FUNCTIONAL CHARACTERIZATION OF THE STRIPED SNAKEHEAD (*CHANNA STRIATA*) DESATURASE GENE, AND ITS IMPLICATION IN LONG-CHAIN POLYUNSATURATED FATTY ACID SYNTHESIS

by

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ABSTRAK

Ikan merupakan sumber utama asid lemak rantai panjang poli-tak-tepu (LC-PUFA) omega-3, antaranya asid eikosapentaenoik (EPA) dan asid dokosaheksaenoic (DHA), yang penting bagi kesihatan optimum manusia. Namun begitu, penggunaan minyak ikan oleh industri akuakultur sebagai sumber lipid dalam makanan ikan dianggap tidak lestari. Minyak daripada tumbuhan mengandungi asid lemak poli-tak-tepu (PUFA) karbon 18 (C18) yang tinggi dan tidak mengandungi LC-PUFA, dicadangkan sebagai alternatif kepada minyak ikan. Oleh itu, pemahaman daripada aspek molekular enzim yang terlibat di dalam laluan biosintesis PUFA C18 kepada LC-PUFA dapat membantu dalam penggunaan minyak tumbuhan di dalam makanan ikan. Kajian ini dijalankan untuk mencirikan fungsi salah satu gen jujukan lengkap desaturase, *fads* (EU 570220), daripada ikan haruan, *Channa striata* (Bloch, 1793), yang merupakan ikan air tawar yang bersifat karnivor; dan untuk menilai peranan gen tersebut dalam penggunaan minyak tumbuhan dalam pemakanan spesis ini. Keputusan daripada kajian ini menunjukkan bahawa ikan haruan mempunyai gen desaturase dwifungsi dengan kebolehan menyahtepukan substrat asid lemak pada kedudukan Δ4 and Δ5. Ini menunjukkan bahawa ikan ini berkemampuan menukar EPA kepada DHA menggunakan laluan yang lebih singkat berbanding laluan biasa. Pengekspresan gen *fads* yang paling tinggi telah dikesan di dalam tisu hati dan otak ikan. Satu eksperimen telah dijalankan secara *in-vivo* dengan penyediaan 4 jenis makanan ikan menggunakan sumber lipid berbeza, iaitu minyak ikan (FO), minyak linseed (LO), minyak jagung (CO), dan campuran minyak linseed and jagung (LOCO), telah mengesan regulasi nutrisi gen *fads* di dalam tisu hati ikan. Didapati penyimpanan EPA dan DHA di dalam otot ikan yang diberi

makanan berasaskan minyak tumbuhan lebih rendah daripada ikan yang diberi makanan berasaskan miyak ikan. Eksperimen susulan kemudiannya dijalankan dengan penggunaan minyak echium, sejenis minyak tumbuhan yang mengandungi asid stearidonik (SDA, 18:4n3) yang tinggi, untuk membekalkan substrat permulaan PUFA C18 yang boleh memintas langkah penyahtepuan enzim Δ6 desaturase di dalam laluan biosintesis dan untuk menentukan jika penyimpanan LC-PUFA dapat ditingkatkan. Lima jenis makanan telah disediakan mengandungi minyak yang berbeza: minyak ikan (100FO), minyak linseed (100LO), minyak echium (100EO), campuran minyak echium dengan linseed (50EOLO) dan campuran minyak echium dengan minyak ikan (50EOFO). Tahap EPA ditingkatkan di dalam hati ikan yang diberi makanan 100EO dan boleh dianggap setanding dengan ikan yang telah diberi makanan yang mengandungi minyak ikan. Tambahan pula, pengekspresan gen *fads* di dalam hati ikan 100EO lebih tinggi berbanding dengan 100FO, menunjukkan aktiviti biosintesis dapat ditingkatkan dengan penambahan SDA sebagai substrat permulaan. Kandungan asid α-linolenik (ALA, 18:3n3) dan asid linoleik (LA, 18:2n6) di dalam otot ikan secara amnya menunjukkan profil asid lemak yang sama seperti makanan. Kandungan LC-PUFA dikesan di dalam otot ikan yang telah memakan makanan berasaskan minyak tumbuhan tetapi pada aras yang rendah daripada makanan yang mengandungi minyak ikan. Penyimpanan DHA paling rendah di dalam otot ikan yang diberi 100LO, ini mungkin menunjukkan bahawa penambahan minyak echium yang kaya dengan SDA berpotensi meningkatkan penyimpanan DHA berbanding dengan ALA yang didapati daripada minyak linseed. Untuk kedua-dua eksperimen pemakanan, tahap DHA di dalam tisu otak tidak menunjukkan perbezaan yang signifikan di antara kesemua jenis makanan. Ini menunjukkan terdapat mekanisma regulasi DHA terutamanya bagi Δ4 desaturase di dalam tisu otak. Secara keseluruhannya, pendekatan molekular dan *in-vivo* telah digunakan untuk menyiasat kebolehan biosintesis LC-PUFA di dalam ikan air tawar bersifat karnivor, dan juga telah menunjukkan potensi penggunaan minyak tumbuhan sebagai sumber lipid di dalam kultur ikan haruan.

FUNCTIONAL CHARACTERIZATION OF THE STRIPED SNAKEHEAD (*CHANNA STRIATA*) DESATURASE GENE, AND ITS IMPLICATION IN LONG-CHAIN POLYUNSATURATED FATTY ACID SYNTHESIS

ABSTRACT

Fish are a major source of beneficial omega-3 long-chain polyunsaturated fatty acids (LC-PUFA) for optimal human health, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). However, the utilization of fish oil as a lipid source in the aquaculture industry as an ingredient in aquafeeds is considered unsustainable. Plant oils are proposed as alternative to fish oil, but are rich in carbon 18 (C18) polyunsaturated fatty acids (PUFA) and devoid in LC-PUFA. Critical enzymes are involved in the biosynthesis pathway of C18 PUFA into LC-PUFA. In fish, molecular characterization of these enzymes is poorly understood in freshwater carnivorous species. This study was undertaken to functionally characterize a previously cloned full length desaturase gene, *fads* (EU 570220) from the freshwater carnivorous striped snakehead fish, *Channa striata* (Bloch, 1793), and to assess its role in utilization of dietary plant oil in this species. Results showed that the striped snakehead fish *fads* gene is a bifunctional gene with capabilities to desaturate Δ4 and Δ5 fatty acid substrates, which indicates that this freshwater carnivorous fish is able to convert EPA into DHA via a more direct route in comparison with the conventional route. At the tissue level, the *fads* gene expression was the highest in the liver and the brain. An *in-vivo* study involving 4 different diets formulated with varying lipid source, fish oil (FO), linseed oil (LO), corn oil (CO) and blend of linseed and corn oils (LOCO) revealed nutritional regulation of the *fads* gene in fish livers. EPA and DHA deposition in muscle of fish fed plant oils based diets was lower than fish oil diet. A subsequent feeding trial was designed with the inclusion of echium oil, a plant oil containing high stearidonic acid (SDA, 18:4n3) which was supplied as a C18 PUFA precursor, bypassing the Δ6 desaturation step in the

