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Special Issue Reprint

Marine Biotechnology for Sustainability of Ecologically Significant Resources

Edited by
Kim Lee Chang and Peter D. Nichols

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Guest Editors

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Cover image courtesy of Kim Jye Lee Chang
Image of an Australian thraustochytrid (TC20) isolate, a single cell long-chain omega-3 oil producer.

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Contents

About the Editors

Kim Lee Chang

Kim Jye Lee Chang is a Senior Research Scientist at Commonwealth Scientific and Industrial Research Organisation (CSIRO) Environment with expertise in marine biotechnology, focusing on the development of microalgae for sustainable biofuels and bioproducts, including omega-3 oils. Over the past decade, Kim has contributed to advancing the understanding of marine protists, particularly thraustochytrids, known for their high lipid production. His research is well-documented in international peer-reviewed journals.

Kim's work includes the discovery of new endemic strains of highly productive thraustochytrids and the optimisation of their growth conditions to enhance biofuel and omega-3 oil yields. His PhD, completed in 2013, was titled "Microalgae—A Renewable Source of Biofuels, Omega-3 Oils, and Other Co-products." Following this, he received the prestigious CSIRO Office of the Chief Executive (OCE) Postdoctoral Fellowship in 2014.

Kim has played a key role in establishing a collaboration between CSIRO and industry partners to develop sustainable omega-3 oils for the global market. His work reflects a strong commitment to advancing sustainable marine biotechnology.

Peter D. Nichols

Peter D. Nichols leads new initiatives with signature lipid technology and environmental applications, and on marine oils, with emphasis on the health-benefitting omega-3 long-chain PUFA containing oils. The research involves detailed characterization of fish-derived and novel microbial oils, process development for utilization of oils, development as part of a CSIRO team of novel land plant sources of long-chain omega-3 oils, and transfer and application of these know-how to industry and community. This research has led to better utilisation of national fisheries resources and wastes and to new oilseed crops. He has contributed to the development of the Australian marine oils industry, with CSIRO research resulting in novel national and international marine oil products (wax ester, omega-3, and shark liver oils). Other innovations have been: development and use of chemical (lipid) signatures in microbial ecology, environmental (e.g., fecal pollution) and food-chain studies and new discoveries and applications in marine biotechnology. He has worked closely with Australian and overseas Universities actively co-supervising and mentoring over 35 PhD students to graduation. He is involved in international collaborations across the above research fields, including sponsoring sabbaticals and visits by overseas scholars.

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Editorial

Marine Biotechnology for Sustainability of Ecologically Significant Resources

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The sustainable production of marine ingredients is increasingly important, valued by manufacturers, government, and authorities, as well as by environmentally aware consumers. This Special Issue invited contributions from authors seeking to highlight developments in the application of biotechnologies for the sustainable production of ecologically significant resources. Twelve out of the fifteen papers received placed an emphasis on n-3 long-chain (LC, $\geq C_{20}$) polyunsaturated fatty acids (n-3 LC-PUFAs) that are used in aquaculture feeds and nutraceutical products. These papers cover the single-cell production and application of n-3 LC-PUFAs; a review of recently achieved oilseed production in n-3 LC-PUFAs, together with recommendations on regulatory matters; a lipidomic investigation into the New Zealand green shell mussel (GSM); a review of the sustainability, use, and efficacy of the GSM; a review on sourcing n-3 LC-PUFAs from globally distributed and presently under-utilised myctophid fishes; a study in northern Australian beef cattle providing insights into IMF deposition and SNP marker-assisted selection for the improvement of the meat's eating quality, emphasising n-3 LC-PUFAs; and a study on the sustainable enzymatic production of omega-3 squid viscera oil. One paper describes the use of sustainably produced krill meal in aquaculture with *Penaeus vannamei*; a second report on krill examines the efforts undertaken in the industry to ensure more sustainable production in the krill fishery. In addition, a review on synthetic chemical surfactants and perspectives on marine-safe biosurfactants, as well as an article on the development and application of a compound-specific stable isotope analysis approach to determine provenance with squalene and shark liver oil, are included. The papers in this Special Issue collectively highlight a range of new and innovative research outcomes, including recent significant achievements in the omega-3 field and in several other marine biotechnologies.

This Special Issue focuses on how marine and associated biotechnologies can allow and enhance social, economic, and environmental sustainability. Marine biotechnologies are not universally applicable, and a combination of ecosystem knowledge and biotechnology in the sustainable production of feeds, fuels, and biochemicals, including nutraceuticals, could complement aquaculture and other applications. There is the potential to enhance future marine spatial planning, incorporating current coastal agriculture and recreational use due to the ongoing growth and expansion of coastal populations and the many activities associated with them. For example, macro- and microalga biotechnologies require both favourable growing conditions and economic bioprocessing for scaling up and the associated downstream processing.

The application of such biotechnologies can also help to reduce waste by recycling nutrients and subsequently reducing greenhouse gas footprints, to achieve social, economic, and environmental sustainability.

This Special Issue originally aimed to showcase technologies from marine spatial planning, life cycle assessment, carbon capture technologies, and nutrient cycling to the

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production of high-value bioproducts, such as bioingredients with health benefits, especially omega (ω) 3 long-chain (LC, $\geq C_{20}$) polyunsaturated fatty acids (n-3 LC-PUFAs), for use in aquafeed and nutraceutical products. The call for papers resulted in a large proportion of the papers submitted covering the n-3 LC-PUFA field, with several of the topics originally planned for not being covered. The Special Issue included ten papers and two review articles exploring a variety of sources of these essential health-benefitting fatty acids. Of these 10 papers, the first five papers on the n-3 LC-PUFA topic cover the use of algal and thraustochytrid biomass produced using a range of systems and approaches. The next five papers cover alternate sources of n-3 LC-PUFA. In the following section, the use of sustainably produced krill meal in aquaculture applications with *Penaeus vannamei* is described, and a communication examines ways of reducing the greenhouse gas emissions from the main operator in the krill fishing industry; such reductions contribute to enhancing sustainability in the fishery. In the final section, one paper reviews synthetic chemical surfactants and offers perspectives on marine-safe biosurfactants; the final contribution show the development and application of compound-specific stable isotope analysis in determining the source and, importantly, the provenance of squalene and shark liver oil. This Special Issue places an emphasis on scientific contributions that enhance social, economic, and environmental sustainability and aims to supplement the gap between current industry practices and R&D efforts to address future global needs.

The contributions to the Special Issue are divided into five sections and commences with an overview editorial (Contribution 1). Many of the papers in this Special Issue then address the topic of the sustainable production of omega-3 long-chain polyunsaturated fatty acids for application in aquaculture. Seafood is the key source of ω3 LC-PUFAs, with aquaculture expected to meet the rising global demand. Given the current high interest in this topic, the papers include a commissioned review by Professor Parrish from Memorial University titled, "Thraustochytrids and algae as sustainable sources of n-3 LC-PUFAs for aquafeeds" (Contribution 2). Limited biosynthesis of the key omega-3 long-chain fatty acids, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), occurs in food webs leading to humans. An often forgotten aspect is that marine fish themselves have high dietary requirements for EPA and DHA. These requirements were traditionally met using what many people see as unsustainably harvested dietary fish oil and fish meal. However, a combination of expanded global aquaculture production, limited supply, increased costs, and environmental concerns has driven research on replacements for these key ingredients. Microalgae, especially thraustochytrids, stand out as resources with high concentrations of EPA and, in particular, DHA. Although they are unicellular, thraustochytrids are not true microalgae, as they are not photosynthetic and are instead microheterotrophs. This feature removes their need for light and facilitates high yields in monoculture for the production of single-cell oils. The availability, in high concentrations, of one or the other of these two long-chain omega-3 fatty acid, permits the calibration of DHA and EPA doses. This is especially useful, as their effects have generally been considered together in medical and aquaculture research. DHA and EPA have different effects on cell function and are precursors to different bioactive components. Using microalgae, heterotrophic dinoflagellates, and thraustochytrids, the importance of DHA has been investigated. DHA has been shown to be essential in optimising growth in the early life stages of scallops and finfish and is preferentially incorporated into fish membrane phospholipids. In addition, in environmental research, the production of microalgae and microheterotrophs can also contribute to the treatment of wastewater, waste gas, and other wastes or byproducts, with such applications further enhancing sustainability and reducing environmental costs in aquaculture.

Dr Guerra from Memorial University, Newfoundland, together with colleagues from several other organisations, examined the effects of replacing fish oil (FO) with a microbial oil (MO) derived from *Schizochytrium* sp. on the lipid composition of Atlantic salmon parr muscle and liver (Contribution 3). A feeding trial was performed to determine the effects of replacing dietary FO with the key n-3 LC-PUFA - DHA, derived from a DHA-rich MO.

The four diets used contained varying levels of FO, MO, and canola oil (CO). The diets were as follows: a control diet with 20% FO, a second control diet with 10% FO and 10% CO, and two experimental diets that completely replaced FO with a low (5%) and high (10%) proportion of MO. The growth parameters (weight gain), lipid class composition, and sterol content of the fish fed the four diets showed no significant differences between treatments over the 16-week trial. In contrast, there were significant differences in the proportions of individual n-3 and n-6 PUFAs in both the muscle and liver tissues; these differences reflected the different diets. The presence of low EPA in the two MO diets did not affect the growth performance of fish; the authors proposed that these data suggest a lower requirement for EPA in the diet and a greater requirement for DHA. The results also show that DHA was present in very high proportions in the cellular membranes, particularly in muscle tissue, with low levels of linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (ALA, 18:3n-3) present. The authors conclude that their findings show that MO derived from *Schizochytrium* sp. (T18) can be a substitute for FO in the feeding of farmed Atlantic salmon.

A paper by Dr Soudant, from the University of Brest, CNRS, and Ifremer in France, and collaborators (Contribution 4), evaluated the biomass of another thraustochytrid species (*Aurantiochytrium mangrovei*) grown on digestate as a sustainable feed ingredient for sea bass (*Dicentrarchus labrax*) juveniles and larvae. The authors produced *Aurantiochytrium mangrovei* biomass via heterotrophic fermentation using a medium containing anaerobic digestion liquid effluent and evaluated the biomass produced, which was rich in n-3 LC-PUFAs and good-quality proteins, as a feed ingredient for sea bass. Bioreactors were used to produce biomass in non-axenic conditions. Sea bass juveniles were fed a control diet or a diet containing 15% freeze-dried *A. mangrovei* biomass. The juvenile survival was 90% on average in both dietary treatments after 38 days. Similar growth was observed between fish fed the two diets, demonstrating the feasibility of replacing 15% of a standard fish feed with *Aurantiochytrium* biomass. The livers of juvenile sea bass fed the *A. mangrovei* diet contained higher proportions of the LC-PUFAs DHA, 22:5n-6 and 20:4n-6, than those fed the control diet, while the proportions of 16:0, 16:1n-7 and 18:1n-9, were lower. Secondary oxidation was measured by determining the content of malonylaldehyde; in the muscle and liver of juveniles fed the microalga diet, malonylaldehyde was higher than in fish fed the control diet, but the differences were not significant. The larval survival was low for all tanks after 41 days of rearing. The inclusion of 15% hydrolysed *A. mangrovei* biomass in the larval microdiet only marginally affected lipid composition and did not impair development in sea bass larvae.

Algal biomass (*Pavlova* sp. 459) was used to replace fishmeal in a trial in which the authors examined the effects on the membrane lipid composition of Atlantic salmon (*Salmo salar*) parr muscle and liver tissues (Contribution 5). The study used a 12-week trial to examine the dietary impact of replacing fishmeal (FM) with algal biomass (AB) derived from *Pavlova* sp. strain CCMP459 (*Pav*459) in Atlantic salmon diets. Three diets were compared: a control diet featuring 20% FM and 7% fish oil (FO), an experimental diet comprising a 50:50 blend of FM and AB *Pav*459 and reduced FO (10% FM, 4.5% FO, 10% AB), and a second experimental diet with the full replacement of FM with AB *Pav*459 and a further reduction in FO (1.75% FO, 20% AB). Replacing FM with AB *Pav*459 showed no significant effects on the growth performance of Atlantic salmon. Fish across all diets exhibited growth exceeding 200% of their initial weight. The total lipid content after the 12-week trial was not significantly different among the diets. The individual proportions of n-3 and n-6 PUFAs varied. FA profiling in muscle and liver tissues showed distinct compositions reflecting different dietary treatments. LA and ALA exhibited higher proportions in the total FA than in membrane lipids. DHA emerged as the predominant FA in the membranes of both liver and muscle tissues. Analysis of the sterol composition in *Pavlova* and salmon muscle tissue showed the presence of important sterols, including conventionally animal-associated cholesterol. This finding emphasises the suitability of microorganisms, such as *Pav*459, for synthesising diverse nutrients. Stable isotope analysis demonstrated the direct incorporation of EPA and DHA from diets into salmon tissues. Notably, minimal biosynthesis from the precursor ALA was observed, reaffirming the

utility of *Pav*459-derived FA. The EPA + DHA proportions in the fillet consistently met daily human consumption requirements across all dietary conditions, supporting the use of *Pav*459 algal biomass as an alternative to FM.

The final paper on microalgal applications, by Ventura and colleagues (Contribution 6), covers the direct use and optimisation of hydrolysis using two commercially available proteases (Papain and Alcalase 2.4L) of thraustochytrid biomass rich in DHA for the production of a new and sustainable ingredient for the aquafeed industry. The hydrolysis conditions did not affect the FA profile or the lipid content of the biomass. The authors conclude that if the improved nutritional value of the solvent-free hydrolysed biomass can provide an advantage in fish fitness and reduce the amount of fish meal and fish oil used, then the treated biomass can be sold at a higher price and can enhance the sustainability of fish aquaculture.

Two papers by Dr Miller of the Cawthron Institute in New Zealand and collaborators present insight into the New Zealand greenshell^{TM} mussel (GSM) (Contribution 7, 8). The first paper provides a comprehensive review of sustainability, traditional use, and efficacy in the GSM. The GSM, *Perna canaliculus*, is Aotearoa/New Zealand's most important aquaculture species, is available in a variety of food and nutritional products worldwide and is a traditional and culturally valuable food of the Māori people (Contribution 7). Following the development of several nutraceutical products (dried powders and extracted oils) in the 1960s, there has been increased interest in the health benefits of GSM products. Omega-3 LC-PUFAs in GSMs have exhibited significant anti-inflammatory activity, and clinical evidence has led to the promotion of GSM powders and oils as treatments for osteoarthritis and rheumatoid arthritis. The authors, in their review, define the nutritional composition of GSMs and describe the sustainability of GSMs and their traditional uses. The review also details the health benefits of GSMs in clinical applications and examines potential mechanisms and molecular pathways initiated by the various bioactive components of GSMs. The second paper by the CSIRO and the Cawthron team provides a lipidomic profile of the GSM as a sustainable source of n-3 LC-PUFAs (Contribution 8). Increases in analytical capabilities have seen lipidomic studies using advanced mass spectral data provide new insights into the content and activity of the lipidome, that is, the totality of all lipids. This paper is described as the first reported characterisation of the GSM lipidome, which provides important and novel information regarding its biology, nutrition, physiology, and epidemiology. In addition to the traditional lipid analytical outputs that are commonly available, new lipidomic capabilities can determine lipid species differences, including between tissues rich in oil. The study identified 16 different lipid species in GSMs, including ceramide aminoethyl phosphonate (CAEP). Many lipid species are differentially expressed between tissues, and correlation analysis demonstrated lipid species associated with the digestive gland that may be obtained from food sources, whilst other lipid species dominated in the mantle or gonad. The authors propose that linking this new information with GSM breeding programmes may assist in the delivery of functional breeding attributes to obtain premium strains for enhanced nutrition and/or extract production.

Bowen Zhang and colleagues from the University of Tasmania and CSIRO reviewed the nutritional composition of myctophid fishes as a key step towards evaluating alternative and sustainable marine-derived food resources (Contribution 9). Such resources are needed as the global human population continues to increase. Marine fishes have long provided essential nutrients, in particular n-3 LC-PUFAs, and proteins, key amino acids, and vitamins both to meet human dietary requirements and for use in feeds in aquaculture and livestock production. Many currently harvested fish stocks are depleted or are seen as being fully exploited. The review shows that oceanic mesopelagic fishes, particularly myctophids (lanternfishes), represent a potentially very large and generally unfished resource for consideration for future use. The authors analysed the literature on the nutritional and biochemical compositions of myctophids as an important first step towards understanding the health benefits and the possible risks of consuming them. Myctophids contain high levels of protein (11–23% wet weight, WW) and a variable lipid content (0.5–26% WW).

In most myctophid species, desirable triacylglycerols or phospholipids dominated over less desirable wax esters, and most species examined had an abundance of health-promoting n-3 LC-PUFAs, such as DHA and EPA. Myctophids, importantly, have only low levels of heavy metals and persistent organic pollutants. Most of the nutritional information available is for species from the Pacific and Southern Oceans and for the genera *Benthosema*, *Electrona*, and *Diaphus*. Overall, myctophids generally possess favourable nutritional profiles. Notwithstanding this, major gaps in knowledge remain regarding their ecology and stock assessment and the economic viability of their harvest. These gaps are currently possible barriers to the future development of sustainable fisheries.

Dr Xue-Rong Zhou from CSIRO and colleagues reviewed the journey, several decades long, from the ocean to the field to develop and successfully obtain new sustainable oilseed sources of n-3 LC-PUFAs (Contribution 10). The authors describe the range of key genes targeted in the development and deregulation of novel oilseed crops producing n-3 LC-PUFAs and their market applications. The genetic engineering of n-3 LC-PUFAs into sustainable oilseed crops such as canola involved the use of multiple-gene pathways to achieve elevated levels of these key nutrients. The R&D was described as extremely challenging. Two decades of collaborative effort have resulted in new oilseed crops being successfully developed, including crops containing a high n-3/n-6 ratio and, in particular, showing fish-oil-like levels of n-3 LC-PUFAs. The deregulation of genetically engineered oilseed crops with such novel nutritional traits is also extremely challenging, and the authors recommend that more trait-based regulations be adopted. Several n-3 LC-PUFA-producing oilseed crops have been approved for large-scale cultivation and, importantly, for applications in feeds in aquaculture application and livestock and for food. These genetically engineered oilseed crops can and will help meet the increasing global market demand for their use in aquaculture and human nutrition. These new oilseed sources of n-3 LC-PUFAs offer a sustainable, safe, scalable, cost-effective, land-based solution to the growing need for these key health-promoting ingredients, which can have critical positive health, economic, and environmental impacts.

The sustainable enzymatic production of omega-3 squid viscera oil was demonstrated by Haque et al. (2024) from Deakin University (Contribution 11). Squid viscera contain up to 10% oil by mass and represent a byproduct available from squid processing. Squid viscera oil contains EPA and DHA and astaxanthin pigment, the latter which has significant anticancer, antioxidant, antidiabetic activity and properties relevant to cardiovascular health. Raw squid viscera oil has a high free fatty acid (FFA) level and conventional alkali-refining results in low yield and loss of the astaxanthin. The authors optimised the lipase-catalysed glycerolysis of the squid viscera oil to convert FFA into acylglycerol using a custom-built one-litre immobilised enzyme reactor and then monitored the reaction progress and assessed its impact on the oil. The lipid classes, fatty acid composition, and astaxanthin levels were determined. Under optimised conditions, FFA was reduced from 40% to <2% in 24 h, with no significant effect on EPA and DHA levels, and astaxanthin was retained. Squid viscera represent a safe and sustainable additional source of marine-derived EPA- and DHA-containing oil.

The final paper on the topic of n-3 LC-PUFAs was written by Professor Malau-Aduli and collaborators at James Cook University, Australia (Contribution 12). The study aimed to identify single-nucleotide polymorphisms (SNPs) in lipogenic genes of northern Australian tropically adapted crossbred beef cattle and to evaluate associations with healthy lipid traits of the *Longissimus dorsi* muscle (loin eye). The authors tested the hypothesis that there are significant associations between SNP loci encoding for the FA binding protein 4 (FABP4), stearoyl-CoA desaturase (SCD), and FA synthase (FASN) genes and n-3 LC-PUFAs, which are beneficial to human health, within the loin eye muscle of crossbred beef cattle. Brahman, Charbray, and Droughtmaster crossbred steers were fed on Rhodes grass hay augmented with lucerne, desmanthus, or a mix of both; the loin eye muscle was then sampled and analysed for its intramuscular fat (IMF), fat melting point (FMP), and FA composition. Polymorphisms in the FABP4, SCD, and FASN genes with significant effects

on lipid traits were identified using next-generation sequencing. Multiple comparisons of genotypes at the SCD g.21266629G>T locus indicated that the TT genotype had significantly higher EPA and DHA, and also higher DPA, than the GG genotype ($p < 0.05$). Significant correlations ($p < 0.05$) between FASN SNP and IMF, SFA, and MUFA were observed. These results provide insights into the contribution of lipogenic genes to IMF deposition and SNP marker-assisted selection for the improvement of the meat's eating quality, with an emphasis on sustainable alternate sources of n-3 LC-PUFAs, in northern Australian tropical crossbred beef cattle.

The use of sustainably produced krill meal in aquaculture applications with *Penaeus vannamei*is described in a paper in the next section by Nunes et al. (Contribution 13). The authors examined the minimum dietary level and mix ratio of krill meal (KRM) and fish meal (FML) to improve feed intake and growth performance in juvenile *Penaeus vannamei.* Shrimp feeds depend on high levels of digestible protein and essential amino acids, which can be sourced from various commercially available feed ingredients. Marine proteins can be used to partially fulfil the requirements for these and other important nutrients. Their utilisation is further influenced by their palatability and growth-promoting effects. However, the continued use of marine ingredients can significantly drive costs in feed formulation, depending on the type and dietary inclusion level. The authors aimed to determine the minimum dietary level of FML and KRM and their best mix ratio to improve feed intake and growth performance in juvenile *Penaeus vannamei*. Diets were formulated with graded FML in combination with KRM, as well as a control diet with FML. Shrimp were stocked in tanks, and after 88 days, their growth performance was determined. Feed preference was assessed over four weeks. No significant differences in survival, yield gain, or feed conversion ratio were observed. Diets with 60 g kg^{-1} FML led to faster growth and a higher feed intake than those with 30, 90, and 120 g kg^{-1} FML. Shrimp on 30 g kg^{-1} FML diets had the lowest body weight (BW), especially with 30–15 (g kg^{-1}, FML–KRM) and 30–30 diets. Shrimp on diets with 90 g kg^{-1} FML outperformed those on diets with 30 g kg^{-1} FML. The control diet delivered a higher shrimp BW than the 30–15 and 30–30 diets, showing similar results to other diets, except for the 60–15 diet. Feed preference was influenced by KRM inclusion; 15 g kg^{-1} KRM resulted in a higher apparent feed intake than 30 and 45 g kg^{-1} KRM. The findings indicate that FML can be effectively reduced by up to 75% when combined with lower levels of KRM. This corresponds with the aquaculture industry's ongoing trend to achieve greater sustainability and cost efficiency through the reduced utilisation of critical marine resources.

The next paper communicates efforts by the main company (Aker BioMarine) in the krill industry to reduce greenhouse gas (GHG) emissions, thereby further improving industry sustainability (Contribution 14). Krill products are used in animal and pet feeds, aquaculture, and human nutrition applications. The background information on fisheries explains that krill fisheries are precautionarily and closely regulated via conservation measures, including oversight by the Committee for the Conservation of Antarctic Marine Living Resources (CCAMLR). The regulated catch is capped at 1% (620,000 MT), of what the estimated total biomass is believed to be in Area 48 around the Antarctic Peninsula. In the communication, demonstration cases are presented (e.g., changing the factory line speed, increasing yield to increase energy efficiency in production, heat recovery for a reduction in energy use) that target GHG reduction; further examples of short- and long-term actions that can minimise the impact of all activities are described.

The closing section of this Special Issue includes two distinct papers, with the first being a review by Fung and Prof. Gutierrez from Heriot Watt University, Scotland, and collaborators, on synthetic chemical surfactants (SCSs) and perspectives on marine-safe biosurfactants (Contribution 15). SCSs are a versatile group of amphiphilic chemical compounds synthesised from fossil fuel precursors, which have been used in a range of industry applications. Their global use is estimated to be over 15 million tons per annum; their use has also resulted in environmental damage and potential toxicological effects to humans and other organisms. Current challenges to both reduce reliance on

petrochemical resources and ensure environmental protection have led to an increased demand for sustainable and environmentally friendly alternatives, such as biosurfactants. Biosurfactants are generally environmentally compatible amphiphilic compounds, are biodegradable, and, importantly, are non-toxic. There is considerable opportunity for microbial biosurfactants to replace SCSs. However, key challenges remain in relation to their low yields and substantial production cost compared to SCSs. The authors discuss the release of SCSs from wastewater treatment plants as the major point source of their release into the ocean and cover the consequences of these pollutants for marine organisms and humans. The opportunity for microbial biosurfactants to be used as a replacement for SCSs is discussed, with a focus on rhamnolipids. Perspectives on current and future work on commercialising microbial biosurfactants are also given.

The final paper by Dr Andy Revill from CSIRO and collaborators demonstrates the use of compound-specific stable carbon isotope analysis (CSIA) of squalene to establish provenance, and thereby ensure sustainability, in the deep-water shark liver oil industry (Contribution 16). Deep-water dogfish (sharks) are caught on Australia's continental shelf as byproducts from fishing for other deep-water species; revenue is derived from their livers, which are sold for their oil, and fillets, which are used for human consumption. Deep-water dogfish utilise their large, oil-rich liver for buoyancy. Livers may account for 20–25% of body weight. A major constituent of dogfish liver oils is squalene, a highly unsaturated triterpenoid ($C_{30}H_{50}$) hydrocarbon, which can be present at up to 90% in the oil; in Australian commercial species, this is generally around 50%. Squalene (and deep-water dogfish liver oil in general) has a traditional and long-standing use in a variety of products, including in nutraceuticals and cosmetics and as an ingredient in the adjuvant used in vaccines. Increasingly, manufacturers are required to, or may seek to, demonstrate product provenance and sustainability. The harvesting of Australia's mid-slope deep-water dogfish is recognised globally as being well managed and sustainable. Therefore, it is extremely important for genuine Australian producers to be able to distinguish their products from other unregulated, unsustainable, and cheaper sources so as to protect Australia's competitive advantages and ensure sustainability. This study sourced deep-water dogfish liver oil samples from Southeast Australia, New Zealand, India, Northeast Africa, and the Arabian Sea. Squalene was isolated via laboratory or commercial processing. CSIA was then performed on the squalene and used to determine the isotopic resolution; the data were then further used to assign the likely origins of a range of off-the-shelf nutraceutical products available in Australian outlets. Squalene sourced and produced from dogfish liver oils harvested from Southeast Australian and New Zealand waters showed $\delta^{13}C$ values distinguishable from all other regions. Most off-the-shelf squalene products claiming to be of Australian origin showed $\delta^{13}C$ values that were very distinct from the range of genuine Southeast Australian- and New Zealand-produced squalene.

The selection of papers in this Special Issue highlights a variety of new and innovative scientific outcomes occurring in the marine biotechnology field across the areas of LC omega-3 oils and their sources and applications, SCSs, and analytical approaches such as CSIA. Future research on these and closely linked topics will assist in furthering our understanding of these important health-promoting ingredients.

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List of Contributions

1. Lee Chang, K.; Nichols, P.D. Marine Biotechnology for Sustainability of Ecologically Significant Resources. *Sustainability* **2024**, *16*.
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Review

Thraustochytrids and Algae as Sustainable Sources of Long-Chain Omega-3 Fatty Acids for Aquafeeds

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Abstract: There is limited ability to biosynthesize long-chain omega-3 fatty acids such as EPA and DHA in food webs leading to humans. Seafood is the key source of ω3 LC-PUFA, with aquaculture expected to meet rising global demand; however, marine fish have a high dietary requirement for EPA and DHA themselves. This was traditionally met using unsustainable dietary fish oil and fish meal, but limited supply and environmental concerns have dictated research on replacements. Among the industrial sources of EPA and DHA, microalgae and especially thraustochytrids stand out as resources with high concentrations. Although unicellular, thraustochytrids are not microalgae as they are not photosynthetic but instead are microheterotrophs. This removes the light requirement and facilitates high yields of monoculture for the production of single-cell oils. The availability, in high concentrations, of usually one or the other essential fatty acid permits a calibration of the EPA and DHA dose, which is especially useful as their effects have mainly been considered together in medical and aquaculture research. EPA and DHA have different effects on cell function and are precursors of different bioactive compounds. Using thraustochytrids, microalgae, and heterotrophic dinoflagellates, the importance of DHA has been investigated. DHA was essential for optimizing the growth of the early life stages of scallops and finfish and was preferentially incorporated into fish membrane phospholipids. The production of microalgae and microheterotrophs can contribute to the treatment of wastewater and waste gas, further enhancing their sustainability and reducing the environmental costs of aquaculture.

Keywords: single-cell oils; microbial lipids; microalgae versus microheterotrophs; DHA versus EPA; environmental bioremediation

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1. Introduction

Lipids are essential for life, providing energy, structure to cell membranes, and metabolic messengers. Among the lipids, long-chain (LC) polyunsaturated fatty acids (PUFAs) and cholesterol are essential for optimal health, and there is particular interest in the intake of omega-3 (ω3) LC-PUFAs in food webs leading to humans. The extent to which ω3 LC-PUFAs intake benefits humans remains controversial [1,2]; nonetheless, positive effects continue to be published on cardiovascular disease [3], stroke risk [4], and psychotic symptoms [5], among many others. A study on all-cause mortality in over 40,000 adults revealed that ω3 LC-PUFAs intake was associated with a lower risk of premature death [1].

Intake of ω3 LC-PUFAs (Figure 1) such as EPA (eicosapentaenoic acid; 20:5ω3) and DHA (docosahexaenoic acid; 22:6ω3) can be determined through blood levels, which permits large studies [1] and even global mapping [6]. Using the Omega-3 Index (O3I), which is the sum of EPA + DHA as a % of total fatty acids in red blood cells, Schuchardt et al. [6] determined that the intake in most countries was low to very low. Aquatic foods are the key source of these essential fatty acids, and aquaculture supplies over half the world's seafood for human consumption, a proportion expected to increase by 17% in 2032 [7]. About two-thirds of all farmed aquatic animals in the world require feeds, and marine fish and salmonids themselves have a high dietary requirement for EPA and DHA

because of their limited ability to biosynthesize them [8]. These dietary requirements were traditionally met using fish oil and fish meal in manufacturing diets, but surging demand for fishmeal and fish oil driven by the fast-growing aquaculture industry has led to increasing prices and pressure to find additional sources for aquafeeds. This in turn is leading to concern regarding sustainability when derived from whole wild fish [7]. Issues around the supply, cost, and sustainability of fish meal and fish oil for aquafeeds have necessitated research on alternative sources for their possible replacement. An increasingly important consideration is the carbon footprint of such replacements.

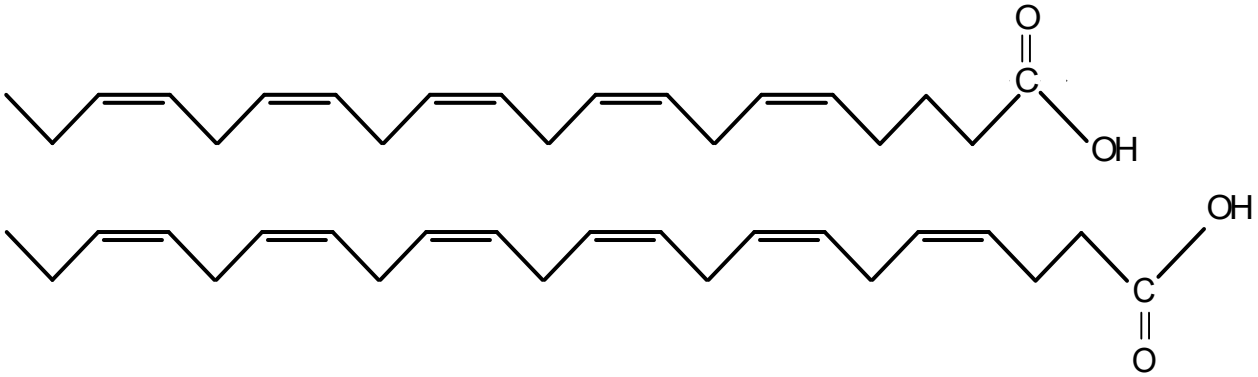

Figure 1. Structures of long-chain ω3 polyunsaturated fatty acids EPA and DHA. Fatty acid notation gives the ratio of carbon atoms to double bonds: 20:5 (**top panel**); 22:6 (**bottom panel**). Eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) are related biochemically because of the location of the first double bond 3 carbons from the methyl end or ω end of the chain.

2. Sources of Omega-3 Long-Chain Polyunsaturated Fatty Acids

Cretton et al. [9] list industrial sources of EPA and DHA arising from oily fish, lean fish, marine by-products, shellfish, heterotrophic dinoflagellates, microalgae, thraustochytrids, and krill. Of the 34 industrial sources, the highest individual amounts occur in the microalgae, which contained up to 4000 mg per 100 g dry mass of EPA and 2700 mg per 100 g dry mass of DHA. Thraustochytrids have even higher amounts: 18,000 mg per 100 g dry mass of DHA [10]. Such remarkably high amounts are achievable from the high DHA proportion among the fatty acids and the very high oil contents of thraustochytrids. Armenta and Valentine [11] list the oil contents (% dry mass) of 13 phototrophic and heterotrophic microorganisms, and the highest range was found in the thraustochytrids. Their oil content was in the range of 50–77% of dry weight, with DHA accounting for greater than 30% of the fatty acids [11]. In the T18 strain of *Schizochytrium* sp., it accounts for greater than 40% [12,13].

Vijayaram et al. [14] list 14 species of algae, both microalgae and macroalgae, and thraustochytrids that have been used to show beneficial effects on aquatic animals in 21 studies. In addition, the cultivation of algae and thraustochytrids can contribute to the treatment of wastewater and waste gas [15–17]. When algae and thraustochytrids were added to aquafeeds, they were shown to increase growth performance and feed conversion efficiency, to enhance the immune system and disease resistance against pathogens, and to improve pigmentation [14].

2.1. Macroalgae and Seagrasses

Macroalgae and seagrasses are often grouped together because they are marine photosynthetic organisms which provide many similar ecosystem services, but seagrasses are vascular plants, while macroalgae are not [18]. However, they are both marine macrophytes and have been studied together as a source of natural products with biological activities [19].

Macroalgae are a source of polysaccharides, peptides, phenolic compounds, and a wide variety of lipids, albeit in small amounts for the latter [20]. They provide polysaccharides

with activity against toxic bacteria found in seawater and estuaries [21] and were found to contain up to 6.2% dry mass lipid in Brazilian red algae [22]. In edible Chilean seaweeds, the major ω3 fatty acid was 18:3ω3 at up to 25% of the fatty acids [23], while the major LC-PUFA was 20:4ω6 at up to 27% of fatty acids in Brazilian red algae [22], and 20:5ω3 (EPA) reached 22% in Brazilian red algae [22].

Maciel et al. [19] reviewed the use of lipidomics in macrophyte research. In general, research into lipid metabolism is moving toward a lipidomic approach, in which comprehensive lipid profiling is combined with sophisticated statistical analysis of the complex data generated (e.g., [24,25]). Organic extracts of seagrasses using different solvents have revealed antimicrobial and anti-inflammatory activities, while glycolipids from green, red, and brown macroalgae had antimicrobial, antitumor, antifungal, and anti-inflammatory activities [19]. Macroalgae have been used as feed supplements for aquatic animals for immune enhancement and improved resistance against viral pathogens [14].

2.2. Microalgae

Microalgae are photosynthetic microorganisms that can be cultivated in freshwater, seawater, or even wastewater to contain 3–75% lipid, 3–80% protein, 1–58% carbohydrate, and 7–34% EPA depending on the species and growth conditions [15]. Microalgae can achieve exceptional bioremediation efficiencies, assimilating nutrients from wastewater [14], all the while converting CO_2 into nutrient-dense food. The administration of microalgae is important in bivalve, fish, and shrimp development, particularly in the early stages [14,26]. They can be added directly to the rearing water or mixed into the feed.

In scallop feeding studies with paired microalgal diets, the combination of the flagellate *Pavlova* sp. (Pav459) and the diatom *Chaetoceros muelleri* provided the best growth rate for larvae, postlarvae, and juveniles [26–28]. The results indicated that DHA and long-chain ω6 PUFAs were essential for optimizing the growth of early life stages of sea scallops and bay scallops. Desaturases from *Pavlova* sp. have also been investigated for the genetic engineering of oilseed crops to produce fish oil levels of ω3 LC-PUFAs [29].

For finfish, microalgae can be used to initiate biofloc systems to maintain water quality [30] and also to be a source of ω3 fatty acids [31]. Biofloc systems are being increasingly used in tilapia culture, and microalgae of the genus *Chlorella* were used by Toyes-Vargas et al. [31] to initiate the biofloc in the recirculation system. Stable isotope analyses revealed that ALA (alpha-linolenic acid; 18:3ω3) from biofloc was involved in the synthesis of ω3 LC-PUFAs in muscle from juvenile tilapia. The flagellate *Pavlova* has also been used for finfish feeding, but indirectly. Atlantic cod larvae (*Gadus morhua*), which were fed rotifers that had *Pavlova* sp. as their main enrichment ingredient, grew better and had a better survival [32]. Similarly, haddock larvae (*Melanogrammus aeglefinus*) reared in the same manner also had a better survival [33].

In order to grow large quantities of photosynthetic microorganisms, there is a wide variety of sophisticated closed photobioreactor (PBR) designs that have been tested, but scaling up is a challenge due to the large capital investments required and large operating costs [34]. Thus, traditional open pond systems remain the most common procedure for the mass culture of autotrophic microorganisms on a commercial basis [35]. Although there are minimal capital and operating costs, and lower energy requirements for culture mixing, there is a long list of substantial intrinsic disadvantages to this approach [36]. Given the challenges with mass photosynthetic production, there has also been considerable interest in heterotrophic production by microorganisms. Some microalgae are able to grow by mixotrophy or even heterotrophy, and the green alga *Chlorella* is an example of one that can grow by autotrophy, mixotrophy, and heterotrophy [36]; however, when grown heterotrophically, it yields three times the biomass than when grown autotrophically. This together with the elimination of the light requirement and ease of control for monoculture has led to considerable interest in the heterotrophic production of single-cell oils [36,37].

2.3. Thraustochytrids and Heterotrophic Dinoflagellates

Thraustochytrids are also microorganisms, but they are incapable of photosynthesis, and so they are not algae [38]. They are often marketed as 'algae' commercially and even called such in the scientific literature, but Leyland et al. [38] are emphatic: "there remains no phylogenetic, biological, or ecological justification for the term 'algae' to be applied to Thraustochytrids or their products". They are eukaryotic microheterotrophs from the kingdom Protista, which means that in addition to not being algae, they are not fungi, plants, or animals. Thraustochytrids belong to the phylum Labyrinthulomycetes, which comprises fungus-like organisms that are mostly unicellular and marine. They are significant decomposers of organic matter through the use of extracellular enzymes and can grow on various industrial and agricultural wastes [39]. They are available commercially from DSM, whose product is DHAgold™, which can be used for human consumption as well as animal feeds, and from Alltech, whose product is All-Grich, targeted for animal feeds [40]. DSM and Evonik established Veramaris® oil, which has been approved for use in aquafeeds. Companies in Atlantic Canada are also targeting the alternative seafood market (Smallfood) and the infant formula market (Mara Renewables Corporation).

Under non-optimized conditions, thraustochytrids can produce total lipid at about 10–50% of biomass, of which 30–70% is DHA [40]. Carbon and nitrogen sources, dissolved oxygen, pH, salinity, temperature, and strain, can be optimized to maximize yields [11,16]. DHA production typically involves a two-stage fermentation with the first nutrient-sufficient stage maximizing biomass production and the second, usually nitrogen-starved but with an excess of the carbon source, causing lipid accumulation [16]. Metabolic engineering has also been used to increase overall fatty acid production, the proportion of PUFAs, and, specifically, the production of DHA [39]. Thraustochytrid-based aquafeeds have been shown to improve growth and immunity in farmed fish [39].

All dinoflagellates arose from photosynthetic ancestors, and about half remain that way, although many of these rely on mixotrophy and use a combination of photosynthesis and heterotrophic nutrition. The other half have lost the ability to photosynthesize and rely on both osmotrophy and phagotrophy [41]. *Crypthecodinium cohnii* can grow in organism-free organic media and forms the basis of the commercial product DHASCO, which improved growth and survival in yellowtail flounder (*Limanda ferruginea*) larvae [42].

3. DHA Versus EPA

Studies on the effects of EPA and DHA intake have mainly considered EPA and DHA together in medical research (e.g., [6,43,44]) and aquaculture research (e.g., [45–47]) because most available sources of EPA and DHA contain significant amounts of both [9]. What is remarkable in the listing of industrial sources of EPA and DHA by Cretton et al. [9] is that less than 20% of the sources contain only EPA or DHA. Oily fish, lean fish, marine by-products, shellfish, and krill all contain significant amounts of EPA and DHA, as do capsules destined for human consumption [48]. By comparison, only one of the species of microalgae, *Phaedactylum tricornutum,* had significant amounts of both EPA and DHA, which depends on the culture conditions [49]. Meanwhile, the remaining six species of industrially available microalgae, thraustochytrids and *Crypthecodinium cohnii*, possessed one or the other essential fatty acid [9].

EPA and DHA have different effects on cell function, which relates to how membrane physico-chemical properties, intracellular signaling pathways, and gene expression are differently affected by these two fatty acids [50]. EPA and DHA affect distinct regions of the membrane lipid bilayer due to differences in the length (C_{20} or C_{22}) and the number of double bonds in their hydrocarbon chains (5 or 6). EPA (20:5ω3) has a more stable and extended structure than DHA (22:6ω3), which contributes to membrane stability as well as the inhibition of lipid oxidation and excessive cholesterol accumulation in membranes [51]. The longer hydrocarbon chain length in DHA leads to increased membrane fluidity.

A well-known effect of the increased intake of C_{20} EPA is that it provides an alternative eicosanoid substrate to C_{20} ARA (arachidonic acid; 20:4ω6). Eicosanoids are highly

bioactive, with roles in inflammation and immune regulation and in various systems [52]. Increased incorporation of EPA in membranes is at the expense of ARA, and EPA results in the production of less potent eicosanoids. While EPA provides an alternative 20-carbon substrate to ARA for oxidation, the oxidation of C_{22} DHA, on the other hand, regulates the resolution of acute inflammation through specialized mediators [53–55].

Fish oils commonly used in aquafeeds contain more EPA than DHA, with ratios that can extend a little beyond two [8]. By using thraustochytrids and heterotrophic dinoflagellates, this ratio can be greatly reversed to investigate the importance of DHA. For example, a dinoflagellate-derived diet with a DHA-to-EPA ratio of eight significantly improved standard length, body depth, and dry weight in yellowtail flounder (*Limanda ferruginea*) larvae [42].

Survival and growth of seabass juveniles fed a diet containing 15% of the thraustochytrid *Aurantiochytrium mangrovei* were the same as in the fish-based control [56]. The *A. mangrovei* was cultured at the pilot scale in two 1000 L cylinders using a manure-based medium, demonstrating environmental bioremediation potential. This diet provided twice the amount of DHA and half the amount of EPA compared to the control. DHA increased significantly in the liver with the *A. mangrovei* diet while EPA remained unchanged; however, in the muscle, there was a significant decrease in the proportion of EPA. The control diet, which contained fish oil and fish meal, had twice the proportion of EPA than there was in the diet containing 15% *A. mangrovei* biomass (3.3 vs. 1.7%), but in the muscle the proportion of EPA was only a third more. As a result, EPA was the same or slightly higher in control tissues, but there was considerably more DHA than EPA in either tissue with *A. mangrovei* feeding.

Sablefish juveniles fed a diet containing 12% *Schizochytrium* sp. meal grew as well as those fed fish meal and fish oil [57]. The *Schizochytrium* diet had 2.5 times the proportion of DHA, while the fish oil and fish meal diet had 7.5 times the proportion of EPA. The fillet fatty acid composition reflected these dietary differences in proportions, although much more closely in the case of fillet DHA proportions. The proportion of DHA was a little over twice the amount with the *Schizochytrium* diet (11.5%) than with the fish oil and fish meal diet (5.4%), while the proportion of EPA with the fish oil and fish meal diet was only 2.5 times more than with the *Schizochytrium* diet. As a result, EPA was just slightly higher than DHA with fish oil and fish meal feeding (6.3 vs. 5.4%), but there was nearly 5-fold the proportion of DHA in the fillet with *Schizochytrium* feeding [57].

The tendency for DHA to be several-fold higher than EPA with thraustochytrid feeding while the levels are similar in the controls extends to tilapia juveniles [58]. In this study, though, the weight gain and protein efficiency ratio were significantly higher with *Schizochytrium* feeding compared to a control diet containing fish oil. It also required significantly less feed to attain a significantly higher final weight, so that the feed conversion ratio was significantly improved with *Schizochytrium* feeding [58].

The clear increase in DHA proportions in cultured finfish fed thraustochytrids reflects the high level in the diets and the high digestibility [12,59,60], but the question remains whether the DHA is incorporated into storage triacylglycerols or membrane phospholipids. This distinction is important not only from the perspective of the animal in terms of membrane structure and function but also for the consumer. ω3 LC-PUFAs bound to phospholipids are more effectively delivered and provide protection against arthritis [61], cardiovascular disease [62,63], and Alzheimer's disease [64].

By isolating the phospholipid fraction by column chromatography prior to derivatization, it is possible to determine the phospholipid fatty acid composition, which can then be compared to the total fatty acid composition obtained without prior fractionation [13]. In an experiment involving different proportions of fish oil and *Schizochytrium* oil fed to Atlantic Salmon parr, there was a continuous increase in the DHA/EPA ratio from the diet to total lipid fatty acids in the liver or muscle and then to phospholipid fatty acids in the liver or muscle [13]. This was true for all diets fed; however, tissues from animals fed the highest amount of *Schizochytrium* had significantly higher ratios than tissues from any of the other

dietary treatments. The DHA/EPA ratio in diets containing fish oil was a low ratio of 0.6, while in tissue phospholipids after feeding fish oil, it ranged from 4 to 5. The DHA/EPA ratio in diets containing microbial lipids ranged from 12 to 19, while in tissue phospholipids after feeding microbial oil, it ranged from 21 to a very high ratio of 32. This continuous increase in the DHA/EPA ratio, going from diet to phospholipid, combined with the dependence of the magnitude of the ratio on the amount of DHA fed, suggests a preference for DHA in phospholipid membranes in fish. In fact, with *Schizochytrium* feeding, over 90% of the phosphatidylcholine molecular species contained a DHA molecule [65]. However, the threshold levels of FPA still need to be maintained for fish health and welfare [66].

4. Conclusions

Industrially available microalgae, e.g., *Nannochloropsis*, thraustochytrids, e.g., *Schizochytrium*, and the heterotrophic dinoflagellate *Crypthecodinium cohnii* possess large amounts of EPA or DHA. Nitrogen supply to cultures is used to manipulate growth and fatty acid synthesis.

While cost is still an impediment to replacing fish products in aquafeeds with those derived from thraustochytrids and algae, it is becoming much less so. The use of waste streams will further reduce costs and enhance sustainability, and the possibility of carefully calibrating ingredient levels of DHA and EPA is an added attraction of these photosynthesis- and non-photosynthesis-derived products. Membrane physico-chemical properties, intracellular signaling pathways, and gene expression are differently affected by these two fatty acids. EPA and DHA are also precursors of different bioactive compounds. Fish feeding studies suggest a preference for DHA over EPA, especially in membranes. Determining the phospholipid composition of aquaculture products is important because ω3 LC-PUFAs bound to phospholipids are more effectively delivered and provide protection against several diseases.

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Effects of Replacing Fishmeal with Algal Biomass (*Pavlova* sp. 459) on Membrane Lipid Composition of Atlantic Salmon (*Salmo salar*) Parr Muscle and Liver Tissues

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Abstract: A 12-week feeding trial examined the dietary impact of replacing fishmeal (FM) with algal biomass (AB) derived from *Pavlova* sp. strain CCMP459 (*Pav*459) in Atlantic salmon diets. Three distinct diets were formulated: a control diet featuring 20% FM and 7% fish oil (FO), an experimental diet incorporating a 50:50 blend of FM and AB *Pav*459 and reduced FO (10% FM; 4.5% FO; 10% AB), and a second experimental diet with full replacement of FM with AB *Pav*459 and further reduction in FO (1.75% FO; 20% AB). Replacing FM with AB *Pav*459 showed no significant effects on the growth performance of Atlantic salmon. Fish across all diets exhibited growth exceeding 200% from their initial weight. Analysis of total lipid content after the 12-week trial revealed no significant differences among the diets. However, individual proportions of omega-3 (ω3) and omega-6 (ω6) fatty acids varied. Fatty acid profiling in muscle and liver tissues showed distinct compositions reflective of dietary treatments. Linoleic acid (LA) and α-linolenic acid (ALA) exhibited higher proportions in total fatty acids than in membrane lipids. Docosahexaenoic acid (DHA) emerged as the predominant fatty acid in the membranes of both liver and muscle tissues. Furthermore, an analysis of sterol composition in *Pavlova* and salmon muscle tissue showed the presence of important sterols, including conventionally animal-associated cholesterol. This emphasizes the suitability of microorganisms, such as *Pav*459, for synthesizing diverse nutrients. Stable isotope analysis demonstrated direct incorporation of eicosapentaenoic acid (EPA) and DHA from diets into salmon tissues. Notably, minimal biosynthesis from the precursor ALA was observed, reaffirming the utility of *Pav*459-derived fatty acids. The EPA+DHA proportions in the fillet consistently met daily human consumption requirements across all dietary conditions, supporting the use of *Pav*459 algal biomass as an alternative to FM.

Keywords: Atlantic salmon; fishmeal; algal biomass; *Pavlova* sp. 459; EPA; DHA; ARA; phospholipid fatty acids; sterols; stable isotopes

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1. Introduction

The consumption of fish is crucial for obtaining omega-3 (ω3) long-chain polyunsaturated fatty acids (LC-PUFA) in human diets. As aquaculture now supplies almost half of the fish and seafood consumed by humans, it plays a vital role in the global food system, the environment, and human health [1,2]. However, carnivorous fish like Atlantic salmon still rely on some inclusion of fish oil (FO) and fish meal (FM) for essential nutrients necessary for their growth and healthy development. The sustainability issues associated

with conventional marine capture fisheries, variable climatic events, and rising prices of FM and FO significantly impact their production [3]. To meet the increasing demand for aquaculture and overcome the limited resources of FM and FO, there is a pressing need for more reliable and sustainable alternative protein and lipid sources. The search for nutritionally suitable substitutes for FM and FO in aquaculture feeds has been a subject of intense research [4], with studies exploring alternatives such as terrestrial plants [5,6], insect meal [7,8], animal by-products [9,10], microalgae [11,12], single-cell protein [13], bioflocs [14], and other protein and lipid sources.

Currently, the inclusion of FM and FO in aquafeeds is optimized to meet the essential amino and fatty acid requirements for fish growth and flesh quality [7]. However, the increasing substitution of ω3 fatty acid-rich marine ingredients with ω6 fatty acid-rich terrestrial ingredients has led to chronically low ω3/ω6 ratios in modern farmed salmonid feeds [15]. This imbalance negatively affects the health of the fish and diminishes the well-established human health benefits associated with the consumption of fatty fish [16]. In this context, the development of novel, low-trophic feedstocks from microalgae has gained attention as potentially ecologically sustainable sources of essential dietary nutrients for aquaculture feeds, provided they can be produced economically [16]. Marine microalgae, including single-cell microbes, are the primary producers of ω3 LC-PUFA in the aquatic environment, continuously supplying eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) that are concentrated through the trophic food chain, as there is limited capacity for synthesizing these beneficial fatty acids by higher trophic organisms [17].

Pavlova sp. strain CCMP459 (referred to as *Pav*459) is a microalga from the phylum Haptista, class Haptophyta, order Pavlovales, family Pavlovaceae, and genus *Pavlova* [18]. Previous studies showed the macronutrient digestibility of intact-cell *Pavlova* sp. 459 meal is high [19] and that *Pav*459 lipid is rich in polyunsaturated fatty acids (PUFA), with over 60% of fatty acids being PUFA, approximately 50% of which are ω3 fatty acids and only around 10% being ω6 fatty acids. Moreover, LC-PUFA, EPA (3% of the biomass), and DHA (1–2% of the biomass) constitute more than 70% of ω3 PUFA with demonstrated high digestibility (>98%) when fed to Atlantic salmon [16,19].

In addition to their significant fatty acid content, microalgae, particularly *Pav*459, have been recognized for their unique sterol composition. Several 4α-methyl sterols, including novel 3,4-dihydroxy-4α-methylsterols known as pavlovols, have been identified in dinoflagellates and microalgae from the genus *Pavlova*. These pavlovols contain an additional hydroxyl group at C-4, a methyl group at C-4, and no nuclear double bonds, with common representatives like 24-methylpavlovol and 24-ethylpavlovol [20].

This study aimed to investigate the effects of replacing FM with *Pav*459 in the diets of farmed Atlantic salmon, specifically focusing on the membrane lipid composition in the liver and muscle tissues. Our main hypothesis was that the substitution of FM with *Pav*459 would induce changes in the proportions of fatty acids present in the salmon tissues and membrane lipids. To test this hypothesis, we conducted a comprehensive 12-week feeding trial, formulating various diets with distinct levels of FM and *Pav*459 and quantifying the total fatty acid and phospholipid fatty acid (PLFA) compositions of the fish liver and muscle tissues. Additionally, we analyzed the sterol composition in *Pavlova* and salmon muscle tissue and utilized bulk carbon stable isotope analysis of *Pav*459 and compound-specific stable isotope analysis (CSIA) to determine the relative contribution of dietary *Pav*459 to the levels of LC-PUFA (EPA, DHA, and ARA) in fish fed the focal diets (FM and AB). This research seeks to expand the understanding of the dietary substitution of traditional marine ingredients with microalgae-based alternatives in aquaculture feeds, particularly focusing on the effects on membrane lipids in Atlantic salmon muscle and liver tissues, which have remained relatively unexplored in previous studies.

2. Materials and Methods

Diet manufacture and feeding trials were done at Dalhousie University, Truro, Nova Scotia.

2.1. Experimental Diets

Experimental diets were formulated by researchers at the National Research Council of Canada and Dalhousie University to assess the impact of substituting fish meal (FM) with algal biomass (AB) *Pav*459 on the lipid composition of Atlantic salmon. Our study employed three distinct dietary treatments: a control diet (FM) containing 7% fish oil (FO), 20% FM, and devoid of AB; a test diet (FM/AB) featuring reduced FO levels and a 50:50 blend of FM and AB *Pav*459 (4.5% FO; 10% FM; 10% AB); and a second test diet, which completely replaced FM with AB *Pav*459 while further reducing FO (1.75% FO; 0% FM; 20% AB). Dietary lipids were additionally sourced from poultry fat and canola oil. All diets were carefully formulated to be isonitrogenous (48% crude protein), isolipidic (23% crude lipid), and isocaloric (23 MJ/kg gross energy) and meet the nutritional requirements of Atlantic salmon as outlined by the National Research Council (NRC) guidelines (2011) [21]. The experimental diets were produced by extrusion at the Chute Animal Nutrition Lab at Dalhousie University Faculty of Agriculture (Truro, Nova Scotia, Canada). The ingredients were provided by Northeast Nutrition (Truro, Nova Scotia, Canada). Further details on production methods of these experimental diets can be found in Wei et al. (2022) [22]. The AB *Pav*459 used in the study was produced at the National Research Council of Canada's Marine Research Station in Ketch Harbour, NS, Canada. Detailed information on the culture production methods can be found in Tibbetts et al. (2020) [16], Wei et al. (2022) [22], and Tibbetts and Patelakis (2022) [19]. For specific details on diet formulations, please refer to Wei et al. (2022) [22] and Table A1 in Appendix A.

2.2. Experimental Fish and Set-Up

Atlantic salmon post-smolts were received from Dartek (Merigomish, NS, Canada). A total of 153 fish were randomly distributed into nine tanks (200 L) in a flow-through freshwater system at Dalhousie University Agricultural Campus (Bible Hill, NS, Canada). Employing a completely randomized design, each tank served as an independent experimental unit, with three replicate tanks per dietary treatment. Atlantic salmon post-smolts (170.1 ± 23.9 g) (mean $\pm$ SD) were fed commercial feed (3 mm) twice a day for two weeks for acclimation after the transfer. The system maintained a constant water flow rate of 2–3 L/min, providing the fish with freshwater at a temperature of 13 °C and oxygen saturation levels of 100%. Temperature and oxygen levels were measured and recorded daily. Fish were hand-fed the experimental test diets 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 a minimum amount of 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.

2.3. Tissue Sampling

To ensure accurate weighing, feed was withheld from the fish for one day prior to the designated sampling day. At week 0 (before the initiation of experimental diets) and at the end of the 12-week trial, five randomly chosen fish per tank were sampled. The ethical treatment of the fish strictly followed the guidelines set forth by the Canadian Council of Animal Care under the approved protocol #2017-84 by the Dalhousie University Faculty of Agriculture Institutional Animal Care Committee. Individual fish were rapidly netted and euthanized with an overdose of anesthetic using tricaine methane sulfonate (TMS222, administered at 150 mg/L) (Sigma Chemicals, St. Louis, MO, USA) buffered using sodium bicarbonate (150 mg/L) (Sigma Chemicals, St. Louis, MO, USA), and clinical signs of death were ensured prior to sampling. The skin was removed from the left side, and subsamples

of white dorsal muscle were collected 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. The sampled tissues were then placed in lipid-clean glass vials with chloroform. The air space was filled with nitrogen before capping the vials and sealing them with Teflon tape. The samples were then stored in a −20 °C freezer until extraction.

2.4. Ethical Approval

The ethical treatment of fish in this study strictly followed the regulations set forth by the Canadian Council of Animal Care. The research was conducted at the Dalhousie University Faculty of Agriculture, with approved Institutional Animal Care Committee Protocol number #2017-84, prioritizing the welfare and ethical treatment of the fish.

2.5. Lipid Extraction

Lipid samples were extracted following the method described by Parrish (1999) [23]. Samples were homogenized in a 2:1 mixture of ice-cold chloroform:methanol using a Tissue Master 125 homogenizer (Omni International, Kennesaw, GA, USA). To achieve a chloroform:methanol:water ratio of 8:4:3, chloroform-extracted water was added. The sample was then 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 (EMD Millipore Corporation, Burlington, MA, USA) was then added back to the extraction test tube, and the entire procedure was repeated three more times. All organic layers were pooled into a lipid-clean vial.

2.6. 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 gas to dryness. Then 1.5 mL of methylene chloride (EMD Millipore Corporation, Burlington, MA, USA) and 3 mL Hilditch reagent were added. The Hilditch reagent was prepared by dissolving 1.5 mL of concentrated sulfuric acid (VWR International, Mississauga, ON, Canada) in 100 mL of methanol (EMD Millipore Corporation, Burlington, MA, USA) that had been dried over anhydrous sodium sulfate (Fisher Scientific Company, Ottawa, ON, Canada). The vial was capped under a nitrogen atmosphere, 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 (Fisher Scientific Company, Ottawa, ON, Canada) was added, followed by 1.5 mL hexane (EMD Millipore Corporation, Burlington, MA, USA). The mixture was shaken, and the upper, organic layer was transferred to a lipid-clean 2 mL vial. The upper, organic layer was then evaporated under a constant stream of nitrogen gas until dry, and the residue was reconstituted with approximately 0.5 mL of hexane. The vial was capped under a nitrogen atmosphere, sealed with Teflon tape, and sonicated for an additional 4 min to ensure proper resuspension of the fatty acids.

2.7. Neutral Lipid/Polar Lipid Separation

The separation of neutral lipids and polar lipids was performed using Strata SI-1 silica tubes (Phenomenex, Torrance, CA, USA) in a vacuum chamber. The silica tube was initially rinsed with 6 mL of methanol, 6 mL of chloroform, and 3 mL of a solvent mixture containing 98:1:0.5 chloroform:methanol:formic acid (Fisher Scientific Company, Ottawa, ON, Canada) through the column into a waste vial. Then the sample extract was applied directly to the silica using a long pipette, and the sample vial was rinsed with a small amount of chloroform. The waste vial was replaced with a lipid-clean 15 mL vial, and 8 mL of the solvent mixture (98:1:0.5 chloroform:methanol:formic acid) was eluted through the

column to collect all the neutral lipid-containing eluent. To recover the acetone-mobile polar lipid (AMPL), a second 15 mL vial was inserted, and the silica gel was rinsed with 6 mL (2 × 3 mL) of acetone (EMD Millipore Corporation, Burlington, MA, USA). The vial containing the AMPL fraction was then replaced with a larger 40 mL vial, and 3 mL of chloroform was passed through the column to remove any remaining acetone. For the elution of phospholipids (PL), two volumes (6 mL) of methanol were used, 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 completely dried in a flash evaporator. The dried lipids were washed into a 15 mL vial using methanol and chloroform. The phospholipid fatty acid (PLFA) was derivatized using the same procedure as the total fatty acid methyl esters (FAME) described in Section 2.6.

2.8. Sterol Derivatization and Analysis

Sterols were derivatized by silylation with N, O-bis-trimethylsilyl trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (Supelco Inc., Bellefonte, PA, USA) to form their corresponding trimethylsilyl (TMS)-ethers [24]. Lipid extracts were evaporated until dry under a stream of nitrogen gas. BSTFA (100 µL) containing 1% TMCS was added to the lipid extract, which was heated at 85 °C for 15 min. Samples were then cooled to room temperature, and excess reagent was evaporated under nitrogen. Hexane/dichloromethane (500 µL, 1:1, by vol) (Sigma Chemicals, St. Louis, MO, USA) was added before analysis by gas chromatography-mass spectrometry (GC-MS).

2.9. 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 were employed, and a three-step development method was followed, as described by Parrish (1999) [23].

Each lipid extract was spotted on an individual rod using a 20 µL Hamilton syringe, and a narrow band was obtained by focusing the sample with a 100% acetone solution. The first development system consisted of a hexane/ethyl ether/formic acid mixture (99:1:0.05). The rods were developed for 25 min, followed by drying in a constant humidity chamber for 5 min, before being developed again in the same solution for 20 min. After the first development, the rods were scanned in the Iatroscan (covering 75% of the rod), which detects the hydrocarbon (HC), steryl ester (SE), and ketone (KET) lipid classes.

Upon completion of the first scan, the rods were dried in a constant humidity chamber for 5 min before proceeding with the second development for 40 min. The second development system comprised a hexane:ethyl ether:formic acid mixture (79:20:1). Following the second development, the rods were scanned in the Iatroscan (covering 89% of the rod) to identify the triacylglycerol (TAG), free fatty acids (FFA), alcohol (ALC), and sterol (ST) lipid classes.

For the third and final separation, the rods were subjected to two rounds of development in 100% acetone for 15 min, dried for 5 min in a constant humidity chamber, and then developed twice for 10 min in a mixture of chloroform:methanol:chloroform-extracted water (50:40:10). After the third development, the rods were scanned in the Iatroscan (covering 100% of the rod) to determine the AMPL and PL lipid classes. The data were collected using Peak Simple software (ver. 3.67, SRI Inc., Torrance, CA, USA), and the Chromarods were calibrated using standards obtained from Sigma Chemicals (Sigma Chemicals, St. Louis, MO, USA).

The fatty acid methyl esters (FAME) samples were analyzed using an HP 6890 gas chromatography (GC)-FID equipped with a 7683 autosampler. A ZB-WAXplus column (Phenomenex) with a length of 30 m and an internal diameter of 0.32 mm was employed. The column temperature was initially set at 65 °C and held for 0.5 min. It was then ramped to 195 °C at a rate of 40 °C/min, held for 15 min, and further ramped to a final temperature of 220 °C at a rate of 2 °C/min. The carrier gas used was hydrogen at a flow rate of

2 mL/min. The injector temperature started at 150 °C and ramped to a final temperature of 250 °C at a rate of 120 °C/min. The detector temperature remained constant at 260 °C. Retention times from standards purchased from Supelco (Supelco Inc., Bellefonte, PA, USA) were used for peak identification. Chromatograms were integrated using Agilent OpenLAB Data Analysis—Build 2.203.0.573 (Agilent Technologies, Inc., Santa Clara, CA, USA). The GC column was periodically checked using a quantitative standard purchased from Nu-Chek Prep, Inc. (product number GLC490) to ensure expected areas were obtained approximately every 300 samples or once a month.

2.10. Compound-Specific Stable Isotope Analysis (CSIA)

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. The analysis included FAME samples from two focal 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). All $\delta^{13}C$ values were calculated relative to the Vienna Pee Dee Belemnite (VPDB) standard using the following equation:

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

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) to correct for the additional methyl group added to fatty acids during transesterification. The correction for the methyl group was applied to all fatty acids using the equation:

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

where n is the number of carbon 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 carbon 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 were 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 GC column, and a thermal conductivity meter (TCD). Additional details on instrumentation and bulk stable isotope results are included in Appendix C.

2.11. Statistical Analysis

The resulting data are reported as mean ± standard deviation. All statistical analyses were conducted using general linear models in Minitab (version 18; Minitab Inc., State College, PA, USA). The model was designed to assess the effects of diet (fixed factor) and nested tank (fixed factor within diet) on different lipid classes and fatty acids (response variables). 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 α = 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 coordinate analysis (PCO) was employed to describe the resemblance and variation in fatty acid composition in muscle and liver tissue. A correlation matrix was plotted on two PCO axes (PCO1 and PCO2) using PRIMER (Plymouth Routines in Multivariate Ecological Research; PRIMER-E Ltd., version 6.1.15, Ivybridge, UK). The similarity of percentages analysis (SIMPER) was utilized to quantify differences among treatments in the fatty acid data. In all cases, the non-parametric Bray-Curtis similarity index was employed.

3. Results

3.1. Pavlova sp. 459 Oil and Experimental Diets Composition

The lipid analysis of *Pav*459 resulted in a total lipid content of 109.6 mg/g *w/w* (Table 1). The predominant lipid class was TAG, comprising 24.7% of the total lipids, followed by AMPL at 22.9%, FFA at 18.5%, PL at 16.6%, and ST at 10.9%. The fatty acid composition of *Pav*459 was primarily composed of PUFA at 64.5%, with saturated fatty acids (SFA) accounting for 23.0% and monounsaturated fatty acids (MUFA) at 11.2% (Table 1). Notably, EPA was the most abundant PUFA at 26.8%, followed by DHA at 13.6%, with arachidonic acid (ARA, 20:4ω6) present in trace amounts at 0.5%. The precursor fatty acids, linoleic acid (LA) and alpha-linolenic acid (ALA), were approximately equal at 3.5% and 3.6%, respectively. The total omega-3 (ω3) fatty acids were four times more prevalent than ω6 fatty acids, resulting in a ω3/ω6 ratio of 4.3.

Table 1. Lipid composition and fatty acid profile of *Pav*459, used in the study [1].

Lipid Class Composition (%)	
Total lipid (mg/g)	109.6 ± 10.4
TAG	24.7 ± 0.4
FFA	18.5 ± 1.1
ST	10.9 ± 1.0
AMPL	22.9 ± 1.7
PL	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

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

An analysis of lipid extracted from *Pav*459 resulted in a total of 11 sterols identified by mass spectrometry and shown in order of retention time in Table 2. The major sterols identified were cholesterol, campesterol, stigmasterol, stigmasta-22-en-3-β-ol, 23,24-dimethylcholest-5-en-3β-ol, 4α-methylporiferast-22-enol, and 24-ethylpavlovol. The 11 GC-MS-determined sterols from the total lipid extract summed to 9.8%, which compares well with the Iatroscan-determined free sterol content.

Table 2. Sterol composition of *Pavlova* sp. 459 lipid extract.

Sterol	Common Name	Formula	MW + TMS	Sterol Content (μg mg^{-1}) of Lipids
Cholest-5-en-3β-ol	Cholesterol	27:1Δ^5	458	8.7
5α-cholestan-3β-ol	Cholestanol	27:0, 5αΔ^0	460	0.5
24-methylcholesta-5,22E-dien-3β-ol	Brassicasterol	28:2, $\Delta^{5,22}$Me^{24}	470	0.7
24-methylcholest-5-en-3β-ol	Campesterol	28:1Δ^5Me^{24}	472	3.8
24-ethylcholesta-5,22E-dien-3β-ol	Stigmasterol	29:2, $\Delta^{5,22}$Et^{24}	484	41.7
24-ethyl-5α-cholest-22E-en-3β-ol *	Stigmasta-22-en-3-β-ol	29:1, 5αΔ^{22}Et^{24}	486	21.6
23,24-dimethylcholest-5-en-3β-ol		29:1Δ^5 Me^{23,24}	486	6.4
4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol	4α-Methylporiferast-22-enol	30:1, 5αΔ^{22}Me^{4}Et^{24}	500	12.3
24-ethyl-5α-cholest-22E-en-3β,4β-diol	4-Desmethyl-22-dehydropavlovol	29:0, 5αΔ^{22}Et^{24}OH	502	0.2
5α-cholestan-3β,4β-diol	24-Methylpavlovol	30:0, 5αΔ°Me^{4,24}OH	504	0.5
4α-methyl-24-ethyl-5α-cholestan-3β,4β-diol	24-Ethylpavlovol	30:0, 5αΔ°Me^{4}Et^{24}OH	518	2.0
Total				9.83%

* or 23, 24-dimethyl-5α-cholesta-22E-en-3β-ol (29:1, 5αΔ^{22}Me^{23,24}).

Specific to the *Pavlova* genus, 24-methylpavlovol and 24-ethylpavlovol, recognized as pavlovols, are 3,4-dihydroxy-4α-methyl sterols with unique features, including an additional hydroxyl group at C-4, a methyl group at C-4, and no nuclear double bonds [20].

The biochemical composition of *Pav*459 resulted in a well-balanced nutritional profile, with a dry matter content of 95%, a protein content of 60.87%, and a crude lipid content of 12.25%, of which 10.68% is esterifiable lipid (Table A2 in Appendix A). The mineral analysis resulted in significant levels of essential elements, including calcium (0.36%), magnesium (0.4%), phosphorous (1.2%), potassium (1.4%), and sodium (1.2%). These minerals are crucial for supporting various physiological functions in aquatic organisms. The carotenoid analysis revealed key compounds with potential immunological benefits, such as astaxanthin, known for its antioxidant properties. In aquaculture, microalgal astaxanthin can enhance immune responses, boost disease resistance, and improve survival rates in aquatic animals. This is achieved through its antioxidative properties, induction of antioxidant enzymes, and modulation of key immunological biomarkers [25]. Astaxanthin content was 9.09 mg/100 g; canthaxanthin was 40.6 mg/100 g; chlorophyll a and b were 2259.69 mg/100 g and 62.1 mg/100 g; and β-carotene was 65.87 mg/100 g. For full details on biochemical composition, please refer to Wei et al. (2022) [22] and Table A2 in Appendix A.

Total lipid content varied among diets, with FM/AB having the highest at 200.0 mg/g *w/w* and FM the lowest at 144.2 mg/g *w/w* (Table 3). TAG was the major lipid class in the diets, comprising 72.7% to 75.1% of total lipids, followed by FFA at 8.5% to 9.1%, PL at 3.1% to 6.3%, AMPL at 5.0% to 8.0%, and ST at 3.5% to 7.5%. Although the PL proportion in the FM/AB diet was twice as low as in other diets, it was not statistically significant. Significant differences were observed for the ST and AMPL lipid classes. The fatty acid composition was predominantly MUFA at 44.8% to 47.1%, followed by PUFA at 31.0% to 32.3%, and SFA at 20.8% to 22.3%. While the EPA and DHA proportions were lower in the AB diet compared with the FM and FM/AB diets, LA and ALA precursors were higher in the AB diet. Despite lower EPA and DHA proportions in the AB diet (~1%), the combined EPA+DHA exceeded the NRC's (2011) [21] recommended minimum levels (0.5–0.8%). ARA

proportions were similar across diets, and ω6 fatty acids prevailed over ω3 fatty acids, resulting in a ω3/ω6 ratio less than 1 across all treatments.

Table 3. Lipid composition and fatty acid profile of diets [1].

	FM	FM/AB	AB
Total lipid (mg/g)	144.2 ± 23.6	200.0 ± 38.2	174.5 ± 34.8
TAG	74.1 ± 1.8	75.1 ± 1.1	72.7 ± 2.2
FFA	9.0 ± 1.1	9.1 ± 0.3	8.5 ± 0.9
ST	3.5 ± 0.4 ^{b}	7.5 ± 0.7 ^{a}	4.7 ± 1.7 ^{b}
AMPL	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/algal biomass *Pav*459; AB = algal biomass *Pav*459. [2] Saturated fatty acid. [3] Monounsaturated fatty acid. [4] Polyunsaturated fatty acid.

3.2. Growth Performance

After a 12-week feeding period, the experimental diets had no negative effects on fish growth. The average weight gain across the diets was approximately 263%, with the salmon showing substantial growth from their initial weight of approximately 170.1 ± 23.9 g. The mean weights of salmon fed the FM, FM/AB, and AB diets reached 451.0 g, 449.7 g, and 440.0 g, respectively, compared with their initial weight of 170.1 g. This growth translated to weight gains of 279.6 g, 276.3 g, and 271.3 g, respectively, with specific growth rates of 1.2% per day. Further details on growth performance, including additional parameters, have been previously published by Wei et al. (2022) [22], and key results are provided in Appendix A.

3.3. Liver Tissue Lipid Classes and Fatty Acid Composition

The initial total lipid content in liver tissue was 18.3 mg/g *w/w*, primarily comprised of neutral lipids (Table 4). Following a 12-week feeding period, total lipid concentration increased uniformly across all dietary treatments (24.4–28.3 mg/g *w/w*), with neutral lipid remaining the dominant component (62.1–65.1%). The major lipid classes included FFA (25.4–30.2%), PL (23.4–25.4%), ST (20.4–23.9%), and TAG (6.8–12.4%). No significant differences were observed for any lipid classes among the dietary treatments.

Table 4. Lipid class and total fatty acid composition of Atlantic salmon liver tissue, prior to feeding experimental diets (initial) and after 12 weeks of feeding experimental diets [1].

	Initial	**FM**	**FM/AB**	**AB**
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 (%)				
TAG	1.5 ± 1.2	12.4 ± 16.3	10.1 ± 10.6	6.8 ± 7.0
FFA	30.8 ± 2.5	30.2 ± 8.8	25.4 ± 6.3	28.7 ± 5.3
ST	26.9 ± 3.9	20.4 ± 4.1	23.9 ± 3.5	21.9 ± 3.6
PL	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/algal biomass *Pav*459; AB = algal biomass *Pav*459. [2] Phospholipid/sterol ratio. [3] Saturated fatty acid. [4] Monounsaturated fatty acid. [5] Polyunsaturated fatty acid.

After 12 weeks of feeding, the fatty acid profile of liver tissue reflected the diet (Table 4). While the differences in total fatty acid proportions were generally small, they were sta-

tistically significant across the dietary treatments. Most notably, significant differences were observed between salmon fed the AB diet and those fed the FM diet, with occasional distinctions between salmon fed the FM/AB and FM diets. The predominant fatty acid composition was PUFA, ranging from 45.4% to 48.3%, followed by MUFA (32.9% to 37.7%) and SFA (16.6% to 18.4%). Compared with the fatty acid proportions at the start of the experiment, most individual fatty acid proportions decreased, except for 18:1ω9, MUFA, LA, ALA, and total ω6 fatty acids in all dietary treatments. DHA emerged as the dominant PUFA in liver tissue, ranging from 19.6% to 23.7%, followed by LA (7.7% to 9.5%), EPA (3.5% to 5.5%), ARA (2.5% to 3.7%), and low levels of ALA (0.9% to 1.1%). The sum of ω3 fatty acids was approximately twice as prevalent as ω6 fatty acids in the livers of salmon fed the FM and FM/AB diets and 1.3 times more prevalent in the livers of salmon fed the AB diet, resulting in a ω3/ω6 ratio exceeding 1 across all dietary treatments.

PCO of week-12 liver tissue total fatty acid composition showed that PCO1 and PCO2 accounted for 80.9% and 12.7% of the variability, respectively (Figure 1). The PCO biplot revealed the highest variation between salmon fed the FM and AB diets. SIMPER analysis (Table A4 in Appendix B) indicated 82.6% similarity within salmon fed the FM diet, 89.1% within salmon fed the FM/AB diet, and 90.4% within salmon fed the AB diet. The greatest dissimilarities were observed between salmon fed the FM and AB diets (16.8%), followed by FM and FM/AB diets (15.4%), and FM/AB and AB diets (10.7%). Primary contributors to similarities and dissimilarities were 18:1ω9, DHA, 16:0, and LA.

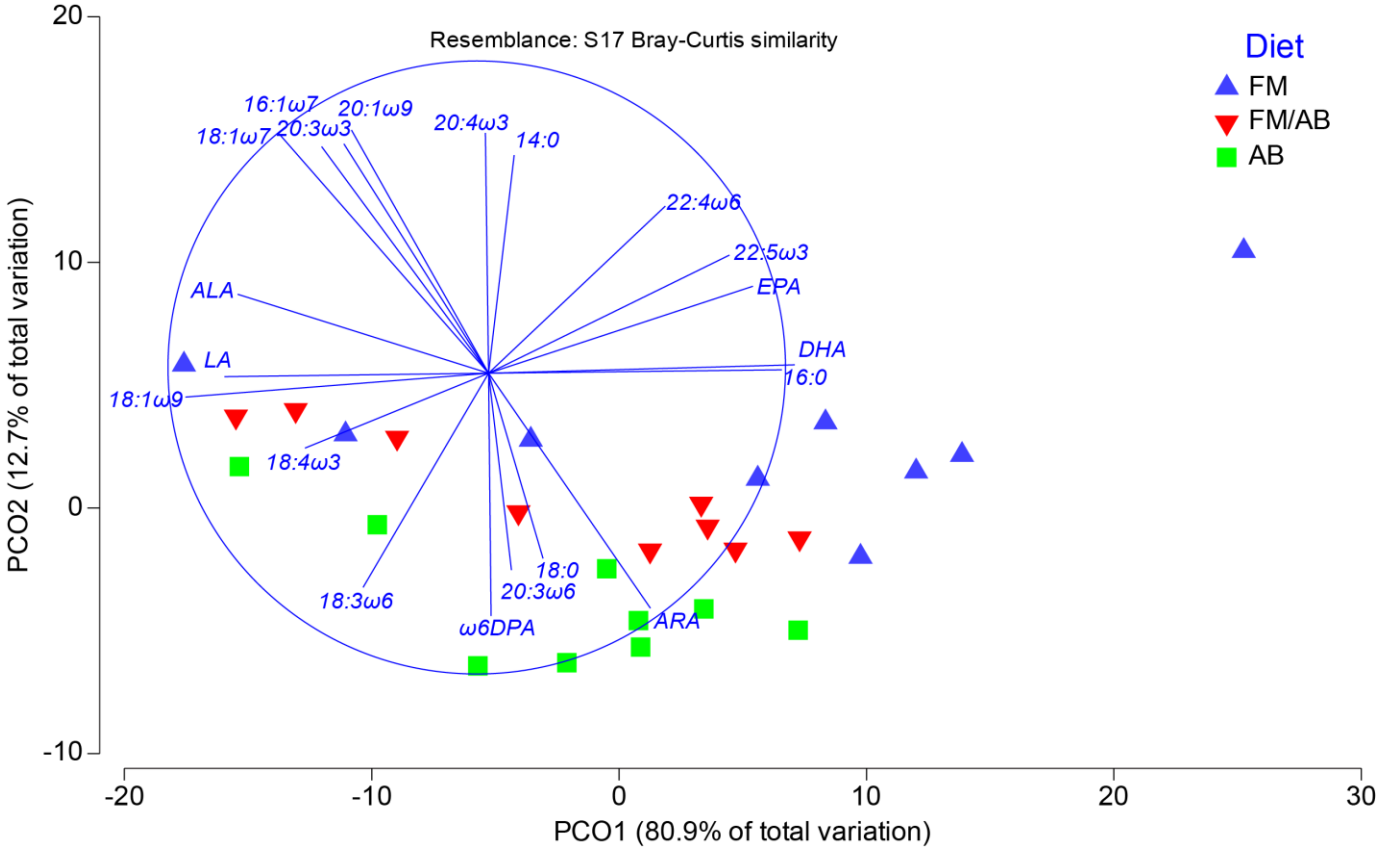

Figure 1. PCO of Atlantic salmon liver tissue total fatty acid composition (%) after 12 weeks of feeding experimental diets.

3.4. Liver Tissue Phospholipid Fatty Acid Composition

Liver tissue PLFA profiles also reflected the diets (Table 5). Although the differences in PLFA proportions among the diets were relatively small, they were statistically significant, particularly between salmon fed the AB diet and salmon fed the FM diet. The liver tissue PLFA profile primarily comprised PUFA (58.5–59.3%), followed by SFA (23.2–24.1%) and

MUFA (17.0–17.4%). DHA emerged as the dominant PUFA in membrane phospholipids, followed by EPA, LA, and ARA. The proportions of EPA and DHA were higher and significantly different in salmon fed the FM and FM/AB diets compared with those fed the AB diet, while the proportion of ARA was higher and significantly different in salmon fed the AB diet compared with those fed the FM and FM/AB diets. Precursors LA and ALA showed higher proportions in storage and lower proportions 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 ω3/ω6 ratio greater than 2 across all dietary treatments. The DHA/EPA ratio was higher and significantly different in salmon fed the AB diet (7.0%) than those fed the FM diet (5.1%). The EPA/ARA ratio was higher and significantly different in salmon fed the FM diet (2.1%) than those fed the AB diet (0.8%). The DHA/ARA ratio was higher and significantly different in salmon fed the FM diet (10.7%) than those fed the AB diet (5.9%).

Table 5. Phospholipid fatty acid composition of Atlantic salmon liver tissue after 12 weeks of feeding experimental diets [1].

	FM	**FM/AB**	**AB**
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/algal biomass *Pav*459; AB = algal biomass *Pav*459. [2] Saturated fatty acid. [3] Monounsaturated fatty acid. [4] Polyunsaturated fatty acid. [5] PUFA/SFA ratio.

PCO of liver PLFA at week 12 revealed that PCO1 and PCO2 accounted for 75.5% and 12.0% of the variability, respectively (Figure 2). The PCO biplot exhibited clear separation between salmon fed different diets, with significant dissimilarity observed between salmon fed the AB and FM diets. Further analysis using SIMPER (Table A5 in Appendix B) showed 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 greatest dissimilarities were observed 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 contributors to the similarities were DHA, 16:0, and 18:1 ω9 across all dietary treatments, while EPA, DHA, 22:5ω6, and ARA were the primary drivers of dissimilarities. The specific order and contribution percentages of these fatty acids to the similarities and dissimilarities varied among the dietary treatments.

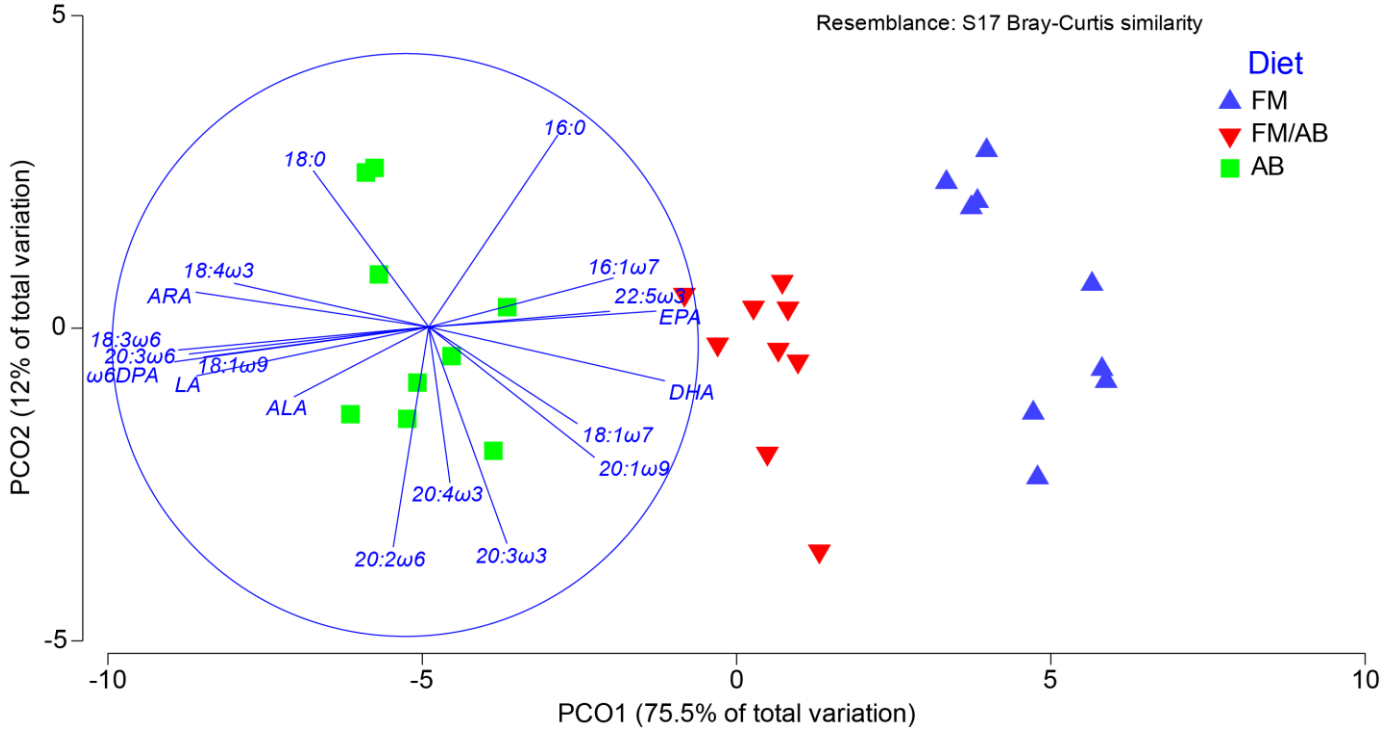

Figure 2. PCO of Atlantic salmon liver tissue phospholipid fatty acid composition (%) after 12 weeks of feeding experimental diets.

3.5. Muscle Tissue Lipid Class and Fatty Acid Composition

The initial total lipid content in muscle tissue at week 0 was 15.8 mg/g *w/w*, primarily consisting of neutral lipids (Table 6). Following 12 weeks of feeding, there was a substantial increase in total lipid concentration in salmon fed the FM diet (70.4 mg/g *w/w*), approximately 4.5 times higher than the initial level. Similarly, salmon fed the FM/AB diet (64.5 mg/g *w/w*) and AB diet (63.4 mg/g *w/w*) exhibited an average 4-fold increase in total lipid concentration (Table 6). However, there were no significant differences in total lipid concentration among the dietary treatments. The composition of muscle tissue lipids was predominantly neutral (61.4–75.5%) across all dietary treatments. Among the lipid classes, TAG was the most abundant at 43.9–54.8%, followed by PL (8.1–20.3%), ST (6.2–11.9%), and FFA (4.1–7.5%). Notably, the proportion of PL in salmon fed the FM/AB diet was significantly lower than the proportion of ST, while the FFA proportion was higher and significantly different compared with salmon fed the FM and AB diets. The ST proportion in salmon fed the AB diet was significantly lower compared with salmon fed the FM and FM/AB diets.

Table 6. Lipid class and total fatty acid composition of Atlantic salmon muscle tissue, prior to feeding experimental diets (initial) and after 12 weeks of feeding experimental diets [1].

	Initial	FM	FM/AB	AB
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}
TAG	56.0 ± 11.6	43.9 ± 10.3	54.8 ± 4.9	49.2 ± 8.6
FFA	5.4 ± 3.4	4.1 ± 1.0 ^{b}	7.5 ± 2.2 ^{a}	4.2 ± 0.7 ^{b}
ST	16.2 ± 7.9	11.0 ± 2.2 ^{a}	11.9 ± 3.8 ^{a}	6.2 ± 4.3 ^{b}
PL	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}
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}
EPA+DHA (mg/g)	2.38 ± 0.96	6.00 ± 2.04	5.37 ± 0.94	4.63 ± 1.08
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/algal biomass *Pav*459; AB = algal biomass *Pav*459. [2] Phospholipid/sterol ratio. [3] Saturated fatty acid. [4] Monounsaturated fatty acid. [5] Polyunsaturated fatty acid.

The fatty acid profile of muscle tissue after the 12-week feeding trial also reflected the diets (Table 6). Although the differences in total fatty acid proportions were relatively small, they were statistically significant across the dietary treatments. Significant differences were observed between salmon fed the AB diet and those fed the FM diet, with occasional significant differences between salmon fed the FM/AB diet and the FM diet. The predominant fatty acid composition in muscle tissue was MUFA (42.1–43.7%), followed by PUFA (35.4–36.4%) and SFA (19.5–21.1%). Similar to liver tissue, most individual fatty acids decreased compared with the initial levels, except for 18:1ω9, MUFA, LA, ALA, and total ω6 fatty acids across all dietary treatments. The dominant PUFA in muscle tissue was the precursor LA (12.8–15.4%), followed by DHA (7.6–9.8%), EPA (2.7–3.9%), and ALA

(2.3–2.7%), while ARA was present in very low proportions (0.6%). The total sum of ω3 fatty acids was approximately equal to or slightly higher than the sum of ω6 fatty acids in salmon fed the FM and FM/AB diets and slightly lower than the sum of ω6 fatty acids in salmon fed the AB diet, resulting in a ω3/ω6 ratio equal to or greater than 1 in salmon fed the FM and FM/AB diets and a ω3/ω6 ratio slightly less than 1 in salmon fed the AB diet.

PCO of muscle total fatty acids at week 12 revealed that PCO1 and PCO2 accounted for 83.8% and 15.5% of the variability, respectively (Figure 3). Unlike liver PLFA, the PCO biplot for muscle total fatty acids did not show clear differentiation between the dietary treatments, although the highest variation was observed between salmon fed the FM and AB diets. SIMPER analysis (Table A6 in Appendix B) demonstrated 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 between 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 of similarities and dissimilarities were 18:1ω9, 16:0, DHA, and LA. The specific order and contribution percentages of these fatty acids to the similarities and dissimilarities varied among the dietary treatments.

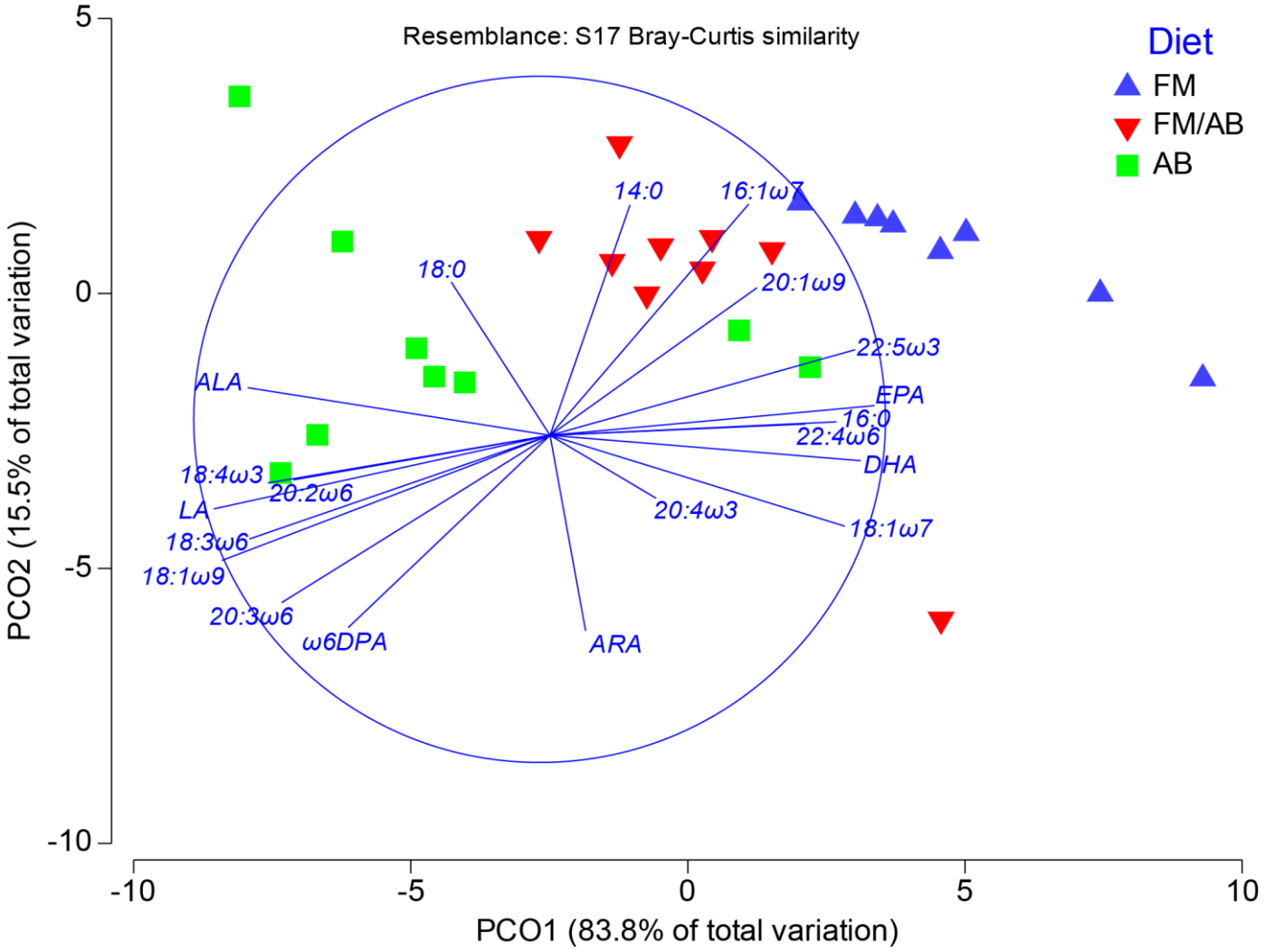

Figure 3. PCO of Atlantic salmon muscle tissue total fatty acid composition (%) after 12 weeks of feeding experimental diets.

3.6. Muscle Tissue Phospholipid Fatty Acid Composition

The muscle tissue PLFA profiles also reflected the dietary treatments (Table 7). Although the differences were minimal, they were statistically significant across the dietary treatments, particularly between salmon fed the FM diet and those fed the AB diet. The predominant composition of muscle tissue PLFA was PUFA (59.2–60.4%), followed by SFA (24.2–25.1%) and MUFA (14.0–15.0%). In contrast to the overall muscle tissue fatty acid composition, the dominant PUFA in the membrane was DHA (33.3–38.2%), followed by EPA (6.7–8.7%), LA (4.1–5.6%), 22:5ω3 (1.7–2.3%), ARA (1.3–1.9%), and ALA (1.1–1.4%). No significant differences were observed in the proportion of DHA among the dietary treatments. However, EPA was significantly higher in salmon fed the FM diet compared with those fed the FM/AB and AB diets, while ARA was significantly higher in salmon fed the AB diet compared with those fed the FM and FM/AB diets. Similarly, the precursors LA and ALA were significantly higher in salmon fed the AB diet compared with those fed the FM and FM/AB diets. The total sum of ω3 fatty acids was approximately 7-fold higher than the sum of ω6 fatty acids in salmon fed the FM diet, approximately 5-fold higher in salmon fed the FM/AB diet, and approximately 4-fold higher in salmon fed the AB diet. As a result, there was a ω3/ω6 ratio greater than 3 across the dietary treatments. Differences in the DHA/EPA ratio, EPA/ARA ratio, and DHA/ARA ratio were mostly minimal but significant across the dietary treatments.

PCO of muscle PLFA at week 12 indicated that PCO1 and PCO2 accounted for 68.7% and 17.7% of the variability, respectively (Figure 4). The muscle PLFA PCO biplot revealed that the main variation was between salmon fed the FM and AB diets. SIMPER analysis (Table A7 in Appendix B) demonstrated 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 similarities in muscle PLFA were DHA, 16:0, and 18:1ω9 across all dietary treatments, while the main drivers for dissimilarities were DHA, 16:0, 18:1ω9, EPA, and 22:5ω6. The specific order and contribution percentages of these fatty acids to similarities and dissimilarities varied among the dietary treatments.

Table 7. Phospholipid fatty acid composition of Atlantic salmon muscle tissue after 16 weeks of feeding experimental diets [1].

	FM	**FM/AB**	**AB**
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

Table 7. *Cont.*

	FM	FM/AB	AB
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/algal biomass *Pav*459; AB = algal biomass *Pav*459. [2] Saturated fatty acid. [3] Monounsaturated fatty acid. [4] Polyunsaturated fatty acid. [5] PUFA/SFA ratio.

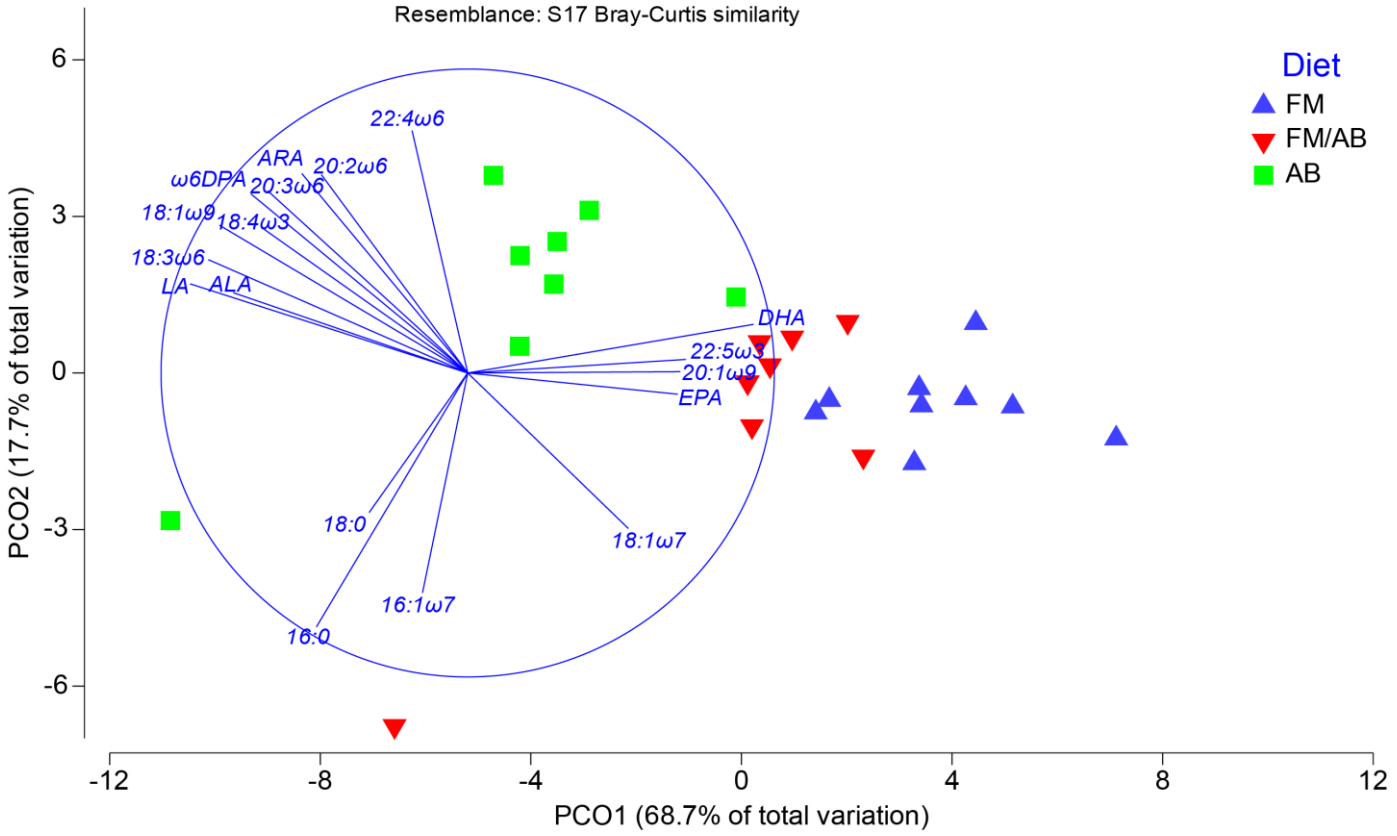

Figure 4. PCO of Atlantic salmon muscle tissue phospholipid fatty acid composition (%) after 12 weeks of feeding experimental diets.

3.7. Muscle Tissue Sterol Composition

The sterols identified in the neutral lipid fraction of Atlantic salmon muscle tissue from a fish fed an AB diet were cholesterol, cholestanol, lathosterol, campesterol, 5α-cholest-8(14)-en-3β-ol, and 23,24-dimethylcholest-5-en-3β-ol (Table 8). Other *Pavlova* sterols were notably absent. The 6 GC-MS-determined sterols from the Atlantic salmon muscle tissue fed an AB diet summed to 4.44%.

Table 8. Sterol composition of the neutral lipid fraction of Atlantic salmon muscle tissue from a fish fed an AB diet.

Sterol	Common Name	Formula	MW	RT (min)	Sterol Content (μg mg^{-1}) of Lipids
Cholest-5-en-3β-ol	Cholesterol	27:1Δ^5	458	47.043	43.9
5α-cholestan-3β-ol *	Cholestanol	27:0, 5αΔ°	460	47.265	tr
5α-cholest-7-en-3β-ol	Lathosterol	27:1Δ^7	458	48.256	0.1
24-methylcholest-5-en-3β-ol *	Campesterol	28:1Δ^5Me^{24}	472	49.125	0.2
5α-cholest-8(14)-en-3β-ol		27:1$\Delta^{8(14)}$	458	49.592	0.2
23,24-dimethylcholest-5-en-3β-ol *		29:1Δ^5 Me^{23,24}	486	50.902	0.1
Total					4.44%

* Also found in *Pavlova lutheri*. Supercripts indicate positions of double bonds when present.

3.8. Compound-Specific Stable Isotope Analysis

The δ^{13}C values for EPA and DHA in the two focal dietary treatments (FM and AB) were significantly different from each other (Table 9). Unfortunately, the δ^{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 fatty acids (TFA) (Table 9) and liver PL (Table 10) 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 TFA (Table 11) and muscle PL (Table 12). 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 TFA between salmon fed the FM diet and salmon fed the AB diet. The δ^{13}C value for ALA was not detectable in the liver tissue. The tissue δ^{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 for *Pav*459.

Table 9. δ^{13}C values (‰) of essential fatty acids for FM and AB diets, liver total fatty acids of fish fed two focal diets (FM and AB), and relative percent contribution (RC) of the AB diet to tissue fatty acids.

Fatty Acids (‰)	FM [1]	AB [1]	*p*-Value	Liver TFA (FM) [1]	Liver TFA (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] Relative contribution of *Pav*459 EPA and DHA.

Table 10. δ^{13}C values (‰) of essential fatty acids for FM and AB diets, liver phospholipids of fish fed two focal diets (FM and AB), and relative percent contribution (RC) of the 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] Relative contribution of *Pav*459 EPA and DHA.

Table 11. δ^{13}C values (‰) of essential fatty acids for FM and AB diets, muscle total fatty acids of fish fed two focal diets (FM and AB), and relative percent contribution (RC) of the AB diet to tissue fatty acids.

