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TITLE

"Sustainable exploitation of discarded fisheries resources and development of added value bioproducts"

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SUMMARY

Abstract

Marine collagen is a precious resource that in recent years has been gaining great success in the cosmetic, food, and biomedical industries thanks to its extreme versatility, bioactive properties, and cytocompatibility with human tissues. Derived mainly from fishing by-products, such as skins, scales, and bones, or from marine organisms obtained from by-catches, such as jellyfish, it represents a safe and sustainable alternative to mammalian collagen. Collagen is the most abundant protein in nature, playing a fundamental role as a mechanical and structural support to tissues.

During the PhD, the jellyfish *Rhizostoma pulmo* collected during a bloom in the Mediterranean Sea was used for collagen extraction using innovative mechanical pre-treatments (e.g., sonication, freeze-drying, and homogenization) to improve yield and reduce the use of chemical solvents. The results showed that sonication significantly increased collagen yield, especially in *R. pulmo* oral arms, with an increase of up to 10-fold compared to traditional methods. On the contrary, freeze-drying and homogenization had no effect. Moreover, the extracted collagen was subsequently characterized by ATR-FTIR and SDS-PAGE, confirming its structure and purity. This study highlighted the effectiveness of sonication as an adjuvant mechanical treatment in improving eco-friendly extraction processes with potential applications in biotechnology. Moreover, collagen extracted from the jellyfish *R. pulmo* was also used to create membranes based on marine collagen and methacrylated chitosan for liver tissue engineering applications. The analyses showed that the addition of marine collagen improves the membranes mechanical properties, hydrophilicity, and porosity, promoting the engraftment of liver cells.

Another study performed during the PhD focused on the extraction, by the classical method, of acid-soluble collagen from sea bass (*Dicentrarchus labrax*) skin, and its use in the development of biocompatible scaffolds based on marine collagen/chitosan, for biomedical applications. The incorporation of marine collagen into the scaffolds improved the mechanical properties, and showed a greater porosity of the scaffolds compared to the scaffolds based on chitosan alone. Also, in vitro cytotoxicity tests confirmed a high cytocompatibility for the murine fibroblast L929 cell line.

On the other hand, marine peptides and hydrolysates also represent an important category of high added value biomolecules derived from fishery by-products and by-catch, such as jellyfish or starfish. In a further study, the bioactive potential of soluble and hydrolysed proteins from the freeze-dried jellyfish *Cotylorhiza tuberculata* collected in the

Mediterranean Sea was explored. Soluble proteins were extracted from the umbrella and oral arms using phosphate-buffered saline, then the insoluble portion was subjected to sequential enzymatic hydrolysis with pepsin and collagenase to obtain hydrolysed proteins. The obtained fractions were fractionated based on the molecular weight through membrane filters with different cut-offs. The fractions were analyzed for their antioxidant, antiproliferative, and anti-inflammatory activities. The ABTS assay revealed strong antioxidant properties in all samples, especially in the collagenase hydrolysed fractions. In vitro cell viability tests on A2058 melanoma and HaCaT cell lines indicated that some fractions showed good antiproliferative activity and low cytotoxic activity. Furthermore, a simulated gastrointestinal digestion model demonstrated that acetic acid hydrolysis of the umbrella fractions reduced IL-6 levels, suggesting anti-inflammatory effects. This study highlighted how peptides from the jellyfish *C. tuberculata* could be used as nutraceutical bioactive ingredients. Similarly, fractions extracted from the digestion of the starfish *Asterias rubens*, performed by three different enzymes, Food Pro PNL, Corolase 8000, and Corolase 7089, were analyzed for their properties. The hydrolysates were produced from raw starfish, deproteinized starfish, and demineralized starfish. The protein purity of the hydrolysates prepared from the deproteinized samples was higher than those prepared from whole starfish, even showing a lower yield. Also in this case, results showed good antioxidant and antiproliferative activity of the hydrolysates, suggesting that starfish could be effectively utilized for nutraceutical and pharmaceutical applications.

Introduction

In recent years, seafood consumption has grown dramatically due to the recognition of fish as an essential part of a balanced diet and healthy lifestyle. According to data from the Food and Agriculture Organization (FAO), the United States and the rest of the world have seen a significant increase in fisheries and aquaculture production, which increased more than eightfold from 1954 to 2014. This increase has been driven by technological advances in the fisheries sector and rapid developments in aquaculture, with global fish production reaching 93.4 million tonnes in 2014. Fisheries and aquaculture provide an estimated global annual fish catch of 214 million tonnes in 2020 (FAO,2022). Between 1961 and 2016, the global fish supply has grown dramatically, with an average annual growth rate of 3.2%. This rate has outpaced the growth of the world population, which has been 1.6%, and has also outpaced the growth of land-based meat, which has been 2.8%. Fish consumption has increased significantly, from 9.0 kg in 1961 to 20.2 kg in 2015 (FAO, 2018). However, in 2020 the global capture fisheries production was 90,3 million tons, a fall of 4% compared with the previous 3 years, probably due to the COVID-19 pandemic that limited fishing operations. Despite this decline, in recent years the trend of catches has fluctuated between 86 and 93 million tonnes since the late 1980s, demonstrating that the long-term trend remains fairly stable (FAO, 2022).

The critical importance of the global fisheries industry for food security, economic development, and cultural heritage (especially in coastal communities) is not a secret. However, a major issue related to this huge seafood consumption is the generation of fish waste, usually related to fisheries by-catches and by-products from fish processing and aquaculture industries. The environmental and economic implications of these issues reflect sustainability and resource efficiency as captured by the European Market Observatory for Fisheries & Aquaculture Products (EUMOFA, 2020). In addition, FAO reported that some fishery discards are still underexploited with economic impacts (FAO, 2020).

For this reason, the next paragraphs will specifically deal with the aspects concerning fishing by-catch and by-product, and how these wastes could intervene in the Blue Economy growth, in a circular economy perspective.

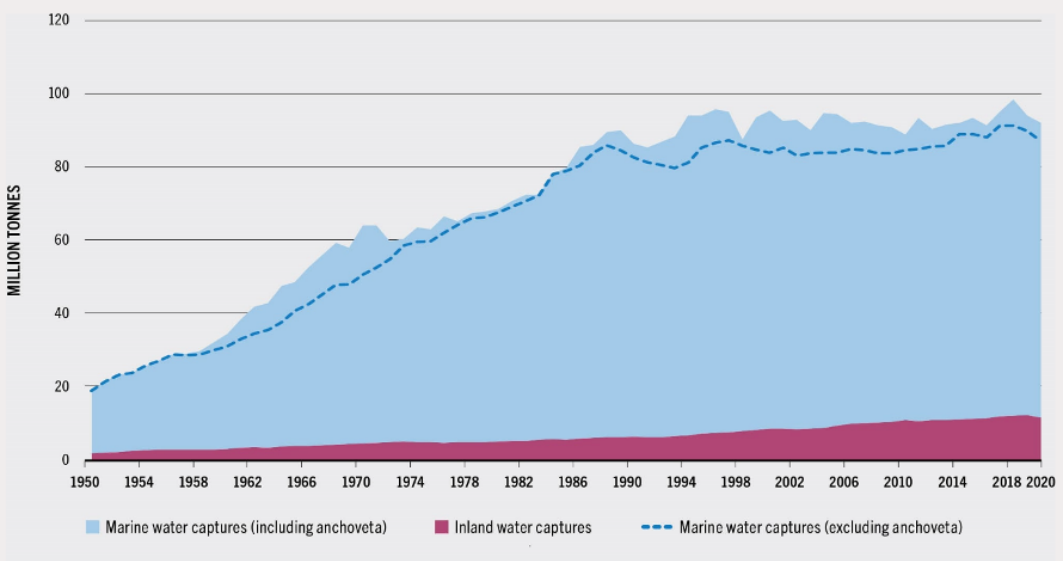

Figure 1 TRENDS IN GLOBAL CAPTURES from (FAO, 2022).

Fishery bycatch

Fishery bycatch in the process of fishing refers to the capture of non-target species. This entails catching under-sized fish or species of fish that are not requested, and many other marine organisms, including mammals, birds, and invertebrates (Hall, Alverson, and Metuzals 2000), with a deep ecological impact. Moreover, bycatch sometimes consists of unwanted endangered and/or vulnerable species, leading to population decline and disruption of marine ecology. For example, in the longline tuna fisheries, sea turtles, dolphins, and sharks are very common as bycatch, with great implications for their populations (Lewison et al. 2004). Many fishing methods produce significant levels of bycatch; for instance, one of the biggest culprits of incidental catch includes bottom trawling, together with longline fishing, which is also known to capture a wide range of unwanted species (Gordon, 1991). Also, bycatch reduces the available target species since a lot of juveniles are caught and killed before their stage of sexual maturity is attained, limiting the potential recovery of fish stocks (FAO, 2020).

Besides ecological impacts, bycatch is a vast economic loss. Non-target species caught accidentally are often discarded into the sea but in most cases do not survive. The FAO estimates that 10-20% of the total catches are being discarded annually, which means waste of marine resources. The implications of fish waste are significant. From an environmental perspective, the decomposition of fish waste contributes to nutrient pollution in marine environments, leading to eutrophication and the creation of dead zones with low oxygen levels that are harmful to marine life (Pauly et al. 2002).

The EUMOFA (2020) report highlights that the European Union fishing sector discards around 25% of its catch as waste, demonstrating the extent of underutilization within regional fisheries.

Economically, bycatch is a huge inefficiency. Fishers discard non-target species, which could otherwise be used for other purposes, such as the production of high-value compounds. Discarding bycatch generates operating costs and reduces the overall value of the catch. For instance, in shrimp trawling up to 90% of the total catch could constitute bycatch and a major portion would be discarded (FAO, 2005).

Several strategies need to be implemented to limit the fishing discards production, such as: i) employing selective fishing equipment for specific fish species to reduce incidental catch. For example, bycatch reduction devices (BRDs), turtle exclusion devices (TEDs), and "BSL" (Bird Scaring Lines) have been useful with the inclusion of Trawl Fishing (Broadhurst 2000; García, Carpio, and Rivas 2024); ii) adopting measures and quotas, and maximizing the effective use of the catches made. The European Union landing obligation or discard ban requires all the catches made to be landed and actually counted as fulfilling the quotas thereby, limiting the practice of by-catch discards. To that extent, the EUMOFA (2020) report highlights that these regulations are essential in promoting compliance and enhancing the sustainable development of European fisheries practices further (EUMOFA, 2020); iii) informing consumers about the benefits of consuming underutilised species could create new markets and reduce waste. Certification programs such as the Marine Stewardship Council (MSC) label (MSC, 2020) promote sustainable fishing practices by providing market benefits to certified products.

Figure 2. A scene of bycatch featuring jellyfish and starfish entangled in fishing nets, created by AI

Jellyfish

Jellyfish bycatch, especially of invasive species, is a recent and growing problem in the Mediterranean, with economic and ecological implications. For example, fishermen report that in the bays of Iskenderun, Mersin, and Antalya the species *Rhopilema nomadica* constitutes about 60% of the total catch in the eastern Mediterranean coast of Turkey (Bodrum, 2011). In the Marmara Sea, during periods of jellyfish proliferation, normal fishing activity was compromised, as explained by (İşinibilir et al. 2024). The nets cast by fishermen were clogged by the amount of jellyfish biomass, making the nets difficult to retrieve from the sea. This resulted in longer and more laborious deployment times of the nets, with increased fuel waste. In a survey conducted in the eastern part of the Sea of Marmara between 2014 and 2022, the species *Aurelia aurita* was found to have a frequency of 72% in trawl nets. In the Moroccan Mediterranean, jellyfish have also negatively affected fishing in the last decade; in M'diq, 86% of the fishermen interviewed reported a seasonal reduction in revenues mainly due to blooms of *Pelagia noctiluca*, *Rhizostoma pulmo* and *Chrysaora hysoscella*. The data showed that the economic loss for the M'diq fleet is approximately 3,26 million dollars per year. In

addition, fishermen complained that the blooms were a damage to the equipment and, also in this case, many additional hours of work were required to restore the nets for normal fishing activities (Mghili, Analla, and Aksissou 2022). Parmieri and collaborators assessed the economic losses due to jellyfish blooms in the Northern Adriatic fisheries of Italy, using Chioggia as a reference port. In 2011, data on vessels, fish catches, fuel costs, and fishing days were collected for the regions of Veneto, Emilia Romagna, and Friuli Venezia Giulia. Data on catches during blooms and non-bloom years were compared and the catches and economic losses were estimated. Results confirmed the negative impact that jellyfish blooms have on fisheries in terms of catches and quantified that the economic losses amounted to 8 million euros per year for the Northern Adriatic trawling fleet. Again, additional costs due to equipment breakage, changes in fishing activity, and wasted fuel should be added (Palmieri et al. 2014). The Israeli Mediterranean coast has also experienced severe jellyfish blooms, causing significant damage to infrastructure, beaches, and particularly to the fishing industry.

In recent years, summer blooms of *Phyllorhiza punctata* and *Cotylorhiza tuberculata* have been recorded (Ghabooli et al. 2011; Angel, Edelist, and Freeman 2016). In Peru the scyphomedusa *Chrysaora plocamia* was fished abundantly as bycatch in 2009, representing around 30% of the catch and leading to an economic loss of 200,000 $ in one month of fishing (Quiñones et al. 2013). Moreover, shrimp fisheries have also suffered large losses due to blooms of the invasive jellyfish *Phyllorhiza punctata*, as well as the scyphomedusa *Lychnorhiza lucerna* (Graham, 2003; Nagata, Haddad, and Nogueira Júnior 2009).

Starfish

Starfish are often caught as bycatch, especially during shrimp fishing. In fact, it is reported that trawling is the main fishing method responsible for the decline of echinoderms (Bergman and Hup, 1992), including starfish that usually live on the seabed (Probert, Mcknight, and Grove 1997; Escolar et al. 2009). During the study in the Santana Archipelago, it was demonstrated how starfish are prey of bycatch due to shrimp fishing. In fact, a total of 158 starfish specimens were caught, representing eight species. The most commonly caught species belonged to three main genera: *Asterina, Astropecten,* and *Luidia*. Data analysis confirmed that the catches did not show a significant trend between the fishing season and the closed fishing season, but rather to the depth to which

the nets reached, in fact most of the catches occurred at depths between 25 and 45 meters (Paula et al. 2022).

In another study, nine types of invertebrates were recorded in the bycatch of trawl nets in the coastal waters of Mallipattinam, Sathubavasathram, and Memesal, southeast coast of India, during the period October 2011 - September 2012. A total of 84 different species were identified as victims of bycatch, including gastropods and echinoderms, such as *Astropecten indicus*, *Stellaster incei*, *Salmasis bicolor*, *Salmasis virgulata* (Prabhu et al. 2013).

Fish by-product

In 2022, 185.4 million tonnes (live weight equivalent) of aquatic animals were harvested globally, and approximately 89% of this amount (164.6 million tonnes) was used for direct human consumption. The remaining 11% (20.8 million tonnes) was used for non-food purposes with low commercial value, with approximately 83% of this share (17 million tonnes) processed into fishmeal and fish oil. The remainder (approximately 4 million tonnes) was mainly used for ornamental fish, aquaculture, bait, pet food or as raw material for direct feed in aquaculture and for livestock and fur farming (FAO, 2024).

Fish by-products are parts of the fish remaining after primary processing, which includes the head, bone, skin, scale, and viscera. These by-products have high potential for value towards diverse uses, different from the traditional ability for human consumption (Jasrotia, Langer, and Dhar 2024). Fish by-products are nutritionally important as a source of protein, fatty acids, and minerals, since the composition is the same as other products used for consumption. For example, fish skin is the richest source of proteins, the trimmings and bones are quite rich in calcium, the head, intestines, and bones are representative of lipids (Kandyliari et al. 2020). Fish meal, obtained from fish parts such as heads and bones, is a key ingredient in animal feed, especially for aquaculture (Metian, 2009). Fish oil extracted from the fatty tissues also serves as a dietary supplement since it is a rich source of omega-3 fatty acids (Alfio, Manzo, and Micillo 2021). Specifically, the average value of all fish by-products, calculated on a dry weight basis, is 49.22-57.92% for protein content, 21.79-30.16% for ash content, and 7.16-19.10% for fat content (Esteban et al. 2007; Vidotti, Viegas, and Carneiro 2003; Abbey et al. 2017).

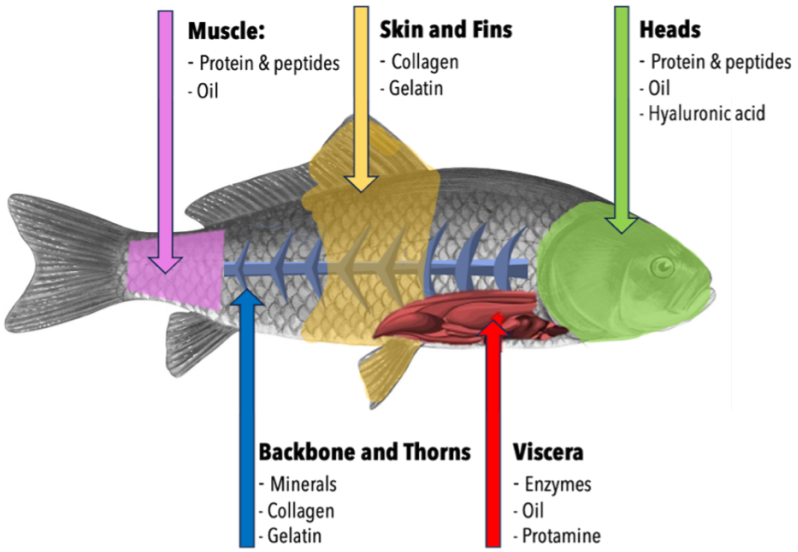

Figure 3. Most common parts of fish and related biomolecules.

Fish Head

Fish heads were widely used in various sectors due to their composition. In the food and cosmetics industry, they are used as a source for collagen, and edible gelatin (Shen et al. 2019). Lipids contained in the heads can be extracted to produce fish oil; several reports indicate that the oil obtained from fish waste is an excellent source of nutrients (Abiona et al. 2021). Moreover, in agriculture, fish heads were used as fertilizers being rich in elements such as nitrogen, phosphorus, and calcium (Zhang, Zhang, and Simpson 2019). In the biotechnology industry, fish heads were exploited for the extraction of enzymes, such as proteases and lipases, which were widely used in industrial production (Shahidi and Janak Kamil 2001).

In addition, the heads from different species of fish were used for the production of fish protein hydrolysate (FPH), converting usually discarded products into products with high added value and high functionality (Naghdi et al. 2023; Panpipat and Chaijan, 2017). For example, seabass *Dicentrarchus labrax* was used for the production of FPH (Valcarcel, Sanz, and Vázquez 2020). In this study, Valcarcel and colleagues produced FPH in 5 L glass reactors, mixing in a ratio 1:1 of by-products and water. A conventional alkaline chemical treatment using NaOH solution for pH control was used. Digestion was performed with Alcalase, and produced FHP was separated from the undigested portion and, subsequently, characterized. The study showed the presence of peptides of variable

size (between 1 and 10 kDa) rich in essential amino acids. The obtained peptides were tested for bioactivity showing good antioxidant and antihypertensive activities, and the improvement in umami flavors thanks to the presence of Glu, Gly, and Ala aminoacids, suggesting a potential nutraceutical application of the peptides (Valcarcel, Sanz, and Vázquez 2020).

Moreover, the eyeball can be used to produce hyaluronic acid. For example, Nile tilapia (*Oreochromis niloticus*) was used to extract hyaluronic acid with excellent physical/chemical properties (Alcântara et al. 2023).

Fishbone

Bones have several applications, for example they can be used as glues, toothpastes, or cosmetics (Olden et al. 2020). Moreover, other applications concern the collagen and gelatin production, as they are formed by structural proteins (Wasswa, Tang, and Gu 2007), up to a yield of 50% of the lyophilized dry weight (Nagai and Suzuki 2000). Fishbone was an important source for biomedical applications due to the presence of hydroxyapatite, in addition to collagen. Due to its tough structure, hydroxyapatite does not break or deform under physiological conditions, being thermodynamically stable at physiological pH, and it is implicated in bone bond formation. Hydroxyapatite is derived from natural materials such as coral and fishbone (Jensen et al. 1996). Interestingly, studies have been conducted to isolate and utilize hydroxyapatite from fish bones to replace synthetic hydroxyapatite (Ozawa and Suzuki, 2002). By heat treatment at high temperature, hydroxyapatite was extracted and, at the same time, the extremely high temperature gave strength to the hydroxyapatite structure (Boutinguiza et al. 2012; Venkatesan and Kim, 2010).

Fish viscera

Fish viscera are rich in proteins, lipids, and bioactive compounds. They are mainly used for the production of proteolytic enzymes, such as pepsin and trypsin, widely used in the food and pharmaceutical industries where they find their use as digestive enzymes. Studies have shown that these enzymes derived from fish guts were highly active even at low temperatures, making them ideal for eco-friendly industrial processes (Shahidi and Janak Kamil 2001; Atef and Mahdi Ojagh, 2017). Viscera is also rich in omega-3 fatty acids (EPA and DHA), and over time various extraction technologies have been developed, using processes such as rendering, pressing, microwave-assisted extraction,

supercritical fluid extraction, solvent extraction, autolysis, and hydrolysis (Bonilla and Hoyos Concha 2018; Ivanovs and Blumberga 2017). Among all, the most promising and eco-friendly methodology is supercritical carbon dioxide, allowing the extraction of compounds sensitive to heat and oxidation, such as polyunsaturated fatty acids (Rubio-Rodríguez et al. 2012; Jamalluddin et al. 2022). Furthermore, the viscera is a good raw material for the production of biogas through anaerobic digestion, a sustainable process that allows recovery of energy from organic waste, with high methane yields (Salam, Sarker, e Rahman 2009)

Fish Skin

Fish skin was a valuable resource due to its richness in collagen and other biomolecules. Widely used for the production of gelatin and marine collagen, as about 30-50% of the fish skin weight is collagen (Karayannakidis and Zotos, 2016). Moreover, fish skin is used in regenerative medicine where it is used for the treatment of burns or wounds, or as a biomaterial for xenotransplants. For example, tilapia skin was sterilized to create dressings that promote healing (Zimba, Rwiza, and Sauli 2024). Furthermore, fish skin could be used as a matrix for the production of bioactive peptides through enzymatic hydrolysis or other methods (Abuine, Rathnayake, and Byun 2019). Many other examples on fish skin applications will be discussed in the thesis.

Collagen

Collagen is the most abundant protein in nature. The collagen family comprises 28 members that contain a triple helical domain. Collagen is a protein required for the formation of the extracellular matrix, and also plays structural roles, contributes to the mechanical properties, organization, and shape of tissues (Ricard-Blum 2011), comprising roughly 20 – 30% of beast (Muller 2003; Stenzel, Miyata, e Rubin 1974). To date, 28 distinct types of collagen have been linked in advanced metazoans (Henriksen e Karsdal 2016)

These collagen types are distributed grounded on their primary structures and forms of supramolecular association. Fibrillar collagens (types I, II, III, V, XI, XXIV, XXVII) are involved in the conformation of cross-striated fibrils. In mature fibrillar collagen motes, the collagenous domain of each α chain features a repeating trinity sequence of Gly-X-Y. Fibrillar collagen (Type I) is the most current protein in animals, and is pivotal for the mechanical parcels of bones, tendons, and skin. Other subfamilies are non-fibrillar

collagens, which have various interruptions in their collagenous disciplines. Non-fibrillar collagens are further divided into several subfamilies (i) network- forming collagens, which include basement membrane collagen (type IV), rounded hair- forming collagen (type VI), anchoring fibril collagen (type VII), short- chain collagens (types VIII, X), which are hexagonal network- forming collagens; (ii) fibril- associated collagens with interrupted triadic helices (types IX, XII, XIV, XX); (iii) collagens with interrupted trio helix- suchlike structures (types XVI, XIX, XXI, XXII); (iv) tube membrane collagens (types XIII, XVII, XXIII, XXV); (v) multiplexing collagens (types XV, XVIII); and other motes with collagenous domains (types XXVI, XXVIII) (Coppola et al. 2020; J. Exposito et al. 2002; J.-Y. Exposito et al. 2010). Partial hydrolysis and thermal denaturation of collagen result in a gelatin product. Gelatin can be an important biopolymer used in different fields, in particular in food industries thanks to its characteristics to improve elasticity, consistency, and stability.

For gelatin production there are two methods used, the acid process to obtain gelatin type A, and alkaline process for the production of gelatin type B. Gelatin from fish waste was commonly obtained from skin and bones (Mariod and Fadul 2013). During gelatin production by hydrolysis, the inter and intra-molecular bonds of collagen filaments are broken (Cole and McGILL, 1988), and the structural characteristics of gelatin depend on the distribution of molecular weight (MW), structure, and composition of each subunity (Mariod and Fadul, 2013). Chemically, gelatin is made of 18 amino acids, where glycine, proline, and hydroxyproline are the most representative compounds (ca. 57%), the remaining 47% are formed by different amino acid families, such as glutamic acid, alanine, arginine, and aspartic acid (Alipal et al. 2021).

Hydrolyzed collagen can also be obtained by the action of enzymes, such as alcalase, papain, pepsin, and others, either in alkaline or acid media, at certain temperatures of incubation that are usually higher than 40 °C. The peptides performing have low molecular weight (0.3 – 8 KDa) (Edgar et al. 2018), and are fluently absorbed by the intestine. More recently, it has also been demonstrated that green and environment-friendly deep eutectic solvents can be employed for the extraction of collagen peptides (Bai, Wei, and Ren 2017).

Different techniques have been proposed to extract collagen from fish waste. Acid assist extraction is the most common system, to obtain the acid-soluble collagen (ASC). This process involves the use of acids, like acetic acid or HCl, which solubilize collagen chains. Is common to use acetic acid from 0.1 M to 0.5 M to extract collagen. Tan and

collaborators studied the extraction yields using different acids (AcOH, citric acid, hydrochloric acid, and lactic acid) at different pH, demonstrating that other acids could also be used for the extraction of collagen from skin (Tan and Chang 2018). In other cases, the collagen extraction was driven by enzymatic reaction. Generally, the most used method uses pepsin which can act on specific regions, promoting solubilization and increasing collagen yield; this collagen is known as pepsin-soluble collagen (PSC) (Jafari et al. 2020). For example, studies conducted on Nibea japonica showed that the collagen yields increased from 66.35% to 79.93% increasing pepsin from 800 to 1200 U/g, reducing also the duration of the extracted collagen (Yu et al. 2018).

Marine Collagen application

Marine collagen from fish waste is gaining increasing interest and is used in various fields, such as tissue engineering, food, pharmaceuticals thanks to its high biocompatibility and low cytotoxicity. Scaffold based on fish collagen finds wide use in tissue engineering, as they are useful in promoting, differentiation process, and in cell adhesion (Xu et al. 2020). For example, scaffold based on collagen from Tilapia fish promoted the cartilage production, both in vitro (using chondrocytes from rabbit auricle) and in vivo (in a rabbit model). In fact, cartilage repair in situ was tested in a rabbit articular defect model, where collagen demonstrated its ability to stimulate cartilage regeneration. Moreover, collagen extracted from tilapia skin showed no cytotoxic side effects in vitro, nor did it provoke an inflammatory response in a mouse model, demonstrating a strong capacity to promote cartilage regeneration in vivo in rabbits. Suzuki and colleagues used Tilapia scale collagen to create scaffolds to engineer oral mucosa. Oral mucosa keratinocytes were seeding onto microstructured tilapia scale collagen scaffolds. The authors also reported a fully differentiated and stratified epithelial layer developed on collagen scaffolds (Suzuki et al. 2020). Wharton gelatine was used also for the production of the electrospun membranes DWJM/PCL. Histological analysis showed lacunae structures and cartilage ECM deposition from tissue formation on the membranes, indicating that these membranes induce the formation of preliminary cartilage-like tissue in vitro (Xu et al. 2020). Moreover, biomimetic nanofibrous membranes were created for periodontal bone regeneration using grass carp scale collagen, hydroxyapatite, and chitosan. This biomimetic coating promoted the osteogenic differentiation of cells. In vivo studies also demonstrated that the membranes are biocompatible and promote cell adhesion, improving the formation of new periodontal

bone in rats (M. Li et al. 2024). Also, Hoyer and colleagues used collagen from the skin of Salmon (*Salmo salar*) to create porous scaffolds for bone regeneration. The scaffolds favored the adherence of mesenchymal cells increasing the cell number, and the differentiation response to the osteogenic stimuli (Hoyer et al. 2012).

Peptides and hydrolysates

Marine environment offers a huge source of bioactive compounds derived from proteins and peptides. Fish protein hydrolysates market size was about USD 420 million globally in 2019 and it is expected to increase at 4.5% CAGR between 2020 and 2026 (https://www.gminsights.com/industry-analysis/fish-protein-hydrolysate-market).

In the last 2 decades, studies relating to marine peptides and proteins have increased enormously, due to their interest in different fields, such as pharmaceutical, nutraceutical and in food industries, because these molecules showed a wide range of bioactivity, depending on their amino acid sequences and structures (Venkatesan and Kim 2010). Many proteins have been isolated from fish processing wastes. Hydrolysates and purified peptides have been isolated from several fish species (e.g. pollack, sole, salmon, skate, halibut, tuna, catfish, skate, crocker, turbot, and hoki), from whole-body fish wastes or from fish by-product, such as frame, scales, bones, head, gonads, and viscera as reported by (Harnedy and FitzGerald 2012; Atef and Mahdi Ojagh 2017).

