UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

TESIS DOCTORAL

Novel fish feeds: trade-offs between sustainability, performance and robustness

Nuevos piensos para peces: compromiso entre sostenibilidad, crecimiento y robustez

D. Rodrigo Canhoto Pinheiro Mendes

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GLOSSARY OF SCIENTIFIC ABBREVIATIONS & TERMS

AA: Amino Acids.

ABW: Average Body Weight.

ADC: Apparent Digestibility Coefficient: measure to assess the proportion of nutrients or energy from the ingested feed that is efficiently absorbed and utilized by the animal.

Animal Welfare: state of an animal, including its physical and mental state.

ANOVA: Analysis of Variance.

Aquaculture: breeding, rearing and harvesting of aquatic organisms.

Bioactive Compounds: components in feed ingredients with health-promoting properties, such as antioxidants, antimicrobials and anti-inflammatory agents.

Biomarkers: molecular or biochemical indicators used to assess physiological responses in an animal, including immune function, oxidative stress and intestinal integrity.

BW: Body Weight.

Carbon Footprint: the total amount of greenhouse gases, including methane and carbon dioxide, that are generated directly or indirectly by human activities.

***cat*:** Catalase: enzyme that catalyses the decomposition of excessive hydrogen peroxide (H_2O_2), providing protection to the cell from the oxidative stress caused by reactive oxygen species (ROS).

CF: Crude Fat.

Circular Economy: an economic model of production and consumption, aimed to reduce and recycle waste, increase the life time of materials and products, promoting sustainability.

***cldn12*:** Claudin 12: protein involved in the structure and function of tight junctions, maintaining epithelial barrier integrity and selective permeability across various tissues.

CL: Crude Lipid.

CO_2: Carbon Dioxide.

***cox2*:** Cyclooxygenase-2: enzyme induced during inflammation that is involved in the production of prostaglandins.

CP: Crude Protein.

CPI: Crude Phosphorus Intake.

CV: Coefficient of Variation.

***dao*:** D-amino oxidase: enzyme involved in the metabolism of D-amino acids, which generates H_2O_2, a ROS involved in several physiological processes including immune response, cell differentiation and proliferation.

DM: Dry Matter.

DO: Dissolved Oxygen.

EAA: Essential Amino Acids: amino acids (*e.g.*, methionine, lysine, leucine) that cannot be synthesized by the body and must be obtained from the diet.

***ef1-α*:** Elongation Factor 1: protein involved in protein synthesis.

Environmental Footprint: measure to calculate the environmental performance of a product, service or organization based on a life cycle approach.

Environmental Sustainability: the ability to maintain an ecological balance and conserve the planet's natural resources, to support the wellbeing of current and future generations.

EPA: Eicosapentaenoic Acid.

Eutrophication: the enrichment of water bodies with nutrients (*e.g.*, nitrogen and phosphorus), that may result in excessive algae growth, which can lead to detrimental effects on aquatic systems.

Extensive Aquaculture: aquaculture systems that rely on natural environmental conditions with minimal human intervention and often with lower stocking densities.

FCR: Feed Conversion Ratio: measure of an animal's efficiency in converting feed mass into body mass. A reduced FCR indicates more efficient conversion.

FE: Feed Efficiency: measure of how effectively an animal converts feed typically into body weight gain.

Feed Digestibility: represents the difference between the amount of feed ingested and the amount of faeces produced. Used to assess how efficiently nutrients or energy from the feed are absorbed and utilized by aquatic organisms.

Fishmeal: proteinaceous flour feed ingredient made from ground fish or fish by-products.

Fish Oil: feed ingredient rich in fatty acids derived from fish.

Fish Robustness: ability of fish to thrive in adverse environmental conditions and resist stressors, including stressful environments and diseases.

FPL: Faecal Phosphorus Losses.

GE: Gross Energy.

Greenhouse Gas Emissions (GHG): gases (*e.g.*, carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O)) that trap heat in the atmosphere, contributing to climate change.

***gpx*:** Glutathione Peroxidase: antioxidant enzyme that protects cells from oxidative damage by catalysing the reduction of excessive H_2O_2.

Growth Trials: long term experiments to evaluate the effect of experimental feeds on fish performance and robustness.

***gsr*:** Glutathione Reductase: antioxidant enzyme that catalyses the reduction of glutathione disulfide (GSSG) into its reduced state (GSH), protecting cells from oxidative stress.

GWP: Global Warming Potential.

***hsp70*:** Heat Shock Protein 70: molecular chaperone and folding catalyst.

HSI: Hepatosomatic Index: the ratio of liver to animal body weight. Can be used to indicate if animal has received proper nutrition.

IBW: Initial Body Weight.

***igm*:** Immunoglobin: glycoproteins involved in immune response by neutralizing antigens.

***il-1β*:** Interleukin-1β: cytokine mainly involved in immune response and inflammation.

Intensive Aquaculture: aquaculture systems that rely heavily on human intervention, often with high stocking densities, aimed to maximize production.

K: Condition Factor: parameter used to assess the overall health of an animal based on the ratio of their body weight relative to length. Can be used to indicate if animal has received proper nutrition.

LAP: Land Animal By-product: residual materials (*e.g.*, blood and bone meal) derived from slaughtered animals that are often not directed for human consumption.

LCA: Life Cycle Assessment: evaluation tool to calculate the environmental impact of products or services throughout their entire lifecycle.

MPL: Metabolic Phosphorus Losses.

***muc13*:** Mucin13: glycoprotein involved in protecting and lubricating epithelial surfaces, playing a role in cellular signalling and maintaining epithelial integrity.

MUFA: Monounsaturated Fatty Acids.

N: Nitrogen.

***nrf2*:** Nuclear Factor Erythroid 2 – Related Factor 2: transcription factor involved in the regulation of antioxidant response elements.

Nutrient Utilization: the efficiency with which nutrients from feed are converted into biomass by an animal.

Nutrient Waste: waste products from aquaculture, primarily as nitrogen and phosphorus, that result from feed digestion and may contribute to environmental pollution.

***ocl*:** Occludin: transmembrane protein involved in the formation and maintenance of tight junctions between epithelial or endothelial cells.

Organic Aquaculture: a form of aquaculture that adheres to strict organic standards and emphasizes animal welfare and environmental sustainability, addressing consumers concerns.

P: Phosphorus.

PCA: Principal Component Analysis.

***pcna*:** Proliferating Cell Nuclear Antigen: protein involved in DNA replication and repair.

PER: Protein Efficiency Ratio: measure to evaluate synthesized protein per protein consumed in the feed.

PPM: Parts Per Million.

PUFA: Polyunsaturated Fatty Acids.

qPCR: Real-time Polymerase Chain Reaction: molecular biology tool used for a rapid determination and quantification of nucleic acid in various biological samples, with diverse applications such as gene expression analysis.

RAS: Recirculating Aquaculture System: type of intensive aquaculture system, where water is continuously filtered and recycled, to provide a constant and controlled environment for the farming of aquatic organisms.

Resource Utilization: utilization of natural resources (*e.g.*, water, energy).

RGR: Relative Growth Rate: represents the rate of increase in percentage of an animal body weight per day.

RNA: Ribonucleic Acid.

ROS: Reactive Oxygen Species: molecules derived from oxygen and formed by redox reactions or by electronic excitation, that can lead to cellular damage.

Nutrient or Energy Retention: efficiency with which nutrients or energy from feed are retained by an organism.

SCM: Single-Cell Microorganisms: microscopic organisms (*e.g.*, yeast, cyanobacteria, algae) that consist of a single cell.

SD: Standard Deviation.

Self-Selection Experiments: experiments where the animal is allowed to freely choose between different experimental feeds to assess their feeding preferences.

SFA: Saturated Fatty Acids.

***sod*:** Superoxide Dismutase: enzyme that catalyses the dismutation of superoxide radicals (O_2^-) into oxygen and H_2O_2, being involved in antioxidant defence.

SPSS: Statistical Package for the Social Sciences.

Sustainability: The ability to maintain or improve environmental, economic, and social conditions, without jeopardizing the ability of future generations to met their own.

TGC: Thermal Growth Coefficient: measure used to assess how the growth rates of an animal account for its size and variations in environmental temperature.

***tgf-β*:** Transforming Growth Factor β: cytokine involved mainly in growth, cell differentiation, and immune function.

***tjp2*:** Tight Junction Protein 2: protein involved in the assembly and function of tight junctions in epithelial and endothelial cells, contributing to maintain barrier integrity.

***tnf-α*:** Tumour Necrosis Factor α: cytokine involved in inflammation and immune system regulation.

Trophic Levels: Classifies the organisms of a food chain on the basis of their feeding behaviour. Low trophic level species are often primary consumers, while high trophic level species are secondary or tertiary consumers.

VFI: Voluntary Feed Intake.

VSI: Viscerosomatic Index: measured used to assess the relative size of visceral organs (*e.g.*, liver, intestine) compared to the body mass of an organism. Can be used to indicate if animal has received proper nutrition.

WBC: Whole-Body Composition: the proportion of different components (protein, lipids, phosphorus, energy, ash) that make up an organism's body. Used to assess if feed was properly utilized and absorbed.

WG: Weight Gain.

WW: Wet Weight.

***18S*:** 18S Ribosomal RNA: component of the ribosome, involved in decoding mRNA and facilitating the assembly of amino acids into proteins during translation.

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INTRODUCTION

1. Introduction

1.1. Aquaculture- a necessary and expanding industry

1.1.1. *The need for novel animal protein sources*

The world population is rising and society's demand for food is constantly increasing, therefore novel protein sources need to be provided. It has been estimated that in 2050 the human global population will reach approximately 9.7 billion people (United Nations, 2019). In addition, the purchasing power of consumers has increased, meaning that the population has recently been able to shift its diets to incorporate a higher proportion of animal protein (FAO, 2024; Naylor et al., 2021). In the future, to fulfil the nutritional requirements of society, the world food supply and production will need to increase by an average of 60% (Hunter et al., 2017). In the past centuries, agriculture was the main source of protein for human consumption (Edwards et al., 2019). However, in some production areas, this sector has reached its limit of intensification and faces several setbacks, such as deterioration of natural resources, negative impacts on biodiversity, overgrazing, as well as concerns about animal welfare, food safety, quality and security (Hampton et al., 2021). In this sense, seafood is a potential complementary protein source for the global population

1.1.2. *Fish as a source of human nutrition*

Seafood represents a healthy source of food worldwide, which has been gaining interest from consumers. Recently, scientific evidence has been emphasizing that the food each individual chooses to eat greatly impacts their health, thus a healthy lifestyle has been promoted, which is mainly based on a varied and fresh diet (Cena and Calder, 2020). One of the items most advertised and encouraged to be consumed is fish (Faletar et al., 2016). Fish is an easily digestible protein source, with low calories and cholesterol, that contains most essential micronutrients, amino acids, minerals (*e.g.*, vitamins A and D, calcium, iodine, zinc, iron) and fatty acids (*e.g.*, long-chain omega-3) (Basto-Silva et al., 2019; Chen et al., 2022; Tomić et al., 2016). For these reasons, studies have shown that consuming fresh fish at least twice a week can help prevent several diseases (*e.g.*, obesity, cardiovascular, nervous and liver) (Chen et al., 2022). This information has been transmitted to consumers, especially from Western

societies, making them more aware of fish nutritional value (Saidi et al., 2022). Moreover, consumer eating habits have been changing towards seafood as a result of increased production and availability, urbanization, rising incomes and demographic changes (*e.g.*, smaller family size) (FAO, 2024; Quagrainie and Shambach, 2021). Currently, more than three billion individuals around the globe depend on fish for at least 20% of their animal protein intake (FAO, 2024). Accordingly, aquatic animal foods supplies 15% of total animal protein and 6% of total protein consumption (FAO, 2024). The average world aquatic food consumption *per capita* has been increasing at around 3% per year, was roughly 9 kg in 1961, rose to 20.7 kg in 2022 and will likely increase to 21.3 in 2032 (FAO, 2024; Figure 1). In this sense, aquaculture – the farming of aquatic organisms - must rise to contribute to food security and meet worldwide fish demand.

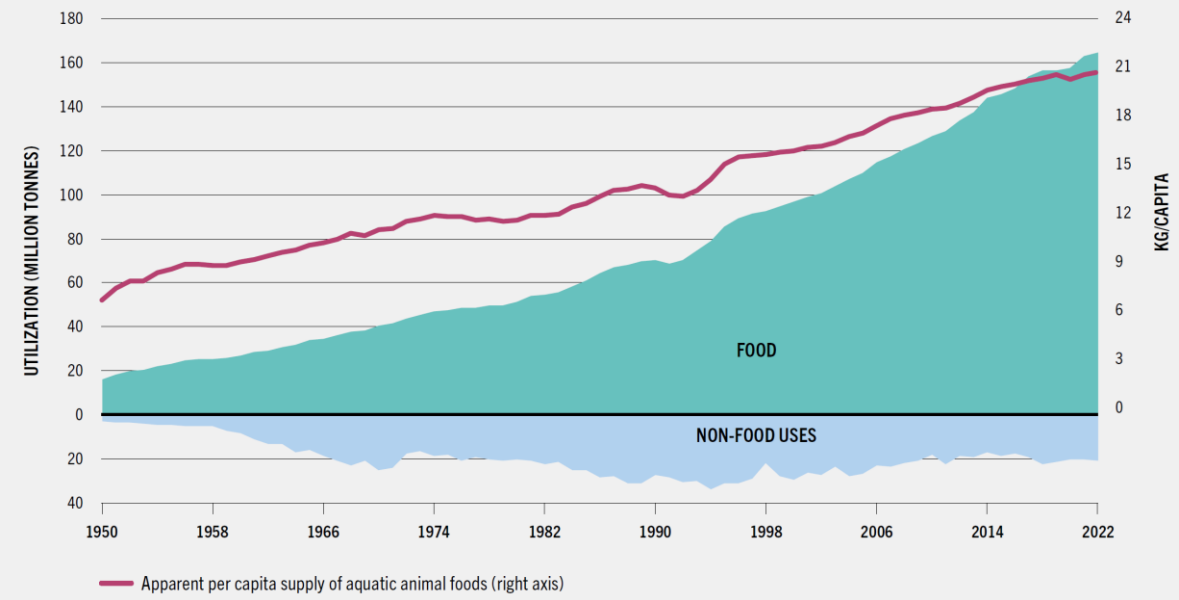

Figure 1 – Utilization of world fisheries and aquaculture production from 1950 to 2022. *Source*: FAO, 2022.

1.1.3. *The importance of aquaculture*

Compared to other food-producing sectors and fisheries, aquaculture is generally an environmentally friendly and sustainable alternative to obtain animal protein. Although not all farmed fish outperform terrestrial animal food products when considering different perspectives of environmental impacts, in general, aquaculture has a lower environmental footprint (*e.g.*, greenhouse

gas emissions (GHG), carbon footprint) and uses some fewer resources (*e.g.*, energy, feed) (Carballeira Braña et al., 2021; Jiang et al., 2022; Naylor et al., 2021). Indeed, when specifically assessing carbon, fish protein derived from aquaculture, produces relatively less carbon dioxide emissions (4–75 kg CO_2 per kg protein) than beef (45–640 kg CO_2 per kg protein) or lamb (51–750 kg CO_2 per kg protein) (Jiang et al., 2022; MacLeod et al., 2020; Parker et al., 2018). Since fish are poikilothermic, have a relatively lighter skeleton and are buoyant, they can save energy, which in turn generally decreases the feed conversion ratio, meaning that less feed is required for the organisms to increase their body weight compared with livestock (Chary et al., 2023; Fry et al., 2018). In the past, wild fisheries captured most of the seafood for human consumption, but this scenario has been changing (Guillen et al., 2019). Global fisheries decreased by 30% from 2000, and are expected to reach ecological limits in 2037 (Froehlich et al., 2018; Jannathulla et al., 2019; Tacon, 2020). In 2021, only around 62.3% of worldwide fish stocks were within biologically sustainable levels (FAO, 2024). Moreover, this activity has become stagnant, having sourced roughly 92.3 million tonnes (Mt) in 2022 and is expected to stabilize at around 94 million Mt in 2032 (FAO, 2024). Consequently, this has resulted in aquaculture production of animal species to surpass for the first time, in 2022, capture fisheries (FAO, 2024; Figure 2). At a global scale, 57% of the total aquatic food available for human consumption is farmed and has been predicted to be 60% in 2032 (FAO, 2024). Thus, aquaculture development and global production have been increasing constantly.

1.1.4. *Aquaculture – global growth, status and production*

Aquaculture has experienced a remarkable development and growth globally, being a high-volume food-producing sector of many species. Although its growth rate has been declining, the industry has been characterised by an annual average growth rate of 5.2%, from 2000 to 2022 (FAO, 2024). Several factors have been responsible for its development. From a socio-economic and demographic perspective, the rising demand and increase in purchasing power from the world population was the main contributor (Sumaila et al., 2022). From a production and supply chain point of view, enhancement in feed nutrition and disease management, improved fish genetics and hatchery

protocols, efficient distribution channels, technological intensification and developments, and labour-saving technology were the main drivers (Ferfolja et al., 2022; Kumar and Engle, 2016). During the 1950s, the annual global production of aquatic animals (without algae) accounted for less than 1 million Mt, while in 2022, that value rose to approximately 94.4 million Mt (USD 296 billion) (FAO, 2024). By far, Asia is the largest producing continent accounting for a global contribution of more than 88%, followed by the Americas (4.6%), Europe (3.7%), Africa (2.5%) and Oceania (0.2%) (FAO, 2024). China (56%) is the uncontested global main producer, followed by India (11%) and Indonesia (6%) (FAO, 2024). Meanwhile, the EU merely contributed 1.2% of the total farming of aquatic animals, with 1.1 million Mt produced, with Spain, France, Greece and Italy being the main producers (FAO, 2024). Globally, 59 million Mt of seafood were produced in inland and 35 million Mt from marine systems (FAO, 2024). Finfish farming represented 65% (62 million Mt) of the total production of aquatic animals (FAO, 2024). From this total, more than 70% was from freshwater (carps, barbel and tilapia – mainly low trophic species) and around 10% of marine species (salmonids, milkfish – high trophic species) (FAO, 2024). At the EU level, molluscs and crustaceans are the main produced organisms (49%), followed by marine (34% with salmonids) and freshwater (10%) finfish species (European Court of Auditors., 2023). The most farmed organisms include mussels, rainbow trout (*Oncorhynchus mykiss*), oysters, gilthead seabream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*), carps and clams (EUMOFA, 2022). However, this global expansion has resulted in some environmental and animal welfare impacts.

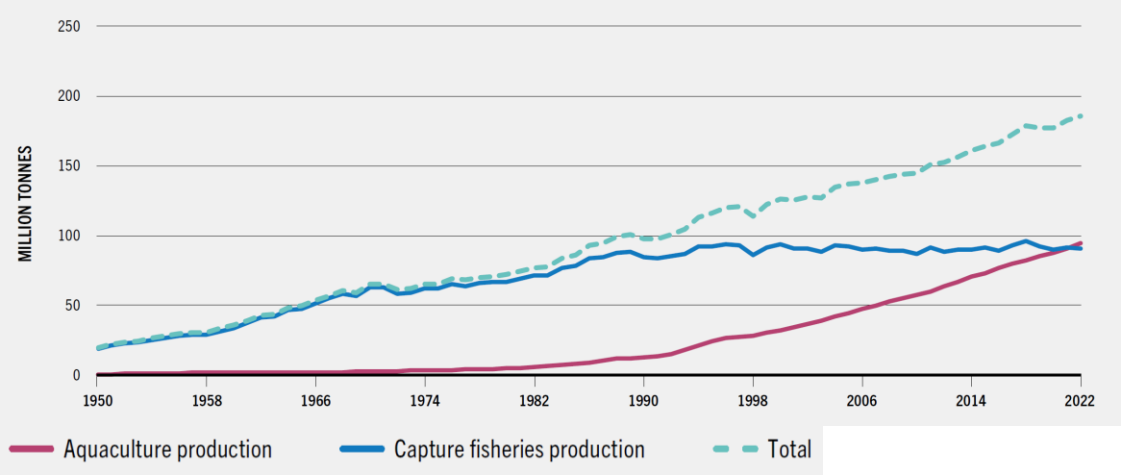

Figure 2 – World capture fisheries and aquaculture production (excluding algae) from 1950 to 2022.

Source: FAO, 2022.

1.2. Environmental sustainability and animal health concerns in aquaculture

1.2.1. *Environmental interactions and implications of aquaculture*

Aquaculture's interaction with multiple terrestrial and aquatic ecosystems can result in a range of ecological implications. The adjacent ecosystems provide resources as inputs (*e.g.*, land, water, energy, feeds) for aquaculture and receive its outputs (*e.g.*, escaped organisms, pathogens, chemotherapeutics, greenhouse gases, organic and inorganic nutrients) (Chary et al., 2023). Consequently, these interactions may lead to various environmental and biological impacts (*e.g.*, introduction of invasive species, alteration of genetic pools, development of antimicrobial resistance, disease outbreaks, habitat and land modification, use of natural resources, depletion of biodiversity, water pollution, eutrophication, climate change), which differ between production sites, especially regarding feeding (fed and unfed), cultivation systems (extensive and intensive), farming technologies (*e.g.*, Recirculating Aquaculture Systems) and farmed species (high, medium or low trophic levels) (Ahmed et al., 2020a; Chary et al., 2023; Xie et al., 2013). A recent report using a FEWC (food-energy-water-carbon) analysis concluded that aquaculture sustainability worldwide has room for improvement, especially in developing countries (main contributors to global production) where there is less resource efficiency and larger environmental impacts (Jiang et al., 2022). Seafood farming

(hatchery and grow-out) and especially feed production have been reported as the stages and processes of aquaculture that mostly contribute to ecological impacts (Bohnes et al., 2019; Newton and Little, 2018). Therefore feeding and nutrition are critical variables that could be optimized to ensure the sustainable development of the industry, however, there are several concerns associated.

1.2.2. *Main concerns related to fish nutrition*

Feed ingredients play a key role in aquaculture. Approximately 70% of aquaculture production of animal species relies on fed organisms, with feed being the main production cost (FAO, 2024; Hua et al., 2019). To meet fish nutritional requirements, particular ingredients, such as marine resources (*e.g.*, fishmeal and fish oil) and mainly plants (*e.g.*, soy), have been commonly used in farmed species' nutrition (Glencross et al., 2024; Little et al., 2016). Although marine ingredients are more prevalent in aquafeeds for high-trophic finfish species (*e.g.*, gilthead seabream), they are also routinely incorporated (inclusion rates of 2–10%) in the diets of low-trophic finfish (*e.g.*, Nile tilapia), which are globally produced at larger volumes (FAO, 2022a; Glencross et al., 2024; Sarker et al., 2020). Marine resources and plants are mainly used due to their availability and particularly regarding fishmeal and oil, due to their nutritional value (balanced amino acid and fatty acid profiles), high palatability, digestibility and absence of antinutritional factors (Colombo, 2020; Jiang et al., 2022; Little et al., 2016; Tacon, 2020). However, these ingredients present questionable environmental concerns (Tacon and Metian, 2015).

Aquafeeds are a major contributor to the carbon footprint and environmental impacts of aquaculture. Feed ingredients can represent on average around 60-70% of the carbon emissions of seafood farming, potentially contributing to climate change (MacLeod et al., 2020). Most studies suggest that the majority of environmental impacts, probably over 90%, are feed-related (Little et al., 2018). The production, processing, and supply chain of feed ingredients are the major contributors to fuel use and greenhouse gases (GHG) emissions (Glencross et al., 2024; Hilborn et al., 2018; MacLeod et al., 2020). Among these parameters, the production (*e.g.*, processing, cooking, drying), blending and transport of feed contributes most to the carbon footprint (60% of GHG emissions) and environmental

impacts (*e.g.*, energy and freshwater use, terrestrial land occupation, water acidification) (Carballeira Braña et al., 2021; MacLeod et al., 2020; McKuin et al., 2022; Vasquez-Mejia et al., 2023). Indeed, these impacts have been accounted, although in different proportions, in marine and plant ingredients.

Although there has been some progress, the sourcing of marine resources for feeds can affect the economic and social strands of aquaculture, and other food producing activities. Marine ingredients are primarily derived from wild stocks of small pelagic fish (*e.g.*, anchovy, herring), which are variable (due to El Niño) and limited, making these ingredients costly and putting pressure on fish stocks (Hilmarsdóttir et al., 2022, 2021; Malcorps et al., 2019). For example, over the past 12 years, European fishmeal prices have risen by an average of 67% and fish oil by 181% (EUMOFA., 2023). Although variations between regions/production sites exist, these ingredients are especially used in developing countries (*e.g.*, West Africa), particularly during the juvenile stages of high trophic level species (Boyd et al., 2020; Hilmarsdóttir et al., 2021; Little et al., 2016; Naylor et al., 2021; Tacon, 2020). This not only increases the pressure on fishery resources, but it impacts food security, nutrition and livelihoods, as catches are turned into fishmeal for export purposes, rather than for human consumption (FAO, 2022a, 2024). Nevertheless, there has been a reduction in the inclusion of fishmeal and fish oil in aquafeeds (more than 50%), which are now seen as strategic ingredients (inclusion levels of less than 10%) and their environmental impacts have been reduced (EUMOFA., 2023; Glencross, 2024 unpublished). This has occurred mainly due to economic reasons, augmented market, social pressure, as well as reduced and/or stabilization of wild fisheries stocks available for fishmeal and oil production (EUMOFA., 2023; Olsen and Hasan, 2012; Tacon and Metian, 2015). In the EU, most of these ingredients are derived from well-regulated wild fish stocks, which are under responsible management practices, certification schemes and regulatory frameworks (EUMOFA., 2023; Hilmarsdóttir et al., 2021; Little et al., 2016). In Europe, consumption of fishmeal has declined around 40% from 2009 to 2022 (EUMOFA., 2023). However, globally in 2020, from the more than 20 million Mt of seafood harvested for non-food purposes from fisheries and aquaculture, 17 million Mt were

used for fishmeal and fish oil (FAO, 2024). Approximately 87% of fishmeal and 74% of fish oil supplies were used to feed aquaculture species, reducing the supply available for other food producing sectors (*e.g.*, pig farming, pet food, poultry) (FAO, 2024; Froehlich et al., 2018). Accordingly, plants have been replacing marine resources in feed formulations, being currently the main ingredients included.

Most aquafeeds contain plant-based ingredients (*e.g.*, soy), however, they also have an environmental impact associated. Although these ingredients may reduce the pressure on fisheries, they may shift resource demand from the oceans onto land (Carballeira Braña et al., 2021; Glencross et al., 2024; Malcorps et al., 2019; Newton and Little, 2018). Plants are already used for direct human consumption and as feed for land animal production (Lathuilliere et al., 2017; Wilfart et al., 2016). Moreover, plant production is associated with a high risk of deforestation, biodiversity loss, land use displacement and non-sustainable resource consumption (*e.g.*, water, and energy) (Lathuilliere et al., 2017; Wilfart et al., 2016; Zortea et al., 2018). Furthermore, the use of fertilisers, pesticides and fossil fuels are also a concern (Malcorps et al., 2019; Wan et al., 2019). Plant production has been reported as contributors to acidification (SO_2 emissions from agricultural production and transportation), eutrophication/water pollution (fertilizer use), as well as land and water use (crop production, fertilizers and processing) (Carballeira Braña et al., 2021; Newton and Little, 2018; Vasquez-Mejia et al., 2023). For example, to produce 1 ton of palm oil is necessary 19.9 m^3 of water while for fish oil 9.0 m^3 are required (Boissy et al., 2011). Moreover, since soy is mainly produced in countries without aquaculture production, the crop needs to be exported, which requires transportation with a high carbon footprint (Malcorps et al., 2019). Imported soybeans for aquafeed are responsible for 75% of aquaculture GHG emissions estimated between 2.9 and 3.8 kg CO^2e kg^{-1} live weight of fish at the farm gate (Ghosh et al., 2020). Therefore, the impacts on the environment and carbon footprint related with feed ingredients must be minimized, but also the health of farmed species must be maintained or improved to cope with farming conditions.

1.2.3. *Increased fish stress and detrimental animal health*

In a way to increase production to meet the growing demand for seafood, aquaculture activities have been intensified, which may affect fish health. In aquaculture, fish are subjected to several stressors, such as handling and transport (Saraiva et al., 2019). A recent paper studied 41 aquaculture species and determined that under current global farming conditions, most fish face stress across the production cycle (Saraiva et al., 2019). A stressful environment can result in a physiological and immunological response from the fish, negatively affecting their wellbeing, as seen in many species such as blunt snout bream (*Megalobrama amblycephala*), Amur sturgeon (*Acipenser schrenckii*), turbot (*Scophthalmus maximus*), thick-lipped grey mullet (*Chelon labrosus*), Nile Tilapia (*Oreochromis niloticus*) and gilthead seabream (*Sparus aurata*) (De las Heras et al., 2015; Ellison et al., 2018; Holhorea et al., 2023; Liu et al., 2016; Ni et al., 2016; Qi et al., 2016). Fish exposed to acute and chronic stress will generate excessive reactive oxygen species (ROS) that will induce oxidative stress and consequently may result in several pathologies, such as cataracts, muscular dystrophy and swim bladder inflammation (Caxico Vieira et al., 2018; Mohanty and Samanta, 2018). Additionally, stress can also induce changes in gut permeability, integrity and barrier function, leading to inflammation and intestinal damage (Lin et al., 2021; Sundh, 2009; Zhang et al., 2020). In stressful situations, fish will need to use energy to cope with the stressors, consequently decreasing growth performance and immune system functions (Dara et al., 2023; Lieke et al., 2020). Accordingly, consumers have been advocating for improved fish health.

1.3. A change towards increasing aquafeeds sustainability

1.3.1. *The crucial role of the consumer*

Seafood consumers' are more knowledgeable about aquaculture, which should not be ignored by the sector. Consumers are becoming more aware about the origin of seafood, food safety, as well as farming conditions, animal welfare, resource utilization, aquafeed production, carbon footprint, climate change and environmental intervention of aquaculture (Feucht and Zander, 2015; López-Mas

et al., 2021; Reig et al., 2019; Zander et al., 2018). Consequently, consumers have been criticising several issues of aquaculture especially the environmental and carbon footprint of aquafeeds and the farming and health conditions of farmed species (Cao et al., 2013; Regueiro et al., 2022). These criticisms create a negative perception and scepticism about aquaculture, which hampers the expansion of the sector (Carrassón et al., 2021; Feucht and Zander, 2017; Regueiro et al., 2022). This is particularly crucial because consumers' purchasing decisions often hinge on whether food production practices align with their preferences and demands (Campbell et al., 2022). Accordingly, although better communication and transparency from the industry to the consumer must occur to better disclose benefits and improve its social acceptance, the sector itself must keep developing while taking more into account its environmental footprint, farming standards and social perception (Ahmed et al., 2020a; Gould et al., 2019; Mente et al., 2011). To align with societal demands, there has been a strong push towards feed concepts within circularity and organic frameworks.

1.3.2. *Circular economy-driven and organic principles*

Circular economy-driven principles have the potential to reduce some of the environmental concerns of aquaculture. These practices create a closed-loop system and can be used to valorise and recycle wastes or side streams, reduce the pressure on natural resources and improve resource and nutrient efficiency/management (Campbell et al., 2022; Chary et al., 2023; Regueiro et al., 2022; Roberts et al., 2015). For example, by-products from agriculture and fisheries could be used as feed ingredients (Newton et al., 2014; Sandström et al., 2022). Using circular economy-driven ingredients in aquafeeds could allow aquaculture and wider food systems to solve waste management issues, minimize the need for raw material inputs and meet the requirements of sustainable ingredients for animal farming (Campanati et al., 2022; Chary et al., 2023; Regueiro et al., 2022; Figure 3). Besides circularity, organic practices may also potentially benefit the environment and the health of farmed organisms.

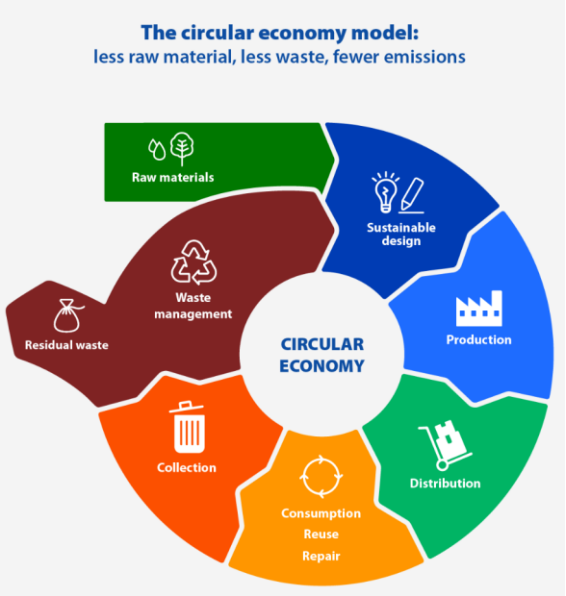

Figure 3 – Infographic explaining the circular economy model. *Source*: European Parliament Research Service.

Organic aquaculture, being derived from organic agriculture and its principles, aims to address the ethical, environmental and food safety concerns of consumers. Organic aquaculture is characterized by a vast range of strict standards, along the entire production chain, most of them based and defined on EU regulations (EC, 2004, 2007, 2008, 2009, 2016; EEC, 1991; EU, 2017, 2018). If the standards are fulfilled, the farming operation guarantees a certificate, such as the EU organic logo (Euro-leaf; Figure 4), which assures the consumer the good governance and benefits of the product (Ahmed et al., 2020a; Bergleiter and Meisch, 2015; EC, 2007; Gould et al., 2019). Consequently, this transparency builds consumer trust and confidence in the quality and integrity of organic aquaculture products (Bergleiter et al., 2009; EC, 2007; Ferfolja et al., 2022; Glebova et al., 2019). Some of the main differences between conventional and organic farming include: lower stocking densities (in most of the species); no use of artificial fertilizers, pesticides, antibiotics and chemotherapeutics; feed must be composed of at least 95% of organic, natural and sustainable ingredients (Busacca and Lembo, 2019; EC, 2007, 2008, 2009; EUMOFA, 2022; Gambelli et al., 2019). All these conditions are expected to solve some of the environmental and farming challenges faced by conventional aquaculture, such as improving water quality, animal welfare and reducing some of the feed environmental impacts (Gould

et al., 2019; Lembo et al., 2018; Prein et al., 2012; Prins et al., 2015). Given the potential benefits of organic and circular economy principles, they have been mainly promoted at the European level.

Figure 4 – European Union organic logo (Euro-leaf). *Source*: EC, 2007.

The EU has prioritized and recognized the need for an environmentally sustainable and animal welfare-friendly aquaculture development, with a specific focus on circularity and organic approaches (Campbell et al., 2022; EC, 2019, 2020a). The European Commission introduced several Strategic Guidelines and actions for a sustainable development of aquaculture and other food-producing sectors, including the European Green Deal (December 2019), Circular Economy Action Plan (March 2020), OrganicTargets4EU (September 2022 - 2026), as well as the Farm to Fork (May 2020), Biodiversity (May 2020) and the Blue Farming strategies (May 2021) (EC, 2019, 2020a, 2020c, 2020b, 2021a; EU, 2022). Several policies and regulations have also been implemented at distinct institutional levels, focusing on environmental, natural resources and biodiversity protection, waste, animal health and welfare, protection of consumers' interest and fair practices in the trade of food and feed (Soininen et al., 2021). In addition, sustainability is becoming a core part of the commercial sector, with many companies setting up specific environmental sustainability programs and reporting mechanisms to demonstrate their commitment and setting targets (IFFO, 2024, unpublished). Therefore, companies have been using a Life Cycle Assessment (LCA) to provide evidence and a comprehensive understanding of the ecological impacts of their novel feeds (Cao et al., 2013; Philis et al., 2019).

1.3.3. *Life Cycle Assessment (LCA)*

A Life Cycle Assessment (LCA) is a well-known complex analytical tool crucial for evaluating and comparing the environmental footprints throughout the aquaculture supply/value chain, making it an indispensable guide for decision-making and policy formulation (ISO, 2006; Philis et al., 2019). The LCA works on the basis of collecting data associated with inputs (resource use)/outputs (emissions), designated material flows and environmental costs from cradle to grave (Avadí et al., 2015; Ellingsen and Aanondsen, 2006; Glencross et al., 2024; Smárason et al., 2017). This method can provide critical information and insights about which processes and areas can be better managed to enhance environmental performance (Philis et al., 2019). It is often the preferred accounting tool to address environmental effects assignable to products and services, due to its holistic approach, preventing the issue of unforeseen consequences in certain situations (Ayer and Tyedmers, 2009). In aquaculture, a LCA most commonly evaluates the indices of global warming potential (GWP), acidification potential (AP), eutrophication potential (EP), as well as land, water and energy use (Bohnes et al., 2019). The GWP is particularly relevant nowadays, given that it has been considered to be the most frightful aspect of climate change, attributed to the emissions of carbon or greenhouse gases (*e.g.*, CO_2), being currently one of the main hot-topics discussed and considered by society (Delistavrou et al., 2023). For all these reasons, LCAs can address if feed formulations, particularly those based on organic or circularity principles, could bring environmental benefits, especially when such feeds incorporate alternative ingredients.

1.4. Alternative feed ingredients: Research on their effects on fish

1.4.1. *Alternative ingredients and their advantages in fish nutrition*

The future of aquaculture hinges on incorporating alternative ingredients in its feeds, to mainly replace marine resources and soy, addressing some of the current aquaculture challenges (Ghamkhar and Hicks, 2020; Verdegem et al., 2023). The search for feasible alternatives has been one of the main focus of research since the beginning of the century, especially since there is a high margin for an

increase in their incorporation levels in aquafeeds (Aragão et al., 2022). Indeed, different regions around the world have adapted to include more alternative ingredients with differing levels of success due to farmed species and available technology (IFFO, 2024, unpublished). Some of the alternative ingredients include non-traditional plant meals (*e.g.*, sunflower, quinoa, rapeseed, lupins), land animal by-products (LAPs; *e.g.*, blood-, feather- and poultry meals), seafood by-products (*e.g.*, fish protein hydrolysates, salmon oil), insect meals (*e.g.*, black soldier fly, *Hermetia illucens* or meal worm*, Tenebrio molitor*) and single-cell microorganisms (SCMs; *e.g.*, bacteria, cyanobacteria, microalgae and yeast) (Figure 5).

The latest data showed that more than 3.1 billion Mt of crops are produced globally per year, from which around 965 million Mt were farmed in the EU, thus being widely available (OECD, 2024). Moreover, the infrastructure and systems for large-scale production and processing of plants are already well-established (Glencross et al., 2024). To improve the use of such ingredients, non-traditional plant species, ideally that are not used in other food/feed producing sectors, could be included in aquafeeds.

LAPs are produced as a direct consequence of terrestrial animal production and the associated meat processing industries (Woodgate et al., 2022). Most LAPs are rendered and originate from lamb, cattle, pig and chicken, representing 17-35% of the edible live animal weight (Marti et al., 2012). In the US and EU alone, more than 40 million tonnes of LAPs are unsuitable for human consumption and are produced yearly, meaning that they are consistently available (Toldrá et al., 2016). These low cost resources make more efficient use of the entire animal carcass and may aid the agricultural and livestock industries in reducing, reutilising and regenerating their waste and nutrients (Sandström et al., 2022; Woodgate et al., 2022). After recent legislations and regulations, following the bovine spongiform encephalopathy outbreak, the use of LAPs in the EU has increased which paved the way for their wider implementation (EC, 2001, 2005, 2013a, 2017a, 2021b).

In 2011, FAO estimated that in fisheries and aquaculture, up to 35% of production (around 60 million Mt) was either lost or wasted (including by-products) every year (FAO, 2011). In the EU, 5.2 thousand Mt/year of seafood by-products (trimmings, offal, by-catch) are generated (Boissy et al., 2011; Lopes et al., 2015; Zhan et al., 2022). Up to 70% of processed fish leftovers (head, fins, scales, skin, bones, blood and viscera) for human consumption or from unintentionally caught species can be converted into by-products for aquafeeds (FAO, 2024; Stevens et al., 2018). In turn, this reduces waste loss, while increasing the value of these products, decreasing economic losses (FAO, 2024; Stevens et al., 2018). Accordingly, the EU has been promoting and highlighting the importance of valuing marine co-products with several initiatives, such as the Landing Obligation, to reach "zero-waste" and "no discards" policies (EC, 2013b; Gasco et al., 2020).

The recent authorization of insect meals in aquafeeds and high consumer acceptance have allowed these ingredients to be potential candidates for aquafeeds (EC, 2017b; Hua et al., 2019; IPIFF, 2018; Liland et al., 2021; Makkar et al., 2014). In addition, although the European production of insects remains low with around 5000 tons produced, it is expected to increase greatly at a growth of 24.4%/year, to at least 1 million Mt produced in 2030 (Gasco et al., 2020; IPIFF, 2018). Insects have shorter production and life cycles, higher fecundity, can be produced intensively and generally require fewer natural resources (*e.g.*, arable land, food and water) than other ingredients (Cadinu et al., 2020). Moreover, they do not enter direct competition for feed with other livestock and can be reared on a variety of low value side streams, such as by-products and organic waste generated from food systems (*e.g.*, industry, livestock, crops), efficiently bio-converting by-products and waste into valuable products (Colombo et al., 2022; Gasco et al., 2020; Hameed et al., 2022; Lu and Serajuddin, 2020; Sandström et al., 2022).

SCMs are a bulk of dried cells mainly produced through fermentation, but also through photosynthesis (Sharif et al., 2021). These ingredients can rapidly grow due to their short doubling time (algae and protists, 2–6 h; bacteria and yeasts, 0.33–2 h) (Adarme-Vega et al., 2012; Bajić et al.,

2022; Matassa et al., 2020; Spalvins et al., 2018). SCMs can be intensively produced year-round and its cultivation systems are amenable to a high degree of automation (Gupta et al., 2022; Sharif et al., 2021). Additionally, SCMs can be produced using waste thermal energy and a wide range of substrates, such as recycled nutrient rich waters, derived from industrial waste streams, gasses and by-products (*e.g.*, municipal waste waters, terrestrial food/feed discards, seafood processing wastes), increasing the utilisation of raw materials and resource efficiency (Campanati et al., 2022; Colombo et al., 2022; Gupta et al., 2022).

Despite there are some differences among the alternative ingredients, generally they have a valuable nutritional profile and may be enriched with functional compounds. Alternative ingredients can be rich in protein, have balanced amino acid profiles and essential vitamins (Ansari et al., 2021; Aragão et al., 2022; Hossain et al., 2024; Little et al., 2016; Turchini et al., 2009). Moreover, some of the ingredients may contain bioactive components (*e.g.*, carotenoids, vitamins, flavonoids, phytosterol and polyphenolic compounds), which possess antimicrobial, antioxidant and/or anti-inflammatory properties (Balakrishnan and Schneider, 2022; Nowak et al., 2016). Nevertheless, the biochemical composition, digestibility and effects of novel feed formulations with alternative ingredients on fish must be thoroughly investigated through growth and self-selection experiments.

Figure 5 – Alternative feed ingredients (quinoa, rendered animal by-products, fish leftovers, black soldier fly, spirulina). *Source*: Unsplash.

1.4.2. *Classical growth and self-selection experiments for the development of novel aquafeeds*

The outcomes of using alternative ingredients in aquafeeds on fish growth, health, utilization and feed digestibility are often addressed using growth trials (Figure 6). Growth rates directly impact aquaculture productivity and profitability. Evaluating the health of fish can be used to identify any adverse effects of the feeds on the immune system or overall well-being (Ciji and Akhtar, 2021; Wang et al., 2023). The intestine and liver have vital physiological functions such as digestion and nutrient absorption, being also the main targets of distinct feed formulations (Aragão et al., 2020, 2022). Thus, it is important to address if the alternative formulations induce consequences on both tissues. Feed utilization and digestibility contribute to fish growth, optimal health and the nutritional quality of the final product (Kokou and Fountoulaki, 2018; Munguti et al., 2020; Teodósio et al., 2020, 2022). The impacts of alternative ingredients on fish performance and robustness have been assessed by many authors (Aragão et al., 2020; Estévez and Vasilaki, 2023; Naya-Català et al., 2021; Pérez-Pascual et al., 2020; Petereit et al., 2022; Tefal et al., 2023a; Vale Pereira et al., 2023). Nevertheless, the screening of potential alternative ingredients could also be tested using less commonly used self-selection experiments.

Figure 6 – Classical growth trial where fish can be fed only one diet.

Self-selection trials could be used to complement growth experiments, particularly to address feed preferences. Fish possess "nutritional wisdom," enabling them to selectively choose and regulate the intake of feeds according to their nutritional needs (Katz, 1937; Raubenheimer and Simpson, 1997; White et al., 2000). Fish dietary choices are governed by a set of mechanisms associated with physiological, behaviour and learning processes (Comesaña et al., 2020; Fortes-Silva et al., 2016; Otero-Rodino et al., 2016; Richter, 1943; Simpson and Raubenheimer, 2001). Since in self-selection experiments fish have at their disposal several feeds (Figure 7), such methodology could be used to allow fish to freely and voluntarily choose their preferred diet while taking into account mainly fish feeding behaviour and learning processes (Fortes-Silva et al., 2016). This methodology can be particularly useful for investigating the acceptance/rejection of ingredients, detecting dietary unbalances (*e.g.*, amino acid deficiencies) and addressing dietary preferences based on the feed intake (Costa et al., 2022; Fortes-Silva et al., 2016).

Figure 7 – Self-feeding trial where fish can "choose" which type of diet to feed on. In this example, three distinct feeds are offered per tank, using three self-feeders.

1.4.3. *Feed intake regulation*

Given its crucial aspect for aquaculture production, feed intake of novel feeds should be addressed, as it is regulated through complex pathways and affected by several feed-related variables. Feed intake can directly influence fish performance, health, nutrient waste and overall farm profitability (Assan et al., 2021; Jobling et al., 2012; Sun et al., 2016). The regulation of feed intake and feeding behaviour is a complex process, driven by two complementary mechanisms: the homeostatic and hedonic pathways (Volkoff, 2019). The homeostatic pathway operates on the basis of physiological needs, with the hypothalamus playing a central role in receiving, integrating, and transmitting internal and external signals related to energy balance and feed intake (Bertucci et al., 2019; Delgado Saavedra et al., 2017). Orexigenic signals (*e.g.*, neuropeptide Y (NPY) and agouti-related peptide (AgRP)) stimulate feeding, while anorexigenic signals (*e.g.*, pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART)) suppress it (Kulczykowska and Sánchez Vázquez, 2010). The hedonic pathway operates based on sensory perception and pleasure, related to the brain's reward

system, and leads to a feeding behaviour based on previously learned experiences, independent of the energy requirements (Rossi and Stuber, 2018). This pathway involves monoamine neurotransmitters (*e.g.*, dopamine, noradrenaline), opioids (*e.g.*, beta-endorphin), and endocannabinoids (*e.g.*, anandamide) (Bojanowska and Ciosek, 2016). Feed intake is influenced by various factors, including nutritional composition and feed palatability (Fortes-Silva et al., 2016; Jobling et al., 2012; Peng et al., 2016). Fish have evolved sophisticated sensing mechanisms to address feed biochemical composition, particularly amino acid availability and detect feeds with inadequate nutritional profiles or with deficiencies in specific amino acids (Calo et al., 2021; Forbes, 2001; Fortes-Silva et al., 2016). This is of high relevancy given the critical role of amino acids in energy metabolism and growth, as an imbalanced amino acid profile can impair protein synthesis, resulting in excessive amino acid catabolism, augmenting nitrogen outputs to the environment (Teodósio et al., 2020, 2022). Feed palatability is particularly relevant to identify if the alternative ingredients manufacturing process or inclusion levels were above a certain threshold tolerable for the fish, which could have affected the orosensory properties (*e.g.*, taste, texture, smell) (Glencross, 2020; Jiang et al., 2022; Lamb, 2001; Morais, 2017; Pérez-Pascual et al., 2020). Therefore, given that each feed formulation is unique and since their effects on fish depend on the rearing conditions, fish developmental stage and species, feeds need to be tested on a case-by-case basis, which is particularly relevant when considering two commercially relevant aquaculture species – freshwater, Nile tilapia – *Oreochromis niloticus* and marine, gilthead seabream – *Sparus aurata*.

1.5. Aquaculture and nutrition of model species

Nile tilapia and gilthead seabream were chosen as model species in this PhD Thesis. Both species make significant contributions to global (tilapia) and European (seabream) aquaculture. Additionally, given their distinct natural environmental conditions (freshwater and marine) and trophic roles (low and high trophic levels), they have different nutritional requirements and physiological characteristics. These differences allow the effects of novel feed formulations to be tested across two diverse scenarios.

