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Andrea Bertini

Towards precision fish farming and low carbon productions in aquaculture: new tools and technologies for a more sustainable, resilient and circular fish farming

Supervisor(s)

Prof. Annachiara Berardinelli, Prof. Alessio Bonaldo

Tutor

Prof. Annachiara Berardinelli

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

AA	Amino acid
ALB	Albumin
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANOVA	Analysis of variance
ARA	Arachidonic acid
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BAPNA	Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride
BHT	Butylated hydroxytoluene
CAT	Catalase
CK	Creatin kinase
CO_2	Carbon dioxide
DGLA	Homo-g-linolenic acid
DHA	Docosahexaenoic acid
DNPH	2,4-dinitrophenylhydrazine
DPH	Days post hatching
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EGTA	Egtazic acid
EPA	Eicosapentaenoic acid
FAMEs	Fatty acids methyl esters
FCR	Feed conversion rate
FFA	Free fatty acid
FFDR	Forage-fish dependency ratio
FI	Feed intake
FIFO	Fish in/ Fish out ratio

FM	Fishmeal
FO	Fish oil
GLE	Gross lipid efficiency
GLOB	Globulin
GPE	Gross protein efficiency
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSI	Hepatosomatic index
ISO	International standard organisation
K	Fulton's condition factor
LCA	Lifecycle assessment analysis
LC-PUFAs	Long chain polyunsaturated fatty acids
LDH	Lactate dehydrogenase
LER	Lipid efficiency rate
LPO	Lipid peroxidation
mtROS	Mitochondrial reactive oxygen species
NMDS	Non-metric multi-dimensional scaling
NMR	Nuclear magnetic resonance
PBS	Phosphate buffer saline
PC	Protein carbonylation
PCA	Principal component analysis
PCBs	Polychlorinated biphenyls
PER	Protein efficiency rate
PERMANOVA	Permutational Multivariate Analysis of Variance
PLS	Partial least square
PM	Poultry meal
PMS	Post-mitochondrial supernatant
POPs	Persistent organic pollutants
PUFA	Polyunsaturated fatty acid
QIM	Quality index method

RGL	Relative gut length
RMSE	Root mean square error
SBM	Soybean meal
SCP	Single cell protein
SGR	Specific growth rate
SMA	Sub miniature version A connector
SWR	Standing wave ratio
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid-reactive substances
TMA	Trimethylamine
TVB-N	Total volatile basic nitrogen
TVC	Total viable count
USD	United States dollar
VNA	Vector network analyser
VSI	Viscerosomatic index
VSWR	Voltage standing wave ratio
WW	Wet weight

ABSTRACT

The exponential growth of aquaculture over the past two decades has generated both optimism and concern among scientists and policy analysts, particularly regarding environmental sustainability. While aquaculture is acknowledged as one of the least impactful forms of animal production, with minimal use of natural resources, chemicals, and antibiotics, persistent environmental challenges demand attention. This thesis addresses a fundamental question: how can we minimise the environmental impact of aquaculture production? A multidisciplinary examination of the entire aquaculture value chain identified critical points where sustainability challenges are most apparent and can be targeted. A recurring theme throughout the thesis revolves around the exploration of alternative ingredients to marine and plant resources, particularly those derived from industrial by–products, such as single–cell proteins and oil–rich microalgae, in alignment with the principles of a circular economy.

The initial study showcased the potential of microalgae, specifically *Nannochloropsis* spp. and *Spirulina* spp., produced by converting waste outputs from a geothermal plant, to replace traditional encapsulated fish oil in the diet of European seabass larvae. This shift not only reduced pressure on wild resources but also allowed for the valorisation of waste streams and decreased downstream costs associated with industrial waste disposal. Furthermore, larvae fed microalgae diets exhibited a favourable long–chain polyunsaturated fatty acids (LC–PUFAs) profile, with notable levels of docosahexaenoic acid (DHA), and lower catalase activity, indicating potential antioxidant effects. In contrast, the second study revealed that *Corynebacterium glutamicum* cell mass, a by–product of industrial amino acid production with high protein content, is not a suitable substitute for fishmeal and soybean meal in the diet of flathead grey mullet. The investigation, encompassing key performance indicators and sophisticated variables like metabolic blood parameters, digestive enzyme

activities, and gut microbiota, hypothesised that the species' digestive system organisation led to incomplete utilisation of the ingredient due to poor lysis of cell–wall components. Despite unexpected outcomes, these trials contribute theoretical foundations for the advancement of sustainable aquafeed formulations.

Transitioning from production to commercialisation, additional environmental challenges were identified, with over 35 % of global fish production wasted or lost due to incorrect storage or problems at the supply chain. The third study introduced a novel, rapid, and non–destructive method for fish freshness evaluation at the commercialisation level. This involved a device with a vector network analyser interfaced with an open coaxial probe, placed in contact with the fish eye, leveraging dielectric properties. The integration with multivariate analysis facilitated the creation of predictive models for storage time and demerit scores of fish spoilage, offering high sensitivity. This innovative approach is poised to assist producers and retailers in the busy commercial setting, addressing a crucial aspect of environmental sustainability in aquaculture.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

In a world where nutritious food are in high demand, fish products represent an essential component of the global food basket. These products offer consumers a diverse array of high–value components, such as essential amino acids, vitamins (particularly A, B and D), minerals and are a primary dietary source of long chain polyunsaturated fatty acids (LC–PUFAs) (Calder, 2018; Mohanty et al., 2019; Golden et al., 2021; Chen et al., 2022). According to the latest FAO report titled "The State of World Fisheries and Aquaculture" (FAO, 2022), the consumption of aquatic foods has shown a consistent upward trend. From 1961 to 2019, it increased by an average annual rate of 3.0 %, surpassing the annual world population growth rate of 1.6 % during the same period. Despite experiencing a slight decline in 2020 due to reduced demand as a consequence of COVID-19 pandemia (Mitra et al., 2023), it is anticipated that consumption will continue to increase until 2030. This growth can be attributed to sociocultural shifts in the recent years, involving changes in consumer preferences towards healthier food, advancements in production technology, increased supplies and global income growth (Menozzi et al., 2020; FAO, 2022).

Against this backdrop, aquaculture has garnered increasing recognition for its essential contribution to global food security and nutrition. Currently, global aquaculture supplies more fish for human consumption than wild capture fisheries (FAO, 2022). With a focus on European market, the total aquaculture production is estimated to be 2.9 million tons in 2022, with 67 % attributed to marine cold–water species, 14 % to freshwater species and 19 % to marine Mediterranean species (FEAP, 2023). Norway emerges as the dominant European producer, contributing 58 % to the total aquaculture supply, primarily driven by Atlantic salmon production. Other countries with significant annual production exceeding 100 thousand tons include Turkey, the United Kingdom and Greece. The primary species cultivated in Europe, listed in terms of production volume, include Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*),

gilthead seabream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*) and carp (*Cyprinus carpio*). Together, these species constitute 95 % of the total aquaculture production in the region (FEAP, 2023).

In recent years, societal awareness of the environmental impact of anthropogenic activities has increased (Carballeira Braña et al., 2021; van Bussel et al., 2022). Consequently, sustainability has evolved into a focal point of debate within contemporary political discourse. This has given rise to a complex and multifaceted arena, encompassing communication, policy formulation, and the active engagement of political entities. The debate on sustainability extends beyond environmental concerns to encompass social and economic dimensions, reflecting a comprehensive approach to addressing the challenges faced by society. The concept of sustainability was defined in the Brundtland Report of 1987 as "meeting the needs of the present generation without compromising the ability of future generations to meet their own needs" (Kuhlman and Farrington, 2010). When this definition is applied to aquaculture sector, sustainability means adopting practices and principles that balance the current needs of the industry with the long–term health of aquatic ecosystems, economic viability, and social equity.

Establishing a universal definition of environmental impact proves challenging due to the diverse ways anthropogenic production can affect various aspects. Recently, environmental impact assessment based on Life Cycle Assessment (LCA) has become increasingly important because it provides a comprehensive view of the environmental impact of a product or process (Ghamkhar et al., 2021; Zoli et al., 2023). As a scientific method, LCA considers all inputs and outputs, allowing for an exhaustive overview of both upstream (material sourcing and delivery) and downstream (product distribution, use, and disposal) impacts associated with any given system. LCA is a data-intensive methodology that considers several impact categories, including climate change, land usage, and water usage. By analysing their relative scores, the aquaculture industry

emerges as one of the least environmentally impactful animal production methods. According to Poore and Nemecek (2018) and Golden et al. (2021), on average, aquaculture records a CO_2 production of 24 grams equivalent per g of protein produced. In contrast, poultry, pork, and beef production demonstrate much higher carbon footprints, with figures standing at 43 grams, 65 grams, and 238 grams of CO_2 equivalent per g of protein, respectively. Furthermore, aquaculture displays a more efficient use of land, requiring notably less space compared to other forms of animal production. It averages 0.04 m^2 per gram of protein, whereas poultry, pork, and beef production demand 0.06, 0.08, and 0.6 m^2 per gram of protein, respectively. However, freshwater usage tends to be higher in aquaculture, with an average of 13.1 litres of water per gram of protein, contrasting with 2.1, 11.2, and 8.5 litres of water per gram of protein for poultry, pork, and beef production, respectively. Nevertheless, advancements in the development of recirculating aquaculture systems (RAS) are expected to significantly mitigate this impact in the future (Zorzi et al., 2023). Moreover, aquaculture production benefits from a favourable feed conversion rate (FCR). This conventional measure of livestock production efficiency calculates the weight of feed intake relative to the biomass gained by farmed animals. FCR is often explained as eFCR (economic), which factors in feed wastage and mortalities, and bFCR (biological), excluding non–consumed feed and production losses from the calculation. The cold–blooded metabolism inherent in fish contributes to a more efficient utilisation of energy. Notably, the average eFCR for global aquaculture production stood at 0.73 in 2020, reflecting an improvement from 0.81 in 2000, with Atlantic salmon weighing in at 1.30. In comparison, traditional terrestrial animal production faces higher eFCRs, requiring 2, 3 and 8 kg of feed to produce 1 kg of poultry, pork and beef respectively (IFFO, 2022).

However, as the aquaculture sector undergoes development and expansion, its impact on the surrounding environment intensifies. At production level, these effects encompasses nutrient

pollution caused from uneaten feed and organic metabolic waste (Herbeck et al., 2013), chemical pollution resulting from various substances utilised in the production process (such as antibiotics and antiparasitic residues) (MacLeod et al., 2020) and the dissemination of farmed fish genes, parasites, and diseases to wild populations (Carballeira Braña et al., 2021). An important indirect consequence of the production process, is the potential impact on aquatic wild ecosystems due to the overexploitation of traditional fishing areas when wild marine resources are used into aquafeed formulations (Colombo et al., 2022; Glencross et al., 2023; Bertini et al., 2023b). When formulating aquafeed, the quantity of wild fish used in the production of farmed fish is a critical parameter affecting sustainability. Several metrics are employed to analyse aquafeed performance, with two common ones being the Fish–In / Fish–Out ratio (FIFO) and the Forage–Fish Dependency Ratio (FFDR). FIFO remains a valuable indicator for assessing the quantity of wild fish utilised to produce farmed fish. It provides a straightforward measure, although it does not consider the value and quality of ingredients, nor does it distinguish from by–products and more sustainable sources from reduction fishery. The formula for this ratio consider the amount of fishmeal and fish oil into aquafeed formulations divided by yield of wild fish to producing fishmeal (22.5 %) and fish oil (5 %) and multiplied for eFCR. On average, in both salmonids and marine finfish species, this ratio decreased from 3.03 and 2.21 in 2000 to 0.93 and 0.75 in 2020, respectively, indicating that nowadays, less than 1 kilogram of wild fish is required to produce 1 kilogram of farmed fish (IFFO, 2022). This reduction has primarily occurred by incorporating more plant–based ingredients, as soybean meal, into formulations despite the rising of concerns related to sustainability and resource efficiency. In fact, several studies on Life Cycle Assessment demonstrated that the substitution of marine ingredients with vegetable ingredients have shifted resource demand from the oceans onto the land with an overall environmental impacts of feed production expected to increase (Wilkinson and Young, 2020; Colombo et al., 2022; Newton et al., 2023).

As a result, the industry's overarching objective is to progressively decrease the reliance on wild marine resources and plant–based ingredients by incorporating novel alternative ingredients. Fish diets of the future are expected to incorporate a broader array of alternative ingredients. Among these alternatives, those derived from bio industrial by–products are emerging as promising options (Glencross et al., 2020; Colombo et al., 2022; Glencross et al., 2023; Bertini et al., 2023b). Industrial by–products offer several advantages, including their non–competition with human nutrition and the presence of an enticing market opportunity, allowing the valorisation of biomass and avoiding costs associated with disposal (Jones et al., 2020; Pereira et al., 2022). This concept of production, defined as "circular economy" or "bioeconomy", encompass the production of renewable biological resources and the conversion of these resources and waste streams into value–added products, including food, feed, bio–based products, and bioenergy (Berbel and Posadillo, 2018; Yarnold et al., 2019; "A European Green Deal," 2021). This approach represents a sustainable option to the unsustainable classic linear economic model of "take–make–dispose". The strategic approach of bioeconomy in terms of by–products utilisation is discussed by Berbel and Posadillo, (2018) in the biomass pyramid value. At its pinnacle, biomass holds the highest value when utilised for pharmaceuticals or bioactive compounds. Following closely, food and animal feed represent the second tier in the value pyramid, while chemicals occupy the third position. By–product biomass is allocated to energy generation, considered the lowest priority, only after fulfilling higher–value applications in the pyramid.

Biomass derived from industry by–products, such as single–cell ingredients (bacterial, fungal, or algal biomasses), typically exhibit favourable profiles for meeting the nutritional requirements of fish in aquafeed formulations (Glencross et al., 2020; Jones et al., 2020; Marchi et al., 2023; Glencross et al., 2023). For instance, bacterial single cell proteins such as *Methylophilus*, *Methylococcus* and *Corynebacterium* biomasses are safe, with high protein content

(> 70 %) and generally presented good digestibility (Lee et al., 2016; Glencross et al., 2020; Marchi et al., 2023). Microalgae single cell ingredients, such as *Nannochloropsis* spp., and cyanobacteria such as *Spirulina* spp., can produce significant sources of long–chain polyunsaturated fatty acids (LC–PUFAs) (Glencross et al., 2020; Conde et al., 2021; Gu et al., 2022). In addition, can be produced on non–arable land, acting as CO_2 sequestrators resulting in a sustainable carbon neutral production (Fernández et al., 2020).

The evaluation of the potential application of this set of ingredients requires comprehensive research do address the potential drawbacks. These challenges may include deficiencies in essential amino acids, such as methionine and lysine, concerns related to palatability, potential problems with digestibility, and the presence of antinutritional factors (Glencross et al., 2020). As such, beyond classical key performance indicators of the farmed animals, more sophisticated aspects such as digestive enzymatic activity, antioxidant status, microbiota, and detailed blood analysis of metabolites need thorough examination to assess their optimal compatibility as novel aquafeed ingredients. Nevertheless, at industrial level, the up–scaling limitation seems to be more an economic problem rather than a technical one. On the one hand, the production of these ingredients presents a significant opportunity for resource utilisation, as they can be cultivated on by–products substrates, in various water environments, without competing for arable land or biodiverse landscapes. On the other hand, the adoption of single–cell ingredients has been slow, primarily due to the substantial capital and operating costs associated with their production. This is because, at the moment the majority of these ingredients are produced by means of capital–Intensive fermentation systems, to exert precise control over the production process (Chua and Schenk, 2017; Soto-Sierra et al., 2018). As a consequence, there is an urgent need to prioritise the development of methods and strategies that enable larger–scale and more cost–effective production, such as open–field systems. In addition, the production of most of these resources

requires the input of various carbon and / or nitrogen resources, among other things, and efforts need to continue to identify ways of reducing these input costs while maintaining qualities and increasing productivity (Glencross et al., 2020).

On the other end of the production chain, the commercialisation of fish products is a significant undertaking. Fish products represent a highly globalised commodity with a commerce value exceeding USD 289.6 billion in 2022, generating a complex supply chain (FAO, 2022). Among the various forms of commerce, globally, whole fresh or live fish is the most consumed. However, due to the highly perishable nature of fish products, their extensive global commerce necessitates a complex organisational apparatus involving producers, commercial retailers and final consumers. In this context, despite significant advancements in processing, refrigeration, and transportation, millions of tonnes of aquatic products are lost or nutritionally compromised annually. On the last before mentioned FAO report on fishery and aquaculture, is estimated that up to 35 percent of the global production faces loss or waste each year (FAO, 2022). A significant portion of fish loss occurs during fishing operations, where undesired fish or parts of the catch are often discarded back into the oceans. However, in developed countries, a substantial quantity of fish is wasted at the retail and household levels (Love et al., 2015; FAO, 2022; Miguéis et al., 2022). Even though the retail stage may generate lower quantity of fish waste, its role as a gatekeeper is crucial, exerting significant influence on both upstream and downstream food handling processes (de Moraes et al., 2020; Miguéis et al., 2022; Bertini et al., 2023a; Tola et al., 2023). It is fair to presume that in the case of aquaculture, a lower amount of fish waste is generated at production level compared to fisheries. This is because the fish harvested in aquaculture are usually pre–sold at the time of harvesting and the presence of a well–developed traceability system contribute to creating a virtuous supply chain. However, the issue of food waste throughout value chain, along with its responsible management, is a focal point in the European Union's transition

process and a priority in the Action Plan for the European green deal ("A European Green Deal," 2021), representing an exciting research line for innovations aimed at tackling the problem.

The intricate processes involved in post–harvest handling and freshness assessment within aquaculture sector highlight the necessity for innovative and easily applicable systems to address the challenges posed by the highly perishable nature of fresh fish. The immediate onset of spoilage activities, driven by autolytic enzymes, high water activity, low pH, and a high content of easily oxidisable LC–PUFAs, creates an optimal environment for bacterial growth. The persistence of active autolytic enzymes post–mortem initiates biochemical and physical changes, including protein degradation and lipid oxidation, leading to the formation of volatile organic compounds that contribute to off–flavours and a decline in sensorial and nutritional quality (Alasalvar et al., 2010; Ghaly, 2010; Bertini et al., 2023a). While traditional methods, such as chemical indicators (e.g., TMA, TVB–N) and sensory evaluations, have proven informative, they suffer from drawbacks such as time consumption, high costs, and destructiveness (Sykes et al., 2009; Grigorakis et al., 2018; Prabhakar et al., 2020). This sets the stage for the development and adoption of advanced, rapid, and non–destructive freshness assessment tools. Technologies like e–sensing techniques, spectroscopic solutions, and dielectric property measurements offer promising alternatives to overcome the limitations of traditional methods, providing real–time insights into nuanced freshness attributes. Spectroscopic techniques, including fluorescence, infrared, hyperspectral imaging, and Raman spectroscopy offer non–invasive means to examine the physicochemical and structural properties of fish samples. Moreover, dielectric properties, measured through techniques like time domain reflectometry and impedance analysers, hold potential as indicators of post–mortem changes (Tito et al., 2012; Dowlati et al., 2013; Nelson, 2015; Rocculi et al., 2019; Franceschelli et al., 2021; Iaccheri et al., 2022). The development of these advanced tools aligns with the overarching goal of reducing food waste. By enabling

efficient, accurate, and non–destructive assessments of freshness, these technologies contribute to improved quality control, enhanced shelf–life predictions, and a more sustainable and resource–efficient utilisation of fish products globally.

Aim of the thesis

Given the aforementioned introduction underscoring the importance of implementing sustainable practices in the aquaculture industry, it becomes imperative to address both aspects of the value chain. This encompasses the journey from production to commercialisation, with a particular emphasis on the health status and quality of the farmed animals. Consequently, the overarching goal of this thesis was to employ a multidisciplinary approach, delving into diverse aspects to enhance understanding and contribute to the advancement of innovative tools and technologies for a more sustainable, resilient, and circular fish farming. The formulation of aquafeed holds a key role in shaping the sustainability, cost–effectiveness, and resource utilisation in aquaculture. In this context, the manipulation of fish diets can actively contribute to this while unlocking also novel market opportunities for by–product valorisation. As such, the first and the second studies conducted in this thesis, focusing on the utilisation into aquafeed formulations of single–cell ingredients derived from industrial by–products, were based on the hypothesis that these innovations could generate positive impacts. Moving beyond production and focusing on the commercialisation of aquaculture products, environmental challenges related to product waste and losses during storage and freshness evaluation have been identified. Over 35 % of global fish products are lost due to incorrect storage or problems in the supply chain, emphasising the need for a higher level of control throughout the value chain (FAO, 2022). In response to this challenge, the third study in this thesis developed a rapid and non–destructive device for evaluating fish freshness. The underlying assumption is that increased control over the entire process can lead to lower waste, thereby enhancing sustainability of the aquaculture industry.

Specific objectives

The specific objectives of the thesis include:

1. To investigate the effects of two microalgal–based aquafeed products, developed through an innovative technology, in comparison to a commercial lipid emulsion control, as live feed enrichments in the diet of European seabass larvae. Besides assessing classical growth performance, this study will investigate antioxidant status and skeletal anomalies in larvae, specifically exploring the impact on larvae development, considering the association between high LC–PUFAs, especially DHA, and lipid peroxidation.

2. To develop sustainable aquafeed for flathead grey mullet by replacing fish meal and soybean meal with single–cell ingredients coming from industrial by–products at different inclusion levels. In order to gain a comprehensive understanding, the study will evaluate the impact on growth, feed utilisation, metabolic parameters, digestive enzymes and gut microbiota.

3. To develop a novel, rapid, and non–destructive method for assessing rainbow trout freshness by utilising dielectric properties. In order to do so, the S11 scattering parameters will be correlated with specific QIM scheme and sensory panel evaluations at different storage times.

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CHAPTER 2. A blend of microalgae and cyanobacteria produced from industrial waste outputs for the enrichments of Artemia salina: effects on growth performance, antioxidant status and anomalies rate of European seabass (*Dicentrarchus labrax*) larvae

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Andrea Bertini, Arianna Marchi, Elisa Benini, Carmen Navarro-Guillén, María J. González, Isabel Medina, Manuel Yúfera, Chrysovalentinos Pousis, Gianluca Ventriglia, Christel Nys, Pier Paolo Gatta, Alessio Bonaldo, Luca Parma

Abstract

The recent fast development of the microalgae–related biotechnologies has enabled the availability of sustainable and nutrients–rich raw materials to be used as substitution of conventional sources in aquafeed formulation. However the cost of energy of the photobioreactors used to produce these microorganisms still constrained their wider inclusion into aquafeed compounds. By exploiting industrial waste outputs, *Nannochloropsis* spp. and *Spirulina* spp. were produced and utilised to formulate two experimental enrichments (LM1 and LM2). During a 60 days trial, their efficacy have been tested as *Artemia* metanauplii enrichments in the diet of European seabass (*Dicentrarchus labrax*) larvae, in comparison to a commercial control. Larvae were fed with isonitrogenous and isolipidic dietary treatments. Each dietary treatment presented a specific profile for the long chain polyunsaturated fatty acids ARA, DHA and EPA. Ratio of DHA/EPA in enriched *Artemia* of 4:1 as in the case of LM1 and LM2 in the presence of 1.4 % of ARA (as a % on total fatty acid) guaranteed equal results to the control which displayed a ratio of DHA/EPA of 2:1 with 2.2 % of ARA. No significant effects of the dietary treatments were detected in final survival, growth performance and incidence of skeletal anomalies. Regarding oxidative status, larvae fed with LM2 enrichment presented lower catalase activity than control larvae with no signs of oxidative damage, suggesting a potential antioxidant effect of LM2. The present study contributes to expanding the existing literature on successful utilisation of microalgae and cyanobacteria, used to produce valuable nutrients, in a perspective of circular nutrients economy.

2.1 Introduction

Aquaculture production of marine fish, such as European seabass (*Dicentrarchus labrax*), relies on the availability of a large number of healthy and fully weaned juveniles for the on–growing phase. The standard hatchery protocol involves feeding newly hatched larvae with live prey for a period before weaning them onto formulated dry feed completely. This initial reliance on live prey is necessary to provide essential nutrients and properly sized food for the small mouths of the altricial marine fish larvae. *Artemia* metanauplii (*Artemia salina)* are extensively used as live prey due to being cost–effective, easy–to–handle and polyvalent (Sorgeloos et al., 2001), despite their natural deficiency in long chain polyunsaturated fatty acids (LC–PUFAs) (Monroig et al., 2006; Ramos-Llorens et al., 2023). To overcome this bottleneck, enrichments are customarily performed to load up *Artemia* metanauplii with the adequate nutritional value for fish larvae (Boglino et al., 2012). In fact, marine fish have a limited capacity to synthesise LC–PUFAs, such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA) (Izquierdo, 2005), although these molecules are important for the synthesis of new cellular structures (Gisbert et al., 2005) and for the primary role on larval survival, growth and immune system modulation (Tocher et al., 2019; Roo et al., 2019; Betancor et al., 2021; Pham et al., 2023). On the other hand, due to their highly peroxidable nature, it has been suggested that an excess of dietary LC–PUFAs, without a proper balance of antioxidant nutrients, can lead to controversial effects on fish oxidative stress (Izquierdo et al., 2013; Betancor et al., 2015; Wischhusen et al., 2022; Pham et al., 2023). Oxidative stress has been linked to morphogenic and skeletogenic alterations during early development stages, which may result in various degree of skeletal anomalies in the larval development of hatchery–produced fish (Conceição et al., 2007; Lall and Lewis-McCrea, 2007; Izquierdo et al., 2013).

Standard LC–PUFAs rich ingredients used by aquaculture industry are derived either from wild marine fish, in the form of fish oil, or from a number of microorganisms including microalgae and cyanobacteria, which are the primary producers of EPA and DHA (Mutale-Joan et al., 2023). On one hand, the utilisation of marine fish oil is posing several environmental and economic concerns as this contributes to the over–exploitation of pelagic fish stocks (Egerton et al., 2020; Glencross et al., 2020) along with presenting very high and unstable prices, due to the competition with human nutrition and other livestock production. In addition, this finite commodity is susceptible to seasonal fluctuation in the fatty acids profile (Gámez-Meza et al., 1999; Zlatanos and Laskaridis, 2007) and to contamination with dioxin–like polychlorinated biphenyls (PCBs), persistent organic pollutants (POPs) and heavy metals, which bioaccumulate in animals at a higher trophic level (Sun et al., 2018; Santigosa et al., 2021; Zatti et al., 2023) and may interfere with normal larval physiological functions (Horri et al., 2018; Bogevik et al., 2023).

On the other hand, the exploitation of microalgae and cyanobacteria can address some of the concerns related to fish oil, despite their production is often constrained by the high costs associated with cultivation. These microorganisms can be produced by farming on non–arable land, reduce water demands and convert CO_2 and other waste products into valuable ingredients (Shah et al., 2018; Yarnold et al., 2019; Chen et al., 2021; Tocher et al., 2019; Conde et al., 2021). They are oxygenic photoautotrophs capable of producing LC–PUFAs together with a diverse array of unique and potent substances (Ma et al., 2020; Nagappan et al., 2021) such as some antioxidant molecules produced by *Spriluina* spp. (Fernandes et al., 2023). Their incorporation into hatchery feeding regimens has been found to exhibit probiotic and antibacterial properties (Vadstein et al., 2018; Ognistaia et al., 2022), resulting in a beneficial effect on larval gut microbiota (Huang et al., 2023), positively influencing the production key performance indicators (Boglino et al., 2012; Paulo et al., 2020; Gui et al., 2022; Pan et al., 2022).

From a production perspective, industrial–scale cultivation of microorganisms can be achieved through outdoor systems, utilising ponds or tanks, or by employing controlled environments such as photobioreactors (Mutale-Joan et al., 2023). While outdoor systems can partially reduce energy costs by using sunlight as an energy input, seasonal environmental variations may affect the composition and quality of microorganism cells, potentially resulting in lower yields (Xu et al., 2020). On the other hand, photobioreactors, offering precise control over environmental conditions, require substantial energy inputs for lighting, temperature regulation, agitation, and nutrient supplementation. In the context of the circular economy, the reutilisation of industrial by–products as a source of energy in more advanced transformation processes is becoming increasingly important. In this context, European Union is funding several projects aimed at bridging the gap between research and industrial application. VAXA (VAXA Technologies Ltd.; Reykjavík, Iceland) has developed a new technology platform perfecting the cultivation of LC–PUFAs rich microorganisms by exploiting geothermal energy produced by a geothermal plant in Iceland. This allows to transform the waste outputs of a geothermal plant, hot and cold water and natural CO_2 emissions, to produce a number of autotrophic microorganisms such as *Nannochloropsis* spp. and *Spirulina* spp. This system results in a year–round microorganisms production, resilient to the environmental variation, free from chemical contamination and with 80 % reduction in production cost due to geothermal energy utilisation.

