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TESI DI LAUREA

REPLACING FISH OIL WITH ALGAE OIL FOR SUSTAINABLE DIET FORMULATION FOR FISH REARED IN RECIRCULATING AQUACULTURE SYSTEMS (RAS)

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INDEX

ABSTRACT

Nowadays fish oil is considered environmental and economical unsustainable due to its heavy reliance on wild catches for the production. However, it remains the primary source of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for farmed fish. Consequently, the replacement of fish oil with vegetable oils in aquaculture diets represents a promising solution, as emerging research highlights their potential and implications. Thus, this thesis investigates the effects of the inclusion of a microalga (*Schizochytrium* sp.) oil in order to replace fish oil in aquafeeds tailored for recirculating aquaculture systems (RAS). In details, three experimental diets were formulated to have only fish oil as fat source (diet FO), an inclusion of the tested microalga oil of 0.4% (diet AO 0.4%) and an inclusion of the tested microalga oil of 2% (diet AO 2%).

A total of 1,080 rainbow trout (*Oncorhynchus mykiss*) and 855 gilthead seabream (*Sparus aurata*) were random distributed into 9 RAS (120 rainbow trout per tank and 95 gilthead seabream per tank) and fed for two months the three experimental diets. At the end of the trial, fish were evaluated for growth performance, fatty acids profile of the fillets and relative lipid quality indices. However, due to an unknown disease affecting the gilthead seabream during the experimental trial, the results related to this species were excluded from this thesis.

Regards growth performance, rainbow trout fed diet AO 2% exhibited a higher final tank biomass (2,384 g) compared to the control group (2,200 g) ($P<0.01$), whereas no difference were found among diets in terms of economic feed conversion ratio (0.93, on average), biologic feed conversion ratio (0.81) and specific growth rate (2.29 %/day) ($P>0.05$). The fatty acid profile analysis showed that fillets from fish fed diet AO 2% resulted in higher EPA (33.9 mg/100g) and DHA (133.6 mg/100g) content than those from fish fed the other diets (18.1 mg/100g and 75.8 mg/100g, respectively) ($P>0.01$). The Atherogenicity Index (0.47%) and the Flesh Lipid Quality Index (20.1%) were higher in fillets from fish fed diet AO 2% compare to those from fish fed the other diets (0.45% and 16.3%, respectively) ($P<0.01$), while Thrombogenicity Index showed no difference. Finally, an economic evaluation was conducted

to help producers and technicians in making the most economically sustainable choices of alternative ingredients that meet the dietary requirements of carnivorous species for optimal RAS performance.

Chapter 1
INTRODUCTION

1. Need for aquafeed: composition and sustainability

In 2020, the total production of aquatic animals was estimated to be around 178 million tonnes, of which almost 50% derived from aquaculture, which had an estimated value of USD 256 billion (FAO, 2022). The aquaculture sector showed an average annual growth rate (AAGR) of 9.5% during the decade 1990-2000 and 4.6% in the decade 2010-2020 (FAO, 2022), that is expected to increase in the following years to follow the population growth and the rise of seafood consumption (Sarker et al., 2016). Specifically, seafood consumption was 9 kg and 20.5 kg per person in 1961 and 2020, respectively (Shah et al., 2018). Furthermore, it is projected that an additional 23 million tonnes of seafood products will have to be supplied in 2030 to meet these demands (Sarker et al., 2016).

The majority of fish produced through aquaculture heavily rely on industrial aquafeed, which amounted to 39,6 million tonnes in 2012. Those quantities are expected to increase, following the growth of the aquaculture sector (Shah et al., 2018).

Aquafeeds are accurately formulated to meet all the nutritional requirement of the selected farmed species in terms of macronutrients (i.e. carbohydrates, proteins and lipids), micronutrients (i.e. vitamins and minerals) and additives (Campbell et al., 2022; Craig et al., 2017).

In details, carbohydrates do not represent essential nutrients (Villasante et al., 2019), whereas they could be stored as glycogen reserves and used as a source of energy when needed (Craig et al., 2017). They are added to the feed pellets mainly as starch, which contributes with favourable binding properties during the technological process of the extrusion of aquafeed (Romano and Kumar, 2019).

Proteins are the most important components in fish diets, although they represent the most expensive ingredients (Craig et al., 2017). They are

composed by different combination of amino acids, ten of which are essential for fish (Li et al., 2021). Lysine and methionine are the most common limiting amino acids (Craig et al., 2017), thus a lack of these could compromise the utilisation of the other amino acids for growth purposes and shift protein catabolism for energy functions (Li et al., 2021).

Then, lipids serve as high-energy nutrients that fish can efficiently utilise for energy production. By incorporating lipids into their diet, fish can reduce their reliance on proteins as an energy source. This reduction not only decreases the cost of aquafeed but also has positive environmental implications by minimising the resources needed for protein production and reducing environmental pollution associated with fish farming. This practice, known as the "protein sparing effect", refers to provide alternative energy sources like lipids (or carbohydrates) in fish diets, thus minimizing the amount of protein required for fish growth (Vergara et al., 1996).

Vitamins are organic compounds that are often not synthetized by fish (Dawood et al., 2018). Two of the most important are vitamin C and vitamin E, respectively a water-soluble and a fat-soluble vitamin (Craig et al., 2017). Both are powerful antioxidants (Bai et al., 2015) and vitamin C is also able to enhance the immune system of fish (Craig et al., 2017; Dawood et al., 2018). Their antioxidant proprieties are also important to prevent the oxidation of lipids and increase the shelf life of aquafeed (Bai et al., 2015).

Minerals are inorganic compounds that are absorbed by the fish through the diet or directly from the water through the gills. They are used for different body functions like osmotic balance and bone formation in the case of macrominerals (e.g. calcium, sodium, chloride, potassium, chlorine, sulphur, phosphorus and magnesium), or for the structural composition of enzymes and for the homeostasis of the hormone system in the case of microminerals (e.g. copper, iron, chromium, iodine, manganese, zinc and selenium) (Craig et al., 2017; Lall and Kaushik, 2021).

Additives are non-functional ingredients added in small quantities for a specific purpose. For example, antioxidants and mould inhibitors are added to aquafeed to preserve the nutritional values before feeding, emulsifier and stabilizers are added to facilitate the pelleting phase, feed stimulants are added to facilitate feed ingestion, and food colorants (i.e. pigments) are added

to increase the acceptance of the final product in some specific cases (e.g. salmonid fillets) by the consumers (Bai et al., 2015).

Nowadays, pelleted aquafeed is obtained from mixing different selected raw materials, through the extrusion process. During this treatment, the raw materials are firstly submitted to a preconditioning phase, during which steam and water are added to reach a moisture content of 10-25% and a temperature at the preconditioner outlet of 70-90°C (Chaabani et al., 2022; Rokey et al., 2010). After that, the mixture goes into the extruder, in which it is treated at increasing temperature (125-150°C) and pressure (34-37 bar) in a short period of time (Alam et al., 2016; Chaabani et al., 2022; Rokey et al., 2010). After the extrusion process, the mixture is forced to pass thorough a die, which cut it into pellet of the selected diameter according to the fish species and growing phase (Chaabani et al., 2022; Rokey et al., 2010). Those pellets experience a sudden decrease in pressure, returning to atmospheric pressure, which causes their expansion. Then, the extruded pellets are dried and cooled before being coated. The coating process consists in adding oils to the pellet. This can be done at atmospheric pressure, by spraying the pellet with the oils and creating a coating which covers the external part of the pellet, or under vacuum conditions, during which the oils are added at low pressure, in order to push them inside the air cells formed inside the pellet after the extrusion phase. This last method allows to add higher quantities of oils to the pellet compared to the addition under atmospheric pressure (Chaabani et al., 2022; Rokey et al., 2010). This kind of treatment makes the pellet more resistant and stable in water (Rokey et al., 2010), which is an important quality traits of aquafeed to consider for recirculating aquaculture systems (RAS). In fact, as detailed later in chapter 1.3., the basic principle of a RAS is that the water is constantly filtered, adjusted and re-used, and therefore the risk of accumulating hazardous compounds derived from aquafeed has to be carefully monitored (Martins et al., 2010).

During the past century, fish oil (hereafter named FO) and fishmeal (hereafter named FM) have been considered the most important ingredients in aquafeed due, respectively, to their high levels of omega-3 long chain polyunsaturated fatty acids (LC-PUFAs), in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and high-value protein content (Shah et. Al 2018). The FO is obtained from cooked fish, which is pressed to obtain an

aqueous solution that will be centrifugated to separate the oil. The FM, instead, is produced by milling and drying entire fish or part of fish (FAO, 2022). Even if a growing proportion of FM and FO is produced starting from fish by-products, the majority is still derived from catches of wild fish (FAO, 2022). In particular, Peruvian anchoveta (*Engraulis ringens*; Jenyns, 1842) from Peru account for almost 80% of all the FO produced (Chauton et al., 2014). This led to the well-known aquaculture paradox: wild fish are caught through fishery industry and used to produce aquafeed to be fed to farmed fish (Naylor et al.,2009).

As mentioned before, nowadays the aquaculture sector account for half of the fish consumed globally (FAO, 2022; Turchini et al., 2009) and this percentage is projected to increase to palliate the decrease of wild catches from fishery and to fulfil the increasing demand of seafood (Turchini et al., 2009). The fast growth of the aquaculture industry is not followed by a comparable increase in the production of FO (Sarker et al., 2016; Turchini et al., 2009). That is linked to the fact that the populations of wild fish used to produce FO are vulnerable to fluctuations in environmental conditions, which makes the catches, and so the supply of FO, not stable (Chauton et al. 2014). Furthermore, those catches are also not expected to increase further, due to the global reduction of marine fish stocks (Sarker et al. 2016). Increased demand and dependence on wild populations for FO production have driven up costs considerably in recent years. (Shah et al. 2018).

