NOVA SCHOOL OF SCIENCE & TECHNOLOGY

Department of Environmental Sciences and Engineering (DCEA)

Eco-innovative biofortified fish product for sustainability:

Nutritional health value and safety

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Master in Marine Biology

Doctorate in Environmental and Sustainability Studies

NOVA University Lisbon

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"*Individually, we are one drop. Together, we are an ocean*."

(Ryunosuke Satoro)

"*The future belongs to those who believe in the beauty of their dreams*."

(Eleanor Roosevelt)

Abstract

Considering the global expansion for food associated with world´s population growth, one of the major problems of the world is the supply of healthy and sustainable diets for all. The development of sustainable aquaculture, one of the fastest-growing food production industry, is gaining interest, and further efforts must be implemented to reduce the dependency on wild fish stocks fishing for feed and to promote management and environmental practices (e.g., reduce waste and water usage). Globally human population has severe deficiencies in some essential health-promoting nutrients like iodine and selenium. Although, seafood is one of the most important sources of these nutrients, it has been demonstrated that, under farming conditions, aquaculture feeds can effectively modulate the nutritional profile of farmed fish. In this context, this PhD thesis aimed to: i) assess the effects of biofortified feeds, using I-rich seaweed and Se-rich yeast, to modulate the elemental composition of edible tissues (muscle) in farmed gilthead seabream (*Sparus aurata*) and common carp (*Cyprinus carpio*), ii) evaluate elements stability during frozen storage and after culinary processing (steaming), iii) assess elements *in vitro* bioacessibility and the potential benefit associated to the consumption of biofortified fish, and iv) screening the application of micro X-ray fluorescence (μ-XRF) spectrometry method to assess elements distribution in fish muscle. Overall, the present biofortified approach resulted in increased essential elements (i.e., I, Se, Fe, Zn) in fish muscle, depending on fish species, feeds supplementation and feeding exposure period. Biofortified seabream and carp maintained their enhanced nutritional value and quality (i.e., increased I and Se contents) after steaming, as well as after 12-months of frozen storage, resulting in increased nutritional contribution (i.e., higher daily intakes of I and Se) through the consumption of fish fillets. The biofortification strategy did not negatively affect essential elements bioaccessibility, and elements bioaccessible concentration were always above 70 %, in biofortified fish fillets (except I bioaccessibility in carp). In addition, through the non-destructive μ-XRF imaging analysis was possible to identified to a limited extent the distribution and elemental accumulation within biofortified fish muscle samples The present results contribute for three United Nations' Sustainable Development Goals (SDGs), since the development of eco-innovative biofortified farmed fish with enhanced nutritional quality will contribute to achieve seafood security and improved nutrition (SDG 2), enabling consumers to address nutritional needs and overcome deficiencies, whereas the use of sustainable, natural, safe, and high-quality ingredients in aquafeeds formulation will promote the better use of resources, responsible consumption and production (SDG 12), as well as to achieve the betterment of life below water (SDG 14).

Keywords: Biofortification, health promoting nutrients, farmed fish, sustainability

RESUMO

Considerando o aumento da procura de alimentos associado ao crescimento da população mundial, um dos principais desafios da atualidade é o acesso a regimes alimentares saudáveis e sustentáveis para todos. O desenvolvimento de aquacultura sustentável tem vindo a ganhar destaque, sendo necessário a promoção de estratégias para reduzir a dependência de subprodutos da pesca na formulação de rações, e implementação de boas práticas de gestão ambiental (ex. reduzir o desperdício e uso de água). A população mundial apresenta graves carências em alguns nutrientes essenciais para a saúde humana, tais como iodo e selénio, sendo o pescado uma das principais fontes destes nutrientes. No entanto, é possível modelar o perfil nutricional do pescado em aquacultura através de dietas compostas. Neste contexto, a presente tese de doutoramento pretendeu: i) avaliar o efeito de rações biofortificadas com algas ricas em I e levedura rica em Se, na composição nutricional do músculo de peixes de aquacultura, nomeadamente dourada (*Sparus aurata*) e carpa (*Cyprinus carpio*), ii) avaliar a estabilidade dos nutrientes durante o armazenamento em congelação e após tratamento culinário (a vapor), iii) avaliar a bioacessibilidade "*in vitro*" dos nutrientes, e o benefício associado ao consumo de peixe biofortificado, e iv) avaliar a aplicação da técnica micro fluorescência de raios X (μ-XRF) na deteção e distribuição dos nutrientes no músculo do peixe. Em geral, a presente estratégia de biofortificação resultou no aumento da retenção de nutrientes essenciais (ex. I, Se, Fe, Zn) no músculo do peixe, estando dependente da espécie, suplementação alimentar (fonte de algas, levedura, biomassa de microalgas) e da duração da alimentação. O elevado valor nutricional e qualidade dos peixes biofortificados manteve-se após a cozedura a vapor e durante 12 meses de armazenamento em congelado. A bioacessibilidade dos nutrientes essenciais no músculo dos peixes biofortificados foi superior a 70 %, excluindo a redução da fração bioacessível de I na carpa. O consumo de dourada e carpa biofortificadas contribui para uma maior ingestão diária de I e Se. Através da análise de imagem μ-XRF foi possível identificar a distribuição e acumulação de cada elemento no músculo do peixe biofortificado. Estes resultados demonstram a eficácia da utilização de ingredientes sustentáveis, naturais, e de elevada qualidade nas rações para a produção de pescado biofortificado que permita aos consumidores cobrir as suas necessidades nutricionais, em concordância com três dos Objetivos de Desenvolvimento Sustentável (ODS), nomeadamente garantir o acesso a alimentos seguros, nutritivos (ODS 2), reduzir o desperdício global de alimentos na produção e consumo (ODS 12) e usar de forma sustentável os oceanos e recursos marinhos (ODS 14).

Palavras chave: Biofortificação, qualidade e valor nutricional, aquacultura, sustentabilidade, saúde humana

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ACRONYMS

A

A	Ampère
AAS	Atomic absorption spectrometry
AI	Adequate intake
ALA	Alpha-linolenic acid (18:3 n-3)
ANOVA	Analysis of variance
AR	Adequate requirement
As	Arsenic

B

B1	Biofortified blend 1
B2	Biofortified blend 2
B3	Biofortified blend 3
BD	Before digestion
BF	Biofortified
BF1	Biofortified B1
BF2	Biofortified B2
BIO	Bioaccessible
BMDL	Benchmark dose lower limit
Br	Bromide
b.w.	body weight

C

Ca	Calcium
$CaCl_2.2H_2O$	Calcium chloride dihydrate
cal	Calories
Cd	Cadmium
$Cd(NO_3)_2$	Cadmium nitrate
CHD	Cardiovascular health diseases
Cl	Chloride
Covid-19	Coronavirus disease 2019
CRM	Certified reference materials
CTR	Control
Cu	Copper
CVD	Cardiovascular diseases
CY	Cooking yield

D

DHA	Docosahexaenoic acid (22:6n-3)
DI	Daily intake
DIT	Monoiodotyrosine
DL	Detection limit
DM	Dry matter
DORM-4	Fish Protein reference material
DORM-2	Dogfish muscle reference material
DRI	Dietary reference intake
DRV	Dietary reference values
d.w.	dry weight

E

EC	European Commission
EDDI	Organic iodine salt
EDXRF	Energy dispersive x-ray Fluorescence
EFSA	European Food Safety Authority
e.g.	for example
EPA	Eicosapentaenoic acid (20:5n-3)
EPPO	IPMA Aquaculture Research Station
ERM®-BB422	Fish muscle reference material
EU	European Union
EUMOFA	European Market Observatory for Fisheries and Aquaculture Products
eV	electron volt

F

FAAS	Flame atomic absorption spectrometry
FAO	Food and Agriculture Organization
FBW	Final body weight
FCR	Feed conversion rate
FCT	Portuguese Foundation for Science and Technology
FELASA	Federation of European Laboratory Animal Science Associations
Fe	Iron

G

g	gram

H

h	hour
HBGVs	Health-based guidance values
HBV_{Se}	Selenium health benefit value
HCl	Hydrochloric acid
Hg	Mercury
HNO_3	Nitric acid
H_2O_2	Hydrogen peroxide

I

I	Iodine
iAs	Inorganic arsenic
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical Emission spectrometry
IDD	Iodine deficiency disorders
i.e.	*Id est* (that is)
IFAD	International Fund for Agricultural Development
IPMA	Portuguese Institute for the Sea and Atmosphere
IQCs	Internal quality controls
IRMM	Joint Research Centre Institute for Reference Materials and Measurements

J

JRC	Joint Research Center

K

K	Potassium
KCl	Potassium chloride
kg	kilograms
KH_2PO_4	Potassium dihydrogen phosphate
KI	Potassium Iodide
KSCN	Potassium thiocyanate
kV	quilovolt

L

L	litre
Lda	Limited liability company
LCPUFA	Long chain polyunsaturated fatty acids
LOD	Limit of Detection
LOQ	Limit of Quantification
LPO	Lipid oxidation

M

m	meter
MDA	Malondialdehyde
MeHg	Metilmercury
Mg	Magnesium
mg	milligram
$MgCl_2$	Magnesium chloride
mL	millilitre
mm	millimetre
Mn	Manganese
MPL	Maximum permissible levels
MUFA	Monounsaturated fatty acids
m/v	mass by volume

N

n	number of individuals
n.a.	Not available
Na	Sodium
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
$Na_2H_2PO_4$	Monosodium phosphate
NaOH	Sodium hydroxide
Na_2SeO_3	Sodium selenite
Na_2SO_4	Sodium sulphate
NBIO	Non bioaccessible
NC	Nutritional contribution
n.d.	Not determined
ng	nanograms
NH_4Cl	Ammonium chloride
No.	Number
n-3 LCPUFA	n-3 long-chain polyunsaturated fatty acids

O

OECD	Organisation for Economic Co-operation and Development

P

P	*P*-value
P	Phosphorous
Pb	Lead
$Pb(NO_3)_2$	Lead nitrate
PCA	Principal component analysis
PET	Polyethylene terephthalate
PPCO	Polypropylene copolymer
PTWI	Provisional tolerable weekly intake
PUFA	Polyunsaturated fatty acids

R

r	Pearson correlation coefficient
rpm	Revolutions per minute
RSD	Relative Standard Deviation

S

S	Sulphur
s/sec	second
SD	Standard deviation
Se	Selenium
SeCys	Selenocysteine
SeMet	Selenomethionine

SeO^-	Selenium trioxide anion
SFA	Saturated fatty acids
SGR	Specific growth rate
sp.	species
spp	Several species
SRM 1566	Oyster tissue reference material
SRM 1571	Orchard leaves reference material

T

TBA	Thiobarbituric acid
TBAR	Thiobarbituric acid reactive substances
TG	Total growth
TMAH	Tetramethylammonium hydroxide
TR	True retention
TRV	True retention values
TWI	Tolerable weekly intake

U

UL	Upper Tolerable Intake Level
UNICEF	United Nations International Children's Emergency Fund
USA	United States of America
USDA	United States Department of Agriculture

W

WFP	World Food Programme
WHC	Water holding capacity
WHO	World Health Organization
w/w	weight by weight
w.w.	wet weight

X

XRF	X-ray Fluorescence

Z

Zn	Zinc
ZUT	West Pomeranian University of Technology in Szczecin

Symbols

α	alpha
°C	Degree Celsius
>	higher than
<	lower than
µ	micro
mol	mole
±	more or less
%	percent
‰	per thousand
Ω	saturation state

1

INTRODUCTION

The 2030 Agenda for Sustainable Development, adopted by all United Nations Member States in 2015, recognized hidden hunger and malnutrition in all forms and dimensions as the greatest global challenge. At a global level, one out of three people in the world suffer from food insecurity and different forms of malnutrition because cannot afford a healthy diet due to income inequality (FAO et al., 2021).

From a public health point of view, micronutrients deficiencies can contribute to several human diseases, including cardiovascular (CVD), endocrine and neurophysiological, and ultimately lead to high rates of morbidity and mortality (Allen et al., 2006; FAO, 2020). To avoid these health problems, regular consumption of seafood products is widely recommended by health authorities, as these products are recognized as healthy food items and important sources of high-quality protein and essential key nutrients, such as n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), vitamins (i.e., A and D), and macro and trace elements (e.g., iodine (I) and selenium (Se)), preventing malnutrition and contributing for the correct imbalanced high-caloric and low-micronutrient diets (EFSA, 2014a, 2015a; FAO, 2020). Global data on seafood consumption shows an increase demand for these products, with the average annual growth rate of total seafood consumption outpacing the annual population growth rate. Still, annual *per capita* fish consumption varies between and within countries, associated to cultural, economic, and geographical factors (FAO, 2020). Most of the European consumers do not meet the dietary recommendations of eating at least two portions of fish (equivalent to 150–300 g) per week to ensure the provision of essential elements (EFSA, 2014a). Over the years, the share of aquaculture production for direct human consumption has been constantly rising, reaching 56% of world fish production (FAO, 2020). In addition, fisheries contribution is

declining associated to biologically unsustainable overfished stocks in the last years (FAO, 2020). Thus, as the world population grows and the subsequent demand for healthy, sustainable, and safe seafood, it is expected that aquaculture production will be the only source of seafood production to fill the supply–demand gap. In fact, improving aquaculture production towards sustainable, cost-effective, and high-quality seafood products is one of the main challenges of the sector. Therefore, the potential to develop tailor-made farmed fish through fortification with natural and sustainable ingredients is gaining interest to improve consumer's health and to reduce all forms of malnutrition.

1.1 Food supply chain: environmental and sustainability challenges

The future of the world depends on healthier, safer, sustainable, and more equitable food diets for all. Nourishing a growing population is now raising added pressure on the planetary ecosystems, and thus feeding a growing population without exhausting the natural resources continues to emerge (FAO, 2022; Gormaz et al., 2014). Human activities are having unprecedented impacts on the environment and global warming, leading to the deforestation, urbanization and agricultural intensification, losses of biodiversity on land and sea, sea levels rise, water resources over-exploitation and increased waste production (Berry, 2019, Downs et al., 2020). With the increasing demand for food supply, land-based expansion and food production are threatening environmental thresholds, raising serious questions on food quality, food safety and resource needs (Berry, 2019, Castello et al., 2020). In this sense, there is a clear need to improve the resilience of food systems and sustainable diets as part of a complex and dynamic food chain that support both environmental and human wellbeing (Downs et al., 2020). To improve sustainable and secure diets, further efforts are needed to shift consumer behaviour towards food waste reduction, less resource-intensive diets and healthy eating patterns (FAO et al., 2021). Moreover. land-based animal products are within the main drivers for increased greenhouse gases (GHG) emissions and carbon footprints. In this regard, environmental and human health are directly linked, and both are strongly affected by dietary changes (Tilman & Clark, 2014). Food loss and waste represents around 24% of the energy content of the food produced and 8% of global GHG (Vågsholm et al., 2020). According to Food and Agriculture of the United Nations (FAO), sustainable diets are those that not only support

environmental sustainability, but also economic, socio-cultural, and human health aspects. Hence, sustainable food security is an important target of the United Nations 2030 Agenda for Sustainable Development, and its 17 Sustainable Development Goals (SDGs) are central to achieve the three dimensions of sustainable development, namely economic, social and environmental growth. In fact, the 2030 Agenda acknowledges the important role of fisheries and aquaculture as relevant food production systems to end hunger (SDG 2 - Zero hunger), ensure food security and livelihoods (SDG 3 - Ensure healthy lives and promote well-being for all at all ages, and SDG 12 - Responsible consumption and production), and for better use of natural resources (SDG 14 - Life below water). Regarding SDG 2, and SDG 3, both the United Nations and the World Health Organization recognized the need to change lifestyle, environmental factors and eating habits, associated with highly processed foods, refined fats and sugars, and meat, which contribute to increased incidences of chronic non-communicable diseases (NCD), especially chronic diseases such as type II diabetes, coronary heart disease and some cancers (FAO et al., 2021). Aquatic foods emerge as a solution for healthy diets, providing several bioavailable nutrients (e.g., iodine, selenium, vitamin B12 and vitamin A, essential fatty acids) essential for physical and cognitive development. Additionally, aquaculture support the provision of sufficient aquatic food for a growing population, ensuring the availability and accessibility of safe and nutritious aquatic food for all (FAO, 2022). In terms of SDG 12, aquaculture allows the implementation of novel solutions to reduce food losses and waste along the value chain, improving the market value of foods (i.e., quantity and quality of foods) (FAO, 2022). Lastely, under the SDG 14, using aquatic nutrient resources more efficiently and developing aquaculture solutions to deliver sustainable diets under changing environmental, social and climate conditions yield healthier ecosystems and richer biodiversity to provide life-sustaining (UN Nutrition. 2021). To foster more resilient aquatic food systems, aquaculture may play an important role on preventing and reducing marine pollution, conservation of the biodiversity by reducing the pressure on biological unsustainable stocks, and supporting equitable development (FAO, 2022), Still, for the long-term sustainability of aquaculture it is crucial the implementation of new and better tools, as well as practices, and to reduce the dependency of the aquaculture feeds, on fishmeal and fish oil extracted from important wild pelagic fish stocks, which is considered the main contributor to undesirable environmental impacts from aquaculture activities (FAO, 2022).

The 17 SDGs are Interconnected by nature and their success relies on the strong Interface between policy management and scientific knowledge. Understanding science-based strategies and improving multidisciplinary research capacities promotes the information and

involvement of the several actors on aquatic food systems (e.g., decision-makers, producers, consumers) by gathering knowledge, supporting innovation and solutions to optimize the marine resources and delivery sustainable and secure diets for world's population under changing environmental, social and climate conditions (FAO, 2022). In this context, the application of the One Health approach to aquaculture, integrating nutritional concerns, biodiversity selection, efficient use and protection of marine resources, and food production systems more resilient to environmental changes, play an increasingly important role in both environmental sustainability and food security (Fiorella et al., 2021, Froehlich et al., 2018, Gormaz et al., 2014).

1.2 Aquaculture role towards the future

The seafood sector (fisheries and aquaculture) has an important role in food security and global supply, accounting for almost 17% of global production of animal protein and 7% of all proteins consumed globally (Castello et al., 2020, FAO, 2022). The world's increasing demand and growing consciousness for natural and sustainable fish products leads to a continuous rising of aquaculture role in providing high quality, nutritious and safe food for human consumption (EUMOFA, 2021; FAO, 2022). While wild fisheries production has been relatively stable and reaching its ecological limits, farmed seafood production can still be further extended with less environmental burdens and human health risks (Costello et al., 2020). In fact, aquaculture production accounted for 56% of total seafood production and 60% of total seafood consumption (EUMOFA, 2021). The expansion of aquaculture is mainly derived by the growth of inland production, which represents 62% of total aquaculture production compared to 38% from marine and coastal aquaculture production (FAO, 2022). Despite this growth, the future expansion of aquaculture production will be bounded by the economic, environmental and technological factors, fundamental to achieve sustainable and equitable global seafood supply (Costello et al., 2020).

1.2.1 Global aquaculture trend, role, and key features

Global aquaculture and fisheries production (excluding algae) has significantly expanded over the years, with an annual growth rate of 3.3% (FAO, 2020). Aquaculture production is growing faster than fisheries (Figure 1.1), though at a slower rate since 2018 (an average of 2.9% per year in 2018–2020 versus an average of 4.6% per year from 2010–2018), mainly due to Covid-19 constraints. On the other hand, global production from fisheries has been relatively

stable, and in 2019 a slight decline of 4.5% was observed relatively to 2018 peak of 96 million tonnes (the highest level ever recorded), followed by a further 2.1% decline in 2020 (FAO, 2020). Despite the continuous increase of global aquaculture production, in the near future the projections of fisheries production will still overtake aquaculture production. Still, such projections take only into account the edible proportion of the seafood production, and the predominance of aquaculture in the production of bivalves and crustaceans results in relatively large proportion of inedible parts. Furthermore, the aquaculture sector has the advantage to ensure greater control over their production enabling to adapt more rapidly and efficiently to changing scenarios of social, economic, and environmental globalization (FAO, 2018, 2020).

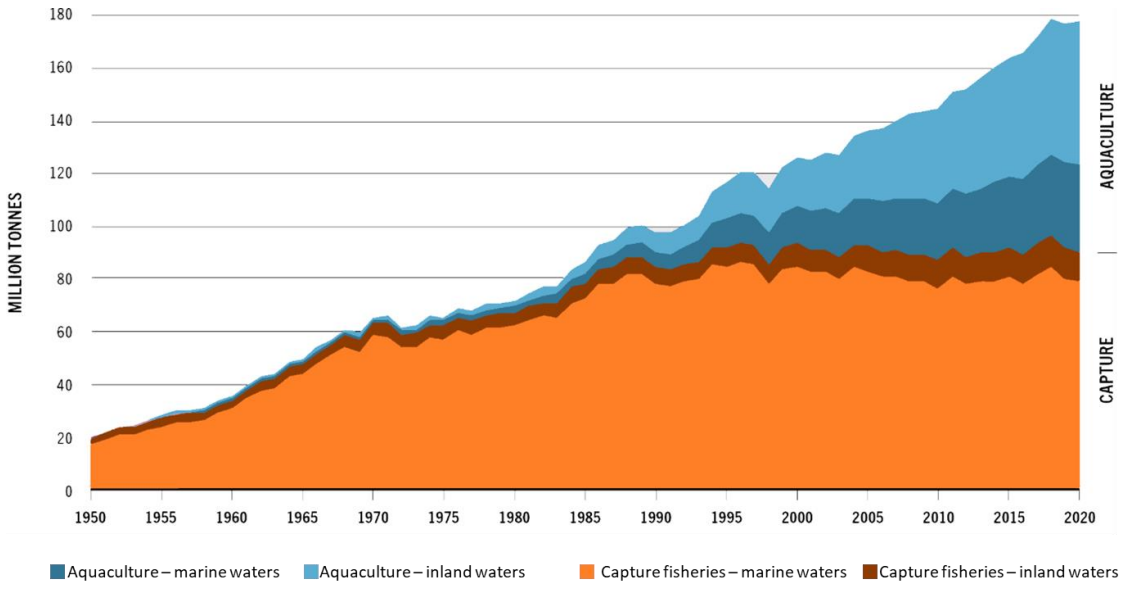

Figure 1.1 - World fisheries and aquaculture production from over the last 70 years. (FAO, 2022)

Since the 80's, fisheries production fluctuated between 86 million tonnes and 93 million tonnes per year, while aquaculture production comprised 87.5 million tonnes of aquatic animals mostly used for human food, and 35.1 million tonnes of marine algae for both food and non-food uses (FAO, 2020). The increased trend of world fisheries and aquaculture productions has been driven mostly by Asian countries, especially China (major producer with a share of 35%) followed by India, Indonesia, Vietnam and Peru, accounting 70% of the world production (Figure 1.2). In contrast, Europe production slightly decreased since 2018, accounting only 10% of the global production and American countries, with ups and downs since the middle 90´s, accounting 12% of the world production. Africa and Oceania share the lowest percentages of

fisheries and aquaculture production, representing 7% and 1%, respectively (EUMOFA, 2021; FAO, 2022).

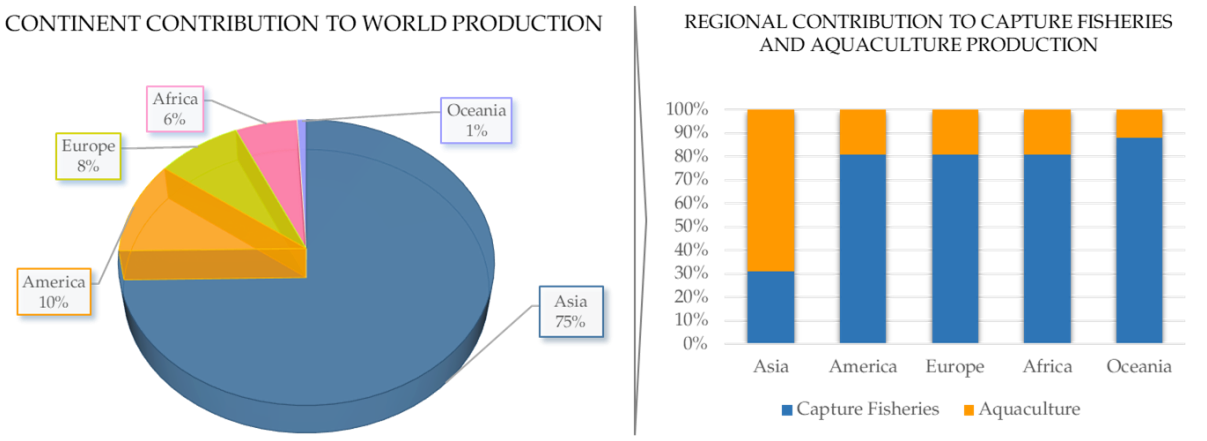

Figure 1.2 - Fisheries and aquaculture production in each continent.

Source: The State of World Fisheries and Aquaculture (FAO, 2020, 2022).

With the world consuming now, more than ever, aquaculture plays a major key in fish availability for human consumption, with a share of 56% and continue to increase (FAO, 2022). In fact, aquaculture allows to expand worldwide fish supply to regions and countries where seafood sources are limited, or non-existing, leading to improved nutrition and food security (FAO, 2020; FAO et al., 2021). Globally, aquaculture production consists mostly by aquatic animals, covering 87.5 million tonnes of production, followed by aquatic algae, covering 35.1 million tonnes (FAO, 2022). In terms of aquatic animals' production, finfish is the most farmed species (66%), followed by molluscs (21%), especially bivalves, crustaceans (11%) and marine invertebrates (1%). Overall, finfish world aquaculture production is dominated by carps, Atlantic salmon and catfish, which accounts for 69% of total finfish production. Farmed seaweeds represents 97% of the total aquatic algae global production, with some species produced primarily for human consumption (e.g., nori and wakame). Worldwide distribution of aquaculture production shows an uneven distribution across regions and even among countries in the same region (FAO, 2022). In fact, among the top ten countries with the highest share in aquaculture around the world, China is the major mainland producer in both animals (56.7%) and algae algal farming (59.5%) (EUMOFA, 2021; FAO, 2022). On the other hand, in Europe, more than 70% of whole EU aquaculture production is mainly achieved by five countries: Spain, United Kingdom, France, Italy and Greece (Figure 1.3). The EU aquaculture production remains similar

in the last ten years, consisting mainly in bivalves farming, especially mussels (36%) and specific farmed fish species, including salmon (15%), trout (14%), seabream and seabass (13%) and carp (6%) (EUMOFA, 2021).

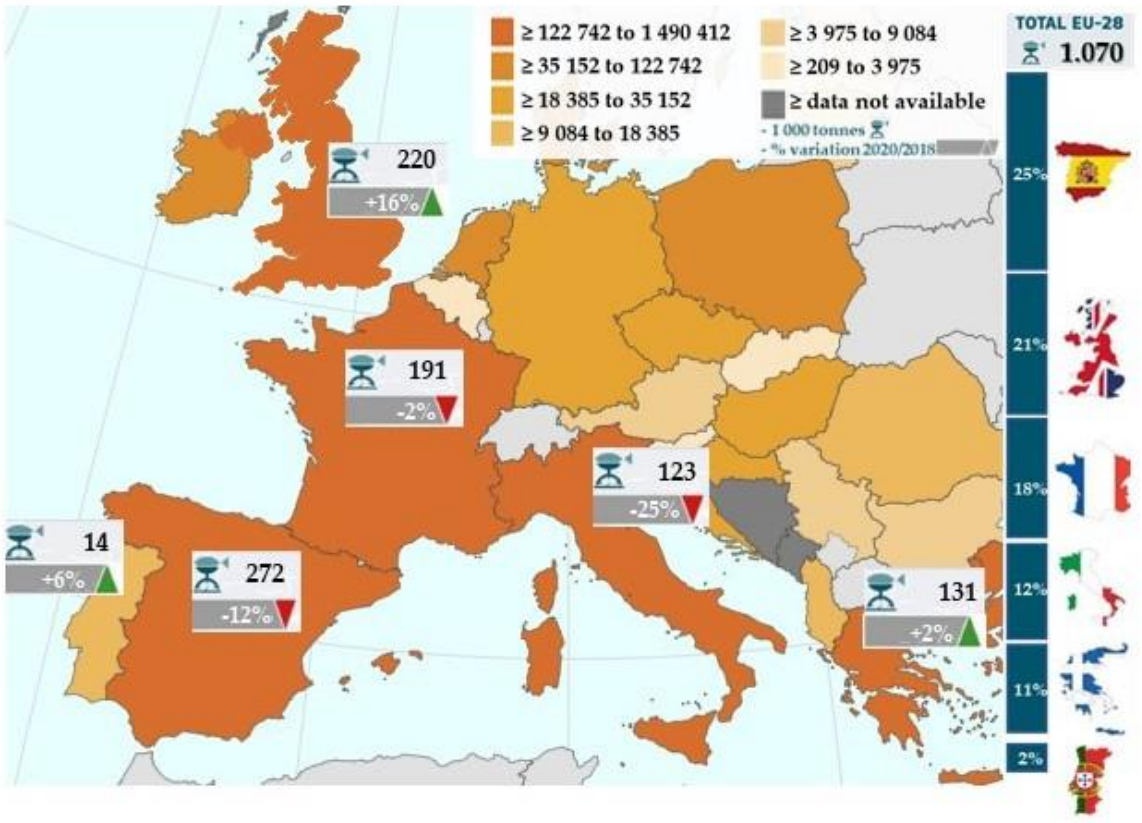

Figure 1.3 - Aquaculture production in the Top 5 European countries and Portugal (adapted from EUMOFA, 2021).

Most global aquaculture production relies on inland aquaculture, accounting 62% of the world's farmed food fish production inland natural water sources, such as rivers and lakes, and fish farms (FAO, 2022). Marine and coastal aquaculture rely on partially or completely artificial structures in coastal areas, coastal ponds, and gated lagoons. Finfish from coastal aquaculture represents 37% of the combined production from marine and coastal aquaculture, whereas finfish from inland aquaculture represents 12% of total inland production. Notably, fish species from aquaculture production have been evolving over the years with the increase of new technologies in the sector, since some species farming conditions are more easily to replicate and control than others. Currently, significant advances in the development and improvement of integrated inland aquaculture farming systems are gaining interest not only due to improved productivity and resource-use efficiency, but also due to its potential to reduce environmental impacts (FAO, 2022).

The average annual growth rate of total seafood consumption has increased at a higher rate than the growth of the world population, and fish contribute to almost 20% of the average *per capita* intake of animal protein (FAO, 2020, 2022). Despite the overall increase in the world seafood *per capita* consumption, upper-middle-income countries experienced a stronger annual growth (3.2%) compared to lower-middle-income countries that experienced slower annual growth (1.9%). Nevertheless, consumers in developing countries revealed higher share of fish protein compared to total animal proteins in their diets relatively to consumers in developed countries (FAO, 2022). In 2019, the highest record of global annual *per capita* consumption of seafood products (20.5 kg) was achieved. The increased demand for seafood products can be associated to several factors, including new technologies in processing and cold chain, improvement in distribution and trade of commodities, and increased awareness of fish health benefits among consumers (FAO, 2022; FAO et al., 2021). However, seafood products consumption rates differ between regions and countries, with higher consumption levels generally associated to coastal areas. Indeed, Asia recorded the highest total fish apparent consumption, with China accounting 36% of the world *per capita* fish consumption, followed by Europe and America (Figure 1.4). Nevertheless, it is worth mentioning that countries with higher consumption levels (more than 50 kg/year) including Portugal, Norway, Greenland, Malaysia, Korea, Iceland, Faroe Islands, and Maldives, in which the last three consumed over 80 kg of seafood *per capita* per year (EUMOFA, 2021; FAO, 2022; OECD/FAO, 2022).

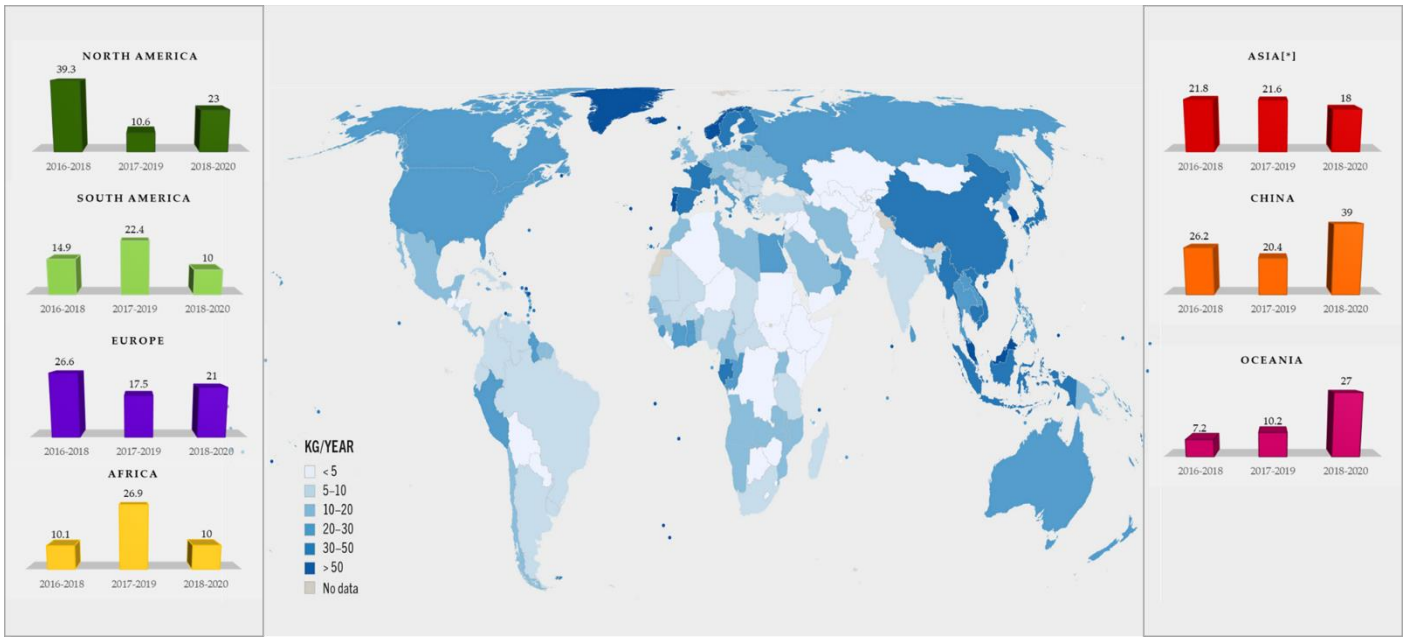

Figure 1.4 - Apparent seafood *per capita* consumption (kg per year) between 2016 and 2020 (adapted from FAO).

Note: data for Asia excludes de average per capita in China, which is represented individually.
Sources: The State of World Fisheries and Aquaculture (FAO, 2020), OECD-FAO Agricultural Outlook OECD Agriculture statistics (OCDE/FAO, 2021)

It is also important to mention that the continuous rising of aquaculture production has been supporting the observed increase in seafood consumption. Moreover, the increased trend for health and nutritious foods habits, accompanied by the increasing attention of consumers and major distributors to the sustainability and security of seafood systems and products, strengthen the aquaculture sector role, by supporting the availability of such products for domestic seafood consumption that meet consumers demand and needs (FAO, 2022).

1.2.2 Aquaculture sustainable development: the 21st century

Sustainable aquaculture development is critical to further increase the global aquaculture production and consumption without exhausting the planet natural resources. By 2030 it is expected that the World population will reach 8.5 billion of people, the annual fish consumption exceeds 21.5 kg *per capita* and an increase in 18% of global fish consumption (FAO, 2022). Additionally, there is a growing awareness of consumers for sustainable, safe and high-quality health habits, driving the development of eco-friendly, traceable, and certified seafood products (FAO et al., 2021). In this sense, the aquaculture sector must start making plans for a sustainable expansion to reduce supply-demand gap for seafood, especially in developing countries, without compromising the existing sources of income (FAO, 2022). Aquaculture feeds are costly resources and the main factor to promote undesirable environmental impacts. In aquaculture, the production of high-value species, such as salmon and seabass (carnivorous species), rely on extruded aquafeeds formulated with large amounts of fishmeal and fish oil derived from wild pelagic fish resources (Boyd et al., 2020; Cabral et al., 2011; FAO, 2022; Nagarajan et al., 2021; Nasopoulou & Zabetakis, 2012). In fact, approximately 10-16% of wild pelagic fish resources are converted in fishmeal and fish oil, posing an increased risk for overfishing of specific fish species for feed proposes (FAO, 2022; Nagarajan et al., 2021). World fishery resources within biologically sustainable levels is one of the major concerns of the century, once by 2019 marine stocks at biologically unsustainable levels increased 35.4% and biologically sustainable stocks have been continuously decreasing (Figure 1.5).

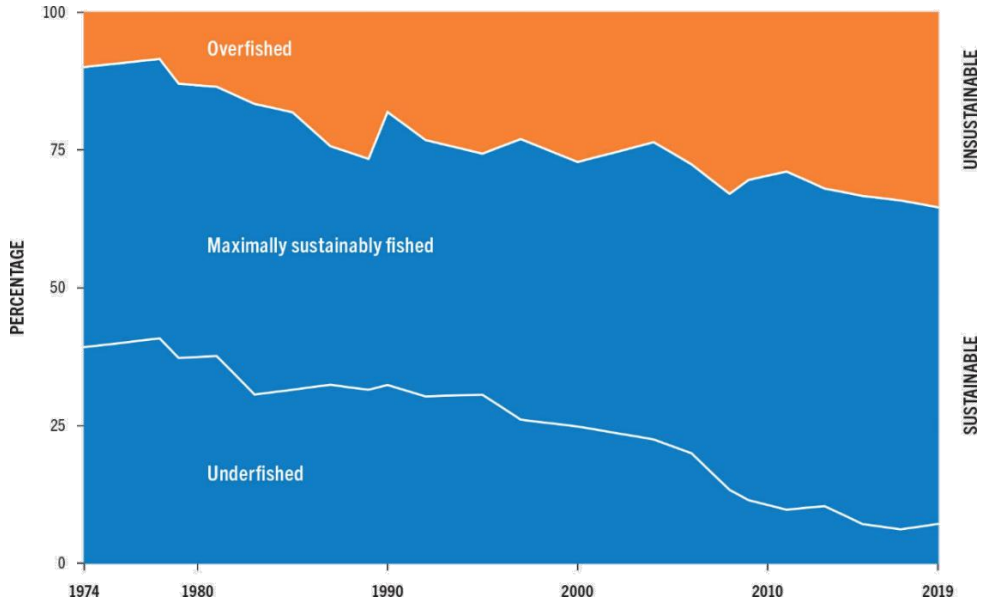

Figure 1.5 - Global trends in world's marine fish stocks, according to sustainable exploitation, between 1974 and 2019 (FAO, 2020).

Fishmeal and fish oil relevance in aquaculture feeds formulation derive from its highly digestible ingredients, including higher contents of essential n-3 LCPUFAs, that play important roles in fish health and growth, as well as in human health (FAO, 2022; Nasopoulou & Zabetakis, 2012). Fishmeal and fish oil are mainly produced from large amounts of whole fish, fish trimmings or other fish processing by-products from different pelagic fish species (FAO, 2022). However, the global production of fishmeal and fish oil seems to have reached their limit of sustainability, leading to a clear downward trend in their inclusion rates in aquaculture feeds formulation (Bell & Waagbø, 2008; Nasopoulou & Zabetakis, 2012). Due to the increasingly demand from the aquafeed industry and overfished stocks, fishmeal and fish oil production has been suffering supply and price variations, leading to a selective use mostly in specific stages of production, such as for hatchery, broodstock or finishing diets (FAO, 2022). In this sense, to support the expansion of the aquaculture sector it is crucial to develop additional and cost-effective sources of feed ingredients to meet the rising demand of the sector and to be less dependent from marine raw materials that are currently unavailable or used elsewhere (Alagawany et al., 2021; Bell & Waagbø, 2008; Cabral et al., 2011; Nasopoulou & Zabetakis, 2012).

In today's world, new trends for achieving synergies between aquaculture and marine ecosystems are being developed to potentially replace the high-cost and unsustainable fishmeal and fish oil ingredients by novel animal feeds, with less environmentally burdensome

ingredients, such as plant by-products, seaweeds, microalgae, insects, animal by-products, and single-cell proteins from bacteria and yeast (Boyd et al., 2020). Although the optimization of sustainable and nutritious feeds is crucial for aquaculture development to increase the share of the sector's output, the new alternatives of protein sources for aquaculture feeds formulation need to be, not only environmentally, but also economically viable. In this sense, several criteria need to be taken into consideration in the formulation of eco-Innovative fish diets, namely, nutritional attributes (i.e., highly digestible, nutritious, and adequate to grow and promote well-being of the farmed species), palatability, storing, handling, and most important life cycle assessment (i.e., environmental and life cycle impacts). Another important factor to improve the efficiency and sustainability of aquaculture sector, consists in reducing the large proportion of the production losses or waste, which accounts for 35% of the global harvest (FAO, 2022). In this context, aquaculture needs to optimize good environmental practices and the efficiency in the use of natural resources by reducing the use of water, land and wild fish use per unit of farmed product output, as well as their dependency of fossil fuel along the entire value chain (Godin et al., 2015). Another important factor that will have great impact on the future of aquaculture is the climate change events. In fact, increasing frequency of warming ocean conditions, ocean acidification, hypoxia, and harmful algal blooms have negative effects on seafood production, resulting in worldwide declines regarding marine aquaculture (Froehlich et al., 2018).

Finding ecologically sustainable and food security development by implementing a system approach that links environmental, human and animal health (One Health Concept), will contribute to provide seafood with acceptable levels of healthy nutrients and bioactive compounds without compromising food security and sustainability of marine resources, as well as improve marine aquaculture resilience to climate change challenges, especially for low-income coastal communities (Fiorella et al., 2021, Gormaz et al., 2014).

1.3 Seafood role in human nutrition

Seafood is one of the most important sources of proteins with high biological value and essential nutrients, representing a crucial food item for approximately 17% of the world's population (about 1.2 billion people) (FAO, 2022). Seafood contains all essential amino acids and is an excellent source of other valuable nutrients, including iodine and selenium, compared to

mammals (Petricorena, 2015; Rehbein & Oehlenschläger, 2009). The regular consumption of seafood products is associated to nutritious diets and healthy dietary behaviours.

1.3.1 Fish nutritional value

The nutritional composition of fish varies greatly among species specimens, age, gender, environment, and season (Petricorena, 2015). In general, fish are naturally identified as a rich source of all amino acids, n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) and minerals such as iodine, selenium, zinc, iron, copper, and calcium (Figure 1.6) (EFSA, 2014a). The main nutrients available in fish muscle are proteins (16–21%), lipids (0.5–2.3%), ash (1.2–1.5%), and water (52–82%). In contrast, carbohydrates (0.5%) and cholesterol (0.04%) contents are usually low (Petricorena, 2015).

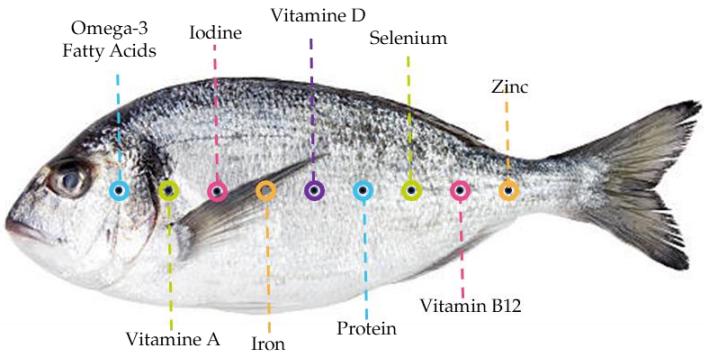

Figure 1.6 - Fish as rich source of essential elements (adapted from FAO, 2020).

Fish protein content is highly digestible due to the low amount of connective tissue (1–2 %) compared to mammals´ meat (10–13 %) (Petricorena, 2015). Among fish muscle proteins, the highly salt soluble myofibrillar proteins correspond to 65–75% of the total muscle protein (Rehbein & Oehlenschläger, 2009). In terms of amino acids, fish has high biological value, containing well-balanced amino acid composition, significant amounts of lysine and leucine (essential), and aspartic and glutamic acids (nonessential) (Petricorena, 2015). On the other hand, fat content shows a great variability depending on species, season, geographic area, age, gender, maturity and feed composition and availability (Zand et al., 2015). Compared to land-based animals' food, fish has lower fat content and higher levels of n-3 LCPUFA, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Zand et al., 2015). As observed in Table 1.1 fish also presents lower cholesterol levels and higher polyunsaturated fatty acids (PUFA) levels than land-based animals. Fish can be classified in three categories considering

their lipid content: a) lean fish, including cod, haddock, and hake, with a lipid content below 2%, b) moderate fatty fish, including seabream, seabass, carp, or trout, with a lipid content between 4% and 8%, and c) fatty fish, including salmon, sardine and mackerel, with a lipid content above 10% (Ackman, 1994). Lean fish species fat content is retained as energy reservoir in the liver and consists mainly of polar lipids, whereas fatty fish species have a higher proportion of neutral lipids (triacylglycerols) being mainly deposited in the lipid bilayer of the cell membranes in the muscle tissue. Fatty fish species also present higher contents of polyunsaturated fatty acids (PUFAs), which are more susceptible to lipid oxidation (Rehbein & Oehlenschläger, 2009).

Table 1.1 - Proximate composition in 100 g (wet weight) of selected food products.

							Fatty acids		
	Energy (kcal)	Water (g)	Ash (g)	Protein (g)	Fat (g)	Cholesterol (mg)	SFA (g)	MUFA (g)	PUFA (g)
Meat and egg									
Beef sirloin steak	212	64	1.00	19.95	14.53	67	5.55	6.13	0.20
Pork loin	106	75	1.06	22.34	1.90	60	0.76	0.85	0.14
Chicken flesh	110	75	1.10	20.33	2.70	65	0.68	0.84	0.65
Egg	143	76	0.85	12.40	9.96	411	3.20	3.63	1.82
Lean fish									
Atlantic Cod	75	81	1.21	17.55	0.58	43	0.10	0.04	0.24
European hake	69	82	1.30	16.30	0.40	n.a.	0.09	0.08	0.11
Moderately fatty fish									
Gilthead seabream	108	77	1.20	16.70	4.00	66	1.67	2.87	2.76
Common carp	127	66	1.46	76.30	5.60	66	1.08	2.33	1.43
Rainbow trout	132	74	1.25	17.94	6.70	n.a.	1.47	2.59	1.93
Fatty fish									
Sardina pilchardus	187	71	1.30	17.90	10.90	28	2.75	2.56	4.07
Mackerel	187	68	1.21	17.83	12.89	66	2.99	5.07	3.55
Atlantic Salmon	228	65	1.60	15.85	18.33	52	2.21	8.05	4.50

n.a. - data not available

Sources: U.S. Department of agriculture, FoodData Central (http://fdc.nal.usda.gov, accessed on 11 September 2022) and Composition Databank (http://frida.fooddata.dk/?lang=en, accessed on 11 September 2022). Note: Values are indicative for each species and may vary depending on the source.

Fish are also a good source of vitamins B (B6 and B12) A and D. Vitamin content in fish is similar to land-based animals, with the exception of vitamin A and D, that are higher in fatty fish (Figure 1.7). In general, vitamins content is mainly associated with the fat content in the fish muscle, as highest levels of fat leads to higher fat-soluble and water-soluble vitamins (Lall, 2003; Rehbein & Oehlenschläger, 2009).

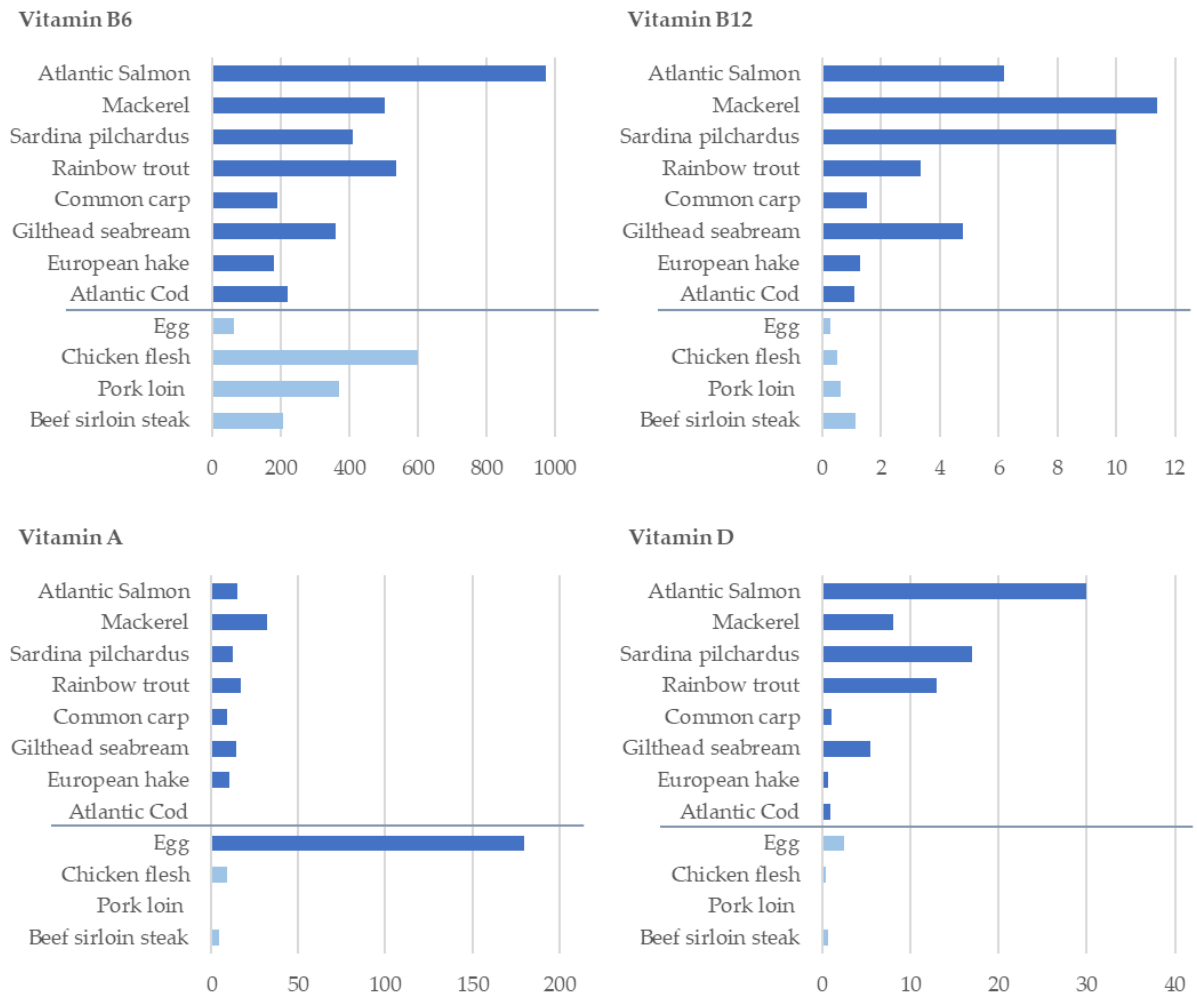

Figure 1.7 - Vitamins content in selected food products (µg per 100 g of edible part)

Sources: U.S. Department of agriculture, FoodData Central (http://fdc.nal.usda.gov, accessed on 11 September 2022) and Composition Databank (http://frida.fooddata.dk/?lang=en, accessed on 11 September 2022). Note: Values are indicative for each species and may vary depending on the source.

The relevance of fish to minerals intakes by humans is mainly due to the high levels of essential elements. Fish assimilate important macro and trace elements through the diet and aquatic environment by gills and skin, and, especially marine species, are a natural source of iodine and selenium (Petricorena, 2015). Iodine (I) and selenium (Se) are two of the most

important elements for human health and well-being, since one-third of the world population present deficiencies of these elements, and it is widely acknowledged that insufficient intake of I and Se leads to several human health disorders and diseases, such as goitre (I deficiency) and autoimmune thyroid conditions as hypothyroidism (I and Se deficiency) (EFSA, 2014b,c). Other important minerals usually found in fish nutritional composition are calcium (Ca), magnesium (Mg), sodium (Na), and potassium (K), as macro elements, and zinc (Zn), copper (Cu), iron (Fe), and manganese (Mn), as trace elements. Notably, fish can be highly relevant in some minerals intake, since some species can be consumed in different presentation formats, including the whole fish and fillets, and both muscle, skin, and liver concentrate most essential macro and trace elements (Petricorena, 2015). Nevertheless, fish elemental contents differ within species and are closely related with seasonal and biological factors (size, age, gender, and sexual maturity), food habits (including the composition and availability) and environmental conditions (water chemistry and temperature) (Godswill et al., 2020). In fact, macro and trace elements concentrations in fish muscle are mainly associated with the rate of availability in the aquatic environment and the metabolic/absorption rate of these nutrients from their diets and from the surrounding water (Lall, 2003). In general, compared to land-based animals, fish is rich in I and Se (Figure 1.8). Moreover, marine species have much higher I and Se contents than freshwater species, since freshwater species acquire these elements by absorption of dietary minerals from the gastrointestinal tract, whereas marine species acquire these elements through the diet and environment (Lall & Kaushik, 2021).

Concerning n-3 LCPUFA, EPA and DHA are key components, which are mainly originated from primary producers (phytoplankton), being available at high levels in marine fish. Both fish and humans have limited capacity to convert the alpha-linolenic acid (ALA, 18:3 n-3) into n-3 LCPUFA, restricting the assimilation of these fatty acids through the food chain (Sargent et al., 2003). In terms of trace elements, Se and I are mostly absorbed by fish from water and secondly by food sources, being two of the most important minerals for all living organism's development and metabolism. Furthermore, Fe is the most abundant trace element, followed by Zn, being assimilated by fish through food and water. Both elements are essential to all cellular and molecular mechanisms, improving fish and human health and well-being (Lall, 2003).

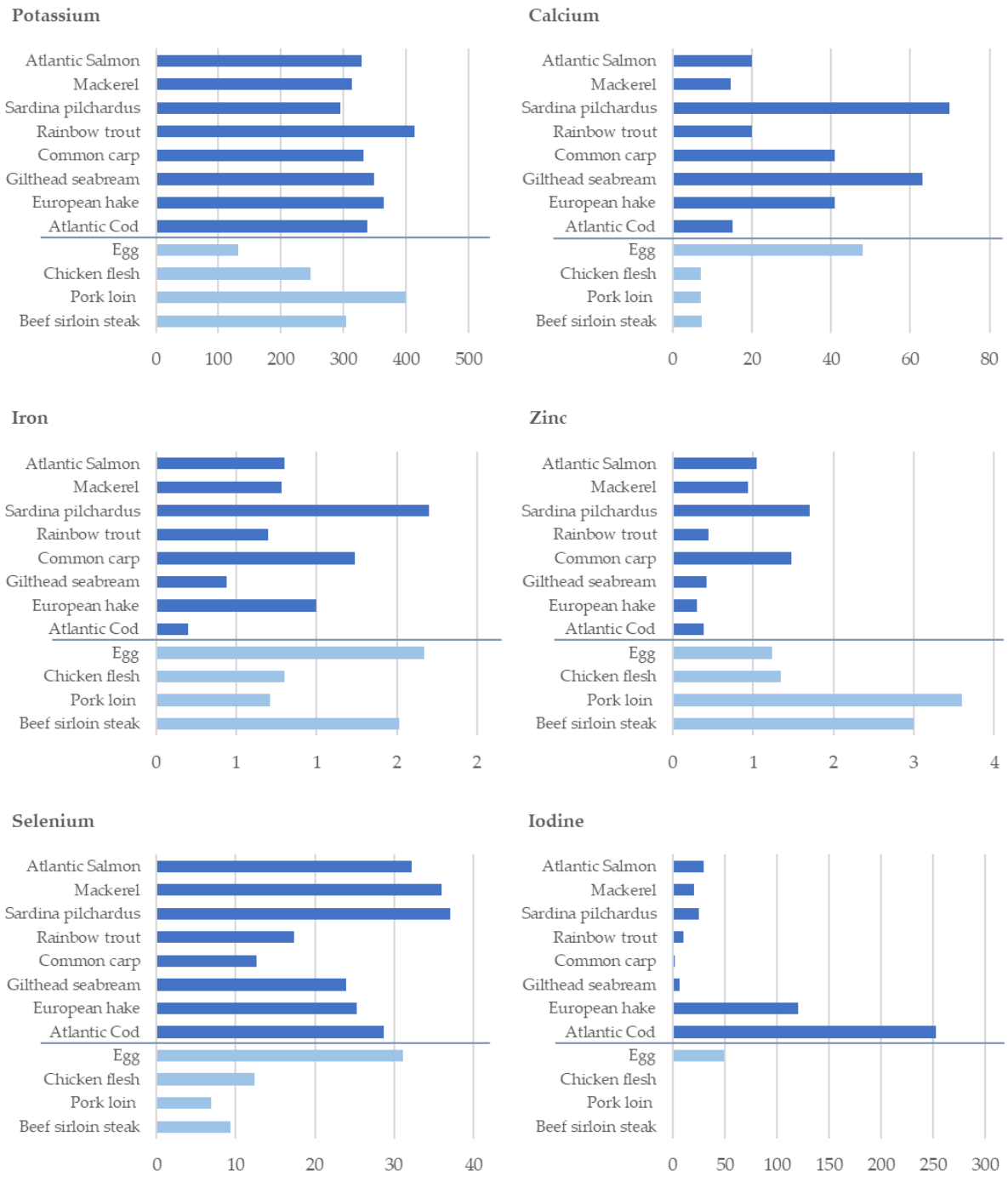

Figure 1.8 - Minerals content in selected animal food products (K, Ca, Fe and Zn in mg per 100 g of edible part; Se and I in µg per 100 g of edible part)

Sources: U.S. Department of agriculture, FoodData Central (http://fdc.nal.usda.gov, accessed on 11 September 2022) and Composition Databank (http://frida.fooddata.dk/?lang=en, accessed on 11 September 2022). Note: Values are indicative for each species and may vary depending on the source.

1.3.2 Fish consumption and health benefits

Fish provides essential nutrients covering human intake requirements allowing to prevent malnutrition and to improve physiological functions and health benefits (FAO/WHO, 2011). Indeed, the dietary habits have significant impact in the world population healthy life (Figure 1.9).

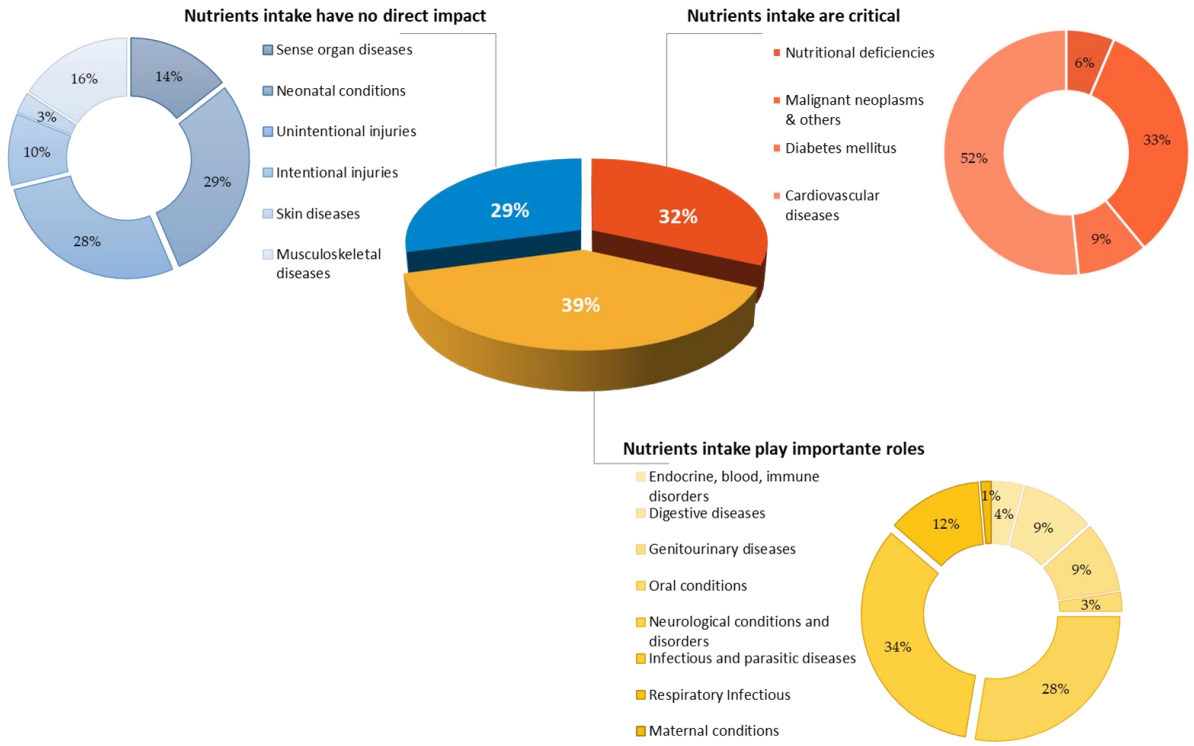

Figure 1.9 - Influence of nutrients in diet-related diseases.

Sources: Global Health Estimates 2019 (WHO, 2020).

The essential role of fish and shellfish in human nutrition are mainly associated to key nutrients, especially n-3 LCPUFA, iodine, selenium, and vitamin D. Most dietary guidelines recommend the consumption of two portions of fish (approximately 300 g) per week, one of which should be a fatty fish, to comply with the nutritional requirements (EFSA, 2014a). Still, for specific population groups, namely children, elderly, and pregnant/lactating women, some health authorities recommended four to seven portions of fish per week to improve functional outcomes of neurodevelopment and cardiovascular health (EFSA, 2014a, 2015a; USDA, 2020). Strong evidences suggest that a daily intake of 200 to 500 mg EPA plus DHA lowers the risk of mortality from cardiovascular health diseases (CHD) and sudden cardiac death by 36% (EFSA,

2014a). A correlation between EPA/DHA intakes and beneficial effects has been documented, particularly on improved carotid artery plaques stability, cell membrane fluidity by changes in phospholipids composition, insulin sensitivity and reduction of inflammatory markers (Hosomi et al., 2012). Moreover, n-3 LCPUFA improve body weight, reduce the risk of obesity, and recent studies suggest that EPA+DHA intake may be involved in neurotransmission and neuroprotection, potentially preventing neurodegenerative conditions, such as Alzheimer's disease (Dyall, 2015). Dietary intake of essential elements, such as iodine, selenium, and iron, are also associated with improved human health benefits (Table 1.2). These elements are of major importance in healthy diets, once they cannot be synthesized by the body and therefore must be taken from food or, in some cases, by supplements (Godswill et al., 2020). Within essential elements, iodine is crucial in human diet, with important roles in thyroid hormones synthesis, as well as in neurological and cognitive neurodevelopment. Marine environment is the major reservoir of I and the highest levels are found in marine algae (seaweeds) (EFSA, 2014b; Lall, 2003). In terms of human diet, both marine fish and shellfish are rich sources of I. The dietary adequate Intakes (AI) for most population groups range between 70 μg day^{-1} and 130 μg day^{-1}, whereas for pregnant/lactating women an AI of 200 μg day^{-1} is recommended due to additional requirements for thyroid hormones synthesis and embryogenesis neurodevelopment (EFSA, 2014b). Insufficient intakes of I result in several iodine deficiency disorders (IDD), including hypothyroidism or hyperthyroidism (goitre), thyroid cancer, mental impairment, and impaired fetal development during pregnancy. Iodine deficiency remains a major public health burden since affects approximately 50 million people all over the world causing preventable brain damage (Godswill et al., 2020). On the other hand, excessive iodine is also associated to sub-clinical thyroid disorders, including increased of autoimmune thyroiditis and the risk of thyroid cancer (EFSA, 2014b). Selenium is a powerful antioxidant element and highly important for many metabolic pathways, including thyroid hormone metabolism, cerebral functions, and immune system (EFSA, 2014c). Moreover, strong evidence suggest that adequate Se intake prevents cardiovascular diseases, risk of cancer and also promote heavy metal detoxification (Zand et al., 2015). On the other hand, excessive Se intakes can be extremely toxic for human health, resulting in selenosis and hair loss, nausea, fatigue, and nerve damages. Selenium dietary adequate Intakes (AI) range between 15 μg day^{-1} and 70 μg day^{-1} for most population groups, whereas for pregnant/lactating women an AI of 85 μg day^{-1} is recommended (EFSA, 2014c). Although most dietary Se is absorbed efficiently (70- 95%), the organic compounds, such as selenomethionine and selenocysteine (animal and plant sources), present higher retention, compared to inorganic compounds, such as selenate and selenite (supplements) (EFSA, 2014c; Zand et al.,

2015). Globally, over 1 billion people is affected by increased risk of cancer, reduced fertility, oxidative stress and immune disorders, due to Se deficiency (Alegría-Torán et al., 2015). Iron and Zn are also essential elements with major roles on human health. Iron is the main compound of haemoglobin (animal red blood cells) and muscle myoglobin, being an essential nutrient for oxygen transport, oxidase activities and cellular energy generation, and playing important roles in cognitive development (EFSA, 2015b; Zand et al., 2015). Iron dietary intake consists in two forms, as haem Fe, which is mainly found in meat, being highly available (25–30%) and non-haem Fe, which is found in both animal and plant products, with wider range of absorption rates (1-10%) (EFSA, 2015b). According to the World Health Organization (WHO) approximately 22.8% of the world population suffers from Fe shortage (anaemia), due to inadequate dietary Fe intake (WHO, 2021). On the other hand, Zn is a component of several enzymes and play important structural and regulatory roles in proteins and gene expression (EFSA, 2014d; Godswill et al., 2020). In addition, Zn is of major importance in antioxidant and anti-inflammatory systems. Nonetheless, limited Zn intake affects both developed and developing countries, and may lead to growth retardation, male hypogonadism cell-mediated immune dysfunction, and abnormal neurosensory changes. In contrast, excessive Zn intake may result in Cu deficiency, which is a vital element for the electron transfer process in the central nervous system (Zand et al., 2015).