Also other marine organisms, such as jellyfish and starfish, are valuable resources of peptides with different biological activities (Teng et al. 2023; Liu et al. 2013). De Domenico for example explored the potential of the jellyfish *R. pulmo* as a source of antioxidant peptides. In particular, fractions obtained, especially those with low molecular weight (<3 kDa) derived from hydrolyzed collagen, showed a high antioxidant activity. Furthermore, in vitro tests on human cells at controlled concentrations did not result toxic (De Domenico et al. 2019). The recent study conducted by Prommasith and collaborators showed bioactive peptides produced by enzymatic hydrolysis of jellyfish *Lobonema smithii* with high levels of immunomodulatory, anticancer, and antioxidant activities. It pointed out their potential applications in functional foods and/or therapeutic products because the peptides had the capability for immune response enhancement, reduction of proliferation of cancerous cells, and mitigation against oxidative stress. The

results suggested that jellyfish derived peptides would be valuable natural ingredients to be utilized in nutraceuticals (Prommasith et al. 2024).

Bioactive peptides are extracted through various methods, including acid-alkaline hydrolysis (using acidic or alkaline reagents), enzymatic hydrolysis (using enzymes to hydrolyze fish waste), and fermentation (using microorganisms as a source of enzymes) (Huang et al. 2015; Abuine, Rathnayake, and Byun 2019). Enzymatic hydrolysis is particularly popular in the food and pharmaceutical industries because the process leaves no residues of organic solvents or toxic chemicals (Kim and Wijesekara, 2010), and is considered the best technique to hydrolyze fish skin without compromising its nutritional value (Huang et al. 2015). Fermentation, another technique for producing peptides naturally, has been used for centuries as a traditional method of preservation, improving the taste of food and increasing its nutraceutical value (Nasri 2019). In literature, many biological activities attributed to peptides from marine species are reported (Zamora-Sillero, Gharsallaoui, and Prentice 2018; Jimenez-Champi et al. 2024; Ishak and Sarbon, 2018)

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Aim of the thesis

The aim of the PhD thesis is to develop a sustainable and low-cost pipeline for the production of high-value products, such as collagen and bioactive molecules, from waste biomass of the fish supply chain, including bycatch (jellyfish or starfish) and by-products of the fish processing industries (skin, bones, scales, fins). The effort is focused on two main focuses: i) the marine soluble-acid collagen extraction and its use for biotechnological applications. Moreover, the attention was also placed on the research for innovative methods, including mechanical treatments to increase the marine collagen yield and limit the consumption of solvents, making the process eco-friendly ii) the production of new bioactive molecules produced by enzymatic hydrolysis of by-catch species, evaluating the biological potential of the peptides through bioactivity assays. In details:

Chapters 1, 2, and 3 were focused on collagen extraction from fishery byproducts (e.g., fish skin) and by-catch species (e.g., jellyfish). Primarily, the characterization of the extracted marine collagen was performed through FTIR, X-ray, SDS-page analysis, showing the high degree of purity of the collagen obtained. The collagen was subsequently used for various biotechnological purposes that will be explained in the various chapters. Collagen extracted from the jellyfish *Rhizostoma pulmo* with a classical method was used for the creation of membranes based on marine collagen and methacrylate chitosan, showing good properties for liver tissue engineering applications (Chapter 2); Collagen extracted from sea bass (*Dicentrarchus labrax*) skin was used for the production of 3D scaffolds based on marine collagen/chitosan, showing an excellent scaffold for cell engraftment with excellent cytocompatibility and excellent mechanical functions for tissue engineering (Chapter 3). Furthermore, different mechanical treatments were tested to optimize the collagen extraction demonstrating that the sonication increases the extraction yield by about 10 times in oral arms jellyfish (Chapter 1).

Chapters 4, and 5 were focused on the production of hydrolysates from bycatch organism (e.g., jellyfish and starfish) and hydrolysates were tested for their bioactivity. The soluble and hydrolysed proteins obtained from the jellyfish *Cotylorhiza tuberculata* were fractionated based on their molecular weight. The fractions showed excellent antioxidant activity, especially for collagen peptides, and good antiproliferative activity (Chapter 4).

Moreover, hydrolysates from the starfish *Asterias rubens* were produced from raw starfish, deproteinized starfish, and demineralized starfish. The digested fractions were tested for their biological activity, showing good results for antioxidant activity, and some fractions showed antiproliferative activity on melanoma cells (Chapter 5).

CHAPTER 1

Development of a sustainable mechanical assisted process for the extraction of marine collagen

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1. Introduction

Collagen is the most abundant protein in nature, formed by three α chains involved in the formation of the triple helix with Gly-Xaa-Ya repeats called collagenous domain (J.-Y. Exposito et al. 2010). Twenty-eight types of collagens have been identified, distributed according to their structures and forms of supramolecular association. Fibrillar collagens (types I, II, III, V, XI, XXIV, XXVII) participate in the conformation of cross-striated fibrils. Type I collagen is the most abundant protein in invertebrates and is very important for the mechanical units of bones, tendons, and skin. On the contrary, the other subfamilies represent non-fibrillar collagens, which have several interruptions in their collagenous domains (J. Exposito et al. 2002; Coppola et al. 2020).

Collagen is one of the most abundant biomolecules in jellyfish. Generally, jellyfish are composed of about 95–98% water by weight, 2–3% salt, and the protein content is about 1.3%, almost all of which is collagen (Khong et al. 2018).

In the last decade, there has been a marked increase in jellyfish blooms caused by several factors, such as rising temperatures, changes in water mass and salinity, and eutrophication (J. Purcell, Uye, e Lo 2007; J. E. Purcell 2005). Intensive fishing activity also favours jellyfish blooms, depleting apex predators in the sea (J. E. Purcell 2005). Moreover, in the same way, the presence of jellyfish blooms, especially during the summer period, causes enormous damage to the fishing sector. Although the information

on the economic consequences of these events in the Mediterranean Sea is relatively limited, the basin is considered the second area most affected by this phenomenon, with about 18% of the global reports regarding the scyphozoans *Aurelia* sp./spp., *Rhopilema nomadica, Pelagia noctiluca*, and *Rhizostoma pulmo* (Bosch-Belmar et al. 2021). On the other side, gelatinous plankton represents a very important source of bioactive molecules that could be used in different fields, from nutraceutical, pharmaceutical, cosmeceutical to medical (De Domenico et al. 2019; Barzideh et al. 2014; Riccio et al. 2022; Ngo et al. 2011; Li et al. 2014).

Usually, the traditional method of collagen extraction from jellyfish involves a pretreatment consisting of the elimination of non-collagenous proteins from the tissue using sodium hydroxide (NaOH), while the subsequent extraction occurs through the use of large amount of 0.5 M acetic acid (Nagai et al. 2000; Barzideh et al. 2014). However, this classical approach is laborious, slow, and yields are extremely low (Barzideh et al. 2014). For this reason, it is important to find new approaches to increase the yield of collagen extracted and reduce, at the same time, the solvent amount used. For example, Khong et al. (2018) improved the collagen extraction method from the jellyfish *Acromitus hardenbergi*. In particular, different physical (e.g., pH adjustment), and mechanical (e.g., homogenization, sonication) pretreatments were added at the acid-assisted extraction, showing increased yield respect to traditional processes of collagen extraction.

In this study, we performed the collagen extraction from *R. pulmo* (Macri 1778) collected during a conspicuous bloom of the species. It is one of the most abundant jellyfish in the Mediterranean Sea (Fuentes et al. 2011), and its numerous interannual fluctuations of jellyfish blooms, especially in coastal areas, make it an excellent species for study, due to the abundant biomass available (Mills 2001; Lilley, Houghton, e Hays 2009).

In particular, the extraction was performed by adding different mechanical treatments, such as homogenization, sonication, freeze-drying, to find the most effective treatment to increase the extraction yield. Moreover, to obtain a more sustainable process, a low amount of acetic acid was also used.

2 Materials and Methods

2.1 Field work

Jellyfish were obtained from the by-catch analysis of commercial fishing activities held in small marinas of the Campania region (Morelli et al. 2024). In particular, the

Rhizostoma pulmo (Macri, 1778) specimens used in the present investigation were fished on the 24th September 2021 during a conspicuous bloom of the species, using trammel nets (1 m in height and ~ 550 m in length; net consisting of an inner panel of 4.8 cm stretched mesh between two panels of 25 cm stretched mesh) deployed on a sandy bottom off the Fusaro Lake mouth (40.8230◦ N, 14.0451◦ E). Soon after disentangling them from nets, jellyfish were stored in single plastic bags filled with seawater and soon transferred into cold storage containers to the Laboratory of Benthos-Napoli (Stazione Zoologica Anton Dohrn, Naples, Italy) for the subsequent laboratory work.

2.2 Jellyfish Acid Soluble Collagen Extraction (ASC) - Conventional method

The collagen was extracted from *R. pulmo* umbrella and oral arms. All procedures were carried out at 4°C according to the method described by (Morelli et al. 2024). Briefly, 80 g of fresh jellyfish umbrella and oral arms were cut into small pieces and used for the extraction separately. To remove non-collagenous proteins, jellyfish were soaked in 0.1 M NaOH with a tissue/solution ratio of 1:1 (w/v), O/N. The treated tissues were washed with water to reach the neutral pH, and then extracted with 0.5 M acetic acid ratio of 1:1 (w/v), stirred for 3 days (twice). The insoluble components were separated with cotton cloth, and the filtrate was centrifuged at 4 °C, 20000 x g, for 1 h. The obtained supernatant containing ASC was precipitated by adding NaCl to a final concentration of 0.9 M. The precipitate was collected by centrifugation at 4 °C, 20000 x g for 1 h. The pellet was dissolved in a minimum volume of 0.5 M acetic acid, and dialyzed against 0.1 M acetic acid, followed by distilled water. The dialysate was freeze dried, and the dry collagen was used for subsequent characterization.

2.3 Jellyfish Acid Soluble Extraction (ASC) - Additional pretreatments

To remove non-collagenous proteins, jellyfish Umbrella and Oral arms were absorbed in 0.1 M NaOH with a tissue/solution ratio of 1:1 (w/v) O/N for each sample and then different pretreatments were used before collagen extraction.

2.3.1 Sonication

Fresh jellyfish umbrella and oral arms (80 g) were extracted as in the paragraph 2.2, with a modification: when the tissues were soaked in 0.5 M acetic acid (tissue/acid solution ratio of 1:1 (w/v)), the mixture was ultrasonicated using an ultrasound (BANDELIN HD 2200) with a radius tip probe (microtip MS 73). The reaction was carried out working at

a single frequency of 20 kHz. To reduce the sample overheating an ice bath was used to maintain the temperature at 4°C. The ultrasound was operated in a pulse mode at 30 sec acting and 10 sec resting time. The treatment was conducted at one level of amplitude 50% for 15 minutes for each sample. After the sonication, the collagen extraction was carried out as previously described in the paragraph 2.2.

2.3.2 Freeze-Dry

Fresh jellyfish umbrella and oral Arms (80 g) were frozen at -80°C and then freeze-dried for 2 days. The lyophilized tissue (1 g) was used for the collagen extraction, as in the paragraph 2.2.

2.3.3 Homogenization

Jellyfish umbrella and oral arms (80 g) were soaked in 0,5 M acetic acid with a tissue/acid solution ratio of 1:1 (w/v) and homogenate using an Homogenizator (AN GNI Lab High Shear Dispersion Homogenizer, M/N- AD300L-H) for 5 min. The homogenized tissues were extracted in 0,5 M acetic acid with a tissue/acid solution ratio of 1:1 for 3 days, and the collagen recovery was performed as described for a conventional method (see paragraph 2.2).

2.4 ATR-FTIR

Fourier-transform infrared (FTIR) spectroscopy in attenuated total reflectance (ATR) mode, using jellyfish umbrella and oral arms collagens, was performed (Thermo Fisher Nicolet IS10, Waltham, MA, United States of America) between 4500 and 500 cm^{-1} with a resolution of 2 cm^{-1}, with each spectrum being the average of 32 scans, to evaluate the presence of collagen's characteristic chemical bounds/groups.

2.5 SDS-PAGE

Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis (SDS–PAGE) was performed by the method of (Laemmli 1970) with modification (Morelli et al. 2024). ASC approximately 10 µg/µL were dissolved in 50 µl of acetic acid, then 50 µl Tris–HCl, pH 8,8 containing 4% SDS were added. The samples were heated at 50°C for 5 min using a thermoblock. Solubilised samples were mixed at 1:1 (v/v) ratio with sample buffer (0.5 M Tris–HCl, pH 6,8, 5% SDS, 20% glycerol, 10% 2-mercaptoethanol), and 10 µl of sample was loaded. Samples were loaded onto a polyacrylamide gel made of

7.5% separating gel and 4% stacking gel, and subjected to electrophoresis at a constant current of 120V. For the visualization of the bands, Spectra Multicolor High Range Protein Ladder (Thermo Fisher Scientific, MA, USA) was used as Marker; the gel was stained with Coomassie Brilliant Blue; Calf skin collagen was used as a standard.

3. Result and discussion

3.1 Jellyfish collection

The barrel jellyfish *Rhizostoma pulmo* is a rhizostome scyphomedusae mostly widespread in the Mediterranean Sea, where it is among the largest invertebrates living in the basin, with a weight that may exceed 10 kilograms in its medusoid form. This form is characterized by a hemispherical umbrella that can be about 60 cm in diameter (exceptionally 90 cm), sometimes higher than broad, whose periphery is deprived of tentacles, replaced by approximately 80–90 lobes distributed on the entire circumference. Under the umbrella, the manubrium is short and massive and has eight long, fused, and unbranched oral arms, whose upper parts have a cauliflower shape, while the ends are smooth and club-like. The colour is usually pale grey to colourless but may also be opalescent-pink milky, while the lobes of the umbrella are blue-purple.

As many jellyfish species, adults of *R. pulmo* occupy a key ecological niche as planktonic predators, feeding mainly on copepods, dinoflagellates, ciliates, and fish eggs (Leoni et al. 2022; Dönmez e Bat 2019). However, before becoming full adults, the species undergoes a complex life cycle that alternates between pelagic and benthic phases, and in general the species is characterized by both sexual and asexual reproduction (Fuentes et al. 2011), with the medusoid form being prominent during the summer seasons and estimated to be one of the most abundant zooplanktonic taxa in both coastal and open-sea environments (Leoni et al. 2021; Fuentes et al. 2011; Reyes Suárez et al. 2022; Pérez-Ruzafa et al. 2002). Despite its rather harmless envenomation potential for humans, the always recurrent outbreaks observed in the last decades in the Mediterranean waters, with extremely large biomass populations, represent a nuisance or a damage for many sea-based human activities (Pagès 2001; Leone et al. 2015; Bosch-Belmar et al. 2021). These mostly include fishery and aquaculture and tourism and leisure, and result in additional management costs to be faced by national and local authorities during the summer seasons. As an example, they affect the fishery and aquaculture activities by reducing the local fish stocks through competition for food or direct predation, clogging nets, and deteriorating the catches, or even by producing mass mortalities in caged fishes; at the

same time, local authorities often set up permanent nets off the main beaches or convert the usual activities of the artisanal fleets into jellyfishing with pelagic nets as to avoid a decrease in visitors and tourists (Pagès 2001; Leone et al. 2015; Bosch-Belmar et al. 2021). Also in the present investigation, jellyfish were collected during a conspicuous bloom of the species, which clogged the nets of the local fishermen. Biometric and average biomass data of the individuals worked here are reported in Table 1.

Table 1. Biometric measures and fresh weights (in grams) of the *R. pulmo* specimens (n = 11) used in the present investigation. Data are reported as range and mean ± standard deviation (SD).

	Umbrella diameter	Umbrella weight	Oral arms weight	Total weight
Range	15–30	153–1258	61–502	214–1742
Mean ± SD	24.04 ± 4.17	679.55 ± 320.74	339.64 ± 152.49	1019.18 ± 455.30

3.2 Marine Collagen Yield

Fresh oral arms and umbrella from *R. pulmo* collected from fisheries by-catch were used to evaluate the amount of collagen extracted by adding different mechanical pretreatments to the traditional process of ASC extraction. In detail, all samples were treated with a 0,1M NaOH solution O/N to eliminate non-collagenous proteins, as the alkalinization is common in many extraction protocols (Coppola et al. 2020). Moreover, mechanical treatments were applied. Therefore, the approaches used include: i) traditional extraction: the extraction was performed by the conventional method, without the addition of any mechanical pretreatment; ii) sonication: samples were sonicated for 15 min as sonication seem to improve the extraction efficiency by breaking the cell structure and making soluble compounds more accessible thanks to the cavitation and shocks created by sonication. Therefore, the sonication causes a collapse of the tissue structures favoring the recovery of the collagen (Khong et al. 2018); iii) freeze-drying: the samples were lyophilized up to complete drying. This treatment was also tested to allow easier conservation of the collected jellyfish, so as not to have to use them for the collagen extraction immediately after their collection; iv) homogenization: homogenization was performed for 5 min. This treatment was included as it allows to obtain a homogeneous dispersion of the tissues favoring the contact with the extraction solvent (Roseboom et al. 2020).

After these treatments, all samples were extracted by using 0,5 M acetic acid, in a ratio 1:1 (v/w) for 3 days, in order to use low amount of solvent for more eco-friendly extraction (Morelli et al., 2024).

The extraction yields, expressed as mg of extracted compound per gram of tissue (mg/g), are reported in the Figure 1 and Table 2; they vary significantly depending on the type of tissues and the treatment applied.

For example, the collagen extraction yield from *R. pulmo* umbrella varied from 1.2 to 2.0 mg/g using the conventional process. Moreover, the yield was similar (1.0-2.0 mg/g) by adding freeze-drying and homogenization as pretreatment. On the contrary, the yield of the collagen extracted from the jellyfish umbrella increased significantly to 9.4-13 mg/g adding the sonication as pretreatment.

Interestingly, the amount of collagen extracted from *R. pulmo* oral arms was greater than that obtained from umbrella, in all methods used. The collagen yield ranged from 4.2 to 8.0 mg/g by using the traditional method, while it reached 30 mg/g by adding the sonication as pretreatment, the highest value observed in the entire experiment. On the contrary, the lowest yields of collagen extracted from oral arms were obtained by using freeze-drying (1.5 mg/g) and homogenization (1-2 mg/g), values similar to the yield obtained from *R. pulmo* umbrella.

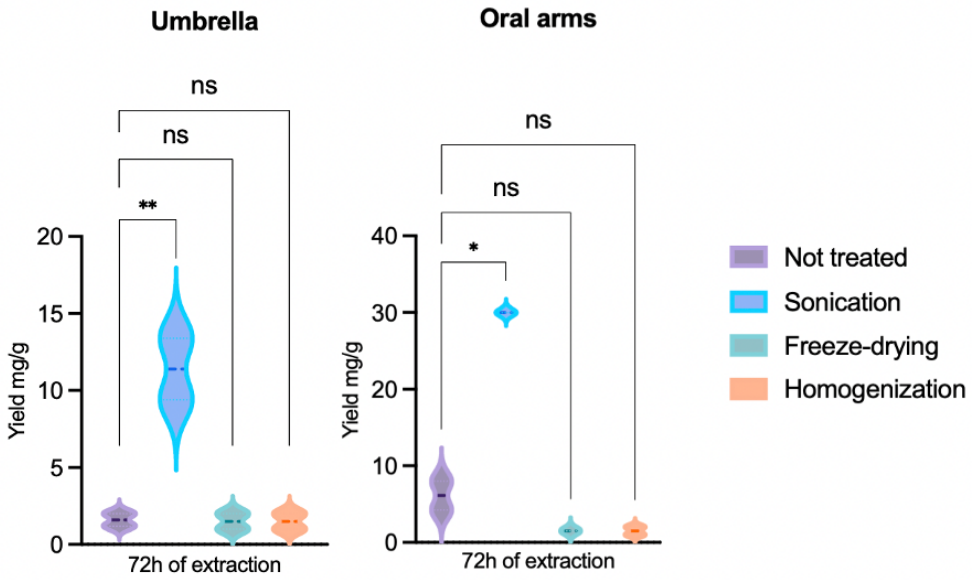

Figure 1. Violin plot of the extraction yields expressed in mg/g of umbrella and oral arms after 72h of extraction. The data show the minimum, maximum, and the average of the collagen extracted using different pretreatments ± standard deviation. Statistical analysis

by ANOVA ordinary one-way Dunnett's MULTIPLE COMPARISON 0.1234 (ns), 0.0332 (*), 0.0021 (**).

Table 2. Yields reported in mg/g of the collagen extracted from umbrella (U) and oral arms (OA), using different pretreatment.

Fresh Jellyfish (80 g)	Chemical pretreatment	Mechanical pretreatment	Extraction	Yield
U	NaOH 0,1M O/N	-	CH3COOH 0,5M 1:1 (V/W) 3 days	1,2 – 2.0 mg/g
U	NaOH 0,1M O/N	SONICATION	CH3COOH 0,5M 1:1 (V/W) 3 days	9,4 – 13,4 mg/g
U	NaOH 0,1M O/N	FREEZE-DRYING	CH3COOH 0,5M 1:1 (V/W) 3 days	1.0 – 2.0 mg/g
U	NaOH 0,1M O/N	HOMOGENIZATION	CH3COOH 0,5M 1:1 (V/W) 3 days	1.0 – 2.0 mg/g
OA	NaOH 0,1M O/N	-	CH3COOH 0,5M 1:1 (V/W) 3 days	4.2 – 8.0 mg/g
OA	NaOH 0,1M O/N	SONICATION	CH3COOH 0,5M 1:1 (V/W) 3 days	30.0 mg/g
OA	NaOH 0,1M O/N	FREEZE-DRYING	CH3COOH 0,5M 1:1 (V/W) 3 days	1.5 mg/g
OA	NaOH 0,1M O/N	HOMOGENIZATION	CH3COOH 0,5M 1:1 (V/W) 3 days	1.0 – 2.0 mg/g

Therefore, the results showed that sonication is the most effective treatment to improve the collagen extraction yield, especially for the *R. pulmo* oral arms extraction, for which it reached a maximum yield of 30 mg/g compared to 4.2-8 mg/g obtained by the traditional collagen extraction, without adding any mechanical treatment. However, the yield was also significantly increased during the extraction of umbrella collagen, where the collagen yield obtained after sonication was 9.4-13 mg/g compared to 1.2-2 obtained in absence of mechanical treatment. These results were consistent with previous studies reporting that sonication has positive effects on collagen extraction (Ata, Kumcuoglu, and Tavman 2022; Khong et al. 2018).

On the contrary, freeze-drying and homogenization do not bring significant improvements in collagen yield from *R. pulmo* compared to the conventional method, without mechanical treatments, and in some cases they even reduced the yield to 1 mg/g.

3.3 Collagen characterization

Type I collagen is the most common type of collagen, abundant in the vertebrate skin, and the triple helix structure of type I collagen is stabilized by interchain hydrogen bonds (Henriksen and Karsdal 2024; Addad et al. 2011). SDS-PAGE analysis of collagens extracted from *R. pulmo* by different methods is reported in Figure 2. The collagen obtained by the conventional method (VII, VIII) shows characteristic heterodimer bands of α1 chains and one α2 chain of type I collagen, with molecular weight and electrophoretic mobility similar to those previously reported for *R. pulmo* (Addad et al. 2011; Morelli et al. 2024). It was noteworthy that the bands show a different electrophoretic mobility compared to calf skin collagen, probably due to the lower content of hydroxyproline, proline, glycine, and glutamic acid residues compared to mammalian type I collagen (Paradiso et al. 2019; Morelli et al. 2024). Further classical bands at molecular weight around 280 kDa and above 300 kDa were visible, and can be assimilated to the dimer α 1 and α 2 chains together or two α 1 chains for the β chain and trimers of α chains for the γ chain, respectively (Akkus, Belaney, e Das 2005).

For the collagen obtained by adding the homogenization (III, IV), although the α1 and α2 bands are visible at the same electrophoretic mobility of the collagen extracted by the traditional method, the α1 chain is less marked. Moreover, there is a greater presence of higher molecular weight bands.

For sonicated collagen (I, II) and freeze-dried collagen (V, VI) the bands were not distinguishable and the lanes were almost empty. This was due to problems of solubility of the samples. In particular, the viscosity of the collagens was extremely increased, and the samples were gelatinous, showing altered conditions of suppleness.

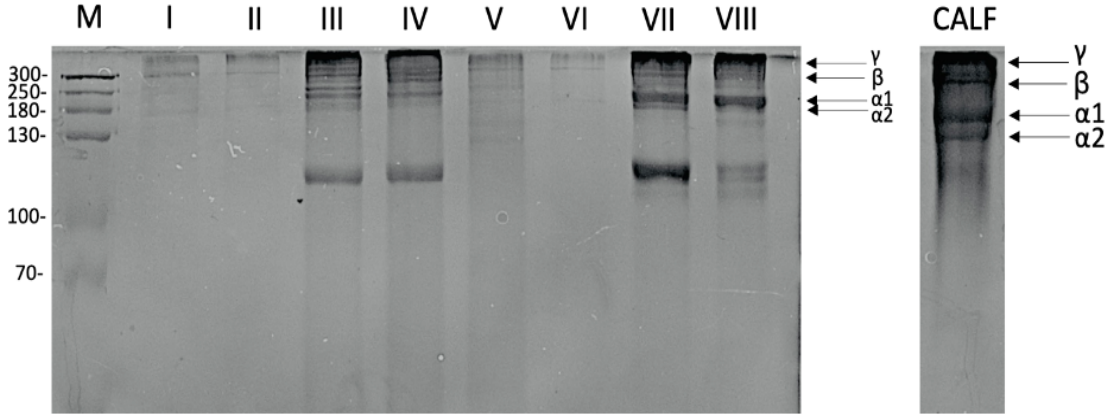

Figure 2. SDS–PAGE (7.5 %) electrophoretic pattern for acid-soluble collagen from *R. pulmo* umbrella and oral arms, extracted by different methods, in presence or absence of

mechanical pretreatments. (M) Molecular weight marker; I) umbrella sonicated II); oral arms sonicated; III) umbrella homogenized; IV) oral arms homogenized; V) umbrella lyophilized; VI) oral arms lyophilized, VII) umbrella without mechanical pretreatments; VIII) oral arms without mechanical pretreatments; (Calf) calf skin collagen, type I.

3.3 ATR-FTIR

The collagens extracted from *R. pulmo* umbrella and oral arms with different protocols were analysed by ATR-FTIR for their secondary structure. The spectra of all collagens ((Figure 3 a) and b) showed similar trends to literature by showing similar bands of type I collagen. These include the main absorption bands of amide A, amide B, amide I, amide II, amide III (Kittiphattanabawon et al. 2005). Amide A band is associated with N–H stretching vibration which occurs in the range 3200–3400 cm−1 and indicates the presence of hydrogen bonds. Amide B band is related to the asymmetrical stretch of CH2 stretching vibration and is observed at ~2928 cm−1. Drastic differences in Amide A and B peak positions among the different collagens were not observed. However, amplitude differences of the Amide A and Amide B found in the different collagens indicated that the secondary structure of collagen could possibly be different. The Amide I peak is associated with C=O stretching vibration or stretching or possibly hydrogen bonding coupled with COO– (1600–1700 cm−1) and is thought to be a sensitive marker for the secondary structure of proteins. For all collagens, Amide I is very prominent and sharp. The Amide II band is associated with N–H bending vibration coupled with C–N stretch (1540–1560 cm−1), indicative of the N–H group being involved in hydrogen bonding. The intensity of some collagens was less than other collagens for Amide II peak, which could possibly indicate less involvement of N–H groups involved in hydrogen bonding and thus a lower stability of triple helix. Amide III is associated with C–N stretching vibration with N–H bend and C–O stretching. Amide III peak (1200 cm-1) and intensity is an important characteristic of collagen. Generally, for collagen, the absorption intensity ratio between Amide III band and the 1450 cm−1 band (CH2 bend) should be 1 for triple helix conformation and 0.5 is usually observed for gelatine. The absorption intensity ratio between Amide III band for all collagens ranges between 0.93 and 0.95. All absorption intensity ratios were way above 0.5, this indicates that the triple helix of all collagen sources was preserved.

