EFFECTS OF REPLACING DIETARY FISH OIL AND FISH MEAL WITH MICROBIAL OIL AND ALGAL BIOMASS ON LIPID CLASS, TOTAL FATTY ACID AND PHOSPHOLIPID FATTY ACID COMPOSITION OF ATLANTIC SALMON LIVER AND MUSCLE TISSUES

by

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ABSTRACT

With the continuous growth of aquaculture comes a growing demand for an alternative lipid source for fish oil (FO) and fish meal (FM) in aquafeeds. Certain microorganisms provide a potential sustainable replacement for FO and FM due to their content of omega-3 (ω3) long-chain polyunsaturated fatty acids, which are essential for the growth and health of fish. Two feeding trials were conducted to determine the effects of replacing FO and FM with oil and biomass from two different microorganism species. The first feeding trial comprised of replacing FO with a microbial oil (MO) derived from a novel strain, *Schizochytrium* sp. (strain T18), in diets for Atlantic salmon. Four experimental diets were developed: a fish oil control diet (FO), a blend of fish oil/canola oil control diet (FO/CO), a fish oil replacement with low proportions of microbial oil diet (LMO), and a fish oil replacement with high proportions of microbial oil diet (HMO). After 16 weeks of feeding, there were no significant effects on growth parameters across the dietary treatments. Fatty acid profiles reflected the diets with DHA being present in high proportions in the tissues, especially in the cellular membrane. The purpose of the second feeding trial was to reduce FO and replacing FM with algal biomass (AB) derived from *Pavlova* sp. strain CCMP459 (*Pav*459) in diets for Atlantic salmon. Three experimental diets were developed: a fish meal control diet (FM), a blend of a fish meal/algal biomass *Pav*459 diet (FM/AB), and a complete FM replacement with algal biomass *Pav*459 diet (AB). After 12 weeks of feeding, again, there was no significant effect on growth paraments, and the fatty acid profiles also reflected those of the diets. The DHA was present in high proportions in the tissues for all dietary treatments, especially the cellular membrane. The stable isotope data suggested a direct integration of EPA and DHA and not biosynthesis from its precursor ALA.

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List of Abbreviations

Δ = delta

ω = omega

ALA = alpha-linolenic acid, 18:3ω3

AMPL = acetone mobile polar lipids

ARA = arachidonic acid, 20:4ω6

CO = canola oil

CSIA = compound-specific stable isotope analysis

DHA = docosahexaenoic acid, 22:6ω3

EFA = essential fatty acid

EPA = eicosapentaenoic acid, 20:5ω3

FFA = free fatty acids

FO = fish oil

FM = fish meal

HUFA = highly unsaturated fatty acids

LA = linoleic acid, 18:2ω6

LC-PUFA = long-chain polyunsaturated fatty acids

MO = microbial oil

ω3 = omega-3

ω6 = omega-6

GC = gas chromatography

MUFA = monounsaturated fatty acids

PL = phospholipids

SFA = saturated fatty acids

ST = sterol

TAG = triacylglycerols

TLC-FID = thin layer chromatography with flame ionized detection (Iatroscan)

WE = wax esters

ww = wet weight

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1. INTRODUCTION

Between 1961 and 2016, the average annual increase in global food fish consumption (3.2%) outpaced population growth (1.6%) and exceeded that of meat from all terrestrial animals combined (2.8%) (FAO, 2018). In 2015, fish accounted for about 17% of animal protein consumed by the global population (FAO, 2018). Besides being a good source of protein, fish also provides valuable lipids as well as minerals and vitamins (Steffens, 2006). Oily fish such as salmon, sardines, trout, mackerel, and tuna are rich sources of omega-3 (ω3) long-chain polyunsaturated fatty acids (LC-PUFA), particularly eicosapentaenoic acid (EPA; 20:5ω3) and docosahexaenoic acid (DHA; 22:6ω3). For nearly a century, linoleic acid (LA; 18:2ω6) and alpha-linolenic acid (ALA; 18:3ω3) have been termed essential fatty acids (EFA) for mammals, however, in marine literature, EPA and DHA are also termed EFA (see section 2.3). Although LA and ALA are termed EFA for mammals, DHA and EPA, together with arachidonic acid (ARA; 20:4ω6), are also important for humans due to their benefits and association with disease prevention such as cardiovascular and inflammatory diseases since humans synthesize them poorly (Sargent et al., 1999; Tocher, 2003; Tocher, 2015).

As the human population continues to expand beyond 7 billion, its reliance on farmed fish production as an important source of protein will also increase (Naylor et al., 2000). Aquaculture is forecast to increase by 62% between 2010 and 2030 in order to supply the increasing fish and seafood demand derived from a steadily growing population and changing consumption patterns (Reverter et al., 2020). However, intensification of aquaculture output requires a greater supply and development of the inputs – mainly feedstuff, their formulations, and optimization (Alhazzaa et al., 2019). In the past, aquaculture feeds largely used fish meal (FM) and fish oil (FO), but with the rapid expansion of aquaculture and a plateau in wild fisheries, these ingredients are no longer

sustainable (Gasco et al., 2018). The search for a sustainable, reliable source of dietary lipids remains a hot topic in aquaculture. FM and FO are still considered the most nutritious and most digestible ingredients for farmed fish feeds, but their inclusion rates in formulated feed for aquaculture have shown a clear downward trend, largely as a result of supply and price variation (FAO, 2018). Nowadays, the inclusion of FO and FM is reduced to the minimum amount able to cover the optimal content of amino acids and other nutrients needed for fish growth and flesh quality; sometimes, the aquafeed industry produces fish diets that are completely free of these marine ingredients (Gasco et al., 2018).

Several studies have investigated terrestrial plants as an alternative lipid source to FO and FM. These studies concluded that although terrestrial plant oils does not affect the growth performance of the fish, the concentration of ω3 LC-PUFA in the flesh was significantly reduced (Bell et al., 2002; Bell et al., 2003; Bransden et al., 2003). This is because terrestrial plant-derived oils do not contain any ω3 LC-PUFA ($\geq C_{20}$), and they can have high amounts of monounsaturated fatty acids (MUFA), high amounts of ω6 PUFA, and low ω3/ω6 ratios (Miller et al., 2007). In general, most fish require LC-PUFA; however, the requirements vary from species to species. Most freshwater fish require LA and ALA as they can convert these precursors to their corresponding ω3 and ω6 LC-PUFA ($\geq C_{20}$), whereas seawater species require EPA, DHA, and ARA due to their inefficiency in converting these precursors to their corresponding ω3 and ω6 LC-PUFA ($\geq C_{20}$) (Tocher, 2003). Almost all fish, as with all vertebrates, potentially have the ability to convert the two basic C_{18} ω6 and ω3 PUFA into the corresponding C_{20} and C_{22} ω6 and ω3 highly unsaturated fatty acids (HUFA) *in vivo* by an alternating succession of elongases and desaturases (Nakamura and Nara, 2004). The degree to which an animal can perform these conversions depends on the relative activities of fatty acid elongases and desaturases, such as Δ^6

and Δ^5, in their tissues, and these activities, in turn, are dependent on the extent to which the species can readily obtain the end products ARA, EPA and DHA preformed from their natural diets (Tocher, 2003). Plants are able to synthesize LC-PUFA *de novo* and interconvert ω3 and ω6 fatty acid families via desaturases with specificity in the Δ^{12} and Δ^{15} positions. Animals have Δ^5, Δ^6, and Δ^9 desaturase enzymes and are unable to synthesize the ω3 and ω6 PUFA *de novo* (O'Keefe, 2002). Therefore, carnivorous fish such as salmon can elongate and desaturate ALA to form EPA and DHA, but they do so with a minimal ability (Sargent et al., 1999; Tocher et al., 2001); therefore, it is essential to maintain high levels of EPA and DHA in the diet.

A more promising alternative lipid source for aquafeed is single-cell microorganisms. Microalgae are unicellular photosynthetic microorganisms recognized as a potentially sustainable source of ω3 LC-PUFA. They are the primary producer of DHA in the marine food web (Abril et al., 2003), and microalgae have the potential to reduce dependence on conventional raw materials in aquafeed (Shah et al., 2018). Although microalgae are recognized as good sustainable lipid sources for aquaculture, efficient large-scale production remains elusive. The use of microalgae in aquaculture is favoured by several factors: microalgae are the natural feeds of many aquaculture species and the basis of the natural food web on which such species depend in the wild. Microalgae have high nutrient value, contain specialty feed components including pigments, essential fatty acids, and vitamins. In many cases, microalgae can be directly used in the aquaculture process without the need for harvesting, processing, or storage (Benemann, 1992). There are numerous studies reporting microalgae as a potential replacement to FO and FM in a diet for different marine species (e.g., Miller et al., 2007; Sarker et al., 2016; Allen et al., 2019; Nagappan et al., 2021). As for this thesis, for the first study, we replaced FO with microbial oil (MO) from a novel strain *Schizochytrium* sp. (T18), often referred to as microalgae in many published papers; and for the

second study, we replaced FM with algal biomass (AB), *Pavlova* sp. strain CCMP459. The objectives of these studies were, in Chapter 3, to evaluate the dietary effect of replacing FO with MO (from *Schizochytrium* sp), and in Chapter 4, to evaluate the dietary effect of replacing FM with AB (from *Pavlova* sp.) in Atlantic salmon muscle and liver tissue composition with a focus on the phospholipid fatty acid composition of membranes.

2. LITERATURE REVIEW

2.1 Lipids in fish

Lipids are hydrophobic compounds that are soluble in organic solvents. They include triacylglycerols (TAG), wax esters (WE), sterols (ST), and phospholipids (PL) (Turchini et al., 2009). Animal lipids, including fish lipids, can be divided into two groups, polar lipids composed principally of PL and neutral lipids composed principally of TAG (Tocher, 2003). TAG constitutes a major class of neutral lipid and consists of three molecules of fatty acids esterified to the three alcohol groups of glycerol (Fig. 2.1) (Tocher, 2003). TAG are the most common energy storage lipids. TAG (~37kJ/g) are more efficient as energy stores than proteins (17kJ/g) and carbohydrates (16 kJ/g) due to the high caloric value per gram (Brindley, 1991; Campbell & Farrell, 2008; McKee & McKee, 2009; Cardoso da Rocha, 2012). WE constitute another class of neutral lipid consisting of a single molecule of a fatty acid esterified to a single molecule of fatty alcohol (Fig. 2.1) (Tocher, 2003). WE generally occur on the surfaces of organisms to protect against water loss, but in some aquatic animals, they are used as metabolic energy reserves (Parrish, 1986). When fish consume WE, they are converted to TAG (Cardoso da Rocha, 2012). ST are characterized by a four-ring core structure called the cyclopentanoperhydrophenanthrene, or steroid, nucleus. ST are monohydroxy alcohols of steroidal structure, with cholesterol being the most common example (Fig. 2.1) (Gropper et al., 2009). ST can exist unesterified as an essential component of cell membranes or in a neutral lipid storage form esterified to a fatty acid (Tocher, 2003). PL is a general term that includes all lipids containing phosphorus (Tocher et al., 2008). However, it is a term often mistakenly equated with phosphoglycerides, the most common of the phospholipids. Phosphoglycerides are a major class of polar lipid characterized by a common backbone of phosphatidic acid, which is L-glycerol 3-phosphate containing two esterified fatty acids (Fig. 2.1)

(Tocher, 2003). PL are essential components of membranes where they share a structural function with sterols (Parrish et al., 2000). In addition to lending structural support to the membrane, they serve as a source of physiologically active compounds such as eicosanoids (Gropper et al., 2009). Lipids can act as vectors for absorption and delivery of lipid-soluble nutrients, including vitamins and carotenoid pigments, as well as provide the precursors for metabolic derivatives such as eicosanoids, docosanoids, hormones, and vitamins (Bell & Koppe, 2010).

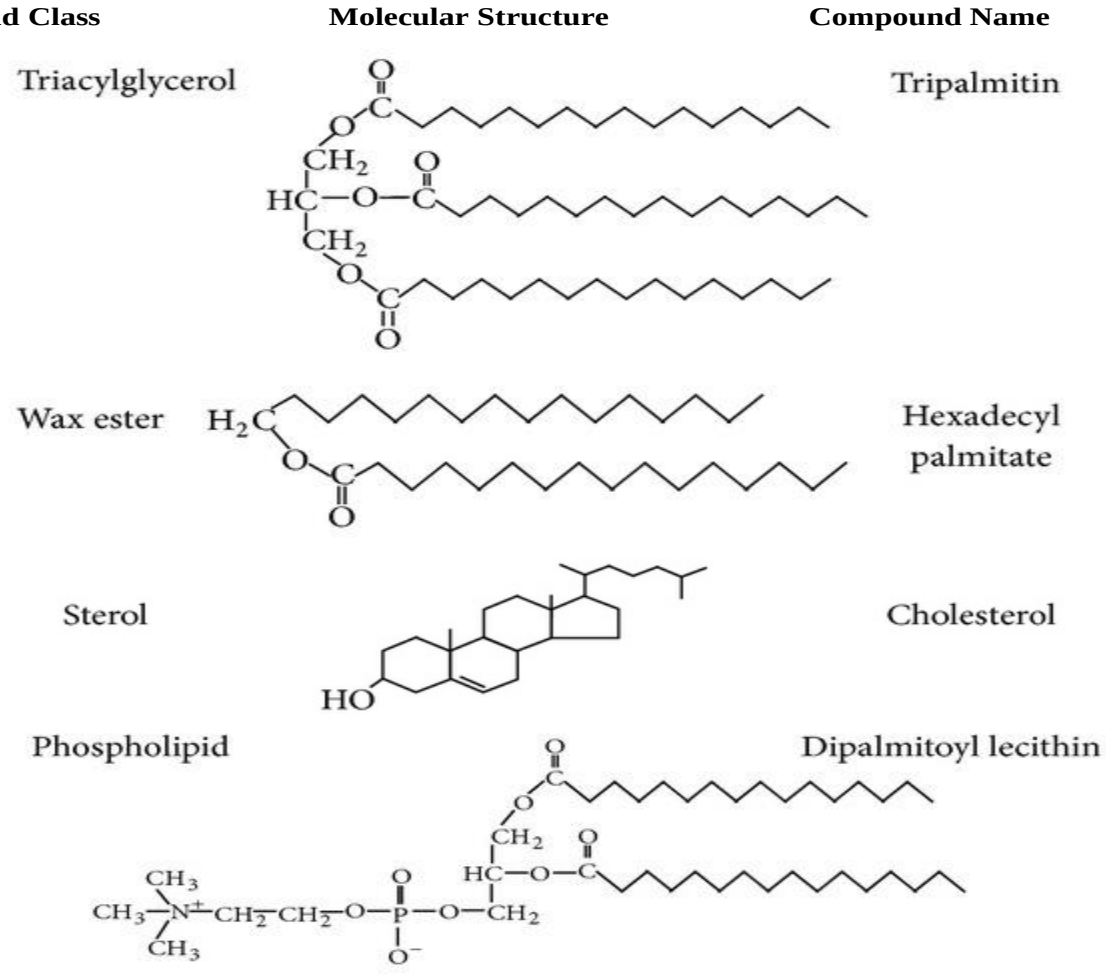


Figure 2.1 Examples of principal lipid classes, their molecular structure and compound name. Triacylglycerol, Wax Ester, Sterol, and Phospholipid (After Parrish, 1988).

2.2 Fatty acids

Fatty acids are the simplest class of lipids. They are composed of a hydrocarbon chain terminating with a carboxylic acid group (Fig. 2.2) (Gropper et al., 2009). They are of vital importance as an energy nutrient, furnishing most of the calories from dietary fat (Gropper et al., 2009). Fatty acids are designated on the basis of their chain lengths, degree of unsaturation (number of carbon-carbon double bonds), and the position of their double bonds (Tocher, 2003). Fatty acids with only single bonds between adjacent carbon atoms are referred to as saturated fatty acids (SFA) (Fig 2.3), whereas those with at least one carbon-carbon double bond (C=C) are called monounsaturated fatty acids (MUFA) (Fig. 2.3). The polyunsaturated fatty acids (PUFA) have two or more C=C and are named according to the position of these bonds and the total chain length (Fig. 2.3) (Ruxton et al., 2004).

Free Fatty Acid

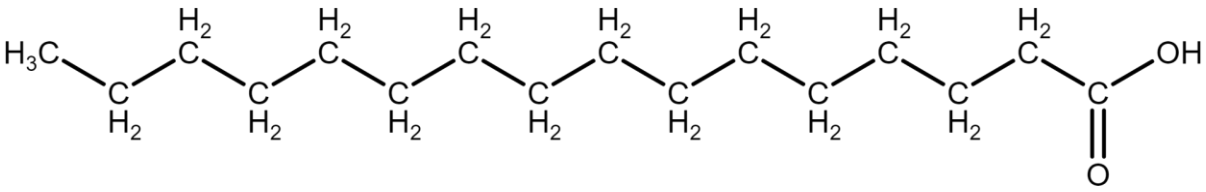
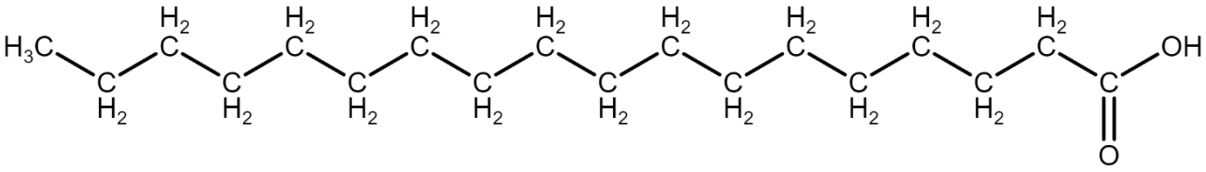


Figure 2.2 Example of free fatty acid (16:0; palmitic acid).

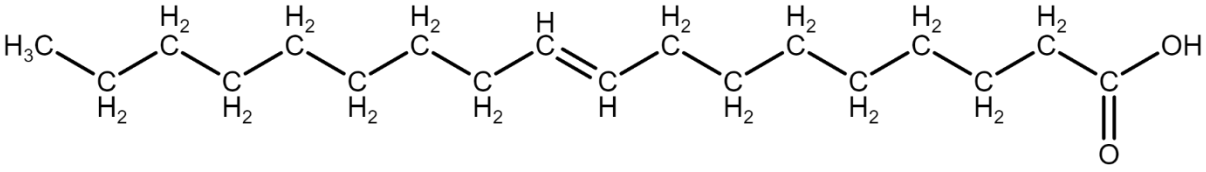
Two systems of notation have been developed to provide a shorthand way to understand the chemical structure of fatty acid (Gropper et al., 2009). The delta (Δ) system of notation has been established to denote the chain length of the fatty acids and the number and position of any C=C that may be present. For example, the notation $18:2^{\Delta 9,12}$ describes LA. The first number, 18 in this case, represents the number of carbon atoms; the number following the colon refers to the total number of double bonds present, and the superscript numbers following the delta symbol

designate the carbon atoms at which the double bonds begin. In this system, the numbering starts from the carboxyl end of the fatty acid. A second commonly used system of notation locates the position of double bonds on carbon atoms counted from the methyl, or omega (ω), end of the carbon chain. For example, the notation for LA would be 18:2ω6. In this system, the total number of carbon atoms in the chain is given by the first number, the number of double bonds is given by the number following the colon, and the location (carbon atom number) of the first double bond is given by the number following ω (Gropper et al., 2009). See Appendix A for additional examples of systems of fatty acid nomenclature.

Saturated fatty acid



Monounsaturated fatty acid



Polyunsaturated fatty acid

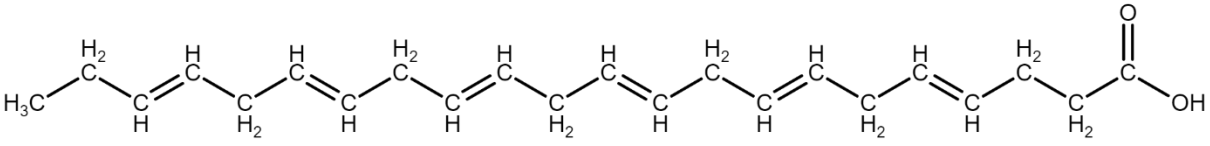


Figure 2.3 Examples of Saturated fatty acids (18:0; stearic acid), Monounsaturated fatty acids (18:1ω9; oleic acid), and Polyunsaturated fatty acids (22:6ω3; DHA)

2.3 Essential fatty acids

In fish, dietary lipids are an important source of EFA for regular growth, health, reproduction, and bodily functions (Turchini et al., 2009). The term "essential fatty acids" means that the organism needs these fatty acids but is unable to synthesize them *de novo* for optimal biological functions. The term "*de novo*" derives from Latin meaning "from the beginning." Theoretically, the only two fatty acids that should be most rigidly termed as essential are LA and ALA, which cannot be biosynthesized *de novo* by fish and other vertebrates (Turchini et al., 2009). In fish nutrition, dietary requirements vary from species to species. Each species has different capacities to biosynthesize LC-PUFA from dietary precursors depending on the presence and expression of genes of fatty acid desaturation and elongation (Tocher, 2003). Top carnivores have limited ability to synthesize LC-PUFA even from C_{18} dietary precursors and require the inclusion of preformed C_{20} and C_{22} LC-PUFA directly in their diet (Alhazzaa et al., 2019). For Atlantic salmon, EPA, DHA, and ARA are considered EFA that need to be supplied in the diet. The biochemical, cellular, and physiological functions of these three PUFAs are broadly the same in fish as in other vertebrates and fall into two categories: (a) an apparently generalized role in maintaining the structural and functional integrity of cell membranes; (b) a more specific role as precursors of the group of highly biologically active paracrine hormones known collectively as eicosanoids (Sargent et al., 1999). Individually, DHA is known to be more critical than EPA as a structural component of the cell membrane, while EPA plays a central role in the regulation of several processes related to immunity and inflammation, and ARA is a major precursor of eicosanoids (Calder, 2006; Gorjão et al., 2009; Horn et al., 2019; Sargent et al., 1999). Eicosanoids are a class of biologically active, oxygenated molecules derived from esters in phospholipids and diacylglycerols of the cell and nuclear membranes. Along with serving as messengers in the central

nervous system, eicosanoids act like local hormones or signalling molecules to control inflammation and immunity (Arts & Kohler, 2009). There are three subgroups of eicosanoids known as prostaglandins, thromboxanes, and leukotrienes (Rodney, 2005; Mckee & Mckee, 2009). EPA, ARA, and dihomo-gamma-linolenic acid (DGLA; 20:3ω6) are the primary fatty acids for the production of eicosanoids; however, EPA and ARA compete between themselves in the production of eicosanoids. Thus, the ω3⁄ω6 dietary ratio will determine the ω3⁄ω6 cellular membranes ratio, which in term will determine which prostaglandin will be more common and it will have consequences for the immune system (Cardoso da Rocha, 2012). ARA-derived eicosanoids promote inflammation (vasodilators), while those from EPA are described as less inflammatory, inactive, or anti-inflammatory (vasoconstrictors) (Arts & Kohler, 2009). The absence of EFA from the diet leads to deficiency symptoms that, in fish, most often include reduced growth and increased mortality (Glencross, 2009; Tocher, 2010).

2.4 Membrane phospholipid

The term "phospholipids" is usually taken to mean phosphoglycerides, which have a common backbone of phosphatidic acid di-fatty acyl esters of l-glycerol 3-phosphate, see Fig. 2.1 (Sargent et al., 2003). The backbone phosphatidic acid can be esterified to the "bases" choline, ethanolamine, serine, and inositol to form the major phosphoglycerides of the tissues of animals, including fish, namely, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) (Sargent et al., 2003). Another phosphorus-containing polar lipid is the sphingolipid, sphingomyelin, a complex lipid based on the long-chain amino alcohol sphingosine (Tocher et al., 2008). Sphingomyelin differs fundamentally from phosphoglycerides in that it is not constituted from phosphatidic acid. It consists of the amino

alcohol “base” sphingosine whose primary alcohol group is esterified to phosphocholine and whose amino group is amide-linked to a long-chain fatty acid, see Fig. 2.4 (Sargent et al., 2003).

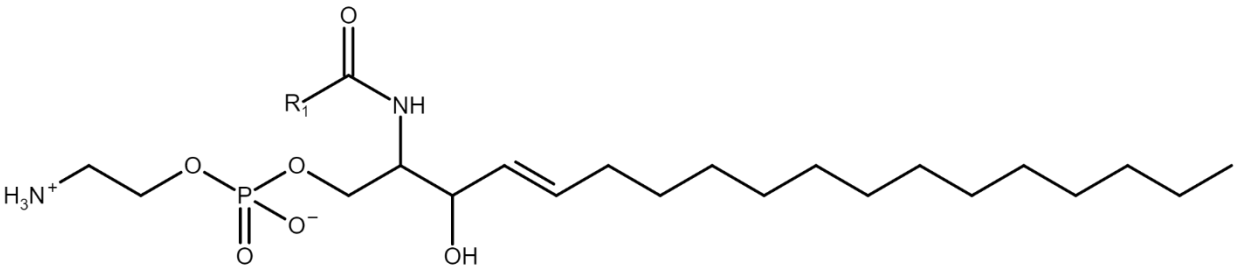


Figure 2.4 Example of sphingolipid (sphingomyelin)

PL makes up most of the lipids of the membrane bilayer and therefore plays an important role in determining the physico-chemical properties of the membrane (Renne & de Kroon, 2018). Central to the physiological functions of PL in cell membranes is their constituent EFA (Sargent et al., 1993). Generally, the highest levels of DHA are contained in PE and PS. PC commonly has the lowest levels of DHA in fish tissues, being rich instead in 16:0 and 18:1ω9. The highest level of ARA and EPA are usually found in PI (Sargent et al., 2003). Cell membrane phospholipids and their fatty acid composition are important in determining the physical characteristics of cell membranes, the manner in which membranes change in response to external stimuli, and the functional activities of membrane-bound proteins (Calder, 2014). Increased abundance of EPA and DHA in the phospholipid membrane may result in several consequences such as increased membrane fluidity, although cells have mechanisms such as modifying membrane cholesterol content to limit this effect. Another consequence as a result of the increased abundance of EPA and DHA, and the associated decreased abundance of ARA, is that the availability of substrates for the synthesis of bioactive lipid mediators is altered. As mentioned above, ARA is quantitatively the major substrate for the biosynthesis of various prostaglandins, thromboxanes, and leukotrienes together termed eicosanoids, which have well-established roles in the regulation of inflammation,

immunity, platelet aggregation, smooth muscle contraction, and renal function (Calder, 2014). Eicosanoid production is associated very broadly with vertebrate animals' responses to a stressful situation and is a normal physiological process, with excess eicosanoid production often occurring in pathological conditions such as cardiovascular and inflammatory diseases (Sargent et al., 2003; Wall et al., 2010).

The fatty acid composition of individual phospholipids varies according to a range of factors. These include the potential selectivity of uptake of certain fatty acids by the cell, the capacity of the cell for *de novo* fatty acid synthesis, potential selectivity for fatty acid incorporation into *de novo* phospholipid synthesis, potential fatty acid exchange reactions between intact phospholipids, and selectivity of incorporation of certain phospholipids into the cell membrane itself (Glencross, 2009).

To summarize, the main roles of phospholipids are: (1) Structural - due to their amphipathic structure (having both hydrophilic and hydrophobic regions), they play a key role in membranes, as well as in digestion and transport of lipids. (2) Regulation of metabolism and physiology due to the fact that PL are important precursors for a range of highly biologically active mediators of metabolism and physiology, including eicosanoids, diacylglycerol (DAG), inositol phosphates, and platelet activating factors. (3) Energy production due to the fact that any lipid class containing fatty acids can act as a source of energy, which is released through ß-oxidation of the acyl chains producing acetyl-CoA and NADH that are further metabolized via the tricarboxylic acid cycle and oxidative phosphorylation, respectively (Tocher et al., 2014). Although PL can be exploited as a source of fatty acids for oxidation, it is generally much more stable than adipose stores reflecting its critical roles in maintaining cell structure and function, with EFA being involved in both of

these roles (Sargent et al., 1993). TAG are the primary class for lipid storage and energy provision (Tocher et al., 2014).

2.5 Lipid metabolism

2.5.1 Synthesis of LC-PUFA

Lipogenesis is the term used to describe the biosynthetic reactions for the formation of new endogenous lipids (Tocher, 2003). *De novo* fatty acid biosynthesis in almost all organisms culminates in the formation of either C_{16} or C_{18} saturated fatty acids. These fatty acids are then modified through a sequence of desaturases and elongases so that an extended range of unsaturated PUFAs are produced (Ratledge, 2004). It is generally accepted that little or no EFA *de novo* synthesis takes place in humans; however, many animals have the capacity to convert ALA to EPA and DHA, and for some animals such as salmonids the efficiency of conversion appears to be low, in particular to DHA (Ratnayake & Galli, 2009). The liver is where most biosynthesis of these fatty acids occurs (Goodridge, 1991 Galindo et al., 2021). In the liver, dietary ALA is first metabolized to stearidonic acid (SDA, 18:4ω3) by the Δ^6 desaturase. This first reaction is a rate-limiting step and competes with the conversion of LA to ARA in ω6 fatty acid metabolism. SDA can be elongated to form eicosatetraenoic acid (ETA, 20:4ω3) that can be desaturated by Δ^5 desaturase to form EPA. At this stage, EPA can be further elongated to form docosapentaenoic acid (DPA, 22:5ω3), and DPA can be converted to DHA by Δ^6 desaturase with the involvement of limited peroxisomal β-oxidation (Scorletti & Byrne, 2013). The two pathways (i.e., ω6 and ω3) are independent of each other, and there are no cross-reactions, see figure 2.5 (Ratnayake & Galli, 2009).

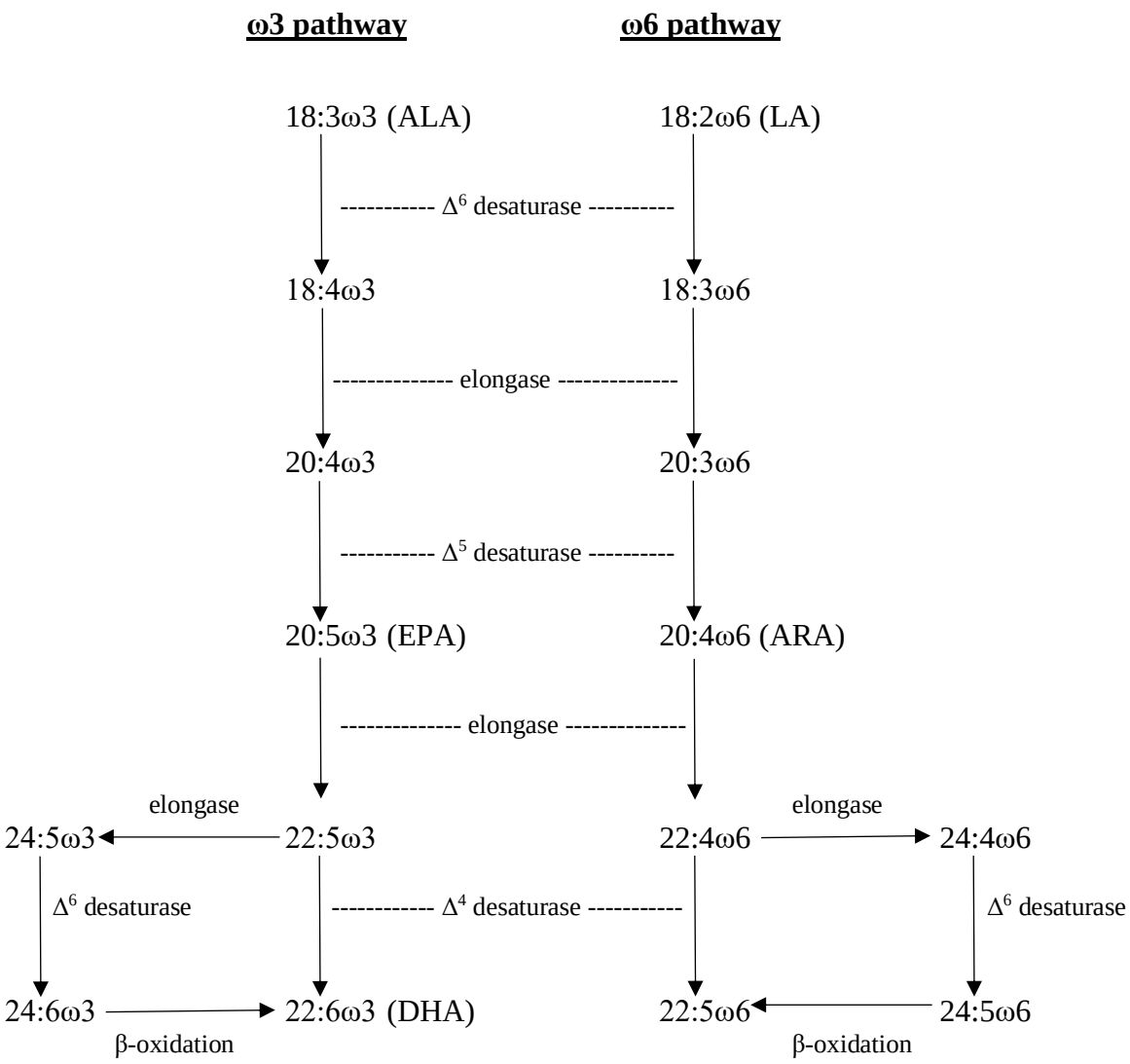


Figure 2.5 Biosynthesis pathway of ω6 and ω3 fatty acids (Modified from Ratnayake & Galli, 2009).