The aim of this study was to examine the effects of two products at development stage (LM1 and LM2) contained different proportions of *Nannochloropsis* spp. and *Spirulina* spp. produced with this more sustainable technology compared to a commercial lipid emulsion control (C), as *Artemia* metanauplii enrichments in the diet of European seabass larvae. Besides their effects on larval survival, growth performance and fatty acid profile, the antioxidant status and the incidence of skeletal anomalies were also evaluated.

2.2 Materials and methods

2.2.1 Larvae origin and rearing methods

European seabass larvae at 7 days post–hatching (DPH) coming from one spawning batch were obtained from a commercial marine hatchery (Ca Zuliani Soc Agricola Srl., Italy). Larvae were transferred to the Laboratory of Aquaculture at the University of Bologna (Cesenatico, Italy) into a 50 L liquid storage bag filled with 1 / 3 seawater and 2 / 3 compressed oxygen, placed inside a polystyrene box for thermal isolation. Once at the laboratory, larvae were randomly allocated into nine 50 L up–welling tanks (2200 larvae $tank^{-1}$) by using volumetric allocation (Parma et al., 2013). Tanks were supplied with seawater and connected to a closed recirculation system consisted of a mechanical sand filter (PTK 1200; Astralpool, Barcelona, Spain), ultraviolet lights (UV PE 45; Sita Srl, Barcelona, Spain) and a biofilter (PTK 1200; Astralpool, Barcelona, Spain). Water exchange rate in the tanks was initially of 0.5 L min^{-1}, increased to 2.5 L min^{-1} following larval development. Photoperiod was set at 12 h light / 12 h darkness regime (with light period from 8 am to 8 pm), through artificial light at < 50 lux at the water surface. Temperature was maintained at 18 ± 2 °C. Dissolved oxygen in the tanks was maintained at > 9 mg/L through an automatic liquid oxygen system connected to a software controller (B&G Sinergia snc, Chioggia, Italy). Sodium bicarbonate was added to keep the pH constant at 7.8 – 8.0 and avoid alkalinity fluctuation. Salinity was maintained at 27 ± 2 g L^{-1}.

2.2.2 Experimental feeding regimes and live prey enrichments

Three different enrichment products were used to enrich *Artemia* metanauplii. Two experimental products, LM1 and LM2, consisting of microalgae and cyanobacteria blends with different proportions of *Nannochloropsis* spp. and *Spirulina* spp. produced by using geothermal resources (VAXA Farm, Reykjavík, Iceland) and a commercial control C (Red Pepper©,

Bernaqua, Olen, Belgium). Both LM1 and LM2 were produced without addition of fish oil, LM2 contained a higher proportion of *Spirulina* spp. compared to LM1. The feeding protocol was set, with some modifications, according to those used in commercial hatcheries for European seabass and based on previous published works (Villamizar et al., 2009; El-Dahhar et al., 2022). Wild caught *Artemia* cysts of EG strain coming from Great Salt lake in USA (Catvis B.V., 's-Hertogenbosch, The Netherlands) were incubated and hatched in seawater (salinity 30 g L^{-1}) at 28 °C for 18 hours. After this, *Artemia* metanauplii were harvested and enriched for 12 hours at 26 °C by transferring metanauplii at a concentration of 300 individuals mL^{-1} to new container filled with seawater and 750 mg L^{-1} of the enriching products. Triplicate groups of larvae were fed with non–enriched *Artemia* nauplii from 7 to 26 DPH (5 nauplii mL^{-1}). Enriched *Artemia* metanauplii were then incorporated from 27 DPH to 60 DPH (7.5 metanauplii mL^{-1} from 27 to 50 DPH and 10 metanauplii mL^{-1} from 51 to 60 DPH). The feeding protocol with *Artemia* metanauplii without including formulated dry feed was maintained until advanced larvae development to avoid any possible bias on the outcomes of this research. The proximate composition and the fatty acids profile of the enriched *Artemia* metanauplii are shown in Table 2.1.

Table 2.1 Proximate analysis (% of dry weight) and fatty acid composition (% of total fatty acids) of the non–enriched *Artemia* nauplii and enriched *Artemia* metanauplii with the experimental enriching products LM1, LM2 and C.

	Non-enriched	LM1	LM2	C	*p* value
Total protein	55.95 ± 0.47	55.18 ± 0.95	56.36 ± 0.62	55.71 ± 1.20	ns
Total lipids	20.07 ± 0.09	23.07 ± 0.32	23.19 ± 0.76	23.90 ± 0.41	ns
Total fatty acids	16.16 ± 0.53	18.24 ± 0.71	18.42 ± 0.25	18.94 ± 0.47	ns

Ash	8.54 ± 0.04	11.66 ± 1.38	10.33 ± 0.21	10.19 ± 0.06	ns
11:0	2.25 ± 0.00	2.29 ± 0.22	2.18 ± 0.08	2.03 ± 0.25	ns
14:0	0.81 ± 0.01	5.60 ± 0.18[b]	5.68 ± 0.09[b]	1.44 ± 0.08[a]	0.000
15:0	0.22 ± 0.05	0.17 ± 0.02[a]	0.18 ± 0.03[a]	0.21 ± 0.01[b]	0.001
16:0	12.04 ± 0.04	12.06 ± 0.11[a]	12.07 ± 0.1[a]	13.15 ± 0.12[b]	0.000
17:0	0.83 ± 0.01	0.67 ± 0.05	0.68 ± 0.00	0.68 ± 0.02	ns
18:0	4.96 ± 0.00	4.46 ± 0.05	4.48 ± 0.03	4.43 ± 0.07	ns
20:0	0.25 ± 0.03	0.28 ± 0.02	0.25 ± 0.08	0.22 ± 0.06	ns
22:0	0.27 ± 0.04	0.40 ± 0.02[a]	0.37 ± 0.04[a]	0.54 ± 0.02[b]	0.000
Σ saturated	21.63 ± 0.23	25.93 ± 0.28[a]	25.89 ± 0.11[a]	22.70 ± 0.41[b]	0.000
14:1 n-5	0.58 ± 0.03	0.41 ± 0.04	0.40 ± 0.03	0.38 ± 0.01	ns
16:1 n-7	2.66 ± 0.00	2.08 ± 0.08[a]	1.98 ± 0.02[b]	2.16 ± 0.03[a]	0.002
16:1 n-9	0.59 ± 0.00	0.46 ± 0.03	0.45 ± 0.01	0.47 ± 0.02	ns
18:1 n-7	7.46 ± 0.24	6.04 ± 0.21	5.94 ± 0.17	6.13 ± 0.11	ns
18:1 n-9	18.9 ± 0.05	15.31 ± 0.16	15.19 ± 0.03	15.35 ± 0.2	ns
20:1 n-9	0.59 ± 0.03	0.53 ± 0.06	0.55 ± 0.07	0.59 ± 0.06	ns
22:1 n-9	0.12 ± 0.01	0.16 ± 0.05	0.16 ± 0.04	0.19 ± 0.09	ns
24:1 n-9	0	0	0	0	ns
Σ monounsaturated	30.9 ± 0.31	24.99 ± 0.39	24.67 ± 0.25	25.27 ± 0.20	ns
16:2 n-6	0.28 ± 0.02	0.09 ± 0.05	0.07 ± 0.02	0.04 ± 0.01	ns
18:2 n-6 (LOA)	6.70 ± 0.04	5.10 ± 0.09[a]	5.09 ± 0.06[a]	5.75 ± 0.08[b]	0.000
18:3 n-6	0.32 ± 0.01	0.29 ± 0.02	0.34 ± 0.05	0.30 ± 0.02	ns
20:2 n-6	0.26 ± 0.01	0.24 ± 0.03	0.24 ± 0.01	0.25 ± 0.02	ns

20:3 n-6 (DGLA)	0.07 ± 0.01	0.11 ± 0.05[a]	0.10 ± 0.01[a]	0.17 ± 0.02[b]	0.002
20:4 n-6 (ARA)	1.35 ± 0.01	1.43 ± 0.03[a]	1.42 ± 0.04[a]	2.21 ± 0.05[b]	0.000
22:4 n-6	0.07 ± 0.04	0.09 ± 0.03	0.07 ± 0.03	0.08 ± 0.01	ns
22:5 n-6 (DPA n-6)	0	1.98 ± 0.10[a]	2.12 ± 0.06[a]	3.22 ± 0.08[b]	0.000
Σ n-6 PUFA	9.05 ± 0.09	9.33 ± 0.09[a]	9.46 ± 0.04[a]	12.02 ± 0.11[b]	0.000
16:3 n-3 (HTA)	0.58 ± 0.02	0.42 ± 0.02	0.42 ± 0.03	0.40 ± 0.02	ns
18:3 n-3 (ALA)	30.58 ± 0.07	22.28 ± 0.24[b]	21.53 ± 0.16[a]	22.47 ± 0.34[b]	0.001
18:4 n-3 (SDA)	4.14 ± 0.09	2.79 ± 0.02[b]	2.64 ± 0.04[a]	2.74 ± 0.04[b]	0.001
20:4 n-3 (ETA)	0.66 ± 0.01	0.61 ± 0.03[a]	0.58 ± 0.02[a]	0.71 ± 0.00[b]	0.000
20:5 n-3 (EPA)	1.70 ± 0.01	2.65 ± 0.04[a]	2.75 ± 0.05[b]	3.94 ± 0.03[c]	0.000
22:5 n-3 (DPA n-3)	0.04 ± 0.05	0.07 ± 0.01[a]	0.06 ± 0.01[a]	0.20 ± 0.06[b]	0.000
22:6 n-3 (DHA)	0.07 ± 0.02	10.43 ± 0.4[b]	11.42 ± 0.15[c]	9.09 ± 0.24[a]	0.000
Σ n-3 PUFA	37.77 ± 0.02	39.25 ± 0.22	39.40 ± 0.12	39.55 ± 0.25	ns
Σ polyunsaturated	46.82 ± 0.04	48.58 ± 0.26[a]	48.85 ± 0.18[a]	51.57 ± 0.27[b]	0.000
n-3/n-6	4.18 ± 0.05	4.21 ± 0.03[b]	4.16 ± 0.02[b]	3.29 ± 0.03[a]	0.000
EPA+DHA	1.77 ± 0.02	13.08 ± 0.40[a]	14.18 ± 0.13[b]	13.03 ± 0.26[a]	0.000
EPA/DHA	-	0.25 ± 0.01[a]	0.24 ± 0.01[a]	0.43 ± 0.01[b]	0.000

Data are given as the mean (n = 4) ± SD. Significant differences among treatments were detected by using one way ANOVA + Tukey's test ($p \leq 0.05$). Values from non-enriched *Artemia* nauplii were not included in the statistic.

2.2.3 Samplings and growth calculations

All experimental procedures were evaluated and approved by the Ethical–Scientific Committee for Animal Experimentation of the University of Bologna, in accordance with

European directive 2010/63/UE on the protection of animals used for scientific purposes. Before each sampling procedure, larvae were euthanised with 300 mg L^{-1} of MS222. At 7, 19, 26, 32, 39, 46 and 54 DPH, 20 larvae tank $^{-1}$ were randomly collected in order to determine wet weight (WW) and dry weight (DW). At the end of the trial (60 DPH), 100 larvae tank $^{-1}$ were collected to determine WW and DW. WW was measured after rinsing larvae with de–ionised water on a 400 µm sieve and removing the excess of moisture away from behind the sieve using lint–free paper towel (Bonaldo et al., 2011). DW was determined by oven–drying the larvae at 70 °C for 12 hours. At the end of the trial, all larvae were removed from each tank and counted to determine survival rate. Larvae were then rinsed in distilled water, and frozen (− 80 °C) until biochemical analyses or fixed in 10 % formalin buffered with phosphate buffer saline (PBS, pH 7.2) for 48 h at 4 °C for skeletal anomalies analysis. Fixed samples were then washed in PBS (pH 7.5) for 48 h at 4 °C and then stored in 70 % ethanol until further processing. Growth rate was calculated as specific growth rate (SGR, % day^{-1}) according to the equation: SGR = 100 * (ln FBW – ln IBW)/days (where FBW and IBW represent the final and the initial body weights, respectively). Survival rate was calculated per tank as a percentage of the initial number of larvae. Growth rate was calculated as specific growth rate (SGR, % day^{-1}) according to the equation:

$$SGR\ (\%\ \mathrm{day}^{-1}) = 100 * \frac{InFBW - lnIBW}{days} \qquad (1)$$

2.2.4 Lipid and fatty acids profiles

Lipids in both *Artemia* nauplii and metanaupli and European seabass larvae were extracted according to (Bligh and Dyer, 1959) by using methanol and chloroform as solvents. Fatty acids were subjected to a methylated ester method (FAMEs) following the procedures as described in (Lepage and Roy, 1986). Briefly, methylated–FAs (FAMEs) were extracted into toluene. The

FAMEs were analysed by gas chromatography (GC/FID, Clarus 500, Perkin-Elmer) using an SP-2330 fused silica capillary column (30 m × 0.25 mm i.d., 0.20 µm; Supelco Inc., Bellefonte, USA).

2.2.5 Evaluation of the antioxidant status

The antioxidant status of the larvae was assessed by measuring catalase activity (CAT), protein carbonylation (PC), lipid peroxidation (LPO) and mitochondrial reactive oxygen species production (mtROS). For the analyses of CAT, PC and LPO, three pools of 3 larvae per replicate tank (n = 9 pools per treatment) were sampled at the end of the growth trial (60 DPH) and snap–frozen in liquid nitrogen. Samples were homogenised in 500 µl ultra–pure water using an Ultra–Turrax® Homogeniser (IKA®-Werke, Germany). One aliquot containing 4 % butylated hydroxytoluene (BHT) in methanol was used for the determination of LPO. The remaining homogenate was diluted (1:1) in 0.2 M K-phosphate buffer, pH 7.4, and centrifuged for 10 min at 10,000 g (4 ºC). The post–mitochondrial supernatant (PMS) was kept in – 80 ºC for the analysis of catalase activity (CAT) and protein carbonylation (PC). For mtROS determination 3 larvae per replicate tank (n = 9 pools per treatment) were sampled and snap–frozen in liquid nitrogen. Samples were homogenised in 200 µl ice–cold mitochondria isolation buffer (225 mM mannitol, 75 mM sucrose, 1 mM EGTA and 4 mM HEPES, pH 7.2). Then, the homogenate was centrifuged for 10 min at 600 g and 4 °C. The supernatant was picked off and centrifuged again for 10 min, at 11,000 g and 4 ºC. The pellet was resuspended in buffer containing 250 mM sucrose and 5 mM HEPES (pH 7.2). Catalase activity (CAT) was determined by measuring decomposition of the substrate H_2O_2 at 240 nm (Clairborne, 1985). Protein carbonylation (PC) was measured by the reaction of 2,4–dinitrophenylhydrazine (DNPH) with carbonyl groups, according to the DNPH alkaline method (Mesquita et al., 2014). Endogenous lipid peroxidation (LPO) was determined by measuring thiobarbituric acid–reactive substances (TBARS) (Bird and Draper, 1984). Mitochondrial reactive oxygen species (mtROS) production was assessed by the dihydrodichloro–

fluorescein diacetate method, H(2)DCF–DA (van der Toorn et al., 2009). The protein content of PMS (CAT, LPO and PC determinations) and mtROS samples was determined according to the Bradford method (Bradford, 1976) using bovine serum albumin as standard. All biomarkers were determined in 96 well flat bottom microplates using a temperature–controlled microplate reader (Synergy H1, BioTek Instrument, Inc., USA).

2.2.6 Evaluation of severe skeletal anomalies

A total of 859 European sea bass larvae at 60 DPH were stained with alcian blue and Alizarin red according to Pousis et al. (2022). Briefly, the specimens were immersed in a solution of 0.5 % KOH and 3 % H2O2 and subjected to prolonged exposure (5–6 hours) to sunlight. Then all the samples were stained in 200 ml cartilage staining solution (120 ml 95 % ethanol, 80 ml glacial acetic acid, 50 mg Alcian blue) and in 200 ml of bone staining solution (60 mg Alizarin red in 200 ml KOH) in the dark for 90 min and 2 hours respectively. After each staining step the samples were washed three times in 0.5 % KOH for 5 minutes. Finally, the samples were subjected to the process of diaphanization by consecutive immersion in containing 0.5 % KOH and increasing glycerol concentrations (0.5 % KOH:glycerol 3:1; 0.5 % KOH:glycerolm1:1; 0.5 % KOH:glycerol 1:3; 100 % glycerol). The duration of the first two steps was about 48 hours while the while the third step was repeated several times until the 0.5 % KOH:glycerol 1:3 solution remained colourless after 24 h of immersion.

Double–stained specimens were observed twice by the same operator, under a stereomicroscope (Leica WILD M3C, Wetzlar, DE), in order to reduce possible errors during the analysis of the samples and all the malformed fish were photographed by a digital camera (DFC 420; Leica Microsystems, Cambridge, UK). For each group, severe skeletal anomalies were recorded according to Prestinicola et al. (2013), and the following variables were calculated: 1) relative frequency (%) of individuals with at least one severe anomaly. 2) severe anomalies load

(number of severe anomalies / number of individuals with severe anomalies). 3) relative frequencies (%) of severe anomalies in the in the four regions of the vertebral column and in the skull (number of severe anomalies in each region/number of total severe anomalies).

2.2.7 Statistical analysis

Significant differences in growth performance, fatty acid profile, antioxidant status and frequencies of severe skeletal anomalies between the groups were determined using one–way ANOVA and, whenever significant differences were identified, means were compared by the Post hoc multiple comparisons Tukey's test ($p \leq 0.05$). Prior to ANOVA, skeletal malformation data were arcsine transform as appropriate for proportion (Sokal and Rohlf, 1969). The assumptions related to data normal distribution and homogeneity of variances were explored through Anderson Darling's test and Levene's test, respectively. In the case data presented a non–normal distribution, significant differences between the groups were determined using Kruskal–Wallis non–parametric test. Analyses were performed using Minitab software (Minitab 19.0.1; Pennsylvania State University, USA). Results are presented as mean ± standard deviation (SD), unless otherwise mentioned.

2.3 Results

2.3.1 Growth performance

Wet weight (WW) and dry weight (DW) measured during the trial of European seabass larvae fed Artemia metanauplii enriched with the different products are shown in Figure 2.1. At the end of the trial no significant differences were detected in specific growth rate (SGR) (7.95 ± 0.31, 7.92 ± 0.14 and 8.33 ± 0.56, respectively in LM1, LM2 and C) and survival rate (65.0 ± 6.9, 68.5 ± 7.1 and 61.5 ± 1.3, respectively in LM1, LM2 and C), among the different groups.

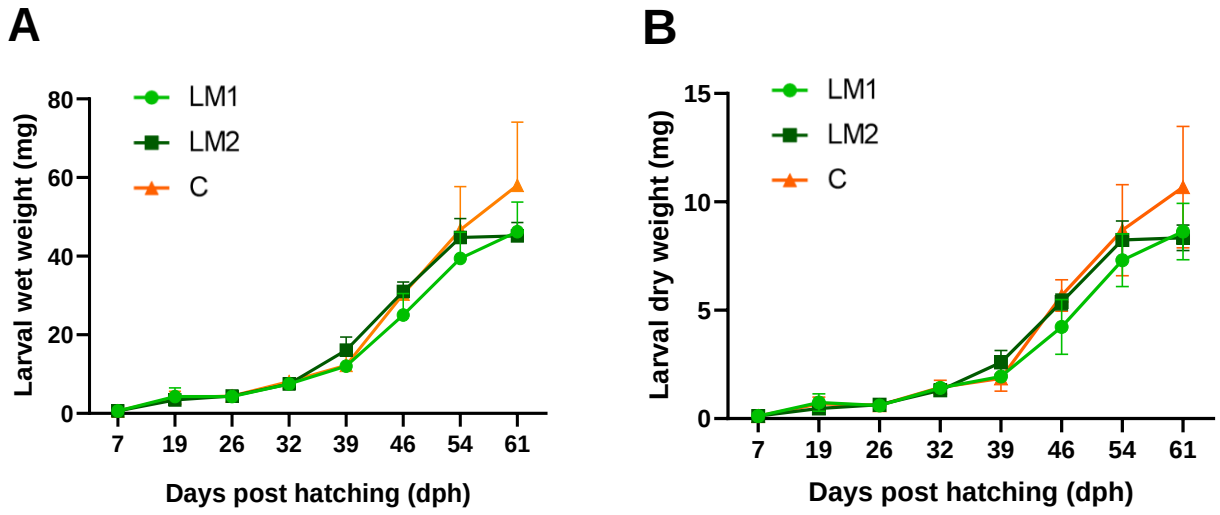

Figure 2.1 Larval wet weight (A) and dry weight (B) of European seabass (*Dicentrarchus labrax*) larvae fed *Artemia* metanauplii enriched with the experimental and control enrichments from 7 to 60 DPH.

2.3.2 Fatty acid profile in *Artemia* metanauplii and larvae

Proximate compositions and fatty acid profiles of *Artemia* metanauplii enriched with the different products are shown in Table 2.1. No significant differences were observed in the proximate composition of *Artemia* metanauplii (total protein, total lipids, total fatty acids and ash) among treatments ($p > 0.05$). For what concern the differences related to LC–PUFAs, significant differences were observed in the proportions of arachidonic (ARA 20:4 n-6) acid, which was found significantly higher in C compared to LM1 and LM2. Eicosapentaenoic acid (EPA 20:5 n-3) was found significantly higher in C compared to LM1 and LM2 and in LM2 compared to LM1. On the contrary, docosahexaenoic acid (DHA 22:6 n-3) was found significantly higher in LM2 compared to LM1 and C and in LM1 compared to C. According to these, the ratio n-3 / n-6 PUFA was significantly higher in *Artemia* metanauplii enriched with LM1 and LM2, the amount of EPA +

DHA was significantly higher in LM2, and the ratio EPA/DHA was significantly higher in the control *Artemia* metanauplii.

Fatty acid profiles of European seabass larvae fed *Artemia* enriched with the different products are shown in Table 2.2. At the end of the trial (60 DPH), no significant differences were detected in the total quantity of saturated, mono unsaturated and total PUFA (n-3 + n-6) fatty acids among larvae from the different treatments groups. With regards to the differences related to LC–PUFAs, these follow the same pattern as the *Artemia* metanauplii treatments. Larvae fed *Artemia* metanauplii enriched with C presented significantly higher arachidonic (ARA 20:4 n-6) end eicosapentaenoic (EPA 20:5 n-3 compared to LM1 and LM2. Docosahexaenoic acid (DHA 22:6 n-3) was found significantly higher in LM1 and LM2 compared to C. Concordantly, the ratio n-3 / n-6 PUFA was significantly higher in larvae fed *Artemia* metanauplii enriched with LM1 and LM2, the amount of EPA + DHA was significantly higher in LM2 compared to LM1 and C and in LM1 compared to C and the ratio EPA/DHA was significantly lower in LM2 compared to LM1 and C and in LM1 compared to C.

Table 2.2 Fatty acid composition (% of total fatty acids) of European seabass larvae fed *Artemia* metanauplii enriched with the experimental enriching products LM1, LM2 and C at the end of the trial.

	LM1	LM2	C	*p* value
11:0	4.32 ± 0.22	3.79 ± 0.20	4.07 ± 0.27	ns
14:0	1.28 ± 0.03^{c}	1.05 ± 0.07^{b}	0.60 ± 0.06^{a}	0.001
15:0	0.25 ± 0.01^{a}	0.27 ± 0.02^{a}	0.29 ± 0.03^{b}	0.007
16:0	17.25 ± 0.24	17.09 ± 0.47	17.40 ± 0.49	ns
17:0	0.92 ± 0.05	0.91 ± 0.03	0.93 ± 0.02	ns

18:0	10.06 ± 0.09	10.30 ± 0.09	9.86 ± 0.16	ns
20:0	0.27 ± 0.03	0.28 ± 0.03	0.27 ± 0.02	ns
22:0	0.52 ± 0.10	0.47 ± 0.05	0.55 ± 0.03	ns
Σ saturated	34.86 ± 0.33	34.16 ± 0.65	33.98 ± 0.69	ns
14:1 n-5	0.14 ± 0.01	0.14 ± 0.02	0.15 ± 0.02	ns
16:1 n-7	0.84 ± 0.02^{ab}	0.78 ± 0.03^{a}	0.89 ± 0.04^{b}	0.031
16:1 n-9	0.53 ± 0.02	0.52 ± 0.02	0.53 ± 0.02	ns
18:1 n-7	5.12 ± 0.11	5.01 ± 0.10	5.25 ± 0.09	ns
18:1 n-9	12.49 ± 0.11	12.27 ± 0.08	12.28 ± 0.12	ns
20:1 n-9	0.81 ± 0.03	0.80 ± 0.03	0.81 ± 0.04	ns
22:1 n-9	0.23 ± 0.03	0.22 ± 0.02	0.25 ± 0.07	ns
24:1 n-9	0.58 ± 0.02	0.62 ± 0.09	0.56 ± 0.03	ns
Σ monounsaturated	20.75 ± 0.21	20.35 ± 0.16	20.72 ± 0.21	ns
16:2 n-6	0.06 ± 0.04	0.05 ± 0.01	0.06 ± 0.01	ns
18:2 n-6 (LOA)	2.62 ± 0.06^{a}	2.56 ± 0.08^{a}	2.94 ± 0.09^{b}	0.011
18:3 n-6	0.14 ± 0.01	0.16 ± 0.03	0.13 ± 0.02	ns
20:2 n-6	0.55 ± 0.07	0.56 ± 0.06	0.57 ± 0.03	ns
20:3 n-6 (DGLA)	0.14 ± 0.02^{a}	0.15 ± 0.03^{a}	0.19 ± 0.02^{b}	0.001
20:4 n-6 (ARA)	4.84 ± 0.08^{a}	4.86 ± 0.14^{a}	5.74 ± 0.11^{b}	0.000
22:4 n-6	0.21 ± 0.03	0.25 ± 0.12	0.24 ± 0.04	ns
22:5 n-6 (DPA n-6)	2.97 ± 0.07^{a}	3.09 ± 0.17^{a}	3.98 ± 0.12^{b}	0.000
Σ n-6 PUFA	11.54 ± 0.21^{a}	11.67 ± 0.22^{a}	13.85 ± 0.19^{b}	0.000
16:3 n-3 (HTA)	0.35 ± 0.08	0.35 ± 0.09	0.32 ± 0.09	ns

18:3 n-3 (ALA)	6.75 ± 0.15	6.22 ± 0.18	6.98 ± 0.22	ns
18:4 n-3 (SDA)	0.55 ± 0.02	0.53 ± 0.03	0.58 ± 0.04	ns
20:4 n-3 (ETA)	0.52 ± 0.14	0.47 ± 0.07	0.50 ± 0.04	ns
20:5 n-3 (EPA)	5.33 ± 0.08^{a}	5.30 ± 0.15^{a}	5.60 ± 0.13^{b}	0.010
22:5 n-3 (DPA n-3)	0.76 ± 0.03^{a}	0.72 ± 0.05^{a}	0.89 ± 0.06^{b}	0.011
22:6 n-3 (DHA)	17.99 ± 0.29^{b}	19.63 ± 0.62^{b}	15.94 ± 0.50^{a}	0.000
Σ n-3 PUFA	32.24 ± 0.29^{a}	33.21 ± 0.70^{a}	30.81 ± 0.63^{b}	0.004
Σ polyunsaturated	43.78 ± 0.38	44.88 ± 0.76	44.66 ± 0.77	ns
n-3/n-6	2.79 ± 0.06^{b}	2.85 ± 0.06^{b}	2.22 ± 0.03^{a}	0.000
EPA+DHA	23.31 ± 0.3^{b}	24.92 ± 0.72^{c}	21.55 ± 0.61^{a}	0.000
EPA/DHA	0.30 ± 0.01^{b}	0.27 ± 0.01^{a}	0.35 ± 0.01^{c}	0.000

Data are given as the mean (n = 3) ± SD. Significant differences among treatments were detected by using one way ANOVA + Tukey's test ($p \leq 0.05$).

