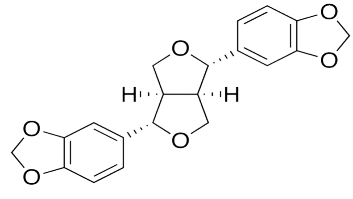

Figure 4. Chemical structure of sesamin.

1.4 Lipids and lipid metabolism in fish

Lipids are a collection of chemically differently structured molecules that possess the characteristic of being soluble in organic solvents, but not in water. The biological functions of lipids include storing energy, signalling, acting as structural components of cell membranes and being a carrier for lipid-soluble micronutrients. Lipids can be classified as acyl residue or neutral-polar, with the latter being more commonly used. Neutral lipids such as FA, monoacylglycerols, diacylglycerols, triacylglycerols (TAG), wax esters and sterols function as energy stores. In the form of TAG, they contain more than twice the amount of energy for the same mass as carbohydrates or protein. Triacylglycerols have a backbone consisting of glycerol molecules, where three molecules of FA are esterified to hydroxyl groups of glycerol in the *sn*-1, *sn*-2, and *sn*-3 positions (Sargent *et al.*, 2002) (Figure 5). The *sn*-2 position of the storage TAG molecule is considered to be the most bioavailable position for the digestion of FA (Miller *et al.*, 2008). In addition, the dietary FA composition can influence TAG more than phospholipids (PL) (Olsen & Henderson, 1997). Phospholipids belong to the group of polar lipids and have a common backbone of phosphatidic acid, which is esterified to choline, ethanolamine, serine or inositol to form phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine or phosphatidylinositol, respectively (Olsen & Henderson, 1989) (Figure 5). Phospholipids are important compounds for cell membrane structures. The composition of the molecular species can be influenced by *e.g.* diet (to a lesser extent than TAG) and has a major effect on the health of the cell and, consequently, on fish health (Bell *et al.*, 1986).

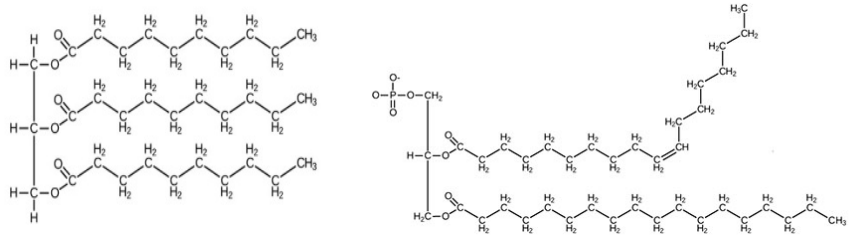

Figure 5. Chemical structure of (left) triacylglycerol (TAG) and (right) phospholipid (PL).

1.4.1 Fatty acids, triacylglycerol and phospholipids in fish

Fatty acids consist of a carbon chain with an aliphatic carboxylic acid at one end and a methyl group at the other. The carbon chain is usually unbranched and has an even number of carbon atoms. They can be classified into different groups on the basis of their chain length, number and position and the

configuration (cis- or trans-) of their double bonds. Fatty acids without a double bond are commonly called saturated FA (SFA), those with one double bond monounsaturated FA (MUFA) and those with more than one double bonds polyunsaturated FA (PUFA). The position of the first double bond, counting from the methyl end of the carbon chain, in PUFA is another important characteristic. If the first double bond is on the third carbon the FA is called n-3 FA, while if it is on the sixth carbon it is called n-6 FA (Figure 6).

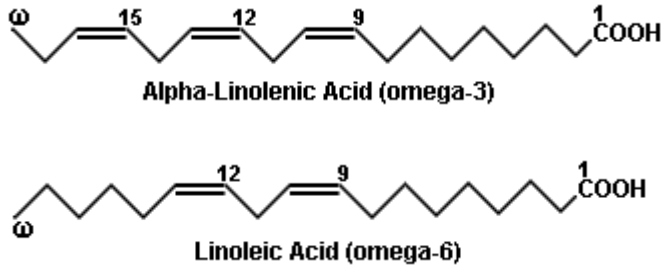

Figure 6. Chemical structure of the essential fatty acids: (above) alpha-linolenic acid (ALA, 18:3n-3) and (below) linoleic acid (LA, 18:2n-6).

Saturated FA and MUFA can be found naturally in fish, with a chain length typically from C_{14} to C_{24}. The SFA levels are similar in TAG and PL, while PL has lower proportions of MUFA compared with TAG. Phospholipids mainly contain C_{16}, C_{18} and, to a lesser extent, C_{20}, whereas in TAG 18:1n-9 is the major FA, followed by 16:0 and 16:1n-7. In fish feeding on zooplankton (rich in 20:1n-9 and 22:1n-11 in the Atlantic Ocean region), this is reflected in abundant levels of those FA in the TAG fraction of MUFA (Sargent *et al.*, 2002). Compared with TAG, PL contain a much higher proportion of PUFA. The C_{18} PUFA in PL is dominated by ALA and LA, while the proportion of LA is greater than ALA. In addition, the major PUFA in PL is DHA, followed by EPA and ARA, while the relative levels vary with the species. The FA pattern, especially PUFA, reflects that of dietary lipid and therefore the ratio of n-3/n-6 PUFA observed in tissue PL is influenced by the amount of n-3 or n-6 PUFA in the diet (Henderson & Tocher, 1987).

1.4.2 Biosynthesis, elongation and desaturation

Fatty acids are created in the cytosol of the liver from the precursor acetyl-CoA through the action of a multi-enzyme complex called fatty acid synthases. In all organisms, including fish, SFA can be biosynthesised *de novo.* Even-number SFA, especially in higher vertebrates, can form monounsaturated FA, *e.g.* 18:1n-9, through the activity of Δ-9 desaturase (Sargent *et al.*, 2002). However,

PUFA cannot be synthesised *de novo* from 18:1n-9 by any vertebrate species due to lack of desaturase enzymes (Zheng *et al.*, 2004; Tocher *et al.*, 1998). Therefore, LA and ALA are essential and need to be included in the diet. Once obtained from the diet, many vertebrates can elongate and desaturate LA and ALA into LCPUFA, such as ARA, EPA and DHA (Figure 7).

Synthesis of EPA is achieved by Δ-6 desaturation from ALA to produce 18:4n-3, which is elongated to 20:4n-3 followed by Δ-5 desaturation. To produce DHA, another two elongation steps, a second Δ-6 desaturation and a chain-shortening step, are required. Synthesis of ARA from LA uses the same enzymes and pathways as for EPA (Morais *et al.*, 2009; Zheng *et al.*, 2004).

It has recently been demonstrated that DHA can be produced directly by Δ-4 desaturation of 22:5n-3 in rabbitfish (*Siganus canaliculatus*) (Li *et al.*, 2010). Furthermore, in some marine fish species, the Δ-6 enzyme has Δ-8 activity and so the first two steps can be reversed in order (Monroig *et al.*, 2011).

The synthesis of LCPUFA occurs in the microsomal fraction of the liver except for the chain-shortening step from 24:6n-3 to DHA, which occurs in the peroxisomes by β-oxidation. The affinity of the enzymes is higher for n-3 series than n-6 series.

