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How do algae oils change the omega-3 polyunsaturated fatty acids market?☆

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Abstract – The health benefits of a diet rich in omega-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA) no longer need to be proven. However, while health authorities attempt to increase the consumption of the n-3 LC-PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), data from the latest intake surveys demonstrate that EPA and DHA consumption is still too low. A push towards greater sustainability, and a rise in vegetarianism are pushing manufacturers to move from traditional fish oils towards alternative sources. Microalgae oils provide a source of n-3 LC-PUFA with a lower environmental impact and are produced using processes that limit damage to the oils. This review aims to report on oleaginous microalgae strains available for n-3 LC-PUFA production, the processes used for their growth and the extraction and refining processes for their oils. It also addresses the challenges inherent in these products and their fabrication, and some of the novel characteristics of microalgal oils, including their very high n-3 LC-PUFA content and the chemical structure of their triglycerides, that lead to exciting opportunities in their use as functional food ingredients.

Keywords: DHA / algae oil / thraustochytrids / *Schizochytrium* / N-3 polyunsaturated fatty acids

Résumé – Comment les huiles de microalgues modifient le marché des acides gras polyinsaturés?
Les bienfaits santé d'une alimentation riche en acides gras polyinsaturés à longue chaîne oméga-3 (AGPI-LC n-3), tels que l'acide eicosapentaénoïque (EPA) et l'acide docosahexaénoïque (DHA) ne sont plus à prouver. Cependant, les données de la dernière enquête de consommation alimentaire en France (INCA3) indiquent que les apports en EPA et DHA sont encore trop faibles au regard des recommandations officielles. En outre, la tendance à une plus grande durabilité et l'augmentation du végétarisme poussent les producteurs à abandonner les traditionnelles huiles de poisson au profit de sources alternatives. Les huiles de microalgues constituent une source d'AGPI-LC n-3 d'intérêt en raison de leur faible impact environnemental et sont produites au moyen de procédés qui permettent de préserver leurs effets santé. Cette revue a pour objectif de faire le point sur les souches de microalgues oléagineuses disponibles pour la production d'AGPI-LC n-3 et de leurs conditions de fermentation, ainsi que des procédés d'extraction et de raffinage de leurs huiles. En plus d'aborder les défis liés à ces produits et à leur production, cette revue dresse un bilan des caractéristiques des huiles de microalgues, notamment leur forte teneur en AGPI-LC n-3 et la structure chimique des triglycérides, qui ouvrent des perspectives intéressantes quant à leur utilisation comme ingrédients alimentaires fonctionnels.

Mots clés : DHA / huile algale / thraustochytrides / *Schizochytrium* / acide gras polyinsaturés oméga-3

1 Introduction

Starting with the key observational epidemiological study conducted on Greenland Eskimo populations in the 1970s, numerous clinical and intervention surveys have demonstrated the nutritional benefits of a diet rich in n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Dyerberg *et al.*, 1975). Indeed, EPA and DHA are involved in all phases of life, from pre- and post-natal development to healthy aging, and in between through the maintenance of an effective immune system, neurological and cardiac functions. In addition, their consumption is linked to a lower risk of developing several pathologies such as neurodegenerative,

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cardiovascular and inflammatory diseases and cancers (Dyall and Michael-Titus, 2008; Riediger *et al.*, 2009; Calder, 2017; Saini and Keum, 2018).

In mammals, even if the liver is able to produce n-3 LC-PUFA from their n-3 precursor α-linolenic acid (ALA), the level of bioconversion is very low, so that endogenous synthesis cannot cover the body's physiological needs for EPA and DHA (Burdge and Wootton, 2002; Arterburn *et al.*, 2006; Blanchard *et al.*, 2013). Thus, n-3 LC-PUFA must be provided from the diet and so EPA and DHA are both classed as conditionally essential fatty acids. France recommends that EPA and DHA are each provided in levels of at least 250 mg per day in the diet but the results from the latest French epidemiological survey (Dubuisson *et al.*, 2017) have shown that in the adult population they are on average consumed at only 117 and 169 mg per day, respectively; this is at least 32% lower than the guidelines (Astorg *et al.*, 2011; *Avis et rapport de l'Anses sur la troisième étude individuelle nationale des consommations alimentaires - INCA 3*, 2017). The deficit is increased to 70% if we consider median consumption indicating that the bulk of the population does not even receive sufficient EPA or DHA to maintain good health. Intake of total lipids was in line with the recommendations at 34% of the total energy intake and thus the challenge is to increase the consumption of EPA and DHA without increasing those of total lipids. It is in this context that oils very rich in n-3 LC-PUFA may be of particular interest.

The main dietary sources of n-3 LC-PUFA are fatty fish and extracted fish oils. However, for reasons associated with (1) negative ecological impacts linked to overfishing, (2) health concerns due to possible contamination by heavy metals in the environment, (3) sensory issues due to the strong smell and flavour of fish, and recently (4) a move away from animal products, the n-3 LC-PUFA market is undergoing a real change in favor of microbial oils such as those extracted from microalgae. The culture of oleaginous microorganisms for production of oil rich in n-3 LC-PUFA is part of the wider search for sustainable and alternative sources of nutrients of interest; research that is leading to new products that are good for both human health and less damaging to the planet.

In 2018, microbial oils accounted for about 1.8% of the volume of the world n-3 LC-PUFA market and 15% of its value (GOED Omega-3, 2020). Between 2018 and 2019, the world volume of algae oils increased from 2009 to 2109 metric tons, an increase of 5%, and market players indicate that this trend is accelerating. Innovation is strong in this space, which is helping to increase the attractiveness of these products in market by increasing quality, reducing costs and improving sustainability. This innovation is illustrated by the annual numbers of publication of patents that have shown an increase of over 175% between 2010 and 2021.

One particular characteristic of microalgae oils that is encouraging their uptake is the high level of n-3 LC-PUFA compared to traditional fish oils. Usually, natural fish oils contain 10 to 30% combined of EPA and DHA and their contents can only be improved by esterification and molecular distillation of the oils followed by re-esterification to triglyceride (TG) molecules (Sehl, 2019), involving solvents and chemical intermediates. Climate change and oceanic warming may even be having a slight downward impact on n-3 LC-PUFA content of natural fish oils, exacerbating the problem (Kang, 2011). In contrast, microalgae have the capacity to produce very high level of EPA and DHA in their lipids, with some species reaching over 60% of total fatty acids as omega-3. Thus, the production of such oils not only has an interest from the point of sustainability, they also allow new nutritional approaches to incorporating n-3 LC-PUFA into the diet.