2.5.2 Lipid digestion, absorption, and transport

2.5.2.1 Digestion

The general mechanisms involved in lipid digestion, absorption, and transport in fish are similar to those described in mammals (Borges et al., 2013). Digestion is the process by which large food molecules are broken into smaller components that are small enough to be absorbed by

the gastrointestinal tract lining (Ratnayake & Galli, 2009). Dietary lipid in fish is largely composed of a combination of TAG and PL, with sterols, free fatty acids, pigments, and waxes comprising additional minor lipid components (Glencross, 2009). The process of digestion converts triacylglycerols into 2-monoacylglycerols, cholesterol esters into cholesterol, and phospholipids into their lyso-derivatives (Brindley, 1991). The hydrophobic nature of lipids makes them difficult to digest; however, difficulties associated with digestion in most animals are usually overcome by emulsification with other lipids or bile salts (Glencross, 2009). Most fish have well-developed biliary emulsifiers (bile salts) produced by the liver and stored in the gall bladder to aid the lipid digestion process (Glencross, 2009). Dietary lipids, primarily TAG, are hydrolyzed in the lumen of the gut by pancreatic lipases to 2-monoacylglycerols and free fatty acids (Sheridan, 1988). It is generally understood that short-chain fatty acids (2–10 carbons) and glycerol are absorbed directly through the brush border of the enterocytes. Long-chain fatty acids (12 and more carbons) are cleaved by lipase and emulsified by bile salts to form negatively charged aggregates called micelles (Rust, 2003). These micelles are then absorbed into the cells of the digestive tissue from the lumen, similar to lipid absorption in most animal cells (Glencross, 2009). Dietary phospholipids, less resistant than biliary phospholipids, are hydrolyzed by activated pancreatic phospholipase A_2 in the presence of trypsin, calcium ions, and bile salts, yielding 1-lysophospholipids and free fatty acids that are absorbed by the intestinal mucosal cells. Cholesterol esters are hydrolyzed by pancreatic cholesterol ester hydrolase, activated by bile salts, into free fatty acids and free cholesterol (Carlier et al., 1991).

2.5.2.2 Absorption

The absorption of the products of lipid digestion has not been as extensively studied in fish as in mammals; however, the basic physical processes, including bile-enhanced emulsification and

transport of the hydrolyzed products in fish, are assumed to be generally similar to that in mammals. Thus, the main hydrolytic products are solubilized or emulsified in bile salt micelles, followed by diffusion to the intestinal mucosa where uptake into the enterocytes occurs, mainly by passive diffusion (Tocher, 2003). As in mammals, lipid absorption in fish occurs predominantly in the proximal part of the intestine, coinciding with the highest lipolytic activity. However, lipid can be absorbed along the entire length of the intestine, although, as with digestive function, in diminishing amounts (Tocher, 2003). In mammals, long-chain fatty acids, 2-monoacylglycerol, lysophospholipids, and cholesterol are mixed with bile salts and lecithin (a PL found in bile) to form micelles, which are polymolecular aggregates of fatty elements clustered together with bile salts in a way that the polar (hydrophilic) ends of the molecules face the water and the non-polar portions (hydrophobic) form the core (Ratnayake & Galli, 2009). The micelles are very small particles (10 - 100 nm) and easily diffuse by passive diffusion, between microvilli of the enterocyte of the intestinal wall and come in close contact with the luminal cell surface (Ratnayake & Galli, 2009).

2.5.2.3 Transport

Similar to absorption, the transport system in fish has also not been as extensively studied as that of mammals. Transport of nutrients from the intestinal lumen into the enterocyte can occur by pinocytosis, simple diffusion, ion exchange, or active transport. Pinocytosis can transport large complex compounds that are later digested intracellularly or used for other purposes such as priming the immune system or recycling components of digestive secretions (enzymes, bile salts, etc.). Diffusion or “nonsaturable” transport is driven by concentration gradients of the nutrient between the lumen and the enterocyte. Ion exchange is selective and functions to maintain the

electrical potential of the tissue. Active transport is selective and often requires a sodium gradient to pump the nutrient across the brush border (Rust, 2003).

2.6 Lipid sources in fish feeds

Fats and oils are lipid sources, fats being the term for lipids that are solid at room temperature and oils being the term for lipids that are liquid at room temperature (Hardy & Barrows, 2003). The main factors determining which lipid source to use in fish feed formulations are the fatty acid composition of the lipid source and its physical characteristics at ambient temperatures, which dictate how it must be stored and handled at the feed mill temperature (Hardy & Barrows, 2003). FM and FO derived from industrial fisheries, e.g., capelin, herring, sand eel, mackerel, anchovy, and sardine fisheries, have been the standard ingredients of bulk feed for intensively farmed fish, above all salmonids and marine fish, for many years (Sargent et al., 2003). FM is a highly regarded source of feed proteins since it is easily digestible and has an excellent composition of essential amino acids. Other essential nutrients present in the meal are the ω3 LC-PUFA, EPA, and DHA (Olsen & Hasan, 2012). FO is the main lipid source in aquafeeds for most species, as it is an excellent source of ω3 EFA (Oliva-Teles, 2012).

Total fish production in 2016 was up 5.0% (54.1 million tonnes) from the previous year, with over 85.7% of fish production being freshwater fish species (mainly carps, tilapia, and catfishes), and to a lesser extent, 9.2% were diadromous fish species (including salmonids, milkfish, eels, etc.), and 5.1% were marine fish species (Tacon, 2018). Since feed represents ~50% of the total operating costs associated with aquatic farming of carnivorous species, the industry has made vigorous efforts to address this dilemma by pursuing and incorporating FM and FO alternatives in their formulations with a broad range of terrestrial animal- and plant-based feed inputs (Tibbetts et al., 2020). Replacing FM and FO is not as simple as replacing them with any

alternative meal or oil. The solution to replacing FM and FO requires retaining as far as possible the health-promoting properties of the end product for the consumer, which means retaining as far as possible the current high levels of EPA and DHA in farmed fish (Sargent et al., 2003).

2.6.1 Terrestrial plant oils

The use of terrestrial plant oils in aquafeed has increased in the past few decades. Palm oil, soybean oil, and canola oil are three major terrestrial plant oils used in aquafeed as alternatives to FO (Alhazzaa et al., 2019). There have been numerous studies that have shown that dietary terrestrial plant oils can successfully replace FO without affecting the health and growth of the fish; however, the ω3 LC-PUFA concentration in the tissue tends to be reduced (Bell et al., 2001, 2003, 2010; Torstensen et al., 2005). Generally, the fatty acid profile of the tissues reflects that of the diets. Unlike FO, which is an excellent source of ω3 PUFA, most terrestrial plant oils are relatively poor sources of ω3 PUFA and completely lack ω3 LC-PUFA. Rather, they are rich sources of ω6 and ω9 fatty acids, mainly LA and 18:1ω9, with the exception of some oilseeds (Hixson, 2014). Hence the low concentration of ω3 LC-PUFA observed in those studies.

2.6.2 Animal fat

Animal lipid sources can generally be divided into those from either marine or terrestrial origin. Alternative marine lipid sources, while providing many of the key attributes of fish oils, also pose similar questions about sustainability issues. Terrestrial lipid sources are recognized as sustainable and are, in fact, increasing in production (Glencross, 2009). Rendered terrestrial animal fats are rich in SFA and MUFA with a low to moderate content of ω6 PUFA and traces of ω3 LC-PUFA (Alhazzaa et al., 2019). When rendered terrestrial animal fats replace up to half of FO in aquafeeds, growth performance tends to be as efficient as those fed exclusively on FO. With higher

levels of FO replacement with rendered terrestrial animal fats, aquafeed formulations must ensure adequate amounts of ω3 and ω6 LC-PUFA are added to maintain efficient growth and health (Alhazzaa et al., 2019).

2.6.3 Microalgae and single-cell oils

Algae are a diverse group of aquatic, photosynthetic organisms generally categorized as either macroalgae (i.e., seaweed) or microalgae (unicellular) (Hemaiswarya et al., 2011). The use of microalgae in aquafeed is not new. Microalgae are widely used in the aquaculture industry, mostly to feed fish, crustaceans, and bivalves directly (Meireles et al., 2003). The PUFA content of microalgae can be modulated by culture conditions, and several studies aimed at the optimization of culture parameters are available (Meireles et al., 2003). Single-cell oils (SCO) are microbial oils considered a promising oil alternative to those from fish and land-based plant sources. The term was created to define oils produced by single-celled microorganisms such as yeasts and moulds (Armenta & Valentine, 2013). However, it has now expanded to include all fatty acid-containing lipids within a single cell, including algal lipids (Ratledge, 2013). Nutritionally, both microalgae oil and SCO are likely to be the best alternative to FO due to their high content of ω3 LC-PUFA. Table 2.1 provides a summary of commonly used microorganism species in aquaculture and their oil content (% dry weight).

Table 2.1: Oil content of several commonly used microalgae and microorganism species in aquaculture (adapted from Armenta & Valentine, 2013; Ryckebosch et al., 2012)

Species	Oil content (% dry weight)
Chlorella sp.	28–32
Isochrysis	7 – 33
Nannochloropsis	16 – 68
Pavlova	7 – 36
Phaeodactylum	15 – 40
Schizochytrium sp.	50–77
Skeletonema	3 – 25
Tetraselmis	6 – 26
Thalassiosira	9 – 26

2.7 Compound-specific stable isotope

Stable isotope analysis can be used to study the food habits of a variety of species. Carbon stable isotope ratios in consumers reflect mainly the photosynthetic pathway used by primary producers. Carbon is a conservative tracer that can be used to trace energy sources in food webs because isotope values increase only slightly between diet and consumer (0–1‰) during trophic transfers (Logan et al., 2008). Bulk isotope analyses are commonly employed to authenticate aquaculture products because marine diets tend to have more positive $\delta^{13}C$ and $\delta^{15}N$ values than terrestrial diets. However, a drawback of bulk isotopes is that environmental factors and diet quality can confound isotope values among different sources and impart variable and relatively poorly constrained isotope fractionations during trophic transfer (Wang et al., 2018), and is also not specific to trace a specific compound which has advantages in tracing fatty acid synthesis and storage. With advancements in instrumentation, researchers are now able to isolate a specific compound, and run a stable isotope analysis, hence the name compound-specific stable isotope analysis (CSIA). The advantage to using the compound-specific stable isotope method is that it uniquely pairs bulk stable isotope and fatty acid profiling to allow for specific quantitative sourcing of each fatty acid. Furthermore, the combination of individual fatty acid composition with stable isotope ratios is a more viable approach than bulk values to identify and differentiate sources of

organic matter (Colombo et al., 2016). In this project, CSIA was applied in Chapter 4 to determine the proportion of synthesized LC-PUFA (EPA, DHA, ARA) in tissues of salmon fed two extreme diets (FM; AB) and to understand the extent these fatty acids were synthesized from their precursors in *Pav*459 or digested and stored from other lipid sources present in the diet.

2.8 Thesis Outline

The goal of this thesis was to study the dietary effect of replacing FO with MO (from *Schizochytrium* sp.) and the dietary effect of reducing FO and replacing FM with AB (from *Pavlova* sp.) on Atlantic salmon muscle and liver tissue composition with a focus on the phospholipid fatty acid composition of membranes. As previously mentioned in section 2.6, one of the goals for the replacement of FO and FM with alternative lipid sources, besides sustainability, is to retain high levels of LC-PUFAs in the tissue for the consumer. Therefore, it was hypothesized that by feeding a DHA-rich MO and an AB to Atlantic salmon will result in tissue levels of LC-PUFAs similar to those found in fish-fed FO and FM diets. Also, in Chapter 4, we conducted a stable isotope analysis to determine the relative contribution of dietary *Pavlova* to the tissues LC-PUFAs. It was hypothesized that there would be direct incorporation of dietary *Pavlova* LC-PUFAs in the tissues, as well as biosynthesis of LC-PUFAs from its respective ω3 and ω6 precursors.

3. EFFECTS OF REPLACEMENT OF FISH OIL WITH MICROBIAL OIL (*SCHIZOCHYTRIUM* SP. T18) ON PHOSPHOLIPID FATTY ACID COMPOSITION OF ATLANTIC SALMON PARR MUSCLE AND LIVER TISSUES

3.1 Abstract

A microbial oil (MO) from *Schizochytrium* sp. (T18), rich in docosahexaenoic acid (DHA), was used as a potential replacement to fish oil (FO) in the diet of farmed Atlantic salmon. A 16-week feeding trial was conducted to investigate the dietary effect of replacing FO with MO on total lipid class and fatty acid composition in Atlantic salmon parr liver and muscle tissues. Four nutritionally balanced diets were formulated with varying levels of FO, MO, and canola oil (CO). A control diet (20% FO), a secondary control diet (10% FO + 10% CO), and two test diets consisted of a complete replacement of FO with a low (5%) and a high (10%) proportion of MO. There were no significant differences in growth parameters among the dietary treatments (81–98 g; weight gain). There were no significant differences in total lipid class composition among the dietary treatments. There were significant differences in proportions of individual ω3 and ω6 fatty acids among the total fatty acids in both muscle and liver tissues reflecting the dietary treatments; however, the proportion of DHA in the tissue depended less on the diet composition. The presence of low EPA in the MO diets did not affect the growth performance of the fish, suggesting a lower requirement for EPA in the diet and a greater necessity for DHA. Furthermore, the phospholipid fatty acids results shared similar patterns as with total fatty acids, but the long-chain polyunsaturated fatty acid precursors, linoleic acid and α-linolenic acid, were present in low proportions, and DHA was present in very high proportions in the cellular membrane, especially in muscle tissue.

3.2 Introduction

Fish oil (FO) is an excellent source of omega-3 (ω3) long-chain polyunsaturated fatty acids (LC-PUFA), and despite its limited supply and continuous cost increase, it remains the primary lipid source for aquafeed. The continuous growth of aquaculture and the constraints that utilization of FO and fish meal impose have resulted in research on alternative and more sustainable lipid sources for aquafeeds. Several studies have been conducted replacing FO with terrestrial plant oils either partially or fully (e.g., Bell et al., 2001; Bell et al., 2003; Torstensen et al., 2005). Generally, most studies have shown that although terrestrial plant oils do not affect the growth parameters of the fish, it does affect the composition of ω3 LC-PUFA in tissues. This is because most terrestrial plant oils are composed mainly of ω6 and ω9 fatty acids and lack the critical long-chain ω3 PUFA that are abundant in FO (Hixson et al., 2013). Marine fish and salmonids require arachidonic acid (ARA, 20:4ω6) and high levels of essential ω3 fatty acids, eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3), as they cannot synthesize them easily (Beheshti Foroutani et al., 2018). Although salmonids have shown the ability to further elongate and desaturate linoleic acid (LA, 18:2ω6) and α-linolenic acid (ALA, 18:3ω3) to form physiologically essential fatty acids (EFA), EPA, DHA, and ARA, they do so with limited capacity (Sargent et al., 1999; Ruyter & Thomassen, 1999). Therefore, it is important to ensure that these EFA are provided in fish diets as they contribute to the health of fish and humans as consumers (Beheshti Foroutani et al., 2018). Studies show that these EFA are essential for normal larval development, fish growth, and reproduction. They are important in the normal development of the skin, nervous system, and visual acuity in fish (Miles & Chapman, 2006). They are also known to provide health benefits to humans as consumers in relation to cardiovascular disease, inflammatory disease, and neurological disorders (Calder, 2004; Dyall & Michael-Titus, 2008; Simopoulos, 2002).

EFA are also components of phospholipids (PL) which play a major role in maintaining membrane fluidity (Kattner & Hagen, 2009). Cellular membranes act not only as a biological barrier but also as a finely tuned system that controls ion permeability, membrane-associated enzymes, receptors, and eicosanoid production. Any alteration of membrane lipid composition may lead to cellular metabolic disorders (Leonardi et al., 1987). Phosphoglycerides, often referred to generally as PL, are characterized by a phosphatidic acid (PA) backbone with two fatty acids esterified at the *sn*-1 and *sn*-2 positions and are the most abundant phospholipids in cell membranes. The major PL classes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI), formed by the esterification of the "bases" choline, ethanolamine, serine, and inositol to the phosphate group of PA (Tocher et al. 2008). Very little is known about molecular species and regiospecificity function in fish. Regiospecificity is the position of individual FA on the glycerol backbone of both storage and membrane lipids (Miller et al., 2006). A common generalization has been that SFA and MUFA are preferentially esterified on position *sn*-1 of PL, with PUFA preferentially esterified on position *sn*-2 (Tocher et al., 2008). To our knowledge, most feeding trials with alternative lipid sources do not quantify PLFA. Therefore, we quantified PLFA to provide a more in-depth study of the influence dietary lipids have on the membranes of Atlantic salmon parr liver and muscle tissues.

Feed composition has changed considerably over the last decades from mainly marine ingredients to an increasing inclusion of plant ingredients (Aas et al., 2019). While terrestrial plant oils can provide digestible energy to the fish, fish health and the consumer products resulting from those fish have become compromised in recent years (Tibbetts et al., 2020b). As an alternative to terrestrial plant oils, attention has turned to marine lipid sources rich in long-chain ω3 PUFA, such

as microalgae and other marine microorganisms, as they show the potential to replace conventional raw materials used in aquafeed (Shah et al., 2018; Sprague et al., 2017).

The microbial oil (MO) used in this study was isolated from a novel strain, *Schizochytrium* sp. (T18), from the group of microorganisms known as thraustochytrids. Thraustochytrids are non-photosynthetic marine protists classified into the class Labryinthula of the kingdom Chromista, including genera such as *Thraustochytrium*, *Aplanochytrium Japonochytrium*, *Ulkenia*, and *Schizochytrium* (Burja et al., 2006). Thraustochytrids are often mistakenly called microalgae when discussing their potential biotechnological applications. Although they are closely related to brown algae, thraustochytrids are not algae, and no literature classifies them as such (Armenta & Valentine, 2013). Among numerous strains, *Schizochytrium* sp. is noteworthy and often considered as a satisfactory alternative to FO due to the advantages of fast growth rate, high productivity, and its lipid profile (Ren et al., 2010). *Schizochytrium* sp. is characterized by high lipid content (55–75% of dry matter) and up to 49% DHA of total lipids and is commonly heterotrophically cultivated for large-scale production (Shah et al., 2018). It is worth noting the low proportion of EPA (0.5%) present in MO and how this might affect growth parameters, the immune system, and lipid deposition in the tissues. The present study aimed to examine the potential of MO as a possible replacement to FO in the diet of farmed Atlantic salmon parr and its influence on the fatty acid composition of the membrane of liver and muscle tissues, in addition to total lipid, lipid class, and total fatty acid compositions of the same tissues.

3.3 Materials and Methods

The feeding study was done in collaboration with our Dalhousie University partners, M.Sc. student Minmin Wei and supervisor Dr. Stefanie Colombo in Truro, Nova Scotia. Diet manufacture and feeding trials were done in Truro.

3.3.1 Experimental diets

The diets used in this experiment were formulated as follows: a control diet (FO) composed of 20% FO; a second control diet (FO/CO) composed of a 50/50 blend of FO (10%) and canola oil (CO) (10%); a test diet (LMO) composed of complete replacement of FO with a lower proportion of MO (5%); a second test diet (HMO) composed of complete replacement of FO with a higher proportion of MO (10%). For extended details on diet formulation, see Table B1 in Appendix B. The four diets were formulated to be isonitrogenous, isocaloric and to meet the nutritional requirements of Atlantic salmon in accordance with National Research Council (NRC), 2011. The MO used in this experiment was provided by Mara Renewables (Dartmouth, NS, Canada).

3.3.2 Experimental fish and set-up

Atlantic salmon parr were received from the Margaree Fish Hatchery (Nova Scotia Department of Fisheries and Aquaculture, Margaree Valley). A total of 360 parr (21.9 ± 4.7 – 26.8 ± 4.1) (mean ± SD) were randomly distributed into 12 tanks (200 L volume) in a flow-through freshwater system at Dalhousie University Agriculture Campus Aquaculture lab (Truro, NS). A completely randomized design was used, and the tank was the experimental unit with three replicates. The salmon were fed commercial feed (3 mm EWOS Vita feed; 43% crude protein, 14% crude fat, maximum 3% fibre) twice a day for a two-week acclimation period after transfer into the system. Fish were hand-fed to satiation with experimental feed for 16 weeks after the initial sampling (week 0) twice a day at 9 AM and 3 PM. Hand feeding was performed carefully to ensure minimal feed waste, and feed consumption was recorded weekly. Mortalities (if any) were weighed and recorded throughout the trial (Wei et al., 2021).

3.3.3 Tissue sampling

Feed was withheld one day before the sampling day for accurate weighing. Five fish per tank were randomly sampled from each tank at week 0 (before feeding experimental diets) and at the end of the trial (week 16). Ethical treatment of fish in this experiment followed guidelines according to the Canadian Council of Animal Care (Dalhousie University Faculty of Agriculture Institutional Animal Care Approved Protocol #2017-84). Individual fish were rapidly netted and euthanized with an overdose of anesthetic using tricaine methane sulfonate (MS222, administered at 150 mg/L) (Sigma Chemicals, St. Louis, MO, USA), and clinical signs of death were ensured prior to sampling. The skin was removed on the left side, and white dorsal muscle was subsampled for subsequent analysis. The skinless dorsal muscle tissue, as well as liver samples, were taken for protein, energy, lipid class, and fatty acid composition analysis. The samples were flash-frozen in liquid nitrogen immediately after sampling and stored at -80°C (Wei et al., 2021). The sampled tissues were then placed in lipid-clean glass vials with chloroform. The air space was filled with nitrogen gas before capping the vials and sealing them with Teflon tape. The samples were then stored in a -20°C freezer until extraction.

3.3.4 Lipid extraction

Lipid samples were extracted according to Parrish (1999). Samples were homogenized using Tissue Master 125 homogenizer (Omni International, Kennesaw, GA, USA) in a 2:1 mixture of ice-cold chloroform:methanol. Chloroform extracted water was added to bring the ratio of cholorform:methanol:water to 8:4:3. The sample was sonicated for 4 min in an ice bath and centrifuged at 5000 rpm for 3 min. The bottom organic layer was removed using a double pipetting technique, placing a long lipid-clean Pasteur pipette inside a short one to remove the organic layer without disturbing the top aqueous layer. Chloroform was then added back to the extraction test

tube, and the entire procedure was repeated three more times. All the organic layers were pooled into a lipid-clean vial.

3.3.5 Fatty Acid Methyl Ester (FAME) Derivatization

To form fatty acid methyl esters (FAME), an aliquot of lipid extract was transferred to a lipid-clean 7 ml vial and evaporated under nitrogen to dryness. Then 1.5 ml of methylene chloride and 3 ml Hilditch reagent were added. The Hilditch reagent is prepared by dissolving 1.5 ml concentrated sulfuric acid in 100 ml methanol that has been dried over anhydrous sodium sulphate. The mixture was capped under nitrogen, then vortexed and sonicated for 4 min before being heated at 100°C for 1 hr. The mixture was allowed to cool to room temperature, and then approximately 0.5 ml saturated sodium bicarbonate solution was added, followed by 1.5 ml hexane. The mixture was shaken, and the upper organic layer was transferred to a lipid-clean 2 ml vial. The upper organic layer was blown dry under a constant stream of nitrogen gas and refilled with hexane to approximately 0.5 ml, capped under nitrogen and sealed with Teflon tape, then sonicated for another 4 min to re-suspend the fatty acids.

3.3.6 Neutral lipid/polar lipid (NL/PL) separation

The NL/PL separation was done using Strata SI-1 silica tubes (Phenomenex, Torrance, CA, USA) in a vacuum chamber. First, the silica tube was rinsed with 6 ml of methanol, 6 ml of chloroform, and 3 ml of a solvent mixture of 98:1:0.5 chloroform:methanol:formic acid through the column into a waste vial. Then the sample extract was directly applied to the silica using a long pipette followed by rinsing the sample vial with a small amount of chloroform. The waste vial was replaced with a lipid-clean 15 ml vial, then 8 ml of the solvent mixture (98:1:0.5 mixture of chloroform: methanol: formic acid) was eluted through the column to collect all neutral lipid-

containing eluent. A second 15 ml vial was replaced to recover the AMPL by rinsing the silica gel with 6 ml (2 x 3 ml) of acetone. The vial containing the AMPL fraction was replaced with a large 40 ml vial, and 3 ml of chloroform was passed through the column to remove any acetone. Phospholipids were eluted with two volumes (6 ml) of methanol followed by 9 ml of a mixture of chloroform:methanol:water (5:4:1). The PL fraction was transferred to a 50 ml round bottom flask and dried completely in a flash-evaporator. The lipids were then washed into a 15 ml vial using methanol and chloroform. The PLFA was derivatized using the same procedure as total FAME (section 3.3.5).

3.3.7 Quantitative lipid analysis

Lipid classes were determined using thin-layer chromatography with flame ionization detection (TLC-FID) in a Mark VI Iatroscan (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan). Silica coated Chromarods, and a three-step development method was used Parrish (1987). Each lipid extract was spotted on an individual rod using a 20 µL Hamilton syringe and then focused to a narrow band using 100% acetone solution. The first development system consisted of hexane/diethyl ether/formic acid mixture (99:1:0.05). The rods were developed for 25 min and dried in a constant humidity chamber for 5 min before developing again in the same solution for 20 min. Upon completion of the first development, the rods were scanned in the Iatroscan (75% of the rod), which detects the hydrocarbon (HC), steryl ester (SE), and ketone (KET) lipid classes. After the first scan, the rods were dried in a constant humidity chamber for 5 min before starting the second development for 40 min. The second development system consists of hexane:diethyl ether:formic acid (79:20:1). On completion of the second development, the rods were scanned in the Iatroscan (89% of the rod) for the triacylglycerol (TAG), free fatty acids (FFA), alcohol (ALC), and sterol (ST) lipid classes. For the third and final development systems, the rods were developed

twice in 100% acetone for 15 min, dried for 5 min in a constant humidity chamber, then developed twice for 10 min in chloroform:methanol:chloroform-extracted water (50:40:10). On completion of the third development, the rods were scanned in the Iatroscan (100% of the rod) for the acetone mobile polar lipid (AMPL) and phospholipid (PL) lipid classes. The data were collected using Peak Simple software (ver. 3.67, SRI Inc., Torrance, CA, USA.). The Chromarods were calibrated using standards from Sigma Chemicals (Sigma Chemicals, St. Louis, MO, USA).

The FAME samples were analyzed on an HP 6890 gas chromatography (GC) FID equipped with a 7683 autosampler. The GC column was a ZB-WAXplus (Phenomenex). The column length was 30 m with an internal diameter of 0.32 mm. The column temperature began at 65^{o}C where it was held for 0.5 min. The temperature ramped to 195^{o}C at a rate of 40^{o}C/min, held for 15 min, then ramped to a final temperature of 220^{o}C at a rate of 2^{o}C/min. This final temperature was held for 0.75 min. The carrier gas was hydrogen flowing at a rate of 2 ml/min. The injector temperature started at 150^{o}C and ramped to a final temperature of 250^{o}C at a rate of 120^{o}C/min. The detector temperature stayed constant at 260^{o}C. Peaks were identified using retention times from standards purchased from Supelco (Superlco Inc., Bellefonte, PA, USA): 37 component FAME mix (Product number 47885-U), Bacterial acid methyl ester mix (product number 47080-U), PUFA 1 (product number 47033) and PUFA 3 (product number 47085-U). Chromatograms were integrated using the Agilent OpenLAB Data Analysis - Build 2.203.0.573 (Agilent Technologies, Inc., Santa Clara, CA, USA). A quantitative standard purchased from Nu-Chek Prep, Inc (product number GLC490) was used to check the GC column about every 300 samples (or once a month) to ensure that the areas returned were as expected.

3.3.8 Statistical analysis

The resulting data were presented as mean ± standard deviation. All statistical analyses were performed using general linear models in Minitab (version 18; Minitab Inc., State College, PA, USA). The model was designed to test diet effect (fixed factor) and nested tank (fixed factor) within diet to detect any tank effects on different lipid classes and fatty acids (response variable). The conditions, selection, and care of the tanks were purposely maintained identical and only applied to this experiment, hence the selection of tank as a fixed factor. Significant difference was set at fixed $\alpha = 5\%$ criterion ($p < 0.05$). Pairwise comparison was performed using Tukey *post hoc* test for multiple comparisons to detect differences between diets. Normality testing was performed using the Anderson–Darling test.

Principal coordinates analysis (PCO) was used to describe the resemblance and variation of the fatty acid composition in the muscle and liver tissue through a correlation matrix plotted by two PCO axes (i.e., PCO1, PCO2) (PRIMER, Plymouth Routines in Multivariate Ecological Research; PRIMER-E Ltd., version 6.1.15, Ivybridge, UK). The similarity of percentages analysis (SIMPER) was used to quantify differences among treatments in fatty acid data. In all cases, the non-parametric Bray-Curtis similarity was used.

3.4 Results

3.4.1 Diet composition

The total lipid composition for MO (determined by TLC-FID) was 753.8 mg/g and 953.4 mg/g (determined gravimetrically). Iatroscan values for aquatic samples are routinely ~90% of those obtained by gravimetry method. Gravimetric values tend to be higher because the Iatroscan determines non-volatile lipids, and it is possible that non-lipid material may be included in

gravimetric determinations (Parrish, 2013). The main lipid class was TAG (76.0%), followed by AMPL (10.4%), FFA (7.0%), PL (3.8%), and ST (1.8%) (Table 3.1). The dominant (>5%) fatty acids were 14:0 (11.0%), 16:0 (26.5%), ω6DPA (7.6%), and DHA (40.7%) (Table 3.1). Total PUFA (50.6%) accounted for half of the total fatty acids, followed by SFA (40.7%) and MUFA (8.7%). MO was rich in DHA (40.7%) and low in EPA and ARA (0.8%; 0.1%), respectively. Additionally, the MO was also high in ω6DPA and made a potential fatty acid biomarker for *Schizochytrium* sp. This biomarker was present in higher proportions in the tissues of salmon fed the MO-containing diets (LMO and HMO) than salmon fed the FO-containing diets (FO and FO/CO). The ω3 composition accounted for 42.4% of total PUFA, 5-fold higher than the ω6 composition, resulting in a 5.1 ω3/ω6 ratio.

The total lipid composition in the diets varied between 206.4 and 269.8 mg/g wet weight (ww) and mostly comprised of neutral lipids (Table 3.2). The main lipid classes were TAG and PL. There was no significant difference in lipid classes between FO-containing and MO-containing diets. Differences in total fatty acid proportions were minimal but often significant. The fatty acid composition of the FO diet was mainly PUFA (41.2%) followed by SFA (29.7%) and MUFA (28.1%), while FO/CO, LMO, and HMO diets were mainly MUFA (39.5 – 49.0%) followed by PUFA (33.4 – 37.5%) and SFA (17.5 – 23.0%) (Table 3.2). EPA and ARA proportions were significantly lower in MO-containing diets compared to FO diets, while the DHA proportion was significantly higher in HMO diet compared to FO diets. The long-chain ω6 and ω3 precursors LA and ALA varied from diet to diet. ω3 FAs were 14-fold more prevalent than ω6 FAs in FO-containing diets and approximately 7-fold more prevalent than ω6 FAs in MO-containing diets.