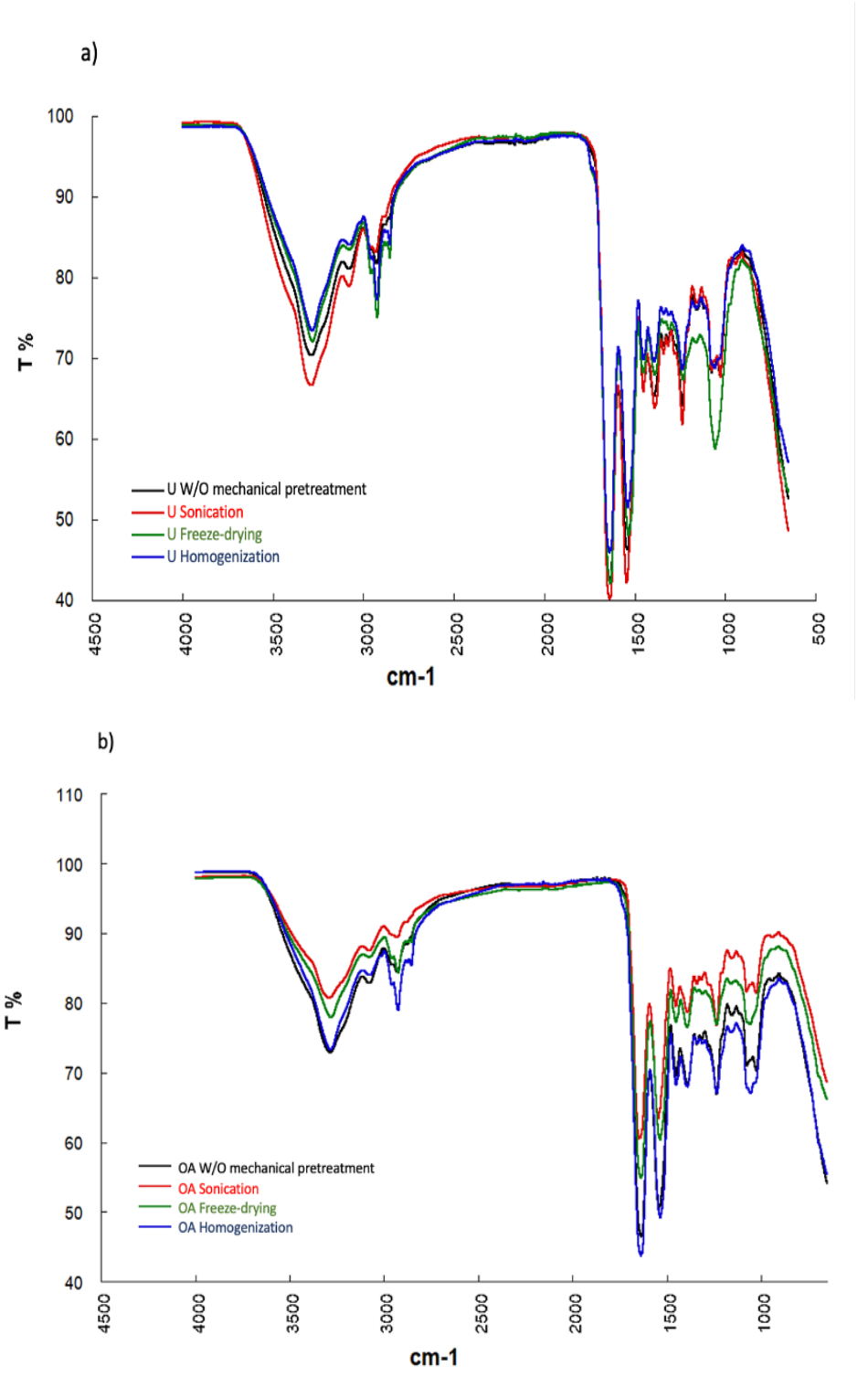

Figure 3. ATR-FTIR spectra of *R. pulmo* collagen extracted from a) umbrella (U) and b) oral arms (OA), by different extraction methods.

Table 3 $\Delta\nu=\nu_{I}-\nu_{II}$, amide III absorbance and absorpition ratios of the collagen sample.

Sample	$\Delta\nu=\nu_{I}-\nu_{II}$	Absorbance		1235 cm-1: 1450 cm-1 ratio
		1235 cm-1	1450 cm-1	
U W/O Mechanical treatment	99	0,64	0,67	0,95
U Sonicated	96	0,62	0,66	0,93
U Freeze-drying	108	0,67	0,68	0,98
U Homogenizated	96	0,68	0,70	0,97
OA W/O Mechanical treatment	91	0,67	0,7	0,95
OA Sonicated	102	0,77	0,79	0,97
OA Freeze-drying	94	0,77	0,78	0,98
OA Homogenizated	106	0,67	0,68	0,98

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CHAPTER 2

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Methacrylated chitosan/jellyfish collagen membranes as cell instructive platforms for liver tissue engineering

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ABSTRACT

Although the multidisciplinary area of liver tissue engineering is in continuous progress, research in this field is still focused on developing an ideal liver tissue template. Innovative strategies are required to improve membrane stability and bioactivity.

In our study, sustainable biomimetic membranes were developed by blending methacrylated chitosan (CSMA) with jellyfish collagen (jCol) for liver tissue engineering applications.

The *in vitro* biological behaviour demonstrated the capability of the developed membranes to create a suitable *milieu* to enable hepatocyte growth and differentiation. The functionalization of chitosan together with the biocompatibility of marine collagen and the intrinsic membrane properties offered the ideal biochemical, topographical, and mechanical cues to the cells. Thanks to the enhanced CSMA/jCol membranes' characteristics, hepatocytes on such biomaterials exhibited improved growth, viability, and active liver-specific functions when compared to the cell fate achieved on CSMA membranes. Our study provides new insights about the influence of membrane properties on liver cells behaviour for the design of novel instructive biomaterials. The enrichment of functionalized chitosan with marine collagen represents a promising and innovative approach for the development of an appropriate platform for hepatic tissue engineering.

1. Introduction

The creation of biomimetic materials for the regeneration and repair of injured tissues and organs remains a challenging issue in biomedical field. Specially, the regulation of specific biomaterial characteristics in the development of ideal tissue and/or organ analogues and their bioactivation through signals, capable of directing cell and tissue fate, represent a crucial challenge in tissue engineering. Membrane-based approaches are successfully employed for the creation of various functional tissue-engineered constructs [1–3]. Polymeric membranes are suitable material for liver tissue engineering owing to their intrinsic characteristics of high selectivity and permeability together with their ability to be easily scaled-up with modulated structural, physicochemical and mechanical properties. It is widely recognized that membranes offer specific cues to hepatocytes for recapitulating the in vivo *milieu* of natural microenvironments, driving three-dimensional (3D) liver tissue formation [4–6]. However, concerted efforts towards innovative strategies and approaches are still needed to improve membrane performance in terms of stability and bioactivity aiming at developing an ideal functional liver tissue for biomedical applications. Natural polymers, such as collagen, and chitosan (CS), exhibit several attractive features for the hepatic tissue regeneration. Their combination offers at the

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cellular interface a proper microenvironment that resembles the natural liver niche. CS is the most common naturally occurring cationic polymer that comes from the *N*-deacetylation of chitin, the second most prevalent biopolymer found in nature. CS has a large number of uses in tissue engineering due to its own features like biodegradability, nontoxicity and biocompatibility. It has a structural similarity to glycosaminoglycans, which are components of the liver extra cellular matrix (ECM), making it an efficient biomaterial for liver tissue engineering [7]. Nevertheless, CS must be modified to balance its hydrophilic character and poor stability before it can be potentially used in tissue engineering. These modifications can be effectively achieved in CS considering that it has many functional groups, including hydroxyl (OH) and amino (NH_2) groups. Hence, its structure can be precisely controlled by physically or chemically crosslinking of the polymer chains. Both strategies show peculiar drawbacks and limitations. Indeed, physical crosslinking does not allow obtaining biomaterials with appropriate structural stability, in high or low pH environments, and controlled biodegradation rates. On the other side, chemically crosslinking is potentially cytotoxic, due to possible presence of unreacted reagents entrapped in the system. Furthermore, it is widely reported that common crosslinkers, such as formaldehyde and glutaraldehyde, are not cell friendly. To overcome these disadvantages, and to produce strong and durable biomaterials, photocrosslinking has emerged as an alternative chemical crosslinking approach in which chemically modified polymer derivatives, such as methacrylated chitosan (CSMA), without using hazardous chemical crosslinkers, are employed [8]. Moreover, the functionalization reaction can occur quickly, at room temperature, and physiological pH [9].

In this work, the idea was to functionalize CS with methacrylic anhydride (MA), obtaining a CSMA polymer that could be photocrosslinked by activating the carbon–carbon double bond using Irgacure 2959 as an initiator to create a free radical under ultraviolet (UV) light. The aim was to improve the stability and better tune the degradation time, providing a more cohesive and stable material. Furthermore, in the recent years, polymer blending is one of the most successful methods for providing suitable polymeric materials with desirable properties for specific tissue engineering applications. Many polymers have been used to improve the properties of CS-based biomaterials. Among them, collagen is particularly interesting as it represents the main structural component of the ECM in all connective tissues and interstitial tissues of the parenchymal organs. Collagen has excellent mechanical strength and viscoelasticity, which allow it to be involved in several biological activities of the human body, such as cell proliferation, migration, and adhesion. This biomaterial has attracted the interest of researchers as it has excellent biocompatibility, low antigenicity, biodegradability, hydrophilicity, and cell adhesive properties for soft tissue engineering applications. Recently, marine organisms have drawn attention as biocompatible, eco-friendly, and safe sources of collagen. Marine collagen removes the risk of transmissible diseases, overcomes the limits of religion and, at the same time, exhibits high similarity in morphology and function with mammal collagen [10]. The growing attention to possible uses of fishing discards, including fishing by-catch as jellyfish, plays an important role in economic growth and sustainable development, as they represent a rich and eco-friendly source of value-added compounds [11]. Many studies have shown that the jellyfish organic matter consists mainly of collagen [12]. Among different species already studied, the scyphomedusan *Rhizostoma pulmo* from the Mediterranean Sea presents high collagen content compared to other jellyfish [13]. Furthermore, the increase in water temperature as a result of global climate change causes seasonal blooms resulting both in direct and indirect negative impacts and an increase in plentiful supply. Studies obtained so far on jellyfish collagen have shown that it exhibits greater cell viability to osteoblasts and fibroblasts compared to other biomaterials, including bovine collagen, as reported by Song et al. [14], probably due to some differences in their amino acid composition. Furthermore, it displays low antigenicity and intrinsic ability to trigger immune reaction in <1 % of the general population [15].

Jellyfish collagen (jCol) was extracted from *R. pulmo* obtained from fishery by-catch, to establish a sustainable process in line with the zero-waste and circular economy procedures. Previous studies have shown that the *R. pulmo* collagen shows a high degree of similarity to the mammalian type I collagen [16] and shares important characteristics with it, such us the ability to support two dimensional (2D) and 3D cell cultures.

Taking advantage of the properties of each compound, namely CSMA and jCol, and to reduce specific limitations of each single material, such as poor mechanical stability, inappropriate degradation rate and wettability, we explored new sustainable bioactive composite membranes for the creation of an in vitro liver tissue analogue. A CSMA membrane and two blend membranes with different percentages of polymers were prepared by phase inversion technique. The membranes were fully characterized to assess their morphology, roughness, wettability, biodegradation, and mechanical properties, which play an important role in guiding hepatocyte differentiation and maintenance of specific functions.

The effects of the different composition-related properties of the developed membranes were analysed on liver cell behaviour by culturing embryonic liver cells up to 18 days. Within the membrane systems cell morphology, viability and functional hepatic differentiation were analysed; albumin production, glycogen storage, and expression of liver specific markers were assessed to address how changes in membrane composition could affect membrane properties, which strongly influence hepatic growth and differentiation. We investigated the role of marine collagen in dictating membrane characteristics and its efficiency in inducing liver tissue regeneration with the final goal to demonstrate the potential of these sustainable biomimetic membranes as new generation of membranes with excellent comprehensive performance.

Research on membranes developed by the enrichment of CSMA with marine collagen to provide suitable hepatic tissue microenvironment for functional hepatocytes is still ongoing. Thus, our research establishes new perspectives in this field. Furthermore, the main novelty of the work is that, for the first time, two techniques—solvent casting for the synthesis of membranes, and photocrosslinking—were coupled as a promising and innovative approach for the creation of novel instructive biomaterials for liver tissue engineering applications.

2. Materials and methods

2.1. Raw material collection

Jellyfish were obtained from the by-catch of commercial fishing activities held in the marinas of Monte di Procida and Mondragone (central-western Mediterranean Sea). In particular, *Rhizostoma pulmo* (Macri, 1778) specimens were collected in September 2021 and June 2022 using trammel nets (1 m in height and ~ 550 m in length; net consisting of an inner panel of 4.8 cm stretched mesh between two panels of 25 cm stretched mesh) fishing passively on a sandy bottom off the Fusaro Lake mouth (40.8230° N, 14.0451° E) and off the Agnena river mouth (41.0743° N, 13.8988° E). Soon after disentangling them from nets, jellyfish were placed into cold storage containers in single plastic bags filled with seawater, and soon transferred to the Laboratory of Benthos-Napoli (Stazione Zoologica Anton Dohrn, Naples, Italy) for subsequent laboratory work.

2.2. Collagen extraction and characterization

All steps of the acid-soluble collagen extraction were carried out on fresh jellyfish, at 4 °C, according to the method described by Addad and collaborators [16], with slight modifications. Briefly, the umbrella and oral arms of each specimen were separated to perform the extraction separately and cut into small pieces. To remove non-collagenous proteins, jellyfish pieces were washed with distilled water and soaked in 0.1 M sodium hydroxide (NaOH, Sigma Aldrich, Milan, Italy) with a wet

tissue/solution ratio of 1:1 (*w/v*) overnight, under continuous stirring. The treated tissues were washed with distilled water until neutral pH was reached and then extracted with 0.5 M acetic acid (CH_3COOH, Sigma Aldrich, Milan, Italy) (1:1 w/v) and stirred for 3 days, twice. The insoluble components were separated with cotton cloth and the filtrates were centrifuged at 20000 xg for 1 h. The acid-soluble collagen was precipitated by adding sodium chloride (NaCl, Sigma Aldrich, Milan, Italy) to a final concentration of 0.9 M. The precipitate was collected by centrifugation at 4 °C, 20000 xg for 1 h. The pellet was dissolved in a minimum volume of 0.5 M CH_3COOH, and dialyzed against 0.1 M CH_3COOH first, subsequently against distilled water, then lyophilised for subsequent uses.

Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis (SDS–PAGE) was performed by the method of Laemmli, with modifications. 1 mg of jCol was dissolved in 100 μL of CH_3COOH, then mixed with 100 μL Tris (hydroxymethyl) aminomethane (THAM) hydrochloride (Tris–HCl, Sigma Aldrich, Milan, Italy), pH 8.8 containing 4 % SDS. The samples were heated at 50 °C for 5 min. Solubilized samples were mixed with the sample buffer 2× (0.5 M Tris–HCl, pH 6.8, 5 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol) and 10 μL of each sample were loaded onto a polyacrylamide gel (7.5 % separating gel and 4 % stacking gel) and subjected to electrophoresis, at 140 *V*. Coomassie Brilliant Blue R-250 was used for the visualization of the bands. Spectra Multicolor High Range Protein Ladder (Thermo Fisher Scientific, MA, United States) was used as protein marker and the type I collagen from Calf skin (Sigma Aldrich, MA, United States) was used as standard.

Fourier-transform infrared (FTIR) spectroscopy in attenuated total reflectance (ATR) mode, using lyophilized jCol samples, was performed (Thermo Fisher Nicolet IS10, Waltham, MA, United States of America) between 4500 and 500 cm^{-1} with a resolution of 2 cm^{-1}, with each spectrum being the average of 32 scans, to evaluate the presence of collagen's characteristic chemical bounds/groups.

X-Ray diffraction analysis (XRD) was exploited to examine the crystal structure of extracted jCol. The lyophilized jCol sample was scanned using an XRD instrument (XPERT-PRO, Malvern Panalytical, United Kingdom) with Cu Kα radiation (λ = 1.54060 Å). Voltage and current were fixed at 40 kV and 30 mA, respectively. The scanning range was set to be between 10° and 80° (2θ) in a fixed time mode at room temperature, meanwhile voltage and current were 0 kV and 30 mA, respectively.

2.3. CSMA synthesis and characterization

CSMA was synthesized according to literature with minor modifications [8]. Briefly, high molecular weight CS (1.5 g) (HM_wCS, M_w - 310,000-375,000 Da, degree of deacetylation >75 %, Sigma Aldrich, Milan, Italy) was first dissolved in 4%*v*/v CH_3COOH (100 mL) overnight at 40 °C under magnetic stirring. 4.6 mol methacrylic anhydride (MA, Sigma Aldrich, Milan, Italy) per mole of CS repeat unit, which is equivalent to 6 mL, were slowly added to the solution. The mixture was maintained at 40 °C, under magnetic stirring, for 12 h, protected from light. Afterwards, the products were redispersed in deionized water (for chromatography LC-MS Grade, conductance at 25 °C ≤ 1 μS/cm, Sigma Aldrich, Milan, Italy), purified by dialysis against distilled water for five days, using dialysis membrane tubes (12–14 kDa molecular weight cut-off; Sigma Aldrich, Milan, Italy) to ensure complete removal of MA and CH_3COOH. During the dialysis, distilled water was refreshed at least twice a day. The material was lyophilized (LaboGene's CoolSafe 55–4 PRO, Bjarkesvej, Denmark) and stored at −80 °C for further use.

^{1}H Nuclear magnetic resonance (^{1}H NMR, Bruker AVIII 400HD, Fällanden, Swiss) was employed to evaluate the functionalization reaction's effectiveness. CS and CSMA (5 mg/mL) were completely dissolved in deuterium oxide (D_2O) acidified with 30 μL trifluoroacetic acid (Sigma Aldrich, Milan, Italy) and allowed to be dissolved overnight at room temperature using a vortex mixer. Afterwards, they were transferred into NMR tubes and analysed at a frequency of 400 MHz. Phase and baseline corrections were applied before elaborating data.

ATR-FTIR spectroscopy was employed to assess the CS functionalization postmethacrylation reaction. CS and CSMA were scanned between 500 and 4500 cm^{-1} with a resolution of 2 cm^{-1}. Data were analysed on the base of the average of six scans per spectrum.

2.4. Preparation of CSMA/jCol membranes

Membranes were prepared in flat configuration by the inverse phase technique. To obtain the membranes, 2 % *w/v* CSMA was dissolved in a CH_3COOH solution (1 M). Polyethylene glycol (PEG, MW 6000 Da; Merck, Italy) at a 4:1 ratio, and Irgacure 2959 (Sigma Aldrich, Milan, Italy) (5 % w/w_{CSMA}) were added to the CSMA solution, as porogen and photocrosslinker, respectively.

Similarly, CSMA/jCol composite membranes were obtained by varying collagen content into the CSMA solution. Briefly, jCol was dissolved in CH_3COOH (0.5 N) to give a final concentration of 2 mg/mL. The collagen solution was added to the CSMA solution, prepared as described above, by mechanical stirring until a good collagen dispersion was obtained. The collagen content was adjusted to the desired percentage. Two blend membranes with different volume percentages (% *v*/v) of polymers, namely 70 % CSMA and 30 % jCol (CSMA/jCol30), and 50 % CSMA and 50 % jCol (CSMA/jCol50) were produced by mixing the corresponding polymeric solutions. The CSMA and CSMA/jCol solutions with different collagen concentration were cast into a petri dish (Ø =90 mm) and dried at room temperature, until complete solvent evaporation. The membranes were neutralized by immersion in a 1 % NaOH solution, and then washed with distilled water.

All membranes were photocrosslinked by using UV-KUB 2 system (λ: 365 nm, P: 1 J/cm^2) (Kloe, France). Lastly, the membranes were sterilized using UV light in a laminar flow chamber for 30 min.

2.5. Characterization of membranes

The morphology of membranes coated with graphite was examined using a High-Resolution Scanning Electron Microscope (HRSEM mod CrossBeam 350 ZEISS – Germany) @ 5 K*V*olt of energy. Images were acquired with secondary electron signal (SE).

The surface topography was characterized by atomic force microscopy (AFM) using a Multimode VIII equipped with a Nanoscope V controller (Bruker, Santa Barbara, CA, United States). The microscope was operated in tapping mode in air using a lever oscillating at a frequency of 150 kHz (Bruker). Images were acquired on 2 × 2 μm^2 areas, with 256 × 256 acquisition points and a scan rate of 1 Hz. Surface roughness was estimated with respect to the average roughness (Ra) and the root mean squared roughness (Rq).

The chemical composition of membranes was characterized by ATR-FTIR analysis. Infrared spectra were collected using Perkin Elmer Spectrum 100 spectrophotometer (Waltham, USA) in the range of 4000–650 cm^{-1} and with a resolution of 4 cm^{-1}.

Membrane thickness was measured with a micrometer (Carl Mahr 40E, Germany). A capillary flow porometer (CFP 1500 AEXL, Porous Materials Inc., PMI, Ithaca, NY, United States) was employed to measure the mean pore diameter of each membrane.

The wettability of the membranes was determined by water contact angle (WCA) measurements with a contact angle meter (KSV Instruments, Ltd., Helsinki, Finland) by using the sessile drop method. The WCA results are the mean of 30 measurements.

The mechanical properties of membranes were assessed using a tensile testing machine (Zwick/Roell Z2.5, Germany). Five samples of each kind of membrane (4 cm × 1 cm) were analysed under uniaxial tension until failure to determine values of Young's modulus (E), ultimate tensile strength (UTS) and elongation at break (ε).

To assess the degradation properties, the membrane samples (2 cm^2) were placed into phosphate buffer solution (PBS, Sigma Aldrich, Milan, Italy), pH = 7.4, containing lysozyme (1 mg/mL, Sigma Aldrich, Milan,

Italy), supplemented with 0.02 % sodium azide (NaN_3, Sigma Aldrich, Milan, Italy), and incubated at 37 °C. Fresh solution was replaced every 6 days. After regular intervals of time, the samples were removed from the enzymatic solution, washed with distilled water, and dried at 37 °C until reaching constant weight. Then, the dissolution index was calculated as:

$$\%S = \frac{W_i - W_d}{W_i} \times 100 \quad (1)$$

where W_i is the sample weight before incubation in enzymatic solution and W_d is the dried sample weight after dissolution test. Three samples of the developed membranes were characterized and the data were presented as average ± SD.

2.6. Liver cell cultures

Rat embryonic liver cells (17 day embryonic liver of Japanese albino rat) (RLC-18), DSMZ, Braunschweig, Germany) were cultured in RPMI medium containing L-glutamine, supplemented with 10 % (*v*/v) heat-inactivated fetal calf serum (FCS, Euroclone, Milan, Italy) and 100 mg/mL penicillin-streptomycin (Sigma Aldrich, Milan, Italy) at 37 °C in a humidified CO_2 incubator (95 % air, 5 % CO_2) and subcultured twice a week using trypsin (0.05 %)/ Ethylenediaminetetraacetic acid (0.025 %) solution (EDTA, Sigma Aldrich, Milan, Italy). The cells were seeded at 9 passages on the different kind of membranes at a density of 1×10^4 cell/cm^2. The culture medium was changed every 48 h.

2.7. Scanning electron microscopy analysis

The morphology of cells cultured on the developed membranes was analized by SEM observation. At day in vitro (DIV) 14, samples were fixed using 3 % *v*/v glutaraldehyde (Sigma Aldrich, Milan, Italy) with 1 % v/v formaldehyde (Sigma Aldrich, Milan, Italy) in PBS for 30 min at 4 °C, then stained with 1 % v/v osmium tetroxide (OsO_4, Sigma Aldrich, Milan, Italy) in PBS for 30 min at 4 °C, rinsed three times with PBS and dehydrated with a graded series of ethanol (10–100 % *v*/v), and left at room temperature to dry.

2.8. Cell viability

Cell viability was evaluated by trypan blue exclusion test. After 7, 13 and 18 days of culture, cells were detached from the different membrane systems by trypsin/EDTA solution (Euroclone, Italy) treatment and centrifuged at approximately 600 x*g* for 5 min at room temperature. The cell pellet was resuspended in culture media and the total number of cells was determined using trypan blue stain (Sigma Aldrich, Milan, Italy) and hemocytometer under a Light Optic Microscope (Axio Vert, Zeiss, Gemany). Cell viability was calculated as the number of viable cells per cm^2.

2.9. Cell immunostaining for laser scanning confocal microscopy

The behaviour of cells in culture on the different membranes was analysed by Laser Scanning Confocal Microscopy (LSCM, Fluoview FV300, Olympus) after the immunostaining of actin cytoskeleton protein, vinculin focal adhesion protein, markers of mature liver, namely albumin and cytokeratin 18 (CK18), and hepatoblasts alfa feto protein marker (AFP).

Hepatocytes in the membrane systems were fixed in 4 % (*w/v*) paraformaldehyde for 15 min, followed by permeabilization and blocking with a solution containing 0.3 % *v*/v Triton X-100 and 10 % v/v foetal bovine serum (FBS, Euroclone, Italy) in PBS for 1 h at 37 °C. Samples were then incubated overnight at 4 °C with the following primary antibodies: anti-vinculin (1:200, Santa Cruz Biotechnology), anti-CK18 (1:200, Santa Cruz Biotechnology), anti-Albumin (1:200, (Bethyl laboratories), anti-AFP (1:200, Santa Cruz Biotechnology). Actin was stained with Alexa 488-conjugated phalloidin (Molecular Probes, Eugene, OR). Secondary antibodies, Cy2™-conjugated Affini Pure donkey anti-rabbit IgG, Cy3™-conjugated Affini Pure donkey anti-mouseIgG and a Cy5™-conjugated Affini Pure donkey anti-goat IgG (1:500, Jackson ImmunoResearch Europe Ltd) were then added for 1 h at room temperature. The nuclei of cells were stained with DAPI (200 ng/mL, Molecular Probes). Finally, samples were rinsed, mounted and observed at LSCM.

Quantitative analysis was performed by calculating the percentage of positive cells for each liver marker proteins (albumin, CK18 and AFP positive nuclei) over the total (DAPI-stained nuclei).

The fluorescence intensity for stained actin and vinculin was calculated on LCSM images of cells at DIV13, in a series of squared areas of 235 × 235 μm of the x–y axis vs the z axis of acquired images, using Fluoview 5.0 software (Olympus Corporation). Each image was composed of 20 slices, having an optical thickness of 0.5 μm. Photomultiplier settings were the same for all acquisitions with each dye. Images were acquired from randomly selected fields.

2.10. Glycogen detection

Cells at the endpoint of the differentiation stage were stained using a Periodic acid-schiff (PAS) kit (Sigma Aldrich, Milan, Italy) for glycogen analysis. Cells were fixed in 4 % formaldehyde, permeabilized with 0.1 % Triton X-100 for 10 min, and then oxidized in 1 % periodic acid for 5 min, rinsed 3 times in deionized water (dH_2O), treated with Schiff's reagent for 15 min, and rinsed in dH_2O for 5–10 min. Samples were counterstained with Mayer's hematoxylin (Sigma Aldrich, Milan, Italy) for 1 min and rinsed in dH_2O and finally, the cells were visualized by light microscopy.

2.11. Albumin synthesis

Liver-specific cellular functions were investigated in terms of the capacity of cells to produce albumin which is integral to hepatocytes function. To this end, the supernatants of cell cultures were collected and stored at −20 °C until analysis. The amount of albumin in the medium was determined by a sandwich enzyme-linked immunosorbent assay (ELISA), as previously reported. 96-well plates were coated with 50 μg/mL rat albumin (Sigma, Milan, Italy) and left overnight at 4 °C. After 4 washes, 100 μL of cell culture supernatant was added to the wells and incubated overnight at 4 °C with 100 μL of anti-rat albumin monoclonal antibody conjugated with horseradish peroxidase (Bethyl Laboratories, Inc., United States). After 4 washes, the substrate buffer containing tetramethylbenzidine and hydrogen peroxide (H_2O_2, Sigma, Milan, Italy) was added for 7 min and the reaction was stopped with 100 μL of 8 N Sulfuric acid (H_2SO_4, Sigma Aldrich, Milan, Italy), followed by absorbance measurements at 450 nm with a Multiskan Ex (Thermo Lab Systems).

2.12. Statistical analysis

Statistical analysis was carried out using one-way ANOVA test followed by Bonferroni *t*-test. A value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Jellyfish collagen (jCol) extraction and characterization

Over the past few years, studies involving the extraction of collagen from marine sources for applications in the nutraceutical, cosmeceutical, and pharmaceutical industries have been increasing, also highlighted by the growing number of scientific publications [17]. In particular, jellyfish collagen (jCol) currently represents an excellent

alternative to commercially available mammalian collagens.

In this work, we obtained the native form of jCol from *R. pulmo* from fisheries by-catch through the acid-solubilized collagen extraction, avoiding the pepsin digestion step (Fig. 1A), as described in Section 2.2. The use of discarded by-catch species is in line with the development of a sustainable process, providing a possible reduction of fishing discards, in accordance with the recent European Common Fisheries Policy (CFP).