The aim of this review is to inform the reader of the state of art in the processes used in the production of microalgae oils rich in n-3 LC-PUFA: particularly those DHA-rich oils currently dominating the market. This includes the choice of strains, to the choice of how these microorganisms are grown and processed through to the refining of crude oil, taking into account customer requirements and the sensitivity of such oils to oxidation.

2 Strains selection and growth mode – the first steps in building a microbial PUFA platform.

2.1 Microalgae producing omega-3s

Oceans are the almost exclusive suppliers of n-3 LC-PUFA for human consumption. Since the 1980s, it has been acknowledged that fatty fish mainly acquire n-3 LC-PUFA from their diet and not by an endogenous synthesis. The majority of EPA and DHA is synthesized at the base of the food chain by many different microalgae species (Doughman *et al.*, 2007; Harwood and Guschina, 2009), which are consumed, and concentrated by the fish. It is apparent that microalgae represent an interesting alternative and vegan source for n-3 LC-PUFA. Nevertheless, several challenges remain to be addressed to ensure that these oils can be produced in sufficient quantity, at a low enough price and in a high-quality form to provide a viable alternative to fish oils. In recent times consumers have become more environmentally driven, and companies have drawn up corporate social responsibility programs to include the consideration of ecological and social issues alongside economic ones, which has facilitated the adoption of n-3 LC-PUFA-rich oils produced by microalgae over those from fish (Schade *et al.*, 2020), however available volume and sale price remain critical.

A wide variety of microalgae species are known to be able to produce n-3 LC PUFA. As a result of screening campaigns, many different candidates have been identified with the ability to grow in laboratory conditions, and with at least the potential to be industrialized. In choosing strains to develop further, many aspects are considered including speed and mode of growth, potential for production of toxins, and sensitivity to environmental factors such as temperature or pH.

Amongst those, which have advanced to development at an industrial scale, are several groups of interest: including Diatoms, Eustigmatophytes, Dinoflagellates and Thraustochytrids. Diatoms like *Phaeodactilum* and *Nitzschia* are microalgae with photosynthetic capacities, but which often also have the capacity to feed on organic matter. They are characterized by an external siliceous envelope. They are known for their low DHA and high EPA content, which can represent on

average around 30% of total fatty acids (Perdana *et al.*, 2021). Eustigmatophytes such as *Nannochloropsis* and *Trachydiscus* are obligate phototrophs and must gain their energy from light. These too produce little to no DHA and their predominant LC-PUFA is EPA. Most Diatoms and Eustigmatophytes produce relatively little triglycerides, and the majority of fatty acids are found in polar membrane lipids. Conversely, Dinoflagellates and Thraustochytrids respectively represented by *Crypthecodinium* and *Schizochytrium/Aurantiochytrium* are known for their higher DHA and their relatively low EPA contents. Indeed, DHA level can reach up to 60% of total fatty acids (Chang *et al.*, 2013). These cells also accumulate non-polar forms (TG) as an energy store and can store these at a significant proportion of total cell mass (Fan *et al.*, 2007). Thraustochytrids are not in fact true microalgae, being protists without plastids or photosynthetic capacity, but in the market and in common parlance oils from these organisms are known as 'algal oils' (Leyland *et al.*, 2017). To avoid being incomplete, they are included loosely in the term "microalgae" in this review.

While most microalgae use fatty acid synthesis pathways similar to those previously described in plant and animal species, two independent metabolic pathways have been identified in Thraustochytrids: the Fatty Acid Synthase (FAS) and Polyketide Synthase (PKS) pathways. The degree of use and the composition of these pathways again varies with species and environmental conditions – some have both, some have either one or the other – and in species where both are present, controls dictating which is used are often complex and not yet fully understood. The FAS pathway, common to most microalgae, is sometimes described as aerobic whereas the PKS pathway is described as anaerobic because the latter does not require the presence of O_2 to carry out the desaturations leading to the LC-PUFA products (Hu *et al.*, 2020). In strains where both are present, it seems that the FAS pathway is implicated in the production of short and medium chain FA while the PKS pathway is responsible for the majority of LC-PUFA synthesis (Morabito *et al.*, 2019).

2.2 Development strategy and scalability

This diversity in n-3 LC-PUFA-producing microalgae has been of great interest and has led to the accumulation of a wealth of scientific knowledge over the years. However, for a microalgae process to be competitive with fish-based processes and meet a growing market demand, it requires a high overall productivity and a certain degree of scalability.

Obviously, one of the most important factors in its success is the strain's capacity to accumulate EPA or DHA; some strains can accumulate lipids up to 70% of their dry mass, depending on culture conditions. In nature this lipid accumulation in microalgae would be a means of storing energy in response to triggers from their environment, often a stress of some kind. In an industrial context, this means that the production process should be designed with the same trigger. This can usually be achieved through a stress of the organism under culture and although different stresses such as phosphate limitation or salt stress may be used, nitrogen limitation is the most commonly used as it is relatively easy to implement and leads to the highest lipid contents (Ren *et al.*, 2014; Sun *et al.*, 2018). It has been previously proposed that the absence of nitrogen in the medium stops the cell division process and redirects the carbon flux from protein and membrane synthesis toward lipid production and accumulation (Morabito *et al.*, 2019).

Culture medium contains both the nutrients that the microalgae need to grow and produce lipids, but also provides an environment that mimics the natural habitat of these organisms. Many microalgae have a marine origin and care must be taken to design culture media that maintains the cells' environmental needs but does not damage equipment or influence later steps of the process. For example, strains may require the osmotic pressure of high salt levels but chloride ions are known to have corrosive effects on stainless steel and therefore should be replaced in the medium with other ions, or at least reduced (Chen *et al.*, 2016). Upstream processes (USP) must also take into account the needs of downstream processes (DSP) and again, the presence of chloride is deleterious when subjecting highly unsaturated oils to deodorization due to their catalytic effect and the high risk of monochloropropanediol (MCPD) synthesis. Therefore, the means of achieving the physiological parameters that have a critical impact on the quality of the product also have to be compatible with the industrial operation of the whole oil production process. In many cases trade-offs have to be made between process performance and product quality; for instance culture temperature has opposing impacts on productivity and the fatty acid profile (Zeng *et al.*, 2011). Higher temperatures generally promote faster growth rates and lipid production, whereas lower temperatures promote the accumulation of LC-PUFA in synthesized lipids. This is a physiological response wherein decreasing temperature decreases membrane fluidity with an impact on the exchange and transport processes. LC-PUFA increase membrane fluidity due to their less ordered structure and thus cells compensate for lower temperatures by increasing the production and incorporation of LC-PUFA into membranes.

