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FROM WASTE TO PROFIT: HIGH BIOLOGICAL VALUE MARINE PRODUCTS, FOR THE VALORIZATION AND RECOVERY OF FISHERIES, AQUACULTURE AND FISH PROCESSING BY-PRODUCTS FOR THE IMPROVEMENT OF THE SUSTAINABILITY OF THE SUPPLY CHAIN

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List of papers

This doctoral thesis is based on the following publications/manuscripts produced during the experimental activity, that are reported in the following chapters:

Chapter 1: Messina, C.M., **Arena, R.**, Manuguerra, S., Barbera, L. La, Curcuraci, E., Renda, G., Santulli, A., 2022. Valorization of Side Stream Products from Sea Cage Fattened Bluefin Tuna (*Thunnus thynnus*): Production and In Vitro Bioactivity Evaluation of Enriched ω-3 Polyunsaturated Fatty Acids. Mar. Drugs 2022, Vol. 20, Page 309 20, 309. https://doi.org/10.3390/MD20050309.

Chapter 2: ***Arena, R.**, Manuguerra, S., Curcuraci, E., Cusimano, M., Lo Monaco, D., Di Bella, C., Santulli, A., Messina, C.M., 2023. Fisheries and aquaculture by-products modulate growth, body composition, and omega-3 polyunsaturated fatty acid content in black soldier fly (*Hermetia illucens*) larvae. Front. Anim. Sci. 4, 1204767. https://doi.org/10.3389/fanim.2023.1204767.

Chapter 3: ***Arena, R.;** Renda, G.; Ottaviani Aalmo, G.; Debeaufort, F.; Maria Messina, C.; Santulli, A. Valorization of the Invasive Blue Crabs (*Callinectes sapidus*) in the Mediterranean: Nutritional Value, Bioactive Compounds and Sustainable By-Products Utilization. Mar. Drugs 2024, 22, 430. https://doi.org/10.3390/md22090430.

Additional papers not included in the thesis which I contributed thanks to application of analyses or technique/protocol I performed, were produced during the doctoral studies:

- Curcuraci, E.; Manuguerra, S.; Messina, C.M.; Arena, R.; Renda, G.; Ioannou, T.; Amato, V.; Hellio, C.; Barba, F.J.; Santulli, A. 2022. Culture Conditions Affect Antioxidant Production, Metabolism and Related Biomarkers of the Microalgae *Phaeodactylum tricornutum*. Antioxidants, 11, 1–17, doi:10.3390/antiox11020411.
- *Arena, R.; de Medeiros, A.C.L.; Secci, G.; Mancini, S.; Manuguerra, S.; Bovera, F.; Santulli, A.; Parisi, G.; Messina, C.M.; Piccolo, G. 2022. Effects of Dietary Supplementation with Honeybee Pollen and Its Supercritical Fluid Extract on Immune Response and Fillet's Quality of Farmed Gilthead Seabream (*Sparus aurata*). Animals, 12, doi:10.3390/ani12060675.
- Bonsignore, M., Concetta, M., Antonio, B., Manuguerra, S., Arena, R., Andrea, S., Maricchiolo, G., Del Core, M., Sprovieri, M., 2022. Chemical and biochemical responses to sub − lethal doses of mercury and cadmium in gilthead seabream (*Sparus aurata*). Chemosphere 307, 135822. https://doi.org/10.1016/j.chemosphere.2022.135822.

- Dhouibi, N., Manuguerra, S., Arena, R., Messina, C.M., Santulli, A., Kacem, S., Dhaouadi, H., Mahdhi, A., 2023. Impact of the Extraction Method on the Chemical Composition and Antioxidant Potency of *Rosmarinus officinalis* L. Extracts. Metabolites 13, 1–11. https://doi.org/10.3390/ metabo13020290.
- Messina, C.M., Manuguerra, S., Arena, R., Espinosa-ruiz, C., Curcuraci, E., Angeles, M., Santulli, A., 2023. Contaminant-induced oxidative stress underlies biochemical, molecular and fatty acid profile changes, in gilthead seabream (*Sparus aurata* L.). Res. Vet. Sci. 159, 244–251. https://doi.org/10.1016/j.rvsc.2023.04.021.
- Mhalhel, K., Arena, R., Rizzo, M., Piccione, G., Aragona, M., Levanti, M., Aragona, F., Arfuso, F., 2024. Potential Implications of Acid-Sensing Ion Channels ASIC2 and ASIC4 in Gonadal Differentiation of *Dicentrarchus labrax* Subjected to Water Temperature Increase during Gonadal Development. Anim. 2024, Vol. 14, Page 1024 14, 1024. https://doi.org/10.3390/ANI14071024.
- Manuguerra, S.; Arena, R.; Curcuraci, E.; Renda, G.; Rannou, M.; Hellio, C.; Messina, C.M.; Santulli, A. In Vitro Potential of Antioxidant Extracts from *Gracilaria gracilis* Cultivated in Integrated Multi-Trophic Aquaculture (IMTA) for Marine Biobased Sector. Water 2024, 16, 2667.

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Summary

The increasing demand for fish products, both for food and non-food purposes, is raising serious concerns for the environmental sustainability of fish production, that is affected by multiple factors as fisheries management, economic competition, and inadequate management of wastes. To address these challenges, the European Green Deal and Sustainable Development Goal 14 of the 2030 Agenda ("Life Below Water") prioritizes the sustainability of the blue economy and the fisheries sector also by the valorization of by-products from fisheries, aquaculture, and fish processing, that represents an effective solution to promote circular blue economy.

My PhD project focused on the valorization of Sicilian fish by-products to contribute to the "zero waste" goal, with the aim to improve the value chain and supporting the achievement of the Sustainable Development Goals, for the recovery of bioactive components of high biological value and their application in other industrial sectors.

The thesis is divided into three parts, in accord with the development of application of bioactive compounds in different industrial sectors: nutraceutical, feed and food.

The part one is related to the valorization of marine by-products in the nutraceutical sector, starting from the side streams from the fisheries and aquaculture value chains, in order to evaluate the possibility of producing tuna oil (TO) as a valuable source of n-3 polyunsaturated fatty acids (PUFA), (**Chapter 1**).

Fish oil is one of the main bioactive components that can be extracted from fish by-products and represents the most important natural source of the essential polyunsaturated fatty acids (PUFAs), of the n-3 series, such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). These essential fatty acids are used both in feed for aquaculture and as oil or food supplement capsules for direct human consumption. In this study, side streams produced during filleting fattened bluefin tuna (*Thunnus thynnus*) were collected and analyzed for proximate composition and fatty acid profile to evaluate the possibility to produce tuna oil (TO) by set-up procedure for enrichment in n-3 PUFA.

The procedure short path distillation (SPD) was set-up and to almost 85% of the PUFA fraction (PUFA-SPe) was enriched. The bioactivity of all the fractions of oils was tested *in vitro* for lipid metabolism, in gilthead sea bream (*Sparus aurata*) fibroblast cell line (SAF-1). TFA and PUFA-SPd upregulated transcription factors (*pparβ and pparγ*) and lipid metabolism-related genes (*D6D, fas, fabp, fatp1, and cd36*), indicating the promotion of adipogenesis. PUFA-SPe treated cells were similar to control. The results indicate that PUFA-SPe modulates fat deposition, contributing to improve health and the quality of its products. **(Chapter 1 - Published chapter: Messina, Arena *et al*. 2022).**

The second part of the thesis is related to the valorization of marine by-products in the aquafeed sector, by focusing its potential use in the modulation of the nutritional profile of one the most studied organism in the past decade for this purpose: the insect *Hermetia illucens*. The use of insects as source of ingredients for aquaculture is highlighted by hundreds of research and papers produced on this subjects.

In this study, by-catch collected from the Mediterranean trawler fishing species, *Parapenaeus longirostris* processing by-products, aquaculture by-products (from sea bass and sea bream filleting), *Thunnus albacares* processing by-products, and *Engraulis encrasicolus* by-products (heads and viscera) were used and incorporated in experimental diets for black soldier fly (BSF) (*H. illucens* L.) to evaluate the effect of their inclusion on the final nutritional profile of BSF larvae **(Chapter 2)**.

The BSF is one of the most promising species for industrial production and animal feed in the world, due to its ability to bioconvert various organic substrates (animal and plant origin) into larval biomass within a 14-day period under optimal growth conditions (temperature of 27°C and humidity of 70%). In recent years, the use of insect meal, mainly as an alternative source of proteins in fishmeal, has also increased in the aquaculture sector. Nevertheless, only a limited number of studies have explored the potential of using BSF as a source of fatty acids due to the low content of n-3 series PUFAs, such as EPA and DHA, which are essential for fish. Recent research has shown that the fatty acid composition of BSF larvae can be modified by utilizing different feed substrates, thereby adding value. The use of marine by-products generated from a diverse supply chain would provide an opportunity to enrich BSF larvae with essential PUFA. The incorporation of marine by-products as feed for BSF would not only reduce food waste but also result in the production of high-quality larvae.

The findings of this study demonstrate that the diverse experimental diets not only influenced the nutrient composition but also the fatty acid profile. A significantly higher n-3 fatty acid content was observed in the fatty acid profile of BSF pre-pupae fed different fish by-products compared to the control. In particular, the pre-pupae that were fed a diet containing by-products of *E. encrasicolus* exhibited the highest levels of EPA, followed by the pre-pupae that were fed diets containing by-products of *T. albacares*, by-catch from Mediterranean trawlers, and aquaculture by-products.

The observed results of this study corroborate the hypothesis that the utilization of marine sources, particularly fish by-products, can improve the nutritional profile of BSF larvae and pre-pupae by providing essential fatty acids, such as EPA and DHA, which are not naturally present in BSF.

This approach not only demonstrates the possibility of incorporating n-3 components into insects, but also highlights the potential of using these insects as a valuable ingredient in aquatic feeds.

Furthermore, this method promotes circular economy pathways at the regional level, in line with the objectives of the European Green Deal (**Chapter 2 - Published chapter: Arena et al., 2023**).

The third part of the thesis reports the valorization of marine by-products in the food sector, specifically in the fish processing industry.

During my PhD, I had the opportunity to work with the company Blu Ocean s.r.l., the industrial partner of the project, was initiated. This company, which has been active in fish processing for almost a century, stands out for its expertise in the sector and its commitment to sustainability and product quality.

In collaboration with Blu Ocean, it was possible to identify some strategies to reduce waste along the supply chain, with the idea to turn residues into useful resources.

Recently, in response to the emergency caused by the blue crab invasion, Blu Ocean has initiated preliminary activities to promote this species by producing new food products. However, the large amount of waste generated requires the implementation of innovative solutions for its valorization.

Given the expertise in the lab on extraction of bioactive compound I studied the composition of the blue crabs by products, focusing on the production of protein hydrolysates.

Blue Crab (*Callinectes sapidus*), native to the western Atlantic Ocean, has recently spread to the Mediterranean Sea, where it is considered one of the 100 most invasive species. This opportunistic species is known for its adaptability to different temperatures and salinities but has a negative impact on biodiversity and human activities, such as fishery and tourism. Despite these negative effects, the blue crab is attracting increasing interest as a food resource due to its high nutritional value. Blue crab meat is rich in protein (14% to 30%) and omega-3 fatty acids (EPA and DHA). The use of this species in the production of new foods could not only mitigate the impact of its invasiveness, but also create new economic opportunities (**Chapter 3)**.

One of the most significant challenges related to this potential resource is the management of waste. The global production of crab shells is estimated to be between 6 and 8 million tons per year. This has the potential to create significant issues surrounding disposal and environmental sustainability.

The low economic value of crab shells often results in their disposal in landfills, highlighting the need for more sustainable solutions. It is imperative that these residues be valorized in order to enhance economic and environmental sustainability. Crab shells and other by-products contain a range of valuable components, including proteins, lipids, chitin, minerals, and pigments, which can be processed into high-value products. While the valorization of crustacean by-products has been widely studied, there is a paucity of research specifically focused on the blue crab (*C. sapidus*). However, studies have indicated that blue crab by-products have the potential to be utilized in various sectors,

thereby reducing environmental impact, promoting a circular economy, and creating new industrial opportunities **(Chapter 3 Published chapter: Arena et al., 2024).**

Given the lack of information on the use of protein hydrolysates derived from the by-products of the blue crab (*C. sapidus*), *in vitro* tests were carried out on these hydrolysates to assess their potential, in order to help local businesses to recover and valorize the by-products of this species according to the principles of the circular economy (**Chapter 4**).

This study first evaluated the yield of blue crab meat, showing that, as indicated in the literature, the yield from processing this crab is very low, resulting in considerable waste, much of which remains underutilized. Blue crab by-products (BCBP) were analyzed to determine their proximate composition, which revealed a high protein content, justifying the study on the valorization of this component. Specially, protein hydrolysates were produced from BCBPs by enzymatic hydrolysis, an environmentally friendly technique that allows the production of protein hydrolysates of high nutritional value. In the present study, the hydrolysates were obtained using the enzymes Alcalase and Protamex and subsequently tested for their antioxidant activity using the DPPH assay and human fibroblast cell (HS-68) tests.

The results showed that the Alcalase enzyme was more effective than Protamex. It achieved a degree of hydrolysis (DH%) of over 20%. This suggests that the resulting hydrolysates may be suitable for utilization as food additives. The two enzymes exhibited high proteolytic activity, as evidenced by SDS-PAGE electrophoresis. The protein hydrolysates derived from the blue crab by-products exhibited notable antioxidant properties, displaying considerable DPPH radical scavenging capacity and moderate reducing power. Furthermore, the hydrolysates demonstrated enhanced viability in HS-68 cells when compared to untreated cells that had been subjected to stress. These results indicate that high-quality bioactive compounds derived from blue crab by-products may have significant applications in the food, nutraceutical, and pharmacological sectors. In particular, for example, they could be incorporated into chitosan-gelatin films as antioxidant food additives, thus producing active food packaging with antioxidant properties.

This study demonstrates how this species can be transformed from a problematic by-product to a valuable resource. (**Chapter4**).

During my research project I had possibility to evaluate the potential of the production of by-products from fisheries, aquaculture, and fish processing. The studies carried out identified some strategies to valorize these by-products by extracting bioactive components useful in areas such as nutraceuticals, animal feed and human nutrition. The achieved results are in line with the objectives and provided basis for further applications and developments in the field of marine biobased production.

Introduction

The PhD project, entitled "***From waste to profit: high biological value marine products, for the valorization and recovery of fisheries, aquaculture and fish processing by-products for the improvement of the sustainability of the supply chain***", as part of the PON "Research and Innovation" 2014-2020 - Action IV.5 "Doctorates on topics Green", fully intercepts the specialization trajectories of the Regional Strategy of Innovation for Intelligent Specialization - S3 Sicily 2021-27 in the agri-food sector, through the development of research and training activities on the following research topics: research, development and production of functional foods; product and process innovation for healthier and sustainable marine foods; innovative and sustainable solutions for packaging and traceability; valorization of by-products of marine supply chains. In addition, the PhD is in line with the technological development paths of national priority identified in the National Strategy for Intelligent Specialization (SNSI), reported in the areas 5.5.3 Health, Food, Quality of Life (nutraceuticals and functional foods) and 5.5.4 Intelligent and Sustainable Industry, Energy and Environment (technologies for biomaterials and bio-based products and biorefineries; systems and technologies for water and waste treatment).

Sustainable fisheries value chains: Implementing the UN 2030 Agenda and the European Green Deal Principles

Global demand for seafood products for both food and non-food purposes continues to grow rapidly (Figure 1), as does concern for the environmental sustainability of fish production, which is fundamental to the food sector (Coppola et al., 2021; FAO, 2022; Messina et al., 2021a).

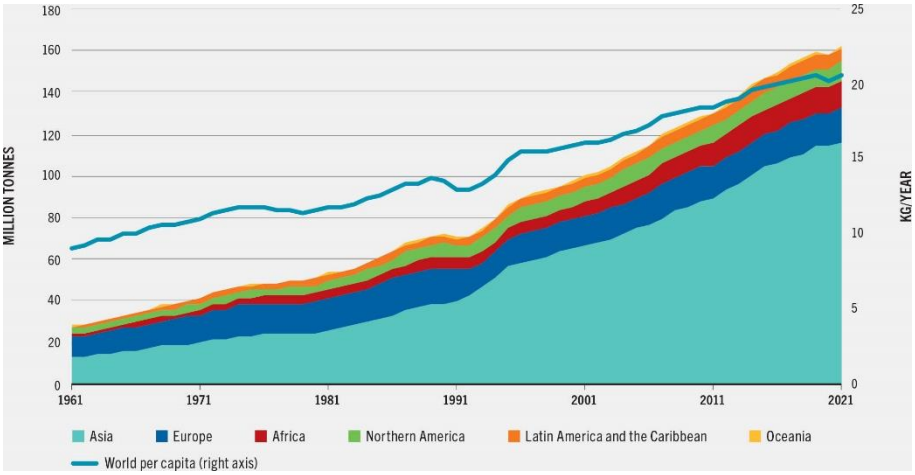

Figure 1. Consumption of aquatic animal foods by region, 1961–2021 (source: Fao, 2024).

The sustainability of fish production is related to several factors, including fisheries management, competition for space, and food waste production and generation, all these aspects are taken into consideration within the UN 2030 Agenda with its 17 Sustainable Development Goals (SDGs) (Figure 2a), (FAO, 2020; Gulseven et al., 2020) and the European Green Deal (Figure 2b), as key policy frameworks for protecting the environment and seeking to minimize risks to climate, human health and biodiversity (European Commission, 2019; Koundouri et al., 2021; United Nations General Assembly, 2015).

(a) *(b)*

Figure 2. (a) Sustainable Development Goals (SDGs) of the United Nations 2030 Agenda (https://commission.europa.eu/strategy-and-policy/sustainable-development-goals/eu-and-united-nations-common-goals-sustainable-future_en); (b) The European Green Deal (https://euinasean.eu/eu-green-deal/).

The 2030 Agenda, signed by 193 UN member States and approved by the UN General Assembly on 25 September 2015, is a programme of action for people, planet, and prosperity (United Nations General Assembly, 2015). The 17 Sustainable Development Goals (SDGs), framed within a broader programme of action comprising 169 targets, aim to end poverty, fight inequality, tackle climate change and build peaceful societies that respect human rights. These goals, to be achieved by 2030, are interconnected and indivisible and balance the three dimensions of sustainable development: economic, social and environmental (United Nations General Assembly, 2015).

The European Union and the United Nations are natural partners in efforts to create a better and safer world for all. Therefore, the European Commission is committed to achieving the goals proposed in the European Green Deal. It is a package of policy initiatives that aims to put the EU on the path to a green transition, with the ultimate goal of achieving climate neutrality by 2050 (European Commission, 2019).

The 2030 Agenda for Sustainable Development (Figure 2a) and the European Green Deal (Figure 2b) include among their objectives the optimization of agri-food production processes. These objectives aim both to meet the growing demand for food and to improve production, economic, and social performance by promoting conscious consumption (European Commission, 2019; Garske et al., 2020; Koundouri et al., 2021; United Nations General Assembly, 2015).

An objective vigorously pursued through the development of prevention, valorization, and management strategies for agri-food supply chains is the reduction of food waste (Papargyropoulou et al., 2014; Priefer et al., 2016; Teigiserova et al., 2020). The reduction of food waste is primarily addressed in Goal 12 (Responsible Consumption and Production), specifically target 12.3 of the 2030

Agenda. This goal is linked to and influences other SDGs such as SDG 2 (Zero Hunger), SDG 6 (Sustainable Water Management), SDG 13 (Climate Change), SDG 14 (Marine Resources), and SDG 15 (Terrestrial Ecosystems, Forests, Biodiversity) (FAO, 2019). In the European Green Deal, the issue of food waste and the strategies implemented to reduce it are addressed in the Farm to Fork Strategy, the Circular Economy Action Plan, and in Supporting Research and Innovation (European Commission, 2019; United Nations General Assembly, 2015).

The reduction of food waste is increasingly promoted at the societal level (Hoehn et al., 2021) since food loss and waste are barriers to global sustainability due to its negative impact on food security, natural resources, the environment and human health (Xue et al., 2017). It is observed that in the food chain, about one third of the global food production is lost or wasted (Figure 3), and food waste accounts for approximately 8-10% of global greenhouse gas emissions (Do Canto et al., 2021; United Nations Environment Programme, 2021).

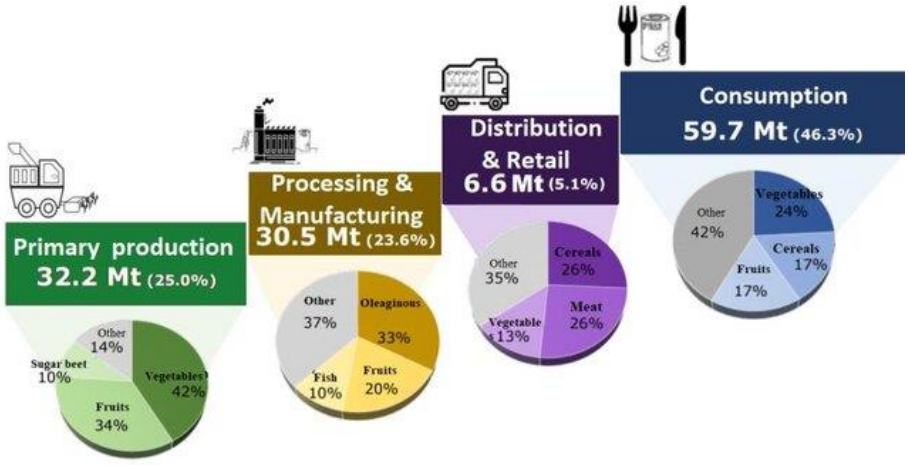

Figure 3. Amount of food waste (in fresh weight) generated during the different stages of the food supply chain (Caldeira et al., 2019; Sanches Lopez et al., 2020).

To reduce food losses and waste, it is crucial to optimize the use of by-products from the main food supply chains, including the fisheries supply chain, through their valorization. This approach aims to achieve the "zero waste" goal, which plays a central role in the circular economy model of production and consumption (Deselnicu et al., 2018; Širá et al., 2022; Venugopal, 2022).

The Circular Economy represents a new economic paradigm based on the principles of diversity, resilience, and inclusive thinking. In this model, the biological, technological, and material spheres interact to transform, distribute, use, and regenerate available resources (Širá et al., 2022).

In the past decade, the European Union has worked to develop detailed strategies based on sustainable economic models (European Commission, 2014) implementing a specific package of directives on the circular economy (Table 1) (Zarbà et al., 2021).

Table 1. The EU Circular Economy Package—Directives (Source: (Zarbà et al., 2021)*).*

Directive No.	Date	Reference
Directive (EU) 2018/849 amending Directive 2000/53/EC on end-of-life vehicles, 2006/66/EC on batteries and accumulators and waste batteries and accumulators, and 2012/19/EU on waste electrical and electronic equipment	30 May 2018	(The European parliament, 2018)
Directive (EU) 2018/850 amending Directive1999/31/EC on the landfill of waste	30 May 2018	(The European parliament, 2018)
Directive (EU) 2018/851amending Directive 2008/98/EC on waste	30 May 2018	(The European parliament, 2018)
Directive (EU) 2018/852 amending Directive 94/62/EC on packaging and packaging waste	30 May 2018	(The European parliament, 2018)

This package clearly defines the concept of the circular economy, which promotes reducing, reusing, and recycling throughout production, distribution, and consumption. This approach contrasts sharply with the traditional linear economy model, characterized by a direct flow of "resources → products → waste" (Figure 4) (Sauvé et al., 2016; Širá et al., 2022).

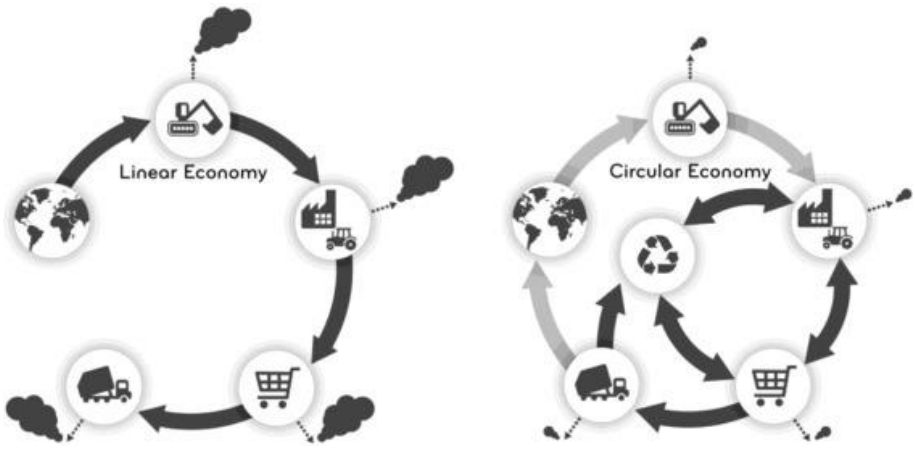

Figure 4. Circular economy vs. linear economy (Sauvé et al., 2016).

According to the European Commission, the circular economy allows "the value of products, materials and resources is maintained in the economy for as long as possible, and the generation of waste minimized". The transition to the circular economy is essential to achieve improved environmental, social and economic sustainability (European Commission, 2015).

In conclusion, both the 2030 Agenda and the European Green Deal place the circular economy at the center of their strategies to address global environmental challenges and promote long-term sustainable development.

The Blue Economy

There is a close correlation between the Circular Economy and the Blue Economy. Both economies share the goal of abandoning traditional economic models in favor of systems that regenerate themselves, promoting reuse, process efficiency and product longevity. The Blue Economy, in particular, aims to protect the oceans (Carta, 2024). The term "Blue Economy" was introduced at the UN Rio+20 Sustainable Development Conference in 2012 (Veríssimo et al., 2021). There are different definitions of the blue economy, as shown in the Table 2. Overall, the definitions in Table 2 are in agreement that the blue economy is a concept related to the sustainable use of the oceans and its resources (Veríssimo et al., 2021).

Table 2. Blue economy definitions. Source (Ogunmakinde et al., 2021; Veríssimo et al., 2021).

Definition	Reference
European Commission "Blue economy encompasses all industries and sectors related to oceans, seas, and coasts, whether they are based directly in the marine environment (e.g., shipping, seafood, energy generation) or on land (e.g., ports, shipyards, coastal infrastructures)".	(European Commission, 2021)
The 'blue economy' comprises the economic activities that create sustainable wealth from the world's oceans and coasts	(Middlebury Institute of International Studies at Monterey, 2018)
"The 'blue economy' is an emerging concept which encourages better stewardship of our ocean or 'blue' resources".	(The Commonwealth, 2018)
"The sustainable use of ocean resources for economic growth, the improvement of livelihoods and jobs, and the health of ocean ecosystems"	(World Bank, 2013)
A blue economy is a long-term strategy aimed at supporting sustainable economic growth through ocean related sectors and activities while improving human well-being and social equity and preserving the environment	(UN DESA, 2014)
The concept of rethinking industrial processes and searching for a viable biological solution that reduces contamination	(Costa et al., 2019)
It has become synonymous with generating wealth from activities related to the oceans while protecting and supporting marine ecosystems	(Phelan et al., 2020)
It arises from the growing worldwide interest in the growth of water-based activities	(Graziano et al., 2019)
It seeks to curb biodiversity loss while stimulating economic development, thereby integrating environmental and economic interests	(Schutter and Hicks, 2019)
It is the mainstream of national development and can integrate land and sea-based socio-economic sustainable development	(Kathijotes, 2013)
It refers to the commercially sustainable development of the oceans	(Kaczynski, 2011)
It has emerged in the last two decades from various forums, but above all from within the policy and practice of environmental development	(Hoegh-Guldberg et al., 2015; Patil et al., 2016; UNECA, 2016)

Ocean aquatic ecosystems are threatened by mismanagement of aquatic resources, pollution and climate change, even though they are an important pillar of the world's food supply and economy (Veríssimo et al., 2021). Taking these aspects into account, to "ensure the sustainability of our blue economy and fisheries sectors" (https://commission.europa.eu/strategy-and-policy/priorities-2019-2024/european-green-deal/protecting-environment-and-oceans-green-deal_en) is one of the European Green Deal priorities and of the 2030 Agenda SDG 14: "Life Below Water" (Selamoglu, 2021).

The Blue Economy concept can be structured around eight main pillars (Veríssimo et al., 2021):

1. Conserve oceans and marine life: Maintaining a healthy ocean and preserving marine life is vital for our survival, in fact oceans provide food and energy.
2. Intelligent transport and shipping: Most shipping is by sea, and there is a need for more economical and environmentally friendly marine technology.
3. Improve aquaculture and fishing industry: There is a need to improve the fish supply chain to reduce waste and increase sustainability.
4. Sustainable use of oceans resources: It is necessary to reduce seafood losses, pressure on marine ecosystems and pollution. The valorization of waste can thus be a source of income
5. Renewable energy: It is possible to harness renewable ocean energy in the form of waves and wind, enabling coastal communities to become energy self-sufficient.
6. Maritime and coastal tourism: Sustainable marine and coastal tourism can help preserve and restore natural landmarks. It is important for restoring aquatic ecosystems, increasing tourist attraction, and maximizing income.
7. Biotechnology: Marine biodiversity is a source of new bioactive biomolecules that can be used in different sectors.
8. Respect tradition: It is important to respect the cultures of coastal communities. Strategies must be developed to protect the environment while preserving traditions (Veríssimo et al., 2021).

The Blue Economy, also called "Ocean Economy," involves commercial ocean-based activities. These industries operate in a range of sectors (Table 3) including food, transport, construction, marine education and research, business, tourism, and energy (Veríssimo et al., 2021).

These sectors play a leading role in the Europe Blue Economy, and Germany, Spain, Italy and France collectively represent the largest contributors to the established sectors of the EU Blue Economy. Together, they account for 61% of gross value added (GVA) and 55% of employment (Langedijk et al., 2024).

Table 3. The Established Blue Economy sectors and their subsectors Source:(Langedijk et al., 2024).

Sector	Sub-Sector
Marine living resources	Primary production
	Processing of fish products
	Distribution of fish products
Marine non-living resources	Oil and gas
	Other minerals
	Support activities
Marine renewable energy	Offshore wind energy
Port activities	Cargo and warehousing
	Port and water projects
Shipbuilding and repair	Shipbuilding
	Equipment and machinery
Maritime transport	Passenger transport
	Freight transport
	Services for transport
Coastal tourism	Accommodation
	Transport
	Other expenditure

As evidenced in "The EU Blue Economy Report 2024" (Langedijk et al., 2024), the Blue Economy in Europe is poised for expansion in the coming years. This growth will be driven by the transition to a sustainable, circular economy, ambitious zero net emissions targets, and innovation and research (Langedijk et al., 2024).

The research sector is a pivotal element in this context, and one programme that merits particular attention at the European level is "Horizon Europe". It represents the European Union's Framework Programme for Research and Innovation for the period 2021-2027. Its objective is to optimize the scientific, technological, economic, and social impact of EU investment in research and innovation. This is pursued through three principal actions: the reinforcement of the Union's scientific and technological foundations, with a view to enhancing competitiveness at the level of all Member States; the provision of support for the EU's strategic priorities, thereby contributing to the formulation of European policies designed to address global challenges, including the Sustainable Development Goals set out in the 2030 Agenda; and finally, the consolidation of the European Research Area (Ricciardiello et al., 2021).

The Blue Economy in Italy represents a sector of fundamental importance, given that the country is surrounded by the sea for approximately 80%. The sea represents a productive resource that is linked to issues of environmental, social, and economic sustainability.

At the national level, to support the transition to a sustainable Blue Economy and to achieve a better balance between opportunities and fisheries capacity, the National Program for the European Maritime, Fisheries and Aquaculture Fund (NP EMFAF) has been established. This program aims to significantly enhance environmental sustainability (EMFAF programmes 2021 - 2027, 2021).

The NP EMFAF addresses three pivotal challenges that must be addressed to facilitate the evolution of the sector by 2030: the green transition, the digital transition, and resilience.

Among the planned interventions, which will be implemented in synergy with the National Plan for Recovery and Resilience (PNRR), the objective is to facilitate the transition of industrial processes towards a circular economy, including the blue economy (Mission 2), and to promote collaboration between businesses and researchers, supporting Horizon Europe partnerships, including the Italian partnership for a sustainable blue economy (Mission 4) (EMFAF programmes 2021 - 2027, 2021).

Among the Italian regions, Sicily is one where the Blue economy has a strong impact on the regional entrepreneurial fabric, with 5% of the total number of companies operating in this sector. The sea is a fundamental resource for Sicily, providing life, food, raw materials, energy, and links with other regions. A wide range of economic activities are based on the sea, including fisheries, transport, shipbuilding, nautical tourism, research, and environmental protection. At the provincial level, the territories where the blue economy is most deeply entrenched are Trapani and Palermo (Regione Siciliana - Assessorato Regionale Delle Attività Produttive, 2021).

In alignment with the PNRR and the national programming of the EMFAF, Sicily implements the strategy of the European Regional Development Fund (ERDF) Regional Programme (RP) at the regional level. This strategy is guided by the Regional Strategy Document (ex DGR 131/22) and aligns with the Regional Strategy for Sustainable Development, which localizes the objectives of the UN 2030 Agenda. The programme focuses on six strategic priorities, including green and digital transition, environmental sustainability, innovation, and social cohesion. Objectives related to the Blue Economy are mainly included in Priority 1 (A more competitive and intelligent Sicily) and Priority 2 (A greener Sicily) of the programme (RP Sicilia ERDF 2021-2027, 2021).

Priority 1 supports research and innovation activities of enterprises in cooperation with universities and research organizations. It also promotes private investments aimed at the industrialization of both public and private research results and technology transfer within the specialization areas outlined in Strategy 3. This priority focuses on sustainable development, the green economy, and the blue economy, with an emphasis on circular economy processes. Priority 2, promotes actions to improve

the environmental sustainability of economic activities, including the Blue Economy, using clean technologies and innovative processes that valorize by-products (RP Sicilia ERDF 2021-2027, 2021). Thus, to ensure the sustainable use of marine resources, it is crucial to make significant investments in infrastructure, coastal protection, and regulatory measures (Langedijk et al., 2024).

Valorization and applications of fish by-products

In 2022, global fisheries and aquaculture production increased by 4% compared to 2020, reaching 185.4 million tonnes of aquatic animals (Figure 5). The growth in fisheries and aquaculture production, and utilization, has led to a significant increase in fish waste generated worldwide from processing (Alfio et al., 2021; Coppola et al., 2021).

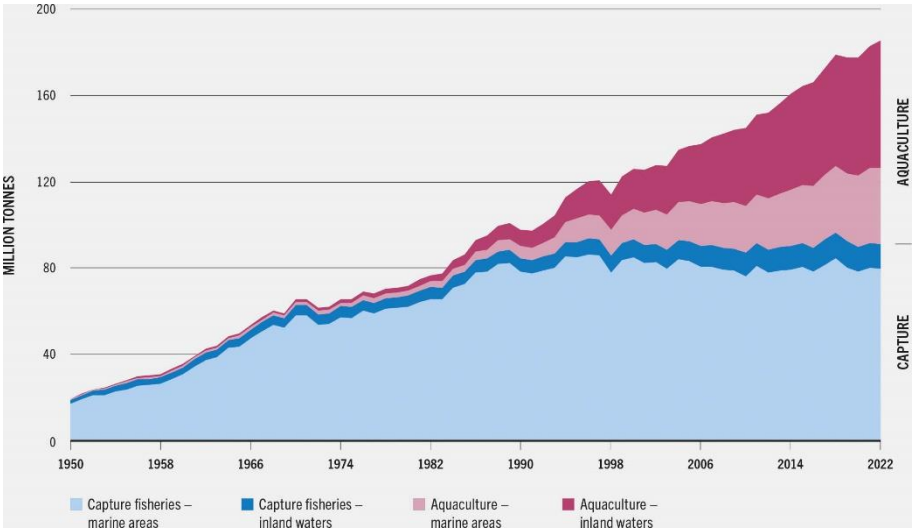

Figure 5.. World fisheries and aquaculture production of aquatic animals; (source: (FAO, 2024).

In the fishery supply chain, the "waste" fraction refers to: 1) by-catch, which includes fish species not directly intended for human consumption, as well as undersized and damaged specimens; 2) by-products from filleting and processing of edible resources from fisheries and aquaculture. (Alfio et al., 2021; Caruso et al., 2020; Debeaufort, 2021).

It is estimated that 7-10 million tonnes of catches are discarded each year (E:C: 2019) (https://oceans-and-fisheries.ec.europa.eu/fisheries/rules/discarding-fisheries_en), representing approximately two-thirds of the total fish caught in the supply chain and posing serious economic and environmental challenges (Caruso et al., 2020; Coppola et al., 2021).

The incidental capture of vulnerable species in fishing gear is a major threat to several taxonomic groups in the Mediterranean. The European Common Fisheries Policy's landing obligation was introduced in 2015 and has been fully implemented since January 2019. Its aim is to eliminate discards by encouraging fishermen to be more selective and avoid unwanted catches, while promoting sustainable fisheries (E:C: 2019).

In terms of seafood processing, it is estimated that 89% of total aquatic animal production is used for human consumption. Of this, 57% is processed, including frozen, prepared, and preserved, and cured

products (Figure 7) (FAO, 2024). It is widely acknowledged that fish processing generates a considerable quantity of by-products. These by-products can account for a significant portion of the total weight of processed fish, ranging from 30% to 70% depending on the species and the processing methods used. For example, by-products obtained from tuna pre-processing can reach 70%, and those from shrimp 40-60% (Abuzar et al., 2023; FAO, 2024; Messina et al., 2021b).