Fibrillar collagen type I is the most abundant protein in vertebrates. It is a heterotrimer consisting of two α1- and one α2-chains, generally of 129 kD and 139 kD, respectively. The SDS-PAGE analysis of extracted jCol showed the characteristic protein bands, corresponding to the molecular mass reported in previous publications on the molecular weight of *R. pulmo* collagen [16,18] (Fig. 1B). Higher molecular weight bands were also observed indicating the presence of β- (approximately 260 kD) and γ- (>350 kD) chains, suggesting the existence of dimers or trimers of α chains, respectively [19]. Moreover, no differences in the protein patterns between *R. pulmo* umbrella or oral arms were observed. It is important to consider that the triple-helical proteins exhibit an apparent electrophoretic mobility that is not related to their molecular masses, due to the low content of hydrophobic residues. As expected

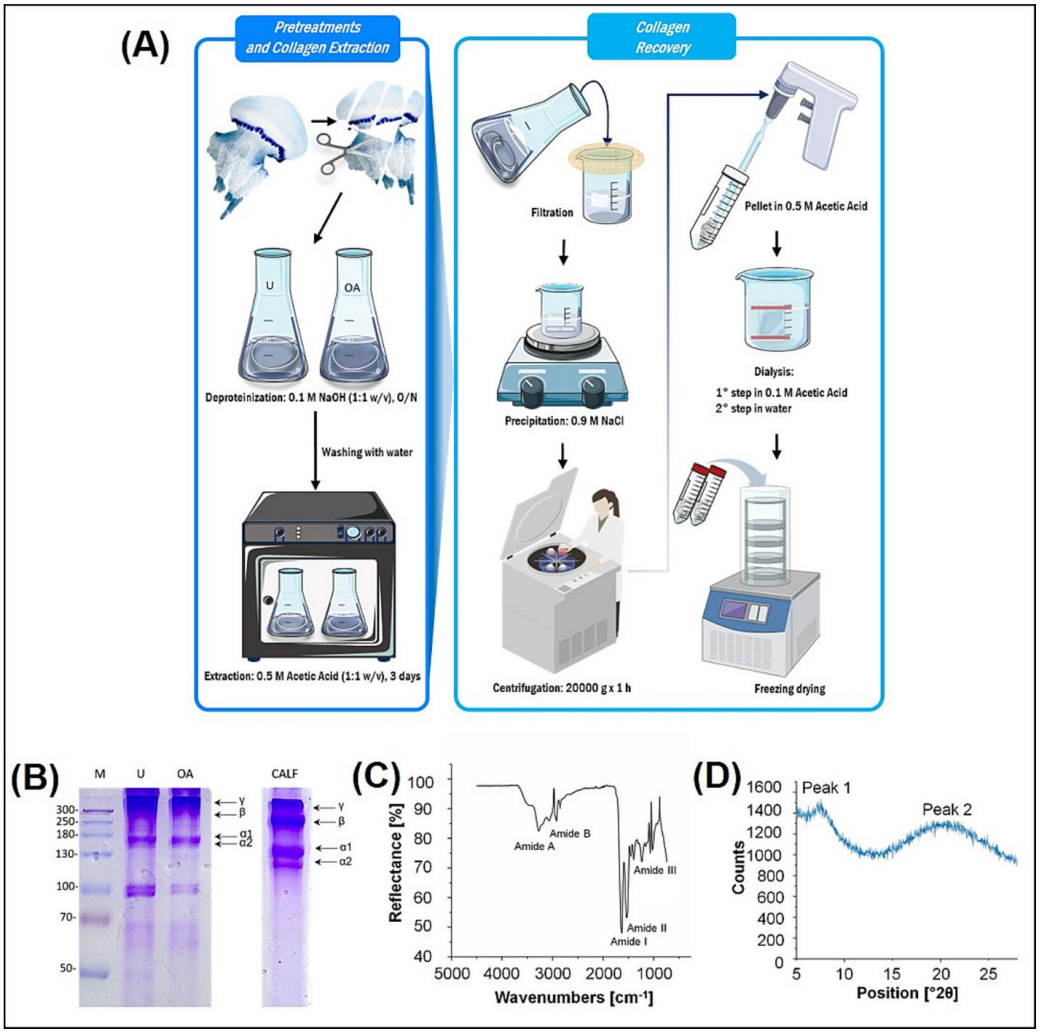

Fig. 1. Jellyfish Collagen (jCol) extraction and characterization.
(A) Representative Scheme of the collagen extraction process from jellyfish.
(B) SDS-PAGE analyses of *R. pulmo* umbrella (U) and oral arms (OA) collagen; Calf type I collagen (CALF); M represents molecular weight markers in kDa.
(C) ATR -FTIR spectrum of jCol between 4500 and 500 cm^{-1}.
(D) X-ray diffraction pattern of jCol.

from the literature, the bands corresponding to the α and β chains of the extracted jCol have a slightly higher apparent molecular mass than the calf counterpart (Fig. 1B). This could be due to the different amino acid composition of *R. pulmo* collagen, containing less hydroxyproline, proline, glycine, and glutamic acid residues than mammalian type I collagen [20]. However, although these residues are involved in the formation and stability of the quaternary structure of type I collagen, they do not appear to influence the structure of collagen [21].

As shown in Fig. 1C, the ATR-FTIR spectrum of the jCol exhibited the representative peaks attributed to amide A, amide B, amide I, amide II, and amide III. For instance, the band position of amide A was found at 3278 cm^{-1} and corresponded to stretching vibrations of N—H, that

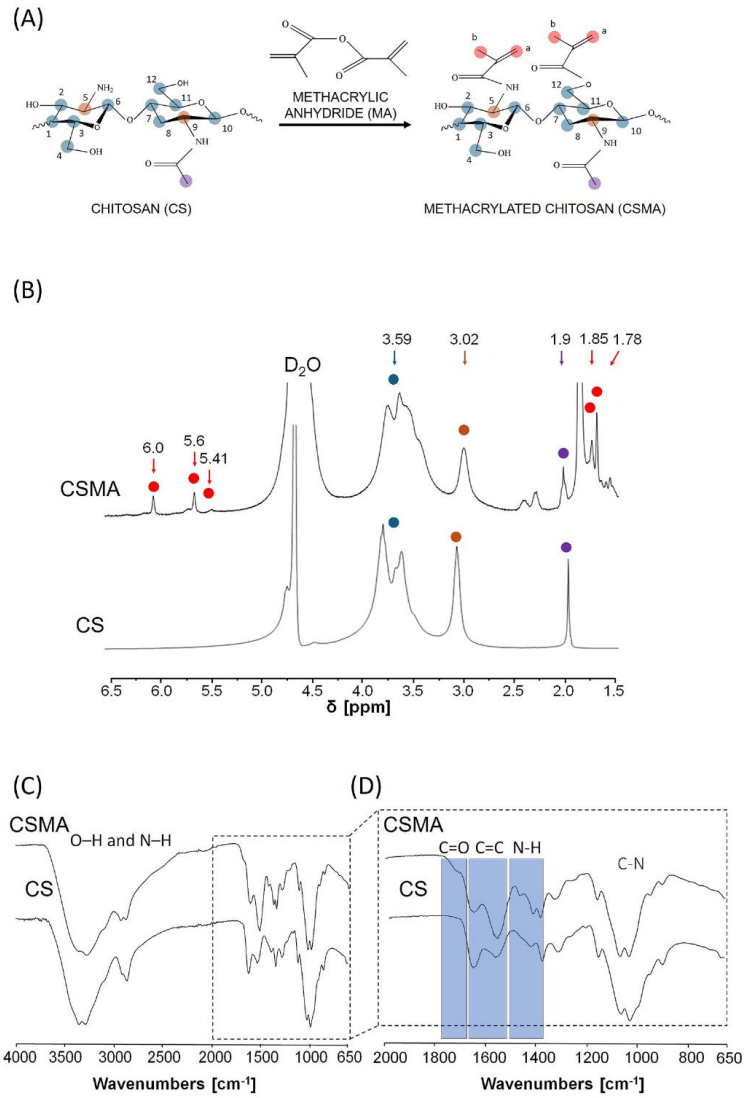

Fig. 2. CSMA synthesis and characterization.
(A) Representative scheme of reaction between Chitosan (CS) and methacrylic anhydride (MA) to obtain methacrylated chitosan (CSMA).
(B) ^{1}H NMR of CS and CSMA.
(C) ATR-FTIR of CS and CSMA between 4000 and 650 cm^{-1}.
(D) ATR-FTIR of CS and CSMA between 2000 and 650 cm^{-1}. Both analyses highlighted the success of the functionalization reaction, reporting the typical functional groups and the moieties introduced by the methacrylation reaction.

generally occur at 3400–3440 cm^{-1} when the N—H group is free from hydrogen bonding [22]. In this work, the band position resulted at low frequency, suggesting that N—H groups were involved in the hydrogen bond formation which helps to hold the triple helical structures together within the collagen molecule [23]. The band position of amide B was observed at 3069 cm^{-1} and correlated to asymmetrical stretching of CH_2 [22]. The peak for amide I is usually observed in the range of 1600–1700 cm^{-1}, which is generated by stretching vibration of C═O in polypeptide backbone of protein. This is the sensitive area of changes of protein secondary structure, and often used for protein secondary structure analysis. The amide I peak of jCol occurred at 1632 cm^{-1}. The presence of amide II was observed from the peak at 1531 cm^{-1} attributed to NH bending vibration coupled with CN stretching. Amide III band was found at wavenumber of 1231 cm^{-1} which corresponds to the stretching vibrations of C—H. Absorption bands around 1390–1455 cm^{-1} were also found. These bands can be ascribed to pyrrolidine ring vibration of hydroxyproline and proline. This result indicated that the triple helical structure of jCol was well preserved during extraction process [24]

The X-ray diffraction pattern of lyophilized jCol is reported in Fig. 1D. There were two diffraction peaks, located at diffraction angles (2θ) of 7.50° and 20.69°, corresponding to the characteristic diffraction peaks of collagen which contains proteins having ordered structure or ordered structure snippet [25]. The first peak, labelled 1, was sharp while the second one, labelled 2 was wide. These peaks correspond to two *d*-spacing at 11.77 (d_1) and 4.28 Å (d_2), which were attributed to the diameter of the triple-helix collagen molecule and the single left-hand helix chain, respectively [26]. The minimum values (d) of the repeat spacing were calculated from the Bragg equation $d(Å) = \lambda/2\sin\theta$ where λ is the X-ray wavelength (1.54 Å) and θ is the Bragg diffraction angle [26]. The obtained results were in accordance with the diameter of a standard collagen molecule with a triple-helix with a single left-handed helix chain. Therefore, it is possible conclude that the collagen after the extraction process was still in its natural conformation and not denatured.

3.2. CSMA synthesis and characterization

In this study, CS was methacrylated by reaction with MA, as schematically reported in Fig. 2A. This strategy has the benefit of allowing chemical crosslinking thanks to the methacrylate group's C═C bond. The success of the methacrylation reaction was confirmed by ^{1}H NMR and ATR-FTIR (Fig. 2B, C and D). The CS spectrum displays the typical CS peaks: the quadruplet peak at $\delta = 3.59$ ppm related to the protons in 1–4, 6–10, 12 positions and the peak at 3.02 ppm ascribed to the protons in 5, 11 positions of the CS ring (Fig. 2B). The peak at $\delta = 1.9$ ppm represents the (*N*-acetyl)-D-glucosamine group. In the CSMA spectrum, new peaks can be observed at $\delta = 6.0$, 5.60 and 5.41 ppm, representing the $= CH_2$ of the methacrylic double bonds, and at $\delta = 1.85$ and 1.78 ppm corresponding to the $-CH_3$ methyl groups of the grafted methacrylated moieties. There are two types of $-CH_3$ signals and three different peaks corresponding to the $= CH_2$ protons, meaning that the methacrylated groups are successfully grafted to both the $-NH_2$ and the −OH groups of the CS [8,27] (Fig. 2B).

ATR-FTIR spectra of CS and CSMA are reported in Fig. 2C and D. Similar regions of both spectra were observed, such as the characteristic broad peak from 3000 cm^{-1} to 3600 cm^{-1}, which indicates the presence of hydroxyl, amino and hydrogen-bonded (−OH) groups, and absorption bands for CS at 1026–1151 cm^{-1} (amine C—N stretch) (Fig. 2C). In both spectra, the peak between 2850 and 2950 cm^{-1} can be ascribed to the CH stretching of the alkyl groups. However, the increase in the intensity of the absorption bands is well evident in CSMA spectrum (Fig. 2C). Particularly, in this spectrum, the presence of a new peak at 1720 cm^{-1}, ascribable to the amide C═O stretching vibrations, can be noticed, and it confirms the methacrylation of CS. Furthermore, the appearance of a double bond signal at 1537–1653 cm^{-1} depicted alkenyl C═C stretch. However, significant changes were observed in the absorption peaks at 1550 cm^{-1} and 1640 cm^{-1}, associated to the amine vibration and methacrylate C═C, respectively, partially overlapping the amide I peak (~1650 cm^{-1}) [8,27] (Fig. 2D).

3.3. Characterization of membranes

In the present study, new biomimetic composite membranes were developed by blending modified CSMA with jCol.

CSMA membrane and CSMA/jCol blended membranes were prepared in flat configuration using the phase inversion technique inducing precipitation by solvent evaporation. For the composite membranes, the jCol solution was added to the CSMA solution by combining the components in two different ratios, 70 % chitosan/30 % collagen, and 50 % chitosan/50 % collagen, generating two different membranes namely CSMA/jCol30 and CSMA/jCol50, respectively. The properties of the developed membranes, including chemical composition, surface morphology, roughness parameters, mean pore size, wettability, mechanical qualities, degradability, and cell growth were all deeply analysed considering their pivotal role for tissue engineering applications, and summarized in Table 1.

Surface topography is an important factor that influences cell adhesion and growth affecting cell-membrane interactions [28]; thus, the surface morphology and roughness of each membrane were also investigated (Fig. 3). SEM observation revealed that CSMA and CSMA/jCol membranes had a homogenous and compact surface looking quite similar (Fig. 3A-C). The smooth surface of the blended membranes suggested that a homogenous blending of the two biopolymers properly occurred in both composite membranes. The successful blending is related to the formation of hydrogen bonding between the NH_2 and OH groups in the functionalised CS and jCol. This result is in line with other studies, in which SEM analysis displayed a very smooth surface of CS/collagen biomaterials [29].

Consistently with SEM results, the AFM analysis highlighted the uniform smooth surface of all the investigated membranes and confirmed the successful combination of marine collagen and functionalized CS (Fig. 3D-F). CSMA membranes exhibited roughness values of 2.9 ± 0.3 nm and 3.7 ± 0.3 nm of Ra and Rq, respectively. Meanwhile, CSMA/jCol membranes highlighted higher values of Ra and Rq, reaching 3.6 ± 0.4 (Ra) and 4.7 ± 0.9 nm (Rq) for CSMA/jCol30 membranes and 3.9 ± 0.4 and 4.7 ± 0.9 nm for CSMA/jCol50, as reported in the Table 1.

Porometer measurements revealed nanopores with diameters of about 26.5 ± 7.0, 47.2 ± 4.2 and 49.7 ± 8.3 nm for CSMA, CSMA/jCol30, and CSMA/jCol50 membranes, respectively. These results suggest that the addition of marine collagen significantly increased the pore size diameter of the pure CSMA membrane, in agreement with other studies [29,30]. Moreover, the increased roughness of CSM/jCol membranes could be ascribable to the presence of increased nanopores on their surface compared to CSMA ones.

ATR-FTIR spectra reported in Fig. 4 A enable to identify the

Table 1
Membrane properties. Thickness; mean pore diameter; Average roughness (R_a) and the root mean squared roughness (R_q); Water Contact Angle (WCA); Young's modulus (E); Elongation at break (ε); Ultimate tensile strength (UTS). Results are expressed as mean value ± standard deviation (SD).

	CSMA	CSMA/jCol30	CSMA/jCol50
Thickness [μm]	27.2 ± 8.0	22.1 ± 4.0	21.7 ± 6.0
Mean Pore Diameter [nm]	26.5 ± 7.0	47.2 ± 4.2	49.7 ± 8.3
Roughness [nm]	Ra: 2.9 ± 0.3 Rq: 3.7 ± 0.3	Ra: 3.6 ± 0.4 Rq: 4.7 ± 0.9	Ra: 3.9 ± 0.3 Rq: 4.9 ± 0.4
WCA [°]	84.4 ± 3.4	76.6 ± 2.6	71.2 ± 6.0
E [MPa]	611.0 ± 81.0	418.0 ± 82.0	364.0 ± 34.0
ε [%]	1.6 ± 0.2	3.0 ± 0.9	4.4 ± 0.9
UTS [MPa]	51.7 ± 6.0	29.9 ± 8.6	22 ± 3.0

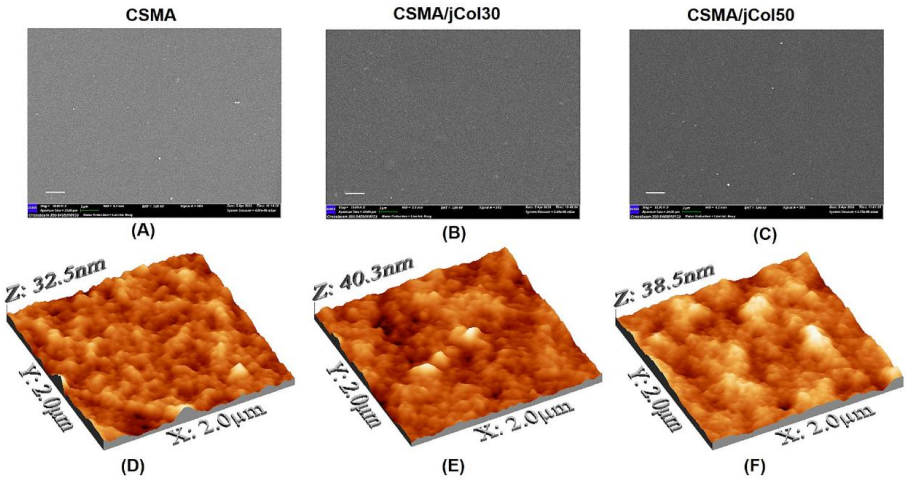

Fig. 3. Membrane surface morphology.
Scanning electron micrographs (A-C) and AFM images (D-F) of the surface of CSMA (A and D), CSMA/jCol30 (B and E) and CSMA/jCol50 (C and F) membranes. Scale bar of SEM micrographs: 2 μm.

vibrational bands corresponding to both polymers' primary functional groups. The spectra related to CSMA/jCol membranes show the characteristic bands of chitosan, with the broad band in the region of 3700–3100 cm^{-1}, ascribed to the axial deformations of the O—H and N—H groups and the region between 2800 and 3000 cm^{-1} from -CH_2 and -CH_3 ones, presented in the polysaccharide structure, as also reported in Fig. 2 C and D. Furthermore, the spectra also show the characteristic peaks at 1640 and 1550 cm^{-1} of C═O and N—H stretching, ascribable to protein amide I and amide II of collagen, as recognized in Fig. 1 C. Even though it is particularly difficult to analyse the peaks due to the partial overlapping, some differences of the intensity of their characteristic peaks were observed between the two compositions, mainly related to the different concentration of both polymers. Particularly, the intensity of those peaks seems slightly higher in CSMA/jCol50 membrane where there was a higher collagen concentration [31].

An important characteristic of these membranes is the transparency that allows a microscopic observation of the cell culture during the experimentation.

Since surface topography influences the physicochemical properties that in turn would affect cell adhesion, we investigated the wettability of the membranes by WCA measurements (Table 1). CSMA membrane displayed values of 84.4 ± 3.4° that indicate a slight hydrophobic character due to the functionalization with MA. The blend with jCol at different concentrations gave arise to a more hydrophilic material. It caused a significant decrease to value of 76.6 ± 2.6° in CSMA/jCol 30 membranes. A further reduction of the WCA occurred by increasing the collagen concentration to 50 % (CSMA/jCol50) and, indeed, this membrane showed a moderate hydrophilic character with a contact angle of 71.2 ± 6°. These findings clearly indicate that marine collagen blending successfully balanced the hydrophobic character of CSMA membranes, leading to the formation of new materials with moderate wettability able to promote cell attachment and growth. As a structural protein, collagen contains many hydrophilic groups (i.e. NH and OH groups), and when it is added to a hydrophobic polymer, like CSMA of our study, the WCA of the pure CS membranes was significantly decreased, obtaining a new material with improved wettability, in good agreement with other papers [29,32].

Due to the need of providing a certain structural stability during culturing *in vitro* and implanting *in vivo*, the mechanical properties play a pivotal role in the engineering of suitable membranes that can significantly affect the specific biological functions of cells. In general, the mechanical strength mainly depends on structural parameters such as pore size and porosity, but it can be strongly affected by the material components and crosslinking. CS is a biomaterial widely used in a variety of biomedical applications, nevertheless, non-crosslinked CS has very poor mechanical features. In our paper, to make CS strong enough to be used in the bioartificial liver, it was functionalized with MA to be photocrosslinked using Irgacure as an initiator under UV light. Young's modulus (E), ultimate tensile strength (UTS) and elongation at break (ε) were assessed via uniaxial tension. These parameters indicate the stiffness, strength, and elasticity of the membrane, and are important mechanical features in supporting the tissue engineering application of the membrane (Table 1). CSMA membrane developed in this study displayed a value of E of 611.0 ± 81.0 MPa, thanks to the MA functionalization. Moreover, marine collagen incorporation induced a decrease in stiffness of the native CSMA membranes, which allowed obtaining membranes with suitable mechanical properties for the specific application [29,33]. Indeed, E values of CSMA/jCol membrane decreased to 418.0 ± 82.0 MPa and 364.0 ± 34.0 for CSMA/jCol30 and CSMA/jCol50 membranes, respectively. Regarding UTS, the same decreasing trend was observed: for CSMA/jCol30 membrane was detected a value of 29.9 ± 8.6 and for the CSMA/jCol50 one, a value of 22 ± 3 MPa, which are significantly lower than that of the native CSMA membrane (51.7 ± 6 MPa). Membranes have different percentage of elongation at break, which was found in the following order CSMA/jCol50 (4.4 ± 0.9 %) > CSMA/jCol30 (3 ± 0.9 %) > CSMA (1.6 ± 0.8 %). Therefore, the marine collagen blend resulted in an enhancement of the elastic properties as demonstrated by the significant decrease of Young's modulus and the tensile strength, and the increase of the elongation at break, in accordance with the literature [29,33]. The improving value was probably related to the formation of an attractive interaction between the functionalized chitosan and marine collagen in the blended membranes since the successful integration of two polymer components depends on the intermolecular interactions, which results in an improved quality of the blend mechanical properties.

Another pivotal factor required for an ideal material for tissue

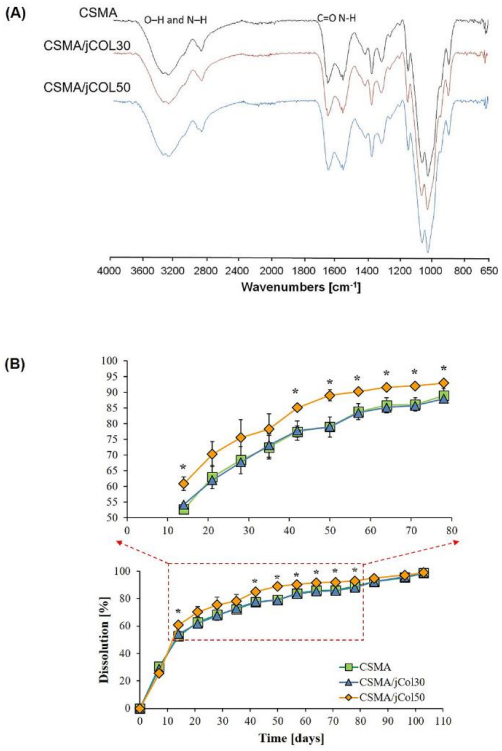

Fig. 4. Chemical composition and degradability of membranes.
(A) ATR-FTIR of CSMA and CSMA/jCol membranes between 4000 and 650 cm^{-1}.
(B) Dissolution behaviour of (□) CSMA, (Δ) CSMA/jCol30 and (◆) CSMA/jCol50 membranes as a function of time. The values expressed as average ± SD are the means of 10 measurements per sample, and data statistically significant were evaluated according to ANOVA followed Bonferroni *t*-test ($p < 0.05$): * vs CSMA and CSMA/jCol30 membranes at the same day.

engineering is an appropriate degradation capacity. Materials should provide sufficient mechanical stability during the time needed for tissue repair and then progressively degrade allowing the new tissue formation. Fig. 4B shows the degradation of membranes in a PBS solution containing lysozyme. Usually, pure CS membranes entirely degrade after few days, while our results highlighted that the modification with MA slowed down the CS degradability by modulating and preserving membrane structural and mechanical integrity for a longer period of time (103 days). Dissolution of all membranes increased with time: over the first 14 days, it was very fast reaching value of 61 ± 2 %, for CSMA/jCol50 membrane, 54 ± 1 % for CSMA/jCol30 membrane and 52 ± 1 % for pure CSMA. Afterwards, membrane dissolution was slower and stable within time, especially from day 42 to day 103, when all membranes were completely dissolved. Interestingly, from day 42 to day 78, CSMA/jCol50 membrane displayed higher values of dissolution ($p < 0.05$) than CSMA and CSMA/jCol30 membranes, probably due to its higher collagen content that contributes to a faster degradation. Thereafter, no differences were observed among the membranes that exhibit the same dissolution percentage, probably related to the CSMA content.

Membrane characterization data highlighted that the addition of marine collagen to CSMA enhanced important properties such as pore size, physicochemical and mechanical characteristics leading to the development of advanced biomimetic membranes that have potential to be used in tissue engineering applications.

3.4. Membrane properties influence liver cell behaviour

Membrane systems are able to promote cell proliferation and differentiation towards the formation of liver tissue [4–6]. They can act as a synthetic ECM thanks to polymer properties (e.g., biocompatibility, biofunctionality) and distinctive intrinsic membrane characteristics such as permeability, selectivity, stability, and a well-defined geometry [5].

In our study, the potential of the membranes produced with CSMA and marine collagen as new generation of sustainable biomimetic materials for liver tissue engineering was addressed by culturing rat embryonic liver cells. Previous papers employed these cells as an alternative model of human liver progenitor cells, since using cells from the foetal human liver is limited by major ethical issues. In those works, the membranes were able to promote the expansion and functional differentiation of rat embryonic liver cells in comparison with traditional substrates [34,35].

Our aim was to validate the suitability of the new marine collagen-blend membranes to support liver tissue engineering with special attention to the influence of membrane properties on liver cells behaviour. Therefore, the ability of the developed membranes to offer to the cells a specific and familiar *milieu* for the embryonic liver cells commitment was deeply investigated.

The biocompatibility was investigated by culturing embryonic liver cells in direct contact with the different membranes. Then, cell viability, morphology and cytoskeleton organization were analysed (Fig. 5). We evaluated the role of membrane properties and collagen content in determining their efficiency for liver tissue regeneration.

SEM analysis, after 14 days of culture, showed that the cellular reorganization over the three substrates was quite similar (Fig. 5A). On membranes, cells adhered, spread, and acquired a polyhedral shape morphology, which is typical of differentiated and mature hepatocytes. Very tight cell-cell interactions with the formation of multilayers were also visible on all membranes. However, morphological cell features changed by modulating the membrane composition and properties. When cells were cultured on CSMA membrane, the most hydrophobic and stiff material, they displayed a very flat and spread morphology, with a nonuniform distribution and empty spaces over the surface (Fig. 5A, Panel I). In the case of composite membranes, the blending with jCol significantly improved mechanical stability, enhancing also hydrophilic properties, thus favouring the interactions with cells. Coherently, as highlighted by Fig. 5A, on CSMA/jCol30 (Panel IV) and CSMA/jCol50 (Panel VI) membranes, cells formed intercellular tight contacts and even the degree of a multi-layered cell aggregation is visibly higher than on CSMA membranes (Panel II), achieving the formation of a complex 3D construct. This is an important result, because it is well known that the cell-cell and cell-material interactions are crucial for the maintenance of hepatocyte phenotype and specific functions. Moreover, blended membranes appeared completely covered by multilayers of cells that assembled themselves in 3D cord-like structures acquiring a tissue-like phenotype with features like that of liver parenchyma [34,35].