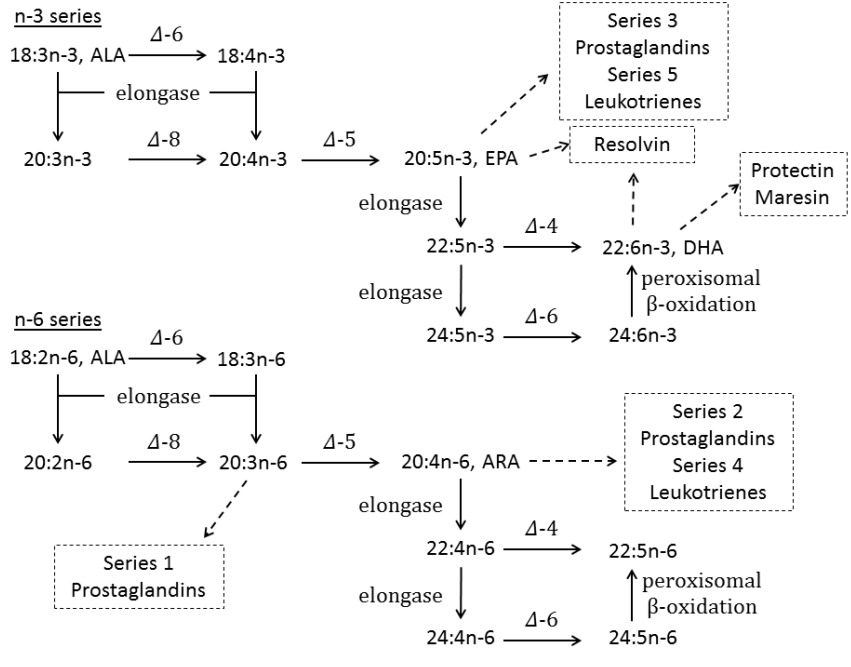

Figure 7. Elongation and desaturation pathway of n-3 and n-6 fatty acids (adapted from Voss *et al.* (1991), Sargent *et al.* (1999), Morais *et al.* (2009), Monroig *et al.* (2011) and reviewed by Calder (2015)).

However, freshwater fish and salmonids are capable of synthesising DHA from ALA, since they possess the elongase and desaturase enzymes necessary for the biosynthesis. In such species, ALA and LA are essential FA in the diets to obtain high conversion to LCPUFA. In other species, including marine fish, the *de novo* FA biosynthetic ability is limited or even zero, due to deficiencies in one or more steps of the pathway. Many species have essential requirements for EPA, DHA and ARA (Sargent *et al.*, 1999).

1.4.3 Physiological role of n-3 and n-6

The three LCPUFA ARA, EPA and DHA have several important functions in fish. The energy obtained from FA is important for growth and development, including reproduction of the fish (Sargent *et al.*, 2002). An important role of ARA, EPA and DHA is *e.g.* maintenance of cell membrane structure and function. However, as described earlier, fish cell membranes generally contain higher levels of EPA and DHA. It has been claimed that the high proportion of DHA in fish tissue is to maintain membrane fluidity at low temperature, and it is also suggested that the fluidity depends on the ratio of MUFA (increased) and SFA (decreased) in response to lower temperature (Sargent *et al.*, 2002). The third main role for C_{20} PUFA, especially ARA and to some extent EPA, is that they act as precursors of eicosanoids (Figure 7) in fish. Eicosanoids, including prostaglandins, leukotrienes, thromboxanes and prostacyclins, are highly biologically active compounds and are produced in virtually every tissue in the body. They are known for being involved in several physiological actions, such as in cardiovascular functions including blood clotting and in the inflammatory and immunological response *etc*. (Sargent *et al.*, 1999). Furthermore, EPA and DHA give rise to anti-inflammatory and inflammation-resolving mediators called resolvins, protectins and maresins as reviewed by Calder (2015) (Figure 7).

1.5 n-3 and n-6 FA in human health

The n-3 LCPUFA, especially EPA and DHA, have been shown to have beneficial effects for human health. The positive effects were first observed in Greenland Eskimos consuming a high seafood diet (Kromann & Green, 1980). They had low rates of coronary heart diseases, asthma, multiple sclerosis and other (Simopoulos, 2008). Over time, more studies have been conducted regarding the health effects of LCPUFA and have shown that these n-3 LCPUFA are important for the prevention of cancer, cardiovascular disease and brain development, and have anti-inflammatory properties (Calder, 2014).

To prevent several diseases, the European Food Safety Authority (EFSA) recommends an intake of 1-2 fatty fish meals per week or 250 mg EPA+DHA per day, with an additional 100-200 mg DHA/day during pregnancy and lactation (EFSA Panel on Dietetic Products, 2010). Other associations have similar recommendations.

A healthy diet includes n-6 and n-3 PUFA in a suitable ratio (Simopoulos, 2002). Wild plants, animals and fish were the main foods for our hunter-gather ancestors and their diet was thus low in total fat and SFA, but high in PUFA, with an equal amount of n-6 to n-3 (ratio thought to have been 1:1) (Figure 8). Over time and with the beginning of industrialisation, the food supply for humans has changed and with it the ratio of n-6 to n-3 FA. It has been estimated that 'Western' diets have an n-6 to n-3 FA ratio greater than 15:1 (Simopoulos, 2008). The higher levels of n-6 FA found in the modern diet are due to the development of the modern vegetable oil industry and increased consumption of cereal grains (Kris-Etherton *et al.*, 2000).

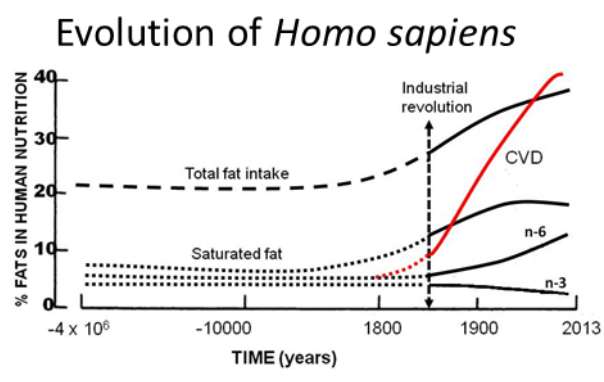

Figure 8. Changes in the composition and representation of fat in human nutrition during evolution in relation to the occurrence of cardiovascular diseases (CVD) (adapted from Leaf and Weber (1987)).

Simultaneously with the increase in the n-6 to n-3 FA ratio, an increase in chronic inflammatory diseases such as non-alcoholic fatty liver disease, cardiovascular disease, obesity, inflammatory bowel disease, rheumatoid arthritis and Alzheimer's disease has been observed (Patterson *et al.*, 2012). Therefore, the Japan Society for Lipid Nutrition and the World Health Organization recommend a n-6 to n-3 FA ratio of 4:1 and 3:1, respectively (Horrocks & Yeo, 1999).

1.6 Metabolomics

Metabolomics is defined as the comprehensive analysis of small metabolites in an organism, tissue or biofluid under a given set of conditions (Goodacre *et al.*, 2004). With this promising and valuable high-throughput tool, it is possible to identify and quantify small organic molecules with a molecular weight of <1.5 kDa, including peptides, sugars, nucleotides, amino acids and lipids, that are detectable in the body (Wishart, 2008).

1.6.1 Metabolomics technologies

Metabolomics strategies aim to detect a broad spectrum of molecules with diverse properties and analyse simultaneously several metabolites in a single spectrum. Therefore, the technique must be sensitive, robust and have the capacity for high-throughput analysis in order to screen a large number of samples (Whitfield *et al.*, 2004). The major techniques that fulfil the requirements are mass spectroscopy (MS) and nuclear magnetic resonance spectroscopy (NMR). The use of NMR for the analysis of biological samples has significant advantages for metabolomics applications, *e.g.* it requires no or little sample preparation and it is highly quantitative and reproducible, which is important for multivariate data analyses. However, there are also certain drawbacks, *e.g.* NMR is not as sensitive as MS and requires a relatively large sample volume (Pan & Raftery, 2007; Whitfield *et al.*, 2004).

1.6.2 Application of metabolomics

The objective with metabolomics is to identify potential small molecule biomarkers that correlate to a disease, drug toxicity, or genetic or environmental variation. The application of metabolomics is constantly progressing.