2.3.3 Oxidative status

Biomarkers related to oxidative status measured on European seabass larvae fed Artemia metanauplii enriched with the different products are shown in Figure 2.2. At the end of the trial (60 DPH), CAT activity was found significantly lower in larvae fed Artemia metanauplii enriched with LM2 compared to C (P = 0.005). No significant differences were detected in mtROS, PC and LPO among experimental groups, although it was observed a trend to higher mtROS and LPO in larvae fed Artemia metanauplii enriched with LM2 and LM1, respectively.

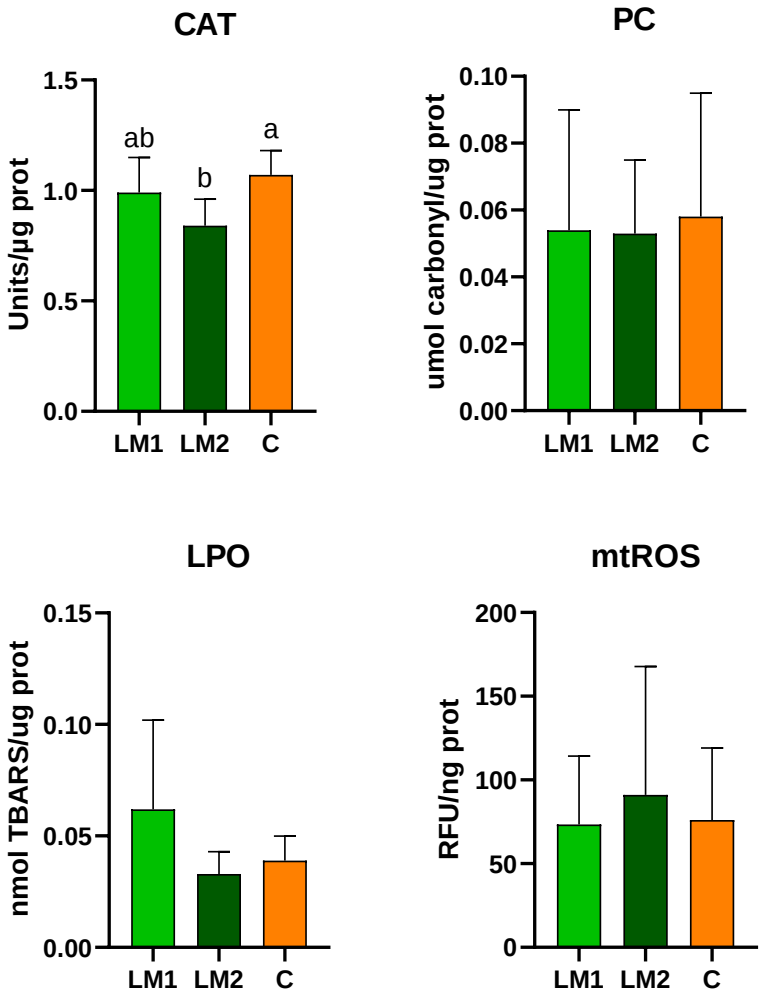

Figure 2.2 CAT (catalase activity), mROS (mitochondrial reactive oxygen species), PC (protein carbonylation) and LPO (lipid peroxidation) levels in European seabass (*Dicentrarchus labrax*) larvae fed *Artemia* metanauplii enriched with the experimental and control enrichments at 60 DPH. Data are given as the mean (n = 3) ± SD. Significant differences among treatments were detected by using one way ANOVA + Tukey's test ($p \leq 0.05$) for CAT, mROS and PC and by Kruskal–Wallis non–parametric test for LPO.

2.3.4 Skeletal anomalies

All larvae with a non–inflated swim bladder (n = 166) were excluded from the analysis and the microscopic analysis to identify skeletal anomalies was performed on the remaining

individuals (n = 693). Representative micrographs of the recorded anomalies in the three analysed groups are reported in Figure 2.3. No significant differences between the three experimental groups were found in the relative frequency (%) of individuals with at least one severe anomaly (LM1 = 47.5 ± 2.1; LM2 = 34.2 ± 10.7 %; C = 37.5 ± 16.4 %; $p > 0.05$) and in the severe anomalies load (LM1 = 2.5 ± 0.1; LM2 = 2.4 ± 0.5; C = 2.7 ± 0.2; $p > 0.05$) (Table 2.3). The relative frequencies of severe anomalies observed in each of the four regions of the vertebral column and in the skull are reported in Figure 2.4. Most of the severe anomalies affected the caudal vertebral region (LM1 = 58.8 ± 13.3; LM2 = 49.8 ± 10.9; C = 41.0 ± 2.7 %), followed by skull anomalies in LM1 (14.7 ± 3.8 %) and LM2 (17.4 ± 9.6 %), and by anomalies of the cranial region of the vertebral column in C (29.1 ± 5.7 %). No statistical differences were observed in the frequency of severe anomalies in any of the examined body regions ($p > 0.05$ for all comparisons).

Table 2.3 Skeletal anomalies of European sea bass larvae fed *Artemia* metanauplii enriched with the experimental enriching products LM1, LM2 and C at the end of the trial.

	LM1	LM2	C	*p* value
Number of examined individuals	195	279	219	ns
Relative frequency of individuals with at least one severe anomaly (%)	47.5 ± 12.1	34.2 ± 10.7	37.5 ± 16.4	ns
Severe anomalies load	2.5 ± 0.1	2.4 ± 0.5	2.7 ± 0.2	ns

Data are mean of three subgroups (tanks) ± SD. No significant differences among LM1, LM2 and C groups were detected (one-way ANOVA, $p > 0.05$).

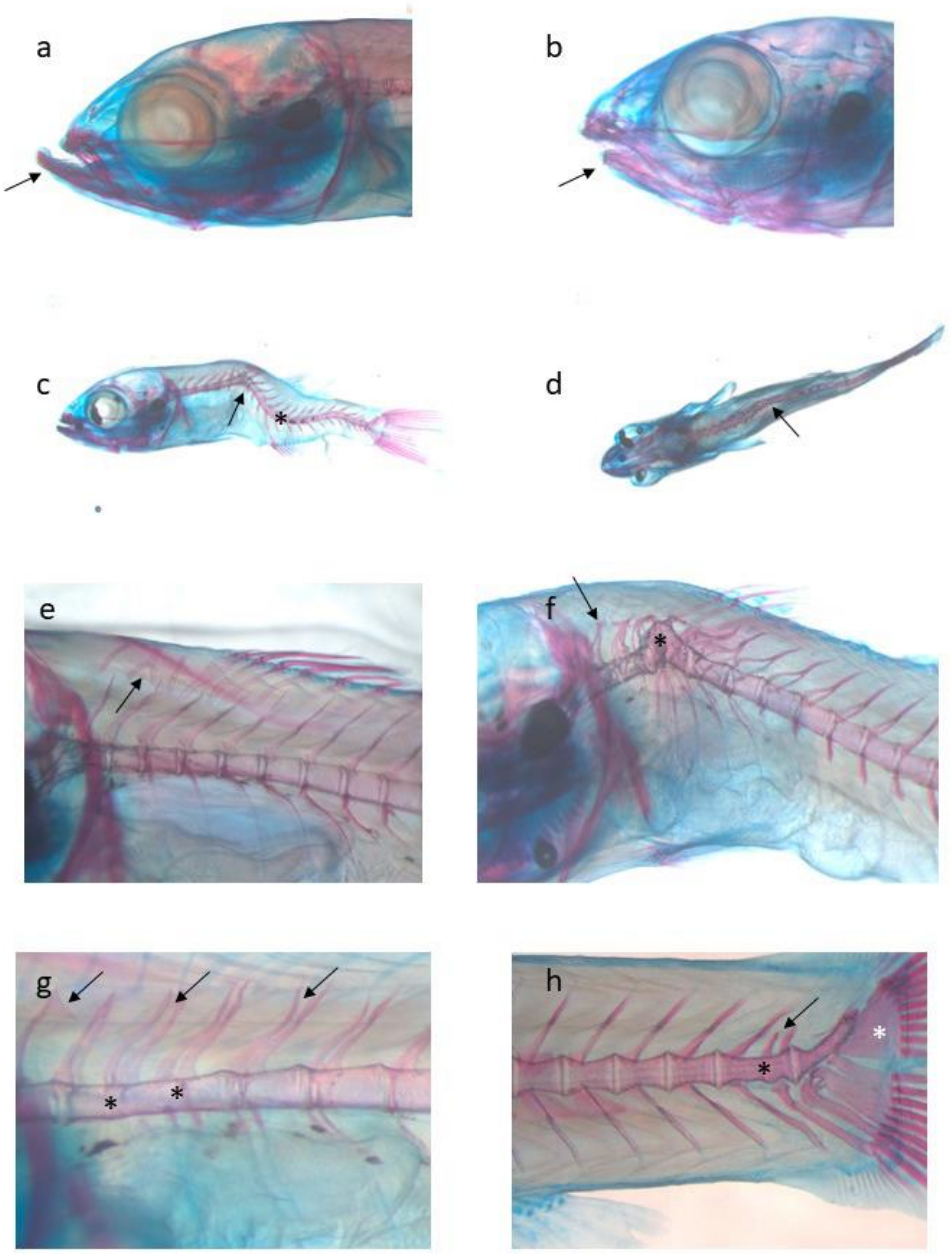

Figure 2.3 Skeletal anomalies in European seabass assessed at 60 DPH. (a) Anomalous dentary (prognathism) (arrow), (b) Anomalous dentary (lower jaw reduction) (arrow). (c) Pre haemal kyphosis (arrow) haemal lordosis (asterisk). (d) Scoliosis between pre haemal and haemal region (arrow). (e) Anomalous (forked) pre dorsal bone. (f) kyphosis, fused vertebrae and deformed bodies of vertebrae (asterisk) between cranial and pre haemal region; anomalous neural spines are also visible (arrow), (g) fused cranial vertebrae (asterisks); anomalous neural spines are also visible (arrows), (h) fused caudal vertebrae (black asterisk); anomalous neural spine (arrow); fused hypural (white asterisk).

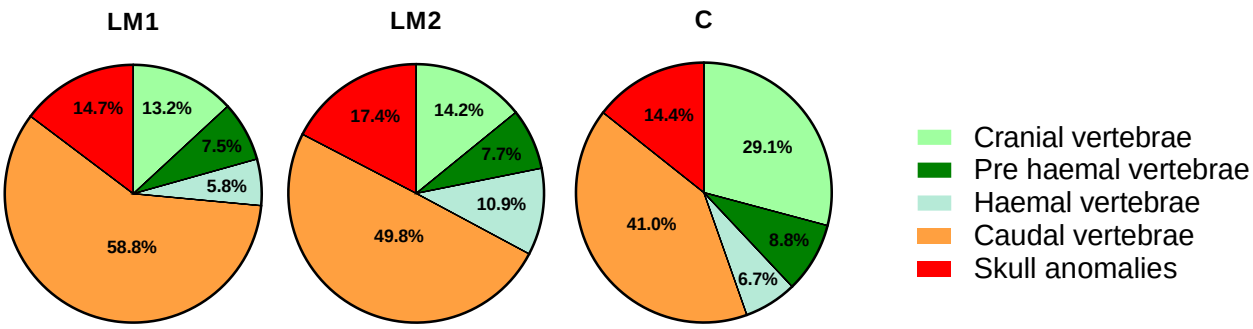

Figure 2.4 Relative frequencies (%) of severe anomalies in the four regions of the vertebral column and in the skull of European seabass fed Artemia metanauplii enriched with the experimental enriching products LM1, LM2 and C at 60 DPH. Data are given as the mean (n = 3) ± SD. No significant differences among LM1, LM2 and C were detected (one-way ANOVA, $p > 0.05$).

2.4 Discussion

One of the most fascinating aspects of phototrophic production of microalgae and cyanobacteria is the important remediation service offered to the environment (Chauton et al., 2015; Fernández et al., 2020; Mishra et al., 2023). By performing photosynthesis, this microorganisms convert inorganic nutrients, carbon dioxide (CO_2) and light radiation into valuable components, such as oils rich in long chain polyunsaturated fatty acids (LC–PUFAs), while releasing oxygen in the environment. In the case of the present study, the use of geothermic resources provides a constant source of heat and CO_2 contributing to reduce 80 % of energy consumption, and at the same time, allowing to obtain a constant product quality all–year round. This is not a minor point because oils derived from wild marine fish suffer from seasonal composition variability which can affect the final fatty acid profile of the enrichment (Gámez-Meza et al., 1999; Zlatanos and Laskaridis, 2007). The present study contributes to expanding the existing literature on successful utilisation of microalgal based products, produced in a perspective of circular nutrients economy (Shah et al., 2018; Tocher et al., 2019; Yarnold et al., 2019; Santigosa

et al., 2021; Chen et al., 2021; Harder et al., 2021; Carvalho et al., 2022; Mota et al., 2023). By feeding European seabass larvae with *Artemia* metanauplii enriched with two experimental products (LM1 and LM2) and a commercial control (C), the same results in terms of growth performance, survival rate and skeletal anomalies were obtained among the experimental groups.

In terms of growth performance indicators, these outcomes are in line with previous studies conducted on European seabass larvae reared with a similar protocol (Sfakianakis et al., 2013; Yan et al., 2019; El-Dahhar et al., 2022; El Basuini et al., 2022). Like the majority of marine fish species, European seabass produce altricial larvae, which typically experience high mortality rates during the initial feeding phase. Compared to previous studies conducted by Vanderplancke et al. (2015) and Darias et al. (2010) reporting a survival rate at 45 DPH of 45 % and 68 % respectively, the observed survival rates in the present study, ranging from 61 to 68 % at 60 DPH, were high in all experimental groups. Larvae mortality occurs both in nature and in captive conditions mostly during the critical phase of yolk sac reabsorption and mouth opening at the beginning of exogenous feeding (Yúfera and Darias, 2007). This is a challenge in fishery biology (Houde, 2008) and the underlying mechanism is not yet completely understood, although feed availability and quality are the main factors explaining mortality in experimental conditions (Cushing, 1973; Malzahn et al., 2022; Benini et al., 2022). Nutritional blunders or deficiency occurring during this delicate phase may impact the correct maturation of the digestive system, the oxidative status and the skeletal ossification of adult fish (Izquierdo et al., 2013; Pham et al., 2023; Wischhusen et al., 2022), therefore it is important to provide larvae with an adequate level of nutrients. Among nutrients, dietary lipids are the main energy source for developing fish larvae. Lipids provide LC–PUFAs, which are needed for the synthesis of new cellular structures (Gisbert et al., 2005) and for the primary role on larval survival, growth and immune system modulation (Tocher et al., 2019; Roo et al., 2019; Betancor et al., 2021; Pham et al., 2023).

In this trial, the three enriched *Artemia* metanauplii dietary treatments were isonitrogenous and isolipidic despite some differences in the fatty acids profiles. Due to the importance of bioactive LC–PUFAs such as DHA, EPA and ARA in larval nutrition (Tocher, 2015), this discussion is focused on the implications related to their contents, overlooking other differences observed in less bioactive fatty acids. With regard to the total amount of n-3 PUFAs evaluated in *Artemia* metanauplii in this trial, no difference among the different dietary treatments were observed. However, within n-3 PUFAs, DHA was more abundant in LM1 and LM2 enriched *Artemia* metanauplii while EPA in C. DHA plays an important role in the development of the visual and neural system of marine fish larvae and its deficiency may affect the development of the predatory behaviour resulting in a lower survival rate (Watanabe, 1993; Izquierdo et al., 2013; Roo et al., 2019; Pham et al., 2023). A functional evidence of DHA importance is represented by the very high content of DHA in marine fish eggs (Anderson et al., 1984; Watanabe, 1993; Xu et al., 2017). Still within n-3 PUFA fatty acid family, EPA plays an important role in larvae metabolism, since it regulates cell membranes integrity and function and enhances their fluidity (Izquierdo, 2005; Hashimoto and Hossain, 2018) much more than arachidonic acid (ARA) (Hagve et al., 1998), but less than DHA (Hashimoto et al., 1999). Moreover, it is considered an important regulator of eicosanoids production (Adam et al., 2017; Sissener et al., 2020), being a major substrate for both cyclooxygenases and lipoxygenase enzymes and the main precursor for leukotriene synthesis in many fish species (Izquierdo, 2005; Montero et al., 2015, 2019). Another metabolically active LC–PUFA from the n-6 family is ARA. In this trial, *Artemia* metanauplii enriched with C showed an increased content of ARA. This is not surprising since ARA is highly present in fish–derived products and C is an encapsulated fish oil-based enrichment specifically supplemented with ARA, as stated in the product label. ARA deficiency in aquafeed formulation become more of a problem with the reduction of fish meal and oil (Araújo et al., 2022). ARA is involved in important physiological and immunological functions in marine fish, such as the

eicosanoids production, being the main precursors for prostaglandin synthesis (Izquierdo, 2005). In addition, ARA is also involved in the stress response (Martins et al., 2013). The concern over n-6 PUFA derived eicosanoids is due to the dogma of n-6 PUFA derived eicosanoids being pro–inflammatory, as opposed to the n-3 PUFA derived being anti–inflammatory (Hundal et al., 2021; Dong et al., 2023). As a consequence, DHA, EPA and ARA are considered essential fatty acids for the majority of marine fish and are required to be supplied in the diet. Marine species do not have sufficient Δ12 (or n-6) and Δ15 (or n-3) desaturases and elongase activities to produce them from oleic (18:1 n-9) acid precursor (Furuita et al., 1996; Izquierdo, 2005; Tocher et al., 2019), hence their requirements have been extensively investigated in many marine species (Watanabe et al., 1989; Izquierdo, 2005; Izquierdo et al., 2013; Navarro-Guillén et al., 2014; Roo et al., 2019; de Mello et al., 2022). An optimal level is set at 0.6 – 2.3 % for DHA, 0.7 – 2.3 % EPA and up to 1 % ARA considered as percentage on dry feed (Izquierdo, 2005), which are met in all experimental groups (data on % of fatty acid/dry feed not shown). What is more difficult is understanding the mechanisms of interaction between the LC–PUFAs. For instance, evidence of competition of LC–PUFAs on the same enzymatic pathways such as lipoxygenases, cyclooxygenases and phospholipases have been reported by several authors (Izquierdo, 2005; Norambuena et al., 2012; Kumar et al., 2016). As a results, when it comes to model the LC–PUFAs requirements, their ratio has to be carefully adjusted. Izquierdo, (2005) and Houston et al. (2017) proposed a ratio of 1:1 (EPA/DHA) with ARA values higher than 0.5 % and a total 3 % of LC–PUFAs, ideal for gilthead seabream growth. Betancor et al. (2016) found reduced growth when juveniles of gilthead seabream were fed diets with high EPA/DHA ratios, emphasising the importance of DHA. A similar superiority of DHA to EPA has been proposed also by Wu et al. (2002), reporting an increased growth in juveniles grouper (*Epinephelus malabaricus*) with lower ratio of EPA/DHA.

The fatty acid profile of European seabass fingerling at 60 DPH reflected the composition of their diets, as observed by several authors (Glencross, 2009; Boglino et al., 2012), with some differences, related to the metabolic transformation occurring in fish, resulting in a compensatory effects between the LC–PUFAs feed input and the final LC–PUFAs output deposited in larvae flesh. For instance, EPA content in *Artemia* metanauplii was 49 % and 43 % more in C compared to LM1 and LM2, respectively, while in larvae was smoothed over to an only 5 % more in C compared to both LM1 and LM2. Likewise, ARA content in *Artemia* metanauplii was 55 % more in C compared to both LM1 and LM2 while in larvae 18 % more in C compared to both LM1 and LM2. Interestingly the ratio of difference in DHA content remained the same in *Artemia* metanauplii and larvae (approximately 14 % and 25 % lower in C compared to LM1 and LM2 respectively in both *Artemia* and larvae). Overall data on LC–PUFAs composition of larvae are in line with previous experiments conducted on gilthead seabream (Koven et al., 2001), European seabass (Gisbert et al., 2005), turbot (*Scophthalmus maximus*) and Senegale sole (*Solea senegalensis*) (Boglino et al., 2012), showing enough nutrients stored for the further development in all experimental groups. This statement is confirmed by the absence of differences, among the dietary treatments, in the n-9 PUFAs (particularly 18:1 n-9 and 20:1 n-9) contents in larvae, which are produced by elongation and increased values were proposed as indicator of LC–PUFAs deficiency in gilthead seabream (Rodriguez et al., 1994) and grouper (Wu et al., 2002).

Excessive LC–PUFAs in diets may lead to increased risk of lipid peroxidation (Betancor et al., 2015; Qian et al., 2015; Adam et al., 2017), which is mediated by free radicals and reactive oxygen species (ROS) production (oxidative factors). Nevertheless, a recent study described the antioxidant protection capacity of EPA and DHA against cellular oxidative stress in humans (Aldhafiri, 2022). In line with this, dietary supplementation with a mixture of EPA + DHA has been reported to exert an antioxidant protective role in fish (Kumar et al., 2022). Balanced

concentrations of anti – and pro–oxidant factors are continuously generated during regular cellular metabolism, which is known as ROS homeostasis (Lushchak, 2016). Between the metabolic antioxidant factors, the enzyme catalase catalyses the decomposition of hydrogen peroxide to water and oxygen, preventing the cascade of oxidation reactions and closing the lipid peroxidation catalytic cycle (Betancor et al., 2012). However, if ROS generation exceeds the antioxidant protection, the imbalance is called oxidative stress and may lead to reduced larval growth (Betancor et al., 2012; Roo et al., 2019), severe dystrophic lesions in the musculature (Betancor et al., 2012) and higher skeletal anomalies rate (Izquierdo et al., 2010). Lipid peroxidation and its by–products, such as protein carbonylation (Suzuki et al., 2010), have been commonly used as biomarkers of oxidative status in fish larvae (Monroig et al., 2006; Betancor et al., 2012; Guerreiro et al., 2022). In this trial, although not statistically significant, larvae from LM2 treatment group showed a trend to higher mitochondrial ROS production. By contrast, this group presented statistically lower catalase activity than larvae from C treatment and did not present any tendency to higher oxidative damage (measured as LPO, PC and larval growth performance). From a nutritional point of view, microalgae and cyanobacteria display diverse bioactive compound profiles, being those with antioxidant properties of particular interest. In that sense, both *Nannochloropsis* spp. and *Spirulina* spp. are rich in the pigments β-carotene and zeaxanthin, although, on a dry weight basis, *Spirulina* spp. contains 2.5 and 15–fold higher β-carotene and zeaxanthin than *Nannochloropsis* spp., respectively (Ghaeni et al., 2015; Bernaerts et al., 2020). In addition, *Spirulina* spp. is also rich in phycocyanin, a blue–coloured photosynthetic pigment with free radical scavenging capacity (Fernandes et al., 2023). Altogether, the higher EPA + DHA content found in *Artemia* enriched with LM2 and the higher content in antioxidant compounds of *Spirulina* spp., results might support a potential preventive effect against oxidative stress of LM2 enrichment (Coulombier et al., 2021; Vignaud et al., 2023). However, further studies are needed to conclusively unravel the antioxidant effects of LM2 blend.

Several skeletal anomalies have been described in marine fish, representing a major problem in aquaculture for the economical, biological and ethical related concerns (Boglione et al., 2013). Anomalies in fish are often associated with a reduced growth and high mortality rate, being a major problem in a hatchery setting due to the derived economical losses. In this trial, the morphological analysis of European seabass did not show an effect of the microalgal enrichments LM1 and LM2 on the total number of severe anomalies and the relative frequencies in the different regions of vertebral column, compared to C. In a wild contest, it is fair to presume that individuals with physical anomalies will have a reduced capacity to swim properly, feed or escape a predator, thus reducing its likelihood to survive. In a survey conducted on wild populations by Boglione et al. (2001) a 4 % of wild gilthead seabream presented some severe anomalies. On the contrary, in a hatchery setting, with controlled environmental conditions, food availability and absence of predation, the anomalies rate is expected to be higher. The findings from this study are in line with surveys conducted by Koumoundouros (2010) and Boglione et al. (2013), reporting an incidence of severe anomalies in Mediterranean marine hatcheries of 30–40 % of the reared fish. A recent study conducted by (Viegas et al., 2023) on European seabass reported an incidence of severe anomalies of 33–37 % at 64 DPH. Even though anomalies in fish have been often associated with nutritional causes such as nutrients deficiency (Boglione et al., 2013) or nutrients unbalancing (Izquierdo et al., 2013; Roo et al., 2019), the severe anomalies observed in this trial are more likely to be attributed to general causes such as tank hydrodynamics, temperature and other abiotic and biotic factors.

Conclusions

Producing microorganisms such as microalgae and cyanobacteria exploiting industrial geothermal waste outputs could mitigate the over–exploitation of marine fish stocks and reduce the costs associate with photobioreactors energy consumption. The results obtained in this study

indicated that using blends of so produced products for the enrichments of Artemia salina produced equal results in terms of growth performance and larvae anomaly rate compared to a commercial control. Each dietary treatment presented a specific accumulation pattern for ARA, DHA and EPA long chain polyunsaturated fatty acids. Ratio of DHA / EPA of 4:1 as in the case of LM1 and LM2 in the presence of 1.4 % of ARA (as a % on total fatty acid) guaranteed equal results to the control which display a ratio of DHA/EPA of 2:1 with 2.2 % of ARA. The fatty acids profile of European seabass larvae sampled at 60 DPH reflected the composition of the diet, showing no signs of LC–PUFAs deficiency in every dietary treatment. Results on the larvae oxidative status suggested that LM2 enrichment product may exert potential preventive effects against oxidative stress, which could be translated into enhanced fish larvae robustness and, in the long–term, improved health status of the fish. After the radiological survey, European seabass larvae at 60 DPH presented a percentage of anomalies ranging from 34 – 47 % in all dietary treatments. The relative frequency of anomaly mainly affected caudal vertebrae, followed by skull, cranial vertebrae, pre– haemal vertebrae and haemal vertebrae in all groups. These findings are in line with other research conducted on the species and on other Mediterranean marine fish.

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CHAPTER 3. 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

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Andrea Bertini, Silvia Natale, Enric Gisbert, Karl B. Andrée, Danilo Concu, Francesco Dondi, Alessandra De Cesare, Valentina Indio, Pier Paolo Gatta, Alessio Bonaldo, Luca Parma

Abstract

The capacity of utilising a single cell protein (SCP) ingredient coming from *Corynebacterium glutamicum* was assessed on adult grey mullet (*Mugil cephalus*) reared in captive conditions. The experiment was carried out using triplicate groups of grey mullet of 68 g average initial body weight. Three diets, SCP0, SCP10 and SCP20 with increasing inclusion of SCP (0 %, 10 % and 20 %) in substitution of soybean, poultry and fish meal were formulated to contain 30 % protein, 10 % fat and 18.5 Mj/kg feed of digestible energy. After 113 days, fish fed SCP diets presented significantly lower growth performance and a significant lower activity of the alkaline proteases and aminopeptidases compared to fish fed diet without SCP inclusion. Gut microbiota appeared modulated by SCP inclusion being dominated at the phylum level by Fusobacteria in fish fed SCP0 (51.1 %), while in fish fed SCP10 (67.3 %) and SCP20 (53.2 %) Proteobacteria was dominant. Data evinces a deficiency in the protein utilisation as a cause of the poor growth performance in fish fed the SCP diets. A hypothesis has been proposed that an incomplete SCP cell–wall lysis accounts for this outcome because of the particular organisation of the digestive system of grey mullet (which lack of an acidic stomach digestion) and the failing in the development of a functional gizzard (no access to sand in captive conditions). Even though the outcomes of this research were quite unexpected, they will improve our knowledge on the digestive system of flathead grey mullet and provide some theoretical basis for an improved development of low FM and SBM aquafeed for the species.