Thus, maximizing production of highly unsaturated oils from microalgae is dependent on the understanding and replication of triggers that are part of the normal adaptative response of the strain to natural stresses, amongst them nutrient limitation leading to intra-cellular lipid accumulation and cold stress to encourage increased production of n-3 LC-PUFA in intracellular lipids.

One of the most important nutrients for the production of lipids is obviously a source of carbon. There are two possibilities when using microalgae; use of inorganic CO_2 *via* photosynthesis or use of an organic carbon source such as a sugar.

As noted above, several species with interesting fatty acid profiles can only be grown under photosynthetic conditions. Photosynthetic production provides certain opportunities including the additional potential to modify cellular metabolism through the choice of the wavelengths used in lighting. It does also present significant challenges: the requirement for light limits growth performance, since increases in algae cell number means more self-shading limiting the available light per cell. This makes it very difficult to achieve high cell densities. Two major types of production platforms are used in photosynthetic production: photobioreactors and opens ponds (Lopes da Silva *et al.*, 2019); the latter have the advantage of

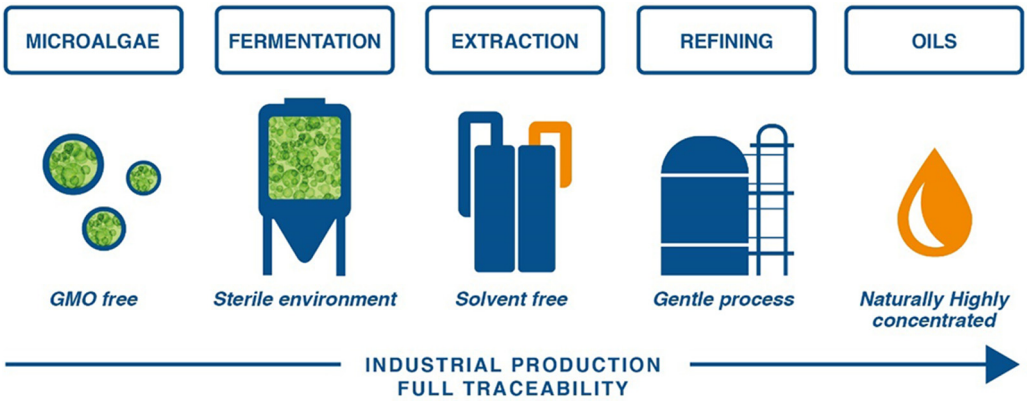

Fig. 1. Representation of a green process of microalgae oil production. The production of refined microalgae oils involves different steps. The first step is the culture of the microalgae strain *via* fermentation including a growth step, and then the accumulation of lipid and n-3 LC-PUFA. At the end of the fermentation, crude oil is extracted with a solventless process. The crude oil obtained is then refined using gentle processes to protect the oil.

low capital cost and easy access to relatively large volumes of culture but are subject to significant environmental variation and as such do not permit sufficient control to ensure a consistent product from the upstream process (USP) of biomass generation. Their outdoor location also leaves them open to environmental contamination, both chemical and biological, and predation of the cultures can lead to significant crop losses. Photobioreactors provide significantly more control over culture parameters and have the potential to operate all year round, especially if using artificial light. Costs of production with photobioreactors are often significantly higher and scaling to the volumes demanded in the omega-3 market can also be tricky with no significant economies of scale being seen.

Under photosynthetic production most species predominantly produce LC-PUFAs in polar lipid form. While these have interesting biological properties, their chemical structure leads to them being difficult to extract from the biomass and organic solvents are often needed. While both acetone and ethanol are approved for use with foodstuffs, their use in lipid recovery is both energy and resource intensive, leading to a significant environmental impact and negating in part the reason for using microalgae.

Heterotrophic production using an organic carbon source in an axenic environment allows greater control of the process and allows high cell densities and productivity to be reached, which has the consequence that the output of the upstream processes is far easier to treat in the downstream, being both more consistent in composition and requiring less dewatering. Scaling of heterotrophic production (known somewhat erroneously as fermentation) is also a well-established process already demonstrated in many biotechnological processes and large volume fermentation sites exist in many areas of the world, including where insufficient sunlight is found for photosynthetic production. Although microalgae have been shown to grow using various carbon sources such as glycerol, ethanol, acetate or even low-cost sources such as molasses (Ren *et al.*, 2013), glucose remains the most easily metabolized substrate maximizing growth performance and lipid production. Moreover, glucose syrup is also the most easily sourced carbon substrate from an industrial point of view. There are environmental impacts associated with the water and fertilizer use in glucose production, but oils produced in this manner still have a lower environmental impact than fish oils. Some organisms capable of heterotrophic growth are also capable of accumulating n-3 LC-PUFA in triglycerides and this form can be significantly easier to extract.

Summarizing, criteria for a microalgae strain to be compatible with an *industrial* n-3 LC-PUFA production process for use as a dietary supplement or ingredient include:

- High biomass productivity;
- Accumulation of significant quantities of LC-PUFA in conditions that are achievable on an industrial scale;
- Biomass production and composition are compatible with downstream requirements for extraction and refining;
- Potential production capacity is available for hundreds or thousands of tons of product per year.

While some companies have made a small success of production *via* photosynthesis, to date, only heterotrophic production of *Crypthecodinium* (De Swaaf *et al.*, 2003) and Thraustochytrids (Lenihan-Geels *et al.*, 2013) has succeeded in producing products that compete with fish oils on both quality and price. Therefore, they are predominant in current n-3 LC-PUFA production processes.

Using a fermentation platform has allowed the whole production chain for microbial oils to be rethought to avoid some of the less environmentally sustainable processes used in the production of fish or vegetable oils. For example, solventless extraction processes can be used to free the oil from the biomass, avoiding the use of organic solvents. Figure 1 represents a classical green process to produce microalgae oil rich in n-3 LC-PUFA, which can be broken down into three sequential steps:

– Fermentation of microalgae strains (upstream process), which increases the cell density, fat content and n-3 LC-PUFA concentration;
– Extraction of fat (downstream process) for crude oil recovery by an efficient and cost-effective extraction process;
– Refining of crude oils to meet customer and regulatory requirements.