Fatty Acids (‰)	FM [1]	AB [1]	*p*-Value	Muscle TFA (FM) [2]	Muscle TFA (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] Relative contribution of *Pav*459 EPA and DHA.

Table 12. δ^{13}C values (‰) of essential fatty acids for FM and AB diets, muscle phospholipids of fish fed two focal diets (FM and AB), and relative percent contribution (RC) of the 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] Relative contribution of *Pav*459 EPA and DHA.

4. Discussion

The study evaluated the effects of replacing FM and reduced FO with the AB of *Pav*459 in the feeds of Atlantic salmon. The results showed that replacing FM with *Pav*459 did not have a significant impact on the growth parameters of the salmon. The experimental feeds had a crude protein content of approximately 49%, which met the minimal 44% digestible protein requirement suggested by the NRC (2011) [21] for salmon weighing 20–200 g, assuming an average protein digestibility of 91.2% measured in salmon fed highly similar *Pav*459 test diets [19].

Although the growth performance of the fish was not significantly affected by replacing FM with *Pav*459, it is important to note that the total oil contribution of *Pav*459 in the diets was 1.2% in the FM/AB diet and 2.4% in the AB diet. The major oil contributors in the experimental diets were 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 [26]. Poultry fat is generally composed of 85–90% total lipid and is rich in MUFA and poor in PUFA with ω6 > ω3 [27,28]. 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 the 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 [29]. Although the oil contribution of *Pav*459 was low, its fatty acid composition was rich in PUFA (64.5%), where the ω3 LC-PUFAs EPA+DHA accounted for 40.4% of total 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 embedded in the membrane, especially 22:5ω6 (particularly concentrated in the phospholipid fractions), which was present in higher proportions in salmon fed the AB diet than in salmon fed the FO diet.

Overall, the fish grew approximately 263% from their initial weight of 170.1 g across the diets, with fish fed all diets achieving a specific growth rate of 1.2%/day and a feed conversion ratio of 0.9 g feed/g gain. This demonstrates that *Pav*459 could be included in the feeds of Atlantic salmon at up to 20% of the diet without compromising growth and

feed utilization 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 partially replaced by different strains of AB in Atlantic salmon diets [30,31], hybrid striped bass diets [32], European seabass [33], and shrimp diets [34], just to mention a few. Although immunology, lipid oxidation, goblet cells, mucus production, and the microbiome were not a focus of this study, other studies that included microalgal biomass in feeds in aquaculture revealed positive results in enhancing immune response [35,36], decreasing nitric oxide [37], increasing goblet cell density in the anterior intestine [38], modulating mucosal immune function, and increasing microbiome diversity indices for microbial communities in the gut of fish fed diets with PUFA-rich microalgae compared with controls [36]. This further supports the potential of marine microalgae as an alternative food source in aquafeeds, either as oil or as biomass.

4.1. Liver Tissue

The liver plays an important role in LC-PUFA biosynthesis and overall body lipid homeostasis in Atlantic salmon [39]. In this study, there were no significant differences in the total lipid composition or the neutral and polar lipid composition of the liver tissue across the dietary treatments. The liver tissue mainly consisted of neutral lipids, with FFA being the dominant lipid class, followed by PL, ST, and TAG (Table 4). Salmon fed the FM diet had higher proportions of TAG and FFA in the liver tissue, while salmon fed the FM/AB diet had higher proportions of PL and ST. The proportion of TAG increased across the dietary treatments but did not show a significant difference at the end. The presence of increased TAG suggests storage of fat in the liver tissue, even though salmon primarily store excess fat in muscle tissue [40].

PL was the second most dominant lipid class in the liver tissue across the dietary treatment, followed by ST. This indicates the importance of membrane material in liver tissue, as both PL and ST play a role in maintaining membrane structure. Cholesterol, a common sterol, modulates the physical properties of membranes [41], and the fluidity of the lipid bilayer depends on the degree of order in the packing of phospholipids [42]. For achieving (or evaluating) fluidity, homeostasis, and plasticity of cellular membranes, we have to look at significant differences in the tissue PL/ST ratio (Table 4) and the membrane P/S fatty acid ratio (Table 5); however, since there were no differences in the tissue PL/ST ratio or membrane P/S fatty acid ratio, adjustments in membrane fluidity likely did not occur in this experiment. The total fatty acid composition of the liver tissue generally reflected the diet composition (Table 4). The dominant fatty acids in liver tissues were 16:0, 18:1ω9, LA, and DHA, with each accounting for more than 4% among all dietary treatments. PLFA composition (Table 5) shared the same dominant fatty acids along with EPA. SIMPER analysis indicated that the dominant fatty acids mentioned above were responsible for the dissimilarities observed in both total fatty acid and PLFA profiles (Tables A4 and A5 in Appendix B). While there were similarities between the total fatty acid profile and PLFA profile, PCO analysis showed that in the liver tissue there was more variation within salmon fed the same diet (80.9% variation) than salmon fed different diets (12.7% variation) (Figure 1). In contrast, for PLFA, there was more variation between salmon fed different diets (75.5% variation) than within the same diet (12.0% variation) (Figure 2).

The proportion of PUFA in liver PL was higher than that in the diet, while MUFA was lower. This suggests a possible preferential β-oxidation of MUFA when they are present in high concentrations in the diet [43,44], and possible elongation and desaturation of 18:3ω3 to maintain cellular function homeostasis [45]. There was no significant change in the proportion of SFA in liver PL. Although *Pav*459 was rich in PUFA, its contribution to the overall oil content in the diets was low (1–2%). Canola oil and poultry fat, the main oil contributors in the diets, are rich in MUFA, which likely influenced the higher proportion of MUFA observed in the diets and reflected in the liver tissue. The proportion of EPA in liver PL did not show a significant variation compared with the diet. However, it is worth

noting that the proportion of DHA in 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 emphasizes the importance of DHA over EPA in the membrane, even though the initial proportion of DHA across the dietary treatments was lower than that of EPA. The proportion of ALA was lower than that in the diet, indicating a possible desaturation and elongation process for the biosynthesis of DHA, as the intermediate step between EPA and DHA, 22:5ω3, doubled in the liver tissue compared with the diet. The lower proportion of ALA could also suggest no incorporation in liver PL.

After 12 weeks of feeding, the DHA:EPA ratio shifted from being less than 1 in the diet to being greater than 1 in the liver tissue, suggesting a higher requirement for DHA than EPA. The levels of ARA in the liver tissue and PL were significantly higher than those in the diet. This could be due to the high proportion of LA in the diet, which decreased after 12 weeks of feeding, indicating a possible desaturation and elongation process for the biosynthesis of ARA. The increase in ARA proportions might suggest the production of pro-inflammatory eicosanoids, but the ω3/ω6 ratio remained greater than 1 across all dietary treatments. EPA and ARA both serve as precursors for the production of eicosanoids, but eicosanoids formed from EPA are considered to be less biologically active than those formed from ARA [46,47]. The direct competition between EPA and ARA leads to inhibition of the formation of eicosanoids from the other [48]. Replacing FM with *Pav*459 resulted in an EPA:ARA ratio less than 1 only in salmon fed the AB diet, which had the highest canola oil content, favoring a more pro-inflammatory eicosanoid production. However, the ω3/ω6 ratio remained greater than 1 across all dietary treatments.

4.2. Muscle Tissue

In this study, there were no significant differences in the total lipid composition of the muscle tissue across the dietary treatments (Table 6). The muscle tissue was predominantly composed of neutral lipids, with TAG being the main lipid class, followed by PL and ST. The proportion of neutral lipids in the muscle tissue was similar between salmon fed the FM and AB diets but significantly higher in salmon fed the FM/AB diet. This may be due to the equal contribution of FM and AB in the diet. The proportion of PL in the muscle tissue was lower in salmon fed the FM/AB diet, but it was the second most dominant lipid class for salmon fed the FM and AB diets.

The fatty acid composition of the muscle tissue reflected the diet (Table 7). The dominant fatty acids in both total fatty acids and PLFA were 16:0, 18:1ω9, LA, and DHA, with each accounting for more than 5% among all dietary treatments. The presence of these fatty acids was the main driver for the dissimilarities observed in both total fatty acids and PLFA, as indicated by SIMPER.

Muscle tissue PL had no significant variation in the proportion of SFA, MUFA, and PUFA among the dietary treatments. The level of DHA in the muscle PL was higher than that in the diet, while EPA was lower. This suggests a preference for DHA over EPA in the muscle membrane and a possible storage of DHA [43]. 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 require a special mechanism [43]. It is also worth noting that there was no noticeable variation in the proportion of ARA in the muscle PL, resulting in an EPA:ARA ratio greater than 1 across all dietary treatments. However, only salmon fed the FM and FM/AB diets maintained a ω3/ω6 ratio greater than 1 in the muscle tissue, suggesting the potential production of anti-inflammatory eicosanoids. Further investigation is needed to draw more definitive conclusions.

Modern diets for farmed salmonids, which are rich in terrestrial plant-based proteins and oils, have posed a challenge by significantly reducing the dietary supply of cholesterol. Traditional fish oils used in salmonid feeds contain approximately 0.5–0.8% cholesterol, while plant oils such as soy, corn, and canola are completely devoid of cholesterol. This dietary shift has led nutritionists to reconsider the conditionally essential nature of dietary cholesterol in fish nutrition [16].

In our analysis of the sterol composition of *Pavlova* and muscle tissue in salmon fed the AB diet, several significant sterols were identified (Tables 2 and 8). The sterols identified in *Pavlova* were cholesterol, cholestanol, brassicasterol, campesterol, stigmasterol, stigmasta-22-en-3-β-ol, 23,24-dimethylcholest-5-en-3β-ol, 4α-methylporiferast-22-enol, 4-desmethyl-22-dehydropavlovol, 24-methylpavlovol, and 24-ethylpavlovol.

While some of these sterols are typically associated with terrestrial plant oils, their presence in microalgae, such as *Pav*459, underscores the adaptability of microorganisms in synthesizing diverse sterols. Particularly noteworthy is the identification of cholesterol, conventionally considered exclusive to animal sources but surprisingly common in microalgae [49,50]. This reveals intricate sterol dynamics within microalgae and their potential significance in providing essential sterols for aquatic organisms. Out of six sterols identified in the muscle tissue, cholestanol, campesterol, and 23,24-dimethylcholest-5-en-3β-ol are also found in *Pavlova lutheri* [50–52]. However, other sterols were notably absent, suggesting either non-transfer or metabolism upon ingestion. On the other hand, lathosterol was not observed in *Pavlova*, suggesting an alternative source in fish muscle.

Prior research has demonstrated that, in addition to being rich in DHA and EPA, the lipid fraction of *Pav*459 microalgae contains approximately 13% sterols, with around 3% of it being cholesterol [49]. This characteristic is somewhat unusual in most plants and phytoplankton, but it is not an isolated finding. Related microalgal species, such as *P. lutheri*, have been reported to accumulate up to 3% of their dry biomass as sterols [52]. Furthermore, research by Volkman et al. (1992) [53] and Vernon et al. (1998) [54] has indicated that cholesterol can comprise up to 75% of the total sterols in certain marine microalgae, a finding recently confirmed by Martin-Creuzburg and Merkel (2016) [55] in a species of *Nannochloropsis*.

From a human health perspective, the muscle tissue of Atlantic salmon, particularly the ω3 LC-PUFA content (such as EPA and DHA), is highly beneficial to human health for the prevention of cardiovascular diseases and many other health benefits [56–58]. 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. Typical recommendations are 300 to 500 mg/day of EPA and DHA provided by two servings of fatty fish/week (one serving is 112 g cooked) [56]. 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 [59–61].

It is important to note that the nutritional composition of the fillet can be influenced by cooking methods. Frying, for example, can significantly alter the lipid content and fatty acid composition of the fish. Baking and grilling are considered cooking methods that result in fewer changes in the fat content and fatty acid composition of the fillet [62].

5. Compound-Specific Stable Isotope Analysis Reveals *Pav*459 Contribution to EPA and DHA in Atlantic Salmon Muscle and Liver tissues

The study investigated the RC of different sources of LC-PUFA in salmon fed focal diets (FM and AB). While the $\delta^{13}C$ values for ARA could not be detected, EPA and DHA were the only detectable LC-PUFA. The sources of EPA and DHA in the diets included (1) the precursor ALA in the diet, FO (herring oil) present in the diet, and *Pav*459 oil in the diet. A two-end-member mixing model was used to determine the relative contributions of *Pav*459 to liver and muscle EPA and DHA. Notably, the AB diet exhibited highly negative $\delta^{13}C$ values for EPA and DHA compared with other measurements, indicating minimal diet-tissue discrimination. Terrestrial plants have a lighter $\delta^{13}C$ value than those of marine origin [63]. The lighter $\delta^{13}C$ values of terrestrial plants compared with marine sources

suggested that ALA is unlikely to be a significant contributor. The *Pav*459 used in the study was cultivated with CO_2-enriched air, which may explain its highly negative bulk stable isotope value [16]. Atmospheric carbon dioxide contains approximately 1.1% of the non-radioactive isotope carbon-13 and 98.9% of carbon-12 [64]. 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 [64]. The highly negative bulk stable isotope value for *Pavlova* could be related to the CO_2 enrichment used in the culture.

As mentioned above, in the second paragraph of the discussion, 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 the 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 [29]. Therefore, the range of levels of EPA and DHA derived from FO in the AB diet will be equal to 0.068–0.266% and 0.035–0.137%, respectively. As for *Pav*459, there was 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 of 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 TFA (Table 9), *Pav*459 showed an RC of 42.7% for EPA and 56.9% for DHA. 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-derived EPA into liver lipids. As for DHA, the average total proportion supplied in the diet by *Pav*459 was 43.0%, which is lower than the RC of *Pav*459 for DHA in the liver TFA. This implies a 132.3% incorporation of *Pav*459-derived DHA into liver lipids. It is worth noting that the 43.0% contribution represents an average total proportion. Therefore, the implication is that nearly all the DHA derived from *Pav*459 was incorporated into liver lipids. The same observation holds true for liver PL (Table 10). For liver PL, the RC of *Pav*459 to EPA and DHA was 41.3% and 54.0%, respectively, with nearly all *Pav*459-derived DHA incorporated. It is noteworthy that the incorporation of DHA consistently exceeded that of EPA and generally surpassed the calculated mean supply of 43.0%, underscoring its greater essentiality.

Although similarities to liver tissue were present, muscle TFA exhibited a lower proportion of EPA and DHA incorporation from *Pav*459. The RC of *Pav*459 to muscle EPA and DHA was 36.6% and 43.1%, respectively, suggesting incorporation of 83.9% and 100.2% of *Pav*459-derived EPA and DHA into muscle lipids, respectively. For muscle PL (Table 12), the RC of *Pav*459 for EPA and DHA was 38.1% and 42.0%, respectively, indicating significant contributions of 87.4% and 97.7% from *Pav*459, respectively.

Overall, the study revealed that *Pav*459, the marine microalgae used in the diets, made a significant contribution to EPA and DHA content in both the liver and muscle tissues of the salmon. Incorporation of *Pav*459-derived EPA and DHA is estimated to range from 94–132% in the liver and liver phospholipids, while muscle tissue also exhibited high, but moderately lower, proportions (83–87%) of *Pav*459-derived EPA but near complete (98–100%) incorporation of *Pav*459-derived DHA. Calculated values of above 100% contribution of *Pav*459-derived DHA as was found in the liver suggest a preferential incorporation of DHA from *Pav*459 over that supplied by the fish oil.

6. Conclusions

This study demonstrated the successful total replacement of FM with *Pav*459 in diets for farmed Atlantic salmon, combined with the reduction of FO to just 1.75%. Overall, the growth performance of the salmon was not significantly affected by the substitutions.

Analyzing the lipid composition of salmon membranes provided an enhanced understanding of the dietary impact. The fatty acid profiles in liver and muscle tissues mirrored the dietary treatments, emphasizing the influence of *Pav*459 on the incorporation of essential fatty acids, particularly DHA. Sterol analysis revealed the presence of important

sterols, like cholesterol, challenging conventional notions and highlighting the applicability of microorganisms like *Pav*459. Stable isotope analysis demonstrated direct incorporation of EPA and DHA from the diets into salmon tissues, with *Pav*459 contributing substantially. This finding, coupled with minimal biosynthesis from the precursor ALA even when it accounted for >1/3 of dietary ω3 FAs, indicates distinct nutritional advantages offered by *Pav*459 algal biomass. The recommended daily intake of DHA+EPA (500 mg/g) was fulfilled across all dietary treatments, reinforcing the potential of *Pav*459 as a sustainable and nutritionally rich alternative to traditional fishmeal sources. This study contributes to the growing body of research on sustainable aquafeeds, highlighting algal biomass as a feed ingredient.

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Appendix A

Table A1. Formulation of diets used in the study (g/kg, as-fed basis) fed to Atlantic salmon.

Ingredient (g/kg) [1]	FM	FM/AB	AB
*Pav*459 algal biomass [2]	0	100	200
Fish meal	200	100	0
Fish (herring) oil	70	45	17.5
Ground wheat	149	117.7	87.8
Soy protein concentrate	84	107	127.5
Corn protein concentrate	140	140	140
Poultry fat	55	67.5	78.75
Canola oil	55	67.5	78.75
Poultry by-product meal	150	150	150
Blood meal	40	40	40
Vitamin/mineral mix [3]	2	2	2
Special pre-mix [4]	20	20	20
Dicalcium phosphate	22	25	30
L-lysine	0.5	3	8.5
L-methionine	1	2.8	3.9
Choline chloride	10.5	10.5	10.5
L-tryptophan	1	2	3
Threonine	0	0	1.8
TOTAL	1000	1000	1000

[1] All ingredients were donated by Northeast Nutrition (Truro, NS, Canada), except soy protein concentrate (President's Choice), which was purchased from Atlantic Superstore (Truro, NS, Canada). [2] Produced at National Research Council of Canada's Marine Research Station (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 A2. Biochemical composition of the AB *Pav*459 used in the study.

Proximate Composition (%)	
Dry matter	95.23
Ash	9.80
Crude protein, N 6.25 × (%)	60.87
Esterifiable lipid	10.68
Crude lipid	12.25
Minerals (%)	
Calcium	0.36
Magnesium	0.41
Phosphorous	1.20
Potassium	1.40
Sodium	1.23
Carotenoids (mg/100 g)	
Astaxanthin	9.09
Canthaxanthin	40.61
Chlorophyll *a*	2259.69
Chlorophyll *b*	62.10
α-carotene	5.39
β-carotene	65.87
Fucoxanthin	630.07
Lutein	266.50
Neoxanthin	6.87
Zeaxanthi	106.25

Table A3. 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 expressed 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/Algal biomass *Pav*459; AB = Algal biomass *Pav*459.

Appendix B

Table A4. 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 A5. Liver phospholipid fatty acids 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 A6. 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 A7. Muscle phospholipid fatty acids 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 similarity: 96.0		**Average dissimilarity = 9.9**		**Average dissimilarity = 9.1**	
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 expressed as %.

Appendix C

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 a 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 SO_2. The resulting gas mixture passed through the reduction reactor (reduced copper) at 650 °C, which reduced nitrogen oxides to nitrogen gas and absorbed 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 ($(NH4)_2SO_4$) and IAEA-N-2 ($(NH_4)_2SO_4$) for nitrogen isotope calibration. NBS-18 ($CaCO_3$), 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 ($(NH4)_2SO_4$), USGS-26 ($(NH4)_2SO_4$), sulfanilamide, and BBOT were used to aide data interpretation of nitrogen isotope analyses. L-glutamic acid and B2155 (protein) were used for both carbon and nitrogen elemental calibration.

Table A8. *Pav*459 bulk stable isotope analysis.

Sample ID	Amount (mg)	Mean δ13CVPDB/‰ of All Analyses [1]	St. Dev. of δ13CVPDB/‰ 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.

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Evaluation of *Aurantiochytrium mangrovei* Biomass Grown on Digestate as a Sustainable Feed Ingredient of Sea Bass, *Dicentrarchus labrax*, Juveniles and Larvae

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Abstract: The use of microalgae as a sustainable source of n-3 long-chain polyunsaturated fatty acids (LC-PUFA) as an alternative to fish oils from small pelagic fish (e.g., anchovy, sardine) has received growing interest in the past few years. The present study aimed to: (i) produce *Aurantiochytrium mangrovei* biomass by heterotrophic fermentation using a medium containing anaerobic digestion liquid effluent, and (ii) evaluate a biomass rich in n-3 LC-PUFA and good quality proteins as a feed ingredient for sea bass juveniles and larvae. Two 800 L bioreactors were used to produce *Aurantiochytrium* biomass in non-axenic conditions. Biomass was then filtered through a crossflow filtration system (300 Kda ceramic membrane) and freeze-dried. Sea bass juveniles (32.7 ± 4.2 g) were fed both a control diet and a diet containing 15% of freeze-dried *A. mangrovei* biomass for 38 days. Juvenile survival percentage was 90% on average in both dietary conditions. Similar growth was observed between fish fed with both diets, demonstrating the feasibility to replace 15% of a standard fish feed by *Aurantiochytrium* biomass. The liver of sea bass juveniles fed with the *A. mangrovei* diet contained significantly higher proportions of 22:6n-3, 22:5n-6, and 20:4n-6 than those fed with the control diet, while the proportions of 16:0, 16:1n-7, and 18:1n-9 were significantly lower. The secondary oxidation, as measured by malonylaldehyde (MDA) content, in the liver and muscle of juveniles fed with the microalgae diet tended to be higher than in fish fed the control diet, but the differences were not statistically significant. Although the larvae survival percentage was low for all the tanks after 41 days of rearing, the inclusion of 15% of hydrolyzed *A. mangrovei* biomass in the larvae micro-diet did not impair the development of sea bass larvae and only marginally affected their lipid composition. In the future, we have to further optimize a sustainable workflow between *Aurantiochytrium* cultivation and fish feed production and confirm the zootechnical and biochemical results.

Keywords: aquaculture; nutrition; sustainability; n-3 long-chain polyunsaturated fatty acids; Thraustochytrids; microalgae; fish oil

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1. Introduction

Capture fisheries and aquaculture accounted for about 179 million tons in 2018, with aquaculture becoming dominant [1]. Farmed fish and shellfish production are major sources of seafood for human consumption, but this production partially depends on fishmeal and fish oil obtained from industrial fisheries of small pelagic fish. Fish meal and fish oil are key ingredients in fish feeds for farmed fish and shellfish species, as they are used as sources of

essential amino acids and n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) [2,3]. Most (74% in 2012) of the world's production of fish oils rich in n-3 LC-PUFA is used for aquaculture. The demand for fish oil is thus growing as the aquaculture sector grows [4] (Tacon and Metian, 2015).

Despite this, fishmeal and fish oil production are reaching their limits in terms of sustainability [5,6]. The use of these limited resources associated with the growing worldwide demand from the aquafeed industry is a threat to natural ecosystems and sustainable aquaculture. Thus, this results in a necessity to find alternative sources of these key ingredients to ensure more sustainable aquaculture production. Furthermore, as fishmeal and fish oil became more expensive and the awareness of aquaculture effect on small pelagic stocks rose, substitution of these ingredients by plant-based and animal-based ingredients increased during the last 30 years, potentially affecting the nutritional composition and value of the farmed fish [7]. Heterotrophic production of n-3 LC-PUFA by marine microorganisms as an alternative to fish oils from small pelagic fish (e.g., anchovy, sardine) has received growing interest in the past few years. The dinoflagellate *Crythecodinium cohnii* and Thraustochytrids are the most commonly grown marine protists for n-3 LC-PUFA production. Although the production cost under controlled heterotrophic fermentation of these microorganisms remains high, the resulting biomass is generally free of contaminants [8].

The Thraustochytrids, most notably the species of *Aurantiochytrium* and *Schizochytrium* genus, are particularly relevant for this type of application, mainly due to their high content in n-3 LC-PUFA [9,10]. Recently, oil and dry biomass from *Schizochytrium* sp. have successfully been incorporated into the diets of marine carnivore finfishes. It was found that substitution of marine fish oils by *Schizochytrium* sp. oil in the diet of Atlantic salmon parr did not impair fish growth [11]. Inclusion of whole *S. limacinum* cell biomass in Atlantic salmon diet was tested at various ratios, with 3–6% significantly improving growth and filet quality [12], 5% resulting in similar performance and a good availability of biomass n-3 LC PUFA [13], and 30% revealing a good digestibility [14]. Dried algae *Schizochytrium* sp. was also shown to be a good source of DHA for seabream *Sparus aurata* larvae [15]. The full replacement of fish oil by a high level of *Schizochytrium* sp. in the diet of Nile tilapia (*Oreochromis niloticus*) allowed for higher lipid digestibility, levels of DHA, and n-3 LC-PUFA as well as weight gain when compared to a control diet containing fish oil [16]. Including 2% of the dried algae *Schizochytrium* sp. in the diet of channel catfish led to a marked increase in n-3 LC-PUFA levels in the fish fillet [17]. A recent study demonstrated that a vegetal oil base supplemented with 5% and 10% of *Schizochytrium limacinum* biomass performed similarly to a fish oil-based diet in terms of growth and feeding efficiencies of *Dicentrachus labrax* juveniles [18].

Beyond providing high-quality n-3 LC-PUFA, *Schizochytrium* biomass could also contribute to the supply of good quality proteins and essential amino acids required in finfish aquaculture nutrition [19,20]. Furthermore, the inclusion of fish protein hydrolysates in marine fish feed improved fish growth, especially at their early stages of development [21]. For example, partial replacement of fishmeal by protein hydrolysates enhanced larval growth and/or survival performance in European sea bass *Dicentrarchus labrax* [22,23]. More recently, the total substitution of fish protein hydrolysate by yeast protein hydrolysate containing 44% of free amino acids (MW < 200 Da), 50% of di- and tripeptides (200 < MW < 500 Da) and 6% of larger polypeptides (500 < MW < 2500 Da) did not affect sea bream (*Sparus aurata*) larval performance [24]. However, the growth and survival of larvae fed with protein hydrolysates may vary according to the nature of the marine protein hydrolysate [25].

Oils and biomass from the industrial production of *Schizochytrium* are already commercialized; however, production costs remain high due to the need for expensive cultivation substrate. The use of industrial by-products and/or waste as carbon and nitrogen sources for microalgae cultivation can reduce production costs [18], but this novel source of nutrients has not been widely considered on an industrial scale [26].

The emergence of a circular economy allowing for the sustainable production of natural raw materials is now a preferred approach within the agricultural and food industries. In this context, the use of nutrient-rich effluents from anaerobic digestion aimed to reduce the cost associated with nutrient supplementation (notably nitrogen) for microalgal cultivation. The main challenge of such a circular economy approach is to identify the limiting nutrients of by-products and/or waste in order to maximize the growth rates of algal cultivated species. To date, most of the research on nitrogen and phosphorus recycling has been conducted at the laboratory scale. Cultivating *Aurantiochytrium* on a medium composed of digestate and a carbon source is possible and has been optimized in non-axenic cultivation conditions at a pilot scale (one m^3) [27,28].

The present study aims to: (i) apply a biomass production process for *Aurantiochytrium mangrovei* (formerly named *Schizochytrium*) by heterotrophic fermentation using a medium containing anaerobic digestion liquid effluent and (ii) evaluate a microalgal biomass rich in n-3 LC-PUFA and good quality protein in the form of feed ingredients for sea bass juveniles (whole biomass) and larvae (hydrolyzed biomass). The applicative objective is to partially replace fishmeal and fish oil ingredients generally obtained from the small pelagic fish industry in fish feed with 15% of microalgae biomass. It is meant to be a first step toward higher replacement percentage.

2. Materials and Methods

2.1. Pilot-Scale Cultivation and Harvesting of Aurantiochytrium Mangrovei on Liquid Effluent from Anaerobic Digestion

2.1.1. Preparation of *A. mangrovei* Inoculum

Initial inoculation was performed using 2 mL of a cryopreserved *A. mangrovei* (RCC893) master cultivation with a concentration of 1×10^7 cells/mL. First, 500 mL flasks were inoculated in 250 mL of Yeast Extract Peptone (YEP) medium containing 15 g/L sea salt (Sigma™, Saint Louis, MO, USA; Sigma S9883, 2 g/L peptone from casein (VWR™ Rosny-sous-bois, France, 84610.0500), 2 g/L yeast extract (VWR™ Rosny-sous-bois, France, 84601.5000) and 20 g/L glucose (TITOL chimica SpA™, Pontecchio Polesine, Italy, Glucose anhydrous pure CA:50-99-7). The culture was then maintained on a shaking platform (100 rpm) for 48 h at 23–25 °C. Then, 2 mL of the first culture was used to inoculate four new flasks in the same conditions for 64 h. The content of four cultures was transferred into two 20 L carboys to inoculate 8 L of YEP medium in each. The carboys were maintained for 24 h on the shaking platform (100 rpm), with air supply (4.8 L/min through a 4 mm diameter tubing) at the base of the carboy. After quality control, the two cultures of 8 L were finally transported from the laboratory (IUEM Plouzané) to the pilot site (Cooperl, Lamballe) for the inoculation of two 800 L poly(methylmethacrylate) (PMMA)-made cylinders for batch cultivation (500 L) at pilot scale.

2.1.2. Preparation of the Digestate for Integration in Culture Medium at Pilot Scale

The digestate used to grow *A. mangrovei* was the result of the anaerobic digestion of pig manure. Prior to microalgal cultivation, the digestate was first centrifuged to eliminate large particles and then using ultra-filtration at a pore size of 300 kDa to sanitize it and remove micro-particles. The composition of the raw digestate can be found in Table S1.

2.1.3. Cultivation of *A. mangrovei* at Pilot Scale

Two 800 L PMMA-made cylinders were used to produce about 10 kg of biomass in non-axenic conditions. Water (for process and cleaning) was supplied by a pump and delivered at the top of the cylinders through a rotating nozzle. Agitation and O_2 supply in each cylinder were provided by airflow bubbling from the bottom of the cylinder, at a rate of 0.4 volume of air per volume of culture per minute (air-lift system). The air was supplied through a ring of 16 mm diameter PVC tubing pierced with 500 1.5 mm diameter holes. All the other necessary inputs were introduced manually at the top of the two cylinders.

The pilot-scale medium for the batch cultivation was composed of industrial glucose syrup (final concentration: 24 g/L ISOSWEET 470 TEREOS), sterilized medium with tryptone and yeast extract at 2 g/L, digestate previously filtered as described above (final concentration 2.5% supplying 48.3 mg N/L, 2.6 mg P/L with N/P ratio of 18.7), and sea salt (final concentration 15 g/L, Le Saunier de Camargue, sel de mer non traité). Two 800 L cylinders were filled with 500 L of the pilot-scale medium and each inoculated with 8 L cultures as prepared above. Silicone-based anti-foam was added (final concentration: 1 mL/L, Antimousse 426R, LABOGROS) as soon as the yeast extract and peptone solution were poured into the cylinder. The microalgae *A. mangrovei* was then cultivated in non-axenic conditions for 62 h. The temperature of the culture was regulated between 28 and 30 °C by immersing a stainless steel electric heater (2 Kw for 500 L) while pH was maintained above 4.5 by regular addition of 10 N NaOH solution. After 62 h of cultivation, the biomass concentration reached about 10 g/L in both cylinders.

2.1.4. Harvesting of *A. mangrovei* Culture at Pilot Scale

Cultures from the two cylinders (1000 L) were pooled and then filtered through a crossflow filtration system. The filtration system from SIVA TM supports 3.5 m^2 of 300 Kda ceramic membrane, in two cartridges supporting 11 channels each (internal diameter 4.6 mm). Filtration conditions were as follows: 4 m/s for retentate velocity on membrane, 2500 L/h for retentate recirculation rate, 0.6 to 0.9 bars for transmembrane pressure, 300 to 180 L/h for permeate flow-rate. During crossflow filtration, samples were taken every hour in order to control the fatty acid composition, notably, their content in n-3 LC-PUFA. The final volume of retentate was approximatively 100 L at 90 g/L (equivalent DW) of microalgae biomass. The harvested biomass was frozen at −20 °C prior to further downstream processing (freeze-drying and enzymatic hydrolysis). The harvested biomass was freeze-dried externally by Eurolyo (https://www.eurolyo.fr/, (accessed on 27 September 2022)) (72 h at −40 °C and 250 µbar).

2.1.5. Monitoring of Culture Concentration and Cellular Parameters

Concentration and cellular parameters (size, complexity, lipid content) of *A. mangrovei* were measured using an Easy-Cyte Plus 6HT flow cytometer (Guava Merck Millipore®, Darmstadt, Germany) equipped with a 488 nm blue laser, detectors of forward (FSC) and side (SSC) light scatter, and three fluorescence detectors: green (525/30 nm), yellow (583/26 nm) and red (680/30 nm). Cell morphological variables, i.e., forward scatter (forward scatter, FSC), side scatter (side scatter, SSC), were used to identify and select the *A. mangrovei* cell population. FSC and SSC give, respectively, information on the relative size and complexity of cells [29]. The flow cytometry measurements were performed on fresh (living) samples. The BODIPY probe (BODIPY 505/515 FL; Molecular Probes, Invitrogen, Eugene OR, USA, final concentration of 10 mM), which stains lipid droplets/bodies within microalgae cells, was used as a proxy of lipid reserves [29]. The green fluorescence emitted is proportional to the quantity of lipid reserve present in the cells. Measurements were performed at a flow rate of 59 µL min $^{-1}$. The concentration of microalgae was given in cells per mL, and cellular parameters (FSC, SCC, and lipid reserve) were expressed in arbitrary units (A.U.).

2.2. Enzymatic Hydrolysis of A. mangrovei Biomass

The freeze-dried biomass was resuspended in distilled water at 10% (*w*/*v*), and it was then hydrolyzed with Alcalase 2.4 L (Sigma-Aldrich, a protease of *Bacillus licheniformis*) at an enzyme/substrate ratio of 1.8%. Hydrolysis was carried out in a 500 mL lab reactor heated with a water bath at 50 °C and stirred at 100 rpm with a 3-bladed propeller. After two hours of hydrolysis, when the degree of hydrolysis was around 14 ± 1%, the biomass was heated at 80 °C for 20 min to inactivate the enzyme.

To obtain enough hydrolyzed biomass (0.5 kg), hydrolysis was repeated ten times. The pooled hydrolysates were freeze-dried for 72 h using a freeze-dryer (Christ Alpha 1–4 LD plus).

2.3. Feed Formulation for Seabass Juveniles and Larvae

2.3.1. Seabass Juveniles Feed

Experimental juvenile diets, namely control and microalgae diets, were prepared in the INRAE facilities (Donzacq, Landes, France) and extruded as pellets of 3 mm. Gelatinized starch was included due to the extrusion process. The compositions of the control and microalgae diets are detailed in Table 1.

Table 1. Formulation and proximal composition of the experimental diets for sea bass juveniles.

	Experimental Juvenile Diets	
Ingredients (% of Dry Weight Diet)	**Control**	**Microalgae**
Fish meal	64	52
Microalgae biomass	0	15
CPSP 90 (pre-digest fishmeal)	10	10
fish oil	1	0
rapeseed oil	1	1
rapeseed lecithin	14	13
Starch	5	5
Vitamin mix [1]	3	3
Mineral mix [2]	1	1
Cellulose	1	0
Total	100	100
Calculated proximal composition (% dry weight)		
Proteins % DW	52.1	48.2
Lipids % DW	18.5	17.8
EPA+DHA % DW	1.1	1.1
EPA+DHA % lipids	6.1	6.0

Dietary ingredients, except microalgae biomass, were commercially obtained. Fishmeal, fish oil, and CPSP 90: Sopropêche 62,126 Wimille; Soya lecithin: Louis François 77,134 Marne La vallée; rapeseed oil: Oleandes, 40,250 Mugron; Starch: Roquette 62,136 Lestrem; Cellulose: Rettenmaier Saint Germain en Laye France 78,100; Vitamins and minerals: INRAE SAAJ 78,530 Jouy en Josas. [1] Per kg of vitamin mix: retinyl acetate, 340 mg; cholecalciferol, 2.5 mg; all-rac-α-tocopherol acetate, 4 g; menadione, 0.1 g; thiamin, 1 g; riboflavin, 2.5 g; D-calcium pantothenate, 5 g; pyridoxine HCl, 1 g; cyanocobalamin, 0.006 g; riacin, 10 mg; folic acid, 0.5 g; biotine, 0.1 g; meso-inositol, 100 g. [2] Per kg of mineral mix: KCl, 90 g; KI, 40 mg; $CaHP0_4$ $2H_2O$, 500 g; NaCl, 40 g; $CuSO_4$ $5H_2O$, 3 g; $ZnSO_4$ $7H_2O$, 4 g; $CoSO_4$ $7H_2O$, 20 mg; $FeSO_4$ $7H_2O$, 20 g; $MnSO_4$ H_2O, 3 g; $CaCO_3$, 215 g; Mg SO_4 $7H_2O$, 124 g; NaF, 1 g.

2.3.2. Seabass Larvae Feed

To produce larvae micro-diets, dietary ingredients (Table 2) were mechanically mixed with water, pelleted and dried at 50 °C for 20 min. The pellets were sieved to obtain two sizes of particles; 125–200 and 200–400 μm. The small micro-diet was used from 16 days after hatching as a co-feed of enriched *Artemia* nauplii at increasing ratios, from 20% to 100% using 20% increments for 5 days. Twenty days after hatching, the larger micro-diet was used until the end of the experiment (41 days post-hatching). Fish larvae were continuously fed in excess for 18 h/day using a belt feeder.

Table 2. Formulation and proximal composition of the experimental larvae micro-diet.

	Experimental Larvae Micro-Diets	
Ingredients (% of Dry Weight Diet)	**Control**	**Microalgae**
Fish meal	62	68
Hydrolyzed microalgae biomass	0	14
CPSP 90 (pre-digest fishmeal)	10	0
Tuna oil	1	0
Soya lecithin	13	13
Vitamin mix [1]	3	3
Mineral mix [2]	3	2
Cellulose	8	0
Total	100	100
Calculated proximal composition (% dry weight)		
Proteins % DW	50.7	50.1
Lipids % DW	16.5	17.6
EPA+DHA % DW	1.1	1.2

Dietary ingredients, except microalgae biomass, were commercially obtained. Fishmeal, fish oil, and CPSP 90: Sopropêche 62,126 Wimille; Soya lecithin: Louis François 77,134 Marne La vallée; rapeseed oil: Oleandes, 40,250 Mugron; Starch: Roquette 62,136 Lestrem; Cellulose: Rettenmaier Saint Germain en Laye France 78,100; Vitamins and minerals: INRAE SAAJ 78,530 Jouy en Josas. [1] Per kg of vitamin mix: retinyl acetate, 340 mg; cholecalciferol, 2.5 mg; all-rac-α-tocopherol acetate, 4 g; menadione, 0.1 g; thiamin, 1 g; riboflavin, 2.5 g; D-calcium pantothenate, 5 g; pyridoxine HCl, 1 g; cyanocobalamin, 0.006 g; riacin, 10 mg; folic acid, 0.5 g; biotine, 0.1 g; meso-inositol, 100 g. [2] Per kg of mineral mix: KCl, 90 g; KI, 40 mg; $CaHP0_4$ $2H_2O$, 500 g; NaCl, 40 g; $CuSO_4$ $5H_2O$, 3 g; $ZnSO_4$ $7H_2O$, 4 g; $CoSO_4$ $7H_2O$, 20 mg; $FeSO_4$ $7H_2O$, 20 g; $MnSO_4$ H_2O, 3 g; $CaCO_3$, 215 g; Mg SO_4 $7H_2O$, 124 g; NaF, 1 g.

2.4. Experimental Design

2.4.1. Sea Bass Juvenile Rearing

Sea bass juveniles, weighing on average 32.7 ± 4.2 g (approximatively 6 months old), were distributed into six 700 L tanks (three tanks per experimental condition) at a density of 100 juveniles per tank. Tanks were continuously filled at a flowrate of 500 L per hour with seawater at a salinity of 35.4 psu on average. Sea water prior to the experiment was passed through two sand filters (~500 µm), heated (tungsten, Plate Heat Exchanger), degassed using a column, filtered using a 2 µm membrane and finally UV sterilized assuring high water quality. Water temperature was increased progressively (17.5 °C to 20.5 °C) during the experiment due to the water network configuration. The fish were subjected to a photoperiod of 12 h:12 h light dark cycle by fluorescent ceiling lights (55–60 Lux). Temperature and water pH were measured weekly, and the tanks were cleaned regularly with a broom after a daily purge of 1/4 of the tank's volume (evacuation of fish feces).

Prior to the experimental feeding with the two diets described in Table 1, juveniles were acclimated for about a week with the fish feed "NEOSTART" 3 mm (Le Gouessant, Lamballe, France). After this acclimation, control and microalgae diets were distributed using belt feeders at 2% of the fish mass per day (ad libitum ratio at the applied rearing temperature [30]). The feeding quantities were adjusted with data obtained after weight measurements on days 14 and 29 of dietary conditioning.

2.4.2. Sea Bass Larvae Rearing

Two days post-hatching (PH) sea bass larvae were purchased from the marine hatchery of Gravelines Ichtus (France) and transferred to LEMAR rearing facility in Plouzané. Larvae were distributed into nine 35 L conical tanks (three tanks per experimental condition) at a density of 3000 larvae per tank. Conical tanks were continuously filled at a flowrate of 30 L per hour with seawater filtered at 10 µm at a salinity of 35.0 psu on average. Photoperiod was also adapted according to standard larval rearing (steady increase from 0.5 to 10 Lux between day 8 PH and day 31 PH, then a step increase to 31 Lux maintained until day 35 PH and a final increase to 59 Lux until day 41 PH). The temperature of each tank was controlled daily until the end of the experiment, showing a steady increase from 17 to 20 °C during the 41 days of the experiment. Tanks were siphoned once per week before micro-diet feeding and every two days after the trial began.

Larvae were fed *Artemia* nauplii (VNBS Brine Shrimp eggs *Artemia* cysts, Vietnam) from mouth opening (day 8 PH) until day 11 PH and then fed one day old *Artemia* nauplii (enriched with Larviva multigain from BioMar, Nersac, France) at day 12 until the co-feeding procedure. To condition larvae to more easily accept the experimental micro-diets, they were co-fed both enriched *Artemia* nauplii and micro-diets starting at day 13 PH. Starting at 16 PH during co-feeding, the micro-diet proportion was increased by a 20% increment each day for 5 days. From day 20 PH until the end of the experiment, the sea bass larvae were fed with the experimental micro-diets using a belt feeder. Feed quantities were calculated empirically: starting at 0.1–0.2 g micro-diet per day per 3000 larvae at day-15 PH and progressively increased up to 0.8–1 g at day 41 PH.

2.5. Sampling and Biometric Measurements

2.5.1. Juvenile Sampling

Prior to each sampling, and after a fast of 24 h, fish weights were recorded on day 0, 14, 29 and at the end of the experiment (day 38). On day 0, all individuals of each condition (100 juveniles) were gradually sedated through an intermediate tank (0.025 g/L of MS222, tricaine methanesulfonate), followed by deeper sedation (0.05 g/L MS222), fished and weighed in accordance with the regulations described in Directive 2010/63/UE. After sedation dissipation in an intermediate bucket, fish were re-introduced to their respective tanks. After 14 and 29 days of rearing, intermediate monitoring of fish weight was limited to a smaller sampling of 30 fish per condition. Mortality, malformations, or other peculiarities (macroscopically and visually assessed) were absent from the three tanks of both dietary conditions. At the end of the experiment, fish anesthesia was executed using euthanasian solution (0.125–0.250 g/L of MS222) according to Directive 2010/63/UE. Liver and muscle from 10 individuals per tank were sampled by dissection. Samples were then immediately submerged in liquid nitrogen and stored at −80 °C. Juvenile liver and muscle samples (5 individuals per tank × 3 tanks for each treatment) were freeze-dried for 72 h and stored at −20 °C until further analysis.

2.5.2. Larvae Sampling

Larvae sampling was performed to verify welfare and larvae development during the first two weeks prior to feeding the experimental micro-diets and two times during the experiment: on days 26 and 31. On day 0, larval density was estimated using alizarin-red staining. Larvae mouth opening and swim bladder formation were checked using a binocular microscope. During the experiment, sampling was processed as follows: water levels in the tanks were lowered, 10 to 15 larvae per condition were sampled using a sieve and sedated and then euthanized by submersion in euthanasian solution (MS222 tricaine methanesulfonate; 50 mg/L for sedation and 2 g/L for euthanization). Larvae were dried on an absorbent paper and then weighed using a precision scale (0.001 g precision). Mortality, malformations, food intake (thanks to larvae transparency) or other peculiarities were assessed under a binocular microscope. For the final sampling at 41 days PH, all remaining larvae of each tank were counted, euthanatized (as mentioned above), frozen in liquid nitrogen and stored at −80 °C. No weight measurement was performed on day 41 to limit air exposure and maximize the quantity and quality of biological material for biochemical analysis. Samples of 10–100 larva (n = 3 tank for those fed with the control diet and n = 3 tanks for those fed hydrolyzed microalgae diets) were freeze-dried for 72 h and stored at −20 °C for further analysis.

2.6. Biochemical Analysis

2.6.1. Lipid Extraction

The dried fish samples were manually grounded using a pestle prior to extraction. Then, 50 mg of powdered juvenile samples, and 10 mg of powdered larvae samples were approximately weighed and extracted in 6 mL of solvent mixture ($CHCl_3$:MeOH, 2:1, *v*:*v*) directly added into glass vials [31]. Extracts were flushed with nitrogen gas, vortexed, and

sonicated for 10 min to ensure complete lipid extraction and stored at −20 °C overnight for further analysis.

Freeze-dried hydrolyzed and non-hydrolyzed microalgae biomasses, as well as experimental diets and micro-diets, were similarly processed as juvenile samples using 50 mg of dried material for lipid extraction.

2.6.2. Lipid Class Analysis

Lipids classes of larvae samples were separated and quantified by high performance thin-layer chromatography (HPTLC). HPTLC glass plates coated with silica were cleaned and then activated by heating them at 120 °C for 30 min. Lipid extracts and a mixture of external standards of known concentrations were spotted on the plates with an automatic TLC sampler ATS4 (CAMAG, Switzerland). Lipid classes were separated by immersion of the plates in solvent mixtures of different polarities, which allow for lipid migration on the plates. First, the plate was immersed in a solution of *methyl acetate:isopropanol:chloroform:methanol:KCl* 0.25% (10:10:10:4:3.6; *v*/*v*), allowing for polar lipid (PL) separation. Subsequently, the plates were immersed first in a solution of *hexane:diethyl ether:acetic acid* (20:5:0.5; *v*/*v*) and then in a solution of *hexane:diethyl ether:* (97:3; *v*/*v*) for neutral lipid (NL) separation. Lastly, the plates were immersed in a solution of $CuSO_4$ 3% and H_3PO_4 (*w*/*v* in distilled water) and then heated at 180 °C for 30 min, to allow for final lipid class revelation. The plates were read using a scanner densitometer set at 370 nm (TLC Scanner 4, CAMAG). Lipid classes were identified and quantified by comparing the retention time and band intensity of each lipid class against those of known external standards using VisionCATS software (v2.4, CAMAG). The mean analytical variability for lipid class quantification was about 15%. Five classes of NL and six classes of PL were quantified: ALC—alcohols, FFA—free fatty acids, FS—free sterols, SE—sterol and wax esters, and TAG—triacylglycerides for NL; LPC—lyso-phosphatidyl choline, PC—phosphatidylcholine, SPG—sphingomyelin, PS—phosphatidylserine PI—phosphatidylinositol, and CL/PE—cardiolipin and phosphatidylethanolamine for PL. Total lipid concentration was computed as the sum of all lipid classes. Concentrations were expressed in $mg \cdot g^{-1}$ of dry weight.

2.6.3. Separation of Polar Lipids (PL) and Neutral Lipids (NL)

To fractionate NL and PL, 1 and 0.2 mL of total lipid (TL) extract (of larvae samples and microalgae biomass as well as the juvenile samples, respectively) were evaporated to dryness under nitrogen, recovered with 3 washes of 0.5 mL of $CHCl_3$:MeOH (98:2 *v*:*v*; final volume 1.5 mL) and spotted at the top of a silica gel column (40 × 4 mm, silica gel 60A 63–200 µm rehydrated with 6% H_2O, 70–230 mesh, Sigma-Aldrich, Darmstadt, Germany). The NL was then eluted using $CHCl_3$:MeOH (98:2 *v*:*v*; 10 mL) and PL fraction with methanol (20 mL). Both were collected in glass vials containing an internal standard (C23:0, 2.3 µg). Lipid fractions were then stored at −20 °C under a nitrogen atmosphere until further analysis.

2.6.4. Fatty Acid Analysis in Total Lipids (TL), Polar Lipids (PL) and Neutral Lipids (NL)

Polar and neutral fractions or total lipid extracts were dried under vacuum with an evaporator (Genevac). Dried lipid fractions were saponified with 1ml of KOH:MeOH (0.5 M) heated for 30 min at 80 °C; then, they were transesterified with 800 µL of MeOH: H_2SO_4 (3.4%; *v*/*v*) heated at 100 °C for 10 min. The fatty acid methyl ester (FAME) formed were recovered in hexane. FAME of the NL of all samples were separated from other unwanted compounds (e.g., sterols and alcohols, potentially present in the NL fraction) using high performance liquid chromatography (HPLC) equipped with two columns (LiChrospher Si 60 and LiChrospher 100 DIOL, both 5 µm). A Dionex HPLC system (P680 pump AS-100 auto sampler, UVD170U UV detector with deuterium lamp, Foxy fraction collector), was used. Details on the analytical methods (i.e., solvent proportions,

flow-rate) can be found in [32]. The purified FAME were recovered in new vials for gas chromatography analysis.

FAME composition was analyzed by gas chromatography coupled to a flame ionization detector (GC-FID; Varian CP8400 gas chromatograph, Agilent). Samples (2 µL) were injected at 250 °C in splitless mode at an oven temperature of 60 °C, with hydrogen as the carrier gas. The GC was equipped with a ZBWAX column (30 m in length, 0.25 mm internal diameter, 0.25 µm film thickness, Phenomenex). The oven temperature was raised to 150 °C at 50 °C·min^{-1}, to 170 °C at 3.5 °C·min^{-1}, to 185 °C at 1.5 °C·min^{-1}, to 225 °C at 2.4 °C·min^{-1} and then to 250 °C at 5.5 °C·min^{-1}. FAME were identified by comparing their retention time to those of an external commercial standard mixture (S37 FAME Mix, PUFA No.1, and PUFA No.3, Supelco) using the software Galaxie 1.9.3.2 (Agilent). FAME peak area was converted into µg of FA based on the peak area of the internal standard C23:0. Concentrations were expressed in mg g^{-1} of wet weight, and FA compositions were expressed in percentage (%).

2.6.5. Analysis of Lipid Peroxidation

The level of lipid peroxidation was assessed by monitoring secondary oxidation using BIOXYTECH Malondialdehyde (MDA) 586TM kit (Tebu-bio, France). Free MDA or, after a hydrolysis step, total MDA (including protein-bound MDA and free MDA) concentration was determined in all samples through spectrophotometry, by measuring the absorbance of an MDA-chromogenic agent adduct at 586 nm. Sample weights were adapted to sample availability: 50 mg for all experimental diets and juvenile sea bass liver samples; 5–10 mg for larvae samples. Values (mean of three-repeated measurements) were expressed as µmol MDA/g DW tissue or diet.

2.6.6. Statistical Analysis

Data normality first was evaluated using the Shapiro–Wilk test, and then a t test for significant differences between dietary conditions in both juvenile and larvae feed trials was performed using Statgraphics Plus statistical software (Manugistics, Rockville, MD, USA). Homogeneity of variances was checked by means of the Barlett test. Differences were considered statistically significant if $p \leq 0.05$.

3. Results

3.1. Cellular Parameters of Microalgae Culture

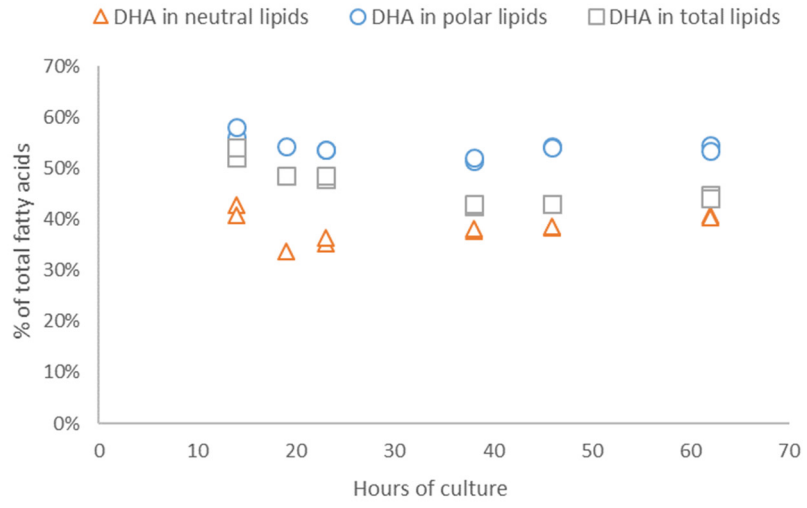
The cell concentration increased rapidly from inoculation (7.5×10^5 cells mL^{-1}) to 38 h (3.0×10^8 cells mL^{-1}), reaching a plateau until the end of the cultivation at 62 h (3.3×10^8 cells mL^{-1}). Concomitantly, the dry matter of the culture increased from 1.4 g/L at 14 h up to 10.5 g/L after 62 h of cultivation (Table S2). Cell size decreased during cultivation, while cell complexity almost doubled between the beginning and the end of cultivation (Table S2). The proxy of cellular lipid content (green fluorescence of BODIPY staining) increased steadily from around 400 A.U. at 14 h up to 3790 A.U. after 46 h of cultivation and then decreased to 2600 A.U. after 62 h of cultivation (Table S2).

3.2. Biochemical Quality of Microalgae Culture and Concentrate

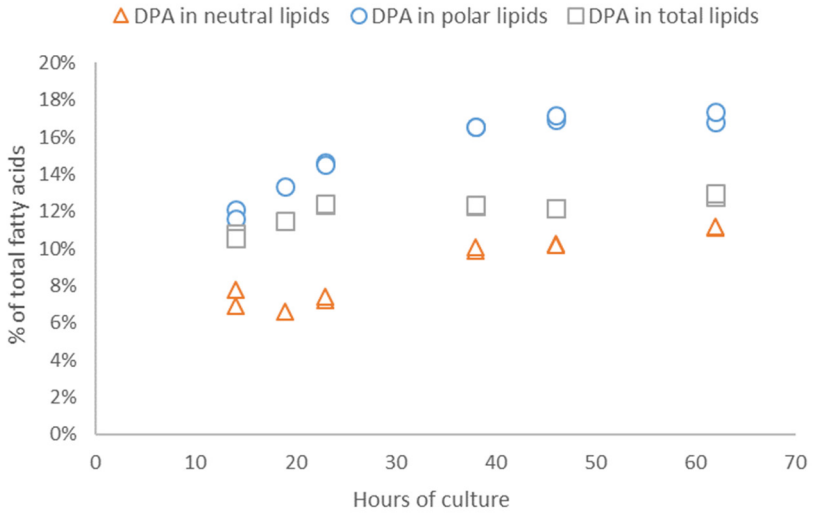
The total fatty acid content in A. mangrovei culture at the pilot scale increased with the duration of cultivation from 0.1 g/L after 14 h post-inoculation to > 2.3 g/L after 38 h post-inoculation and then slightly decreased to 1.6 g/L after 62 h of cultivation (Table S3). Total fatty acid content per DW increased from 8.6% at 14 h to 27.1% after 38 h and then decreased down to 15.3% at 62 h. Lipids were dominated by polar lipids (PL) during the first 23 h (73–74% of total lipids) and thereafter by neutral lipids (NL) until the end of the cultivation, accounting for more than 80% of total lipids (Table S3).

The percentage of DHA over total fatty acids, which is the targeted valuable compound, remained stable during the cultivation in both PL and NL (52–57% and 36–42%, respectively) (Figure 1A). When NL and PL were combined, the percentage of DHA over total fatty acids

ranged from 43% to 53% (Figure 1A). The second most dominant PUFA in A. mangrovei biomass was the n-6 DPA (22:5n-6), increasing from 12% to 17% in PL and from 7% to 11% in NL during the culture growth (Figure 1B, Table S3).



(**a**)



(**b**)

Figure 1. Percentages of the major PUFA, DHA (**a**) and DPA (**b**), expressed as percentage of total fatty acids in polar, neutral, and total lipids of Aurantiochytrium mangrovei biomass collected at 14, 19, 23, 38, 46, and 62 h of cultivation with yeast extract peptone medium supplemented with 2.5% digestate (Cooperl, France). At each sampling, the presented two values were obtained from the two 800 L cylinder cultures performed concomitantly.

At the end of cultivation (62 h), the biomass was concentrated to 90 g/L using crossflow filtration. The hourly control of fatty acid composition during filtration revealed that the PUFA content in the biomass was only slightly impacted by the harvesting process (Figure S1). The percentage of DHA in NL ranged between 39.4% and 42.8% independently of sampling time (every hour for five hours) and slightly decreased in PL from 60.0% to 53.1% after 5 h of cross filtration (Figure S1). When combining both NL and PL fractions, the percentage DHA in total lipids was 43.5% on average during the whole concentration process. The n-6 DPA was stable in both fractions at about 20% in PL and 11% in NL (Figure S1).

After cross-filtration, the biomass was freeze-dried. The final dried biomass contained 11% fats (8.3% total fatty acids), 6% carbohydrates, 33% proteins and 18% ash. Fatty acid and amino acid compositions are presented in Table S4. The fatty acid profile was dominated by the DHA, 16:0, and 22:5n-6 (40.5%, 34.3% and 12.3%, respectively) and remained very similar to the profile of the biomass right after the harvesting process (data not shown). The predominant amino acids were glutamic acid and aspartic acid above 10% followed by threonine, valine, leucine, lysine, arginine, serine, glycine, and alanine above 5%. Methionine, isoleucine, histidine, proline, and cysteine were below 5%.

3.3. Biochemical Quality of Experimental Juvenile Diets and Larvae Micro-Diets

As described in the material and methods, the fish feed formulation was complex and included several commercial ingredients. Including 15% of microalgae biomass in the fish feed allowed for replacement of the fish oil (usually supplied at 1% DW of fish feed), part of the fishmeal (reduced by 12%, from 64% to 52% DW) as well as part of the lecithin (reduced from 14% to 13% DW). Cellulose was not added into the experimental microalgae fish feed.

The fatty acid compositions of both fish feeds (control and microalgae) were compared (Table 3). The LC-PUFA DHA and 22:5n-6 contents were respectively two and four-fold higher in the microalgae diet than in the control diet. The saturated FA, 15:0 and 17:0, were also found in higher proportions in the microalgae diet, 3.4 and 1.8-fold, respectively. Furthermore, the LC-PUFA 20:5n-3 and 22:5n-3 were in lower proportions in the microalgae diet, 1.9 and 1.5-fold, respectively. The 16:1n-7, the C16 PUFA, and the 18:2n-4 were also found in lower proportions in the microalgae diet. The sum of the essential LC-PUFA EPA+DHA was 7.8% and 11.0% in the control and microalgae diets, respectively. Both were higher than expected according to the calculation in Table 3. The inclusion of the microalgae biomass in the fish diet could increase the DHA level and DHA/EPA ratio in the fish feed (from 4.4 to 9.0% and from 1.3 to 4.5%, respectively).

Total MDA (including MDA bound to proteins plus free MDA) and free MDA were slightly higher in the microalgae diet (2.0 and 1.0 µmol MDA/g DW, respectively) as compared to the control diet (1.5 and 0.75 µmol MDA/g DW) (Figure 2).

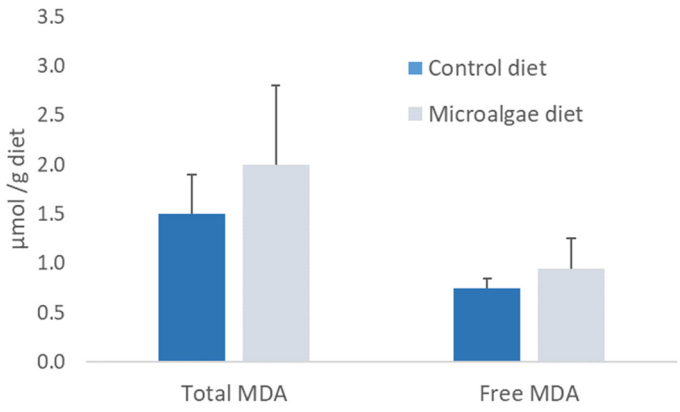


Figure 2. Total and free malondialdehyde (MDA) of control and microalgae diets for sea bass juveniles.

Fatty acid composition of the larvae control micro-diet was dominated by 16:0, 18:1n-9 and 18:2n-6 accounting for 20%, 17% and 25%, respectively (Table S5). The n-3 LC-PUFA, DHA and EPA were found at 44%, 8% and 4%, respectively. The n-3 LC-PUFA content of the hydrolyzed microalgae micro-diet was slightly lower than in the control micro-diets (41% vs. 44%) with DHA at 7%.

The larvae micro-diet containing 15% of hydrolyzed microalgae biomass had a higher level of total MDA (almost two-fold) than the control micro-diets (Figure 3). However, the content of free MDA was similar in the two tested micro-diets (Figure 3).

Table 3. Fatty acid composition of the control and microalgae diets for sea bass juveniles.

% of Total FA	Control Diet		Microalgae Diet	
	Mean	S.D.	Mean	S.D.
14:0	1.6	0.1	1.6	0.1
15:0	0.2	0.0	0.8	0.0
16:0	12.1	0.1	17.4	0.2
17:0	0.2	0.0	0.3	0.0
18:0	1.8	0.0	1.7	0.0
20:0	0.2	0.0	0.2	0.0
16:1n-7	2.2	0.1	1.4	0.0
18:1n-7	2.6	0.1	2.3	0.1
18:1n-9	39.2	0.2	35.5	0.2
20:1n-11	0.5	0.0	0.4	0.0
20:1n-7	0.1	0.0	0.1	0.0
20:1n-9	1.9	0.1	1.5	0.1
22:1n-11	1.8	0.1	1.6	0.1
22:1n-9	0.3	0.0	0.2	0.0
24:1n-9	0.7	0.0	0.6	0.0
16:2n-4	0.2	0.0	0.1	0.0
16:3n-4	0.1	0.0	0.0	0.0
16:4n-1	0.2	0.0	0.1	0.0
18:2n-4	0.1	0.0	0.0	0.0
18:2n-6	19.4	0.4	17.3	0.2
18:3n-3	3.2	0.0	2.8	0.0
18:4n-3	0.6	0.0	0.4	0.0
20:4n-3	0.2	0.0	0.2	0.0
20:4n-6	0.4	0.0	0.4	0.0
20:5n-3	3.3	0.0	1.7	0.1
22:5n-3	0.5	0.0	0.3	0.0
22:5n-6	0.4	0.1	1.8	0.1
22:6n-3	4.1	0.2	7.8	0.3
Total Branched	0.3	0.0	0.2	0.0
Total SFA	16.2	0.1	22.2	0.3
Total MUFA	49.9	0.4	44.0	0.3
Total PUFA	33.6	0.4	33.5	0.6
DHA/EPA	1.2	0.0	4.5	0.1

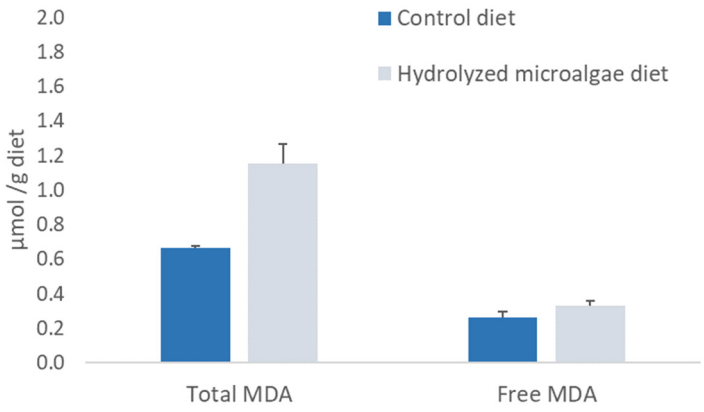


Figure 3. Total and free malondialdehyde (MDA) of sea bass larvae control and hydrolyzed microalgae micro-diets.

3.4. Sea Bass Juveniles Feeding Trial

After Sea bass juveniles were fed for 38 days, similar growth was observed in juveniles fed with both the control and microalgae diets (Figure 4), and survival percentage was 90% for both dietary conditions. This nutritional trial demonstrated the feasibility to replace 15% of a standard fish feed with microalgal biomass.

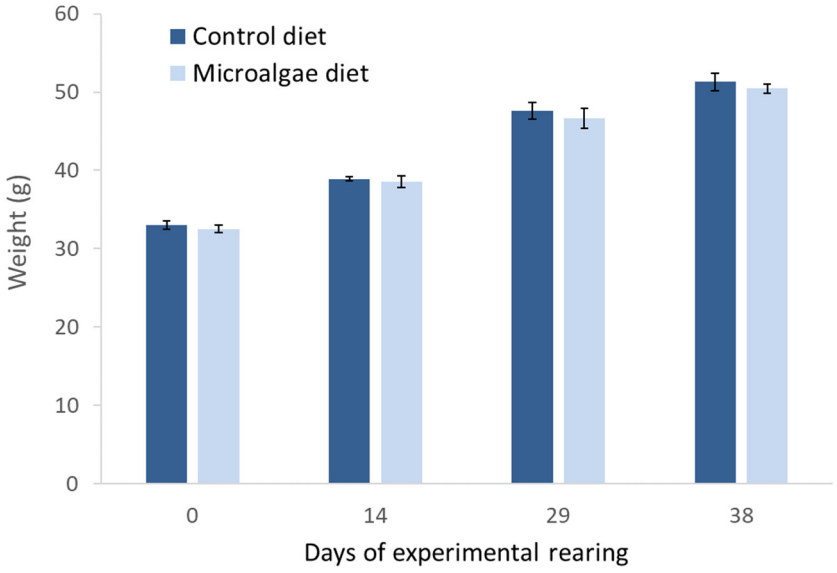


Figure 4. Growth of sea bass, Dicentrarchus labrax, juveniles fed with control and microalgae diets (described in Material and Methods) for 38 days.

After 38 days of dietary conditioning, the liver composition in FA of sea bass juveniles was significantly affected by the diets ($p < 0.05$; Figure 5). In the liver of fish fed with the microalgae diet, the content of DHA, 22:5n-6, 20:4n-6 over total fatty acids increased significantly, while the content of 16:0, 16:1n-7, and 18:1n-9 decreased significantly. Consequently, the PUFA proportion increased in juveniles fed with the microalgae diet as compared to the control diet (24% vs. 15.7%, respectively), and saturated and monounsaturated FA percentages decreased from 29.6% and 49% to 25.1% and 43.3%, respectively. A similar dietary imprint was observed in the juvenile muscle but to a lesser extent. Only the 18:1n-9, 20:1n-9 and the EPA proportions decreased significantly.

The total MDA and free MDA contents in the liver of juveniles fed with the microalgae diet tended to be higher than in the liver of fish fed with the control diet (Figure 6A), but the differences were not statistically significant. In muscle, only the free MDA tended to be higher in the muscle of juveniles fed with the microalgae diet than in those fed with the control diet (Figure 6B).

3.5. Sea Bass Larvae Feeding Trial

The larvae feed trial using control and hydrolyzed microalgae micro-diets started 20 days after hatching. Some mortalities, independent of dietary conditions, occurred when larvae were between 12 and 20 days post-hatching (during co-feeding with micro-diet from 20 to 100%) and between 31 and 37 days post-hatching (during feeding with 100% micro-diet). The measurement of larvae weight after 26 and 31 days of rearing revealed a large variability between the three tank replicates within one condition (Figure 7).

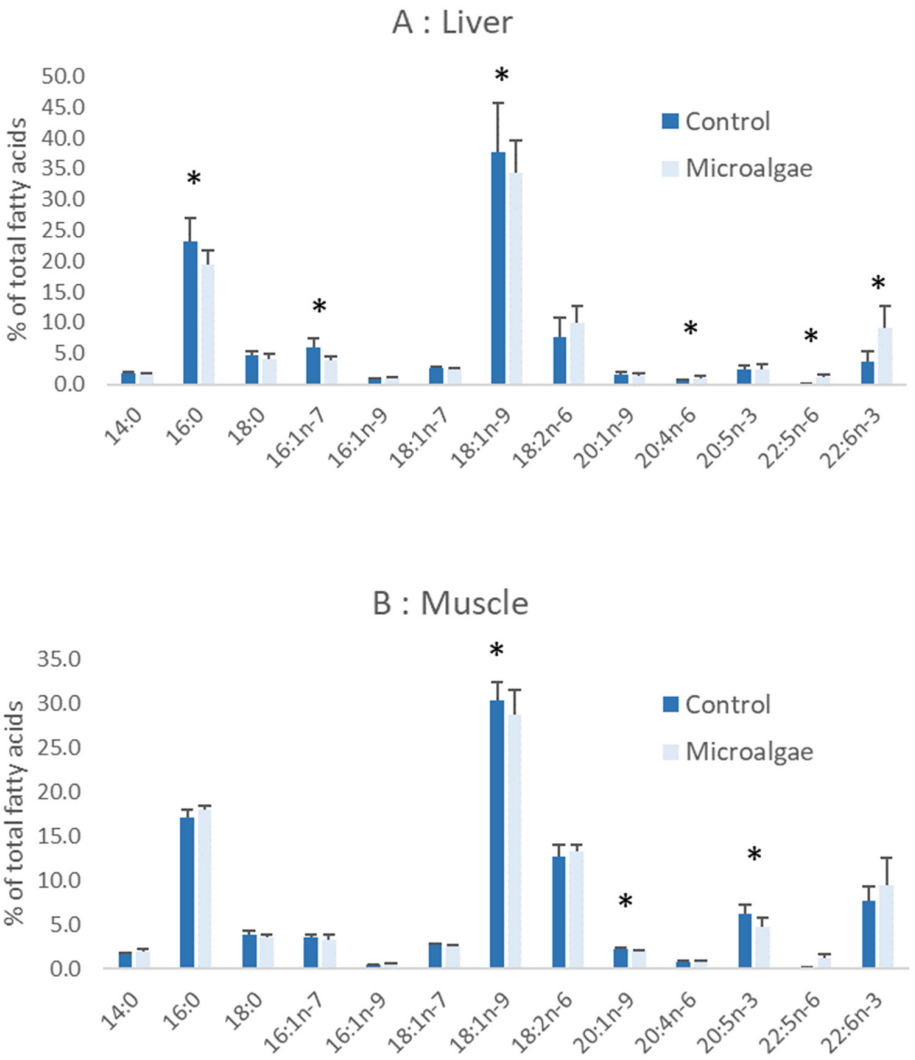


Figure 5. Fatty acid composition (expressed in percentage of total lipid fatty acids) of liver (**A**) and muscle (**B**) of sea bass juveniles fed control and microalgae diets for five weeks and a half. * indicates the significant differences between both treatments.

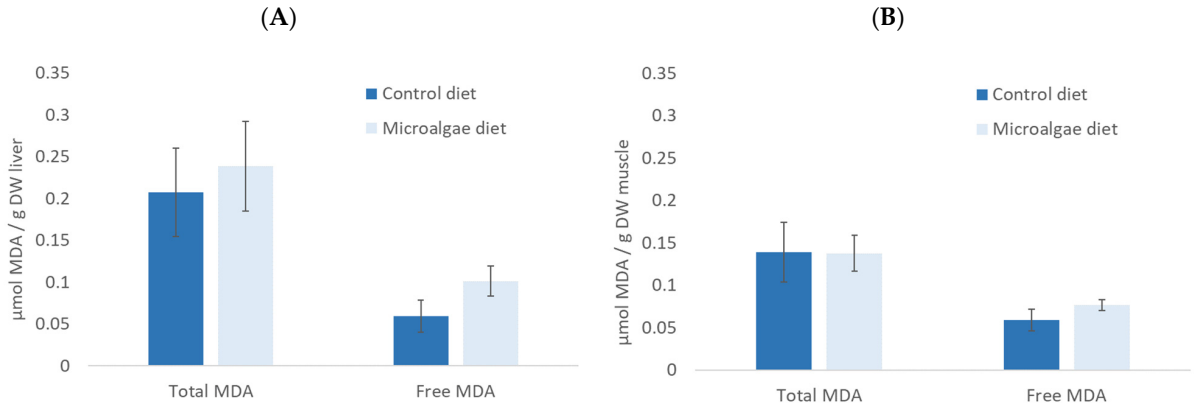


Figure 6. Total and free malondialdehyde (MDA) in the liver (**A**) and muscle (**B**) of sea bass juveniles fed control and microalgae diets for 5 weeks and a half.

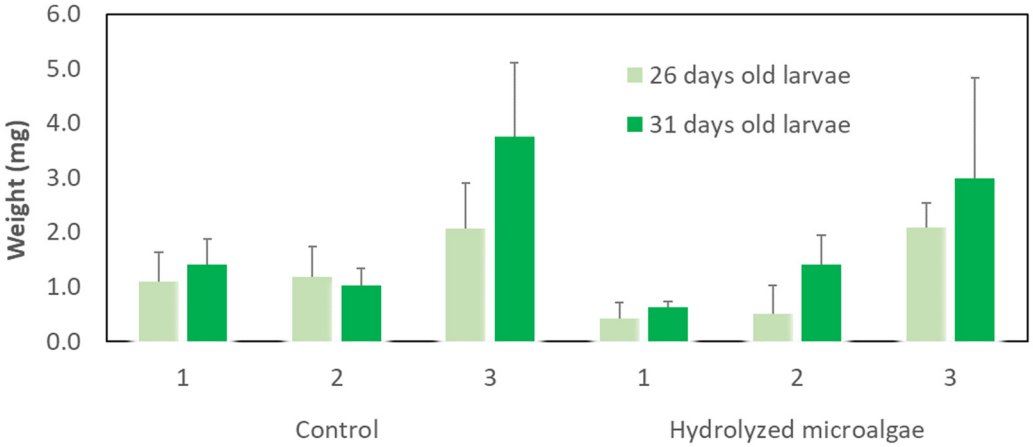


Figure 7. Weights of 26- and 31-day-old sea bass (Dicentrarchus labrax) larvae fed with the control, and hydrolyzed microalgae diets, in their respective 3 tank replicates.

After 41 days of rearing, the survival percentage was low for all the tanks (Table 4) and below 5% for most tanks. The highest number of live larvae was reported in one control tank having a survival percentage of 26%. Nevertheless, the chi-2 test comparing the six tanks did not reveal a statistically significant difference between dietary conditions.

Table 4. Survival of sea bass, Dicentrarchus labrax, larvae fed with control, and hydrolyzed microalgae diets from day 20 to 41 after hatching.

Micro-Diet Conditioning	Tank Number	Survival %
Control	1	1.4
	2	2.7
	3	26.4
Hydrolyzed microalgae	1	0.9
	2	1.7
	3	2.5

The statistical comparison of the fatty acid compositions in neutral and polar lipids of larvae according to the tested diets revealed only a few differences (Tables S6 and S7). The level of reserve lipids (47% of the FA present as neutral lipids) was higher in the larvae fed with the control micro-diet (Table S6). The percentage of saturated fatty acids in neutral lipids was higher in the larvae fed with the hydrolyzed microalgae micro-diet. The 18:2n-6, the most abundant FA in the neutral lipids, tended to be in higher proportion in larvae fed with the control diet while the 22:5n-6 tended to be higher in larvae fed with the hydrolyzed micro-diet (Table S6). The amount of DHA associated with neutral lipids expressed as mg/g DW was higher in the larvae fed with the control micro-diet (3.1 vs. 2.0 mg/DW, respectively), while the DHA percentage of total fatty acids was lower (9.2% vs. 9.9%, respectively) (Table S6).

In polar lipids, the proportions of 20:2n-6 and 22:5n-6 were significantly higher in larvae fed with the hydrolyzed micro-diet than in larvae fed with the control micro-diet (Table S7). The percentage of 15:0 in both neutral and polar lipids was higher in larvae fed with the hydrolyzed microalgae micro-diet than in larvae fed with the control micro-diet (Tables S6 and S7).

The level of secondary oxidation in the larvae partially reflects the level of secondary oxidation of their respective micro-diets, but differences were smaller and not statistically significant (Figure 8). The larvae fed with the control diet tended to have a lower level of total and free MDA than those fed with the hydrolyzed microalgae micro-diet (Figure 8).

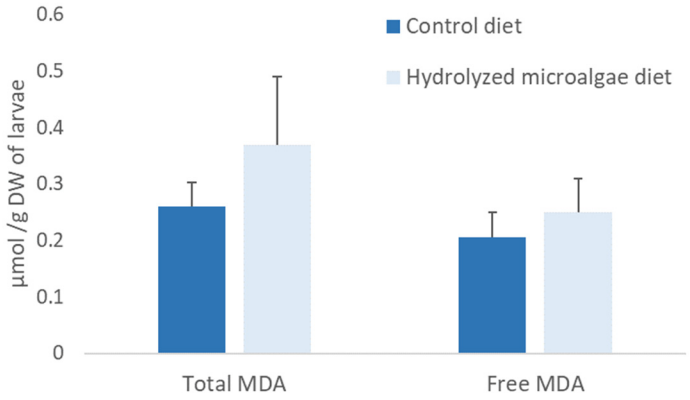


Figure 8. Total and free MDA of sea bass larvae fed control, and hydrolyzed microalgae diets from day 20 to 41 after hatching.

4. Discussion

The use of *Aurantiochytrium mangrovei* produced on a digestate-based medium was investigated as a microalgal ingredient in the nutrient formulation of sea bass (*Dicentrarchus labrax*) fish feed for juveniles and larvae. *Aurantiochytrium mangrovei* was selected in this study due to its richness in long-chain n-3 polyunsaturated fatty acids, especially DHA (or 22:6n-3). The sea bass juvenile experimental feed included 15% of non-hydrolyzed biomass. An inclusion level of 15% was targeted to have a significant impact on fishmeal and fish oil replacement in fish feed. The seabass larvae experimental feed included the same level (15%) of *A. mangrovei* biomass but in a hydrolyzed form, as protein hydrolysates were previously shown to enhance larval growth and/or survival performance of European sea bass *Dicentrarchus labrax* larvae.

4.1. From Microalgae Biomass Production to Fish Feed Formulation

4.1.1. Cellular and Biochemical Changes during Batch Cultivation

The Thraustochytrid *Aurantiochytrium mangrovei* was cultivated according to cultivation optimization performed by De La Broise et al. [28]. The culture grew exponentially for 38 h, resulting in up to 3.0×10^8 cell mL^{-1}, and reached a plateau until the end of the cultivation (62 h), obtaining 10.4 g L^{-1} of equivalent dry biomass.

Cell complexity and cellular neutral lipid content (as estimated by Bodipy staining) increased similarly to cell density, suggesting that increased complexity (or granularity) was likely related to lipid accumulation in the ageing culture. Bodipy fluorescence dye preferentially stained neutral (reserve) lipids [29]. Thus, it can be assumed that the increase in measured green fluorescence after Bodipy staining reflected the lipid body accumulation within the cells as it followed the increase in lipid content in the biomass (total fatty acid content per DW) (Tables S2 and S3). The cellular lipid content and lipid accumulation in biomass were particularly high after 38 h of cultivation, when vegetative division stopped or drastically slowed down. At this stage, most of the lipids were found in the form of neutral lipids (>80%). The predominant lipid class of neutral lipids is triacylglycerides in *Aurantiochytrium mangrovei* (data not shown).

An increase in lipid body was previously reported by Morita et al. [33] during synchronous growth of *Schizochytrium limacinum* SR21. Similarly, during the fed-batch fermentation of *Schizochytrium* sp., Zhao et al. [34] reported that lipid bodies were small during the lag phase, started to increase in number during the balanced growth phase and merged into larger lipid bodies during the lipid accumulation stage. They established a linear relationship ($R^2 > 0.98$) between the increase in cells full of lipid body and the lipid content of the biomass.

In our experiment, cellular lipid reserve (measured as green fluorescence after Bodipy staining) decreased between 46 and 62 h, suggesting that cells started to consume their energy reserve. It likely corresponded to the lipid turnover stage described by Zhao

et al. [34]. The authors observed a decrease in cells full of lipid body between 96 and 120 h of cultivation when glucose and nitrogen in the medium were almost exhausted.

The decrease in cell size reported in the present study when the culture is ageing may reflect the decrease in large zoosporangia and their replacement by cells full of lipid bodies as reported by Zhao et al. [34].

The fatty acid composition of our biomass was very similar to previous studies on Thraustochytrid [9,34–36], with 22:6n-3 (DHA) and 22:5n-6 (n-6 DPA) as major PUFA. In our study, 22:6n-3 (DHA) and 22:5n-6 (n-6 DPA) ranged from 43% to 53% and from 12% to 17% of total fatty acids, respectively. The DHA percentage in polar lipids (phospholipids) (52–57%) was consistently higher than in neutral lipids (36–42%). As DHA in the form of phospholipids is nutritionally preferred to DHA in the form of triacylglycerides, this could have some implication in future industrial cultivation strategies attempting to favor a biomass richer in phospholipids.

4.1.2. Biomass Downstream Processing

Following the cultivation of *A. mangrovei*, the influence of biomass concentration using cross-flow filtration (from 10 to 90 g L^{-1}) on the n-3 LC-PUFA was evaluated. Results showed that only the DHA in polar lipids slightly decreased from 60% to 53% while neutral lipids remained stable at 41%. This may reflect that DHA in the form of phospholipids are more sensitive to degradation and/or oxidation than in the form of triacylglycerides. The freeze-drying process allowed for the preservation of DHA content in the microalgal biomass to 40.5%, slightly lower than in the concentrated biomass. In addition to supplying high-quality n-3 LC-PUFA, the microalgae biomass contained all the essential amino acids required in finfish aquaculture in similar proportions to those found in fishmeal [37] (Table S8).

4.1.3. Fish Feed Formulation and Composition

The fish feed including the dried microalgal biomass was enriched with C22 PUFA, 22:6n-3 and 22:5n-6. It allowed the 22:6n-3/20:5n-3 to more than triple, reaching up to 4.5. It is widely recognized that this ratio has to be above 2 to ensure good survival and growth in farmed finfish. The fatty acids found in higher proportions in the control diet as compared to the microalgae reflect the natural origin of the fishmeal and oil. The C16 PUFA, the 16:1n-7 and 20:5n-3 are usually found in diatoms [38], which are at the base of the trophic chain supporting small pelagic fish generally harvested in upwelling zones [39].

Similar to other oleaginous microalgae species, the proportion of reserve lipids increased when the culture aged and reached the stationary phase. In the present study, the culture was stopped at the late exponential/early stationary phase in order to maintain a high level of n-3 LC-PUFA in the form of the phospholipids (29–36%). Kissinger et al. [19] highlighted that fishmeal is also important in supplying essential n-3 LC-PUFA for marine carnivores in the form of phospholipids, especially when the diets contain little or no fish oil. Commercially available *Schizochytrium* biomass is largely dominated by n-3 LC-PUFA in the form of triacylglycerides (data not shown). The biomass produced in the present study contained 20% of n-3 LC-PUFA in the form of phospholipids, which may represent a nutritional benefit, especially for fish in their early development stages. It could be interesting to shorten the cultivation duration in order to favor the amount of phospholipids in the biomass.

Although the inclusion of dried microalgae biomass at 15% in the fish diet resulted in an improvement of the essential n-3 LC-PUFA, 22:6n-3 + 20:5n-3, this may result in a higher susceptibility of this diet to oxidation [40]. Total and free MDA increased by 30% in the microalgae diet as compared to the control diet. Nevertheless, it did not appear to affect the zootechnical performance of sea bass juveniles.

The higher MDA content (secondary oxidation) in the microalgae diet (fed to Juveniles) may have non-exclusive origins. Firstly, the harvested biomass was not protected by the addition of any antioxidant, while this is generally the case for commercially available

ingredients containing a high proportion of PUFA. Secondly, after its manufacturing, the microalgae diet contained more long-chain PUFA (12.2%) than the control diet (8.9%). This may have increased the "targets" for oxidation during diet manufacturing. Although a high oxidation level of the microalgae diets did not impair the growth performance, the addition of commercial antioxidants could be considered to preserve the nutritional quality of the microalgal biomass.

The *Aurantiochytrium*-produced biomass was richer (in % DW) in proteins as compared to commercial Thraustochytrid biomass (30% vs. <10%, data not shown). This allows for a higher proportion of fishmeal to be replaced, potentially up to 30–40%, as it is well balanced in terms of essential amino acids in addition to its high DHA content. The amino acid profile of the dried microalgae biomass was very similar to the fishmeal amino acid profile (Table S4). Although the dried microalgae biomass contained fewer proteins (around 30%) than fishmeal (60–70%), it is a very good source of essential amino acids for the sea bass diet. To increase the inclusion percentage of dried microalgae biomass, an increase in the microalgal protein content would be necessary by optimizing cultivation conditions and maintaining the exponential phase. Additionally, the harvesting conditions could also be improved by coupling cross-flow filtration with centrifugation, improving solid matter percentage (up to 20%). This would reduce the ash content of the biomass prior to drying and allow for limiting the ash content in formulated diets.

4.2. Nutritional Value of Microalgae for Sea Bass Juveniles

Substitution of 15% of a standard juvenile sea bass feed resulted in similar growth performance after 38 days of experiment. The assimilation of the microalgal lipids was confirmed by monitoring specific FA. The C22 PUFA 22:6n-3 and 22:5n-6 increased 2.5- and 14.5-fold in the liver of fish fed with the microalgae diet. The FA supplied by the microalgae diet more intensively imprinted the liver than the muscle, probably linked to the liver being directly bound to the digestive system. It can also be expected that muscle, a bigger tissue, could take more time to reflect dietary changes. Overall, the results have demonstrated that the microalgal biomass was well incorporated/assimilated in juvenile tissues.

Similarly, in the literature, the inclusion of *Schizochytrium* sp. in the fish diet up to 16% of DW (fully replacing fish oil) resulted in higher weight gain and DHA deposition, as well as higher DHA/EPA in Nile Tilapia fillets [16]. Additionally, *Schizochytrium limacinum* has been proven effective as the main lipid source in diets for giant grouper without significantly affecting fish performance or condition [20].

Channel catfish (*Ictalurus punctatus*) fed with diets containing 1.0% and 1.5% dried algae *Schizochytrium* gained significantly more weight than fish fed with diets containing 0% and 0.5% dried algae [17]. Concomitantly, the content of 22:6n-3 and 22:5n-6 of channel catfish fillet increased as dietary levels of dried algae increased. Change in PUFA composition was also observed in salmon fed a diet with Thraustochytrid oil, revealing higher percentages of 22:6n-3 and 22:5n-6 (33% and 7% in white muscle, respectively) as compared to salmon fed a control diet with fish oil (20.4% and 0.4%, respectively).

Although arachidonic acid (20:4n-6) was absent from the microalgal biomass, its percentage increased in the liver of fish under the microalgae diet. This likely reflects the retro-conversion (or beta oxidation) of the 22:5n-6 into 20:4n-6. Similar observations were reported by Miller et al. [11], in which a higher concentration of arachidonic acid found in red and white muscles of salmon fed a diet containing Thraustochytrid oil originated from the retro-conversion of DPA-6 (22:5n-6) present in high proportions in such oil. Similarly, both ARA (20:4n-6) and DPA (22:5n-6) contents in seabream larvae fed *Schizochytrium*-containing diets were higher than in larvae fed with the standard diet with fish oil [15]. The authors also suggested that DPA was retro-converted to ARA by oxidation. *Schizochytrium* oil and biomass may have an additional benefit to DHA supply by contributing to ARA supply.

Hart et al. [14] reported a very good digestibility of *Schizochytrium* sp. biomass by Atlantic salmon (*Salmo salar*) juveniles. Apparent digestibility coefficients of protein, lipid, and gross energy of *Schizochytrium* biomass were 93.9%, 67.1% and 70%, respectively. PUFA

and DHA were more digestible in the diet containing *Schizochytrium* biomass (96.7% and 96.3%) than in the reference diet (96% and 93.1%, respectively). A blend of soybean meal, soy protein concentrate and algal meal from *Schizochytrium limacinum* could replace 40% of marine proteins from fishmeal and squid meal [20].

As fatty acids with a high degree of saturation are more prone to lipid peroxidation, MDA was measured in the juveniles' liver and muscle at the end of the dietary experiment. Feeding the sea bass juveniles with the microalgae diet tended to increase the amount of total and free MDA, showing that the higher oxidation level of the microalgae biomass diet was translated into in the fish tissues fed this diet.

Although tested in shrimp diets, the inclusion of commercial Thraustochytrid biomass from 2.5% to 7.5% did not result in an increase in MDA content in the tail muscle of shrimp [41]. However, commercial Thraustochytrid biomasses are generally protected from oxidation by the addition of antioxidants.

Some studies explored the change of microbial composition upon feeding with a diet containing Thraustochytrid biomass. The partial substitution of fish oil component by the microalgae *Schizochytrium limacinum* (5% inclusion) in the diet of the farmed rainbow trout (*Oncorhynchus mykiss*) resulted in a greater level of microbial diversity in the distal intestinal microbiota than in the trout fed with a control diet without microalgae [42]. Inclusion of Thraustochytrid biomass in the Tilapia fish diet modulated its microbiome and increased the red blood cell and lymphocyte concentrations [43]. It would be interesting to confirm similar effects in sea bass fed with a diet enriched with Thraustochytrid biomass.

4.3. Nutritional Value of Microalgae for Sea Bass Larvae

To maintain fast growth and optimal visual and neural development during the early life stages, fish larvae require a high level of n-3 LC-PUFA and especially DHA [44,45]. Thus, we tested the influence of substituting fish oil in sea bass larvae with DHA-containing Thraustochytrid biomass. As the inclusion of fish protein hydrolysates in marine fish feed improves larvae fish growth and survival [21], we included hydrolyzed microalgae biomass in the micro-diet. Tanks receiving the micro-diet containing 15% of hydrolyzed microalgae biomass performed similarly to the tanks receiving the control micro-diets. The fact that the 18:2n-6 in neutral and polar lipids (Tables S5 and S6), as well as triacylglycerides in neutral lipids (Table S9), were found in higher proportions in larvae fed with control micro-diets as compared to larvae fed with hydrolyzed microalgae diet may reflect slightly better assimilation of the artificial micro-diet.

As the inclusion of 15% of hydrolyzed microalgae biomass in the micro-diet did not impair the development of sea bass larvae, it is tempting to consider that the hydrolyzed microalgae biomass has the potential to substitute both fish oil and pre-digest fishmeal (CPSP 90) in larvae artificial micro-diets. Nevertheless, the low general survival percentages of larvae in this dietary conditioning experiment limit further exploitation of the obtained biochemical results. In the present study, high mortalities occurred when an artificial micro-diet was introduced in their regime. Feeding with artificial micro-diets, particularly before the complete maturation of the digestive functions, led to poor larval performance compared to live prey [21,23]. Survival percentages between 15–35% were usually reported in the present experimental rearing facility with sea bass larvae fed exclusively with an artificial micro-diet as compared to >50% for larvae fed with live prey [21,46]. The commercial hatcheries introduce artificial food generally around the 30th day of development of sea bass larvae [47] in order to ensure good survival, which may suggest that we should not have tested our experimental feed in only 13-day-old larvae. Nevertheless, this could only partially explain the low survival percentages reported in the present study. Such low survival may also reflect some anterior weakness or weakening due to their transportation from the marine hatchery of Gravelines Ichtus (France). Although the experimental system used allows for good larval growth and survival, the rearing conditions for this batch of larvae may have been sub-optimal.

5. Conclusions

The feed trials on sea bass juveniles and larva indicated that microalgal biomass can partially replace fishmeal (pre-digest fishmeal) and fish oil in sea bass feed. This allowed for an equivalent growth rate and improved DHA delivered to sea bass juveniles. However, the high performance variability between larvae rearing tanks did not allow for a clear and definitive conclusion and would require further testing, introducing the experimental feed later in the sea bass feeding sequence, around day 20 of development. Overall, feeding carnivore fish species with diets containing Thraustochytrid oil or biomass increased the amount of DHA and n-3 LC-PUFA in fish fillets, which would make them nutritionally beneficial to the human consumer.

Considering that fish oil has higher levels of EPA than DHA, the inclusion of Thraustochytrid oil or biomass may improve the DHA/EPA ratio as required in certain aquaculture applications such as in marine fish larvae rearing in husbandry. Beyond supplying a high level of n-3 LC-PUFA, Thraustochytrid may also bring other nutritional compounds such as essential amino acids, vitamins, and pigments. Thus, it cannot be excluded that part of the ability of Thraustochytrid biomass to replace fish meal and oil may also be due to other nutrients than n-3 LC-PUFA.

Finally, the introduction of digestate as a source of nitrogen in the culture medium resulted in good quality microalgae biomass, with the needed biochemical composition, and including it in fish feed to replace fish meal and oils in fish feed did not cause any detrimental effect on the animals. However, European regulation states that animal-based digestates cannot be used for feed production, including aquafeed (de la Broise et al., 2022). Today, only crop-based digestates can be used in the developed process. Alternatively, other by-products from the food industry could be investigated as potential sources of carbon and/or nitrogen to produce Thraustochytrid biomass.

In the future, further optimization of the workflow between digestate or by-product processing, microalgae cultivation and fish feed production needs to be performed. Additionally, we will need to confirm the zootechnical and biochemical results.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su142114573/s1. Table S1: Chemical characterization of digestate from COOPERL Organic biological waste (pig manure), Brittany (FR) https://www.nweurope.eu/projects/project-search/alg-ad-creating-value-from-waste-nutrients-by-integrating-algal-and-anaerobic-digestion-technology/publications/the-alg-ad-project-reports-and-deliverables/ (accessed on 27 September 2022). Table S2. Cellular parameters (concentration, size, complexity, lipid content) as measured by flow cytometry on two 500 L culture tanks. Table S3. Fatty acid composition (expressed as % of total fatty acids in the fraction; n = 2 batches) in neutral lipids (NL) and polar lipids (PL) and percentage of total lipids per dry weight of *Aurantiochytrium mangrovei* cultivated on yeast extract peptone medium enriched with liquid effluent from anaerobic digestion. Figure S1. Percentages of the major PUFA, DHA (A) and DPA (B), expressed as percentage of total fatty acids in polar, neutral, and total lipids of *Aurantiochytrium mangrovei* biomass collected every hour during 5 h of cross filtration. Table S4. Amino acid and fatty acid profiles of the dried microalgae biomass included in the experimental microalgae diet for juveniles and micro-diet for larvae. Table S5. Fatty acid composition expressed in percentage of the control, and hydrolyzed micro-diets for sea bass larvae. Table S6. Fatty acid composition of neutral lipids (expressed as % of total fatty acids of the fraction) of sea bass (*Dicentrarchus labrax*) larvae fed control, and hydrolyzed microalgae diets 41 days after hatching. Table S7. Fatty acid composition of polar lipids (expressed as % of total fatty acids of the fraction) of sea bass (*Dicentrarchus labrax*) larvae fed control, and hydrolyzed microalgae diets 41 days after hatching. Table S8. Comparison of the amino acid profile (expressed in % of total amino acids) of *Aurantiochytrium mangrovei* with this of fish meal. Table S9. Lipid class composition of sea bass (*Dicentrarchus labrax*) larvae fed control and hydrolyzed microalgae diets 41 days after hatching.

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Effects of Replacement of Fish Oil with Microbial Oil (*Schizochytrium* sp. T18) on Membrane Lipid Composition of Atlantic Salmon Parr Muscle and Liver Tissues

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Abstract: A 16-week feeding trial was conducted to investigate effects of replacing dietary fish oil (FO) with docosahexaenoic acid (DHA)-rich microbial oil (MO) from *Schizochytrium* sp. (T18) on membrane lipid composition of Atlantic salmon parr liver and muscle tissues. Four nutritionally balanced diets were formulated with varying levels of FO, MO, and canola oil (CO), including a control diet with 20% FO, a secondary control diet with 10% FO and 10% CO, and two experimental diets that completely replaced FO with a low (5%) and high (10%) proportion of MO. No significant differences were observed in growth parameters (81–98 g; weight gain), total lipid class composition, and total sterol content among the dietary treatments. However, there were significant differences in the proportions of individual ω3 and ω6 fatty acids in both liver and muscle tissues, reflecting the different dietary treatments. Notably, the presence of low eicosapentaenoic acid (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. The results also showed that DHA was present in very high proportions in the cellular membrane, particularly in muscle tissue, with low levels of linoleic acid and alpha-linolenic acid. Overall, the findings suggest that MO derived from *Schizochytrium* sp. (T18) could be a potential substitute for FO in the diet of farmed Atlantic salmon.

Keywords: Atlantic salmon; fish oil; microbial oil (*Schizochytrium* sp. T18); EPA; DHA; ARA; polar lipid fatty acids; sterols

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1. 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 [1–3]. 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 ω3 LC-PUFA that are abundant in FO [4]. 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, DHA and ARA are also termed EFA. 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 [5]. However, 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 [6]. Top carnivores have limited ability to synthesize LC-PUFA even from dietary precursors and require the inclusion of LC-PUFA directly in their diet [7]. For Atlantic salmon, EPA, DHA, and ARA are considered EFA that need to be supplied in the diet, although they can synthesize them when large amounts of ALA and LA are provided [8]. 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 [9]. They are also known to provide health benefits to humans as consumers in relation to cardiovascular disease, inflammatory disease, and neurological disorders [10–12].

Feed composition has changed considerably over the last decades from mainly marine ingredients to an increasing inclusion of plant ingredients [13]. While terrestrial plant oils can provide digestible energy to fish, fish health and the consumer products resulting from those fish have become compromised in recent years [14]. 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 potential to replace conventional ingredients used in aquafeed [15,16].

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 [17]. 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 [18]. Among numerous strains, *Schizochytrium* sp. is noteworthy and often considered a satisfactory alternative to FO due to the advantages of fast growth rate, high productivity, and its lipid profile [19]. *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 [15]. It is worth noting the low proportion of EPA (0.5%) present in MO and how this might affect the growth parameters, the immune system, and lipid deposition in the tissues. The present study builds on the findings of Wei et al. (2021) [20], which demonstrated the potential of MO as a substitute for FO in the diet of farmed Atlantic salmon parr and its effect on total fatty acid concentrations. The current study provides a more comprehensive investigation of the impact of dietary lipids on the membranes of Atlantic salmon parr liver and muscle tissues by quantifying phospholipid fatty acids (PLFA) and sterols. This addresses a significant research gap in the field, as most feeding trials that incorporate alternative lipid sources do not evaluate PLFA, cholesterol, and phytosterols. Furthermore, we compare the PLFA proportions with total fatty acid proportions to gain a deeper understanding of the dietary impact on fish lipid composition.

2. Materials and Methods

Diet manufacture and feeding trials were done at Dalhousie University, Truro, Nova Scotia.

2.1. 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%); an experimental diet (LMO) composed of complete replacement of FO with a lower proportion of MO (5%); a second experimental diet (HMO) composed of complete replacement of FO with a higher proportion of MO (10%). For extended details on diet formulation, see Table A1. 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 [21]. The MO used in this experiment was provided by Mara Renewables (Dartmouth, NS, Canada).

2.2. Experimental Fish and Set-Up

Atlantic salmon parr were received from the Margaree Fish Hatchery (Nova Scotia Department of Fisheries and Aquaculture, Margaree Valley, NS, Canada). Fish were inspected by the provincial government veterinarian (Department of Fisheries and Aquaculture) prior to transfer to Dalhousie University Agriculture Campus Aquaculture lab. The fish received a health status permit certificate (pathogen and disease free) that allowed transfer from the provincial hatchery to the university. A total of 360 parr (21.9 $\pm$ 4.7–26.8 $\pm$ 4.1 g) (mean $\pm$ SD) were equally and randomly distributed into 12 tanks (200 L volume) in a flow-through freshwater system at Dalhousie University Agriculture Campus Aquaculture lab (Truro, NS, Canada). 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 until visual 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. Tanks were checked for mortalities twice daily throughout the trial. Temperature and dissolved oxygen were measured and recorded daily. Weekly measurements included pH and total gas pressure.

2.3. Tissue Sampling

Feed was withheld one day before sampling 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 was buffered using sodium bicarbonate (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. The sampled tissues were then placed in lipid-clean glass vials with chloroform. The air space was filled with nitrogen before capping the vials and sealing them with Teflon tape. The samples were then stored in a -20 °C freezer until extraction.

2.4. Ethical Approval

Ethical approval for the treatment of fish in this study was obtained in accordance with guidelines set forth by the Canadian Council of Animal Care. The study was conducted at the Dalhousie University Faculty of Agriculture, and the Institutional Animal Care Approved Protocol number was #2017-84. All efforts were made to ensure the welfare and ethical treatment of the fish used in this experiment.

2.5. Lipid Extraction

Lipid samples were extracted according to Parrish (1999) [22]. 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 (EMD Millipore Corporation, Burlington, MA, USA) was then added back

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

2.6. 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 (EMD Millipore Corporation, Burlington, MA, USA) and 3 mL Hilditch reagent were added. The Hilditch reagent is prepared by dissolving 1.5 mL concentrated sulfuric acid (VWR International, Mississauga, ON, Canada) in 100 mL methanol (EMD Millipore Corporation, Burlington, MA, USA) that has been dried over anhydrous sodium sulphate (Fisher Scientific Company, Ottawa, ON, Canada). The mixture was capped under nitrogen, then vortexed and sonicated for 4 min before being heated at 100 °C for 1 h. The mixture was allowed to cool to room temperature, and then approximately 0.5 mL saturated sodium bicarbonate solution (Fisher Scientific Company, Ottawa, ON, Canada) was added, followed by 1.5 mL hexane (EMD Millipore Corporation, Burlington, MA, USA). 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.

2.7. Sterol Derivatization

Derivatization of sterols was performed according to Hailat & Helleur (2014) [23] by silylation with *N, O*-bis-trimethylsilyl trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (Supelco Inc., Bellefonte, PA, USA) to form their corresponding trimethylsilyl (TMS)-ethers. Lipid extracts were evaporated until dryness under a stream of nitrogen. 100 µL of BSTFA containing 1% TMCS was added to the lipid extract and heated at 85 °C for 15 min. Samples were then cooled to room temperature and excess reagent was evaporated under nitrogen gas. 500 µL of hexane/dichloromethane (1:1, by vol) (Sigma Chemicals, St. Louis, MO, USA), was added followed by addition of 100 µL of 100 mg/L of 5α-androstanol (Steraloids Inc., Newport, RI, USA) as internal standard and then stored at −20 °C until analysis by gas chromatography-mass spectrometry (GC-MS) and Gas Chromatography with Flame Ionization Detection (GC-FID).

2.8. 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 (Fisher Scientific Company, Ottawa, ON, Canada) through the column into a waste vial. Then the sample extract was directly applied to the silica using a long pipette followed by rinsing of 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 acetone-mobile polar lipid (AMPL) by rinsing the silica gel with 6 mL (2 × 3 mL) of acetone (EMD Millipore Corporation, Burlington, MA, USA). 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 2.6).

2.9. 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 according to Parrish (1987) [24]. 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/ethyl 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. On 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 consisted of hexane:ethyl 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). After the third development, the rods were scanned in the Iatroscan (100% of the rod) for the 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 °C where it was held for 0.5 min. The temperature ramped to 195 °C at a rate of 40 °C/min, held for 15 min, then ramped to a final temperature of 220 °C at a rate of 2 °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 °C and ramped to a final temperature of 250 °C at a rate of 120 °C/min. The detector temperature stayed constant at 260 °C. Peaks were identified using retention times from standards purchased from Supelco (Supelco 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.