3.1 Introduction

Flathead grey mullet (*Mugil cephalus*) is an omnivorous and detritivores fish species which has been selected as suitable for the diversification of Mediterranean aquaculture both in monoculture and polyculture systems due to its low trophic position (Vallainc et al., 2021; Solovyev and Gisbert, 2022). Grey mullet presents a rapid growth, a good adaptability to captivity and high market price of its salted and dried roe, known as "bottarga" (Whitfield et al., 2012). In addition, being a consumer of the lower trophic layers, its protein requirement is considered to be lower when compared to strict carnivorous farmed Mediterranean species. For instance, the protein content of standard aquafeed formulations for gilthead sea bream and European sea bass is normally around 45 % (Busti et al., 2020a; Pelusio et al., 2022), while for flathead grey mullet can be as low as 30 % (De Carvalho et al., 2010; Talukdar et al., 2020).

Among the protein sources commonly used in aquafeeds, fishmeal (FM) is recognised as the most suitable in terms of nutritional value. However, the increased demand and the decline of the marine fishery stocks limit its application in aquafeeds (Glencross et al., 2016; Pereira et al., 2022). Alternatives to FM are conventionally represented by plant–based ingredients such as soybean meal (SBM), although with the presences of certain drawbacks (Colombo et al., 2022; Newton et al., 2023). As an example, SBM which is largely used into aquafeed formulation as a FM substitute, has a high carbon footprint if it is derived from crops grown on land converted from forest or savannah (Wilkinson and Young, 2020). In addition, its massive use into aquafeeds goes in direct competition with human consumption, as SBM is a key element in human diets.

In a circular economy, aquafeed ingredients derived from industry by–products such as single-cell ingredients are in the spotlight as a promising option for the sustainability of the aquaculture industry (Colombo et al., 2022; Glencross et al., 2020). Single–cell ingredients are a relatively broad class of materials including bacterial, fungal and microalgal–derived products,

which have shown potential as a source of protein, lipids or specific nutritional components in aquafeed ingredients (Pereira et al., 2022; Glencross et al., 2020). Among such "circular" ingredients, bacterial single cell protein (SCP) presents a high percentage of crude protein with a favourable amino acid profile, along with vitamins and other functional compounds (Glencross et al., 2020; Jones et al., 2020). Bacteria may be produced using a wide range of substrates and nutrients ranging from industrial and agricultural residues to bioindustry by–products (Jones et al., 2020); thus, allowing the valorisation of waste streams and reducing the downstream costs related to industrial waste disposal (Pereira et al., 2022). Among different SCP sources, *Corynebacterium glutamicum* is a Gram–positive bacteria generally regarded as safe (GRAS organism) (Cheng et al., 2021; Liu et al., 2022), which is commonly used to produce amino acids (AA). After the AA production, filtered *C. glutamicum* cell mass, contains high protein content (>70 %) and a suitable AA profile to be used as a replacement of conventional protein sources in livestock nutrition (Zhang et al., 2013). In aquaculture nutrition, it's application has been successfully proven in gilthead seabream even at high inclusion level (Marchi et al. 2023). In general, digestibility and palatability of bacterial SCP is considered good; however, variable performances may appear between the tested bacteria genus (Bratosin et al., 2021; Glencross et al., 2020), the application of post–production cell lysis processes (e.g. autolysis or hydrolysis) (Glencross et al., 2023; Teuling et al., 2019; Schøyen et al., 2005) and the aquaculture species (Marchi et al., 2023; Zamani et al., 2020; Hamidoghli et al., 2019; Marit Berge et al., 2005).

To the best of our knowledge there is no literature to date concerning the application of a bacterial SCP in the diet of flathead grey mullet in captive conditions. In the present study, with the aim of providing a theoretical basis for the development of zero FM and SBM aquafeed for mullet, *C. glutamicum* was used to replace these conventional ingredients at three different inclusion levels. The effects of its inclusion in flathead grey mullet were evaluated on classical key

performance indicators like growth performance, feed utilisation, and other variables like metabolic blood parameters, digestive enzyme activities and gut microbiota in order to evaluate holistically the effects of this new sustainable ingredient in fish nutrition.

3.2 Materials and methods

3.2.1 Experimental diets

C. glutamicum SCP was utilised as a replacement of the conventional ingredients soybean meal (SBM), poultry meal (PM) and fishmeal (FM) in the diet of flathead grey mullet. This ingredient consisted of bulk dried bacterial single cells composed of 73.5 ± 1.5 crude protein, 3.7 ± 0.6 crude lipid, 8.8 ± 0.4 moisture and 4.6 ± 0.3 ash (NT70, M.I. Gordini srl, Bologna, Italy). Three isonitrogenous, isolipidic and isoenergetic experimental diets were formulated to contain increasing inclusion levels of bacterial SCP: SCP0 (0 % SCP), SCP10 (10 % SCP) and SCP20 (20 % SCP). In the SCP20 diet, both SBM and FM were completely replaced by the tested bacterial SCP, whereas PM was significantly reduced. Extruded diets (pellet size: 1.5 mm) were manufactured by SPAROS Lda (Olhão, Portugal). The contents of protein, lipid and energy in the diet formulations were decided on the basis of several research works (Talukdar et al., 2020; De et al., 2012). As observed by Ramos-Júdez and Duncan (2022), grey mullet requires small size pellet because of its particular feeding behaviour. The species do not chew and break the pellet into smaller particles but capture and keep it in the oral cavity or spit it out and recapture before ingestion. The ingredients and the proximate composition of the diets were shown in Table 3.1.

Table 3.1 Ingredients and proximate composition of the experimental diets.

	SCP0	SCP10	SCP20
Ingredient, % of the diet			
SCP meal[1]	0.00	10.00	20.00
SBM meal[2]	30.00	9.10	0.00
Fish meal[3]	3.00	3.00	0.00
Poultry meal[4]	9.73	9.73	5.53
Feather meal hydrolysate[5]	5.00	5.00	5.00
Wheat meal[6]	39.90	50.27	54.54
Fish oil[7]	3.45	3.50	3.75
Soybean oil[8]	3.45	3.50	3.75
Dicalcium phosphate (DCP)[9]	2.99	2.99	3.99
L-Lysine[10]	0.82	1.20	1.60
L-Threonine[10]	0.00	0.00	0.10
L-Tryptophan[10]	0.10	0.15	0.18
DL-Methionine[11]	0.30	0.30	0.30
Vit & Min Premix[12]	1.00	1.00	1.00
Vitamin E50[13]	0.05	0.05	0.05
Antioxidant[14]	0.20	0.20	0.20
Yttrium oxide[15]	0.01	0.01	0.01
Proximate composition, % on a wet weight basis			
Crude protein	31.87	31.37	31.31
Crude fat	9.41	9.89	10.24

Moisture	8.88	9.43	8.61
Ash	7.16	6.31	5.70
Energy (Mj/kg feed)	18.5	18.7	18.8

[1]SCP, *C. glutamicum* single cell protein, 73.5 % crude protein (CP), 3.7 % crude fat (CF), NT70, M.I. Gordini srl, Italy; [2]SBM, Soybean meal, 44 % CP, 3.5 % CF, Ribeiro & Sousa Lda, Portugal; [3]Fishmeal 61.2 % CP, 8.4 % CF, Conserveros Reunidos S.A., Spain; [4]Poultry meal, 62.4 % CP, 12.5 CF, SAVINOR UTS, Portugal; [5]Feather meal hydrolysate, 88.8 % CP, 1.6 % CF, Empro Europe NV, The Netherlands; [6]Wheat meal, 11.7 % CP, 1.6 % CF, Molisur, Spain; [7]Fish oil, 98.1 % CF, Sopropêche, France; [8]Soybean oil, 98.6 % CF, JC Coimbra, Portugal; [9]DCP, dicalcium phosphate, Premix Lda, Portugal; [10]L-Lysine, L-Threonine and L-Tryptophan, Ajinomoto EUROLYSINE S.A.S, France; [11]DL-Methionine, RHODIMET NP99, ADISSEO, France; [12]Vitamin and Mineral Premix, Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamine, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg^{-1} 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; Premix Lda, Portugal. [13]Vitamin E50, ROVIMIX E50, DSM nutrition products, Switzerland; [14]Antioxidant, VERDILOX, Kemin Europe NV, Belgium; [15]Yttrium oxide, Sigma Aldrich, USA.

3.2.2 Fish rearing and feeding trial

Flathead grey mullet born in captivity at International Marine Centre (Oristano, Italy) were delivered to the Laboratory of Aquaculture at the University of Bologna (Cesenatico, Italy). At the beginning of the trial, fish (average body weight = 67.90 ± 15.46 g) were randomly distributed into 9 x 800 L fiberglass square tanks (45 fish/tank, each diet assigned in triplicate), connected to a closed recirculation unit. This unit consisted of a mechanical sand filter (PTK 1200; Astralpool,

Barcelona, Spain), ultraviolet lights (UV PE 45; Sita Srl, Barcelona, Spain) and a biofilter (PTK 1200; Astralpool, Barcelona, Spain). Water consisted of a mixture of marine and freshwater (salinity 7 ± 2 ‰) (Cardona, 2006), the water exchange rate was kept at 100 % / h in each tank and the overall water renewal in the system was at 5 % daily. Temperature was kept at 26.8 ± 2.5 °C, photoperiod was maintained at 12 h light / 12 h dark through artificial light and oxygen saturation at 100 % by an integrated control system. Nitrogen compounds (ammonia, nitrite and nitrate) and pH were daily monitored and kept at the optimum range for the fish welfare. Feed was provided to apparent satiation by oversupplying the feed (10 % of the daily ration) by automatic feeders once a day for six days a week. Each meal lasted 6 hours to provide fish a continuous feed supply over daily hours. The uneaten pellets of each tank were collected three times daily at each meal (2, 4 and 6 hours after the beginning of meal), dried overnight at 105 °C, and deducted for overall calculations following the method as described in (Bonvini et al., 2018).

3.2.3 Samplings

Before each sampling procedure, fish were either anaesthetised (100 mg L^{-1}) or euthanised (300 mg L^{-1}) by MS222. At the end of the experiment (day 113), all fish were anesthetised and individually weighted. The proximate composition of the carcasses was determined at the beginning of the experiment on a pooled sample of 10 fish and on a pooled sample of 3 fish per tank (9 fish per diet) at the end of the experiment, this information was later used for determining efficiency of nutrient (protein and lipid) uptake. Biometric indices [total body length, SL (cm); wet weight, BW (g); viscera and liver weight (g) and gut length (cm)] were recorded on 3 fish per tank (9 fish per diet). At the end of the experiment, fish were fed to satiety on a 6–hour meal and sampled for blood chemistry, gut microbiota and digestive enzyme's activity. Blood was collected from the caudal vein from 6 fish per tank (18 fish per diet) and transferred to a clot activator tube (Vacutest Kima Srl, Padova, Italy). As soon as the blood clotted it was centrifuged at 3,000 x g for 10 min

at 4 °C, serum aliquots removed and stored at – 80 °C for chemical analysis. At the same time, 250 mg of hindgut content from 3 fish per tank (9 fish per diet) was collected aseptically by gently stripping the final 5 cm of the gut into a sterile tube and stored at – 80 °C for gut microbiota analysis. At the same time, samples for assessing the activity of digestive enzymes were also collected. Being that the duration of a meal was 6 hours, these samples were collected from fish over 0 to 6 hours after the end of the meal. To do so, the whole gastro–intestinal tract and stomach were dissected from 2 fish per tank (6 fish per diet) for the analysis of pancreatic enzymes and placed into resealable bags and snap frozen at − 80 °C, until their analysis. All experimental procedures were evaluated and approved by the Ethical–Scientific Committee for Animal Experimentation of the University of Bologna, in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes.

3.2.4 Calculations

The indices employed for evaluating somatic growth and feed utilisation were specific growth rate (SGR), feed intake (FI), feed conversion rate (FCR), viscerosomatic index (VSI), hepatosomatic index (HSI), relative gut length (RGL), Fulton's condition factor (K), protein efficiency rate (PER), lipid efficiency rate (LER), gross protein efficiency (GPE) and gross lipid efficiency (GLE). Formulae are outlined as follows:

$$SGR\ (\%\ \text{day}^{-1}) = 100 * \frac{InFBW - lnIBW}{days} \quad (2)$$

$$FI\ (\text{g feed/fish day}^{-1}) = 100 * \frac{feed\ intake/((FBW + IBW)/2)}{days} \quad (3)$$

$$FCR\ = \frac{feed\ intake}{weight\ gain} \quad (4)$$

$$VSI(\%) = 100 * \frac{viscera\ weight}{body\ weight} \quad (5)$$

$$HSI(\%) = 100 * \frac{liver\ weight}{body\ weight} \quad (6)$$

$$RGL(\%) = 100 * \frac{gut\ length}{body\ length} \quad (7)$$

$$K = 100 * \frac{FBW}{body\ length^3} \quad (8)$$

$$PER = \frac{FBW - IBW}{protein\ intake} \quad (9)$$

$$GPE = 100 * \frac{(\%FBprotein * FBW) - (\%IBprotein * IBW)}{total\ protein\ intake} \quad (10)$$

$$GLE = 100 * \frac{(\%FBlipid * FBW) - (\%IBlipid * IBW)}{total\ lipid\ intake} \quad (11)$$

3.2.5 Analytical methods

Diets and whole carcasses were analysed for moisture, nitrogen (N), lipid and ash contents. Moisture was calculated by gravimetric analysis following oven drying at 105 °C overnight. Protein level was determined as total nitrogen (N * 6.25) after performing the Kjeldahl method. Lipid level was determined according to Bligh and Dyer's extraction method (Bligh and Dyer, 1959). Ash content was determined by gravimetric analysis following incineration of the sample in a muffle furnace at 450 °C overnight. Diets' buffering capacity was defined as the slope of the titration curve from the initial pH of the diet to a value below 3. The procedure consisted in suspending 1 g of the diet in 5 mL of distilled water, keeping it magnetically stirred, then adding successive aliquots of HCl and recording the pH 30 s after each addition of HCl as described in Márquez et al. (2012) and Parma et al. (2019). All pH measurements were performed by using a Hanna HI 991002 pH–meter (Hanna instruments, Woonsocket, USA). Metabolic blood parameters were determined in a 500 µL serum sample by using an automated analyser (AU 480; Beckman

Coulter Inc., Brea, USA) with dedicated methods (Olympus system reagent, OSR). The type of reaction used in the assay and the OSR identification number were reported in brackets following the reported parameters. Metabolic blood parameters include glucose (hexokinase reaction, OSR6121), urea (urease reaction, OSR6134), uric acid (uricase reaction, OSR6198), bilirubin (colorimetric reaction, OSR6112), cholesterol (enzymatic method, OSR6116), high–density lipoprotein (HDL, enzymatic method, OSR6187), triglycerides (TG, enzymatic method, OSR61118), lactic acid (enzymatic method, OSR6193), total protein (biuret method, OSR6132), albumin (ALB, bromocresol green method, OSR6102), aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), creatine kinase (CK) and lactate dehydrogenase (LDH) (enzymatic reaction, OSR6009, OSR6170, OSR6004, OSR6179 and OSR6128, respectively), total calcium (Ca total, oCPC colorimetric reaction, OSR6113), calcium ion (Ca^{2+}, arsenazo colorimetric reaction), phosphorus (P, molybdate reaction, OSR6122), magnesium (Mg, xylidin blue reaction, OSR6189), iron (Fe), sodium (Na^{+}), potassium (K^{+}) and chloride (Cl) (ion selective electrode indirect method). The following ratios were also calculated: albumin (ALB) / globulin (GLOB) and Na / K, as well as the Ca * P.

3.2.6 Digestive enzyme activity

The quantification of the activity of several digestive enzymes (total alkaline protease, trypsin, chymotrypsin, leucine aminopeptidase, α–amylase and bile salt–activated lipase) were conducted on independent samples coming from 2 fish per tank (6 fish per diet). Digestive enzymes activity was quantified according to the methods described in Gisbert et al. (2018) and samples handled according to Solovyev and Gisbert (2016) in order to prevent their degradation during storage and handling. Briefly, samples were homogenised by using an Ultra–Turrax T25 basic (IKA©-Werke, Germany). After, 0.50 g of sample were adjusted in 30 volumes (v/w) of ice–cold mannitol (50 mM mannitol, 2 mM Tris-HCl buffer; pH 7.0), following the addition of 0.1 M

of CaCl2. Samples were re–homogenised for 30 seconds and sonicated for 90 seconds. The resultant crude tissue extract was centrifuged at 9,000 * g (10 min at 4 °C) and then, 1 ml of supernatant was stored at − 80 °C in Eppendorf tube until enzyme quantification. Total alkaline proteases were quantified using 0.5 % (w/v) azo–casein as substrate in 50 mM Tris-HCl buffer (pH 8.0). One unit of total alkaline proteases per mL (U) was defined as 1 μmol azocasein hydrolysed per min and mL of extract at λ = 366 nm (García-Carreño and Haard, 1993). Trypsin activity was assayed using BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide) as substrate (Holm et al., 1988). Chymotrypsin activity was quantified using suc-AAPF-pNA (N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide) as substrate (Decker, 1977). The quantification of trypsin and chymotrypsin is achieved from the cytosolic extraction and following their kinetic curve in the spectrophotometer (λ = 405 nm, 5 min). The activity of α–amylase was determined using 0.3 % starch dissolved in Na2HPO4 buffer (pH 7.4) as substrate and its activity (U) was defined as the mg of starch hydrolysed per min and mL of extract (λ = 580 nm) (Métais and Bieth, 1968). Bile salt–activated lipase activity was measured using p–nitrophenyl myristate as substrate in 0.25 mM Tris–HCl (pH 7.9), 0.25 mM 2–methoxyethanol and 5 mM sodium cholate buffer (Iijima et al., 1998). The activity of bile salt–activated lipase (U) was defined as the μmol of substrate hydrolysed per min and mL of extract (λ = 405 nm). Leucine aminopeptidase activity was determined using leucine–alanine as substrate in 50 mM Tris–HCl buffer (pH 8.0); one unit of enzyme activity (U) was defined as 1 nmol of the hydrolysed substrate per min per ml of tissue homogenate (λ = 530 nm) (Nicholson and Kim, 1975). All enzyme activities were expressed as total specific activity (U mg protein^{-1}) and soluble protein in enzyme extracts was quantified by means of the Bradford's method, using bovine serum albumin as standard (Bradford, 1976). The activity of all enzymes was determined at 25 ºC in triplicate per sample (methodological replicates).

3.2.7 Gut bacterial community DNA extraction, sequencing and bioinformatic analysis

For gut microbiota studies, the DNA was extracted from each sample as previously described in De Cesare et al. (2019). Briefly, 250 mg of hindgut contents were suspended in 1 mL lysis buffer (500 mM NaCl, 50 mM Tris–HCl, pH 8.0, 50 mM EDTA, 4 % SDS) with steel beads and vigorously shaken using a MM400 mixer mill (Retsch GmbH, Haan, Germany) for 2 minutes at a frequency of 30 Hz. Samples were then heated at 70 °C for 15 min and further centrifuged to separate the DNA from the bacterial cellular debris. This process was repeated on the remaining cellular debris with a second 0.3 mL aliquot of lysis buffer. Then, samples were subjected to 10 M v/v ammonium acetate precipitation, followed by isopropanol precipitation and a 70 % ethanol wash and resuspended in 0.1 mL 1X Tris–EDTA (Sigma, Milan, Italy). Samples were treated with DNase–free RNase (Roche, Milan, Italy) and incubated overnight at 4 °C, before being processed following the instructions of the QIAmp DNA Stool Mini Kit (Qiagen, Milan, Italy). DNA libraries were prepared following the Illumina 16S Library preparation protocol, amplifying the variable V3 and V4 regions of the 16S rRNA in order to obtain a single amplicon of approximately 460 bp. Sequencing was performed in 251 * 2 paired–end in the Illumina MiSeq with the MiSeq Reagent kit v2 500 cycles. Sequencing produced an average of 14.0 MB per sample.

All the samples were analysed using a bioinformatics pipeline based on QIIME2 (http://qiime.org/) except two SCP0 samples (i.e. 6_SCP0 and 7_SCP0) with a low sequence output that were discarded. First, the sequences were imported as paired–end reads and processed with the dada2 algorithm in order to perform the denoising and to merge forward and reverse sequences per each pair. By this, we retained about 70 % of input sequences per sample homogeneously along the sample groups (diets). The taxonomic classification of cleaned data was performed by applying the VSEARCH–based classifier implemented in QIIME and adopting the Greengenes 13_8 97 % OUT dataset as reference. High variability among samples within

treatment groups required that the data set be adjusted for this. To do so alpha diversity of all samples was calculated. Samples with an alpha diversity not respecting 25–percentile were not considered and are not present in the figure (i.e. 2_SCP0, 1_SCP10, 7_SCP10, 8_SCP10, 9_SCP10 and 4_SCP20).

3.2.8 Statistical analysis

Significant differences in growth performance, nutritional indices, metabolic serum parameters and digestive enzyme activities between dietary groups were determined using one way ANOVA ($p \leq 0.05$). The assumptions related to data normal distribution and homogeneity of variances were explored through Anderson Darling's test and Levene's test, respectively. Analyses were performed using Minitab software (Minitab 19.0.1; Pennsylvania State University, USA).

For the microbiota analysis, mean values for the relative frequency of abundance of each taxonomic level in each sample were compared using the two–sided t–test on R software (package stats, function t.test). The R environment was also adopted to perform the alpha and beta diversity analysis using the package vegan and respectively the functions diversity and vegdist. For alpha diversity the indices Shannon, Simpson and Fisher were calculated. Beta diversity was explored by using a non–metric multi–dimensional scaling (NMDS). Significant separation between groups was observed with PERMANOVA using the package Adonis2 in R; data comply with the assumption related to data homogeneity of multivariate dispersion. On each statistical analysis the p values ≤ 0.05 were considered statistically significant while $0.05 < p$ value ≤ 0.1 were considered borderline (tendency). Results are presented as mean ± standard deviation (SD), unless mentioned otherwise.

3.3 Results

3.3.1 Growth performance and feed utilisation

Growth performances of flathead grey mullet fed diets differing in their content in bacterial SCP are shown in Table 3.2. At the end of the experiment, grey mullet fed the experimental diets, presented significant differences in terms of growth performance and feed utilisation indicators ($p \leq 0.05$). Fish fed SCP0 showed significantly higher FBW and SGR compared to fish fed SCP10 and SCP20 diets. Fish fed the SCP20 diet showed significantly lower values for these two parameters in comparison to the other groups. Values of FI and FCR of fish fed the SCP0 diet were significantly lower in comparison to fish fed SCP10 and SCP20, whereas fish from the SCP20 feeding regimen showed significantly higher values for the two parameters ($p \leq 0.05$).

Table 3.2 Growth performance and feed utilisation of flathead grey mullet (*Mugil cephalus*) fed the experimental diets.

	SCP0	SCP10	SCP20	*p* value
IBW (g)	67.65 ± 0.47	68.15 ± 0.34	68.18 ± 0.22	0.2071
FBW (g)	182.13 ± 8.27^{a}	137.54 ± 10.32^{b}	108.70 ± 2.57^{c}	0.0001
SGR (% day^{-1})	0.88 ± 0.04^{a}	0.62 ± 0.07^{b}	0.41 ± 0.02^{c}	0.0001
FI (g feed/fish Day^{-1})	1.45 ± 0.04^{a}	1.59 ± 0.07^{b}	1.92 ± 0.04^{c}	0.0001
FCR	1.80 ± 0.10^{a}	2.69 ± 0.36^{b}	4.80 ± 0.12^{c}	0.0000
Survival (%)	98.5 ± 2.6	99.3 ± 1.3	97.0 ± 3.4	0.5868

Data are given as the mean (n = 3) ± SD. Different superscript letters in the same line, indicate significant differences among treatments ($p \leq 0.05$).

3.3.2 Biometric and nutritional indices, and body composition

Data on biometric indices, body composition and nutritional indices are shown in Table 3.3. At the end of the experiment, no significant differences among dietary groups were observed in terms of VSI, HSI and RGL values ($p > 0.05$). In contrast, K values from fish fed the SCP20 diet were significantly lower to those found in fish from the SCP0 group ($p \leq 0.05$), whereas fish fed the SCP10 diet showed intermediate values which are found to be non–statistically different from the groups SCP0 and SCP20. The content of protein, lipid and moisture was not significantly affected by the diet ($p > 0.05$). On the contrary, the ash carcass content was significantly different only between the groups SCP0 and SCP20 ($p > 0.05$). Nutritional indices like PER, LER, GPE and GLE from fish fed the SCP0 diet were significantly higher in comparison to fish fed SCP10 and SCP20 diets ($p \leq 0.05$), whereas fish fed the SCP20 diet showed significantly lower values for all nutritional indices.

Table 3.3 Biometric indices, body composition and nutritional indices of flathead grey mullet (*Mugil cephalus*) fed the experimental diets.

	SCP0	SCP10	SCP20	*p* value
Biometric indices				
VSI	5.20 ± 0.60	5.79 ± 0.63	5.00 ± 1.19	0.1480
HSI	1.07 ± 0.24	0.97 ± 0.15	1.10 ± 0.29	0.5059
RGL	2.02 ± 0.26	1.94 ± 0.18	1.77 ± 0.32	0.1344
K	1.14 ± 0.06^{a}	1.08 ± 0.07^{ab}	1.06 ± 0.04^{b}	0.0336
Whole body composition %				
Protein	18.09 ± 0.08	17.65 ± 0.11	17.70 ± 0.76	0.4506

Lipid	13.60 ± 0.84	13.83 ± 0.94	11.61 ± 1.50	0.0984
Ash	3.30 ± 1.00[a]	4.52 ± 1.00[ab]	4.75 ± 0.08[b]	0.0443
Moisture	64.65 ± 1.05	63.35 ± 0.73	65.50 ± 2.21	0.2734
Nutritional indices				
PER	1.7 ± 0.1[a]	1.2 ± 0.2[b]	0.6 ± 0.0[c]	0.0001
LER	5.8 ± 0.3[a]	3.8 ± 0.6[b]	1.9 ± 0.1[c]	0.0001
GPE	32.3 ± 1.7[a]	21.7 ± 3.5[b]	11.9 ± 0.9[c]	0.0001
GLE	75.4 ± 9.6[a]	49.2 ± 14.7[b]	11.4 ± 7.9[c]	0.0012

Data are given as the mean (n = 3; n = 9 for VSI, HSI, RGL and K value) ± SD. Different superscript letters in the same line, indicate significant differences among treatments ($p \leq 0.05$).

3.3.3 Feed buffer capacity

Feed buffer capacity is shown in figure 3.1. The three experimental diets SCP0, SCP10 and SCP20 presented a significant difference in pH of 6.00 ± 0.01, 5.62 ± 0.00 and 5.22 ± 0.00, respectively ($p \leq 0.05$). Similarly, the diet buffer capacity (as mmol of HCl to lower 1 g of the diet to pH 3) significantly decreased by the inclusion of the SCP ingredient being 0.64 ± 0.01, 0.59 ± 0.00 and 0.52 ± 0.01 mmol of HCl for SCP0, SCP10 and SCP20 diets, respectively.

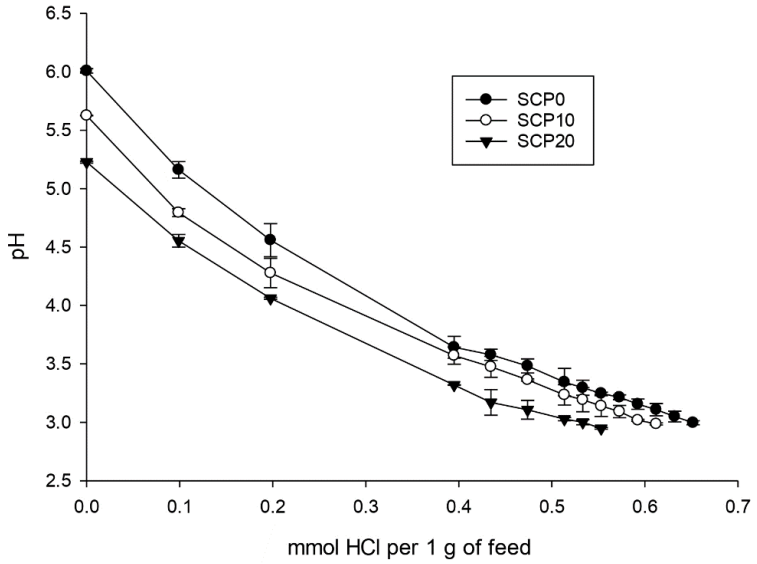

Figure 3.1 Plot showing changes in pH with the addition of HCl for the three diets considered (SCP0, SCP10 and SCP20). Data are shown as mean ± SD (n = 3).