For these reasons, the inclusion rates of FM and FO in compound feeds have shown a downward trend, with a more selective use during specific stages of production, like in hatcheries, bloodstocks and finishing diets (FAO, 2022). Despite the reduction in the use, FO is no longer sustainable when considering future needs of the aquaculture industry, and therefore some alternatives have to be found (Turchini et al. 2009).

2. Algae oil as a sustainable alternative in aquafeed

In 1994, the maximum amount (i.e. 30 million tonnes) of marine fish allocated to the production of FO was reached. After that peak, this production declined down to 16 million tonnes in 2020 (FAO, 2022), mainly because of the lack of catches of wild marine fish (Sarker et al., 2016). Nowadays, the global supply of FO stabilises at around 1 million tonnes and the majority of it (i.e. 70%) is destined to the aquaculture industry (Chauton et al., 2014). According to future projections, if the use of FO by the aquaculture industry remains unchanged, demand will exceed supply by 2030. This fact, together with the relative increase in the prices of the raw materials and environmental concerns, has led to a reduction in the use of FO in aquafeed (Campbell et al., 2022).

Following this decreasing trend, many alternative raw materials have been tested to replace FO: mainly of plant origin, like soybean oil, sunflower oil and cottonseed oil (Santigosa et al., 2021; Shah et al., 2018; Turchini et al., 2009), but also of animal origin, like lard, poultry fat and tallow (Turchini et al., 2009). Even if these ingredients could represent promising alternatives because of their competitive price, wide availability and good potential as sources of energy (Carvalho et al., 2020; Shah et al., 2018; Turchini et al., 2009), they still do not represent a complete substitution of FO, mainly because of their low content in omega-3 PUFAs, EPA and DHA, along with the presence of several antinutritional factors (Sarker et al., 2016; Santigosa et al., 2021; Turchini et al., 2009).

In details, DHA and EPA are important for the growth of the fish, since deficiencies in these fatty acids could slow down this process, and for their survival, since they could increase the functionality of the skin as a barrier to pathogens and, overall, increase the immunological competency of farmed fish (Bou et al., 2017). They also have a role in determining the intestinal microbiota composition and the intestinal health of the fish, in neurological development, and in reproduction (Santigosa et al., 2021; Santigosa et al., 2023).

Moreover, among the long chain omega-3 PUFAs (LC-PUFAs), EPA and DHA provide several benefits also to human health (Santigosa et al., 2021; Pike and Jackson 2010). In particular, they have the potential to alleviate inflammatory disorders and increase cardiac health by reducing mortality by congenital heart disease (CHD), myocardial infraction and strokes (Pike and Jackson,

2010). Those benefits in the cardiovascular system are linked to the enrichment of the cell-membrane phospholipids with EPA and DHA, which increase the arrhythmic threshold, decrease blood pressure and improve endothelial and arterial functions (Pike and Jackson, 2010). Since EPA and DHA are important components of the brain and nervous tissues, an important role is expected to be played during the neurological development, including cognitive functions (Pike and Jackson 2010; Santigosa et al., 2023).

In aquaculture, freshwater species have lower requirements of LC-PUFAs, like DHA and EPA, because they have elongase and desaturase enzymes which are able to synthetize them starting from linolenic acid. On the other hand, saltwater fish lack of those enzymes and thus they are forced to absorb EPA and DHA through the diet (Craig et al, 2017). In addition, high content of EPA and DHA is considered a favourable characteristic of the final product, so it is crucial that the replacement of FO with alternative oils does not compromise the efficiency of absorption of those fatty acids (Glencross et al., 2003; Ng et al., 2007). Thus, ingredients with high levels of EPA and DHA could be used to produce the so-called “finishing diets”, which are diet specifically formulated for the administration in the last period of fish rearing in order to include in the flesh of fish some precious compounds like PUFAs (Ng et al., 2007).

Among the FO alternatives, microalgae are at the base of the marine food chain (Guedes and Malcata, 2012) and they are naturally rich in EPA and DHA, since they are the primary producers of those fatty acids, which then accumulated along the food chain (Chauton et al., 2014). Their industrial production shows several advantages compared to other ingredients: they can grow in a wide range of conditions, they have a higher biomass production compared to terrestrial plants, they have very simple nutritional requirements, and their availability does not depend on wild catches (Shah et al., 2018). In aquafeed formulation, microalgae can be easily integrated into a fish diet as unprocessed raw material (Chauton et al., 2014; Satigosa et al., 2021), whereas microalgae processed as oils can be more easily included as direct substitutes of FO (Tacher et al., 2019). They can also be easily combined with other oils to optimise the fatty acid profile of a diet, allowing a more flexible formulation and production of fish feed (Carvalho et al., 2022). Moreover, by integrating microalgae oils as raw materials in a fish diet, they can provide several high-value compounds, like astaxanthin, phycocyanin and PUFAs

(Chen et al., 2021; Reyes-Becerril et al., 2013; Shah et al., 2018). On the other hand, they could also expose the fish to the risk of ingesting hazardous compounds, such as heavy metals, and anti-nutritional factors linked to the presence of their not digestible cell wall (Chen et al., 2021).

As described in Table 1.1, different species of microalgae can contain different types of PUFAs. For example, algae from the genus *Schizochytrium* and *Crypthecodinium* are rich in DHA; algae from the genus *Nannochloropsis* are rich in EPA; whereas algae from the genus *Porphyridium* are rich in arachidonic acid (ARA) (Nagappan et al., 2021). In particular, *Schyzochytrium* sp. are characterized by a lipid content up to 55-75% (of which about 49% is DHA) (Shah et al., 2017).

Table 1.1. a) Levels of linolenic acid (C 18:3), EPA (C 20:5), DHA (C 22:6) and ARA (C 20:4) of soybean oil and of some of the most widely used microalgae species as alternatives to FO (data from Alves et al., 2008; Chi et al., 2022; Hill et al., 2008; Nagappan et al., 2021).

	Fatty Acids (% on wet weight)			
	Linolenic acid (C 18:3)	EPA (C 20:5)	DHA (C 22:6)	ARA (C 20:4)
Soybean oil	6.8-8	n.d.	n.d.	2.9
Porphyridium oceanicum	n.d.	6.1	n.d.	6
Nannochloropsis oceanica	0.5-0.8	23.5-28.9	n.d.	3.7-7.5
Schyzochytrium sp.	0-0.6	0.7	36.2-37.6	0.5

In literature, the replacement of FO with algal oil rich in EPA and DHA has been already proven to be feasible in many fish species (Santigosa et al., 2023). In particular, a total substitution of FO with algae oil during the post-smolt phase of Atlantic salmon (*Salmo salar,* Linnaeus, 1758) makes no difference in terms of zootechnical parameters compared to FO-based diets, and also improves the EPA and DHA profile of the fillets (Santigosa et al., 2023). Moreover, a mixture of algal oil, rich in DHA and EPA, and poultry oil can perform as a complete substitute of FO in diets for Gilthead seabream (*Sparus* aurata, Linnaeus, 1758), supporting a good growth of the fish and ensuring high

nutritional values of the fillets (Carvalho et al. 2020). The same substitution was demonstrated to enhance neurogenesis and neural activity in juvenile Gilthead seabream (Carvalho et al., 2022). In addition, it has been proved that the use of microalgal oil did not compromise feed intake, growth performance and health of rainbow trout (*Oncorhynchus mykiss*; Walbaum, 1792) fed FO-free diets, whereas the content of EPA and DHA in the fillets was enhanced, reflecting the fatty acids composition of the diet (Santigosa et al., 2020).

3. Recirculating aquaculture systems

Recirculating Aquaculture Systems (RAS) are land-based aquaculture setups which could be placed outdoor or indoor (Martins et al., 2010). The basic principle of these type of systems is the continuous re-use of farmed water after specific waste treatments (Martins et al., 2010) in order to regenerate water with optimal quality for the rearing fish. In a RAS, the waste products mainly consist of metabolic wastes (Ahmed and Turchini, 2021), like carbon dioxide and ammonia, but also faeces, suspended solids and dissolved organic matter, derived from uneaten or undigested pellets (Brengballe, 2022). As showed in Figure 1.1., the basic structure of a RAS is formed by rearing tanks, a mechanical filter, a biological filter and a degasser, which remove carbon dioxide and favour the diffusion of oxygen in the water (Brengballe, 2022). In details:

1. The tanks should be designed to meet the needs of the fish in term of shape, water depth and size, to ensure the welfare of the farmed fish and enhance the entire system productivity (Brengballe, 2022). Bottom dwelling fish, like turbot (*Scophthalmus maximus*) and sole (*Solea solea*), take benefit from tank with a high surface area, whereas pelagic fish like salmon (*Salmo salar*) take benefit from tank with a higher water volume (Brengballe, 2022). Circular tanks are more commonly used in aquaculture, due to their favourable structural proprieties and hydrodynamic characteristics that allow an easy removal of uneaten

pellets, faeces and suspended solids (Brengballe, 2021; Malone, 2013). On the other hand, rectangular tanks allow efficient space utilization and easy harvest of the fish, but they present several structural weaknesses compared to circular tanks, which forces the use of more resistant materials for their construction (e.g. fiberglass, concrete etc.) and a greater attention to their cleaning (Malone, 2013).