For the quantification of sterols, a Varian CP-3800 GC/FID with a CP-8400 autosampler was used. Detection of sterols was performed using a DB-5 column. Analysis was run in splitless mode, with helium as the carrier gas at a pressure of 14.0 psi. Ten µL of sample was injected at an injector temperature of 290 °C. The initial temperature of the oven was set at 80 °C and held for 1 min. The oven temperature was then increased at a rate of 50 °C/min until it reached 200 °C. The rate was then decreased to 4 °C/min to reach a final temperature of 305 °C and held there for a period of 5 min. The total run time per sample was 34.65 min. A detector temperature of 315 °C was used. 5α-androstanol was used as the internal standard. Ratios of standard peaks to internal standard peaks were determined by integration and plotted against ratios of concentration of internal standard to sterol standard to generate calibration curves.

2.10. Statistical Analysis

The resulting data are 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 on 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. Results

The objective of this study was to investigate the potential of MO from *Schizochytrium* sp. (T18) as a replacement for FO in the diet of farmed Atlantic salmon parr. Specifically, we aimed to determine the effect of replacing FO with MO on membrane lipid composition in the liver and muscle tissues of Atlantic salmon parr. We hypothesized that replacing FO with MO would result in changes in the proportions of individual fatty acids in the membrane lipids of the fish. To test this hypothesis, we conducted a 16-week feeding trial in which we formulated four nutritionally balanced diets with varying levels of FO, MO, and CO and quantified the PLFA and sterol compositions of the fish liver and muscle tissues.

3.1. 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 [25]. The main lipid class was TAG (76.0%), followed by AMPL (10.4%), FFA (7.0%), PL (3.8%), and ST (1.8%) (Table 1). The dominant (>5%) fatty acids were 14:0 (11.0%), 16:0 (26.5%), ω6DPA (7.6%), and DHA (40.7%) (Table 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 making it 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 in 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 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 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 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 ± 0.0
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.

The MO sample was derivatized to its TMS-ethers and the sterol TMS-ethers identified were cholesterol, lathosterol, brassicasterol, 24-methylenecholesterol, 24-methylenelophenol, stigmasterol, and spinasterol. The peaks were identified by comparing the relative retention times with those of standards and confirmed by their corresponding mass spectra. In the FO diet, four phytosterols were identified; cholesterol, campesterol, 23,24-dimethylcholest-5-en-3β-ol, and 24-ethyl-5α-cholest-7-en-3β-ol (Table 3). In the FO/CO diet, these same phytosterols were also identified, with the addition of stigmasterol and brassicasterol. In the MO-containing diets, the same four phytosterols as were present in the control were identified, as well as stigmasterol. Additionally, brassicasterol was detected in both the LMO and HMO diets. In the HMO diet, one other phytosterol, lathosterol, was also detected. This sterol was one of the phytosterols detected in the original MO analysis. Statistical analysis indicated that there were no significant differences between the amounts of sterol present in each diet. Total sterol amounts ranged from 815 μg/g for the LMO diet to 1024 μg/g in the HMO diet.

Table 2. Lipid composition of experimental diets [1].

	FO	FO/CO	LMO	HMO
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 = 9 per treatment). Means with different superscripts indicate significant differences ($p < 0.05$) based on Tukey's post-hoc 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. Sterol composition of experimental diets expressed in μg/g [1].

	FO	FO/CO	LMO	HMO
Cholesterol	736 ± 229	551 ± 167	304 ± 151	349.8 ± 58.7
Brassicasterol	-	84.0 ± 13	56.9 ± 27	23.9 ± 13.8
Lathosterol	-	-	-	57.6 ± 9.1
Campesterol	58.9 ± 21	163 ± 53	129 ± 44	210.9 ± 58.7
Stigmasterol	-	102 ± 33	63.9 ± 22	38.0 ± 16.3
23,24-dimethylcholest-5-en-3β-ol	209 ± 87	427 ± 139	166 ± 58	247.7 ± 73.6
24-ethyl-5α-cholest-7-en-3β-ol	76.9 ± 28	71.2 ± 17	94.9 ± 19	96.1 ± 20.9
Total Sterol	1081 ± 91	1398 ± 70	815 ± 54	1024 ± 72.5

[1] Values are means ± standard deviation (n = 3 per treatment). FO = fish oil; FO/CO = fish oil/canola oil; LMO = low microbial oil; HMO = high microbial oil.

3.2. 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) [20], and key results are attached in Appendix A.

3.3. Liver Tissue Lipid Classes and Fatty Acid Composition

Initial liver tissue content was 34.4 mg/g wet weight (ww) total lipid, and it was mostly composed of polar lipid (Table 4). 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 4). 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%).

Table 4. 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}

Table 4. *Cont.*

	Initial	FO	FO/CO	LMO	HMO
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 post-hoc 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.

After 16 weeks of feeding, the fatty acid profile mostly reflected that of the fish fed diets, except the relative proportions of SFA, MUFA, and especially PUFA (Table 4). 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 (Figure 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 (Tables A4 and A5 in Appendix B) 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 B.

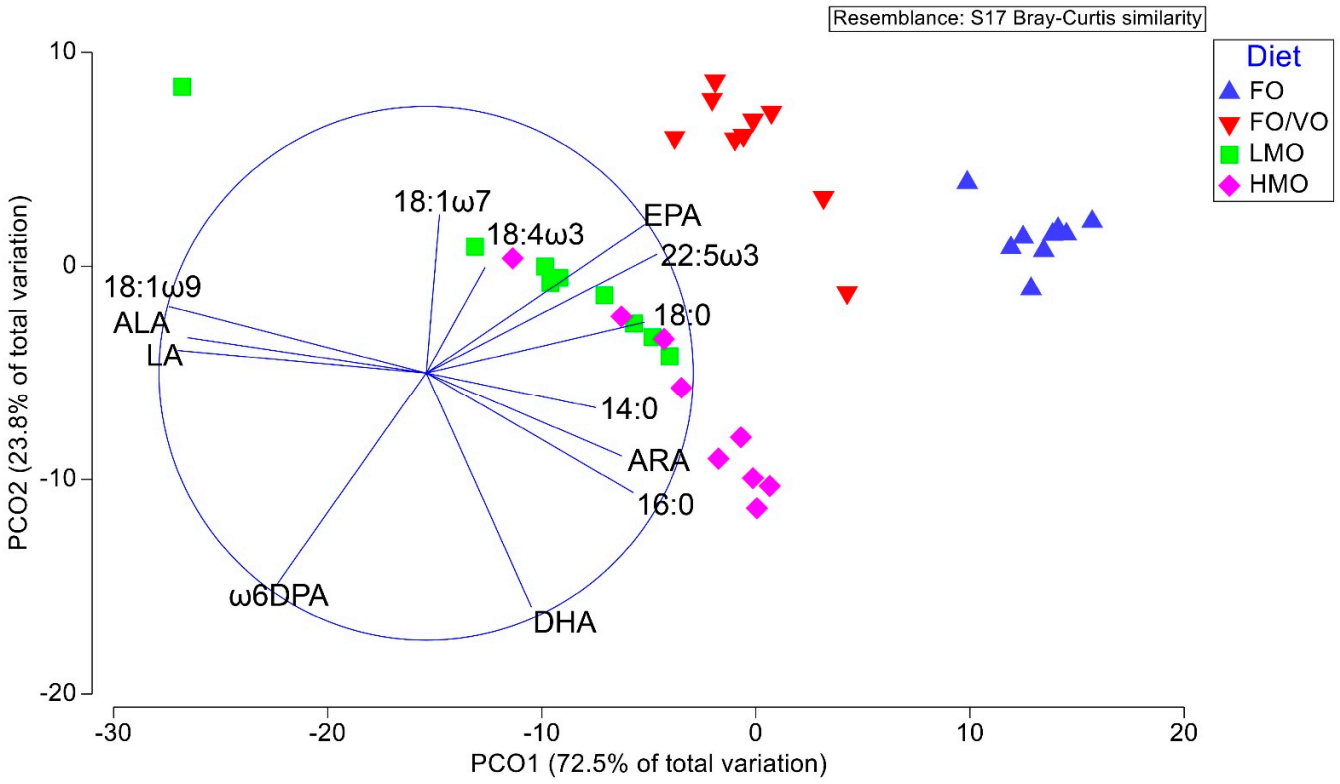

Figure 1. Principal coordinates analysis (PCO) of Atlantic salmon liver tissue total fatty acid composition (%) after 16 weeks of feeding experimental diets.

3.4. 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 5). Differences in EPA, DHA and ARA 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.

Table 5. 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 post-hoc 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.

Principal coordinates analysis of week-16 liver PLFA showed PCO1 and PCO2 (Figure 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 (Tables A6 and A7 in Appendix B) 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 B.

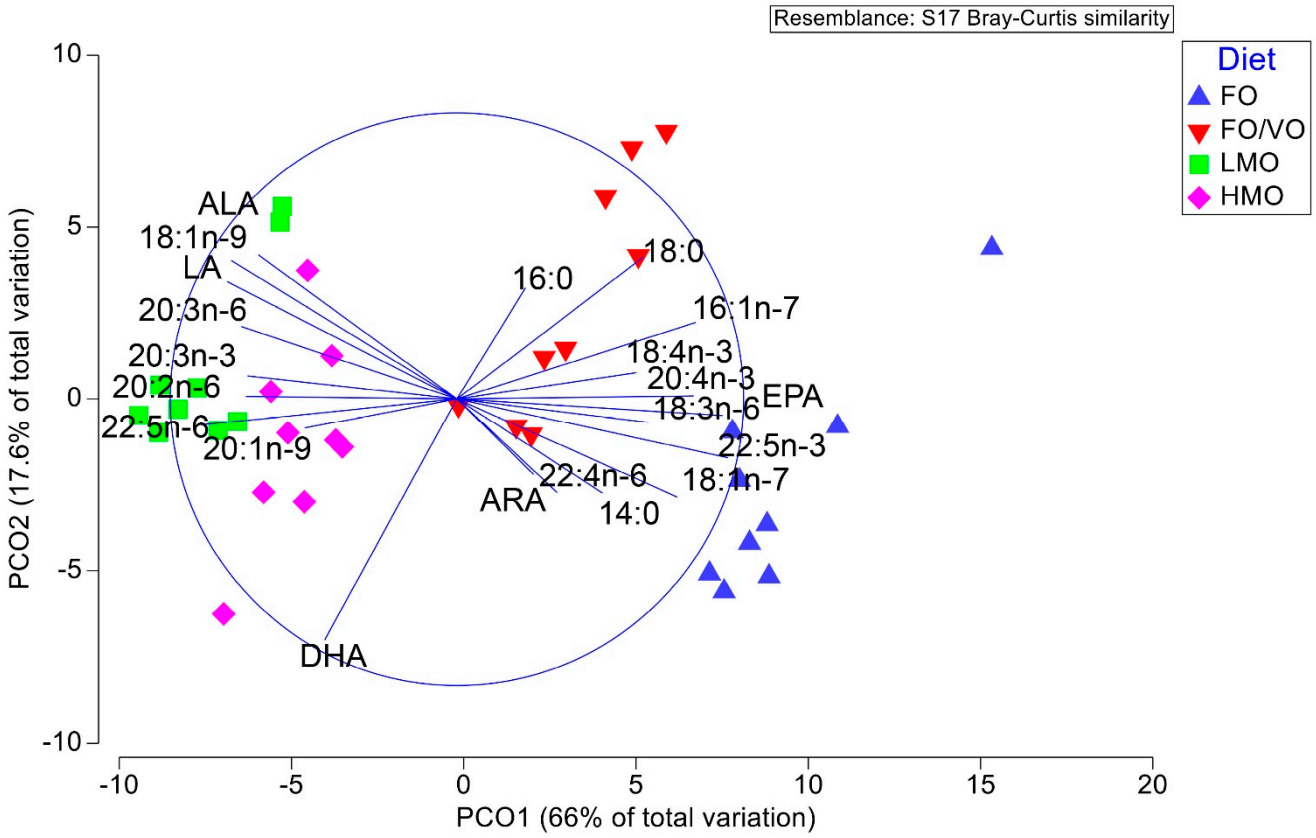

Figure 2. Principal coordinates analysis (PCO) of Atlantic salmon liver tissue phospholipid fatty acid composition (%) after 16 weeks of feeding experimental diets.

3.5. 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 6). 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 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 6). 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 EPA, DHA and ARA 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.

Table 6. 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 post-hoc 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.

Principal coordinates analysis of week-16 muscle total fatty acids showed PCO1 and PCO2 (Figure 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 (Tables A8 and A9 in Appendix B) 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 B.

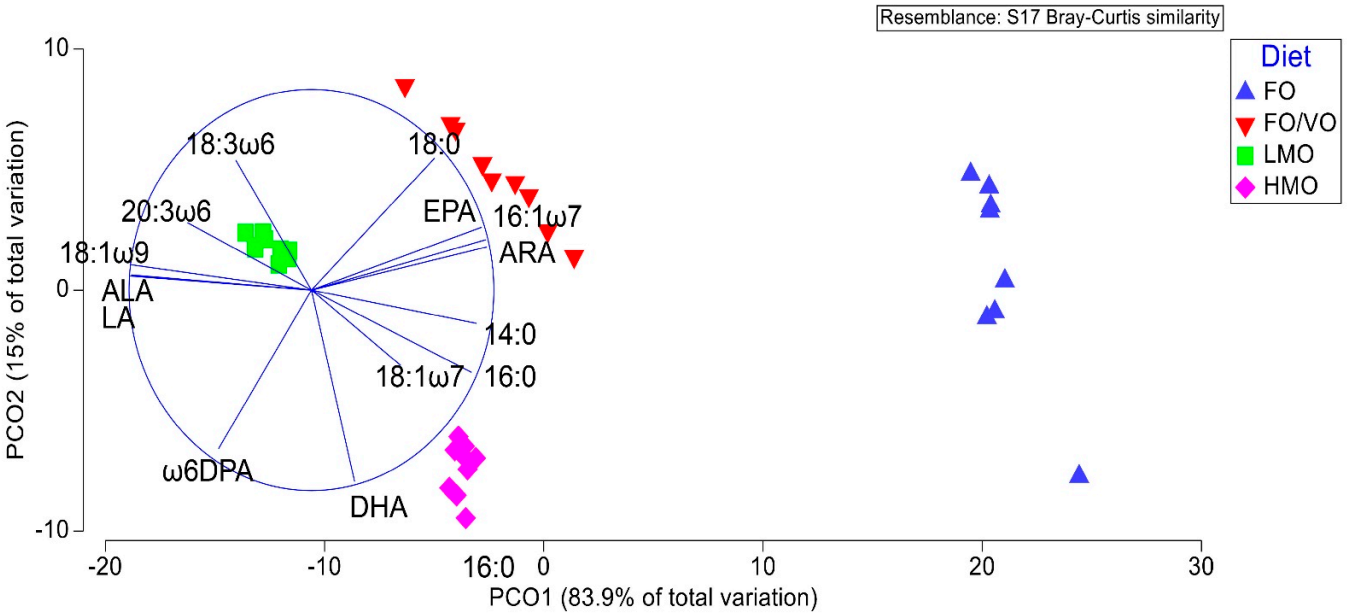

Figure 3. Principal coordinates analysis (PCO) of Atlantic salmon muscle tissue total fatty acid composition (%) after 16 weeks of feeding experimental diets.

3.6. 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 7). 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 (Figure 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 (Tables A10 and A11 in Appendix B) 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 B.

Table 7. Phospholipid fatty acid composition of Atlantic salmon muscle tissue after 16 weeks of feeding experimental diets [1].

	FO	FO/CO	LMO	HMO
		Fatty acid 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 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 post-hoc 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.

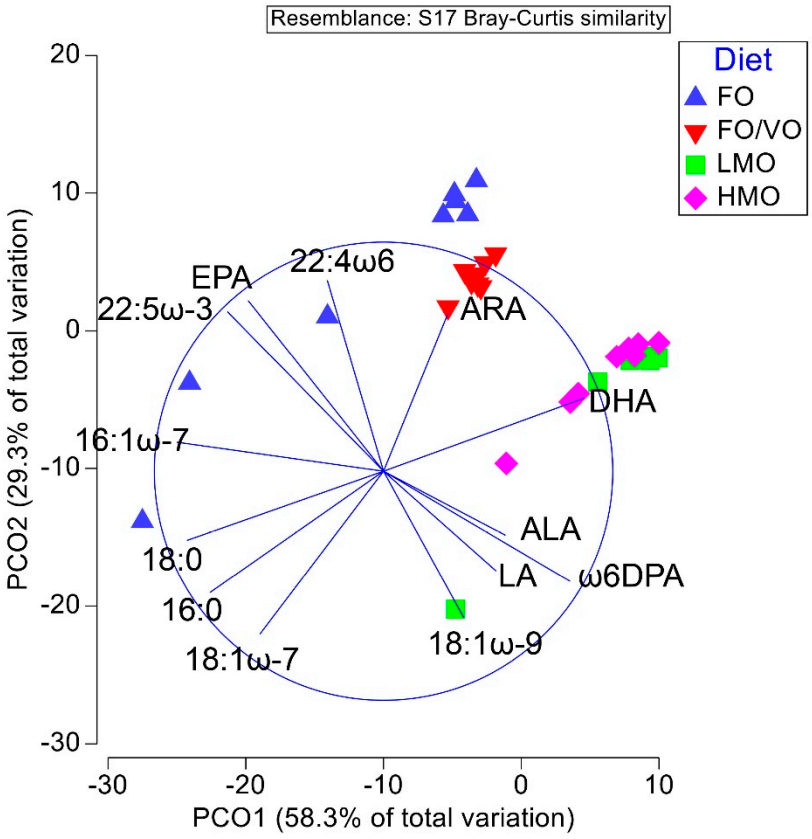

Figure 4. Principal coordinates analysis (PCO) of Atlantic salmon muscle tissue phospholipid fatty acid composition (%) after 16 weeks of feeding experimental diets.

3.7. Muscle Tissue Sterol Composition

After 16 weeks of feeding, the sterols identified in the muscle tissue extracts were cholesterol, cholestanol, campesterol, stigmasterol, and dinosterol. Cholesterol content was significantly higher in salmon fed MO-containing diets than in salmon fed the FO/CO diet, but not higher than in salmon fed the FO diet. Salmon fed the LMO diet had cholestanol, a derivative of cholesterol, present in all samples, and was highest in salmon fed the FO diet. Campesterol, which was also identified in the diet samples, was present in all samples, and the highest amount was found in salmon fed the LMO diet. Similarly, stigmasterol was present in all samples, with the highest amount being found in salmon fed the LMO diet. Dinosterol, a high molecular weight sterol, was present in all samples as well, with a higher amount found in salmon fed the LMO diet. Statistically significant differences were not found in muscle tissue concentrations of cholestanol, campesterol, stigmasterol or dinosterol.

Significant differences were found when sterol data were compared as proportions of total sterols (Figure 5). Brassicasterol, lathosterol, 23,24-dimethylcholest-5-en-3β-ol, or 24-ethyl-5α-cholest-7-en-3β-ol, which were identified in the experimental diets, were not present in the muscle tissue samples. Lathosterol was detected in only salmon fed the HMO diet at 5.6% and was not present in any of the muscle tissues. Campesterol was present in all diets at an average of 13.4% and all muscle tissues with an average of 7.2%, but there were no significant differences among them. Stigmasterol was present in most diets and all muscle tissue at low (<8%) levels but there were also no significant differences between treatments. Dinosterol was detected at even lower levels ($\leq$7%) in muscle only, but mean values were not significantly different to zero (Table 8).

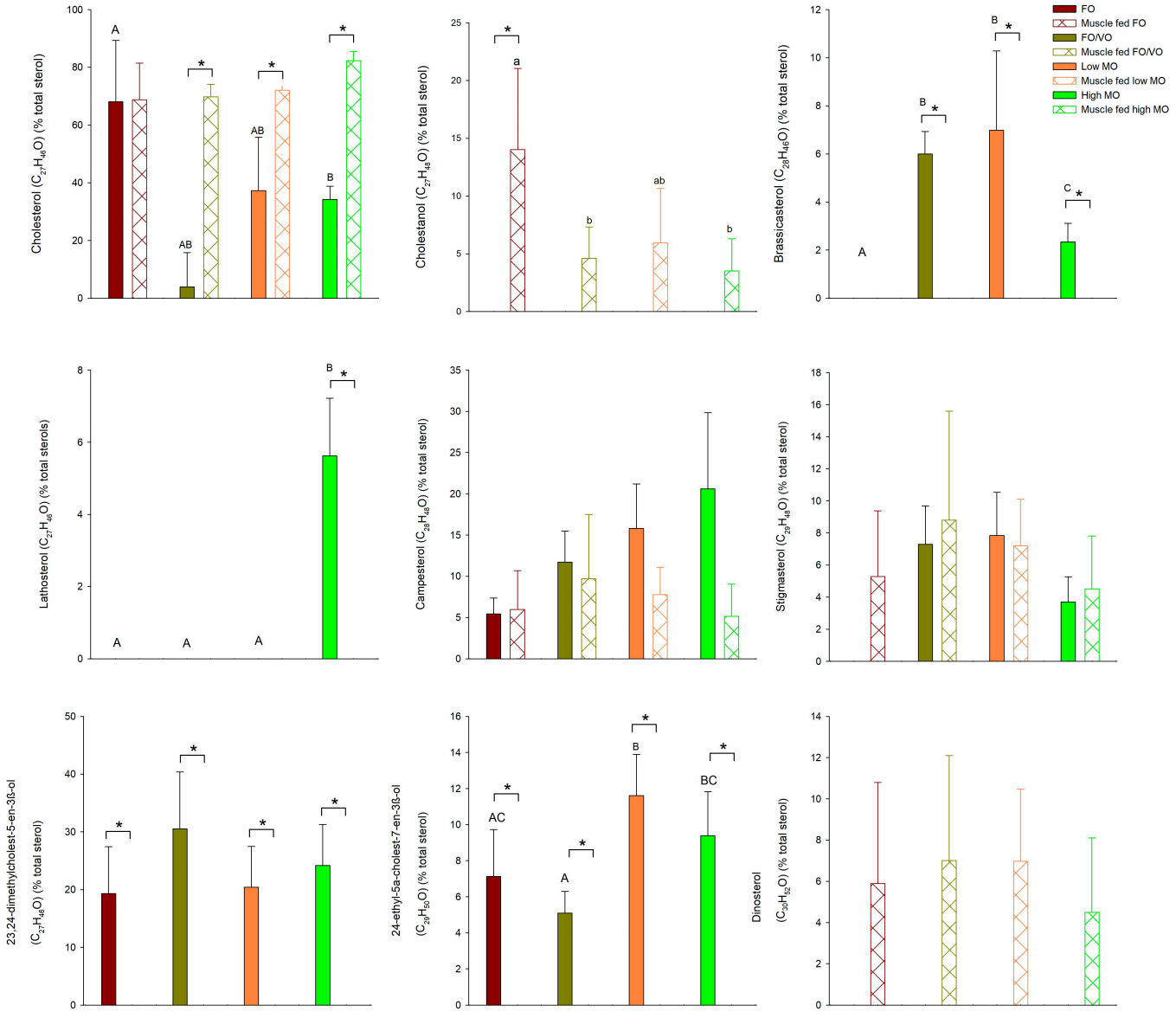

Figure 5. Identified sterols (in % of total sterols) found in experimental diets and corresponding muscle tissue from fish fed the control and experimental microbial oil diets. Upper case letters indicate significant differences in diet proportions; lower case letters in muscle proportions. Asterisks indicate significant differences between diet and muscle.

Table 8. Sterol composition of Atlantic salmon muscle tissue after 16 weeks of feeding experimental diets expressed in µg/g [1].

	FO	FO/CO	LMO	HMO
Cholesterol	89.9 ± 16.6 [a]	53.9 ± 3.35 [b]	119 ± 2.57 [a]	117 ± 4.38 [a]
Cholestanol	18.3 ± 9.19	3.57 ± 2.07	9.8 ± 7.79	5.03 ± 3.94
Campesterol	7.8 ± 6.07	7.5 ± 6.01	12.8 ± 5.45	7.31 ± 5.54
Stigmasterol	6.9 ± 5.38	6.83 ± 5.24	11.9 ± 4.93	6.41 ± 4.63
Dinosterol	7.7 ± 6.50	5.40 ± 3.91	11.5 ± 5.72	6.40 ± 5.07
Total Sterol	131 ± 9.8	77 ± 5.6	165 ± 5.3	142 ± 4.7

[1] Values are means ± standard deviation (n = 5 per treatment). Means with different superscripts indicate significant differences ($p < 0.05$) based on Tukey's post-hoc test following a general linear model analysis; FO = fish oil; FO/CO = fish oil/canola oil; LMO = low microbial oil; HMO = high microbial oil.

4. 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) [21] the dietary requirement for salmon is 0.5–1.0% EPA + DHA, which was recently reviewed by Qian et al. (2020) [26], who concluded that the minimum requirement is 0.5% EPA + DHA of dry diet. It is worth noting that the EPA + DHA requirement has not been de-coupled, since it is unknown what the requirements are for EPA and DHA separately. The EPA + DHA composition of the experimental diets (Table 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 [20]. Previous studies using MO from *Schizochytrium* sp. in diets for Atlantic salmon showed positive growth performance [27,28], and a high digestibility [14].

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 was 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.

4.1. Liver Tissue

The liver is considered an important site for LC-PUFA synthesis and lipid metabolism in Atlantic salmon [29]. Replacing dietary FO with MO did not significantly change the total lipid composition in the liver tissue (Table 4). 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 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 to establish proper membrane permeability and fluidity [30]. 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 4), as well as the P/S ratio in the liver PL (Table 5), 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 1) than liver PLFA (Figure 2). The PUFA proportion in the liver PL was noticeably higher than that of the diet (Table 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 [31,32].

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 [33]. 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.

4.2. 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 content in 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 A1 in Appendix A). 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 6). TAG are the primary class for lipid storage and energy provision, and the major lipid storage site for Atlantic salmon is the muscle tissue [34,35]. 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 they have an inverse relationship [36,37]. 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 6). A similar difference was also observed for P/S ratio in the muscle PL (Table 7). 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.

The distribution of total fatty acids (Table 6) and PLFA (Table 7) shared similarities as both reflected the diets, the PCO analysis showed a higher variation in muscle total fatty acid (Figure 3) than muscle PLFA (Figure 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 [29,38,39]. 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 Δ4 double bond in DHA requires a special mechanism [40].

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 [41]. Recently, Yeo & Parrish (2021) identified a relatively smaller number of PS and PI molecular species was in salmon muscle tissue compared to PC and PE [42]. 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 [40,43]; 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 [36,40]. PC and PE are the dominant PL classes in most eukaryotic membranes [42]. 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.

Five sterols were identified in the muscle tissue: cholesterol, cholestanol, campesterol, stigmasterol, and dinosterol. Cholesterol was present in all dietary treatments, with the highest amounts being found in MO-containing diets, however not at significantly higher levels than in salmon fed the FO diet. They were significantly higher than in the FO/CO-containing diet, indicating the cholesterol can be obtained from MO and incorporated by the fish. There were no significant differences in the amounts of the other sterols as compared to the control diets. Sissener et al. (2018) looked at cholesterol and phytosterol retention levels. Campesterol was the predominant sterol in all samples, and they found that the retention of campesterol correlated negatively with both dietary cholesterol and dietary phytosterol content. Retention of brassicasterol correlated negatively with dietary phytosterol content but not with dietary cholesterol [44]. Brassicasterol was not detected in the muscle tissue samples from this MO trial, however campesterol was detected in all samples. It does not appear that phytosterol retention increased or decreased in any of the samples from the MO trial, as there was no significant difference among the treatments. Sissener et al. (2018) determined that dietary phytosterols did not seem to affect cholesterol absorption or tissue cholesterol levels, and also did not affect tissue phytosterol levels [44]. Cholestanol was present in muscle from all diets and is a reduced form of cholesterol. Campesterol was also present in all samples, as was seen in the diets, and can be attributed to the wheat content of the diets. Stigmasterol was present in all diets, with no significant

difference among the treatments. Dinosterol was present in all diets and is common in dinoflagellates. Total sterol amounts ranged from 77 μg/g for muscle tissue from salmon fed the FO/CO diet to 165 μg/g in the muscle tissue from salmon fed the LMO diet. The low amount in the FO/CO diet can be attributed to the decrease in cholesterol. The muscle samples with the highest total sterol content were from salmon fed the LMO diet, which was the opposite of what was seen in the diet samples. The total sterol concentration averaged 0.13 mg/g across all treatments. Miller et al. (2007) conducted a study replacing fish oil with thraustochytrid *Schizochytrium* sp. oil in Atlantic salmon diets and found an average sterol content of 0.26 mg/g, with no significant difference among treatments [28]. There was no significant difference in the total sterol content among the muscle from fish fed the experimental diets versus muscle from fish fed the control diets.

Although there were significant differences in the proportions of cholesterol in the diets (Figure 5), there were no significant differences in the cholesterol in the muscle tissues. The FO/CO and two MO tissues had cholesterol proportions which were all significantly higher than in the diets. Sissener et al. (2018) found that the retention of cholesterol proved a high extent of de novo production in Atlantic salmon when fed low dietary levels, which could explain the similar effect seen here [44]. Cholestanol and dinosterol were only present in the tissue samples and were not present in the diets. Brassicasterol, 23,24-dimethylcholest-5-en-3β-ol, and 24-ethyl-5α-cholest-7-en-3β-ol were detected in the diets, but not in the muscle tissues. While campesterol and stigmasterol were present in both diets and tissue samples, there were no significant differences between the treatments. Brassicasterol is typically well absorbed, however Hamada et al. (2006) showed that campesterol was much more solubilized in the micelles than brassicasterol which may have influenced the absence of brassicasterol in the tissues while campesterol remained stable [45]. Six sterols were identified in the MO, four of which were detected in the diets, and two of these were subsequently found in the muscle tissue. There were also two different sterols that were found in the muscle tissue that were not in the diets or the MO. This indicates that sterols in MO can be transferred to the fish by consumption, but not all sterols were found to do so. Also, phytosterols can be metabolized to other sterols by the fish, as was seen with cholesterol and cholestanol.

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) [46]. 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 [47]. 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 [48]. However, it is worth mentioning that few lipid changes have been observed during frying for fish with a high-fat content [49,50]. Appendix C 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 [48].

5. Conclusions

This study aimed to investigate the potential use of MO as a replacement for FO in the diet of farmed Atlantic salmon, with a focus on the impact of dietary lipids on the membranes of liver and muscle tissues. Our results demonstrated that *Schizochytrium* sp. (T18)-derived MO can replace FO in the diet of Atlantic salmon without negative effects on growth and fatty acid composition of tissues. The fatty acid profiles of the tissues reflected their respective dietary treatments, and liver and muscle PL showed variations in response to dietary MO, reflecting the functions of each tissue. In addition, our findings revealed that the proportions of EPA and DHA in the tissue were dependent on the diet composition, with less necessity for EPA and more necessity for DHA. The presence of certain sterols in the MO that were also detected in muscle tissue indicated that some sterols could be transferred by consumption, while others cannot. These results provide important insights into the use of MO as a potential alternative to FO in the diet of farmed Atlantic salmon.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Formulation of experimental diets (g/kg as fed basis) containing microbial oil (MO), fish oil (FO), or fish oil/canola oil (FO/CO) blend, fed to Atlantic salmon.

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
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, NS, Canada). [2] Produced by Mara Renewables (Dartmouth, 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 A2. Chemical composition of experimental diets [1].

	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 *post hoc* test following a one-way ANOVA. FO = fish oil; FO/CO = fish oil/canola oil; LMO = low microbial oil; HMO = high microbial oil.

Table A3. Growth performance of Atlantic salmon fed experimental diets for 16 weeks.

	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 $(cm)^3 \times 100$, 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 × 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 B

Table A4. 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 A5. 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 A6. 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 A7. 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 A8. 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 A9. 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 A10. 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 A11. 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 C

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) [49].

Table A12. Moisture and fat content of fishes [1]. (Adapted with permission from [49]).

	Raw	Cooked	Warm Held
Salmon			
Moisture	69.3 [a] ± 0.06	56.3 [b] ± 1.09	51.2 [c] ± 0.20
Fat	12.0 [a] ± 0.15	14.8 [a] ± 0.30	15.9 [a] ± 1.66
Mackerel			
Moisture	61.0 [a] ± 0.10	64.6 [b] ± 0.40	61.1 [a] ± 0.02
Fat	16.0 [a] ± 0.20	7.25 [b] ± 0.24	9.33 [c] ± 0.08
Sardines			
Moisture	74.0 [a] ± 0.48	56.7 [b] ± 0.01	57.5 [b] ± 0.19
Fat	4.0 [a] ± 0.20	13.3 [b] ± 0.20	11.8 [c] ± 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 ($p \leq 0.05$).

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Hydrolysis Optimization of Thraustochytrid (Heterotrophic Protist) Biomass for the Production of a New and Sustainable Ingredient for the Aquafeed Industry

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Abstract: Thraustochytrids biomass, rich in docosahexaenoic acid (DHA), could be a sustainable ingredient for the aquafeed industry. However, its production cost renders it less competitive than fishmeal and fish oil from wild-caught fish. This study proposes optimizing the hydrolysis conditions of thraustochytrid biomass to generate an ingredient with improved properties thanks to the production of peptides with different biological activities. The improved nutritional value of the biomass could justify its use to decrease the amount of wild-caught fish in aquafeed, which would enhance the sustainability of fish aquaculture. First, two commercial proteases, Papain and Alcalase 2.4L, were compared for their capacity to hydrolyze the biomass. The best degree of hydrolysis (19 ± 1%) was obtained with Alcalase 2.4L, which was then used for the optimization of the pH, enzyme concentration and reaction time using response surface methodology. The results showed that the highest concentration of peptides and DHA in the aqueous phase was obtained with a pH, enzyme concentration and reaction time of 7.5, 2.7% and 205 min, respectively. If thraustochytrid hydrolysates prove to improve fish fitness, this mild and free-solvent process protocol could be used to produce a sustainable ingredient for aquafeed.

Keywords: thraustochytrids; peptides; enzymatic hydrolysis; DHA; Alcalase

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1. Introduction

n−3 long-chain polyunsaturated fatty acids (n−3 LC PUFAs) such as docosahexaenoic acid (C22:6n−3, DHA) are an important component of aquafeed formulations for aquaculture due to the important role they play in the regulation and protection of the cardiovascular and nervous systems in humans [1], the final consumers of fish. To date, the main sources of DHA for the aquafeed industry have come from wild pelagic fish resources [2]. However, the production of aquaculture is expected to double by 2050, having a negative impact on wild fish stocks [2]. Therefore, new sustainable alternatives to these limited resources are needed to cope with the growing demand from the aquafeed industry.

Thraustochytrids are heterotrophic protists present in marine food webs [3] which have received considerable attention for their capacity to produce DHA [4,5]. These oleaginous microorganisms are one of the potential alternatives to fish oil. However, one of the main obstacles to the use of thraustochytrid biomass in animal nutrition is its production cost, which is primarily attributed to the cost of the substrate used for cultivation [6]. To make the production of this biomass more profitable, several strategies are presented in the literature,

including the use of low-cost substrates such as industrial by-products [7]. Another strategy is to increase the nutritional quality of the biomass. While the processes used for this may make the biomass more expensive than fishmeal or fish oil, the improved nutritional quality will justify its use in aquaculture and decrease the amount of wild-caught fish in aquafeed, making the aquaculture industry more sustainable. In this study, we explored the valorization of the protein fraction of thraustochytrids, which is rarely exploited in scientific studies or industrial processes.

The nutritional value of thraustochytrid proteins could be increased through enzymatic hydrolysis, a mild and solvent-free process. In this process, proteolytic enzymes bind to proteins and break the peptide bonds, resulting in the formation of peptides of different sizes. Protein hydrolysates have already been proven to improve the fishmeal of sea bass (*Dicentrarchus labrax*) larvae, allowing for better growth rates and survival and reducing malformations [8,9]. Moreover, enzymatic hydrolysis ameliorated the digestibility of *Nannochloropsis oceanica* in the diets of sea bass juveniles [10]. This process may not only improve the digestibility of the biomass but may also result in the production of bioactive peptides. These short amino acid (AA) chains, usually composed of 2–20 AAs (or in the range of 200 to 2000 Daltons), can have different nutritional (and health) benefits, including anti-thrombotic, mineral binding, antioxidant, antihypertensive, anti-microbial, anti-cancer and/or immuno-modulatory properties, among others [11–13].

Although enzymatic hydrolysis is a complex bioprocess, by selecting the best hydrolysis conditions and the degree of hydrolysis (DH), it is possible to control the molecular weight of the produced peptides and thus design the properties of the final product [14,15]. Controlling the DH is important, as it determines the extent to which the native protein is broken down into peptides. If the DH is too low, the quantity of the desired peptides will be low. However, a too high DH or a complete hydrolysis of the biomass can lead to the destruction of the peptides and their activity, as well as a production of free AAs, which are absorbed more slowly than peptides [16]. Thus, the process may become inefficient or economically irrelevant.

Several factors can affect the DH, such as the type of protease, enzyme-to-substrate ratio (E/S), pH, temperature, time of reaction, agitation or the presence of endogenous enzymes [17]. The choice of the protease is one of the key steps in the proteolysis process. Some studies have already practiced enzymatic hydrolysis on thraustochytrid biomass, and many of them have used Alcalase [18,19]. This protease is a serine endopeptidase produced by the fermentation of a selected strain of *Bacillus licheniformis*, and it has been widely used for the production of bioactive peptides from different substrates [17]. It has a broad working pH and temperature range; however, it has an optimal activity at a pH between 7 and 9 and at a temperature between 30 and 65 °C. Other studies also used Papain, a cysteine endopeptidase derived from papaya (*Carica papaya*). This enzyme has been extensively used for the production of antioxidant peptides from different substrates, such as fish residues or microalgae [20,21]. Compared to Alcalase, Papain has a lower optimum pH but a similar temperature range.

The objective of this study was to optimize the enzymatic hydrolysis conditions of thraustochytrid biomass in order to obtain a hydrolysate with a high content of peptides between 200 and 2000 Da and DHA in the same aqueous phase (AP) and a DH between 10% and 20%.

2. Materials and Methods

2.1. Chemicals

The chemicals and reagents used in this study were of analytical grade. Potassium phosphate monobasic, potassium phosphate dibasic, (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), fluorescein sodium salt, 2,2′-azobis 2-amidinopropane dihydrochloride (AAPH) and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trifluoroacetic acid was purchased from Fischer Scientific (Waltham, MA, USA). For SEC-HPLC analysis, ten molecular standards were used for the column calibration

(Sigma-Aldrich, St. Louis, MO, USA): Glycine (75 Da; G7126), L-threonine (119 Da; T8625), L-asparagine (132 Da; A0884), Leupeptin (463 Da; L9783), Substance P F1–7 (900 Da; S6272), Luteinizing Hormone-Releasing Hormone (LH-RH) (1182 Da; L7134), Substance P acetate (1347 Da; S6883), Neurotensin (1672 Da; N6383), Insulin (3495 Da; I6383) and Cytochrome C (12,327 Da; C2037).

2.2. Biological Material

The substrate used in this study was the commercial thraustochytrid biomass sold under the name of DHA Gold by the company DSM (Heerlen, the Netherlands). DHA Gold is the dried biomass of the whole cells of *Schyzochytrium* sp. (ATCC PTA 9695) used in animal nutrition products and complemented with natural antioxidants (lecithin, rosemary extract, mono- and diglycerides and citric acid). This biomass containing a high DHA percentage can be easily purchased in large quantities and stored at 4 °C until use. The protocol performed with this biomass could be easily applied to any other thraustochytrid biomass.

2.3. Comparison of the Effect of Two Proteases for the Digestion of Thraustochytrid Biomass

Two commercial enzymes, Papain and Alcalase 2.4L, were first compared for their capacity to hydrolyze the thraustochytrid biomass. As mentioned in the introduction, these two enzymes have been used for thraustochytrid biomass hydrolysis, but it is not clear which one is the best for peptide production. The DH (measured using the pH-Stat method) and the molecular weight of the peptides released with both proteases and the control condition (SEC-HPLC) were compared in order to decide which enzyme would be used for hydrolysis optimization. Papain (EC 3.4.22.2, Sigma-Aldrich, St. Louis, MO, USA) has optimal activity at 65 °C and in a pH range of 6–7. The batch used had a proteolytic activity of 17.7 $U \cdot mg^{-1}$ and a concentration of 19.9 $mg \cdot mL^{-1}$. Alcalase 2.4L (EC 3.4.21.62, Sigma-Aldrich, St. Louis, MO, USA) has an optimal pH and temperature of 7–9 and 30–65 °C, respectively, and a density of 1.25 $g \cdot mL^{-1}$, and its declared proteolytic activity is 2.4 $U \cdot g^{-1}$.

For these experiments, the working pH was set at 6.8. This pH condition is not in the optimal pH range for Alcalase 2.4L; however, since the thraustochytrid biomass used in this study has a pH of 4, this allowed us to compare Alcalase 2.4L acidic hydrolysis with that of Papain (which has a more acidic optimal pH range than Alcalase 2.4L). The enzymes were used at a concentration of 0.8 $U \cdot L^{-1}$. All the experiments were performed in a 500 mL double-walled glass reactor (Pyrex) with a biomass concentration of 100 $g \cdot L^{-1}$ in distilled water. The temperature was set at 50 °C, and the biomass was stirred with a 3-bladed propeller (Janke & Kunkel, IKA-Labortechnik, Staufen im Breisgau, Germany). The hydrolysis was conducted for 2 h. Control conditions without enzymes was performed with the same hydrolysis conditions. The experiments were conducted in triplicate for each protease tested and the control conditions. At the end of the hydrolysis, the enzymes were inactivated in a water bath at 80 °C for 20 min. The hydrolysate was cooled to room temperature and freeze-dried for 96 h (freeze-drier Christ Alpha 1–4 LD plus, Bioblock Scientific, Illkirch, France) before storage at −20 °C until further analysis.

2.4. Optimization of Enzymatic Hydrolysis Conditions of Thraustochytrid Biomass by RSM

Based on the comparison of the two enzymes, the study was pursued using Alcalase 2.4L. Hydrolysis were conducted in a 500 mL double-walled reactor with a biomass concentration of 100 $g \cdot L^{-1}$ in distilled water at 50 °C and terminated through the inactivation of the enzyme, as described above (80 °C, 20 min). The different experimental conditions were analyzed using RSM and CCD. This type of design comprises a two-level factorial design with central points that are repeated three times to improve the predictive capability of the model. Axial points were added to the experimental design to permit the estimation of the curvature of the response surface. The factors evaluated to optimize the hydrolysis were E/S (from 0.6% to 3%), where the substrate is the content of protein in the biomass,

the pH of the reaction (between 7 and 8) and the reaction time (60 min to 180 min). The ranges proposed for each variable were determined based on preliminary experiments. A plan of 17 experiments was designed (Table 1), and the results were analyzed in order to optimize 3 criteria. The first one was to maximize the peptide area (mAU · min) in the range of 2000–200 Da in the AP. This variable will be referred to as PEP concentration. In this study, it was assumed that the peptide area measured in mAU · min is an indicator of peptide concentration, and therefore a higher area means a higher concentration. This concept is valid in the case of injection at the same volume (which is the case in this study). The second criterion was to maximize the DHA percentage in the total lipids of the AP (TL-DHA) with the objective of obtaining a product rich in both bioactive peptides and DHA, giving an added value to the hydrolysate. Finally, the third criterion was to obtain a DH (pH-Stat) between 10% and 20%. These values were chosen based on preliminary tests, which showed that these DH values allowed an optimal production of peptides at the desired molecular weight range without creating too many free AAs.

Table 1. Experimental matrix of thraustochytrid biomass hydrolysis by Alcalase 2.4L with real and centered reduced values.

Experiment	pH		Time (min)		E/S (%)	
	Real Value	Centered Reduced Values	Real Value	Centered Reduced Values	Real Value	Centered Reduced Values
1	7.0	−1	60	−1	0.6	−1
2	7.5	0	120	0	0.1	−1.68
3	7.0	−1	60	−1	3.0	1
4	7.5	0	205	1.68	1.8	0
5	7.5	0	120	0	3.5	1.68
6	7.0	−1	180	1	3.0	1
7	7.5	0	35	−1.68	1.8	0
8	8.0	1	180	1	0.6	−1
9	8.0	1	180	1	3.0	1
10	7.0	−1	180	1	0.6	−1
11	7.5	0	120	0	1.8	0
12	7.5	0	120	0	1.8	0
13	6.8	−1.68	120	0	1.8	0
14	7.5	0	120	0	1.8	0
15	8.2	1.68	120	0	1.8	0
16	8.0	1	60	−1	0.6	−1
17	8.0	1	60	−1	3.0	1

The Statgraphics software (Statgraphics 18, FRANCESTAT, Neuilly-sur-Seine, France) was used to create the CCD and determine the optimum conditions to maximize the response variables. The ANOVA test, the F test and the determination coefficient R^2 were used to analyze the adequacy of the model and the statistical significance. For each criterion, the model proposes a second-order polynomial equation to calculate the predicted values considering the quadratic effects of each factor, the interactions between different factors and the linear relationships between them.

2.5. Model Validation

The model was validated by performing hydrolysis with a combination of factors that were convenient in view of industrial, economic and sustainability criteria. The hydrolysis for validation was conducted in triplicate, and the observed values were compared to the values predicted by the model equations. A control without enzymes was also performed in triplicate. After enzyme inactivation, as described above, the biomass was separated in aliquots to analyze the different components of the hydrolysate. One aliquot was freeze-dried without any other manipulation (whole hydrolysate, WH). A second aliquot was

centrifugated at 15,000× *g* during 30 min (Centrifuge 5810R, Eppendorf, Germany) to obtain a sample composed of the AP and an emulsion (AP + E), a sample composed of the AP alone and a sample of the non-hydrolyzed biomass (bottom phase, BP). Finally, a sample of the AP was centrifuged at 10,000× *g* for 30 min using an 10,000 NMWL Amicon Centrifugal Filter Unit (Merck KGaA, Darmstadt, Germany) to discard molecules larger than 10 kDa (AP 10 kDa).

2.6. Monitoring of Enzymatic Hydrolysis with the pH-Stat Method

The DH was determined using the pH-Stat method. The DH is measured by the sodium hydroxide titration of the amino groups liberated during hydrolysis in an alkaline medium and calculated according to Equation (1) [22]:

$$DH(\%) = \frac{B \times N_b}{\alpha \times Mp \times h_{tot}} \times 100 \quad (1)$$

where B is the volume of the titrant (mL), N_b its normality (meq $\cdot$ mL^{-1}), α is the mean degree of dissociation of α-amino groups (Table 2), M_p is the protein mass (g) and h_{tot} is the number of peptide bonds per gram of proteins. The value for h_{tot} (6.41) was calculated from the characterization of the total AA profile. The pH of the hydrolysis was maintained with NaOH 0.5 N using a high-end potentiometric titrator (902 Titrando, Metrohm Ltd., Herisau, Switzerland).

Table 2. α values for each pH condition.

pH	α
6.5	0.20
6.8	0.33
7	0.44
7.5	0.72
8	0.89
8.2	0.93

2.7. Size Exclusion HPLC (SEC-HPLC)

Freeze-dried samples were diluted at 10% (W/W) in MilliQ Water, agitated for 15 min at 100 rpm and centrifuged at 10,000× *g* for 30 min using an 10,000 NMWL Amicon centrifugal filter unit (Merck KGaA, Darmstadt, Germany) to discard molecules larger than 10 kDa (AP 10 kDa). The molecular weight profile of the samples was then analyzed by SEC-HPLC using a Superdex 30 Increase 10/300 GL column with a fractionation range from 100 to 7000 Da (10 × 300–310 nm, Cytiva, Marlborough, MA, USA). A UHPLC Dionex Ultimate 3000 Series system (Thermo Fisher Scientific, MA, USA) equipped with a photodiode array detector (DAD-3000, Thermo Fisher Scientific, MA, USA) was used. The mobile phase was composed of water with 0.1% trifluoroacetic acid and 30% acetonitrile, the flow rate was set at 0.5 mL $\cdot$ min^{-1} and the absorbance was measured at 214 nm. For this analysis, 50 µL of the samples was injected, and the elution time was 60 min. After each analysis, the data were processed using Chromeleon 0.7 software (Thermo Fisher Scientific, MA, USA).

2.8. Biochemical Analysis of the Biomass

Analyses of AAs and proteins were performed by a subcontractor (Upscience, Saint-Nolff, France).

2.9. Lipid Extraction and Fatty Acid (FA) Analysis

Lipids were extracted from the freeze-dried biomass (20 mg for the biomass before hydrolysis and the WH and 50 mg for the AP of the hydrolyzed thraustochytrid biomass) as described in [23], using chloroform instead of dichloromethane. After gravimetric

determination of the total lipids, the neutral and polar lipids were separated from the total lipid extract (TL) by solid-phase extraction as described in [24] with slight modifications. Briefly, 0.25 µL of TL was evaporated to dryness under nitrogen, recovered with 3 washes of 0.5 mL of $CHCl_3$:MeOH (98:2 *v/v*; final volume 1.5 mL) and spotted at the top of a silica gel column. The neutral lipid fraction was eluted with 10 mL of $CHCl_3$:MeOH (98:2, *v/v*) and the polar fraction with 20 mL of MeOH. After the addition of an internal standard (23:0, in free FA form), both lipid fractions were dried in an EZ-2 evaporator (Genevac, Ipswich, UK) and hydrolyzed in 1 mL of KOH-MeOH (0.5 M) for 30 min at 80 °C. The samples were then transesterified with 1.6 mL of MeOH:H_2SO_4 (3.4%; *v/v*) for 10 min at 100 °C. The FA methyl ester (FAME) were recovered in hexane and analyzed by gas chromatography coupled to a flame ionization detector (GC-FID; Varian CP8400 gas chromatograph, Agilent, CA, USA). FAME were identified by comparing their retention time to those of external commercial standard mixtures (S37 FAME Mix, PUFA No.1, and PUFA No.3, Supelco, PA, USA) using the software Galaxie 1.9.3.2 (Agilent). FAME peak area was converted into µg of FA based on the peak area of the internal standard C23:0, and the FAs contents were expressed in mg $\cdot$ 100 g^{-1} DW and as a percentage (%) of total FAs.

2.10. Determination of Antioxidant Activity

The AP + E, BP and AP 10 kDa fractions of the hydrolysate and the control without enzymes in validation conditions were tested for their antioxidant activity using the oxygen absorbance capacity (ORAC) assay. This method is based on the oxidation of a fluorescent probe, fluorescein, through the addition of a free radical generator (2,2′-azobis 2-amidinopropane dihydrochloride, AAPH) that quenches the fluorescein over time. The antioxidant molecules present in the sample block the generation of free radicals until the antioxidant activity of the sample is depleted. Briefly, 25 µL of the sample and 150 µL of fluorescein ($4 \cdot 10^{-9}$ M) diluted in phosphate buffer (75 mM, pH 7.4) were placed in the well of a plate (black 96-well plates—FLUOTRAC 200; Greiner Bio-One, Kremsmünster, Austria). The plates were incubated at 37 °C for 10 min. After 10 min of incubation, the oxidation of the fluorescein was initiated through the addition of 25 µL of 153 mM AAPH diluted in phosphate buffer (pH 7.4). The fluorescence decay was monitored every minute for 150 min using a fluorescence plate reader (Infinite M Plex, TECAN, Männedorf, Switzerland) at excitation and emission wavelengths set at 485 nm and 520 nm, respectively. The plate was automatically shaken for 10 s before each reading. Each extract was tested in triplicate. Trolox, a water-soluble vitamin E analog, diluted in phosphate buffer, was used to establish a calibration curve with 6 concentrations (200, 100, 50, 25, 12.5, 6.25 µM). The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve. The net AUC of the sample was calculated by subtracting the AUC of the blank. The regression equation between the net AUC and Trolox concentration was determined. The results are expressed as Trolox equivalents (µmol TE $\cdot$ g^{-1} of sample).

3. Results and Discussion

3.1. Biochemical Characterization of Thraustochytrid Biomass

The biomass used in this study contained a high fat content (42.2 g $\cdot$ 100 g^{-1}) and a low protein content (12 g $\cdot$ 100 g^{-1}). The major FAs were the saturated FA 16:0 (25.6% of total FAs) and the PUFAs 22:6n−3 (DHA, 42% of total FAs) and 22:5n−6 (17% of total FAs) (Table 3). The main AAs were glutamic acid (19.2% of total AAs) and aspartic acid (11.2% of total AAs), followed by arginine (7.6% of total AAs) and leucine (8.3% of total AAs) (Table 3). The composition of the biomass used in this study coincided with the data reported in other studies using *Schizochytrium* spp. [25–28], which indicates that the protocol developed in this study could be easily adapted to other thraustochytrid biomass. However, the protein content of the biomass was low compared to that of other species of microalgae such as *Chlorella* spp. (up to 58% protein/DW) and *Scenedesmus* spp. (up to 56% protein/DW) [29]. The low protein content in thraustochytrid biomass will result in a concomitant low concentration of peptides. To overcome this limitation, the protein can be

separated from the rest of the biomass in order to only hydrolyze that fraction [18]. This approach would result in a higher concentration of peptides, leading to increased biological activity due to such fraction. However, this method needs an additional extraction step, increasing the overall cost of the process. In this study, we opted to hydrolyze the whole biomass without pre-treatment in order to obtain a product rich in PUFAs and peptides at the same time with potential enhanced bioactivity.

Table 3. Total amino acid (AA) and fatty acid (FA) profiles of thraustochytrid biomass.

AA	Total AAs %	FAs	Total FAs %
	Mean ± S.D		**Mean ± S.D**
aspartic acid	11.2 ± 0.92	C14:0	7.3 ± 0.6
threonine	4.9 ± 0.41	C15:0	0.3 ± 0.5
serine	5.1 ± 0.41	C16:0	25.6 ± 2.0
glutamic acid	19.2 ± 1.54	C17:0	0.1 ± 0.5
proline	4.1 ± 0.31	C18:0	0.6 ± 0.5
glycine	5.4 ± 0.41	C16:1	0.3 ± 0.5
alanine	7.0 ± 0.51	C18:1	0.9 ± 0.5
cysteine	1.6 ± 0.31	C18:2n−6	0.4 ± 0.5
valine	5.4 ± 0.41	C18:3n−6	0.2 ± 0.5
methionine	2.5 ± 0.31	C18:4n−3	0.3 ± 0.5
isoleucine	4.3 ± 0.31	C20:3n−6	0.4 ± 0.5
leucine	8.3 ± 0.61	C20:4n−3	0.8 ± 0.5
tyrosine	3.4 ± 0.31	C20:4n−6	0.4 ± 0.5
phenylalanine	4.4 ± 0.31	C20:5n−3	1.1 ± 0.5
histidine	1.8 ± 0.31	C22:5n−3	0.3 ± 0.5
lysine	4.3 ± 0.31	C22:5n−6	16.6 ± 1.3
arginine	7.6 ± 0.61	C22:6n−3	41.9 ± 3.4
Total ($g \cdot 100\ g^{-1}$)	9.76	Total ($g \cdot 100\ g^{-1}$)	37.3

3.2. Comparison of the Effect of Alcalase 2.4L and Papain for the Digestion of Thraustochytrid Biomass

Several commercial enzymes are now available and can be used to break down the proteins of thraustochytrids. In this study, we decided to use Alcalase 2.4L, which has already been proven to be efficient in producing bioactive peptides from various substrates [17], and Papain, which is an enzyme that can work at a more acidic pH than Alcalase 2.4L and is therefore closer to the natural pH of the thraustochytrid biomass.

To determine the efficiency of the enzymes, the DH and peptide molecular weight of the hydrolysate produced were evaluated. With an enzyme concentration of $0.8\ U \cdot L^{-1}$, a pH of 6.8 and 2 h of reaction time, a DH of 19 ± 1% was obtained with Alcalase 2.4L, while with Papain, the biomass was almost not hydrolyzed (DH 0.2 ± 1%). This was confirmed by the SEC-HPLC results. Figure 1 shows the chromatographic profiles of the Alcalase 2.4L and Papain hydrolysates and the control. No increase in peptide concentration was observed with the addition of Papain, as its chromatogram profile is identical to that of the control without enzymes. There is evidence that Papain can successfully extract the oil from *Schizochytrium* spp. lipid droplets after cell disintegration by ultrasound [30] and that it can also hydrolyze *Aurantiochytrium limacinum* protein residue after oil extraction [18]. However, it seems that without pre-treatment of the biomass, this enzyme does not work as well as Alcalase 2.4L. Although Alcalase 2.4L is an enzyme whose optimum is in the pH range of 7 to 8, this study demonstrates that even at a low pH, this enzyme is more efficient than other enzymes whose pH optimum is more acidic. However, even if the DH obtained with Alcalase 2.4L is within the values targeted by this study, it is important to optimize the hydrolysis conditions in order to maximize the results with a minimal enzyme concentration (3% of the protein content in this experiment) due to economic (since this is the most expensive factor in this kind of process) and sustainability issues.

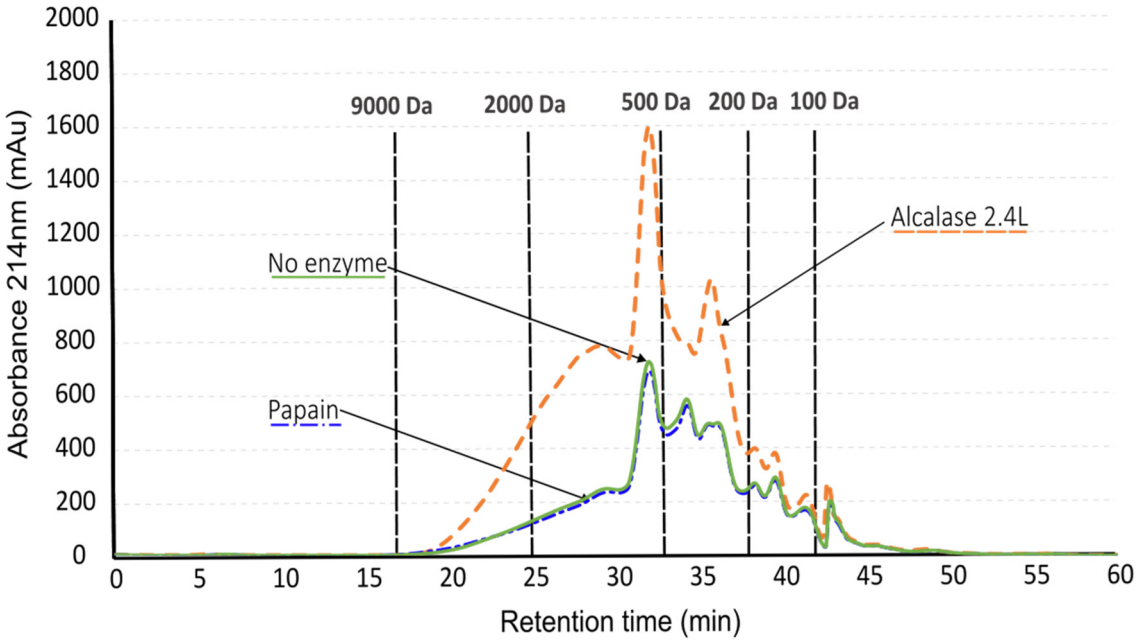

Figure 1. SEC-HPLC chromatogram of thraustochytrid hydrolysate with Alcalase 2.4L (orange dashed line), Papain (blue dot-dashed line) and the control without enzyme (green solid line). 50 µL of sample (10 kDa fraction of the hydrolysate) were injected.

3.3. Optimization of the Hydrolysis Conditions with Alcalase 2.4L Using Response Surface Methodology

The optimization of (A) pH, (B) reaction time and (C) E/S for the enzymatic hydrolysis using Alcalase 2.4L was performed using a CCD. The PEP and TL-DHA concentrations in the AP, and the DH were the three criteria chosen to evaluate the optimal hydrolysis conditions.

The analysis of the variance of the model was carried out with the centered reduced values of the variables (Table 4) to obtain a better assessment of the model's significance. The *p*-value of the three factors studied was significant (p-value < 0.01), indicating that the models had high significance levels. Moreover, the adjusted R-squared (R^2) indicates that the model explains 93.6% of the variability in the DH and 98.7% of the variability in PEP concentration, meaning that the model has a good capacity to predict the response for new observations when changing these factors. The adjusted R-squared of the TL-DHA concentration was lower (77.1%), meaning that the model has a lower predictive power for this response. This is because the concentration of TL-DHA in the AP did not vary substantially with the different hydrolysis conditions and was around 11 ± 2% considering all the experiments performed.

Table 4. Analysis of variance of the central composite design to optimize the degree of hydrolysis (DH), the peptide (PEP) concentration and the total lipid DHA (TL-DHA) of thraustochytrid hydrolysate.

Factor	F-Ratio	*p*-Value	R^2	Adjusted R^2
DH	27.0	0.0001	97.2	93.6
PEP	135.6	<0.0001	99.4	98.7
TL-DHA	7.0	0.0089	90.0	77.1

To obtain a DH between 10 and 20% and to maximize PEP and TL-DHA concentrations in the AP, the factors were optimized, taking into account their real values. Moreover, some of the terms that did not have a significant effect on the response were removed from the equation to simplify the model without affecting its accuracy or predictive power. The

analysis of variance of the terms that were used to predict the response of DH, PEP and DHA is shown in Table S1. Pareto charts (Figure 2) show factors with the highest effect on each response and whether their effect is positive or negative. E/S (Enzyme) is the term that has the strongest positive effect on the three responses. However, the quadratic term Enzyme × Enzyme has a significant negative effect on the DH and PEP concentration, meaning that this factor has a maximum value at which the DH and PEP concentration start to decrease. The time of reaction (Time) also has a significant positive effect for the three responses, while pH has a significant positive effect only for PEP concentration, and it is the term with the weakest effect on the response. Finally, the interaction term pH × Enzyme has a significant negative effect on the TL-DHA concentration; this indicates that when the pH is increased, the E/S should be decreased (and the other way around) to maximize the TL-DHA concentration in the AP.

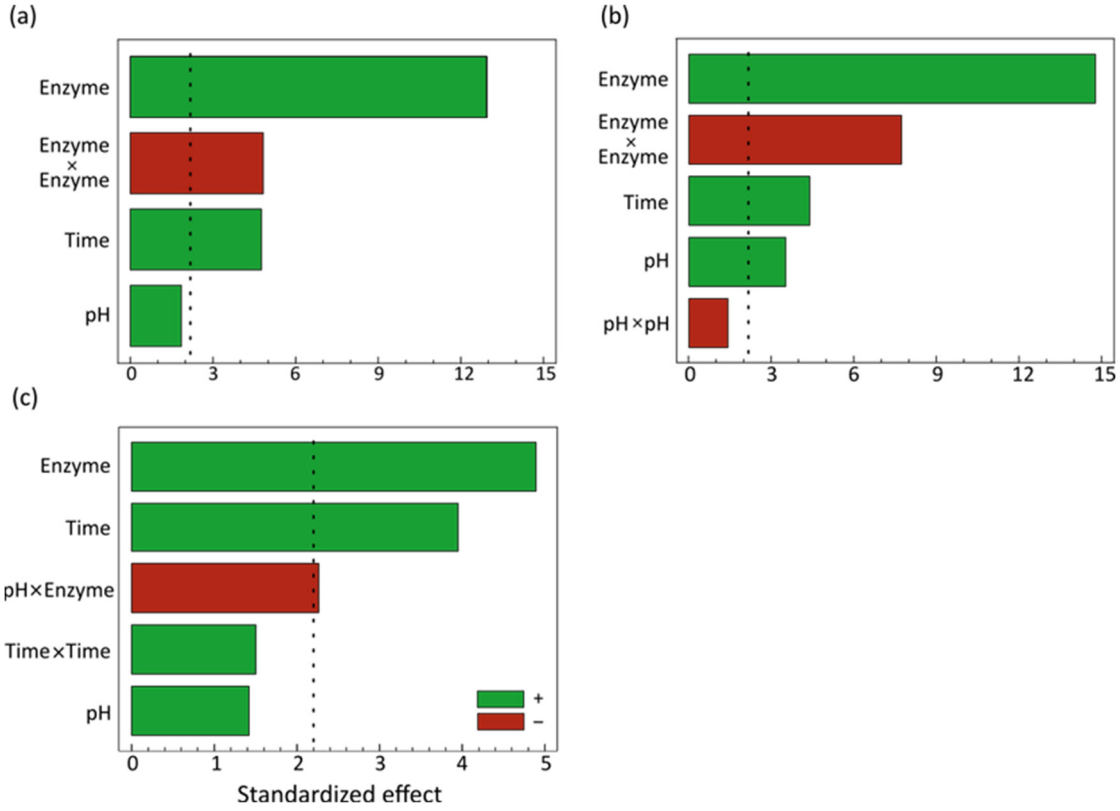

Figure 2. Pareto charts showing the effect of the different factors (enzyme-to-substrate ratio (Enzyme), time and pH of hydrolysis) and the interactions and quadratic effects on (**a**) degree of hydrolysis (DH), (**b**) peptide (PEP) concentration and (**c**) total lipid DHA (TL-DHA) of thraustochytrid hydrolysate. The vertical-dashed lines represent the significance level (p = 0.05). The green bars show a positive effect, while the red bars show a negative effect.

The response surfaces (Figure 3) show that increasing the hydrolysis time leads to an increase in the response; however, it should be noted that the maximum hydrolysis time evaluated in this study was 205 min and is not possible to conclude that longer hydrolysis would be advantageous. Longer reaction times could lead to an excessively high DH, causing the enzymes to begin attacking the peptides formed and increasing the concentration of free AAs in the AP. Given that free AAs have a lower nutritional quality, it is necessary to find a balance between producing as many peptides of the desired size as possible while limiting the increase in the free AA concentration [16,31]. Regarding E/S, the optimal value is approximately 3%. Increasing E/S beyond this value when the hydrolysis time is at its maximum and the pH is 7.5 can negatively impact the PEP concentration and the DH. Furthermore, the pH also has a positive effect on the PEP concentration;

however, its effect is quadratic, and values higher than 8.0 may lead to a decrease in the PEP concentration. This can be explained by the fact that changes in pH can alter the charge distribution and conformation of the substrate and the enzymes used, resulting in different interactions between the two [22].

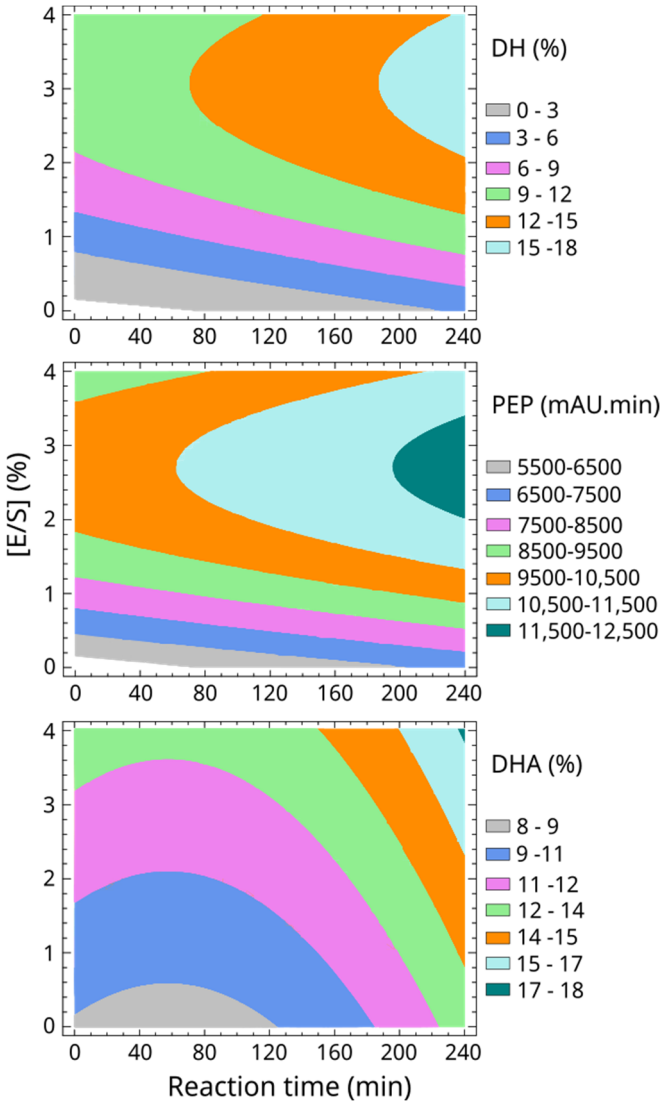

Figure 3. Estimated response surface contours showing the effect of the interaction between two independent variables (enzyme-to-substrate (E/S) ratio and hydrolysis reaction time) on the degree of hydrolysis (DH), peptide (PEP) concentration and total lipid DHA (TL-DHA) of thraustochytrid hydrolysate when the third variable is kept at the intermediate level. In this case, the pH was kept at 7.5.

As shown in Table 5, the optimal time of reaction and E/S conditions are similar for the three responses. However, the pH values are slightly different due to the quadratic effect on the PEP concentration. Since multi-response optimization is not possible with this type of design, several aspects had to be taken into consideration to choose the conditions for model validation.

The optimum TL-DHA concentration is obtained with a neutral-acid pH, while a more basic pH is needed to optimize the DH. It was decided to continue the validation of the model using a pH of 7.5 since this diminution of the reaction pH will only decrease the PEP concentration by 1.5% (11,573 mAU · min), but less NaOH would be added during the reaction, which is preferable for industrial transposition (scale-up). Regarding E/S,

the optimal value for model validation was set at 2.7%. Although the model suggests a higher E/S to increase the DH and TL-DHA concentration, the enzyme quadratic effect may negatively affect the PEP concentration. Finally, the time was set at 205 min, which is the value proposed for the optimization of the three responses.

Table 5. Calculated values of pH, time of hydrolysis and enzyme-to-substrate ratio (Enzyme) to optimize the degree of hydrolysis (DH), the peptide (PEP) concentration and the total lipid DHA (TL-DHA) of thraustochytrid hydrolysate and the equations used to predict the responses with the response surface methodology.

	pH	Time (min)	E/S (%)	Optimum Value	Equation
DH	8.2	204.8	3.1	16%	$DH = -11.8 + 1.2 \times pH + 0.02 \times Time + 8.5 \times Enzyme - 1.4 \times Enzyme^2$
PEP	8.0	204.8	2.7	11,749 mAU · min	$PEP = -41{,}657.4 + 11{,}704.8 \times pH + 7.5 \times Time + 3766.1 \times Enzyme - 732.3 \times pH^2 - 695.4 \times Enzyme^2$
TL-DHA	6.8	204.8	3.5	15%	$TL\text{-}DHA = -0.1 + 0.03 \times pH - 0.0001 \times Time + 0.09 \times Enzyme - 0.01 \times pH \times Enzyme + 0.000001 \times Time^2$

3.4. Validation of the Model for the Prediction of DH, PEP Concentration and TL-DHA of Thraustochytrid Hydrolysate

The validation was performed using a pH of 7.5, a reaction time of 205 min and an E/S of 2.7%. With these conditions, the predicted values were 15.3% for the DH, 11,573 mAU · min for the PEP concentration and 14.1% for the TL-DHA concentration in the AP. The chromatogram obtained using SEC-HPLC (Figure 4) reveals a significant production of peptides due to the addition of Alcalase 2.4L. The analysis of the area of each peptide fraction (Table 6) demonstrated a significant increase in the molecular weight fractions between 2000 and 200 Da in the Alcalase 2.4L-treated samples, which is the fraction that was targeted for maximization in the RSM.

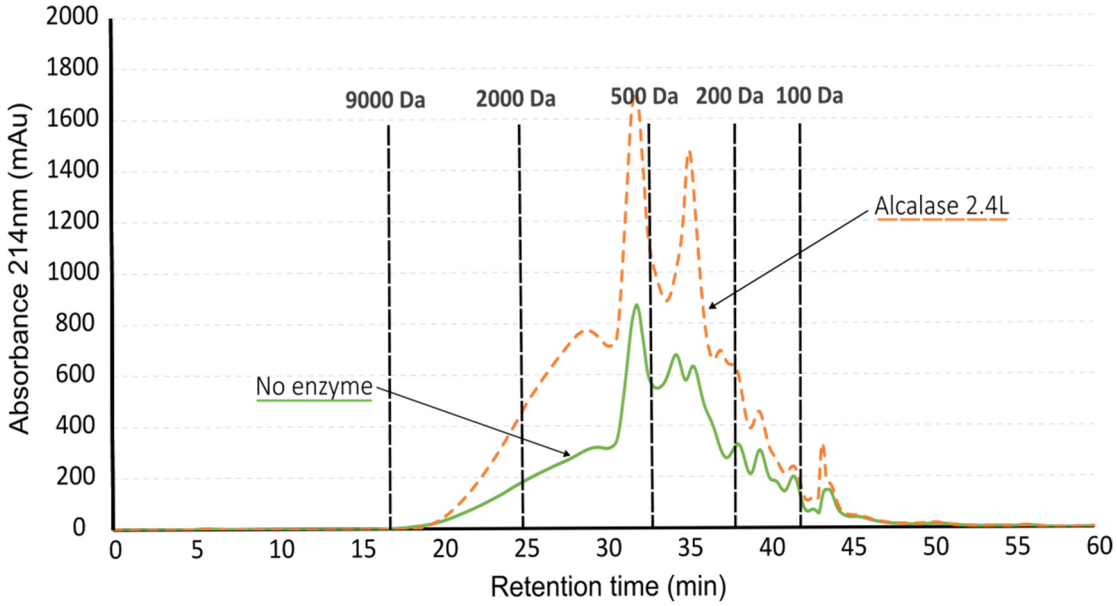

Figure 4. SEC-HPLC chromatogram of thraustochytrid hydrolysate in validation conditions (pH: 7.5; reaction time: 205 min; E/S: 2.7%) with Alcalase 2.4L (orange dashed line) and the control without enzyme (green solid line). A total of 50 µL of sample was injected.

Table 6. Area (mAU · min) and variation (%) of peptide fractions of thraustochytrid hydrolysate produced using a pH of 7.5, a reaction time of 205 min and an E/S of 2.7% with Alcalase 2.4L and the control without enzyme.

Molecular Weight (Da)	No Enzyme (mAU · min)	Alcalase 2.4L (mAU · min)	Increase/Decrease (Expressed as %)
9000–6000	16.3	10.2	−38%
6000–3000	244.8	462.4	89%
3000–2000	372.8	875.0	135%
2000–1500	360.2	970.9	170%
1500–1000	626.2	1591.9	154%
1000–500	1998.5	4151.8	108%
500–200	2459.2	4513.8	84%
200–100	802.4	1274.3	59%
<100	264.6	448.1	69%

Within this range, the 2000–1500 Da fraction displayed the highest percentage variation (170% increase), followed by the 1500–1000 Da (154% increase) and 1000–500 Da (108% increase) fractions. Moreover, the 9000–6000 Da fraction shows a reduction of 38%, which provides evidence that larger molecules were effectively digested by Alcalase 2.4L, leading to the generation of smaller and potentially bioactive compounds within the desired molecular weight range.

With the validation conditions, the observed PEP concentration was 11,228 mAU · min. Although the observed value is 3% lower than the predicted value, the validation experiment confirmed the performance of the model for PEP concentration prediction. Moreover, a TL-DHA concentration of 23 ± 1% was obtained, which is higher than the predicted value (+8%), indicating that we managed to optimize the TL-DHA concentration in the AP. Finally, a DH of 18.3 ± 0.25% was obtained. This value was also higher than the predicted value (+3%) but in the range of what we were expecting.

3.5. Lipid Composition of the Hydrolysate

The commercial value of thraustochytrid biomass lies in its lipid content and composition. Those parameters were studied in depth for the hydrolyzed biomass to see whether the process could have a negative impact on its PUFA content and profile. In the WH, the lipid content was conserved after hydrolysis (40.0 ± 2.4% of DW), and the FA profile was not affected by the hydrolysis conditions used as compared with the biomass before hydrolysis (Table 3). The major FAs were DHA and 16:0, and their contents were not modified by hydrolysis (Figure 5). In the AP, the total lipid content was 14 ± 0.3% of DW.

The AP was enriched in polar lipids (44 ± 5% of total lipids; 6.2 g · 100 g^{-1} DW), while the WH contained only 6 ± 0.3% of these lipids as polar lipids (2.4 g · 100 g^{-1} DW). Moreover, from the 22.7 ± 0.8% TL-DHA found in the AP, 9 ± 3.6% of the DHA was in the form of polar lipids. In *Schizochytrium* spp., the main polar lipids are the phospholipids (PLs) phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol [32–35]. Even if lipids are generally hydrophobic molecules, PLs have a polar head that renders them amphiphilic, making them soluble in an aqueous medium. A study with sardine viscera showed that Alcalase or Protamex were better at extracting PLs compared to other enzymes, such as Flavourzyme. In the same study, 74% of the PLs were found in the AP of the hydrolysate, while the oily fraction and sludge contained less than 20% each [36]. These results are interesting since there is evidence that PL ingestion is more efficient in the protection of brain activity than the ingestion of triglycerides [37]. Several studies have also demonstrated that marine fish and larvae assimilate PLs better than triglycerides [38–41]. A study by Cahu et al. (2003) [38] examined the impact of diets with different PL concentrations and PL/neutral lipid (NL) values on the development of sea bass larvae. The results indicated that larvae fed diets with higher levels of PLs and higher PL/NL values showed better growth rates and survival rates and less malformations

than those fed diets with lower levels of PLs and lower PL/NL values. These beneficial effects of PLs are partly associated with better transport of FAs to the rest of the organs by lipoproteins, which are vesicles formed by PLs. These results indicate that the AP of the hydrolyzed thraustochytrid biomass could be an ingredient of great interest for the aquafeed industry due to its high content of DHA in the form of PLs (DHA-PLs) and its high PL/NL value. This enrichment in DHA-PLs was not an objective at the time the experiment was designed, so it is possible that the extraction yield could be improved by modifying the unitary operations of the process.

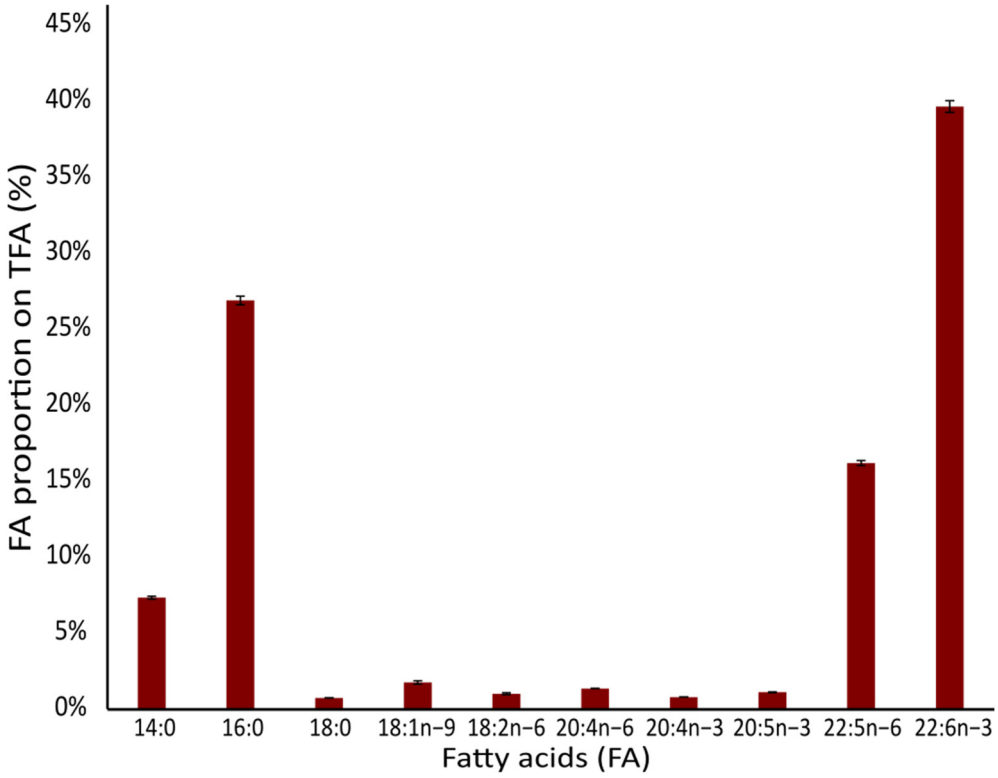

Figure 5. Fatty acid composition of thraustochytrid hydrolysate in validation conditions (pH: 7.5; reaction time: 205 min; E/S: 2.7%) with Alcalase 2.4L and the control without enzyme. Mean ± S.D.

3.6. Determination of Antioxidant Activity with ORAC Assay

Antioxidant activity has already been demonstrated for thraustochytrid biomass and has been attributed to its high content of unsaturated FAs, tocopherols, phenolic compounds, flavonoids, carotenoids and sterols [42–45]. In addition, some studies have demonstrated the production of antioxidant peptides by enzymatic hydrolysis of thraustochytrid biomass [18,46]. Incorporated into fish feed, these antioxidant peptides could promote the antioxidant defense system of fish and improve their survival, resulting in a reduction in or the elimination of synthetic antioxidants in fishmeal [47,48].

In this study, the in vitro antioxidant activity was determined on the hydrolysate fractions AP + E, BP and AP 10 kDa produced under the model validation conditions. The AP + E fraction contains all the hydrolyzed soluble molecules and lipids extracted from the biomass. The BP fraction contains the non-hydrolyzed proteins and other insoluble molecules, and the AP 10 kDa fraction only contains dissolved molecules smaller than 10 kDa, including peptides produced through hydrolysis. The WH and the AP 10 kDa fraction produced without enzymes (AP 10 kDa—no enzymes) were also analyzed. All the fractions analyzed had high antioxidant activity and presented no significant differences (Figure 6), meaning that the peptides produced through hydrolysis do not bring additional antioxidant activity or are not concentrated enough to reveal their antioxidant effect. Moreover, it must be noted that the biomass used in this study contains added

natural antioxidants, so they might also contribute to the total antioxidant capacity of the biomass. However, for all the fractions, the activity was measured for the fraction containing molecules smaller than 10 kDa. It is possible that with further fractionation (for example, of the 2000–200 Da fraction), the antioxidant activity of the produced peptides would be more evident. Moreover, in this study, the antioxidant activity was determined using the ORAC method since it is the most widely used method in the food industry. However, this method only measures peroxyl radicals, so it is important to study the activity of other potential antioxidant compounds that could be found in thraustochytrid hydrolysates by means of other methods (DPPH and hydroxyl radical scavenging ability, ABTS assay, FRAP assay, etc.). Finally, it is interesting to note that hydrolysis does not seem to destroy the molecules with antioxidant activity already present in thraustochytrid commercial biomass.

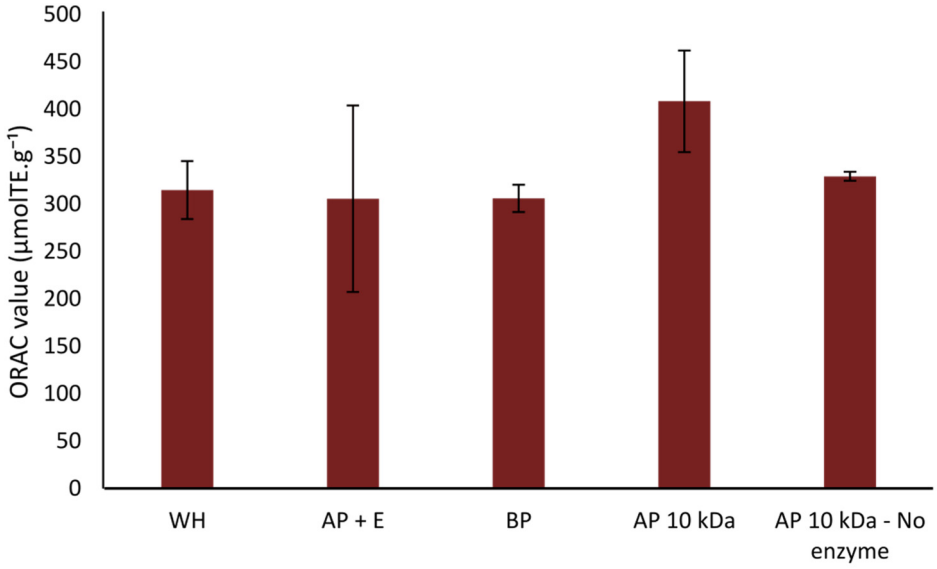

Figure 6. Antioxidant activity (ORAC) of the whole hydrolysate (WH) and its different fractions: aqueous phase + emulsion (AP + E); bottom phase (BP); aqueous phase filtered at 10 kDa (AP 10 kDa). The values of the aqueous phase filtered at 10 kDa of the control are also represented (AP 10 kDa—no enzymes). Mean ± S.D.

4. Conclusions

This study demonstrated that Alcalase 2.4L was more efficient than Papain for the hydrolysis of thraustochytrid commercial biomass, even at a low pH. A CCD was performed to optimize the pH, the E/S and the time of reaction of the hydrolysis using Alcalase 2.4L. The validation of the model was performed using a pH of 7.5, a reaction time of 205 min and an E/S of 2.7%. With these conditions, the PEP concentration and the TL-DHA content in the AP were maximized (11,228 mAU · min and 23%, respectively), and a DH of 18% was obtained. These results confirmed the predictive capacity of the CCD model.

The hydrolysis conditions proposed in this study did not affect the FA profile or the lipid content of the biomass and promoted an enrichment of the AP with PLs. Because of its high content of peptides and promising DHA-PL content, the AP produced from thraustochytrid biomass could be a sustainable ingredient in the aquafeed industry. A tendency to increase the antioxidant activity was observed for the hydrolysis carried out with Alcalase 2.4L, but more conclusive results could be obtained by analyzing the antioxidant activity with other methods and with better fractionation (by molecular weight) of the newly produced peptides. Moreover, this hydrolysate could have more interesting nutritional properties than non-hydrolyzed biomass.

In conclusion, if the hydrolysate produced in this study could provide an advantage for fish fitness when incorporated into aquafeed and help to reduce the amount of fishmeal

and fish oil used, then the treated biomass could be sold at a higher price to obtain better profitability and sustainability of aquafeed. In that case, this study offers a protocol that could be easily adapted to most thraustochytrid biomass.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/su16146052/s1. Table S1: Analysis of variance for the factors affecting the degree of hydrolysis (DH), peptide (PEP) concentration and total lipid DHA (TL-DHA) of thraustochytrid hydrolysate.

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Conflicts of Interest: Author Cloé Oroy was employed by the company Symrise Pet Food. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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sustainability

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Review

Greenshell Mussel Products: A Comprehensive Review of Sustainability, Traditional Use, and Efficacy

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Abstract: Greenshell™ mussels (GSMs), *Perna canaliculus*, are Aotearoa/New Zealand's most important aquaculture species and is sold as a variety of food products worldwide. GSMs are a traditional and culturally valuable food of the Māori people. Following the development of a series of nutraceutical products (dried powders and extracted oils) by the GSM aquaculture industry in the 1960s, there has been an increased scientific interest in the clinical health benefits of GSM products. Omega-3 polyunsaturated fatty acids in GSMs have exhibited significant anti-inflammatory activity, and the clinical evidence has led to GSM powders and oils being extensively promoted as treatments for rheumatoid arthritis and osteoarthritis. This review defines the nutritional composition of GSMs and describes the sustainability of GSMs and their traditional uses. The review also details the health benefits of GSMs in clinical applications and identifies potential mechanisms and molecular pathways initiated by the various bioactive components of GSMs.

Keywords: green-lipped mussel; greenshell mussel; marine bioactive; arthritis; omega-3 polyunsaturated fatty acids

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1. Introduction

Green-lipped mussels, trademarked as Greenshell™ mussels (GSMs), *Perna canaliculus*, are a bivalve mollusc indigenous to the inshore coastlines of Aotearoa/New Zealand (NZ). GSMs are Aotearoa/NZ's most important aquaculture species and are characterised by a green colouration of the shell and a larger size than other major commercial mussel species, such as the blue mussel (*Mytilus galloprovincialis*). The annual revenue from GSMs exported to the international food market was worth over NZD 303 M in 2021 [1]. GSMs are commercially farmed for food as well as for nutraceutical products in the form of oil extracts and powders [2].

GSMs have been a long-established kaimoana (seafood) in Aotearoa/NZ, traditionally eaten by Māori after intertidal collection. Mātaitai (seafood, shellfish, fish or other food collected from the approximate intertidal zone of the sea) was a prized taonga (treasure) to Māori people. Traditional indigenous uses and practices of preparing kūkū (GSMs) have been recorded as far back as the 1700s throughout Aotearoa/NZ and the Pacific. The kūkū (also known as kūtai and kūkūtai) was regarded as a staple part of the diet of coastal Māori people. Its benefits were traditionally recognised, and more recently, scientists have attempted to understand its effectiveness in supporting health and preventing disease. In the accounts of Sydney Parkinson, the artist aboard Captain Cook's vessel *Endeavour*, wrote in 1769, "We traded with them [the Māori] for cloth, crayfish, and mussels" [3]. In 1777, William Anderson, the surgeon aboard Cook's vessel *Resolution*, wrote, "The rocks

are abundantly furnished with great quantities of excellent mussels" [3]; thus, GSMs were one of the first internationally traded products from NZ.

The GSM aquaculture industry was developed during the 1960s and established in 1969 following the collapse of dredge fisheries; initially, the industry met local demand but has since grown to become a major exporter. GSMs are now an established export product with frozen half-shell products successfully selling in the competitive and cost-sensitive food service sector. GSMs are also used to produce high-value nutraceuticals, including oil extracts and mussel powders, which are sold worldwide. Furthermore, GSMs have become a key ingredient in pet foods, particularly for companion animals [4]. In the last decade, several unique mussel powder and oil products based on different extraction and drying technologies have come onto the market.

The earliest clinical studies focused on the potential of GSMs in the treatment of arthritis [5–7] before more broadly assessing its anti-inflammatory effects [8–10]. The potential health benefits and biological activity of GSMs are generally accepted to be derived from lipid and some carbohydrate components. The long-chain omega-3 polyunsaturated fatty acids (PUFAs) present in GSMs include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These PUFAs can effectively compete with arachidonic acid (AA) as a substrate for cyclooxygenase (COX)-2 and 5-lipoxygenase (5-LO) enzymes due to their structural similarity. However, they produce fewer inflammatory prostaglandins, broncho-constricting agents (leukotrienes) and tumour-promoting agents, such as 5-hydroxyeicosatetraenoic acid (5-HETE), in various immune cell types [11]. Instead, EPA and DHA generate potent anti-inflammatory E-series resolvins [12]. There are relatively few reports on the bioactivity of other lipid components of GSMs; for example, the furan fatty acids (FuFA) [13] or the novel anti-inflammatory omega-3 PUFAs, such as 5,9,12,15-octadecatetraenoic acid, 5,9,12,16-nonadecatetraenoic acid, 7,11,14,17-eicosatetraenoic acid and 5,9,12,15,18-heneicosapentaenoic acid [14,15]. In addition, glucosamine and chondroitin, which are a monosaccharide and polysaccharide, respectively, are found in GSMs as a component of glycosaminoglycans (GAGs) [16]. Due to their role as matrix components of cartilage tissue, they have generally been studied in the context of osteoarthritis (OA) treatment. Some studies indicated the promotion of chondrocyte proliferation, proteoglycan production and inhibition of cartilage degradation by these components [17].

Whole-GSM powder contains a mixture of proteins, lipids and carbohydrates with a complex profile of omega-3 PUFAs, sterols, GAGs and several vitamins and minerals. It appears that the combination of these compounds acts additively, or perhaps even synergistically, providing additive bioactivity. To support this observation, a clinical trial demonstrated that a combination of both omega-3 PUFAs and glucosamine provided a greater response in terms of improving the OA symptoms than glucosamine alone [18]. Further in vitro studies are needed to clarify the mechanisms by which the whole-GSM extract acts at the cellular and molecular levels.

This review comprehensively examines the production, composition and function of GSMs and explores their traditional use by the indigenous Māori population and communities. The Section 1 of the review covers the GSM content and nutritional composition and is followed by a description of its sustainability and traditional uses. The Section 2 identifies its bioactive components, their bioavailability and the details of the health benefits of GSMs in clinical applications. The Section 5 highlights the potential mechanisms and molecular pathways of each bioactive component in the context of the discovered health benefits.

2. Greenshell^{TM} Mussel Industry, Traditional Use, Sustainability and Products

The GSM nutraceutical industry has grown significantly since its small beginnings in the 1970s, exporting NZD 13.4 M (368 tonnes) of dried powder and NZD 46.0 M (21.6 tonnes) of extracted oil in 2021 [1]. The industry consists of a number of differently sized businesses that use a variety of techniques to produce a range of products, which are primarily oils, dried powders and defatted powders. It is vital that the highest quality mussels are used to produce nutraceutical products.

GSMs are opened with physical, steam or pressure assistance to release the meat from the shell. The GSM meat is then frequently homogenised and dried by either freeze-drying or bead-assisted flash drying. Extracting the lipids from GSMs creates challenges, as the lipid content is low, being approximately 6–10% of the dried product, and consists of a mixture of lipid types. The majority are polar lipids, including phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidylserine (PS), ceramides, acyl carnitines, their lysophospholipid precursors, and plasmanyl and plasmenyl orthologues. The fatty acid profile of the polar lipid fraction of mussel oil also has moderate levels (approximately 5–10% of the fatty acids profiles) of dimethylacetals of aliphatic aldehydes, a methylated component of a plasmalogen [19]. In addition, the mixture contains non-polar fats, such as triacylglycerols, diacylglycerols, sterols and free fatty acids [20]. Extraction techniques have been developed using solvents such as ethanol or super-critical CO_2 extraction (SCO_2) to isolate the lipid fraction from the protein and carbohydrate fractions. Each business has developed its own systems to produce nutraceutical products, which are often highly dissimilar to each other. The active components in GSM powder include the well-known omega-3 PUFAs, an array of bioactive lipids and GAGs, but they also include components such as glycogen, selenium, iron, iodine, phosphorus, niacin and vitamin B_{12} [21].

GSM powders and oils are sold under a broad range of brand names. GSM lipid extract, trademarked as PCSO-524™ (Lyprinol®), is produced by MacLab (Nelson, Aotearoa/NZ) in partnership with Pharmalink International Ltd., headquartered in Hong Kong. MacLab has been producing GSM nutraceutical products since 1973, and its products are sold under the trade names OmegaXL®, BioLex™, Biolane® and Seatone®. GlycOmega™ PLUS is marketed by Aroma NZ Ltd. (Christchurch, Aotearoa/NZ). Enzaq, an extraction company based in Blenheim, Aotearoa/NZ, has commercially produced mussel powders since its first GSM concentrate in 1967. Enzaq and its facility were purchased by the fishing and aquaculture company Sanford Limited in 2017. Recently, Sanford introduced two new brands, the mussel powder product PernaUltra™ and the lipid extract product PernaGold™. CFARMX, based in Motueka, Aotearoa/NZ, produces mussel powders as well as peptide and oil products and is partnered with the privately-owned Aotearoa/NZ-based seafood and agribusiness company Talley's Group Ltd. Based in Christchurch, Aotearoa/NZ, Waitaki Biosciences produces mussel powder and oil under the brand name PernaTec®. United Fisheries, also based in Christchurch, is a family-owned fishing company that produces GSM powders under the brand Nutri Zing. There are also several other companies in Aotearoa/NZ, including DryFoods in Havelock and Kōrure and Bio-Mer in Christchurch, producing mussel-based nutraceutical products.

2.1. Traditional Use of Greenshell™ Mussels 'Ka Whakangotea Ki Te Wai o Te Kākahi . . . ' (It Was Suckled on the Juice of the Freshwater Shellfish...)

This Māori proverb (whakataukī) references the cultural child-rearing practice of feeding the kākahi to young infants as an acceptable means of food to prime an infant's stomach in its formative years. Research conducted by Whaanga, Wehi [22] considered how whakataukī, such as the proverb cited above, were informed by oral traditions relating to the marine and freshwater practices of Māori.

A century ago, Best [23] observed and documented the traditional preparation methods of the Māori to cook shellfish in a steam earth oven (umu) or preserve them through a drying process. This drying method involved both curing and dehydrating the kūkū before storage in a pātaka, a raised storehouse [23]. Later, the kūkū were re-steamed prior to eating. This method sought to lock in the nutritional quality of mussels whilst extending the shelf life of the kai and embellishing its mana, which encapsulates prestige, authority, status and spiritual power as both an important and lasting food source and a tradeable asset. Kūkū and many other types of seafood were prepared this way, and these methods are still practised in some hapū (subtribe or sections of a large kinship group in traditional Māori communities) of Aotearoa/NZ.

Many of these Māori practices are similar to native Hawaiian culture, which also has a rich traditional mātauranga (the modern term for the traditional knowledge of the Māori people) base pertaining to kūkū and other marine food sources that encompasses their appropriate nutritional context, uses and values. A similar steaming method was specifically used to prepare pipi (oysters) and kio-nahawele (common mussels) for chiefs and children in pre-European Hawaii. The meat was removed from the shell, salted and placed in a nest of fibres from 'ahu'awa, a sedge plant, to drain overnight. The next morning, the pipi were wrapped in clean leaves and then placed in an umu or on a fire for cooking before eating or drying [24]. These cultural practices of early pacific communities indicate a very nuanced knowledge of the nutritional value and methods required to enhance the durability of kūkū.

The authors acknowledge that Te Ao Māori is a culture whose knowledge is recorded and transferred through oral and practical traditions as opposed to written traditions. Therefore, academic literature is not an accurate measure of recorded mātauranga Māori. It is also important to recognise that although researchers such as Best [23] recorded vast traditional content, knowledge and practices, the nature of their recording was abstract and translative through the English language. It is a mere glimpse of the totality of the knowledge base and knowledge of hapū-specific oral traditions such as te reo, tikanga, kawa and whakarite, which encompass "what we say, what we do, how we do it and how we regulate it," is required to further unlock the potential of the information found in this review.

Toroi–The Traditional Method of Fermentative Preservation

Toroi is the Māori term that describes a fermentation process that is a traditional method of preserving seafood. It is commonly known as preserved pickled kūkū or mussels, and the usual ingredients are mussels and pūhā (*Sonchus oleraceus*), a native leafy green vegetable, or watercress (*Nasturtium officinale*) as an alternative. Traditionally, toroi is a method used to preserve fish and other shellfish in addition to kūkū (both marine and freshwater), and it used a variety of traditional vegetables in the pickling process, including pūhā, pikopiko (bush asparagus-*Gastro obscura*) and tī kōuka (cabbage tree-*Cordyline australis*); watercress, dandelion and other vegetables were also used following their introduction to Aotearoa/NZ [25]. These preserved foods were a common delicacy favoured by central inland hapū, who had limited access to fresh marine resources. Fish and shellfish were traditionally packed into kono or oko, which are woven baskets and wooden bowls, respectively, and stored in pātaka or other traditional food storage areas. These were used both as a food source and as a commodity for trade. Toroi is now often prepared using glass preserving jars.

A common method of preparation is as follows: boil chopped pūhā for 30–60 min; drain; mix in chopped mussels that have been steamed open [25–27]. The pūhā and mussel mixture is then packed into preserving jars. Retained mussel juice and plant juice are added to the jar, and the jar is then overlain with fat or topped with a screwcap lid to seal the mixture airtight. The toroi mixture is then stored under cool, dry conditions. Kūkū preserved using the process of toroi is suggested to use lactic acid fermentation, which changes the pH of the food to reduce microbial growth [28]. As a means of ensuring food safety, the toroi should be made with mussels sourced from reputable and regulated sources, and, as an extra precaution, the storage of toroi should be maintained at chilled temperatures (<5 °C) until it is consumed.

2.2. Sustainability of GSM Production

Shellfish aquaculture is considered one of the most sustainable forms of aquaculture, as it has little to no negative effect on the environment [29,30]. Shellfish and GSMs are extractive species that filter nutrients from the water column; therefore, they do not require any feed inputs during cultivation. These species can also provide important coastal ecosystem functions by contributing to habitat and removing accumulated nitrogen and

phosphorous from the ambient environment when harvested [31]. Each mussel can filter up to 350 L of water per day [32], and in doing so, they remove suspended solids, bioremediate land run-off and aquaculture outputs; the improvements to water clarity allow light to penetrate, which enables the growth of algae and sea grass, and thus the key primary producers of the marine food chain can thrive [33].

The aquaculture production of extractive species, including molluscs and algae, has doubled in volume since the year 2000 [30]. A recent life cycle assessment (LCA) of Aotearoa/NZ mussels commissioned by the country's Ministry for Primary Industries (MPI) modelled that farmed mussels in Aotearoa/NZ have a lower carbon footprint per 100 g of protein than all other animal protein sources in the country, which is comparable to producing tofu (Figure 1) [34]. Although the industry has the potential to further reduce its carbon footprint, a core reason for its low impact is that it does not have to source, produce and ship feed for these animals. Instead, their filter feeding from the surrounding marine ecosystem of phytoplankton, microalgae and other particulates, including macroalgae detritus and particulate matter, allows for rich and diverse sources of essential macro- and micro-nutrients. For example, GSMs obtain long-chain (carbon length $\geq$ 20 -LC)-PUFAs directly from ocean-sourced microalgae rather than bioaccumulation lower trophic levels of the food chain, as is the case for many commercial fish species. This, in turn, ensures a rich and diverse lipid profile in GSMs. Recently a non-targeted lipidomic study of a GSM lipid fraction identified over 750 individual lipids species (data not shown; manuscript in preparation by M Miller et al.). As consumers eat the whole GSM, including the digestive gland, they also consume the contents of the mussels' last meals as well as their microbiome. Therefore, part of the nutritional benefit of the GSM comes directly from the phytoplankton and other particulates they consume.