1.5.1. *Nile Tilapia (Oreochromis niloticus)*

Nile Tilapia (Figure 8) is one of the most robust and main farmed species globally, with its production being fundamental for many livelihoods. The warm freshwater cichlid is indigenous to eastern, central and western Africa and at least one coastal river in the Middle East (McAndrew and Beveridge, 2000; Philippart and Ruwet, 1982; Trewavas, 1983). Nile tilapia is highly adapted to tropical, subtropical and temperate environments, being tolerant to a wide range of environmental conditions (temperature, salinity, pH, dissolved oxygen) (El-Sayed and Fitzsimmons, 2023). Moreover, tilapia is characterized by several cultivation advantages, such as high growth rates, stress and disease resistance, robustness to handling procedures, early sexual maturation, high prolificacy, short generation time, trophic plasticity and great acceptance of feeds (Assefa, 2015; El-Sayed, 2019a; Pullin and Capili, 1988). For all these reasons, they have been introduced in several other regions of the globe, especially in Southeast Asia and the Americas, mainly for fisheries and aquaculture enhancement (El-Sayed and Fitzsimmons, 2023). Globally, Nile tilapia is the third most farmed freshwater fish species in aquaculture, accounting for roughly 10% of total finfish production corresponding to around 5 million metric tonnes farmed (FAO, 2024). More than 100 countries are producing this species, from which China and Indonesia (Asia), Egypt (Africa) and Brazil (South America) are the main producers (FAO, 2022a). Traditionally, tilapia was farmed in extensive and semi-intensive systems in Egypt since 4000 BC (El-Sayed and Fitzsimmons, 2023). Currently, due to advances in culture systems, nutrition and market demand, intensive farming is often more used using ponds, raceways, tanks, recirculating aquaculture systems (RAS) and cages (El-Sayed, 2006; El-Sayed and Fitzsimmons, 2023). In Africa, it is often the most important food-fish in inland aquaculture production (FAO, 2020). Furthermore, in the developing and least developed countries, tilapia is an affordable protein source and has played a significant role in rural development and poverty alleviation, addressing food security (El-Sayed, 2019a; El-Sayed and Fitzsimmons, 2023). In general, to achieve optimum growth, commercial feeds used for tilapia farming have crude protein (CP) and crude fat (CF) contents that range from 25% to 45% and 5 to 15%, respectively (Abdel-Ghany et al., 2021; Sayed,

2018). In 2017, aquafeeds for the farming of tilapia represented 17.9% of the total aquafeed production, with most feeds having low (<10%) or no inclusion of marine resources (Boyd et al., 2020; Sarker et al., 2020; Teodósio et al., 2020). When screening for alternative ingredients for tilapia farming, one must consider their environmental integration and costs given the important role of tilapia as a food source and rural development in the communities (El-Sayed and Fitzsimmons, 2023). Therefore, the identification of environmentally-friendly and affordable feed ingredients would improve the long-term sustainability of tilapia culture, concerning resource utilisation and lowering feeding costs (Mmanda et al., 2020). Although it does not have the same role globally, gilthead seabream is particularly relevant in Southern Europe.

Figure 8 – Nile tilapia (*Oreochromis niloticus*). *Source*: Kee Sau Suan.

1.5.2. *Gilthead seabream (Sparus aurata)*

Gilthead seabream (Figure 9) farming is well-established and the species is fundamental for the Mediterranean (aqua)culture. The marine subtropical fish inhabits the warm coastal waters of the Black and Mediterranean seas, as well as the North and Eastern Atlantic Ocean (Mhalhel et al., 2023). Its a species with fast growth, high robustness and plasticity, able to resist diet changes and microbial outbreaks (Mhalhel et al., 2023). Additionally, gilthead seabream presents high survival, reproductive success and a reliable supply of juveniles (Manchado et al., 2016). In the Mediterranean, intensive gilthead seabream production began in the early 1980s mainly after improving reproduction techniques (*e.g.*, using light control), disease prevention and knowledge regarding larval rearing conditions and nutritional requirements (Laird, 2001; Moretti et al., 1999). Gilthead seabream farming

sector is currently one of the main Mediterranean aquaculture species, with an annual global production of approximately 280 thousand metric tonnes (FAO, 2022a). The leading six producers worldwide are Turkey, Greece, Egypt, Tunisia, Spain and Italy (EUMOFA, 2023). Currently, the species is part of the gastronomic culture and seafood economy of Mediterranean countries (Pérez-Lloréns et al., 2021). Although extensive and semi-intensive production in coastal lagoons and earthen ponds occurs, most of the production is in intensive systems using sea cages, land-based tanks or RAS (Sanches-Fernandes et al., 2022). Given its feeding habits and physiology, the species require a high inclusion of dietary crude protein and fat in the diets (Wilson, 1991). The CP content in seabream feeds can range from 40% to 55%, while CF between 15% and 25% (Makled et al., 2017). Moreover, its feeds rely on traditional sources, especially on a high inclusion of marine resources, thus it can be challenging to incorporate higher percentages of alternative ingredients, although there is more room for improvement (Aragão et al., 2020; Estruch et al., 2018).

Figure 9 – Gilthead seabream (*Sparus aurata*). *Source*: Unknown.

OBJECTIVES

2. Objectives

This PhD Thesis aims to develop a set of circular economy-driven (eco-efficient) or organic frameworks fish feeds, aligned with societal demands, that include non-traditional plants (*e.g.*, sunflower, quinoa, rapeseed, lupins), land animal by-products (*e.g.*, blood-, feather- and poultry meals), seafood by-products (*e.g.*, fish protein concentrate, salmon oil), insect meals (*e.g.*, black soldier fly, *Hermetia illucens* or meal worm*, Tenebrio molitor*) and single-cell microorganisms (*e.g.*, bacteria, cyanobacteria, microalgae and yeast). Furthermore, the global warming potential, as well as the acceptance and effects (*e.g.*, on feed intake, performance, gut health, immune condition and oxidative status) of these innovative feeds was investigated, using two commercially relevant aquaculture species, Nile tilapia (*Oreochromis niloticus*) and gilthead seabream (*Sparus aurata*). For this purpose, the following specific goals were pursued (Figure 10):

1) Formulate a new generation of organic or eco-efficient fish feeds that align with societal concerns, concomitant with reducing the use of traditional feed ingredients (*e.g.*, marine resources and soy) (**Chapters I, II and III**).

2) Investigate Nile tilapia and gilthead seabream feeding behaviour and feed preferences for the new generation of organic or eco-efficient feeds using the self-selection methodology (demand-feeders) (**Chapter I**).

3) Assess the digestibility of such novel diets and the effects on key performance indicators (*e.g.*, weight gain, feed conversion ratio, feed intake), fish robustness (*e.g.*, gut health, immune condition and oxidative status) and feed utilization (whole-body composition, retention) in juvenile Nile tilapia and gilthead seabream, through long-term experimental studies (**Chapters II and III**).

4) Evaluate the global warming potential (GWP) of the novel feeds through an LCA with economic allocation (**Chapter III**).

This PhD Thesis has been accomplished with the support of an Industrial ITN (EASYTRAIN) with the major supervision of an industrial partner (SPAROS), which ensured the direct application of the

results. The findings of this research will provide a comprehensive evaluation of the acceptance, effects and environmental impacts of socially-acceptable aquafeeds. Overall, this PhD Thesis will shed light on aquaculture fish' nutrition, fish feeding behaviour, physiology, health and feeds carbon footprint.

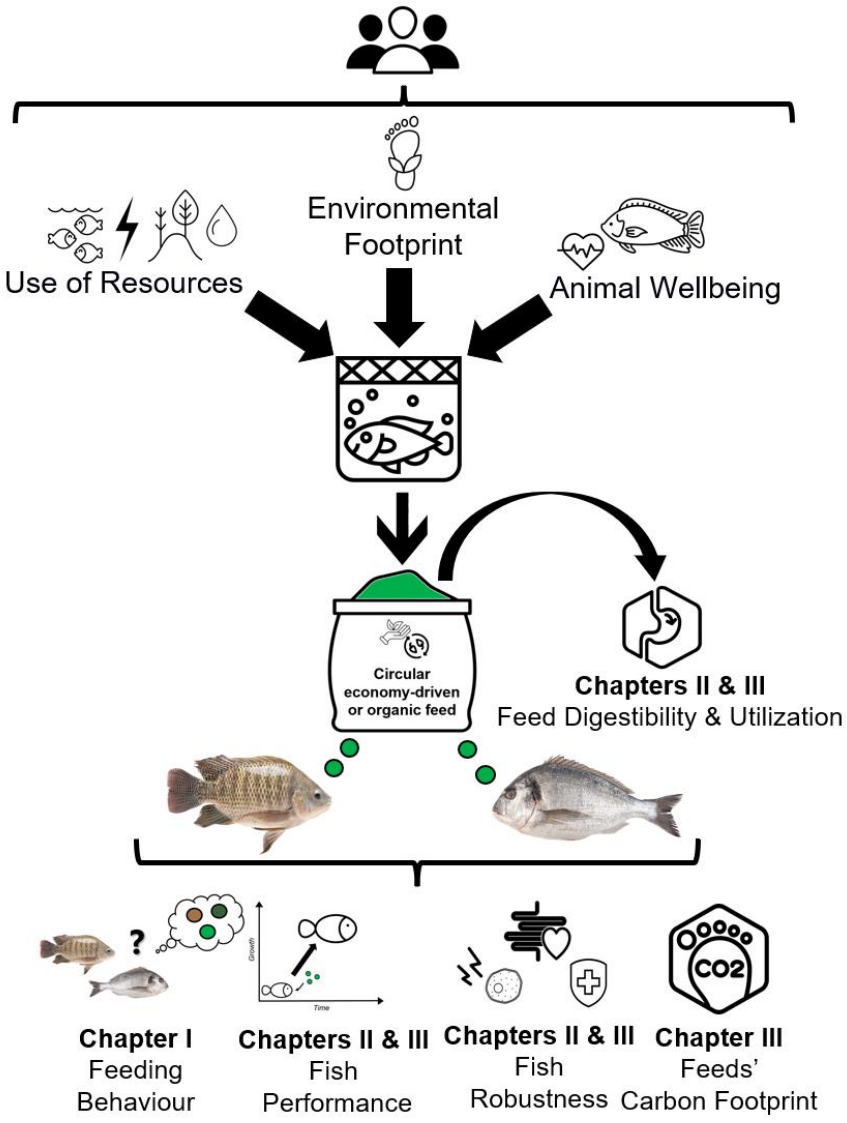

Figure 10 - Schematic overview of the thesis objectives and experimental chapters.

Experimental Chapters

Chapter I

Nile Tilapia and Gilthead Seabream Dietary Self-Selection of Alternative Feeds

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Chapter I

ABSTRACT

Classical assessments of new fish feeds are anthropocentric, focusing mainly on growth. Although this methodology is accurate, it does not consider the fish' perspective. This study aimed to investigate the behavioural responses and feed preferences of Nile tilapia - *Oreochromis niloticus* and gilthead seabream - *Sparus aurata,* through a self-selection trial using self-feeders. Both species were offered three feeds: a control (PD) commercial-like feed and two diets (ORG1 and ORG2) formulated with different inclusions of alternative ingredients to address some of the current environmental concerns and/or ethical issues often associated with commercial formulations. Three groups of tilapia with an average weight of 163.0 g ± 4.3 g (mean ± SD) and four groups of seabreams with 174.7 g ± 27.0 g were tested. Tilapia exhibited a preference for ORG2 (46.5%), influenced by the sensory properties of the feed and post-ingestion signals. Seabream did not show a preference for any feed. These findings highlight the effectiveness of self-selection experiments in allowing fish to express their feeding behaviour and preferences. Therefore, this approach should be considered in the initial screening and design of new aquaculture feeds and ingredients.

Keywords: Animal Behaviour; Fish Physiology; Self-selection; Alternative Feeds; Nile tilapia; Gilthead seabream.

Chapter I

INTRODUCTION

In the wild, since no single feed supplies all essential nutrients, most fish show dietary selection and pick up different items to create a complete and balanced diet according to their physiological needs to survive (Huntingford, 2020). Fish are able to select and regulate the intake of macronutrients and energy through a process known as "nutritional wisdom" (Luz et al., 2018; Raubenheimer and Simpson, 1997; Simpson and Raubenheimer, 2001). In order to restore the metabolic balance as a result of a nutritional challenge, "specific hungers" have the ability to sense and ingest particular nutrients and/or substances in diets (White et al., 2000). Therefore, fish select their feed based on a series of complex regulatory mechanisms, associated with physiological, learning, and behavioural processes, involving hormonal and neural activities in the brain, gastrointestinal tract and liver (Comesaña et al., 2020; Forbes, 2001; Fortes-Silva et al., 2016; Otero-Rodino et al., 2016; Richter, 1943; Simpson and Raubenheimer, 2001). Accordingly, fish feeding behaviour is a relevant characteristic that should be considered when farming aquaculture species.

Aquaculture plays a major role in society by providing to the growing world population a vital source of animal protein, however its development can be hampered by its feeds (FAO, 2022a). Although aquafeeds have been commonly based on marine (*e.g.*, fishmeal and fish oil) and plant-based sources (*e.g.*, soy), these ingredients often encompass questionable environmental implications (*e.g.*, resource consumption, global warming) that concern consumers (Hilmarsdóttir et al., 2022; Little et al., 2016). The inclusion rates of these ingredients have dropped considerably over the past years and varying degrees of success in reducing their environmental impacts have been achieved (Colombo et al., 2022; Glencross et al., 2024; Little et al., 2016). Nevertheless, research has focused on finding more alternative ingredients (*e.g.*, single-cell microorganisms - bacteria, cyanobacteria, microalgae and yeast- and non-traditional plant meals - sunflower, quinoa, rapeseed, lupins) that could address societal demands and potentially reduce some of the negative environmental impacts of the sector (Glencross et al., 2024; Hilmarsdóttir et al., 2022; Newton et al., 2023). Therefore, when developing feeds with alternative ingredients, it is necessary to address their effects on fish.

Chapter I

The classic approach to investigate the effects of new diets occurs through growth experiments mainly based on physiological mechanisms, however solely this method might not totally reveal the full picture (Roy et al., 2020). Although this methodology is accurate and have yielded considerable knowledge on animal production, it has several disadvantages (*e.g.*, time-consuming, expensive) and does not consider fish preferences (Brännäs and Strand, 2015; Filho et al., 2018; Fortes-Silva et al., 2016). Conversely, self-selection methods allow fish to freely and voluntarily accept the given diet, while taking into account fish feeding behaviour and learning processes (Fortes-Silva et al., 2016). Furthermore, this methodology allows fish to choose which feeds better suits their nutritional and energetic needs (Fortes-Silva et al., 2016). Additionally, it can also be used to investigate the detection and acceptance/rejection of feed additives, toxic substances and antinutritional factors (*e.g.*, as phytic acid and phytate) (Costa et al., 2022; Fortes-Silva et al., 2016). Therefore, to provide a more complete perspective on the potential of novel diets, growth experiments could be complemented with self-selection methods.

Self-feeders have proven to be useful to investigate fish feed intake regulation and dietary preferences. This methodology allows fish to evaluate and sense the organoleptic properties (*e.g.*, taste, texture, smell), nutritional and ingredient composition of the feeds (Filho et al., 2018; Fortes-Silva et al., 2016; Raubenheimer et al., 2012). Self-feeders have been used and validated for Nile tilapia (*Oreochromis niloticus*) and gilthead seabream (*Sparus aurata*), but also for several other species, such as goldfish (Carassius auratus), sharpsnout seabream (*Diplodus puntazzo*) and rainbow trout (*Oncorhynchus mykiss*) (Atienza et al., 2004; Fortes-Silva and Sánchez-Vázquez, 2012; Montoya et al., 2012; Pratiwy and Kohbara, 2018; Puchol et al., 2022; Sánchez-Vázquez et al., 1998; Yamamoto et al., 2001). However, knowledge about fish behaviour and its controls have not yet been totally understood and should be further explored, especially when considering the initial screening and design of potential new aquaculture feeds (Fortes-Silva et al., 2012; Pratiwy and Kohbara, 2018; Puchol et al., 2022).

Chapter I

The present work aimed to investigate the acceptability, behavioural response, feed intake regulation and dietary preferences of two commercially important fish species - freshwater (Nile tilapia) and marine (gilthead seabream) – using self-feeders, to test the acceptance of non-conventional diets.

MATERIALS AND METHODS

Formulation and analysis of the diets

Three experimental diets (PD, ORG1 and ORG2; pellet size: 4mm) for each species (Nile tilapia and gilthead seabream) were formulated and produced by SPAROS Lda (Olhão, Portugal). A control diet (PD) was formulated to mimic current commercial feeds. The remaining two diets (ORG1 and ORG2) were formulated to include alternative non-traditional ingredients (*e.g.*, single-cell microorganisms, sunflower, quinoa, rapeseed, lupins) to address some of the current environmental concerns and/or ethical issues often associated with ingredients present in traditional commercial formulations. The ingredient selection (Tables 1 and 2) was chosen based within an organic framework (ingredients that can be found on the market as organic), on market availability and nutritional composition. The inclusion levels were adjusted for each species, according to existing knowledge on tolerance to different ingredients as well as their nutritional and especially amino acid requirements, without compromising fish growth, development, and welfare. Initially, a pilot-scale twin-screw extruder (CLEXTRAL BC45, France) equipped with a screw diameter of 55.5 mm was used to manufacture the feeds. A temperature range of 105–110 °C was used for the extrusion process. All batches of extruded feeds were dried in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom).

Feed samples were grounded and analysed for dry matter (105 °C for 24 h), gross energy in an adiabatic bomb calorimeter (Werke C2000, IKA, Germany), crude protein by the Kjeldahl method (automatic flash combustion; Leco FP-528, Leco, St. Joseph, USA) (N × 6.25%), lipid content by diethyl ether extraction (Soxtherm Multistat/SX PC, Gerhardt, Königswinter, Germany; 150 °C) and ash by heating in a muffle furnace (Nabertherm L9/11/B170, Germany) at 450 °C for 24 h. All diets were

formulated to be isonitrogenous (crude protein of ~ 33.8% and 41.7% as fed, for tilapia and seabream, respectively) and isoenergetic (gross energy of ~ 18.9 kJ/g and 22.2 kJ/ as fed, for tilapia and seabream, respectively) (Tables 1 and 2).

The total amino acid content of the experimental feeds was determined through a series of analytical procedures. Initially, samples underwent hydrolysis in aqueous hydrochloric acid. For cysteine, cystine, and methionine, prior oxidation with hydrogen peroxide and formic acid at cold temperature was performed. Following this, sample pH adjustment, volume adjustment with loading buffer, and filtration were carried out. Amino acid separation occurred using an amino acid analyzer, with detection facilitated through post-column derivatization with ninhydrin reagent and measurement at wavelengths of 440 nm and 570 nm. Tryptophan quantification involved high-performance liquid chromatography (HPLC), with preliminary exposure to alkaline hydrolysis. Separation on AAA occurred via a sodium cation exchange column, with post-column derivatization using O-Phtahalic aldehyde (OPA) and detection through fluorescence at wavelengths of 338/425 nm. Amino acid profiles of the experimental diets given to both species are presented in Tables 3 and 4. Although in ORG1 the diets exhibited lower methionine levels, the amino acid requirement was fulfilled.

Table 1 – Diet formulation (% inclusion levels) and proximate composition (% as fed) of the experimental diets (PD, ORG1 and ORG2) for Nile tilapia (*Oreochromis niloticus*).

Ingredients (% inclusion levels)	PD	ORG1	ORG2
Fishmeal	5.00		
Poultry meal	5.00		
Brewer's yeast	5.00	10.00	10.00
Spirulina		3.50	7.00
Pea protein concentrate		5.50	2.25
Wheat gluten		3.50	7.00
Corn gluten meal	11.60		
Soybean meal	18.00	18.00	
Rapeseed meal	6.50	6.50	13.00
Sunflower meal	3.25	7.50	15.00
Wheat meal	27.75	6.70	9.35
Rice bran full fat	10.00	10.00	10.00
Corn meal		6.95	6.95
Quinoa		5.00	5.00
Faba beans		9.00	7.00
Vitamin and mineral premix	1.00	1.00	1.00
Choline chloride	0.20	0.20	0.20
Antioxidant powder (Verdilox)	0.20	0.20	0.20
Mono-calcium phosphate	1.80	2.15	2.05
L-Lysine	0.30		
DL-Methionine	0.10		
Fish oil	0.90	1.00	1.00
Soybean oil	3.40	3.30	3.00
Proximate Composition (% as fed)	**PD**	**ORG1**	**ORG2**
Dry matter (DM)	92.10	91.74	90.58
Ash	6.27	6.33	6.22
Crude protein	33.50	34.06	33.75
Crude fat	8.43	7.63	6.75
Gross energy (kJ/g^{-1})	19.33	19.24	18.18

All values are reported as mean of duplicate analyses.

Table 2 – Diet formulation (% inclusion levels) and proximate composition (% as fed) of the experimental diets (PD, ORG1 and ORG2) for gilthead seabream (*Sparus aurata*).

Ingredients (% inclusion levels)	PD	ORG1	ORG2
Fishmeal Super Prime	20.00	20.00	20.00
Poultry meal	10.00		
Brewer's yeast		5.00	5.00
Spirulina		3.50	7.00
Pea protein concentrate		16.50	11.50
Wheat gluten			3.00
Corn gluten meal	8.00		
Soybean meal	16.00	16.00	
Rapeseed meal	3.30		3.30
Sunflower meal	6.00		10.00
Wheat meal	5.90	5.55	
Wheat bran		5.00	5.00
Quinoa		5.00	5.00
Faba beans	7.00	7.00	7.00
Whole peas	7.00		7.00
Vitamin and mineral premix	1.00	1.00	1.00
Choline chloride	0.20	0.20	0.20
Antioxidant powder (Verdilox)	0.20	0.20	0.20
Mono-calcium phosphate	0.70	1.05	1.00
L-lysine	0.30		
DL-methionine	0.10		
Fish oil	4.60	4.60	4.60
Soybean oil	9.70	9.40	9.20
Proximate Composition (% as fed)	**PD**	**ORG1**	**ORG2**
Dry matter (DM)	94.22	95.11	91.50
Ash	7.07	6.36	6.33
Crude protein	40.91	43.03	41.01
Crude fat	18.43	16.63	15.22
Gross energy (kJ/g^{-1})	22.20	22.47	22.00

All values are reported as mean of duplicate analyses.

Table 3 – Amino acid profile (g/100g fed basis) of the experimental diets for Nile tilapia (*Oreochromis niloticus*).

Amino Acids (g/100g fed basis)	PD	ORG1	ORG2
Arginine	1.77	2.19	2.01
Histidine	0.74	0.77	0.76
Lysine	1.79	1.77	1.46
Threonine	1.24	1.25	1.27
Tryptophan	0.36	0.41	0.40
Isoleucine	1.32	1.36	1.30
Leucine	3.11	2.42	2.36
Valine	1.55	1.58	1.60
Methionine	0.75	0.52	0.59
Phenylalanine	1.62	1.55	1.53
Cysteine + Cystine	0.52	0.53	0.54
Tyrosine	1.14	1.16	1.12
Aspartic Acid	2.68	2.98	2.54
Glutamic Acid	6.46	6.32	6.95
Alanine	1.95	1.55	1.60
Glycine	1.63	1.48	1.54
Proline	2.22	1.93	2.04
Serine	1.62	1.61	1.60

All values are reported as mean of duplicate analyses.

Table 4 – Amino acid profile (g/100g fed basis) of the experimental diets for gilthead seabream (*Sparus aurata*).

Amino Acids (g/100g fed basis)	PD	ORG1	ORG2
Arginine	2.80	2.75	2.69
Histidine	0.93	1.01	0.94
Lysine	2.69	2.84	2.55
Threonine	1.59	1.61	1.63
Tryptophan	0.47	0.49	0.50
Isoleucine	1.62	1.78	1.68
Leucine	3.36	3.15	2.96
Valine	1.97	2.07	2.08
Methionine	0.96	0.74	0.92
Phenylalanine	1.88	1.88	1.77
Cysteine + Cystine	0.52	0.51	0.49
Tyrosine	1.39	1.42	1.34
Aspartic Acid	3.69	4.31	3.74
Glutamic Acid	6.63	6.66	6.76
Alanine	2.41	2.17	2.15
Glycine	2.36	2.00	2.02
Proline	2.18	1.74	1.82
Serine	1.81	1.88	1.84

All values are reported as mean of duplicate analyses.

Fish and husbandry conditions

Fish were reared and handled by trained scientists and following the Spanish legislation on Animal Welfare and Laboratory Practices, while the experimental protocol was approved by the National Committee of the University of Murcia on Ethics and Animal Welfare under the Guidelines of the European Union Council on the protection of animals used for experimental purposes (Directive 2010/63/EU).

Nile tilapia (*Oreochromis niloticus*) were provided by the University of Murcia from a mono-sex male population (offspring tilapia, GMT®). The experiment was carried out in the chronobiology laboratory at the University of Murcia. At the start of the study, 33 fish were randomly distributed in three homogeneous groups (CV < 4%), with an average initial body weight of 163.0 g ± 4.3 g (mean ± SD), into indoor fiberglass tanks of 300 L in a recirculating aquaculture system (RAS). Each tank contained 11 tilapia and was equipped with a protein skimmer, as well as mechanical, biological, UV filtered and aerated water. Fish were allowed to acclimate to laboratory conditions for at least 2 weeks, during which time they were fed a commercial diet (Skretting TI-3 (3.2mm); with % DM: 32.0% crude protein, 6.0% crude fat and 5.8% crude fibre), which was supplied by hand until visual satiation once a day. Abiotic parameters, feed intake and mortality were measured and recorded daily. A photoperiod of 12h:12h (09h00 to 21h00 lights on) light/dark period was maintained during the study. Average water temperature was 29.0 ± 1.0 °C, pH of 7.2 ± 0.2, dissolved oxygen of 6.9 ± 0.4 ppm and ammonic nitrogen of 0.7 ± 1.0 mg/l. The experiment lasted for 36 days.

Gilthead seabream (*Sparus aurata*) were provided by IMIDA from San Pedro del Pinatar (Spain). The experiment was performed at the Aquaculture Laboratory located in Algameca (Cartagena, Spain). Four groups (CV ~ 15%) of 8 fish with an average initial individual weight of 174.7 g ± 27.0 g were maintained in indoor fiberglass tanks of 150 L in a in a flow-through system. Each tank was equipped with a protein skimmer, as well as mechanical, biological, UV filtered and aerated water. Fish were allowed to acclimate to laboratory conditions for at least 2 weeks, during which time they were fed a commercial diet (Skretting L-4 Alterna 2P; with % DM: 46.5% crude protein, 20.0% crude

fat and 3.4% crude fibre), which was supplied by hand until visual satiation once a day. Abiotic parameters, feed intake and mortality were measured and recorded daily. The animals were kept with a photoperiod of 12h:12h (09h00 to 21h00 lights on) light/dark period at an average water temperature of 27.0 ± 1.0 °, salinity of 37 ± 1.0 ppm, pH of 7.6 ± 1.0 for pH and dissolved oxygen of 6.3 ± 0.5 ppm. The experiment lasted for 67 days.

Experimental setup

The experimental setup of both experiences is present in Figures 1 and 2. The experiments were performed in accordance with Fortes-Silva et al. (2012). Three self-feeders provided by the University of Murcia were equipped in each tank. The position of each diet (PD, ORG1 and ORG2) on the feeders also varied between tanks, to avoid a possible positional effect. The feeding systems were connected with an electric transformer (one for five self-feeders). Each of them was composed of a trigger (a switch with rubber tip), actuated by the fish, placed 2 cm above the water surface, connected to an electromagnet, and a feeder (EHEIM 3581, Deizisau, Germany) that delivered a predetermined amount of feed (1 pellet= 0.04 g) after each trigger actuation and electromagnet activation. To determine the daily intake, every day the feed remaining in the feeder was weighed at the same time (11:30) and subtracted with the total number of grams given the previous day, before refilling the feeder recipient for the next day. After percentages of the offered diets exhibited a statistically significant difference for one feed, diets were switched between feeders to provide a challenge for the fish, reduce the possible preference and influence for a particular string sensor or relative position of the self-feeders. In the case of seabream, on day 50 a fasting period of 10 days started as a challenge test to motivate the fish to choose a diet.

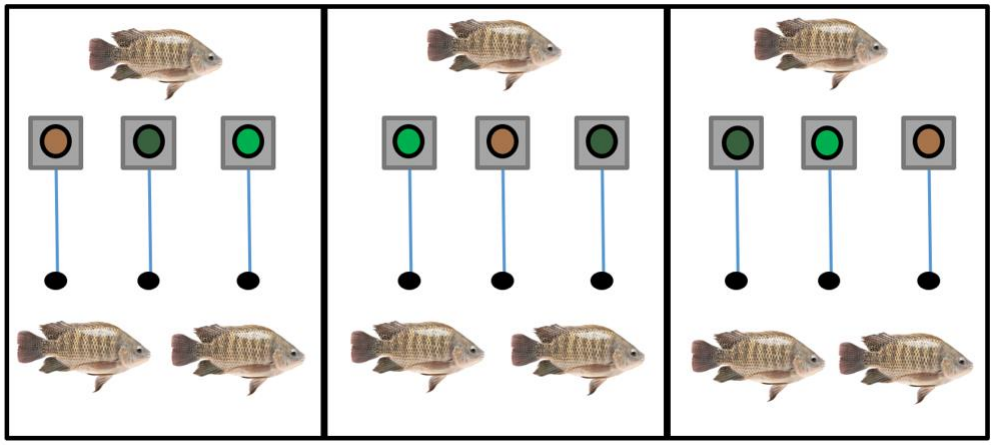

Figure 1 - Experimental setup using Nile tilapia (*Oreochromis niloticus*). Each of the three feeders inside each tank, contained a specific feed (PD, ORG1 and ORG2).

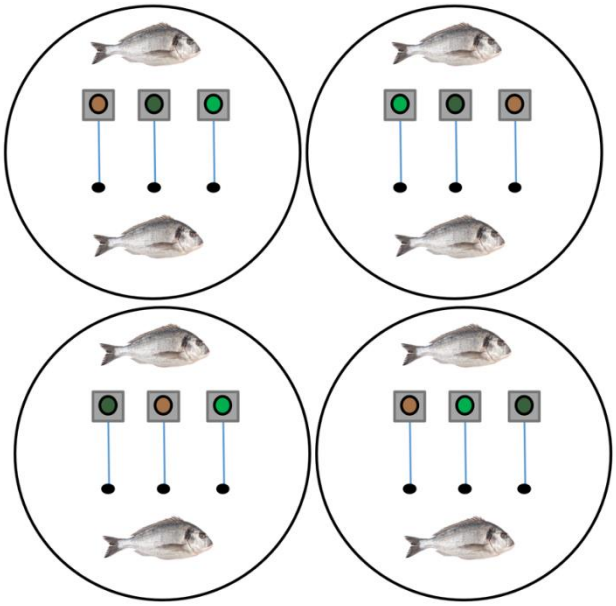

Figure 2 - Experimental setup using gilthead seabream (*Sparus aurata*). Each of the three feeders inside each tank, contained a specific feed (PD, ORG1 and ORG2).

Data analysis and statistics

The statistical analysis was performed with the IBM SPSS software, version 23.0. The experimental unit considered was the tank (n = 3 for tilapia and 4 for gilthead seabream). The relative selection of each diet was expressed as a percentage of the total feed consumed, considering the total diets as 100%. The feed intakes were expressed as total grams of feed ingested/% of body weight. *Arcsine* transformations of feed intake percentages were performed. In the days where a specific diet was significantly selected, the percentages of feed consumed were compared by one-way ANOVA, followed by a Tukey's post-hoc test to examine significant pair-wise comparisons, before meeting criteria for normality and homogeneity using Shapiro – Wilk and Levene's test, respectively. The statistical significance was considered at $P < 0.05$.

RESULTS

Nile tilapia reached a final body weight of 194.7 ± 3.9 g , all fish survived and on average feed consumed daily represented 0.75% of average body weight/day. Uneaten and wasted feed was negligible, only around 2% of the total given feed, thus the amount of feed demanded by the fish was almost entirely ingested. The dietary preference (Fig. 3) in self-feeders initially demonstrated an adaptation period to the feeders of around 5 days. During this time, fish preferred the position of specific feeders, rather than the feed itself, but quickly changed their behaviour. All diets were chosen similarly for several days before an increase in preference for diet ORG2 was observed during three consecutive days (with an average of 46.5%; $p < 0.05$). Throughout the same period diets PD and ORG1 were preferred on average 28.9% and 24.7%, respectively. After diets were switched between feeders on day 22, another period of equal preference remained, while from day 30 until the end of the experiment, diet ORG2 was once again mainly chosen (between 40.7% and 56.0%; $p < 0.05$).

Diets PD (0.24 grams/% BW) and ORG1 (0.21 grams/% BW;) were consumed 38.5% and 46.2%, respectively, less ($p < 0.001$) than diet ORG2 (0.39 grams/% BW;) during days with statistically significant differences (Fig. 4).

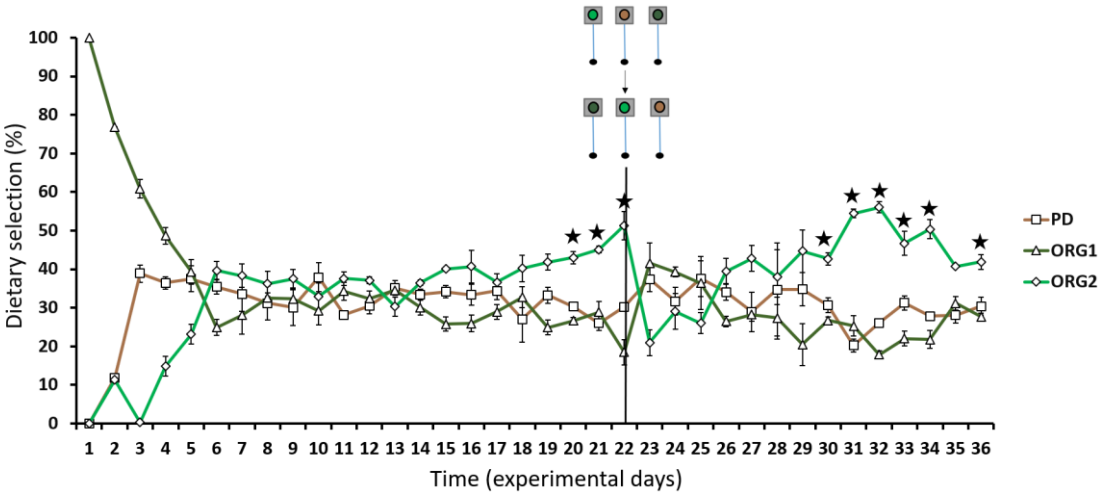

Figure 3 - Evolution of average daily intake (% total grams of feed ingested) of three diets (PD, ORG1 and ORG2) by Nile tilapia over 36 days. Diets were changed between feeders on day 22. Lines represent the mean counts ± SD (*n* = 3 tanks). Stars represent significant differences (one-way ANOVA, $p < 0.05$).

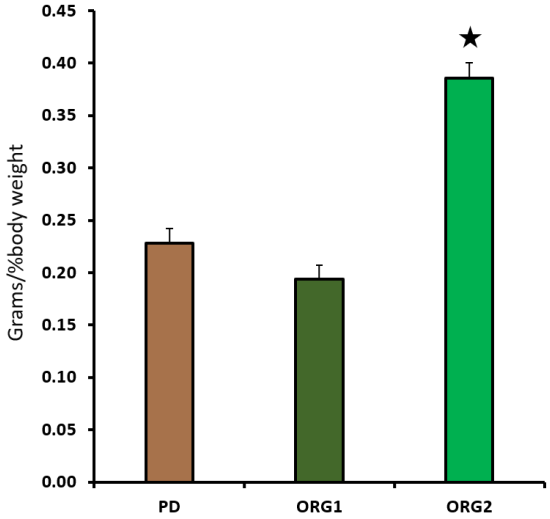

Figure 4 - Average daily intake (total grams of feed ingested per percentage of body weight) of three diets (PD, ORG1 and ORG2) by Nile tilapia only during days with statistically significant differences. Bars represent the mean counts ± SD (*n* = 3 tanks). The star represents significant differences (one-way ANOVA, $p < 0.0001$).

Gilthead seabream final weight was 264.4 ± 29.5 g, no mortality was recorded and average feed consumption was 1.21% of the animal's biomass. Compared to tilapia, seabream wasted slightly more feed (<5%). From the initial days of the study, fish exhibited a clear preference for diet ORG1 (between 65.50% and 83.45%; $p < 0.05$). However, after diets were switched between feeders, the preference for diet ORG1 fell, while for diet PD and ORG2 increased (Fig. 5). For several days, no statistically significance was achieved. Then, fish were fasted for 10 days, starting on day 50, as a challenge test to motivate them to choose a diet, according to Aranda et al. (2001). Nevertheless, even after this approach, a consistent preference was not achieved ($p > 0.05$).

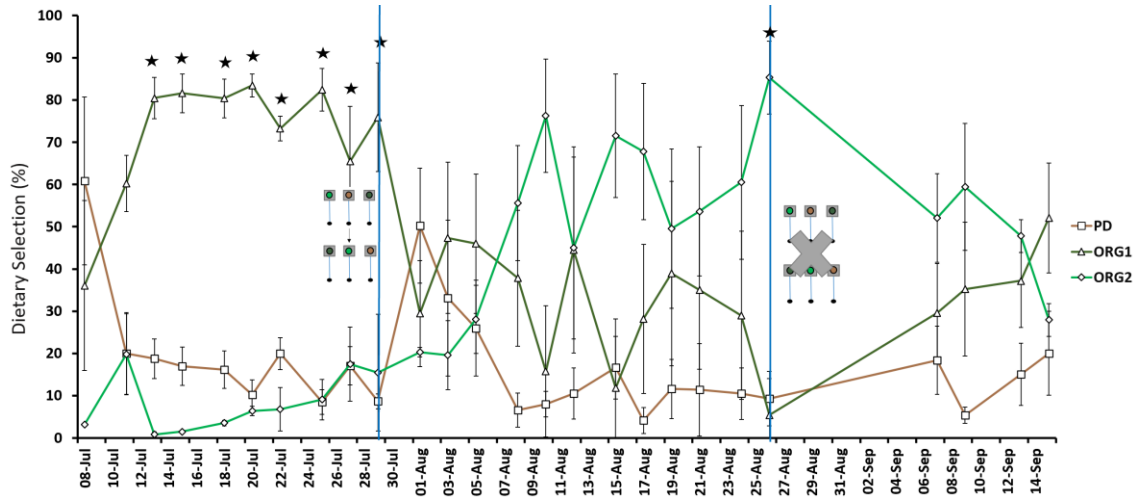

Figure 5 - Evolution of average daily intake (% total grams of feed ingested) of three diets (PD, ORG1 and ORG2) by gilthead seabream over 67 days. Diets were changed between feeders on day 22. Fish were fasted for 10 consecutive days starting on day 50 . Lines represent the mean counts ± SD (n = 4 tanks). Stars represent significant differences (one-way ANOVA, $p < 0.05$).

DISCUSSION

Fish can choose which feed items to ingest mainly based on size, palatability, ingredients and proximal composition (Raubenheimer et al., 2012). Fish are also able to identify and evaluate distinct amino acid profiles between diets (Fortes-Silva et al., 2012). All feeds were formulated to contain the minimum requirements of every essential amino acid (EAA). However, diet ORG1 presented the lowest

levels of methionine. Although methionine was near the lowest requirement, it was enough to fulfil the species physiological state for normal growth (NRC, 2012). Nevertheless, it is possible that this decrease in methionine could have affected fish dietary choice, particularly in tilapia, coupled with diet palatability and ingredient composition, as all feeds had the same size, were isonitrogenous and isoenergetic.

Tilapia consistently preferred ORG2 feed, influenced by learning-reward behaviour, post-ingestion signals and the orosensory properties of the diet. In a population, there may be only one dominant fish that is curious enough to pull the trigger, but if rewarded, this information may be socially transmitted, learned and repeated by all individuals (Millot et al., 2014). On the initial days of the experiment tilapia showed a preference for one feeder, which changed after some time after assessing the content of the other feeders, demonstrating their exploratory and learning behaviour, as shown by Figueiredo et al. (2023). A similar situation was recorded using European seabass (*Dicentrarchus labrax*), which exhibited a preference for one of the self-feeders (Aranda et al., 2000). Nile tilapia chose diet ORG2 with an intake 0.75% of fish weight/day and most pellets were consumed (less than 2% of the total given feed was wasted). Similarly, Fortes-Silva et al. (2012) reported a negligible food waste of 1% with tilapia. Pratiwy et al. (2017) tested the growth performance of Nile tilapia reared under self-feeding systems and showed feed intake values of around 1.85%/body weight. Fish had to evaluate the quality and nutritional composition of the feeds, based on a wide range of physiological processes. Accordingly, the choice for diet ORG2 was presumably based on tilapia nutritional needs (post-ingestive and/or post-absorption) coupled with feed organoleptic characteristics (texture, flavour and odour), which after evaluation, was probably more able to satisfy their physiological state (Brännäs and Strand, 2015; Fortes-Silva et al., 2016). Likewise, other studies with European seabass and tilapia reported a similar behaviour (Fortes-Silva et al., 2016, 2012; Rubio et al., 2006). It is important to note since fish required almost three weeks to exhibit a preference and no feed was predominantly chosen from the beginning of the study, it can reflect the less clear differences between feeds. In a study by Carlberg et al. (2015), Arctic charr (*Salvelinus alpinus*) took 9

days to define a pattern. Fortes et al. (2010) noted that tilapia clearly preferred, since the beginning of the experiment, diets containing phytase and that after switching feeds, the pattern was re-established only after 3 days. In the present study, after feed was switched between feeders, tilapia also resumed and sustained the previous pattern of selection of diet ORG2, while maintaining a constant consumption of other diets, meaning that the fish established levels of consumption for each feeds. However, once again they took some time (9 days), pointing out the effect of the minor differences between the diets. These findings are in accordance with Fortes et al. (2010), who reported that a diet with 1500 IU kg^{-1} phytase was preferred throughout the trial, even after switching feeders. Conversely to tilapia, gilthead seabream was not able to select a feed.

Gilthead seabream did not show a consistent preference for any particular feed, which can be due to several factors. The experiment was performed with rapidly growing juveniles at a high water temperature during summer. Since it was a flow-through aquaculture system, in this scenario, the homeostatic system, which is associated with high energetic demands, may have overridden the hedonic regulation of feeding behaviour, preventing seabream from efficiently discriminating between diets (Puchol et al., 2022). Another possible explanation for the lack of diet discrimination is because all three experimental diets were nutritionally similar to the previously fed commercial feed, meaning that fish were familiar with it and did not notice enough differences (Pulido-Rodriguez et al., 2021). There was a higher variation on the daily intake of feeds compared with tilapia, which could be related with the more curious and aggressive behaviour of seabream towards feed (Puchol et al., 2022). There are few studies available regarding seabream using self-feeders. Nevertheless, it was shown that fish with around 250g could select a diet with distinct oxidation levels of dietary lipids after 10 days with a preference of 82% and 7 days after switching feeders with an average intake of 1.57%/body weight (Montoya et al., 2011). Similarly to what occurred with tilapia, it is possible that on the initial days seabream were preferring a specific feeder on each tank that, by coincident, contained diet ORG1. A specific feeder was also selected on the initial experimental days with European seabass and gilthead seabream by Aranda et al. (2000) and Montoya et al. (2011), respectively. Therefore, it was necessary

to change the positions of the feeders to assert dietary preferences and avoid any preference for a specific position as it was noted by Puchol et al. (2022). Indeed, after changing the position of the feeders, seabream decreased their intake for ORG1 and never achieved a clear preference for any of the given feeds (Montoya et al., 2011). Montoya et. al. (2011) observed two selection patterns after changing the position of the feeders: some fish groups resumed their selection for a specific diet, while the other groups did not show a clear preference for any diet until they were subjected to a 3-week fasting period. In the present study, seabream were also fasted, during 10 days, aiming to define a feeding pattern as the physiological state of fish caused by oxidative stress due to fasting would reinforce their selection behaviour (Montoya et al., 2011). However, seabream were not observed to define a preference, as the compensatory bite activity increase was not enough. Conversely, after a fasting period of 6 and 15 days, European seabass increased the intake for diets richer in protein and energy to recover their metabolic status (Aranda et al., 2001; Vivas et al., 2003). Although there was not a defined pattern, the general performance of the fish was not a concern.

The feed intake and growth rates obtained in our trials were in general lower compared to performance experiments, as it was expected. It should be noted that fish sizes differ among experiments, which in turn directly affects their intake requirements and growth rates. Moreover, the diets were not formulated with the goal of optimizing fish growth but rather to study fish behaviour. Indeed, experiments on dietary selection do not necessarily correlate the most selected diet with optimal performance (Fortes-Silva et al., 2012; Gélineau et al., 1998; Montoya et al., 2011; Santos et al., 2019; Tidwell et al., 1991). Moreover, a lower fish performance can be related with the adaptation of fish to use self-feeders, where some animals in the same group may better assimilate the self-feeding system than others (Ferrari et al., 2014; Tidwell et al., 1991). Even in growth experiments, although a diet is formulated to provide maximum performance, when given the opportunity, fish might not prefer that formula and reduce their intake (de la Higuera, 2001). Nevertheless, the lower performance indicators obtained in our experiments were not a concern, especially as no mortality occurred and fish still gained weight.

CONCLUSIONS

The main purpose of this research was to assess the feeding behaviour and ability of Nile tilapia and gilthead seabream to self-select their preferred diets. In one hand, tilapia was able to show a preference and selected one of the given feeds by sensing its orosensory properties and formulation and as well as based on post-ingestion and absorption signals, confirming their ability to choose a specific feed. On the other hand, gilthead seabream did not show a consistent preference for any diet. Accordingly, self-selection studies based on fish "nutritional wisdom", allow fish to exhibit their behaviour, thus they may be considered in the initial screening of potential new aquaculture feeds, with alternative, ingredients before being used commercially.

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REFERENCES

Aranda, A., Sanchezvazquez, F., Madrid, J., 2001. Effect of short-term fasting on macronutrient self-selection in sea bass. Physiology & Behaviour 73, 105–109. https://doi.org/10.1016/S0031-9384(01)00464-4

Aranda, A., Sánchez-Vázquez, F.J., Zamora, S., Madrid, J.A., 2000. Self-design of fish diets by means of self-feeders: validation of procedures. Journal of Physiology and Biochemistry. 56, 155–166. https://doi.org/10.1007/BF03179782

Atienza, M.T., Chatzifotis, S., Divanach, P., 2004. Macronutrient selection by sharp snout seabream (*Diplodus puntazzo*). Aquaculture 232, 481–491.

Brännäs, E., Strand, Å., 2015. A test of “nutritional wisdom” in perch (*Perca fluviatilis*) by self-selection of encapsulated macronutrients. Applied Animal Behaviour Science 171, 219–225. https://doi.org/10.1016/j.applanim.2015.08.009

Carlberg, H., Cheng, K., Lundh, T., Brännäs, E., 2015. Using self-selection to evaluate the acceptance of a new diet formulation by farmed fish. Applied Animal Behaviour Science 171, 226–232. https://doi.org/10.1016/j.applanim.2015.08.016

Colombo, S.M., Roy, K., Mraz, J., Wan, A.H.L., Davies, S.J., Tibbetts, S.M., Øverland, M., Francis, D.S., Rocker, M.M., Gasco, L., Spencer, E., Metian, M., Trushenski, J.T., Turchini, G.M., 2022. Towards achieving circularity and sustainability in feeds for farmed blue foods. Reviews in Aquaculture raq.12766. https://doi.org/10.1111/raq.12766

Comesaña, S., Conde-Sieira, M., Velasco, C., Soengas, J.L., Morais, S., 2020. Oral and pre-absorptive sensing of amino acids relates to hypothalamic control of food intake in rainbow trout. Journal of experimental biology 223, jeb221721.

Costa, L.S., Guilherme, H.O., Bahiense, R.N., Santos, F.A.C., Gamarano, P.G., Olmeda, J.F.L., Ribeiro, P.A.P., Luz, R.K., 2022. Effect of protein on the post-ingestive response of tilapia fed encapsulated diets. Aquaculture 556, 738289. https://doi.org/10.1016/j.aquaculture.2022.738289

de la Higuera, M., 2001. Effects of Nutritional Factors and Feed Characteristics on Feed Intake, in: Houlihan, D., Boujard, T., Jobling, M. (Eds.), Food Intake in Fish. Blackwell Science Ltd, Oxford, UK, pp. 250–268. https://doi.org/10.1002/9780470999516.ch11

FAO, 2022. The State of World Fisheries and Aquaculture 2022. FAO. https://doi.org/10.4060/cc0461en

Figueiredo, L.G., Santos, F.A.C., Torres, I.F.A., Boaventura, T.P., Luz, R.K., 2023. Behaviour of personality-screened juvenile *Oreochromis niloticus* in different challenges: Bold fish present more exploratory capacity and learning to face imposed challenges. Anais da Academia Brasileira de Ciências. 95, e20210976. https://doi.org/10.1590/0001-3765202220210976

Filho, E.C.T.N., Mattos, B.O., Santos, A.A., Barreto, K.A., Albinati, R.C.B., Oliveira Vidal, L.V., Sánchez-Vázquez, F.J., Fortes-Silva, R., 2018. Geometric approach to evaluate the energy/protein balance in tambaqui (*Colossoma macropomum*): Can fish ensure nutritional intake targets by post ingestion signals? Aquaculture Nutrition 24, 741–747. https://doi.org/10.1111/anu.12602

Forbes, J., 2001. Consequences of feeding for future feeding. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 128, 461–468.