For example, to produce oils from the DHA ORIGINS® line, Fermentalg uses a proprietary heterotrophic strain FCC3204 which is part of the *Aurantiochytrium* genus. This strain was isolated from a mangrove ecosystem and is the result of years of non-GMO lab selection and adaptation to fine-tune its growth performance, lipid accumulation and fatty acid profile. It has an optimal growth temperature around 30 °C, which allows growth rates of around 0.45 h^{-1} during the exponential growth phase. Both energy and a carbon source are provided by glucose syrup during the fed-batch production process. The strain has been adapted to grow with very low chloride concentrations and, eventually, it has been possible to entirely eliminate NaCl from the media, thus avoiding the corrosion risk to the stainless-steel equipment used in the production process and reducing the risks of unwanted chemical reactions during refining. As is the case in many fermentation processes, we have chosen to separate our process into two main parts. The first part consists in increasing the concentration of catalytic biomass (with a relatively low lipid level). During this stage, the applied culture conditions, which include non-limiting nutrients, high oxygen provision and warmer temperatures, favor high glucose consumption and biomass accumulation rates. When the biomass concentration is satisfactory, a nitrogen deficiency is then applied to trigger the accumulation of intracellular lipids under TG form until the end of the fermentation. During the last hours of fermentation the temperature may also be decreased to optimize DHA levels. By combining our strain and the optimized conditions of our fermentation process, it became possible to reach an overall biomass productivity of over 1 g. L^{-1}. h^{-1} with DHA making up around 70% of the total fatty acids (FA); as a consequence the oils also contain low levels of saturated fatty acids.

3 Extraction process: from fermentation broth to crude oil

3.1 Oxidation of crude oil: a primary consideration

Since microalgae oils are very rich in LC-PUFA, they are also very sensitive to oxidation phenomena with a much higher density of double bonds in the oils. Consequently the risk of initiation of oxidation and the kinetics of propagation steps are significantly increased compared to vegetable oils or even normal fish oils (Frankel *et al.*, 2002; Jacobsen, 2010). Indeed, to our chagrin we have observed that oxidation of oil residues can generate sufficient heat that even small amounts of process wastes can spontaneously catch fire! If uncontrolled, reactions of the oil during processing can lead to an increase in oxidative indices (peroxide value and *para*-anisidine value) and the formation of undesirable fishy and rancid off-flavors. It is therefore critical that, throughout the process of extraction and refining of microalgal oils, particular attention is given to the limitation and control of oxidation, and if necessary, the integration of process steps designed to remove undesirable compounds without damaging oil quality. For fermentation processes, control is such that this can begin from the moment that the growth is complete and the cells have no further need for oxygen. The use of nitrogen blanketing and antioxidants from the very early stages of harvest has a beneficial effect on oil quality whilst vacuum can also be used during refining to prevent contact with oxygen. These precautions are even more important when pro-oxidative factors are present, as in the case during an aqueous extraction process. Here, crude oil will be in contact with the culture medium containing all the compounds used for the growth of microalgae biomass, but while, numerous strains need iron or traces of copper for growth, these metals are also known to initiate the oxidation reactions of n-3 LC-PUFA.

3.2 Dewatering to facilitate oil extraction

Due to their size, growth environment and mixture of lipid types, microalgae present unique challenges when it comes to the extraction of their oils and classical oilseed processes (*i.e.*, pressing or solid/ liquid extraction) cannot be applied. Novel techniques specific to algae have thus been developed.

At harvest, the microalgal biomass has a very high water content from the culture medium (which can be more than 90% of the total) and so once lipid-rich biomass has been produced with all the desired parameters (*i.e.*, dry cell weight, fat content, n-3 LC-PUFA concentration) the first step of crude oil recovery is often a reduction in water content. Different methods including flocculation, filtration and centrifugation may all be used to increase the density of cells to facilitate the further oil recovering (Molina Grima *et al.*, 2003). Full drying using a drum dryer, vacuum dryer or spray-dryer for example may be a prerequisite for solvent extraction of the lipids, but the energy requirements are an important additional cost, and often significantly increases the environmental impact of the process reducing its sustainability due to the impacts of the energy production (Lardon *et al.*, 2009). In addition, the lipids rich in LC-PUFA, which are the reason for producing the algae in the first place, are very sensitive to oxidation. The high temperatures required for evaporating water, often in a stream of air, impose a significant additional risk of oxidation and lipids extracted after drying tend to have a lower quality.

3.3 Cell lysis as an aid to oil extraction

The cell wall or membrane of microalgae can present a significant barrier to oil extraction and cell lysis is often an integral part of the extraction process, even when solvent-based techniques are to be used (Halim *et al.*, 2012). The microscopic size of microalgae is significantly smaller than that of sunflower or rapeseeds and makes traditional shredding and pressing equipment unsuitable; other techniques are required to break open the cells and free the oil.

Mechanical techniques such as bead milling, microwave-assisted disruption, ultrasonication, and high-pressure homogenisation, or chemical or enzymatic lysis are used to break the

cell wall and release the lipids enclosed within the biomass. Extraction using gentle techniques such as enzymes are often the most efficient technique overall since mechanical disruption can often lead to the creation of emulsions whose destabilisation is subsequently a challenge (Vian *et al.*, 2013) leading to a reduction of yield and an increase of oil oxidation.

Since cell membranes of microalgae cells are mainly composed of proteins and polysaccharides, proteases and carbohydrases are widely used in the literature and at the industrial scale, due to their capacity to hydrolyse macromolecular structures within the cell wall and membrane (Lin *et al.*, 2018) and their relative low cost. The choice of enzyme(s) and reaction conditions are tailored to the type of algae for maximum efficacy against the types of structures to be found enclosing the cell. For example, lipids from *Scenedesmus* sp. were extracted at a high rate (86%) at 30–50 °C and pH 3.5–4.5 using a mix of cellulase, pectinase and hemicellulase. Controlling the dry matter content, the temperature and the enzyme concentration were key to high yields (Huo *et al.*, 2015). Using cellulase and protease made possible the extraction of a third to a half of lipids from *Chlorella vulgaris*, *Nannocloropsis* sp. and *Scenedesmus dimorphus* but reduced pH, optimised enzyme rate and extended lysis duration were necessary (Liang *et al.*, 2012). Some types of cells can be lysed with just proteases, for example oil may be extracted from *Schizochytrium* sp. using an alkaline protease (3%), at 55 °C over 9 h (Lin *et al.*, 2018).