Table 1.2 - Dietary recommendations of selected essential trace elements and their potential benefits to human health.

Trace element	*RDA (mg day^{-1})*	*UL (mg day^{-1})*	*Principal dietary sources*	*Principal role/Health benefits*
I	0.070-0.150	0.200-0.600	Seaweed, seafood, eggs, iodized salt, grains	Energy metabolism and prevention of IID (i.e., goitre, cretinism, impaired mental development)
Se	0.015-0.070	0.060-0.300	Bread, cereal, meat, seafood dairy products, eggs	Antioxidant enzymes activity, regulation of thyroid hormone action
Fe	6-8	30-45	Meat, seafood, nuts, beans, dark chocolate	Cellular metabolism (as part of cytochrome enzymes) and prevents microcytic hypochromic anaemia
Zn	2.4-12.7	7-25	Oysters, red meat, nuts, whole grains, poultry, dairy products	Protein synthesis, control of differentiation, immune system function, acts in many enzymes involved in macronutrient metabolism and in sexual maturation. Required for normal flavour sensation

Sources: EFSA, 2014b, 2014c, 2014d, 2015b; Zand et al., 2015

There is evidence that a diversified seafood-based diet is the best approach to comply with consumer's nutritional requirements and needs, with positive impacts in preventing several diseases (Figure 1.10). In addition, the consumption of seafood is regarded as the main natural source of health-valuable nutrients (n-3 LCPUFA, I and Se), being increasingly recommended as the natural alternative to the ingestion of food supplements (Hosomi et al., 2012; Larsen et al., 2011).

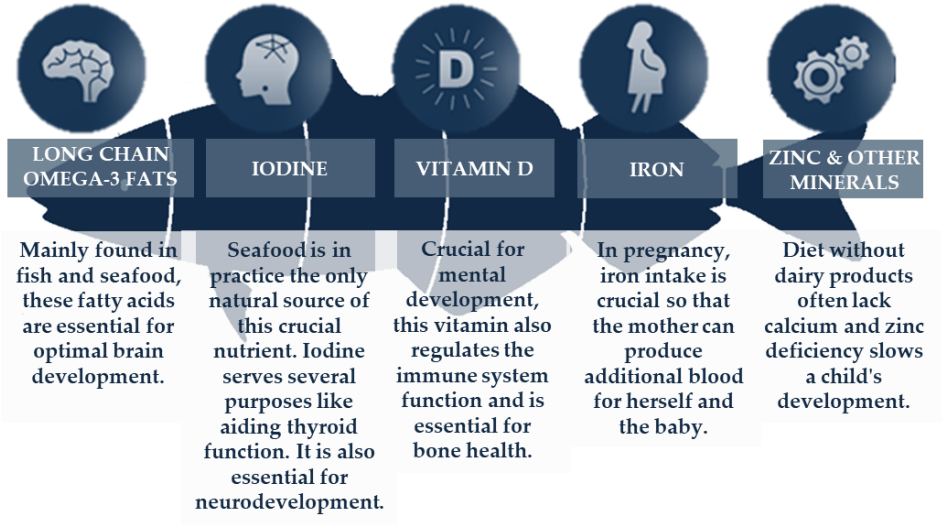

Figure 1.10 - Health benefits associated with the consumption of a marine fish-based diet. Adapted from The State of World Fisheries and Aquaculture 2022 (FAO, 2020).

1.4 Biofortification: improving farmed fish nutritional value

The concept of fortified food is usually based in the addition of one or more nutrients to particular foods (vehicles) to enhance its nutritional composition in amounts that are close to those provided by a good, well-balanced diet (FAO et al., 2021). In this context, the potential to develop farmed eco-innovative products with improved nutritional value and targeting specific population groups, such as tailor-made farmed fish, is gaining interest. It is known that, under farming conditions, fishmeal and supplements can effectively modulate the composition of fish edible part in terms of essential nutrients for optimal fish nutrition and welfare (Allen et al., 2006; Saltzman et al., 2013; Tocher, 2015). So far, aquaculture feeds have mostly targeted fish welfare and productivity, without considering benefits for consumers' health. Therefore, the development of innovative tailor-made farmed fish integrating consumers' dietary needs

through the incorporation of sustainable and natural ingredients when designing aquafeeds, unlocks the possibility to create novel food products that are safe, nutritious, and able to increase the trust of consumers in farmed products.

1.4.1 Tailoring farmed fish with health-valuable nutrients

Several research has been developed to design specially formulated foods that promote optimal health and reduce the risk of disease (Allen et al., 2006). In terms of seafood, different approaches are being developed to effectively modulate fish fillets with bioactive fatty acids (Dantagnan et al., 2009; Ramos et al., 2008; Rosa et al., 2010), selenium (Cotter et al., 2009; Elia et al., 2011; Pacitti et al., 2015; Ramalho Ribeiro et al., 2017; Schram et al., 2010) and iodine (Julshamn et al., 2006; Ramalho Ribeiro et al., 2015; Valente et al., 2015). Such approaches include the supplementation of aquafeeds, using microalgae blends (EPA and DHA-rich microalgae), seaweed (I-rich macroalgae) and yeast (Se-rich yeast) as ingredients in feeds formulation (Dantagnan et al., 2009; FAO, 2018; Ramalho Ribeiro et al., 2017). As previously mentioned, the current trend in aquaculture production is to replace fishmeal and fish oil (due to the high cost and unsustainability of marine-derived ingredients) by sustainable protein and lipid sources. Vegetable ingredients have been widely used as cost-effective alternative sources to reduce the dependency from marine ingredients (Cabral et al., 2011; Gouveia & Davies, 2000; Kaushik et al., 2004), despite still being often characterized by having high protein fibre contents, low levels of n-3 LCPUFA, I and Se, as well as delivering nutrients with low absorption rates for carnivorous fish (FAO, 2018; Van Paemel et al., 2010). On the other hand, marine ingredients such as microalgae and seaweeds (macroalgae) are natural sources of bioactive compounds, antioxidants, vitamins (e.g., A, B12, D and folic acid), minerals (e.g., I, Se, Fe and Ca) and phytochemicals (Alagawany et al., 2021; Ramos et al., 2008; Roohinejad et al., 2017). The potential of using seaweeds and seaweed extracts as functional ingredients to improve health-related properties of several food products are gaining interest (Roohinejad et al., 2017). Previous studies suggest the inclusion of seaweed species as alternative ingredients in diets for several fish species. The inclusion of *Gracilaria bursa-pastoris* (red algae), *Ulva rigida* (green algae) and *Gracilaria cornea* (red algae) revealed no adverse effects on growth performance and feed utilization efficiency in European sea bass juveniles (Valente et al., 2006). In contrast, the inclusion of *Macrocystis pyrifera* (brown algae) contributed to increased levels of PUFAs, especially EPA and DHA, in rainbow trout (Dantagnan et al., 2009). The inclusion of *Gracilaria vermiculophylla* (red algae) resulted in decreased lipid content and increased iodine content in rainbow trout (Valente et al., 2015). At last, the inclusion of *Laminaria digitata* (brown algae)

contributed to increased iodine levels in chars (Schmid et al., 2003), gilthead seabream (Ramalho Ribeiro et al., 2015) and in rainbow trout (Ramalho Ribeiro et al., 2017). Within seaweed, brown algae are important sources of fucoxanthin, that possess anti-cancer, anti-obesity, and anti-inflammatory effects, and *Laminaria* spp., is an important source of I, Mg, Ca and vitamins A and B (Pereira, 2011). On the other hand, it is known that some seaweed species exhibits high affinity to accumulate inorganic arsenic (iAs), thus the potential risk of human exposure to this toxic element through dietary sources (Alves et al., 2018). Previous studies demonstrated that aquafeeds supplemented with organic iodized salt (EDDI) and inorganic potassium iodide (KI) resulted in increased I content in gilthead seabream fish muscle (Ramalho Ribeiro et al., 2015) and in Atlantic salmon (Julshamn et al., 2006). Successful Se fortification was also achieved with organic Se sources, such as SeMet in rainbow trout (Rodríguez & Rojas, 2014) and in grouper, as well as with an inorganic Se source (Na_2SeO_3) in juvenile grouper (Lin, 2014). Moreover, multiple fortification of I and Se from organic sources (I-rich seaweed and Se-rich yeast, respectively) resulted in increased contents of both elements in trout fillets without altering sensorial traits (Ramalho Ribeiro et al., 2017). The advantage of using sustainable sources from natural ingredients (biofortification), such as seaweed and yeast, is related to their higher nutrients' bioavailability (Rider et al., 2009) and the potential use as functional ingredients (Figure 1.11).

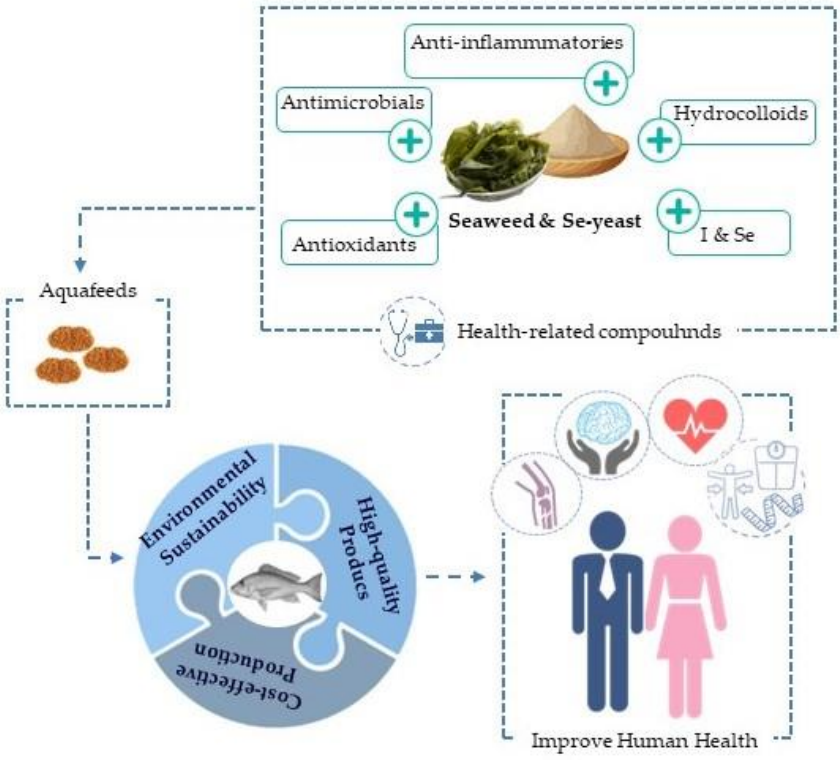

Figure 1.11 - Application of seaweed and selenised-yeast ingredients to develop biofortified farmed fish products

Although fish fortification through aquafeed modulation might be a feasible, cost-effective, and sustainable strategy to improve farmed fish nutritional quality, some limitations still exist. Food fortification effectiveness depends on several factors, including the nature of the food vehicle, the fortified nutrient origin, dose, and form (inorganic versus organic), and the potential interactions with other compounds during fortification processes (Allen et al., 2006; Pinkaew & Karrila, 2015; Ramalho Ribeiro et al., 2017). Additionally, the bioavailability of fortified nutrients and interactions between multiple nutrients fortification (mixtures) can adversely affect nutrients stability and the organoleptic qualities of the fortified food (Allen et al., 2006). This is not simple since antagonistic (e.g., Zn and Cd) or synergistic (e.g., Fe and Cu) relationships among nutrients occur. Other interactions between elements, such as Se high affinity for certain toxic elements (e.g., Hg) result in Se protective effect against toxic compounds by forming complexes with decreased availability (Alves et al., 2018; Lall, 2003; Lall & Kaushik, 2021; Ralston et al., 2016). In addition, a complex interaction between Se and I may occur, since Se is an essential component of the enzyme tetraiodothyronine 5'-deiodinase, which is involved in I metabolism (Carvalho et al., 2015). Moreover, direct positive interactions between Se, Zn and Cu have been recognized in structural processes, as well as I interactions with Zn and Fe in the

thyroid function (Rizzi et al., 2005). In this context, when developing biofortified seafood products the interaction of minerals with other nutrients should be considered due to their lability and tendency to form chemical bonds (Lall & Kaushik, 2021), as well as the potential of toxicities through excess intakes of some nutrients (i.e., I, Se, and Fe) (EFSA, 2014c, 2015b).

1.4.2 Biofortified fish as functional food to consumers' healthier diets

Accordingly with health authorities, functional foods are those that when consumed as part of a varied diet on a regular basis, provide health benefits beyond the supply of essential nutrients (e.g., vitamins and minerals) (Hasler, 2002). Food fortification is one of the most valid approaches to improve the nutritional status of individuals or targeted population groups, having the potential to provide the fastest supply of micronutrients at adequate levels to improve micronutrient status with minimal risk to health (Allen et al., 2006). Compared to supplements (e.g., pills), food biofortification has the advantage of containing "natural" or near natural levels of micronutrients, and when consumed on a regular basis will maintain more efficient and effective the nutrients supply compared to intermittent supplements (Allen et al., 2006). Still, to be considered as functional food, biofortified seafood products need to present added physiologic benefits, enabling the consumers to have healthier life (i.e., reducing chronic disease risk) without changing their eating behaviour (Hasler, 2002). As previously mentioned, fish is one of the most healthier food item, and the potential to tailor farmed fish muscle properties with health beneficial compounds may promote fish as an excellent carrier of health-valuable nutrients (Siró et al., 2008). Seafood, especially marine species, significantly contribute to the daily intake of multiple beneficial compounds, especially n-3 LCPUFA, I and Se. Nevertheless, even essential elements can display toxicity in concentrations above the required levels for organisms' biological functions. In this sense, health authorities set dietary reference intakes (DRIs) for minerals as recommendations for healthy individuals, considering the nutritional value of a food item as the equilibrium between nutrients intake (i.e., nutrients levels in food) and nutrients assimilation (i.e., nutrients levels that are absorbed and become available for use and storage in the body).

The perceived healthiness of fish products combined with sustainable and natural ingredients enrichment are key factors for consumer acceptance of biofortified farmed fish products as novel functional foods in a growing market (Krutulyte et al., 2011; Ramalho Ribeiro et al., 2019; Siró et al., 2008). Currently, consumers receptivity and purchase intention rise when functional foods are associated with natural sources with less manipulation and additives (Bearth et al., 2014; Doherty et al., 2021). Moreover, in terms of consumers acceptance of functional

foods, the natural aspect and taste overlap healthiness and convenience properties, revealing the potential of seafood biofortification strategies by natural enhancement of an already present bioactive compound in the food matrix through growing conditions (Luten et al., 2008). Understanding the benefit of biofortified fish products consumption and the nutritional, physiological or other health advantage (i.e., health claims) over non-biofortified fish, supported by scientific evidence, may promote consumers' trust in innovative and sustainable farmed fish products (European Union, 2007; Urala & Lähteenmäki, 2007; Verbeke et al., 2005).

1.4.3 *In vitro* digestion of dietary compounds

The potential impact of foods on human health is related to the fraction of nutrients and bioactive compounds transferred from ingested foods into the body (Fernández-García et al., 2009). Therefore, knowing the fraction of nutrients and bioactive compounds released from the food matrix into gastrointestinal tract, becoming available for intestinal absorption (i.e., bioaccessibility) and reach the systemic circulation (blood stream) to be distributed to organs and tissues, becoming available to manifest its bioactivity and utilization in normal physiological functions (i.e., bioavailability) (Fernández-García et al., 2009; Wu & Chen, 2021). Consequently, establishing nutrients bioaccessibility is a crucial information to estimate nutrients supplementation effectiveness in designing biofortified food products to meet consumers nutritional requirements and needs (Wu & Chen, 2021). It is scientifically recognized that claims of nutritional content are mainly related to bioaccessibility and bioactive assessment. Moreover, nutrients bioaccessibility are influenced not only by the food matrix, but also by compounds, as well as synergistic and antagonistic interactions between nutrients and/or food components (Fernández-García et al., 2009). For example, Se organic compounds, namely selenomethionine (SeMet) and selenocysteine (SeCys), which were identified in several fish species, presented higher rates of gastrointestinal absorption, compared to the inorganic forms, such as selenate or selenite (Alegría-Torán et al., 2015; Luten et al., 2008). Indeed, Se-enriched yeast showed higher bioavailability than sodium selenite (Godin et al., 2015). On the other hand, the most bioavailable form of iodine is the inorganic form iodide (I^-), which is the predominant compound in marine species (i.e., fish and seaweeds) and the most bioavailable form for humans (Blikra et al., 2022; Doh & Park, 2018; EFSA, 2014b), while organic iodine, such as monoiodotyrosine (DIT) has lower bioavailability (Hou, 2009). In terms of elements Interactions, previous studies showed that Se reduce heavy metals (e.g., Hg, As) bioaccessibility, while sulphur (S) and fibres may reduce Se bioavailability due to the competition between chemically similar elemental species for specific binding sites and carriers. Moreover, possible interaction

between Se and I may occur, affecting each element metabolism and absorption directly or indirectly due to functional interrelation (Alegría-Torán et al., 2015).

Different approaches have been used to measure the bioaccessible content, differing between *in vivo* and *in vitro* methods (Cardoso et al., 2015). Within *in vivo* methods, two methodologies can be applied: 1) balance studies, which determines the bioaccessible fraction by the distinction between the input and excreted amounts of a nutrient; and 2) tissue concentration, which consists in the control of nutrients concentration in the plasma/serum (Cardoso et al., 2015; Fernández-García et al., 2009). Both methodologies require either human or animal experimental subjects and raise ethical, technical and cost constrains (Fernández-García et al., 2009). On the other hand, *in vitro* methods are a cost-effective alternative to *in vivo* methods, being less expensive, rapid, energy saving and allowing to control the experimental conditions for better reproducibility (Cardoso et al., 2015). Static and dynamic *in vitro* digestion models have been developed to simulate the human digestion process considering the three areas of the human gastrointestinal (GI) tract (mouth, stomach, and intestine) (Cardoso et al., 2015; Torres-Escribano et al., 2011). The static methodologies sequentially simulating oral, gastric, and intestinal phases with digestive juices (including the relevant enzymes in all steps, Figure 1.12) have been the most used and reliable models to evaluate nutrients bioaccessibility in seafood (Alves et al., 2018; Cardoso et al., 2015; Torres-Escribano et al., 2011). During the *in vitro* digestion models differences in enzymes, pH, salt concentrations and digestion time affect the simulating conditions. In this sense for better reproducibility, a standardized static *in vitro* digestion model was developed by the international research network INFOGEST simulation an oral, gastric and small intestinal digestion phase in sequence with controlled conditions of temperature and pH (Brodkorb et al., 2019; Wu & Chen, 2021). Although the *in vitro* static models are more convenient and cheap, and therefore widely use on a daily basis, the dynamic methodologies attempt to overcome the inherent limitations of static methods by reproducing the GI tract dynamic aspects, by simulating shear, mixing, hydration, or peristalsis conditions, mimicking the *in vivo* physical processes (Cardoso et al., 2015; Fernández-García et al., 2009). Noteworthy, *in vitro* digestion models provide results that can be correlated to *in vivo* studies, especially in terms of minerals content (Cardoso et al., 2015).

Thus, nutrients bioaccessibility is a key aspect in foods nutritional composition, given information on the amount of nutrients that is absorbed effectively. More importantly, when developing biofortified seafood products, bioacessibility provides valuable information on nutrients biofortification levels (e.g., if is sufficient, insufficient, or excessive), and if the selected matrix, as well as the nutrients chemical form used, are the most suitable ones (Fernández-García et al., 2009).

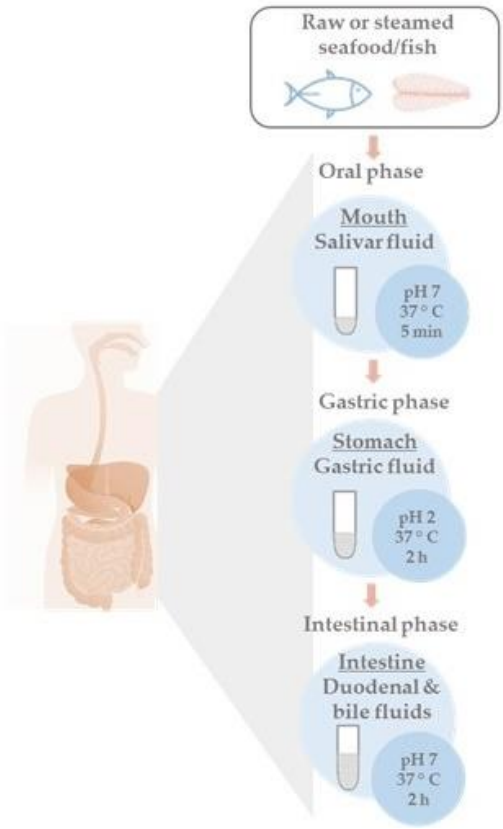

Figure 1.12 - Diagram overview of the static *in vitro* digestion model (adapted from Alves et al., 2018 and Minekus et al., 2014).

1.4.4 Farmed fish processing

Fish nutritional quality and safety may be influenced by several diverse factors, including origin, feed, and farming conditions. In terms of fish quality attributes, the main organoleptic properties (i.e., appearance, freshness, flavour, aroma, texture) and nutritional value (i.e., essential nutrients contents and toxic compounds) can be also influenced by processing and cooking procedures (Barciela-Alonso & Bermejo-Barrera, 2015; Grigorakis, 2010). Biofortified food linked with seafood processing is being optimized, especially regarding the appropriate levels,

stability, and interactions of fortified nutrients during commercial and household processing (Allen et al., 2006). In general, except for sushi, most fish products are consumed after cooking to improve palatability (i.e., flavour and taste) and to prolong products shelf life. During culinary procedures (e.g., boiling, grilling, frying, roasting, steaming) several physicochemical reactions occur improving fish digestibility and safety (Oliveira et al., 2019; Sobral et al., 2018). On the other hand, heating can lead to nutritional and quality losses, associated with muscle shrinkage through proteins denaturation, affecting the aggregation of sarcoplasmic protein and leading to the disruption of cell membrane structures and loss in water holding capacity (WHC) and leaching of water-soluble nutrients (Barciela-Alonso & Bermejo-Barrera, 2015; Blikra et al., 2020). Consequently, such changes, generally, result in fish muscle with increased tough texture and hardness (Abraha et al., 2018; Blikra et al., 2020). In general, cooking decreases moisture content and increase total protein and lipid contents. Still, cooking methods have different time and temperature parameters, that are the main factors affecting fish nutritional composition and quality. In fact, higher temperatures increase proteins denaturation, as well as loss of essential nutrients, such as minerals, vitamins, and amino acids (Abraha et al., 2018). For example, frying results in a significant decrease in the mineral content in farmed seabream due to minerals leaching into the cooking medium during exposure to oil and heat (Mnari et al., 2012). Boiling also leads to minerals losses in farmed seabream (Mnari et al., 2012) and rainbow trout (Gokoglu et al., 2004), mainly associated with minerals ex-change between fish and the water medium. On the other hand, steaming results in increased mineral content in several fish species (Alves et al., 2018). Overall, steaming has been pointed out as the healthier cooking option, since induces less changes in food nutritional quality compared with other culinary procedures such as frying or grilling (Alves et al., 2018; Barbosa et al., 2018; Maulvault et al., 2012, 2013). In terms of physical changes, cooking may also have impact on foods texture and colour.

Processing methods such as freezing also influence fish nutritional quality. Currently, there is a growing demand towards tailor-made, easy-to-prepare, ready-to-eat and ready-to-cook food products with extended shelf-life, where frozen products account 35% of fish and fish products trade (FAO, 2020). Frozen storage affects the quality of fish not only at physical levels (e.g., texture and colour), but also at chemical (e.g., nutrients contents) and enzymatic levels (Abraha et al., 2018). Frozen storage has the capacity to extend the shelf-life for of the products for long periods, but depending on several factors, such as the initial condition of fish and the frozen storage conditions (i.e., temperature, time elapsed between harvest and freezing, fish species, freezing speed) (Abraha et al., 2018; Sikorski & Sun Pan, 1994). The main changes occurring in frozen fish products are rancidity (i.e., lipid oxidation), toughening

(protein dehydration and denaturation), discoloration and desiccation (freezer burn). Additionally, nutrients interactions and their impact on organoleptic properties of fish products may affect their nutritional quality (Burgaard & Jørgensen, 2011). During frozen storage, fish muscle protein denaturation is the main chemical and structural property affecting texture, WHC, colour and flavour of frozen fish and fish products (Abraha et al., 2018; Duarte et al., 2020). Time of freezing and type of frozen storage also affects the quality of fish products. In fact, slow freezing can result in high ice crystal formation and, leading to structural damages of cell membranes, proteins denaturation, increased drip loss, WHC loss and textural changes (Nakazawa & Okazaki, 2020). Freeze-thawing process has also a direct impact on chemical reactions and muscle degradation through the formation of formaldehyde, lipid oxidation (LPO) and protein denaturation and hydrolysis, and consequently resulting in nutritional quality and antioxidant activity losses (Abraha et al., 2018; Aubourg et al., 2004; Schubring, 2005; Sikorski & Sun Pan, 1994). Additionally, decreased WHC and substantial loss of water from the fish muscle during thawing can lead to important losses of minerals combined with muscle proteins denaturation (Prego et al., 2020). Nevertheless, scientific evidence demonstrated that adequate treatments such as glazing (i.e., application of a layer of ice on the surface of a frozen product by spraying, brushing on water or dipping) and/or packaging in polystyrene bags may delay quality deterioration during frozen storage and improve the final product shelf-life up to 12 months when stored at – 20 °C (Duarte et al., 2020; Naseri et al., 2020).

Overall, different processing methods (i.e., culinary treatment, freezing and frozen storage) extend shelf life of fish products, but also affect their nutritional quality and organoleptic properties due to protein denaturation, enzymatic changes, digestibility, as well as oxidation and loss of nutrients. Consequently, quality and shelf life of processed fish products is an important criterion for consumer's acceptance of novel seafood products, such as biofortified fish.

1.4.5 Analytical methods

The analytical methodology is a key factor in food composition data, and the choice of the appropriate analytical method is crucial to ensure reliable and accurate results. In food composition analysis the method selection should be based on the following attributes: 1) reliability (specificity, accuracy, precision and sensitivity), and 2) practicability (speed, costs, technical skill requirements, dependability and laboratory safety) (Greenfield & Southgate, 2003). Within traditional methods considered appropriated for macro and trace elements analyses in food products inductively coupled plasma mass spectrometry (ICP-MS) is considered

an accurate and precise method for multi-elemental analyses, due to its well-known analytical characteristics, namely very low detection limits, multi-element quantification capabilities, the possibility of measuring several samples and isotope ratios, and the easiness to couple with chromatographic techniques (Alonso et al., 2015). Like most methods for inorganic compounds analyses, requires the extraction or destruction of the organic matter from the food matrix, removing potential sources of interference and providing concentrated form of inorganic matter (Greenfield & Southgate, 2003). Similar to ICP-MS, inductively coupled plasma optical emission spectrometry (ICP-OES) is a powerful technique to measure multi-elemental analyses in higher quantities in food products (Alonso et al., 2015).

Recently, there is a growing interest for alternative and emerging methods that provide non-destructive, high repeatability, faster analysis and low-cost techniques (Hassoun & Karoui, 2017). In this regard, fluorescence techniques, such as micro X-ray fluorescence techniques (μ-XRF) and energy dispersive X-ray fluorescence (EDXRF), provide fast and environmentally friendly alternative methods for essential and toxic elements analysis, providing a typical spectrum of each element (fingerprint) of the sample by controlling the wavelength or the photons energy (de la Guardia & Garrigues, 2015). New developments in the X-ray fluorescence spectrometry have also been made to provide two- and three-dimensional elemental imaging (2D/3D spatial elemental distribution measurement) that enable the quantification of elemental distributions within biological samples (Dias et al., 2015). Furthermore, X-ray fluorescence techniques have the advantage of presenting portable systems and no sample preparation is needed. Both characteristics are crucial for *in situ* analysis, making this technique a big support for fast environmental answers (Carvalho et al., 2020; Machado, et al., 2020; Pessanha et al., 2016).

1.5 Thesis aim and experimental approach

1.5.1 Hypothesis and objectives

Fish provides 17% of animal protein supply for the global human population. (FAO, 2022). Considering the urgent need to meet the global demand for seafood products, and the recognised importance of this food source for human health, the developing of tailor-made farmed fish biofortified with natural and sustainable ingredients presents itself as an important approach. In this sense, improving aquaculture production towards sustainable, cost-effective, feasible and high-quality seafood products is one of the main challenges of the sector to

overcome consumers nutritional deficiencies and to improve consumer's confidence in eating farmed products, while ensuring animal welfare.

To address this research topic there is an urgent need to understand if the incorporation of natural and sustainable ingredients in aquaculture feeds designing, ensures, on the one hand, more sustainable production of farmed fish, and on the other hand is able to fulfil the nutritional requirements of the population. Within this context, the present PhD thesis aims at providing an important contribution to develop an innovative tailor-made farmed fish biofortified with natural and sustainable ingredients with potential market in Europe. Particularly, addressing the efficacy of I and Se incorporation in farmed fish fed with natural ingredients. Specifically, it is aimed to:

1. Assessing the efficacy of I and Se incorporation in farmed fish fed with natural ingredients and the effect on elemental composition of biofortified fish fillets;
2. Evaluating the stability of biofortified I and Se in fish fillets during frozen storage and during culinary (steaming);
3. Understanding how biofortification influenced the bioaccessibility of different elements;
4. Validating a non-invasive method to map different elements in the body of biofortified farmed fish.

Hence, these objectives are framed by the specific research questions:

1. *Are I and Se levels effectively enhanced in farmed gilthead seabream and common carp muscle tissue through I-rich seaweed and Se-rich yeast enriched diets?*
2. *Is farmed fish nutritional profile affected using different biofortified dietary strategies?*
3. *Are biofortified nutrients stability affected by processing procedures, such as frozen storage and steam-cooking processing?*
4. *How is the bioaccessibility of elements affected by the biofortification strategy?*
5. *Is x-ray fluorescence spectrometry a suitable tool to map the distribution of elements in biofortified fish?*

1.5.2 Experimental approach

To address these objectives, three enriched diets were developed with different levels of biofortification, using I-rich seaweed and Se-rich yeast, commercially viable from the economic point of view compared to a conventional commercial diet. For each fish species, a control diet

(CTR) was formulated and manufactured by a specialized feed producing company (SPAROS, Lda) considering the nutritional requirements of adult gilthead seabream and common carp.

Adult specimens of two of the most commercially relevant farmed fish species in Europe were used as biological models for biofortification approach assays. Gilthead seabream (*Sparus aurata*) and common carp (*Cyprinus carpio*) are within the top ten species produced and consumed in Mediterranean countries and in central Europe, respectively (Figure 1.13). In fact, gilthead seabream and common carp represents, respectively 10% and 5% of European aquaculture production, sharing 7% (gilthead seabream) and 6% (common carp) of European total apparent seafood consumption (EUMOFA, 2021).

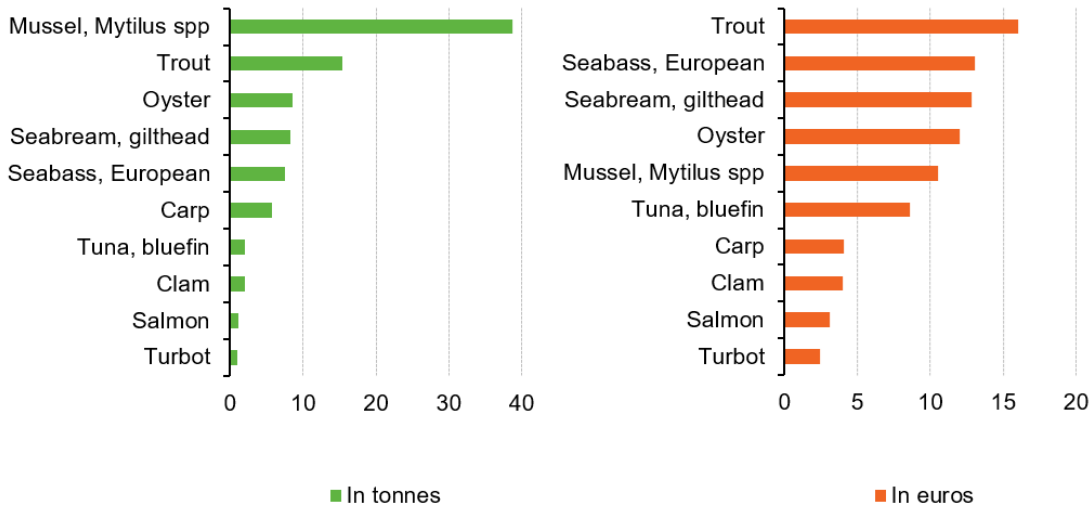

Figure 1.13 - Main species in aquaculture production in volume and value in Europe Union 2021 (Eurosat, fish_aq2a).

1.5.3 Thesis structure

The present PhD dissertation comprises seven chapters, including the general introduction, five chapters corresponding to the multidisciplinary research approach (four manuscripts and a short communication), and the final chapter with the general discussion and final remarks. The five chapters regarding the multidisciplinary research approach are interlinked and intended to establish the efficacy of the biofortification strategy along the food chain, meaning the evaluation at the farming production level (i.e., influence of the feed ingredients in fish fillet composition), along the processing chain (i.e., effect of storage and culinary treatment) and consumers health benefits (i.e., nutritional value).

In **Chapter 2**, , three enriched diets (B1, B2 and B3) were formulated based on control (CTR) diet and tested, each being supplemented with different blends of I-rich seaweed (0.40% in B1 and B2; 0.80% in B3 for seabream and 0.541% in B1, B2 and B3 for carp) and Se-rich yeast (0.015% in B1 and B2; 0.035% in B3 for seabream and 0.010% for carp), taking into consideration the maximum limit levels set by authorities (20 mg I kg^{-1} of feed and 2 mg Se Kg^{-1} of feed). To assess essential and toxic elements accumulation in biofortified fish muscle fillets through different dietary strategies, each diet was tested in triplicate and the feeding experimental trials lasted a minimum of three months, mimicking a finishing diet to be added at the end of the production stage, i.e., just before fish reaches market size. Animal condition was regularly monitored, and final sampling of whole fish was carried out per treatment (following the three R's principle: ethical testing in animal experimentation) through typical aquaculture commercial practices. Then, in Chapter 3 and 4, the stability of biofortified nutrients in fish fillets during frozen storage and during culinary (steaming) processing were evaluated in two experimental trials, considering the deposition levels of essential elements in biofortified fish muscle. The effect of steam-cooking procedure in the stability of elemental composition (i.e., I, Se, Cu, Zn, Fe, Ca, K) and fish nutritional quality was evaluated in biofortified fish for two enriched diets (**Chapter 3**), taking into consideration the previous results (**Chapter 2**) from elements deposition rate from feed to fish muscle. For each species, individually fish fillets were steamed at 105 ºC for 15 min in an oven. The steam-cooking procedure was employed since is the most reliable culinary treatment, once it allows to maintain the cooking temperature constant during all time and it is widely recognized as one of the healthiest culinary procedures. The effect of frozen storage in physicochemical quality changes (i.e., macro, trace and toxic elements content, lipid oxidation (LPO), water-holding capacity (WHC), instrumental colour and texture) was evaluated in biofortified fish from the most enriched diet (B3) (**Chapter 4**). Fish fillets or whole fish were freeze, glazed and frozen simulating the conventional industrial processing pathway. Frozen storage was carried at -20 ºC for 12-months, and samples were taken every 45 days of storage. Finally, with the purpose to evaluate the effect of biofortification on bioaccessibility of essential elements for consumers, an *in vitro* assay simulating the human digestion process was implemented (**Chapter 5**). For this trial, fish fed with a conventional commercial diet and fish fed with the best biofortification blend for each species were tested. In addition, the differences in elements distribution (multielement mapping) in biofortified and non-biofortified fish fillets was evaluated using the micro-Energy Dispersive X-Ray Fluorescence (μ-EDXRF) (**Chapter 6**). In this chapter, a preliminary study was carried out to evaluate the suitability of μ-EDXRF technique,

as non-destructive method, to assess elemental distribution with micrometer resolution for essential elements of biological interest.

Elemental composition in feeds and fish muscle (**Chapter 2-6**) was carried out through two validated methodologies: inductively coupled plasma mass spectrometry (ICP-MS) and energy dispersive x-ray fluorescence technique (EDXRF).

2

Enriched Feeds with Iodine and Selenium from Natural and Sustainable Sources to Modulate Farmed Gilthead Seabream (*Sparus Aurata*) and Common Carp (*Cyprinus Carpio*) Fillets Elemental Nutritional Value

In this chapter you will find the Manuscript:

Vera Barbosa, et al. (2020). Enriched feeds with iodine and selenium from natural and sustainable sources to modulate farmed gilthead seabream (*Sparus aurata*) and common carp (*Cyprinus carpio*) fillets elemental nutritional value. Food Chem. Toxicol. 140, 111330. DOI: 10.1016/J.FCT.2020.111330.

Abstract

Developing tailor-made fortified farmed fish is a promising solution to overcome nutritional deficiencies and increase consumer confidence in these products. This study evaluated the supplementation of three fortified diets with I-rich seaweed and selenised-yeast on essential and toxic elements levels in gilthead seabream (*Sparus aurata*) and common carp (*Cyprinus carpio*). Fortified diets resulted in increased I, Se, and Fe in fish muscle. Biofortified seabream and carp revealed lower Cu and Br. The reduction of fishmeal and fish oil in fortified diets resulted in lower Hg and Cd in seabream muscle. Contrarily, fortified diets increased As and Hg in carp fillets. The consumption of 150 g of fortified seabream enabled a significantly higher contribution to the daily recommended intake (DRI) of I (10%) and Se (76%) than non-fortified fish, whereas fortified carp fulfilled 23% of I DRI and 91% of Se DRI. Moreover, the exposure to Pb decreased with the consumption of biofortified seabream (23-82% $BMDL_{01}$) and carp (26-92% $BMDL_{01}$). These results support the strategy of developing eco-innovative biofortified farmed fish using sustainable, natural, safe, and high-quality ingredients in feeds, to enable consumers to overcome nutritional deficiencies without significantly increased feed costs.

Keywords: biofortification, sugar kelp, selenised-yeast, seabream, carp, essential and toxic elements

1. Introduction

The regular consumption of seafood products is recommended widely by health authorities globally, as these products are recognized as nutritionally important foods that not only improve human health, but also help prevent chronic pathologies, such as cardiovascular diseases, diabetes, and obesity (Cotter et al., 2009; FAO/WHO, 2011). The essential role of seafood in human nutrition can be attributed to the composition of proteins, vitamins (i.e., D, A and B12), n-3 long-chain polyunsaturated fatty acids (n-3 PUFA), mainly EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), iodine (I), selenium (Se) and iron (Fe) (EFSA, 2014a). Most dietary guidelines recommend the consumption of two portions of fish (approximately 300 g) per week, one of which should be an oily fish, to comply with nutritional requirements (EFSA, 2014a; Gidding et al., 2009). In 2016, 88% of seafood production was consumed directly and fish provided almost 20% of animal protein consumed globally (FAO, 2018). The increased demand for fish products, associated with a decline in fisheries, globally, has led to more emphasis on aquaculture products, which make up 60% of total fish consumption, but is expected to continue rising (FAO, 2018). One third of global population have severe nutritional deficiencies, particularly iodine (I), selenium (Se) and iron (Fe), which result in impaired endocrine, neurophysiological, and immunological function (Cotter et al., 2009; Pinkaew & Karrila, 2015). Thus, the potential to develop tailor-made fortified farmed fish products with adequate levels of essential nutrients using feed with sustainable, natural, safe, and high-quality ingredients has gained in importance (Allen et al., 2006; Ramalho Ribeiro et al., 2019; Saltzman et al., 2013). Different strategies have been developed to improve nutritional quality and safety of seafood, including fortification of fish fillets with bioactive fatty acids (Dantagnan et al., 2009; Ramos et al., 2008; Rosa et al., 2010), selenium (Cotter et al., 2009; Schram et al., 2010) and iodine (Ramalho Ribeiro et al., 2015; Schmid et al., 2003; Valente et al., 2015), through aquaculture feed modulation. Currently, the main challenge in aquaculture feed formulation is in the replacement of costly and unsustainable marine-derived resources (i.e., fishmeal and fish oil) with cost-effective and sustainable natural ingredients from plant sources, without compromising farmed fish growth, nutritional quality, and safety (FAO, 2018). In this context, novel approaches are being developed in aquaculture, including the use of seaweed (iodine-rich macroalgae), microalgae (EPA and DHA-rich microalgae) and yeast (selenised-yeast) as feed supplements (Dantagnan et al., 2009; FAO, 2018; Ramalho Ribeiro et al., 2017). Despite some advances in the development of biofortified seafood products, some limitations still exist. In fact, fortification efficiency for a given nutrient in aquaculture feed depends on different aspects, such as

origin, dose, and form (inorganic versus organic), as well as potential interactions with other compounds during fortification processes (Pinkaew & Karrila, 2015; Ramalho Ribeiro et al., 2017). Although fish fortification might be a feasible, cost-effective, and sustainable strategy to overcome nutrient deficiencies, toxicities through excess intakes of some nutrients (i.e., Se and Fe) needs to be considered when developing biofortified feeds (EFSA, 2014c, 2015b). Similarly, the potential for contamination of feeds with toxic elements (e.g., inorganic arsenic, organic mercury, cadmium, and lead) present in some ingredients (e.g., in seaweeds) and, subsequently, farmed seafood products, needs to be managed. This is not simple since supplementation with some nutrients (e.g., Se) can protect against toxic compounds that occur naturally in fish feeds (e.g., Hg) (Alves et al., 2018; Ralston et al., 2016).

The present study aimed to assess the effects of biofortified feeds, using iodine-rich seaweed and selenised-yeast, to modulate essential and toxic elemental composition (i.e., I, Se, Cu, Fe, Br, As, Hg, Cd, Pd) in edible tissues (fillets) in two of the most commonly farmed fish species in Europe, namely gilthead seabream (*Sparus aurata*) and common carp (*Cyprinus carpio*), and to assess the potential benefit-risk associated to the consumption of biofortified farmed fish.

2. Material and Methods

2.1.Control and enriched diets

For each species, a control diet (CTR) was formulated and manufactured by SPAROS Lda (Olhão Portugal), a specialized feed producing company, considering the nutritional requirements of adult gilthead seabream and common carp. For gilthead seabream, a CTR diet was formulated with moderate levels of fishmeal (15%), fish oil (5.45%) and vegetable oils (2.81% soybean, 5.61% rapeseed and 0.94% linseed). Based on the CTR formulation, three enriched diets (B1, B2 and B3) supplemented with different blends of iodine-rich macroalgae (0.40% in B1 and B2; 0.80% in B3) and selenised-yeast (0.015% in B1 and B2; 0.035% in B3) were also manufactured. Additionally, the three enriched diets were formulated with a 5% replacement of fishmeal by a blend of microalgae (*Tetraselmis* sp., *Chlorella* sp., *Schizochytrium* sp.), as well as reduction of vegetable oils (up to 0.65% of soybean, up to 1.29% of rapeseed and up to 0.22% of linseed in B2 and B3). Noteworthy, B1 diet also contained less fish oil (1.09%) (Annex A.I Table S. 2. 1). For common carp, a CTR diet formulation was based on moderate levels of fishmeal (5%), plant raw materials and vegetable oils (3% soybean and rapeseed oil). Based on

CTR formulation, three enriched diets (B1, B2 and B3) were supplemented with iodine-rich macroalgae (0.541%), selenised-yeast (0.010%) and a DHA-rich microalgae (*Schizochytrium* sp.; 3.125% in B1; 1.563% in B2), or salmon oil (2.1% in B3). In addition, a 2.5% replacement of fishmeal was achieved with blends of microalgae (1% *Spirulina* sp. and 1% *Chlorella* sp.), as well as a reduction of soybean oil (100% in B1 and B2; 1% in B3). Different levels of rapeseed oil were included in the enriched diets, specifically 1.1% (B1), 2.1% (B2) and 1% (B3) (Annex A.I Table S. 2. 2). All enriched diets formulations took into consideration the current maximum authorized contents of total iodine (20 mg kg^{-1}) and selenium (0.5 mg kg^{-1}) in European fish feeds (EFSA, 2005, 2006) and were produced by extrusion process at SPAROS, Lda facilities (Olhão, Portugal).

2.2. Experimental trial and sampling

The gilthead seabream trial was conducted at the Portuguese Institute for the Sea and Atmosphere aquaculture research station (EPPO-IPMA, Olhão, Portugal), whereas the common carp trial was conducted at the aquaculture facilities of the West Pomeranian University of Technology Szczecin (ZUT, Szczecin, Poland). Both trials were performed in compliance with the European guidelines on protection of animals used for scientific purposes (EC, 2010 — Directive 2010/63/EU).

Gilthead seabream specimens were distributed to 12 circular fiberglass tanks (1500 L), in a flow-through water circulation system (salinity: 35 ‰; temperature: 24–25 °C; dissolved oxygen 5.6 ± 0.9 mg L^{-1}) and subjected to natural photoperiod summer conditions (14 light/10 dark). Each experimental feed was tested in triplicate tanks (n = 50 fish per replicate/tank) and fish were fed four times a day with 1.3-2% of the biomass for a period of 72 days. Common carp specimens were distributed to floating set of 12 cages (net volume of 3 m^3 each). Each experimental feed was tested in triplicate cages (n = 100 fish per replicate/cage) and fish were fed three times a day with 2% of the biomass for a period of 98 days. No mortality was observed during either trial. For each species and treatment/feed, 15 fish (5 per replicate tank or cage) were collected at the beginning (i.e., day 0, corresponding to baseline) and at the end of the trials (i.e., after 72 days for gilthead seabream and 98 days for common carp). In both trials, fish were fasted for 24 hours before being sacrificed, by immersion in chilled seawater (gilthead seabream) or in chilled freshwater (common carp) following the procedure usually performed in commercial fish farms. All fish were measured, weighted (morphometric data is presented in Annex A.I Table S. 2. 3), and a portion of muscle tissue was collected and skinned. For each species, treatment, and sampling time, three composite samples were prepared using five

skinless fish muscles randomly grouped for each treatment. Samples were stored at – 80 °C until further analysis (Figure 2.1). Growth performance parameters were calculated, including total growth (TG), feed conversion rate (FCR) and specific growth rate (SGR).

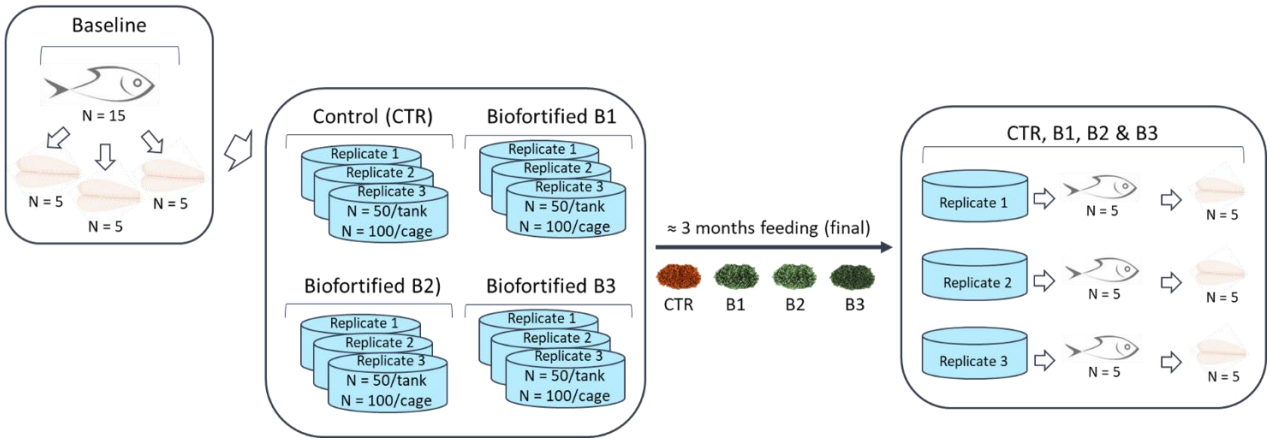

Figure 2.1 - Experimental design

2.3. Essential and toxic elements analysis

2.3.1. Total mercury (Hg)

Mercury concentrations (Hg) in fillets samples and feeds (CTR, B1, B2 and B3) were determined by atomic absorption spectrometry (AAS), using an automatic Hg analyser (AMA 254, LECO, USA) according to Maulvault et al. (2015). Briefly, 10 mg of feed (dry weight; d.w.) or fish muscle (wet weight; w.w.) sample was dried and combusted at 700 °C. The dissolved elemental mercury (Hg) was pre-concentrated, released and detected at 254 nm. Mercury concentrations were calculated from linear calibration with a Hg(II) nitrate standard solution (1000 mg L^{-1}; Merck) diluted in nitric acid (0.5 mol L^{-1}; Merck) at concentrations between 0.10 and 40 ng Hg.

2.3.2. Cadmium (Cd) and lead (Pb)

Cadmium (Cd) and lead (Pb) concentrations were determined using flame atomic absorption spectrometry (FAAS; Varian Spectre AA 20, Australia) according to Maulvault et al. (2015). Briefly, 5 g of fish muscle (w.w.) or feed (d.w.) sample (CTR, B1, B2, B3) was dry-ashed at 500 °C and dissolved in concentrated nitric acid (65% w/w, Merck). Cd and Pb were detected at 228 nm (Cd) and 283 nm (Pb), respectively, and concentrations were determined by linear calibration with standard solutions [$Cd(NO_3)_2$ and $Pb(NO_3)_2$ in 0.5 M HNO_3, 1 g L^{-1}; Merck)].

2.3.3. Iodine (I), selenium (Se) and arsenic (As)

Iodine (I), selenium (Se), and arsenic (As) were determined by inductively coupled plasma mass spectrometer (ICP-MS) (Thermo X series II, Thermo Fisher Scientific, Waltham, USA), after acid (Se and As) or alkaline digestion (I) of the samples. Se and As contents were determined according to the European standard procedure EN 15763:2009 (European Committee for Standardization, 2009). For each sample, 0.5 g of feed (d.w.) or fish muscle (w.w.) was weighed into 50 mL polypropylene DigiTUBEs (SCP Science, Quebec, Canada) and digested overnight in 7 mL of nitric acid (60% ultrapure w/w). Then, 1 mL of hydrogen peroxide (30% w/w, Merck) was added, and digestion carried out in a 48-well heating block (DigiPREP, SCP Science, Courtaboeuf, France) for 3.5 h at 85 ºC. After cooling, the digests were diluted up to 25 mL (fish) or 50 mL (feed) with MilliQ water and kept at 5 ± 3 °C until ICP-MS analysis (Coelho et al., 2017). Determination of I was performed separately, in compliance with the European Standard procedure EN 15111:2007 (European Committee for Standardization, 2007). Briefly, 0.7 g of fish muscle (w.w.) or 0.5 g of feed (d.w.) sample was weighed into 50 mL of polypropylene DigiTUBEs (SCP Science, Quebec, Canada) and digested with 9 mL of tetramethylammonium hydroxide (TMAH; 25%, Fluka, St. Gallen, Switzerland) in ultra-pure water (1:8 ratio) using a 48-well heating block (DigiPREP, SCP Science, Courtaboeuf, France) for 3 h at 90 ºC. After cooling, the digests were diluted up to 25 mL (fish) and 50 mL (feed) with MilliQ, transferred to centrifuge tubes and centrifuged at 15,550 × g (15 min at 20 ºC). The supernatant was filtered through a 0.45 µm syringe filter (Millipore) before ICP-MS analysis (Delgado et al., 2019). ICP-MS operating conditions were optimized daily (Annex A.I Table S. 2. 4). Quantification was achieved by linear calibration using standard solutions prepared from single-element high-purity ICP stock standards for iodine (Inorganic Ventures, Christiansburg, Virginia), selenium and arsenic (SCP Science, Marktoberdorf, Germany), ranging between 1 and 50 µg g^{-1} of I, 0.5 and 5 µg g^{-1} of Se and 0.25 and 2.5 µg g^{-1} of As.

2.3.4. Chloride (Cl), potassium (K), calcium (Ca), manganese (Mn), iron (Fe), copper (Cu), zinc (Zn) and bromide (Br)

Macro (Cl, K, Ca) and trace elements (Mn, Fe, Cu, Zn, Br) were quantified using a micro-Energy Dispersive X-Ray Fluorescence (µ–EDXRF) method (Mangueze et al., 2018). Feed and freeze-dried fish muscle samples were dried and ground. Around 1 g of the resulting powder was pressed (10 tons, 2 min) to form a cylindrical pellet of 2 cm in diameter. Considering the morphology of the powder and the capacity to be compacted, preparation of the pellets was performed without addition of a binder. Pellets were placed in the focal spot (25 µm) of the

μ–EDXRF system (M4 TornadoTM, Bruker, Germany). This spectrometer consists of an air-cooled micro-focus side window Rh-anode X-ray tube, powered by a low-power HV generator. The system featured a poly-capillary X-ray optics, which allowed a spot size of 25 μm at the sample. The X-ray generator was operated at 50 kV and 300 μA without the use of filters in open air. Detection of fluorescence radiation was performed using an energy-dispersive silicon drift detector, XFlashTM, with 30 mm^2 of sensitive area and energy resolution of 142 eV, for an energy of 5.9 keV (corresponding to Mn Kα).

2.3.5. Quality control

All reagents used in the analyses were high analytical grade and water was ultra-purified (< 18 MΩ cm) using a Milli-Q-Integral system (Merck, Germany). Internal quality controls (IQCs), prepared from independent stock standards, were used daily to verify the accuracy of the calibration curve for all elements. Reference materials for quality assurance included Orchard Leaves (SRM 1571) from the National Institute of Standards and Technology (Gaithersburg, EUA), fish protein (DORM-4) and dogfish muscle (DORM-2) from the National Research Council of Canada (Ontario, Canada), and fish muscle (ERM®-BB422) from the European Commission – Joint Research Centre Institute for Reference Materials and Measurements (IRMM) (Geel, Belgium). Detailed information about quality assurance, including the limit of quantification (LOQ) and detection (LOD), are shown in Table 2.1. Values obtained in the present study were in agreement with the certified values.

Table 2.1 - Mean certificate and measured concentrations (µg g^{-1} in dry weight) and the associated relative standard deviation (RSD) in certified reference materials (CRM). Limit of detection (LOD) and limit of quantification (LOQ) for each element and analytical.

Elements	Analytical method	Type	CRM Certificate value (µg g^{-1})	Measured value (µg g^{-1})	LOD (µg g^{-1})	LOQ (µg g^{-1})
Hg	AAS	DORM-4	0.412 ± 0.036	0.397 ± 0.006	0.004	0.01
Cd	FAAS	DORM-2	0.065 ± 0.007	0.064 ± 0.008	0.002	0.006
Pb	FAAS	DORM-2	0.043 ± 0.008	0.042 ± 0.005	0.002	0.006
As	ICP-MS	ERM®-BB422	12.7 ± 0.7	12.0 ± 0.2	0.003	0.013
I*	ICP-MS	ERM®-BB422	1.40 ± 0.40	1.23 ± 0.02	0.01 (0.068)	0.036 (0.25)
Se	ICP-MS	ERM®-BB422	1.33 ± 0.13	1.21 ± 0.02	0.007	0.025
Cl	µ-EDXRF	SRM 1571	700	600 ± 100	100	-
K	µ-EDXRF	SRM 1571	14700 ± 300	13500 ± 1300	20	-
Ca	µ-EDXRF	SRM 1571	20900 ± 300	19500 ± 2000	30	-
Mn	µ-EDXRF	DORM-4	3.6 ± 0.3	4.0 ± 0.8	5	-
		SRM 1571	91 ± 4	88 ± 4		
Fe	µ-EDXRF	DORM-4	142 ± 10	150 ±15	5	-
		SRM 1571	300 ± 20	298 ± 10		
Cu	µ-EDXRF	DORM-4	2.3 ± 0.2	2.4 ± 0.8	1	-
		SRM 1571	12 ± 1	13 ± 1		
Zn	µ-EDXRF	DORM-4	27 ± 2	28 ± 3	1	-
		SRM 1571	25 ± 3	24 ± 2		
Br	µ-EDXRF	SRM 1571	10	11 ± 1	1	-

*Iodine values for fish matrix and in parentheses for feed matrix
AAS (Atomic absorption spectroscopy); FAAS (Flame atomic absorption spectrometry); ICP-MS (Inductively coupled plasma mass spectrometer); DORM-4 (Fish protein); DORM-2 (Dogfish muscle); ERM®-BB422 (Fish muscle); SRM 1571 (Orchard leaf).

2.4. Health benefit value and risk-benefit balance

2.3.6. Se:Hg molar ratio and Se health benefit value (HBV_{Se})

For each feed/treatment, Se:Hg molar ratio was calculated from Se and Hg concentrations in µg g^{-1} by converting values to µmol kg^{-1} (µM) and dividing by molecular weights (200.59 for Hg and 78.96 for Se). Se health benefit value (HBV_{Se}) was calculated according to the following formula (Ralston et al., 2016):

$$HBV_{Se} = \left(\frac{Se - Hg}{Se}\right) \times (Se + Hg),$$

where Se and Hg were the concentrations in µmol kg^{-1}

2.3.7. Consumer risk benefit balance

Consumer risk-benefit associated with ingestion of 150 g of biofortified fish fillets was evaluated based on the available health-based guidance values (HBGVs), as follow: i) percentages of adequate intakes (AI) for I, Se, Fe, Cu, K and Cl (EFSA, 2014b,c, 2015b,c, 2016, 2019); ii) percentages of the adequate requirement (AR) for Ca and Zn (EFSA, 2014d, 2015d); iii) percentage of tolerable weekly intakes (TWIs) for Hg and Cd (EFSA, 2011, 2012a); and iv) Benchmark Dose Lower Limit (BMDL) for Pb (EFSA, 2010). In addition, where the percentage of AI/AR was greater than 100%, upper tolerable levels (ULs), i.e., maximum levels intake of essential elements unlikely to result in adverse effects for the general population were also calculated. Since there is no tolerable intake level set for total arsenic (PTWI of 15 μg kg−1 body weight (b.w.) was no longer appropriate; EFSA, 2009), and the most toxic and regulated form of As (i.e., inorganic As) were not analysed, this element was not included in these approach. Bromide was not considered either, as no reference value for intakes is available.

2.5. Statistical analysis

Data were analysed for distribution and variance homoscedasticity using Kolmogorov–Smirnov and Levene's tests, respectively. Growth performance and differences in elements content among feeds and fillets (CTR, B1, B2 and B3) were analysed by One-way ANOVA, followed by Tukey's post-hoc test for pair wise multiple comparisons. When ANOVA assumptions were not met, the Kruskal–Wallis test was performed followed by non-parametric multiple comparison test. Significance level was assigned at 0.05 and analyses were carried out using STATISTICA™ (Version 7.0, StatSoft Inc., Tulsa, Oklahoma, USA).

3. Results

1.1. Gilthead seabream and common carp growth performance

At the end of the trial, gilthead seabream approximately doubled their initial weight, reaching a final body weight (FBW) ranging from 531 to 578 g, whereas common carp more than tripled their initial body weight, reaching a FBW ranging from 1217 to 1338 g (Table 2.2). Gilthead seabream fed with the biofortified diet B3 presented significantly lower growth performance with decreased FBW and increased feed conversion ratio (FCR), compared to fish fed with CTR, B1 and B2 diets. On the other hand, I and Se biofortified diets led to a significant

increase of FBW and no differences were found in growth response indices (TG, FCR and SGR) of common carp fed with the different dietary strategies (Table 2.2).

Table 2.2 - Growth performance of gilthead seabream and common carp from the different treatments (average ± standard deviation).

	CTR	B1	B2	B3
Gilthead seabream				
IBW[1] (g)	371 ± 15	379 ± 2	376 ± 13	370 ± 3
FBW[2] (g)	626 ± 5[b]	623 ± 7[b]	623 ± 3[b]	589 ± 5[a]
TG[3] (%)	69 ± 5	64 ± 2	66 ± 6	59 ± 2
FCR[4]	1.87 ± 0.16[a]	1.83 ± 0.04[a]	1.90 ± 0.20[a]	2.38 ± 0.35[b]
SGR[5] (%/d)	6.22 ± 0.08	6.22 ± 0.01	6.22 ± 0.06	6.25 ± 0.03
Common carp				
IBW[1] (g)	301 ± 29	295 ± 15	295 ± 15	292 ± 20
FBW[2] (g)	1085 ± 16[a]	1193 ± 76[b]	1189 ± 50[b]	1218 ± 36[b]
TG[3] (%)	263 ± 35	306 ± 38	304 ± 12	319 ± 40
FCR[4]	1.52 ± 0.12	1.45 ± 0.11	1.45 ± 0.09	1.39 ± 0.11
SGR[5] (%/d)	1.29 ± 0.10	1.40± 0.10	1.40 ± 0.03	1.43 ± 0.10

Different letters (a-d) indicate significant differences between treatments (CTR – control; B1 – biofortification blend 1; B2 - biofortification blend 2; B3 - biofortification blend 3) for each species.
[1] Initial mean body weight, [2] Final mean body weight, [3] Total growth: (wet weight gain/IBW) x 100, [4] Feed conversion ratio: dry feed intake/wet weight gain, [5] Specific growth rate:(Ln FBW− Ln IBW) × 100 / feeding days.

1.2. Gilthead seabream elemental composition of diets and fillets

Biofortified diets (B1, B2 and B3) presented significantly higher contents of I, Se, Fe (B1, B3), Zn (B1, B2), Br (B3) and Cl (B3), as well as significantly lower contents of Hg, Cd (B1), Pb (B1) and Ca (B3) compared to the control diet (Figure 2.2). The composition of the biofortified diets (B1, B2 and B3) led to a significantly increased Se, Cl (B3), I (B3) and Fe (B1, B3) contents in biofortified gilthead seabream fillets, compared to fish fed with CTR diet, and significantly decreased Ca (B1), Br (B3), Cu (B2, B3), Hg (B1, B2, B3) and Pb (B3) contents. Overall, control (CTR) fillets revealed the highest contents of Ca and Hg and the lowest contents of Se and Fe. B1 fillets statistically revealed higher contents of Zn and Cu and the lowest contents of Cd. B2 fillets significantly revealed the lowest contents of Cl. At last, B3 fillets showed statistically the highest Cl, I, Se, Fe and Cd contents and the lowest Br and Pb contents. Control fillets (CTR) showed higher levels of I and Ca deposition, whereas higher levels of Se (B2, B3), Fe (B3), Cu (B1), As (B2), Cd (B3), Pb (B1, B2), Cl (B1) and K (B1, B2, B3) deposition were observed in biofortified fillets.