To further characterize the liver cell behaviour on these membranes, immunocytochemistry investigation was carried out by LSCM. Consistent with SEM analysis, confocal images (Fig. 5B) indicated that membranes supported adhesion and growth of liver cells. Staining for the cytoskeletal protein actin (in green) and the focal adhesion marker, vinculin (in red), demonstrated the overall morphology of the cells cultured and the organization of the cytoskeletal proteins on CSMA and CSMA/jCol membranes. Cells grown on CSMA (Fig. 5B, Panel I), CSMA/jCol30 (Fig. 5B, Panel II) and CSMA/jCol50 membranes (Fig. 5B, Panel III), appeared well spread and acquired a polyhedral shape morphology

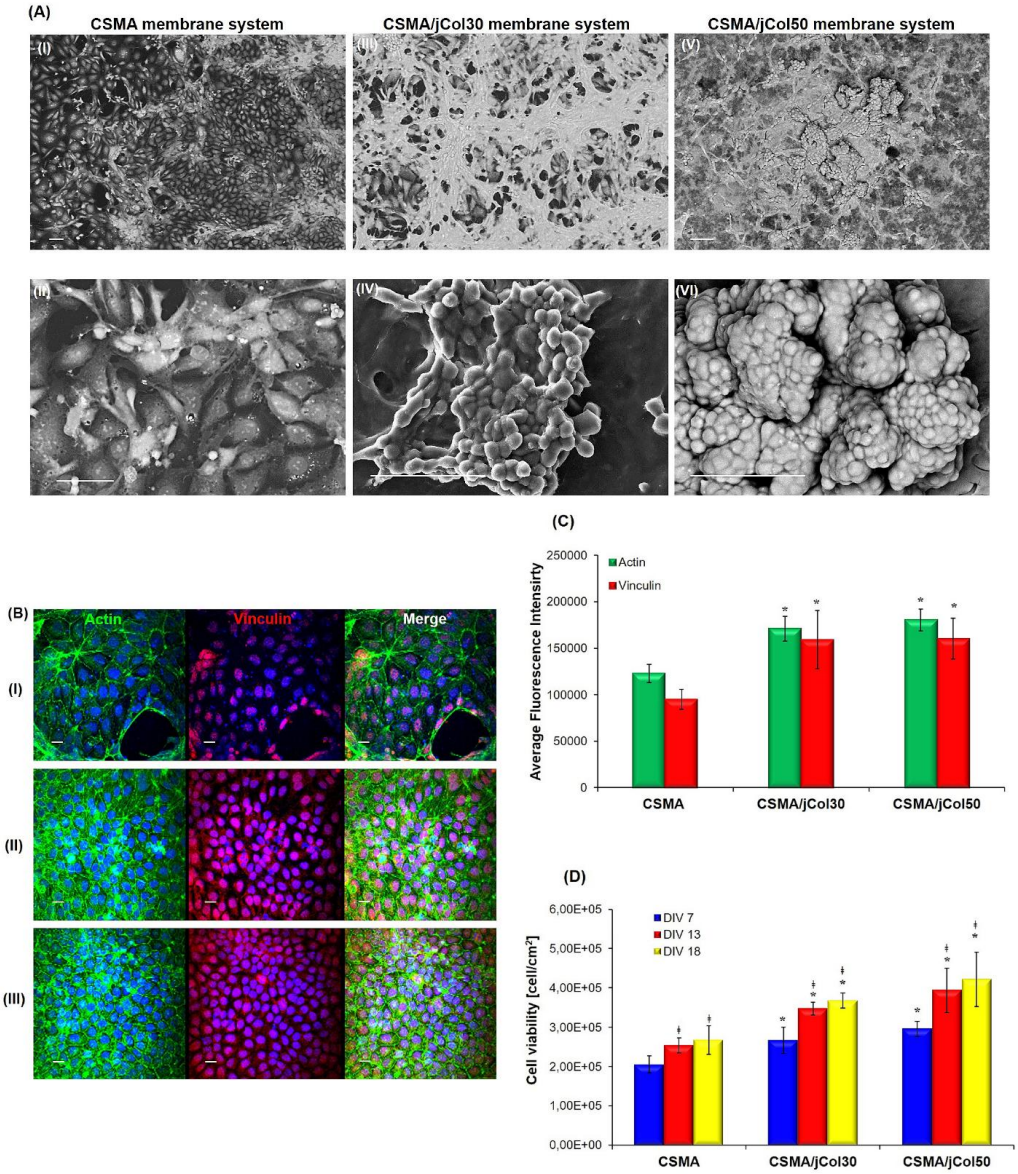

Fig. 5. Behaviour of embryonic liver cells in the different membrane systems.
(A) SEM images at different magnifications of liver cells at DIV14 on: (I-II) CSMA, (III-IV) CSMA/jCol30, (V-VI) CSMA/jCol50 membranes. Scale bar: 50 μm.
(B) Confocal laser micrographs of liver cells after 14 days of culture in the different membrane systems (I) CSMA, (II) CSMA/jCol30, (III) CSMA/jCol50 membranes. Cells were stained for actin (green), vinculin (red) and nuclei (blue). Scale bar: 20 μm.
(C) Average fluorescence intensity for stained actin (green bar) and vinculin (red bar) of cells at DIV13 on the different membranes. Data statistically significant according to ANOVA followed Bonferroni t-test; *$p < 0.05$ vs CSMA membrane.
(D) Changes in cell viability of liver cells after 7, 13 and 18 days of culture in the different membrane systems. The values expressed as average ± SD are the means of 5 experiments and data statistically significant were evaluated according to ANOVA followed Bonferroni t-test ($p < 0.05$): * vs CSMA membrane at the same DIV; ǂ vs the same membrane at DIV7.

as hallmark of differentiated liver cells. On all kinds of membranes, the expression and pattern distribution profile of actin and vinculin showed up a cell displacement according to a parenchymal-like structure (Fig. 5B). A dot-like distribution of vinculin also highlighted the numerous cell−cell and cell−membrane interactions. In order to evaluate the changes in the expressions of cytoskeletal and focal adhesion markers, quantitative analysis in terms of the fluorescence intensity of actin and vinculin was then performed (Fig. 5C). The fluorescence intensity of both investigated markers was significantly higher in cells cultured on blended membranes with respect to CSMA membranes. The permissive surrounding within these membrane systems favoured specific liver features development, recapitulating those of the natural liver.

These results were confirmed by cell viability data. As shown in Fig. 5D, the number of viable cells on each membrane significantly increased ($p < 0.05$) after 18 days in vitro, suggesting that these biomaterials have appropriate features in terms of biocompatibility and nontoxic response that validate their use for the realization of promising tissue engineered liver constructs. Our investigation showed a distinct biocompatibility degree for the different membranes. The highest cell viability was assessed on membranes with the highest collagen content (CSMA/jCol50 membranes). Cell number on these membranes was similar to the ones on CSMA/jCol30 membranes by day 7; cell expansion within time was quite comparable on these two surfaces. Interestingly, at each culture time point, cell viability on both marine collagen-blended membranes was significantly higher than on CSMA membrane. It was noticed that after 18 days of culture on CSMA/jCol30 ($3.7 \pm 0.2 \times 10^5$ cell/cm^2) and CSMA/jCol50 ($4.2 \pm 0.7 \times 10^5$ cell/cm^2) membranes, the cells showed 37 % and 57 % higher viability than on the pure CSMA membrane ($2.7 \pm 0.4 \times 10^5$ cell/cm^2), respectively. This agrees with SEM and LSCM analysis: the cells on blended membranes were very close to each other then, they communicated more easily and could proliferate to a much higher number of cells compared to CSMA membrane where cells did not completely cover their surface. These results are strictly related to the high biocompatibility of collagen and to its good and uniform dispersion in the CSMA solution, which improved hydrophilicity and mechanical stability of the blended membranes. It is reported that the adhesion of hepatocytes to collagen substrate regulates functional behaviour. Furthermore, adhesion, morphological features, and viability of cells are strongly affected by the properties of the membrane on which they are seeded, as suggested by previous works, showing a relationship between cell adhesion and membrane surface hydrophilicity [29]. In this scenario, our CS-based membranes offered the cells a matrix that is similar to glycosaminoglycans of the liver ECM and favoured the initial step of cell adhesion due to interaction of its protonated NH_2 groups with anionic functional groups on the cellular membrane. However, despite the promising effect of collagen and CS-based materials in hepatocyte cultures, only a few studies described the impact of CS/collagen composites for liver tissue engineering [7,36–37] and even less work is focused on marine collagen [32,38]. For this reason, the present work is pioneering investigation that reports the combination of marine collagen with CSMA as support for the culture of liver cells.

3.5. Membranes boost hepatocyte differentiation and specific liver tissue functions

The capability of the different membrane systems to boost liver differentiation was further investigated by observing the expression profile of distinctive hepatic lineage markers. LSCM analysis was carried out after immunostaining of albumin, a typical marker of mature hepatocytes, cytokeratin 18 (CK18), a marker expressed by several liver cell types, including bile ductal epithelial cells and hepatocytes, and alfa-fetoprotein (AFP), a serum glycoprotein produced by liver during fetal life, a typical marker of hepatoblasts and foetal hepatocytes.

The initial cell suspension before seeding was AFP-positive with a very low expression of albumin (data not shown), as reported in a previous paper [35]. In our study, after 14 days of culture on the developed membranes, cells displayed low AFP expression, while albumin and CK18 were highly expressed and widely distributed, mainly localized on the cell membrane, which is consistent with their native distribution in liver [39]. In line with other studies [35–36,40], cells cultured onto the membranes lost their progenitor characteristics related to the AFP expression and gained more differentiated features, as a result of the high expression of albumin and CK18, markers of mature liver cells. These findings suggest that all membranes promoted cell differentiation towards a liver phenotype.

In addition, to investigate cell functionality within the different membrane systems, we tested the ability of the differentiating cell populations to store glycogen, a characteristic of functional hepatocytes. Cells at DIV 14 were stained for cytoplasmic glycogen using the PAS staining procedure (Fig. 6A). Cytoplasm of majority of the cells was stained pink to dark red-purple, indicating the capacity to store glycogen like functional hepatocytes. Hence, consistent with the acquisition of expression of mature hepatic markers, the differentiating cells cultured on all the developed membranes, also exhibited a progressive gain of hepatic functionality in terms of glycogen storage.

To ascertain any differences among the developed membranes in dictating the hepatic differentiation, a quantitative analysis of liver functional phenotype was performed (Fig. 6B). The profile expression patterns for each of the above-mentioned liver markers was quantified and expressed as the percentage of positive cells over the total number of cells within the investigated image field (Fig. 6B). The analysis revealed that, after 14 days, the percentage of albumin-positive cells on CSMA/jCol30 and CSMA/jCol50 was 93.0 ± 3.0 % and 96.0 ± 2.0 %, respectively, which are values significantly higher compared with the CSMA membranes (71.0 % ± 4.0 %). The number of cells expressing CK18 was significantly higher than the one calculated for other membranes with a percentage in the following order: CSMA/jCol50 (79.0 ± 9.0 %) > CSMA/jCol30 (64.0 ± 7.0 %) > CSMA (41.0 ± 1.0 %). Interestingly, the values referred to the membrane with the highest collagen content resulted 2-fold higher than the pure CSMA membrane. Although the percentage of AFP-positive cells on CSMA membrane was kept at low level (17.0 ± 1.0 %), it was significantly higher than on collagen-blended membranes and the values followed the order CSMA > CSMA/jCol50 (9.0 ± 1.0 %) > CSMA/jCol30 (7.0 ± 3.0 %). This may indicate that the state of some cells on CSMA membrane was still at an early stage of differentiation.

Since albumin is a specific and the most abundant protein that can only be synthesized by functioning mature hepatocytes, we examined the cells grown onto the different membranes for albumin secretion, to determine whether the hepatocytes were functionally active. We found that the albumin synthesis rate was maintained throughout the 18-day experiment period and increased continuously over time in all membrane systems, as shown in Fig. 6C. Therefore, the data highlight that all investigated membranes are able to trigger specific metabolic liver functions. During the first 8 days albumin secretion was stable at values of 374.0 ± 8.0 pg/h on CSMA, 458.0 ± 22.0 pg/h on CSMA/jCol30 and 499.0 ± 69.0 pg/h on CSMA/jCol50 membrane, respectively. Then, for each membrane, it rapidly increased reaching its peak values at day 13 and remaining at these levels up to the end of culture. Indeed, at this time frame, the level of albumin synthesis by cells on CSMA membrane (928.0 ± 90.0 pg/h) and collagen blended membrane (1190.0 ± 30.0 pg/h on CSMA/jCol30 and 1203.0 ± 38.0 pg/h on CSMA/jCol50 membrane) was 2-fold and 3-fold higher than those at day 8, respectively. A slight further increase was observed during the last days of culture. Collagen blended membranes enabled albumin synthesis at a greater extent with respect CSMA membrane showing the prominent impact of collagen on albumin secretion, in agreement with previous results [7]. In fact, our data evidence that, for each day of culture, cells on the membrane with the highest collagen content, had the highest metabolic activity. The values of albumin synthesis are significantly higher on blended collagen membranes than those observed when cells

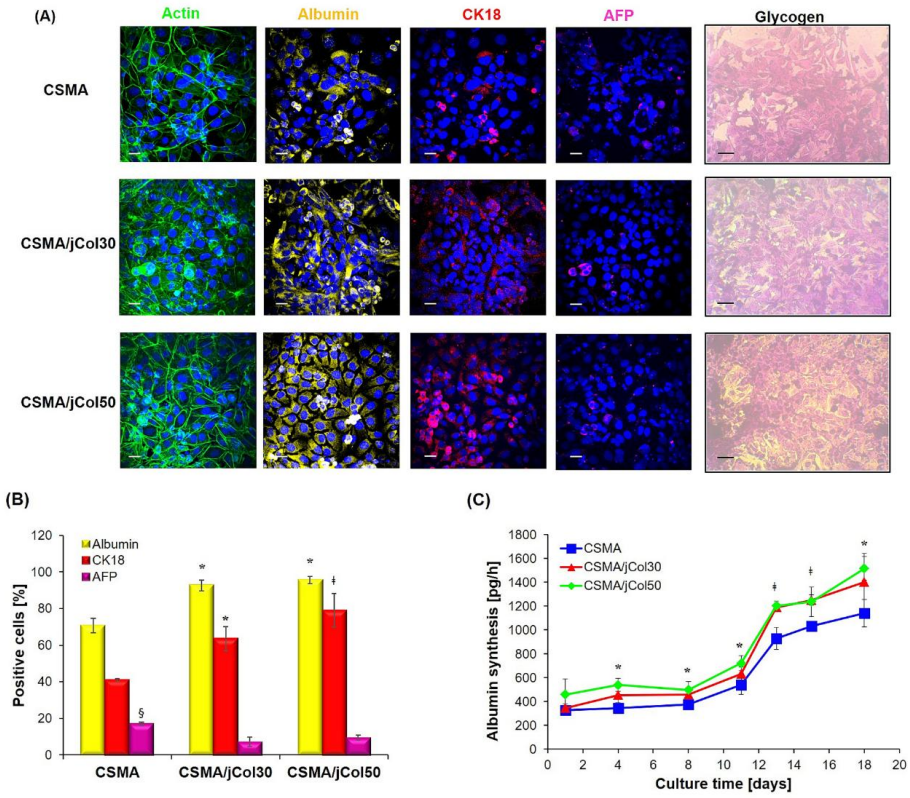

Fig. 6. Liver specific-functions in the different membrane systems.
(A) Confocal laser micrographs of liver cells after 14 days of culture in the different membrane systems. Distribution pattern profile of actin (green), and hepatocytes markers albumin (yellow), CK18 (red) and AFP (magenta). Nuclei are counterstained with DAPI (blue). Scale bar: 20 μm. Light microscope images of Periodic acid–Schiff (PAS) staining of hepatocytes used to detect glycogen storage, scale bar 50 μm.
(B) Quantitative analysis of positive cells for each liver-specific marker. Results are expressed as percentage of positive cells over total and are reported as average ± SD of the means of 5 experiments. Data statistically significant were evaluated according to ANOVA followed Bonferroni t-test ($p < 0.05$): * vs CSMA membrane; ǂ vs CSMA and CSMA/jCol30 membranes; § vs CSMA/jCol30 and CSMA/jCol50 membranes.
(C) Albumin synthesis of liver cells cultured in the different membrane systems. The values expressed as average ± SD are the means of 5 experiments and data statistically significant were evaluated according to ANOVA followed Bonferroni t-test ($p < 0.05$): Within the sane membrane system, albumin synthesis at DIV 13, 15 and 18 is significative vs DIV 1, 4, 8 and 11. *CSMA/jCol50 vs CSMA membrane system at the same DIV; ǂ CSMA/jCol50 and CSMA/jCol30 vs CSMA membrane system at the same DIV.

were cultured on CSMA membranes, consistently with the results of albumin immulocalization data.

4. Conclusions

In this work, we developed sustainable biomimetic membranes by blending of CSMA with jCol and evaluated their properties and biocompatibility for liver tissue engineering applications.

The overall dataset corroborates the complete achievement of differentiation pattern of liver cells cultured on the developed membranes, especially on CSMA/jCol blended membranes. Our approach, based on the combination of functionalized chitosan with marine collagen, significantly improved hepatic differentiation and generated a more reliable *in vitro* tool resembling liver for tissue engineering applications. Furthermore, although many researchers have tried to maintain hepatocyte functions with different culture conditions, our study is the first to show that CSMA/jCol membranes provide a valuable *in vitro* microenvironment, which is able to promote hepatocyte differentiation.

The present work also contributed to highlight the relevance of marine discards for the development of high value-added products, and particularly the use of marine collagen as a good alternative to currently used commercial materials of mammalian origin. In the future, these most promising membranes should be tested *in vivo* to better understand their suitability for the regeneration of liver tissue. These engineered constructs have the potential to address the complex challenges of liver diseases and transplantation, significantly reducing the demand for donor organs, which are often in short supply, and mitigating the risks associated with transplantation.

CRediT authorship contribution statement

Sabrina Morelli: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ugo D'Amora:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Antonella Piscioneri:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Maria Oliviero:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Stefania Scialla:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Alessandro Coppola:** Writing – review & editing, Writing – original draft, Methodology. **Donatella De Pascale:** Writing – review & editing, Writing – original draft, Funding acquisition. **Fabio Crocetta:** Writing – review & editing, Writing – original draft, Methodology. **Maria Penelope De Santo:** Writing – review & editing, Methodology, Formal analysis. **Mariano Davoli:** Methodology. **Daniela Coppola:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Loredana De Bartolo:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data presented in this study are available on request from the corresponding author.

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CHAPTER 3

Marine collagen from fish by-product for the production of a promising 3D scaffold in tissue engineering

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1. Introduction

Collagen is the most abundant protein in nature (Ricard-Blum 2011), and it is a major component of the extracellular matrix of organisms (Maroušek et al. 2015). It is an extremely versatile protein thanks to its biocompatibility and biodegradability, and for this reason it is the object of study in many applications. For example, collagen has been studied for years in the cosmetics industry for its anti-aging properties. In fact, collagen-based creams are widely used to improve skin texture by counteracting the signs of aging (Isnaini et al. 2024). Moreover, a recent study conducted on the use of collagen as dietary supplements in athletes highlighted that these supplements are useful not only for stimulating the production of endogenous collagen, but also for reducing pain and aiding recovery from injuries, such as Achilles tendinopathy. In addition, collagen intake complemented by training promotes resistance and muscle strength (Gołda et al. 2024).
Collagen is also a natural thickener used in several products, such as sauces, ice creams, and desserts, due to its ability to improve texture without compromising flavor (Gómez-Guillén et al. 2011). It is also an effective carrier for the delivery of various agents, including genes, drugs, proteins, and growth factors, and it can be used to produce biomaterials with different characteristics. In particular, collagen nanoparticles offer significant advantages over other polymeric nanoparticles, both natural and synthetic. These include excellent biocompatibility and biodegradability, low antigenicity, reduced toxicity, and a high potential for cationic charge density and their nature allows them to be easily dispersed in aqueous solutions (El-Sawah et al. 2024; Arun et al. 2021).
Collagen-based scaffolds find their greatest use in the biomedical industry where they are used in the regeneration of various tissues, such as cartilage (Allan et al. 2021), bone

(Ge et al. 2020; Bermueller et al. 2013), and cornea (Rana et al. 2023). Moreover, these scaffolds functionalized with Cu^{2+} chelated EGCG nanoparticles promote wound healing, thanks to their anti-inflammatory, vascularizing, antimicrobial activities, proving to be a promising material for wound dressings (Ma et al. 2024).

The skin represents the largest organ of the human body, and is the first barrier that protects the individual from external insults. Following damage, a skin lesion is autonomously repaired through biological actions that lead to complete healing (Bielefeld, Amini-Nik, e Alman 2013). Many times, due to extensive damage such as severe burns, the tissue is unable to regenerate on its own (Shahrokhi, Arno, e Jeschke 2014; Snyder, Sullivan, e Schoelles 2012).

In this regard, tissue engineering represents a valid ally in serious wounds requiring tissue regeneration. In particular, tissue engineering aims to reconstruct damaged tissues or organs (Vacanti e Langer 1999), such as skin, through the use of biomaterials and bioengineering to create 3D models for tissue regeneration (Barros et al. 2021).

The 3D biomaterial scaffolds have been widely studied for tissue regeneration (Domingos et al. 2013). There are many products already on the market today based on mammalian collagen. However, recent studies are increasingly focusing on marine collagen, due to its exceptional biocompatibility, low antigenicity, and high degradability (Cho et al. 2014; Kittiphattanabawon et al. 2005). The shift from mammalian collagen to marine collagen is primarily due to issues related to religious restrictions and the possible transmission of diseases related to mammal-derived products, including bovine spongiform encephalopathy, avian and swine influenza, and tooth-and-mouth disease in bovine, pig, and buffalo (Song et al. 2006).

Fish collagen has been widely used in numerous tissue engineering applications, including skin, bone, and cartilage regeneration (Li et al. 2020; Diogo et al. 2020; Elkasabgy, Mahmoud, e Shamma 2018; Nie et al. 2019; Elango et al. 2016). Wound regeneration also plays a crucial role in biomedical research (Ramanathan et al. 2020; Feng et al. 2020), where scaffolds based on fish collagen are very promising.

In this context, the attention has recently been given to fishery by-products, including skin, scales, bones, fins, and heads, that represent a rich and not fully exploited source of high-added value bioactive compounds, including collagen (Rustad, Storrø, e Slizyte 2011; Nagai 2000). The use of fishery by-products fits perfectly with the concept of circular bioeconomy and zero-waste which aim to minimize waste generation by reusing and valorizing resources.

In the fishery industry, up to 50% of the weight of processed fish is considered waste, which is usually disposed of with high environmental and economic costs (Kim e Mendis 2006). Reusing of these by-products can therefore contribute to the development of new solutions with a reduced environmental impact (Pauly et al. 2002; Venslauskas et al. 2021; EUMOFA, 2020).

In this work, we extracted marine collagen from discarded sea bass (*Dicentrarchus labrax* (Linnaeus 1758)) skin. Sea bass is a species widely present in the Mediterranean, farmed and fished globally (Fuentes et al. 2010; Pérez-Ruzafa e Marcos 2014); it produces a large amount of waste from fish processing and aquaculture companies, as well as from the fish market. We hereby explored the production of scaffolds based on marine collagen for potential uses in would engineering.

It is known that collagen can also be combined with other substances which improve its essential mechanical properties. In particular, in recent years, there has been an increased focus on materials based on the natural biopolymer chitosan, for the development of matrices for tissue engineering. It is suitable for biomedical and clinical applications thanks to its biological and structural properties (Khor e Lim 2003), such as biocompatibility, biodegradability, non-toxicity, and low immunogenicity (Pillai, Paul, e Sharma 2009; Baharlouei e Rahman 2022). Chitosan is formed by deacetylation of chitin, a structural element found in the exoskeleton of crustaceans such as shrimp, crab, and lobster, and it is the most abundant amino polysaccharide in the world, composed of glucosamine and N-acetyl glucosamine units linked by β (1–4) glycosidic bonds (Levengood e Zhang 2014; Pillai, Paul, e Sharma 2009). Being created from crustacean exoskeletons it fits perfectly with the idea of the work of creating scaffolds with biomaterials of marine origin; furthermore, thanks to its structure it is extremely useful for providing stability and compactness to collagen which alone would be inefficient from a structural point of view due to its delicacy and sponginess (Da Cunha et al. 2023).

2. Materials and methods

2.1 Seabass skin preparation and acid soluble collagen (ASC) extraction

Seabass by-products were purchased from local markets in Naples, Italy. Fish skin was washed with cold water, descaled, cut into small pieces (about 1 cm^2), air dried for 3 h, and then used for the extraction.

The acid soluble collagen (ASC) was extracted from fish skin samples according to the method described by (Kittiphattanabawon et al. 2005), with some modifications. All

procedures were carried out at 4 °C. Initially, the fish skin was subjected to two different pre-treatment steps. In particular, to remove non-collagenous proteins, fish skin was soaked in 0.1 M NaOH with a ratio of 1:50 (w/v) for 24 h, twice. Then, the skin was washed with water to reach a neutral pH. To remove lipids, the skins were soaked in 10% isopropyl alcohol, with a skin/solution ratio of 1:10 (w/v) for 2 days, changing the solution every day. Then, the skin was washed with water until reaching neutral pH, and used for the extraction of collagen by using 0.5 M acetic acid, with a ratio of 1:50 (w/v) for 3 days, twice. The insoluble components were separated with cotton cloth, and the filtrate was centrifuged at 4 °C, 20000 x g for 1 h. The obtained supernatant was precipitated by adding NaCl (NaCl, Sigma Aldrich, Milan, Italy) to a final concentration of 2.6 M and Tris HCl 0.05 M. The precipitate containing collagen was collected by centrifuging at 4 °C, 20000 g for 1 h. The pellet was dissolved in a minimum volume of 0.5 M acetic acid, and dialyzed against 0.1 M acetic acid, followed by distilled water. The dialysate was freeze-dried, and subsequently stored at –20 °C.

2.2 SDS-PAGE

SDS–PAGE was performed by the method of (Laemmli 1970) with modification (Morelli et al. 2024). 1 mg of sea bass collagen was dissolved in 100 µL of CH_3COOH 0.5 M, then mixed with 100 µL Tris-HCl (Sigma Aldrich, Milan, Italy), pH 8.8 containing 4% SDS. Solubilised samples were heated at 50 °C for 5 minutes, and then mixed with the sample buffer 2X (0.5 M Tris–HCl, pH 6.8, 5% SDS, 20% glycerol, 10% 2-mercaptoethanol). Samples were loaded onto a polyacrylamide gel made of 7.5% separating gel and 4% stacking gel, and subjected to electrophoresis at a constant current of 120 V. Gel was stained with Coomassie Brilliant Blue and the Spectra Multicolor High Range Protein Ladder (Thermo Fisher Scientific, MA, United States) was used as Marker. Calf skin collagen (Sigma Aldrich, MA, United States) was used as standard.

2.3 Chitosan/collagen scaffold preparation

A controlled interconnected porous chitosan/collagen scaffold was prepared by freeze-drying process. Chitosan (Sigma Aldrich, low molecular weight, Mw 50–190 kDa based on viscosity, degree of deacetylation ≥75 %)) and extracted sea bass skin collagen were dissolved in 0.5 M of acetic acid (CH3COOH, Sigma Aldrich - ACS reagent, ≥ 99.8 %). The slurry was mixed using a magnetic stirrer. The resulting suspension was dropped in a Petri dish plate (Ø =90 mm) and frozen in a controlled way, 4 °C for 30 minutes, -20

°C for 1.5 hour, and -80 °C overnight, then freeze-dried under vacuum in a SCANVAC COOLSAFE (Labogene Scandivian by Design freeze dryer - Bjarkesvej, Denmark). Two different concentrations of chitosan (20 and 40 mg/mL) and two concentrations of collagen (5 and 10 mg/mL) were considered.

Neat and chitosan/collagen cylindrical scaffolds (d = 6 mm, h = 4 mm) were obtained by cutting the membrane using biopsy punches and were soaked in 5 mg/mL sodium tripolyphosphate (TPP, Sigma Aldrich technical grade, 85%) for 6 hours at 4 °C. After this period, scaffolds were rinsed in ultrapure water to remove the excess of salt until pH 7 and, finally, submitted to a second step of freeze-drying. The nomenclature of the samples is reported in Table 1.

Table 1. Nomenclature of the scaffolds

Sample	Chitosan (CS) (mg/mL)	Collagen (COL) (mg/mL)
CS2	20	-
CS2COL0,5	20	5
CS2COL1	20	10
CS4	40	-
CS4COL0,5	40	5
CS4COL1	40	10

2.4 Characterization of scaffold

2.4.1 Physicochemical Analysis

Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy (Thermo Fisher Nicolet IS10, Waltham, MA, United States of America) was employed to assess the composition of the polymer blend as well as the crosslinking. The polymer was scanned from 500 to 4500 cm^{-1} with a resolution of 2 cm^{-1}.

2.4.2 Morphological Analysis

3D scaffolds were observed by scanning electron microscopy (SEM, FEI Quanta 200 FEG, Hillsboro, OR, United States of America). Lyophilized samples were coated with an ultrathin layer of Au/Pt by using an ion sputter and observed by SEM.

2.4.3 Swelling Behaviour

The swelling behaviour was assessed by immersion of the scaffolds in phosphate buffer (PBS, Sigma Aldrich, 10 mM, pH 5.0 and 8.0 at 37 ◦C a). Scaffolds were weighted before immersion (W0). At different time points, the scaffolds were dabbed on a filter paper to remove excess solvent and weighted (Wt). The test was carried out until saturation. Water uptake was defined as follows:

W (%) = Wt − W0/W0*100

Three swelling experiments were performed for each sample.

2.4.4 Dynamic mechanical analysis

TA-Q800 (TA-Instrument, New Castle, DE, United States of America) was employed to perform dynamic mechanical analysis (DMA) of the scaffolds. The frequency was set at 1.0 Hz, which simulates the physiological stride. Furthermore, an amplitude of 100 µm in compression, a preload of 0.001 N and a force track of 125% were adopted. Tests were carried out in a closed chamber in a wet state, and at room temperature. Sample dehydration was negligible as the measurement procedure required only a few minutes.

2.5 cell viability

2.5.1 In vitro indirect cytotoxic Assay

Mouse adipose tissue fibroblasts, L929 cells, were cultured in low-glucose DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% Pen-Strep solution in a humidified incubator, at 37 °C, and 5% CO_2.

To estimate cell viability, L929 cells were seeded into a 96-well microtiter plate at a density of 1x10^4 cells/well, and incubated at 37 °C. After 24 h, cells were treated with 100 ml of medium previously acclimated with the sea bass collagen. In detail, 2.5 ml low-glucose DMEM, was conditioned overnight by immersing 100 mg of each type of prepared scaffold, according to the ISO 10993-5 guidelines. Then, the medium was centrifuged at 14000 rpm for 15 minutes, and the supernatant was used as culture medium. Cells were incubated for 24 h at 37 °C. Each condition was tested in triplicate. After 24 h of treatment, cell viability was assessed using the 3-(4,5-dimethyl-2-thizolyl)-2,5-diphenyl-2H-tetrazolium bromide MTT assay (Mosmann 1983). 10 µl of MTT/PBS solution (0.5 mg/ml) was added into each well and incubated for 3 h at 37°C in a humidified atmosphere. The reaction was stopped by removal of the supernatant and the

formazan crystals were dissolved with 100µL of isopropanol for 1 h. Absorbance was measured at 570 nm by using a microplate reader (Biotek Synergy HT Multi-Mode Microplate Reader, Agilent). Cell survival was expressed as the percentage of viable cells compared to untreated control cultures.