3.4 Lipid recovery using solvent: a solid/liquid extraction process

Conventional processes for recovering intra-cellular lipids using organic solvents are widely described in the literature (Medina *et al.*, 1998; Vian *et al.*, 2013; Dvoretsky *et al.*, 2016). However, these techniques have safety issues requiring specialized explosion-proof zones and energy required for drying and solvent recovery is high. Traces of hexane, ethanol or other food-grade solvents in oil extracts, whilst never usually at harmful levels, are nonetheless poorly perceived by consumers and "solvent-free" processes give products an additional advantage in the marketplace. Where possible, therefore, the market is moving away from solvent-extraction, although for some polar lipid-rich mixtures this is still unavoidable.

An alternative method of solvent extraction is the use of supercritical fluids. Often using CO_2 as the primary solvent, this type of extraction is seen as "greener" and "solvent-free" as it leaves no residues in the extracted material. Supercritical CO_2 is already used for many types of aromatic plants, exotic fruits and spices, most often to extract antioxidant molecules, demonstrating that this type of process is applicable to sensitive molecules (Herrero *et al.*, 2006). The use of a co-solvent such as ethanol may be required in order to extract more-polar molecules and coextraction with $scCO_2$/Ethanol has been applied to vegetable oilcake to recover oil that has resisted the traditional upstream extraction process (Bardeau *et al.*, 2015). Due to the high pressures involved, capital costs of equipment for supercritical extraction are high and so this process is currently reserved for very high value molecules, but groups are investigating other supercritical solvents in order to bring down costs and make the techniques more available (Catchpole *et al.*, 2012).

The production of oils rich in LC-PUFA from microalgae is part of a move towards more sustainable sourcing and the use of organic solvents for microbial oil extraction, which is perceived as being contrary to this approach, is therefore being phased out wherever possible.

3.5 Lipid recovery without drying: a liquid/liquid extraction process

For biomasses rich in non-polar lipids an alternative is to break open cells in an aqueous environment and separate the oil that is released. The aqueous environment of the lysed cell mixture is complex, containing both molecules derived from the growth medium and from the contents of the cell. While it is a well-known truism that oil and water don't mix, this complex mixture include peptides, polar and non-polar lipids and carbohydrates, many of which can act to stabilize emulsions, which inhibits the easy separation and extraction of the oil droplets from the cells. Fortunately, in some cases these appear to be mainly stabilized by proteins and protein residues and proteases can aid in the destabilization of the emulsion. For example, in a recent study conducted by Xue *et al.* (2021), an increase of liquid to solid ratio, the enzyme concentration and the reaction time increase the activity of papain against *Schizochytrium* sp. cells and improved the ratio of non-emulsified oil to emulsified and consequently favored the availability of oil for extraction. Fermentalg, along with other leading producers of oils from Thraustochytrids, has developed its own method of enzymatic lysis followed by emulsion resolution, and this is now routinely used industrially to free oil from cultures tens or hundreds of thousands of liters at a time.

Extraction into an aqueous environment also means that the oil is in close proximity to several pro-oxidative compounds such as metal ions or dissolved oxygen. As mentioned previously, LC-PUFAs are particularly sensitive to oxidation and the presence of these compounds could initiate oxidation reactions leading to the production of an oxidized oil, non-compliant with customer and regulatory requirements and with impaired organoleptic qualities. Therefore, the contact time between oil and water after lipids release should be minimized to protect LC-PUFA in the crude oil from oxidation. Separation of oil and water medium is generally carried out using classical liquid/liquid methods such as centrifugation. During these processes contact with oxygen in the air should be avoided by using an inert gas, typically nitrogen, or a vacuum to fill the tanks and containers (Winwood, 2013).

In some cases, especially where the extract is rich in polar lipids, the final product form may be a crude extract. However, for other oils, especially those that are predominantly triglycerides, a refining step is used to further improve the quality of the oil and ensure long-term stability.

4 Refining of crude algae oil: a purification step of oil

All crude oils (vegetable, animal or algae oils), with the exception of virgin oils and lipid mixtures with a high polar-lipid content, are refined to improve their organoleptic properties (appearance, smell, taste) and the safety of the

edible oils. Notably, refining processes are used to remove chemical contaminants (*e.g.*, pesticides, heavy metals, high molecular weight molecules), molecules that can affect the refining process (free fatty acids, phospholipids (PL), monoglycerol) or that impact the visual appearance of the oil (waxes, wax esters, highly saturated TG). The level of refining required may be determined by the market expectations or the regulations for the final use (such as infant food, food and beverages, or dietary supplements).

A traditional refining process includes several steps, each removing different undesirable compounds: degumming, neutralization, bleaching, winterization or fractionation and then deodorization. This order is relatively well preserved, whatever the source of the crude oils, since subsequent steps may be adversely affected by molecules removed in early steps. In some cases steps may be omitted. For example if appropriately extracted, crude algae oils extracted using an aqueous solventless processes can contain sufficiently low levels of polar compounds like phospholipids and free FA (FFA) (Nyam *et al.*, 2009) that the degumming and neutralization steps can be avoided. Reducing the number of process steps reduces the oxidation risk to the oil, and the environmental impact of the process (no caustic soda in wash water), which also improves the refining yield, has a positive impact on process time and thus reduces costs.

The refining process of a crude algae oil starts with a bleaching step in which bleaching agents (activated earth and/or activated carbon) remove some pigments, secondary oxidation products and traces of PL and metals by molecular adsorption reactions (Vaisali *et al.*, 2015). Due to their activation, acid-bleaching earths can also decompose peroxides into smaller molecules (*e.g.*, aldehydes, ketones, acids) (Zschau, 2001; Silva *et al.*, 2014), which can then be removed by adsorption on clays, reducing the oxidative index of the oil. Bleaching agents are removed by a simple filtration step conducted under nitrogen.