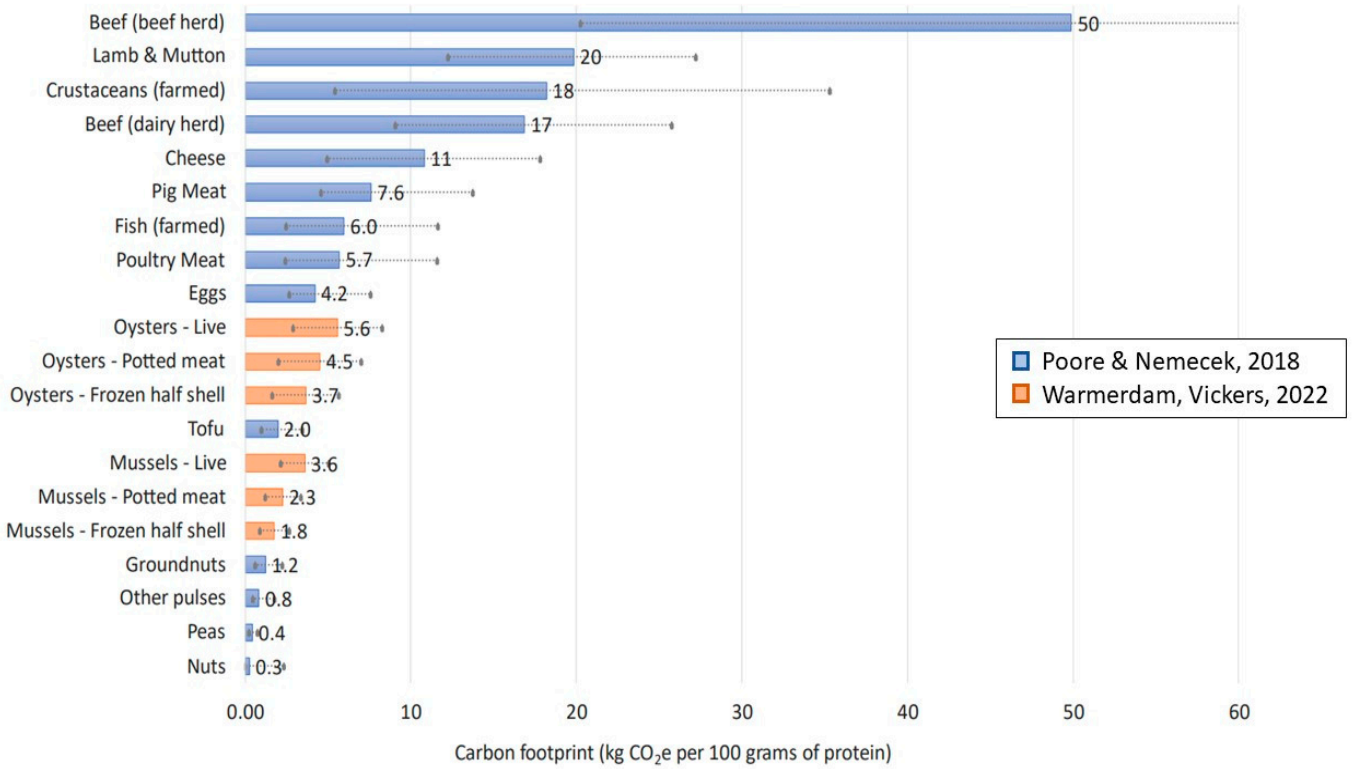

Figure 1. 'Cradle-to-retail' carbon footprint of high proteins food sources (domestic markets) from Warmerdam, Vickers [34] and data from Poore and Nemecek [35]. Taken with permission from Warmerdam, Vickers [34].

The sustainability of nutraceuticals from GSMs is superior compared to other marine resources primarily because of the sustainability and low-carbon nature of the raw

material [34]. However, producing oils and powder products does add environmental and carbon costs. The drying of marine products is an energy-intensive process [36]. Drying can cost approximately 20–30% of the total energy consumed to produce the various products [37,38]. Freeze drying has two energy-intensive stages, the freezing time and the vacuum drying time; however, it is the best technique in terms of preventing oxidative stress and protecting the bioactivity of the raw material due to low processing temperatures, which range from −2 to −10 °C, and the virtual absence of air/oxygen during processing [39]. Spray drying is potentially more energy efficient than freeze drying, as it removes the freezing stage and is much faster [40]. The extraction of lipid fractions has a further impact on the carbon footprint. Supercritical CO_2 (SCO_2) extraction of lipids has many benefits because it is highly selective, highly efficient and uses a short extraction time compared with other extraction methods. CO_2 as a solvent is inert, cheap, available, odourless, tasteless and partially recyclable. However, there are higher energy costs associated with creating the high pressures involved in SCO_2 extraction [41]. The solvent extraction of lipids has lower energy costs, but solvents such as ethanol need to be removed from the product to ensure consumer safety, although they can be recycled through the process.

2.3. Nutritional Composition of GSM

The composition of fresh, whole GSM is comprised of approximately 12–14% protein, 3–6% carbohydrate, 2–3% ash, 1.6–2.2% fat and 76–82% moisture (Figure 2) [19]. The composition of dried whole meat is comprised of approximately 36–67% protein, 10–25% carbohydrate, 2–25% ash, 6–12% fat and 0–5% moisture (Figure 2) [42]. These proportions vary across GSM and GSM nutraceutical products and depend on the season of harvest, aquaculture location, mussel diet and the procedure used to produce the powder [19]. The content of carbohydrates, fats and proteins in GSMs show major fluctuations across the season, with the lowest levels occurring during the winter and the higher levels occurring in the other seasons. Notably, fat content undergoes a 3.4-fold change over the calendar year, varying from 0.6 to 2.6 g/100 g wet weight [19]. This seasonal variation in the proximate composition of GSMs is mainly related to their reproductive cycle and controlled by water temperature, which is linked to food availability in their habitat; food availability is critical to maintaining energy requirements for spawning [19]. In addition, GSMs contain significant amounts of minerals, including iron, zinc, magnesium, calcium and iodine, making it a food rich in micronutrients [2].

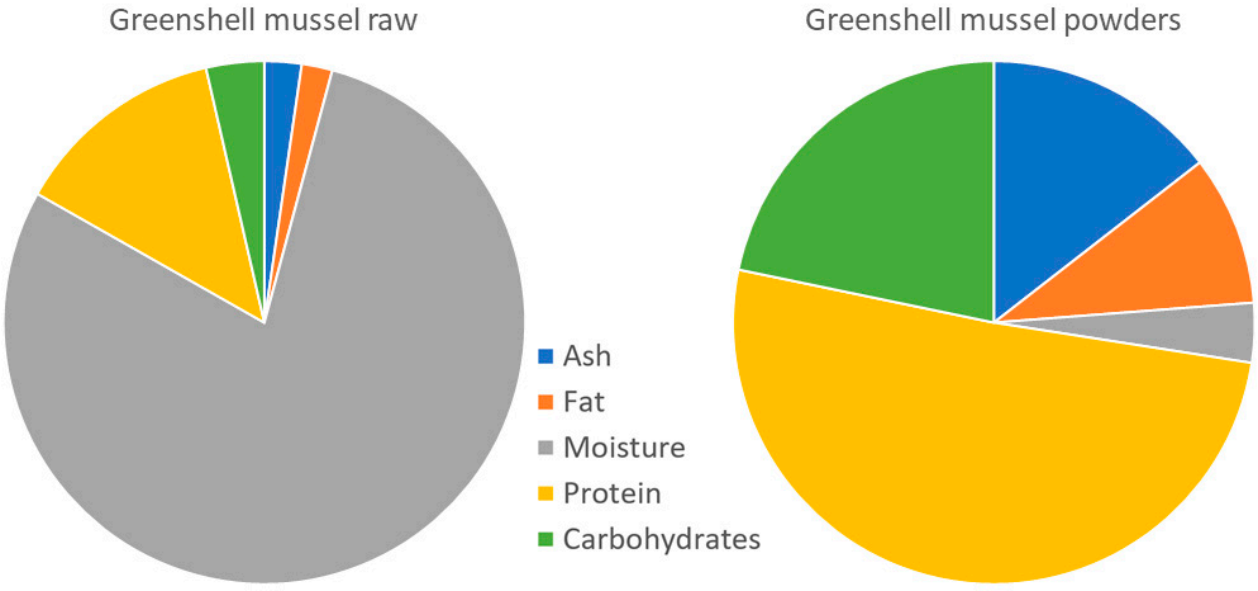

Figure 2. The composition of fresh, whole Greenshell mussels (GSMs) and GSM powders. Data taken from Miller and Tian [19] and Miller [42].

There is a strong industry and research focus on the lipid fraction of GSMs. It contains unique anti-inflammatory omega-3 PUFAs that have four double bonds and carbon chain lengths of 18, 19, 20 or 21 carbon atoms; respectively, these are 5,9,12,15-octodecatetraenoic acid (OTA, C18:4), 5,9,12,16-nonadecatertraenoic acid (C19:4), 7,11,14,17-eicosatetraenoic acid (ETA, C20:4) and 5,9,12,15,18-heneicosapentaenoic acid (C21:5). These four PUFAs have been detected in GSM lipid extracts, but not often reported in fish oil or other marine sources [14]. ETA, a structural isomer of arachidonic acid (AA), is the predominant type of PUFA detected in the lipid extract [18]. It has been shown that the COX inhibition activity of GSM oil extract is predominantly due to the free fatty acid fraction, with the greatest activity residing in the PUFA class [24]. Other fatty acids such as non-methylene interrupted (NMI), particularly 20:2 NMI and 22:2 NMI, are present in minor amounts of 1–3 g/100 g of total lipid extract, but they have potential novel biological mechanisms and bioactivity [43]. Plasmalogens, a subclass of phospholipids that include a vinyl–ether bond at the sn-1 position and polyunsaturated fatty acid at the sn-2 position, are common in GSM oil fractions, making up approximately 3–12% of total lipids [19,44]. Plasmalogens have potential health benefits for Alzheimer's and respiratory diseases. Furan fatty acids (FuFA) are another potent antioxidant and anti-inflammatory fatty acid class detected in a GSM product in one study [17], although the product in that study contained olive oil, which is a known source of FuFA [26]. Recently, FuFA were not detected when screening the extracts or raw products of GSMs (unpublished data).

2.4. Bioavailability of Bioactive Components in GSM Products

The components of GSMs that have clearly and consistently demonstrated bioactivity are omega-3 PUFAs in the lipid portion of the mussel and glucosamine and chondroitin sulphate in the carbohydrate portion of the mussel. The latter is primarily in the form of polysaccharides and are sometimes bound to polypeptides, forming a glycoprotein [45]. Glucosamine is a monosaccharide and forms one of the building blocks of GAGs, including chondroitin sulphate. Because of its large size, chondroitin sulphate is poorly bioavailable in its intact form when consumed orally. Its low bioavailability of 5–15% in the small intestine results in approximately 90% remaining unabsorbed; the remaining unabsorbed portion must be degraded by the gut bacteria in order to be absorbed [46]. Thus, degradation by gut microbiota plays a vital role in the bioavailability of chondroitin sulphate in the host [47].

The bioavailability of long-chain omega-3 PUFAs is affected by many factors. Following oral intake of omega-3 PUFAs in food or nutraceuticals, they are initially emulsified in the stomach; omega-3 PUFAs then enter the small intestine, where they are cleaved off from their TG bond to form free fatty acids and 2-monoacylglyceride (2-MAG). Free omega-3 fatty acids and 2-MAG are taken up by enterocytes and then re-esterified to form TGs, which are incorporated into chylomicrons; omega-3 PUFAs are then transferred to the lymphatic system and finally to the blood circulation, where they reach target tissues and become incorporated into cell membranes [48]. In general, EPA and DHA that are bound to triglycerides (TGs) are more effectively absorbed than the free forms bound to ethyl esters. Moreover, the fat content of food plays an important role in the absorption rate [48]. Therefore, the delivery format of a GSM product influences intestinal absorption of its omega-3 PUFAs. A study by Miller et al. [49] established the bioavailability of EPA and DHA in four orally administrated GSM products: phospholipid-rich oil, half-shell unprocessed whole mussel, TAG-rich powder and a food ingredient. Although the EPA content was consistent and all four formats were incorporated into identical whole meals of leek and potato soup, the half-shell whole mussel and powder formats resulted in a 20% greater increase in plasma EPA concentration compared to the oil format. In contrast, DHA and total n-3 LC PUFA plasma exposure parameters were not statistically different across the four GSM products. It is likely that lipid class has an impact on EPA bioavailability, but other factors, such as processing and extraction methods, may also play a role. The

bioavailability of the other components of GSMs (other lipids, vitamins, minerals and bioactive peptides) is yet to be determined.

3. The GSM Health Benefits: Evidence from Clinical Studies

The potent anti-inflammatory bioactivity of GSM lipids has made it an attractive nutraceutical treatment for various inflammatory conditions, including arthritis. Glucosamine and chondroitin sulphate from GSMs and other sources have also been marketed for decades as treatments for arthritic conditions and joint health. There are a number of potential mechanisms underlying the protective effect of the bioactive compound present in GSM (Figure 3). The following sections describe the established GSM health benefits and the cellular and molecular mechanisms by which these compounds exert their bioactive properties.

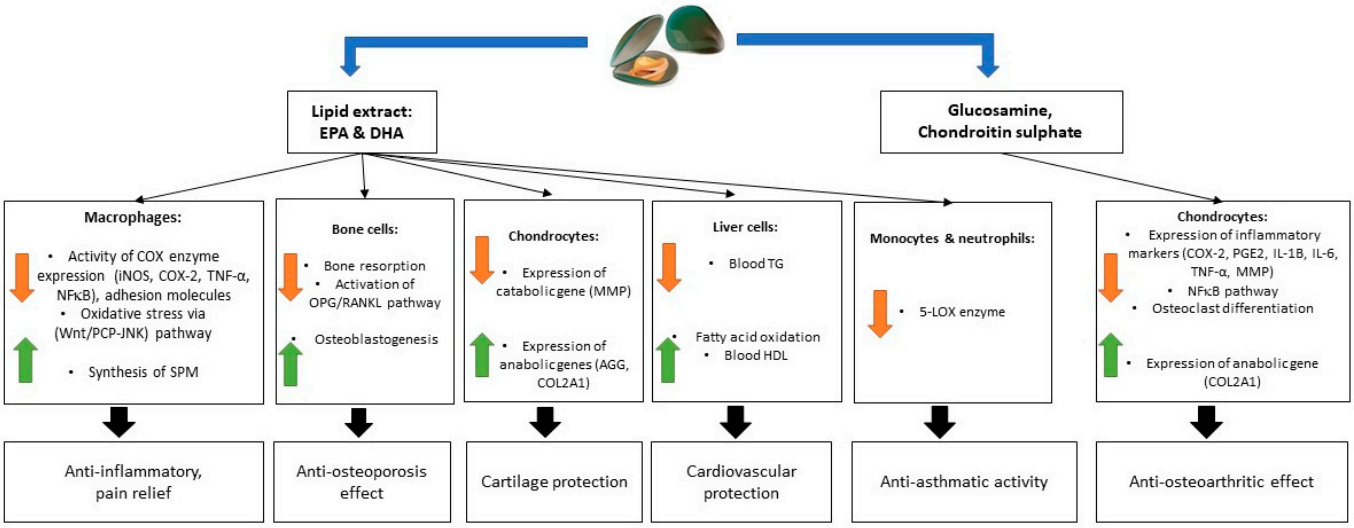

Figure 3. Potential mechanisms underlying the protective effect of bioactive compounds present in Greenshell™ mussels. AGG, aggrecan; COX, cyclooxygenase; COL2A1, collagen Type II alpha 1 chain; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HDL, high-density lipoprotein; IL-1B, interleukin-1 beta; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; LOX, lipoxygenases; MMP, matrix metalloproteinase; NFκB, nuclear factor kappa B; TNF-α, tumour necrosis factor alpha; OPG, osteoprotegerin; PGE2, prostaglandin E2; RANKL, receptor activator of NF-κB ligand; SPM, specialised pro-resolving mediators; TG, triglycerides; Wnt/PCP-JNK, Wnt/planar cell polarity-c-Jun N-terminal kinase.

3.1. Osteoarthritis, Rheumatoid Arthritis and Osteoporosis

The early clinical trials [7,50] and the majority of the subsequent studies found in the published literature have established the efficacy of GSM extract on arthritis symptoms, with the assumption being that the anti-inflammatory PUFAs in GSM reduced chronic inflammation and, thus, joint pain [7,50]. Freeze-dried GSM powder at a dose of 3 g/day for 3 months improved OA symptoms [51] and was equally effective as glucosamine sulphate at the same dose [52]. Similarly, the GSM oil extract Lyprinol at four capsules per day (200 mg of GSM oil extract/day) resulted in OA pain relief in several studies [53,54], with higher efficacy compared to fish oil [55]. BioLex, a dose of four capsules per day (600 mg/day) for 3 months, showed no effect, possibly because of the high severity of symptoms in the participants, and furthermore, this formula contains fewer bioactive lipids than Lyprinol [56]. Based on our recent systematic review and an earlier study, both GSM oil and the powder format have proven to be equally effective in reducing OA symptoms [50,57].

In studies of rheumatoid arthritis (RA), GSM powder supplementation (300–1180 mg/day) resulted in no improvement [6,58,59], whereas Lyprinol combined with fish oil (140 and

1832 mg/day, respectively) reduced RA symptoms. The improvement of RA symptoms with the oil extract but not with the powder [60] is probably due to RA patients' higher requirements of omega-3 PUFAs to modify their autoimmune-related inflammation [61]. In addition, GSM oil and powder have shown promise as protective agents against proteoglycan and collagen degradation in animal studies. For example, GSM oil (PCSO-524) fed to dogs with OA at 5mg/kg body weight once daily reduced cartilage degradation and the release of WF6, an epitope of chondroitin sulphate; in contrast, fish oil fed at 1000 mg/dog twice daily had no significant protective effect [62]. More recently, GSM powder administrated at 5% of the diet, providing 1% of the total dietary fat and 33% of dietary protein, was shown in an obese rat model of metabolic OA to reduce both the production of C-telopeptide collagen type II (CTX-II), a biomarker of type II collagen degradation, and the severity of cartilage damage in knee joints [63]. Similarly, oral supplementation of whole GSM powder at 3 g/day demonstrated a reduction in degradation of type II collagen in overweight and obese postmenopausal women and demonstrated a clinical benefit on overall joint pain as self-reported by the Visual Analogue Scale (VAS) [64]. However, in this study and in the above-mentioned study using the obese rat model of metabolic OA [63], no significant decreases in inflammatory cytokines were observed. This suggests that GSMs may act within the joint microenvironment rather than at the systemic level [64].

Animal and human studies indicate that omega-3 PUFAs can also influence bone health. Clinical trials have demonstrated that treatment with EPA and DHA decreased bone turnover markers [65], inhibited bone resorption [66] and protect against bone mineral density loss in postmenopausal women [67]. Dietary intake of omega-3 and -6 PUFAs affects the fatty acid composition of the bone, thus modulating the synthesis of PGE2, a potent stimulator of bone resorption [68]. In murine models of high-fat diet-induced obesity [69] and senile osteoporosis [70], supplementation with fish oil prevented adipose tissue expansion in the bone marrow as well as hematopoietic bone marrow atrophy and, ultimately, bone loss.

The bioactivities of glucosamine and chondroitin sulphate in promoting joint health and cartilage protection have been documented in human studies. In studies in athletes such as soccer players and cyclists, the administration of glucosamine at 1.5 or 3 g/day for 3 months reduced the urinary level of the cartilage degradation marker CTX-II in a dose-dependent fashion, but these doses had no effect on bone formation and bone resorption markers [71,72]. Glucosamine may have potential benefits in the treatment of osteoporosis and bone maintenance; however, to date, positive results in bone health and osteogenesis have been limited to experimental models. For example, a study in a rat model of combined ovariectomy-induced osteoporosis and OA induced by anterior cruciate ligament transection showed that supplementation with a mixture of glucosamine and chondroitin sulphate effectively protected both cartilage and bone. The mixture was administered at 140 or 175 mg/kg/day, which would equate to a very achievable dose of 1200–1500 mg/day for the average adult human. In this rat model, the treatment significantly reduced cartilage and proteoglycan depletion by 80%. The bone microarchitecture, assessed by micro-computed tomography (microCT), exhibited positive improvements in bone structure, and these correlated with an increased ratio of osteoprotegerin (OPG): receptor activator for nuclear factor kappa B ligand (RANKL) [73]. The OPG/RANKL system is a critical molecular pathway that induces osteoclast bone resorption and bone loss. OPG neutralises the effect of RANKL and suppresses bone resorption; therefore, an increase in the ratio of OGP:RANKL reduces the rate of bone resorption [74].

3.2. Metabolism, Chronic Inflammation and Cardiovascular Disease

There is evidence that GSM extracts effectively modulate the metabolism associated with obesity in rodent models. Increased lean mass and decreases in fat mass gain, body weight and visceral fat were observed in obese rats fed with GSM powder or oil [63,75]. Similarly, blue mussel powder improved metabolic parameters in rats [76]. In a recent study, a lipid-lowering effect was reported when GSM powder comprised 25–45% of the

basal diet of rats; this dietary intervention reduced low-density lipoprotein cholesterol (LDL-C) and lipid peroxidation in liver tissue and increased glutathione and glutathione peroxidase antioxidant activities [77]. The same study also revealed an increase in antithrombotic activity and prolonged coagulation time, which could be beneficial for patients who need to take blood thinners, such as warfarin, to prevent the formation of intravascular thromboses. These highly promising data will undoubtedly generate interest for further investigation into whole and fractionated GSMs; future studies should identify its potential uses in humans for the treatment or prevention of cardiovascular disease and numerous other disorders related to oxidative stress and inflammation.

It is well documented that a diet rich in EPA and DHA, which are the most abundant types of fatty acid in GSM, lowers the risk of cardiovascular disease, which is a leading cause of death in Western countries [78]. A previous intervention study showed intake of blue mussels (75 g/day) as a meal elevated EPA and DHA in red blood cells and plasma, which correlates with a lower risk of cardiovascular disease [79]. Habitual intake of fish oil and marine foods rich in omega-3 PUFAs has also been associated with reduced systemic inflammation [78,80]. Daily doses of EPA and DHA at 0.7 or 1.8 g/day for 8 weeks have been shown to clinically reduce blood pressure, which plays a role in lowering the risk of cardiovascular disease [81]. In addition to omega-3 PUFAs, the GAG components of GSM have been shown to provide protective cardiovascular health benefits. For example, long-term intake of glucosamine was associated with decreased cardiovascular mortality [82] and a reduction in C-reactive protein (CRP) [83], which is a biomarker for systemic inflammation.

3.3. Asthma and Airway Inflammation

Lyprinol, a GSM oil product rich in EPA and DHA, has been investigated in asthma and airway inflammation in four studies, and two of these studies showed positive outcomes. Lyprinol improved asthma symptoms and airway inflammation in asthmatic adults when taken at a dose of 4 to 8 capsules per day (each capsule contained 50 mg of GSM oil extract and 100 mg of olive oil) for 8 and 3 weeks, respectively [84,85]. However, children with moderate to severe asthma showed no significant difference between Lyprinol and olive oil treatments (4 capsules daily), although children with moderate asthma did improve with Lyprinol treatment [86]. Lyprinol taken at eight capsules per day for 12 weeks did not improve respiratory muscle function in non-asthmatic runners [87]. Due to the limited number of studies, it is likely but not conclusively proven that Lyprinol is effective in the treatment of asthmatic adults and children with mild to moderate symptoms. No other components of GSMs have been studied for bioactivity in asthma and airway inflammation.

3.4. Exercise-Induced Muscle Damage and Inflammation

There are four published clinical studies assessing the effects of Lyprinol on muscle damage induced by exercise, of which three reported a beneficial effect. Lyprinol administrated to nonprofessional runners at 400 mg/day for 11 weeks resulted in less soreness after running, with a greater effect observed in less well-trained runners [88]. Lyprinol, taken at 1200 mg/day for 26 days after muscle damage had occurred resulted in reductions in a circulating biomarker of muscle damage and inflammation, muscle pain, loss of strength, range of motion in the knee and peripheral fatigue in untrained men [89]. ESPO-572, a mixture of 75% Lyprinol and 25% krill oil, taken at 600 mg/day for 26 days prior to conducting physical exercise, was equally effective as Lyprinol at a matching dose in reducing physical symptoms of muscle damage in untrained men [90]. However, Lyprinol at a lower dose of 200 mg/day for 8 weeks provided no observable benefits in male athletes [91]. Similarly, several intervention studies have demonstrated the positive effects of EPA and DHA on delaying muscle soreness and decreasing the elevation in muscle damage markers [92–94]. In contrast, clinical trials using non-lipid components of GSM, specifically glucosamine and chondroitin sulphate, did not reduce muscle soreness, a muscle damage biomarker, or inflammation related to exercise even when provided at 1500–3600 mg/day; in fact,

glucosamine increased muscle pain [95,96]. In general, the current evidence supports the effectiveness of GSM in mitigating exercise-induced muscle damage in untrained individuals, with the bioactive factors likely being EPA and DHA lipids.

3.5. Dysbiosis and Colorectal Cancer

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used for the treatment of inflammatory diseases. The mechanism of action of NSAIDs is based on the inhibition of prostaglandin biosynthesis via inhibiting COX enzyme activity. There are two isoforms of this enzyme: COX-1 is responsible for the protection of gastric mucosa [97], while COX-2 is induced by cell damage and produces proinflammatory prostaglandins. NSAID-induced gastropathy, such as gastric ulcers, is caused by the inhibition of COX-1 [98,99]. Freeze-dried GSM powder orally administered to rats on NSAIDs with carrageenan-induced paw oedema, which is used as a model of arthritis, produced anti-inflammatory effects and reduced gastric ulcer formation [100]. This is explained by the fact that GSM lipids have stronger inhibitory effects on COX-2 (25%) compared to COX-1 (12%), and thus their overall effect protects the gastric mucosal lining [101]. Two clinical studies showed that GSM powder reduced the required dose of NSAIDs and improved gastrointestinal symptoms associated with NSAIDs in patients with knee OA [52,102]. In addition, GSM powder and glucosamine supplementation positively altered gut microbiome profiles that correlated with decreases in inflammation and OA symptoms [52].

These findings suggest that the anti-inflammatory and pain-reducing effects of GSM and glucosamine may be partially due to their effect on gut microbiota. Supplementation with GSM powder and glucosamine sulphate (3 g/day for 12 weeks) notably reduced the abundance of *Clostridium* and increased short-chain fatty acid-producing bacteria such as *Lactobacillus*, *Streptococcus* and *Eubacterium* species in the gut. Specifically, supplementation with GSM in particular increased *Bifidobacterium* and *Enterococcus* and decreased yeast species [52]. The matching effects of GSM and glucosamine on gut microbes imply that glucosamine or other indigestible compounds in GSM powder provide a substrate for gut bacteria and promote prebiotic activity. This could lead to the protection of the gut barrier [103] and increase the bioavailability of chondroitin sulphate and glucosamine, which may add to the therapeutic efficacy of GSMs and alleviate OA symptoms [104]. How intestinal microbiota metabolise GSM powder has not been fully investigated; however, in vitro [105] and in vivo [106] studies have confirmed that omega-3 PUFAs and GAG compounds present in GSM can alter the profile of the microbiome. For example, supplementation with EPA and DHA has been shown to promote the abundance of commensal and short-chain fatty acid-producing bacteria such as *Bifidobacterium* and *Lactobacillus* in healthy middle-aged people [107].

It is important to note that gut microbiota has been implicated in colorectal carcinogenesis. There is evidence that the use of glucosamine and chondroitin sulphate supplements lowers the risk of colorectal cancer [108]. Recently, an animal study using low-molecular-weight chondroitin sulphate effectively prevented the growth of colorectal tumour cells by inhibiting cell proliferation and inducing apoptosis [109]. Increasing the abundance of short-chain fatty acid-producing bacteria via supplementation with whole GSM or an isolate of its prebiotic components may be the mechanism by which it provides a beneficial effect in protecting against colorectal cancer.

3.6. Attention Deficit Hyperactivity Disorder (ADHD)

One published study provided Lyprinol (200 mg of GSM oil per day via three to four capsules) to children with ADHD in a 14-week trial. The data from this study identified a promising effect of GSM oil in treating inattention and hyperactivity, particularly in children with less severe symptoms [110]. The marine omega-3 PUFA EPA, alone or combined with DHA in the form of fish oil, has also shown benefits in children and adults with ADHD [111]. The findings of these two studies show insufficient evidence to determine the benefit of GSM in treating ADHD, and further research is warranted in this field.

4. Potential Mechanisms and Molecular Pathways of GSM Components

4.1. Cellular and Molecular Mechanisms of Lipid Components of GSM

The lipid fraction of GSM, particularly EPA and DHA, are the major components that are assumed to be responsible for GSM's anti-inflammatory and other beneficial effects observed in patients with arthritis, asthma and cardiovascular disease [2,112]. To date, the role of GSM lipid extracts or fractions has not been directly investigated for a cardioprotective effect. However, there are data from several in vitro studies using individual omega-3 PUFAs, and in particular, EPA, which support this hypothesis. EPA has shown anti-inflammatory effects in macrophages via the inhibition of the expression of adhesion molecules and monocyte chemoattractant protein 1 (MCP-1), a molecule that regulates the migration and infiltration of monocytes and macrophages, as well as the inhibition of the synthesis of metalloproteinases, which are enzymes that accumulate in and promote the formation of atherosclerotic plaques [113]. Another potential mechanism to explain the cardioprotective role of EPA is through its influence on the Wnt/planar cell polarity-c-Jun N-terminal kinase (Wnt/PCP-JNK) pathway in macrophages, which is involved in oxidative stress and inflammation [114]. Furthermore, dietary EPA and DHA reduce fasting and postprandial plasma TG levels and are approved for the treatment of hypertriglyceridemia, as they reduce very-low-density lipoprotein (VLDL)-TG production by increasing hepatic fatty acid oxidation, which reduces the TG content in liver cells. The reduction in postprandial plasma VLDL-TG concentrations is explained by an increase in lipoprotein lipase activity and enhanced chylomicron clearance [115]. Data from studies on omega-3 PUFAs treatment in patients with non-alcoholic fatty liver disease suggest that the resultant reduction in liver fat content was related to changes in DHA intake, while the reduction in fasting plasma TG concentration was mainly associated with EPA intake [116].

EPA and DHA have been positively associated with high-density lipoprotein (HDL) functionality by improving HDL size and altering their lipid content, antioxidant capacity and enzyme composition [117]. EPA and DHA can reduce blood pressure and increase vasodilation. The vasodilatory effects of EPA and DHA on vascular smooth muscle cells are largely facilitated through the opening of conductance calcium-activated potassium channels (BKCa), ATP-sensitive potassium channels (KATP) and members of the Kv7 family of voltage-activated potassium channels, leading to vasodilation and relaxation [118]. In addition, these fatty acids can protect vascular cells by mitigating the proinflammatory reactions that occur with hypertension [119].

The main mechanism of action underpinning the anti-asthmatic activity of GSM lipids is their ability to reduce leukotrienes, such as the bronchiole constrictor eicosanoid, by inhibiting 5-lipoxygenase (5-LOX) in monocytes and neutrophils [14]. With respect to muscle recovery, GSM oil contains an ample amount of fatty acids bound to phospholipids (approximately 77–82%), which gives it anabolic properties that have been shown to enhance muscle recovery by facilitating muscle protein synthesis [120]. Due to limited data, the mechanism of action by GSM lipids in reducing ADHD symptoms is still unknown; however, it has been proposed that its anti-inflammatory activity and ability to decrease the ratio of AA to EPA may lead to improvement in associated symptoms [121].

There are several mechanisms proposed for the anti-arthritic effects of GSM oil. Its lipids are well documented as having an inhibitory effect on COX-2 and the 5-LOX cascade to suppress the inflammatory response [101]. Specialised pro-resolving mediators (SPMs) are compounds enzymatically derived from EPA and DHA when they are metabolised by LOX. These novel anti-inflammatory molecules, which include resolvins, maresins and protectins, promote the resolution of inflammation, tissue healing and relief of chronic pain in rheumatic diseases [122,123]. Another mechanism for the anti-inflammatory effect of GSM lipids is the suppression of gene expression of inducible nitric oxide synthase (iNOS) and COX-2, resulting in reduced levels of nitric oxide (NO) and prostaglandin E2 (PGE2), two mediators of the inflammatory response. GSM lipids also down-regulate the expression of the proinflammatory cytokines tumour necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β and suppress the nuclear factor kappa B (NF-kB) signalling pathway and

phosphorylation of mitogen-activated protein kinases (MAPKs) in macrophage cells [124]. The enhanced expression of these proinflammatory cytokines and activation of NF-kB signalling in macrophages that reside in synovial membranes have both been associated with the development of OA [125]. Thus, inhibiting the NF-kB pathway in macrophages by GSM lipids is another likely mechanism by which they modify OA disease incidence and progression.

Plasmalogens are a class of glycerophospholipids found in GSMs and other marine bivalves, accounting for 10–35% of their total lipids [126,127]. Plasmalogens are unique phospholipids, being characterised by a vinyl ether at the sn-1 position attached to saturated and monounsaturated fatty acids (C16:0, C18:0 and C18:1), while PUFAs, specifically DHA (C22:6 omega-3) or arachidonic acid (C20:4 omega-6), are found in the sn-2 position [128,129]. Plasmalogens are present in cell membranes where they serve as endogenous antioxidants due to their vinyl ether double bond, protecting the PUFAs in the sn-2 position from oxidative stress [130]. Low levels of plasma ethanolamine plasmalogens have been linked with Alzheimer's disease and cognition deficit, and there is growing interest in clinical interventions using plasmalogens as potential therapeutics for Alzheimer's [131].

The treatment of chondrocytes, the cells that produce cartilage, with omega-3 PUFAs from GSM oil versus krill oil and versus fish oil demonstrated overall down-regulation in the expression of the catabolic genes matrix metalloproteinase (MMP)-1, MMP-3 and MMP-13, which code for enzymes that degrade collagen; the data showed up-regulation in the expression of anabolic genes that produce aggrecan (AGG) and the alpha-1 subunit of collagen type II (COL2A1), which are components of healthy cartilage [132]. The PUFAs also reduced the release of sulfated glycosaminoglycans (s-GAGs); however, for this effect, fish oil and krill oil were superior to GSM oil, a variation that is likely due to the differences in their forms of lipid classes. In krill and GSMs, the omega-3 PUFAs are mainly in the form of phospholipids, while in fish oil, these are in the form of either triacylglycerols or fatty acid esters. With respect to individual fatty acids, EPA was more effective than DHA in promoting anabolic activity and reducing catabolic activity. However, omega-3 PUFAs were unable to reduce the expression of inflammatory cytokines IL-1β and TNF-α in chondrocytes.

A non-polar lipid fraction of GSM oil, which is rich in free fatty acids, was shown to possess potent anti-osteoclastogenic activity. Osteoclasts are cells that degrade and resorb bone. In an in vitro study using the murine macrophage cell line RAW 264.7 stimulated with RANKL to induce osteoclastogenesis, cells treated with the non-polar GSM lipid extract significantly inhibited osteoclast differentiation, the production of tartrate-resistant acid phosphatase (TRAP) enzyme that degrades bone, and the number of TRAP-containing cells, which in vivo would equate to a decrease in osteoclast activity and bone resorption [133]. The non-polar lipids also diminished the formation of actin rings in osteoclasts, which is an important element of bone resorption. In addition, this treatment down-regulated mRNA expression of several genes related to osteoclast function and bone digestion, including cathepsin K, carbonic anhydrase II (CA II), MMP-9 and nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), which is a master transcription factor of osteoclast differentiation. DHA and EPA in the form of free fatty acids are likely responsible for this anti-osteoclastogenic effect. Further studies are needed to confirm this bioactivity of non-polar GSM lipids on bone metabolism in humans.

EPA and DHA from marine sources have been shown to positively affect bone metabolism. The osteoprotegerin (OPG)/RANKL signaling pathway is the main mediator of osteoclastogenesis. AA and its metabolite PGE2 are the main stimulators of RANKL expression, leading to enhanced bone resorption. Krill oil, which contains abundant omega-3 EPA and DHA, suppresses the osteoclastogenesis-related OPG/RANKL pathway by decreasing the secretion of PGE2 and its receptor EP4 [134]. Moreover, omega-3 PUFAs can regulate bone formation and the differentiation of osteoblasts. In vitro evidence from bone marrow mesenchymal stem cells treated with krill oil showed an increase in osteogenesis via increasing Runx2 expression, a transcription factor that promotes osteoblastogenesis, as

well as in vivo evidence of krill oil downregulating PPARγ and adipogenesis in a mouse model of postmenopausal osteoporosis [135]. There is also evidence that omega-3 PUFAs can act as PPAR ligands and modulate osteoclast formation. In a study by Kasonga, Kruger [136], DHA and EPA activated PPARα and PPARγ to a larger extent than PPAR β/σ in the human CD14+ osteoclast cell line. This study showed that PPAR activation exerted an inhibitory effect on osteoclastogenesis via the modulation of RANKL signaling [136].

4.2. Cellular and Molecular Mechanisms of Glucosamine and Chondroitin Sulphate and Bioactive Peptides

Whole GSM powder typically contains 3% GAGs [2]. There is only one in vivo study in which glycogen isolated specifically from GSMs produced an anti-inflammatory effect in rats with induced footpad oedema; however, the anti-inflammatory effect disappeared with further hydrolysis of protein, suggesting that a protein component of glycogen was responsible for the effect [137]. The existing in vitro studies on glucosamine and chondroitin sulphate used samples obtained from bovine and other marine sources; there are no data available specifically on GAGs derived from mussels. Nevertheless, as the origin of GAGs has not been shown to impact their bioactivity, GAGs present in mussels are likely to possess similar bioactivity to bovine GAGs.

As the main component of the extracellular matrix in cartilage, GAGs have been widely studied for joint and cartilage protective properties [138]. The potential anti-inflammatory and chondroprotective effects of glucosamine on human osteoarthritic chondrocytes and the possible mechanisms have been investigated. Glucosamine sulphate reduced chondrocyte expression and the release of COX-2, PGE2, IL-1β, IL-6, TNF-α and MMPs, while it up-regulated COL2A1 [139,140]. It also reduced apoptosis and promoted both chondrocyte proliferation and proteoglycan production [140,141]. The effects of glucosamine sulphate are mediated through the inhibition of NF-kB activity [142]. However, chondrocyte and synoviocyte cell cultures treated with glucosamine at physiologically relevant concentrations found in serum and synovial fluid did not show the same impact on proteoglycan production or MMP-13 synthesis; however, the results showed a decrease in the production of PEG2 in chondrocytes, suggesting that oral supplementation with glucosamine at clinically relevant concentrations may reduce inflammation in joint disease [143]. In an in vitro study using human primary osteoclasts cultured in a dynamic three-dimensional system to resemble the in vivo bone microenvironment, treatment with glucosamine sulphate decreased osteoclast cell differentiation and function; in addition, osteoclasts isolated from patients with OA were more sensitive and responsive to glucosamine compared to osteoclasts from healthy donors [144].

In a clinical trial, participants regularly taking a glucosamine supplement or a chondroitin sulphate supplement, or both, were found to have lower biomarkers of systemic inflammation compared to non-users [145]. In addition to possessing chondroprotective and anti-inflammatory activities, the GAGs present in GSMs may have health benefits in preventing sarcopenia, a state of high-fat mass and relatively low muscle mass that commonly occurs with ageing. Several studies have shown an association and comorbid interaction of sarcopenia with musculoskeletal diseases such as OA and osteoporosis [146,147]. The mechanisms of action of GAGs on the pathophysiology of sarcopenia have been partially identified. These compounds inhibit the proinflammatory NF-kB that is activated in muscle atrophy. Furthermore, they supply building blocks for the regeneration of connective tissue surrounding myocytes [148].

The proteins from mussels have also been investigated to identify bioactive peptides. Antioxidant, antimicrobial and angiotensin-converting enzyme (ACE)-inhibitory activities are the main bioactive features that have been found for mussel peptides [149]. ACE is the enzyme that converts angiotensin I into angiotensin II, which constricts blood vessels and increases blood pressure; therefore, inhibiting ACE is a target for hypertension treatment [150]. To date, only one published study has characterised bioactive peptides from GSMs [151]. This study singled out one bioactive peptide isolated from enzymatic hydroly-

sis by pepsin after 30 min. Named GPH, the peptide possessed the highest antioxidant and ACE inhibitory activities but no antimicrobial activity. Other peptides demonstrated both antioxidant and antihypertensive bioactivities, but to a lesser degree than GPH. This study also showed that the antioxidant and ACE inhibitory activities were predominantly in peptides with a molecular weight of less than 5 kD. Analysis of the amino acid composition for these peptides showed that hydrophobic amino acids, such as glycine, valine, lysine, isoleucine and alanine, were present in higher quantities compared to polar and charged amino acids; in addition, the hydrophobic amino acids appeared to have contributed to the observed bioactivity. In this study, GSM peptides did not show any antimicrobial activity; the authors suggest potential antimicrobial peptides from GSMs are more likely to be present in the haemolymph and would best be extracted using organic solvents. Bioactive peptides with potent antioxidant or ACE inhibitory activity have enormous potential for the treatment of cardiovascular diseases, and it would be of interest to confirm the in vitro findings with GPH by conducting further investigations in vivo.

5. Conclusions

Greenshell^{TM} mussels, kūkū, a kaimoana species endemic to Aotearoa/NZ, have for centuries been consumed, either fresh, dried or pickled, for their health-promoting bioactivity as well as for their nutritive content. GSMs are the basis of the country's highly sustainable aquaculture industry and a major export product. Although GSMs are low in fat and carbohydrates and high in protein, it is their non-protein components that are of the most value due to their bioactivity. The lipid fraction of GSMs is rich in omega-3 PUFAs, including DHA and EPA, which have been shown to provide protection against inflammation, osteoarthritis, osteoporosis and cardiovascular disease by modulating well-defined cellular signaling pathways. GSM lipid products, such as Lyprinol, have been proven in multiple clinical trials to be efficacious and have been marketed primarily to osteoarthritis patients for decades; in recent years, research has focused on the lipids' bioactive effects in asthma and muscle damage. Glucosamine and chondroitin sulphate have also been shown to protect against osteoarthritis and other joint disorders by providing the building blocks needed to produce new cartilage and by modulating cellular functions. GSMs contain other unusual components, including plasmalogens and unique lipids, which are likely to have novel bioactivities as well as at least one novel peptide with antioxidant and antihypertensive bioactivity. Further investigations will likely identify additional bioactive components and health benefits of GSM.

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Abbreviations

2-monoacylglyceride (2-MAG); 5,9,12,15-octodecatetraenoic acid (OTA); 5-hydroxyeicosatetraenoic acid (5-HETE); 5-lipoxygenase (5-LO); 7,11,14,17-eicosatetraenoic acid (ETA); Aggrecan (AGG); Alpha-1 subunit of collagen type II (COL2A1); Angiotensin-converting enzyme (ACE); Aotearoa—New Zealand (NZ); Arachidonic acid (AA); ATP-sensitive potassium channels (KATP); Attention deficit hyperactivity disorder (ADHD); Calcium-activated potassium channels (BKCa); Carbonic anhydrase II (CA II); Collagen Type II alpha 1 chain (COL2A1); C-reactive protein (CRP); C-telopeptide collagen

type II (CTX-II), Cyclooxygenase (COX); Docosahexaenoic acid (DHA); Eicosapentaenoic acid (EPA); Furan fatty acids (FuFA); Glycosaminoglycans (GAGs); GreenshellTM mussel (GSM); High-density lipoprotein (HDL); Inducible nitric oxide synthase (iNOS); Interleukin 6, IL-6; Interleukin-1 beta, IL-1B; Lipoxygenases; LOX; Low-density lipoprotein cholesterol (LDL-C); Matrix metalloproteinase (MMP); Ministry for Primary Industries (MPI); Non-methylene interrupted (NMI); Nonsteroidal anti-inflammatory drugs (NSAIDs); Nuclear factor kappa B (NFkB); Nuclear factor of activated T-cells cytoplasmic 1 (NFATc1); Osteoarthritis (OA); Osteoprotegerin (OPG); Phosphatidylcholine (PC); Phosphatidylethanolamine (PE); Phosphatidylglycerol (PG); Phosphatidylinositol (PI); Phosphatidylserine (PS); Polyunsaturated fatty acids (PUFAs); Prostaglandin E2 (PGE2); Receptor activator of NF-kB ligand (RANKL); Rheumatoid arthritis (RA); Specialised pro-resolving mediators (SPM); Sulfated glycosaminoglycans (s-GAGs); Supercritical CO2 (SCO2); Tartrate-resistant acid phosphatase (TRAP); Triglycerides (TGs); Tumour necrosis factor alpha (TNF-α); Very-low-density lipoprotein (VLDL); Visual Analogue Scale (VAS); Wnt/planar cell polarity-c-Jun N-terminal kinase (Wnt/PCP-JNK).

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A Lipidomic Profile of a Sustainable Source of Omega-3 Long-Chain Polyunsaturated Fatty Acids, Greenshell Mussels™, *Perna canaliculus*

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Abstract: Greenshell mussel (GSM- *Perna canaliculus*) is the most important aquaculture species in New Zealand and produces one of the most expensive bioactive lipid extracts on the nutraceutical market. There have been numerous studies on the composition of GSM as well as pre-clinical and clinical studies on the efficacy of GSM extracts and foods. With increases in analytical capabilities, lipidomic studies using advanced mass spectral data may provide new insight into the content and activity of the lipidome, the totality of all lipids, of GSM. This study is the first reported characterisation of the GSM lipidome which may disclose important novel information regarding its nutrition, biology, physiology, and epidemiology. This study adds to the traditional lipid analytical outputs with new lipidomic capabilities to interrogate the lipid species differences between tissues rich in oil. We have identified 16 different lipid species in GSM including ceramide aminoethyl phosphonate (CAEP). Many lipid species are differentially expressed between tissues and correlation analysis demonstrates lipid species associated with the digestive gland that may be obtained from food sources, whilst other lipid species are dominant in the mantle or gonad. Linking this new information to the GSM breeding programmes may deliver functional breeding attributes to deliver premium strains for enhanced nutrition and/or extract production.

Keywords: green-lipped mussels; greenshell™ mussels lipidomics; sustainable; bivalve; aquaculture; mass spectrometry

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1. Introduction

The Greenshell™ mussel (GSM), is endemic to the coastlines of New Zealand. The mussels are farmed in waters that provide all the nutrition required for their growth. GSM is New Zealand's leading aquaculture species, in terms of both total volume and value. In 2022 the New Zealand GSM industry produced revenue of ~USD 192M from 33,237 tonnes of exported products [1]. GSMs are sold as food and are also used to produce high-value nutraceuticals including oil extracts and freeze-dried mussel powders, for example, Lyprinol®. GSM lipids are extracted by supercritical CO_2 with/without ethanol as a co-solvent or by using chemical extraction methods to produce nutraceutical oils. To the best of our knowledge, GSM oil is the most valuable marine oil (by price) in the world (~US$1220/kg in 2023). Studies have shown that lipid extracted from GSM has numerous health benefits, including the ability to reduce inflammation, reduce pain, and aid mobility [2–9] recently reviewed by Miller, et al. [10]. This high price is driven not only by the consumer benefits but by the high cost of extraction and production of GSM oil.

The GSM lipid fraction contains a high proportion of omega-3 long-chain ($C \geq 20$) polyunsaturated fatty acids (omega-3 LC-PUFAs), predominantly docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), which are split between the triacylglycerol and polar lipid classes [11–14]. Further, it contains a series of novel lipids not found

in commodity marine oil such as anchovies, tuna, and sardine, including non-methylene-interrupted (NMI)-FA, fatty aldehydes (FALD), plasmalogens and phytosterols [12]. The lipid content of GSM, and the lipid classes and fatty acid (FA) profile of GSM oil, are affected by many factors, including the season, location, and the types and amounts of algae consumed [12,13].

GSMs are filter feeders and obtain lipids directly from the microalgae they consume. GSMs are considered to be one of the most sustainable sources of omega-3 LC-PUFA available as they are farmed, rather than wild-harvested, and do not require any dietary inputs for their nutrition [10]. Recently there have been attempts to understand the capacity that invertebrates have in the production of omega-3 LC-PUFA [15]. Functional gene characterisation of the freshwater mollusk Unionida (*Elliptio complanata*) demonstrated a series of desaturases that have the ability to produce omega-3 LC-PUFA de novo [15]. This finding indicates mussels may have the capacity to make significant amounts of omega-3 LC-PUFA and possibly other fatty acids endogenously rather than obtaining them from dietary sources.

Understanding the physiology and nutritional requirements of GSM might result in higher yields for the nutraceutical industry and better human health outcomes for consumers. Lipids are important components of cellular membranes and are integral to various physiological processes like reproduction, growth, immunological responses, and energy reserves. The lipid diversity, particularly for intact lipids, in GSM is poorly understood as research has focused on traditional techniques of FAME analysis by gas chromatography (GC) or GC-mass spectrometry and lipid class assessed by thin layer chromatography–flame ionisation detection (TLC-FID i.e., Iatroscan™) [11–14]. Technological advancements have provided tools to unveil the diversity in intact lipids. These advances include ultra-high performance liquid chromatography (UHPLC) and new column phases as well as improvements to mass spectroscopy such as the electrospray ionisation source (ESI), further developments in multidimensional and high-resolution mass spectrometry, and incremental improvements in data analysis (recently reviewed by [16]). Lipidomics, a branch of metabolomics, is the science targeting the lipidome (e.g., the totality of biological lipids in an organism). Understanding the lipidome for GSM could disclose important information regarding the nutrition, biology, physiology, genetics, and epidemiology of bivalves. It may also aid research looking at the efficacy of GSM extracts in pre-clinical and clinical studies [17–19]. This knowledge, once applied, has the potential to improve GSM production, analytics, clinical outcomes, and the resilience of the GSM industry to environmental changes.

In this study, we compare the results of three oil-rich tissues from GSM using both a traditional analysis of lipids and a lipidomics method that uses a C_{30} reverse phase UHPLC column that has the capacity to separate not only lipid classes and species but also iso-baric lipid species in a single LC-MS/MS analysis.

2. Materials and Methods

2.1. Greenshell Mussels (GSM) Sampling

GSMs were provided by SpatNZ (Nelson NZ). Nine GSM were weighed, measured (length and width at widest point), shucked, and then the mussel meat and shell weights were recorded. Three tissues were dissected from each mussel: gonads, mantle, and digestive gland as per Miller et al. [12]. Only female mussels of harvestable fatness and gonads containing both eggs and storage reserves were used in this trial. Gonad maturity was scored using the visual grading system defined by Buchanan [20].

2.2. Traditional Lipid Chemistry Analysis

Lipids from the GSM samples were extracted using a modified Folch methodology [21]. In brief, mussel homogenate (1–2 g) was extracted in 20 mL of chloroform/methanol (2:1) with constant mixing for 20 min, and phase splitting was achieved by the addition of

4 mL of NaCl solution in H_2O (0.9%, w/v). The organic lower phase was collected and concentrated under nitrogen.

Lipid classes were analysed with an Iatroscan MK V thin-layer chromatography-flame ionisation detector (TLC-FID) (Iatron Laboratories, Tokyo, Japan). Samples were spotted onto silica gel SIII Chromarods (5-µm particle size) and developed in a glass tank lined with pre-soaked filter paper. The solvent system used for lipid separation was hexane/diethyl ether/acetic acid (60:17:0.1, $v/v/v$). After development for 25 min, the Chromarods were dried with hot air and analysed immediately to minimise absorption of atmospheric contaminants. Lipid classes were quantified using Clarity software (DataApex; Prague, The Czech Republic). The FID was calibrated for each lipid class using the following compounds: phosphatidylcholine, cholesterol, oleic acid, hydrocarbon (squalene), and triglyceride (TG, Glyceryl Trioleate). All standards were purchased from Sigma Aldrich (Auckland, New Zealand).

An aliquot of the total lipid extract (TLE) from each sample type was trans-methylated in methanol/chloroform/hydrochloric acid (10:1:1, $v/v/v$) for 1 h at 100 °C. After the addition of water, the mixture was extracted three times with hexane/chloroform (4:1, v/v) to obtain fatty acid methyl esters (FAME). Samples were made up to 1 mL with an internal injection standard (19:0 FAME). FAME samples were run in accordance with AOAC official methods 963.22 "Methyl Esters of fatty acids in oils and fats". In brief, FAME was analysed by gas chromatography (GC) performed using an Agilent 6890 with an Agilent SP-2560 silica capillary column (100 m v 0.25 mm i.d., 0.2 µm film thickness) and flame ionisation detection (FID). Samples (1 µL) were injected via a split injector at 260 °C. The column temperature program was: 220 °C at 17 min then raise by 2.8 °C min^{-1} to 240 °C and hold for 5 min. Nitrogen was the carrier gas. GC results were typically repeatable to within ±5% of the individual component area for replicate analyses. Supelco 37 Component FAME (Auckland, Sigma Aldrich) Mix was utilised for verification of FAME identification.

2.3. Lipidomic Analysis

Samples were analysed on a ThermoScientific Orbitrap Fusion tribrid mass spectrometer coupled with a ThermoScientific Vanquish UHPLC system based on the methods of Rampler [22]. Briefly, 2 µL of the sample was injected onto a Vanquish C_{30} column (ThermoFisher, Waltham, MA, USA) at a flow rate of 0.26 mL/min and initial composition of 30% solvent B. The composition was raised to 43% B at 2 min then 55% at 2.1 min, 21 min 65% B, 18 min 85% B, 20 min 100% B, 25 min 100% B, returning to 30% B at 25.1 min until the end of the analysis at 30 minutes. The source conditions for the H-ESI source were spray voltage 3500 V in positive ion mode or 2400 V in negative ion mode. The gas pressures were set to sheath gas, 35 arb units, auxiliary gas 5 arb units, sweep gas 1 arb units, and both the vaporiser temperature and ion transfer tube were 300 °C. The mass spectrometer was operated in both positive and negative ion modes with the MS scan conducted in the Orbitrap set at 120,000 resolution and a scan range between 250–1500 m/z in positive ion mode and 320–2000 m/z in negative ion mode with the automatic gain control (AGC) target of 4.0 × 105. MS/MS analysis was conducted in the ion trap, ions that reached an intensity greater than 5.0 × 103 were isolated using the quadrupole set at 0.7 m/z, the HCD collision energy was set at 25, 30 and 35% in positive ion mode, and 30% in negative ion mode, and the mass was excluded for 5 seconds. Phosphatidylcholine analysis was conducted by a triggered MS/MS if the positive daughter ion was 184 m/z, the parent ion was isolated using the quadrupole and subjected to CID fragmentation at 32%. Similarly, the neutral loss of fatty acid on a triacylglycerol also triggers MRM using CID at 35% to confirm TAG species.

Orbitrap fusion data were analysed using LipidSearch software (version 4.1.3, ThermoFisher) using the product search feature with the precursor tolerance set to 5.0 ppm and product tolerance set to 0.5 Da to reflect the use of the ion trap for the MS/MS analysis. The orbitrap database was selected. In the first pass of the analysis, all lipid classes were selected and the ions were -H^-, +$HCOO^-$, -$2H^-$ for negative ion mode and +H^+, +$NH4^+$

in positive ion mode. Compound Discoverer was also used to further identify unknowns. After successive rounds of data analysis with both LipidSearch and Compound Discoverer, lipid species were identified using LipidSearch algorithm to search the MS/MS spectra and create positive identification of the lipid species. The MS/MS data provided sufficient information to determine the acyl chain composition of each lipid species but not the stereospecific numbering (sn) of the acyl chain position on the 3 carbon glycerol backbone and thus denoted as "_" between acyl chains ("/" is used when sn position is known). Retention time and m/z graphs were plotted to establish the ordered elution of lipids.

Further analysis was conducted using in-house R scripts (R version 4.2.1, using the tidyverse package [23,24]) for data normalisation of Compound Discoverer (ThermoFisher) results and to aid in the identification of lipid species. Probabilistic quotient normalisation (PQN) was applied to normalise the data without the potential introduction of artificial correlations in the data [25]. Missing values were removed from the data analysis. Statistical analysis and plotting were conducted using the R packages stats, emmeans, ggplot2, cowplot, GGally, ggfortify, and RColorBrewer packages. The significance between tissues was determined for compounds that were present in more than half of the samples and were present in all three tissues using ANOVA and Tukey HSD of the log-transformed data. Principal component analysis (PCA) was conducted for singular value decomposition using the "prcomp" function in the R Stats package. Correlation analysis was conducted using the "pairs" function in the R graphics package and the Pearson correlation coefficient was calculated using the "cor" function in the R Stats package.

3. Results

The mussels selected for analysis were large females (average shell length 113 $\pm$ 11 mm, width 51$\pm$ 5 mm) with marketable plumpness and colour (gonad visual grading score 6.6 $\pm$ 0.4). Gonads, mantle, and digestive glands were chosen as organs of interest as they have been previously shown to contain the majority of the lipid content and are important in digestion and reproduction [12]. The tissues tested made up about 45% of the total wet weight of the GSM, relatively clean samples could be dissected out representing the bulk of each organ. The remainder includes muscle, foot, gills, digestive tract heart, and some remnants of the target tissues.

3.1. Traditional Lipid Chemistry Analysis

The lipid content, lipid class, and fatty acid content are shown in Table 1. This traditional way to present the lipid data gives the overall content class and fatty acid profile of GSM. The gonad had the highest lipid content (4.8 g/100 g) of the three organs, while the mantle was leaner with only 1.6 g/100 g lipid. The lipid class analysis, by TLC-FID, showed that the majority of the lipid in all three organs was polar lipids.

Table 1. Traditional analysis of the three organs of Greenshell mussel for proximate composition, lipid class, and fatty acid analysis.

	Mantle	**Digestive Gland**	**Gonad**	**Mantle**	**Digestive Gland**	**Gonad**
Composition		Whole tissue			Extracted oil	
Moisture (g/100 g of Organ)	81.2	72.5	68.9	NA	NA	NA
Lipid (g/100 g of Organ)	1.6	4.2	4.8	NA	NA	NA
Lipid (g/100 g of GSM)	0.28	0.45	0.77	NA	NA	NA
Proportion of organ in the Mussel	16.1	17.7	10.7	NA	NA	NA
Lipid class content		mg/100 g of GSM			g/100 g of oil	
Hydrocarbon/nonpolar lipids	1.9	1.9	2.1	0.7	0.4	0.3
Free Fatty Acids	2.7	4.0	1.3	1.0	0.9	0.2
Triacylglycerols	27.2	138.7	219.8	9.8	31.0	28.4

Table 1. *Cont.*

	Mantle	Digestive Gland	Gonad	Mantle	Digestive Gland	Gonad
Sterols	6.7	5.4	9.2	2.4	1.2	1.2
Polar lipids	238.2	297.3	541.9	86.2	66.4	70.1
Fatty acid content (mg/100 g of GSM)		mg/100g of GSM			% Fatty acids	
C14:0 myristic acid	9.3	16.9	30.9	4.2	4.7	5.0
C15:0 pentadecanoic acid	1.2	1.4	3.2	0.6	0.4	0.5
C16:0 palmitic acid	32.6	49.4	98.7	14.7	13.8	16.0
C16:1 palmitoleic acid	16.2	36.7	55.8	7.3	10.3	9.0
C16:2n4 hexadecadienoic acid	1.3	4.2	4.9	0.6	1.2	0.8
C17:0 heptadecanoic acid	1.5	1.8	4.0	0.7	0.5	0.6
C18:0 stearic acid	8.7	13.6	22.7	3.9	3.8	3.7
C18:1n7 vaccenic acid	6.0	9.5	19.5	2.7	2.7	3.2
C18:1n9c oleic acid	2.6	4.9	6.8	1.2	1.4	1.1
C18:1t elaidic acid	0.2	0.2	0.9	0.1	0.1	0.1
C18:2n6c linoleic acid	3.8	4.9	9.3	1.7	1.4	1.5
C18:3n3 alpha linolenic acid (ALA)	1.4	2.4	4.9	0.7	0.7	0.8
C18:3n6 gamma linolenic (GLA)	0.3	0.6	1.3	0.1	0.2	0.2
C18:3n4 octadecatrienoic acid	4.1	5.0	10.4	1.9	1.4	1.7
C18:4n3 stearidonic acid (SDA)	3.9	9.1	16.3	1.8	2.5	2.6
C20:0 arachidic acid	0.1	0.3	0.3	0.0	0.1	0.0
C20:1 gadoleic acid	0.9	0.9	1.4	0.4	0.3	0.2
C20:3n3 cis-11, 14, 17-eicosatrienoic acid	0.1	0.5	0.8	0.0	0.1	0.1
C20:3n6 cis-8, 11, 14-eicosatrienoic acid	0.6	1.2	2.5	0.3	0.3	0.4
C20:4n3 eicosatetraenoic acid	0.6	1.9	2.3	0.3	0.5	0.4
C20:4n6 arachidonic acid (AA)	4.5	5.3	8.2	2.1	1.5	1.3
C20:5n3 eicosapentaenoic acid (EPA)	45.5	94.9	157.9	20.6	26.5	25.5
C22:5n3 docosapentaenoic acid (DPA)	3.7	6.7	9.1	1.7	1.9	1.5
C22:6n3 docosahexaenoic acid (DHA)	28.2	30.7	67.0	12.8	8.6	10.8
Fatty acid classes		mg/100 g of GSM			% Fatty acids	
∑SFA	53.6	83.7	160.1	24.2	23.4	25.9
∑MUFA	26.0	52.2	84.4	11.8	14.6	13.6
∑PUFA	98.2	167.3	294.6	44.4	46.7	47.6
∑n-3 PUFA	83.5	146.1	258.1	37.8	40.8	41.7
∑n-6 PUFA	9.3	12.0	21.2	4.2	3.4	3.4
Other Fatty acids		mg/100 g of GSM			mg/100 g of GSM	
16:0 FALD	0.6	0.6	0.9	0.3	0.2	0.2
18:0 FALD	13.5	13.6	15.6	6.1	3.8	2.5
16:0 OH	1.8	0.6	8.1	0.8	0.2	1.3
20:2 NMI	3.5	5.7	10.8	1.6	1.6	1.7
22:2 NMI	4.2	4.8	7.4	1.9	1.3	1.2

Note: Left column describes the lipid content in totality (mg per 100 g of mussel) of the mussel and the right column describes the extracted oil (g per 100 g of oil). NA; not applicable. GSM = Greenshell Mussel (n = 9), SFA = Sum of Saturated fatty acids, MUFA = Sum of Monounsaturated fatty acids, PUFA = Sum of Polyunsaturated fatty acids, n-3 = omega-3, n-6 = omega-6, c, cis; FALD, fatty aldehydes; NMI, non-methylene interrupted; OH, Hydroxy fatty acids.

3.2. Lipidomic Analysis

Analysis of the LC-MS/MS chromatograms identified 16 different lipid classes, not including ether and vinyl ether-linked hydrocarbon chains. Following lipid identification, the normalised data were $\log_2$ transformed and principal components analysis was used to determine if the lipid composition was different between the three tissue types. PCA analysis (Figure 1a) using the complete dataset shows a clear separation between the different tissue types of GSM. The first component shows a clear separation between the digestive gland (red) and the other two tissue types, accounting for almost 60% of the

differences. The second principal component accounted for about 17% of the differences and demonstrates the differences between the mantle (blue) and gonad (green).

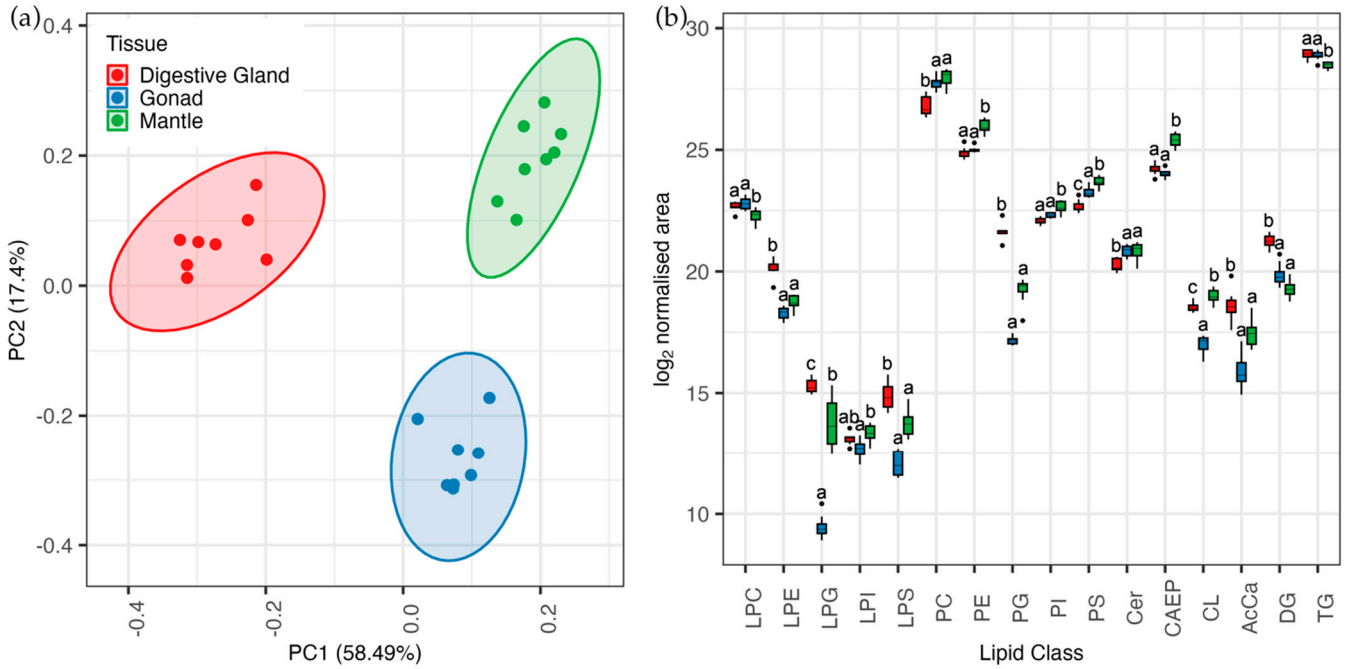

Figure 1. Panel (**a**) Principal Component Analysis of identified lipids from GSM. Panel (**b**) plot of summed lipid class data. AcCa Acetyl Carnitine, CAEP ceramide aminoethyl phosphonate, Cer, Ceramide, CL Cardiolipin, DG diarylglycerol, LPC lyso phosphatidylcholine, LPE lyso phosphatidylethanolamine, LPG lyso phosphatidylglycerol, LPI. Digestive gland (red), gonad (blue), and mantle (green). Box plots show the median, first and third quartiles, minimum and maximum, different letters above indicate a significant ($p < 0.05$) difference to the other tissues within the same lipid class.

We then compared the lipid classes using a more traditional analysis and combined all of the lipid species within a class; the lyso phospholipids were separated from the diacyl, ether, and vinyl ether phospholipids. As there were no lipid standards for the ceramide aminoethyl phosphonate (CAEP) at the time of analysis, the normalised $\log_2$ transformed area was used to produce a box plot of the lipid class data (Figure 2). Consistent with the lipid class data (from Table 1), there is significantly less TG in the mantle compared to the digestive gland and gonad. The difference in the polar lipid distribution (mantle 86.2 > gonad 70.1 > digestive gland 66.4 g/100 g of oil) is most likely due to significantly less phosphatidylcholine (PC) in the digestive gland and significantly more phosphatidylethanolamine (PE) and CAEP in the mantle. There is a similarity in the distribution of the lyso-phospholipids and acylcarnitine (AcCa) species, indicating phospholipid breakdown/remodeling for AcCa formation and energy production. Cardiolipin, a mitochondrial lipid, is significantly lowest in the gonad and significantly highest in the mantle.

The major lipid class of the GSM chloroform extract for each of the three different tissue types is the triglyceride (TG) species. Over 200 TG species were identified, predominantly acyl-glycerides with very few ether TG species identified. Twenty-five different TG species make up approximately 50% of the TG oil composition of each tissue (Figure 2). The use of a C30 column has enabled the baseline separation of many TG isomers in GSM oil with a relatively fast gradient. The chromatographic separation of lipid species increases the complexity of the lipidome by separating isobaric lipid species [26]. In the twenty-five most abundant TGs in GSM oil, we have identified isobaric species of 52:7, 54:7, and 56:7 that contain either an EPA or DHA. These TG species are baseline separated compared to using

a similar gradient with a more conventional separation on a C18 or C8 column where the isobaric species usually overlap.

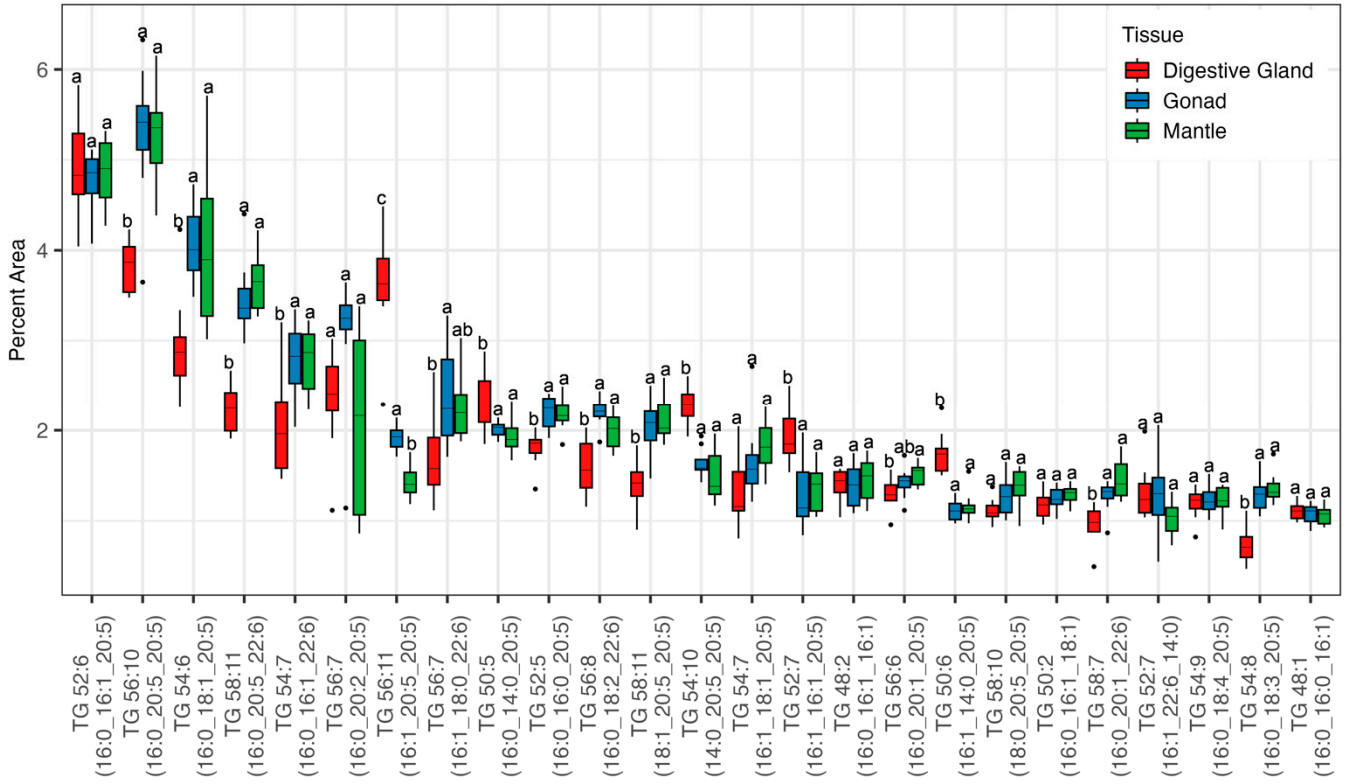

Figure 2. The top 25 most abundant triglyceride (TG) species in GSM tissues. Digestive gland (red), gonad (blue), and mantle (green). Lipid species names on the x-axis indicate the sum total of carbons in the acyl chains with the number of double bonds. The lipid species acyl chain composition is shown in brackets, the underscore is used to denote that the sn-position of each acyl chain is unknown. Box plots show the median, first and third quartiles, minimum and maximum, different letters above indicate a significant ($p < 0.05$) difference to the other tissues for each lipid species.

The top five most abundant TG all contain both palmitic acid and EPA: TG 52:6 (16:0_16:1_20:5), TG 56:10 (16:0_20:5_20:5), TG 54:6 (16:0_18:1_20:5), TG 58:11 (16:0_20:5_22:6) and TG 56:7 (16:0_20:2_20:5). In the digestive gland, TG 56:11 (16:1_20:5_20:5) and TG 56:7 (16:0_20:2_20:5) are more abundant than the DHA containing species. There were few significant differences in the percentage of TG species between gonad and mantle tissues, the only significant difference was between TG 56:11 (16:1_20:5_20:5) which is significantly lower in the mantle. For sixteen of the top 25 most abundant TG species, there is a significant difference between the digestive gland and one or both of the other tissues. There is significantly more TG in the digestive gland in five species which all contain either 16:1 or 14:0 fatty acids, namely TG 56:11 (16:1_20:5_20:5), TG 50:5 (16:0_14:0_20:5), TG 54:10 (14:0_20:5_20:5), TG 52:7 (16:1_16:1_20:5) and TG 50:6 (16:1_14:0_20:5). All of the five TG species that contain DHA (22:6) are lower in the digestive gland than in both other tissues. EPA is found in at least one position in 15 of the top 25 most abundant TG species, whereas DHA is only found in 5 lipid species, although not all species contain long-chain fatty acids. However, medium-chain fatty acids are found in all of the top 25 most abundant lipid species, with palmitic acid found in 16 species.

Sixteen diglyceride (DG) species were identified and were all diacyl species with no ether lipids. The two most abundant were DG 36:6 (16:1_20:5) and DG 40:10 (20:5_20:5) shown in Figure 3a which were both significantly more abundant in the digestive gland. Other species with higher abundance in the digestive gland all contained fatty acids 14:0, 16:2, 18:2, or 18:4 which are consistent with the observations for the TG species. Species

with FA 20:1 (20:1_20:4, 20:1_20:5, and 20:1_22:6) were all significantly higher in the mantle. The remaining species with a DHA (22:6) moiety were all significantly more abundant in the mantle than in the digestive gland. Acylcarnitine species (Figure 3b) associated with β-oxidation and lipid breakdown [27], show a strong preference for the degradation and mitochondrial translocation of the high-energy unsaturated and monounsaturated species, with the highest amount in total occurring in the digestive gland. Surprisingly, the AcCa 14:0 has a relatively low distribution in the digestive gland. AcCa 16:0 is lowest in the mantle and AcCa 16:1 is lowest in the gonad. There is little difference between the relative percentage distribution of AcCa in tissues and also no difference in AcCa 18:0.

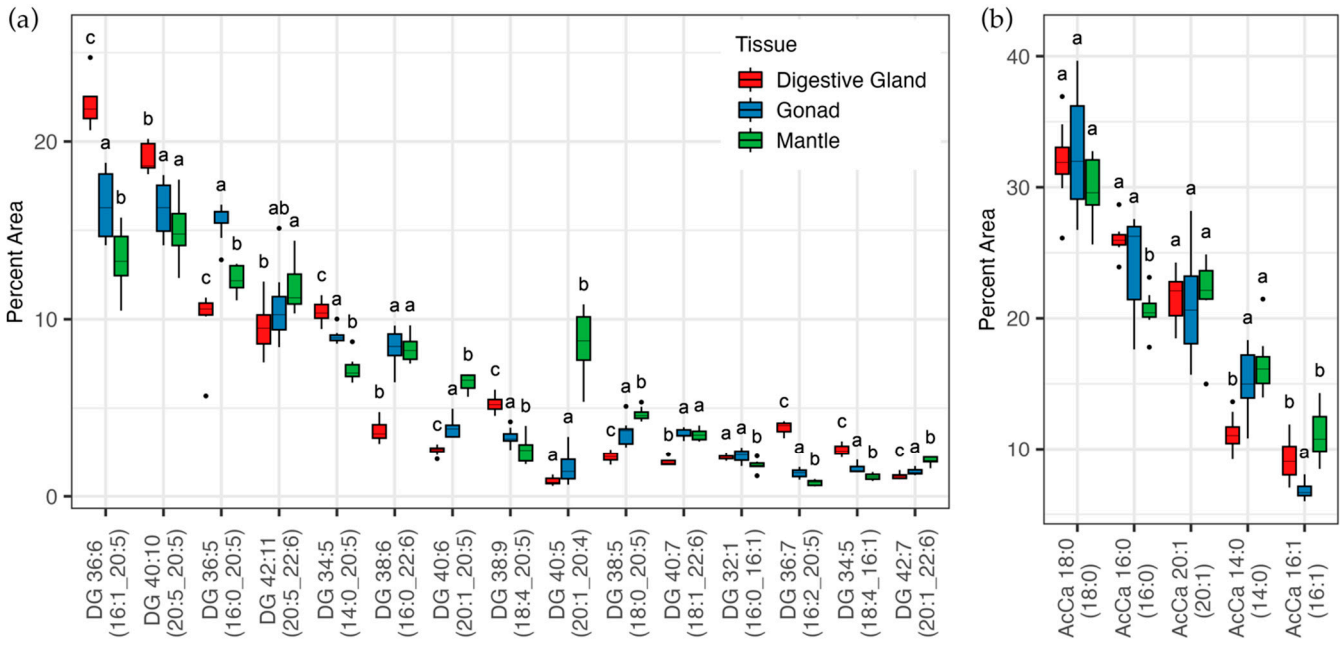

Figure 3. The lipid species distribution of diglyceride (DG), panel (**a**), and acylcarnitine (AcCa), panel (**b**). Both plots are expressed as the relative percentage of each lipid class. Only the top 15 most abundant DG species are shown. All of the AcCa species identified are shown. Digestive gland (red), gonad (blue), and mantle (green). Box plots show the median, first and third quartiles, minimum and maximum, different letters above indicate a significant ($p < 0.05$) difference to the other tissues for each lipid species.

To investigate the relationship between the DG and TG pools, we conducted a correlation analysis between the most abundant lipid species that show differential expression between the tissues (Figure 4). This analysis shows a strong positive correlation between di-EPA TG species with the two TG species containing either 16:0 and 18:1 (TG 58:11(18:1_20:5_20:5) and TG 56:10 (16:0_20:5_20:5)) showing a very strong positive correlation with each other and with the two species containing either 14:0 or 16:1 (TG 54:10 and TG 56:11) having a strong negative correlation. The predominant positive correlation was between TG 56:11 (16:1_20:5_20:5), TG 54:10 (14:0_20:5_20:5), TG 50:6 (16:1_14:0_20:5), TG 50:5 (16:0_14:0_20:5) and TG 52:7 (16:1_16:1_20:5), which all have significantly higher abundance in the digestive gland than in the gonad and mantle; correlation coefficient greater than 0.60.

The correlation of the major DG species with corresponding TG species shows the two major DG species correlate strongly with TG. The most abundant DG species (DG 36:6 (16:1_20:5), has a strong correlation (0.89) with TG 56:11 (16:1_20:5_20:5). Whereas DG 40:10 (20:5_20:5) has the strongest correlation, 0.83, with TG (14:0_20:5_20:5). These species are all characterised by a significantly higher percent in the digestive gland and in the case of 16:1 containing TG 56:11 almost half as much in both the gonad and mantle. Conversely, the DHA containing DG 16:0_22:6 has a strong negative correlation with the

EPA containing DG species (DG 36:6 -0.847 and DG 40:10, -0.828), and has a very strong correlation (>0.90) with TG (16:0_20:5_22:6) which indicates that this TG species may have 20:5 in the sn-3 position.

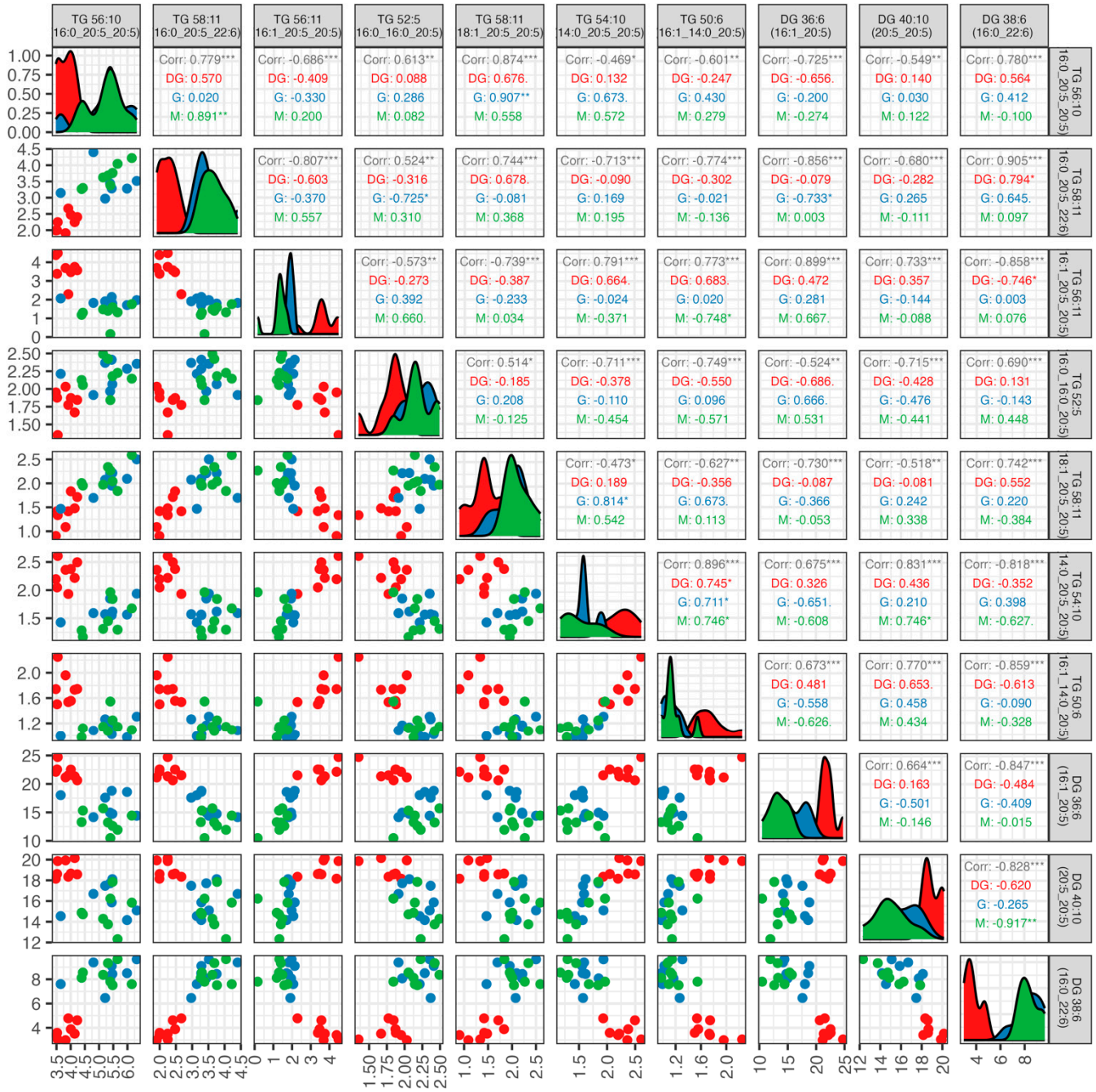

Figure 4. Correlation plot of TG species, with a limited number of species included for visual purposes. Values in grey represent the overall correlation, coloured values represent the correlation within the tissue as Figure 2. Values from 0 to 0.16 have none to negligible correlation, 0.20 to 0.29 weak, 0.30 to 0.39 moderate, 0.40 to 0.69 strong, and values above 0.70 have a very strong correlation. Significance of the correlation shown with stars following each value; *** $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; '.', $p < 0.10$. Negative values have a negative correlation coefficient. Charts on a diagonal depict the distribution of concentrations for each TG/DG species in each tissue.

Liquid chromatography of oil extracted from GSM tissues showed a number of large peaks which elute in the polar lipid fraction of the chromatogram that were not identified

using standard databases. These lipid species ionised in both positive and negative ion modes and were determined to be CAEP species [28]. GSMs have significant levels of ceramide and CAEP and there are significant differences in the abundances of CAEP between GSM tissues with a major accumulation in the mantle (Figure 5a). Twenty-one ceramide species were identified with the most abundant shown in Figure 5b. The most abundant species of both CAEP and ceramide were the 1,3 dihydroxy- (d)35:3 (d19:3_16:0) which accounted for approximately 30% of the abundance for each class and significantly more in the mantle than the other two tissues. The predominant sphingoid bases for both CAEP and ceramide are d19:3, d18:2, and d18:3. The next most abundant CAEP species were CAEP d34:2 and CAEP d36:3 which accounted for around 10% more than the other CAEP species and were less abundant in the mantle. For the ceramides, the species distribution slowly tapered from Cer d34:2 (d18:2_16:0) which accounted for approximately 10% of the ceramide pool.

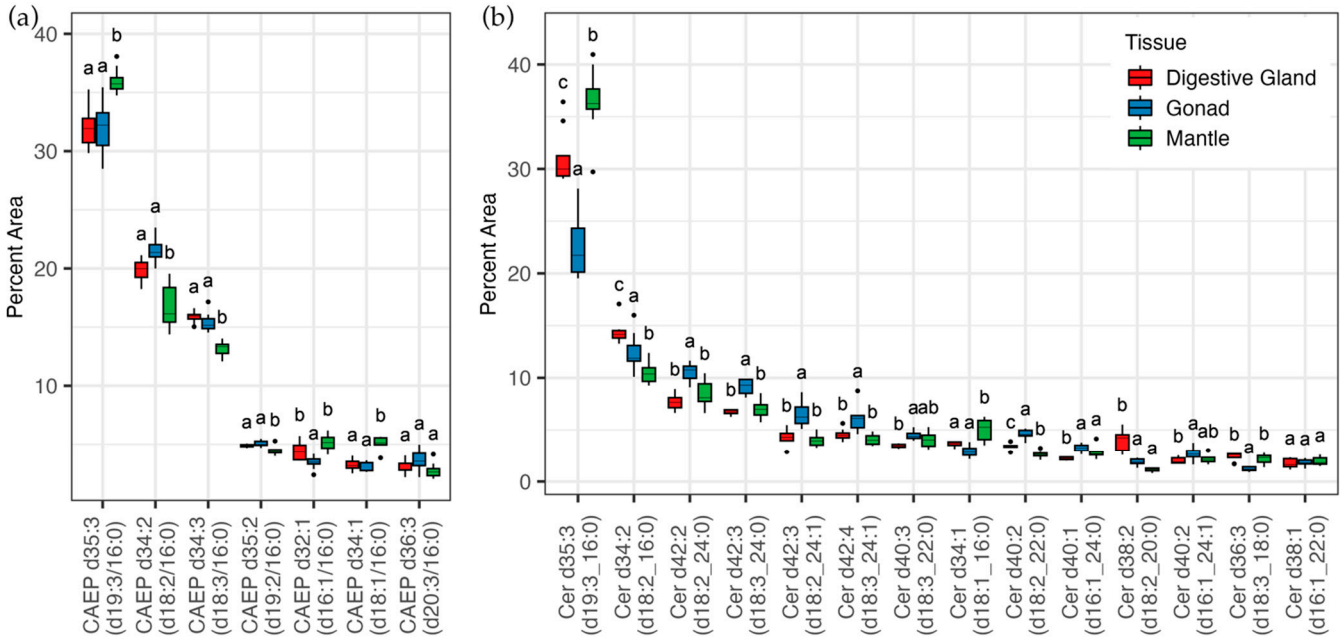

Figure 5. The distribution of ceramide aminoethyl phosphonate (CAEP) species, panel (**a**) and ceramide (Cer) species, panel (**b**). Both plots are expressed as the relative percentage of each lipid class. Digestive gland (red), gonad (blue), and mantle (green). Box plots show the median, first and third quartiles, minimum and maximum, different letters above indicate a significant ($p < 0.05$) difference to the other tissues for each lipid species.

The phosphatidylcholine (PC) species are typically the most identified phospholipid species in biological tissue by lipidomic techniques. This is partly due to their ease of ionisation and also the fragmentation of phosphocholine which gives a unique diagnostic ion of 184 m/z. In GSM tissues, 81 PC species were identified and they were predominantly acyl species, although ether (e) and plasmalogen (p, vinyl ether) linked species were also identified. PC 36:5 (16:0_20:5) was almost twice as abundant as the other species accounting for approximately 20% of the PC pool (Figure 6a), although it was significantly lower in the mantle. Less than 10% each of another two palmitic-containing species PC 38:6 (16:0_22:6) and PC 32:1 (16:0_16:1) had only a small difference between the tissues. The ether-linked containing PC species were characterised by some of the more abundant species being more abundant in the mantle, namely the DHA ether-containing species (14:0e_22:6) and (16:0e_22:6) (Figure 6b).

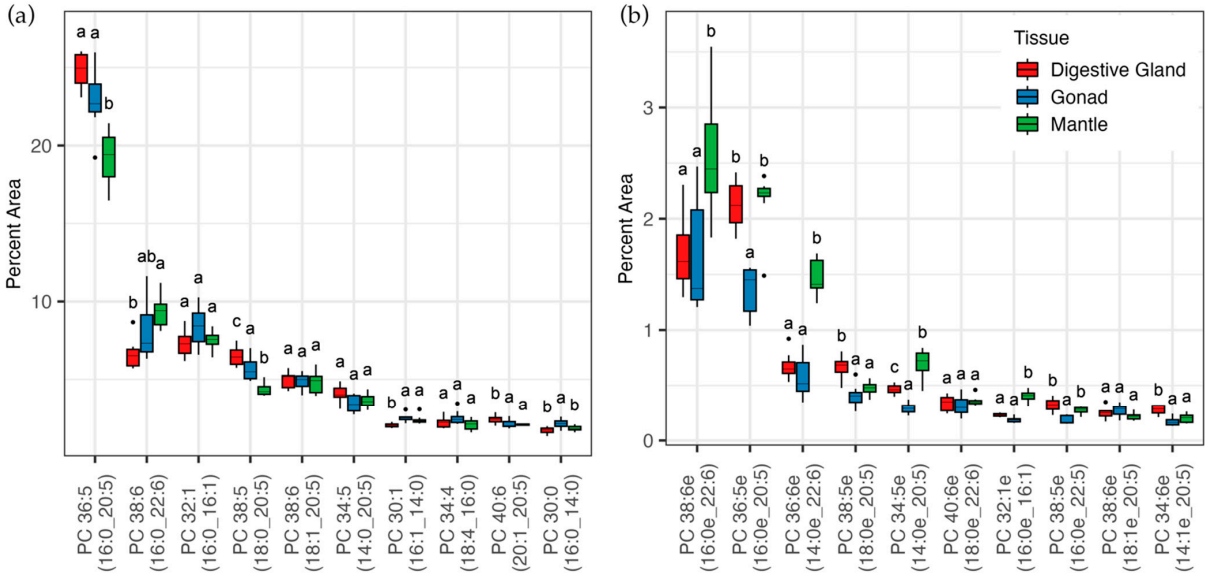

Figure 6. The distribution of phosphatidylcholine (PC) species with acyl bonds, panel (**a**), and PC species with ether bonds, panel (**b**). Both plots are expressed as the relative percent of the PC class. Digestive gland (red), gonad (blue), and mantle (green). Box plots show the median, first and third quartiles, minimum and maximum, different letters above indicate a significant ($p < 0.05$) difference to the other tissues for each lipid species.

Phosphatidylethanolamine (PE) is easily detected in positive ion mode through the neutral loss of the head group (141 m/z). Negative ion fragmentation results in the predominant loss of the fatty acid from the sn-2 position, enabling confirmation of the fatty acid composition. We identified 29 PE species including ether and plasmalogen species. Unusually, the ether-linked PE species were more abundant than the acyl-linked species. For the acyl species (Figure 7a) only PE 38:5 was significantly higher in the digestive gland. The two palmityl containing species, together with a LC-PUFA, were both significantly higher in the gonad along with PE 34:5 (14:0_20:5). Three species were significantly higher in the mantle containing the acyl pairs (18:0_22:6, 17:0_20:5 and 16:0_16:1). For the ether-linked PE species (Figure 7b), three species were dominant in abundance; these were all annotated as having a plasmalogen 18:0p and an LC-PUFA, either (20:5, 22:5 or 22:6). The mantle differs more significantly to the other two tissues, particularly with almost 10% less PE 38:5p (18:0_20:5) and significantly more PE 40:6p (18:0p_22:6).

The phospholipids phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidylinositol (PI) are all detected through negative ion mode and fragmentation gives rise to the loss of acyl chains again allowing composition determination. These phospholipids are not only less dominant than PC and PE but have lower signal intensity, and therefore it is harder to detect low-abundance species. Eleven PS species were annotated, the two most abundant species containing either DHA or EPA with stearic acid (Figure 8a). The DHA-containing species were more abundant in the mantle and EPA species were significantly more abundant in the gonad. The PG distribution was entirely different for each tissue (Figure 8b), only seven PG species were identified. In the digestive gland, PG was almost entirely PG 32:0 (16:0_16:0) with less than 5% PG 34:1. PG 34:1 (16:0_16:1) was the most predominant PG species in both the gonad and mantle. However, the mantle had a more even distribution with PG 34:2, PG 36:2, and PG 36:6. The mantle also had significantly more di EPA and di-DHA PG than both other tissues. Figure 8c shows the 6 most abundant PI species. In total, there were 16 PI species annotated. PI 40:5 (20:1_20:4) is significantly higher in the mantle than in the other tissues. It accounts for almost 35% of the total PI species in the mantle, with >10% more than the other tissues. This corresponds with an almost similar increase in the corresponding DG species.

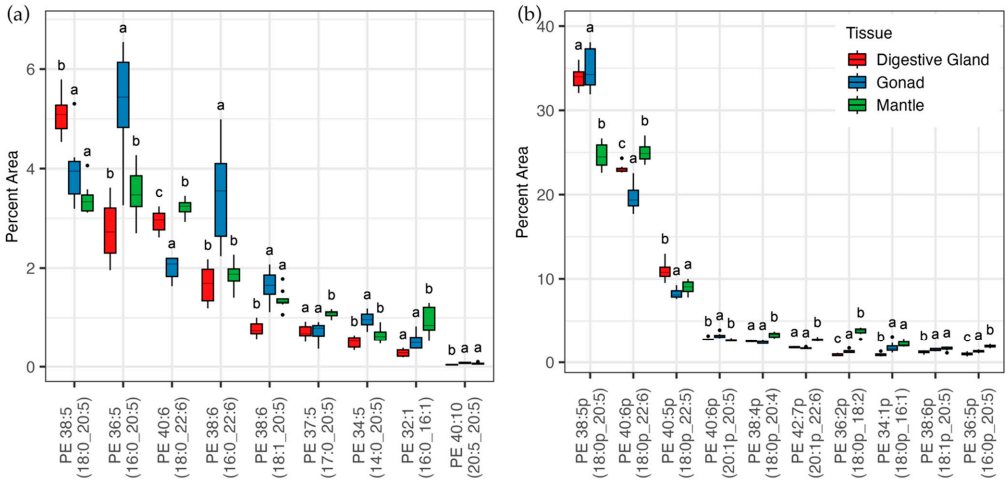

Figure 7. The distribution of phosphatidylethanolamine (PE) species with acyl bonds, panel (**a**), and PE species with ether bonds, panel (**b**). Both plots are expressed as the relative percent of the PE class. Digestive gland (red), gonad (blue), and mantle (green). Box plots show the median, first and third quartiles, minimum and maximum, different letters above indicate a significant ($p < 0.05$) difference to the other tissues for each lipid species.

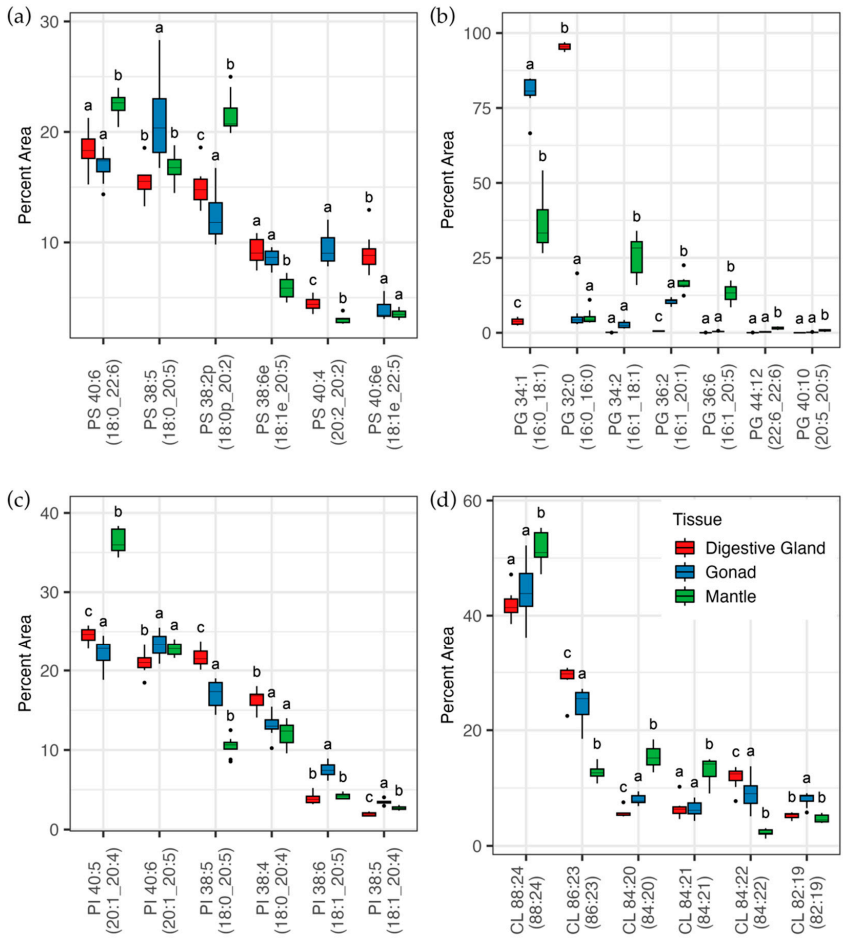

Figure 8. The distribution of phosphatidylserine (PS) species, panel (**a**); phosphatidylglycerol (PG), panel (**b**); phosphatidylinositol (PI), panel (**c**) and cardiolipin (CL) panel (**d**). For cardiolipins refer to the text for the acyl chain composition. Digestive gland (red), gonad (blue), and mantle (green). Box plots show the median, first and third quartiles, minimum and maximum, different letters above indicate a significant ($p < 0.05$) difference to the other tissues for each lipid species.

Cardiolipin (CL) species are associated with the inner membrane of the mitochondria and can be detected in both positive ion mode $[M+H]^+$ and negative ion mode $[M-H]^-$ and $[M-2H]^2$. The daughter ions in negative ion mode are the diagnostic free fatty acids and in positive ion mode are fragmented into two DAG -H_20^+. Some indication of the composition can be gleaned although the exact location of the acyl chains cannot be confirmed. The CL species containing four DHA moieties, CL 88:24 (22:6/22:6/22:6/22:6), accounted for between 40-50% of the CL in GSM. This along with the other species that contain 3 DHA moieties and a medium chain FA CL 84:21 (18:3_22:6_22:6_22:6) and CL 84:20 (18:2_22:6_22:6_22:6) were significantly more abundant in the mantle. The two species that were significantly less in the mantle were both EPA-containing species CL 86:23 (20:5_22:6_22:6_22:6) and CL 82:19 (18:2_20:5_22:6_22:6). The final species containing an equal mixture of both EPA and DHA, CL 84:22 (20:5/22:6/20:5/22:6), is proportionally more abundant in the gonad. These results indicate potential remodeling or differences in the bioavailability of the precursors in the different tissues.

4. Discussion

Marine bivalves such as mussels, oysters, and scallops are a sustainable source of both protein and omega-3 LC-PUFAs. As filter feeders, they grow and survive on microorganisms from the marine environment having one of the lowest carbon footprints of animal protein sources [29]. The waste shells have been used successfully for redeveloping habitats through artificial reefs [30]. The ecosystem services range from filtering and cleaning water enabling more light (decreasing turbidity) and food for benthic organisms and improving the water quality, and may be used for the restoration of the seabed [31]. If 1% of the suitable tropical coastlines were used for farming of bivalves they could supply the protein demands of over 700 million people, approx. 3.8 Mt of omega-3 [32].

Both the traditional lipid analysis and lipidomic analysis of the three tissues of GSM shed light on their biological function from their environmental conditions to the transfer of nutrients for biological function and replication, through to their potential health benefits. GSM obtain all their nutrients from their immediate environment and are responsive to environmental changes and stressors. Seasonal changes, spawning events, and environmental stressors will heavily influence tissues such as the digestive gland and gonad. In this study, the digestive gland has the highest concentration of triglyceride in its oil (TG 31 g/100 g oil), presumably due to the high lipid concentration in the diet and a high lipid concentration of the organ (4.1 mg/100 g).

Due to the high amount of lipids in the gonad, 57% of the total TG comes from the gonad fraction. This is important as it is the TG fraction that is extracted by the supercritical extraction process preferentially over the PL when no co-solvent is utilised. Therefore, the gonad fat and TG content of GSM are vital to the yield of oil for commercial nutraceutical producers.

The polar lipid classes are not differentiated through the TLC-FID analysis performed here. There are methods available to determine broad PL classes, but often involve multiple developments of the TLC rods and multiple analyses. From experience this multiple development approach can be quite variable and hard to achieve good repeatability on samples. Other methods such as LC-MS lipidomics shown in this study and NMR are better techniques to look at PL classes; however, both techniques have limitations.

The majority of the fatty acids in GSMs are PUFA with the omega-3 dominating (high omega-3/omega-6 ratio ~9–12). GSMs have been shown to be a good source of omega-3 LC-PUFA and are thought to obtain these important lipids directly from the microalgal source, although recent studies indicate that these invertebrates may have the capability to endogenously produce omega-3 LC PUFA. Interestingly the proportion of DHA in the mantle was higher (12 g/100 g FA) and EPA lower (20.6 g/100 g) than determined for the other organs. Traditional lipid analysis has been used to approximate diet through signature lipid comparisons. The EPA to DHA ratio is ~2 to 3, indicating greater diatom to flagellates in the GSM diet which is supported by a high 16:1/16:0 ratio [33]. This is

higher in the gonad and digestive gland than in previous reports [12,14] and could be due to changes in storage or utilisation in the different organs, seasonal or spatial differences, or possible changes in diet.

This traditional analysis of GSM is in the same format as many other previous research [12–14] which makes comparisons between studies possible, however traditional analysis lacks the depth and resolution of lipidomic analysis. The lipidomic analysis shows higher DG and lyso-phosopholipids than occur in the other tissues. These observations and a lower abundance of phospholipids suggest that lipases are involved in the acyl chain cleavage of TG to DG and phospholipids to lyso-phospholipids. The acylcarnitines are also increased in the digestive gland, showing a need for energy production and the breakdown of the saturated and monounsaturated fatty acids. Acylcarnitines are predominantly involved in lipid degradation via the beta-oxidation pathway, where they transport acyl chains into the mitochondrial matrix for degradation. Acylcarnitines may be a stress biomarker in bivalves as they are up-regulated as they go into a stress response and move to anaerobic metabolism [34]. Lipidomic analysis may provide greater depth of metabolic activity and future studies could identify biomarkers for environmental and stress conditions.

The most abundant triglyceride in both the gonad and mantle was TG 56:10 (16:0_20:5_20:5) which has a significant negative correlation with the second most abundant lipid in the digestive gland which was TG 56:11 (16:1_20:5_20:5). This corresponds with significantly higher concentrations of DG 36:6 (16:1_20:5) and DG 40:10 (20:5_20:5) in the digestive gland. This inverse relationship is suggesting lipid remodeling mechanisms are involved in the removal and modification of dietary lipids rich in myristic (14:0) and palmitoleic (16:1) fatty acids. Elongation and desaturation of lipids can take place on phosphatidylcholine, where PC 36:5 (16:0_20:5) accounts for over 20% of the PC content and is most abundant in the digestive gland. Remodeling of lipids would account for the increases in palmitic (16:0), stearic (18:0), oleic (18:1), and DHA (22:6) species seen in DG and TG species in the mantle and gonad.

Lipidomics may provide key information regarding the diet and location or the capacity to edit, modify and selectively utilise/translocate particular lipid species. A higher percent of 14:0, 16:1, and 18:4 DG and TG species in the digestive gland could indicate a diet of either dinoflagellates or brown algae [35]. This would change seasonally and would also depend on the particular species found in the waters. The distribution and ratio of TG to phospholipid are in accordance with the organ's function, namely that the mantle, which is involved in environmental sensing and provides an outer coat for the other organs, contains a high PL low TG ratio in the oil. The gonad which requires large energy stores for reproduction has a higher concentration of TG and the digestive gland contains microbial food sources rich in oil. Phospholipids are the predominant polar lipids and biologically are the main component of cell membranes. The phospholipid class and acyl chain structure determine its shape and functionality. PC and PE are usually the most abundant phospholipid in all organisms and the major structural components of biological membranes. The relative sum abundance of the results obtained in this study is consistent with these observations. PC is usually associated with the outer membrane of cells and involved in the remodeling, elongation, and desaturation of lipids, while PE has a more conical shape which is important for the more concave inner membrane. The overall phospholipid composition of GSM is consistent with the phospholipid composition of *Mytilus edulis* [36,37] having predominant PC and PE as the predominant classes with a high proportion of plasmalogen PE species and only small numbers of PI, PS, and PG species identified. A higher proportion of plasmalogen derived 18:0 FALD [11] is found in the mantle which is consistent with the higher concentrations of PE in the tissue and high concentrations of 18:0 plasmalogens in the PE class.