2.6 Turbidimetric assay

For turbidimetric assay, *Staphylococcus aureus* ATCC® 6538 and *Candida albicans* were plated on commercial Mueller Hinton (MH) and YPD (10 g/L Yeast extract, 20 g/L Bacto peptone, 20 g/L Dextrose (glucose)) agar plate, and incubate overnight at 37 °C. Then, 2-3 colonies of *S. aureus* were transferred in 3 ml of MH and incubated in agitation overnight at 37°C, while 1-2 colonies of *C. albicans* were dissolved in a sterile water directly from the agar plate just before the test. The turbidimetric assay tubes were prepared with *S. aureus (*4x10^6 CFU/ml) or *C. albicans* (3x10^5 CFU/mL) in 2 ml of MH and YPD medium, respectively. Then, 10 mg of each scaffold (previously sterilized by UVC for 30 minutes) were immersed in the experimental tube and incubated at 37 °C overnight. Tubes without scaffold were used as bacteria growth controls; gelatin methacryloyl (GelMA) (Yue et al. 2015) was used as a negative control.

3. Results

3.1 Collagen extraction from sea bass skin

ASC extraction by acetic acid is one of the most used methods to obtain collagen from marine organisms (Arumugam et al. 2018). The collagen yield is dependent on the concentration of the solvent used up to a maximum concentration of 0.6 M, above this concentration the collagen yield decreases ((Wang et al. 2014)). In this study, acetic acid at the concentration of 0.5 M in a ratio of 1:50 (w/v) was used for the ASC extraction from skin of *D. labrax*. The extraction with acetic acid (1:50 v/w) was performed twice, and called I and II extraction. The final yield of sea bass collagen extracted from skin was 17.8% (wet weight basis), according to studies previously reported (Sinthusamran, Benjakul, e Kishimura 2013). Type I collagen is the most common type of collagen in nature, found primarily in the vertebrate skin, consisting of a triple helix structure stabilized by interchain hydrogen bonds between glycine and amide groups in the polypeptide chains (Henriksen e Karsdal 2024). SDS-PAGE analysis (Figure 1) showed characteristic bands of type I collagen, formed by an heterotrimer of two α1 chains and one α2 chain, with a molecular weight of approximately 135 kDa and 120 kDa,

respectively, corresponding to the molecular mass reported in previous publication on sea bass skin collagen (Sinthusamran, Benjakul, e Kishimura 2013). Bands at higher molecular weights were also observed, at around 250 kDa for the β band, and above 300 kDa for the γ band, assimilable at the presence of dimers formed by α 1 and α 2 chains together or two α 1 chains (β chain) and trimers formed by 3 α chains (γ chain), respectively (Akkus, Belaney, e Das 2005). Moreover, no difference in the protein patterns between first and second collagen extraction was observed.

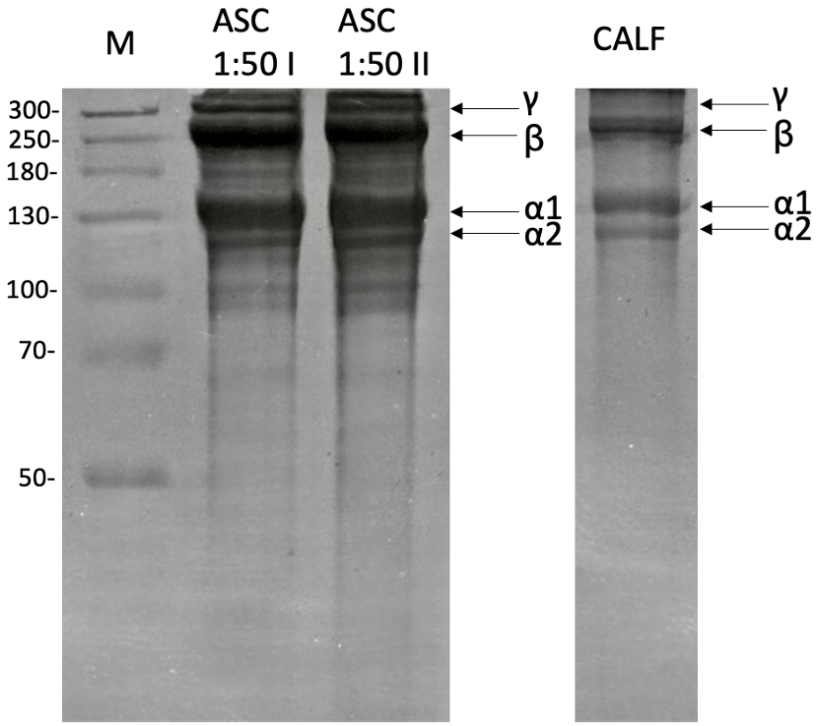

Figure 1. SDS–PAGE (7.5 %) electrophoretic pattern for acid-soluble collagen. (M) Molecular weight marker; (ASC 1:50 I, ASC 1:50 II) collagen extracted by the first and the second extraction, respectively; (Calf) calf skin collagen, type I.

3.2 Scaffolds preparation and characterization

In this study, different type of chitosan/sea bass collagen scaffolds were prepared by using a freeze-drying process, as described in the paragraph 2.3, by using two concentrations of chitosan (20 - 40 mg/mL) and collagen (5 - 10 mg/mL).

Figure 2 shows the effect of TPP crosslinking on CS scaffolds. In particular, the characteristic bands of CS were represented. The bands between 3500 and 3000 cm^{-1} correspond to -OH and -NH stretching frequencies. It is possible to observe the bands of

C-H stretching in the range 2920–2875 cm^{-1}; C = O stretching of acetylate groups (amide I) at 1650 cm^{-1}; N-H bending of primary amine at 1550 cm^{-1}.

Meanwhile, the spectrum of TPP is characterized by the bands centered at 880 cm^{-1} attributed to the antisymmetric stretching of the P-O-P bond. The band centered at 1095 cm^{-1} can be attributed to the symmetric and antisymmetric stretching of the PO_3 group, the one of 1150 cm^{-1} related to the symmetric and antisymmetric stretching of the -PO_2 group, and 1215 cm^{-1}, relative to the P = O stretching (Silvestro et al. 2020).

By comparing the spectra of CS2 and CS4 scaffold (crosslinked with TPP) with that of the pristine CS, several variations can be noticed due to the cross-linking reaction. Indeed, in the spectra of the modified scaffolds, besides the peak at 880 cm^{-1} typical of TPP molecule, a new band at 1215 cm^{-1}, related to the antisymmetric stretching vibrations of -PO2 groups of TPP ions, is present as an index of the formation of ionic interactions between CS and TPP. Due to the low degree of cross-linking this latter peak was not very pronounced in the spectrum of sample CS2 compared to CS4 (Fernandes et al. 2011; Ferreira Tomaz et al. 2018).

Figure 3 reports the FTIR spectra of CS2 and CS4 with collagen 0.5% w/v and 1.0 % w/v. The Amide A position was found at 3300 cm^{-1} from sea bass collagen, which represents the absorption band of N–H stretching, and indicates the presence of hydrogen bonds. The amide B band of the collagen was observed at 2932 cm^{-1}, which is related to the CH_2 asymmetric stretch. The peak of Amide I is mostly associated with the stretching vibrations of the carbonyl groups (C=O bond) along the polypeptide backbone. The Amide I band of collagen was observed at 1631-1642 cm^{-1}. The amide II band of collagen appeared at 1547 cm^{-1} and amide III band was observed at 1236–1237 cm-1, resulting from NH bend coupled with CN stretch and NH bend.

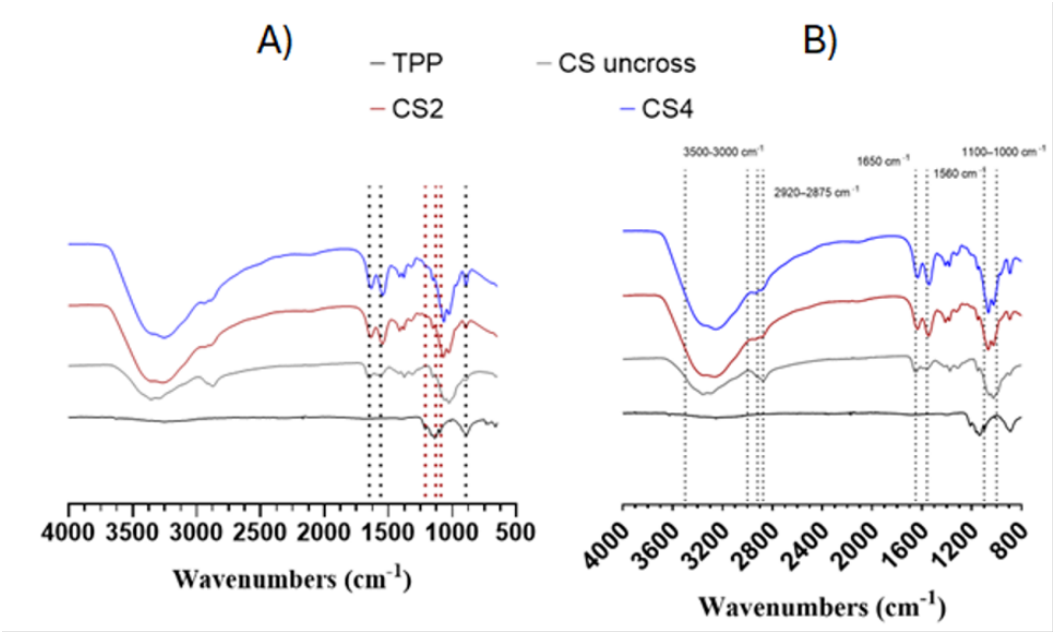

Figure 2. ATR-FTIR spectra of: sodium triphosphate (TPP), chitosan scaffolds at 2% w/v and 4% w/v crosslinked with TPP (CS2 and CS4, respectively) and CS uncrosslinked (CS uncross) between (A) 4000 and 500 cm^{-1}, and (B) 2000 and 800 cm^{-1}.

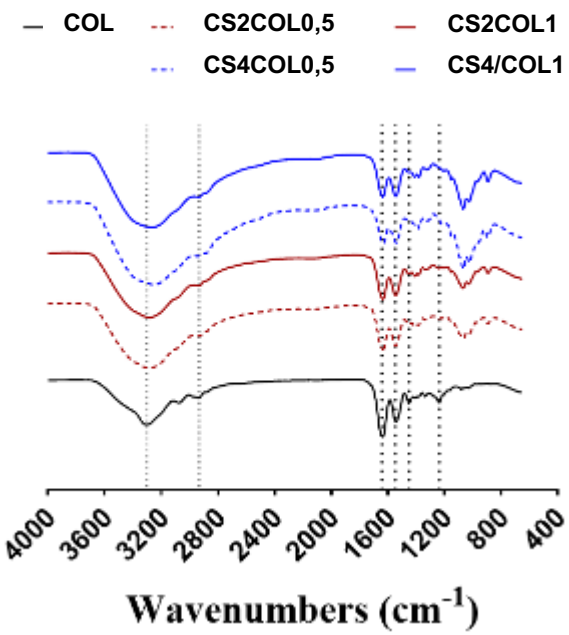

Figure 3. ATR-FTIR spectra of: Collagen (COL), CS2 and CS4 with sea bass collagen (0.5% w/v and 1.0% w/v) between 4000 and 500 cm^{-1}.

Figures 4 and 5 report the swelling scaffolds ability. In particular, it is clear that by increasing the chitosan concentration the swelling ratio decreased. Furthermore, the addition of sea bass collagen increased the swelling ratio. At pH=5.0, the swelling ratio

was slightly higher for all the compositions. This different behaviour could be ascribed to the known dissolution phenomenon of chitosan, being the pH lower than its pKa (≈ 6.3–6.5) (Dacrory et al. 2024).

As a wound dressing or scaffold, the materials should have mechanical properties that can withstand external forces maintaining their structural integrity. It is widely recognized that skin has a Young's modulus in the range of 10 kPa to 50 kPa. Previous studies have reported that regeneration is enhanced under conditions where i) the bulk modulus of the scaffold exceed that of host dermal tissue (50 kPa), and ii) scaffold persists throughout all stages of the remodeling process (Guo et al. 2015). Dynamic mechanical analysis (DMA) results showed that the storage modulus is strongly influenced by both chitosan and collagen concentrations. Indeed, by increasing chitosan concentration, it varied from 12.6 kPa to 27.2 kPa. Meanwhile, by increasing marine collagen concentration the modulus increased from 18.3 to 29.2 kPa (CS2) and from 31.9 to 51.6 kPa (CS4). Such high elastic moduli was useful considering the significant forces occurring during wound healing (Berry et al. 2023), and they have also been demonstrated to favorably stimulate fibroblasts growth (Hadjipanayi, Mudera, e Brown 2009).

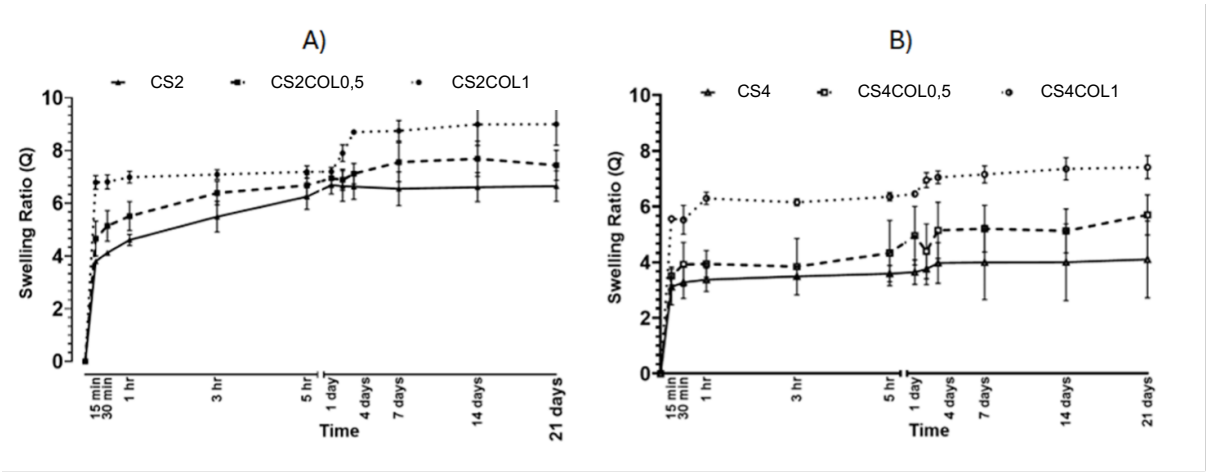

Figure 4. Swelling behavior of CS2 and CS4 scaffolds with and without collagen (0.5% w/v and 1.0% w/v) at pH=5.

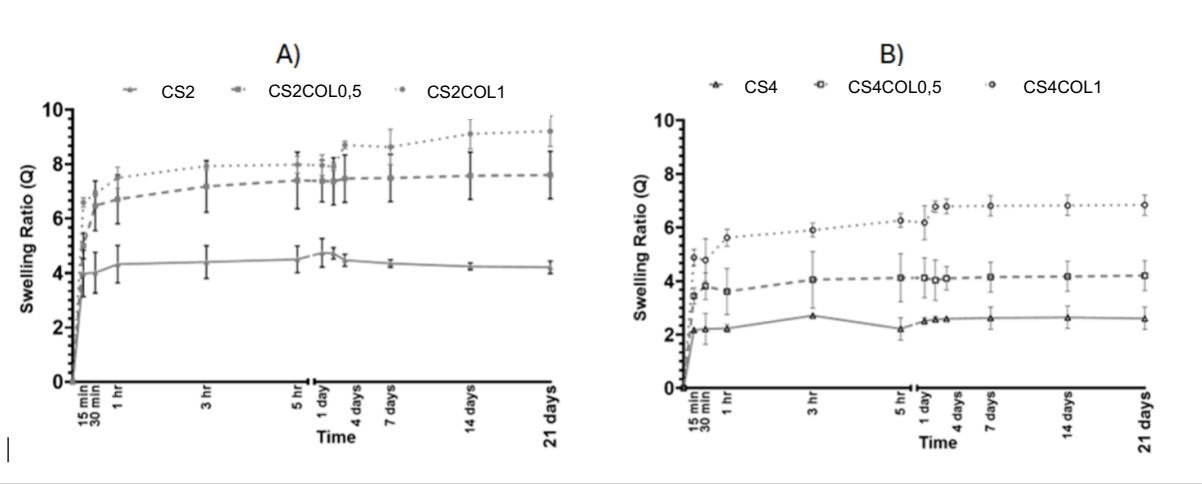

Figure 5. Swelling behavior of CS2 and CS4 scaffolds with and without collagen (0.5% w/v and 1.0% w/v) at pH=8.

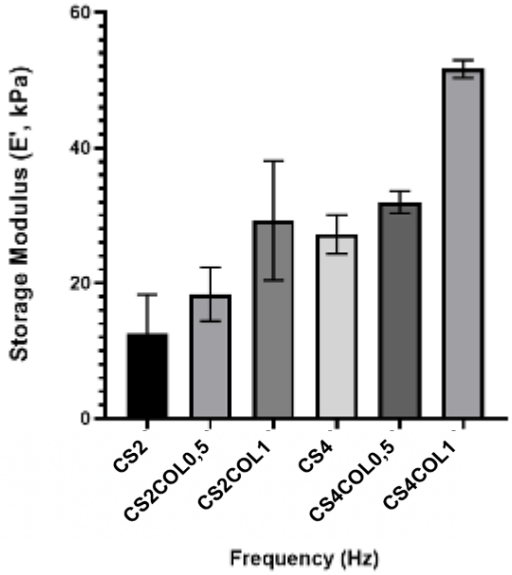

Figure 6. Results from Dynamic mechanical analysis (DMA) performed on CS2 and CS4 scaffolds with and without collagen (0.5% w/v and 1.0% w/v). Results are expressed as mean value ±standard deviation.

Figure 7 shows SEM micrographs of the chitosan and chitosan/collagen scaffolds. Open pore structure with a high degree of interconnectivity can be observed. For neat CS, by increasing the chitosan concentration, CS4 highlighted a denser and more compact polymer network compared to CS2, with smaller and homogeneously distributed pores. It can be observed that the addition of collagen to chitosan increased both pore aperture

and porosity of the scaffolds, for both studied compositions. The same trend was reported in the literature (Tan, Krishnaraj, e Desai 2001; Fernandes et al. 2011). In its natural state, the collagen triple helix structure is held together by direct chemical bonds, hydrogen bonds, and water bridged crosslinks. The large number of amino groups in chitosan chains seems to reinforce the fibers by anchoring them in place and functions as crosslink to increase the overall matrix integrity.

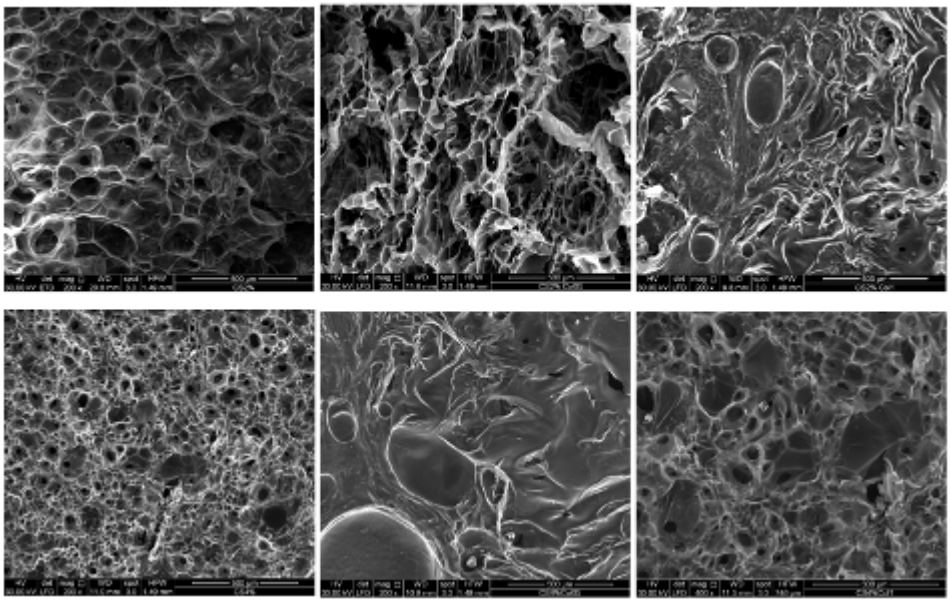

Figure 7. SEM analysis performed on CS2 and CS4 scaffolds with and without collagen (0.5% w/v and 1.0% w/v).

3.3 In vitro cytotoxic assay

The scaffolds were tested for their possible cytotoxic activity against murine fibroblast L929 cells. For this assay, 100 mg of each scaffold were soaked in 2,5 ml low-glucose DMEM overnight to test if the produced scaffolds can release cytotoxic compounds in the media. Then, the supernatant was used as a media (conditioned media) for the cell growing. For the control only low-glucose DMEM was used. After 24 h of incubation at 37 °C cell survival was measured by MTT test. The screening was performed in triplicate. As shown in Figure 8, after 24 h of treatment, conditioned media with scaffold did not show any cytotoxic activity on L929 cells compared with the control. On the contrary, the cells treated with media conditioned with scaffold CS2COL0,5, CS2COL1, CS4 and CS4COL1 showed an increase of the growth up to 175%, compared with control. This result indicates that these scaffolds could have a pro-proliferative activity.

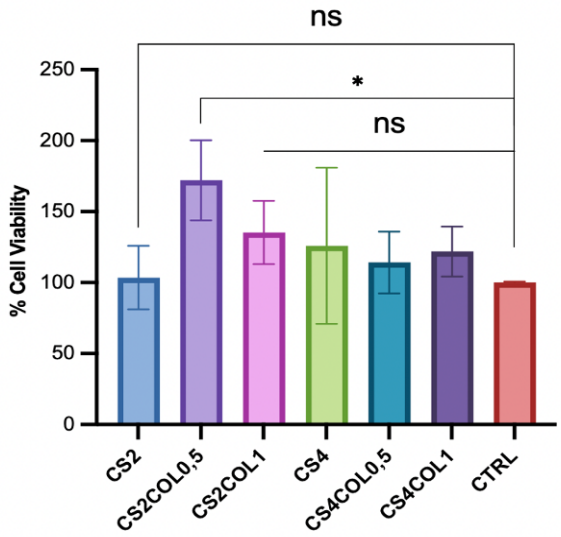

Figure 8. *In vitro* cytotoxic assay on L929 cell line (murine fibroblast); CTRL: respective control using unconditioned culture medium. Results are expressed as percentage of cell survival after 24 h exposure (n = 3).

3.4 Turbidimetric assay

The scaffolds were tested for their antimicrobial activity against two human pathogens, the gram-positive bacterium *S. aureus* and the fungus *C. albicans*. Figure 9 shows that, after 24 h incubation, microbial growth was absent in the presence of scaffolds in all tubes, except for CS4 (tube 6) in presence of *S. aureus* (Figure 9a). On the contrary, microbial growth was evident in absence of scaffolds (tubes 1) or in presence of GelMA (tubes 2) known to have no antimicrobial activity (Vargas-Alfredo et al. 2022). Although growth can be seen for CS4 (tube 6) in presence of *S. aureus*, it is much lower than the growth control (Tubes 1 and 2) (Figure 9a).

Literature reports that chitosan is considered a potent antimicrobial material; however, the mechanism of action is still cloudy, and different possible mechanisms of action are reported. The most acceptable involves the electrostatic charges of the positively charged NH^{3+} groups of chitosan that could interact with the negative charges of bacterial cell membranes, leading to permeability of the membrane wall, thus causing osmotic imbalances or hydrolysis of peptidoglycan with consequent release of cellular material. Another mechanism of action could involve the chitosan binding to microbial mRNA, inhibiting protein synthesis. Chitosan would be able to migrate into the nucleus and interact with the nucleic acids of microorganisms (Goy, Britto, e Assis 2009; Nasaj et al.

2024). Therefore, our preliminary results show that the addition of marine collagen to chitosan-based scaffolds maintains the already known antimicrobial activity of chitosan.

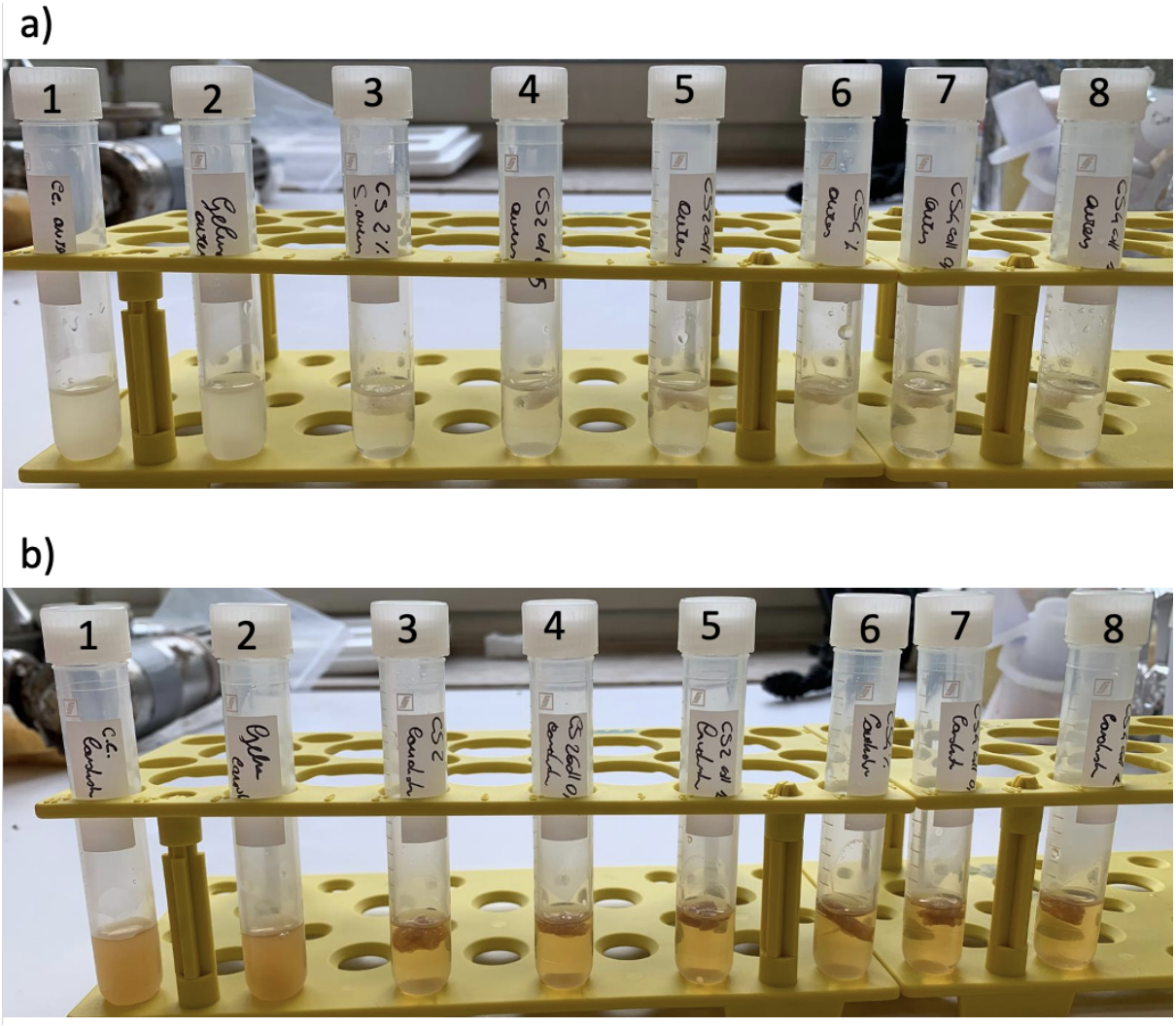

Figure 9. Turbidimetric assay on a) *S. aureus* and b) *C. albicans*. 1) absence scaffold (growth control); 2) GelMA (negative control); 3) CS2; 4) CS2COL0,5; 5) CS2COL1; 6) CS4; 7) CS4COL0,5; 8) CS4COL1.

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CHAPTER 4

Mediterranean Jellyfish *Cotylorhiza tuberculata* as a potent source of bioactive molecules

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1. Introduction

In the last decades, the occurrence of gelatinous plankton is increasing sharply in the marine realm due to a combination of factors, which include climate change with increased temperatures, variations in water mass, salinity, and eutrophication (Jennifer E. Purcell 2005; J. Purcell, Uye, and Lo 2007), and various anthropic disturbances or disappearance of apex predators from the environment (Boero et al. 2008; Utne-Palm et al. 2010; Jennifer E. Purcell e Arai 2001; J. Purcell, Uye, and Lo 2007). Such increase has also led to a higher amount of jellyfish within the bycatch, thus negatively impacting fisheries and aquaculture activities (Bosch-Belmar et al. 2021), and somehow increasing pollution, as these catches are generally discarded as waste (Hall, Alverson, e Metuzals 2000).

In recent years, the recycling of these fishery wastes is becoming a key topic to be solved (Coppola et al. 2021). Several studies have shown that bycatch species can be an excellent source of bioactive molecules (Mutalipassi et al. 2021; Ozogul et al. 2021; Jimenez-Champi et al. 2024). For example, jellyfish, with their high protein and collagen content, are the subject of growing interest due to their potential applications in the functional foods, cosmetics, and pharmaceutical industries. In particular, as highlighted by previous studies (Raposo et al. 2022; J.E. Purcell, Baxter, and Fuentes 2013), jellyfish offer a promising and sustainable nutritional source. In Asia, jellyfish have been consumed as food for centuries, usually dried and salted, and are still used today in soups and salads, especially in China, Japan, and Korea (Edelist et al. 2021). Moreover,

recently, considering the increasing interest in jellyfish due to their nutritional properties, and the need to find new uses for bycatch species, Western researchers are also exploring new methods to make them more palatable to consumers (Omori e Nakano 2001; Raposo et al. 2022).