Table 3.1: Lipid class and total fatty acid composition of the microbial oil, *Schizochytrium* sp. (T18), used in the study[1]

Lipid class composition (%)	
Total lipid (mg/g)[2]	953.4 ± 5.0
Triacylglycerol	76.0 ± 4.4
Free fatty acids	7.0 ± 3.2
Sterols	1.8 ± 0.7
Acetone mobile polar lipids	10.4 ± 4.9
Phospholipid	3.8 ± 1.1
Fatty acid composition (%)	
14:0	11.3 ± 0.1
16:0	26.5 ± 0.4
18:0	1.0 ± 0.0
Total SFA[3]	40.7 ± 0.5
16:1ω7	4.6 ± 0.1
18:1ω9	1.0 ± 0.0
18:1ω7	3.0 ± 0.1
Total MUFA[4]	8.7 ± 0.1
18:2ω6 (LA)	0.4 ± 0.0
18:3ω6	0.1 ± 0.0
20:4ω6 (ARA)	0.1 ± 0.0
22:5ω6 (ω6DPA)	7.6 ± 0.0
18:3ω3 (ALA)	0.1 ± 0.0
18:4ω3	0.2 ± 0.0
20:4ω3	0.5 ± 0.0
20:5ω3 (EPA)	0.8 ± 0.0
22:5ω3	0.1 ± 0.02
22:6ω3 (DHA)	40.7 ± 0.3
Total PUFA[5]	50.6 ± 0.4
Total ω3	42.4 ± 0.3
Total ω6	8.2 ± 0.1
ω3/ω6 ratio	5.1 ± 0.0
EPA+DHA	41.5 ± 0.3
DHA/EPA ratio	51.9 ± 0.1

[1]Data expressed as percent lipid or fatty acid methyl ester (FAME); Values are means ± standard deviation (n=3 per treatment).
[2]Data determined gravimetrically.
[3]Saturated fatty acid.
[4]Monounsaturated fatty acid.
[5]Polyunsaturated fatty acid.

Table 3.2: Lipid composition of experimental diets[1].

	FO	FO/CO	LMO	HMO
Lipid class composition (%)				
Total lipid (mg/g)	206.4 ± 17.9	269.8 ± 12.8	240.5 ± 68.4	220.5 ± 48.1
Triacylglycerol	69.0 ± 6.3^{ab}	66.4 ± 1.2^{b}	77.3 ± 2.9^{a}	74.5 ± 4.3^{ab}
Free fatty acids	7.4 ± 1.2	6.3 ± 0.3	5.7 ± 0.3	6.6 ± 0.9
Sterol	1.5 ± 0.2	1.7 ± 0.5	2.2 ± 0.2	1.8 ± 0.7
Phospholipid	16.1 ± 2.7^{ab}	19.9 ± 0.9^{a}	12.3 ± 2.8^{b}	13.3 ± 3.7^{ab}
Fatty acid composition (%)				
14:0	6.1 ± 0.1^{a}	3.3 ± 0.1^{c}	2.6 ± 0.0^{d}	4.5 ± 0.2^{b}
16:0	18.0 ± 0.1^{a}	12.4 ± 0.1^{c}	11.4 ± 0.1^{d}	15.1 ± 0.3^{b}
18:0	4.0 ± 0.1^{a}	3.2 ± 0.1^{c}	2.3 ± 0.0^{d}	2.0 ± 0.0^{b}
Total SFA[2]	29.7 ± 0.3^{a}	20.2 ± 0.1^{c}	17.5 ± 0.1^{d}	23.0 ± 0.3^{b}
16:1ω7	6.8 ± 0.1^{a}	4.0 ± 0.1^{b}	2.0 ± 0.0^{d}	2.8 ± 0.2^{c}
18:1ω9	12.8 ± 0.2^{d}	32.5 ± 0.1^{b}	41.0 ± 0.1^{a}	29.6 ± 0.8^{c}
18:1ω7	2.4 ± 0.0	2.3 ± 0.1	2.3 ± 0.1	2.5 ± 0.1
20:1ω9	1.9 ± 0.1	1.9 ± 0.1	1.6 ± 0.0	1.7 ± 0.5
Total MUFA[3]	28.2 ± 0.3^{d}	43.9 ± 0.5^{b}	49.0 ± 0.1^{a}	39.5 ± 1.1^{c}
18:2ω6 (LA)	8.1 ± 0.0^{c}	14.6 ± 0.1^{b}	17.6 ± 0.1^{a}	14.2 ± 0.5^{b}
18:3ω6	0.2 ± 0.1^{a}	0.1 ± 0.0^{b}	0.0 ± 0.0^{b}	0.1 ± 0.1^{b}
20:3ω6	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
20:4ω6 (ARA)	1.0 ± 0.1^{a}	0.6 ± 0.1^{b}	0.2 ± 0.0^{c}	0.2 ± 0.0^{c}
22:4ω6	0.1 ± 0.0^{a}	0.1 ± 0.0^{ab}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
22:5ω6 (ω6DPA)	0.3 ± 0.0^{c}	0.2 ± 0.1^{c}	1.4 ± 0.0^{b}	2.7 ± 0.1^{a}
18:3ω3 (ALA)	1.2 ± 0.1^{c}	3.8 ± 0.0^{b}	4.9 ± 0.0^{a}	3.6 ± 0.2^{b}
18:4ω3	2.0 ± 0.0^{a}	1.1 ± 0.1^{b}	0.1 ± 0.0^{c}	0.2 ± 0.0^{c}
20:4ω3	0.6 ± 0.1^{a}	0.3 ± 0.1^{b}	0.1 ± 0.0^{c}	0.2 ± 0.0^{bc}
20:5ω3 (EPA)	12.8 ± 0.0^{a}	6.7 ± 0.3^{b}	0.7 ± 0.6^{c}	0.8 ± 0.1^{c}
22:5ω3	1.6 ± 0.0^{a}	0.8 ± 0.0^{b}	0.1 ± 0.0^{c}	0.1 ± 0.0^{c}
22:6ω3 (DHA)	8.0 ± 0.1^{b}	4.3 ± 0.1^{c}	8.1 ± 0.1^{b}	15.0 ± 0.6^{a}
Total PUFA[4]	41.2 ± 0.4^{a}	35.3 ± 0.5^{c}	33.4 ± 0.1^{d}	37.5 ± 1.1^{b}
Total ω3	27.0 ± 0.2^{a}	17.4 ± 0.4^{c}	14.0 ± 0.1^{d}	19.9 ± 0.6^{b}
Total ω6	9.9 ± 0.2^{d}	15.7 ± 0.1^{c}	19.3 ± 0.1^{a}	17.4 ± 0.6^{b}
ω3/ω6 ratio	2.7 ± 0.1^{a}	1.1 ± 0.0^{b}	0.7 ± 0.0^{c}	1.1 ± 0.0^{b}
EPA+DHA	20.8 ± 0.1^{a}	11.0 ± 0.4^{c}	8.7 ± 0.1^{d}	15.9 ± 0.5^{b}
EPA+DHA g/kg feed	3.73 ± 0.4	2.53 ± 0.1	1.87 ± 0.5	3.10 ± 0.8
DHA/EPA ratio	0.6 ± 0.0^{c}	0.6 ± 0.0^{c}	12.2 ± 1.1^{b}	18.6 ± 3.3^{a}
EPA/ARA ratio	12.5 ± 0.8^{a}	11.1 ± 1.6^{a}	3.4 ± 0.3^{b}	3.6 ± 0.5^{b}
DHA/ARA ratio	7.8 ± 0.5^{c}	7.0 ± 0.9^{c}	40.8 ± 2.9^{b}	66.7 ± 6.7^{a}

[1]Data expressed as percent lipid or fatty acid methyl ester (FAME); Values are means ± standard deviation (n=3 per treatment). Means with different superscripts indicate significant differences ($p < 0.05$) based on Tukey's *posthoc* test following a general linear model analysis; FO = fish oil; FO/CO = fish oil/canola oil; LMO = low microbial oil; HMO = high microbial oil.

[2]Saturated fatty acid.

[3]Monounsaturated fatty acid.

[4]Polyunsaturated fatty acid.

3.4.2 Growth performance

There were no significant differences in all measured (weight, length, weight gain) and calculated (condition factor, visceral somatic index, specific growth rate, apparent feed intake, and feed conversion rate) parameters among the dietary treatments, resulting in over 300% growth from their initial weight (~25 g). There were no mortalities throughout the study. The full details for growth performance, colour, and texture analysis were published in Wei et al. (2021), and the results are attached in Appendix B.

3.4.3 Liver tissue lipid classes and fatty acid composition

Initial liver tissue composition contained 34.4 mg/g wet weight (ww) total lipid, and it was mostly composed of polar lipid (Table 3.3). After 16 weeks of feeding, there was no major significant change in total lipids among the dietary treatments (35.0 – 37.4 mg/g ww) (Table 3.3). The tissue was mostly composed of polar lipid in all dietary treatments. PL was the only lipid class that increased in total proportion in the liver tissue of salmon fed the FO/CO diet (79.3%) and decreased in salmon fed the LMO diet (75.5%).

After 16-weeks of feeding, the fatty acid profile mostly reflected that of the fish fed diets, except the relative proportions of MUFA, PUFA, and SFA (Table 3.3). The fatty acid composition of the salmon fed the FO diet was mostly PUFA (58.5%), followed by SFA (24.3%) and MUFA (16.9%), while salmon fed the FO/CO, LMO, and HMO diets were mostly PUFA (52.8 – 55.9%), followed by MUFA (22.4 – 28.0%) and SFA (19.1 – 21.6%). DHA was the dominant EFA; however, differences in EFA proportion including LC ω6 and ω3 precursors, LA and ALA, were observed between salmon fed the FO diet and other dietary treatments. The LC-PUFA ω6DPA was higher in salmon fed MO-containing diets (LMO, 3.3%; HMO, 4.4%) than salmon fed FO-

containing diets (FO, 0.6%; FO/CO, 0.4%). The ω3 fatty acids were ~4-fold higher than ω6 fatty acids in salmon fed FO-containing diets and ~ 2-fold higher than ω6 fatty acids in salmon fed MO-containing diets. Compared to week-0, salmon fed the FO diet had the highest increase in ω3 fatty acid proportion (47.0%), and it was the only treatment that had a decrease in ω6 fatty acid proportion (9.9%). On the other hand, salmon fed the LMO diet had the highest increase in ω6 fatty acid proportion (18.2%), and it was the only treatment with a decrease in ω3 fatty acid proportion (34.5%).

Principal coordinates analysis of week-16 liver total fatty acids showed PCO1 and PCO2 (Fig. 3.1) accounted for 72.5% and 23.8% of variability, respectively. The PCO biplot showed a higher variation between salmon fed the FO diet and salmon fed the LMO diet and less variation between salmon fed the MO-containing diets. SIMPER analysis (Table C1 and Table C2 in Appendix C) showed there was an average of 93% similarity within groups of the same dietary treatments and different percentages of dissimilarities between salmon fed different diets. The highest dissimilarity was between salmon fed the FO and LMO diet (23.8%), confirming the spatial distribution observed in the PCO biplot. The second highest dissimilarity was between salmon fed the FO diet and HMO diets (18.9%), which makes sense based on the PCO biplot. The top driver for the similarity within the diet groups was DHA across all dietary treatments, and the top drivers for the dissimilarities between different treatments varied among 18:1ω9, EPA, and DHA. For extended details on average similarities and dissimilarities results, see Appendix C.

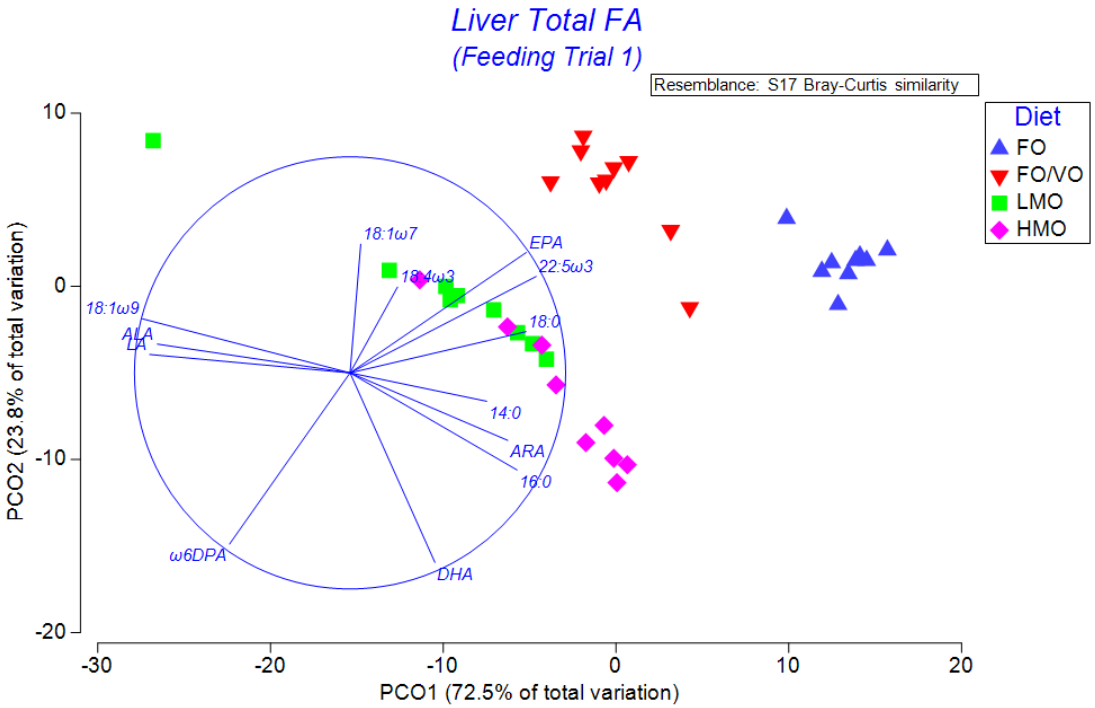


Figure 3.1: Principal coordinates analysis (PCO) of Atlantic salmon liver tissue total fatty acid composition (%) after 16 weeks of feeding experimental diets

3.4.4 Liver tissue phospholipid fatty acid composition

The PLFA composition was mainly PUFA (55.0 – 56.7%) followed by SFA (25.6 – 29.3%) and MUFA (13.6% - 17.9%) (Table 3.4). Differences in EFA proportion, including LC ω6 and ω3 precursors, LA and ALA, were minimal across the dietary treatments except for EPA. Salmon fed FO-containing diets had significantly higher EPA proportions (FO, 8.0%; FO/CO, 6.7%) than salmon fed MO-containing diets (LMO, 1.7%; HMO, 1.4%). The LC-PUFA ω6DPA was also found embedded in the membrane in higher proportions in salmon fed MO-containing diets (LMO, 4.1%; HMO, 4.7%) than salmon fed FO-containing diets (FO, 0.6%; FO/CO, 0.5%). The ω3 fatty acids were ~4-fold higher than ω6 fatty acids in salmon fed FO-containing diets and ~2-fold higher than ω6 fatty acids in salmon fed MO-containing diets. The DHA/EPA ratio was significantly higher in salmon fed MO-containing diets (LMO, 21.2%; HMO, 27.8%) than salmon fed FO-

containing diets (FO, 4.4%; FO/CO, 5.0%). The EPA/ARA ratio was significantly higher in salmon fed FO-containing diets (FO, 2.0%; FO/CO, 1.9%) than salmon fed MO-containing diets (LMO, 0.5%; HMO, 0.4%). As for the DHA/ARA ratio, differences were minimal among the dietary treatments.

Principal coordinates analysis of week-16 liver PLFA showed PCO1 and PCO2 (Fig. 3.2) accounted for 66% and 17.6% variation, respectively. The PCO biplot showed that the highest variation in liver PL was between salmon fed the FO and LMO diets. SIMPER analysis (Table C3 and Table C4 in Appendix C) showed that there was an average of 94% similarity within the same dietary groups, and also confirmed spatial distributions in the PCO biplot in that the highest dissimilarity was between salmon fed the FO and LMO diets (17.8%) and the lowest dissimilarities was between salmon fed the LMO and HMO diets (7.9%). The top driver for the similarities in the liver PL was DHA, and the top driver for the dissimilarities varied among 18:1ω9, EPA, and DHA. For extended details on average similarities and dissimilarities results, see Appendix C.

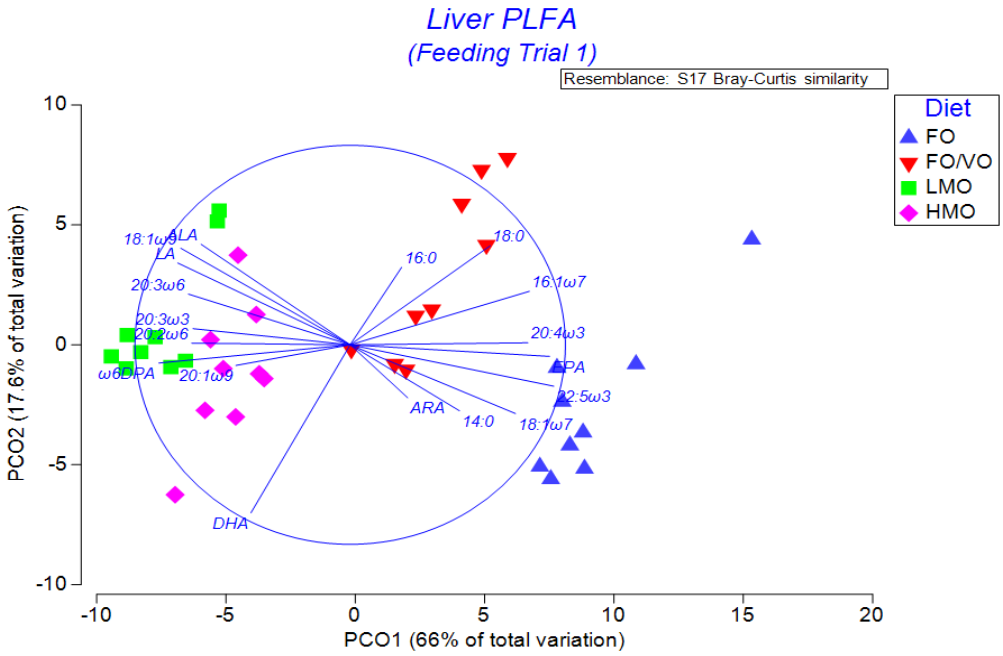


Figure 3.2: Principal coordinates analysis (PCO) of Atlantic salmon liver tissue phospholipid fatty acid composition (%) after 16 weeks of feeding experimental diets

Table 3.3: Lipid class and total fatty acid composition of Atlantic salmon liver tissue, prior to feeding experimental diets and after 16 weeks of feeding experimental diets[1]

	Initial	FO	FO/CO	LMO	HMO
Lipid composition (%)					
Total lipid (mg/g)	34.4 ± 8.7	35.03 ± 4.28	37.44 ± 6.74	37.41 ± 6.22	35.77 ±4.72
Neutral lipid	34.0 ± 9.2	16.8 ± 4.4	15.6 ± 3.3	18.9 ± 5.5	17.6 ± 4.4
Polar lipid	66.0 ± 9.2	83.2 ± 4.4	84.4 ± 3.3	81.1 ± 5.5	82.4 ± 4.4
Lipid class composition (%)					
Triacylglycerol	5.7 ± 10.0	0.1 ± 0.1^{b}	0.7 ± 0.8^{ab}	0.4 ± 0.6^{ab}	2.8 ± 4.2^{a}
Free fatty acids	18.5 ± 3.1	7.8 ± 2.9	7.6 ± 2.3	9.3 ± 1.3	7.9 ± 2.5
Sterol	9.1 ± 2.5	7.4 ± 2.8	5.2 ± 1.5	5.9 ± 1.8	5.6 ± 1.4
Phospholipid	58.0 ± 10.7	77.4 ± 8.0	79.3 ± 4.9	75.5 ± 8.7	78.3 ± 5.5
PL/ST ratio	6.7 ± 1.8	13.7 ± 11.6	16.6 ± 4.8	13.6 ± 3.9	14.9 ± 4.0
Fatty acid composition (%)					
14:0	1.3 ± 0.2	1.9 ± 0.2^{a}	1.4 ± 0.1^{c}	1.2 ± 0.1^{d}	1.7 ± 0.2^{b}
16:0	14.3 ± 1.9	17.0 ± 1.4^{a}	14.6 ± 1.4^{b}	14.1 ± 2.3^{b}	15.7 ± 1.3^{ab}
18:0	5.2 ± 0.5	4.8 ± 0.6^{a}	4.0 ± 0.4^{b}	3.3 ± 0.3^{c}	3.5 ± 0.4^{bc}
Total SFA[2]	21.7 ± 2.0	24.3 ± 1.6^{a}	20.4 ± 1.8^{bc}	19.1 ± 2.4^{c}	21.6 ± 1.6^{b}
16:1ω7	2.4 ± 0.6	2.0 ± 0.3^{a}	1.5 ± 0.2^{b}	0.9 ± 0.2^{c}	1.1 ± 0.2^{c}
18:1ω9	17.8 ± 4.6	9.3 ± 0.9^{c}	17.3 ± 1.5^{ab}	20.8 ± 4.4^{a}	15.8 ± 2.9^{b}
18:1ω7	2.6 ± 0.5	2.4 ± 0.3	2.2 ± 0.3	2.0 ± 0.4	2.1 ± 0.3
Total MUFA[3]	27.4 ± 6.6	16.9 ± 1.6^{c}	24.9 ± 2.5^{ab}	28.0 ± 6.2^{a}	22.4 ± 3.6^{b}
18:2ω6 (LA)	6.8 ± 2.2	3.5 ± 0.2^{a}	6.6 ± 0.5^{b}	8.3 ± 0.8^{c}	6.4 ± 1.2^{b}
18:3ω6	0.3 ± 0.2	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{ab}	0.1 ± 0.0^{b}
20:3ω6	0.9 ± 0.2	0.3 ± 0.1^{d}	0.8 ± 0.1^{b}	1.3 ± 0.2^{a}	0.6 ± 0.1^{c}
20:4ω6 (ARA)	3.6 ± 0.7	4.0 ± 0.3^{a}	3.1 ± 0.4^{b}	3.0 ± 0.6^{b}	3.3 ± 0.7^{ab}
22:4ω6	0.2 ± 0.1	0.5 ± 0.1^{a}	0.4 ± 0.2^{b}	0.1 ± 0.0^{c}	0.1 ± 0.0^{c}
22:5ω6 (ω6DPA)	0.5 ± 0.1	0.6 ± 0.1^{c}	0.4 ± 0.0^{c}	3.3 ± 0.4^{b}	4.4 ± 0.4^{a}
18:3ω3 (ALA)	0.9 ± 0.2	0.3 ± 0.0^{c}	1.1 ± 0.1^{ab}	1.3 ± 0.2^{a}	1.0 ± 0.3^{b}
18:4ω3	0.5 ± 0.2	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
20:4ω3	0.6 ± 0.2	0.8 ± 0.1^{a}	0.6 ± 0.1^{b}	0.3 ± 0.1^{c}	0.2 ± 0.0^{d}
20:5ω3 (EPA)	5.8 ± 1.1	9.0 ± 0.9^{a}	6.7 ± 1.0^{b}	1.6 ± 0.4^{c}	1.5 ± 0.2^{c}
22:5ω3	1.5 ± 0.3	3.6 ± 0.2^{a}	1.9 ± 0.2^{b}	0.4 ± 0.1^{c}	0.4 ± 0.1^{c}
22:6ω3 (DHA)	27.0 ± 5.7	32.8 ± 0.9^{ab}	29.8 ± 2.0^{b}	30.5 ± 4.2^{b}	35.8 ± 3.0^{a}
Total PUFA[4]	50.6 ± 4.6	58.5 ± 0.6^{a}	54.4 ± 1.4^{b}	52.8 ± 3.9^{b}	55.9 ± 2.4^{ab}
Total ω3	36.6 ± 6.2	47.0 ± 0.9^{a}	40.4 ± 1.6^{b}	34.5 ± 4.2^{c}	39.2 ± 2.8^{b}
Total ω6	12.9 ± 1.9	9.9 ± 0.5^{a}	13.0 ± 0.4^{b}	18.2 ± 0.7^{c}	16.5 ± 0.5^{d}
ω3/ω6 ratio	2.9 ± 0.7	4.8 ± 0.3^{a}	3.1 ± 0.2^{b}	1.9 ± 0.3^{d}	2.4 ± 0.2^{c}
EPA+DHA	32.8 ± 6.6	41.8 ± 0.9^{ab}	36.4 ± 1.8^{b}	32.1 ± 4.5^{a}	37.3 ± 3.1^{ab}
DHA/EPA ratio	4.7 ± 0.8	3.7 ± 0.4^{c}	4.6 ± 0.9^{c}	19.2 ± 3.2^{b}	24.6 ± 2.6^{a}
EPA/ARA ratio	1.6 ± 0.2	3.3 ± 0.4^{a}	2.1 ± 0.3^{a}	0.6 ± 0.2^{b}	0.5 ± 0.1^{b}
DHA/ARA ratio	7.6 ± 0.6	8.4 ± 0.7^{b}	9.6 ± 1.4^{ab}	10.3 ± 1.7^{ab}	11.1 ± 1.9^{a}

[1]Data expressed as percent lipid or fatty acid methyl ester (FAME); Values are means ± standard deviation (n=9 per treatment). Means with different superscripts indicate significant differences ($p < 0.05$) based on Tukey's *posthoc* test following a general linear model analysis; FO = fish oil; FO/CO = fish oil/canola oil; LMO = low microbial oil; HMO = high microbial oil. [2]Saturated fatty acid. [3]Monounsaturated fatty acid. [4]Polyunsaturated fatty acid.

Table 3.4: Phospholipid fatty acid composition of Atlantic salmon liver tissue after 16 weeks of feeding experimental diets[1]

	FO	FO/CO	LMO	HMO
Fatty acid composition (%)				
14:0	1.7 ± 0.2^{a}	1.3 ± 0.1^{b}	1.1 ± 0.2^{b}	1.6 ± 0.1^{a}
16:0	20.3 ± 1.8	19.3 ± 2.1	18.9 ± 2.0	20.9 ± 1.3
18:0	6.5 ± 1.2^{a}	6.1 ± 1.4^{a}	4.9 ± 0.5^{b}	5.6 ± 1.0^{ab}
Total SFA[2]	29.3 ± 2.8^{a}	27.3 ± 3.4^{ab}	25.6 ± 2.5^{b}	28.9 ± 2.0^{a}
16:1ω7	1.6 ± 0.4^{a}	1.4 ± 0.4^{b}	0.8 ± 0.2^{c}	1.0 ± 0.2^{c}
18:1ω9	7.3 ± 0.3^{d}	11.5 ± 0.3^{b}	13.1 ± 0.4^{a}	10.6 ± 0.9^{c}
18:1ω7	2.0 ± 0.1^{a}	1.6 ± 0.2^{b}	1.4 ± 0.1^{c}	1.5 ± 0.1^{bc}
20:1ω9	1.1 ± 0.2^{b}	1.5 ± 0.5^{ab}	1.7 ± 0.5^{a}	1.5 ± 0.4^{ab}
Total MUFA[3]	13.6 ± 0.5^{c}	17.5 ± 1.1^{a}	17.9 ± 0.8^{a}	15.7 ± 1.3^{b}
18:2ω6 (LA)	2.7 ± 0.2^{a}	4.9 ± 0.5^{b}	6.4 ± 0.3^{c}	4.7 ± 0.6^{b}
18:3ω6	0.1 ± 0.0^{a}	0.1 ± 0.0^{ab}	0.1 ± 0.0^{bc}	0.1 ± 0.0^{c}
20:2ω6	0.9 ± 0.1^{c}	1.6 ± 0.5^{b}	2.1 ± 0.6^{a}	1.6 ± 0.4^{ab}
20:3ω6	0.3 ± 0.1^{d}	0.8 ± 0.1^{b}	1.4 ± 0.3^{a}	0.6 ± 0.1^{c}
20:4ω6 (ARA)	4.1 ± 0.3	3.6 ± 0.5	3.7 ± 0.6	3.7 ± 0.4
22:4ω6	0.2 ± 0.1^{a}	0.2 ± 0.1^{ab}	0.1 ± 0.0^{ab}	0.1 ± 0.0^{b}
22:5ω6 (ω6DPA)	0.6 ± 0.1^{c}	0.5 ± 0.1^{c}	4.1 ± 0.2^{b}	4.7 ± 0.3^{a}
18:3ω3 (ALA)	0.2 ± 0.0^{c}	0.6 ± 0.1^{ab}	0.7 ± 0.1^{a}	0.5 ± 0.1^{b}
20:3ω3	0.1 ± 0.0^{b}	0.2 ± 0.1^{a}	0.2 ± 0.1^{a}	0.2 ± 0.0^{a}
20:4ω3	0.5 ± 0.1^{a}	0.5 ± 0.1^{a}	0.3 ± 0.1^{c}	0.1 ± 0.0^{b}
20:5ω3 (EPA)	8.0 ± 1.1^{a}	6.7 ± 1.2^{b}	1.7 ± 0.4^{c}	1.4 ± 0.2^{c}
22:5ω3	2.9 ± 0.2^{a}	1.7 ± 0.2^{b}	0.4 ± 0.1^{c}	0.3 ± 0.1^{c}
22:6ω3 (DHA)	34.6 ± 2.6^{ab}	32.5 ± 2.9^{b}	35.1 ± 1.5^{ab}	37.1 ± 2.6^{a}
Total PUFA[4]	56.7 ± 3.0	55.0 ± 3.7	56.5 ± 2.4	55.4 ± 2.7
PUFA/SFA ratio	2.0 ± 0.3	2.1 ± 0.4	2.2 ± 0.3	1.9 ± 0.2
Total ω3	46.7 ± 2.7^{a}	42.5 ± 3.2^{b}	38.4 ± 1.6^{c}	39.7 ± 2.7^{bc}
Total ω6	8.9 ± 0.4^{a}	11.7 ± 0.7^{b}	17.8 ± 1.3^{c}	15.5 ± 0.6^{d}
ω3/ω6 ratio	5.3 ± 0.4^{a}	3.6 ± 0.2^{b}	2.2 ± 0.2^{d}	2.6 ± 0.2^{c}
EPA+DHA	42.6 ± 2.6^{a}	39.2 ± 3.1^{b}	36.8 ± 1.5^{b}	38.4 ± 2.7^{b}
DHA/EPA ratio	4.4 ± 0.7^{c}	5.0 ± 1.1^{c}	21.2 ± 4.1^{b}	27.8 ± 2.8^{a}
EPA/ARA ratio	2.0 ± 0.4^{a}	1.9 ± 0.4^{a}	0.5 ± 0.2^{b}	0.4 ± 0.1^{b}
DHA/ARA ratio	8.5 ± 0.9	9.2 ± 1.7	9.7 ± 1.8	10.3 ± 1.6

[1]Data expressed as percent lipid or fatty acid methyl ester (FAME); Values are means ± standard deviation (n=9 per treatment). Means with different superscripts indicate significant differences ($p < 0.05$) based on Tukey's *posthoc* test following a general linear model analysis; FO = fish oil; FO/CO = fish oil/canola oil; LMO = low microbial oil; HMO = high microbial oil.

[2]Saturated fatty acid.

[3]Monounsaturated fatty acid.

[4]Polyunsaturated fatty acid.

3.4.5 Muscle tissue lipid class and fatty acid composition

Initial muscle tissue contained 9.9 mg/g ww total lipid, and it was mostly composed of polar lipid (Table 3.5). After 16-weeks of feeding, there was a ~5-fold increase in total lipid in salmon fed the FO diet (45.4 mg/g ww), and a ~4-fold increase in salmon fed the FO/CO, LMO, and HMO diets (36.1 – 38.3 mg/g ww). There was a significant difference in total lipid concentration between salmon fed the FO diet and salmon fed the FO/CO diet. The lipid class composition of salmon fed the FO diet was mostly composed of polar lipids, while interestingly, salmon fed the FO/CO, LMO, and HMO diets were mostly composed of neutral lipids. The dominant lipid classes in the muscle tissue were TAG (40.4 – 57.4%) and PL (21.9 – 36.9%). The TAG proportion increased in all salmon fed diets while the PL proportion decreased in all treatments. Salmon fed the FO diet had the lowest TAG and the highest PL proportion, and it was significantly different from other treatments.

After 16-weeks of feeding, the muscle tissue fatty acid profile mostly reflected the diets, except the relative proportions of MUFA, PUFA, and SFA (Table 3.5). The fatty acid composition of the salmon fed the FO and HMO diets were mostly PUFA (FO, 47.7%; HMO, 42.4%) followed by MUFA (FO, 28.6%; HMO, 37.7%) and SFA (FO, 23.0%; HMO, 19.7%), while salmon fed the FO/CO and LMO diets were mostly MUFA (FO/CO, 41.3%; LMO, 45.6%), followed by PUFA (FO/CO, 40.0%; LMO, 38.2%) and SFA (FO/CO, 18.3%; LMO, 16.1%). There were significant differences in EFA proportions, including ω6 and ω3 precursors, LA and ALA, across the dietary treatments, especially between salmon fed the FO diet and the salmon fed the FO/CO, LMO, and HMO diets. DHA was not always the dominant EFA; it was only the dominant EFA in salmon fed the FO and HMO diets, while LA was the dominant EFA in salmon fed the FO/CO and LMO diets. The EPA proportion was significantly higher in salmon fed FO-containing diets (FO, 3.1%;

FO/CO, 1.3%) than in salmon fed MO-containing diets (LMO, 0.3%; HMO, 0.3%). The LC-PUFA ω6DPA was higher in salmon fed MO-containing diets (LMO, 1.5%; HMO, 2.6%) than salmon fed FO-containing diets (FO, 0.3%; FO/CO, 0.3%). The ω3 fatty acids were ~3-fold higher than ω6 fatty acids in salmon fed FO-containing diets and ~2-fold higher than ω6 fatty acids in salmon fed MO-containing diets.