Not all coloured compounds need to be removed. Crude oil extracted from algae can often be highly pigmented due to the accumulation of high amounts of carotenoids. For some Thraustochytrid species this is mainly astaxanthin and β-carotene (Tab. 2), which can be retained in oil during the extraction process. Carotenoids are important natural antioxidants reacting as reactive oxygen and radical scavengers and quenchers of UV irradiation (Subagio and Morita, 2001; Guerin *et al.*, 2003). It has been proposed that carotenoids increase during the cell lipid accumulation step of fermentation to protect PUFAs against oxidation reactions (Morabito *et al.*, 2019). Retaining these compounds during bleaching is thus a further way to protect PUFA-rich oils against oxidation. Using different refining parameters it is possible to produce, from a single crude algae oil, different color refined oils (Fig. 2). Thus, DHA ORIGINS® 550-O (refined orange oil; Fermentalg) is produced by a soft refining process, ensuring high quality and a good sensorial experience while retaining the pigments. At the opposite end of the spectrum, the production process of DHA ORIGINS® 550-Y (refined yellow oil; Fermentalg) consists in the total removing of carotenoids during the bleaching step with the same quality and sensorial goals.

Different research groups have demonstrated that Thraustochytrid strains are able to produce sterols and squalene during the exponential phase step of the fermentation (Morabito *et al.*, 2019), notably stigmasterol, cholesterol and ergosterol. These sterols, along with TGs with high saturated FA levels have a high melting point and thus are likely to crystallize at room temperature, reducing the clarity of the oil. For applications where oil clarity is important, these high melting point compounds are removed by a fractionation process, which consists of gradually cooling to control the crystallization of these components in the oil followed by their removal using filtration at low temperature.

The refining process of algae oil ends with a deodorization step whose aim is to remove low molecular weight, volatile compounds by steam distillation at high temperature (180–200 °C) and very high vacuum (< 5 mbars). During this final step, compounds causing off-flavors (*e.g.*, aldehydes, ketones) resulting from lipid and protein degradations, residual FFAs and remaining pigments are volatilized, thus improving the organoleptic properties of the refined oil. For consumer acceptance, the deodorized oil obtained must have the lowest possible intensity of both smell and taste as one of the major complaints surrounding omega-3 supplements is the presence of a fishy aftertaste or fishy burps.

In the case of oils highly rich in LC-PUFAs, such as microalgae oils, a particular attention must be paid to the deodorization temperature. At temperatures above 200 °C many sensitive compounds including EPA, DHA and antioxidants (tocopherols, carotenoids) are degraded by cyclisation, decomposition and polymerization (Fournier *et al.*, 2006), and thus deodorization should ideally be carried out at lower temperatures. This also reduces the risk of MCPD and glycidyl ester (GE) formation during the process.

After the refining process is completed, the refined oil may be supplemented with different antioxidants such as tocopherols, ascorbyl palmitate and rosemary extract, to improve its shelf life and guarantee the best sensorial experience of oil throughout its shelf life.

5 Composition of algae oils

Characterization of microalgae oils demonstrates that these oils are not just fish oils from another source but can have unique properties in term of lipid composition, n-3 LC-PUFA content chemical structure of TG and sensorial experience.

The vast majority of algal oil available in today's market is derived from the family of Thraustochytrids. While these share some similarities, the composition of the oils is intimately linked to the strains used and also to the fermentation conditions used in their production (Wang and Wang, 2012; Quilodrán *et al.*, 2020). The refined algae oils from the DHA ORIGINS® line (Fermentalg) may be chemically characterized in term of their fatty acid (FA) profile (Tab. 1) and their lipid and sterol profile and carotenoid content (Tab. 2).

The fatty acid (FA) profile of algae oils is far simpler than the profile seen in fish oils. Indeed, analysis of refined oil from DHA ORIGINS® line revealed that there are only 3 major FA: palmitic acid (16:0), 22:5 n-6 and DHA (22:6 n-3) representing near to 90% of total FA of oil total lipids. In fish oils, there is significant variability, even within species and more than ten FA can be recovered at significant proportions (Brockerhoff *et al.*, 1968; Aursand *et al.*, 1995; Belarbi *et al.*, 2000). In addition to having a simpler FA profile, the n-3 LC-PUFA

Table 1. Main fatty acid profile (%) of two representative batches of refined oils from DHA ORIGINS® 550 range (Fermentalg).

	DHA ORIGINS® 550-O	DHA ORIGINS® 550-Y
Fatty acid profile (%)		
16:0	12.2	11.8
18:0	0.5	0.8
18:1 n-9	2.7	4.0
18:2 n-6	0.6	0.7
18:3 n-3	0.2	0.3
20:4 n-3	0.7	0.7
20:4 n-6	0.1	0.2
20:5 n-3	0.5	0.6
22:5 n-6	14.1	13.3
22:6 n-3	64.8	63.3
Fatty acid quantification (mg/g as FA)		
20:5 n-3	4.2	5.4
22:6 n-3	597.0	596.5

DHA ORIGINS® 550-O is produced using a soft-refining process resulting in the retention of carotenoids. DHA ORIGINS® 550-Y is produced using a refining process that removes all the pigments from the crude oil.

Table 2. Lipid and sterol profile (%), sterol content (g/ kg of oil) and carotenoid content (mg/ kg) of two representative batches of refined oils from DHA ORIGINS® 550 range (Fermentalg).

	DHA ORIGINS® 550-O	DHA ORIGINS® 550-Y
Lipid profile (%)		
Sterol esters	< 0.1	< 0.1
Free sterols	0.8	0.6
Triglycerides	99.0	99.1
Free fatty acids	< 0.1	< 0.1
Diglycerides	0.2	0.3
Monoglycerides	< 0.1	< 0.1
Sterols content (g/kg oil)	7.9	7.7
Sterol profile (%)		
Cholesterol	42.2	52.6
Ergostadienol	2.7	4.2
δ-7-cholesterol	2.1	1.9
Ergosterol	5.2	3.5
Stigmasterol	2.3	10.3
Stigmastadienol	37.8	21.9
Stigmastenol	7.7	5.6
Carotenoid content (mg/kg)		
Astaxanthine	8.0	0.0
β-carotene	4.0	0.0

DHA ORIGINS® 550-O is produced using a soft-refining process resulting in the retention of carotenoids. DHA ORIGINS® 550-Y is produced using a refining process that removes all the pigments from the crude oil.

Fig. 2. Crude and refined oils from DHA ORIGINS® 550 range (Fermentalg). From a single crude oil, different refined oils in term of color could be obtained. (a) crude algal oil; (b) refined orange oil (DHA ORIGINS® 550-O); (c) refined decoloured oil (DHA ORIGINS® 550-Y).