One important area in which metabolomics is making a significant contribution is in clinical applications such as clinical toxicology and chemistry (Wishart, 2008). A number of diseases, *e.g.* atherosclerosis, type 2 diabetes mellitus and cancer, may result from a chronic imbalance in the normal metabolism. Therefore, to detect new biomarkers of diseases, metabolomics strategies are applied in clinical research. Validated biomarkers are important for diagnostic procedures, prognostic evaluation and monitoring of treatments (Whitfield *et al.*, 2004).

Another major application of metabolomics is in pharmaceutical research such as drug discovery and especially toxicology. It is of high interest to determine at an early stage the disturbances in metabolite populations of tissues and body fluids after giving various chemicals and drugs to animals (Whitfield *et al.*, 2004).

Metabolomics can be also used to study the function of genes in plants, yeasts and experimental animals. With this metabolite profiling technique, it is possible to obtain important information on unexpected metabolic changes by biotic and abiotic factors, but also to characterise the phenotype of specific genes (Robertson, 2005; Whitfield *et al.*, 2004).

Metabolomics applied in nutrition and food has received increased interest over the past few years. It makes it possible to develop a more detailed and comprehensive molecular picture of food composition, food quality, food consumption and molecular biological changes of different diets (Wishart, 2008). A high number of NMR-based metabolomics analyses have been conducted with natural food, spices and beverages, such as milk (Sundekilde *et al.*, 2013), tomato juice (Tiziani *et al.*, 2006), coffee (Kwon *et al.*, 2015), cheese (Piras *et al.*, 2013), chicken (Ruiz-Aracama *et al.*, 2012), *etc*.

1.6.3 Use of metabolomics in fish studies

Use of the NMR-based metabolomics approach is also important in aquatic ecosystems, *e.g*. for exploring the effects of pharmaceutical and other chemicals on fish, but also to improve knowledge about the basic physiology of fish, diseases and other issues (Asakura *et al.*, 2014; Dove *et al.*, 2012; Picone *et al.*, 2011; Aursand *et al.*, 2007).

The ^{1}H NMR technology is a well-established technique for exploring metabolic changes in fish liver (Abro *et al.*, 2014; Bankefors *et al.*, 2011; Kullgren *et al.*, 2010), white muscle (Bankefors *et al.*, 2011; Kullgren *et al.*, 2010; Gribbestad *et al.*, 2005) and plasma (Kokushi *et al.*, 2012; Kullgren *et al.*, 2010), in order to study the response to different diets. For example, analyses of white muscle can give insights into changes in muscle metabolism and, more importantly, can indicate possible effects on the final flesh quality of the fish depending on the diet. The liver is the key organ in connection with biosynthesis and metabolism, while plasma analysis can give insights into small intermediary metabolites. Analysis of extracts of white muscle and liver can provide information regarding FA distribution (lipid phase = non polar) and small metabolites (aqueous phase = polar).

The metabolites detected can give insights into *e.g*. changes in energy metabolism (alanine, valine, isoleucine, glucose, glycogen), homeostasis (trimethylamine n-oxide, betaine, taurine), protein breakdown and single carbon metabolism (betaine, n,n-dimethylglycine, sarcosine).

2 Objectives

The overall aim of this thesis was to investigate the effects of alternative plant, microbial and marine feed compounds on the lipid profile and metabolic footprint in Atlantic salmon and Arctic charr. This was accomplished by performing *in vivo* studies with fish fed different diets and sampling liver and white muscle tissues and plasma for lipid analyses (lipid content, fatty acid composition, lipid classes) and metabolomics analyses (aqueous and chloroform phase).

Specific objectives were to:

- Study the effect of sesamin in diets with addition of vegetable oils with different n-6/n-3 FA ratio on the fatty acid composition of Atlantic salmon (Paper I).
- Explore changes in the liver and white muscle metabolic profile of Atlantic salmon fed different levels of sesamin in vegetable oil-based diets with varying n-6/n-3 FA ratio (Paper II).
- Determine the effect of alternative dietary sources (zygomycete fungi, yeast, mussel and krill) in aquafeeds on the lipid profile of liver and white muscle tissues of Arctic charr (Papers III, IV).
- Study the liver, white muscle tissue and plasma metabolome of Arctic charr fed an alternative microbial and marine dietary source (zygomycete fungi, yeast, mussel and krill) using a metabolomics approach (Papers III, IV).

3 Materials and methods

This chapter provides a short description of the material and methods used in the studies included in the thesis. For a more detailed description of each method, see Papers I-IV. A summary of study design is provided in Table 1.

Table 1. *Summary of study design for Papers I-IV*

Study	I	II	III	IV
Species	Atlantic salmon	Atlantic salmon	Arctic charr	Arctic charr
Initial size (g)[a]	104.6 ± 9.9	104.6 ± 9.9	48.1 ± 0.32	103.7 ± 2.68
Sample size (g)[a]	553.6 ± 88.1	546.5 ± 86.0	124.4 ± 1.01	256.9 ± 13.0
Trial length	4 months	4 months	14 weeks	15 weeks
Treatment	Sesamin	Sesamin	MM	MM
	S0-0 g/kg feed	S0-0 g/kg feed	EY	FKM
	SL-1.16 g/kg feed	SL-1.16 g/kg feed	NY	
	SH-5.8 g/kg feed	SH-5.8 g/kg feed	ZYG	
Vegetable oil	Mixture of rapeseed, linseed and palm oil	Mixture of rapeseed, linseed and palm oil	-	Rapeseed oil
	V0.5-n-6/n-3=0.5	V0.5-n-6/n-3=0.5		
	V1-n-6/n-3=1	V1-n-6/n-3=1		
Sample (n/group)	Liver (8)	Liver(6)	Liver (9)	Liver (8)
	White muscle (6)	White muscle (6)	White muscle (9)	White muscle (8)
				Plasma (8)
Analyses	Lipid content	Metabolomics	Lipid content	Lipid content[b]
	Fatty acids		Fatty acids	Fatty acids[b]
			Lipid classes[b]	Lipid classes[b]
			Metabolomics	Metabolomics

Abbreviations: MM – mussel meal, EY – extracted yeast (*Saccharomyces cerevisiae*) meal, NY – non-extracted yeast meal, Zyg – zygomycete fungi meal; FKM – mixture of krill and fish meal.
[a]Mean value ± SD for Papers I, II and IV, mean value ± SEM for Paper III.
[b]Lipid analyses performed only in liver.

3.1 Study design

In Papers I and II, Atlantic salmon (*Salmo salar*) with an initial body weight of 105 g were individually tagged and distributed in groups of 35 fish into seven tanks (500 L, provided with flow-through seawater at 12 °C). The fish were fed six experimental diets based on rapeseed, linseed and palm oil, with a FO diet as the control. Each diet was formulated to give an n-6/n-3 FA ratio of either 0.5 (V0.5) or 1 (V1). The different ratios were combined with three different levels of sesamin; high dose (SH) = 5.8 g/kg feed, low dose (SL) = 1.16 g/kg feed and a control without sesamin (S0) = 0 g/kg feed (Figure 9).