3.3.4 Serum metabolic parameters

The results of serum metabolic parameters are shown in Table 3.4. The levels of glucose, total calcium, Ca^{2+}, Fe, K^{+} and the result of Ca * P were significantly higher in fish fed the SCP0 diet compared to those fed with SCP10 and SCP20 diets ($p \leq 0.05$). By contrast, the ratio Na^{+} / K^{+} was significantly lower in fish fed the SCP0 diet compared to those from SCP10 and SCP20 groups ($p \leq 0.05$). Urea tended to be higher ($p = 0.0533$) in fish fed SCP10 and SCP20 diet compared to the SCP0 group. On the contrary, the serum enzyme alkaline phosphatase (ALP) tended to be higher ($p = 0.0652$) in fish fed the SCP0 diet with respect to their congeners fed SCP10 and SCP20 diets. No significant differences between the treatments were observed for uric acid, total bilirubin, cholesterol, HDL, TG, lactic acid, TP, ALB, AST, ALT, CK, LDH, P, Mg, Na^{+}, Cl and the ratio ALB / GLOB.

Table 3.4 Serum metabolites, enzymes and electrolytes values for flathead grey mullet (*Mugil cephalus*) fed the experimental diets.

	SCP0	SCP10	SCP20	*p* value
Serum metabolites				
Glucose (mg dL^{-1})	200.72 ± 39.43[a]	141.17 ±51.08[b]	137.67 ± 39.43[b]	0.0001
Urea (mg dL^{-1})	2.48 ± 0.60	2.67 ± 0.70	3.05 ± 0.72	0.0533
Uric acid (mg dL^{-1})	0.45 ± 0.23	0.35 ± 0.27	0.48 ± 0.45	0.4729
Bilirubin (mg dL^{-1})	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	0.6419
Cholesterol (mg dL^{-1})	285.56 ± 32.58	291.94 ± 36.56	265.61 ± 41.45	0.0942
HDL (mg dL^{-1})	108.22 ± 11.62	112.83 ± 13.83	103.50 ± 13.96	0.1028
TG (mg dL-^{1})	999.55 ± 289.76	778.82 ± 383.44	767.50 ± 233.61	0.1179
Lactic acid (mg dL^{-1})	73.45 ± 11.11	71.09 ± 16.75	69.84 ± 12.67	0.7271
Total protein (g dL^{-1})	3.77 ± 0.33	3.85 ±0.38	3.70 ± 0.35	0.4358
ALB (g dL^{-1})	1.17 ± 0.11	1.20 ± 0.11	1.13 ± 0.11	0.1760
ALB/GLOB	0.45 ± 0.01	0.45 ± 0.02	0.44 ± 0.02	0.1916
Serum enzymes				
AST (U L^{-1})	64.28 ± 68.24	45.00 ± 53.21	55.61 ± 44.85	0.5884
ALT (U L^{-1})	8.28 ± 7.91	6.33 ± 3.09	6.67 ± 2.91	0.4894
ALP (U L^{-1})	137.28 ± 47.69	105.56 ± 46.02	107.11 ± 40.02	0.0652
CK (U L^{-1})	5076.28 ± 5687.75	3606.50 ± 6691.27	5471.22 ± 5167.40	0.6082
LDH (U L^{-1})	1064.22 ± 1225.05	717.22 ± 1102.87	992.78 ± 707.11	0.5727
Serum electrolytes				
Ca total (mg dL^{-1})	16.85 ± 0.93[a]	16.01 ± 0.86[b]	15.54 ± 0.88[b]	0.0002
Ca^{2+} (mg dL^{-1})	14.51 ± 0.99[a]	13.63 ± 0.96[b]	13.17 ± 0.95[b]	0.0005

P (mg dL^{-1})	11.71 ±1.03	11.66 ± 1.35	11.25 ± 1.13	0.4474
Mg (mg dL^{-1})	3.39 ± 0.30	3.46 ± 0.63	3.26 ± 0.32	0.3820
Fe (ug dL^{-1})	209.44 ± 77.79^{a}	167.61 ± 29.32^{b}	138.22 ± 23.98^{b}	0.0004
Na^{+} (mEq L^{-1})	157.83 ± 2.04	158.61 ± 3.60	158.83 ± 4.37	0.6649
K^{+} (mEq L^{-1})	2.58 ± 0.71^{a}	1.92 ± 0.43^{b}	1.79 ± 0.26^{b}	0.0001
Cl (mEq L^{-1})	128.73 ± 2.99	129.04 ± 4.66	130.23 ± 3.59	0.4655
Ca * P	170.28 ± 22.17^{a}	159.17 ± 24.04^{ab}	148.72 ± 22.93^{b}	0.0258
Na / K	65.33 ± 17.27^{a}	85.94 ± 15.49^{b}	90.50 ± 12.50^{b}	0.0001

Data are given as the mean (n = 18) ± SD. Different letters indicate significant difference ($p \leq 0.05$) between treatments. HDL, High–Density Lipoprotein; TG, triglycerides; ALB, albumin; GLOB, globulin; AST, aspartate aminotransferase; ALT, alanine transaminase; ALP, alkaline phosphatase; CK, creatine kinase; LDH, lactate dehydrogenase; Ca total, total calcium; Ca^{2+}, calcium ion; P, inorganic phosphorus; Mg, magnesium; Fe, iron; Na^{+}, sodium; K^{+}, potassium; Cl, chloride.

3.3.5 Activity of digestive enzymes

The specific activity of total alkaline proteases, trypsin, chymotrypsin, leucine aminopeptidase, α–amylase and bile salt–activated lipase are shown in Figure 3.2. The activity of pancreatic and intestinal proteases decreased in fish fed SCP diets. Specifically, total alkaline proteases, trypsin, chymotrypsin and leucine aminopeptidase activities were significantly higher in fish fed the SCP0 diet compared to fish fed SCP10 and SCP20 diets ($p = 0.0011$, $p = 0.009$, $p = 0.0034$ and $p = 0.0021$ respectively). No differences were detected in the activity of both α–amylase and bile salt–activated lipase between the dietary treatments.

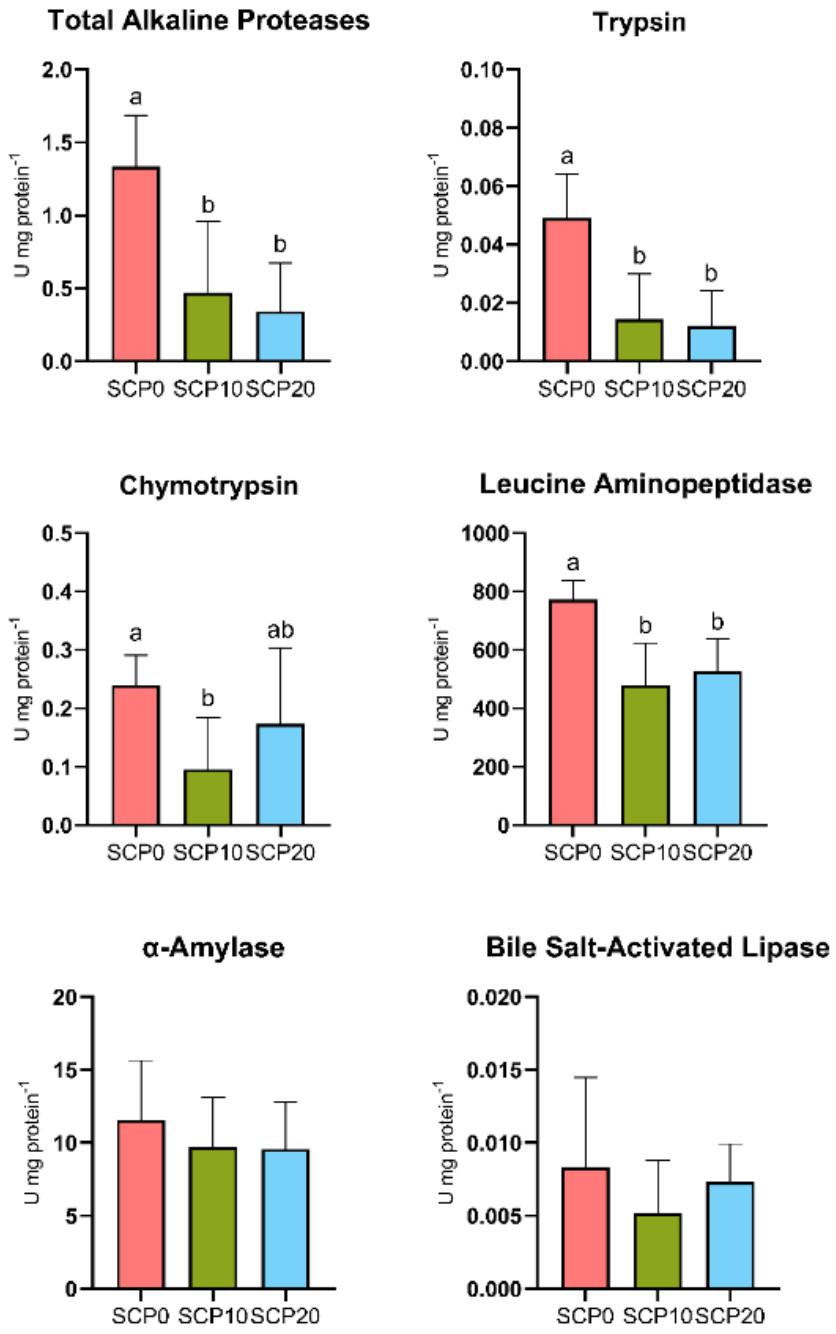

Figure 3.2 Digestive enzymes activity of flathead grey mullet (*Mugil cephalus*) fed experimental diets after 113 days. Enzymes activity was quantified on the whole stomach and intestine on 2 independent samples per tank (6 samples per diet). Different letters indicate significant difference ($p \leq 0.05$) between treatments.

3.3.6 Gut bacterial community profiles

Gut bacterial community profiles at phylum and at genus level are shown in Figure 3.3. Because the variability of the samples was high even within the same group, data were adjusted based on the distribution of the alpha diversity. As shown in Table 3.5, after parametric t–test between the groups, some significant differences appeared in the composition of gut microbiota. At the phylum level, gut microbiota was dominated by Fusobacteria in fish fed the SCP0 diet (51.1 ± 24.1 %) while the most abundant phylum was Proteobacteria in fish fed SCP10 (67.3 ± 16.6 %) and SCP20 (53.2 ± 20.3 %) diets. Firmicutes was present at a much lower relative abundance in all groups, and it was significantly higher in fish from the SCP10 group compared to their congeners fed the SCP0 diet (p = 0.0134), and almost significantly higher in fish fed SCP20 compared to SCP0 (p = 0.0852), but not between the groups fed both SCP diets (p = 0.2465). Samples 2_SCP10, 3_SCP10 and 6_SCP10, and 2_SCP20, 3_SCP20, 8_SCP20, and 9_SCP20 included to a greater or lesser amount Actinobacteria, which is the corresponding phylum for *C. glutamicum* (data not shown). For some samples this phylum was even a dominant as can be expected. The differences observed at the phylum level were maintained at the genus level, mainly due to the differences in the relative abundance of *Cetobacterium* spp. This genus appears to be more abundant in fish fed the diet without inclusion of bacterial SCP. In particular, as shown in Table 3.5, its abundance is significantly higher in fish fed SCP0 compared to SCP10 (p = 0.0055) and in fish fed SCP10 compared to SCP20 (p = 0.0382) and almost significantly higher in fish fed SCP0 compared to SCP20 (p = 0.0743). Other statistically significant differences observed at the genus level included *Corynebacterium* spp., which is higher in fish fed the SCP20 diet compared to those from the SCP0 group (p = 0.0222) and almost significantly higher in fish fed the SCP10 diet compared to the SCP0 group (p = 0.0991). Furthermore, *Streptococcus* spp. was statistically higher in fish fed the SCP10 diet compared to the control group (SCP0 diet) (p = 0.0502) and in

fish fed the SCP20 diet compared to the SCP0 group ($p = 0.0432$). No significant differences were observed in the alpha diversity between fish fed different diets for the three diversity indices considered (i.e. Shannon, Simpson and Fisher diversity) (Figure 3.4). The gut microbiota variations between samples (beta–diversity) were plotted on a two–dimensional non-metric multidimensional scaling (NMDS) plot based on Bray–Curtis values. According to the findings, groups showed a significant variation in terms of overall gut microbiota composition (PERMANOVA, $p = 0.012$) (Figure 3.5).

Table 3.5 Table showing the relative abundance of bacteria taxa at phylum and genus level found in the gut of flathead grey mullet (*Mugil cephalus*) fed experimental diets.

Taxonomy	Relative abundance (% ± SD)			*p* value		
	SCP0	SCP10	SCP20	SCP0 – SCP10	SCP0 – SCP20	SCP10 – SCP20
Phylum level						
Fusobacteria	51.1 ± 24.1	4.0 ± 4.3	25.5 ± 23.1	0.0055	0.0743	0.0382
Proteobacteria	34.1 ± 24.1	67.3 ± 16.6	53.2 ± 20.3	0.0100	0.0740	0.2064
Firmicutes	1.2 ± 2.0	6.7 ± 3.0	4.3 ± 0.3	0.0134	0.0852	0.2465
Genus level						
Cetobacterium	51.1 ± 24.1	4.0 ± 4.3	25.5 ± 23.1	0.0055	0.0743	0.0382
Vibrionaceae_ unclassified	5.5 ± 7.0	0.0 ± 0.1	14.0 ± 19.8	0.1152	0.2902	0.0851
Aeromonadaceae_ unclassified	12.7 ± 14.2	0.5 ± 0.3	3.3 ± 4.7	0.0901	0.1833	0.1311
Corynebacterium	0.2 ± 0.5	9.4 ± 9.6	4.8 ± 4.4	0.0991	0.0222	0.3682

Rhodobacter	0.3 ± 0.5	0.9 ± 1.0	2.1 ± 3.6	0.2621	0.0672	0.3281
Chlamydiaceae_ unclassified	0.2 ± 0.3	1.4 ± 1.6	0.9 ± 0.9	0.2182	0.0961	0.5552
Streptococcus	0.1 ± 0.2	1.4 ± 0.7	0.9 ± 0.4	0.0502	0.0432	0.2453
Lactobacillus	0.1 ± 0.2	0.6 ± 0.4	0.2 ± 0.3	0.0734	0.4322	0.1433
Faecalibacterium	0.0 ± 0.0	0.5 ± 0.4	0.4 ± 0.4	0.0901	0.0573	0.6929
Propionibacterium	0.1 ± 0.2	0.4 ± 0.2	0.4 ± 0.5	0.0951	0.2365	0.9761

Only taxa presenting a p value ≤ 0.1 (after parametric t–test) in at least one group examined were included. Differences were explored through parametric t–test among three groups, SCP0 – SCP10, SCP0 – SCP20 and SCP10 – SCP20. Data are presented as % of relative abundance ± SD.

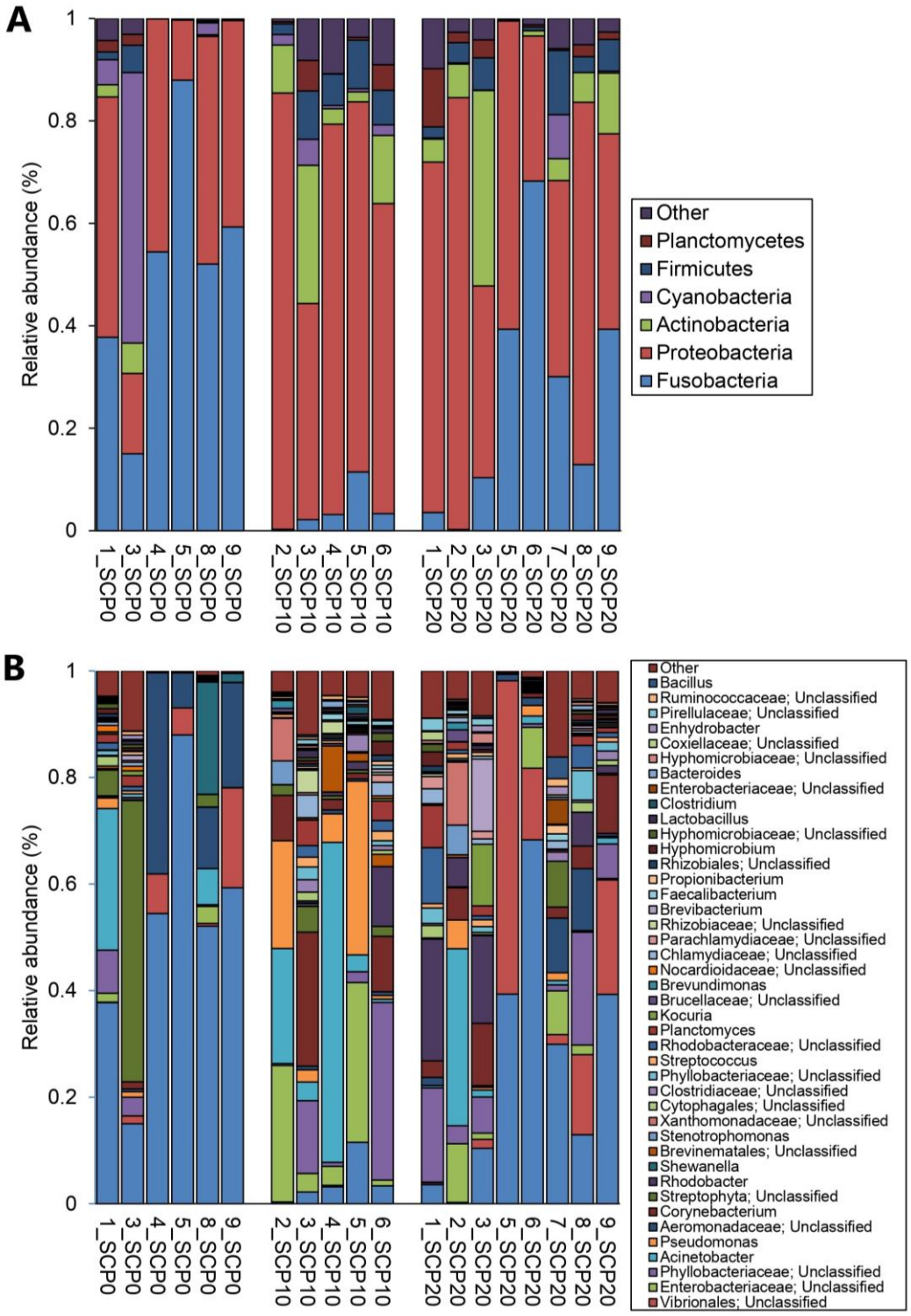

Figure 3.3 Relative abundance (%) of gut microbiota composition at phylum (A) and genus (B) levels measured on flathead grey mullet (*Mugil cephalus*) fed experimental diets after 113 days. Microbiota analysis were performed individually on a sample of hindgut content for each fish. Only taxa with relative abundance > 0.1 % in at least 1 sample were included.

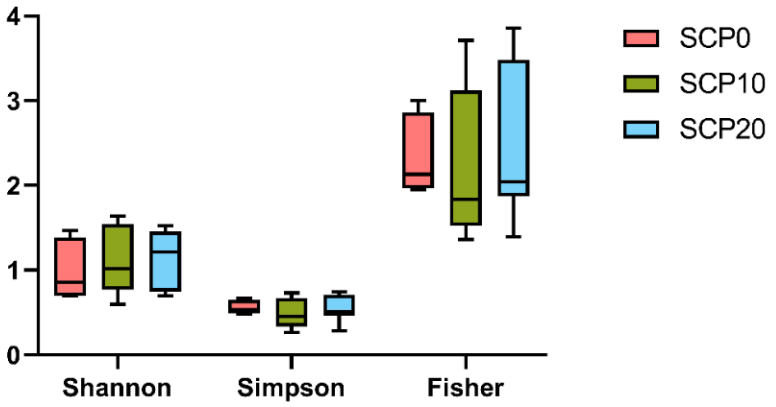

Figure 3.4 Boxplot showing alpha diversity in the flathead grey mullet (*Mugil cephalus*) gut microbiota calculated with different indexes (i.e. Shannon diversity, Simpson diversity and Fisher diversity). No significant differences ($p \leq 0.05$) were obtained after parametric t–test.

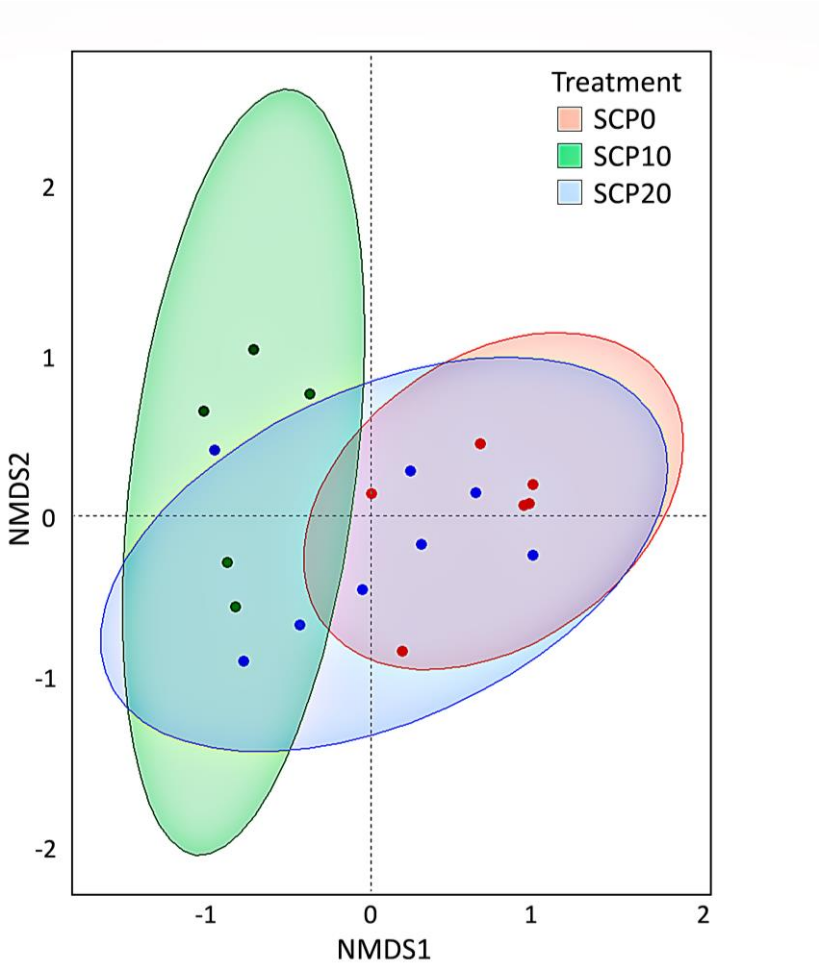

Figure 3.5 Two–dimension non–metric multidimensional scaling (NMDS) plot based on Bray–Curtis distance of flathead grey mullet (*Mugil cephalus*) gut microbiota fed experimental diets

after 113 days. Red dots are for gut microbiota communities found in fish fed SCP0, green dots for communities in fish fed SCP10 and blue dots for communities in fish fed SCP20. A significant separation among groups was observed ((p value = 0.012; Permutational Multivariate Analysis of Variance (PERMANOVA) (Adonis2)). Data comply with the assumption related to homogeneity of multivariate dispersion (p value = 0.3544).

3.4 Discussion

The present study sought to define the capacity of flathead grey mullet, reared in captive conditions, in utilising a SCP ingredient derived from *C. glutamicum* in substitution of SBM, FM and PM. By using diets with equivalent levels of protein, lipid and digestible energy but different levels of bacterial SCP inclusion, a clear effect was seen at the end of the experiment. In particular, fish fed the SCP diets presented significantly lower growth performances, and feed and protein utilisation in comparison with fish fed control diet. To the best of our knowledge, there is no literature to date investigating the effects of such an ingredient in the diet of flathead grey mullet. Despite of this, the findings of this trial were quite unexpected, since representatives from the family mugilidae are able to utilise a variety of ingredients in captive conditions such as plant meals (Liu et al., 2021; Gisbert et al., 2016; Luzzana et al., 2005), algae (Rosas et al., 2019) and yeast (Luzzana et al., 2005; Wassef et al., 2001). In addition, considering the assumption that wild fish represent a reference for the development of formulated diets, and it has been reported previously that bacteria account for 15–30 % of the organic carbon in the stomach content of this species (Crosetti and Blaber, 2015; Moriarty, 1976), the results from the current nutritional study were puzzling.