Principal coordinates analysis of week-16 muscle total fatty acids showed PCO1 and PCO2 (Fig. 3.3) accounted for 83.9% and 15.0% variability, respectively. There was a clear variability among different dietary groups with the largest variability being between salmon fed the FO and LMO diets. SIMPER analysis (Table C5 and Table C6 in Appendix C) showed an average of 97% similarity within the same dietary group and confirmed the spatial distribution in the PCO biplot that the highest dissimilarity was between salmon fed the FO and LMO diets (33.5%). The top driver for the similarities varied among 18:1ω9 and 16:0, while the top driver for the dissimilarities between different treatments varied among 18:1ω9 and DHA. For extended details on average similarities and dissimilarities results, see Appendix C.

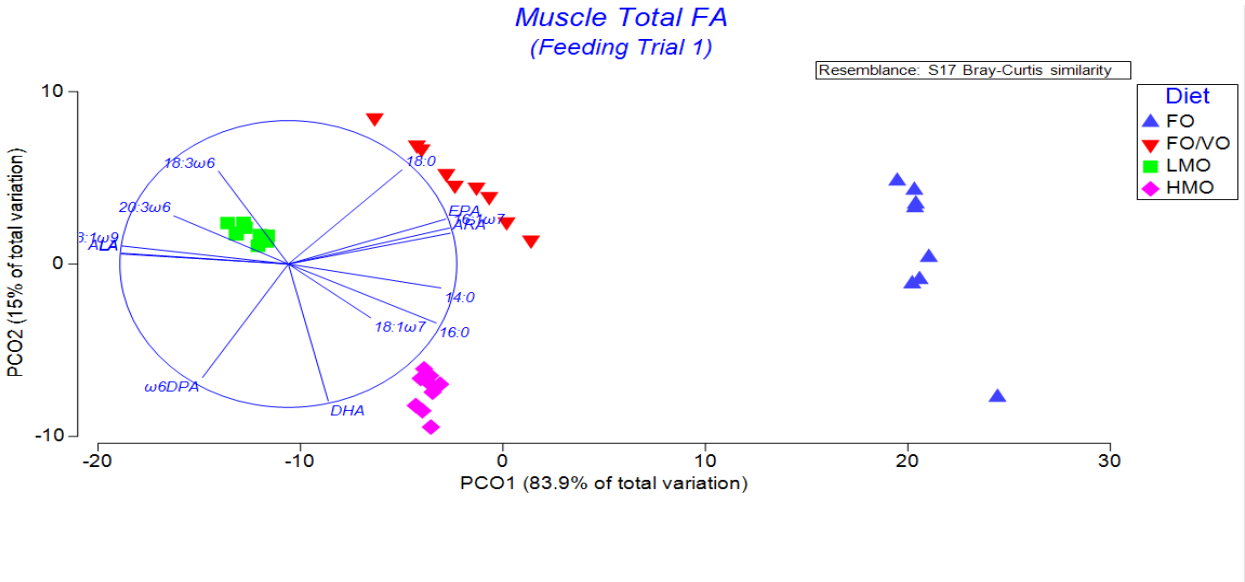


Figure 3.3: Principal coordinates analysis (PCO) of Atlantic salmon muscle tissue total fatty acid composition (%) after 16 weeks of feeding experimental diets

3.4.6 Muscle tissue phospholipid fatty acid composition

The PLFA composition was mostly PUFA (54.2 – 59.8%) followed by SFA (24.2 – 32.7%) and MUFA (12.7% - 17.7%) (Table 3.6). DHA was the dominant EFA, followed by EPA and ARA. Salmon fed FO-containing diets had significantly higher EPA proportions (FO, 8.7%; FO/CO, 8.8%) than salmon fed MO-containing diets (LMO, 1.8%; HMO, 1.4%). The LC-PUFA ω6DPA was also found embedded in the membrane at higher proportions in salmon fed MO-containing diets (LMO, 3.9%; HMO, 4.0%) than salmon fed FO-containing diets (FO, 0.5%; FO/CO, 0.6%). The ω3 fatty acids were ~10-fold higher than ω6 fatty acids in salmon fed the FO diet were ~7-fold higher than ω6 fatty acids in salmon fed the FO/CO diet, ~4-fold more prevalent than ω6 fatty acids in salmon fed the LMO diet and were ~5-fold more prevalent than ω6 fatty acids in salmon fed the HMO diet. The DHA/EPA ratio was significantly higher in salmon fed MO-containing diets (LMO, 22.1%; HMO, 31.6%) than those containing FO (FO, 3.8%; FO/CO, 4.1%). The EPA/ARA ratio was significantly higher in salmon fed FO-containing diets (FO, 7.5%; FO/CO, 6.5%) than those containing MO (LMO, 1.5%; FO/CO, 1.3%). The DHA/ARA ratio was significantly higher in salmon fed MO-containing diets (LMO, 42.4%; HMO, 32.4%) than those containing FO (FO, 29.0%; FO/CO, 26.3%).

Principal coordinates analysis of week-16 muscle PLFA showed PCO1 and PCO2 (Fig. 3.4) accounted for 58.3% and 29.3% variability, respectively. The PCO biplot showed that the highest variation in muscle PL was between salmon fed the FO and LMO diets. Visually the variability was not as clear as the muscle total fatty acids; however, the PCO biplot still showed that the main dissimilarity was between salmon fed the FO and LMO diets and also indicated that salmon fed the LMO and HMO diets were much more similar. SIMPER analysis (Table C7 and Table C8 in Appendix C) showed an average of 94.0% similarity for FO/CO, LMO, and HMO

dietary groups and an 85.6% similarity for the FO dietary group. The highest dissimilarity was between the FO and LMO dietary groups (23.6%), which confirms the spatial distribution in the PCO biplot. The second highest dissimilarity was between FO and HMO dietary groups (21.6%), and the lowest dissimilarity was between the LMO and HMO dietary groups. The top driver for the similarities within the dietary groups was DHA, while the top driver for the dissimilarities between different treatments varied among DHA and EPA. For extended details on average similarities and dissimilarities results, see Appendix C.

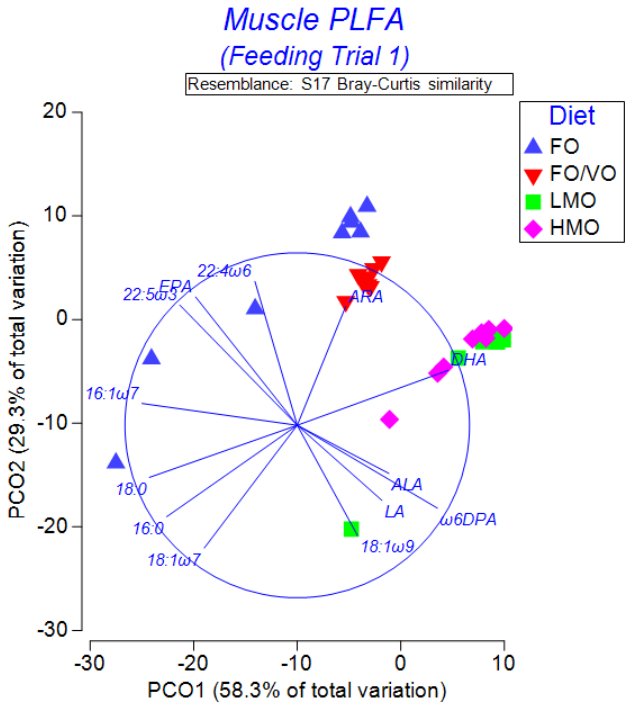


Figure 3.4: Principal coordinates analysis (PCO) of Atlantic salmon muscle tissue phospholipid fatty acid composition (%) after 16 weeks of feeding experimental diets

Table 3.5: Lipid class and total fatty acid composition of Atlantic salmon muscle tissue, prior to feeding experimental diets and after 16 weeks of feeding experimental diets[1]

	Initial	FO	FO/CO	LMO	HMO
Lipid composition (%)					
Total lipid (mg/g)	9.9 ± 3.2	45.4 ± 11.1^{a}	36.1 ± 10.8^{b}	38.3 ± 7.0^{ab}	37.4 ±6.6^{ab}
Neutral lipid	27.9 ± 9.7	47.8 ± 16.8^{b}	60.8 ± 12.9^{a}	65.8 ± 6.1^{a}	60.6 ± 8.7^{a}
Polar lipid	72.1 ± 9.7	52.2 ± 16.8^{a}	39.2 ± 12.9^{b}	34.2 ± 6.1^{b}	39.4 ± 8.7^{b}
Lipid class composition (%)					
Triacylglycerol	16.0 ± 9.7	40.4 ± 14.5^{b}	53.1 ± 13.9^{a}	57.4 ± 6.7^{a}	51.9 ± 8.2^{a}
Free fatty acids	4.3 ± 2.2	4.3 ± 1.5	4.0 ± 1.1	5.6 ± 1.0	5.5 ± 1.6
Sterol	6.8 ± 1.8	0.9 ± 0.6^{b}	1.8 ± 0.8^{a}	1.2 ± 0.3^{ab}	0.8 ± 0.4^{b}
Phospholipid	68.8 ± 11.9	36.9 ± 15.4^{a}	23.7 ± 13.4^{b}	21.9 ± 8.2^{b}	23.3 ± 6.1^{b}
PL/ST ratio	10.6 ± 2.1	43.2 ± 39.3^{a}	14.0 ± 7.7^{b}	19.6 ± 9.1^{b}	27.5 ± 21.1^{ab}
Fatty acid composition (%)					
14:0	1.5 ± 0.4	4.3 ± 0.3^{a}	2.5 ± 0.2^{c}	1.9 ± 0.1^{d}	3.1 ± 0.2^{b}
16:0	14.8 ± 0.9	14.9 ± 0.5^{a}	12.2 ± 0.5^{c}	11.1 ± 0.4^{d}	13.5 ± 0.3^{b}
18:0	4.5 ± 0.4	2.9 ± 0.1^{a}	2.8 ± 0.1^{a}	2.3 ± 0.1^{b}	2.1 ± 0.1^{c}
Total SFA[2]	21.5 ± 0.9	23.0 ± 0.4^{a}	18.3 ± 0.4^{c}	16.1 ± 0.5^{d}	19.7 ± 0.6^{b}
16:1ω7	2.7 ± 0.8	6.2 ± 0.4^{a}	3.5 ± 0.2^{b}	1.9 ± 0.1^{d}	2.6 ± 0.1^{c}
18:1ω9	13.9 ± 3.1	14.5 ± 0.8^{d}	30.6 ± 1.6^{b}	36.8 ± 0.4^{a}	28.7 ± 0.7^{c}
18:1ω7	2.6 ± 0.2	2.8 ± 0.1^{a}	2.7 ± 0.1^{b}	2.7 ± 0.0^{b}	2.8 ± 0.0^{a}
Total MUFA[3]	23.9 ± 5.6	28.6 ± 1.6^{d}	41.3 ± 2.1^{b}	45.6 ± 0.4^{a}	37.7 ± 0.9^{c}
18:2ω6 (LA)	6.7 ± 1.3	8.6 ± 0.6^{c}	13.5 ± 0.6^{b}	15.7 ± 0.4^{a}	13.4 ± 0.2^{b}
18:3ω6	0.2 ± 0.1	0.3 ± 0.0^{bc}	0.3 ± 0.0^{b}	0.3 ± 0.0^{a}	0.2 ± 0.0^{c}
20:3ω6	0.5 ± 0.1	0.2 ± 0.0^{c}	0.2 ± 0.0^{b}	0.4 ± 0.0^{a}	0.2 ± 0.0^{bc}
20:4ω6 (ARA)	1.8 ± 0.4	1.0 ± 0.0^{a}	0.6 ± 0.1^{b}	0.5 ± 0.0^{c}	0.5 ± 0.0^{c}
22:5ω6 (ω6DPA)	0.6 ± 0.1	0.3 ± 0.0^{c}	0.2 ± 0.0^{d}	1.5 ± 0.0^{b}	2.6 ± 0.1^{a}
18:3ω3 (ALA)	1.3 ± 0.2	1.2 ± 0.1^{d}	3.3 ± 0.2^{b}	3.8 ± 0.1^{a}	3.1 ± 0.1^{c}
18:4ω3	0.7 ± 0.2	1.6 ± 0.1^{a}	1.0 ± 0.1^{b}	0.5 ± 0.0^{c}	0.3 ± 0.0^{d}
20:4ω3	0.7 ± 0.1	0.8 ± 0.0^{a}	0.5 ± 0.0^{b}	0.3 ± 0.0^{c}	0.3 ± 0.0^{c}
20:5ω3 (EPA)	6.8 ± 1.1	9.6 ± 0.7^{a}	4.7 ± 0.5^{b}	0.8 ± 0.0^{c}	0.9 ± 0.2^{c}
22:5ω3	2.1 ± 0.2	3.3 ± 0.1^{a}	1.6 ± 0.1^{b}	0.3 ± 0.0^{c}	0.3 ± 0.0^{c}
22:6ω3 (DHA)	29.8 ± 5.9	15.6 ± 2.0^{b}	10.7 ± 2.3^{c}	12.6 ± 0.5^{c}	19.4 ± 1.3^{a}
Total PUFA[4]	53.4 ± 5.4	47.7 ± 1.7^{a}	40.0 ± 2.0^{c}	38.2 ± 0.6^{c}	42.4 ± 1.4^{b}
Total ω3	41.9 ± 6.4	33.1 ± 2.4^{a}	22.3 ± 2.6^{b}	18.5 ± 0.5^{c}	24.5 ± 1.4^{b}
Total ω6	10.4 ± 1.2	10.9 ± 0.6^{d}	15.6 ± 0.6^{c}	19.2 ± 0.4^{a}	17.6 ± 0.2^{b}
ω3/ω6 ratio	4.2 ± 1.0	3.1 ± 0.4^{a}	1.4 ± 0.2^{b}	1.0 ± 0.0^{c}	1.4 ± 0.1^{b}
EPA+DHA	36.5 ± 6.8	25.2 ± 2.5^{c}	15.4 ± 2.7^{c}	13.4 ± 0.5^{b}	20.3 ± 1.4^{a}
DHA/EPA ratio	4.4 ± 0.5	1.6 ± 0.1^{a}	2.3 ± 0.3^{c}	15.7 ± 1.1^{c}	21.7 ± 3.3^{b}
EPA/ARA ratio	3.8 ± 0.4	10.2 ± 0.5^{a}	7.4 ± 0.4^{b}	1.8 ± 0.1^{c}	1.9 ± 0.3^{c}
DHA/ARA ratio	16.4 ± 2.4	16.4 ± 1.9^{c}	16.7 ± 2.3^{c}	27.9 ± 1.9^{b}	40.1 ± 3.0^{a}
DHA+EPA/112 g	273.3	924.0	467.0	467.0	670.9

[1]Data expressed as percent lipid or fatty acid methyl ester (FAME); Values are means ± standard deviation (n=9 per treatment). Means with different superscripts indicate significant differences ($p < 0.05$) based on Tukey's *posthoc* test following a general linear model analysis; FO = fish oil; FO/CO = fish oil/canola oil; LMO = low microbial oil; HMO = high microbial oil. [2]Saturated fatty acid. [3]Monounsaturated fatty acid. [4]Polyunsaturated fatty acid.

Table 3.6: Phospholipid fatty acid composition of Atlantic salmon muscle tissue after 16 weeks of feeding experimental diets[1]

	FO	FO/CO	LMO	HMO
Fatty acids composition (%)				
14:0	1.7 ± 0.5^{a}	1.0 ± 0.1^{b}	1.0 ± 0.3^{b}	1.2 ± 0.3^{b}
16:0	25.8 ± 8.3^{a}	19.2 ± 1.6^{b}	19.4 ± 2.9^{b}	21.7 ± 2.7^{ab}
18:0	4.4 ± 1.2^{a}	3.6 ± 0.3^{ab}	3.2 ± 0.6^{b}	3.4 ± 0.5^{b}
Total SFA[2]	32.7 ± 10.0^{a}	24.4 ± 1.8^{b}	24.2 ± 3.8^{b}	26.9 ± 3.3^{ab}
16:1ω7	1.9 ± 0.4^{a}	1.3 ± 0.1^{b}	0.8 ± 0.1^{c}	1.0 ± 0.1^{bc}
18:1ω9	6.8 ± 1.3^{c}	11.0 ± 0.9^{b}	13.3 ± 1.9^{a}	9.9 ± 1.2^{b}
18:1ω7	2.4 ± 0.5	2.2 ± 0.1	2.4 ± 0.3	2.3 ± 0.3
20:1ω9	0.5 ± 0.1^{ab}	0.5 ± 0.1^{b}	0.6 ± 0.1^{a}	0.4 ± 0.1^{b}
Total MUFA[3]	12.6 ± 2.6^{c}	15.7 ± 1.1^{ab}	17.7 ± 2.6^{a}	14.3 ± 1.6^{bc}
18:2ω6 (LA)	2.3 ± 0.2^{a}	4.4 ± 0.5^{b}	5.4 ± 0.5^{c}	3.7 ± 0.3^{d}
18:3ω6	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{b}
20:2ω6	0.3 ± 0.0^{c}	0.5 ± 0.1^{b}	0.7 ± 0.1^{a}	0.4 ± 0.1^{b}
20:3ω6	0.1 ± 0.0^{c}	0.4 ± 0.1^{b}	0.6 ± 0.1^{a}	0.2 ± 0.0^{c}
20:4ω6 (ARA)	1.1 ± 0.3^{b}	1.4 ± 0.1^{a}	1.2 ± 0.1^{ab}	1.1 ± 0.1^{b}
22:4ω6	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.1 ± 0.1^{b}	0.1 ± 0.0^{b}
22:5ω6 (ω6DPA)	0.5 ± 0.1^{b}	0.6 ± 0.0^{b}	3.9 ± 0.5^{a}	4.0 ± 0.4^{a}
18:3ω3 (ALA)	0.5 ± 0.0^{a}	1.7 ± 0.1^{b}	2.0 ± 0.2^{c}	1.2 ± 0.1^{d}
18:4ω3	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}	0.2 ± 0.0^{b}	0.1 ± 0.0^{c}
20:3ω3	0.1 ± 0.0^{c}	0.2 ± 0.0^{b}	0.2 ± 0.0^{a}	0.1 ± 0.0^{c}
20:4ω3	0.6 ± 0.1^{a}	0.7 ± 0.1^{a}	0.4 ± 0.1^{b}	0.2 ± 0.0^{c}
20:5ω3 (EPA)	8.7 ± 2.1^{a}	8.8 ± 0.7^{a}	1.8 ± 0.3^{b}	1.4 ± 0.2^{b}
22:5ω3	3.6 ± 0.6^{a}	2.9 ± 0.2^{b}	0.6 ± 0.1^{c}	0.4 ± 0.1^{c}
22:6ω3 (DHA)	33.7 ± 9.5^{b}	35.8 ± 1.5^{b}	39.8 ± 6.0^{ab}	45.0 ± 4.5^{a}
Total PUFA[4]	54.2 ± 12.6	59.8 ± 1.3	58.0 ± 6.4	58.7 ± 4.8
PUFA/SFA ratio	1.9 ± 0.8^{b}	2.5 ± 0.2^{a}	2.5 ± 0.5^{a}	2.2 ± 0.4^{ab}
Total ω3	48.0 ± 12.4	50.8 ± 1.2	45.1 ± 6.3	48.5 ± 4.7
Total ω6	4.7 ± 0.3^{a}	7.5 ± 0.7^{b}	12.0 ± 0.6^{c}	9.5 ± 0.6^{d}
ω3/ω6 ratio	10.2 ± 2.3^{a}	6.8 ± 0.6^{b}	3.8 ± 0.5^{c}	5.1 ± 0.6^{c}
EPA+DHA	42.4 ± 11.5	44.6 ± 1.2	41.6 ± 6.3	46.4 ± 4.7
DHA/EPA ratio	3.9 ± 0.4^{c}	4.1 ± 0.4^{c}	22.1 ± 1.5^{b}	31.6 ± 1.7^{a}
EPA/ARA ratio	7.5 ± 0.4^{a}	6.5 ± 0.3^{b}	1.5 ± 0.2^{c}	1.3 ± 0.2^{c}
DHA/ARA ratio	29.0 ± 3.3^{bc}	26.3 ± 2.6^{c}	32.4 ± 3.9^{b}	42.4 ± 4.8^{a}

[1]Data expressed as percent fatty acid methyl ester (FAME); Values are means ± standard deviation (n=9 per treatment). Means with different superscripts indicate significant differences ($p < 0.05$) based on Tukey's *posthoc* test following a general linear model analysis; FO = fish oil; FO/CO = fish oil/canola oil; LMO = low microbial oil; HMO = high microbial oil.

[2]Saturated fatty acid.

[3]Monounsaturated fatty acid.

[4]Polyunsaturated fatty acid.

3.5 Discussion

In this study, the MO used was isolated from a novel strain, *Schizochytrium* sp. (T18), which is rich in DHA and low in EPA. According to NRC (2011) the dietary requirement for salmon is 0.5-1.0% EPA+DHA, which was recently reviewed by Qian et al. (2020), who concluded that the minimum requirement is 0.5% EPA+DHA. It is worth noting that the EPA+DHA requirement has not been de-coupled, since are unknown what the requirements are for EPA and DHA separately. The EPA+DHA composition of the experimental diets (Table 3.2) in this study exceeds the minimum requirement suggested by NRC; therefore, EPA+DHA was not a limiting factor for growth. Despite the low proportion of EPA in the diets, the fish grew over 300% from their initial weight and, numerically, salmon fed the LMO and HMO diets gained 14% and 18%, respectively, more weight than the salmon fed the FO or FO/CO diets, within the 16-week period (Wei et al., 2021). Previous studies using MO from *Schizochytrium* sp. in diets for Atlantic salmon showed positive growth performance (Li et al., 2009; Miller et al., 2007) and a high digestibility (Tibbetts et al., 2020b).

Studies often refer to EPA+DHA as one component of the dietary requirement; however, most of these studies provided little to no information as to which fatty acid was more important for different biological functions. The present study demonstrates that DHA-rich MO from *Schizochytrium* sp. (T18) is an effective alternative lipid source for farmed Atlantic salmon parr reared in freshwater and that low dietary EPA (LMO, 0.16%; HMO, 0.20%) and high dietary DHA (LMO, 1.97%; HMO, 3.87%) relative to control diet (FO: 1.81% EPA, 1.05% DHA; FO/CO: 0.97% EPA; 0.56% DHA) did not impact growth performance. The total fatty acid profile reflected the diets, and the quantification of PLFA showed similar patterns as with the total fatty acid composition, where DHA was present in a very high proportion in the membrane, especially in

muscle tissue. Additionally, *Schizochytrium* sp. had high proportions of ω6DPA, which reflected in the muscle and liver tissues of salmon fed the MO-containing diets. The LC-PUFA ω6DPA was also found embedded in the membrane in higher proportions in salmon fed the MO-containing diets than salmon fed the FO-containing diets. Replacing dietary FO with MO had clear effects on PLFA compositions of both liver and muscle tissues, although the magnitude of the effects varied between the tissues.

3.5.1 Liver tissue

The liver is considered an important site for LC-PUFA synthesis and lipid metabolism in Atlantic salmon (Betancor et al., 2014). Replacing dietary FO with MO did not significantly change the total lipid composition in the liver tissue (Table 3.3). The majority of the lipid classes were similar across the dietary treatments, except for TAG, where it differed significantly between salmon fed the FO diet and salmon fed the HMO diet. HMO feeding showed higher TAG than FO feeding indicating that the excess lipid was likely stored as TAG in the liver tissue instead of being metabolized for energy. However, PL was the dominant lipid class in the liver, accounting for ~78% total lipid across the dietary treatments suggesting that there was more membrane material in the liver than in the muscle (~37% in FO fed fish and ~23% in FO/CO, LMO, HMO fed fish). There was a much greater proportion of PL and ST in the liver tissue than in the diet suggesting the accumulation and retention of these classes in the liver. (Table 3.2). Both PL and ST play a major role in maintaining the structure of the membrane. Cholesterol is an essential structural component of animal cell membranes that is required in order to establish proper membrane permeability and fluidity (Morzycki, 2014). However, to assess adjustments of cellular fluidity, it is necessary to look at significant differences in PL/ST ratio and the membrane PUFA/SFA (P/S) ratio. The PL/ST ratio (Table 3.3), as well as the P/S ratio in the liver PL (Table 3.4), showed no

significant difference across the dietary treatments. Also, the inverse relationship between PL/ST ratio and P/S ratio was not consistent across the dietary treatments. However, there are suggestions of possible adjustments to optimize fluidity in the membrane, as salmon fed the FO/CO diet had the highest PL/ST ratio and the second highest P/S ratio, and salmon fed the LMO diet had the lowest PL/ST ratio but the highest P/S ratio.

While similarities were observed between liver tissue total fatty acid profile and PLFA profile, the PCO analysis showed a higher variation between the diets for liver total fatty acids (Figure 3.1) than liver PLFA (Figure 3.2). The PUFA proportion in the liver PL was noticeably higher than that of the diet (Table 3.2), while the MUFA proportion was noticeably lower, suggesting possible ß-oxidation of these fatty acids. SFA proportions in liver PL were higher in salmon fed the FO/CO, LMO, and HMO diets and lower in salmon fed the FO diet than that of the diet. Replacing dietary FO with MO, rich in DHA, resulted in higher DHA proportions for the PLFA in salmon fed MO-containing diets compared to FO-containing diets. In contrast, the EPA proportion was lower in salmon fed MO-containing diets than in salmon fed FO-containing diets. This might be an indication that retro-conversion from DHA to EPA did not occur and also highlights the importance of DHA in the liver membrane compared to EPA. The levels of ARA in liver PL were higher than EPA (ARA>EPA) in salmon fed MO-containing diets despite both EPA and ARA being significantly lower in the diet. This could be an indication of elongation and desaturation from LA to ARA but not from ALA to EPA. Both EPA and ARA serve as precursors of eicosanoid biosynthesis, and there is direct substrate competition between the two fatty acids, where the increase in one results in the decrease of the other (Bell et al., 1994, Calder, 2006). Changes in the ω3/ω6 ratio can affect eicosanoid production. Increased consumption of ω3 LC-PUFA reduces the synthesis of ω6 LC-PUFA derived pro-inflammatory eicosanoids and elevates

the production of anti-inflammatory eicosanoids from ω3 PUFA (Hixson et al., 2014). The EPA/ARA ratio in salmon fed MO-containing diets was lower compared to salmon fed FO-containing diets. This may suggest the production of pro-inflammatory eicosanoids; however, the ω3/ω6 ratio remained >1 across the dietary treatments. The impact of the low diet and tissue EPA/ARA ratio on the salmon immune system requires further investigation. The DHA/ARA ratio remained >1, indicating the important role of DHA in membranes.

3.5.2 Muscle tissue

Replacing FO with MO in the diet of Atlantic salmon parr resulted in no significant difference in total lipid composition in the muscle tissue between salmon fed MO-containing diets and salmon fed the FO and FO/CO diets. However, the total lipid between salmon fed the FO diet and salmon fed the FO/CO diet was different. This could be due to the equal concentration of FO and CO in the FO/CO diet compared to no CO in the FO diet (Table B1 in Appendix B). Unlike liver tissue, the muscle lipids were mainly composed of TAG (40% in FO fed fish; >50% in FO/CO, LMO, HMO fed fish) (Table 3.5). TAG are the primary class for lipid storage and energy provision, and the major lipid storage site for Atlantic salmon is the muscle tissue (Tocher et al., 2008; Zhol et al., 1995). Although PL was not as dominant in the muscle compared to the liver, it was still present in a high proportion (40% in FO fed fish; ~23% in FO/CO, LMO, HMO fed fish). The neutral and polar composition of the muscle tissue highlighted the difference between salmon fed the FO diet and salmon fed the FO/CO, LMO, and HMO diets, where the lipid in salmon fed the FO diet was mainly composed of polar lipids (52%), while the other treatments were mainly composed of neutral lipid (>60%). The difference between salmon fed the FO diet and the other treatments were also detected in TAG and PL lipid classes. However, salmon fed the FO diet were not significantly different from salmon fed the LMO and HMO diets for ST. Both PL and ST play

essential roles in maintaining membrane fluidity, where when the proportion of one goes up, the other goes down (Stillwell & Wassall, 2003; Bell & Koppe, 2014). Variation in PL/ST ratio was observed across the dietary treatments, where salmon fed the FO diet had the highest PL/ST ratio (43.2%), while salmon fed the FO/CO diet had the lowest PL/ST ratio (14.0%). Also, a significant difference for PL/ST ratio was observed between salmon fed the FO diet and salmon fed the FO/CO and LMO diets (Table 3.5). Similar difference was also observed for P/S ratio in the muscle PL (Table 3.6). An inverse relationship between PL/ST ratio and P/S ratio was observed in a way that salmon fed the FO diet had the highest PL/ST ratio and the lowest P/S ratio, while salmon fed the FO/CO diet had the lowest PL/ST ratio and one of the two equally highest P/S ratios. Salmon fed the LMO diet had a similar P/S ratio to salmon fed the FO/CO diet, but the muscle had the second lowest PL/ST ratio. Given that these two counteract each other, it could indicate an adjustment to minimize fluidity effects of diet-induced changes to membranes.

Although the distribution of total fatty acids (Table 3.5) and PLFA (Table 3.6) shared similarities as both reflected the diets, the PCO analysis showed a higher variation in muscle total fatty acid (Figure 3.3) than muscle PLFA (Figure 3.4). The excess of DHA in the MO diets resulted in high DHA proportions being incorporated into muscle tissue. The DHA proportion in muscle PL was higher than that of the diet, demonstrating the importance of DHA in the membrane. In contrast, the level of EPA was lower in salmon fed MO-containing diets compared to salmon fed FO-containing diets. It is worth noting that the concentration of EPA was low in MO treatments; however, no signs of retro-conversion from DHA to EPA in any appreciable amounts were observed since EPA remained low in the muscle. Similar to other published studies where dietary DHA was present in excess, DHA was the preferred fatty acid to be accumulated in the tissues, while EPA was probably used for energy production or biosynthesis of DHA (Betancor et al.,

2014; Codabaccuss et al., 2012; Emery et al., 2016). EPA is more readily ß-oxidized by mitochondria than DHA, primarily due to DHA being a poor substrate for ß-oxidation due to the fact that insertion and removal of the Δ^4 double bond in DHA requires a special mechanism (Sargent et al., 2003).

It was observed in the liver tissue and within liver PL that ARA>EPA in salmon fed MO-containing diets; however, in the muscle tissue and within muscle PL, ARA<EPA across all dietary treatments with possible connections to energy production and storage versus inflammation and immunity, and was therefore primarily stored in the liver. In terms of regiospecificity, ARA is known to be located almost exclusively in the *sn*-2 position of the glycerol of PI, which has critical roles in many areas of cellular signal transductions (Bell & Sargent, 2003). Recently Yeo & Parrish (2021) identified that a relatively smaller number of PS and PI molecular species was in salmon muscle tissue compared to PC and PE. Therefore, this could indicate why there is a lower proportion of ARA in the muscle tissue compared to the liver tissue. The regiospecificity of DHA and EPA is also generally at the *sn*-2 position (Miller et al., 2006; Sargent et al., 2003); however, new incoming DHA from the diet has a preference to be incorporated into PE, while high levels of EPA can be found in PI (Sargent et al., 2003; Stillwell & Wassall, 2003). PC and PE are the dominant PL classes in most eukaryotic membranes (Yeo & Parrish, 2021). Unlike liver tissue, the EPA/ARA ratio in salmon fed MO-containing diets remained >1. The ω3/ω6 ratios also remained >1 across the dietary treatments, perhaps suggesting the production of anti-inflammatory eicosanoids with consequent effects on immunity. The DHA/ARA ratio for the muscle PL also remained >1, but it is worth noting the ratio was 3-4 times higher than liver PL.