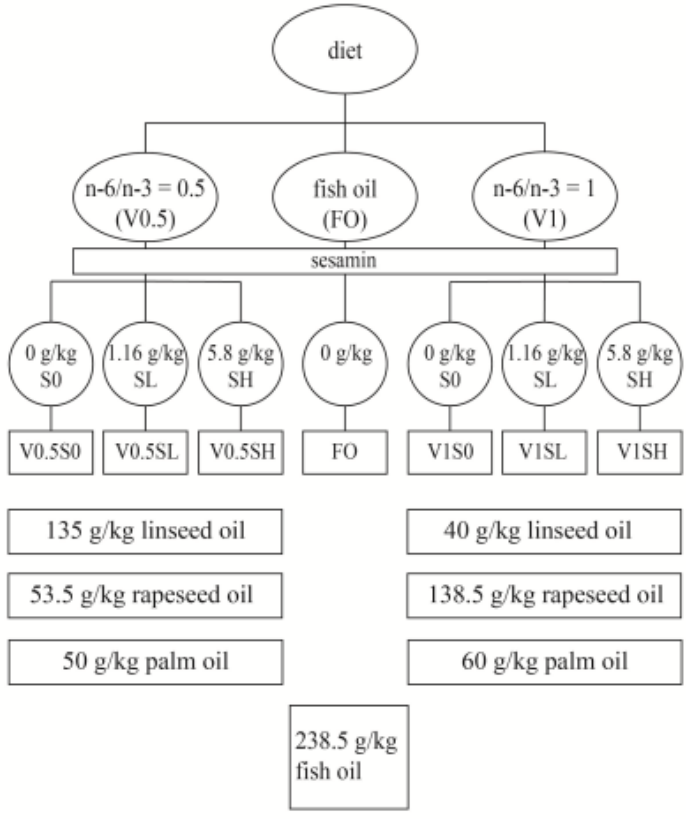

Figure 9. Schematic presentation of diet codes, vegetable oil (VO)/fish oil (FO) and sesamin (S) level in the experimental diets.

The experimental diets were fed to the fish *ad libitum* twice per day for 4 months (November-March). At harvest, the fish had an average final body weight of 554 g (Paper I) or 547 g (Paper II). In Paper II the FO group was excluded from the analyses, hence the lower final body weight.

In Paper III, Arctic charr (*Salvelinus alpinus*) with an initial body weight of 48 g were individually tagged and distributed in triplicate groups of 50 fish into 15 experimental tanks (700 L, provided with flow-through freshwater at 7 °C). The fish were fed four experimental diets and one reference diet (REF) containing FM as the main protein source. In the experimental diets, 40% of the crude protein content of FM was replaced with one of four alternative protein sources: blue mussel (MM), non-extracted yeast (*Saccharomyces cerevisiae*, NY), extracted yeast (EY) and zygomycete fungi (ZYG). The fish were fed for 14 weeks (March-June) at a growth rate of 1% BW/day using automatic feeders. At the end of the feeding trial, the fish had an average final body weight of 124 g.

In Paper IV, 12 juvenile Arctic charr (initial body weight 104 g) were distributed into three experimental tanks (700 L, provided with flow-through freshwater at 11 °C). The fish were fed a FM diet as control and two experimental diets containing either mussel meal (MM) or a mixture of FM and krill meal (KM) as protein source. Rapeseed oil was used in all three diets as the oil source. The diets were fed to the fish *ad libitum* for 15 weeks (May-August) using band feeders mounted on the tanks. At harvest, the fish had an average final body weight of 257 g.

3.2 Sample collection

For collection of tissues (n=8 in Papers I, II and IV, n=9 in Paper III) and plasma (n=8 Paper IV) for analyses, the fish were anaesthetised with ethylene glycol monophenyl ether (5 mL/L in Papers I and II) or tricaine methane sulphonate (100mg/L in Paper III, 30 mL/L in Paper IV) and euthanised with a blow to the head (Papers I, II and IV) or killed by cutting the spinal cord (Paper III). Liver and white muscle tissue were dissected from each fish, frozen in liquid nitrogen and stored at -80°C for lipid analyses (Papers I, III and IV) and metabolomics analyses (Papers II, III and IV). Blood samples were taken from caudal vessels with heparinised syringes. Plasma was obtained after centrifugation (12000*g*, 12 min, 4 °C) and stored at -80 °C until metabolomics analyses (Paper IV).

3.3 Lipid analyses

3.3.1 Lipid extraction and fatty acid analyses

Total lipids (Papers I, III, IV) from diets, liver and white muscle were homogenised and extracted in hexane:isopropanol (3:2, v/v) according to the method of Hara and Radin (1978). The lipid content was determined after

drying and weighing the lipids (Table 2). Total lipids in liver and white muscle were separated by thin-layer chromatography (TLC) into PL and TAG following the method of Pickova *et al.* (1997). Fatty acid methyl esters (FAME) from the diets, total lipids, PL and TAG were prepared with BF_3 from liver and white muscle (Appelqvist, 1968) (Table 2) and analysed by gas chromatography (GC) using a CP 3800 instrument equipped with flame ionisation detector and split injector and fitted with a fused silica capillary column BPX 70 (SGE, Austin, Texas, USA), with length 50 m, id 0.22 mm and 0.25 μm film thickness (Fredriksson Eriksson & Pickova, 2007). The peaks were identified by comparison with the standard GLC-68A (Nu-check Prep, Elysian, USA). Peak areas were integrated using Galaxie chromatography software version 1.9 (Varian AB, Stockholm, Sweden). Fatty acids were quantified using an internal standard (C17:1; methyl-15-methylheptadecanoate, Larodan Fine Chemicals AB, Malmö, Sweden).

Table 2. *Summary of different lipid analyses employed in Papers I, III and IV*

Study	I		III		IV
	Liver	Muscle	Liver	Muscle	Liver
Lipid content	x	x	x	x	x
FA composition					
Total lipids			x	x	x
TAG	x	x			x
PL	x	x			x
Lipid classes			x		x

3.3.2 Lipid class composition of total lipids

Lipid class composition (Table 2) was analysed according to Mraz and Pickova (2009) with minor modifications. Extracted lipid samples were applied on pre-coated silica gel 60 HPTLC plates (20 cm x 10 cm, 0.2 mm layer; Merck, Darmstadt, Germany) using a Camag ATS 4 automatic TLC sampler. The lipid classes were separated using a Camag ADC 2 developing chamber with mobile solvent hexane-diethylether-acetic acid (85:15:2, v:v:v). Phosphomolybdenic acid/ethanol was used for derivatisation. After drying the plates in the oven, they were scanned with a detection scanner (Camag TLC scanner 3) at a wavelength of 650 nm. The lipid classes were identified by comparison with an external standard (TLC 18-5A; Nu-check Prep, Elysian, Minnesota, USA), integrated with Wincats software package (Camag, Switzerland) and expressed as percentage of height.

3.4 ^{1}H NMR-based metabolomics analyses

The ^{1}H NMR analyses of liver and white muscle samples (Papers II, III and IV) were performed according to Moazzami *et al.* (2011) and those of plasma samples (Paper IV) according to Tiziani *et al.* (2008), with small modifications. In Paper II, the aqueous supernatant from the liver was collected, mixed with 600 µL sodium phosphate buffer (0.25M, pH=7.0), dried and afterwards reconstituted by adding 595 µL D_2O and 5 µL sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate in D_2O (TSP-d_4, 23.2 mmol/L, Cambridge Isotope Laboratories, Andover, MA, USA). The aqueous supernatant of white muscle was collected, dried and then re-dissolved in 240 µL Millipore water, 280 µL phosphate buffer (0.25M, pH=7.0), 50 µL D_2O and 30 µL TSP-d_4 as internal standard.

In Papers III and IV, the dried supernatant of the aqueous phase was re-dissolved in 280 µL phosphate buffer (0.25M, pH=7.0) and 240 µL Millipore water. Nanosep® centrifugal filters with 3kDa cutoff (Pall Life Science, Port Washington, NY, USA) were used to filter the samples. Phosphate buffer (0.25M, pH=7.0), D_2O (50 µL), TSP-d_4 (30 µL, 23.2 mmol/L) and Millipore water were added to bring the samples to a final volume of 600 µL and they were then analysed by NMR.

The chloroform supernatant was dried and re-dissolved in 580 µL $CDCl_3$ (99.96 atom-% D) and 20 µL CD_3OD (99.8 atom-% D) as internal standard (Papers II and IV) or 600 µL $CDCl_3$ (Paper III) and analysed by NMR.