Recently, there has been a growing interest in the research and development of functional foods, nutraceuticals, and food supplements (Ngo et al. 2011); enriching foods with nutraceuticals is becoming an increasingly popular approach to offer healthy food products. In this regard, studies are increasing regarding the production of bioactive peptides as possible functional ingredients in medicine and food industries, due to their important physiological functions (e.g. antioxidant capacity), and whose range of bioactivity depends on their amino acid sequences and structures (Venkatesan et al. 2017; Sarmadi e Ismail 2010).

Actually, a good amount of bioactive hydrolysates/peptides have been isolated from several jellyfish species (Cadar et al. 2024; Barzkar et al. 2024). The characterization of edible jellyfish, including different Mediterranean species (e. g. *Aurelia* sp., *Cotylorhiza tuberculata*, and *Rhizostoma pulmo*), demonstrated the presence of a protein content up to 40% of their dry weight in collagen, representing a rich source of bioactive peptides and hydrolysates, with many beneficial health applications (Raposo et al. 2022; Leone et al. 2015). For example, jellyfish seems have significant implications in the pharmaceutical sector as its peptides showed potential for inhibiting angiotensin-I-converting enzyme (ACE) (Li et al. 2014), antimicrobial activity (Ovchinnikova et al. 2006), immune-stimulation effects (Sugahara et al. 2006), anti-proliferative activity (Riccio et al. 2022; De Domenico et al. 2019).

It is known that the production of reactive oxygen species (ROS) is one of the major causes of various human degenerative diseases associated with mechanisms of oxidative stress, due to attack the membrane lipids, protein, and DNA (Ahmed e Mohammed 2020). In this regard, several studies have demonstrated promising in-vitro radical scavenger activity of peptides isolated from different jellyfish species, such as *Rhopilema esculentum* and *R. pulmo*, suggesting jellyfish as a rich source of antioxidant peptides (De Domenico et al. 2019; Li et al. 2014; Teng et al. 2023; Zhuang et al. 2010).

Similarly, studies on *C. tuberculata* from the Mediterranean Sea also showed a high amount of bioactive peptides, including antioxidant peptides, opening the door to the use of *C. tuberculata* in the Western market as a possible high value-added food, and a high level of total phenols, probably due to the rigidity of the mesoglea, suggesting that these

compounds may play a role in the anatomical adaptation of the jellyfish (Leone et al. 2015). Moreover, antioxidant capacity was observed in *Aurelia* sp., *C. tuberculata*, and *R. pulmo*, both in protein and non-protein extracts, and in hydrolyzed fractions. In particular, the two latter species showed significantly higher antioxidant activity, probably due to the presence in these two species of high content of amino acids usually correlated to antioxidant activity.

Moreover, the effects of heat treatment on jellyfish as a first processing step for food or nutraceutical purposes were also evaluated (Leone et al. 2019). In particular, it was examined how different heat treatment methods affect the content of antioxidants, phenolic compounds, and proteins in different common Mediterranean jellyfish. For example, after heat treatment – 100 °C for 10 min to mimic jellyfish boiling – the antioxidant activity increased in *C. tuberculata* oral arms, suggesting that this treatment could be effective as a food processing method.

In this study, new and deeper information regarding the bioactivities of *C. tuberculata* proteins and hydrolysates will be discussed, for the production of possible functional foods. The fried egg jellyfish *C. tuberculata* (Macri, 1778) is a common inhabitant of the Mediterranean Sea, where its blooms are increasing. It lives in both coastal and open-sea environments, where it occupies a key ecological niche as a planktonic predator, feeding mainly on diatoms, ciliates, molluscan larvae, and copepods (Pérez-Ruzafa et al. 2002; Kikinger 1992).

The production of biologically active peptides is generally carried out by several methods, including acid-alkaline hydrolysis (using acidic or alkaline reagents), enzymatic hydrolysis (using digestive enzymes), and fermentation (using microorganisms as a source of enzymes) (Coppola et al. 2021). Interestingly, enzymatic hydrolysis is particularly popular in the food and pharmaceutical industries, as the process leaves no residues of toxic chemicals and solvents (Kim e Wijesekara 2010), and not compromising the nutritional value (Huang et al. 2015). For this reason, in this study, the protein extraction of *C. tuberculata* was performed by PBS, followed by the production of hydrolysed proteins by sequential enzymatic hydrolysis, using pepsin and collagenase, to study the possible use of this jellyfish for the production of functional foods. Moreover, the obtained samples were fractionated based on the molecular weight, and the fractions analyzed for their antioxidant and antiproliferative activity. In addition, to use the bioactive proteins/hydrolysates of *C. tuberculata* in food applications, it was investigated how they could influence the inflammatory state of the human intestine.

2. Results and Discussion

2.1. C. tuberculata Collection and Soluble/Hydrolysed Proteins Extraction

C. tuberculata is a rhizostome jellyfish mostly widespread in the Mediterranean Sea. Its medusoid form can reach about 40 centimeters in diameter and is characterized by a brownish/whitish flat and disc-shaped umbrella, whose margin is typically jagged and that possesses a darker colored and slight convex protuberance in the middle of it. Under the umbrella, eight main oral arms branch off from the four lobes of the mouth, each of them bifurcating near its base and then branching several more times. The species also has several smaller and club-shaped tentacles, each of them bearing disk-like ends that are blue/purple in color. As many jellyfish species, *C. tuberculata* undergoes a complex life cycle involving both sexual and asexual phases, with the medusoid form having been always prominent from July to November (Avian 1986). However, in last decades, and mostly as a result of the global warming, with always milder winters and hotter summers, blooms of *C. tuberculata* are rapidly increasing, and medusae of the species have been occurring all over the year and in always higher abundances (Ruiz, Prieto, and Astorga 2012; Prieto et al. 2010). Apart from the variegate ecological impacts of these jellyfish outbreaks, they may also have negative consequences on many sea-based human activities, including fishery/aquaculture and tourism/leisure, with cascade effects on human economy, including higher management costs to be faced by national and local authorities (Pagès 2001; Leone et al. 2015; Bosch-Belmar et al. 2021).

For our study, *C. tuberculata* was collected from the bycatch of commercial fishing activities held in Naples, in the central-western Mediterranean Sea (Italy), to explore its potential as a food supplements.

In a previous study conducted by Leone and collaborators (Leone et al. 2015), the whole jellyfish *C. tuberculata* showed high antioxidant activity, especially when extracted in PBS (approx. 25,000 nmol of TE/g of dry weight), compared to hydroalcoholic solvents, e.g., methanol and ethanol (approx. 8,000-10,000 nmol of TE/g of dry weight). However, it is known that in total extracts, the bioactivity of a compound can be masked, or in any case be lower, compared to enriched fractions or pure compounds. For this reason, in this study, we performed a purification step of the samples by filtration, as done for the purification of other bioactive peptides from jellyfish, such as *R. esculentum* (Li et al. 2014) and *R. pulmo* (De Domenico et al. 2019). Moreover, we present the bioactivities in umbrella and oral arms separately, to understand if they are attributable to both jellyfish parts.

Therefore, freeze-dried umbrella and oral arms samples of jellyfish *C. tuberculata* were separately subjected to aqueous protein extraction using PBS, to extract the water-soluble protein fraction, considering that treatments with methanol or ethanol limit the protein extraction in the jellyfish. On the contrary, the pretreatment based on ethanol and methanol seemed to be a good strategy for fats and pigments removal. In particular, *C. tuberculata* hydroalcoholic extracts contained carotenoids and fatty acids, probably derived by algal symbionts (Leone et al. 2015).

In addition, the remaining insoluble portion was subjected to sequential enzymatic digestion with pepsin and, subsequently, collagenase, as previously reported (Leone et al. 2015), to obtain soluble pepsin- and collagenase-digested hydrolyzed proteins, respectively. Sequential enzymatic hydrolysis, in which each step involves the use of a single and commercial enzyme, rather than the combined use of multiple proteases, is dictated by the need to find an easily reproducible and controllable process, to obtain a relatively controlled peptide composition (De Domenico et al. 2019).

The soluble portions were fractionated based on molecular weight, using membranes filtration with different cutoff (30 kDa, 10 kDa, 3 kDa). For each sample, four subfractions were obtained, separated based on their molecular weight: > 30 kDa, between 30 and 10 kDa, between 10 and 3 kDa, and <3 kDa, as reported previously (Ruiz, Prieto, e Astorga 2012; Prieto et al. 2010). After fractionation and freeze-drying, PBS soluble fractions accounted for the majority of the weight, and it was approx. 67.7% and 73.5% for the umbrella and oral arms, respectively. Moreover, soluble pepsin- and collagenase-digested hydrolyzed proteins were 25.6% and 6.7% for the umbrella, and 19.6% and 6.9% for the oral arms, respectively.

All fractions obtained after filtration were tested for in vitro antioxidant and antiproliferative activity on A2058 melanoma cell cultures. Furthermore, the cellular cytotoxicity of the samples was also evaluated on human keratinocyte HaCaT cells.

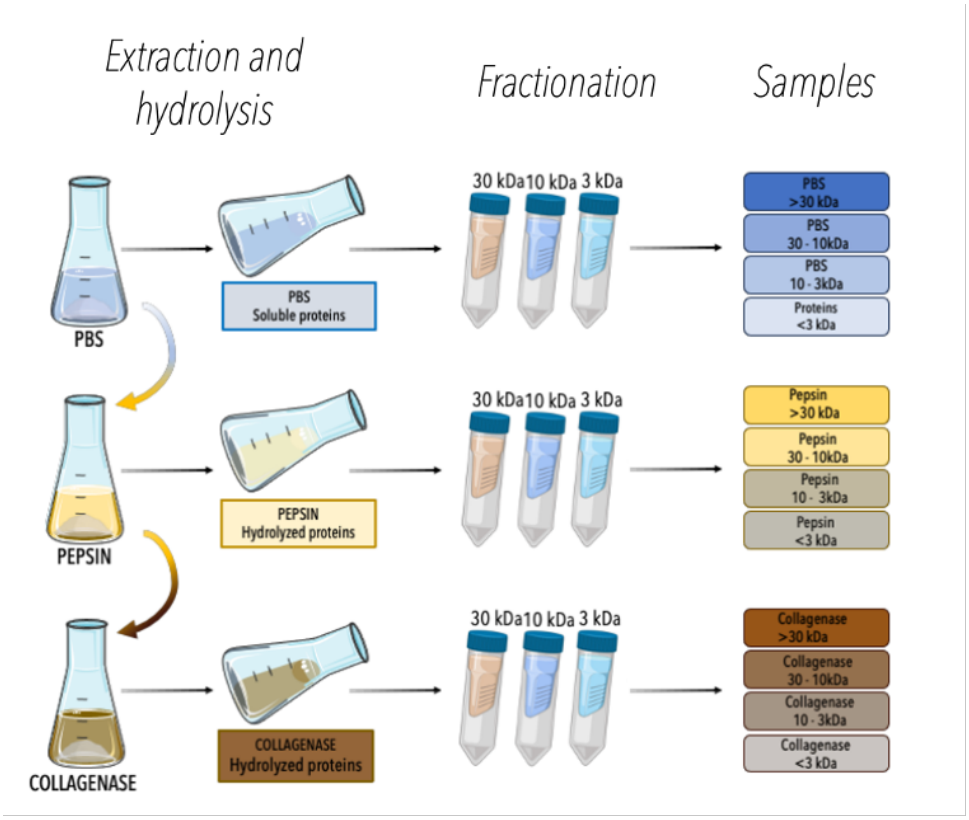

Figure 1. Schematic representation of different steps of soluble proteins extraction, sequential hydrolysis, and fractionation based on molecular weight of the proteins of *C. tuberculata* umbrella and oral arms, by membrane filtration. On the right of the diaphragm are reported the names of the fractions obtained, used in the following paragraphs.

2.2 In vitro Antioxidant activity

Recently, peptides obtained from marine organisms have been increasingly useful in various fields - such as cosmetics and nutraceuticals, especially for their antioxidant properties (De Domenico et al. 2019; Liu et al. 2016; Wu et al. 2015). In this study, the soluble and hydrolysed protein fractions obtained from *C. tuberculata* umbrella and oral arms (see paragraphs 2.1) were analyzed for their radical scavenging activity by ABTS (2,20-Azinobis (3-ethylben-zothiazoline-6-sulfonic acid) diammonium salt] assay, a spectrophotometric method widely used for antioxidant natural products discovery. However, the fraction including soluble proteins at molecular weight < 3 kDa were not tested due to their high salt content. Similarly, also fractions including pepsin- and collagenase-hydrolysed peptides at molecular weight > 30 kDa were not considered, as

they conteined the pepsin (approx. 34.5 kDa) and the collagenase (approx. 68–130 kDa) enzymes, respectively.

The antioxidant activity was reported as nmol of Trolox equivalents (TE) per gram of dry weight, and shown in Figure 2.

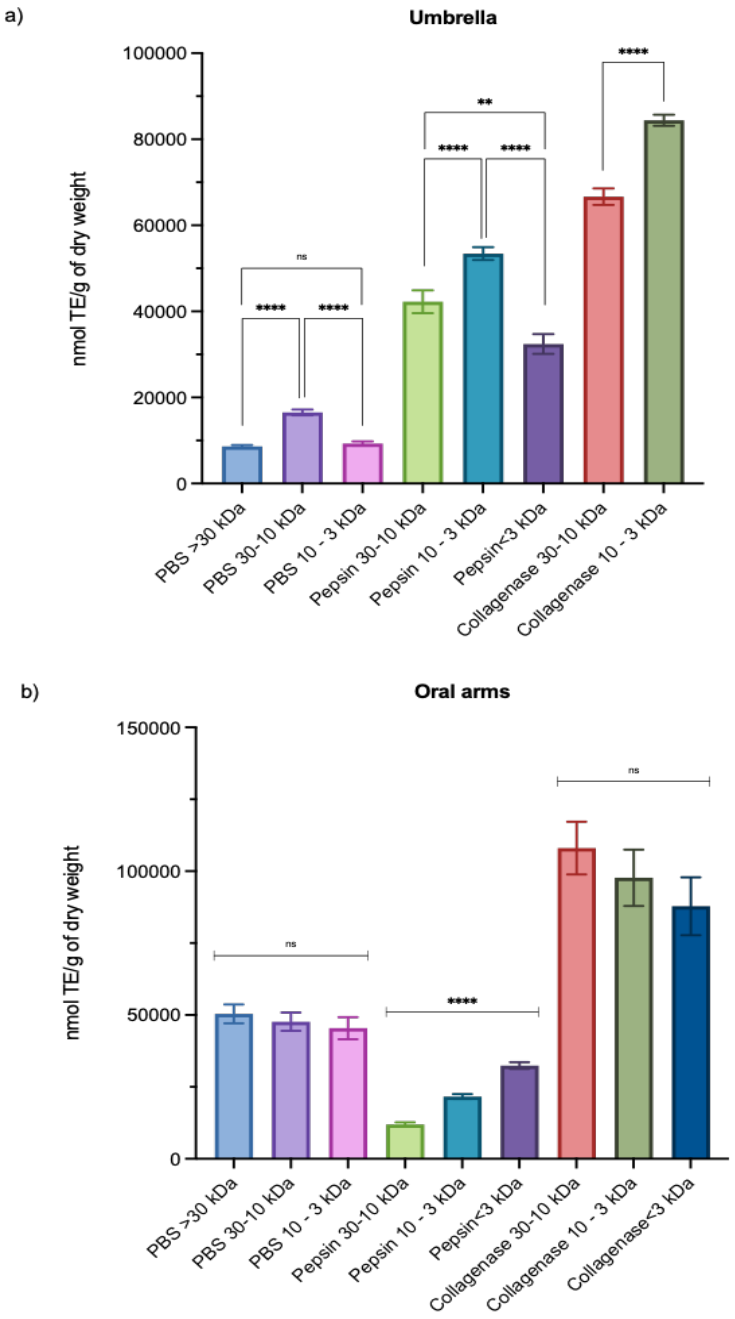

Figure 2. In vitro antioxidant activity of *C. tuberculata* a) umbrella and b) oral arms. Fractions of soluble protein extracted in PBS with different molecular weight: PBS > 30 kDa, PBS 30-10 kDa, PBS 10-3 kDa; Fractions of soluble pepsin-hydrolysed proteins with different molecular weight: Pepsin 30-10 kDa, Pepsin 10-3 kDa, Pepsin < 3 kDa;

Fractions of soluble collagenase-hydrolysed proteins with different molecular weight: Collagenase 30-10 kDa, Collagenase 10-3 kDa, Collagenase < 3 kDa. Data are the mean values of three independent experiments performed in three technical replicates. The antioxidant activity is expressed as nmol of TE per g of dry weight ± standard deviation. ANOVA ordinary one-way Bonferroni's MULTIPLE COMPARISON 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0,0001 (****).

The results showed antioxidant activity in all fractions tested. Regarding the soluble proteins extracted in PBS, the antioxidant activity was much higher in the protein fractions obtained from *C. tuberculata* oral arms, than in the umbrella. However, the antioxidant activity in the aqueous extraction may also be due to the production of antioxidants from the endosymbiotic microalgal organisms (zooxanthellae) of dinoflagellates that *C. tuberculata* hosts in its endodermal tissue (kim et al. 2000). The presence of endosymbiotic dinoflagellates associated with the jellyfish in the first vital states represent an important biological aspect linked to the success of *C. tuberculata* populations, as it promotes its maturation (Kikinger 1992; Leone et al. 2013).
In addition, the good antioxidant activity found in the soluble fractions of *C. tuberculata* oral arms could be due to the protein toxins abundantly present both in the nematocysts in the ectodermal and in the mucus of *C. tuberculata*, as already reported for the jellyfish *R. pulmo* (De Domenico et al. 2019). The hydrolysates obtained from digestion with pepsin showed a lower activity in oral arms *C. tuberculata*, in all the subfractions at different molecular weights, compared to umbrella *C. tuberculata* digested in the same manner.
Collagenase digestion showed the best antioxidant results in all samples, suggesting that collagen peptides are involve in the antioxidant activity, may depending on the amino acid composition of the peptides (Nurilmala et al. 2020). Type I collagen consists of two α1 chains and one α2 chain, containing the internal triple helical regions, and the N- and C-terminal telopeptides. For this reason, although pepsin hydrolysis does not produce peptides with high antioxidant activity, it may be important for the production of bioactive collagen-based peptides by the telopeptides removal. As previously reported (Qian et al. 2017), digestion with pepsin leads to excision of the N-terminal telopeptide, favoring a good digestion by collagenase.

2.3 In vitro anti-proliferative test of C. tuberculata hydrolysates

The soluble proteins and hydrolysates derived from the umbrella and oral arms of the jellyfish *C. tuberculata* were tested for their possible anti-proliferative activity against human malignant melanoma cells A2058 and human spontaneously immortalized keratinocytes HaCaT using the MTT test. The two cell lines were incubated in presence of three concentrations for each sample (10 µg/mL, 50 µg/mL, and 100 µg/mL) and in absence of them. After 24 h (Figures 3 and 5) and 72 h (Figures 4 and 6) of incubation at 37°C with the samples, cell survival was measured by MTT test. The screening was performed using three biological replicates.

Regarding the soluble proteins extracted in PBS, as shown in Figure 3, at the end of the 24 h treatment with *C. tuberculata* umbrella proteins and hydrolysates, between the tested samples, PBS 10 – 3 kDa showed a slight antiproliferative activity against A2058 melanoma cells. In fact, a reduction in cell viability to 46% was observed with 100 µg/ml of PBS 10 – 3 kDa treatment, compared to 100% viability of the untreated. PBS 10 – 3 kDa did not show any cytotoxicity on normal HaCaT cells. Moreover, for compound PBS 10 – 3 kDa the anti-proliferative activity also seemed to follow a dose-dependent trend. Regarding soluble pepsin-hydrolysed proteins, both Pepsin 10 – 3 kDa and <3 kDa showed a reduction in cell viability below 50% at treatment with 100 µg/ml, while on normal HaCaT cells, viability with the same treatment induced a reduction of cell viability to about 75%. However, both pepsin-hydrolysed samples did not show a dose-dependent activity. Finally, regarding soluble collagenase-hydrolysed proteins, only Collagenase 30 – 10 kDa showed a reduction of cell viability below 50% without dose-dependent effect.

To validate the observed results at 24 h treatment with *C. tuberculata* umbrella samples, the anti-proliferative activity under the same experimental conditions on HaCaT and A2058 cells, was also tested for 72 h, as shown in Figure 4.

The soluble proteins extracted in PBS 10 - 3 KDa after 72h of treatment seemed to restore the decrease in vitality observed at 24h. After 72 h of treatment, the soluble pepsin-hydrolysed proteins showed a reduction of cell proliferation on A2058 below 50% at 10 µg/ml and 50 µg/ml. The fraction Collagenase 30 - 10 kDa showed a viability percentage below 50% in A2058 at 10 and 50 µg/mL, the fraction Collagenase <3 only at 10 µg/mL, while the fraction Collagenase 10 - 3 kDa did not show significant results. On normal HACAT cells only the fraction Pepsin 10 – 3 kDa induced a reduction below 50%, while the other samples did not show significant variations.

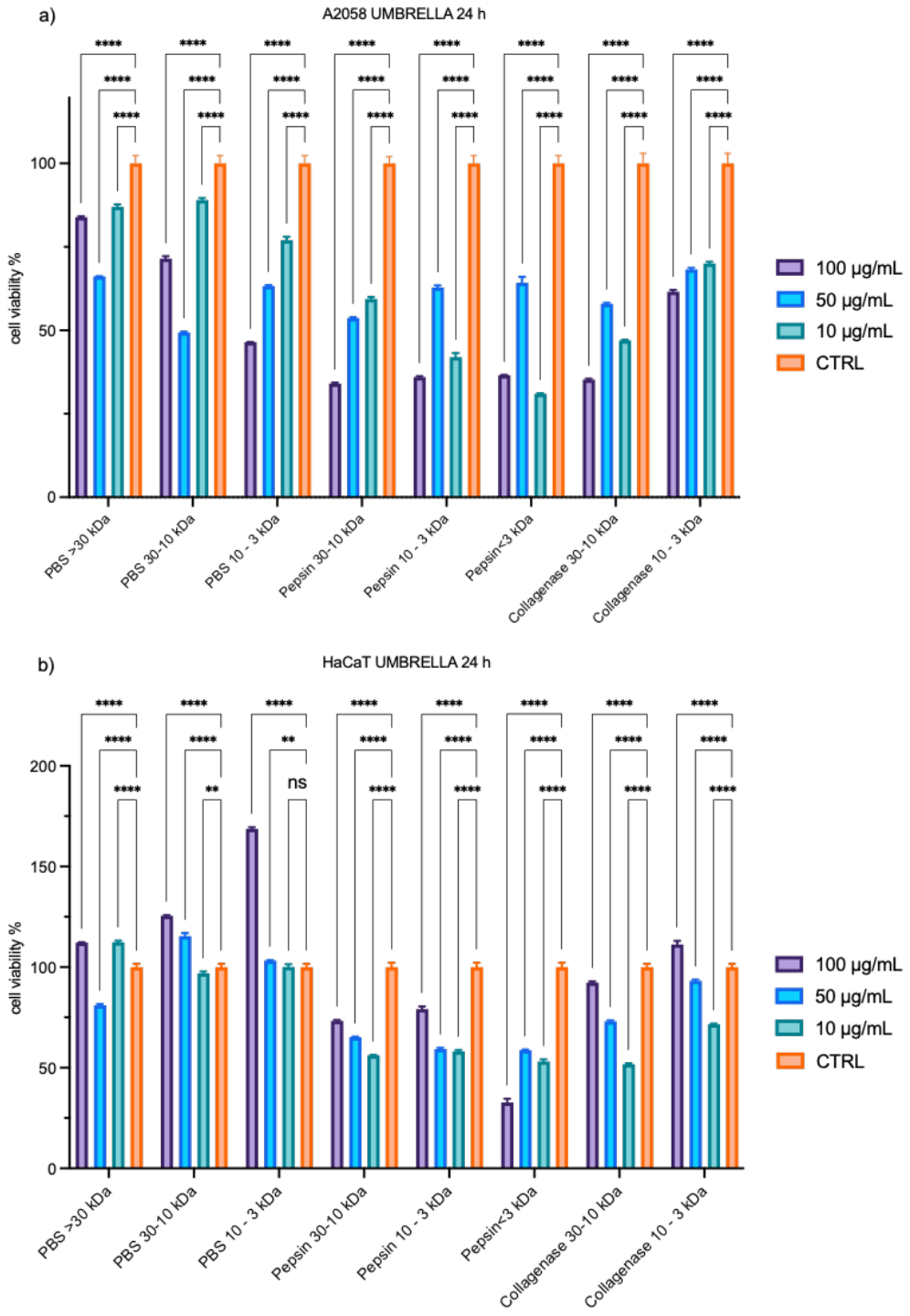

Figure 3. In vitro anti-proliferative assay. The histograms show anti-proliferative effects of *C. tuberculata* hydrolysates extracted from umbrella, on a) A2058 cell line (malignant melanoma) and b) HaCaT (control cell line). Results are expressed as percentage of cell survival after 24 h exposure (n = 3). Cell growth was expressed as the percentage of cell viability with respect to the control for each concentration ANOVA 2WAY Dunnett's MULTIPLE COMPARISON 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0,0001 (****)

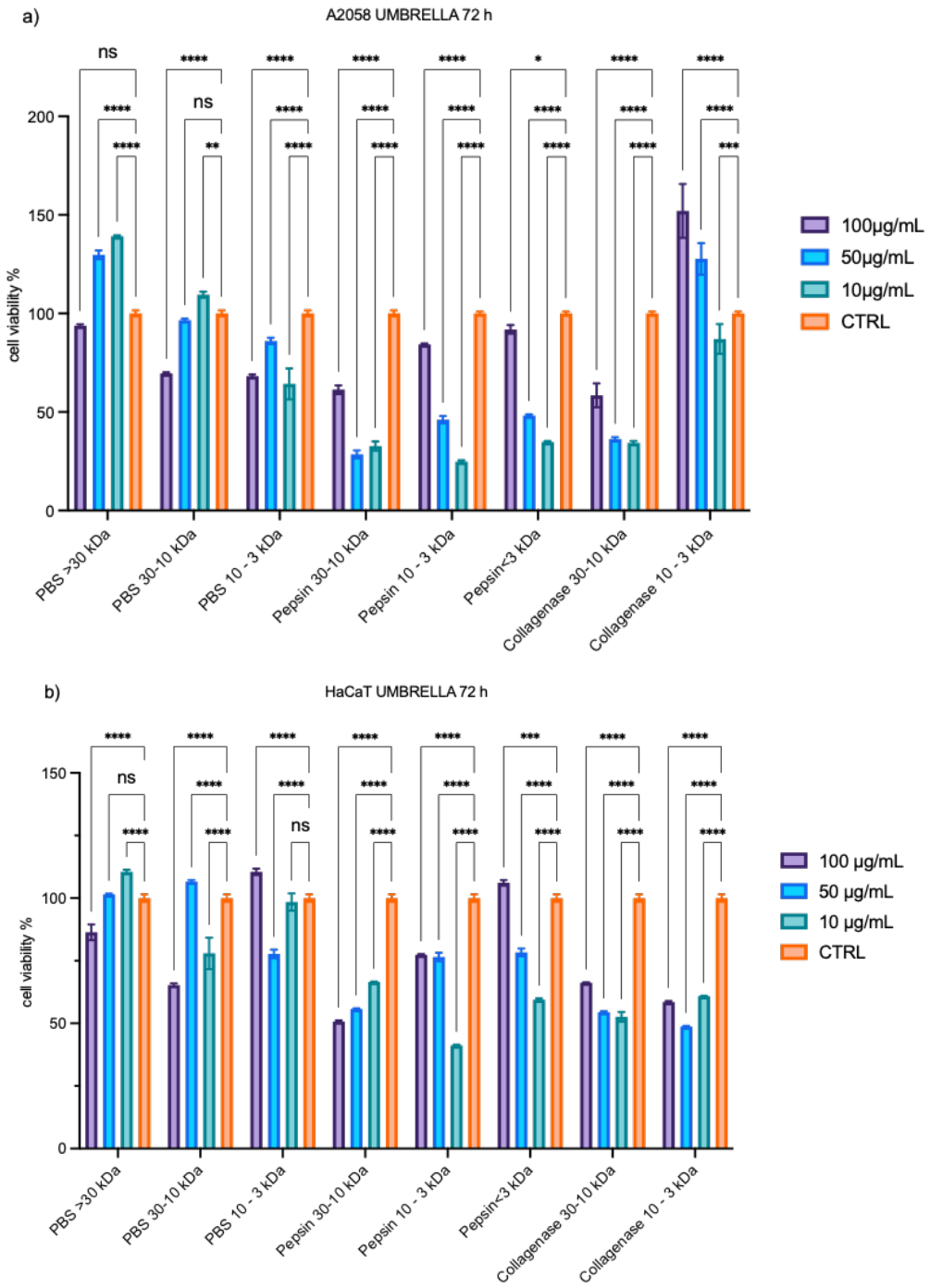

Figure 4. In vitro Anti-proliferative assay. The histograms show anti-proliferative effects of *C. tuberculata* hydrolysates extracted from umbrella, on a) A2058 cell line (malignant melanoma) and b) HaCaT (control cell line) and the respective controls only with the culture medium. Results are expressed as percentage of cell survival after 72 h exposure (n = 3). Cell growth was expressed as the percentage of cell viability with

respect to the control for each concentration ANOVA 2WAY DUNNETT'S MULTIPLE COMPARISON 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0,0001 (****).