2. The mechanical filtration is needed to remove the organic waste products such as faeces and suspended solids (Brengballe, 2022). This process is commonly carried out by a drum filter, in which the water is filtered through an internal micro screen (60-200 micron) (Dolan et al., 2013), that is fixed and partially submerged on a rotating drum (Dolan et al., 2013; Xiao et al., 2019). To remove fine particles, which are not stopped by the drum filter, a foam separator (namely 'skimmer') can be used. This instrument is more commonly used in marine RAS and works by injecting air in the water. This will create tiny bubbles that attach surface-active substances creating a foam that can be removed (Xiao et al., 2019).
3. The biological filtration aims at removing the harmful ammonia (NH_3) produced by the fish and excreted in the water through the gills (Brengballe, 2022; DeLong and Losordo, 2012). Ammonia is toxic to fish and can cause reduced appetite, reduced growth and even death at high concentration (DeLong and Losordo, 2012). The process to remove ammonia is carried out by ammonia-oxidizing bacteria (i.e. *Nitrosomonas* genus), which convert it first into nitrite (NO_2^-), which is still toxic to the fish, and then by nitrite-oxidizing bacteria (i.e *Nitrobacter* genus) which convert nitrite into harmless nitrate (NO_3^-) (Brengballe, 2022; DeLong and Losordo, 2012). The biofilter treatment capacity is determined by the number of bacteria inside it: the higher the surface area of the biofilter the higher the amount of ammonia converted in nitrate (Guerdot et al., 2009; Xiao et al., 2019). Thus, in order to increase as much as possible the surface on which bacteria can grow, biofilters are generally established using plastic media (e.g. 'Bio-Balls') which maximise the surface area per m^3 (Brengballe, 2022).
4. Depending on the requirements of the RAS structure, other equipment can be added, like oxygen enrichment or UV treatment (Brengballe,

2022). For example, an UV treatment (240 and 280 nm) could be useful for RAS-farmed fish because it could prevent the accumulation of coliform and heterotrophic pathogens bacteria by damaging their DNA (Brengballe, 2022; Gullian et al., 2012).

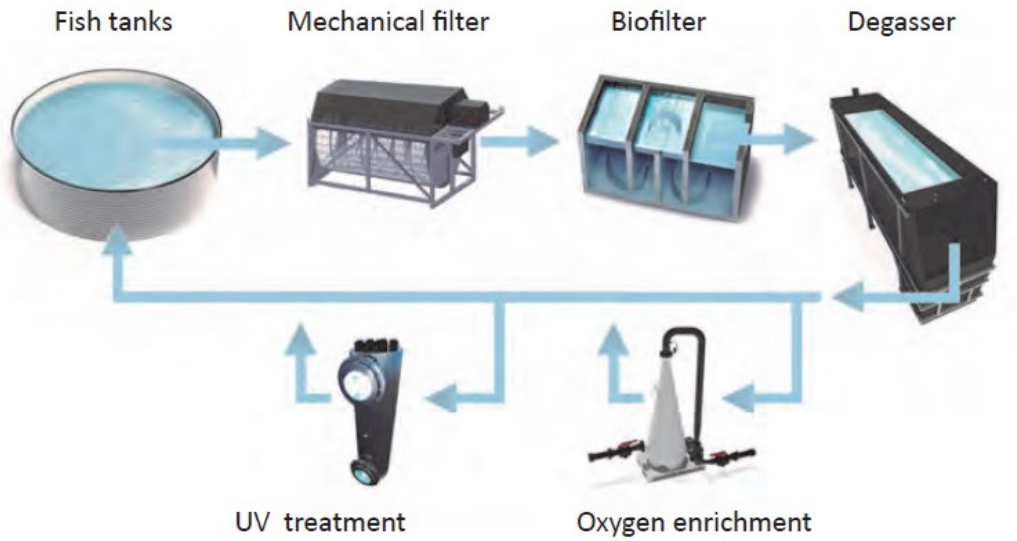

Figure 1.1: Basic component of a Recirculating Aquaculture Systems (RAS) (Brengballe, 2022)

One of the main advantages of RAS is the reduced water consumption, i.e. 0.1 m^3/kg of water per fish produced, compared to traditional systems that normally utilise 45 m^3/kg of water per fish produced in extensive systems and 30 m^3/kg of water per fish produced in flow-through systems (Ahmed and Turchini, 2021; Gullian et al., 2012). Furthermore, thanks to the inherent design of the system, RAS could improve waste management and nutrient recycling, ensure a high standard of hygiene, and control the spread of disease and biological pollution (Ahmed and Turchini, 2021). Due to the precise control of all the water parameters, RAS allow to achieve the ideal environmental conditions for the reared species, enabling the farmers to achieve higher stocking densities and thus higher productivity (EcoPlan International, 2008; Gullian et al., 2012). Moreover, the stable environmental conditions allow the achievement of profitable feed conversion ratio (FCR) and specific growth rate (SGR) all year-round (Masser et al., 1999; Murray et al., 2010). In other words, fish could reach the market size in shorter time (Murray

et al., 2014). In addition, the low requirement of RAS in terms of water exchange allows the production of fish near the markets, reducing the CO_2 emissions linked to the transportation (Maritns et al., 2010; Masser et al., 1992).

Despite the abovementioned pros, up to now RAS contributes to the total aquaculture production with little quantities compared to traditional systems, such as flow-through systems and open water systems (Ahmed and Turchini, 2021). Compared to a RAS, flow-through systems require less treatments to ensure a high-quality water, but these systems need high amount of water and farmers have little control over water chemistry and temperature. On the other hand, open systems have a constant water temperature, large space availability and low costs for pumping water, but they are dependent on weather conditions and are not always accessible, which makes it difficult to monitor the fish and manage them on a daily basis (EcoPlans International, 2008).

Then, the main disadvantages of a RAS are the high initial capital costs (Martins et al., 2010) and the high operating and personnel costs, which limit the production to high-value species reared at high stocking densities (Ahmed and Turchini, 2021). Other problems are related to the technological complexity of the system and the risk of exposing fish to alterations in water chemistry that could lead to large economic setbacks (EcoPlans International, 2008).

4. Rainbow trout as a model organism for freshwater RAS

Rainbow trout (*Oncorhynchus mykiss*; Wolbaum, 1792) is a species belonging to the order *Salmoniformes* and to the family *Salmonidae* (Fishbase, 2024), which include also Atlantic salmon (*Salmo salar*) (Hardy, 2002). It is characterised by a fusiform body with a silver colouration of the ventral side, which becomes blue to olive green on the dorsal side. In correspondence of the lateral line there is a pink band. The colour can vary according to the

environment, the size and the sexual maturation (FAO, 2009; Fishbase, 2024) (Figure 1.2).

Figure 1.2: Rainbow trout (*Oncorhynchus mykiss*; Wolbaum, 1792) (https://commons.wikimedia.org/w/index.php?curid=394359)

It is native of the North Pacific region, from Alaska to California, and in the area of the Kamchatka peninsula and the Okhotsk sea drainage (Hardy 2002; Fornshell, 2002; FAO, 2009). Since 1874, rainbow trout was present in every continent, except for Antarctica, where it was introduced for recreational fishing purposes and for aquaculture (FAO, 2009).

The first records of rainbow trout farming date back to 1870 in California (USA) (Fornshell, 2002; Sun et al., 2023). Rainbow trout farming in Europe started in 1885 in England and then the industry rapidly expanded to Denmark, Germany and Italy (Sun et al., 2023). Nowadays, trout are the 15th fish species farmed in the world (Sun et al., 2023). In 2019 the global annual production accounted for 940,000 tonnes, of which 97% was represented by *Oncorhynchus mykiss* species, a 21% increase compared to 2015 (D'Agaro et al., 2022). The major global producers are Norway, Chile and Turkey (D'Agaro et al., 2022). In North America, Great Britain, France and Italy, trout farming is carried out mainly in freshwater flow-through systems (Fornshell, 2002; Hardy, 2002; Samuel-Fitwi et al., 2012), but they are raised also in RAS (Samuel-Fitwi et al., 2012). In Scandinavian countries and in Chile, after a first period in freshwater, the growing phase is commonly carried out in marine cages allowing a larger harvesting size (Hardy, 2002; Sun et al., 2023). Several characteristics makes rainbow trout suitable for rearing in captivity: the reproduction phase is easy to manage and the fries are large, thus they are able to accept commercial

feed from the first feeding; they can tolerate a wide range of environmental temperatures and conditions; and final products are highly requested in the market (Hardy, 2002).

The production cycle starts with the eggs fertilisation and it is generally followed by one month of incubation and hatching, then three month of fry rearing and 10-12 month of growing phase to reach the marketable size (300-350 g) in freshwater (D'Agaro et al., 2022; FAO, 2009). In marine cages the growth is generally faster and trout can be harvested at 3 kg after 18 months (FAO, 2009). In fact, most rainbow trout strains can adapt to live in saltwater once they reach the post juvenile stages, following a slow increase in salinity in their rearing water (Hardy, 2002). Trout reared in marine waters, namely "steelhead trout", generally grow faster obtaining a weight of 7-10 kg within a year, compared to the 3 kg of the freshwater strains in the same period (FAO, 2009; Sun et al., 2023).

Rainbow trout, as other salmonids species, are predatory fish that occupy the top of the food chain (Kamalam et al., 2020; Panserat et al., 2013). Depending on the environmental temperature, rainbow trout needs 75-100 kJ per day per kg of body weight of energy for the maintenance needs of the body (Kamalam et al., 2020; Panserat et al., 2013), whereas others surplus calories acquired by the diet will be used for growth purposes. As other fish, trout do not have any dietary requirement for carbohydrates (Craig et al., 2017, Gatlin, 2010; Panserat, 2013), since they mainly rely on proteins and lipids to produce energy (Panserat et al., 2013).