In our experiment, feed intake was higher in fish fed diets containing bacterial SCP inclusion. Published studies have revealed contradictory effects of SCP sources on feed intake in fish (Glencross et al., 2020; Aas et al., 2006). Those that found a positive correlation of SCP

inclusion on feed intake, suggested that the high content of nucleotides (e.g. inosinic acid and inosine) presented in bacterial SCP may act as an attractant enhancing the palatability of the diet (Hossain et al., 2020; Gamboa-Delgado and Márquez-Reyes, 2018). However, our results were in agreement with previous studies conducted on rainbow trout (*Oncorhynchus mykiss*) (Zamani et al., 2020; Perera et al., 1995), Atlantic salmon (*Salmo salar*) (Marit Berge et al., 2005) and Pacific white shrimp (*Penaeus vannamei*) (Hamidoghli et al., 2019), in which inclusion of dietary bacterial SCP did not affect or enhance feed intake but lead to a poorer growth performance. This suggests that in our case the issue with SCP from *C. glutamicum* was not due to its palatability, but rather to the utilisation potential of the ingredient. Across a range of SCP sources, bacteria are generally recognised as relatively digestible, and their inclusion has been successfully demonstrated in several major finfish species of commercial interest without affecting growth or fish health (Glencross et al., 2020; Jones et al., 2020). In particular, the same SCP ingredient was tested by Marchi et al. (2023) on gilthead seabream, reporting no negative effects on the growth performance and an interesting probiotic effect on the gut microbiota, even at a high substitution rate. However, still some inconsistency appeared in the data being perceived as a limiting factor for their broader application (Glencross et al., 2023). Some works suggest that the thickness of the cell–wall may be a constraint for the digestibility of a number of SCP sources (Teuling et al., 2019; Ritala et al., 2017; Tibbetts et al., 2017; Yamada and Sgarbieri, 2005; Ugalde and Castrillo, 2002). *C. glutamicum* is gram–positive bacteria with a thick cell wall made out of a complex structure of peptidoglycan and arabinogalactan (Cheng et al., 2021; Eggeling and Sahm, 2001). In general, finfish can cope well with cell–wall lysis by producing acidic secretion of HCl and thus decreasing the stomach pH to values below 3 (Lobel, 1981). By observing the digestive system of grey mullet, the species presented a stomach with an alkaline pH (approx. 8.5), the same as the intestine (Payne, 1978). In the absence of a specific acid secretion of HCl in the stomach, the species has evolved relying on a mechanical trituration of feed particles carried out in the pyloric stomach, called the

gizzard (Crosetti and Blaber, 2015; Lobel, 1981; Payne, 1978). A gizzard–like pyloric stomach is frequent in those species that eat detritus and flocculant benthic biofilm (e.g. algae, bacteria and non–living organic matter) such as *Plotosus lineatus* (Bowen, 2022), *Heterotis niloticus* (Agbugui et al., 2021), *Salmo stomachicus* (Wilson and Castro, 2010) and *Dorosoma cepedianum* (Watson et al., 2003). The wall of the gizzard is made of rings of smooth, non–striated muscle fibres and often contains sand or small debris to assist in grinding plant and fibrous material (Crosetti and Blaber, 2015). A work conducted by Sánchez et al. (1993) hypothesised that the poor growth that occurred in grey mullet fed a microalgae–based diet may have been due to the lack of access to sand in captive conditions, therefore preventing the development of a functional gizzard. To the best of our knowledge, no other scientific publications dealt with the possible effects of a non–functional gizzard on diet digestion in finfish species. On the other hand, this phenomena is well described in poultry nutrition in which it is known that the access to structural components such as whole cereals or coarse fibres, improve growth and nutrient absorption in chickens by enhancing gizzard functionality (Idan et al., 2023; Svihus, 2014). Flathead grey mullet used in this experiment were raised in a captive condition in fiberglass tanks, therefore having no access to sand or coarse material and possibly impairing the proper development of a functional gizzard. Thus, the lower growth performance observed in fish fed SCP may be related to poor nutrient digestion caused by an incomplete lysis of the *C. glutamicum* cell–wall, as described previously (Sánchez et al., 1993). The hypothesis of a deficiency in nutrient digestion appears to be reasonably acceptable after a look to the digestive enzyme activity. SCP inclusions lead to a reduction in the specific activity of the pancreatic and intestinal proteases (e.g. trypsin, chymotrypsin and leucine aminopeptidase), which is well correlated with an impaired protein absorption and low growth performance (Gisbert et al., 2016; Silva et al., 2010). A similar reduction in the activity of digestive proteases has been observed in carnivorous fish when fed too high inclusion of plant ingredients and associated with poor growth and histopathological changes (e.g. enteritis) (Willora et al., 2022; Chikwati et al.,

2013; Rodiles et al., 2012; Silva et al., 2010). How was suggested in a work conducted by Pujante et al. (2016) on thick–lipped grey mullet (*Chelon labrosus*) the enzymatic digestion of proteins mainly takes place in the anterior intestine by the action of alkaline proteases. A functional evidence for this is evinced from the lack of acid proteases activity (e.g. pepsin) in flathead grey mullet (Gisbert et al., 2016). Alkaline proteases are most active at a pH range from 8 to 10 (Sanatan et al., 2013; Liew et al., 2013; Nasri et al., 2011). Among them, trypsin activates other enzymes initially generated as inactive zymogens, such as chymotrypsin and therefore is considered a key enzyme in protein digestion (Asgeirsson et al., 1989). Trypsin and chymotrypsin catalyse the hydrolysis of the bonds of peptides into smaller dipeptides and oligopeptides which are split by the action of leucine aminopeptidase into free amino acids that can be absorbed into the enterocytes (Willora et al., 2022). In marine finfish with a functional gastric stomach, the acidity of the chyme entering from the stomach into anterior intestine is buffered by the secretion of HCO_3 from the pancreas, which create the optimal pH condition for the activation of the alkaline proteases (Parma et al., 2019; Márquez et al., 2012; Wilson et al., 2002). It is interesting to note that in our trial, we have observed a decreased pH and buffer capacity in the diets containing the SCP from *C. glutamicum*. These findings are in line with a previous study conducted by Prodanovic and Sredanovic (2005) reporting that the buffer capacity of yeast SCP is significantly lower than that of SBM (9.15 and 21.15, respectively), as well as the pH (5.84 and 6.58, respectively) probably due to the higher content of nucleic acids, as suggested in a work conducted by Ritala et al. (2017). We do not know if this could have contributed to an inhibitory effect on the activity of alkaline proteases; however, it is likely that in our experiment the intestinal pH of grey mullet decreased as a function of bacterial SCP inclusion. The activity of the alkaline proteases is mediated by a complex cascade process involving pH, temperature and the concentration of ions. In fish fed SCP diets, we have also found significantly lower concentrations of ions of calcium (Ca^{2+}) and potassium (K^{+}) in the blood. Ca^{2+} is recognised as a trypsin activator, since its presence strengthens

the interactions inside protein molecules and enhances thermal stabilisation of this pancreatic enzyme (Bezerra et al., 2005), while K^+ has an effect on the activation of leucine aminopeptidase (Liew et al., 2013). No differences were observed in both α–amylase and bile salt–activated lipase activity between fish fed the different diets. If compared to carnivorous species, the higher α–amylase activity found in flathead grey mullet, broadly hints that carbohydrates may be preferred as a protein sparing substrate as it has been previously postulated by several authors (Solovyev and Gisbert, 2022; Calixto da Silva et al., 2020; Pujante et al., 2016).

If we consider the other performance indicators, we observed that fish fed the diet with the highest inclusion of SCP also showed a significantly lower Fulton's condition factor (K). This length–weight relationship can be correlated with the wellness conditions of the fish, and its fluctuations with the interaction between feeding condition and energy reserves (Datta et al., 2013; Robinson et al., 2008; Yulianto et al., 2020). Because of the issue with nutrient digestion in fish fed SCP diets, flathead grey mullet belonging to these groups appeared underfed with lower energy reserves and therefore with a lower flesh / skeleton ratio, which also explain the increased content of ash in the body of this group of fish. In terms of nutritional indices, which are used for comparing the nutritional values of different ingredient sources, they appeared to be significantly decreased by SCP inclusion, which is reflected by the feed conversion rate and the growth. On the other hand, gross protein and lipid efficiency (32.3 ± 1.7 and 75.4 ± 9.6 %, respectively) of fish fed the SBM diet were encouraging in order to further design compound feeds for this low trophic species. With a focus on the protein utilisation, grey mullet fed a diet with only 3 % fishmeal, were able to convert almost 1/3 of the dietary protein into edible fillet. This ratio appears similar to that found for gilthead seabream (Busti et al., 2020a) and rainbow trout (Pelusio et al., 2020) and it is somewhat higher to previous findings with European seabass (Bonvini et al., 2018; Parma et al.,

2020; Pelusio et al., 2022). These results may indicate that is possible to further decrease the level of fishmeal in aquafeeds for this species.

As mentioned previously, the SCP additive contains a high level of nucleic acids, mainly RNAs (Ritala et al. 2017). In our trial, we have seen an increasing concentration of urea in the blood of the fish fed SCP diets. This phenomena has been described in previous works and is ascribed to the catabolism of pyrimidines, which are presents in nucleic acid molecules (Marchi et al., 2023; Lin et al., 2022; Agboola et al., 2021). High concentrations of ammonia metabolites in the blood may also interfere with protein, fat and carbohydrate metabolism (Rumsey et al., 1992), which may also explain the lower performance of flathead grey mullet fed bacterial SCP diets in comparison to the control group. In our trial, the blood glucose concentration found in the fish appears higher than values found in literature for the same species (Calixto da Silva et al., 2020; Wanshu, 1992). Despite this, the significantly lower concentration of blood glucose in fish fed SCP diets is likely to support the hypothesis of there being a problem with the digestion of this ingredient rather than a pathological condition. Starvation or underfeeding conditions are generally associated with a decreased blood glucose level in many fish species (Polakof et al., 2012). The enzyme activity of the marker enzymes in the blood associated to liver damage (aspartate transaminase and alanine transaminase) and tissue damage (creatine kinase and lactate dehydrogenase) did not show any difference among the fish groups in our trial. Interestingly, the activity of the enzyme alkaline phosphatase showed a trend and appeared to be higher in fish fed the diet with no inclusion of SCP. This difference may be related to the higher nutrient absorption in this group of fish, since the enzyme is involved in the absorption and transport of lipid and carbohydrates from the intestine, and its activity is positively correlated with food ingestion and growth rates (Lallès, 2020; Lemieux et al., 1999).

The gut microbiota of fish plays an important role in regulating nutrient digestion, immune responses, disease resistance, and the colonisation of potential pathogens (Xie et al., 2021; Busti et al., 2020b; Pelusio et al., 2020; Gu et al., 2017; Parma et al., 2016). The composition of bacterial communities is strongly determined by the environment conditions both internal and external such as salinity (Kivistik et al., 2020) and temperature (Sepulveda and Moeller, 2020; Thompson et al., 2017), the immune / health status (Kashinskaya et al. 2019; Parshukov et al. 2019), the host species considered and their dietary components (Kashinskaya et al. 2018). In particular, the composition of flathead grey mullet microbiota in response to different dietary ingredients is a relatively unknown topic. While there is a published study conducted by Le and Wang (2020) aimed at the characterisation of the microbiota from wild flathead grey mullet, information is lacking for fish reared in captive conditions fed formulated diets. Our finding displayed Fusobacteria and Proteobacteria as the most abundant phyla, accounting for more than 70 % of the total variability in all groups. Inclusion of *C. glutamicum* in the diet of flathead grey mullet promoted a shifting between the two phyla, increasing the amount of Proteobacteria over the Fusobacteria (with presence of Actinobacteria expected, particularly in the SCP10 and SCP20 groups). Using as a reference the gut microbiota observed in wild fish, with Proteobacteria and Firmicutes as the most abundant phyla (Le and Wang, 2020), it is interesting to note that this appears more similar to fish fed the SCP diets even though these groups displayed severely reduced growth performances. Proteobacteria are found to be dominant in the gut microbiota of several fish species fed marine–derived ingredients (Gu et al., 2017; Kim et al., 2021). The phylum Firmicutes accounts for only a minor part of the variability of the gut microbiota in all groups, even though it was more abundant in fish fed SCP supplemented diets. This phylum, and also especially the lactic acid bacteria, often dominate the composition of the microbiome of fish fed plant–based diets (Busti et al., 2020b; Pelusio et al., 2020), and it is normally associated with improved nutrient digestion (Marchi et al., 2023; Parma et al., 2020, 2016). In our trial, fish fed the diet without inclusion of SCP displayed

an increased dominance of the phylum Fusobacteria, which is totally represented by the genus *Cetobacterium.* This genus has been found to be dominant in the gut of many freshwater species and its presence is associated with a positive impact on the digestion and metabolic efficiency of the host (Xie et al., 2021). At the genus level, we have seen an increased abundance of *Streptococcus* in fish fed SCP diets. Some species of streptococci, as potential spore forming species, may represent carry–over of bacteria from the SCP feed additive (Karlsen et al. 2022), as some feed components are now shown to bias the interpretation of microbiome analyses by acting as the vectors for introduction of species to the intestinal microbiome of the host. Moreover, several bacterial species within the genus *Streptococcus* spp. have been reported as important pathogens of fish species (Halimi et al., 2020; Ringø et al., 2018). Although without a significant difference, in fish fed SCP diets we have also seen an increasing trend for the family Vibrionaceae, a family associated with important pathogens of fish (Sepúlveda et al., 2022; Zhang et al., 2020). On the other hand, fish fed diets with inclusion of SCP showed in their gut microbiota a decreasing trend in the family Aeromonadaceae, while there was a trend toward increasing in the relative abundances of *Rhodobacter*, *Corynebacterium* and *Lactobacillus*. Different species of *Aeromonas* are described as important fish pathogens (Soto-Dávila et al., 2022; Zmysłowska et al., 2009). *Rhodobacter* and *Lactobacillus* are considered among the most promising probiotics used in aquaculture (Cao et al., 2022; El-Saadony et al., 2021; Ringø et al., 2018). *Corynebacterium*, as expected, was found significantly higher in fish fed SCP diets and the presence of some species has been associated with a probiotic effect (Marchi et al., 2023; Horn et al., 2022). Along these same lines of investigation of probiotics are studies that evince the quorum sensing interactions that bacterial food components can have (Davares et al. 2022). Quorum sensing utilises small molecules like acyl–homoserine lactones and oligopeptides for intercellular signalling. An unrefined product like SCP may include bioactive molecules with potential for interspecies bacterial communication. When using quorum sensing bacteria not only communicate with

members of the same species but may also “eavesdrop” on the “conversation” of other species and modulate their behaviour in response to signal molecules they do not synthesise (Federle et al. 2003). This can have unexpected consequences on diverse assemblages of bacteria from unique hosts, perhaps even some degree of dysbiosis and poor fitness of the microbiota, which could lead to some of the poor performance indicators found in this study. Overall the resulting microbiota data will contribute to improved characterisation of the taxonomic diversity of the gut bacterial community from grey mullet, which is essential for understanding the dynamics of the diet–host–gut–microbiota axis.

Conclusions

These results indicated that flathead grey mullet reared under captive conditions demonstrate incomplete utilisation of a protein source derived from *C. glutamicum* SCP. Probably due to the particular organisation of the digestive system of flathead grey mullet there was an incomplete protein digestion of SCP due to poor lysis of cell–wall components (this being due to lack of an acidic stomach digestion) and also associated with the captive conditions (failure in the development of a functional gizzard). Based on our assessment, the diet had significant negative effects on individual variables in analysis of both digestive enzymes and gut microbiota.

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CHAPTER 4. Non–destructive rainbow trout (*Oncorhynchus mykiss*) freshness estimation by using an affordable open–ended coaxial technique

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Andrea Bertini, Eleonora Iaccheri, Martina Magnani, Anna Badiani, Alessio Bonaldo, Luigi Ragni, Annachiara Berardinelli

Abstract

In the present work, a non–destructive device set up for a rapid and reliable freshness assessment of rainbow trout during 10 days of storage in ice was evaluated. The device was characterised by a Vector Network Analyser interfaced with an open coaxial probe to be placed in contact with the fish eye. The acquisition of the reflected scattering parameter (*S11*), which is the ratio between the amplitude of the reflected and the incident signal, was assessed in the 50 kHz – 3 GHz spectral range. *S11* is composed of a real part and an imaginary part, and both parts were used to predict Quality Index Method (QIM) for freshness evaluations. Partial Least Squares regression (PLS) predictive models of the demerit scores related to fish eye attributes (eye pupil and eye shape) and the day of storage were set up. The main results showed that both the real and imaginary parts of the *S11* decrease as a function of storage time. The combination with multivariate analysis allowed to set up predictive models of the storage time and the demerit scores with R^2 values up to 0.946 (RMSE = 0.88 days) and 0.942 (RMSE = 3.17 demerit scores related to the fish eyes attributes), respectively (external validation). According to our results, the proposed cheap solution appears a useful tool for the freshness assessment of rainbow trout.

4.1 Introduction

Fish products provide consumers with a variety of high–value components, such as essential amino acids, n-3 fatty acids, and important micronutrients. Therefore their consumption, production, and commercialisation are increasing worldwide (Calder, 2018; Chen et al., 2022; Hoffmire et al., 2012; Mohanty et al., 2019). Aquaculture production of rainbow trout (*Oncorhynchus mykiss*) in the EU reached about 210 thousand tons in 2019, being the most valuable freshwater species on the continent (FEAP, 2019). Rainbow trout can be found on European markets all year round, presented in several ways, whole or filleted, fresh or smoked. Whole fresh portion trout is by far the more commercialised, however, fresh fish is highly perishable product with a very short shelf–life. Spoilage activities in fish start immediately after harvesting as a result of the presence of autolytic enzymes, high water activity, low pH, and high content of unsaturated fatty acids, which create the optimal conditions for bacterial growth (Alasalvar et al., 2010; Ghaly et al., 2010; Lougovois, 2005). In particular, autolytic enzymes that remain active after the death of the fish are responsible for several biochemical and physical changes resulting in the protein degradation and lipid oxidation (Ghaly et al., 2010). These processes also involve the formation of volatile organic compounds responsible for off–flavours and the deterioration of sensorial and nutritional quality (Whitfield, 1999). The above cited changes can be partially slowed down by the storage under controlled temperatures (e.g. melting ice) and the application of strict inspection controls (Badiani et al., 2013). According to the European Commission (EC) regulation No 853/2004 (European Parliament, 2004), “Fresh fishery products mean unprocessed fishery products, whether whole or prepared, including products packaged under vacuum or in modified atmosphere, that has not undergone any treatment to ensure preservation other than chilling”. As a result, freshness is one of the major contributors to the quality of fish as well as the major attribute which influence the market value and the consumers’

willingness to buy fresh fish (Freitas, Vaz-Pires and Câmara, 2021). In addition, freshness is associated with important food safety concerns as it is directly linked with several health risks for consumers (Olafsdóttir et al., 1997; Rocculi et al., 2019). Traditional freshness analytical methods are based on quantitative chemical, microbiological and physical techniques aimed at assessing different parameters recognised as indicators of fish post–mortem changes. Among chemical indicators, trimethylamine (TMA), total volatile basic nitrogen (TVB–N), free fatty acids (FFA), thiobarbituric acid (TBA) and K value generated from the autolytic decomposition of adenosine triphosphate (ATP) are well correlated with fish spoilage. Total viable bacteria count (TVC) and the alterations in colour and texture are respectively microbial and physical indicators of fish spoilage. Generally, these methods are time–consuming, expensive, and destructive (Prabhakar et al., 2020).

Sensory evaluation is a commonly used method for the assessment of fish freshness. In European Union, its application in fish inspection services is defined in the European Commission (EC) Regulation No 2406/96 (European Parliament, 1996) (García et al., 2017; Luten and Martinsdottir, 1997). This regulation lays down a freshness scheme, based on sensory evaluation, to distinguish fish products freshness during commercialisation into three categories (i.e. EXTRA, A and B). More precise and worldwide recognised is the quality index method (QIM) sensory evaluation (Freitas, Vaz-Pires and Câmara, 2021). This method is based on a scheme developed by the Tasmanian Food Research Unit (TFRU) (Bremner, 1985) and is specifically designed for each fish species. Several QIM schemes have been developed for the main commercial fish species including Atlantic salmon (Sveinsdottir et al., 2003), cod (Cardenas Bonilla et al., 2007) and sea bream (Sant'Ana et al., 2011). This method assigns demerit scores (ranging from 0 for fresh products to 2 or 3 according to freshness) to several spoilage–related attributes such as colour, smell and texture changing of a particular fish species. The sum of all scores gives the Quality

Index demerit score, the lower the score the fresher the fish. Developing a QIM for a particular fish species involves the selection of the more appropriate attributes in order to observe a linear increase in the Quality Index demerit score with the storage time. The application of this method is inexpensive, non–destructive and rapid, but it requires a trained panel (Bernardi et al., 2013; Freitas et al., 2021).

The need for a rapid, cheap, and non–destructive method for freshness evaluation of fish products is pushing research to find alternatives. The state of the art of indirect and non–invasive techniques applied to fish freshness assessment mainly covers e–sensing techniques, optical spectroscopic solutions, and nuclear magnetic resonance (NMR) (Franceschelli et al., 2021). These approaches are commonly combined with statistical algorithms able to extrapolate qualitative and quantitative models describing the variability occurring in the fish during storage. E–sensing techniques, including electronic noses (El Barbri et al., 2008) and tongues (Ruiz-Rico et al., 2013), colorimetric sensor array (Morsy et al., 2016) and computer vision systems (Rocculi et al., 2019), are deeply investigated because of their ability to simulate the human sensorial perceptiveness. In addition, the physicochemical and structural properties of the fish samples have been researched by modelling information coming from Fluorescence (Omwange et al., 2020), Infrared (Tito et al., 2012), Hyperspectral Imaging (Shao et al., 2023) and Raman spectroscopy (Herrero, 2008). Indirect techniques cover also those based on fish dielectric properties explored by a few works conducted by using the time domain reflectometry technique (Kent et al., 2004), impedance analysers (Wang et al., 2008), commercial freshness meters (Vaz-Pires et al., 2008) and open–ended coaxial probe (Iaccheri et al., 2022). As well known, dielectric properties such as dielectric constant and loss factor change according to the physical and chemical characteristics of the food product, being mainly influenced by the moisture content (Ragni et al., 2016, 2017). According to Iaccheri et al. (2022), dielectric constant and loss factor of anchovy eyes, acquired in the 250 to

2400 MHz spectral range, decrease as a function of storage time. Authors attribute this behaviour, to a reduction of the moisture on the surface of the fish eyes and to other modifications occurring in fish post–mortem. In addition, the observed slight decrease in the dielectric constant in relation to frequencies evidences the presence of γ–dispersion related to dielectric relaxation of hydration (Gabriel et al., 1983). In this way, eye dehydration occurring during storage may be monitored by using dielectric spectroscopy and correlated with the freshness of the fish.

This study represents a preliminary step in the exploration of rainbow trout (*Oncorhynchus mykiss*) freshness through the application of a rapid and cheap spectroscopic technique based on the fish's dielectric properties. Measurements of real and imaginary parts of the reflection (*S11* scattering parameter) acquired on fish eye at different storage times were combined with those obtained through the application of a specific QIM scheme by a trained sensory panel. Outcomes from this study can be useful for the setting up of a novel rapid and non–destructive method for fish freshness evaluation, which will support producers and retailers in the busy commercial setting.

4.2 Materials and methods

4.2.1 Storage condition and experimental plan

Fresh–farmed rainbow trout (*Oncorhynchus mykiss*) from a commercial producer located in the north of Italy (Trentino province, Madonna di Campiglio area, 46° 14' 00'' N, 46° 14' 00'' E) were delivered to the Laboratory of Fish Quality Evaluation at the University of Bologna (Cesenatico, Italy) for three distinct storage trials between April and June 2021, following the procedure described in detail by Luten et al. (2006) in "Seafood Research from Fish to Dish – Quality, Safety and Processing of Wild and Farmed Fish". The fish had been slaughtered by ice–killing (hypothermia), packed with flaked ice into polystyrene boxes and delivered to the

laboratory within 3 – 4 h from harvesting. Upon arrival, the fish were randomly divided into batches of 8 individuals, covered with ice, and stored gutted in a refrigerator set at 0 ± 1 °C. On the day of analysis, one batch was removed from the refrigerator. Each fish was coded, its mass (g) and its length (mm) were measured and then placed randomly on white trays on the evaluation desks. The first two storage trials were conducted as preliminary tests with the aim of setting up the Quality Index scheme. In detail, for each storage trial, a total of 80 trout were considered and 8 fish for each day of storage (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days) were assessed. For the third storage trial, a total of 48 trout were considered and 8 fish for each day of storage (0, 2, 4, 6, 8, and 10 days) were assessed. At each considered day of storage, sensory evaluation, Torrymeter assessment and spectral acquisitions were carried out. The length of storage was chosen based on several research works, evidencing that, based on microbiological and sensorial data, the endpoint of whole rainbow trout edibility, whenever stored in ice, is between 9 and 12 days (Rezaei et al., 2008; Tavakoli et al., 2018). Moreover, as laid down by the trout producer, a maximum shelf–life of 7 days from harvesting is to be considered.

4.2.2 Sensory evaluation

Sensory evaluation was performed by a trained panel of four assessors (sex ratio 1:1, 25:45 age range) according to the Quality Index Method (QIM). This sensory approach is based on the freshness assessment scheme proposed for rainbow trout by (Grigorakis et al., 2018) and previously developed for Atlantic salmon by Sveinsdottir et al. (2003). Some modifications to the original scheme were implemented during the two preliminary storage trials, to best match the spoilage features occurring in the rainbow trout used in this experiment. In the way it is structured, and provided that fish is continuously kept under melting ice, the QIM demerit score is linearly related to the storage time. Modifications were addressed to those attributes that least fit the linear model in terms of R^2. In detail, the attribute “texture”, composed of two description points in the

original scheme, was increased to four. The attribute “abdomen odour” was removed as redundant, because the panellists were not able to discriminate any differences from the general “skin odour.” In addition, some minor adjustments were applied to the description of the attributes, to better match the external features of the rainbow trout used in this experiment. The so–developed QIM scheme is shown in Table 4.1. Sensory evaluation was conducted in a laboratory that complies with the general criteria for the design of sensory analysis rooms proposed by the International Standard Organisation (ISO) (ISO 8589:2014, Sensory analysis – General guidance for the design of test rooms). Evaluations were conducted at room temperature under a white–fluorescent light (Philips Master TL–D 36 W / 65, average intensity of 1200 lux on the working area, colour temperature of 6500 K). Assessors kept a one–meter distance from each other while working.

4.2.3 Torrymeter freshness assessment

Changes in chemical and physical properties occurring during storage as a consequence of spoilage were assessed by using a commercial Fish Freshness Meter “295 – Torrymeter” (Distell, Fauldhouse, West Lothian, UK), according to the procedure described by Lougovois, Kyranas and Kyrana (2003). By means of two pairs of concentrically arranged electrodes, the meter converts the electronically measured phase angle between the current (A) and voltage (V) on a 0 – 18 scale that increases with fish freshness. Before sensory evaluation, two measurements were taken on the left and the right side of the fish by applying the Fish Freshness Meter probe directly in contact with the portion of the skin just behind the gill cover, above the lateral line. Before each measurement, the electrodes were cleaned to remove debris (scales and mucus) that could compromise the measurement. The meter readings were read on the digital display.

Table 4.1 Quality index method scheme developed for rainbow trout (*Oncorhynchus mykiss*).

Quality parameter	Description	Demerit
Skin		
Colour	Pearl-shiny, green glass spots on the back	0
	Less shiny, slight metallic-pink discoloration on the side	1
	Greenish colour spread, pink-orange discoloration on the side and gill cover	2
	Green-grey discoloration and attenuation of the pink-orange nuance on the lateral side and gill cover	3
Mucus	Clear, shiny, and abundant	0
	Less clear, more viscous, and less abundant	1
	Milky, coagulated, and scarce	2
Odour	Fresh grass, river weed, cucumber	0
	Neutral to metallic smell	1
	Metallic, damp cellar and slight musty smell	2
	Oxidised blood smell, sour and decaying vegetation	3
Texture	Rigor	0
	Post rigor, firm, elastic	1
	Less firm, less elastic	2
	Soft	3
Abdomen		
Colour	Pure white / silver flecks, solid	0
	Whitish / greyish discoloration, slight longitudinal depression	1

	Yellowish / dark grey discoloration, evident longitudinal depression	2
Eyes		
Pupil	Limpid, black with metal shiny	0
	Lightly opaque, greyish discoloration	1
	Mat, grey	2
Shape	Convex shape	0
	Flat	1
Gills		
Colour	Slightly sunken	2
	Bright red, burgundy (Pantone 19-1617)	0
	Marsala red (Pantone 18-1438) and apical discoloration	1
	Totally discoloured, grey-yellowish colour	2
Mucus	Glossy, clear	0
	Filamentous, milky	1
	Yellow, clotted, and filamentous	2
Odour	Fresh grass, river weed	0
	Neutral to metallic smell	1
	Oxidised blood, fish bowels, mushroom smell	2
	Rotten, sulphurous, decaying vegetation	3
Total demerit points		0 – 24

4.2.4 Spectral acquisition

Spectral acquisitions were conducted immediately after the sensory evaluation procedure by placing a coaxial probe in contact with the fish eyes. For each fish, both right and left eyes were acquired. The layout of the instrumental chain is shown in Figure 4.1.

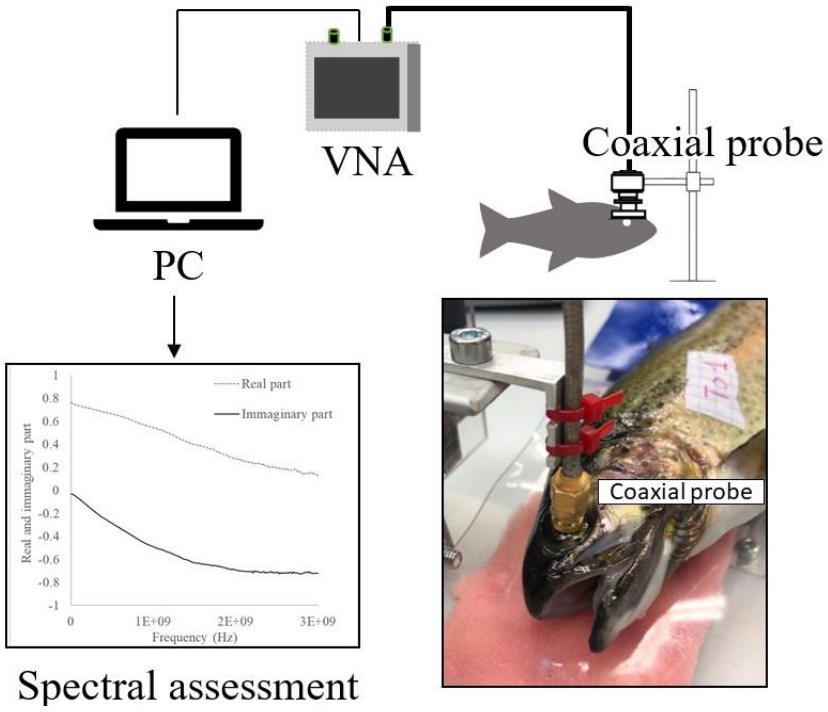

Figure 4.1 Setup of the instrumental chain with typical signal output and a picture of the contact between fish eye and the coaxial probe.