The plasma samples were filtered (Nanosep® centrifugal filters) and mixed with 50 µL phosphate buffer (0.25M, pH=7.0), 15 µL D_2O, 15 µL Millipore water and 10 µL TSP-d_4 as internal standard to give a total volume of 170 µL.

The NMR spectra of liver and white muscle were determined in 5-mm outer diameter NMR tubes and the spectra of plasma in 3-mm outer diameter NMR tubes using a Bruker 600MHz (Karlsruhe, Germany), with the exception of liver samples from Paper II, for which a 400 MHz spectrometer was used (Table 3). The NMR spectra were measured using different temperatures, scans, data points, spectral width, acquisition time and relaxation delay depending on the different extracts (water and chloroform phase from liver and muscle) and plasma of the different studies. For details, see Table 3.

Table 3. *Summary of NMR settings used for liver (L), muscle (M) and plasma analyses in the two different phases (water or chloroform) in Papers II, III and IV*

Study	I			III				IV				
Phase	Water		Chloro-form	Water		Chloro-form		Water		Chloro-form		
Sample	L	M	L	L	M	L	M	L	M	L	M	Plasma
NMR (MHz)	400	600	400	600				600				
Temp. (°C)	25			25		20		25		20		25
Scans	400	128	180	128				128				512
Data points	32,768	65,536		65,536				65,536				
Spectral width (Hz)	8012.82	17,942	8223.68	17,942		12,019		17,942		12,019		17,942
Acquisition time (s)	2	1.83	4	1.83		2.72		1.83		2.72		1.83
Relaxation delay (s)	3	4	3	4		3		4		3		4

The data were processed using Bruker Topspin 2.0 software and Fourier-transformed after multiplication by line broadening of 0.3 Hz. The data were subsequently referenced to standard peak TSP-d_4 at 0.0 ppm in the water phase and to methyl signals at 3.31 ppm (Papers II and IV) or to the chloroform peak at 7.24 ppm (Paper III) in the chloroform phase. The ^{1}H NMR signals were identified using the NMR Suite 7.5 library (ChenomX Inc, Edmonton, AB, Canada), the Human Metabolome Database (www.hmdb.ca), and previous literature (Bankefors *et al.*, 2011; Moazzami *et al.*, 2011; Gribbestad *et al.*, 2005). For the quantitative analyses (profiling approach), the metabolites were identified by overlapping with standard spectra and their concentrations (µmol/g tissue) were calculated using the NMR Suite 7.5 library after accounting for overlapping signals.

3.5 Statistical analyses

The data were processed using the data analysis software SAS (Statistical Analysis System; SAS Institute Inc., Cary, NC, USA, version 9.2 in Papers I and II, version 9.3 in Papers III and IV). The data were checked for normal distribution (Anderson-Darling test), followed by a test of equal variance (Barlett or Levene test). If not normally distributed, they were log transformed and re-tested. In addition, for the metabolomics data a Mann-Whitney test was used when the distribution was skewed (Papers II, III, IV). The data were analysed by analysis of variance (ANOVA) in a general linear model (GLM), followed by Tukey's post hoc test with statistical level of significance p=0.05. After ANOVA analyses, Bonferroni correction (p=0.05 divided by the number of variables) (Papers II and IV) or false discovery rate (FDR; α=0.05, number

of metabolites = number of variables) (Paper III) was used to account for multiple testing and to obtain stronger, truly significant results. All lipid and metabolomics values are presented as mean ± standard deviation (Papers I and II) or ± standard error of mean (Papers III and IV).

4 Summary of results

4.1 Growth performance

In Papers I and II, the high inclusion rate of sesamin had a significant negative effect on final body weight and specific growth rate compared with fish fed without sesamin.

In Paper III, the 40% crude protein replacement of FM in the REF diet with ZYG and EY resulted in lower final body weight, specific growth rate and weight gain. No effect was seen in MM and NY fish.

In Paper IV, growth performance was not affected by partly replacing FM with KM or totally replacing FM with MM.

4.2 Lipid analyses

4.2.1 Paper I

The lipid content in the white muscle of Atlantic salmon was ~1.6% regardless of treatment, while the liver lipid content increased significantly, from ~5% to ~7.4% (V0.5SH) and ~8% (V1SH) compared with fish in the groups without sesamin.

The FA composition in tissues from fish fed the FO diet was significantly different from that from fish fed VO. Apart from PL in the liver for V0.5 fish, SFA, EPA (except PL) and DHA were significantly higher in FO groups compared with VO groups. Sesamin inclusion had a greater effect on FA composition of the liver than of white muscle in fish fed VO diets. The inclusion of sesamin in VO diets had no effect on DHA, irrespective of n-6/n-3 FA ratio and tissue analysed, compared with fish fed without sesamin supplementation. The EPA level decreased with the high inclusion of sesamin in white muscle with an n-6/n-3 FA ratio of 0.5 and in the liver at both n-6/n-3 FA ratios (except low ratio of TAG fraction). The high level of sesamin with

an n-6/n-3 FA ratio of 1 in white muscle resulted in an increase in LA, total PUFA and total n-6 and a decrease in total MUFA. When comparing the two n-6/n-3 FA ratios of muscle tissue, V1 groups had higher percentages of MUFA and n-6, and lower of percentages of PUFA and n-3.

Diets with a low n-6/n-3 FA ratio of 0.5 and low sesamin supplementation increased the percentage of LA, ALA, PUFA, n-3 and n-6 in both liver fractions, while high sesamin addition elevated LA, ALA, MUFA and n-6 and decreased the percentage of n-3 in PL. Diets with high n-6/n-3 FA ratio and high sesamin addition resulted in an increased percentage of LA and a decreased percentage of ARA in the PL fraction in the liver. Low n-6/n-3 FA ratio gave higher levels of ALA, SFA (PL fraction), PUFA (TAG fraction) and n-3 (TAG fraction) and lower levels of MUFA, ARA (PL fraction), DHA (PL fraction) and n-6 (PL fraction).

4.2.2 Paper III

The lipid content of Arctic charr muscle was ~1.75% regardless of treatment, while the hepatic fat content was significantly lower in ZYG fish (4.85%), compared with the other four groups (~8.5%).

Analyses of the composition of lipid classes in the liver of fish fed the five experimental diets showed that ZYG fish had highest percentage of PL and cholesterol, and the lowest percentage of TAG.

Determination of FA composition showed that FM replacement affected liver lipids more than muscle lipids, as evidenced by higher levels of LA, ARA, n-6 PUFA, DHA, n-3 PUFA and total PUFA and a lower level of MUFA, while in the muscle LA and n-6 PUFA decreased in ZYG fish.

4.2.3 Paper IV

The lipid content was ~94.5 µg/µL for liver and ~43 µg/µL for muscle of Arctic charr, irrespective of treatment. The hepatic lipid class composition did not differ between the three groups.

The FA composition was more affected by partial replacement of FM with KM than total replacement of FM with MM in liver. The FKM fish had a higher percentage of SFA (TL and TAG), EPA, DHA (TAG) and n-3 PUFA (TAG) and a lower percentage of MUFA (TAG), LA (PL and TAG), ARA and n-6 PUFA compared with FM fish. The MM fish had higher levels of SFA (TAG) and ARA (TAG) and lower levels of n-6 PUFA (TAG), DHA (TL and TAG), n-3 PUFA (TL) and PUFA (TAG) compared with FM fish.

4.3 Metabolomics analyses (Papers II-IV)

A summary of the metabolites in the aqueous phase that were affected by the dietary treatments in Papers II, III and IV is presented in Table 4.