The phosphonate (carbon–phosphate bond) is uncommon in land-based organisms and is more prevalent in marine environments. Through genome sequencing the enzyme responsible for the biosynthesis of the C-P bond, phosphoenolpyruvate mutase pepM has

been found in approximately 5% of the bacterial genomes and also identified in the sea snail (*Lottia gigantean*) and sea anemone (*Nematostella vectensis*) [38]. As filter feeders, GSM would likely sequester CAEP or its precursor, 2-aminoethyl phosphonate, from its diet or symbionts. The phosphonates are a group of compounds with potential bioactive properties and have been reported for their antibacterial, antiparasitic, and herbicidal properties [39]. While the bioactivities of some phosphono-peptides and other phosphonates are known, the biological properties of phosphonolipids are not well studied [40].

Cardiolipins are found in the inner mitochondrial membrane of all tissues, although they were originally discovered in heart tissue. They are essential for the formation and stability of Complex III and IV super complex of the mitochondrial electron transport chain [41] and play a major role in other mitochondrial functions such as ATP synthase and the proton gradient, cytochrome C release and apoptosis, and mitochondrial formation and dynamics [42]. In GSM the mantle has significantly more CL than the digestive gland and both these tissues are significantly higher than the gonad (Figure 1b.), indicating that both the digestive gland and mantle are involved in energy production, whereas the gonad is more likely involved in energy storage. The acyl chain composition lacks the diversity of other lipids and is usually quite specific. Tafazzin remodels cardiolipin in a coenzyme A independent fashion; enzyme studies with *Drosophila tafazzin* show a high specificity for 18:2 and that there is negligible arachidonyl activity [43]. Of particular interest is the observation that the GSM cardiolipins all contain DHA and EPA, with very few other lipid species present. As with *Drosophila* and mammalian cardiolipin, there are very few cardiolipin species, with the predominant species being CL 88:24 (22:6_22:6_22:6_22:6). In blue mussel, both CL 88:24 and 88:23 were reported [36], whereas both 18:2 and 18:3 containing species were most abundant in the mantle of GSM. Overall, the results are of particular interest to the evolution of mitochondria and their adaption to different environments (membrane structure is important and potentially related to enzyme activity) and are of further interest regarding the environment and nutrient uptake for marine organisms.

All bivalves including GSMs are a sustainable source of omega-3 LC-PUFA which are in high demand globally due to their numerous health benefits. While tuna oil contains mainly TG and krill oil is a mixture of TG and phospholipid, with the phospholipid fraction in the form of phosphatidylcholine, GSM oil has a mixture of both TG and phospholipid with a wider range of PL lipid classes and many more novel FA including NMIs, plasmalogens, and FALD. In terms of human diet and health benefits, LC-PUFA-containing phospholipids may have more health benefits through alternate uptake mechanisms and can cross the blood-brain barrier [44]. Plasmalogen PE species with EPA have been shown to efficiently increase brain omega-3 PUFA concentration [45]. The phospholipid PUFA species have been shown to be quickly taken up in the brain and other tissues where PUFA deficiency is occurring. However, DHA in the form of non-esterified fatty acids may be the most efficient form of DHA, being supplied by adipose through the storage of dietary TG's [46]. Long-term dietary-derived DHA in the form of TG may lead to longer-term outcomes through the release of non-esterified fatty acids. A higher EPA to DHA ratio has also been associated with increased health outcomes through a decrease in C-reactive protein and decreases in inflammation [47]. The balanced distribution of phospholipid and TG may provide these fatty acids through multiple uptake mechanisms, with a higher ratio of EPA to DHA leading to greater health benefits.

The CAEP lipids are the third most abundant phospholipid in some mussel species [48]. CAEP d35:3 (d19:3_16:0) and CAEP d34:2 (d18:2:16:0) have also been identified as the major CAEP in blue mussels and may assist in the adaptation to thermal stress [28]. There is little clinical data or reports on the health benefits of these lipids. However, it has been demonstrated that CAEP is readily hydrolysed to ceramide and sphingoid bases in the mouse gut [49], which would give CAEPs similar bioactivity to other sphingolipids. Dietary sphingomyelin has been reported to augment acute and chronic inflammation, obesity, and gut inflammation [50]. This appears in contradiction to the fact that sphingosine-1-phosphate is known to be a pro-inflammatory compound and an increase in sphingolipids

should therefore increase this pro-inflammatory response. Polyunsaturated sphingoid bases, that are derived from GSM and other marine CAEP, are unable to form sphingosine-1-phosphate and thus may be protective against ulcerative colitis and other inflammatory colon cancers [51].

5. Conclusions

This paper focused on a qualitative analysis of the different GSM tissues to provide new results on the distribution of a range of lipid classes in this unique and commercially harvested species, and to demonstrate the utility of lipidomics alongside traditional lipid profiles in such research and development. The traditional lipid profile is a concise data set and easily comparable to published results in lipid or broad nutritional studies. Conversely, lipidomic analysis in marine organisms is still in its infancy. Many standards are available but are more suited for the fatty acid composition of mammalian and plant-based lipids. Other lipids such as the CAEP have no available standards and are not present in lipidomic reference datasets, creating further challenges for studying lipidomics in marine systems. The lipidomic data set highlights the complexity of the lipidome and allows a more detailed examination of the roles of specific lipids in metabolism and function. This could assist studies aimed at understanding the molecules and physiological processes underpinning the bioactivity of GSM powders and oils (10), and could ultimately help breeding programmes to produce GSM strains with enhanced bioactivity.

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Review

Evaluating Alternative and Sustainable Food Resources: A Review of the Nutritional Composition of Myctophid Fishes

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Abstract: Additional and alternative sustainable food resources are needed as the global human population increases. Marine fishes have long provided essential nutrients, such as omega-3 long-chain (≥C20) polyunsaturated fatty acids (n-3 LC-PUFA), protein, and vitamins to meet human dietary requirements and feed for agricultural production. Many current commercial fish stocks are depleted or fully exploited, but oceanic mesopelagic fishes, particularly the myctophids (lanternfishes), represent a potentially very large and unfished resource. This review analysed the literature on nutritional and biochemical compositions of myctophids as a first step towards understanding the health benefits and risks of consuming them. We found that myctophids have high levels of protein (11–23% wet weight, WW) and variable lipid content (0.5–26% WW). In most species, desirable triacylglycerols or phospholipids dominated over less-desirable wax esters, and most have abundant amounts of health-promoting n-3 LC-PUFA, such as DHA and EPA. Myctophids have low levels of heavy metals and persistent organic pollutants. Most nutritional information is available for species from the Pacific and Southern Oceans and for the genera *Benthosema*, *Electrona*, and *Diaphus*. Myctophids generally possess favourable nutritional profiles, but major gaps in knowledge regarding their stock assessment, ecology and the economic viability for their harvest are barriers to developing sustainable fisheries.

Keywords: protein; omega-3 oils; contaminants; mesopelagic; lipids; fishery

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1. Introduction

As the global human population increases, the demand for food and feed resources is escalating proportionally. Marine fishes are food with exceptional nutritional value that has been recognised for their positive impact on human health [1,2]. It has been reported that fish consumption represents about 20% of human intake of animal protein worldwide. The high-quality proteins in fish contain many essential amino acids that are vital for human growth and maintenance [3]. Fish constitutes a major dietary source of polyunsaturated fatty acids (PUFAs) [4], particularly the omega-3 (n-3) long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA, also termed LC omega-3 oils), including eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). These essential fatty acids are crucial to human brain development and contribute to the maintenance of cardiovascular health [5,6], and can provide protection against coronary heart disease and ischemic stroke. Fish is also considered a valuable dietary source of micronutrients, such as vitamin D, calcium, selenium, iodine, and zinc [7], which are essential for maintaining physiological function [8].

Worldwide, many coastal demersal and pelagic fisheries are at maximum harvest capacity and, in some instances facing the threat of collapse [9,10]. The decline in overall biomass can be attributed to a variety of factors, including overfishing, habitat destruction,

pollution, and, more recently, climate change [11,12]. Declining fish stock and biomass can have cascading effects through the food chain and alter the structure and functioning of entire ecosystems [12,13]. Such ecological changes affect not only the amount of food available for human consumption, but also the nutritional quality. Growing concerns for current coastal and terrestrial food systems mean that new sources of nutrition are needed.

The mesopelagic zone (200–1000 m) contains a large diversity and abundance of small, migratory organisms that serve a key role in regulating the carbon and nutrient cycles globally [14,15]. Myctophid fishes are among the most abundant and widely distributed taxa, as well as one of the most diverse families of the teleost fishes [16], with over 250 species in 33 genera [17] (one species is illustrated in Figure 1). While there are order-of-magnitude disparities in the estimates of the worldwide biomass of mesopelagic fishes, the resource is prospectively very large at 1 to ~16 Gt (10^9 tonnes) [17–22] and made up mostly of myctophids. These data suggest that myctophids could potentially contribute to meeting the increasing global demand for nutrients derived from marine sources. Myctophids are also thought to be a promising future source of health-benefiting LC omega-3 oils, protein, and essential minerals and vitamins [23]. As a mid-trophic and oceanic resource, they are reported to have low levels of toxins and heavy metals, including mercury, lead, arsenic, and cadmium [23]. There are, however, a number of reports which indicate that some myctophids can have undesirable features (e.g., high wax esters; see Section 2.1) and that there are challenges associated with processing myctophids on a large scale which may hinder their use as a food source [24,25].

Figure 1. A common myctophid species found in the Southern Ocean is *Electrona antarctica* (from Nansy Phleger).

The primary aims of this review were to (i) examine the nutritional composition of myctophid fishes caught in different oceans to assess their suitability for human consumption; and (ii) summarise aspects of myctophid ecology, including knowledge gaps relevant to the future exploitation of myctophid resources. For each nutritional component, we summarise the available data in the most widely recognised unit of analysis (typically expressed in wet mass as a concentration or relative percentage) and for whole specimens of species.

2. Nutritional Profiles

2.1. Lipids & Fatty Acids

Lipids are the densest form of stored energy and provide at least two-thirds more energy per gram than proteins or carbohydrates [26]. The need for fish oil in recent years has grown mostly due to growth in the aquafeed (aquaculture) and nutraceutical industries [27,28]. The price of fish oil has increased from around 750 USD/ton to 3000 USD/ton over the last 15 years [29,30]. Between 2009 and 2013, there was a considerable increase in the real prices of fish oil, which subsequently declined until 2017, although have since increased again [30]. The fish oil price has increased by 45.1% in the past decade, and fish oil production will grow by 0.8% p.a. by 2030 [30]. Global prices for fishmeal have also increased, athough they are highly variable and dependent on the amount of oil (lipid) and its composition [30]. The most desirable characteristics of these fish oils are high amounts of triacylglycerols (TAG) and n-3 LC-PUFA, particularly DHA and EPA, which are essential dietary components for human and animal health.

The lipid class composition of fish oil is also an important feature, as they have distinct effects on human and animal health. Certain types of fats (e.g., TAG-based saturated and trans fats) can increase the risk of heart disease and other health problems [31,32]. Wax ester (WE) is a class of lipids that are considered less desirable for human consumption due to their propensity to cause keriorrhea, a digestive disorder characterised by diarrhoea and abdominal discomfort, and limited digestibility [16,33]. However, WE have potential applications in various industries, including cosmetics (such as occurs for jojoba oil [23]), food (such as the coating material [34]), and biofuel industries [35].

Our assessment of the literature showed that the lipid content in whole individuals of myctophid species varies widely from 0.5 to 26% wet weight (WW), with those characterised by high lipid content (>10% WW) and dominated by TAG considered most desirable for food nutrition (Table 1). Such high variations between species have been reported in regional studies (e.g., [36] showed a range of 4.4–26.1%). Some species have high lipid content (>10%), which is higher than many other commercially valuable fish, including mackerel icefish [24] and capelin, sand lance, and squid in the North Pacific [37]. To date, few studies have examined the influence of geographical location on the lipid content and composition of myctophid species [24]. In a regional comparison study, significant differences in the lipid composition of myctophids were reported between those sampled in the Indian and Pacific sectors of the Southern Ocean [24]. In a study characterising the lipid of 20 myctophid species in both the subarctic and tropical Pacific Ocean, it was found that those sampled in tropical waters had much less lipid and were typically not dominated by WE or TAG [38]. Our assessment of the available literature also suggests that myctophids harvested in polar regions mostly have intermediate to high lipid content (>5% WW), with only one species, *Gymnoscopelus fraseri* from Macquarie Ridge, reported to have low lipid content (3.6% average WW) [24]. In contrast, there is more variability in lipid content reported between species of myctophids from temperate and tropical ecosystems; this seems to be attributed to phylogeny in addition to geographical location and perhaps time of capture. For one of the most studied genera of myctophids, *Diaphus*, we found that lipid content varies considerably with examples of both high and low lipid content in almost all ocean bodies assessed, but the lipid composition is consistently dominated by TAG. We found that no studies have explored long-term (decadal) trends in the lipid content of any myctophid species. Some studies have explored short-term temporal variability in the lipid compositions of myctophids noting seasonal [39] and interannual differences [38].

Myctophids have variable lipid class compositions and typically are either TAG or WE dominant (Table 1), although many species have substantial amounts of phospholipids (PL) [38,40]. Except for several species (e.g., *Electrona antarctica*, Figure 1), which have a high WE content [41], TAG is the dominant lipid class in most of the Southern Ocean myctophid species studied to date [24,33,42–44]. In temperate and tropical myctophids, lipid class composition seems to differ among tax; fewer species were dominated by either WE or TAG, but rather lipid class profiles consisted of a combination of TAG and PL [36,38,40].

A spatial comparison study looked at the effect of vertical distribution and migration and suggested that myctophids that are WE-dominated do not undergo large vertical migrations [38]. Species that are dominant in WE (Table 1) may be a problem for both direct human consumption and for use in aquafeeds, as most humans and many fish species have limited capacity to utilise them [16].

Our analysis of literature reporting fatty acid composition for whole myctophids found that they have higher levels of DHA than EPA and that there are large variations between species and study regions (Table 2). Across all the study regions, levels of EPA ranged from 0 to 1042 mg/100 g (0 to 7.7% of total fatty acids) WW, while DHA ranged from 298 to 2016 mg/100 g (2.1 to 25.1%) WW. Genera with the highest relative amounts of DHA include a *Diaphus* species in the subantarctic Pacific Ocean [38] and *Gymnoscopelus fraseri* in the Pacific section of the Southern Ocean [24]. Species highest in relative levels of EPA included *Lampanyctus australis* [40] from the South Pacific Ocean. Most studies show that myctophids are dominated by monosaturated fatty acids (MUFA), such as 18:1 and 22:1. There is evidence to suggest that the fatty acid composition of myctophids is strongly aligned with the lipid class [24,33,42]. Higher relative levels of EPA were found in the WE–rich species, while DHA was higher in TAG–rich species [33,38,41]. Palmitic acid (16:0) and DHA also seem to be more abundant in myctophid species with high levels of PL [45].

Table 1. Overview of the total lipid content and TAG & WE dominance in myctophid taxonomic groups (up to genus level) in relation to oceanic regions. Lipid content is expressed as a percentage of wet weight (% WW), while TAG & WE are expressed as a percentage of total lipid content. Dominance classifications are based on mean reported values. References for taxa with moderate levels of total lipid content (5–10% WW) reported are not included.

	Location	Genera	Catch Year	Refs.
High lipid (>10% WW)	Indian Ocean	*Diaphus*	2009	[46]
		Diaphus	2011	[47] ^
	Pacific Ocean	*Diaphus*	2012	[39]
		Ceratoscopelus, *Lampadena*, *Lampanyctus*, *Notoscopelus*, *Protomyctophum*, *Stenobrachius*, *Symbolophorus*	1994	[38]
		Ceratoscopelus, *Notoscopelus*, *Symbolophorus*, *Diaphus*, *Myctophum*, *Lampanyctus*, *Protomyctophum*, *Stenobrachius*	1995	[36]
		Lampanyctus, *Triphoturus*, *Symbolophorus*	1975–1979	[48]
		Diaphus, *Protomyctophum*, *Symbolophorus*	2005	[40]
	Atlantic Ocean	*Benthosema*	2015–2018	[49,50]
	Subarctic	*Diaphus*, *Lampanyctus*, *Notoscopelus*, *Stenobrachius*, *Symbolophorus*	1992–1994	[38]
	Southern Ocean	*Electrona*, *Gymnoscopelus*, *Krefftichthys*, *Protomyctophum*	1995	[42]
		Metelectrona	1987	[51]
		Electrona, *Gymnoscopelus*	1999, 2008	[24,43]
		Electrona	2009–2012	[44]
Low lipid (<5% WW)	Indian Ocean	*Myctophum*	2012	[47] ^
		Diaphus, *Benthosema*, *Myctophum*	2013	[52]
	Pacific Ocean	*Benthosema*, *Diaphus*	2012	[39]
		Diaphus	1994	[38]

Table 1. *Cont.*

	Location	Genera	Catch Year	Refs.
Low lipid (<5% WW)	Pacific Ocean	*Benthosema*, *Ceratoscopelus*, *Diaphus*, *Hygophum*, *Lampadena*, *Myctophum*	1993	[38]
		Lampanyctus	1995	[36]
		Bolinichthys, *Diaphus*, *Gonichthys*, *Hygophum*, *Myctophum*, *Notoscopelus*, *Protomyctophum*, *Taaningichthys*, *Tarletonbeania*, *Lampanyctus*, *Ceratoscopelus*	1975–1979	[48]
		Diaphus, *Electrona*, *Hygophum*, *Lampanyctus*, *Lampanyctodes*, *Lampichthys*, *Metelectrona*, *Nannobrachium*	2005	[40]
	Atlantic Ocean	*Lampanyctus*, *Hygophum*	1968	[53]
	Southern Ocean	*Gymnoscopelus*	1999	[24]
TAG dominant (>60%)	Pacific Ocean	*Ceratoscopelus*, *Symbolophorus*	1993	[38]
		Bolinichthys, *Lampanyctus*, *Symbolophorus*	1975–1979	[48]
		Diaphus, *Lampadena*, *Lampanyctus*, *Lampichthys*, *Notoscopelus*, *Protomyctophum*	1994	[38]
		Diaphus, *Lampanyctus*, *Notoscopelus*, *Symbolophorus*	2005	[40]
		Benthosema, *Diaphus*	2012	[39,47] ˆ
	Indian Ocean	*Myctophum*, *Diaphus*	2011	[47] ˆ
	Subarctic	*Diaphus*, *Lampanyctus*, *Symbolophorus*	1992	[38]
	Southern Ocean	*Electrona*, *Gymnoscopelus*	1999	[24,33]
WE dominant (>40%)	Pacific Ocean	*Lampanyctus*, *Stenobrachius*	1994	[38]
		Lampanyctus, *Triphoturus*	1975–1979	[48]
		Nannobrachium	2005	[40]
	Atlantic Ocean	*Benthosema*	2015–2016	[50]
	Subarctic	*Lampanyctus*, *Stenobrachius*	1992	[38]
	Southern Ocean	*Electrona*, *Gymnoscopelus*, *Krefftichthys*	1999	[24,33,42]

ˆ myctophid fish in this study were filleted, deskinned and homogenised for lipid analysis.

Table 2. Reported ranges of mean values of EPA and DHA (as % of total fatty acids, wet weight) in the whole body of myctophid species studied in different regions.

Ocean	Region	# Species Assessed	EPA	DHA	Reference
Indian	Arabian Sea	1	3.8	9.3	[46]
	Arabian Sea	4	4.1–7.0	7.6–20.2	[52]
	Arabian Sea	3	4.3–5.8	9.8–15.9	[47] ˆ
Pacific	Papua New Guinea	9	0–1.5	3.8–10.3	[38]
	Subantarctic	17	0–2.2	10.1–23.9	[38]
	Coast of Japan	3	0.9–1.5	6.9–18.5	[39]
	Tasman Sea	12	3.0–7.2	7.4–19.8	[40]
Atlantic	Norwegian fjords	1	6.2	10.4	[49]
	North-eastern	4	1.0–4.8	2.1–17.34	[54]
Southern Ocean	Kerguelen Plateau	6	0.7–1.1	5.5–12.2	[53]
	Macquarie Island	2	1.2–1.4	18.9–20.5	[53]
	Kerguelen	5	0.3–4.6	3.9–7.4	[42]
	Antarctic Peninsula	1	1.7–6.9	2.9–8.8	[41]
	Heard Island	15	4.1–7.7	6.3–17.0	[55]

ˆ myctophid fish in this study were filleted, deskinned and homogenised for lipid analysis.

2.2. Proteins & Amino Acids

Marine fish have long been recognised as a good source of high-quality protein that contains all the essential amino acids required for optimal health benefits [56–59]. Protein content is also an important factor in determining economic value [60,61]. Protein derivatives from fish, including mince, surimi, protein isolate, hydrolysate, and powder, have been used in the food industry [23]. From the available literature (Table 3), we found that the total protein content of myctophids varies from 11% to 23% (WW) [23,25,62–67], which has been shown to be similar or higher than other commercial fish and shellfish [68]. Differences between myctophid species have been noted [23,63,64,66], especially in the two highly abundant genera, *Diaphus* and *Lampanyctus.* For instance, *Diaphus hudsoni* (off the south-west coast of India, [66]) and *Diaphus watasi* (Arabian Sea, [65]) exhibit protein contents of 14% and 21% (WW), respectively, while *Diaphus luetkeni* and *Diaphus effulgens* both have protein contents of 16.5% (WW) (Arabian Sea, [69]). The majority of protein research conducted on myctophids (Table 3) has been focused on the Indian Ocean, such as the Gulf of Oman [63] and Arabian Sea [23,52,65], and the western Pacific (southwestern Taiwan) [67]. However, a large knowledge gap exists for myctophid species inhabiting other oceanic regions, where limited research has been carried out.

The amino acid composition of 13 myctophid species, expressed as a percentage of protein (g/100 g of total protein), has been reported, with total amino acid content ranging from 40% to 52% WW (Figure 2) [25,62–65]. No significant differences were observed between the amino acid content of migratory and non-migratory myctophids from the sub-arctic and tropical Pacific Ocean [64]. Essential amino acids, including valine, methionine, leucine, isoleucine, tyrosine, phenylalanine, histidine, lysine, threonine, and tryptophan, exhibited the highest concentrations in *Diaphus effulgens* (52%), followed by *Diaphus watasei* (47%), *Benthosema fibulatum* (44%), *Myctophum obtusirostre* (43%), *Diaphus hudsoni* (42%), and *Diaphus luetkeni* (39%) [25,62–65]. Leucine, lysine and arginine, which play a vital role in calcium absorption and metabolism [70,71] in humans, are the most dominant amino acids in myctophids (Figure 2), while histidine is also high in *Diaphus effulgens* [66].

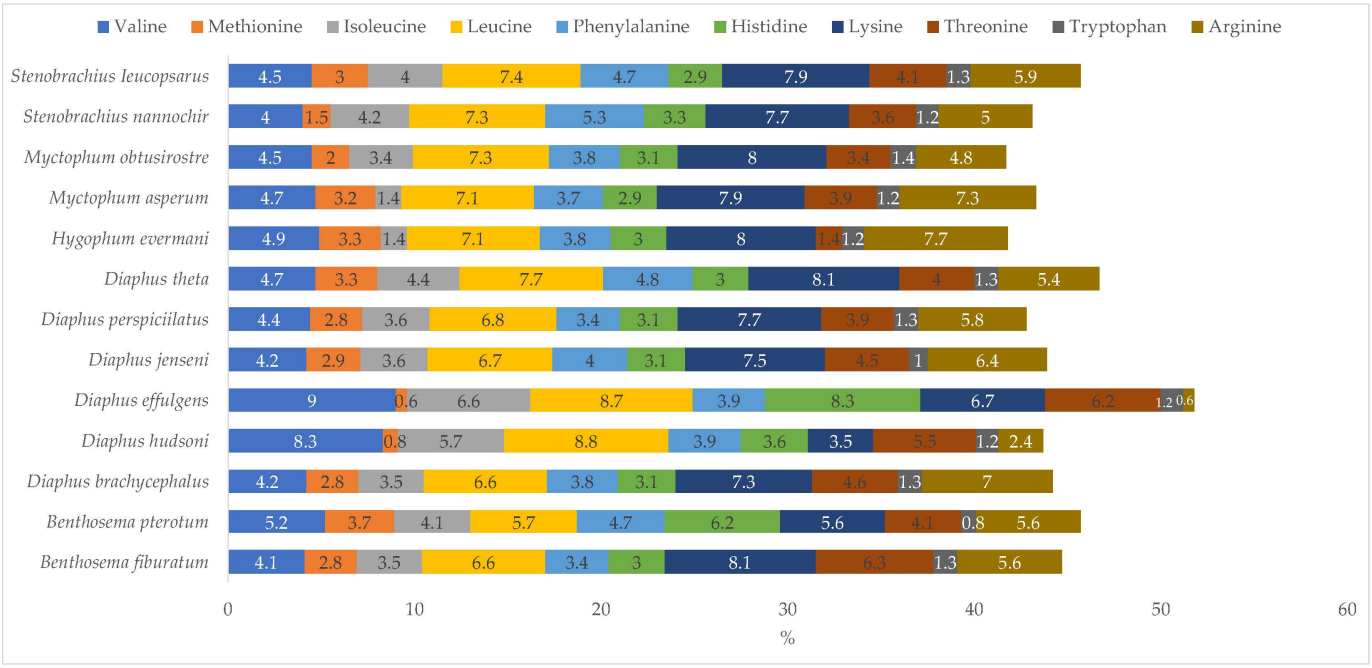

Figure 2. Amino acid composition (%, g/100 g of total amino acid, wet weight) of 13 myctophid species caught from the Indian Ocean [25,62,64,65] and trophic Pacific Ocean [63] and Northern Atlantic Ocean [50].

Table 3. Overview of protein content (percentage of wet weight; WW) of myctophids from the Indian, Pacific and Atlantic Oceans.

Ocean	Genus	Protein (% WW)	Catch Year	Reference
Indian	*Benthosema*	16.1–18.6		[23,52]
	Ceratoscopelus	11.5	1979	[63]
	Diaphus	13.3–21.4	1979, 2009, 2012	[46,52,63,65,66]
	Lampadena	11.5	1979	[63]
	Lampanyctus	12.1–13.4	1979	[63]
	Myctophum	19.3–22.3		[52,65]
	Notoscopelus	13.5	1979	[63]
	Stenobrachius	12.5–12.8	1979	[63]
	Symbolophorus	12.3	1979	[63]
Western Pacific	*Benthosema*	14.4–15.0		[67]
Northern Atlantic	*Benthosema*	41.1	2015–2016	[50]

2.3. Minerals and Vitamins

Fish are considered a vital source of dietary essential minerals and trace elements [72–75], which are more readily absorbed and utilised by humans in comparison to alternative food sources. This includes calcium, which is fundamental to skeletal growth and various physiological functions [50], as well as magnesium, a key nutrient required for metabolic processes [54]. Moreover, iron is an essential mineral that is critical for the production of red blood cells and oxygen transport in fish [1]. Myctophids sampled in the northern Indian Ocean have higher levels of calcium (>900 mg/100 g), phosphorus (500 mg/100 g), potassium (>300 mg/100 g), and iodine (>100 µg/100 g), compared with small pelagic fish (such as *Fringescale sardinella*) and benthic fish species (such as Bombay duck, *Harpadon nehereus*) [76]. Among known edible species of myctophids, *Diaphus watasei* has been identified as having a relatively high mineral content, specifically in terms of potassium and calcium, with concentrations of 35.3 and 47.3 mg/100 g, respectively [69]. Another abundant species, *Benthosema glaciale*, in the Northern Atlantic also has been reported to have good levels of favourable macro minerals and trace elements [50].

Table 4 presents an overview of the macro minerals and trace elements found in myctophid species, including *Benthosema glaciale* [50,76] and *Benthosema fibulatum* [76]. The macro minerals analysed included calcium, phosphorus, magnesium, sodium, and potassium, while the trace elements included iron, manganese, zinc, copper, selenium, nickel, lead, and iodine. The results show that *Benthosema glaciale* has higher levels of calcium, phosphorus, and potassium, while *Benthosema fibulatum* has higher levels of magnesium, copper, and iodine. The recommended intake (RI) values suggest that these myctophid species can be a valuable source of these essential minerals and trace elements for human consumption, especially for meeting dietary requirements for calcium, phosphorus, and iodine [77]. The levels of trace elements found in both species fall within the acceptable ranges recommended by health organisations, making them safe for human consumption.

Fish is also considered an excellent source of soluble vitamins, including vitamins A, D, and E, in addition to vitamin B12 which are important for energy production, functioning of the nervous systems, and metabolic processes [78,79]. Myctophids can provide a rich source of soluble vitamins, particularly vitamins A and E [47,80]. Past research suggests that myctophids have higher levels of vitamin A1 (>100 µg/100 g raw, edible part) and vitamin B12 (6.2 µg/100 g raw, edible part on average), while vitamin A2 and D were found to be significantly lower in demersal fish (<0.5 µg/100 g raw, edible part) [76]. A study conducted on myctophids in the Norwegian fjords also revealed very high levels of vitamin A, particularly in the head and viscera [49]. Despite their potential as a nutrient-rich food source, the vitamin composition of myctophids remains poorly documented, and more comprehensive research is required to assess their potential, both for direct human consumption and as feed ingredients in aquaculture.

Table 4. Summary of macro minerals: calcium (Ca), phosphorus (P), magnesium (Mg), sodium (Na), potassium (K), and trace elements: iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), selenium (Se), nickel (Ni), lead (Pb), iodine (I) in myctophid species (*Benthosema glaciale* and *Benthosema fibulatum*). The recommended intake (RI) per day for these minerals and trace elements is also provided [77].

		Benthosema glaciale		*Benthosema fibulatum*		**RI (mg)**
Catch year		2015, 2016	2018	2018	2018	
Ocean Location		Atlantic	Indian	Atlantic	Indian	
Reference		[50]	[76]	[49]	[76]	[77]
Macro minerals (mg/g DW)	Ca	12.1		5.0	9.4	540–900
	P	8.3		3.8	5.8	420–700
	Mg	1.2		0.7	0.6	80–350
	Na	5.4		3.9	2.3	
	K	7.5		2.6	3.0	2900–3510
Trace elements (mg/kg DW)	Fe	51.6		10.8	25.0	5–15
	Mn	2.8				
	Zn	36.0		8.0	15.0	5–12
	Cu	3.0				0.3–1.0
	Se	1.7	0.06	0.6	0.1	0.015–0.06
	Ni	0.9				
	Pb	0.4				
	I		0.04		0.2	0.05–0.15

2.4. Contaminants

Knowledge about contaminants in any food source is important for a proper assessment of potential health risks associated with human consumption, regulatory compliance, and consumer awareness. Food safety regulations, as dictated by the European Union or the US food safety regulations, have been set particularly tight according to recommended intake levels. Reporting of contaminant concentrations in myctophids is presently limited, but is available for toxic metals (e.g., mercury) [81–85] (Table 5) and plastic-associated chemicals (e.g., bisphenol A (BPA), alkylphenol ethoxylates (APEs), pesticides, polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs)) [86–93] (Table 6).

From our assessment of the literature, ten regional studies have reported total mercury (THg) concentrations in myctophid species from the Atlantic, Southern and Pacific Oceans. This research has provided evidence that myctophids contain very low levels of Hg, with mean values consistently lower than 0.5 μg/g DW (Table 5), which is below the tolerable daily intake (TDI) of 0.05 mg per day [94]. THg ranges in myctophids are similar across the globe ranging between 0.03 and 0.45 μg/g DW in the North Atlantic Ocean [95–99], 0.03 and 0.42 μg/g DW in the Southern Ocean [82,84,100], and between 0.04 to 0.32 μg/g in the Southwest Pacific Ocean [40]. Among the genera studied, the highest Hg concentrations were observed in species from the genera *Benthosema*, *Ceratoscopelus*, and *Gymnoscopelus* from the Atlantic Ocean and the Southern Ocean. A study in the North Atlantic Ocean showed that Hg levels in an abundant myctophid species, *Benthosema glaciale*, had not changed between 1936 to 1993, but that there was significant regional differences [98]. Other regional studies have shown evidence that inter-specific variations in THg levels of myctophid are linked to size [83] and vertical distribution [101], with higher levels reported in larger and deeper dwelling species. We found only one study that has reported the proportion of THg that consists of the toxic methylmercury form in myctophids from the Mediterranean Sea and indicated that it ranges from ~70–82% [102].

We reviewed five scientific studies that have examined concentrations of plastic-associated chemicals in myctophids (Table 6). These studies show that for most congeners, concentrations range from non-detectable amounts to levels that are above guidelines and tolerable daily intake levels. Between different species, the reported ranges of the concentrations of bisphenol A (BPA) and alkylphenols, alkylphenol ethoxylates (APEs) are

comparable, while there seems to be more between species variability for alkylphenols. Total polychlorinated biphenyls (PCBs) concentrations range between 0.02–5.97 ng/g WW, with the highest reported levels in *Myctophum* species from the western North Pacific [90]. Reported levels of total polybrominated diphenyl ethers (PBDEs) are highly variable in the North Pacific Gyre, while notably lower in other regional studies.

Table 5. Summary of total mercury (THg) concentration range (ug/g dry weight; DW) in myctophids of various genera from different oceanic regions. * Values were converted from wet to dry weight, assuming a water content of 75%.

Genus	THg	Location	Catch Year	Reference
Benthosema	0.11–0.45	NW Atlantic Ocean	1936–1993	[98]
Bolinichthys	0.02–0.2	N Pacific Ocean,	2007–2011	[95]
	0.16	NW Atlantic Ocean	1971	[95,96]
Ceratoscopelus	0.21–0.42	N Atlantic Ocean	1971, 1978	[96,97]
Diaphus	0.10–0.11	NW Atlantic Ocean	1971–1974	[95,96]
Electrona	0.05–0.27	Southern Indian Ocean, Southern Ocean	1997–1999, 2007–2013, 1995	[82,84,97,100]
	0.08–0.20 *	SW Pacific Ocean	2005–2006	[40]
Gymnoscopelus	0.06–0.424	Southern Ocean	2015–2016, 2007–2011, 1997–1998	[82,84,100]
Hygophum	0.25–0.30	NW Atlantic Ocean	1973–1974	[95,96]
	0.08–0.16 *	SW Pacific Ocean	2005–2006	[40]
Krefftichthys	0.03–0.05	Southern Ocean	2007–2008	[84]
Lampanyctus	0.16–0.34	NW Atlantic Ocean	1974	[96]
	0.12–0.28 *	SW Pacific Ocean	2005–2006	[40]
Lampichthys	0.08–0.20 *	SW Pacific Ocean	2005–2006	[40]
Lobianchia	0.20–0.24	NW Atlantic Ocean	1973–1974	[95,96]
Myctophum	0.08–0.32	N Atlantic Ocean	1994, 2001–2010	[97,103]
Nannobranchium	0.28–0.32 *	SW Pacific Ocean	2005–2006	[40]
Notoscopelus	0.03–0.24	NW, NE Atlantic Ocean	1974, 2001–2003	[96,99]
	0.08–0.12 *	SW Pacific Ocean	2005–2006	[40]
Protomyctophum	0.06–0.10	S Indian Ocean, Southern Ocean	1997–1999, 2007–2009	[84,100]
Symbolphorus	0.04–0.24 *	SW Pacific Ocean	2005–2006	[40]

Table 6. Comparison of plastic concentrations (ng/g wet weight; WW), including bisphenol A (BPA), alkylphenols, alkylphenol ethoxylates (APEs), polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs), in myctophids from different oceans and the corresponding tolerable daily intake (TDI) (nd is non-detectable).

Location	North Pacific Ocean	Western North Atlantic Ocean	North Pacific Gyre	Southern Ocean	TDI
Species **Catch Year**	*Myctophum nitidulum* 2009	*Taaningichthys crenularis* 2009	*Not specified* 2010	*Gymnoscopelus nicholsi* 2003	
BPA	nd-5.10	nd-7.70	nd-6.20		4 ng/g [104]
Alkylphenols	nd-47	nd-62	nd-10.80		
APEs	nd-11	nd-8.70	nd-5.40		
PCBs	0.55–3.40	0.84–1.30	0.02–5.97		1–4 ng/g [105]
PBDEs	0.01–0.05	0.05–0.59	0.002–11.20	0.09 ± 0.02	0.15 ng/g [106]
Reference	[90]	[90]	[87]	[91]	

3. Considerations for Future Exploitation of Myctophids

The above section reviewed literature reporting nutritional information for myctophids from around the globe. While the information suggests that many myctophid species can provide a nutritionally valuable alternative food resource for human consumption or agriculture production, other factors will determine the viability of fisheries to exploit them. Here we briefly summarise some of the knowledge of myctophid ecology that is relevant to developing fisheries for myctophids and other mesopelagic fishes: identifying suitable target species, biodiversity and distribution, biomass estimates, growth and life history, and the ecosystem.

3.1. Species Diversity and Distribution

Our review demonstrates important interspecies differences in the nutritional composition of myctophids, indicating that any potential future exploitation may need to consider relevant factors at the species level. This may be challenging because, with 250 recorded species, the Family Myctophidae is one of the most diverse fish families in the world's oceans [16]. Several regional surveys and studies have reported on species diversity: in the Indian Ocean, 137 myctophid species in 28 genera have been reported, with the most abundant species from the genera *Diaphus* and *Lampanyctus* [107]. In areas of the eastern tropical northern Atlantic Ocean, over 80 species of myctophids have been identified, with the genera *Benthosema*, *Myctophum*, *Diaphus*, *Lampanyctus*, and *Hygophum* most prevalent [107]. In parts of the Pacific Ocean, approximately 60 species of myctophids are recorded to occur: *Myctophum asperum*, *Diaphus theta,* and *Lampanyctus festivus* are particularly abundant in the western Pacific; *Ceratoscopelus warmingii*, *Hygophum hygomii*, and *Diaphus* spp. are abundant in the southern region [108,109] and *Hygophum hygomii* in the northern region [16,110,111]. In the Southern Ocean, abundant species of myctophids include *Electrona antarctica* (Figure 1), *Gymnoscopelus braueri*, *G. ophisthopetrus*, *G. nicholsi*, *Krefftichthys anderssoni*, *Protomyctophum bolini* and *P. tensioni* [24].

A distinct characteristic of many myctophid species is their diel vertical migration. This phenomenon describes night-time ascent by large fractions of the populations of many migratory species from their daytime residence depths below 200 m (mostly below 400 m) to surface or near-surface depths (0–100 m) to feed [17,112,113]. Patterns of migration vary between species and locations with factors, including life history stage, sex, latitude, hydrography, topography, and season believed to influence them [111,114,115]. While strong currents and eddies may affect the horizontal distributions of mesopelagic fishes at local or regional scales, their distributions generally coincide with those of major water masses [17]. This interplay between environmental factors and inherent biological characteristics shapes both the migratory behaviour and biomass distribution of myctophids [17,112,113] and the suitability of environmental habitats [101,107–109,116]. These regional, seasonal and daily patterns of habitat use, which are often species-specific, have important implications for the availability of myctophids to fishing fleets.

3.2. Life History and Growth

The majority of myctophids, in common with numerous other marine fish species, are presumed to be broadcast spawners, reproducing via the release of planktonic eggs and larvae that drift with ocean currents, where fertilisation takes place [117]. Both male and female myctophids are non-guarding pelagic spawners, with females being oviparous [16]. Various myctophid species display sexual dimorphism and size disparities, with some smaller myctophid species exhibiting equal sex ratios and no size differences between the sexes [17,62,118,119]. As myctophid larvae mature, they transition from the epipelagic zone (0–200 m) to deeper waters. These young fish possess the ability to navigate density gradients, including the thermocline and halocline, which typically impede mixing through physical processes [120]. In tropical and temperate waters, reproductive activity can vary seasonally, contributing to the variation observed in body size and individual biomass [121].

Myctophids are generally short-lived compared to many deep-sea and coastal fish species; their lifespans range from less than a year to 8 years, depending on the species and geographical locality [17,118,119,122] (Table 7). Daily growth increments in otoliths have been utilised to confirm the cyclical pattern of vertical migration in various species, with most migratory myctophids exhibiting a standard asymptotic growth curve [123]. There is a correlation between growth rate and vertical migration behaviour, with daily vertically migrating species exhibiting comparatively slower growth rates than non-migratory species [122]. Maximum age varies with genera; for example, species of *Lampanyctus* have been documented to reach a maximum age of 4.5 years in the Pacific Ocean [118,124], while *Lampanyctus* species in the Atlantic Ocean can reach 5.5 to 6 years [118,125]. Short-lived species may be suited to commercial harvest, but the substantial influence of the surrounding marine environment, likely temperature, on growth and longevity has direct implications for potential fishery productivity.

Table 7. Overview of maximum age (yr: year; d: day) and geographical distribution of selected myctophids from the available literature.

	Genera	Max Age	Ocean Region	Ref.
<2 yr	*Benthosema*; *Diaphus*; *Lepidophanes*	325 d; 362 d; 439 d	Atlantic Ocean	[126]
	Ceratoscopelus; Stenobrachius	416 d; 541 d		[127,128]
	Myctophum	440 d	Pacific Ocean	[129]
	Tarletonbeani	504 d		[130]
	Benthosema	~1 yr	Indian Ocean	[131]
	Myctophum	1 yr	Atlantic Ocean	[132]
	Protomyctophum	1.25 yr		[133]
2–4 yr	*Ceratoscopelus*	2 yr	Atlantic Ocean	[134]
	Kreffichthys	2 yr	Southern Ocean	[125]
	Diaphus	2.5 yr	Pacific Ocean	[135]
	Lampanyctodes	3 yr		[136]
	Electrona	3.5 yr	Southern Ocean	[137]
4–6 yr	*Benthosema*	4.5 yr	Atlantic Ocean	[138]
	Lampanyctus; *Triphoturus*	4.5 yr; 5 yr	Pacific Ocean	[118,124]
	Benthosema	5 yr	Atlantic Ocean	[131]
	Lampanyctus	5.5–6 yr		[118,125]
6–8 yr	*Gymnoscopelus*	6 yr	Southern Ocean	[125]
	Benthosema, *Gymnoscopelus*	7 yr	Atlantic Ocean	[139,140]
	Benthosema	7 yr	Pacific Ocean	[141]
	Stenobrachius	7.5 yr	Atlantic Ocean	[118]
	Stenobrachius	8 yr	Atlantic Ocean	[142]

3.3. Role in the Ecosystem

The ecological characteristics and trophic interactions of myctophids and the importance of their roles within the mesopelagic ecosystem have been long recognised [143,144], but many key knowledge gaps remain. Myctophids have been identified as key prey items of commercially important species, including tropical tunas [145,146] and Southern Ocean toothfish [147]. In the northeastern Pacific, dolphins have been reported to consume significant amounts of myctophids [148]. In the Southern Ocean, myctophids contribute a large proportion of the diet of squid [149], penguins and other seabirds [150–152] and marine mammals [143]. Despite the established importance of myctophids in other ecosystems, there is currently limited information available on their ecological role within the subarctic or within tropical regions.

As critical components of marine food webs, myctophids bridge the gap between primary producers and higher trophic-level predators by consuming mesozooplankton [36,119,152,153]. Myctophids exhibit opportunistic predation on a di-

verse array of prey, including copepods, ostracods, euphausiids, hyperiid amphipods, chaetognaths, pteropods, fish eggs, and fish larvae [17,131]. There is evidence to suggest that diet changes with size and age; for example, early-stage *Electrona antarctica* feed on sinking particles [154], copepods and hyperiid amphipods, while the larger fish feed on euphausiids [155]. Despite their ecological importance, the full extent of trophic interactions and dietary preferences of most myctophid species are not yet comprehensively understood. In addition to their contribution to the food web, these mesopelagic fish are integral to biogeochemical cycling in the open ocean [25]. Through diel vertical migration and the generation of sinking faeces, myctophids provide a conduit for the significant export of nutrients, trace elements and organic matter between the surface and deep ocean [156]. The many vital ecological roles of myctophids and their importance in the diets of higher-order and often charismatic predators, together with many knowledge gaps, indicate there will be complex environmental challenges to operating and managing future fisheries according to principles of ecological sustainability.

3.4. Biomass and Fishery Potential

The global biomass of mesopelagic animals, including myctophids, has been estimated using several methods: pelagic trawl surveys [17], acoustic surveys [18,19,157], modelling [20–22] and remotely operated vehicles [158–160]. The first (in 1980) [17] was based on a compilation of trawl survey data from many ocean regions. It estimated total mesopelagic fish biomass to be approximately 1 Gt; myctophids formed a large fraction of biomass in all regions surveyed and comprised over 500 million tonnes in total [17]. More recent global estimates are based on acoustic surveys (9 to 15 Gt [18,19]) and modelling studies (0.73 to 16 Gt [20–22]). The large variations in estimates between methods are due to a number of factors, including underestimation due to net avoidance, assumptions and parameter choice in models, geographic coverage and extrapolation, and interpretation of acoustics data, for example, whether animal groups, such as siphonophores, are included from the fish fraction [19]. Irrespective of the variation in estimates, the total standing stock of mesopelagic fishes, including myctophids, appears considerably larger than any other commercial fish stock.

Areas of concentration of myctophids in regional estimates from trawl catches, for example, in the western Arabian Sea (100 million tonnes [161]) and the Southern Ocean (containing 70–200 million tonnes [151]), indicate there may be prospective locations in which commercial fisheries for myctophids could be developed. There has been interest in and development of commercial mesopelagic fisheries over the last few decades, but these attempts have, however, not yielded economically viable outcomes in the long-term [162,163]. The former Soviet Union started commercially fishing myctophid fishes (predominantly *Diaphus coeruleus* and *Gymnoscopelus nicholsi*) in the Southwest Indian Ocean and Southern Atlantic in 1977, and catches peaked in 1992 with a total harvest of 51,680 tonnes [164]. However, the fishery was eventually abandoned due to low profits [165]. The shelf-edge myctophid *Lampanyctodes hectoris* was targeted by a purse seine fishery in South African waters with a peak annual catch of 42,560 tonnes in 1973, but the fishery discontinued in the mid-1980s due to the low efficiency of catch [166]. During the 1980s, a rigorous investigation was carried out to assess the possibility of developing a sustainable commercial mesopelagic fishing sector in the northern Arabian Sea [131,167]. Similar trends have been reported for Icelandic fisheries [168] targeting the small hatchetfish, *Maurolicus muelleri*. Substantial quantities (46,000 and 18,000 tonnes) were caught by pelagic trawling operations in 2009 and 2010, respectively, but the fishery was discontinued because alternative target species were preferred for economic reasons [164].

In more recent years, as the demand for and price of LC omega-3 oils has grown, there has been renewed interest in commercially fishing mesopelagic resources, particularly in nations with strong aquaculture industries, such as Norway [164,169] and Peru [163]. Despite this, the commercial exploitation of mesopelagic fish species continues to face challenges in achieving long-term economic viability [168,170].

4. Future Prospects and Conclusions

Future food requirements for the increasing human population will include additional and alternative fish resources to meet nutritional demands via direct human consumption of fish and indirectly through fish products fed to other domesticated animals. The estimated biomass of myctophids represents a very large resource relative to other fishery stocks and therefore has the potential to contribute to this need. Our review found that most myctophid species have abundant protein and LC omega-3 oil content, along with low levels of contaminants. Several species were found to have very desirable lipid profiles, with many high in DHA, though some species contain large amounts of less desirable wax esters that may impact their practical utilisation. Myctophids also seem to contain good levels of total protein high in arginine and leucine amino acids, in addition to essential trace elements and macro minerals. These findings emphasise the potential of myctophids to provide a valuable source of nutrition for human consumption and to assist agriculture (including aquaculture) production. Our review of the main nutritional components of myctophids, matched with other data, including the price of fish oils and fishmeal, should assist any future assessments of the economic feasibility of harvesting these fishes as an alternative resource. We also demonstrated some major gaps in the knowledge of the nutritional composition of myctophids: a general lack of measurements on the composition of minerals and vitamins in most species, and geographical gaps, including a lack of any protein or contaminant data for myctophids in the Indian Ocean.

Despite the nutritional suitability of myctophids for human consumption, there are barriers to developing sustainable fisheries for them due to major gaps in knowledge regarding their stock assessment and ecology. Estimates of myctophid stock size have an order-of-magnitude variation [164]. Their trophic pathways, links to primary production and roles in biogeochemical cycling are largely unknown [162] despite their being forage species to many high-order fishes, birds and marine mammal predators. Although there is increased demand for LC omega-3 oils, efficient and cost-effective commercial harvesting and processing methods are required for potential myctophid fisheries to attain commercial viability.

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Review

New Sustainable Oil Seed Sources of Omega-3 Long-Chain Polyunsaturated Fatty Acids: A Journey from the Ocean to the Field

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Abstract: Omega-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (ω3 LC-PUFA) play a critical physiological role in health and are nutritionally important for both humans and animals. The abundance of marine-derived resources of the health-benefitting ω3 LC-PUFA is either static or in some cases declining. This review focuses on the development and deregulation of novel oilseed crops producing ω3 LC-PUFA and their market applications. Genetic engineering of ω3 LC-PUFA into sustainable oilseed crops involving multiple-gene pathways to reach fish oil-like levels of these key nutrients has been extremely challenging. After two decades of collaborative effort, oilseed crops containing fish oil-levels of ω3 LC-PUFA and importantly also containing a high ω3/ω6 ratio have been developed. Deregulation of genetically engineered crops with such novel nutritional traits is also challenging and more trait-based regulations should be adopted. Some ω3 LC-PUFA-producing oilseed crops have been approved for large-scale cultivation, and for applications into feed and food. These genetically engineered oilseed crops can and will help meet the increasing market demand for aquaculture and human nutrition. These new oil seed sources of ω3 LC-PUFA offer a sustainable, safe, cost-effective, and scalable land-based solution, which can have critical and positive health, economic, and environmental impacts.

Keywords: aquafeed; DHA; DPA; EPA; ω3 LC-PUFA; oilseed crops; nutrition; regulatory; sustainability

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1. Introduction

Omega-3 long-chain polyunsaturated fatty acids (ω3 LC-PUFA, defined as containing 20 or more carbon atoms), including eicosapentaenoic acid (EPA, 20:5ω3), docosapentaenoic acid (DPA, 22:5ω3), and docosahexaenoic acid (DHA, 22:6ω3), are beneficial to human health throughout the whole lifespan [1]. They are essential components of cell membranes important for cell function as well as precursors for biologically active signalling molecules in mammals.

DHA is one of the most important ω3 LC-PUFA. Sub-optimal levels of DHA in the human body are associated with an increased risk of several diseases [2]. Ghasemi Fard et al. [3] provided a comprehensive collection of evidence and a critical summary of the documented physiological effects of high DHA fish oils on human health. The positive effects of EPA and DHA have been reported across a range of degenerative and inflammatory disorders such as heart disease, stroke, rheumatoid arthritis, asthma and some cancers, diabetes mellitus, multiple sclerosis, dementia, and clinical depression [2,4,5]. EPA- and in particular DHA-rich oils are also important in infant nutrition, with DHA present in high concentrations in the brain and retina, and these two key LC-PUFA are important in the development, health, and enhanced functioning of these and other organs [6–8].

Similar to DHA and EPA, ω3 DPA is gaining increasing recognition and importance because of its unique properties. ω3 DPA is the precursor of many lipid mediators involved in the pro-resolution of inflammation with specific effects compared to other ω3 LC-PUFA [9]. The presence of ω3 LC-PUFA in human tissues and its relative abundance in human milk have long served as clues to its importance in human health. It is increasingly recognized as an important part of our diet. Numerous trials have demonstrated a clear link between ω3 DPA intake and better health, while multiple in vitro and in vivo studies have shown direct effects of ω3 DPA on inflammation, improved plasma lipid profile, and cognitive function [10,11]. Morin et al. [12] reported that ω3 LC-PUFA monoacylglycerides (MAG) were found to be better absorbed in cultured human colorectal cancer cells compared to the corresponding free fatty acids. Furthermore, that study demonstrated that ω3 DPA-MAG had increased anti-proliferative and pro-apoptotic effects, decreased cell proliferation and induced apoptosis, when compared to DHA-MAG and EPA-MAG. Recently, Ghasemi Fard et al. [13] summarised the physiological effect, delivery, fatty acid metabolism, and bioavailability of ω3 DPA.

ω3 LC-PUFA are also essential for fish development [14]. They are nutritionally important for the survival, growth, and general health of aquaculture species, particularly at the larval stage. Reduced accumulation of ω3 LC-PUFA in farmed fish also decreases the nutritional value of the final product [15,16].

The current principal sources of ω3 LC-PUFA for human consumption are wild-caught marine fish species, krill, and some algae. The increasing demand for these fatty acids has contributed in some regions to overfishing of many source species, generating a huge negative environmental impact [17]. In addition, global warming leading to an increase in water temperature, depending on the climate scenario and location, could result in a 10 to 58% loss of globally available DHA by 2100 [18]. The ω3 LC-PUFA in these fish species are accumulated up the food web, primarily originating from microalgae. While aquaculture is an alternative way to replace the wild fish stocks for human consumption of ω3 LC-PUFA, farmed fish need sustainable sources of ω3 LC-PUFA in their diet for their development and growth. This requirement constrains the impact that aquaculture per se can have on mitigating the decline in wild fish stocks, including in some cases due to unsustainable harvesting of wild fisheries.

Fermentation of microalgae containing ω3 LC-PUFA has also been seen as a potential solution in this area. However, growing microalgae heterotrophically has its own challenges, including energy consumption, the high capital investment required for large-scale fermentation facilities, reproducibility and consistency of production, efficiency of cell breaking, high production cost, and other factors. The development of algal-derived single cell oils will not be covered further in this review, and background on and an example of such research is covered in this Special Issue by Soudant et al. 2023 [19].

2. Development of Oilseed Crops with Fish Oil-like Levels of ω3 LC-PUFA

The above challenges have led to the exploration of alternative and sustainable approaches. Metabolic engineering of land-based oilseed crops to produce fish oil levels of ω3 LC-PUFA is one of the most striking and ambitious examples of such a strategy [20]. High oil yield and relatively low production costs of oilseed crops can provide an economic and sustainable production platform for oil containing ω3 LC-PUFA. For example, canola (*Brassica napus* L.) picks itself as a potential oil platform for EPA and DHA production. Canola seed yields have been reported up to 4 T/ha with 40–45% seed oil content. It has broad agronomic and geographic adaptation, considerable genetic resources, and substantially developed germplasms. These aspects make canola the second largest oilseed crop (behind soybean), producing 84.8 million metric tons (MMT) globally in 2022/23 [21] and representing an ideal vehicle for producing ω3 LC-PUFA.

LC-PUFA can be synthesised by two distinct pathways: the aerobic pathway utilizing fatty acid desaturases and elongases, and the anaerobic polyketide synthase (PKS) pathway [22]. The aerobic pathway uses sequential oxygen-dependent desaturation and

elongation steps coupled with electron flow (Figure 1A). The same set of desaturases and elongases can synthesise ω6 DPA or ω3 DHA from the ω6 substrate linoleic acid (LA, 18:2ω6) or the ω3 substrate α-linolenic acid (ALA, 18:3ω3), respectively. The PKS pathway synthesises LC-PUFA directly from malonyl-CoA and acetyl-CoA without the need for oxygen for desaturation (Figure 1B) [23,24]. Such a complex pathway makes it challenging to engineer for achieving the desired high levels of specific end products. In the last two decades, these two distinct pathways have been introduced into oilseeds to produce ω3 LC-PUFA including EPA, ω3 DPA, and DHA [25–27]. The introduced aerobic pathway required genes for the five desaturation and/or elongation steps from ALA to DHA, while the introduced PKS pathway contained several genes for multiple domains (Figure 1). Most recently, production of ω3 docosatrienoic acid (DTA, 22:3ω3) in *B. carinata* was achieved by introducing a minimal single elongase from the plant *Eranthis hyemalis* that can elongate a wide range of PUFA, thus converting plant endogenous ALA to ω3 eicosatrienoic acid (ETA, 20:3ω3) then further converting ETA to DTA [28]. Generally, the production of ω3 LC-PUFA with the PKS pathway has resulted in only low levels of products [25]; however, efforts with the aerobic pathway have produced ω3 LC-PUFA at the same levels as found in wild fish oils [20,29].

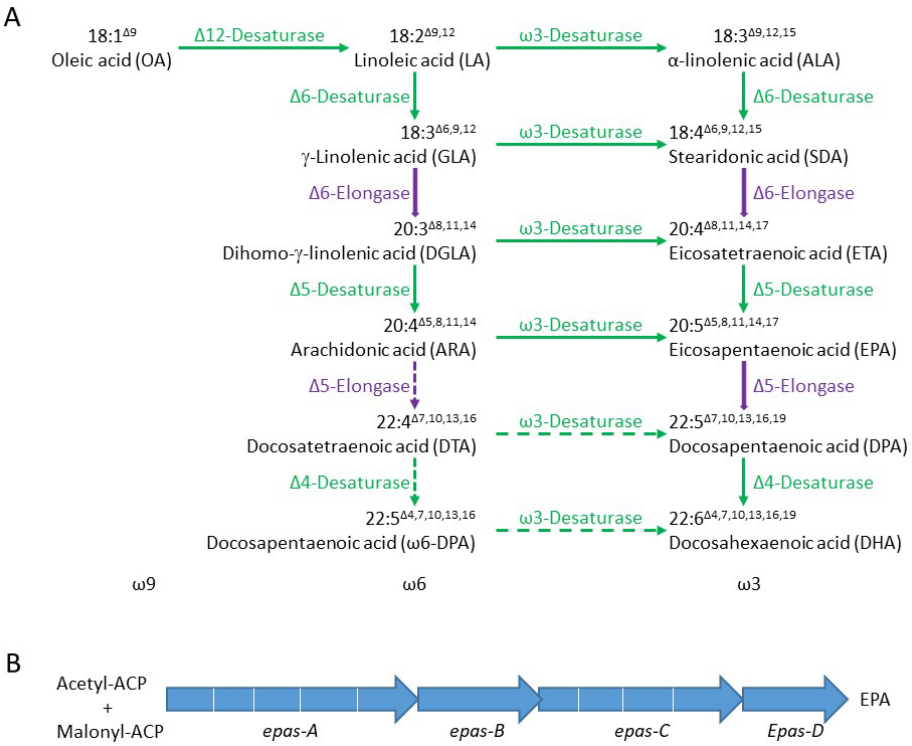

Figure 1. Biosynthetic pathways of LC-PUFA. (**A**) The aerobic pathway using specific desaturases (green) and elongases (purple) converts both ω6 LA or ω3 ALA to ω6 DPA or ω3 DHA, respectively. The dashed arrows are minor activity steps of the pathway in microalgae and engineered oil crops. (**B**) EPA polyketide synthase pathway from *Shewanellea oneidensis* [24] is composed of huge enzyme complexes with multiple domains as divided by bars.

Since the earlier demonstration of successful metabolic engineering of EPA or DHA production at low levels in yeast or seed oils [23,30–32], development of commercially sustainable oilseed crops with fish oil-like levels of ω3 LC-PUFA has been one of the main targets by a range of researchers and companies in the last two decades and longer in some cases. Previous reviews of the research and development on oilseed sources of ω3 LC-PUFA are available in [20,33–36].

Several of the research efforts have ceased, and/or not achieved what were seen as very difficult aims. The collaboration between CSIRO, the Grains Research and Develop-

ment Corporation (GRDC), and Nuseed has continued and has successfully developed a genetically engineered canola, event NS-B5ØØ27-4, that produces ω3 LC-PUFA containing oil with levels of 9.7% DHA, 1% DPA, and 0.5% EPA [27]. An ongoing breeding program aims to further increase ω3 LC-PUFA levels in the oil. The project was initiated by CSIRO researchers in 1997, although it took several years to build momentum. The research team comprised: marine microalgae researchers, plant geneticists, plant breeders, marine oil chemists, food technologists, and other specialists. One key aspect was that the CSIRO research team accessed a unique selection of microalgae from the CSIRO-based Australian National Algae Culture Collection (http://www.csiro.au/ANACC (accessed on 1 June 2023)). The algae collection had been established at CSIRO for strategic research on algal chlorophyll and carotenoid pigments as applied in biological oceanographic research.

Another collaboration between BASF and Cargill has also generated a transgenic canola, event LBFLFK, that produces 0.3% DHA, 2% DPA, and 4% EPA in refined, bleached, and deodorized oil [37]. These two events (CSIRO-Nuseed and BASF-Cargill) will be covered in this paper, with emphasis on the former project. Key desaturase and elongase enzymes identified, validated (in yeast and plant models), and developed and used in the CSIRO-Nuseed project are listed in Table 1.

Table 1. DHA biosynthesis enzymes. In the isolation of an efficient synthesis pathway, key desaturase and elongase enzymes were isolated from strains held in the CSIRO-based Australian National Algae Culture Collection (http://www.csiro.au/ANACC (accessed on 1 June 2023)).

Enzyme	Conversion	Comment	References
Micromonas persilla Δ6-desaturase	18:3ω3 to 18:4ω3 (ALA to SDA)	Use of a marine microalgae Δ6-desaturase with a higher preference for ω3 substrate than ω6 substrate	[38]
Pyraminomas cordata Δ6-elongase	18:4ω3 to 20:4ω3 (SDA to ETA)	High conversion efficiency of SDA to ETA via Δ6-elongase	[39]
Pavlova salina Δ5-desaturase	20:4ω3 to 20:5ω3 (ETA to EPA)	Demonstrated the acyl-CoA desaturation ability	[39,40]
Pyraminomas cordata Δ5-elongase	20:5ω3 to 22:5ω3 (EPA to ω3 DPA)	Highly efficient Δ5-elongase targeted to maximise the elongation from EPA to ω3 DPA	[39]
Pavlova salina Δ4-desaturase	22:5ω3 to 22:6ω3 (ω3 DPA to DHA)	Demonstrated the acyl-CoA desaturation ability	[40]
ω3 desaturases from various sources	conversion of ω6 PUFA and ω6 LC-PUFA to ω3 PUFA and ω3 LC-PUFA	Results in very low amounts of ω6 fatty acids and contributed to the high ω3/ω6 ratio	[41]

Multiple attempts have been made to achieve the fish oil-like levels of ω3 LC-PUFA for commercialisation. The first consideration was to enhance the fatty acid flux from oleic acid (OA, 18:1ω9) to ω3 ALA by introducing a yeast Δ12-desaturase and ω3-desaturase, in addition to the endogenous Δ12-desaturase and Δ15-desaturase. ω3-Desaturases can convert a range of ω6 fatty acids including LA, γ-linolenic acid (GLA, 18:3ω6), dihomo-γ-linolenic acid (DGLA, 20:3ω6), arachidonic acid (ARA, 20:4ω6), docosatetraenoic acid (DTA, 22:4ω6), and docosapentaenoic acid (ω6 DPA, 22:5ω6) into the corresponding ω3 fatty acids with different substrate preferences [41]. The introduced ω3-desaturase maximally converts ω6 LA to ω3 ALA, thus making higher levels of ω3 substrate available for the biosynthesis pathway. The remaining low amount of LA is used for synthesis of the downstream ω6 LC-PUFA, which can also be converted to their ω3 counterparts by ω3-desaturase. This resulted in very low amounts of ω6 fatty acids and contributed to the high ω3/ω6 ratio [27] that is desired for both human and fish health.

The second consideration was to use a marine microalgae *Micromonas pusilla* Δ6-desaturase with a higher preference for the ω3 substrate than the ω6 substrate [38]. The combined effect of the enhanced fatty acid flux from OA to ALA, and the ω3 substrate preference of the Δ6-desaturase, led to the elevated production of ω3 fatty acids at the early steps of the biosynthetic pathway.

The third consideration was to use acyl CoA desaturases for subsequent steps in the pathway rather than phosphatidylcholine (PC) type desaturases to avoid excessive acyl shuffling between acyl-CoA and acyl-PC pools, as the fatty acid elongation occurs in acyl-CoA pools. Phylogenetic analysis of amino acid sequences showed that the *M. pusilla* Δ6-desaturase, *Pavlova salina* Δ5- and Δ4-desaturases used in the ω3 LC-PUFA containing canola event, NS-B5ØØ27-4, clustered with other demonstrated acyl-CoA desaturases. *M. pusilla* Δ6-desaturase has been demonstrated to have acyl-CoA desaturation ability [38]. *P. lutheri* Δ4-desaturase, from a very closed related species to *P. salina*, has also been shown to desaturate acyl-CoA substrates [42].

The fourth consideration was to use a highly efficient Δ5-elongase from the microalga *Pyramimonas cordata* to maximise the elongation from EPA to ω3 DPA. Earlier proof of concept work had expressed the DHA biosynthetic pathway containing *P. salina* Δ5-elongase in *Arabidopsis* and successfully produced DHA in seed oil, but only at low levels (<1%). The conversion rate of the Δ5-elongation step was the major bottleneck, with the efficiency lower than 20% [31]. The *P. cordata* Δ5-elongase showed much higher efficiency for elongating EPA to ω3 DPA in yeast cells [39] than the *P. salina* Δ5-elongase [43]. The superior conversion efficiency of *P. cordata* Δ5-elongase was confirmed to be as high as 90% in *Arabidopsis* seeds [44].

In addition, the large T-DNA vector consisting of seven genes in the DHA biosynthetic pathway plus a selection marker had been carefully designed with multiple seed-specific promoters. The promoter expression timing was an important factor to reduce accumulation of intermediate fatty acids. The direction of gene expression cassettes and the inclusion of non-coding spacers between cassettes was another consideration designed to maximise the gene expression levels. Finally, thousands of lines were created having stable inserts with relatively low copies of T-DNA. The selected elite canola event, NS-B5ØØ27-4, contains a multi-copy of the full transgene construct at one locus plus an extra partial T-DNA insertion at another locus effectively acting as a 'booster' for the full pathway with increased gene dosage.

In the case of canola event LBFLFK, gene dosage with multiple copies of alternate genes for the same enzymatic activity was a contributing factor [20]. The transgene cassette had a total of 12 genes for ω3 LC-PUFA biosynthesis, with two copies inserted in the canola chromosome. Increased gene dosage has also been applied in engineering EPA production in the yeast *Yarrowia lipolytica* by integrating five to seven copies of each desaturase or elongase gene, with a total number of 24 desaturase/elongase genes for maximised EPA accumulation [45]. These approaches collectively provide successful strategies for metabolic engineering that utilise complex multiple gene pathways.

In addition to canola, other oilseed crops such as *Camelina sativa* have been engineered for the production of ω3 LC-PUFA oil using a similar approach. A transgene cassette expressing five genes or seven genes for ω3 LC-PUFA biosynthesis from OA in *C. sativa* resulted in 24% EPA or 11% EPA and 8% DHA in seed oil, respectively [46]. Petrie et al. [47] describe the production of fish oil-like levels (>12%) of DHA in *C. sativa* seed oil and achieving a high ω3/ω6 ratio. The same T-DNA vector for producing fish oil-like levels of DHA in canola [27] was used to engineer DHA production in *B. juncea*, with up to 17% DHA produced in the T_4 seed oil of some *B. juncea* lines. Interestingly, some lines with a truncation of the T-DNA insert that eliminated the Δ4-desaturase activity stably accumulated 12% of ω3 DPA [48]. This was the first example of land plant-based oil seed ω3 DPA production, and was 2–3 times higher than most other natural sources. An exception for the occurrence of ω3 DPA is the abalone, a group of small to very large marine gastropod molluscs in the family Haliotidae, which can contain elevated ω3 DPA, e.g., 13–14% [49]. The distribution of abalone globally is restricted to a limited number of countries, with only a comparatively small harvest available. Abalone would not be able to serve as a sustainable large-scale source of ω3 DPA.

The development of a new and sustainable source of ω3 DPA further demonstrated the capability of engineering a complex metabolic pathway in different oilseed crops. This

demonstration also offered a sustainable source of ω3 DPA, which is currently only available from wild oceanic species or seals with limited quantities for commercial use. Studies have also shown that DPA is much more effective at reducing the risk of cardiovascular disease, and that DPA is more effective than EPA at promoting endothelial migration [10]. A 1:1 ratio (*w*/*w*) of DHA to DPA in *B. juncea* seed oil has also been achieved [48]. The combination of DHA and DPA may be an excellent future dietary means for promoting cardiovascular health. Other attempts for producing stearidonic acid (SDA, 18:4ω3), a medium chain length ω3 PUFA, were also reported in linseed [50] and soybean [51]; the latter has been deregulated in the USA.

3. The Challenge of Deregulation

Development of oilseed crops with fish oil-like levels of ω3 LC-PUFA generates one of the most novel nutritional traits through genetic engineering, which is regulated in many production and import markets. Although the terms "genetic modification" or "genetically modified organism" are commonly used to refer to agricultural biotechnology or its products, USA regulatory authorities, such as the USDA and FDA, do not recommend using these terms because they are less accurate and could encompass the broader spectrum of genetic alterations, including some traditional and new breeding technologies [52]. In addition, according to the US National Bioengineered Food Disclosure Standard, the "bioengineered" distinction is now required for mandatory compliance in the United States [53]. In this review, the term "genetic engineering" has been used to describe the technology. Genetically engineered crops have established a history of safe use since their first recorded commercialization in the mid-1990's [54]. Since then, most countries that either produce or import biotechnology products have developed and implemented rigorous policies to regulate genetically engineered crops and their derived products. Although safety assessments should be science-based without geographic borders, the country- or regional-based regulatory systems have indeed created many challenges for approving and adopting biotechnology products on a broader scale and in a timely fashion. Different regulations and measurements about genetically engineered products also create complexities for the movement of agricultural commodities between countries and have a significant impact on international trade.

In addition to the challenges that are caused by various regulatory requirements in different jurisdictions, nutritionally enhanced products face unique challenges for deregulation because nutritional quality or traits are generally more complex and involve multiple genes to function. As of today, most approved and commericalized genetically engineered crops are focused on agronomically improved traits, such as herbicide tolerance or insect resistance. The introduction of a single gene to express the specific protein for the intended use makes it relatively easy to characterize the insertion structure, protein safety, and interactions with the environment. In fact, many of the regulatory data requirements for risk assessments are established based on these single-gene traits, such as determining the impact on non-targeted organisms and weediness potential. However, for nutritionally enhanced traits, the introduction of a multiple gene pathway generates new challenges for characterization. For example, a detailed molecular characterization is required for regulatory approval, and traditionally it has relied on Southern blot analysis to establish transgene locus and copy number along with targeted sequencing of PCR products spanning any inserted DNA. However, Southern blot analysis is labor intensive and time-consuming; therefore, it can be very expensive for characterizing complex events that contain multiple genes and/or multiple copies.

The use of sequencing-based technologies, such as whole-genome sequencing and junction sequence analysis bioinformatics, proves to be more efficient and effective at identifying and characterizing complex events and provides in-depth sequence-level information [55–57]. Therefore, for nutritional traits obtained with a multiple-gene pathway, next-generation sequencing is the preferred and recommended approach for molecular characterization. Further, unlike the insect resistance or herbicide tolerance traits, nu-

tritional quality traits generally do not present plant pest properties; therefore, risk assessment should be trait-based. Some regulatory requirements for assessing impacts on non-target organisms or weediness potential might not be relevant or applicable to some nutritional traits.

The ω3 LC-PUFA are marine-sourced nutrients and the multiple-gene pathway for their biosynthesis that exists in algae is not native to higher plants, making genetic engineering the only viable tool to produce these valuable LC-PUFA in oilseed crops, such as canola. Currently, there are two new genetically engineered canola events that have been developed to produce ω3 LC-PUFA. One is the canola event NS-B5ØØ27-4, also known as DHA canola, described above. The other is the canola event LBFLFK from BASF. Both events have multiple genes introduced from yeast and microalgae to express various desaturases and elongases that are required to convert OA to ω3 LC-PUFA in canola seed.

One important aspect of a risk assessment is to evaluate the safety of newly introduced proteins in genetically engineered crops. The desaturases and elongases introduced in ω3 LC-PUFA oilseed crops are integral transmembrane proteins, which are intractable proteins, making them extremely difficult to isolate and purify for protein characterization and safety studies [58].

Another challenge of characterizing transmembrane proteins is to achieve a suitable level of recombinant protein expression and purity to successfully generate antibodies [59]. Moreover, these introduced desaturases and elongases share high levels of amino acid sequence homology between individual desaturases or elongases. This makes it difficult to quantify the individual expressed protein levels in the transgenic crops using conventional Western blot analysis. Western blot analysis is only possible if the antibody against the specific desaturase or elongase is available, as antibodies may suffer from cross-reactivity. A targeted LC-MS/MS method was developed to simultaneously quantify the seven biosynthetic pathway enzymes in DHA canola [60]. The targeted peptides for LC-MS/MS detection can be defined in silico for each specific protein. Even a single amino acid residue variation in a highly homologous region of two proteins can be detected separately with a highly selective and sensitive multiple reaction monitoring mass spectrometry assay. The absolute quantification of the targeted peptides was achieved by adding synthetic peptides identical in sequence but labelled with stable isotopes at the C-terminal arginine or lysine leading to a mass shift of +10 or +8 daltons. This approach confirmed that the enzymes that drive the production of DHA using seed-specific promoters were detected only in the seed of DHA canola at low levels [60]. A similar LC-MS/MS approach was applied for determining the in vitro protein digestibility of these transmembrane proteins, using simulated gastric conditions [61]. It has been shown that all the enzymes in the biosynthetic pathway were readily digested.

Desaturases and elongases are naturally found in a wide range of organisms with a long history of safe use. They are rapidly digestible and heat-instable, and no safety concerns were identified from the bioinformatics analysis and protein characterization [62]. Therefore, there is little value in conducing animal toxicity studies. Nevertheless, some jurisdictions require protein toxicity studies despite of the weight of evidence of their safety. Considering it is impractical to conduct such studies using purified proteins, alternative approaches using crude proteins or whole foods, such as meal and oil, should be accepted.

To address the different requirements for safety assessments in different jurisdictions, comprehensive safety evaluations for these new ω3 LC-PUFA canola events have been conducted using the weight of evidence approach. This included a detailed molecular characterization, a safety assessment of gene sources, a history of safe use, in silico bioinformatic analysis for allergenicity and toxicity potential, in vitro digestibility and stability studies, comparable agronomic field studies and compositional analysis, dietary exposure evaluations, toxicity testing, and animal feeding studies, amongst others. All the studies confirmed that these two ω3 LC-PUFA canola events (NS-B5ØØ27-4 and LBFLFK) were as substantially equivalent as conventional canola varieties; the only difference observed was the expected oil profile change [37,62]. Fish feeding trials also demonstrated that DHA

canola oil [62–64] or *Camelina* EPA oil [65–68] were safe and effective alternatives to fish oil in the fish diet. Another study using ω3 LC-PUFA canola oil to replace menhaden fish oil in shrimp diets also showed no significant differences in shrimp performance metrics [69].

DHA canola has been approved for food, feed, and cultivation in Australia, Canada, and the USA. Given its unique total omega-3 profile for human nutrition and health, Nutriterra® Total Omega-3, the brand name of DHA canola oil for the human consumption market from Nuseed, was also recognized as a new dietary ingredient by the US FDA, allowing its use as a nutraceutical supplement in the USA. The canola event LBFLFK was also approved for cultivation, food, and feed use in Canada and the USA. Both ω3 LC-PUFA canola events are targeted to substitute fish oil in aquaculture feed. Most recently, Nuseed's Aquaterra® omega-3 canola oil was authorized for use in fish feed in Norway, making history as the first biotech product to receive approval in this market, the world's largest salmon producer.

It is also important to point out that, unlike input traits, nutritionally enhanced crops are typically produced under the identity preservation (IP) system to maintain their product identity and integrity. For example, Nuseed's DHA canola is produced under a closed-loop stewardship IP system to closely track and manage its ω3 LC-PUFA trait from seed to oil.

4. Market Demand and Consumer Acceptance for ω3 Canola Oil and Applications

The necessity for novel ω3 sources is driven by rapidly increasing demand and urgent environmental challenges. Fish oil is the historic primary source of ω3 LC-PUFA and is typically sourced through wild-caught fish. About 75% of this oil is directed to the aquaculture industry with the remainder going to human nutrition markets [70]. Looking closer at these multiple channels reveals the market readiness and opportunity for ω3 canola oils.

A key aspect for market consideration is consumer opinion. In the early stages of the CSIRO research, consumer preference trials were performed in Australia, the USA, Europe, and Asia [71–73]. When conducted up to 15 years ago, these studies concluded that a large proportion of the population are accepting of genetically engineered land plants producing omega-3 LC-PUFA oils that provided a health benefit, that were supported by health claims from a trusted source, and were indirectly consumed (e.g., food for farming fish).

5. Aquaculture

All feeds manufactured for use in salmonid aquaculture contain marine ingredients. Although marine ingredients are renewable resources, the supply cannot keep up with the rapid growth of aquaculture. The aquaculture industry has expanded in the past twenty years, and now delivers about 50% of all seafood for human consumption [74], but wild-caught fisheries have maintained overall static production levels since the late 1980s. This creates a shortage of raw marine materials and challenges the economic and environmental sustainability of fisheries and aquaculture [74].

The declining availability of marine-sourced oils against the increased demand has led to significant changes to fish feed formulations; specifically, higher incorporation of plant-derived and animal by-products, where approved, are replacing marine ingredients [16]. In Norway, the marine oil content of feeds declined from 24% in 1990 to 10.9% in 2013 [75]. Mowi's annual report for 2019 indicates their use of fish oil in salmon diets was 10% [76]. Mowi is also by far the world's largest Atlantic salmon farmer with harvest volumes of 466,000 tonnes in 2021, equivalent to a global market share of approximately 20% [77]. The Chilean average marine oil incorporation in fish feeds was 7% in 2017 [78].

The composition of dietary fatty acids affects many aspects of fish growth and health. According to Robert [79], nutritionally compromised diets often increase a species' susceptibility to infectious diseases. There is now growing concern about the effect that low availability of omega-3 fatty acids could have on the ability of fish to survive disease or environmental challenges.

Unsurprisingly, a decline in EPA and DHA in salmon feed formulations resulted in a reduced omega-3 content in salmon fillets [14,15], reducing the nutritional value of salmon for human consumption. Consumers are also increasingly concerned for the environmental impact of aquaculture, in particular the dependency on foraged fish to produce farmed fish. These are measured in the industry as the Fish In–Fish Out ratio (FIFO) and the Foraged Fish Dependency Ratio (FFDR), and salmon producers are under pressure to reduce these metrics without compromising omega-3 nutrition for fish welfare and human nutrition.

For all these reasons, aquaculture is enthusiastically embracing the innovation of ω3 canola oils. This uptake is represented in Figure 2, with the application of the DHA canola now occurring. Aquaterra® Advanced Omega-3, the DHA canola oil being marketed by Nuseed, contains significant levels of DHA and other ω3 PUFA and ω3 LC-PUFA (35% of total fatty acids). Oil harvested from 1–2 ha DHA canola contained the same amount of DHA that would be available from 10,000 kg of wild caught fish (Figure 2). The oil also contains high levels of ALA and has a much higher ω3/ω6 ratio in comparison with conventional canola oil [27,37,62]. The unique combination of high DHA, high ALA, and a high ω3/ω6 ratio are the major attributes of Aquaterra®, which led to impressive results in a commercial scale feeding trials and industry recognition [62,80].

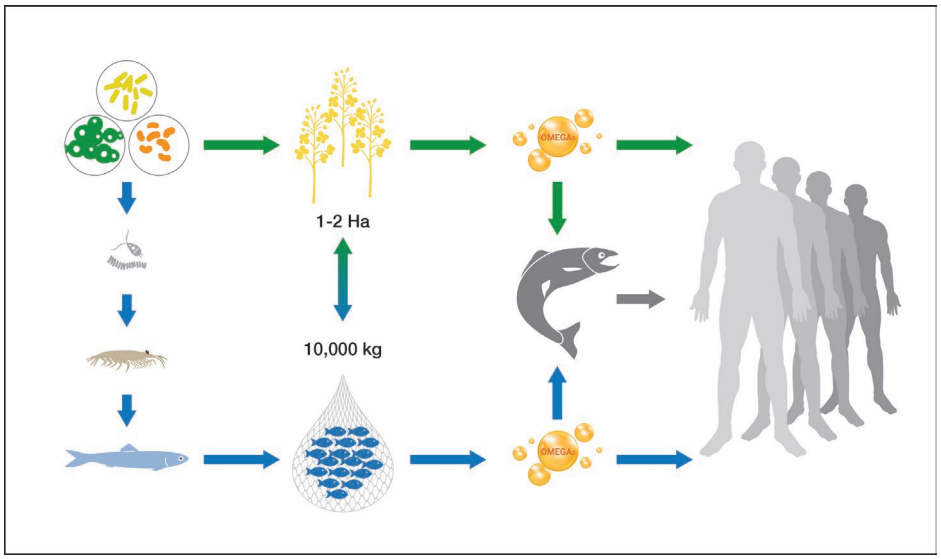

Figure 2. The combined sources of ω3 LC-PUFA are shown, with the traditional main sources (left and bottom, arrows in blue) being seafood and microalgae. New sources of ω3 LC-PUFA are now available through the availability of genetically engineered oilseed plants for use in both feed and food/supplement applications; these are indicated along the top row in green.

6. Human Nutrition

While most of the oil harvested from wild-caught fish is used in aquaculture, the global human nutrition market for ω3 LC-PUFA currently requires over 115,000 MT each year, and this is projected to grow at 2.1% annually [81]. The human nutrition market is experiencing the same supply constraints as the aquaculture industry, which leaves supplement manufacturers in need of sustainable alternative sources to meet current demand. There is also significant interest in plant-based omega-3 sources. A survey conducted from 1200 vitamin consumers discovered 64% would prefer a plant-based alternative, driven by a combination of organoleptic and environmental concerns [82]. These findings are in line with the CSIRO consumer research on oilseed-derived omega-3 sources noted earlier [71–73].

ω3 LC-PUFA containing oils from genetically engineered *Camelina* or canola have been confirmed as effective replacements for fish oil as a dietary source of ω3 LC-PUFA for mice, rats [83,84], and humans [85–88]. Nutriterra® is expeller-pressed from DHA canola seed,

then refined, bleached, and deodorized. Like Aquaterra®, it delivers a unique omega-3 profile and a beneficial (high) ω3/ω6 ratio, with a milder sensory experience than occurs for marine-based ω3 LC-PUFA. The Nutriterra® product demonstrated bioavailability and efficacy in a human clinical trial [89]. Sixteen weeks of daily supplementation of the DHA canola oil was found to be safe and well-tolerated [89]. Additionally, Nutriterra® is certified by Friend of the Sea [90] to provide consumer confidence in the sustainability claims.

7. Conclusions and Perspective

Production of sustainably grown and harvested ω3 LC-PUFA canola events is now a commercial reality (Figure 2), and importantly, it can provide triple bottom-line benefits (economic, social, and environmental) for the world. The global market for the ω3 LC-PUFA oils containing EPA and DHA is estimated to be USD$3B. As demand for DHA and EPA increases, there are significant prospects for the global expansion of the ω3 LC-PUFA containing canola crops. For example, a DHA canola crop of just 1.25 M ha (~3.5% of global canola production) would be required to replace 50% of fish oil currently sold globally. It is further estimated that 2.5 M ha of the DHA canola crop can provide equivalent amounts of DHA to that currently harvested from all fish oils [91].

Crop-based production of ω3 LC-PUFA will enable increased consumption of omega-3 oils by consumers. A report [92] on the global burden of human disease estimated that there was a USD$31B cost in terms of productivity and 1.4 B premature deaths due to ischemic heart disease associated with low intake of DHA and EPA. The report estimates that with the use of dietary supplements of DHA and EPA, pre-term births could be significantly reduced, resulting in USD$6.1B in healthcare savings in the USA and USD$51M in Australia. These dietary supplements could also reduce heart disease in adults; this benefit is associated with an estimated USD$485M in savings.

In terms of environmental considerations, nearly 60% of wild-caught fish is used to provide omega-3 oil for the burgeoning global aquaculture industry. One widely cited paper describes the amount of large predatory fish in the oceans as being at only 10% of pre-industrial times [17]. One of the authors claimed in a later publication that the state of the oceans, combined with the impact of climate change and the effect of these factors on fish stocks, were such that 'all commercial fish and seafood may collapse' by 2048 [93]. A land-based sustainable and scalable source of the health-benefitting DHA and EPA (Figure 2) can play a critical role in providing nutritional security and reducing the chance of depleting wild fish stocks.

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Sustainable Enzymatic Production of Omega-3 Oil from Squid Viscera

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Abstract: Fish oils are widely consumed around the world to increase omega-3 fatty acid intake. Due to negative impacts on marine resources and ecosystems from an increasing demand for fish, alternative sustainable sources are under investigation. Squid viscera contains up to 10% oil by mass and is available as a byproduct from squid processing. Squid viscera oil is a source of EPA and DHA and contains the xanthophyll carotenoid astaxanthin, known for its significant anticancer, antioxidant, antidiabetic, and cardiovascular properties. In the raw form, squid viscera oil has a high free fatty acid (FFA) content, so conventional alkaline refining results in low yield and loss of astaxanthin. As a higher-yielding alternative, the current study optimized lipase-catalyzed glycerolysis of squid viscera oil to convert FFA into acylglycerol using a custom-built one-liter immobilized enzyme reactor. To monitor the reaction progress and assess its impact on the oil, we analyzed lipid classes, fatty acid composition and astaxanthin levels. Under optimized conditions, FFA was reduced from 40% to 2.7% in 10 h and 1.7% in 24 h, with no significant effect on EPA and DHA levels, and astaxanthin being retained. Squid viscera presents a safe and sustainable additional source of marine-derived EPA and DHA oil.

Keywords: omega-3 oils; marine; lipase; fish oil; sustainable; nutritional; astaxanthin

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1. Introduction

Long-chain omega-3 polyunsaturated fatty acids (PUFAs) including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are beneficial to human health [1]. EPA and DHA are precursors to endogenous anti-inflammatory mediators that are required for optimum health [2]. EPA and DHA are implicated in fetal development, healthy aging, lowering plasma triglycerides and cholesterol, and protection against chronic diseases including type 2 diabetes mellitus, ischemic heart disease, cancer, high blood pressure, depression, Alzheimer's, and cardiovascular diseases [3–5].

EPA and DHA are abundant in the marine environment, including fatty fish and algae, which are the primary sources of these PUFAs in the human diet [6]. Approximately 85% of the world's population does not consume the recommended levels of EPA and DHA for optimal health, so marine oils are widely used as nutritional supplements and also as starting materials for some pharmaceutical products [7–9]. Due to the limited source of fish oil, there is interest in additional sources of EPA and DHA, particularly sustainable sources such as microalgae, plants and fisheries by-products [10–13]. Fishery by-products can comprise 50% or more of the total fish mass, of which a significant portion is disposed of in landfills with negative environmental and economic impacts.

Squid, or calamari, is a popular seafood widely consumed around the world. Typically, the squid body and tentacles are commercially recovered for consumption, and by-products including viscera are discarded. The viscera consists of approximately 10% oil which is high in omega-3 fatty acids, particularly DHA [14]. Depending on species and harvest location, the oil contains 15 to 20% EPA and 20 to 25% DHA, making it a promising marine source of PUFAs for nutraceuticals or pharmaceuticals [14,15]. Squid oil contains higher DHA levels than standard anchovy oil (180 mg EPA/120 mg DHA), a more balanced ratio of EPA to DHA compared with tuna oil (50 mg EPA/20 mg DHA and considerably higher EPA and DHA than krill oil. Therefore, squid oil offers a high omega-3 alternative to fish and krill oil for nutritional supplement purposes, with further research needed to determine an appropriate dose. From the delivery of DHA perspective, squid oil delivers significantly more DHA than either standard anchovy oil or krill oil per gram of oil. Although some algal oils have high levels of DHA the scale-up of these oils is relatively expensive and, in some cases, involves genetically modified organisms. Also, squid oil is from waste viscera and so offers an environmentally friendly alternative to other oils. In addition to potential use in nutritional supplements, squid oil could be microencapsulated or emulsified for food use with appropriate stabilizers. Squid oil also contains astaxanthin, a xanthophyll carotenoid known for its significant anticancer, antioxidant, antidiabetic and cardiovascular properties [16]. Astaxanthin is added to animal, fish and human foods to impart desirable color or health properties [17,18], hence its presence in squid oil provides a potential health benefit advantage.

Producing a nutritional oil from squid viscera oil requires several processing steps including oil extraction and neutralization of free fatty acids [19]. Depending on the oil properties additional steps of bleaching, winterization and deodorization may be necessary [19]. Conventional neutralization of oils involves alkali deacidification (alkali refining), which can reduce the non-glyceride FFAs to as low as 0.03% [20]. However, the high levels of FFA in squid oil result in low recovery after alkali refining and loss of astaxanthin [20]. However, the removal of FFA is important for stability and sensory acceptance, particularly for nutritional oils [21,22].

Enzymatic glycerolysis of FFAs is an alternative greener method of FFA removal that results in the conversion of FFA to acylglycerol and no loss of yield [20,23,24]. The enzyme process is favored industrially over the chemical-based process for the formation of acylglycerols from FFA in fish oil manufacturing due to the high catalytic efficiency and mild reaction conditions of the enzyme process. Enzymatic acylglycerol formation for fish oil results in higher acylglycerol yields at lower temperatures, less pressure and shorter time than required for the chemical process. In addition, the lipase-catalyzed process can be carried out at close to a 1:1 ratio of glycerol and FFA, whereas the chemical process requires high levels of FFA to drive the reaction forward since it is an equilibrium reaction [25,26]. However, due to the high cost of enzymes, glycerolysis is not viable unless the enzyme can be multiply reused, which requires immobilization of the enzyme. Using immobilized enzymes in glycerolysis offers additional benefits including greater enzyme stability and the elimination of enzyme recovery and purification steps [27,28]. Previous studies have found immobilized enzymes are effective for both vegetable and fish oil esterification reactions, with the enzyme remaining active over multiple cycles [29–32].

This study investigates the use of immobilized lipase for the enzymatic glycerolysis of crude squid viscera oil to produce an alternative to fish oil for omega-3 nutritional applications. A custom-designed one-litre reactor was used to isolate the immobilized enzyme from the stirrer for ease of recycling over multiple cycles. Normal stirring results in the breakdown of the support material and the inability to reuse the enzyme. This work explores the impact of key parameters influencing the reaction and optimizes these for the squid-oil acyglycerolysis process, including choice of enzyme, enzyme amount, substrate ratios, operating temperature, reaction time, mixing speed and removal of water during the reaction. The reaction was optimized for acylglycerol levels while also minimizing damage to sensitive components, particularly EPA, DHA and astaxanthin. The developed process

is the first reported one-pot reactor-based lipase re-esterification of natural squid oil that retains the natural triacylglyceride component while converting the FFA component to acylglycerol resulting in no loss of yield and greater than 95% acylglycerol product.

2. Materials and Methods

2.1. Materials

Crude squid viscera oil from the commercial arrow squid (*Nototodarus gouldi*) was provided by Mantzaris Fisheries Pty Ltd., North Geelong, Australia. Glycerol was purchased from EnviroChem International Pty Ltd., Melbourne, Australia, and immobilized enzyme (Lipozyme RMIM, Novozym 435 and Lipozyme TLIM) was sourced from Novozymes Australia Pty Ltd., North Rocks, Australia. Molecular sieves, HPLC grade hexane, heptane, ethyl acetate and diethyl ether, astaxanthin and phenolphthalein indicator were sourced from Sigma Aldrich, Castle Hill, Australia. Acetone, isopropyl alcohol and methanol were purchased from Chem Supply, Gillman, Australia and toluene from Thermofisher Scientific, Melbourne, Australia. Potassium hydroxide was supplied by Unilab Chemicals and Pharmaceuticals Pvt Ltd., Mumbai, India.

2.2. Design and Working Principle of the One-Liter Reactor

Neutralization of the squid viscera oil was carried out using a custom-built one-liter jacketed reactor. The reactor is jacketed and consists of a reaction chamber at the top (3/4 of reactor volume) with a stirrer placed in the upper reaction chamber and a lower enzyme compartment (1/4 of reactor volume). The two chambers are separated by two layers of stainless-steel mesh which holds the immobilized enzyme and separates it from the stirrer to minimize enzyme support breakdown. An overhead stirrer (IKA RW20 digital, John Morris Scientific, Melbourne, Australia) was used to mix the reactor contents and a peristaltic pump (Masterflex L/S, Cole Parmer Instrument Company, Chatswood, Australia) with PVC tubing was used to recirculate the reaction mixture, enabling either or both to be applied depending on the degree of mixing required. Normally both stirring and recirculation were required for adequate mixing and contact with the enzyme. The temperature was controlled by circulating water through the reaction chamber jacket using a water bath (Temperature RATEK Instrument Pty Ltd., Boronia, Australia). Molecular sieves were loaded into the reaction chamber to remove water as it formed, as an alternative to vacuum if required. To monitor the reaction progress, 5 mL of oil/glycerol mixture was sampled by disconnecting the PVC tubing at the required timepoints. Reaction and reactor operation parameters were optimized to achieve a minimal residual free fatty acid level in the final product (see Table 1 for detailed operating conditions).

2.3. Determination of Lipid Classes by TLC-FID

Lipid class analysis was carried out using TLC-FID (Iatroscan) following a previously described method with minor modifications [33]. Briefly, oil samples were dissolved in heptane (10 mg of oil in 2 mL heptane), then 1 µL from each sample was spotted onto chromarods along the line of origin on the rod holder for separation. The rods were then developed with hexane/diethyl ether/acetic acid (60:17:0.2, $v/v/v$) for 22 min. The rods were scanned using the TLC-FID (Iatroscan MK6, Iatron Laboratories Inc., Tokyo, Japan) with settings 2.0 L/min air flow rate, 160 mL/min hydrogen flow rate and scan speed 30 s/scan. TLC standards from Nu-Chek Prep were used to identify each lipid class.

Table 1. Working conditions of the reactor.

Parameters	Working Conditions *
Starting conditions	1% (*m*/*v*) molecular sieves, 50 °C reactor temperature, 1% (*m*/*v*) enzyme, oil: glycerol 3:1, stirring speed 400 rpm
Molecular sieves	(i) Without molecular sieves (M. S.) (ii) 1% (*m*/*v*) M. S. added at commencement of reaction. (iii) 2% (*m*/*v*) M. S. added at commencement of reaction. (iv) 1% M.S. added at commencement, then 0.20% extra at 2, 4, 6, 8 and 10 h
Reactor operating temperature	40 °C, 50 °C, 60 °C (non-preheated substrate, 1% *m*/*v* M. S.)
Pre-heating reaction substrate	(i) Non-preheated (ii) Preheated (reaction at 50 °C, 1% M. S.)
Enzyme	0.50%, 1%, 2% and 3% (*m*/*v*) of the total substrate (reaction at 50 °C, 1% M. S., pre-heated substrate)
Oil to glycerol ratio	(i) Oil:glycerol = 4:1 (ii) Oil:glycerol = 3:1 (iii) Oil:glycerol = 2:1 (iv) Starting oil:glycerol = 3:1, then extra 75 mL (9.50% of the starting substrate volume), 50 mL (6.25%) and 50 mL (6.25%) glycerol added at 2, 4 and 6 h (Reaction at 50 °C, 1% M. S., pre-heated substrate, 1% enzyme)
Recovery and re-using of glycerol	(i) Oil:glycerol = 1.67:1, fresh glycerol (ii) Oil:glycerol = 1.67:1, used glycerol (iii) Starting oil:glycerol = 3:1 (fresh), extra 75 mL (9.50%), 50 mL (6.25%) and 50 mL (6.25%) glycerol (used) at 2, 4 and 6 h (Reaction at 50 °C, 1% M. S., pre-heated substrate, 1% enzyme)
Stirring/Mixing speed	(i) 400 rpm (ii) 800 rpm (iii) First 2 h 400 rpm, after 2 h 600 rpm (iv) First 2 h 400 rpm, after 2 h 800 rpm (Reaction at 50 °C, 1% M. S., pre-heated substrate, 1% enzyme)

* Condition limits were identified based on preliminary runs to define usable ranges.

2.4. Fatty Acid Composition by GC-FAMEs

The fatty acid composition of oil samples was determined using gas chromatography-fatty acid methyl esters (GC-FAMEs) with flame ionization detection (FID) as previously described [34]. For each sample 10 mg of oil was dissolved in 1 mL toluene, and 200 µL of internal standard (5 mg/mL methyl nonadecanoate in toluene) and 200 µL of antioxidant (1 mg/mL 2,6-di-tert-butyl-4-methylphenol (butylated hydroxytoluene; BHT in toluene)) were added. To this, 2 mL of acidified methanol (prepared by adding 1 mL acetyl chloride drop wise to 10 mL methanol on ice) was added and mixed with the solutions and left overnight at 50 °C in a sealed tube. After cooling, 5 mL sodium chloride solution (5% *m*/*v*) was added. The fatty acid methyl esters were then extracted twice with heptane (5 mL) and the heptane layer was washed with 5 mL potassium bicarbonate solution (2% *m*/*v*). Finally, the heptane layer was dried over sodium sulphate and analyzed using an Agilent 6890 gas chromatography with flame ionization detector (FID) equipped with a BPX70 SGE column (30 m, 0.25 mm i.d., 0.25 µm film thickness). The oven was programmed from 140 °C (5 min hold) to 220 °C (5 min hold) at a rate of 4 °C/min for a total run time of 30 min. A volume of 1 µL of each sample was injected with a split ratio of 50:1 (injector temperature, 250 °C). Helium was used as the carrier gas (1.5 mL/min, constant flow) and the detector gases were 30 mL/min hydrogen, 300 mL/min air and 30 mL/min nitrogen. Peak areas were integrated using ChemStation software version A.06.01 and corrected using theoretical relative FID response factors [35]. The GC fatty acids standards used were a mixture of saturated, monounsaturated and polyunsaturated fatty acids ranging from carbon 4 to 24.

An internal standard, acetyl chloride and BHT were sourced from Sigma Aldrich, Castle Hill, Australia.

2.5. Determination of Astaxanthin Content by HPLC

The original and esterified squid oils were analyzed for astaxanthin content using HPLC as previously described with minor modifications [36]. Approximately 0.5 g of oil sample was diluted in a mixture of petroleum ether:acetone:water (15:75:10). The sample was then sonicated for 30 min in an ultrasonic bath and centrifuged for 20 min at 4200 rpm. Then 2 mL acetone was added, and the sample centrifuged for an additional 20 min. The supernatant was then filtered through a 0.45 μm syringe filter and 10 μL injected on a reversed-phase column (Luna 5u C18(2) 100 A, 260 × 4.6 mm, Phenomenex Pty. Ltd., Lane Cove West, Australia). An Agilent 1260 Series HPLC system equipped with solvent degasser, quaternary pump, autosampler, thermostated column compartment and diode array detector was used for analysis. Methanol, ethyl acetate and water were used as gradient mobile-phase systems, initially at a ratio of 88:10:2 (methanol/ethyl acetate/water) for 10 min at 0.75 mL/min. Over the next 20 min the flow rate was adjusted to 1.5 mL/min and the mobile phase composition changed to 48:50:2. These conditions were held for an additional 10 min, then the column was re-equilibrated for 15 min for an overall run time of 55 min. Column temperature was maintained at 23 °C. The diode array detector was set to 477 nm for detection of astaxanthin.

The percent loss of astaxanthin due to different operating conditions and time was calculated using Equation (1).

$$\text{Loss of astaxanthin (\%)} = [\text{A\_original} - \text{A\_test}]/(\text{A\_original}) \times 100 \quad (1)$$

where,

A_original = Area under peak produced from the original (non-esterified) squid oil
A_test = Area under peak produced from the esterified squid oil

2.6. Determination of Acid Value

Acid values of the squid oil samples were determined following the AOAC International 2009 titration method. Approximately 0.5 g of each of the samples were measured into a 250 mL conical flask, in which 125 mL of solvent mixture (isopropyl alcohol and toluene, 1:1) was added. The mixture was titrated against 0.1 M potassium hydroxide in presence of 1% phenolphthalein indicator solution in isopropyl alcohol. The titration end point was reached when the solution turned to pink color and persisted for at least 30 s. The acid value was determined using the Equation (2).

$$\text{Acid value, mg KOH per g of test sample} = [(V - B) \times M \times 56.1]/W \quad (2)$$

where,

V = Volume of standard alkali used in the titration, mL
B = Volume of standard alkali used for the blank titration, mL
M = Molarity of KOH, the standard alkali
W = Mass of test sample, g

2.7. Statistical Analysis of Data

Experiments were carried out in triplicate and the average values are reported in the article. The significant difference between the two mean values was determined by using a one-way analysis of variance at a 95% confidence level ($p < 0.05$). The inbuilt program in Microsoft ® Excel for Microsoft 365 MSO (Version 2303 Build 16.0.16227.20202) was used for this purpose.

3. Results and Discussion

3.1. Crude Squid Viscera Oil

The crude squid viscera oil supplied by Mantzaris Fisheries was characterized for lipid class, EPA and DHA content, and acid value (Table 2). The oil contained 40.3% FFA, 5.5% monoacylglycerols (MAG), 10.1%, diacylglycerols (DAG) and 41.6% triacylglycerols (TAG). It contained approximately 2.6% other lipid classes which may include esters, phospholipids, sterols, and other minor lipid classes, which were similar to previously reported compositions [14]. The EPA and DHA contents of the crude oil were 167.6 mg/g and 167.1 mg/g oil, respectively. The acid value of the supplied oil was 79.6 mg KOH/g of the sample. Astaxanthin content of the squid viscera oil was 22.5 mg/kg.

Table 2. Percent lipid classes and omega-3 fatty acids content in the crude squid viscera oil.

Crude Squid Oil	% Lipid Classes (with Standard Deviation Values)				Omega-3 Fatty Acids, mg/g Oil		Acid Value (mg KOH/g of Oil)
	FFA	TAG	DAG	MAG	EPA	DHA	
	40.3 ± 2.91	41.6 ± 2.18	10.1 ± 1.09	5.5 ± 0.81	167.6 ± 4.07	167.1 ± 3.29	79.6 ± 2.19

3.2. Optimizing the Enzymatic Glycerolysis of Squid Viscera Oil

3.2.1. Selection of Enzyme

Three commercially available immobilized lipases were trialed for the enzymatic glycerolysis of squid viscera oil: Lipozyme TLIM from *Thermomyces lanuginosus*, Lipozyme RMIM from *Rhizormucor miehei* and Novozym 435 from *Candida antarctica*. Each of these enzymes complies with the recommended food grade specifications of the joint FAO/WHO expert committee on Food Additives (JEFCA) and the Food Chemical Codex (FCC). The three enzymes were compared by stirring under a vacuum in a glass bottle for 24 h at 60 °C using a 2:1 crude oil to glycerol ratio, 1% *m*/*v* enzyme, and 1% *m*/*v* molecular sieves. Lipozyme RMIM and Novozym 435 both provided greater than 98% conversion of FFA's to acylglycerol, compared to only a 4.4% conversion for Lipozyme TLIM (Figure 1).