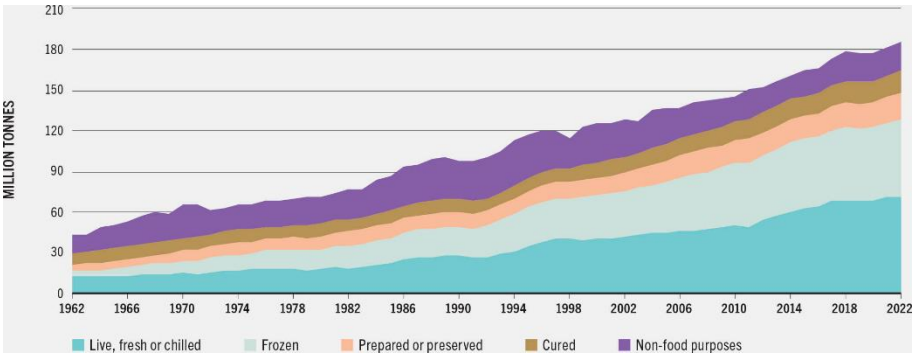

Figure 6. Utilization of world fisheries and aquaculture production of aquatic animals (source: (FAO, 2024).

Fishery by catch and seafood processing by-products represent not only a challenge in terms of management and disposal, but also an opportunity to develop innovative solutions (FAO, 2024; Välimaa et al., 2019; Al Khawli et al., 2019; Galanakis, 2012; Kim and Mendis, 2006; Ozogul et al., 2021; Rustad et al., 2011).

The valorization of fish waste (Figure 7) should be strongly promoted to achieve the "zero waste" goal, in line with the principle of the circular economy, and in addition, the valorization of waste should be carried out using technologies with a low environmental impact (Alfio et al., 2021; Caruso et al., 2020; Coppola et al., 2021; Nawaz et al., 2020). To maximize the effective use of fish waste and achieve the zero-waste goal, it is crucial not only to understand the composition of the biomass (in this case, fish waste from different sources), but also to assess the potential market value and applications of the biomolecules, chemicals and other by-products that can be isolated or converted from each type of waste (Veríssimo et al., 2021).

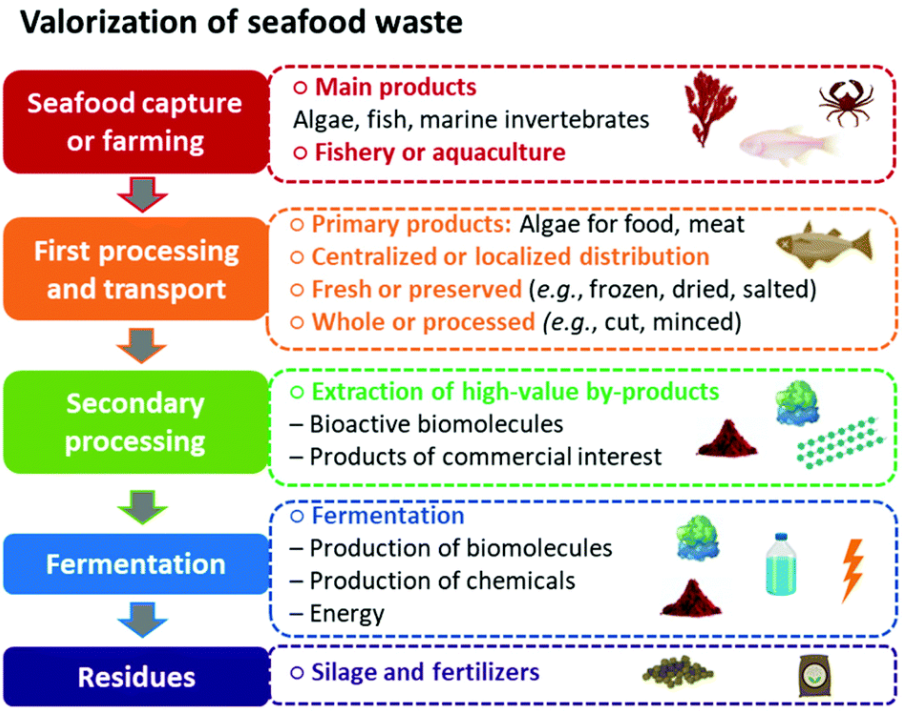

Figure 7. Valorization of seafood waste with a biorefinery approach (Veríssimo et al., 2021).

Fish by-products and bioactive compound

Fish by-products from fisheries, aquaculture and processing include of a variety of tissues and organs that can be divided into two groups: easily degradable products with high enzyme content (viscera and blood) and less perishable products (bones, head, and skin) (Al Khawli et al., 2019; Caruso et al., 2020). Heads, viscera, skin, bones and scales, accounting respectively for the 9-12%, 12-18%, 1-3%, 9-15% and 5% of the total fish weight (Figure 8), are recognized as valuable source of bioactive compounds (Al Khawli et al., 2019; Villamil et al., 2017). These include, essential polyunsaturated fatty acids (n-3 PUFA), bioactive peptides, oligosaccharides, enzymes, water-soluble minerals and antioxidants (Figure 8), which can be extracted by both conventional and advanced green technologies. These bioactive compounds have been shown to have beneficial effects on human health, such as anti-tumor, anti-depressant, anti-diabetic, anti-hyperglycemic, anti-hypertensive, anti-inflammatory, antimicrobial, antioxidant, anti-proliferative, anti-rheumatic and immunomodulatory properties, and are therefore used in the pharmaceutical, nutraceutical and cosmeceutical industries (Al Khawli et al., 2019; Ishak and Sarbon, 2018; Zamora-Sillero et al., 2018). These ingredients can also be used in the feed and food industries (Al Khawli et al., 2019; Alfio et al., 2021; Caruso et al., 2020; Coppola et al., 2021; Debeaufort, 2021; Kim and Mendis, 2006; Messina et al., 2021a, 2021b; Nawaz et al., 2020; Ozogul et al., 2021; Venugopal, 2021).

In addition, many molecules derived from marine by-products are particularly promising for the development of active packaging, such as biopolymers, bioactive agents, inorganic compounds, fibers, or nano- and micro-objects. The use of these bioactive components in this sector can not only extend the shelf life of food products, but also reduce the dependence on traditional plastics, thus facilitating recycling and improving the overall sustainability in packaging (Debeaufort, 2021).

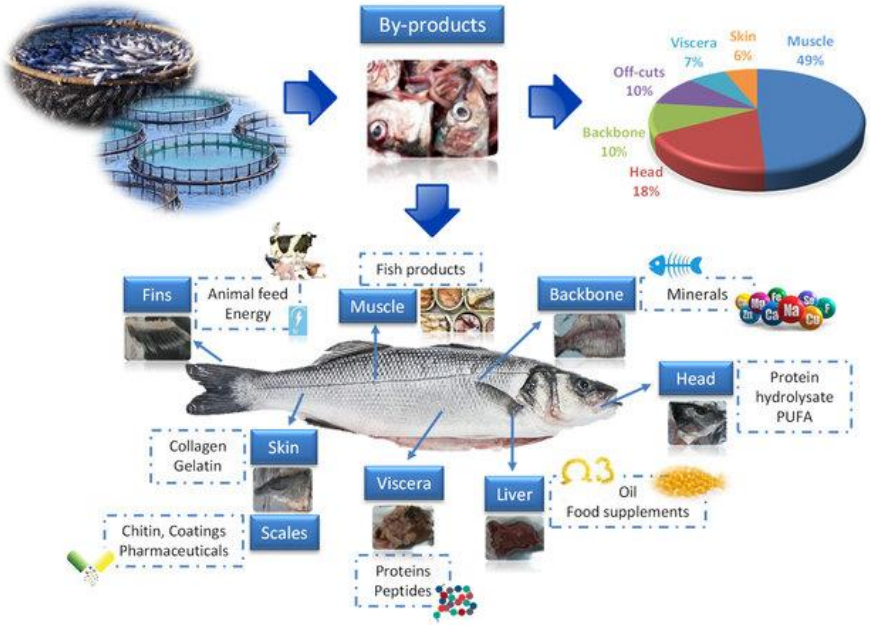

Figure 8. Fish by-products and bioactive compound (Source: (Al Khawli et al., 2019)*).*

The list of high value components that could be obtained from fish by-products is reported in Table 4.

Table 4. Content of high value components in fishery by-products (modified from Caruso et al., (2020).

Fishery By-Products	High Value Components	Content (% w/w)
Cod liver, mackerel oil	Polyunsaturated fatty acids-PUFA (n3 and n6)	50–80% in cod liver, 23% are w3 PUFA
Fish skin, scales, and bones	Hydroxyapatite	60–70% in bones, up to 50% in scales
Fish skin, scales, and bones	Collagen and gelatin	Up to 80% in skin, up to 50% in scales
Fish viscera	Enzymes	
Fish viscera	Polyunsaturated fatty acids-PUFA (n3)	50–60% in farmed cilthead sea bream viscera
White fish flesh residues	Free aminoacids	0.8–2% of taurin, 2.7% of creatine (on dry matter)

The main bioactive components that could be extracted from fish by-products are:

- *Lipids*. Fish by-products are a valuable source of high-quality fish oil, as they contain lipids (2-30%) in the form of oil, the concentration of which varies depending on the fish species (Caruso et al., 2020). Fish oil is particularly rich in n-3 long-chain polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These PUFAs, essential for fish, are synthesized in microalgae and accumulate significantly in fish along the food chain (Messina et al., 2021a). Fish oil can be extracted from fish by-products using a variety of techniques, ranging from chemical and enzymatic processes, cooking and pressing to more green technologies such as microwaves and supercritical fluids. The choice of the most appropriate method depends on a variety of factors, particularly the nature of the by-products and the final use of the oil (Coppola et al., 2021).

  The two main fish oil applications are feed/food supplements and biofuel production (Coppola et al., 2021). The main sectors requiring high quality oil are aquaculture and nutraceuticals. Since n-3 PUFA cannot be synthesized by human and are crucial for health, n-3 fatty acid concentrates are in high demand by the pharmaceutical and food industries to produce drugs and supplements (Caruso et al., 2020). Their beneficial bioactivities include prevention of atherosclerosis, arrhythmias, reduced blood pressure, reducing symptoms in asthma patients, alleviating the symptoms of cystic fibrosis, improving survival of cancer patients, reduction in cardiovascular disease and improved learning ability (Coppola et al., 2021; Khoddami, 2012; Kim and Mendis, 2006; Kim and Venkatesan, 2014; Messina et al., 2021a).

  Another relevant application of oil derived from fish by-products, is the production of environmentally friendly fuels, especially biodiesel (Coppola et al., 2021; Martins et al., 2015).

- *Proteins*. Fish by-products contain significant amounts of high-quality protein, due to the presence of essential amino acids such as lysine, valine, and phenylalanine. The protein composition of fish varies according to species and season. In fish muscle the principal proteins are grouped into three major groups according to their solubility: myofibrillar, sarcoplasmic and stromal proteins (Della Malva et al., 2018; Lionetto and Esposito Corcione, 2021). The technique for the extraction of proteins and amino acids is enzymatic hydrolysis (Caruso et al., 2020). With denaturing conditions required for their solubilisation and extraction, due to their structure and localization (Lionetto and Esposito Corcione, 2021).

  Fish protein is generally utilized as fishmeal, fish sauce and silage (Caruso et al., 2020), and due to their nutritional values, non-toxicity, biodegradability, and ability to form gels are used such biomaterials in the food industry (Lionetto and Esposito Corcione, 2021).

- *Bioactive peptides*. Marine organisms may be an excellent source of bioactive peptides (Harnedy and Fitzgerald, 2011). Hydrolysates and peptides have been isolated from different fish by-products using several extraction methods. Among these, acid-alkaline hydrolysis (using acid or alkaline reagents), enzymatic hydrolysis (using enzymes to hydrolyze fish waste) and fermentation (using microorganisms as enzyme sources) are the main techniques used. Enzymatic hydrolysis is particularly favored for the extraction of bioactive peptides for the food and pharmaceutical sectors, as it does not leave any residues of organic solvents or toxic chemicals. After the extraction phase, the peptides are purified and analyzed using techniques such as ultrafiltration, chromatography, and mass spectrometry (Coppola et al., 2021).

  Peptides contained in proteins extracted from fish by-products have antihypertensive, antithrombotic, immunomodulatory and antioxidant properties and have been used to prevent and treat several human pathologies (Caruso et al., 2020; Coppola et al., 2021; Messina et al., 2021b). Furthermore, peptides can be used not only in the health sector but also in the food and nutraceutical industries (Thirukumaran et al., 2022; Venugopal, 2022). One potential application of protein hydrolysates is their incorporation into edible films, which can protect foods from free radical-mediated oxidative damage during storage (Debeaufort, 2021; Hajji et al., 2021). These peptides are also increasingly used in the cosmetic sector, where they are incorporated into various formulations related to wrinkles and skin elasticity (Messina et al., 2021b; Thirukumaran et al., 2022; Venugopal, 2022).
- *Enzymes.* Fish by-products, particularly internal organs such as the stomach, pancreas, and intestines, contain many enzymes that are active at low temperatures, have high catalytic activities at relatively low concentrations and are stable in a wide pH and temperature range (Coppola et al., 2021). The largest group of enzymes found naturally in fish are proteases, which utilize different mechanisms of action to catalyze the hydrolysis of peptide bonds. Among these, the most studied include pepsin, trypsin, chymotrypsin, and collagenase (Coppola et al., 2021). Additionally, fish are rich in lipases, which are glycerol ester hydrolases that catalyze the hydrolysis of ester bonds in substrates including triglycerides, phospholipids, cholesterols, and vitamin esters. Lipases exhibit specificity in fatty acid, alcohol type and stereospecificity (Coppola et al., 2021).

  Proteases are the most important enzymes in the total enzyme market, mainly used in the food industry and as ingredients in detergents. Due to their properties, proteases derived from fish by-products can find applications in various industries such as detergents, food, pharmaceuticals, and agrochemicals (Coppola et al., 2021; Thirukumaran et al., 2022).

- *Collagen.* Fish by-products, due to their safety and solubility in neutral salt solutions and dilute acids, are a reliable source for obtaining collagen. Collagen is the major structural protein in fish skin, scales, bones, and fins. Fish collagen has important properties, such as more efficient absorption into the body and higher bioavailability than bovine or porcine collagen (Coppola et al., 2021; Sripriya and Kumar, 2015). However, the development of a sustainable extraction process for its commercial use is the main problem associated with the use of fish collagen (Carvalho et al., 2018). Several methods have been used to extract collagen from fish by-products. The most common is acid extraction, which uses acids (such as acetic acid or HCl) to solubilize the collagen chains (Coppola et al., 2021). Another technique is based on the use of pepsin to extract collagen. This technique involves treating the collagen fibers with pepsin to cleave specific regions to promote solubilization and increase the yield of collagen extraction (Coppola et al., 2021; Jafari et al., 2020).

  Partial hydrolysis and thermal denaturation of collagen lead to the production of gelatin. Collagen and gelatin properties are of significant importance for many application fields, representing excellent functional molecules for the cosmetic, pharmaceutical, biomaterial, food, and nutraceutical industries (Coppola et al., 2021; Debeaufort, 2021; Jafari et al., 2020).
- *Chitin and chitosan derivatives.* Chitin, a linear N-acetyl-d-glucosamine polysaccharide with β-(1-4) linkage, is the second most abundant natural polymer after cellulose and it is the most prevalent natural polymer from marine invertebrate shells. It can be extracted from marine by-products, especially from the shell of crustaceans and mollusks (Coppola et al., 2021; Hajji et al., 2021; Ozogul et al., 2021; Veríssimo et al., 2021). The conventional method for obtaining this biopolymer is chemical extraction, but this technique is environmentally unfriendly (Pachapur et al., 2016). This method involves an initial alkali treatment for deproteination followed by a hydrochloric acid treatment for demineralization (Ozogul et al., 2021; Venugopal, 2021). The green extraction methods used to obtain chitin, which overcome the chemical extraction drawbacks, include biological (such as lactic acid fermentation and other miscellaneous fermentations) and enzymatic treatments (Cahú et al., 2012; Hajji et al., 2014; Paul et al., 2015). Other innovative extraction techniques are based on the use of autoclaves (Al Hoqani et al., 2020; Sedaghat et al., 2017), microwaves (EL Knidri et al., 2019), or ultrasound (del Carmen Borja-Urzola et al., 2020; Huang et al., 2020).

  Since chitin is insoluble in water, to increase its solubility, it is possible to remove a large proportion of its acetyl groups by deacetylation and convert it into chitosan (Ozogul et al., 2021) by chemical (more widely used but not environmentally friendly) and enzymatic

extraction methods (Ozogul et al., 2021). Chitosan is a nontoxic, biodegradable polysaccharide with the ability to adsorb and chelate (Parthiban et al., 2017).

Chitin and Chitosan, due to their ability to form polyoxysalts and films, to chelate metals and their optical structure characteristics, are used in various fields, including clinical, cosmetic, food, pharmaceutical, agricultural, aquaculture, and environmental applications (Coppola et al., 2021; Debeaufort, 2021; Parthiban et al., 2017).

- *Natural pigments*. The main pigments in seafood by-products are carotenoids. These pigments, present in shellfish, krill, shrimp, crab, crayfish, and in salmon and trout, provide red and orange colors to some foods. Carotenoids are a large group of organic and lipophilic pigments known for their biological activities and their beneficial effects on human health in the prevention and treatment of a wide range of diseases (Messina et al., 2021b; Ozogul et al., 2021; Venugopal, 2022, 2021; Veríssimo et al., 2021). Astaxanthin is a carotenoid that is present in higher amounts in the crustaceans by-products such as lobster, shrimp, crab, and krill. It has antioxidant, anti-tumor, anti-inflammatory, anti-diabetic, immunomodulatory, and neuroprotective effects (Messina et al., 2021b).

  Astaxanthin can be extracted from crustacean by-products by different organic solvents (ethanol, hexane, petroleum ether, methanol, acetone, and chloroform) (Ozogul et al., 2021) or "green" solvents such as vegetable oils and their methyl esters or cod liver (Messina et al., 2021b; Parjikolaei et al., 2016; Razi Parjikolaei et al., 2017, 2015; Sachindra and Mahendrakar, 2005; Shahidi and Synowiecki, 1991; Yara-Varón et al., 2017). Furthermore, the "green extraction" of astaxanthin could also be carried out using supercritical CO_2 extraction (Ahmadkelayeh and Hawboldt, 2020; Al Khawli et al., 2019; Messina et al., 2021b, 2019; Radzali et al., 2014; Razi Parjikolaei et al., 2017).

  Due to their bioactive activity, carotenoids, and particularly astaxanthin, extracted from crustacean by-products can be used in various sectors such as nutraceuticals, cosmetics, food, and aquaculture (Messina et al., 2021b; Ozogul et al., 2021; Venugopal, 2022, 2021; Veríssimo et al., 2021).

Waste, by-products and end-of-waste legislation in the seafood supply chain

By applying circular economy principles, resource management can be efficiently improved, and the life and use of a product can be extended through recovery, reuse, and recycling processes. Waste can be transformed through a supply chain and become an input that feeds other production chains (Sehnem et al., 2021). In fact, waste still contains high valuable components that could be recovered, and these bioactive compounds could be used in different industrial sectors (cosmetics and nutraceuticals, food preservation and packaging products, pharmaceuticals, etc.) (Alfio et al., 2021; Caldeira et al., 2020; Coppola et al., 2021).

It is important to define when a waste can be considered by-product rather than end-of-waste. Waste, according to Article 3 of the Directive 2008/98/EC (European Commission, 2008) is defined:

> *"means any substance or object which the holder discards or intends or is required to discard".*

The Directive establishes a hierarchy to be applied in waste management (Article 4 (European Commission, 2008)) (Deselnicu et al., 2018; Zarbà et al., 2021) (Figure 9).

Figure 9. The waste hierarchy. (Source: https://environment.ec.europa.eu/topics/waste-and-recycling/waste-framework-directive_en.).

Thus, waste management plays a key role, following the EU waste hierarchy (Figure 9) that priorities prevention, preparation for re-use, recycling, and energy recovery as alternatives to disposal (Deselnicu et al., 2018; Zarbà et al., 2021). The ability to utilize production scraps as a raw material leads to a reduction in waste and less pressure on virgin raw materials, with a positive impact on the environment (Zarbà et al., 2021).

Directive (EU) 2018/851 (European Commission, 2018) amending Directive 2008/98/EC as part of a package of measures on the circular economy, strengthens the rules on waste prevention and

clarifies the "waste hierarchy" (Zarbà et al., 2021). According to the principle of the "waste hierarchy" (Figure 9), prevention is the pillar of this directive, supported by provisions on "extended producer responsibility". The directive also defined and clarified the definitions of "*by-products"* and "*end of waste status"* (Zarbà et al., 2021).

By-product and end-of-waste are two different concepts that are regulated differently, although both aim to reduce and reuse waste (Zarbà et al., 2021).

By-product is (Art. 184-bis, point 1 of Legislative Decree no. 152/2006 (Decreto Legislativo, 2006))

> *"(a) the substance or object originates in a production process, of which it is an integral part, and whose primary purpose is not the production of that substance or object;*
> *(b) further use of the substance or object is certain, in the same or a subsequent production or use process, by the producer or a third party;*
> *(c) the substance or object can be used directly without any further processing other than normal industrial practice;*
> *(d) further use is lawful, i.e., the substance or object fulfils all relevant product, environmental and health protection requirements for the specific use and will not lead to overall adverse environmental or human health impacts."*

The following conditions must be met in order for production residues to be considered as by-products rather than waste:

> *"(a) the substance or the object originates from a production process of which it forms an integral part and whose primary purpose is not the production of that substance or object;*
> *(b) it is certain that the substance or object will be used during the same or subsequent production or use by the manufacturer or by a third party;*
> *(c) the substance or object may be used directly without any further treatment other than normal industrial practice;*
> *(d) further use is legal, that is, the substance or object satisfies, for the specific use, all relevant product and health and environmental protection requirements and will not lead to overall negative impacts on the product, Environment, or human health"* (M.A.T.T.M, 2016).

According to the waste hierarchy (Figure 9), by-product represents the first level (prevention) because it has characteristics that make it possible to use it in another process and it cannot be defined as waste. It is the responsibility of the producer to decide whether to dispose of or use the production residues and to ensure that these residues meet all the necessary legal requirements. If one of these elements is not satisfied, the substance or object cannot be considered a by-product but is classified as waste (Zarbà et al., 2021).

However, if the waste undergoes recovery operations (preparing for re-use, second level of the waste hierarchy), it acquires a new status that makes it usable in other production processes: this is the end-of-waste status (Zarbà et al., 2021).

According to Art. 184-ter, of Legislative Decree no. 152/2006 (Decreto Legislativo, 2006) the end-of-waste status is defined as follows:

> *"A waste shall cease to be waste, when it has undergone a recovery operation, including recycling and preparing for re-use, and complies with specific criteria to be developed in accordance with the following conditions:*
> *(a) the substance or object is intended to be used for specific purposes;*
> *(b) a market or demand exists for such a substance or object;*
> *(c) the substance or object meets the technical requirements for the specific purposes and complies with existing regulations and standards applicable to products;*
> *(d) the use of the substance or object will not result in overall adverse environmental or human health impact"*

Therefore, as by-products and end-of-waste status are legally different, they need to be managed differently, taking into account what is defined by current regulations (Legislative Decree no. 152/2006 (Decreto Legislativo, 2006) (Zarbà et al., 2021).

By-products generated in the seafood supply chain are part of a specific category defined as *animal by-products not intended for human consumption.* These by-products are covered by the Regulation (EC) No 1069/2009 laying down health rules concerning animal by-products and derived products to reduce and prevent risks to public and animal health.

Animal by-products (ABPs) are defined as "*entire bodies or parts of animals which are not intended for human consumption*", and consequently, derived products are defined as "*items obtained from processing of animal by-products*" (EC No 1069/2009, 2009).

According to the regulation, ABPs are classified into three categories in relation to the health risk to humans, animals, and the environment. ABPs from the fisheries sector are included in category 3, the lowest risk category (EC No 1069/2009, 2009). ABPs from the fisheries sector include:

- *aquatic animals, and parts of such animals, except sea mammals, which did not show any signs of disease communicable to humans or animals;*
- *animal by-products from aquatic animals originating from establishments or plants manufacturing products for human consumption;*
- *the following material originating from animals which did not show any signs of disease communicable through that material to humans or animals:*

    - *shells from shellfish with soft tissue or flesh.*
- *aquatic and terrestrial invertebrates other than species pathogenic to humans or animals* (EC No 1069/2009, 2009).

Regulation (EC) No 1069/2009, (2009) lays down the regulatory principles and implementing procedures for the identification, classification, collection, transport, storage, processing, disposal, movement, import and export of ABPs. The Regulation lays down several obligations that operators must comply with, e.g., operators must ensure that animal by-products and derived products are accompanied during transport by a commercial document which must include information on the origin, destination, quantity, and description of these products (EC No 1069/2009, 2009).

Regulation (EC) No 1069/2009, (2009) also defines the health requirements for the disposal and use of ABPs according to their category. Category 3 ABPs from aquatic animals may be disposed of as waste by incineration, recovery, ensiled, composted, or transformed into biogas. In addition to these methods, it is also possible to request the authorization of alternative methods of use or disposal of ABPs, which can be initiated by the Commission, a Member State or a third party by means of an application. The application, together with an assessment report, is sent to the European Food Safety Authority (EFSA), which issues an opinion on the application. The entire by-product chain is under the supervision of the EFSA (EC No 1069/2009, 2009).

Objective of the study

The seafood sector in Sicily represents a fundamental component of the regional economy, and tradition, to the presence of fishery and fish processing industries, mainly in the geographic regions of Trapani, Palermo and Agrigento, and aquaculture plants. This sector produces by-products that are not well managed and which represent a resource for circular economy interventions.
The research sector provides fundamental tools for resource valorization by identifying innovative strategies and technologies that can be applied to support the circular economy. However, one of the main weaknesses of circular economy interventions is the effective transfer of these techniques to enterprises.

The aim of my PhD project was to develop techniques and processes for the valorization of fisheries, aquaculture and processing by-products through the recovery of bioactive components of high biological value and their use in other industrial sectors.
My project, is part of the PON “Research and Innovation” 2014-2020 - Action IV.5 "Doctorates on topics Green", in line with the European Green Deal and the Agenda 2030 SDGs, and the circular economy principles, that want to enhance the sustainability of the Sicilian seafood supply chain.

The project was developed in collaboration with the industrial partner Blu Ocean s.r.l., a company located in Western Sicily, specialized in III and IV class fish products. Furthermore, part of the project actives on the potential use of marine bioactive compounds in the bioactive packaging industry, was developed, at the University of Burgundy/AgroSupDijon Institute, under the supervision of Professor Frédéric Debeaufort, in the Unit Food and Microbiological Processes (UMR-PAM A02. 102)

The initial phase of the study was the identification of the most reliable and accessible sources of by-products from aquaculture, bycatch, and processing industries, to characterize their quality from a nutritional and biochemical point of view. The second phase of the study involved the optimization of extraction procedures, and the identification of bioactive compounds present in the by-products. This process enabled the isolation of molecules of interest, which are crucial for application in various fields. In the final phase, the extracted bioactive compounds were subjected to *in vitro* testing to determine their potential utility in feed, nutraceuticals, and other non-food applications. A specific focus was placed on the potential utilization of the extracted components within the nutraceutical, animal feed, and food sectors. For this reason, my thesis was divided into three principal sections (Figure 10):

- **Part 1 - Valorization of marine by products in nutraceutical sector**
- **Part 2 - Valorization of marine by products in aqua feed sector**
- **Part 3 - Valorization of marine by products in food sector**

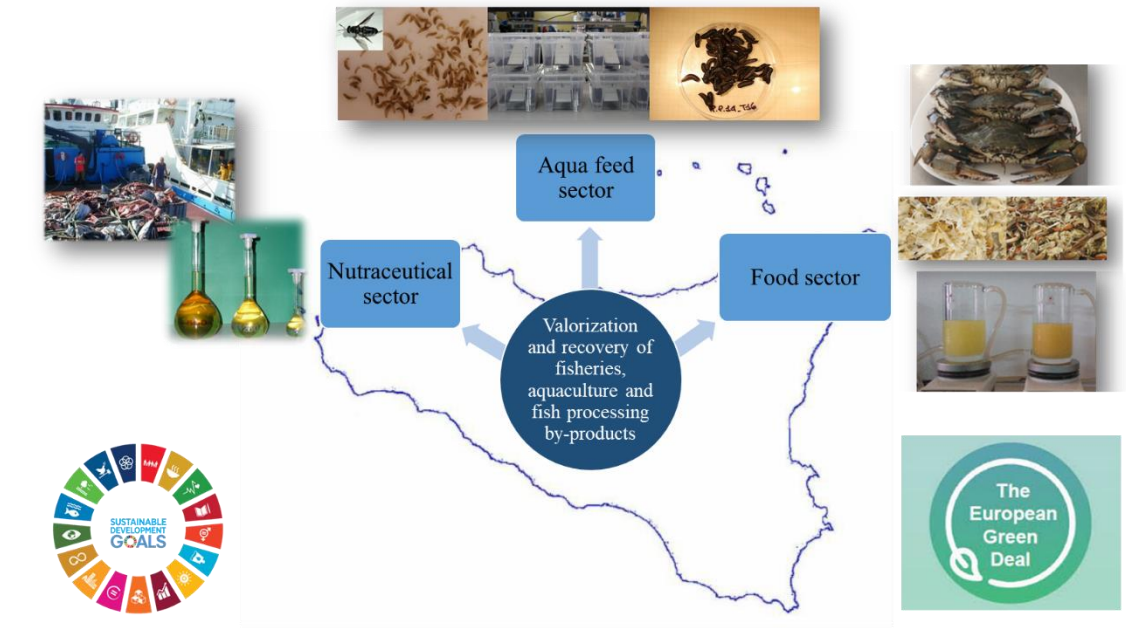

Figure 10. The potential uses of marine by-product extracts, focusing on their relevance in three sectors: nutraceuticals, animal feed and food.

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Part 1 - Valorization of marine by products in nutraceutical sector

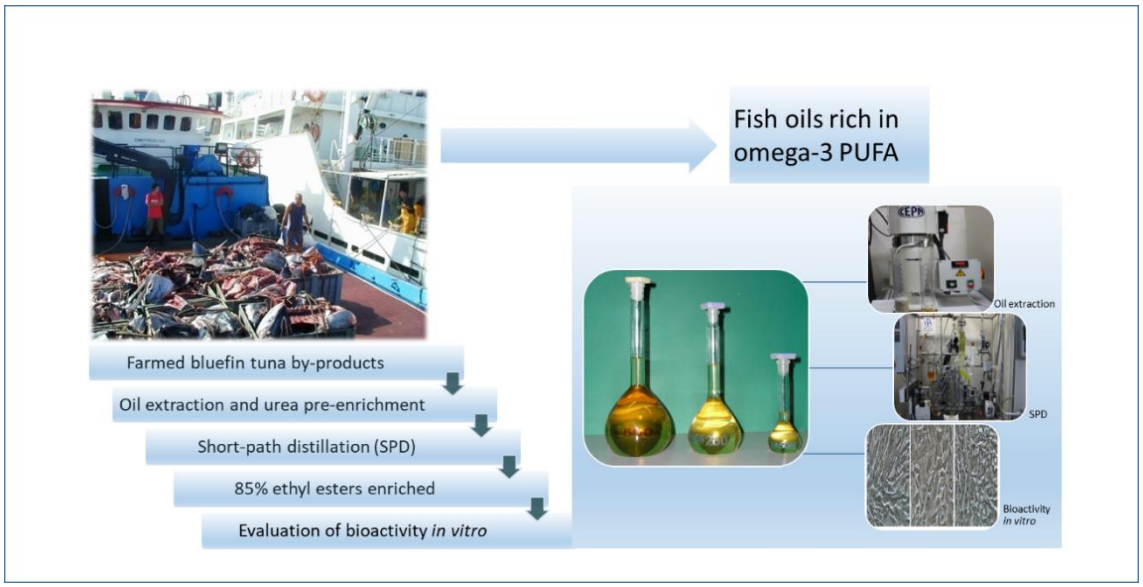
Chapter 1. Valorization of side stream products from sea cage fattened bluefin tuna (*Thunnus thynnus*): production and in vitro bioactivity evaluation of enriched n-3 polyunsaturated fatty acids

In this chapter, the side streams from bluefin tuna (*Thunnus thynnus*) filleting were identified and selected as reliable biomasses for potential utilization. Due to the high lipid content of bluefin tuna, its side streams could be an optimal resource for fish oil extraction. Indeed, the proximate composition and fatty acid profile of the bluefin tuna side streams were analyze, confirming their suitability for fish oil production. Using well-established techniques (Messina et al., 2021a), oil extracted from side stream of bluefin tuna was refined and enriched in omega-3 fatty acids by urea complexation (PUFA-Ue) and short path distillation (SPD). The omega-3-enriched oil, obtained from the side streams of farmed bluefin tuna, were tested *in vitro* to investigate the effect on adipogenesis and their potential use in fish feed formulation. The results showed that these ethyl esters could be utilized in fish feed formulations to prevent excessive fat deposition, contributing to improving both aquaculture sustainability and the quality of its products.

The results of this study are included in the published paper:

Messina, C.M., Arena, R., Manuguerra, S., Barbera, L. La, Curcuraci, E., Renda, G., Santulli, A., 2022. Valorization of Side Stream Products from Sea Cage Fattened Bluefin Tuna (*Thunnus thynnus*): Production and In Vitro Bioactivity Evaluation of Enriched ω-3 Polyunsaturated Fatty Acids. Mar. Drugs 2022, Vol. 20, Page 309.

https://doi.org/10.3390/MD20050309



Graphical abstract "Valorization of Side Stream Products from Sea Cage Fattened Bluefin Tuna (Thunnus thynnus): Production and In Vitro Bioactivity Evaluation of Enriched ω-3 Polyunsaturated Fatty Acids. Mar. Drugs 2022, Vol. 20, Page 309".

1.1. Abstract

The valorization of side streams from fishery and aquaculture value-chains is a valuable solution to address one of the challenges of the circular economy: turning wastes into profit. Side streams produced after filleting of sea cage fattened bluefin tuna (*Thunnus thynnus*) were analyzed for proximate composition and fatty acid profile to evaluate the possibility of producing tuna oil (TO) as a valuable source of n-3 polyunsaturated fatty acids (PUFA) and testing its bioactivity in vitro. Ethyl esters of total fatty acids (TFA), obtained from TO, were pre-enriched by urea complexation (PUFA-Ue) and then enriched by short path distillation (SPD) up to almost 85% of the PUFA fraction (PUFA-SPe). The bioactivity of TFA, PUFA-SPe, and ethyl esters of depleted PUFA (PUFA-SPd) were tested in vitro, through analysis of lipid metabolism genes, in gilthead sea bream (*Sparus aurata)* fibroblast cell line (SAF-1) exposed to oils. TFA and PUFA-SPd upregulated transcription factors (*pparβ* and *pparγ*) and lipid metabolism-related genes (*D6D*, *fas*, *fabp*, *fatp1*, and *cd36*), indicating the promotion of adipogenesis. PUFA-SPe treated cells were similar to control. PUFA-SPe extracted from farmed bluefin tuna side streams could be utilized in fish feed formulations to prevent excessive fat deposition, contributing to improving both the sustainability of aquaculture and the quality of its products.

Keywords: side streams; n-3 fatty acids; tuna fish oils; SAF-1 cell line; biomarkers; lipid metabolism; adipogenesis

1.2. Introduction

Global fish production, according to FAO statistics (FAO, 2020), reached about 179 million tons in 2018; 156 million tons were used for human consumption, and 22 million tons were for non-food uses, mainly to produce fishmeal and fish oil.

Fish processing and its significant expansion have led to increasing amounts of side stream (about 60% of processed fish) (de la Fuente et al., 2021; FAO, 2020; Thirukumaran et al., 2022). The worldwide production of fishery wastes has many implications for environmental and economic management sustainability and the protection of marine resources (Caruso et al., 2020).

The need to combine the sustainable management of marine resources with strong actions to recover the intrinsic value of side streams from fisheries and aquaculture is becoming a priority (Coppola et al., 2021; Messina et al., 2021a, 2021b). Actually, these side streams may represent unused or underutilized resources that still contain a wide number of components with high nutritional value (Galanakis, 2012; Kim and Mendis, 2006; Rustad et al., 2011). There is a growing interest in research and industry for the sustainable use of fisheries and marine organisms and processing side streams for the extraction of high biological value molecules (Caruso et al., 2020; García-de-Vinuesa et al., 2021; Kim and Mendis, 2006; Messina et al., 2021b, 2021a, 2013; Pateiro et al., 2020; Ucak et al., 2021), especially side streams which can be used to obtain molecules of biochemical interest such as fatty acids, peptides, antioxidants, and bioactive metabolites (Aspevik et al., 2021; Caruso et al., 2020; García-de-Vinuesa et al., 2021; Gnanavel et al., 2019; Lapi et al., 2021; Mayer and Gustafson, 2006; Messina et al., 2013), which can be utilized in nutraceutical, pharmaceutical, and animal feed fields (Caruso et al., 2020; Kim and Mendis, 2006; Messina et al., 2021b, 2021a; Pateiro et al., 2020; Ucak et al., 2021).

The use of side streams and their valorization might lead to a reduction in product losses and food waste, generating economic development from a resource that would otherwise be discarded, ensuring the achievement of the “zero waste” goal (Alfio et al., 2021; Coppola et al., 2021; Messina et al., 2021a; Regueiro et al., 2021; Stübler et al., 2020).

The valorization of biological waste from fishery and aquaculture is fundamental for implementing circularity in the bioeconomy and minimizing the environmental impact (Alfio et al., 2021; Coppola et al., 2021; Messina et al., 2021a; Regueiro et al., 2021; Stübler et al., 2020).

Processing side streams are an optimal resource for fish oil extraction (Alfio et al., 2021; Caruso et al., 2020; Messina et al., 2021a; Šimat et al., 2019), which composition varies considerably depending on the species and fishery season (Kim and Mendis, 2006).