Fortes-Silva, R., Kitagawa, A., Sanchez Vazquez, F.J., 2016. Dietary self-selection in fish: a new approach to studying fish nutrition and feeding behaviour. Reviews in Fish Biology and Fisheries 26. https://doi.org/10.1007/s11160-015-9410-1

Fortes-Silva, R., Rosa, P.V., Zamora, S., Sánchez-Vázquez, F.J., 2012. Dietary self-selection of protein-unbalanced diets supplemented with three essential amino acids in Nile tilapia. Physiology & Behaviour 105, 639–644. https://doi.org/10.1016/j.physbeh.2011.09.023

Fortes-Silva, R., Sánchez-Vázquez, F., 2012. Use of self-feeders to evaluate macronutrient self-selection and energy intake regulation in Nile tilapia. Aquaculture 326, 168–172.

Gélineau, A., Corraze, G., Boujard, T., 1998. Effects of restricted ration, time-restricted access and reward level on voluntary food intake, growth and growth heterogeneity of rainbow trout (*Oncorhynchus mykiss*) fed on demand with self-feeders. Aquaculture 167, 247–258.

Glencross, B., Ling, X., Gatlin, D., Kaushik, S., Øverland, M., Newton, R., Valente, L.M.P., 2024. A SWOT Analysis of the Use of Marine, Grain, Terrestrial-Animal and Novel Protein Ingredients in Aquaculture Feeds. Reviews in Fisheries Science & Aquaculture 1–39. https://doi.org/10.1080/23308249.2024.2315049

Hilmarsdóttir, G.S., Ögmundarson, Ó., Arason, S., Gudjónsdóttir, M., 2022. Identification of environmental hotspots in fishmeal and fish oil production towards the optimization of energy-related processes. Journal of Cleaner Production 343, 130880. https://doi.org/10.1016/j.jclepro.2022.130880

Huntingford, F.A., 2020. Fish Behaviour: Determinants and Implications for Welfare, in: Kristiansen, T.S., Fernö, A., Pavlidis, M.A., van de Vis, H. (Eds.), The Welfare of Fish, Animal Welfare. Springer International Publishing, Cham, pp. 73–110. https://doi.org/10.1007/978-3-030-41675-1_4

Little, D.C., Newton, R.W., Beveridge, M.C.M., 2016. Aquaculture: a rapidly growing and significant source of sustainable food? Status, transitions and potential. Proceedings of the Nutrition Society 75, 274–286. https://doi.org/10.1017/S0029665116000665

Luz, R.K., Boluda-Navarro, D., Sánchez-Vázquez, F.J., Fortes-Silva, R., Portella, M.C., Zamora Navarro, S., Madrid, J.A., 2018. European seabass (*Dicentrarchus labrax*) ability to discriminate between diets made with different dietary fat sources. Aquaculture Nutrition 24, 65–73. https://doi.org/10.1111/anu.12534

Millot, S., Nilsson, J., Fosseidengen, J.E., Bégout, M.-L., Fernö, A., Braithwaite, V.A., Kristiansen, T.S., 2014. Innovative behaviour in fish: Atlantic cod can learn to use an external tag to manipulate a self-feeder. Animal Cognition 17, 779–785.

Montoya, A., Martins, D.A., Yúfera, M., Sánchez-Vázquez, F.J., 2011. Self-selection of diets with different oil oxidation levels in gilthead seabream (*Sparus aurata*). Aquaculture 314, 282–284. https://doi.org/10.1016/j.aquaculture.2011.01.041

Montoya, A., Zamora, S., Sánchez-Vázquez, F.J., 2012. Dietary selection by gilthead sea bream (*Sparus aurata*) provided with unbalanced mixed-macronutrient feeds dispensed from self-feeders. Aquaculture 358–359, 35–40. https://doi.org/10.1016/j.aquaculture.2012.06.008

Newton, R.W., Maiolo, S., Malcorps, W., Little, D.C., 2023. Life Cycle Inventories of marine ingredients. Aquaculture 565, 739096. https://doi.org/10.1016/j.aquaculture.2022.739096

NRC, 2012. National Research Council (NRC): Nutrient requirements of fish and shrimp: The National Academies Press, Washington, D.C., 2011, 376 + XVI pp, £128 (Hardback), ISBN: 978-0-309-16338-5. Aquaculture International 20, 601–602. https://doi.org/10.1007/s10499-011-9480-6

Otero-Rodino, C., Libran-Perez, M., Velasco, C., Alvarez-Otero, R., Lopez-Patino, M.A., Míguez, J.M., Soengas, J.L., 2016. Glucosensing in liver and Brockmann bodies of rainbow trout through glucokinase-independent mechanisms. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 199, 29–42.

Pratiwy, F.M., Kohbara, J., 2018. Dualistic feeding pattern of Nile tilapia (*Oreochromis niloticus,* L.*)* reared under different self-feeding system conditions. Aquaculture Research 49, 969–976. https://doi.org/10.1111/are.13544

Puchol, S., Leal, E., Angotzi, R., Rosel, J., Morais, S., Cerdá-Reverter, J.M., 2022. Dietary discrimination using a dual-choice self-feeding system in seabream (*Sparus aurata*). Aquaculture 559, 738449. https://doi.org/10.1016/j.aquaculture.2022.738449

Pulido-Rodriguez, L.F., Cardinaletti, G., Secci, G., Randazzo, B., Bruni, L., Cerri, R., Olivotto, I., Tibaldi, E., Parisi, G., 2021. Appetite regulation, growth performances and fish quality are modulated by alternative dietary protein ingredients in gilthead sea bream (*Sparus aurata*) culture. Animals 11, 1919.

Raubenheimer, D., Simpson, S., Sánchez-Vázquez, J., Huntingford, F., Kadri, S., Jobling, M., 2012. Nutrition and diet choice. Aquaculture and Behaviour. Wiley-Blackwell 150–82.

Raubenheimer, D., Simpson, S.J., 1997. Integrative models of nutrient balancing: application to insects and vertebrates. Nutrition Research Reviews. 10, 151–179. https://doi.org/10.1079/NRR19970009

Richter, C.P., 1943. Total self-regulatory functions in animals and human beings. Harvey Lecture Series 38, 1942–1943.

Roy, J., Mercier, Y., Tonnet, L., Burel, C., Lanuque, A., Surget, A., Larroquet, L., Corraze, G., Terrier, F., Panserat, S., Skiba, S., 2020. Rainbow trout prefer diets rich in omega-3 long chain polyunsaturated fatty acids DHA and EPA. Physiology & Behaviour 213, 112692. https://doi.org/10.1016/j.physbeh.2019.112692

Rubio, V.C., Sánchez-Vázquez, F.J., Madrid, J.A., 2006. Influence of nutrient preload on encapsulated macronutrient selection in European sea bass. Physiology & Behaviour 89, 662–669. https://doi.org/10.1016/j.physbeh.2006.08.005

Sánchez-Vázquez, F., Yamamoto, T., Akiyama, T., Madrid, J., Tabata, M., 1998. Selection of macronutrients by goldfish operating self-feeders. Physiology & Behaviour 65, 211–218.

Santos, F.A.C., Fortes-Silva, R., Costa, L.S., Luz, R.K., Guilherme, H.O., Gamarano, P.G., Oliveira, C.G., Santos, W.M., Ribeiro, P.A.P., 2019. Regulation of voluntary protein/energy intake based practical diet composition for the carnivorous neotropical catfish *Lophiosilurus alexandri*. Aquaculture 510, 198–205. https://doi.org/10.1016/j.aquaculture.2019.05.038

Simpson, S.J., Raubenheimer, D., 2001. A framework for the study of macronutrient intake in fish: A framework for nutrient regulation. Aquaculture Research 32, 421–432. https://doi.org/10.1046/j.1365-2109.2001.00593.x

Tidwell, J., Webster, C., Knaub, R., 1991. Seasonal production of rainbow trout, *Oncorhynchus mykiss* (Walbaum), in ponds using different feeding practices. Aquaculture Research 22, 335–342.

Vivas, M., Sánchez-Vázquez, F.J., García, B., Madrid, J.A., 2003. Macronutrient self-selection in European sea bass in response to dietary protein or fat restriction: Protein or fat restriction in macronutrient self-selection in sea bass. Aquaculture Research 34, 271–280. https://doi.org/10.1046/j.1365-2109.2003.00799.x

White, B.D., Porter, M.H., Martin, R.J., 2000. Protein selection, food intake, and body composition in response to the amount of dietary protein. Physiology & Behaviour 69, 383–389. https://doi.org/10.1016/S0031-9384(99)00232-2

Yamamoto, T., Shima, T., Furuita, H., Suzuki, N., Sánchez-Vázquez, F.J., Tabata, M., 2001. Self-selection and feed consumption of diets with a complete amino acid composition and a composition deficient in either methionine or lysine by rainbow trout, *Oncorhynchus mykiss* (Walbaum): Self-feeding of amino acid-deficient diets by trout. Aquaculture Research 32, 83–91. https://doi.org/10.1046/j.1355-557x.2001.00007.x

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Socially-acceptable feed formulations may impact the voluntary feed intake and growth, but not robustness of Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Society is becoming more demanding with aquaculture environmental footprint, resource usage and animal wellbeing. In order to potentially mitigate these concerns, feed formulations could be based on eco-efficient (circular economy-driven), or organic ingredients. This study aimed to investigate the performance, feed utilization, and health status of juvenile Nile tilapia (*Oreochromis niloticus*) when fed with such novel feeds. The growth trial lasted for 8 weeks, and fish had an initial weight of 31.0 ± 0.5 g (mean ± SD). Fish were fed until visual satiation, in quadruplicate, with one of three isonitrogenous and isoenergetic experimental feeds: a commercial-like feed without fishmeal (PD), a diet based on ingredients compatible with organic certification (ORG) or a feed formulated using circular economy-driven subproducts and emergent ingredients (ECO). Fish fed ECO showed a tendency for decreased feed intake, while ORG fish significantly reduced their intake compared to those fed PD. Consequently, fish fed ECO exhibited almost half the growth than those fed PD, while ORG fish almost did not increase their weight. ECO and ORG diets had a lower digestibility for protein, lipid and energy when compared to PD. Feed utilization of fish fed ECO or ORG was also lower than those fed PD. From the health-related genes analysed, only glutathione reductase (*gsr*) showed statistically significant differences, being more expressed in fish fed ECO than those fed PD. Thus, even when such novel formulations induced extreme effects on voluntary feed intake, their impact is noted only in fish growth, but not in its robustness.

Keywords: Feed Intake; Palatability; Eco-efficient Feeds; Organic Feeds; Fish Welfare; Nile Tilapia.

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INTRODUCTION

Aquaculture provides a vital source of animal protein to the world population. In 2020, 56% of the total aquatic food available for human consumption production was farmed, and has been predicted to increase to 59% by 2030 (FAO, 2022b). Nile tilapia (*Oreochromis niloticus*) is one of the most cultivated finfish worldwide and an affordable protein source, being highly relevant for addressing food security, especially in developing countries (El-Sayed, 2019b). To ensure that future aquatic food global demand is met, the industry must intensify its production (Cottrell et al., 2020). However, this process can be hampered by ingredient availability and is facing challenges related with its social sustainability.

Environmental sustainability and animal welfare are pivotal concerns in aquaculture to reduce the risk of pathogen outbreaks, improve societal perceptions, and empower consumer choices. Consumers are increasingly aware of issues such as resource depletion and environmental intervention (Feucht and Zander, 2015; López-Mas et al., 2021; Zander et al., 2018). Moreover, there are ethical concerns about animal welfare and health in seafood production, which lead to a strong reduction in the use of antibiotics and other therapeutic drugs in many countries (Ellingsen et al., 2015; Reig et al., 2019; Zander et al., 2018). Society expects organisms to be farmed in a responsible manner, with transparent and sustainable practices, that minimize negative environmental impacts, prioritize aquatic animals well-being, as well as promote sustainable resource use and circularity principles (Bjørhusdal and Haugen, 2023; Ellingsen et al., 2015; Regueiro et al., 2022; Stentiford et al., 2020). Accordingly, optimizing fish nutrition presents one of the potential approaches to improve aquaculture environmental performance and ethical treatment of animals, also enhancing consumer trust and confidence in the industry.

Although nutrition plays a crucial role in ensuring the welfare and health of farmed organisms, fish feeding has always an associated environmental impact. Approximately 70% of farmed aquatic animals must be fed, thus most of aquaculture relies on formulated feeds (FAO, 2022b). Though marine

ingredients are more prevalent in aquafeeds for high-trophic finfish species (*e.g.*, gilthead seabream), they are also routinely incorporated (inclusion rates of 2–10%) in the diets of low-trophic finfish (*e.g.*, Nile tilapia), which are globally produced at larger volumes (FAO, 2022b; Sarker et al., 2020). While common feed ingredients, including marine and plant-based sources, continue to play a significant role in the environmental footprint of the industry, variations exist among regions and production sites (Malcorps et al., 2019; Newton et al., 2023). The degree of success in addressing environmental impacts varies due to the implementation of responsible management practices, certification schemes and regulatory frameworks (*e.g.*, European Union (EU) policies) (EUMOFA, 2023; Hilmarsdóttir et al., 2021; Little et al., 2016). Nevertheless, there is still room for improvement and novel aquafeed formulations must be based on environmentally friendly concepts, while reducing the use of ingredients that present questionable environmental concerns.

To address some of the challenges of aquaculture, there is a growing emphasis on integrating eco-efficient (circular economy) or organic principles to develop socially-acceptable feed formulations. Eco-efficiency strategies prioritize resource conservation, waste management and by-product valorisation (Chary et al., 2023; Do Vale Pereira et al., 2023; Hoerterer et al., 2022; Petereit et al., 2022). Similarly, circular economy principles aim to close resource loops, while organic production addresses ethical, environment and food safety concerns, guided by strict standards, regulations and certification schemes (Ahmed et al., 2020; Regueiro et al., 2022). Accordingly, several countries and the EU have been emphasizing aquaculture environmental sustainability through various initiatives, including the European Green Deal and Farm to Fork strategy (EC, 2020b, 2021a). In addition, these approaches address criticisms from researchers, activists and NGOs, while fulfilling the requirements of knowledgeable consumers (Cao et al., 2013; Regueiro et al., 2022). One possible way to implement this framework, could be through the identification of alternative ingredient formulations that would generate novel fish feeds.

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Alternative ingredients, such as land animal by-products (LAPs; blood-, feather- and poultry meals), insect meals (*e.g.*, black soldier fly, *Hermetia illucens* or meal worm*, Tenebrio molitor*), single-cell microorganisms (*e.g.*, bacteria, cyanobacteria, microalgae and yeast) and non-traditional plant meals (*e.g.*, sunflower, rapeseed, lupins) rise as possible solutions to mitigate some of the current bottlenecks of aquaculture (Aragão et al., 2022; Hoerterer et al., 2022; Petereit et al., 2022; Vale Pereira et al., 2023). These alternative ingredients generally present environmental advantages for the sector, but often need a more thorough evaluation (Gephart et al., 2021; Stevens et al., 2018). They align with principles of circularity, contribute to waste reduction, may require minimal resource consumption, promote resource regeneration and valorise side streams (Little et al., 2016; Newton and Little, 2018). For instance, the cyanobacteria spirulina (*Arthrospira platensis*) can be produced in low-cost open pond technologies using nutrient-rich wastewater or carbon dioxide emissions from nearby industries, aligning with circularity (Mosha, 2019; Viswanaathan et al., 2022). Quinoa (*Chenopodium quinoa*) can be cultivated without the need for synthetic fertilizers or pesticides and using minimal water, reducing the use of resources (Valdivia-Cea et al., 2021). While the nutritional composition of alternative ingredients in aquafeeds can vary significantly, some may offer valuable nutrients such as high crude protein content, balanced amino acid profiles and essential vitamins (Han et al., 2021; Hossain et al., 2024). Moreover, these ingredients may contain bioactive components (*e.g.*, carotenoids, vitamins, flavonoids, phytosterol and polyphenolic compounds), which possess antimicrobial, antioxidant and/or anti-inflammatory properties (Balakrishnan and Schneider, 2022; Nowak et al., 2016). All these characteristics can promote fish health, immune function and weight gain, as demonstrated by Ahmed et al. (2020), Aragão et al. (2020), Tippayadara et al. (2021), Velasquez et al. (2016a) and Zhang et al. (2014). Despite these theoretical benefits and potential, it is important to note that the inclusion of alternative ingredients may not be well accepted by the fish affecting feed intake.

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Feed intake is a crucial aspect of aquaculture production, directly influencing growth performance, fish health and overall farm profitability. Fish have the ability to regulate their feed intake, which is influenced by various factors, including environmental conditions, feed palatability, orosensory properties, and nutritional composition (Fortes-Silva et al., 2016). A nutritionally imbalanced feed will be avoided and can negatively impact fish performance, health or welfare (Assan et al., 2021; Conceição et al., 2012; Fortes-Silva et al., 2016; Teodósio et al., 2020). Antinutritional factors (ANFs; protease inhibitors, tannins, lectins, phytates), commonly present in plants, can interfere with feed intake, but also in nutrient absorption and utilization, leading to reduced growth and impaired immune function (Conceição et al., 2012; Jannathulla et al., 2019; Krogdahl et al., 2010; Magbanua and Ragaza, 2024). Studies have shown that some ingredients and combinations, above a certain threshold, may lead to lower feed intake. For example, inclusions of rapeseed meal of more than 7% in the diets of *Oreochromis niloticus* fingerlings resulted in a decrease in weight gain and feed intake (Sallam et al., 2021). Indeed, reduced feed intake may result in insufficient nutrient intake and impact fish intestine, which is one of the primary targets of dietary changes and has a pivotal role in fish metabolism/digestion (Aragão et al., 2020; Segner et al., 2012). In turn, these consequences can affect fish vital physiological functions, impair growth performance, result in abnormal behaviour, negatively impact welfare, stress response and increase fish susceptibility to diseases (Assan et al., 2021). Accordingly, as aquafeed formulations evolve to address sustainability concerns, understanding if feeds are well accepted by the fish becomes of high relevancy.

The present work aimed to investigate the impact of novel diets, without fishmeal and wild fish oil (replaced with salmon oil as a by-product from salmon processing industry), and formulated within eco-efficient (circular economy-driven), or organic frameworks, on performance, feed utilization and health of juvenile Nile tilapia (*Oreochromis niloticus*).

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MATERIALS AND METHODS

Experimental diets

Three experimental diets: practical (PD), organic (ORG) and eco-efficient (ECO) were formulated and produced by SPAROS Lda (Olhão, Portugal). The formulation concept and ingredient selection (Table 1) was based within an eco-efficient and organic framework (ingredients that can be found on the market as organic), on market availability and nutritional composition. A commercial-like feed, without fishmeal (practical diet, PD) served as control. The organic (ORG) feed was based on ingredients compatible with organic certification (including spirulina and quinoa). The eco-efficient (ECO) was formulated using circular economy-driven subproducts (*e.g.*, poultry and feather meal) and emergent ingredients (*e.g.*, spirulina, insect meal, quinoa). In particular, spirulina had inclusion levels of 10% and 2.5%, quinoa 5% and 2.5%, rapeseed meal 26% and 13%, and brewer's yeast 10% and 5%, in ORG and ECO, respectively. All diets were formulated to be isonitrogenous (crude protein of ~ 39.4% as fed) and isoenergetic (gross energy of ~ 19.2 kJ/g as fed) (Table 1). Amino acid profiles are presented in Table 2. The dietary treatments (PD, ORG and ECO) were randomly assigned to replicate tanks (n = 4 replicates per dietary treatment).

Table 1 – Diet formulation (% inclusion levels) and proximate composition (% as fed) of the experimental diets (PD, ORG and ECO) for Nile tilapia (*Oreochromis niloticus*).

Ingredients (% inclusion levels)	PD	ORG	ECO
Poultry meal[a]	5.00		2.50
Porcine blood meal[b]			5.00
Feathermeal hydrolysate[c]			5.00
Insect meal[d]			7.50
Microbial biomass[e]			5.50
Brewer's yeast[f]		10.00	5.00
***Spirulina*[g]**		10.00	2.50
Soy protein concentrate[h]	5.00		
Pea protein concentrate[i]		5.00	
Corn gluten meal[j]	12.00		
Soybean meal[k]	25.00	12.50	
Rapeseed meal[l]	13.00	26.00	13.00
Sunflower meal[m]	7.50	15.00	15.00
Wheat (whole)[n]	13.90		15.61
Rice bran[o]	9.78	9.78	
Quinoa[p]		5.00	2.50
Whole peas[q]			11.00
Vitamin and mineral premix[r]	1.00	1.00	1.00
Choline chloride[s]	0.20	0.20	0.20
Antioxidant powder[t]	0.20	0.20	0.20
Mono-calcium phosphate[u]	2.55	2.00	2.75
L-Lysine[v]	0.30		0.30
DL-Methionine[w]	0.15		0.22
Yttrium oxide[x]	0.02	0.02	0.02
Salmon oil[y]	2.00	2.00	2.00
Rapeseed oil[z]	2.40	1.30	3.20
Proximate Composition (% as fed)	**PD**	**ORG**	**ECO**
Dry matter (DM)	94.77	93.49	93.93
Ash	7.07	7.32	6.86
Crude protein	38.63	39.65	40.02
Crude fat	8.60	8.58	8.95
Total phosphorus	1.41	1.54	1.48
Gross energy (kJ/g^{-1})	19.24	19.14	19.32

All values are reported as mean of duplicate analyses.

[a] Poultry meal: 62.4% CP, 12.5% CF; SAVINOR UTS, Portugal.

[b] Porcine blood meal: 89.1% CP, 0.4% CF; SONAC BV, The Netherlands.

[c] Feathermeal hydrolysate EM'PAQ: 88.8% CP, 1.6% CF; Empro Europe, The Netherlands.

[d] Insect meal (*Hermetia illucens*), PROTE-IN HP55: 57.8% CP, 8.5% CF.

[e] Microbial biomass (*Corynebacterium glutamicum*), Aminopro NT70: 74.1% CP, 3.1% CF, MAZZOLENI SPA, Italy.

[f] Brewer's yeast: 38.9% CP, 4.5% CF; Premix Lda, Portugal.

[g] *Spirulina* (*Arthrospira platensis*): 72.1% CP, 1.0% CF, Sopropêche, France.

[h] Soy protein concentrate, Soycomil P: 62.2% CP, 0.7% CF; ADM, The Netherlands.

[l] Pea protein concentrate, Lysamine GPS: 78.1% CP, 8.3 % CF, Roquette, France.

[j] Corn gluten meal: 61.2% CP, 5.2 % CF, COPAM, Portugal.

[k] Solvent extracted soybean meal: 43.8% CP, 3.5 % CF, Ribeiro & Sousa Lda., Portugal.

[l] Solvent extracted rapeseed meal: 34.3 %CP, 2.1 % CF, Ribeiro & Sousa Lda., Portugal.

[m] Solvent extracted dehulled sunflower meal, HiPro: 42.9 % CP, 3.8% CF, AGP Slovakia, s.r.o, Slovakia.

[n] Wheat (whole): 11.7 % CP, 1.6% CF, Molisur, Spain.

[o] Rice bran full-fat: 12.6% CP; 15.5% CF, Casa Lanchinha, Portugal.

[p] Quinoa seeds (*Chenopodium quinoa*): 14.0% CP, 5.6% CF, Comfeipas Lda., Portugal

[q] Whole peas: 19.6% CP, 2.2% CF, Ribeiro & Sousa Lda., Portugal.

[r] Vitamin and mineral premix, WISIUM MIX AQUA 1.5%: PREMIX Lda, Portugal. Vitamins (IU or mg/Kg diet): DL-alphatocopherol acetate, 100mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamine, 30 mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1 mg; nicotidin acid, 200 mg; folic acid, 15mg; ascorbic acid, 1000 mg; inositol, 500mg; biotin, 3 mg; calcium panthotenate, 100mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): cobalt

carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate. 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings.

[s] Choline chloride 50%: ORFFA, The Netherlands.

[t] Antioxidant powder, VERDILOX: Kemin Europe NV, Belgium.

[u] Mono-calcium phosphate, ALIPHOS MONOCAL: 22.7% P, 17.5% Ca, ALIPHOS, Belgium.

[v] L-Lysine 99%: Ajinomoto EUROLYSINE S.A.S, France.

[w] DL-Methionine 99%: Rhodimet NP99, ADISSEO, France.

[x] Yttrium oxide, Amperit: Höganäs Germany GmbH, Germany.

[y] Salmon oil: 98.3% CF, 4.6% EPA; 5.2% DHA, Sopropêche, France.

[z] Rapeseed oil: 98.2% CF, JC Coimbra, Portugal.

Table 2 – Amino acid composition (g/100g fed basis) of the experimental diets (PD, ORG and ECO) for Nile tilapia (*Oreochromis niloticus*).

Amino Acids (g/100g fed basis)	PD	ORG	ECO
Arginine	2.24	2.57	2.33
Histidine	0.97	0.95	0.89
Lysine	2.03	2.00	2.12
Threonine	1.43	1.60	2.16
Tryptophan	0.42	0.51	0.46
Isoleucine	1.60	1.62	1.62
Leucine	3.54	2.89	2.93
Valine	1.88	1.98	2.13
Methionine	0.80	0.70	0.87
Phenylalanine	1.94	1.75	1.74
Cysteine + Cystine	0.66	0.65	0.70
Tyrosine	1.37	1.30	1.21
Aspartic Acid	3.36	3.48	3.08
Glutamic Acid	7.38	6.77	5.93
Alanine	2.10	2.02	2.11
Glycine	1.82	1.92	2.12
Proline	2.35	1.92	2.20
Serine	1.86	1.82	1.99
Taurine	0.02	<0.002	0.01

All values are reported as mean of duplicate analyses.

Initially, all powder ingredients were mixed accordingly to target formulation in a double-helix mixer (model 500L, TGC Extrusion, France) and grounded (below 4.00 mm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Diets (pellet size: 4.0 mm) were manufactured with a twin-screw extruder (model BC45, Clextral, France) with a screw diameter of 55.5 mm. Extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling, oils were added by vacuum coating (model PG-10VCLAB, Dinnissen, The Netherlands). Coating conditions were: pressure (700 mbar); spraying time under vacuum (approximately 90 sec) and return to atmospheric pressure (120 sec). Immediately after coating, feeds were packed in sealed plastic buckets and shipped to the research site where they were stored at

room temperature, in a cool and aerated emplacement. Representative samples of each diet were taken for proximate composition and amino acids analyses.

Fish husbandry

Growth trial

The trial was carried out at the University of Trás-os-Montes e Alto Douro (UTAD, Vila Real, Portugal), by trained scientists (following category B FELASA recommendations) according to the European Parliament and European Union Council guidelines on the protection of animals used for scientific purposes (Directive 2010/63/EU, 2010).

Male juvenile Nile tilapia (*Oreochromis niloticus*) were transferred to the experimental facilities by a duly authorized carrier. During the acclimation period fish were fed by hand, *ad libitum*, twice a day, with a commercial diet (Standard 4 Orange, Sorgal, Portugal; 43% CP, 17% CF).

At the start of the study, the animals were randomly distributed into 12 homogeneous groups (CV < 2%), with an initial weight of 31.0 ± 0.5 g (mean ± SD) and condition factor (K) of 1.8 ± 0.2, in indoor fiberglass tanks of 300 L in a recirculating aquaculture system (RAS). Each tank had an initial stocking density of 3.4 kg/m^3 and contained 30 fish. Further, 8 fish from each replicate tank (n = 32 fish per dietary treatment) were anaesthetised (400mg/L of 2-phenoxyethanol; Sigma-Aldrich, Spain) and carefully PIT-tagged under the dorsal muscle to allow identification of individuals for further measurements. All tanks were covered with a net to prevent escapes. The RAS was equipped with a mechanical filter, a submerged biological filter, UV sterilizer and an aeration mechanism for oxygenation. Abiotic parameters, feed intake and mortality were measured and recorded daily, with further removal of dead fish. Average dissolved oxygen in water was 4.2 ± 1.1 mg/L and temperature 24.4 ± 1.3 °C. A 12h:12h (08h00 to 20h00 lights on) light/dark photoperiod was maintained during the study. Experimental diets were supplied daily by hand until apparent visual satiation two times per day (10h00 and 15h00). Distributed feed was quantified throughout the study. The trial lasted for 55 days.

Digestibility trial

For the digestibility trial, five homogeneous groups of 10 fish (49.4 ± 0.5 g) were distributed in five cylinder-conical tanks of 50 L. Fish were fed by hand, twice a day, during the morning (09h00 and 10h00), until apparent satiation. After an adaptation period of three days, faeces collection started. Each day after feeding, tanks were thoroughly cleaned to remove any uneaten feed and fish left undisturbed until afternoon with clean water (24 °C) and aeration. Further, a recipient was placed in the water outlet at the bottom of the tank, which collected faeces through settling decantation. At the end of the day (17h00) the recipient was removed, the faeces collected and stored at -20 °C until analysis. All feeds were formulated to contain 0.02% of yttrium oxide (Y_2O_3) as an inert marker, which allowed to determine the apparent digestibility coefficients (ADC's) of the dietary nutrients by an indirect method, according to the following formula:

Apparent digestibility coefficients (%), ADC's of dietary nutrients and energy (Maynard et al., 1979)**:**

$$\text{ADC (\%)} = 100 \times \left[1 - \frac{\text{dietary marker (\%)}}{\text{faecal marker (\%)}} \times \frac{\text{faecal nutrient or energy content}}{\text{dietary nutrient or energy content}}\right]$$

ADC (%) of dry matter (DM):

$$\text{ADC (\%)} = 100 \times \left[1 - \frac{\text{dietary marker (\%)}}{\text{faecal marker (\%)}}\right]$$

Sample collection

In all samplings, fish were previously fasted for 24 h, before being individually weighed, measured, and euthanised with a lethal dose of anaesthetic (900mg/L of 2-phenoxyethanol; Sigma-Aldrich, Spain). Prior to the beginning of the experiment, 10 fish (30.1 ± 8.9 g) from the initial stock were pooled and frozen at − 20 °C for subsequent whole-body composition analysis. At the end of the study, a pool of 6 fish from each replicate tank (*n* = 4 pools per dietary treatment) was sampled and frozen at − 20 °C for whole body composition analysis. Further, the viscera and liver of 3 PIT-tagged fish per replicate (*n* = 12 fish per dietary treatment) were carefully sampled and weighed for determination of viscerosomatic (VSI) and hepatosomatic (HSI) indexes. From the same fish, anterior intestine was also

carefully dissected and preserved in RNA *later* (Sigma Aldrich, Spain) at -80 °C until further genetic analysis.

Key Performance Indicators

At the start of the experiment, after five weeks and at the end, fish were counted and bulk weighted to determine growth performance, feed utilization and nutrient retention indicators as follows:

Weight gain (%IBW; WG) = 100 × wet weight gain (g) × initial biomass $(g)^{-1}$

Where wet weight gain (g) = final biomass (g) – initial biomass (g)

Relative growth rate (%.day^{-1}; RGR) (Ricker, 1958) = 100 × (e^{g} – 1)

Where *g* = [ln (final body weight (g)) – ln (initial body weight (g)) × number of feeding $days^{-1}$]

Feed conversion ratio (FCR) = apparent feed intake (g) × wet weight gain $(g)^{-1}$

Voluntary feed intake (%ABW^{-1}.day^{-1}; VFI) = relative growth rate × feed conversion $ratio^{-1}$

Protein efficiency ratio (PER) = wet weight gain (g) × crude protein intake (g DM)$^{-1}$

Viscerosomatic index (%; VSI) = 100 × viscera weight (g) × body weight $(g)^{-1}$

Hepatosomatic index (%; HSI) = 100 × liver weight (g) × body weight $(g)^{-1}$

Condition factor (K) = 100 × body weight (g) × total $length^{3}(cm)^{-1}$

Nutrient retention (% digestible intake; NR) = 100 × (final whole-body protein, lipid or energy content – initial whole-body protein, lipid or energy content) × (crude protein, crude lipid or gross energy $intake^{-1}$ × ADC% of protein, lipid, or energy)

Analytical Procedures

Analysis of the diets, whole-fish and faeces were made in duplicates and following the methodology described by AOAC (Association of Official Analytical Chemists, 2007). All samples were

freeze-dried and ground until a homogeneous powder was obtained. Dry matter after drying at 105 °C for 24 h; total ash by combustion (550 °C during 12 h) in a muffle furnace (Nabertherm L9/11/B170, Germany); crude protein (N×6.25) by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection with a Leco N Analyzer (Model FP-528, Leco Corporation, USA); crude lipid by petroleum ether extraction (40-60 °C) using a Soxtec™ 2055 Fat Extraction System (Foss, Denmark), with prior acid hydrolysis with 8.3 M HCl; gross energy in an adiabatic bomb calorimeter (Werke C2000, IKA, Germany); total phosphorus according to ISO 27085:2009 by ICP-AES methodology; phosphorus in the feeds was determined by a colorimetric method involving a wet ashing step followed by phosphorous measurement with 1-amino-2-naphthol-4-sulfonic acid-molybdate in a microplate reader at 660 nm (Brooks et al., 2001); yttrium concentration in feed and faeces was determined by atomic absorption spectrometry (SpectrAA 220 FS, Varian) (Reis et al., 2008).

To determine the total amino acid content of the experimental feeds, samples were initially hydrolysed in aqueous hydrochloric acid. For cysteine and cystine, and methionine, samples were previously oxidized with hydrogen peroxide and formic acid at cold temperature. Subsequently, the sample pH was adjusted, brought to volume with loading buffer and filtered. Amino acids were separated in an amino acid analyzer and the detection was carried out using post column derivatisation with ninhydrin reagent and 440 and 570 nm. Tryptophan was quantified using high-performance liquid chromatography (HPLC), before being exposed to alkaline hydrolysis. Extraction of free taurine was performed with metaphosphoric acid and protein precipitation with centrifugation. Separation occurred on AAA by sodium cation exchange column, post column derivatisation with O-Phtahalic aldehyde (OPA) and detection by fluorescence at 338/425 nm.

Reverse transcription–quantitative real-time PCR (qPCR)

Samples from the anterior intestine from two fish per replicate (*n* = 7-8 per dietary treatment) were analysed. To extract total RNA, samples were initially thawed and homogenised using a

TissueLyser II (Star-Beater, VWR, USA) with 1 ml of Tri Reagent (Sigma-Aldrich, Spain), according to manufacturer's instructions.. Total RNA quality and integrity was determined by denaturing agarose gel electrophoresis, while concentration and purity were based on absorbance at 260 nm and ratios at 260:280 and 260:230 nm, using a Nanodrop OneC (Thermo Fisher Scientific, USA). Complementary DNA (cDNA) synthesis was performed by reverse transcription of 1000 ng of total RNA using the RevertAid H Minus First Strand Kit (Thermo Fisher Scientific), according to the manufacturer's protocol. Real-time PCR (RT-PCR) was performed in a CFX384 Real Time PCR detection system (Bio-Rad, Hercules, USA) with PowerTrack™ SYBR™ Green chemistry (Thermo Fisher Scientific), using specific primers (Table 3). Primers for each gene were designed using the Geneious Prime version 2023.1 (https://www.geneious.com) based on sequences from the GenBank database (NCBI; Clark et al., 2016). PCR efficiency was determined using five-point standards curves of 3-fold dilution series (1:3 to 1:243) of pooled cDNA. For the intestinal epithelial integrity, the expression levels of several genes were analysed: D-amino oxidase (*dao*), occludin (*ocl*) and tight junction protein 2 (*tjp2*). The biomarkers for oxidative status/stress were catalase (*cat*), glutathione peroxidase (*gpx*), glutathione reductase (*gsr*), nuclear factor erythroid 2 – related factor 2 (*nrf2*) and heat shock protein 70 (*hsp70*). Genes analysed for immune condition were tumour necrosis factor (*tnf-α*), interleukin-1β (*il-1β*) and transforming growth factor β (*tgf-β*). The RT-PCR assays were run in duplicates in a 10 µl volume containing 2 µl of cDNA, 0.625 µl of each specific forward and reverse primers at 10 µM, 5 µl of PowerTrack™ SYBR™ Green Master Mix (Thermo Fisher Scientific) and 1.75 µl of nuclease-free water. The amplification protocol was set as follows: an initial denaturation step of 2 min at 95 °C, followed by 40 cycles of denaturation for 5 sec at 95 °C and 30 sec at 58 °C for annealing/extension. Negative controls without sample templates were consistently executed for each primer set. The specificity of reactions was confirmed through the examination of melting curves, using ramping rates of 0.5 °C/5 sec, across a temperature span of 60-95 °C. Gene expression levels were normalised using a reference

housekeeping gene, the elongation factor 1 *α* (*ef1-α*). The relative mRNA expression of the target genes was calculated according to the Pfaffl method (Pfaffl, 2004).

Table 3 - Sequences of primers used in qPCR.

Gene	Forward Primer Sequence (5′ → 3′)	Reverse Primer Sequence (5′ → 3′)	NCBI GenBank Accession Number
dao	CAACCTTTGCAGTGAACCCG	TCACTCCCCTCTTTCGCAAC	XM_005473333
ocl	TCAGATGAGCAGCGCAGAAA	TTCCAGTGCGTCCAACTCTC	XM_005476075
tjp2	GCTACATGGACTCCGGCTAC	GCGATCTGGGCTGTACTCTC	XM_025908597
cat	TCCATTCCCAGAAGCGCAAT	ATTCATGTGACGGTGGCCAT	XM_019361816
gpx	ACTTCCATTCCCCTGCGATG	GCTTGTAAGGTTCCCCGTCA	NM_001279711
gsr	CAGCAGGAAGAGTCAGTGCA	ACCCATCTTGATGGCCACAG	XM_013271309
nrf2	TCTCAGCCCGATGACAGAGA	GTGCTGACCACTGCTCTCTT	XM_003447296
hsp70	CCAAAAGGTGTCCAACGCTG	CCCCACCCAGGTCAAAGATC	NM_001279671
tnf-α	ATGGCAGAAGGATGTGGACC	GACCATGGGATGCGAAGACA	XM_013266976
il-1β	CATGTCTTGCCGCATGGAAG	GTTCAACGGGCTGGTTTTCC	XM_005457887
tgf-β	CACGCTGAAGGACAAATGGC	TCACAGTACCGCCGAAGTTC	NM_001311325
ef1-α	TTGAGAAGGAAGCCGCTGAG	GCTGGTCTCGAACTTCCACA	AB075952

Abbreviations: *dao:* D-amino oxidase; *ocl*: occludin; *tjp2:* tight junction protein 2; *cat*: catalase; *gpx*: glutathione peroxidase; *gsr:* glutathione reductase; *nrf2*: nuclear factor erythroid 2 – related factor 2; *hsp70:* heat shock protein 70; *tnf-α*: tumour necrosis factor; *il-1β*: interleukin-1β; *tgf-β*: transforming growth factor β; *ef1-α:* elongation factor 1 *α.*

Data analysis and statistics

All statistical analyses were performed using the computer package IBM SPSS version 26.0. Results are expressed as mean ± standard deviation (mean ± SD). When needed, data were previously transformed using *arcsine* (Ennos, 2007) or added an arbitrary value to ensure values were positive (retention data), and after tested for normality and homogeneity using Shapiro – Wilk and Levene's test, respectively. Thereafter, data was analysed by one-way ANOVA followed by Tukey post hoc test or by non-parametric Kruskal-Wallis followed by Dunn's post hoc test (if ANOVA assumptions were not

met), to identify differences among the experimental dietary treatments. The level of significance used was $P < 0.05$ for all statistical tests**.**

RESULTS

Apparent digestibility coefficients of diets

Apparent digestibility coefficients (ADCs) of dry matter, nutrients and energy of experimental diets are presented in Table 4. There were significant differences ($p < 0.05$) regarding all analysed parameters, except phosphorus ($p = 0.247$), with diet PD presenting higher ADCs particularly for protein ($p = 0.018$), lipids ($p = 0.005$) and energy ($p = 0.043$). Conversely, diet ECO exhibited the lowest values for dry matter ($p = 0.040$), protein and energy.

Table 4 – Apparent digestibility coefficients (ADCs; %) of nutrients and energy of experimental diets (PD, ORG and ECO) given to Nile tilapia (*Oreochromis niloticus*) for 55 days

	Diets			
	PD	**ORG**	**ECO**	***p* value**
Dry matter (DM; %)	66.0 ± 0.8^{a}	64.0 ± 2.3^{ab}	59.0 ± 4.7^{b}	0.040
Protein (%)	85.3 ± 0.6^{a}	81.0 ± 0.4^{ab}	75.4 ± 4.5^{b}	0.018
Lipids (%)	95.6 ± 0.2^{a}	92.8 ± 0.8^{b}	93.8 ± 1.2^{b}	0.005
Phosphorus (%)	67.8 ± 2.0	68.8 ± 2.2	70.8 ± 2.6	0.247
Energy (%)	74.8 ± 0.9^{a}	73.4 ± 1.5^{ab}	67.1 ± 4.0^{b}	0.043

Data are presented as mean ± standard deviation ($n = 4$ for diet PD and $n = 3$ for diets ORG and ECO). Different superscripts within the same row indicate significant differences (one-way ANOVA; $p < 0.05$) between dietary treatments.

Growth performance, feed utilization and somatic indices

The performance indicators of fish fed the experimental diets are presented in Table 5. Fish did not respond in the same way to the diets offered. Although fish were fed until satiation, the voluntary feed intake (VFI) was lower and feed conversion ratio (FCR) was higher in fish fed ORG compared with those fed PD ($p = 0.010$ for VFI and $p = 0.012$ for FCR). Diet PD was well accepted, but diet ECO and especially ORG received a negative response from the fish, which directly affected their performance. Accordingly, final body weight (FBW) and relative growth rate (RGR) were significantly

affected by the different experimental diets (p = 0.007 for FBW and p < 0.001 for RGR). Fish that were fed diet PD exhibited the highest weight gain (3.5 - fold increase), those fed with ECO roughly doubled their initial body weight, while fish fed ORG did not show a noticeable growth. Similarly, the final body weight of fish fed PD was 1.7-fold and 3.3-fold higher than ECO and ORG fish (p = 0.007), respectively. Moreover, the RGR was much higher in fish fed diet PD than the other two dietary treatments (p < 0.001). The protein efficiency ratio (PER) also presented significant differences (p < 0.001), being higher in PD fish, followed by ECO and ORG. The viscerosomatic (VSI) and hepatosomatic indices (HSI) were similar in all dietary treatments (p = 0.273 and p = 0.092, respectively), while the condition factor (K) differed (p < 0.02), being lower in ORG fish compared to those fed PD. During the study, average survival was high (~99%) and unaffected by the dietary treatments (p = 0.368).

Table 5 - Growth performance, feed utilization and somatic indices of Nile tilapia (*Oreochromis niloticus*) fed with three different experimental diets (PD, ORG and ECO) for 55 days.

	Diets			
	PD	**ORG**	**ECO**	***p* value**
FBW (g)	107.8 ± 6.1^{a}	32.7 ± 1.3^{b}	62.7 ± 5.4^{ab}	0.007
RGR (%.day^{-1})	2.3 ± 0.1^{a}	0.1 ± 0.0^{c}	1.3 ± 0.2^{b}	<0.001
VFI (%ABW.day^{-1})	2.1 ± 0.2^{a}	0.02 ± 0.01^{b}	0.9 ± 0.3^{ab}	0.010
FCR	1.1 ± 0.1^{b}	7.1 ± 2.3^{a}	1.5 ± 0.4^{ab}	0.012
PER	2.3 ± 0.2^{a}	0.3 ± 0.3^{c}	1.7 ± 0.4^{b}	<0.001
VSI (%)	7.8 ± 0.7	7.2 ± 0.6	8.1 ± 0.7	0.273
HSI (%)	1.8 ± 0.1	1.0 ± 0.2	1.2 ± 0.5	0.092
K	1.9 ± 0.1^{a}	1.7 ± 0.2^{b}	1.8 ± 0.0^{ab}	0.014

Data are presented as mean ± standard deviation (n = 4 replicates per dietary treatment, except in RGR, VFI and FCR for ORG, where n = 3). Different superscripts within the same row indicate significant differences (Kruskal-Wallis ANOVA; p < 0.05) between dietary treatments. Abbreviations: FBW: Final body weight; RGR: Relative growth rate; VFI: Voluntary Feed Intake; FCR: Feed conversion ratio; PER: Protein efficiency ratio; VSI: Viscerosomatic index; HSI: Hepatosomatic index, K: Condition factor.

Whole body composition and retention

Data on the whole-body composition of fish at the beginning and end of the study are presented in Table 6. In all analysed parameters, except ash, the dietary treatments had an impact on the body composition, where fish fed diet PD exhibited higher concentrations of all nutrients, dry matter and energy ($p < 0.004$).

Table 6 - Whole-body composition (% wet weight) of Nile tilapia (*Oreochromis niloticus*) fed with three different experimental diets (PD, ORG and ECO) for 55 days.

		Diets			
(% WW)	**Initial**	**PD**	**ORG**	**ECO**	***p* value**
Dry matter (DM; %)	26.0 ± 0.8	29.8 ± 1.6^{a}	22.0 ± 1.6^{b}	25.3 ± 1.9^{b}	<0.001
Protein (%)	14.8 ± 0.3	16.7 ± 1.1^{a}	13.8 ± 0.5^{b}	15.0 ± 0.8^{b}	0.003
Lipid (%)	6.3 ± 0.2	9.2 ± 1.4^{a}	3.1 ± 1.0^{c}	5.9 ± 0.6^{b}	<0.001
Ash (%)	4.1 ± 0.3	3.4 ± 0.3	3.9 ± 0.3	3.2 ± 0.7	0.174
Energy (kJ/g)	6.1 ± 0.1	7.3 ± 0.3^{a}	4.4 ± 0.4^{c}	5.8 ± 0.4^{b}	<0.001

Data are presented as mean ± standard deviation ($n = 4$ pools per dietary treatment). Different superscripts within the same row indicate significant differences (one-way ANOVA; $p < 0.05$) between dietary treatments (without considering initial values).

Ash, protein, lipid and energy retentions of fish fed with the different diets are shown in Figure 1. The rates reflect the tendency of the distinct feed intakes between feeds, thus all values where higher in fish fed diet PD and lower in those fed ORG ($p = 0.018$ for ash, $p = 0.007$ for protein, $p = 0.007$ for lipids and $p = 0.007$ for energy). The latter revealed negative results, in particular -1.7% for protein, -16.5% for energy and -78.5% for lipid.

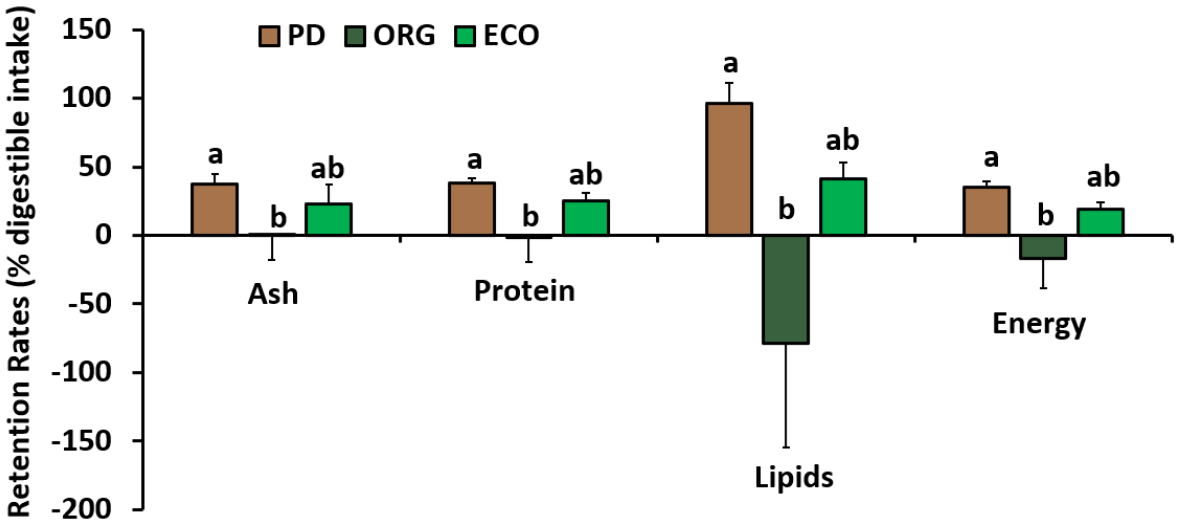

Figure 1 – Nutrient or energy retentions (% digestible intake) of protein, lipid and energy of experimental diets (PD, ORG and ECO) given to Nile tilapia (*Oreochromis niloticus*) for 55 days. Data are presented as mean ± standard deviation (n = 4). Different letters indicate significant differences (Kruskal-Wallis; $p < 0.05$) between dietary treatments.

Relative gene expression

Figure 2 shows the relative expression of genes from the anterior intestine of Nile tilapia juveniles at the end of the experiment. Dietary treatments did not show statistically significant differences between them ($p > 0.05$; ranging from 0.196 to 0.780), with the exception of glutathione reductase (*gsr*), which was more expressed in fish fed diet ECO than the control group ($p = 0.014$).