The dietary requirements of proteins for rainbow trout vary according to the life stage (Hardy, 2002), but in general they are high (FAO, 2009; Panserat et al., 2013). In particular, the requirements will be higher in young fish (45-50%) and lower in growing stages (42-48%) (Hardy, 2002). Regarding the aminoacids requirements, rainbow trout need the same 10 essential aminoacids of all other vertebrates (Hardy, 2002; Kamalam et al., 2020; Panserat et al., 2013). Arginine, lysine, methionine and tryptophan are considered the limiting aminoacids (Hardy, 2002). In details, for an optimal utilization of the dietary protein content, the ratio between essential aminoacids and non-essential aminoacids should be 57:43 (Kamalam et al., 2020).

Lipids are another major source of energy in rainbow trout and they can be introduced in high quantities in salmonids diets (Panserat et al., 2013). A day-

to-day diet for trout contains 16-24% of lipids (Kamalam et al., 2020) and the eventual excess will be accumulated mainly as perivisceral fat, but also as subcutaneous and intramuscular adipose tissue (Panserat et al., 2013). Like for proteins, lipids requirements vary according to the life stage, with lower requirements for young fish (16-18%) and higher requirements in growing diets (18-24%) (Hardy, 2002). As described before, lipids are the main source of essential fatty acids (Hardy, 2002). Unlike many marine species, rainbow trout is able to desaturate and elongate the linolenic acid to produce EPA and DHA (Barry and Truskenski, 2020; Hardy, 2002; Panserat et al., 2013), therefore their requirements can be fulfilled with an inclusion of 1% of linolenic acid or 0.4-0.5% of EPA and DHA. In other words, the dietary inclusion of PUFAs is not strictly necessary, but it could give a series of energetic advantages, since rainbow trout do not have to produce EPA and DHA by themselves (Barry and Trushenski, 2020).

All the fat-soluble vitamins (e.g. D, E, K and A) are considered necessary for rainbow trout (Phillips and Brockway, 1957). On the other side, the researchers especially focused on the requirements for vitamin E (Hardy, 2002) because of its powerful antioxidant action, which has the potential to enhance fish growth performance, fillet quality and shelf life (El-Sayed and Izquierdo, 2021). In details, requirements for vitamin E vary according to the concentration of PUFAs in the diet, ranging from 25 to 100 mg per kg, whereas for the other vitamins, less studies are available (Hardy, 2002).

Literature about requirements of water-soluble vitamins is easily available and some recommendations of their inclusion in rainbow trout diets are provided (Hardy, 2002).

As for minerals, rainbow trout require the same ones as terrestrial animals, but all fish can acquire minerals from the rearing water (Hardy, 2002; Lall and Kaushik, 2021), thus the inclusion on the feed is usually limited into five trace minerals: copper, iodine, iron, manganese and zinc (Hardy, 2002).

As for dietary additives, wild trout feed normally with zooplankton as fry, then they move to insects, crustaceans and other fish as they grow (Hardy, 2002). In wild condition, the orange-pink colouration of the meat is achieved after feeding on crustacea containing pigments, such as carotenoids. The same colouration can be achieved in aquaculture thanks to the administration of a special feed which include synthetic pigments as astaxanthin (FAO, 2009).

5. Gilthead seabream as a model organism for marine RAS

Gilthead seabream (*Sparus aurata*; Linnaeus, 1758) is a teleost fish belonging to the family *Sparidae* (Fishbase, 2024). It is characterised by an oval and laterally compressed body of a silver-grey colouration. A large black blotch covers the origin of the lateral line and the upper part of the opercula, which is bordered on the underside by a reddish area. In addition, a golden band is visible between the eyes and a series of thin, longitudinal dark lines cover the sides of the body (FAO, 2005) (Figure 1.3).

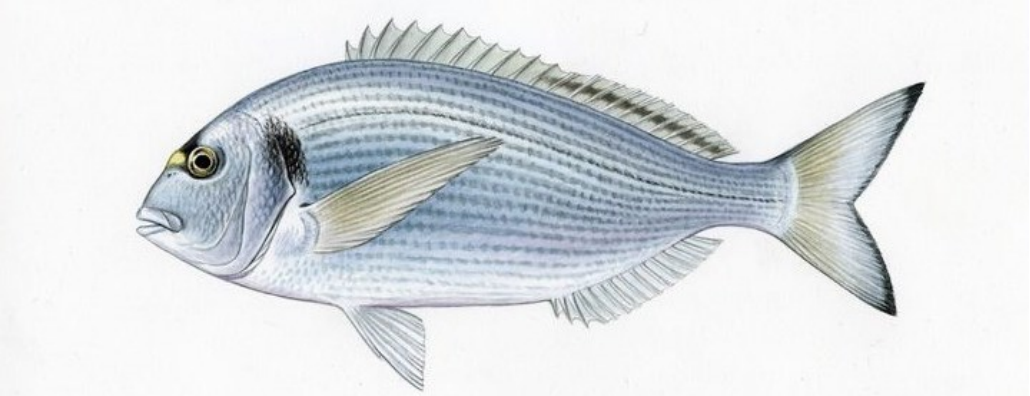

Figure 1.3: Gilthead seabream (*Sparus aurata*; Linnaeus, 1758) (https://www.pinterest.it/pin/315814992612985746/)

The natural habitat matches the Mediterranean Sea and the eastern Atlantic Ocean, from the British islands to the Canary Islands, while it is rarer in the Black sea (FAO, 2005). It is an euryhaline and eurythermal species (Mhalhel et al., 2023), which lives preferentially in seagrass meadow or sandy bottom, moving from shallow and protected coastal areas during summer, to deeper marine waters during winter (Sola et al., 2007).

Together with European seabass (*Dicentrarchus labrax*; Linnaeus, 1758), gilthead seabream is the second farmed fish in Europe in terms of value, after Atlantic salmon (Ferreira et al., 2012; Llorente et al., 2020). Almost all of the

production is carried out in Mediterranean countries, with Turkey and Greece as the main producers (Mhalhel et al., 2023). The trade is also carried out mainly in the Mediterranean area, with Turkey, Italy, Egypt, France and Spain as the main markets (Llorente et al., 2020). Traditionally, gilthead seabream is farmed under extensive conditions in coastal lagoon, like in the case of Italian "Vallicoltura" or Egyptian "Hosha" (FAO, 2005; Sola et al., 2007). In those systems, the fish are captured as juveniles, taking advantage of their natural movement towards coastal lagoons during spring and summer (FAO, 2005; Mhalhel et al., 2023). From 1980 successful breeding programs have been established (Mhalhel et al., 2023; FAO, 2005; Llorente et al., 2020) allowing the development of intensive rearing systems (FAO, 2005; Llorente 2020), in fact the enhancement of their production in sea cages and closed systems began from 1990 (Mhalhel et al., 2023). Nowadays, the most common systems to farm gilthead seabream is represented by sea cages placed in sheltered areas, even if the increasing competition for coastal areas tends to move the production to offshore cages or inland systems (e.g. RAS) in the next future (Mazes et al., 2011).

The production cycle starts with the hatching of eggs, which happen two days after their fertilization (Mhalhel et al., 2023). During the first 4 days from hatching the larvae rely on the yolk sac to take nutrients (Mazes et al., 2011; Mhalhel et al., 2023). Then gilthead seabreams start to feed on live feed: firstly their diet is composed only on rotifers and then, from 17-19 days after hatching, also on artemia nauplii (Mazes et al., 2011). Rotifers and artemia are both enriched with lipid sources in order to enhance their fatty acid content (Koven, 2002). Compounds feed will be administrated gradually in the last period of the larval stage, together with live feed (Mazes et al., 2011). Sixty days after hatching, compound feed will be the only source of feed (Mazes et al., 2011; Mhalhel et al., 2023). At 2-5 g the fish are moved to fattening facilities, where they reach the marketable size (300-500 g) in 18-24 month, according to the rearing conditions (Mhalhel et al., 2023).

Wild gilthead seabreams are mainly carnivorous, feeding on bivalves and gastropods (Mhalhel et al., 2023; Fishbase, 2024), but it can change its feeding habit according to the available resources (Mhalhel et al., 2023) and occasionally have and herbivorous behaviour (Fishbase, 2024).

As for the nutritional requirements, even if gilthead seabreams are able to digest starch better than other carnivorous species like salmonids (Enes et al., 2011), they have a restricted ability to digest carbohydrates (Koven, 2002). Therefore, the main source of non-protein energy is represented by lipids, and carbohydrates inclusions should be lower than 15-20% (Mhalhel et al., 2023; Koven, 2002).

Gilthead seabreams have higher needs of protein compared to salmonids (Peres and Oliva-Teles, 2009), which normally account for 40-50% in grow-out diets, while in juvenile reach almost 60% (Koven, 2002). These requirements will vary according to the size of the fish, the quality of the protein source and the levels of non-protein energy (i.e. lipids and carbohydrates) in the feed (Mhalhel et al., 2023). As for the essential aminoacids, the main limiting ones are arginine, lysine, methionine and tryptophan, as it is for rainbow trout (Hardy, 2002; Koven, 2002).

The lipid requirements vary between 12 and 24 % of the diet, with a content of EPA and DHA around 1% (i.e. 0.7% DM of EPA and 0.6% DM of DHA) in juveniles (Magalhães et al., 2020; Koven, 2002), and between 1.5% of EPA and 2.7% of DHA in grow-out diets (Koven, 2002). It has been shown that DHA contributes more to the development of gilthead sea bream larvae than EPA and a dietary inclusion level of 1-1.2% ARA can promote their survival and rapid growth (Koven, 2002; Magalhães et al., 2020).

Literature on vitamins requirements focused mainly on vitamin C (Mhalhel et al., 2023), which cannot be synthesized by most teleost fish, and vitamin E, which inclusion levels vary according to the inclusion levels of PUFAs, as for rainbow trout (Koven, 2002; Mhalhel et al., 2023).