The proportion of EPA+DHA in the muscle tissue, commonly referred to as the fillet, is important for human consumption. Atlantic salmon is considered to be part of a healthy diet,

primarily due to its high content ω3 PUFA, which are known to be beneficial for the prevention and treatment of coronary disease. According to the American Dietetic Association/Dietitians of Canada, the daily recommendation is 500 mg/day of EPA+DHA provided by two servings of fatty fish/week (one serving is 112 g cooked) (Kris-Etherton et al., 2009). Our data show DHA+EPA/112 g (uncooked) would provide 924 mg per serving from salmon fed the FO diet, 467 mg from salmon fed the FO/CO and LMO diets, and 670.9 mg from salmon fed the HMO diet. Although salmon fed the FO diet had the highest EPA+DHA/112 g (uncooked) per serving, salmon fed the HMO diet also fulfills the 500 mg/day recommendation. Depending on different ways of cooking fish, the nutritional composition of the fillet can change based on the cooking method applied. Generally, most information about PUFA content is available for raw fish; thus, the consumer has little knowledge about the nutritive values of cooked fish (Bhouri et al., 2010). Deep-frying fish induces the largest change in fish lipids due to the absorption of high amounts of frying oil, such as vegetable oil which contains high amounts of ω6 fatty acids, thus resulting in an increased content of ω6 fatty acids (Moradi et al., 2011). However, it is worth mentioning that few lipid changes have been observed during frying for fish with a high-fat content (Candela et al., 1998; Mai et al., 1978). Appendix D compares the moisture and fat content of different fatty fishes when raw, cooked (deep-fried), and held warm. Oven baking resulted in loss of water with a consequent increase in protein, fat, and ash content. In contrast, grilling resulted in an increase in total lipids and ω3 PUFA, presumably due to the decrease in tissue water content (Moradi et al., 2011).

3.6 Conclusion

This study demonstrated that dietary MO from *Schizochytrium* sp. (T18) can be used to replace FO in the diet of farmed Atlantic salmon without negatively impacting fish growth and

fatty acid composition of the tissues. The fatty acid profiles reflected their respective dietary treatments. There were variations in response to dietary MO in liver and muscle PL, reflecting the functions of each tissue. Muscle total fatty acids had higher proportions of LA and ALA than did liver total fatty acids, while the DHA proportion was higher in the liver total fatty acids than muscle total fatty acids. The proportions of LA and ALA were lower in both liver and muscle PL than total fatty acids, while the DHA proportion was higher in both liver and muscle PL than the total fatty acids. The proportion of EPA in the tissue was dependent on the diet composition, while the proportion of DHA in the tissue was not dependent on the diet composition, suggesting less necessity for EPA in the diet and more necessity for DHA. The EPA+DHA daily recommendation of 500 mg/g was fulfilled by feeding salmon FO and HMO diets.

4. EFFECTS OF REPLACING FISH MEAL WITH *PAVLOVA* 459 SP. ALGAL BIOMASS ON LIPIDS AND PHOSPHOLIPID FATTY ACIDS IN ATLANTIC SALMON MUSCLE AND LIVER TISSUES

4.1 Abstract

A 12-week feeding trial was conducted to investigate the dietary effect of replacing fish meal (FM) with algal biomass (AB) derived from *Pavlova* sp. strain CCMP459 (*Pav*459). Three experimental diets were formulated to replace FM and partially replace fish oil (FO) with AB *Pav*459: a control diet (20% FM; 7% FO); an experimental diet composed of 50/50 FM and AB *Pav*459, and reduced FO (10% FM; 4.5% FO; 10% AB); a second experimental diet composed of full replacement of FM with AB *Pav*459 and reduced FO (1.75% FO; 20% AB). Replacing traditional FM with AB *Pav*459 did not significantly affect the growth parameters of Atlantic salmon, resulting in fish growth of over 200% from their initial weight across all dietary treatments. After 12-weeks of feeding, there were no significant differences in total lipid composition among the treatments; however, there were significant differences in individual proportions of ω3 and ω6 fatty acids among the treatments. Overall, the total fatty acids and phospholipid fatty acid composition in both muscle and liver tissues reflected the dietary treatments. The precursors, linoleic acid (LA) and α-linolenic acid (ALA), were present in a higher proportion in total fatty acid composition than in membrane lipids. Docosahexaenoic acid (DHA) was the dominant fatty acid in the membrane of both liver and muscle tissues. Furthermore, the compound-specific stable isotope analysis suggested direct incorporation of eicosapentaenoic acid (EPA) and DHA into the tissue from the diets and little biosynthesis from the precursor ALA. The EPA+DHA proportion in the fillet satisfied the daily requirement for human consumers across the treatments, further supporting the use of *Pav*459 as an alternative to FM.

4.2 Introduction

Fish is the most important food source of omega-3 (ω3) long-chain polyunsaturated fatty acids (LC-PUFA) in human diets. Aquaculture now accounts for more than half of the fish and seafood consumed by humans and therefore plays an increasingly important role in the global food system, the environment, and human health (Fry et al. 2016; FAO, 2020). Carnivorous fish such as Atlantic salmon require nutrients found in fish oil (FO) and fish meal (FM) for normal growth and development. However, the production of FM and FO is significantly affected by sustainability issues of marine capture fisheries, variable climatic events, and increasing prices of FM and FO (Bandara, 2018). With the continuous growth of aquaculture and limited FM and FO resources comes the growing demand for alternative protein and lipid sources that are reliable and sustainable. Finding nutritionally appropriate and sustainable alternatives to FM and FO for use in aquaculture feeds is an area of intense research (Stenberg et al., 2019). There have been numerous studies in past years regarding substituting FM and FO with alternative lipid sources from terrestrial plants (Mundheim et al., 2004; Burr et al., 2012), insect meal (Henry et al., 2015; Belghit et al., 2019), animal by-products (Fowler, L. G., 1991; Galkanda-Arachchige et al., 2020), microalgae (Kiron et al., 2016; Sprague et al., 2015), and other protein and lipid sources just to mention a few.

Nowadays, the inclusion of FM and FO in aquafeeds is reduced to a minimum amount able to cover the requirement for essential amino acids and fatty acids and other nutrients needed for fish growth and flesh quality (Gasco et al., 2018). With increasing substitution of ω3 fatty acid-rich marine ingredients with ω6 fatty acid-rich terrestrial ingredients, the chronically low ω3/ω6 ratios in modern farmed salmonid feeds are having negative health effects on the fish themselves, and also have a detrimental effect on the well-established dietary human health benefits normally

associated with consumption of fatty fish (Tibbetts et al., 2020a). Under such circumstances, novel low-trophic feedstocks produced from microalgae are becoming attractive as potentially more ecologically sustainable alternative sources of essential dietary nutrients for aquaculture feeds if they can be produced economically (Tibbetts et al., 2020a). Microalgae, along with other single-cell microbes, are the primary producers of ω3 LC-PUFA in the aquatic environment, providing a continual supply of eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) that is concentrated through the trophic food chain where there is limited capacity to synthesize these beneficial fatty acids (Sprague et al., 2017). *Pavlova* sp. strain CCMP459 (henceforth referred to as *Pav*459) is from the phylum Haptista, class Haptophyta, order Pavlovales, family *Pavlovaceae*, and genus *Pavlova* (Schoch et al., 2020). Tibbetts et al. (2020a) reported that *Pav*459 lipid was high in PUFA (> 60% of fatty acids), where ~50% were ω3 fatty acids, and only ~10% were ω6 fatty acids. In addition, 81% of ω3 PUFA were comprised of essential LC-PUFA, EPA at 3% of the meals, and DHA at 2% of the meals (Tibbetts et al., 2020a).

For this study, the objective was to determine the effect of reducing FO and a partial and total replacement of dietary FM with an algal biomass (AB) *Pav*459 on lipid class, total fatty acid, and phospholipid fatty acid (PLFA) composition of Atlantic salmon liver and muscle tissues. In addition, a bulk carbon stable isotope analysis on *Pav*459 and compound-specific stable isotope analysis (CSIA) on muscle and liver tissues was used as a tool to determine the relative contribution of dietary *Pav*459 to tissues LC-PUFA (EPA, DHA, ARA) in fish fed the two extreme diets (FM; AB). To our knowledge, there are no published studies investigating the effects of replacing FM with AB on membrane lipids of Atlantic salmon muscle and liver tissues. Indeed, very few feeding trial studies go to the extent of quantifying PLFA, and even fewer do CSIA on the same tissues.

4.3 Materials and Methods

Similar to chapter 3, this feeding study was also done in collaboration with our Dalhousie University partners, M.Sc. student Minmin Wei and supervisor Dr. Stefanie Colombo in Truro, Nova Scotia. Feeding trials were undertaken Dalhousie University Agricultural Campus in Bible Hill, NS.

4.3.1 Experimental diets

For this experiment, three experimental treatments were formulated as follows: a control diet (FM) included 7% FO, 20% FM, and 0% AB; a test diet (FM/AB) with reduced FO and 50/50 FM and AB *Pav*459 (4.5 % FO; 10% FM; 10% AB); a second test diet with reduced FO and 100% replacement of FM with AB *Pav*459 (1.75% FO; 0% FM; 20% AB). All diets were formulated to be isonitrogenous, isocaloric and to meet the nutritional requirements of Atlantic salmon (National Research Council (NRC), 2011). The AB *Pav*459 was produced at the National Research Council (Ketch Harbour, NS, Canada). Extended details on culture production methods can be viewed in Tibbetts et al. (2020a). For extended details on diet formulations, see Table D1 in Appendix D.

4.3.2 Experimental fish and tank set-up

Atlantic salmon post-smolts were received from Dartek (Merigomish, NS, Canada). A total number of 153 fish were randomly distributed into nine tanks (200 L) in the flow-through freshwater system at Dalhousie University Agricultural Campus (Bible Hill, NS, Canada). The experiment was a completely randomized design, and the tank was the experimental unit with three replicates. Atlantic salmon post-smolts (170.1 ± 23.9 g) (mean ± SD) were fed commercial feed (3 mm) twice a day for two weeks for acclimation after the transfer. The system was supplied with 13°C freshwaters with 100% oxygen saturation at a flow rate of 2-3 L/min. Temperature and

oxygen levels were measured and recorded daily. Fish were hand-fed experimental feed for 12 weeks after the initial sampling (week 0) twice a day at 9:00 and 15:00 until the fish were satiated. The hand-feeding was performed carefully to ensure no feed or minimum feed was wasted. Feed consumption was recorded weekly for each tank. The system was exposed to a natural photoperiod (16 h light: 8 h dark). Tanks were purged daily to remove fecal material. Mortalities were checked twice daily. Ethical treatment of fish in this experiment followed guidelines according to the Canadian Council of Animal Care (Dalhousie University Faculty of Agriculture Institutional Animal Care Approved Protocol #2019-135) (Wei et al., 2022).

4.3.3 Tissue sampling

Tissue sampling followed a similar procedure as in Section 3.3.3. Briefly, three fish per tank were randomly sampled from each tank at week 0, and five fish per tank were sampled at the end of the trial (week 12). Individual fish were rapidly netted and euthanized with TMS222 (150 mg/L). Skinless dorsal muscle and liver samples were collected for lipid class and fatty acid composition analysis. The tissue samples were flash-frozen in liquid nitrogen immediately after sampling and stored temporarily in a -80°C freezer. Upon arrival at the Ocean Sciences Centre (St. John's, NL, Canada), the sampled tissues were placed in a lipid-cleaned vial with chloroform and stored in a -20°C freezer.

4.3.4 Lipid extraction

Lipid samples were extracted as in section 3.3.4. Briefly, the samples were homogenized in a 2:1 mixture of ice-cold chloroform:methanol. Chloroform extracted water was added to bring the ratio of cholorform:methanol:water to 8:4:3. The samples were sonicated in an ice bath and centrifuged at 4000 rpm. A double pipetting technique was used to remove the bottom organic

layer and transferred into a lipid-cleaned vial. Chloroform was then added back to the extraction test tube, and the entire procedure was repeated three more times.

4.3.5 Fatty acid methyl ester (FAME) derivatization

Fatty acid methyl ester (FAME) derivatization followed a similar procedure as in Section 3.3.5. Briefly, lipid extracts were transesterified using methylene chloride and Hilditch reagent for 1 hr at 100°C. After allowing the mixture to cool, saturated sodium bicarbonate solution was added, followed by hexane. The upper organic layer was transferred to a lipid-clean vial, blown dried, and refilled with hexane. The vial was capped and sealed with Teflon tape, then sonicated to re-suspend the fatty acids.

4.3.6 Neutral lipid/polar lipid (NL/PL) separation

The neutral and polar lipids were separated as in Section 3.3.6. Briefly, the separation was done in a vacuum chamber using different solvents and solvent mixtures. All neutral lipid was washed with a solvent mixture (98:1:0.5 mixture of chloroform: methanol: formic acid) and collected in a lipid-clean vial. Acetone-mobile polar lipid (AMPL) was eluted with acetone and collected in a different lipid-clean vial. Phospholipid (PL) was first eluted with methanol, followed by a mixture of chloroform:methanol:water into a different lipid-clean vial. The PL fraction was transferred to a round-bottom flask and dried completely in a flash-evaporator. The lipids were then washed into a different lipid-clean vial using methanol and chloroform. The PLFA were derivatized using sulfuric acid and methanol for 1 hour at 100°C, as in section 4.3.5.

4.3.7 Quantitative lipid class analysis

Quantitative analysis followed similar procedures as in Section 3.3.7. Briefly, lipid classes were determined using TLC on silica-coated Chromarods and a three-step development method.

This was followed by Iatroscan FID. After the first development system, HC, SE, and KET lipid classes were detected. The second development system separated TAG, FFA, ALC, and ST lipid classes. The third development system separated AMPL and PL lipid classes.

All FAME samples were analyzed on an HP 6890 GC-FID equipped with a 7683 autosampler. Peaks were identified using retention times from standards purchased from Supelco: 37 component FAME mix, bacterial acid methyl ester mix, PUFA 1, and PUFA 3. Chromatograms were integrated using the Agilent OpenLAB Data Analysis.

4.3.8 Compound Specific Stable Isotope Analysis

The $\delta^{13}C$ ($^{13}C/^{12}C$) values of identified FAME were measured and analyzed using an Agilent 6890N gas chromatograph coupled via a GC Combustion III interface to a Delta V Plus isotope ratio mass spectrometer (Thermo Fisher Scientific, Mississauga, ON, Canada) at the Core Research Equipment and Instrument Training Network (CREAIT Network) of Memorial University. FAME samples from two extreme diets (FM, AB; n=3), FAME samples from muscle total fatty acids (FM, AB; n=9), six randomly selected FAME samples from muscle PLFA (FM, AB; n=3), FAME samples from liver PLFA (FM, AB; n=9), and six randomly selected FAME samples from liver total fatty acids (FM, AB; n=3) were analyzed. All $\delta^{13}C$ values were calculated relative to the Vienna Pee Dee Belemnite (VPDB) standard using the following equation:

$$\delta^{13}C = \left[\frac{R(sample)}{R(standard)} - 1\right] * 1000$$

where R is the ratio of $^{13}C/^{12}C$. An aliquot of the methanol used during the FAME derivatization of fatty acids was collected and analyzed for $\delta^{13}C$ composition at the University of Ottawa (Ottawa, ON, Canada) in order to correct for the additional methyl group added to fatty acids

during transesterification. This correction was applied to all fatty acids using the following equation:

$$\delta^{13}C = (n+1)[\delta^{13}C_{FAME}] - n[\delta^{13}C_{FFA}]$$

where n is the number of C atoms in the fatty acid.

Finally, a two-end-member mixing model was used to determine the relative contributions to liver and muscle EPA, DHA, and ARA in the two dietary treatments:

$$\delta^{13}C\ Tissue_{,k} = X_k\delta^{13}C_{pre} + (1 - X_k)\delta^{13}C_{LC-PUFA}$$

where X_k is the proportion of precursor C contribution to k, the fatty acid of interest (i.e., EPA or DHA). $\delta^{13}C_{pre}$ is the isotopic signature of precursor, while $\delta^{13}C_{LC\text{-}PUFA}$ is the isotopic signature of EPA or DHA in each of the two diets.

The bulk carbon stable isotopes was analyzed in an Elemental Analyzer (EA) system (NA1500; Carlo-Erba) consisting of an autosampler, an oxidation reactor (oven), a reduction reactor, a water trap, a gas chromatography (GC) column, and a thermal conductivity meter (TCD). Extended details on instrumentation and bulk stable isotope results are attached in Appendix G.

4.3.9 Statistical analysis

Similar to Section 3.3.8, the resulting data were presented as mean ± SD. Grubbs' outlier tests were performed, and lipid data from one tank had more than 50% of the data with an identified outlier. All lipid data from fish in this tank were removed. All statistical analyses were performed using general linear models on Minitab with one-way ANOVA. The model was designed to test diet effect (fixed factor) and nested tank (fixed factor) within diet for tank effect on different lipid classes and fatty acids (response variable). Significant difference were set at fixed $\alpha = 5\%$ criterion

($p < 0.05$). Pairwise comparisons were performed using Tukey post hoc test for multiple comparisons to detect differences between diets. Normality testing was performed using the Anderson–Darling test.

4.4 Results

4.4.1 Diet composition

The total lipid content of *Pav*459 was 109.6 mg/g ww (Table 4.1). The main lipid class was TAG (24.7%), followed by AMPL (22.9%), FFA (18.5%), PL (16.6%) and ST (10.9%). The fatty acid composition of *Pav*459 was mainly composed of PUFA (64.5%) followed by SFA (23.0%) and MUFA (11.2%) (Table 4.1). The dominant EFA was EPA (26.8%), followed by DHA (13.6%), and very low ARA (0.5%). *Pav*459 had approximately the same proportion of the precursors LA (3.5%) and ALA (3.6%). The total sum of ω3 fatty acids was 4-fold more prevalent than ω6 fatty acids.

The total lipid content in the diets varied between 144.2 and 200.0 mg/g ww (Table 4.2). Lipid classes in the diets were mainly composed of TAG (72.7% - 74.1%), followed by FFA (8.5% - 9.1%), PL (3.1% - 6.3%), AMPL (5.0% - 8.0%) and ST (3.5% - 7.5%). The PL proportion in FM/AB diet was 2-times lower than the other dietary treatments; however, it was not significantly different. Significant differences were present for ST and AMPL lipid classes. Differences in total fatty acid proportions were small, but often significant (Table 4.2). The fatty acid composition of the diets was mainly MUFA (44.8% – 47.1%), followed by PUFA (31.0% - 32.3%) and SFA (20.8% - 22.3%). The EPA and DHA proportions were significantly lower in AB diet compared to FM and FM/AB diets; however, the precursors LA and ALA were significantly higher in AB diet than FM and FM/AB diets. Despite the low EPA and DHA proportion in AB diet, the

EPA+DHA proportion was higher than the minimum satisfactory levels (0.5 – 2.0%) recommended by NRC (2011). The ARA proportion was similar across the dietary treatments. The total sum of ω6 fatty acids was more prevalent than ω3 fatty acids across the dietary treatments resulting in a >1 ω6/ω3 ratio.

Table 4.1: Lipid class and fatty acid composition of the AB *Pav*459 used in the study[1]

Lipid class composition (%)	
Total lipid (mg/g)	109.6 ± 10.4
Triacylglycerol	24.7 ± 0.4
Free fatty acids	18.5 ± 1.1
Sterol	10.9 ± 1.0
Acetone mobile polar lipids	22.9 ± 1.7
Phospholipid	16.6 ± 1.4
Fatty acid composition (%)	
14:0	15.6 ± 0.1
15:0	0.2 ± 0.0
16:0	7.3 ± 0.2
Total SFA[2]	23.0 ± 0.2
16:1ω7	6.4 ± 0.0
16:1ω5	3.7 ± 0.0
18:1ω9	0.2 ± 0.0
18:1ω7	0.1 ± 0.0
Total MUFA[3]	11.2 ± 0.2
16:2ω4	2.2 ± 0.0
18:2ω6 (LA)	3.5 ± 0.1
18:3ω6	0.4 ± 0.0
20:4ω6 (ARA)	0.5 ± 0.5
22:5ω6 (ω6DPA)	7.2 ± 0.1
18:3ω3 (ALA)	3.6 ± 0.1
18:4ω3	6.3 ± 0.1
20:5ω3 (EPA)	26.8 ± 0.1
22:5ω3	0.0 ± 0.0
22:6ω3 (DHA)	13.6 ± 0.2
Total PUFA[4]	64.5 ± 0.3
Total ω3	50.3 ± 0.3
Total ω6	11.7 ± 0.4
ω3/ω6 ratio	4.3 ± 0.2
EPA+DHA	40.4 ± 0.3
DHA/EPA ratio	0.5 ± 0.0

[1]Data expressed as % diet (wet weight), values are means (n=3 per treatment) ± standard deviation.
[2]Saturated fatty acid
[3]Monosaturated fatty acid
[4]Polyunsaturated fatty acid

Table 4.2: Lipid composition of experimental diets[1]

Lipid class composition (%)			
	FM	**FM/AB**	**AB**
Total lipid (mg/g)	144.2 ± 23.6	200.0 ± 38.2	174.5 ± 34.8
Triacylglycerol	74.1 ± 1.8	75.1 ± 1.1	72.7 ± 2.2
Free fatty acids	9.0 ± 1.1	9.1 ± 0.3	8.5 ± 0.9
Sterol	3.5 ± 0.4^{b}	7.5 ± 0.7^{a}	4.7 ± 1.7^{b}
Acetone mobile polar lipids	5.7 ± 0.8^{b}	5.0 ± 0.7^{b}	8.0 ± 1.0^{a}
Phospholipid	6.3 ± 1.7	3.1 ± 0.5	6.1 ± 2.2
Fatty acid composition (%)			
14:0	2.3 ± 0.4	1.9 ± 0.5	2.2 ± 0.1
16:0	14.9 ± 0.7	13.9 ± 0.8	14.0 ± 0.1
18:0	4.0 ± 0.2	4.3 ± 0.6	3.7 ± 0.0
Total SFA[2]	22.3 ± 0.9	21.4 ± 0.6	20.8 ± 0.1
16:1ω7	4.7 ± 0.2^{a}	3.9 ± 0.5^{ab}	3.8 ± 0.0^{b}
16:1ω5	0.1 ± 0.0^{c}	0.2 ± 0.0^{b}	0.4 ± 0.0^{a}
18:1ω9	31.6 ± 0.3^{c}	36.2 ± 1.2^{b}	38.7 ± 0.3^{a}
18:1ω7	2.6 ± 0.0^{a}	2.5 ± 0.0^{b}	2.3 ± 0.0^{c}
20:1ω9	2.0 ± 0.1^{a}	1.5 ± 0.3^{b}	0.6 ± 0.1^{c}
Total MUFA[3]	44.8 ± 0.6	47.1 ± 1.6	46.8 ± 0.2
16:2ω4	0.4 ± 0.0^{a}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}
18:2ω6 (LA)	14.6 ± 0.2^{c}	16.0 ± 0.7^{b}	18.8 ± 0.0^{a}
18:3ω6	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.1 ± 0.0^{b}
20:3ω6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4ω6 (ARA)	0.4 ± 0.0^{a}	0.4 ± 0.0^{b}	0.3 ± 0.0^{b}
22:5ω6 (ω6DPA)	0.1 ± 0.0^{c}	0.4 ± 0.0^{b}	0.6 ± 0.0^{a}
18:3ω3 (ALA)	2.8 ± 0.1^{b}	3.2 ± 0.3^{b}	3.9 ± 0.0^{a}
18:4ω3	0.9 ± 0.0^{b}	0.9 ± 0.1^{b}	1.2 ± 0.0^{a}
20:4ω3	0.3 ± 0.0^{a}	0.2 ± 0.0^{b}	0.1 ± 0.0^{c}
20:5ω3 (EPA)	5.3 ± 0.2^{a}	4.4 ± 0.3^{b}	3.8 ± 0.0^{c}
22:5ω3	0.7 ± 0.0^{a}	0.4 ± 0.0^{b}	0.2 ± 0.0^{c}
22:6ω3 (DHA)	4.4 ± 0.2^{a}	3.2 ± 0.2^{b}	1.9 ± 0.1^{c}
Total PUFA[4]	32.3 ± 0.8	31.0 ± 1.3	32.0 ± 0.2
Total ω3	14.8 ± 0.5^{a}	12.4 ± 0.7^{b}	11.1 ± 0.1^{c}
Total ω6	16.1 ± 0.2^{c}	17.5 ± 0.7^{b}	20.4 ± 0.1^{a}
ω3/ω6 ratio	0.9 ± 0.0^{a}	0.7 ± 0.0^{b}	0.5 ± 0.0^{c}
EPA+DHA	9.7 ± 0.4^{a}	7.5 ± 0.4^{b}	5.6 ± 0.1^{c}
DHA/EPA ratio	0.8 ± 0.0^{a}	0.7 ± 0.1^{b}	0.5 ± 0.0^{c}
EPA/ARA ratio	12.5 ± 0.4	12.3 ± 1.0	11.7 ± 0.9
DHA/ARA ratio	10.4 ± 0.2^{a}	8.8 ± 0.1^{b}	5.7 ± 0.4^{c}

[1]Data expressed as % lipid or fatty acid methyl ester (FAME). Values are means ± standard deviation (n=3 per treatment). Means with different superscripts indicate significant differences ($p < 0.05$) based on Tukey's *posthoc* test following a general linear model. FM = fish meal (control); FM/AB = fish meal/AB *Pav*459; AB = AB *Pav*459.
[2]Saturated fatty acid.
[3]Monounsaturated fatty acid.
[4]Polyunsaturated fatty acid.

4.4.2 Growth performance

The experimental diets were formulated to provide adequate nutrients and energy to Atlantic salmon. After 12 weeks of feeding, the dietary treatments had no negative effect on fish growth, resulting in approximately 263% growth from their initial weight (~170.1 ± 23.9 g) across the diets. Salmon fed the FM, FM/AB, and AB diet gained mean weights of 279.6, 276.3, 271.3 g, respectively, compared to the initial weight of 170.1 g. The full details for growth performance is part of a paper being prepared by our partners in Truro, Nova Scotia, as a collaboration on this project. However, the Results Tables are attached in Appendix D.

4.4.3 Liver tissue lipid class and fatty acid composition

Week-0 liver tissue total lipid was 18.3 mg/g ww, composed mostly of neutral lipid (Table 4.3). After 12 weeks of feeding, the total lipid concentration increased similarly across all dietary treatments (24.4 – 28.3 mg/g ww) (Table 4.3). The tissue lipid composition remained mainly neutral lipid in all dietary treatments. The dominant lipid class was FFA (25.4 – 30.3%), followed by PL (23.4 – 25.4%), ST (18.4 – 23.9%), and TAG (6.8 – 12.4%). There was no significant difference for any lipid classes among the dietary treatments.

After 12-weeks of feeding, the fatty acid profile for liver tissue reflected the diets (Table 4.3). Differences in total fatty acid proportions were small but significant across the dietary treatments. Significant differences were mostly observed between salmon fed the AB diet and salmon fed the FM diet. Occasionally, significant differences between salmon fed the FM/AB diet and salmon fed the FM diet were present. The fatty acid composition was mostly PUFA (45.4 – 48.3%), followed by MUFA (32.9 – 37.7%) and SFA (16.6 – 18.4%). Almost all individual fatty acid proportions decreased compared to week-0 fatty acid proportions, except for 18:1ω9, MUFA,

LA, ALA, and total ω6 among all dietary treatments. The dominant EFA was DHA (19.6 – 23.7%), followed by the precursor, LA (7.7 – 9.5%), EPA (3.5 – 5.5%), ARA (2.5 – 3.7%), and there was low ALA (0.9 – 1.1%). The sum of ω3 fatty acids was approximately 2-fold more prevalent than ω6 fatty acids in salmon fed the FM and FM/AB diets and approximately a third more prevalent than ω6 fatty acids in salmon fed the AB diet, resulting in a >1 ω3/ω6 ratio across the dietary treatments.

Principal coordinates analysis of week-12 liver total fatty acids showed PCO1 and PCO2 (Fig. 4.1) accounted for 80.8% and 12.4% of the variability, respectively. The PCO biplot showed that the highest variation in liver total fatty acids was between salmon fed the FM and AB diets. SIMPER analysis (Table E1 in Appendix E) showed an 82.6% similarity within salmon fed the FM diet, an 89.1% similarity within salmon fed the FM/AB diet, and a 90.4% similarity within salmon fed the AB diet. The dietary treatments with the highest dissimilarities were salmon fed the FM and AB diets (16.8%), followed by salmon fed the FM and FM/AB diets (15.4%), and salmon fed the FM/AB and AB diets (10.7%). The main drivers for the similarities and dissimilarities were 18:1ω9, followed by DHA, 16:0, and LA.

4.4.4 Liver tissue phospholipid fatty acid composition

Liver tissue PLFA profiles also reflected the diets (Table 4.4). Differences in PLFA proportion across the dietary treatments were small but significantly different, especially between salmon fed the AB diet and salmon fed the FM diet. The liver tissue PLFA comprised mainly of PUFA (58.5 – 59.3%), followed by SFA (23.2 – 24.1%) and MUFA (17.0 – 17.4%). DHA was the dominant EFA in the membrane phospholipid, followed by EPA and ARA. The EPA and DHA proportions were higher and significantly different in salmon fed the FM and FM/AB diets than salmon fed the AB diet, while the ARA proportion was higher and significantly different in salmon

fed the AB diet than salmon fed the FM and FM/AB diets. As for the precursors, LA was also present in high proportion in storage but present in low proportion in the membrane. Similarly, ALA was also higher in storage and nearly less than half in the membrane. The total sum of ω3 fatty acids was approximately 3-fold more prevalent than ω6 fatty acids in salmon fed the FM and FM/AB diets, and approximately 2-fold more prevalent than ω6 fatty acids in salmon fed the AB diet, resulting in a >1 ω3/ω6 ratio across the dietary treatments. The DHA/EPA ratio was higher and significantly different in salmon fed the AB diet (7.0%) than salmon fed the FM diet (5.1%). The EPA/ARA ratio was higher and significantly different in salmon fed the FM diet (2.1%) than salmon fed the AB diet (0.9%). The DHA/ARA ratio was higher and significantly different in salmon fed the FM diet (10.7%) than salmon fed the AB diet (5.9%).

Principal coordinates analysis of week-12 liver PLFA showed PCO1 and PCO2 (Fig. 4.2) accounted for 75.5% and 12.0% of the variability, respectively. The liver PLFA PCO biplot clearly showed that the main variation was between salmon fed different diets, with salmon fed the AB diet being significantly different than salmon fed the FM diet. SIMPER analysis (Table E2 in Appendix E) demonstrated that there was a 96.1% similarity within salmon fed the FM diet, a 97.0% similarity within salmon fed the FM/AB diet, and a 96.6% similarity within salmon fed the AB diet. The highest dissimilarities were between salmon fed the FM and AB diets (10.3%), followed by salmon fed the FM/AB and AB diets (6.4%), and salmon fed the FM and FM/AB diets (5.5%). The main drivers for the similarities were DHA, 16:0, and 18:1ω9 across all dietary treatments, and the main drivers for the dissimilarities were EPA, DHA, 22:5ω6, and ARA. The order and the contribution percentage to which these fatty acids affected the similarities and dissimilarities varied across the dietary treatments.