biosynthesis pathway and to evaluate if it would improve LC-PUFA deposition. Five diets were designed containing different oils: fish oil (100FO), linseed oil (100LO), echium oil (100EO), blends of linseed and echium oils (50EOLO) and blends of echium oil and fish oil (50EOFO). When fish were fed diet 100EO, the EPA levels in their liver was raised and was comparable to those which were fed diets containing fish oil. In addition, the expression of *fads* gene in liver of 100EO fish was higher compared to 100FO, indicating higher endogenous biosynthesis activity with the inclusion of SDA as precursor. Fatty acid profiles of α-linolenic acid (ALA, 18:3n3) and linoleic acid (LA, 18:2n6) content in fish muscle generally reflected the dietary treatment. LC-PUFA content was detected in muscle of all fish fed the plant oil diets but in levels lower than fish oil diets. DHA content was lowest in muscle of fish fed diet 100LO, suggesting that inclusion of SDA-rich echium oil potentially improved DHA deposition in comparison with ALA from linseed oil. Interestingly, for both feeding trials, DHA levels in brain tissues showed no significant difference amongst different dietary treatment, indicating important DHA regulating mechanism, in particular Δ4 desaturase in neural tissues. On the whole, molecular and *in-vivo* approach was utilized to investigate the LC-PUFA biosynthesis capacity in a freshwater carnivorous fish, and additionally highlighted the potential of using plant oil as lipid source in striped snakehead fish culture.

CHAPTER 1

INTRODUCTION

1.1 Overview

In recent times, lifestyle influenced conditions such as obesity and cardiovascular diseases are commonplace, even sometimes regarded as the norm in some communities. Due to rise in health issues, there is a steady scramble to improve eating habits and live an active lifestyle to reduce risk of diseases. Being healthy ensures well-being and is one of the aspects of happiness to humans. Dietary nutrient intake of humans plays a big part in influencing health and diseases. One such group of nutrients is the lipid. Lipids are a wide group of compounds which are soluble in organic solvents and insoluble in water, serving both structural and metabolic functions in organisms. Ingested lipids are broken down in the body by enzymes called lipases produced in the pancreas. Fatty acids are derived from triglycerides or phospholipids, and are considered the building blocks of dietary lipids. Fatty acids are carboxylic acids which can be classified as saturated, monounsaturated and polyunsaturated depending on their molecular structure. Studies have been conducted on dietary intake of humans, and it has been recommended that saturated and *trans*-fatty acids be replaced with unsaturated fatty acids to prevent cardiovascular diseases (Erkkila et al., 2008). Inclusion of polyunsaturated fatty acids into the diet is capable to lower blood pressure, reduce atherosclerosis and alleviate depression.

Natural sources of polyunsaturated fatty acids (PUFA) can be obtained by consuming plants, oil seeds and fatty fish, among others. Plant and oil seeds such as flaxseed (linseed), sunflower, evening primrose oil, sesame, soybean and corn oils contain high amount of PUFA, however they are more concentrated in carbon 18 (C18) PUFA, which are considered precursors in the long-chain polyunsaturated fatty acids (LC-PUFA) biosynthesis pathway. LC-PUFA are fatty acids containing 20 or more carbons in their molecular

structure, are usually found in abundance in fish. In the context of this study, mention of LC-PUFA means eicosapentaenoic acid (EPA, 20:5n3), docosahexaenoic acid (DHA, 22:6n3) and arachidonic acid (ARA, 20:4n6). Humans have a limited capacity to synthesize LC-PUFA from shorter chain precursors, and fish have become vitally important as the only significant source of dietary LC-PUFA (Leaver et al., 2008b). This has led to humans relying on the aquatic environment to fulfil the nutritional demand of health promoting LC-PUFA.

Today, we are in a situation where the wild fisheries are being fully exploited or overharvested, yet the demand for seafood continues to peak. The demand for fish as food is not only to satisfy the increasing human population, but also to satisfy the nutritional requirements as consuming fish has shown to have health benefits. As the demand increases, wild fish supplies are depleting. Unsustainable fishing practices have placed heavy emphasis on aquaculture to meet the global shortfalls in the supply of fish and seafood for human consumption. Aquaculture activities are widespread worldwide and involve a diverse range of fish and other aquatic organisms. Aquaculture has been defined in many ways, one of them being the rearing of aquatic organisms under controlled or semi-controlled conditions. A simpler way of calling this principle is 'underwater agriculture', as proposed by Stickney (2005). It may seem that aquaculture is the answer to the world's problem on replenishing fish as food to the human consumer; however, there is a downside to this industry. The farming of fish is not a sustainable practice due to the large requirement of ingredients in aquafeeds to supply protein and lipid, which are derived from fish itself. Fish meal (protein source) and fish oil (lipid source) production is a commodity obtained from wild fish stocks, particularly small, bony, pelagic marine fish which have been deemed unsuitable for human consumption, also known as industrial fish or feed grade fish (sometimes called trash fish), such as anchovy, mackerel, herring and sardine, and are currently being depleted in an attempt to supply the dietary LC-PUFA for the farmed species (Naylor et al., 2009). Besides being able to fulfil the LC-PUFA requirements, these ingredients are incorporated into

aquafeeds as they are highly digestible, palatable and increase feed appeal especially for carnivorous species (Schipp, 2008).

The utilization of fish meal and fish oil derived from wild fisheries halts the ability of aquaculture to solve the issue of overfishing and exploitation of natural resources from our oceans; this is an unsustainable vicious circle which will continue if no immediate action is taken. The availability of these resources are also dictated by the climate, whereby a major drop in supply was detected in the event of 1998 El Niño (Schipp, 2008). Decreasing global availability and high variable prices of fish meal and oil are also factors which have forced the aquaculture industry to investigate the possibilities of alternative dietary protein and lipid sources (Tacon and Metian, 2008, Turchini et al., 2009). In comparison to wild fish, the relative abundance of plants and their renewable ability makes plant/vegetable oils a sustainable candidate as lipid source in aquafeeds. To date, there have been numerous studies conducted to replace fish oil with alternative oils in various fish cultures. Fish oil has been partially or completely replaced by plant oils such as olive, linseed, corn, soya and palm oils, and the effect on fish evaluated.

However, a new problem arises with this solution, sending researchers to find answers to the surfaced problem. Plant oils are rich in C18 PUFA but devoid of LC-PUFA. Conversely, fish oil is rich in beneficial LC-PUFA and low in C18 PUFA. This shows a completely opposite fatty acid profile displayed by these two types of oils. Hence, replacing fish oil with plant oils in fish feed may affect the deposition of LC-PUFA in the muscle of cultured fish, as it is inevitable that the flesh fatty acid profiles are usually a reflection of the dietary fatty acid profile. Previous work has shown that Atlantic salmon can survive and grow on a plant oil-based diet, but the concentration of EPA and DHA in their muscle was reduced (Tocher et al., 2003b). This not only is a deleterious effect on the fish, but it affects the total omega-3 LC-PUFA which is available for the human consumer. The benefits of fish consumption would prove to be futile.