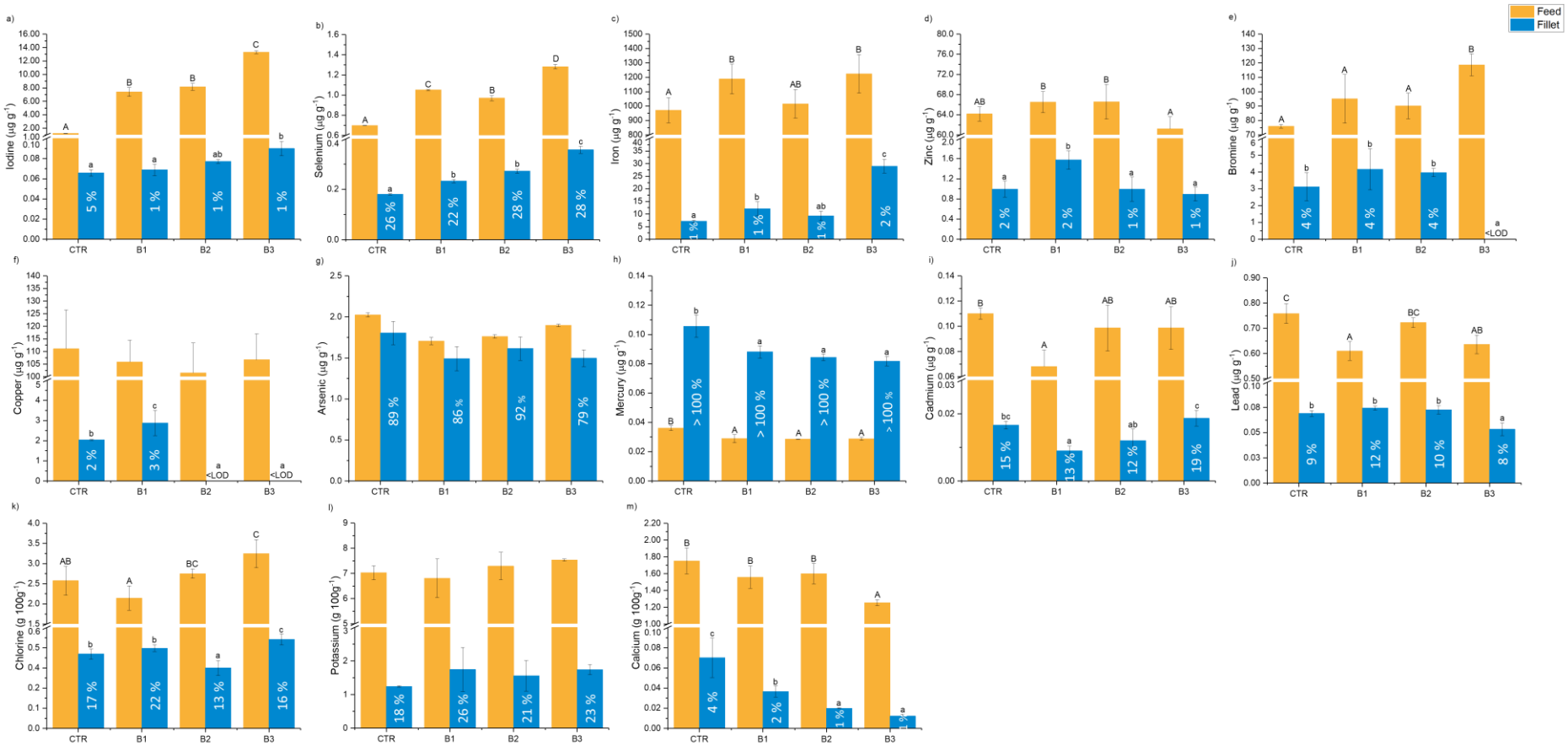

Figure 2.2 - Levels of trace elements (a - Iodine, b - Selenium, c - Iron, d - Zinc, e - Bromide, f – Copper; in µg g^{-1}), toxic elements (g – Arsenic, h - Mercury, i – Cadmium, j – lead; in µg g^{-1}) and macro elements (k - Chlorine, l – Potassium, m – Calcium; in g 100 g^{-1}), in gilthead seabream diets (average ± SD, in dry weight) and fillets (average ± SD, in wet weight); and percentages of element deposition in fish fillet from each diet . Different capital letters (A - D) represents significant differences ($p < 0.05$) in elements concentration between diets (CTR- control, B1 - biofortified B1, B2 - biofortified B2, B3 - biofortified B3), whereas small letters (a - d) represents significant differences ($p < 0.05$) between fillets (CTR, B1, B2, B3).

1.3. Common carp elemental composition of diets and fillets

Biofortified diets (B1, B2 and B3) revealed significantly higher contents of I, Se, Fe, Zn, Br, Cu, As, Hg, Cd and Cl and significantly lower contents of K compared to the control diet (Figure 2.3). The composition of biofortified diets (B1, B2 and B3) significantly increased I, Se, Zn, As and Hg contents in common carp fillets compared to control (CTR) and significantly decreased the contents of Ca (B1, B3), K (B1), Br, Cu and Pb (B1). Overall, control fillets (CTR) revealed the highest Br, Cu and Pb contents and the lowest I, Se, Zn, As and Hg contents. B1 fillets statistically revealed the lowest K and Pb contents. B2 fillets revealed the highest Hg contents. Control fillets (CTR) showed higher deposition of Se, Fe, Zn, Br, Cu, as, Cd, Pb, Cl, and Ca, whereas the biofortified fillets presented higher deposition of I and K (B2, B3).

1.4. Health benefit value and risk-benefit balance

Biofortification of gilthead seabream led to a significant increase of the Se:Hg molar ratios, with the highest value observed in fillets from B3 (11.11 ± 0.83) treatment, and the lowest in CTR (4.37 ± 0.26) treatment (Table 2.3). In contrast, the Se:Hg molar ratio decreased significantly in biofortified common carp , with the lowest value in B2 (8.55 ± 0.18) treatment (Table 2.3). HBV_{Se} followed a similar trend, with significant higher values observed in biofortified gilthead seabream fillets (highest value observed in fish from B3 treatment). In contrast, HBV_{Se} values in common carp fillets were not significantly different among treatments (CTR, B1, B2 and B3; Table 2.3).

Table 2.3 - Se:Hg molar ratio and selenium health benefit value (HBV_{Se}) in gilthead seabream and common carp fillets from the different treatments (average ± standard deviation).

	Se:Hg	HBV_{Se}
Gilthead seabream		
CTR	4.37 ± 0.26^{a}	2.18 ± 0.02^{a}
B1	6.73 ± 0.42^{b}	2.89 ± 0.09^{b}
B2	8.19 ± 0.40^{c}	3.39 ± 0.10^{c}
B3	11.11 ± 0.83^{d}	4.48 ± 0.18^{d}
Common carp		
CTR	13.85 ± 1.46^{b}	1.18 ± 0.06
B1	9.08 ± 0.63^{a}	1.51 ± 0.04
B2	8.55 ± 0.18^{a}	1.70 ± 0.10
B3	9.52 ± 0.74^{a}	1.65 ± 0.13

Different letters (a-d) indicate significant differences between treatments (CTR – control; B1 – biofortification blend 1; B2 - biofortification blend 2; B3 - biofortification blend 3) for each specie.

The nutritional contributions to the dietary reference values (DRVs) set for adults, children (1-3 years) and pregnant/lactating women achieved through consumption of 150 g of gilthead seabream or common carp fillets are presented in Table 2.4. Overall, biofortified gilthead seabream from B3 treatment contributes to higher intakes of I (7-10% of AI), Se (63-76% of AI for adults and pregnant/lactating; and >100% of AI (59% UL) for children), Fe (>100% of AI and 10% of UL), K (65->100% of AI)) and Cl (26-31% of AI) and lower intakes of Hg (4-16% of TWI) and Pb (23-24% of $BMDL_{01}$ for adults and pregnant/lactating, and 82% of $BMDL_{01}$ for children). Higher intakes of Cu (27-41% of AI) and Zn (3-4% of AR) and lower intake of Cd (1-3% of TWI) were associated with the consumption of 150 g of gilthead seabream fillets from B1 treatment. However, higher intakes of Ca (14-18% of AR) were provided by fillets from CTR treatment. Biofortified common carp from B2 treatment contributes to higher intakes of I (16-23% of AI), Se (24-91% of AI), Cl (5-6% of AI) and Zn (33-24% of AR) and lower intakes of Cd (1-3% of TWI) and Pb (22-23% of $BMDL_{01}$ for adults and pregnant/lactating, and 77% of $BMDL_{01}$ for children). Conversely, common carp fillets from B3 treatment contributes to higher intake of Fe (97% of AI for adults, and >100% of AI (7% UL) for pregnant/lactating and children) and Cd (1-4% of TWI). As for CTR carp fillets, it is worth mentioning that such consumption provides lower intakes of Hg (1-3% of TWI), as well as higher intakes of Ca (25-32% of AR) and Pb (>100% of $BMDL_{01}$, 26% UL for Children).

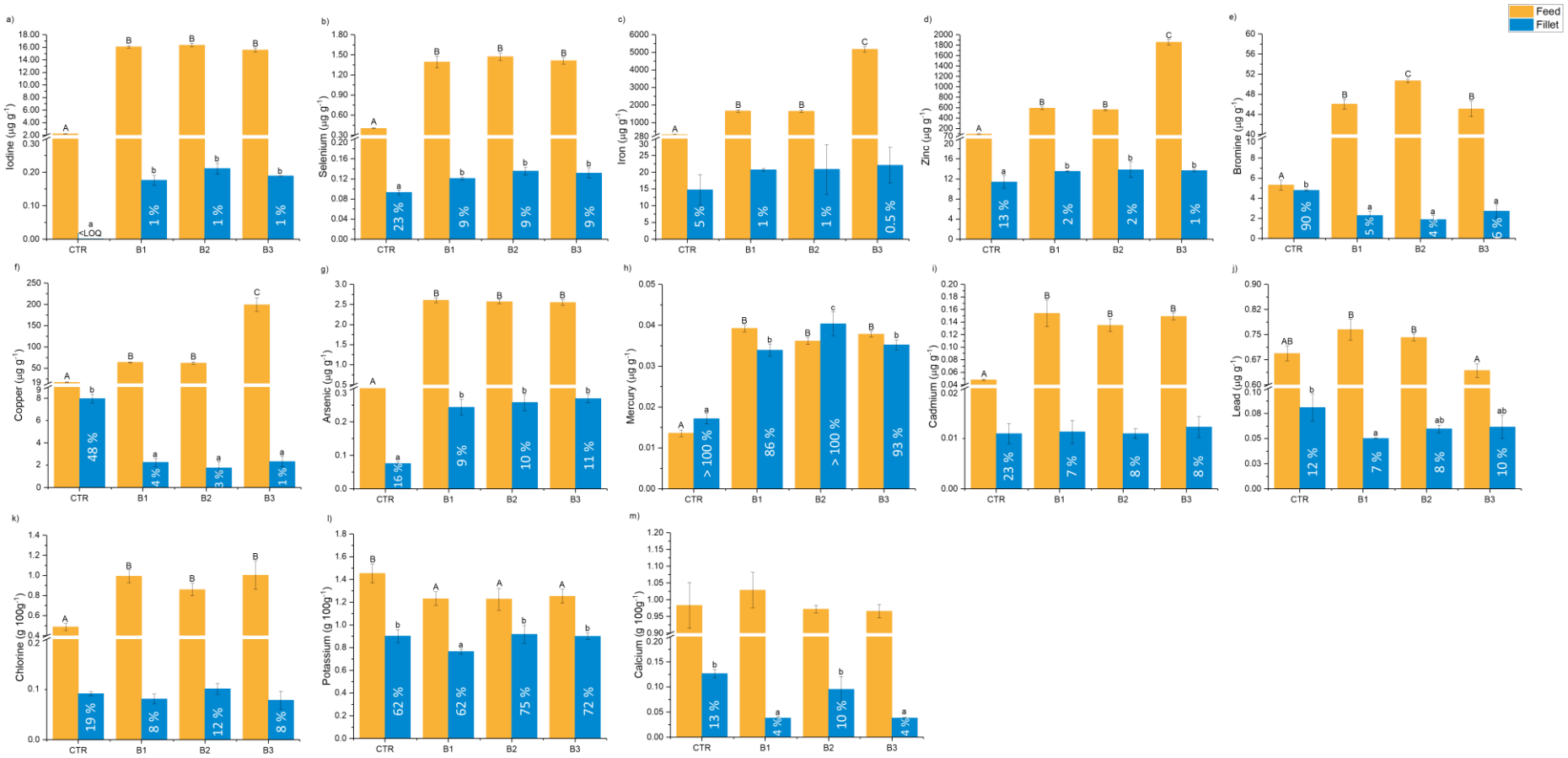

Figure 2.3 - Levels of trace elements (a - Iodine, b - Selenium, c - Iron, d - Zinc, e - Bromide, f – Copper; in µg g^{-1}), toxic elements (g – Arsenic, h - Mercury, i – Cadmium, j – Lead; in µg g^{-1}) and macro elements (k - Chlorine, l – Potassium, m – Calcium; in g 100 g^{-1}) in common carp diets (average ± SD, in dry weight) and fillets (average ± SD, in wet weight); and percentages of element deposition in fish fillet from each diet. Different capital letters (A-D) represent significant differences ($p < 0.05$) in elements concentration between feed (CTR- control, B1 - biofortified B1, B2 - biofortified B2, B3 - biofortified B3), whereas small letters (a - d) represent significant differences ($p < 0.05$) between fillets (CTR, B1, B2, B3).

Table 2.4 - Target elements percentage of the health-based guidance values (HBGVs) set by EFSA, considering the consumption of a portion of 150 g of fish fillet.

	I[1]			Se[1]			Fe[1]			Cu[1]			K[1]			Cl[1]		
	Adults	Children	Pregnant	Adults	Children	Pregnant	Adults	Children	Pregnant	Adults	Children	Pregnant	Adults	Children	Pregnant	Adults	Children	Pregnant
Gilthead seabream																		
CTR	7	7	5	39	120 (30)	32	31	118 (2)	37	19	29	20	53	156[5]	47	21	26	21
B1	7	8	5	50	155 (39)	41	53	202 (4)	63	27	41	29	75	218[5]	65	23	28	23
B2	8	9	6	58	181 (45)	48	41	155 (3)	48	0	0	0	67	195[5]	58	17	21	17
B3	9	10	7	76	238 (59)	63	128 (10)	483 (10)	150 (10)	0	0	0	75	218[5]	65	26	31	26
Common carp																		
CTR	2	2	1	20	62	16	65	246 (5)	76	75	114 (16)	80	39	113[5]	34	4	5	4
B1	18	20	13	26	81	21	91	345 (7)	107 (7)	21	32	23	33	96	29	4	5	4
B2	21	23	16	29	91	24	92	348 (7)	108 (7)	17	25	18	39	115[5]	34	5	6	5
B3	19	21	14	28	88	23	97	368 (7)	114 (7)	22	33	23	39	113[5]	34	4	5	4

	Ca[2]			Zn[2]			Hg[3]			Cd[3]			Pb[4]		
	Adults	Children	Pregnant	Adults	Children	Pregnant	Adults	Children	Pregnant	Adults	Children	Pregnant	Adults	Children	Pregnant
Gilthead seabream															
CTR	14	18	14	2	3	2	6	20	6	1	5	1	30	106 (22)	31
B1	7	9	7	4	4	3	5	17	5	1	3	1	32	114 (24)	33
B2	4	5	4	2	3	2	5	16	5	1	4	1	31	112 (23)	33
B3	2	3	2	2	2	2	4	16	5	2	6	2	23	82	24
Common carp															
CTR	25	32	25	28	32	20	1	3	1	1	3	1	35	125 (26)	36
B1	8	10	8	33	38	24	2	7	2	1	3	1	22	77	23
B2	19	24	19	33	38	24	2	8	2	1	3	1	26	92	27
B3	8	10	8	33	38	24	2	7	2	1	4	1	26	95	28

[1] Percentages were calculated according to the adequate intakes (AI) as well as the tolerable upper intake level (UL; in parenthesis) set by EFSA (2014b, 2014c, 2015b, 2015c, 2016, 2019). [2] Percentages were calculated according to the adequate requirement (AR) set by EFSA (2014d, 2015d); [3] Percentages were calculated according to the tolerable weekly intake (TWI) set by EFSA (2011, 2012a); [4] Percentages were calculated according to the benchmark dose lower limit ($BMDL_{01}$) as well as the margin of exposure (MOE; in parenthesis) set by EFSA (2010). Data was calculated using adults (> 18 years), children (1-3 years) and pregnant/lactating women's (18-35 years) mean body weights in Europe (body weight: 70, 13 and 67 kg, respectively; EFSA, 2012b). CTR – Control treatment; B1 – treatment B1; B2 - treatment B2; B3 - treatment B3. [5] No tolerable upper intake level (UL) has been set for potassium by EFSA due to insufficient data (EFSA, 2016a).

4. Discussion

4.1. Biofortified diets enhanced I and Se content in gilthead seabream and common carp

The different strategies used in formulation of the three biofortified diets for gilthead seabream and common carp in the present study, revealed differences in dietary elemental composition, and ultimately in fish fillets nutritional value. Fish is a rich source of essential elements, such as iodine and selenium, with wild species presenting, in general, higher I and Se contents than farmed products (EFSA, 2005) and usually with marine species with higher I levels than freshwater species (5 to 10-fold) (Haldimann et al., 2005; Julshamn et al., 2001). However, the potential to modulate fillet compositions through addition of natural ingredients from sustainable sources to aquaculture feeds is attractive, as it could be an efficient strategy to enhance health-valuable nutrients to address consumers' needs (Cotter et al., 2009). In line with previous studies, the results demonstrate that dietary inclusion of iodine-rich seaweed, microalgae and selenised-yeast supplementation can effectively enhance iodine and selenium contents in fish fillets, but also affect the composition of other essential nutrients as well as potentially toxic compounds. Indeed, increased iodine contents in gilthead seabream (Ramalho Ribeiro et al., 2015), rainbow trout (Ramalho Ribeiro et al., 2017; Valente et al., 2015) and chars (Schmid et al., 2003) achieved through dietary supplementation using iodine-rich seaweed (*L. digitata*) has been previously reported. The present study demonstrated that both gilthead seabream and common carp fillets were successfully enriched through dietary supplementation with I-rich seaweed, specifically *L. digitata*. However, the biofortification strategy was more effective in common carp (incorporation of 0.54% of *L. digitata* as part of the diet) than in gilthead seabream (incorporation of 0.8% of *L. digitata* as part of the diet), resulting in a 11-fold increase and a 1.4-fold increase in I contents, respectively, in relation to non-biofortified fish (CTR). Higher I contents representing a 6.5-fold increase in I contents compared with control fish were previously reported by Ramalho Ribeiro et al. (2015) in gilthead seabream fillets (0.84 μg g^{-1}) using the biofortification approach, but I was supplied above the current legal levels (incorporation of 10% of *L. digitata* and feeding period of 118 days in the previous study, against 0.8% and feeding period of 72 days in the present study). Increased I contents were also observed in previous studies focused

on freshwater species biofortified with the same seaweed species (Ramalho Ribeiro et al., 2017). For instance, rainbow trout (*Oncorhynchus mykiss*) and char (*Salvelinus* sp.) fillets I contents increased 6-fold and 4-fold, respectively, in relation to fillets of fish feed with the non-supplemented diet (Ramalho Ribeiro et al., 2017; Schmid et al., 2003). However, what was noteworthy about this study was that despite being different species (thus having different feed conversion rates), the feeding period for rainbow trout was same duration as the present study with common carp (91 day of feeding trial), while the char trial had a longer feeding period (i.e., 270 days), which might explain the different outcomes. Furthermore, Valente et al. (2015) showed that inclusion of a red seaweed species (5% of *Gracilaria vermiculophylla*) in rainbow trout diet was associated with a 2-fold increase in fillet I content (0.21 μg g^{-1}).

In terms of Se biofortification, dietary supplementation with 0.035% and 0.010% of selenised-yeast, respectively, was more effective in gilthead seabream (2-fold increase) than in common carp (1.4-fold increase). A previous study also reported successful Se fortification in rainbow trout fish fillets using a similar dietary approach (supplementation with selenised-yeast) for approximately the same period of time (91 days of feeding trial), though higher Se contents in fillets were observed (2.9-fold increase; Ramalho Ribeiro et al., 2017). Although the previous studies have used dietary supplementation with I-rich seaweed and/or selenised-yeast, to author's knowledge none have addressed novel dietary strategies by combining I-rich seaweed and selenised-yeast supplementation and replacement of fish (i.e., fishmeal and fish oil) and plant raw material (i.e., vegetable oils). Several studies have reported efficient fortification of fish fillets using I and Se from organic and inorganic sources. For example, with gilthead seabream feeds supplemented with organic Iodized salt (EDDI, 23 mg kg^{-1}) and inorganic potassium iodide (KI, 26 mg kg^{-1}), feeding over 118 days, led to a 1.3-fold increase in fillet I content, in relation to the non-supplemented feed (Ramalho Ribeiro et al., 2015). Increased I contents were also reported in Atlantic salmon fed with diets supplemented with 40 or 80 mg kg^{-1} of KI over 150 days, representing 3-fold and 2.4-fold increase, respectively (Julshamn et al., 2006). Successful Se fortification of fillets from fish fed with organic Se sources, such as SeMet, was achieved in rainbow trout (2-fold increase) after 40 days of feeding trial (Rodríguez & Rojas., 2014) and in grouper (22-fold increase) after 56 days (Lin, 2014). In comparison, increased Se content in fillets was also achieved with an inorganic Se source (Na_2SeO_3) in juvenile grouper fed for 56 days, resulting in a 7-fold increase relative to non-supplemented diet (Lin, 2014). However, the use of I and Se from organic sources, such as

seaweed and selenised-yeast, is advantageous due to their higher bioavailability (Rider et al., 2009) and potential as functional ingredients. The fact that I and Se come from natural and sustainable sources further reinforces their potential in dietary biofortification (Ramalho Ribeiro et al., 2015). However, the effectiveness of biofortification with I and Se depends on fish and seaweed species, and selenised-yeast used as well as the duration of feeding exposure (Ramalho Ribeiro et al., 2017).

Lower growth performance was observed in gilthead seabream fed with higher levels of dietary supplementation with iodine-rich seaweed and selenised-yeast (0.8% and 0.035%, respectively), associated to lower FBW and higher FCR (fish consumed more feed but grew less). Similarly, lower gilthead seabream growth performance was reported with an experimental diet including a mixture of ingredients, such as micro- and macroalgae, insect meals and yeast (Aragão et al., 2020). In contrast, a diet supplemented with 10% of *Laminaria digitata* showed no negative effects in gilthead seabream FBW and FCR (Ramalho Ribeiro et al., 2015). Such results may be associated with higher levels of selenised-yeast fortification, since impaired intestinal barrier function was reported in fish fed yeast-based diets (Aragão et al., 2020). On the other hand, the different dietary strategies showed no adverse effects on common carp growth performance. Indeed, biofortified diet B3 resulted in higher FBW, probably due to salmon oil supplementation (2.1%), which is an important source of omega-3 long chain polyunsaturated fatty acids (mainly DHA). It is widely known that enriched DHA diets promote fish growth (Bell & Waagbø, 2008). Nevertheless, nutrients digestibility and utilization of such ingredients from fortified diets is still unclear and further research is required.

4.2. Effects of biofortified diets on toxic element contents of fish fillets

The dietary supplementation with iodine-rich seaweed and selenised-yeast was in addition to inclusion of different amounts of algae meal and reduced fishmeal and vegetable oils relative to control diets. In terms of gilthead seabream biofortified diets, the reduction of fishmeal (5%) resulted in lower Hg contents and the reduced fish oil (approximately 1%) lowered Cd and Pb contents (B1 diet). Less exposure to Hg, Cd and Pb has been previously reported with the replacement of fish raw materials with vegetable protein sources (Berntssen et al., 2010; Dórea, 2006). Interestingly, in common carp biofortified diets, a significant reduction in Pb content was observed only in B3, most likely

due to the reduction in rapeseed oil content (1% relative to CTR and up to 3.1% relative to B2), since some plant protein sources are potential sources of Cd, Pb and Cu (Berntssen et al., 2010). On the other hand, the inclusion of microalgae blends was associated with higher contents of Fe and Zn in common carp biofortified diets, but not in gilthead seabream biofortified diets. These results might be explained by different mineral compositions and absolute concentrations in microalgae species, since *Spirulina* sp. (1% in common carp) is a functional food with higher nutrient contents than *Tetraselmis* sp, (0.5% in gilthead seabream) (Liestianty et al., 2019; H. Pereira et al., 2019). Additionally, supplementation of fish diets with the seaweed *L. digitata* enhanced I and Fe contents in both gilthead seabream and common carp fillets, as well as As content in common carp biofortified fillets. Seaweed species are widely recognized as important sources of I and Fe (Pereira, 2011), but also tend to accumulate As (Alves et al., 2018). Noteworthy, despite the reduction of fishmeal (2.5%) in common carp biofortified diets, increased Hg content was observed in common carp biofortified fish fillets. In fact, the biofortified diet with increased rapeseed oil (2.1% in B2) was associated with highest Hg content in common carp fillets. Both plant oils, including rapeseed oil, and mineral mixtures used in aquaculture feeds can be a route for chemical contamination, especially Hg (Peacock, 2013). On the other hand, despite containing less rapeseed oil (1%), in biofortified diet B3, common carp fillets fed with biofortified diet B3 presented higher Hg contents, possibly due to the supplementation with salmon oil (2.1%), which as a marine origin oil can contain high Hg levels (FAO/WHO, 2008). Contrasting with a previous study in rainbow trout fillets where the contents of different elements, including Fe, Zn and K, were unaffected by dietary supplementation with seaweed *L. digitata* and selenised-yeast (Ramalho Ribeiro et al., 2017), the present results suggest that both I and Se supplementations affected the contents of these elements in both gilthead seabream and common carp fillets. Several studies have demonstrated that Se supplementation exerts an antagonistic effect on toxic elements exposure, mainly Hg, As, Cd and Pb (Ralston et al., 2016; Rastogi et al., 1976; Zwolak, 2020). Furthermore, Se has an important role in the sequestration of some elements including Cu, Pb and Br, providing an efficient natural detoxification mechanism to reduce exposure to these harmful elements (Schrauzer, 2009). In addition, seaweeds are rich in polyphenols, which have the ability to bind and consequently reduce the bioavailability and the uptake of a range of metal elements (Roohinejad et al., 2017). Still, whereas decreased Cu contents were observed in common carp fillets fed with biofortified diets with increased Se contents, in gilthead seabream this was observed in fish fed

with B2 and B3 diets, but not in B1 diet, highlighting the importance of further research into fish Cu metabolism.

4.3. Biofortified farmed fish increases nutritional benefits to human without increas*ing toxic elements exposure*

Over the recent years, consumer awareness and, in some cases, preference for healthier and sustainable food products has increased. Therefore, the potential to develop eco-innovative seafood products, through biofortification should convey the delicate balance between quality (health-promoting nutrients) and safety (contaminant free content). Based on EC Regulation 882/2004, the biofortified feeds used in the present study had As, Hg, Cd and Pb contents below the maximum permissible levels (MPL; As = 25 mg kg^{-1}, Hg = 0.5 mg kg^{-1}, Cd = 1 mg kg^{-1}, Pb = 5 mg kg^{-1}). In addition, fillets from fish fed any of the biofortified diets had toxic element contents below the MPL set by the Regulation (EC) No 1881/2006 (Hg = 0.5 mg kg^{-1}, Cd = 0.05 mg kg^{-1}, Pb = 0.3 mg kg^{-1}), demonstrating that the biofortification strategies used improved the quality (i.e., nutritional contents) without compromising safety (elements concentrations).

The Se:Hg molar ratio is an essential criterium for evaluating the human health risks associated with Hg exposure and molar ratios above 1 indicate that Se compensates for the presence of any Hg (Ralston et al., 2016). The present study shows that both gilthead seabream and common carp from all treatments (CTR and biofortified) had Se:Hg molar ratios greater than 1. Still, biofortification improve gilthead seabream Se:Hg molar ratios, but the opposite was observed for common carp. Such differences might be explained by decreased Hg contents in gilthead seabream fillets, as result of the lower Hg contents in biofortified feeds (B1-B3) associated with reduced levels of fishmeal and fish oil. However, in common carp the reduction in fishmeal was not associated with reduced Hg contents in biofortified feeds, meaning increased Hg content in fillets might be related to rapeseed and salmon oil dietary supplementation. In addition, positive HBVSe values obtained in the present study indicated that the consumption of both biofortified gilthead seabream and common carp fillets reduced the negative effects associated with Hg exposure (Alves et al., 2018; Ralston et al., 2016) but, once again, biofortified gilthead seabream fillets offered higher Se-related beneficial effects than biofortified common carp.

Regarding the recommended dietary intakes, the present results showed that the consumption of 150 g of biofortified gilthead seabream fillets provides a higher contribution to the daily adequate intake (AI) set for I, Se, K and Cl. Despite exceeding the AI for Se in children and Fe in all groups, values were still below the upper limit levels (ULs) set for both elements. The biofortification strategies also contributed to decreased exposure to toxic elements, such as Hg, Cd and Pb.

Similarly, results from common carp, showed that the consumption of biofortified fillets improved the contribution to the daily AI set for I, Se, and Fe. Biofortified carp fillets also yielded a lower exposure to Hg, Cd and Pb. Considering the balance between essential and toxic elements (i.e., benefit-risk relationship), particular attention should be given to gilthead seabream biofortification strategies that avoid exceeding the UL set for Se in children. Moreover, without further work, consumption of either biofortified species would have to be parsimonious to avoid exceeding the ULs set for Fe in all demographic groups. Nevertheless, the benefits of the biofortification strategies used in this study outweigh the apparent risks, since increased intakes of I and Se offer added value for consumers' diets without increased exposure to toxic elements.

5. Conclusions

The results demonstrated that biofortification strategies, specifically the incorporation of iodine-rich seaweed (*L. digitata*) and selenised-yeast in gilthead seabream and common carp feeds can contribute to nutritional enrichment of fish fillets (i.e., enhanced I, Se, and Fe contents) without compromising consumer safety (i.e., exposure to Hg, Pb and Cd). To the authors' knowledge, the effects of different dietary strategies combining the replacement of fish-based raw materials (i.e., fishmeal and oil) with vegetable sources (i.e., I-rich macroalgae, Se-yeast, microalgae meals, salmon oil and vegetable oils) has been evaluated for the first time based on fillets composition in two model species, marine gilthead seabream and freshwater common carp. Iodine fortification was more efficient in common carp (more than 100% increase in B1, B2 and B3), whereas Se biofortification was more significant in gilthead seabream (98% increase in B3 treatment). Moreover, Se and Fe nutritional contributions were highly relevant, whereas I nutritional contribution could still be further improved. Based on current recommendations for toxic elements, parsimonious consumption of either species would be advised, since adverse

health effects from Pb potential exposure in children cannot be excluded. Increased Se contents in gilthead seabream fillets resulted in significantly higher selenium health benefit value (HBV_{Se}).

The present study clearly showed the importance of developing eco-innovative and cost-effective biofortified fish products and its potential in achieving sustainable, safe, and high-quality production of farmed seafood in Europe, overcoming nutritional deficiencies and meeting consumers' dietary needs more widely. However, further studies should be undertaken integrating different seaweed species and vegetable sources and improving the digestibility of these ingredients for, thus improving bioaccessibility and uptake fish. Furthermore, assessing the effects of cooking procedures, bioaccessibility and bioavailability of biofortified fish products will provide more realistic data for consumers risk-benefit assessment. In addition, further research is needed to evaluate not only the effective nutrient deposition in fillets (retention from feed to fillet), but also how fortification may affect fish welfare, the environmental costs, the economic feasibility (consumers acceptance) and the ecological footprint to enable the validation of alternative and commercial aquaculture feeds.

Ethical statement

Fish trials were conducted according to legal regulations (EU Directive 2010/63) and approved by the Ethical Committee of the EPPO-IPMA and ZUT, overseen by the National Competence Authority. All researchers and technicians involved in the maintenance, handling and sampling of live animals were certified in Laboratory Animal Sciences, by the Federation of European Laboratory Animal Science Associations (FELASA).

3

Effects of Steaming on Health-Valuable Nutrients from Fortified Farmed Fish Gilthead Seabream (*Sparus Aurata*) and Common Carp (*Cyprinus Carpio*) as Case Studies

In this chapter you will find the Manuscript:

Vera Barbosa, et al. (2021). Effects of steaming on health-valuable nutrients from fortified farmed fish: Gilthead seabream (*Sparus aurata*) and common carp (*Cyprinus carpio*) as case studies. Food Chemical Toxicology, 152, 1112218. DOI: 10.1016/j.fct.2021.112218.

Abstract

Fish fortification with iodine-rich macroalgae (*Laminaria digitata*) and Selenium-rich yeast is expected to promote nutritional added value of this crucial food item, contributing to a healthy and balanced diet for consumers. However, it is not known if steaming can affect these nutrient levels in fortified fish. The present study evaluates the effect of steaming on nutrients contents in fortified farmed gilthead seabream (*Sparus aurata*) and common carp (*Cyprinus carpio*). Fortified seabream presented enhanced I, Se, and Fe contents, whereas fortified carp presented enhanced I, Se, and Zn contents. Steaming resulted in increased I and Se contents in fortified seabream, and increased Fe and Zn levels in fortified carp, with higher elements true retention values (TRVs >90%). The consumption of 150 g of steamed fortified seabream contributes to a significant daily intake (DI) of I (up to 12%) and Se (up to >100%). On the other hand, steamed fortified carp contributes to 19-23% of I DI and 30% to 71% of Se DI. These results demonstrate that steaming is a healthy cooking method, maintaining the enhanced nutritional quality of fortified fish. Moreover, the present fortification strategy is a promising solution to develop high-quality farmed fish products to overcome nutritional deficiencies.

Keywords: selenium, iodine, fortification, steaming, seabream, carp

1. Introduction

The 21st century global challenges include those related with environmental changes and worldwide population nutritional deficiencies (United Nations, 2020). Several scientific evidence demonstrate that seafood consumption have been associated with beneficial effects for human health, when consumed at least twice a week (EFSA, 2015a; Luten et al., 2008). Fish contains many nutrients required to address micronutrient deficiencies (i.e., iodine, iron, and selenium) that affects 30% of the world's population (FAO, 2018). In addition, several evidence stress the beneficial health effects of fish consumption in mental health and in the prevention of cardiovascular diseases (Luten et al., 2008; Pinkaew & Karrila, 2015). Currently, there is a growing trend to develop tailor-made fish products by including natural ingredients with health-promoting nutrients to meet consumers' nutritional requirements and the growing health consciousness for sustainable, natural, safe and high-quality food (FAO, 2018). Several studies demonstrate that the natural enhancement of aquaculture feeds with health-promoting nutrients is an important strategy to produce sustainable, healthy/nutritious fortified farmed fish products (Barbosa et al., 2020; Cotter et al., 2009; Ramalho Ribeiro et al., 2015, 2017; Ramos et al., 2008; Saltzman et al., 2013; Valente et al., 2015). Within the context of functional food, fortified fish products are a potential strategy to improve consumers diets, providing beneficial health effects beyond the provision of essential nutrients (e.g., vitamins and minerals), when consumed as part of a diversified diet approach (Hasler, 2002; Luten et al., 2008). Nevertheless, the success of fish fortification as functional food depends on the combination of its efficacy (enhancement of active components linked to increased health benefits and disease risk reduction) and consumes' acceptance (Hasler, 2002; Ramalho Ribeiro et al., 2019). Moreover, consumer's demand for healthier, natural, and cost-effective fortified farmed fish products, foster the aquaculture sector to design and produce novel fish products using more sustainable and natural ingredients in feeds formulation (Ramalho Ribeiro et al., 2019). The use of different ingredients from algae and plant or non-animal sources in fish feed formulation, especially I-rich seaweed, EPA and DHA-rich microalgae and Se-rich yeast, plays an important role in the aquaculture sector, promoting the development of eco-innovative fortified fish products and the reduction of production costs and wastes (FAO, 2018; Sidari & Tofalo, 2019). A previous study demonstrated the efficacy of fish fortification with health-valuable nutrients through the incorporation of I-rich seaweed (*Laminaria digitata*) and Se-rich yeast in gilthead seabream and common carp feeds, resulting in enhanced I, Se, and iron (Fe) contents in fish muscle, without compromising consumer safety (Barbosa et al., 2020). Indeed, the

replacement of fishmeal and fish oil by microalgae blends, I-rich macroalgae and Se-rich yeast result in less exposure to toxic elements, mainly Hg, Cd and Pb (Barbosa et al., 2020).

Gilthead seabream (*Sparus aurata*) and common carp (*Cyprinus carpio*) are two of the most intensively farmed species in Europe, being mostly produced and consumed in Mediterranean countries and in central Europe, respectively (European Commission, 2020). As a matter of fact, gilthead seabream and common carp represents, respectively 10% and 5% of European aquaculture production, sharing 7% (gilthead seabream) and 6% (common carp) of European total apparent consumption (EUMOFA, 2019). Despite the increase trend in global fish consumption and the beneficial effects associated with seafood diets, I deficiency is a major concern of European authorities with critical consequences in neurological development, especially in children (FAO, 2018; WHO, 2013). Moreover, Se deficiency has been implicated in cardiovascular diseases, infertility, and hypothyroidism (Martins et al., 2011), while Fe deficiency is one of the world's most common disorders that lead to anaemia (Kongkachuichai et al., 2002). Since I and Se are not naturally found in the human body, the main source of these minerals for humans is the diet, particularly seafood (Bevis, 2015).

In general, most seafood is only consumed after cooking and therefore it is important to take into consideration the diversity and effect of culinary procedures when estimating nutrients daily intakes. Several culinary methods, such as boiling, grilling, frying, steaming, and roasting, are usually used to cook fish before consumption, and vary according to the region, local traditions, and cultural heritages (Sobral et al., 2018). Although cooking procedures improves fish digestibility and safety in terms of pathogenic microorganisms (Oliveira et al., 2019; Sobral et al., 2018), it can also lead to potential changes in the nutritional value (Alves et al., 2018; Karimian-Khosroshahi et al., 2016; Oliveira et al., 2019; Tontisirin et al., 2002). Indeed, the content of nutrients in cooked fish may increase or decrease compared to the raw counterpart, depending on the culinary procedures used (Badiani et al., 2013; Karimian-Khosroshahi et al., 2016). Overall, thermal processing is associated to water-soluble nutrients (i.e., vitamins C and B) leaching (Karimian-Khosroshahi et al., 2016). Regarding minerals, both increases or decreases in its content (i.e., Ca, Cu, Fe, I and Se) have been reported in fish, though varying with fish species and cooking methods (Alves et al., 2018; Sobral et al., 2018). For example, steaming results in increased Zn (hake, mackerel, plaice, and seabream), Se (mussels and octopus), Na, K, Fe and Cu (seabream) contents (Alves et al., 2018; Mnari et al., 2012). On the other hand, boiling and microwave cooking results in decreased K and increased Zn contents in rainbow trout, while grilling results in increased Cu content in seabream (Gokoglu et al., 2004; Mnari et al., 2012). Still, steaming has been pointed out as the healthier option and generally inducing

less changes in the product nutritional content compared to other culinary procedures such as frying or grilling (Alves et al., 2018; Maulvault et al., 2012, 2013).

The effects of culinary treatments on enhanced health-valuable nutrients in fortified fish products have not been previously studied. Moreover, most available studies assessing the effects of cooking methods on nutrient contents in seafood did not consider the use of the true retention values (TRVs) approach, which allows to provide more accurate knowledge on nutrients content after culinary procedures (Bognár & Piekarski, 2000). Hence, the present work aims to: (1) evaluate the effects of steaming on essential nutrients contents (i.e., I, Se, Cu, Zn, Fe, Ca, K) in gilthead seabream and common carp fish muscle (fillets) fortified with I-rich seaweed (*L. digitata*) and Se-rich yeast as feed ingredients; and (2) provide the most accurate data on nutrients contribution to the dietary reference values (DRVs) by using true retention (TR) calculations.

2. Material and Methods

2.1. Experimental diets

For each species, three experimental diets were formulated, a control diet (CTR), considering the nutritional requirements of adult gilthead seabream and common carp, and two enriched diets supplemented with different blends of I-rich macroalgae and Se-rich yeast (BF1 and BF2, respectively). Based on the control formulation, gilthead seabream enriched diets were formulated targeting increased I levels, supplied from *L. digitata* (0.40% in BF1 and 0.80% in BF2) and increased Se levels, supplied through Se-rich yeast (0.02% in BF1 and 0.04% in BF2). Additionally, enriched seabream diets were formulated with a 5% replacement of fishmeal by a blend of microalgae (*Tetraselmis* sp., *Chlorella* sp., *Schizochytrium* sp.) and with the reduction of vegetable oils levels (1.05% in BF1 and 2.15% in BF2). The enriched BF1 diet also contained less fish oil (1.09%; Table 3.1). Concerning common carp, the enriched diets were formulated based on control diet (CTR), targeting increased I levels, supplied from *L. digitata* (0.54%) and increased Se levels, supplied from Se-rich yeast (0.01%). Enriched carp diets were formulated with a 2.5% replacement of fishmeal by a blend of microalgae (*Spirulina* sp. and *Chlorella* sp.) and the enriched BF1 diet was supplemented with DHA-rich microalgae (1.56% *Schizochytrium* sp.), whereas the enriched BF2 diet was supplemented with salmon oil (2.10%) from salmon industry by-products. In addition, the enriched BF1 diet contained higher levels of rapeseed oil (5.1%) and lower levels of soybean oil (0%), whereas the enriched BF2 diet contained lower

levels of rapeseed and soybean oils (2%; Table 3.1). Experimental extruded diets were manufactured by SPAROS, Lda (Olhão, Portugal) and the enriched diets formulations took into consideration the current maximum authorized contents of total I (20 mg kg^{-1}) and Se (0.5 mg kg^{-1}) in fish feeds (EFSA 2014a,b).

2.2. Growth trial and sampling

The trial with gilthead seabream was conducted at the Aquaculture Research Station (EPPO-IPMA, Olhão, Portugal) of IPMA, whereas the common carp trial was conducted at the Fisheries Research Station (FRS-ZUT Nowe Czarnowo, Poland). Both trials were performed in compliance with the European guidelines on protection of animals used for scientific purposes (European Commission, 2007). The experimental design is schematized in Figure 3.1. Nine homogenous groups of 50 gilthead seabream each, with a mean initial body weight of 374 ± 9 g were distributed in 1500 L circular fiberglass tanks, supplied with flow-through seawater circulation (salinity: 35‰; temperature: 24–25 °C; dissolved oxygen 5.6 ± 0.9 mg L^{-1}) and subjected to natural photoperiod summer conditions (14 h light/10 h dark). Each experimental treatment was tested in triplicate tanks (n = 150 fish per treatment) over 72 days. Common carp specimens, with a mean initial body weight of 296 ± 10 g were distributed in a floating set of nine cages with 3000 L each (n = 100 fish per cage), placed in the cooling water discharge channel of the Dolna Odra power plant. Each experimental treatment was tested in triplicate tanks (n = 300 fish per treatment) over 98 days. For each species, fish were hand-fed to apparent satiety in three to four daily meals with 1.3-2.0 % of the biomass. during the experimental period, mimicking the final stage of the production (i.e., just before reaching market size). No mortality was observed during either trial. Final samplings were done 24 h following the last meal and 15 fish per treatment (5 per replicate tank or cage) were sacrificed by immersion in chilled seawater (gilthead seabream) or freshwater (common carp) following the commercial procedures employed in fish farms. Both gilthead seabream and common carp skinless fish muscle were collected at the start and at the end of the trial (n = 3 pools of 5 fish each). All fish were measured, weighted (morphometric data in Annex A.II Table S. 3. 1) and at the end of the trial one fish fillet collected was used for culinary steam-cooking procedure assessment (steaming) and the other fillet for raw assessment. All fish samples were homogenized with a grinder (Retasch Grindomix GM200, Germany) using polypropylene cups and stainless-steel knives at 10,000 *g* until complete visual disruption of the tissue and stored at – 80 °C until further analysis.

Table 3.1 - Ingredients and proximate composition (%) of the experimental diets (CTR - control, BF1 – fortified diet B1, BF2 - fortified diet B2) for gilthead seabream (*S. aurata*) and common carp (*C. carpio*).

	Gilthead seabream			Common carp		
Ingredients (%)	**CTR**	**BF1**	**BF2**	**CTR**	**BF1**	**BF2**
Fishmeal 70 [1]	15.00	10.00	10.00	-	-	-
Fishmeal 60 [2]	-	-	-	5.00	2.50	2.50
Fish protein concentrate [3]	2.50	2.50	2.50	-	-	-
Porcine blood meal [4]	2.50	2.50	2.50	2.00	2.00	2.00
Microalgae meal (*Tetraselmis* sp.) [5]	-	0.50	0.50	-	-	-
Microalgae meal (*Spirulina* sp.) [6]	-	-	-	-	1.00	1.00
Microalgae meal (*Chlorella* sp.) [7]	-	5.00	5.00	-	1.00	1.00
Microalgae meal (*Schizochytrium* sp.) [8]	-	3.20	3.20	-	1.56	-
Soy protein concentrate [9]	17.00	17.00	17.00	2.50	2.50	2.50
Corn gluten meal [10]	8.00	8.00	8.00	4.00	4.00	4.00
Soybean meal 48 [11]	8.00	8.00	8.00	-	-	-
Soybean meal 44 [12]	-	-	-	25.00	25.00	25.00
Rapeseed meal [13]	-	-	-	7.00	7.00	7.00
Sunflower meal [14]	-	-	-	12.50	12.50	12.50
Corn meal [15]	-	-	-	2.50	2.50	2.50
Wheat meal [16]	16.60	14.40	14.00	22.50	21.80	22.40
Wheat glúten [17]	12.00	12.00	12.00	-	-	-
Wheat bran [18]	-	-	-	5.00	5.00	5.00
Fish oil [19]	5.45	4.36	5.45	-	-	-
Salmon oil [20]	-	-	-	-	-	2.10
Soybean oil [21]	2.81	2.49	2.16	3.00	-	2.00
Rapeseed oil [21]	5.61	4.98	4.32	3.00	5.10	2.00
Linseed oil [21]	0.94	0.83	0.72	-	-	-
Vitamins and minerals premix [22]	1.10	1.10	1.10	1.00	1.00	1.00
Betaine HCl [23]	-	-	-	0.10	0.10	0.10
Binder [24]	1.00	1.00	1.00	1.00	1.00	1.00
Macroalgae meal (*Laminaria digitata*)[25]	-	0.40	0.80	-	0.54	0.54
Antioxidant [26]	0.20	0.20	0.20	0.20	0.20	0.20
Sodium propionate [27]	0.10	0.10	0.10	0.10	0.10	0.10
Monoammonium phosphate [28]	0.50	0.50	0.50	-	-	-
Sodium phosphate [29]	-	-	-	2.10	2.10	2.10
Selenised yeast [30]	-	0.02	0.04	-	0.01	0.01
L-Taurine [31]	0.40	0.50	0.50	-	-	-
L-Tryptophan [32]	0.10	0.10	0.10	0.20	0.20	0.20
DL-Methionine [33]	0.20	0.30	0.30	0.60	0.60	0.60
L-Lysine [34]	-	-	-	0.70	0.70	0.70
Dry matter (DM), %	7.90 ± 0.00	8.10 ± 0.00	8.10 ± 0.01	5.30 ± 0.01	6.40 ± 0.02	8.30 ± 0.02
Crude protein, % DM	46.00 ± 0.10	45.70 ± 0.20	45.50 ± 0.10	30.20 ± 0.20	30.40 ± 0.10	30.30 ± 0.10
Crude fat, % DM	17.20 ± 0.10	17.30 ± 0.10	17.30 ± 0.10	8.10 ± 0.10	8.00 ± 0.10	8.10 ± 0.20
Ash, % DM	5.30 ± 0.00	5.30 ± 0.01	5.30 ± 0.01	4.40 ± 0.10	7.20 ± 0.20	7.20 ± 0.10
Iodine, mg kg^{-1} DM	1.24 ± 0.02	7.38 ± 0.66	13.3 ±0.2	2.22 ± 0.03	16.30 ± 0.30	15.60 ± 0.30
Selenium, mg kg^{-1} DM	0.70 ± 0.00	1.05 ± 0.01	1.28 ± 0.02	0.40 ± 0.01	1.47 ± 0.05	1.41 ± 0.05

1CONRESA 70: 47.4% crude protein (CP), 817.5% crude fat (CF), Conserveros Reunidos S.A., Spain; 2CONRESA 60: 61.2% crude protein (CP), 8.4% crude fat (CF), Conserveros Reunidos S.A., Spain; 4Porcine blood meal: 89% CP, 1% CF, SONAC BV, The Netherlands; 5Tetraselmis meal: 72% CP, 1% CF, Willows Ingredients Ltd, Ireland; 6Spirulina meal: 72% CP, 1% CF, Willows Ingredients Ltd, Ireland; 7Chlorella meal: 62% CP, 9% CF, ALLMICROALGAE, Portugal; 8ALL-G RICH (Schizochytrium), Alltech Portugal; 9Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands; 10Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal; 11Solvent extracted soybean meal: 43.8% CP, 3.3% CF, CARGILL, Spain; 12Solvent extracted soybean meal: 43.8% CP, 3.3% CF, CARGILL, Spain; 13Defatted rapeseed meal: 32.7% CP, 4.1% CF, Ribeiro & Sousa Lda, Portugal; 14Defatted sunflower meal: 29.1% CP, 1.8% CF, Ribeiro & Sousa Lda, Portugal; 15Corn meal: 8% CP, 3.7% CF, Ribeiro & Sousa Lda, Portugal; 16Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal; 17Wheat glúten; 18Wheat bran: 14.9% CP, 4.0% CF, Cerealis Moagens S.A., Portugal; 19Fish oil; 20Sopropêche; 21Henry Lamotte Oils GmbH, Germany; 22INVIVONSA Portugal SA, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 500mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate,7.5mg; sodium chloride, 400mg; excipient wheat middling's; 23ORFFA, The Netherlands; 24CELATOM FP1SL (diatomite), Angelo Coimbra S.A., Portugal; 25Dry Laminaria digitata: 5.4% CP, 0.5% CF, 3700 mg iodine/kg, Agrimer, France; 26VERDILOX, Kemin Europe NV, Belgium; 27PREMIX LDA., Portugal; 29Vadequimica, Spain; 30ALKOSEL R397: 2200 mg selenium/kg, Lallemand, France; 31L-Taurine; 32TrypAMINO 98%, Evonik Nutrition & Care GmbH, Germany; 33DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany; 34L-Lysine HCl 99%: Ajinomoto Eurolysine SAS, France.

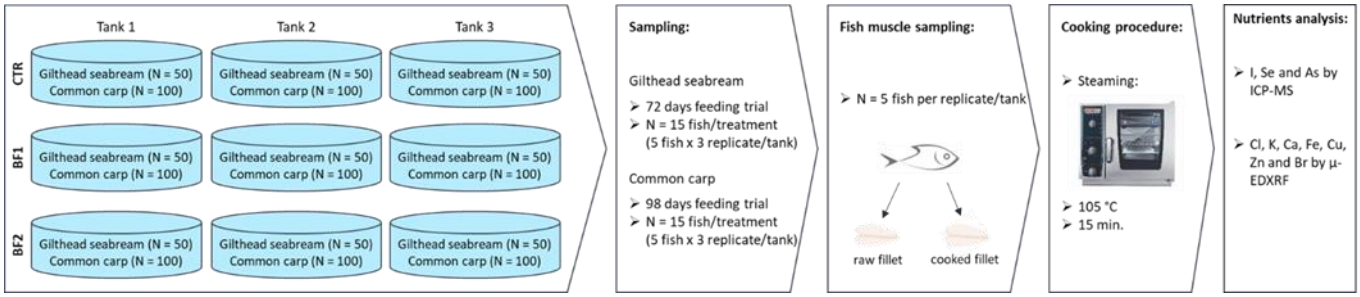

Figure 3.1 - Experimental design

2.3. Culinary steam-cooking procedure

For each treatment and species, fish muscle was individually wrapped up in aluminium foil and steamed in an oven (Combi-Master CM 6, Rational GroßkÜcken Technik GmbH, Germany) at 105 °C during 15 min. After steaming, fish muscle samples were cooled at room temperature. The final weight was registered to obtain the relevant cooking yield (CY = 100 × steamed weight / raw weight), as the percentage ratio between cooked and raw fish muscle weight (Annex A.II Table S. 3. 1).

2.4. Analytical methods

2.4.1. Elemental composition

Iodine (I), selenium (Se) and arsenic (As) were determined in fish muscle samples by inductively coupled plasma mass spectrometer (ICP-MS; Thermo X series II, Thermo Fisher Scientific, Waltham, USA) according to Barbosa et al. (2020). Iodine (I) content was quantified according to the EN 15111:2007 (European Committee for Standardization, 2007) and Se and As according to the EN 15763:2009 (European Committee for Standardization, 2009). Briefly, the alkaline digestion (for I) was performed by a 48-well graphite heating block (DigiPREP, SCP Science, Courtaboeuf, France) with tetramethylammonium hydroxide (TMAH; Fluka, St. Gallen, Switzerland) solution 25% (v/v), whereas the acid digestion (for Se and As) was performed overnight with 60% (v/v) ultrapure nitric acid solution, followed by a 48-well graphite heating block (DigiPREP, SCP Science, Courtaboeuf, France) with hydrogen peroxide solution 30% (v/v, Merck). ICP-MS operating conditions were optimized daily, and the quantification was done by linear calibration using standard solutions of I, Se and As prepared from single elements high purity ICP stock standards (Inorganic Ventures and SCP Science, respectively), ranging between 1 and 50 µg g^{-1} for I, 0.5 and 5 µg g^{-1} for Se and 0.25 and 2.5 µg g^{-1} for As (Coelho et al., 2017; Delgado et al., 2019).

Chlorine (Cl), potassium (K), calcium (Ca), iron (Fe), copper (Cu), zinc (Zn) and bromide (Br) were determined by micro-energy dispersive X-ray fluorescence spectrometry (μ-EDXRF) according to Reboredo et al. (2020). Briefly, feed, and freeze-dried fish muscle samples were dried and ground for 2 min under 10 tons pressure to make a cylindrical pellet with a diameter of 20 mm and a thickness of 1 mm. The energy μ-EDXRF spectra were acquired by a polarized geometry, secondary target, and high energy XRF spectrometer. The characteristic radiations emitted by each element in the sample were detected by a Si(Li) detector with 30 mm^2 of sensitive area, 142 eV resolution at 5.9 keV cooled by liquid nitrogen. The acquisition time of each spectrum was adjusted for each secondary target and the operating conditions of the X-ray tube were 50 kV, 300 μA. The spectra were evaluated using the fundamental parameters method.

2.4.2. Quality Assurance

All reagents used in the analyses were of high analytical grade and water was ultra-purified (< 18 MΩ cm) using a Milli-Q-Integral system (Merck, Germany). Analytical quality was assessed through reference materials including Oyster tissue (SRM 1571) from the National Institute of Standards and Technology (Gaithersburg, EUA) and fish muscle (ERM®-BB422) from the European Commission – Joint Research Centre Institute for Reference Materials and Measurements (IRMM) (Geel, Belgium). The obtained values agreed with certified values. Detailed information about quality assurance, including the limit of quantification (LOQ) and detection (LOD), are shown in Table 3.2.

2.5. True Retention (TR)

The TR (%) for each element was calculated using the following formula (USDA, 2008):

$$TR = \left(\frac{mean\ content\ of\ element\ in\ cooked\ food}{mean\ content\ of\ element\ in\ raw\ food}\right) \times CY,$$

where CY = cooking yield

Table 3.2 - Average certificate and measured concentrations (µg g^{-1} dry matter) and the associated relative standard deviation (RSD) in certified reference materials (CRM). Limit of detection (LOD) and limit of quantification (LOQ) for each element and analytical

Elements	Analytical method	Type	CRM Certificate value (µg g^{-1})	Measured value (µg g^{-1})	LOD (µg g^{-1})	LOQ (µg g^{-1})
As	ICP-MS	ERM®-BB422	12.7 ± 0.7	12.0 ± 0.2	0.003	0.013
I*	ICP-MS	ERM®-BB422	1.40 ± 0.40	1.23 ± 0.02	0.010 (0.068)	0.036 (0.25)
Se	ICP-MS	ERM®-BB422	1.33 ± 0.13	1.21 ± 0.02	0.007	0.025
Cl	µ-EDXRF	SRM 1571	700	600 ± 100	100	-
K	µ-EDXRF	SRM 1571	14700 ± 300	13500 ± 1300	20	-
Ca	µ-EDXRF	SRM 1571	20900 ± 300	19500 ± 2000	30	-
Fe	µ-EDXRF	DORM-4	142 ± 10	150 ±15	2	-
		SRM 1571	12 ± 1	13 ± 1		
Cu	µ-EDXRF	DORM-4	2.3 ± 0.2	2.4 ± 0.8	1	-
		SRM 1571	12 ± 1	13 ± 1		
Zn	µ-EDXRF	DORM-4	27 ± 2	28 ± 3	1	-
		SRM 1571	25 ± 3	24 ± 2		
Br	µ-EDXRF	SRM 1571	10	11 ± 1	1	-

*Iodine values for fish matrix and in parentheses for feed matrix
ICP-MS (Inductively coupled plasma mass spectrometer); µ-EDXRF (micro energy dispersive X-ray fluorescence spectrometry); ERM®-BB422 (Fish muscle CRM, Joint Research Centre (JRC), Brussels); SRM 1571 (Orchard leaf National Institute of Standards and Technology, EUA); DORM-4 (Fish Protein CRM, National Research Council of Canada, Canada).

2.6. Nutritional Contribution (NC)

The NC of steamed fish muscle was calculated considering the consumption of 150 g of fish and the dietary reference values (DRVs) recommended by the European Food Safety Authority (EFSA), according to the following formula:

$$NC\ (\%) = 100\ \times \frac{(C\ \times M)}{DRV}$$

where C = concentration of the element in mg kg^{-1}; M = typical meal portion in kg (0.150 kg for adults and pregnant women and 0.075 kg for children); DRV = adequate intake (AI; mg day^{-1}) for I, Se, Fe, Cu, Cl or K (EFSA, 2014b,c, 2015b,c, 2016, 2019) and adequate requirement (AR; mg day^{-1}) for Ca or Zn (EFSA, 2014d, 2015d). Since the reference value for total As (PTWI of 15 µg kg^{-1} body weight) is no longer appropriate (EFSA, 2009), and the most toxic and regulated form of As (i.e. inorganic As) was not analysed, this element was not included in these approach. Moreover, Br was not considered either, as no reference value is available.

2.7. Statistical analysis

Data were analysed for distribution and variance homoscedasticity using Kolmogorov–Smirnov and Levene's tests, respectively. The t-test student for dependent samples was performed to test significant differences between elements content in raw and steamed fish, for each treatment (CTR, BF1 and BF2). Whenever data (or transformed data) did not meet the normality and variance homoscedasticity assumptions, non−parametric Mann–Whitney U test was used. Furthermore, differences in fish muscle elements content among treatments (CTR, BF1 and BF2) were analysed by One-way ANOVA, followed by Tukey's post-hoc test for pair wise multiple comparisons. When ANOVA assumptions were not met, the Kruskal–Wallis test was performed followed by non-parametric multiple comparison test. Significance level was assigned at 0.05. Samples were also discriminated by multivariate parametric methods where the principal component analysis (PCA) was carried out to compute the linear combinations of the elements retained in each treatment. All analyses were carried out using STATISTICA™ (Version 7.0, StatSoft Inc., Tulsa, Oklahoma, USA).

3. Results

3.1. Essential elements composition in raw and steamed fortified farmed fish

Significantly higher CY were observed in fortified gilthead seabream (84% for BF2 and 87% for BF1) compared to fortified common carp (80% for BF2 and 81% for BF1) (Annex A.II Table S. 3. 1).

Fortified gilthead seabream (BF1 and BF2) presented significantly higher contents of I and Se, compared to the CTR (Table 3.3). Additionally, higher contents of Fe (BF1 and BF2) and Zn (BF1) were found in fortified fillets, compared to the CTR. On the other hand, fortified BF2 fillets presented significantly lower contents of Cu and Br (<LOD) compared to CTR fillets, while fortified BF1 fillets presented significantly higher contents of Cu and Br compared to CTR fillets. Steaming significantly increased I content in gilthead seabream fillets in all treatments (CTR, BF1 and BF2), as well as Se content in fortified BF2 fillets, resulting in TRs above 100% and 93%, respectively. Contrarily, Fe content significantly decreased in fortified BF2 fillets after steaming (69% TR), while Cu and Br contents significantly decreased in fortified BF1 and CTR reaching levels below LOD). Concerning macro elements, fortified gilthead seabream (BF2) presented

significantly higher Cl contents compared to the CTR. On the other hand, fortified fillets (BF1 and BF2) presented statistically lower levels of Ca compared with CTR fillets. Steaming significantly decreased Cl (BF1 and BF2) and Ca (CTR and BF1), with TRs ranging from 68% (BF1) to 73% (BF2 and CTR) for Cl and from 60% (BF1) to 65% (CTR and BF2) for Ca. Overall, among all macro elements the lowest TR was observed for Ca in all steamed fillets. In terms of As (toxic element), fortified gilthead seabream fillets (BF1 and BF2) presented significantly lower contents compared to CTR fillets. Statistically lower TRs were found for macro (Cl, K and Ca), trace (Se, Fe and Zn) and toxic (As) elements in fortified BF1 fish fillets. On the other hand, significantly higher I TRs were found in fortified BF1 fillets after steaming (Table 3.3).

Fortified common carp also presented significantly higher contents of I and Se (Table 3.4). Additionally, statistically higher levels of Zn, As (raw and steamed BF1 and BF2), and Fe (only steamed BF2) were found in fortified fillets, compared with non-fortified fish. In contrast, fortified BF2 fillets (raw and steamed) presented significantly lower Ca content compared with the CTR. Fortified fillets (BF1 and BF2) presented significantly lower contents of Cu and Br compared to CTR fillets (raw and steamed). Concerning the steaming effect, in terms of trace elements, steaming significantly increased Fe and Zn contents (CTR and BF2), with TRs above 100% for Fe and around 90% for Zn. In contrast, Cu content significantly decreased after steaming in the CTR (TR of 65%), as well as As content in fortified fillets (BF1 and BF2 with TR of 59% and 62%, respectively). Concerning macro elements, steaming significantly increased Cl (CTR and BF1; TR of 95% and >100%, respectively) and significantly decreased K (CTR and BF2, TR of 68% and 73%, respectively) and Ca (CTR and BF2, TR of 64% and 63%, respectively) contents. Likely to gilthead seabream, among macro elements the lowest TR was observed for Ca. Lower TRs were found for macro (Cl and Ca in BF2), trace (Se and Br in BF2, Fe and Zn in BF1) and toxic (As in BF1) elements in fortified common carp fillets. On the other hand, steamed BF1 fillets revealed higher TRs of Cl, K, Cu and Br, whereas steamed BF2 fillets revealed higher TRs of I, Fe and Zn (Table 3.4).

Table 3.3 - Concentrations of macro, trace and toxic elements and true retention (TR) of gilthead seabream (*S. aurata*) fed with different experimental diets (CTR - control, BF1 – fortified diet B1, BF2 - fortified diet B2)

	CTR		TR (%)	BF1		TR (%)	BF2		TR (%)
	Raw	Steam		Raw	Steam		Raw	Steam	
Macro elements (mg 100 g^{-1})									
Cl	444 ± 31^{a}	377 ± 8^{A}	73	477 ± 20^{a}	372 ± 5^{A}*	68	530 ± 33^{b}	461 ± 4^{B}*	73
K	1244 ± 24	1444 ± 163	100	1747 ± 371	1596 ± 57	79	1742 ± 150	1683 ± 148	81
Ca	70 ± 19^{c}	52 ± 3^{C}*	65	37 ± 6^{b}	25 ± 1^{B}*	60	12 ± 2^{a}	9.5 ± 0.1^{A}	65
Trace elements (mg kg^{-1})									
I	0.07 ± 0.00^{a}	0.10 ± 0.00^{A}*	125	0.07 ± 0.01^{a}	0.11 ± 0.00^{B}*	134	0.09 ± 0.00^{b}	0.12 ± 0.00^{C}*	110
Se	0.18 ± 0.00^{a}	0.18 ± 0.01^{A}	88	0.23 ± 0.01^{b}	0.23 ± 0.00^{B}	86	0.36 ± 0.01^{c}	0.40 ± 0.00^{C}*	93
Fe	7.1 ± 0.5^{a}	7.8 ± 0.6^{A}	95	12.1 ± 2.8^{b}	9.6 ± 0.6^{A}	69	29.0 ± 2.8^{c}	23.9 ± 3.4^{B}*	69
Cu	2. 0 ± 0.0^{b}	<LOD*	n.d.	2.9 ± 0.6^{c}	<LOD*	n.d.	<LOD^{a}	<LOD	n.d.
Zn	1.0 ± 0.2^{a}	1.1 ± 0.0	93	1.6 ± 0.2^{b}	1.3 ± 0.0	71	0.9 ± 0.1^{a}	1.1 ± 0.2	106
Br	3.1 ± 0.2^{b}	<LOD*	n.d.	4.2 ± 0.2^{c}	<LOD*	n.d.	<LOD^{a}	<LOD	n.d.
Toxic elements (mg kg^{-1})									
As	1.8 ± 0.1	1.9 ± 0.1B	91	1.5 ± 0.2	1.5 ± 0.0^{A}	88	1.5 ± 0.1	1.6 ± 0.0^{A}	87

Values are mean ± standard deviation, in wet weight. Different superscript small letters represent statistical differences ($p < 0.05$) between treatments (CTR, BF1, BF2) in raw fish fillets and different superscript capital letters represent statistical differences ($p < 0.05$) between treatments in steamed fish fillets. * represent statistical differences ($p < 0.05$) between raw and steamed fish filets in each treatment. <LOD, below the detection limit

Table 3.4 - Concentrations of macro, trace and toxic elements and true retention (TR) of common carp (*C. carpio*) fed with different experimental diets (CTR - control, BF1 – fortified diet B1, BF2 - fortified diet B2)

	CTR		TR (%)	BF1		TR (%)	BF2		TR (%)
	Raw	Steam		Raw	Steam		Raw	Steam	
Macro elements (mg 100 g^{-1})									
Cl	92 ± 4	106 ± 5^{B*}	95	101 ± 11	148 ± 3^{C*}	118	84 ± 11	85 ± 9^{A}	81
K	902 ± 57	746 ± 26*	68	918 ± 79	841 ± 51	74	900 ± 30	821 ± 17*	73
Ca	126 ± 9^{b}	98 ± 25^{B*}	64	95 ± 26^{b}	75 ± 5^{B}	64	38 ± 1^{a}	30 ± 2^{A*}	63
Trace elements (mg kg^{-1})									
I	<LOQ^{a}	<LOQ^{A}	n.d.	0.21 ± 0.02^{b}	0.23 ± 0.02^{B}	87	0.19 ± 0.00^{b}	0.21 ± 0.02^{B}	89
Se	0.09 ± 0.00^{a}	0.10 ± 0.01^{A}	87	0.14 ± 0.01^{b}	0.14 ± 0.01^{B}	84	0.14 ± 0.00^{b}	0.14 ± 0.00^{B}	80
Fe	14.7 ± 1.8	20.9 ± 2.5^{A*}	117	20.9 ± 4.6	23.4 ± 1.1^{A}	91	22.1 ± 5.4	35.2 ± 0.5^{B*}	128
Cu	8.0 ± 0.4^{b}	6.3 ± 0.0^{B*}	65	1.8 ± 0.4^{a}	1.6 ± 0.0^{A}	74	2.3 ± 0.5^{a}	2.1 ± 0.1^{A}	73
Zn	11.4 ± 1.2^{a}	12.7 ± 0.4^{A*}	91	13.8 ± 1.6^{b}	14.9 ± 0.9^{B}	87	13.7 ± 0.2^{b}	15.8 ± 0.2^{B*}	93
Br	4.8 ± 0.1^{b}	4.6 ± 0.3^{B}	79	1.9 ± 0.4^{a}	1.9 ± 0.1^{A}	83	2.7 ± 0.4^{a}	2.5 ± 0.0^{A}	73
Toxic elements (mg kg^{-1})									
As	0.08 ± 0.00^{a}	0.07 ± 0.00^{A}	72	0.26 ± 0.02^{b}	0.19 ± 0.01^{B*}	59	0.27 ± 0.01^{b}	0.21 ± 0.00^{B*}	62

Values are mean ± standard deviation, in wet weight. Different superscript small letters represent statistical differences ($p < 0.05$) between treatments (CTR, BF1, BF2) in raw fish fillets and different superscript capital letters represent statistical differences ($p < 0.05$) between treatments in steamed fish fillets. * represent statistical differences ($p < 0.05$) between raw and steamed fish filets in each treatment. n.d., not determined. <LOQ, below the quantification limit.