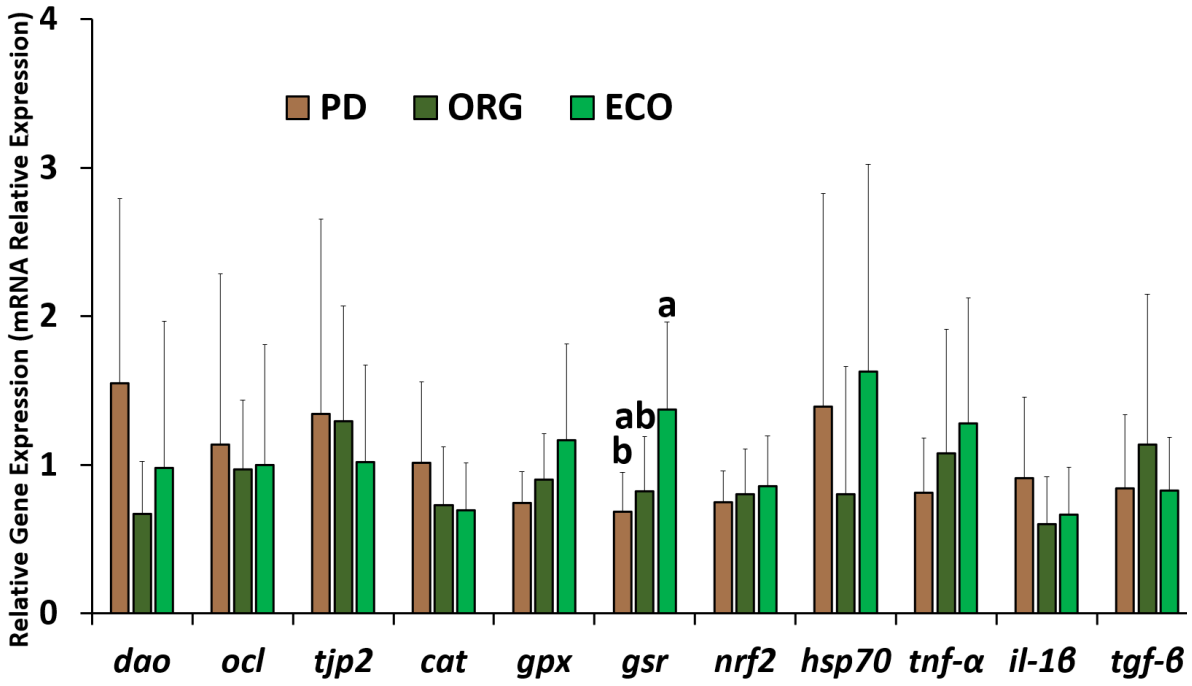

Figure 2 – Relative expression (mRNA relative expression) of genes encoding for intestinal epithelial integrity (*dao*, *ocl* and *tjp2*), oxidative status/stress (*cat*, *gpx*, *gsr*, *nrf2* and *hsp70*) and immune condition (*tnf-α*, *il-1β* and *tgf-β*) in juvenile Nile tilapia (*Oreochromis niloticus*) fed with three diets (PD, ORG and ECO) over 55 days. Data are presented as mean ± standard deviation (n = 7 for CTRL and n = 8 for ORG and ECO). Different letters indicate significant differences (one-way ANOVA; $p < 0.05$) between dietary treatments. Abbreviations: *dao:* D-amino oxidase; *ocl*: occluding; *tjp2:* tight junction protein 2; *cat*: catalase; *gpx*: glutathione peroxidase; *gsr:* glutathione reductase; *nrf2*: nuclear factor erythroid 2 – related factor 2; *hsp70:* heat shock protein 70; *tnf-α*: tumour necrosis factor; *il-1β*: interleukin-1β; *tgf-β*: transforming growth factor β.

DISCUSSION

Diet formulation and fish performance

All feeds were formulated without fishmeal and wild fish oil (replaced with salmon oil as a by-product from salmon processing industry),as they may raise environmental concerns and/or ethical issues, which mirrors farming practices of tilapia and usually does not compromise feed utilization. This is in line with commercial tilapia feeds, that have low or no inclusion of both ingredients, especially

with the goal of limiting production costs and to keep tilapia an affordable food item mainly in developing countries (Zhou and Yue, 2010). Such formulations have been tested previously without adverse impacts on tilapia performance, as seen by El-Saidy and Gaber (2003) and Teodósio et al. (2020). All diets were formulated with a mix of distinct inclusions of plant ingredients. A mixture of plant sources can decrease the nutritional imbalances of individual species and improve the nutritionally profile of the feeds (Aragão et al., 2022; Conceição et al., 2012; Oliva-Teles et al., 2015; Soltan et al., 2023). Several studies reported partial or full replacements of fishmeal with a plant mixture in tilapia feeds without a negative effect on fish performance and robustness (Agbo et al., 2015; El-Saidy and Gaber, 2003; González-Félix et al., 2010). Since soy may lead to environmental concerns (*e.g.*, deforestation), soy inclusion was reduced in ORG and ECO diets and replaced with alternative ingredients.

To replace traditional ingredients with alternative ingredients based on organic and circularity frameworks, the inclusion of several constituents from PD diet had to be modified in ORG and ECO diets. This change has considered the known species nutritional requirements, while maintaining a balanced amino acid profile and proximal composition. Since diets were isonitrogenous and isoenergetic, the dietary macronutrient profiles were most likely not responsible for the distinct performance results. The tested inclusion levels of some alternative ingredients differed between ORG and ECO diets, which was likely the main cause for the lower fish performance results. Furthermore, while the digestibility of ORG was similar to PD and although ECO exhibited slightly lower digestibility than the other feeds, the differences were marginal and within normal ranges. In this sense, we hypothesize that the lower intake and consequent decreased growth rate of fish fed ORG and ECO diets, were mainly related with palatability due to high inclusion levels of (a) particular ingredient(s) above a tolerable threshold for the fish.

Diet palatability

Since most the ingredients were included within known tolerable inclusion levels, the lower palatability was likely affected by specific ingredients. According to the literature, it seems minimal and unlikely that brewer's yeast, peas and sunflower meal inclusion levels have affected ORG or ECO palatability, as these ingredients area often palatable and their inclusion levels were below the limits by which fish intake can be negatively affected (Christopher et al., 2020; Desouky et al., 2023; Madalla An, 2014; Nogales Mérida et al., 2010; Ozório et al., 2012; Pereira-da-Silva and Pezzato, 2000; Schulz et al., 2007). For the same reasons and due to the extensive use in aquafeeds, a possible effect of exclusive ingredients from the ECO diet, including LAPs, insect meal and microbial biomass, was most likely marginal (Alves et al., 2019; Bertini et al., 2023; Bureau, 2010; Colombo et al., 2022; Hua et al., 2019; Tacon et al., 2009; Vale Pereira et al., 2023). Regarding spirulina, a recent work showed that tilapia preferred feeds with spirulina included at 7% rather than at 3.5% (Mendes et. al. submitted). Other studies reported no differences or an increase in tilapia intake when spirulina was included between 2.5 to 12% (Abdel-Warith and Elsayed, 2019; AlMulhim et al., 2023; Al-Zayat, 2019; Youssef et al., 2023). Since spirulina was included at 2.5% and 10% in diets ECO and ORG respectively, the effects of this emergent ingredient are unlikely to have affected palatability. Therefore, rapeseed meal and/or quinoa were likely the main responsibles for the lower ECO and ORG palatability.

The inclusion levels of rapeseed meal could have affected ORG palatability. Rapeseed meal was present at 26% on diet ORG, double the inclusion from ECO and PD. Literature has shown that rapeseed meal contains ANFs, precisely sinapine and glucosinolates (*e.g.*, progoitrin), that give a bitter taste, decreasing palatability (Clandinin, 1961; Enami, 2010; McCurdy and March, 1992; Montoya-Camacho et al., 2019). Consequently, this may led to fish avoiding diets with a higher inclusion of this ingredient as they can discriminate the presence of bitter flavours (Morais et al., 2019; Puchol et al., 2022). Zhou and Yue (2010) reported that an inclusion of more than 19% of rapeseed meal in hybrid tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) feeds, reduced intake, performance and feed

utilization, likely due to the toxic impacts of ANFs. Replacement of fishmeal by rapeseed meal higher than 10% or 20% in the diets of *O. niloticus* fingerlings resulted in a significant decrease in weight gain and feed intake (Sallam et al., 2021). Indeed, due to feed intake limitations, some authors proposed that rapeseed meal to be included in fish diets at levels ranging from 10 to 20% (Nemati Shizari, 2014; Piedad-Pascual, 2000). Apart from rapeseed, quinoa also has a bitter nature.

Quinoa was present in both diets ORG and ECO, at an inclusion of 5% and 2.5% respectively, which could have been above the tolerable limit for the fish. The seed is rich in ANFs (*e.g.*, quinine and saponins), particularly found in the outer coating that serve as a natural mechanism against pests (Trigo et al., 2018). These compounds are often associated with having a bitter nature and can be highly aversive, deterrent, toxic and harmful for the fish, interfering with quinoa's palatability, significantly limiting the sensory acceptance of quinoa (Molina-Poveda et al., 2017; Rigos et al., 2013; Song et al., 2024). However, in some cases quinoa was not detrimental for the farmed species. Supplementation up to 30% did not significantly changed the FCR in tilapia (Timaná Morales et al., 2022). Feed intake was not significantly affected by the inclusion of quinoa to replace oat grains in goldfish (*Carassius auratus*) (Değirmencioğlu, 2023). Distinct results can be related with different quinoa varieties and processing methodologies, such as pressure cooking, that can be used to inactivate ANFs (Adeniji et al., 2007; Değirmencioğlu, 2023). Although in the present study quinoa was heat treated, the temperature used may have not eliminate completely the ANFs, thus a possible impact of quinoa in palatability must be considered (Mhada et al., 2020; Sharma et al., 2022).

Diet digestibility

The ADCs of protein, energy and lipid were all slightly lower in diet ECO compared to PD and although it can be difficult to identify a single cause to justify these values, they are unlikely a concern. The lower digestibility can be attributed to varying proportions of different ingredients. ECO had lower plant sources than PD, which was likely the main reason for the lower ADCs of energy and protein, as lower trophic level finfish species, such as tilapia, have a well-adapted long gastrointestinal tract to

digest plants (Fetahi and Getahun, 2020). The lower ADCs of lipids may be related to several factors, such as phospholipids and fatty acid composition (*e.g.*, chain length, level of incorporation in dietary fat, degree of unsaturation, melting points), as well as proportion of saturated and unsaturated (monounsaturated (MUFA) and polyunsaturated (PUFA) - docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)) fatty acids. Although the fatty acid profile was not evaluated, the values of MUFA, PUFA and EPA+DHA were all estimated and considered during formulation to be similar between dietary treatments. In order to provide more precise reasons for the lower ADCs, it would be needed to analyse the dietary carbohydrates and fatty acid profiles, as well as address the digestibility of raw individual ingredients (Hoerterer et al., 2022; Tefal et al., 2023a; Telles, 2021). Nevertheless, the protein and lipid ADCs of ECO, ORG and PD diets were all above 75% and 90%, respectively, which are in line with the recommended values of 75%–95% and 85%-95% (National Research Council, 1993). Hence, the reduced digestibility observed in the ECO diet was unlikely a major factor for the reduced performance, and possibly, it also did not significantly affect feed utilization.

Whole-body composition and retentions

Whole-body composition and retentions were negatively affected in fish fed ORG and ECO compared to those fed PD, which are likely related with the distinct feed intakes (Ahmed, 2007). Since feed intake was reduced, fish oxidized their lipid storages to obtain energy and maintain their vital processes, structure and functionality of cell membranes (Adebayo et al., 2000; Gallardo-Collí et al., 2020; Marais and Kissil, 1979). Since energy was being obtained from fat, it also decreased. Consequently, moisture increased as lipids were replaced with water in the muscle (Wang et al., 2000). Similar patterns in body composition were observed in tilapia and other fish species when their intake was reduced (Dong et al., 2017; Lui et al., 2020; Rodde et al., 2021; Sarsangi Aliabad et al., 2022; Xiao et al., 2013). However, in most of the studies, body protein composition was not affected, meaning that in the present study particularly the slight decrease in protein in fish fed ORG was an indication

that the feed intake was close to maintenance level and that the animals were only eating to survive (Dong et al., 2016; Salze et al., 2014). As a result, there could have been some impacts on fish health and condition.

Fish health and somatic indices

Given that from the molecular biomarkers assessed for intestinal epithelial integrity, immune condition and oxidative status, only glutathione reductase (*gsr*) was significantly affected by the dietary treatments, the overall health status of the fish does not seem to have been compromised. The expression of *gsr*, an important biomarker for oxidative status, was upregulated in fish fed ECO compared to those fed PD. Glutathione reductase is a crucial enzyme that plays a pivotal role in maintaining cellular redox homeostasis and antioxidant defence systems in fish (Couto et al., 2016). From the formulation of this feed, the inclusion levels of spirulina and quinoa could have had a higher impact on the antioxidant activity, as both are rich in bioactive compounds with antioxidant properties (*e.g.*, carotenoids, saponins, phycoerythrin and phycocyanin) (Abdelkhalek et al., 2017; Fischer et al., 2017; Kumar et al., 2022). Quinoa increased the antioxidant status when included at 10% and 31% in tilapia and rats, respectively (Ahmed et al., 2020; Pasko et al., 2010). Spirulina at inclusion levels ranging from 0.5% to 45%, improved the antioxidant capacity of several fish species (Abdelkhalek et al., 2017; Abdel-Warith and Elsayed, 2019; Kim et al., 2013; Rosas et al., 2019). The suggestion that fish health was likely not affected in ORG and ECO, despite their lower feed intake, based on gene expression results, is also supported by the somatic indices.

VSI and HSI were similar between dietary treatments, while K was slightly lower in ORG fish, meaning that fish robustness was likely not compromised. VSI and HSI are often used to evaluate the nutritional and physiological state of fish metabolism (Dawood, 2016; Velasquez et al., 2016b). Since these indexes did not differ between dietary treatments, it indicates that the capacity for nutrient absorption and fish metabolism were not significantly affected, also suggested by the absence of different expressions in health-related genes. The condition factor provides insights into the wellbeing,

nutritional status and growth of fish (Paredes-Trujillo et al., 2021). Although K differed between dietary treatments, the differences were small and the values were considered normal, when compared to the ones reported by Asmamaw et al. (2019), Ighwela et al. (2011) and Keyombe et al. (2017). Therefore, it is interesting to note that although fish fed diet ECO and particularly ORG ate much less than those fed PD, their health condition was not affected, meaning that in the case of ORG, they were eating to maintain their body weight and survive.

CONCLUSIONS

Clearly, more detailed studies are still necessary to optimize organic and eco-efficient formulations frameworks before they are to be implemented commercially for tilapia. When addressing societal concerns on feed formulations, one must consider the balance between environmental and social sustainability with fish performance and wellbeing. Despite being fed until apparent satiation, the three aquafeed formulations had significantly distinct impacts on juvenile Nile tilapia performance, whereas fish fed ORG and ECO showed considerably lower growth. Diet ORG exhibited reduced palatability likely due to the inclusion levels of specific ingredients on the feeds, most likely quinoa and rapeseed, or their combination with the other ingredients used, which greatly decreased feed intake. Still, fish appeared to have voluntarily chosen feed intake levels close to those required for maintenance as there was no weight loss. The evaluation of the impacts of the diets on gut epithelium integrity, immune condition, oxidative status and somatic indices revealed no major impacts. Therefore, despite the decreased feed intake, and reduced feed utilization, the alternative feed formulations affected tilapia growth but not robustness.

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REFERENCES

Abdelkhalek, N.K.M., Eissa, I.A.M., Ahmed, E., Kilany, O.E., El-Adl, M., Dawood, M.A.O., Hassan, A.M., Abdel-Daim, M.M., 2017. Protective role of dietary Spirulina platensis against diazinon-induced Oxidative damage in Nile tilapia; *Oreochromis niloticus*. Environmental Toxicology and Pharmacology 54, 99–104. https://doi.org/10.1016/j.etap.2017.07.002

Abdel-Warith, A.-A.A., Elsayed, E.A., 2019. Use of Arthrospira platensis as a Feed Additive to Improve Growth Performance, Feed Utilization, Body Composition, and Immune Response of Nile Tilapia, 78.

Adebayo, O., Balogun, A., Fagbenro, O., 2000. Effects of feeding rates on growth, body composition and economic performance of juvenile clariid catfish hybrid (female *Clarias gariepinus*× male *Heterobranchus bidorsalis*). Journal of Aquaculture in the Tropics 15, 109–117.

Adeniji, C.A., Fakoya, K.A., Omamohwo, V.R., 2007. Partial Replacement of Soybean Cake with Amaranth us spinosus Leaf Meal in the Diet of Nile Tilapia (*Oreochromis niloticus*). Biological Sciences - PJSIR 50, 335–338.

Agbo, N.W., Madalla, N., Jauncey, K., 2015. Mixtures of oilseed meals as dietary protein sources in diets of juvenile Nile tilapia (*Oreochromis niloticus* l.). Journal of Science and Technology (Ghana) 35, 11–24. https://doi.org/10.4314/just.v35i3.2

Ahmed, I., 2007. Effect of ration size on growth, body composition, and energy and protein maintenance requirement of fingerling Indian major carp, *Labeo rohita* (Hamilton). Fish Physiology and Biochemistry 33, 203–212.

Chapter II

Ahmed, N., Thompson, S., Turchini, G.M., 2020. Organic aquaculture productivity, environmental sustainability, and food security: insights from organic agriculture. Food Security 12, 1253–1267. https://doi.org/10.1007/s12571-020-01090-3

Ahmed, S.A.A., Abd El-Rahman, G.I., Behairy, A., Beheiry, R.R., Hendam, B.M., Alsubaie, F.M., Khalil, S.R., 2020. Influence of Feeding Quinoa (*Chenopodium quinoa*) Seeds and Prickly Pear Fruit (*Opuntia ficus indica*) Peel on the Immune Response and Resistance to *Aeromonas sobria* Infection in Nile Tilapia (*Oreochromis niloticus*). Animals 10, 2266. https://doi.org/10.3390/ani10122266

AlMulhim, N.M., Virk, P., Abdelwarith, A.A., AlKhulaifi, F.M., 2023. Effect of incorporation of Spirulina platensis into fish diets, on growth performance and biochemical composition of Nile Tilapia, *Oreochromis niloticus*. The Egyptian Journal of Aquatic Research 49, 537–541. https://doi.org/10.1016/j.ejar.2023.08.008

Alves, D.R.S., de Oliveira, S.R., Luczinski, T.G., Paulo, I.G.P., Boscolo, W.R., Bittencourt, F., Signor, A., 2019. Palatability of Protein Hydrolysates from Industrial Byproducts for Nile Tilapia Juveniles. Animals 9, 311. https://doi.org/10.3390/ani9060311

Aragão, C., Cabano, M., Colen, R., Fuentes, J., Dias, J., 2020. Alternative formulations for gilthead seabream diets: Towards a more sustainable production. Aquaculture Nutrition 26, 444–455. https://doi.org/10.1111/anu.13007

Aragão, C., Gonçalves, A.T., Costas, B., Azeredo, R., Xavier, M.J., Engrola, S., 2022. Alternative Proteins for Fish Diets: Implications beyond Growth. Animals 12, 1211. https://doi.org/10.3390/ani12091211

Asmamaw, B., Beyene, B., Tessema, M., Assefa, A., 2019. Length-weight relationships and condition factor of Nile tilapia, Oreochromis niloticus (Linnaeus, 1758) (Cichlidae) in Koka Reservoir, Ethiopia Volume 4, 5–6.

Assan, D., Huang, Y., Mustapha, U.F., Addah, M.N., Li, G., Chen, H., 2021. Fish Feed Intake, Feeding Behavior, and the Physiological Response of Apelin to Fasting and Refeeding. Frontiers in Endocrinology (Lausanne) 12, 798903. https://doi.org/10.3389/fendo.2021.798903

Association of Official Analytical Chemists, 2007. Official Methods of Analysis of AOAC International. Association of Official Analysis Chemists International 1.

Balakrishnan, G., Schneider, R.G., 2022. The Role of Amaranth, Quinoa, and Millets for the Development of Healthy, Sustainable Food Products—A Concise Review. Foods 11, 2442. https://doi.org/10.3390/foods11162442

Bertini, A., Natale, S., Gisbert, E., Andrée, K.B., Concu, D., Dondi, F., De Cesare, A., Indio, V., Gatta, P.P., Bonaldo, A., Parma, L., 2023. Exploring the application of Corynebacterium glutamicum single cell protein in the diet of flathead grey mullet (*Mugil cephalus*): effects on growth performance, digestive enzymes activity and gut microbiota. Frontiers in Marine Science. 10, 1172505. https://doi.org/10.3389/fmars.2023.1172505

Bjørhusdal, V., Haugen, S., 2023. Circular Economy Implications for Sustainable Supply Chain Practices: Comparative case study within the Norwegian fish farming industry.

Brooks, S.P.J., Oberleas, D., Dawson, B.A., Belonje, B., Lampi, B.J., 2001. Proposed phytic acid standard including a method for its analysis. Journal of AOAC International 84. https://doi.org/10.1093/jaoac/84.4.1125

Bureau, D., 2010. Feather meal. Improving its nutrient value characterization for fish. Render 39, 14–16.

Cao, L., Diana, J.S., Keoleian, G.A., 2013. Role of life cycle assessment in sustainable aquaculture. Reviews in Aquaculture 5, 61–71. https://doi.org/10.1111/j.1753-5131.2012.01080.x

Chary, K., Van Riel, A., Muscat, A., Wilfart, A., Harchaoui, S., Verdegem, M., Filgueira, R., Troell, M., Henriksson, P.J.G., De Boer, I.J.M., Wiegertjes, G.F., 2023. Transforming sustainable

aquaculture by applying circularity principles. Reviews in Aquaculture raq.12860. https://doi.org/10.1111/raq.12860

Christopher, R.B., Ahilan, B., Cheryl, A., Samuel, M., 2020. Sunflower meal as an alternative protein source to replace soybean meal in the diet of GIFT strain of Nile tilapia *Oreochromis niloticus*. Indian Journal of Fisheries 67. https://doi.org/10.21077/ijf.2020.67.3.91750-09

Clandinin, D.R., 1961. Rapeseed Oil Meal Studies. Poultry Science 40, 484–487. https://doi.org/10.3382/ps.0400484

Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Sayers, E.W., 2016. GenBank. Nucleic Acids Research 44, D67–D72. https://doi.org/10.1093/nar/gkv1276

Colombo, S.M., Roy, K., Mraz, J., Wan, A.H.L., Davies, S.J., Tibbetts, S.M., Øverland, M., Francis, D.S., Rocker, M.M., Gasco, L., Spencer, E., Metian, M., Trushenski, J.T., Turchini, G.M., 2022. Towards achieving circularity and sustainability in feeds for farmed blue foods. Reviews in Aquaculture raq.12766. https://doi.org/10.1111/raq.12766

Conceição, L.E.C., Aragão, C., Dias, J., Costas, B., Terova, G., Martins, C., Tort, L., 2012. Dietary nitrogen and fish welfare. Fish Physiol Biochem 38, 119–141. https://doi.org/10.1007/s10695-011-9592-y

Cottrell, R.S., Blanchard, J.L., Halpern, B.S., Metian, M., Froehlich, H.E., 2020. Global adoption of novel aquaculture feeds could substantially reduce forage fish demand by 2030. Nature Food 1, 301–308.

Couto, N., Wood, J., Barber, J., 2016. The role of glutathione reductase and related enzymes on cellular redox homoeostasis network. Free Radical Biology and Medicine 95, 27–42. https://doi.org/10.1016/j.freeradbiomed.2016.02.028

Dawood, M.A.O., 2016. Effect of various feed additives on the performance of aquatic animals.

Değirmencioğlu, T., 2023. Possibility of using quinoa (*Chenopodium quinoa*) as an alternative energy source in the goldfish (*Carassius auratus auratus*) diet. Journal of Advanced Research in Natural and Applied Sciences 9, 457–464. https://doi.org/10.28979/jarnas.1168626

Desouky, A., Hwihy, H., Shaban, W., Azab, A., 2023. Evaluating of Pea Peels Meal as a Fishmeal alternative in Formulated Diet Ingredients of *Oreochromis niloticus*. Egyptian Journal of Aquatic Biology and Fisheries 27, 725–737. https://doi.org/10.21608/EJABF.2023.330193

Do Vale Pereira, G., Teixeira, C., Couto, J., Dias, J., Rema, P., Gonçalves, A.T., 2023. Dietary Protein Source Affects the Interplay between Gut Microbiota and Host Performance in Nile Tilapia (preprint). Biology and Life Sciences. https://doi.org/10.20944/preprints202311.0543.v1

Dong, C., He, G., Mai, K., Zhou, H., Xu, W., 2016. Palatability of water-soluble extracts of protein sources and replacement of fishmeal by a selected mixture of protein sources for juvenile turbot (*Scophthalmus maximus*). Journal of Ocean University of China 15, 561–567.

Dong, G., Yang, Y., Yao, F., Chen, L., Yue, D., Yu, D., Huang, F., Liu, J., Liu, L., 2017. Growth performance and whole-body composition of yellow catfish (*Pelteobagrus fulvidraco Richardson*) under feeding restriction. Aquaculture Nutrition 23, 101–110.

EC, 2021. A new strategic vision for sustainable aquaculture production and consumption in the European Union: blue farming in the European Green Deal. Publications Office, LU.

EC, 2020. COMMUNICATION FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT, THE COUNCIL, THE EUROPEAN ECONOMIC AND SOCIAL COMMITTEE AND THE COMMITTEE OF THE REGIONS A Farm to Fork Strategy for a fair, healthy and environmentally-friendly food system.

Ellingsen, K., Grimsrud, K., Nielsen, H.M., Mejdell, C., Olesen, I., Honkanen, P., Navrud, S., Gamborg, C., Sandøe, P., 2015. Who cares about fish welfare? A Norwegian study. British Food Journal 117, 257–273.

El-Saidy, D.M.S.D., Gaber, M.M.A., 2003. Replacement of fish meal with a mixture of different plant protein sources in juvenile Nile tilapia, *Oreochromis niloticus* (L.) diets: Plant protein mixture in Nile tilapia diets. Aquaculture Research 34, 1119–1127. https://doi.org/10.1046/j.1365-2109.2003.00914.x

El-Sayed, A.-F.M., 2019. Tilapia culture. Academic press.

Enami, H.R., 2010. A Review of Using Canola/Rapeseed Meal in Aquaculture Feeding. Journal of Fisheries and Aquatic Science 6, 22–36. https://doi.org/10.3923/jfas.2011.22.36

Ennos, R., 2007. Statistical and data handling skills in biology. Pearson Education.

EUMOFA, 2023. The EU fish market: 2023 edition.

FAO, 2022. The State of World Fisheries and Aquaculture 2022: towards blue transformation. FAO, S.l.

Fetahi, T., Getahun, A., 2020. Food and feeding habits of juvenile and adult Nile tilapia, *Oreochromis niloticus* (L.)(Pisces: Cichlidae) in Lake Ziway, Ethiopia. SINET Ethiopian Journal of Science 43, 25–33.

Feucht, Y., Zander, K., 2015. Of earth ponds, flow-through and closed recirculation systems — German consumers' understanding of sustainable aquaculture and its communication. Aquaculture 438, 151–158. https://doi.org/10.1016/j.aquaculture.2015.01.005

Fischer, S., Wilckens, R., Jara, J., Aranda, M., Valdivia, W., Bustamante, L., Graf, F., Obal, I., 2017. Protein and antioxidant composition of quinoa (*Chenopodium quinoa* Willd.) sprout from seeds submitted to water stress, salinity and light conditions. Industrial Crops and Products 107, 558–564. https://doi.org/10.1016/j.indcrop.2017.04.035

Fortes-Silva, R., Kitagawa, A., Sanchez Vazquez, F.J., 2016. Dietary self-selection in fish: a new approach to studying fish nutrition and feeding behavior. Reviews in Fish Biology and Fisheries 26. https://doi.org/10.1007/s11160-015-9410-1

Gallardo-Collí, A., Pérez-Fuentes, M., Pérez-Rostro, C.I., Hernández-Vergara, M.P., 2020. Compensatory growth of Nile tilapia *Oreochromis niloticus* , L. subjected to cyclic periods of

feed restriction and feeding in a biofloc system. Aquac Res 51, 1813–1823. https://doi.org/10.1111/are.14530

Gephart, J.A., Henriksson, P.J.G., Parker, R.W.R., Shepon, A., Gorospe, K.D., Bergman, K., Eshel, G., Golden, C.D., Halpern, B.S., Hornborg, S., Jonell, M., Metian, M., Mifflin, K., Newton, R., Tyedmers, P., Zhang, W., Ziegler, F., Troell, M., 2021. Environmental performance of blue foods. Nature 597, 360–365. https://doi.org/10.1038/s41586-021-03889-2

González-Félix, M.L., Perez-Velazquez, M., Villalba-Villalba, A.G., Civera-Cerecedo, R., Ezquerra, J.M., Goytortúa-Bores, E., 2010. Tailoring a diet for Nile tilapia (*Oreochromis niloticus*) culture in northwest Mexico. Journal of Marine Science and Technology 18, 674–681.

Han, P., Li, Jingjing, Zhong, H., Xie, J., Zhang, P., Lu, Q., Li, Jun, Xu, P., Chen, P., Leng, L., Zhou, W., 2021. Anti-oxidation properties and therapeutic potentials of spirulina. Algal Research 55, 102240. https://doi.org/10.1016/j.algal.2021.102240

Hilmarsdóttir, G.S., Ogmundarson, Ó., Arason, S., Gudjónsdóttir, M., 2021. Efficiency of fishmeal and fish oil processing of different pelagic fish species: Identification of processing steps for potential optimization toward protein production for human consumption. Journal of Food Processing and Preservation 45. https://doi.org/10.1111/jfpp.15294

Hoerterer, C., Petereit, J., Lannig, G., Johansen, J., Conceição, L.E.C., Buck, B.H., 2022. Effects of dietary plant and animal protein sources and replacement levels on growth and feed performance and nutritional status of market-sized turbot (*Scophthalmus maximus*) in RAS. Frontiers in Marine Science 9.

Hossain, Md.S., Small, B.C., Kumar, V., Hardy, R., 2024. Utilization of functional feed additives to produce cost-effective, ecofriendly aquafeeds high in plant-based ingredients. Reviews in Aquaculture 16, 121–153. https://doi.org/10.1111/raq.12824

Hua, K., Cobcroft, J.M., Cole, A., Condon, K., Jerry, D.R., Mangott, A., Praeger, C., Vucko, M.J., Zeng, C., Zenger, K., Strugnell, J.M., 2019. The Future of Aquatic Protein: Implications for Protein

Sources in Aquaculture Diets. One Earth 1, 316–329. https://doi.org/10.1016/j.oneear.2019.10.018

Ighwela, K.A., Ahmed, A., Abol-Munafi, A., 2011. Condition Factor as an Indicator of Growth and Feeding Intensity of Nile Tilapia Fingerlings (*Oreochromis niloticus*) Feed on Different Levels of Maltose.

Jannathulla, R., Rajaram, V., Kalanjiam, R., Ambasankar, K., Muralidhar, M., Dayal, J.S., 2019. Fishmeal availability in the scenarios of climate change: Inevitability of fishmeal replacement in aquafeeds and approaches for the utilization of plant protein sources. Aquaculture Research 50, 3493–3506. https://doi.org/10.1111/are.14324

Keyombe, J.L., Malala, J.O., Waithaka, E., Lewo, R.M., Obwanga, B.O., 2017. Seasonal changes in length-weight relationship and condition factor of Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) (Cichlidae) in Lake Naivasha, Kenya. International Journal of Aquatic Biology 5. https://doi.org/10.22034/ijab.v5i1.231

Kim, S.-S., Rahimnejad, S., Kim, K.-W., Lee, K.-J., 2013. Partial Replacement of Fish Meal with Spirulina pacifica in Diets for Parrot Fish (*Oplegnathus fasciatus*). Turk. J. Fish. Aquat. Sci. 13. https://doi.org/10.4194/1303-2712-v13_2_01

Krogdahl, Å., Penn, M., Thorsen, J., Refstie, S., Bakke, A.M., 2010. Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. Aquaculture research 41, 333–344.

Kumar, Agam, Ramamoorthy, D., Verma, D.K., Kumar, Arvind, Kumar, N., Kanak, K.R., Marwein, B.M., Mohan, K., 2022. Antioxidant and phytonutrient activities of *Spirulina platensis*. Energy Nexus 6, 100070. https://doi.org/10.1016/j.nexus.2022.100070

Little, D.C., Newton, R.W., Beveridge, M.C.M., 2016. Aquaculture: a rapidly growing and significant source of sustainable food? Status, transitions and potential. Proceedings of the Nutrition Society 75, 274–286. https://doi.org/10.1017/S0029665116000665

López-Mas, L., Claret, A., Reinders, M.J., Banovic, M., Krystallis, A., Guerrero, L., 2021. Farmed or wild fish? Segmenting European consumers based on their beliefs. Aquaculture 532, 735992.

Lui, T.A., Da Silva, W.P., Nervis, J.A.L., Brum, J.M.D., Bittencourt, F., Neu, D.H., Boscolo, W.R., 2020. Food restriction in Nile tilapia juveniles (*Oreochromis niloticus*). Spanish Journal of Agricultural Research 18, e0607. https://doi.org/10.5424/sjar/2020183-15639

M. Al-Zayat, A., 2019. Effect of various levels of Spirulina (*Arthrospira platensis*) as feed supplement on growth performance, feed utilization , immune response and hematology of the Nile tilapia (*Oreochromis niloticus*) fingerlings. Egyptian Journal of Aquatic Biology and Fisheries. 23, 361–370. https://doi.org/10.21608/ejabf.2019.54036

Madalla An, N.J., 2014. Effects of Soaked Pigeon Peas on the Growth of Nile Tilapia (*Oreochromis niloticus* L) Fingerlings. Journal of Fisheries and Livestock Production 03. https://doi.org/10.4172/2332-2608.1000125

Magbanua, T.O., Ragaza, J.A., 2024. Selected dietary plant-based proteins for growth and health response of Nile tilapia *Oreochromis niloticus*. Aquaculture and Fisheries, Special Issue: Friendly nutritional strategies for sustainable aquaculture 9, 3–19. https://doi.org/10.1016/j.aaf.2022.04.001

Malcorps, W., Kok, B., van 't Land, M., Fritz, M., van Doren, D., Servin, K., van der Heijden, P., Palmer, R., Auchterlonie, N.A., Rietkerk, M., 2019. The sustainability conundrum of fishmeal substitution by plant ingredients in shrimp feeds. Sustainability 11, 1212.

Marais, J.F.K., Kissil, G.Wm., 1979. The influence of energy level on the feed intake, growth, food conversion and body composition of *Sparus aurata*. Aquaculture 17, 203–219. https://doi.org/10.1016/0044-8486(79)90124-8

Maynard, L., Loosli, J., Hintz, H., Warner, R., 1979. Digestive processes in different species. Animal nutrition 7, 21–46.

McCurdy, S.M., March, B.E., 1992. Processing of canola meal for incorporation in trout and salmon diets. Journal of the American Oil Chemists' Society 69, 213–220. https://doi.org/10.1007/BF02635889

Mhada, M., Metougui, M.L., El Hazzam, K., El Kacimi, K., Yasri, A., 2020. Variations of Saponins, Minerals and Total Phenolic Compounds Due to Processing and Cooking of Quinoa (*Chenopodium quinoa* Willd.) Seeds. Foods 9, 660. https://doi.org/10.3390/foods9050660

Molina-Poveda, C., Cárdenas, R., Jover, M., 2017. Evaluation of amaranth (*Amaranthus caudatus* L.) and quinoa (*Chenopodium quinoa*) protein sources as partial substitutes for fish meal in *Litopenaeus vannamei* grow-out diets. Aquaculture Research 48, 822–835. https://doi.org/10.1111/are.12926

Montoya-Camacho, N., Marquez-Ríos, E., Castillo-Yáñez, F.J., Cárdenas López, J.L., López-Elías, J.A., Ruíz-Cruz, S., Jiménez-Ruíz, E.I., Rivas-Vega, M.E., Ocaño-Higuera, V.M., 2019. Advances in the use of alternative protein sources for tilapia feeding. Reviews in Aquaculture 11, 515–526. https://doi.org/10.1111/raq.12243

Mosha, S., 2019. The Signifiance of Spirulina Meal on Fishmeal Replacement in Aquaculture: A Review.

National Research Council, 1993. Nutrient Requirements of Fish. National Academies Press, Washington, D.C. https://doi.org/10.17226/2115

Nemati Shizari, F., 2014. Rapeseed cake as a feed ingredient for Nile tilapia : responses to replacing protein from soybean meal with rapeseed cake, and fine milling and autoclaving of the rapeseed cake (Master thesis). 87. Norwegian University of Life Sciences, Ås.

Newton, R.W., Little, D.C., 2018. Mapping the impacts of farmed Scottish salmon from a life cycle perspective. The International Journal of Life Cycle Assessment 23, 1018–1029. https://doi.org/10.1007/s11367-017-1386-8

Newton, R.W., Maiolo, S., Malcorps, W., Little, D.C., 2023. Life Cycle Inventories of marine ingredients. Aquaculture 565, 739096. https://doi.org/10.1016/j.aquaculture.2022.739096

Nogales Mérida, S., Tomás-Vidal, A., Martínez-Llorens, S., Jover Cerdá, M., 2010. Sunflower meal as a partial substitute in juvenile sharpsnout sea bream (*Diplodus puntazzo*) diets: Amino acid retention, gut and liver histology. Aquaculture 298, 275–281. https://doi.org/10.1016/j.aquaculture.2009.10.025

Nowak, V., Du, J., Charrondière, U.R., 2016. Assessment of the nutritional composition of quinoa (*Chenopodium quinoa* Willd.). Food Chemistry 193, 47–54. https://doi.org/10.1016/j.foodchem.2015.02.111

Oliva-Teles, A., Enes, P., Peres, H., 2015. Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous fish, in: Feed and Feeding Practices in Aquaculture. Elsevier, pp. 203–233. https://doi.org/10.1016/B978-0-08-100506-4.00008-8

Ozório, R.O.A., Portz, L., Borghesi, R., Cyrino, J.E.P., 2012. Effects of Dietary Yeast (*Saccharomyces cerevisia*) Supplementation in Practical Diets of Tilapia (*Oreochromis niloticus*). Animals 2, 16–24. https://doi.org/10.3390/ani2010016

Paredes-Trujillo, A., Velázquez-Abunader, I., Papiol, V., del Rio-Rodríguez, R.E., Vidal-Martínez, V.M., 2021. Negative effect of ectoparasite burdens on the condition factor from farmed tilapia *Oreochromis niloticus* in the Yucatan, Mexico. Veterinary Parasitology 292, 109393. https://doi.org/10.1016/j.vetpar.2021.109393

Pasko, P., Barton, H., Zagrodzki, P., Izewska, A., Krosniak, M., Gawlik, Malgorzata, Gawlik, Maciej, Gorinstein, S., 2010. Effect of diet supplemented with quinoa seeds on oxidative status in plasma and selected tissues of high fructose-fed rats. Plant foods for human nutrition 65, 146–151.

Pereira-da-Silva, E., Pezzato, L., 2000. Response of Nile Tilapia (*Oreochromis niloticus*) to the Attraction and Palatability of the Used Ingredients in the Feeding of Fishes. Revista Brasileira de Zootecnia 29, 1273–1280.

Petereit, J., Hoerterer, C., Bischoff-Lang, A.A., Conceição, L.E.C., Pereira, G., Johansen, J., Pastres, R., Buck, B.H., 2022. Adult European Seabass (*Dicentrarchus labrax*) Perform Well on Alternative Circular-Economy-Driven Feed Formulations. Sustainability 14, 7279. https://doi.org/10.3390/su14127279

Pfaffl, M.W., 2004. Quantification strategies in real-time PCR. IUL Biotechnology. Ser. 5.

Piedad-Pascual, F., 2000. Handbook on ingredients for aquaculture feeds. Kluwer Academic Publishers.

Regueiro, L., Newton, R., Soula, M., Méndez, D., Kok, B., Little, D.C., Pastres, R., Johansen, J., Ferreira, M., 2022. Opportunities and limitations for the introduction of circular economy principles in EU aquaculture based on the regulatory framework. Journal of Industrial Ecology 26, 2033–2044. https://doi.org/10.1111/jiec.13188

Reig, L., Escobar, C., Carrassón, M., Constenla, M., Gil, J.M., Padrós, F., Piferrer, F., Flos, R., 2019. Aquaculture perceptions in the Barcelona metropolitan area from fish and seafood wholesalers, fishmongers, and consumers. Aquaculture 510, 256–266.

Reis, P.A., Valente, L.M.P., Almeida, C.M.R., 2008. A fast and simple methodology for determination of yttrium as an inert marker in digestibility studies. Food Chemistry 108. https://doi.org/10.1016/j.foodchem.2007.11.060

Ricker, W.E., 1958. Handbook of Computations for Biological Statistics of Fish Populations. Fisheries Research Board of Canada.

Rigos, G., Karagouni, E., Kyriazis, I., Athanasiou, E., Grigorakis, K., Kotou, E., Katharios, P., 2013. In vitro and in vivo evaluation of quinine in gilthead sea bream, Sparus aurata naturally infected

with the ciliate *Cryptocaryon irritans*. Aquaculture 416–417, 185–191. https://doi.org/10.1016/j.aquaculture.2013.09.021

Rodde, C., Vandeputte, M., Trinh, T.Q., Douchet, V., Canonne, M., Benzie, J.A.H., de Verdal, H., 2021. The Effects of Feed Restriction and Isolated or Group Rearing on the Measurement of Individual Feed Intake and Estimation of Feed Conversion Ratio in Juvenile Nile Tilapia (*Oreochromis niloticus*) for Selective Breeding Purposes. Frontiers in Genetics 11.

Rosas, V.T., Monserrat, J.M., Bessonart, M., Magnone, L., Romano, L.A., Tesser, M.B., 2019. Fish oil and meal replacement in mullet (*Mugil liza*) diet with *Spirulina* (*Arthrospira platensis*) and linseed oil. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 218, 46–54. https://doi.org/10.1016/j.cbpc.2018.12.009

Sallam, E.A., Matter, A.F., Mohammed, L.S., Azam, A.E., Shehab, A., Mohamed Soliman, M., 2021. Replacing fish meal with rapeseed meal: potential impact on the growth performance, profitability measures, serum biomarkers, antioxidant status, intestinal morphometric analysis, and water quality of *Oreochromis niloticus* and *Sarotherodon galilaeus* fingerlings. Veterinary Research Communications 45, 223–241. https://doi.org/10.1007/s11259-021-09803-5

Salze, G., Alami-Durante, H., Barbut, S., Marcone, M., Bureau, D.P., 2014. Nutrient deposition partitioning and priorities between body compartments in two size classes of rainbow trout in response to feed restriction. British Journal of Nutrition 111, 1361–1372. https://doi.org/10.1017/S000711451300384X

Sarker, P.K., Kapuscinski, A.R., McKuin, B., Fitzgerald, D.S., Nash, H.M., Greenwood, C., 2020. Microalgae-blend tilapia feed eliminates fishmeal and fish oil, improves growth, and is cost viable. Scientific Reports 10, 19328. https://doi.org/10.1038/s41598-020-75289-x

Sarsangi Aliabad, H., Naji, A., Mortezaei, S.R.S., Sourinejad, I., Akbarzadeh, A., 2022. Effects of restricted feeding levels and stocking densities on water quality, growth performance, body

composition and mucosal innate immunity of Nile tilapia (*Oreochromis niloticus*) fry in a biofloc system. Aquaculture 546, 737320. https://doi.org/10.1016/j.aquaculture.2021.737320

Schulz, C., Wickert, M., Kijora, C., Ogunji, J., Rennert, B., 2007. Evaluation of pea protein isolate as alternative protein source in diets for juvenile tilapia (*Oreochromis niloticus*). Aquaculture Research 38, 537–545. https://doi.org/10.1111/j.1365-2109.2007.01699.x

Segner, H., Sundh, H., Buchmann, K., Douxfils, J., Sundell, K.S., Mathieu, C., Ruane, N., Jutfelt, F., Toften, H., Vaughan, L., 2012. Health of farmed fish: its relation to fish welfare and its utility as welfare indicator. Fish physiology and biochemistry 38, 85–105.

Sharma, S., Kataria, A., Singh, B., 2022. Effect of thermal processing on the bioactive compounds, antioxidative, antinutritional and functional characteristics of quinoa (*Chenopodium quinoa*). LWT 160, 113256. https://doi.org/10.1016/j.lwt.2022.113256

Soltan, N.M., Soaudy, M.R., Abdella, M.M., Hassaan, M.S., 2023. Partial dietary fishmeal replacement with mixture of plant protein sources supplemented with exogenous enzymes modify growth performance, digestibility, intestinal morphology, haemato-biochemical and immune responses for Nile tilapia, *Oreochromis niloticus*. Animal Feed Science and Technology 299, 115642. https://doi.org/10.1016/j.anifeedsci.2023.115642

Song, L.M., Yu, Y., Du, L.D., Ji, X.Y., Gao, H., Cai, Y.Q., Li, C.J., Xue, P., 2024. Does saponin in quinoa really embody the source of its bitterness? Food Chemistry 437, 137872. https://doi.org/10.1016/j.foodchem.2023.137872

Stentiford, G., Bateman, I., Hinchliffe, S., Bass, D. 1, Hartnell, R., Santos, E., Devlin, M., Feist, S., Taylor, N., Verner-Jeffreys, D., 2020. Sustainable aquaculture through the One Health lens. Nature Food 1, 468–474.

Stevens, J.R., Newton, R.W., Tlusty, M., Little, D.C., 2018. The rise of aquaculture by-products: Increasing food production, value, and sustainability through strategic utilisation. Marine Policy 90, 115–124.

Tacon, A.G., Metian, M., Hasan, M.R., 2009. Feed ingredients and fertilizers for farmed aquatic animals: sources and composition. Food and Agriculture Organization of the United Nations (FAO).

Tefal, E., Jauralde, I., Tomás-Vidal, A., Martínez-Llorens, S., Peñaranda, D.S., Jover-Cerdá, M., 2023. New Organic Raw Materials for Gilthead Seabream (*Sparus aurata*) Feeding and the Effects on Growth, Nutritive Parameters, Digestibility, and Histology. Fishes 8, 330. https://doi.org/10.3390/fishes8060330

Telles, M., 2021. Efeito da fortificação de Spirulina maxima em rações para tilápia do Nilo (*Oreochromis niloticus*): análise bromatológica e avaliação da digestibilidade.

Teodósio, R., Engrola, S., Colen, R., Masagounder, K., Aragão, C., 2020. Optimizing diets to decrease environmental impact of Nile tilapia (*Oreochromis niloticus*) production. Aquaculture Nutrition 26, 422–431. https://doi.org/10.1111/anu.13004

Timaná Morales, M.F., Naspiran-Jojoa, D.C., Mafla-Mejía, S.M., Imúes-Figueroa, M.A., Guerrero-Romero, C.L., 2022. Efecto de la inclusión de diferentes niveles de quinoa (*Chenopodium quinoa*) sobre el crecimineto de tilapia nilotica (*Oreochromis niloticus*). REVIP 8, 7. https://doi.org/10.22267/revip.2181.25

Tippayadara, N., Dawood, M.A.O., Krutmuang, P., Hoseinifar, S.H., Doan, H.V., Paolucci, M., 2021. Replacement of Fish Meal by Black Soldier Fly (*Hermetia illucens*) Larvae Meal: Effects on Growth, Haematology, and Skin Mucus Immunity of Nile Tilapia, *Oreochromis niloticus*. Animals 11, 193. https://doi.org/10.3390/ani11010193

Trigo, M., Rodríguez, A., Dovale, G., Pastén, A., Vega-Gálvez, A., Aubourg, S.P., 2018. The effect of glazing based on saponin-free quinoa (*Chenopodium quinoa*) extract on the lipid quality of frozen fatty fish. LWT 98, 231–236. https://doi.org/10.1016/j.lwt.2018.08.031

Valdivia-Cea, W., Bustamante, L., Jara, J., Fischer, S., Holzapfel, E., Wilckens, R., 2021. Effect of Soil Water Availability on Physiological Parameters, Yield, and Seed Quality in Four Quinoa Genotypes (*Chenopodium quinoa* Willd.). Agronomy 11, 1012. https://doi.org/10.3390/agronomy11051012

Vale Pereira, G. do, Conceição, L.E.C., Soares, F., Petereit, J., Buck, B.H., Johansen, J., Dias, J., Faccenda, F., 2023. Alternative Feed Formulations Impact Growth Performance, Flesh Quality and Consumer Acceptance of Rainbow Trout (*Oncorhynchus mykiss*). Journal of Marine Science and Engineering 11, 1135. https://doi.org/10.3390/jmse11061135

Velasquez, S.F., Chan, M.A., Abisado, R.G., Traifalgar, R.F.M., Tayamen, M.M., Maliwat, G.C.F., Ragaza, J.A., 2016a. Dietary Spirulina (*Arthrospira platensis*) replacement enhances performance of juvenile Nile tilapia (*Oreochromis niloticus*). Journal of Applied Phycology 28, 1023–1030. https://doi.org/10.1007/s10811-015-0661-y

Velasquez, S.F., Chan, M.A., Abisado, R.G., Traifalgar, R.F.M., Tayamen, M.M., Maliwat, G.C.F., Ragaza, J.A., 2016b. Dietary Spirulina (*Arthrospira platensis*) replacement enhances performance of juvenile Nile tilapia (*Oreochromis niloticus*). Journal of Applied Phycology 28, 1023–1030. https://doi.org/10.1007/s10811-015-0661-y

Viswanaathan, S., Perumal, P.K., Sundaram, S., 2022. Integrated Approach for Carbon Sequestration and Wastewater Treatment Using Algal–Bacterial Consortia: Opportunities and Challenges. Sustainability 14, 1075. https://doi.org/10.3390/su14031075

Wang, Y., Cui, Y., Yang, Y., Cai, F., 2000. Compensatory growth in hybrid tilapia, *Oreochromis mossambicus*× *O. niloticus*, reared in seawater. Aquaculture 189, 101–108.