Fish oils represent the main natural sources of long-chain n-3 polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), both used in aquaculture (feeds) and for direct human consumption (nutritional supplement capsules) (Caruso et al., 2020; Shepherd and Jackson, 2013). Furthermore, n-3 PUFA are of great interest to the pharmaceutical and food industries (Caruso et al., 2020; Ferraro et al., 2010). In fact, thanks to the numerous studies that have shown the beneficial effects on human health, there has been an exponential growth in the market of the n-3 PUFA for human consumption (Mentoor et al., 2019; Messina et al., 2021a; Mozaffarian and Wu, 2011; Omabe et al., 2015; Rimm et al., 2018; Schley et al., 2007; Siddiqui et al., 2008; Tarasiuk et al., 2018; Zamora-Sillero et al., 2019; Zárate et al., 2017). Even research for alternative lipid sources has grown considerably in recent years. Among them, vegetable oils, alternative marine oils (krill, copepod, and amphipod oils), etc. (Ofori-Mensah et al., 2020) are the main lipid resources for aquatic fodder.

The increasing utilization of vegetable oils in fish feed has caused a decrease in EPA and DHA, affecting lipid metabolism, fish fillet composition, and fatty acid quality (Antraco et al., 2021). Vegetable oils are rich in α-linolenic acid (ALA, 18:3n-3), and marine fish have a low capacity to convert ALA into EPA and DHA, which are essential for them (Ofori-Mensah et al., 2020; Tocher et al., 2019).

Developing strategies to prevent excessive fat deposition in farmed fish could be useful for their welfare, increasing the final product quality (Salmerón, 2018), and also enhancing the sustainability of the aquaculture industry (Todorčević et al., 2008). Fish oil, rich in n-3 fatty acid, is known to regulate both the storage and secretory functions of adipose tissue (Martins et al., 2020; Murali et al., 2014). It may also contain trans and saturated fatty acids that can have negative effects; therefore, it is needed to increase the n-3 PUFA levels, particularly EPA and DHA, through enrichment. (Wayan Suriani and Komansilan, 2019). There are several methods to enrich n-3 PUFA, such as chromatographic separation, fractional or molecular distillation, low-temperature crystallization urea complexation, supercritical CO_2 extraction, and enzymatic purification (Kahveci and Xu, 2011; Li et al., 2019; Tang et al., 2011).

Enrichment by urea complexation, using inexpensive solvents and simple equipment, is an efficient method for DHA enrichment. Furthermore, this technique has been shown to protect DHA from autoxidation (Chakraborty et al., 2010; Gámez-Meza et al., 2003; Li et al., 2019; Tang et al., 2011).

Short-path distillation (SPD) allows the distillation of temperature-sensitive products. This process is suitable for the treatment of lipids as it is a continuous separation process working under vacuum conditions with low evaporation temperature and short sample residence time (Xu, 2019).

For the n-3 PUFA enrichment, a combination of techniques, such as urea complexation followed by molecular distillation, are often used (Solaesa et al., 2016).

The aim of this study was to valorize the side streams obtained by the filleting of bluefin tuna (*Thunnus thynnus*) specimens BFT-F fattened in a sea cage in Castellammare del Golfo (Italy) by the production of n-3 polyunsaturated fatty acids tuna oil (TO). BFT fattening in the Mediterranean Sea was set up as a seasonal activity intended to introduce tuna from the wild into sea cages for three months, feed them with highly energetic fish to increase the body fat percentage, and obtain a better yield in the brief fishing period (Mylonas et al., 2010). In our study, BFT side streams (heads, fins, tails, and individual organs and tissues) were analyzed for proximate composition and fatty acid (FA) profile and for tuna oil (TO) extraction. Total fatty acids (TFA) extracted from the oil were enriched trough the combination of two techniques: urea complexation (PUFA-Ue), followed by short path distillation (SPD) (PUFA-SPe). The effects of TFA, PUFA-SPe, and ethyl esters depleted PUFA (PUFA-SPd), were tested in vitro in gilthead sea bream *(Sparus aurata)* fibroblast cell line (SAF-1) to investigate the effect on adipogenesis and their potential use in fish feed formulation.

1.3. Materials and Methods

1.3.1. Sampling

By-products of BFT-F were sampled during harvesting and filleting on board a fishing boat, together with the by-products of BFT-W and YFT-W, collected from a fishery.

For each lot, twelve samples were done (n = 12).

BFT-F, BFT-W, and YFT-W were placed in cold containers and transported to the laboratory (less than one hour), and stored at −80 °C until analyses.

1.3.2. Proximate Composition and FA Profile

Proximate composition and FA profile were analyzed from the by-products of BFTF, BFT-W, and YFT-W, homogenated on ice by Ultraturrax T25 (IKA, Labortechnik, Staufen, Germany). Each homogenate was used to assess the moisture content by the AOAC method (AOAC, 1990) and the crude protein content was found by the Kjeldahl method, multiplying the measured total nitrogen by the conversion factor 6.25 (AOAC, 1992). The total lipids were determined according to Folch et al. (Folch et al., 1957) and the FA methyl esters were obtained according to Lepage and Roy (Lepage and Roy, 1984), and their profile was obtained by gas chromatography, as described in Messina et al. (2013).

The fatty acid profile was utilized to evaluate the following indices:

- the polyene index (PI) (Equation (1)), used as a measure of PUFA damage (Lubis and Buckle, 1990):

$$PI= ((20:5n-3+ C22:6n-3)/C16:0) \quad (1)$$

where C20:5n-3 represents EPA, C22:6n-3 DHA and C16:0 palmitic acid.

- the atherogenicity index (AI) (Equation (2)), is a nutritional quality index for assessing the risk of platelet aggregation (Ulbricht and Southgate, 1991):

$$AI = (C12:0 + 4 \times C14:0 + C16:0)/(MUFA + PUFA) \quad (2)$$

where C12:0 represents lauric acid, C14:0 miristyc acid, C16:0 palmitic acid, MUFA monounsaturated fatty acid and PUFA polyunsaturated fatty acid.

- the thrombogenicity index (TI) (Equation (3)) [101], is a nutritional quality index representing the potential to form clots in the blood vessels

$$TI = [(C14:0 + C16:0 + C18:0)/(0.5 \times MUFA + 0.5 \times Totn-6 + 3 \times Totn-3 + (Totn-3/Totn-6))] \quad (3)$$

where C14:0 represents miristyc acid, C16:0 palmitic acid, C18:0 stearic acid, MUFA is monounsaturated fatty acid, Totn-6 the total amount of omega 6 fatty acid and Totn-3 the total amount of omega 3 fatty acids.

1.3.3. Extraction of Crude Oil

TO was extracted from 10 kg BFT-F side stream batches by wet extraction (Messina et al., 2021a; Sathivel et al., 2003).

Preheated distilled water was added to the ground BFT-F side stream at a 1:2 *w/v* ratio, and the mixture was incubated in a 50 L steel reactor equipped with an internal heating coil. Extraction was performed under constant agitation at 60 °C for 30 min, with optimal yields condition reported by Messina et al. (Messina et al., 2021a). Extraction mixtures were filtered on a 125 μm mesh sieve to remove the coarse particulates. The filtrate liquid phase was centrifuged at a centrifugal force of 40,000× *g* by a continuous tubular centrifuge (CEPA, Carl Padberg, Zentrifugenbau GmbH, Lahr/Schwarzwald, Germany) equipped with a separating cylinder (type TR).

The extraction mixture was fed at the bottom of the cylinder by a Masterflex L/S peristaltic pump equipped with tubing L/S 18 (Cole-Parmer s.r.l., Mi, Italy), with a throughput of 0.03 $L \cdot min^{-1}$. This

configuration allowed us to separate contemporary and continuous solids (retained in the cylinder), a heavy liquid phase (containing protein and cellular end tissue debris), and a light liquid phase (containing TO) that exited the cylinder in two separate fluxes. TO samples were refined as described by Messina et al. (2021a) and stored at −20 °C in 2.5 L dark bottles under nitrogen.

1.3.4. Assessment of Oil Quality

The quality of TO was evaluated by monitoring PV, TBARS, ρ-AV, TOTOX, and free fatty acid (FFA)% as described by Messina et al. (Messina et al., 2021a). Commercial cod liver oil was used as the control oil (CO) (Pearson, Italy).

1.3.5. Urea PUFA Enrichment

The batches of extracted tuna oil (2.5 L) were trans-esterified to obtain TFA without the use of any solvent other than ethanol, as reported by Vazquez and Akoh (2010). During transesterification, all liquid phase separations were carried out by a continuous tubular centrifuge equipped with a separating cylinder, as described above by Messina et al. (2021a).

TFA were mixed with ethanol and urea, a simple method for obtaining concentrated PUFA (Hsieh et al., 2005). SFA and MUFA bind easily to urea and crystallize at low temperatures. Crystals were removed by centrifugation and subsequently recovered with n-hexane to determine saturated residual fraction. PUFA-Ue supernatant was recovered with n-hexane and evaporated to obtain a concentrated sample. The PUFA-Ue fractions obtained after treatment with urea were diluted in c-hexane to final a 1% concentration for gas-chromatographic analysis.

The variation in the percentages of SFA, MUFA, and PUFA were calculated by equation (4):

$$\% \text{ FA variation} = (\Sigma\%\text{FA-Ue} - \Sigma\%\text{FA-TFA})/\Sigma\%\text{FA-TFA} \times 100 \qquad (4)$$

where Σ%FA-Ue is the percentage of FAs after urea complexation. Σ%FA-TFA is the percentage of FAs in the TFA (Crexi et al., 2012).

1.3.6. Shorth Path Distillation PUFA Enrichment

Batches of 2 L of PUFA-Ue were distilled by SPD using the VLK 70-4 molecular distillation unit (VTA Gmbh, Niederwinkling, Germany) with an evaporating surface of 4.8 dm^2.

Before PUFA enrichment, to remove impurities and any solvent traces, PUFA-Ue underwent a degassing step.

PUFA-Ue samples, preheated to 40 °C, were loaded into the feed vessel (at 40 °C) by a peristaltic pump. Distillation trials were run utilizing the following operating conditions: feeding vessel at 40 °C, condenser at 25 °C; evaporator at 160 °C (120 °C for degassing); feeding rate of 300 mL/h (500 mL/h for degassing); roller speed of 400 rpm; and vacuum of < 0.01 mbar (5 mbar for degassing).
A second enrichment test was performed by processing the ethyl esters at the evaporation temperature (160 °C), at which the highest PUFA concentration was obtained, and three cycles were repeated at the same temperature on the same enriched fraction to obtain a further concentration.
PUFA-SPe (heavy phase) and PUFA-SPd (distilled phase) were collected, and yields were determined gravimetrically.
In order to evaluate the enrichment process for every 1.0 L PUFA-Ue feed and at the end of distillation, aliquots of the two separated phases were diluted at 1% in c-hexane to analyze FA profiles by GC.
On the basis of the FA profile, the following indices were calculated:

- EPA and DHA%.
- FA ratio (R) (Equation (5)) (Liang and Hwang, 2000):

$$R = (C20:5n\text{-}3 + C22:6n\text{-}3)/(16:0 + 18:1n9) \quad (5)$$

  where C20:5n-3 represents EPA, C22:6n-3 DHA, 16:0 palmitic acid and 18:1n9 oleic acid.
- Enrichment factor for EPA, DHA, and PUFA.
- Ratio of total PUFA to total SFA (PUFA/SFA).

1.3.7. Sparus aurata fibroblast (SAF-1) cell culture

The SAF-1 cell line (ECACC No. 00122301) was cultured at 25 °C in Leibovitz L-15 medium supplemented with 2% l-glutamine, 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin and 15% foetal bovine serum (all reagents from Sigma-Aldrich, Saint Louis, MO, USA). Cells were seeded in 12-well plates (Nunc, Germany) (500,000 cells/well) and incubated for 24 h.
After 24 h, the cells were treated with TFA, PUFA-SPe, and PUFA-SPd dissolved in ethanol at a concentration of 5 µg/mL in the medium, with a final solvent concentration of 0.1% (*v/v*), and left to incubate for 48 h. As attested by routine internal procedures (Messina et al., 2021a), ethanol did not exert any detrimental effects when used as a vehicle.
Then, the medium was removed and cells were washed using PBS, and 1 mL of PUREzol (Bio-Rad, Hercules, CA, USA) was added.

1.3.8. Image Acquisition

Cells were observed daily using an inverted microscope Nikon Eclipse Ti-S (Nikon Instrument Inc., Melville, NY, USA), and the images were obtained with a Nikon DS-L3 digital camera (Nikon Corporation, Tokyo, Japan) and the DS-L3 Digital Camera Controller acquisition software. The images represent the SAF-1 cells observed with phase-contrast microscopy at 40X magnification.

1.3.9. RNA isolation and Rt-qPCR

The total RNA was isolated from SAF-1 cells using the RNA extraction kit (Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules, CA, USA). Then, 1 µg of the total RNA was converted into cDNA using the 5X iScript Reaction Mix Kit (Bio-Rad, Hercules, CA, USA). RT-qPCR was performed in a 20 µL reaction system using the 1X IQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The relative mRNA level of a target gene was quantified using the comparative cycle threshold ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001). The *18s* of the endogenous reference and the relative quantification of (*pparβ, pparγ, D6D, fas, cd36, fatp1*, and *fabp1*) gene expression was evaluated after normalization with the reference genes. Data processing and statistical analyses were performed using the CFX Manager Software (Bio-Rad, Hercules, CA, USA). The primers used are shown in Table1.

Table 1. S. aurata primer sequences used for real-time PCR.

Gene	F/R Primer sequence (5'–3')
pparβ	Fw CAGGTGACTTTGCTGAAGTA
	Rv TAAAGGGCTTTCTTAAGCTG
pparγ	Fw CTCAAGAGTCTCAGGAAACC
	Rv GATAATGACAGCCAGAAACA
D6D	Fw TACCTTCGCTACCTGTGCTG
	Rv TTGCAGGTGGTTGTAACTG
fas	Fw TGCCATTGCCATAGCACTCA
	Rv ACCTTTGCCCTTTGTGTGGA
cd36	Fw GTCGTGGCTCAAGTCTTCCA
	Rv TTTCCCGTGGCCTGTATTCC
fatp1	Fw CAACAGAGGTGGAGGGCATT
	Rv GGGGAGATACGCAGGAACAC
fabp1	Fw CATGAAGGCGATTGGTCTCC
	Rv GTCTCCAAGTCTGCCTCCTT
18s	Fw TGTGCCGCTAGAGGTGAAATT
	Rv GCAAATGCTTTCGCTTTCG

1.3.10. Statistical Analysis

Results are reported as mean ± standard deviation. A comparison of the two treatments was conducted using the Student's t-test. Statistical differences among different treatments were evaluated for each parameter with analysis of variance (ANOVA). Student-Newman-Keuls post-hoc tests were performed in order to make multiple comparisons between experimental groups. The degree of heterogeneity was measured by the Cochran test (Underwood, 1997). Differences were considered statistically significant when $p < 0.05$. Data were processed by Statistica (version 8.0, Statsoft, Inc., Tulsa, OK, USA).

1.4. Results and Discussion

1.4.1 Recovery of processing side stream from sea cage fattened bluefin tuna

Raw fish oil quality depends mainly on the raw material used for its production: the better the quality, the fattier and fresher the fish. The lipid content and the proximate composition of fish side stream changes according to the species, season, and growing conditions (Šimat et al., 2020).

Results were compared with those obtained from the analysis of side streams of fresh wild bluefin tuna (BFT-W) and frozen yellowfin tuna (*T. albacares*) (YFT-W).

The obtained results (Table 2) showed a higher total lipid content in BFT-F compared to BFT-W and YFT-W (Table 2).

Table 2. Proximate composition (mean ± SD) (g/100g) of total side stream minced from wild (BFT-W), farmed (BFT-F-<60kg; BFT-F->200kg) bluefin tuna, and wild yellowfin tuna (YFT-W).

	Moisture	Ash	Total Lipid	Protein
BFT-W	62.1 ± 2.0^{c}	4.8 ± 0.4^{a}	19.6 ± 1.5^{b}	13.3 ± 3.4^{a}
BFT-F >200kg	46.8 ± 1.8^{a}	5.52 ± 2.1^{a}	32.1 ± 2.1^{c}	14.2 ± 1.8^{a}
BFT-F<60kg	57.1 ± 1.1^{b}	4.25 ± 0.5^{a}	22.5 ± 1.2^{b}	19.2 ± 2.4^{b}
YFT-W	61.0 ± 1.5^{c}	6.4 ± 1.1^{b}	10.8 ± 0.8^{a}	20.5 ± 3.2^{c}

Means in the same column with different superscript letters are significantly different ($p < 0.05$).
The data are reported as mean ± standard deviation, n = 12.

The high lipid content observed in the BFT-F side streams was in agreement with Šimat et al., (2020) and were determined by the rearing and feeding conditions that are responsible for the increased fat deposition (Mylonas et al., 2010). This observation highlights the suitability of this matrix for fish oil production (Šimat et al., 2020).

Since BFT-F, due to its high lipid content, represents the most suitable matrix for fish oil extraction, the fatty acid profile was evaluated (Table 3).

Table 3. FA profile (relative percentage with respect to total FAs) of total pooled side streams from BFT-F>200kg.

FA	BFT-F
14:0	5.79±0.18
16:0	20.16±0.96
16:1n -7	6.84±0.45
16:2n-4	1.03±0.02
16:3n-4	0.56±0.19
18:0	4.43±0.13
18:1n-9	15.71±0.61
18:1n-7	6.78±0.27
18:2n-6	1.22±0.03
18:3n-3	6.03±0.59
20:1n-9	2.77±0.09
20:4n-6	0.54±0.01
20:4n-3	0.29±0.01
20:5n-3 (EPA)	9.93±0.36
22:1n-11	4.30±0.57
22:6n-3 (DHA)	13.64±0.56
SFA	30.38±0.71
MUFA	36.40±1.36
PUFA	33.22±1.72
Tot n-3	29.89±1.49
Tot n-6	1.75±0.03
EPA+DHA	23.57±0.92
PI	1.17±0.09
AI	0.62±0.01
AT	0.24±0.01

The data are reported as mean ± standard deviation, n = 12.
SFA Saturated fatty acids; MUFA Monounsaturated fatty acids; PUFA Polyunsaturated fatty acids; PI polyene index; AI atherogenicity index; TI thrombogenicity index.

Monounsaturated fatty acids (MUFA) were the most abundant class of total fatty acids (36.40±1.36%), the major fatty acid was 18:1n-9. The second most abundant class was PUFA (33.22±1.72%) followed by saturated fatty acids (SFA) (30.38±0.71%) where the most important SFA was the palmitic acid (C16:0; 20.16±0.96) (Table 3).

Regarding PUFA, bluefin tuna is considered a good source of n-3 fatty acids, especially DHA, in fact DHA (13.64±0.56%) was higher than EPA (9.93±0.36%) (Table 3). The sum of DHA and EPA reached about 24%. Similar results were observed in wild and farmed bluefin tuna muscle (Topic Popovic et al., 2012).

These results (Table 3) show that BFT-F side streams side streams have significant EPA and DHA contents, confirming their possible utilization for the extraction of oils rich in n-3 fatty acids (Schneider, 2013).

1.4.2. Extraction, yield and quality of tuna oil (TO)

TO extraction was performed at 60 °C for 30 min, identified as optimal conditions by Messina et al. (2021a) and subsequently refined. The yield obtained was 75±4.52%, higher compared to Ferdosh et al. (2013) from long-tail tuna (*Thunnus tonggol*) heads. This difference depends on species, by-products used and the extraction technique employed. De la Fuente et al. (2022) reported that fish oil extracted from Atlantic salmon by-products showed different yields in relation to by-product used (57±1%, 56±2% and 77±2% for spines, heads and viscera, respectively).

In our study, high lipid content in the side streams by BFT-F (Table 2) combined with the extraction efficiency resulted in high yields.

The quality results of TO and their comparison with cod liver oil (CO) are shown in Table 4.

Table 4. TO quality determined by: Peroxide Value: PV; ρ-Anisidine: ρ-AV; Thiobarbituric acid reactive substances: TBARS; Total Oxidation Value: TOTOX; Acid value and Free Fatty Acid percentage: FFA%. Commercial Cod liver oil (CO) was used as control.

	TO	CO
PV (meqO_2/kg)	2.96±0.55^b	2.10±0.53^a
ρ-AV	12.93±1.53^b	4.96±0.89^a
TBARS (MDA µg/g)	11.81±2.45^b	5.51±0.81^a
TOTOX	20.80±0.88^b	10.83±0.24^a
Acid value (FFA%)	2.25±0.32^b	0.50±0.04^a

Different superscript letters in the same raw indicate significant differences ($p < 0.05$). The data are reported as mean ± standard deviation (n = 12).

Among the quality parameters for oil, the peroxide value (PV) was evaluated. This parameter allows for the assessment of the rancidity of the oil (Othman and Ngassapa, 2010), monitoring the formation of hydroperoxides (Bako et al., 2017; Šimat et al., 2019).

The PV obtained was 2.96 meqO_2/kg, which is comparable to the PV values obtained by Šimat et al. (2020, 2019), in oil obtained from tuna by-products. However, the PV value for TO is higher than that of CO, which had a PV of 2.10±0.53. This indicates that TO shows a slightly higher degree of oxidation than CO, but both the CO and the TO may be suitable for human consumption as it is below ≤5 meq O_2/kg (Codex Alimentarius Commission, 2017; Šimat et al., 2019).

Other parameters related to the oxidation were evaluated, such as ρ-anisidine (ρ-AV), thiobarbituric acid reactive substances (TBARS), and total oxidation value (TOTOX) (Table 4). Compared to commercial cod liver oil (CO), the TO had higher levels of ρ-AV, TBARS and TOTOX, indicating a higher level of oxidation. The observed values were lower than those obtained by Šimat et al. (2020, 2019) on tuna by-products, but were comparable to the values observed by Franklin et al. (2020) in oil extracted from yellowtail fish waste by supercritical CO_2 extraction. A lower TBARS value is related to at the use adsorbents, such as charcoal powder and Fuller's earth, known for their capacity

to adsorb primary and secondary oxidation compounds (Chakraborty and Joseph, 2015). In addition, the ρ-AV, 12.93 ± 1.53, was below the limit for the acceptability of fish oil for human consumption, equal to ≤20% (Crexi et al., 2012).

The low values (2.25%) of FFA in TO confirmed that the temperatures during extraction and refining did not cause significant hydrolysis, as observed by Šimat et al (2020, 2019). The observed value was less than 3% as recommended for edible oils (Codex Alimentarius Commision, 2017; EFSA Panel on Biological Hazards, 2010). It is important to obtain low FFA values since high values might lead to difficulties in the n-3 extraction (Bako et al., 2017; Šimat et al., 2019).

1.4.3. Pre-Enrichment by Urea Complexation

Total methyl esters FAs were extracted, ethyl esters (TFA) were produced by transesterification of the FAs, and preliminary fractionation by urea complexation was carried out.

Through urea complexation, a simple, quick, and efficient technique, it was possible to separate FAs according to their degree of unsaturation (González-Fernández et al., 2020; Magallanes et al., 2019). It was observed that the urea complexation treatment was efficient in extracting polyunsaturated fatty acid methyl esters and used methanol as a solvent (Lopes da Silva et al., 2018). This technique is easy to scale-up, it is environmentally sustainable, and it has been used to valorize byproducts of fish in the canning industry (Lopes da Silva et al., 2018).

FA profiles are shown in Table 5.

The TO showed a predominance of MUFA and PUFA content (Table 5) greater than 50% of total FA, suggesting that oils extracted from BFT-F side streams are a rich source of unsaturated FA, as reported by Šimat et al. (2020).

The variation in the percentage of PUFA and SFA in PUFA-Ue was calculated according to Equation (4) (Section 1.3.5.). An increase equal to 86% was observed in PUFA content, while a 45% decrease was observed in SFA. In particular, an increase in DHA (22:6n-3) was observed, with a final value equal to 22.50 ± 0.45% (Table 5).

A significant increase in PUFA through this selective enrichment was previously observed by several authors (Crexi et al., 2012; González-Fernández et al., 2020; Magallanes et al., 2019; Wayan Suriani and Komansilan, 2019).

The use of this technique is advantageous not only for its operational simplicity but also because it is environmentally friendly, as inexpensive and renewable materials (urea and ethanol or methanol as a solvent) are used (González-Fernández et al., 2020; Hayes, 2006).

Polyene index (PI), which indicates PUFA damage and oxidation, showed a significant increase ($p < 0.05$) after urea pre-enrichment.

In general, a decrease in PI values in fish oil suggests the degradation of PUFA (Šimat et al., 2020); in our study, the increase in PI was related to the significant increase in PUFA, particularly DHA (Table 5).

Table 5. FA profile (relative percentage with respect to total FAs) of total methyl esters in Tuna Oil (TO), total FA ethyl esters (TFA), and samples pre enriched by urea complexation (PUFA-Ue).

FA	TO	TFA	PUFA-Ue
14:0	6.82±0.11^{b}	6.99±0.10^{b}	4.37±0.18^{a}
16:0	21.15±0.31^{c}	19.38±0.22^{b}	9.21±0.23^{a}
16:1n-7	7.27±0.09^{c}	6.24±0.09^{b}	4.09±0.06^{a}
16:2n-4	1.53±0.19^{b}	1.34±0.11^{b}	0.27±0.03^{a}
16:3n-4	0.84±0.13^{b}	0.79±0.03^{b}	0.41±0.08^{a}
18:0	3.75±0.22^{b}	2.85±0.12^{a}	3.73±0.05^{b}
18:1n-9	15.42±0.4^{c}	13.80±0.3^{b}	8.52±0.35^{a}
18:1n-7	6.66±0.13^{b}	6.96±0.31^{b}	3.67±0.28^{a}
18:2n-6	1.21±0.02^{a}	3.25±0.08^{b}	5.29±0.09^{c}
18:3n-6	0.44±0.29^{a}	0.99±0.25^{a}	2.50±0.12^{b}
18:3n-3	0.92±0.09^{a}	0.89±0.15^{a}	8.47±0.1^{b}
20:1n-9	3.00±0.42^{a}	3.04±0.18^{a}	3.66±0.34^{a}
20:4n-6	0.59±0.04^{a}	0.34±0.21^{a}	2.38±0.22^{b}
20:4n-3	0.44±0.08^{a}	0.20±0.13^{a}	1.78±0.07^{b}
20:5n-3	9.73±0.35^{a}	10.26±0.05^{a}	10.96±0.03^{b}
22:1n-11	4.84±0.35^{a}	5.02±0.32^{a}	5.06±0.18^{a}
22:1n-9	0.16±0.05^{a}	0.14±0.03^{a}	0.16±0.04^{a}
22:4n-6	0.41±0.10^{a}	1.37±0.29^{b}	1.53±0.09^{c}
22:5n-3	1.21±0.05^{a}	1.25±0.09^{a}	1.43±0.012^{b}
22:6n-3	13.60±0.08^{a}	14.90±0.16^{b}	22.50±0.45^{c}
SFA	31.72±0.90^{a}	29.23±1.21^{a}	17.31±1.51^{b}
MUFA	37.36±1.88^{c}	35.19±1.18^{b}	25.16±1.18^{a}
PUFA	30.92±1.08^{a}	35.58±1.19^{b}	57.53±1.35^{c}
Tot n-3	25.90±0.65^{a}	27.50±0.95^{b}	45.14±1.75^{c}
Tot n-6	2.20±0.38^{a}	4.96±0.34^{b}	9.20±0.86^{c}
EPA+DHA	23.33±0.98^{a}	25.15±0.75^{b}	33.46±1.02^{c}
PI	1.10±0.02^{a}	1.30±0.03^{b}	3.63±0.25^{c}
AI	0.71±0.09^{b}	0.67±0.09^{b}	0.32±0.02^{a}
TI	0.29±0.04^{b}	0.27±0.02^{b}	0.11±0.01^{a}

Different superscript letters in the same row indicate significant differences ($p < 0.05$). The data are reported as mean ± standard deviation, n = 12.

The lipid quality indices, atherogenetic index (AI), and the thrombogenetic index (TI), which indicate the overall dietary quality of lipids and their potential effect on coronary heart disease prevention (Görgün and Zengin, 2015; Jankowska et al., 2010), showed an improvement in oil quality, highlighting significantly lower values due to the significant increase in PUFA and decrease in SFA and MUFA ($p < 0.05$) (Görgün and Zengin, 2015) (Table 5).

1.4.4. PUFA Enrichment by SPD

The PUFA-Ue was subjected to three cycles of SPD to enrich the PUFA content in the PUFA-SPe fraction, via the elimination of the fraction containing short-chain fatty acid ethyl esters (PUFA-SPd). Table 6 shows the differences among the PUFA-Ue, PUFA-SPe, and PUFA-SPd fatty acid profiles.

Table 6. FA profile (relative percentage with respect to total FAs) of the oils, pre enriched by urea (PUFA-Ue), enriched in PUFA (PUFA-SPe), and exhausted in fatty acid ethyl esters (PUFA-SPe), obtained by short path distillation (SPD) at 160 °C.

FA	PUFA-Ue	PUFA-SPe	PUFA-SPd
14:0	4.37±0.18^{b}	0.86±0.03^{a}	6.39±0.49^{c}
16:0	9.21±0.23^{b}	2.23±0.06^{a}	14.38±0.53^{c}
16:1n-7	4.09±0.06^{b}	0.99±0.01^{a}	5.99±0.12^{c}
16:2n-4	0.27±0.03^{b}	0.22±0.01^{b}	0.07±0.03^{a}
16:3n-4	0.41±0.08^{b}	0.26±0.00^{a}	0.80±0.12^{c}
18:0	3.73±0.05^{b}	1.64±0.06^{a}	4.37±0.24^{c}
18:1n-9	8.52±0.35^{b}	2.39±0.19^{a}	14.58±0.11^{c}
18:1n-7	3.67±0.28^{b}	0.95±0.03^{a}	4.52±0.14^{c}
18:2n-6	5.29±0.09^{b}	3.14±0.06^{a}	5.29±0.67^{a}
18:3n-6	2.50±0.12^{b}	1.48±0.16^{a}	2.86±0.23^{b}
18:3n-3	8.47±0.10^{b}	8.33±0.10^{b}	4.99±0.28^{a}
20:1n-9	3.66±0.34^{b}	1.29±0.06^{a}	5.70±0.27^{a}
20:4n-6	2.38±0.22^{b}	2.63±0.13^{b}	0.48±0.08^{a}
20:4n-3	1.78±0.07^{b}	1.77±0.00^{b}	0.85±0.06^{a}
20:5n-3	10.96±0.03^{b}	17.59±0.59^{c}	4.85±0.45^{a}
22:1n-11	5.06±0.18^{b}	2.10±0.06^{a}	5.20±0.17^{b}
22:1n-9	0.16±0.04^{a}	0.79±0.00^{b}	0.26±0.05^{a}
22:4n-6	1.53±0.09^{b}	1.86±0.03^{c}	0.68±0.13^{a}
22:5n-3	1.43±0.12^{a}	2.97±0.20^{b}	1.49±0.01^{a}
22:6n-3	22.50±0.45^{b}	46.51±0.34^{c}	16.25±0.14^{a}
SFA	17.31±1.51^{b}	4.73±0.03^{a}	25.13±0.20^{c}
MUFA	25.16±1.18^{b}	8.51±0.10^{a}	36.26±0.42^{c}
PUFA	57.53±1.35^{b}	86.76±0.07^{c}	38.61±0.22^{a}
Tot n-3	45.14±1.75^{b}	77.17±0.24^{c}	28.44±0.52^{a}
Tot n-6	9.20±0.86^{c}	7.63±0.20^{b}	6.44±0.87^{a}
EPA+DHA	33.46±1.02^{b}	64.10±0.25^{c}	21.09±0.31^{a}
PI	3.63±0.25^{b}	28.81±0.95^{c}	1.47±0.03^{a}
IA	0.32±0.02^{b}	0.06±0.00^{a}	0.53±0.02^{c}
IT	0.11±0.01^{b}	0.02±0.00^{a}	0.23±0.01^{c}
R	1.89±0.23^{b}	13.95±0.83^{c}	0.73±0.01^{a}
EPA Enrichment Factor	1.00±0.07^{b}	1.54±0.05^{c}	0.90±0.08^{a}
DHA Enrichment Factor	1.00±0.07^{b}	1.99±0.01^{c}	0.69±0.01^{a}
PUFA Enrichment Factor	1.00±0.07^{b}	1.45±0.02^{c}	0.70±0.01^{a}
PUFA/SFA	3.32±0.25^{b}	18.35±0.10^{c}	1.54±0.01^{a}

Different superscript letters in the same row indicate significant differences $p < 0.05$). The data are reported as mean ± standard deviation, n = 12.

Using the SPD technique, SFA and short-chain fatty acids were distilled into the light phase (Oliveira and Miller, 2014; Solaesa et al., 2016). In fact, in PUFA-SPe, a significant decrease ($p < 0.05$) of SFA (4.73 ± 0.03%) was observed compared to PUFA-Ue (17.31 ± 1.51%). In PUFA-SPd, the SFA amount was significantly increased ($p < 0.05$) compared to PUFA-Ue, reaching a value of 25.13 ± 0.20% (PUFA-SPd) (Table 6).

The SPD temperature used (160 °C), defined in previous studies (Messina et al., 2021b, 2021a), allowed us to obtain a PUFA content of 86.76 ± 0.07% (Table 6). PUFA were increased in the PUFA-SPe (from 57.53% to 86.76%), while those in the PUFA-SPd fraction were depleted (38.61%) (Table 6).

Higher temperatures (around 200 °C), might compromise PUFA, causing their degradation (Liang and Hwang, 2000; Marsol-Vall et al., 2020).

The increase in PUFA in PUFA-SPe was confirmed by the R-parameter, indicating the ratio of the sum of EPA (20:5n-3) and DHA over the sum of 16:0 and 18: 1 (Liang and Hwang, 2000). R increased significantly ($p < 0.05$) in PUFA-SPe (13.95 ± 0.84), while decreased in PUFA-SPd (0.73 ± 0.01) ($p < 0.05$) in respect to PUFA-Ue (Table 6).

In addition, in PUFA-SPe, a significant increase of EPA (17.59 ± 0.59%) and DHA (46.51 ± 0.34) values were observed. Furthermore, EPA Enrichment Factor (1.54 ± 0.05) DHA Enrichment Factor (1.99 ± 0.01), and PUFA Enrichment Factor (1.45 ± 0.02) showed a significant increase value ($p < 0.05$) compared to PUFA-Ue.

One of the main advantages of SPD is that it does not require chemical treatments during processing, and refined fish oils can be obtained as described by Oliveira and Miller, (2014). Solaesa et al. (2016) demonstrated that SPD could contribute to the concentration of omega- 3 PUFA obtained from an enzymatic glycerolysis of sardine oil.

The results obtained suggest that SPD is an effective separation technology that can be used to concentrate PUFA, particularly EPA and DHA, as ethyl esters from fish oil (Messina et al., 2021a; Oliveira and Miller, 2014; Solaesa et al., 2016; Valverde et al., 2013; Wang et al., 2012; Zhang et al., 2018).

1.4.5. Effects of Fatty Acids Ethyl Esters on Lipid Accumulation and Related Genes, in SAF-1 Cell Lines

The development of new strategies to increase sustainable fish production and ensuring a high-quality product is among the objectives of the aquaculture sector (Riera-Heredia et al., 2020). Adjusting the dietary fatty acid composition by using different fish oils might be important to improve the flesh lipid content in fish. The mechanisms of how fatty acids affect fat deposition in fish flesh are not

clearly known. In vitro systems are useful tools to identify the possible adipogenic effects of dietary lipids in fish feeds (Cheng and Chen, 2015).

The fatty acid composition in fish tissue depends mainly on dietary fatty acids. The replacement of dietary fish oils with vegetable oils that are devoid of PUFA, alters the fatty acid composition, production value, and β-oxidation capacity (Huang et al., 2010).

In our study on the *Sparus aurata* cell line, changes in response to the treatments were observed, starting from cellular morphology. Figure 1 shows that cells treated with PUFASPe showed lower lipid accumulation than those exposed to PUFA-SPd. PUFA-SPd treated cells were more rounded with an enlarged cytoplasm, losing the fibroblastic shape and a significant lipid accumulation, suggesting that PUFA-SPd might stimulate fat uptake and fat cytoplasm accumulation. These findings are in agreement with the antiadipogenic effects of PUFA during pre-adipocyte differentiation in cobia (*Rachycentron canadum*) (Cheng and Chen, 2015), Atlantic salmon (*Salmo salar*) (Vegusdal et al., 2003) and rainbow trout (*Oncorhynchus mykiss*) (Riera-heredia et al., 2020).

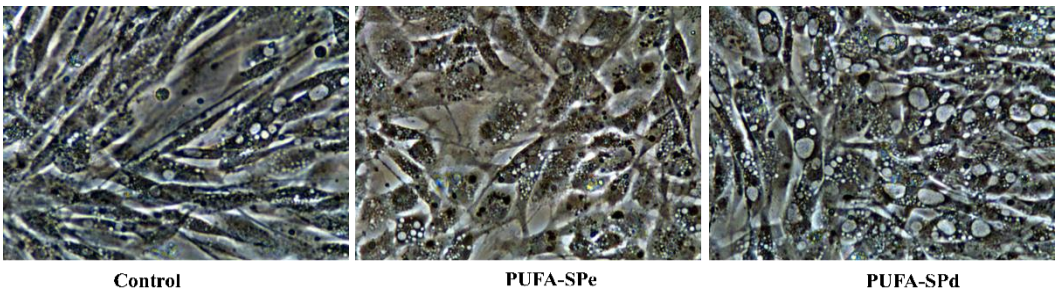


Figure 1. Representative phase-contrast images at 40× magnification (scale bar =100 μm). Untreated cells (Control), SAF-1 cell treated with enriched in PUFA (PUFA-SPe), and exhausted in fatty acid ethyl esters (PUFA-SPd).