PCA analysis revealed a notable separation between gilthead seabream and common carp (PC1) related to different elements contents (Figure 3.2). In addition, for common carp, two groups were clearly identified according with fish diets, first group comprises the fortified fillets (BF1 and BF2) and the second group comprise CTR fillets (PC2). On the other hand, in gilthead seabream, a clear distinction between the most fortified treatment (BF2) from the less fortified treatment (BF1) and CTR was observed (PC2). In terms of culinary treatments, steamed fillets were clearly separated from raw fillets in the CTR and fortified BF2 common carp fillets, whereas no clear separation between raw and steamed fillets was found for seabream in all treatments. Se, Cl, K and As were the main elements influencing the differences between gilthead seabream and common carp. On the other hand, I and Fe were the main elements responsible for the distinction between treatments (CTR, BF1 and BF2). Concerning the culinary treatments in common carp, Cu and Br were the major elements responsible for the separation between raw and steamed CTR fillets, whereas Fe was the major contributor for the separation between raw and steamed BF2 fillets (Figure 3.2).

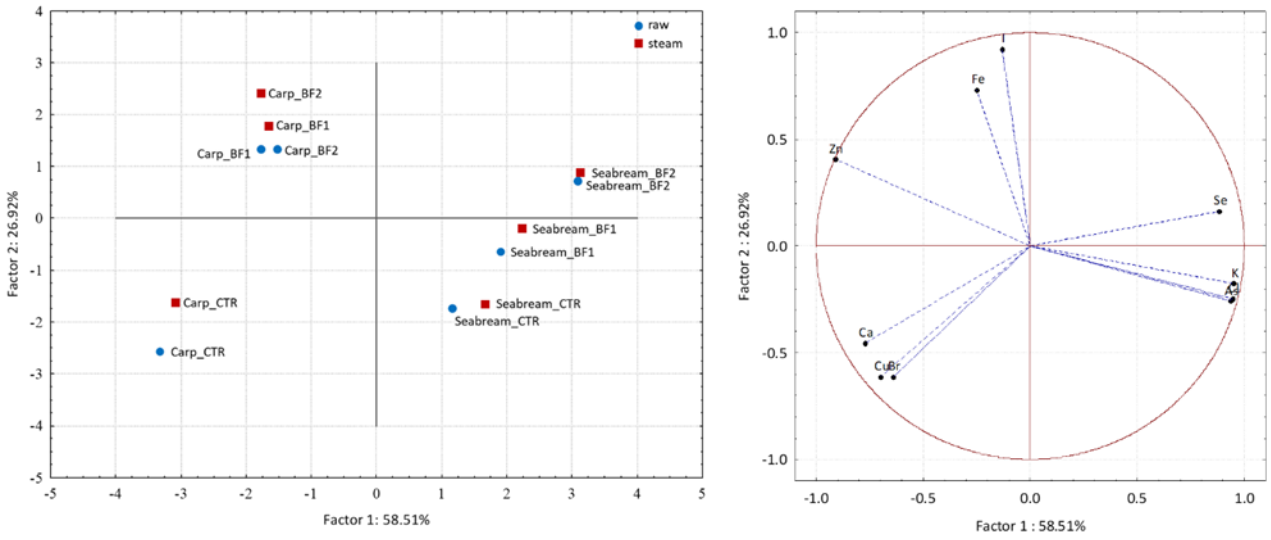

Figure 3.2 - Score plot of first two principal components (PC1 and PC2) for the nutrients composition in raw and steamed gilthead seabream (*S. aurata*) and common carp (*C. carpio*) fed with different experimental diets. PC1 and PC2 explained 85.43% variance. CTR - control, BF1 - fortified B1, BF2 – fortified B2.

3.2. Nutritional contribution of fortified farmed fish

The consumption of 150 g (adults and pregnant women) and 75 g (children) portion of steamed fortified BF2 gilthead seabream fillets contributed to higher intakes of I (from 9% for pregnant women to 12% for adults) and Se (from 70% for pregnant women to more than 100% for children) (Table 3.5). Moreover, steamed BF2 fillets contributed to higher intake of Fe (more than 100% for all population groups), compared to BF1 and CTR fillets. Yet, despite exceeding the daily adequate intake, fortified BF2 fillets contributed to 50% of Se upper intake level (UL) for children and to 8% of Fe UL for adults/pregnant women and 6% of UL for children. On the other hand, the consumption of steamed fortified fillets (BF1 and BF2) contributed to lower intake of Ca (5% and 2% for all population groups, respectively), compared to CTR fillets (11% for adults/pregnant women and 10% for children). The consumption of gilthead seabream fillets (fortified and CTR) also exceeded K daily adequate intake (AI) for children (Table 3.5). Yet, due to insufficient data, no UL exists for this element (EFSA, 2016).

The consumption of 150 g (adults and pregnant women) and 75 g (children) of steamed fortified common carp fillets (BF1 and BF2) contributed to higher intakes of I (from 16% of AI for pregnant women to 23% for adults) and Se (from 24% for pregnant women to 71% for children), compared to CTR fillets (Table 3.5). Additionally, the consumption of steamed BF2 fillets contributed to higher intakes of Zn (from 28% for pregnant women to 38% for adults). Contrarily, both fortified BF1 and BF2 fillets contributed to lower intakes of Cu (from 15% % for adults to 23% for children). Despite exceeding the daily AI, fortified BF1 and BF2 fillets contributed, respectively, to 8% and 12% of Fe UL for adults/pregnant women and to 6% and 9% Fe UL for children. In terms of macro elements, the consumption of steamed fortified fillets (BF1 and BF2) contributed to lower intakes of Ca (BF2: 6% for all population groups; BF1: 14% for children and 15% for adults/pregnant women) (Table 3.5).

Table 3.5 - Nutritional contribution (%) of steamed gilthead seabream (*S. aurata*) and common carp (*C. carpio*) in terms of essential elements in different population groups, considering the consumption of a portion of 150 g of fish for adults and pregnant women, and with the consumption of 75 g of fish for children.

			DRVs (mg day^{-1})[4]	Gilthead seabream			Common carp		
				CTR	BF1	BF2	CTR	BF1	BF2
Macro elements									
Cl	Adults[1]/Pregnant women[2]	AI	3100	18 ± 3	18 ± 0	22 ± 0	5 ± 0	7 ± 0	4 ± 0
	Children[3]	AI	1700	17 ± 3	16 ± 0	20 ± 0	5 ± 0	7 ± 0	4 ± 0
K	Adults[1]	AI	3500	62 ± 7	68 ± 2	72 ± 6	32 ± 1	36 ± 2	35 ± 1
	Pregnant women[2]	AI	4000	54 ± 6	60 ± 2	63 ± 6	28 ± 1	32 ± 2	31 ± 1
	Children[3]	AI	800	> 100	> 100	> 100	70 ± 2	79 ± 5	77 ± 2
Ca	Adults[1]/Pregnant women[2]	AR	750	11 ± 1^{b}	5 ± 0^{a}	2 ± 0^{a}	20 ± 0^{c}	15 ± 1^{b}	6 ± 0^{a}
	Children[3]	AR	390	10 ± 1^{b}	5 ± 0^{a}	2 ± 0^{a}	19 ± 0^{c}	14 ± 1^{b}	6 ± 0^{a}
Trace elements									
I	Adults[1]	AI	0.15	10 ± 0^{a}	11 ± 0^{b}	12 ± 0^{c}	n.d^{a}	23 ± 2^{b}	21 ± 2^{b}
	Pregnant women[2]	AI	0.20	7 ± 0^{a}	8 ± 0^{b}	9 ± 0^{c}	n.d^{a}	17 ± 1^{b}	16 ± ^{1b}
	Children[3]	AI	0.09	8 ± 0^{a}	9 ± 0^{b}	10 ± 0^{c}	n.d^{a}	19 ± 2^{b}	18 ± 1^{b}
Se	Adults[1]	AI	0.07	40 ± 1^{a}	50 ± 1^{b}	85 ± 2^{c}	21 ± 1^{a}	30 ± 2^{b}	30 ± 0^{b}
	Pregnant women[2]	AI	0.085	33 ± 1^{a}	41 ± 0^{b}	70 ± 2^{c}	17 ± 1^{a}	25 ± 1^{b}	24 ± 0^{b}
	Children[3]	AI	0.015	92 ± 3	> 100 (29 ± 0)	> 100 (50 ± 1)	48 ± 3^{a}	71 ± 4^{b}	69 ± 0^{b}
Fe	Adults[1]	AI	3.4	31 ± 2^{a}	54 ± 12^{a}	> 100 (8 ± 1)^{b}	92 ± 10	> 100 (8 ± 0)	> 100 (12 ± 0)
	Pregnant women[2]	AI	2.9	37 ± 3^{a}	63 ± 15^{a}	> 100 (8 ± 1)^{b}	> 100 (7 ± 1)	> 100 (8 ± 0)	> 100 (12 ± 0)
	Children[3]	AI	0.6	98 ± 8	> 100 (2 ± 0)	> 100 (6 ± 1)	> 100 (5 ± 1)	> 100 (6 ± 0)	> 100 (9 ± 0)
Cu	Adults[1]	AI	1.6	n.d.	n.d.	n.d.	59 ± 0^{b}	15 ± 0^{a}	20 ± 1^{a}
	Pregnant women[2]	AI	1.5	n.d.	n.d.	n.d.	63 ± 0^{b}	16 ± 0^{a}	21 ± 1^{a}
	Children[3]	AI	0.7	n.d.	n.d.	n.d.	68 ± 0^{b}	18 ± 0^{a}	23 ± 1^{a}
Zn	Adults[1]	AR	6.2	3 ± 0	3 ± 0	3 ± 1	31 ± 1^{a}	36 ± 2^{ab}	38 ± 1^{b}
	Pregnant women[2]	AR	8.6	2 ± 0	2 ± 0	2 ± 0	22 ± 1^{a}	26 ± 2^{ab}	28 ± 0^{b}
	Children[3]	AR	3.6	2 ± 0	3 ± 0	2 ± 0	26 ± 1^{a}	31 ± 2^{ab}	33 ± 0^{b}

Values are mean ± standard deviation. The Nutritional contribution (NC; %) are presented for 1adults (> 18 years) with mean body weight in Europe (70 kg), 2children (1–3 years) with mean body weight in Europe (13 kg) and 3pregnant/lactating women with mean body weights in Europe (67 kg) set by EFSA (2012b). 4The Dietary Reference Values (DRVs) are presented as Adequate Intakes (AI) for I (EFSA, 2014b), Se (EFSA, 2014c), Fe (EFSA, 2015b), Cu (EFSA, 2015c), Cl (EFSA,2019) and K (EFSA, 2016), as well as the tolerable upper intake level (UL; in parenthesis) and adequate requirement (AR) for Ca (EFSA, 2015d) and Zn (EFSA, 2014d). n.d., not determined due to contents bellow the detection limit (<LOD). No tolerable upper intake level (UL) has been set for K by EFSA due to insufficient data (EFSA, 2016). Different superscript small letters represent statistical differences (p < 0.05) between treatments (CTR, BF1, BF2). CTR – control; BF1 – fortified B1; BF2 – fortified B2.

4. Discussion

4.1. Effect of steaming on elements content in fortified farmed fish

The incorporation of iodine-rich seaweed (*L. digitata*) and Se-rich yeast in gilthead seabream and common carp feeds resulted in enhanced content of most essential elements, especially I and Se. It is known that culinary treatments, particularly those that require heat, can strongly affect fish nutritional composition depending on the temperature and duration of the cooking process (Barbosa et al., 2018). In line with previous studies (Ramalho Ribeiro et al., 2015), the results demonstrate that steaming significantly increased I content in gilthead seabream, but not in common carp fillets, compared to raw products. Such results may be explained by the fact that fortified common carp presented lower retention of I after streaming, associated to lower cooking yield (lower ratio of the amount of the edible portion that results from raw products), compared to fortified gilthead seabream. In general, lower cooking yields result from the damaging and solubilization of higher proportion of musculature connective tissue and dehydration of the muscle fibrils (Oliveira et al., 2019). Interestingly, increased I content was also previously reported in steamed anchovy and whiting, which presented lower contents in raw, compared to decreased I content in steamed horse mackerel, bluefish, Atlantic bonito and striped red mullet, which presented higher contents in raw meat (Erkan, 2011). Similarly, steaming increased Se content in most fortified gilthead seabream fillets (BF2), but not in fortified common carp. Increased Se content was previously reported in blue shark after grilling and steaming, which is associated to water loss during culinary treatment (Matos et al., 2015); whereas no statistically significant differences between Se content after cooking were reported in sardine, mackerel, hake and scabbardfish (Martins et al., 2011). Previous authors explained that I and Se are mainly bound to proteins (Hou, 2009; Vicente-Zurdo et al., 2019) and, therefore less prone to leaching during mild cooking procedures, such as steaming. In general, gilthead seabream presents higher protein and fat contents, whereas common carp presents higher moisture contents (Huss, 1995). Additionally, increased I and Se contents after fish cooking have been associated with the concentration of these elements due to water losses (Alves et al., 2018; Erkan, 2011; Martins et al., 2011). Nevertheless, the present study demonstrated that steaming has no detrimental effect in enhanced I and Se contents in fortified fish fillets from both species.

In terms of other essential nutrients contents, steaming resulted in increased Cl, Fe and Zn in common carp fillets, but not in gilthead seabream. Increased Fe and Zn contents have been previously reported in fried gilthead seabream (Mnari et al., 2012), whereas steaming resulted in increased Zn content in plaice, mackerel, and hake (Alves et al., 2018). In contrast, steaming resulted in decreased Cl and Fe contents in fortified gilthead seabream fillets, as well as Cu and Br contents in both fortified and non-fortified fillets. On the other hand, steaming resulted in decreased As content in fortified common carp fillets, Cu content in non-fortified fillets and K content in both fortified and non-fortified fillets. Interestingly, Ca content decreased after steaming in fortified and non-fortified fillets from both species. During thermal processing, the solubilisation of some minerals, such as the divalent elements, may occur due to muscle proteins denaturation (Kong et al., 2007; Mohan et al., 2008). The denaturation of sarcoplasmic and myofibrillar proteins results in the disconnection and dehydration of the muscle fibrils, leading to protein structural changes and decreased stability to form complexes protein-mineral complexes, and to consequent solubilisation of some minerals, such as Ca and Mg, intrinsically linked to fish muscle sarcoplasmic and myofibrillar proteins (Bastías et al., 2017; Ochiai & Ozawa, 2020). Previous studies also reported different changes in elements content, likely related with fish species and the different culinary treatments used. For example, boiling resulted in increased Ca content (Karimian-Khosroshahi et al., 2016), as well as decreased Zn and K contents (Gokoglu et al., 2004) in rainbow trout, as well decreased Ca, K, Fe and Zn contents in gilthead seabream (Mnari et al., 2012) and decreased K content in kutum roach (Hosseini et al., 2014). Furthermore, decreased contents of K and Zn were observed in grilled gilthead seabream (Mnari et al., 2012) and rainbow trout (Gokoglu et al., 2004), respectively, while increased content of K was observed in African catfish (Ersoy & Özeren, 2009) and rainbow trout (Gokoglu et al., 2004) after grilling. Frying increased Cu content in kutum roach (Hosseini et al., 2014), Cu and Ca content in rainbow trout (Gokoglu et al., 2004; Karimian-Khosroshahi et al., 2016), whereas decreased contents of Ca and Zn in fried rainbow trout (Gokoglu et al., 2004). Microwaving increased K content in rainbow trout (Gokoglu et al., 2004; Karimian-Khosroshahi et al., 2016), as well as K and Ca contents in African catfish (Ersoy and Özeren, 2009). Contrarily, increased content of K and decreased content of Fe were reported in kutum roach after microwave cooking (Hosseini et al., 2014). Furthermore, decreased content of Ca, K, Fe and Zn was also reported in gilthead seabream after oven-cooking (Mnari et al., 2012). Both losses and concentrations of macro and trace elements are mainly associated to

water loss, as result of the evaporation, dehydration of muscle fibrils, and probably to some heat-induced protein denaturation during steaming, leading to minerals leaching from water protein structures or by the concentration of minerals due to weight loss (Oliveira et al., 2019; Sobral et al., 2018). The present results contribute with relevant data, highlighting that the elemental composition is closely related to cooking procedures, as well as to the initial elemental content in raw fish and therefore being species-specific, as reported in the literature (Mnari et al., 2012; Petricorena, 2015). In fact, comparing the elemental composition between each treatment (CTR, BF1 and BF2), a different pattern was observed for each species with results showing a clear distinction between common carp and gilthead seabream. Moreover, the different fortification strategies contributed to distinct effects on fish elemental composition, whereas the steam cooking treatment seems to have less influence on fillets elemental composition, especially in gilthead seabream. However, other factors related to species-specific may also influence the different elemental profiles. For example, Ramalho Ribeiro et al. (2015) reported increased I content after steaming in gilthead seabream fish with similarly final body weight (488-506 g compared to 491-525 g from the present study), despite the different origin (i.e., farmed in different aquaculture stations). In contrast, Mnari et al. (2012) reported increased Fe and Zn contents in wild and farmed gilthead seabream with lower body weight (71 ± 1 g and 85 ± 2 g, respectively), compared to the present study (decreased Fe and Zn in farmed gilthead seabream with 549 – 525 g of body weight). Additionally, considering different species and different origins, but specimens' similar sizes different patterns in minerals contents was observed. For example, with similar body weight (1 - 1.3 kg), rainbow trout specimens (Karimian-Khosroshahi et al., 2016) and katum rach specimens (Hosseini et al., 2014), from different origins presented increased Ca content after cooking whereas decreased Ca content was observed in steamed common carp, suggesting that fish elemental composition is also dependent on specimens' origins and sizes.

The nutrients true retention (TR) is an important method for the determination of nutrients content in cooked foods, considering changes in weight and nutrient composition during cooking (Bognár & Piekarski, 2000). Most macro and trace elements TR values, with the exception of Ca, were approximately in the same range to those estimated and found by Bognár (2002), reflecting the differences associated to specific cooking yields. Noteworthy, TR values nearly 100% indicate that the nutrient is less prone to leaching or degradation process during cooking (Badiani et al., 2013), which is the case of most trace elements. Moreover, in line with previous studies, Ca was the least retained

element in both gilthead seabream and common carp (Badiani et al., 2013), showing to be the element with higher losses during culinary procedures. Fortified gilthead seabream fillets (BF2) revealed the highest TRs for most trace elements (I, Se, Zn), combined with the lowest TR of toxic element (As). Similarly, fortified common carp fillets (BF1), revealed higher TRs of macro (Cl, K) and trace elements (I, Se, Fe, Cu and Br), with the lowest TR for the toxic element (As), demonstrating that steaming affected differentially the elements content with potentially added value to fortified fish products.

4.2. Fortified farmed fish improve nutritional benefits to human health

The consumption of a usual portion of 150 g of steamed fortified gilthead seabream for adults and pregnant women and 75 g for children contributes to increased NC of macro (Cl and K), and trace (Se and Fe) elements. Similarly, the consumption of 150 g of fortified common carp also improved the NC of macro (Cl and K) and trace (I, Se, Fe and Zn) elements. A previous study assessed the nutritional value of gilthead seabream fortified with *L. digitata* and found that the consumption of 160 g of steamed seabream fillet covered about 80% of I daily AI for adults (Ramalho Ribeiro et al., 2015). Yet, it is worth mentioning that *L. digitata* was supplied at much higher levels (i.e., nine times more). Increased NC of I (12.4% of AI for adults) and Se (97.8% of AI for adults) was also reported in rainbow trout fillets fortified with I-rich seaweed (*L. digitata*) and Se-rich yeast (Ramalho Ribeiro et al., 2017). In comparison, fortified rainbow trout fillets showed higher NC of Se (+12.8%) and Zn (+2.1%) and lower NC of K (-55.8%) and Fe (-100%) than fortified gilthead seabream, as well as higher NC of Se (+67.8%) and lower NC of I (-10.6%), K (-19.8%), Fe (-100%) and Zn (-30.9%) than fortified common carp. Although, it is worth mentioning that in the previous study the nutritional value was assessed in raw rainbow trout fillets and that I-rich seaweed and Se-rich yeast were incorporated at different levels from the present study (0.365% of *L. digitata* and 1% of Se-rich yeast in the previous study, against 0.8% of *L. digitata* and 0.035% of Se-rich yeast in gilthead seabream and 0.54% of *L. digitata* and 0.1% of Se-rich yeast in common carp). To the author's knowledge, no studies addressed the health nutritional value of fortified fish fillets considering the effect of culinary procedures in a wide range of essential nutrients. Only the influence of steaming to the levels of essential and toxic elements was assessed in several fish species available in European markets (Alves et al., 2018). Considering the

results from this study, higher NC of Se and Fe is achieved by the consumption of fortified gilthead seabream and fortified common carp relatively to five fish species (plaice, hake, tuna, mackerel, and monkfish; Alves et al., 2018). Additionally, comparing to the previous study of Alves et al., (2018), fortified common carp contributed to higher NC of I relatively to hake and mackerel, and of Zn comparing to hake, tuna, mackerel, plaice, and monkfish.

The present results clearly demonstrate that fortification strategies with iodine-rich seaweed (*L. digitata*) and Se-rich yeast in gilthead seabream contributes to reduce Se and Fe deficiencies in target population groups. In contrast, fortified common carp contributes to reduce I and Fe deficiencies of consumers. Despite the benefits of fortification strategies used in this study outweigh the apparent risks, since increased intakes of I and Se offer added value for consumers' diets, parsimonious consumption of common carp should be considered particularly for children to avoid exceeding the UL set for Se. Additionally, particular attention should be given to fortification strategies of both species to avoid exceeding ULs set for Fe to all population groups.

5. Conclusions

This study provides new insights into the effect of steaming in nutritional enrichment of fortified gilthead seabream and common carp fillets. The dietary strategies assessed through the supplementation with I-rich macroalgae and Se-rich yeast, revealed to be highly efficient in gilthead seabream Se fortification (more than 90% increase) and in common carp iodine fortification (more than 100% increase). Results clearly indicate that steaming can indeed affect macro, trace, and toxic elements contents, being strongly related with the chemical properties of each element and fish species. Steaming resulted in significant increased contents of I and Se in fortified gilthead seabream fillets, as well as in significant decreased contents of Cl, Fe, Cu and Br. On the other hand, steaming resulted in significant increased contents of Fe, Zn and Cl in fortified common carp fillets, as well as in significant decreases in K and As contents. In both fortified fish species, steaming significantly decreased Ca content. Additionally, the main essential elements (I, Se, and Fe) NC were improved with fortified fish fillets. Yet, whereas I nutritional contribution could still be further improved, particular attention should be given to Fe and Se nutritional contribution to avoid exceeding the current recommendations. The findings of the present study clearly demonstrate the great potential of the studied fortification

strategies to reduce essential elements deficiencies in consumers, especially those associated with I, Se and Fe, and the related adverse disorders/diseases. Moreover, fish fortification seems to be an excellent strategy to enhance the nutritional quality of farmed fish products, and steaming can be considered as a suitable cooking procedure for a healthy consumption. Nonetheless, future studies regarding elements bioaccessibility and bioavailability of fortified fish will provide more insights for the realistic assessment on nutritional benefits to human health of fortification strategies with natural ingredients from sustainable sources.

Ethical statement

Fish trials were conducted according to legal regulations (EU Directive 2010/63) and approved by the Ethical Committee of the EPPO-IPMA and ZUT, overseen by the National Competence Authority. All researchers and technicians involved in the maintenance, handling and sampling of live animals were certified in Laboratory Animal Sciences, by the Federation of European Laboratory Animal Science Associations (FELASA).

4

PHYSICOCHEMICAL PROPERTIES OF IODINE AND SELENIUM BIOFORTIFIED *SPARUS AURATA* AND *CYPRINUS CARPIO* DURING FROZEN STORAGE

In this chapter you will find the Manuscript:

Vera Barbosa, et al. (2022). Physicochemical properties of iodine and selenium biofortified *Sparus aurata* and *Cyprinus carpio* during frozen storage. Food Chemistry, 397, 133780. DOI: 10.1016/j.foodchem.2022.133780.

Abstract

Fish biofortification with natural ingredients like iodine-rich macroalgae and selenised-yeast is an excellent strategy to enhance the nutritional quality of farmed fish. This study aimed to assess the effect of frozen storage during 12-months on physicochemical quality of biofortified seabream (*Sparus aurata*) and carp (*Cyprinus carpio*). Frozen storage reduced iodine content in biofortified seabream fillets (17%), as well as selenium content in biofortified carp fillets (24%). Yet, biofortified fillets still presented enhanced iodine and selenium contents at the end of the storage period. Increased lipid oxidation (3.45 mg MDA kg^{-1} for seabream and 2.41 mg MDA kg^{-1} for carp) and decreased water holding capacity (23-29% for seabream and 14-23% for carp) was observed during storage, whereas major changes in colour and texture occurred after 45 days (seabream) and 225 days (carp) of storage. In general, biofortified fillets maintained their nutritional value and quality after 360 days of frozen storage.

Keywords: macro, trace, and toxic elements; iodine and selenium biofortification; frozen storage, quality changes; seabream; carp

1. Introduction

Farmed gilthead seabream (*Sparus aurata*) and common carp (*Cyprinus carpio*) are two of the most valuable commercial species in Europe, being highly appreciated in Mediterranean countries and in central European countries markets, respectively (FAO, 2018). In fact, these species have become two of the main products of European aquaculture, representing 10% (gilthead seabream) and 5% (common carp) of the whole European aquaculture production and 7% (gilthead seabream) and 6% (common carp) of European total apparent consumption (EUMOFA, 2019). Marine fish species are considered rich sources of bioavailable micronutrients that are often deficient in other food products (Hicks et al., 2019). On the other hand, freshwater fish species are excellent sources of proteins and vitamins, but poor sources of essential unsaturated fatty acids (i.e., docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA)), in comparison with marine fish (EUMOFA, 2019). Therefore, fish-based food strategies are widely recommended to address the main micronutrient deficiencies and to improve human health (Allen et al., 2006). Over 2 billion people in developing countries have nutrients malnutrition, with iron (Fe), iodine (I), zinc (Zn), and vitamin A deficiencies among the most prevalent worldwide (Allen et al., 2006). These deficiencies may have adverse effects on human health, and ultimately, to high rates of morbidity and mortality among most vulnerable populations groups, especially elderly and children (Allen et al., 2006). Although fish consumption has the potential to contribute to increased micronutrients supply, its nutritional composition depends on specimens' origin and feeding pathway (Hicks et al., 2019). Aquaculture is a key resource to meet increasing seafood demand due to its potential to develop tailor-made biofortified fish products addressing consumers' nutritional requirements through sustainable, natural, safe, and high-quality feeds (Allen et al., 2006; FAO, 2018). Previous studies demonstrated that fish biofortification strategies, including bioactive fatty acids (Dantagnan et al., 2009; Rosa et al., 2010), iodine (Ramalho Ribeiro et al., 2015; Valente et al., 2015) and selenium (Ramalho Ribeiro et al., 2017; Schram et al., 2010) in feed modulation can contribute to further improve the nutritional quality of fish fillets. A recent study clearly demonstrated the efficacy of fish fortification using sustainable and natural ingredients in feeds formulation, namely I-rich seaweed, EPA+DHA-rich microalgae and Se-rich yeast, promoting the development of biofortified fish products with enhanced health-valuable nutrients without compromising consumer safety and fish well-being, as well as the production costs (Barbosa et al., 2020).

In the modern world, consumers' demand towards tailor-made, easy-to-prepare, ready-to-eat and ready-to-cook food products with extended shelf-life is gaining importance, with

frozen products representing 31% of share of fish and fish products trade (FAO, 2018). Freezing represents one of the most used methods for fish preservation intended for human consumption (FAO, 2018; Naseri et al., 2020). Cold storage allows to extend seafood shelf-life due to the prevention of microbial growth and enzymatic activity that trigger deterioration (Nakazawa & Okazaki, 2020). During frozen storage, chemical and structural changes may occur in the edible portion of fish, mainly protein dehydration and denaturation, ice crystals formation, lipid oxidation, rancidity formation, and textural changes (i.e., lower myofibrils water retention capacity) (Duarte et al., 2020). The rate of such changes depends on several factors including biological status and size, muscle type, husbandry (diet, handling, slaughtering stress), and post-mortem treatments (freezing rate, storage temperature and time and thawing method) (Burgaard & Jørgensen, 2011; Duarte et al., 2020; Nakazawa & Okazaki, 2020). Quick freezing of fresh fish results in the formation of smaller ice crystals, reduction of myofibril protein molecules dehydration and maintenance of the water retention capacity of the muscle tissue (Duarte et al., 2020). On the other hand, slow freezing will produce large and irregular ice crystals, leading to the destruction of muscle cells, fast protein denaturation and reduced water holding capacity (I after thawing, which will compromise the sensory acceptance of food (Nakazawa & Okazaki, 2020). The combination of ice crystal formation and proteins denaturation decrease WHC of the fish muscle, disturbing the complete restoring during thawing, reducing moisture content, and compromising frozen products quality (Nakazawa & Okazaki, 2020). Moreover, under frozen conditions the formation of formaldehyde and lipids oxidation (LPO), as well as hydrolysis occur, accelerating protein denaturation and leading to nutritional and antioxidant partial losses (Aubourg et al., 2004; Nakazawa & Okazaki, 2020). Previous reports demonstrated that some whole frozen fish species maintain sensory attributes up to 9 months when stored at temperatures below −30 °C, whereas shorter storage periods (up to 6 months) were advised for fish fillets (Duarte et al., 2020). Therefore, adequate treatments such as glazing (i.e., application of a layer of ice on the surface of a frozen product by spraying, brushing on water or dipping) and/or packaging in polystyrene bags may protect the fish product surface from dehydration, oxidation and, ultimately, quality losses, improving the final product shelf-life up to 12 months when stored at − 20 °C (Duarte et al., 2020; Naseri et al., 2020). Additionally, thawing conditions (i.e., time and temperature) are important factors that affect the quality of frozen fish products due to its influence on dynamics of chemical reactions and muscle degradation (Nakazawa & Okazaki, 2020). During thawing enzymatic reactions, including glycolysis, proteolysis, lipolysis, and histamine formation are activated triggering quality losses, by decreasing the water-binding capacity and texture deterioration (Schubring, 2005). Decreased WHC and

substantial loss of water from the fish muscle during thawing, can lead to important losses in elements content associated with muscle proteins denaturation (Prego et al., 2020).

Although several studies have focused their attention on seafood quality changes during frozen storage, the specific effects of this preservation method on essential elemental composition of fortified fish products have not yet been addressed. On the other hand, it has been demonstrated that fish processing, such as steam-cooking, affect essential elements contents of biofortified fish products (Barbosa et al., 2021). Moreover, frozen storage may lead to quality-related changes, such as loss of physical (e.g., texture) and chemical properties (e.g., nutrients contents). Additionally, nutrients interactions and their impact on organoleptic qualities of the fish products may affect the nutritional quality of farmed fish products (Burgaard & Jørgensen, 2011). In this context, understanding nutrients stability throughout the shelf-life preservation of biofortified fish products plays an important role to validate fish biofortification as a potential strategy to tackle the prevalence of micronutrient deficiencies in human populations. For this reason, the assessment of frozen storage stability of iodine and selenium biofortification, and the impact on biofortified fish quality, will provide more insights on nutritional benefits and economic feasibility (consumers acceptance) of fish biofortification strategies. The present work aimed to evaluate the effects of frozen storage during 12 months on physico-chemical quality changes (i.e., macro, trace, and toxic elements content, LPO, WHC, colour, texture) of farmed gilthead seabream and common carp fillets biofortified with iodine-rich seaweed (*L. digitata*) and selenium-rich yeast as feed ingredients.

2. Material and Methods

2.1. Fish biofortification trials and sampling

Gilthead seabream and common carp trials were conducted at the Aquaculture Research Station of Portuguese Institute for the Sea and Atmosphere (IPMA-EPPO, Olhão, Portugal) and Fisheries Research Station (Nowe Czarnowo, Poland), respectively. Both trials were performed in compliance with the European guidelines on protection of animals used for scientific purposes (European Commission, 2007). Six homogeneous groups of 50 gilthead seabream each, with a mean initial body weight of 374 ± 9 g were distributed in 1500 L circular fiberglass tanks, supplied with flow-through seawater circulation (salinity: 35‰; temperature: 24 – 25 °C; dissolved oxygen 5.6 ± 0.9 mg L^{-1}) and subjected to natural photoperiod summer conditions (14 light/10 dark). Common carp specimens, with a mean initial body weight of 296 ± 10 g, were

distributed in a floating set of six cages (n = 100) of 3 m^3 each, placed in the cooling water discharge channel of the Dolna Odra power plant. For each species, the trial comprised two experimental diets: a control diet (CTR) considering the nutritional requirements of adult fish and an enriched diet (BF), based on the control formulation, supplemented with I-rich macroalgae (iodine target level of 13.3 ± 0.2 mg kg^{-1} and 15.6 ± 0.3 mg kg^{-1}, for gilthead seabream and common carp, respectively) and Se-rich yeast (selenium target level of 1.28 ± 0.02 mg kg^{-1} and 1.41 ± 0.05 mg kg^{-1}, for gilthead seabream and common carp, respectively) blends (Annex A.III Table S. 4. 1) manufactured at SPAROS, Lda (Olhão, Portugal). Each experimental treatment (BF and CTR diet) was tested in triplicate tanks over 72 days (gilthead seabream) or cages over 98 days (common carp), representing the effect of a finishing diet. At the end of the trials, 24h following the last meal, 30 fish per treatment (10 per replicate tank or cage) were slaughtered by immersion in chilled seawater (gilthead seabream) or freshwater (common carp) following the procedure usually performed in commercial fish farms, individually weighed, and stored at 4 °C. Gilthead seabream specimens mean final weigh ranged from 620 ± 57 g (BF) to 568 ± 63 (CTR), whereas common carp from 1129 ± 165 g (BF) to 919 ± 118 g (CTR).

2.2. Freezing, glazing and frozen storage

Gilthead seabream preparation, freezing, glazing and frozen storage were performed at GELPEIXE (Loures, Portugal), simulating the conventional industrial process. Fish were washed and cleaned. Fish skin-on fillets were collected, deep frozen in an industrial cryogenic tunnel (Praxair 0.6 x 6 m, TBM S.A.), immersed in water at 4 °C for 30 s, and immediately transferred to the cryogenic tunnel to form a layer of glaze (with a glazing target of 6 - 10 % accordingly with the commercial practices). For each treatment (CTR and BF), fish fillets were randomly packed in polyethylene terephthalate (PET) bags (n = 3 fillets in each package), sealed (Lovero Bag Sealer Sk-410, Korea) and stored in cartoon packages at – 20 °C in a freezing chamber for 12 months. Similarly, common carp whole specimens were washed, cleaned and frozen following the conventional industrial process. Glazed common carp specimens were randomly packed in PET bags (n = 3 fish in each package), sealed (Multivac A 300/52, Wolfertschweden, Germany), stored in cartoon packages at – 20 °C in the freezing chamber for 12 months and fish fillets were prepared whenever required. For each treatment (CTR and BF), samples (n = 6 fish fillets) were taken before (day 0) and after 45, 135, 225 and 360 days of frozen storage.

2.3. Analyses

Frozen samples were thawed under refrigeration (4 ± 1 °C) for approximately 12h, and fish fillets were divided in sections for further analysis (Figure 4.1). For each treatment (BF and CTR), physicochemical quality of gilthead seabream and common carp six fillets were assessed on day 0 and after 45, 135, 225 and 360 days of storage. For elemental composition analyses, skinless fish fillets portions (n = 3 pools of 2 fillets each) were homogenized with a grinder (Retasch Grindomix GM200, Germany) using polypropylene cups and stainless-steel knives at 10,000 *g* until complete visual disruption of the tissue and stored at – 80 °C until further analysis. Lipid oxidation (LPO) and WHC of gilthead seabream and common carp were evaluated in six skinless fish fillets portions, whereas colour and texture (hardness) were assessed in six skin-on fillets (in order to reduce samples manipulation) portions. All analyses were performed at least in duplicate.

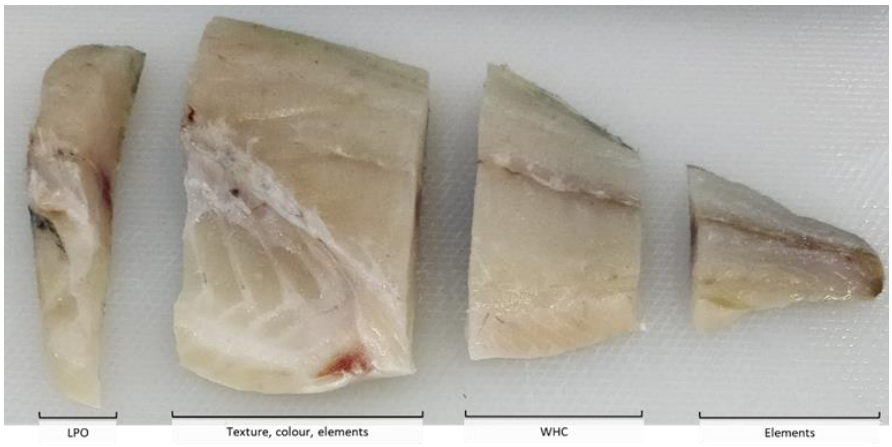

Figure 4.1 - Scheme of seabream fish fillet sections for each analysis. Both fillets were used for elemental composition and other quality assessment. LPO – lipid oxidation (TBAR), WHC – water holding capacity.

2.3.1. Elemental composition

2.3.1.1. Iodine, selenium and arsenic

Iodine (I), selenium (Se) and arsenic (As) were determined by inductively coupled plasma mass spectrometer (ICP-MS; Thermo X series II, Thermo Fisher Scientific, Waltham, USA), according to Barbosa et al. (2020). Iodine (I) content was quantified according to the EN15111:2007 (European Committee for Standardization, 2007), whereas Se and As were determined following the EN15763:2009 (European Committee for Standardization, 2009). Briefly, the alkaline digestion (for I) was performed by a 48-well graphite heating block (DigiPREP, SCP Science, Courtaboeuf, France) with tetramethylammonium hydroxide (TMAH; Fluka, St. Gallen,

Switzerland) solution 25% (v/v), whereas the acid digestion (for Se and As) was performed overnight with 60% (v/v) ultrapure nitric acid solution, followed by a 48-well graphite heating block (DigiPREP, SCP Science, Courtaboeuf, France) with hydrogen peroxide solution 30% (v/v, Merck). ICP-MS operating conditions were optimized daily, and the quantification was Ione by linear calibration using standard solutions of I, Se and As prepared from single elements high purity ICP stock standards (Inorganic Ventures and SCP Science, respectively), ranging between 1 and 50 µg g^{-1} for I, 0.5 and 5 µg g^{-1} for Se and 0.25 and 2.5 µg g^{-1} for As.

2.3.1.2. Iron, zinc, copper, bromide, chlorine, potassium and calcium

Iron (Fe), copper (Cu), zinc (Zn), bromide (Br), chlorine (Cl), potassium (K) and calcium (Ca), were determined by energy dispersive X-ray fluorescence spectrometry (EDXRF) according to (Shaltout et al. (2020). Briefly, freeze-dried muscle samples were ground for 2 min under 10 tons to make a cylindrical pellet with a diameter of 20 mm and a thickness of 1 mm. The energy EDXRF spectra were acquired by a polarized geometry, secondary target, and high energy XRF spectrometer (Epsilon 5, PANalytical, Netherlands). The characteristic radiations emitted by each element in the sample were detected by secondary targets (CaF_2, Ge and Mo). A Germanium detector with a nominal resolution of 144 eV for Mn-Kα was used for recording the X-ray fluorescence spectra and the acquisition time of each spectrum was daily adjusted for each secondary Target and the operating conditions.

2.3.1.3. Quality assurance

All reagents used in the analyses were of high analytical grade and water was ultra-purified (< 18 MΩ cm) using a Milli-Q-Integral system (Merck, Germany). Analytical quality was assessed through reference materials including oyster tissue (SRM 1566b) from the National Institute of Standards and Technology (Gaithersburg, EUA), dogfish muscle (DORM-2) from the National Research Council of Canada (Ontario, Canada) and fish muscle (ERM®-BB422) from the European Commission – Joint Research Centre Institute for Reference Materials and Measurements (IRMM) (Geel, Belgium). The obtained values agree with certified values. Detailed information about quality assurance, including the limits of quantification (LOQ) and detection (LOD), is presented in Annex A.III Table S. 4. 2.

2.3.2. Lipid oxidation

Lipid oxidation (LPO) was determined by the 2-thiobarbituric acid index (TBA). Thiobarbituric acid reactive substances (TBARs) were determined according to the Vyncke method

modified by Ke et al. (1984). Briefly, TBARs were determined in duplicate in trichloroacetic acid ≥98% (7.5% m/v, Merck) extracts of homogenized samples by the spectrophotometric method (530 nm). Results were expressed as mg of malonaldehyde (MDA) kg^{-1} of sample and calculated using a standard curve prepared with five different concentrations of 1,1,3,3-tetraethoxypropane (≥ 96%, Sigma-Aldrich).

2.3.3. Glazing water

Glazing water was determined according to the NP 4355:2002 (Portuguese Committee for Standardization, 2002). Briefly, fish samples were removed from the freezer and immediately weighted (Wi). Then, the frozen sample was immersed into a water bath, containing an amount of fresh water equal to about 10 times the declared weight of the product, at 20 ± 1°C for about 1 min until all visible ice-glaze was removed. Finally, fish samples were carefully dipped dry (without pressure) with a cotton rag and the net-weight determined (Wf). The percentage of glazing was calculated according to the following equation:

$$Glazing\ (\%) = \left(\frac{Wi - Wf}{Wi}\right) \times 100$$

2.3.4. Water holding capacity and moisture

Water holding capacity (WHC) was determined according to Estévez et al. (2021). Each sample analysed (approximately 2 g) comprised 3 slices of independent fish fillets. The slices were chopped into small cubes (3 × 3 mm), wrapped in two overlaid Whatman No.1 filter papers (previously weighted) and centrifuged at 3000 *g* for 10 min at 18 °C (Kubota 6800, Kubota Corp., Tokyo, Japan). After centrifugation, the sample was removed, and filter papers were weighed. Samples WHC was calculated by the weight of the liquid released and expressed as the amount of water retained by the sample using the following equation:

$$WHC\ (\%) = \left(\frac{Ws \times \left(\frac{H}{100}\right) - (Wf - Wi)}{Ws \times \left(\frac{H}{100}\right)}\right) \times 100$$

Where:

Ws = weight of sample analysed (approximately 2 g)

Wf and *Wi* = weight of filter papers after (f) and before (i) centrifugation (*g*), respectively

H = Sample moisture (%) determined by weight loss during freeze-drying at − 50 °C, 1.1 Pa of vacuum pressure, for 48h (Power Dry 150 LL3000, Heto, Czech Republic).

2.3.5. Colour

Colour determination was performed in fillets surface (dorsal) using the model CR-410 (Konica Minolta Camera, Co, Japan), previously calibrated with a white standard plate (L^*= 97.79, a^*= - 0.02, b^* = 1.84). The L^*, a^* and b^* coordinates from CIELAB system were recorded. In this system, L^* denotes lightness on a scale of 0 (black) to 100 (white); a^* values describe the intensity from green (-) to red (+); and b^* values from blue (-) to yellow (+). Whiteness (WI) and chroma value (C^*) (colour intensity) were calculated according to Schubring (2005) and International Commission on Illumination (Hermann, 2001), respectively by the following equations:

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

$$C^* = \sqrt{(a^{*2} + b^{*2})}$$

2.3.6. Texture

Hardness analysis of fish muscle samples was carried out on a TA.XTplus analyser (Stable Micro Systems, Surrey, UK) using a 5 kg load cell and the TA.XTplus software. Fish muscle was cut into slices (20 mm thickness and 60 mm length) and samples were compressed up to 50% of the original height with a spherical probe of 12.5 mm diameter and applying a constant speed of 1 mm s^{-1} in the centre of the sample. Hardness results were expressed in newtons (N).

2.4. Statistical analysis

The distribution and variance homoscedasticity of the data were analysed using Kolmogorov–Smirnov and Levene's tests, respectively. The influence of frozen storage time (0, 45, 135, 225, 360 days) on physicochemical quality parameters were analysed for each treatment (CTR and BF) by one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for pair wise multiple comparisons. Whenever data (or transformed data) did not meet the normality and variance homoscedasticity assumptions, the Kruskal–Wallis test was performed followed by non-parametric multiple comparison test. Significant differences between treatments (CTR and BF) in each frozen storage time were tested by t-test student. Furthermore, when t-test student assumptions were not met, nonparametric Mann–Whitney U test was used. Significance level was assigned at 0.05. Pearson or Spearman correlations were used to correlate

physicochemical quality data (P <0.05). All analyses were carried out using STATISTICA™ (Version 7.0, StatSoft Inc., Tulsa, Oklahoma, USA).

3. Results

3.1. Trace, macro and toxic elements

Generally, the elements content in gilthead seabream fillets showed some stability during frozen storage, yet occasionally some fluctuations were observed. During storage of biofortified (BF) fillets, a significant decrease in I and Br contents was observed after 135 and 45 days, respectively. Then, in both cases, the values remained constant until the end of storage (Figure 4.2). On the other hand, non-biofortified (CTR) fillets showed a significant decrease in Br and Ca contents after 45 days. Decreased Fe content was observed during storage, though only significantly in BF fillets at day 225 and in CTR fillets from day 135 to 225, compared to days 0 and 45 of storage. BF fillets revealed significantly higher contents of I during frozen storage (except at 225 days) and Se along the storage period, as well as K content at days 0 and 225, compared to CTR fillets. In contrast, significantly lower content of As was observed in BF fillets at day 45, compared to CTR fillets. Before storage (day 0), BF fillets presented significantly higher content of Fe in relation to CTR. Frozen storage decreased Cu content (< LOQ) in both BF and CTR fillets over the entire storage period (data not shown).

Concerning common carp, occasionally mineral changes with no specific trend were observed during frozen storage. Biofortified (BF) fillets presented significantly lower content of As, as well as higher content of Cl after 135 and 45 days of storage, respectively (Figure 4.3). Significantly higher Fe content was observed in BF fillets at days 45 and 360, as well as Ca content after 360 days, compared to day 0 (before frozen storage). On the other hand, CTR fillets showed lower Se content after day 360 and As content at days 135 and 360, compared to day 0. Biofortified fillets revealed significantly higher contents of I, Se, Zn, Br and As along the whole storage period (from day 0 to 360), compared to CTR fillets. Moreover, BF fillets presented significantly higher contents of Fe (at days 0, 45 and 360), Cl (at day 135) and Ca (at day 225), as well as significantly lower contents of K (at day 135) and Ca (at day 45), compared to CTR. Additionally, a positive and significant (P <0.05) correlation between Se and As contents during frozen storage was only observed in BF fillets (r=0.99).

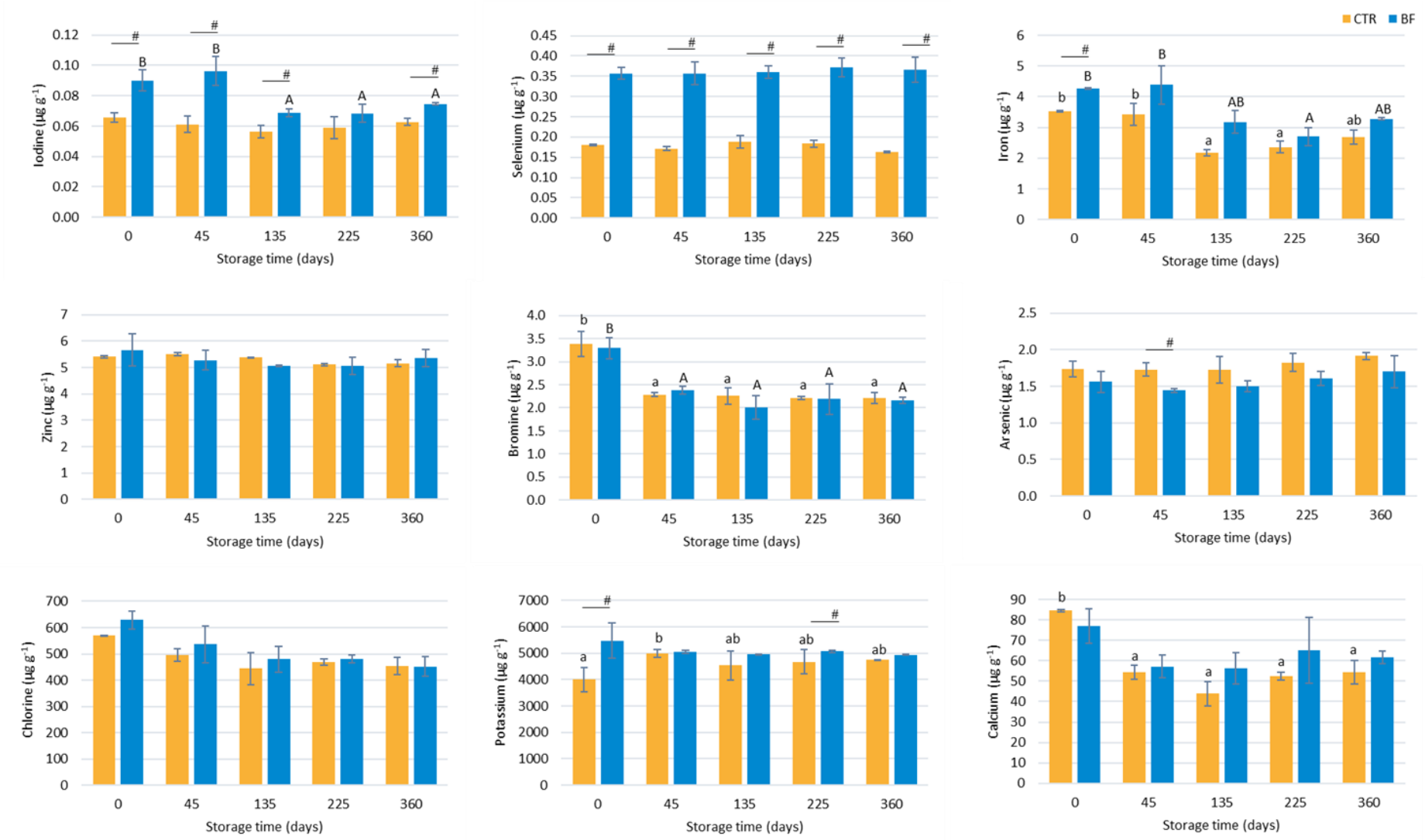

Figure 4.2 - Levels of trace (iodine, selenium, iron, zinc, bromine; in µg g^{-1}), toxic (arsenic; in µg g^{-1}) and macro (chlorine, potassium, calcium, in µg g^{-1}) elements in biofortified (BF) and non-biofortified (CTR) gilthead seabream fillets (average ± SD, in wet weight) during frozen storage at – 20 °C. Different lower-case and upper-case letters indicate significant differences ($P < 0.05$) between times (0, 45 135, 225 and 360 days) in CTR and BF fish fillets, respectively. For each frozen storage time, # represents significant differences ($P < 0.05$) between CTR and BF fish fillets.

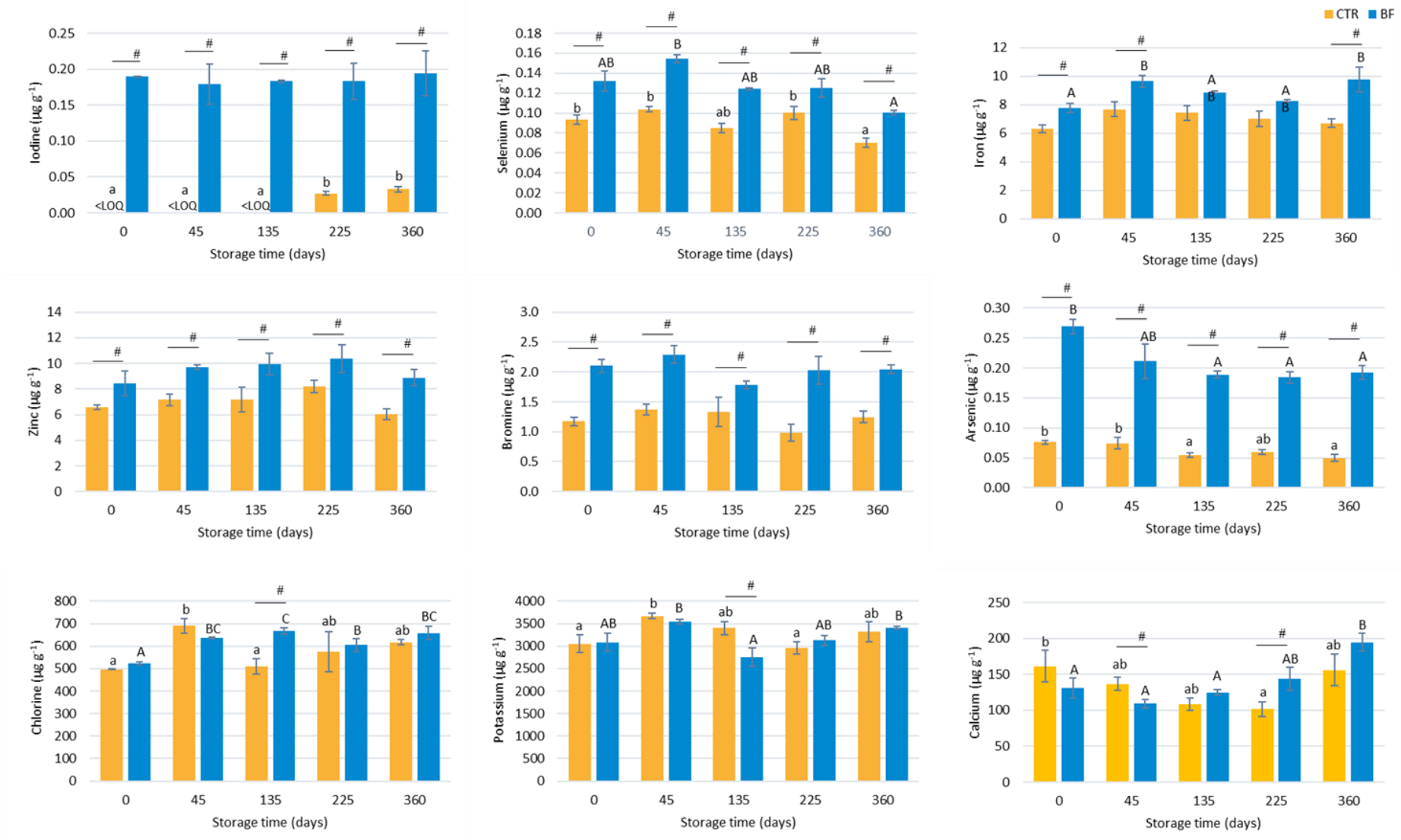

Figure 4.3 - Levels of trace (iodine, selenium, iron, zinc, bromine; in µg g^{-1}), toxic (arsenic; in µg g^{-1}) and macro (chlorine, potassium, calcium, in µg g^{-1}) elements in biofortified (BF) and non-biofortified (CTR) common carp fillets (average ± SD, in wet weigh) during frozen storage at – 20 °C. Different lower-case and upper-case letters indicate significant differences ($P < 0.05$) between times (0, 45 135, 225 and 360 days) in CTR and BF fish fillets, respectively. For each frozen storage time, # represents significant differences ($P < 0.05$) between CTR and BF fish fillets.

3.2. Glazing water, WHC, moisture and lipid oxidation (TBAR)

Glazing water percentages ranged between 9 ± 1 and 13 ± 1 in CTR gilthead seabream fillets and between 10 ± 1 and 13 ± 2 in BF gilthead seabream fillets. WHC significantly decreased immediately after 45 days and then stabilized throughout the storage period in both BF and CTR fish fillets (Table 4.1). Biofortified fillets presented significantly lower WHC at 45 and 360 days of storage, compared to CTR fillets. A positive and significant (P <0.05) correlation between WHC and Br was observed in CTR (r=0.94) and BF (r=0.60) fillets. Regarding moisture, in general, no significant differences were observed during frozen storage, yet BF fillets presented a higher content at 135 and 225 days compared to day 0 of storage. Both BF and CTR fillets showed significantly increased lipid oxidation throughout the storage period, presenting similar thiobarbituric acid reactive substances (TBARs) formation rates (x = 0.0058 for BF and x = 0.0062 for CTR). Furthermore, BF fillets revealed significantly higher TBARs values before frozen storage (day 0) and at 360 days of storage compared to CTR fillets (Figure 4.4).

Table 4.1 - Water holding capacity (WHC, %) and moisture (%) of gilthead seabream and common carp fillets (CTR – control, BF – biofortified) during frozen storage at –20 °C.

Storage time (days)	WHC		Moisture	
	CTR	BF	CTR	BF
Gilthead seabream				
0	61 ± 4 ^{c}	58 ± 4 ^{B}	69 ± 1	65 ± 6 ^{A}
45	49 ± 3 ^{ab}	44 ± 2 $^{A\#}$	68 ± 1	67 ± 1 ^{AB}
135	51 ± 2 ^{b}	48 ± 4 ^{A}	69 ± 1	72 ± 3 ^{B}
225	45 ± 3 ^{a}	47 ± 4 ^{A}	70 ± 0	72 ± 1 ^{B}
360	47 ±3 ^{ab}	41 ± 4 $^{A\#}$	71 ± 1	70 ± 1 ^{AB}
Common carp				
0	58 ± 2 ^{c}	51 ± 2 $^{C\#}$	77 ± 1 ^{ab}	77 ± 1
45	57 ± 4 ^{c}	53 ± 4 ^{C}	77 ± 1 ^{ab}	77 ± 0
135	49 ± 3 ^{b}	48 ± 3 ^{BC}	77 ± 2 ^{ab}	78 ± 6
225	43 ± 3 ^{a}	41 ± 2 ^{A}	79 ± 0 ^{b}	79 ± 0
360	44 ± 3 ^{a}	44 ± 3 ^{AB}	76 ± 1 ^{a}	77 ± 1

Results are given as average values ± standard deviation.
Different lower-case and upper-case superscript letters indicate significant differences (P < 0.05) in CTR and BF fish fillets, respectively, during frozen storage (0, 45, 135, 225 and 360 days). For each frozen storage time, # represent significant differences (P < 0.05) between CTR and BF fish fillets.

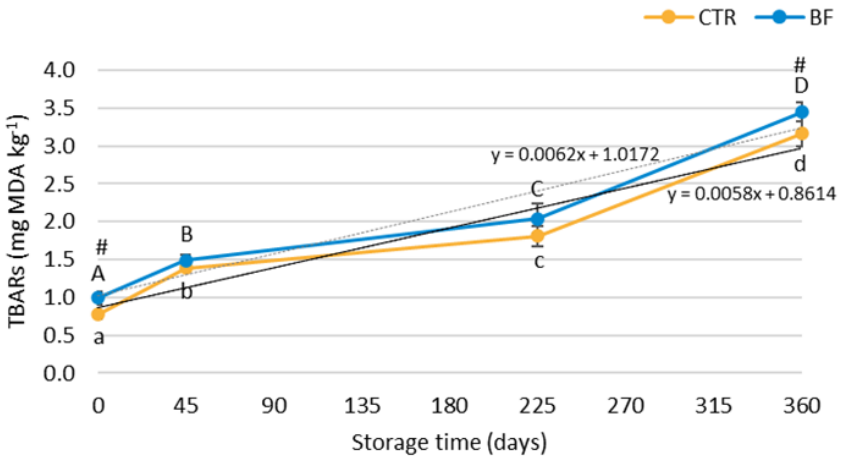

Figure 4.4 - Thiobarbituric acid reactive substances (TBAR, in wet weight) values in gilthead seabream (CTR – control, BF – biofortified) during frozen storage at – 20 °C. Different lower-case and upper-case letters indicate significant differences ($P < 0.05$) in CTR and BF fish fillets, respectively during frozen storage (0, 45 135, 225 and 360 days). For each frozen storage time, # represent significant differences ($P < 0.05$) between CTR and BF fish fillets.

Concerning common carp, glazing water percentages ranged between 5 ± 0.1 and 6 ± 0.3 in CTR fillets and between 4 ± 0.4 and 6 ± 0.4 in BF fillets. WHC significantly decreased after 225 days in BF and fish fillets and after 135 days of frozen storage in CTR fillets (Table 4.1). Biofortified fillets presented significantly lower WHC before storage (day 0), compared to CTR fillets. Overall, no significant differences were observed in moisture content during the frozen storage, yet such content were higher at 225 days compared to 360 days (end of storage time) in CTR fillets. Additionally, a positive and significant correlation between WHC and As was found in CTR (r=0.81) and BF (r=0.66) fillets. Both BF and CTR fillets had significantly increased lipid oxidation after 225 days of frozen storage, presenting equal TBARs formation rates (x = 0.0043 for BF and CTR). Additionally, BF fillets revealed significantly lower TBARs values during frozen storage (except at 360 days) compared to CTR fillets (Figure 4.5).

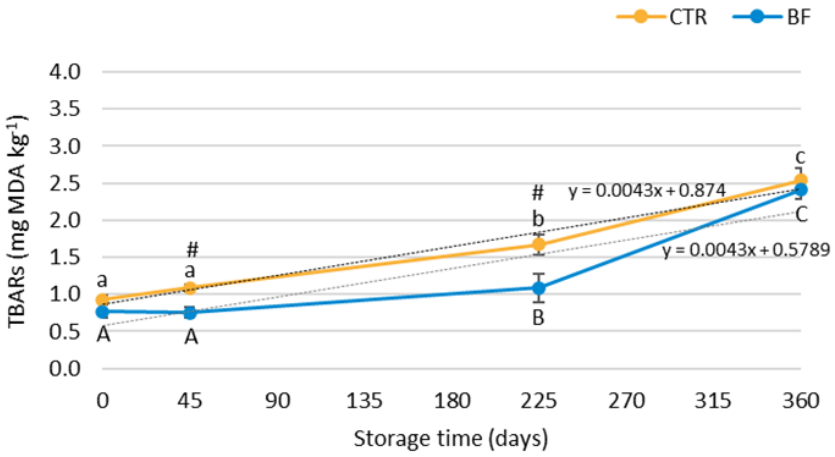

Figure 4.5 - Thiobarbituric acid reactive substances (TBAR, in wet weight) values in common carp (CTR – control, BF – biofortified) during frozen storage at – 20 °C. Different lower-case and upper-case letters indicate significant differences ($P < 0.05$) in CTR and BF fish fillets, respectively during frozen storage (0, 45 135, 225 and 360 days). For each frozen storage time, # represent significant differences ($P < 0.05$) between CTR and BF fish fillets.