The solution to this issue would have to be sourced out at a deeper level. To ensure aquaculture continues as a sustainable industry, the understanding of fish LC-PUFA biosynthesis at a molecular level must be gained. Biosynthesis of LC-PUFA consists of 2 separate pathways for the omega-3 and omega-6 fatty acids. Plant oils containing C18 PUFA are a rich source of LC-PUFA precursors such as α-linolenic acid (ALA, 18:3n3) and linoleic acid (LA, 18:2n6). ALA and LA will go through sequential desaturation and elongation steps involving 2 types of enzymes – the desaturase and elongase. The primary step will convert ALA and LA into stearidonic acid (SDA, 18:4n3) and γ-linolenic acid (GLA, 18:3n6) respectively via a Δ6 desaturase enzyme. These products will then be elongated into C20 fatty acids then subjected to Δ5 desaturation to form EPA and ARA. In the omega-3 pathway, EPA can be further elongated to docosapentaenoic acid (DPA, 22:5n3) which could be converted into DHA through 2 different routes. The conventional way (also known as the 'Sprecher pathway') previously reported in vertebrates, states that DPA goes through an elongation to form tetracosapentaenoic acid (24:5n3), then Δ6 desaturated into tetracosahexaenoic acid (24:6n3), goes through 2 carbon chain shortening through β-oxidation and finally forms DHA. The alternative and shorter route would be a direct conversion of DPA into DHA via a Δ4 desaturase, which has so far been reported in few other teleosts (Li et al., 2010, Morais et al., 2012). By knowing the presence and function of the desaturase and elongase genes involved in the LC-PUFA biosynthesis pathway in fish, it can then be determined whether the C18 PUFA precursors provided by plant oils in aquafeeds can be effectively utilized by fish to deposit beneficial LC-PUFA in their flesh.

1.2 Problem statement

Functional characterization of desaturase and elongase enzymes has been evaluated in many marine and freshwater teleosts, however most of them belong to the temperate climate; and in comparison, only few studies have been done on tropical species. Thus far, no extensive functional characterization and nutritional regulation studies have been carried out on a freshwater carnivorous fish.

In this present study, the fish investigated is the striped snakehead fish (*Channa striata*), a freshwater carnivore. This fish is popular as food in Thailand, Indo-China and Malaysia. In Malaysia, locally known as 'ikan haruan', it is not only good for consumption but it is also regarded as traditional medicine by some communities. In reference available from the Food and Agriculture Organization of the United Nations (FAO) web database, the *C. striata* fact sheet has shown an increase in global aquaculture production since 1950, and shows the highest production beginning 2004 up till 2012. This freshwater fish has potential to be a good source of omega-3 fatty acids and protein for human consumption, especially in inland areas where obtaining marine fish and seafood can be a challenge or expensive. Striped snakehead fish has also been extensively researched for their effects on wound healing and medicinal purposes. It was reported that the fatty acid and amino acid content in the mucus and flesh of these fish possess anti-inflammatory properties which promote wound healing (Mat Jais et al., 1994). Other studies have looked into protein and lipid requirement of *C. striata* growth, but research on the molecular capacity for LC-PUFA biosynthesis and deposition has yet to be undertaken. Understanding their ability of endogenous LC-PUFA biosynthesis from a molecular platform is worth being studied at a deeper level and could potentially promote sustainable farming of this species.

1.3 Objectives

It is proposed here to utilize molecular tools to study one of the genes involved in the LC-PUFA biosynthesis pathway of the striped snakehead fish. A preliminary study was conducted and published on the full length desaturase gene of the striped snakehead fish (Jaya-Ram et al., 2011). The main objective of this present study is to functionally characterize this full length desaturase gene *in-vitro* employing a heterologous expression in yeast assay. Subsequently, desaturase gene expression in different tissues will be evaluated to identify tissues which are highly involved in LC-PUFA biosynthesis. Plant oils with different fatty acid profiles will be investigated as fish oil replacement in aquafeeds and the desaturase gene expression will be evaluated *in-vivo.*

The objectives of this study are summarized as follows:

1. To characterize a full length desaturase gene and understand its function *in-vitro.*
2. To identify the tissue distribution of the desaturase gene in the striped snakehead fish.
3. To understand the nutritional regulation of the desaturase gene *in-vivo* by means of employing a feeding trial replacing fish oil with commonly used plant oils in aquaculture.
4. To investigate the effectiveness of a plant oil containing high stearidonic acid (an omega-3 C18 PUFA precursor), as potential for fish oil replacement in striped snakehead fish aquafeed.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter reviews literature topics which are relevant to this research. A general overview of important nutrients required by fish, such as protein and lipid usages in aquaculture is highlighted in the beginning. It is then followed by an explanation on fatty acids, their nomenclature and main classification. Subsequently, the pathways involved in biosynthesis of long-chain polyunsaturated fatty acids and their enzymes are reviewed. The chapter then moves into the implication of lipid and fatty acids in aquaculture, and also the alternative lipid sources which may replace fish oil in aquafeeds.

2.2 Nutrient requirement of fish in aquaculture

In aquaculture, fish usually consume manufactured artificial feed instead of consuming natural prey organisms or foliage. Aquafeeds are normally designed to contain a range of essential and non-essential nutrients for maintenance, movement, normal metabolic functions, growth and energy. Feed requirements of fish vary in quantity and quality according to their digestive anatomy, feeding habits, size, reproductive state and sometimes also affected by environmental temperature. Nutrients such as protein, lipid and carbohydrates are considered the macronutrients which provide fish with energy. Micronutrients, which are trace or small amounts of minerals and vitamins, are also essential for fish (Gonzalez and Allan, 2007).

Proteins are major organic material in fish tissue which provides fish with amino acids. The protein is digested and released as free amino acids, which are absorbed from the intestinal tract and distributed by the blood to organs and tissues. These amino acids are then

used by tissues to synthesize new proteins. A continuous intake of protein is required by fish as amino acids are utilized to build new proteins for growth and reproduction or to replace existing proteins (Wilson, 2002). Carbohydrates include fibre, starches and sugars. This group of nutrients is considered the cheapest source of energy and inclusion in the diet may lead to a reduced feed cost. However, most fish, especially carnivorous species, have limited natural access to carbohydrates and are better adapted to use proteins and lipids at the digestive and metabolic levels (Stone, 2003, Wilson, 1994).