Table 3. Molecular TG species (%) and main fatty acid positions on the glycerol backbone of a representative batch of DHA ORIGINS® 550-Y oil.

TG species	Refined oil
22:6/14:0/14:0	1.0
22:6/14:0/16:0 or 22:6/16:0/14:0	3.1
22:6/16:0/16:0	19.4
22:6/16:0/18:0 or 22:6/18:2/16:0	1.9
22:6/16:0/20:5	3.9
22:6/16:0/20:4	3.1
22:6/16:0/22:6	32.9
22:6/22:6/22:6	16.6
22:6/22:5/22:6	18.1

TG species were analyzed by thin layer chromatography and mass spectrometry. Data represented fatty acids esterified in the *sn-1*/*sn-2*/*sn-3* positions on the glycerol backbone.

concentration, notably DHA, is generally much higher than LC-PUFA levels of fish oil. High levels of LC-PUFA in a small volume of oil are far more acceptable to the consumer, and many fish oil supplements on the market are transformed through chemical and physical concentration steps to reduce the amount of oil needed for a meaningful dose. This is unnecessary for most microalgae oils, which can naturally reach very high levels of DHA.

TG molecules from DHA ORIGINS® 55O-Y oil were analyzed by mass spectrometry to determine the main FA positions on the glycerol backbone and molecular species (Tab. 3). All TG species had at least one DHA molecule at the external (*sn-1/3*) position of the glycerol backbone. Nearly 77% of TG species have one molecule of DHA (mono-DHA), 21% have two DHA (di-DHA) and 1% three DHA (tri-DHA). The latter represents the only TG species with DHA at the internal (*sn-2*) position of the glycerol backbone. This TG profile is closer to that observed in marine mammalian oils, like seal or whale oils, in which DHA is exclusively located at the *sn-1/3* positions. In fish oils, nearly 50% of DHA is located at the *sn-2* position (Brockerhoff *et al.*, 1968; Aursand *et al.*, 1995).

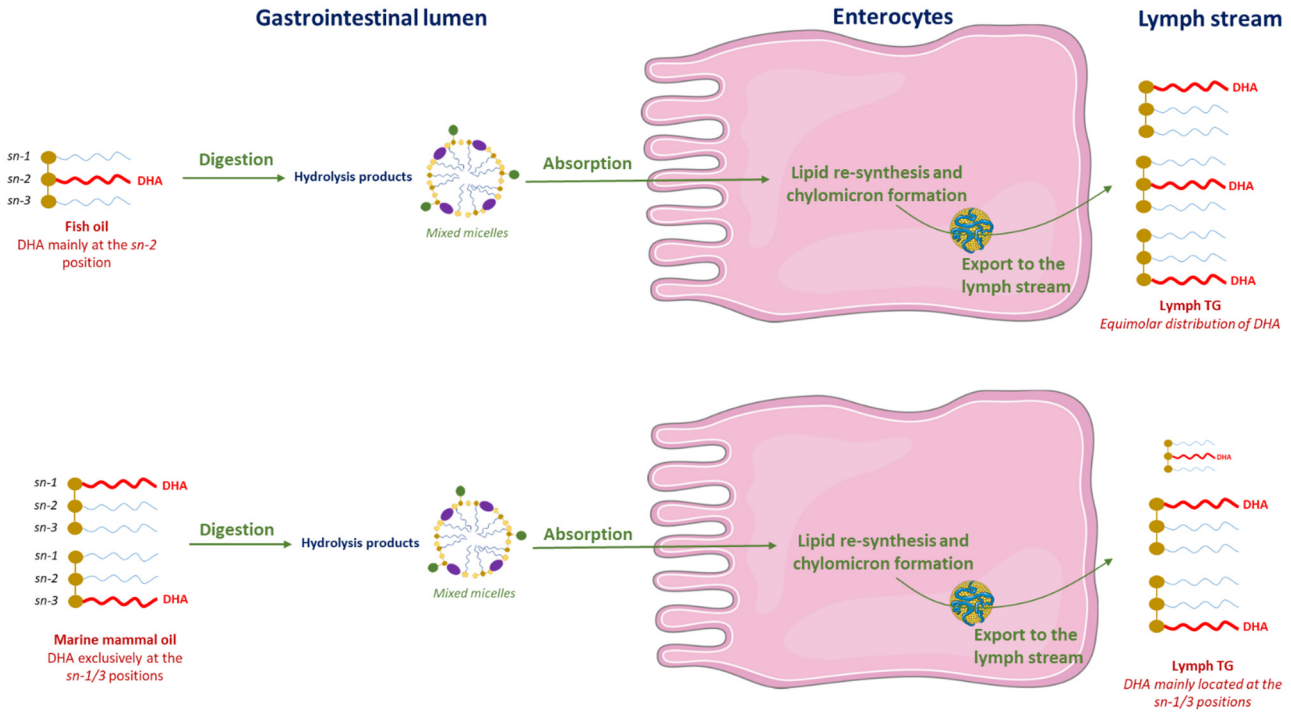

Fig. 3. Dietary TG DHA position impact on lymph TG DHA position. After gastro-intestinal digestion and absorption, DHA is incorporated into TG in enterocytes during re-synthesis of lipids. Neo-synthesized lipids are compacted with PL and proteins to form chylomicrons which are then exported to the lymph stream. When DHA is provided by fish oil (DHA mainly at the *sn-2* position), DHA is equally distributed between the 3 positions in lymph TG. However, when DHA is supplied from a marine mammal oil (DHA exclusively at the *sn-1/3* positions), its position is mainly preserved so that DHA remains at *sn-1/3* position of lymph TG.

While "lipids" may include polar lipids such as phospholipids, what consumers recognize as "oil" is mainly composed of TG (generally > 95% for refined products). Free sterols and diglycerides often represent less than 1% of the oil volume in a refined product. Interestingly, in Thraustochytrid oils, the sterol profile contains compounds traditionally associated with both animal and vegetable sources (Bai *et al.*, 2021). Indeed, the major sterol is often cholesterol (animal), but more than 50% of total free sterol of algae oils is composed of phytosterols (plant). Nonetheless, the profile is dominated by only a few types; cholesterol, stigmastadienol and stigmastatrienol make up more than 70% of total sterols (Wang and Wang, 2012; Tab. 2).