Adipogenesis is a consequence of normal cell turnover because of the need to store energy (Salmerón, 2018), and high concentrations of fatty acids play an essential role in adipocyte differentiation (Bou et al., 2017). Very little is known about the regulation and development of adipose tissue in fish. n-3 PUFAs (EPA and DHA) are able to inhibit adipocyte differentiation and decrease lipid accumulation through the down-regulation of certain transcriptional factors or lipolytic genes (Li et al., 2017).

In this study, lipid metabolism-related genes were analyzed to investigate how the predominant fatty acids contained in TFA, PUFA-SPe, and PUFA-SPd, affect molecular pathways associated with lipid accumulation *in vitro*.

Peroxisome proliferator-activated receptors (PPAR) are nuclear receptor proteins that function as fatty acid-activated transcription factors with a key regulatory role in lipid metabolism (Torstensen et al., 2009). *pparβ* and *pparγ* are target genes involved in adipocyte differentiation and in lipid

storage (Lei et al., 2016; Torstensen et al., 2009). In our study, *pparβ* and *pparγ* genes were significantly upregulated ($p < 0.05$) in SAF-1 cells treated with TFA and PUFA-SPd, compared to notreated cells (control) (Figure 2). *pparβ* and *pparγ* mRNA levels in cells treated with PUFASPe were similar to the control (Figure 2). Li et al. (2015) reported that dietary fish oil decreased *pparγ* gene expression in intraperitoneal fat of *Ctenopharyngodon idellus*, suggesting that n-3 PUFA could inhibit lipid accumulation by affecting the expression of lipid-metabolism-related genes.

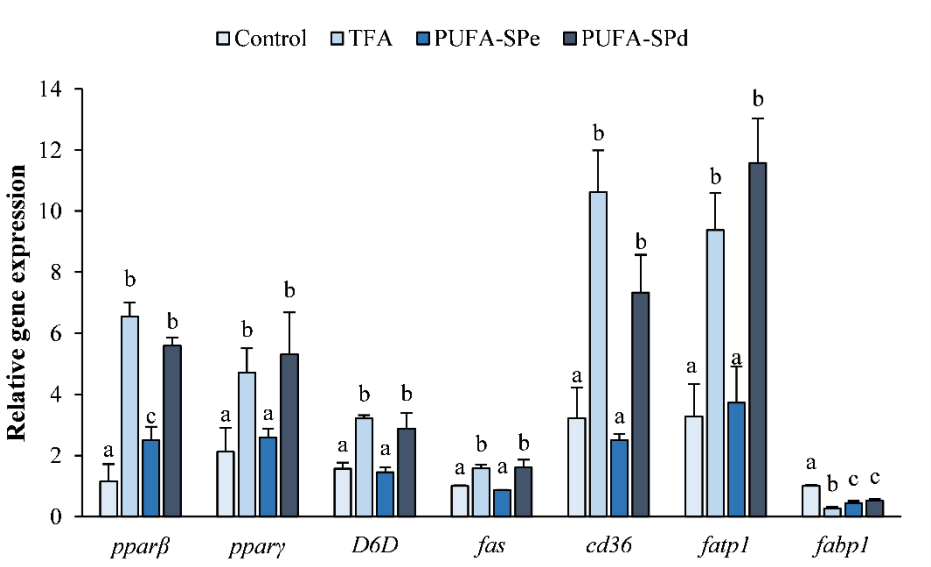


Figure 2. Relative expression of transcription factors (pparβ and pparγ), lipid metabolism-related genes (D6D and fas) and fatty acid transporters (cd36, fatp1, fabp1) in SAF-1 cell line treated or not (Control) with total FA ethyl esters (TFA), enriched in PUFA (PUFA-SPe), and exhausted in fatty acid ethyl esters (PUFA-SPd). Date are shown as mean ± SEM (n = 3). Different letters indicate significant differences among treatments (p < 0.05). Peroxisome proliferator-activated receptor β (pparβ), peroxisome proliferator-activated receptor γ (pparγ), Delta 6 desaturase (D6D), fatty acid synthase (fas), fat translocase/cluster of differentiation (cd36), fatty acid transporter protein 1 (fatp1) and fatty acid binding protein (fabp1).

These results are in accordance with the anti-adipogenic effect of EPA and DHA observed in several in vitro studies: *pparγ* results showed that reduced preadipocyte 3T3- L1 induced differentiation in the presence of EPA and DHA alone or combined, indicating that both FA attenuated adipogenesis (Barber et al., 2013; Martins et al., 2020). Even in fish adipocytes cells, PUFA suppressed the expression of *pparβ*, and fatty acid transport protein (*fatp1*) during differentiation (Huang et al., 2010). Although, other authors have demonstrated a pro-adipogenic effect of these two fatty acids in similar mammalian cell line models (Murali et al., 2014; Prostek et al., 2014; Wójcik et al., 2014). Nevertheless, the effect of fatty acids in the modulation of adipogenesis and lipid accumulation in fish is very complex and not completely understood yet.

The lipid composition of an organism is not only dependent on the dietary lipids ingested but also on the capacity of the species to transform these lipids through desaturation and elongation pathways (Vagner and Santigosa, 2011). Marine fish have a lower capacity for the bioconversion of 18C fatty acid precursors into PUFA, hence requiring the preformed PUFA in their diet (Vagner and Santigosa, 2011). Delta 6 desaturase (D6D) is a key enzyme in the biosynthesis of n-3 PUFA from precursors (Huang et al., 2016; Ofori-Mensah et al., 2020). Seiliez et al. (2003) reported that *D6D* gene expression in sea bream liver was high in fish fed with a PUFA-free diet (containing vegetable oil as a lipid source).

In the current study, the *D6D* gene expression was significantly upregulated ($p < 0.05$) in cells treated with TFA and PUFA-SPd compared to the control (Figure 2), whereas in the PUFA-SPe treatment, it was similar to the control (Figure 2). These results suggest that *D6D* expression could be inhibited by high levels of dietary DHA content, as reported in rainbow trout (Gregory et al., 2016), and upregulated by a vegetable oil replacement diet in meagre *Argyrosomus regius* (Silva-Brito et al., 2016).

In agreement with these studies, an increase of *D6D* activity was recorded in gilthead seabream fed with vegetable oil (Ofori-Mensah et al., 2020) and Atlantic salmon fed with diets containing linseed oil compared to fish oil (Vagner and Santigosa, 2011), suggesting that the high PUFA levels present in fish oil might suppress *D6D* activity, decreasing the ability of marine fish to desaturate 18C fatty acids precursors.

The fatty acid synthase (*fas*) gene is a marker of de novo lipogenesis involved in the conversion of acetyl-CoA and malonyl-CoA to palmitate (Wang et al., 2021). Cells incubated with TFA and PUFA-SPd were significantly upregulated ($p < 0.05$) compared to the control (Figure 2). Whereas *fas* mRNA levels in SAF-1 cells treated with PUFA-SPe were similar to the control (Figure 2), this was perhaps due to direct inhibition of de novo synthesis caused by the addition of PUFA into the culture medium (Riera-Heredia et al., 2019). These results suggest that the addition of PUFA to the diet could inhibit the lipogenic pathway, restraining lipid synthesis (Basto-Silva et al., 2020; Riera-heredia et al., 2020).

Among genes related to uptake and transport of fatty acid, we considered fat translocase/cluster of differentiation (*cd36*), fatty acid transporter protein 1 (*fatp1*), and fatty acid-binding protein 1 (*fabp1*). *Cd36* is an integral membrane glycoprotein that transports fatty acids into adipocytes (Martins et al., 2020). *Fatp1* are membrane-bound fatty acid uptake proteins that have an important role in responding to changes in metabolic activity (Torstensen et al., 2009). Finally, *fabp1* is a protein that transports fatty acids through the cytoplasm (Sun et al., 2003; Torstensen et al., 2009).

In our experiment, both *cd36* and *fatp1* resulted in significantly upregulated TFA and PUFA-SPd treated cells, with respect to the control (Figure 2), highlighting the important role in the uptake of

fatty acids from the environment (Riera-Heredia et al., 2019). Whereas the PUFA-SPe treated cells were similar to the control (Figure 2). Our results are in accordance with Martins et al. (Martins et al., 2020), reporting that EPA and DHA treatment, alone or combined, downregulated *cd36* mRNA levels in 3T3-L1 cells, reducing fatty acid transport into adipocytes.

The gene *fabp1* was downregulated in all treatments compared to the control (Figure 2). These observations might indicate that n-PUFA reduces fatty acid uptake and transport activity (Riera-Heredia et al., 2019), affecting the catabolism of fatty acids (Torstensen et al., 2009).

Our *in vitro* observations confirm, as previously reported (Messina et al., 2021a), that dietary PUFA have several beneficial properties for farmed fish, affecting lipid metabolism, with a possible effect on fat deposition in fish fillets. Therefore, regulating the composition of fatty acids in the diet could improve the lipid deposition in various tissues and, consequently, the lipid profile of the edible parts of fish, contributing to ameliorating the acceptance of consumers.

1.5. Conclusions

The utilization of farmed bluefin tuna side streams could be linked to the fish processing sector, generating development and efficiency from a resource that would otherwise be discarded. Reducing food losses and waste, as well as valorizing food waste, is fundamental to achieving zero waste.

PUFA-SPe extracted from farmed bluefin tuna side streams could be utilized in fish feed formulations to prevent excessive fat deposition, contributing to improving both aquaculture sustainability and the quality of its products. In addition, the n-3 PUFA side stream of farmed bluefin tuna could contain other compounds such as collagen, minerals, and proteins with a high biological value that could be obtained after the extraction of the oil for their application in animal feed, cosmeceutical, and nutraceutical fields.

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Part 2 - Valorization of marine by products in aqua feed sector

Chapter 2. Fisheries and aquaculture byproducts modulate growth, body composition, and omega-3 polyunsaturated fatty acid content in black soldier fly (*Hermetia illucens*) larvae

In this chapter I have focused on the valorization of fish by-products in the aquafeed sector.
By-catch from the Mediterranean trawler fishing species, *Parapenaeus longirostris* processing by-products, aquaculture by-products (from sea bass and sea bream filleting), *Thunnus albacares* processing by-products and *Engraulis encrasicolus* by-products (heads and viscera) were analyzed. The analysis focused on evaluating their proximate composition and fatty acid profile, and their use as food substrate in an experimental diet to feed *Hermetia illucens*, in order to improve its fatty acid profile. *H. illucens* is a promising species to produce protein-rich ingredients for animal feed, but its low content of n-3 polyunsaturated fatty acids (PUFAs) limits its use in fish feed.
To address this, I investigated the incorporation of fish by-products into BSF diets to improve their nutritional value, particularly in terms of beneficial fatty acids such as EPA and DHA.
Obtained results showed that *E. encrasicolus* processing by-products, by-catch from Mediterranean trawling fisheries, *T. albacares* processing by-products, and aquaculture processing by-products used to feed *H. illucens* could be interesting feed substrates for the rearing of this species. In fact, by using these by-products, it was possible to modulate the lipid and fatty acid profile of *H. illucens* larvae, significantly increasing their n-3 fatty acid content. This highlighted their potential as an alternative sustainable source of fishmeal for commercial aquaculture feed formulations.
The results of this study are included in the published paper:

Arena, R., Manuguerra, S., Curcuraci, E., Cusimano, M., Lo Monaco, D., Di Bella, C., Santulli, A., Messina, C.M., 2023. Fisheries and aquaculture by-products modulate growth, body composition, and omega-3 polyunsaturated fatty acid content in black soldier fly (*Hermetia illucens*) larvae. Front. Anim. Sci. 4, 1204767.

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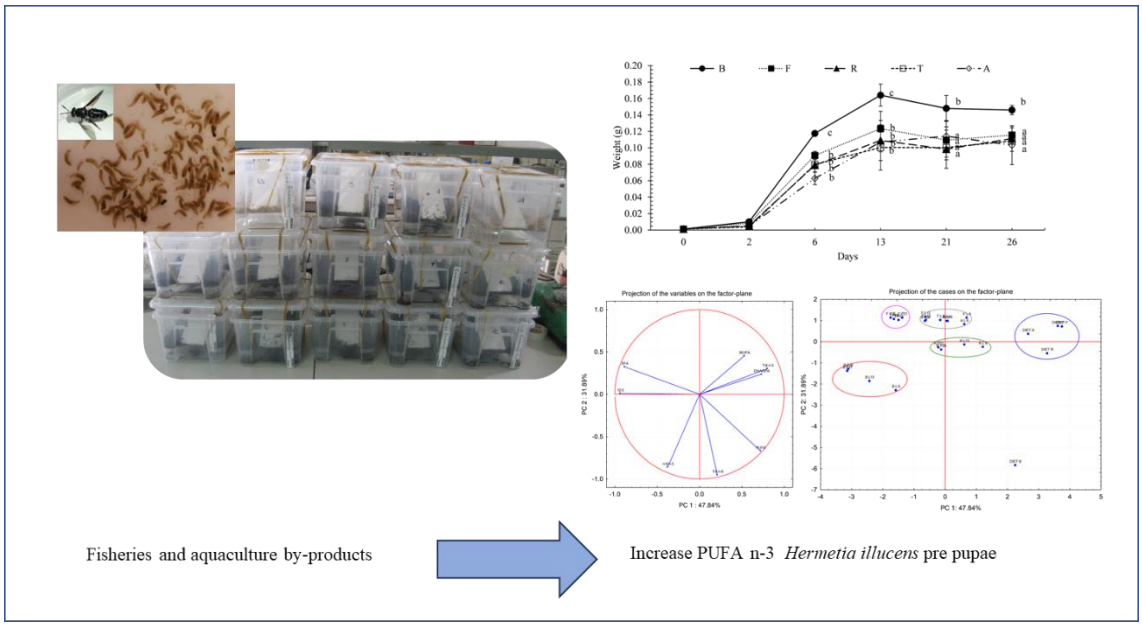

Graphical abstract "Fisheries and aquaculture by-products modulate growth, body composition, and omega-3 polyunsaturated fatty acid content in black soldier fly (Hermetia illucens) larvae. Front. Anim. Sci. 4, 1204767".

2.1. Abstract

The black soldier fly (BSF) (*Hermetia illucens L.*) is one of the most promising species for the production of ingredients, mainly protein, useful for animal feed formulation, owing to its ability to convert organic waste into biomass with a high nutritional value. However, the low percentage of n-3 series polyunsaturated fatty acids (PUFAs) in its fatty acid profile is a limiting factor for the utilization of BSF in fish feed. Recent studies have highlighted that wastes derived from different agro-food value chains could affect the nutritional composition of BSF larvae, depending on the composition of the wastes used as larvae growing substrate. Due to the significant amount of n-3 PUFA in marine products, both in edible produce and in by-products, in this study, different sources of fish by-products were included in experimental diets for BSF to evaluate the effect of its addition on the final nutritional profile of BSF pre-pupae.

One control diet and five experimental diets were prepared to feed the BSF larvae: wheat bran as the control diet (diet B), bycatch from Mediterranean trawl fisheries (diet F), *Parapenaeus longirostris* processing by-products (diet S), aquaculture processing by-products (diet R), *Thunnus albacares* processing by-products (diet T), and *Engraulis encrasicolus* processing by-products (diet A). In this study, the effects of the different diets were analyzed on the growth, body composition, and fatty acid profile of BSF larvae and pre-pupae. The obtained results showed that the different experimental diets affected total lipids content and fatty acids composition, when compared with the control. A significant increase in eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) in BSF larvae and

pre-pupae fed with all fish by-products was observed when compared with those fed with diet B, in particular in larvae and pre-pupae fed with diet A, demonstrating that the utilization of fish processing by-products is a suitable solution for improving the nutritional value of insects as ingredients in aqua feeds. The reuse of marine by-products can contribute to the industry's "zero waste" goal, increasing the sustainability of the fishery value chain and the formulation of new valuable products.

Keywords: *Hermetia illucens*, black soldier fly, fatty acids, marine by-products, PUFA, EPA, DHA.

2.2. Introduction

Global fisheries and aquaculture production increased in 2020 due to the constant expansion of aquaculture (FAO, 2022), which reached the value of 49.2% in 2020 on the global production of aquatic animals (FAO, 2022). The growth of global aquaculture production has consequently led to an increase in the demand for fishmeal (FM) and fish oil (FO), which are indispensable ingredients for satisfying fish nutritional requirements. As the production of FM and FO fluctuates according to catches, the decrease in catches reported over the past 3 years (–4.0%) (FAO, 2022), has led to a gradual decrease in the supply of FM and FO, with a consequent increase in price and availability. This situation has stimulated the need to find additional or alternative sources of these ingredients for the sustainable growth of aquaculture (Ayoola, 2010; Barroso et al., 2014; Belghit et al., 2019, 2018; FAO, 2020; Hoc et al., 2021; Liland et al., 2017; Maiolo et al., 2020).

In recent years, insects have received growing attention as a food source, owing to their potential as a sustainable, alternative source of proteins in FM for commercial aqua feed formulations and to their relatively easy cultivation (Alfiko et al., 2022; Govorushko, 2019; Huang et al., 2019; Ng et al., 2001; Osimani et al., 2017; Purschke et al., 2018).

Insects are good sources of proteins, fats, minerals, vitamins, and energy (Gasco et al., 2020; Rumpold and Schlüter, 2013). Traditionally used as a food in different countries, their cultivation and commercialization has recently become of global interest as a source of protein and fat for human food and animal feed (Starčević et al., 2017).

Since the 1970s, insects have been studied as a protein source in animal feed, mainly due to their ability to convert food waste (e.g., vegetable, fruit, factory, and animal tissue waste) into high-quality protein (Belghit et al., 2019; Calvert et al., 1969; Ichhponani and Malik, 1971; Newton et al., 1977; Oonincx et al., 2019; Phelps et al., 1975; Teotia and Miller, 1974). An important group of insects, able to consume different decaying organic substrates for their larval development, are known as

bioconverter species. Among bioconverter insects, the most promising species for industrial production and animal feed in the world is *Hermetia illucens* (order Diptera, family Stratiomyidae), better known as the black soldier fly (BSF) (Müller et al., 2017; Surendra et al., 2016). The BSF life cycle, which lasts from 6 to 7 weeks (Maroušek et al., 2022), can be divided into four stages: egg, larva, pre-pupa, and adult (Li et al., 2011; Liu et al., 2017). From the pre-pupa stage, the organism stops feeding in preparation for metamorphosis and uses the nutrients stored from the larval stage (Sheppard et al., 2002).

BSF larvae under optimal growth conditions (27°C temperature and 70% humidity) are able to bioconvert different organic substrates (of both animal and vegetable origin) into larval biomass in just 14 days (Franco et al., 2021). Among the large variety of organic materials that BSF is able to feed on, agro-food waste is receiving increasing attention (Jucker et al., 2017; Nguyen et al., 2015). Through the consumption of organic waste material, the insect gains energy and nutrients useful for its growth and then converts itself into a biomass that can be used as feed for other livestock and human food (Franco et al., 2021). Thus, the BSF can be reared as a feed ingredient [approved by the Association of American Feed Control Officials and by the European Food Safety Authority (EFSA)] using approved feed-grade materials, including food waste and by-products of food production (Lähteenmäki-Uutela et al., 2021; Maroušek et al., 2022). Several EU member countries have requested a food safety evaluation by the EFSA of new substrates to be used for BSF feeding (Lähteenmäki-Uutela et al., 2021).

In recent years, the use of insect-based meal in aquaculture has grown exponentially, due to its nutritional value, including as a protein source (Belghit et al., 2019, 2018; Guerreiro et al., 2020; Karapanagiotidis et al., 2023; Ogunji et al., 2006). Several studies have reported on the importance of BSF as a source of protein (Chaklader et al., 2021, 2020; Maiolo et al., 2020); however, few studies have reported on the possibility of exploiting BSF as a source of fatty (Barroso et al., 2019; Hossain et al., 2023). The low levels of n-3 series polyunsaturated fatty acids (PUFAs), such as the two PUFAs essentials for fish, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in the lipids of BSF is one of the limiting factors for its utilization in diets for farmed fish (Oonincx et al., 2020). In fact, this nutritional gap could compromise the growth, health, and quality of farmed fish (Guerreiro et al., 2020; Karapanagiotidis et al., 2014; Kroeckel et al., 2012; Sealey et al., 2011).

Recent studies have shown that the fatty acid composition of BSF larvae can be modified through the use of different feeding substrates, acquiring a new value (Barroso et al., 2019; Bava et al., 2019; Franco et al., 2021; Hoc et al., 2021; Liland et al., 2017; Lopes et al., 2020; Spranghers et al., 2017; Starcevic et al., 2019; Surendra et al., 2016). In view of this, the possibility to enrich the concentration of essential PUFAs in BSF larvae with marine based diets is an attractive option for obtaining n-3-

enriched larvae (Barroso et al., 2019). Thus, the interest in insects is related not only to their intrinsic nutritional composition (Hwangbo et al., 2009; Ramos-Elorduy et al., 2002; Wang et al., 2007), but also to the possibility of enriching them with other nutrients and micronutrients (St-Hilaire et al., 2007; Mlcek et al., 2014; Hoc et al., 2021; Ferrari et al., 2022).

The process of bioconversion via BSF is a very attractive option, as it could potentially solve two problems: organic waste management and the ability to satisfy global food demand without competing with food crops for land use (Chavez, 2021; Salomone et al., 2017; Surendra et al., 2016). Under this framework, the use of marine by-products generated from a diverse supply chain could be an important resource for high-nutritional-value components, such as n-3 PUFAs and proteins of high biological value (Caruso et al., 2020; Messina et al., 2022, 2021a, 2021b; Ucak et al., 2021). The use of these by-products and their valorization could lead to a reduction in food losses and waste, ensuring the achievement of the industry's "zero-waste" goal (Alfio et al., 2021; Coppola et al., 2021; Kee et al., 2023).

Recently, some researchers have highlighted the possibility of rearing BSF on a diet including marine wastes and by-products such as aquaculture waste (Lopes et al., 2020), (Magee et al., 2021), carp mince (Chaklader et al., 2021), fish protein hydrolysates (from yellowtail kingfish, carp, and tuna) (Chaklader et al., 2020) and discarded round sardinella (*Sardinella aurita*) (Barroso et al., 2019). These diets improved process performance in BSF larvae composting and improved larval quality, which was characterized by high levels of fat and protein, suitable for farmed animal feeds including pig, chicken, and fish feeds (Barroso et al., 2019; Chaklader et al., 2021; Lopes et al., 2020; Magee et al., 2021).

Owing to the increasing interest in the farming of this insect for diverse purposes in the sector of food and feed, and the increase in interventions aimed at recycling by-products from marine sectors to reach zero waste, in this present work we studied the effects of different fish by-products, obtained from the fishery and aquaculture value chain, on growth, body composition, and modulation of fatty acid profile in BSF larvae and pre-pupae. Our aim was to determine if this solution could be a reasonable method for implementing n-3 components in the body composition of these insects, which are intended as new ingredients for aqua feeds, promoting circular economy pathways at a regional level in accordance with the European Green Deal.

2.3. Materials and methods

2.3.1. Larvae rearing and experimental diets

H. illucens larvae were purchased alive from Smart Bugs s.s. (Ponzano Veneto, Italy). The experiment was performed in an acclimatized environment at 26 ± 1°C and 65 ± 5% humidity.

Larvae were kept in transparent containers (28 cm × 20 cm × 14 cm) fitted with lids, a drainage system, and a collection system for the pre-pupae.

Air circulation was enabled through holes in the lower (1 mm diameter) and upper (2–2.5 mm diameter) sections of the boxes.

The experiment was set up to administer one control wheat bran-based diet (B) and five experimental diets, including diverse sources of fish by-products (25%) collected from fisheries, aquaculture facilities, and processing plants located in the region of Trapani (western Sicily, Italy). Each treatment was performed in three replicates. Four hundred 6-day-old BSF larvae were allowed in each container (1,200 larvae for each treatment), as described in the literature (Grossule and Lavagnolo, 2020); substrates were weighed and distributed among the feeding containers in order to provide a daily ration equal to 100 mg/larva/day (Diener et al., 2009) (Table 1). The BSF larvae were fed for 26 days and were sampled at 6, 13, 21, and 26 days.

Table 1. Percentage of inclusion of fish by-products in the experimental diets provided to BSF larvae.

Diets	B (%)	F (%)	S (%)	R (%)	T (%)	A (%)
Diet P (Inert component)	75	75	75	75	75	75
Diet B	25	0	0	0	0	0
Diet F	0	25	0	0	0	0
Diet S	0	0	25	0	0	0
Diet R	0	0	0	25	0	0
Diet T	0	0	0	0	25	0
Diet A	0	0	0	0	0	25

P: peat; B: wheat bran; F: by-catch sea trawl fishery; S: *P. longirostris* processing by-products; R: aquaculture processing by-products; T: *T. albacares* processing by-products; A: *E. encrasicolus* processing by-products.

The five experimental diets were as follows: diet F, comprising fishery bycatch from the Mediterranean trawler fishing species *Trachurus trachurus* (Atlantic horse mackerel), *Merluccius merluccius* (European hake), *Spicara flexuosa* (Mediterranean picarel), *Munida* sp. (squat-lobsters), *Helicolenus dactylopterus* (blackbelly rosefish), *Phycys blennoides* (greater forkbeard), *Galeus melastomus* (blackmouth catshark), *Gadiculus argenteus* (Silvery pout); diet S, comprising *Parapenaeus longirostris* (deep-water rose shrimp) processing by-products; diet R, comprising aquaculture byproducts (from sea bass and sea bream filleting); diet T, made from *Thunnus albacares* (yellowfin tuna) processing by-products; and diet A, comprising *Engraulis encrasicolus* (European

anchovy) byproducts (heads and viscera) from specimens collected at the Trapani fish market. *P. longirostris* and *T. albacares* processing by-products from companies in the province of Trapani. All the materials were oven dried at 60°C for 48 hours and then ground. The obtained meal was stored under vacuum at −20°C. Different byproducts and wheat bran were mixed into the peat in the ratios indicated in Table 1. The water content was increased to 75% (Diener et al., 2009). The choice of peat as an inert component was suggested owing to its easy availability, homogeneity of composition, and high degree of sustainability. The proximate composition and fatty acid profile of the experimental diets are shown in Table 2 and Table 3.

Diet aliquots were prepared to be added to the substrate at startup and then every 2 days as a supplement to the diet until the end of the trial (Diener et al., 2009; Parra Paz et al., 2015).

Table 2. Proximate composition (g/100g wet), chitin (g/100g wet), energy content (kJ/100g) of experimental diets provided to BSF larvae.

	Diet B	**Diet F**	**Diet S**	**Diet R**	**Diet T**	**Diet A**
Total lipid	2.48±0.17	9.55±0.17	5.72±0.45	27.97±0.15	9.32±0.59	12.87±0.64
Moisture	9.54±0.01	4.89±0.17	6.89±0.11	2.64±0.04	2.32±0.26	3.45±0.03
Protein	14.50±0.05	69.73±0.02	25.27±0.48	41.60±0.71	73.26±1.67	49.52±0.61
Ash	3.91±0.04	14.84±0.33	34.04±0.31	18.87±0.14	13.18±0.61	27.46±0.37
Carbohydrates	50.00±0.03	n.d.	n.d.	n.d.	n.d.	n.d.
Chitin	n.d.	n.d.	17.90±0.29	n.d.	n.d.	n.d.
Energy	1177.90±6.96	1543.20±6.64	945.55±6.01	1741.99±12.31	1590.19±33.10	1318.08±24.81

Each value represents the mean of three independent determinations. B: wheat bran; F: by-catch sea trawl fishery; S: *P. longirostris* processing by-products; R: aquaculture processing by-products; T: *T. albacares* processing by-products; A: *E. encrasicolus* processing by-products. n.d.= not detectable.

Table 3. Fatty acids profile (% of total FA) of experimental diets provided to BSF larvae.

Fatty acid	**DIET B**	**DIET F**	**DIET S**	**DIET R**	**DIET T**	**DIET A**
8:0	0.15±0.07	0.04±0.00	0.02±0.01	0.02±0.02	0.05±0.00	0.04±0.04
10:0	n.d.	n.d.	0.03±0.03	n.d.	n.d.	0.03±0.01
11:0	0.01±0.01	n.d.	n.d.	n.d.	n.d.	n.d.
12:0	0.02±0.00	0.10±0.01	0.31±0.23	0.05±0.01	0.07±0.01	0.31±0.01
13:0	n.d.	0.03±0.00	0.01±0.00	0.01±0.00	0.04±0.00	0.13±0.00
14:0	0.15±0.00	2.64±0.04	1.18±0.06	2.23±0.15	2.91±0.09	5.72±0.14
15:0	0.21±0.01	0.61±0.00	0.67±0.00	0.24±0.01	1.24±0.05	1.26±0.03
16:0	13.86±0.67	20.57±0.25	13.96±0.25	12.56±0.16	24.82±0.45	25.81±0.55
16:1n-7	0.25±0.05	4.34±0.05	4.26±0.06	3.66±0.15	4.69±0.07	3.06±0.08
16:2n-4	0.06±0.01	0.74±0.09	0.37±0.01	0.33±0.01	1.10±0.13	0.91±0.03
17:0	0.18±0.01	0.75±0.01	1.09±0.04	0.25±0.01	1.78±0.09	1.52±0.04
16:3n-4	0.06±0.01	0.72±0.00	0.95±0.01	0.38±0.03	0.97±0.04	0.68±0.02
18:0	1.39±0.03	6.24±0.02	5.64±0.18	3.14±0.14	8.19±0.08	6.33±0.14
18:1n-9	19.56±0.30	22.81±0.43	16.98±0.66	36.40±0.45	18.04±0.49	17.29±0.19
18:1n-7	1.19±0.10	3.29±0.02	4.72±0.18	3.00±0.04	3.06±0.04	2.92±0.00
18:2n-6	55.88±0.29	1.41±0.11	1.87±0.20	19.76±0.08	1.72±0.25	3.08±0.29
18:3n-6	n.d.	0.17±0.01	0.36±0.01	0.05±0.01	0.56±0.20	0.24±0.01
18:3n-4	n.d.	0.23±0.01	n.d.	0.04±0.05	0.23±0.06	0.26±0.01
18:3n-3	3.63±0.07	0.60±0.01	0.27±0.06	3.77±0.06	0.41±0.06	1.00±0.06
18:4n-3	n.d.	0.40±0.00	0.21±0.00	0.52±0.01	0.33±0.01	0.78±0.00
20:1n-9	0.97±0.09	2.43±0.06	1.89±0.51	2.65±0.08	1.41±0.00	1.00±0.02
20:4n-6	n.d.	1.46±0.02	6.07±0.08	0.38±0.01	1.74±0.05	1.51±0.01
20:4n-3	0.20±0.28	0.79±0.00	0.31±0.01	0.46±0.00	0.30±0.01	0.36±0.01
20:5n-3	0.38±0.05	5.11±0.05	12.69±0.18	2.19±0.00	2.99±0.13	6.05±0.07
22:1n-11	n.d.	0.41±0.03	0.55±0.39	1.09±0.06	0.15±0.03	0.29±0.01
22:1n-9	n.d.	0.21±0.24	0.26±0.04	0.40±0.03	0.10±0.00	0.13±0.01
22:4n-6	n.d.	0.35±0.01	0.79±0.00	0.12±0.04	0.34±0.05	0.16±0.03
22:5n-6	n.d.	0.89±0.04	1.24±0.03	0.13±0.02	2.02±0.04	0.81±0.00
22:5n-3	0.34±0.08	1.85±0.33	1.37±0.01	1.48±0.02	1.13±0.03	0.81±0.09
22:6n-3	n.d.	20.83±0.58	21.93±0.75	4.71±0.02	19.60±0.20	17.54±0.15
SFA	15.98±0.70	30.96±0.23	22.92±0.19	18.49±0.14	39.11±0.77	41.14±0.88
MUFA	21.97±0.54	33.49±0.70	28.66±0.33	47.21±0.33	27.46±0.49	24.68±0.27
PUFA	60.55±0.06	35.55±0.47	48.42±0.51	34.30±0.18	33.43±0.28	34.17±0.61
Tot n-3	4.55±0.24	29.57±0.21	36.78±0.62	13.12±0.10	24.76±0.26	26.54±0.35
Tot n-6	55.88±0.29	4.28±0.18	10.33±0.10	20.43±0.01	6.38±0.39	5.79±0.26
n-6/n-3	12.30±0.71	0.28±0.01	0.28±0.01	1.56±0.01	0.26±0.02	0.22±0.01
unknown	1.51±0.11					
DHA/EPA	0.00±0.00	4.08±0.16	1.73±0.08	2.15±0.02	6.56±0.22	2.90±0.01

Each value represents the mean of three independent determinations. B: wheat bran; F: by-catch sea trawl fishery; S: *P. longirostris* processing by-products; R: aquaculture processing by-products; T: *T. albacares* processing by-products; A: *E. encrasicolus* processing by-products. SFA Saturated fatty acids; MUFA Monounsaturated fatty acids; PUFA Polyunsaturated fatty acids. n.d.= not detectable.

2.3.2. BSF growth performance

In order to determine the growth rate, 10 larvae at 2, 6, and 13 days and 10 pre-pupae at 21 and 26 days from each replicate were randomly sampled and weighed individually. The "pre-pupae" stage was defined as the mobile, non-feeding, final larval stage of BSF, as defined in the literature (Harnden and Tomberlin, 2016; Martínez-Sánchez et al., 2011; Myers et al., 2008; Tomberlin et al., 2009). Prepupae were identified by a dark brown–black dorsal and ventral integument and reduced, darkened mouth parts (Harnden and Tomberlin, 2016; May, 1961). Larvae and pre-pupae were immediately frozen at –20°C for subsequent chemical analysis.

2.3.3. Proximate composition

The proximate composition of both diets and of BSF was measured as follows: the total lipids (TL) were determined according to Folch et al. (1957); moisture and ash content was determined using the AOAC method (AOAC, 1990); total nitrogen was determined using the Kjeldahl method (AOAC, 1992); and crude protein (P) and chitin (Q) content were determined by applying the equations:

$$P = \frac{(Nt * Cq + k - 100) * Cp}{Cq - Cp} \quad (1)$$

$$Q = \frac{(Nt * Cp + k - 100) * Cq}{Cp - Cq} \quad (2)$$

used by Díaz-Rojas et al. (2006), where Nt is the total nitrogen content, K is the sum of total lipid, moisture and ash, and Cp and Cq are the conversion coefficients that relate to the mass fraction of nitrogen with protein and chitin (Díaz-Rojas et al., 2006). The protein content in the literature is mainly based on nitrogen content, using the nitrogen-to-protein conversion factor (Cp) of 6.25 (Gnaiger and Bitterlich, 1984; Woyewoda et al., 1986), while the value of Cq is 14.5 (Díaz-Rojas et al., 2006; Messina et al., 2019).

Carbohydrate content was determined following the method described by Dubois et al. (1956).

Caloric content was measured as total energy content using an isoperibolic oxygen bomb calorimeter (model 6200, Parr Instrument Company, USA).

The composition of experimental diets varies considerably according to the species used and the current fishing season (Kim and Mendis, 2006).

2.3.4. Fatty acid profile

Total fatty acid (FA) methyl esters were determined from the total lipid content, following the method described by Lepage and Roy (1984) and analyzed using the conditions described by Messina et al. (2013), employing a Perkin Elmer (Waltham, MA, USA) CLARUS 580 instrument equipped using a silica capillary column (30 m × 0.32 mm, df 0.25 mm, Omegawax 320, Supelco, Bellefonte, PA, USA). Individual FAME were measured by the comparison of known standards (a mix of PUFA 1, PUFA 2, and PUFA 3 oil, Supelco).

2.3.5. Statistical analysis

Statistical differences were evaluated for each parameter at each time point by diet using analysis of variance (ANOVA). The differences among the mean values were assessed using the Student–Newman–Keuls test. The degree of heterogeneity was measured by the Cochran test (Underwood et al., 1997). A principal component analysis (PCA) was performed to highlight the differences in fatty acid profiles in BSF larvae and pre-pupae fed different diets. The ANOVA and PCA were performed using STATISTICA (version 8.0, Statsoft Inc. USA).

2.4. Results

2.4.1. Effects of the experimental diets on BSF growth performance

The growth performance of BSF larvae during the experiment is shown in Figure 1. Larvae fed diet S died after a few days and did not develop into pre-pupae (data not shown). The best growth performance was recorded in larvae fed the control diet (diet B), which weighed significantly more than the other larvae and prepupae ($p < 0.05$) fed marine by-products. The larvae and pre-pupae fed the experimental diets (diet F, diet R, diet T, and diet A) did not differ regarding growth performance (Figure 1).

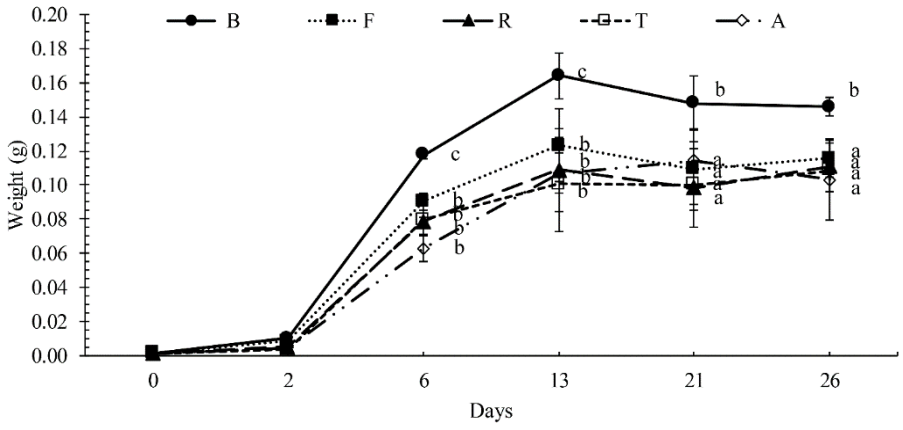

Figure 1. Growth performance of BSF fed with B: wheat bran; F: by-catch from sea trawling fishery; S: Parapenaeus longirostris processing byproducts; R: aquaculture processing by-products; T: Thunnus albacares processing by-products; A: Engraulis encrasicolus processing by-products. Each value represents the mean of three independent determinations. Different letters within the same day indicate significant differences ($p < 0.05$) within treatments.