Minerals requirements are not fully known for gilthead seabream, but it has been proved that an increasing substitution of FM and FO oil with plant-based ingredients requires a mineral dietary supplementation (Mhalhel et al., 2023).

Chapter 2
OBJECTIVE OF THE STUDY

Aquaculture currently contributes around 50 per cent of the global supply of seafood products, a figure that is set to grow further in response to human population growth and the concomitant decline in wild fish stocks. A major challenge to the sustainability of aquaculture relates to the reliance on wild fish for the production of fish oil, which is a key component of the diet of farmed fish. Fish oil is not only a vital source of energy, but also the main supplier of long-chain polyunsaturated fatty acids (LC-PUFA), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are essential for optimal health and growth of aquaculture species.

However, the rising costs of fish oil and increasing environmental concerns have prompted a reduction in its inclusion in animal feed. As a result, alternative lipid sources of animal or plant origin have been incorporated. These alternatives can meet the energy needs of farmed fish, but generally lack significant amounts of LC-PUFA, such as EPA and DHA. Therefore, it is imperative to identify new ingredients that can increase the LC-PUFA content in feed without further increasing the use of fish oil. Microalgae oils represent a promising solution, as they are the original producers of these fatty acids in the marine ecosystem and several lipid-rich microalgae species have been identified for their favourable EPA and DHA contents.

Thus, the aim of this thesis was to evaluate the feasibility of incorporating a microalgal oil, derived from specific strains of the genus *Schizochytrium* sp. (developed by Veramaris Biotechnology Research Centre), at inclusion rates of 0.4% and 2% in rainbow trout (*Oncorhynchus mykiss*) and sea bream (*Sparus aurata*) diets. At the end of the feeding trial, growth performance was assessed by calculating the specific growth rate and feed conversion ratio. Furthermore, a fatty acid profile analysis aimed at monitoring the accumulation of EPA and DHA in the finished product was performed. Finally, an economic evaluation was made to understand the feasibility of including this microalgal oil in commercial diets for fish farmed in RAS.

Chapter 3
MATERIALS AND METHODS

1. Experimental facilities and equipment

The trial was conducted at the NaturAlleva VRM s.r.l. facility located in Cologna Veneta during 2 months. In an indoor location, two independent RAS units were installed to farm rainbow trout in freshwater RAS, and gilthead seabream in marine RAS.

Each RAS unit was composed of the following elements:

- one sump of 9 m^3 in concrete (Lamar; Udine, Italy);
- three pumps (Lowara ESHE 50-250/30/P45RSSZ; Vicenza, Italy), of which one is active;
- one chiller (TECO TK15K; Ravenna, Italy);
- one UV filter (EtaPlus PE22; Stuttgart, Germany);
- two digital fluximeter (GF Signet Flow Integral System with 9900 Transmitter; Schaffhaused, Switzerland);
- one biofilter of 1.8 m^3 in fiberglass (LAMAR; Udine, Italy);
- one distribution tank in fiberglass (LAMAR; Udine, Italy);
- one drum filter (CM Aqua HEX Drum Filter Model F; Copenhagen, Denmark);
- twenty-four tanks of 0.5 m^3 in fiberglass (LAMAR; Udine, Italy) each one equipped with 1 LED light, 1 tube for oxygen supply, 1 tube for air supply, and 1 analogical fluximeter (GF variable area fluximeter type 123; Schaffhausen, Switzerland) set to have a water flow of 500 l/h.

The sump collected the water coming from the tanks; once it had been cleaned by the drum filter. From here it was pumped first in the UV filter and then in the biofilter through a pump. The biofilter was placed at a higher level compared to the tanks, so that no other pump was necessary to move the water. From the biofilter the water flowed to the distribution tank, which

distributed the water by fall to the rearing tanks. The first of the two digital fluximeters was placed between the sump and the biofilter, and was set to provide a water flow of 40 000 l/h. The second was placed right after the distribution tank and it was set to provide a water flow of 20 000 l/h.

The photoperiod was fixed in both units to have 10 hours of light and 14 hours of dark. In particular, the lights turned on at 7:00 and reached the maximum intensity at 7:30; the intensity started to be reduced at 16:30 to be completely turned off at 17:00.

Throughout the experiment, several parameters were constantly monitored through the use of probes. In particular, water conductivity was controlled through two probes (Mettler Toledo InPro 7100i/12/120/4435; Urdof, Switzerland) positioned after the tanks and in the distribution tank. The pH and water temperature were measured in each tank and right before the drum filtration through multiparametric probes (Mettler Toledo INPRO 325x; Urodf, Switzerland). Oxygen probes (Mettler Toledo InPro6960i/12/320; Urodof, Switzerland) were present in each tank and in the distribution tank, and turbidity was measured right before the drum filter through a probe (B&C electronics TU8355; Carnate, Italy).

Every week, samples of water were taken to measure the content of ammonium (Hach Lange LCK 304; Berlin, Germany), nitrite (Hach Lange LCK 341 and Hach Lange LCK 342; Berlin, Germany) and nitrates (Hach Lange LCK 340; Berlin, Germany) through the use of a spectrophotometer (Hach Lange DR 3900; Berlin, Germany).

Rainbow trout was moved by an authorised truck from the Edmund Mach Foundation in San Michele all'Adige (Trento, Italy), while the gilthead seabream was purchased and flight-shipped from Avromar Aquaculture (Kastella, Greece). Once arrived in NaturAlleva facility, both species observed a quarantine period of two weeks before being collocated into the tanks. In details, 2880 rainbow trout were distributed in the 24 freshwater tanks (i.e. 120 fish per tank), whereas 2280 gilthead seabream were distributed in the 24 marine tanks (i.e. 95 fish per tank).

2. Experimental diets

For the experimental trial, three isolipidic (EE 16.3% on dry matter, DM), isonitrogenous (CP 53.7% DM) and isoenergetic (DE 18.6% MJ/Kg) diets of 2-mm diameter were formulated in order to meet all the nutritional requirements of rainbow trout and gilthead seabream (NRC, 2011). From a control diet, which included only fish oil (FO) as the only lipid source (namely Diet FO), FO was partially replaced in the other two diets with algal oil (AO), i.e. 0.4% inclusion of algal oil (namely Diet AO 0.4%) and 2% inclusion of algal oil (namely Diet AO 2%). The algal oil was produced by Veramaris Biotechnology Research Centre, using microalgae of the genus *Schyzochytrium* sp., and was characterized by high levels of DHA and EPA (i.e. 15.7% and 39.8% respectively, and 65% EPA+DHA). Other ingredients were included at the same level for all the three diets. Ingredient and proximate composition of the three diets are shown in Table 3.1, whereas the fatty acid profile of the three diets is showed in Table 3.2. Regards this latter point, diet FO had a content of EPA of 98.9 mg/100g and of DHA of 105.3 mg/100g, whereas diet AO 0.4% had a content of EPA of 108.2 mg/100g and of DHA of 118.4 mg/100g, and diet AO 2% had a content of EPA of 131.6 mg/100g and of DHA of 167.2 mg/100g. Before the starting of the trial, all fishes were fed a standard diet (EE 18% DM; CP 54% DM; DE 20.3 MJ/Kg) of 1.5 mm diameter for one month of adaptation to the system.

During the first weeks of the trial, the gilthead seabreams were affected by an unidentified disease that caused inappetence and severe mortality. Therefore, the trial with sea bream was stopped and, in this thesis, the abovementioned feed formulations will be considered only as for the effects on growth performance, fatty acid profile and lipids quality indexes of rainbow trout fillets.

Table 3.1. Ingredients (% as fed) and proximate composition of the experimental diets fed rainbow trout for the two-months trial.

	Diet		
	FO	**AO 0.4%**	**AO 2%**
Ingredients (% as fed)			
Fishmeal	40.9	40.9	40.9
Fish Oil (FO)	**10.2**	**9.8**	**8.2**
Algal oil (AO)	**0.0**	**0.4**	**2.0**
Marine zooplankton meal	5.3	5.3	5.3
Wheat meal	2.6	2.6	2.6
Corn gluten	22.0	22.0	22.0
Vital wheat gluten	10.6	10.6	10.6
Soy protein concentrate	2.64	2.64	2.64
Beta glucans-yeast extract	0.02	0.02	0.02
Emulsifier (E484)	0.16	0.16	0.16
Monoammonium phosphate	0.70	0.70	0.70
Taurine	0.79	0.79	0.79
Pea starch	1.32	1.32	1.32
Shrimp hydrolysate	1.00	1.00	1.00
Vitamin and mineral premix*	1.78	1.78	1.78
Proximate composition			
Dry matter (%)	89.6	89.6	89.6
Crude protein (%)	53.7	53.7	53.7
Crude fat (%)	16.3	16.3	16.3
Ash (%)	8.00	8.00	8.00
Fibre (%)	0.41	0.41	0.41
Gross energy (MJ/kg)	20.4	20.4	20.4

*Vitamin and mineral premix (quantities in 1 kg of mix): Vitamin A, 4000,000 IU; Vitamin D3, 800,000 IU; Vitamin C, 25,000 mg; Vitamin E, 15,000 mg; Inositol, 15,000 mg; Niacin, 12,000 mg; Choline chloride, 6000 mg; Calcium Pantothenate, 3000 mg; Vitamin B1, 2000 mg; Vitamin B3, 2000 mg; Vitamin B6, 1800 mg; Biotin, 100 mg; Manganese, 9000 mg; Zinc, 8000 mg; Iron, 7000 mg; Copper, 1400 mg; Cobalt, 160 mg; Iodine, 120 mg; Anticaking and antioxidant + carrier, making up to 1000 g; Nucleotides, 0.06%;

Essential oils, 0.01%, Oligosaccharides, 0.35%; Whey protein concentrate, 0.02%; Antioxidant, 0.1%; Stay C35%, 0.14%; Tributyrin, 0.4%.