6 What about the bioavailability of microalgal n-3 LC PUFA?

Most of the work to date studying the ingestion and uptake of n-3 LC PUFA has been performed with fish oils. There remains some uncertainty if these results can be directly transposed to microalgal oils given the slight differences in physicochemical composition between the two sources. Comparison between polar lipid forms from photosynthetic algae and fish oils in triglyceride form is complex due to the potential differences in their ability to emulsify in the gastrointestinal tract, and differences in uptake measurement.

Nonetheless, several studies have looked specifically at microalgal n-3 LC-PUFA in oil form and have demonstrated that PUFA are both taken up and have beneficial effects in the body (Lane *et al.*, 2014; Li *et al.*, 2021); intake of microalgal DHA (0.2–1.0 g/day) over 4 weeks resulted in a dose-dependent increase in the DHA content of plasma and erythrocyte lipids, and reduced ARA levels in the same tissues (Arterburn *et al.*, 2007). Likewise, consumption of 0.94 to 1.5 g microalgal DHA/day over 4 to 8 weeks doubled plasma DHA content and reduced plasma TG by 14 to 23% at the end of the studies (Geppert *et al.*, 2006; Sanders *et al.*, 2006), with best effects in patients with the highest triglyceridemia (Geppert *et al.*, 2006). These data have been supported by a study conducted with hypertriglyceridemic subjects who had taken 2.4 g of microalgal DHA/day for 14 weeks. Doubled plasmatic DHA level was detected associated with an improvement of cardiovascular risk factor because of the plasmatic TG and LDL-C reduction (Maki *et al.*, 2014). However, no differences were observed in comparison to the group supplemented with fish oil. All these studies seem to demonstrate that bioavailability of DHA from algae oil is at least equal to that from fish oil.

Nevertheless, among factors influencing the bioavailability of FA, the chemical structure of dietary TG is of particular significance. It has been previously demonstrated that the DHA position in dietary TG directly influences its bioaccessibility (*i.e.*, digestion and intestinal absorption), which is the first stage of systemic bioavailability (Chernenko *et al.*, 1989; Christensen and Høy, 1996; Yoshida *et al.*, 1996; Porsgaard and Høy, 2000). Other studies have indicated that the position could also influence the tissues in which the DHA is finally incorporated (Sehl, 2019).

In vitro studies have demonstrated that increased levels of EPA and DHA in the *sn-1/3* positions of TG reduced pancreatic lipase activity due to a steric hindrance (Bottino *et al.*, 1967; Akanbi *et al.*, 2014). These preliminary results suggested that oils with DHA at the *sn-1/3* positions of TG could theoretically be less digestible than fish oils. However, *in vivo* studies conducted in rats using seal and whale oils *vs.* fish oils have shown that DHA digestion and intestinal absorption were only minimally influenced by the differences in chemical structure between these food sources.

DHA position in the glycerol backbone of dietary TG has an important influence on lipid synthesis occurring in enterocytes after intestinal absorption. In rats fed with seal or whale oils (DHA exclusively at the *sn-1/3* positions), these positions were conserved in the triglycerides of chylomicrons leading to the synthesis of lymph TG with DHA also mainly located at the *sn-1/3* positions (Christensen and Høy, 1996). In fish oil groups, where DHA is primarily found in the *sn*-2 position, DHA appears to have been redistributed in chylomicron TG and only 30% of DHA was detected at the *sn-2* position in lymph. (Fig. 3). It has been proposed that DHA position in chylomicron TG directly impacts its metabolic fate (Ackman, 1988): DHA at the *sn-2* position would go to the liver whereas DHA at the *sn-1/3* position could be a source for extra-hepatic tissues like brain and retina. In this regard, dietary sources of DHA (algae oil *vs.* fish oil) could influence its metabolic fate and eventual incorporation into tissues where DHA is of structural importance (Sehl, 2019).

Despite these interesting indicators, further studies are still needed to better understand the impact of DHA position in the glycerol backbone on its bioavailability and use in the body. Particularly, it would be of great interest to study how fermentation conditions could influence the DHA position in TG, how its position in TG impacts its use within the body and thus how this could be used to target DHA to different tissues. This study would allow to better target the therapeutic effects of DHA by driving its tissue accretion.

7 Can microalgae oils help increase n-3 LC-PUFA uptake?

EPA and DHA are conditionally essential fatty acids; and while conversion of plant-derived ALA into EPA and DHA in the liver occurs, it does so at too low a rate to supply physiological requirements for n-3 LC-PUFA. To maintain a healthy level, n-3 LC-PUFA must be directly supplied by the diet, which today is mainly sourced from fatty fish (*e.g.*, salmon, tuna, sardine, mackerel).

There are many barriers to increasing uptake, with sustainability, a dislike for fish or fish-taste (either personal or ethical) and ease of access being amongst the front runners.

Vegetarians and vegans, who do not eat fish generally cannot meet their recommended intake of EPA and DHA as revealed by lower plasmatic status of n-3 LC-PUFA in comparison to omnivores (Rosell *et al.*, 2005; Saunders *et al.*, 2013; Harris, 2014). Since microalgae are non-animal, and in some cases are considered by the public as microscopic plants, these oils offer an excellent alternative to traditional fish oils. In this context, vegetarians and vegans, especially specific populations with increased requirements such as pregnant and nursing women, can enjoy the n-3 LC-PUFA health benefits in accordance with their ethical standards.

Thanks to the degree of protection, which can be provided during the extraction and refining process, and in contrast to fish oils, refined microalgae oils are generally not exposed to significant oxidation prior to consumption. They are thus not characterized by marine smell and taste notes (often described as fishy or painty) and instead have more vegetable and nutty notes. Microalgae oils overcome classical objections based on the fishiness of other omega-3 sources. Their superior organoleptic properties provide an opportunity for expanded incorporation into food applications and thus increased consumption amongst a wider public. While prices for microbial oils remain higher than fish oils, technological improvements, increased volumes of production and increases in the n-3 LC PUFA content of microbial oils are working to drive the price per kg of omega-3s down and as the availability of fish oils becomes more limited the gap is closing fast.

Microbial sources of n-3 LC PUFA thus provide a real opportunity for increasing uptake and helping to address the nutritional needs of the population.

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Conflict of interest

Anthony Sehl, Emma Caderby, Sammy Bouhouda, Hywel Griffiths and Sonia Da Rocha Gomes are employees of Fermentalg.

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