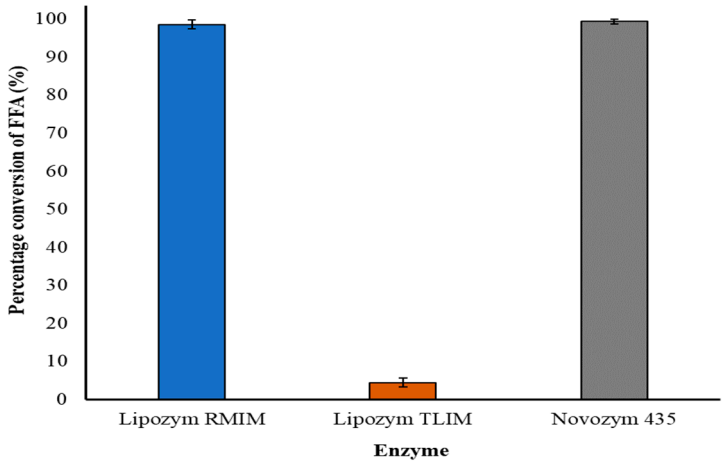

Figure 1. Comparison of the effectiveness of three lipases to convert FFA to acylglycerol for crude squid oil, using conditions of 2:1 crude oil to glycerol ratio, 1% *m*/*v* enzyme, 1% *m*/*v* molecular sieves at 60 °C for 24 h. Results are mean values of three replicates.

To select between Lipozym RMIM and Novozym 435 we used the glass bottle reaction system to compare the effect of temperature, molecular sieves, and reaction time on the degree of acylglycerol formation with each enzyme. Novozym 435 gave a more complete reaction in the absence of molecular sieves, however, Lipozym RMIM reacted more effectively at the lower temperatures of 40 and 50 °C, so the latter was selected for a more energy-efficient reaction.

3.2.2. Removal of Water Formed during the Reaction

The glycerolysis of squid viscera oil releases water as a reaction product, which hinders the reaction progress and needs to be removed as the reaction progresses, to drive the equilibrium reaction forward [20]. Two methods were investigated for the removal of water, vacuum, and molecular sieves. At the 1-L scale, molecular sieves were the more effective, although it is the less scalable option [24,37,38]. We compared the effect of adding up to 2% m/v low-cost inert aluminosilicate beads, either before or during the reaction (detailed in Table 1). The other parameters were selected based on the preliminary enzyme screening experiments: stirring at 400 rpm in the custom-designed reactor at 50 °C, 3:1 oil to glycerol and 1% m/v Lipozym RMIM enzyme. A reaction time of 24 h was selected for all experiments to ensure reaction completion. The experimental data (Figure 2A) show that the addition of molecular sieves resulted in a faster reaction and a 2% improvement in FFA after 8 h and 1.5% after 24 h, irrespective of the quantity added. Despite the relatively minor improvement, we elected to continue using 1% m/v molecular sieves for the remaining optimization experiments.

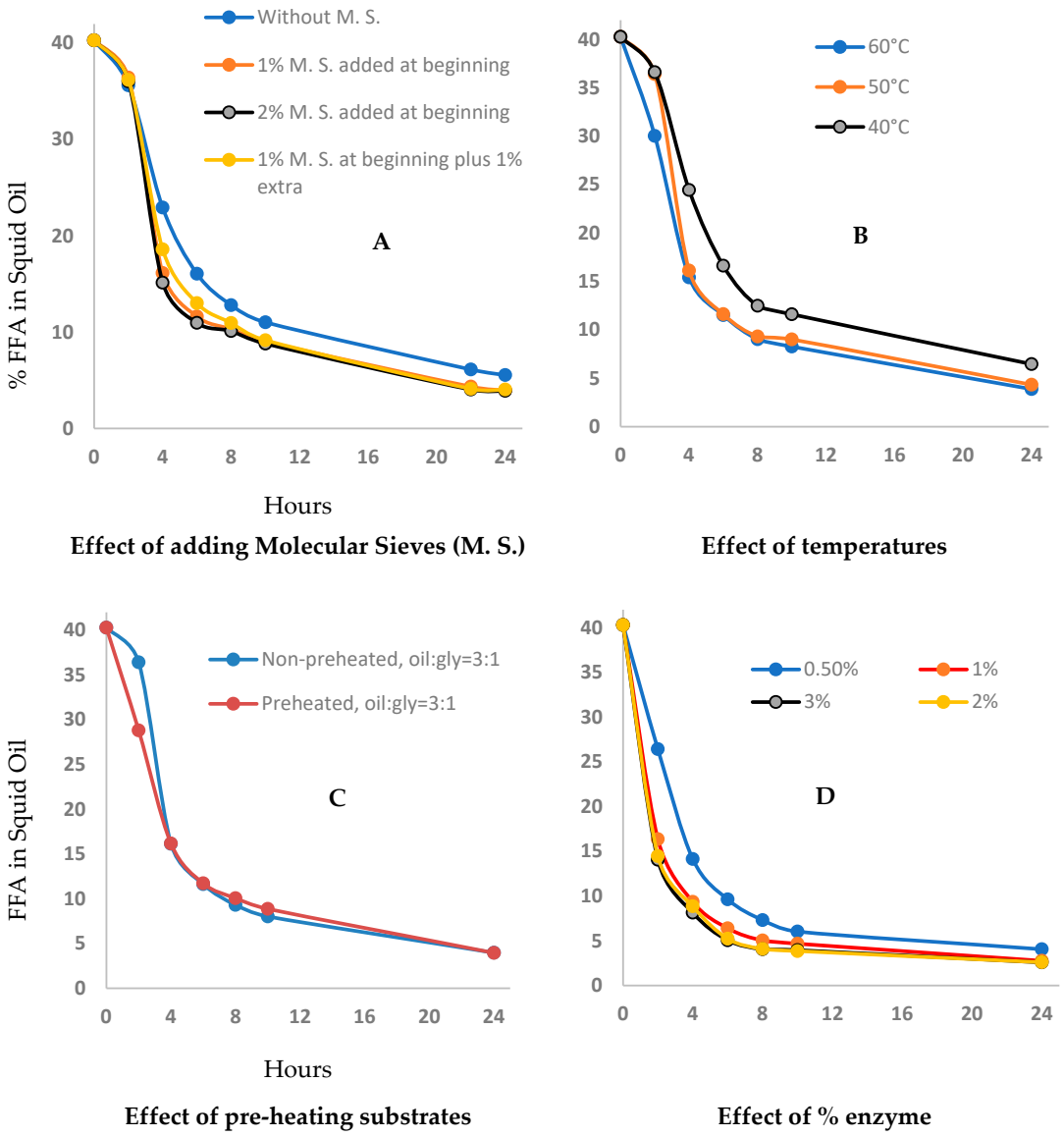

Figure 2. Optimization of (**A**) molecular sieves (**B**) reaction temperature (**C**) pre-heating of substrate and (**D**) percent enzyme for esterification process.

3.2.3. Effect of Reaction Temperature

Reaction temperature influences kinetics, enzyme longevity, energy costs and hence environmental impact for the FFA to acylglycerol conversion process. With these factors in mind, we compared reaction rate and yield at 40, 50 and 60 °C (starting from room temperature, Figure 2B), with stirring at 400 rpm, 1% *m*/*v* molecular sieves, 3:1 oil to glycerol and 1% *m*/*v* enzyme. At 40 °C the conversion rate of FFA to acylglycerol (TAG, DAG and MAG) was slower than at 50 °C and 60 °C. Although the reaction at 60 °C started earlier than at 50 °C, there were no significant differences between these two temperatures from 4 h onwards. After 8 h at 40 °C there was 11.6% FFA remaining, compared to 9% at the two higher temperatures and at 24 h the 40 °C reaction still had a 2% higher residual FFA content. Consequently, we selected 50 °C as the optimum temperature for the enzymatic esterification of squid oil to minimize damage to the enzyme and energy usage, while maximizing product quality. This temperature is consistent with previous work for lipase-catalyzed esterification of fish and microalgae oils [39].

3.2.4. Influence of Pre-Heating Substrates

From Figure 2B, we can see an initial lag in the reaction rates at 40 °C and 50 °C compared to the trial at 60 °C. Given each trial commenced at room temperature the enzyme activity is initially low until sufficient heat is transferred to the reaction mixture from the jacketed reactor, which occurs more rapidly at 60 °C. To avoid the initial lag in reaction rate we compared the effect of pre-heating the substrate to the optimized temperature of 50 °C, with the additional parameters held consistent: stirring at 400 rpm, 50 °C, 3:1 oil to glycerol and 1% *m*/*v* enzyme (Figure 2C). Despite an initial increase in the rate of reaction gained from pre-heating the substrates, the results show an almost identical FFA level remaining in the product from 4 h into the reaction. For further trials we elected to retain the pre-heating step, to remove any possible variation from differences in the starting room temperature.

3.2.5. Effect of Immobilised Enzyme Ratio

An enzyme loading from 0.5% *w/v* to 3% *w/v* was compared for the glycerolysis reaction (Figure 2D), with consistent parameters of stirring at 400 rpm, 50 °C with pre-heated reactants, 3:1 oil to glycerol and 1% *m*/*v* molecular sieves. At 0.5% *w/v* the conversion of FFA to acylglycerol was slower compared to the higher enzyme quantities, among which there was no significant difference (4 to 5% FFA after 8 h and 2.6 to 2.8% of the residual FFA after 24 h). We selected 1% *m*/*v* as the optimized enzyme level, balancing cost and yield, which is consistent with results from a previous study using sardine oil [20].

3.2.6. Substrate Ratio

To optimize the substrate ratio, we compared crude oil to glycerol ratios of 4:1, 3:1 and 2:1 for the effect on the rate and extent of FFA conversion. Consistent parameters were selected of stirring at 400 rpm, 50 °C with pre-heated reactants, 1% *m*/*v* enzyme and 1% *m*/*v* molecular sieves. A 1:1 oil to glycerol was also trialed but resulted in a mixture too viscous to mix effectively. Our results presented in Figure 3A show that the highest substrate ratio of 4:1 oil–glycerol resulted in a slower rate of reaction and final product with 5% more FFA after 8 h and 3% more FFA after 24 h, compared to both 3:1 and 2:1 ratios. This finding is consistent with a similar study indicating that a glycerol/PUFA 1.2:3 ratio was optimum for lipase-catalyzed esterification [39].

We also compared the reaction progression as extra glycerol was added at different time points, with the aim of driving the esterification reaction forward faster. The reaction started with a 3:1 oil-to-glycerol ratio, and an extra 9.5% (75 mL) was added at 2 h and an extra 6.25% (50 mL) after 4 and 6 h. These conditions resulted in an increased reaction rate, with residual FFA at 4% within 8 h and 1.8% in 24 h. However, a significant amount of glycerol remains unreacted requiring removal at the completion of the reaction. This was achieved through phase separation and centrifugation of the glycerol and oil product.

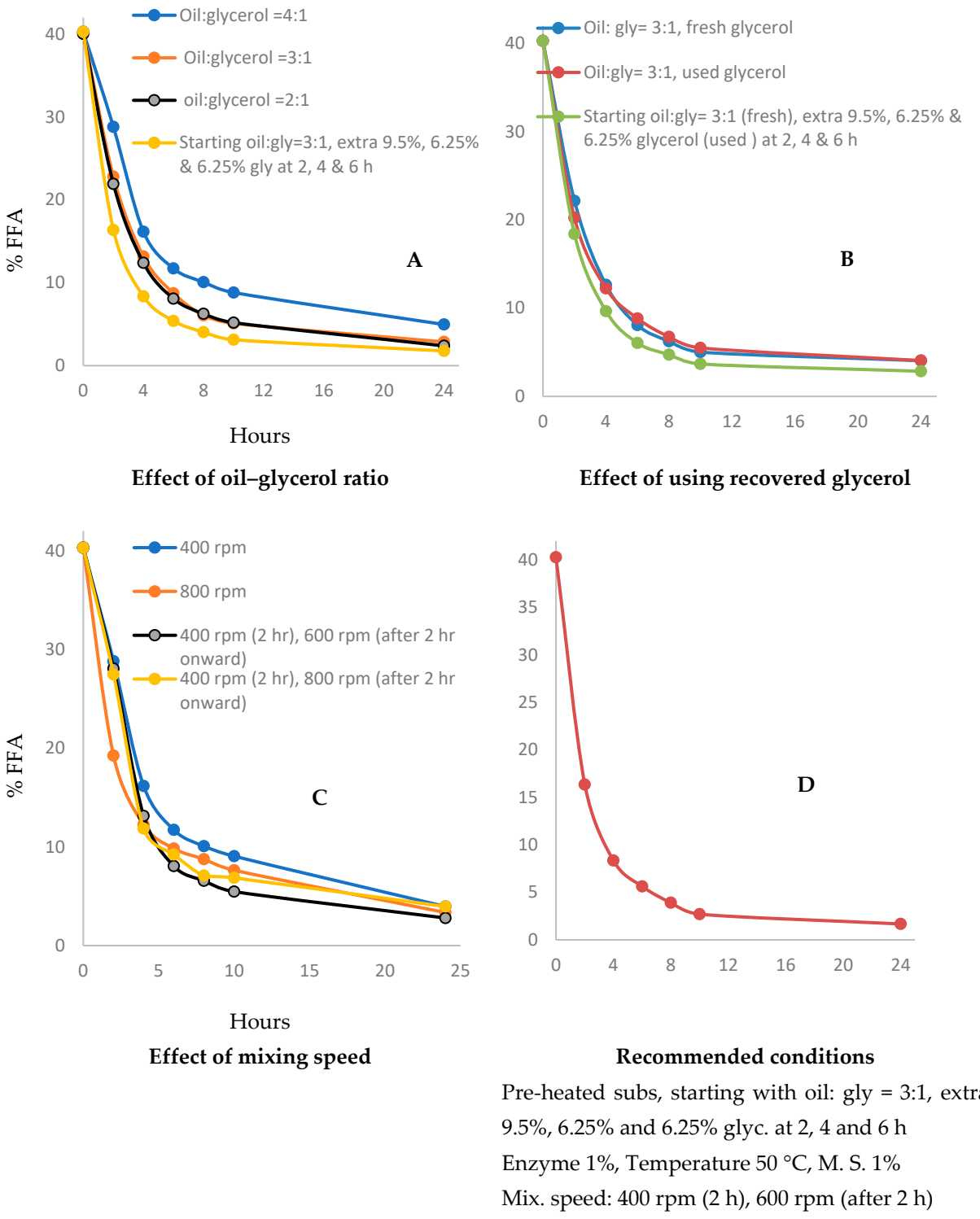

Figure 3. Effect of (**A**) oil to glycerol ratio (**B**) reusing of glycerol and (**C**) mixing speed for esterification process, (**D**) recommended conditions for the best performance of the lab-scale reactor.

3.2.7. Recovery and Reutilization of Glycerol

To reduce the cost of substrates for the glycerolysis reaction, we trialed recovering unreacted glycerol and reusing it in a subsequent trial. We compared reactions using a 3:1 oil-to-glycerol ratio with recovered glycerol only to a 3:1 oil-to-glycerol ratio starting with fresh material and adding recovered glycerol at 2-, 4- and 6-h timepoints (Figure 3B). In both cases, our reaction data show a reaction rate and degree of FFA neutralization

comparable to using fresh substrate only. We concluded that using fresh glycerol, recovered glycerol or a combination of both is suitable to reduce input material costs.

3.2.8. Reaction Mixing Speed

To further increase kinetics and reduce the reaction time for commercial viability we studied the effect of mixing speed in the 1 L vessel. Five different mixing speeds of 400, 500, 600, 700 and 800 rpm were compared under conditions of 1% (*m*/*v*) enzyme, 1% (*m*/*v*) molecular sieves, oil to glycerol ratio 3:1, temperature of 50 °C with pre-heated reactants (Figure 3C). The reaction rate was slowest at 400 rpm and highest at 800 rpm, although after 6 h the difference was within 2%, and within 1.25% after 8 h. We also compared the effect of increasing the mixing speed to 600 or 800 rpm after the first two hours and found both trials significantly increased the percent FFA conversion. Using a low speed (400 rpm) for the first 2 h and then to moderate high (600 rpm) for the remainder of the reaction showed the most complete neutralization between 6 and 24 h. Previous studies indicated mixing speed ranges from 300 to 700 rpm were suitable for maximum glycerolysis of conjugated linoleic acid catalyzed by immobilized enzyme [40,41].

3.2.9. Optimised Glycerolysis Conditions

Figure 3D shows optimized reaction conditions for the glycerolysis reaction, where the pre-heated substrate of 3:1 oil/glycerol was reacted with an extra 175 mL (22% of the starting substrate volume) of glycerol being added progressively at 2,4 and 6 h (75 mL, 50 mL and 50 mL, respectively). The mixing speed started at 400 rpm and increased to 600 rpm after two hours. The reaction temperature was 50 °C with preheated substrates, 1% (*m*/*v*) enzyme and 1% (*m*/*v*) molecular sieves. Under these conditions, a reaction time of 10 h was sufficient to reduce FFA levels to 2.7%.

3.3. Product Characterisation

The neutralized squid viscera oil was analyzed for lipid classes, fatty acid compositions, acid values and astaxanthin content to confirm its suitability as a nutritional lipid.

3.3.1. Lipid Classes

The changes in lipid classes during the glycerolysis reaction of squid oil under optimized conditions are shown in Table 3. As shown, after 24 h of reaction FFA was reduced from 40.3% to 1.8%, DAG increased from 10.1% to 48.8% and MAG increased from 5.5% to 25.9%. Total glycerides increased from 57.2% to 96.3% over the course of the reaction. The unidentified lipids reduced from 2.6 to 1.9%. The low levels of FFA achieved compare favorably with levels achieved in fish oil re-esterification and also when lipases have been applied to reduce fish oil acidity [25,42].

Table 3. Changes of lipid classes with reaction time under optimized conditions.

Hours	% Lipid Classes (with Standard Deviation Values)				
	FFA	**TAG**	**DAG**	**MAG**	**Other Lipid Classes**
0	40.3 ± 2.9	41.6 ± 2.2	10.1 ± 1.1	5.5 ± 0.8	2.6 ± 0.4
2	16.4 ± 2.1	33.0 ± 2.6	34.3 ± 2.2	14.7 ± 1.8	1.7 ± 0.3
4	8.4 ± 0.9	25.2 ± 2.0	42.6 ± 2.0	22.2 ± 1.8	1.7 ± 0.3
6	5.4 ± 0.5	23.0 ± 1.6	45.9 ± 2.2	24.0 ± 1.1	1.7 ± 0.2
8	4.0 ± 0.4	22.3 ± 2.2	47.1 ± 2.2	24.8 ± 2.0	1.8 ± 0.3
10	3.1 ± 0.3	22.1 ± 1.2	47.2 ± 1.7	24.9 ± 2.1	1.7 ± 0.2
24	1.8 ± 0.1	21.5 ± 2.1	48.8 ± 2.5	25.9 ± 1.7	1.9 ± 0.3

3.3.2. Fatty Acid Compositions

Fatty acid analysis was carried out using GC-FAMES (Table 4), which showed the squid oil had 45.8% PUFA, 26.4% MUFA and 25.0% SFA. These were unchained from the starting material within experimental error and consistent with literature fatty acid compositions for squid oil [43]. Individual fatty acids were essentially unchanged after glycerolysis. These results compare favorably with fish oil re-esterification, particularly chemical methods that result in some polymerization of PUFA [26].

Table 4. Fatty acid compositions of the squid oil before and after esterification.

Fatty Acid Components	% Fatty Acids (Results Are Mean Values of Three Replicates)	
	Crude Squid Oil Input	Esterified Squid Oil Using the Optimized Condition for 24 h
Myristic acid, C14:0	4.77	4.82
Tetradecanoic acid, C14:1n5c	0.52	0.53
Pentadecylic acid, C15:0	0.49	0.47
Palmitic acid, C16:0	13.44	12.74
Palmitoleic acid, C16:1n7c	5.42	5.58
Stearic acid, C18:0	1.72	1.61
Oleic acid, C18:1n9c	16.48	16.90
Linoleic acid (LA), C18:2n6c	2.43	2.49
α-Linoleic acid (ALA), C18:3n3	1.74	1.74
Arachidic acid, C20:0	4.57	4.66
Eicosenic acid, C20:1n9	4.02	4.16
Arachidonic acid, C20:4n6	1.30	1.34
Eicosapentaenoic acid, (EPA) C20:5n3	20.13 ± 1.76	20.48 ± 1.13
Docosahexaenoic acid, (DHA) C22:6n3	20.17 ± 1.83	20.30 ± 1.95
Saturated Fatty Acids (SFA)	24.99 ± 1.41	24.30 ± 1.16
MUFA	26.44 ± 1.92	27.17 ± 1.70
PUFA	45.77 ± 1.68	46.35 ± 2.50

3.3.3. Astaxanthin Content after Glycerolysis

Astaxanthin content was compared for the crude input squid oil and product prepared under optimized conditions, with fresh and recovered glycerol (Figure 4A). After a reaction time of 24 h the astaxanthin content was reduced by approximately 50% showing some loss of this oxidatively sensitive carotenoid. To further investigate this partial degradation, we analyzed the change in astaxanthin content over time using the optimized conditions set (Figure 4B). Significantly, within the first 10 h, there was only a 12% reduction in astaxanthin, while the glycerolysis reaction was mostly complete. High astaxanthin degradation is observed after 24- and 48-h reactions, with the change modelled by a 2^{nd}-order polynomial trendline with a 0.965 R2 value. Consequently, we recommend a reaction time of 10 h or less to achieve the optimum balance between neutralization and retention of EPA, DHA and astaxanthin. Oils containing EPA and DHA such as fish oil, algal oil and squid oil normally require added antioxidants for stabilization. The presence of astaxanthin, a natural antioxidant, appears to be important for stabilizing the oil and so its retention is not only important for potential health benefits, but also for oil stability.

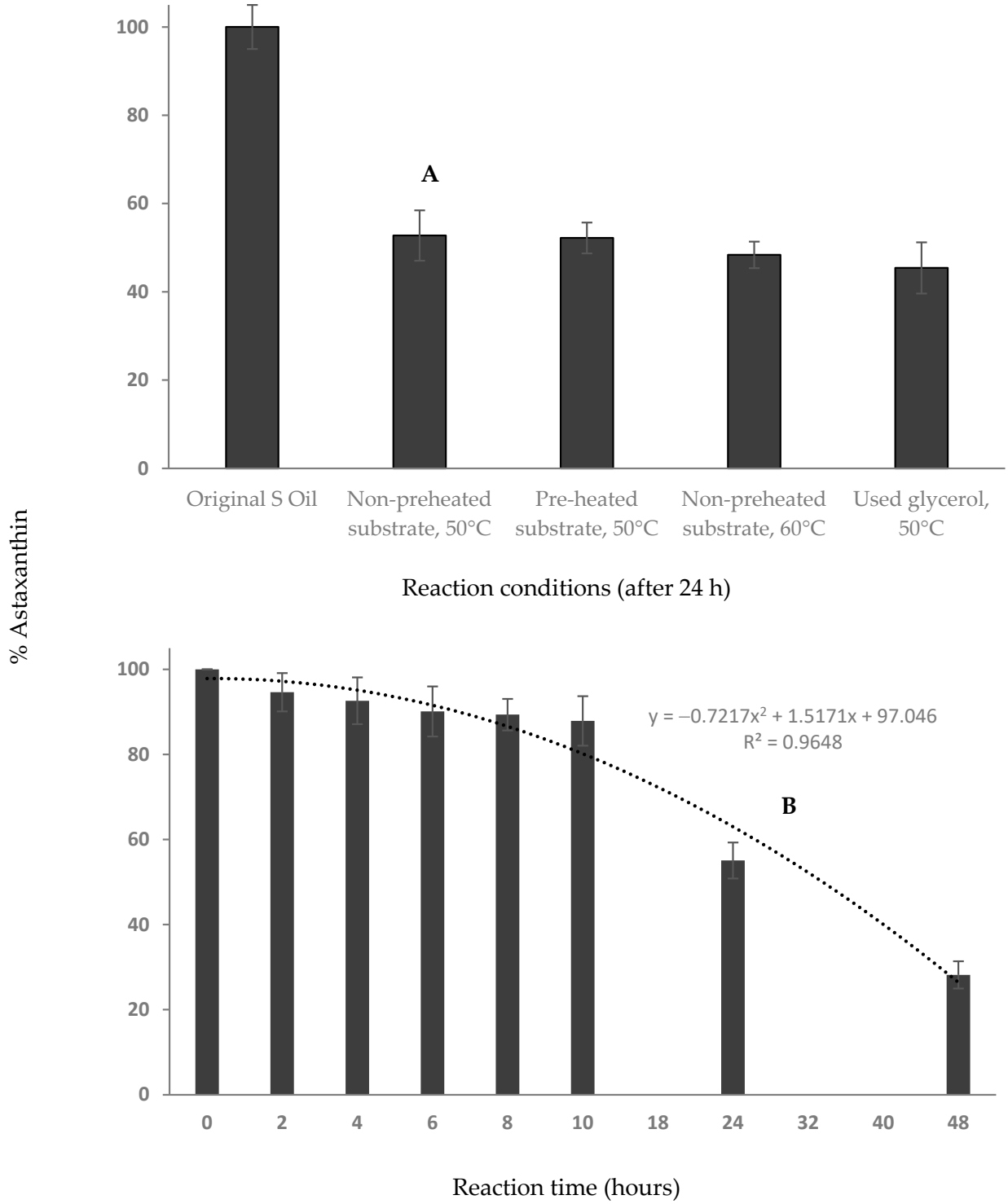

Figure 4. Effect of (**A**) different reaction conditions (**B**) reaction time (under optimized conditions) on the astaxanthin content of squid oil during esterification process.

3.3.4. Acid Value

The acid value of an oil is used commercially as a measure of free fatty acid levels [42,44]. Consistent with the lipid class data obtained from TLC-FID, acid values of the squid oil decreased as the reaction progressed (Figure 5), from an initial acid value of 79.62 mg KOH/g oil to 6.78 mg KOH/g oil after 10 h and 3.54 mg KOH/g oil after 24 h.

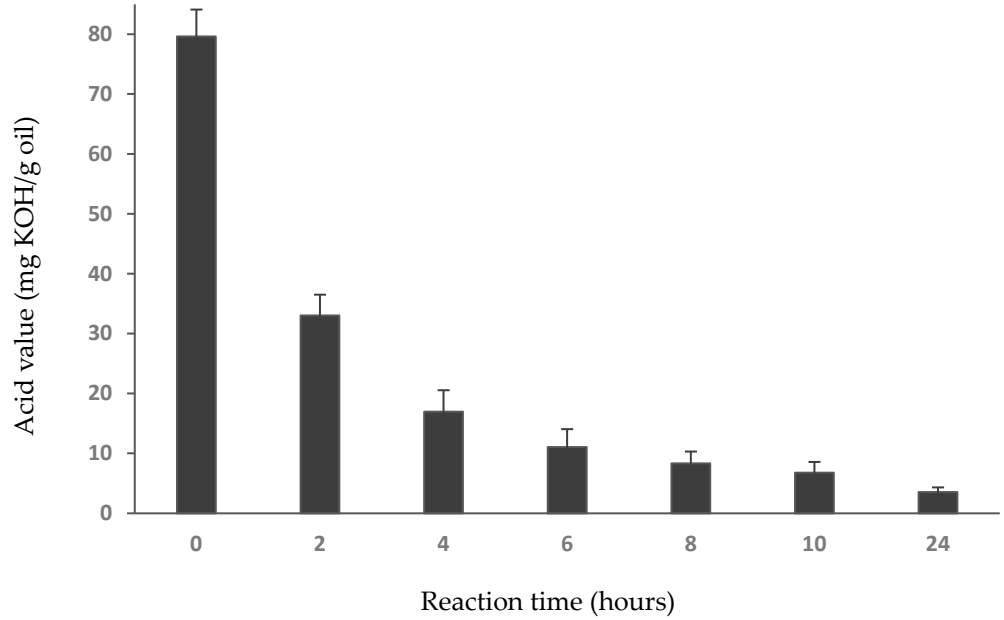

Figure 5. Decrease of Acid Value of squid oil with increasing esterification time.

4. Conclusions

A custom-built one-liter reactor was used to optimize the lipase-catalyzed neutralization of a marine oil rich in the omega-3 fatty acids EPA and DHA derived from squid waste to produce a nutritional oil. Optimized conditions include using 1% m/v lipozyme RMIM, and 1% m/v molecular sieves, commencing with a 3:1 oil-to-glycerol ratio and adding additional glycerol at 2, 4 and 6 h. The reaction proceeded faster at 50 °C, when reactants were pre-heated separately and the stirring speed was 400 rpm for the first 2 h, before being increased to 600 rpm. Under the optimized conditions, greater than 93% of the free fatty acids were converted to acylglycerol in 10 h and 96% in 24 h, with a corresponding decrease in acid values. Dietary significant PUFAs including EPA and DHA were unchanged after the reaction, at 46.5 $\pm$ 1.0%. Astaxanthin was mostly retained after 10 h of reaction (88% retained) but decreased significantly after 24 h reaction (50%). This study shows that high FFA-containing oils, particularly squid viscera oil, can be effectively converted to high acylglycerol oils using an immobilized lipase reactor system, with retention of EPA, DHA and astaxanthin.

Author Contributions: M.A.H., B.J.H. and C.J.B. designed the experiments and confirmed the experimental protocols. C.J.B., T.O.A. and M.S. designed and built the lab-scale reactor. M.A.H., T.O.A. and B.J.H. conducted the laboratory experiments. M.A.H. and B.J.H. prepared the draft of the manuscript. All co-authors contributed by editing and checking. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original data presented in the study are included in the article; further inquiries can be directed to the corresponding author.

Conflicts of Interest: Author Moninder Sachar was employed by Mantzaris Fisheries. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Towards Sustainable Sources of Omega-3 Long-Chain Polyunsaturated Fatty Acids in Northern Australian Tropical Crossbred Beef Steers through Single Nucleotide Polymorphisms in Lipogenic Genes for Meat Eating Quality

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Abstract: This study aimed to identify single nucleotide polymorphisms (SNP) in lipogenic genes of northern Australian tropically adapted crossbred beef cattle and to evaluate associations with healthy lipid traits of the *Longissimus dorsi* (loin eye) muscle. The hypothesis tested was that there are significant associations between SNP loci encoding for the fatty acid binding protein 4 (FABP4), stearoyl-CoA desaturase (SCD) and fatty acid synthase (FASN) genes and human health beneficial omega-3 long-chain polyunsaturated fatty acids (ω3 LC-PUFA) within the loin eye muscle of northern Australian crossbred beef cattle. Brahman, Charbray, and Droughtmaster crossbred steers were fed on Rhodes grass hay augmented with desmanthus, lucerne, or both, for 140 days and the loin eye muscle sampled for intramuscular fat (IMF), fat melting point (FMP), and fatty acid composition. Polymorphisms in FABP4, SCD, and FASN genes with significant effects on lipid traits were identified with next-generation sequencing. The GG genotype at the FABP4 g.44677239C>G locus was associated with higher proportion of linoleic acid than the CC and CG genotypes ($p < 0.05$). Multiple comparisons of genotypes at the SCD g.21266629G>T locus indicated that the TT genotype had significantly higher eicosapentaenoic, docosapentaenoic, and docosahexaenoic acids than GG genotype ($p < 0.05$). Significant correlations ($p < 0.05$) between FASN SNP and IMF, saturated and monounsaturated fatty acids were observed. These results provide insights into the contribution of lipogenic genes to intramuscular fat deposition and SNP marker-assisted selection for improvement of meat-eating quality, with emphasis on alternate and sustainable sources of ω3 LC-PUFA, in northern Australian tropical crossbred beef cattle, hence an acceptance of the tested hypothesis.

Keywords: meat fatty acids; omega-3 fatty acids; intramuscular fat; next-generation sequencing; tropical beef cattle; marker-assisted selection

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1. Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), sustainable diets are protective and respectful of biodiversity and ecosystems, nutritionally

adequate, healthy, safe, accessible, culturally acceptable, economically fair, and affordable [1]. The fatty acid composition and intramuscular fat (IMF) content of beef contribute to sustainability due to their significant influences on shelf life [2], eating quality [3], and human health [4]. Studies suggest that dietary supplementation [5,6], nutritional alteration [7], and selective breeding [8] are management tools for manipulating meat fatty acid composition and beef quality. The nutritional composition of the diet is known to influence meat fatty acid composition and has been a subject of many literature reviews [9–15]. However, dietary manipulation of meat quality and fatty acid profile is challenging in ruminants due to rumen microbial lipolysis [16,17] and biohydrogenation of unsaturated to saturated fatty acids [18]. Muscle fatty acid composition is less diet-dependent and more largely regulated by key lipogenic enzymes in fatty acid metabolism [19,20].

Genetic selection and breeding of beef cattle provide a long-term, cumulative, and permanent approach to improving meat fatty acid composition because of their moderate to high heritability [21–26]. Heritability estimates of 0.47 was reported for total polyunsaturated fatty acids in Japanese Black cattle [27]. The identification of single nucleotide polymorphisms (SNP) in genes encoding key enzymes and proteins involved in fatty acids metabolism may improve the current fundamental understanding of underpinning genetic variants controlling muscle fatty acid composition. Several studies have shown that SNP can be used as genetic markers for improving IMF and muscle fatty acid composition in ruminant livestock. For instance, associations were reported between the growth hormone g.253 locus SNP with C14:0, C16:1, and C18:0 concentrations in Japanese Black cattle [28]; multiple autosomal SNP loci with C14:0, C16:0, and C18:0 concentrations in Nellore bulls [29]; the diacylglycerol O-acyltransferase 1 gene SNP K232A and c.947 of μ-calpain gene with IMF in meat of five beef cattle breeds in Sweden [30]; and multiple SNP in the hormone-sensitive lipase gene with IMF of the Qinchuan and Nanyang cattle [31]. Furthermore, the stearoyl-CoA desaturase (SCD) g.23881050T>C locus was significantly associated with IMF, C22:6ω-3, and C22:5ω-3, fatty acid binding protein 4 (FABP4) g.62829478A>T locus with IMF and fatty acid synthase (FASN) g.12323864A>G locus with C18:3ω-3, C18:1ω-9, C18:0, and C16:0 concentrations in Tattykeel Australian White lamb [32].

Three known candidate genes were selected for a targeted next-generation sequencing (NGS) of SNP in this study based on current knowledge of allelic substitutions encoding the FASN, SCD, and FABP4 genes. The FASN is a complex homodimeric enzyme that regulates biosynthesis of long-chain FA, and has been reported to be associated with fatty acid composition in Korean [33], crossbred Jersey and Limousin [34], Japanese Black and Limousin crossbred [20,35,36], and Angus [20] cattle. Moreover, SNP in the gene encoding stearoyl-CoA desaturase (SCD), a rate-limiting enzyme that catalyses monounsaturated fatty acid (MUFA) synthesis, is reported to influence fat melting point (FMP), SFA, MUFA, and polyunsaturated fatty acid (PUFA) composition in beef [37–40]. The fatty acid binding protein 4 (FABP4) functions include fatty acid uptake, transport, and metabolism [41], and the influence of FABP4 genotypes on fatty acid composition is documented [42]. For instance, the GG genotype of the c.388G>A, c.408G>C, and c.456A>G SNP had higher MUFA composition in Korean cattle compared to the other genotypes [43].

Meat quality measurements are often attained after slaughter making it difficult to predict meat quality in living animals [44,45]. Pewan et al. (2021) demonstrated that a combination of laboratory-based IMF, FMP, and fatty acid analyses of samples obtained through a minimally invasive biopsy sampling and next-generation sequencing of polymorphisms in lipid metabolism genes is a suitable method to directly quantify the genetic worth of live animals for IMF and fatty acid composition. Therefore, this study aimed to identify targeted SNP in lipid metabolism related genes of tropically adapted crossbred beef cattle of northern Australia and determine associations with loin eye muscle fat characteristics.

2. Materials and Methods

All the study protocols followed the Australian code of practice for the care and use of animals for scientific purposes [46] and were approved by the Commonwealth Scientific and Industrial Research Organisation Animal Ethics Committee (Approval Number 2019-38).

2.1. Animals, Diets and Experimental Design

Sample size determination, animal management, diet compositions, and experimental design were previously described [47,48], and will not be repeated herein. In summary, 48 Charbray, Brahman, and Droughtmaster crossbred steers (28–33 months old steers with an initial average liveweight of 332 $\pm$ 21 kg) were fed on isonitrogenous diets of Rhodes grass hay augmented with either desmanthus, lucerne, or both for 140 days in a completely randomised design. Steers were group-housed in 12 open outdoor pens and had unlimited access to clean water and mineral blocks with a five to ten per cent allowance for daily feed refusal. At the end of the study, steers were divided into two groups based on liveweight. The heavier steers (453 $\pm$ 15 kg) were transported to a commercial abattoir and slaughtered without feedlot finishing, while the lighter steers (406 $\pm$ 25 kg) were transferred to a commercial feedlot for finishing.

2.2. Loin Eye Muscle Sampling and Chemical Analysis

A minimally invasive biopsy technique was used to collect loin eye muscle samples from the 12th–13th rib interface of the steers transported to the feedlot after forage-feeding phase according to the protocol described earlier [49]. Samples from the steers slaughtered immediately after the forage-feeding phase were collected from the 12th–13th rib interface of the chilled carcasses 12 h after slaughter. The IMF of the biopsy and carcass samples was extracted as described by Flakemore et al., (2014) [50], and FMP was determined with the slip-point method [51]. The fatty acid composition was evaluated using a gas chromatography-mass spectrometry procedure [52].

2.3. Blood Sampling and Genomic DNA Extraction

Blood samples were collected into 10 mL EDTA-containing vacutainer tubes (BD, Sydney, Australia) via jugular venipuncture, transported in dry ice and stored at -80 °C until needed for laboratory analysis. Blood samples were thawed at room temperature and genomic DNA was extracted from a 2 mL aliquot using the NucleoSpin Blood Kit (Macherey-Nagel GmbH and Co. KG, Duren, Germany) according to the manufacturer's instructions. DNA yield and purity were determined with NanoDrop ND-1000 (Thermo Fisher Scientific Australia Pty Ltd., Scoresby, VIC, Australia).

2.4. Primer Design, Amplification of Target Genes, Clean-Up of PCR Products, Library Preparation, Sequencing and Data Analysis

The procedures were carried out as described previously [32] with slight modifications on the gene amplification conditions. The target genes were amplified using the primer sequences presented in Table S1 and the gel image of the amplification products is presented in Supplementary Figure S1. The amplification reactions were executed in a SimpliAmp Thermal Cycler (Thermofisher Scientific, Scoresby, VIC, Australia) in a total volume of 50 µL consisting of 25 µL of PCR master mix, 100 ng of DNA template, and 0.5 µM of each primer in a 3-step procedure: single initial denaturation at 98 °C for 1 min, 35 cycles of denaturation, annealing and extension at 98 °C for 15 s, 60 °C for 15 s, and 72 °C for 9 min, respectively, followed by a final extension at 72 °C for 9 min and a 4 °C hold. The FASN gene was amplified with PrimeSTAR GXL Master Mix (TaKaRa Bio Inc., Kusatsu, Shiga, Japan) in a 2-step protocol. The amplification reaction mix consisted of 1.25 units of polymerase, 10 µL of 5 $\times$ buffer, 0.2 µM of each primer, 200 µM of dNTP mixture, and 100 ng of DNA template in a total volume of 50 µL. The amplification reaction conditions included initial denaturation for 1 min at 98 °C and 30 cycles of denaturation and annealing combined with extension at 98 °C for 10 s and 68 °C for 10 min, respectively. The Hereford cattle breed

sequences NC_ 037353.1, NC_ 037346.1, and NC_ 037341.1 obtained from the GenBank database were used as the SCD, FASN, and FABP4 reference sequences, respectively.

2.5. Calculations and Statistical Analysis

Data analyses and the plotting of figures were conducted with the R software v.4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). The GDIcall online calculator (http://www.msrcall.com/Gdicall.aspx (accessed on 14 January 2022)) was used to calculate SNP polymorphism information content (PIC). Hardy-Weinberg equilibrium (HWE) and expected heterozygosity (He) were calculated according to the methods described by Nei and Roychoudhury (1974) [53]. The HWE was tested for each identified SNP locus with the Chi-square test. Summary statistics including range, means, and standard deviations were computed and checked for data entry errors and outliers. The degree of linkage disequilibrium between each pair of loci was examined with distance-based hierarchical clustering of SNP loci [54] and the results presented as dendrograms and heatmaps. Linear correlations between genomic variants and muscle IMF, FMP, and fatty acid composition were estimated with Spearman's ρ correlations. Generalised least square procedure was used to fit linear models to investigate SNP associations with the loin eye muscle IMF, FMP, and fatty acid composition. Differences between means were compared using the Tukey-adjusted multiple comparisons test with a threshold for significance set at $p < 0.05$.

3. Results

3.1. Genetic Diversity of the Identified Single Nucleotide Polymorphisms

In total, 88 SNP, comprising 16, 42 and 30 SNP for FABP4, SCD, and FASN genes respectively, were identified (Supplementary Table S2). Thirty-five of the 88 SNP were not found in the Bovine Genome Variation Database (BGVD) (http://animal.nwsuaf.edu.cn/code/index.php/BosVar, (accessed on 28 January 2022)), and were deemed novel. All SNP had 0.11–0.50 minor allele frequency, 0.20–0.50 He, and 0.18–0.38 PIC. All the SNP were in HWE except FASN g.50784824G>A (rs209227647), g.50785253C>T (novel), g.50786977A>G (novel), g.50788575T>C (rs41919993), and g.50790973C>A (rs109149276) ($p \leq 0.04$). Many of the identified SNP were located in the introns. The distance-based hierarchical clustering of SNP loci indicated the presence of linkage disequilibrium between SNP loci (Figures S1–S4). The FABP4 SNP loci formed three clusters but g.44677611G>C (rs41729172) was not in linkage disequilibrium with other FABP4 loci (Supplementary Figure S1). A similar trend was observed for the SCD gene SNP loci (Supplementary Figure S2) but not for the FASN gene. All the FASN SNP loci were in linkage disequilibrium with at least one other locus (Supplementary Figure S3). Only nine SNP were non-synonymous amino acid substitutions (Table 1).

Table 1. Single nucleotide polymorphisms of the FABP4, SCD, and FASN genes, protein coding sequence positions and non-synonymous amino acid substitutions.

Gene [1]	SNP (Variant ID) [2]	PCS Position [4]	Amino Acid Substitution
FABP4	g.44677959 T>C (rs110757796)	220	Isoleucine to Valine
SCD	g.21272422 C>T (rs41255693)	878	Alanine to Valine
FASN	g.50782773 G>A (rs715140536)	1243	Alanine to Threonine
	g.50784533 C>G (rs481622676)	2066	Alanine to Glycine
	g.50784824 G>A (rs209227647)	2252	Arginine to Histidine
	g.50786496A>G [3]	3145	Serine to Glycine
	g.50788575T>C (rs41919993)	4168	Tyrosine to Histidine
	g.50789448C>T (rs516607144)	4693	Leucine to Phenylalanine
	g.50790973C>A (rs109149276)	5572	Leucine to Isoleucine

[1] FABP4: Fatty acid binding protein 4, SCD: Stearoyl-CoA desaturase, FASN: Fatty acid synthase. [2] SNP: Single nucleotide polymorphism. Variant dbSNP ID are based on the Bovine Genome Variation Database (BGVD). [3] SNP not listed in BGVD. [4] PCS: Protein coding sequence.

3.2. Correlations between Single Nucleotide Polymorphisms, Intramuscular Fat, Fat Melting Point, and Fatty Acid Composition

The clustering patterns of the SNP loci among steers with regard to the FABP4, SCD, and FASN genes are presented in Figures 1A, 2A and 3A, respectively. There was less variability in heterozygosity and homozygosity of closely related individuals for the FASN gene SNP compared to the FABP4 and SCD genes. Most SNP were in linkage disequilibrium but a few SNP depicted no linkage (Figures 1B, 2B and 3B). Four FABP4 gene SNP–g.44677205A>G (rs109388335), g.44677239C>G (rs110383592), g.44678114G>C (novel), and g.44678641T>C (rs110490217) were positively correlated with linoleic acid (C18:2n6). One SNP (g.44680048A>G; rs468994137) on the other hand, was negatively correlated with linoleic acid concentration, while g.44677587G>C (rs723716479) was positively correlated with conjugated linoleic acid (CLA) (Figure 1B; $p < 0.05$). Fourteen SCD SNP were negatively correlated, while g.21266629G>T (novel) and g.21271645G>A (rs380628677) were positively correlated with eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), docosahexaenoic acid (DHA) (Figure 1B; $p < 0.05$). No correlation was observed between FABP4 and SCD SNP with IMF, FMP, SFA, or MUFA. In contrast, FASN g.50784242C>T (rs800844468) SNP was positively correlated with IMF, palmitic acid (16:0), oleic acid (18:1), SFA, and MUFA (Figure 3B; $p < 0.05$). CLA concentration was negatively correlated with g.50787886A>G (novel), g.50788691T>C (rs526036338), and g.50788956C>T (novel) while g.50792445C>T (novel) was negatively correlated with EPA and the sum of EPA and DHA ($p < 0.05$). Several fatty acids were positively correlated ($p < 0.05$), and no significant negative correlations between quantified fatty acids were observed. Highly positive correlations ($\geq$0.7) between palmitic acid, palmitoleic acid, stearic acid, and oleic acid with the CLA, SFA, and MUFA levels were observed ($p < 0.01$).

(**A**)

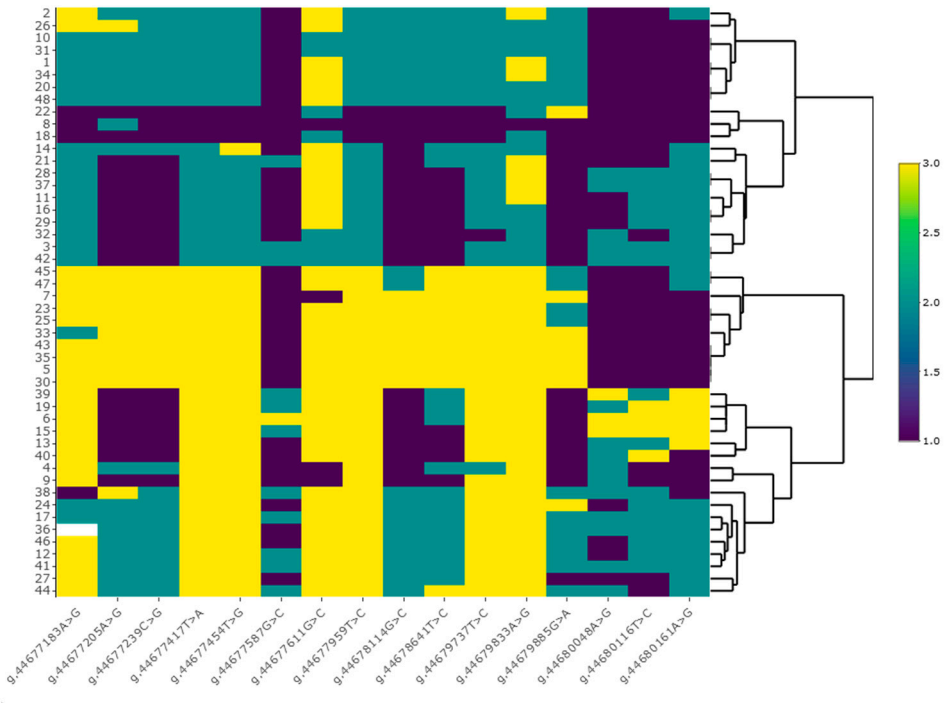

Figure 1. *Cont.*

(**B**)

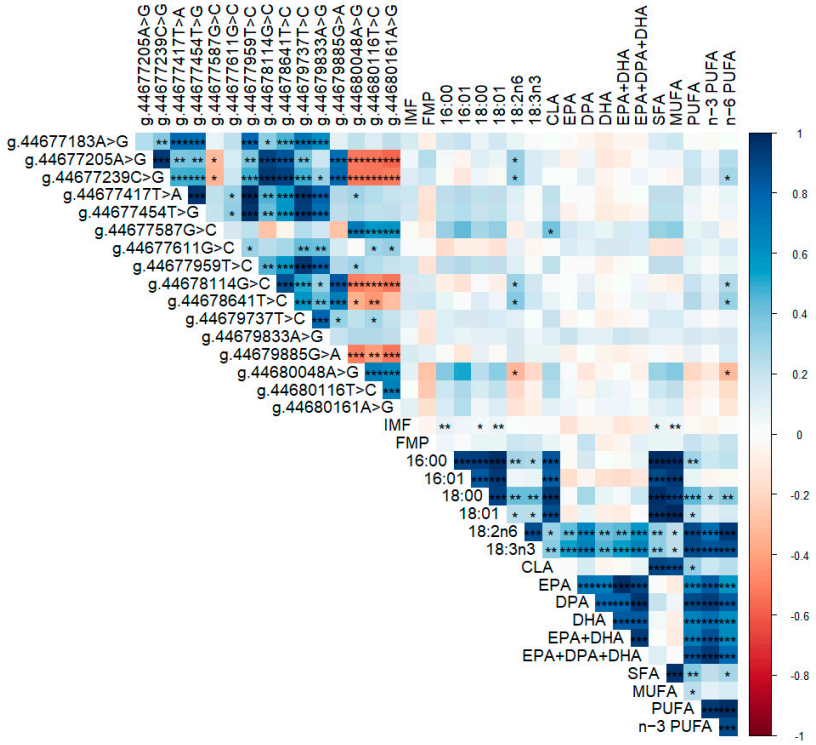

Figure 1. Single nucleotide polymorphisms in the FABP4 gene. (**A**) Clustering map of genetic variants; homozygotes similar to the reference sequence genotype (Hereford), heterozygotes and alternative allele homozygotes. (**B**) Correlations between SNP and IMF, FMP and fatty acids. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

(**A**)

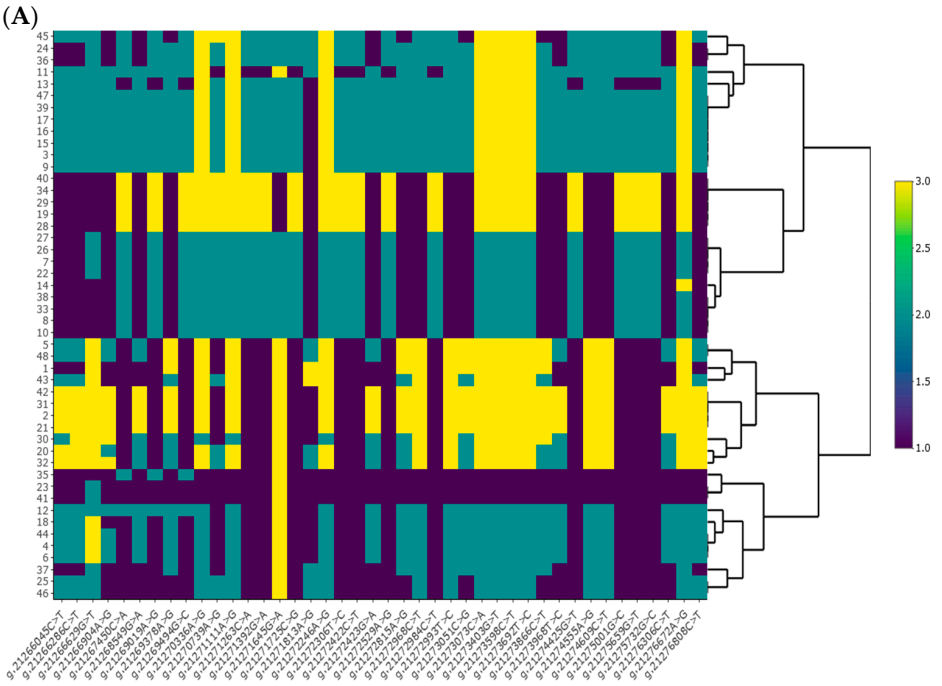

Figure 2. *Cont.*

(**B**)

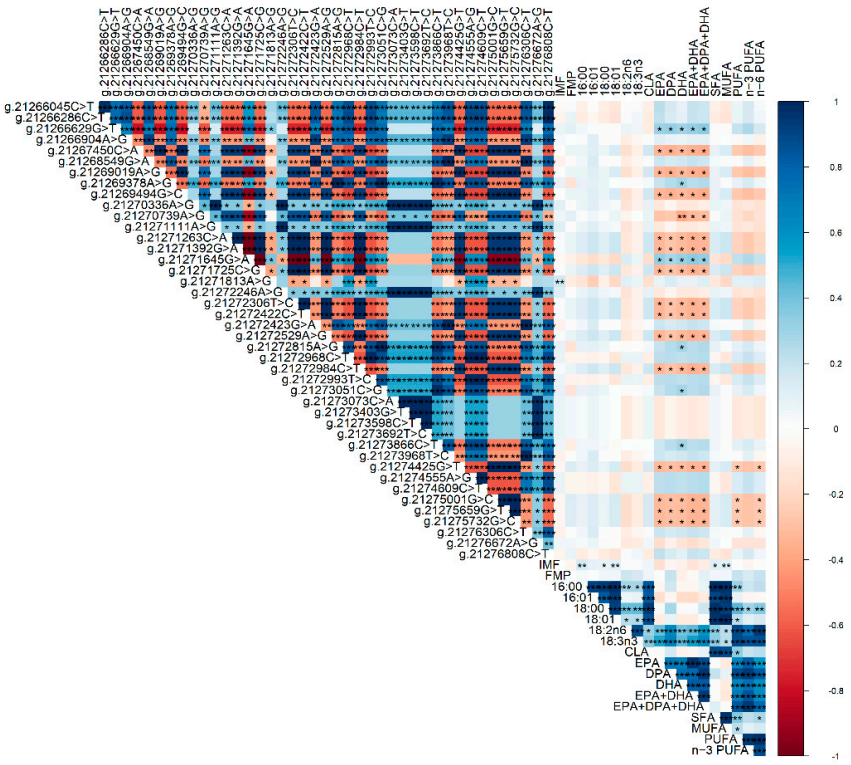

Figure 2. Single nucleotide polymorphisms in the SCD gene. (**A**) Clustering map of genetic variants; homozygotes similar to the reference sequence genotype (Hereford), heterozygotes and alternative allele homozygotes. (**B**) Correlations between SNP and IMF, FMP and fatty acids. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

(**A**)

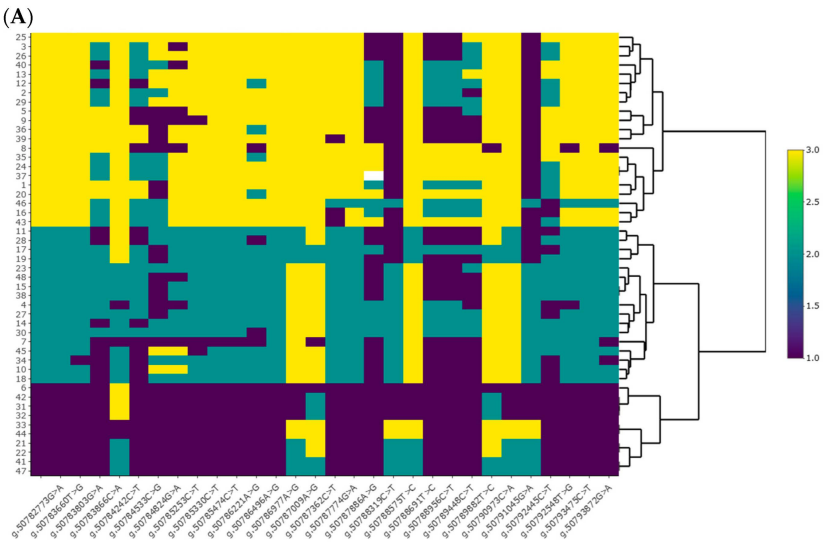

Figure 3. *Cont*.

(**B**)

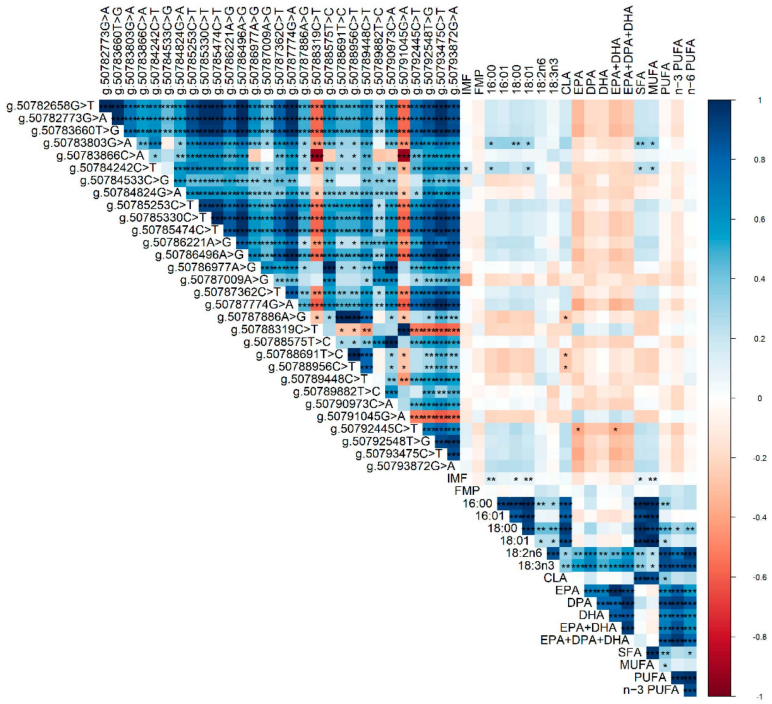

Figure 3. Single nucleotide polymorphisms on the FASN gene. (**A**) Clustering map of genetic variants; homozygotes similar to the reference sequence genotype (Hereford), heterozygotes and alternative allele homozygotes. (**B**) Correlations between SNP and IMF, FMP and fatty acids. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3.3. Associations between Single Nucleotide Polymorphisms, Intramuscular Fat, Fat Melting Point, and Fatty Acid Composition

Associations between FABP4 g.44677239C>G (rs110383592), SCD g.21266629G>T (novel), and FASN g.50783803G>A (novel) are presented in Table 2. No significant associations were observed between the FASN g.50783803G>A with IMF, FMP, or any fatty acid examined. However, FABP4 g.44677239C>G was significantly associated with linoleic acid ($p = 0.03$). Linoleic acid was lower for the CC than the GG genotypes at 45.8 ± 10.88 mg/100 g and 54.5 ± 7.3 mg/100 g, respectively ($p = 0.02$), but CG was not significantly different from the homozygotes (Figure 4). Significant associations between the SCD g.21266629G>T SNP with DPA, DHA, EPA+DHA, and EPA+DPA+DHA were observed ($p \leq 0.02$). Multiple comparisons in Figure 5 indicate that EPA, DPA, and DHA were significantly higher for the TT compared to the GG genotypes ($p \leq 0.03$). The DHA level was lower ($p = 0.02$) while EPA and DPA tended to be lower for the TT compared to the GT variants ($p \leq 0.08$). No significant difference was observed for EPA, DPA, and DHA in GT compared to GG variants ($p \geq 0.47$). The IMF and FMP levels were not associated with either FABP4 g.44677239C>G, SCD g.21266629G>T, or FASN g.50783803G>A ($p \geq 0.38$).

Table 2. Least Square Means ± SD of loin eye muscle IMF (%), FMP (°C) and fatty acid concentrations (mg/100 g fresh muscle) by genotype at the FABP4 g.44677239C>G, SCD g.21266629G>T and FASN g.50783803G>A SNP loci.

Gene/SNP [1] **FABP4 g.44677239C>G**	**Total (*n* = 48)**	**CC (*n* = 19)**	**CG (*n* = 19)**	**GG (*n* = 10)**	***p*-Value** [2]
IMF	2.3 ± 0.75	2.1 ± 0.62	2.5 ± 0.9	2.2 ± 0.67	0.38
FMP	43.9 ± 4.79	42.7 ± 4.58	44.6 ± 4.83	44.9 ± 5.13	0.53
16:0 (Palmitic acid)	209.1 ± 149.68	194.3 ± 113.24	238.7 ± 185.99	179.5 ± 133.59	0.72

Table 2. *Cont.*

Gene/SNP [1] FABP4 g.44677239C>G	Total (*n* = 48)	CC (*n* = 19)	CG (*n* = 19)	GG (*n* = 10)	*p*-Value [2]
16:1 (Palmitoleic acid)	34.6 ± 34.72	40.5 ± 47.35	34.5 ± 25.6	23.3 ± 16.87	0.52
18:0 (Stearic acid)	128.6 ± 78.22	119.2 ± 66.31	139.4 ± 83.68	125.1 ± 92.35	0.61
18:1 (Oleic acid)	263.1 ± 195.31	244.6 ± 143.89	302.4 ± 245.96	221.4 ± 170.23	0.74
18:2ω6 (Linoleic acid)	50.1 ± 10.2	45.8 ± 10.88[a]	52.0 ± 9.62 [ab]	54.5 ± 7.3[b]	0.03
18:3ω3 (α-linolenic acid)	16.3 ± 3.14	15.7 ± 3.69	16.4 ± 2.85	16.9 ± 2.73	0.73
CLA	4.3 ± 3.33	4.2 ± 3.11	4.4 ± 3.49	4.1 ± 3.77	0.79
EPA	9.4 ± 2.18	9.2 ± 2.13	9.8 ± 2.31	9.1 ± 2.12	0.58
DPA	14.0 ± 3.37	13.0 ± 3.61	15.0 ± 2.64	13.9 ± 3.92	0.12
DHA	2.3 ± 0.8	2.3 ± 0.93	2.5 ± 0.69	2.2 ± 0.79	0.28
EPA+DHA	11.8 ± 2.77	11.5 ± 2.81	12.3 ± 2.76	11.4 ± 2.86	0.33
EPA+DPA+DHA	25.8 ± 5.79	24.6 ± 5.88	27.4 ± 5.23	25.3 ± 6.53	0.14
SFA	376.9 ± 254.18	351.5 ± 201.61	420.0 ± 298.96	340.8 ± 260.83	0.75
MUFA	313.4 ± 228.69	294.7 ± 173.9	358.2 ± 285.22	261.9 ± 199.01	0.69
PUFA	142.8 ± 26.51	134.2 ± 28.71	149.6 ± 23.23	146.3 ± 25.98	0.41
ω3 PUFA	48.2 ± 8.92	47.0 ± 10.06	49.9 ± 7.84	47.5 ± 8.96	0.55
ω6 PUFA	79.6 ± 16.18	73.0 ± 18.67	83.6 ± 13.12	84.5 ± 13.17	0.11
SCD g.21266629 G>T	**Total (*n* = 48)**	**GG (*n* = 11)**	**GT (*n* = 22)**	**TT (*n* = 15)**	
IMF	2.3 ± 0.75	2.2 ± 0.55	2.2 ± 0.62	2.5 ± 1.03	0.64
FMP	43.9 ± 4.79	42.9 ± 3.64	44.1 ± 6.17	44.3 ± 3.26	0.78
16:0 (Palmitic acid)	209.1 ± 149.68	182.2 ± 111.02	201.0 ± 116.16	240.2 ± 209.36	0.86
16:1 (Palmitoleic acid)	34.6 ± 34.72	27.4 ± 16.93	38.4 ± 44.8	34.2 ± 27.7	0.91
18:0 (Stearic acid)	128.6 ± 78.22	125.6 ± 76.49	122.6 ± 60.52	139.2 ± 102.52	0.82
18:1 (Oleic acid)	263.1 ± 195.31	241.6 ± 164.92	255.0 ± 152.87	290.1 ± 266.99	0.82
18:2ω6 (Linoleic acid)	50.1 ± 10.2	50.0 ± 5.35	48.0 ± 10.93	53.2 ± 11.52	0.66
18:3ω3 (α-linolenic acid)	16.3 ± 3.14	16.5 ± 2.66	15.5 ± 3.24	17.1 ± 3.28	0.49
CLA	4.3 ± 3.33	4.7 ± 4.46	4.3 ± 2.71	4.0 ± 3.4	0.57
EPA	9.4 ± 2.18	8.6 ± 1.61 [a]	9.2 ± 2.43 [ab]	10.3 ± 1.93 [b]	0.08
DPA	14.0 ± 3.37	12.9 ± 1.78 [a]	13.4 ± 3.75 [ab]	15.7 ± 3.2 [b]	0.03
DHA	2.3 ± 0.8	2.1 ± 0.51 [a]	2.2 ± 0.88 [a]	2.8 ± 0.74 [b]	0.02
EPA+DHA	11.8 ± 2.77	10.8 ± 1.89 [a]	11.4 ± 3.07 [ab]	13.1 ± 2.48 [b]	0.03
EPA+DPA+DHA	25.8 ± 5.79	23.7 ± 3.21 [a]	24.8 ± 6.31 [ab]	28.9 ± 5.51 [b]	0.02
SFA	376.9 ± 254.18	345.6 ± 210.17	360.6 ± 197.48	422.7 ± 348.72	0.89
MUFA	313.4 ± 228.69	287.2 ± 191.76	303.9 ± 181.66	345.7 ± 310.88	0.85
PUFA	142.8 ± 26.51	138.0 ± 15.98	137.8 ± 29.03	153.7 ± 26.98	0.31
ω3 PUFA	48.2 ± 8.92	45.5 ± 5.37	47.2 ± 10.49	51.8 ± 7.77	0.12
ω6 PUFA	79.6 ± 16.18	78.5 ± 7.52	75.9 ± 19.07	85.9 ± 15.07	0.40
FASN g.50783803G>A	**Total (*n* = 48)**	**GG (*n* = 20)**	**GA (*n* = 20)**	**AA (*n* = 8)**	
IMF	2.3 ± 0.75	2.2 ± 0.71	2.3 ± 0.60	2.5 ± 1.14	0.49
FMP	43.9 ± 4.79	44.8 ± 3.56	43.1 ± 6.01	43.8 ± 3.62	0.40
16:0 (Palmitic acid)	209.1 ± 149.68	161.3 ± 84.44	211.4 ± 131.91	323.2 ± 248.45	0.24
16:1 (Palmitoleic acid)	34.6 ± 34.72	26.0 ± 13.60	41.8 ± 46.84	45.3 ± 31.24	0.17
18:0 (Stearic acid)	128.6 ± 78.22	103.2 ± 38.01	129.8 ± 76.27	189.3 ± 123.57	0.28
18:1 (Oleic acid)	263.1 ± 195.31	196.9 ± 114.57	279.5 ± 185.13	389.2 ± 309.04	0.16
18:2ω6 (Linoleic acid)	50.1 ± 10.20	50.5 ± 10.29	48.9 ± 9.88	52.1 ± 11.72	0.95
18:3ω3 (α-linolenic acid)	16.3 ± 3.14	16.3 ± 3.00	16.1 ± 3.34	16.8 ± 3.32	0.87
CLA	4.3 ± 3.33	3.4 ± 1.70	4.8 ± 4.01	5.6 ± 4.49	0.35
EPA	9.4 ± 2.18	9.9 ± 2.25	9.0 ± 2.05	9.3 ± 2.40	0.52
DPA	14.0 ± 3.37	14.7 ± 2.78	13.1 ± 3.45	14.5 ± 4.40	0.53
DHA	2.3 ± 0.80	2.4 ± 0.78	2.3 ± 0.8	2.1 ± 0.91	0.48

Table 2. *Cont.*

Gene/SNP [1] FABP4 g.44677239C>G	Total (n = 48)	CC (n = 19)	CG (n = 19)	GG (n = 10)	p-Value [2]
EPA+DHA	11.8 ± 2.77	12.3 ± 2.89	11.4 ± 2.5	11.4 ± 3.25	0.44
EPA+DPA+DHA	25.8 ± 5.79	27.1 ± 5.48	24.6 ± 5.34	26.0 ± 7.57	0.48
SFA	376.9 ± 254.18	294.0 ± 135.9	381.0 ± 229.59	574.6 ± 417.76	0.28
MUFA	313.4 ± 228.69	235.3 ± 132.97	332.1 ± 217.83	464.1 ± 359.61	0.20
PUFA	142.8 ± 26.51	143.9 ± 22.70	139.2 ± 25.97	148.9 ± 37.51	0.90
ω3 PUFA	48.2 ± 8.92	48.9 ± 8.18	47.4 ± 9.19	48.9 ± 10.91	0.82
ω6 PUFA	79.6 ± 16.18	81.5 ± 14.45	76.8 ± 16.70	79.6 ± 19.88	0.89

[1] IMF, intramuscular fat; FMP, fat melting point; CLA, conjugated linoleic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. SFA is the sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, and 21:0; MUFA is the sum of 14:1,16:1ω13t, 16:1ω9, 16:1ω7, 16:1ω7t, 16:1ω5c, 17:1ω8, 18:1ω7t,18:1ω5, 18:1ω7, 18:1ω9, 18:1a, 18:1b, 18:1c, 19:1a, 19:1b, 20:1ω11, 20:1ω9, 20:1ω7, 20:1ω5, 22:1ω9, 22:1ω11, and 24:1ω9; PUFA is the sum of 18:4ω3, 18:3ω6, 18:2ω6, 18:3ω3, 20:2ω6, 20:3, 20:3ω6, 20:4ω3, 20:4ω6, 20:5ω3, 22:4ω6, 22:5ω3, 22:5ω6, and 22:6ω3; ω3 PUFA is the sum of 18:3ω3, 18:4ω3, 20:4ω3, 20:5ω3, 22:5ω3, and 22:6ω3; ω6 PUFA is the sum of 18:2ω6, 18:3ω6, 20:2ω6, 20:3ω6, 20:4ω6, 22:4ω6, and 22:5ω6. [ab] Means followed by different lowercase superscripts differ significantly. [2] ANOVA p-value.

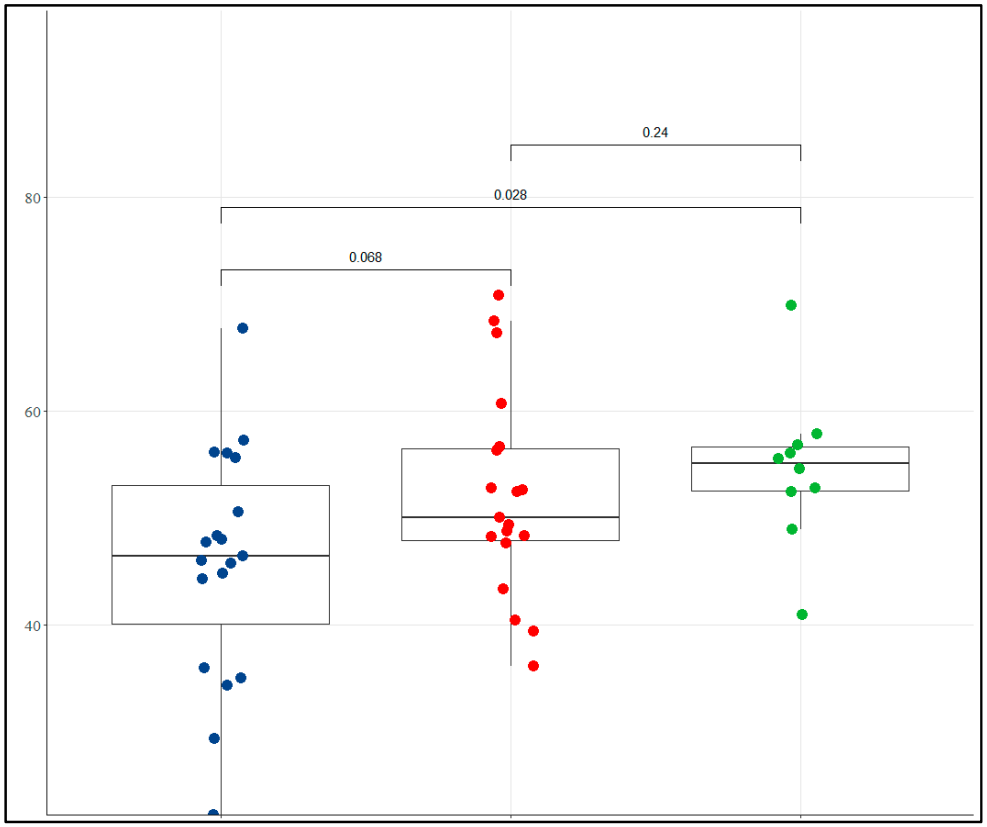

Figure 4. Multiple comparisons of loin eye muscle linoleic acid content between genotype variants at the FABP4 g.44677239C>G SNP locus CC (●), CG (●), and GG (●).

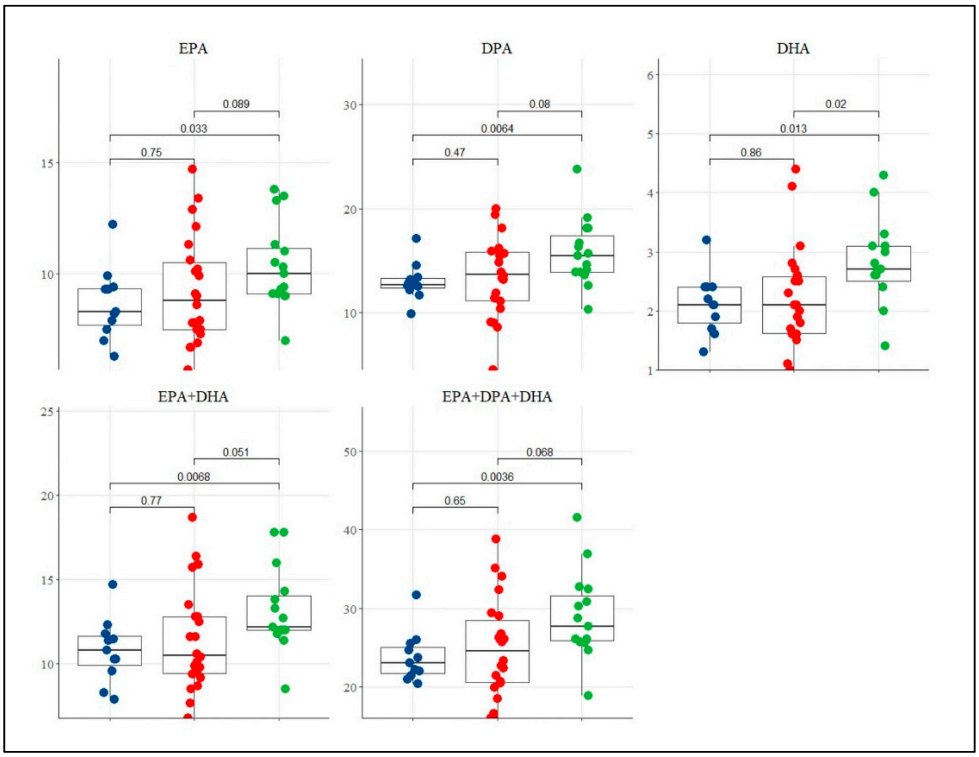

Figure 5. Multiple comparisons of loin eye muscle EPA, DPA, DHA, EPA+DHA, and EPA + DPA + DHA content between genotype variants at the SCD g.21266629G>T SNP locus GG (●), GT (●) and TT (●).

4. Discussion

Meat fatty acid composition influences meat shelf life, eating quality and consumers' health [55–57]. Although many studies have reported that diet modulation influences meat fatty acid composition, it is more difficult in ruminants compared to monogastric livestock due to microbial lipolysis [16,17] and biohydrogenation of unsaturated to saturated fatty acids in the rumen [18]. As a result, meat fatty acids are more saturated in ruminant than in monogastric animals [58,59]. On the other hand, studies have reported that fatty acid composition is heritable [21,27,60,61]. A recent study by Sakuma et al. [60] reported medium to high heritability estimates of 0.48 to 0.85 for six out of the eight fatty acids analysed. Therefore, there is an increased research interest in breeding, selecting and producing farm animals with desirable fatty acid composition [62].

Selection and breeding provide a long-term alternative to improving marbling level [63], and meat fatty acid composition [64,65]. Several SNP in genes encoding key enzymes and proteins involved in fatty acid metabolism have been reported as potential genetic markers for the improvement of IMF and fatty acid composition in different cattle breeds [33,35,41]. This study examined SNP in the FABP4, SCD, and FASN genes of northern Australian tropical crossbred beef cattle and identified SNP with significant influences on fatty acid composition of the loin eye muscle.

4.1. Fatty Acid Binding Protein 4 Gene Polymorphisms

The FABP4 gene is an important protein for long-chain fatty acid transport in mammals, and its polymorphism is associated with growth, fat deposition, and carcass traits in cattle [66–68]. Substitution of the G to C allele of the g.44677587 (rs723716479) locus was positively correlated with CLA, previously inversely linked with the risk of colorectal and breast cancer in some population-based studies [69]. The observed trend where the homozygous GG variant had the highest linoleic acid levels (almost 10 mg/100 g higher than homozygous CC in the g.44677239C>G loci) may indicate higher inflammatory eicosanoids synthesis. Linoleic acid is a building block in the synthesis of arachidonic acid,

the precursor for prostaglandins and other inflammatory eicosanoids [70]. In contrast to findings of this study, variation in the g.44677959T>C (c.220) influenced palmitoleic acid in Japanese Black cattle [71]. This discrepancy may be due to epistatic interaction of the g.44677959T>C locus with polymorphisms at another locus in line with the observations of Xu et al., (2021) [72] on the effect of polymorphisms on FABP4 protein structure. They reported that the wild type protein with isoleucine in amino acid 74 had 58.33% sheet and 29.55% loop interactions that changed to 59.09% sheet and 28.79% loop when isoleucine was substituted with valine. This discrepancy may also be due to breed differences since the Japanese Black cattle are reported to be genetically predisposed to producing carcass lipids with higher concentration of MUFA, including palmitoleic acid, compared to other cattle breeds such as Japanese Brown, Holstein or Charolais steers, likely due to the activity of the delta 9 desaturase enzyme on palmitic acid [73,74].

4.2. Stearoyl-CoA Desaturase Gene Polymorphisms

For most diets, approximately 70% to 95% and 85% to 100% ω6 PUFA and ω3 PUFA, respectively, are hydrogenated in the rumen [75]. As a result, fatty acids are absorbed almost entirely as SFA and biohydrogenation intermediates comprising conjugated di- or trienoic fatty acids and trans-11 fatty acids, notably trans-vaccenic acid, due to chemical reduction of unsaturated fatty acids in the rumen by microorganisms in ruminants [75,76]. Therefore, the composition of fatty acids stored in the fat depots mirror the action of SCD on fatty acids substrates [77]. The enzyme SCD catalyses the desaturation of SFA and MUFA by inserting a cis-double bond in the delta (Δ) 9 position of SFA substrates, with a higher preference for palmitic acid and stearic acid substrates transformed into palmitoleic acid and oleic acid, respectively [62,77,78]. Nucleotide substitution of C with T identified in the fifth exon of bovine SCD gene at the 878 CDS causes the replacement of the amino acid alanine with valine [37]. The replacement caused significantly higher MUFA and lower FMP in *M. trapezius* of CC compared to TT genotype cattle [37]. Similarly, Flekvieh bulls with the CC genotype had lower SFA and higher MUFA compared to the TT, but CC and the CT genotypes were similar [39]. The TT genotype of Chinese Simmental cattle were reported to have lower IMF compared to the CC genotype, but no difference was found between the heterozygous (CT) and either of the homozygous genotypes [79]. Additionally, the SNP had a significant association with stearic acid, oleic acid, SFA, and MUFA in Japanese black cattle with higher MUFA and lower SFA reported in animals with the CC variant [78]. In contrast, the SNP (g.21272422C>T) did not have significant effect on palmitic acid, stearic acid, palmitoleic acid, or oleic acid in the present study. However, findings of this study concur with a previous study that reported no effect of the SNP with palmitic acid, stearic acid, palmitoleic acid, or oleic acid in Canadian Angus and Charolais-based commercial crossbred beef steers [40]. Moreover, Dujková et al., (2015) [80] found that the SNP did not influence fatty acid composition in Aberdeen Angus and Blonde d'Aquitaine cattle. Unsaturated fatty acids are synthesized through the activity of Δ5, Δ6 or Δ9 desaturases [81], hence the difference between studies may be due to the activity of other desaturases or other genes [76,82]. The SCD genotype was reported to explain only 4% of the MUFA composition in Japanese Black cattle [37], and 5% in MUFA and 4% oleic acid variation, respectively, in Wagyu × Limousin cattle [38]. The significant correlations between EPA, DPA, and DHA with at least 16 SCD SNP observed in this study corroborate the findings of a previous study in sheep that recorded significant correlations between two SCD SNP and ω3 long-chain PUFA [32]. The three ω3 long-chain PUFA are synthesized from alpha-linolenic acid through the activity of Δ6 desaturase and Δ5 desaturase among other enzymes, but not Δ9 desaturase since ALA already has a double bond between C9 and C10 [83,84]. Therefore, the correlation may be due to linkage disequilibrium between the SCD SNP and other loci responsible for the synthesis of ω3 long-chain PUFA. Nonetheless, the significant correlations of SCD SNP with the EPA, DPA and DHA with no influence on the SFA and MUFA observed in this study suggests that the SNP can be used as markers to select cattle for improved health beneficial ω3 long-chain PUFA with no negative influence

on meat-eating quality denoted by the lack of correlation with oleic acid; the most abundant fatty acid in beef that is reported to improve fat softness and meat palatability [85].

Seafood sources including fish, crustaceans, and molluscs are recognized as the best dietary sources of long-chain ω3 oils [86]. However, sustainability of seafood as a source of ω3 LC-PUFA is threatened by the global decline in wild-harvest fish stocks [87], high cost of seafood [88], and low availability of seafood in many geographical locations [89]. On the other hand, beef contributes significantly to meat intake as it is the third most consumed meat in the world at 6.3 kg per capita [90]. Therefore, the significant correlations of SCD SNP with the EPA, DPA, and DHA suggests that marker assisted selection can be used to provide a sustainable source of dietary ω3 LC-PUFA in communities where beef constitutes a significant proportion of the diet.

4.3. Fatty Acid Synthase Gene Polymorphisms

The FASN gene is located in the BTA19 region where quantitative trait loci affecting milk fat content, meat fatty acid composition and related traits had been previously identified [36,91]. The enzyme FASN catalyses the de novo synthesis of palmitic acid, a substrate for palmitoleic acid synthesis through desaturation, and stearic acid through elongation [92–94]. Genome-wide association studies with varying breeds of cattle have reported significant effect of FASN SNP on intramuscular composition of SFA, MUFA, and linoleic acid [91,95–98]. Previous studies had reported that FASN polymorphism significantly influenced the intramuscular composition of oleic acid, SFA and MUFA in Fleckvieh bulls [41], and palmitic acid, palmitoleic acid, oleic acid, SFA, and MUFA of the intramuscular adipose tissue in Japanese Black cattle [36,82,99]. Zhang et al., (2008) [20] reported an additive effect of the g.17924A>G SNP on fatty acid composition, where the G allele was associated with higher MUFA and lower SFA compared to the A allele in purebred Angus bulls. The SNP also influenced palmitoleic acid and oleic acid composition in commercial crossbred beef steers [40], and palmitic acid, palmitoleic acid, oleic acid, total MUFA, SFA, and marbling score in Korean cattle [100–102]. In addition, FASN polymorphisms influenced SFA, MUFA, and PUFA in Chinese Holstein cattle [98]. Oh et al., (2012) reported associations of five FASN exonic SNP with intramuscular fatty acid composition in Korean cattle. These findings align with this current study where FASN g.50784242C>T was positively correlated with IMF, palmitic acid, oleic acid, SFA, and MUFA, while g.50783803G>A was correlated with palmitic acid, stearic acid, oleic acid, SFA and MUFA. Majority of the previous studies suggested that polymorphisms influenced the tissue fatty acid composition through amino acids substitutions on the b-ketoacyl reductase domain and the thioesterase domain by changing the spatial structure of the substrate-binding site [36,100,102]. However, the g.50784242C>T was a synonymous mutation, while g.50783803G>A was in the intron, thus they did not influence the production of missense codons, but may have exerted their effect by changing the splicing regulatory sequences [33]. The effect of g.50784242C>T on palmitic acid, oleic acid, SFA and MUFA may be due to the differences in IMF content. A review by De Smet et al. [103] reported a linear increase in SFA and MUFA expressed in mg/100g muscle (r = 0.98) with IMF content.

Tissue CLA is primarily derived from endogenous synthesis from *trans-11* C18:1 (vaccenic acid) by the SCD activity [104], and to a lesser extent, as an intermediate of microbial fatty acid biohydrogenation in the rumen [105,106]. In this study, SNP of the g.50787886A>G, g.50788691T>C, and g.50788956C>T loci were found to be correlated with high CLA levels. Although these polymorphisms were either synonymous or located in the intron, they may have influenced the function of FASN in palmitic acid synthesis, the substrate for *trans-11* C18:1 and subsequently CLA synthesis. These findings suggest that these loci may be used to select cattle with high CLA composition, a fatty acid associated with lower risk for atherosclerosis, diabetes and cancer [69,106]. Put together, these findings indicate that polymorphisms on the FASN gene can be used to select individuals for improved IMF and fatty acid composition of northern Australian tropical crossbred beef cattle.

5. Conclusions

This study aimed to investigate the targeted identification of SNP in the FABP4, SCD, and FASN genes and their associations with fatty acid composition in the loin eye muscle of northern Australian tropical crossbred beef cattle. Single nucleotide polymorphisms on the FABP4 gene significantly influenced linoleic acid, SCD was associated with long-chain n3 PUFA and FASN impacted IMF, SFA, MUFA, CLA, and EPA compositions. These findings not only provide insights into the genetic role of SNP in fat deposition and lipid metabolism in tropical crossbred cattle of northern Australia, but also their potential use in marker-assisted selection and breeding for improved meat-eating quality. The tested hypothesis of significant associations between SNP loci encoding for the fatty acid binding protein 4, stearoyl-CoA desaturase and fatty acid synthase genes and human health beneficial ω3 long-chain polyunsaturated fatty acids within the loin eye muscle of northern Australian crossbred beef cattle is therefore acceptable. This is the first study that demonstrates the presence of single nucleotide polymorphisms in lipogenic genes in northern Australian crossbred beef cattle which constitute over 50% of Australian beef production and exports.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su14148409/s1, Table S1: Primer sequences for target gene amplification; Figure S1: Gel image of the amplification products of the three target genes visualised in 0.8% agarose gel.; Table S2: Genetic variants detected in the FABP4, SCD and FASN genes of tropically adapted crossbred steers; Figure S2: Correlation coefficients for all pairs of SNP loci of the FABP4 gene. The rectangles represent distance-based clustering of SNP loci; Figure S3: Correlation coefficients for all pairs of SNP loci of the SCD gene. The rectangles represent distance-based clustering of SNP loci; Figure S4: Correlation coefficients for all pairs of SNP loci of the FASN gene. The rectangles represent distance-based clustering of SNP loci.

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Institutional Review Board Statement: The study was conducted in accordance with the CSIRO Animal Ethics Committee approved guidelines (approval number 2019-38, issued on the 20 February 2020) and the Australian code of practice for the care and use of animals for scientific purposes.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available from the corresponding author on request.

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The Minimum Dietary Level and Mix Ratio of Krill Meal and Fish Meal to Elicit Feed Intake and Growth Performance in Juvenile *Penaeus vannamei*

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Abstract: Shrimp feeds depend on high levels of digestible protein and essential amino acids, which can be sourced from various commercially available feed ingredients. Marine proteins can be used to partially fulfill the requirements of these and other important nutrients. Their utilization is further influenced by their palatability and growth-promoting effect. However, marine ingredients can significantly drive costs in feed formulation depending on the type and dietary inclusion level. This study aimed to determine the minimum dietary level of fish meal (FML) and krill meal (KRM) and their mix ratio to elicit feed intake and growth performance in juvenile *Penaeus vannamei*. Ten diets were formulated with graded FML (90, 60, 30 g kg^{-1}) in combination with 15, 30, and 45 g kg^{-1} KRM and a control diet with 120 g kg^{-1} FML. Shrimp (1.28 ± 0.08 g body weight, BW) were stocked in seventy 1-m^3 tanks (135 animals m^{-2}), and after 88 days, their growth performance was determined. Feed preference was assessed through two-by-two comparisons in twenty 0.5 m^3 tanks over four weeks. No significant differences in survival (93.9 ± 4.5%), gained yield (1235 ± 92 g m^{-2}), and feed conversion ratio (1.47 ± 0.09) were observed. Diets with 60 g kg^{-1} FML led to faster growth and higher feed intake than 30, 90, and 120 g kg^{-1} FML. Shrimp on 30 g kg^{-1} FML diets had the lowest BW, especially with 30–15 (FML-KRM) and 30–30 diets. Diets with 90 g kg^{-1} FML outperformed 30 g kg^{-1} FML. The control diet delivered a higher shrimp BW than diets 30–15 and 30–30, showing similar results to other diets except 60–15. Feed preference was influenced by KRM inclusion, with 15 g kg^{-1} KRM resulting in higher apparent feed intake than 30 and 45 g kg^{-1}. The findings indicate that FML can be effectively reduced by up to 75% when combined with lower levels of KRM. This corresponds with the industry's ongoing trend to achieve greater sustainability and cost efficiency through the reduced utilization of critical resources.

Keywords: krill meal; mix ratio; optimization; feed preference; growth enhancement; sustainable feed

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1. Introduction

Low-trophic-level aquaculture has been identified as an important part of the puzzle for future food security, providing essential nutrients to a growing population [1]. Aquaculture has also demonstrated its ability to boost local per capita consumption of aquatic food while presenting itself as more sustainable compared to the production of other farmed animals [2]. Global production of farm-raised whiteleg shrimp, *Penaeus vannamei*, has significantly increased in recent years, reaching 5.8 million MT in 2020 [1]. This has led to a temporary oversupply in the market, with falling prices in recent years making the industry less profitable. Nevertheless, recent reports suggest a more promising outlook for the coming years [3], with anticipated further growth driven by technological advancements, increased political attention, and growing consumer interest in the industry [1,3].

As the industry aims to adapt, production and economic efficiency are in focus. In addition, to allow for sustainable growth, there is an increased emphasis on reduced use of marine resources from fish stocks that are either overfished or fished to the limit [4]. Thus, feeds become a crucial element for cost rationalization and the optimization of ingredients for more sustainable industry growth, as they may represent over 40% of overall shrimp production costs. Practical feed formulation is conducted on a least-cost basis, allowing the meeting of targeted levels of essential nutrients required by farmed animals for maximum growth at the lowest possible economic cost [5]. Nevertheless, high feed costs and the overuse of some ingredients may arise from over-formulating with levels of essential nutrients beyond the animal's requirements and/or the inclusion of costly and/or less sustainable ingredients.

Shrimp feeds depend on high levels of digestible protein and essential amino acids (EAAs), which can be sourced from various commercially available feed ingredients. Marine proteins obtained from fish, squid, krill, and other crustaceans can be used to partially fulfill the requirements of these and other important nutrients. The utilization of marine proteins is further influenced by their ability to elicit chemoattraction and stimulate feeding in marine shrimp [6–10]. These factors collectively contribute to a growth-promoting effect in penaeid shrimp, sometimes correlated to the presence of unidentified growth factors [6,11–13]. However, marine ingredients can significantly drive costs in feed formulation depending on the type and dietary inclusion level. In addition, there is a significant risk that fish meal used in shrimp feeds originates from unsustainable fisheries [14,15]. Despite several studies supporting the reduction or complete removal of fish meal from shrimp feeds [16–23], this ingredient persists in usage in commercial feed formulations. The challenge arises from the difficulty of replicating the performance of marine ingredients in shrimp feeds, which demonstrates the importance of identifying their optimal combinations [16–20,22]. In these studies, krill meal has been included as a marine resource. Krill meal is a well-managed and sustainably fished resource [24], that has proven to be a highly effective ingredient in shrimp feeds [10,13,23].

Chemoattractants and palatability enhancers, collectively referred to as feeding effectors (meals and hydrolysates made from krill, squid, and fish), are added to shrimp feeds at levels ranging from 5 to 50 g kg^{-1} (g kg^{-1} of the diet on an as-is basis [8,10,12]). However, most published studies have evaluated their efficacy individually or in diets with low concentrations of marine ingredients [7–10]. This differs from practical feed formulas, which employ a combination of ingredients from various origins and chemical profiles [5], potentially resulting in antagonist or synergist effects on shrimp feeding responses. The modulation of behavioral olfactory responses, either through stimulation or suppression, has been documented in the Caribbean spiny lobster, *Panulirus argus*, using chemical binary mixtures, mostly synthetic amino acids [25–27]. These studies suggest that the physiological response arising from mixture interactions deviates from predictions based on individual responses to the components within the mixture, both in terms of quality and intensity [27]. Hence, it is crucial to investigate whether these interactions exist among protein ingredients used in practical shrimp feeds, particularly those derived from marine sources, to identify opportunities for reducing their usage for sustainability concerns and to enhance economic results. The present study aimed to determine the minimum dietary concentration of fish meal and krill meal, as well as their mix ratio, to stimulate feed intake and growth performance of juvenile *P. vannamei*.

2. Materials and Methods

2.1. Experimental Design

In this study, ten practical diets were formulated to contain graded levels of fish meal (FML) and krill meal (KRM). FML was included at 90, 60, and 30 g kg^{-1} (g kg^{-1} of the diet, as-is), each paired with 15, 30, and 45 g kg^{-1} KRM. This resulted in nine combinations of FML and KRM. A diet with 120 g kg^{-1} FML and no KRM was used as a control. Juvenile *P. vannamei* were stocked in outdoor tanks and fed four times daily in

feeding trays over a continuous 88-day rearing period. At harvest, shrimp were counted, weighed on an electronic scale, and their survival, growth performance, yield, and feed efficiency were determined. Subsequently, feed preference was assessed by measuring the shrimps' apparent feed intake. Diets with different levels of KRM within the same FML inclusion were confronted two-by-two in 30 tanks of 0.5 m^3 over 10 days.

2.2. Rearing System, Water Preparation, and Management

Shrimp were raised in 70 independent round tanks of 1.0 m^3 (h = 0.84 m, d = 1.06 m, bottom A = 0.89 m^2), allowing seven replicate tanks per dietary treatment. Outdoor tanks were sheltered under a roof with a 70% dark sunblock shade cloth to protect from a water temperature exceeding 30 °C. Tanks were fitted with a perforated lid on top to prevent shrimp from escaping. Each tank was also equipped with an individual water inlet and outlet, as well as an aeration system. The system operated under a minimum water exchange condition, without any water interexchange between rearing tanks over the complete rearing cycle. Weekly water exchange was carried out using sand-filtered seawater mixed with groundwater. Continuous aeration was provided by an air diffusing system made with 0.5 m aeration tubing (Aero-Tube™, Tekni-Plex Aeration, Austin, TX, USA), which rested near the bottom of each tank, but opposed to the feed delivery point. A 150-kvA (Kilo Volt Amperes or 120 kW) diesel generator was used as a backup power supply in case of power failure.

Rearing tanks, aeration equipment, and feeding trays were thoroughly cleaned and disinfected before preparing the water. High-pressure jet cleaning was used on the tank walls, followed by manual removal of any residues. Tanks were treated with a sulfuric acid-based descaling agent, left for 24 h, and then disinfected using peracetic acid. After drying, tanks were filled with water at 11 g L^{-1} salinity, achieved by mixing groundwater at 5 g L^{-1} salinity with previously disinfected seawater at 35 g L^{-1} salinity. Seawater was previously disinfected using sodium hypochlorite at 30 ppm. Once tanks were filled, culture water was prepared by fertilizing it with a commercial probiotic containing a blend of microorganisms (*Bacillus* spp., *Lactobacillus* spp., and *Saccharomyces cerevisiae*). This probiotic mix, along with sugar-cane molasses, wheat bran, and tap water, underwent fermentation with aeration for 24 h. After sieving to eliminate solids, this mixture was added to each tank at a rate of 50 g m^{-3} daily for a week. Strong aeration was maintained in rearing tanks throughout mixing during the water preparation process.

During shrimp rearing, water was exchanged on a weekly basis at 14% of total tank volume by draining bottom water and replacing it with clean brackish water. Water pH, temperature, and salinity were measured daily in each tank. Average values reached a mean (±standard deviation) of 8.3 ± 0.2, 28.0 ± 0.7 °C, and 11.2 ± 2.8 g L^{-1}, respectively. Dissolved oxygen was kept saturated with a continuous aeration on the tank bottom.

2.3. Diet Formulation

FML used in this study was produced from the byproducts obtained during processing of farmed Atlantic salmon (Pesquera Pacific-Star, Puerto Montt, Chile). KRM was obtained from the commercial fisheries of Antarctic krill (*Euphausia superba*) in the Antarctic Atlantic (from fishing Area 48) processed whole on board (QRILL™ Aqua, Aker Biomarine Feed Ingredients AS, Lysaker, Norway).

Ten practical grower diets for juvenile *P. vannamei* were formulated for this study (Table 1). A basal diet containing 120 g kg^{-1} (g kg^{-1} of the diet, as-is) FML was initially designed (diet 120–0, mix ratio of 100%). From this diet, nine other diets were formulated to contain 90, 60, and 30 g kg^{-1} FML, each in combination with 15, 30, and 45 g kg^{-1} KRM. This resulted in FML-KRM dietary levels (and mix ratios, in %) of 90–15 (17), 90–30 (33), 90–45 (50), 60–15 (25), 60–30 (50), 60–45 (75), 30–15 (50), 30–30 (100), and 30–45 (150), respectively. The inclusions of soybean meal, wheat flour, and wheat gluten meal were fixed at 380, 300, and 20 g kg^{-1}, respectively, across all diets. To maintain a uniform dietary crude protein (CP) content, the inclusion of soy protein concentrate was adjusted relative

to FML and KRM levels. Similarly, the total dietary content of methionine (Met) and lysine (Lys) was balanced with DL-Methionine and L-Lysine. Our previous study has shown that under green-water culture conditions, shrimp growth performance is maximized with a total dietary Met (Met + Cysteine, Cys) and Lys of 8.2 (12.9 g kg^{-1} of the diet, as-is) and 17.8 g kg^{-1}, respectively, with a minimum of 60 g kg^{-1} FML [22]. To counteract the biased influence of Met (Met + Cys) on shrimp growth, diets were structured with levels of these two AAs marginally below specifications. Salmon oil and soy lecithin oil were used to meet the minimum required levels of essential fatty acids (EFAs) and phospholipids, respectively. Their dietary inclusion also varied according to FML and KRM levels.

Table 1. Ingredient and proximate composition (g kg^{-1}, as-is) of experimental diets.

Ingredients	**Diets/Ingredient Composition (g kg^{-1}, as-is)**									
Fish meal (g kg^{-1})	120		90			60			30	
Krill meal (g kg^{-1})	0	15	30	45	15	30	45	15	30	45
Mix ratio	100	17	33	50	25	50	75	50	100	150
Soybean meal [a]	380.0	380.0	380.0	380.0	380.0	380.0	380.0	380.0	380.0	380.0
Wheat flour [b]	300.0	300.0	300.0	300.0	300.0	300.0	300.0	300.0	300.0	300.0
Soy protein concentrate [c]	70.5	87.3	73.8	60.3	117.6	104.1	90.6	147.9	134.4	120.9
Salmon meal [d]	120.0	90.0	90.0	90.0	60.0	60.0	60.0	30.0	30.0	30.0
Krill meal [e]	-	15.0	30.0	45.0	15.0	30.0	45.0	15.0	30.0	45.0
Wheat gluten meal [f]	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Salmon oil	37.9	37.5	36.6	35.8	37.9	37.1	36.2	38.3	37.5	36.6
Cassava starch	20.0	15.5	16.9	18.4	9.4	10.9	12.4	3.4	4.9	6.4
Calcium carbonate	15.0	16.5	15.5	14.5	19.1	18.0	17.0	21.6	20.6	19.5
Soy lecithin oil	9.4	10.0	8.3	6.7	12.1	10.5	8.9	14.3	12.7	11.0
Sodium monophosphate [g]	8.7	9.2	10.0	10.7	9.0	9.8	10.5	8.8	9.6	10.3
Salt	6.7	7.2	7.2	7.2	7.7	7.7	7.7	8.2	8.2	8.2
Synthetic binder [h]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin–mineral premix [i]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
DL-Methionine [j]	1.6	1.8	1.6	1.5	2.0	1.9	1.8	2.3	2.2	2.0
Stay C, 35% [k]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Choline chloride [l]	0.8	0.7	0.8	0.8	0.5	0.6	0.7	0.4	0.5	0.5
L-Lysine [m]	0.4	0.4	0.2	0.1	0.6	0.5	0.3	0.8	0.7	0.5
Proximate composition (%, as-is)										
Dry matter	865.0	869.5	863.6	862.2	861.7	877.2	879.1	891.9	882.9	887.9
Crude protein	347.2	341.2	342.3	339.5	341.4	337.9	345.8	349.5	349.2	351.2
Total lipids	73.7	69.5	70.6	64.7	52.1	65.2	63.0	66.4	62.5	66.1
Total fiber	21.3	23.6	22.2	23.7	26.7	26.7	24.5	27.6	24.6	28.8
Crude ash	68.5	71.0	71.0	72.1	70.3	71.7	71.8	73.3	72.0	73.9
Nitrogen-free extract [n]	354	364	358	362	371	376	374	375	375	368
Gross energy (MJ kg^{-1})	16.9	16.9	16.8	16.6	16.2	16.9	17.0	17.4	17.0	17.0

[a] Bunge Alimentos S.A. (Luiz Eduardo Magalhães, Brazil). 898.1 g kg^{-1} dry matter (DM), 46.06 g kg^{-1} CP, 1.60 g kg^{-1} ether extract (EE), 5.70 g kg^{-1} total fiber, 6.79 g kg^{-1} ash, 0.51 g kg^{-1} methionine (Met), 1.27 g kg^{-1} M+C, 2.90 g kg^{-1} lysine (Lys), 1.80 g kg^{-1} threonine (Thr). [b] Bunge Alimentos S.A. (Gaspar, Brazil). 87.32 g kg^{-1} DM, 12.44 g kg^{-1} CP, 1.55 g kg^{-1} EE, 0.37 g kg^{-1} total fiber, 0.81 g kg^{-1} ash, 0.21 g kg^{-1} Met, 0.61 g kg^{-1} M+C, 0.28 g kg^{-1} Lys, 0.45 g kg^{-1} Thr. [c] X-Soy 600®, CJ Selecta S.A. (Araguari, Brazil). 93.04 g kg^{-1} DM, 62.24 g kg^{-1} CP, 1.20 g kg^{-1} EE, 4.60 g kg^{-1} total fiber, 6.60 g kg^{-1} ash, 0.81 g kg^{-1} Met, 1.68 g kg^{-1} M+C, 3.76 g kg^{-1} Lys, 2.40 g kg^{-1} Thr. [d] Pesquera Pacific Star (Puerto Monte, Chile). 89.42 g kg^{-1} DM, 63.86 g kg^{-1} CP, 9.30 g kg^{-1} EE, 0.14 g kg^{-1} total fiber, 16.20 g kg^{-1} ash, 1.65 g kg^{-1} Met, 2.35 g kg^{-1} M+C, 4.19 g kg^{-1} Lys, 2.35 g kg^{-1} Thr. [e] QRILL™ Aqua, Aker BioMarine Feed Ingredients AS (Lysaker, Norway). 93.53 g kg^{-1} DM, 57.42 g kg^{-1} CP, 22.00 g kg^{-1} EE, 3.51 g kg^{-1} total fiber, 12.23 g kg^{-1} ash, 1.57 g kg^{-1} Met, 1.98 g kg^{-1} M+C, 4.00 g kg^{-1} Lys, 2.41 g kg^{-1} Thr. [f] Agridient, Inc. (Farmington Hills, MI, USA). 90.91 g kg^{-1} moisture, 79.19 g kg^{-1} CP, 3.96 g kg^{-1} EE, 0.18 g kg^{-1} total fiber, 0.82 g kg^{-1} ash, 1.24 g kg^{-1} Met, 2.68 g kg^{-1} M+C, 1.33 g kg^{-1} Lys, 2.16 g kg^{-1} Thr. [g] Monobasic sodium phosphate. 0.60 g kg^{-1} calcium, 20.70 g kg^{-1} phosphorous, 14.12 g kg^{-1} available phosphorous. [h] Nutri-Bind Aqua Veg Dry, Nutri-Ad International NV (Dendermonde, Belgium). Synthetic pellet binder consisting of calcium lignosulfonate (94.00 g kg^{-1}) and guar gum (6.00 g kg^{-1}). [i] Rovimix 2050 Px Camarões VitMin (SAM) VM25L3 (BR4418A025). DSM Produtos Nutricionais Brasil Ltd.a. (São Paulo, Brazil). Guarantee levels per kg of product: vitamin A, 2,996,333 IU; vit. D3, 1,080,066 IU; vit. E, 22,344.50 mg; vit. K3,

7350 mg; vit. B1, 14,560 mg; vit. B2, 13,200 m g; vit. B5, 45,070 mg; vit. B6, 14,560 mg; vit. B12, 7.63 mg; folic acid, 1870 mg; nicotinic acid, 26,350 mg; biotin, 381 mg; inositol, 83,000 mg; Cu, 11,000 mg; I, 500 mg; Mn, 5000 mg; Se, 134 mg; Zn, 31,000 mg; Co, 1350 mg. [j] MetAMINO®, Evonik Nutrition & Care GmbH (Hanau, Germany). DL-Methionine, Feed Grade 990 g kg^{-1}. [k] Rovimix® Stay C® 35. Minimum of 350 g kg^{-1} of phosphorylated vitamin C activity. DSM Nutritional Products AG (Schweiz, Switzerland). [l] Zouping Jujia Choline Industrial Co., Ltd. (Handian Industrial Zone, Zouping County, Shandong, China). 600 g kg^{-1} of active choline. [m] Biolys®, Evonik Nutrition & Care GmbH (Hanau, Germany). L-Lysine 546 g kg^{-1}. [n] Calculated by difference [DM − (CP + EE + total fiber + ash)].