Table 3.2. Fatty acid profile (mg/100 g as fed) of the experimental diets fed rainbow trout for the two-months trial.

	Diet		
	FO	**AO 0.4%**	**AO 2%**
Fatty acids (mg/100 g as fed)			
C 12:0	1.99	1.78	1.84
C 14:0	65.4	69.1	73.4
C 15:0	5.16	5.12	5.75
C 16:0	270.1	276.3	292.9
C 17:0	7.85	7.29	8.18
C 18:0	58.5	53.1	53.8
C 18:1 ω9	381.1	354.5	320.8
C 18:1 ω7	42.2	45.1	42.4
C 18:2 ω6c (linoleic acid, LA)	291.3	267.2	212.2
C 20:0	5.01	4.09	4.46
C 18:3 ω3 (alpha-linolenic acid, ALA)	49.1	41.8	36.4
C 20:1 ω9	41.0	53.1	45.9
C 18:4 ω3	17.6	19.5	19.9
C 20:2 ω6	6.07	5.71	5.45
C 20:3 ω6	1.31	1.55	1.75
C 22:0	2.68	2.07	1.88
C 20:3 ω3+C 20:4 ω6	12.2	10.9	14.8
C 22:1 ω11	34.2	51.0	43.4
C 22:1 ω9	5.40	7.02	6.20
C 20:4 ω3 (eicosatetraenoic acid, ETA)	6.81	6.91	7.72
C 20:5 ω3 (EPA)	**98.9**	**108.2**	**131.6**
C 22:4 ω6	4.43	4.51	5.82
C 24:1 ω9	6.92	8.42	7.72
C 22:5 ω6	3.44	3.03	6.89
C 22:5 ω3	14.3	13.3	18.4
C 22:6 ω3 (DHA)	**105.3**	**118.4**	**167.2**
Sum ω3	298.1	313.6	388.6
Sum ω6	312.7	287.5	239.5

ω3:ω6 ratio	0.95	1.09	1.62

3. In vivo recordings

Both rainbow trout and gilthead seabream underwent a 14-day quarantine period to ensure the absence of pathologies before the beginning of the experimental trial. At the start of the trial, the fish were weighed before being introduced into the rearing tanks. Specifically, groups of 10 fish were removed from the quarantine tanks, placed into pre-weighed buckets, weighed, and then distributed into the rearing tanks. Each tank was stocked with 120 individual rainbow trout and 95 individual gilthead seabream, ensuring uniform initial biomass across all tanks. The tank biomass was checked every two weeks until the end of the trial. In detail, in one tank at a time, the water level was lowered until the fish could be collected and a dose of anaesthetic (Tricaine methasulfunate, 10 mg/l concentration) was added to the remaining water in the tank to achieve mild sedation of the fish. Then all the fish from the tank were placed in a separate bucket and weighed with a scale (KERN PNJ12000-1M; Balingen, Germany). Finally, the fish were returned to the corresponding tank after filling it with clean water.

The fish were hand-fed based on a theoretical feed ration calculated using internal data from Naturalleva VRM s.r.l., determined by the Daily Feed Intake (DFI) and the Thermal Growth Coefficient (TGC) (data not shown). Therefore, it was possible to calculate the exact amount of feed given to the fish, excluding eventual leftovers.

Knowing the initial and final weight of the fish, and the weight of the feed that was administered, the Specific Growth Rate (SGR) and the Economic and Biological Feed Conversion Ratio (FCReco and FCRbio) were calculated using the following formulas:

$$\text{Specific growth ratio (SGR)} = \frac{\ln\ (\text{Final Weight}) - \ln\ (\text{Initial Wieght})}{\text{Days}} \times 100$$

$$\text{Feed conversion ratio (FCReco)} = \frac{\text{Feed Administrated}}{(\text{Final Weight of alive fish} - \text{Initial Weight})}$$

$$\text{Feed conversion ratio (FCRbio)} = \frac{\text{Feed Administrated}}{(\text{Final Weight of total fish} - \text{Initial Weight})}$$

4. Analytical procedures

5.1. Determination of total fat content

The fat content of the three experimental diets and fish fillets was determined using a Soxhlet SER 148 extractor (VELP Scientific, Bohemia, NY, U.S.A.) following method #920.39 (AOAC). Specifically, four fish per tank (i.e. 12 per experimental diet) were sampled and filleted. All fillets from fish fed the same diet were shredded to obtain a homogeneous mixture and divided into three groups, resulting in three samples per diet. Each sample was dried at 50°C overnight before undergoing an initial extraction using petroleum ether as the solvent. Hydrolysis was then performed to break the bonds between lipids and other nutrients (i.e. proteins, carbohydrates, and metals). Following hydrolysis, a second extraction using petroleum ether was conducted.

5.2. Determination of fatty acid profile and lipid quality indexes

The methodology for the fatty analysis was based on Bligh and Dyer (1959) and Medina et al. (2007). The first part involved the extraction of fatty acids from the sample in the form of glycerol esters, while the second part required methylation to obtain the free esters.

The extraction process began by grinding and weighing 0.25 g of the feed sample into a 36 ml Pyrex bottle. Subsequently, 8 ml of methanol and 4 ml of dichloromethane were added to the bottle, which was then shaken for 1 minute using a vortex mixer (VELP Scientific TX4 Digital IR Vortex Mixer; Bohemia, NY, U.S.A.). Following this, 4 ml of dichloromethane and 5 ml of demineralized water were added, and the sample was centrifuged for 5 minutes at 3000 rpm (Remi Neya-8; Vasai, India). The extraction process for fillet samples differed slightly: 2 g of fillets were weighed into a 36 ml Pyrex bottle, to which 4 ml of methanol and 4 ml of dichloromethane were immediately added. The samples were homogenized for 1 minute using a

homogenizer (VELP Scientific OV5 Homogenizer; Bohemia, NY, U.S.A.). Next, 4 ml of dichloromethane and 4 ml of demineralized water were added, and the samples were homogenized again before being centrifuged at 3000 rpm for 5 minutes. From this point, the procedure was the same for both fillet and feed samples. The extraction process resulted in three phases: an upper methanol phase, a middle sample phase, and a lower dichloromethane phase. The upper phase was removed, and the lower phase was transferred to a smaller Pyrex test tube. A small amount of sodium sulfate was added, and the sample was shaken for 10 seconds using the vortex mixer. After filtration, the sample was ready for methylation or storage in the freezer.

For the methylation step, 0.5 ml of the sample was transferred to a 36 ml Pyrex bottle and dried under nitrogen to remove the solvent. Then, 2 ml of toluene and 10 ml of methanol containing 0.1% sulphuric acid were added. Next, 0.5 ml of an internal standard (C19:0, 0.4 mg/ml in toluene) was added, and the sample was incubated at 50°C for 18-20 hours. After incubation, the sample was cooled to room temperature, and 15 ml of 5% NaCl solution was added. The sample was shaken for 10 seconds with the vortex mixer and centrifuged for 5 minutes at 3000 rpm, resulting in the toluene and methylated compounds separating into the upper phase. Finally, the upper phase was transferred into two vials for gas chromatography analysis using a Shimadzu GC-2025 (Kyoto, Japan).

To assess the nutritional quality of the fatty acids contained in the fillets, several lipid quality indexes were calculated, including the Atherogenicity Index (AI), Thrombogenicity Index (TI), and Flesh Lipid Quality Index (FLQ).

The Atherogenicity index reflects the ratio between the main unsaturated fatty acids and the main saturated fatty acids, giving an indication of the tendency of the lipids to adhere to cells of the immune-circulatory system (Bruce and Core, 2004; Lunn and Theobald, 2006; Ulbtricht and Southgate, 1991). On the other hand, the Thrombogenicity index provide an estimation of the formation of platelets in the blood vessels (Ulbtricht and Southgate, 1991). The Flesh Lipid Quality index evaluates the ratio between EPA + DHA and the total fatty acids contents as an estimation of the quality of the lipids, i.e. higher the values higher the quality (Abrami et al., 1992).

Those three indexes were calculated by using the following formulas (Nava et al., 2023):

$$\text{Atherogenicity Index (AI)} = \frac{\text{C12:0} + (4 \times \text{C14:0}) + \text{C16:0}}{\Sigma\text{n6PUFA} + \Sigma\text{MUFA} + \Sigma\text{n3PUFA}}$$

$$\text{Thrombogenicity Index (TI)} = \frac{\text{C14:0} + \text{C16:0} + \text{C18:0}}{0.5 \times \Sigma\text{n6PUFA} + 0.5 \times \Sigma\text{MUFA} + 3 \times \Sigma\text{n3PUFA} + (\frac{\text{n3PUFA}}{\text{n6PUFA}})}$$

$$\text{Flesh Lipid Quality (FLQ)} = 100 \times \frac{\text{EPA} + \text{DHA}}{\%\text{ of total fatty acids}}$$

5. Economic evaluation

In order to achieve an economical evaluation of the feed formulation tested in this experimental trial, the following formulas were used to calculate the production costs for 1 kg of fish (Fanizza et al., 2023), the Economic Conversion Ratio (ECR) and the Economic Profit Index (EPI) (Martinéz-Llorens et al., 2007):

$$\text{Fish cost to produce 1 kg of fish} = \text{Feed Cost} \times \text{FCR}$$

$$\text{Economic Conversion Ratio (ECR)} = \frac{\text{Feed Production Cost}}{\text{Weight Gain}}$$

$$\text{Economic Profit Index (EPI)} = (\text{Final Weight} \times \text{Price of the Fish}) - (\text{ECR} \times \text{Weight Gain})$$

The production costs of the feed were calculated at NaturAlleva VRM s.r.l. using the costs of the raw materials and excluding the labour, packaging and transportation costs. The price of fish was obtained from European Market Observatory for Fishery and Aquaculture (EUMOFA, 2024). For the calculation of abovementioned indexes, an average of these prices was taken. In particular, for rainbow trout, the price considered was 3.64 EUR/kg.