In general, lipids are a group of compounds which are not soluble in water but can be solubilized in organic solvents. There are eight primary categories of lipids: fatty acids, glycerolipids, glycerophospholipids, sterols, prenols, sphingolipids, saccharolipids and polyketides (Fahy et al., 2005). They provide energy for growth, reproduction and migration, membrane structural components, essential fatty acids, precursors of eicosanoids required for regulatory processes and assist in the uptake of lipid soluble nutrients (Tocher, 2003). Fatty acids act as precursors to several metabolic derivatives like eicosanoids, docosanoids, hormones and vitamins (Bell and Koppe, 2011). Lipids, specifically triglycerides, are catabolized into fatty acids. Following absorption, fatty acids are then resynthesized into lipids which form droplets (Heird and Lapillonne, 2005). These lipid droplets are circulated in the fish blood system. In order to be used, they must again be broken down to their constituent fatty acids. Fatty acids are then used for synthesis of membranes or further degraded for energy. Lipids contain more energy per unit weight than any other dietary component, and are used efficiently by fish as energy sources. Besides providing energy, they are source of hydrophobic components for the synthesis of macromolecules (Jump et al., 1999). The degradative pathways of amino acids, simple sugars and fatty acids will eventually reach a common intermediate compound, acetyl coenzyme-A (acetyl CoA). Acetyl CoA enters the citric acid cycle, which in turn is linked to the process of oxidative phosphorylation. The result is the production of CO_2, the consumption of O_2 and the liberation of energy, which is then stored as high-energy phosphate molecules, adenosine

triphosphate (De Silva and Anderson, 1995). It should be highlighted that the culture of fish is mainly to provide humans with dietary source of omega-3 long-chain polyunsaturated fatty acids (LC-PUFA). In the context of this study, importance is given to literature review on dietary lipids, especially polyunsaturated fatty acids (PUFA), and will be further elaborated in sections which follow, as the core of this study grapples with the issue of fish oil replacement in aquafeeds.

2.3 Fatty acids

Fatty acids are carboxylic acids with a typical RCOOH structure, including a methyl end, a hydrocarbon chain (R) and a carboxylic terminus (-COOH) (Tvrzicka et al., 2011). They are designated on the basis of their chain lengths, degree of unsaturation (number of double bonds) and the position of the double bond. Usually, fatty acids molecules contain even numbers of carbon atoms in straight chains, commonly 14 to 24, and may be saturated or unsaturated (Christie and Han, 2010). Fatty acids have both systemic names and a common name, for example octadecanoic acid corresponds to stearic acid (18:0). They are often represented in a shorthand notation formula as follows:

CN:p n-x

whereby, carbon number (CN) indicates the number of carbon atoms, p indicates the degree of unsaturation representing the number of double bonds, and x is the position of the first double bond from the methyl terminus (n) or also called the omega end of the molecule (IUPAC-IUB, 1978, Tvrzicka et al., 2011). For example, 18:1n7 and 18:1n9 indicate that these fatty acids possess 18 carbon atoms and have one double bond (monounsaturated) at the position of carbon number 7 and 9 respectively, calculated from the methyl end of the molecule. Alternatively, these fatty acids could be written as 18:1Δ11 and 18:1Δ9 respectively. This notation uses Δ to signify the position of the double bond from the carboxyl end of the molecule.

2.3.1 Saturated fatty acids

The simplest forms of fatty acids have no double bonds in their carbon chains and are considered saturated. They are notated as the number of carbons followed by a zero, 16:0, 18:0 and 20:0, indicating a molecule with 16, 18 and 20 carbon atoms respectively. The most abundant saturated fatty acids (SFA) in animal fats, including fish lipids are 16:0 and 18:0 (Fig 2.1), although a range of carbon chain lengths of C2 to C36 can be found. SFA containing 12 and more carbons in their chains (12:0 onwards) have relatively high melting points. Animal fats and palm oil or coconut oil are high in SFA and tend to be in solid form at room temperature.

Long chain saturated fatty acids include lauric (12:0), myristic (14:0), palmitic (16:0) and stearic acids (18:0) have been reported to have significant negative effects in humans. Consumption of these long chain SFA may increase cholesterol levels, especially the low density lipoprotein (LDL) cholesterol which is connected to increase in coronary heart disease mortality (Damude and Kinney, 2008, Erkkila et al., 2008, Tvrzicka et al., 2011).

2.3.2 Monounsaturated fatty acids

This group of fatty acids contain only one double bond and make up a high proportion of the total fatty acids in most natural lipids. Normally the double bond in monounsaturated fatty acids (MUFA) can take the form of the *cis*-configuration (*Z*-configuration) or in the *trans*-configuration (*E*-configuration) (Christie and Han, 2010). Oleic acid or 18:1n9 is commonly found and abundant MUFA in plant and animal tissues. It may comprise 30 – 40% of total fatty acids in animal adipose fats and 20 – 80% in seed oils. Olive oil contains around 78% of oleic acid and is reported to have nutritional benefits. An example of high olive oil consumption is in the Mediterranean diet (Christie and Han, 2010). The high content of MUFA in olive oil is shown to be cardio-protective, resulting in

increased high density lipoprotein (HDL) and reduced LDL cholesterol (Kremmyda et al., 2011). Similar to SFA, fatty acids in the form of *trans*-configurations, especially those from industrial hydrogenation processes, are also considered to have negative cardiovascular effects.

2.3.3 Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFA) are essential dietary components for all vertebrates, as they are unable to be synthesized *de novo* from MUFA (Bell and Tocher, 2009). PUFA are defined as fatty acids containing two or more double bonds. The entire structure of a particular PUFA can be defined by specifying the position of the first double bond relative to the methyl end. Hence, in 18:3n3 (α-linolenic acid, ALA), the first double bond is situated three carbon atoms from the methyl end of the molecule, which can also be written as 18:3Δ9,12,15. The n-nomenclature is more convenient and commonly used compared to the Δ-nomenclature. The Δ-nomenclature is generally used for specifying fatty acid desaturase activities. Therefore, fatty acid desaturases which introduce double bonds five or six carbons from the carboxylic terminus of the molecule are termed Δ5 and Δ6 desaturases respectively (Tocher, 2003).

There are two principle groups of PUFA occurring in nature which are derived from ALA and LA (Fig. 2.1), namely the omega-3 and omega-6 respectively. Both ALA and LA can be synthesized in plants but not in animal tissues and are therefore deemed as essential fatty acids. However, both of these PUFA are considered as precursors in the long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis pathway. LC-PUFA are molecules containing 20 or more carbon atoms, coupled with 3 or more double bonds (Bell and Koppe, 2011). The three most important LC-PUFA in vertebrates are eicosapentaenoic acid (EPA, 20:5n3), docosahexaenoic acid (DHA, 22:6n3) and arachidonic acid (ARA, 20:4n6) (Fig. 2.1). LC-PUFA are reported to have beneficial effects in human diseases and conditions such