In Paper II, the metabolomics analyses of aqueous extracts showed that fish fed the high sesamin diet had an increase in some metabolites in the liver (*i.e.* leucine, valine, carnitine, creatine, glucose, glycogen and two unknown signals) and in muscle (*i.e.* lactate, creatine/phosphocreatine and nucleosides). The analyses of chloroform extracts showed an increase in all FA except DHA and EPA and a decrease in n-3 FA, phosphatidylcholine and glyceryls of lipids and phospholipids for the high inclusion of sesamin, irrespective of the n-6/n-3 FA ratio.

In Paper III, the replacement of 40% crude protein from FM in the REF diet with alternative meals had an effect on several metabolites in the polar phase of the liver and muscle. Compared with in the liver of REF fish, creatine was lower in the other four groups, while 3-aminoisobutyrate was higher in MM fish, o-phosphocholine in ZYG fish, and betaine, n,n-dimethylglycine and sarcosine in NY fish. In the muscle, higher levels of 2-aminobutyrate, alanine, betaine, formate, isoleucine, proline and valine and lower levels of hydroxyproline in EY fish compared with REF fish were observed, while higher concentrations of 3-aminoisobutyrate and malonate were found in MM fish. Furthermore, betaine and n,n-dimethylglycine increased in NY fish and 3-aminoisobutyrate, anserine and pantothenate increased, while hydroxyproline decreased, in ZYG fish compared with REF fish. The metabolomics analyses of the chloroform extracts showed higher percentages of n-3 FA, EPA, DHA, PUFA and phosphatidylcholine/-ethanolamine in ZYG fish compared with REF fish. In addition, signals assigned to all FA except EPA and DHA, unsaturated FA and glyceryl of lipids were lower in ZYG fish than REF fish. The metabolomics analyses of the chloroform phase in muscle tissue showed no differences between the diets.

In Paper IV, the substitution of FM partly with KM or totally with MM gave similar results in tissue and plasma metabolites (betaine, n,n-dimethylglycine, sarcosine (liver and plasma), n-methylhydantoin (liver and muscle), glycine (muscle and plasma), asparagine (liver and plasma) and trimethylamine n-oxide (TMAO) (muscle and plasma). Furthermore, the aqueous liver analyses showed higher concentrations of alanine in FKM fish and lower levels of uridine in FKM fish and MM fish compared with FM fish. In muscle, acetate and glucose were higher and creatinine lower in FKM fish and glucose, lysine and beta-alanine were higher in MM fish compared with FM fish. The plasma analyses revealed higher choline concentrations in FKM fish and lower taurine and o-acetylcarnitine concentrations in FKM fish and

MM fish compared with FM fish. The NMR-based metabolomics analyses of chloroform liver extracts showed higher percentages of all FA, especially n-3 FA, EPA, DHA, unsaturated FA and glyceryl of lipid for FKM fish compared with the other two groups. In addition, MM fish had higher percentages of all FA except n-3 FA. Fish fed the FKM and MM diets had a decreased percentage of phosphatidylcholine. The metabolomics analyses of the chloroform phase in muscle tissue showed no differences between the diets.

Table 4. *Summary of metabolites in the aqueous phase of Atlantic salmon (Paper II) and Arctic charr (Papers III and IV) increased (plain type) or decreased (in italics) by the experimental diet compared with the control*

	Paper II		Paper III			Paper IV	
	SH	MM	EY	NY	ZYG	FKM	MM
Liver	Leucine	Aminoisobutyrate	*Creatine*	*Creatine*	*Creatine*	*Alanine*	Asparagine
	Valine	*Creatine*		Betaine	O-phosphocholine	Betaine	Betaine
	Carnitine			Dimethylglycine		Dimethylglycine	Dimethylglycine
	Creatine			Sarcosine		*Methylhydantoin*	*Methylhydantoin*
	Glucose					Sarcosine	Sarcosine
	Glycogen					*Uridine*	*Uridine*
Muscle	Lactate	Aminoisobutyrate	Aminobutyrate	Betaine	Aminoisobutyrate	Acetate	Betaine
	Creatine/	Malonate	Alanine	Dimethylglycine	Anserine	Betaine	Glucose
	Phosphocreatine		Betaine		Pantothenate	*Creatinine*	Lysine
	Nucleosides		Formate		*Hydroxyproline*	Glucose	Dimethylglycine
			Isoleucine			*Glycine*	*Methylhydantoin*
			Proline			Dimethylglycine	*TMAO*
			Valine			*Methylhydantoin*	Beta-alanine
			Hydroxyproline			TMAO	
Plasma						Asparagine	Asparagine
						Betaine	Betaine
						Choline	Dimethylglycine
						Glycine	*O-acetylcarnitine*
						Dimethylglycine	Sarcosine
						O-acetylcarnitine	*Taurine*
						Sarcosine	*TMAO*
						Taurine	
						TMAO	

Abbreviations: SH – high sesamin diet, MM – mussel meal, EY – extracted yeast (*Saccharomyces cerevisiae*) meal, NY – non-extracted yeast meal, Zyg – zygomycete fungi meal; FKM – mixture of krill and fish meal.

5 General discussion

5.1 Growth performance

Replacement of FO with VO did not affect growth performance in Paper I, which is in agreement with previous findings in Atlantic salmon (Torstensen *et al.*, 2005; Bell *et al.*, 2003; Caballero *et al.*, 2002; Bell *et al.*, 2001; Rosenlund *et al.*, 2001). Growth performance was negatively affected (in a dose-dependent manner) with inclusion of sesamin. The highest inclusion of sesamin reduced weight gain and specific growth rate (SGR) of the fish in Papers I and II. This contradicts growth performance results reported for advanced juvenile teleost species (Trattner *et al.*, 2011; Mraz *et al.*, 2010; Trattner *et al.*, 2008a), but is in agreement with results found for early juvenile barramundi (*Lates calcarifer*) (Alhazzaa *et al.*, 2012). Furthermore, the reduced growth performance in high sesamin groups is in agreement with findings for other bioactive compounds in Atlantic salmon, *e.g.* tetradecylthioacetic acid (TTA) at 12 °C (Moya-Falcon *et al.*, 2004) and dodecylthioacetic acid and TTA at 5 °C (Kleveland *et al.*, 2006).

Replacement of FM with ZYG in the diet of Arctic charr in Paper III had a negative effect on final body weight and SGR, which was confirmed in later work by Langeland (2014), but contradicted findings in previous studies where FM was replaced with 19% zygomycetes as a feed ingredient in Atlantic salmon (Bankefors *et al.*, 2011) and 60% zygomycetes as a feed ingredient in Arctic charr (Abro *et al.*, 2014). Furthermore, growth performance was decreased in fish fed extracted yeast (EY), while non-extracted yeast (NY) did not induce any growth change. In contrast to these findings, Øverland *et al.* (2013) observed reduced growth in Atlantic salmon fed 34.5% non-extracted yeast.

5.2 Effects on lipid profile

5.2.1 Total lipid content and lipid class composition

The white muscle lipid content in fish in Papers I and II was unaffected by the inclusion of sesamin in the diets. However, a significant increase in lipid content was found in the liver of Atlantic salmon fed a high sesamin diet, confirming previous findings in rats (Ashakumary *et al.*, 1999). In contrast to our results, Moya-Falcon *et al.* (2004) reported that the liver lipid content was unaffected by the addition of another lipid metabolism modulating substance, tetradecylthioacetic acid, to Atlantic salmon diets.

In Paper III, the total lipid content of white muscle was unaffected, while the replacement of FM resulted in decreased liver lipid content and levels of TAG and increased percentage of PL and cholesterol in ZYG fish compared with REF fish. It is known that PL serve as cell membrane constituents, whereas TAG are mainly used for energy purposes. Therefore, the lower lipid content in the liver and the lower final body weight of ZYG fish seem to be in agreement with the lower TAG percentage found in the liver of these fish, suggesting either lower TAG deposition or greater use of energy stores.