2.4.2. Proximate composition of the pre-pupae

The proximate composition of the BSF pre-pupae, analyzed at the end of the experiment, is shown in Table 4. Since larvae fed diet S died after few days into the experiment, their proximate composition is not reported among the pre-pupae results. The different experimental diets affected the proximate composition of the BSF pre-pupae (Table 4). Total lipid content was highest in pre-pupae fed diet R (P26-R, 28.05 ± 1.90 g/100 g) ($p< 0.05$) (Table 4), which aligns with diet R having the highest energy content of (Table 2). The pre-pupae fed diets B, F, T, and A did not differ significantly in lipid content (ranging between 24.1 and 25.51 g/100 g dw). The highest protein content was observed in pre-pupae fed diet B (P26-B, 39.93 g/100 g) ($p< 0.05$) (Table 4). The pre-pupae fed experimental diets showed a comparable total protein content, ranging from 34.29 to 34.98 g/100 g (Table 4). Ash content was significantly higher ($p< 0.05$) in pre-pupae fed diets T, A, and F than in those fed the control diet (diet B); no significant differences were observed between diet R and diet B. Carbohydrates, despite being absent in all diets except diet B (Table 2), ranged from 22.86 g/100 g (P26-B) to 26.34 g/100 g (P26-F) in the BSF (Table 4).

Table 4. Proximate composition (g/100g dw), chitin (g/100g dw), energy content (kJ/100g) of pre-pupae analyzed at the end of the experiment (P26).

	P26-B	**P26-F**	**P26-R**	**P26-T**	**P26-A**
Total lipid	25.51±1.05^{ab}	25.27±0.83^{ab}	28.05±1.90^{b}	25.23±1.32^{ab}	24.10±1.04^{a}
Protein	39.93±0.13^{b}	34.94±0.22^{a}	34.83±0.23^{a}	34.47±0.43^{a}	34.29±0.65^{a}
Ash	4.23±0.07^{a}	7.25±0.02^{b}	6.16±0.35^{ab}	8.30±3.13^{b}	7.82±1.21^{b}
Carbohydrates	22.86±0.74^{a}	26.44±0.72^{c}	24.73±0.32^{b}	25.92±0.36^{c}	26.37±0.25^{c}
Chitin	7.46±0.25^{b}	8.19±0.15^{c}	6.10±0.11^{a}	6.03±0.19^{a}	5.99±0.14^{a}
Energy	2011.13±32.57	1978.20±25.33	2050.32±78.53	1960.21±46.28	1926.64±46.51

Each value represents the mean of three independent determinations. Different letters in the same row indicate significant differences ($P<0.05$). B: wheat bran; F: by-catch sea trawl fishery; R: aquaculture processing by-products; T: *T. albacares* processing by-products; A: *E. encrasicolus* processing by-products.

Similarly, the levels of chitin in BSF body composition was comparable regardless of its concentration in diets, ranging between 6 and 8.1 g/100 g (Table 4). Analysis of body energy content showed no significant differences among the pre-pupae fed the experimental diets and control diet (Table 4).

2.4.3. FA profile

The FA profiles of the BSF larvae and pre-pupae fed different diets are shown in Tables 5–10 and Figure 2. The larvae fed the experimental diets reflected the FA composition of the diets, showing significant differences ($p< 0.05$) when compared with the larvae fed diet B.

The FA composition of the larvae and pre-pupae was dominated mainly by saturated fatty acids (SFAs) and increased in the pre-pupae (Tables 5–10, Figure 2). In particular, lower values were observed in the larvae and pre-pupae fed fish by-products (diet F, diet R, diet T, and diet A), than in those fed the control diet (diet B) ($p< 0.05$) (Tables 5–10, Figure 2.). The predominant fatty acid in the SFA was lauric acid (12:0), which increased significantly in content ($p< 0.05$) during the growth phase in all treatments. In particular, it was observed that larvae and pre-pupae fed control diet (diet B) had the highest value of lauric acid, reaching 46.58 ± 0.19% in P26 (Table 5 and Table 10.). Significantly lower values ($p< 0.05$) were observed in the larvae and pre-pupae fed fish by-products, particularly in those fed diet A. However, after 26 days, the pre-pupae (P26) fed fish by-products showed a lauric acid concentration above 20%. The larvae and prepupae fed diet A showed a higher concentration of palmitic acid (16:0) (Table 9 and Table 10) due to the higher amount present in the diet (Table 2).

Monounsaturated FA (MUFA) represented the second most abundant class of FA in almost all larvae and in all analyzed prepupae. L6 showed the highest MUFA content (Tables 5–9, Figure 2). A decrease in MUFAs occurred during the growth phase due to a reduction in oleic acid (18:1n-9), while palmitoleic acid (16:1 n-7) remained almost constant over time (Tables 5–9, Figure 2). Pre-pupae fed the experimental diets had significantly higher MUFA content ($p< 0.05$) than the larvae fed diet B (Table 10).

Similarly, polyunsaturated FA (PUFA) concentrations in the BSF larvae and pre-pupae decreased during growth (Tables 5–9). Linoleic acid (LA, 18:2n6) was the most represented fatty acid in BSF larvae and pre-pupae fed diets B and R (Table 5 and Table 7). In contrast, pre-pupae fed diets A, T, and F showed significantly lower LA values ($p< 0.05$) (Table 10). These larvae and pre-pupae fed diets A, T, and F also demonstrated an improvement in the concentration of eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3) (Table 6, Table 8 and Table 9). The most remarkable result was obtained for EPA, which reached 4.33% in P26 fed diet A and was undetected in P26 fed the control diet (diet B) (Table 10). Regarding DHA, the highest value was observed in L6 fed diet F, and levels then decreased during the growth phase. Larvae and pre-pupae fed diet A showed an average DHA content equal to 2% in all experiments (Table 9). DHA was not detected in larvae and pre-pupae fed diet B (Table 5). Larvae and pre-pupae fed diets A, F, and T showed higher Tot n-3 content than Tot n-6 and, consequently, the n-6/n-3 ratio was significantly lower in BSF larvae and pre-pupae ($p< 0.05$) than in BSF larvae and pre-pupae fed the control diet (diet B) (Table 5-10, Figure 2).

Table 5. FA profile (% fatty acids/total FA) of larvae (L) and pre-pupae (P) of BSF, analysed at 6-13- 21 and 26 days, fed diet B.

Fatty acid	Larvae 6 days	Larvae 13 days	Prepupae 21 days	Prepupae 26 days
8:0	0.05±0.01[a]	0.10±0.02[c]	0.08±0.02[b]	0.14±0.00[d]
10:0	0.29±0.08[a]	0.60±0.03[b]	0.77±0.03[c]	0.89±0.08[d]
11:0	0.01±0.00	0.01±0.00	0.02±0.00	0.02±0.00
12:0	28.23±0.14[a]	36.47±0.62[b]	47.14±1.51[c]	46.58±0.19[c]
13:0	0.02±0.00[a]	0.05±0.00[b]	0.05±0.00[b]	0.06±0.00[c]
14:0	6.52±0.43[a]	7.11±0.19[b]	8.00±0.13[c]	7.88±0.03[c]
15:0	0.32±0.05[a]	0.46±0.04[c]	0.38±0.03[b]	0.38±0.00[b]
16:0	15.72±0.15[c]	13.73±0.13[b]	11.65±0.44[a]	11.77±0.09[a]
16:1n-7	3.16±0.60[a]	3.80±0.26[b]	3.55±0.18[ab]	3.90±0.15[b]
16:2n-4	0.04±0.00[b]	0.01±0.02[a]	0.01±0.01[a]	0.05±0.00[b]
17:0	0.35±0.01[b]	0.39±0.02[c]	0.28±0.02[a]	0.29±0.00[a]
16:3n-4	0.21±0.10	0.31±0.08	0.26±0.02	0.25±0.00
18:0	2.65±0.18[c]	1.84±0.03[b]	1.23±0.08[a]	1.12±0.02[a]
18:1n-9	16.40±0.19[c]	12.27±0.16[b]	9.35±0.45[a]	9.04±0.04[a]
18:1n-7	1.30±0.02[c]	1.09±0.07[b]	0.73±0.05[a]	0.68±0.06[a]
18:2n-6	20.21±0.52[d]	14.69±0.22[c]	12.86±0.22[b]	12.09±0.05[a]
18:3n-6	0.07±0.01[c]	n.d.[a]	0.03±0.00[b]	0.03±0.00b
18:3n-4	n.d.	n.d.	0.01±0.01	n.d.
18:3n-3	1.54±0.09[d]	1.03±0.03[c]	0.91±0.02[b]	0.84±0.00[a]
18:4n-3	n.d.[a]	n.d.[a]	0.19±0.02[c]	0.12±0.00[b]
20:1n-9	0.55±0.02[c]	1.02±0.00[d]	0.00±0.00[a]	0.52±0.00[b]
20:4n-6	n.d.	n.d.	0.02±0.03	0.06±0.00
20:4n-3	n.d.	n.d.	n.d.	n.d.
20:5n-3	n.d.[a]	0.06±0.02[b]	0.11±0.03[c]	0.21±0.00[d]
22:1n-11	n.d.	n.d.	n.d.	n.d.
22:1n-9	n.d.	n.d.	n.d.	n.d.
22:4n-6	n.d.	n.d.	n.d.	n.d.
22:5n-6	n.d.	n.d.	n.d.	n.d.
22:5n-3	n.d.	n.d.	n.d.	n.d.
22:6n-3	n.d.	n.d.	n.d.	n.d.
SFA	54.16±0.20[a]	60.76±0.34[b]	69.59±1.08[c]	69.18±0.08[c]
MUFA	21.42±0.83[c]	18.17±0.36[b]	13.63±0.68[a]	14.16±0.12[a]
PUFA	22.07±0.70[d]	16.11±0.16[c]	14.42±0.19[b]	13.67±0.06[a]
Tot n-3	1.54±0.09[c]	1.09±0.04[a]	1.21±0.03[b]	1.17±0.01[b]
Tot n-6	20.28±0.51[d]	14.69±0.22[c]	12.92±0.19[b]	12.20±0.05[a]
n-6/n-3	13.18±0.41[b]	13.53±0.32[b]	10.65±0.39[a]	10.47±0.00[a]
unknown	3.04±1.03[a]	4.97±0.18[b]	2.36±0.21[a]	2.99±0.26[a]
DHA/EPA	n.d.	n.d.	n.d.	n.d.

Each value represents the mean of three independent determinations. Different letters in the same row indicate significant differences ($P<0.05$). n.d.= not detectable.

Table 6. FA profile (% fatty acids/total FA) of larvae (L) and pre-pupae (P) of BSF, analysed at 6-13- 21 and 26 days, fed diet F.

Fatty acid	Larvae 6 days	Larvae 13 days	Prepupae 21 days	Prepupae 26 days
8:0	0.07±0.01^{a}	0.11±0.01^{b}	0.10±0.01^{b}	0.14±0.03^{c}
10:0	0.10±0.00^{a}	0.38±0.02^{b}	0.51±0.13^{c}	0.59±0.04^{c}
11:0	0.04±0.01	0.02±0.01	0.03±0.01	0.03±0.02
12:0	12.96±0.14^{a}	25.87±0.69^{b}	33.60±0.64^{c}	38.23±0.75^{d}
13:0	0.05±0.01	0.05±0.01	0.08±0.02	0.07±0.03
14:0	4.19±0.21^{a}	5.89±0.13^{b}	6.85±0.93^{c}	6.91±0.06^{c}
15:0	0.65±0.03^{b}	1.51±0.22^{c}	0.47±0.00^{a}	0.38±0.12^{a}
16:0	19.51±0.26^{c}	16.42±0.17^{b}	15.11±0.21^{a}	14.55±1.02^{a}
16:1n-7	7.38±0.38^{c}	7.48±0.04^{c}	6.81±0.03^{b}	5.63±0.46^{a}
16:2n-4	0.44±0.01^{c}	0.35±0.00^{b}	0.29±0.01^{a}	0.24±0.08^{a}
17:0	0.90±0.03^{d}	0.60±0.03^{c}	0.50±0.02^{b}	0.40±0.13^{a}
16:3n-4	1.10±0.01^{c}	0.93±0.10^{b}	0.85±0.05^{b}	0.55±0.27^{a}
18:0	3.98±0.10^{c}	2.89±0.13^{b}	2.10±0.17^{a}	2.02±0.05^{a}
18:1n-9	28.32±0.14^{d}	24.68±0.35^{c}	21.58±0.61^{b}	19.95±0.38^{a}
18:1n-7	2.92±0.24^{c}	2.06±0.02^{b}	1.43±0.20^{a}	1.39±0.19^{a}
18:2n-6	1.44±0.06^{b}	1.04±0.11^{a}	1.01±0.01^{a}	1.07±0.08^{a}
18:3n-6	0.09±0.00	0.08±0.00	0.07±0.03	0.19±0.15
18:3n-4	0.11±0.01^{b}	0.05±0.02^{a}	0.08±0.03^{a}	0.05±0.04^{a}
18:3n-3	0.46±0.02	0.36±0.04	0.36±0.00	0.44±0.12
18:4n-3	0.54±0.03	0.70±0.22	0.67±0.03	0.59±0.05
20:1n-9	0.91±0.05	0.63±0.01	0.44±0.11	0.83±0.53
20:4n-6	0.77±0.03^{c}	0.59±0.06^{b}	0.49±0.08^{ab}	0.39±0.21^{a}
20:4n-3	0.20±0.01^{b}	0.13±0.02^{a}	0.07±0.09^{a}	0.10±0.01^{a}
20:5n-3	4.48±0.26^{b}	3.74±0.26^{ab}	3.43±0.45^{a}	3.03±0.99^{a}
22:1n-11	0.08±0.00^{b}	n.d.^{a}	n.d.^{a}	n.d.^{a}
22:1n-9	0.05±0.01^{b}	n.d.^{a}	n.d.^{a}	n.d.^{a}
22:4n-6	0.07±0.00^{b}	n.d.^{a}	n.d.^{a}	n.d.^{a}
22:5n-6	0.24±0.01^{c}	0.13±0.02^{b}	n.d.^{a}	n.d.^{a}
22:5n-3	0.34±0.00^{b}	0.31±0.11^{b}	0.08±0.12^{a}	0.15±0.01^{a}
22:6n-3	4.20±0.22^{c}	2.41±0.39^{b}	1.56±0.31^{a}	1.21±0.47^{a}
SFA	42.45±0.07^{a}	53.73±0.67^{b}	59.34±1.77^{c}	63.31±1.50^{d}
MUFA	39.66±0.69^{d}	34.86±0.40^{c}	30.27±0.92^{b}	27.80±0.64^{a}
PUFA	14.48±0.08^{c}	10.82±0.88^{b}	8.96±1.11^{a}	8.00±1.77^{a}
Tot n-3	10.21±0.02^{c}	7.66±0.78^{b}	6.17±0.99^{a}	5.51±1.40^{a}
Tot n-6	2.62±0.03^{c}	1.84±0.19^{b}	1.57±0.12^{a}	1.65±0.02^{a}
n-6/n-3	0.26±0.00	0.24±0.00	0.26±0.02	0.31±0.08
unknown	3.41±0.70^{c}	0.58±0.19^{a}	1.43±0.26^{b}	0.89±0.37^{ab}
DHA/EPA	0.94±0.10^{b}	0.34±0.49^{a}	0.24±0.34^{a}	0.39±0.03^{a}

Each value represents the mean of three independent determinations. Different letters in the same row indicate significant differences ($P<0.05$). n.d.= not detectable.

Table 7. FA profile (% fatty acids/total FA) of larvae (L) and pre-pupae (P) of BSF, analysed at 6-13- 21 and 26 days, fed diet R.

Fatty acid	Larvae 6 days	Larvae 13 days	Prepupae 21 days	Prepupae 26 days
8:0	0.06±0.02^{a}	0.11±0.06^{ab}	0.10±0.03^{ab}	0.16±0.05^{b}
10:0	0.04±0.02^{a}	0.19±0.06^{ab}	0.31±0.05^{b}	0.53±0.21^{c}
11:0	0.02±0.01	0.02±0.02	0.02±0.02	0.02±0.02
12:0	7.23±0.48^{a}	15.98±0.52^{b}	23.63±0.22^{c}	24.12±0.86^{c}
13:0	0.03±0.01^{a}	0.02±0.00^{a}	0.03±0.00^{a}	0.07±0.04^{b}
14:0	3.11±0.09	3.92±0.50	4.87±0.49	5.22±0.40
15:0	0.40±0.12	0.30±0.05	0.26±0.00	0.37±0.17
16:0	17.72±0.71^{c}	14.35±0.18^{b}	13.57±0.16^{a}	12.89±0.84^{a}
16:1n-7	4.83±0.86	5.09±0.21	4.60±0.15	4.90±0.96
16:2n-4	0.28±0.07^{b}	0.22±0.01^{ab}	0.17±0.00^{a}	0.21±0.05^{ab}
17:0	0.55±0.20^{b}	0.32±0.03^{a}	0.26±0.01^{a}	0.37±0.13^{a}
16:3n-4	0.48±0.17	0.39±0.00	0.33±0.00	0.52±0.32
18:0	3.51±0.04^{c}	2.23±0.16^{b}	1.74±0.07^{a}	1.83±0.13^{a}
18:1n-9	33.99±0.84^{c}	33.36±0.38^{c}	26.96±0.61^{b}	25.74±0.47^{a}
18:1n-7	2.39±0.18^{c}	1.90±0.04^{b}	1.36±0.02^{a}	1.30±0.02^{a}
18:2n-6	14.65±0.37^{c}	13.86±0.13^{b}	13.36±0.16^{a}	14.29±0.50^{bc}
18:3n-6	0.33±0.03^{b}	0.27±0.05^{ab}	0.22±0.03^{ab}	0.17±0.12^{a}
18:3n-4	n.d.	n.d.	n.d.	n.d.
18:3n-3	1.98±0.64	2.07±0.02	2.01±0.02	2.05±0.21
18:4n-3	0.40±0.01	0.49±0.03	0.44±0.01	0.53±0.15
20:1n-9	1.45±0.05^{c}	1.36±0.23^{c}	1.08±0.06^{b}	0.41±0.02^{a}
20:4n-6	0.34±0.11	0.19±0.04	0.19±0.01	0.33±0.21
20:4n-3	0.04±0.05^{a}	0.14±0.03^{b}	0.06±0.08^{a}	0.09±0.01^{ab}
20:5n-3	2.09±0.07	2.12±0.04	1.96±0.10	2.06±0.33
22:1n-11	0.05±0.01	0.03±0.05	0.02±0.03	0.02±0.03
22:1n-9	0.03±0.01^{b}	0.01±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}
22:4n-6	n.d.	n.d.	n.d.	n.d.
22:5n-6	n.d.	n.d.	n.d.	n.d.
22:5n-3	0.25±0.06	0.11±0.16	0.14±0.00	0.15±0.01
22:6n-3	1.16±0.35	0.67±0.05	0.42±0.00	0.93±0.74
SFA	32.66±0.27^{a}	37.44±0.84^{b}	44.81±0.40^{c}	45.59±0.53^{d}
MUFA	42.75±0.13^{d}	41.75±0.50^{c}	34.03±0.39^{b}	32.39±0.41^{a}
PUFA	21.99±0.38^{c}	20.56±0.39^{b}	19.31±0.07^{a}	21.36±0.95^{c}
Tot n-3	5.91±0.34	5.61±0.14	5.04±0.05	5.82±1.00
Tot n-6	15.32±0.28^{d}	14.33±0.21^{b}	13.77±0.12^{a}	14.80±0.41^{c}
n-6/n-3	2.60±0.10	2.56±0.02	2.73±0.05	2.59±0.52
unknown	2.59±0.03^{c}	0.25±0.05^{a}	1.85±0.06^{b}	0.66±0.83^{a}
DHA/EPA	0.55±0.15^{b}	0.31±0.03^{ab}	0.21±0.01^{a}	0.43±0.29^{ab}

Each value represents the mean of three independent determinations. Different letters in the same row indicate significant differences ($P<0.05$). n.d.= not detectable.

Table 8. FA profile (% fatty acids/total FA) of larvae (L) and pre-pupae (P) of BSF, analysed at 6-13- 21 and 26 days, fed diet T.

Fatty acid	Larvae 6 days	Larvae 13 days	Prepupae 21 days	Prepupae 26 days
8:0	0.07±0.00^{a}	0.12±0.0^{c}	0.09±0.00^{b}	0.14±0.02^{d}
10:0	0.11±0.09^{a}	0.44±0.19^{b}	0.47±0.14^{b}	0.56±0.11^{b}
11:0	0.02±0.01	0.02±0.01	0.03±0.00	0.03±0.01
12:0	18.73±0.77^{a}	23.03±0.87^{b}	33.54±0.38^{c}	35.28±0.42^{d}
13:0	0.05±0.02^{a}	0.08±0.01^{b}	0.09±0.00^{b}	0.10±0.01^{b}
14:0	4.02±0.73^{a}	5.81±0.65^{b}	6.45±0.83^{b}	6.70±0.78^{b}
15:0	0.71±0.22^{a}	0.94±0.00^{b}	0.86±0.06^{ab}	0.88±0.00^{ab}
16:0	19.80±0.24^{b}	19.55±0.07^{b}	17.11±0.17^{a}	16.97±0.29^{a}
16:1n-7	8.41±0.39^{b}	7.90±0.21^{b}	6.97±0.45^{a}	6.69±0.68^{a}
16:2n-4	0.45±0.09^{a}	0.56±0.06^{b}	0.46±0.06^{a}	0.43±0.04^{a}
17:0	1.12±0.42	1.27±0.10	1.01±0.15	0.98±0.14
16:3n-4	1.12±0.57	1.54±0.09	1.35±0.01	0.66±0.91
18:0	4.33±0.04^{c}	3.64±0.36^{b}	2.63±0.46^{a}	2.44±0.39^{a}
18:1n-9	23.95±0.86^{c}	21.70±0.60^{b}	18.27±0.41^{a}	18.77±0.03^{a}
18:1n-7	2.76±0.42^{c}	1.98±0.11^{b}	1.40±0.16^{a}	1.28±0.13^{a}
18:2n-6	1.46±0.16^{b}	1.20±0.07^{a}	1.06±0.02^{a}	1.22±0.16^{a}
18:3n-6	0.31±0.08^{b}	0.23±0.02^{a}	0.22±0.01^{a}	0.22±0.01^{a}
18:3n-4	n.d.	n.d.	n.d.	n.d.
18:3n-3	0.41±0.30	0.22±0.00	0.17±0.00	0.22±0.04
18:4n-3	0.45±0.08^{a}	0.55±0.07^{b}	0.58±0.01^{b}	0.60±0.06^{b}
20:1n-9	1.06±0.93	0.34±0.01	0.24±0.03	0.22±0.02
20:4n-6	0.76±0.32	0.93±0.10	0.82±0.06	0.82±0.06
20:4n-3	0.06±0.08	0.03±0.05	0.00±0.00	0.06±0.00
20:5n-3	2.82±0.14^{a}	3.27±0.25^{b}	3.06±0.10^{b}	3.11±0.09^{b}
22:1n-11	0.04±0.06	n.d.	n.d.	n.d.
22:1n-9	0.68±0.92	n.d.	n.d.	n.d.
22:4n-6	n.d.	n.d.	n.d.	n.d.
22:5n-6	1.08±0.96^{a}	0.28±0.04^{b}	n.d.^{a}	n.d.^{a}
22:5n-3	0.04±0.06^{a}	0.17±0.04^{c}	0.10±0.00^{b}	0.11±0.02^{b}
22:6n-3	2.26±0.71^{b}	2.37±0.35^{b}	1.72±0.29^{ab}	1.51±0.23^{a}
SFA	48.97±0.51^{a}	54.89±1.21^{b}	62.28±0.51^{c}	64.08±0.53^{d}
MUFA	36.90±1.73^{c}	31.94±0.48^{b}	26.89±0.16^{a}	26.97±0.56^{a}
PUFA	11.22±0.55^{b}	11.33±1.00^{b}	9.55±0.54^{a}	8.95±1.09^{a}
Tot n-3	6.04±0.43^{ab}	6.61±0.76^{b}	5.64±0.39^{a}	5.61±0.24^{a}
Tot n-6	3.61±0.55^{c}	2.63±0.09^{b}	2.11±0.09^{a}	2.26±0.10^{ab}
n-6/n-3	0.60±0.13^{b}	0.40±0.03^{a}	0.37±0.01^{a}	0.40±0.04^{a}
unknown	2.91±0.68^{d}	1.84±0.26^{c}	1.27±0.20^{b}	n.d.^{a}
DHA/EPA	0.80±0.21^{b}	0.72±0.05^{b}	0.56±0.07^{a}	0.48±0.06^{a}

Each value represents the mean of three independent determinations. Different letters in the same row indicate significant differences ($P<0.05$). n.d.= not detectable.

Table 9. FA profile (% fatty acids/total FA) of larvae (L) and pre-pupae (P) of BSF, analysed at 6-13- 21 and 26 days, fed diet A.

Fatty acid	Larvae 6 days	Larvae 13 days	Prepupae 21 days	Prepupae 26 days
8:0	0.04±0.01[a]	0.12±0.00[c]	0.08±0.00[b]	0.11±0.02[c]
10:0	0.02±0.00[a]	0.15±0.07[a]	0.07±0.00[a]	0.33±0.14[b]
11:0	0.01±0.00	0.01±0.00	0.01±0.00	0.02±0.01
12:0	2.48±0.59[a]	10.76±0.74[b]	10.56±0.25[b]	20.12±1.03[c]
13:0	0.11±0.02[a]	0.13±0.00[b]	0.14±0.00[c]	0.16±0.01[d]
14:0	4.68±0.21[a]	6.66±0.52[b]	7.22±0.09[c]	7.66±0.57[c]
15:0	1.47±0.09[b]	1.37±0.04[b]	1.42±0.07[b]	1.27±0.04[a]
16:0	28.85±1.72[c]	26.46±0.72[b]	27.03±0.85[b]	23.74±0.89[a]
16:1n-7	8.03±0.21	8.09±0.36	8.20±0.87	7.81±0.29
16:2n-4	0.61±0.02[b]	0.53±0.06[a]	0.52±0.03[a]	0.50±0.02[a]
17:0	1.75±0.14[c]	1.43±0.03[b]	1.41±0.08[b]	1.10±0.08[a]
16:3n-4	1.63±0.00[b]	1.56±0.05[b]	1.43±0.11[b]	0.67±0.94[a]
18:0	5.06±0.38[c]	3.86±0.01[b]	3.77±0.25[b]	2.65±0.18[a]
18:1n-9	27.54±0.25[c]	23.97±0.27[b]	24.46±0.61[b]	21.45±0.61[a]
18:1n-7	2.69±0.13[d]	2.29±0.12[c]	2.05±0.09[b]	1.47±0.06[a]
18:2n-6	3.58±0.09[b]	2.26±0.29[a]	2.45±0.05[a]	2.36±0.07[a]
18:3n-6	0.30±0.01[b]	0.13±0.01[a]	0.14±0.00[a]	0.13±0.01[a]
18:3n-4	n.d.	n.d.	n.d.	n.d.
18:3n-3	0.69±0.01	0.57±0.04	0.64±0.01	0.40±0.44
18:4n-3	0.31±0.44[a]	0.67±0.06[b]	0.73±0.08[b]	0.80±0.01[b]
20:1n-9	0.42±0.02[b]	0.40±0.02[b]	0.37±0.03[b]	0.25±0.05[a]
20:4n-6	0.89±0.04[b]	0.72±0.09[a]	0.77±0.01[a]	0.73±0.05[a]
20:4n-3	0.08±0.02	0.08±0.02	0.09±0.00	0.09±0.01
20:5n-3	4.48±0.22[b]	3.92±0.40[a]	4.21±0.29[ab]	4.33±0.16[ab]
22:1n-11	0.03±0.04	n.d.	n.d.	n.d.
22:1n-9	0.04±0.06	n.d.	n.d.	n.d.
22:4n-6	n.d.	n.d.	n.d.	n.d.
22:5n-6	0.09±0.12[ab]	0.14±0.02[b]	n.d.[a]	n.d.[a]
22:5n-3	0.09±0.11	0.14±0.04	0.13±0.01	0.11±0.02
22:6n-3	1.52±2.15	2.39±0.31	2.07±0.01	1.73±0.30
SFA	44.48±1.97[a]	50.95±0.53[b]	51.71±1.58[b]	57.17±0.59[c]
MUFA	38.75±0.67[c]	34.75±0.77[b]	35.08±1.36[b]	30.98±0.44[a]
PUFA	14.27±3.00	13.10±1.24	13.21±0.22	11.85±0.15
Tot n-3	7.18±2.94	7.77±0.83	7.87±0.36	7.46±0.94
Tot n-6	4.86±0.08[b]	3.25±0.41[a]	3.39±0.00[a]	3.22±0.13[a]
n-6/n-3	0.74±0.29[b]	0.42±0.01[a]	0.43±0.02[a]	0.43±0.04[a]
unknown	2.50±0.36[c]	1.20±0.06[b]	n.d.[a]	n.d.[a]
DHA/EPA	0.33±0.46	0.61±0.02	0.49±0.04	0.40±0.06

Each value represents the mean of three independent determinations. Different letters in the same row indicate significant differences ($P<0.05$). n.d.= not detectable.

Table 10. FA profile (% fatty acids/total FA) of pre-pupae analyzed at the end of the experiment (P26).

Fatty acid	B	F	R	T	A
8:0	0.14±0.00	0.14±0.03	0.16±0.05	0.14±0.02	0.11±0.02
10:0	0.89±0.08^{c}	0.59±0.04^{b}	0.54±0.21^{b}	0.56±0.11^{b}	0.33±0.14^{a}
11:0	0.02±0.00	0.03±0.02	0.02±0.02	0.03±0.01	0.02±0.01
12:0	46.62±0.19^{e}	38.23±0.75^{d}	24.12±0.86^{b}	35.28±0.42^{c}	20.11±1.01^{a}
13:0	0.06±0.00^{a}	0.07±0.03^{a}	0.07±0.04^{a}	0.10±0.01^{a}	0.15±0.01^{b}
14:0	7.89±0.03^{c}	6.91±0.06^{b}	5.22±0.40^{a}	6.70±0.78^{b}	7.66±0.56^{c}
15:0	0.38±0.00^{a}	0.38±0.12^{a}	0.37±0.17^{a}	0.88±0.00^{b}	1.27±0.05^{c}
16:0	11.78±0.09^{a}	14.55±1.02^{c}	12.89±0.84^{b}	16.97±0.29^{d}	23.73±0.92^{e}
16:1n-7	3.91±0.15^{d}	5.63±0.46^{a}	4.90±0.96^{a}	6.69±0.68^{b}	7.81±0.28^{c}
16:2n-4	0.05±0.00^{a}	0.24±0.08^{b}	0.21±0.05^{b}	0.43±0.04^{c}	0.50±0.02^{d}
17:0	0.29±0.00^{a}	0.40±0.13^{a}	0.37±0.13^{a}	0.98±0.14^{b}	1.10±0.08^{b}
16:3n-4	0.25±0.00	0.55±0.27	0.52±0.32	0.66±0.91	0.66±0.94
18:0	1.12±0.02^{a}	2.02±0.05^{b}	1.83±0.13^{b}	2.44±0.39^{c}	2.65±0.18^{c}
18:1n-9	9.05±0.04^{a}	19.95±0.38^{c}	25.75±0.47^{e}	18.77±0.03^{b}	21.43±0.63^{d}
18:1n-7	0.69±0.06^{a}	1.39±0.19^{b}	1.30±0.02^{b}	1.28±0.13^{b}	1.47±0.06^{b}
18:2n-6	12.10±0.05^{d}	1.07±0.08^{a}	14.30±0.50^{c}	1.22±0.16^{a}	2.36±0.07^{b}
18:3n-6	0.03±0.00^{a}	0.19±0.15^{b}	0.17±0.12^{b}	0.22±0.01^{b}	0.13±0.01^{b}
18:3n-4	n.d.	0.05±0.04	n.d.	n.d.	n.d.
18:3n-3	0.84±0.00^{b}	0.44±0.12^{a}	2.05±0.21^{c}	0.22±0.04^{a}	0.40±0.44^{a}
18:4n-3	0.12±0.00^{a}	0.59±0.05^{b}	0.53±0.15^{b}	0.60±0.06^{b}	0.80±0.01^{c}
20:1n-9	0.52±0.00^{ab}	0.83±0.53^{b}	0.41±0.02^{a}	0.22±0.02^{a}	0.25±0.05^{a}
20:4n-6	0.06±0.00^{a}	0.39±0.21^{b}	0.33±0.21^{b}	0.82±0.06^{c}	0.73±0.05^{c}
20:4n-3	n.d.^{a}	0.10±0.01^{c}	0.09±0.01^{c}	0.06±0.00^{b}	0.09±0.01^{c}
20:5n-3	0.21±0.00^{a}	3.03±0.99^{c}	2.06±0.33^{b}	3.11±0.09^{c}	4.33±0.16^{d}
22:1n-11	n.d.	n.d.	n.d.	n.d.	n.d.
22:1n-9	n.d.	n.d.	n.d.	n.d.	n.d.
22:4n-6	n.d.	n.d.	n.d.	n.d.	n.d.
22:5n-6	n.d.	n.d.	n.d.	n.d.	n.d.
22:5n-3	n.d.	0.15±0.01^{b}	0.15±0.01^{b}	0.11±0.02^{a}	0.11±0.02^{a}
22:6n-3	n.d.	1.21±0.47^{ab}	0.93±0.74^{a}	1.51±0.23^{ab}	1.73±0.30^{b}
SFA	69.18±0.08^{d}	63.31±1.50^{c}	45.59±0.53^{a}	64.08±0.53^{c}	57.17±0.59^{b}
MUFA	14.16±0.12^{a}	27.80±0.64^{c}	32.39±0.41^{e}	26.97±0.56^{b}	30.98±0.44^{d}
PUFA	13.67±0.06^{c}	8.00±1.77^{a}	21.36±0.95^{d}	8.95±1.09^{a}	11.85±0.15^{c}
Tot n-3	1.17±0.01^{a}	5.51±1.40^{b}	5.82±1.00^{b}	5.61±0.24^{b}	7.46±0.94^{c}
Tot n-6	12.20±0.05^{d}	1.65±0.02^{a}	14.80±0.41^{e}	2.26±0.10^{b}	3.22±0.13^{c}
n-6/n-3	10.47±0.00^{c}	0.31±0.08^{a}	2.59±0.52^{b}	0.40±0.04^{a}	0.43±0.04^{a}
unknown	2.99±0.26^{c}	0.89±0.37^{b}	0.66±0.83^{ab}	n.d.^{a}	n.d.^{a}
DHA/EPA	n.d.	0.39±0.03	0.43±0.29	0.48±0.06	0.40±0.06

Each value represents the mean of three independent determinations. Different letters in the same row indicate significant differences (P<0.05). B: wheat bran; F: by-catch sea trawl fishery; R: aquaculture processing by-products; T: *T. albacares* processing by-products; A: *E. encrasicolus* processing by-products. n.d.= not detectable.

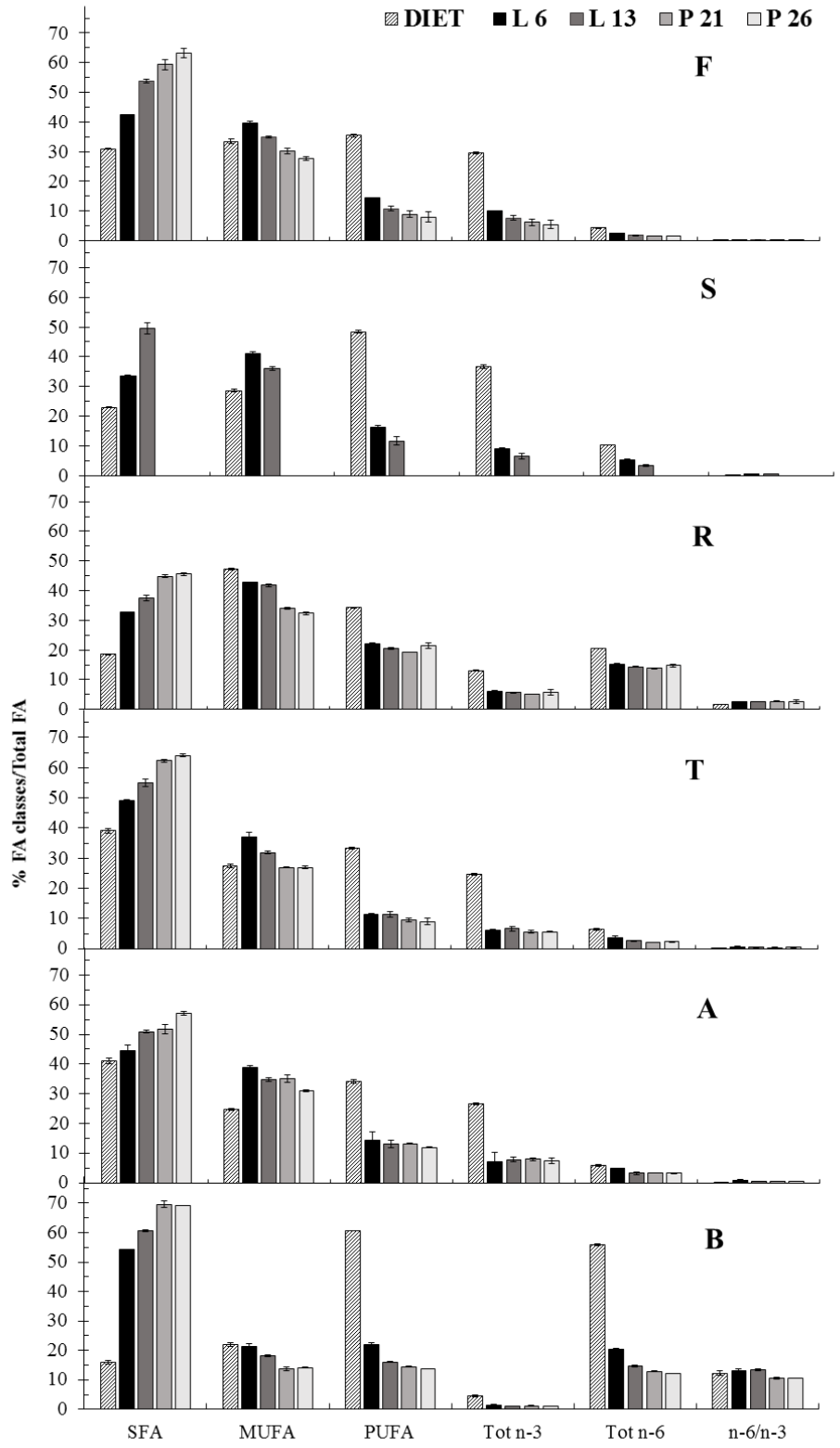

Figure 2. FA classes composition (% fatty acids/ total FA) of experimental diets larvae (L) and pre-pupae (P) of BSF, analysed at 6-13- 21 and 26 days. Each value represents the mean of three independent determinations. B: wheat bran; F: by-catch sea trawl fishery; S: P. longirostris processing by-products; R: aquaculture processing by-products; T: T. albacares processing by-products, A: E. encrasicolus processing by-product.