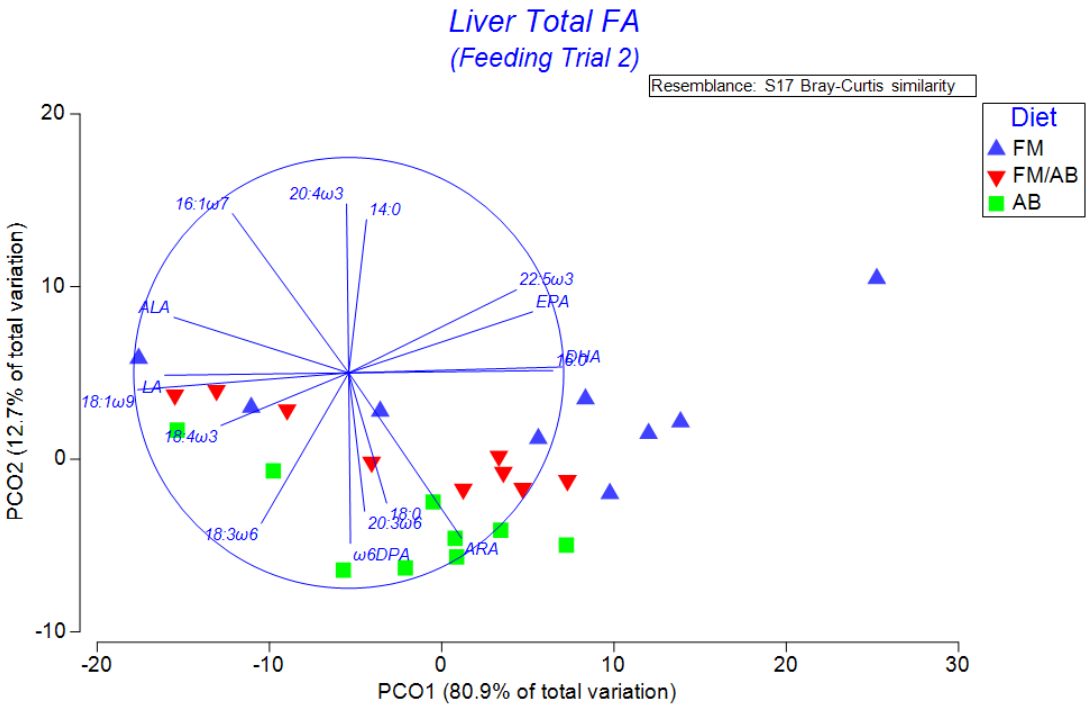


Figure 4.1: Principal coordinates analysis (PCO) of Atlantic salmon liver tissue total fatty acid composition (%) after 12 weeks of feeding experimental diets

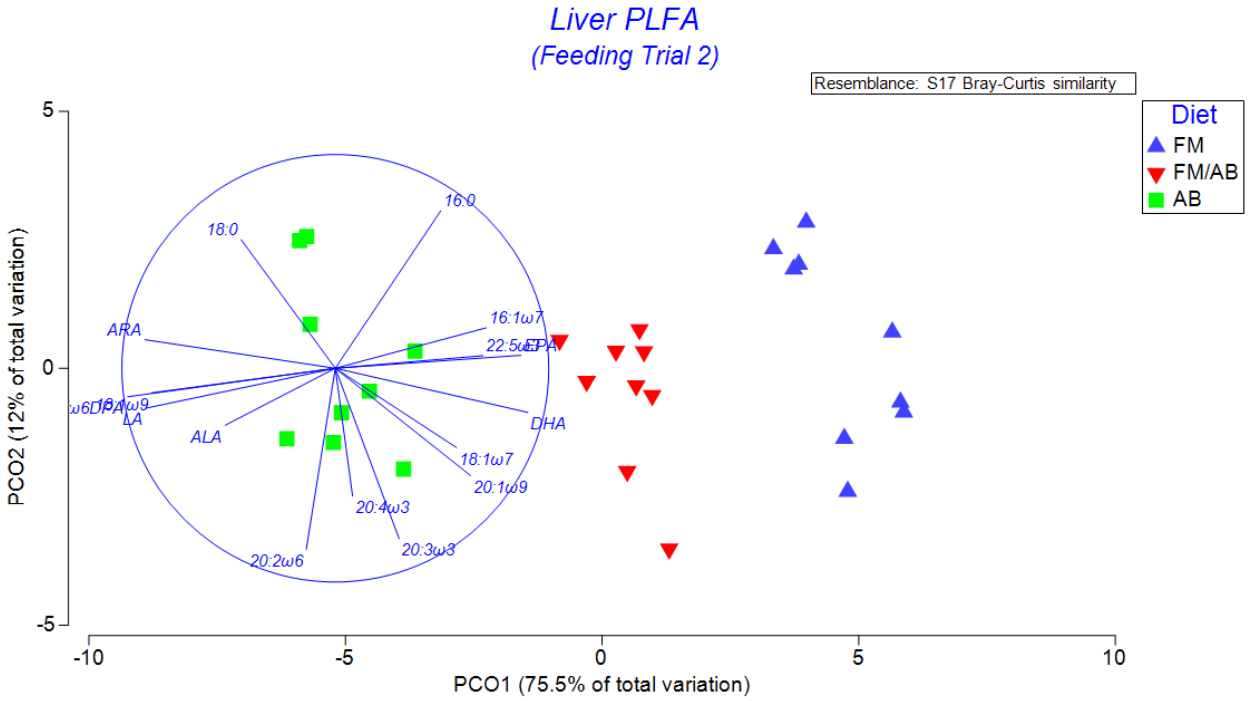


Figure 4.2: Principal coordinates analysis (PCO) of Atlantic salmon liver tissue phospholipid fatty acid composition (%) after 12 weeks of feeding experimental diets

Table 4.3: Lipid class and total fatty acid composition of Atlantic salmon liver tissue, prior to feeding experimental diets and after 12 weeks of feeding experimental diets[1]

	Initial	FM	FM/AB	AB
Lipid composition (%)				
Total lipid (mg/g)	18.3 ± 4.5	28.3 ± 4.9	24.4 ± 4.5	26.3 ± 8.3
Neutral Lipid	60.7 ± 4.6	65.1 ± 5.8	62.1 ± 6.5	62.1 ± 4.7
Polar Lipid	39.3 ± 4.6	34.9 ± 5.8	37.9 ± 6.5	37.9 ± 4.7
Lipid class composition (%)				
Triacylglycerol	1.5 ± 1.2	12.4 ± 16.3	10.1 ± 10.6	6.8 ± 7.0
Free fatty acids	30.8 ± 2.5	30.2 ± 8.8	25.4 ± 6.3	28.7 ± 5.3
Sterol	26.9 ± 3.9	20.4 ± 4.1	23.9 ± 3.5	21.9 ± 3.6
Phospholipid	26.7 ± 3.2	23.4 ± 5.4	25.4 ± 5.3	24.1 ± 4.3
PL/ST ratio[2]	1.0 ± 0.2	1.2 ± 0.1	1.1 ± 0.2	1.1 ± 0.1
Fatty acid composition (%)				
14:0	1.5 ± 0.1	1.2 ± 0.2^{a}	1.1 ± 0.1^{ab}	1.0 ± 0.1^{b}
16:0	18.0 ± 1.3	12.6 ± 2.3	11.1 ± 1.8	11.2 ± 1.4
18:0	5.6 ± 0.5	4.2 ± 0.5	3.9 ± 0.4	4.4 ± 0.8
Total SFA[3]	25.7 ± 0.9	18.4 ± 2.3	16.6 ± 2.2	17.1 ± 1.9
16:1ω7	2.4 ± 0.2	2.5 ± 0.5^{a}	2.4 ± 0.3^{ab}	2.0 ± 0.2^{b}
16:1ω5	0.1 ± 0.0	0.1 ± 0.0^{c}	0.2 ± 0.0^{b}	0.3 ± 0.1^{a}
18:1ω9	11.4 ± 1.6	23.0 ± 9.9	28.0 ± 5.6	27.5 ± 4.4
18:1ω7	2.5 ± 0.2	2.7 ± 0.3	2.6 ± 0.3	2.4 ± 0.4
Total MUFA[4]	17.6 ± 2.3	32.9 ± 11.2	37.7 ± 7.0	36.0 ± 5.5
18:2ω6 (LA)	4.3 ± 0.3	7.7 ± 1.7^{b}	9.2 ± 1.0^{ab}	9.5 ± 1.2^{a}
18:3ω6	0.2 ± 0.0	0.2 ± 0.0^{c}	0.3 ± 0.0^{b}	0.4 ± 0.1^{a}
20:3ω6	0.9 ± 0.2	1.2 ± 0.3^{b}	1.4 ± 0.1^{b}	2.2 ± 0.3^{a}
20:4ω6 (ARA)	4.6 ± 0.6	2.5 ± 0.8^{b}	2.7 ± 0.8^{b}	3.7 ± 0.7^{a}
22:5ω6 (ω6DPA)	0.9 ± 0.1	0.4 ± 0.1^{c}	1.0 ± 0.2^{b}	1.8 ± 0.3^{a}
18:3ω3 (ALA)	0.5 ± 0.1	0.9 ± 0.3	1.1 ± 0.2	1.0 ± 0.2
18:4ω3	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
20:4ω3	0.5 ± 0.1	0.7 ± 0.2^{a}	0.6 ± 0.1^{ab}	0.5 ± 0.1^{b}
20:5ω3 (EPA)	7.4 ± 1.2	5.5 ± 1.2^{a}	4.0 ± 0.9^{b}	3.5 ± 0.8^{b}
22:5ω3	2.7 ± 0.5	1.3 ± 0.2^{a}	0.9 ± 0.1^{b}	0.8 ± 0.2^{b}
22:6ω3 (DHA)	32.5 ± 1.8	23.7 ± 8.2	20.3 ± 4.5	19.6 ± 3.6
Total PUFA[5]	56.7 ± 1.7	48.3 ± 9.0	45.4 ± 5.1	46.7 ± 3.9
Total ω3	43.9 ± 1.8	32.6 ± 9.2	27.6 ± 5.2	26.0 ± 4.2
Total ω6	11.6 ± 0.9	14.1 ± 1.2^{c}	16.5 ± 0.5^{b}	19.7 ± 0.5^{a}
ω3/ω6 ratio	3.8 ± 0.4	2.3 ± 0.7^{a}	1.7 ± 0.3^{b}	1.3 ± 0.2^{b}
EPA+DHA	39.9 ± 1.7	29.2 ± 9.3^{a}	24.3 ± 5.4^{b}	23.1 ± 4.3^{b}
DHA/EPA ratio	4.5 ± 0.9	4.2 ± 0.7^{b}	5.1 ± 0.5^{a}	5.7 ± 0.5^{a}
EPA/ARA ratio	1.6 ± 0.2	2.3 ±0.5^{a}	1.5 ± 0.3^{b}	0.9 ± 0.2^{c}
DHA/ARA ratio	7.2 ± 1.1	9.5 ± 1.2^{a}	7.7 ± 1.0^{b}	5.3 ± 0.5^{c}

[1]Data expressed as % lipid or fatty acid methyl ester (FAME). Values are means ± standard deviation (n=9 per treatment). Means with different superscripts indicate significant differences ($p < 0.05$) based on Tukey's *posthoc* test following a general linear model. FM = fish meal (control); FM/AB = fish meal/AB *Pav*459; AB = AB *Pav*459. [2]Phospholipid/sterol ratio [3]Saturated fatty acid. [4]Monounsaturated fatty acid. [5]Polyunsaturated fatty acid.

Table 4.4: Phospholipid fatty acid composition of Atlantic salmon liver tissue after 12 weeks of feeding experimental diets[1]

	FM	FM/AB	AB
Fatty acid composition (%)			
14:0	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
16:0	17.4 ± 0.8^{a}	16.8 ± 0.7^{ab}	16.3 ± 0.8^{b}
18:0	5.2 ± 0.9	5.0 ± 0.3	5.7 ± 0.6
Total SFA[2]	24.1 ± 1.3	23.2 ± 1.0	23.3 ± 1.1
16:1ω7	1.4 ± 0.2^{a}	1.2 ± 0.1^{b}	1.0 ± 0.1^{c}
16:1ω5	0.1 ± 0.0^{c}	0.1 ± 0.0^{b}	0.2 ± 0.0^{a}
18:1ω9	11.5 ± 0.4^{c}	12.2 ± 0.3^{b}	12.9 ± 0.5^{a}
18:1ω7	1.5 ± 0.4^{a}	12.2 ± 0.3^{ab}	12.9 ± 0.5^{b}
20:1ω9	1.6 ± 0.3^{a}	1.4 ± 0.3^{ab}	1.2 ± 0.2^{b}
Total MUFA[3]	17.0 ± 0.6	17.2 ± 0.7	17.4 ± 0.5
18:2ω6 (LA)	4.5 ± 0.2^{c}	5.4 ± 0.3^{b}	6.0 ± 0.3^{a}
18:3ω6	0.1 ± 0.0^{c}	0.2 ± 0.0^{b}	0.3 ± 0.0^{a}
20:2ω6	1.5 ± 0.3	1.6 ± 0.2	1.6 ± 0.3
20:3ω6	1.3 ± 0.2^{c}	1.8 ± 0.2^{b}	2.7 ± 0.3^{a}
20:4ω6 (ARA)	3.4 ± 0.4^{c}	4.2 ± 0.6^{b}	5.5 ± 0.6^{a}
22:4ω6	0.2 ± 0.1^{a}	0.1 ± 0.0^{b}	0.2 ± 0.1^{a}
22:5ω6 (ω6DPA)	0.6 ± 0.0^{c}	1.7 ± 0.1^{b}	3.1 ± 0.1^{a}
18:3ω3 (ALA)	0.4 ± 0.0^{b}	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}
18:4ω3	0.0 ± 0.0^{b}	0.1 ± 0.0^{b}	0.1 ± 0.0^{a}
20:3ω3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4ω3	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
20:5ω3 (EPA)	7.2 ± 0.7^{a}	5.7 ± 0.6^{b}	4.6 ± 0.6^{c}
22:5ω3	1.6 ± 0.2^{a}	1.2 ± 0.1^{b}	1.1 ± 0.2^{b}
22:6ω3 (DHA)	36.1 ± 1.1^{a}	35.2 ± 1.0^{a}	32.0 ± 0.7^{b}
Total PUFA[4]	58.5 ± 1.2	59.3 ± 0.9	59.1 ± 1.0
P/S ratio[5]	2.4 ± 0.2	2.6 ± 0.1	2.5 ± 0.2
Total ω3	45.9 ± 1.2^{a}	43.3 ± 1.0^{b}	39.0 ± 0.8^{c}
Total ω6	11.7 ± 0.3^{c}	15.1 ± 0.5^{b}	19.4 ± 0.5^{a}
ω3/ω6 ratio	3.9 ± 0.2^{a}	2.9 ± 0.1^{b}	2.0 ± 0.1^{c}
EPA+DHA	43.2 ± 1.0^{a}	40.9 ± 1.0^{b}	36.7 ± 0.8^{c}
DHA/EPA ratio	5.1 ± 0.5^{b}	6.2 ± 0.7^{a}	7.0 ± 0.8^{a}
EPA/ARA ratio	2.1 ± 0.4^{a}	1.4 ± 0.3^{b}	0.8 ± 0.1^{c}
DHA/ARA ratio	10.7 ± 1.6^{a}	8.5 ± 1.3^{b}	5.9 ± 0.6^{c}

[1]Data expressed as % lipid or fatty acid methyl ester (FAME). Values are means ± standard deviation (n=9 per treatment). Means with different superscripts indicate significant differences ($p < 0.05$) based on Tukey's *posthoc* test following a general linear model. FM = fish meal (control); FM/AB = fish meal/AB *Pav*459; AB = AB *Pav*459.
[2]Saturated fatty acid.
[3]Monounsaturated fatty acid.
[4]Polyunsaturated fatty acid.
[5]PUFA/SFA ratio.

4.4.5 Muscle tissue lipid class and fatty acid composition

Week-0 muscle tissue total lipid was 15.8 mg/g ww and composed mainly of neutral lipid (Table 4.5). After 12 weeks of feeding, there was approximately a 4.5-fold increase in total lipid in salmon fed the FM diet (70.4 mg/g ww), and an average of a 4-fold increase from salmon fed the FM/AB diet (64.5 mg/g ww) and AB diet (63.4 mg/g ww) (Table 4.5). There was no significant difference in total lipid concentration across the dietary treatments, and the tissue composition was mostly neutral lipids (61.4 – 75.5%) across the dietary treatments. The dominant lipid class was TAG (43.9 – 52.5%), followed by PL (8.1 – 20.3%), ST (6.2 – 11.9%), and FFA (4.1 – 7.5%). It is important to point out that the PL proportion in salmon fed the FM/AB diet was lower than the ST proportion. Significant differences were present for FFA, ST, and PL lipid classes. The PL proportion in salmon fed the FM/AB diet was low and significantly different to salmon fed the FM and AB diets, while the FFA proportion was higher and significantly different to salmon fed the FM and AB diets. The ST proportion in salmon fed the AB diet was low and significantly different to salmon fed the FM and FM/AB diets.

After 12-weeks of feeding, the muscle tissue fatty acids profile reflected the diets (Table 4.5). Similar to liver tissue, differences in total fatty acid proportions were small but significant across the dietary treatments. Significant differences were observed between salmon fed the AB diet and salmon fed the FM diet, and occasionally, significant differences between salmon fed the FM/AB diet and salmon fed the FM diet were observed. However, the fatty acid composition in the muscle tissue was mostly MUFA (41.6 – 44.7%), followed by PUFA (35.1 – 36.4%) and SFA (18.5 – 22.6%). Similar to liver tissue, almost all individual fatty acids decreased compared to week-0 except for 18:1ω9, MUFA, LA, ALA, and total ω6 across all dietary treatments. The dominant EFA was the precursor LA (12.4 – 15.4%), followed by DHA (7.6 – 9.1%), EPA (2.7 –

3.7%), ALA (2.3 – 2.7%), and there was very low ARA (0.5 – 0.7). The total sum of ω3 fatty acids was approximately 1-fold more prevalent than ω6 fatty acids in salmon fed the FM and FM/AB diets, and approximately 1-fold less prevalent than ω6 fatty acids in salmon fed the AB diet, resulting in a >1 ω3/ω6 ratio in salmon fed the FM and FM/AB diets and a >1 ω6/ω3 ratio in salmon fed the AB diet.

Principal coordinates analysis of week-12 muscle total fatty acids showed PCO1 and PCO2 (Fig. 4.3) accounted for 67.5% and 27.0% of the variability, respectively. The PCO biplot for muscle total fatty acids did not show a clear variation between the dietary treatments as the one observed in liver PLFA. However, the highest variation observed was between salmon fed the FM and AB diets. SIMPER analysis (Table E3 in Appendix E) demonstrated that there was a 90.7% similarity within salmon fed the FM diet, a 95.3% similarity within salmon fed the FM/AB diet, and a 92.8% similarity within salmon fed the AB diet. The dietary treatments with the highest dissimilarities were salmon fed the FM and AB diets (13.6%), followed by salmon fed the FM and FM/AB diets (8.8%), and salmon fed the FM/AB and AB diets (7.9%). The main drivers for the similarities and dissimilarities were 18:1ω9, 16:0, DHA, and LA. The order and the contribution percentage to which these fatty acids affected the similarities and dissimilarities varied across the dietary treatments.

4.4.6 Muscle tissue phospholipid fatty acid composition

Muscle tissue PLFA profiles also reflected the diets (Table 4.6). Differences were minimal but significant across the dietary treatments, especially in salmon fed the FM diet and salmon fed the AB diet. The muscle tissue PLFA was mostly composed of MUFA (41.6 – 44.7%) followed by PUFA (35.1 – 36.4%) and SFA (18.5 – 22.6%). Unlike the muscle tissue total fatty acid composition, the dominant EFA in the membrane was DHA (33.3 – 38.2%), followed by EPA (6.7

– 8.7%), LA (4.1 – 5.6%), ARA (1.3 – 1.9%), and ALA (1.1 – 1.4%). There was no significant difference in DHA proportion among the dietary treatments. Although present in low proportion, EPA was higher and significantly different in salmon fed the FM and diet than salmon fed the FM/AB and AB diets, while ARA was higher and significantly different in salmon fed the AB diet than salmon fed the FM and FM/AB diets. Similarly, the precursors LA and ALA were significantly higher in salmon fed the AB diet than salmon fed the FM and FM/AB diets. The total sum of ω3 fatty acids was approximately a quarter more prevalent than ω6 fatty acids in salmon fed the FM and FM/AB diets, and approximately a fifth less prevalent than ω6 fatty acids in salmon fed the AB diet. This resulted in a >1 ω3/ω6 ratio in salmon fed the FM and FM/AB diets and a >1 ω6/ω3 ratio in salmon fed the AB diet. Differences in DHA/EPA ratio, EPA/ARA ratio, and DHA/ARA ratio were mostly minimal but significant across the dietary treatments.

Principal coordinates analysis of week-12 muscle PLFA showed PCO1 and PCO2 (Fig. 4.4) accounted for 84.1% and 11.4% of the variability, respectively. The muscle PLFA PCO biplot showed that the main variation was more between salmon fed the FM and AB diets. SIMPER analysis (Table E4 in Appendix E) demonstrated that there was a 96.5% similarity within salmon fed the FM diet, an 87.0% similarity within salmon fed the FM/AB diet, and a 94.8% similarity within salmon fed the AB diet. The highest dissimilarity was between salmon fed the FM/AB and AB diets (10.8%), followed by salmon fed the FM and FM/AB diets (9.9%), and salmon fed the FM and AB diets (9.1%). The main drivers for the similarities in the muscle PL were DHA, 16:0 and 18:1ω9 across all dietary treatments, and the main drivers for the dissimilarities were DHA, 16:0, 18:1ω9, EPA, and 22:5ω6. The order and the contribution percentage to which these fatty acids affected the similarities and dissimilarities varied across the dietary treatments.

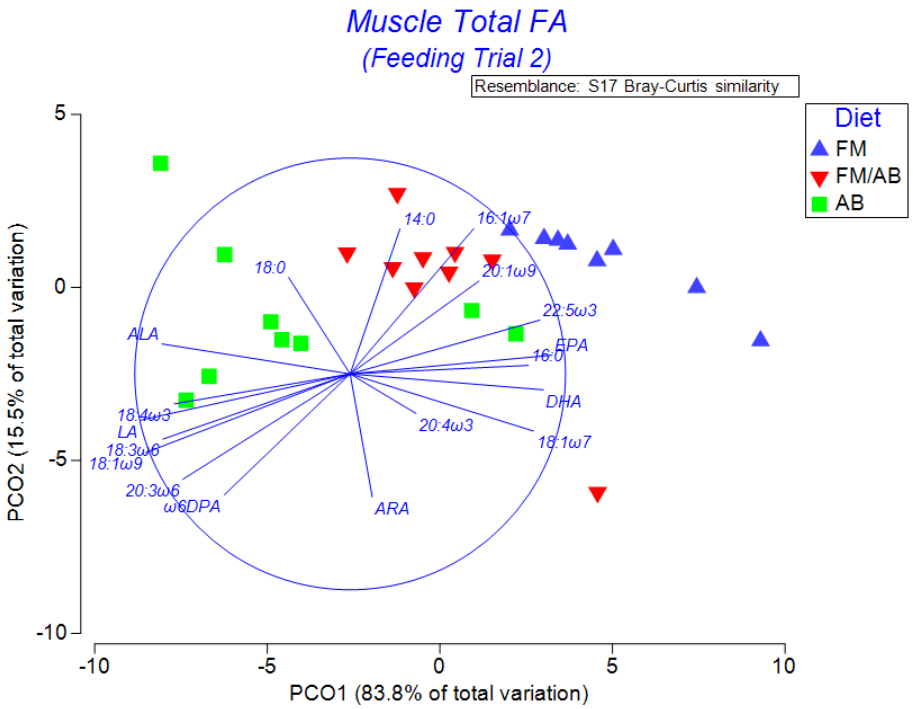


Figure 4.3: Principal coordinates analysis (PCO) of Atlantic salmon muscle tissue total fatty acid composition (%) after 12 weeks of feeding experimental diets

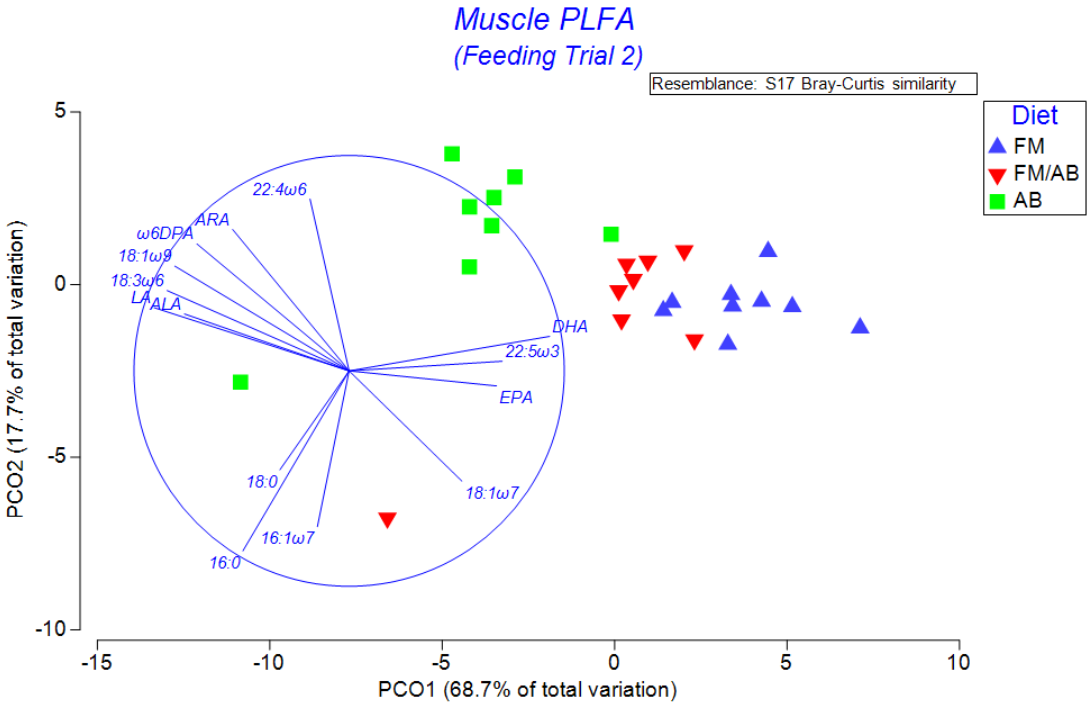


Figure 4.4: Principal coordinates analysis (PCO) of Atlantic salmon muscle tissue phospholipid fatty acid composition (%) after 12 weeks of feeding experimental diets

Table 4.5: Lipid class and total fatty acid composition of Atlantic salmon muscle tissue, after 12 weeks of feeding experimental diets[1]

	Initial	FM	FM/AB	AB
Lipid composition (%)				
Total lipid (mg/g)	15.8 ± 6.7	70.4 ± 15.9	64.5 ± 12.7	63.4 ± 12.5
Neutral Lipid	78.9 ± 7.1	61.4 ± 9.0^{b}	75.5 ± 7.7^{a}	62.8 ± 10.2^{b}
Polar Lipid	5.6 ± 5.5	38.6 ± 9.0^{a}	24.5 ± 7.7^{b}	37.2 ± 10.2^{a}
Lipid class composition (%)				
Triacylglycerol	56.0 ± 11.6	43.9 ± 10.3	54.8 ± 4.9	49.2 ± 8.6
Free fatty acids	5.4 ± 3.4	4.1 ± 1.0^{b}	7.5 ± 2.2^{a}	4.2 ± 0.7^{b}
Sterol	16.2 ± 7.9	11.0 ± 2.2^{a}	11.9 ± 3.8^{a}	6.2 ± 4.3^{b}
Phospholipid	15.6 ± 8.2	20.3 ± 5.1^{a}	8.1 ± 5.2^{b}	18.4 ± 7.4^{a}
PL/ST ratio[2]	1.1 ± 0.6	2.0 ± 0.1^{b}	0.8 ± 0.7^{b}	4.5 ± 2.7^{a}
Fatty acid composition (%)				
14:0	3.6 ± 2.2	2.3 ± 0.1	2.2 ± 0.2	2.1 ± 0.1
16:0	16.0 ± 2.5	14.7± 0.4^{a}	14.0 ± 0.6^{ab}	13.3 ± 0.5^{b}
18:0	3.7 ± 0.2	3.3 ± 0.1	3.5 ± 0.3	3.5 ± 0.6
Total SFA[3]	24.2 ± 4.7	21.1 ± 0.4^{a}	20.4 ± 0.5^{ab}	19.5 ± 0.8^{b}
16:1ω7	6.1 ± 2.0	4.6 ± 0.3^{a}	4.4 ± 0.3^{ab}	3.5 ± 0.1^{b}
16:1ω5	0.1 ± 0.0	0.1 ± 0.0^{c}	0.2 ± 0.0^{b}	0.3 ± 0.1^{a}
18:1ω9	18.5 ± 2.1	30.1 ± 1.4^{b}	33.2 ± 0.8^{a}	35.0 ± 2.4^{a}
18:1ω7	3.4 ± 0.6	2.2 ± 0.3	2.0 ± 0.3	1.7 ± 0.4
Total MUFA[4]	33.6 ± 2.8	42.1 ± 1.6	43.7 ± 0.6	43.4 ± 2.3
18:2ω6 (LA)	8.1 ± 0.6	12.8 ± 0.6^{a}	14.1 ± 0.4^{b}	15.4 ± 0.7^{c}
18:3ω6	0.2 ± 0.0	0.4 ± 0.1^{c}	0.6 ± 0.1^{b}	0.7 ± 0.1^{a}
20:3ω6	0.3 ± 0.0	0.4 ± 0.0^{c}	0.5 ± 0.1^{b}	0.7 ± 0.1^{a}
20:4ω6 (ARA)	1.0 ± 0.2	0.6 ± 0.1^{b}	0.6 ± 0.0^{ab}	0.6 ± 0.1^{a}
22:5ω6 (ω6DPA)	0.5 ± 0.3	0.2 ± 0.1^{c}	0.4 ± 0.0^{b}	0.8 ± 0.1^{a}
18:3ω3 (ALA)	1.5 ± 0.1	2.3 ± 0.1^{b}	2.4 ± 0.2^{b}	2.7 ± 0.1^{a}
18:4ω3	1.0 ± 0.2	0.9 ± 0.1^{c}	1.1 ± 0.1^{b}	1.2 ± 0.1^{a}
20:4ω3	0.8 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.1
20:5ω3 (EPA)	4.8 ± 1.0	3.9 ± 0.4^{a}	2.9 ± 0.1^{ab}	2.7 ± 0.5^{b}
22:5ω3	2.0 ± 0.4	1.3 ± 0.1^{a}	1.0 ± 0.1^{b}	0.9 ± 0.2^{b}
22:6ω3 (DHA)	17.4 ± 4.9	9.8 ± 1.6	8.3 ± 0.5	7.6 ± 2.1
Total PUFA[5]	41.3 ± 6.0	36.3 ± 1.6	35.4 ± 0.3	36.4 ± 2.3
Total ω3	28.0 ± 6.2	19.2 ± 2.0	16.8 ± 0.5	15.9 ± 2.7
Total ω6	10.8 ± 0.7	15.2 ± 0.5^{c}	16.8 ± 0.8^{b}	19.2 ± 0.7^{a}
ω3/ω6 ratio	2.6 ± 0.6	1.3 ± 0.2^{a}	1.0 ± 0.1^{a}	0.8 ± 0.2^{b}
EPA+DHA	22.2 ± 5.9	13.7 ± 2.0^{a}	11.2 ± 0.5^{b}	10.3 ± 2.6^{b}
DHA/EPA ratio	3.6 ± 0.5	2.5 ± 0.3^{b}	2.9 ± 0.1^{a}	2.8 ± 0.3^{a}
EPA/ARA ratio	4.8 ± 0.3	7.0 ± 0.7^{a}	5.2 ± 0.3^{b}	4.2 ± 0.4^{c}
DHA/ARA ratio	17.1 ± 2.1	17.3 ± 1.1^{a}	14.9 ± 0.7^{a}	11.6 ± 1.6^{b}
DHA+EPA/112 g	266.6	672.0	601.4	518.6

[1]Data expressed as % lipid or fatty acid methyl ester (FAME). Values are means ± standard deviation (n=9 per treatment). Means with different superscripts indicate significant differences ($p < 0.05$) based on Tukey's *posthoc* test following a general linear model. FM = fish meal (control); FM/AB = fish meal/AB *Pav*459; AB = AB *Pav*459. [2]Phospholipid/sterol ratio. [3]Saturated fatty acid. [4]Monounsaturated fatty acid. [5]Polyunsaturated fatty acid.

Table 4.6: Phospholipid fatty acid composition of Atlantic salmon muscle tissue after 12 weeks of feeding experimental diets[1]

	FM	FM/AB	AB
Fatty acid composition (%)			
14:0	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
16:0	19.7 ± 0.6	20.4 ± 2.3	20.0 ± 1.2
18:0	3.3 ± 0.2	3.4 ± 0.7	3.3 ± 0.3
Total SFA[2]	24.2 ± 1.3	25.1 ± 2.8	24.2 ± 0.9
16:1ω7	1.5 ± 0.2^{ab}	1.4 ± 0.3^{a}	1.3 ± 0.1^{b}
16:1ω5	0.1 ± 0.0^{c}	0.1 ± 0.0^{b}	0.2 ± 0.0^{a}
18:1ω9	9.6 ± 0.6	10.1 ± 0.7	11.2 ± 0.5
18:1ω7	1.8 ± 0.1	1.6 ± 0.1	1.5 ± 0.1
20:1ω9	0.4 ± 0.0^{a}	0.3 ± 0.1^{ab}	0.3 ± 0.0^{b}
Total MUFA[3]	14.0 ± 0.9	14.2 ± 1.0	15.0 ± 0.7
18:2ω6 (LA)	4.1 ± 0.3^{c}	4.7 ± 0.3^{b}	5.6 ± 0.4^{a}
18:3ω6	0.1 ± 0.0^{c}	0.2 ± 0.0^{b}	0.2 ± 0.0^{a}
20:2ω6	0.5 ± 0.1^{b}	0.5 ± 0.1^{ab}	0.6 ± 0.0^{a}
20:3ω6	0.5 ± 0.1^{c}	0.8 ± 0.1^{b}	1.1 ± 0.2^{a}
20:4ω6 (ARA)	1.3 ± 0.1^{b}	1.5 ± 0.2^{b}	1.9 ± 0.1^{a}
22:4ω6	0.2 ± 0.0^{ab}	0.2 ± 0.0^{b}	0.2 ± 0.0^{a}
22:5ω6 (ω6DPA)	0.7 ± 0.1^{c}	1.6 ± 0.2^{b}	2.7 ± 0.4^{a}
18:3ω3 (ALA)	1.1 ± 0.1^{b}	1.3 ± 0.1^{ab}	1.4 ± 0.1^{a}
18:4ω3	0.3 ± 0.0^{b}	0.4 ± 0.1^{b}	0.5 ± 0.1^{a}
20:3ω3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4ω3	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
20:5ω3 (EPA)	8.7 ± 0.5^{a}	7.0 ± 0.2^{b}	6.7 ± 0.7^{b}
22:5ω3	2.3 ± 0.2^{a}	1.8 ± 0.2^{b}	1.7 ± 0.2^{b}
22:6ω3 (DHA)	38.2 ± 1.9	36.9 ± 3.4	33.3 ± 3.1
Total PUFA[4]	60.4 ± 1.5	59.2 ± 3.6	59.3 ± 1.0
P/S ratio[5]	2.4 ± 0.1	2.4 ± 0.3	2.5 ± 0.1
Total ω3	51.7 ± 1.6^{c}	48.6 ± 3.4^{b}	45.9 ± 1.4^{a}
Total ω6	7.4 ± 0.4	9.4 ± 0.5	12.3 ± 0.7
ω3/ω6 ratio	7.0 ± 0.5^{a}	5.2 ± 0.4^{b}	3.7 ± 0.3^{c}
DHA/EPA ratio	4.4 ± 0.4^{b}	5.3 ± 0.5^{a}	5.0 ± 0.7^{ab}
EPA/ARA ratio	6.5 ± 0.8^{a}	4.6 ± 0.5^{b}	3.5 ± 0.4^{b}
DHA/ARA ratio	28.5 ± 2.3^{a}	24.4 ± 1.8^{b}	17.5 ± 1.4^{c}

[1]Data expressed as % lipid or fatty acid methyl ester (FAME). Values are means ± standard deviation (n=9 per treatment). Means with different superscripts indicate significant differences ($p < 0.05$) based on Tukey's *posthoc* test following a general linear model. FM = fish meal (control); FM/AB = fish meal/AB *Pav*459; AB = AB *Pav*459.
[2]Saturated fatty acid.
[3]Monounsaturated fatty acid.
[4]Polyunsaturated fatty acid.
[5]PUFA/SFA ratio.