3.3. Colour and texture

Gilthead seabream presented significantly lower *a** values (redness) in both BF (after 135 days) and CTR (after 45 days) fillets, as well as significantly higher *b** values (yellowness) over the storage period (after 45 days up to 360 days) in both BF and CTR fillets (Table 4.2). Regarding *C** values, a significant increase was observed after 45 days of storage in both BF and CTR fillets. Then, the colour intensity generally remained stable until the end of storage. Significantly higher *L** values (lightness) were only observed in BF fillets at day 45 of storage. Higher *WI* (whiteness) values were observed in BF fillets at day 45 of storage, compared to days 135 and 360 of storage. Biofortified (BF) gilthead seabream fillets presented significantly higher lightness, redness, whiteness, and colour intensity than CTR fillets only at 45 days of storage. Before storage (day 0), BF fillets presented significantly lower *a** values (redness) and higher *b** values (yellowness), compared to CTR fillets.

Table 4.2 - Colour (colour coordinates, whiteness and chroma) and texture (hardness, N) values found in gilthead seabream and common carp fillets (CTR – control, BF – biofortified) during frozen storage at – 20 °C.

Storage time (days)	Colour										Texture	
	*L**		*a**		*b**		*WI*		*C**		Hardness	
	CTR	BF	CTR	BF	CTR	BF	CTR	BF	CTR	BF	CTR	BF
Gilthead Seabream												
0	65 ± 2 ^{ab}	64 ± 1 ^{A}	5.3 ± 0.6 ^{c}	4.3 ± 0.5 $^{B\#}$	4.0 ± 0.6 ^{a}	5.2 ± 0.9 $^{A\#}$	65 ± 2	64 ± 1 ^{AB}	6.3 ± 1.0 ^{a}	6.9 ± 1.0 ^{A}	3.2 ± 0.3 ^{b}	3.3 ± 0.4 ^{B}
45	65 ± 3 ^{ab}	75 ± 4 $^{B\#}$	2.4 ± 0.3 ^{b}	5.2 ± 0.2 $^{C\#}$	8.4 ± 1.1 ^{b}	9.2 ± 0.8 ^{B}	64 ± 3	71 ± 4 $^{B\#}$	8.8 ± 0.5 ^{b}	10.8 ± 0.6 $^{C\#}$	2.4 ± 0.3 ^{a}	2.6 ± 0.3 ^{A}
135	62 ± 2 ^{a}	61 ± 4 ^{A}	1.7 ± 0.2 ^{a}	1.6 ± 0.2 ^{A}	8.3 ± 0.9 ^{b}	9.0 ± 0.8 ^{B}	61 ± 2	60 ± 4 ^{A}	8.8 ± 0.8 ^{b}	9.1 ± 0.8 ^{BC}	2.7 ± 0.3 ^{ab}	2.8 ± 0.3 ^{AB}
225	66 ± 2 ^{b}	67 ± 1 ^{A}	1.4 ± 0.2 ^{a}	1.3 ± 0.2 ^{A}	11.1 ± 1.4 ^{c}	10.1 ± 0.9 ^{B}	64 ± 2	65 ± 1 ^{AB}	11.1 ± 1.3 ^{b}	9.9 ± 1.1 ^{BC}	2.7 ± 0.3 ^{ab}	2.8 ± 0.5 ^{AB}
360	63 ± 2 ^{ab}	64 ± 2 ^{A}	1.3 ± 0.2 ^{a}	1.0 ± 0.1 $^{A\#}$	9.3 ± 1.4 ^{bc}	8.4 ± 1.2 ^{B}	62 ± 2	63 ± 2 ^{A}	9.7 ± 0.9 ^{b}	8.6 ± 1.3 ^{AB}	2.8 ± 0.4 ^{ab}	2.9 ± 0.4 ^{AB}
Common carp												
0	49 ± 2 ^{a}	52 ± 2 $^{AB\#}$	15 ± 2	15 ± 1	7.2 ± 1.0	5.0 ± 0.1 $^{A\#}$	46 ± 3 ^{a}	50 ± 2 $^{AB\#}$	17 ± 2 ^{b}	16 ± 2	1.3 ± 0.2 ^{a}	1.6 ± 0.3 $^{A\#}$
45	52 ± 3 ^{a}	47 ± 3 $^{A\#}$	11 ± 1	13 ± 2 $^{\#}$	7.3 ± 0.4	6.0 ± 0.8 $^{AB\#}$	53 ± 3 ^{bc}	46 ± 2 $^{A\#}$	13 ± 1 ^{a}	15 ± 2	1.7 ± 0.2 ^{a}	1.9 ± 0.2 ^{A}
135	55 ± 2 ^{ab}	53 ± 2 ^{AB}	12 ± 1	13 ± 1	7.5 ± 0.5	7.0 ± 0.7 ^{BC}	49 ± 3 ^{ab}	51 ± 3 ^{AB}	15 ± 1 ^{ab}	14 ± 2	1.8 ± 0.3 ^{a}	2.0 ± 0.2 $^{A\#}$
225	56 ± 4 ^{ab}	55 ± 4 ^{B}	13 ± 1	14 ± 1 $^{\#}$	7.4 ± 0.4	7.6 ± 0.6 ^{BC}	53 ± 4 ^{bc}	52 ± 2 ^{AB}	15 ± 0 ^{ab}	16 ± 1	2.9 ± 0.5 ^{b}	2.8 ± 0.4 ^{B}
360	60 ± 3 ^{b}	57 ± 3 ^{B}	12 ± 1	13 ± 1	8.5 ± 0.7	8.0 ± 0.7 ^{C}	57 ± 3 ^{c}	55 ± 4 ^{B}	15 ± 1 ^{ab}	15 ± 1	2.9 ± 0.5 ^{b}	2.7 ± 0.3 ^{B}

Results are given as average values ± standard deviation.

Different lower-case and upper-case superscript letters indicate significant differences ($P < 0.05$) in CTR and BF fish fillets, respectively, during frozen storage (0, 45, 135, 225 and 360 days). For each frozen storage time, $^{\#}$ represent significant differences ($P < 0.05$) between CTR and BF fish fillets.

Common carp presented a significant increase of b^*values (yellowness) in BF fillets after 135 days of storage (Table 4.2). Lower WI (whiteness) values were observed in BF fillets at day 45 of storage, compared to day 360 of storage. In contrast, CTR fillets presented higher L^* values (lightness) after 360 days of storage, increased *WI* (whiteness) after 45 days of storage and lower C^* values at day 45 of storage. Biofortified (BF) common carp fillets presented significantly lower lightness, yellowness, and whiteness, as well as higher redness, than CTR fillets only at 45 days of storage. Before storage (day 0), BF fillets presented significantly higher L^*values (lightness) and whiteness, as well as b^*values (yellowness), compared to CTR fillets.

Concerning textural properties, a significant decrease in hardness was observed in both BF and CTR gilthead seabream fillets after 45 days of frozen storage, and then the values remained stable until the end of storage (Table 4.2). In contrast, a significant increase in hardness was observed in both BF and CTR common carp fillets after 225 days of frozen storage. In addition, common carp BF fillets presented significantly higher hardness values before storage (day 0) and at 135 days of storage, compared to CTR fillets.

4. Discussion

4.1. Trace, macro and toxic elements

The present biofortification strategies (i.e., incorporation of iodine-rich seaweed and Se-rich yeast in aquafeeds) resulted in an enhanced content of I and Se in fish fillets, as previously reported (Barbosa et al., 2020). Additionally, higher As content in biofortified (BF) common carp fillets resulted from the dietary supplementation with seaweed, since seaweed species tend to accumulate As. On the other hand, higher contents of Fe, Zn and Br in biofortified common carp fillets resulted by the inclusion of *Spirulina* sp. microalgae blends in biofortified diets (Barbosa et al., 2020). Still, biofortified (BF) and non-biofortified (CTR) gilthead seabream and common carp fillets revealed distinct patterns in terms of elemental contents, likely as a result of losses during thawing (Arannilewa et al., 2006; Malik et al., 2021; Prego et al., 2021). This aspect may be explained by a decrease in the binding forces between minerals and water from the muscle, causing nutrients losses (Gökoğlu & Yerlikaya, 2015b). Several studies reported the effect of frozen storage on fish chemical composition, particularly on lipids and protein contents (Dang et al., 2017; Jiang et al., 2020; Nasopoulou et al., 2012; Popelka et al., 2014;

Romotowska et al., 2017). Yet, to the best of the author's knowledge, few studies addressed the effect of such preservation method on essential elements, including I and Se, in fish species, and no studies focused on mineral concentrations stability in fortified fish products during long-term storage. Biofortified gilthead seabream fillets showed a significant decrease of I content after 135 days of frozen storage, and then stabilized throughout the storage period, probably due to some volatilization (Todorov & Gray, 2016). It seems that chemical form of the element and the ability of each fish species to absorb inorganic elements from their diet could also affect the mineral contents of fish fillets during frozen storage. Losses of elements observed during freezing may also be related with the release from fish muscle into the surrounding aqueous medium after thawing (Malik et al., 2021). Karl, et al. (2005) demonstrated that I content significantly decreased in Atlantic cod fillets after deep-freezing at –40 °C and subsequent thawing, with higher I concentrations found in the drip compared to fillets. Despite the decrease found in biofortified gilthead seabream fillets after 135 days of frozen storage, I contents were always higher in biofortified fillets than in non-biofortified fillets in both species. On the other hand, Se content seems to be relatively stable during storage, since no significant changes were observed in both biofortified and non-biofortified gilthead seabream fillets, whereas a significant decrease in Se content was observed in both biofortified and non-biofortified common carp fillets only at 360 days of storage. Noteworthy both gilthead seabream and common carp biofortified fillets always presented significantly higher Se content along the frozen storage period, compared to non-biofortified fish fillets. A previous study also reported no specific upward or downward trends for several essential elements, including Na, Mg, P, S, Fe, Cu and Se, in Atlantic chub mackerel stored at –18 °C for 15 months (Prego et al., 2021). The present study reports a reduction in Fe, Cu and Br in biofortified gilthead seabream fillets during frozen storage, which is in line with a previous one, where frozen storage of freshwater fish species significantly decreased Fe (in fillets of Forskal's catfish after 30 days of storage at –18 °C) and Cu contents (in fillets of Nile perch over the 45 days storage period, in wahrindi after 15 and 45 days of storage and in Forskal's catfish after 30 days of storage at –18 °C) (Malik et al., 2021). Freezing frequently produces intracellular ice crystals leading to highly concentrated intracellular medium, and ultimately to osmotic imbalance, resulting in intracellular water flow, from inside to outside, alongside with nutrients, during the thawing process (Wolfe & Bryant, 2001). Increased and decreased Ca content was also previously reported in Atlantic chub mackerel during frozen storage (Prego et al., 2021), and in Forskal's catfish at the end of

the storage period (Malik et al., 2021). Additionally, no changes in Ca content were reported in Nile tilapia stored at –18 °C for 60 days (Arannilewa et al., 2006) and in wahringi, Forskal's catfish and Nile perch stored at –18 °C for 45 days (Malik et al., 2021). Moreover, Sharaf (2013) did not observed a clear relationship between the mineral content (Cu, Zn, Fe, Ca, K) and freezing time in frozen Nile tilapia muscle at –18 °C for 8 weeks. Additionally, the present study demonstrated that frozen storage decreased As content in biofortified common carp fillets, whereas increased As content was previously reported in Atlantic chub mackerel during frozen storage (Prego et al., 2021). Therefore, changes in terms of elemental content in frozen fish are mainly associated with the species, as well as storage period and conditions (Malik et al., 2021). Moreover, some of the observed fluctuations in elements content in gilthead seabream and common carp fillets during frozen storage could be related with different fish specimens used in each sampling period, since natural differences (e.g., stage of development/size, metabolism) inherent to each individual may occur. The present results shown that both biofortified and non-biofortified gilthead seabream fillets can be stored at –20 °C for at least 45 days without any changes in essential elements levels when stored under conventional processing industrial conditions. Afterwards, despite maintaining a high nutritional quality, biofortified fillets lost I and Br content and non-biofortified fillets lost Fe, Br and Ca content. On the other hand, biofortified and non-biofortified common carp whole fish can be stored at –20 °C for at least 225 days without no detrimental effect in essential elements stability, since only afterwards both biofortified and non-biofortified fillets lost Se content. Overall, biofortified gilthead seabream and common carp fillets still maintained enhanced nutritional quality compared to non-biofortified fillets, particularly due to significantly higher I and Se levels during the 360 days of frozen storage at –20 °C.

4.2. Glazing water, WHC and moisture

Previous studies have shown that glazing fish fillets can reduce dehydration, leading to less freezer-burns and also a delay in lipid hydrolysis and oxidation reactions during frozen storage (Gonçalves & Junior, 2009; Soares et al., 2013; Wang et al., 2020). In both biofortified and non-biofortified gilthead seabream fillets, glazing percentages where in general within the normal ranges of glaze content (8–12 %) (Vanhaecke et al., 2010), and never exceeded the maximum glazing percentage (up to 20 %) considered as reasonable to guarantee the final quality of seafood products (Gonçalves & Junior, 2009;

Vanhaecke et al., 2010). On the other hand, biofortified common carp whole fish presented lower glazing percentages than gilthead seabream fillets, still near the 6 % threshold for adequate protection. Variations in glazing percentages between different batches at different times of frozen storage were also previously reported due to irregular glaze layer application difficult to control (Vanhaecke et al., 2010). In contrast to the present results, decreased moisture content during frozen storage was observed in non-glazed fish species, namely in mango tilapia fillets stored at –18 °C up to 60 days (Arannilewa et al., 2006) and in wahrindi, Forskal's catfish, and Nile perch at –18 °C up to 45 days (Malik et al., 2021). On the other hand, in line with the current results, reduced WHC during freezing and frozen storage was also previously reported by other authors (Burgaard, 2010; Burgaard & Jørgensen, 2011; Naseri et al., 2020; Tan et al., 2019; Wang et al., 2020; Zang et al., 2017). In general, biofortified and non-biofortified fillets presented similar WHC. WHC is associated to muscle proteins properties, and lower water-binding ability is primarily due to denaturation and aggregation of proteins (especially myosin) and oxidative changes, during the freezing-thawing process (Burgaard & Jørgensen 2011; Dang et al., 2017). Decreased WHC in fish stored between –10 to –20 °C is likely due to the formation of ice crystals in the muscle, leading to mechanical damage of cells tissue and denaturation of myofibril proteins, resulting in incomplete restoration of the muscle tissue during thawing, which can reduce the amount of water retained after thawing (Dang et al., 2017; Nakazawa & Okazaki, 2020). The slight decrease in WHC in both gilthead seabream and common carp fillets suggests that glazing process was effective to maintain their quality during frozen storage.

4.3. Lipid oxidation (TBAR)

Fish muscle lipid oxidation was quantified by the determination of MDA content, which is the final product of lipid oxidation. In line with previous studies, an increase of TBARs values was observed in glazed tuna stored at –18 °C for 180 days (Wang et al., 2020), rainbow trout at –20 °C for 18 months (Burgaard & Jørgensen 2011), Atlantic herring at –25 °C for 14 months (Dang et al., 2017), Atlantic mackerel at –25 °C for 9 months (Romotowska et al., 2017), and horse mackerel at –20 °C for 12 months (Aubourg et al., 2004). Furthermore, Calanche and co-authors (2019) reported increased TBARs values in gilthead seabream stored at –30 °C for 1 month after 5, 9, 11 and 18 days, with highest values (1.5 mg kg^{-1} MDA) than the present values obtain for biofortified and non-

biofortified gilthead seabream fillets (1.5 mg kg^{-1} MDA and 1.4 mg kg^{-1} MDA, respectively) stored at –20 °C for 45 days. On the other hand, increased TBARs values were also observed in common carp (Hematyar et al., 2018) stored at –20 °C for 24 weeks, with lowest MDA values (0.1 mg kg^{-1} in semi-frozen samples) than those obtained in the present study for biofortified and non-biofortified common carp fillets (0.8 and 1.8 mg kg^{-1} MDA, respectively) stored at –20 °C for 45 days. Nevertheless, glazed silver carp stored at –3 °C for 30 days, showed higher values (3.0 mg kg^{-1} MDA) than the present results obtained for biofortified and non-biofortified common carp fillets (2.4 mg kg^{-1} MDA and 2.5 mg kg^{-1} MDA, respectively) during 360 days of frozen storage. Increased lipid oxidation may be associated with the transformation of peroxides into aldehyde compounds that are end-products of lipid oxidation in the presence of oxygen and pro-oxidant molecules (Duarte et al., 2020; Gökoğlu & Yerlikaya, 2015a). Nevertheless, glazing or coating layers prevent the penetration of oxygen into tissues, delaying lipid oxidation during frozen storage (Gökoğlu & Yerlikaya, 2015a; Soares et al., 2013; Tolstorebrov et al., 2016). Despite TBARs values of 1–2 mg MDA kg^{-1} of fish muscle are usually regarded as the limit beyond which fish quality deteriorates significantly and meat present rancid flavour and odour (Connell, 1990), 8 mg MDA per kg of fish muscle was set as acceptable for human consumption (Schormüller, 1969). In the present study, results never exceeded the maximum limit of MDA acceptable for human consumption. Considering the range of values referred by Schormüller (1969), TBARs values were close to 2 mg MDA kg^{-1} after 225 days of storage (2.0 mg MDA kg^{-1} for biofortified fillets and 1.8 mg MDA kg^{-1} for non-biofortified fillets), whereas all TBARs values were close to 3 mg MDA kg^{-1} (3.5 mg MDA kg^{-1} for biofortified fillets and 3.2 mg MDA kg^{-1} for non-biofortified fillets) by the end of the frozen storage period, indicating a good quality of the gilthead seabream fillets frozen at –20 °C for 360 days. Concerning common carp fillets, all TBARs values were close to 2 mg MDA kg^{-1} (2.4 mg MDA kg^{-1} for biofortified fillets and 2.5 mg MDA kg^{-1} for non-biofortified fillets) by the end of the frozen storage period, indicating a very good quality for 360 days of frozen storage. In general, similar TBARs formation (i.e., development rates) during frozen storage was observed in biofortified and non-biofortified fillets. The values of TBARs obtained in common carp fillets were lower than those found in gilthead seabream, which may be due to whole fish frozen storage versus fish fillets.

4.4. Colour and texture

Colorimetric measurements revealed that the frozen storage period significantly influenced lightness (*L**), redness (*a**) and yellowness (*b**) values, mainly in both gilthead seabream and common carp biofortified fillets. Fish muscle colour is a crucial factor for consumer acceptance, and fresh gilthead seabream is recognized for its white and shiny flesh (Tsironi et al., 2020), whereas common carp fish muscle presents a reddish colour. The increase in lightness (L*) and whiteness values found in our study agrees with previous results (Agüeria et al., 2016). At 45 days of storage the maximum of lightness and whiteness was observed in biofortified gilthead seabream fillets, values significantly higher compared to those observed in non-fortified fillets. On the other hand, both biofortified and non-biofortified common carp fillets revealed an increase of lightness and whiteness, generally, after 225 days of frozen storage. Increased lightness during frozen storage was also reported in glazed fillets of Atlantic herring stored at –20 °C for 52 weeks (Cavonius & Undeland, 2017), in common carp fillets stored at –20 °C after the 3rd, 8th, and 16th week of storage (Hematyar et al., 2018) and in Atlantic cod fillets stored at –20 °C during 13 months (Schubring, 2005). Overall, a decrease in redness (*a**) and increase in yellowness (*b**) were observed in gilthead seabream, reflected on increased intensity of colour (*C**) without affecting the whiteness. On the other hand, an increase of lightness (*L**) was observed in common carp fillets, resulting in higher whiteness at the end of the frozen storage period. Similar results were also observed in previous studies. Indeed, both increase and decrease in redness was observed in gilthead seabream (whole fish) stored at –20 °C for 360 days (Huidobro & Tejada, 2004). Furthermore, increased *b** values were also observed in gilthead seabream (whole fish) up to 100 days of frozen storage (Huidobro & Tejada, 2004), and both increase and decrease in yellowness were also reported in glazed fillets of Atlantic herring during storage at –20 °C (Cavonius & Undeland, 2017), while no changes were observed in common carp fillets (Hematyar et al., 2018). Additionally, Naseri et al. (2020) reported decreased *L**, *a** and *b** values in glazed rainbow trout after 10 days of storage at –18 °C. Colour changes, during freezing-thawing processes, can be associated to myoglobin autoxidation and metmyoglobin production resulting from lipid oxidation and pigment degradation (Alsailawi et al., 2020; Hematyar et al., 2018; Naseri et al., 2020). Nevertheless, glazing can retard lipid oxidation during frozen storage, protecting fish muscle from colour changes (Tan et al., 2019), and therefore extend the quality of fillets.

In terms of texture, in general it was observed a decreasing trend in hardness during frozen storage of gilthead seabream fillets, where the lowest values were found in the first 45 days. Similar results were previously reported in glazed bigeye tuna stored at –18 °C for 180 days (Wang et al., 2020), grass carp fillets stored at –18 °C for 5 months (Jiang et al., 2020), and gilthead seabream stored in ice for 24 days (Alasalvar et al., 2001). Furthermore, a significant decrease in firmness was found in common carp fillets after 1 week of frozen storage at –20 °C, being constant afterwards until 23 weeks of storage (Hematyar et al., 2018), and in glazed and non-glazed rainbow trout after 10 days of storage at –18 °C (Naseri et al., 2020). In contrast, a significant increase of hardness was observed in both biofortified and non-biofortified common carp after 225 days of storage at –20 °C. An increase of hardness followed by a decrease was reported in Atlantic herring during storage at –25 °C (Szczepanik et al., 2010), and its decrease followed by a subsequent increase was reported in Nile tilapia stored at –18 °C for 150 days (Subbaiah et al., 2015). Hardness of fish fillets after frozen-thawing process is mainly affected by the formation of ice crystals and protein denaturation (Hematyar et al., 2018; Jiang et al., 2020). The formation of ice crystals during freezing and frozen storage may lead to structural damage of myofibrillar cells, resulting in decreased mechanical strength of connective fish muscle tissue after thawing (Hematyar et al., 2018; Wang et al., 2020). However, increased hardness of fish muscle can be related to the loss of WHC and the ratio between salt-soluble protein and total proteins (Subbaiah et al., 2015; Alsailawi et al., 2020). Furthermore, in general, glazing probably reduce the denaturation of myofibrillar proteins, maintaining the textural properties (Naseri et al., 2020; Wang et al., 2020), and consequently the quality of fish fillets during frozen storage.

5. Conclusions

This study provides new insights about the effect of frozen storage in the quality assessment (in terms of elemental composition, WHC, lipid oxidation, colour, and texture) of biofortified gilthead seabream and common carp fillets. Overall, the biofortification strategies through the dietary supplementation with the incorporation of I-rich seaweed (*L. digitata*) and Se-rich yeast in gilthead seabream and common carp did not relevantly affect the studied quality parameters during frozen storage. In fact, biofortified gilthead seabream and common carp presented significantly higher contents of iodine and

selenium over the frozen storage period, and therefore potentially improved nutritional benefits, compared to non-biofortified fish. No specific trend was observed in elements contents and colour attributes during frozen storage, yet decreased WHC and increased lipid oxidation (TBARs) in both biofortified and non-biofortified during frozen storage. In general, the main quality changes in gilthead seabream fillets started after 45 days of storage, whereas the main physicochemical changes in common carp fillets occurred after 225 days of storage.

Overall, the industrial frozen storage did not compromise the nutritional benefits and quality of the tested biofortified gilthead seabream and common carp, hence and according to the parameters analysed, namely enhanced I and Se contents, TBARs, WHC, texture and colour, biofortified fillets maintained good nutritional quality and storage stability during the 360 days of storage at –20 °C.

Ethical statement

Fish trials were conducted according to legal regulations (EU Directive, 2010/63) and approved by the Ethical Committee of the EPPO-IPMA, overseen by the National Competence Authority. All researchers and technicians involved in the maintenance, handling and sampling of live animals were certified in Laboratory Animal Sciences, by the Federation of European Laboratory Animal Science Associations (FELASA).

5

In Vitro Bioaccessibility of Macro and Trace Elements in Biofortified and Conventional Farmed Gilthead Seabream (*Sparus Aurata*) and Common Carp (*Cyprinus Carpio*)

In this chapter you will find the Manuscript:

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Abstract

Biofortification is a promising strategy to improve the nutrient profile of farmed fish but requires consideration of the nutrient bioaccesible fraction. In this study, the in vitro bioaccessibility of macro and trace elements was investigated in biofortified and conventional farmed gilthead seabream and common carp, also considering the effect of cooking (by steaming). Biofortification enhanced iodine and selenium levels in seabream and carp fillets. Steaming increased iodine and selenium contents in biofortified seabream, and increased selenium and decreased copper levels in biofortified carp. Higher iodine bioaccessibilty (> 80%) was observed in biofortified seabream compared to biofortified carp (45%). In both species, selenium, and iron bioaccessibility was above 70%. Upon steaming iodine, calcium, and iron bioaccessibility decreased in seabream, while selenium and calcium bioaccessibility decreased in carp. The consumption of steamed biofortified seabream and carp contributes to significantly higher daily intakes of iodine (up to 12% and 9%, respectively) and selenium (up to 54% and above 100%, respectively), without increased exposure to arsenic. The present study demonstrates the potential of developing innovative biofortified farmed fish using natural sustainable feed ingredients to improve the intake of important nutrients for human health.

Keywords: biofortification; macro and trace elements; in vitro digestion, seafood

1. Introduction

Seafood-based diets are associated to health benefits in view of their being valuable source of essential nutrients, such as long chain polyunsaturated n-3 fatty acids, vitamin D, selenium, and iodine (EFSA, 2015d; Guérin et al., 2011). A regular and balanced consumption of seafood may overcome widespread nutritional deficiencies and is recommended during pregnancy for the positive impact on functional outcomes of children's neurodevelopment and in adulthood to lower the risk of cardiovascular diseases (EFSA, 2014d; FAO/WHO, 2011). However, one third of the world's population still suffers from nutritional deficiencies, particularly of iron, iodine, selenium, zinc, and vitamin A, which are elements related with impaired neurophysiological and immunological functions during the most crucial stages of human growth (FAO, 2020; FAO et al., 2021). Significant efforts have been put into finding alternative resources to reduce hunger, improve food security, nutrition and promote food systems sustainability (Bellia et al., 2021; FAO, 2020). Aquaculture offers the opportunity to produce seafood with additional health benefits by tailoring the nutritional contents of farmed species through the incorporation of functional components in feeds (Barbosa et al., 2020; Bellia et al., 2021; Ferreira et al., 2020; Ramalho Ribeiro et al., 2017; Valente et al., 2015). Successful biofortification (i.e., improvement of the nutritional profile of fish using diets supplemented with natural ingredients) was achieved in farmed gilthead seabream (*Sparus aurata*) and common carp (*Cyprinus carpio*) through the incorporation of I-rich seaweed (*Laminaria digitata*) and Se-enriched yeast in aquaculture feeds, resulting in enhanced I, Se, and Fe contents in fish muscle (Barbosa et al., 2020). Nevertheless, it is well acknowledged that the level of a nutrient in a portion of seafood does not allow to predict the amount of such nutrient that will be released from the food matrix and become available for absorption across the human intestinal epithelium during the digestion process, i.e., the bioaccessible fraction (Alves et al., 2018; Cardoso et al., 2015; Marques et al., 2011; Versantvoort et al., 2005). Bioaccessibility analysis plays an important role in risk-benefit assessment of the consumption of specific food or diets, since nutrients uptake depends not only on the ingested amount of a specific food item, but also on nutrients bioaccessibility (Cardoso et al., 2015; Girard et al., 2018; Hu et al., 2019). Several *in vitro* models have been developed to simulate the human digestion process, with static methodologies sequentially simulating oral, gastric and intestinal phases with digestive juices (including the relevant enzymes in all steps) being the most used and reliable models to evaluate nutrients bioaccessibility in seafood (Alves et al., 2018; Cardoso et al., 2013; Torres-Escribano et al., 2011). Also, in vitro models are a cost-effective alternative to in vivo methods, since are less expensive,

rapid, energy saving and allow controlling the experimental conditions for better reproducibility (Cardoso et al., 2015; He et al., 2010). This has led to an international effort to standardize them (Brodkorb et al., 2019; Minekus et al., 2014). Previous studies have used in vitro digestion methodologies to evaluate nutrients bioaccessibility in seafood and showed that oral bioaccessibility varies with the biochemical composition of the food matrix, processing, or preparation (Alves et al., 2018; Costa et al., 2016; Guérin et al., 2011; Lei et al., 2013; Maulvault et al., 2011; Moreda-Piñeiro et al., 2012). For instance, Alves and co-authors (2018) found that the cooking process reduces the bioaccessibility of toxic elements in seafood (i.e., MeHg and Cd), while a wider variability was found for essential elements (i.e., increased in fish for Zn, decreased in mussel for Fe and unchanged in fish and shellfish for Se and I). Despite attention has been recently given to trace elements bioaccessibility, including selenium and its species in seafood (Cabañero et al., 2007; Moreda-Piñeiro et al., 2011, 2013b), to our knowledge, the existing information on iodine bioaccessibility in fish is still scarce (Ferraris et al., 2021). I and Se are essential nutrients for neurological and thyroid development, and seafood is a prominent dietary source (Bevis, 2015; FAO, 2020). Therefore, integrating bioaccessibility in the evaluation of the supply of these nutrients via seafood is of utmost importance. Indeed, the few available studies addressing Se and I bioaccessibility in fortified foodstuff focused on vegetables (do Nascimento da Silva et al., 2017; Hu et al., 2019; Pedrero et al., 2006).

In this context, the aim of the present study was to: 1) investigate the bioaccessibility of macro and trace elements (calcium, potassium, bromine, copper, iodine, iron, selenium, zinc, and the non-essential element arsenic), in gilthead seabream and common carp biofortified with I-rich seaweed (*L. digitata*) and Se-enriched yeast as feed ingredients with respect to non-biofortified counterparts; 2) evaluate the effect of steam-cooking on the bioaccessibility of macro and trace elements; and 3) assess the nutrients intake provided by the consumption of these fish items in relation to the relevant dietary reference values (DRVs). Seabream (*S. aurata*) and common carp (*C. carpio*) were selected as models since they are two of the most relevant and consumed farmed fish species (10% and 5% of European aquaculture production, respectively; 7% and 6% of European total apparent consumption, respectively) in Mediterranean countries and in central Europe (EUMOFA, 2021).

2. Material and Methods

2.1. Growth trials and sampling

For each species, the feeding trial comprised two diets: a control diet (CTR), consisting in a commercial feed formulation covering the nutritional requirements for adult gilthead seabream and common carp, and an experimental biofortified feed (BF), supplemented with I-rich seaweed (*L. digitata*) and Se-enriched yeast blends (Annex A.IV Table S. 5. 1). Gilthead seabream BF diet contained moderate levels of fishmeal (10%) and fish oil (3.8%) and was supplemented with a blend of microalgae (*Chlorella* sp., *Tetraselmis* sp., *Schizochytrium* sp.), macroalgae (*L. digitata*) and selenised-yeast. Concerning common carp BF diet, half of the fishmeal was replaced with a blend of microalgae (*Spirulina* sp., *Chlorella* sp.), macroalgae (*L. digitata*) and selenised-yeast, and vegetable oil was replaced by salmon oil extracted from by-products of farmed Atlantic salmon. Experimental extruded diets were manufactured by SPAROS, Lda (Olhão, Portugal) and enriched diets formulations took into consideration the current maximum authorized contents of total I (20 mg kg^{-1}) and Se (0.5 mg kg^{-1}) in fish feeds (EFSA, 2005, 2006).

The trial with gilthead seabream was conducted at SKALOMA farm facilities (Greece), whereas the common carp trial was conducted at the Fisheries Research Station of West Pomeranian University of Technology in Szczecin (Poland). Both trials were performed in compliance with the European guidelines on protection of animals used for scientific purposes (European Commission, 2007). Gilthead seabream specimens with an average initial body weight of 424 ± 21 g were distributed into a set of three cages (n = 490 fish per cage) placed in a coastal fish farm (39° 40' 16.77" N 20° 04' 22.70" E) and subjected to natural photoperiod (from August till November) with water temperature average of 23.4 ± 2.4 °C and a mean salinity of 36 ‰. The experimental feeding trial was tested in triplicate for 90 days (simulating a finishing diet). On the other hand, the common carp study was carried in a total of 6 cuboid cages of 3 m^3 placed in an earthen pond (53° 42′ 5.99′′ N 15° 21′ 22.19′′ E). Each cage was stocked with 100 fish (average initial body weight of 250 ± 10 g), and the feeding trial was conducted in triplicate for 116 days. Fish were hand-fed with equal portions, according to standard practices at the fish farms, and no mortality was observed during both gilthead seabream and common carp trials. For each species, final samplings were done 24 h after the last meal and 24 fish per treatment (CTR and BF) were sacrificed by immersion in chilled seawater (seabream) or freshwater (carp) following the commercial procedures employed in fish farms. Both gilthead seabream and common carp skinless fish muscle were collected at the start (n = 3 pools of 3 fish

each) and at the end of the trial (n = 3 pools of 8 fish each per treatment). All fish were measured, weighted (Table 5.1) and at the end of the trial one fish fillet (per specimen) was used for culinary steam-cooking, whereas the other fillet was used for raw assessment. All fish samples were homogenized with a grinder (Retasch Grindomix GM200, Germany) using polypropylene cups and stainless-steel knives at 10,000 *g* until complete visual disruption of the tissue and were stored at − 80 °C until further analysis.

2.2. Steam-cooking procedure and moisture content

For each treatment and species, fish muscle samples were individually wrapped up in aluminium foil and steamed in an oven (Combi-Master CM 6, Rational GroßkÜcken Technik GmbH, Germany) at 105 °C for 15 min. After steaming, fish muscle samples were cooled at room temperature. The final weight was registered to obtain the relevant cooking yield (CY), as the percentage ratio between cooked and raw fish muscle weight (Table 5.1). The true retention (TR, %) for each element was calculated using following the formula (USDA, 2008):

$$TR = \left(\frac{mean\ content\ element\ in\ cooked\ food}{mean\ content\ element\ ras\ food}\right) \times CY,$$

where CY = cooking yield

Table 5.1 - Gilthead seabream (*S. aurata*) and common carp (*C. carpio*) biometric information before and after the feeding trial and fish muscle moisture (%) content before and after the culinary treatment.

				Muscle fillet			
	n	Total weight (g)	Total lenght (cm)	Moisture Raw (%)	Moisture Steamed (%)	Weight loss (%)	CY (%)
Gilthead seabream							
Baseline	9	413 ± 33	30 ± 1	72 ± 1	n.d.	n.d.	n.d.
CTR	24	598 ± 76	32. ± 1	71 ± 1	71 ± 1	8 ± 1	92 ± 4
BF	24	572 ± 74	32 ± 1	71 ± 1	70 ± 1	8 ± 1	92 ± 4
Common carp							
Baseline	9	261 ± 51	25 ± 2	81 ± 1	n.d.	n.d.	n.d.
CTR	24	1295 ± 90	37 ± 4	75 ± 1	71 ± 2	10 ± 1	92 ± 8
BF	24	1359 ± 37	37 ± 3	76 ± 1	73 ± 1	9 ± 2	92 ± 4

n, number of specimens analysed; n.d., not determined; CY, cooking yield; CTR, control diet; BF, biofortified diet.

2.3. *In vitro* human digestion model

2.3.1. Reagents

The reagents used to prepare the digestion fluids (Annex A.IV Table S. 5. 2) were the following: Inorganic: NaCl (Merck, 99.5% m/v), $NaHCO_3$ (Merck, 99.5% m/v), $CaCl_2{\cdot}2H_2O$ (Sigma, C3881), KCl (Merck, 99.5% m/v), KSCN (Sigma, P2713), NaH_2PO_4 (Merck, 99.5% m/v), Na_2SO_4 (Merck 90% m/v), NH_4Cl (Riedel-de Haen, 99.5% m/v), KH_2PO_4 (Merck, 99.5% m/v), $MgCl_2$ (Riedel-de Haen, 99.5% m/v), HCl (Merck, 37% m/v); Organic: urea (Sigma, U5128), glucose (Sigma, G5400), glucuronic acid (Sigma, G5269), D-(+)-Glucosamine hydrochloride (Sigma, G1514), uric acid (Sigma, U2625), albumin from bovine serum (Sigma, A7906), α-amylase, from *Aspergillus oryzae* (Sigma, 86250), mucin from porcine stomach (Sigma, M2378), pepsin from porcine stomach mucosa (Sigma, P7125), lipase from porcine pancreas type II (Sigma, L3126), pancreatin from porcine pancreas (Sigma, P1625), trypsin from porcine pancreas (Sigma, T0303), α-chymotrypsin from bovine pancreas (Sigma, C4129), and bile porcine extract (Sigma, B8631).

2.3.2. *In vitro* digestion procedure

Each raw and steamed fish sample was digested in duplicate using the same in vitro digestion protocol described by Alves et al. (2018). Briefly, 1.5 g of each fish homogenized sample were digested in Nalgene™ high-speed PPCO centrifuge tubes at 37 ºC using a Rotary Tube Mixer with Disc (25 rpm; LSCI, Portugal) in an incubator (Genlab, UK). The digestion steps were performed as follow: i) oral phase, where 4 mL of saliva fluid was added to the fish sample and incubated for 5 min at pH 7.0 ± 0.2; ii) gastric phase, where 8 mL of gastric fluid was added to the oral phase and incubated for 2 h at pH 2.0 ± 0.2); and iii) intestinal phase, where 8 mL of duodenal fluid and 4 mL of bile fluid were added to the gastric phase and incubated for 2 h at pH 7.0 ± 0.2 (digestion fluids composition are described in Annex A.IV Table S. 5. 2). Enzyme degradation/inhibition was prevented by preparing each digestion fluid immediately before starting the digestion protocol, and the pH was adjusted immediately before each digestion step with NaOH (1 M) or HCl (1 M). At the end of the digestion, the pro-cess was stopped by placing the reaction tubes on ice, followed by centrifugation at 2750 g for 10 min at 10 ºC to separate the bioaccessible fraction (i.e., supernatant; BIO) from the sample pellet (non-bioaccessible fraction - NBIO). Negative controls containing the di-gestion fluids without fish sample were also performed. BIO and NBIO fractions were kept at − 80 ºC until analysis.

2.4. Analytical determination

2.4.1. Moisture content

Moisture content was determined in raw and steamed samples according to the Association of Official Analytical Chemists methods (AOAC, 2005). Briefly, moisture was determined by oven (ULE 500, Memmert, Schwabach, Germany) drying of sample overnight at 105 ± 1 °C.

2.4.2. Essential and toxic elements

Essential and toxic elements were quantified in biofortified (BF) and non-biofortified (CTR) fish muscle samples (raw and steamed) before (BD) and after the in vitro digestion procedure. Each element in the bioaccessible (BIO) fraction (%) was calculated as the following ratio:

$$Bioaccessibility\ (\%) = \frac{BIO\ \times 100}{BD}$$

where BIO corresponds to the element levels detected in bioaccessible fraction and BD corresponds to the element levels detected in the sample before digestion.

2.4.2.1. Iodine (I), selenium (Se) and arsenic (As)

I and Se were determined by inductively coupled plasma mass spectrometry (ICP-MS). Raw and steamed fish muscle samples (BD) were homogenized, and 1 g was placed in high-pressure Teflon containers with 3 ml of HNO_3 67-69% v/v (ultrapure grade, Carlo Erba, Rodano, Italy) and 1 mL of H_2O_2 30% v/v (ultrapure grade, Sigma-Aldrich, Darmstadt, Germany). Samples were digested in a microwave system (UltraWAVE Single Reaction Chamber Microwave Digestion System, Milestone, Bergamo, Italy) with the following program: a) 23 mins to reach 240 °C; b) 10 mins at 240 °C (maximum power 1400 W); and c) 30 min depressurization and cooling to reach room temperature. I was determined by quadrupole ICP-MS using a Nexion 350D ICP-MS (Perkin Elmer, Waltham, MA, U.S.A.) equipped with a quartz concentric nebulizer and a cyclonic spray chamber (Waltham, MA, U.S.A.), whereas for Se a triple quadrupole ICP-MS/MS (Agilent 8800, Agilent Technologies Inc., Tokyo, Japan) equipped with a PFA (perfluoroalkoxy) concentric nebulizer and a double-pass PFA spray chamber cooled to 2 °C was used. The latter instrument was operated in MS/MS reaction mode by using oxygen as a reaction gas in mass shift; the analytical masses (SeO^+) were m/z = 94 and 96, which provided interference-free conditions. The quantitative determinations were carried out by the standard

addition method. For iodine, an instrument tuning was performed daily prior to analysis to get the highest sensitivity at ^{127}I. Standards and samples were prepared in 1.5% (v/v) ammonia (Sigma Aldrich, Darmstadt, Germany) and 1% (v/v) isopropanol (Sigma Aldrich, Darmstadt, Germany), and quantitative determinations were carried out by external calibration. The bioaccessible fraction were diluted with the same mixture and analysed with the standard addition method. To prevent memory effects in I determination, tetramethylammonium hydroxide (TraceSelect, Sigma Aldrich, Darmstadt, Germany) 0.5% (v/v) was used for rinsing the sample introduction system. For both I and Se, the standard solutions were obtained by diluting stock solutions (1 g L^{-1}, High-Purity, Charleston, SC, USA) with high-purity deionized water obtained from a Milli-Q Element system (Millipore, Molsheim, France).

For As, raw and steamed fish muscle subsamples (BD) were homogenized and weighed (0.5 g) into 50 mL polypropylene DigiTUBEs (SCP Science, Quebec, Canada) and digested overnight in 7 mL of nitric acid (60% ultrapure w/w). Then, 1 mL of hydrogen peroxide (30% w/w, Merck) was added, and the digestion was carried out in a 48-well heating block (DigiPREP, SCP Science, Courtaboeuf, France) for 3.5 h at 85 °C. After cooling, the digests were diluted to 25 mL with MilliQ water. The bioaccessible fraction (BIO) was diluted in MilliQ water, filtered through 0.45 mm filters, and analysed by ICP-MS (Thermo X series II, Thermo Fisher Scientific, Bremen, Germany). ICP-MS operating conditions were optimized daily, and the quantification was done with a linear calibration curve using standard solutions from single elements high purity ICP stock standards (Inorganic Ventures and SCP Science) (Barbosa et al., 2020).

2.4.2.2. Potassium (K), calcium (Ca), iron (Fe), copper (Cu), zinc (Zn) and bromide (Br)

K, Ca, Br, Cu, Fe and Zn were determined in raw and steamed fish muscle samples (BD) by energy dispersive X-ray fluorescence spectrometry (EDXRF). Briefly, freeze-dried muscle samples were ground for 2 min under 10 tons to make a cylindrical pellet with a diameter of 20 mm and a thickness of 1 mm. high-energy 3-D optics XRF spectrometer (Epsilon 5, PANalytical, Netherlands). It is equipped with a 600W Sc/W-target X-ray tube with a beam spot diameter of about 18mm. Between the X-ray tube and the specimen, a set of secondary targets is inserted in XYZ polarization geometry offering, mainly, the monochromatization of the exciting beam. In this study CaF2, Ge and Mo secondary targets were selected. A Germanium detector with a nominal resolution of 140 eV for Mn-Kα was used for recording the X-ray fluorescence spectra and the acquisition time of each spectrum was adjusted for each secondary target and the operating conditions (Manousakas et al., 2018). For K and Fe, bioaccessible

fractions (BIO) were determined using an ICP-OES (Thermo iCAP 6000 series), with radial and axial configuration. ICP-OES instrumental operating conditions were the following: Auxiliar Flow: 0.5 L min^{-1}, Plasma Orientation: radial or axial, RF power: 1200 W, Peristaltic pump's speed (Flush pump rate and analysis pump rate): 50 rpm, Pump stabilization time: 5 sec, Integration time in UV and Visible: 15 and 10 sec. For Cu, Zn and Br, bioaccessible fractions (BIO) were diluted in Milli-Q water, filtered through 0.45 µm filters before ICP-MS analyses (ThermoX Series II, Thermo Fisher Scientific, Bremen, Germany). ICP-MS operating conditions were optimized daily, and standard solutions from single elements high purity ICP stock standards (Inorganic Ventures and SCP Science) were used.

2.4.2.3. Quality control

All reagents used in the analyses were of high analytical grade and water was ultra-purified (18.2 MΩ cm) using a Milli-Q-Integral system (Merck, Germany). Analytical quality was assessed through reference materials, including fish muscle (ERM®-BB422) from the European Commission – Joint Research Centre Institute for Reference Materials and Measurements (IRMM) (Geel, Belgium), dogfish muscle (DORM-2) from the National Re-search Council of Canada (Ontario, Canada) and oyster tissue (SRM 1566b) from the National Institute of Standards and Technology (Gaithersburg, EUA). The obtained values agreed with certified values (Annex A.IV Table S. 5. 3). Limits of detection (LODs) and quantification (LOQs) are presented in Annex A.IV Table S. 5. 3. The LOD was assigned to the detection limit (DL) of the calibration curve (DL = 3×standard deviation (σ) of response at the zero-concentration level) and the LOQ was calculated as (3 × LOD).

2.5. Nutritional contribution (NC)

The NC of biofortified (BF) and non-biofortified (CTR) fish muscle consumption was calculated according to reference values set for individual adults (> 18 years old), pregnant women and children (1-3 years) by the European Safety Authority and to the following formula:

$$NC\ (\%) = 100\ \times \frac{(C\ \times M)}{DRV}$$

where C = concentration of the element in µg g^{-1}; M = typical meal portion in g (150 g for adults and pregnant women and 75 g for children); DRV = adequate intake (AI) for I, Se, Cu and K (EFSA, 2014b, 2014c, 2015c, 2016) or population reference intake (PRI) for Ca, Fe and Zn (EFSA, 2014d, 2015b, 2015d). Whenever the NC was greater than 100%, the tolerable upper

intake level (UL), i.e., the maximum levels of total chronic daily intake (from all sources) which is not expected to pose a risk of adverse health effects to humans was also considered. Total As (TAs) concentrations were used to estimate the concentration of the chemical species of greater toxicological concern, i.e., inorganic As (iAs), under a worst case scenario where 5% of TAs in fish is in the form of iAs (Julshamn et al., 2012). For assessing consumers' health risks, the margin of exposure (MOE) with respect to the lower bound of the range of benchmark dose lower confidence limit ($BMDL_{01}$) for inorganic As (iAs) of 0.3 to 8 μg kg^{-1} body weight (b.w.) per day (EFSA, 2009), was considered.

2.6. Statistical analysis

Data were analysed for normality of distribution and homoscedasticity using Kolmogorov–Smirnov and Levene's tests, respectively, and data were Log-transformed, whenever necessary, to comply with the assumptions of normality (Kolmogorov–Smirnov's test) and homogeneity of variances (Levene's test). The effect of diet (BF and CTR) and culinary treatment (raw or steamed) on fish fillets elements content was tested by factorial analysis of variance (ANOVA). Post-hoc Tukey HSD test was applied in group multiple comparisons to identify significant differences. Statistical significance was set at $P < 0.05$ for all analyses using STATISTICA™ (Version 7.0, StatSoft Inc., Tulsa, Oklahoma, USA).

3. Results

3.1. Macro and trace elements in biofortified farmed fish

BF gilthead seabream fillets presented significantly higher contents of I and Se compared to CTR fillets (Figure 5.1, Annex A.IV Table S. 5. 4). Additionally, steaming significantly increased I, Se, Cu and Zn contents only in BF fillets. Higher TR values of I, Se, Cu and Zn were found in BF fillets (>100%), while CTR fillets showed higher TR values of As and Ca (>100%).

Concerning common carp, BF fillets presented statistically higher contents of I, Se, Zn, Cu and Br compared to CTR (Figure 5.2, Annex A.IV Table S. 5. 4). For Cu, this higher content was found only in raw BF fillets compared to CTR. Steaming significantly increased I and As content in CTR fillets, as well as Se content in BF fillets with TRs values above 100%. In contrast, steaming significantly decreased Cu content in BF fillets, where the lowest TR value (66%) was observed.

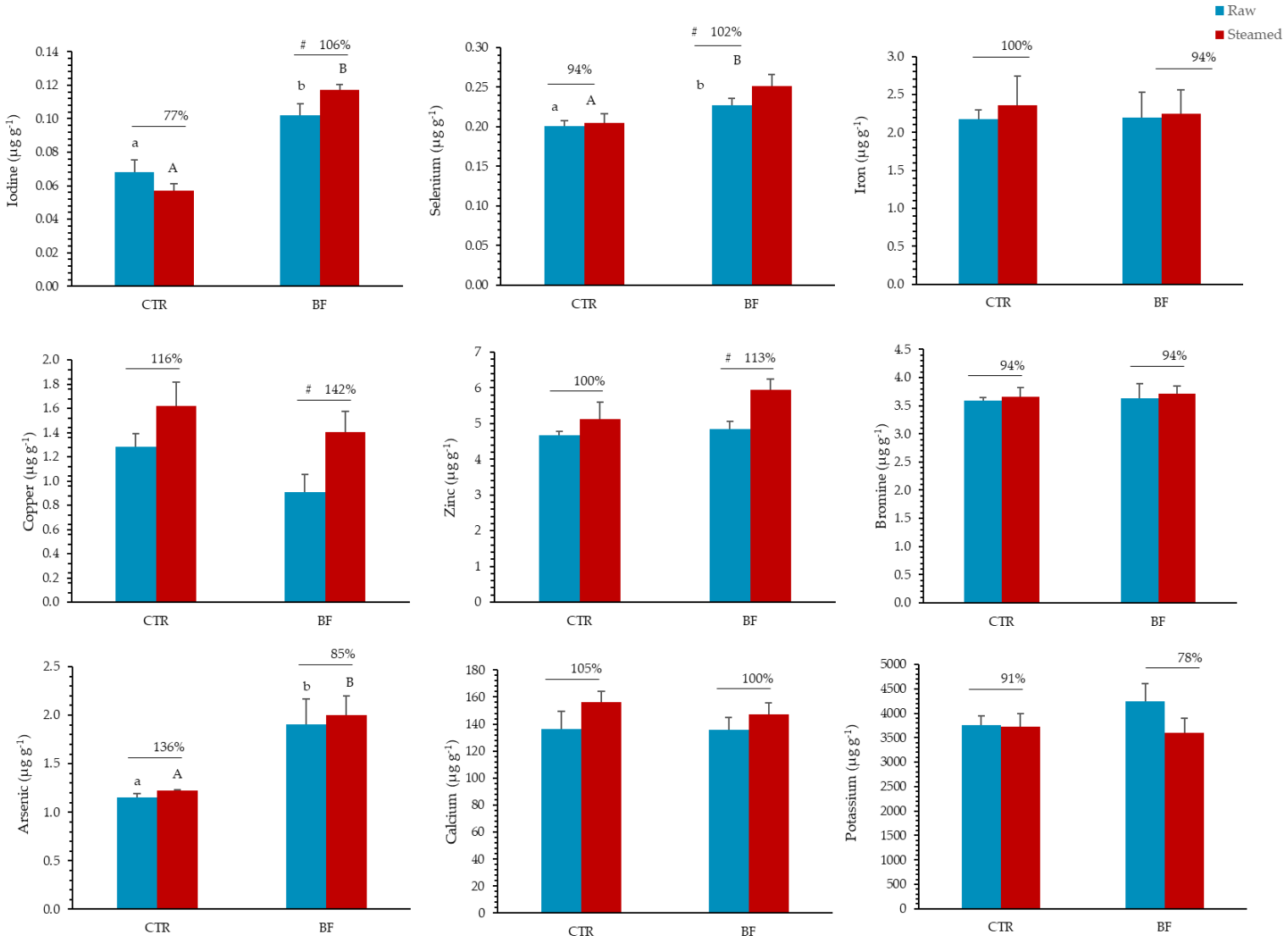

Figure 5.1 - Levels of trace (iodine, selenium, iron, zinc, copper, bromine; in µg g^{-1}), toxic (arsenic; in µg g^{-1}) and macro (potassium, calcium; in µg g^{-1}) elements biofortified (BF) and non-biofortified (CTR) gilthead seabream fillets (average ± SD, in wet weight) prior to in vitro digestion, and percentages of element true retention (TR) values in fish fillet after steaming. Different lower-case and upper-case letters indicate significant differences ($P < 0.05$) between CTR and BF fish fillets, in raw and steamed samples, respectively. For each treatment (CTR and BF), # represents significant differences ($P < 0.05$) between raw and steamed fillets

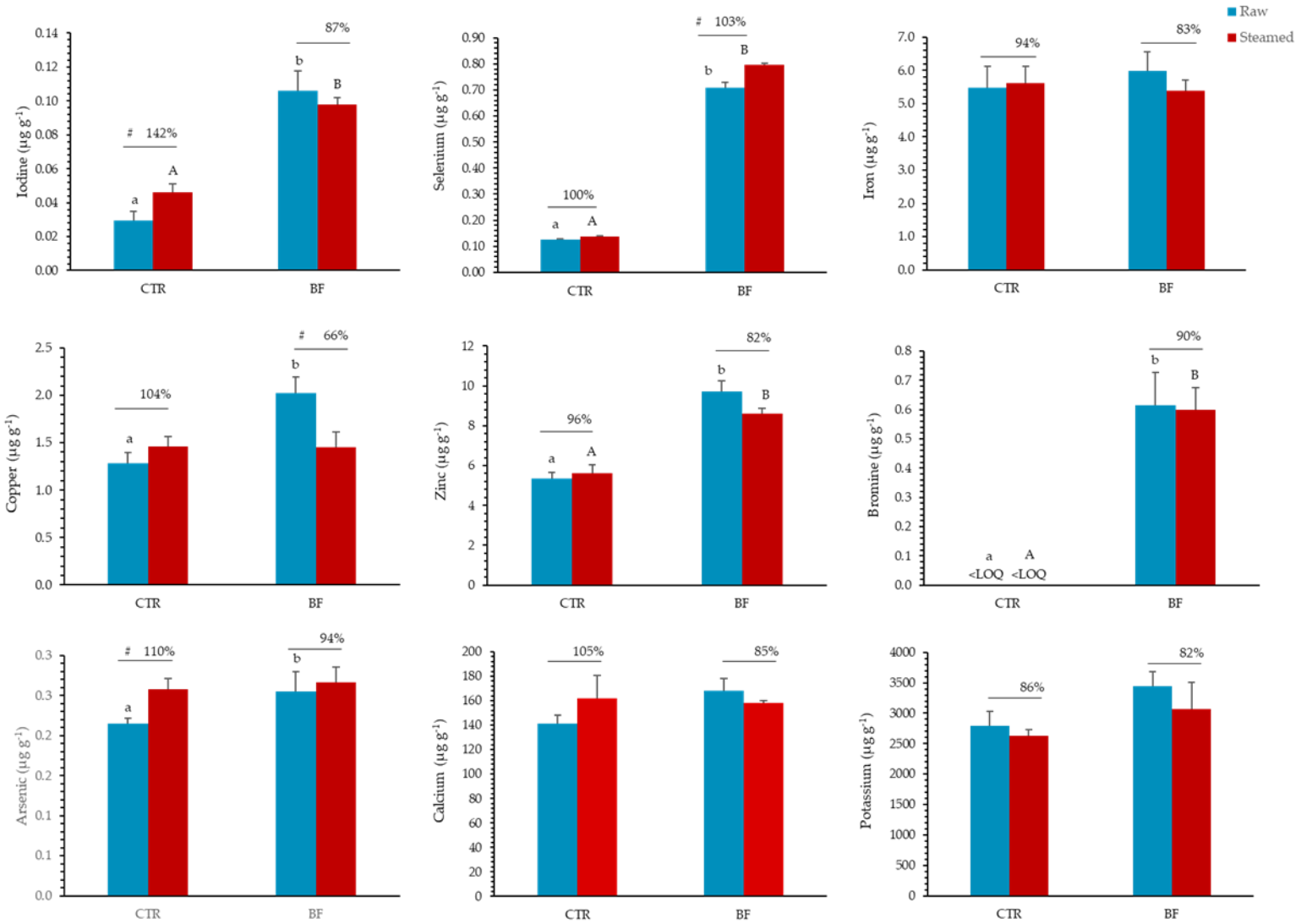

Figure 5.2 - Levels of trace (iodine, selenium, iron, zinc, copper, bromine; in µg g^{-1}), toxic (arsenic; in µg g^{-1}) and macro (potassium, calcium; in µg g^{-1}) elements biofortified (BF) and non-biofortified (CTR) common carp fillets (average ± SD, in wet weight) prior to in vitro digestion, and percentages of element true retention (TR) values in fish fillet after steaming. Different lower-case and upper-case letters indicate significant differences ($P < 0.05$) between CTR and BF fish fillets, in raw and steamed samples, respectively. For each treatment (CTR and BF), # represents significant differences ($P < 0.05$) between raw and steamed fillets.

3.2. Bioaccessibility of trace and macro elements in biofortified farmed fish

In both biofortified (BF) and non-biofortified (CTR) gilthead seabream fillets, I, Se, Fe, Zn, and K bioaccessibility was higher than 60% regardless of the culinary treatment (Figure 5.3, Annex A.IV Table S. 5. 5). The most bioaccessible elements were I and Zn, and Fe in raw fillets only. Yet, significantly lower K bioaccessibility was only observed in raw BF fillets compared to CTR. Steaming significantly decreased I (BF), Fe (CTR and BF) and Ca bioaccessibility (CTR and BF).

Regardless of the culinary treatment, for common carp fillets, I bioaccessibility was lower than 50% (CTR and BF), while Se and K bioaccessibilities varied between 50% (CTR) and 70% (BF), and Fe and Zn bioaccessibilities were higher than 70% (Figure 5.4, Annex A.IV Table S. 5. 5). BF fillets presented significantly higher bioaccessibility of I compared to CTR (< LOQ), as well as Se (in steamed fillets). In contrast, Ca bioaccessibility was significantly lower in CTR and BF fillets after steaming.

Bioaccessible Cu and Br values for both gilthead seabream (CTR and BF) and common carp (CTR and BF) fillets could not be determined as the results were below the limit of quantification (Annex A.IV Table S. 5. 5).

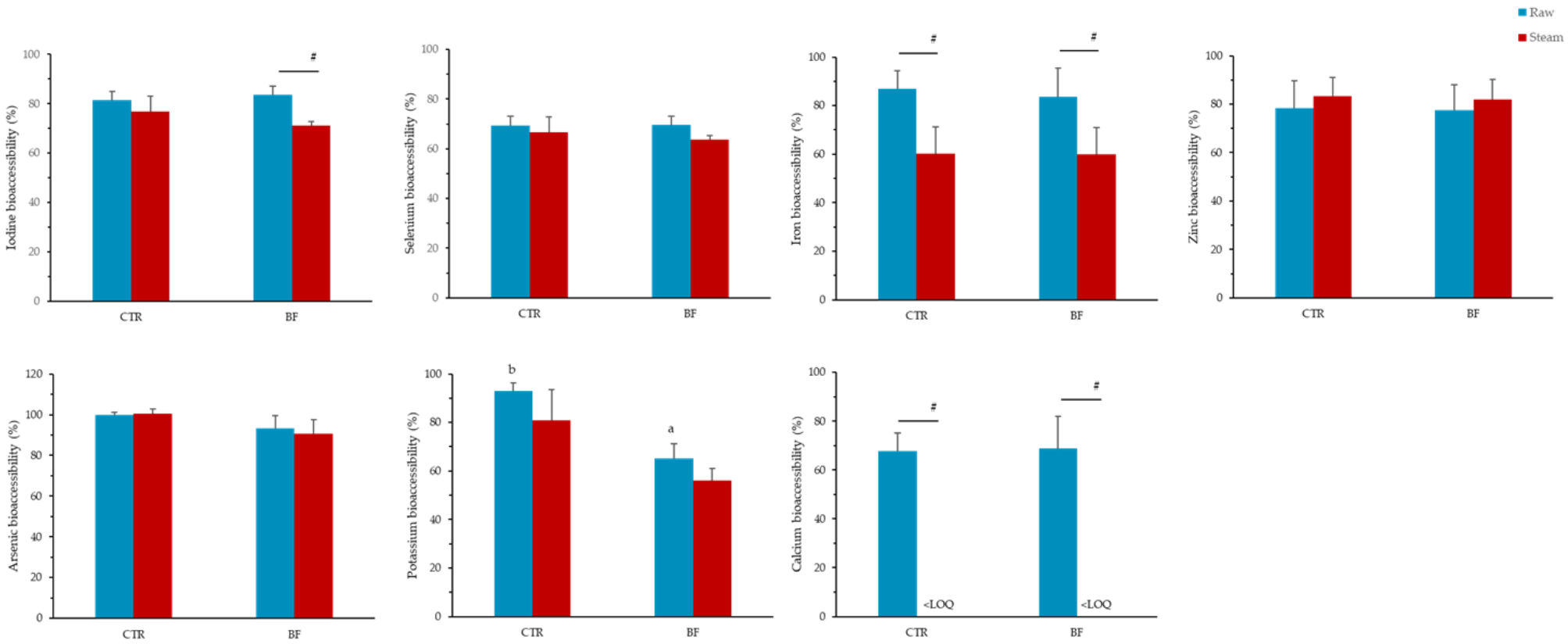

Figure 5.3 - Bioaccessibility (%) of trace (iodine, selenium, iron, zinc), toxic (arsenic) and macro (potassium, calcium) elements in biofortified (BF) and non-biofortified (CTR) gilthead seabream fillets (average ± SD). Different lower-case and upper-case letters indicate significant differences ($P < 0.05$) between CTR and BF fish fillets, in raw and steamed samples, respectively. For each treatment (CTR and BF), $^{\#}$ represents significant differences ($P < 0.05$) between raw and steamed fillets.

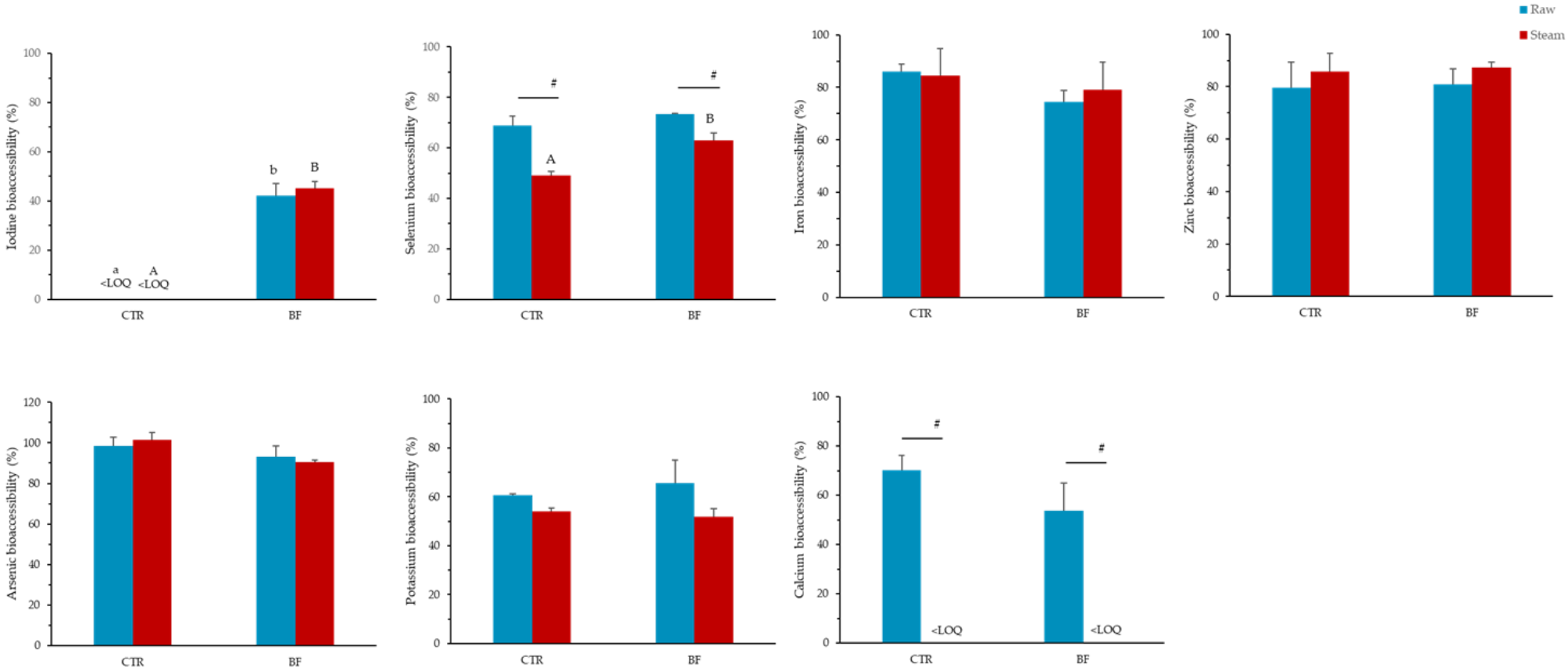

Figure 5.4 - Bioaccessibility (%) of trace (iodine, selenium, iron, zinc), toxic (arsenic) and macro (potassium, calcium) elements in biofortified (BF) and non-biofortified (CTR) common carp fillets (average ± SD). Different lower-case and upper-case letters indicate significant differences ($P < 0.05$) between CTR and BF fish fillets, in raw and steamed samples, respectively. For each treatment (CTR and BF), # represents significant differences ($P < 0.05$) between raw and steamed fille

3.3. Nutritional contribution of biofortified farmed fish consumption

The consumption of 150 g (adults and pregnant women) and 75 g (children) portion of raw and steamed BF gilthead seabream fillets contributed to higher intakes of I (up to 10% for children and up to 12% for adults) and Se (up to 44% for pregnant women and more than 100% for children), compared to CTR fillets (Table 5.2). Additionally, steaming significantly increased Se intake in BF fillets for all population groups, as well as Cu intake in both CTR and BF fillets. Despite exceeding the daily adequate intake, both CTR and BF fillets were within Se UL for children (up to 26% for raw and up to 31% for steamed). In terms of elements bioaccessibilty, BF fillets (raw and steamed) contributed to higher intakes of I (up to 6% for pregnant women and up to 9% for adults) and Se (up to 28% for pregnant women and up to 80% for children).