Xiao, J.-X., Zhou, F., Yin, N., Zhou, J., Gao, S., Li, H., Shao, Q.-J., Xu, J., 2013. Compensatory growth of juvenile black sea bream, *Acanthopagrus schlegelii* with cyclical feed deprivation and refeeding. Aquaculture Research 44, 1045–1057. https://doi.org/10.1111/j.1365-2109.2012.03108.x

Youssef, I.M.I., Saleh, E.S.E., Tawfeek, S.S., Abdel-Fadeel, A.A.A., Abdel-Razik, A.-R.H., Abdel-Daim, A.S.A., 2023. Effect of Spirulina platensis on growth, hematological, biochemical, and immunological parameters of Nile tilapia (*Oreochromis niloticus*). Tropical Animal Health and Production 55, 275. https://doi.org/10.1007/s11250-023-03690-5

Zander, K., Risius, A., Feucht, Y., Janssen, M., Hamm, U., 2018. Sustainable Aquaculture Products: Implications of Consumer Awareness and of Consumer Preferences for Promising Market Communication in Germany. Journal of Aquatic Food Product Technology 27, 5–20. https://doi.org/10.1080/10498850.2017.1390028

Zhang, Z., Xu, L., Liu, W., Yang, Y., Du, Z., Zhou, Z., 2014. Effects of partially replacing dietary soybean meal or cottonseed meal with completely hydrolyzed feather meal (defatted rice bran as the carrier) on production, cytokines, adhesive gut bacteria, and disease resistance in hybrid tilapia (*Oreochromis niloticus* ♀ × *Oreochromis aureus* ♂). Fish & Shellfish Immunology 41, 517–525. https://doi.org/10.1016/j.fsi.2014.09.039

Zhou, Q.-C., Yue, Y.-R., 2010. Effect of replacing soybean meal with canola meal on growth, feed utilization and haematological indices of juvenile hybrid tilapia, *Oreochromis niloticus*×*Oreochromis aureus*. Aquaculture Research 41, 982–990. https://doi.org/10.1111/j.1365-2109.2009.02381.x

Chapter III

Impact of organic-based and circular economy-driven feeds on performance and robustness of juvenile gilthead seabream (Sparus aurata)

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ABSTRACT

To align with societal demands, aquaculture needs to enhance the environmental performance of its feeds, while ensuring the health of farmed organisms. Accordingly, novel formulations could be based on organic or circular economy-driven ingredients. This study aimed to evaluate the global warming potential (GWP) and digestibility of such feeds and their effects on performance, feed utilization and health status of juvenile gilthead seabream (*Sparus aurata*) under grow-out conditions and after exposure to a challenge event (overcrowding). Three isonitrogenous and isoenergetic experimental diets were formulated: a control (CTRL) commercial-like feed; an organic (ORG) diet based on ingredients compatible with organic certification, with limited inclusion of animal proteins; an eco-efficient (ECO) feed using circular economy-driven subproducts, with limited inclusion of fishmeal. The GWP of each feed was calculated using the Life Cycle Impact Assessment methodology. Seabream were fed three times daily, in triplicate, according to a feeding table during a growth period of 9 weeks and challenge period of 2 weeks, with an initial density of 12.5 kg/m^3 (from 8 kg/m^3 at the end of the growth stage). Although at the end of the growth period final body weight was higher in fish fed diets CTRL and ECO than ORG fish, all dietary groups increased their initial body weight at least three-fold. Fish health and overall robustness to stress was maintained in the growth and challenge periods based on the performance results and relative expression of molecular biomarkers for gut health, oxidative status and immune condition. All feeds were efficiently utilized and highly digestible. Despite having a higher GWP, ORG and ECO feeds may provide societal benefits, and particularly ORG fish can increase phosphorus retention. Organic and circular economy-driven feeds have potential to address consumer concerns and decrease some of the environmental impacts of aquaculture, while ensuring good fish performance and robustness.

Keywords: Aquafeeds; Global Warming Potential; Fish Performance; Fish Robustness; Gilthead Seabream.

Chapter III

INTRODUCTION

Nowadays, society is more demanding with the sustainability of food and aquaculture products (Feucht and Zander, 2015; López-Mas et al., 2021; Reig et al., 2019; Zander et al., 2018). Consumers are particularly concerned with interventions in the environment (*e.g.*, the organic food movement), carbon footprint and the health of farmed aquatic animals (Cao et al., 2013; Regueiro et al., 2022). Consumers' purchasing decisions often hinge on whether food production practices align with their preferences and concerns (Campbell et al., 2022). In this respect, aquaculture must promote its societal perception, improve its environmental performance and enhance farming conditions (Mente et al., 2011). Particularly, this goes in accordance with the objectives of the European Union (EU), which aim to increase aquaculture production focusing on environmental sustainability, food safety and ethical standards, while considering and meeting consumer demands (Campbell et al., 2022; EC, 2019, 2020). Accordingly, the industry needs to address several concerns, including the environmental sustainability of aquafeeds.

Aquaculture is focused on reducing the environmental impacts of its feeds, which remains a serious issue. There has been some progress to reduce the reliance and environmental footprint of commonly used feed ingredients (*e.g.*, marine and soy-based sources), mainly through the implementation of responsible management and sourcing practices, certification schemes, regulatory frameworks and technological advancements (Glencross et al., 2024; Newton et al., 2023). However, the progress in adopting environmentally sustainable practices varies across different regions. Despite notable advancements, aquafeeds production continue to play a significant role in contributing to the industry's environmental impacts, particularly in terms of resource consumption and/or global warming (Hilmarsdóttir et al., 2021; Little et al., 2016; Malcorps et al., 2019; Newton et al., 2023). Feed ingredients can represent around 60-70% of the carbon emissions of seafood farming (MacLeod et al., 2020). Soybean usage in aquafeeds has been estimated to account for up to 75% of aquaculture greenhouse gases (GHG) emissions, estimated to be between 2.9 and 3.8 kg CO_2 eq kg^{-1} live weight of

fish at the farm gate (Ghosh et al., 2020). Hence, a possible strategy to enhance consumer perception and potentially reduce some of aquaculture environmental impacts, could be through the development of alternative feed formulations.

Organic and circular economy (eco-efficient) based feed ingredients are well perceived by society and may provide some environmental benefits. Organic formulations address environmental, ethical and food safety concerns, widely regarded by society (Altintzoglou et al., 2013). Especially circular economy feed concepts may reduce forage fish demand, promote system-wide performance and enhance resource efficiency (Cottrell et al., 2020; Gephart et al., 2021; Stevens et al., 2018). However, these benefits vary significantly from production sites and need a thorough evaluation, particularly regarding the global warming potential (GWP).

The GWP is a relevant measure to indicate the environmental friendliness of aquafeeds. Nowadays, the GWP is particularly relevant, as it is considered a good indicator of climate change due to carbon or greenhouse gas emissions (*e.g.*, CO_2) (Delistavrou et al., 2023). The GWP can be assessed through a Life Cycle Assessment (LCA), similar to what has been done by Basto-Silva et al. (2022) and Bergman et al. (2024) with gilthead seabream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*) feeds, respectively. This assessment is particularly relevant when feed formulations are based on distinct types of alternative ingredients.

Potential alternative ingredients must be widely available, while promoting fish performance and considering the environmental footprint and animal welfare. Suitable alternatives could include land animal by-products (LAPs; *e.g.*, blood-, feather- and poultry meals), single cell microorganisms (SCMs; *e.g.*, microalgae, yeast, bacteria) and some vegetables (*e.g.*, potato, pea, sunflower, rapeseed, wheat, corn). LAPs are affordable, widely available and can reduce waste generation while valorising side streams (Colombo et al., 2022; Glencross et al., 2024; Sandström et al., 2022; Toldrá et al., 2016). SCMs have rapid growth, can be intensively produced year-round and its cultivation systems are amenable to a high degree of automation (Gupta et al., 2022; Sharif et al., 2021). Methods, infrastructure and

systems for large-scale production and processing of vegetables are already well-established (Glencross et al., 2024). All these ingredients and their combinations have shown positive results in fish performance and robustness (Aragão et al., 2020; Estévez and Vasilaki, 2023; Naya-Català et al., 2021; Pérez-Pascual et al., 2020; Petereit et al., 2022; Tefal et al., 2023a; Vale Pereira et al., 2023). However, the effects of feed formulations in fish, must be monitored on a case-by-case basis. This is especially important since some ingredients and combinations, at certain inclusion levels, may bring disturbances and malfunctions that can affect nutrient absorption and immune response (Aragão et al., 2020, 2022). Particular attention should be given to the intestine, as the tissue is one of the primary targets of dietary changes and given its key role in fish digestion/metabolism (Aragão et al., 2020, 2022). This is relevant in commercially important species, such as gilthead seabream (*Sparus aurata*), a pivotal farmed fish part of the gastronomic culture and seafood economy of the Mediterranean, with an annual global production of approximately 280 thousand metric tonnes (FAO, 2022; Pérez-Lloréns et al., 2021).

This study aimed to evaluate the digestibility and effects of feeds formulated within circular economy-driven (eco-efficient) or organic frameworks, on performance, feed utilization and health status of juvenile gilthead seabream (*Sparus aurata*) under grow-out conditions. At the end of the growth period, fish were exposed to a chronic challenge event (overcrowding) to assess their resilience. The global warming potential of the feeds was also evaluated.

Chapter III

MATERIALS AND METHODS

Experimental diets

Three experimental diets: control (CTRL), organic (ORG) and eco-efficient (ECO) were formulated and produced by SPAROS Lda (Olhão, Portugal). Powder ingredients were mixed accordingly to a target formulation in a double-helix mixer (model 500L, TGC Extrusion, France) and grounded (below 2.0 mm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Diets (pellet size: 2.0 mm) were extruded with a twin-screw extruder (model BC45, Clextral, Firminy, France) and dried in a convection oven (OP 750-UF, LTE Scientific, Oldham, UK). After cooling, oils were added to the pellets by vacuum coating (model PG-10VCLAB, Dinnisen, Sevenum, The Netherlands). Throughout the duration of the study, experimental diets were stored inside plastic buckets at room temperature in a cool and aerated storage room. Representative samples from each diet were collected and analysed for proximate composition and amino acid profile analyses.

The formulation concept and ingredient selection (Table 1) was based within a circular economy or organic framework (ingredients that can be found on the market as organic), on market availability and nutritional composition to fulfil the known nutritional requirements of juvenile gilthead seabream. The control feed (CTRL) was formulated to mimic a commercial formulation, being soy-free and with medium levels of LAPs. The organic (ORG) feed was designed to include ingredients compatible with organic certification, SCMs (microalgae, yeast) and with limited inclusion of fishmeal and LAPs, which were replaced with plant sources (*e.g.*, pea protein, potato protein, rapeseed meal and oil). The eco-efficient (ECO) feed was formulated with ingredients similar to those used in the CTRL feed, but with higher inclusion levels of LAPs (feathermeal hydrolysate, poultry meal and poultry blood meal) and limited fishmeal. All diets were formulated to be isonitrogenous (crude protein of ~51% as fed) and isoenergetic (gross energy of ~22.2 kJ/g as fed) (Table 1). The global warming potential (GWP) of each feed is presented in Table 1. Amino acid profiles are presented in Table 2. The

dietary treatments (CTRL, ORG and ECO) were randomly assigned to replicate tanks (*n* = 3 replicates per dietary treatment).

Table 1 - Diet formulation (% inclusion levels), proximate composition (% as fed) and global warming potential (GWP; kg CO_2 eq/ton feed) of the experimental diets (CTRL, ORG and ECO) for gilthead seabream (*Sparus aurata*).

Ingredients (% inclusion levels)	CTRL	ORG	ECO
Fishmeal Super Prime[1]	15.00	15.00	
Fishmeal[2]	5.00		5.00
Fish protein hydrolysate[3]	3.00		3.00
Poultry meal[4]	15.00		20.00
Poultry blood meal[5]	3.00		5.00
Feathermeal hydrolysate[6]	5.00		10.00
Microbial meal[7]	4.00		4.00
Brewer's yeast[8]		5.00	
***Arthrospira platensis*[9]**		5.00	
Potato protein concentrate[10]		8.90	
Pea protein concentrate[11]		11.00	
Wheat gluten[12]		11.00	
Corn gluten meal[13]	8.00		5.70
Guar korma[14]	5.00	9.50	5.00
Rapeseed meal[15]		4.50	
Sunflower meal[16]	3.00		6.00
Wheat meal[17]	13.48	7.58	12.63
Whole peas[18]	5.50	5.50	5.50
Vitamin and mineral premix[19]	1.00	1.00	1.00
Choline chloride[20]	0.20	0.20	0.20
Antioxidant[21]	0.20	0.20	0.20
Mono-calcium phosphate[22]		1.10	1.65
L-Lysine[23]			0.50
DL-Methionine[24]			0.05
Yttrium oxide[25]	0.02	0.02	0.02
Algae meal (*Schyzochytrium* spp)[26]			1.30
Rapeseed lecithin[27]	0.50	0.50	0.50
Fish oil[28]	4.45	7.00	4.45
Salmon oil[29]	8.65		8.30
Rapeseed oil[30]		7.00	

Proximate Composition (% as fed)	CTRL	ORG	ECO
Dry matter (DM)	94.31	96.91	95.56
Ash	7.76	7.03	5.76
Crude protein	50.61	51.05	51.40
Crude fat	17.66	17.93	17.80
Total phosphorus	1.10	1.02	1.31
Gross energy (kJ/g^{-1})	21.80	22.2	22.54
GWP (kg CO_2 eq/ton feed)	1407	1961	1550

Proximate composition values are reported as mean of duplicate analyses.

[1] Fishmeal Super Prime, Diamante: 66.3% CP, 11.5% CF; Pesquera Diamante, Peru.

[2] Fishmeal, CONRESA: 61.2% CP, 8.4% CF; Conserveros Reunidos S.A., Spain.

[3] Fish protein hydrolysate, CPSP90: 82.6% CP, 9.6% CF; Sopropêche, France.

[4] Poultry meal: 62.4% CP, 12.5% CF; SAVINOR UTS, Portugal.

[5] Poultry blood meal: 90.0% CP, 1.0% CF, ECB COMPANY SRL A S.U, Italy.

[6] Feathermeal hydrolysate EM'PAQ: 88.8% CP, 1.6% CF; Empro Europe, The Netherlands.

[7] Microbial meal (*Corynebacterium glutamicum*), Aminopro NT70: 74.1% CP, 3.1% CF, MAZZOLENI SPA, Italy.

[8] Brewer's yeast: 38.9% CP, 4.5% CF; Premix Lda, Portugal.

[9] *Arthrospira platensis*: 72.1% CP, 1.0% CF, Sopropêche, France.

[10] Potato protein concentrate, Protamyl: 77.0% CP, 1.4% CF, AVEBE, The Netherlands.

[11] Pea protein concentrate, Lysamine GPS: 78.1% CP, 8.3 % CF, Roquette, France.

[12] Wheat gluten, VITAL: 80.4% CP, 5.8 % CF, Roquette, France.

[13] Corn gluten meal: 61.2% CP, 5.2 % CF, COPAM, Portugal.

[14] Guar korma, Seah International, France.

[15] Solvent extracted rapeseed meal: 34.3 %CP, 2.1 % CF, Ribeiro & Sousa Lda., Portugal.

[16] Solvent extracted dehulled sunflower meal, HiPro: 42.9 % CP, 3.8% CF, AGP Slovakia, s.r.o, Slovakia.

[17] Wheat meal: 11.7 % CP, 1.6% CF, Molisur, Spain.

[18] Whole peas: 19.6% CP, 2.2% CF, Ribeiro & Sousa Lda., Portugal.

[19] Vitamin and mineral premix, WISIUM MIX AQUA 1.5%: PREMIX Lda, Portugal. Vitamins (IU or mg/Kg diet): DL-alphatocopherol acetate, 100mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamine, 30 mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1 mg; nicotidin acid, 200 mg; folic acid, 15mg; ascorbic acid, 1000 mg; inositol, 500mg; biotin, 3 mg; calcium panthotenate, 100mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate. 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings.

[20] Choline chloride 50%: ORFFA, The Netherlands.

[21] Antioxidant, VERDILOX: Kemin Europe NV, Belgium.

[22] Mono-calcium phosphate, ALIPHOS MONOCAL: 22.7% P, 17.5% Ca, ALIPHOS, Belgium.

[23] L-Lysine 99%: Ajinomoto EUROLYSINE S.A.S, France.

[24] DL-Methionine 99%: Rhodimet NP99, ADISSEO, France.

[25] Yttrium oxide, Amperit: Höganäs Germany GmbH, Germany.

[26] Algae meal (*Schizochytrium* spp.): 11% CP, 49.4% CL, 16% DHA, Allmicroalgae, Portugal.

[27]Rapeseed lecithin, CANOLECITHIN F60: 94% CL, Novastell, France.

[28] Fish oil: 98.1% CL, 16% EPA; 12% DHA, Sopropêche, France.

[29] Salmon oil: 98.3% CL, 4.6% EPA; 5.2% DHA, Sopropêche, France.

[30] Rapeseed oil: 98.2% CL, JC Coimbra, Portugal.

Table 2 - Amino acid composition (mg AA g^{-1} dry weight) of the experimental diets (CTRL, ORG and ECO) for gilthead seabream (*Sparus aurata*).

Amino Acids (mg AA g^{-1} dry weight)	CTRL	ORG	ECO
Arginine	31.20	33.79	33.01
Histidine	11.26	11.60	10.97
Lysine	26.33	29.11	27.86
Threonine	23.82	21.23	24.76
Isoleucine	21.86	24.23	22.08
Leucine	39.16	38.54	39.47
Valine	25.84	26.66	27.77
Methionine	8.61	10.27	10.31
Phenylalanine	22.78	25.25	23.66
Cystine	8.42	8.93	10.94
Tyrosine	15.91	19.91	18.17
Aspartic Acid + Asparagine	38.34	44.84	37.48
Glutamic Acid + Glutamine	71.44	94.45	70.44
Alanine	29.29	24.96	28.91
Glycine	33.14	27.15	34.78
Proline	30.36	31.40	33.61
Serine	25.02	25.13	30.65
Taurine	1.95	0.97	1.64

All values are reported as mean of duplicate analyses.

Life Cycle Impact Assessment (LCA)

Global Warming Potential (GWP) was calculated using the Life Cycle Impact Assessments (LCA) methodology with economic allocation. Agribylase, Ecoinvent and Global Feed LCA Institute (GFLI) databases, as well as information present in literature and CarbonCloud (www.carboncloud.com; CarbonCloud, Sweden) were used as sources of background data regarding raw materials. Although this data can present some level of uncertainty, the most accurate information was used. The system boundary was set to include the grow-out, fishery activities or production of feed ingredients (including energy usage), as well as processing and transportation from production to processing locations and final product from factory to markets. Ingredient mixing and pelletization were not considered.

Chapter III

Fish husbandry

Fish feeding

Before starting the experiment, a tailored feeding table was generated according to the species requirements, with the goal of ensuring optimum fish performance under summer temperature conditions and avoid overfeeding. The FiT Feeding Tables™ (www.sparos.pt/products/#fit) tool developed by SPAROS and RIASEARCH' was used. As input data, the experimental conditions (fish body weight, water temperature) from a previous study (Ramos-Pinto et al., 2019) performed in the same fish facilities and season (summer) with gilthead seabream were considered. This choice was made because the experimental conditions were expected to be similar to those of the present trial. Data on diet proximate composition and digestibility were defined based the feeds used. Subsequently, the table was generated using the Energy and Protein fluxes (EP model) defined by Nobre et al. (2019) and derived from the bioenergetic factorial approach (Lupatsch, 2003). The same table was used for all feeds (CTRL, ORG and ECO) since the differences in proximate composition were marginal. The daily feeding rate values obtained were then applied to the thermal growth coefficient (TGC) model, using the formula provided by Besson et al.(2016), in order to estimate fish growth and thereby determine the amount of feed that would be given to each tank daily. The TGC model allows to predict fish daily body weight based on the relationship between water temperature and fish weight allometry (Sun and Wang, 2024). Therefore, to obtain accurate estimations of the feed that would be given, water temperature was measured daily, and two intermediate sampling points were performed to adjust the fish biomass on each tank.

Growth period

The trial was carried out at the Ramalhete Experimental Research Station of the Centre of Marine Sciences of Algarve (CCMAR, Faro, Portugal). Trained scientists performed the trial, following the European Directive 2010/63/EU of European Parliament and of the Council of European Union on the protection of animals used for scientific purposes, being approved by the Committee of Ethic and

Animal Experimentation of CCMAR. The CCMAR facilities and their staff are certified to house and conduct experiments with live animals ('group-1' license by the 'Direção Geral de Veterinaria', Ministry of Agriculture, Rural Development and Fisheries of Portugal).

Gilthead seabream (*Sparus aurata*) juveniles were supplied from a commercial farm and transported to the to the Research Station by an authorized carrier. No mortality or pathological signs were observed in association to transport. During the acclimation period of three weeks, fish were fed twice a day using a commercial diet (Standard 4 Orange, Sorgal, Portugal; 43% CP, 17% CF, according to manufacturer data).

At the start of the trial, fish with an average initial weight of 14.1 ± 0.02g (mean ± SD), were randomly distributed to form 9 homogenous groups (CV < 5%) in outdoor cylinder fiberglass tanks of 500 L. Each replicate tank had 90 fish and an initial stocking density of 2.5 kg/m^3. From the initial stock, 42 fish, that resembled the experimental population, were measured and weighed individually to obtain the initial condition factor. Tanks were supplied with flow-through, gravel-filtered, aerated seawater and subjected to natural photoperiod changes through summer conditions (April-August). Abiotic parameters (temperature: mean 23.5 ± 2.1 °C, ranging from 27.3 °C to 18.8 °C; salinity: 37.8 ± 0.4 ‰; oxygen saturation: 96.3 ± 1.4 %), feed intake and mortality were measured and recorded daily. Fish were fed by hand one of the experimental diets (CTRL, ORG or ECO) using a feeding table as guideline, three times per day from Monday to Saturday (09h45, 11h45, 16h00) and twice on Sundays (09h45, 11h45). If fish were satisfied before all the predetermined feed was given, the leftover weight was recorded. The growth period lasted for 65 days.

Challenge period

Using the same experimental setup and fish that remained on each tank (average *n* = 68 fish/tank) at the end of the growth period, gilthead seabream with an initial weight of 45.9 ± 3.0 g were exposed to chronic crowding stress for two weeks. This was achieved by decreasing the tank water volume to 250 L, consequently increasing the density to 12.5 kg/m^3 (from 8 kg/m^3 at the end of the growth

period). The feeding regimes were kept, and fish were fed similarly to the growth period. Abiotic parameters (temperature: 23.9 ± 0.9 °C; salinity: 38.4 ± 0.4‰; oxygen saturation: 93.7 ± 1.8 %), feed intake and mortality were measured and recorded daily.

Digestibility trial

Nine cylinder-conical fiberglass tanks (*n* = 3 per dietary treatment) of 100 L with homogeneous groups of 20 fish (~25 g each) were fed by hand with the experimental diets (CTRL, ORG and ECO), twice a day (09h45 and 12h00), to apparent satiety. After an adaptation period of three weeks, faeces collection started. Every day, half an hour after feeding, tanks were thoroughly cleaned to remove any uneaten pellets and a recipient, covered with ice packs, was inserted at the water outlet channel at the bottom of the tank to collect faeces by a settling decantation system. On the following day, faeces were collected from each tank and frozen at − 20 °C until analysis. Further, the faecal samples were analysed to indirectly determine the apparent digestibility coefficients (ADCs) of the dietary nutrients using yttrium oxide (Y_2O_3), an inert marker, according to the following formula:

Apparent digestibility coefficients (%), ADC's, of dietary nutrients and energy (Maynard et al., 1979)**:**

$$\text{ADC (\%)} = 100 \times \left[1 - \frac{\text{dietary marker (\%)}}{\text{faecal marker (\%)}} \times \frac{\text{faecal nutrient or energy level}}{\text{dietary nutrient or energy level}}\right]$$

ADC (%) of dry matter (DM):

$$\text{ADC (\%)} = 100 \times \left[1 - \frac{\text{dietary marker (\%)}}{\text{faecal marker (\%)}}\right]$$

Sample collection

All samplings were performed within 24 h following the last meal and fish euthanised with a lethal dose of anaesthetic (1000 mg/L; 2-phenoxyethanol, Sigma-Aldrich, Spain). At the beginning of the growth period, 15 fish from the initial stock were pooled and stored at − 20 °C for subsequent analysis of whole-body composition. At the end of the growth period, sampled fish (*n* = 12 fish per replicate tank) were individually measured and weighted, before their viscera and liver were carefully sampled

and weighed for determination of the somatic indices. From these fish, 6 were pooled (*n* = 3 pools per dietary treatment) and frozen at − 20 °C for analysis of whole-body composition. At the end of both growth and challenge periods, 6 fish from each replicate tank (*n* = 18 fish per dietary treatment) were dissected for the anterior intestine. A small section of the tissue was preserved in RNA *later* (Sigma Aldrich, Spain) until analysed for gene expression. Further, all samples were kept at − 80 °C until further analysis.

Key performance indicators

At the start of the experiment, on day 19, 40 and at the end of the growth and challenge periods, fish were counted, and bulk weighted to monitor growth performance, feed utilization and retention indicators as follows:

Weight gain (%IBW; WG) = 100 × wet weight gain (g) × initial biomass $(g)^{-1}$

Where wet weight gain (g) = final biomass (g) – initial biomass (g)

Relative growth rate (%.day^{-1}; RGR) (Ricker, 1958) = 100 × (e^{g} – 1)

Where *g* = [ln (final body weight (g)) – ln (initial body weight (g)) × number of feeding $days^{-1}$]

Feed conversion ratio (FCR) = apparent feed intake (g) × wet weight gain $(g)^{-1}$

Voluntary feed intake (% BW.day^{-1}; VFI) = relative growth rate × feed conversion $ratio^{-1}$

Protein efficiency ratio (PER) = wet weight gain (g) × crude protein intake (g DM)$^{-1}$

Viscerosomatic index (%; VSI) = 100 × viscera weight (g) × body weight $(g)^{-1}$

Hepatosomatic index (%; HSI) = 100 × liver weight (g) × body weight $(g)^{-1}$

Condition factor (K) = 100 × body weight (g) × total $length^{3}(cm)^{-1}$

Crude phosphorus intake (mg.kg^{-1}.d^{-1}; CPI) = 1000 × (phosphorus intake (g DM) × biomass weight $(kg)^{-1}$ × number of feeding $days^{-1}$)

Phosphorus (P) gain (mg.kg^{-1}.d^{-1}; PG) = 1000 × (final whole-body P content (% DM) – initial whole-body P content (%DM) × biomass weight (kg)$^{-1}$ × number of feeding days^{-1})

Faecal phosphorus (P) losses (mg.kg^{-1}.d^{-1}; FPL) = crude P intake (mg.kg^{-1}.d^{-1}) × apparent digestibility coefficient of P (%)

Metabolic phosphorus (P) losses (mg.kg^{-1}.d^{-1}; MPL) = crude P intake (mg.kg^{-1}.d^{-1}) – (P gain (mg.kg^{-1}.d^{-1}) + faecal P losses (mg.kg^{-1}.d^{-1})

Nutrient or energy retention (% digestible intake; NR) = 100 × (final whole-body protein, lipid, or energy content – initial whole-body protein, lipid or energy content) × (crude protein, crude lipid, or gross energy intake^{-1} × ADC% of protein, lipid, or energy)

Analytical procedures

Diet samples, faeces and whole-fish were freeze dried and grounded until a homogeneous powder was obtained. Chemical analyses were made in duplicates and following the methodology described by AOAC (Association of Official Analytical Chemists, 2007): dry matter after drying at 105 °C for 24 h; total ash by combustion (550 °C during 12 h) in a muffle furnace; crude protein (N × 6.25) by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection with a Leco N Analyzer (Model FP-528, Leco Corporation, USA); crude lipid by petroleum ether extraction (40-60 °C) using a Soxtec™ 2055 Fat Extraction System (Foss, Denmark), with prior acid hydrolysis with 8.3 M HCl; gross energy in an adiabatic bomb calorimeter (Werke C2000, IKA, Germany);

Phosphorus concentrations in diets, faeces and whole-fish, as well as yttrium concentrations in diets and faeces were initially determined by weighting (50-125mg) dry samples in quartz vessels. Samples were then digested in 6 mL of nitric acid (HNO_3 tracer grade, 70%) in a Discovery SP-D microwave digestion unit according to the following program: 200 °C; 4 min ramp; 3 min hold. The samples were then cooled to room temperature, and a final volume of 10 mL was achieved by adding ultrapure water. Subsequently, samples were diluted in ultrapure water and the standard curves prepared. Mineral quantification was performed by MP-AES (Agilent, model 4200) at 371nm for

phosphorus and 214nm for yttrium. Blank samples containing only the decomposition acid were included to measure the matrix effects of decomposition, which were subtracted from every element in each sample.

Experimental diets were analysed for total amino acid content according to Aragão et al. (2020). Briefly, samples underwent acid hydrolysis (6 M HCl at 116 °C for 48 h in nitrogen-flushed glass vials) and were then pre-column derivatised with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA). Analyses were done by ultra-high-performance liquid chromatography (UPLC) in a Waters reversed-phase amino acid analysis system, using norvaline as an internal standard. The resulting chromatograms were analysed with EMPOWER software (Waters, USA).

Reverse transcription–quantitative real-time PCR (qPCR)

Samples from the anterior intestine from 3 fish per replicate (*n* = 9 per dietary treatment) at the end of the growth and challenge periods were analysed. Total RNA was extracted using the Maxwell® RSC simplyRNA Tissue Kit (Promega, Madison, USA) according to the manufacturer's instructions. Total RNA quality and integrity was determined by denaturing agarose gel electrophoresis, while concentration and purity were based on absorbance at 260 nm and ratios at 260:280 and 260:230 nm, using a Nanodrop OneC (Thermo Fisher Scientific, Waltham, USA). Complementary DNA (cDNA) synthesis was performed by reverse transcription of 1000ng of total RNA using the RevertAid H Minus First Strand Kit (Thermo Fisher Scientific), according to the manufacturer's protocol. Real-time PCR (RT-PCR) was performed in a CFX384 Real Time PCR detection system (Bio-Rad, Hercules, CA, USA) with PowerTrack™ SYBR™ Green chemistry (Thermo Fisher Scientific), using specific primers (Table 3). Primers for each gene were designed using the Geneious Prime version 2023.1 (www.geneious.com) based on sequences from the GenBank database (NCBI; Clark et al., 2016). Biomarkers for the oxidative status include superoxide dismutase (*sod*), catalase (*cat*), glutathione peroxidase (*gpx*) and nuclear factor erythroid 2 – related factor 2 (*nrf2*). Regarding immune

condition, interleukin-1β (*il-1β*), immunoglobin (*igm*) and cyclooxygenase-2 (*cox2*) were analysed. The intestinal epithelial integrity was assessed for mucin13 (*muc13*), claudin 12 (*cldn12*), tight junction protein 2 (*tjp2*), occludin (*ocl*) and proliferating cell nuclear antigen (*pcna*). The RT-PCR assays were run in duplicates in a 10 µl volume containing 2 µl of cDNA, 0.625 µl of each specific forward and reverse primers at 10 µM, 5 µl of PowerTrack™ SYBR™ Green Master Mix (Thermo Fisher Scientific) and 1.75 µl of nuclease-free water. The amplification protocol was set as follows: an initial denaturation step of 2 min at 95 °C, followed by 40 cycles of denaturation for 5 sec at 95 °C and 30 sec at 55, 57 or 59 °C for annealing/extension, depending on the primer specificity. Negative controls without sample templates were consistently executed for each primer set. The specificity of reactions was confirmed through the examination of melting curves, using ramping rates of 0.5 °C/ 5 sec, across a temperature span of 60-95 °C. Gene expression levels were normalised using the geomean from two reference housekeeping genes, elongation factor 1 *α* (*ef1-α*) and 18S ribosomal RNA (*18S*). The relative mRNA expression of the target genes was calculated according to the Pfaffl method (Pfaffl, 2004). The relative gene expressions of fish fed each diet were analyzed at the end of the growth and challenge periods. At the end of the growth period, gene expressions were calculated using the CTRL dietary treatment as reference. To compare the expressions pre and post stress, the expressions at the end of the growth period were used as reference.

Table 3 - Sequences of primers used in qPCR.

Gene	Forward Primer Sequence (5' → 3')	Reverse Primer Sequence (5' → 3')	NCBI GenBank Accession Number
sod	TCACAGGAGAAATCAAAGGGCT	GGACCGCCATGATTCTTACCAT	JQ308832
cat	CGACATGGTGTGGGACTTCT	CGCTCACCATTGGCATTGAC	JQ308823
gpx	TTTACGCCCTGACAGCCAAT	AGTAACGACTGTGGAGCTCG	KC201352
nrf2	TGAAGGAGGAGAAGGAGCGT	AGTACTCGGACGGCGAGTAT	XM_030427725
il-1ß	TCCAAGCTTGCATCTGGAGG	GCTGAAGGGAACAGACACGA	AJ277166
igm	GACAACCTCAGCGTCCTTCA	CTTTTGAGTCTGCAGCGTCG	JQ811851
cox2	GACATCATCAACACTGCCTCC	GATATCACTGCCGCCTGAGT	AM296029
muc13	CTGTCTACTGAACGGGGCAA	ATTCTGTCACTGAACGCCGT	JQ277713
cldn12	AGCCGTATTTGCCTGTCCAG	CGTAACTTTGTGAGGGGGCA	XM_030393069.1
tjp2	CTGCTGGATGTGACACCCAA	GGCGATCCTCTGTCTCAAGG	XM_030417304.1
ocl	TACGGTGGAATCGGAGGGAA	CTGGTGAGACACGACGATGA	JQ692876
pcna	TCATGATCTCCTGCGCCAAG	CAAAGATCAGCTGGACGGGT	KF857335
ef1-α	GGAGATGCACCACGAGTCTC	GCGTTGAAGTTGTCAGCTCC	AF184170
18S	TGCAGAATCCTCGCCAGTAC	GGTGAGCCCGGATCTTCTTC	AM490061

Abbreviations: *sod*: superoxide dismutase; *cat*: catalase; *gpx*: glutathione peroxidase; *nrf2*: nuclear factor erythroid 2 – related factor 2; *il-1ß*: interleukin-1β; *igm*: immunoglobin; *cox2*: cyclooxygenase-2; *muc13*: mucin13; *cldn12*: claudin 12, *tjp2*: tight junction protein 2, *ocl*: occludin; *pcna*: proliferating cell nuclear antigen; *ef1-α:* elongation factor 1 *α*; *18S*: 18S ribosomal RNA.

Data analysis and statistics

All results are expressed as mean ± standard deviation (mean ± SD). Data were checked for normality and homogeneity of variances with Shapiro-Wilk and Levene's test, respectively. Results expressed as percentages (VFI, RGR, VSI, HSI, survival, ADCs, whole-body composition, retentions) were, prior to statistical analysis, transformed using *arcsine* square root. Relative gene expression data were transformed by a Box-Cox transformation. When conditions were met, a One-way ANOVA followed by Tukey's multiple-comparison test were used to identify differences among groups. If

conditions were not verified, a non-parametric Kruskal-Wallis followed by a Dunn's post-hoc pairwise comparison test were performed instead. To identify specific differences in the relative gene expression before and after the stress, a one-way ANOVA planned contrasts analysis (Myers et al., 2010) was planned *a priori*. Here we planned the following contrasts: 1 – across all dietary treatments at the end of the growth and challenge periods, to identify which relative genes expressions were affected by the overcrowding event, regardless of the dietary treatment; 2,3,4 - each dietary treatment independently (CTRL, ORG, ECO) at the end of the growth and challenge periods, to identify which genes' relative expression were significantly affected by the challenge period within each dietary treatment. To complement such analysis and understand the overall response of fish, we followed an integrative approach through an exploratory multivariate analysis. Here, we integrated the relative expression of biomarkers associated with oxidative status, immune condition, intestinal epithelium integrity and homeostasis in a principal component analysis (PCA) using RStudio (Boston, USA). Since the PCA is an unsupervised exploratory technique, it was used to identify dataset underlying structures that could reveal if there was an association between overall response to stress patterns and diets, and also to highlight the main variables that had the most influence on the data. Accordingly, for the PCA analysis, the standard *prcomp* function in R was applied to the auto-scaled matrices, while score plots for the first two principal components (PC1 and PC2) were generated using the *ggbiplot* and *factoextra* packages. PC1 and PC2 were chosen as the main principal components given that their eigen values accounted for most of the dataset variability. The score plots included confidence ellipses representing 95% confidence intervals around the centroid of each data cluster. The *fviz_cos2* function was used to view the quality of representation (*cos2*) of the variables in the principal components. The scores from the retrieved principal components were further analysed as new variables, which underwent a Box-Cox transformation and were subsequently analysed using a Student's t test to identify differences between the growth and challenge periods. The level of significance considered

was $P < 0.05$ for all statistical tests. Statistical analyses were performed using the computer package IBM SPSS Statistics version 26.0 and RStudio version 4.2.

RESULTS

Apparent digestibility coefficients of diets

Nutrient and energy ADCs are presented in Table 4. Diet ORG showed significantly higher ADCs of protein ($p = 0.001$) and phosphorus ($p = 0.023$) compared to the other feeds. Dry matter ($p = 0.516$), lipids ($p = 0.330$) and energy ($p = 0.999$) were similar between ORG and CTRL. Diet ECO showed a similar digestibility to diet CTRL regarding protein ($p = 0.066$) and phosphorus ($p = 0.985$), while dry matter ($p = 0.001$), lipids ($p = 0.001$) and energy ($p = 0.003$) were significantly higher in CTRL.

Table 4 - Apparent digestibility coefficients (ADCs; %) of nutrients and energy of experimental diets (CTRL, ORG and ECO) given to gilthead seabream (*Sparus aurata*) during the growth and challenge periods.

	Diets			
	CTRL	**ORG**	**ECO**	***p* value**
Dry matter (DM; %)	80.6 ± 3.5[a]	79.2 ± 3.1[a]	61.8 ± 9.9[b]	0.003
Protein (%)	94.7 ± 1.1[b]	96.6 ± 0.5[a]	89.6 ± 2.9[b]	0.001
Lipids (%)	98.5 ± 0.3[a]	98.3 ± 0.2[a]	96.4 ± 0.7[b]	0.002
Phosphorus (%)	66.6 ± 9.0[b]	77.5 ± 2.8[a]	67.2 ± 7.8[b]	0.023
Energy (%)	94.0 ± 1.3[a]	94.3 ± 0.7[a]	86.4 ± 3.7[b]	0.003

Data are presented as mean ± standard deviation ($n = 3$). Different superscripts within the same row indicate significant differences (Kruskal-Wallis; $p < 0.05$) between dietary treatments.

Growth performance, feed intake and somatic indices

All fish increased their initial body weight at least three-fold (Table 5). There were no statistical differences regarding the relative growth rate (RGR; $p = 0.061$) among dietary treatments. Nevertheless, the final body weight (FBW) of fish fed ECO and CTRL diets were higher than ORG fish ($p = 0.002$). A similar pattern was observed on the voluntary feed intake (VFI; $p = 0.046$) that consequently reflected a higher feed conversion ratio (FCR; $p = 0.046$) in fish fed ORG compared to CTRL and ECO fish (Table 5). The dietary treatments had no significant impact ($p > 0.05$) regarding the protein

efficiency ratio (PER; p = 0.051), viscerosomatic index (VSI; p = 0.245), hepatosomatic index (HIS; p = 0.757) and condition factor (K; p = 0.174). Similarly, survival was high (>92%) and not affected by the dietary treatments (p = 0.109).

Table 5 - Growth performance, feed intake and somatic indices of gilthead seabream (*Sparus aurata*), after 65 days of feeding (growth period) with three different experimental diets (CTRL, ORG and ECO).

	Diets			
	CTRL	**ORG**	**ECO**	***p* value**
FBW (g)	47.8 ± 1.4[a]	42.6 ± 0.3[b]	48.8 ± 1.6[a]	0.002
RGR (%.day^{-1})	1.9 ± 0.1	1.7 ± 0.0	1.9 ± 0.0	0.061
VFI (%BW.day^{-1})	1.8 ± 0.2[a]	1.4 ± 0.0[b]	1.8 ± 0.1[a]	0.046
FCR	1.1 ± 0.1[b]	1.2 ± 0.0[a]	1.1 ± 0.0[b]	0.046
PER	1.9 ± 0.1	1.6 ± 0.0	1.9 ± 0.1	0.051
VSI (%)	7.7 ± 0.7	8.2 ± 0.2	7.6 ± 0.3	0.245
HSI (%)	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.0	0.757
K	1.4 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	0.174
Survival (%)	96.7 ± 0.1	96.7 ± 1.9	92.9 ± 3.5	0.109

Data are presented as mean ± standard deviation (n = 3 replicates per dietary treatment). Different superscripts within the same row indicate significant differences (one-way ANOVA; $p < 0.05$) between dietary treatments. Abbreviations: FBW: Final body weight; RGR: Relative growth rate; VFI: Voluntary feed intake; FCR: Feed conversion ratio; PER: Protein efficiency ratio; VSI: Viscerosomatic index; HSI: Hepatosomatic index, K: Condition factor.

Fish whole body composition, retentions and phosphorus balance

Regarding data on fish whole-body composition and retentions, most of the nutrients or energy showed no statistically significant differences among dietary treatments in the analysed parameters (Table 6). The only exception was phosphorus retention, which was significantly higher in fish fed ORG than those fed CTRL (p=0.033).

Table 6 - Whole-body composition (% wet weight) and retention of nutrients and energy (% digestible intake) in gilthead seabream (*Sparus aurata*), after 65 days of feeding (growth period) with three different experimental diets (CTRL, ORG and ECO).

		Diets			
	Initial	**CTRL**	**ORG**	**ECO**	***p* value**
Whole body composition (% wet weight)					
Dry matter (DM; %)	26.8 ± 1.1	29.5 ± 0.2	30.2 ± 0.8	30.1 ± 0.4	0.308
Protein (%)	14.9 ± 0.6	15.3 ± 0.4	15.8 ± 0.2	15.8 ± 0.2	0.066
Lipid (%)	7.0 ± 0.3	8.7 ± 0.7	9.3 ± 0.5	8.9 ± 0.4	0.428
Ash (%)	3.9 ± 0.0	4.2 ± 0.4	4.4 ± 0.3	4.2 ± 0.2	0.697
Phosphorus (%)	0.9 ± 0.0	0.8 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.073
Energy (kJ/g)	5.8 ± 0.1	6.5 ± 0.2	6.9 ± 0.3	6.7 ± 0.1	0.208
Retention (% digestible intake)					
Protein (%)	-	28.1 ± 2.1	25.8 ± 0.9	27.8 ± 1.6	0.061
Lipid (%)	-	48.9 ± 6.4	47.1 ± 2.4	48.2 ± 1.6	0.434
Phosphorus (%)	-	65.4 ± 10.3^{b}	78.6 ± 7.6^{a}	67.4 ± 6.8^{ab}	0.033
Energy (%)	-	28.7 ± 2.2	27.2 ± 1.6	27.7 ± 1.4	0.362

Data are presented as mean ± standard deviation (*n* = 3 pools per dietary treatment). Different superscripts within the same row indicate significant differences (one-way ANOVA or Kruskal-Wallis; $p < 0.05$) between dietary treatments.

The daily phosphorus balance is presented in Figure 1. The dietary treatments only had a significant effect on the daily phosphorus faecal loss ($p < 0.001$). Fish fed with ECO diet had significantly higher faecal phosphorus losses (79.0 ± 2.0 mg P kg^{-1} d^{-1}) than those fed with CTRL (65.8 ± 2.5 mg P kg^{-1} d^{-1}) and ORG (42.5 ± 0.8 mg P kg^{-1} d^{-1}). Phosphorus gain (p = 0.111) ranged from 163.0 ± 18.3 mg P kg^{-1} d^{-1} in the ECO treatment to 129.2 ± 19.6 mg P kg^{-1} d^{-1} in the CTRL treatment. Metabolic phosphorus losses were very small and comparable among diets (p = 0.972).

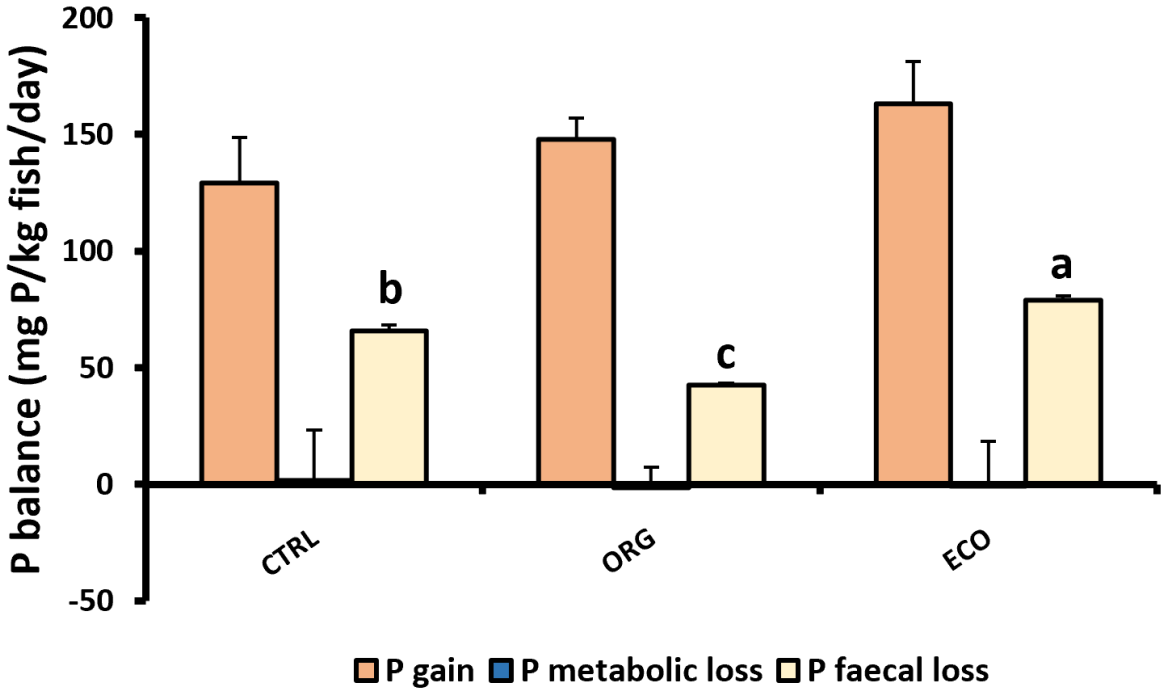

Figure 1 - Daily phosphorus (P) balance (mg P/kg fish/day) in gilthead seabream (*Sparus aurata*), after 65 days of feeding (growth period) with three different experimental diets (CTRL, ORG and ECO). Data are presented as means ± standard deviation (n = 3). Different letters indicate significant differences (one-way ANOVA; $p < 0.05$) between dietary treatments among the same fraction.

Molecular biomarkers analysis

Most of the molecular biomarkers at the end of the growth period were not significantly affected by the dietary treatments (Table 7). The exceptions were interleukin-*1β* (*il-1β*) which was less expressed in fish fed ECO than those fed CTRL (p = 0.040), immunoglobulin M (*igm*), where fish fed ORG and ECO showed an up-regulation ($p < 0.001$) when compared to those fed CTRL and mucin 13 (*muc13*) which was more expressed in fish fed ECO when compared to both CTRL and ORG treatments (p=0.004).

To analyze the effects of the challenge period, the planned contrasts for one-way ANOVA revealed that the overcrowding stressful event significantly affected the relative expression of several genes (Fig. 2). The first contrast compared the expression of all genes in all dietary treatments pre and post stress, revealing that *cat* (p = 0.001), *gpx* ($p < 0.001$), *il-1β* ($p < 0.001$), *igm* ($p < 0.001$), *cox2* (p =

0.001), *cldn12* (p = 0.004) and *tjp2* (p < 0.001) were significantly down-regulated following the challenge period. Fish fed the CTRL diet showed a down-regulation of *il-1β* (p = 0.001), while *pcna* was up-regulated (p = 0.001) following the challenge period. Fish fed ORG had *cat* (p = 0.019), *gpx* (p = 0.021), *igm* (p < 0.001), *cox2* (p = 0.026), *cldn12* (p = 0.035) and *tjp2* (p = 0.015) significantly downregulated when comparing the expression values between fish sampled at the end of the growth and challenge periods. Similarly, *cat* (p = 0.030), *gpx* (p = 0.002), *il-1β* (p = 0.019), *igm* (p < 0.001), *cox2* (p = 0.001), *cldn12* (p = 0.032), *tjp2* (p = 0.001) and *ocl* (p = 0.002) in fish fed ECO had their relative expression decreased after the stress event.