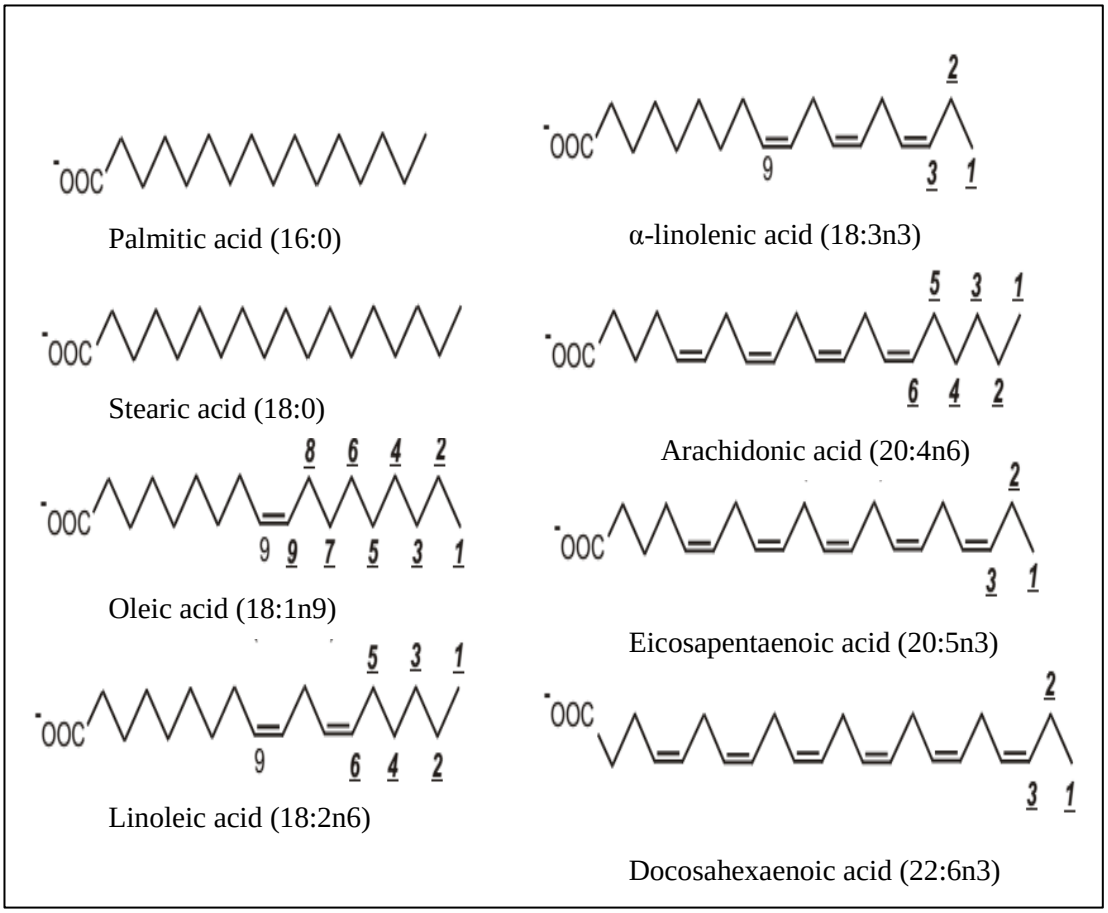

Figure 2.1: Structural formulae and short hand notation of fatty acids (Tvrzicka et al., 2011).

as cardiovascular and inflammatory disorders, neural development and neurological conditions (Calder, 2006, Horrobin, 1993, Simopoulos, 1991). Both EPA and ARA are precursors for biologically active docosanoids and eicosanoids that are vital components of cell membranes and play many dynamic roles in mediating and controlling a wide array of cellular activities (Ahlgren et al., 2009). The importance of LC-PUFA in health has prompted various studies to evaluate the pathways of endogenous biosynthesis of these components, especially in fish, the main source of dietary LC-PUFA for humans.

2.4 LC-PUFA biosynthesis pathway

ALA and LA cannot be synthesized by any vertebrates, and is required to be supplemented via dietary intake. This is because vertebrates lack the Δ12 and Δ15 desaturase enzymes required to produce these 18 carbon (C18) PUFA from oleic acid (Fig. 2.2) (Torstensen and Tocher, 2011, Wallis et al., 2002). Conversely, LC-PUFA can be synthesized by vertebrates, including fish, from omega-3 and 6 precursors ALA and LA, provided they possess functional desaturase and elongase genes, however, this capacity varies among species.

The biosynthesis of LC-PUFA from C18 PUFA involves pathways with a series of microsomal fatty acid desaturation and elongation steps (Fig. 2.3). The synthesis of ARA is achieved with a Δ6 fatty acyl desaturase enzyme activity on LA, forming γ-linolenic acid (GLA, 18:3n6) which will be elongated to C20 PUFA with the addition of two carbon atoms. Dihomo-γ-linolenic acid (DGLA, 20:3n6) was then finally desaturated by Δ5 desaturase to produce ARA (Zheng et al., 2004a). The pathway for EPA synthesis from ALA involves the same enzymes and is essentially similar as ARA synthesis. However, synthesis of DHA could occur via two separate routes. One of the routes involves two elongation steps from EPA, forming 22:5n3 (docosapentaenoic acid, DPA) then 24:5n3 (tetracosapentaenoic acid). Subsequently going through Δ6 desaturation, producing 24:6n3 (tetracosahexaenoic acid)

and finally chain shortened via β-oxidation to eventually form DHA. Studies done on rat liver reported that mammals generally produce DHA through this route, which is deemed as the 'Sprecher pathway' (Sprecher et al., 1995). Other studies done on rainbow trout and zebrafish further confirmed that desaturation of 24:5n3 to 24:6n3 was functional, and it was thought that this was the sole predominant way DHA was formed in vertebrates (Buzzi et al., 1997, Tocher et al., 2003a).

A ground breaking discovery was made of a functional Δ4 desaturase in the rabbitfish (*Siganus canaliculatus*), a marine herbivore, which indicated a direct production of DHA by desaturation of DPA (Li et al., 2010). Soon after, another marine carnivore, the Senegalese sole (*Solea senegalensis*) was reported to possess the Δ4 desaturase as well (Morais et al., 2012). This finding shed some light on the capacity of certain vertebrate species to synthesize DHA via a more direct path; however it was unclear how widespread the presence of a Δ4 desaturase was in vertebrates. By understanding the molecular mechanisms of the desaturase and elongase genes in the LC-PUFA biosynthesis pathway and its regulation in fish, this will enable the manipulation and optimization of the dietary fatty acids in aquafeeds provided to fish, especially in respect to the replacement of unsustainable and rising costs of lipid sources in aquaculture (Zheng et al., 2004a).