Concerning common carp, the consumption of 150 g (adults and pregnant women) and 75 g (children) portion of raw and steamed BF fillets contributed to higher intakes of I (up to 8% for pregnant women and up to 11% for adults) and Se (higher than 100% for all population groups), compared to CTR fillets (Table 5.2). Despite exceeding the daily adequate intake, both raw and steamed BF carp fillets were within Se UL for all population groups (up to 40% for adults/pregnant women and up to 99% for children). Steaming significantly increased Se intake in BF fillets for all population groups. Additionally, the consumption of raw BF fillets contributed to higher intakes of Cu for all population groups (from 10% for pregnant women to 22% for children) and steaming significantly reduced Cu intakes (7% for pregnant women and 16% for children). Higher intakes of Zn were also observed for the consumption of raw and steamed BF fillets (up to 8% for pregnant women and up to 23% for adults). In terms of elements bioaccessibilty, BF fillets (raw and steamed) contributed to higher intakes of I (up to 3% for pregnant women and up to 5% for adults) and Se (up to 92% for pregnant women and up to 65% of UL for children).

Table 5.2 - Nutritional contribution (%) of non-biofortified (CTR) and biofortified (BF) gilthead seabream (*S. aurata*) and common carp (*C. carpio*) in terms of essential elements in different population groups, considering the consumption of a portion of 150 g of fish for adults and pregnant women, and with the consumption of 75 g of fish for children.

		Gilthead seabream								**Common carp**							
		CTR[1]		BF[1]		CTR[2]		BF[2]		CTR[1]		BF[1]		CTR[2]		BF[2]	
		raw	steamed	raw	steamed	raw	steamed	raw	steamed	raw	steamed	raw	steamed	raw	steamed	raw	steamed
Trace elements																	
I	Adults[4]	6.8 ± 0.7^{a}	5.7 ± 0.4^{A}	10 ± 1^{b}	12 ± 0^{B}	5.6 ± 0.8^{a}	4.4 ± 0.4^{A}	8.6 ± 0.8^{b}	8.3 ± 0.1^{B}	3.1 ± 0.5^{a}	4.6 ± 05^{A}	11 ± 1^{b}	9.8 ± 0.4^{B}	n.d.^{a}	n.d.^{A}	4.5 ± 0.8^{b}	4.3 ± 0.1^{B}
	Pregnant women[5]	5.1 ± 0.6^{a}	4.3 ± 0.3^{A}	7.7 ± 0.5^{b}	8.8 ± 0.2^{B}	4.2 ± 0.6^{a}	3.3 ± 0.3^{A}	6.4 ± 0.7^{b}	6.2 ± 0.1^{B}	2.2 ± 0.4^{a}	3.4 ± 0.4^{A}	8.0 ± 0.9^{b}	7.3 ± 0.3^{B}	n.d.^{a}	n.d.^{A}	3.4± 0.8^{b}	3.2 ± 0.1^{B}
	Children[6]	5.7 ± 0.6^{a}	4.8 ± 0.3^{A}	4.8 ± 0.3^{b}	9.8 ± 0.3^{B}	4.7 ± 0.6^{a}	3.7 ± 0.3^{A}	7.1 ± 0.8^{b}	6.9 ± 0.1^{B}	2.5 ± 0.4^{a}	3.8 ± 0.4^{A}	8.9 ± 0.9^{b}	8.2 ± 0.3^{B}	n.d.^{a}	n.d.^{A}	3.8 ± 0.9^{b}	3.5 ± 0.1^{B}
Se	Adults[4]	43 ± 1^{a}	44 ± 2^{A}	49 ± 2^{b}	54 ± 3$^{B\#}$	30 ± 1^{a}	29 ± 0^{A}	34 ± 2^{b}	34 ± 2^{B}	27 ± 1^{a}	29 ± 1^{A}	> AI (35 ± 1)^{b}	> AI (40 ± 0)$^{B\#}$	18 ± 1^{a}	14 ± 0^{A}	> AI (26 ± 1)^{b}	> AI (25 ± 1)^{B}
	Pregnant women[5]	35 ± 1^{a}	36 ± 2^{A}	40 ± 2^{b}	44 ± 3$^{B\#}$	25 ± 1^{a}	24 ± 0^{A}	28 ± 2^{b}	28 ± 2^{B}	22 ± 1^{a}	24 ± 1^{A}	> AI (35 ± 1)^{b}	> AI (40 ± 0)$^{B\#}$	15 ± 1^{a}	12 ± 0^{A}	92 ± 3^{b}	89 ± 4^{B}
	Children[6]	> AI (25 ± 1)^{a}	> AI (28 ± 1)^{A}	> AI (26 ± 1)^{b}	> AI (31 ± 2)$^{B\#}$	70 ± 2^{a}	68 ± 1^{A}	79 ± 5^{b}	80 ± 5^{B}	63 ± 2^{a}	69 ± 2^{A}	> AI (88 ± 3)^{b}	> AI (99 ± 2)$^{B\#}$	43 ± 3^{a}	34 ± 1^{A}	> AI (65 ± 2)^{b}	> AI (63 ± 3)^{B}
Fe	Adults[4]	3.1 ± 0.2	3.2 ± 0.5	3.1 ± 0.5	3.1 ± 0.4	2.5 ± 0.2	2.1 ± 0.2	2.7 ± 0.3	2.1 ± 0.1	7.5 ± 0.9	7.7 ± 0.7	8.2 ± 0.8	7.3 ± 0.5	6.7 ± 0.8	6.1 ± 0.3	5.7 ± 0.2	2.3 ± 3.2
	Pregnant women[5]	2.1 ± 0.1	2.2 ± 0.4	2.1 ± 0.3	2.1 ± 0.3	1.7 ± 0.1	1.4 ± 0.2	1.9 ± 0.2	1.4 ± 0.1	5.1 ± 0.6	5.3 ± 0.5	5.6 ± 0.5	5.1 ± 0.3	4.6 ± 0.8	4.2 ± 0.2	3.9 ± 0.1	1.6 ± 2.2
	Children[6]	2.3 ± 0.1	2.5 ± 0.4	2.4 ± 0.4	2.4 ± 0.3	2.1 ± 0.1	1.6 ± 0.2	2.1 ± 0.1	1.6 ± 0.1	5.9 ± 0.7	6.1 ± 0.6	6.4 ± 0.6	5.8 ± 0.4	5.3 ± 0.6	4.8 ± 0.2	4.5 ± 0.1	1.8 ± 2.6
Cu	Adults[4]	11 ± 1	15 ± 2$^{\#}$	8.5 ± 1.4	13 ± 2$^{\#}$	n.d.	n.d.	n.d.	n.d.	12 ± 1^{a}	14 ± 1	18 ± 2^{b}	14 ± 2$^{\#}$	n.d.	n.d.	n.d.	n.d.
	Pregnant women[5]	12 ± 1	16 ± 2$^{\#}$	9.1 ± 1.4	14 ± 2$^{\#}$	n.d.	n.d.	n.d.	n.d.	13 ± 1^{a}	15 ± 1	19 ± 1^{b}	15 ± 2$^{\#}$	n.d.	n.d.	n.d.	n.d.
	Children[6]	13 ± 1	17 ± 2$^{\#}$	9.8 ± 1.6	15 ± 2$^{\#}$	n.d.	n.d.	n.d.	n.d.	14 ± 1^{a}	16 ± 1	21 ± 2^{b}	16 ± 2$^{\#}$	n.d.	n.d.	n.d.	n.d.
Zn	Adults[4]	7.5 ± 0.1	8.2 ± 0.8	7.7 ± 0.4	9.5 ± 0.5	5.9 ± 3.4	7.0 ± 0.6	6.2±0.2	7.7±0.5	8.6 ± 0.5^{a}	9.0 ± 0.6^{A}	16 ± 1^{b}	14 ± 1^{B}	6.8 ± 0.4^{a}	7.5 ± 0.6^{A}	13 ± 1^{b}	12 ± 1^{B}
	Pregnant women[5]	7.7 ± 0.2	8.4 ± 0.8	8.0 ± 0.4	9.8 ± 0.5	6.1 ± 0.1	7.3 ± 0.6	6.4±0.2	7.9±0.5	8.8 ± 0.5^{a}	9.3 ± 0.7^{A}	16 ± 1^{b}	14 ± 1^{B}	7.1 ± 0.6^{a}	7.8 ± 0.6^{A}	13 ± 1^{b}	12 ± 1^{B}
	Children[6]	8.2 ± 0.2	8.9 ± 0.8	8.4 ± 0.4	10 ± 0.5	6.4 ± 0.1	7.7 ± 0.7	7.7±0.7	8.4±0.5	9.4 ± 0.5^{a}	9.8 ± 0.7^{A}	17 ± 1^{b}	15 ± 1^{B}	7.5 ± 0.6^{a}	8.2 ± 0.6^{A}	14 ± 1^{b}	13 ± 1^{B}
Macro elements																	
K	Adults[4]	16 ± 1	16 ± 1	18 ± 2	15 ± 1	15 ± 1	13 ± 1	12 ± 1	8.8 ± 0.9	12 ± 1^{a}	11 ± 1	15 ± 1^{b}	13 ± 2	7.1 ±0.21	5.9 ± 0.1	10 ± 1	7.4 ± 0.1
	Pregnant women[5]	14 ± 1	14 ± 1	16 ± 1	13 ± 1	13 ± 1	11 ± 1	11 ± 1	7.7 ± 0.8	11 ± 1^{a}	10 ± 1	13 ± 1^{b}	12 ± 1	6.1 ± 0.2	5.2 ± 0.1	10 ± 1	6.5 ± 0.1
	Children[6]	35 ± 2	35 ± 3	40 ± 3	34 ± 3	33 ± 1	28 ± 2	27 ± 1	19 ± 2	26 ± 2^{a}	25 ± 1	32 ± 2^{b}	29 ± 4	15 ± 1	13 ± 1	18 ± 2	16 ± 1
Ca	Adults[4]/Pregnant women[5]	2.2 ± 0.2	2.5 ± 0.1	2.1 ± 0.1	2.3 ± 0.1	1.5 ± 0.2	n.d.$^{\#}$	1.4 ± 0.1	n.d.$^{\#}$	2.2 ± 0.1	2.6 ± 0.3	2.7 ± 0.2	2.5 ± 0.1	1.6 ± 0.1	n.d.$^{\#}$	1.5 ± 0.1	n.d.$^{\#}$
	Children[6]	2.3 ± 0.2	2.6 ± 0.1	2.3 ± 0.1	2.5 ± 0.1	1.6 ± 0.2	n.d.$^{\#}$	1.5 ± 0.1	n.d.$^{\#}$	2.3 ± 0.1	2.7 ± 0.3	2.8 ± 0.2	2.6 ± 0.1	1.6 ± 0.1	n.d.$^{\#}$	1.6 ± 0.1	n.d.$^{\#}$
Toxic element																	
iAs	Adults[4]	1.0 ± 0.1	1.0 ± 0.1	1 ± 0	2 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
	Pregnant women[5]	1.0 ± 0.1	1.0 ± 0.1	2 ± 0	2 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
	Children[6]	2.3 ± 0.1	2.4 ± 0.1	4 ± 0	4 ± 0	2 ± 0	3 ± 0	4 ± 0	3 ± 0	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1

[1]Values calculated without considering element bioaccessibility; [2]Values calculated considering element bioaccessibility. Values are mean ± standard deviation. The Nutritional contribution (NC; %) are presented for [3]adults (> 18 years) with mean body weight in Europe (70 kg), [4]pregnant/lactating women with mean body weights in Europe (67 kg) and [5]children (1–3 years) with mean body weight in Europe (13 kg) set by EFSA (2012b). The percentages of NC were calculated accordingly the Dietary Reference Values (DRVs), namely Adequate Intakes (AI) or population reference intake (PRI), as well as the tolerable upper intake level (UL; in parenthesis) and benchmark dose lower confidence limit (BMDL01) set by EFSA (2009, 2014a, 2014b, 2014c 2015a,2015c, 2015d, 2016, 2019. Different lower-case and upper-case letters represent statistical differences ($P < 0.05$) between CTR and BF fish fillets, in raw and steamed samples, respectively. [#]represents significant differences ($P < 0.05$) between raw and steamed fillets

4. Discussion

4.1. Effects of the biofortification strategy on elements content in farmed fish fillets

In line with previous studies undertaken at pilot scale, the incorporation of iodine-rich seaweed (*L. digitata*) and Se-rich yeast in gilthead seabream and common carp feeds resulted in enhanced content of most essential elements, especially I and Se, in fish fillets (Barbosa et al., 2020, 2021). Increased I contents were also observed in previous studies focused on marine species, namely gilthead seabream (*S. aurata*), and freshwater species, namely rainbow trout (*Oncorhynchus mykiss*) and char (*Salvelinus sp.*), biofortified with I-rich seaweed supplemented diets. Successful Se biofortification was also reported in rainbow trout (*O. mykiss*) using a similar dietary approach (Ramalho Ribeiro et al., 2017). The present biofortification strategy (incorporation of approximately 0.5% of *L. digitata* and 0.03% Se-yeast as part of the diet) was more effective in common carp (3.6-fold increase in I and 5.7-fold increase in Se) than in gilthead seabream (1.5-fold increase in I and 1.1-fold increase in Se) in comparison with non-biofortified fish (CTR). This does not appear to depend on the lower I and Se levels in fillets of conventional common carp only. It is known that I and Se biofortification effectiveness depends on fish and seaweed species (i.e., origin and size), and Se-rich yeast used, as well as the duration of feeding exposure (Barbosa et al., 2020; Ramalho Ribeiro et al., 2017). Indeed, different effectiveness of biofortification with I and Se using the same approach (incorporation of *L. digitata* and Se-yeast as part of the diet) was previously reported in the same fish species. For instance, the incorporation of higher percentages of *L. digitata* (0.8%) as part of the diet, resulted in lower enhancement of I content in gilthead seabream fillets (1.4-fold increase), which may be explained by the initial I concentration in the seaweed specimens used resulting in different I levels in feeds (13.3 ± 0.2 mg kg^{-1} versus 20.4 ± 0.6 mg kg^{-1}). Moreover, the incorporation of lower percentages of Se-rich yeast (0.01%) as part of the diet, resulted in lower enhancement of Se content in common carp fillets (1.4-fold increase). In terms of other elements content, similar to the author's previous study, BF gilthead seabream and common carp fillets presented higher content of As, likely due to the supplementation of fish diets with the seaweed *L. digitata*, this since seaweed species are known to naturally accumulate arsenic (Alves et al., 2018). Also, the inclusion of

microalgae blends with different mineral compositions and absolute concentrations, especially *Spirulina* sp. (Pereira et al., 2019), resulted in higher contents of Zn, Cu, Br, K and Ca in BF common carp fillets, in contrast to gilthead seabream. In line with previous studies (Barbosa et al., 2021; Ramalho Ribeiro et al., 2015), the results demonstrate that steaming significantly increase I content, but only in BF gilthead seabream fillets and in CTR common carp fillets. On the other hand, Alves and co-authors (2018) reported that steaming not affect I content in hake (*Merlucius australis*), monkfish (*Lophius piscatorius*), mackerel (*Scomber scombrus*), tuna (*Katsuonus pelamis*), and plaice (*Pleuronectes platessa*). Increased Se content was also previously reported in steamed BF gilthead seabream (Barbosa et al., 2021), in boiled, grilled and roasted gilthead seabream (Afonso et al., 2018), and in blue shark (*Prionace glauca*) after grilling and steaming (Matos et al., 2015), which is likely associated with water loss during culinary treatment (Alves et al., 2018; Erkan, 2011; Martins et al., 2011). Higher retention of I and Se (TR $\geq$ 80%) after streaming are mainly associated to higher cooking yields (CY > 90%) and by the fact that these elements are mainly bound to proteins, being less prone to leaching during steam-cooking (Barbosa et al., 2021; Oliveira et al., 2019; Vicente-Zurdo et al., 2019). Overall, elements true retention after steaming were high, indicating that this culinary procedure has no detrimental effect in elements content in BF fish fillets from both species. In contrast with author's previous study, BF gilthead seabream presented higher cooking yield (CY of 92% versus 84%), resulting in higher elements retention after steaming, and ultimately less minerals leaching from muscle (Bastías et al., 2017). Indeed, no significant changes were observed in gilthead seabream fillets moisture composition after steaming, indicating that steam cooking has less influence in fillets elemental composition. In contrast, despite BF common carp fillets also presented higher CY (92% versus 80%), lower retention of Fe, Zn and Cu were observed, reflecting in losses during steam-cooking. In fact, decreased moisture content was observed in common carp fillets after steaming, which may explain some minerals leaching due to water loss, evaporation, and/or dehydration (Oliveira et al., 2019; Sobral et al., 2018). The present results demonstrate that the biofortification strategy contributed to enhance farmed fish nutritional quality and steaming is a healthy cooking method, maintaining enhanced health-valuable nutrients. Nevertheless, fish elemental composition is closely related with specimen's origin, size, and initial elemental content, being species-specific, as reported earlier (Barbosa et al., 2020, 2021; He et al., 2010; Mnari et al., 2012; Petricorena, 2015).

4.2. Macro and trace elements bioaccessibility in biofortified farmed fish

Fish contains several essential elements and is a good source of some of them, especially I, Se, and to a certain extent Fe (partially present in haem form), that have vital roles in human health (Cilla et al., 2019; EFSA, 2014a; Gharibzahedi & Jafari, 2017). In this sense, developing and designing biofortification strategies considering economical and sustainable solutions is a cost-effective measure to supply essential nutrients to the global population (FAO et al., 2021). Still, nutrients absorption from biofortified food is overall limited, especially in seafood. Bioaccessibility is the major determinant to be investigated in this respect since a nutrient must be first released from the food matrix by the digestive process to become available for absorption in the human intestine. In general, the bioaccessibility of essential elements in BF and CTR gilthead seabream and common carp were above 60%, except I bioaccessibility in common carp (less than 50%). Previous studies also reported overall good bioaccessibility of I and Se with some variability depending on the fish species and culinary treatment. For example, bioaccessibility of I reached 98% in blue whiting (*Micromesistius poutassou*) (Ferraris et al., 2021), whereas it was only 47% in tuna (*K. pelamis*) (Alves et al., 2018). Similarly, a variable bioaccessibility of Se has been reported, in a range from 59% in tuna (*K. pelamis*) to 76% in swordfish (*Aphanopus carbo*), 83% in sardine (*Sardina pilchardus*) (Cabañero et al., 2004), 87% in monkfish (*L. piscatorius*) (Alves et al., 2018), 90% in gilthead seabream (*S. aurata*) (Afonso et al., 2018), and in blue shark (*P. glauca*) (Matos et al., 2015). Higher bioaccessibility of I and Se may be explained by the strong affinity of these elements to soluble proteins that are easily digestible by digestive enzymes (Afonso et al., 2018). One the other hand, the differences may also be related with fish proximate chemical composition, since proteins and fat content may affect I and Se solubility, thus affecting enzymes efficiency (Cabañero et al., 2004; Doh et al., 2019). To what extent this effect observed in the in the *in vitro* digestion procedure is relevant in vivo (i.e., in humans) needs to be ascertained. Steaming induced a decrease in I bioaccessibility only in BF gilthead seabream fillets, but not in CTR gilthead seabream and common carp. Similarly, steaming did not affect I bioaccessibility in tuna (*K. pelamis*) (Alves et al., 2018). In contrast, steaming decreased Se bioaccessibility in common carp fillets (BF and CTR), but not in gilthead seabream. Decreased Se bioaccessibility was also reported after steaming in blue shark (*P. glauca*) (Matos et al., 2015) and plaice (*P. platessa*) (Alves et al., 2018), whereas

increased Se bioaccessibility was reported in steamed mackerel (*S. scombrus*) (Alves et al., 2018), and in boiled, grilled, and roasted gilthead seabream (*S. aurata*) (Afonso et al., 2018). Overall, cooking procedures may lead to a decrease in elements bioaccessibility due to the elemental leaching from protein complexes because of highly digestible and unstable proteins losses associated with moisture and muscle tissues protein denaturation (Amiard et al., 2008). On the other hand, muscle myofibrils denaturation and contraction, may result in insoluble protein-elements complexes, leading to less digestible and bioavailable nutrients in the muscle tissues (Amiard et al., 2008; Doh et al., 2019; He et al., 2010).

Concerning other elements, higher bioaccessibility of Fe (up to 80% in seabream and up to 70% in carp) and Zn (up to 70% in seabream and up to 80% in carp) were observed compared to previous studies. In fact, bioaccessibility of Fe reached 69% in tuna (*K. pelamis*) (Alves et al., 2018), up to 58% in seabass (*Lateolabrax japonicus*) and 52% in red seabream (*Pagrosomus major*) (He et al., 2010). On the other hand, Zn bioaccessibility reached up to 70% in seabass (*L. japonicus*) and up to 67% in red seabream (*P. major*), as well as 71% in hake (*M. australis*), 40% in plaice (*P. platessa*) and 28% in tuna (*K. pelamis)* (Alves et al., 2018). Similarly, Fe bioaccessibility significantly decreased in steamed red seabream (*P. major*) (He et al., 2010) and Zn bioaccessibility increased in steamed hake (*M. australis*), in plaice (*P. platessa*) and in tuna (*K. pelamis*) (Alves et al., 2018). In contrast to the present results, higher bioaccessibility of K (80%) was reported in raw and cooked tilapia (*O. niloticus*) (Santos et al., 2022). Still, the bioaccesibility of K was relatively high, reaching 65% in gilthead seabream and 66% in common carp. The high bioaccesibility of K may be explained by the fact that this element occurs in food matrix as simple ions, being easily solubilized into the gastrointestinal tract (Santos et al., 2022). Additionally, in contrast with the present study, Cu bioaccessibility was reported in seabass (*L. japonicus*) and red seabream (*P. major*) that ranged between 70% and 85% (He et al., 2010). On the other hand, lower Cu bioaccessibility (40%) was reported in tuna (*K. pelamis*); whereas due to inconclusive results Cu bioaccessibility was not determined for the other fish species (Alves et al., 2018). Lower Cu bioaccessibility may be related with this element storage in the form of less easily degraded complexes and digestible proteins by metallothionein and insoluble ligands, especially after cooking (Amiard et al., 2008). Moreover, previous studies also reported higher variabilities of Ca bioaccessibility, ranging from 94% in sea bass to 20% in sardine (Hernández-Olivas et al., 2020), to less than 45% in raw and cooked tilapia (*Oreochromis niloticus*) (Santos et al., 2022). Such

variability may be explained by the fact that this element is mainly found in complex molecules that are only partially soluble within the intestinal lumen (Hernández-Olivas et al., 2020; Santos et al., 2022). Moreover, species-specific protein content could also exert a salting-out effect when free amino acids are in salt forms, leading to poor solubility of Ca species (Moreda-Piñeiro et al., 2013a) and therefore bioaccessibility analysis.

Nevertheless, elemental bioaccessibility depends not only on the food matrix, but especially on nutrients chemical structure changes, mobility, and solubility (Doh et al., 2019; Liu et al., 2017). In terms of the steam-cooking effect on elemental bioaccessibility, the present results indicate a species-specific effect in accordance with previous findings (He & Wang, 2013). The different biofortification approaches for gilthead seabream and common carp contributed to distinct effects on fish elemental composition and the steam-cooking treatment does not seem to remarkably affect fillets elemental composition (see TRs values). In general, BF fillets elemental bioaccessibility was above 65%, except for I and Ca in common carp. Steam-cooking affected differentially the elemental bioaccessibility, demonstrating that, excluding I in common carp, Ca was the least digestible element in steamed BF fish fillets from both species.

4.3. Nutritional benefits of biofortified farmed fish consumption to human health

Considering the consumption of a portion of 150 g for adults/pregnant women and 75 g for children, steamed BF gilthead seabream and common carp contributed to significantly increased NC of I and Se, compared to CTR fish. These results are consistent with the authors' previous findings (Barbosa et al., 2021). In comparison with the previous study, BF gilthead seabream fillets presented similar NC of I (12% for adults, 9% for pregnant women and 10% for children) and lower NC of Se (54% for adults, 44% for pregnant women and 31% of UL for children). Yet, it is worth mentioning that *L. digitata* and Se-rich yeast were supplemented in gilthead seabream diets at lower levels (i.e., 0.5% against 0.8% and 0.03% against 0.04%, respectively). On the other hand, BF common carp fillets showed lower NC of I (10% for adults, 7% for pregnant women and 8% for children), but higher NC of Se (more than 100% for all population groups). In this case, *L. digitata* was supplemented in fish diets at the same levels (0.54%), while Se-rich yeast was supplemented at higher levels (0.03% against 0.01%). Furthermore, in line with our previous study, increased NC of Zn was observed in BF common carp fillets compared to CTR.

Nevertheless, lower NC of Fe (up to – 11%), Cu (up to – 7%) and Zn (up to – 41%) were achieved, compared to previous results (Barbosa et al., 2021). Moreover, lower exposure to iAs intakes have also been previously reported in several fish species (Barbosa et al., 2018; Cano-Sancho et al., 2015). Such results are supported by the fact that the major arsenic form in fish is the organic forms AsBet, wich is not metabolized in humans being excreted unchanged, and therefore pose no toxicological concern (EFSA, 2009). When it comes to elements bioaccessibility, a reduction was observed in all elements NC in both gilthead seabream and common carp, compared to elements NC before the *in vitro* digestion process, being consistent with previous findings in hake (*M. australis*), tuna (*K. pelamis*), monkfish (*L. piscatorius*), mackerel (*S. scombrus*), and plaice (*P. platessa*) (Alves et al., 2018). Despite the observed reduction in NC based on elements bioaccessibility, BF fish fillets from both species still presented enhanced nutritional value for consumer´s diets, providing increased intakes of I and Se, two of the most important nutrients required for human health and well-being. Furthermore, the consumption of BF gilthead seabream and common carp fillets seems to be a good alternative to improve consumers I intakes without exceeding the maximum levels set by the authorities, compared to seaweed (*L. digitata*) consumption, that may increase consumers risk of exceeding the UL set for I (Alves et al., 2018). Considering the present results, higher NC of I and Se are reached through the consumption of BF gilthead seabream and higher NC of I, Se and Zn are achieved through the consumption of BF common carp. Therefore, the present biofortification approaches with I-rich seaweed (*L. digitata*) and Se-rich yeast in gilthead seabream and common carp contribute to reduce I and Se deficiencies in target population groups, whereas parsimonious consumption of BF common carp should be considered to avoid exceeding the UL set for Se (0.3 mg day^{-1} for adults and pregnant women, 0.06 mg day^{-1} for children from 1 to 3 years old).

5. Conclusions

The dietary strategies assessed through the supplementation with I-rich seaweed and Se-rich yeast were highly efficient in both gilthead seabream and common carp produced at commercial scale. Biofortified gilthead seabream and common carp fillets revealed enhanced I and Se contents and steaming resulted in increased or decreased contents, depending on the chemical properties of each element and fish species.

Additionally, the bioaccessibility of I, Se, Fe and As was above 80% in BF gilthead seabream fillets, whereas BF common carp fillets revealed lower I bioaccessibility (42%) and up to 70% of Se, Fe, Zn and As bioaccessibility. Steaming affected differentially the elements bioaccessibility. In fact, bioaccessibility of I and Fe were reduced after steaming in BF gilthead seabream fillets, whereas Se bioaccessibility was reduced in BF common carp fillets. In biofortified fish species, steaming significantly decreased Ca bioaccessibility. Generally, BF gilthead seabream and common carp fillets improved the nutritional contribution of I and Se. Still, particular attention should be given to Se intakes to avoid exceeding the current UL recommendations. This study clearly reveals that biofortification strategies are excellent solutions to reduce essential elements deficiencies in consumers, especially concerning I and Se. Moreover, most essential elements are maintained at high concentrations after steam-cooking and digestion, preserving the enhance nutritional quality of biofortified fish. Further studies on different biofortification strategies (i.e., supplementation by other natural ingredients from sustainable sources) and elements bioavailability, as well as spatial distribution of elements in biofortified fish fillets will be relevant to provide more accurate insights to develop eco-innovative and cost-effective biofortified farmed fish with nutritional benefits to human health.

Ethical statement

Fish trials were conducted according to legal regulations (EU Directive, 2010/63) and approved by the Ethical Committee of SKALOMA SA and ZUT, overseen by the National Competence Authority. All researchers and technicians involved in the maintenance, handling and sampling of live animals were certified in Laboratory Animal Sciences, by the Federation of European Laboratory Animal Science Associations (FELASA).

6

Mapping the Distribution of Ca, Fe, K and Zn In Biofortified Gilthead Seabream and Common Carp Fish Muscle Through X-Ray Fluorescence Spectrometry

In this chapter you will find the Short Communication [Submitted]:
Vera Barbosa, et al. (2023). Mapping the distribution of Ca, Fe, K and Zn in biofortified gilthead seabream and common carp fish muscle through X-ray fluorescence spectrometry. Submitted at Journal of the Science of Food and Agriculture.

Abstract

Currently, there is a growing demand for healthy and sustainable food products associated to worlds population expansion. Despite seafood products are widely recognized as healthy food items and important sources of essential key nutrients, one third of the world population suffer from food insecurity and different forms of malnutrition. Hence, developing tailor-made fortified farmed fish is a promising solution to overcome nutritional deficiencies and increase consumer confidence in these products. The aim of the present study is to evaluate the differences in elements distribution in biofortified and non-biofortified fish fillets from gilthead seabream and common carp, using the micro X-ray fluorescence (μ-XRF) technique. The μ-XRF provide multielement mapping with the advantage of non-destructive and low-cost analyses. Results showed that calcium was mainly accumulated in skeleton tissues; whereas iron, potassium and zinc were more or less uniformly distributed in the fish muscle. Biofortified gilthead seabream fillets showed higher concentration of iron in specific areas of the sample, compared to control; whereas biofortified common carp fillets seemed to show higher concentration of zinc in specific areas of the sample, compared to control. This study demonstrates that the micro-X-ray analysis is a suitable technique to assess elemental distribution with micrometer resolution for essential elements of biological interest. The advantage of μ-XRF spectrometers for predicting the elemental distribution in biological systems compared to other techniques relies in the simplicity of operation, fast elemental analysis, no complex sample preparation, and no sample destruction.

Keywords: μ-XRF, elements imaging, biofortification, gilthead seabream, common carp.

1. Introduction

With the increase of the world's population and the subsequent increase in seafood consumption, the development of eco-innovative aquaculture solutions is very important for human nutrition and environmental sustainability (FAO, 2020). Seafood is a rich source of essential nutrients for human health, and it has been demonstrated that aquaculture feeds can effectively modulate the nutritional profile of fish (Cotter et al., 2009; Tocher, 2015). In this sense, the development of tailor-made farm fish using sustainable marine resources added to aquafeeds, unlocks the possibility to create innovative biofortified farmed fish products that may promote consumers' health and cope with dietary deficiencies observed in the human populations worldwide (Barbosa et al., 2020; Ramalho, 2019; Roohinejad et al., 2017). In fact, recent studies showed that biofortified fish fillets through the incorporation of iodine-rich seaweed (*Laminaria digitata*) and selenium-rich yeast are a sustainable and natural solution to pro-vide higher intakes of essential elements, especially iodine, selenium, iron, and zinc (Barbosa et al., 2020; Ramalho Ribeiro et al., 2017). Nevertheless, as the benefits of essential nutrients intake become clearer, it is not only important to quantify the elemental composition of biofortified fish fillets (e.g., essential elements content in fish muscle), but also to determine where each element is localized/prevalent (i.e., elements deposition and accumulation) in the biofortified fish fillets compared to non-biofortified fillets.

Energy Dispersive X-Ray Fluorescence (EDXRF) has been widely used for qualitative and quantitative elemental composition analysis in biological tissues, due to its non-destructive features, fast detection, high sensitivity, and reduced costs (Carvalho et al., 2001, 2005; Carvalho et al., 2020; de la Guardia & Garrigues, 2015). Recent developments of dynamic, fast, and non-destructive scanning technique trough bench-top X-Ray Fluorescence spectrometry provides two- and three-dimensional elemental imaging (2D/3D spatial elemental distribution measurement), as well as the quantification of elemental distributions within the sample at trace level detection limit (de Samber et al., 2008; Dias et al., 2015).

The present preliminary study aimed to evaluate the differences in essential elements distribution in farmed gilthead seabream and common carp fish muscle (fillets) biofortified with I-rich seaweed (*L. digitata*) and Se-rich yeast as feed ingredients.

2. Material and Methods

2.1. Experimental feeding trial and sampling

For each fish species, two experimental diets were formulated, a control diet (CTR), considering the nutritional requirements of adult gilthead seabream and common carp, and one enriched diet supplemented with different blends of I-rich macroalgae and Se-rich yeast (biofortified, BF). Based on the CTR formulation, gilthead seabream BF diet was formulated targeting increased I levels, supplied from *L. digitata* (0.80%) and increased Se levels, supplied through Se-rich yeast (0.04%). Additionally, BF seabream diet was formulated with a 5% replacement of fishmeal by a blend of microalgae (*Tetraselmis* sp., *Chlorella* sp., *Schizochytrium* sp.). Concerning common carp, the BF diets was formulated based on CTR diet, targeting increased I levels, supplied from *L. digitata* (0.54%) and increased Se levels, supplied from Se-rich yeast (0.01%). BF carp diets was formulated with a 2.5% replacement of fishmeal by a blend of microalgae (*Spirulina* sp. and *Chlorella* sp.). Experimental extruded diets were manufactured by SPAROS, Ltda (Olhão, Portugal) and BF diet formulations took into consideration the current maximum authorized contents of total I (20 mg kg^{-1}) and Se (0.5 mg kg^{-1}) in fish feeds (EFSA, 2005, 2006).

The experimental trial with seabream was carried at the Aquaculture Research Station (EPPO-IPMA, Olhão, Portugal) of IPMA, while the experimental trial with carp was performed at the Fisheries Research Station (FRS-ZUT Nowe Czarnowo, Poland). Both trials were performed in compliance with the European guidelines on protection of animals used for scientific purposes (European Commission, 2007). For each species, fish were randomly distributed in fiberglass tanks (seabream) or in a floating set of cages (carp) and each experimental diet was tested in triplicate for approximately 3-months period. Fish were hand-fed to apparent satiety in three to four daily meals with 1.3–2.0% of the biomass. during the experimental period, mimicking the final stage of the production (i.e., just before reaching market size). No mortality was observed during either trial. Final samplings were done 24 h following the last meal and fish were sacrificed by immersion in chilled seawater (gilthead seabream) or freshwater (common carp) following the commercial procedures employed in fish farms.

2.2. Elemental mappings

For the µ-XRF analysis, seabream and carp specimens for each treatment (BF and CTR) were labelled, filleted, and transversely and longitudinal cross-sections from the fish muscle (fillet) were cut using a microtome (sectioned with an average thickness of about 2.0 ± 0.1 mm). Then each section was lyophilized for 24 h and directly mounted in a mylar sheet attached to slide frames (50 × 50 mm,

Figure 6.1 and Figure 6.2). Each sample was analysed using µ-XRF system M4Tornado (Bruker, Germany) accordingly to Pessanha et al. (2016). Briefly, detection of fluorescence radiation was performed by an energy dispersive silicon drift detector with 30 mm^2 sensitive area and energy resolution of 142 eV for Mn Ka. The µ-XRF generator was operated at 50 kV and 600 µA and a composition of filters (100 µm Al/50 µm Ti/25 µm Cu) was used to reduce background and improve detection limits. Elemental mappings were performed under 20 mbar vacuum conditions and directly on the sectioned samples using a lateral step size of 15 µm and µ-XRF provided analytical point spectra and elemental maps of essential nutrients, including Ca, Fe, K and Zn distribution.

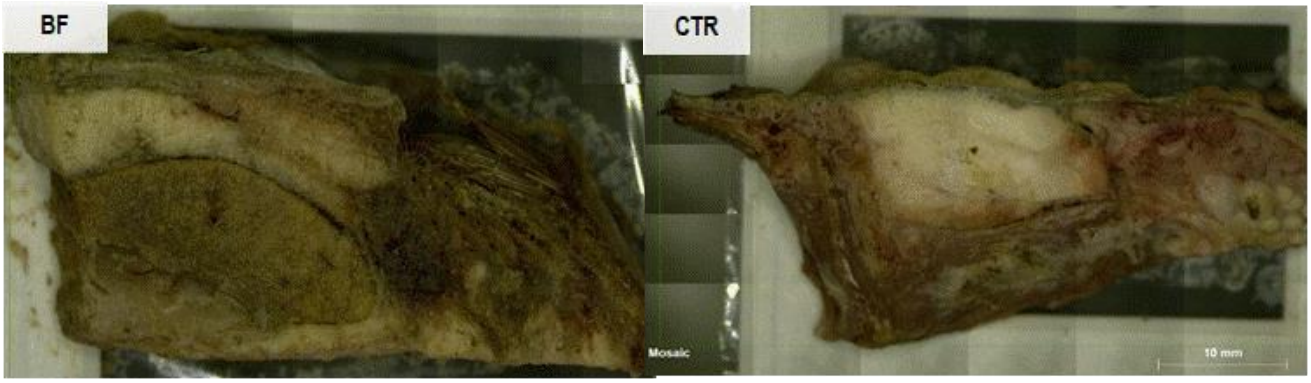

Figure 6.1 - Gilthead seabream cross-sections from the fish muscle (BF – biofortified, CTR- control)

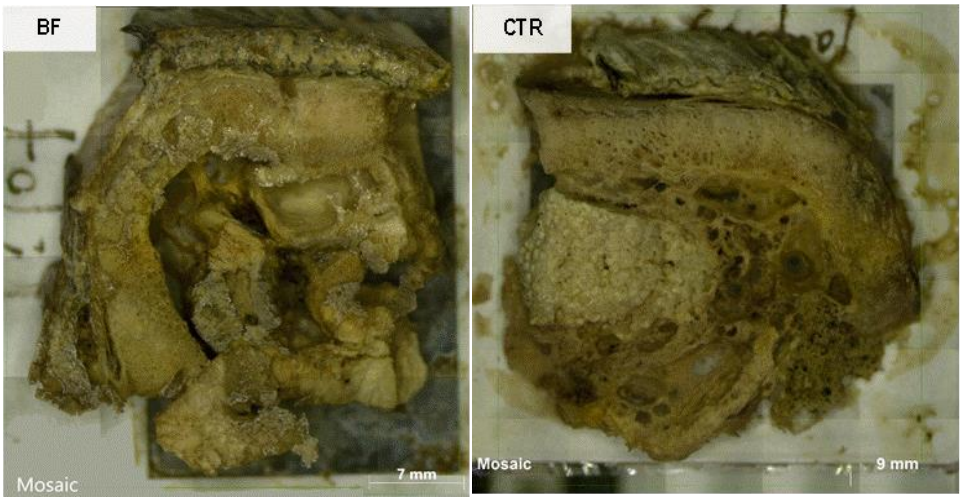

Figure 6.2 - Common carp cross-sections from the fish muscle (BF – biofortified, CTR- control).

Quantitative analyses were performed using MQuant, an in-built software of the M4 TORNADO system. This software allows spectra deconvolution, peak fitting and quantification using the Fundamental Parameters (FP) method based on the Sherman's equation (Sherman, 1955) after ad-hoc input of the sample's matrix. The sample's mean Z was determined comparing the Comp-ton-to-Rayleigh peak ratios in the spectra with the Compton-to-Rayleigh of the calibration curve deter-mined by Machado et al. (2020). Considering the elements present in biological samples matrices, this mean Z corre-sponds to a sample matrix of 20% C, 3% N, 10% H and 60% O. Limits of Detection (LoD), Limits of Quantification (LoQ) (van Grieken & Margui, 2013), and validation results for the reference material Oyster Tissue SRM 1566b were determined and are presented in Table 6.1.

Table 6.1 - Limits of Detection (LoD), limits of quantification (LoQ), accuracy and uncertainty obtained for certified reference materials using the M4 Tornado (in μg g^{-1}).

	LoD	LoQ	Certified value	Obtained value
S	5.3 ± 0.1	15.9 ± 0.3	6890 ± 14	5600 ±100
Cl	4.2 ± 0.1	12.6 ± 0.3	5140 ± 10	4140 ±80
K	3.4 ± 0.1	10.2 ± 0.3	6520 ± 90	6700 ± 200
Ca	2.8 ± 0.2	8.4 ± 0.6	838 ± 20	780 ± 20
Fe	1.7 ± 0.1	5.1 ± 0.3	206 ± 7	220 ± 5
Cu	0.80 ± 0.05	2.4 ± 0.1	71 ± 2	65 ± 2
Zn	0.90 ± 0.06	2.7 ± 0.1	1424 ± 46	1370 ± 30

3. Results & Discussion

The present biofortification strategies (i.e., incorporation of I-rich seaweed and Se-rich yeast in aquafeeds) appeared to not have a negative impact in the elemental distribution of Ca, K, Fe and Zn, since both biofortified (BF) and non-biofortified (CTR) presented similarities on the elemental concentration distributions in fish muscle samples. The elemental distribution of Ca, K, Fe and Zn in both BF and CTR gilthead seabream fish muscle sections are presented in Figure 6.3. It is possible to see that Ca is mainly concentrated in the areas of the skeleton spines and in scales at the mesoderm layer of fish skin (identified with white boxes), regardless the biofortification treatment. Indeed, it is known that fish uptake Ca mostly from water exposure through gills, scales, and skin (Flik et al., 1995). Additionally, bones and scales are considered the main reservoirs of Ca which makes them a physiologically important source of this element in fish metabolism (Rotllant et al., 2005). Regarding K, Fe and Zn, these elements were accumulated throughout the fish muscle. Yet, while K and Zn are more or less uniformly distributed, Fe appeared to be more concentrated in specific areas of the sample (identified with white boxes), that may be related to the surrounding areas of the sarcoplasm of the skeletal muscle cells. Despite the elemental distribution pattern of the observed elements is similar between BF and CTR samples, in line with authors previous study BF fillets shows higher concentration of Fe compared to CTR, associated with higher levels of Fe in the BF diets (Barbosa et al., 2020). In fact, food is the main source of Fe for fish metabolism and Fe uptakes occurs primarily in the intestinal mucosa. The absorbed Fe is mainly found in form of heme compound in myoglobin (muscle) and haemoglobin (blood cells) (Lall & Kaushik, 2021).

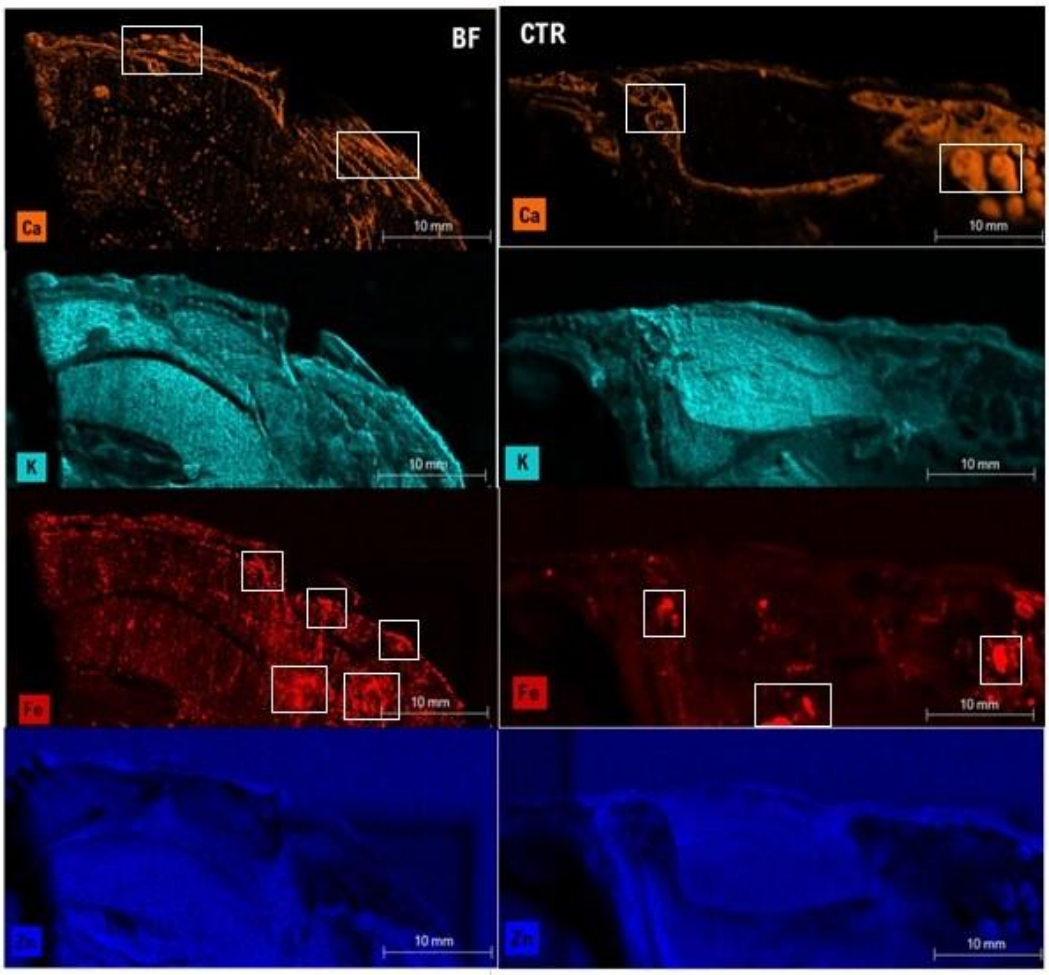

Figure 6.3 - Elements (Ca, K, Fe, Zn) mapping obtained using the microanalytical system. Analyses in cross-sections samples from biofortified (BF) and control (CTR) gilthead seabream fish muscle. The colour scheme is the following: orange – calcium, light blue – potassium, red – iron, blue – zinc. White boxes identified areas with increased intensity of colour.

Concerning common carp elemental distribution of Ca, K, Fe and Zn in BF and CTR fish muscle sections can be observed in Figure 6.4. Similar to gilthead seabream, Ca is mainly concentrated in skeleton spines and scales at the mesoderm layer of both BF and CTR fish skin (identified with white boxes). K and Fe are more or less uniformly distributed throughout the fish muscle, regardless the treatment (BF or CTR). On the other hand, Zn appeared to be more concentrated in specific areas of the fish muscle (identified with white boxes), that seemed to be near the abdominal cavity where the haematopoietic organs are located, in both CTR and BF samples. It is known that fish accumulate higher amount of Zn in viscera, associated with detoxification and excretion processes in these organs (Lall & Kaushik, 2021), which may explain the concentration of this elements in the cavity area where the liver and kidney are located.

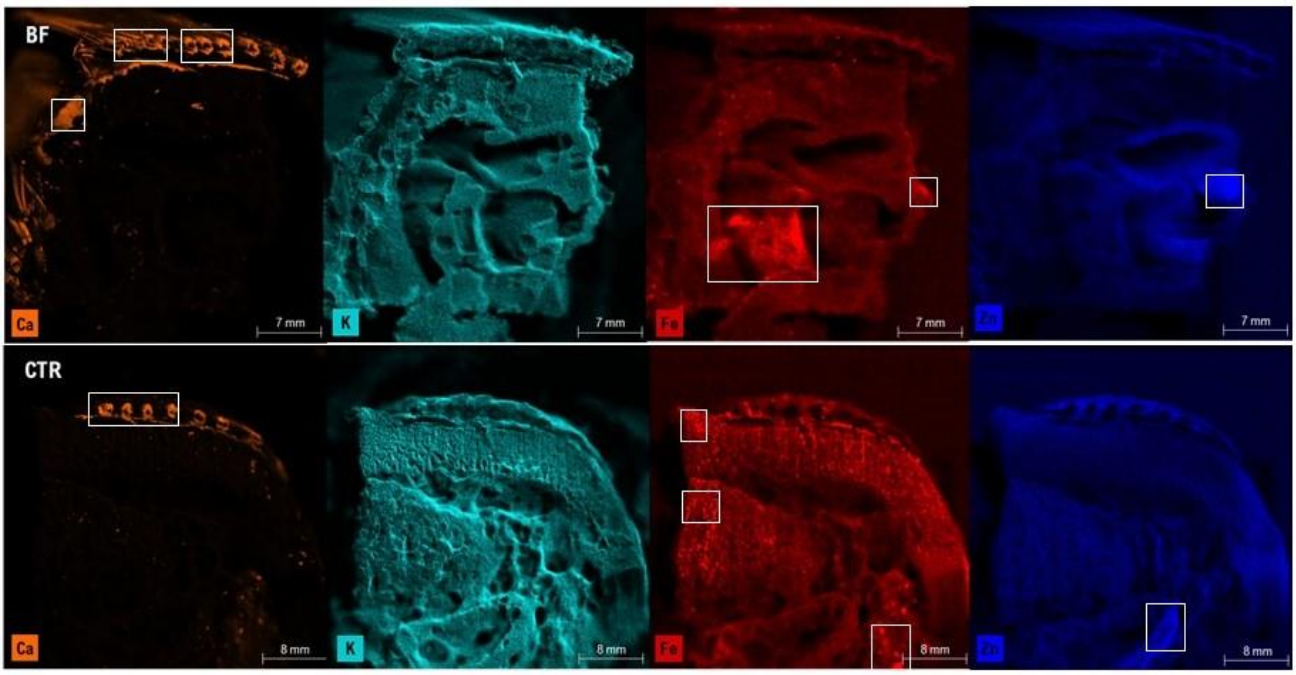

Figure 6.4 - Elements (Ca, K, Fe, Zn) mapping obtained using the microanalytical system. Analyses in cross-sections samples from biofortified (BF) and control (CTR) common carp fish muscle. The colour scheme is the following: orange – calcium, light blue – potassium, red – iron, blue – zinc. White boxes identified areas with increased intensity of colour.

The observed differences between species elements distribution and accumulation may be related with different fish species intrinsic factors, such as stage of development/size and metabolism inherent to each individual (marine versus freshwater species). Additional, factors such as: i) heterogeneous samples surface roughness, due to the freeze-drying procedure, and ii) difficulty to prepare homogeneously fish muscle transverse and longitudinal cross-section samples using a microtome that may result in elements scanning at different levels. Still, in line with previous studies the μ-XRF system M4Tornado imaging analysis is advantageous for quick preliminary assessments of elemental distribution in biological samples (Dias et al., 2015; Leitão et al., 2022; Pessanha et al., 2016). However, in comparison the present study revealed some limitations of the technique, and further research is needed for the optimization of fish muscle transverse and longitudinal cross-section samples since uneven, and thicker sections may pose some constrains for mapping analyses and quantitative analyses, and for the quantification of elements present in lower trace amounts (Leitão et al., 2022; Pessanha et al., 2016).

4. Conclusions

This study demonstrates that the micro-X-ray analyses is a suitable technique for elemental distribution with micrometer resolution for essential elements of biological interest. No differences were observed in the elemental distribution pattern between biofortified and non-biofortified fish muscle samples for both species, Yet, biofortified samples showed higher elements accumulation (i.e., higher signals/intensity of elements colour). Overall, the present results indicate a tendency of higher accumulation of Ca in the skeleton spines and scales of the fish, whereas K, Fe and Zn seems to be more or less uniformly accumulated throughout the fish muscle, regardless the biofortification strategy. The advantage of the μ-XRF spectrometers for predicting the elemental distribution in biological systems compared to other XRF based techniques relies in the simplicity of operation, fast elemental analysis, no sample preparation or destruction and low-cost.

Ethical statement

Fish trials were conducted according to legal regulations (EU Directive, 2010/63) and approved by the Ethical Committee of SKALOMA SA and ZUT, overseen by the National Competence Authority. All researchers and technicians involved in the maintenance, handling and sampling of live animals were certified in Laboratory Animal Sciences, by the Federation of European Laboratory Animal Science Associations (FELASA).

7

GENERAL DISCUSSION AND FINAL REMARKS

7.1 General Discussion

Being one of the most resource-efficient industries, aquaculture has the potential to be the main supplier of seafood. The expansion of aquaculture production has triggered some concerns regarding the environmental sustainability and costs (i.e., pollution from effluent discharge, escape of farmed organism affecting wild-stocks, and overexploitation of wild stocks of under valorised species for aquafeeds) (Naylor et al., 2021). It is known that aquaculture is still dependent on natural resources, and aquaculture feeds (aquafeeds) represent a key component in aquaculture production. In this sense, over the last years, increased efforts have been applied in fish nutrition research to improve feeding efficiency through the replacement of unsustainable resources, namely fishmeal (10%-20% of the feed formulation) and fish oil (5-15% of feed formulation), by alternative and sustainable resources (Arshad et al., 2022; FAO, 2022; Fiorella et al., 2021; Naylor et al., 2021). However, this trend may have implications in the nutritional composition of fish, and ultimately in the expected beneficial effects for consumers, since fish by-products are natural sources of health-valuable nutrients, such as I, Se, and n-3 PUFA. In this regard, the development of tailor-made farmed fish targeting consumers dietary needs and the environmental issues are gaining more interest towards the objectives of Blue Transformation (Figure 7.1). Throughout the present PhD thesis, a multidisciplinary research approach was applied to assess the efficacy and the nutritional added value of nutrients biofortification strategies in farmed fish, considering the consumer's nutritional needs and the environmental sustainability (e.g., use of sustainable natural ingredients in aquafeeds formulation).

Figure 7.1 - Blue Transformation strategic objectives in support of 2030 Agenda for Sustainable Development (adapted from FAO, 2022).

7.1.1 Biofortified feeds using I-rich seaweed and Se-rich yeast effectively modulate the nutritional composition of farmed fish.

The traditional concept of food fortification consists in the addition of exogenous target nutrients to the foodstuffs when they are being processed (FAO et al., 2021). In aquaculture, a biofortification approach through the addition of natural feed ingredients to aquafeeds allows to tailor (biofortify) the composition of farmed fish in terms of selected nutritional health-valuable compounds, without compromising consumers' perception of naturalness of the product (Ramalho Ribeiro et al., 2019).

For the biofortification approaches assessed in this thesis, two of the most relevant farmed species produced in Europe were chosen, namely gilthead seabream (a marine species) and common carp (a freshwater species), allowing the comparation between species with distinct physiologic characteristics, and therefore differences on muscle (fillet) deposition from dietary intakes of target nutrients biofortification. The target nutrients (I and Se) were selected based on three criteria:

1) nutrients with relevant health benefits to mitigate several segments of the human population inadequate intakes;
2) availability of natural and sustainable raw materials sources to reduce the dependence on finite marine harvested resources such as fishmeal and fish oil;
3) consumers acceptance (e.g., naturalness and healthiness) and market feasibility (production costs, safety, and manufacturing process).

The use of natural sources for iodine (i.e., I-rich seaweed) and selenium (i.e., Se-rich yeast) not only promotes the concept of natural and sustainable products, but also increase the potential of bio-efficacy in fish.

The dietary use of the I-rich seaweed *L. digitata* was demonstrated as a highly effective strategy to significantly enhance I content in common carp fillets, while I biofortification could still be further improved in gilthead seabream fillets (Chapter 2). In fact, the incorporation of 0.54% of *L. digitata* as part of the common carp diet resulted in a 11-fold increase of fillets iodine content in relation to non-biofortified fish (Figure 2.3), whereas the incorporation of 0.8% of *L. digitata* as part of the gilthead seabream diet resulted in a 1.4-fold increase in relation to non-biofortified fish (Figure 2.2). In contrast, the dietary supplementation with Se-rich yeast was a more effective strategy to enhance Se content in gilthead seabream fillets than in common carp fillets. In common carp, a diet supplemented with 0.035% of Se-rich yeast resulted in a 2-fold increase in relation to non-biofortified fish (Figure 2.3), while in gilthead seabream, a diet supplemented with 0.010% of Se-rich yeast resulted in a 1.4-fold increase in relation to non-biofortified fish (Figure 2.2).

Similarly, to previous studies, I supplementation reached much lower levels in biofortified fish (0.09 μg g^{-1} for seabream and 0.21 μg g^{-1} for carp) compared to the threshold levels found in wild fish species (12.7 μg g^{-1}), and the I content in fish fillets does not seem to proportionally increased to the supplemented levels in feeds (Julshamn et al., 2006; Ramalho Ribeiro et al., 2017). Concerning Se supplementation, accumulation levels of these element in biofortified fish fillets (0.4 μg g^{-1} for seabream and 0.14 μg g^{-1} for carp) were also far below to the threshold levels found in other wild fish species (1.07 μg g^{-1}) (Lavilla et al., 2008; Moreda-Piñeiro et al., 2013b). Such results, demonstrate that increased levels of I and Se in fish feed led to increased accumulation in fish muscle, though the deposition rates are not proportional to the dietary levels, and may be highly related to fish species (e.g., metabolic rates, size, age), feeding period and the elements bioavailable forms (organic and inorganic) (Ramalho Ribeiro et al., 2017). In addition, the replacement of fishmeal and fish oil by microalgae meal in the formulation of feeds resulted in lower accumulation of some toxic elements. It is known that some plant oils (e.g., rapeseed oil), mineral mixtures and marine sources (i.e., fishmeal and fish oil) used in aquaculture feeds can be a route for some chemical contamination, such as Hg, Cd, Pb and Cu (Berntssen et al., 2010; Peacock, 2013). On the other hand, the inclusion of microalgae blends resulted in enhanced Fe and Zn contents, depending on the microalgae species

used, since *Spirulina* sp. (common carp) is a functional food with higher nutritional value than *Tetraselmis* sp. (gilthead seabream) (Liestianty et al., 2019; Pereira et al., 2019). In addition, increased Se supplementation exerts an antagonistic effect on toxic elements exposure, such as Hg, As, Cd and Pb, since Se plays an important role in detoxification mechanisms (Ralston et al., 2016; Zwolak, 2020). In fact, the biofortification strategy resulted in Se:Hg molar ratios greater than 1, as well as in positive HBV_{Se} values, indicating that the consumption of both biofortified gilthead seabream and common carp fillets reduced the negative effects associated with Hg exposure (Table 2.3). Still, biofortified gilthead seabream fillets offered higher Se-related beneficial effects than biofortified common carp. Therefore, when designing the farmed fish biofortification approach, it is essential to consider the balance between requirement and excess/toxicity, potential interactions between elements, tissues metabolic pathways, aquatic environment, as well as fish species, age, size and gender (Lall, 2003).

To a large extent, the success of biofortification strategies not only rely on the effective nutrient deposition in fish fillets (retention from feed to fillet), but also in the production viability (e.g., fish welfare), economic feasibility (e.g., costs), and environmental issues (e.g., raw material and resources). Within the various biofortification strategies tested, the incorporation of I-rich seaweed (*L. digitata*) and Se-rich yeast in gilthead seabream and common carp feeds has the potential to develop biofortified famed fish products as promising solution to meet consumers' dietary needs (enhanced I and Se contents) without increasing the exposure to toxic elements at different cost-effective levels (balance between production costs, environmental sustainability and nutritional enhancement) (Table 7.1).

Table 7.1 - Feasibility evaluation of the three biofortification strategies (B1, B2 and B3) in gilthead seabream and common carp

	Gilthead Seabream			Common carp		
	B1	**B2**	**B3**	**B1**	**B2**	**B3**
FEED						
raw materials availability	YES	YES	YES	YES	YES	YES
production costs	+ 4%	+ 11 %	+ 17%	+ 24%	+ 17%	+ 5%
manufacturing process	no dif.	no dif.	no dif.	no dif.	no dif.	no dif.
legal compliance	YES	YES	YES	YES	YES	YES
ENVIRONMENT						
sustainable ingredients						
▪ microalgae meal	+ 8.7%	+ 8.7%	+ 8.7%	+ 5.12%	+ 3.56%	+ 2.0%
▪ **seaweed (*L. digitata*)**	+ 0.4%	+ 0.4%	+ 0.8%	+ 0.54%	+ 0.54%	+ 0.54%
▪ **selenized yeast**	+ 0.015%	+ 0.015%	+ 0.035%	+ 0.01%	+ 0.01%	+ 0.01%
unsustainable ingredients						
▪ fishmeal	- 5%	- 5%	- 5%	- 2.5%	- 2.5%	- 2.5%
▪ fish oil	- 1.09%	no dif.	no dif.	n.a.	n.a.	n.a.
FARMING						
zootechnical indices	OK	OK	↙ GP	OK	OK	OK
nutrients biofortification						
▪ **iodine**	+ 4%	+ 17%	+ 37%	+ 100%	+ 100%	+ 100%
▪ **selenium**	+ 29%	+ 51%	+ 98%	+ 30%	+ 46%	+ 41%
▪ iron	+ 70%	+ 31%	+ 100%	no dif.	no dif.	no dif.
▪ zinc	no dif.	no dif.	no dif.	+ 18%	+ 21%	+ 20%
legal compliance (essential & toxic elements levels)	YES	YES	YES	YES	YES	YES
CONSUMERS						
naturalness & healthiness	YES	YES	YES	YES	YES	YES
safety	YES	YES	YES	YES	YES	YES
COST-EFFECTIVE	**Moderate**	**High**	**Low**	**Moderate**	**Moderate**	**High**

B1 - biofortified diet B1, B2 - biofortified diet B2, B3 - biofortified diet B3
no dif. - no differences, n.a. - not applicated
GP - fish growth performance

Despite being highly effective for gilthead seabream fillet biofortification, the biofortified B3 diet (supplementation with 0.08% I-rich seaweed and 0.035% Se-rich yeast) showed some impacts on formulation costs and in fish growth performance, leading to lower feasibility in terms of industrial application. Regarding common carp biofortification strategies, the significant increase in the formulation costs observed in biofortified diets B1 and B2 (mainly associated with the costs of the microalgae meal supplementation with, respectively, 3.12% and 1.56% of *Schizochytrium* sp.) increase the risk for an unsuccessful implementation of these two strategies in terms of market application. To achieve the balance on the cost-effective production of high quality and safe biofortified farmed fish products, with environmental sustainability standards, the practical application of a biofortification strategy, although with benefits to the human health, should not generate detrimental effects on the environment. In this sense, the supplementation with 0.01% Se-rich yeast and 0.4% I-rich seaweed (*L. digitata*) results in the most cost-effective approach for industrial production to biofortify gilthead seabream fillets, whereas similar supplementation (0.01% Se-rich yeast and 0.5% I-rich seaweed) together with salmon by-products oil (2.1%) results in the most cost-effective scenario to biofortify common carp fillets.

7.1.2 Biofortified farmed fish fillets maintained enhanced nutritional value after processing

Another important aspect is to understand the effect of processing, such as culinary treatment and frozen storage, in biofortified fish products nutritional quality, since on one hand, most seafood is only consumed after cooking, and on the other hand, freezing represents one of the most used methods for fish preservation. In these studies, the choice of biofortified strategies were based in the following criteria: the most biofortified diet (B3 for seabream and B2 for carp) for both steam-cooking and frozen storage, and 2) the most economic and sustainable biofortified diet (B1 for seabream and B3 for carp). The nutritional quality evaluation of biofortification strategies used for gilthead seabream and common carp demonstrated that biofortified fish fillets through the dietary supplementation with the incorporation of I-rich seaweed (*L. digitata*) and Se-rich yeast maintained their nutritional quality parameters after steaming and during frozen storage (Chapter 3 and 4).

Regarding the effect of steam-cooking procedure on enhanced health-valuable nutrients in biofortified gilthead seabream and common carp, no detrimental effect was observed in the enhanced contents of I and Se in the biofortified fillets, indicating that steaming is a healthy cooking method. Comparing the elemental composition between the different biofortification strategies, a different pattern was observed for each species with results showing a clear distinction between gilthead seabream and common carp (Figure 3.2). In fact, steaming resulted in increased contents of I and Se, as well as decreased contents of Cl, Fe, Cu and Br in biofortified gilthead seabream fillets (Table 3.3), whereas increased contents of Fe, Zn and Cl, as well as decreased contents of K and As, were observed in biofortified common carp fillets after steaming (Table 3.4). Nutrients losses and concentrations are mainly associated to water loss, as a result of evaporation, dehydration of muscle fibrils, and probably to some heat-induced protein denaturation during steaming, leading to minerals leaching from water protein structures or by the concentration of minerals due to weight loss (Alves et al., 2018; Erkan, 2011; Martins et al., 2011; Oliveira et al., 2015; Sobral et al., 2018). Additionally, increased I and Se contents after cooking may be explained by the fact that these elements are mainly bound to proteins and, therefore less prone to leaching during mild cooking procedures, such as steaming (Hou, 2009; Vicente-Zurdo et al., 2019). Therefore, the different biofortification strategies contributed to distinct effects on fish elemental composition, whereas the steam cooking treatment seems to have less influence on fillets elemental composition, especially in gilthead seabream.