Due to the selection of appropriate biomarkers and quality indicators, PCA allowed us to appreciate the differences among samples. PCA was applied to fatty acid classes (Figure 3). Eight variables (12:0, SFA, MUFA, PUFA, Tot n-3, Tot n-6, DHA/EPA, and n-6/n-3) were considered for the analysis; PCA was used to examine 25 cases consisting of the average fatty acid values of the diets and of the BSF larvae and pre-pupae fed different diets. The analysis generated a small number of linear combinations on the eight variables and three principal components with an eigenvalue greater than one were identified. These three components could explain the 95.51% of the variance of the original variables.

A

B

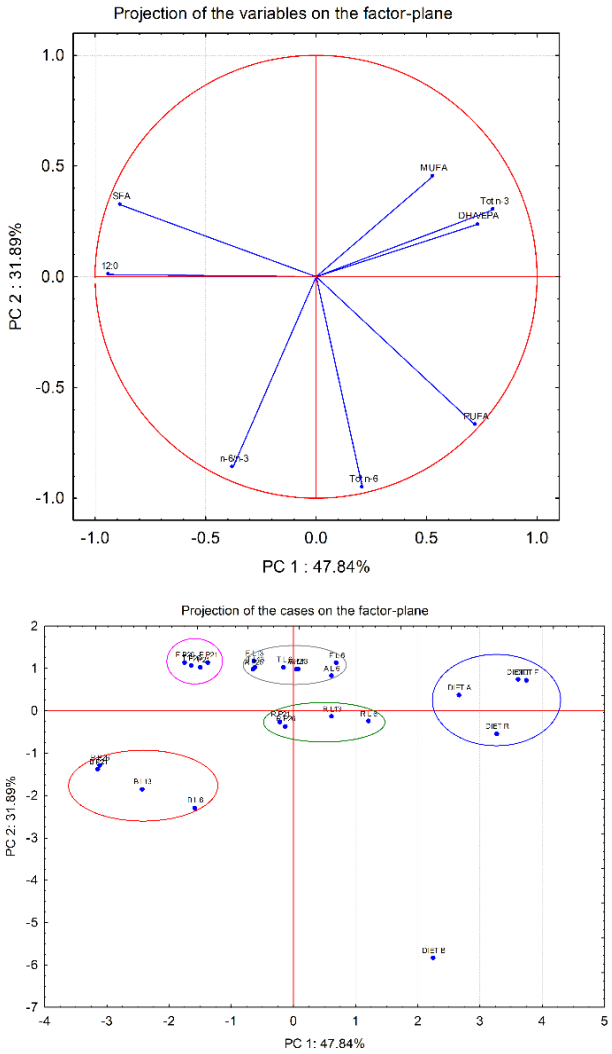

Figure 3. Principal component analysis (PCA) obtained from correlation of eight components (12:0, SFA, MUFA, PUFA, Tot n-3, Tot n-6, DHA/EPA, and n6/n-3) and 25 cases determined on larvae and pre-pupae fed with different diets. (A) Correlation circle; (B) factor-plan. Red circle: larvae and pre-pupae fed diet B; blue circle: experimental diets; green circle: larvae and pre-pupae fed diet R; purple circle: pre-pupae fed diet F and T; gray circle: larvae and pre-pupae fed diet A and larvae fed diets F and T.

PCA was performed considering the first two principal components (PCs) (Figure 3); this analysis explained 79.73% of the variability in the original data Figure 2). The first principal component (PC1) explained 47.84% of the combined variance and the second component (PC2) explained 31.89% (Figure 3).

The correlation circle (Figure 3A) showed the correlation of the eight variables in the experimental cases. In particular, a positive correlation was observed between 12:0 and SFA; in fact, 12:0 was the predominant fatty acid in the SFA class. Figure 3B shows the factorial plane of the samples, identifying distinct groups that characterize the different larvae and pre-pupae fed different diets.

It is can be seen that larvae fed diet B were spatially distanced from the other samples (Figure 3B). As shown in the correlation circle Figure 3A), these samples were mainly influenced by 12:0, which showed the highest values in P26 fed diet B (Table 10).

PCA allowed to discriminate how larvae are influenced by their diet (Figure 3B) since the beginning of feeding. The larvae and prepupae fed diet R are grouped together, distinguishing them from larvae and pre-pupae fed other diets; this is influenced by MUFA, as can be observed from the circle of correlations (Figure 3A).

2.5. Discussion

This study investigated the effects of different fish by-products, characterized by a wide variability in chemical composition, on the development and nutritional composition of BSF larvae and pre-pupae at the laboratory scale. The use of bioconverting insects, such as BSF, allows for the use and valorization of by-products to produce larvae enriched with protein and fat. The obtained results, although not derived from an industrial production process, provide an important insight into the potential sustainable uses for BSF in aquaculture. The use of marine by-products as feed for BSF larvae supports a circular system, enabling the achievement of "zero-waste" goals.

2.5.1. Effects of the experimental diets on BSF larvae development

Larvae fed the different experimental diets (Figure 1) showed a lower growth rate than larvae fed wheat bran (diet B). As reported by other authors (Grossule et al., 2020; Grossule and Lavagnolo, 2020), these results could reflect the different availability of carbon and degradable nutrients in the feeding substrates employed, thus confirming the predominant role of bran as a source of carbon and nutrients (Grossule et al., 2020; Grossule and Lavagnolo, 2020).

Larvae fed diet S died after few days of the trial, probably due to the high level of chitin, which has negative effects on growth, food efficiency, and nutrient (particularly protein) digestibility (Guerreiro

et al., 2020; Marono et al., 2015; Olsen et al., 2006; Shiau and Yu, 1999) or due to the lowest energy content of diet S among the diets (Starcevic et al., 2019) (Table 2).

2.5.2. Proximate composition of the pre-pupae

Recently, interest in the utilization of BSF larvae as a raw material for animal feed has increased, mainly due to its potential as a sustainable source of high-quality protein (Kroeckel et al., 2012; Liland et al., 2017; Veldkamp et al., 2012).

BSF is a scavenger, commonly used to accelerate the composting of organic material, as it can efficiently utilize organic resources, such as fruit, vegetable, and meat waste (Čičková et al., 2015; Surendra et al., 2016) and, depending on the growing medium, can modulate its own body composition, reaching a high concentration of lipids (>30% of dry weight (dw) and proteins (around 40% of dw) (Diener et al., 2009; St-Hilaire et al., 2007).

As reported by Cammack and Tomberlin (2017) diet does not have a significant impact on adult life-history traits; in fact, BSF feeds only in the larval stage. Dietary composition affects larval growth and performance (Nairuti et al., 2022).

The employed by-products in this study demonstrated an influence on the nutrient profiles of the BSF pre-pupae. Obtained results showed the highest lipid content in pre-pupae fed diet R (28.05 ± 1.90 g/100 g dw), which showed the highest lipid content (27.97 ± 0.15 g/100 g wet) of the experimental diets. The lipid content in insects is largely dependent on their diets and stage of development (Stanley-Samuelson and Dadd, 1983) and it is affected by the composition of the rearing substrate (Franco et al., 2021). In fact, the lipid content can vary by 15–49% of the total dry weight (Franco et al., 2021). This confirms that rearing BSF pre-pupae on substrates with a high lipid content resulted in higher lipid and lower protein contents in BSF pre-pupae, as reported by Nairuti et al., (2022)

The pre-pupae fed with experimental diets showed a protein content ranging from 34.29 to 34.94 g/100 g. The highest protein content was observed in pre-pupae fed diet B (P26-B, 39.93 g/ 100 g), in accordance with the values reported in literature (31.9%–46.3%) (Barroso et al., 2014; Diener et al., 2009; Sánchez-Muros et al., 2014; Surendra et al., 2016). The method used to determine the protein content of BSF prepupae larvae is based on the percentage of nitrogen, also taking into account the fraction of nitrogen attributable to chitin, as described in the Materials and methods section. This method, as reported in the literature (Díaz-Rojas et al., 2006; Messina et al., 2019), allows a correct estimation of the protein percentage and the accuracy of the results. The values obtained in our study are in accordance with those reported by Boulos et al. (2020) and Janssen et al. (2017), using a different method.

The varying proximate composition of BSF fed with various diets confirms the entirely generalist nature of this insect and therefore its plasticity in the use of food waste of varying origins and compositions, and confirms its ability to skillfully convert this food waste into nutrients (Cammack and Tomberlin, 2017). Although BSF exhibits high plasticity, some substrates appear to perform less well in the growth processes of reared larvae, as observed in larvae reared on diet S ((Guerreiro et al., 2020; Marono et al., 2015; Olsen et al., 2006; Shiau and Yu, 1999). Numerous studies are in progress to define which substrates are most suitable for the optimal growth of this species in qualitative and quantitative terms (Ribeiro et al., 2022).

Our results indicate the high potential of insects as alternative sources of new and renewable animal proteins and fat (Finke, 2002; Gobbi et al., 2013; Rumpold and Schlüter, 2013; Sánchez-Muros et al., 2014; Zielińska et al., 2015).

2.5.3. FA profile

BSF larvae have been reared mainly for their protein content (Halloran et al., 2016; Nogales-Mérida et al., 2019) and only recently have attracted interest for their lipid content (Barroso et al., 2019; Hossain et al., 2023; Rodrigues et al., 2022; Smetana et al., 2019; Spranghers et al., 2017).

Our results show that the FA profile of the different provided diets influenced the FA profiles of the BSF larvae and partially affected the fatty acid profile of the pre-pupae, as shown in the PCA analysis (Figure 3), which is in agreement with Barroso et al. (2019) and Spranghers et al. (2017). In the pre-pupae the main fatty acid was C12:0, even if this fatty acid was contained in the substrates only in trace amounts. Obtained results, in accordance with Barroso et al. (2019), highlighted the higher C12:0 content in pre-pupae fed the control diet than in pre-pupae fed with fish by-product substrates; indeed, several studies show that larvae fed on substrates containing cereals, fruit, and vegetables show the lauric acid highest content (Rodrigues et al., 2022). High lauric acid content could be due to the bioconversion of carbohydrates to lipids (Guil-Guerrero et al., 2018). Lauric acid represents the main lipid component of BSF and it is converted by animals and humans into monolaurin (antiviral, antibacterial, and antiprotozoal glyceride) (Leong et al., 2015; Ushakova et al., 2016). A high amount of lauric acid enables the larvae to survive in substrates with temperatures above 40°C, as lauric acid gives the larvae a solid consistency that is not subject to the rapid oxidation of fats (the melting point of lauric acid is 43.2°C) (Ushakova et al., 2016).

BSF pre-pupae fed different fish by-products showed higher contents of n-3 fatty acids than the control, in agreement with recent studies (Barroso et al., 2019; Ewald et al., 2020; Rodrigues et al., 2022). In particular, pre-pupae fed diet A had the highest levels of EPA, followed by pre-pupae fed diets T, F, and R (Table 10). Regarding DHA, the pre-pupae accumulated a maximum of 1.73% DHA

(P26 diet A), whereas DHA constituting 17.54% of the total FA content was detected in diet A (Table 10). In addition, the n-6/n-3 values decreased in the larvae and pre-pupae fed diets R, A, T, and F (Table 5-10, Figure 2) when compared with control, as was also reported by Barroso et al. (2019). Since the essential fatty acids EPA and DHA, which belong to marine environments, are not found in BSF (Barroso et al., 2019), our results, which show an increase of EPA and DHA in BSF larvae and pre-pupae fed fish by-products, confirm that marine sources, in particular fish by-products, are capable of enriching BSF with these fatty acids (Barroso et al., 2019; Ewald et al., 2020; Rodrigues et al., 2022; Sealey et al., 2011; St-Hilaire et al., 2007).

2.6. Conclusions

The aim of this study was to investigate the effects of different fishery and aquaculture by-product diets on the growth, body composition, and fatty acid profile of BSF larvae and pre-pupae, with the aim of converting organic waste into high-value biomass as part of a "zero-waste" approach. Our results show that *E. encrasicolus* processing by-products, by-catch from a sea trawling fishery, *T. albacares* processing by-products, and aquaculture processing by-products could be interesting feeding substrates for the rearing of *H. illucens*. Using these by-products, it was possible to modulate the lipid and fatty acid profile of BSF larvae, increasing their level of n-3 fatty acids. This possibility represents added value to BSF as an organism capable of modulating its lipid and fatty acid composition for its utilization as an alternative source of ingredients for aquaculture feeds.
Furthermore, the utilization of these by-products could support circular economy pathways, contributing to the reduction of food losses and waste.

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Part 3 - Valorization of marine by products in food sector

Sustainability and innovation in the seafood supply chain: mapping by-product oand application

The increase in global fish production, as reported by the FAO (2024), represents a significant challenge for the fish processing industry, that generates considerable biomass, that ranges from 30% to 70%, depending on the species and the processing (Abuzar et al., 2023; FAO, 2024; Messina et al., 2021).

During my project I had the opportunity to work with the company Blu Ocean s.r.l., a company committed to maximizing sustainability in the production processes.

Furthermore, Blu Ocean is involved in a supply chain project approved by decree of the Ministry of Agriculture that is aimed at increasing the environmental, economic, and social sustainability of the "multi-species fishing system of excellence".

Working in partnership with the company, it has been possible to identify and implement effective strategies to reduce waste along the supply chain. Particular attention has been paid to the management and fate of waste, mapping the production and plan some actions aimed to valorize it.

In order to map the biomass of by-products and classify it, I realized a questionnaire that was provide to the industry (Table 1 and Table 2).

Table 1. General survey on production data of residual processing fractions (wastes, by-products and offcuts) of processed fish products (from minimal processing to the fourth stage).

Main species of origin of processing fractions of different categories	□ _____
Amount of raw material processed annually (kg)	□ 1-10 Kg □ 10-50 kg □ 50-100kg □ 100-200kg □ over 300 kg
Production residue components	□ Heads, bones, skin □ Viscera □ Carapace, claws and other_____
Amount of production residues annually (kg)	□ 1-10 Kg □ 10-50 kg □ 50-100kg □ 100-200kg □ over 300 kg
Actual destination	□ Disposal of waste □ Other_____

Table 2. Identification of reuse processes for production residues.

Can by-products be obtained from the production process (Legislative Decree 152/2006, art. 184-bis)?	□ Yes □ Not
Can production residues that cannot be classified as by-products be recovered from the production process (end-of-waste or energy material)? (Legislative Decree 152/2006 Article 184-ter (End of waste status))	□ Yes □ Not

The survey showed that the company produces more than 300 tonnes of residues per year, mainly consisting of heads, bones, skin, and guts, mainly from the processing of salmon, sea bass and sea bream (from the minimum to the fourth processing stage). Waste is generated as a result of the processing of ready-to-eat and ready-to-cook products.

The company has optimized the valorization of by-products thanks to the purchase of a pulping machine, financed by a project supported by the Region of Sicily. The use of a pulping machine in the mechanical processing of fish has significantly increased yield and efficiency by mechanically separating the meat from the bones, skin and other parts of the fish (Sampels, 2015). This innovative process has allowed the recovery of side streams that would otherwise have been discarded, enabling the production of a wide range of high value-added fish products, including hamburgers, fish patties and the like. Despite the increased efficiency, the process still generates around 12 tonnes of waste per month.

Currently, these residues are disposed of as waste, but they have the potential to be classified as by-products. If properly managed and utilized, they could be redirected to other sectors for reintegration into the production cycle, thus contributing to greater sustainability and waste reduction.

The company is strongly committed to improving the management of its waste and is actively investigating ways to improve its processing systems. It is even considering the purchase of minimal waste processing technologies that would allow it to convert these residues into valuable raw materials. These by-products could be redirected to companies specializing in the extraction of high value components for the pharmaceutical, nutraceutical or other specialist industries.

This approach is in line with the principles of a circular economy, which promotes sustainability while significantly reducing waste. By exploring opportunities to reuse these materials - for example in the production of animal feed, biofuels or bioactive compounds - the company could not only improve its environmental footprint but also create new revenue streams.

As part of a co-creation activity with my research group and the company Blue Ocean, an activity was initiated to valorize the blue crab as a potential resource, with the production of crab cakes, crab-based pasta toppings and blue crab burgers. However, a significant problem is the large amount of waste produced and the need to valorize it.

Application of by-products in bioactive packaging

Blue crab by-products from Blu Ocean processing were used to extract protein hydrolysates by an enzymatic hydrolysis process (Chapter 4). These hydrolysates showed high antioxidant activity, suggesting their potential for applications in various fields, including nutraceuticals, cosmeceuticals and food. In particular, similar results were observed for crustacean protein hydrolysates, they could be incorporated into chitosan-gelatin films to produce active food packaging with antioxidant properties (Hajji et al., 2021).

Today, the food industry relies heavily on packaging for product preservation. Packaging, mainly derived from petrochemicals, has become fundamental to the sector, and is used in a wide range of products. However, the environmental impact and end-of-life management of these materials pose significant challenges, making the development of more sustainable and environmentally friendly solutions urgent (Debeaufort, 2021). As a result, packaging has become a social issue.

In order to minimize the environmental impact and reduce packaging waste, manufacturers are exploring two main directions when selecting packaging materials: reusable and biodegradable packaging (Debeaufort, 2021).

In this regard, the optimal packaging solution would be biodegradable, exhibiting barrier properties, recyclable, derived from renewable sources, and produced without the utilization of food resources (Debeaufort, 2021).

The utilization of food waste and by-products for the development of sustainable packaging represents a rapidly evolving field of research, which is attracting growing interest from both the food processing and packaging material industries (Debeaufort, 2021).

Many of bioactive compounds have the potential to be used in the development of active packaging materials (Benbettaïeb et al., 2019b, 2019a; Debeaufort, 2021).

Such bioactive compounds include chitosan (obtained by deacetylation of crustacean chitin), gelatin (extracted from fish and cephalopod skins), and fish myoproteins. These compounds have film-forming properties, which can be cross-linked through physical or chemical treatments to produce biopolymers and provide structural properties of packaging films (Benbettaïeb et al., 2016; Benbettaïeb et al., 2015; Debeaufort, 2021; Guerrero et al., 2020; Kchaou et al., 2018).

Furthermore, peptides and some enzymes derived from fish by-products, which exhibit antioxidant properties, can be integrated into biopolymers to inhibit the oxidative degradation of films and prevent food oxidation. Additionally, they possess antimicrobial and healing properties, which are beneficial for the development of active dressings and packaging in the food and pharmaceutical industries (Debeaufort, 2021; Hajji et al., 2021; Kchaou et al., 2020).

Scientific research has demonstrated the feasibility of valorizing fish by-products for active and sustainable packaging applications. However, several obstacles must be overcome for their effective implementation. These include organizing the collection of recyclable waste, developing industrial-scale extraction processes, controlling costs to remain competitive with existing packaging systems, managing health risks throughout the supply chain, adapting regulations, and increasing collaboration among scientists, producers, clients, and governments. It is also crucial to engage and educate consumers to accept future solutions, even if they are less effective but more sustainable. Overcoming these obstacles, biodegradable and bio-based packaging, based on current knowledge, could represent a very near future (Debeaufort, 2021).

Chapters 3 and 4 discuss in detail the strategies and solutions adopted to manage and valorize the by-products of blue crab processing, showing how this species can be turned from a problem into a resource.

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Chapter 3. Valorization of the invasive blue crabs *(Callinectes sapidus*) in the Mediterranean: nutritional value, bioactive compounds and sustainable by-products utilization

In this chapter, I have written a review highlighting the potential of the blue crab (*Callinectes sapidus*) as both a valuable food resource, due to its high nutritional content, and as a source of by-products containing proteins, lipids, chitin and minerals that can be valorized to improve both economic and environmental sustainability.

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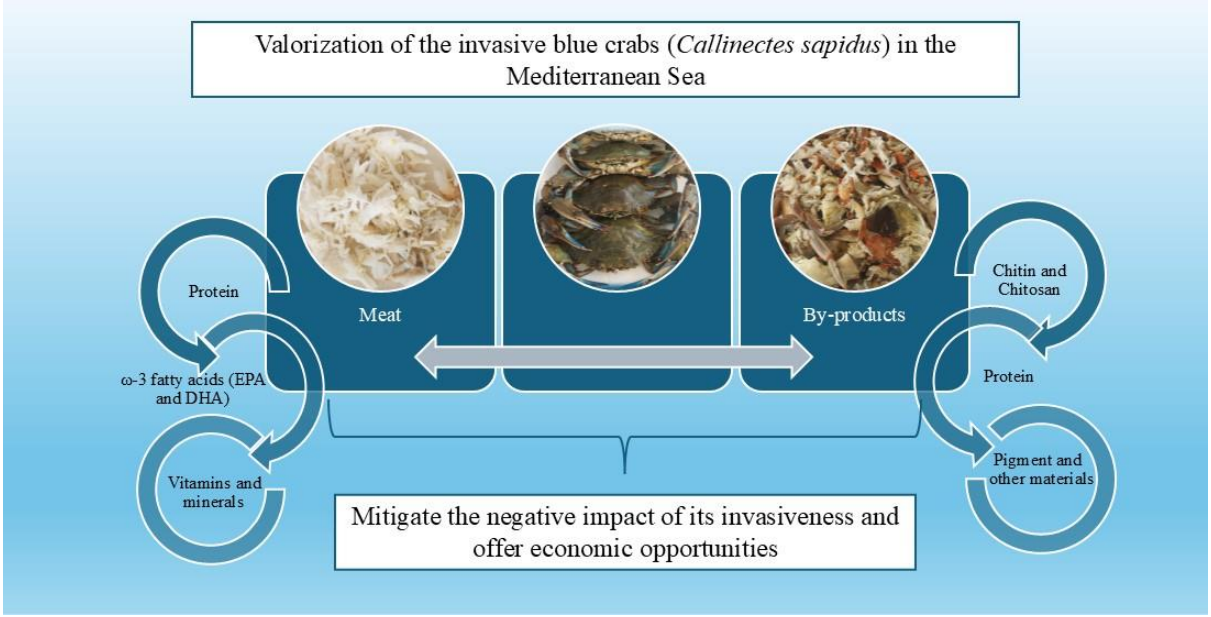

Graphical abstract "Valorization of the Invasive Blue Crabs (Callinectes sapidus) in the Mediterranean: Nutritional Value, Bioactive Compounds and Sustainable By-Products Utilization. Mar. Drugs 2024, 22, 430".

3.1. Abstract

The blue crab (*Callinectes sapidus*), originally from the western Atlantic Ocean, has recently spread to the Mediterranean and is now considered one of the one hundred most invasive species in that region. This opportunistic species, known for its adaptability to different temperatures and salinities, negatively impacts biodiversity and human activities such as fishing and tourism in the Mediterranean. However, the blue crab is gaining interest as a potential food resource due to its high nutritional value and delicate, sweet flavor. Its meat is rich in protein (14% to 30%), omega-3 fatty acids (EPA and DHA) and other essential nutrients beneficial for human health such as vitamins, and minerals. Utilizing this species in the production of new foods could help mitigate the negative impact of its invasiveness and offer economic opportunities. One challenge with this potential resource is the generation of waste. Approximately 6–8 million tonnes of crab shells are produced worldwide each year, leading to disposal problems and concerns regarding environmental sustainability. To improve economic and environmental sustainability, there is a need to valorize these residues, which are an important source of proteins, lipids, chitin, minerals, and pigments that can be processed into high-value-added products. However, especially in areas with industrial pollution, attention should be paid to the heavy metal (Cd and As) contents of blue crab shells. Studies suggest that blue crab by-products can be used in various sectors, reducing environmental impacts, promoting a circular economy, and creating new industrial opportunities.

Keywords: *Callinectes sapidus*; blue crabs; nutrition value; by-products

3.2. Introduction

The blue crab (*Callinectes sapidus* Rathbun, 1896 (Brachyura: Portunidae)), native to the western Atlantic Ocean (Kevrekidis et al., 2023), is an opportunistic euryhaline species whose life cycle occurs in both brackish and marine habitats, including estuaries, lagoons, and other coastal habitats (Mancinelli et al., 2021; Taybi and Mabrouki, 2020).

Blue crabs spend most of their life cycle in estuarine habitats and their distribution depends on temperature, salinity and dissolved oxygen (Kevrekidis et al., 2023). Temperature plays a key role in the life cycle of this species. Juvenile and adult blue crabs have a tolerance range of 0–39 °C, which may depend on several factors, including the ability of the blue crab to acclimate to both temperature and salinity (Kevrekidis et al., 2023; Tagatz, 1969). During the warm season, egg production and incubation occur, while feeding, movement, and moulting cease when temperatures drop below 10

°C (Hines et al., 2010). In terms of salinity, blue crabs use various salinity regimes to complete their life cycle. For example, they use low-salinity environments for mating and as nursery habitats, and seawater salinity levels for larval growth (Marchessaux et al., 2022). Therefore, blue crabs can tolerate and grow in a wide range of environmental salinities (Kevrekidis et al., 2023). Furthermore, blue crabs disperse along a salinity gradient according to sex and life stage: males and juveniles prefer oligohaline waters upstream, while adult females select areas with meso-and polyhaline salinities (Taylor and Fehon, 2021). With regard to the parameter of dissolved oxygen (DO), it was demonstrated that this has a significant effect on the growth and abundance of blue crabs. Levels of DO below 2–4 mg/L were shown to induce an avoidance response in crabs, while exposure to levels below 1.2 mg/L was observed to result in mortality (Taylor and Fehon, 2021).

The successful spread of blue crabs across regions is due to their biological characteristics: rapid growth rates, opportunistic feeding (on vegetation, detritus, polychaetes, molluscs, crustaceans and fish), high reproductive rate, generalist habitat uses, long-distance larvae dispersal, and effective physical and aggressive behaviours, including scavenging and cannibalistic (Kampouris et al., 2019; Kevrekidis et al., 2023; Mancinelli et al., 2017; Marchessaux et al., 2023; Streftaris and Zenetos, 2006). Due to these characteristics, the blue crab, accidentally introduced in the early 20th century in the Mediterranean Sea, likely through ballast water (Nehring, 2011) and deliberately introduced in the Aegean Sea in the 1930s (Zotti et al., 2016b), has since spread widely in the Mediterranean, the Black Sea, and along the European coasts of the Atlantic Ocean (Mancinelli et al., 2021). Thus far, blue crabs are considered one of the hundred “worst invasive” alien marine species in the Mediterranean Sea, with a high impact on ecosystem biodiversity and human activities (fisheries, aquaculture, tourism) (Khamassi et al., 2022; Piras et al., 2019). This species has the potential to cause radical changes in the composition and structure of marine communities in coastal zones by competing with native species for resources (Clavero et al., 2022; Kevrekidis et al., 2023). It has a severe impact on artisanal fisheries, reducing both the quantity and quality of catches, and negatively affecting overall income (Marchessaux et al., 2023). Despite the negative impacts, the blue crab could be considered a new source of income since, considering its high nutritional value (Khamassi et al., 2022; Mancinelli et al., 2017; Marchessaux et al., 2023; Nuñez et al., 2012), its harvesting could improve the economic situation of both the local fishing (Khamassi et al., 2022; Mancinelli et al., 2017) and processing sectors through its utilization in local industries and the valorization of by-products (Khamassi et al., 2022; Mancinelli et al., 2017).

3.3. Commercial Value and Management Approach

The blue crab holds substantial commercial value, particularly in its native range along the western Atlantic Ocean and the Gulf of Mexico. In these regions, it is a cornerstone of the fishing industry, contributing significantly to local economies through job creation in harvesting, processing, distribution and sales sectors (NOAA, 2023).

In the Mediterranean Sea, the blue crab has become an invasive species, posing significant ecological and economic challenges, particularly in regions such as Sicily (Marchessaux et al., 2023; Tarkan et al., 2021).

The blue crab represents a double-edged sword for local ecosystems and economies. Ecologically, it can alter local marine life by preying on native species and competing for resources, reducing biodiversity and affecting fisheries. In addition, blue crab management is costly, as traditional disposal methods are expensive and laborious, with a low yield of usable meat.

On the other hand, turning the invasive blue crab into a commercial resource offers a novel solution in the European context to mitigate its negative impacts. By developing products such as crab patties, pasta sauces, and crab burgers, businesses can create economic value from what is otherwise considered a nuisance. This approach aligns with broader strategies seen in other regions, where invasive species are harvested for food, pharmaceuticals, or cosmetic products, thereby controlling their populations while generating revenue. This approach was successfully implemented in Sicily where a seafood enterprise has integrated innovative practices to manage and utilize the blue crab, developing a range of blue crab-based products. These efforts have focused on creating value-added products tailored to both local and national markets, despite the inherent challenges associated with the species' low meat yield and high processing costs.

The effective commercialization of blue crab products necessitates robust infrastructure and advanced technological applications. Significant investments have been made in novel preservation methods, such as modified atmosphere packaging, to extend the shelf life of blue crab products. Additionally, the implementation of mechanized processing systems is emphasized to reduce labour costs and enhance operational efficiency.

While the current infrastructure adequately supports existing operations, several challenges persist, including the need for more efficient processing technologies and improved integration within supply chain and logistics frameworks. Future plans are directed towards upgrading facilities to enhance both production capacity and operational efficiency, reflecting a broader industry trend towards modernization and technological integration within the seafood sector.

Regulatory frameworks play a critical role in shaping the economic viability of utilizing blue crabs. Until recently, the use of specific fishing gear and the classification of blue crabs as a marketable species were major regulatory hurdles. For instance, the Veneto Region received a special allowance to use dredges within three miles of the coast to prevent damage to mussel beds. These regulatory adjustments are crucial for enabling businesses to legally and efficiently harvest blue crabs.

Market perception and consumer acceptance are also vital. In Sicily, the blue crab is not yet seen as a significant threat, but its potential to become one is recognized. Developing a market for blue crab products involves not only creating appealing products but also educating consumers about the benefits of using this invasive species as a sustainable food source.

3.4. Nutritional Aspects of *C. sapidus*

In the Mediterranean, there is a growing interest in the potential of the blue crab as a food resource due to its high nutritional value (Khamassi et al., 2022; Mancinelli et al., 2017; Marchessaux et al., 2023; Nuñez et al., 2012). The meat, characterized by a delicate and sweet flavour and sold at a high market price (averaging USD 35.84 per pound (Martínez et al., 2017)), is rich in essential nutrients such as omega-3 fatty acids, vitamins, and minerals. These nutritional components, beneficial for human health, combined with the low fat content, contribute to improving cardiovascular performances in terms of heart health, brain function, and overall well-being (Nanda et al., 2021).

The proximate composition in terms of moisture, lipid, protein, and ash, as well as the main fatty acids reported by different studies on the edible meat of the blue crab, are summarized in Table 1 Differences in biochemical composition among individuals of the same species can be attributed to various factors, such as habitat influence, which is related to different diets with different biochemical compositions that directly affect the crab, exposure to different environmental conditions, and resource availability (Nanda et al., 2021). Seasonal and climatic changes also play a fundamental role in shaping the biochemical composition of this species. Environmental factors such as temperature, salinity, light, and nutrient availability throughout the year can impact their metabolism and biochemical processes, leading to variations in their composition. Biological differences, such as size, age, sex, maturity stage, gametogenesis, and spawning cycle, can also influence the biochemical composition of the blue crab (Jiang et al., 2020; Martínez et al., 2017; Nanda et al., 2021; Tufan, 2023; Wu et al., 2020).

In terms of nutritional composition, blue crab meat is rich in protein, typically ranging from 14% to 30%. This protein content is influenced by seasonal variations, with the lowest levels observed in autumn for both males and females and the highest values in the spring season, which could be due

to the change in the carapace of blue crabs in autumn (Ayas, 2016). On this basis, the consumption of 50 g of *C. sapidus* meat can meet the World Health Organisation's (WHO) recommended daily requirement of essential amino acids for an adult (FAO/WHO/UNU, 1985; Küçükgülmez et al., 2006) *C. sapidus*, like other crustaceans, has a low lipid content, typically between 0.4% and 4% (Table 1), making it suitable for low-fat diets (Çelik et al., 2004; Gökoolu and Yerlikaya, 2003; Khamassi et al., 2022; Küçükgülmez et al., 2006). The difference in lipid content between males and females is consistent across all seasons, with females consistently having significantly higher lipid content than males. Furthermore, both male and female crabs have higher lipid content in winter (Alparslan et al., 2022; Ayas, 2016). This is probably due to a metabolic strategy used by crustaceans to store high-energy compounds that will be useful during winter (Alparslan et al., 2022; Luvizotto-Santos et al., 2003). Furthermore, a markedly elevated fat content was observed in leg meat relative to claw meat (Ayas, 2016; Kuley et al., 2008). This phenomenon can be attributed to a number of factors influencing the lipid content in seafood, including environmental conditions, seasonality, sex and sexual variations related to reproduction, and the carapace moulting period (Ayas, 2016).

Regarding fatty acids profile, the predominant class of fatty acids is polyunsaturated fatty acid (PUFA) in all seasons. These include arachidonic acid (ARA; C20:4 n6), eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA 22:6 n-3) (Alparslan et al., 2022; Ayas, 2016; Çelik et al., 2004; Khamassi et al., 2022; Kuley et al., 2008).

Several studies have shown that blue crab, despite its low fat content, is an excellent source of n-3 PUFA, particularly EPA and DHA, throughout the year, for both sexes and in all muscle types. Its consumption could meet the recommended dietary requirement of n-3 fatty acids (DHA and EPA) in the human diet (Alparslan et al., 2022; Ayas, 2016; Çelik et al., 2004; Gökoolu and Yerlikaya, 2003; Khamassi et al., 2022; Kuley et al., 2008; Tufan, 2023).

Despite the high nutritional value of blue crab meat, the marketing and processing of this species are closely linked to meat yield, which is very low for this species. Meat yield depends on several factors, such as sex and processing methods. In fact, male crabs generally have a higher yield than females, as the latter consume more energy for vitellogenesis during the reproductive period. The yield also varies depending on whether the blue crabs are fresh or cooked and whether the product is processed mechanically or manually (Khamassi et al., 2022; Martínez-Maldonado et al., 2020; Martínez et al., 2017; Pinheiro et al., 2015). It was observed that using mechanical processing of the blue crab and rock crab by-products, after hand picking, can increase the amount of meat available for the production of new food products by up to 20% (Galetti, 2010; Galetti et al., 2017; Gates and Parker, 1993, 1992; Murphy et al., 2003).

Table 1. Proximate and fatty acids composition of edible blue crabs.

	Moisture (%)	Lipid (%)	Protein (%)	Ash (%)	Fatty Acids %	Season	Geographic area	References
Crab meat	78,71		18,40	2,32	-	-	local market (Madrid, Spain)	(Martínez et al., 2017)
Crab meat (cooked)	80,17		16,46	2,32	-	-	local market (Madrid, Spain)	(Martínez et al., 2017)
Claw meat	83.1	0.64	15.0	1.39	-	Summer	Gulf of Antalya, the coast of Western Mediterranean of Turkey	(Gökoolu and Yerlikaya, 2003)
Body meat	81.58	0.79	14.71	1.89	-	Summer	Gulf of Antalya, the coast of Western Mediterranean of Turkey	(Gökoolu and Yerlikaya, 2003)
Claw meat (cooked)	78.02	0.44	19.55	2.13	-	Winter	Akyazan lagoon on the coast of the eastern Mediterra- nean Sea of Turkey	(Küçükgülmez et al., 2006)
Breast meat (cooked)	79.05	0.44	18.10	2.03	-	Winter	Akyazan lagoon on the coast of the eastern Mediterra- nean Sea of Turkey	(Küçükgülmez et al., 2006)
Claw meat	80.12	-	15.13	1.63	-	Summer	Acquatina Lagoon (SE Italy)	(Zotti et al., 2016a)
Breast meat (cooked)	-	-	-	-	n3PUFA=17.0; ARA=5.02; EPA=8.4; DHA=6.8,	Winter	Off the Mediterranean coast of Akyazan Lagoon Karataş/Turkey	(Çelik et al., 2004)
Claw meat (cooked)	-	-	-	-	n3PUFA=18.1; ARA=7.48; EPA=10.6; DHA=5.9,	Winter	Off the Mediterranean coast of Akyazan Lagoon Karataş/Turkey	(Çelik et al., 2004)
Lump crab meat (female)	74.33	1.24	21.64	2.18	SFA=25.24; MUFA=26.24; PUFA=38.94; ARA=4.93; EPA=17.89; DHA=12.02	Spring	Eastern Mediterranean Sea	(Ayas, 2016)
Claw crab meat (female)	72.48	0.80	24.33	2.24	SFA=23.65; MUFA=28.04; PUFA=37.88; ARA=6.38; EPA=16.89; DHA=10.34	Spring	Eastern Mediterranean Sea	(Ayas, 2016)

Table 1. (continue).