The Vector Network Analyser (VNA Nano V2, HCXQS in collaboration with OwOComm, China) is interfaced with an open coaxial probe connected to the instrument via port CH0 through a semi–rigid coaxial cable for high frequencies (50 Ohm). The cable instrument and probe were assembled using specially made supports. The open coaxial probe was obtained starting from a female SMA connector, for the electronic boards, by turning and subsequent surface gilding of the

brass. The VNA selected for the present research is quite a cheap portable instrument available on the market (about 100 €). Nano VNA V2 is a two–port vector network analyser, CH0 and CH1 (reflection/transmission), designed for frequencies from 50 kHz to 3 GHz. Antenna analysers, such as Nano VNA, are designed to measure impedance (in ohms) and "standing wave ratio" (SWR). In a transmission line, the so–called VSWR (Voltage Standing Wave Ratio), also referred to simply as SWR is a parameter that indicates the ratio between the maximum value and the minimum value of the magnitude of the voltage along the line. At a fixed frequency, SWR is a measure of the impedance mismatch between the transmission line and its load. The higher the SWR, the greater the mismatch. The minimum value of the SWR, which corresponds to the condition of perfect adaptation for which the impedances of the transmission line and the load are equal to the absence of a reflected wave, is equal to 1. Finite values greater than 1 indicate a mismatch with the presence of a standing wave due to partial reflection from the load. SWR, therefore, is a measure of the impedance mismatch between the antenna and the receiver. Reflected spectra are reported as the input port voltage reflection coefficient (*S11*). The relationship between (*S11*) and SWR is previously reported by Franceschelli et al. (2023) and it is given by the following formulae:

$$SWR = \frac{1 + |S11|}{1 - |S11|} \tag{12}$$

$$|S11| = \sqrt{(Re(S11))^2 + (Im(S11))^2} \tag{13}$$

The scattering parameter *S11* is composed of real (Re(*S11*)) and imaginary (Im(*S11*)) parts. In the present research, only the reflection port, CH0, was used and the scattering parameters *S11* (reflection), real and imaginary, were measured. The assembled instrumental chain was calibrated to remove the three typical systematic errors in one–port measurements: directivity, source match, and reflection tracking (Chen et al., 2004). For the purpose, its own commercial calibration kit

(SMA-type 50 Ohm, HCXQS in collaboration with OwOComm, China) was used, accounting for open, short, and load calibration acquisitions and corrections. The spectra were acquired with the Nano-VNA-saver software (GNU, General Public License, version 0.3.8, Rune Broberg) for the whole frequency range 50kHz-3GHz, averaging three consecutive acquisitions of 301 spectral points.

4.2.5 Statistical analysis

Significant differences in mass (g), length (mm), QIM, and Torrymeter scores acquired during the third storage trial, were explored between means during the storage by using a one–way analysis of variance (ANOVA) and Tukey's Post hoc test ($p \leq 0.05$). The assumptions related to data normal distribution and homogeneity of variances were explored through Anderson Darling's test and Levene's test, respectively. The coefficient of determination R^2 obtained from a linear regression between QIM attributes, QIM scores, and days of storage was explored and discussed. In addition, QIM scheme attributes were analysed by principal component analysis (PCA) to better evidence the role of each sensorial attribute in the discrimination of the samples according to the storage time and their relationships. ANOVA, linear regression, and PCA analyses were performed with Minitab statistical analysis platform (Minitab 19.0.1, Pennsylvania State University, USA). For both real and imaginary parts of *S11*, average values of measurement conducted on right and left eyes were used as independent variables for Partial Least Squares regression analysis (PLS) with the aim of setting up predictive models of the demerit scores related to the fish eyes attributes (as a sum of the total demerit points) and of the day of storage. Data were arranged in a 48 (samples) * 301 (spectral variables) matrix. Loading weights were exploited to select the spectral range, considering that higher values related to the frequency portion greatly contribute to explaining the variability. In this way, the lower frequencies were not considered for the analysis, and the selected frequency range was 1.2 – 3 GHz. The calibration sample set was used for

computing the calibration models. Validation was then performed to well understand how the developed model would perform with unknown samples. Test set validation was applied. Seven samples that were not included in the training stage were randomly selected and used to validate the models. The procedure was repeated ten times and results in terms of coefficient of determination (R^2), Root Mean Square Error (RMSE), and significative PLS components (PCs) both for calibration and validation were reported and discussed. PLS analysis was performed with statistical analysis software (Unscrambler software, version 9.7, CAMO, Oslo, Norway).

4.3 Results and discussion

4.3.1 External appearance

Examples of pictures of rainbow trout (*Oncorhynchus mykiss*) captured during the storage trial and after spectral assessments are shown in Figure 4.2. Ice chilling is the most popular storage method used in the fishery and aquaculture industry to delay the onset of spoilage, which is related to oxidative, enzymatic, and microbial degradation (Badiani et al., 2013; Kontominas et al., 2021). As expected, during the analysed 10 days of ice storage, changes in the eye, skin, and gill appearance were observed. The discoloration of the rainbow trout skin that occurred over the storage time can be explained by the oxidation of the carotenoid pigments. Astaxanthin, an abundant carotenoid pigment in fish, is converted into colourless carbonyl compounds by a lipoxygenase enzyme present in the skin tissue and atmospheric oxygen (Lougovois, 2005). Colour fading is amplified by the coagulation of mucus, due to the dehydration of the mucin glycoprotein, which modifies the reflection of the light on the fish skin (Lougovois, 2005). More generally, oxidative process occurring in fish during spoilage involves the reaction of unsaturated fatty acids with atmospheric oxygen to form hydroperoxides. These are decomposed to carbonyl compounds (e.g. aldehydes and ketones), which are responsible also for the off–flavour appearance

(Kontominas et al., 2021; Nie et al., 2022). Off–flavours are intensified by the autolytic degradation of nucleotides, the bacterial reduction of trimethylamine oxide (TMAO) to trimethylamine (TMA) (Barrett and Kwan, 1985) and other microbial spoilage process (e.g. ammonia and hydrogen sulphide producing bacteria) (Gram, 1992; Lougovois, Kyranas, and Kyrana, 2003). The odour of rainbow trout assessed during the storage trial was of fresh vegetables notes (e.g. cucumber and river weeds) until 4 days of storage. Following 2 days where the odour appeared as neutral or slightly metallic until reaching the characteristic off–flavours (e.g. oxidised blood, decaying vegetation) after 6 days of storage. The colour of the gills assessed in rainbow trout appeared bright red or burgundy (Pantone 19–1617) with a glossy and clear mucus until 2 days of storage. As time increased, gill the colour passed from marsala red (Pantone 18–1438) with apical discoloration and a filamentous and milky mucus on day 4. After 8 days of storage, mainly because of microbial growth and blood oxidation, gills acquired a grey–yellowish colour with yellow coagulated mucus. The eyes pupil tended to be grey and opaque over the period of storage. This could be attributed to the internal chemical changes which lead to external changes such as colour and appearance in fish post mortem (Dowlati et al., 2013). Moreover, with increasing storage time, the shape of the eye gradually passed from being flat to concave (sunken), it is reported in the literature (Lougovois, 2005). Regarding the texture, rainbow trout presented a firm and hard consistency, often in rigor mortis, until 2 days of storage. A progressive post–mortem softening of the fish muscle was observed during the storage, as a result of enzymatic degradation generated by the activity of endogenous proteases on myofibrillar proteins (Cheng et al., 2014).

4.3.2 Sensorial evaluation and Torrymeter freshness assessment

Mean values of the mass (g), length (mm), QIM, and Torrymeter scores acquired during the third storage trial are summarised in Table 4.2. The table also shows the results of the Analysis of Variance conducted within the same analysed parameter according to the storage time. As

expected, storage time significantly influences both QIM ($p \leq 0.01$) and Torrymeter scores ($p \leq 0.01$). After post hoc test, non–significant differences in QIM mean values emerged between samples stored for 4 and 6 days. The QIM scheme developed in this work, shown in Table 4.1, reached its highest score after 10 days of storage and consisted of ten attributes for a total of 24 demerit scores. Other QIM schemes developed for rainbow trout focused on the degradation of the sensorial characteristic on longer storage periods such as 17 days (Grigorakis et al., 2018) and 12 days (Diler and Genç, 2018). However, the shelf–life of rainbow trout is considered to be somewhat shorter (Rezaei et al., 2008; Tavakoli et al., 2018). This is confirmed in this experiment by the onset of an unpleasant smell, which appeared after 6 days of storage, and become unacceptable on day 10. Significant differences were observed in Torrymeter mean values between samples at 0, 2, 4 and samples at 8 and 10 days of storage. Differently from the QIM, Torrymeter assessment appears to discriminate trout samples only into two clusters of freshness; respect to day 0, the first significant differences between means appeared only starting from 6 days of storage. Torrymeter is a useful tool to evaluate fish freshness over several days, although less discriminative on shorter storage time (Cheyne, 1975). According to the manufacturer's with regard to Atlantic salmon (*Salmo salar*) stored in ice, a Torrymeter score above 12 corresponds to a fish harvested for less than 3 days and to an "Extra" grade in the EC Regulation No 2406/96 (European Parliament, 1996). A Torrymeter score between 9 and 10 corresponds to a 5 – 8 days fish and to an "A" grade in the EC Regulation No 2406/96 (European Parliament, 1996). Results of the coefficient of determination R^2 obtained from linear regression models between each QIM quality parameter, QIM total, and days of storage are shown in Table 4.3. The results refer to both models calculated starting from all the considered samples or the mean values. By considering linear models obtained by using all samples, the gill colour / appearance and gill mucus attributes were characterised by the lowest R^2 values. As evidenced in Table 4.3 and Figure 4.3, a coefficient

of determination R^2 of 0.948 was observed for QIM total demerit point. Passing to the models obtained from mean values, the R^2 values increased as expected.

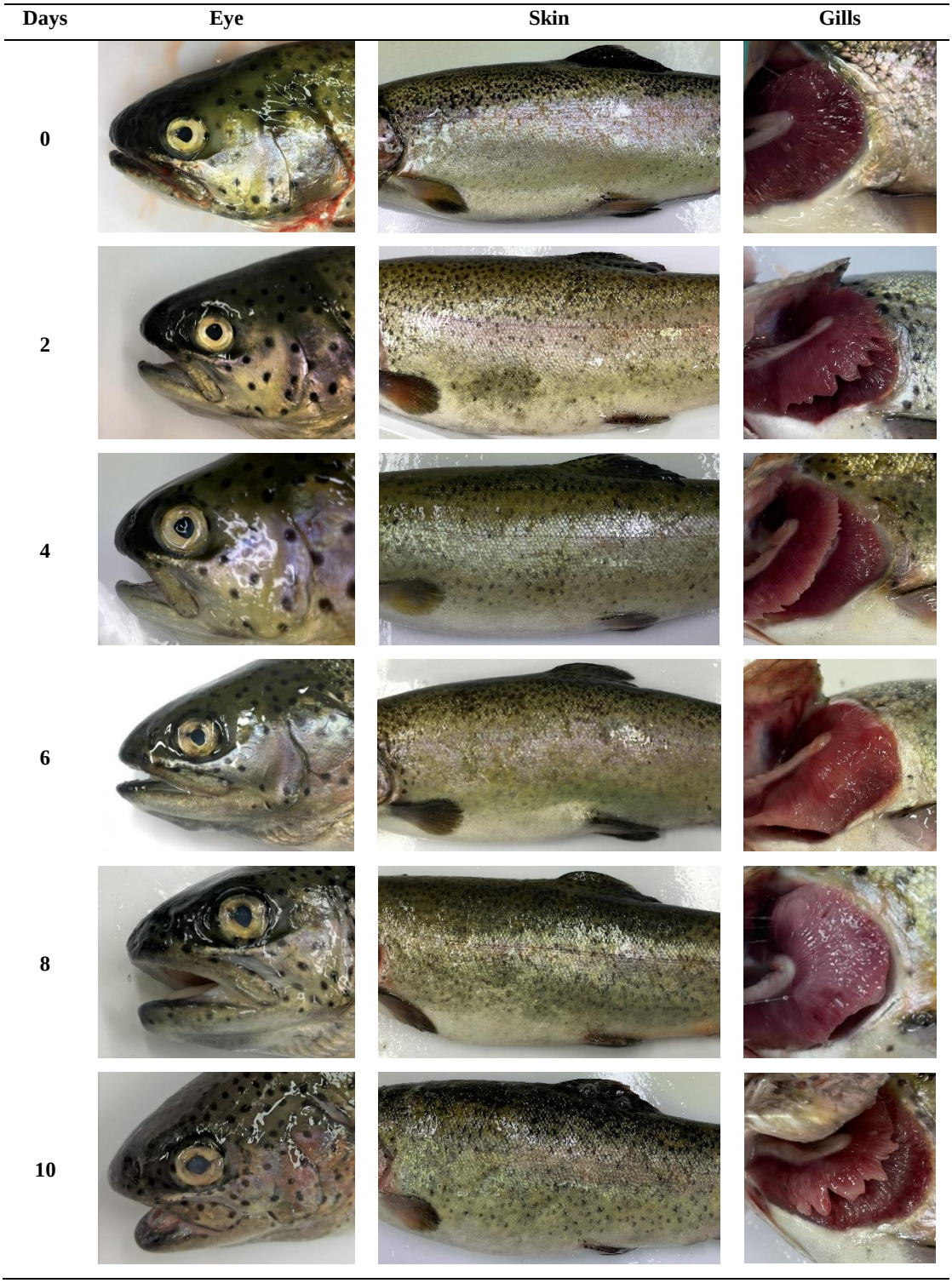

Figure 4.2 Examples of rainbow trout (*Oncorhynchus mykiss*) appearance during 10 days of ice storage acquired after spectral assessments.

Table 4.2 Mass, length, QIM and Torrymeter scores of rainbow trout (*Oncorhynchus mykiss*) during 10 days of storage in ice.

Storage (days)	Mass (g)	Length (mm)	QIM	Torrymeter
0	307.4 ± 23.6	299 ± 6	0.4 ± 0.5^{a}	13.1 ± 0.8^{a}
2	332.4 ± 62.5	314 ± 17	5.0 ± 1.5^{b}	13.1 ± 0.7^{a}
4	356.3 ± 43.5	312 ± 6	11.4 ± 2.3^{c}	12.6 ± 0.8^{ab}
6	361.6 ± 61.2	312 ± 16	13.9 ± 2.4^{c}	11.6 ± 1.3^{bc}
8	338.3 ± 51.8	307 ± 14	19.5 ± 1.6^{d}	10.5 ± 1.1^{c}
10	328.0 ± 313.0	300 ± 5	22.5 ± 1.0^{e}	11.0 ± 1.0^{c}

Data are given as the mean (n = 8) ± SD. Different superscript letters, within column, indicate significant differences among samples during storage ($p \leq 0.05$).

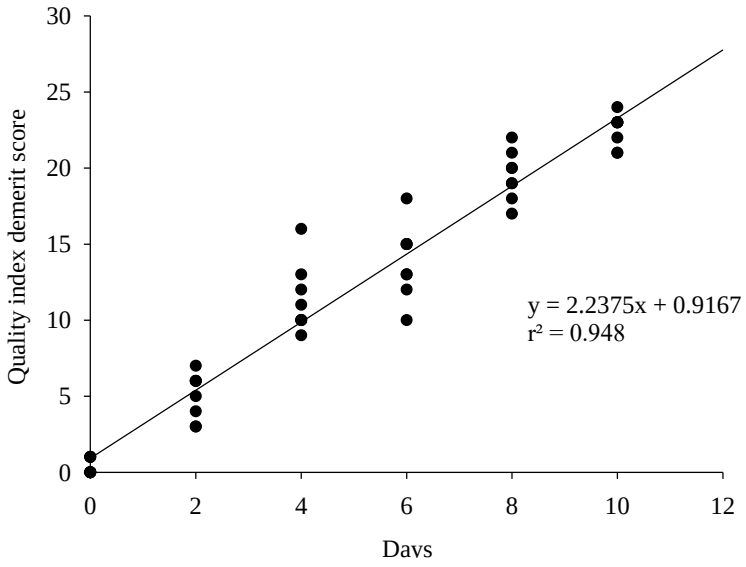

Figure 4.3 Linear regression between quality index demerit score and storage time assessed during the third storage trial with the QIM scheme developed for rainbow trout (*Oncorhynchus mykiss*).

Table 4.3 Coefficient of determination (R^2) for each quality parameter assessed during 10 days of ice storage with the QIM scheme developed for rainbow trout (*Oncorhynchus mykiss*).

Quality attribute	R^2 (all samples)	R^2 (mean values)
Skin colour	0.788	0.942
Skin mucus	0.821	0.942
Skin odour	0.809	0.936
Texture	0.819	0.936
Abdomen colour	0.751	0.961
Eyes pupil	0.816	0.941
Eyes shape	0.819	0.979
Gills colour	0.506	0.982
Gills mucus	0.592	0.876
Gills odour	0.848	0.979

4.3.3 Principal component analysis

The biplot obtained from Principal Component Analysis (PCA), conducted by using the QIM quality parameters measured during the third storage trial and the storage time, is shown in Figure 4.4. The first component (PC1) accounted for 79.96 % of the variability and appeared to model differences related to the storage time while the second component (PC2), accounting for 5.31 %, seems to explain the variability between samples within the same storage time. As indicated in Figure 4.4, PC1, associated with storage time, appears less influenced by the gill colour/appearance and gill mucus quality attributes compared to the other analysed attributes. On

the contrary, gill colour/appearance is the quality attribute that affect PC2 and therefore the variability between samples on the same day of storage.

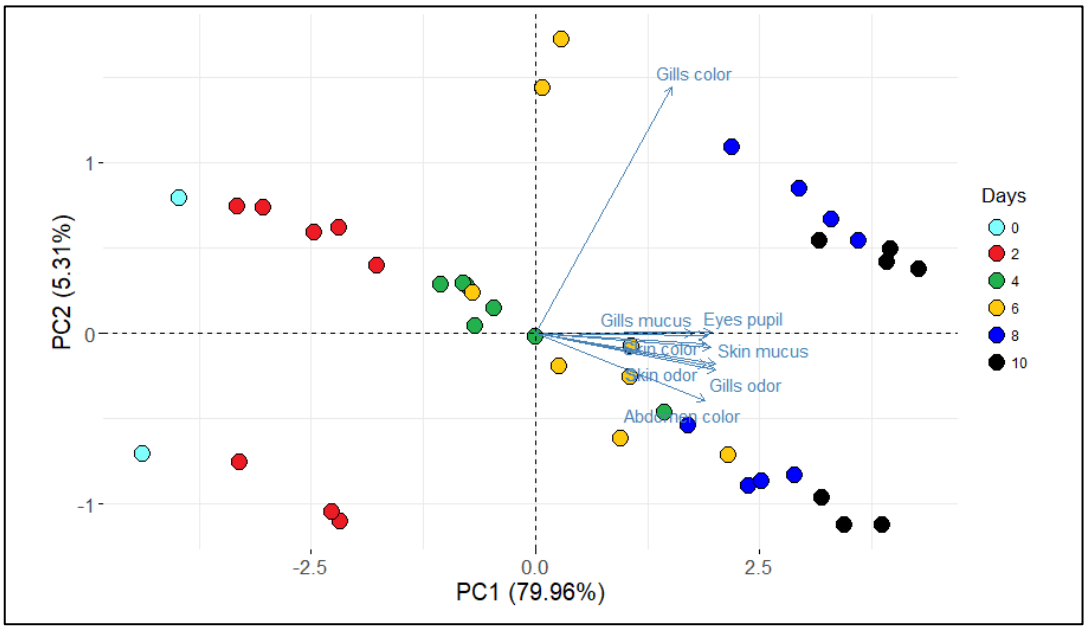

Figure 4.4 Principal Component Analysis (PCA) biplot including all quality parameters evaluated in the QIM scheme developed for rainbow trout (*Oncorhynchus mykiss*) during the storage time.

4.3.4 Spectral assessments

The open–ended coaxial technique measures the scattering parameters *S11* as a function of sample response. In particular, the reflected signal is caused by an impedance mismatch, a discontinuity at the end of the cable. The dielectric properties of the sample are derived from this discontinuity. As the dielectric permittivity, *S11* is a complex number composed of a real and an imaginary part. Both real and imaginary parts are related to the magnitude and the phase of the reflected electromagnetic wave. The real and imaginary parts of the scattering parameter *S11* (reflection) is shown in Figure 4.5 for trout eyes samples during storage time. Averaged spectra of trout eyes were shown as a function of the sum of QIM total demerit points related to trout eyes, such as shape and pupils' attributes.

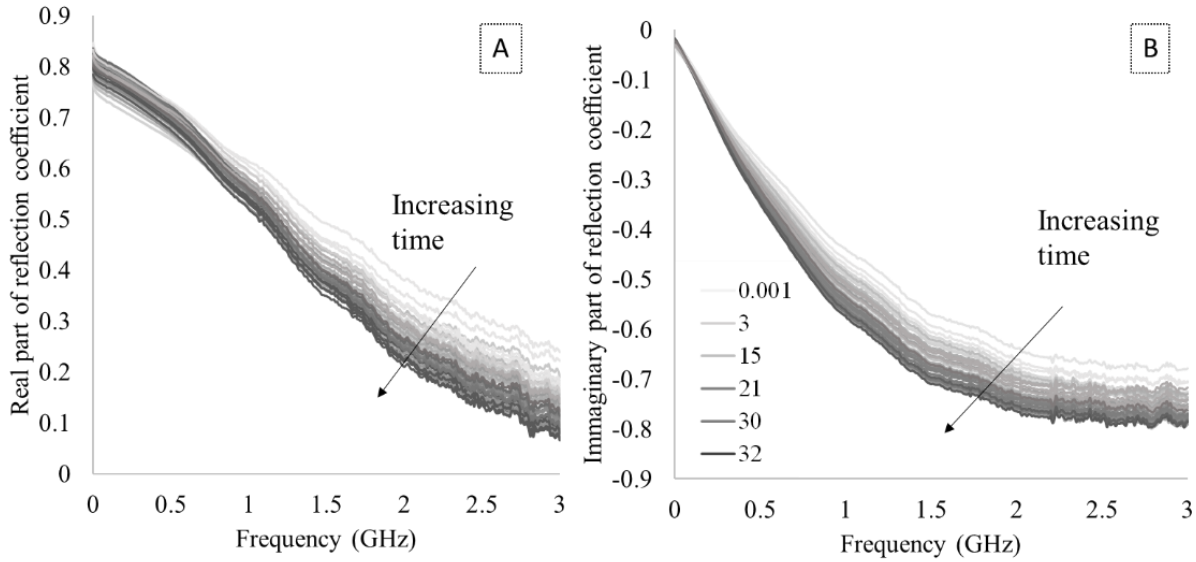

Figure 4.5 Real (A) and imaginary (B) part of *S11* as a function of different storage time. The legend reports the sum of QIM total demerit point related to trout eyes.

Figure 4.5 showed spectra variability also as a function of storage time. Particularly, the low portion of the frequency range shows less spectral variability than that of high frequencies, both for the real and imaginary part of reflection. Physical–chemical modification induced by post–mortem mechanisms influences the spectral response, as also previously reported for anchovy eyes by Iaccheri et al., (2022). According to the authors, a dehydration of fish eyes during storage time leads to a decrease in dielectric constant and loss factor, which is evidenced by a decrement in both real and imaginary parts of permittivity. In our trial, the real and imaginary parts of *S11* spectra were used as predictors (x–variables) for PLS regression for QIM total demerit point of eyes and storage time estimation, the responses (y–variables). Results in terms of R^2, RMSE, and PCs for both calibration and test set validation models are reported in Tables 4.4, and 4.5. PLS–validated regression model gave considerable results in terms of the coefficient of determination and related errors. Particularly, PLS models demonstrated very good prediction ability in terms of R^2 0.946, 0.945 and RMSE 0.88, 0.79 (mean values for the real and imaginary

part respectively), and R^2 0.942, 0.940, and RMSE 3.17, 3.03 (mean values for the real and imaginary part respectively), for storage time and QIM total demerit point of eyes respectively. As an example, predicted versus observed values of one PLS–validated model for both estimations of QIM total demerit point of eyes and storage time were shown in Figures 4.6 and 4.7. Predicted versus observed values confirm the ability of the proposed instrumental chain based on a coaxial probe to estimate both storage time and QIM total demerit point of eyes. The spectral results obtained in our trial, can be compared with the relationship evidenced in the above cited previous research work between anchovy eyes freshness and changes in the dielectric properties (Iaccheri et al., 2022), where a discrimination of fish as a function of freshness was evidenced after a cluster analysis. Moreover, as shown in Table 4.4, the storage time estimation of the present device appears to produce a maximum RMSE value of 1.1 and 1.03 days for real and imaginary part respectively. As a comparison, the discrimination capability of Torrymeter observed in our trial could be as high as 4 days, as shown in Table 4.2. This freshness assessment technique demonstrated its non–inferiority when compared to other sensors assessing the interaction between electromagnetic waves and fish eyes during storage. As an examples Shao et al. (2023), investigated the potentiality of a 400–1000 nm hyperspectral imaging technique in combination with multivariate data analysis for the prediction of freshness of yellow croaker and showed an R^2 = 0.90 after PLS validation. Furthermore, good correlations (R^2 > 0.99 in calibration) between fish eyes colorimetric attributes and storage time were observed also in several research works aimed at setting up methods based on machine vision systems (Wu et al., 2019; Rocculi et al., 2019). Overall, the presented results show that the technique is promising for the freshness assessment of rainbow trout, even though some possible pitfalls can be verified. Attention should be paid when the coaxial probe is put in contact with fish eyes, only a gentle pressure should be applied to prevent damages. In addition, being the dielectric responses matrix dependent, the so developed model is intrinsically species specific being as much reliable only on rainbow trout. As such,

dedicated measures should be developed to build new model for each species of fish. Despite this, the instrumental method is affordable, rapid, simple, and non–destructive avoiding the possible personal impact that sensory evaluation could subjected.

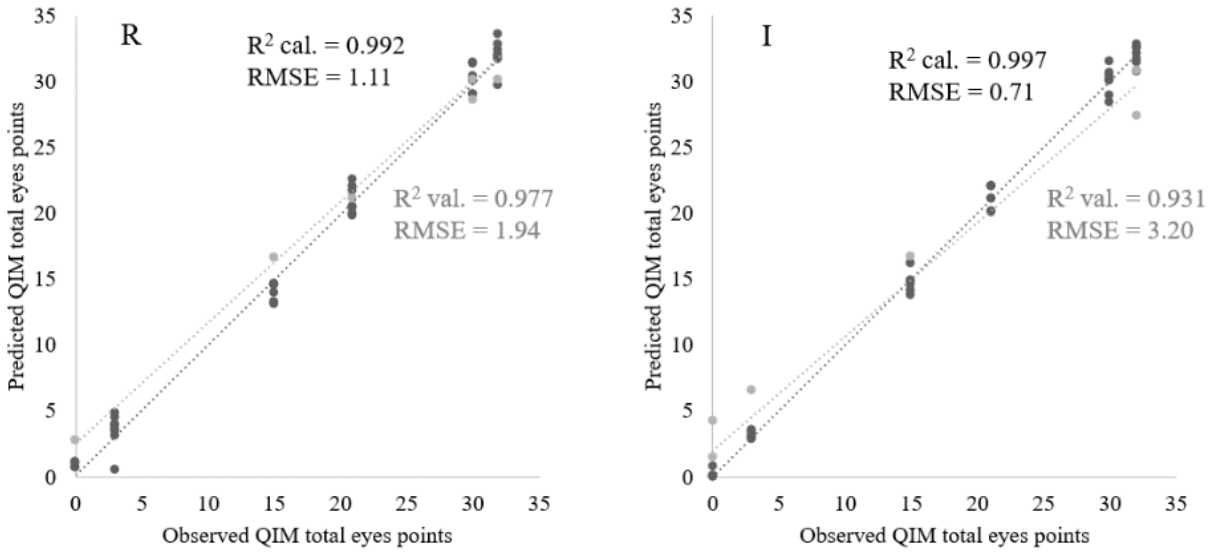

Figure 4.6 Predicted versus observed values for QIM estimation by using the real (R) and imaginary (I) part as predictors. Validation procedure number 7 for R and 9 for I, see Table 4.4.

Table 4.4 PLS results both for real and imaginary part of the reflection scattering signal *S11*, used as predictors in multivariate model for QIM estimation.