4.4.7 Compound-specific stable isotope analysis

The $\delta^{13}C$ values for EPA and DHA in the two extreme dietary treatments (FM and AB) were significantly different from each other (Table 4.7). Unfortunately, the $\delta^{13}C$ values for ARA in the diets were not detectable; therefore, the relative contribution (RC) in the tissues was undetermined. There was a significant difference for ALA between the two dietary treatments, but there was no significant difference for LA. There were significant differences for EPA, DHA, and ARA in both liver total lipid (TL) (Table 4.7) and liver PL (Table 4.8) between salmon fed the FM diet and salmon fed the AB diet. Similarly, in the muscle tissue, there were also significant differences for EPA, DHA, and ARA in both muscle TL (Table 4.9) and muscle PL (Table 4.10). As for the precursors LA and ALA, there was a significant difference for LA in the liver PL, and a significant difference for ALA in muscle TL between salmon fed the FM diet and salmon fed the AB diet. The $\delta^{13}C$ value for ALA was not detectable in the liver tissue. The tissue $\delta^{13}C$ values for EPA and DHA in the AB treatment were substantially more negative than any ALA measurement, which suggests ALA is unlikely to be a significant contributor; therefore, the two-end-member mixing model was based on *Pav*459 bulk stable isotope data (-55.7 ± 0.4‰), in the absence of CSIA data of *Pav*459.

Table 4.7: $\delta^{13}C$ values (‰) of essential fatty acids for FM and AB diets, liver total lipids of fish fed two extreme diets (FM & AB), and relative percent contribution (RC) of AB diet to tissue fatty acids

Fatty acids (‰)	FM[1]	AB[1]	p-value		Liver TL (FM)[1]	Liver TL (AB)[1]	p-value	RC (%)[2]
LA	-26.1 ± 0.4	-25.8 ± 0.0	0.322		-25.6 ± 0.1	-25.9 ± 0.2	0.127	
ALA	-31.8 ± 0.2	-30.9 ± 0.1	<0.05		ND	ND		
ARA	ND	ND			-23.5 ± 0.5	-28.4 ± 0.4	<0.05	
EPA	-24.8 ± 0.7	-39.6 ± 0.1	<0.05		-24.8 ± 0.3	-38.0 ± 1.2	<0.05	42.7%
DHA	-24.4 ± 0.1	-36.0 ± 0.2	<0.05		-24.4 ± 0.2	-35.6 ± 0.6	<0.05	56.9%

ND = not detectable
[1]Values expressed as mean ± standard deviation (n=3)
[2]RC of *Pav*459 EPA and DHA

Table 4.8: $\delta^{13}C$ values (‰) of essential fatty acids for FM and AB diets, liver phospholipids of fish fed two extreme diets (FM & AB), and relative percent contribution (RC) of AB diet to tissue fatty acids

Fatty acids (‰)	FM[1]	AB[1]	p-value		Liver PL (FM)[2]	Liver PL (AB)[2]	p-value	RC (%)[3]
LA	-26.1 ± 0.4	-25.8 ± 0.0	0.322		-25.1 ± 0.4	-26.0 ± 0.4	<0.05	
ALA	-31.8 ± 0.2	-30.9 ± 0.1	<0.05		ND	ND		
ARA	ND	ND			-24.1 ± 0.7	-28.3 ± 1.0	<0.05	
EPA	-24.8 ± 0.7	-39.6 ± 0.1	<0.05		-24.8 ± 1.1	-37.6 ± 0.2	<0.05	41.3%
DHA	-24.4 ± 0.1	-36.0 ± 0.2	<0.05		-24.2 ± 1.0	-35.0 ± 1.5	<0.05	54.0%

ND = not detectable
[1]Values expressed as mean ± standard deviation (n=3)
[2] Values expressed as mean ± standard deviation (n=9)
[3]RC of *Pav*459 EPA and DHA

Table 4.9: $\delta^{13}C$ values (‰) of essential fatty acids for FM and AB diets, muscle total lipids of fish fed two extreme diets (FM & AB), and relative percent contribution (RC) of AB diet to tissue fatty acids

Fatty acids (‰)	FM[1]	AB[1]	p-value		Muscle TL (FM)[2]	Muscle TL (AB)[2]	p-value	RC (%)[3]
LA	-26.1 ± 0.4	-25.8 ± 0.0	0.322		-26.7 ± 0.4	-27.0 ± 0.5	0.128	
ALA	-31.8 ± 0.2	-30.9 ± 0.1	<0.05		-30.6 ± 0.4	-31.4 ± 0.2	<0.05	
ARA	ND	ND			-24.5 ± 0.5	-28.0 ± 0.4	<0.05	
EPA	-24.8 ± 0.7	-39.6 ± 0.1	<0.05		-25.2 ± 0.4	-36.1 ± 1.3	<0.05	36.6%
DHA	-24.4 ± 0.1	-36.0 ± 0.2	<0.05		-25.3 ± 0.4	-32.9 ± 1.5	<0.05	43.1%

ND = not detectable
[1]Values expressed as mean ± standard deviation (n=3)
[2]Values expressed as mean ± standard deviation (n=9)
[3]RC of *Pav*459 EPA and DHA

Table 4.10: $\delta^{13}C$ values (‰) of essential fatty acids for FM and AB diets, muscle phospholipids of fish fed two extreme diets (FM & AB), and relative percent contribution (RC) of AB diet to tissue fatty acids

Fatty acids (‰)	FM[1]	AB[1]	p-value		Muscle PL (FM)[1]	Muscle PL (AB)[1]	p-value	RC (%)[2]
LA	-26.1 ± 0.4	-25.8 ± 0.0	0.322		-25.6 ± 0.4	-26.3 ± 0.2	0.073	
ALA	-31.8 ± 0.2	-30.9 ± 0.1	<0.05		-30.7 ± 1.2	-30.6 ± 0.3	0.836	
ARA	ND	ND			-23.8 ± 0.4	-27.5 ± 0.2	<0.05	
EPA	-24.8 ± 0.7	-39.6 ± 0.1	<0.05		-25.2 ± 0.1	-36.6 ± 0.7	<0.05	38.1%
DHA	-24.4 ± 0.1	-36.0 ± 0.2	<0.05		-25.5 ± 0.4	-32.7 ± 1.0	<0.05	42.0%

ND = not detectable
[1]Values expressed as mean ± standard deviation (n=3)
[2] RC of *Pav*459 EPA and DHA

4.5 Discussion

The present study evaluated the effects of replacing FM and reduced FO with the AB of *Pav*459 in the feeds of Atlantic salmon. Replacing traditional FM with *Pav*459 did not significantly affect the growth parameters of Atlantic salmon. The experimental feeds had a protein content of ~49%, which is above the minimal 44% digestible protein requirement for salmon weighing 20-200 g, suggested by NRC (2011). It is important to mention that while replacing FM with *Pav*459 did not have a significant impact on the growth performance of the fish, the total oil contribution of that *Pav*459 in the diets accounted for 1.2% in the FM/AB diet and 2.4% in the AB diet. The major oil contributor in the experimental diets was canola oil (6.8% for FM/AB; 7.9% for AB), poultry fat (5.9% for FM/AB; 6.9% for AB), and herring oil (4.5% for FM/AB; 1.8% for AB). Generally, canola oil in aquafeed is 95-98% TAG where 5-7% is SFA, 55-72% is MUFA, 19-23% is ω6 PUFA, and 6-12% is ω3 PUFA (Turchini & Mailer, 2011). Poultry fat is generally composed of 85-90% total lipid and is rich in MUFA and poor in PUFA with ω6 > ω3 (Bureau & Meeker, 2010; Campos et al., 2019). Herring oil is rich in TAG, generally contributing in excess of 90% of the total fatty acid composition. FO is best known and highly regarded for their high proportions of ω3 LC-PUFA with levels of EPA and DHA ranging from 3.9-15.2% and from 2.0-7.8%, respectively (De Silva et al., 2011). Although the oil contribution of *Pav*459 was low, *Pav*459 fatty acid composition was rich in the PUFA (64.5%), where EPA+DHA accounted for 40.4% of FAME. Additionally, we identified a few fatty acid biomarkers for *Pav*459, which were 16:1ω5, 16:2ω4, stearidonic acid (SDA, 18:4ω3) and 22:5ω6. These biomarkers were found in the tissues and also were found embedded in the membrane, especially 22:5ω6, which was present in higher proportions in salmon fed the AB than salmon fed the FO diet.

Overall, the fish grew ~263% from their initial weight of 170.1 g across the diets, suggesting that *Pav*459 could be included in the feeds of Atlantic salmon without compromising growth parameters. The results of the present study are similar to other feeding trial studies in which the authors did not detect any significant effects on growth parameters when FM was replaced by different strains of AB in Atlantic salmon diets (Peterson et al., 2019; Sørensen et al., 2016), hybrid striped bass diets (Perez-Velazquez et al., 2019), and shrimp diets (Pakravan et al., 2017) just to mention a few. This further supports the potential of marine microalgae as an alternative lipid source in aquafeed either as oil or as biomass.

4.5.1 Liver tissue

The liver plays an important role in LC-PUFA biosynthesis and overall body lipid homeostasis in Atlantic salmon (Betancor et al., 2014). There was no significant difference in total lipid composition for the liver tissue across the dietary treatments (Table 4.3), and similarly, there was no significant difference in the neutral and polar composition of liver tissue. The liver tissue was mostly composed of neutral lipids with FFA as the dominant lipid class, followed by PL, ST, and TAG. Salmon fed the FM diet had the highest proportions of TAG and FFA, while salmon fed the FM/AB diet had the highest proportions of PL and ST in the liver tissue. The proportion of TAG increased across the dietary treatments but was not significantly different at the end. The presence of increased TAG in the liver tissue indicates storage instead of metabolism for energy, even though salmon store excess fat in the muscle tissue, unlike cod which stores excess fat in the liver tissue (Zhol et al., 1995). PL was the second most dominant lipid class (23 – 25%) followed by ST (18 – 24%), indicating the importance of membrane material in liver tissue. Both PL and ST play an important role in maintaining the structure of the membrane. A primary role of cholesterol is to modulate the physical properties of membranes (Stillwell & Wassall, 2003). The

fluidity of the lipid bilayer depends primarily upon the degree of order in the packing of its constituent phospholipids. (Lund et al., 1999). Fish can regulate membrane fluidity by altering the composition of SFA and unsaturated fatty acids. For adjustments of cellular fluidity, we have to look at significant differences in the tissue PL/ST ratio (Table 4.3) and the membrane P/S fatty acid ratio (Table 4.4); however, no significant differences were present.

The total fatty acid composition generally reflects the diet (Table 4.3). The dominant fatty acids in liver tissue were 16:0, 18:1ω9, LA, and DHA (>5% among all dietary treatments). PLFA composition (Table 4.4) shared the same dominant fatty acids plus EPA. Based on PCO analysis, the same dominant fatty acids listed above were the drivers for the dissimilarities observed in total fatty acid and PLFA. While similarities were observed between total fatty acid profile and PLFA profile, the PCO analysis also showed that for total fatty acids, there was more variation within salmon fed the same diet rather than salmon fed different diets, and for PLFA there was more variation between salmon fed different diets than salmon fed the same diets. The proportion of PUFA in the liver PL was noticeably higher than that of the diet (Table 4.2), while MUFA was noticeably lower, indicating a possible preferential β-oxidation of these fatty acids when present in high concentrations in the diet (Sargent et al., 2003; Stubhaug & Torstensen, 2007). There was no significant change in SFA proportion in liver PL. *Pav*459 was rich in PUFA; however, its oil contribution was only 1-2%. Canola oil and poultry fat were the main oil contributors in the diets, and as highlighted above, they are rich in MUFA, hence, the high proportion of MUFA in the diets and its reflection in the tissues. There was no significant variation of EPA proportion compared to that of the diet, but it is worth highlighting that the DHA proportion in the liver PL was 8-fold higher in salmon fed the FM diet, 11-fold higher in salmon fed the FM/AB diet, and 17-fold higher in salmon fed the AB diet than that of the diet. This highlights the importance of DHA over EPA

in the membrane, even though the initial DHA proportion across the dietary treatments was lower than EPA proportions. The ALA proportion was also lower than that of the diet, indicating a possible desaturation and elongation for the biosynthesis of DHA, as the intermediate step between EPA and DHA, the 22:5ω3, double in the liver tissue than that of the diet. After 12-weeks of feeding, the DHA:EPA ratio went from a <1 in the diet to a >1 in the tissue, suggesting a higher necessity for DHA than EPA. The levels of ARA in the liver tissue and PL were also significantly higher than that of the diet. This is possibly due to the high proportion of LA, which after 12-weeks of feeding was lower than that of the diet, indicating a possible desaturation and elongation for the biosynthesis of ARA. The increase in ARA proportions might suggest a possible production of pro-inflammatory eicosanoids; however, the ω3/ω6 ratio remained >1 across the diets. Both EPA and ARA play similar roles as the precursor in the production of eicosanoids; however, those eicosanoids formed from EPA are considered to be less biologically active than eicosanoids formed from ARA. The direct competition between EPA and ARA results in one inhibiting the formation of eicosanoids from the other. Replacing FM with AB *Pav*459 resulted in a positive ARA:EPA ratio in salmon fed the AB diet only. The AB diet had the highest canola oil content favouring a more pro-inflammatory eicosanoid production. However, the ω3/ω6 remained >1 across all dietary treatments. As mentioned earlier, the DHA:ARA ratio remained >1 across the diets, indicating the importance of DHA in membranes

4.5.2 Muscle tissue

The muscle tissue is often referred to as the storage tissue in Atlantic salmon. Atlantic salmon deposits fat/oil mainly in the visceral adipose tissue and myosepta/connective tissue sheets in muscle, being stored as TAG to be used in times of food deprivation (Yeo & Parrish, 2020). Replacing FM with *Pav*459 resulted in no significant difference in total lipid composition among

the treatments (Table 4.5). The muscle tissue was mainly composed of neutral lipid, which accounted for 61-63% in salmon fed the FM and AB diets, and 75% in salmon fed the FM/AB diet. Interestingly, there was no significant difference between salmon fed the FM diet and salmon fed the AB diet for the neutral and polar lipid composition; however, they were both significantly different from salmon fed the FM/AB diet. The reason for this is unclear, but perhaps it is due to the equal contribution of FM and AB in the diet. The main lipid class in the muscle tissue was TAG, followed by PL and ST. The ST lipid class composition was similar between salmon fed the FM diet and salmon fed the FM/AB diet, but significantly different than salmon fed the AB diet. It is worth noting that feeding salmon the FM/AB diet resulted in low membrane material, as the PL proportion (8.1%) was the lowest across the dietary treatments and was lower than the ST proportion (11.9%). The decrease in PL proportion was balanced with increases in TAG, FFA and ST suggesting changes in metabolism and fluidity. The PL proportion was still the second dominant lipid class for salmon fed the FM and AB diet (20.3% and 18.5%) despite the low PL proportion observed for salmon fed the FM/AB diet. Regarding a possible membrane fluidity, there was a significant difference in the muscle tissue PL/ST ratio (Table 4.5) across the dietary treatments, but no significant difference was present in P/S ratio (Table 4.6) in the muscle PL, suggesting a possible adjustment to fluidity in the membrane based on the dietary treatments.

The muscle tissue total fatty acid (Table 4.5) and PLFA (Table 4.6) reflected the diets. The dominant fatty acids in muscle tissue and PL were 16:0, 18:1ω9, LA, and DHA (>5% among all treatments). Unlike liver PL, muscle PL EPA was not present in high proportions (>5%). According to PCO analysis, the same dominant fatty acids listed above were the main drivers for the dissimilarities observed in total fatty acids, and with inclusion of EPA and 22:5ω6, they were the main drivers for the dissimilarities observed in PLFA. Interestingly, different than liver tissue,

muscle PLFA had more variation within salmon fed the same diet, and muscle total fatty acids had more variation between salmon fed different diets. There was no major change in total SFA, MUFA and PUFA proportion in the muscle PL, probably suggesting that there was no preferential *β*-oxidation of these fatty acids. The level of DHA in the muscle PL was higher than that of the diet, while EPA was lower. This might be an indication of preference of DHA over EPA in the membrane, as well as a preference of DHA for storage, while EPA was either used for biosynthesis of DHA or used in *β*-oxidation for energy production (Sargent et al., 2003). EPA is a better substrate for *β*-oxidation by mitochondria than DHA due to the fact that insertion and removal of the Δ^4 double bond in DHA requires a special mechanism (Sargent et al., 2003). This might also be a case of no retro-conversion from DHA to EPA. In a recent study done by Metherel et al. (2019), the author concluded that the increases in EPA upon DHA feeding are the result of slowed EPA metabolism and not retro-conversion of DHA to EPA (Metherel et al., 2019). We cannot make a full conclusion on this topic, but it is worth investigating in the future. Unlike liver tissue, there was no noticeable variation for ARA proportion in the muscle PL, resulting in a >1 EPA:ARA ratio across all dietary treatments. However, the ω3/ω6 ratio remained >1 only in salmon fed the FM and FM/AB diets. Salmon fed the AB diet had a >1 ω6/ω3 ratio suggesting a possible production of pro-inflammatory eicosanoids. Eicosanoid actions are determined by the ratio of ARA to EPA in cellular membranes, this in turn being determined by the dietary intake of ω6 and ω3 PUFA (Sargent et al., 2003). A further investigation will be necessary to make a more definitive conclusion.

The muscle tissue also referred to as fillet, is important for the consumer. It is well known that ω3 LC-PUFA, particularly EPA+DHA, are beneficial to human health against the prevention of cardiovascular diseases and many other health benefits (Kris-Etherton et al., 2002; Ruxton et

al., 2005; Rimm et al., 2018). To ensure a high level of deposition of these fatty acids into the fillet tissue, current commercial aquafeed formulations for Atlantic salmon tend to contain a physiological excess of ω3 LC-PUFA (Quian et al., 2020). By replacing FM with *Pav*459, the EPA+DHA proportion in the fillet was 6.00 mg/g in salmon fed the FM diet, 5.37 mg/g in salmon fed the FM/AB diet, and 4.63 mg/g in salmon fed the AB diet. A number of countries (Canada, Sweden, United Kingdom, Australia, Japan) made formal population-based dietary recommendations for ω3 fatty acids. Typical recommendations are 300 to 500 mg/day of EPA+DHA provided by two servings of fatty fish/week (one serving is 112 g cooked) (Kris-Etherton et al., 2002). Our data shows DHA+EPA/112 g (uncooked) would provide per serving 672 mg from salmon fed the FM diet, 601.4 mg from salmon fed the FM/AB diet, and 518.6 mg from salmon fed the AB diet, which falls within the daily recommendation. However, changes in PL proportion were observed in the muscle tissue from one dietary treatment to another, which may provide added benefits to human health, since there is evidence that PLs ω3 PUFAs (such as EPA and DHA) are more efficiently incorporated into tissue membranes and at much lower doses than TAGs ω3 PUFAs (Küllenberg et al., 2012; Murru et al., 2013; Lordan et al., 2017). It is worth pointing out that the way fish is cooked can change the nutritional composition of the fillet, as some cooking methods may be better than others. Overall, frying produces higher changes in fish lipids than other cooking methods. Changes during frying depend on the fat content of fish, frying oil composition, and types of frying technology. Baking showed the least changes in fat content and fatty acid composition of fillets. Grilling resulted in an increase in total lipids and ω3 PUFA, presumably due to the decrease in tissue water content (Moradi et al., 2011). Table F1 in Appendix F compares moisture and fat of salmon, mackerel, and sardines when raw, cooked (deep pan-fried), and held warm.

4.6 Benefits of using CSIA

The main goal for using CSIA in this study was to determine the relative contribution (RC) of different sources of LC-PUFA (EPA, DHA, ARA) in salmon fed the two extreme diets (FM and AB). However, the $\delta^{13}C$ values for ARA were not detectable, hence, not being possible to determine the relative contribution of its source in the tissues. The only detectable LC-PUFA were EPA and DHA, and there were three possible sources of EPA and DHA: (1) the precursors ALA in the diet; (2) the FO (herring oil) present in the diet; (3) and *Pav*459 oil in the diet. A two-end-member mixing model was used to determine the relative contributions of *Pav*459 to the liver and muscle EPA and DHA. It is important to point out that the AB diet $\delta^{13}C$ values for EPA and DHA are all highly negative compared to other $\delta^{13}C$ values, and that FM diet $\delta^{13}C$ EPA and DHA values are the same as liver values, suggesting little diet-tissue discrimination. Because tissue $\delta^{13}C$ values for AB diet EPA and DHA are always substantially more negative than those for any ALA measurement, ALA is unlikely to be a significant contributor. Terrestrial plants have a lighter $\delta^{13}C$ value than those of marine origin (Phillips et al., 2005). According to Tibbets et al. (2020a), the marine microalgae used in this study (*Pav*459) was cultivated with CO_2-enriched (1%) air (Tibbets et al., 2020a). Atmospheric carbon dioxide contains approximately 1.1% of the non-radioactive isotope carbon-13 and 98.9% of carbon-12 (O'Leary, 1988). A more negative $\delta^{13}C$ means more ^{12}C or lighter in mass; a more positive $\delta^{13}C$ means more ^{13}C or heavier (O'Leary, 1988). The highly negative bulk stable isotope value for *Pavlova* could be related to the CO_2 enrichment used in the culture.

As mentioned in section 4.5, the total oil contribution of *Pav*459 from the 20% algal biomass in the AB diet was 2.4%, and the total oil contribution of FO (herring oil) in AB diet was 1.75%. The levels of EPA and DHA in herring oil range from 3.9-15.2% and from 2.0-7.8%,

respectively (De Silva et al., 2011). Therefore, the range of levels of EPA and DHA derived from FO in AB diet will be equal to 0.068% - 0.266% and 0.035% - 0.137%, respectively. As for *Pav*459, there was a 26.8% EPA and 13.6% DHA in the AB diet, which results in the levels of EPA and DHA derived from *Pav*459 in the diet being equal to 0.129% and 0.065%, respectively. To simplify calculations, a mean from the range of levels of EPA and DHA derived from FO in the AB diet was used to calculate the total proportion of EPA and DHA supplied in the diet. Therefore, the average total proportion of EPA and DHA supplied in the diet from *Pav*459 was 43.6% and 43.0%, respectively.

In the liver TL (Table 4.7), the RC of dietary *Pav*459 to tissue EPA and DHA was 42.7% and 56.9%, respectively. Since the average total proportion of EPA supplied in the diet from *Pav*459 was 43.6%. This implies an almost complete (42.7/43.6 = 97.9%) incorporation of *Pav*459 into liver lipids. As for DHA, the average total proportion of DHA supplied in the diet from *Pav*459 was 43.0% which is less than the RC of *Pav*459 to DHA in the liver TL. However, the 43.0% was an average total proportion, so again the implication is that almost all *Pav*459 DHA was incorporated into liver lipids. This is also true for liver PL and muscle TL (see Table 4.8 and Table 4.9). It is noteworthy that DHA incorporation was consistently higher than EPA incorporation and that it was usually higher than the calculated mean supply of 43.0%, pointing to its greater essentiality. In the liver PL (Table 4.8), the RC of dietary *Pav*459 to EPA and DHA in the membrane was 41.3% and 54.0%, respectively. The average total proportion of EPA and DHA supplied in the diet from *Pav*459 was 43.6% and 43.0%, respectively. This also implies the almost complete incorporation of *Pav*459 EPA into liver PL and that almost all *Pav*459 DHA was incorporated into liver PL.

Although similarities to liver tissue were present, the muscle tissue had a lesser proportion of EPA and DHA incorporated from *Pav*459. In the muscle TL (Table 4.9), the RC of dietary *Pav*459 to tissue EPA and DHA was 36.6% and 43.1%, respectively. The average total proportion of EPA supplied in the diet from *Pav*459 was 43.6%, which implies that about 83.9% (36.6/43.6 = 83.9%) incorporation of dietary *Pav*459 EPA into muscle lipids. As for DHA, the average total proportion of DHA supplied in the diet from *Pav*459 was 43.0% which is 0.1% less than the RC of dietary *Pav*459 to DHA in the muscle TL. Therefore, the implication is that almost all *Pav*459 DHA was incorporated into muscle lipids.

In the muscle PL (Table 4.10), the RC of dietary *Pav*459 to EPA and DHA in the membrane was 38.1% and 42.0%, respectively. The average total proportion of EPA supplied in the diet from *Pav*459 was 43.6%, which would result in 87.4% (38.1/43.6 = 87.4%) of EPA to be derived from *Pav*459. For DHA, the average total proportion of DHA supplied in the diet from *Pav*459 was 43.0%, which would result in 96.3% of DHA to be derived from *Pav*459.

4.7 Conclusion

This was a novel study completely replacing fish meal with AB *Pav*459 in diets for farmed Atlantic salmon. Overall, it demonstrated that replacing traditional FM with *Pav*459 did not significantly affect the growth parameters of Atlantic salmon, but the total fatty acid and PLFA composition in both muscle and liver tissues reflected the respective dietary treatments. Replacing FM with *Pav*459 had clear effects on the membrane composition of both liver and muscle tissues; however, the magnitude of the effect varied between the tissues. The variations in proportions of ω3 and ω6 fatty acids reflected the functions of the tissue, e.g., the DHA proportion was higher in the muscle membrane than the liver membrane. There was a higher preference for storage for DHA, while EPA was probably used for β-oxidation or biosynthesis of DHA. The precursor LA

was present in high proportion in storage but in low proportion in the membrane. The CSIA suggested direct incorporation of EPA and DHA into the tissue from dietary *Pav*459. The DHA+EPA/112 g (uncooked) daily recommendation of 500 mg/g was fulfilled by all dietary treatments.

5. GENERAL CONCLUSION AND FUTURE PERSPECTIVE

Two experimental feeding trial studies were conducted to evaluate the effects of replacing FO and FM with alternative lipid sources derived from two different strains of microorganisms. The main objective of the studies were the dietary effect on lipid classes and fatty acid composition with emphasis on the phospholipid fatty acid composition.

In the first study, FO was replaced with MO derived from *Schizochytrium* sp. (T18). The MO used in the study was high in DHA and low in EPA, which would additionally provide us insight into the requirements of EPA and DHA in the Atlantic salmon diet. The replacement of FO with MO resulted in no significant effects on growth parameters. The fatty acid composition of liver and muscle tissues reflected the dietary treatments, with DHA being present at higher levels in both tissues, especially in the cellular membrane. Our results showed that there is a higher necessity for DHA and less necessity for EPA in the membrane. In the membrane, the precursors LA and ALA were present in low proportions and while DHA was present in high proportions. The high content of DHA in the diet implies an effect on membrane fluidity. Despite the general indication of a mostly >1 ω3/ω6 ratio, and our results suggesting a possible synthesis towards a pro-inflammatory eicosanoid in the liver tissue and a possible synthesis towards an anti-inflammatory eicosanoid in the muscle tissue for MO fed diets, additional studies in this area are warranted. For human consumption, the proportion of EPA+DHA in the fillet fulfilled the daily recommendation when FO is replaced by a lower and higher proportion of MO. Therefore, MO is a great replacement candidate for FO in the diet of Atlantic salmon, at least in parr.

In the second study, FO proportion was reduced, and FM was replaced by AB derived from *Pavlova* sp. strain CCMP459 (*Pav*459). The total oil contribution of *Pav*459 was 2.4% in the AB diet. The major oil contributor in this study came from canola oil, poultry fat, and herring oil.

However, the use of *Pav*459 signifies a reduction in the use of FM and FO and reliance on wild fisheries. Similar to the first study, replacing FM with *Pav*459 resulted in no significant effects on growth parameters. Tissue fatty acids generally reflected the diets, with DHA also present at high levels in both tissues, especially in the membrane. This further showed a higher requirement for DHA over EPA and a possible preference of MUFA and EPA for β-oxidation. The precursor LA was present in high proportion in storage lipid but in low proportions in the membrane lipid. Despite a >1 ω3/ω6 ratio, the ARA proportion increased in the liver tissue while it remained about the same in the muscle tissue. Due to the regiospecificity of ARA, this is a further indication of the higher role of ARA in inflammation and immunity and a lesser role in storage and energy production. However, additional study is warranted. The fatty acids composition suggested a possible synthesis of EPA and DHA from precursor ALA; however, CSIA suggested direct incorporation of EPA and DHA from the diet into the tissue. The EPA+DHA proportion in the fillet also satisfied the daily recommendation for *Pav*459 fed diets. Therefore, *Pav*459 is a great candidate for replacing or reducing FM usage in the diet of Atlantic salmon post-smolt.

Future research on replacing FO and FM with MO and/or *Pav*459 will likely need to focus on (1) combining both dietary ingredients and analyzing its impact on health parameters of the fish, total lipids, and fatty acids composition, as well as EPA+DHA proportion in the fillet for human consumption; (2) further research on genetics and immune system of the fish when fed either or both combined diets.

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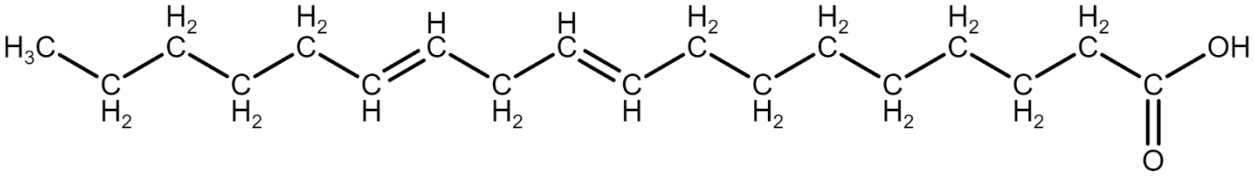
APPENDIX A

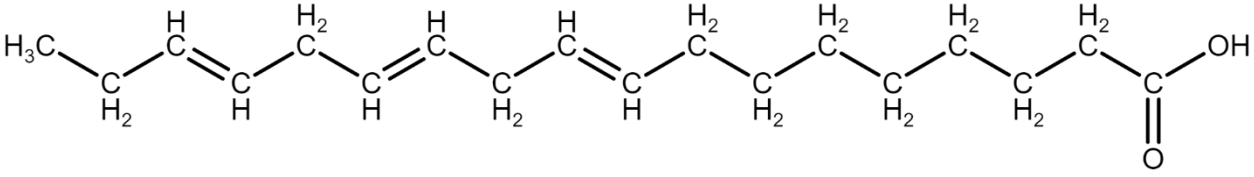
Table A1: Summary of systems of fatty acid nomenclature

	Delta (Δ) nomenclature	Omega (ω) nomenclature
LA	$18{:}2^{\Delta 9,12}$	18:2ω6
ALA	$18{:}3^{\Delta 9,12,15}$	18:3ω3
ARA	$20{:}4^{\Delta 5,8,11,14}$	20:4ω6
EPA	$20{:}5^{\Delta 5,8,11,14,\ 17}$	20:5ω3
DHA	$22{:}6^{\Delta 4,7,10,13,16,19}$	22:6ω3

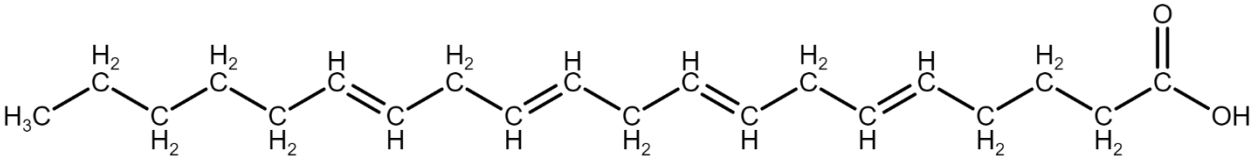
Linoleic acid



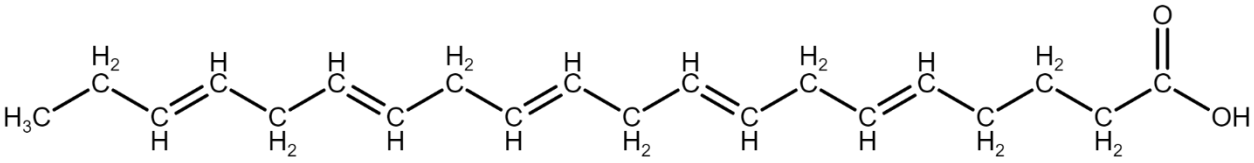
Alpha-linolenic acid



Arachidonic acid



Eicosapentaenoic acid



Docosahexaenoic acid

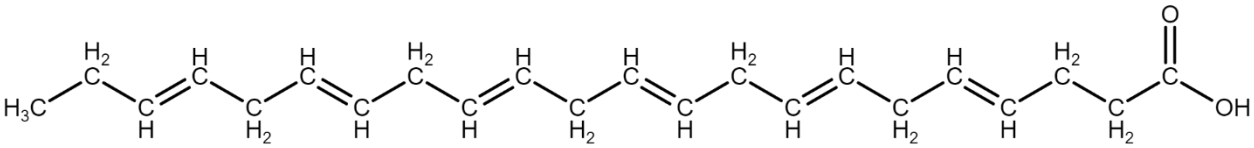


Figure A1 Example of essential fatty acids molecular structure; Linoleic acid (18:2ω6; LA), alpha-linolenic acid (18:3ω3; ALA), arachidonic acid (20:4ω6; ARA), eicosapentaenoic acid (20:5ω3; EPA), and docosahexaenoic acid (22:6ω3; DHA).