Concerning frozen storage stability, no specific trend in elements contents and colour attributes was observed during the frozen storage period (12-month). Yet, distinct patterns in terms of elemental contents, lipid oxidation (LPO), and texture were observed between the two species, due to the different storage conditions (i.e., fish fillets frozen storage in seabream versus whole fish frozen storage in carp). In fact, gilthead seabream fillets can be stored at –20 °C for at least 45 days without any changes in essential elements levels, and only afterwards some losses in I content were observed in biofortified fillets (Figure 4.2). On the other hand, common carp whole fish can be stored at –20 °C for at least 225 days without no detrimental effect in essential elements stability, and only afterwards some losses of Se content were observed in both biofortified and non-biofortified fillets (Figure 4.3). Nevertheless, both gilthead seabream and common carp biofortified fillets maintained their enhanced nutritional value compared to non-biofortified fillets, particularly due to significantly higher I and Se levels during the 360 days of

frozen storage at –20 °C. Nutrients losses of elements contents during freezing are probably related to the decrease in the binding forces between minerals and water from the fish muscle, and consequently released into the surrounding aqueous medium after thawing (Gökoğlu & Yerlikaya, 2015b; Malik et al., 2021). In terms of lipid oxidation (LPO), as expected, an increasing trend was observed in both species during the frozen storage period, still, gilthead seabream fillets presented higher levels of LPO (3.45 mg MDA kg^{-1}, Figure 4.4) compared to common carp (2.41 mg MDA kg^{-1}, Figure 4.5). Increased LPO is associated with the transformation of peroxides into aldehyde compounds that are end products of lipid oxidation in the presence of oxygen and pro-oxidant molecules (Duarte et al., 2020; Gökoğlu & Yerlikaya, 2015a), but can be delayed by glazing or coating layers (Gökoğlu & Yerlikaya, 2015a; Soares et al., 2013; Tolstorebrov et al., 2016). In addition, during frozen storage, a decrease of hardness was observed in gilthead seabream fillets after 45 days of storage, while an increase of harness was observed in common carp fillets after 225 days of storage (Table 4.2). It is known that during freezing and frozen storage, the formation of ice crystals, which leads to structural damage of myofibrillar cells, results in decreased mechanical strength of connective fish muscle tissue and loss of muscle water-holding capacity after thawing (Alsailawi et al., 2020; Hematyar et al., 2018; Wang et al., 2020). In general, biofortified fish fillets maintained their nutritional benefits and quality during the 360 days of storage at –20 °C. Nonetheless, most quality changes occurred after 45 days of storage in gilthead seabream fillets and after 225 days of storage in common carp fillets.

7.1.3 Biofortified farmed fish fillets consumption improve iodine and selenium nutritional contribution to human health benefits

From a consumers' perspective it is not only important to have information regarding the biofortification level (i.e., enhanced content of health-valuable nutrients), but also the nutritional contribution of biofortified fish products to the dietary reference values (DRVs). Moreover, it is well acknowledged that the level of a nutrient present in seafood or any other food may not reflect the amount of such nutrient that will become available for absorption during the digestion process in the human gastrointestinal tract (Alves et al., 2018; Demarco et al., 2022; Fernández-García et al., 2009; Ferraris et al., 2021; Marques et al., 2011; Van de Wiele et al., 2007). For this reason, to assess nutrients bioaccessibility a standardized *in vitro* digestion methodology was used to simulate the human digestion

process, and the nutritional contribution of conventional and biofortified fish fillets was evaluated for the health-valuable nutrients, including steam-cooking procedure and bioaccessibility (Chapter 5). For this study, the feeding trials for each fish species comprised two diets: a conventional diet currently used by the aquaculture industry and a high cost-effective biofortified diet (BF), formulated considering the results from the feasibility assessment of the biofortified B1, B2 and B3 diets (Table 7.1).

In line with previous studies, except for iodine bioaccessible fraction in carp (≤ 45%), I, Se, Fe, and Zn was always above 70% in both species, regardless the biofortification strategy (Figure 5.3 and Figure 5.4). The lower I bioaccessibility in common carp may be related by the initial low content of this element in the raw matrix, even though biofortified fillets presented an increase of more than 100% compared to conventional fish fillets. Steam-cooking affected differentially the elemental bioaccessibility, but overall, biofortified fillets elemental bioaccessibility was above 65% (except for I in carp) after steaming. Cooking procedures usually lead to a decrease in elements bioaccessibility due to the leaching of unbound elements or of elements in protein complexes as a result of protein denaturation (Amiard et al., 2008).

The nutritional contribution of the conventional and biofortified fillets was calculated for the essential elements considering an average meal portion size of 150 g fillets for adults and pregnant women, or 75 g fillets for children. (1-3 years). The nutritional value of food is related with its contribution to fulfil the dietary reference intakes (DRI) of a specific nutrient, through the consumption of a typical portion meal. Steamed biofortified gilthead seabream and common carp fillets highly improved the nutritional contribution of I and Se compared to the conventional fish fillets. Interestingly, higher nutritional contributions are reached through the consumption of biofortified common carp fillets (up to 81% of I DRI and more than 100% of Se DRI), whereas the consumption of biofortified gilthead seabream fillets contribute up to 6% increase of I DRI and up to 10% increase of Se DRI (Table 5.2). Additionally, despite the slightly reduction of the nutritional value after the *in vitro* digestion process (elements bioaccessibility), biofortified fish fillets still presented improved nutritional contribution of I and Se compared to conventional fish fillets.

Overall, the present biofortification approach with I-rich seaweed (*L. digitata*) and Se-rich yeast in gilthead seabream and common carp may contribute to reduce I and Se suboptimal intakes in target population groups. Nevertheless, for common carp a fine-tuning of Se biofortification, with lower supplemented levels, seems advisable to avoid exceedance of the UL upon regular consumption, considering the Se intake from the rest of the diet.

In terms of functional foods concept, biofortified fish products may have an important role by offering health benefits beyond their nutritional value when introduced as part of a diversified diet on a regular basis, since I and Se are essential nutrients for the neurological and thyroid development, as well as for lowering the risk of cardiovascular diseases. Moreover, accordingly to the European Regulation No 1924/2006, food products can be promoted in the markets, with nutritional claims as "source of" when contains at least 15% of the DRI in 100g of product, "high in" when contains at least twice the value of "source of" and "increased" when the product meets the conditions for the claim 'source of' and the increase in content is at least 30%. In this context, biofortified gilthead seabream fillets can be labelled as "source of Se" and common carp fillets as "increased Se", but no claims can be made for the enhanced content of I in the biofortified fillets of both species.

7.1.4 Micro-X-ray fluorescence spectrometry as a suitable tool to map the distribution of elements in fish muscle samples

Through the non-destruction and low-cost micro-X-ray spectrometry (μ-XRF) analyses, it was possible to assess the elemental distribution (elements colour map) in both biofortified and non-biofortified fish fillets (Chapter 6). Despite, similar elemental distribution pattern was observed for both biofortified and non-biofortified fish muscle samples of gilthead seabream and common carp, in line with our previous results, higher elements accumulation (i.e., higher signals/intensity of elements colour) was observed in biofortified fish fillets compared to non-biofortified fillets. Mapping the elemental distributions allowed to provide insightful information regarding the tissues/regions where each element is more accumulated. Preliminary results demonstrated that Ca is mainly accumulated in the skeleton spines and scales regions, whereas K, Fe and Zn seems to be distributed throughout the fish muscle (Figure 6.3 and Figure 6.4). Nevertheless, further research is still needed to improve the detection efficiency, since the μ-XRF imaging

analyses showed some limitations for the quantification of the corresponding distributions map of elements present in trace amounts, such as I and Se. Furthermore, in contrast to previous studies where XRF imaging system elemental maps were acquired for whole zebrafish (Leitão et al., 2022) and human tooth (Dias et al., 2015), for larger samples such as seabream and carp fillets elemental maps were only possible for smaller cross-sections of the fish muscle. Still, compared to other XRF based techniques the advantage of μ-XRF system M4Tornado imaging analysis for predicting the elemental distribution in biological systems relies in the simplicity of operation (i.e., fast elemental analysis, no sample destruction and low-cost), and showed to be advantageous for a quick preliminary assessment of elemental distribution in biological samples.

7.2. Final Remarks and Future Perspectives

The present PhD demonstrated that aquaculture feeds can effectively modulate the nutritional profile of fish, targeting not only fish welfare, but also the nutritional benefits to consumers' health (i.e., complying with the Dietary Reference Values). Designing aquafeeds integrating sustainable ingredients and the consumers' dietary needs perspective, unlocks the potential of developing natural, sustainable, and environmentally friendly tailor-made fortified fish with premium nutritional quality. Such innovative fish is expected to address the human population deficiencies in some essential health-promoting nutrients, particularly iodine, selenium, and iron, which are more accentuated in pregnant woman, youth, and elderly people, where barriers to the consumption of seafood are more pronounced.

The findings gathered in this PhD dissertation have a considerable impact and applicability in aquaculture sector, tackling three of the "Transforming our world: the 2030 Agenda for Sustainable Development" goals, namely, Goal 2 - End hunger, achieve food security and improved nutrition and promote sustainable agriculture; Goal 12 - Ensure sustainable consumption and production patterns, and Goal 14 - Conserve and sustainably use the oceans, seas and marine resources for sustainable development, through the demonstration and validation of innovative, sustainable, nutritious and safe seafood products from aquaculture production.

The results from the four trials clearly evidence the importance of developing eco-innovative and cost-effective biofortified fish products and its potential in achieving sustainable, safe, and high-quality production of farmed seafood in Europe, overcoming nutritional deficiencies and meeting consumers' dietary needs more widely. Moreover, the main findings of this PhD thesis allow to provide answers to the main research questions initially proposed (Chapter 1).

1. Are I and Se levels effectively enhanced in farmed gilthead seabream and common carp muscle tissue through I-rich seaweed and Se-rich yeast enriched diets? And is farmed fish nutritional profile affected using different biofortified dietary strategies?

The feeding trials with three biofortified diets through the incorporation of I-rich seaweed (*L. digitata*) and Se-rich yeast in gilthead seabream and common carp feeds (see Chapter 2), clearly demonstrate the nutritional enrichment of fish fillets, especially in terms of I, Se, and Fe contents through the different dietary strategies. Nevertheless, the effects of different dietary strategies combining the replacement of fish-based raw materials (i.e., fishmeal and fish oil) with vegetable sources (i.e., I-rich macroalgae, Se-yeast, microalgae meals, and vegetable oils) resulted in different biofortification efficiencies in both fish species (i.e., marine gilthead seabream versus freshwater common carp). In this way further knowledge on elements uptake and bioavailability during fish digestion process is needed to evaluate not only the effective nutrient deposition in fillets (retention from feed to fillet), but also how fortification may affect fish welfare.

2. Are biofortified nutrients stability affected by processing procedures, such as frozen storage and steam-cooking processing?

This PhD results showed that steam-cooking procedures can affect macro, trace, and toxic elements contents (see Chapter 3), and that fish fillets quality changes (in terms of elemental composition, WHC, lipid oxidation, colour, and texture) during frozen storage (see Chapter 4), were strongly related with the chemical properties of each element and fish species. Nevertheless, biofortification strategies through dietary supplementation of I-rich seaweed and Se-rich yeast in gilthead seabream and common carp did not relevantly affect quality parameters during steaming or frozen storage. In fact, biofortified fillets potentially improve the nutritional benefits, maintaining the enhanced contents of I and Se after steaming and during the frozen storage period, compared to non-biofortified fish.

3. How is the bioaccessibility of elements affected by the biofortification strategy?

In terms of biofortified elements content that are available for absorption after digestion (Chapter 5), results showed that the present biofortification strategies improved I, Se, and Fe nutritional contributions, with overall higher elements bioaccessibility in gilthead seabream, compared to common carp. Additionally, while I biofortification can still be further improved, particular attention should be given to Se intakes to avoid exceeding the current dietary recommendations.

4. Is x-ray fluorescence spectrometry a suitable tool to map the distribution of elements in biofortified fish?

Understanding how and the extent of elemental distribution pattern and prevalence in fish tissues using non-invasive elemental mapping techniques (Chapter 6), has the potential to provide new insights in the role of nutrients metabolism and therefore, the efficacy of the biofortification strategy. Still, preliminary results showed that within the framework of multi-elemental mapping analysis, biological tissues constitute a major research challenge, due to the rough surface and non-uniform thickness samples, especially for elements present in trace amounts like I and Se, as well as, for large samples such as longitudinal cross sections of whole body of adult fish.

In conclusion, the dietary strategies assessed through the biofortification with different blends (%) of I-rich seaweed and Se-rich yeast were highly efficient in both gilthead seabream and common carp, being an excellent way to reduce essential elements deficiencies in consumers, especially for I and Se. Moreover, the present biofortification strategy does not seem to negatively affect essential elements intakes during the digestion process and steam-cooking can be considered a healthy culinary procedure, maintaining the enhanced nutritional quality of biofortified fish. Nevertheless, future studies still need to be undertaken considering not only different biofortification strategies (i.e., supplementation with other natural ingredients from sustainable sources) and other fish species, but also understanding how fortification may affect fish welfare and elements bioavailability. In addition, studies on the potential toxic effects of feeds supplementation by *in vitro* assays using primary fish cells and genotoxicity, considering elements speciation, should be considered for potential cytotoxicity and risk assessment.

Currently, the development of sustainable, innovative, and cost-effective farmed fish with nutritional benefits to human health is still a challenge for the aquaculture industry, therefore further evaluation of environmental costs, and economic feasibility (consumers acceptance) will be relevant to provide accurate insights to validate biofortified farmed fish with nutritional benefits to human health. This will be crucial to further develop the aquaculture sector towards food security and to deliver sufficient, healthy, safe, nutritious, and affordable nutrients in the world, through the development of eco-innovative, cost-effective, and premium biofortified fish products to the growing market of healthy and functional foods. Such strategy will improve the socio-economic and environmental sustainability of European seafood aquaculture production systems.

Bibliography

Abraha, B., Admassu, H., Mahmud, A., Tsighe, N., Shui, X. W., & Fang, Y. (2018). Effect of processing methods on nutritional and physico-chemical composition of fish: a review. MOJ Food Processing & Technology, 6(4). https://doi.org/10.15406/mojfpt.2018.06.00191.

Ackman, R. G. (1994). Seafood lipids. In F. Shahidi & J. R. Botta (Eds.), Seafoods: Chemistry, Processing Technology and Quality (pp. 34–48). Springer US. https://doi.org/10.1007/978-1-4615-2181-5_4.

Afonso, C., Costa, S., Cardoso, C., Coelho, I., Castanheira, I., Lourenço, H., Gonçalves, S., Oliveira, R., Carvalho, M. L., Martins, M. F., Bandarra, N. M., & Nunes, M. L. (2018). Bioaccessibility in risk-benefit analysis of raw and cooked seabream consumption. Journal of Food Composition and Analysis, 68, 118–127. https://doi.org/10.1016/j.jfca.2016.10.003.

Agüeria, D., Sanzano, P., Vaz-Pires, P., Rodríguez, E., & Yeannes, M. I. (2016). Development of Quality Index Method Scheme for Common Carp (*Cyprinus carpio*) Stored in Ice: Shelf-Life Assessment by Physicochemical, Microbiological, and Sensory Quality Indices. Journal of Aquatic Food Product Technology, 25(5), 708–723. https://doi.org/10.1080/10498850.2014.919975.

Alagawany, M., Taha, A. E., Noreldin, A., El-Tarabily, K. A., & Abd El-Hack, M. E. (2021). Nutritional applications of species of *Spirulina* and *Chlorella* in farmed fish: A review. Aquaculture, *542*, 736841. https://doi.org/10.1016/j.aquaculture.2021.736841.

Alasalvar, C., Taylor, K. D. A., Öksüz, A., Garthwaite, T., Alexis, M. N., & Grigorakis, K. (2001). Freshness assessment of cultured sea bream (*Sparus aurata*) by chemical, physical and sensory methods. Food Chemistry, 72(1), 33–40. https://doi.org/10.1016/S0308-8146(00)00196-5.

Alegría-Torán, A., Barberá-Sáez, R., & Cilla-Tatay, A. (2015). Bioavailability of minerals in foods. In M. de la Guardia & S. Garrigues (Eds.), Handbook of Mineral Elements in Food (pp. 41–67). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118654316.ch3.

Allen, L., Benoist, B. de, Dary, O., & Hurrell, R. (2006). Guidelines on Food Fortification with Micronutrients. In WHO; FAO. World Health Organization and Food and Agriculture Organization of the United Nations. https://doi.org/10.1242/jeb.02490.

Alonso, J. I. G., Marchante-Gayón, J. M., & Moldovan, M. (2015). New developments in food analysis by ICP-MS. In M. de la Guardia & S. Garrigues (Eds.), Handbook of Mineral Elements in Food (pp. 239–262). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118654316.ch12.

Alsailawi, H. A., Mudhafar, M., & Abdulrasool, M. M. (2020). Effect of Frozen Storage on the Quality of Frozen Foods—A Review. Journal of Chemistry and Chemical Engineering, 14(3), 86–96. https://doi.org/10.17265/1934-7375/2020.03.002.

Alves, R. N., Maulvault, A. L., Barbosa, V. L., Fernandez-Tejedor, M., Tediosi, A., Kotterman, M., van den Heuvel, F. H. M., Robbens, J., Fernandes, J. O., Romme Rasmussen, R., Sloth, J. J., & Marques, A. (2018). Oral bioaccessibility of toxic and essential elements in raw and cooked commercial seafood species available in European markets. Food Chemistry, 267, 15–27. https://doi.org/10.1016/j.foodchem.2017.11.045.

Amiard, J.-C., Amiard-Triquet, C., Charbonnier, L., Mesnil, A., Rainbow, P. S., & Wang, W.-X. (2008). Bioaccessibility of essential and non-essential metals in commercial shellfish from Western Europe and Asia. Food and Chemical Toxicology, 46(6), 2010–2022. https://doi.org/10.1016/j.fct.2008.01.041.

AOAC, 2005. Official Methods of Analysis of AOAC International. In: Latimer, G., Horwitz, W. (Eds.), AOAC, 18th ed. AOAC International, Gaithersburg, pp. 3000.

Aragão, C., Cabano, M., Colen, R., Fuentes, J., & Dias, J. (2020). Alternative formulations for gilthead seabream diets: Towards a more sustainable production. Aquaculture Nutrition, 26(2), 444–455. https://doi.org/10.1111/anu.13007.

Arannilewa, S. T., Salawu, S. O., Sorungbe, A. A., & Ola-Salawu, B. B. (2006). Effect of frozen period on the chemical, microbiological and sensory quality of frozen Tilapia fish (*Sarotherodun galiaenus*). Nutrition and Health, 18(2), 185–192. https://doi.org/10.1177/026010600601800210.

Aubourg, S. P., Piñeiro, C., & González, M. J. (2004). Quality loss related to rancidity development during frozen storage of horse mackerel (*Trachurus trachurus*). Journal of the American Oil Chemists' Society, 81(7), 671–678. https://doi.org/10.1007/s11746-004-960-1.

Badiani, A., Stipa, S., Bitossi, F., Pirini, M., Bonaldo, A., Gatta, P.P., Rotolo, M., & Testi, S. (2013). True retention of nutrients upon household cooking of farmed portion-size European sea bass (*Dicentrarchus labrax L.*). LWT - Food Science and Technology, 50 (1), 72–77. https://doi.org/10.1016/j.lwt.2012.06.026.

Barbosa, V., Maulvault, A. L., Alves, R. N., Kwadijk, C., Kotterman, M., Tediosi, A., Fernández-Tejedor, M., Sloth, J. J., Granby, K., Rasmussen, R. R., Robbens, J., De Witte, B., Trabalón, L., Fernandes, J. O., Cunha, S. C., & Marques, A. (2018). Effects of steaming on contaminants of emerging concern levels in seafood. Food and Chemical Toxicology, 118, 490–504. https://doi.org/10.1016/j.fct.2018.05.047.

Barbosa, V., Maulvault, A.L., Anacleto, P., Santos, M., Mai, M., Oliveira, H., Delgado, I., Coelho, I., Barata, M., Araújo-Luna, R., Ribeiro, L., Eljasik, P., Sobczak, M., Sadowski, J., Tórz, A., Panicz, R., Dias, J., Pousão-Ferreira, P., Carvalho, M.L., Martins, & M., Marques, A. (2020). Enriched feeds with iodine and selenium from natural and sustainable sources to modulate farmed gilthead seabream (*Sparus aurata*) and common carp (*Cyprinus carpio*) fillets elemental nutritional value. Food and Chemical Toxicology, 140, 111330. https://doi.org/10.1016/J.FCT.2020.111330.

Barbosa, V., Maulvault, A.L., Anacleto, P., Santos, M., Mai, M., Oliveira, H., Delgado, I., Coelho, I., Barata, M., Araújo-Luna, R., Ribeiro, L., Eljasik, P., Sobczak, M., Sadowski, J., T´orz, A., Panicz, R., Dias, J., Pousão-Ferreira, P., Carvalho, M.L., Martins, M., & Marques, A. (2021). Effects of steaming on health-valuable nutrients from fortified farmed fish: Gilthead seabream (*Sparus aurata*) and common carp (*Cyprinus carpio*) as case studies. Food Chemical Toxicology, 152, 1112218. https://doi.org/10.1016/j.fct.2021.112218.

Barciela-Alonso, M. C., & Bermejo-Barrera, P. (2015). Variation of food mineral content during industrial and culinary processing. *In* M. de la Guardia & S. Garrigues (Eds.), Handbook of Mineral Elements in Food (pp. 163–176). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118654316.ch8.

Bastías, J. M., Balladares, P., Acuña, S., Quevedo, R., & Muñoz, O. (2017). Determining the effect of different cooking methods on the nutritional composition of salmon (*Salmo salar*) and chilean jack mackerel (*Trachurus murphyi*) fillets. PLOS ONE, 12(7), e0180993. https://doi.org/10.1371/journal.pone.0180993.

Bearth, A., Cousin, M.-E., & Siegrist, M. (2014). The consumer's perception of artificial food additives: Influences on acceptance, risk and benefit perceptions. Food Quality and Preference, 38, 14–23. https://doi.org/10.1016/j.foodqual.2014.05.008.

Bell, J. G., & Waagbø, R. (2008). Safe and Nutritious Aquaculture Produce: Benefits and Risks of Alternative Sustainable Aquafeeds. In M. Holmer, K. Black, C. M. Duarte, N. Marbà, & I. Karakassis (Eds.), Aquaculture in the Ecosystem (pp. 185–225). Springer Netherlands. https://doi.org/10.1007/978-1-4020-6810-2_6.

Bellia, C., Timpanaro, G., Scuderi, A., & Foti, V. T. (2021). Assessment of Several Approaches to Biofortified Products: A Literature Review. Applied System Innovation, 4(2), 30. https://doi.org/10.3390/asi4020030.

Berntssen, M. H. G., Julshamn, K., & Lundebye, A.-K. (2010). Chemical contaminants in aquafeeds and Atlantic salmon (*Salmo salar*) following the use of traditional- versus alternative feed ingredients. Chemosphere, 78(6), 637–646. https://doi.org/10.1016/j.chemosphere.2009.12.021.

Berry, E. M. (2019). Sustainable Food Systems and the Mediterranean Diet. Nutrients, 11(9):2229. https://doi.org/10.3390/nu11092229.

Bevis, L. E. M. (2015). Soil-to-Human Mineral Transmission with an Emphasis on Zinc, Selenium, and Iodine. Springer Science Reviews, 3(1), 77–96. https://doi.org/10.1007/s40362-014-0026-y.

Blikra, M. J., Hodnefjell, Å. V., Feyissa, A. H., & Skipnes, D. (2020). Dimensional change and cook loss during heating of fish: Problem formulation and semi-empirical modeling approach. Journal of Food Engineering, 281, 110004. https://doi.org/10.1016/j.jfoodeng.2020.110004.

Blikra, M. J., Henjum, S., & Aakre, I. (2022). Iodine from brown algae in human nutrition, with an emphasis on bioaccessibility, bioavailability, chemistry, and effects of processing: A systematic review. Comprehensive Reviews in Food Science and Food Safety, 21(2), 1517–1536. https://doi.org/10.1111/1541-4337.12918.

Bógnar, A. (2002). Tables on Weight Yield of Food and Retention Factors of Food Constituents for the Calculation of Nutrient Composition of Cooked Foods (Dishes); BFE-R-02-03; Berichte der Bundesforschungsanstalt für Ernährung: Karlsruhe, Germany, 2002. http://www.fao.org/uploads/media/bognar_bfe-r-02-03.pdf (accessed on 20 December 2020).

Bognár, A., & Piekarski, J. (2000). Guidelines for recipe information and calculation of nutrient composition of prepared foods (Dishes). Journal of Food and Composition Analysis, 13, 391–410. https://doi.org/10.1006/jfca.2000.0922.

Boyd, C. E., D'Abramo, L. R., Glencross, B. D., Huyben, D. C., Juarez, L. M., Lockwood, G. S., McNevin, A. A., Tacon, A. G. J., Teletchea, F., Tomasso, J. R., Tucker, C. S., & Valenti, W. C. (2020). Achieving sustainable aquaculture: Historical and current perspectives and future needs and challenges. Journal of the World Aquaculture Society, 51(3), 578–633. https://doi.org/10.1111/jwas.12714.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A. R., Martins, C., Marze, S., & Recio, I. (2019). INFOGEST static *in vitro* simulation of gastrointestinal food digestion. Nature Protocols, 14(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1.

Burgaard, M. G., & Jørgensen, M. B. (2011). Effect of Frozen Storage Temperature on Quality-Related Changes in Rainbow Trout (*Oncorhynchus mykiss*). Journal of Aquatic Food Product Technology, 20(1), 53-63. https://doi.org/10.1080/10498850.2010.538894.

Cabañero, A. I., Madrid, Y., & Cámara, C. (2004). Selenium and mercury bioaccessibility in fish samples: an *in vitro* digestion method. Analytica Chimica Acta, 526(1), 51–61. https://doi.org/10.1016/j.aca.2004.09.039.

Cabañero, A. I., Madrid, Y., & Cámara, C. (2007). Mercury–Selenium Species Ratio in Representative Fish Samples and Their Bioaccessibility by an *In Vitro* Digestion Method. Biological Trace Element Research, 119(3), 195–211. https://doi.org/10.1007/s12011-007-8007-5.

Cabral, E. M., Bacelar, M., Batista, S., Castro-Cunha, M., Ozório, R. O. A., & Valente, L. M. P. (2011). Replacement of fishmeal by increasing levels of plant protein blends in diets for Senegalese sole (*Solea senegalensis*) juveniles. Aquaculture, 322–323, 74–81. https://doi.org/10.1016/j.aquaculture.2011.09.023.

Calanche, J., Tomas, A., Martinez, S., Jover, M., Alonso, V., Roncalés, P., & Beltrán, J. A. (2019). Relation of quality and sensory perception with changes in free amino acids of thawed seabream (*Sparus aurata*). Food Research International, 119, 126–134. https://doi.org/10.1016/j.foodres.2019.01.050.

Cano-Sancho, G., Perelló, G., Maulvault, A. L., Marques, A., Nadal, M., & Domingo, J. L. (2015). Oral bioaccessibility of arsenic, mercury and methylmercury in marine species commercialized in Catalonia (Spain) and health risks for the consumers. Food and Chemical Toxicology, 86, 34–40. https://doi.org/10.1016/j.fct.2015.09.012.

Cardoso, C., Afonso, C., Lourenço, H., & Nunes, M. L. (2013). Seafood consumption health concerns: The assessment of methylmercury, selenium, and eicosapentaenoic+docosahexaenoic fatty acids intake. Food Control, 34(2), 581–588. https://doi.org/10.1016/j.foodcont.2013.06.001.

Cardoso, C., Afonso, C., Lourenço, H., Costa, S., & Nunes, M. L. (2015). Bioaccessibility assessment methodologies and their consequences for the risk–benefit evaluation of food. Trends in Food Science & Technology, 41(1), 5–23. https://doi.org/10.1016/j.tifs.2014.08.008.

Carvalho, M. L., Casaca, C., Marques, J. P., Pinheiro, T., & Cunha, A. S. (2001). Human teeth elemental profiles measured by synchrotron x-ray fluorescence: dietary habits and environmental influence. X-Ray Spectrometry, 30(3), 190–193. https://doi.org/10.1002/xrs.487.

Carvalho, M. L., Santiago, S., & Nunes, M. L. (2005). Assessment of the essential element and heavy metal content of edible fish muscle. Analytical and Bioanalytical Chemistry, 382(2), 426–432. https://doi.org/10.1007/s00216-004-3005-3.

Carvalho, D. C., Coelho, L. M. M., Acevedo, M. S. M. S. F., & Coelho, N. M. M. (2015). The oligoelements. *In* M. de la Guardia & S. Garrigues (Eds.), Handbook of Mineral Elements in Food (pp. 109–122). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118654316.ch5.

Carvalho, P. M. S., Pessanha, S., Machado, J., Silva, A. L., Veloso, J., Casal, D., Pais, D., & Santos, J. P. (2020). Energy dispersive X-ray fluorescence quantitative analysis of biological samples with the external standard method. Spectrochimica Acta Part B: Atomic Spectroscopy, 174, 105991. https://doi.org/10.1016/j.sab.2020.105991.

Cavonius, L. R., & Undeland, I. (2017). Glazing herring (*Clupea harengus*) fillets with herring muscle press juice: effect on lipid oxidation development during frozen storage. International Journal of Food Science and Technology, 52(5), 1229–1237. https://doi.org/10.1111/ijfs.13399.

Cilla, A., Barberá, R., López-García, G., Blanco-Morales, V., Alegría, A., & Garcia-Llatas, G. (2019). Impact of processing on mineral bioaccessibility/bioavailability. In Innovative Thermal and Non-Thermal Processing, Bioaccessibility and Bioavailability of Nutrients and Bioactive Compounds (pp. 209–239). Elsevier. https://doi.org/10.1016/B978-0-12-814174-8.00007-X.

Coelho, I., Rego, A., Gueifão, S., Nascimento, A. C., & Castanheira, I. (2017). Application of chemometric methods for multi-elemental characterization of fruit juices and nectars analysed in the Portuguese Total Diet Studies pilot study. Journal of Chemometrics, 31(2), e2868. https://doi.org/10.1002/cem.2868.

Connell, J. J. (1990). Methods of assessing and selecting for quality. *In*: Control of Fish Quality, (3rd Ed., pp. 122–150). Fishing News Books, Oxford.

Costa, S., Afonso, C., Cardoso, C., Oliveira, R., Alves, F., Nunes, M. L., & Bandarra, N. M. (2016). Towards a deeper understanding of fatty acid bioaccessibility and its dependence on culinary treatment and lipid class: a case study of gilthead seabream (*Sparus aurata*). British Journal of Nutrition, 116(10), 1816–1823. https://doi.org/10.1017/S0007114516003780.

Costello, C., Cao, L., Gelcich, S., Cisneros-Mata, M. Á., Free, C. M., Froehlich, H. E., Golden, C. D., Ishimura, G., Maier, J., Macadam-Somer, I., Mangin, T., Melnychuc, M. C., Miyahara, M., de Moor, C. L., Naylor, R., Nøstbakken, L., Ojea, E., O'Reilly, E., Parma, A. M., Plantinga, A. J., Thilsted, S. H., & Lubchenco, J. (2020). The future of food from the sea. Nature 588, 95–100. https://doi.org/10.1038/s41586-020-2616-y.

Cotter, P. A., McLean, E., & Craig, S. R. (2009). Designing Fish for Improved Human Health Status. Nutrition and Health, 20(1), 1–9. https://doi.org/10.1177/026010600902000101.

Dang, H. T. T., Gudjónsdóttir, M., Karlsdóttir, M. G., Van Nguyen, M., Romotowska, P. E., Tómasson, T., & Arason, S. (2017). Influence of Temperature Stress on Lipid Stability of Atlantic Herring (*Clupea harengus*) Muscle During Frozen Storage. Journal of the American Oil Chemists' Society, 94(12), 1439–1449. https://doi.org/10.1007/s11746-017-3053-4.

Dantagnan, P., HernÃ¡ndez, A., Borquez, A., & Mansilla, A. (2009). Inclusion of macroalgae meal (Macrocystis pyrifera) as feed ingredient for rainbow trout (*Oncorhynchus mykiss*): effect on flesh fatty acid composition. Aquaculture Research, 41(1), 87–94. https://doi.org/10.1111/j.1365-2109.2009.02308.x.

de la Guardia, M., & Garrigues, S. (2015). X-ray. In M. de la Guardia & S. Garrigues (Eds.), Handbook of Mineral Elements in Food (pp. 285–300). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118654316.ch14.

de Samber, B., Silversmit, G., Evens, R., de Schamphelaere, K., Janssen, C., Masschaele, B., van Hoorebeke, L., Balcaen, L., Vanhaecke, F., Falkenberg, G., & Vincze, L. (2008). Three-dimensional elemental imaging by means of synchrotron radiation micro-XRF: Developments and applications in environmental chemistry. Analytical and Bioanalytical Chemistry, 390(1), 267–271. https://doi.org/10.1007/s00216-007-1694-0.

Delgado, I., Ventura, M., Gueifão, S., Coelho, I., Nascimento, A. C., Silva, J. A. L., & Castanheira, I. (2019). 12th IFDC 2017 special issue – Iodine, selenium and iron contents in Portuguese key foods as consumed. Journal of Food and Composition Analysis, 79, 39–46. https://doi.org/10.1016/j.jfca.2019.03.004.

Dias, A. A., Carvalho, M., Carvalho, M. L., & Pessanha, S. (2015). Quantitative evaluation of ante-mortem lead in human remains of the 18th century by triaxial geometry and bench top micro X-ray fluorescence spectrometry. Journal of Analytical Atomic Spectrometry, 30(12), 2488–2495. https://doi.org/10.1039/c5ja00340g.

do Nascimento da Silva, E., Aureli, F., D'Amato, M., Raggi, A., Cadore, S., & Cubadda, F. (2017). Selenium Bioaccessibility and Speciation in Selenium-Enriched Lettuce: Investigation of the Selenocompounds Liberated after *in Vitro* Simulated Human Digestion Using Two-Dimensional HPLC-ICP-MS. Journal of Agricultural and Food Chemistry, 65(14), 3031–3038. https://doi.org/10.1021/acs.jafc.7b01188.

Doh, H. S., & Park, H. J. (2018). Speciation of Bio-Available Iodine in Abalone (*Haliotis discus hannai*) by High-Performance Liquid Chromatography Hyphenated with Inductively Coupled Plasma-Mass Spectrometry Using an *In Vitro* Method. Journal of Food Science, 83(6), 1579–1587. https://doi.org/10.1111/1750-3841.14173.

Doh, H., Lee, M. H., & Park, H. J. (2019). Effect of different cooking methods on the content and bioaccessibility of iodine components in abalone (*Haliotis discus hannai*). Food Chemistry, 301, 125197. https://doi.org/10.1016/j.foodchem.2019.125197.

Doherty, A., Wall, A., Khaldi, N., & Kussmann, M. (2021). Artificial Intelligence in Functional Food Ingredient Discovery and Characterisation: A Focus on Bioactive Plant and Food Peptides. Frontiers in Genetics, 12. https://doi.org/10.3389/fgene.2021.768979.

Dórea, J. G. (2006). Fish Meal in Animal Feed and Human Exposure to Persistent Bioaccumulative and Toxic Substances. Journal of Food Protection, 69(11), 2777–2785. https://doi.org/10.4315/0362-028X-69.11.2777.

Downs, S. M., Ahmed, S., Fanzo, J., & Herforth, A. (2020). Food Environment Typology: Advancing an Expanded Definition, Framework, and Methodological Approach for Improved Characterization of Wild, Cultivated, and Built Food Environments toward Sustainable Diets. Foods, 9(4):532. https://doi.org/10.3390/foods9040532.

Duarte, A. M., Silva, F., Pinto, F. R., Barroso, S., & Gil, M. M. (2020). Quality Assessment of Chilled and Frozen Fish — Mini Review. Foods, 9, 1739. https://doi.org/10.3390/foods9121739.

Dyall, S. C. (2015). Long-chain omega-3 fatty acids and the brain: a review of the independent and shared effects of EPA, DPA and DHA. Frontiers in Aging Neuroscience, 7(52), 1–15. https://doi.org/10.3389/fnagi.2015.00052.

EFSA. (2005). Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed on the request from the Commission on the use of iodine in feedingstuffs. EFSA Journal, 168, 1–42. https://doi.org/hhttps://doi.org/10.2903/j.efsa.2005.168.

EFSA. (2006). Opinion of the Panel on additives and products or substances used in animal feed (FEEDAP) on the safety and efficacy of the product Selenium enriched yeast (*Saccharomyces cerevisiae* NCYC R397) as a feed additive for all species in accordance with Regulation. EFSA Journal, 430, 1–23. https://doi.org/10.2903/j.efsa.2007.430.

EFSA, (2009). EFSA Panel on Contaminants in the Food Chain (CONTAM); Scientific Opinion on Arsenic in Food. EFSA Journal, 7(10):1351, 199. https://doi.org/10.2903/j.efsa.2009.1351.

EFSA. (2010). EFSA Panel on Contaminants in the Food Chain (CONTAM); Scientific Opinion on Lead in Food. EFSA Journal, 8(4):1570, 151. https://doi.org/10.2903/j.efsa.2010.1570.

EFSA. (2011). EFSA Panel on Contaminants in the Food Chain (CONTAM); Scientific Opinion on tolerable weekly intake for cadmium. EFSA Journal, 9(2):1975, 19. https://doi.org/10.2903/j.efsa.2011.1975.

EFSA. (2012a). EFSA Panel on Contaminants in the Food Chain (CONTAM); Scientific Opinion on the risk for public health related to the presence of mercury and methylmercury in food. EFSA Journal, 10(12):2985, 241. https://doi.org/10.2903/j.efsa.2012.2985.

EFSA. (2012b). Scientific Committee; Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data. EFSA Journal 2012, 10(3):2579, 32 pp. http://doi:10.2903/j.efsa.2012.2579.

EFSA. (2014a). EFSA NDA Panel (EFSA Panel on Panel on Dietetic Products Nutrition and Allergies), Scientific Opinion on health benefits of seafood (fish and shellfish) consumption in relation to health risks associated with exposure to methylmercury. EFSA Journal, 12(7):3761, 80. https://doi.org/10.2903/j.efsa.2014.3761.

EFSA. (2014b). EFSA NDA Panel (EFSA Panel on Panel on Dietetic Products Nutrition and Allergies); Scientific Opinion on Dietary Reference Values for iodine. EFSA Journal, 12(5):3660, 57. https://doi.org/10.2903/j.efsa.2014.3660.

EFSA. (2014c). EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies); Scientific Opinion on Dietary Reference Values for selenium. EFSA Journal, 12(10):3846, 3846. https://doi.org/10.2903/j.efsa.2014.3846.

EFSA. (2014d). EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies); Scientific Opinion on Dietary Reference Values for zinc. EFSA Journal, 12(10):3844, 76. https://doi.org/10.2903/j.efsa.2014.3844.

EFSA. (2015a). EFSA Scientific Committee Statement on the benefits of fish/seafood consumption compared to the risks of methylmercury in fish/seafood. EFSA Journal, 13(1):3982, 36. https://doi.org/10.2903/j.efsa.2015.3982.

EFSA. (2015b). EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies); Scientific Opinion on Dietary Reference Values for iron. EFSA Journal, 13(10):4254, 115. https://doi.org/10.2903/j.efsa.2015.4254.

EFSA. (2015c). EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies); Scientific Opinion on Dietary Reference Values for copper. EFSA Journal, 13(10):4253, 51. https://doi.org/10.2903/j.efsa.2015.4253.

EFSA. (2015d). Panel on dietetic products, nutrition and allergies (NDA); scientific opinion on dietary reference values for calcium. 2015. EFSA Journal 13(5):4101, 82. https://doi.org/10.2903/j.efsa.2015.4101.

EFSA. (2016). EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies); Scientific Opinion on Dietary Reference Values for potassium. EFSA Journal, 14(10):4592, 56. https://doi.org/10.2903/j.efsa.2016.4592.

EFSA. (2019). EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), Scientific Opinion on Dietary Reference Values for chloride. EFSA Journal, 17(9):5779, 24. https://doi.org/10.2903/j.efsa.2019.5779.

EFSA (2022). Protocol for the intake assessments performed in the context of the revision of Tolerable Upper Intake Levels for selected nutrients. EFSA supporting publication 2022:e200801,15 pp. https://doi:10.2903/sp.efsa.2022.e200801.

Elia, A. C., Prearo, M., Pacini, N., Dörr, A. J. M., & Abete, M. C. (2011). Effects of selenium diets on growth, accumulation and antioxidant response in juvenile carp. Ecotoxicology and Environmental Safety, 74(2), 166–173. https://doi.org/10.1016/j.ecoenv.2010.04.006.

Erkan, N. (2011). Iodine content of cooked and processed fish in Turkey. International Journal of Food Science and Technology, 46 (8), 1734–1738. https://doi.org/10.1111/j.1365-2621.2011.02684.x.

Ersoy, B., & Özeren, A. (2009). The effect of cooking methods on mineral and vitamin contents of African catfish. Food Chemistry, 115 (2), 419–422. https://doi.org/10.1016/j.foodchem.2008.12.018.

Estévez, A., Camacho, C., Correia, T., Barbosa, V., Marques, A., Lourenço, H., Serrano, C., Sapata, M., Duarte, M.P., Pires, C., Gonçalves, A., Nunes, M.L., & Oliveira, H. (2021). Strategies to reduce sodium levels in European seabass sausages. Food and Chemical Toxicology, 153, 112262. https://doi.org/10.1016/j.fct.2021.112262.

EUMOFA. (2019). European Market Observatory for Fisheries and Aquaculture Products; the Eu Fish Market, 2019 Edition. https://doi.org/10.2771/168390.

EUMOFA. (2021). The EU fish market 2021 edition. Publications Office of the European Union. https://doi.org/10.2771/563899.

European Commission. (2007). Commission Recommendation of 18 June 2007 on guidelines for the accomodation and care of animals used for experimental and other scientific purposes (2007/526/EC). In Official Journal of the European Union (Vol. L197, Issue 806). https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:197:0001:0089:EN:PDF. (Accessed 20 November 2021).

European Committee for Standardization. (2007). ES EN 15111:2007. Foodstuffs. Determination of Trace Elements. Determination of Iodine by ICP-MS (Inductively Coupled Plasma Mass Spectrometry). European Committee for Standardization: Brussels, Belgium, pp. 1–16.

European Committee for Standardization. (2009). ES EN 15763:2009. Foodstuffs—Determination of Trace Elements—Determination of Arsenic, Cadmium, Mercury and Lead in Foodstuffs by Inductively Coupled Plasma Mass Spectrometry (ICPMS) after Pressure Digestion. European Committee for Standardization: Brussels, Belgium, pp. 1–22.

European Union. (2007). Regulation (EC) No. 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods. In Official Journal of the European Union OJ L12.

European Union. (2019). Agriculture, forestry and fishery statistics, Eurostat, 2019 Edition. https://ec.europa.eu/eurostat/documents/3217494/10317767/KS-FK-19-001-EN-N.pdf/742d3fd2-961e-68c1-47d0-11cf30b11489?t=1576657490000 (accessed on 10 January 2021).

FAO. (2018). The State of World Fisheries and Aquaculture 2018 - Meeting the Sustainable Development Goals. Food and Agriculture Organization of the United Nations, Rome pp. 227. https://doi.org/10.1093/japr/3.1.101.

FAO. (2020). The State of World Fisheries and Aquaculture 2020. Sustainability in action. Food and Agriculture Organization of the United Nations, Rome. https://doi.org/10.4060/ca9229en.

FAO, IFAD, UNICEF, WFP, & WHO. (2021). The State of Food Security and Nutrition in the World 2021. FAO. https://doi.org/10.4060/cb4474en.

FAO/WHO. (2006). Guidelines on Food Fortification with Micronutrients Rome. Food and Agriculture Organization of the United Nations; World Health Organization, Geneva, pp. 341.

FAO/WHO. (2008). 2008. Animal Feed Impact on Food Safety Report of the FAO/WHO Expert Meeting Rome. Organization of the United Nations; World Health Organization, Geneva, pp. 45.

FAO/WHO. (2011). Report of the Joint FAO/WHO Expert Consultation on the Risks and Benefits of Fish Consumption. Food and Agriculture Organization of the United Nations; World Health Organization Fisheries and Aquaculture Report, Rome, Geneva, pp. 50.

Fernández-García, E., Carvajal-Lérida, I., & Pérez-Gálvez, A. (2009). In vitro bioaccessibility assessment as a prediction tool of nutritional efficiency. Nutrition Research, 9(11), 751-760. https://doi.org/10.1016/j.nutres.2009.09.016.

Ferraris, F., Iacoponi, F., Raggi, A., Baldi, F., Fretigny, M., Mantovani, A., & Cubadda, F. (2021). Essential and toxic elements in sustainable and underutilized seafood species and derived semi-industrial ready-to-eat products. Food and Chemical Toxicology, 154, 112331. https://doi.org/10.1016/j.fct.2021.112331.

Ferreira, M., Larsen, B. K., Granby, K., Cunha, S. C., Monteiro, C., Fernandes, J. O., Nunes, M. L., Marques, A., Dias, J., Cunha, I., Castro, L. F. C., & Valente, L. M. P. (2020). Diets supplemented with *Saccharina latissima* influence the expression of genes related to lipid metabolism and oxidative stress modulating rainbow trout (*Oncorhynchus mykiss*) fillet composition. Food and Chemical Toxicology, 140, 111332. https://doi.org/10.1016/j.fct.2020.111332.

Fiorella, K. J., Okronipa, H., Baker, K., & Heilpern, S. (2021). Contemporary aquaculture: implications for human nutrition. Current Opinion in Biotechnology, 70, 83-90. https://doi.org/10.1016/j.copbio.2020.11.014.

Flik, G., Verbost, P. M., & Bonga, S. E. W. (1995). 12 Calcium Transport Processes in Fishes. Fish Physiology, 14(C), 317–342. https://doi.org/10.1016/S1546-5098(08)60251-4.

Froehlich, H. E., Gentry, R. R., & Halpern, B. S. (2018). Global change in marine aquaculture production potential under climate change. Nature Ecology & Evolution, 2, pages1745–1750. https://doi.org/10.1038/s41559-018-0669-1.

Gharibzahedi, S. M. T., & Jafari, S. M. (2017). The importance of minerals in human nutrition: Bioavailability, food fortification, processing effects and nanoencapsulation. Trends in Food Science & Technology, 62, 119–132. https://doi.org/10.1016/j.tifs.2017.02.017.

Gidding, S. S., Lichtenstein, A. H., Faith, M. S., Karpyn, A., Mennella, J. A., Popkin, B., Rowe, J., Van Horn, L., & Whitsel, L. (2009). Implementing American Heart Association Pediatric and Adult Nutrition Guidelines. Circulation, 119(8), 1161–1175. https://doi.org/10.1161/CIRCULATIONAHA.109.191856.

Girard, C., Charette, T., Leclerc, M., Shapiro, B. J., & Amyot, M. (2018). Cooking and co-ingested polyphenols reduce in vitro methylmercury bioaccessibility from fish and may alter exposure in humans. Science of The Total Environment, 616–617, 863–874. https://doi.org/10.1016/j.scitotenv.2017.10.236.

Godin, S., Fontagné-Dicharry, S., Bueno, M., Tacon, P., Jesu Prabhu, P. A., Kaushik, S., Médale, F., & Bouyssiere, B. (2015). Influence of Dietary Selenium Species on Selenoamino Acid Levels in Rainbow Trout. Journal of Agricultural and Food Chemistry, 63(28), 6484–6492. https://doi.org/10.1021/acs.jafc.5b00768.

Godswill, A. G., Somtochukwu, I. V., Ikechukwu, A. O., & Kate, E. C. (2020). Health Benefits of Micronutrients (Vitamins and Minerals) and their Associated Deficiency Diseases: A Systematic Review. International Journal of Food Sciences, 3(1), 1–32. https://doi.org/10.47604/ijf.1024.

Gokoglu, N., Yerlikaya, P., & Cengiz, E. (2004). Effects of cooking methods on the proximate composition and mineral contents of rainbow trout (*Oncorhynchus mykiss*). *Food Chemistry*, *84*(1), 19–22. https://doi.org/10.1016/S0308-8146(03)00161-4.

Gökoğlu, N., & Yerlikaya, P. (2015a). Freezing and frozen storage of fish. *In*: Seafood Chilling, Refrigeration and Freezing (1st Edition), pp. 186–207). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118512210.ch8.

Gökoğlu, N., & Yerlikaya, P. (2015b). Thawing of fish. In Seafood Chilling, Refrigeration and Freezing (1st Edition, pp. 208–227). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118512210.ch9.

Gonçalves, A. A., & Gindri Junior, C. S. G. (2009). The effect of glaze uptake on storage quality of frozen shrimp. Journal of Food Engineering, 90, 285–290. https://doi.org/10.1016/j.jfoodeng.2008.06.038.

Gormaz, J. G., Fry, J. P., Erazo, M., & Love, D. C. (2014). Public Health Perspectives on Aquaculture. Current Environmental Health Reports, 1(3):227-238. doi: 10.1007/s40572-014-0018-8.

Gouveia, A., & Davies, S. (2000). Inclusion of an extruded dehulled pea seed meal in diets for juvenile European sea bass (*Dicentrarchus labrax*). Aquaculture, 182(1–2), 183–193. https://doi.org/10.1016/S0044-8486(99)00246-X.

Grigorakis, K. (2010). Effects of Nutrition and Aquaculture Practices on Fish Quality. In C. Alasalvar, F. Shahidi, K. Miyashita, & U. Wanasundara (Eds.), Handbook of Seafood Quality, Safety and Health Applications (pp. 82–95). Wiley-Blackwell. https://doi.org/10.1002/9781444325546.ch7.

Greenfield, H., & Southgate, D. A. T. (2003). Food Composition Data, 2nd Edition: Production, Management and Use. Elsevier Science Publishers, FAO, Rome, 288 pp.

Guérin, T., Chekri, R., Vastel, C., Sirot, V., Volatier, J., Leblanc, J., & Noël, L. (2011). Determination of 20 trace elements in fish and other seafood from the French market. Food Chemistry, 127(3), 934–942. https://doi.org/10.1016/j.foodchem.2011.01.061.

Haldimann, M., A., A., Blanc, A., & Blondeau, K. (2005). Iodine content of food groups. Journal of Food Composition and Analysis, 18(6), 461–471. https://doi.org/10.1016/j.jfca.2004.06.003.

Hasler, C. M. (2002). Functional Foods: Benefits, Concerns and Challenges—A Position Paper from the American Council on Science and Health. The Journal of Nutrition, 132(12), 3772–3781. https://doi.org/10.1093/jn/132.12.3772.

Hassoun, A. & Karoui, R. (2017) Quality evaluation of fish and other seafood by traditional and nondestructive instrumental methods: Advantages and limitations, Critical Reviews in Food Science and Nutrition, 57:9, 1976-1998, https://doi.org/10.1080/10408398.2015.1047926.

He, M., Ke, C.-H., & Wang, W.-X. (2010). Effects of Cooking and Subcellular Distribution on the Bioaccessibility of Trace Elements in Two Marine Fish Species. Journal of Agricultural and Food Chemistry, 58(6), 3517–3523. https://doi.org/10.1021/jf100227n.

He, M., & Wang, W.-X. (2013). Bioaccessibility of 12 trace elements in marine molluscs. Food and Chemical Toxicology, 55, 627–636. https://doi.org/10.1016/j.fct.2013.01.046.

Hematyar, N., Masilko, J., Mraz, J., & Sampels, S. (2018). Nutritional quality, oxidation, and sensory parameters in fillets of common carp (*Cyprinus carpio L.*) influenced by frozen storage (–20 °C). Journal of Food Processing and Preservation, 42, 1–13. https://doi.org/10.1111/jfpp.13589.

Hermann, C. (2001). The International Commission on Illumination - CIE: What It Is and How It Works. Symposium - International Astronomical Union, 196, 60-68. https://doi.org/10.1017/S0074180900163831.

Hernández-Olivas, E., Muñoz-Pina, S., Andrés, A., & Heredia, A. (2020). Impact of elderly gastrointestinal alterations on *in vitro* digestion of salmon, sardine, sea bass and hake: Proteolysis, lipolysis and bioaccessibility of calcium and vitamins. Food Chemistry, 326, 127024. https://doi.org/10.1016/j.foodchem.2020.127024.

Hicks, C. C., Cohen, P. J., Graham, N. A. J., Nash, K. L., Allison, E. H., D'Lima, C., Mills, D. J., Roscher, M., Thilsted, S. H., Thorne-Lyman, A. L., & MacNeil, M. A. (2019). Harnessing global fisheries to tackle micronutrient deficiencies. Nature 574, 95–98 (2019). https://doi.org/10.1038/s41586-019-1592-6.

Hosomi, R., Yoshida, M., & Fukunaga, K. (2012). Seafood Consumption and Components for Health. Global Journal of Health Science, 4(3), 72–86. https://doi.org/10.5539/gjhs.v4n3p72.

Hosseini, H., Mahmoudzadeh, M., Rezaei, M., Mahmoudzadeh, L., Khaksar, R., Khosroshahi, N. K., & Babakhani, A. (2014). Effect of different cooking methods on minerals, vitamins and nutritional quality indices of kutum roach (*Rutilus frisii kutum*). Food Chemistry 148, 86–91. https://doi.org/10.1016/j.foodchem.2013.10.012.

Hou, X. (2009). Iodine Speciation in Foodstuffs, Tissues, and Environmental Samples: Iodine Species and Analytical Method. In V. R. Preedy, G. N. Burrow, & R. R. Watson (Eds.), Comprehensive Handbook of Iodine (pp 139–150), Academic Press. https://doi.org/10.1016/B978-0-12-374135-6.00015-7.

Hu, L., Fan, H., Wu, D., Wan, J., Wang, X., Huang, R., Liu, W., & Shen, F. (2019). Assessing bioaccessibility of Se and I in dual biofortified radish seedlings using simulated *in vitro* digestion. Food Research International, 119, 701–708. https://doi.org/10.1016/j.foodres.2018.10.049.

Huidobro, A., & Tejada, M. (2004). Gilthead seabream (*Sparus aurata*): Suitability for freezing and commercial alternatives. Journal of the Science of Food and Agriculture, 84, 1405–1413. https://doi.org/10.1002/jsfa.1827.

Huss, H. H. (1995). Quality and Quality Changes in Fresh Fish. Fisheries Technical Paper no. 348. Rome, Italy, FAO. http://www.fao.org/3/V7180E/V7180E00.htm (accessed on 20 December 2020).

Jiang, Q., Yin, T., Yang, F., Yu, D., Xu, Y., Tie, H., Gao, P., Wang, B., & Xia, W. (2020). Effect of freezing methods on quality changes of grass carp during frozen storage. Journal of Food Process Engineering, 43, 1–11. https://doi.org/10.1111/jfpe.13539.

Julshamn, Kaare, Dahl, L., & Eckhoff, K. (2001). Determination of Iodine in Seafood by Inductively Coupled Plasma/Mass Spectrometry. Journal of AOAC INTERNATIONAL, 84(6), 1976–1983. https://doi.org/10.1093/jaoac/84.6.1976.

Julshamn, K., Maage, A., Waagbo, R., & Lundebye, A.-K. (2006). A preliminary study on tailoring of fillet iodine concentrations in adult Atlantic salmon (*Salmo salar L.*) through dietary supplementation. Aquaculture Nutrition, 12(1), 45–51. https://doi.org/10.1111/j.1365-2095.2006.00380.x.

Julshamn, Kaare, Nilsen, B. M., Frantzen, S., Valdersnes, S., Maage, A., Nedreaas, K., & Sloth, J. J. (2012). Total and inorganic arsenic in fish samples from Norwegian waters. Food Additives and Contaminants: Part B Surveillance, 5(4), 229–235. https://doi.org/10.1080/19393210.2012.698312.

Karimian-Khosroshahi, N., Hosseini, H., Rezaei, M., Khaksar, R., & Mahmoudzadeh, M. (2016). Effect of Different Cooking Methods on Minerals, Vitamins, and Nutritional Quality Indices of Rainbow Trout (*Oncorhynchus mykiss*). International Journal of Food Properties, 19, 2471–2480. https://doi.org/10.1080/10942912.2015.1039028.

Karl, H., Basak, S., Ziebell, S., & Quast, P. (2005). Changes of the Iodine Content in Fish during Household Preparation and Smoking. Deutsche Lebensmittel-Rundschau, 101(10), 431–436.

Kaushik, S. J., Covès, D., Dutto, G., & Blanc, D. (2004). Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*. *Aquaculture*, *230*(1–4), 391–404. https://doi.org/10.1016/S0044-8486(03)00422-8.

Ke, P.J., Cervantes E, & Robles-Martinez C. (1984). Determination of thiobarbituric acid reactive substances (TBARS) in fish tissue by an improved distillation-spectrophotometric method. Journal of the Science of Food and Agriculture, 35(11), 1248-1254. https://doi.org/10.1002/jsfa.2740351117.

Kong, F., Tang, J., Rasco, B., & Crapo, C. (2007). Kinetics of salmon quality changes during thermal processing. Journal of Food Engineering 83 (4), 510-520. https://doi.org/10.1016/j.jfoodeng.2007.04.002.

Kongkachuichai, R., Napatthalung, P., & Charoensiri, R. (2002). Heme and nonheme iron content of animal products commonly consumed in Thailand. Journal of Food Composition Analysis, 15 (4), 389–398. https://doi.org/10.1006/jfca.2002.1080.

Krutulyte, R., Grunert, K. G., Scholderer, J., Lähteenmäki, L., Hagemann, K. S., Elgaard, P., Nielsen, B., & Graverholt, J. P. (2011). Perceived fit of different combinations of carriers and functional ingredients and its effect on purchase intention. Food Quality and Preference, 22(1), 11–16. https://doi.org/10.1016/j.foodqual.2010.06.001.

Lall, S. P. (2003). The Minerals. In J. E. Halver & R. W. Hardy (Eds.), Fish Nutrition (3 eds, pp. 259–308). Elsevier. https://doi.org/10.1016/B978-012319652-1/50006-9.

Lall, S. P., & Kaushik, S. J. (2021). Nutrition and Metabolism of Minerals in Fish. Animals, 11(9), 2711. https://doi.org/10.3390/ani11092711.

Larsen, R., Eilertsen, K., & Elvevoll, E. O. (2011). Health benefits of marine foods and ingredients. Biotechnology Advances, 29(5), 508–518. https://doi.org/10.1016/j.biotechadv.2011.05.017.

Lei, B., Chen, L., Hao, Y., Cao, T., Zhang, X., Yu, Y., & Fu, J. (2013). Trace elements in animal-based food from Shanghai markets and associated human daily intake and uptake estimation considering bioaccessibility. Ecotoxicology and Environmental Safety, 96, 160–167. https://doi.org/10.1016/j.ecoenv.2013.06.032.

Leitão, R. G., Silva, M. P., Diniz, M., & Guerra, K. (2022). Mapping the distribution of mercury (II) chloride in zebrafish organs by benchtop micro-energy dispersive X-ray fluorescence: A proof of comcept. Journal of Trace Elements in Medicine and Biology, 69, 126874. https://doi.org/10.1016/j.jtemb.2021.126874.

Liestianty, D., Rodianawati, I., Arfah, R. A., Assa, A., Patimah, Sundari, & Muliadi. (2019). Nutritional analysis of *spirulina* sp to promote as superfood candidate. IOP Conference Series: Materials Science and Engineering, 509(1), 012031. https://doi.org/10.1088/1757-899X/509/1/012031.

Lin, Y.-H. (2014). Effects of dietary organic and inorganic selenium on the growth, selenium concentration and meat quality of juvenile grouper *Epinephelus malabaricus*. Aquaculture, 430, 114–119. https://doi.org/10.1016/j.aquaculture.2014.03.048.

Liu, C., Lin, H., Mi, N., Xu, Y., Song, Y., Liu, Z., & Sui, J. (2017). Effect of thermal processing on the concentration and bioaccessibility of rare earth elements in seaweed and oyster. Journal of Food Processing and Preservation, 41(6), e13259. https://doi.org/10.1111/jfpp.13259.

Luten, J., Schram, E., & Elvevoll, E. (2008). Tailor-made functional seafood for consumers: dietary modulation of selenium and taurine in farmed fish, in: Øyvind Lie (Eds.), Improving Farmed Fish Quality and Safety, Woodhead Publishing Series in Food Science, Technology and Nutrition, Woodhead Publishing, pp 343-362, https://doi.org/10.1533/9781845694920.2.343.

Machado, J., Carvalho, M., Elix, A. F., Doutel, D., José, J. J., Santos, P., Carvalho, M. L., & Pessanha, S. (2020). Accuracy improvement in XRF analysis for the quantification of elements ranging from tenths to thousands mg g^{-1} in hu-man tissues using different matrix reference mate-rials. https://doi.org/10.1039/d0ja00307g.

Malik, I. A., Elgasim, E. A., Adiamo, O. Q., Ali, A. A., & Mohamed Ahmed, I. A. (2021). Effect of frozen storage on the biochemical composition of five commercial freshwater fish species from River Nile, Sudan. Food Science and Nutrition, 9, 3758–3767. https://doi.org/10.1002/fsn3.2340.

Mangueze, A. V. de J., Pessoa, M. F. G., Silva, M. J., Ndayiragije, A., Magaia, H. E., Cossa, V. S. I., Reboredo, F. H., Carvalho, M. L., Santos, J. P., Guerra, M., Ribeiro-Barros, A. I., Lidon, F. C., & Ramalho, J. C. (2018). Simultaneous Zinc and selenium biofortification in rice. Accumulation, localization and implications on the overall mineral content of the flour. Journal of Cereal Science, 82, 34–41. https://doi.org/10.1016/j.jcs.2018.05.005.

Manousakas, M., Diapouli, E., Papaefthymiou, H., Kantarelou, V., Zarkadas, C., Kalogridis, A.-C., Karydas, A.-G., & Eleftheriadis, K. (2018). XRF characterization and source apportionment of PM10 samples collected in a coastal city. X-Ray Spectrometry, 47, 190–200. https://doi.org/10.1002/xrs.2817.

Marques, A., Lourenço, H. M., Nunes, M. L., Roseiro, C., Santos, C., Barranco, A., Rainieri, S., Langerholc, T., & Cencic, A. (2011). New tools to assess toxicity, bioaccessibility and uptake of chemical contaminants in meat and seafood. Food Research International, 44(2), 510–522. https://doi.org/10.1016/j.foodres.2010.12.003.

Martins, C. T., Almeida, C. M. M., & Alvito, P. C. (2011). Selenium Content of Raw and Cooked Marine Species Consumed in Portugal. Food Analytical Methods, 4(1), 77–83. https://doi.org/10.1007/s12161-009-9119-7.

Matos, J., Lourenço, H. M., Brito, P., Maulvault, A. L., Martins, L. L., & Afonso, C. (2015). Influence of bioaccessibility of total mercury, methyl-mercury and selenium on the risk/benefit associated to the consumption of raw and cooked blue shark (*Prionace glauca*). Environmental Research, 143, 123–129. https://doi.org/10.1016/j.envres.2015.09.015.

Maulvault, A. L., Machado, R., Afonso, C., Lourenço, H. M., Nunes, M. L., Coelho, I., Langerholc, T., & Marques, A. (2011). Bioaccessibility of Hg, Cd and As in cooked black scabbard fish and edible crab. Food and Chemical Toxicology, 49(11), 2808–2815. https://doi.org/10.1016/j.fct.2011.07.059.

Maulvault, A. L., Anacleto, P., Machado, R., Amaral, A., Carvalho, M. L., Lourenço, H. M., Nunes, M. L., & Marques, A. (2012). Effect of sex, maturation stage and cooking methods on the nutritional quality and safety of black scabbard fish (*Aphanopus carbo Lowe*, 1839). Journal of the Science of Food and Agriculture, 92(7), 1545–1553. https://doi.org/10.1002/jsfa.4741.

Maulvault, A. L., Cardoso, C., Nunes, M. L., & Marques, A. (2013). Risk–benefit assessment of cooked seafood: Black scabbard fish (*Aphanopus carbo*) and edible crab (*Cancer pagurus*) as case studies. Food Control, 32(2), 518–524. https://doi.org/10.1016/j.foodcont.2013.01.026.

Maulvault, A. L., Anacleto, P., Barbosa, V., Sloth, J. J., Rasmussen, R. R., Tediosi, A., Fernandez-Tejedor, M., van den Heuvel, F. H. M., Kotterman, M., & Marques, A. (2015). Toxic elements and speciation in seafood samples from different contaminated sites in Europe. Environmental Research, 143, 72–81. https://doi.org/10.1016/j.envres.2015.09.016.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., & Brodkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food – an international consensus. Food Function, 5, 1113. https://doi.org/10.1039/C3FO60702J.

Mnari B., A., Zaghbib, S. F., Dhibi, M., Harzallah, H. J., Dabbou, S., El Cafsi, M., Hammami, M., & Chaouch, A. (2012). Effects of different cooking methods on the mineral contents of wild and farmed sea bream (*Sparus aurata*). International Journal of Food Science & Technology, 47(9), 1964–1969. https://doi.org/10.1111/j.1365-2621.2012.03057.x.