6. Statistical analysis

Data related to growth performance of rainbow trout, fatty acids profile of fish fillets, and fillet quality indexes were submitted to ANOVA and analysed using the PROC GLM, considering the diet as main effect. The Bonferroni test was used to compare least square means. Differences among least square means with $P<0.05$ were assumed to be statistically significant.

Chapter 4
RESULTS AND DISCUSSION

1. Growth performance

Survival rate did not differ among diets (79.1% on average) (P>0.05). No difference was found between the initial tank biomass (670 g on average) of the three experimental diets (P>0.05), whereas the final tank biomass was higher in tanks with trout fed the diet AO 2% (2,384 g) compared to those fed the diet FO (2,200 g), while diet AO 0.4% performed in between (P<0.05). No differences were found in terms of weight gain (76.0 %, on average), specific growth rate (2.28 %/day), economic feed conversion ratio (0.90) and biologic feed conversion ratio (0.80) among the three experimental diets (P>0.05) (Table 4.1).

Table 4.1. Growth performance of rainbow trout fed the three experimental diets for two-months trial.

	Diets			P-value	RMSE
	FO	**AO 0.4%**	**AO 2%**		
Survival (%)	81.0	77.3	79.0	0.17	4.83
Initial tank biomass (g)	680	663	694	0.57	34.1
Final tank biomass (g)	2,200[a]	2,234[ab]	2,384[b]	0.01	58.0
Weight gain (%)	75.0	77.0	77.0	0.16	1.27
Specific growth rate (SGR, %/day)	2.20	2.34	2.33	0.17	0.09
Economic Feed conversion ratio	1.00	0.92	0.88	0.35	0.09
Biological Feed conversion ratio	0.87	0.79	0.76	0.14	0.06

RMSE: Root mean square error. Values in the same row with different superscript are significantly different *(P<0.05)*

In line with the results of our study, literature on rainbow trout reported no significant differences in specific growth rate and feed conversion ratio

between fish oil-based diets and diets with increasing inclusion (1.4%-10%) of algae meals (*Macrocystis pyrifera, Durvillea antartica, Scenedesmus* sp., *Spirulina platensis*) (Dantagnan et al., 2009; Quiñones et al., 2021; Skalli et al., 2020; Teimouri et al., 2015; Tómas-Almenar et al., 2018). Similarly, Osmond et al. (2021) and Santigosa et al. (2020) found no impairments on growth performance of rainbow trout fed diets with different inclusion (2.5%-10%) of algae oils (*Schizochytrium* sp.), both alone or in combination with vegetable oils, with respect to the fish oil-based control diet.

On the other hand, an impairment of growth performance has been shown? in rainbow trout fed diets including different species of algae (*Nannochloropsis* sp.; *Isochrysis* sp.; *Schizochytrium* sp.) and camelina oil as fish oil substitutes (Sarker et al., 2020). In details, rainbow trout fed the algae oil-based diet exhibited a higher feed conversion ratio and lower final biomass compared to those fed the fish oil-based diet, although the specific growth rate did not differ among diets. These results could be explained by a decrease in feed intake, likely due to the low palatability of high inclusion rates of vegetable oils in fish diets (Sarker et al., 2020).

Another study reported a lower specific growth rate in fish fed the algae-free diet compared to those fed diets with an inclusion of 5% and 10% of algae meal, whereas the feed conversion ratio did not differ among diets. The enhanced values in specific growth rate of fish fed the algae-based diets was attributed to the presence of vitamins, minerals, and essential amino acids in the microalgal biomass (Chen et al., 2021).

Contrasting results has been observed with an inclusion rate of 1% of algal oil (*Schizochytrium* sp.) in diets for rainbow trout, which led to a higher specific growth rate and a lower feed conversion ratio compared to a control diet without algae inclusions (Lee et al., 2022). However, the same study reported that an inclusion rate of 2% of algal oil did not compromise the specific growth rate and feed conversion ratio with respect to the fish oil-based diet, findings that align more closely with those of this study.

2. Fatty acid profile and quality indexes

As showed in Table 4.2, the linoleic (LA) and alpha-linolenic (ALA) acids content in the fillets of fish fed diet FO and diet AO 2% did not differ (94.1 mg/100 g and 14.9 mg/100 g on average on fresh fillets, respectively), while fillets of fish fed diet AO 0.4% showed lower levels (58.2 mg/100 g of LA on fresh fillets and 7.91 mg/100 g of ALA on fresh fillets) ($P<0.05$).

Higher contents of eicosatetraenoic acid (ETA) were found in fillets of fish fed diet AO 2% (5.36 mg/100 g on fresh fillets) compared to those fed diet AO 0.4% (2.60 mg/100 g on fresh fillets), while fillets of fish fed diet FO showed intermediate levels of ETA (3.91 mg/100 g on fresh fillets) ($P<0.05$).

For both EPA and DHA no differences were found among fillets of fish fed diet FO and those fed diet AO 0.4% (18.1 mg/100 g and 75.8 mg/100 g on average on fresh fillets, respectively), while fillets of fish fed diet AO 2% showed higher content of both EPA (33.9 mg/100 g on fresh fillets) and DHA (133.6 mg/100 g on fresh fillets) ($P<0.05$) (Table 4.2).

Table 4.2. Total fat content (% wet weight) and fatty acids (mg/100 g wet weight) of the fillet of rainbow trout fed the three experimental diets for the two-months trial.

	Diets			P-value	RMSE
	Control	**AO 0.4%**	**AO 2%**		
Total fat content (% wet weight)	6.62[ab]	4.70[a]	8.38[b]	0.01	0.97
Fatty acids (mg/100 g wet weight)					
C 12:0	0.47[ab]	0.26[a]	0.59[b]	0.02	0.10
C 14:0	21.2[ab]	15.5[a]	29.9[b]	0.01	3.76
C 15:0	1.72[ab]	1.28[a]	2.41[b]	0.01	0.29
C 16:0	121.1[ab]	90.6[a]	162.4[b]	0.01	20.2
C 17:0	2.29[ab]	1.62[a]	3.12[b]	0.01	0.36
C 18:0	25.0[ab]	18.4[a]	31.7[b]	0.03	4.34
C 18:1 ω9	162.8	107.7	147.3	0.07	23.6
C 18:1 ω7	18.3	13.4	56.4	0.33	35.1
C 18:2 ω6c (linoleic acid, LA)	95.3[b]	58.2[a]	92.9[b]	0.01	11.6
C 20:0	2.23[ab]	1.45[a]	2.78[b]	0.01	0.32
C 18:3 ω3 (alpha-Linolenic acid, ALA)	14.9[b]	7.91[a]	14.4[b]	0.01	1.77
C 20:1 ω9	14.6[ab]	12.1[a]	20.0[b]	0.02	2.40
C 18:4 ω3	4.18[ab]	2.47[a]	5.57[b]	0.02	0.91
C 20:2 ω6	5.67[b]	3.73[a]	5.50[b]	0.04	0.79
C 20:3 ω6	2.70[b]	1.71[a]	2.44[ab]	0.03	0.33
C 22:0	0.66[b]	0.41[a]	0.74[b]	0.01	0.07
C 20:3 ω3+C 20:4 ω6	5.48[ab]	3.84[a]	7.36[b]	0.01	0.79
C 22:1 ω11	7.88[a]	7.56[a]	13.2[b]	0.01	1.57
C 22:1 ω9	1.64[ab]	1.39[a]	2.37[b]	0.02	0.29
C 20:4 ω3 (eicosatetraenoic acid, ETA)	3.91[ab]	2.60[a]	5.36[b]	0.01	0.60
C 20:5 ω3 (EPA)	**20.2[a]**	**15.9[a]**	**33.9[b]**	**0.01**	**3.34**
C 22:4 ω6	1.86[a]	1.29[a]	2.68[b]	0.01	0.30
C 24:1 ω9	2.50[a]	2.17[a]	3.62[b]	0.01	0.41

C 22:5 ω6	1.97[a]	1.44[a]	3.64[b]	0.01	0.34
C 22:5 ω3	6.41[a]	4.65[a]	10.8[b]	0.01	0.90
C 22:6 ω3 (DHA)	**83.2[a]**	**68.4[a]**	**133.6[b]**	**0.01**	**13.5**

RMSE: Root mean square error. Values in the same row with different superscript are significantly different *(P<0.05)*

Regards the quality indexes of lipids, the Atherogenicity index was higher in fillets of fish fed diet AO 2% (0.47) than in those fed diet FO (0.43), while fillets of fish fed diet AO 0.4% performed in between (0.46) (P<0.05) (Table 4.3). The Thrombogenicicty index showed no differences between fillets of fish fed the three experimental diets (0.29 on average) (P>0.05). The Flesh Lipid Quality index was higher in fillets of fish fed diet AO 2% (20.1) respect to those fed the other diets (16.3 on average) (P<0.05) (Table 4.3).

Table 4.3. Atherogenicicity, Thrombogenicity and Flesh Lipid Quality indexes of the fillet of rainbow trout fed the three experimental diets for the two-months trial.