The PCA revealed that fish response was affected by the crowding environment (Fig. 3). When assessing data structure through PCA, the PC1 and PC2 accounted for 58.3% of the dataset variability. The PC1 alone accounted for 40.8% and was strongly related with stress (*e.g.*, before and after stress groups' pattern distinguished along PC1). This was loaded mainly by the lower relative expression of *cat*, *gpx*, *il-1β*, *cox2*, *cldn12*, *tjp2* and *ocl* mainly from fish fed ORG and ECO. This differential response of fish before and after stress was confirmed with significant differences between the groups' scores (p < 0.001; supplementary data). When assessing the impact of each diet on the overall response pattern before and after the stress based on the scores retrieved for each group, fish fed CTRL or ECO diet had no differences, whereas fish fed ORG had different scores before and after stress (p = 0.048).

Table 7 – mRNA relative expression of genes of the anterior intestine of gilthead seabream (*Sparus aurata*) juveniles, after 65 days of feeding (growth period) with three different experimental diets (CTRL, ORG and ECO).

	Diets			
	CTRL	**ORG**	**ECO**	***p* value**
Oxidative Status (mRNA relative expression)				
sod	1.0 ± 0.3	1.0 ± 0.3	1.1 ± 0.2	0.640
cat	1.5 ± 1.3	2.0 ± 1.3	1.9 ± 0.9	0.559
gpx	0.9 ± 0.5	1.0 ± 0.8	1.3 ± 0.8	0.468
nrf2	1.1 ± 0.6	0.9 ± 0.3	0.9 ± 0.2	0.757
Immune Condition (mRNA relative expression)				
il-1β	1.0 ± 0.3^{a}	0.8 ± 0.5^{ab}	0.6 ± 0.4^{b}	0.040
igm	0.9 ± 0.6^{b}	13.8 ± 1.2^{a}	13.2 ± 1.0^{a}	<0.001
cox2	1.1 ± 0.5	1.1 ± 0.9	0.9 ± 0.6	0.781
Epithelium Integrity (mRNA relative expression)				
muc13	1.1 ± 0.4^{b}	1.1 ± 0.4^{b}	1.8 ± 0.4^{a}	0.004
cldn12	1.2 ± 0.7	1.4 ± 1.1	1.0 ± 0.4	0.962
tjp2	1.1 ± 0.6	1.8 ± 1.2	1.7 ± 1.0	0.353
ocl	1.2 ± 0.7	1.6 ± 1.1	1.9 ± 0.7	0.330
pcna	1.1 ± 0.4	1.2 ± 0.3	1.2 ± 0.5	0.564

Data are presented as mean ± standard deviation (n = 9). Different letters indicate significant differences (one-way ANOVA; $p < 0.05$) between dietary treatments. Abbreviations: *sod*: superoxide dismutase; *cat*: catalase; *gpx*: glutathione peroxidase; *nrf2*: nuclear factor erythroid 2 – related factor 2; *il-1β*: interleukin-1β; *igm*: immunoglobin; *cox2*: cyclooxygenase-2; *muc13*: mucin13; *cldn12*: claudin 12, *tjp2*: tight junction protein 2, *ocl*: occludin; *pcna*: proliferating cell nuclear antigen.

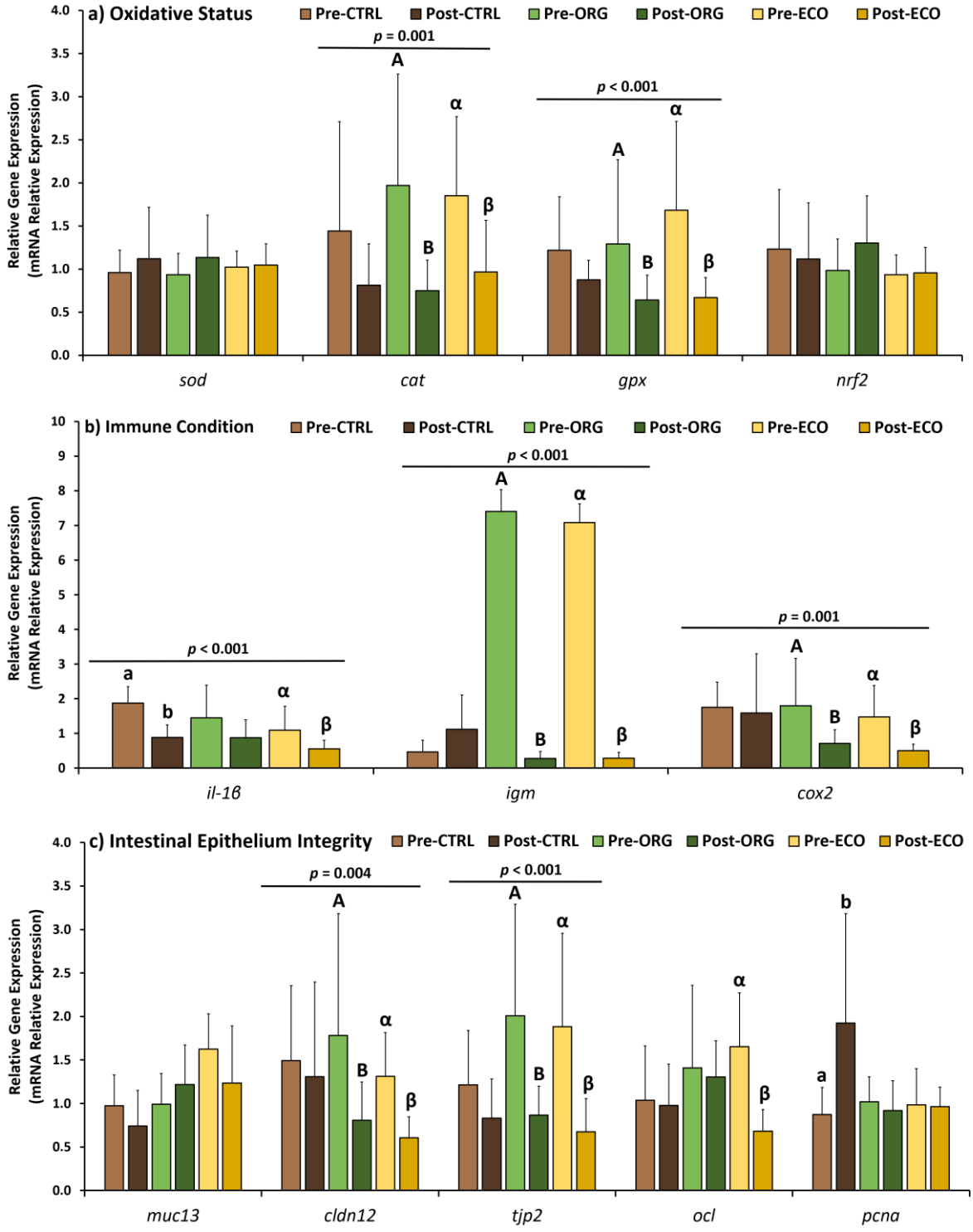

Figure 2 - Relative expression (mRNA relative expression) of genes encoding for **a)** oxidative status (*sod*, *cat*, *gpx* and *nrf2*), **b)** immune condition (*il-1β, igm* and *cox2*) and **c)** intestinal epithelium integrity (*muc13*, *cldn12*, *tjp2*, *ocl* and *pcna*) in the anterior intestine of gilthead seabream (*Sparus aurata*) juveniles, after feeding with three different experimental diets (CTRL, ORG and ECO), analysed pre- and post-stress. Data are presented as mean ± standard deviation (*n* = 9). Different

letters (CTRL – a and b, ORG – A and B, ECO – α and β) indicate significant differences (planned contrasts one-way ANOVA; $p < 0.05$) between sampling periods (growth vs challenge) among the same dietary treatment. Genes within lines on top with *p* value indicate significant differences (planned contrasts one-way ANOVA; $p < 0.05$) between sampling periods (growth vs challenge) while considering all dietary treatments. Molecular biomarkers abbreviations same as in Table 7.

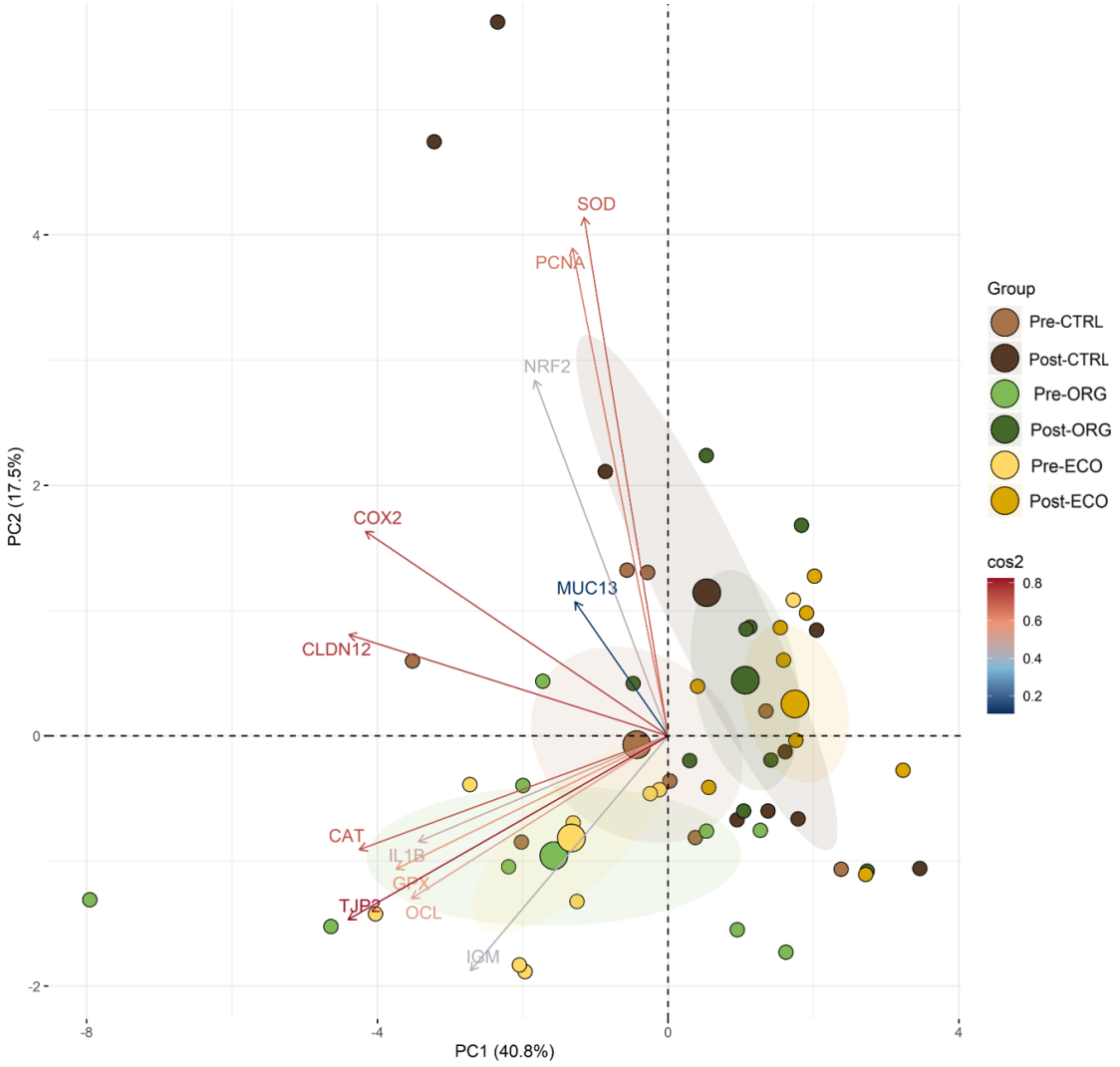

Figure 3 - Score plot from the Principal Component Analysis (PCA) based on the relative gene expression in the anterior intestine of gilthead seabream (*Sparus aurata*) juveniles, after feeding with three different experimental diets (CTRL, ORG and ECO), analysed pre- and post-stress. The first two principal components (PC1 and PC2) are shown on the axis. Scores were grouped by experimental diet (CTRL, ORG, ECO), pre- and post-stress. Each point represents the projection of an individual sample in the PC1 and PC2 axes. The ellipses represent 95% confidence intervals

around the centroid (larger point) of each data cluster. Cos2 scale indicates variable loadings. Abbreviations: Pre-CTRL, Post-CTRL, Pre-ORG, Post-ORG, Pre-ECO, Post-ECO: fish fed diet CTRL, ORG or ECO, respectively, at the end of the growth (pre-) or challenge (post-) period. Molecular biomarkers abbreviations same as in Table 7.

DISCUSSION

To attain a sustainable development, while ensuring the performance and robustness of farmed organisms, aquaculture should formulate feeds considering alternative ingredients that align with societal concerns. Additionally, these new formulations ideally should encompass ingredients that could potentially aid to decrease the industry environmental footprint. In this study, diet formulations based on organic or circular economy-driven frameworks were tested. At the end of the growth period and regardless of the diet, fish health was maintained, based on the relative gene expression and somatic indices, while achieving growth increases of at least three-fold. Although fish fed ORG showed lower growth, the VSI, HSI and K were similar to those from CTRL and ECO fish, suggesting a proper nutrition. Some of these values are slightly different from previous data on seabream fed alternative and organic-based diets (Aragão et al., 2020; Estruch et al., 2020; Tefal et al., 2023b). However, in the present study, values were within the expected range and differences could be attributed to fish size, experimental conditions and feeding regimes (Piccolo et al., 2007, 2017). Furthermore, there was no clear evidence that the chronic overcrowding event negatively affected the overall fish stress response. The experimental feeds were highly digestible, and no differences were observed in feed utilization between experimental treatments. Feed intake is usually regulated by feed proximal composition and palatability (Peng et al., 2016). Feeds were formulated to fulfil the known species nutritional requirements, being isonitrogenous and isoenergetic. Moreover, although the fatty acid profile was not evaluated, the values of eicosapentaenoic + docosahexaenoic acids (EPA+DHA), monounsaturated (MUFA) and total polyunsaturated fatty acids (PUFA) were all estimated and considered during formulation to fulfil the nutritional requirements in all diets. Accordingly, the potential effects of

biochemical composition on the lower fish performance of fish fed ORG are unlikely. Therefore, feed palatability appears to be the most possible cause for the reduced intake.

Particularly the inclusion of plant ingredients, might have been the main responsible for the reduced ORG palatability, decreased intake and consequently lower growth. Compared with CTRL and ECO feeds, ORG had higher amount of plant proteins. A reduction in feed intake and growth has been observed when fish are fed with plant-rich feeds in gilthead seabream, European seabass (*Dicentrarchus labrax*), channel catfish (*Ictalurus punctatus*) and Atlantic salmon (*Salmo salar*) (Abasubong et al., 2021; Pérez-Pascual et al., 2020; Pratoomyot et al., 2010; Randazzo et al., 2023). Conversely, a plant mixture did not alter or even increased intake and growth of juvenile seabream in other studies (Aragão et al., 2020; Dias et al., 2009; Monge-Ortiz et al., 2016). Different results can be attributed to differences in fish size, formulations, ingredient processing and inclusion levels of plants (Christopher et al., 2020; Estruch et al., 2020). Diet ORG could have had a bitter taste and astringent properties, making fish avoid it as they can discriminate the presence of bitter flavours (Enami, 2010; Morais et al., 2019; Puchol et al., 2022). In addition, the presence of antinutritional factors (ANFs; tannins, phytic acid, saponin and gossypol), as well as changes in pellet properties (*e.g.*, hardness, colour) due to a higher inclusion of particular vegetables sources (*e.g.*, spirulina, rapeseed meal) could have also impacted feed palatability and intake (Hardy and Kaushik, 2021; Jiang et al., 2022; Krogdahl et al., 2010; Pérez-Pascual et al., 2020). Regardless of the lower palatability of ORG, all experimental feeds were highly digestible.

All ADCs of the experimental feeds were considered to be high and normal for the species, with only ECO being less digestible than the other feeds, likely due to the inclusion of LAPs and algae meal (NRC, 2012, 1993). LAPs must undergo harsh processing conditions (*e.g.*, heat treatment) during the rendering process that can affect digestibility (Hatlen et al., 2015). Moreover, LAPs can have a low nutritional value, mainly due to presence of connective tissue, skin, high keratin content, and could induce negative impacts on the activities of digestive enzymes, as they may contain protease and lipase

inhibitors (Fasakin et al., 2005; Gisbert et al., 2018; Karapanagiotidis et al., 2019; Nengas et al., 1999; Touhata et al., 2017). Literature has reported that *Schizochytrium* can affect lipid and protein digestibility as it contains lipase and phospholipase inhibitors, as well as due to its complex cellulosic cell wall integrity, which may not be efficiently disrupted during processing and feed production or can resist digestion by digestive enzymes in the fish intestinal tract (Bitou et al., 1999; Kousoulaki et al., 2015; Mayer et al., 1993; Zhang, 2013). Energy digestibility was likely indirectly affected by protein and lipid ADCs, and was in agreement with other studies using alternative formulations (Pérez-Pascual et al., 2020; Tefal et al., 2023a; Vale Pereira et al., 2023). Nevertheless, the observed ECO digestibility differences did not affect feed utilization.

From the whole-body composition, and nutrient and energy retentions, only phosphorus retention differed between dietary treatments, which may suggest that fish physiological mechanisms efficiently utilized feed and metabolic functions remained unaffected by the dietary treatments. No changes in body composition were observed when alternative and eco-organic feeds were fed to gilthead seabream and red seabream (*Pagrus major*) (Gunathilaka et al., 2023; Tefal et al., 2023b). In the case of ECO, it is likely that fish exhibited the ability to compensate for lower diet digestibility through efficient metabolic processes (Fontinha et al., 2021; Psofakis et al., 2020). Similarly, a previous study showed that a feed rich in LAPs also had lower protein digestibility, but showed increased retention, no effects on the whole-body composition of juvenile seabream and resulted in better fish growth than the control treatment (Aragão et al., 2020). Similarly to feed utilization being unaffected by the dietary treatments, fish robustness was also maintained.

At the end of the growth period, in agreement with overall performance, from all the assessed markers, only the expression of *il-1β*, *igm,* and *muc13* were affected by the dietary treatments, suggesting that the experimental feeds maintained fish health status. The cytokine *il-1β* is involved in early immune response to stress, so its downregulation in fish fed ECO could suggest a dampening of a possible deteriorating health effect induced by the stressor (Harris and Bird, 2000; Mokhtar et al.,

2023). Immunoglobulin is a class of antibodies produced by B cells, that trigger and play a crucial role in early humoral immune regulation, tolerance and response against pathogens or environmental stress (Estensoro et al., 2012; Mu et al., 2022; Salinas, 2015). In this sense, an increase in expression of *igm* could suggest a stronger and enhanced adaptive immunity of fish fed ORG and ECO (Harris and Bird, 2000; Mokhtar et al., 2023; Tort, 2011). A study reported an upregulation of *igm* in the anterior intestine of juvenile seabream when fed a diet rich in LAPs (Naya-Català et al., 2021). Mucin13 is involved in protecting and lubricating the epithelial surfaces of various organs, including the intestine (Adamek et al., 2017; Dhanisha et al., 2018; Maher et al., 2010). A higher expression of *mucin13* in ECO fish can indicate an enhancement of the barrier function of the mucosal surfaces and increased protection of the intestinal epithelium against bacteria-, virus- or pH-derived damages, also contributing to a thicker layer (Estruch et al., 2018; Lang et al., 2007; Naya-Català et al., 2021; Pérez-Sánchez et al., 2013). In addition to the growth period, fish were exposed to a chronic challenge event.

After a chronic challenge, planned contrasts and PCA analysis revealed distinct responses in fish before and after the stress, however an overall downregulation of the genes may be just a new allostatic equilibrium. In medium or long-term stressful situations, the relative expression of immune-related, antioxidant and epithelium integrity genes were affected and decreased in the intestine of common carp (*Cyprinus carpio*), darkbarbel catfish (*Pelteobagrus vachelli*), Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*) and gibel carp (*Carassius gibel)* (Dai et al., 2023; Dawood et al., 2022; Gonçalves et al., 2019; Sundh, 2009; Wang et al., 2023). Stress is a immunosuppressor process in chronic stressful periods, hence fish have a higher energy demand that is primarily directed towards coping mechanisms (Tort, 2011; Tort et al., 2004). Therefore, it is common for the molecular machinery responsible for oxidative stress management, immune modulation and intestinal barrier integrity to reduce activity, as these processes are energy-demanding (Harris and Bird, 2000; Mokhtar et al., 2023; Tort, 2011; Tort et al., 2004). Planned contrasts indicated that fish fed ECO and ORG diets had more downregulated genes compared to fish fed CTRL, indicating a stronger downregulation following

stress. At least in ECO feed, such differences could be related with the lower digestibility levels that could result in lower energy available in stressed fish. However, when assessing the overall response integrating all markers using the PCA analysis, it was not significantly different (PC1 scores not significant before and after stress). Nevertheless, the health measurement in the present study was only at the molecular level. Still, there was no indication that gene downregulation in fish fed ECO and ORG feeds reflected in a less robust fish, particularly since fish continued to eat and grow, which suggests that seabream were coping effectively with the stress. In the future, incorporating disease response into similar studies would provide an additional perspective on fish health status. Besides fish robustness, some of the possible environmental impacts of aquafeeds were assessed.

In addition to having no negative effects on fish performance and health, the experimental feeds could provide some environmental consequences. ORG and ECO feeds exhibited a higher global warming potential (GWP) than CTRL due to differences in feed composition. ORG and ECO had higher inclusions of by-products and side streams (LAPs, salmon oil, brewer's yeast and microbial meal), algae (*Arthrospira platensis* and *Schyzochytrium*) and plant-based sources (*e.g.*, potato protein concentrate, wheat gluten, corn gluten meal), along with the reduced inclusion of marine ingredients (fishmeal, fish protein hydrolysate and fish oil). LAPs, SCMs and plants can significantly contribute to carbon emissions due to their production (*e.g.*, fertilizer usage, risk of deforestation), processing (*e.g.*, rendering, grinding, drying) and transport (Campos et al., 2020; Glencross et al., 2024; Maiolo et al., 2020; Malcorps et al., 2019; Newton et al., 2023; Zortea et al., 2018). Atlantic Salmon, rainbow trout, European seabass and meagre (*Argyrosomus regius*) feeds richer in plants, had a higher GWP than others with a higher inclusion of marine resources (Boissy et al., 2011; Konstantinidis et al., 2021). Other studies reported that marine ingredients have a remarkably low carbon footprint and are even considered to be among the most sustainable of all global fisheries (Hilborn et al., 2022; Newton et al., 2023; Parker et al., 2018). Although ORG and ECO showed a higher GWP, it may not necessarily bring a higher environmental impact, as this topic includes many other variables (*e.g.*, eutrophication,

acidification, resource usage, land use) out of the scope of this study. Moreover, the GWP of LAPs, SCMs and other ingredients with high energy requirements, tends to decrease dramatically when clean and renewable energies, within the societal urge for decarbonization, are used (Bartek et al., 2021; Glencross et al., 2024; Saracevic et al., 2019). In addition, despite the higher GWP, it is relevant to note that the inclusion of particularly LAPs and SCMs, could indirectly improve system-wide performance, valorise side streams and by-products from other industries, increase resource efficiency and reduce waste (Agboola et al., 2021; Campos et al., 2020; Glencross et al., 2024; Jones et al., 2020). In fact, phosphorus retention of fish fed diet ORG was higher than CTRL fed fish, which can be related with the higher digestibility of diet ORG (Idris and Patang, 2023; Vale Pereira et al., 2023). Such improvement in retention, could reduce the discharge of phosphorus into the environment, reducing potential negative ecological issues (Chary et al., 2023).

CONCLUSIONS

This study showed that while the organic feed may slightly reduce fish performance, due to lower intake and palatability, likely caused by plant ingredients, it still allowed fish to increase their initial body weight three-fold, in line with the other dietary treatments. Despite the lower digestibility of the eco-efficient feed, all feeds were easily digestible and efficiently utilized. In addition, metabolic functions and fish health remained unaffected throughout the growth period, while overall fish robustness to stress was not affected after the chronic stressful event. Although the novel feeds have a higher GWP associated, they can promote system-wide performance and their environmental footprint can be reduced depending on the type of substrate, carbon and energy sources used. In fact, the organic feed can reduce phosphorus releases into the environment. Organic and circular economy-driven feeds have potential to address consumer concerns and mitigate some of the potential environmental impacts of aquaculture, without compromising fish performance and robustness.

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REFERENCES

Abasubong, K.P., Cheng, H., Li, Z., Wang, C., Huang, Y., Liu, W., Zhang, D., Zhu, X., Jiang, G., 2021. Effects of replacing fish meal with plant proteins at different dietary protein levels on growth and feed intake regulation of juvenile channel catfish *Ictalurus punctatus*. Aquaculture Research 52, 4911–4922. https://doi.org/10.1111/are.15325

Adamek, M., Hazerli, D., Matras, M., Teitge, F., Reichert, M., Steinhagen, D., 2017. Viral infections in common carp lead to a disturbance of mucin expression in mucosal tissues. Fish & Shellfish Immunology 71, 353–358.

Agboola, J.O., Øverland, M., Skrede, A., Hansen, J.Ø., 2021. Yeast as major protein-rich ingredient in aquafeeds: a review of the implications for aquaculture production. Reviews in Aquaculture 13, 949–970. https://doi.org/10.1111/raq.12507

Altintzoglou, T., Honkanen, P., van Haaster-de Winter, M., Olesen, I., 2013. Consumer aspects: Report on consumer aspects related to European organic aquaculture. Deliverable D3.1 [WWW Document]. Nofima. URL https://nofima.com/publication/1482266/ (accessed 7.4.22).

Aragão, C., Cabano, M., Colen, R., Fuentes, J., Dias, J., 2020. Alternative formulations for gilthead seabream diets: Towards a more sustainable production. Aquaculture Nutrition 26, 444–455. https://doi.org/10.1111/anu.13007

Aragão, C., Gonçalves, A.T., Costas, B., Azeredo, R., Xavier, M.J., Engrola, S., 2022. Alternative Proteins for Fish Diets: Implications beyond Growth. Animals 12, 1211. https://doi.org/10.3390/ani12091211

Association of Official Analytical Chemists, 2007. Official Methods of Analysis of AOAC International. Association of Official Analysis Chemists International 1.

Bartek, L., Strid, I., Henryson, K., Junne, S., Rasi, S., Eriksson, M., 2021. Life cycle assessment of fish oil substitute produced by microalgae using food waste. Sustainable Production and Consumption 27, 2002–2021. https://doi.org/10.1016/j.spc.2021.04.033

Basto-Silva, C., García-Meilán, I., Couto, A., Serra, C.R., Enes, P., Oliva-Teles, A., Capilla, E., Guerreiro, I., 2022. Effect of Dietary Plant Feedstuffs and Protein/Carbohydrate Ratio on Gilthead Seabream (*Sparus aurata*) Gut Health and Functionality. Fishes 7, 59. https://doi.org/10.3390/fishes7020059

Bergman, K., Woodhouse, A., Langeland, M., Vidakovic, A., Alriksson, B., Hornborg, S., 2024. Environmental and biodiversity performance of a novel single cell protein for rainbow trout feed. Science of The Total Environment 907, 168018. https://doi.org/10.1016/j.scitotenv.2023.168018

Besson, M., Vandeputte, M., Van Arendonk, J.A.M., Aubin, J., De Boer, I.J.M., Quillet, E., Komen, H., 2016. Influence of water temperature on the economic value of growth rate in fish farming:

The case of sea bass (*Dicentrarchus labrax*) cage farming in the Mediterranean. Aquaculture 462, 47–55. https://doi.org/10.1016/j.aquaculture.2016.04.030

Bitou, N., Ninomiya, M., Tsujita, T., Okuda, H., 1999. Screening of lipase inhibitors from marine algae. Lipids 34, 441–445. https://doi.org/10.1007/s11745-999-0383-7

Boissy, J., Aubin, J., Drissi, A., Van Der Werf, H.M.G., Bell, G.J., Kaushik, S.J., 2011. Environmental impacts of plant-based salmonid diets at feed and farm scales. Aquaculture 321, 61–70. https://doi.org/10.1016/j.aquaculture.2011.08.033

Campbell, K.B., McLean, E., Barrows, F.T., 2022. In Pursuit of Fish-Free Feeds: A Multi-Species Evaluation. Fishes 7, 336. https://doi.org/10.3390/fishes7060336

Campos, I., Valente, L.M.P., Matos, E., Marques, P., Freire, F., 2020. Life-cycle assessment of animal feed ingredients: Poultry fat, poultry by-product meal and hydrolyzed feather meal. Journal of Cleaner Production 252, 119845.

Cao, L., Diana, J.S., Keoleian, G.A., 2013. Role of life cycle assessment in sustainable aquaculture. Reviews in Aquaculture 5, 61–71. https://doi.org/10.1111/j.1753-5131.2012.01080.x

Chary, K., Van Riel, A., Muscat, A., Wilfart, A., Harchaoui, S., Verdegem, M., Filgueira, R., Troell, M., Henriksson, P.J.G., De Boer, I.J.M., Wiegertjes, G.F., 2023. Transforming sustainable aquaculture by applying circularity principles. Reviews in Aquaculture raq.12860. https://doi.org/10.1111/raq.12860

Christopher, R.B., Ahilan, B., Cheryl, A., Samuel, M., 2020. Sunflower meal as an alternative protein source to replace soybean meal in the diet of GIFT strain of Nile tilapia *Oreochromis niloticus*. Indian Journal of Fisheries. 67. https://doi.org/10.21077/ijf.2020.67.3.91750-09

Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Sayers, E.W., 2016. GenBank. Nucleic Acids Research 44, D67–D72. https://doi.org/10.1093/nar/gkv1276

Colombo, S.M., Roy, K., Mraz, J., Wan, A.H.L., Davies, S.J., Tibbetts, S.M., Øverland, M., Francis, D.S., Rocker, M.M., Gasco, L., Spencer, E., Metian, M., Trushenski, J.T., Turchini, G.M., 2022.

Towards achieving circularity and sustainability in feeds for farmed blue foods. Reviews in Aquaculture raq.12766. https://doi.org/10.1111/raq.12766

Cottrell, R.S., Blanchard, J.L., Halpern, B.S., Metian, M., Froehlich, H.E., 2020. Global adoption of novel aquaculture feeds could substantially reduce forage fish demand by 2030. Nature Food 1, 301–308.

Dai, C., Zheng, J., Qi, L., Deng, P., Wu, M., Li, L., Yuan, J., 2023. Chronic stress boosts systemic inflammation and compromises antiviral innate immunity in *Carassius gibel*. Frontiers in Immunology 14, 1105156. https://doi.org/10.3389/fimmu.2023.1105156

Dawood, M.A.O., Alkafafy, M., Sewilam, H., 2022. The antioxidant responses of gills, intestines and livers and blood immunity of common carp (*Cyprinus carpio*) exposed to salinity and temperature stressors. Fish Physiology and Biochemistry 48, 397–408. https://doi.org/10.1007/s10695-022-01052-w

Delistavrou, A., Tilikidou, I., Papaioannou, E., 2023. Climate change risk perception and intentions to buy consumer packaged goods with chemicals containing recycled CO_2. Journal of Cleaner Production 382, 135215. https://doi.org/10.1016/j.jclepro.2022.135215

Dhanisha, S.S., Guruvayoorappan, C., Drishya, S., Abeesh, P., 2018. Mucins: Structural diversity, biosynthesis, its role in pathogenesis and as possible therapeutic targets. Critical reviews in oncology/haematology 122, 98–122.

Dias, J., Conceição, L.E.C., Ribeiro, A.R., Borges, P., Valente, L.M.P., Dinis, M.T., 2009. Practical diet with low fish-derived protein is able to sustain growth performance in gilthead seabream (*Sparus aurata*) during the grow-out phase. Aquaculture 293, 255–262. https://doi.org/10.1016/j.aquaculture.2009.04.042

EC, 2020. COMMUNICATION FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT, THE COUNCIL, THE EUROPEAN ECONOMIC AND SOCIAL COMMITTEE AND THE COMMITTEE OF THE REGIONS EU Biodiversity Strategy for 2030 Bringing nature back into our lives.

EC, 2019. COMMUNICATION FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT, THE EUROPEAN COUNCIL, THE COUNCIL, THE EUROPEAN ECONOMIC AND SOCIAL COMMITTEE AND THE COMMITTEE OF THE REGIONS The European Green Deal.

Enami, H.R., 2010. A Review of Using Canola/Rapeseed Meal in Aquaculture Feeding. Journal of Fisheries and Aquatic Science 6, 22–36. https://doi.org/10.3923/jfas.2011.22.36

Estensoro, I., Calduch-Giner, J.A., Kaushik, S., Pérez-Sánchez, J., Sitjà-Bobadilla, A., 2012. Modulation of the IgM gene expression and IgM immunoreactive cell distribution by the nutritional background in gilthead sea bream (*Sparus aurata*) challenged with *Enteromyxum leei* (Myxozoa). Fish & Shellfish Immunology 33, 401–410. https://doi.org/10.1016/j.fsi.2012.05.029

Estévez, A., Vasilaki, P., 2023. Organic production of gilthead sea bream (*Sparus aurata*) using organic certified green pea protein and seaweed. Effects on growth, feed conversion and final product quality. Aquaculture 571, 739490. https://doi.org/10.1016/j.aquaculture.2023.739490

Estruch, G., Collado, M.C., Monge-Ortiz, R., Tomás-Vidal, A., Jover-Cerdá, M., Peñaranda, D.S., Pérez Martínez, G., Martínez-Llorens, S., 2018. Long-term feeding with high plant protein based diets in gilthead seabream (*Sparus aurata*, L.) leads to changes in the inflammatory and immune related gene expression at intestinal level. BMC Veterinary Research 14, 302. https://doi.org/10.1186/s12917-018-1626-6

Estruch, G., Martínez-Llorens, S., Tomás-Vidal, A., Monge-Ortiz, R., Jover-Cerdá, M., Brown, P.B., Peñaranda, D.S., 2020. Impact of high dietary plant protein with or without marine ingredients in gut mucosa proteome of gilthead seabream (*Sparus aurata*, L.). Journal of Proteomics 216, 103672. https://doi.org/10.1016/j.jprot.2020.103672

FAO, 2022. The State of World Fisheries and Aquaculture 2022. FAO. https://doi.org/10.4060/cc0461en

Fasakin, E., Serwata, R., Davies, S., 2005. Comparative utilization of rendered animal derived products with or without composite mixture of soybean meal in hybrid tilapia (*Oreochromis niloticus*× *Oreochromis mossambicus*) diets. Aquaculture 249, 329–338.

Feucht, Y., Zander, K., 2015. Of earth ponds, flow-through and closed recirculation systems — German consumers' understanding of sustainable aquaculture and its communication. Aquaculture 438, 151–158. https://doi.org/10.1016/j.aquaculture.2015.01.005

Fontinha, F., Magalhães, R., Moutinho, S., Santos, R., Campos, P., Serra, C.R., Aires, T., Oliva-Teles, A., Peres, H., 2021. Effect of dietary poultry meal and oil on growth, digestive capacity, and gut microbiota of gilthead seabream (*Sparus aurata*) juveniles. Aquaculture 530, 735879. https://doi.org/10.1016/j.aquaculture.2020.735879

Gephart, J.A., Henriksson, P.J.G., Parker, R.W.R., Shepon, A., Gorospe, K.D., Bergman, K., Eshel, G., Golden, C.D., Halpern, B.S., Hornborg, S., Jonell, M., Metian, M., Mifflin, K., Newton, R., Tyedmers, P., Zhang, W., Ziegler, F., Troell, M., 2021. Environmental performance of blue foods. Nature 597, 360–365. https://doi.org/10.1038/s41586-021-03889-2

Ghosh, A., Misra, S., Bhattacharyya, R., Sarkar, A., Singh, A.K., Tyagi, V.C., Kumar, R.V., Meena, V.S., 2020. Agriculture, dairy and fishery farming practices and greenhouse gas emission footprint: a strategic appraisal for mitigation. Environmental Science and Pollution Research 27, 10160–10184. https://doi.org/10.1007/s11356-020-07949-4

Gisbert, E., Nolasco, H., Solovyev, M., 2018. Towards the standardization of brush border purification and intestinal alkaline phosphatase quantification in fish with notes on other digestive enzymes. Aquaculture 487, 102–108.

Glencross, B., Ling, X., Gatlin, D., Kaushik, S., Øverland, M., Newton, R., Valente, L.M.P., 2024. A SWOT Analysis of the Use of Marine, Grain, Terrestrial-Animal and Novel Protein Ingredients in Aquaculture Feeds. Reviews in Fisheries Science & Aquaculture 1–39. https://doi.org/10.1080/23308249.2024.2315049

Gonçalves, A.T., Núñez-Acuña, G., Détrée, C., Gallardo-Escárate, C., 2019. Coding/non-coding cross-talk in intestinal epithelium transcriptome gives insights on how fish respond to stocking density. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 29, 14–23. https://doi.org/10.1016/j.cbd.2018.10.005

Gunathilaka, B.E., Jeong, S.-M., Cho, M.-U., Kim, K.-W., Hur, S.-W., Lee, S., You, S.-G., Lee, S.-M., 2023. Effects of Dietary Fish Meal Replacement with Alternative Protein Ingredients and Their Combinations on Growth Performance, Feed Utilization, Fillet Composition, and Biochemical Parameters of Red Seabream (*Pagrus major*). Aquaculture Nutrition 2023, e8883739. https://doi.org/10.1155/2023/8883739

Gupta, A., Barrow, C.J., Puri, M., 2022. Multiproduct biorefinery from marine thraustochytrids towards a circular bioeconomy. Trends in Biotechnology 40, 448–462.

Hardy, R.W., Kaushik, S.J., 2021. Fish nutrition. Academic press.

Harris, J., Bird, D.J., 2000. Modulation of the fish immune system by hormones. Veterinary immunology and immunopathology 77, 163–176.

Hatlen, B., Jakobsen, J.-V., Crampton, V., Alm, M., Langmyhr, E., Espe, M., Hevrøy, E.M., Torstensen, B.E., Liland, N., Waagbø, R., 2015. Growth, feed utilization and endocrine responses in Atlantic salmon (*Salmo salar*) fed diets added poultry by-product meal and blood meal in combination with poultry oil. Aquaculture Nutrition 21, 714–725. https://doi.org/10.1111/anu.12194

Hilborn, R., Buratti, C.C., Díaz Acuña, E., Hively, D., Kolding, J., Kurota, H., Baker, N., Mace, P.M., De Moor, C.L., Muko, S., Osio, G.C., Parma, A.M., Quiroz, J., Melnychuk, M.C., 2022. Recent trends in abundance and fishing pressure of agency-assessed small pelagic fish stocks. Fish and Fisheries 23, 1313–1331. https://doi.org/10.1111/faf.12690

Hilmarsdóttir, G.S., Ogmundarson, Ó., Arason, S., Gudjónsdóttir, M., 2021. Efficiency of fishmeal and fish oil processing of different pelagic fish species: Identification of processing steps for

potential optimization toward protein production for human consumption. Journal of Food Processing and Preservation. 45. https://doi.org/10.1111/jfpp.15294

Idris, A.P.S., Patang, P., 2023. Influence of Different Protein Doses on Digestibility Levels and Food Retention in Reared Eel Fish (Anguilla sp.). Egyptian Journal of Aquatic Biology and Fisheries. 27, 1007–1024. https://doi.org/10.21608/ejabf.2023.333000

Jiang, W., Miao, L., Lin, Y., Ci, L., Liu, B., Ge, X., 2022. Spirulina (*Arthrospira*) platensis as a protein source could improve growth, feed utilisation and digestion and physiological status in juvenile blunt snout bream (*Megalobrama amblycephala*). Aquaculture Reports 22, 100932.

Jones, S.W., Karpol, A., Friedman, S., Maru, B.T., Tracy, B.P., 2020. Recent advances in single cell protein use as a feed ingredient in aquaculture. Current Opinion in Biotechnology 61, 189–197. https://doi.org/10.1016/j.copbio.2019.12.026

Karapanagiotidis, I.T., Psofakis, P., Mente, E., Malandrakis, E., Golomazou, E., 2019. Effect of fishmeal replacement by poultry by-product meal on growth performance, proximate composition, digestive enzyme activity, haematological parameters and gene expression of gilthead seabream (*Sparus aurata*). Aquaculture Nutrition 25, 3–14. https://doi.org/10.1111/anu.12824

Konstantinidis, E., Perdikaris, C., Ganias, K., 2021. Life cycle assessment of seabass and meagre in marine cage farming: From feeding plant to harvesting. Mediterranean Marine Science 22, 125–136. https://doi.org/10.12681/mms.25052

Kousoulaki, K., Østbye, T.-K.K., Krasnov, A., Torgersen, J.S., Mørkøre, T., Sweetman, J., 2015. Metabolism, health and fillet nutritional quality in Atlantic salmon (*Salmo salar*) fed diets containing n-3-rich microalgae. Journal of Nutritional Science 4, e24. https://doi.org/10.1017/jns.2015.14

Krogdahl, Å., Penn, M., Thorsen, J., Refstie, S., Bakke, A.M., 2010. Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. Aquaculture research 41, 333–344.

Lang, T., Hansson, G.C., Samuelsson, T., 2007. Gel-forming mucins appeared early in metazoan evolution. Proceedings of the National Academy of Sciences (PNAS). U.S.A. 104, 16209–16214. https://doi.org/10.1073/pnas.0705984104

Little, D.C., Newton, R.W., Beveridge, M.C.M., 2016. Aquaculture: a rapidly growing and significant source of sustainable food? Status, transitions and potential. Proceedings of the Nutrition Society 75, 274–286. https://doi.org/10.1017/S0029665116000665

López-Mas, L., Claret, A., Reinders, M.J., Banovic, M., Krystallis, A., Guerrero, L., 2021. Farmed or wild fish? Segmenting European consumers based on their beliefs. Aquaculture 532, 735992.

Lupatsch, I., 2003. Factorial Approach to Determining Energy and Protein Requirements of Gilthead seabream (*Sparus aurata*) for Optimal Efficiency of Production.

MacLeod, M.J., Hasan, M.R., Robb, D.H.F., Mamun-Ur-Rashid, M., 2020. Quantifying greenhouse gas emissions from global aquaculture. Scientific Reports 10, 11679. https://doi.org/10.1038/s41598-020-68231-8

Maher, D., Gupta, B., Ebeling, M., Nagata, S., Jaggi, M., Chauhan, S.C., 2010. MUC13 (mucin 13, cell surface associated). http://AtlasGeneticsOncology. org 1023.

Maiolo, S., Parisi, G., Biondi, N., Lunelli, F., Tibaldi, E., Pastres, R., 2020. Fishmeal partial substitution within aquafeed formulations: life cycle assessment of four alternative protein sources. The International Journal of Life Cycle Assessment 25, 1455–1471. https://doi.org/10.1007/s11367-020-01759-z

Malcorps, W., Kok, B., van 't Land, M., Fritz, M., van Doren, D., Servin, K., van der Heijden, P., Palmer, R., Auchterlonie, N.A., Rietkerk, M., 2019. The sustainability conundrum of fishmeal substitution by plant ingredients in shrimp feeds. Sustainability 11, 1212.

Mayer, A.M.S., Paul, V.J., Fenical, W., Norris, J.N., de Carvalho, M.S., Jacobs, R.J., 1993. Phospholipase A2 inhibitors from marine algae.

Maynard, L., Loosli, J., Hintz, H., Warner, R., 1979. Digestive processes in different species. Animal nutrition 7, 21–46.

Mente, E., Karalazos, V., Karapanagiotidis, I.T., Pita, C., 2011. Nutrition in organic aquaculture: an inquiry and a discourse: Nutrition in organic aquaculture. Aquaculture Nutrition 17, e798–e817. https://doi.org/10.1111/j.1365-2095.2010.00846.x

Mokhtar, D.M., Zaccone, G., Alesci, A., Kuciel, M., Hussein, M.T., Sayed, R.K., 2023. Main components of fish immunity: An overview of the fish immune system. Fishes 8, 93.

Monge-Ortiz, R., Martínez-Llorens, S., Márquez, L., Moyano, F.J., Jover-Cerdá, M., Tomás-Vidal, A., 2016. Potential use of high levels of vegetal proteins in diets for market-sized gilthead sea bream (*Sparus aurata*). Archives of Animal Nutrition 70, 155–172. https://doi.org/10.1080/1745039X.2016.1141743

Morais, S., Angotzi, R., Cerdá-Reverter, J.M., Rosel, J., Puchol, S., 2019. Evaluating feed discrimination in seabream *Sparus aurata* using a dualchoice self-feeding system.

Mu, Q., Dong, Z., Kong, W., Wang, X., Yu, J., Ji, W., Su, J., Xu, Z., 2022. Response of immunoglobulin M in gut mucosal immunity of common carp (*Cyprinus carpio*) infected with *Aeromonas hydrophila*. Frontiers in Immunology 13, 1037517. https://doi.org/10.3389/fimmu.2022.1037517

Myers, J.L., Well, A.D., Lorch Jr., R.F., 2010. Research design and statistical analysis, 3rd ed, Research design and statistical analysis, 3rd ed. Routledge/Taylor & Francis Group, New York, NY, US.

Naya-Català, F., do Vale Pereira, G., Piazzon, M.C., Fernandes, A.M., Calduch-Giner, J.A., Sitjà-Bobadilla, A., Conceição, L.E.C., Pérez-Sánchez, J., 2021. Cross-Talk Between Intestinal Microbiota and Host Gene Expression in Gilthead Sea Bream (*Sparus aurata*) Juveniles:

Insights in Fish Feeds for Increased Circularity and Resource Utilization. Frontiers in Physiology 12. https://doi.org/10.3389/fphys.2021.748265

Nengas, I., Alexis, M.N., Davies, S.J., 1999. High inclusion levels of poultry meals and related byproducts in diets for gilthead seabream *Sparus aurata* L. Aquaculture 179, 13–23. https://doi.org/10.1016/S0044-8486(99)00148-9

Newton, R.W., Maiolo, S., Malcorps, W., Little, D.C., 2023. Life Cycle Inventories of marine ingredients. Aquaculture 565, 739096. https://doi.org/10.1016/j.aquaculture.2022.739096

Nobre, A.M., Valente, L.M.P., Conceição, L., Severino, R., Lupatsch, I., 2019. A bioenergetic and protein flux model to simulate fish growth in commercial farms: Application to the gilthead seabream. Aquacultural Engineering 84, 12–22. https://doi.org/10.1016/j.aquaeng.2018.11.001

NRC, 2012. National Research Council (NRC): Nutrient requirements of fish and shrimp: The National Academies Press, Washington, D.C., 2011, 376 + XVI pp, £128 (Hardback), ISBN: 978-0-309-16338-5. Aquaculture International 20, 601–602. https://doi.org/10.1007/s10499-011-9480-6

NRC, 1993. National Research Council. Nutrients requirements of fish. National Academy Press. 114p.

Parker, R.W., Blanchard, J.L., Gardner, C., Green, B.S., Hartmann, K., Tyedmers, P.H., Watson, R.A., 2018. Fuel use and greenhouse gas emissions of world fisheries. Nature Climate Change 8, 333–337.

Peng, K., Shirley, D.C., Xu, Z., Huang, Q., McAllister, T.A., Chaves, A.V., Acharya, S., Liu, C., Wang, S., Wang, Y., 2016. Effect of purple prairie clover (*Dalea purpurea Vent*.) hay and its condensed tannins on growth performance, wool growth, nutrient digestibility, blood metabolites and ruminal fermentation in lambs fed total mixed rations. Animal Feed Science and Technology 222, 100–110. https://doi.org/10.1016/j.anifeedsci.2016.10.012

Pérez-Lloréns, J.L., Acosta, Y., Brun, F.G., 2021. Seafood in Mediterranean countries: A culinary journey through history. International Journal of Gastronomy and Food Science 26, 100437. https://doi.org/10.1016/j.ijgfs.2021.100437

Pérez-Pascual, D., Estellé, J., Dutto, G., Rodde, C., Bernardet, J.-F., Marchand, Y., Duchaud, E., Przybyla, C., Ghigo, J.-M., 2020. Growth Performance and Adaptability of European Sea Bass (*Dicentrarchus labrax*) Gut Microbiota to Alternative Diets Free of Fish Products. Microorganisms 8, 1346. https://doi.org/10.3390/microorganisms8091346

Pérez-Sánchez, J., Estensoro, I., Redondo, M.J., Calduch-Giner, J.A., Kaushik, S., Sitjà-Bobadilla, A., 2013. Mucins as Diagnostic and Prognostic Biomarkers in a Fish-Parasite Model: Transcriptional and Functional Analysis. PLoS ONE 8, e65457. https://doi.org/10.1371/journal.pone.0065457

Petereit, J., Hoerterer, C., Bischoff-Lang, A.A., Conceição, L.E.C., Pereira, G., Johansen, J., Pastres, R., Buck, B.H., 2022. Adult European Seabass (*Dicentrarchus labrax*) Perform Well on Alternative Circular-Economy-Driven Feed Formulations. Sustainability 14, 7279. https://doi.org/10.3390/su14127279

Pfaffl, M.W., 2004. Quantification strategies in real-time PCR. IUL Biotechnology. Ser. 5.