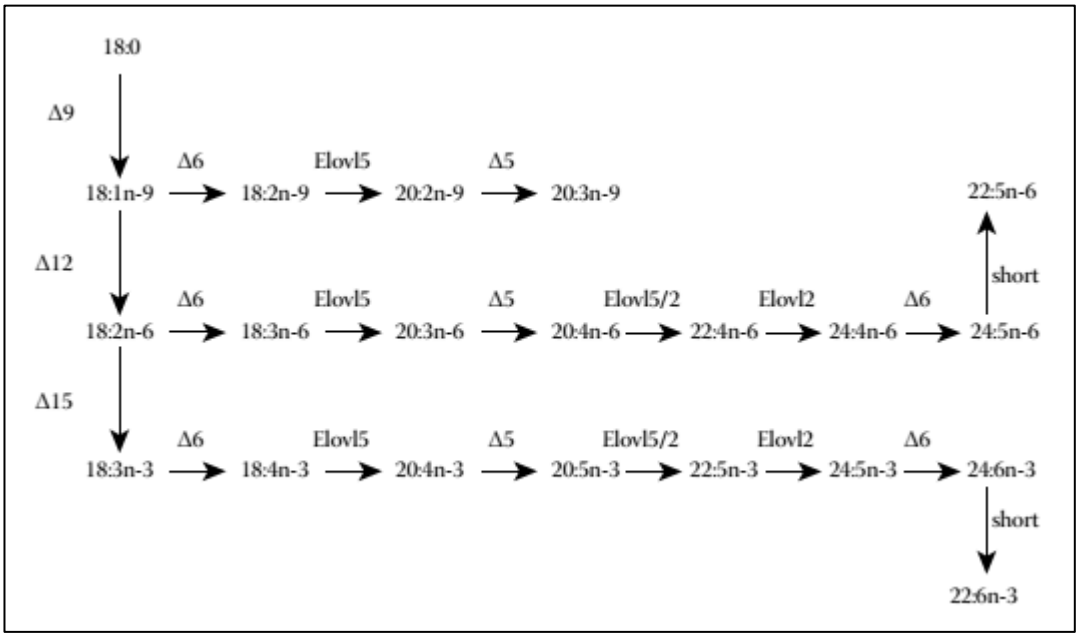

Figure 2.2: A general representation of long-chain polyunsaturated fatty acids (LC-PUFA) biosynthesis pathways encompassing omega-3, 6 and 9 groups. Vertebrates are unable to form 18:2n6 and 18:3n3 as they do not possess the Δ12 and Δ15 fatty acyl desaturase (only found in plants and some invertebrates). Δ9 represents stearoyl CoA desaturase, Δ5 and Δ6 indicate fatty acyl desaturase activities. Elovl2 and Elovl5 indicate activity of PUFA elongation and 'short' indicates peroxisomal chain shortening (Torstensen and Tocher, 2011).

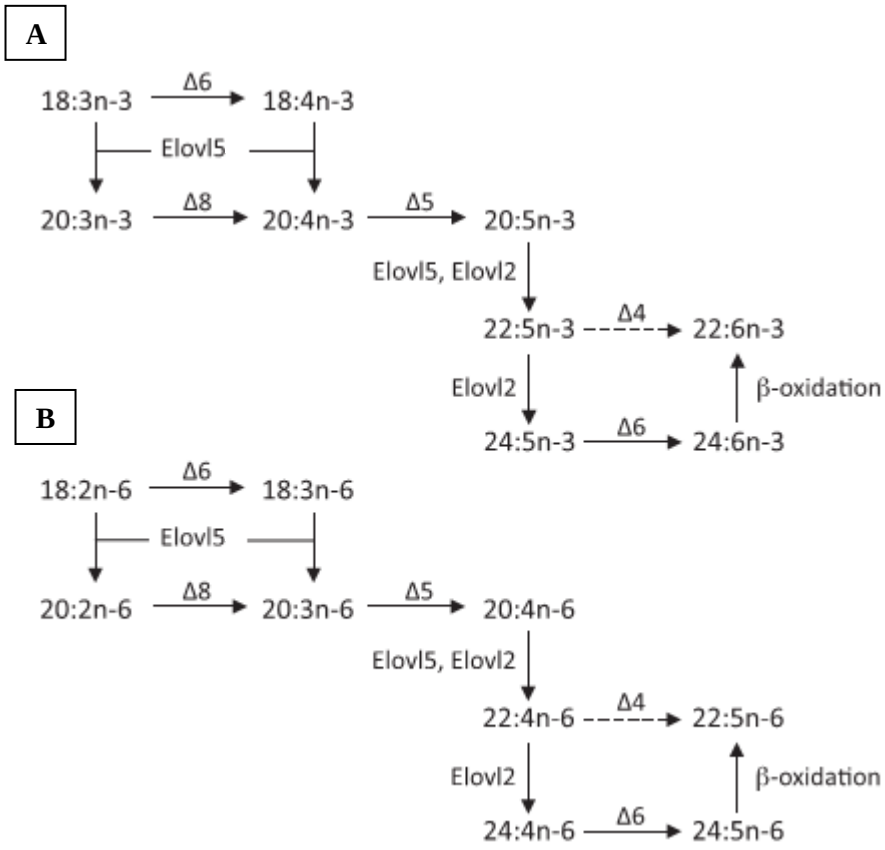

Figure 2.3: Long-chain polyunsaturated fatty acids (LC-PUFA) biosynthesis from C18 PUFA precursors in vertebrates. Pathway begins with C18 PUFA 18:3n3 for the omega-3 pathway (A) and 18:2n6 for the omega-6 pathway (B). Δ4, Δ5, Δ6 and Δ8 represent fatty acyl desaturases; Elovl2 and Elovl5 indicate elongation, while β-oxidation is a chain shortening step. Figure adapted from (Monroig et al., 2011).

2.4.1 Desaturase

The fatty acyl desaturase enzymes constitute a highly diversified family, including at least 10 different types of regioselectivities, such as Δ4, Δ5, Δ6, Δ8, Δ9, Δ10, Δ11, Δ12, Δ13 and Δ15, some of which sequences are significantly close to one another (Sperling et al., 2003). Fatty acyl desaturases catalyze the introduction of a double bond into an acyl chain, their sequences are characterized by three histidine box motives, two transmembrane regions, and N-terminal cytochrome b_5 domains containing the haem-binding motif HPGG (Zheng et al., 2004a). Hashimoto et al. (2008) reported that desaturases are divided into four functional subfamilies, comprising of i) First Desaturase, introducing the first double bond into the saturated fatty acid chain; ii) Omega Desaturase, introducing a double bond between an existing double bond and the acyl end (contains Δ12 and Δ15 desaturases); iii) Front-End Desaturase, introducing a double bond between an existing double bond and the carboxyl end (includes Δ4, Δ5, Δ6 and Δ8 desaturases) ; and iv) Sphingolipid Desaturase, whose sole function is the sphingolipid Δ4.

In recent years, significant progress has been made in characterizing fatty acid desaturases involved in LC-PUFA biosynthesis. Complementary deoxyribonucleic acid (cDNA) for genes in the pathway have been cloned from several fish species and functionally characterized by heterologous expression in yeast (*Saccharomyces cerevisiae*) (Tocher et al., 1998, Torstensen and Tocher, 2011). Full length cDNA for Δ6 desaturases have been isolated from the filamentous fungus *Mortierella alpina*, the nematode *Caenorhabditis elegans*, rat, mouse and human (Aki et al., 1999, Cho et al., 1999, Napier et al., 1998, Zheng et al., 2004a). The Δ6 desaturase has the reputation of being the rate-limiting enzyme in the LC-PUFA biosynthesis pathway as it is responsible for the first desaturation step of ALA and LA, and also the involvement to form DHA via the Sprecher pathway from EPA. The same enzyme serves for both reactions (Tocher, 2010, Zheng et al., 2009a). Heterologous expression studies of human and rat Δ6 desaturases confirms that the same enzymes are active on C18 and C24 fatty acids (de Antueno et al., 2001).