5.2.2 Fatty acid composition

It is well known that changes in the tissue FA profile are usually explained by dietary FA profile, since they are closely related to each other (Bell *et al.*, 2004; Torstensen *et al.*, 2004). Thus, in Paper I a significantly higher percentage of MUFA was observed in the TAG fraction of both liver and white muscle of Atlantic salmon fed the VO diet, with its high n-6/n-3 FA ratio, compared with the FO diet, as a result of the higher amount of MUFA in the VO diet. Furthermore, the VO diet contained higher levels of LA and ALA and lower levels of DHA than the FO diet, which was also reflected in the fatty acid profile of fish fed those diets. The lower proportion of EPA and DHA in VO fish is in agreement with findings in several previous studies where FO was replaced with a VO such as rapeseed oil, palm oil, camelina oil, linseed and/or olive oil (Hixson *et al.*, 2014; Pettersson *et al.*, 2009; Turchini & Francis, 2009; Jordal *et al.*, 2007; Bell *et al.*, 2004; Torstensen *et al.*, 2004).

The addition of sesamin to the diets affected the FA involved in the synthesis of DHA, *e.g.* 20:3n-3 increased, while EPA and 22:5n-3 decreased. However, DHA was not affected, which contradicts previous findings of a significant increase in DHA in white muscle of rainbow trout (Trattner *et al.*, 2008a) and in Atlantic salmon hepatocytes (Trattner *et al.*, 2008b) on addition of a dietary sesamin/episesamin mixture and in TL of barramundi after inclusion of sesamin in combination with echium oil in the diet (Alhazzaa *et al.*, 2012). The different

responses to sesamin could be explained by several factors: i) the physiological response to sesamin may vary between fish sizes and species. Freshwater fish have higher capacity to convert ALA to DHA than seawater fish (Sales, 2010; Zheng *et al.*, 2004); ii) within the same species, the variation in the response to sesamin may depend on age, gender and possible environmental conditions such as temperature and feed composition. The fish in Papers I and II had a size of ~105g and were reared in tanks with flow-through seawater at 12 °C for 4 months, while Alhazzaa *et al.* (2012) studied juvenile barramundi (~0.6g) in brackish water at 30 °C for 15 days and Trattner *et al.* (2008a) studied rainbow trout (~35g) in tanks with a water temperature of 10 °C for 35 days; iii) we used pure sesamin in combination with rapeseed, palm and linseed oil, whereas Trattner *et al.* (2008a) used a equi-mixture of sesamin/episesamin in combination with mixed oil (linseed and sunflower oil) or linseed oil for rainbow trout and Alhazzaa *et al.* (2012) used sesamin in combination of echium oil. Episesamin has previously been shown to be a stronger lipid modulator of enzyme activity than sesamin (Kiso *et al.*, 2005) in mammals. Therefore, the presence of episesamin or the right combination of sesamin with a vegetable oil in the diet of fish may be important for modulation of FA composition.

Replacement of FM with zygomycete fungi meal had a major impact on the lipid content and thereby the DHA proportion in ZYG fish compared with REF fish, resulting in a 54% increase in DHA proportion. This is in line with the almost 50% lower lipid content commonly found in storage lipids in comparison with PL. The increased DHA level may be explained by the low lipid content, an assumption which is supported by the high PL level found in the liver of ZYG fish, which results in conversion of DHA for membrane use. These findings are in agreement with those in a previous study by Pan (2013), who found that an experimental diet containing 23% zygomycete fungi increased DHA level in the PL fraction of white muscle in Arctic charr.

In Paper IV, a higher FA content of 14:0, 16:0, 16:1n-7, 18:1n-7 and EPA were found in the diet FKM fed to Arctic charr and in the liver of those fish, as a consequence of *Euphausia* species feeding on phytoplankton, which generally contain higher amounts of those FA (Saito *et al.*, 2002). It is well known that salmonids have the capacity to adjust the FA composition by selective oxidation and/or desaturation and elongation processes (Tocher, 2003). The FKM diet had a threefold higher EPA level compared with FM, which was also reflected in an increased EPA percentage in the fish liver. However, the DHA content in the FKM diet was almost twice as high as with FM, but only the TAG fraction had a 100% higher value of DHA in fish liver. The higher levels of EPA and DHA in the liver tissue compared with the diets is in agreement with Suontama *et al.* (2007), who found similar results in muscle of Atlantic salmon and Atlantic halibut.

Surprisingly, although mussels also feed on phytoplankton (Lindahl *et al.*, 2005), this was not reflected in the FA profile of the MM diet or, consequently, in the liver of Arctic charr. The EPA content was higher in the diet compared with FM, while the liver was unaffected. The DHA level was similar in the diets, but decreased (TL and TAG fraction) in the liver of fish fed the MM diet. This might indicate that less peroxisomal beta-oxidation took place, while more microsomal beta-oxidation was used for energy, as shown by lower DHA levels in the TAG fraction. On the other hand, fish fed the MM diet had a higher fat content (although the difference was not significant), which might explain the higher level of storage lipids and the lower percentage of DHA found in those fish.

5.3 Metabolomics

In this thesis, ^{1}H NMR-based metabolomics analyses were carried out to study the metabolic response in liver, white muscle and plasma. The results showed that fish metabolism was affected by the replacement of FM with alternative microbial, marine and plant ingredients.

5.3.1 Changes associated with dietary ingredients

It is well known that dietary ingredients can be reflected in fish tissues or biofluids. Thus, the increased levels of alanine, isoleucine, proline and valine observed in the muscle of EY fish (Paper III) might be due to higher levels of those amino acids in the EY diet compared with the control diet. Furthermore, higher levels of 3-aminoisobutyrate were observed in the muscle of MM fish (Paper III), which is in agreement with Awapara and Allen (1959), who reported the presence of this metabolite in blue mussel. In Paper IV, MM had the highest dietary amino acid content, as reflected in high amounts of *e.g.* asparagine and asparate, glycine, lysine, cysteine and methionine. The higher levels of asparagine (liver and plasma) and lysine (muscle) in MM fish might have been due to higher availability of these metabolites in the MM diet compared with the FM diet, but surprisingly glycine showed no response to diet.

The elevated levels of betaine and its by-products (n,n-dimethylglycine and sarcosine) found in MM fish and FKM fish in Paper IV could be because the tissues of small molluscs and crustaceans generally contain higher amounts of betaine, while it is absent or present in very low concentrations in teleost species (Carr *et al.*, 1996).

The replacement of FM with 100% MM and 50% KM affected TMAO in Paper IV. It serves as an oxygen donor under anoxic stress, protects cells against osmotic stress and prevents oxidative damage (Martinez *et al.*, 2005; Zerbst-

Boroffka *et al.*, 2005). Jiang *et al.* (2014) reported that crustaceans have a higher TMAO content than teleost fish, followed by shellfish, which was also shown in this thesis. Furthermore, higher levels of taurine, another osmolyte, antioxidant and feeding stimulator, have been observed in marine invertebrates such as blue mussel, whereas FM (El-Sayed, 2014) and Antarctic krill (Chi *et al.*, 2013) have much lower taurine concentrations. However, these results were not evident in this thesis, where FKM fish and MM fish had lower plasma taurine levels compared with FM fish.

5.3.2 Changes associated with energy metabolism

The replacement of FM with alternative ingredients had an effect on several metabolites (*e.g*. Paper II: leucine, valine, carnitine, creatine, glucose, glycogen, lactate and nucleosides; Paper III: alanine, isoleucine, valine and proline; Paper IV: alanine, glycine and glucose). Those changes might suggest disturbance of protein biosynthesis and catabolism, as well as energy metabolism pathways leading to the tricarboxylic acid (TCA) cycle.