	Moisture (%)	Lipid (%)	Protein (%)	Ash (%)	Fatty Acids %	Season	Geographic area	References
Lump crab meat (male)	74.98	1.22	21.41	2.25	SFA=24.74; MUFA=23.22; PUFA=42.64; ARA=5.84; EPA=17.91; DHA=14.13	Spring	Eastern Mediterranean Sea	(Ayas, 2016)
Claw crab meat (male)	74.65	0.92	22.25	2.07	SFA=23,00; MUFA=25.86;, PUFA=41.13; ARA=7.68; EPA=16.95; DHA=12.22	Spring	Eastern Mediterranean Sea	(Ayas, 2016)
Lump crab meat (female)	76.01	0.86	20.79	2.09	SFA=28.25; MUFA=25.39; PUFA=39.25; ARA=5.29; EPA=17.48; DHA=13.07	Summer	Eastern Mediterranean Sea	(Ayas, 2016)
Claw crab meat (female)	73.36	0.81	23.32	2.35	SFA=27.02; MUFA=27.33; PUFA=39.25; ARA=6.11; EPA=17.83; DHA=11.55	Summer	Eastern Mediterranean Sea	(Ayas, 2016)
Lump crab meat (male)	77.36	1.01	19.19	2.33	SFA=28.63; MUFA=22.64; PUFA=41.85; ARA=6.07; EPA=16.32; DHA=15.40	Summer	Eastern Mediterranean Sea	(Ayas, 2016)
Claw crab meat (male)	76.46	0.69	20.25	2.48	SFA=27.38; MUFA=25.75; PUFA=40.10; ARA=7.29; EPA=15.92; DHA=12.81	Summer	Eastern Mediterranean Sea	(Ayas, 2016)
Lump crab meat (female)	80.83	0.89	16.61	1.53	SFA=27.41; MUFA=27.48; PUFA=39.77; ARA=6.10; EPA=19.19; DHA=10.64	Autumn	Eastern Mediterranean Sea	(Ayas, 2016)
Claw crab meat (female)	76.73	0.63	20.17	2.06	SFA=28.00; MUFA=31.08; PUFA=34.22; ARA=6.66; EPA=15.05; DHA=8.70	Autumn	Eastern Mediterranean Sea	(Ayas, 2016)
Lump crab meat (male)	77.64	0.98	19.48	1.72	SFA=26.04; MUFA=25.04; PUFA=41.76; ARA=5.79; EPA=19.75; DHA=11.72	Autumn	Eastern Mediterranean Sea	(Ayas, 2016)
Claw crab meat (male)	79.64	0.58	17	2.13	SFA=25.85; MUFA=30.46; PUFA=37.06; ARA=6.65; EPA=16.75; DHA=9.02	Autumn	Eastern Mediterranean Sea	(Ayas, 2016)

Table 1. (continue).

	Moisture (%)	Lipid (%)	Protein (%)	Ash (%)	Fatty Acids %	Season	Geographic area	References
Lump crab meat (female)	76.45	1.21	20.48	1.76	SFA=21.63; MUFA=22.89; PUFA=41.60; ARA=8.96; EPA=18.50; DHA=9.83	Winter	Eastern Mediterranean Sea	(Ayas, 2016)
Claw crab meat (female)	75.06	0.84	21.80	2.08	SFA=22.09; MUFA=23.11; PUFA=39.60; ARA=9.98; EPA=16.91; DHA=8.60	Winter	Eastern Mediterranean Sea	(Ayas, 2016)
Lump crab meat (male)	77.09	1.51	19.20	1.83	SFA=24.82; MUFA=23.84; PUFA=44.01; ARA=7.45; EPA=19.25; DHA=13.02	Winter	Eastern Mediterranean Sea	(Ayas, 2016)
Claw crab meat (male)	75.66	1.04	21.05	2.06	SFA=23.64; MUFA=27.81; PUFA=40.14; ARA=9.24; EPA=17.56; DHA=9.35	Winter	Eastern Mediterranean Sea	(Ayas, 2016)
Cephalothorax muscle (female)	68.8	2	26.56	-	SFA=30.31; MUFA=26.06; PUFA=43.64; ARA=7.18; EPA=15.64; DHA=13.27	Summer	Southern lagoon of Tunis (north-east of Tunisia) during	(Khamassi et al., 2022)
Chelipeds muscle (female)	70.3	3.69	23.71	-	SFA=31.64; MUFA=26.35; PUFA=42.01; ARA=7.43; EPA=13.83; DHA=13.78	Summer	Southern lagoon of Tunis (north-east of Tunisia) during	Khamassi et al., 2022)
Cephalothorax muscle (male)	67.4	2.91	27.05	-	SFA=32.04; MUFA=23.80; PUFA=44.15; ARA=7.21; EPA=15.20; DHA=13.29	Summer	Southern lagoon of Tunis (north-east of Tunisia) during	Khamassi et al., 2022)
Chelipeds muscle (male)	68.7	2.86	25.77	-	SFA=31.74; MUFA=25.59; PUFA=42.67; ARA=9.21; EPA=12.58; DHA=11.25	Summer	Southern lagoon of Tunis (north-east of Tunisia) during	Khamassi et al., 2022)
Body meat (female)	67.54	1.62	26.51	1.16	SFA=25.98; MUFA=22.04; PUFA=41.41; ARA 8.71; EPA=15.20; DHA=11.43	-	Akyatan Lagoon in the south of the Mediterranean Sea	(Kuley et al., 2008)
Claw meat (female)	68.12	1.12	29.59	1.58	SFA=24.75; MUFA=23.57; PUFA=41.60; ARA=10.89; EPA=13.11; DHA=11.23	-	Akyatan Lagoon in the south of the Mediterranean Sea	(Kuley et al., 2008)

Table 1. (continue).

	Moisture (%)	Lipid (%)	Protein (%)	Ash (%)	Fatty Acids %	Season	Geographic area	References
Body meat (male)	66.40	1.64	30.31	1.10	SFA=26.76; MUFA=21.79; PUFA=39.73; ARA=7.73; EPA=13.61; DHA=13.03	-	Akyatan Lagoon in the south of the Mediterranean Sea	(Kuley et al., 2008)
Claw meat (male)	66.59	1.22	31.03	1.32	SFA=24.62; MUFA=23.84; PUFA=40.98; ARA=10.59; EPA=13.0; DHA=10.84	-	Akyatan Lagoon in the south of the Mediterranean Sea	(Kuley et al., 2008)
Body meat (female)	67.99	2.02	27.69	1.15	SFA=31.84; MUFA=7.55; PUFA=43.78; ARA=9.49; EPA=16.29; DHA=9.45	-	Hurma Strait in the south of the Mediterranean Sea	(Kuley et al., 2008)
Crab meat	83.59	1.80	9.12	1.22	SFA=2733.67 mg/100g of sample; UFA=1061.93 mg/100g of sample	Summer	Baltim Fish Market, Kafar Elshaikh, Egypt during	(Elsayed et al., 2020)
Carapace meat (male)	77.69	0.69	18.97	2.39	-	-	Fatsa, Ordu, Türkiye	(Tufan, 2023)
Left Claw meat (male)	78.32	0.59	18.69	2.38	-	-	Fatsa, Ordu, Türkiye	(Tufan, 2023)
Right Claw meat (male)	78.58	0.58	18.26	2.35	-	-	Fatsa, Ordu, Türkiye	(Tufan, 2023)
Legs meat (male)	77.49	0.46	19.22	2.34	-	-	Fatsa, Ordu, Türkiye	(Tufan, 2023)
Carapace meat (female)	76.73	0.92	19.93	2.29	-	-	Fatsa, Ordu, Türkiye	(Tufan, 2023)
Left Claw meat (female)	77.85	0.79	18.75	2.32	-	-	Fatsa, Ordu, Türkiye	(Tufan, 2023)
Right Claw meat (female)	78.62	0.77	18.05	2.31	-	-	Fatsa, Ordu, Türkiye	(Tufan, 2023)
Legs meat (female)	76.98	0.63	19.72	2.30	-	-	Fatsa, Ordu, Türkiye	(Tufan, 2023)
Claw and body meat (female)		0.9	-	-	SFA=29.0; MUFA=21.7; PUFA=39.5; ARA=6.8; EPA=15.0; DHA=12.2	Autumn	Koycegiz Lagoon (Mugla, Turkey)	(Alparslan et al., 2022)
Claw and body meat (female)	-	1.3	-	-	SFA=26.9; MUFA=22.6; PUFA=39.5; ARA=8.1; EPA=14.1; DHA=12.3	Winter	Koycegiz Lagoon (Mugla, Turkey)	(Alparslan et al., 2022)

Table 1. (continue).

	Moisture (%)	Lipid (%)	Protein (%)	Ash (%)	Fatty Acids %	Season	Geographic area	References
Claw and body meat (female)	-	0.9	-	-	SFA=26.2; MUFA=21.2; PUFA=41.1; ARA=6.7; EPA=15.0; DHA=13.8	Spring	Koycegiz Lagoon (Mugla, Turkey)	(Alparslan et al., 2022)
Claw and body meat (female)	-	0.9	-	-	SFA=30.2; MUFA=21.7; PUFA=40.4; ARA=7.9; EPA=16.3; DHA=11.1	Summer	Koycegiz Lagoon (Mugla, Turkey)	(Alparslan et al., 2022)
Claw and body meat (male)	-	0.8	-	-	SFA=28.01; MUFA=21.4; PUFA=41.7; ARA= 8.0; EPA=14.5; DHA=13	Autumn	Koycegiz Lagoon (Mugla, Turkey)	(Alparslan et al., 2022)
Claw and body meat (male)	-	1.2	-	-	SFA=253; MUFA=22.6; PUFA=41.6; ARA=7.7; EPA=14.0; DHA=14.9	Winter	Koycegiz Lagoon (Mugla, Turkey)	(Alparslan et al., 2022)
Claw and body meat (male)	-	0.8	-	-	SFA=26.3; MUFA=18.5; PUFA=46.5; ARA=7.4; EPA=16.9; DHA=15.9	Spring	Koycegiz Lagoon (Mugla, Turkey)	(Alparslan et al., 2022)
Claw and body meat (male)	-	0.8	-	-	SFA=29.23; MUFA=18.9; PUFA=43.1; ARA=8.5; EPA=16.0; DHA=13.0	Summer	Koycegiz Lagoon (Mugla, Turkey)	(Alparslan et al., 2022)
Crab muscle (female)	75.14	3.75	9.38	-	-	-	El-Gamil is an outlet located along the northeast side of Lake Manzala, within the coastal sand bar 5 km west of Port Said on the Mediterranean Sea	(Zakzok et al., 2022)
Crab muscle (male)	74.38	4.77	11.51	-	-	-	El-Gamil is an outlet located along the northeast side of Lake Manzala, within the coastal sand bar 5 km west of Port Said on the Mediterranean Sea	Zakzok et al., 2022)
Raw crab meat	81.17	1.06	16.22	1.45	-		Köyceğiz Dalyan	(Alparslan et al., 2023)

Table 1. (continue).

	Moisture (%)	Lipid (%)	Protein (%)	Ash (%)	Fatty Acids %	Season	Geographic area	References
Steam-cooked crab meat	76.88	1.46	18.05	1.88	-		Köyceğiz Dalyan	(Alparslan et al., 2023)

ARA: arachidonic acid; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; MUFA: Monounsaturated fatty acid; PUFA: Polyunsaturated fatty acid; SFA: Saturated fatty acid.

The potential applications of crab meat in producing crab-based products have been extensively studied in various crab species (Nanda et al., 2021). These studies have shown promising results, indicating that the same approach can be applied to the blue crab (Nanda et al., 2021). By recognizing the blue crab as a new food source and exploring its potential applications, we can effectively tackle the challenges posed by its invasive nature (Marchessaux et al., 2024). Moreover, this approach opens up exciting economic opportunities and promotes sustainable practices in the Mediterranean region. By utilizing the blue crab in the production of crab-based products, it is possible not only to reduce the negative impact of its invasive presence but also to contribute to the local economy and foster environmentally friendly practices.

3.5. Blue Crab By-Products Valorization

3.5.1. Shell Valorization

Globally, around 6-8 million tons of crab shells are produced each year, causing disposal problems and environmental concerns (Su et al., 2019; Vidal et al., 2022). Due to its low economic value and high disposal costs, most of this waste is actually disposed of in landfills. To improve the economic and environmental sustainability of crab shell residues, it is important to valorize this biomass and no longer consider it merely waste to be disposed of (Gao et al., 2016; Su et al., 2019; Vidal et al., 2022; Zapantioti et al., 2022).

Crab shells and other crab by-products are an important source of proteins, lipids, chitin, minerals (mainly calcium and magnesium carbonates) and pigments that can be processed into high-value products (Abd El-Ghany et al., 2023; Antunes-Valcareggi et al., 2017; Maschmeyer et al., 2020; Nanda et al., 2021; Su et al., 2019; Venugopal, 2022; Vidal et al., 2022). Although the valorization of crustacean by-products has been widely discussed (Ahmadkelayeh and Hawboldt, 2020; Ali et al., 2020; Azelee et al., 2023; Cahú et al., 2012; Caruso et al., 2020; Cretton et al., 2021; Dey and Dora, 2014; Gildberg and Stenberg, 2001; Guerard et al., 2007; Gunasekaran et al., 2015; Hajji et al., 2021; Hong et al., 2018; Jiang et al., 2017; Khiari et al., 2020; Kim et al., 2016; Maia et al., 2023; Mao et al., 2017; Messina et al., 2021; Parthiban et al., 2017; Pinheiro et al., 2021; Pires et al., 2017; Rodrigues et al., 2021; Sánchez-Camargo et al., 2012; Syama Dayal et al., 2013; Vidal et al., 2022; Yoon et al., 2013; Zou et al., 2021), very few studies have focused specifically on blue crab (*C. sapidus*) (Abd El-Ghany et al., 2023; Akbay et al., 2022; Antunes-Valcareggi et al., 2017; Baron et al., 2017; Félix-Valenzuela et al., 2001; Jabeen et al., 2021; Kaya et al., 2016; Metin et al., 2019; Montoya et al., 2021; Nekvapil et al., 2021, 2023; Pinzaru et al., 2024; Zapantioti et al., 2022). These by-products hold significant potential for the extraction of valuable compounds, including chitin,

which is of particular interest for various industrial applications. Table 2 details the studies conducted on blue crab by-products.

The crustacean shells of blue crabs are primarily composed of chitin (Azelee et al., 2023), a linear Nacetyl-d-glucosamine polysaccharide with β-(1-4) linkage, insoluble in water (Mauricio-Sánchez et al., 2018), making it the second most abundant biopolymer in the world after cellulose. Chitin is found in the exoskeletons of insects, the cell walls of fungi and algae, and marine invertebrates like crustaceans, including shrimp and crabs (Jabeen et al., 2021; Metin et al., 2019; Ray et al., 2019; Xu et al., 2020). As shown in Table 2, most studies on blue crab (*C. sapidus*) by-products have focused on chitin extraction (Antunes-Valcareggi et al., 2017; Jabeen et al., 2021; Metin et al., 2019; Pinzaru et al., 2024). Chitin and its derivatives have attracted significant interest in research and industry due to their multiple applications in different sectors (food, agriculture, pharmaceutical and chemical) (Ali et al., 2020; Asif et al., 2020; Jabeen et al., 2021; Oyeleye and Normi, 2018), making it important to identify new resources (Kaya et al., 2016). Studies on blue crab by-products have shown that they are a good source of chitin (Antunes-Valcareggi et al., 2017; Jabeen et al., 2021; Kaya et al., 2016; Metin et al., 2019; Pinzaru et al., 2024).

Chitin was obtained by treating blue crab shells with alkalis and acids to remove proteins and minerals. This method allowed for obtaining high-quality chitin, with a recovery rate of 10-14% (Antunes-Valcareggi et al., 2017; Jabeen et al., 2021; Kaya et al., 2016; Metin et al., 2019; Pinzaru et al., 2024).

By deacetylating chitin, chitosan is obtained — a polysaccharide with many applications in different fields (clinical, cosmetic, food, pharmaceutical, agricultural, aquaculture and environmental) due to its non-toxicity, biodegradability, and adsorption and chelating ability (Parthiban et al., 2017). Chitosan can also be used as an alternative antimicrobial agent as it possesses antimicrobial activity against many bacteria, fungi and yeasts (Kaya et al., 2016). Furthermore, chitosan has excellent film-forming properties, making it suitable for applications in the food industry to preserve the quality of various foods (Aider, 2010; Baron et al., 2017; Lorevice et al., 2016; Ouattara et al., 2000).

Baron et al. (Baron et al., 2017), Kaya et al. (Kaya et al., 2016), and Metin et al. (Metin et al., 2019) obtained chitosan from blue crab by-products with yields ranging from 7 to 14%. These authors showed that chitosan derived from blue crab by-products exhibited antimicrobial activity against human and fish pathogens and was also a good source of antioxidants (Kaya et al., 2016; Metin et al., 2019). Consequently, it could be considered a natural alternative to commercial chitosan and synthetic antimicrobials (Kaya et al., 2016; Metin et al., 2019), with potential application as a biopolymer in the food industry (Metin et al., 2019). In addition, chitosan-based biofilms have shown properties that allow their future use as coatings or packaging materials for food products (Baron et al., 2017).

Blue crab (*C. sapidus*) shells are a valuable source of high-nutritional-value compounds, including proteins, which represent approximately 30% of their content (Antunes-Valcareggi et al., 2017; Félix-Valenzuela et al., 2001). These proteins could serve as excellent precursors for bioactive peptides derived through the protein hydrolysis process (Azelee et al., 2023; Messina et al., 2021). Protein hydrolysates and peptides obtained from crustacean byproducts have shown significant health benefits, particularly in the prevention of chronic diseases (Azelee et al., 2023; Mahgoub et al., 2023). Despite the crucial importance of protein hydrolysates, current literature includes only one study highlighting the possibility of obtaining protein hydrolysates from blue crab by-products (Antunes-Valcareggi et al., 2017). This represents a research and development opportunity to further explore the potential of blue crab by-products not only as a source of high quality protein but also as nutraceutical ingredients that can significantly contribute to human health.

Other studies have focused on various innovative and sustainable applications of blue crab shells. One such study investigated the production of new carbonaceous materials with high adsorption capacity, showing that carbonaceous materials pyrolyzed at 800°C have the highest adsorption rate (Zapantioti et al., 2022). These carbonaceous materials can be used as effective adsorbents to reduce contaminants in the marine ecosystem and mitigate environmental pollution (Zapantioti et al., 2022). Another study showed that the three-dimensional nanostructured natural material derived from blue crabs retains its nanomorphology even after mechanical grinding or heat treatment, making it compatible with a wide range of industrial processes (Nekvapil et al., 2019). This result paves the way for numerous innovative applications based on nanoscale porosity and chemical composition of materials after industrial processing steps.

The potential use of blue crab shells as an alternative filler material in the rubber industry was also investigated. The results showed that incorporating these by-products into the ethylene propylene diene monomer (EPDM) matrix improved the rheological, mechanical and thermal properties of the composite. Specifically, the addition of up to 12 phr of BCS to the EPDM matrix was found to increase the T50 temperature, tensile strength and cross-link density of the composite (Akbay et al., 2022). These biodegradable and bio-based materials may prove to be significant substitutes, offering economic and environmental benefits.

Finally, the biogenic particulate matter derived from blue crab shells was investigated to determine its potential future use in composite formulations for high-value-added products. The analyses showed that these powders meet the legal standards for fertilizers in terms of composition and microbiological content, making them potentially useful as biofertilisers and other sustainable products. However, special attention should be paid to the content of heavy metals in crab shells, especially Cd and As, in areas where industrial pollution is known to occur [88].

Table 2. Blue crab by-products, bioactive compounds extracted/isolated and their uses.

Blue crab by-products	Bioactive compounds extracted or products	Parameters evaluated	References
Shell waste	Astaxanthin (extracted with a mixture of supercritical CO, and ethanol as a co-solvent	Chemical composition of demineralized crab shell waste	(Félix-Valenzuela et al., 2001)
Shell	Chitin	Comparison in the chitin extracted from blue edible crab and market purchased chitin.	(Jabeen et al., 2021)
Shells and tongs	Chitosan to produce chitosan:pectin (Ch:P) based films	Chitosan production and characterization Film characterizations	(Baron et al., 2017)
Shell wastes	Chitin and Chitosan	Yield, Moisture and Ash Content of Chitin and Chitosan	(Metin et al., 2019)
Shells	Chitin and Chitosan	Characterization of chitin and chitosan through FTIR, TGA, XRD, and SEM analyses. Determination of antimicrobial and antioxidant activities of chitosan.	(Kaya et al., 2016)
Shells, pincers , and legs	Produce new carbonaceous materials with high adsorption capacity	The adsorption properties of the materials were investigated	(Zapantioti et al., 2022)
Shells	Production of Biogenic Micropowders of Crab Shells.	Characterization of Biogenic Micropowders of Crab Shells.	(Nekvapil et al., 2019)
Shell	Ethylene pro- pylene diene termonomer (EPDM) and EPDM with different blue crab shell (BCS) incorporated into rubber matrix	The effect of carbon black and blue crab shell content within the Ethylene pro- pylene diene termonomer matrix on the rheological, mechani-cal, morphological, and thermal properties	(Akbay et al., 2022)
Shells waste	Plant biofertilizer	Crab Shell Powdering Technical Aspects. Macronutrients, heavy metals, bacterial content, and antimicrobial properties were tested	(Nekvapil et al., 2021)
Food waste from cooked crabs	Chitin-based polymeric materials	The process of waste shells demineralization and the final products of the treatment were investigated using various Raman techniques and technologies, cross-validating the results with FT-IR, XRD and SEM-EDX techniques.	(Pinzaru et al., 2024)

Table 2. (continue).

Blue crab by-products	Bioactive compounds extracted or products	Parameters evaluated	References
Carapaces and legs	Astaxanthin Chitin Hydrolysates protein	Characterization of blue crab processing waste Enzymatic hydrolysis Astaxanthin recovery and antioxidant activity Chitin characterization	(Antunes-Valcareggi et al., 2017)
Exoskeleton (carapace and legs)	Astaxanthin	Recovery and quantification of astaxanthin, antioxidant and anti-inflammatory activity Antimicrobial activity of extracted astaxanthin	(Abd El-Ghany et al., 2023)
Exoskeletons	Astaxanthin	Quantification of astaxanthin, extracted with organic solvents (ethyl acetate, acetone, cyclohexane, isopropyl alcohol, hexane, heptane, and a combination of hexane-acetone-ethanol-toluene solvents), by high-performance liquid chromatography analysis.	(Montoya et al., 2021)
Exoskeleton powder	Carotenoids	Composition and morphology of the exoskeleton powder analysed by SEM, Raman spectroscopy, FTIR and XRD.	(Nekvapil et al., 2023)

3.5.2. Exoskeleton and Cephalothorax Valorization

The exoskeleton and cephalothorax of the blue crab are composed of carotenoids, particularly astaxanthin (Abd El-Ghany et al., 2023; Antunes-Valcareggi et al., 2017; Félix-Valenzuela et al., 2001; Montoya et al., 2021; Nekvapil et al., 2023). Astaxanthin is the major carotenoid pigment naturally occurring in marine crustaceans, known for its antioxidant and anti-inflammatory properties (Abd El-Ghany et al., 2023; Aneesh et al., 2022), and for its beneficial effects on human and animal health, which has significantly increased its demand in the market (Abd El-Ghany et al., 2023; Aneesh et al., 2022; Siahaan et al., 2022).

Astaxanthin was extracted from blue crab by-products using organic solvent extraction techniques (Abd El-Ghany et al., 2023; Antunes-Valcareggi et al., 2017; Montoya et al., 2021; Nekvapil et al., 2023) and with supercritical fluids (SFE) using ethanol as co-solvent (Félix-Valenzuela et al., 2001). As observed by Abd El-Ghany et al. (2023), the rigid structure of the blue crab shell, which includes complex proteins and chitin, makes the extraction process difficult. To improve the efficiency of astaxanthin extraction, these authors pre-treated the blue crab by-products with chitinolytic and/or proteolytic microorganisms. Other authors, on the other hand, demineralized the sample before astaxanthin extraction (Antunes-Valcareggi et al., 2017; Félix-Valenzuela et al., 2001), thereby increasing the availability of carotenoids and facilitating their extraction with organic solvents (Antunes-Valcareggi et al., 2017). Mixtures of acetone/hexane (1:1 v/v) (Abd El-Ghany et al., 2023) and hexane–acetone–ethanol–toluene (Montoya et al., 2021) have shown better efficiency. These studies (Abd El-Ghany et al., 2023; Antunes-Valcareggi et al., 2017; Félix-Valenzuela et al., 2001; Montoya et al., 2021; Nekvapil et al., 2023) highlighted the potential use of blue crab by-products as a valuable source of carotenoids, particularly astaxanthin. The recovered astaxanthin can be used in various sectors, including food, pharmaceuticals and cosmetics,
providing a sustainable and natural alternative to synthetic carotenoids.

These studies demonstrate that blue crab by-products can be employed in various sectors to generate bioactive compounds, thereby reducing environmental impact and advancing a circular economy. Additionally, this approach increases the economic value of marine by-products and creates new avenues for industrial growth.

3.6. The Potential Future of Blue Crab Valorization

Addressing the challenges posed by the blue crab invasion also requires social innovation, which involves new strategies, concepts, and ideas that meet social needs and create new social relationships or collaborations. This can include community-based management programs, educational initiatives,

and public-private partnerships. Community-Based Management Programs can engage local communities in the management and utilization of blue crab populations. By involving fishermen, local businesses, and residents in decision-making processes, these programs can ensure that the solutions are locally appropriate and sustainable. For instance, fishermen can be trained to catch blue crabs using methods that minimize ecological damage, while local businesses can be incentivized to develop and market blue crab products. Similarly, educational Initiatives raise awareness about the blue crab issue and its potential solutions can inform the public about the ecological impacts of the blue crab and the benefits of its commercial utilization. Schools, community centre, and media can play pivotal roles in disseminating this information, thereby fostering a culture of sustainability and environmental stewardship. Also, collaboration between government agencies, private enterprises, and academic institutions can drive innovation and investment in blue crab management and utilization. Such partnerships can facilitate research and development, streamline regulatory processes, and provide funding for new initiatives. For example, partnerships can support the development of new processing technologies or the exploration of alternative uses for blue crab by-products. By integrating social innovation into the management of the blue crab invasion, it is possible to create comprehensive and sustainable solutions that not only address the ecological and economic challenges but also enhance community resilience and social cohesion.

3.7. Conclusions

The blue crab (*C. sapidus*) is a significant invasive species in the Mediterranean Sea, presenting both challenges and opportunities. On the one hand, its presence disrupts local ecosystems, leading to negative impacts on biodiversity and human activities such as fishing and tourism. The species competes with native marine life for resources, potentially leading to declines in native populations and altering the ecological balance.

On the other hand, the blue crab's high nutritional value offers a unique opportunity to turn this invasive species into an economic asset. Its meat is rich in proteins, omega-3 fatty acids, and other essential nutrients, making it a valuable resource for the local fishing and industrial processing sectors. Additionally, the valorization of blue crab by-products—such as proteins, lipids, chitin, minerals, and pigments—holds promise for various industries, including food, pharmaceuticals, and cosmetics. However, care must be taken to avoid the possible presence of heavy metals (Cd and As) in blue crab shells in areas subject to industrial pollution. This approach not only helps mitigate the environmental impact of blue crab invasions but also promotes a circular economy by transforming waste into high-value products.

However, the process of integrating blue crab into local economies is not without its challenges. The low meat yield and high processing costs can make it difficult to commercialize on a large scale. Furthermore, while the potential benefits of utilizing blue crab by-products are significant, more research is needed to fully understand and optimize the bioactivity and industrial applications of these components.

Addressing the blue crab invasion effectively requires a balanced approach that combines economic utilization with ecological management. Social innovation plays a critical role in this effort, with strategies such as community-based management programs, educational initiatives, and public–private partnerships being essential. Engaging local communities, fostering education, and promoting collaboration between various stakeholders can help develop sustainable solutions that not only mitigate the ecological impact of the blue crab but also enhance economic resilience.

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Chapter 4. Valorization of blue crab (*Callinectes sapidus*) by-products: Improving economic and environmental sustainability through protein hydrolysates with bioactive properties.

In order to promote the valorization of marine by-products in the food sector, I analyzed blue crab by-products from fish processing. Blue Ocean S.r.l. supplied blue crab shells and legs for analysis of proximate composition and fatty acid profile. These by-products proved to be a reliable protein source, with hydrolysates exhibiting high antioxidant activity, making them suitable for applications in nutraceuticals, cosmeceuticals, and other industries.
The results of this study are presented in detail in this chapter.

4.1. Abstract

The blue crab, *Callinectes sapidus*, is an opportunistic invasive species in the Mediterranean, known for its impact on biodiversity, fisheries, and tourism. However, this species could be considered as a valuable food resource for the Mediterranean area due to the high nutritional value of its meat, although the yield obtained from its processing is very low, resulting in a significant production of waste, which is largely underutilized. Therefore, it is crucial to valorize this unused biomass to improve its economic and environmental sustainability. This study aimed to valorize the by-products of the blue crab (BCBP), according to the principles of the circular economy, by producing protein hydrolysates and testing their bioactivity *in vitro*. BCBP hydrolysates were obtained by enzymatic hydrolysis using Alcalase and Protamex enzymes and were tested *in vitro* to evaluate antioxidant activity by DPPH and reducing power assays and using human skin fibroblasts (HS-68). The highest degree of enzymatic hydrolysis was achieved by the enzyme Alcalase, whereas both enzymes showed efficient enzymatic reactions, as evidenced by SDS-PAGE electrophoresis. BCBP hydrolysates showed antioxidant power and protective effects against oxidative damage in HS-68. These results suggest that high quality bioactive compounds for food, nutraceutical and pharmaceutical applications can be obtained from BCBP and that these by-products can be utilized on a larger scale, suggesting a strategy to increase their utilization and sustainability.

Keywords: Blue crabs by-products; protein hydrolysates; antioxidant activity; HS-68 cell line

4.2. Introduction

The Atlantic blue crab (*Callinectes sapidus* Rathbun, 1896 (Brachyura: Portunidae)) is native to the western Atlantic Ocean from Uruguay to Nova Scotia (Kevrekidis et al., 2023) and was accidentally introduced in the early twentieth century in the Mediterranean Sea, probably through ballast water (Nehring, 2011) and deliberately introduced in the Aegean Sea in the 1930s (Zotti et al., 2016). Since then, it has spread widely in the Mediterranean, the Black Sea, and along the European coasts of the Atlantic Ocean (Mancinelli et al., 2021).

Blue crabs are considered one of the 100 "worst invasive" alien marine species in the Mediterranean Sea, with a high impact on ecosystem biodiversity and human activities (fisheries, aquaculture, tourism) (Arena et al., 2024; Khamassi et al., 2022; Piras et al., 2019).

It is an opportunistic euryhaline species and its life cycle takes place in both brackish and marine habitats, inhabiting estuaries, lagoons, and other coastal habitats (Mancinelli et al., 2021; Taybi and Mabrouki, 2020). Its successful spread across regions is due to its biological characteristics: rapid growth rates, opportunistic feeding (vegetables, detritus, polychaetes, molluscs, crustaceans and fish), high reproductive rate, generalist habitat use, long-distance dispersal of larvae, and effective physical and aggressive behaviour exhibiting scavenging and cannibalistic behaviour (Kampouris et al., 2019; Kevrekidis et al., 2023; Mancinelli et al., 2017; Marchessaux et al., 2023; Streftaris and Zenetos, 2006). Artisanal fishery is strongly affected by this species, as reported by Marchessaux et al., (2023), who highlighted a lesser caught of fish by fishermen in the presence of blue crabs and that the quality and value of the catches were reduced, negative affecting the overall income (Marchessaux et al., 2023). Despite the negative impacts, by promoting and developing fisheries and management strategies, this species could be considered as a new source of income due to its high nutritional value (Arena et al., 2024; Khamassi et al., 2022; Mancinelli et al., 2017; Marchessaux et al., 2023; Nuñez et al., 2012).

Atlantic blue crab meat typically contains 76-80% moisture, approximately 19-14% protein and between 0.5 and 0.8% lipids and highly unsaturated fatty acids, particularly arachidonic acid (ARA; C20:4 n6), eicosapentaenoic acid (EPA; 20:5 n3) and docosahexaenoic acid (DHA 22:6 n3), making it an excellent source of many nutrients important for human health (Alparslan et al., 2022; Arena et al., 2024; Ayas, 2016; Çelik et al., 2004; Gökoolu and Yerlikaya, 2003; Jiang et al., 2020; Khamassi et al., 2022; Kuley et al., 2008; Martínez et al., 2017; Nanda et al., 2021; Tufan, 2023; Wu et al., 2020).

Despite having a high nutrition value, its meat yield is very low (10-15%), and it is strictly dependent on the used process methods (mechanical vs. manual processes) (Khamassi et al., 2022; Martínez-

Maldonado et al., 2020; Martínez et al., 2017; Pinheiro et al., 2015). In fact, it was observed that the use of the mechanical process in the blue crab and rock crab by-products after hand picking was able to yield up to 20% additional meat to produce new food products (Galetti, 2010; Galetti et al., 2017; Gates and Parker, 1993, 1992; Murphy et al., 2003).

The low meat yield is associated with a large amount of waste, in fact, globally, approximately 6-8 million tons of crab shells are produced each year, and a large amount is cast off into the soil environment. Crab waste is not fully utilized and is considered a cheap material, but it appears very important to valorize this unused biomass to improve the economic and environmental sustainability (Arena et al., 2024; Gao et al., 2016; Su et al., 2019; Vidal et al., 2022; Zapantioti et al., 2022).

Nowadays, crustacean by-products and its valorization is widely discussed (Ahmadkelayeh and Hawboldt, 2020; Ali et al., 2020; Cahú et al., 2012; Cretton et al., 2021; Dey and Dora, 2014; Espinosa-Ruiz et al., 2021; Gunasekaran et al., 2015; Hong et al., 2018; Jiang et al., 2017; Khiari et al., 2020; Kim et al., 2016; Maia et al., 2023; Mao et al., 2017; Parthiban et al., 2017; Pinheiro et al., 2021; Pires et al., 2017; Rodrigues et al., 2021; Sánchez-Camargo et al., 2012; Syama Dayal et al., 2013; Vidal et al., 2022; Zou et al., 2021), but very few studies have been carried out on Atlantic blue crab (*C. sapidus*) by-products (Abd El-Ghany et al., 2023; Akbay et al., 2022; Antunes-Valcareggi et al., 2017; Arena et al., 2024; Baron et al., 2017; Camargo et al., 2021; Félix-Valenzuela et al., 2001; Jabeen et al., 2021; Kaya et al., 2016; Metin et al., 2019; Montoya et al., 2021; Nekvapil et al., 2023, 2021; Pinzaru et al., 2024; Zapantioti et al., 2022). Since Atlantic blue crab by-products is rich in chitin, proteins, unsaturated fatty acids, minerals, and carotenoids such as astaxanthin (Antunes-Valcareggi et al., 2017; Su et al., 2019), its reuse is crucial for the promotion of the sustainable use of aquatic resources, particularly waste products, in accordance with the principles of the “blue economy” (Jolaosho et al., 2023) and its application in different industrial sectors, such as pharmaceutical and food industries (Antunes-Valcareggi et al., 2017; Arena et al., 2024; Su et al., 2019; Yan and Chen, 2015).

Given the abundance of this species in the Mediterranean and its detrimental effects on the ecosystem, blue crabs could be considered a new food resource, and their exploitation could improve the economic situation of local fishing sectors (Khamassi et al., 2022; Mancinelli et al., 2017). Fishing incentive strategies should be identified to promote the consumption of this new resource, its utilization and processing in local industries and the valorization of the by-product.

Considering the scarcity of information on the activity of the bioactive compounds of the blue crab *C. sapidus*, the aim of this study was to evaluate the production of protein hydrolysates and to test their bioactivity in vitro, for pharmaceutical applications.

4.3. Materials and Methods

4.3.1. Raw material collection

Blue crab (*C. sapidus*) by-products (BCBP), consisting of crab shells and legs, were supplied by a fish processing industry in the city of Palermo, Sicily. At the factory, the crabs were boiled in water at 100°C for 20 minutes. The claws, legs and body meat were removed by hand. The extracted meat was weighed for yield (Y) analysis using the equation

$$Y = M/WW\text{-}100 \quad (1)$$

where M is weight of meat removed and WW is total wet weight (Yomar-Hattori et al., 2006).
The BCBP are stored in cold containers, transported to the laboratory, and frozen at −20 °C. The BCBP were rinsed and dried in a ventilated thermostat at 37°C for 24 hours and ground. Sample portions of 250 g were vacuum-packed in plastic bags and stored at -80°C.