Piccolo, G., De Riu, N., Tulli, F., Cappuccinelli, R., Marono, S., Moniello, G., 2007. Somatic indexes, chemical-nutritive characteristics and metal content in caught and reared sharpsnout seabream (*Diplodus puntazzo*). Italian Journal of Animal Science 6, 351–360. https://doi.org/10.4081/ijas.2007.351

Piccolo, G., Iaconisi, V., Marono, S., Gasco, L., Loponte, R., Nizza, S., Bovera, F., Parisi, G., 2017. Effect of *Tenebrio molitor* larvae meal on growth performance, *in vivo* nutrients digestibility, somatic and marketable indexes of gilthead sea bream (*Sparus aurata*). Animal Feed Science and Technology 226, 12–20. https://doi.org/10.1016/j.anifeedsci.2017.02.007

Pratoomyot, J., Bendiksen, E.Å., Bell, J.G., Tocher, D.R., 2010. Effects of increasing replacement of dietary fishmeal with plant protein sources on growth performance and body lipid composition of Atlantic salmon (*Salmo salar* L.). Aquaculture 305, 124–132. https://doi.org/10.1016/j.aquaculture.2010.04.019

Psofakis, P., Karapanagiotidis, I.T., Malandrakis, E.E., Golomazou, E., Exadactylos, A., Mente, E., 2020. Effect of fishmeal replacement by hydrolyzed feather meal on growth performance, proximate composition, digestive enzyme activity, haematological parameters and growth-related gene expression of gilthead seabream (*Sparus aurata*). Aquaculture 521, 735006. https://doi.org/10.1016/j.aquaculture.2020.735006

Puchol, S., Leal, E., Angotzi, R., Rosel, J., Morais, S., Cerdá-Reverter, J.M., 2022. Dietary discrimination using a dual-choice self-feeding system in seabream (*Sparus aurata*). Aquaculture 559, 738449. https://doi.org/10.1016/j.aquaculture.2022.738449

Ramos-Pinto, L., Martos-Sitcha, J.A., Reis, B., Azeredo, R., Fernandez-Boo, S., Pérez-Sánchez, J., Calduch-Giner, J.A., Engrola, S., Conceição, L.E.C., Dias, J., Silva, T.S., Costas, B., 2019. Dietary tryptophan supplementation induces a transient immune enhancement of gilthead seabream (*Sparus aurata*) juveniles fed fishmeal-free diets. Fish & Shellfish Immunology 93, 240–250. https://doi.org/10.1016/j.fsi.2019.07.033

Randazzo, B., Di Marco, P., Zarantoniello, M., Daniso, E., Cerri, R., Finoia, M.G., Capoccioni, F., Tibaldi, E., Olivotto, I., Cardinaletti, G., 2023. Effects of supplementing a plant protein-rich diet with insect, crayfish or microalgae meals on gilthead sea bream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) growth, physiological status and gut health. Aquaculture 575, 739811. https://doi.org/10.1016/j.aquaculture.2023.739811

Regueiro, L., Newton, R., Soula, M., Méndez, D., Kok, B., Little, D.C., Pastres, R., Johansen, J., Ferreira, M., 2022. Opportunities and limitations for the introduction of circular economy principles in

EU aquaculture based on the regulatory framework. Journal of Industrial Ecology 26, 2033–2044. https://doi.org/10.1111/jiec.13188

Reig, L., Escobar, C., Carrassón, M., Constenla, M., Gil, J.M., Padrós, F., Piferrer, F., Flos, R., 2019. Aquaculture perceptions in the Barcelona metropolitan area from fish and seafood wholesalers, fishmongers, and consumers. Aquaculture 510, 256–266.

Ricker, W.E., 1958. Handbook of Computations for Biological Statistics of Fish Populations. Fisheries Research Board of Canada.

Salinas, I., 2015. The Mucosal Immune System of Teleost Fish. Biology 4, 525–539. https://doi.org/10.3390/biology4030525

Sandström, V., Chrysafi, A., Lamminen, M., Troell, M., Jalava, M., Piipponen, J., Siebert, S., Van Hal, O., Virkki, V., Kummu, M., 2022. Food system by-products upcycled in livestock and aquaculture feeds can increase global food supply. Nature Food 3, 729–740. https://doi.org/10.1038/s43016-022-00589-6

Saracevic, E., Koch, D., Stuermer, B., Mihalyi, B., Miltner, A., Friedl, A., 2019. Economic and Global Warming Potential Assessment of Flexible Power Generation with Biogas Plants. Sustainability 11, 2530. https://doi.org/10.3390/su11092530

Sharif, M., Zafar, M.H., Aqib, A.I., Saeed, M., Farag, M.R., Alagawany, M., 2021. Single cell protein: Sources, mechanism of production, nutritional value and its uses in aquaculture nutrition. Aquaculture 531, 735885.

Stevens, J.R., Newton, R.W., Tlusty, M., Little, D.C., 2018. The rise of aquaculture by-products: Increasing food production, value, and sustainability through strategic utilisation. Marine Policy 90, 115–124.

Sun, X., Wang, Y., 2024. Growth models in aquaculture for hybrid and natural groupers based on early development stage. Aquaculture 578, 740026. https://doi.org/10.1016/j.aquaculture.2023.740026

Sundh, H., 2009. Chronic stress and intestinal barrier function: Implications for infection and inflammation in intensive salmon aquaculture.

Tefal, E., Jauralde, I., Tomás-Vidal, A., Martínez-Llorens, S., Peñaranda, D.S., Jover-Cerdá, M., 2023a. New Organic Raw Materials for Gilthead Seabream (*Sparus aurata*) Feeding and the Effects on Growth, Nutritive Parameters, Digestibility, and Histology. Fishes 8, 330. https://doi.org/10.3390/fishes8060330

Tefal, E., Tomás-Vidal, A., Martínez-Llorens, S., Jauralde, I., Sánchez-Peñaranda, D., Jover-Cerdá, M., 2023b. Effects of Eco-Organic Feed on Growth Performance, Biometric Indices, and Nutrient Retention of Gilthead Seabream (*Sparus aurata*). Sustainability 15, 10750. https://doi.org/10.3390/su151410750

Toldrá, F., Mora, L., Reig, M., 2016. New insights into meat by-product utilization. Meat science 120, 54–59.

Tort, L., 2011. Stress and immune modulation in fish. Developmental & Comparative Immunology 35, 1366–1375.

Tort, L., Balasch, J., MacKenzie, S., 2004. Fish health challenge after stress. Indicators of immunocompetence. Contributions to Science 2, 443–454.

Touhata, K., Koshiishi, T., Satoh, S., Ishida, N., 2017. Evaluation of gastric evacuation and digestion of extruded pellet composed of a single protein source for juvenile red seabream <I>*Pagrus major*</I>. https://doi.org/10.11233/aquaculturesci.65.1

Vale Pereira, G. do, Conceição, L.E.C., Soares, F., Petereit, J., Buck, B.H., Johansen, J., Dias, J., Faccenda, F., 2023. Alternative Feed Formulations Impact Growth Performance, Flesh Quality and Consumer Acceptance of Rainbow Trout (*Oncorhynchus mykiss*). Journal of Marine Science and Engineering 11, 1135. https://doi.org/10.3390/jmse11061135

Wang, M., Liao, S., Zang, X., Fu, Z., Yin, S., Wang, T., 2023. Long-term hypoxia stress-induced oxidative stress, cell apoptosis, and immune response in the intestine of *Pelteobagrus vachelli*. Fish Physiology and Biochemistry 49, 585–597. https://doi.org/10.1007/s10695-023-01204-6

Zander, K., Risius, A., Feucht, Y., Janssen, M., Hamm, U., 2018. Sustainable Aquaculture Products: Implications of Consumer Awareness and of Consumer Preferences for Promising Market Communication in Germany. Journal of Aquatic Food Product Technology 27, 5–20. https://doi.org/10.1080/10498850.2017.1390028

Zhang, C., 2013. Determination of the Digestibility of a Whole-Cell DHA-Rich Algal Product and Its Effect on the Lipid Composition of Rainbow Trout and Atlantic Salmon.

Zhang, W., Tan, B., Deng, J., Dong, X., Yang, Q., Chi, S., Liu, H., Zhang, S., Xie, S., Zhang, H., 2021. Mechanisms by Which Fermented Soybean Meal and Soybean Meal Induced Enteritis in Marine Fish Juvenile Pearl Gentian Grouper. Frontiers in Physiology 12.

Zortea, R.B., Maciel, V.G., Passuello, A., 2018. Sustainability assessment of soybean production in Southern Brazil: A life cycle approach. Sustainable Production and Consumption 13, 102–112.

General Discussion

4. General Discussion

Aquaculture is an essential sector to ensure global food security that must prioritize sustainability while considering its environmental, economic and social impacts. Circular economy-driven and organic aquafeeds address some environmental and ethical concerns that currently can hamper the development of the industry. In addition, these feeds align with consumers preferences and comply with national and international policies and priorities, including those of the EU. Nonetheless, before a full commercial implementation, these feeds require a comprehensive evaluation of their environmental footprint and impacts on the performance and robustness of farmed organisms. This PhD thesis has undertaken an holistic approach investigating the feeding behaviour (Chapter I), performance and feed intake (Chapter I, II and III), feed digestibility (Chapter II and III), feed utilization (Chapter II and III) and robustness (Chapter II and III) of Nile tilapia and gilthead seabream fed circular economy-driven or organic feeds. Additionally, the global warming potential of such feeds was assessed (Chapter III).

4.1. Feeding behaviour

Assessing fish dietary preferences is a novel approach to investigate feed acceptance, since fish is asked to freely choose their preferred diet. In Chapter I, using self-feeders, Nile tilapia exhibited a dietary preference for a specific feed formulated with alternative ingredients (*e.g.*., spirulina, sunflower, quinoa, rapeseed, lupins) that address some of the current environmental concerns and/or ethical issues associated with traditional commercial formulations. This preference was based on fish nutritional needs (post-ingestive and/or post-absorption) and regardless of feed organoleptic characteristics (texture, flavour and odour) (Fortes et al., 2016). Additionally, tilapia avoided feeds with a slight reduction of methionine, revealing that fish can sense in detail the nutritional composition of the diets. Conversely, in the self-selection trial, gilthead seabream showed no feed preferences (Chapter I), which could be due to absence of major differences in taste, smell and texture of the diets or an incapability of noticing any differences. However, these results indicate that self-feeding trials

should be included during the initial screenings of alternative feed ingredients as they allow fish to express their natural feeding behaviours and provide valuable insights on feed regulation and intake.

4.2. Fish performance and feed intake

Feed intake significantly impacts various aspects of aquaculture production, including fish growth and health, being affected, among others by feed formulation. Underfeeding may result in increased aggression among individuals, lower performance and lead to wellbeing concerns, while overfeeding results in feed waste and higher feed conversion ratios (Føre et al., 2016; Huntingford et al., 2012). In Chapters II and III, two feed concepts (ORG - based on ingredients compatible with organic certification and ECO - formulated using circular economy-driven emergent ingredients including by-products from fisheries, aquaculture and agriculture) were tested for Nile tilapia and gilthead seabream. In Chapter II, tilapia significantly reduced their intake of the ORG feed, which contained quinoa and spirulina, compared to the control diet. This contrasted with the self-feeder trial (Chapter I), where tilapia preferred feeds containing both ingredients. Such discrepancy between trials could have been due to various factors such as experimental conditions, fish developmental stage, ingredient batches, feed formulations and feeding regimes. In Chapter III, gilthead seabream fed with ORG (with spirulina) and ECO (no spirulina or quinoa) feeds, only had a lower intake of ORG compared to fish fed CTRL or ECO feeds. In both species, the lower intakes were likely related with a reduced feed palatability, and/or bitter nature of the feeds, due to the inclusion levels of specific ingredients, including quinoa, rapeseed meal, spirulina and plant sources, or their combination with the other ingredients included. In the case of tilapia, the inclusion levels are a strong variable as during the growth trial (Chapter II), fish fed ECO, which contained quinoa, rapeseed meal and spirulina as in ORG, but in a lower amount, had a similar intake to those fed CTRL. In turn, the feed intake affected fish performance in both trials, but with distinct degrees of severity.

For novel fish feeds to be used commercially, they must maintain or enhance fish performance, which is fundamental for farm profitability. Nile tilapia fed ORG had lower growth compared to fish fed CTRL, likely due to reduced feed intake (Chapter II). Nevertheless, the growth of fish fed ECO was not

significantly different from the control treatment. For gilthead seabream, only the ORG feed resulted in slightly lower performance, while the ECO feed sustained an equal growth to fish fed the control diet (Chapter III). Other studies using alternative eco-efficient or organic-based ingredients have also reported distinct effects on performance (Aragão et al., 2020; Estévez and Vasilaki, 2023, 2023; Naya-Català et al., 2021; Pérez-Pascual et al., 2020; Petereit et al., 2022; Tefal et al., 2023a, 2023a; Vale Pereira et al., 2023). Different results can be attributed to differences in fish species, sizes, formulations, ingredient batches and processing, experimental conditions and feeding regimes (Christopher et al., 2020; Estruch et al., 2020). In any case, the reduced performance was not related with nutritional deficiencies or feed digestibility.

4.3. Feed digestibility

Feed digestibility affects growth, ensures efficient nutrient utilization and impacts feed metabolic waste. In Chapters II and III, feed digestibly was assessed for tilapia and gilthead seabream feeds. Although some eco-efficient feeds had reduced digestibility compared to the control diets, the apparent digestibility coefficients remained high, indicating that the novel formulations were easily digestible and unlikely to have negatively impacted fish performance. The causes of a slightly reduced digestibility can be challenging to identify; however the incorporation of LAPs, plants and microalgae could have played a role. Regardless of the feed ADCs, feed utilization was mainly affected when feed intake was reduced.

4.4. Feed utilization

Whole body composition and nutrient/energy retention provide information about fish metabolism and feed utilization efficiency, which affect metabolic nutrient waste. In Chapter III, gilthead seabream showed differences only in phosphorus retention between dietary treatments, while whole-body composition and nutrient/energy retention of tilapia were negatively affected by the ORG and ECO feeds (Chapter II). The reduced feed utilization in tilapia, was most likely directly related with the reduced feed intake, “forcing” the fish to oxidize their lipid reserves, which consequently affected energy composition. Notably, protein content was only slightly reduced,

indicating that tilapia were primarily eating feed to maintain weight. Despite these effects on feed utilization, fish robustness may have not been compromised.

4.5. Fish robustness and somatic indices

Nutrition should maintain/enhance fish robustness, ensure welfare (addressing consumers concerns) and allow them to cope with adverse situations that may occur during the production cycle. Based on the assessed biomarkers for immune condition, oxidative status and gut epithelium integrity/permeability, fish health was barely affected by the ORG and ECO feeds (Chapters II and III). In the tilapia trial (Chapter II), from the eleven analysed biomarkers, only glutathione reductase, an important biomarker for oxidative status, was affected by the dietary treatments, being upregulated in fish fed ECO compared to those fed the control diet. This upregulation could have been induced by the antioxidant properties of quinoa and spirulina. Changes in only one biomarker, likely suggest that fish health was not significantly affected by the alternative feeds. In the gilthead seabream trial (Chapter III), the relative gene expression of molecular biomarkers of the anterior intestine were assessed at the end of the growth period and after exposure to a challenge period (overcrowding). Fish health and robustness were maintained in the growth and challenge periods. Although in the latter period an overall downregulation of many genes occurred, it likely does not necessarily indicate a declined fish health and may be just a new allostatic equilibrium. Other studies have also observed effects on fish health, induced by alternative feeds, in European seabass (*Dicentrarchus labrax*), yellowtail kingfish (*Seriola lalandi*), and rainbow trout (*Oncorhynchus mykiss*) (Dam et al., 2020; Estruch et al., 2018; Lazzarotto et al., 2018; Naya-Català et al., 2021; Torrecillas et al., 2017). For example, adult gilthead seabream, fed with a strict plant protein-based diet, had a lower expression in genes related to pro-inflammatory response and immune condition (Estruch et al., 2018). Feeds based on processed animal proteins, insect meal, yeast and microbial biomasses, fed to juvenile seabream, induced inflammatory systemic markers (Naya-Català et al., 2021).Besides the biomarkers, fish somatic indices and condition factor where only slightly affected by the experimental feeds.

VSI, HSI, and K can be used to understand if fish are receiving adequate nutrition and serve as an indicator of fish welfare. Although gilthead seabream fed the ORG diet showed slightly lower performance, the somatic indices and condition factor were similar to those of fish fed the CTRL and ECO diets (Chapter III). Regarding tilapia, the VSI and HSI were similar to those of fish fed the CTRL and ECO diets, while K was only slightly reduced in fish fed ORG compared to the other dietary treatments, despite the reduced performance (Chapter II). This small decrease may indicate that tilapia ate primarily to maintain their body weight and survive. Since there were no other changes in the indices, it may indicate that the physiological functions and health of tilapia may not have deteriorated. Furthermore, the VSI, HSI, and K for both species were normal and similar to previous published data (Aragão et al., 2020; Asmamaw et al., 2019; Estruch et al., 2020; Ighwela et al., 2011; Keyombe et al., 2017; Piccolo et al., 2017, 2007; Tefal et al., 2023b). Differences among studies can be the result of distinct fish size, developmental stage, experimental conditions, feed formulations and feeding regimes (Piccolo et al., 2007, 2017).

4.6. Feeds' global warming potential

Estimating aquafeeds' GWP provides a glimpse of their total environmental impact, which is relevant for consumers and stakeholders, as well as sustainable aquaculture development. In Chapter III, the GHG emissions of seabream feeds were as follows: the ORG feed (1961 kg CO2 eq/ton product) had the highest GWP, followed by ECO (1550 kg CO2 eq/ton product) and CTRL (1407 kg CO2 eq/ton product) diets. A similar assessment for tilapia feeds showed a slightly different scenario, where the ORG feed had the highest CO_2 emissions (1991 kg CO_2 eq/ton product), followed by the CTRL (1918 kg CO_2 eq/ton product) and ECO (1774 kg CO_2 eq/ton product) feeds (Mendes et al., unpublished data). Plant sources (*e.g.*, pea protein concentrate, wheat, rapeseed meal, sunflower meal, whole peas) were the main ingredients for both species and were also the primary contributors to total GWP (Fig. 1). Plants are associated with deforestation, transportation, processing, as well as heavy use of energy-intensive fertilizers, herbicides, and pesticides, all of which contribute to the carbon footprint (Glencross et al., 2024; Malcorps et al., 2019; Newton et al., 2023; Zortea et al., 2018). A higher GWP

in plant-based feeds has also been noted in aquafeeds for Atlantic salmon, rainbow trout, European seabass and meagre (Boissy et al., 2011; Konstantinidis et al., 2021). Nevertheless, the GWP is only one of the several aspects (*e.g.*, eutrophication, acidification, resource usage, land use) that a LCA can determine (Glencross et al., 2024; Newton et al., 2023). Hence, the higher GWP of the socially-acceptable feeds tends to bring, but does not necessarily mean, greater environmental impacts. Besides, the GWP of SCMs, LAPs and other ingredients that require energy-demanding processes, are highly dependent on the type of energy sources used (Bartek et al., 2021; Glencross et al., 2024). In the context of innovation and decarbonization of society, more greener energies are likely to be used, thus likely reducing the GWP.

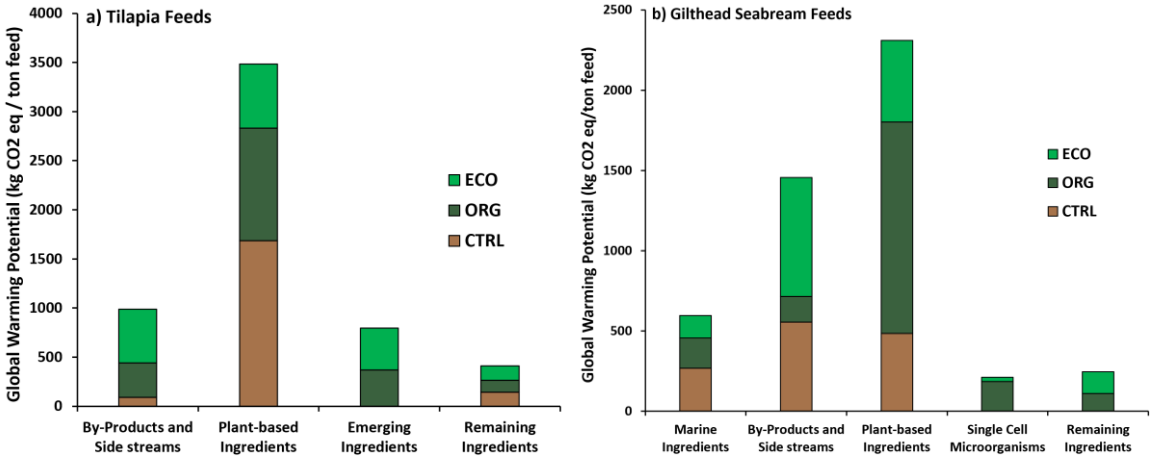

Figure 1 – Contribution of each type of ingredient for the total GWP (kg CO_2 eq/ton product) of a) tilapia and b) gilthead seabream feeds (CTRL, ORG and ECO).

This PhD Thesis provides a broaden evaluation and understanding of the acceptability, digestibility, utilization, effects and global warming potential of novel organic or circular economy-driven feeds on Nile tilapia and gilthead seabream aquaculture. This holistic approach highlighted the potential trade-offs between the environmental impacts and physiological responses of farmed organisms to alternative aquafeeds, which are crucial for the sustainable development of aquaculture.

Conclusions & Future Perspectives

5. Conclusions & Future Perspectives

This PhD Thesis attained the following conclusions:

1. Nile tilapia showed an ability to self-select a diet, expressing their feeding behaviour. Therefore, self-selection experiments may be considered as a possible tool for an initial screening of new aquaculture feed ingredients (**Chapter I**).

2. The socially-acceptable feed formulations induced extreme effects on the voluntary feed intake of Nile tilapia, due to reduced palatability, and resulted in poor feed utilization. Nevertheless, diet digestibility was high and the impact was only noted in fish performance, but not on robustness (**Chapter II**).

3. For gilthead seabream, the organic and circular economy-driven feeds were easily digestible and efficiently utilized by the fish, ensuring good fish performance and robustness, even after a chronic stressful event (**Chapter III**).

4. The organic and circular economy-driven tilapia feeds, and the organic seabream feed had the highest GWP. However, in the long-term, such diets may promote a better system-wide performance (**Chapter III**).

The main findings of this PhD Thesis suggested several approaches to deepen the tailoring of aquafeeds, so to become increasingly sustainable. Feed formulations can be optimized by carefully selecting ingredients and setting limits to their inclusion levels, incorporating distinct combinations of protein sources or adding feed attractants to enhance feed palatability and acceptability. Analysing the fatty acid profiles and the digestibility of individual ingredients could provide more accurate insights about feed utilization and overall digestibility. Assessing other environmental impacts of feed ingredients, including eutrophication, acidification, resource usage and/or land use, would provide a more holistic view of the environmental footprint of aquafeeds. Furthermore, when determining the environmental impacts of the feed ingredients, it should be a priority that the assessments are carried out using the same rules and framework, to minimize the variations and uncertainty, especially if the

data are received from external sources. In the future, it is recommended that more self-feeding and growth studies should be conducted on other commercially important species (*e.g.*, salmon and carp) in response to alternative aquafeeds, as such studies would take into account the differences in the species' digestive systems, physiological responses, metabolic rates and feeding behaviours. To allow the inclusion of alternative ingredients, additional funding, better regulatory frameworks, increased consumer acceptance and education, enhanced cost-effective production and processing techniques, as well as more access to renewable energies would be beneficial. Ultimately, a holistic and transparent approach that balances the nutrition, feeding behaviour, performance and robustness of farmed species with the environmental, social and economic sustainability of aquaculture is necessary to ensure the long-term development of the sector, as a vital source of animal protein for the booming global population.

GENERAL BIBLIOGRAPHY

6. General Bibliography

Abdel-Ghany, H.M., Salem, M.E.-S., Ezzat, A.A., Essa, M.A., Helal, A.M., Ismail, R.F., El-Sayed, A.-F.M., 2021. Effects of different levels of dietary lipids on growth performance, liver histology and cold tolerance of Nile tilapia (*Oreochromis niloticus*). Journal of Thermal Biology 96, 102833. https://doi.org/10.1016/j.jtherbio.2020.102833

Adarme-Vega, T.C., Lim, D.K., Timmins, M., Vernen, F., Li, Y., Schenk, P.M., 2012. Microalgal biofactories: a promising approach towards sustainable omega-3 fatty acid production. Microbial cell factories 11, 1–10.

Ahmed, N., Thompson, S., Turchini, G.M., 2020. Organic aquaculture productivity, environmental sustainability, and food security: insights from organic agriculture. Food Security 12, 1253–1267. https://doi.org/10.1007/s12571-020-01090-3

Ansari, F.A., Guldhe, A., Gupta, S.K., Rawat, I., Bux, F., 2021. Improving the feasibility of aquaculture feed by using microalgae. Environmental Science and Pollution Research 28, 43234–43257. https://doi.org/10.1007/s11356-021-14989-x

Aragão, C., Cabano, M., Colen, R., Fuentes, J., Dias, J., 2020. Alternative formulations for gilthead seabream diets: Towards a more sustainable production. Aquaculture Nutrition 26, 444–455. https://doi.org/10.1111/anu.13007

Aragão, C., Gonçalves, A.T., Costas, B., Azeredo, R., Xavier, M.J., Engrola, S., 2022. Alternative Proteins for Fish Diets: Implications beyond Growth. Animals 12, 1211. https://doi.org/10.3390/ani12091211

Asmamaw, B., Beyene, B., Tessema, M., Assefa, A., 2019. Length-weight relationships and condition factor of Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) (Cichlidae) in Koka Reservoir, Ethiopia Volume 4, 5–6.

Assan, D., Huang, Y., Mustapha, U.F., Addah, M.N., Li, G., Chen, H., 2021. Fish Feed Intake, Feeding Behavior, and the Physiological Response of Apelin to Fasting and Refeeding. Frontiers in Endocrinology (Lausanne) 12, 798903. https://doi.org/10.3389/fendo.2021.798903

General Bibliography

Assefa, W.W., 2015. The food and feeding ecology of Nile tilapia, *Oreochromis niloticus*, in Lake Hayq, Ethiopia. International Journal of Ecology and Environmental Sciences 41, 55–65.

Avadí, A., Pelletier, N., Aubin, J., Ralite, S., Núñez, J., Fréon, P., 2015. Comparative environmental performance of artisanal and commercial feed use in Peruvian freshwater aquaculture. Aquaculture 435, 52–66.

Ayer, N.W., Tyedmers, P.H., 2009. Assessing alternative aquaculture technologies: life cycle assessment of salmonid culture systems in Canada. Journal of Cleaner production 17, 362–373.

Bajić, B., Vučurović, D., Vasić, Đ., Jevtić-Mučibabić, R., Dodić, S., 2022. Biotechnological Production of Sustainable Microbial Proteins from Agro-Industrial Residues and By-Products. Foods 12, 107. https://doi.org/10.3390/foods12010107

Balakrishnan, G., Schneider, R.G., 2022. The Role of Amaranth, Quinoa, and Millets for the Development of Healthy, Sustainable Food Products—A Concise Review. Foods 11, 2442. https://doi.org/10.3390/foods11162442

Bartek, L., Strid, I., Henryson, K., Junne, S., Rasi, S., Eriksson, M., 2021. Life cycle assessment of fish oil substitute produced by microalgae using food waste. Sustainable Production and Consumption 27, 2002–2021. https://doi.org/10.1016/j.spc.2021.04.033

Basto-Silva, C., Guerreiro, I., Oliva-Teles, A., Neto, B., 2019. Life cycle assessment of diets for gilthead seabream (*Sparus aurata*) with different protein/carbohydrate ratios and fishmeal or plant feedstuffs as main protein sources. The International Journal of Life Cycle Assessment 24, 2023–2034. https://doi.org/10.1007/s11367-019-01625-7

Bergleiter, S., Berner, N., Censkowsky, U., Julià-Camprodon, G., 2009. Organic aquaculture 2009 - production and markets. Naturland e.V. & and Organic Services GmbH, München.

Bergleiter, S., Meisch, S., 2015. Certification Standards for Aquaculture Products: Bringing Together the Values of Producers and Consumers in Globalised Organic Food Markets. Journal of

Agricultural and Environmental Ethics 28, 553–569. https://doi.org/10.1007/s10806-015-9531-5

Bertucci, J.I., Blanco, A.M., Sundarrajan, L., Rajeswari, J.J., Velasco, C., Unniappan, S., 2019. Nutrient regulation of endocrine factors influencing feeding and growth in fish. Frontiers in Endocrinology 10, 83.

Bohnes, F.A., Hauschild, M.Z., Schlundt, J., Laurent, A., 2019. Life cycle assessments of aquaculture systems: a critical review of reported findings with recommendations for policy and system development. Reviews in Aquaculture 11, 1061–1079. https://doi.org/10.1111/raq.12280

Boissy, J., Aubin, J., Drissi, A., Van Der Werf, H.M.G., Bell, G.J., Kaushik, S.J., 2011. Environmental impacts of plant-based salmonid diets at feed and farm scales. Aquaculture 321, 61–70. https://doi.org/10.1016/j.aquaculture.2011.08.033

Bojanowska, E., Ciosek, J., 2016. Can we selectively reduce appetite for energy-dense foods? An overview of pharmacological strategies for modification of food preference behavior. Current Neuropharmacology 14, 118–142.

Boyd, C.E., D'Abramo, L.R., Glencross, B.D., Huyben, D.C., Juarez, L.M., Lockwood, G.S., McNevin, A.A., Tacon, A.G.J., Teletchea, F., Tomasso Jr, J.R., Tucker, C.S., Valenti, W.C., 2020. Achieving sustainable aquaculture: Historical and current perspectives and future needs and challenges. Journal of the World Aquaculture Society 51, 578–633. https://doi.org/10.1111/jwas.12714

Busacca, E., Lembo, G., 2019. EU Regulation on Organic Aquaculture, in: Lembo, G., Mente, E. (Eds.), Organic Aquaculture. Springer International Publishing, Cham, pp. 23–39. https://doi.org/10.1007/978-3-030-05603-2_2

Cadinu, L.A., Barra, P., Torre, F., Delogu, F., Madau, F.A., 2020. Insect rearing: Potential, challenges, and circularity. Sustainability 12, 4567.

Calo, J., Blanco, A.M., Comesaña, S., Conde-Sieira, M., Morais, S., Soengas, J.L., 2021. First evidence for the presence of amino acid sensing mechanisms in the fish gastrointestinal tract. Scientific Reports 11, 4933. https://doi.org/10.1038/s41598-021-84303-9

Campanati, C., Willer, D., Schubert, J., Aldridge, D.C., 2022. Sustainable Intensification of Aquaculture through Nutrient Recycling and Circular Economies: More Fish, Less Waste, Blue Growth. Reviews in Fisheries Science & Aquaculture 30, 143–169. https://doi.org/10.1080/23308249.2021.1897520

Campbell, K.B., McLean, E., Barrows, F.T., 2022. In Pursuit of Fish-Free Feeds: A Multi-Species Evaluation. Fishes 7, 336. https://doi.org/10.3390/fishes7060336

Campos, I., Valente, L.M.P., Matos, E., Marques, P., Freire, F., 2020. Life-cycle assessment of animal feed ingredients: Poultry fat, poultry by-product meal and hydrolyzed feather meal. Journal of Cleaner Production 252, 119845.

Cao, L., Diana, J.S., Keoleian, G.A., 2013. Role of life cycle assessment in sustainable aquaculture. Reviews in Aquaculture 5, 61–71. https://doi.org/10.1111/j.1753-5131.2012.01080.x

Carballeira Braña, C., Cerbule, K., Senff, P., Stolz, I., 2021. Towards Environmental Sustainability in Marine Finfish Aquaculture. Frontiers in Marine Science 8. https://doi.org/10.3389/fmars.2021.666662

Carrassón, M., Soler-Membrives, A., Constenla, M., Escobar, C., Flos, R., Gil, J.M., Luzón, V., Piferrer, F., Reig, L., 2021. Information impact on consumers' perceptions towards aquaculture: Dismantling the myth about feeds for farmed fish. Aquaculture 544, 737137. https://doi.org/10.1016/j.aquaculture.2021.737137

Caxico Vieira, C.A.S., Vieira, J.S., Bastos, M.S., Zancanela, V., Barbosa, L.T., Gasparino, E., Del Vesco, A.P., 2018. Expression of genes related to antioxidant activity in Nile tilapia kept under salinity stress and fed diets containing different levels of vitamin C. Journal of Toxicology and Environmental Health, Part A 81, 20–30. https://doi.org/10.1080/15287394.2017.1401968

General Bibliography

Cena, H., Calder, P.C., 2020. Defining a Healthy Diet: Evidence for the Role of Contemporary Dietary Patterns in Health and Disease. Nutrients 12, 334. https://doi.org/10.3390/nu12020334

Chary, K., Van Riel, A., Muscat, A., Wilfart, A., Harchaoui, S., Verdegem, M., Filgueira, R., Troell, M., Henriksson, P.J.G., De Boer, I.J.M., Wiegertjes, G.F., 2023. Transforming sustainable aquaculture by applying circularity principles. Reviews in Aquaculture raq.12860. https://doi.org/10.1111/raq.12860

Chen, J., Jayachandran, M., Bai, W., Xu, B., 2022. A critical review on the health benefits of fish consumption and its bioactive constituents. Food Chemistry 369, 130874. https://doi.org/10.1016/j.foodchem.2021.130874

Christopher, R.B., Ahilan, B., Cheryl, A., Samuel, M., 2020. Sunflower meal as an alternative protein source to replace soybean meal in the diet of GIFT strain of Nile tilapia *Oreochromis niloticus*. Indian Journal of Fisheries. 67. https://doi.org/10.21077/ijf.2020.67.3.91750-09

Ciji, A., Akhtar, M.S., 2021. Stress management in aquaculture: A review of dietary interventions. Reviews in Aquaculture 13, 2190–2247.

Colombo, S.M., 2020. Chapter 2 - Physiological considerations in shifting carnivorous fishes to plant-based diets, in: Benfey, T.J., Farrell, A.P., Brauner, C.J. (Eds.), Fish Physiology, Aquaculture. Academic Press, pp. 53–82. https://doi.org/10.1016/bs.fp.2020.09.002

Colombo, S.M., Roy, K., Mraz, J., Wan, A.H.L., Davies, S.J., Tibbetts, S.M., Øverland, M., Francis, D.S., Rocker, M.M., Gasco, L., Spencer, E., Metian, M., Trushenski, J.T., Turchini, G.M., 2022. Towards achieving circularity and sustainability in feeds for farmed blue foods. Reviews in Aquaculture raq.12766. https://doi.org/10.1111/raq.12766

Comesaña, S., Conde-Sieira, M., Velasco, C., Soengas, J.L., Morais, S., 2020. Oral and pre-absorptive sensing of amino acids relates to hypothalamic control of food intake in rainbow trout. Journal of experimental biology 223, jeb221721.

Costa, L.S., Guilherme, H.O., Bahiense, R.N., Santos, F.A.C., Gamarano, P.G., Olmeda, J.F.L., Ribeiro, P.A.P., Luz, R.K., 2022. Effect of protein on the post-ingestive response of tilapia fed

encapsulated diets. Aquaculture 556, 738289. https://doi.org/10.1016/j.aquaculture.2022.738289

Dam, C.T.M., Ventura, T., Booth, M., Pirozzi, I., Salini, M., Smullen, R., Elizur, A., 2020. Intestinal Transcriptome Analysis Highlights Key Differentially Expressed Genes Involved in Nutrient Metabolism and Digestion in Yellowtail Kingfish (*Seriola lalandi*) Fed Terrestrial Animal and Plant Proteins. Genes 11, 621. https://doi.org/10.3390/genes11060621

Dara, M., Carbonara, P., La Corte, C., Parrinello, D., Cammarata, M., Parisi, M.G., 2023. Fish Welfare in Aquaculture: Physiological and Immunological Activities for Diets, Social and Spatial Stress on Mediterranean Aqua Cultured Species. Fishes 8, 414. https://doi.org/10.3390/fishes8080414

De las Heras, V., Martos-Sitcha, J.A., Yúfera, M., Mancera, J.M., Martínez-Rodríguez, G., 2015. Influence of stocking density on growth, metabolism and stress of thick-lipped grey mullet (*Chelon labrosus*) juveniles. Aquaculture 448, 29–37.

Delgado Saavedra, M.J., Cerdá Reverter, J.M., Soengas, J.L., 2017. Hypothalamic Integration of Metabolic, Endocrine, and Circadian Signals in Fish: Involvement in the Control of Food Intake.

Delistavrou, A., Tilikidou, I., Papaioannou, E., 2023. Climate change risk perception and intentions to buy consumer packaged goods with chemicals containing recycled CO2. Journal of Cleaner Production 382, 135215. https://doi.org/10.1016/j.jclepro.2022.135215

EC, 2021a. A new strategic vision for sustainable aquaculture production and consumption in the European Union: blue farming in the European Green Deal. Publications Office, LU.

EC, 2021b. Commission Regulation (EU) 2021/1372 of 17 August 2021 amending Annex IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards the prohibition to feed non-ruminant farmed animals, other than fur animals, with protein derived from animals (Text with EEA relevance), OJ L.

EC, 2020a. COMMUNICATION FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT, THE COUNCIL, THE EUROPEAN ECONOMIC AND SOCIAL COMMITTEE AND THE COMMITTEE OF THE REGIONS EU Biodiversity Strategy for 2030 Bringing nature back into our lives.

EC, 2020b. COMMUNICATION FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT, THE COUNCIL, THE EUROPEAN ECONOMIC AND SOCIAL COMMITTEE AND THE COMMITTEE OF THE REGIONS A Farm to Fork Strategy for a fair, healthy and environmentally-friendly food system.

EC, 2020c. COMMUNICATION FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT, THE COUNCIL, THE EUROPEAN ECONOMIC AND SOCIAL COMMITTEE AND THE COMMITTEE OF THE REGIONS A new Circular Economy Action Plan For a cleaner and more competitive Europe.

EC, 2019. COMMUNICATION FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT, THE EUROPEAN COUNCIL, THE COUNCIL, THE EUROPEAN ECONOMIC AND SOCIAL COMMITTEE AND THE COMMITTEE OF THE REGIONS The European Green Deal.

EC, 2017a. Commission Regulation (EU) 2017/1017 of 15 June 2017 amending Regulation (EU) No 68/2013 on the Catalogue of feed materials (Text with EEA relevance.).

EC, 2017b. COMMISSION REGULATION (EU) 2017/ 893 - of 24 May 2017 - amending Annexes I and IV to Regulation (EC) No 999 / 2001 of the European Parliament and of the Council and Annexes X, XIV and XV to Commission Regulation (EU) No 142 / 2011 as regards the provisions on processed animal protein.

EC, 2016. Commission Implementing Regulation (EU) 2016/673 of 29 April 2016 amending Regulation (EC) No 889/2008 laying down detailed rules for the implementation of Council Regulation (EC) No 834/2007 on organic production and labelling of organic products with regard to organic production, labelling and control (Text with EEA relevance), OJ L.

EC, 2013a. Commission Regulation (EU) No 56/2013 of 16 January 2013 amending Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down

rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies Text with EEA relevance, OJ L.

EC, 2013b. Regulation (EU) No 1380/2013 of the European Parliament and of the Council of 11 December 2013 on the Common Fisheries Policy, amending Council Regulations (EC) No 1954/2003 and (EC) No 1224/2009 and repealing Council Regulations (EC) No 2371/2002 and (EC) No 639/2004 and Council Decision 2004/585/EC.

EC, 2009. Commission Regulation (EC) No 710/2009 of 5 August 2009 amending Regulation (EC) No 889/2008 laying down detailed rules for the implementation of Council Regulation (EC) No 834/2007, as regards laying down detailed rules on organic aquaculture animal and seaweed production, OJ L.

EC, 2008. Commission Regulation (EC) No 889/2008 of 5 September 2008 laying down detailed rules for the implementation of Council Regulation (EC) No 834/2007 on organic production and labelling of organic products with regard to organic production, labelling and control, OJ L.

EC, 2007. Council Regulation (EC) No 834/2007 of 28 June 2007 on organ... - EUR-Lex [WWW Document]. URL https://eur-lex.europa.eu/legal-content/en/LSU/?uri=CELEX%3A32007R0834 (accessed 4.20.22).

EC, 2005. Commission Regulation (EC) No 1292/2005 of 5 August 2005 amending Annex IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards animal nutrition (Text with EEA relevance), OJ L.

EC, 2004. Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules COMMUNICATION FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT, THE COUNCIL, THE EUROPEAN ECONOMIC AND SOCIAL COMMITTEE AND THE COMMITTEE OF THE REGIONS on a new approach for a sustainable blue economy in the EU Transforming the EU's Blue Economy for a Sustainable Future, OJ L.

EC, 2001. Regulation (EC) No 999/2001 of the European Parliament and of the Council of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies.

Edwards, P., Zhang, W., Belton, B., Little, D.C., 2019. Misunderstandings, myths and mantras in aquaculture: Its contribution to world food supplies has been systematically over reported. Marine Policy 106, 103547. https://doi.org/10.1016/j.marpol.2019.103547

EEC, 1991. Council Regulation (EEC) No 2092/91 of 24 June 1991 on organic production of agricultural products and indications referring thereto on agricultural products and foodstuffs, OJ L.

Ellingsen, H., Aanondsen, S.A., 2006. Environmental impacts of wild caught cod and farmed Salmon-a comparison with chicken (7 pp). The International Journal of Life Cycle Assessment 11, 60–65.

Ellison, A.R., Uren Webster, T.M., Rey, O., Garcia de Leaniz, C., Consuegra, S., Orozco-terWengel, P., Cable, J., 2018. Transcriptomic response to parasite infection in Nile tilapia (*Oreochromis niloticus*) depends on rearing density. BMC genomics 19, 1–12.

El-Sayed, A.-F.M., 2019. Tilapia culture. Academic press.

El-Sayed, A.-F.M., 2006. Tilapia culture in salt water: environmental requirements, nutritional implications and economic potentials. Avances en Nutricion Acuicola.

El-Sayed, A.-F.M., Fitzsimmons, K., 2023. From Africa to the world—The journey of Nile tilapia. Reviews in Aquaculture 15, 6–21. https://doi.org/10.1111/raq.12738

Estévez, A., Vasilaki, P., 2023. Organic production of gilthead sea bream (*Sparus aurata*) using organic certified green pea protein and seaweed. Effects on growth, feed conversion and final product quality. Aquaculture 571, 739490. https://doi.org/10.1016/j.aquaculture.2023.739490

Estruch, G., Collado, M.C., Monge-Ortiz, R., Tomás-Vidal, A., Jover-Cerdá, M., Peñaranda, D.S., Pérez Martínez, G., Martínez-Llorens, S., 2018. Long-term feeding with high plant protein based

diets in gilthead seabream (*Sparus aurata*, L.) leads to changes in the inflammatory and immune related gene expression at intestinal level. BMC Vet Res 14, 302. https://doi.org/10.1186/s12917-018-1626-6

Estruch, G., Martínez-Llorens, S., Tomás-Vidal, A., Monge-Ortiz, R., Jover-Cerdá, M., Brown, P.B., Peñaranda, D.S., 2020. Impact of high dietary plant protein with or without marine ingredients in gut mucosa proteome of gilthead seabream (*Sparus aurata*, L.). Journal of Proteomics 216, 103672. https://doi.org/10.1016/j.jprot.2020.103672

EU, 2022. Transformation scenarios for boosting organic farming and organic aquaculture towards the Farm-to-Fork targets | OrganicTargets4EU Project | Fact Sheet | HORIZON [WWW Document]. CORDIS | European Commission. URL https://cordis.europa.eu/project/id/101060368 (accessed 1.25.24).

EU, 2018. Regulation (EU) 2018/848 of the European Parliament and of the Council of 30 May 2018 on organic production and labelling of organic products and repealing Council Regulation (EC) No 834/2007, OJ L.

EU, 2017. Regulation (EU) 2017/625 of the European Parliament and of the Council of 15 March 2017 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products, amending Regulations (EC) No 999/2001, (EC) No 396/2005, (EC) No 1069/2009, (EC) No 1107/2009, (EU) No 1151/2012, (EU) No 652/2014, (EU) 2016/429 and (EU) 2016/2031 of the European Parliament and of the Council, Council Regulations (EC) No 1/2005 and (EC) No 1099/2009 and Council Directives 98/58/EC, 1999/74/EC, 2007/43/EC, 2008/119/EC and 2008/120/EC, and repealing Regulations (EC) No 854/2004 and (EC) No 882/2004 of the European Parliament and of the Council, Council Directives 89/608/EEC, 89/662/EEC, 90/425/EEC, 91/496/EEC, 96/23/EC, 96/93/EC and 97/78/EC and Council Decision 92/438/EEC (Official Controls Regulation)Text with EEA relevance., OJ L.

EUMOFA., 2023. Fishmeal and fish oil: production and trade flows in the EU. Publications Office, LU.

General Bibliography

EUMOFA, 2023. The EU fish market: 2023 edition.

EUMOFA, 2022. Organic Aquaculture in the EU. Publications Office, LU.

European Court of Auditors., 2023. EU aquaculture policy: stagnating production and unclear results despite increased EU funding. Special report 25, 2023. Publications Office, LU.

Faletar, I., Cerjak, M., Kovačić, D., 2016. Fresh seafood consumption determinants and inventory satisfaction. MESO : The first Croatian meat journal XVIII, 97–101.

FAO, 2024. The State of World Fisheries and Aquaculture 2024. FAO. https://doi.org/10.4060/cd0683en

FAO, 2022. The State of World Fisheries and Aquaculture 2022. FAO. https://doi.org/10.4060/cc0461en

FAO, 2020. The State of World Fisheries and Aquaculture 2020. FAO. https://doi.org/10.4060/ca9229en

FAO, C., 2011. Global food losses and food waste: extent, causes and prevention; study conducted for the International Congress Save Food! at Interpack 2011, [16 - 17 May], Düsseldorf, Germany. Presented at the International Congress Save Food!, Food and Agriculture Organization of the United Nations, Rome.

Ferfolja, M., Cerjak, M., Matulić, D., Maksan, M.T., 2022. Consumer Knowledge and Perception about Fresh Fish from Organic Farming in Croatia. Croatian Journal of Fisheries 80, 7–16. https://doi.org/10.2478/cjf-2022-0002

Feucht, Y., Zander, K., 2017. Aquaculture in the German print media. Aquaculture international 25, 177–195.

Feucht, Y., Zander, K., 2015. Of earth ponds, flow-through and closed recirculation systems — German consumers' understanding of sustainable aquaculture and its communication. Aquaculture 438, 151–158. https://doi.org/10.1016/j.aquaculture.2015.01.005

Forbes, J., 2001. Consequences of feeding for future feeding. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 128, 461–468.

Føre, M., Alver, M., Alfredsen, J.A., Marafioti, G., Senneset, G., Birkevold, J., Willumsen, F.V., Lange, G., Espmark, Å., Terjesen, B.F., 2016. Modelling growth performance and feeding behaviour of Atlantic salmon (*Salmo salar* L.) in commercial-size aquaculture net pens: Model details and validation through full-scale experiments. Aquaculture 464, 268–278.

Fortes-Silva, R., Kitagawa, A., Sanchez Vazquez, F.J., 2016. Dietary self-selection in fish: a new approach to studying fish nutrition and feeding behavior. Reviews in Fish Biology and Fisheries 26. https://doi.org/10.1007/s11160-015-9410-1

Froehlich, H.E., Jacobsen, N.S., Essington, T.E., Clavelle, T., Halpern, B.S., 2018. Avoiding the ecological limits of forage fish for fed aquaculture. Nature Sustainability 1, 298–303.

Fry, J.P., Mailloux, N.A., Love, D.C., Milli, M.C., Cao, L., 2018. Feed conversion efficiency in aquaculture: do we measure it correctly? Environmental Research Letters 13, 024017.

Gambelli, D., Vairo, D., Solfanelli, F., Zanoli, R., 2019. Economic performance of organic aquaculture: A systematic review. Marine Policy 108, 103542. https://doi.org/10.1016/j.marpol.2019.103542

Gasco, L., Acuti, G., Bani, P., Dalle Zotte, A., Danieli, P.P., De Angelis, A., Fortina, R., Marino, R., Parisi, G., Piccolo, G., Pinotti, L., Prandini, A., Schiavone, A., Terova, G., Tulli, F., Roncarati, A., 2020. Insect and fish by-products as sustainable alternatives to conventional animal proteins in animal nutrition. Italian Journal of Animal Science 19, 360–372. https://doi.org/10.1080/1828051X.2020.1743209

Ghamkhar, R., Hicks, A., 2020. Comparative environmental impact assessment of aquafeed production: Sustainability implications of forage fish meal and oil free diets. Resources, Conservation and Recycling 161, 104849. https://doi.org/10.1016/j.resconrec.2020.104849

Ghosh, A., Misra, S., Bhattacharyya, R., Sarkar, A., Singh, A.K., Tyagi, V.C., Kumar, R.V., Meena, V.S., 2020. Agriculture, dairy and fishery farming practices and greenhouse gas emission footprint: a strategic appraisal for mitigation. Environmental Science and Pollution Research 27, 10160–10184. https://doi.org/10.1007/s11356-020-07949-4

Glebova, I.A., Larionova, A.A., Zaitseva, N.A., Grunina, A.A., Chvyakin, V.A., Takhumova, O.V., Glagoleva, L.E., 2019. Organic Aquaculture as a Promising Direction for the Production of Organic Food 7.