Mohan, M., Ramachandran, D., Sankar, T.V., & Anandan, R. (2008). Physicochemical characterization of muscle proteins from different regions of mackerel (*Rastrelliger kanagurta*). Food Chemistry, 106 (2), 451-457. https://doi.org/10.1016/j.foodchem.2007.05.024.

Moreda-Piñeiro, J., Moreda-Piñeiro, A., Romarís-Hortas, V., Moscoso-Pérez, C., López-Mahía, P., Muniategui-Lorenzo, S., Bermejo-Barrera, P., & Prada-Rodríguez, D. (2011). *In-vivo* and *in-vitro* testing to assess the bioaccessibility and the bioavailability of arsenic, selenium and mercury species in food samples. TrAC Trends in Analytical Chemistry, 30(2), 324–345. https://doi.org/10.1016/j.trac.2010.09.008.

Moreda-Piñeiro, J., Moreda-Piñeiro, A., Romarís-Hortas, V., Domínguez-González, R., Alonso-Rodríguez, E., López-Mahía, P., Muniategui-Lorenzo, S., Prada-Rodríguez, D., & Bermejo-Barrera, P. (2012). Trace metals in marine foodstuff: Bioavailability estimation and effect of major food constituents. Food Chemistry, 134(1), 339–345. https://doi.org/10.1016/j.foodchem.2012.02.165.

Moreda-Piñeiro, J., Moreda-Piñeiro, A., Romarís-Hortas, V., Domínguez-González, R., Alonso-Rodríguez, E., López-Mahía, P., Muniategui-Lorenzo, S., Prada-Rodríguez, D., & Bermejo-Barrera, P. (2013a). ICP-MS for the determination of selenium bioavailability from seafood and effect of major food constituents. Microchemical Journal, 108, 174–179. https://doi.org/10.1016/j.microc.2012.10.019.

Moreda-Piñeiro, J., Moreda-Piñeiro, A., Romarís-Hortas, V., Domínguez-González, R., Alonso-Rodríguez, E., López-Mahía, P., Muniategui-Lorenzo, S., Prada-Rodríguez, D., & Bermejo-Barrera, P. (2013b). In vitro bioavailability of total selenium and selenium species from seafood. Food Chemistry, 139(1–4), 872–877. https://doi.org/10.1016/j.foodchem.2013.01.116.

Nagarajan, D., Varjani, S., Lee, D.-J., & Chang, J.-S. (2021). Sustainable aquaculture and animal feed from microalgae – Nutritive value and techno-functional components. *Renewable and* Sustainable Energy Reviews, 150, 111549. https://doi.org/10.1016/j.rser.2021.111549.

Nakazawa, N., & Okazaki, E. (2020). Recent research on factors influencing the quality of frozen seafood. Fisheries Science, 86(2), 231–244. https://doi.org/10.1007/s12562-020-01402-8.

Naseri, M., Abedi, E., Vafa, S., & Torri, L. (2020). Comparative investigation of physico-chemical and sensory properties of glazed and non-glazed frozen rainbow trout (*Oncorhynchus mykiss*) thawed with different methods by principal component analysis. Iran Agricultural Research, 39(1), 45–58. https://doi.org/10.22099/iar.2020.33677.1354.

Nasopoulou, C., & Zabetakis, I. (2012). Benefits of fish oil replacement by plant originated oils in compounded fish feeds. A review. *LWT*, *47*(2), 217–224. https://doi.org/10.1016/j.lwt.2012.01.018.

Nasopoulou, C., Demopoulos, C. A., & Zabetakis, I. (2012). Effect of freezing on quality of sea bass and gilthead sea bream. European Journal of Lipid Science and Technology, 114, 733–740. https://doi.org/10.1002/ejlt.201100255.

Naylor, R.L., Hardy, R.W., Buschmann, A.H. Bush, S.R., Cao, L., Klinger, D. H., Little, D. C., Lubchenco, J., Shumway, S. E., & Troell, M. (2021). A 20-year retrospective review of global aquaculture. Nature 591, 551–563. https://doi.org/10.1038/s41586-021-03308-6.

Ochini, Y. & Ozawa, H. (2020). Biochemical and physicochemical characteristics of the major muscle proteins from fish and shellfish. Fisheries Science, 86, 729–740. https://doi.org/10.1007/s12562-020-01444-y.

OECD/FAO. (2022). *OECD Agriculture Statistics*. OECD-FAO Agricultural Outlook OECD Agriculture Statistics (Database). https://doi.org/10.1787/agr-data-en.

Oliveira, H., Muniz, J.A., Bandarra, N.M., Castanheira, I., Coelho, I.R., Delgado, I., Gonçalves, S., Lourenço, H.M., Motta, C., Duarte, M.P., Nunes, M.L., & Gonçalves, A. (2019). Effects of industrial boiling on the nutritional profile of common octopus (*Octopus vulgaris*). Foods, 8(9), 1–15. https://doi.org/10.3390/foods8090411.

Pacitti, D., Lawan, M. M., Sweetman, J., Martin, S. A. M., Feldmann, J., & Secombes, C. J. (2015). Selenium Supplementation in Fish: A Combined Chemical and Biomolecular Study to Understand Sel-Plex Assimilation and Impact on Selenoproteome Expression in Rainbow Trout (*Oncorhynchus mykiss*). PLOS ONE, 10(5), e0127041. https://doi.org/10.1371/journal.pone.0127041.

Peacock, B., 2013. Animal feed contamination effects on livestock and food safety (2010). In: Fink-Gremmels, J., Woodhead (Eds.), Publishing Ltd., 80 High Street, Sawston, Cambridge CB22 3HJ, UK. Int J Dairy Technol, vol. 66. pp. 302–303. https://doi.org/10.1111/1471-0307.12012.

Pedrero, Z., Madrid, Y., & Cámara, C. (2006). Selenium Species Bioaccessibility in Enriched Radish (*Raphanus sativus*): A Potential Dietary Source of Selenium. Journal of Agricultural and Food Chemistry, 54(6), 2412–2417. https://doi.org/10.1021/jf052500n.

Pereira, H., Silva, J., Santos, T., Gangadhar, K. N., Raposo, A., Nunes, C., Coimbra, M. A., Gouveia, L., Barreira, L., & Varela, J. (2019). Nutritional Potential and Toxicological Evaluation of *Tetraselmis* sp. CTP4 Microalgal Biomass Produced in Industrial Photobioreactors. Molecules, 24(17), 3192. https://doi.org/10.3390/molecules24173192.

Pereira, L. (2011). A Review of the Nutrient Composition of Selected Edible Seaweeds. In V. H. Pomin (Ed.), Seaweed: Ecology, Nutrient Composition and Medicinal Uses (pp. 15–47). Nova Science Publishers, Inc. https://www.emerald.com/insight/content/doi/10.1108/NFS-07-2014-0063/full/html.

Pessanha, S., Carvalho, M., Carvalho, M. L., & Dias, A. (2016). Quantitative analysis of human remains from 18th–19th centuries using X-ray fluorescence techniques: The mysterious high content of mercury in hair. Journal of Trace Elements in Medicine and Biology, 33, 26–30. https://doi.org/10.1016/J.JTEMB.2015.08.004.

Petricorena, Z. C. (2015). Handbook of Food Chemistry. In P. C. K. Cheung (Ed.), Handbook of Food Chemistry. Springer, Berlin, Heidelberg, 1–28. https://doi.org/10.1007/978-3-642-41609-5.

Pinkaew, S., & Karrila, T. T. (2015). Key properties of iodine-, iron- and zinc- fortified fish cracker: effects of ambient shelf storage on iodine retention and quality indicators. International Journal of Food Science & Technology, 50(9), 1979–1987. https://doi.org/10.1111/ijfs.12871.

Popelka, P., Marcinčák, S., Maskal'ová, I., Guothová, L., & Čertík, M. (2014). Comparison of the chemical composition and nutritional values of fresh and frozen rainbow trout. Slovenian Veterinary Research, 51(2), 73–80.

Portuguese Committee for Standardization. (2002). NP 4355:2002. Produtos da pesca e da aquicultura. Determinação do peso líquido escorrido e do teor em água de vidragem dos produtos congelados/ultracongelados, vidrados. Instituto Português da Qualidade, Caparica, Portugal, 6pp.

Prego, R., Vázquez, M., Cobelo-García, A., & Aubourg, S. P. (2020). Macroelements and Trace Elements Content in Brine-Canned Mackerel (*Scomber colias*) Subjected to High-Pressure Processing and Frozen Storage. Foods, 9(12), 1868. https://doi.org/10.3390/foods9121868.

Prego, R., Martínez, B., Cobelo-García, A., & Aubourg, S. P. (2021). Effect of High-Pressure Processing and Frozen Storage Prior to Canning on the Content of Essential and Toxic Elements in Mackerel. Food and Bioprocess Technology, 14, 1555–1565. https://doi.org/10.1007/s11947-021-02657-4.

Ralston, N. V. C., Ralston, C. R., & Raymond, L. J. (2016). Selenium Health Benefit Values: Updated Criteria for Mercury Risk Assessments. Biological Trace Element Research, 171(2), 262–269. https://doi.org/10.1007/s12011-015-0516-z.

Ramalho Ribeiro, A., Gonçalves, A., Colen, R., Nunes, M. L., Dinis, M. T., & Dias, J. (2015). Dietary macroalgae is a natural and effective tool to fortify gilthead seabream fillets with iodine: Effects on growth, sensory quality and nutritional value. Aquaculture, 437, 51–59. https://doi.org/10.1016/j.aquaculture.2014.11.028.

Ramalho Ribeiro, A., Gonçalves, A., Bandarra, N., Nunes, M. L., Dinis, M. T., Dias, J., & Rema, P. (2017). Natural fortification of trout with dietary macroalgae and selenised-yeast increases the nutritional contribution in iodine and selenium. Food Research International, 99, 1103–1109. https://doi.org/10.1016/j.foodres.2016.10.030.

Ramalho Ribeiro, A., Altintzoglou, T., Mendes, J., Nunes, M. L., Dinis, M. T., & Dias, J. (2019). Farmed fish as a functional food: Perception of fish fortification and the influence of origin – Insights from Portugal. Aquaculture, 501, 22–31. https://doi.org/10.1016/j.aquaculture.2018.11.002.

Ramos, A., Bandarra, N. M., Rema, P., Vaz-Pires, P., Nunes, M. L., Andrade, A. M., Cordeiro, A. R., & Valente, L. M. P. (2008). Time course deposition of conjugated linoleic acid in market size rainbow trout (*Oncorhynchus mykiss*) muscle. Aquaculture, 274(2–4), 366–374. https://doi.org/10.1016/j.aquaculture.2007.11.040.

Rastogi, S. C., Clausen, J., & Srivastava, K. C. (1976). Selenium and lead: Mutual detoxifying effects. Toxicology, 6(3), 377–388. https://doi.org/10.1016/0300-483X(76)90041-X.

Reboredo, F.H., Barbosa, A., Silva, M.M. Carvalho, M.L., Santos, J.P., Pessoa, M.F., Lindon, F., Ramalho, J.C., & Guerra, M. (2020). Mineral Content of Food Supplements of Plant Origin, by Energy Dispersive X-ray Fluorescence: A Risk Assessment. Exposure and Health 12, 917–927. https://doi.org/10.1007/s12403-020-00354-9.

Rehbein, H., & Oehlenschläger, J. (2009). Basic facts and figures. In H. Rehbein & J. Oehlenschläger (Eds.), Fishery Products: Quality, Safety and Authenticity (pp. 1–18). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781444322668.

Rider, S. A., Davies, S. J., Jha, A. N., Fisher, A. A., Knight, J., & Sweetman, J. W. (2009). Supra-nutritional dietary intake of selenite and selenium yeast in normal and stressed rainbow trout (*Oncorhynchus mykiss*): Implications on selenium status and health responses. Aquaculture, 295(3–4), 282–291. https://doi.org/10.1016/j.aquaculture.2009.07.003.

Rizzi, L., Bargellini, A., Borella, P., & Mordenti, A. (2005). The role of selenium and iodine in controlling some egg minerals. Italian Journal of Animal Science, 4, 504–506. https://doi.org/10.4081/ijas.2005.2s.504.

Rodríguez P., H., & Rojas M., S. (2014). Efecto de dietas enriquecidas con vitamina e y selenio orgánico en el comportamiento productivo y calidad funcional del filete de trucha arco iris (*Oncorhynchus mykiss*). Revista de Investigaciones Veterinarias Del Perú, 25(2), 213–225. https://doi.org/10.15381/rivep.v25i2.8494.

Romotowska, P. E., Gudjónsdóttir, M., Karlsdóttir, M. G., Kristinsson, H. G., & Arason, S. (2017). Stability of frozen Atlantic mackerel (*Scomber scombrus*) as affected by temperature abuse during transportation. LWT - Food Science and Technology, 83, 275–282. https://doi.org/10.1016/j.lwt.2017.05.024.

Roohinejad, S., Koubaa, M., Barba, F. J., Saljoughian, S., Amid, M., & Greiner, R. (2017). Application of seaweeds to develop new food products with enhanced shelf-life, quality and health-related beneficial properties. *Food Research International*, *99*, 1066–1083. https://doi.org/10.1016/j.foodres.2016.08.016.

Rosa, R., Andrade, A. M., Bandarra, N. M., & Nunes, M. L. (2010). Physiological and biochemical effects of conjugated linoleic acid and its use in aquaculture. Reviews in Aquaculture, 2(2), 59–72. https://doi.org/10.1111/j.1753-5131.2010.01021.x.

Rotllant, J., Redruello, B., Guerreiro, P. M., Fernandes, H., Canario, A. V. M., & Power, D. M. (2005). Calcium mobilization from fish scales is mediated by parathyroid hormone related protein via the parathyroid hormone type 1 receptor. Regulatory Peptides, 132(1–3), 33–40. https://doi.org/10.1016/J.REGPEP.2005.08.004.

Saltzman, A., Birol, E., Bouis, H. E., Boy, E., De Moura, F. F., Islam, Y., & Pfeiffer, W. H. (2013). Biofortification: Progress toward a more nourishing future. Global Food Security, 2(1), 9–17. https://doi.org/10.1016/j.gfs.2012.12.003.

Santos, H. M., de Higuera, J. M., & Nogueira, A. R. (2022). *In vitro* bioaccessibility of essential minerals from raw and cooked Tilapia fillet: Method validation and analysis by synchronous vertical dual view ICP OES. Food Chemistry Advances, 1, 100080. https://doi.org/10.1016/j.focha.2022.100080.

Sargent, J. R., Tocher, D. R., & Bell, J. G. (2003). The Lipids. *In* J. E. Halver & R. W. Hardy (Eds.), Fish Nutrition (3 eds, pp. 181–257). Elsevier. https://doi.org/10.1016/B978-012319652-1/50005-7.

Schmid, S., Ranz, D., He, M. L., Burkard, S., Lukowicz, M. V., Reiter, R., Arnold, R., Le Deit, H., David, M., & Rambeck, W. A. (2003). Marine algae as natural source of iodine in the feeding of freshwater fish - A new possibility to improve iodine supply of man. Revue de Medecine Veterinaire, 154(10), 645–648.

Schormüller, J. (1969). Fette und Lipoide (Lipids). *In*: Acker, L., Bergner, K.-G., Diemair, W., Heimann, W., Kiermeier, F., Schormüller, J., Souci, S.W. (Eds.), Handbuch der Lebensmittel Chemie. Springer Verlag, Berlin, Germany, pp. 872–878.

Schram, E., Schelvis-Smit, R. A. A. M., Van Der Heul, J. W., & Luten, J. B. (2010). Enrichment of the African catfish *Clarias gariepinus* (Burchell) with functional selenium originating from garlic: effect of enrichment period and depuration on total selenium level and sensory properties. Aquaculture Research, 41(6), 793–803. https://doi.org/10.1111/j.1365-2109.2009.02356.x.

Schrauzer, G. N. (2009). Selenium and selenium-antagonistic elements in nutritional cancer prevention. Critical Reviews in Biotechnology, 29(1), 10–17. https://doi.org/10.1080/07388550802658048.

Schubring, R. (2005). Changes in texture, water holding capacity, colour and thermal stability of frozen cod (*Gadus morhua*) fillets: Effect of frozen storage temperature. Deutsche Lebensmittel-Rundschau, 101(11), 484–493.

Shaltout, A. A., Harfouche, M., Ali, S. S. M., Karydas, A. G., Kregsamer, P., Wobrauschek, P., Streli, C., Abd-Elkader, O. H., Yassin, M. A., & El Orabi, N. F. (2020). Elemental composition and source apportionment of atmospheric aerosols collected from urban and residential areas of Jordan using multi-secondary targets energy dispersive X-ray fluorescence. Spectrochimica Acta Part B: Atomic Spectroscopy, 170, 105900. https://doi.org/10.1016/j.sab.2020.105900.

Sharaf, M. M. (2013). Influence of domestic freezing on the biochemical composition and mineral contents of fish muscles. Egyptian Academic Journal of Biological Sciences B Zoology, 5(1), 11–16. https://doi.org/10.21608/eajbsz.2013.13506.

Sherman, J. (1955). The theoretical derivation of fluorescent X-ray intensities from mixtures. Spectrochimica Acta, 7(C), 283–306. https://doi.org/10.1016/0371-1951(55)80041-0.

Sidari, R., & Tofalo, R. (2019). A Comprehensive Overview on Microalgal-Fortified/Based Food and Beverages. Food Reviews International, 35 (8), 778–805. https://doi.org/10.1080/87559129.2019.1608557.

Sikorski, Z. E., & Sun Pan, B. (1994). Preservation of seafood quality. In F. Shahidi & J. R. Botta (Eds.), Seafoods: Chemistry, Processing Technology and Quality (pp. 168–195). Springer US. https://doi.org/10.1007/978-1-4615-2181-5_10.

Siró, I., Kápolna, E., Kápolna, B., & Lugasi, A. (2008). Functional food. Product development, marketing and consumer acceptance — A review. Appetite, 51(3), 456–467. https://doi.org/10.1016/j.appet.2008.05.060.

Soares, N. M., Mendes, T. S., & Vicente, A. A. (2013). Effect of chitosan-based solutions applied as edible coatings and water glazing on frozen salmon preservation - A pilot-scale study. Journal of Food Engineering, 119(2), 316–323. https://doi.org/10.1016/j.jfoodeng.2013.05.018.

Sobral, M. M. C., Cunha, S. C., Faria, M. A., & Ferreira, I. M. (2018). Domestic Cooking of Muscle Foods: Impact on Composition of Nutrients and Contaminants. Comprehensive Reviews in Food Science and Food Safety, 17(2), 309–333. https://doi.org/10.1111/1541-4337.12327.

Sterniša, M., Dvořak, P., Lunda, R., Linhartova, Z., Možina, S. S., & Mraz, J. (2018). Bleeding of common carp (*Cyprinus carpio*) improves sensory quality of fillets and slows oxidative and microbiological changes during refrigerated aerobic storage. Food Technology and Biotechnology, 56(4), 524–532. https://doi.org/10.17113/ftb.56.04.18.5792.

Subbaiah, K., Majumdar, R. K., Choudhury, J., Priyadarshini, B. M., Dhar, B., Roy, D., Saha, A., & Maurya, P. (2015). Protein Degradation and Instrumental Textural Changes in Fresh Nile Tilapia (*Oreochromis niloticus*) during Frozen Storage. Journal of Food Processing and Preservation, 39, 2206–2214. https://doi.org/10.1111/jfpp.12465.

Szczepanik, G., Kryza, K., & Ostrowska, M. (2010). Influence of method of packing on physicochemical and textural changes in atlantic herring during frozen storage. Acta Scientiarum Polonorum: Technologia Alimentaria, 9(4), 425–440.

Tan, M., Li, P., Yu, W., Wang, J., & Xie, J. (2019). Effects of Glazing with Preservatives on the Quality Changes of Squid during Frozen Storage Mingtang. Applied Sciences, 9, 3847. https://doi.org/10.3390/app9183847.

Tilman, D., & Clark, M. (2014). Global diets link environmental sustainability and human health. *Nature*, 515, 518–522. https://doi.org/10.1038/nature13959Tocher, D. R. (2015). Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. Aquaculture, 449, 94–107. https://doi.org/10.1016/j.aquaculture.2015.01.010.

Todorov, T. I., & Gray, P. J. (2016). Analysis of iodine in food samples by inductively coupled plasma-mass spectrometry. PhyFood Addit Contam Part A Chem Anal Control Expo Risk Assess, 33(2), 282–290. https://doi.org/10.1080/19440049.2015.1131337.

Tolstorebrov, I., Eikevik, T. M., & Bantle, M. (2016). Effect of low and ultra-low temperature applications during freezing and frozen storage on quality parameters for fish. International Journal of Refrigeration, 63, 37–47. https://doi.org/10.1016/j.ijrefrig.2015.11.003.

Tontisirin, K., Nantel, G., & Bhattacharjee, L. (2002). Food-based strategies to meet the challenges of micronutrient malnutrition in the developing world. Proceedings of the Nutrition Society, 61 (2), 243–250. https://doi.org/10.1079/pns2002155.

Torres-Escribano, S., Denis, S., Blanquet-Diot, S., Calatayud, M., Barrios, L., Vélez, D., Alric, M., & Montoro, R. (2011). Comparison of a static and a dynamic in vitro model to estimate the bioaccessibility of As, Cd, Pb and Hg from food reference materials *Fucus* sp. (IAEA-140/TM) and Lobster hepatopancreas (TORT-2). Science of The Total Environment, 409, 604–611. https://doi.org/10.1016/j.scitotenv.2010.10.021.

Tsironi, T. N., Stoforos, N. G., & Taoukis, P. S. (2020). Quality and Shelf-Life Modeling of Frozen Fish at Constant and Variable Temperature Conditions. Foods, 9, 1893. https://doi.org/10.3390/foods9121893.

United Nations. (2020). The sustainable development goals report 2020, The sustainable development goals, United Nations Publications. https://doi.org/10.29171/azu_acku_pamphlet_k3240_s878_2016 (accessed 18 December 2020).

UN Nutrition. (2021). The role of aquatic foods in sustainable healthy diets. Discussion Paper. Rome, FAO. www.unnutrition.org/wp-content/uploads/FINAL-UN-Nutrition-Aquatic-foods-Paper_EN_.pdf.

Urala, N., & Lähteenmäki, L. (2007). Consumers' changing attitudes towards functional foods. Food Quality and Preference, 18(1), 1–12. https://doi.org/10.1016/j.foodqual.2005.06.007.

USDA. (2008). Composition of foods raw, processed, prepared. In USDA National Nutrient Database for Standard Reference, Release 27. http://www.ars.usda.gov/ba/bhnrc/ndl (accessed 1 December 2020).

USDA. (2020). *Dietary Guidelines for Americans 2015-2020* (8 eds). U.S. Department of Health and Human Services and U.S. Department of Agriculture. http://health.gov/dietaryguidelines/2015/guidelines/.

Vågsholm, I., Shah Arzoomand, N., & Boqvist, S. (2020). Food Security, Safety, and Sustainability - Igg the Trade-Offs Right. Frontiers in Sustainable Food Systems, 4, 16. https://doi.org/10.3389/fsufs.2020.00016.

Valente, L. M. P., Gouveia, A., Rema, P., Matos, J., Gomes, E. F., & Pinto, I. S. (2006). Evaluation of three seaweeds *Gracilaria bursa-pastoris*, *Ulva rigida* and *Gracilaria cornea* as dietary ingredients in European sea bass (*Dicentrarchus labrax*) juveniles. Aquaculture, 252(1), 85–91. https://doi.org/10.1016/j.aquaculture.2005.11.052.

Valente, L. M. P., Rema, P., Ferraro, V., Pintado, M., Sousa-Pinto, I., Cunha, L. M., Oliveira, M. B., & Araújo, M. (2015). Iodine enrichment of rainbow trout flesh by dietary supplementation with the red seaweed Gracilaria vermiculophylla. Aquaculture, 446, 132–139. https://doi.org/10.1016/j.aquaculture.2015.05.004.

Valente, L. M. P., Rema, P., Ferraro, V., Pintado, M., Sousa-Pinto, I., Cunha, L. M., Oliveira, M. B., & Araújo, M. (2015). Iodine enrichment of rainbow trout flesh by dietary supplementation with the red seaweed *Gracilaria vermiculophylla*. *Aquaculture*, *446*, 132–139. https://doi.org/10.1016/j.aquaculture.2015.05.004.

van Grieken, R., & Margui, E. (2013). X-Ray Fluorescence Spectrometry and Related Techniques: an introduction. Momentum Press. https://doi.org/10.5643/9781606503935.

Van Paemel, M., Dierick, N., Janssens, G., Fievez, V., & De Smet, S. (2010). Selected trace and ultratrace elements: Biological role, content in feed and requirements in animal nutrition – Elements for risk assessment. EFSA Supporting Publications, 7(7):1132. https://doi.org/10.2903/sp.efsa.2010.EN-68.

Vanhaecke, L., Verbeke, W., & De Brabander, H. F. (2010). Glazing of frozen fish: Analytical and economic challenges. Analytica Chimica Acta, 672, 40–44. https://doi.org/10.1016/j.aca.2010.03.045.

Verbeke, W., Sioen, I., Pieniak, Z., Van Camp, J., & De Henauw, S. (2005). Consumer perception versus scientific evidence about health benefits and safety risks from fish consumption. Public Health Nutrition, 8(4), 422–429. https://doi.org/10.1079/PHN2004697.

Versantvoort, C. H. M., Oomen, A. G., Van De Kamp, E., Rompelberg, C. J. M., & Sips, A. J. A. M. (2005). Applicability of an *in vitro* digestion model in assessing the bioaccessibility of mycotoxins from food. Food and Chemical Toxicology, 43(1), 31–40. https://doi.org/10.1016/j.fct.2004.08.007.

Vicente-Zurdo, D., Gómez-Gómez, B., Pérez-Corona, M. T., & Madrid, Y. (2019). Impact of fish growing conditions and cooking methods on selenium species in swordfish and salmon fillets. Journal of Food Composition and Analysis, 83, 103275. https://doi.org/10.1016/j.jfca.2019.103275.

Wang, J., Yu, W., & Xie, J. (2020). Effect of Glazing with Different Materials on the Quality of Tuna During Frozen Storage. Foods, 9(231), 1–16. https://doi.org/10.3390/foods9020231.

WHO. (2013). Salt Reduction and Iodine Fortification Strategies in Public Health; Report of a Joint Technical Meeting Convened by the World Health Organization and the George Institute for Global Health in Collaboration with the International Council for the Control of Iodine Deficiency Disorders Global Network, World Health Organization WHO, Sydney, Australia, 2013. https://apps.who.int/iris/bitstream/handle/10665/101509/9789241506694_eng.pdf.

WHO. (2021). WHO Global Anaemia estimates, 2021 Edition. WHO Global Anaemia Estimates, 2021 Edition. https://www.who.int/data/gho/data/themes/topics/anaemia_in_women_and_children.

Wolfe, J., & Bryant, G. (2001). Cellular cryobiology: thermodynamic and mechanical effects. International Journal of Refrigeration, 24, 438–450. https://doi.org/10.1016/S0140-7007(00)00027-X.

Wu, P., & Chen, X. D. (2021). Validation of in vitro bioaccessibility assays — a key aspect in the rational design of functional foods towards tailored bioavailability. Current Opinion in Food Science, 39, 160-170. https://doi.org/10.1016/j.cofs.2021.03.002.

Zand, N., Christides, T., & Loughrill, E. (2015). Dietary intake of minerals. In M. de la Guardia & S. Garrigues (Eds.), Handbook of Mineral Elements in Food (eds, pp. 23–39). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118654316.ch2.

Zang, J., Xu, Y., Xia, W., & Jiang, Q. (2017). The impact of desmin on texture and water-holding capacity of ice-stored grass carp (Ctenopharyngodon idella) fillet. International Journal of Food Science and Technology, 52(2), 464–471. https://doi.org/10.1111/ijfs.13302.

Zwolak, I. (2020). The Role of Selenium in Arsenic and Cadmium Toxicity: an Updated Review of Scientific Literature. Biological Trace Element Research, 193(1), 44–63. https://doi.org/10.1007/s12011-019-01691-w.

A

ANNEXES

Annex I

SUPPLEMENTARY INFORMATION FOR CHAPTER 2

Enriched feeds with iodine and selenium from natural and sustainable sources to modulate farmed gilthead seabream (*Sparus aurata*) and common carp (*Cyprinus carpio*) fillets elemental nutritional value

Table S. 2. 1 - Ingredients and proximate composition (%) of the experimental diets (CTR - control, B1 – biofortified B1, B2 - biofortified B2, B3 - biofortified B3) for gilthead seabream (*S. aurata*)

Ingredients (%)	CTR	B1	B2	B3
Fishmeal 70 [1]	15.000	10.000	10.00	10.000
Fish protein concentrate[2]	2.500	2.500	2.500	2.500
Porcine blood meal [3]	2.500	2.500	2.500	2.500
Microalgae meal (*Tetraselmis* sp.) [4]	-	0.500	0.500	0.500
Microalgae meal (*Chlorella* sp.) [5]	-	5.000	5.000	5.000
Microalgae meal (*Schizochytrium* sp.) 6	-	3.200	3.200	3.200
Soy protein concentrate [7]	17.000	17.000	17.000	17.000
Corn gluten meal [8]	8.000	8.000	8.000	8.000
Soybean meal 48 [9]	8.000	8.000	8.000	8.000
Wheat meal [10]	16.600	14.425	14.435	14.015
Wheat glúten [11]	12.000	12.000	12.000	12.000
Fish oil [12]	5.450	4.360	5.450	5.450
Soybean oil [13]	2.805	2.490	2.160	2.160
Rapeseed oil [13]	5.610	4.980	4.320	4.320
Linseed oil [13]	0.935	0.830	0.720	0.720
Vitamins and minerals premix [14]	1.100	1.100	1.100	1.100
Binder [15]	1.000	1.000	1.000	1.000
Macroalgae meal (*Laminaria digitata*) [16]	-	0.400	0.400	0.800
Antioxidant [17]	0.200	0.200	0.200	0.200
Sodium propionate [18]	0.100	0.100	0.100	0.100
Monoammonium phosphate [19]	0.500	0.500	0.500	0.500
Selenised yeast [20]	-	0.015	0.015	0.035
L-Taurine [21]	0.400	0.500	0.500	0.500
L-Tryptophan [22]	0.100	0.100	0.100	0.100
DL-Methionine [23]	0.200	0.300	0.300	0.300

[1]CONRESA 70: 47.4% crude protein (CP), 817.5% crude fat (CF), Conserveros Reunidos S.A., Spain; [2] [3]Porcine blood meal: 89% CP, 1% CF, SONAC BV, The Netherlands; [4]Tetraselmis meal: 72% CP, 1% CF, Willows Ingredients Ltd, Ireland; [5]Chlorella meal: 62% CP, 9% CF, ALLMICROALGAE, Portugal; [6]ALL-G RICH (Schizochytrium), Alltech Portugal; [7]Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands; [8]Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal; [9]Solvent extracted soybean meal: 43.8% CP, 3.3% CF, CARGILL, Spain; [10]Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal; [11] Wheat glúten:; [12]Fish oil; [13]Henry Lamotte Oils GmbH, Germany; 14INVIVONSA Portugal SA, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 500mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate,7.5mg; sodium chloride, 400mg; excipient wheat middling's; [15]CELATOM FP1SL (diatomite), Angelo Coimbra S.A., Portugal; [16]Dry Laminaria digitata: 5.4% CP, 0.5% CF, 3700 mg iodine/kg, Agrimer, France; [17]VER-DILOX, Kemin Europe NV, Belgium; [18]PREMIX LDA., Portugal; [19] [20]ALKOSEL R397: 2200 mg selenium/kg, Lallemand, France; [21]L-Taurine; [22]TrypAMINO 98%, Evonik Nutrition & Care GmbH, Germany; [23]DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany.

Table S. 2. 2 - Ingredients and proximate composition (%) of the experimental diets (CTR - control, B1 – biofortified B1, B2 - biofortified B2, B3 - biofortified B3) for common carp (*C. carpio*)

Ingredients (%)	CTR	B1	B2	B3
Fishmeal 60 [1]	5.000	2.500	2.500	2.500
Porcine blood meal [2]	2.000	2.000	2.000	2.000
Microalgae meal (*Spirulina* sp.) [3]	-	1.000	1.000	1.000
Microalgae meal (*Chlorella* sp.) [4]	-	1.000	1.000	1.000
Microalgae meal (*Schizochytrium* sp.) [5]	-	3.125	1.563	-
Soy protein concentrate [6]	2.500	2.500	2.500	2.500
Corn gluten meal [7]	4.000	4.000	4.000	4.000
Soybean meal 44 [8]	25.000	25.000	25.000	25.000
Rapeseed meal [9]	7.000	7.000	7.000	7.000
Sunflower meal [10]	12.500	12.500	12.500	12.500
Wheat meal [11]	22.500	21.224	21.786	22.349
Wheat bran [12]	5.000	5.000	5.000	5.000
Corn meal [13]	2.500	2.500	2.500	2.500
Salmon oil [14]	-	-	-	2.100
Soybean oil [15]	3.000	-	-	2.000
Rapeseed oil [15]	3.000	4.100	5.100	2.000
Vitamins and minerals premix [16]	1.000	1.000	1.000	1.000
Betaine HCl [17]	0.100	0.100	0.100	0.100
Binder [18]	1.000	1.000	1.000	1.000
Macroalgae meal (*Laminaria digitata*) [19]	-	0.541	0.541	0.541
Antioxidant [20]	0.200	0.200	0.200	0.200
Sodium propionate [21]	0.100	0.100	0.100	0.100
Sodium phosphate [22]	2.100	2.100	2.100	2.100
Selenised yeast [23]	-	0.010	0.010	0.010
L-Lysine [24]	0.700	0.700	0.700	0.700
L-Tryptophan [25]	0.200	0.200	0.200	0.200
DL-Methionine [26]	0.600	0.600	0.600	0.600

1 CONRESA 60: 61.2% crude protein (CP), 8.4% crude fat (CF), Conserveros Reunidos S.A., Spain; 2 Porcine blood meal: 89% CP, 1% CF, SONAC BV, The Netherlands; 3 Spirulina meal: 72% CP, 1% CF, Willows Ingredients Ltd, Ireland; 4 Chlorella meal: 62% CP, 9% CF, ALLMICROALGAE, Portugal; 5 ALL-G RICH (Schizochytrium), Alltech Portugal; 6 Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands; 7 Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal; 8 Solvent extracted soybean meal: 43.8% CP, 3.3% CF, CARGILL, Spain; 9 Defatted rapeseed meal: 32.7% CP, 4.1% CF, Ribeiro & Sousa Lda, Portugal; 10 Defatted sunflower meal: 29.1% CP, 1.8% CF, Ribeiro & Sousa Lda, Portugal; 11 Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal; 12 Wheat bran: 14.9% CP, 4.0% CF, Cerealis Moagens S.A., Portugal; 13 Corn meal: 8% CP, 3.7% CF, Ribeiro & Sousa Lda, Portugal; 14 Sopropêche, France; 15 Henry Lamotte Oils GmbH, Germany; 16 INVIVONSA Portugal SA, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 500mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate,7.5mg; sodium chloride, 400mg; excipient wheat middling's; 17 ORFFA, The Netherlands; 18 CELATOM FP1SL (diatomite), Angelo Coimbra S.A., Portugal; 19 Dry Laminaria digitata: 5.4% CP, 0.5% CF, 3700 mg iodine/kg, Agrimer, France; 20 VERDILOX, Kemin Europe NV, Belgium; 21 PREMIX LDA., Portugal; 22 Vadequimica, Spain; 23 ALKOSEL R397: 2200 mg selenium/kg, Lallemand, France; 24 L-Lysine HCl 99%: Ajinomoto Eurolysine SAS, France; 25 TrypAMINO 98%, Evonik Nutrition & Care GmbH, Germany; 26 DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany.

Table S. 2. 3 - Gilthead seabream (*S. aurata*) and common carp (*C. carpio*) initial (Baseline) and final (in each treatment: CTR, B1, B2, B3) total length (cm) and weight (g)

Species	Treatment	n	Total length (cm)	Total weight (g)	Moisture (%)
Gilthead seabream	Baseline	15	31 ± 2 (28 - 34)	491 ± 68 (380 – 584)	69 ± 3.9
	CTR	15	33 ± 1 (31 - 36)	578 ± 70 (483 – 692)	69 ± 0.6
	B1	15	32 ± 1 (30 - 35)	531 ± 74 (427 – 664)	68 ± 0.5
	B2	15	33 ± 2 (30 - 35)	574 ± 70 (460 – 677)	69 ± 1.1
	B3	15	33 ± 2 (30 - 36)	578 ± 62 (463 – 666)	69 ± 0.9
Common carp	Baseline	15	29 ± 3(26 – 37)	333 ± 44 (250 – 400)	78 ± 0.3
	CTR	15	40 ± 2 (37 – 43)	1236 ± 108 (1027 – 1443)	78 ± 1.0
	B1	15	41 ± 1 (40 – 42)	1226 ± 106 (1095 – 1397)	78 ± 0.3
	B2	15	40 ± 2 (37 – 42)	1217 ± 105 (1045 – 1440)	77 ± 1.3
	B3	15	41 ± 2 (37 – 43)	1338 ± 112 (1133 – 1493)	78 ± 0.7

Treatment, baseline (initial) and at the end of the feeding trial (final) in control diet (CTR) and three different fortified diets (B1 - biofortified B1, B2 - biofortified B2, B3 - biofortified B3); n, number of specimens analysed; total length (cm) and total weight (g) – mean ± SD (range minimum and maximum).

Table S. 2. 4 - ICP-MS operating conditions

Pole Bias	- 0.1
Hexapole Bias	- 3.0
Nebuliser	0.82
Forward power	1404
Horizontal	87
Vertical	389
Cool	13.0
Auxiliary	0.85
Sampling depht	80
Standard resolution	125
High resolution	130
Analogue detector	1770
PC detector	3210

Table S. 2. 5 - Gilthead seabream (*S. aurata*) and common carp (*C. carpio*) fillets initial (Baseline) and final (CTR) elemental composition.

	I (µg g^{-1})	Se (µg g^{-1})	Fe (µg g^{-1})	Zn (µg g^{-1})	Br (µg g^{-1})	Cu (µg g^{-1})	As (µg g^{-1})	Hg (µg g^{-1})	Cd (µg g^{-1})	Pb (µg g^{-1})	Cl (g 100 g^{-1})	K (g 100 g^{-1})	Ca (g 100 g^{-1})
Gilthead seabream													
Baseline	0.06 ± 0.004	0.18 ± 0.01	7.4 ± 1.6	0.9 ± 0.1	3.3 ± 0.2	2.4 ± 0.04	1.8 ± 0.06	0.1 ± 0.006	0.01 ± 0.002	0.06 ± 0.001	0.2 ± 0.01[a]	1.7 ± 0.3	0.07 ± 0.004
CTR	0.07 ± 0.003	0.18 ± 0.002	7.1 ± 0.5	1.0 ± 0.1	3.1 ± 0.4	2.0 ± 0.02	1.8 ± 0.1	0.1 ± 0.008	0.02 ± 0.001	0.07 ± 0.003	0.4 ± 0.03[b]	1.2 ± 0.02	0.07 ± 0.02
Common carp													
Baseline	0.01 ± 0.001	0.07 ± 0.002[a]	10 ± 1.5	9 ± 0.4[a]	1.1 ± 0.3[a]	1.3 ± 0.1[a]	0.03 ± 0.002[a]	< LOQ[a]	0.01 ± 0.002	0.08 ± 0.01	0.09 ± 0.03	0.8 ± 0.01	0.04 ± 0.0005[a]
CTR	0.02 ± 0.001	0.09 ± 0.005[b]	15 ± 1.6	11 ± 1.2[b]	4.8 ± 0.1[b]	8 ± 0.4[b]	0.08 ± 0.004[b]	0.02 ± 0.001[b]	0.01 ± 0.002	0.08 ± 0.02	0.09 ± 0.004	0.9 ± 0.06	0.13 ± 0.01[b]

Different letters (a, b) represents significant differences ($p < 0.05$) between fish fillets from baseline (initial) and in the final of the experimental feeding trial with control (CTR) diet. Values are average ± standard deviation in wet weight

Annex II

SUPPLEMENTARY INFORMATION FOR CHAPTER 3

Effects of steaming on health-valuable nutrients from fortified farmed fish: gilt-head seabream (*Sparus aurata*) and common carp (*Cyprinus carpio*) as case studies.

Table S. 3. 1 - Gilthead seabream (*S. aurata*) and common carp (*C. carpio*) biometric information moisture (%) content before and after the feeding trial.

	n	Total length (cm)	Total weight (g)	Muscle fillet					
				Raw weight (g)	Moisture Raw (%)	Moisture Steam (%)	Steam weight (g)	Weight loss (%)	CY (%)
Gilthead seabream									
Baseline	15	31 ± 2	491 ± 68	n.d.	69 ± 4$^{\#}$	n.d.	n.d.	n.d.	n.d.
CTR	15	33 ± 1	549 ± 52	96 ± 19	69 ± 1$^{\#}$	68 ± 0$^{\#}$	83 ± 17	14 ± 2$^{\#}$	86 ± 2$^{\#}$
BF1	15	32 ± 1	517 ± 55	92 ± 18	68 ± 0$^{\#}$	68 ± 1	80 ± 17	13 ± 2$^{\#}$	87 ± 3$^{\#}$
BF2	15	33 ± 2	525 ± 49	98 ± 14	69 ± 1$^{\#}$	68 ± 1$^{\#}$	84 ± 11	14 ± 2$^{\#}$	84 ± 5$^{\#}$
Common carp									
Baseline	15	29 ± 3	333 ± 44	n.d.	78 ± 0§	n.d.	n.d.	n.d.	n.d.
CTR	15	40 ± 2	1236 ± 108	139 ± 27	75 ± 3§	73 ± 1§	114 ± 22	19 ± 2§	81 ± 2§
BF1	15	39 ± 2	1138 ± 108	90 ± 16	75 ± 1§	70 ± 2§	74 ± 13	19 ± 2§	81 ± 2§
BF2	15	41 ± 2	1338 ± 112	129 ± 44	76 ± 1§	73 ± 0§*	103 ± 35	20 ± 3§	80 ± 3§

n, number of specimens analysed; n.d., not determined; CY, cooking yield; CTR, control diet; BF1, fortified diet B1; BF2, fortified diet B2. The symbols (§, #) indicate significant differences ($p < 0.05$) between species, whereas * represent statistical differences ($p < 0.05$) between raw and steamed fish filets in each treatment.

Annex III

SUPPLEMENTARY INFORMATION FOR CHAPTER 4

Physicochemical properties of iodine and selenium biofortified *Sparus aurata* and *Cyprinus carpio* during frozen storage.

Table S. 4. 1 -Ingredients and proximate composition (%) of the experimental diets (CTR - control, BF – fortified diet) for gilthead seabream (*S. aurata*) and common carp (*C. carpio*).

	Gilthead seabream		Common carp	
Ingredients (%)	**CTR**	**BF**	**CTR**	**BF**
Fishmeal 70 [1]	15.00	10.00	-	-
Fishmeal 60 [2]	-	-	5.00	2.50
Fish protein concentrate [3]	2.50	2.50	-	-
Porcine blood meal [4]	2.50	2.50	2.00	2.00
Microalgae meal (*Tetraselmis* sp.) [5]	-	0.50	-	-
Microalgae meal (*Spirulina* sp.) [6]	-	-	-	1.00
Microalgae meal (*Chlorella* sp.) [7]	-	5.00	-	1.00
Microalgae meal (*Schizochytrium* sp.) [8]	-	3.20	-	-
Soy protein concentrate [9]	17.00	17.00	2.50	2.50
Corn gluten meal [10]	8.00	8.00	4.00	4.00
Soybean meal 48 [11]	8.00	8.00	-	-
Soybean meal 44 [12]	-	-	25.00	25.00
Rapeseed meal [13]	-	-	7.00	7.00
Sunflower meal [14]	-	-	12.50	12.50
Corn meal [15]	-	-	2.50	2.50
Wheat meal [16]	16.60	14.00	22.50	22.40
Wheat glúten [17]	12.00	12.00	-	-
Wheat bran [18]	-	-	5.00	5.00
Fish oil [19]	5.45	5.45	-	-
Salmon oil [20]	-	-	-	2.10
Soybean oil [21]	2.81	2.16	3.00	2.00
Rapeseed oil [21]	5.61	4.32	3.00	2.00
Linseed oil [21]	0.94	0.72	-	-
Vitamins and minerals premix [22]	1.10	1.10	1.00	1.00
Betaine HCl [23]	-	-	0.10	0.10
Binder [24]	1.00	1.00	1.00	1.00
Macroalgae meal (*Laminaria digitata*) [25]	-	0.80	-	0.54
Antioxidant [26]	0.20	0.20	0.20	0.20
Sodium propionate [27]	0.10	0.10	0.10	0.10
Monoammonium phosphate [28]	0.50	0.50	-	-
Sodium phosphate [29]	-	-	2.10	2.10
Selenised yeast [30]	-	0.04	-	0.01
L-Taurine [31]	0.40	0.50	-	-
L-Tryptophan [32]	0.10	0.10	0.20	0.20
DL-Methionine [33]	0.20	0.30	0.60	0.60
L-Lysine [34]	-	-	0.70	0.70
Dry matter (DM), %	7.90 ± 0.00	8.10 ± 0.01	5.30 ± 0.01	8.30 ± 0.02
Crude protein, % DM	46.00 ± 0.10	45.50 ± 0.10	30.20 ± 0.20	30.30 ± 0.10
Crude fat, % DM	17.20 ± 0.10	17.30 ± 0.10	8.10 ± 0.10	8.10 ± 0.20
Ash, % DM	5.30 ± 0.00	5.30 ± 0.01	4.40 ± 0.10	7.20 ± 0.10
Iodine, mg kg^{-1} DM	1.24 ± 0.02	13.3 ±0.2	2.22 ± 0.03	15.60 ± 0.30
Selenium, mg kg^{-1} DM	0.70 ± 0.00	1.28 ± 0.02	0.40 ± 0.01	1.41 ± 0.05

1CONRESA 70: 47.4% crude protein (CP), 817.5% crude fat (CF), Conserveros Reunidos S.A., Spain; 2CONRESA 60: 61.2% crude protein (CP), 8.4% crude fat (CF), Conserveros Reunidos S.A., Spain; 4Porcine blood meal: 89% CP, 1% CF, SONAC BV, The Netherlands; 5Tetraselmis meal: 72% CP, 1% CF, Willows Ingredients Ltd, Ireland; 6Spirulina meal: 72% CP, 1% CF, Willows Ingredients Ltd, Ireland; 7Chlorella meal: 62% CP, 9% CF, ALLMICROALGAE, Portugal; 8ALL-G RICH (Schizochytrium), Alltech Portugal; 9Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands; 10Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal; 11Solvent extracted soybean meal: 43.8% CP, 3.3% CF, CARGILL, Spain; 12Solvent extracted soybean meal: 43.8% CP, 3.3% CF, CARGILL, Spain; 13Defatted rapeseed meal: 32.7% CP, 4.1% CF, Ribeiro & Sousa Lda, Portugal; 14Defatted sunflower meal: 29.1% CP, 1.8% CF, Ribeiro & Sousa Lda, Portugal; 15Corn meal: 8% CP, 3.7% CF, Ribeiro & Sousa Lda, Portugal; 16Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal; 17Wheat glúten; 18Wheat bran: 14.9% CP, 4.0% CF, Cerealis Moagens S.A., Portugal; 19Fish oil; 20Sopropêche; 21Henry Lamotte Oils GmbH, Germany; 22INVIVONSA Portugal SA, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 500mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate,7.5mg; sodium chloride, 400mg; excipient wheat middling's; 23ORFFA, The Netherlands; 24CELATOM FP1SL (diatomite), Angelo Coimbra S.A., Portugal; 25Dry Laminaria digitata: 5.4% CP, 0.5% CF, 3700 mg iodine/kg, Agrimer, France; 26VERDILOX, Kemin Europe NV, Belgium; 27PREMIX LDA., Portugal; 29Vadequimica, Spain; 30ALKOSEL R397: 2200 mg selenium/kg, Lallemand, France; 31L-Taurine; 32TrypAMINO 98%, Evonik Nutrition & Care GmbH, Germany; 33DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany; 34L-Lysine HCl 99%: Ajinomoto Eurolysine SAS, France.

Table S. 4. 2 - Average certificate and measured concentrations (µg g^{-1} DM) and the associated relative standard deviation (RSD) in certified reference materials (CRM). Limit of detection (LOD) and limit of quantification (LOQ) for each element and analytical method

Elements	Analytical method	CRM			LOD	LOQ
		Type	Recovery (%)	RSD (%)	(µg g^{-1})	(µg g^{-1})
As	ICP-MS	ERM®-BB422	94	1.7	0.003	0.013
I*	ICP-MS	ERM®-BB422	88	1.6	0.01 (0.068)	0.03 (0.25)
Se	ICP-MS	ERM®-BB422	91	1.7	0.007	0.025
Cl	EDXRF	SRM 1566	90	5.5	50	150
K	EDXRF	SRM 1566	92	5.6	20	60
Ca	EDXRF	SRM 1566	68	4.9	9.28	27.85
Fe	EDXRF	SRM 1566	99	5.2	0.40	1.21
		DORM-2	124	5.7		
Cu	EDXRF	SRM 1566	100	7.9	0.41	1.23
		DORM-2	98	21.7		
Zn	EDXRF	SRM 1566	101	5.8	0.54	1.62
		DORM-2	117	10.0		
Br	EDXRF	SRM 1566	98	9.3	0.21	0.64

*Iodine values for fish matrix and in parentheses for feed matrix

ICP-MS (Inductively coupled plasma mass spectrometer); EDXRF (energy dispersive X-ray fluorescence spectrometry) ERM®-BB422 (Fish muscle); SRM 1566b (Oyster Tissue); DORM-2 (Dogfish muscle)

Annex IV

SUPPLEMENTARY INFORMATION FOR CHAPTER 5

In Vitro bioaccessibility of macro and trace elements in biofortified and conventional farmed gilthead seabream (*Sparus aurata*) and common carp (*Cyprinus carpio*)

Table S. 5. 1 -Ingredients and proximate composition (%) of experimental diet (BF – biofortified diet) for gilthead seabream (*S. aurata*) and common carp (*C. carpio*).

Ingredients (%)	Gilthead seabream	Common carp
Fishmeal 70 [1]	10.00	-
Fishmeal 60 [2]	-	2.50
Fish protein concentrate [3]	2.50	-
Porcine blood meal [4]	2.50	2.00
Microalgae meal (*Tetraselmis sp.*) [5]	0.03	-
Microalgae meal (*Spirulina sp.*) [6]	-	1.00
Microalgae meal (*Chlorella sp.*) [7]	2.50	1.00
Microalgae meal (*Schizochytrium sp.*) [8]	2.30	-
Soy protein concentrate [9]	17.00	2.50
Corn gluten meal [10]	8.00	4.00
Soybean meal 48 [11]	12.00	-
Soybean meal 44 [12]	-	25.00
Rapeseed meal [13]	-	7.00
Sunflower meal [14]	-	12.50
Corn meal [15]	-	2.50
Wheat meal [16]	13.13	22.33
Wheat gluten [17]	12.50	-
Wheat bran [18]	-	5.00
Fish oil [19]	3.80	-
Salmon oil [20]	-	6.10
Soybean oil [21]	2.30	-
Rapeseed oil [21]	6.10	-
Linseed oil [21]	0.80	-
Vitamins and minerals premix [22]	1.10	1.00
Betaine HCl [23]	-	0.10
Binder [24]	1.00	-
Binder [25]	-	1.00
Macroalgae meal (*Laminaria digitata*) [26]	0.50	0.54
Antioxidant [27]	0.20	0.20
Sodium propionate [28]	0.10	0.10
Monoammonium phosphate [29]	1.00	-
Sodium phosphate [30]	-	2.10
Selenised yeast [31]	0.03	0.03
L-Taurine [32]	0.50	-
L-Tryptophan [33]	0.02	0.20
DL-Methionine [34]	0.10	0.60
L-Lysine [35]	-	0.70
Dry matter (DM), %	8.0 ± 0.0	6.2 ± 0.0
Crude protein, % DM	19.9 ± 0.2	16.9 ± 2.0
Crude fat, % DM	14.8 ± 0.1	7.9 ± 0.2
Ash, % DM	8.4 ± 0.0	7.2 ± 0.7
Iodine, mg kg^{-1} DM	20.4 ± 0.6	24.9 ± 0.6
Selenium, mg kg^{-1} DM	1.4 ± 0.1	2.0 ± 0.1

[1] NORVIK LT: 70.6% crude protein (CP), 5.8% crude fat (CF), Sopropêche, France; [2] CONRESA 60: 61.2% crude protein (CP), 8.4% crude fat (CF), Conserveros Reunidos S.A., Spain; [3] CPSP90: 86% CP, 6% CF, Sopropêche, France; [4] Porcine blood meal: 89% CP, 1% CF, SONAC BV, The Netherlands; [5] Tetraselmis spp. meal: 23% CP, 6.2% CF, ALLMICROALGAE, Portugal; [6] Spirulina meal: 72% CP, 1% CF, Willows Ingredients Ltd, Ireland; [7] Chlorella meal: 62% CP, 9% CF, ALLMICROALGAE, Portugal; [8] ALL-G RICH (Schizochytrium), Alltech Portugal; [9] Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands; [10] Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal; [11] Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, CARGILL, Spain; [12] Solvent extracted soybean meal: 43.8% CP, 3.3% CF, CARGILL, Spain; [13] Defatted rapeseed meal: 32.7% CP, 4.1% CF, Ribeiro & Sousa Lda, Portugal; [14] Defatted sunflower meal: 29.1% CP, 1.8% CF, Ribeiro & Sousa Lda, Portugal; [15] Corn meal: 8% CP, 3.7% CF, Ribeiro & Sousa Lda, Portugal; [16] Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal; [17] VITEN: 82% CP, 2.1% CF, Roquette, France; [18] Wheat bran: 14.9% CP, 4.0% CF, Cerealis Moagens S.A., Portugal; [19] Sopropêche, France; [20] Sopropêche, France; [21] Henry Lamotte Oils GmbH, Germany; [22] INVIVONSA Portugal SA, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 500mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate,7.5mg; sodium chloride, 400mg; excipient wheat middling's; [23] ORFFA, The Netherlands; [24] Guar gum, SEAH International, France; [25] CELATOM FP1SL (diatomite), Angelo Coimbra S.A., Portugal; [26] Dry Laminaria digitata: 5.4% CP, 0.5% CF, 3700 mg iodine/kg, Agrimer, France; [27] VERDILOX, Kemin Europe NV, Belgium; [28] PREMIX LDA., Portugal; [29] Windmill AQUAPHOS, 26% P, ALIPHOS, Belgium; [30] Vadequimica, Spain; [31] ALKOSEL R397: 2200 mg selenium/kg, Lallemand, France; [32] L-Taurine 98%, ORFFA, The Netherlands; [33] TrypAMINO 98%, Evonik Nutrition & Care GmbH, Germany; [34] DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany; [35] L-Lysine HCl 99%: Ajinomoto Eurolysine SAS, France;

Table S. 5. 2 - Composition of simulated digestion fluids used in the in vitro digestion protocol. The volumes (mL) and mass (mg) are calculated for a volume of 100 mL for each simulated digestion fluid

Components (stock concentration)	Oral phase	Gastric phase	Intestinal phase	
	Saliva	Gastric juice	Duodenal juice	Bile
	Volume of stock (mL)	Volume of stock (mL)	Volume of stock (mL)	Volume of stock (mL)
Inorganic and organic components				
KCl (89.6 g L^{-1})	2.0	1.8	1.2	0.8
KSCN (20 g L^{-1})	2.0	-	-	-
NaH_2PO_4 (88.8 g L^{-1})	2.0	0.6	-	-
Na_2SO_4 (57 g L^{-1})	2.0	-	-	-
NaCl (175.3 g L^{-1})	0.4	3.1	8.0	6.0
$NaHCO_3$ (84.7 g L^{-1})	4.0	-	1.33*	13.6
$CaCl_2.2H_2O$ (22.2 g L^{-1})	-	3.6	0.144*	0.080*
NH_4Cl (30.6 g L^{-1})	-	2.0	-	-
KH_2PO_4 (8 g L^{-1})	-	-	2.0	-
$MgCl_2$ (5 g L^{-1})	-	-	2.0	-
Urea (25 g L^{-1})	1.6	0.7	0.8	2.0
Glucuronic acid (2 g L^{-1})	-	2.0	-	-
Glucose (65 g L^{-1})	-	2.0	-	-
Glucoseamine hydrochloride (33 g L^{-1})	-	2.0	-	-
	Mass of stock (mg)	Mass of stock (mg)	Mass of stock (mg)	Mass of stock (mg)
Bioactive components				
α-amylase	90	-	-	-
Uric acid	3	-	-	-
Mucin	5	-	-	-
BSA	-	200	200	360
Pepsin	-	500	-	-
Pancreatin	-	600	1800	-
Lipase	-	-	300	-
Trypsin	-	-	3.2	-
α-chymotrypsin	-	-	34.8	-
Bile	-	-	-	6000

* Volume added to each in vitro digestion reaction (mixture of simulated digestion fluid and food) – as precipitation may occur.

Table S. 5. 3 - Average certificate and average measured concentrations (µg g^{-1} dry weight) and the associated relative standard deviation (RSD) in certified reference materials (CRM). Limits of detection (LOD) and limits of quantification (LOQ) for each element and limits of quantification (LOQ) for each element and analytical method.

Elements	Type	CRM				Fish samples (BD)		Bioaccessible fraction (BIO)	
		Certificate (µg g^{-1})	Measured (µg g^{-1})	Recovery (%)	RSD (%)	LOD (µg g^{-1})	LOQ (µg g^{-1})	LOD (µg g^{-1})	LOQ (µg g^{-1})
I	ERM®-BB422	1.40 ± 0.40	1.31 ± 0.13	88	1.6	0.013	0.025	0.013	0.025
Se	ERM®-BB422	1.33 ± 0.13	1.39 ± 0.01	91	1.7	0.009	0.018	0.009	0.018
As	ERM®-BB422	12.7 ± 0.7	12.0 ± 0.2	94	1.7	0.003	0.013	0.001	0.003
K	SRM 1566	9690	8890 ±500	92	5.6	20	60	8.0	25
Ca	SRM 1566	1500	1015 ± 50	68	4.9	9.28	27.85	17	50
Fe	DORM-2	142	176 ± 10	124	5.7	0.40	1.21	0.2	0.5
Cu	DORM-2	2.34	2.3 ± 0.5	98	21.7	0.41	1.23	0.3	0.8
Zn	DORM-2	25.6	30 ± 3	117	10.0	0.54	1.62	0.01	0.04
Br	SRM 1566b	55	54 ± 5	98	9.3	0.21	0.64	0.4	1.2

ERM®-BB422: fish muscle, SRM 1566b: oyster tissue, DORM-2: dogfish muscle.; BD: before digestion, BIO: bioaccessible

Table S. 5. 4 - Concentrations of trace (iodine, selenium, iron, zinc, copper, bromine) and macro (calcium, potassium) elements in raw and steamed samples prior to *in vitro* digestion (μg g^{-1} wet weight, average ± standard deviation).

		Iodine (μg g^{-1})			Selenium (μg g^{-1})			Iron (μg g^{-1})		
		Raw	Steamed	TR (%)	Raw	Steamed	TR (%)	Raw	Steamed	TR (%)
Gilthead seabream	CTR	0.068 ± 0.007^{a}	0.057 ± 0.004^{A}	77	0.201 ± 0.007^{a}	0.205 ± 0.011^{A}	94	2.17 ± 0.13	2.36 ± 0.39	100
	BF	0.102 ± 0.007^{b}	0.117 ± 0.003$^{B\#}$	106	0.227 ± 0.009^{b}	0.251 ± 0.015$^{B\#}$	102	2.20 ± 0.34	2.25 ± 0.31	94
Common carp	CTR	0.03 ± 0.005^{a}	0.046 ± 0.005$^{A\#}$	142	0.125 ± 0.004^{a}	0.137 ± 0.004^{A}	100	5.47 ± 0.66	5.61 ± 0.52	94
	BF	0.106 ± 0.012^{b}	0.098 ± 0.004^{B}	85	0.708 ± 0.02^{b}	0.795 ± 0.008$^{B\#}$	103	5.99 ± 0.57	5.38 ± 0.34	83

		Zinc (μg g^{-1})			Copper (μg g^{-1})			Bromine (μg g^{-1})		
		Raw	Steamed	TR (%)	Raw	Steamed	TR (%)	Raw	Steamed	TR (%)
Gilthead seabream	CTR	4.68 ± 0.09	5.11 ± 0.49	100	1.28 ± 0.11	1.62 ± 0.20	116	3.59 ± 0.05	3.66 ± 0.17	94
	BF	4.83 ± 0.22	5.94 ± 0.30$^{\#}$	113	0.91 ± 0.14	1.40 ± 0.17$^{\#}$	142	3.63 ± 0.26	3.71 ± 0.14	94
Common carp	CTR	5.36 ± 0.30^{a}	5.63 ± 0.40^{A}	96	1.28 ± 0.11^{a}	1.46 ± 0.11	104	LOQ^{a}	LOQ^{A}	n.a.
	BF	9.71 ± 0.55^{b}	8.62 ± 0.25^{B}	82	2.02 ± 0.17^{b}	1.45 ± 0.16$^{\#}$	66	0.62 ± 0.11^{b}	0.60 ± 0.07^{B}	90

		Calcium (μg g^{-1})			Potassium (μg g^{-1})		
		Raw	Steamed	TR (%)	Raw	Steamed	TR (%)
Gilthead seabream	CTR	136 ± 13	156 ± 8	105	3764 ± 177	3723 ± 274	91
	BF	136 ± 9	147 ± 8	100	4245 ± 355	3599 ± 297	78
Common carp	CTR	141 ± 7	162 ± 19	105	2797 ± 237	2623 ± 111	86
	BF	168 ± 10	158 ± 2	87	3445 ± 236	3063 ± 449	82

Different lower-case and upper-case letters indicate significant differences ($P < 0.05$) between non-biofortified (CTR) and biofortified (BF) fish fillets, in raw and steamed samples, respectively. For each treatment (CTR and BF), # represents significant differences ($P < 0.05$) between raw and steamed fillets. TR (%), represents the percentage of element true retention values in fish fillet after steaming. n.a. – not available.

Table S. 5. 5 - Bioaccessibility (%) of trace (iodine, selenium, iron, zinc, copper, bromine) and macro (calcium, potassium) elements in raw and steamed samples (average ± standard deviation).

		Iodine (%)		Selenium (%)		Iron (%)		Zinc (%)	
		Raw	Steamed	Raw	Steamed	Raw	Steamed	Raw	Steamed
Gilthead seabream	CTR	81.3 ± 3.7	76.8±6.2	69.4 ± 1.1	66.6 ± 3.7	86.9 ± 7.6	60.1 ± 11.2$^{\#}$	78.4 ± 11.2	83.4 ± 7.7
	BF	83.6±3.4	71.1±1.6$^{\#}$	69.7±3.3	63.7 ± 0.4	83.7 ± 11.8	59.9±11.2$^{\#}$	77.5±10.4	82.0 ± 8.3
Common carp	CTR	LOQ^{a}	LOQ^{A}	68.8 ± 3.8	49.1 ± 1.5$^{\#}$	85.9 ± 2.9	84.5 ± 10.3	79.6 ± 9.8	85.7 ± 7.1
	BF	42.2 ± 5.0^{b}	45.2 ± 2.8^{B}	73.4 ± 0.3	63.1 ± 2.8$^{\#}$	74.4 ± 4.4	79.1 ± 10.6	80.9 ± 5.8	87.4 ± 2.0

		Copper (%)		Bromine (%)		Calcium (%)		Potassium (%)	
		Raw	Steamed	Raw	Steamed	Raw	Steamed	Raw	Steamed
Gilthead seabream	CTR	n.d.	n.d.	n.d.	n.d.	67.8 ± 7.3	LOQ$^{\#}$	93.0 ± 3.2^{a}	80.9 ± 12.7
	BF	n.d.	n.d.	n.d.	n.d.	64.0 ± 6.0	LOQ$^{\#}$	65.1 ± 6.2^{b}	56.0 ± 4.9
Common carp	CTR	n.d.	n.d.	n.d.	n.d.	68.1 ± 3.3	LOQ$^{\#}$	60.7 ± 0.6	54.1 ± 1.5
	BF	n.d.	n.d.	n.d.	n.d.	55.8 ± 8.3	LOQ$^{\#}$	65.6 ± 9.4	51.9 ± 3.4

n.d – not determined, inconclusive results due to low or not detected values in bioaccessible fractions.

Different lower-case and upper-case letters indicate significant differences ($P < 0.05$) between non-biofortified (CTR) and biofortified (BF) fish fillets, in raw and steamed samples, respectively. For each treatment (CTR and BF), # represents significant differences ($P < 0.05$) between raw and steamed fille

(2023)

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Eco-innovative biofortified fish products with sustainable and healthy nutrients: Eco-toxicology and nutritional value