	Diets			P-value	RMSE
	Control	**AO 0.4%**	**AO 2%**		
Atherogenicity Index (AI)	0.43[a]	0.46[ab]	0.47[b]	0.01	0.01
Thrombogenicity Index (TI)	0.29	0.30	0.27	0.11	0.01
Flesh Lipid Quality (FLQ)	15.6[a]	17.0[a]	20.1[b]	0.01	1.11

RMSE: Root mean square error. Values in the same row with different superscript are significantly different *(P<0.05)*

In studies examining the substitution of fish oil with a mixture of different microalgae (*Nannochloropsis* sp., *Isochrysis* sp., *Schizochytrium* sp.) and vegetable oils in rainbow trout feed (9%-12%) variable results were observed as for the quality of the fillets. In contrast of the results found in this study, DHA levels in the fillets did not differ in trout fed diets with an inclusion of microalgae oil, while EPA levels were higher in fillets from fish fed diet without the substitution of fish oil (Sarker et al., 2020) which was attributed to a reduction in digestibility of algal cells, particularly those species high in EPA (Sarker et al., 2020), which could lead to a lower content of EPA in the final product. Consistently, another study testing a diet with a 5% inclusion of microalgae meal (*Scenedesmus* sp.) reported lower EPA content in fillets of

fish fed the microalgae diet compared to those of fish fed diet without the microalgae inclusion (Skalli et al., 2020). On the other hand, higher DHA levels in fillets of fish fed the microalgae diet were found (Skalli et al., 2020).

In contrast with the result found in this thesis, Quiñones et al. (2021) and Serrano et al. (2021) found higher or comparable EPA levels and no differences in DHA levels between a fish oil-based diet and diets including 1.5%-17% of algae meals derived from different species such as *Schizochytrium limacium*, *Nannochloropsis oceanica*, and *Durvillea antartica*. These variations in EPA and DHA levels could be due to differences in the fatty acid profile of the used algae (Chen et al., 2021; Dantagnan et al., 2009; Quiñones et al., 2021; Serrano et al., 2021; Skalli et al., 2020) or the preferential storage of DHA in muscle tissue, while EPA can be more easily oxidized by salmonids species (Serrano et al., 2021).

In line with our findings, other studies focusing on diets including 1.4%-6% of microalgae meals (*Macrocystis pyrifera* and *Tribonema ultriculosum*) showed higher levels of both EPA and DHA compared to the diet including only fish oil as fat source (Chen et al., 2021; Dantagnan et al., 2009). Furthermore, similar results were reported in studies where microalgae oil (*Schizochytrium* sp.) were included from 1% to 7.5%. Specifically, DHA level was higher in fillets of fish fed the diets with microalgae oil, whereas the EPA level was lower respect to those fed the algae-free diet. This reduction could be explained to the lower levels of EPA in these microalgae diets due to differences in algae strains (Lee et al., 2022; Osmond et al., 2021). Comparable results of those found in this work were obtained for other salmonid species (*Atlantic salmon*) fed diets with increasing inclusion levels of algal oils (2.6%-13% *Schizochytrium* sp.) (Miller et al., 2007; Santigosa et al., 2023). In details, higher DHA levels were reported in fillet from fish fed diets with microalgae oils. On the other side of the coin, literature reported either lower (Zatti et al., 2023) and higher (Santigosa et al., 2023) EPA levels in fillet of fish fed diets with microalgae oils. The lower EPA levels might be explained by better DHA retention or higher precursor (i.e. alpha-linolenic acid, C18:3ω) inclusions (Zatti et al., 2023).

Regarding alpha-linolenic acid (ALA) and linoleic acid (LA), studies on salmonid diets including different rate (5-17%) of microalgae meals (*Scenedesmus* sp.; *Schizochytrium limacium*; *Nannochloropsis oceanica*; *Tribonema ultriculosum*) reported lower levels of these fatty acids in the fillets

compared to those of fish fed a fish oil-based diet (Chen et al., 2021; Serrano et al., 2021; Skalli et al., 2020). However, a study on rainbow trout fed diets with increasing levels (1.5%-6%) of algae meal (*Durvillea antartica*) showed results comparable to those of this thesis, with no differences compared to fillets from fish fed diets with fish oil as the sole fat source (Quiñones et al., 2021). Moreover, studies on rainbow trout and Atlantic salmon fed diets with an inclusion of 6%-11% of microalgae oil (*Schizochytrium* sp.) reported increased levels of both alpha-linolenic acid and linoleic acid in the fillets of the two species (Osmond et al., 2021; Zatti et al., 2021).

Regarding the fatty acid quality in fish fillets, in contrast with the results of this thesis, studies evaluating fish diets with an inclusion of 1.5%-10% of algae meal (*Durvillea antartica* and *Spirulina platensis*) reported a reduction in the Atherogenicity Index and Thrombogenicity Index with increasing levels of microalgae inclusion (Quiñones et al., 2021; Teimouri et al., 2015). Furthermore, a lower Atherogenicity Index was also reported in studies on other salmonid species (*Atlantic salmon*) fed a diet with 10% inclusion of algae meals (*Nannochloropsis gaditana*; *Tisochrysis lutea*; *Rhodomonas lens*; *Isochrysis galbana*) (Estévez et al., 2022). Regarding the Thrombogenicity Index, the above mentioned study on Atlantic salmon fed a diet with 10% inclusion of algae meals reported results similar to this thesis, with no differences among diets with and without algae meals inclusion. On the other hand, studies evaluating the Flesh Lipid Quality Index in fillet of fish fed diet with an algae inclusion showed no differences compared to those fed diets including only fish oil as fat ingredient (Estévez et al., 2022).

Similar results to those found in this thesis were also obtained in an experimental trial with Atlantic salmon fed diet with an inclusion rate of 4% of algae oil (*Schizochytrium* sp.), which reported no differences in the Thrombogenicity Index (Leyton et al., 2024). In the same study, the Atherogenicity Index was lower in fillets made from fish fed the diet including the algal oil, differing from the findings of this experimental trial (Leyton et al., 2024).

3. Economic evaluation

Given the dependence of most farmed fish on industrial feeds, aquatic feed production is expected to increase in the coming years (Shah et al., 2018) following the rise of aquaculture production (FAO, 2022; Shah et al., 2018). This increase must be sustainable from both ecological and economic perspectives. Therefore, it is essential to replace less sustainable ingredients, such as fish oil, with alternative raw materials that are not only more environmentally sustainable, but also provide comparable profitability in fish production. Microalgae production is considered environmentally sustainable (Shah et al., 2018), but remains associated with high economic costs (Beal et al., 2018). For these reasons, this thesis includes an economic evaluation of the three experimental diets tested.

Based on the growth performances, the costs of feed and the selling price of rainbow trout it was possible to calculate a cost of 1.67 EUR to produce 1 kg of fish fed diet FO, 1.54 EUR to produce 1 kg of fish fed diet AO 0.4% and 1.61 EUR to produce 1 kg of fish fed diet AO 2%. The Economic conversion ratio was 1.95 EUR/kg for diet FO, 1.78 EUR/kg for diet AO 0.4% and 1.86 EUR/kg for diet AO 2%.

Finally, the economic profit index was 5.10 EUR per kg of fish produced for diet FO, 5.33 EUR per kg of fish produced for diet AO 0.4% and 5.27 EUR per kg of fish produced for diet AO 2% (Table 4.4).

Table 4.4. Economic indexes calculated based on the cost of the three experimental diets.

	Diet		
	FO	**AO 0.4%**	**AO 2%**
Cost to produce 1 kg of fish (EUR/kg)	1.67	1.54	1.61
Economic Conversion Ratio (EUR/kg fish)	1.91	1.78	1.86
Economic Profit Index (EUR/kg fish)	5.10	5.33	5.27

Feed production cost at production plant gate: costs of labour, packaging and transport are not included.

Chapter 5
CONCLUSIONS

The expected growth of the aquaculture industry must be sustained by an increase in the production of aquafeed, consequently raising the demand for raw materials such as fish oil. Fish oil primarily depends on wild fish stocks for its production, posing sustainability challenges and instabilities of supply chains due to the susceptibility of wild populations to environmental fluctuations. This has led to a reduction in the use of fish oils in favour of more reliable and sustainable alternatives, particularly vegetable oils. While vegetable oils can support fish growth by providing high energy levels, they lack in certain valuable compounds such as long-chain polyunsaturated fatty acids (LC-PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are crucial for both fish and human health.

Thus, the aim of this thesis was to evaluate the feasibility of incorporating two different rates (0.4% and 2%) of *Schyzochytrium* sp. microalgae oil into aquafeed formulations to reduce the use of fish oil and enhance the levels of EPA and DHA in the diet of fish reared in recirculating aquaculture systems (RAS), and ultimately in their fillets.

At the end of the experimental trial, fish fed the highest inclusion level of microalgae oil exhibited higher final tank biomass, although specific growth rate (SGR) and feed conversion ratio (FCR) showed no significant differences compared to fish fed diet without microalgae inclusion. The fatty acid profile of the fillets showed higher accumulation of both EPA and DHA in fish fed the diet with an inclusion of 2% of microalgae oil.

Given the importance of these fatty acids for human health, several lipid quality indexes were calculated. Notably, the Flesh Lipid Quality index indicated higher lipid quality in the fillets of fish fed the 2% microalgae oil diet compared to other diets, despite these fillets also exhibiting a higher Atherogenicity index.

Overall, the findings from this thesis suggest the potential of microalgae oil to effectively enhance the LC-PUFA content in aquafeeds, offering a sustainable alternative to fish oil. This could mitigate the environmental impact associated with fish oil utilisation and provide a viable economic option for commercial fish farming operations. Thus, the successful integration of microalgae oil into aquaculture diets represents a significant step towards more sustainable and nutritionally balanced aquafeeds.

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