Leucine and valine are branched-chain essential amino acids particularly involved in energy metabolism (Kimball & Jefferson, 2006). Both amino acids play a role in the TCA cycle, as leucine can be degraded to form *e.g*. acetoacetate and acetyl-CoA and valine acts as an intermediary metabolite to form succinyl-CoA. Therefore, the increase in both leucine and valine with the higher inclusion rate of sesamin in the diets in Paper II might reflect inhibition of the TCA metabolic pathway generating energy, which is in line with the lower growth rate found in those fish.

Fish have a limited ability to metabolise glucose for energy purposes (Enes *et al.*, 2009). However, it has been shown that in the liver of trout fed a carbohydrate-rich diet, glucose in excess may be stored as glycogen (Hemre *et al.*, 2002). Glycogen level was also higher in the liver of fish fed the high sesamin diet in Paper II. The excess of both glucose and glycogen may also suggest that the TCA metabolic pathway was inhibited, indicating the inability of the liver of fish fed a high sesamin diet to generate energy and reducing power through this pathway. The high inclusion of sesamin elevated the level of creatine, which functions as a cell energy shuttle (Owen & Sunram-Lea, 2011), as a possible response to energy stress. This might also explain the lower growth rate found in those fish. Moreover, in Paper II a higher level of carnitine in the liver of fish fed the high sesamin diet was observed, which is in agreement with Ide *et al.* (2009a), who found that dietary sesamin in rodents increased the hepatic carnitine concentration, which might explain the higher fat content in the liver of fish.

These findings raise the questions of whether sesamin can be used as a bioactive compound for improving growth and lipid metabolism in aquafeeds. In a previous study analysing the *in vitro* effect of sesamin on CYP1A activity, which is a biomarker for xenobiotic compounds, it was found that sesamin decreased the CYP1A activity in a concentration-dependent manner (Wagner *et al.*, 2013). Furthermore, a previous *in vivo* study showed up-regulation of CYP1A activity with sesamin addition (Zlabek *et al.*, 2015). Therefore it can be concluded that sesamin has an effect on fish liver and, in the amount used in this thesis, is a xenobiotic compound in Atlantic salmon.

The replacement of FM with EY in Paper III affected the level of proline. This metabolite, which can act as an antioxidant, is a substrate for glucose synthesis. Stimulated degradation of proline and increased activity of proline oxidase (a mitochondrial inner membrane enzyme which can generate ATP when proline is further metabolised via TCA) in response to stress, including nutritional stress, was shown in a previous study (Pandhare *et al.*, 2009). Therefore, a potential lack of nutrients when FM is replaced with EY might cause nutritional stress, as demonstrated in increased proline levels and also in lower final body weight of EY fish compared with REF fish.

Lower levels of alanine (liver) and glycine (muscle and plasma) and higher levels of glucose (muscle), metabolites correlated to energy metabolism, were observed in FKM fish compared with FM fish in Paper IV. Alanine is a major glucogenic precursor in all mammals and an important energy substrate in fish, while glycine is involved in gluconeogenesis for energy production, single carbon metabolism and fat digestion (Li *et al.*, 2009). Glucose metabolism increases FA synthesis, which is in agreement with the findings of increased FA, including unsaturated FA, glyceryl of lipids, EPA and DHA, obtained for chloroform liver extract analyses using the metabolomics approach.

5.3.3 Changes associated with single carbon metabolism

Replacement of FM with EY (Paper III), NY (Paper III), FKM (Paper IV) and 100% MM (Paper IV) gave similar results in the tissues and plasma metabolites (betaine, n,n-dimethylglycine and sarcosine). Betaine, which comes from the diet or by oxidation of choline, is an organic osmolyte which is essential for proper liver function and is important in protein and energy metabolism. This metabolite increased in liver and plasma of NY (Paper III), FKM (Paper IV) and MM (Paper IV) fish and in the muscle of EY (Paper III), NY (Paper III), FKM (Paper IV) and MM fish (Paper IV). Betaine can be de-methylated to produce n,n-dimethylglycine and to simultaneously convert homocysteine via single carbon metabolisms to methionine (Lever & Slow, 2010). This thesis showed an increase

in n,n-dimethylglycine in NY (Paper III), FKM (Paper IV) and MM fish (Paper IV). Moreover, n,n-dimethylglycine can be further metabolised to sarcosine. Sarcosine increased in the liver (Papers III and IV) and plasma (Paper IV) samples of NY, FKM and MM fish, but was unaffected in muscle samples. This is in agreement with Van Waarde (1988), who concluded that the conversion from choline to glycine in little skate (*Raja erinacea*) does not occur in skeletal muscle, but only in the kidney and liver.

6 Main findings and conclusions

The results of this thesis showed that replacement of fishmeal (FM) and fish oil (FO) with alternative feed sources, such as zygomycete fungi, yeast, krill, mussels and plants, as dietary ingredients in the feed of Atlantic salmon and Arctic charr can cause a number of changes in the lipid and metabolic profile. The following main findings and conclusions can be drawn from the results obtained:

- Some of the diets tested were identified as a good source of single carbon metabolism precursors.

- Salmonid growth was negatively affected by zygomycete fungi, extracted yeast or a high level of sesamin.

- Sesamin had a dose-dependent minor effect on FA composition, *e.g*. LA and ALA increased in the liver and TAG fraction of muscle, EPA decreased in the muscle and DHA was unaffected by inclusion of sesamin.

- High inclusion of sesamin affected several metabolites (*e.g*. glucose, glycogen, leucine, valine, creatine, carnitine, lactate, nucleosides) in Atlantic salmon. These metabolites are mainly associated with energy metabolism, suggesting that high sesamin levels affect fish liver and muscle metabolism.

- Fish fed a diet including zygomycete fungi meal had a lower lipid and MUFA content and a higher level of n-3, n-6 PUFA and DHA in the liver compared with fish fed a reference FM-based diet.

- Replacing FM with non-extracted yeast had the greatest effect on the liver of Arctic charr, while with extracted yeast the muscle was more affected. There was an increase in *e.g*. betaine, formate, valine, isoleucine, alanine, and proline, suggesting a change in muscle metabolism in Arctic charr.

- Replacing FM with 40% mussel meal had a minor effect on lipid metabolism, while total replacement resulted in a decreased DHA level in TL and in the TAG fraction.

- Krill meal represents a valuable source of n-3 PUFA and resulted in higher levels of EPA and DHA in fish.

- Changes in the metabolic profile of fish fed 100% mussel meal diet and 50% krill meal diet compared with fish fed the FM diet might reflect the dietary ingredients of those diets.

7 Future perspectives

This thesis presents novel information on the effects of alternative raw materials on the protein and lipid metabolism of salmonids. Information was obtained about FA, lipid classes, lipid content and metabolic fingerprint. Based on the results presented, the following investigations are of interest in order to find alternative feed ingredients for the diets of farmed fish in order to support sustainable aquaculture:

- Evaluation of the impact of different levels of dietary inclusion of microbial (*e.g.* zygomycete fungi, yeast) or novel marine materials (*e.g.* krill, mussel, invertebrates in general) on the growth performance, CYP 450, lipid and metabolic profile of salmonids.

- Analysis of the impact of microbial and novel marine sources on fish metabolism, especially lipid metabolism, in different tissues and biofluid (plasma, brain, red muscle).

- Determination of the effect of microbial and novel marine materials in the feed on fish tissues at molecular level, including genomics and proteomics.

- Application of the knowledge obtained from studies on fish metabolites and their response to microbial, marine and plant feed compounds in order to understand and assess food quality and the effect on human nutrition.

- Evaluation of biomarkers to assess the sensory and nutritional quality of fish as healthy food for human consumption.

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