The capability of LC-PUFA production from C18 PUFA varies between fish species (Sargent et al., 1999, Sargent et al., 2002, Tocher et al., 2006b). The first Δ6 desaturase cDNA cloned and characterized in teleosts were from the zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*) (Hastings et al., 2001, Seiliez et al., 2001). It was discovered that zebrafish displayed a bifunctional desaturase with the ability of Δ5 and Δ6 activity. Besides zebrafish, the Δ6 desaturase was discovered in the freshwater fish, carp (*Cyprinus carpio*) and Nile tilapia (*Oreochromis niloticus*) (Tanomman et al., 2013, Zheng et al., 2004a).

Extensive work has been done on marine fish in comparison to the freshwater fish; findings from functional characterization studies mainly surfaced more Δ6 desaturases. Among some of the fish species with Δ6 desaturase are European sea bass (*Dicentrarchus labrax*), northern bluefin tuna (*Thunnus thynnus*), Atlantic cod (*Gadus morhua*) and pike eel (*Muraenesox cinereus*) (González-Rovira et al., 2009, Kim et al., 2013, Morais et al., 2011, Tocher et al., 2006a). Among the other Δ6 desaturases in marine fish such as the gilthead sea bream (*Sparus aurata*), cobia (*Rachycentron canadum*), Asian sea bass (*Lates calcarifer*) and turbot (*Psetta maximus*) (Seiliez et al., 2003, Mohd-Yusof et al., 2010, Zheng et al., 2004a, Zheng et al., 2009a), it was recently discovered that this enzyme also had the ability to desaturate Δ8 fatty acid substrates in all of these fish (Monroig et al., 2011, Tu et al., 2012). The zebrafish bifunctional Δ6/Δ5 desaturase also had the Δ8 desaturase ability as is in the mammalian Δ6 desaturase. Expression of the baboon Δ6 desaturase cDNA in yeast promoted the Δ8 desaturation of 20:2n6 and 20:3n3 to 20:3n6 and 20:4n3 respectively. It is still unclear how widespread the Δ8 desaturase pathway is among vertebrates (Monroig et al., 2011, Park et al., 2009).

In salmonids, the anadromous Atlantic salmon (*Salmo salar*), begins its life in freshwater and migrates to the sea before returning to the freshwater to breed. It was reported that this is the only fish which possesses separate (unifunctional) Δ5 desaturase besides Δ6 (Monroig et al., 2010). Elsewhere, rabbitfish (*Siganus canaliculatus*), a marine herbivore

was shown to have Δ4/Δ5 bifunctional desaturase and a Δ6/Δ5 desaturase (also shows some Δ8 activity) (Li et al., 2010, Monroig et al., 2011). The discovery of the Δ4 desaturase in this teleost indicated that a direct production of DHA by desaturation of 22:5n3 was possible in some vertebrates. This finding was soon ensued by a discovery of another bifunctional Δ4/Δ5 in a marine fish, the Senegalese sole (*Solea senegalensis*) (Morais et al., 2012). Just like the Δ8 desaturase, Δ4 desaturases were a new discovery in teleosts and how widespread they are is still yet to be discovered.

2.5 Implication of lipid and fatty acids in aquaculture

The main source of health promoting LC-PUFA for human nutrition comes from the consumption of fish. Due to unsustainable fishing practices, we now have to rely heavily on aquaculture to meet the demands of a growing population. Capture fisheries are not expected to increase production, and the aquaculture industry has been challenged to rise up to the occasion (Turchini et al., 2009). With the increase in demand of fish and seafood, aquaculture practices intensified and advanced in a global effort. Initially the cultured species were focused on the lower trophic chain; however this was inadequate to meet the growing demands. Besides this, community and consumer preference dictated the increase in culture of carnivorous, high valued species such as salmonids (Tacon and Metian, 2009).

Following the intensification in aquaculture of carnivorous and other consumer desired species, there was a major resource input required in form of aquafeeds to sustain these practices. Therefore, this situation led to the utilization of fish meal as protein source in fish feed as it had several benefits: easily digestible, contains essential amino acids, increases palatability and it was already in use for livestock feed (De Silva et al., 2011). The dependence on fish oil came into prominence only in the last decade, as human nutrition and well-being was highlighted and was connected to the importance of the omega-3 LC-PUFA, EPA and DHA. It was recognised that fish were the optimal source of the LC-PUFA, and

also combats several human health pathologies stemming from imbalance or a lack of these fatty acids, demand for fish remained high (Calder, 2006, De Silva et al., 2011). Carnivorous fish species such as salmon and other 'high-end' marine fish (sea bass, cod, barramundi), may have more than four times the omega-3 content of fish such as carp, and are becoming more sought after for their health giving benefits (Schipp, 2008).

The majority of the world's industrial fisheries are located in the Pacific ocean off South America (40% global supply) and in Scandinavian countries such as Denmark, Iceland and Norway (5% global supply) (Schipp, 2008). Fishmeal and fish oil are produced from any type of fish or seafood often termed 'industrial fish'/'feed grade fish', or sometimes called 'trash fish'. The fact that it is called 'trash fish' implies that these fish have little value, however this implication is misleading (Tacon and Forster, 2003). These trash fish consist of any type of marine wild-caught fish, small in size, bony and oily, which are usually deemed unsuitable for direct human consumption (Allan, 2004). In actual fact, fish which are used for fishmeal and fish oil production, are eaten in small quantities, but not considered prime food, some examples of these fish: Peruvian anchovy (*Engraulis ringens*), herring (*Clupea harrengus*), capelin (*Mallotus* spp.) and mackerel (*Trachurus/Scomber* spp.) (De Silva et al., 2011, Tacon, 2005). However, there is no significant human consumption for 90% of the fish caught for fishmeal and fish oil production. These fish are usually caught in mass numbers and is often difficult and expensive to process them so that they remain fit for human consumption (FAO, 1986).

Global fish oil production has fluctuated significantly over the years, indicating the volatility in this production area. Even though the global fish supplies have been relatively static, fish oil utilization has displayed a significant increase (Turchini et al., 2011). Fish oil and fish meal prices have been fluctuating widely too (Naylor et al., 2009). Climate change also impacts the fish oil supplies and prices, whereby the El Niño has shown to affect wild fisheries. Fish oil is still widely used in aquaculture due to their high proportion of omega-3

LC-PUFA. Besides beneficial fatty acids, fish oils also are a natural source of vitamins A and D, and small concentrations of vitamin E (De Silva and Anderson, 1995).