	Total QIM estimation	R^2 calibration	RMSE calibration	R^2 validation	RMSE validation	PCs
	1	0.993	1.09	0.912	3.64	6
Real	2	0.997	1.41	0.914	4.43	6
part of *S11*	3	0.997	0.72	0.956	2.83	6
	4	0.994	0.97	0.945	3.32	6

	5	0.993	1.09	0.910	3.77	6
	6	0.991	1.15	0.981	2.11	6
	7	0.992	1.11	0.977	1.94	6
	8	0.989	1.36	0.950	2.92	6
	9	0.996	0.84	0.928	3.40	6
	10	0.997	0.71	0.943	3.36	6
	1	0.998	0.54	0.911	3.18	9
	2	0.996	0.82	0.936	3.04	8
	3	0.996	0.78	0.960	3.01	8
	4	0.996	0.85	0.949	2.26	8
Imaginary	5	0.993	1.05	0.939	3.17	7
part of *S11*	6	0.987	1.45	0.969	2.18	6
	7	0.997	0.57	0.900	4.57	9
	8	0.985	1.52	0.962	2.72	6
	9	0.997	0.71	0.931	3.20	8
	10	0.990	1.25	0.946	3.02	7

From 1 to 10: validation procedures (each validation was conducted by considering a combination of seven fish).

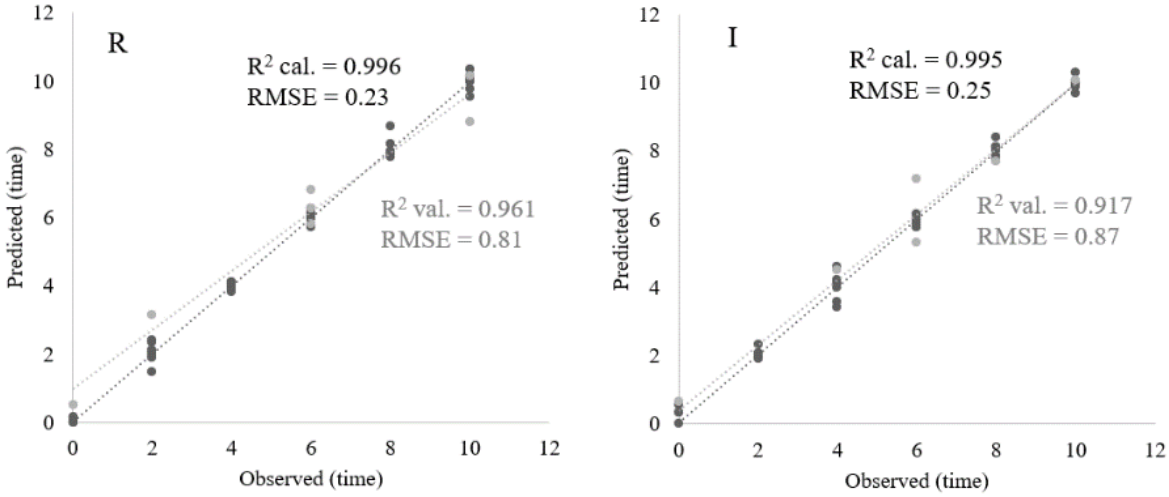

Figure 4.7 Predicted versus observed values for storage time by using the real (R) and imaginary (I) part as predictors. Validation procedure number 1 for R and 6 for I, see Table 4.5.

Table 4.5 PLS results both for real and imaginary part of the reflection scattering signal *S11*, used as predictors in multivariate model for storage time estimation.

	Storage time estimation	R^2 calibration	RMSE calibration	R^2 validation	RMSE validation	PCs
Real part of *S11*	1	0.996	0.23	0.961	0.81	7
	2	0.980	0.47	0.947	1.10	7
	3	0.928	0.96	0.938	0.98	5
	4	0.994	0.28	0.945	0.79	6
	5	0.996	0.22	0.960	0.82	7
	6	0.993	0.30	0.912	1.05	6
	7	0.936	0.93	0.945	0.78	6
	8	0.982	0.47	0.957	0.85	5
	9	0.991	0.34	0.934	0.90	6

	10	0.995	0.24	0.956	0.73	7
Imaginary part of *S11*	1	0.991	0.32	0.967	0.76	7
	2	0.999	0.11	0.918	1.00	10
	3	0.994	0.27	0.970	0.61	7
	4	0.997	0.21	0.953	0.73	8
	5	0.993	0.28	0.947	0.85	7
	6	0.995	0.25	0.917	0.87	7
	7	0.989	0.37	0.920	0.96	6
	8	0.986	0.43	0.983	0.37	6
	9	0.982	0.47	0.964	0.73	6
	10	0.991	0.32	0.906	1.03	7

From 1 to 10: validation procedures (each validation was conducted by considering a combination of seven fish).

Conclusions

A cheap and non–destructive device combined with multivariate statistical tools was set up aiming at exploring its ability in predicting the freshness of rainbow trout during storage of 10 days in ice. The proposed solution was based on PLS–validated regression models obtained by considering both real and imaginary part spectra of scattering parameter S11 acquired in the 50 kHz – 3 GHz frequency range as independent variables. The acquisitions were conducted by placing a coaxial probe connected with VNA in contact with the fish eye. The day of storage and the demerit point calculated from a specific Quality Index Method scheme was chosen as dependent variables. According to the results, apart from gill colour/appearance and gill mucus, all the considered sensorial attributes well describe the degradative changes occurring in the fish

during the storage in ice. Both real and imaginary part spectra also contain information related to these changes. The tested device appears to be able to predict the storage time with an R^2 value of up to 0.946 and RMSE of 0.88 days and the total demerit scores associated with fish eye with an R^2 value of 0.946 and RMSE of 3.17. This simple solution can play a big role in the post–harvest processes, contributing to higher product quality and safety and supporting producers and retailers during the qualitative inspections.

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OVERALL CONSIDERATIONS

The rapid growth of the aquaculture industry, driven by the global demand for nutritious food, has prompted a greater focus on sustainable expansion. This doctoral thesis aimed to contribute to the advancement of sustainable aquaculture production by investigating innovative tools and technologies, fostering sustainability, resilience, and circular practices. The sustainability challenges in the aquaculture sector are likely to be intricately connected to the ongoing efforts to identify and develop alternative ingredients to fish meal and fish oil for aquafeeds. This pursuit aligns with broader sustainability goals, including reducing pressure on marine resources, minimising the ecological footprint of aquaculture, and addressing concerns related to overfishing and environmental impact. In addition, improving the organisation of the aquaculture supply chain is another critical component of enhancing sustainability in the aquaculture sector. A well–organised supply chain contributes to more efficient resource utilisation, reduced environmental impact, and fish products waste. As such, the work performed in this thesis was mainly applied with two main research areas: 1) aquaculture nutrition, in chapter 2 and 3, where a combination of biological and physiological tools, along with sophisticated analyses, were applied to the application of single–cell ingredients derived from industrial by–products as aquafeed ingredients. 2) food science and engineering, in chapter 4, in which the focus was on developing innovative freshness evaluation methods based on dielectric properties with the aim at enhancing control over the supply chain, contributing to the reduction of food waste.

In chapter 2, the study investigates the remediation services provided by aquafeed contained *Nannochloropsis* spp. and *Spirulina* spp. single cell ingredients, produced utilising geothermal by–product resources for sustained heat and CO_2 supply. The study's primary focus is understanding their applications in the diet of European seabass larvae as live feed enrichments compared to a commercial control. Results indicate similar outcomes in growth, survival, and skeletal anomalies across all groups. The study explores the impact of these enrichments on the

LC–PUFAs composition in *Artemia* metanauplii and larvae, considering the competitive metabolism of these essential fatty acids. Furthermore, it delves into the potential risk of lipid peroxidation due to excessive LC–PUFAs and the role of antioxidant factors, contained in microalgae, in mitigating oxidative stress. The findings suggest that single–cell microalgae enrichment may represent a valuable alternative to conventional marine resources into marine larvae aquafeed products.

However, in chapter 3, the investigation into the utilisation of another single–cell ingredient source, derived from *C. glutamicum* in flathead grey mullet diets revealed some unexpected findings. This ingredient, previously successfully applied to carnivorous marine species, faced unforeseen challenges in protein digestion when applied to a lower trophic animal like flathead grey mullet. The study revealed potential factors contributing to these challenges, including the absence of an acidic digestion and a functional gizzard in flathead grey mullet. The findings underscored the importance of species–specific considerations in the application of alternative ingredients, emphasising the need for tailored approaches in sustainable aquafeed development. As a matter of fact, different farmed species, even those with similar trophic levels, may respond differently to alternative aquafeeds ingredients due to anatomical, physiological and/or metabolic differences.

In chapter 4, was validated a non–destructive device utilising a Vector Network Analyser and an open coaxial probe for the freshness assessment of rainbow trout during a 10–day storage period in ice. The device measures the reflected scattering parameter (S11) in the 50 kHz – 3 GHz spectral range, focusing on both real and imaginary parts. After using a combination of statistical models, the approach showed a strong correlation between S11 measurements and the freshness indicators. However, the model proposed appeared to be species specific for rainbow trout as dielectric properties are matrices dependant and may vary depending on fish size, species and

preservation method. In addition, while the proposed device is deemed a cost–effective solution, the practical implementation in real–world settings, such as fish processing facilities, remains to be explored. Considerations regarding ease of use, maintenance, and integration into existing quality control processes are essential for the successful adoption of such a tool.

Overall, the presented thesis, provide valuable insights for innovative and sustainable approaches in aquafeed production, emphasising the potential of single–cell ingredients, and advocates for the adoption of rapid and non–destructive techniques to enhance quality assessment in the ever–evolving landscape of the aquaculture industry.

ANNEXES

First insight into diversity and structure of the gut microbiota of flathead grey mullet (*Mugil cephalus*) fed very low fishmeal diet

Poster presentation at XX INTERNATIONAL SYMPOSIUM ON FISH NUTRITION AND FEEDING, Towards precision fish nutrition and feeding, Sorrento, Italy, 5 - 9 June 2022

Andrea Bertini, Danilo Concu, Silvia Natale, Valentina Indio, Alessandra De Cesare, Luca Parma, Annachiara Berardinelli, Alessio Bonaldo

Abstract

Flathead grey mullet (*Mugil cephalus*) is a promising species for the sustainable aquaculture transition due to the low dietary requirement of protein and lipid and the favourable conversion of those into edible fillet. Gut microbiota structure in farming condition is often associated with nutrients absorption and fish health however this data are not available yet on grey mullet. The present work aimed at exploring the diversity and the structure of the gut microbiota of grey mullet fed a plant–based diet with a very low (3 %) fish–meal inclusion. As such, bacterial DNA from the hindgut content was sequenced (MiSeq Illumina) by targeting the V3–V4 regions of the 16S rRNA gene. The most abundant phyla found in grey mullet gut were Fusobacteria and Proteobacteria. The first was strongly represented by *Cetobacterium* which have been found to be dominant in the gut of many freshwater species and have a positive impact in the digestion and metabolism efficiency of the host (Xie et al., 2021). The second, have been found to be dominant in the gut microbiota of the majority of fish fed marine–derived ingredients (Kim et al., 2021). Interestingly the phylum Firmicutes was not found as abundant as expected. This phylum, especially lactic acid bacteria, often dominated the gut of fish fed plant–based diet (Busti et al., 2020; Pelusio et al., 2020). Although preliminary, the resulting data contribute to the deep characterisation of the taxonomic diversity of the grey mullet gut bacterial community which is essential for understanding the dynamics of the diet–host–gut microbiota axis.

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Characterisation of the gut microbiota of flathead grey mullet (*Mugil cephalus*) fed bacterial single cell protein

Oral presentation at AQUACULTURE EUROPE 2022, Innovative solutions in a changing world, Rimini, Italy, 27-30 September 2022

Andrea Bertini, Silvia Natale, Danilo Concu, Valentina Indio, Alessandra De Cesare, Francesco Dondi, Luca Parma, Pier Paolo Gatta, Alessio Bonaldo

Introduction

Flathead grey mullet (*Mugil cephalus*) is a promising omnivorous fish species for a sustainable Mediterranean aquaculture diversification due to the low dietary requirement of nutrients and the high market price of its roe (bottarga) (Solovyev and Gisbert, 2022). Bacterial single cell ingredients produced by fermenting industry by-products represent a sustainable alternative of soybean meal (SBM) and fishmeal (FM) (Glencross et al., 2020). The application of bacterial single cell protein (SCP) ingredient were assessed on the blood biochemistry parameters and the gut microbiota of flathead grey mullet. Gut microbiota structure in farming condition is often associated with nutrients absorption and fish health. To the best of our knowledge this is the first characterisation of grey mullet gut microbiota diversity and structure under captive conditions and fed different diets.

Material and methods

Two isonitrogenous and isoenergetic experimental diets containing different SCP and SBM inclusion (SCP0, 0 % SCP and 30 % SBM; SCP10, 10 % SCP and 9 % SBM) were tested on triplicated groups of 45 fish (initial weight: 67.9 g) reared in a RAS over a period of 113 days. SCP ingredient consisted of a bulk of dried bacterial (*Corynebacterium glutamicum*) single cell (73.5 % crude protein). Feed was provided to apparent satiation on a 6 hours meal once a day.

Somatometric indices, blood metabolic parameters and gut microbiota (GM) (MiSeq Illumina protocol, targeting V3–V4 regions of 16S rRNA gene) were assessed on 12 fish per treatment. Differences among treatments were considered significant at $p \leq 0.05$.

Results and discussions

At the end of the trial, no significant diet effect were detected on somatometric indices. The levels of glucose, creatine, Ca^{2+}, Fe, K^{+} and the ratio Na / K were found significantly higher in the fish fed SCP0 compared to SCP10 ($p \leq 0.05$). No significant differences between the treatments were observed for the other blood metabolic parameters considered. With regards to the gut microbiota, the most abundant phyla found in grey mullet gut were Fusobacteria and Proteobacteria (shown in Figure 6.1). These two taxa represent about 87 % of the whole GM in fish fed SCP0 where Fusobacteria were more abundant, while 83 % in fish fed SCP10 where Proteobacteria were more abundant, although no significant differences were detected. The phyla Actinobacteria and Firmicutes seems to be higher in fish fed SCP10 ($p = 0.1210$ and $p = 0.0592$ respectively). Actinobacteria represented by *Corynebacterium* genus agreed with the dietary inclusion of bacterial SCP from *C. glutamicum*, while Firmicutes, especially lactic acid bacteria, have been associated with a beneficial effect and a healthy gut ecosystem. With a focus on the class level, specific compositional differences, although non–significant, where detected in fish fed SCP10 which present high abundance of bacilli ($p = 0.1702$) and clostridia ($p = 0.0621$). These two classes of bacteria include some promising probiotics which have been shown to be beneficial for the immune response and disease resistance in cultured fish (Guo et al., 2020; Kuebutornye et al., 2019). Overall in both groups, the more abundant genus was *Cetobacterium*, which have been found to be dominant in the gut of many freshwater species and have a positive impact on the digestion and metabolism efficiency of the host. Finally (shown in Figure 6.2), no difference in the gut ecosystem diversity were detected at phylum and genus level between the two groups,

however at species level there is a tendency for higher diversity in fish fed SCP10 (α–diversity; $p = 0.1280$).

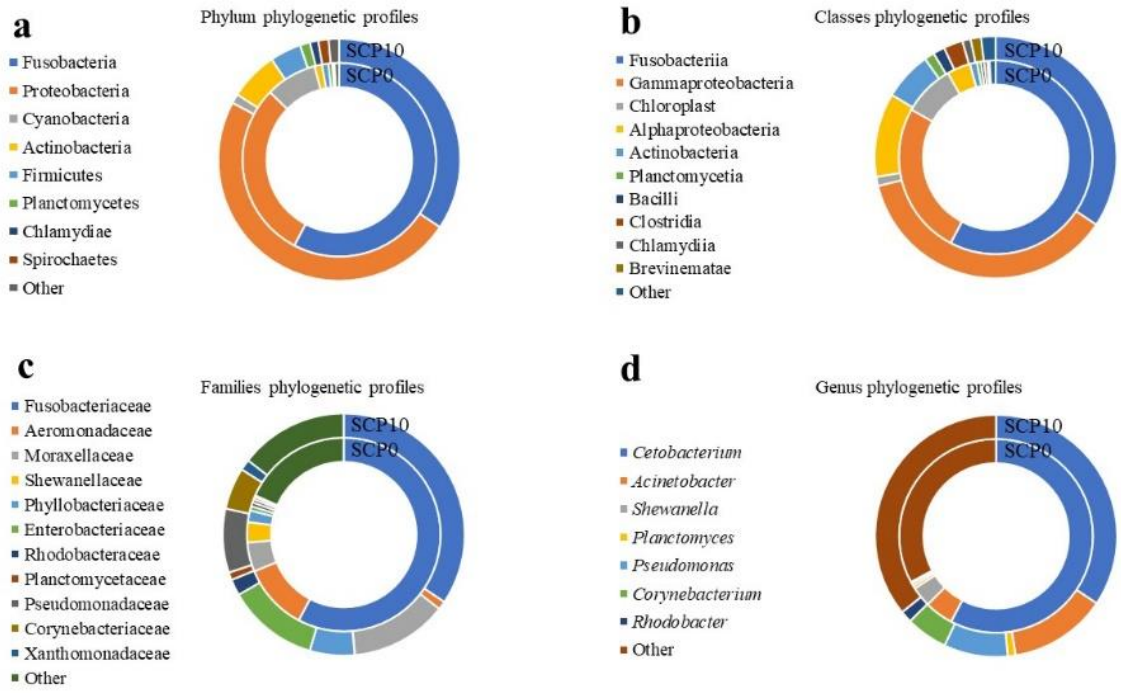

Figure 6.1 Average phylogenetic profiles of grey mullet gut microbiota at phylum (a), classes (b), families (c) and genus (d) level.

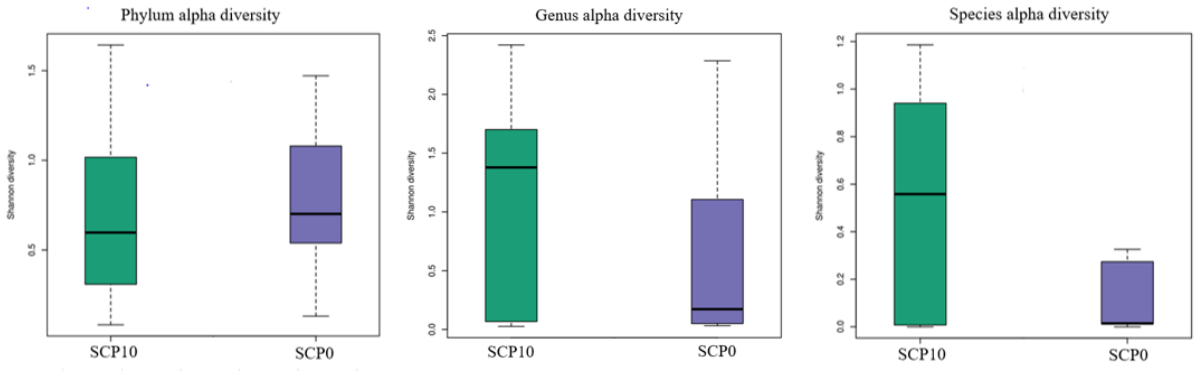

Figure 6.2 Shannon alpha diversity of grey mullet gut microbiota at phylum (left), genus (centre) and species (right) level.

Conclusions

Bacterial SCPs are promising circular ingredients which also present a probiotic effect. As such, further research is needed to better understand their application on grey mullet diet. Moreover, data from the gut microbiota contribute to the deep characterisation of the taxonomic diversity of the grey mullet gut bacterial community which is essential for understanding the dynamics of the diet–host–gut microbiota axis.

Acknowledgments

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Exploring European seabass (*Dicentrarchus labrax*) eicosanoids metabolism when fed diets with increasing inclusion of free catches ingredients after stress exposure

Poster presentation at AQUACULTURE EUROPE 2023, Balanced diversity in Aquaculture development, Vienna, Austria, 18 - 21 September 2023

Andrea Bertini, Kasidis Chaiyasut, Richard Broughton, Annachiara Berardinelli, Monica Betancor, Alessio Bonaldo

Introduction

Inclusions of fish meal (FM) and oil (FO) from trimmings by–products and oil–rich microalgae (MIC) into aquafeed formulations, can advance sustainability by valorising industry waste streams and reducing the utilisation of marine wild resources. However, these ingredients may present differences in long–chain polyunsaturated fatty acids (LC–PUFA) (Sprague et al., 2016) and contain higher levels of bones and ashes with a lower availability of nutrients (Albrektsen et al., 2022), compared to marine wild one. LC–PUFA such as eicosapentaenoic (EPA), docosahexaenoic (DHA) and arachidonic (ARA) are precursors of eicosanoids, which are key protagonists as a mediators of inflammation and in the immune response modulation. Marine fish have a limited capacity to synthesise LC–PUFA from C–18 precursors, thus it is important to understand how “free–catches” diets may affect eicosanoid production, particularly in relation to a stressful situation. In this trial, the effects of five diets, formulated with increasing inclusions of fish meal and oil from trimmings by–products and microalgae, were assessed on the European seabass (*Dicentrarchus labrax*) eicosanoids production by exploring both molecular markers and the eicosanoids metabolites found in anterior kidney samples.

Material and methods

Five diets (FMFO100–MIC0, FMFO50–MIC0, FMFO50–MIC50, FMFO0–MIC50 and FMFO0–MIC100), formulated to totally replace marine wild ingredients with increasing inclusion of FM and FO from trimmings by–products and MIC, were tested on triplicate groups of 45 fish (initial weight: 46.66 ± 0.04 g) reared in a RAS over a period of 88 days. At the end of the trial fish were exposed to a confinement challenge by increasing stocking density to 70 kg / m3 for 2 hours. Samples of anterior kidney were collected from 2 fish per tank (n = 6) at 0, 2 and 24 hours after the challenge. The expression of the selected genes involved in eicosanoids metabolism (EP2, Alox5, Alox12, Alox15 and P450) were evaluated on anterior kidney samples by quantitative real–time polymerase chain reaction method, as described in (Betancor et al., 2021). Eicosanoids metabolites were detected in anterior kidney samples by using HPLC–MS technique. Statistical analysis were performed by means of two–ways ANOVA ($p \leq 0.05$) in order to evidence significant differences among dietary treatments, time and their interaction. Starting from metabolite responses, explorative multivariate PCA were considered in order to discriminate between treatments and the role of each metabolite and their relationship.

Results and discussion

At the end of the trial no significant diet effect were detected on the growth performances among the dietary treatments (data not shown). Regardless of some differences in the fatty acids proportions of the diets, the markers evaluated to assess European seabass eicosanoids metabolism appeared similar among dietary treatments. With regards to the molecular markers evaluated in this trial, only the expression of the gene P450 at time 0 was found significantly different among the treatments. This was found higher in fish fed diets with higher inclusion of microalgae. No other significant difference in gene expression was observed between the diets at the other sampling times (time 2 and 24). The two–way ANOVA model reveals a significant influence of

time on the relative expression level of EP2, Alox5, Alox12 and Alox15. After HPLC–MS analysis, 23 eicosanoids metabolites were found in European seabass anterior kidney. Explorative PCA analysis has highlighted some relationship between the metabolites and the diets. Overall, these results suggest the potential of using completely free catches aquafeed formulations in the diet of European seabass. Moreover, modelling the eicosanoids metabolites and their LC–PUFA precursors can contribute to the deep characterisation of the biological pathways involved in the eicosanoids production.

Acknowledgements

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Non–destructive method for the assessment of fish quality: a promising tool for the aims of sustainable aquaculture project EIT food "Just add water"

Poster presentation at AQUACULTURE EUROPE 2021, Oceans of opportunities, Funchal, Madeira, 4 - 7 October 2021

Andrea Bertini, Eleonora Iaccheri, Annachiara Berardinelli, Alessio Bonaldo, Francesco Parrino

Introduction

In recirculating aquaculture system (RAS), the accumulation of geosmin and 2–methylisoborneol (MIB) in the flesh of the fish, results in off–flavours and odours which are some of the main issues limiting the expansion of this technology (Schrader et al., 2010; Azaria and van Rijn, 2018; Davidson et al., 2020). A promising water treatment unit based on the synergic integration of TiO2 photocatalysis and ozonation could improve the oxidation rate of these compounds and could be used for the purification of the rearing water (Parrino et al., 2014, 2015; Camera-Roda et al., 2019). The research is part of the EIT Food "Just add water" project granted by the European commission in 2021. The project aims to demonstrate that the use of recirculating systems in industrially relevant environments is economically feasible, environmentally sustainable, and respectful of the fish welfare and quality. This work represents a preliminary step in the exploration of fish quality through the setting up of a non–destructive system based on dielectric spectroscopy, able to discriminate between fishes according to storage times and conditions.

The interaction between an electric field and a material is described by the dielectric permittivity ε^*, a complex number characterised by a real (ε') and an imaginary part (ε''). The real part of the dielectric permittivity, related to the transmission and reflection processes, represents a measure of how much energy is stored in a material while the measure of how

dissipative or lossy a material is represented by the imaginary part. These parameters are influenced by different food attributes as the moisture content and water activity, chemical composition and physical structure (Nelson, 2010). Fish dielectric properties are reviewed by Franceschelli et al. (2021).

Material and methods

Physical and chemical changes occurring in the fish during the storage was explored by using a non–destructive tool based on the dielectric property analysis. In detail, dielectric constant ($\varepsilon^{\wedge\prime}$) and loss factor ($\varepsilon^{\wedge\prime\prime}$) of trout eyes were acquired during storage by using an open–ended coaxial probe (DAKS–3.5 probe, Speag). The probe was connected to a VNA (Vector Network Analyser, Copper Mountains) and interfaced by USB with PC (DAK Software Installer 2.6.1.7). The calibration of the instrumental chain was conducted through the customised calibration kit (Speag DAK–3.5 / 1.2 Shorting Block, Metallic Strip Sets, and 0.6 L of Tissue Simulating Liquid). Acquisitions were carried out at 23 ± 1 °C on 7 fishes in the GHz frequency range during 5 days of storage at 0 ± 0.5 °C.

Results

Dielectric constant and loss factor of trout eyes for all the considered storage time were shown in Figure 6.3. As can be observed, there is a clear trend in the spectral behaviour according to storage time. The dielectric constant and loss factor show spectral variability as a function of eyes changes. An increase in the storage time involves a decrease in dielectric constant and loss factor of fish eyes. The phenomenon can be explained by the moisture reduction occurring in the surface of trout eyes following post–mortem processes.

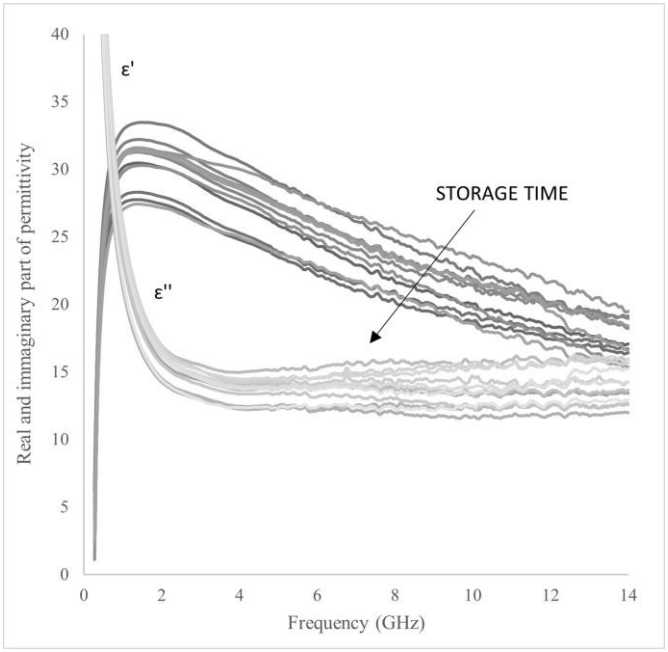

Figure 6.3 Dielectric constant (ε^′) and loss factor (ε^′′) measured on trout eye during storage time.

Conclusions

Dielectric constant and loss factor of trout eyes appeared to describe main changes occurring in the fish during storage. Further steps may include the validation of the techniques with other fish species and different rearing conditions. The setting up of a non–destructive and rapid tool will represent an important step in the project development.

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