2.4. Feed Manufacturing and Chemical Analysis

Diets were manufactured at LABOMAR's experimental feed mill facility using a laboratory pelleting machine (model EX MICRO, Exteec Máquinas, Ribeirão Preto, Brazil). First, all dried raw materials were ground to less than 300 microns (mesh #48). Next, ingredients were weighed to a 0.01 g precision on an electronic scale following formula specifications. All micro-ingredients (vitamins, minerals, synthetic binder, crystalline AAs) were mixed with a 1 kg sample of all dried macro-ingredients in a Y-mixer (model MA201/5MO, Marconi Equipamentos para Laboratórios Ltd.a., Piracicaba, Brazil) for 10 min at 30 RPM. This mix was then combined with all other macro-ingredients (dry and liquids) and mixed for 10 min in a planetary mixer with freshwater until a feed dough was formed. The feed dough was then pressed through a plastic net to obtain small chunks of moist feed for extrusion. For feed cooking and extrusion, a pellet mill was used and adjusted to operate at a maximum temperature of 95 °C. The die and knife of the pelleting machine were first adjusted to produce pellets of 1.80 ± 0.07 mm in diameter by 5.86 ± 0.80 mm in length (n = 30). To obtain pellets with a consistent moisture content, the feed was dried at 60 °C using a convection oven for a maximum period of 3 h. After an initial 30 min drying, batches of 5 kg of feed were transferred to a pot for steam-cooking for 10 min under 95 °C. Post-cooked pellets were then subjected to final drying in the convection oven until a moisture content of 125.9 ± 11.2 g kg^{-1} (n = 10) was reached. Moisture content of pellets was kept as consistent as possible by taking feed samples at 15 min intervals during drying. Samples were analyzed with a halogen rapid moisture analyzer.

Finished diets were chemically analyzed [28]. Dry matter (DM) was determined by drying samples in a convection oven for 24 h at 105 °C. The Dumas combustion method was applied to analyze CP (AOAC 968.06), while total lipids were determined through acid hydrolysis (AOAC 954.02). Ash content was determined by burning samples in a muffle furnace at 600 °C for 2 h (AOAC 942.05) and crude fiber by enzymatic–gravimetric determination (AOAC 992.16). AA and FA compositions were determined using high-performance liquid chromatography [29,30] and high-resolution gas chromatography (GC) with a flame ionization detection fitted with a capillary GC column, respectively.

Diets reached a mean (±standard deviation, sd) CP and total lipid content of 344.5 ± 4.7 and 65.4 ± 5.8 g kg^{-1} (g kg^{-1} of the diet, as-is), respectively (Table 1). Total dietary Met (Met + Cys), Lys, and threonine (Thr) content reached 6.2 ± 0.2 (10.6 ± 0.4 g kg^{-1}), 18.4 ± 0.6, and 12.9 ± 0.4 g kg^{-1}, respectively (Table 2). The dietary FA profile changed as FML was replaced for KRM (Table 3). The total dietary concentration of eicosapentaenoic acid (EPA, 20:5n-3) tended to increase with higher inclusions of KRM, from a low of 1.4 g kg^{-1} in diet 120–0 to a high of 2.9 g kg^{-1} in diet 6–4.5. The total dietary content of docosahexaenoic acid (DHA, 22:6n-3) was more consistent across diets, varying between 2.1 and 2.8 g kg^{-1}. The total dietary polyunsaturated (PUFA) and highly unsaturated fatty acid (HUFA) content remained above 19.9 and 4.0 g kg^{-1}, respectively, across all diets.

Table 2. Amino acid (g kg^{-1} of the diet, as-is) composition of experimental diets. CV, coefficient of variation (%).

Composition	**Diets/Amino Acid Composition (g kg^{-1}, as-is)**										
% FML	120		90			60			30		CV (%)
% KRM	0	15	30	45	15	30	45	15	30	45	
Essential Amino Acids (EAA)											
Arginine	20.4	21.7	20.0	19.2	20.3	20.7	20.6	21.5	20.3	20.9	3.5
Histidine	7.7	8.1	7.1	7.4	7.7	7.9	8.0	7.9	7.9	8.0	4.0
Isoleucine	13.3	14.1	13.2	12.4	13.6	13.3	13.2	13.9	13.8	14.1	3.9
Leucine	24.0	25.4	24.0	22.6	23.9	24.3	24.1	24.8	23.9	24.7	3.0
Lysine	18.6	19.3	17.9	17.3	18.2	18.2	18.3	19.2	18.1	18.4	3.2
Methionine	6.3	6.5	6.3	6.1	5.8	6.4	6.1	6.3	5.8	6.2	3.8
Met + Cys [a]	10.9	11.1	10.5	10.2	10.3	10.8	10.6	10.9	10.0	10.4	3.3
Phenylalanine	15.3	16.2	14.7	14.4	15.5	15.7	15.5	16.1	15.5	16.0	3.7
Threonine	12.9	13.5	13.3	12.3	12.7	13.1	13.0	13.0	12.5	12.6	2.9
Valine	14.2	15.0	14.1	13.1	14.3	14.1	13.8	14.4	14.3	14.8	3.7
Non-Essential Amino Acids (NEAA)											
Alanine	15.7	16.3	14.9	14.7	14.9	15.3	15.3	15.0	14.3	14.8	3.7
Aspartic acid	30.5	31.9	31.9	29.1	30.6	31.6	31.3	31.8	30.7	31.8	2.9
Cystine	4.6	4.6	4.2	4.1	4.5	4.4	4.5	4.6	4.2	4.2	4.5
Glycine	17.5	17.7	15.9	16.0	15.7	16.2	16.1	14.8	14.7	15.2	6.3
Glutamic acid	63.8	66.4	59.9	58.7	63.6	64.1	62.9	66.3	63.2	65.6	4.0
Hydroxyproline	1.8	1.4	1.5	1.3	1.0	1.0	0.9	0.6	0.6	0.6	39.2
Proline	19.1	20.1	19.3	17.8	18.5	19.1	18.9	19.1	18.7	19.4	3.2
Serine	15.4	16.3	15.6	14.5	15.2	15.8	15.5	15.8	15.0	15.4	3.2
Taurine	0.8	0.8	0.9	0.9	0.6	0.7	0.8	0.1	0.5	0.6	35.9
Tyrosine	10.4	10.9	10.2	9.8	10.3	10.6	10.5	10.8	10.5	10.8	3.1
∑ EAA [b]	132.7	139.8	130.6	124.8	132.0	133.7	132.6	137.1	132.1	135.7	3.0
∑ NEAA	179.6	186.4	174.3	166.9	174.9	178.8	176.7	178.9	172.4	178.4	2.9
∑ EAA + NEAA	312.3	326.2	304.9	291.7	306.9	312.5	309.3	316.0	304.5	314.1	2.9

[a] Methionine + cysteine. [b] Tryptophan not analyzed.

Table 3. Fatty acid composition (g kg^{-1} of the diet, as-is basis) of experimental diets.

Fatty Acid	**Diets/Fatty Acid Composition (g kg^{-1}, as-is)**									
% FML	120		90			60			30	
% KRM	0	15	30	45	15	30	45	15	30	45
4:0	-	0.1	0.1	<0.01	0.1	0.1	0.1	0.1	0.1	-
6:0	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
8:0	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
10:0	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
11:0	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
13:0	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
14:0	1.1	01.1	1.4	1.5	1.2	1.3	1.6	1.2	1.3	1.4
15:0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
16:0	12.2	10.3	11.0	10.6	10.7	10.6	11.3	11.3	11.6	10.8
17:0	0.2	0.1	0.1	0.1	0.2	0.1	0.1	0.2	0.2	0.1
18:0	4.6	3.0	2.9	4.6	3.1	2.9	3.0	3.4	3.3	2.9
20:0	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
21:0	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
22:0	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
23:0	-	-	-	-	-	-	-	-	-	0.1
24:0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
15:1	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
16:1n-7	1.5	1.4	1.6	1.6	1.5	1.5	1.7	1.5	1.6	1.6
18:1n-9	24.1	18.9	19.8	24.1	20.2	19.4	20.1	22.0	22.3	20.4

Table 3. *Cont.*

Fatty Acid	Diets/Fatty Acid Composition (g kg^{-1}, as-is)									
20:1n-9	1.0	0.9	0.9	1.0	0.9	0.9	0.9	1.0	1.0	1.0
22:1n-9	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
24:1n-9	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
18:2n-6	19.4	17.9	18.2	19.4	17.8	16.6	16.6	19.2	18.7	16.4
20:2n-6	0.5	0.4	0.4	0.5	0.4	0.4	0.4	0.5	0.5	0.4
18:3n-6	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
20:3n-6	0.2	0.1	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2
20:4n-6	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
18:3n-3	3.1	2.8	2.8	3.1	2.8	2.7	2.7	3.1	3.0	2.7
20:3n-3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
20:5n-3	1.4	1.9	2.5	1.4	2.1	2.4	2.9	1.8	2.2	2.4
22:2n-6	0.1	-	-	0.1	-	-	-	-	-	-
22:6n-3	2.3	2.1	2.5	2.3	2.4	2.5	2.8	2.4	2.7	2.6
∑n-3 [a]	6.9	6.9	7.9	6.9	7.4	7.7	8.5	7.4	8.0	7.8
∑n-6 [b]	20.5	18.6	19.1	20.5	18.7	17.4	17.5	20.2	19.7	17.3
∑SFA [c]	18.7	15.2	16.1	17.4	15.9	15.6	16.7	16.8	17.1	15.9
∑MUFA [d]	27.3	21.7	22.9	27.4	23.2	22.4	23.3	25.1	25.5	23.6
∑PUFA [e]	23.4	21.4	21.8	23.4	21.4	20.0	20.1	23.2	22.6	19.9
∑HUFA [f]	4.0	4.1	5.2	4.0	4.7	5.1	5.9	4.4	5.1	5.2

[a] n-3, 18:3n-3, 20:3n-3, 20:5n-3, 22:6n-3. [b] n-6, 18:2n-6, 20:2n-6, 18:3n-6, 20:3n-6, and 20:4n-6. [c] SFA, saturated fatty acids: 4:0, 6:0, 8:0, 10:0, 11:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0, 22:0, 23:0, and 24:0. [d] MUFA, monounsaturated fatty acids: 15:1, 16:1, 18:1, 20:1, 22:1, and 24:1. [e] PUFA, polyunsaturated fatty acids: 18:2n-6, 20:2n-6, 18:3n-6, 18:3n-3, 20:3n-6, and 20:3n-3. [f] HUFA, highly unsaturated fatty acids: 20:4n-6, 20:5n-3, 22:2n-6, and 22:6n-3.

2.5. Shrimp Stocking

The shrimp species used in this trial was the Pacific whiteleg shrimp, *P. vannamei*, purchased as post-larvae (PL) from a commercial hatchery (Atlantico Larvicultura Ltd.a., Beberibe, Brazil) distant 63 km from the lab. A total of 100,000 PLs at the age of PL10 with 2.7 ± 0.2 mg body weight (BW) were transported to the lab in nine 15-L plastic bags (741 PLs L^{-1}). Plastic bags contained seawater saturated with pure dissolved oxygen. At arrival, shrimp were acclimated to temperature, pH, and salinity and stocked in nursery tanks. A shrimp sample containing approximately 1000 animals was collected for RT-PCR (Real-Time Polymerase Chain Reaction) to screen for the following viruses: White Spot Syndrome (WSS), Infectious Hypodermal and Hematopoietic Necrosis (IHHN), and Infectious Myonecrosis (IMN). RT-PCR results indicated shrimp were free from these viruses.

PLs were nursery-reared in five 23-m^3 tanks with a commercial crumbled diet containing a minimum of 40% CP. Once shrimp reached 1.28 ± 0.08 g BW ($p > 0.005$, $n = 140$, one-way Analysis of Variance, ANOVA), they were transferred to seventy 1-m^3 tanks under 135 shrimp m^{-2} (120 shrimp tank^{-1}), allowing seven replicate tanks per diet. Shrimp were first acclimated during 19 days with a commercial 35%-CP feed (Camanutri 35, Neovia Nutrição e Saúde Animal Ltd.a., São Lourenço da Mata, Brazil) when they reached 2.87 ± 0.45 g. Animals were fed on the experimental diets from the 20th to the 88th day of rearing.

2.6. Feeding

Shrimp were fed daily, including Sundays, exclusively in feeding trays measuring 14.3 cm in diameter and borders with 3.5 cm in height. Trays were installed in the middle of each tank bottom at a density of one unit per tank. At each feeding time, feeding trays were checked for feed remains, which were collected for weighing and disposal. Feed delivery and collection of feed remains in feeding trays occurred at the following times: 1st meal: 07:00 a.m.–10:00 am; 2nd meal: 10:00 a.m.–01:00 p.m.; 3rd meal: 01:00 p.m.–04:00 p.m.; and

4th meal: 04:00 p.m.–07:00 a.m. Daily rations were split as 25, 15, 15, and 45% at the 1st, 2nd, 3rd, and 4th feeding times, respectively.

Over the 20-day acclimation period, shrimp were first fed a fixed daily ration that varied from 12.0 to 15.0 g of feed per tank (3.8–8.5% of the estimated stocked shrimp biomass). Starting on the 21st day of rearing, the daily rations were calculated based on the equation MM = $0.0931BW^{0.6200}$, where MM is the maximum amount of feed that can be consumed daily by an individual with a specific BW Daily meals were reduced by 30% across all treatments to achieve an FCR of 1.5. Feeding trays were inspected daily to check for dead animals, which were collected and discarded. Dead animals were not replaced throughout the culture period. Starting on the 19th day of rearing and then on a biweekly basis, five shrimp from each tank were captured, and their BW was determined. Until the next weight check, feed ratio increased, assuming individual mean daily weight shrimp gains for each tank from previous week, maintaining a fixed 0.21% daily drop in survival.

2.7. Shrimp Growth Performance

At harvest, all live shrimp were counted and individually weighed to a 0.01 g precision Ohaus Adventurer, model ARA520, Toledo do Brasil Indústria de Balanças Ltd.a., São Bernardo do Campo, Brazil). Final shrimp survival (S, %) was calculated as $S = \left(\frac{POPf}{POPi}\right) \times 100$, where POPi represents the number of stocked shrimp, and POPf represents the number of shrimp at harvest. The weekly weight gain (WWG, g $week^{-1}$) was determined by the formula

$$WWG = \left(\frac{BWf - BWi}{t}\right) \times 7,$$

where

BWi is the wet shrimp body weight (BW, g) at stocking, BWf is the final shrimp BW at harvest, and t is the number of days in culture.

The gain in shrimp yield (YIE, g of shrimp biomass gained m^{-2}) was determined as

$$YIE = \frac{BIOf - BIOi}{\text{tank bottom area}(m^2)},$$

where

BIOi denotes the initial shrimp biomass per tank (g), BIOf denotes the final shrimp biomass (g), and tank bottom area denotes 0.89 m^2.

FCR was calculated by dividing the total inputs of feed (g, as-is basis) delivered per tank during the entire rearing period by the total gained shrimp biomass per tank (g, as-is). The apparent feed intake (AFI, g of feed delivered divided by the number of stocked shrimp) was calculated by dividing the total amount of feed delivered (g) by the number of stocked shrimp.

2.8. Shrimp Whole Body CP Content and Protein Deposition

At stocking and harvest, live shrimp were collected for CP (AOAC 968.06) analysis of their whole body. At stocking, a total of 1 kg of shrimp was collected for analysis. At harvest, a composite sample consisting of 70 shrimp from each dietary treatment (10 shrimp per tank) was prepared for analysis. The head-on shell-on (HOSO) shrimp were freeze-dried, ground, and blended. Deposition of dietary protein (%) was calculated as (NRC 2011)

$$\text{Deposition (\%)} = \frac{(\text{final shrimp BW} \times \text{CP in shrimp at harvest}) - (\text{initial shrimp BW} \times \text{CP in shrimp at stocking}) \times 100}{(\text{CP in feed} \times \text{AFI})}.$$

2.9. Feed Preference

Feed preference was assessed by simultaneously confronting two individual diets and measuring their relative apparent feed intake (AFI, %). In this study, 30 tanks of 0.5 m^3

were stocked with juvenile shrimp of 12.46 ± 2.05 g (n = 1200) under 71 animals m^{-2} (40 shrimp $tank^{-1}$). Two feeding trays (141 mm^2 in surface area) were placed in each tank with individual diets. Feeding trays were simultaneously immersed in water and rested in the tank bottom near the side walls of the tank and away from the aeration area. During the observation period, shrimp were fed in excess so that feed remains were always available for collection. Feed delivery and collection of uneaten feed took place at the following times: 1st meal: 07:30 a.m.–08:30 a.m.; 2nd meal: 01:30 p.m.–02:30 p.m., respectively. Shrimp were fed for eight consecutive days. After one hour of feed delivery, feeding trays were recovered, feed remains were collected, dried in a convection oven at 105 °C for 24 h, and weighed. AFI (% of the total meal consumed) was calculated as (total amount of dried feed remains collected, in g/total amount of dried feed delivered, in g) × 100. The total amount of feed consumed was calculated on a DM basis by subtracting the amount of dried feed delivered from the dried amount of feed leftovers. Feed moisture content was determined by drying five samples of 3 g of each diet type in a convection oven at 105 °C for 24 h.

2.10. Statistical Analysis

Homogeneity of variance was examined for all data by using Bartlett-Box F and Cochran's C tests. Kurtosis and skewness and their standard error (i.e., s.e. kurtosis and s.e. skewness) were applied to the data as measures of asymmetry and tests of normality. When needed, data were transformed to a log(x) scale to normalize and homogenize the variances and to meet statistical assumptions. The effect of the dietary inclusion and mix ratios of FML and KRM on shrimp final survival, gained yield, weekly growth, AFI, FCR, and protein deposition were analyzed through two-way ANOVA. The differences in the mean values of shrimp BW and CP content in HOSO shrimp between dietary treatments were analyzed with one-way ANOVA. When significant differences were detected, they were compared two-by-two with Tukey's test. The significant level of 5% was set in all statistical analyses. The statistical package IBM® SPSS® Statistics 23.0 (SPSS Inc., Chicago, IL, USA) was used.

3. Results

The HOSO shrimp CP content at harvest reached 795.4 ± 10.8 g kg^{-1} (dry matter basis, n = 30) with a protein deposition of 36.04 ± 1.96% (n = 70). No statistical effect of FML and KRM dietary level or their mix ratio was detected over these parameters ($p > 0.05$). Final shrimp survival achieved 93.9 ± 4.5% (mean ± sd), and it remained unaffected by the dietary inclusion or different ratios of FML and KRM (Table 4, $p > 0.05$). A similar result was found for gained shrimp yield (1235 ± 92 g m^{-2}) and FCR (1.47 ± 0.09). Shrimp grew from a minimum of 0.68 ± 0.05 g $week^{-1}$ (diet 30–30) to a maximum of 0.76 g $week^{-1}$ (diets 60–15 and 60–30). Both shrimp weekly growth and AFI were significantly influenced by the dietary inclusion of FML ($p < 0.05$). An inclusion of 60 g kg^{-1} FML, regardless of KRM inclusion, resulted in faster shrimp growth and higher AFI compared to 30 g kg^{-1}. No statistical differences could be detected in these parameters among the other dietary inclusion levels of FML and KRM or their interaction ($p > 0.05$).

The final shrimp BW exceeded 10 g for all dietary groups except for shrimp fed the 30–30 diet. Final BW was significantly influenced by dietary treatment (Figure 1, $p < 0.05$). Shrimp fed diets with 30 g kg^{-1} FML, regardless of KRM inclusion, achieved the lowest BW, particularly when fed 30–15 and 30–30 diets. However, combining 30 g kg^{-1} FML with 45 g kg^{-1} KRM (30–45 diet) was as effective as diets 120–0 or 90–15 in terms of shrimp BW ($p > 0.05$). There was no difference in BW within the 60 g kg^{-1} FML group. Shrimp BWs from the 60–30 and 60–45 diets were comparable to the 120–0 diet, whereas only the 60–15 diet outperformed the former. Increasing FML from 30 to 60 g kg^{-1} significantly enhanced shrimp BW regardless of KRM inclusion. There was no significant increment in shrimp BW when FML was increased from 60 to 90 g kg^{-1}. All diets with 90 g kg^{-1} FML outperformed those with 30 g kg^{-1} FML. Shrimp demonstrated comparable performance in the final BW, matching that of diet 120–0 and the group of diets containing 60 g kg^{-1}

FML (Table 4). The only exception was the combination of 90–15, which showed an inferior BW in comparison to 60–15. The control diet, with 120 g kg^{-1} FML and no KRM, only outperformed diets 30–15 and 30–30. It performed similarly to all other diets, except when compared to shrimp fed the diet 60–15 ($p < 0.05$).

Table 4. Growth performance (mean ± standard deviation) and protein retention (%) in juvenile *P. vannamei* fed diets with different combinations (g kg^{-1} of the diet, as-is) of fish meal (FML) and krill meal (KRM). Shrimp were raised for 88 days under 135 animals m^{-2} and fed the experimental diets for 70 days. Each value represents the mean (±standard deviation, sd) of seven rearing tanks.

g kg^{-1} of the Diet		Shrimp Growth Performance						Protein Deposition (%)
FML	KRM	Initial Body Weight (mg)	Final Survival (%)	Gained Yield (g m^{-2})	Growth (g $week^{-1}$)	AFI [a] (g Feed per Stocked Shrimp)	FCR [b]	
120	0	1.31 ± 0.07	95.1 ± 3.9	1252 ± 97	0.73 ± 0.08	13.6 ± 1.0	1.46 ± 0.05	34.88 ± 1.22
90	15	1.27 ± 0.10	94.3 ± 2.1	1237 ± 101	0.73 ± 0.05	13.3 ± 0.4	1.46 ± 0.11	35.62 ± 2.18
	30	1.27 ± 0.08	93.9 ± 1.8	1258 ± 55	0.74 ± 0.04	13.5 ± 0.4	1.45 ± 0.03	36.19 ± 1.10
	45	1.24 ± 0.07	94.2 ± 3.2	1260 ± 110	0.74 ± 0.07	13.4 ± 0.8	1.43 ± 0.05	36.73 ± 1.60
60	15	1.28 ± 0.09	89.6 ± 8.1	1224 ± 112	0.76 ± 0.06	13.8 ± 0.6	1.53 ± 0.13	36.14 ± 1.45
	30	1.28 ± 0.08	92.8 ± 4.4	1261 ± 107	0.76 ± 0.09	13.6 ± 0.8	1.45 ± 0.09	37.27 ± 2.52
	45	1.33 ± 0.07	93.3 ± 5.7	1269 ± 63	0.75 ± 0.05	13.6 ± 0.7	1.45 ± 0.08	37.25 ± 2.01
30	15	1.33 ± 0.07	93.9 ± 4.4	1179 ± 123	0.69 ± 0.07	13.1 ± 0.5	1.51 ± 0.12	35.24 ± 2.53
	30	1.28 ± 0.07	94.9 ± 5.1	1166 ± 73	0.68 ± 0.05	12.9 ± 0.4	1.50 ± 0.10	34.79 ± 2.20
	45	1.27 ± 0.10	96.5 ± 4.3	1233 ± 47	0.71 ± 0.04	12.9 ± 0.3	1.41 ± 0.07	36.32 ± 1.68
Mean ± sd		1.28 ± 0.08	93.9 ± 4.5	1235 ± 92	0.73 ± 0.06	13.4 ± 0.6	1.47 ± 0.09	36.04 ± 1.96
Two-Way ANOVA		-	-	-	0.004; 6 > 3;	0.004; 6 > 3;	-	-
FML		-	0.093	0.084	3 = 12 = 9; 12, 9 = 6	3 = 12 = 9; 12, 9 = 6	0.539	0.077
KRM		-	0.356	0.378	0.876	0.855	0.066	0.194
FML × KRM		-	0.803	0.904	0.927	0.908	0.682	0.838

[a] Apparent feed intake (AFI, g) is the amount of feed delivered (as-is) divided by the number of stocked shrimp.
[b] Feed conversion ratio.

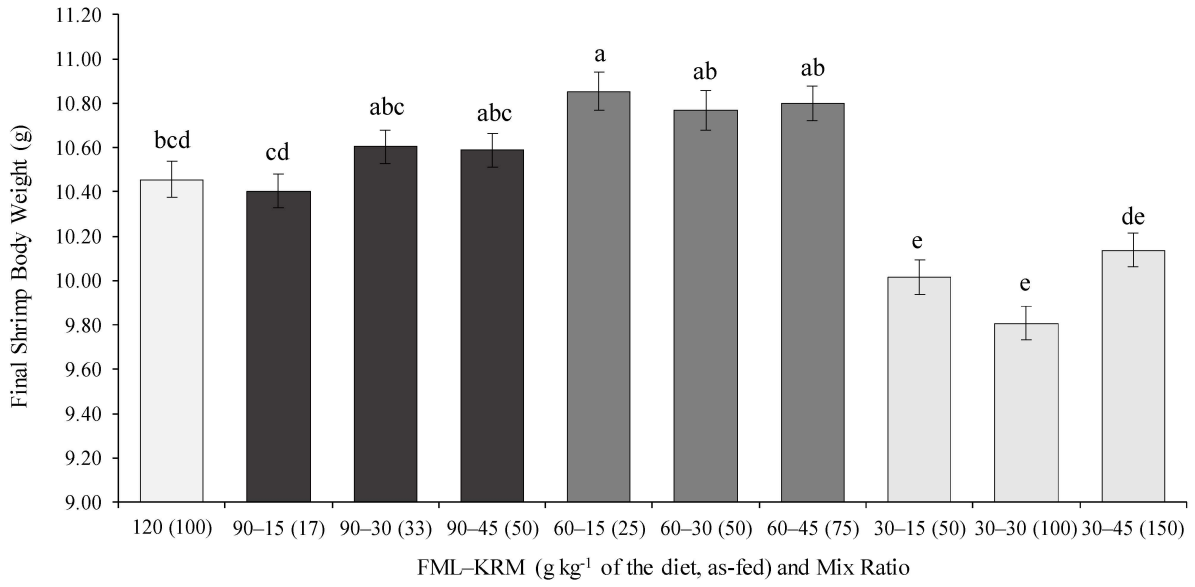

Figure 1. Mean (±standard error) body weight (BW) of *P. vannamei* after 88 days of rearing under 135 shrimp m^{-2}. Shrimp were raised with diets with different dietary levels of fish meal (FML) and krill meal (KRM) and mix ratio (in parentheses). Each column is the mean BW obtained from seven rearing tanks. Common letters indicate non-statistically significant differences according to Tukey's test at $\alpha = 0.05$ significant level.

Feed preference was significantly affected by the dietary inclusion of KRM regardless of FML level (Figure 2). Two-by-two comparisons indicated that the dietary inclusion of KRM at 15 g kg^{-1} resulted in a higher AFI (%) than at 30 and 45 g kg^{-1} under all FML levels ($p < 0.05$, Student's *t*-test). Diets with 30 g kg^{-1} KRM inclusion also resulted in a greater feed preference than at 45 g kg^{-1}.

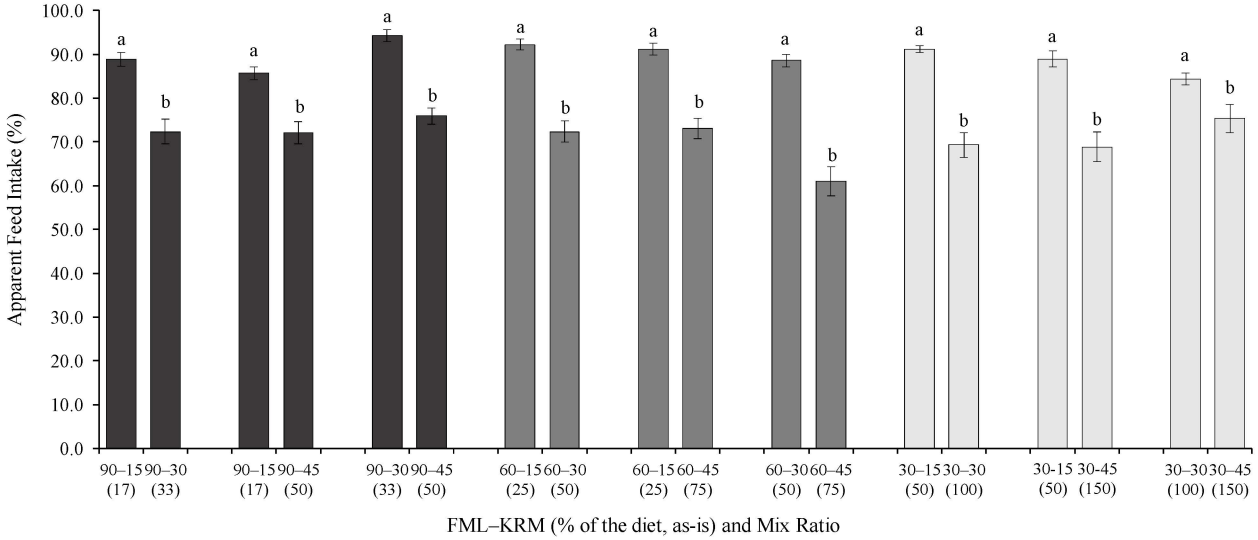

Figure 2. Two-by-two comparisons of relative apparent feed intake (AFI, %) for juvenile *P. vannamei* fed diets with different levels of fish meal (FML) and krill meal (KRM) and their mix ratio (in parentheses). Diets were confronted against each other for two weeks in nine 0.5-m^{-3} tanks using two feeding trays per tank. Each bar represents the mean (±standard error) of 140 individual measurements of feed intake. Different letters indicate statistically significant differences in AFI between diets at the $\alpha = 0.05$ level in accordance with the Student's *t*-test.

4. Discussion

The present study has indicated that a combination of 60–15 g kg^{-1} FML-KRM was able to deliver a higher final shrimp BW than 120 g kg^{-1} FML alone. Shrimp performance (survival, growth, yield, FCR, and final BW) exhibited similarity between diets containing FML–KRM in combinations such as 30–45, 60–30, 60–45, and 120–0. This corresponds to a reduction of up to 75% and 37.5% in the dietary utilization of FML and the sum of marine proteins, respectively. These findings are in line with the work of Nunes and Masagounder [22]. These authors reported that reducing the dietary inclusion of FML from 180 to 60 g kg^{-1} had no detrimental effect on shrimp overall performance if total dietary Met levels were kept at 8.2 g kg^{-1} (as-is).

Most of the published studies have evaluated KRM in diets that combined high levels of plant and/or animal proteins with low inclusions of FML [7,9,10,31] or in diets that were not thoroughly balanced for essential nutrients [7,9,23,31,32]. In the latter case, KRM is generally added to the diets as a supplementary ingredient or as an FML protein replacement. Even if formulas are designed to be isonitrogenous and/or isolipidic, these approaches will likely elevate the levels of EAAs, EFAs, and other nutrients if not appropriately balanced. Such conditions should favor an increased shrimp growth performance, especially when nutrients in diets used for comparison purposes are restrained. In our work, we sought to balance EAAs and EFAs across all diets, limiting the supply of dietary Met. Previous studies have shown that shrimp growth performance is maximized with a total dietary Met (Met + Cys) of 8.1 g kg^{-1} (12.8 g kg^{-1}) depending on FML level [22]. Our results indicated that even when EAAs and EFAs are well-balanced with Met (Met + Cys) marginally restrained at 6.2 ± 0.2 g kg^{-1} (10.6 ± 0.4 g kg^{-1}), KRM has a growth-promoting effect on juvenile *P. vannamei*.

However, these inclusion levels and ratios should not be viewed as fixed recommendations for whiteleg shrimp diets. The adoption of various other ingredients and dietary nutrient levels can introduce effects different from those in the present study. For example, higher dietary inclusions of KRM, more than 15 g kg^{-1}, have been reported to have a growth and immune enhancement effect in juvenile *P. vannamei*. Ambasankar et al. [23] raised juvenile *P. vannamei* (initial BW = 0.5 g) with diets containing a combination of KRM at 0, 20, 40, and 60 g kg^{-1} with 60 or 120 g kg^{-1} FML (Indian fish meal with 614 g kg^{-1} CP and 76 g kg^{-1} lipid). They reported the highest final shrimp survival and BW when KRM was used at 40 and 60 g kg^{-1}, irrespective of the FML level. The authors also observed that shrimp fed diets containing 120 g kg^{-1} FML and 60 g kg^{-1} KRM showed a higher up-regulation of immune parameters, such as a prophenoloxidase-activating enzyme, serine protease, and superoxide dismutase. In this work of Ambasankar et al., the diets remained isonitrogenous (ranging from 355.0 $\pm$ 1.4 to 363.9 $\pm$ 2.4 g kg^{-1}, as-is) and isolipidic (53.6 $\pm$ 1.3 to 58.8 $\pm$ 1.0 g kg^{-1}). Nevertheless, the dietary levels of EPA (20:5n-3) gradually increased with higher doses of KRM. Additionally, since diets were not designed for EAA balance, it is likely that their concentrations also increased in response to higher inclusions of KRM. A similar case was observed in Soares et al. [9] work with juvenile *P. vannamei* (initial BW = 0.15 $\pm$ 0.01 g). The authors added 10, 20, and 40 g kg^{-1} (as-is) KRM to an FML-deprived diet containing 540 g kg^{-1} soybean meal. They reported an increase in food consumption with 20 and 40 g kg^{-1} KRM diets, but only the latter diet improved shrimp growth. While in this case, diets were also kept isonitrogenous and isolipidic, and other dietary nutrients probably increased in response to higher inclusions of KRM.

KRM and FML are both known to act as strong chemoattractants for juvenile whiteleg shrimp [31]. These ingredients are reported to contain a high concentration of low molecular weight compounds that act as chemical drivers for shrimp feeding stimulation [10]. Our results indicated that a dietary inclusion of 15 g kg^{-1} of KRM and 60 g kg^{-1} FML at a 25% mix ratio in a practical diet for juvenile whiteleg shrimp can be more effective in terms of feed preference than higher inclusions of both ingredients. Other studies have found that KRM can successfully stimulate behavioral feeding activity in *P. vannamei* when included at a dietary level of 10 to 30 g kg^{-1} in FML-challenged diets [8,31]. Nevertheless, the effectiveness of feeding effectors varies [8,10,31], as does their minimum concentration needed to boost feed attractability and palatability [7,8,31]. This is likely because the amount of stimulatory compounds (free amino acids, nucleotides, peptides) diverges widely among ingredients [10]. For example, Derby et al. [7], working with juvenile *P. vannamei,* reported that 10, 30, and 60 g kg^{-1} KRM added to a feed deprived of FML enhanced the palatability (i.e., consumption) of pellets in a concentration-dependent fashion. In gelatin-based pellets, Nunes et al. [8] reported that 10 g kg^{-1} of condensed fish soluble protein is more effective in terms of attractiveness and palatability for juvenile *P. vannamei* than 5 g kg^{-1}. In our study, a mixture of FML and KRM, without the inclusion of any other animal protein, appeared to generate a synergistic effect in enhancing shrimp feed preference, eliminating the need for additional quantities of these ingredients. A better understanding of the presence of chemical feed drivers in marine proteins, their threshold concentrations to stimulate feeding, and synergistic interactions will allow further reductions in their usage while optimizing shrimp feed intake and growth performance.

5. Conclusions

Our study emphasized the synergistic impact of combining precise dietary levels and mix ratios of FML and KRM for whiteleg shrimp grower diets. The findings indicate that when essential nutrients are evenly balanced across diets, lower doses of KRM and FML at specific ratios can be equally effective in generating positive feeding stimuli and promoting shrimp growth performance as higher ones. Furthermore, our findings demonstrated that FML can be effectively reduced by up to 75% when combined with lower levels of KRM. This corresponds with the industry's ongoing trend to achieve greater sustainability

and cost efficiency through the reduced utilization of critical resources. Further studies are necessary to better predict the dosage of feeding effectors based on the levels and interactions of chemical drivers of shrimp feeding stimuli. This may allow nutritionists to include feeding effectors based on targeted formulated levels and ratios of key drivers of shrimp feeding stimulation rather than relying on product-specific dietary inclusions.

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Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: L.B. and R.D. are employed by Aker BioMarine Feed Ingredients AS, Norway, which has provided the krill meal.

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Communication

Sustainability Activities in a Hard-to-Abate Industry—A Real-Life Example

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Abstract: Marine sources of omega-3, proteins, and other nutrients are in increasing demand, while supply is struggling to meet this demand increase. A key focus for meeting the need for marine ingredients for human and animal nutrition is ensuring sustainable sourcing for both the oceans as well as other production types. Aker BioMarine is harvesting and producing marine ingredients from krill and this communication is intended to showcase how the harvesting and production of such ingredients are performed in a sustainable way. This communication is written to describe the krill fisheries' management, to provide demonstration cases from CO_2 hot spotting, and show how results from these are used to target GHG emission reduction. The initiatives that are taken to ensure sustainable fishing and production, as well as examples of short- and long-term actions to reduce and minimize the impact of all activities, are provided.

Keywords: sustainability; fisheries certification; CO_2 mapping; life cycle analysis; EPD; CSRD

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1. Introduction

Antarctic krill (*Euphausia superba*) inhabits the Southern Ocean encircling Antarctica. Once they reach adulthood, krill take on a shrimp-like appearance, measuring up to 6 cm in length, and form sizable gatherings facilitating harvesting [1]. Despite their relatively large size, they consume tiny free-floating nano- and microplankton. This is made possible by their specialized filtering system, featuring a mesh with dimensions of 2–3 µm [2]. Thanks to their diet primarily consisting of algae, krill accumulate abundant nutrients while maintaining low levels of pollutants, owing to their low position on the food chain.

Krill meal (KM), derived from finely ground krill, holds significance in diverse feed applications for pets, or in fish and shrimp aquaculture. Characterized by a protein content of approximately 60%, exhibiting a balanced amino acid profile and a lipid content of, on average, 25%, KM can play a valuable role in animal nutrition. In the context of human consumption, the lipid fraction undergoes extraction to yield krill oil (KO), containing around 40% phospholipids (PLs) [3,4] with the majority of long-chain omega-3 polyunsaturated fatty acids (n − 3 PUFAs), such as eicosapentaenoic acid (EPA, 20:5n − 3) and docosahexaenoic acid (DHA, 22:6n − 3), residing within the phosphatidylcholine (PC) configuration [5,6]. In contrast, the omega-3 fatty acids of fish oil are incorporated into triglycerides. There is evidence that the differences between the molecular forms of omega-3 fatty acids (triglycerides and ethyl-esters in fish oil, and phospholipids in krill oil) are important. Studies conducted on various species, including dogs [7], piglets [8], baboons [9], mice [10], rats [11], and humans [12], have demonstrated that the phospholipid omega-3 delivery form has the potential to enhance omega-3 tissue integration when compared to triglycerides. Furthermore, the scientific evidence suggests that phospholipids and choline, as found in krill oil, offer numerous health benefits on their own [13–15]. Notably, krill also

contains a potent antioxidant in the form of astaxanthin [16], which is of relevance to protect the PUFAs from oxidative degradation, while concurrently enhancing the characteristic pink pigmentation in salmon.

From an aquaculture perspective, krill products are further valuable due to their content of bioactive constituents, including cholesterol, minerals, and vitamins [6], as well as water-soluble, low-molecular-weight feed attractants encompassing free amino acids, nucleotides, nucleosides, quaternary ammonium compounds, phospholipids (PLs), biogenic amines, and chitin. These compounds collectively enhance the attractability and palatability of diets [17,18].

Studies have shown that krill oil can support the optimization of cell membranes, improve nutrient levels in the body, and exhibit anti-inflammatory, inflammation-resolving, and antioxidant properties [19,20]. This is especially significant in conditions that impact the heart, liver, brain, and joints, as well as those following intense exercise or as a result of aging, which commonly exhibit decreased levels of the nutrients found in krill oil.

In a world with a rapidly increasing population, seafood and ingredient production from the ocean represents a key part of the solution for a sustainable food system for the future [21,22]. Omega-3 and marine proteins are in increasingly short supply, and supplying these ingredients from sustainably managed fisheries is key. The unique composition of krill products makes the harvesting of krill appealing and addresses the n − 3 PUFA and marine proteins' shortage in the world, since the availability of marine ingredients derived from capture fisheries is unlikely to increase much beyond its present extraction rate due to the stock capacity and increased management of resources [23,24].

AKBM strongly believes that krill is a piece of the solution for future food and feed, and that the documented affect from krill products underlines the importance of using the right ingredients for the right markets. Among all the multicellular animal species on Earth, Antarctic krill stands out as one of the most abundant, displaying a biomass of between 300 to 500 million metric tons [25]. Krill is a closely and very sustainably managed species with a quota for harvesting set to well below 1% of the estimated biomass. Krill plays an important role in both human and animal nutrition [26,27], and the sustainable and correct use of krill is part of supporting future sustainable food systems.

AKBM owns and operates three fishing vessels that both harvest and process the krill on board to yield krill meal. The fishing vessels remain on the fishing fields during the fishing season, which lasts around 11 months, and during that period, AKBM's transportation vessel goes to and from the fishing fields to collect meal produced on board the vessel, as well as to provide the fishing vessels with needed supplies. The meal is offloaded and stored in the logistics hub in Montevideo, Uruguay, before it is either shipped directly to customers around the world, or to AKBM's factory in Houston, Texas, USA. In Houston, omega-3- and phospholipid-rich krill oil is extracted (KO). The division between meal shipped directly to customers and to lipid extraction depends on the biological seasonal variations of the krill fisheries, but in general, about 10% of the produced meal is used for omega-3 oil extraction, while the rest is shipped directly to customers. The majority of KM customers are in the aquaculture and pet industry. Figure 1 illustrates the process and various products produced on the boats and up to oil extraction. The further processing, established as described below, is not included in this illustration as it is still being established.

Upon extracting the KO from the KM, a residual protein-rich meal remains. This meal is either shipped to customers directly for use in aquaculture or to a newly established factory in Ski in Norway. The factory in Ski is established in order to maximize the value of the protein-rich meal, which is a side stream of the oil extraction. The side stream retains the food grade classification. A process to produce a nutritious protein product for human consumption has been established. Utilizing this protein fraction directly to human consumption without taking it via a feed fits well into the sustainable utilization of biomass by employing the food first principle [28]. The factory is in the process of establishing production, thus sustainability data for the production are being collected and will be added to the AKBM reporting when the data are available. The factory is built with specific

focus on energy efficiency and resource optimization with low water usage. The factory also has focused on total biomass utilization, thus any waste fraction from the protein production will be utilized, either in feed, as a fermentation substrate, as a bio-stimulant or other. The results from these efforts will be communicated as they are mapped [29].

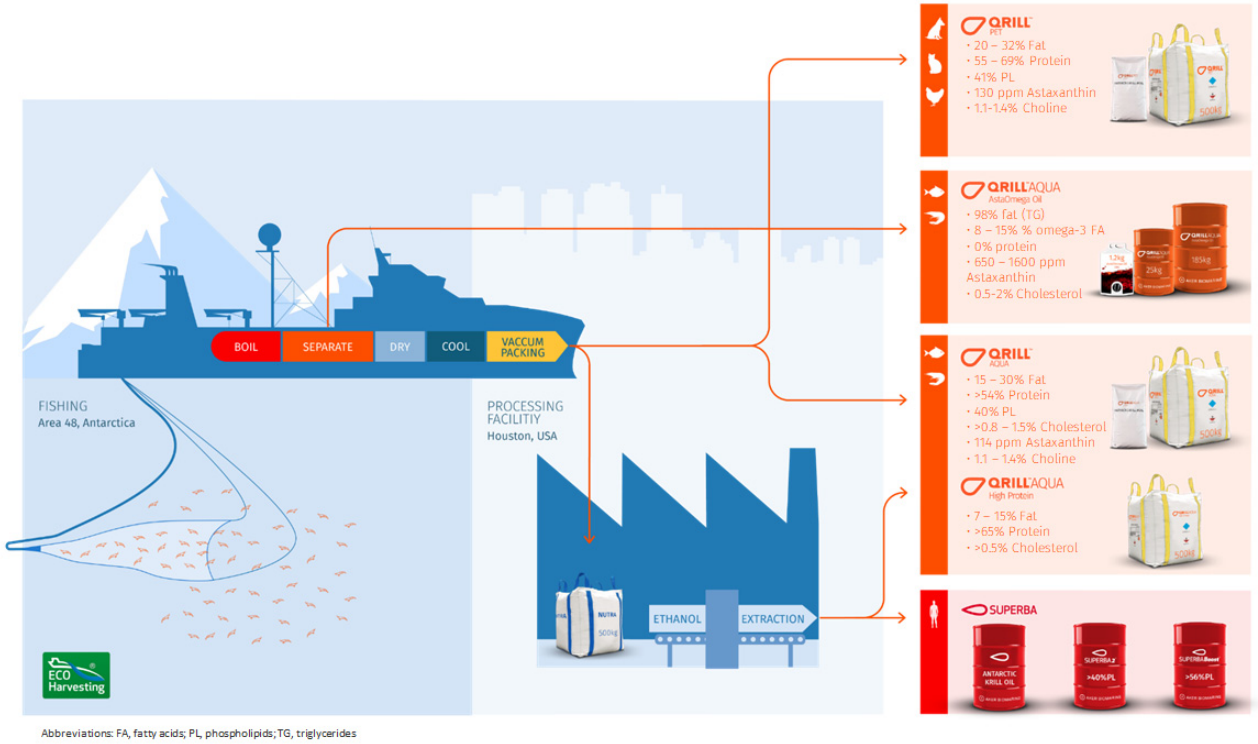

Figure 1. Illustration showing the process from fishing, production on-board, and further processing to yield products to different markets and for different applications.

Aker BioMarine (AKBM) stands for 65–70% of the global catch of krill [30], thus the efforts made by AKBM are important to ensure that the harvesting and utilization of krill is conducted in a sustainable manner [29]. This communication is composed in order to showcase how the company is working to improve sustainability in harvest and production as well as in conducting business. As a company, AKBM is committed to ensuring that resources from the ocean are used in a sustainable way and for the optimal purpose. The significance of improved harvesting methods, product quality, and ecosystem conservation is discussed, emphasizing advanced monitoring, data incorporation, and technological innovations. In addition, CO_2 hotspot mapping and how to use data to steer the reduction of the environmental footprint is discussed. Sustainable production and business operations also include many social and economic aspects which will be touched upon at the end of this communication, together with the resource utilization and circularity of the materials used. In this communication, activities to certify the fisheries' activities, as well as the process for accounting for greenhouse gas (GHG) emissions, are described. A selection of the activities conducted for reducing the energy consumption of fisheries and during on-board production is described in detail to showcase how the work can be conducted. The activities described will not be a fully exhaustive description of all activities conducted in order be as energy effective as possible, but rather representative examples of how innovation and development has guided the improved and more sustainable fisheries and production.

2. Certifications and Documentation of Sustainable Fisheries' Activities

A key focus for AKBM is to make sure that fishing activities do not impact the Antarctic ecosystem. Therefore, the independent certifications of AKBM activities from external certification bodies are ensured. MSC is the only global wild-capture certification program

that meets the best practice requirements set by the UN Food and Agriculture Organization, Global Sustainable Seafood Initiative (GSSI), and ISEAL. AKBM has been MSC-certified since 2010 and part of Friends of the Sea since 2016. MSC certification is carried out every fifth year, where the fishing operations are measured against MSC's Fishery Standard requirements and verified by an independent third-party reviewer, a Conformity Assessment Body (CAB). A surveillance audit is undertaken every year. Aker BioMarine is certified according to the fisheries standard and the chain of custody standard [31]. Friends of the Sea (FoS) has developed an additional sustainability standard for wild-caught fisheries, which was last updated in 2023. FoS, like MSC, requires the certification process to be conducted through an independent certification body [32]. The external certification structure is important as it provides an external evaluation of AKBM activities and allows the benchmarking of activities towards other fisheries.

Performing activities in the Antarctic comes with the responsibility of stringent control and constant adherence to current regulations, standards, and scientific findings. The Antarctic fishery, and under that, the krill fishery, is governed by CCAMLR, the commission for the conservation of Antarctic marine living resources. CCAMLR has been active since 1980 and is the institution that holds the most knowledge about the Antarctic ecosystem. The scientific committee evaluates the status and management of the fisheries on an annual basis. The evaluation is based on up-to-date scientific evaluations as well as data collected during real time or semi-real time monitoring of all fisheries conducted during the fishing season. Catch limits in each fishery are agreed using decision rules that ensure the long-term sustainability of the fishery. These limits and the other operational aspects defined in the conservation measures determine when, where, and how fisheries are conducted in order to manage the potential impacts on the ecosystem. These regulations are usually specific to a fishing season, and currently apply to toothfish, icefish, and krill fisheries. Other fisheries have operated at various times in the past and are no longer active.

Antarctic krill is harvested in the area around the Antarctic Peninsula, called Area 48. The fishery is precautionary and closely regulated by conservation measures (CM). These measures are CM 51-01 and CM 51-07 [33,34]. The total biomass in the Antarctic is estimated to be around 300 to 500 million tons [25]. Krill reproduces at an exceptionally high rate. The krill biomass was surveyed by CCAMLR in 2019 and estimated to be about 63 million metric tons. The regulated catch is capped at 620,000 MT which is less than 1% of what the estimated total biomass is believed to be in Area 48. Under this quota system, the biomass has been stable in its density and distribution across the estimates at 60.3 million tons in 2000 and 62.6 million tons in 2019, as reported by CCAMLR [26,35]. The conservative catch limits and the observed trends in biomass management contribute to the well-regulated and underutilized nature of krill stocks. Another study, using ten years of fresh acoustic biomass monitoring data, also confirms the consistently high densities of krill on smaller scales. This study presents a 10-year time-series on smaller scales (South Orkney) that confirms that the annual krill catches are kept well below the upper precautionary level for the area [27]. The last 15 years have been marked by more regular big-scale krill surveys in Antarctica which have not detected any systematic change in the krill population. As such, the conclusion by Krafft et al. [35], who organized the last big-scale CCAMLR krill survey, is that "active acoustic techniques from larger vessels currently remain the only practical krill surveying option that can synoptically sample at the scales considered here during most sea-states". As an alternative to larger vessels, unmanned vehicles are now also emerging as a viable option for survey and biomass mapping [36]. A more recent time-series published in 2023 has confirmed the significant concentration of krill and a fishery operating well below the upper precautionary limit [27]. A recent study confirms whales' return to ancestral feeding grounds in the Antarctic Peninsula. High densities and the re-establishment of historical behaviors indicate a recovering population [37].

The krill fishery has very low bycatch rates. A study published in Fisheries Management and Ecology [26] concludes that the bycatch in the Antarctic krill fishery (range is 0.1–2.2%) is lower than other trawl fisheries globally (range 10–55%). AKBM employs

Eco-Harvesting technology, a technology that was developed and patented by the company. The technology ensures that by-catching is avoided through exclusion devices, as well as gentle harvesting through slow-speed mid-water trawling with a modern pump technology that conserves both the biomass we harvest and the ecosystem around.

The monitoring of the fisheries is performed using information reported to the Secretariat in real-time and other short intervals during the fishing season. AKBM is the only krill operator that has internationally independent observers deployed on board vessels during 100% of the fishery operations who report directly to CCAMLR. This is an important step to ensure the transparency of operations. The status and management of the fisheries is reviewed annually by the Scientific Committee and its specialist working groups use the best available science and information, including detailed data from the fisheries and fishery surveys, and the CCAMLR Scheme of International Scientific Observation (SISO). Member countries maintain complementary management strategies in areas under their jurisdiction in the Convention Area, including waters adjacent to the Prince Edward and Marion Islands (South Africa), and Crozet and Kerguelen Islands (France).

In addition to the compulsory monitoring and reporting, transparency and adherence to the scientific work and measures in the Antarctic are key commitments for AKBM. AKBM facilitates science through providing scientists with time on vessels to perform research, and we share data from all our fisheries. AKBM also contributes annually to the Antarctic wildlife research fund (AWR) [38], which facilitates and promotes research on the Antarctic ecosystem to promote a healthy and resilient ecosystem.

Industrial fishing companies have come together to form ARK—the association of responsible krill harvesting companies—where the primary goal is to develop practices that ensures the long-term sustainability of both the krill fisheries as well as the ecosystem in the Antarctic. This has, for example, resulted in voluntary measures with the seasonal closure of areas to protect penguin breeding grounds [39].

3. Accounting, Reporting, and Reduction of Greenhouse Gas Emissions from Industrial Activities

One key focus of this communication is the demonstration on how to reduce emissions from fisheries and production activities in AKBM. In order to start reduction efforts, an important first step is to establish CO_2 accounting and standardized reporting that enables AKBM to know which activities have the most impact on our emissions, as well as communicate our impact factors and reduction efforts in a way that is easily understood by our stakeholders. GHG emissions in the AKBM value chain are measured using the standards and guidance stated in The Greenhouse Gas Protocol, where scopes 1, 2, and 3 emissions are mapped and converted to CO_2 equivalents using calculated conversion factors or available conversion factors [40]. The conversion factors used in GHG accounting were either calculated specifically for our vessels and operations or obtained from suppliers. AKBM-specific conversion factors were calculated by DNV, Det Norske Veritas, an accredited certification organization. GHG accounting is audited annually by a certified accounting body [40,41]. To structure direct efforts towards GHG reduction, a value chain approach was adopted. By employing the GHG protocol, AKBM has been able to hot-spot the value chain and identify where in the value chain activities have the highest impact in terms of GHG emission. These activities can then be targeted specifically for the reduction of CO_2 emissions. Figure 2 is a representative example of how GHG emissions are distributed along the value chain. The figure shows that the majority of emissions are related to the fisheries' operations. The harvest and production label represents the activities conducted by the active three fishing vessels, while the transportation to/from the fishing field represents the GHG emissions from the transportation vessel that transports products produced on-board to the logistics hub.

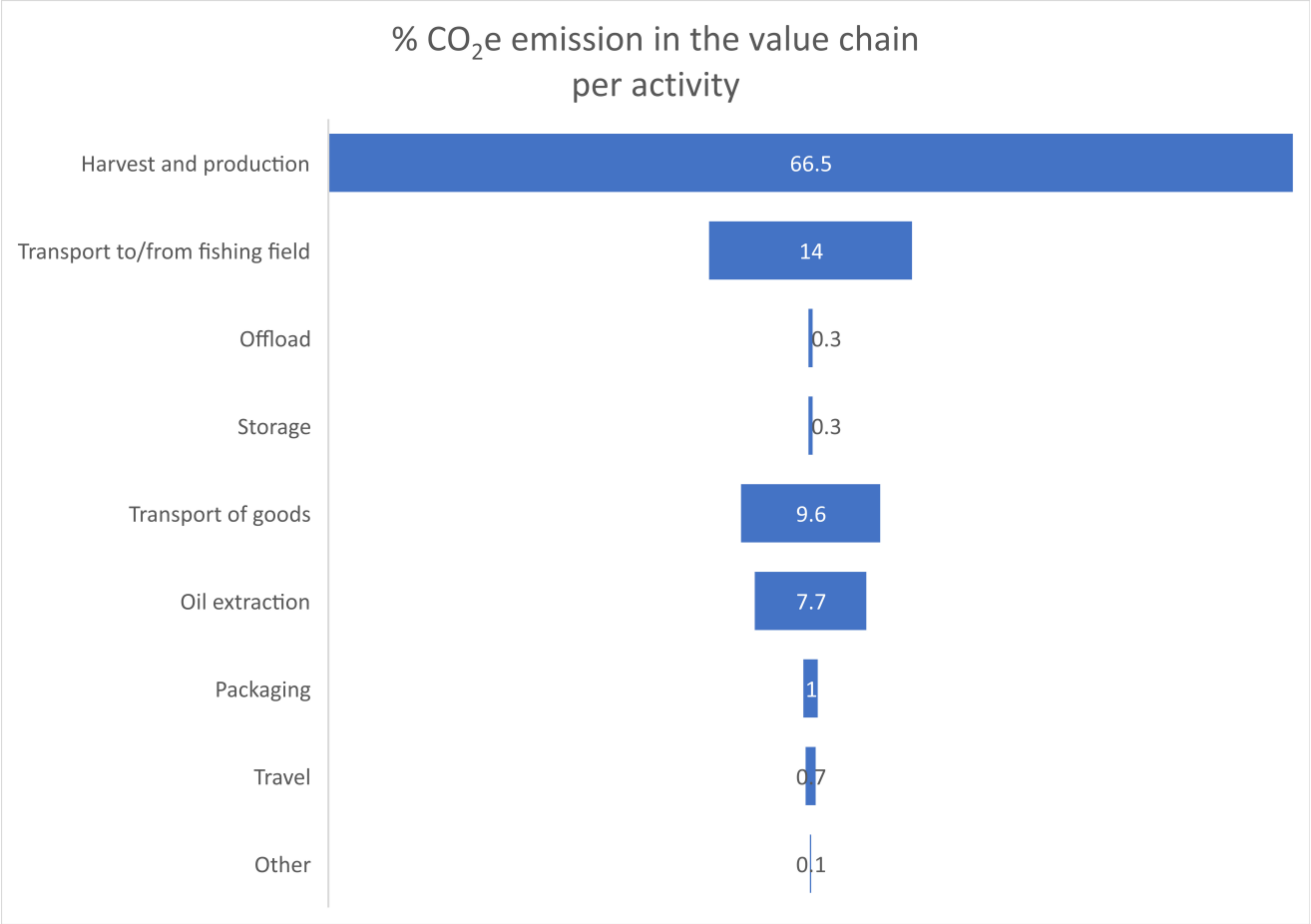

Figure 2. This figure shows the GHG emissions mapped in AKBM value chain as % division per activity. The four most emitting activities are harvest and production, which represent the emissions from our three fishing vessels. The second most emitting activity is the transport of meal to and from the fishing fields to our logistics hub. The third is transportation of goods performed by our transport suppliers from our logistics hub to customers globally, while extraction of omega-3 oil in our factory in Houston represents the fourth most intensive activity.

Most fisheries today are dependent on fossil fuels for their operations [42] which makes fisheries and on-board production activities something that is considered hard to abate. Decarbonizing the fishing industry has been identified as a bottleneck in many reports and studies [42,43]. The implementation of the use of alternative fuel sources is a challenge in fisheries, especially in long-distance fisheries. There are many different reasons for this, including access to the fuels and infrastructure, the retrofitting and safety of new fuels, regulations, and the energy density of the alternatives [36,42,43]. Alternative fuels will be needed in the future in order to reduce GHG emissions to very low levels; however, in the meantime, digital tools, life cycle analysis, and process analysis, combined with process optimization are valuable tools to reduce GHG emissions [36,42,43], and are being employed in AKBMs' operations. The reduction in the energy consumption per unit of product produced is essential to reduce the reliance on any fuel and improve fishing and production operations. In AKBM's annual report, the total GHG emission for all operations is reported together with the GHG emission per meal produced on board [29]. Improvement efforts during fishing and production are based on the analysis of fuel consumption during vessel operations, as can be seen in Figure 3. The figure illustrates that the activity that requires the most fuel, and thus is the most GHG-intensive in the value chain, is when the vessels are actively fishing and producing meal on-board. During fishing and production activities, the split between fuel usage for propulsion and the running

factory is estimated to be about a four-to-six ratio. This is based on measurements from the factory and overall fuel usage. By implementing improvements to the on-board production, the energy requirements per unit of product can be improved. This will thereby reduce the GHG intensity of production. Similarly, by optimizing fishing operations, energy consumption connected with the searching and catch of krill (propulsion) can be reduced. This will reduce the searching for krill and steaming portion of Figure 3, and similarly to the factory and processing improvement, reduce the GHG intensity of production. Below, the activities and estimated effect of the improvements with respect to energy consumption during fishing and on-board production are described as demonstration cases.

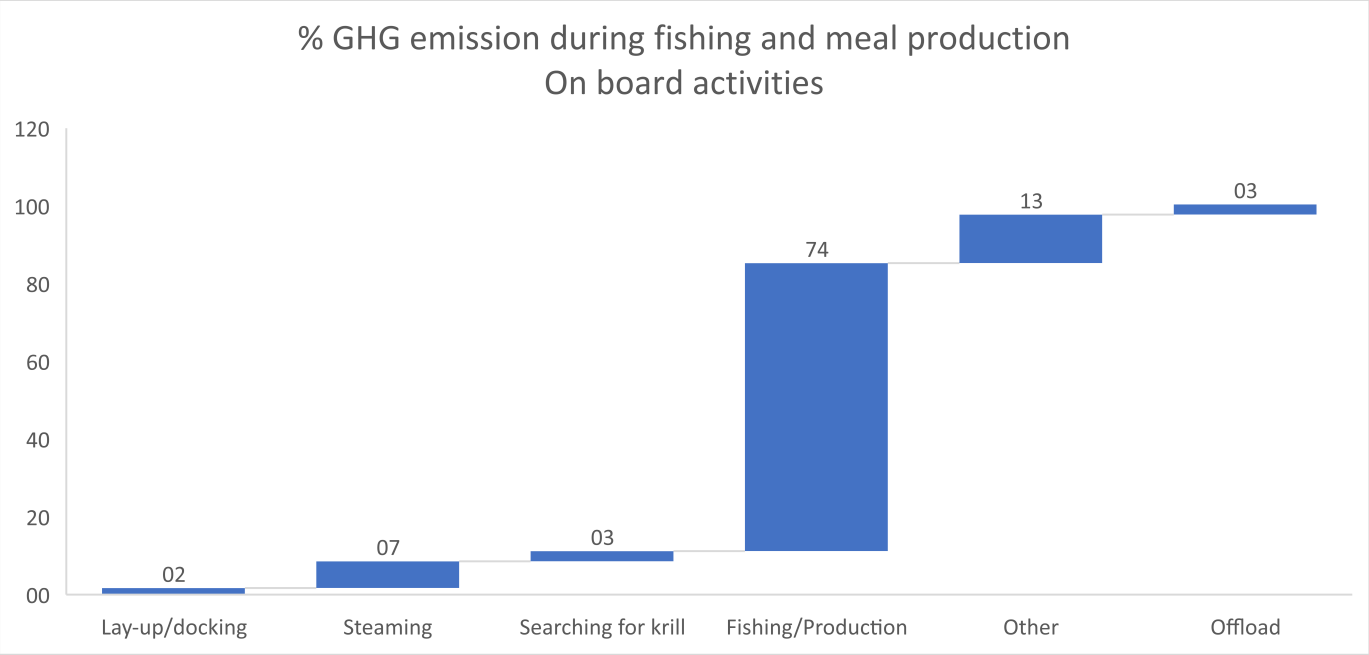

Figure 3. Description of how GHG emissions are linked to the activities on the fishing vessels. This plot shows that when the boats are harvesting and producing, the majority of GHG emissions are generated. The steaming represents when the vessels are going between different fishing areas or between the logistics hub and the fishing fields. The "other" category can be periods were boats are not fishing due to weather or some activities that are difficult to label in the remaining categories.

4. On-Board Factory Improvements to Reduce GHG Emissions

Meal production in the factory on board has a significant impact on the total emissions, as seen in Figures 2 and 3. A project was initiated to investigate whether optimizing the way the factories are operated can reduce the fuel usage. All three AKBM fishing vessels have meal processing on board. Each vessel has three lines of production, except for the smallest vessel which has two lines. The production lines on the different vessels are considered similar enough for the validation of process optimization on one of the vessels to be used as a demonstration case.

5. Demonstration Case 1—Investigating Factory Line Speed Effect on Energy Efficiency

In the first demonstration case, a project was initiated to evaluate whether the factory line speed could be optimized to reduce GHG emissions. The fuel efficiency in the factory was investigated in order to evaluate the effect of the speed and usage of factory lines in the on-board factory. The fuel efficiency was compared based on the total fuel consumption per produced unit of meal when one, two, or three factory lines were run in parallel. In this case, data from the vessel Antarctic Endurance were used. This vessel has installed several sensors on various infrastructure in the production line which allows for more accurate calculations of the effects of modifications to the way the factory operates. Factories are

operated based on the mass of krill fished and the quality assurance requirements, including the temperature and holding time. Unlike shore-based factories, the feeding into the factory varies with harvesting rates and the amount of krill and the holding time in the tanks. Thus, the factory must be able to operate as efficiently as possible at various feed rates.

In production, raw krill enters the factory where it is weighed and then routed into one of three holding tanks, representing the start of the factory line. The factory used has three lines, these are called A, B, and C in this demonstration case. The speeds of the factory lines are between 40 and 100 Hz, which amounts to between 10 and 20 tons of krill per hour. The speed of each line is described as the speed of the lamellar pumps pumping the krill to the pre-heating stage, where the biomass is pre-heated to about 55 °C before it is moved to the steam cooker to reach and hold 95 °C according to preset quality assurance parameters. The preheater uses indirect heating (hot water) while the steam cooker uses direct heating with steam injections. After steam cooking, the krill is decanted using a decanter to separate the liquids, containing mainly water and some lipids, from the solid mass. The solids are further dried using a disc or vacuum dryer to a dryness of about 8%. From the liquids, the lipids can be separated from the water phase. These lipids are rich in triglycerides and astaxanthin and can be collected separately or reintroduced in the krill meal. The water phase, often called stick water, can either be discarded, or in the case of this vessel, routed to an evaporator to isolate the proteinaceous biomass in the stick water. The evaporation can either be reintroduced into the meal or collected separately. The reintroduction of stick water into the meal is a separate initiative to reduce fuel consumption per unit of meal produced, as described below. Dried meal is then packed in either 500 kg or 25 kg bags based on customer specifications. The preheating, cooking, and drying steps are the most energy-consuming steps. The decanter uses little energy in comparison; however, overloading the decanter can lead to a loss of separation efficiency, and thus will impact the yield of the production.

The total factory speed was calculated by adding the speed of each line A, B, and C together. In the comparisons, one line running at 80 Hz is the same factory speed as two lines running at 40 Hz. Similarly, three lines running at 50 Hz is the same factory speed as one line running at 70 Hz and one other running at 80 Hz. In these calculations, Hz is used as a measure for speed. All three pumps in the demonstration factory are the same size with a similar performance. The data used in the calculations were flow scale data into the factory (Kg/min), the speed of the lamellar pumps into each factory line (Hz), the fuel consumption from fuel flow meters (L/h), the decanter differential bowl speed (rpm), the disc dryer motor amp (A), the steam consumption for each dryer (Kg/h), and the steam consumption for each steam cooker (Kg/h). The data were also verified against other available production data sources including packaging and QA/QC data. Due to the different granularities of the data and some shifts in responses, some data were sliced and shifted, but these modifications were verified using logged production and sales data.

In the analyses, only the speed and number of lines operated were investigated, and the temperature set points for various infrastructure were not a part of the scope. Production within a three-hour period was compared. The fuel consumption in the factory was evaluated based on both feed-in and product-out of the factory in order to make comparable data. This was denoted fuel per meal efficiency and measured in liters of fuel per Kg of product produced. The energy metrics were evaluated at different factory speeds for various line configurations. Due to possible minor differences, the number and combination of lines were considered. For a factory with three parallel production lines, there are seven unique combinations which were denoted A, B, C, AB, AC, BC, and ABC.

The analysis shows that the energy efficiency, as measured in the fuel per product, improves when the factory line speed is reduced and, conversely, that the energy efficiency decreases with the increasing factory line speed. The same trend is shown when two factory lines are run slowly compared to one line running fast, and three factory lines running slowly compared to two running fast. This is illustrated in Figure 4, where the fuel consumption per unit of meal produced versus the speed and number of factory

lines is shown. This analysis results in a new tool for monitoring the factory line speed with the resulting fuel per meal production unit so that the factory workers can monitor the operations and adjust the line speed to optimize fuel usage. As the tool has been recently developed, the impact of optimizing just the line speed needs further analysis, but preliminary estimates indicate that just line speed optimization can result in about a 1% reduction in fuel usage in total. This is a rough estimate based on several factors, including the total fuel consumption in the factory and historical catch and production data showing the amount of production time line adjustments which are possible. In the maximal effect, the fuel consumption per Kg meal produced can be improved by almost 70% according to Figure 4, from the least energy efficient production speed to the most energy efficient production speed. Based on Figures 2 and 3, factory processing accounts for almost 50% of the total AKBM fuel consumption. However, it needs to be taken into account that line speed setting is dependent on how much krill is being fished, and thus it is not always possible to run the lines at a low speed, and the average fuel consumption per unit produced is an average of what is seen in Figure 4.

As the tool is further implemented, the impact can be estimated more accurately, and the effects of other factory optimizations can be evaluated and implemented. While the impact is not large for the line speed alone, there is a possibility that other improvement efforts can yield additional GHG savings. The implementation of these types of efforts are especially important as they are ways of reducing GHG emissions without capex, and they save on costs as they reduce the energy usage; thus they are truly valuable tools for many factories.

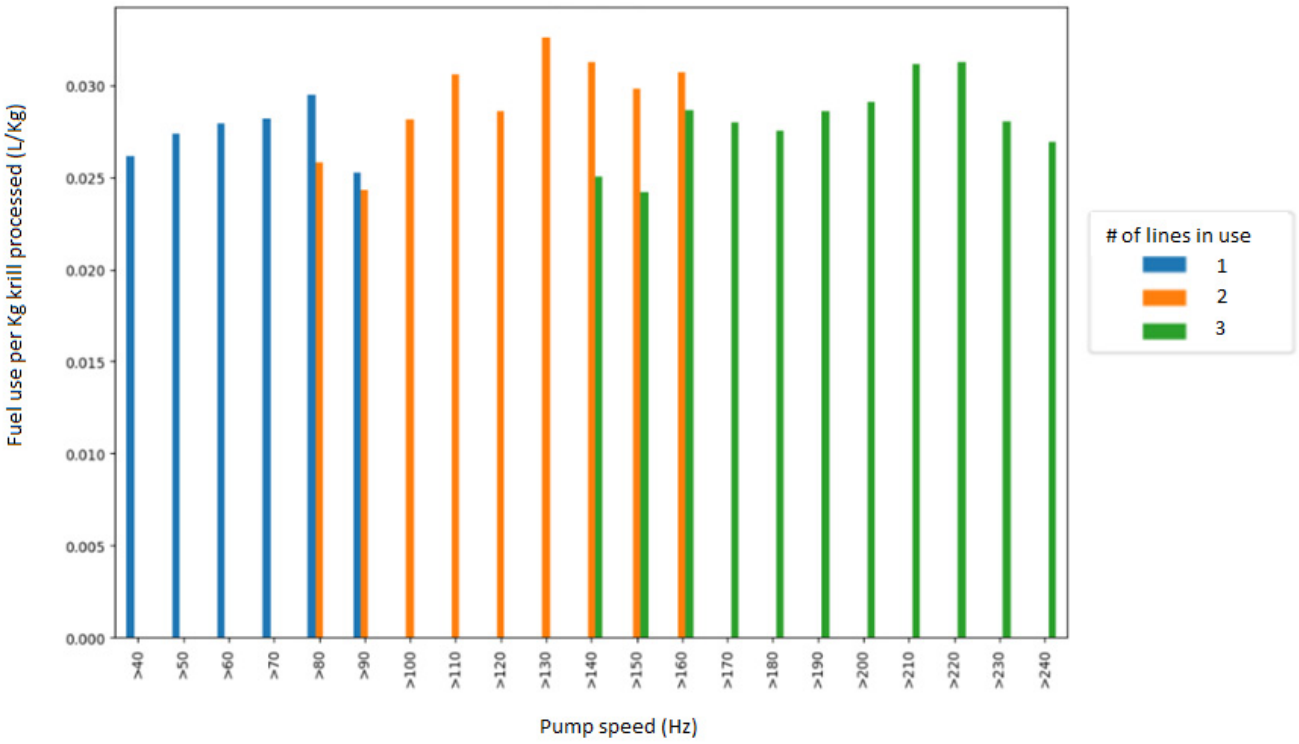

Figure 4. Fuel use during production when factory lines are run at various speed. Blue for one line in use, orange for two lines and green for three lines. The fuel used is assessed in terms of how much fuel is used in the factory per unit of krill that is processed depending on how fast the pumps into the factory are going. The analysis shows that running two or three lines at slower speed results in less fuel consumption per unit meal than running one or two lines at high speed. The output of the factory does not change, thus optimizing the factory line speed is a tool in reducing the GHG emissions from fisheries and production.

6. Demonstration Case 2—Increasing Yield to Increase Energy Efficiency of Production

Producing more product without increasing the energy consumption is an efficient improvement step. In order to increase the yield during meal production, stick water from the decanter was concentrated using a stick water evaporator. Heat used in the evaporator was waste heat generated in the factory during meal drying, thus minimizing the energy consumption during stick water inclusion into the meal. To evaluate the effectiveness of stick water inclusion, production yield was calculated. The yield was calculated as a % of the meal produced based on the mass of the raw krill fed into the factory. The total fuel consumption per meal produced compared to the production without stick water was then used as a measure of the GHG improvement during production. The catch and production data have been analyzed to determine if including stick water in the produced meal improves the GHG emission per unit of produced meal and to compare the fuel usage and production yield when stick water was not included in the meal in the same factory. Stick water inclusion in meal production is still being developed and the effect of the process is being investigated. Therefore, further monitoring and analysis is needed to determine the long-term effect. The early analysis indicates that the yield can be increased by as much as 2% with the same input energy. The effect of a 2% yield increase on one vessel amounts to about 1.4% of the total GHG emissions in AKBM, based on the division of fuel usage in the value chain, as illustrated in Figures 2 and 3.

7. Demonstration Case 3—Heat Recovery in Factories for Reduction of Energy Usage

During production, one of the steps is preheating the krill before steam cooking, as described above. In one vessel, an energy-saving measurement was implemented and evaluated by using waste heat in the factory to pre-heat the krill before cooking. In this process, waste heat from the steam cooking was routed to the pre-heater, replacing the need for boiler steam to pre-heat krill before cooking as described above, and thus reducing the energy needed for generating heat for the pre-heating step. The fuel consumption per produced meal was then calculated and the fuel used before and after routing waste heat to the pre-heating stage was compared and evaluated. The calculations were carried out in the same way for the factory optimization and the stick water inclusion, as demonstrated in cases 1 and 2, where the fuel usage per unit of meal produced was the measure. Demonstration case 3 was performed on a different vessel than the vessel investigating factory optimization and stick water inclusion. The results are estimated to a reduction of about 7% in the factory energy usage for running the factory during krill meal production. The fraction of energy usage in the factory with respect to the total fishing and production contribution of the energy to the factory depends on several input factors including the catch rates and factory speed, but a yearly average is illustrated in Figures 2 and 3. From this figure it can be deducted that a 7% energy usage reduction in all factories can represent up to 4% of the total fuel consumption for the vessels, and just under 3% of the total emissions from the value chain. This is based on the calculations that harvesting and production represent just under 70% of the total AKBM GHG emissions. While this setup and these measurements of effects are still under development, the extensive use of waste heat and factory improvements and optimization will result in a significant reduction in the total GHG emissions.

8. Optimizing Searching for Krill in Order to Reduce Fuel Consumption in Fisheries through Development of a Digital Tool

In order to reduce fuel consumption by optimizing the search for krill, a digital tool was developed in AKBM. The tool enables captains to use data-driven predictions to improve the harvesting efficiency. An improved efficiency in the fisheries results in reduced fuel usage per harvested unit of krill. The model is based on 10+ years of harvesting history combined with open sources' indirect data. The machine learning model predicts the likelihood of finding harvestable krill resources at different locations of the fishing grounds at any given time. Based on the predictions of the model, the captains can move the fishing

vessels to a location with a high probability of having harvestable krill resources. During fishing, it is a likely scenario that krill is highly abundant in the area where krill is actively fished, but the area is geographically very large and the stocks are spread out over a large area. Fishing is performed where the krill are in schools, and due to the size of the fishing grounds as well as the unpredictable behavior of the schools, it is very difficult to predict the exact location of any given school. This can result in large variations in catch volumes. The fishing vessels are optimized to harvest and produce krill meal, and thus using the vessels to search for krill does not result in the optimal usage of the vessels. The fuel usage dedicated to the activity of searching for krill is not straight forward to calculate, but it is estimated that searching for krill alone stands for just under 3% of the fuel usage for the fishing and production part of AKBM activities. Some of the fuel usage attributed to steaming and the category called other can also be involved in the searching processes as these are hard to separate (see Figure 3).

An application was thus designed and given the name "Krillviz" to minimize the search carried out by fishing vessels, and to provide better decisions during searching and fishing operations. The application combines a predictive model with unmanned energy-efficient drones equipped with echosounder and fish-finder tools. The model predicts the best location to fish and the drone is deployed to confirm that the location is good enough to move the big vessel to harvest in the predicted location.

The application and technology are still under implementation and test evaluation, but the data used are both direct and indirect data. Direct observations are data from echosounders located on the harvesting vessels, transport vessels, and unmanned vehicles. The echosounders used are Kongsberg Simrad DS 80 Sounders using 38, 70, and 120 Hz frequencies. The data are synced in near-real-time amongst the fleet and allow captains to obtain a better overview of the whereabouts of krill schools at any given time. Indirect observation data are historical catch data reported to CCAMLR from all active fishing vessels. In addition to these satellite data, weather and ocean models and research data are used. In building the model, data from all input have been combined and processed and a predictive model provides a local biomass estimate on a small scale where the fishing vessels are active. Fishing data are aggregated to daily data and catches from one day are combined with echosounder data and used to train the predictive model. In addition to the catch data, sea ice data [44] and models developed to predict copepod movement are combined in the predictive model. The exact predictive model used in the in-house predictive model is proprietary, but representative principles of modeling are given by Slagstad et al. [45].

The application together with the unmanned vehicle was tested early in 2024, and it has been demonstrated that information from the application enabled the boats to significantly improve their efficiency. In the first use case, autonomous vehicles were deployed from vessels that were actively fishing, and by combining the data, the application was able to predict that a repositioning of only 10–20 km would improve harvesting. Upon repositioning, it was determined that the harvesting efficiency went up to towards 100%. In addition, to reduce the GHG footprint of the fishing and production, using the application can also be of use for the management of the fisheries.

The prediction and monitoring of the krill biomass is not only of value for making the fishery more energy efficient; the data from the model can also be used in biomass estimations, scientific research for monitoring and following the krill biomass, as well as providing more data for quota setting. AKBM shares data from the application to further scientific research and ensure the best management of the fisheries.

9. On-Shore-Production Plant Improvements for Reduction of GHG Emissions

Part of onshore production activities in AKBM include oil extraction in a dedicated factory situated in Houston, TX, USA. To reduce the GHG emissions of this extraction factory, a number of activities are being initiated in the plant. The plant's energy usage and CO_2 equivalent emissions are monitored using the GHG protocol [40]. An in-house

data collection and analysis application has been developed in order to optimize the various steps involved in oil extraction. The aim is to reduce the input factors including electricity, water, and chemicals, while maximizing the output of the extracted oil. While the full description of the factory and developing on-going measures is not included in this communication, the principles of using an energy audit on a factory scale is well described in a case study from the food industry presented by Almomani et al. [46]. As seen from Figure 3, about 7.7% of the GHG emissions from AKBM activities are a result of processes to extract oil. The extraction process involves several steps, and in a similar way to the factory optimization described for the on-board process, the optimization of each stem can result in a more energy-efficient process. The GHG emissions from the factory are attributed to electricity usage and natural gas usage. The most applicable approaches for AKBM include switching, where possible, the energy usage from gas to electricity, as well as sourcing green electricity from the grid which is more readily available than green gas. Process efficiency is a key tool to improve the footprint of production. In the last year, due to several ongoing projects, periodic factory shut down has been conducted. These variations in factory running have complicated the quantification of the effects on emissions of ongoing improvement projects. More accurate reporting will be available upon continuous production over time.

An important tool to reduce the impact of factory operations is that the input factors are gas and electricity, which allows for low-emitting alternatives. These alternatives include green certificates for electricity and gas. Electricity can be produced using technologies like solar and wind, which results in a clean energy source. Purchasing green electricity and gas will allow us to reduce the GHG emissions by up to 7.7% of the total GHG impact of the value chain. Low emission energy production is a technological field in fast development, and it is fair to assume that low or no GHG-emitting electricity will be readily available in the future. As the efficiency efforts continue in the factory, the % impact from the unit on the entire value chain will likely be reduced, and the aim is to eliminate the GHG impact from Houston production by the end of 2029.

10. Industry Collaboration and Business Innovation for Scope 3 Reduction

Reductions in scope 3 emissions are activities that are performed in collaboration with our suppliers. The transport of goods and packaging is the largest contributor to scope 3 emissions in AKBM. Scope 3 reduction is reliant on collaboration and progress in the shipping industry. Challenges, opportunities, and regulatory and industry activities for the decarbonization of the shipping industry are described at length in the recent literature [47–49]. A number of different actions are taken for scope 3 reduction emissions, including optimizing freight routes and working with our suppliers to introduce green fuel on their shipping routes. AKBM has also been committed to an initiative named The First Movers Coalition, where over 90 large industry players have committed to use their combined purchasing power to drive green technology, including green shipping, forward [50]. The transportation of goods in AKBM is mostly conducted by sea, and while sea transport is today a GHG heavy industry, there are significant efforts being put in place to decarbonize the industry, including policies, MGO initiatives, green corridors, technical developments, and market initiatives [51–53]. Many of the shipping companies have also set their own targets, and while the solutions are not in place today, it is expected that significant improvements in the GHG from global shipping can be realized within the next decade [51–53]. Shipping currently accounts for approximately 9.6% of the total value chain emissions, thus a potential reduction of about 9% total value chain reduction is achievable through the de-carbonization of the shipping industry; however, the timing of the realization is yet to be determined.

The second largest scope 3 emission in AKBM is packaging. To reduce the footprint of the packaging materials, AKBM has been and continues to switch to materials for packaging that are less GHG intensive when being produced, as well as better suited for recycling such that reuse into new materials can be facilitated. The main type of packaging used is

big bags for meal production on board as well as steel drums for oil production. Plastic products that are used in fishing and aquaculture have been identified as high-quality with respect to recycling and thus are suitable raw materials in other production processes, including furniture, construction, and others [54]. The recycling and reuse of plastic is a separate industry and thus not a core business for AKBM [55]. Plastic waste from the fishing trawls as well as used krill meal packaging bags are handled by AION, a spin-off company of AKBM since 2020. This company has established circularity as a service for several different industries, transforming used plastics into new products including trays for cafeterias, shopping baskets for grocery stores, and pallets for transportation to replace wooden once, to name a few of the products created from used plastic products [56]. All products from AION represent GHG savings compared to conventional products. These savings are reported by AION.

11. LCA and EPD Analysis and Usage for Reporting, Benchmarking, and Improvement

An LCA analysis was conducted on the krill meal produced on-board which landed in Montevideo. The life cycle assessment (LCA) analysis was conducted according to the following Product Category Rules (PCR): preparation used in animal feeding for food-producing animals, PCR 2016:03, V.2.0, UN CPC 233. 2021-09-10. The PCR review was conducted by The Technical Committee of the International EPDR System. The LCA was conducted and given accountability by Riga Technical University, Institute of Energy Systems and Environment, Riga, Latvia. The independent third-party verification of the declaration and data was carried out according to ISO 14025:2006 and approved by The International EPDR System. The full EPD report is available at www.environdec.com (accessed on 9 June 2023) or by request from Aker BioMarine. In addition to the EPD according to the abovementioned PCR, an economic and mass allocation was performed by the LCA responsible.

The EPD format was chosen as it is used globally and well established, and there is an available PCR that can be used for ingredients for feed from marine-harvested processed products. Many feed producers are increasingly also adhering to the European version of environmental product declarations, named the PEF, or the Product Environmental Footprint. Product category rules for PEF declarations are named PEFCRs, and a suitable PEFCR is currently not available for the type of ingredient that AKBM produces, but as this becomes available, AKBM is aiming to develop this type of documentation as well. The two types of environmental documentation, EPD and PEF, are not directly comparable, and discrepancies are found with respect to the allocation and cut-off rules, as well as the modeling approach. The impact categories also differ to some degree [57]. Thus, a direct one-to-one comparison with a PEF declaration is not possible, but both allow for an evaluation of many levels of impact as well as the general trending of the product over time. Figure 5, demonstrates the system boundaries and the life cycle stages in the EPD in order to evaluate the environmental impact categories, and shows the upstream, core, and downstream processes included. It can be seen that the impact has been calculated for meal delivered to the AKBM logistics hub in Montevideo, Uruguay. The reason the impact was determined here was that KM customers are located at different locations globally, and thus an impact category for delivered meal would have to be calculated for each location. Therefore, AKBM communicates to customers the cut-off for the environmental impact, and thus the customer can add the impact for shipping into their calculations if required. Figure 6 and Table 1 show the LCA results for the different environmental performance indicators in the EPD preparation. The LCA was performed using 2022 data. A simple estimation of the same model for 2020 data indicated a total reduction in 36% of all impact categories, and an 18% reduction in GHG emissions for these two years. The results provide a trend to AKBM that can be used to continue efforts that have proven to be positive for GHG reductions.

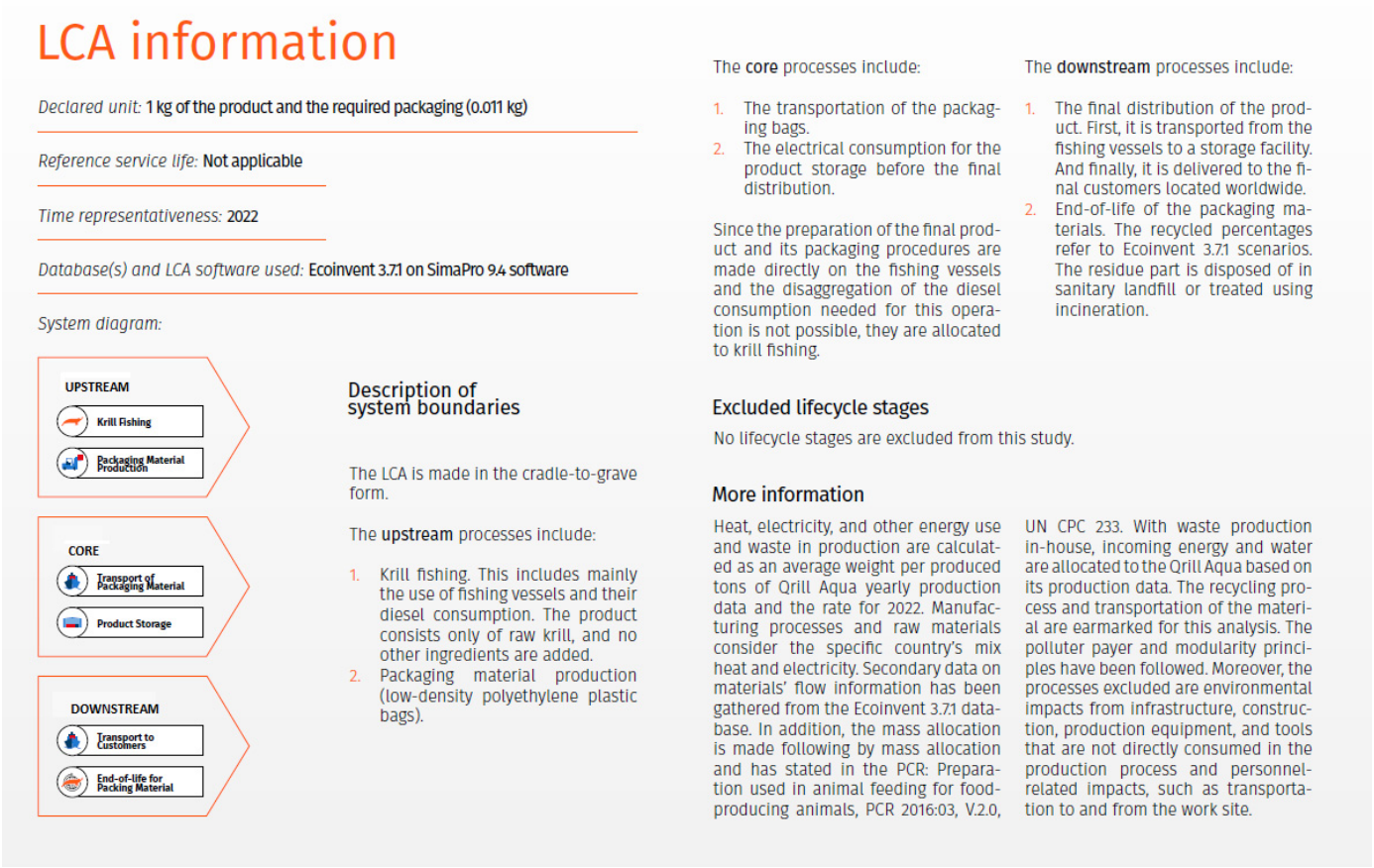

Figure 5. Explanation of EPD boundaries, upstream, core, and downstream process. The figure is taken from the full EPD report which is published on the Environdec platform.

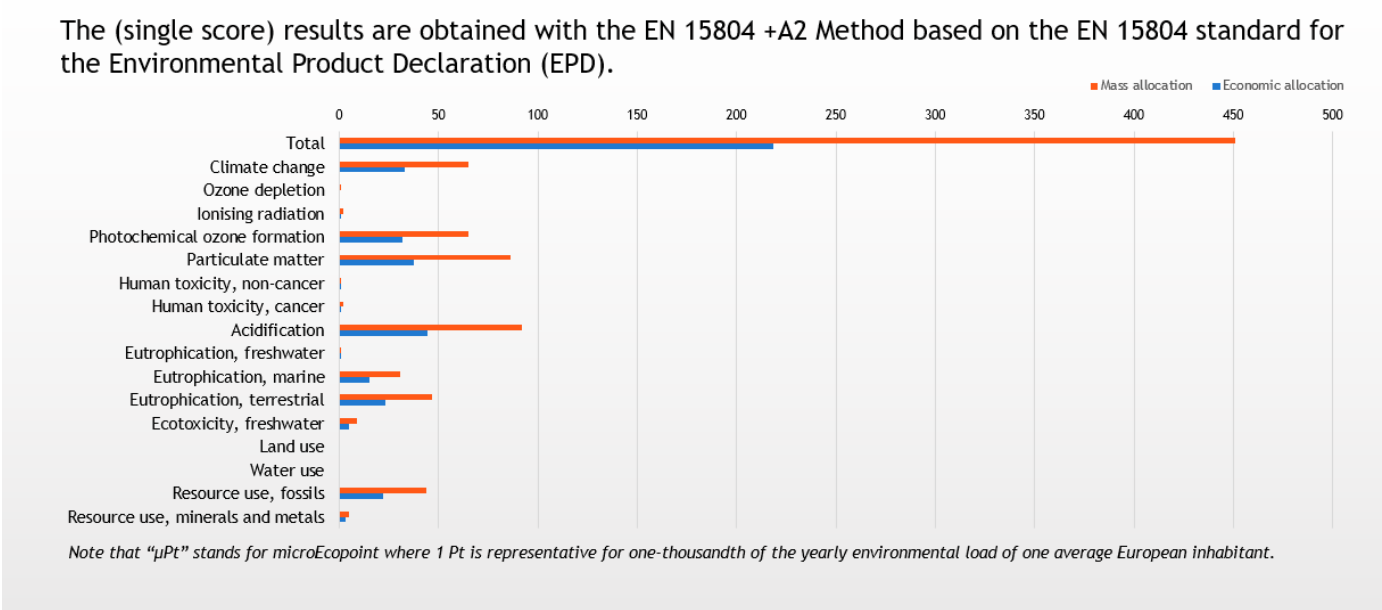

Figure 6. Single-score results for environmental impact categories in the EPD document. The scores are given an allocation based on either weight or economic value.

Table 1. Results from EPD LCA analysis performed for the EPD documentation.

Impact Category	Unit	Upstream Process	Core Process	Down-Stream Process	Total
Climate change	kg CO_2 eq	2.167804798	0.000963664	0.33325149	2.50
Ozone depletion	kg CFC11 eq	4.70743×10^{-7}	1.11436×10^{-10}	6.81531×10^{-8}	0.00
Ionizing radiation	kBq U-235 eq	0.134154272	3.49934×10^{-5}	0.019095853	0.15
Photochemical ozone formation	kg NMVOC eq	0.049017605	8.38491×10^{-6}	0.006185716	0.06
Particulate matter	disease inc.	5.61536×10^{-7}	5.83287×10^{-11}	9.31648×10^{-9}	0.00
Human toxicity, non-cancer	CTUh	1.1664×10^{-8}	8.39881×10^{-12}	2.8711×10^{-9}	0.00
Human toxicity, cancer	CTUh	1.5362×10^{-9}	8.9062×10^{-13}	2.11849×10^{-10}	0.00
Acidification	mol H+ eq	0.074575478	1.27365×10^{-15}	0.007954411	0.08
Eutrophication, freshwater	kg P eq	4.78856×10^{-5}	7.12363×10^{-8}	1.53321×10^{-5}	0.00
Eutrophication, marine	kg N eq	0.018014463	3.10007×10^{-6}	0.00231181	0.02
Eutrophication, terrestrial	mol N eq	0.197199905	3.43268×10^{-5}	0.025481248	0.22
Ecotoxicity, freshwater	CTUe	16.76788855	0.013391508	3.248236109	20.03
Land use	Pt	3.938592129	0.034794937	0.600144136	4.57
Water use	m3 depriv.	0.049251675	5.58782×10^{-5}	0.002584578	0.05
Resource use, fossils	MJ	30.10881641	0.007909807	4.025804073	34.14
Resource use, minerals and metals	kg Sb eq	2.68935×10^{-6}	1.11956×10^{-8}	1.43815×10^{-6}	0.00
Climate change—Fossil	kg CO_2 eq	2.166509948	0.000701092	0.332312631	2.50
Climate change—Biogenic	kg CO_2 eq	0.001047109	0.000262178	0.000771701	0.00
Climate change—Land use and LU change	kg CO_2 eq	0.000247732	3.93806×10^{-7}	0.00016715	0.00
Human toxicity, non-cancer—organics	CTUh	2.00818×10^{-10}	3.48608×10^{-13}	4.63541×10^{-11}	0.00
Human toxicity, non-cancer—inorganics	CTUh	5.99729×10^{-9}	2.01547×10^{-12}	1.60061×10^{-9}	0.00
Human toxicity, non-cancer—metals	CTUh	5.51445×10^{-9}	6.05727×10^{-12}	1.23175×10^{-9}	0.00
Human toxicity, cancer—organics	CTUh	1.08864×10^{-9}	1.9825×10^{-13}	4.91373×10^{-11}	0.00
Human toxicity, cancer—inorganics	CTUh	0	0	0	0.00
Human toxicity, cancer—metals	CTUh	4.47564×10^{-10}	6.9237×10^{-13}	1.62711×10^{-10}	0.00
Ecotoxicity, freshwater—organics	CTUe	1.850321125	0.00048013	0.280209497	2.13
Ecotoxicity, freshwater—inorganics	CTUe	4.919523892	0.001475054	0.799102561	5.72
Ecotoxicity, freshwater—metals	CTUe	1.00×10^{1}	1.14×10^{-2}	2.17×10^{0}	12.18

12. Discussions

This communication describes a representative collection of activities AKBM has started and continues to conduct to ensure that the company's sustainability profile and efforts are aligned with a sustainable future. Not all measures and possibilities towards improving the footprint of krill fisheries and production are included, rather a collection of initiatives towards different areas. An important goal is ensuring that the fishing activities that AKBM and the industry as a whole are conducting are safe and sustainable. In addition, AKBM is focused on reducing the environmental impact, improving the societal impact and adhering to regulatory and market expectations in terms of governance, reporting, and communication. The impact of the described sustainability efforts in this communication describes the current situation, and improvements and innovations are continuously being made and implemented to drive forward the improvement efforts, thus this communication is a snapshot of current activities in the company. Some efforts will not result in concrete measurable effects with respect to reduced emissions or a specific impact. However, trends in the right direction can be measured, and continued data will enable a more precise measure of impacts in the future.

Much focus in this communication has been kept on activities that target biodiversity and ecosystem conservation in our fisheries, as well as activities that reduce the GHG footprint of AKBM operations. Environmental impact monitoring and improvement is just one leg of conducting sustainable operations and ensuring that the products produced are of value to society and justify the fisheries and production operations.

Krill products are important in both human and animal nutrition. Starting with aquaculture, krill products are sustainably sourced essential marine ingredients that are and will increasingly be scarce in the future. Both proteinaceous and omega-3 ingredients

will be in need. Marine ingredients contain important marine proteins and omega-3 that aquaculture feeds need. However, many marine-harvested species come from fish stocks that are fished beyond the stock capacity, and the availability of wild-caught marine ingredients is not likely to increase in the future [23,24]. Providing an ingredient from a marine-harvested species that is harvested from very well managed and conservative fisheries is important to enable growth in the industry.

Another important part of delivering a sustainable ingredient to aquaculture is providing an ingredient that improves the performance of the farm. Many trials have demonstrated that adding a relatively small amount of krill to the feed leads to an increased appetite, growth and feed utilization, improved health and robustness, as well as to a better quality product with a better yield. This supports the better feed utilization and reduces the impact of health treatments in the farm; in addition, it supports the better use of the products produced. All of these benefits support sustainable farming.

As aquaculture is growing, it is essential that the aquaculture industry can use feed ingredients that support the health and well-being of the farmed animals as well as allow for the increased use of novel and alternative ingredients to support the increased feed need. Many novel ingredients have shown challenges with palatability and digestibility. Krill inclusion in the feed can alleviate this, and AKBM firmly believes that krill is an important puzzle in the introduction of novel ingredients [58–60].

Krill also is a source of omega-3 that is high in phospholipids, which are essential for not only animal, but also human health. A number of studies have demonstrated the beneficial effects of omega-3 from krill in humans and pets in addition to the effects seen in aquaculture. Utilizing omega-3 in preventive health for humans and pets is an essential step to reducing the impact of health treatments and reduced public health issues [61–63]. In addition, marine omega-3 is a superior ingredient, and like omega-3 to aquaculture, many fisheries suffer from over fishing and stock reductions, thus sourcing sustainably is essential to provide enough omega-3 to the market.

Transparency and reporting have become and will become even more important going forward. Being transparent with reporting and sharing successes and challenges within and between industries is a driving force towards more sustainable operations. AKBM reports on activities in the annual report to track and demonstrate progress over time. Reporting according to established frameworks allows for the comparison and benchmarking of activities of different industry players. In addition to environmental reporting, financial reporting including taxonomy scoring, as implemented in the EU [64,65], and reporting on socially responsible activities is essential [29]. Recently, to allow for a better comparison between sectors, the Corporate Sustainability Reporting Directive (CSRD) has been established to harmonize reporting across industries [66]. AKBM is constantly developing their reporting structure to adhere to current reporting requirements. The thorough and comprehensive periodical revision of all ESG ambitions, actions, and the following transition plans is also performed. These efforts are reported in the annual report [29].

13. Conclusions

This communication aims at providing a representative and descriptive overview of sustainability efforts in the value chain of a hard-to-abate fishing industry. The emphasis has been made on how fisheries and production has been investigated, managed, modified, and reported to reduce the impact of activities, while providing an important marine ingredient for the future. Management and reporting systems have been described and examples of concrete activities and modification in AKBM operations to reduce GHG emissions have been given. This communication provides discussions around building a transition plan for sustainable operations in the future based on the results of innovation. The landscape of technology is rapidly changing and AKBM keeps an updated, adaptable, and dynamic approach to utilizing and developing a data-driven technology landscape to improve the operations and reduce the impacts. There is still a considerable effort to be

undertaken to reach the needed level of impact in the future; however, many successful initiatives have proven that it will be possible to operate sustainably going forward.

Author Contributions: R.D. and L.B. has participated in manuscript conceptualization, writing, and revising. P.S. has provided data and insight for the fisheries and policy section. T.R., V.A. and S.G. have provided data for the methods implemented to improve harvesting and production on board as well as on land. All authors have read and agreed to the published version of the manuscript.

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Review

From Wastewater Treatment Plants to the Oceans: A Review on Synthetic Chemical Surfactants (SCSs) and Perspectives on Marine-Safe Biosurfactants

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Abstract: Synthetic chemical surfactants (SCSs) are a versatile group of amphiphilic chemical compounds synthesized from fossil fuel precursors which have found use in various industrial applications. Their global usage is estimated to be over 15 million tons annually, which has resulted in unabated environmental damage and potential toxicological effects to humans and other organisms. Current societal challenges to ensure environmental protection and reduce reliance on finite resources have led to an increased demand for sustainable and environmentally friendly alternatives, such as biosurfactants, to replace these toxic pollutants. Biosurfactants are biodegradable, non-toxic, and generally environmentally compatible amphiphilic compounds. Although there is enormous potential for microbial biosurfactants to replace SCSs, the key challenge limiting their commercialization relates to their low yields and substantial cost for production compared to that for the SCSs. In this review, we discuss the release of SCSs, with wastewater treatment plants (WWTPs) as the major point source of their release into the ocean, and we then delve into the consequences of these pollutants on marine organisms and humans. We then explore microbial biosurfactants as a replacement for SCSs, with a focus on rhamnolipids, and end with some perspectives on current and future work for commercializing microbial biosurfactants.