The negative aspect of fish oil properties is that it may contain persistent organic pollutants, consisting of a wide range of lipophilic compounds including polychlorinated biphenyls (PCB), organochlorine pesticides, polycyclic aromatic hydrocarbons, and dioxins. PCB present the greatest risk as they affect reproductive viability in humans and also increase the incidence of cancer (De Silva et al., 2011). These pollutants typically originate as a result of human industrial activity. They tend to accumulate in the lipid component of fish tissues due to their lipophilic nature, and subsequently remain in extracted fish oil. This may seem like a threat to human health in general if fish affected with pollutants were consumed (Bell et al., 2005b).

It is generally acknowledged that food production has to be sustainable, and resource usage is of utmost importance. In recent times, public attention has focused heavily on global warming and climate change impacts on the planet, and its consequences on food production. Aquaculture, currently accounts for 50% of the global fish supply, has to develop adaptive and alternative measures to ensure that it continues to meet the increasing global food fish needs (De Silva et al., 2011). The usage of alternative oils has been studied in farming of many different fish species to alleviate the pressure of excessive fish oil usage and to move towards sustainable aquaculture (Francis et al., 2006, Turchini et al., 2009, Turchini et al., 2011).

2.6 Alternative lipid sources in aquaculture

Plant based oils have been investigated intensively as alternatives to fish oil in aquafeeds. Oil sources obtained from plants are considered renewable and therefore a sustainable commodity. However, the unfeasible aspect in plant oils is their fatty acid profile which is devoid of LC-PUFA, but high in C18 PUFA precursors (Bell et al., 2001, Bell et al.,

2003b). Therefore, it is crucial to understand the LC-PUFA biosynthesis in the cultured fish species in order to successfully utilize plant oils in aquafeeds to ensure efficient usage of the dietary PUFA provided. Studies on marine fish species have been conducted over the years and have demonstrated that partial plant oil replacements have demonstrated promising results without affecting growth in sharp snout sea bream (*Diplodus puntazzo*), gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), red sea bream (*Pagrus aurata*) and turbot (*Psetta maxima*) (Caballero et al., 2003, Glencross et al., 2003, Izquierdo et al., 2003, Izquierdo et al., 2005, Montero et al., 2005, Mourente and Bell, 2006, Piedecausa et al., 2007, Regost et al., 2003).

Accordingly, studies performed on freshwater and salmonid species have also demonstrated the possibility of high inclusion levels of plant oil without compromising growth rate of fish. Feeding Atlantic salmon with fish oil alone or blends of fish oil and sunflower oil, or linseed oil and sunflower oil did not show differences in growth (Menoyo et al., 2007). Other studies utilizing soybean, linseed, rapeseed, palm and sunflower oils have been evaluated and showed no reduction in growth or feed utilization, but affected tissue fatty acid and lipid composition, which reflected that of the dietary oils used (Bahurmiz and Ng, 2007, Bell et al., 2003b, Bransden et al., 2003, Turchini et al., 2006b). Conversely, it has been reported that 100% replacement of fish oil with plant oil, therefore lacking LC-PUFA, caused severe heart lesions, thinning of ventricular walls and muscle necrosis and influences the development of arteriosclerotic changes in the Atlantic salmon (Bell et al., 1991, Seierstad et al., 2005).

As plant oils are less favourable in perspective of fatty acid profile, there have been several investigations to find other lipid sources. Promising opportunities exists with single cell organisms and also genetically modified terrestrial plants (Turchini et al., 2012). Single cell oils may include bacteria, fungi, heterotrophic and photosynthetic microalgae, which provide a novel and renewable source of omega-3 LC-PUFA (Miller et al., 2011). Fermentation technology had been employed to produce alternative LC-PUFA from

microalgae through heterotrophic cultivation under sterile conditions where lipid accumulation can be tailored. It has been reported that DHA can be produced in high concentrations by the dinoflagelate *Crypthecodinium cohnii* and the protist *Schizochytrium sp*. Therefore, these unlimited, producible microalgae can supply a constant lipid source for fish feeds. Another study suggests that fungi, *Mortierella alpina* was a suitable source of LC-PUFA which benefited striped bass larvae. They affirm the potential usage of single cell heterotrophs as partial or complete replacement for fish-based ingredients in aquafeeds (Ganuza et al., 2008, Harel et al., 2002).

2.7 Plant oils

The following section lists out some of the plant oils utilized or have potential to be used in aquafeeds as fish oil replacers. This list is non exhaustive and aims to provide information in the perspective of fatty acid profile of the oils.

2.7.1 Saturated fatty acids oils

Coconut oil and palm kernel oil are commonly called lauric oils, as they are high in short and medium chain fatty acids, in particular lauric acid (12:0). They also contain a significant amount of other saturated fatty acids 14:0, 16:0 and 18:0. Palm oil contains about 48% saturated fatty acids and low concentrations of PUFA. Crude palm oil is obtained from the mesocarp of palm fruit and palm kernel oil is obtained from the kernels. Palm oil is the most produced plant oil in the world, and is relatively cheap in comparison with the other oils. Some fish species are able to effectively utilize high levels of palm oil in their diets as dietary energy and fatty acid sources without adverse effects on growth and feed utilization efficiency (Ng and Gibon, 2011, Ng et al., 2007).

2.7.2 Monounsaturated fatty acids oils

MUFA are easily digestible, good energy source, and their deposition into fish flesh is considered to be less detrimental than other fatty acid classes from the human nutrition perspective. Olive oil and avocado oil are high in oleic acid (80 – 93% of total fatty acids). Olive oil is more commonly used as direct human consumption; it is also expensive to process and must be consumed within a reasonable amount of time to benefit from its flavours and nutritional qualities.

Among this class of plant oil, rapeseed oil is the most produced and consumed. Rapeseed oil is obtained from *Brassica* sp. plants from the USA, Canada and Australia. It was initially high in erucic acid (ERA, 22:1n9), up to 50% of total fatty acids and high oleic acid (18:1n9). Fatty acid digestibility is directly determined by the chain length and level of unsaturation, thus SFA-rich oils are more difficult to digest (Ng et al., 2004). ERA is easily metabolized to produce energy, however it was reported that excessive dietary intake may cause cardiac lesions. Therefore, cultivars of rapeseed containing reduced amount of ERA were developed by selective breeding procedures. Hence, the name 'canola' oil was coined, derived from 'CANadian Oil, Low Acid'. Another high oleic acid canola cultivar that has been investigated in aquaculture is the monola oil (Turchini et al., 2013). The use of canola has been abundantly studied, in rainbow trout and Atlantic salmon (Bell et al., 2003a, Caballero et al., 2002, Menoyo et al., 2007). Canola represents one of the most commonly used fish oil replacement in aquafeed. However, MUFA-rich oils cannot completely replace fish oil in aquafeeds, as this can negatively impact the fulfilment of essential fatty acid requirements. Other MUFA-rich oils like rice bran oil is in use in India and China could potentially gain importance in the future as aquafeed raw materials (Turchini and Mailer, 2011).