Glencross, B., Ling, X., Gatlin, D., Kaushik, S., Øverland, M., Newton, R., Valente, L.M.P., 2024. A SWOT Analysis of the Use of Marine, Grain, Terrestrial-Animal and Novel Protein Ingredients in Aquaculture Feeds. Reviews in Fisheries Science & Aquaculture 1–39. https://doi.org/10.1080/23308249.2024.2315049

Glencross, B.D., 2020. A feed is still only as good as its ingredients: An update on the nutritional research strategies for the optimal evaluation of ingredients for aquaculture feeds. Aquaculture Nutrition 26, 1871–1883.

Gould, D., Compagnoni, A., Lembo, G., 2019. Organic aquaculture: Principles, standards and certification, in: Organic Aquaculture. Springer, pp. 1–22.

Guillen, J., Natale, F., Carvalho, N., Casey, J., Hofherr, J., Druon, J.-N., Fiore, G., Gibin, M., Zanzi, A., Martinsohn, J.Th., 2019. Global seafood consumption footprint. Ambio 48, 111–122. https://doi.org/10.1007/s13280-018-1060-9

Gupta, A., Barrow, C.J., Puri, M., 2022. Multiproduct biorefinery from marine thraustochytrids towards a circular bioeconomy. Trends in Biotechnology 40, 448–462.

Hameed, A., Majeed, W., Naveed, M., Ramzan, U., Bordiga, M., Hameed, M., Ur Rehman, S., Rana, N., 2022. Success of Aquaculture Industry with New Insights of Using Insects as Feed: A Review. Fishes 7, 395. https://doi.org/10.3390/fishes7060395

Hampton, J.O., Hyndman, T.H., Allen, B.L., Fischer, B., 2021. Animal Harms and Food Production: Informing Ethical Choices. Animals (Basel) 11, 1225. https://doi.org/10.3390/ani11051225

Hilborn, R., Banobi, J., Hall, S.J., Pucylowski, T., Walsworth, T.E., 2018. The environmental cost of animal source foods. Front Ecol Environ 16, 329–335. https://doi.org/10.1002/fee.1822

Hilmarsdóttir, G.S., Ögmundarson, Ó., Arason, S., Gudjónsdóttir, M., 2022. Identification of environmental hotspots in fishmeal and fish oil production towards the optimization of

energy-related processes. Journal of Cleaner Production 343, 130880. https://doi.org/10.1016/j.jclepro.2022.130880

Hilmarsdóttir, G.S., Ogmundarson, Ó., Arason, S., Gudjónsdóttir, M., 2021. Efficiency of fishmeal and fish oil processing of different pelagic fish species: Identification of processing steps for potential optimization toward protein production for human consumption. Journal Food Processing and Preservation. 45. https://doi.org/10.1111/jfpp.15294

Holhorea, P.G., Naya-Català, F., Belenguer, Á., Calduch-Giner, J.A., Pérez-Sánchez, J., 2023. Understanding how high stocking densities and concurrent limited oxygen availability drive social cohesion and adaptive features in regulatory growth, antioxidant defense and lipid metabolism in farmed gilthead sea bream (*Sparus aurata*). Frontiers in Physiology 14, 1272267. https://doi.org/10.3389/fphys.2023.1272267

Hossain, Md.S., Small, B.C., Kumar, V., Hardy, R., 2024. Utilization of functional feed additives to produce cost-effective, ecofriendly aquafeeds high in plant-based ingredients. Reviews in Aquaculture 16, 121–153. https://doi.org/10.1111/raq.12824

Hua, K., Cobcroft, J.M., Cole, A., Condon, K., Jerry, D.R., Mangott, A., Praeger, C., Vucko, M.J., Zeng, C., Zenger, K., Strugnell, J.M., 2019. The Future of Aquatic Protein: Implications for Protein Sources in Aquaculture Diets. One Earth 1, 316–329. https://doi.org/10.1016/j.oneear.2019.10.018

Hunter, M.C., Smith, R.G., Schipanski, M.E., Atwood, L.W., Mortensen, D.A., 2017. Agriculture in 2050: Recalibrating Targets for Sustainable Intensification. BioScience 67, 386–391. https://doi.org/10.1093/biosci/bix010

Huntingford, F., Jobling, M., Kadri, S. (Eds.), 2012. Aquaculture and Behavior, 1st ed. Wiley. https://doi.org/10.1002/9781444354614

Ighwela, K.A., Ahmed, A., Abol-Munafi, A., 2011. Condition Factor as an Indicator of Growth and Feeding Intensity of Nile Tilapia Fingerlings (*Oreochromis niloticus*) Feed on Different Levels of Maltose.

General Bibliography

IPIFF, 2018. The European insect sector today: challenges, opportunities and regulatory landscape.

ISO, I., 2006. ISO 14040. Environmental management–Life cycle assessment–Principles and framework (ISO 14040: 2006).

Jannathulla, R., Rajaram, V., Kalanjiam, R., Ambasankar, K., Muralidhar, M., Dayal, J.S., 2019. Fishmeal availability in the scenarios of climate change: Inevitability of fishmeal replacement in aquafeeds and approaches for the utilization of plant protein sources. Aquaculture Research 50, 3493–3506. https://doi.org/10.1111/are.14324

Jiang, Q., Bhattarai, N., Pahlow, M., Xu, Z., 2022. Environmental sustainability and footprints of global aquaculture. Resources, Conservation and Recycling 180, 106183. https://doi.org/10.1016/j.resconrec.2022.106183

Jobling, M., Alanärä, A., Noble, C., Sánchez-Vázquez, J., Kadri, S., Huntingford, F., 2012. Appetite and feed intake. Aquaculture and Behavior 183–219.

Katz, D., 1937. Animals and Men: Studies in Comparative Psychology, First edition. ed. Longmans, Green & Co, London.

Keyombe, J.L., Malala, J.O., Waithaka, E., Lewo, R.M., Obwanga, B.O., 2017. Seasonal changes in length-weight relationship and condition factor of Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) (Cichlidae) in Lake Naivasha, Kenya. International Journal of Aquatic Biology 5. https://doi.org/10.22034/ijab.v5i1.231

Kokou, F., Fountoulaki, E., 2018. Aquaculture waste production associated with antinutrient presence in common fish feed plant ingredients. Aquaculture 495, 295–310. https://doi.org/10.1016/j.aquaculture.2018.06.003

Konstantinidis, E., Perdikaris, C., Ganias, K., 2021. Life cycle assessment of seabass and meagre in marine cage farming: From feeding plant to harvesting. Mediterranean Marine Science 22, 125–136. https://doi.org/10.12681/mms.25052

Kulczykowska, E., Sánchez Vázquez, F.J., 2010. Neurohormonal regulation of feed intake and response to nutrients in fish: aspects of feeding rhythm and stress. Aquaculture Research 41, 654–667. https://doi.org/10.1111/j.1365-2109.2009.02350.x

Kumar, G., Engle, C., 2016. Technological Advances that Led to Growth of Shrimp, Salmon, and Tilapia Farming. Reviews in Fisheries Science & Aquaculture 24, 136–152. https://doi.org/10.1080/23308249.2015.1112357

Laird, L., 2001. Mariculture Overview.

Lamb, C.F., 2001. Gustation and feeding behaviour. Food intake in fish 108–130.

Lathuilliere, M.J., Miranda, E.J., Bulle, C., Couto, E.G., Johnson, M.S., 2017. Land occupation and transformation impacts of soybean production in Southern Amazonia, Brazil. Journal of cleaner production 149, 680–689.

Lazzarotto, V., Médale, F., Larroquet, L., Corraze, G., 2018. Long-term dietary replacement of fishmeal and fish oil in diets for rainbow trout (*Oncorhynchus mykiss*): Effects on growth, whole body fatty acids and intestinal and hepatic gene expression. PLoS ONE 13, e0190730. https://doi.org/10.1371/journal.pone.0190730

Lembo, G., Jokumsen, A., Spedicato, M.T., Facchini, M.T., Bitetto, I., 2018. Assessing stakeholder's experience and sensitivity on key issues for the economic growth of organic aquaculture production. Marine Policy 87, 84–93.

Lieke, T., Meinelt, T., Hoseinifar, S.H., Pan, B., Straus, D.L., Steinberg, C.E.W., 2020. Sustainable aquaculture requires environmental-friendly treatment strategies for fish diseases. Reviews in Aquaculture 12, 943–965. https://doi.org/10.1111/raq.12365

Liland, N.S., Araujo, P., Xu, X.X., Lock, E.-J., Radhakrishnan, G., Prabhu, A.J.P., Belghit, I., 2021. A meta-analysis on the nutritional value of insects in aquafeeds. JIFF 7, 743–759. https://doi.org/10.3920/JIFF2020.0147

Lin, G., Li, S., Huang, J., Gao, D., Lu, J., 2021. Hypoosmotic stress induced functional alternations of intestinal barrier integrity, inflammatory reactions, and neurotransmission along gut-brain

axis in the yellowfin seabream (*Acanthopagrus latus*). Fish Physiology and Biochemistry 47, 1725–1738.

Little, D.C., Newton, R.W., Beveridge, M.C.M., 2016. Aquaculture: a rapidly growing and significant source of sustainable food? Status, transitions and potential. Proceedings of the Nutrition Society 75, 274–286. https://doi.org/10.1017/S0029665116000665

Little, D.C., Young, J.A., Zhang, W., Newton, R.W., Al Mamun, A., Murray, F.J., 2018. Sustainable intensification of aquaculture value chains between Asia and Europe: A framework for understanding impacts and challenges. Aquaculture 493, 338–354. https://doi.org/10.1016/j.aquaculture.2017.12.033

Liu, B., Jia, R., Han, C., Huang, B., Lei, J.-L., 2016. Effects of stocking density on antioxidant status, metabolism and immune response in juvenile turbot (*Scophthalmus maximus*). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 190, 1–8.

Lopes, C., Antelo, L.T., Franco-Uría, A., Alonso, A.A., Pérez-Martín, R., 2015. Valorisation of fish by-products against waste management treatments–Comparison of environmental impacts. Waste management 46, 103–112.

López-Mas, L., Claret, A., Reinders, M.J., Banovic, M., Krystallis, A., Guerrero, L., 2021. Farmed or wild fish? Segmenting European consumers based on their beliefs. Aquaculture 532, 735992.

Lu, Q., Serajuddin, M., 2020. Emerging Technologies, Environment and Research for Sustainable Aquaculture. BoD–Books on Demand.

MacLeod, M.J., Hasan, M.R., Robb, D.H.F., Mamun-Ur-Rashid, M., 2020. Quantifying greenhouse gas emissions from global aquaculture. Scientific Reports 10, 11679. https://doi.org/10.1038/s41598-020-68231-8

Maiolo, S., Parisi, G., Biondi, N., Lunelli, F., Tibaldi, E., Pastres, R., 2020. Fishmeal partial substitution within aquafeed formulations: life cycle assessment of four alternative protein sources. The International Journal of Life Cycle Assessment 25, 1455–1471. https://doi.org/10.1007/s11367-020-01759-z

Makkar, H.P.S., Tran, G., Heuzé, V., Ankers, P., 2014. State-of-the-art on use of insects as animal feed. Animal Feed Science and Technology 197, 1–33. https://doi.org/10.1016/j.anifeedsci.2014.07.008

Makled, S.O., Hamdan, A.M., El-Sayed, A.-F.M., Hafez, E.E., 2017. Evaluation of marine psychrophile, Psychrobacter namhaensis SO89, as a probiotic in Nile tilapia (*Oreochromis niloticus*) diets. Fish & Shellfish Immunology 61, 194–200. https://doi.org/10.1016/j.fsi.2017.01.001

Malcorps, W., Kok, B., van 't Land, M., Fritz, M., van Doren, D., Servin, K., van der Heijden, P., Palmer, R., Auchterlonie, N.A., Rietkerk, M., 2019. The sustainability conundrum of fishmeal substitution by plant ingredients in shrimp feeds. Sustainability 11, 1212.

Manchado, M., Planas, J.V., Cousin, X., Rebordinos, L., Claros, M.G., 2016. Current status in other finfish species: Description of current genomic resources for the gilthead seabream (Sparus aurata) and soles (*Solea senegalensis* and *Solea solea*), in: Genomics in Aquaculture. Elsevier, pp. 195–221.

Marti, D.L., Johnson, R.J., Mathews Jr, K.H., 2012. Where's the (not) meat? Byproducts from beef and pork production. Journal of Current Issues in Globalization 5, 397.

Matassa, S., Papirio, S., Pikaar, I., Hülsen, T., Leijenhorst, E., Esposito, G., Pirozzi, F., Verstraete, W., 2020. Upcycling of biowaste carbon and nutrients in line with consumer confidence: the "full gas" route to single cell protein. Green Chemistry 22, 4912–4929.

McAndrew, B.J., Beveridge, M.C., 2000. Tilapias: biology and exploitation. Kluwer Academic Publishers.

McKuin, B.L., Kapuscinski, A.R., Sarker, P.K., Cheek, N., Colwell, A., Schoffstall, B., Greenwood, C., 2022. Comparative life cycle assessment of heterotrophic microalgae *Schizochytrium* and fish oil in sustainable aquaculture feeds. Elementa: Science of the Anthropocene 10, 00098. https://doi.org/10.1525/elementa.2021.00098

Mhalhel, K., Levanti, M., Abbate, F., Laurà, R., Guerrera, M.C., Aragona, M., Porcino, C., Briglia, M., Germanà, A., Montalbano, G., 2023. Review on Gilthead Seabream (*Sparus aurata*)

Aquaculture: Life Cycle, Growth, Aquaculture Practices and Challenges. Journal of Marine Science and Engineering 11, 2008. https://doi.org/10.3390/jmse11102008

Mmanda, F., Lundh, T., Norman Haldén, A., Mtolera, M., Kitula, R., Lindberg, J., 2020. Replacing fish meal with locally available feed ingredients to reduce feed costs in cultured Nile tilapia (*Oreochromis niloticus*). Livestock Research for Rural Development 32.

Mohanty, D., Samanta, L., 2018. Dietary supplementation of *Spirulina* ameliorates iron-induced oxidative stress in Indian knife fish *Notopterus Notopterus*. Environmental Toxicology and Pharmacology 61, 71–78. https://doi.org/10.1016/j.etap.2018.05.007

Morais, S., 2017. The Physiology of Taste in Fish: Potential Implications for Feeding Stimulation and Gut Chemical Sensing. Reviews in Fisheries Science & Aquaculture 25, 133–149. https://doi.org/10.1080/23308249.2016.1249279

Moretti, A., Fernandez-Criado, M.P., Betillart, R., 1999. Manual on hatchery production of Seabass and Gilthead Seabream: Volume 2.

Munguti, J.M., Kirimi, J.G., Obiero, K.O., Ogello, E.O., Kyule, D.N., Liti, D.M., Musalia, L.M., 2020. Aqua-feed wastes: Impact on natural systems and practical mitigations—A review. Journal of Agricultural Science 13, 111.

Naya-Català, F., do Vale Pereira, G., Piazzon, M.C., Fernandes, A.M., Calduch-Giner, J.A., Sitjà-Bobadilla, A., Conceição, L.E.C., Pérez-Sánchez, J., 2021. Cross-Talk Between Intestinal Microbiota and Host Gene Expression in Gilthead Sea Bream (*Sparus aurata*) Juveniles: Insights in Fish Feeds for Increased Circularity and Resource Utilization. Frontiers in Physiology 12. https://doi.org/10.3389/fphys.2021.748265

Naylor, R.L., Hardy, R.W., Buschmann, A.H., Bush, S.R., Cao, L., Klinger, D.H., Little, D.C., Lubchenco, J., Shumway, S.E., Troell, M., 2021. A 20-year retrospective review of global aquaculture. Nature 591, 551–563. https://doi.org/10.1038/s41586-021-03308-6

Newton, R., Telfer, T., Little, D., 2014. Perspectives on the utilization of aquaculture coproduct in Europe and Asia: prospects for value addition and improved resource efficiency. Critical reviews in food science and nutrition 54, 495–510.

Newton, R.W., Little, D.C., 2018. Mapping the impacts of farmed Scottish salmon from a life cycle perspective. The International Journal of Life Cycle Assessment 23, 1018–1029. https://doi.org/10.1007/s11367-017-1386-8

Newton, R.W., Maiolo, S., Malcorps, W., Little, D.C., 2023. Life Cycle Inventories of marine ingredients. Aquaculture 565, 739096. https://doi.org/10.1016/j.aquaculture.2022.739096

Ni, M., Wen, H., Li, J., Chi, M., Bu, Y., Ren, Y., Zhang, M., Song, Z., Ding, H., 2016. Effects of stocking density on mortality, growth and physiology of juvenile Amur sturgeon (*Acipenser schrenckii*). Aquaculture Research 47, 1596–1604.

Nowak, V., Du, J., Charrondière, U.R., 2016. Assessment of the nutritional composition of quinoa (*Chenopodium quinoa* Willd.). Food Chemistry 193, 47–54. https://doi.org/10.1016/j.foodchem.2015.02.111

OECD, 2024. OECD (2024), Crop production (indicator). doi: 10.1787/49a4e677-en (Accessed on 03 February 2024) [WWW Document]. the OECD. URL http://data.oecd.org/agroutput/crop-production.htm (accessed 2.3.24).

Olsen, R.L., Hasan, M.R., 2012. A limited supply of fishmeal: Impact on future increases in global aquaculture production. Trends in Food Science & Technology 27, 120–128.

Otero-Rodino, C., Libran-Perez, M., Velasco, C., Alvarez-Otero, R., Lopez-Patino, M.A., Míguez, J.M., Soengas, J.L., 2016. Glucosensing in liver and Brockmann bodies of rainbow trout through glucokinase-independent mechanisms. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 199, 29–42.

Parker, R.W., Blanchard, J.L., Gardner, C., Green, B.S., Hartmann, K., Tyedmers, P.H., Watson, R.A., 2018. Fuel use and greenhouse gas emissions of world fisheries. Nature Climate Change 8, 333–337.

Peng, K., Shirley, D.C., Xu, Z., Huang, Q., McAllister, T.A., Chaves, A.V., Acharya, S., Liu, C., Wang, S., Wang, Y., 2016. Effect of purple prairie clover (*Dalea purpurea Vent*.) hay and its condensed tannins on growth performance, wool growth, nutrient digestibility, blood metabolites and ruminal fermentation in lambs fed total mixed rations. Animal Feed Science and Technology 222, 100–110. https://doi.org/10.1016/j.anifeedsci.2016.10.012

Pérez-Lloréns, J.L., Acosta, Y., Brun, F.G., 2021. Seafood in Mediterranean countries: A culinary journey through history. International Journal of Gastronomy and Food Science 26, 100437. https://doi.org/10.1016/j.ijgfs.2021.100437

Pérez-Pascual, D., Estellé, J., Dutto, G., Rodde, C., Bernardet, J.-F., Marchand, Y., Duchaud, E., Przybyla, C., Ghigo, J.-M., 2020. Growth Performance and Adaptability of European Sea Bass (*Dicentrarchus labrax*) Gut Microbiota to Alternative Diets Free of Fish Products. Microorganisms 8, 1346. https://doi.org/10.3390/microorganisms8091346

Petereit, J., Hoerterer, C., Bischoff-Lang, A.A., Conceição, L.E.C., Pereira, G., Johansen, J., Pastres, R., Buck, B.H., 2022. Adult European Seabass (*Dicentrarchus labrax*) Perform Well on Alternative Circular-Economy-Driven Feed Formulations. Sustainability 14, 7279. https://doi.org/10.3390/su14127279

Philippart, J., Ruwet, J., 1982. Ecology and distribution of tilapias In. Pullin, RSV; Lowe-McConnell, RH (eds.) pp. 55-59. The Biology and Culture of Tilapias. International Center for Living Aquatic Resources, Manila.

Philis, G., Ziegler, F., Gansel, L.C., Jansen, M.D., Gracey, E.O., Stene, A., 2019. Comparing Life Cycle Assessment (LCA) of Salmonid Aquaculture Production Systems: Status and Perspectives. Sustainability 11, 2517. https://doi.org/10.3390/su11092517

Piccolo, G., De Riu, N., Tulli, F., Cappuccinelli, R., Marono, S., Moniello, G., 2007. Somatic indexes, chemical-nutritive characteristics and metal content in caught and reared sharpsnout seabream (*Diplodus puntazzo*). Italian Journal of Animal Science 6, 351–360. https://doi.org/10.4081/ijas.2007.351

Piccolo, G., Iaconisi, V., Marono, S., Gasco, L., Loponte, R., Nizza, S., Bovera, F., Parisi, G., 2017. Effect of *Tenebrio molitor* larvae meal on growth performance, *in vivo* nutrients digestibility, somatic and marketable indexes of gilthead sea bream (*Sparus aurata*). Animal Feed Science and Technology 226, 12–20. https://doi.org/10.1016/j.anifeedsci.2017.02.007

Prein, M., Bergleiter, S., Ballauf, M., Brister, D., Halwart, M., Hongrat, K., Kahle, J., Lasner, T., Lem, A., Lev, O., Morrison, C., Shehadeh, Z., Stamer, A., Wainberg, A., 2012. Organic aquaculture: the future of expanding niche markets, in: Proceedings of the Global Conference on Aquaculture 2010. pp. 549–568.

Prins, H., Ur, L.W., Stokkers, R., Ur, L.W., Hoste, R., Ur, L.W., Immink, V., Ur, L.W., 2015. Farm economics and competitiveness of organic aquaculture 92.

Pullin, R., Capili, J., 1988. Genetic improvement of tilapias: problems and prospects. Presented at the The Second International Symposium on Tilapia in Aquaculture. ICLARM Conference Proceedings. 15, pp. 259–266.

Qi, C., Xie, C., Tang, R., Qin, X., Wang, D., Li, D., 2016. Effect of stocking density on growth, physiological responses, and body composition of juvenile blunt snout bream, *Megalobrama amblycephala*. Journal of the World Aquaculture Society 47, 358–368.

Quagrainie, K.K., Shambach, A.M., 2021. Aquaculture Markets in the Twenty-First Century 8.

Raubenheimer, D., Simpson, S.J., 1997. Integrative models of nutrient balancing: application to insects and vertebrates. Nutrition Research Reviews 10, 151–179. https://doi.org/10.1079/NRR19970009

Regueiro, L., Newton, R., Soula, M., Méndez, D., Kok, B., Little, D.C., Pastres, R., Johansen, J., Ferreira, M., 2022. Opportunities and limitations for the introduction of circular economy principles in EU aquaculture based on the regulatory framework. Journal of Industrial Ecology 26, 2033–2044. https://doi.org/10.1111/jiec.13188

Reig, L., Escobar, C., Carrassón, M., Constenla, M., Gil, J.M., Padrós, F., Piferrer, F., Flos, R., 2019. Aquaculture perceptions in the Barcelona metropolitan area from fish and seafood wholesalers, fishmongers, and consumers. Aquaculture 510, 256–266.

Richter, C.P., 1943. Total self-regulatory functions in animals and human beings. Harvey Lecture Series 38, 1942–1943.

Roberts, C.A., Newton, R., Bostock, J., Prescott, S., Honey, D.J., Telfer, T., Walmsley, S.F., Little, D.C., Hull, S.C., 2015. A risk benefit analysis of mariculture as a means to reduce the impacts of terrestrial production of food and energy. Scottish Aquaculture Research Forum.

Rossi, M.A., Stuber, G.D., 2018. Overlapping brain circuits for homeostatic and hedonic feeding. Cell metabolism 27, 42–56.

Saidi, A., Sacchi, G., Cavallo, C., Cicia, G., Di Monaco, R., Puleo, S., Del Giudice, T., 2022. Drivers of fish choice: an exploratory analysis in Mediterranean countries. Agricultural and Food Economics 10, 29. https://doi.org/10.1186/s40100-022-00237-4

Sanches-Fernandes, G.M.M., Sá-Correia, I., Costa, R., 2022. Vibriosis Outbreaks in Aquaculture: Addressing Environmental and Public Health Concerns and Preventive Therapies Using Gilthead Seabream Farming as a Model System. Frontiers in Microbiology 13.

Sandström, V., Chrysafi, A., Lamminen, M., Troell, M., Jalava, M., Piipponen, J., Siebert, S., Van Hal, O., Virkki, V., Kummu, M., 2022. Food system by-products upcycled in livestock and aquaculture feeds can increase global food supply. Nature Food 3, 729–740. https://doi.org/10.1038/s43016-022-00589-6

Saraiva, J.L., Arechavala-Lopez, P., Castanheira, M.F., Volstorf, J., Heinzpeter Studer, B., 2019. A global assessment of welfare in farmed fishes: The FishEthoBase. Fishes 4, 30.

Sarker, P.K., Kapuscinski, A.R., McKuin, B., Fitzgerald, D.S., Nash, H.M., Greenwood, C., 2020. Microalgae-blend tilapia feed eliminates fishmeal and fish oil, improves growth, and is cost viable. Scientific Reports 10, 19328. https://doi.org/10.1038/s41598-020-75289-x

Sayed, A., 2018. Optimum Crude Protein Requirement of the Fingerlings Nile Tilapia (Oreochromis niloticus). Biological Sciences 2, 1. https://doi.org/10.31058/j.bs.2018.21001

Sharif, M., Zafar, M.H., Aqib, A.I., Saeed, M., Farag, M.R., Alagawany, M., 2021. Single cell protein: Sources, mechanism of production, nutritional value and its uses in aquaculture nutrition. Aquaculture 531, 735885.

Simpson, S.J., Raubenheimer, D., 2001. A framework for the study of macronutrient intake in fish: A framework for nutrient regulation. Aquaculture Research 32, 421–432. https://doi.org/10.1046/j.1365-2109.2001.00593.x

Smárason, B.Ö., Ögmundarson, Ó., Árnason, J., Bjornsdottir, R., Davidsdottir, B., 2017. Life cycle assessment of Icelandic arctic char fed three different feed types.

Soininen, N., Romppanen, S., Huhta, K., Belinskij, A., 2021. A brake or an accelerator? The role of law in sustainability transitions. Environmental Innovation and Societal Transitions 41, 71–73.

Spalvins, K., Zihare, L., Blumberga, D., 2018. Single cell protein production from waste biomass: comparison of various industrial by-products. Energy procedia 147, 409–418.

Stevens, J.R., Newton, R.W., Tlusty, M., Little, D.C., 2018. The rise of aquaculture by-products: Increasing food production, value, and sustainability through strategic utilisation. Marine Policy 90, 115–124.

Sumaila, U.R., Pierruci, A., Oyinlola, M.A., Cannas, R., Froese, R., Glaser, S., Jacquet, J., Kaiser, B.A., Issifu, I., Micheli, F., Naylor, R., Pauly, D., 2022. Aquaculture over-optimism? Frontiers in Marine Science 9.

Sun, M., Hassan, S.G., Li, D., 2016. Models for estimating feed intake in aquaculture: A review. Computers and Electronics in Agriculture 425–438. https://doi.org/10.1016/j.compag.2016.06.024

Sundh, H., 2009. Chronic stress and intestinal barrier function: Implications for infection and inflammation in intensive salmon aquaculture.

Tacon, A.G., 2020. Trends in global aquaculture and aquafeed production: 2000–2017. Reviews in Fisheries Science & Aquaculture 28, 43–56.

Tacon, A.G.J., Metian, M., 2015. Feed Matters: Satisfying the Feed Demand of Aquaculture. Reviews in Fisheries Science & Aquaculture 23, 1–10. https://doi.org/10.1080/23308249.2014.987209

Tefal, E., Jauralde, I., Tomás-Vidal, A., Martínez-Llorens, S., Peñaranda, D.S., Jover-Cerdá, M., 2023a. New Organic Raw Materials for Gilthead Seabream (*Sparus aurata*) Feeding and the Effects on Growth, Nutritive Parameters, Digestibility, and Histology. Fishes 8, 330. https://doi.org/10.3390/fishes8060330

Tefal, E., Tomás-Vidal, A., Martínez-Llorens, S., Jauralde, I., Sánchez-Peñaranda, D., Jover-Cerdá, M., 2023b. Effects of Eco-Organic Feed on Growth Performance, Biometric Indices, and Nutrient Retention of Gilthead Seabream (*Sparus aurata*). Sustainability 15, 10750. https://doi.org/10.3390/su151410750

Teodósio, R., Aragão, C., Conceição, L.E.C., Dias, J., Engrola, S., 2022. Amino Acid Metabolism in Gilthead Seabream Is Affected by the Dietary Protein to Energy Ratios. Aquaculture Nutrition 2022, 1–10. https://doi.org/10.1155/2022/8230704

Teodósio, R., Engrola, S., Colen, R., Masagounder, K., Aragão, C., 2020. Optimizing diets to decrease environmental impact of Nile tilapia (*Oreochromis niloticus*) production. Aquaculture Nutrition 26, 422–431. https://doi.org/10.1111/anu.13004

Toldrá, F., Mora, L., Reig, M., 2016. New insights into meat by-product utilization. Meat science 120, 54–59.

Tomić, M., Matulić, D., Jelić, M., 2016. What determines fresh fish consumption in Croatia? Appetite 106, 13–22. https://doi.org/10.1016/j.appet.2015.12.019

Torrecillas, S., Caballero, M.J., Mompel, D., Montero, D., Zamorano, M.J., Robaina, L., Rivero-Ramírez, F., Karalazos, V., Kaushik, S., Izquierdo, M., 2017. Disease resistance and response against *Vibrio anguillarum* intestinal infection in European seabass (*Dicentrarchus labrax*) fed low

fish meal and fish oil diets. Fish & Shellfish Immunology 67, 302–311. https://doi.org/10.1016/j.fsi.2017.06.022

Trewavas, E., 1983. Tilapiine fishes of the genera Sarotherodon, Oreochromis, and Danakilia. 583.

Turchini, G.M., Torstensen, B.E., Ng, W.-K., 2009. Fish oil replacement in finfish nutrition. Reviews in Aquaculture 1, 10–57. https://doi.org/10.1111/j.1753-5131.2008.01001.x

United Nations, 2019. World population prospects Highlights, 2019.

Vale Pereira, G. do, Conceição, L.E.C., Soares, F., Petereit, J., Buck, B.H., Johansen, J., Dias, J., Faccenda, F., 2023. Alternative Feed Formulations Impact Growth Performance, Flesh Quality and Consumer Acceptance of Rainbow Trout (*Oncorhynchus mykiss*). Journal of Marine Science and Engineering 11, 1135. https://doi.org/10.3390/jmse11061135

Vasquez-Mejia, C.M., Shrivastava, S., Gudjónsdóttir, M., Manzardo, A., Ögmundarson, Ó., 2023. Current status and future research needs on the quantitative water use of finfish aquaculture using Life Cycle Assessment: A systematic literature review. Journal of Cleaner Production 425, 139009. https://doi.org/10.1016/j.jclepro.2023.139009

Verdegem, M., Buschmann, A.H., Latt, U.W., Dalsgaard, A.J.T., Lovatelli, A., 2023. The contribution of aquaculture systems to global aquaculture production. Journal of the World Aquaculture Society 54, 206–250. https://doi.org/10.1111/jwas.12963

Volkoff, H., 2019. Fish as models for understanding the vertebrate endocrine regulation of feeding and weight. Molecular and Cellular Endocrinology 497, 110437. https://doi.org/10.1016/j.mce.2019.04.017

Wan, A.H., Davies, S.J., Soler-Vila, A., Fitzgerald, R., Johnson, M.P., 2019. Macroalgae as a sustainable aquafeed ingredient. Reviews in Aquaculture 11, 458–492.

Wang, B., Thompson, K.D., Wangkahart, E., Yamkasem, J., Bondad-Reantaso, M.G., Tattiyapong, P., Jian, J., Surachetpong, W., 2023. Strategies to enhance tilapia immunity to improve their health in aquaculture. Reviews in Aquaculture 15, 41–56.

White, B.D., Porter, M.H., Martin, R.J., 2000. Protein selection, food intake, and body composition in response to the amount of dietary protein. Physiology & Behavior 69, 383–389. https://doi.org/10.1016/S0031-9384(99)00232-2

Wilfart, A., Espagnol, S., Dauguet, S., Tailleur, A., Gac, A., Garcia-Launay, F., 2016. ECOALIM: A Dataset of Environmental Impacts of Feed Ingredients Used in French Animal Production. PLoS ONE 11, e0167343. https://doi.org/10.1371/journal.pone.0167343

Wilson, R.P., 1991. Handbook of nutrient requirements of finfish. CRC press Boca Raton.

Woodgate, S.L., Wan, A.H.L., Hartnett, F., Wilkinson, R.G., Davies, S.J., 2022. The utilisation of European processed animal proteins as safe, sustainable and circular ingredients for global aquafeeds. Reviews in Aquaculture 14, 1572–1596. https://doi.org/10.1111/raq.12663

Xie, B., Qin, J., Yang, H., Wang, X., Wang, Y.-H., Li, T.-Y., 2013. Organic aquaculture in China: A review from a global perspective. Aquaculture 414–415, 243–253. https://doi.org/10.1016/j.aquaculture.2013.08.019

Zander, K., Risius, A., Feucht, Y., Janssen, M., Hamm, U., 2018. Sustainable Aquaculture Products: Implications of Consumer Awareness and of Consumer Preferences for Promising Market Communication in Germany. Journal of Aquatic Food Product Technology 27, 5–20. https://doi.org/10.1080/10498850.2017.1390028

Zhan, J., Lu, J., Wang, D., 2022. Review of shell waste reutilization to promote sustainable shellfish aquaculture. Reviews in Aquaculture 14, 477–488.

Zhang, H., Ran, C., Teame, T., Ding, Q., Hoseinifar, S.H., Xie, M., Zhang, Z., Yang, Y., Olsen, R.E., Gatlin, D.M., 2020. Research progress on gut health of farmers teleost fish: a viewpoint concerning the intestinal mucosal barrier and the impact of its damage. Reviews in Fish Biology and Fisheries 30, 569–586.

Zortea, R.B., Maciel, V.G., Passuello, A., 2018. Sustainability assessment of soybean production in Southern Brazil: A life cycle approach. Sustainable Production and Consumption 13, 102–112.

ANNEXES

7. Annexes

7.1. Scientific Publications

1) Mendes, R., Conceição, L.E.C., Dias, J., Engrola, S., Sánchez-Vázquez, F.J. Nile tilapia and gilthead seabream dietary self-selection of alternative feeds. Fish Physiology and Biochemistry (2024). Published.

2) Mendes, R., Rema, P., Dias, J., Gonçalves, A.T., Teodósio, R., Engrola, S., Sánchez-Vázquez, F.J., Conceição, L.E.C. Socially-acceptable feed formulations may impact the voluntary feed intake and growth, but not robustness of Nile tilapia (*Oreochromis niloticus*). Fishes MDPI (2024). Published.

3) Mendes, R., Teodósio, R., Dias, J., Gonçalves, A.T., Speranza, L., Magalhães, S., Aires, T., Sánchez-Vázquez, F.J., Conceição, L.E.C., Engrola, S. Impact of organic-based and circular economy-driven feeds on performance and robustness of juvenile gilthead seabream (*Sparus aurata*). Submitted.

7.2. Congress Contributions

1) Mendes, R., Teodósio R., Dias, J., Gonçalves, A.T.G., Speranza L., Magalhães S., Aires T., Vázquez, F.J.S., Conceição L.E.C., Engrola, S., 2024. Impact of organic-based and circular economy-driven feeds on performance and robustness of juvenile gilthead seabream (*Sparus aurata*). AQUA 2024, Copenhagen, Denmark. **Oral communication**.

2) Mendes, R., Conti, F., Pintos, S., Teodósio R., Rema, P., Dias, J., Gonçalves, A.T.G., Vera L.M., López-Olmeda J.F., Engrola, S., Bertolucci C., Vázquez, F.J.S., Conceição L.E.C., 2024. Influencia de piensos socialmente sostenibles en la ingesta voluntaria de alimento, crecimiento, robustez y respuesta al estrés de la tilapia del Nilo (*Oreochromis niloticus*). XIX Congreso Nacional de Acuicultura 2024, Las Palmas de Gran Canaria, Spain. **Poster**.

3) Mendes, R., Rema, P., Dias, J., Gonçalves, A.T.G., Teodósio R., Engrola, S., Vázquez, F.J.S., Conceição L.E.C., 2023. Can eco-efficient, circular economy-driven and organic formulations improve the performance and robustness of Nile tilapia? Aquaculture Europe 2023, Vienna, Austria. **Oral communication**.

4) Mendes, R., Conceição L.E.C., Vázquez, F.J.S., 2022. The use of diet encapsulation as a valuable tool to identify the potential of novel fish diets, Aquaculture Europe 2022, Rimini, Italy. **Oral communication & Poster**.

5) Mendes, R., Soares, F., Silva, T., Tulli, F., Conceição L., Nobre, A., 2021. The use of a nutrient-based model as an additional tool in determining amino acid requirements. International Symposium on Fish Nutrition and Feeding (online), Busan, South Korea. **Poster**.

6) Mendes, R., Soares, F., Silva, T., Nobre, A., Conceição L.E.C., 2021. The use of a nutrient-based model as a decision tool to support the design of nutritional experiments, Aquaculture Europe 2021, Funchal, Portugal. **Poster**.

RESUMEN EN CASTELLANO

8. Resumen en Castellano

El objetivo de esta tesis doctoral es desarrollar un conjunto de piensos basados en la economía circular (ecoeficientes) o en marcos orgánicos que incluyan, en particular, harinas vegetales no tradicionales (p. ej., girasol, quinoa, colza, altramuces), subproductos de animales terrestres (p. ej., harinas de sangre, plumas y aves), subproductos del mar (p. ej., concentrado de proteínas de pescado, aceite de salmón), harinas de insectos (p. ej., mosca soldado negra, *Hermetia illucens*, o gusano de la harina, *Tenebrio molitor*) y microorganismos unicelulares (p. ej., bacterias, cianobacterias, microalgas y levaduras). Además, se investigó el potencial de calentamiento global, así como la aceptación y los efectos (p. ej., en la ingesta de alimentos, el crecimiento, la salud intestinal, la condición inmunológica y el estado oxidativo) de estos peinsos innovadores, utilizando dos especies de acuicultura comercialmente relevantes, la tilapia del Nilo (*Oreochromis niloticus*) y la dorada (*Sparus aurata*). Así, se persiguieron los siguientes objetivos específicos:

1) Formular una nueva generación de piensos orgánicos o ecoeficientes para peces que respondan a las preocupaciones de la sociedad y reduzcan el uso de ingredientes tradicionales para piensos (p. ej., recursos marinos y soja) (**Capítulos I, II y III**).

2) Investigar el comportamiento alimentario de la tilapia del Nilo y la dorada, así como sus preferencias por la nueva generación de piensos orgánicos o ecoeficientes, utilizando una metodología de autoselección (alimentadores a demanda) (**Capítulo I**).

3) Evaluar la digestibilidad de las dietas y sus efectos sobre los indicadores clave de rendimiento (aumento de peso, índice de conversión alimentar, ingesta de alimento), la robustez de los peces (salud intestinal, estado inmunitario y estado oxidativo) y la utilización del alimento (composición corporal, retención) en juveniles de tilapia del Nilo y dorada, mediante estudios experimentales a largo plazo (**Capítulos II y III**).

4) Evaluar el potencial de calentamiento global (GWP) de los nuevos piensos un LCA con asignación económica (**Capítulo III**).

Esta Tesis Doctoral se ha realizado con el apoyo de una ITN Industrial (EASYTRAIN) y con la supervisión principal de un socio industrial (SPAROS), lo que ha garantizado la aplicación directa de los resultados por parte de la industria. Los resultados de esta investigación proporcionarán una evaluación exhaustiva de los efectos y las repercusiones medioambientales de piensos acuícolas socialmente aceptables. En general, esta tesis doctoral arrojará luz sobre la nutrición, comportamiento alimentario, fisiología y salud de los peces de acuicultura alimentadas con estos nuevos piensos, así como sobre su huella medioambiental.

Para cumplir estos objetivos se desarrollaron diversos experimentos que aparecen organizados en 3 capítulos experimentales, los cuales aparecen resumidos a continuación.

Capítulo I: **Autoselección alimentaria de la tilapia del Nilo y la dorada con piensos alternativos**

Las evaluaciones clásicas de los nuevos piensos para peces son antropocéntricas y se centran principalmente en el crecimiento. Aunque esta metodología es precisa, no tiene en cuenta la perspectiva de los peces. El objetivo de este estudio era investigar las respuestas conductuales y las preferencias alimentarias de la tilapia del Nilo (*Oreochromis niloticus*) y la dorada (*Sparus aurata*) mediante un ensayo de autoselección con alimentadores a demanda. A ambas especies se les ofrecieron tres piensos: uno de control (PD) de tipo comercial y dos dietas (ORG1 y ORG2) formuladas con diferentes inclusiones de ingredientes alternativos para abordar algunas de las actuales preocupaciones medioambientales y/o cuestiones éticas a menudo asociadas con las formulaciones comerciales. Se probaron tres grupos de tilapias con un peso medio de 163.0 g ± 4.3 g (media ± DE) y cuatro grupos de doradas con 174.7 g ± 27.0 g. La tilapia mostró preferencia por el ORG2 (46.5%), influida por las propiedades sensoriales del alimento y las señales posteriores a la ingestión. La dorada no mostró preferencia por ningún alimento. Estos resultados ponen de relieve la eficacia de los experimentos de autoselección para permitir a los peces expresar su comportamiento alimentario y

sus preferencias. Por lo tanto, este enfoque debería tenerse en cuenta en la selección inicial y el diseño de nuevos piensos e ingredientes para la acuicultura.

<u>Capítulo II</u>: Las fórmulas de piensos socialmente aceptables pueden influir la ingesta voluntaria de alimento y el crecimiento, pero no la robustez de la tilapia del Nilo (*Oreochromis niloticus*)

La sociedad es cada vez más exigente con la huella medioambiental de la acuicultura, el uso de recursos y el bienestar de los animales. Para mitigar estas preocupaciones, las fórmulas de los piensos podrían basarse en ingredientes ecoeficientes (basados en la economía circular) u orgánicos. El objetivo de este estudio era investigar el rendimiento, la utilización del alimento y el estado de salud de los juveniles de tilapia del Nilo (*Oreochromis niloticus*) alimentados con estos nuevos piensos. El ensayo de crecimiento duró 8 semanas y los peces tenían un peso inicial de 31.0 ± 0.5 g (media ± DE). Los peces fueron alimentados hasta la saciedad visual, por cuadruplicado, con uno de los tres piensos experimentales isonitrogenados e isoenergéticos: un pienso de tipo comercial sin harina de pescado (PD), una dieta basada en ingredientes compatibles con la certificación ecológica (ORG) o un pienso formulado utilizando subproductos e ingredientes emergentes impulsados por la economía circular (ECO). Los peces alimentados con ECO mostraron una tendencia a la disminución de la ingesta de alimento, mientras que los peces alimentados con ORG redujeron significativamente su ingesta en comparación con los alimentados con PD. En consecuencia, los peces alimentados con ECO mostraron casi la mitad de crecimiento que los alimentados con PD, mientras que los peces ORG prácticamente no aumentaron de peso. Las dietas ECO y ORG presentaron una digestibilidad más baja de proteínas, lípidos y energía que las dietas PD. La utilización del alimento de los peces alimentados con ECO u ORG también fue inferior a la de los alimentados con PD. De los genes relacionados con la salud analizados, sólo la glutatión reductasa (*gsr*) mostró diferencias estadísticamente significativas, siendo más expresada en los peces alimentados con ECO que en los alimentados con PD. Así pues, aun cuando tales formulaciones novedosas indujeron efectos extremos en la ingesta voluntaria de alimento, su impacto sólo se dejó notar en el crecimiento de los peces, pero no en su robustez.

Resumen en Castellano

<u>Capítulo II</u>: Impacto de la alimentación ecológica y basada en la economía circular en el rendimiento y la robustez de juveniles de dorada (*Sparus aurata*)

Para adaptarse a las demandas de la sociedad, la acuicultura necesita mejorar el huella medioambiental de sus piensos, garantizando al mismo tiempo la salud de los organismos cultivados. Con este objetivo, las formulaciones novedosas podrían basarse en ingredientes orgánicos o impulsados por la economía circular. El objetivo de este estudio era evaluar el potencial de calentamiento global (GWP) y la digestibilidad de dichos piensos, así como sus efectos sobre el rendimiento, la utilización de los alimentos y el estado de salud de los juveniles de dorada (*Sparus aurata*) en condiciones de crecimiento y tras la exposición a un desafío (hacinamiento). Se formularon tres dietas experimentales isonitrogenadas e isoenergéticas: un pienso de control (CTRL) de tipo comercial; una dieta ecológica (ORG) basada en ingredientes compatibles con la certificación ecológica, con inclusión limitada de proteínas animales; y un pienso ecoeficiente (ECO) que utilizaba subproductos impulsados por la economía circular, con inclusión limitada de harina de pescado. El GWP de cada pienso se calculó utilizando la metodología de Evaluación del Impacto del Ciclo de Vida con asignación económica. Se alimentó a juveniles de dorada tres veces al día, por triplicado, según una tabla de alimentación durante un periodo de crecimiento de 9 semanas y un periodo de desafío de 2 semanas, con una densidad inicial de 12.5 kg/m^3 (a partir de 8 kg/m^3 al final de la fase de crecimiento). Aunque al final del periodo de crecimiento el peso corporal final fue mayor en los peces alimentados con las dietas CTRL y ECO que en los alimentados con ORG, todos los peces aumentaron su peso corporal inicial al menos tres veces. La salud de los peces se mantuvo durante el periodo de crecimiento y la resistencia general al estrés no se vio afectada tras el evento estresante crónico. Todos los alimentos se utilizaron eficazmente y presentaron coeficientes de digestibilidad altos y normales. A pesar de tener un GWP más alto, los piensos ORG y ECO pueden proporcionar beneficios medioambientales, y en particular los peces ORG pueden aumentar la retención de fósforo. Los piensos orgánicos y de economía circular tienen potencial para responder a las preocupaciones de los

consumidores y disminuir algunos de los impactos ambientales de la acuicultura, al tiempo que garantizan un buen rendimiento y robustez de los peces.

Basado en los resultados obtenidos de los capítulos experimentales, esta tesis doctoral alcanzó las siguientes conclusiones:

1) La tilapia del Nilo mostró una capacidad para seleccionar su dieta, expresando su comportamiento alimenticio. Por lo tanto, los experimentos de auto demanda pueden considerarse una herramienta posible para una evaluación inicial de nuevos ingredientes para alimentos en acuicultura (**Capítulo I**).

2) Las formulaciones de alimentos socialmente aceptables indujeron efectos extremos en la ingesta voluntaria de alimento de la tilapia del Nilo, debido a la reducción de la palatabilidad, lo que resultó en una pobre utilización del alimento. No obstante, la digestibilidad de la dieta fue alta y el impacto solo se observó en el rendimiento del pez, pero no en su robustez (**Capítulo II**).

3) En el caso de la dorada, los alimentos basados en la economía circular y orgánica fueron fácilmente digestibles y eficientemente utilizados por los peces, asegurando un buen rendimiento y robustez incluso después de un evento de estrés crónico (**Capítulo III**).

4) Los alimentos para tilapia basados en la economía circular y orgánica, así como el alimento orgánico para dorada, presentaron el mayor GWP (potencial de calentamiento global). Sin embargo, a largo plazo, tales dietas pueden promover un mejor rendimiento a nivel de sistema (**Capítulo III**).

Los principales hallazgos de esta Tesis Doctoral sugieren varios enfoques para profundizar en la personalización de los alimentos para acuicultura, con el fin de que sean cada vez más sostenibles. Las formulaciones de alimentos pueden optimizarse seleccionando cuidadosamente los ingredientes y estableciendo límites en sus niveles de inclusión, incorporando combinaciones distintas de fuentes

de proteínas o añadiendo atrayentes alimentarios para mejorar la palatabilidad y aceptación de los alimentos. Analizar los perfiles de ácidos grasos y la digestibilidad de los ingredientes individuales podría proporcionar una visión más precisa sobre la utilización de los alimentos y su digestibilidad global. Evaluar otros impactos ambientales de los ingredientes de los alimentos, incluyendo la eutrofización, la acidificación, el uso de recursos y/o el uso del suelo, proporcionaría una visión más holística de la huella ambiental de los alimentos para acuicultura. Además, al determinar los impactos ambientales de los ingredientes alimentarios, debería ser prioritario que las evaluaciones se realicen utilizando las mismas reglas y marco, para minimizar las variaciones y la incertidumbre, especialmente si los datos provienen de fuentes externas. En el futuro, se recomienda que se realicen más estudios de auto-alimentación y crecimiento en otras especies de importancia comercial (por ejemplo, salmón y carpa) en respuesta a alimentos alternativos para acuicultura, ya que dichos estudios tendrían en cuenta las diferencias en los sistemas digestivos, respuestas fisiológicas, tasas metabólicas y comportamientos alimenticios de las especies. Para permitir la inclusión de ingredientes alternativos, serían beneficiosos un mayor financiamiento, mejores marcos regulatorios, una mayor aceptación y educación por parte del consumidor, técnicas de producción y procesamiento más rentables, así como un mayor acceso a energías renovables. En última instancia, un enfoque holístico y transparente que equilibre la nutrición, el comportamiento alimenticio, el rendimiento y la robustez de las especies cultivadas con la sostenibilidad ambiental, social y económica de la acuicultura es necesario para asegurar el desarrollo a largo plazo del sector, como una fuente vital de proteína animal.