Keywords: synthetic chemical surfactants (SCSs); biosurfactants; rhamnolipids (RLs); wastewater treatment (WWT); marine environment

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1. Introduction

Synthetic chemical surfactants (SCSs) are a broad group of chemical compounds that are widely used in the pulp and paper industry, in oil recovery, as antimicrobial agents for fruit and vegetable preservation, in the manufacture of paints, pesticides, fungicides and herbicides, in the treatment and dyeing of fabrics and leather, as additives in lubricating oils, cosmetics and personal hygiene products, as well as in domestic and industrial cleaning products, amongst other applications [1–4]. According to a report by Allied Market Research [5], the surfactant market in 2019 was estimated at USD 41 billion and the expected Compound Annual Growth Rate (CAGR) was 5.3% from 2020 to 2027, reaching USD 58.5 billion. The report also states that the group of anionic surfactants are the market leaders, with its main representative being linear dodecylbenzene sulfonates (LAS), followed by non-ionic surfactants whose main representatives are nonylphenol ethoxylates (NPEO) and linear alcohol ethoxylates (LAE). The entry of SCSs into the environment occurs from a range of sources, the most important of which are wastewater treatment plants (WWTP) [6,7] where these chemicals, depending on their susceptibility to microbial

degradation, may be partially degraded into by-products [8]. Global surfactant usage is estimated to be over 15 million tons annually. Since numerous consumer products are marketed as down-the-drain disposable, most end up in WWTPs [9]. However, WWTPs do not effectively remove SCSs, mainly because these compounds and their by-products are not readily agglomerated and are subsequently sedimented out into the sludge fraction. Whilst LAS are mostly removed in WWTPs via effective microbial biodegradation processes, NPEOs are less biodegradable. NPEOs themselves show little toxicity, but their byproducts, mainly nonyl and octyl phenols, are toxic and can be readily absorbed by suspended soils [10]. This results in the unmitigated release of large quantities of SCSs from WWTP discharge points where these compounds and their by-products find their way into rivers and coastal areas and spread further out to sea [11–14]. These SCSs are potentially some of the most concerning pollutants that are released into the environment from WWTPs, which will be discussed in this review together with their partial degradation by-products. Since many types of SCSs are toxic and can remain in the environment undegraded for many years, their impact on marine ecosystems remains largely unknown. There is currently no effective technology available that can be implemented into a WWTP to remove SCSs, and as such this pollution problem continues unabated.

Growing public awareness about marine pollution and the risks it can pose to humans and other life has led to increased interest toward the use of naturally derived, biological surfactants (i.e., biosurfactants) which are commonly associated with low toxicity, high biodegradability, better environmental compatibility, and may be sustainably sourced compared to SCSs which are produced via organo-chemical synthesis in a laboratory or industrial chemical plant [15]. Despite the advantages of biosurfactants over SCSs, their commercialization for use in consumer products has been limited, mainly due to their cost of production and the relatively low yields produced in comparison to the production of SCSs. The most extensively studied biosurfactant for biotechnological applications are rhamnolipids (RL), due to their physiochemical properties and potential to reach high fermentation titers [16], but their entry into the market is still constrained by low yields and the fact they are derived from pathogenic bacterial species.

In this review, we discuss WWTPs as a major point source for the release of SCSs into the ocean, as well as some of the most important types of SCSs based on the risks they pose to the environment and to humans. We explore biosurfactants as replacements for SCSs as a way to help mitigate the risks, with a focus on RLs for applications in marine-safe personal care products, and also discuss the pathways of current and future work for increasing the uptake of this important biosurfactant class.

2. Priority SCSs of Concern and Pathways of Entry to the Ocean

The pathway for entry of SCSs into the environment is generally the same almost anywhere in the world, regardless of climate, geographical location, or social and cultural habits. The process begins with the industrial manufacture of the SCSs, followed by their use and then discarding them by some form or another (commonly down roadside or household drains). These pollutants find their way to a WWTP or, if such infrastructure is not available, into streams and rivers [17,18]. Whilst a fraction of SCSs and their by-products end their journey on land, the final destination for the bulk of these chemicals is the ocean (Figure 1), mainly from the effluents of WWTPs [19–22]. Direct discharge of SCSs into the environment is also possible, for example, as in the case of pesticide application to agricultural land, and the spraying of synthetic chemical dispersants on the sea surface to combat oil spillage.

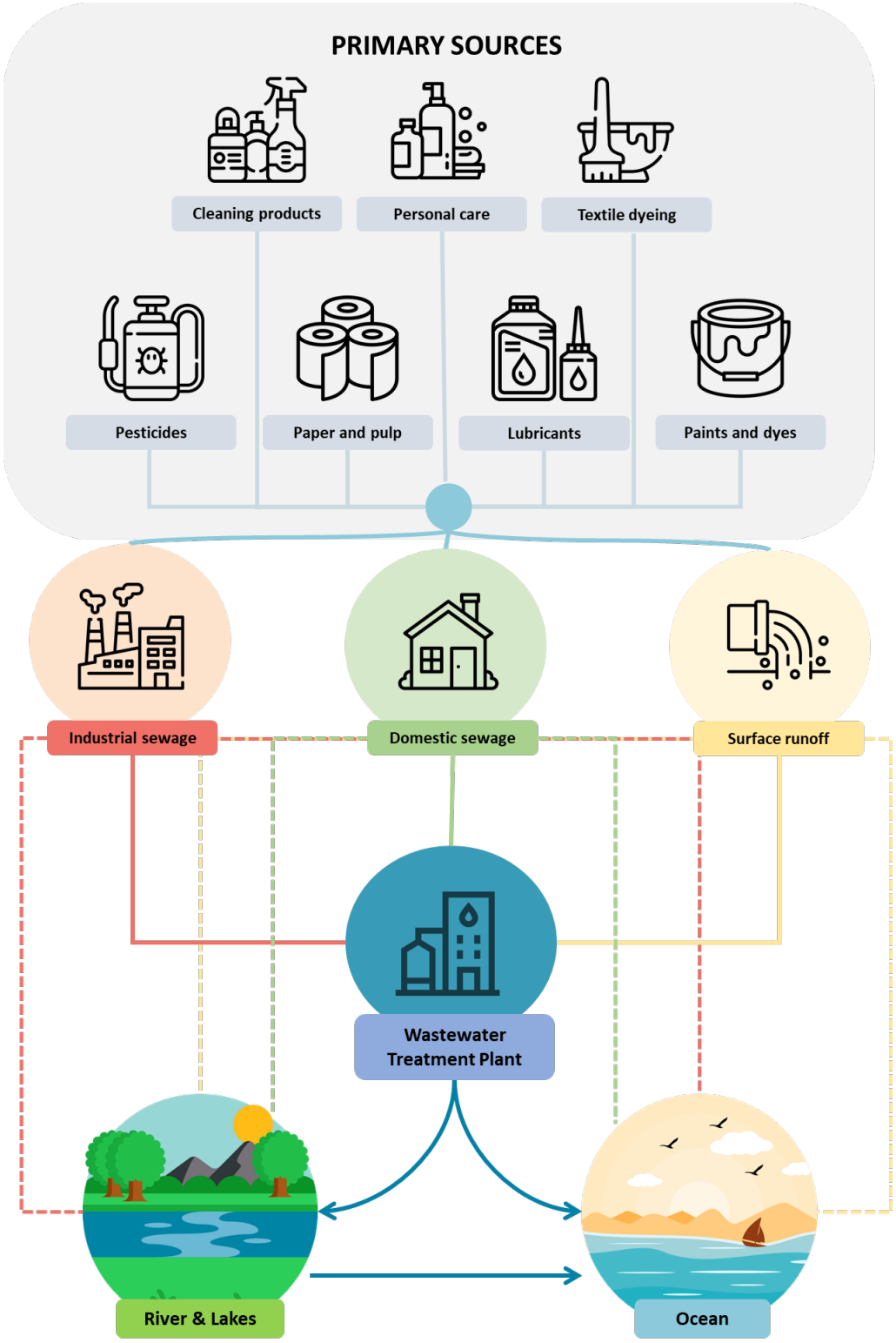

Figure 1. Pathways for entry of synthetic chemical surfactants (SCSs) into the oceans. Solid lines represent treated discharge through a WWTP; dotted lined represent direct discharge of untreated source SCSs into the environment.

2.1. LAS, NPEO, and Their By-Products in WWTPs

The first widely commercialized class of SCS was linear alkylbenzene sulfonates (LAS) (Figure 2), which has increased in use over the years [23]. Due to extensive use for many

years, there is concern regarding its long-term presence in the environment and its toxic effects to a range of organisms. LAS are a class of anionic surfactants consisting of a hydrophilic sulfonate head-group and a hydrophobic alkylbenzene tail. First introduced in the 1930s in the form of branched alkylbenzene sulfonates (BAS), LAS is one of the oldest and most widely used SCSs. It is used in numerous consumer goods that include personal care products (e.g., toothpaste, shampoos, soaps) and household care products (e.g., dishwashing liquids, laundry detergents, spray cleaners) [24]. Following environmental concerns, BAS were replaced with LAS during the 1960s [25], and since then production of LAS has increased significantly, from approximately 1 million tons in 1980 to 3.5 million tons in 2016, making them the most produced anionic surfactant after soaps.

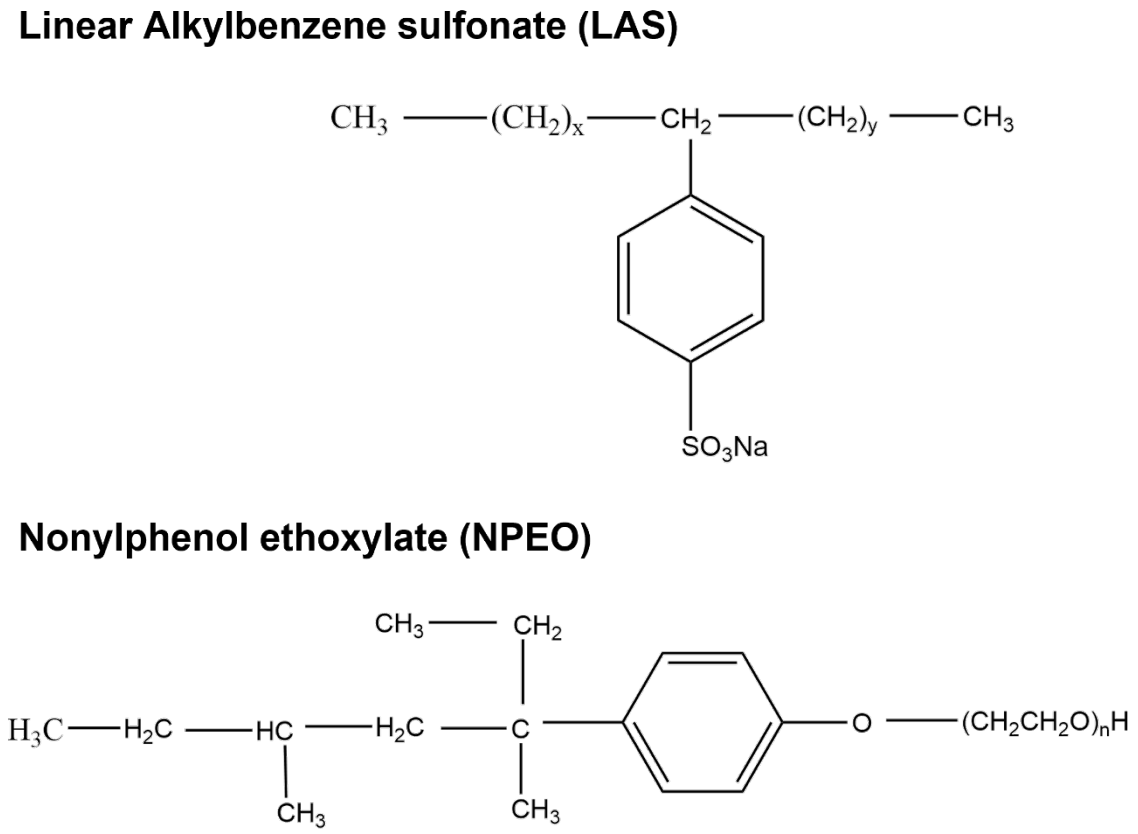

Figure 2. Chemical structure of Linear Alkylbenzene sulfonate (LAS) and Nonylphenol ethoxylate (NPEO).

Since the beginning of the commercial introduction of synthetic surfactants, they have since been detected in the environment at increasing concentrations. Decades following its commercial introduction, several researchers reported the occurrence of LAS in different environmental matrices. For instance, McAvoy et al. [26] monitored fifty sewage treatment plants in the USA and found that the average concentration of influent LAS was 5 mg L^{-1}, with an average removal of 77% to 99%. Tabor and Barber [27] evaluated the occurrence of LAS in the Mississippi River (USA), which is the largest river in North America and its basin covers 41% of the US territory; the concentration of LAS along the river ranged from 0.1 to 28.2 μg L^{-1}. In a study by González-Mazo et al. [28], the behavior of LAS was evaluated in a coastal zone of the Bay of Cádiz (southwest Spain) under the effect of strong tidal currents and directly receiving untreated wastewater discharge from a population of approximately 100,000 inhabitants, and the occurrence of dissolved LAS was reported to range from 2.7 to 1687.2 μg L^{-1}, while the LAS adsorbed to suspended solids ranged from 14.4 to 5941.0 μg L^{-1}. Sakai et al. [29] reported the occurrence of LAS in two rivers in Malaysia, with concentrations ranging from 3.3 to 2406.8 μg L^{-1} in the Langat River, and from 1.9 to 1044.8 μg L^{-1} on the Selangor River.

Another important group of SCSs that are prevalently in WWTP effluent discharge and of concern are the nonylphenol ethoxylates (NPEO) (Figure 2). These are commercially important, non-ionic surfactants which are used in detergents, paints, pesticides, personal care products, plastics, and a variety of other products and applications. The European Union prohibits their use due to their effects on human health and to the environment [30,31]. The main issue regarding this group of SCSs is that during their biodegradation, some recalcitrant by-products are generated, such as 4-nonylphenol (4-NP) and 4-octylphenol (4-OP), which can act as endocrine disrupters [32–34]. The occurrence of 4-NP has been reported in tap water [35,36] and across various environmental matrices, such as in rain and snow [37], and in waters and sediments of rivers [38–41], lakes [42], estuaries [43,44], and marine environments [45]. Concentrations differ depending on the discharge source and location. 4-NP has been detected in rivers at 0.015–2.25 μg L^{-1} [46] to 15 μg L^{-1} [19], whereas in river sediments concentrations can be higher, reaching 5100 mg kg^{-1} [47]. In 2021, Lalonde and Garron [48] assessed the freshwater environment at 35 sites in Canada and reported concentrations of 4-NP, nonylphenol monoethoxylate (NP_1EO), nonylphenol diethoxylate (NP_2EO), and octylphenols ranging from 1.29 to 477.22 ng L^{-1}. Based on the dominant activities present upstream in their watersheds, the sampling sites were categorized into the following groups: mixed use, municipal wastewater treatment plant (MWWTP)-associated, textile mill, urban, and reference sites. The study found that 4-NP and 4-OP activities were detected more frequently in sites associated with urban and effluent launch from WWTP.

Surface runoff can also carry SCSs (and other pollutants) from urban areas, due to the use of these compounds in a diverse range of products for use outdoors, such as lubricants used for motor vehicles [49]. In 2021, Zhao et al. [50] investigated the occurrence of 4-NP and 4-OP in Pearl River in rainfall runoff, and the maximum 4-NP concentrations found were 14.5 μg L^{-1} in surface water and 3.1 μg g^{-1} in sediments. According to their study, the mass loads from runoff rainfall were 3–62 times higher than those from WWTP effluents, suggesting runoff from rainfall is an important source of SCSs into receiving waters. In a study by Salgueiro-González et al. [39], the concentrations of 4-NP and 4-OP were measured in water and sediment collected along the Minho River estuary in Portugal. It was reported that these NPEO degradation by-products were present in almost all samples and was directly related to the release of treated effluent into the river from a WWTP. The concentrations of 4-NP and 4-OP ranged, from 0.05 μg L^{-1} to 0.888 μg L^{-1} in liquid samples, and from 13 ng g^{-1} to 4536 ng g^{-1} dry weight in sediment samples.

Compared to anionic and non-ionic surfactants, cationic surfactants represent a smaller class of surfactants. It is worth noting that cationic surfactants such as quaternary ammonium compounds (QACs) have been identified as emerging contaminants in sewage sludge and estuarine sediments with values above 100 μg g^{-1} [51]. Although cationic surfactants are not widely used, their higher sorption capacity and poor anaerobic biodegradation are the reasons for such high concentrations in sewage-impacted estuarine sediments [52]. Cationic surfactants are used in personal care products such as hair conditioners, as quaternary ammoniums are positively charged which attracts to negatively charged hair providing a very effective binding to allow absorption of conditioning compounds to act [53]. Since such products are disposed down the drain, cationic surfactants can accumulate and become persistent in the environment considering the increased consumer use of such products. More research is necessary to monitor the accumulation of cationic surfactants compared to anionic and non-ionic surfactants since their levels are rising [51].

2.2. Fate of SCSs through WWTPs

Due to the widespread and extensive use of SCSs in many products and their applications worldwide, their presence in sewage effluent is omnipresent. As such, assessing pollutant loads in effluent treatment processes needs to account for the presence of these chemicals. The aim of a WWTP is to significantly reduce the quantity of carbonaceous (organic; predominantly determined as biological oxygen demand (BOD)) materials, as well as pathogens and, where sensitive waters are involved, nitrogen (N) and phosphorus (P)

compounds prior to being discharged into receiving systems [54,55]. If these materials are not removed, this will result in their release in large quantities into the environment which can have deleterious effects on dissolved oxygen concentrations, as well as the trophic state and ultimately the well-being of the flora and fauna in receiving waters [56]. There are technologies capable of effectively eliminating these compounds, but this requires high investment, increased operating costs, and specialized staff. The most promising technologies include advanced oxidative processes (AOP), ozonation associated with hydrogen peroxide, photocatalysis, micellar enhanced ultra-filtration (MEUF), as well as more current methods such as adsorption on activated carbon, nanofiltration in membranes, reverse osmosis, chlorination, and reactors with UV lamps [3]. Implementation of such technologies occurs at a much slower rate compared to the increasing industrial manufacture, commercial and consumer use of SCSs.

A WWTP works as a "factory" that, by established microbial consortia, executes a diversity of metabolic pathways that direct energy from organic matter to a central metabolic pathway, ending up in products such as gases (methane and carbon dioxide) and new biomass. An in-depth description on the workings of a WWTP is outside the scope of this review, so the reader is referred to other works that focus on this topic (e.g., [57]). In this WWTP "factory", the consortia of microbial "employees" prioritize the use of simpler and easily metabolizable substrates over more complex ones, such as SCSs, that metabolically require more energy to break them down by biological systems, as can be seen principally in microorganisms. For example, with respect to LAS, the most widely used SCS, its biodegradation begins with the hydroxylation of the alkyl chain attached to the aromatic ring [58] with successive β-oxidations, followed by desulfonation, and finally the cleavage of the ring [59]. This process is dependent on oxygen which, under anaerobic conditions, is generated from water molecules via dioxygenases which are subsequently added to the alkyl chain via hydroxylases. As an alternative to this route, Lara-Martín et al. [60] reported that the degradation of alkylphenols can be initiated by the addition of fumarate to the alkyl chain instead of via hydroxylation. The following steps proceed via the conventional β-oxidations leading to the formation of benzoyl-CoA and cleavage of the aromatic ring. These degradation steps are specific for each type of SCS chemical and may vary according to the type of WWTP since each treatment facility will vary in microbial consortia, as well as geochemical and abiotic conditions. Due to these reasons, it is difficult to remove SCSs in conventional WWTP, and the majority of these chemicals end up passing through wastewater facilities and are released into receiving waters.

The average influent and effluent LAS concentrations going through WWTPs have been monitored in different countries, as summarized in Figure 3. Whilst the majority of LAS is removed in WWTPs, a proportion of effluent LAS passes through WWTP and ultimately enters the ocean. Considering the scale of products containing LAS, and although concentrations of effluent LAS appear to be minimal, the volume of effluent LAS released into the oceans each year around the world is staggering. Considering its very slow rate of biodegradation under environmental conditions, its environmental accumulation will be expected to result in deleterious consequences to marine life. It is worth bearing in mind that the figures are approximate values since treatment types vary from one WWTP to another, due to different methods and country legislations. These values are likely to be a great underestimate in comparison to the actual amounts, emphasizing the greater concern of SCS effluents into the ocean and the consequences and ramifications associated with this. In effect, it is largely not possible to remove SCSs using conventional WWT systems to curb their release into the environment. Additional infrastructure is required to be implemented to a plant in order to more effectively capture these chemicals, either by physically separating them out of the wastewater, or chemically or biologically breaking them down into innocuous end products before they are released into effluent streams. However, this increases the operational complexity and cost of the treatment process, which is a major impediment toward this end. To circumvent this problem, one way is to address the problem at the source. For example, recent years have seen manufacturers

of SCSs investing into less harmful (i.e., biosurfactants) replacements for these chemicals. Consumer awareness about the impacts of SCSs (and other anthropogenic pollutants) to the environment and to human health has spurred manufacturers to invest in biosurfactants (discussed below).

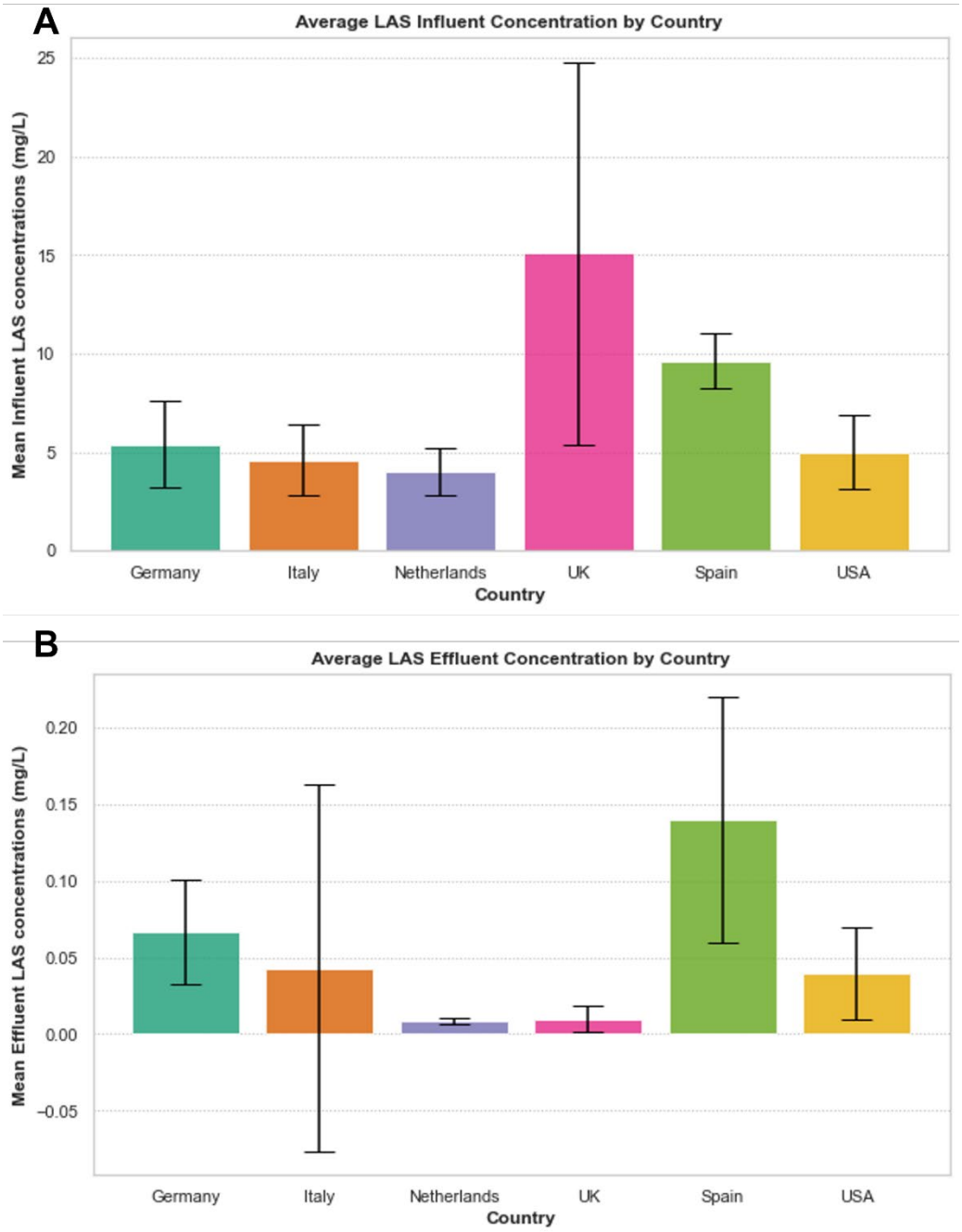

Figure 3. Mean influent (**A**) and effluent (**B**) values of LAS concentration (mg/L) in WWTPs across different countries (Germany, Italy, The Netherlands, UK, Spain, and USA). Error bars represent standard deviation. Values are taken from Feijtel et al., 1996 [61] and McAvoy et al., 1993 [26]. Values are approximate and will vary from one WWTP to another as well as country. Negative standard deviation error bar in (**B**) Italy is likely due to a lack of data in day one of the monitoring study due to major plant disturbance from data obtained by Di Corcia et al., 1994 [62].

3. Environmental Risks of SCSs on Aquatic Organisms and Humans

SCSs and their degradation by-products have been shown to exhibit toxicological effects on humans and upon the full trophic range of aquatic organisms. Once in the environment, these chemicals can cause deleterious consequences to living cells, such as impairing cell membrane function [63]. Table 1 presents some of the reported effects of LAS, which is the most widely used and arguably the most concerning SCS, and 4-NP which is the most common SCS degradation by-product detected in WWTPs and released into receiving waters. An important concern relates to WWTPs located upstream of aquaculture farms, considering the ease at which by-products of some types of SCSs, like 4-NP, become transported through the trophic chain and may end up on a plate for human consumption.

Table 1. Effects of LAS and 4-NP on aquatic species.

Organism	Surfactant	Effect	Dose	Ref.
Acartia tonsa	LAS	Larval mortality and reduced development rate.	0.54 mg L^{-1} *	[64]
Oncorhynchus mykiss	LAS	Hypertrophy of the lamellar gill epithelium and reduction of swimming ability.	0.2 mg L^{-1} $^{+}$	[65]
Daphnia magna	LAS	50% mortality (EC50) during 48 h of exposure.	3.54 mg L^{-1} **	[66]
Lithobates catesbeianus	LAS	Enlargement of liver sinusoids and vacuolization of hepatocytes.	0.5 mg L^{-1} *	[67]
Lithobates catesbeianus	LAS	Hypertrophy of the myocardium.	0.5 mg L^{-1} *	[68]
Channa punctatus	4-NP	High DNA damage. Genotoxicity in gills, liver and kidney tissue.	630 µg L^{-1} *	[69]
Labeo rohita	4-NP	Swelling and rupture of the secondary gill lamella, necrosis in liver tissue, reduction of hemoglobin, and blood erythrocytes.	54.8 µg L^{-1} *	[70]
Salmo trutta caspius	4-NP	Injuries to branchial tissues and intestinal tissues.	100 µg L^{-1} *	[34]
Salmo trutta caspius	4-NP	Change in estradiol and testosterone levels, histopathological lesions in the liver and kidney.	100 µg L^{-1} *	[71]
Lithobates catesbeianus	4-NP	Oocyte atrophy, erythrocyte nuclear abnormality, darkening of skin pigmentation.	100 µg L^{-1} *	[72]
Oreochromis niloticus	4-NP	Increased frequency of reproductive stages.	0.032 mL L^{-1} *	[73]
Oreochromus spilurs	4-NP	Altered liver architecture, with lysis, core loss, necrosis and fat infiltration.	15 µg L^{-1} $^{++}$	[74]
Heteropneustes fossilis	4-NP	High accumulation in the brain, followed by gills, liver, kidney, ovary, and muscles.	164 µg L^{-1} *	[75]
Danio rerio	4-NP	Testicular damage and reduced sperm density.	150 µg L^{-1} $^{++}$	[76]
Clarias gariepinus	4-NP	DNA damage and erythrocyte apoptosis.	100 µg L^{-1} $^{++}$	[77]
Rana catesbeiana	4-NP	Abnormalities in the nucleus of erythrocytes.	100 µg L^{-1} *	[78]

* = LC50, $^{+}$ = NOEC (no observed effect concentration), ** = EC50, $^{++}$ = Not determined.

LAS has been shown to directly affect biological activity by binding with enzymes, proteins, and phospholipids, altering their function and, consequently, triggering undesirable biochemical reactions and promoting metabolic imbalance [79]. Kusk and Petersen [64] evaluated the acute toxicity of LAS for the crustacean species *Acartia tonsa* (during 48 h and 18% salinity) and verified a mortality rate of 50% (LC50) at a LAS concentration of 2.1 mg L^{-1}. The authors also found that a concentration of 0.54 mg L^{-1} caused a 50% larval mortality rate and a reduced development rate. Hofer et al. [65] studied the chronic effects of LAS in rainbow trout (*Oncorhynchus mykiss*) at a concentration of 0.2 mg L^{-1} over 54 days and observed hypertrophy of the lamellar gill epithelium and reduction of swimming ability.

As mentioned before, the degradation by-products of SCSs can pose a greater risk to the environment as they are more toxic than their respective precursor parent compound. This has been reported in the cases of 4-NP and 4-OP [69–71,75,80], which are generated from the degradation of the surfactant NPEO [7,30,47,81–84]. NPEO is a non-ionic surfactant widely used in the production of detergents, lubricants, antistatic agents, high-performance textile cleaning agents, pesticides, antioxidants for rubber production, and lubricating oil additives [85]. The alkylphenols are a group of SCSs with an enormous diversity of compounds, each with distinct characteristics [86,87]. Their degradation produces by-

products that are more harmful than their respective parent compound [88], and have a high capacity for bioaccumulation [42,43,89], allowing them to be easily transported through the trophic chain and to potentially end up in food for human consumption. NPEO has nine carbons attached to the aromatic ring in the *para* position in relation to the *n*-ethoxylated group. When these NPOEs reach a WWTP, they may be partially degraded by microorganisms, forming 4-NP and 4-OP. In addition, genotoxic effects have been reported in test organisms like fish [34,42,69–71], birds [90], plants [46,91,92], rats [93–96], and human cells [33,97–99]. The diffusion of these compounds in the environment is so high that levels of these by-products have also been detected in umbilical cord blood samples [99] and urine [100,101], and they have been associated with increased childhood obesity and early puberty.

Concerns about NPEO emerged when Soto et al. [102] observed that 4-NF, generated from the degradation of NPEO, was able to mimic estrogen and act as an endocrine disruptor, inducing the proliferation of mammary tumor cells. This motivated several other research groups to investigate the xenobiotic effects of 4-NP, leading several research groups to confirm the ability of this degradation by-product to mimic the female hormone 17β-estradiol [30,47,88,97,103,104]. Furthermore, it was observed that the recalcitrance of 4-NP is greater with the decrease in the number of ethoxylated groups [30,83,105]. 17β-estradiol is a natural hormone that influences the development and maintenance of female sex characteristics [106]. Because of this, 4-NP is expected to trigger a variety of reactions in exposed organisms. 4-NP can bind to the same receptors as the hormone 17β-estradiol, generating competition for binding to the estrogen receptor due to similarities in its chemical structure [30]. The same functional regions for protein synthesis are activated by both compounds. However, different biological responses are provoked, such as reduction of hemoglobin and blood erythrocytes [70], oocyte atrophy [72], increased frequency of reproductive stages [73], testicular damage and reduced sperm density [76], and apoptosis and DNA damage [69,77]. Some of the effects of 4-NP on aquatic organisms are presented in Table 1.

Shirdel et al. [71] evaluated the effects of 4-NP (1–100 μg L^{-1}) on the levels of plasma reproductive hormones and liver antioxidant enzymes, as well as the histopathology of reproductive and non-reproductive organs of brown trout (*Salmo trutta Caspius*). After 21 days of exposure, the authors found an increase in the concentration of estradiol in the plasma of both male and female organisms and a reduction in antioxidant enzymes in the liver. Also observed were histopathological lesions in liver and kidney tissues, such as congestion, cytoplasmic degeneration, hypertrophy, necrosis, nuclear degeneration, pyknosis, and vacuolar degeneration. In male organisms, different levels of hyperemia in the testes, a decrease in seminiferous lobe thickness, and spermatogonial degeneration were observed.

The effects of SCS by-products in humans (in vivo) and on human cell lines have been reported and have highlighted some concerning effects that included decreased secretion of oxytocin [97], increased proliferation of aberrant cells [33], childhood obesity [100], and increase in inflammatory processes [107] (Table 2). Bechi et al. [97] studied the effects of SCS by-products in human placental cells and observed that, even at low concentrations (0.022–220 ng L^{-1} of 4-NP), there was a reduction in oxytocin secretion, which inferred the potential of these chemicals to lead to pregnancy interruption and other complications. Forte et al. [33] analyzed the effects of NPEO on lymph node carcinoma of the prostate (LNCaP) cell line and found that it induced proliferation of LNCaP and increased the expression of estrogen receptor α (ERα) and its translocation from the cytoplasm to the nucleus. Moreover, the authors found NPEO also resulted in up-regulation of key target genes involved in the cell cycle and inflammation processes.

Table 2. Effects of 4-Nonylphenol (4-NP) and 4-Nonylphenol (4-NP) on human and in vitro human cells.

Source	Surfactant	Effect	Dose	Ref.
In vitro				
Human placenta	4-NP	Decreased secretion of oxytocin, which may lead to complications during pregnancy.	0.022–220 ng L^{-1}	[97]
Human prostate	4-NP	Increased proliferation of aberrant cells. Cell cycle dysregulation.	0.22 mg L^{-1}	[33]
Human cell lines				
Urine and serum samples	4-NP and 4-OP	Childhood obesity in biological samples of young girls.	3.95–44.15 ng mL^{-1}	[100]
Urine	4-NP	Decreased age at menarche estimates in breast development, pubic hair, and menstruation status in girls.	5.03 ng mL^{-1}	[101]
Adenocarcinoma prostate cells	4-NP	Induced cell proliferation and key target genes involved in cell cycle and inflammation process.	22.04 ng mL^{-1}	[107]

Consumption of SCSs by humans is a common occurrence, especially in societies in which plastic containers and packaging are usually used for storage of food and drink, and is related to certain symptoms, diseases, and defects in adults, children, and unborn babies. SCSs are used in the synthesis of polymers for the manufacture of plastic containers and packaging and, as such, they can be released into food and water and in turn ingested by humans. Loyo-Rosales et al. [108] evaluated the migration of NPEOs in plastic bottles used to store drinking water. The authors observed concentrations ranging from 180 to 300 ng L^{-1} in drinking water stored in plastic containers made of HDPE (high-density polyethylene), PET (polyethylene terephthalate), and PVC (polyvinyl chloride) bottles purchased at a supermarket. In a study by Chen et al. [99], the authors reported 1.82 to 211 ng of 4-NP g^{-1} plasma in cord blood samples from 174 human fetuses, with the highest concentrations detected in samples from pregnant women residing in metropolitan areas. The authors posited that through repeated consumption of food contained in plastic containers, fetuses of pregnant women are more likely to encounter high exposure levels to SCSs due to transplacental absorption, and the accumulation of these chemicals due to inefficient detoxification mechanisms in the fetus. In another study, Choi et al. [100] studied the association between obesity in young girls and several endocrine-disrupting chemicals (EDCs), including 4-NP. The concentration of 4-NP (and other EDCs tested) was lower in serum and urine in the control group compared to that in the obese group, and the results showed a statistically significant relationship to childhood obesity. Similarly, Hou et al. [101] investigated the exposure effects of NP on obesity and pubertal maturity to compare the body sizes of general adolescents in Taiwan. Urine samples were analyzed from 270 children aged 6 to 15 years old, and the study showed that 4-NP exposure was positively correlated with abdominal obesity, including skinfold thickness, waist circumference, waist-to-height and waist-to-hip ratios, and indicated a dose–response relationship. The pathway of SCSs affecting human and aquatic organisms has been summarized in Figure 4.

Consumer products, including personal care products like shampoo, are important sources of human exposure to SCSs and harmful chemicals including volatile organic chemicals (VOCs). Diethanolamine (DEA) is widely used to precipitate diethanolamides and diethanolamine salts of long-chain fatty acids used in soaps and surfactants in various products including shampoos, conditioners, and cosmetics. This chemical is banned in the EU and Canada in cosmetics due to its ability to react with other ingredients in products to form carcinogenic chemicals [109]. In the study by Knox et al., 2023 [109], it was found that many consumer products sold in California contained chemicals associated with cancer, reproductive harm, and developmental harm. Considering the extortionate usage of personal care products, these chemicals will accumulate individually as well as in combination which could be very harmful. These findings emphasize the need for safer products for consumers to ensure humans are not exposed to such risks. Safer products

not only have to be safe for humans, but also marine organisms. Not only will suitable replacements need to be safe and non-toxic, but also function as suitable replacements for the purpose of the product, at the same extent if not better. In a study by BIOWAYS assessing public perception of biobased products, 50.2% of respondents consider biobased products to be as good as conventional products and 53.1% were willing to pay more for a biobased product if they have the same functionality and properties of a fossil-fuel-derived product [110]. There is clearly a market for biobased personal care products, but such products will need to be suitable alternatives to conventional ones already being used.

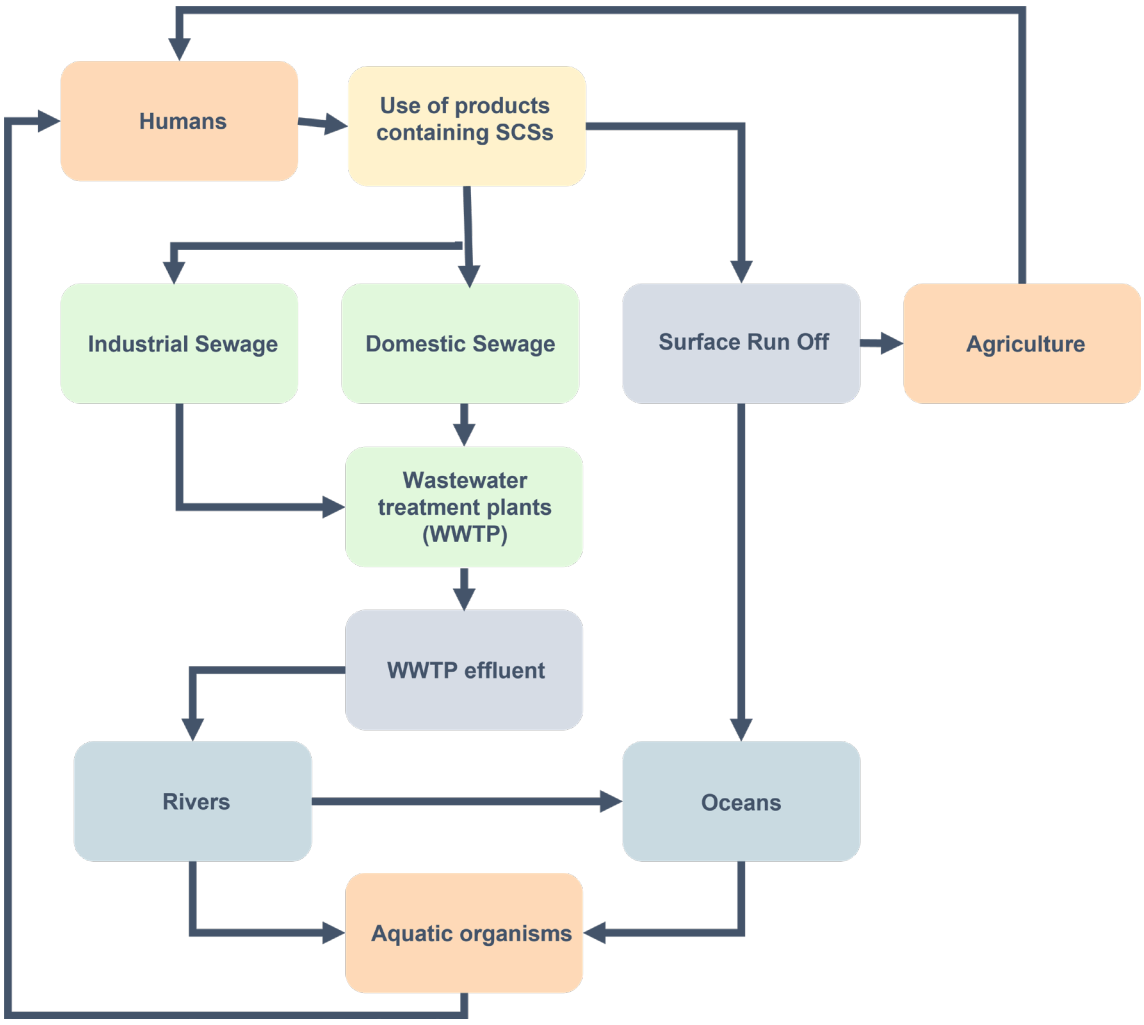

Figure 4. Flowchart showing the pathway of SCSs to the oceans, humans, and aquatic organisms. Orange represents sources; yellow represents the use of products containing SCSs as the source activity, green represents wastewater containing SCSs and treatment of wastewater, blue represents water bodies where SCSs are present.

4. The Need for Greater Biosurfactant Uptake by Industry: Toward a Turn in the Tide for Reducing the Entry of SCSs into the Ocean

As noted earlier, the volume of SCSs entering the marine environment annually is enormous, and their toxic effects to aquatic organisms (including terrestrial model organisms) has been extensively researched and documented. Since enormous quantities of healthcare products (soaps, shampoos, detergents, cosmetics etc.) are flushed down domestic drainage systems, WWTPs are a significant point source of SCS release to the oceans due to their inability to capture and remove these chemicals [111]. To-date, most of the research to improve the capture and removal of SCSs in WWTPs has focused on

installing new infrastructure for the treatment of wastewater. However, this approach is somewhat synonymous with the age-old idiom "treat the symptom, not the disease". It would be far more effective to begin to address the problem at the root cause, in this case to reduce the quantities of SCSs used in commercial and industrial products by replacing them with innocuous or, at least, less toxic alternatives that do not harm marine life and accumulate in the food chain where they could find a route to human consumption.

There are countless reviews on biosurfactants (also referred to more generally as surface-active agents) which also include their higher-molecular-weight sister group called the bioemulsifiers. As such, we will not dwell or go into any lengthy description about these molecules in this review, except to provide a glance-over on the basic nature of these molecules which will become useful when we discuss a special class of biosurfactants; the RLs.

Briefly, biosurfactants are like SCSs in that they are surface-active and amphipathic compounds, but the difference is that the former are derived from biological sources, principally microorganisms and plants, but also animals [112]. Because of their amphipathic nature, biosurfactants can dissolve in both polar and non-polar solvents, and as such they are able to interface between aqueous and non-aqueous solvents/substances [113]. The effectiveness of these chemicals is determined by their ability to lower the surface tension (ST) of water, or to lower the interfacial tension (IFT) between two immiscible phases (e.g., of a non-polar and polar liquid) [113]. The ST is a measure of the energy (per unit area) that is required to increase the surface area of a liquid by lowering of the intermolecular forces between water molecules. An effective biosurfactant is able to lower the ST of water from 72 to <35 mN/m, whereas the IFT should be lowered from 40 to 1 mN/m in the case of a water and non-aqueous (e.g., *n*-hexadecane) mixtures [114]. Further to the description on what defines an efficient biosurfactant, it is one that has a low critical micelle concentration (CMC)—the CMC is the minimum concentration required to initiate micelle formation and generally correlates with the ST and IFT [114]. A low CMC means that less biosurfactant is required to reduce the ST or IFT. Biosurfactants are biodegradable, less toxic, and highly specific in comparison to SCSs. Most biosurfactants are thermostable and pH resistant. For example, a psychrophilic strain of *Arthrobacter protophormiae* produced a biosurfactant that was resistant to temperatures over the range of 30–100 °C and resistant to high sodium chloride concentrations of 100 gL^{-1} [115]. Biosurfactants are also biodegradable under aerobic, anaerobic, and anoxic conditions. The biodegradation of biosurfactants occurs in two stages: firstly, the hydrocarbon chains are broken causing a structural change and thereby the loss the amphiphilicity of the biosurfactant and the second stage is the conversion of the byproducts in the first stage into water, minerals, and CO_2 [116]. Biosurfactants have a higher degradation potential than SCSs and also display the capacity to reduce the risk of their accumulation in the environment [117]. They also display high specificity due to their complex structures and specific functional groups, allowing them to be used in highly specific applications. The combination of these characteristics distinguishes biosurfactants from SCSs, allowing them to have wider applications across multiple industrial processes. Various biosurfactants and their associated applications, advantages, and disadvantages have been summarized in Table 3.

Table 3. Various microbial biosurfactants and their associated applications, advantages, and challenges.

Biosurfactant						
Group	**Sub Group**	**Class**	**Application**	**Advantages**	**Challenges**	**References**
Low-molecular weight surface active agents	Glycolipids	Rhamnolipids	Pharmaceutical, personal care, agriculture, enhanced oil recovery	High emulsifying properties, low to no toxicity, highly biocompatible and biodegradable	Primary producer pathogenic (*Pseudomonas aeruginosa*), high cost of production	[16,118,119]
		Sophorolipids	Cleaning products, agriculture, pharmaceutical, cosmetic, enhanced oil recovery	Excellent surface activity, biodegradable, low toxicity, high biocompatibility	Some toxicity to physiological systems, high cost of production, strong foam formation, very low productivity	[120,121]
		Trehalolipids	Bioremediation, enhanced oil recovery, cosmetic, pharmaceutical	Excellent surface activity, excellent emulsifier	Low production yields, cell-bound biosurfactant, expensive recovery methods	[122,123]
	Lipopeptides and Lipoproteins	Surfactin	Bioremediation, pharmaceutical, enhanced oil recovery, food, cosmetics	Excellent surface activity, excellent emulsifier	Low production yield, high cost of production, complex regulation of biosynthesis	[124,125]
		Viscosin	Pharmaceutical, agriculture, enhanced oil recovery	Excellent surface activity, excellent emulsifier, inhibit metastatic cancer cell migration	Low production yield, high cost of production	[126,127]
	Fatty acids, neutral lipids and phospholipids	Fatty acids, neutral lipids and phospholipids	Food industry, gene carrier system	Excellent surface activity	Low production yield, high cost of production	[128,129]
High-molecular weight surface active agents	Polymeric surfactants	Emulsan Biodispersan Alasan Liposan	Medicine, pharmaceuticals, cosmetics, agriculture, water purification, electronic, enhanced oil recovery	Excellent emulsifiers, various micelle assembling properties	High diversity poses challenges in characterization	[130]
	Particulate surfactants	Vesicles and fimbriae	Alkane uptake in microbial cells	Not studied	Not well-studied for industrial applications	[113]
		Whole cells	Not studied	Not studied	Not studied	

Owing largely to their enormous genomic diversity, microorganisms are recognized as the most sustainable and commercially promising source for biosurfactants. To-date, bacteria and yeast have been the main focus of this enterprise as many species of these organisms have been shown to produce biosurfactants with different structures, chemistries, and functional qualities for potential commercial application, and indeed a few have reached a commercial endpoint. These compounds are found forming structural components of microbial cells, such as of cell walls, or they are excreted extracellularly out of the cell [131]. With respect to their structure and chemical composition, biosurfactants vary greatly depending on the producing microbial species, and, broadly speaking, they are classified based on their chemical charge (i.e., anionic, non-ionic, or cationic) or molecular weight [132–134]. Based on the latter, biosurfactants are classified as either low molecular-weight (LMW) surfactants, which reduce surface tension between two immiscible liquids, or high molecular weight (HMW) emulsifiers, which enable the formation of oil-in-water or water-in-oil emulsions and are also referred to as polymeric surfactants (or bioemulsifiers) that are commonly composed of exopolysaccharides (EPS) with/without protein and lipid.

There is a major market opportunity, societal need, and increasing consumer pull for scientifically proven, eco-compatible products. As a case example, in the personal care market, no products in the USD 29 billion shampoo global market can claim to be safe to marine life based on robust multi-species ecotoxicity testing. Current marketing and product labelling by many industries of their personal care products is flooded with 'green washing', with claims such as "reef safe", but where the evidence is lacking. As the world becomes increasingly conscious of the damage inflicted on marine life by pollutants, including plastics from fishnets, bags, and other sources, liquid pollutants, such as SCSs, have become a focus of significant attention [3,135]. Up to 82,000 personal care ingredients flow into the ocean, even after wastewater treatment—shampoos account for a significant proportion of that outflow by virtue of their frequent use by the world's population [135,136]. Significant quantities of shampoo-derived chemicals enter the oceans from coastal populations through so-called 'grey water' outflows, even after water treatment [3]. Fossil fuel-based surfactants are used most often in shampoos as active ingredients, and have been demonstrated to be damaging to marine life [17,137,138]. Yet, to-date, no commercial retailer or supplier has provided substantiated evidence of the true eco-compatibility of surfactant ingredients used in shampoos, let alone for the other ingredients in shampoo formulations, such as by adopting ecotoxicity testing with different, and relevant, model species.

One of the key challenges for the 21st century is to reduce dependence on finite supplies of fossil fuels (oil, coal, gas) by moving toward the use of renewable and sustainable sources to supply our energy needs and the wide range of materials and fine chemicals that are largely still derived from crude oil and its derivatives. Under current climate change scenarios, even plant and animal sources used for supplying industrial materials and fine chemicals are non-sustainable since they can be seriously affected by political upheavals and meteorological events. As noted earlier, SCSs are synthesized from organo-chemical synthesis using fossil fuel-based precursors [139], which is problematic, not only because they are derived from a non-renewable resource, but also because of their proven or perceived toxicological effects to humans and to the environment [140] compared with their biogenic counterparts, the biosurfactants [141]. Biosurfactants, which are of biological origin, have gained increasing interest in recent years, mainly driven by changing government legislation requiring a shift toward industrial use of renewable and less toxic compounds, and an increasing consumer demand for natural and 'environmentally-friendly' ingredients [142]. An important trend in the household products, personal care products, food, and healthcare industries is the adoption of 'natural' ingredients that are eco-compatible (i.e., biodegradable and non-toxic to life) and, where possible, with perceived benefits to the wellbeing of consumers.

For commercial exploitation, microorganisms offer a reliable and sustainable alternative for producing biosurfactants compared to surfactants derived via organo-chemical synthesis, or deriving these chemicals from plant or animal sources [143]. In contrast to biosurfactants derived from plant and animal sources, which can be hampered by lim-

ited production (e.g., low crop yields), political constraints, and increasing energy and transport costs, biosurfactants produced by microorganisms offer a much more sustainable source. Additionally, microorganisms possess enormous genetic diversity, which offers considerable promise in identifying novel compounds, including biosurfactants. The peer review literature and worldwide patent databases contain a plethora of reports and descriptions of biosurfactants produced by many different species of microorganisms, yet notably less than a handful have ever reached the market. In other words, the uptake of microbial-produced biosurfactants by industry still significantly falls short compared to that for the SCSs, and this is largely due to the economics of producing these chemicals—specifically, upscaling the process for biosurfactant production is rarely economically viable. The price of microbial-produced biosurfactants is approximately USD 20–25 per kg^{-1}, which is 20–30% more expensive in comparison to their synthetic counterparts at USD 1–3 per kg^{-1} [144]. There are two major reasons for this, the first of which relates to insufficient production, as, quite often, yields from microbial cells fall short of satisfying industrial demand [145]. While it is well established that growth parameters (e.g., pH, temperature, aeration, agitation, O2/CO2 levels, C:N ratios, substrate composition/concentration etc.) affect biosurfactant production during fermentation [146], a major reason preventing their commercialization is still the low final quantities that are produced per liter volume of fermentation broth—for example, in the case of the RLs, often anywhere from mg/L to <2 g/L of fermentation medium, even under optimized fermentation conditions [147].

The other major reason is that in many cases, the biosurfactant-producing organisms are pathogenic, or they can become pathogenic under certain conditions, which prevents their usage in a range of applications. For example, high water content cosmetic products, such as shampoos, shower gels, creams, and dental products, are susceptible to microbial contamination of pathogenic microorganisms, including *P. aeruginosa* and *Burkholderia* species, which in turn pose potential harm to the user [148]. Recently in the UK, a batch of Vernacare personal cleaning products were recalled due to *P. aeruginosa* contamination and resulted in a stoppage of their production until the issue had been resolved [149]. With these concerns regarding natural microbial contamination of pathogenic strains in cosmetics products, there is more incentive to not implement the use of pathogenic biosurfactant-producing organisms in commercial products destined for human use or consumption. Attempts also to modify genetic/physiological processes, such as quorum sensing to upregulate biosurfactant production of RLs [150], have to a point been successful, but have not been able to yield the quantities required to supply the market to replace fossil fuel-based surfactants, or biosurfactants sourced from non-sustainable sources. It is important to note that microbial biosurfactants are and will remain more expensive than conventional fossil fuel-derived SCSs for the foreseeable future. Currently, their use can therefore only be justified on the basis of added functionality, synergistic interaction with other ingredients, or PR value. All easily produced microbial biosurfactants are commercialized (Table 4). Hence, the most important limitation in getting a microbial biosurfactant commercialized has been the economics associated with their production.

More so than environments on *terra firma*, including freshwater, the marine environment remains a highly promising source for the discovery of biosurfactants and other novel natural products, particularly from extreme habitats, such as hydrothermal vents, polar regions, and the deep sea [151]. Due to its vastness (>70% of the Earth's surface area), variable depth (<1 m down to ca. 10 km), and physical and chemical conditions, scientists and explorers across the many fields in oceanography still consider the oceans a frontier for discovery. This has much to do with the wider range of abiotic conditions found in marine environments than on land. Genetic diversity is also enormous in the oceans, as microbial cell abundances in seawater are estimated to average 105 cells/mL with an average taxonomic diversity of 1000 species/mL [152]. Oil-degrading microorganisms, in particular, are recognized for producing these types of surface-active compounds, principally as a way to gain access to utilizable hydrocarbons from oil as a source of their carbon and energy. In this way, many species of microorganisms thrive in oil spills, pro-

ducing biosurfactants (or bioemulsifiers) that reduce the interfacial tension of the oil, and resulting in smaller oil droplet sizes, as well as increasing the solubility of hydrocarbon species in the oil [153,154]. Bioemulsifiers, which are excreted by many species of marine bacteria, are high-molecular-weight macromolecules, commonly exopolysaccharides (EPS) with/without other associated chemical groups, and which have amphiphilic qualities that contribute to many functions in the marine environment [155]. Like biosurfactants, bioemulsifiers can increase the bioavailability of poorly soluble hydrophobic substrates, which in the event of an oil spill will facilitate oil hydrocarbon dispersion, emulsification, and ultimately biodegradation [156]. Marine environments are a particularly rich source for discovering biosurfactant- and bioemulsifier-producing microorganisms (Table 5). Despite this, the rate of interest for exploring the marine environment for microbial biosurfactants had only increased in recent years, as there are considerably far fewer publications on this compared to biosurfactants from terrestrial microbes [154].

Table 4. Microbial biosurfactant-producing companies and their applications.

Company	Location(s)	Biosurfactant	Application
TeeGene Biotech	Teeside, UK	Rhamnolipid/lipopeptide	Pharmaceutical, cosmetics, antimicrobial, anti-cancer ingredients
AGAE Technologies LLC	Corvallis, OR, USA	Rhamnolipid	Pharmaceutical, cosmetics, personal care, bioremediation
Jeneil Biosurfactant Co. LLC	Saukville, WI, USA	Rhamnolipid	Cleaning products, EOR
Paradigm Biomedical Inc.	New York, NY, USA	Rhamnolipid	Agriculture, cosmetics, EOR, bioremediation, food products, pharmaceutical
Unilever and Evonik	London, UK/ Slovenská Ľupča, Slovakia	Rhamnolipid	Personal care products, cleaning products
Altinbio Scientific Pvt. Ltd.	Navi Mumbai, India	Rhamnolipid/Surfactin	Personal care, cleaning products, agriculture, wastewater treatment
MG Intobio Co., Ltd.	Incheon, Korea	Sophorolipid	Cosmetics, personal care
Synthezyme LLC	Rensselaer, NY, USA	Sophorolipid	Cleaning products, cosmetics, food products, crude oil emulsification
Ecover Belgium	Malle, Belgium	Sophorolipid	Cleaning products, cosmetics, bioremediation
Kaneka Co.	Tokyo, Japan	Sophorolipid	Cosmetics, personal care
Groupe Soliance	Pleumeur-Bodou, France	Sophorolipid	Cosmetics
Lion Corporation	Tokyo, Japan	Methyl-ester sulfonate	Cleaning products, detergents
Fraunhofer IGB	Stuttgart, Germany	Glycolipid	Pharmaceuticals, washing detergent

Table 5. Microbial biosurfactant-producing bacteria from marine environments. The high-molecular-weight bioemulsifiers are included since these types of compounds have amphipathic properties that can serve as ingredients in personal care products.

Environment	Organism	Type of Biosurfactant/Bioemulsifier	Ref.
Sea water/sediment	*Alcanivorax borkumensis*	Glucose-lipid	[157]
Oil-contaminated site	*Alcanivorax dieselolei*	Glycolipid	[158]
Hydrothermal vent	*Alteromonas infernus*	Acidic EPS *	[159]
Hydrothermal vent	*Alteromonas macleodii*	Sulfated EPS *	[160]
Coastal pond	*Cobetia* sp. MM1IDA2H-1	Lipidic surfactant	[161]
Arctic marine sediment	*Colwellia psychrerythraea* 34H	Capsular EPS *	[162]
Marine sediment	*Enterobacter cloaceae* 71a	EPS *	[163]
Seawater	*Flexibacter* sp. TG382	Glycoprotein EPS *	[164]
Oil-contaminated site	*Gordonia amicalis* LH3	Rhamnolipid	[165]
Hypersaline soils	*Halomonas eurihalina*	Sulfated heteropolysaccharide *	[166]
Shoreline sediment	*Idiomarina* sp. 185	Glycolipid	[167]
Surface seawater	*Marinobacter* sp. MCTG107b	Rhamnolipid	[168]
Orthogenic soil	*Pantoea* sp. A-13	Glycolipid	[169]
Surface seawater	*Paracoccus* sp. MJ9	Rhamnolipid	[170]
Surface seawater	*Planococcus* sp. XW-1	Glycolipid	[171]
Seawater	*Pseudomonas* sp. MCTG214(3b1)	Rhamnolipid	[172]
Hydrothermal vent	*Pseudoaltermonas* sp. 93	Glycolipid EPS *	[167]
Hydrothermal vent	*Vibrio diabolicus* HE800	EPS *	[173]

* High-molecular-weight bioemulsifier.

Gene transfer is highly frequent amongst marine microbial communities, and as such these organisms are provisioned with a higher genomic flexibility that allows them to adapt to varying environmental conditions [174]. This high gene transfer frequency could allow unique biochemistry and novel characteristics, ideal for biotechnological applications. For example, biosurfactants from psychrophilic microorganisms have been explored in formulations for washing products since they can work efficiently at lower temperatures [175]. Particularly as consumer goods companies are aiming for products to be energy saving and reduce environmental impact, such characteristics are beneficial to meet such criteria [175]. Marine bacteria are generally mesophilic, which could be advantageous to industrial production since less energy is required and does not require high-temperature production. In addition, it has been noted that biosurfactants produced from mesophiles have high levels of thermo-stability [154], a characteristic that could be advantageous in industrial applications. Marine-derived biosurfactants have also been shown to have antimicrobial, anti-adhesive, and biofilm disrupting properties, and for these reasons could be suitable for cosmetics and personal care products [176]. As such, biosurfactants are considered viable replacements to SCSs, yet the marine environment remains relatively unexplored for microorganisms that produce these molecules.

5. The Rhamnolipids: Speeding Up the Turn in the Tide?

Rhamnolipids (RLs) are glycolipids, one of the major categories of biosurfactants. Global interest in RL production has been on the rise due to their broad range of applications in various industries and their excellent physicochemical properties [16,177]. RLs are low-molecular-weight compounds of relatively simple molecular structure—i.e., they are composed of one or two rhamnose sugars with up to two ß-hydroxy fatty acid tails ranging in chain length (from 8 to 16 carbons). Thus, their synthesis is achieved using either just two or three separate enzymes (RhlA and RhlB, and/or RhlC), as shown in Figure 5. The first two enzymes are encoded by the genes *rhlA* and *rhlB*, respectively, and are located on a single operon alongside an AHL-mediated quorum sensing system (*rhlR*, *rhlRI*), whereas the third enzyme is encoded by *rhlC*, and is located ca. 2.5 Mb downstream of the *rhl* operon. In comparison to other glycolipid surfactants, RLs are unique as they contain a hydrophilic group consisting of either one or two (L)-rhamnose molecules with a glycosidic link to a hydrophobic group made up of one or two β-hydroxy fatty acids. RLs have two forms dependent on the presence of one or two rhamnose molecules (Figure 5) and are known as mono-RLs and di-RLs, respectively [178].

Among the various different types of biosurfactants, RLs stand apart [177]. This is because high yields of production can be achieved after relatively short incubation periods and since the primary producer is *P. aeruginosa*, it is easy to cultivate and is a well-understood bacterial species [179]. Compared to other biosurfactants (tannins, saponin, lecithin), rhamnolipids showed the best emulsifying properties in distilled water and seawater [180]. They are widely recognized in industry as eco-compatible, 'green' replacements of the classic fossil fuel-based surfactants. A major and recent advancement in the commercialization of RLs was the partnership between Evonik and Unilever to build the world's first industrial-scale production plant for RLs [181]. The partnership dated back several years, when Unilever developed the first household cleaning product, Quix, containing biosurfactants which was distributed in Chile in 2019. The success of Quix led to another pilot release of a biosurfactant-based hand dishwash in Vietnam. Following this, both companies intended to distribute these on a global scale, leading to a large investment between EVONIK and Unilever in the construction of a new million-euro production plant in Slovakia for, largely, RLs production. This demonstrates significant industry-led commitment to these biomolecules by major global players.

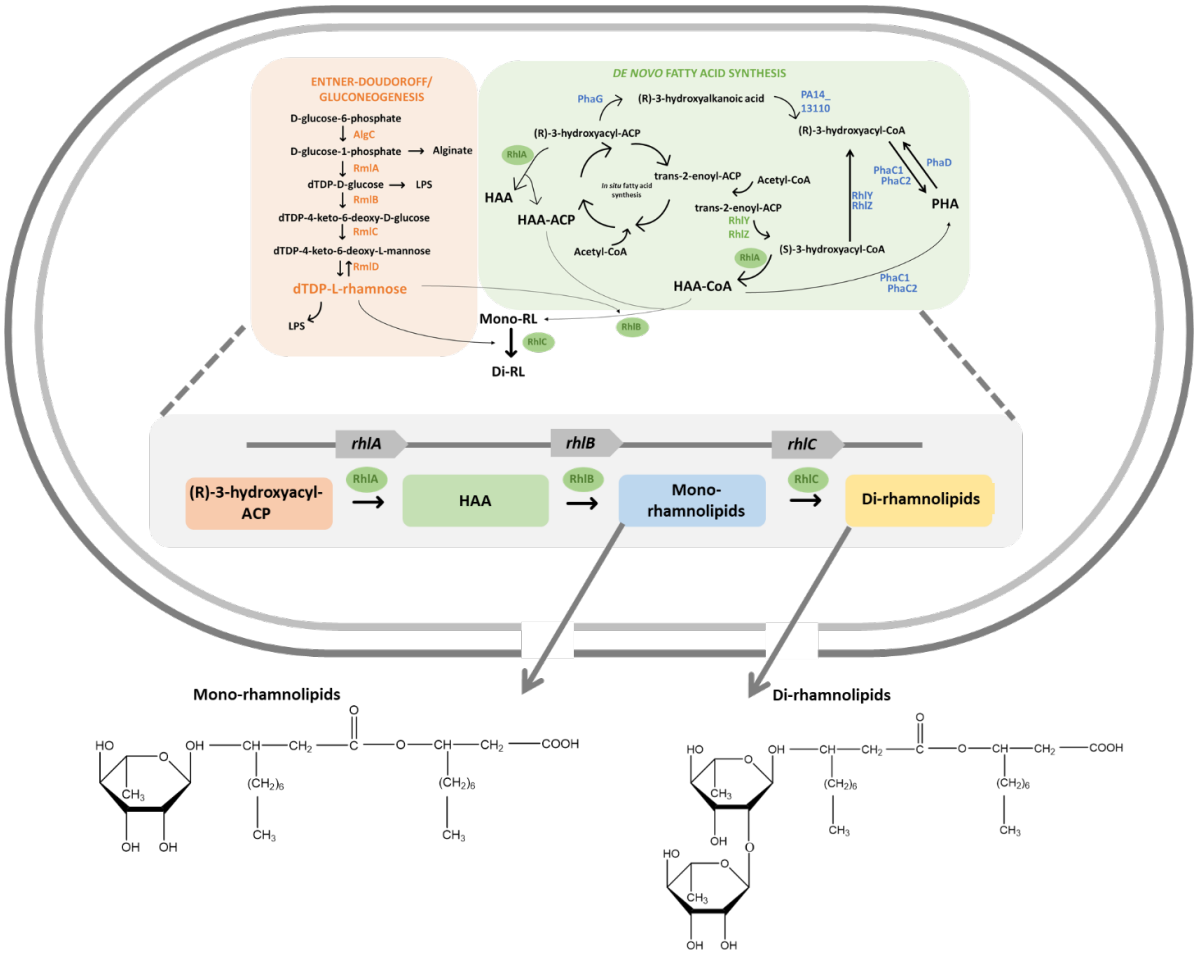

Figure 5. Pathway for the synthesis of mono-and di-rhamnolipids.

Other companies around the world that have also invested into research for producing RLs are TeeGene Biotech (Teeside, UK), AGAE Technologies (Corvallis, OR, USA), Jeneil (Saukville, WI, USA), and Paradigm Biomedical Inc. (New York, NY, USA) Table 4). However, production yields of RLs by these companies is limited to approximately a couple of grams per L of fermentation volume, as the producing strains have been stretched as far as they can be optimized for maximal RL synthesis, and because genetic engineering attempts to obtain overexpressing strains have been unsuccessful. Quite recently, only EVONIK has been successful in cloning the RL synthesis pathway from *P. aeruginosa* into the overexpression host *Escherichia coli*.

It also accomplished RL-overexpression using the chassis *Pseudomonas putida*, but only overexpression of RL-producing genes in the host *E. coli* resulted in market entry for these compounds. For other companies to source this RL product, this comes with a cost higher than from having this ingredient autonomy via inhouse production. From a company's financial perspective, vertical integration and supply line control are valuable and preferred over depending on other suppliers.

RLs have found usage in enhanced oil recovery (EOR) [182], agriculture [183], pharmaceuticals and therapeutics [184], detergent compositions, laundry products, shampoos and soaps [185], cosmetics and skin treatments [186], and show low toxicity and antimicrobial activities against both Gram-positive and Gram-negative bacteria [187], and as such show promise for uses in pharmaceuticals and therapeutics [184]. The antimicrobial activities of RLs are also a favorable property as it acts as a preservative, thereby removing the requirement to add additional preservative ingredients to extend the shelf-life of a product.

The RL market is expected to reach over USD 145 million by 2026 with a compound annual growth rate of >5% [188].

Until recently, RLs were known to be produced by principally *P. aeruginosa*, with some strains producing mono- or di-RLs, or both. Historically, RLs were first described just over 70 years ago from *P. aeruginosa* [189], thereafter becoming the key species in RL research. Despite the vast number of reports in the literature describing RL-producing bacteria [190], it is important to note that some publications misinform on the production of RLs from "non-*Pseudomonas*" strains, either because the strains were incorrectly identified or their produced biosurfactants were misidentified as RLs [191]. The second major microbial RL-producers comprise species of *Burkholderia* [192–195], and a few other members of the class *Gammaproteobacteria*, mainly species of *Pseudoxanthomonas* [196], *Pantoea* [197], and *Acinetobacter* and *Enterobacter* [198], and have since been identified as RL producers. In addition to varying, albeit low, quantities of RLs produced by these organisms, their potential as pathogens in humans, animals, or plants has made them unappealing for commercial development in various applications, especially where human/animal contact or ingestion is involved [190,199].

Whilst RL-producing microorganisms are ubiquitous across different environments, generally they have been isolated from terrestrial habitats. In two recent keystone publications, RLs were found to be produced by two new strains of marine bacteria that are not related to pathogenic *P. aeruginosa*. 16S rRNA gene sequencing identified one of the strains to represent a new species of *Pseudomonas* (strain MCTG214(3b1)) that is unrelated to pathogenic *P. aeruginosa* [172]. The strain was found to express RL synthesis genes *rhlA* and *rhlB*, although no *rhlC* homologue was identified. The study also found the strain produced a significantly high level of short-chain di-RLs, the first to have been described in a non-pathogenic marine *Pseudomonas* species [172]. The other study identified a RL-producing *Marinobacter* species, and optimization of culturing conditions to optimize RL production found that it could be cultured in cheap and renewable feedstocks [168]. In these studies, RL synthesis by each of the two strains was confirmed by analytical methods (e.g., NMR and GC/MS) and bio-molecular tools (e.g., qPCR) [168,172], and their produced RLs were subsequently shown to be non-cytotoxic or mutagenic [200]. These characteristics present these two strains as excellent candidates for the development of a microbial chassis for the heterologous overexpression of RLs. These RL-producing strains, which were isolated from the marine environment, represent the first non-pathogenic RL-producers, as *P. aeruginosa* and the other above-named RL-producers are known pathogens or can turn pathogenic under certain conditions [168]. This opens up a huge opportunity to develop the overexpression of these biomolecules for use in applications whose doors have for so long been closed off because the producing organisms, or the native sources of RLs, display pathogenicity. The overexpression of RL production using the genetic pathway encoded by organisms other than pathogenic *P. aeruginosa* (cf. production of RLs by EVONIK in collaboration with UNILEVER) has not been completed before, thus representing a unique opportunity (discussed in the next section below) to attempt this and which could open new opportunities for using RLs in a much wider field of applications than is currently happening.

6. Genetic Engineering: The Most Promising Road to Market?

Microbial biosurfactants have enormous potential in commercial products, and many published reports proclaim biosurfactants from microorganisms as viable alternatives to replacing fossil fuel-derived SCSs. However, the major setback to their commercialization is the cost competitiveness compared to SCSs which, quite often, the latter are significantly cheaper to produce. All current commercially available microbial biosurfactants—i.e., sophorolipids, mannosylerythritol lipids, RLs, and surfactin—are derived from industrial fermentation based on renewable raw materials. These four biosurfactant types currently occupy only small market niches because their upscaled production, via mainly optimization of the fermentation conditions, has been marginal compared to the non-optimized

strain. To obtain higher yields (from mg/L to g/L of biosurfactant per L of fermentation medium), history shows that genetic engineering, such as cloning of the respective biosynthetic pathway in a competent host, is duly necessary to circumvent the limitations that are inherent in the wild-type producing strain. However, the expertise in genetic engineering and funding to support this is commonly a limiting factor to achieving this.

Before delving into the genetic engineering possibilities, we would like to summarize some of the options that have commonly been attempted for upscaling the production of biosurfactants, including that of the RLs, as these options are generally cheaper and systematic for almost any lab around the world to use. The biggest limitation in introducing biosurfactants into consumer products is cost, and this can only be achieved when profit is achieved. In general, studies have determined mg/L yields of biosurfactant, which is extortionately low compared to g/L yields of SCSs [154]. Microbial production of biosurfactants can be increased by optimization of growth conditions, but the cost of raw materials for RL fermentation is estimated to be approximately 50% of the total production costs, and as such this makes it difficult for these and other biosurfactant classes to compete economically with SCSs [144]. Many studies have investigated the use of waste and renewable substrates as a carbon source (e.g., mannitol, glucose, glycerol, *n*-paraffin, *n*-alkane, polycyclic aromatic hydrocarbons, or vegetable oils) to reduce the production cost of RLs [201–204]. The use of such waste materials has other benefits, such as in circularity of economics and waste management. Despite this, not much progress has been made on commercial upscaling of biosurfactant production using waste feedstocks. It is also noteworthy that whilst many published reports on biosurfactants note that the cost of raw or waste feedstocks contribute a significant cost to the overall economics of production, this is possibly still a small fraction compared to the costs of energy required for prolonged periods, as when running a multi-day fermentation.

Another way of reducing total production costs is by addressing the downstream process as this has the largest impact, accounting for approximately 80% of the total production costs [205]. The recovery and purification of RLs usually involves the use of acid precipitation and organic solvent extraction [147,205]—chemicals which, in large volumes, contribute a significant expense from their purchase and also for their appropriate disposal. In addition, the use of such chemicals can result in reduced biosurfactant activity and undesirable product aggregation [206], as may be affected by the biosurfactant molecule's ionic charge, water solubility, and location of the product [15]. How much processing is involved will of course depend on the intended application of the respective biosurfactant, and as such affect the cost of production [207]. For low-cost applications which do not require a high-purity product, such as in oil recovery, RLs can be recovered directly from fermentation broth, and there is research exploring the use of a cell-free fermentation broth in place of purified RLs for industrial applications [208]. A low-cost, integrated foam fractionation process has also been proposed, resulting in relatively high-purity RLs [209].

Of all avenues to tackle the problem of production costs, genetic engineering is unquestionably the most promising, and history holds testament to this by the fact that most antibiotics, therapeutics, pharmaceuticals, and other compounds are today produced by a recombinant overexpression system. As noted earlier, *P. aeruginosa* is the most studied RL-producing strain, and as such this species has been the focus for increasing RL yields by genetic engineering. The two main approaches in this respect have involved either the introduction of genes that promote RL production, or the deletion of genes that inhibit RL production. The introduction of the strong promoter of the *oprL* gene to the RL genes *rhlAB* increased the copy number of *rhlAB* genes and the engineered strain produced a higher yield of RLs [210]. Alternatively, it was found that deletion of the *cplX* and *lon* genes, which expresses the negative regulators of quorum sensing *in P. aeruginosa*, was found to increase RL production since RL biosynthesis genes are closely linked to the quorum-sensing mechanism in this organism [211]. Whilst these are promising approaches, as they have led to reported increases in RL yields, there is still the problem relating to the pathogenicity of the strain which can severely limit its application, especially in certain

industrial sectors. Yet even with non-pathogenic strains of marine or terrestrial origin, quite often the biosurfactants they produce have not reached a commercial endpoint due to low yields, as well as insufficient structural elucidation and uncharacterized genes [154]. One method of tackling this could be by removing the pathogenic trait of the strain [16]. The synthesis of pyocyanin, a secondary metabolite of *P. aeruginosa* which interferes with multiple cellular functions, is the cause of its pathogenicity [212] and inhibition of its synthesis is suggested to remove its pathogenic trait. This could be achieved by inhibiting the synthesis of the precursors involved in the expression of *phzABCDEFG* and *phzHMS*, which are the genes involved in the synthesis of pyocyanin [213]. More research would be required to assess the effects of removing the pathogenicity of the strain on RL production for the recombinant strain to have potential in industrial applications.

E. coli is one of the most characterized and commonly used chassis in recombinant technology. Undoubtedly, it has played a pivotal role in developing the tools we use today in synthetic biology since *E. coli* was one of the primary species these tools were built upon [214]. For the recombinant production of RLs, as a microbial chassis *E. coli* could be suitable since it has been highly characterized and proven to be useful for many industrial biotechnological applications. However, there is an apparent requirement for new chassis to advance metabolic engineering across numerous applications. In recent decades, bacterial species, including *P. putida* and *Bacillus subtilis*, have been extensively characterized with many defined genetic toolkits developed for these species for metabolic engineering [215,216]. For example, *P. putida* KCTC1067 expressing both *rhlAB* and *rhlI* was reported to produce 7.3 g of RL per L of fermentation medium [217]. Although, it should be noted that it has been found to be difficult to overproduce RLs in heterologous hosts. This was made evident when comparing the results of one study aiming to express *rhlAB* to produce mono-RLs and HAAs, which resulted in 0.25 g/L using an *E. coli* chassis, and 0.6 g/L with *Pseudomonas fluorescens* [218], to another study where both *rhlAB* and *rhlBDAC* were introduced into *E. coli*, and a yield of 120.6 mg/L was achieved [219]. Since the precursors to RL biosynthesis are derived from central metabolic pathways native to *P. aeruginosa*, heterologous hosts may not have the essential genes required to synthesize these precursors, and as such it is more difficult to overproduce RLs. Considering these limitations, a highly characterized pseudomonad would be a more suitable option for the heterologous expression of RLs compared to *E. coli*, and other non-pseudomonad species, since the central metabolic pathways will have more similarity or compatibility. Once a suitable candidate for heterologous expression of RL genes has been identified and with more research optimizing the strain for RL production, it could be possible to develop an industrially viable process for high RL production in the future [220].

7. Conclusions

The abundance of products and widespread applications of SCSs means their presence in sewage effluent is omnipresent. In the Mississippi River alone, concentrations of LAS along the river ranged from 0.1 to 28.2 $\mu g\ L^{-1}$ [27] indicating the volume of SCSs entering the marine environment is extensive and is even likely to be undervalued. SCSs are detrimental to aquatic organisms, human health, and the environment in general, as they can cause complications in pregnancy, obesity, and cancer [97,100,107]. The occurrence of these chemicals in the environment is mainly associated with their incomplete removal at WWTPs, leading to their unmitigated release, including that of their by-products, into the oceans. Proper removal of SCSs at WWTPs is one approach to tackle this problem, but it would be far more effective to replace SCSs with a greener and environmentally friendly alternative(s), particularly with increasing public demand for this considering these pollutants are recognized to inflict detrimental damage to marine life and ultimately find a route to human consumption. As well as the toxicity of SCSs, there is a worldwide push to move away from a dependence on finite resources, including fossil fuels, to produce materials and chemicals like SCSs. Surfactants from biogenic sources (biosurfactants), and in particular the RLs, are seen as promising alternatives to SCSs since they are biodegradable, non-toxic,

and can be produced from renewable resources—ideal characteristics toward implementing more eco-conscious products into industrial and domestic usage.

From a production standpoint, microbial biosurfactants are more sustainable than those from other sources. Microorganisms are also more genetically diverse than, for example, higher animals and plants, and different species of microbes have been shown to be excellent sources of novel biosurfactants. Despite this, the uptake of microbial biosurfactants by industry falls significantly short compared to their synthetic chemical counterparts, due mainly to the costs of production and low yields, making them economically uncompetitive compared to SCSs. In order for microbial biosurfactants to reach a commercial endpoint, production costs and yield must be optimized. To achieve this, marine microorganisms have been considered a highly promising source for the discovery of biosurfactants, as well as other types of biomolecules. Whilst there are several methods that are currently being explored to reduce production costs, such as via the use of waste and renewable substrates/feedstocks as a carbon source for the producing organisms, or by optimizing the downstream process, the most promising approach going forward in this respect is considered to be genetic engineering. Various genetic engineering approaches, particularly for microbial RLs production, have been explored in order to improve yield, such as via the deletion of non-competitive genes or the introduction of genes to promote RLs synthesis. However, much more research is needed for achieving the overexpression of RLs to meet commercial demand. The heterologous expression of RL genes derived from marine organisms could be a promising solution to the limitations of microbial biosurfactant production, but a highly characterized synthetic biology chassis will need to be identified for an industrially viable process for high RL production. Advancements in synthetic biology coupled with the growing demand for biosurfactants will undoubtedly see RLs become more commercially viable, perhaps not so far into the future.

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Using Compound-Specific Carbon Stable Isotope Analysis of Squalene to Establish Provenance and Ensure Sustainability for the Deep-Water Shark Liver Oil Industry

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Abstract: Deep-water dogfish (sharks) are caught on Australia's continental shelf as by-products to other deep-water species with revenue derived from fillets for human consumption and from the livers which are sold for their oil content. Deep-water dogfish utilise a large oil-rich liver for buoyancy, which may account for 20–25% of their body weight. An important constituent of certain dogfish liver oil is squalene, a highly unsaturated triterpenoid ($C_{30}H_{50}$) hydrocarbon which in some species can be up to 90% of the oil, though in the Australian commercial species it is typically around 50%. Squalene (and deep-water dogfish liver oil in general) has a long-standing high value in products, such as cosmetics and nutraceuticals. Manufacturers are increasingly required to demonstrate the sustainability of products, and this is integral to the importance of demonstrating product provenance. Australia's mid-slope deep-water dogfish fishery is recognised globally as well managed and sustainable; therefore, it is important to be able to distinguish products derived from these regions from other unregulated, unsustainable and cheaper sources in order to protect Australia's competitive advantages and ensure sustainability. In this study, we have sourced deep-water dogfish liver oil samples originating from Southeast Australia, New Zealand, India, Northeast Africa and the Arabian Sea. The squalene was isolated by commercial or laboratory processing. A compound-specific carbon stable isotope analysis of the derived squalene was then used to determine isotopic resolution and assign the likely origins of a variety of commonly available off-the-shelf nutraceuticals in Australian outlets. Squalene sourced and produced from Southeast Australian and New Zealand dogfish liver oils showed $\delta^{13}C$ values in the range of −22.1 to −24.2‰, with all other squalene samples distinguishable at −19.9 to −20.7‰. Many of the off-the-shelf squalene products claiming to be of Australian origin showed $\delta^{13}C$ values very distinct from the range of the genuine Southeast Australian- and New Zealand-produced squalene.

Keywords: dogfish; squalene; compound-specific stable isotope analysis (CSIA); isotopes; provenance

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1. Introduction

Shark liver oil is a generic term used to describe the marine lipids derived from the livers of certain deep-water dogfish (*Squalidae*, a family of sharks) which comprise diacylglyceryl ethers, triacylglycerols, and a major hydrocarbon, squalene [1]. Squalene is a triterpene hydrocarbon ($C_{30}H_{50}$; 2,6,10,14,18,22-tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (6E,10E,14E,18E)) first discovered in 1916 in shark liver oil from a deep-water dogfish (*Squalus* spp.) [2]. The livers from certain deep-water dogfish provide the largest natural source of squalene, with up to 700–800 mg/g of oil (70–80%) [1], while olive oil can contain 0.4–0.6% (of oil) [3]. Other natural sources include grape seed oil, soybean oil and peanut oil [4]

Approximately 90% of global squalene production is hydrogenated to form squalane and used largely as a moisturiser base in high-quality cosmetics, with around 9% used in the nutraceutical industry [5,6]. The nutraceutical use of squalene has suggested anti-cancer, anti-ageing and antioxidant properties, but there is limited published evidence for any direct health benefits [7]. Pharmaceutical interest in squalene is increasing with high purity squalene proving to be effective as an adjuvant in vaccines [8,9]. There is interest in developing alternative microbial or bio-synthetic sources for squalene [10]; however, deep-water dogfish liver oil and olive oil remain the two primary sources, with deep-water dogfish liver oil presently remaining the preferred source [3,9].

Deep-sea dogfish may generally be characterised by slow growth, late maturation and low levels of reproduction, with just under 20% of extant species of sharks (including selected deep-water dogfish) and rays listed as "threatened" [11]. Thus, it is essential that the catch and utilisation of these species is carefully managed to ensure sustainability. Although some of these species may be caught as by-products in Australia, they are also target species in all unregulated fisheries (e.g., African, Indian, Indonesian and Filipino dogfish fisheries), resulting in an unconstrained and under-reported harvest in these regions [12].

1. In Southern Australia, deep-water dogfish have historically been caught on the slopes of the continental shelf with the catch divided into two groups which are sustainably managed by the Australian Fisheries Management Authority (AFMA) [13,14]: Upper-slope (shallower) species comprise the gulper sharks (*Centrophorus* spp.) and green eye species (*Squalus* spp.). Individuals in this group typically have life histories with slow maturity and low fecundity. Three species (*Centrophorus harrissoni*, *Centrophorus moluccensis* and *Centrophorus zeehaani*) from this group are listed as conservation-dependent under the Australian Environmental Protection and Biodiversity Conservation (EPBC) Act and several have poor (threatened) International Union for the Conservation of Nature (IUCN) listings. The AFMA has in place a rebuilding strategy for several of these sharks.
2. The mid-slope (deeper) species are managed by the AFMA as the 'Deep-water Shark Basket' (DWSs). DWSs are a management basket of 18 species from five families. More than 90% of those harvested are a single species, *Deania calcea* (aka 'Birdbeak Dogfish'/'Brier Shark'/'Shovelnose Spiny Dogfish'), which has an IUCN listing of "least concern", is not EPBC listed and is not subject to a rebuilding plan. DWSs have significantly higher productive life histories than gulper sharks.

Deep-water dogfish in category #2 above are consistently caught as an element of both mixed and target species fisheries (such as blue grenadier (hoki), ling and others). Their trunks are utilised for fillets for human consumption and the livers for squalene production, which provide approximately 40% and 60% of revenue, respectively, to the industry, though this will vary with market fluctuations. The main harvest species, *Deania calcea*, has a lower squalene content (c.a. 50%) than some other deep-water dogfish [1] thus production costs are higher. Squalene has regular, long-standing, and reliable markets around the world. This makes the supply of squalene from a sustainably managed resource vulnerable to a high supply of cheaply produced oil from poorly regulated/unregulated and unsustainable fisheries.

To ensure the viability of a sustainable deep-water dogfish resource, it is imperative to be able to demonstrate provenance of supply. Stable isotope analysis has become an increasingly important tool in determining the authenticity and traceability of agricultural products, particularly for terrestrial systems (e.g., [15,16]), and has been successfully used to assign regions of origin to commercially important products, such as beef [17], wine [18] and honey [19]. This technique relies on the fact that the isotopic ratios of many elements vary by geographic region due to a combination of geology and hydrology [16,20]. In the marine environment, the application of stable isotopes to establish provenance may be more problematic since both water masses and animals are generally not static. To date, there are few reported marine applications of the technique being used for determining

provenance compared with terrestrial environments, although stable isotope analysis has demonstrated some success in the task of distinguishing wild caught versus aquaculture production [21,22]. It has also been possible to assign geographical origins to prawn production [23,24]. To our knowledge, no techniques have previously existed or been applied for the separation of the source of origin of squalene from the different regions of the world oceans.

An important requirement for this technique to be successful is the use of material free from contamination to establish reference libraries for the point of origin and, when required, to ensure only the ingredient of interest is being measured. To this end, in recent years, there has been an increase in the use of compound-specific isotope analysis (CSIA) in which compounds in a mixture are separated and their individual isotopic ratio determined. This technique has the ability to introduce an extra level of specificity to the analysis by targeting one or more individual compounds known to be endemic to the product of interest. CSIA to date has generally involved the use of carbon isotopes of volatiles and fatty acids in products, such as scotch whiskey [25], cocoa butter [26] and olive oil [27]. Relevant to this study, this technique was successfully utilised to distinguish between squalene derived from olive oil and deep-water dogfish liver oil [3].

There are well established gradients in the carbon isotopes of marine phytoplankton with temperature, and therefore latitude, in the southern hemisphere (e.g., [28]), but this relationship is less obvious in the northern hemisphere (e.g., [29]). Given deep-sea dogfish are not generally regarded to be highly mobile, we hypothesised that there is the potential for these latitudinal differences in phytoplankton isotopes to be reflected in deep-water dogfish liver oil and therefore their squalene. These differences would allow differentiation between squalene derived from deep-water dogfish caught in southern latitudes (Australia and New Zealand) and those caught in less well-regulated fisheries within the warmer waters of the Indian Ocean.

2. Materials and Methods

Samples of deep-water dogfish liver oil of confirmed origin were sourced from Southeast Australia, New Zealand, Northeast Africa, the Arabian Sea and India (Figure 1; Table 1). Australia squalene is derived from dogfish raw materials available for harvest mostly from Southeast Australia, as outlined in Figure 1. Samples of raw oils containing squalene were supplied by traders who are active with local fishermen in their areas. Oil samples were large batches (typically 5–15 tonnes, representing many thousands of dogfish covering both sexes, seasons and age ranges). All oil samples were prepared by maceration of the livers, settling and separation of the oil followed by filtration. Molecular distillation and filtration over activated carbon was then used to obtain 99.85% purity squalene. In addition, we analysed a range of squalene capsules, commercially available and purchased in Australia, with a view to assessing their likely origin (Table 2).

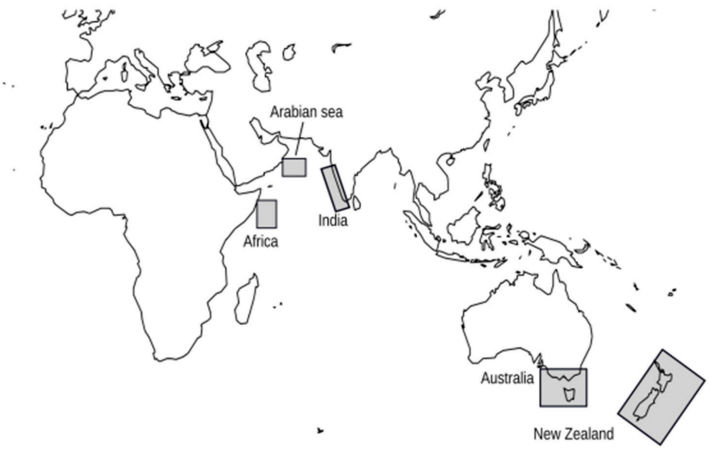

Figure 1. Approximate geographical areas of deep-sea dogfish fisheries sampled in this study.

Table 1. CSIA results for squalene isolated from deep-sea dogfish liver oil with a known origin. AUS denotes Southeast Australian, NZ denotes New Zealand. $\delta^{13}C$ values are means of duplicate or triplicate analyses, SD = standard deviation.

Oil Sample Origin	$\delta^{13}C$ (‰ vs. V-PDB [1])	SD
AUS	−22.6	0.3
AUS	−22.6	0.1
AUS	−22.5	0.1
AUS	−22.6	0.2
AUS	−22.5	0.1
AUS	−22.6	0.2
AUS	−22.3	0.2
NZ	−22.5	0.3
NZ	−24.2	0.1
NZ	−22.3	0.1
NZ	−22.4	0.1
NZ	−22.1	0.2
NZ	−22.4	0.2
NZ	−22.4	0.2
India	−20.4	0.2
Northeast Africa	−20.6	0.1
Northeast Africa	−20.7	0.1
Northeast Africa	−20.6	0.2
Arabian Sea	−19.9	0.2
Arabian Sea	−20.1	0.2

[1] The standard Vienna PeeDee Belemenite (V-PDB).

Table 2. CSIA results for squalene isolated from capsules commercially available in Australia. $\delta^{13}C$ values are means of duplicate or triplicate analyses, SD = standard deviation.

Capsule	Product Origin Information [1]		$\delta^{13}C$ (‰ vs. V-PDB [2])	SD
	Australian Made [3]	Australian Squalene [4]		
1	Y	n.s.	−21.1	0.1
2	Y	n.s.	−20.8	0.1
3	Y	n.s.	−21.1	0.1
4	Y	n.s.	−21.2	0.0
5	Y	n.s.	−22.0	0.1
6	n.s.	n.s.	−21.1	0.2
7	Y	n.s.	−21.3	0.5
8	Y	Y	−21.2	0.1
9	Y	Y	−21.6	0.3
10	Y	Y	−22.0	0.1
11	n.s.	n.s.	−20.5	0.1
12	n.s.	n.s.	−20.5	0.0

Table 2. *Cont.*

Capsule	Product Origin Information [1]		$\delta^{13}C$ (‰ vs. V-PDB [2])	SD
	Australian Made [3]	Australian Squalene [4]		
13	Y	n.s.	−21.1	0.1
14	n.s.	n.s.	−21.1	0.2
15	n.s.	n.s.	−20.8	0.2
16	Y	n.s.	−21.5	0.1
17	Y	n.s.	−21.1	0.1

[1] Obtained from manufacturer product website; [2] The standard Vienna PeeDee Belemenite (V-PDB); [3] Australian made does not currently have to include Australian-derived squalene; [4] Product website states oil is from dogfish caught in waters around Australia; Y = Specified as Australian made or sourced; n.s. = not specified.

2.1. Squalene Isolation

One drop of each sample was transferred to a 1.8 mL vial and made up to 1.5 mL with dichloromethane (DCM). The sample was reduced under nitrogen gas, dissolved in hexane, then transferred to a 1 g silica chromatographic column and the hydrocarbon fraction eluted with 10 mL of hexane and collected into a 50 mL round bottom flask. Most of the solvent was removed under vacuum. The sample was transferred to a 12 mL test tube and then blown to dryness under a stream of nitrogen gas. To ensure adequate purity for the subsequent instrumental analysis, the samples were then saponified using 3 mL of methanolic potassium hydroxide (5% KOH in 80:20 methanol:Milli-q water) by heating for 2 h at 80 °C with vortex mixing every $\frac{1}{2}$ h for 5 s. Samples were then cooled and 1 mL of Milli-q water and 1.5 mL of hexane:DCM (4:1) was added to all samples, which were then vortexed for 5 s and centrifuged at 2500 rpm for 3 min. The top hexane layer was carefully removed and transferred to a 1.8 mL GC vial and the solvent was removed under a stream of nitrogen gas. This was repeated with a further two extractions and the solvent layers were combined. Samples were dissolved in 1.5 mL of DCM and then an aliquot of 200 µL was transferred to a second vial, dissolved in 1.5 mL of DCM and analysed for purity by gas chromatography (GC).

GC was performed on an Agilent Technologies 7890A GC (Santa Clara, CA, USA) equipped with a non-polar Equity-1 fused silica capillary column (15 m × 0.1 mm i.d., 0.1 mm film thickness), flame ionisation detector and split/splitless injector. Samples were injected in splitless mode at an oven temperature of 120 °C, and after injection, the oven temperature was increased to 270 °C at 10 °C min^{-1} and then to 310 °C at 5 °C min^{-1}. Peaks were quantified with Agilent Technologies ChemStation software Rev. C.01.10 [201] (Santa Clara, CA, USA).

2.2. Compound-Specific Stable Isotope Analysis

Compound-specific isotope ratio mass spectrometry was performed using a Thermo Scientific Trace Ultra GC (Waltham, MA, USA), which was coupled via a Finnigan MAT GC combustion interface and Conflo IV to a Thermo Scientific Delta V plus isotope ratio mass spectrometer. The GC was equipped with a 60 m J&W HP-5, 0.32 mm i.d. column with He as the carrier gas. Samples (0.5 µL) were injected split (1:7) via a hot split/splitless injector (250 °C). The initial oven temperature of 45 °C was maintained for 1 min and then increased at 30 °C min^{-1} to 140 °C, then at 3 °C min^{-1} to 310 °C when it was held for 10 min. Combustion of individual peaks to CO_2 was achieved in a combustion reactor at 940 °C. Samples were analysed in either duplicate or triplicate depending on the agreement between analyses and were co-injected with a squalane internal standard with known $\delta^{13}C$ value. Squalane (Sigma, St. Louis, MO, USA, Lot 10K0167) was used in this instance, as it elutes close to squalene and was not present in the samples.

Isotope values are reported relative to the standard Vienna PeeDee Belemenite (V-PDB) and expressed as parts per thousand (‰) using:

$$\delta^{13}C = \left(\left[\frac{R_{sample}}{R_{standard}} \right] - 1 \right) \times 10^3$$

R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ ratio in the sample and standard, respectively. Average standard deviation for triplicate analyses was 0.2‰. The standard deviation across all co-injected squalane analyses was 0.5‰ (n = 60).

2.3. Statistical Analysis

Data were transferred to the R statistics package (ver. 3.4.0) operating within RStudio (ver. 1.0.153) and plotted using the ggplot2 package (Vienna, Austria). A linear regression comparing Southeast Australia, New Zealand, the north-west Indian Ocean/Arabian Sea (Arabian Sea, India, Northeast Africa) samples and capsules was applied with region/capsules as a categorical factor.

3. Results and Discussion

Carbon utilised by primary producers (microalgae and phytoplankton) in marine ecosystems is primarily derived from dissolved inorganic carbon that has a $\delta^{13}C$ value around 0‰ [30]. In comparison, primary producers in terrestrial ecosystems primarily derive carbon from atmospheric CO_2 with an approximate $\delta^{13}C$ value of −8‰ [31]. During photosynthesis further isotope C fractionation occurs, resulting in clear differentiation between terrestrial plants (−9 to −38‰) [32,33] and marine phytoplankton and microalgae (−15 to −22‰) [30,33].

As carbon moves through the marine food chain to higher trophic levels, further isotopic fractionation occurs, with an approximately 0.8 to 1.5‰ increase in ^{13}C value per trophic level [30]. The difference in carbon sources as well as trophic discrimination in the dogfish results in an isotope distinction between squalene derived from olive oil (−26.8 to −29.3‰) [3] and from deep-sea dogfish liver oil (−19.9 to −24.2‰, Table 1).

Compound-specific carbon isotope values for squalene isolated from deep-water dogfish liver oils with a known origin and contained within commercially available capsules are shown in Tables 1 and 2 and Figure 2. Squalene originating from deep-water dogfish caught in Southeast Australia and New Zealand are clearly separated from that originating from other fisheries by >1‰. This is also well above the average analytical error of ±0.2‰ for within sample replicates (n = 3) and ±0.5‰ for the total number of squalane standard co-injections (n = 60). A linear regression showed that the ^{13}C values were significantly higher in the Arabian Sea, Northeast Africa and India region compared to Southeast Australia ($p < 0.0001$). New Zealand was not significantly different from Southeast Australia ($p = 0.73$). An average carbon isotope value by CSIA for olive oil-derived squalene of −27.9‰ (±0.5‰) was reported [3], which, as expected, is significantly more depleted in ^{13}C compared to marine-derived squalene and would be easily distinguishable.

The isotopic separation observed between the southern high latitudes squalene samples (−22.1 to −24.2‰) sourced from Australia and New Zealand from those closer to the equator and those in the northern hemisphere (−19.9 to −20.7‰) is most likely primarily driven by the well-established relationship between southern hemisphere water temperature and CO_2 solubility and subsequent planktonic isotope fractionation [28]; phytoplankton from high southern latitudes have significantly more negative $\delta^{13}C$ values. However, it is worth noting that this relationship is not as strong in the northern hemisphere, where other factors probably also play a part. Thus, while the isotopic differences exhibited here are strong, we have little information on how consistent they might be over longer time periods due to seasonal effects or possible small shifts in the location of where the dogfish are caught within a "region". While it seems apparent that, based on the samples analysed to date, the exact region of origin for squalene is difficult to assign on a global scale, there is very strong evidence that it is possible to ascertain whether

marine-derived squalene is sourced from within the Southeast Australian/New Zealand region currently and therefore from a well-regulated and sustainable fishery.

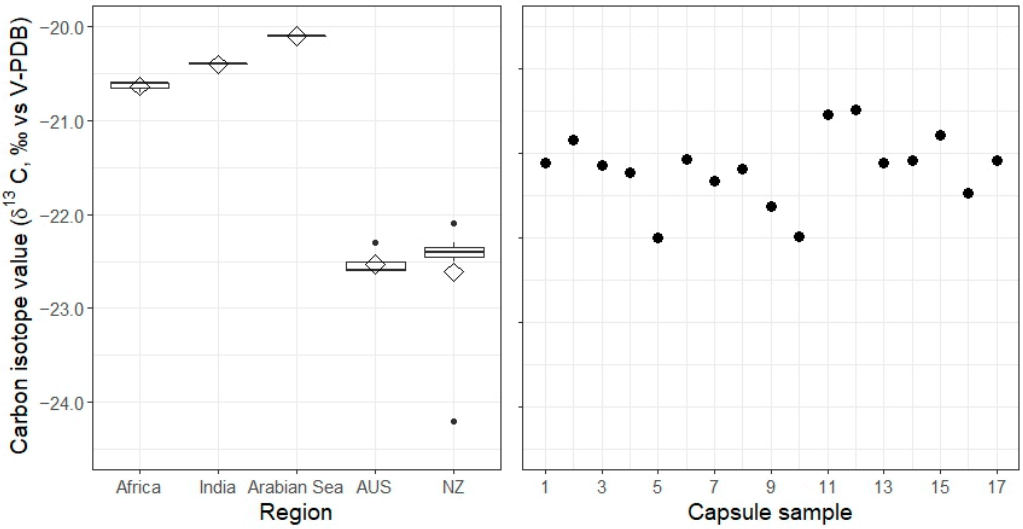

Figure 2. Compound-specific carbon isotope values for squalene isolated from deep-sea dogfish liver oil with a known origin (**left**) and samples from a range of commercially available squalene capsules (**right**). AUS denotes Southeast Australia, NZ denotes New Zealand. Boxplot: Boxes = upper and lower quartiles; horizontal line = median; vertical lines = value range; ◇ = mean; gong = outliers.

Stable isotopes are a conservative tracer (e.g., [34]); thus, for example, squalene which is derived from a mix of both Southeast Australian and alternatively sourced deep-water dogfish liver oils will possess a δ^{13}C value which reflects the relative proportion of each source. Based on the above, it would appear that only two of the capsule samples analysed (numbers 5 and 10) are potentially using squalene primarily derived from the Southeast Australia or New Zealand regions. Of the three products claiming to be sourced from Australian waters (as opposed to Australian made; numbers 8, 9 and 10), only one (number 10) appears to be using oil from Southeast Australia. The average isotopic value of the capsules is different than the Australian ($p < 0.00001$), New Zealand ($p < 0.00001$) and the Northeast Africa, India and Arabian Sea ($p < 0.0006$) squalene. The results strongly indicate that the majority of capsules analysed are either using squalene derived from other regions or from a mixture of sources. The less negative the δ^{13}C value, the lower the possible proportion of squalene obtained from the Australian and/or New Zealand regions. Clearly, none of the capsules analysed here are using squalene derived from olive oil (−26.8 to −29.3‰) [3].

Compound-specific isotope analysis is more technical and expensive compared to the more traditional bulk material methods utilising an elemental analyser coupled to the isotope ratio mass spectrometer (EA-IRMS). Both types of analyses were performed on samples and clearly demonstrated the potential effect of impurities with values obtained by EA-IRMS varying from those using CSIA on the same sample by up to 1‰ [3]. Thus, for analysing relatively pure squalene samples (for example, capsules), results from EA-IRMS may provide sufficient resolution to distinguish olive oil-derived squalene from marine sources. At this stage, the use of EA-IRMS is unlikely to be sufficient for distinguishing between Australian/New Zealand sources and those from other fisheries. To use EA-IRMS it would be necessary to ensure >90% purity of the squalene and even then, based on the data from [3], confidence limits would still be reduced. The analytical preparation used in this study routinely achieves a high level of purity which then ensures optimal compound-specific carbon isotope results by GC-IRMS. In addition, GC-IRMS requires several orders of magnitude less material than EA-IRMS, allowing for a much smaller scale of analytical preparation, particularly when analysing raw deep-water dogfish liver oil. In some instances (e.g., cosmetics), GC-IRMS alone may be sufficient with no sample

pre-treatment following a solvent extraction [3]. Other complementary approaches might also be used to assist in determining where the current tested products originate from.

4. Conclusions

The CSIA technique appears particularly promising with respect to assigning a South-east Australian/New Zealand origin for squalene which is beneficial in ensuring deep-water dogfish-derived squalene is being sourced from robustly managed and sustainable fisheries. Similarly, as new and emerging sources for squalene are developed (e.g., olive oil, microalgae, synthetic production), there will continue to be a need to ensure only sustainable sources are being utilised. Compound-specific carbon isotope analysis would appear to be an ideal tool in that it facilitates the analysis of squalene in a wide range of matrices, from raw deep-water dogfish liver oil and other potential sources of squalene to final products, such as those used in cosmetics and nutraceuticals.

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