APPENDIX B

The feed was produced at the Chute Nutrition lab at Dalhousie University Faculty of Agriculture (Truro, Nova Scotia, Canada). The feed ingredients were provided by Northeast Nutrition (Truro, Nova Scotia, Canada). The ingredients were mixed by Hobart Commercial Mixer (The Hobart Manufacturing Co. LTD. Ontario, Canada) and the mix was steam pelleted into 3 mm and 5 mm pellets by Laboratory Pellet Mill (California Pellet Mill Co., California, US). The pellets were dried in the oven for 4.5 h at 65 ◦C and sifted. The feed was transferred to the Aquaculture lab at Dalhousie University Faculty of Agriculture directly after production and stored at -20 ◦C until feeding. Salmon were fed 3 mm pellets at the beginning of the study and then switched to 5 mm pellets when they grew larger (Wei et al., 2021).

Table B1: Formulation of experimental diets (g/kg as fed basis) containing microbial oil (MO), fish oil (FO), or FO/canola oil (CO) blend, fed to Atlantic salmon (After Wei et al., 2021).

Ingredient (g/kg)[1]	FO	FO/CO	LMO	HMO
Fish meal	150	150	150	150
Fish oil (Herring)	200	100	0	0
Microbial oil (MO)[2]	0	0	50	100
Canola oil	0	100	150	100
Ground wheat	117.5	117.5	117.5	117.5
Empyreal (corn protein concentrate)	250	250	250	250
Poultry byproduct	170	170	170	170
Soybean meal	80	80	80	80
Vitamin/mineral mix[3]	2	2	2	2
Dicalcium phosphate	20	20	20	20
Special premix[4]	2.5	2.5	2.5	2.5
Lysine HCL	5	5	5	5
Choline chloride	3	3	3	3
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DHA (%)	1.05	0.56	1.97	3.87
EPA (%)	1.806	0.966	0.16301	0.20003
DHA+EPA (%)	2.86	1.53	2.13	4.07

[1]All ingredients were supplied and donated by Northeast Nutrition (Truro, Nova Scotia, Canada)

[2]Produced by Mara Renewables (Dartmouth, Nova Scotia, Canada)

[3]Vitamin/ mineral mix contains (/kg): zinc, 77.5 mg; manganese, 125 mg; iron, 84 mg; copper, 2.5 mg; iodine, 7.5 mg; vitamin A, 5000 IU; vitamin D, 4000 IU; vitamin K, 2 mg; vitamin B12, 4 µg; thiamine, 8 mg; riboflavin, 18 mg; pantothenic acid, 40 mg; niacin, 100 mg; folic acid, 4 mg; biotin, 0.6 mg; pyridoxine, 15 mg; inositol, 100 mg; ethoxyquin, 42 mg; wheat shorts, 1372 mg.

[4]Special premix contains (/kg): selenium, 0.220 mg; vitamin E, 250 IU; vitamin C, 200 mg; astaxanthin, 60 mg; wheat shorts, 1988 mg.

Table B2: Chemical composition of experimental diets[1] (Wei et al., 2021)

Chemical composition (%)				
	FO	**FO/CO**	**LMO**	**HMO**
Dry matter	93.6	93.9	94.6	94.1
Crude protein	46.9	47.5	45.4	46.3
Crude fat	25.7	25.1	25.6	27.7
Ash	6.7	6.5	6.9	6.6

[1] Data express as % diet (wet weight), values are means (n=3 per treatment) ± standard deviation. Means with different superscripts indicate significant differences among treatment diets based on Tukey's *posthoc* test following a one-way ANOVA. FO, fish oil; FO/CO, fish oil/canola oil; low MO, low microbial oil; high MO, high microbial oil.

Table B3: Growth performance of Atlantic salmon fed experimental diets for 16 weeks (Wei et al., 2021)

	FO	**FO/CO**	**LMO**	**HMO**
Initial weight[1]	25.0 ± 4.2	22.3 ± 3.5	21.9 ± 4.7	26.8 ± 4.1
Final weight[2]	106.6 ± 10.2	120.8 ± 8.7	115.2 ± 5.1	123.5 ± 2.4
Weight gain[3]	81.6 ± 9.8	98.6 ± 6.6	93.3 ± 5.9	96.7 ± 2.3
Initial length[1]	13.9 ± 0.7	13.3 ± 0.7	16.2 ± 5.4	14.1 ± 0.6
Final length[2]	21.3 ± 2.1	22.2 ± 2.0	21.9 ± 1.7	22.4 ± 2.0
Initial CF[4]	0.92 ± 0.08	0.94 ± 0.09	0.93 ± 0.07	0.96 ± 0.07
Final CF[4]	1.08 ± 0.07	1.09 ± 0.08	1.07 ± 0.07	1.07 ± 0.11
Initial VSI[5]	9.6 ± 2.4	9.5 ± 2.1	11.9 ± 3.6	9.7 ± 1.7
Final VSI	9.9 ± 0.6	9.4 ± 0.8	10.1 ± 1.1	9.3 ± 0.9
SGR[6]	1.3 ± 0.9	1.5 ± 0.1	1.5 ± 0.2	1.4 ± 0.02
AFI[7]	76.8 ± 5.7	84.9 ± 6.8	77.9 ± 1.9	87.5 ± 1.3
FCR[8]	0.90 ± 0.19	0.86 ± 0.03	0.84 ± 0.03	0.90 ± 0.04

[1]Initial measurements are mean ± standard deviation, body weight (g/fish), fork length (cm/fish), $n = 5$.
[2]Final measurements are mean ± standard deviation, body weight (g/fish), fork length (cm/fish), $n = 15$.
[3]Weight gain (g/fish) = final weight – initial weight.
[4]Condition factor = body weight (g)/length $(cm)^3 x100$, calculated by individual fish, the mean was obtained from 5 fish in each treatment.
[5]Visceral somatic index (%) = 100×(viscera mass/body mass).
[6]Specific growth rate (%/day) = (ln (final body weight) – ln (initial body weight)) / number of days in period x 100.
[7]Apparent feed intake (g/fish) = (total feed consumed, g)/ (number of fish per tank).
[8]Feed conversion rate = (feed intake, g/fish)/(weight gain, g/fish).

APPENDIX C

Table C1: Feeding trial 1 liver total fatty acids average similarities results[1]

FO		FO/CO		LMO		HMO	
Average similarity: 96.0		Average similarity: 94.0		Average similarity: 91.1		Average similarity: 92.6	
FAs	**Contribution**	**FAs**	**Contribution**	**FAs**	**Contribution**	**FAs**	**Contribution**
DHA	35.46	DHA	31.68	DHA	32.12	DHA	37.72
16:0	17.73	18:1ω9	18.19	18:1ω9	21.03	16:0	16.56
18:1ω9	9.66	16:0	15.17	16:0	14.39	18:1ω9	15.57
EPA	9.28	LA	6.97	LA	8.82	LA	6.30

[1]SIMPER data expressed as %

Table C2: Feeding trial 1 liver total fatty acids average dissimilarities results[1]

FO & FO/CO		FO & LMO		FO/CO & LMO		FO & HMO		FO/CO & HMO		LMO & HMO	
Average dissimilarity = 14.7		Average dissimilarity = 23.8		Average dissimilarity = 13.5		Average dissimilarity = 18.9		Average dissimilarity = 14.3		Average dissimilarity = 11.5	
FAs	**Contrib.**	**FAs**	**Contrib.**	**FAs**	**Contrib.**	**FAs**	**Contrib.**	**FAs**	**Contrib.**	**FAs**	**Contrib.**
18:1ω9	28.70	18:1ω9	25.18	EPA	19.20	EPA	20.64	DHA	22.50	DHA	25.21
DHA	12.18	EPA	16.04	18:1ω9	14.53	18:1ω9	17.77	EPA	18.80	18:1ω9	24.76
LA	11.33	LA	10.50	DHA	13.27	ω6DPA	10.49	ω6DPA	14.35	16:0	10.11
16:0	9.22	22:5ω3	6.99	ω6DPA	11.06	DHA	10.05	18:1ω9	10.74	LA	8.91
EPA	8.35	16:0	6.63	16:0	7.69	22:5ω3	8.70	16:0	6.69	ω6DPA	4.78
22:5ω3	6.21	DHA	6.15	LA	6.25	LA	8.09	-	-	-	-

[1]SIMPER data express as %

Table C3: Feeding trial 1 liver phospholipid average similarities results[1]

FO		FO/CO		LMO		HMO	
Average similarity: 94.7		Average similarity: 93.2		Average similarity: 95.3		Average similarity: 94.7	
FAs	**Contribution**	**FAs**	**Contribution**	**FAs**	**Contribution**	**FAs**	**Contribution**
DHA	36.64	DHA	34.27	DHA	36.65	DHA	38.39
16:0	21.26	16:0	20.05	16:0	18.98	16:0	21.79
EPA	8.18	18:1ω9	12.58	18:1ω9	13.73	18:1ω9	10.87
18:1ω9	7.82	EPA	6.66	LA	6.65	-	-

[1]SIMPER data express as %

Table C4: Feeding trial 1 liver phospholipid average dissimilarities results[1]

FO & FO/CO		FO & LMO		FO/CO & LMO		FO & HMO		FO/CO & HMO		LMO & HMO	
Average dissimilarity = 10.8		Average dissimilarity = 17.8		Average dissimilarity = 12.3		Average dissimilarity = 15.2		Average dissimilarity = 12.6		Average dissimilarity = 7.9	
FAs	**Contrib.**	**FAs**	**Contrib.**	**FAs**	**Contrib.**	**FAs**	**Contrib.**	**FAs**	**Contrib.**	**FAs**	**Contrib.**
18:1ω9	20.26	EPA	18.35	EPA	20.76	EPA	22.74	EPA	21.8	DHA	17.27
DHA	16.34	18:1ω9	16.72	ω6DPA	14.81	ω6DPA	14.15	DHA	19.8	16:0	17.09
16:0	10.61	LA	10.63	DHA	13.43	18:1ω9	11.21	ω6DPA	17.18	18:1ω9	15.93
LA	10.48	ω6DPA	10.17	16:0	9.53	DHA	10.8	16:0	9.39	LA	10.79
EPA	7.78	22:5ω3	7.38	18:1ω9	6.58	22:5ω3	8.85	22:5ω3	5.79	18:0	6.74
18:0	6.94	16:0	6.85	LA	6.26	LA	6.80	-	-	20:3ω6	5.10

[1]SIMPER data express as %

Table C5: Feeding trial 1 muscle total fatty acids average similarities results[1]

FO		FO/CO		LMO		HMO	
Average similarity: 96.3		Average similarity: 95.7		Average similarity: 98.3		Average similarity: 97.9	
FAs	**Contribution**	**FAs**	**Contribution**	**FAs**	**Contribution**	**FAs**	**Contribution**
16:0	16.82	18:1ω9	33.1	18:1ω9	38.57	18:1ω9	30
DHA	16.61	LA	14.7	LA	16.27	DHA	19.79
18:1ω9	16.24	16:0	13.29	DHA	12.97	16:0	14.16
EPA	10.6	DHA	10.35	16:0	11.48	LA	14.11
LA	9.50	-	-	-	-	-	-
16:1ω7	6.86	-	-	-	-	-	-

[1]SIMPER data express as %

Table C6: Feeding trial 1 muscle total fatty acids average dissimilarities results[1]

FO & FO/CO		FO & LMO		FO/CO & LMO		FO & HMO		FO/CO & HMO		LMO & HMO	
Average dissimilarity = 24.0		Average dissimilarity = 33.5		Average dissimilarity = 12.4		Average dissimilarity = 26.1		Average dissimilarity = 12.8		Average dissimilarity = 12.9	
FAs	**Contrib.**	**FAs**	**Contrib.**	**FAs**	**Contrib.**	**FAs**	**Contrib.**	**FAs**	**Contrib.**	**FAs**	**Contrib.**
18:1ω9	36.41	18:1ω9	35.5	18:1ω9	26.31	18:1ω9	28.96	DHA	36.06	18:1ω9	32.47
EPA	11.24	EPA	14.12	EPA	16.56	EPA	17.87	EPA	15.51	DHA	27.51
DHA	11.2	LA	11.33	DHA	10.62	LA	9.98	ω6DPA	9.66	16:0	9.95
LA	11.19	16:1ω7	6.94	LA	9.21	DHA	8.25	18:1ω9	8.65	LA	8.92
-	-	16:0	6.17	16:1ω7	7.03	16:1ω7	7.54	16:0	5.67	-	-
-	-	-	-	22:5ω3	5.69	-	-	-	-	-	-

[1]SIMPER data expressed as %

Table C7: Feeding trial 1 muscle phospholipid average similarities results[1]

FO		FO/CO		LMO		HMO	
Average similarity: 85.6		Average similarity: 96.0		Average similarity: 93.2		Average similarity: 93.6	
FAs	**Contribution**	**FAs**	**Contribution**	**FAs**	**Contribution**	**FAs**	**Contribution**
DHA	35.03	DHA	37.65	DHA	40.93	DHA	46.46
16:0	26.05	16:0	19.7	16:0	19.89	16:0	22
EPA	9.1	18:1ω9	11.3	18:1ω9	13.66	18:1ω9	10.08
-	-	EPA	9.1	-	-	-	-

[1]SIMPER data express as %

Table C8: Feeding trial 1 muscle phospholipid average dissimilarities results[1]

FO & FO/CO		FO & LMO		FO/CO & LMO		FO & HMO		FO/CO & HMO		LMO & HMO	
Average dissimilarity = 14.5		Average dissimilarity = 23.6		Average dissimilarity = 14.5		Average dissimilarity = 21.6		Average dissimilarity = 16.1		Average dissimilarity = 10.3	
FAs	**Contrib.**	**FAs**	**Contrib.**	**FAs**	**Contrib.**	**FAs**	**Contrib.**	**FAs**	**Contrib.**	**FAs**	**Contrib.**
DHA	25.43	DHA	19.00	EPA	25.01	DHA	28.47	DHA	30.06	DHA	33.25
16:0	24.98	16:0	16.06	DHA	23.6	EPA	17.34	EPA	23.72	16:0	17.28
18:1ω9	15.14	EPA	15.03	ω6DPA	11.62	16:0	15.06	ω6DPA	11.02	18:1ω9	16.71
LA	7.6	18:1ω9	14.32	18:1ω9	8.5	ω6DPA	8.44	16:0	9.41	LA	8.67
-	-	ω6DPA	7.34	22:5ω3	8.46	18:1ω9	7.79	-	-	-	-

[1]SIMPER data expressed as %

APPENDIX D

The feed was produced at the Chute Nutrition lab at Dalhousie University Faculty of Agriculture (Truro, Nova Scotia, Canada). The ingredients were provided by Northeast Nutrition (Truro, Nova Scotia, Canada). The ingredients were mixed using a Hobart Commercial Mixer (The Hobart Manufacturing Co. LTD. Ontario, Canada) and the mix was steam pelleted into 4 mm pellets by a Laboratory Pellet Mill (California Pellet Mill Co., California, US). The pellets were dried in the oven for 4.5 h at 65°C and sifted. The feed was transferred to the Aquaculture lab at Dalhousie University Faculty of Agriculture directly after production and stored at -20°C until feeding.

Table D1: Formulation of diets used in the study (g/kg, as-fed basis) fed to Atlantic salmon

Ingredient (g/kg)[1]	**FM**	**FM/AB**	**AB**		**FM (%)**	**FM/AB (%)**	**AB (%)**
*Pav*459 algal biomass[2]	0	100	200		0	10	20
Fish meal	200	100	0		20	10	0
Fish (herring) oil	70	45	17.5		7	4.5	1.75
Ground wheat	149	117.7	87.8		14.9	11.77	8.78
Soy protein concentrate	84	107	127.5		8.4	10.7	12.75
Empyreal	140	140	140		14	14	14
Poultry fat	55	67.5	78.75		5.5	6.75	7.875
Canola oil	55	67.5	78.75		5.5	6.75	7.875
Poultry by-product meal	150	150	150		15	15	15
Blood meal	40	40	40		4	4	4
Vitamin/mineral mix[3]	2	2	2		0.2	0.2	0.2
Special pre-mix[4]	20	20	20		2	2	2
Dicalcium phosphate	22	25	30		2.2	2.5	3
L-lysine	0.5	3	8.5		0.05	0.3	0.85
L-methionine	1	2.8	3.9		0.1	0.28	0.39
Choline chloride	10.5	10.5	10.5		1.05	1.05	1.05
L-tryptophan	1	2	3		0.1	0.2	0.3
Threonine	0	0	1.8		0	0	0.18
TOTAL	1000	1000	1000		100	100	100

[1]All ingredients were donated by Northeast Nutrition (Truro, NS), except soy protein concentrate (President's Choice), which was purchased from Atlantic Superstore (Truro, NS).
[2]Produced at National Research Council (Ketch Harbour, NS, Canada).
[3]Vitamin/ mineral mix contains (/kg): zinc, 77.5 mg; manganese, 125 mg; iron, 84 mg; copper, 2.5 mg; iodine, 7.5 mg;

vitamin A, 5000 IU; vitamin D, 4000 IU; vitamin K, 2 mg; vitamin B12, 4 µg; thiamine, 8 mg; riboflavin, 18 mg; pantothenic acid, 40 mg; niacin, 100 mg; folic acid, 4 mg; biotin, 0.6 mg; pyridoxine, 15 mg; inositol, 100 mg; ethoxyquin, 42 mg; wheat shorts, 1372 mg.
[4]Special premix contains (/kg): selenium, 0.220 mg; vitamin E, 250 IU; vitamin C, 200 mg; astaxanthin, 60 mg; wheat shorts, 1988 mg.

Table D2: Proximate composition of the AB *Pav*459 used in the study

Proximate composition (%)	
Dry matter	95.23
Ash	9.80
Total nitrogen	9.74
Crude protein, N 6.25 × (%)	60.87
Crude protein, N × 4.78[3]	46.55
Esterifiable lipid	10.68
Crude lipid	12.25

Table D3: Nutritional composition of diets[1]

	FM	**FM/AB**	**AB**
Proximate composition (%)			
Dry matter	94.4 ± 0.3[a]	93.7 ± 0.0[b]	91.5 ± 0.1[c]
Crude protein	48.0 ± 0.3	48.5 ± 0.4	49.0 ± 0.3
Crude fat	22.9 ± 0.4	22.9 ± 0.2	23.0 ± 0.1
Ash	8.2 ± 0.1[a]	7.6 ± 0.2[b]	6.7 ± 0.1[c]
Minerals[1]			
Calcium (%)	2.1 ± 0.06[a]	1.7 ± 0.0[b]	1.3 ± 0.0[c]
Potassium (%)	0.5 ± 0.0	0.6 ± 0.0	0.6 ± 0.0
Magnesium (%)	0.3 ± 0.0[b]	0.1 ± 0.0[a]	0.1 ± 0.0[a]
Phosphorus (%)	1.6 ± 0.0[a]	1.5 ± 0.2[b]	1.3 ± 0.0[c]
Sodium (%)	0.4 ± 0.0[b]	0.4 ± 0.2[ab]	0.5 ± 0.0[a]
Copper (ppm)	11.7 ± 3.	12.5 ± 0.5	18.0 ± 0.8
Iron (ppm)	489.5 ± 6.0[c]	880.5 ± 10.2[b]	1318.6 ± 0.2[a]
Manganese (ppm)	27.2 ± 2.0[c]	33.9 ± 0.5[b]	43.0 ± 0.0[a]
Zinc (ppm)	179.4 ± 8.4[a]	150.1 ± 1.3[b]	120.8 ± 3.4[c]

[1]Data express as % diet (wet weight), values are means (n=3 per treatment) ± standard deviation. Means with different superscripts indicate significant differences among treatment diets based on Tukey's posthoc test following a one-way ANOVA. Means with different superscripts indicate significant differences based on Tukey's posthoc test following a GLM; FM, fish meal (control); FM/AB, fish meal/AB; AB, AB

APPENDIX E

Table E1: Feeding trial 2 liver total fatty acids SIMPER results[1]

FM		**FM/AB**		**AB**	
Average similarity: 82.6		Average similarity: 89.1		Average similarity: 90.4	
FAs	**Contribution**	**FAs**	**Contribution**	**FAs**	**Contribution**
DHA	23.85	18:1ω9	28.95	18:1ω9	28.62
18:1ω9	21.94	DHA	20.58	DHA	20.09
16:0	14.33	16:0	11.75	16:0	11.93
LA	8.52	LA	10.08	LA	10.03
EPA	6.15	-	-	-	-

FM & FM/AB		**FM & AB**		**FM/AB & AB**	
Average dissimilarity = 15.4		Average dissimilarity = 16.8		Average dissimilarity = 10.7	
FAs	**Contribution**	**FAs**	**Contribution**	**FAs**	**Contribution**
18:1ω9	31.30	18:1ω9	27.33	18:1ω9	26.28
DHA	26.68	DHA	24.57	DHA	21.92
16:0	8.47	16:0	7.20	16:0	8.48
LA	6.91	LA	6.99	LA	5.86
-	-	EPA	6.59	ARA	5.76
-	-	-	-	EPA	4.89

[1]SIMPER data expressed as %

Table E2: Feeding trial 2 liver phospholipid fatty acid SIMPER results[1]

FM		**FM/AB**		**AB**	
Average similarity: 96.1		Average similarity: 97.0		Average similarity: 96.6	
FAs	**Contribution**	**FAs**	**Contribution**	**FAs**	**Contribution**
DHA	37.96	DHA	36.72	DHA	33.58
16:0	18.23	16:0	17.38	16:0	16.79
18:1ω9	12.01	18:1ω9	12.82	18:1ω9	13.41
EPA	7.28	EPA	5.71	LA	6.16
-	-	-	-	18:0	5.64

FM & FM/AB		**FM & AB**		**FM/AB & AB**	
Average dissimilarity = 5.5		Average dissimilarity = 10.3		Average dissimilarity = 6.4	
FAs	**Contribution**	**FAs**	**Contribution**	**FAs**	**Contribution**
EPA	13.64	DHA	20.19	DHA	25.35
DHA	12.38	EPA	12.59	ω6DPA	10.89
ω6DPA	10.23	ω6DPA	12.18	ARA	10.34
16:0	8.94	ARA	10.30	EPA	9.31
LA	8.68	LA	7.45	16:0	7.59
ARA	8.58	18:1ω9	7.06	20:3ω6	6.95
18:1ω9	7.52	20:3ω6	6.75	-	-
18:0	7.38	-	-	-	-

[1]SIMPER data expressed as %

Table E3: Feeding trial 2 muscle total fatty acids SIMPER results[1]

FM		**FM/AB**		**AB**	
Average similarity: 90.7		Average similarity: 95.3		Average similarity: 92.8	
FAs	**Contribution**	**FAs**	**Contribution**	**FAs**	**Contribution**
18:1ω9	31.13	18:1ω9	35.00	18:1ω9	38.49
16:0	17.20	16:0	15.20	LA	17.12
LA	14.05	LA	14.83	16:0	11.40
DHA	9.08	DHA	9.04	DHA	7.31

FM & FM/AB		**FM & AB**		**FM/AB & AB**	
Average dissimilarity = 8.8		Average dissimilarity = 13.6		Average dissimilarity = 7.9	
FAs	**Contribution**	**FAs**	**Contribution**	**FAs**	**Contribution**
18:1ω9	28.19	18:1ω9	25.86	18:1ω9	21.66
DHA	15.14	16:0	13.83	DHA	17.41
LA	9.78	DHA	11.84	16:0	16.11
16:0	8.35	LA	11.53	LA	10.94
14:0	7.18	16:1ω7	6.39	18:0	5.88
16:1ω7	6.53	14:0	4.91	-	-

[1]SIMPER data expressed as %

Table E4: Feeding trial 2 liver phospholipid fatty acid SIMPER results[1]

FM		**FM/AB**		**AB**	
Average similarity: 96.5		Average similarity: 87.0		Average similarity: 94.8	
FAs	**Contribution**	**FAs**	**Contribution**	**FAs**	**Contribution**
DHA	40.08	DHA	34.69	DHA	34.82
16:0	20.50	16:0	23.54	16:0	21.25
18:1ω9	10.00	18:1ω9	11.79	18:1ω9	12.09
-	-	-	-	EPA	6.89

FM & FM/AB		**FM & AB**		**FM/AB & AB**	
Average dissimilarity = 9.9		Average dissimilarity = 9.1		Average dissimilarity = 10.8	
FAs	**Contribution**	**FAs**	**Contribution**	**FAs**	**Contribution**
DHA	31.05	DHA	28.61	DHA	35.42
16:0	20.33	18:1ω9	11.44	16:0	18.61
EPA	12.11	ω6DPA	11.28	18:1ω9	10.32
18:1ω9	9.17	EPA	11.15	ω6DPA	6.03
-	-	LA	8.31	-	-

[1]SIMPER data express as %

APPENDIX F

Candela et al. (1998) keynotes:

Fish samples: salmon (*Salmon salar*), Spanish mackerel (*Scomberomorus commersoni*), and sardine (*Sardine pilchardus*). Fish samples (corresponding to six helpings of ≈150 g each) were cooked by a catering industry firm following their usual procedure. They were introduced into an industrial deep fryer for 5 min with cooking oil (sunflower oil) at 180 °C. After draining, a quantity corresponding to three helpings was homogenized and immediately analyzed. The rest of the samples were introduced into a thermal unit used by the company for distribution. The internal temperature of food was 65°C. After 3 h, the samples were homogenized and analyzed. Raw samples were analyzed in the same way. Each parameter was analyzed four times for each batch. Moisture content was calculated by drying (ISO, 1973a).

Table F1: Moisture and fat content of fishes[1] (Adapted from Candela et al., 1998)

	Raw	Cooked	Warm held
Salmon			
Moisture	$69.3^{a} \pm 0.06$	$56.3^{b} \pm 1.09$	$51.2^{c} \pm 0.20$
Fat	$12.0^{a} \pm 0.15$	$14.8^{a} \pm 0.30$	$15.9^{a} \pm 1.66$
Mackerel			
Moisture	$61.0^{a} \pm 0.10$	$64.6^{b} \pm 0.40$	$61.1^{a} \pm 0.02$
Fat	$16.0^{a} \pm 0.20$	$7.25^{b} \pm 0.24$	$9.33^{c} \pm 0.08$
Sardines			
Moisture	$74.0^{a} \pm 0.48$	$56.7^{b} \pm 0.01$	$57.5^{b} \pm 0.19$
Fat	$4.0^{a} \pm 0.20$	$13.3^{b} \pm 0.20$	$11.8^{c} \pm 0.20$

[1]All values referred to g/100 g of food (mean ± standard derivations). Values in the same row bearing different letters are significantly different ($p \leq 0.05$).

Appendix G

The entire EA was flushed continuously with helium gas (He) at a rate of 90 to 110 mL min^{-1}. Each tin capsule with its content was individually dropped onto the oxidation reactor at a temperature of 1050 °C, with simultaneous injection of oxygen and quick flushing with He. This sequence triggered flash combustion at 1800 °C between the tin capsule and oxygen, creating combustion gases that were pushed through an oxidation catalyst (chromium trioxide, CrO3) to ensure complete oxidation of the sample and silvered cobaltous/cobaltic oxide, which removes halides and SO2. The resulting gas mixture passed through the reduction reactor (reduced copper) at 650 °C, which reduces nitrogen oxides to nitrogen gas and absorbs oxygen. The gases then passed through a magnesium perchlorate ($Mg(ClO_4)_2$ water trap, after which the remaining gases (N2, CO2) entered a 3 m stainless steel GC column (QS 50/80; Poropak) at 40 to 100 °C. The individual gases were separated as they moved through the GC column. Upon reaching the TCD, they were detected as separate gas peaks; first N_2, then CO_2. From the TCD, He carried the gases to a ConFloIII interface (Finnigan, Thermo Electron Corporation), which has split tubes open to the atmosphere, which allows a portion of the He and combustion gases to enter directly into the ion source of the mass spectrometer (MS) (DeltaVPlus; Thermo Scientific) via fused glass capillaries. During operation, He from the EA flowed continuously into the MS. All gases exiting the EA also entered the ion source, but the instrument only recorded signals for the gases of interest, as defined through the software by instrument configuration. Internal and external reference material was used to calibrate MS data. EDTA #2 and D-Fructose were used for carbon isotope calibration and IAEA-N-1 ((NH4)2SO4) and IAEA-N-2 ((NH4)2SO4) for nitrogen isotope calibration. NBS-18 (CaCO3), B2150 (high organic sediment), B2151 (high organic sediment), and B2105 (Cystine) were used to aide data interpretation of carbon isotope analyses, and sorghum

flour, B2153 (low organic soil), USGS-25 ((NH4)2SO4), USGS-26 ((NH4)2SO4), sulfanilamide, and BBOT to aide data interpretation of nitrogen isotope analyses. L-glutamic acid and B2155 (protein) were used for both carbon and nitrogen elemental calibration.

Table G1: *Pavlova*459 sp. bulk stable isotope analysis

Sample ID	Amount (mg)	Mean $\delta^{13}C_{VPDB}$/‰ of All Analyses[1]	St. Dev. of $\delta^{13}C_{VPDB}$/‰ of All Analyses[2]
L-glutamic acid	1.124	-26.67	0.07
L-glutamic acid	1.201	-26.67	0.07
L-glutamic acid	1.186	-26.67	0.07
L-glutamic acid	1.143	-26.67	0.07
Blank capsule	0.000		
Blank capsule	0.000		
Blank capsule	0.000		
EDTA #2	1.285	-40.38	0.08
EDTA #2	1.159	-40.38	0.08
D-Fructose	1.197	-10.53	0.08
D-Fructose	1.121	-10.53	0.08
L-glutamic acid	1.697	-26.67	0.07
L-glutamic acid	1.566	-26.67	0.07
L-glutamic acid	0.958	-26.67	0.07
L-glutamic acid	0.658	-26.67	0.07
L-glutamic acid	0.415	-26.67	0.07
Pav 459-1	1.258	-56.17	
EDTA #2	1.280	-40.38	0.08
D-Fructose	1.175	-10.53	0.08
Pav 459-2	1.215	-55.60	
Pav 459-3	1.273	-55.30	
L-glutamic acid	1.152	-26.67	0.07
EDTA #2	1.246	-40.38	0.08
D-Fructose	1.188	-10.53	0.08
L-glutamic acid	1.159	-26.67	0.07

$\delta^{13}C_{VPDB}$/‰ of Peak is the isotope ratio determined from the valid peak for the individual sample analysis.
[1]Is the average isotope ratio of all valid analyses of a sample within a run. If this cell is empty, the individual result is considered to be invalid.
[2]If a sample has more than one valid analysis in a run, this number will be the standard deviation of all of the valid analyses.