4.3.2. Evaluation of proximate composition and fatty acid profile of blue crab by-products

Analysis for proximate composition and fatty acid profile were carried out in three replicates. The moisture and ash content were determined using AOAC method (AOAC, 1990), total lipid by Folch method (Folch et al., 1957), Crude protein, calculated from total nitrogen determined by the Kjeldahl method (AOAC, 1992), was multiplied by a conversion factor of 6.25, corrected to account for the presence of nitrogen in chitin, according to the following formula (Khiari et al., 2020):

$$Protein\ content\ (\%\ dw) = (Total\ N - Chitinous\ N) * 6{,}25 \quad (2)$$

Chitinous N was determined as follows (Khiari et al., 2020):

$$Chitinous\ N\ (\%\ dw) = \frac{Chitin\ content * 6{,}89}{100} \quad (3)$$

Where 6.89% is the theoretical nitrogen factor corresponding to completely acetylated chitin (Khiari et al., 2020; Rutledge, 1971; Sajomsang and Gonil, 2010).
Chitin was obtained from BCPB. The sample was demineralized by acid treatment, then deproteinized by alkaline treatment, decolorized with a sodium hypochlorite solution, and dried at 60°C for 4 h in a forced-air oven according to Bolat et al. (2010). After drying, the chitin yield, expressed as a percentage of total BCPB, was determined.

Fatty acids were extracted and transesterified according to Lepage and Roy (1984) and determined using gas chromatography (Messina et al., 2013).

4.3.3. Enzymatic Hydrolysis

Enzymatic hydrolysis of BCBP was performed in three replicates. The reaction was carried out on 10 g of BCBP in distilled water (1:10 BCP weight/ water volume), in a 250 mL vessel with continuous stirring and pH monitoring according to Messina et al. (2015). Two different proteases (peptidases) (Alcalase and Protamex, Sigma-Aldrich) were used at a ratio of 3% enzyme/substrate (E/S), following the optimal conditions for enzyme activity, according to the manufacturers, 53°C and a pH of 9.0 and 8.5 for Alcalase and Protamex, respectively. The pH was adjusted with 5 N NaOH.

The degree of hydrolysis (DH%) of each enzyme was determined directly every 15 min for 265 min, and applying the equation used by Dumay et al. (2006) (Messina et al., 2021). The enzymatic activity was stopped at 90 °C for 5 min and the samples were centrifuged at 7142 g force for 15 min at 4 °C. The supernatants were lyophilized for further determinations and stored at 4 °C (De Holanda and Netto, 2006; Messina et al., 2021).

The concentration of proteins in BCPB hydrolysates was measured according to the Lowry method (Lowry et al., 1951), using different concentrations of bovine serum albumin as standard.

4.3.4. Protein Hydrolysates Characterization and Assessment of Antioxidant Activity

Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For evaluation of the relative molecular mass of the proteins present in the samples, both BCBP and hydrolysates were separated using SDS-PAGE. The concentration of total proteins in all samples was determined using the method of Lowry et al. (1951), using BSA (bovine serum albumin) Sigma-Aldrich) as standard.

Aliquots of 60 µg of protein, diluted with a Laemmli buffer (Sigma-Aldrich) anddenatured for 5 min at 90 ◦C, were loaded onto a gradient polyacrylamide minigel (4–15%) (Bio-Rad, Hercules, CA, USA) and subjected to electrophoresis at 20 mA for about 2 h. A mix of standard proteins with relative molecular masses varying between 220 and 8 kDa (mERK) was run simultaneously into the gel. After the electrophoretic run, the gel was stained with Coomassie Blue (Bio-Rad, Hercules CA, USA). The image of the gel was acquired and elaborated using the software Image Lab 4.1 (Bio-Rad, Hercules CA, USA) (Messina et al., 2021).

DPPH Radical Scavenging Activity

The total antioxidant power of the obtained hydrolysates was measured using the DPPH assay (Messina et al., 2021). A total of 0.4 mL of different concentrations of 0.5, 1, 2.5, 5 and 10 mg/mL were prepared by diluting the stock solutions in water (30 mg/mL), then they were mixed with 1.6 mL of 100 µM DPPH in 96% ethanol to start the reaction. The reaction mixture was kept at room temperature and the absorbance was measured at 517 nm after 30 min. Gallic acid (Sigma-Aldrich) was used as a positive control.

The scavenging activity was determined using the following equation (3):

$$I\% = 1 - \left(\frac{A_{sample}}{A_{blank}}\right) * 100 \qquad 4)$$

Reducing power

The reducing power of the hydrolysates was assessed according to the spectrophotometric iron reduction method (Manuguerra et al., 2018; Messina et al., 2019b) Different concentrations of hydrolysates BCBP (0.5, 1, 2.5, 5 and 10 mg/mL) were prepared. Phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (1% w/v) were mixed with the hydrolysates extract (1:1:1 v/v/v) and incubated at 50°C for 20 min. Equal amount of Trichloroacetic acid (1% w/v) was added, and centrifugatates at 3000 rpm for 10 min. At the upper layer of hydrolysate solution, distilled water and $FeC1_3$ (0.1% w/v) were added (1:1:2 v/v/v). The absorbance at 700 nm was measured and gallic acid (GAE) was used as a positive control.

Cell culture

Human skin fibroblast (HS-68) (ECACC n. 89051701, Sigma®) were grown as a monolayer in flasks, using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 µg/mL penicillin–streptomycin, incubated in a humidified atmosphere at 5% CO_2, 95% air and 37 °C, under sterile conditions.

Protective effects of BCBP hydrolysates against oxaditive stress

Confluent cells were trypsinized and seeded in a 96-well plate at a concentration of 7.5×10^4 cells/well and incubated for 24 h. Different concentrations of BCBP hydrolysates obtained from Alcalase and Protamex (0.5-50 mg/mL dissolved in sterile distilled water and filtered in 0.22 µm Millipore membrane) (Millex® Merck Millipore, Darmstadt, Germany), were added to cells and incubated for 24 h, to individuate the adequate concentration of hydrolysates that does not determine

significant toxicity, useful to realize experiments of induction of oxidative stress. After 24 h, the cell viability was measured using the 3-(4,5-dimethyl-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay, according to Mossman's method (Mosmann, 1983), as reported by Messina et al. (2019a).
After the individuation of the range of concentration that does not induce significant cell mortality, cells were again seeded on 96 well plate and treated with concentration 0.5 mg/ml of BCBP hydrolysates obtained from Alcalase and Protomax for 24h. In addition, a set of samples represented the control were maintained only with culture medium and another subset of samples were treated with synthetic antioxidant N-acetyl cysteine (NAC, 0.5 mM) (Merck KGaA Darmstadt, Germany).
After 24 h, all samples, except the control, were exposed to the chemical promoter of oxidative stress, hydrogen peroxide (10 µM) according to a previous standardized protocol (Abbes et al., 2013; Manuguerra et al., 2018; Messina et al., 2016), and left to incubate at 37°C for 2 h. The cell viability was measured using the MTT method according to Mosmann (1983). Results were expressed as percentage of viable cells in respect to the control. Each experiment of viability was carried on in triplicate.

4.3.5. Statistical Analysis

Statistical analysis was performed using SPSS for Windows® (version 20.0, SPSS Inc., Chicago, IL, USA). All analyses were carried out in triplicate. Results are expressed as mean ± standard deviation. Homogeneity of variance was confirmed by the Levene test. Data were subjected to one-way analysis of variance (ANOVA), and Student-Newman-Keuls or Games-Howell post-hoc tests were performed to make multiple comparisons between experimental groups. The significance level was 95% ($p < 0.05$) in all cases.

4.4. Results and discussion

4.4.1. Yield, proximate composition, and fatty acid profile of blue crab by-products

Blue crab meat is rich in nutrients (Alparslan et al., 2022; Arena et al., 2024; Ayas, 2016; Çelik et al., 2004; Gökoolu and Yerlikaya, 2003; Jiang et al., 2020; Khamassi et al., 2022; Kuley et al., 2008; Martínez et al., 2017; Nanda et al., 2021; Tufan, 2023; Wu et al., 2020), but has a low yield, resulting in significant waste. The main problem in crab meat production is the process of meat removal (Yomar-Hattori et al., 2006). In our study, where the meat was removed by hand, the meat yield was 21.87 ±2.38 (Table 1), according to Yomar-Hattori et al., (Yomar-Hattori et al., 2006). This resulted in a high amount of waste (72.45 ±4.08) (Table 1) that can be used as by-products because as noted,

blue crab by-products are rich in chitin, protein, minerals, fatty acids, and carotenoids (Abd El-Ghany et al., 2023; Antunes-Valcareggi et al., 2017; Arena et al., 2024; Camargo et al., 2021; Nanda et al., 2021).

Table 1. Yield and waste (mean ± SD) (%) of blue crabs obtained from the fish processing industry.

	%
Yield meat	21.87 ±2.38
Waste	72.45 ±4.08

The proximate composition of BCBP (dw) is shown in Table 2. A high ash content was found (35.38±0.30), lower than that observed by Antunes-Valcareggi et al. (Antunes-Valcareggi et al., 2017) (56.2 ± 1.2%). The high ash content does not allow the direct use of these by-products as meal for fish in aquaculture, because when the ash content exceeds 12%, the growth of the fish is compromised, since its high amount reduces the digestibility of other ingredients in the diet (Cretton et al., 2021).

Table 2. Proximate composition (dw) (mean ± SD) of blue crab byproducts BCBP.

Components	**%**
Ash	35,38±0,30
Total Lipid	2.57±0.09
Protein	31,21±1,12
Chitin	10,57±0,66

A lipid content of 2.41± 0.08% was observed, in accordance with Antunes-Valcareggi et al. (2017), but higher respect to Félix-Valenzuela, et al., (2001) where a lipid content of 0.41±0.01% was reported. Not many results on the composition of blue crab by-products can be found in the literature. The low lipid content was also observed in other crab by-products such as the southern king crab (*Lithodes santolla)* (0.5%) (Cretton et al., 2021), green crab shells (*Carcinus maenas*) (0.37-0.67%) (Naczk et al., 2004), hard shell (2.41± 0.30%) and soft-shell crabs (1.50± 0.7%) of *Portunus sanguinolentus* (Sudhakar et al., 2009). In general, lipid content ranging from 0.2 to 17% has been observed in crustacean by-products (Ahmadkelayeh and Hawboldt, 2020; Pinheiro et al., 2021). This variability depends on the species, the geographical location of the fishery, and the nature of the by-products (Pinheiro et al., 2021).

The fatty acids (FA) profile of BCBP is shown in Table 3.

Table 3. FA profile (% of total FA) of blue crabs by products (BCBP).

FA	BCBP
14:0	1.53±0.40
16:0	15.17±1.85
16:1n-7	5.01±0.68
16:2n-4	0.29±0.04
17:0	1.26±0.09
16:3n-4	0.84±0.08
16:4n-1	0.12±0.03
18:0	7.35±0.08
18:1n-9	11.86±0.43
18:1n-7	4.27±0.52
18:2n-6	2.66±0.07
18:2n-4	0.36±0.02
18:3n-6	0.13±0.12
18:3n-4	0.22±0.01
18:3n-3	0.80±0.03
18:4n-3	0.27±0.02
18:4n-1	0.11±0.02
20:0	0.77±0.06
20:1n-9	3.45±0.23
20:2n-6	1.28±0.09
20:3n-6	0.41±0.03
20:4n-6	6.04±0.36
20:4n-3	0.32±0.13
20:5n-3	11.03±0.65
22:0	0.82±0.19
22:1n-11	0.22±0.06
22:1n-9	0.09±0.04
21:5n-3	0.31±0.09
22:4n-6	1.20±0.15
22:5n-6	0.85±0.11
22:5n-3	2.22±0.29
22:6n-3	9.75±1.13
SFA	27.21±2.15
MUFA	24.90±0.75
PUFA	38.90±2.69
Tot n-3	24.40±2.07
Tot n-6	12.56±0.77
other PUFA	1.94±0.16
NI	8.99±0.39

The data are reported as mean ± standard deviation, n = 12. SFA Saturated fatty acids; MUFA Monounsaturated fatty acids; PUFA Polyunsaturated fatty acids; NI not identified.

The FA composition of BCBP was mainly dominated by polyunsaturated fatty acids (PUFA) (38.90±2.69) (Table 3) with eicosapentaenoic acid (EPA, 20:5n3 - 11.03±0.65%) and docosahexaenoic acid (DHA, 22:6n3 - 9.75±1.13%) being the major PUFA (Table 3). The high

content of n-3 polyunsaturated fatty acids (n-3 PUFA) (24.40±2.07) (Table 3), especially EPA and DHA, suggests the potential use of these matrices as a source of n-3 PUFA as observed in other crustaceans species (Ahmadkelayeh and Hawboldt, 2020; Messina et al., 2021; Sánchez-Camargo et al., 2012).

The second most abundant class was saturated FA (SFA) (27.21±2.15) (Table 3) with palmitic acid (C16:0 - 15.17±1.85) was the main SFA. Monounsaturated fatty acids (MUFA) (24.90±0.75) were the last class of fatty acids, in order of abundance, with oleic acid (C18:1 n-9 - 11.86±0.43) being the most important MUFA.

High levels of protein (31,21±1,12) was observed (Table 2). Antunes-Valcareggi et al. (2017), and Félix-Valenzuela, et al., (2001) similar to other studies values of proteins (respectively 33±1 and 33.8±2.3%). Similar protein content has been observed in other crab wastes (Parthiban et al., 2017).

Proteins and their derivatives, such as isolates, concentrates, hydrolysates, peptides, or amino acids, are a major component of crustacean by-products (Arena et al., 2024). A high protein content (25-50%) (Pinheiro et al., 2021; Rodrigues et al., 2021) is generally observed in crustacean by-products, and it is very important to valorize this component. Studies conducted on crustacean by-products have focused on identifying and characterizing potentially active peptides that could be used to prevent various pathologies, produce functional foods, and create edible films (Giordano et al., 2018; Hajji et al., 2021; Jabeur et al., 2022; Pinheiro et al., 2021).

In addition to protein, chitin is a critical component of the by-products of crustaceans. In our study, a chitin content of 10,57±0,66% was observed (Table 2) in agreement with other studies (Arena et al., 2024; Jabeen et al., 2021; Kaya et al., 2016; Metin et al., 2019) on the same species. A similar value has been reported in other crab species (Khiari et al., 2020; Parthiban et al., 2017; Pires et al., 2017), although different contents were observed depending on the parts of the crab analyzed (Pires et al., 2017).

4.4.2. Enzymatic Hydrolysis

In this study, protein hydrolysates were obtained by enzymatic hydrolysis using commercially available proteases according to a standardized protocol (Messina et al., 2021) previously applied to other fishery species (Messina et al., 2021). This method is environmentally friendly for deproteinizing crustacean by-products and allows the production of protein hydrolysates with high nutritional value (Antunes-Valcareggi et al., 2017; Cahú et al., 2012; Gildberg and Stenberg, 2001; Simpson et al., 1998).

The enzymes used for the hydrolysis of blue crab by-products were Alcalase and Protamex. Alcalase, derived from *Bacillus licheniformis,* showed higher efficiency in hydrolyzing the total proteins of

BPBC, as reflected by the highest degree of hydrolysis (DH%) compared to Protamex (Figure 1), suggesting a specific enzyme affinity for the substrate (Antunes-Valcareggi et al., 2017). The maximum degree of hydrolysis (DH%) (between 14% of the enzyme Protamex and 23% of the enzyme Alcalase) was reached after 175 min of reaction for Protamex and 205 min for Alcalase (Figure 1). Further analyses will be carried out to evaluate the specific aspects of enzyme saturation kinetics based on the Michaelis-Menten equation.

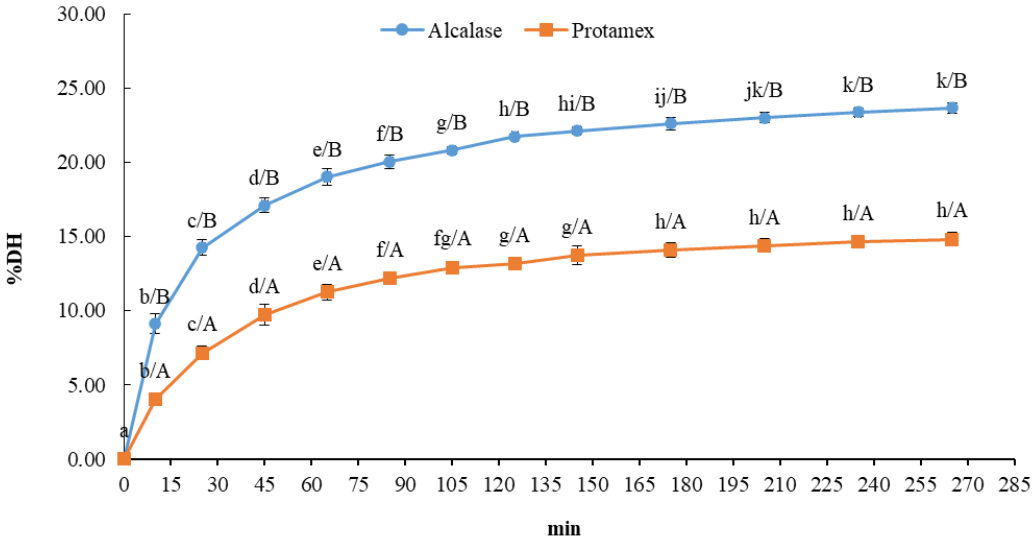


Figure 1. Degree of protein hydrolysis (% DH) for BCBP, using Alcalase and Protamex. Different lowercase letters indicate significant differences between different time points for each enzyme (a, b, c...: $p < 0.05$), different uppercase letters indicate significant differences between different enzymes (A; B; ...$p < 0.05$).

Studies carried out on other marine products and by-products have shown a higher efficiency of the enzyme Alcalase compared to Protamex (Antunes-Valcareggi et al., 2017; Camargo et al., 2021; Dey and Dora, 2014; Gunasekaran et al., 2015; Messina et al., 2021; Shaibani et al., 2020). Thus, Alcalase appears to be one of the most effective enzymes in the preparation of fish and crustacean protein hydrolysates due to its high proteolytic activity on different protein sources (Camargo et al., 2021; Dey and Dora, 2014; Ovissipour et al., 2009; Rajapakse et al., 2005; Shaibani et al., 2020).

DH% is an important parameter of protein hydrolysate, and a high DH%, above 10%, indicates a higher production of peptides in solution, with increased protein solubility and thus the possibility of recovering the protein for use as a food additive (Camargo et al., 2021; Zamora-Sillero et al., 2017). Our results, with DH% above 10%, showed how BPBC can be valorized to produce high value-added products, and its protein hydrolysate could be used as a food additive.

In addition to their use as food additives, protein hydrolysates are of great interest to the pharmaceutical and seafood industries due to their bioactivity (Camargo et al., 2021; Shaibani et al., 2020).

4.4.3. Hydrolysates Characterization and Bioactive Properties of the Protein Fractions

SDS PAGE

The SDS-PAGE electrophoresis (Figure 2) showed that the electrophoretic pattern of the protein hydrolysates compared to the total proteins extracted had a progressive loss of the bands at higher molecular mass, attesting to the efficiency of the reaction. The enzymes Alcalase and Protamex were observed to exhibit high proteolytic activity, as evidenced by the presence of peptides with a molecular mass of 65 kDa and 12 kDa. At a higher degree of hydrolysis, the bands with greater mass begin to disappear; this confirmed the fact that the molecular weight of the peptides formed by hydrolysis is associated with the degree of hydrolysis.

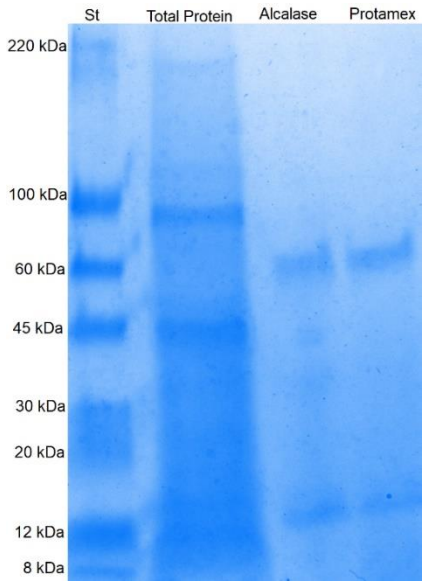


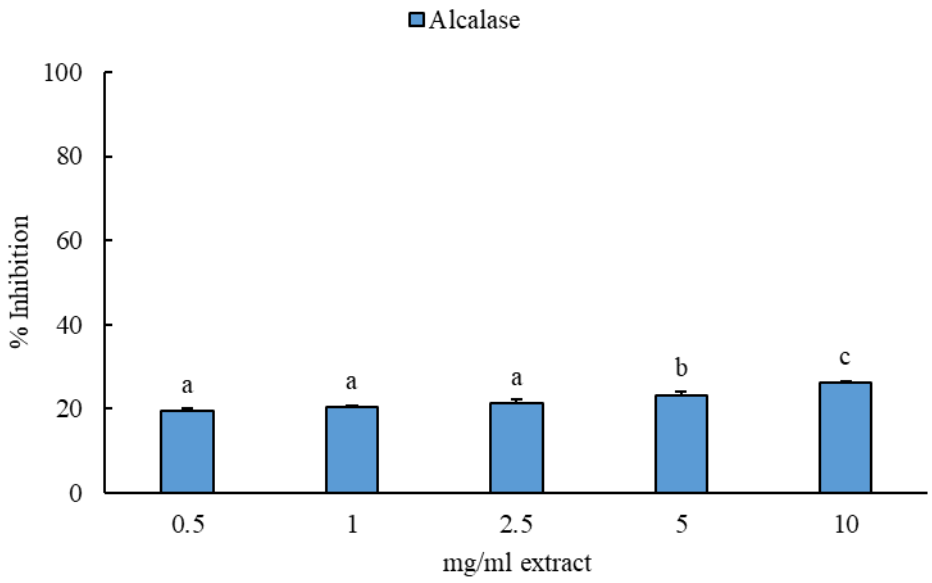
Figure 2. SDS-PAGE of the total proteins and proteins hydrolisates, obtained with the enzymes Alcalase and Protamex from by-products of the blue crab (BCBP) powder at the end of the enzymatic process.

Antioxidant activity

The protein hydrolysates analyzed in this study showed the ability to eliminate DPPH radicals (Figure 3a and 3b).

At the concentrations of 0.5 and 1 mg/ml, no statistically significant differences were observed for either hydrolysate obtained with Alcalase (Figure 3a) and Protamex (Figure 3b). Both Alcalase and

Protamex exhibited their maximum radical inhibition potential at the highest tested concentration of 10 mg/ml (Figures 3a and 3b).



a)

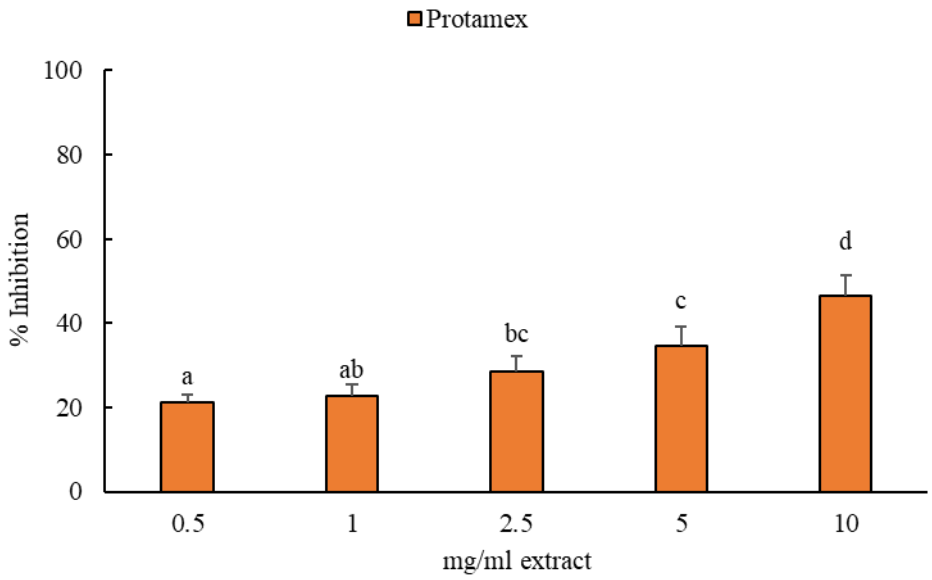


Figure 3. 1,1-diphenyl-2-picryhydrazyl (DPPH) radical inhibition (%) of protein hydrolysates BCBP at different concentrations (0.5, 1, 2.5, 5 and 10 mg/mL extract) obtained by hydrolysis with Alcalase (a) and Protamex (b). Different letters indicate significant differences indicate significant differences between the different concentrations of extract tested for each enzyme (a; b; c... $p < 0.05$); values are mean (6 replicates) ± standard deviation.

The DPPH assay showed that the BCBP protein hydrolysate has antioxidant properties and could act as a good electron donor, reacting with free radicals, terminating the radical chain reaction, and converting them into stable molecules as observed in other crab (Camargo et al., 2021; Jiang et al., 2017) and shrimp by-products (Dey and Dora, 2014; Kim et al., 2016; Messina et al., 2021; Zou et

al., 2021). BCBP protein hydrolysate such as may be able to reduce oxidative stress, given the ability of antioxidants to retard or inhibit the oxidation of oxidizable substrates (Aklakur, 2018; Camargo et al., 2021; Di Bernardini et al., 2011; Nikoo et al., 2019; Zamora-Sillero et al., 2018).

Since both enzymes show the same inhibitory power at the lowest concentrations (Figure 3a and 3b), they could be good candidates to produce antioxidant peptides.

BCPB hydrolysates were tested using the reducing power assay, which is used to assess the ability of an antioxidant to donate an electron or hydrogen (Guerard et al., 2007; Jiang et al., 2017). In this assay, the ability to reduce iron (Fe^{3+}) to ferrous (Fe^{2+}) is tested. Studies have shown that there is a direct correlation between antioxidant activities and the reducing power of certain bioactive compounds (Guerard et al., 2007; Jiang et al., 2017; Yoon et al., 2013).

Obtained results (Figure 3) showed no significant differences between the BCBP hydrolysates obtained with Alcalase and Protamex. Both showed moderate reducing power compared to gallic acid (GAE), used as a positive control.

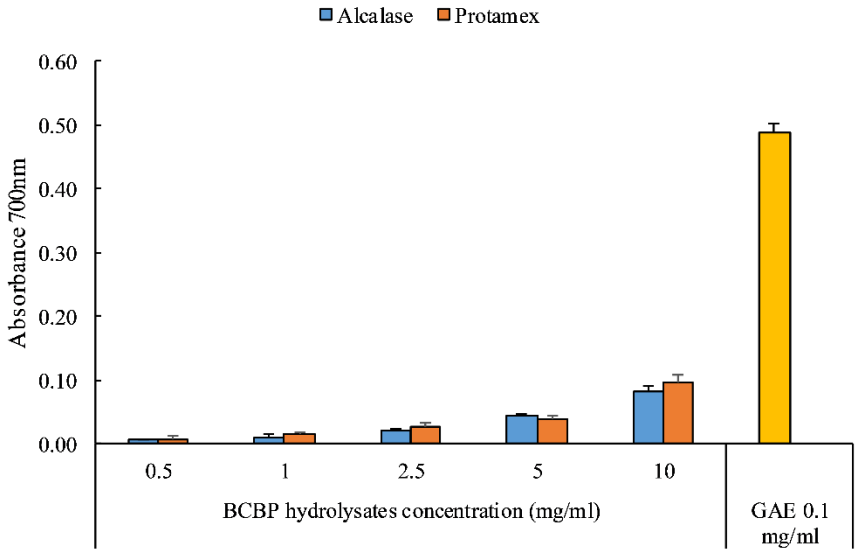


Figure 4. Reducing power of BCBP hydrolysates of protein hydrolysates BCBP at different concentrations (0.5, 1, 2.5, 5 and 10 mg/mL extract) obtained by hydrolysis with Alcalase and Protamex . Gallic acid equivalents (GAE 0.1 mg/ml), values are mean (3 replicates) ± standard deviation.

Antioxidant Activity of Hydrolyzed Fractions in Human skin fibroblast (HS68)

The HS68 cell line was treated with BCBP hydrolysates obtained from the enzymes Alcalase and Protamex at concentrations of 0.5- 5-10-15-25-50 mg/ml for 24 hours.

A significant decrease in cell viability was observed in a dose-dependent manner at concentrations of 10, 15, 25 and 50 mg/ml of BCBP hydrolysates produced by both enzymes, compared to the control group (Figure 5). Similar results were reported by Camargo et al. (Camargo et al., 2021) in pre-

osteoblasts cells treated with protein hydrolysates from *Callinectes ornatus*, obtained from the same enzymes, that showed a decrease in the cellular viability with a dose-dependent profile (Camargo et al., 2021).

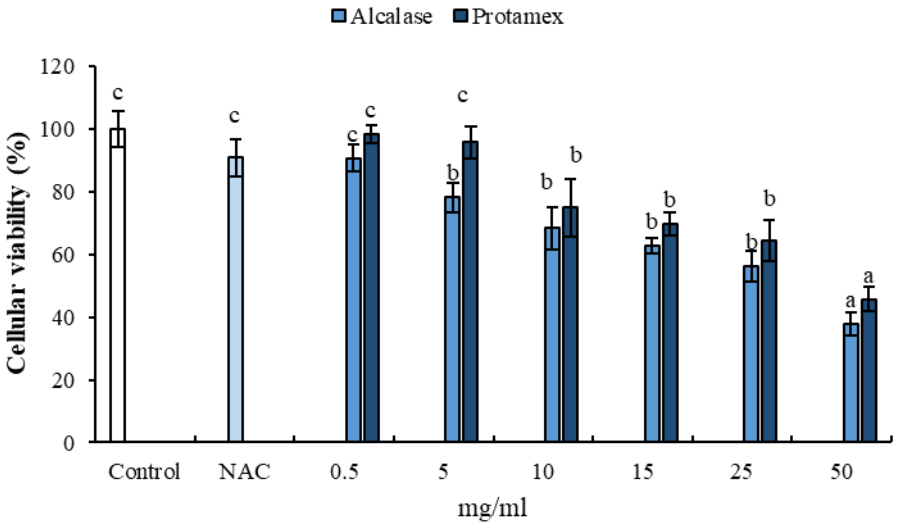


Figure 5. Cytotoxicity effect of BCBP hydrolysates at different concentrations (mg/ml) in HS68 by MTT assay (all data are presented as mean (5 replicates) ± standard deviation). Control: untreated cells, NAC: cells pretreated with the synthetic antioxidant N-acetyl cysteine; 0.5, 5, 10, 15, 25 and 50: cells treated with different concentrations of BCBP hydrolysates (mg/ml extract) for 24 h.

The lower concentration (0.5 mg/ml) of both BCBP hydrolysates obtained from the Alcalase and Protamex enzymes did not affect cell viability (Figure 5).

Consequently, the latter, which showed the same antioxidant activity with both Alcalase and Protamex (Figure 3 and Figure 4), was selected for further investigation into the potential protective effects of hydrolysates against oxidative stress induced by a chemical inducer such as hydrogen peroxide. The results reported in Figure 6 showed that cells pretreated with concentrations 0.5 mg/ml of BCBP hydrolysates produced by both enzymes and subsequently exposed to hydrogen peroxide, showed a higher viability than untreated stressed cells (+HP), which is comparable to cells treated with the synthetic antioxidant NAC (Figure 6).

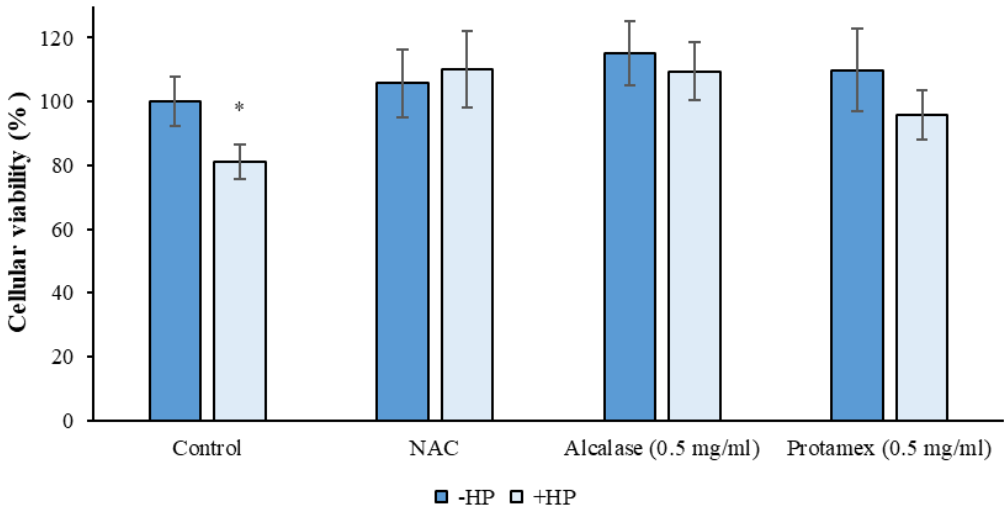


Figure 6. Effect of BCBP hydrolysates obtained from Alcalase and Protomax (0.5 mg/ml) on HS68 fibroblast cells exposed to oxidative stress, induced by H2O2 (+ HP). Control: untreated cells; NAC: cells treated with N-acetyl cysteine. Alcalase: cells pretreated with BCBP hydrolysates obtained by hydrolysis with Alcalase. Protamex: cells pretreated with BCBP hydrolysates obtained by hydrolysis with Protamex. (The bars represent the mean (5 replicates) ± standard deviation). Statistical differences ($p < 0.05$) vs Control (-HP) are indicated by "".*

The findings suggest that BCBP exerts an antioxidant effect on oxidative damage.

No results were found in the specialized literature regarding antioxidant activity of Hydrolyzed fractions in Human Fibroblast of blue crab waste.

However, the high level of bioactivity observed in the protein hydrolysates derived from blue crab by-products is in accordance with the findings reported for other protein hydrolysates of marine origin derived from enzymatic hydrolysis, which have demonstrated marked antioxidant activity against free radicals and pro-oxidative metal ions (Messina et al., 2021; Nikoo et al., 2023). These compounds therefore have the potential to be used as natural antioxidants in various sectors such as nutraceuticals, cosmeceuticals, and food, helping to protect against free radical-induced ageing processes (Messina et al., 2021; Nikoo et al., 2023). These hydrolysates, as observed for crustacean protein hydrolysates, could be incorporated into chitosan-gelatin films to produce antioxidant composite films used as active food packaging (Hajji et al., 2021).

4.5. Conclusions

The blue crab (*C. sapidus*), an alien species in the Mediterranean, has the potential to be exploited by local companies as a food resource. However, a considerable quantity of waste is generated, which is currently not fully utilized. This study has shown that blue crab by-products can be effectively converted into valuable protein hydrolysates through enzymatic processes.

The findings demonstrate that these protein hydrolysates have remarkable antioxidant activity, suggesting their potential use as natural antioxidants in various sectors, including food, nutraceuticals, cosmeceuticals, and even pharmaceuticals. The utilization of such hydrolysates could help to extend product shelf-life, improve food preservation, and provide health benefits related to protection against oxidative stress.

These findings highlight the possibility for local enterprises to adopt innovative strategies for waste management through the recovery and valorization of blue crab by-products. This approach is in line with the principles of the circular economy, which prioritizes the reduction of waste and the optimization of resource utilization. By transforming what is typically regarded as waste into valuable products, companies can not only mitigate environmental impact but also generate new economic opportunities.

4.6. References

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Concluding remarks

The European Green Deal identifies some key priorities, including the transition towards a circular economy, the improvement of food waste management, and the assurance of the sustainability of the blue economy and the fisheries sector. My research project is aligned with these priorities, investigating innovative for resource valorization and waste management in the fisheries sector.

My research activity, within the PON "Research and Innovation" 2014-2020 - Action IV.5 "Doctorates on topics Green", was focused on the valorization of Sicilian fish by-products. The objective was to develop techniques and processes for the valorization of fisheries, aquaculture and processing by-products, through the recovery of bioactive components of high biological value and their application in other industrial sectors. The study aimed to provide a comprehensive evaluation of the potential of each supply chain to ensure a stable and continuous production of ingredients derived from fishery, aquaculture and processing by-products.

In this study reliable sources were identified and selected from

- the side streams produced after filleting of sea cage fattened bluefin tuna (*T. thynnus*)
- by-catch from the Mediterranean trawler fishing species,
- *Parapenaeus longirostris* processing by-products,
- aquaculture by-products (from sea bass and sea bream filleting),
- *Thunnus albacares* processing by-products,
- *Engraulis encrasicolus* by-products (heads and viscera),
- blue crab (*C. sapidus*) by-products.

The studies carried out have identified effective strategies for the valorization of these by-products, extracting bioactive components for use in areas such as nutraceuticals, animal feed and human nutrition. Ethyl esters enriched with n-3 polyunsaturated fatty acids have been obtained from these by-products, which could be employed in fish feed formulations, thereby improving the sustainability and quality of aquaculture products (Messina et al., 2022). Furthermore, these by-products could potentially be used as feed substrates for *H. illucens* larvae, improving their lipid profile and significantly increasing their n-3 fatty acid content. This result demonstrated that *H. illucens* larvae fed with by-products from fisheries, aquaculture and processing represent a sustainable and alternative source to fishmeal for feed formulations in aquaculture (Arena et al., 2023). Finally, the by-products obtained during my research represent a source of high-quality protein hydrolysates with antioxidant properties, useful for food preservation and protection against oxidative stress.

In conclusion, the results of this research demonstrate the importance of developing innovative strategies and technologies for the valorization of by-products from the fish supply chain, in accordance with the principles of a circular economy and the objectives set by the European Green

Deal. Furthermore, the effective transfer of these results to companies in the fisheries sector can contribute to the improvement of economic and environmental sustainability, waste management strategies and resource valorization in companies.

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