Regarding proteins and hydrolysates obtained from *C. tuberculata* oral arms the results are reported in Figures 5 and 6. Results showed that, after 24 h treatment, soluble proteins extracted in PBS presented a general reduction of A2058 cell viability. All samples reduced the viability below 50% except for the fraction PBS >30 kDa. Moreover, soluble pepsin-hydrolysed proteins reduced the cell viability but no one below 50%, while for soluble collagenase-hydrolysed proteins, only the fraction Collagenase 30 - 10 kDa induced a reduction below 50% at both 10 µg/ml and 100 µg/ml.

At 72 h of treatment with soluble collagenase-hydrolysed proteins extracted from the oral arms of *C. tuberculata*, it can be observed a situation completely different respect to the treatment at 24 h. All subfractions had a cytotoxicity on HaCaT immortalized keratinocytes and pro-proliferative activity on A2058 melanoma cells.

As it is evident, the graphs show that some fractions have cytotoxic activity not only on melanoma cells but also on normal cells. On the other hand, for some of the soluble proteins and hydrolysed proteins, an increase in the percentage of cell viability of melanoma A2058 cells is observed after treatment with the sample. This is due to increased proliferation versus control of untreated cells and HaCaT control cells. This could in some way be because in cancer cells both the content and distribution of collagen itself coordinate a series of activities in the cell such as mutations, activation of signal transduction pathways, metastases (Xu et al. 2019). Therefore, the anti or pro-proliferative activity of the extracts obtained from the umbrella and the oral arms of *C. tuberculata* on the two cell lines tested, could be explained by the fact that the hydrolysates, comprising protein fragments with low molecular weight (León-López et al. 2019), could be constituted by a peptide part implicated in the previously explained mechanisms and others, those with anti-proliferative activity on cancer cells, peptides that have no control over pro-cancer pathways.

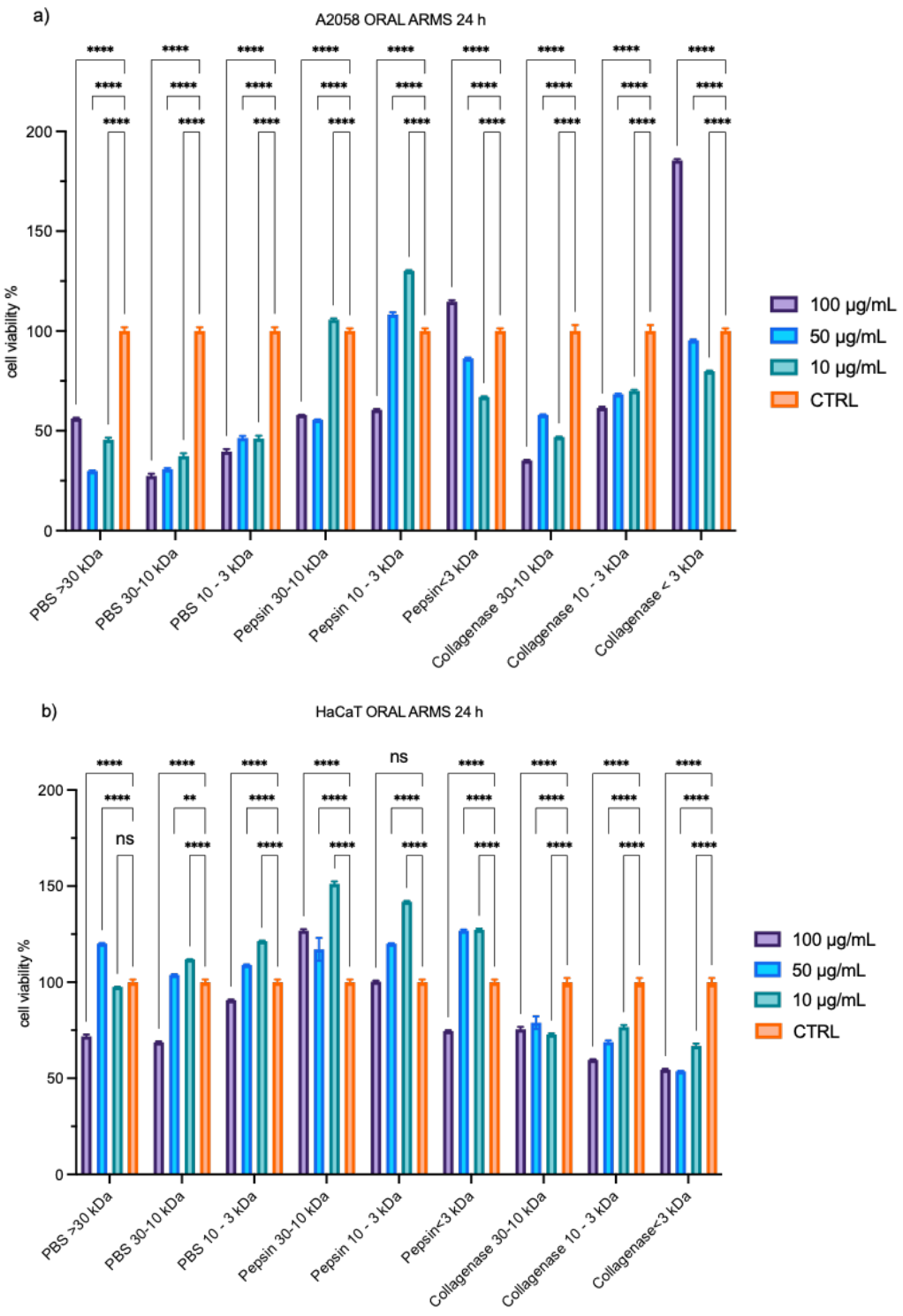

Figure 5. In vitro Anti-proliferative assay. The histograms show antiproliferative effects of *C. tuberculata* hydrolyzed extracted from oral arms, on a) A2058 cell line (melanoma) and b) HaCaT (control cell line) and the respective controls only with the culture medium. Results are expressed as percentage of cell survival after 24h exposure (n = 3). Cell growth was expressed as the percentage of cell viability with respect to the control for

each concentration ANOVA 2WAY Dunnett's MULTIPLE COMPARISON 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0,0001 (****)

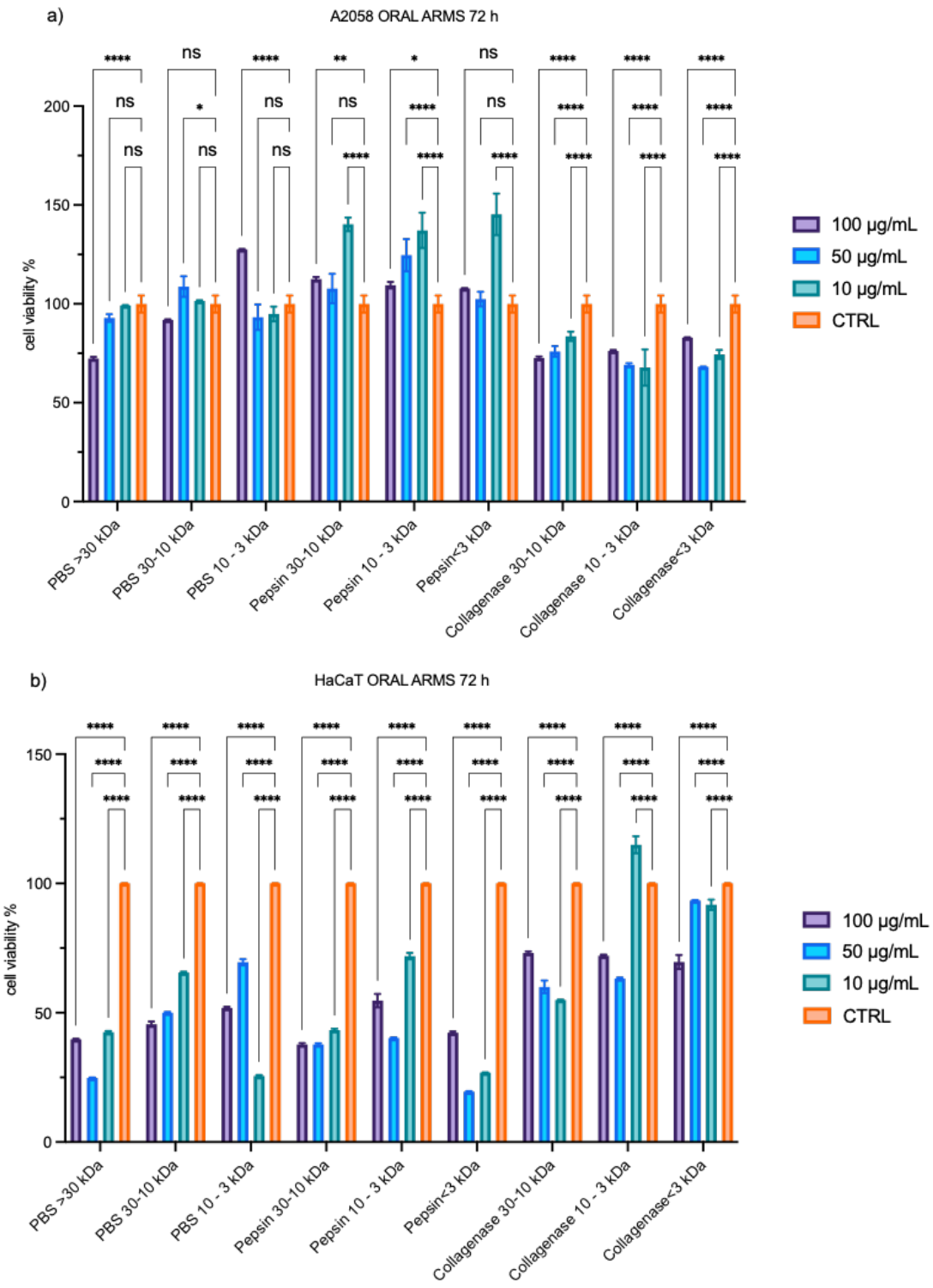

Figure 6. In vitro Antiproliferative assay. The histograms show antiproliferative effects of *C. tuberculata* hydrolysates extracted from oral arms, on a) A2058 cell line (melanoma) and b) HaCaT (control cell line) and the respective controls only with the

culture medium. Results are expressed as percentage of cell survival after 72h exposure (n = 3). Cell growth was expressed as the percentage of cell viability with respect to the control for each concentration ANOVA 2WAY Dunnett's MULTIPLE COMPARISON 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0,0001 (****)

2.4 In-vitro anti-inflammatory effect on Caco-2 cells

In an attempt to investigate how hydrolyzed proteins jellyfish could affect the inflammatory state of the human intestine, we used the human intestinal Caco-2 cell model in combination with a simulated gastrointestinal digestion protocol, commonly used to study effects of foods and nutrients on e.g. absorption, permeability, and inflammation. Caco-2 cell epithelia were pre-incubated with digested hydrolyzed jellyfish fractions for two hours to simulate bowel exposure to jellyfish (eating). Then after the jellyfish digests were removed, the intestinal cells were given a mild cocktail of inflammatory cytokines (TNFα and IL-1β) to address the question if jellyfish pre-exposure can protect the cells against inflammation, in this study we chose the chronic inflammation marker IL-6 as endpoint.

The Figure 7 shows that the acetic acid hydrolysis (HAc) decreased IL-6 (9,95% ±5,9%, p=0,04) suggesting a beneficial anti-inflammatory effect on the cells, inspite the very low concentration of added jellyfish umbrella fractions (0.1 mg/ml). This effect could not be seen with the Jellyfish oral arms fraction. It was also observed that neither PBS or collagenase treatment had a significant anti-inflammatory effect. Indeed, collagenase processing significantly increased IL-6 levels. Since all hydrolysates were exposed to a simulated GI digestion including pepsin and pancreatin treatment, the HAc treatment as such seemed to be a beneficial factor for anti-inflammatory activity. However, we have not investigated if this is specifically an effect by acetic acid or if e.g. another weak acid or strong ones could have the same or similar effects.

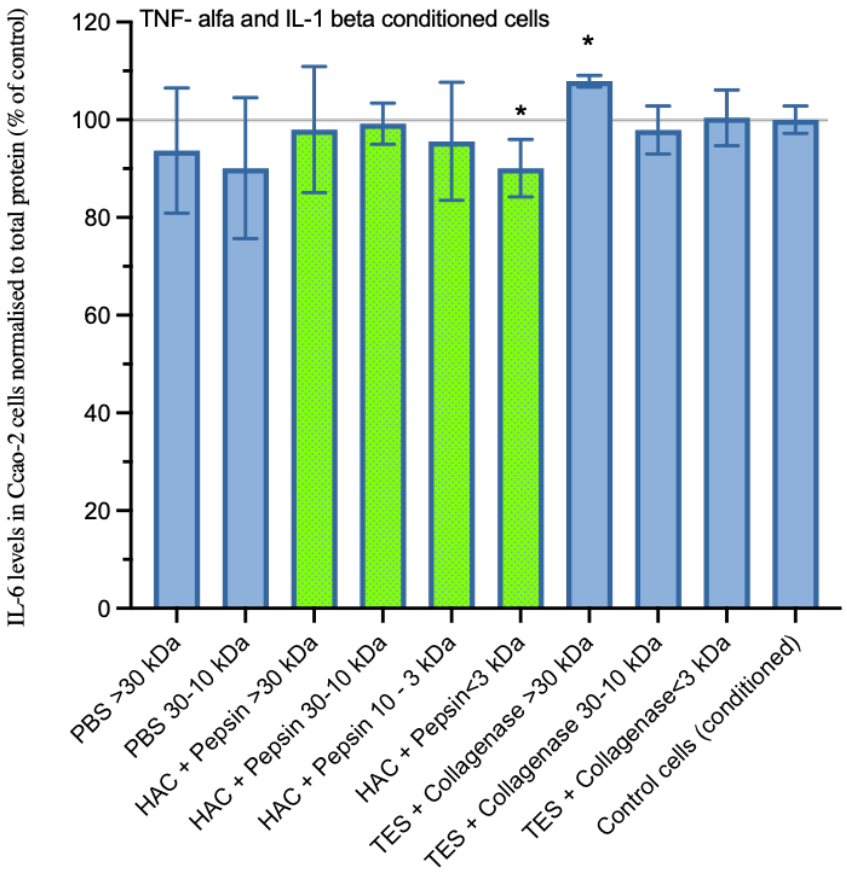

Figure 7. IL-6 response in post-confluent intestinal Caco-2 cells (pre-treated with low dose TNF-alpha and IL1-beta) exposed to in vitro GI-digested hydrolysed Jellyfish at 0.1 mg/ml (*C. tuberculata* umbrella). Data are means of two experiments in duplicates

3. Materials and Methods

3.1 Chemicals and Equipment

Amicon® Ultra-30K, 10K, 3K device, purchased from Merck (Darmstadt, Germany), Pepsin from porcine gastric mucosa (≥2500 U/mg), Collagenase from Clostridium histolyticum [0.5–5.0 furylacryloyl-Leu-Gly-Pro-Ala (FALGPA) units/mg solid, ≥125 were purchased from Sigma-Aldrich (Milan, Italy), TES(2-{[1,3-Dihydroxy-2-(hydroxymethyl)-2-propanyl]amino}ethanesulfonic acid buffer), Germany). Potassium persulfate (dipotassium peroxdisulfate) purchased from Sigma-Aldrich (St. Louis, MO, USA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and ABTS [2,20-Azinobis (3-ethylben-zothiazoline-6-sulfonic acid) diammonium salt], were obtained from Santa-Cruz Biotechnology (CA, USA), phosphate buffered saline (PBS), Human melanoma cell (A2058; ATCCR CRL-11147™), human spontaneously immortalized keratinocytes from adult skin (HaCaT), and Human intestinal Caco-2

(HTB-37) were purchased from (ATCC, Manassas, VA, USA); DMSO and 3-(4,5-dimethyl-2-thizolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT); A2231,0001, were purchased from (Applichem Panreac Tischkalender, Darmstadt, GmbH), trypan blue, alpha amylase (105U/ml; SigmaAldrich, Schnelldorff, Germany), bile and pancreatine from porcine pancreas 8X USP, were purchased from (SigmaAldrich, Schnelldorff, Germany), MEM, FBS were purchased from (Gibco; Thermo Fisher Scientific; Waltham, MA, USA), ELISA kit.

3.2 Raw Material Sampling

Jellyfish were obtained from the bycatch of commercial fishing activities held in the marina of Monte di Procida (Naples, central-western Mediterranean Sea). In particular, two *C. tuberculata* (Macri, 1778) specimens were collected on the 31st August 2021 using trammel nets (1 m in height and ~550 m in length; net consisting of an inner panel of 4.8 cm stretched mesh between two panels of 25 cm stretched mesh) fishing passively on a sandy bottom off the Fusaro Lake (40.8230° N, 14.0451° E). Soon after disentangling them from nets, jellyfish were placed into cold storage containers in single plastic bags filled with seawater, and soon transferred to the Laboratory of Benthos - Napoli (Stazione Zoologica Anton Dohrn, Naples, Italy) for subsequent laboratory work.

3.3 Protein Extraction and Sequential Hydrolysis

Three grams of lyophilized *C. tuberculata* umbrella (U) and oral arms (OA) tissue were used for the protein extraction and production of hydrolysates, as described by (De Domenico et al. 2019). Briefly, umbrella and oral arms were separately extracted by stirring of the sample with 16 volumes (w/v) of PBS (phosphate buffer saline), pH 7.4, at 4 °C for 2 h. The soluble proteins were separated from the insoluble portion by centrifugation at 9000 × g for 30 min, at 4 °C, and the supernatant was collected and subjected to filtration through membranes (see paragraph 3.X). Then, the insoluble pellet was treated with sequential enzymatic hydrolyses. In particular, the pellet was hydrolysed by pepsin (1 mg/mL) in 0.5 M acetic acid (enzyme/substrate ratio of 1:50 (w/w)) under stirring for 48 h at 4°C, centrifugated at 9000× g for 30 min, and the supernatant containing the pepsin-hydrolyzed material was used for fractionation by membrane filtration. The pellet was washed with bi-distilled water twice, and subjected to a digestion with collagenase (6 mg/mL in TES buffer 50 mM, pH 7.4, and 0.36 mM of CaCl2) using an enzyme/substrate ratio of 1:50 (w/w), under stirring 5 h at 37 °C. The

sample was centrifuged at 9000 × g for 30 min, and the supernatant containing the soluble collagenase-hydrolyzed material was collected and used for filtration.

3.4 Fractionation by Membrane Filtration

Umbrella and oral arms were centrifuged at 4°C through membrane filters with different molecular weights. each sample was filtered almost totally with Amicon® Ultra 30K device (Merck) by centrifugation at 4000× g; the volume retained in the tube contained compounds with MW higher than 30kDa. The filtrates with compounds less than 30kDa were fractionated using Amicon® Ultra 10K device (Merck) in the same manner to obtain the 10–30kDa fraction in the retentate. Finally, the filtrates containing compounds lower than 10kDa were centrifuged using Amicon® Ultra 3K device (Merck) at 4000× g to obtain in the portion not eluted fractions with 10 < MW < 3kDa and MW < 3kDa.

3.5 ABTS Radical Scavenging Activity Assay

The scavenging activity of the ABTS radical was determined according the method provided by Ahn (Ahn et al. 2004). Briefly, an ABTS stock solution (7 mM ABTS, in water) was mixed with 2.45 mM K2S2O8 (final concentration) and incubate in a dark room for 16–18 h at room temperature, allowing ABTS radical cation generation. Then, the ABTS•+ was mixed with ddH2O in order to create the working solution with an absorbance about 0.7 ± 0.05 at 734 nm. 4 µL (50 mg/ml) of each sample were added in 196 µL of ABTS•+ working solution, mixed and hold in the dark at 25–27 ◦C for 7 min; the absorbance was read at 734 nm. 96-well microplates were used for test. Results were expressed as nmol of Trolox Equivalents per mg of proteins (nmol TE/mg protein).

3.6 Determination of cell density by trypan blue dye assay

In a microtube, 1 part of 0.4% trypan blue and 1 part of cell suspension were mixed. The mixture was loaded into a Bürker chamber and, within 5 min of mixing, the dead cells and the total number of cells were counted to evaluate the percentage of viable cells (León-López et al. 2019).

3.7 In vitro Anti-proliferative Assay

Human cells were bought at ATCC (https://www.atcc.org). Human melanoma cell (A2058; ATCCR CRL-11147™) and human spontaneously immortalized keratinocytes from adult skin (HaCaT;) were cultured in DMEM high glucose supplemented with 10%

Fetal Bovine Serum, 1% L-glutamine, 1% Pen-Strep solution in a humidified incubator at 37°C and 5% CO2. To estimate the in vitro anti-proliferative effects HaCaT and A2058 cells were seeded in 96-well microtiter plate at density of 1x104 cells/well and incubated at 37°C to allow for cell adhesion in the plates. After 24h, the medium was replaced with fresh medium containing increasing concentrations of the extracts (10 µg/mL, 50 µg/mL, and 100 µg/mL) dissolved in sterilized water (millieQ), and further incubated for 24 and 72 h. Each concentration was tested at least in triplicate. After 24 and 72 h of treatment, cell viability was assessed using the MTT test 3-(4,5-dimethyl-2-thizolyl)-2,5-diphenyl-2H-tetrazolium bromide; A2231,0001, Applichem Panreac Tischkalender, Darmstadt, GmbH) (Baudelet et al.,2013; Russo et al., 2016; Martinez et al., 2022). MTT/PBS solution (0.5 mg/ml) was then added to the wells and incubated for 3 h at 37°C in a humidified atmosphere. The reaction was stopped by removal of the supernatant and the formazan products was dissolved with 100µL of isopropanol. Absorbance was measured at OD = 570 nm using a microplate reader (MultiskanTM FC Microplate Photometer, Thermo Fisher Scientific, Waltham, MA, United States). The assay was performed according to the manufacturer's instructions. Cell survival was expressed as a percentage of viable cells in the presence of the tested samples, with respect to untreated control cultures. Extracts with cell viabilities above 50% were not considered active.

The percentage of cell viability was calculated as follows: mean (A570–A630) and compared to cells supplemented with media alone. Values shown in the plot are mean ± SD of sixfold determinations. Mean, standard deviation and statistical analysis was calculated on biological triplicates using GraphPad Prism8 software (GraphPad, San Diego, CA, USA).

3.8 Simulated gastrointestinal digestion

Lyophilized hydrolyzed umbrella and oral arms fractions of *C. tuberculata* were resuspended in an alpha amylase solution (3 mL) containing NaCl (140mM), KCL (5mM), alpha amylase (105U/ml; SigmaAldrich, Schnelldorff, Germany) dissolved in MilliQ water. The heterogenous oral digests (at pH 7.0) were mixed on a rotary shaker (200 rpm, 37 °C, 2 minutes). The gastric digestion phase was started by adding an isotonic salt solution (7 ml) consisting of NaCl (140mM), KCL (5mM), Pepsin (16 000 U). HCl (1M, 1 mL) was added to lower the pH (the pH in the blank digests were pH 1.0). The gastric digests (8 ml) were incubated at 37°C for 60 min at 200 rpm. Starting the intestinal digestion phase, the pH was raised to 5.7 by dropwise addition of NaHCO3

(1M). Volumes needed differed between the samples and were between 2-3 ml (2 ml for the digest control). Simulating the post-pancreas part of the duodenum, bile-pancreatin solution (2.5 mL) were added to each digest containing 10.6 mg/ml of bile and 3.5 mg/ml pancreatin (8X USP, SigmaAldrich, Schnelldorff, Germany). pH was increased to 7 and the volumes were brought up to 13 ml by adding the isotonic salt solution. The digests were mixed on a rotary shaker (200 rpm, 37°C, 120 min).

3.9 Cell culture and experiments

Human intestinal Caco-2 cells (HTB-37; ATCC, Manassas, VA, USA) were continuously cultured in MEM, 10% FBS (Gibco; Thermo Fisher Scientific; Waltham, MA, USA) at 95% air, 5% CO2, 37°C. The medium was changed three times a week. And the cells were passaged at 60- 80% confluence. For experiments, cells (100 000/well) were plated in 24-well plates (MEM, FBS 10%, Normocin™ 0.2% (Invivogen, Toulose, France)). 14 days post-seeding, the medium was changed to MEM FBS 5%, Normocin™ 0.2%. 24 hours later, the experiments were initiated by adding the digested hydrolysates directly to the medium, to achieve a final concentration of 0.1 mg/mL (dry weight of each hydrolysate). 2 hours later, the digest-containing media were aspirated, and the cells were washed with PBS. Fresh medium (MEM FBS 5%, Normocin™ 0.2%) containing cytokines TNFα (50ng/ml, #210-TA-020/CF, R&D Systems) and IL-1β (2ng/ml, #201-LB-005/CF, R&D Systems) were added. After 24 hours, the cells were washed with PBS and then lysed in ice-cold RIPA buffer (100μl). The cell lysates were collected and stored at -80°C until analysis. Total protein was measured with the BCA assay (Pierce) to be used for normalization purposes and the inflammatory marker IL-6 was measured (in cell lysates) using a commercial ELISA kit.

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CHAPTER 5

Characterization and application of protein hydrolysates from common starfish (*Asterias rubens*) extracted using different enzymes

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1. Introduction

Protein hydrolysates are produced by breaking down proteins chemically or biologically into peptides of different sizes. They have been prepared from various sources including plant, dairy, chicken, fishes, and shellfishes. Apart from having many functional properties, protein hydrolysates shown to have bioactivities such as antihypertensive, antagonists, immunomodulatory, anti-thrombotic, antioxidant, anti-cancer, and anti-microbial activities (Clare & Swaisgood, 2000; Elias et al., 2008; Shahidi & Zhong, 2008). The protein hydrolysates also play an important role in nutrition as di and tri-peptides present are readily absorbed than the whole proteins from which they are produced from (Di Pasquale, 1997). Marine by-products, including whole body, head, skin, scales, etc., have been utilized for the production of hydrolysates having different types of bioactivities and functional properties. For example, the protein hydrolysates from freshwater carp (*Catla catla*) showed antioxidant activities and functional properties, such as emulsion and foaming capacity (Elavarasan et al., 2012). Similarly, the protein hydrolysates from parrotfish (*Chlorurus sordidus*) head also showed antioxidant activity (Prihanto et al., 2019), as well as jellyfish hydrolysates that represent a good source of bioactive molecules, including antioxidants and antiproliferative compounds (De Domenico et al. 2019; Leone et al. 2015). Apart from fish and fish by-products, by-catches, such as sea cucumbers, have been used for the production of bioactive protein hydrolysates, such as Angiotensin-I Converting Enzyme (ACE)

Inhibitory and anti-hypertensive effect (Vishkaei et al., 2016). Many bioactive compounds derived from echinoderms have been discovered over the years and showed different applications in the treatment of various diseases (Samuel, Prince, e P. 2011). For example, Solstad and collaborators (Solstad et al. 2016) led to the characterization of new antimicrobial peptides derived from the organism *Echinus esculentus*, EeCentrocins 1 and 2, and EeStrongylocin 2. These peptides presented 6-Br-Trp post-translational modifications, conferring strong antimicrobial activity against two clinically relevant species, *Staphylococcus aureus* and *Escherichia coli*.

Marine peptides extracted from marine organisms, such as marine echiuroid worm (Jo et al., 2009), starfish (Koyama et al., 1998), blue mussel (Jung & Kim, 2009), yellowfin sole (Rajapakse et al., 2005), and ark shell (Jung et al., 2007) also shown anti-coagulant activity, and no cytotoxic effects, suggesting a potential use as functional ingredients in nutraceuticals or pharmaceuticals.

The starfish *Asterias pectinifera* is a voracious feeder known to cause problems in aquaculture industry. It has recently been identified as an eco-friendly source of non-toxic and highly water-soluble low-molecular-weight collagen peptides, which promote wound healing, bone regeneration, and skin protection. Moreover, collagen peptides produced from *A. pectinifera* have been used for encapsulating elastic nanoliposomes for cosmetic applications (Han, Won, Yang & Kim, 2021). Interestingly, saponins were also extracted from the starfish *A. pectinifera* and *Asterias amurensis*, and compared with the commercial saponin Quil-A; the authors demonstrated immunomodulatory effects, suggesting their possible use as adjuvants in vaccine formulations (Kawase et al. 2011)

The common starfish *Asterias rubens* is an invasive species creating problems in mussel farming, on the west coast of Sweden which is harvested as by-catch along with the mussel and thrown away as waste. It is reported that the starfish *A. rubens* and *Marthasterias glacialis* are predators of scallops and can cause losses of up to 11% of young scallops in sea farming (Magnesen e Redmond 2012). This starfish has successfully been utilized as a source of collagen in our previous study (Vate, Undeland & Abdollahi, 2022), and could be a good source of peptides having unique bioactivities. Hence, the present study aimed to produce protein hydrolysates from common starfish using different enzymes, investigating the different bioactivities.

2. Materials and methods

2.1 Raw Materials

Fresh common starfish (*A. rubens*) were collected from local mussel farmers in Gothenburg, Sweden. The starfish were covered with ice and transported to the lab. They were washed with cold water upon arrival in the lab, packed, and stored at -80 °C until use.

2.2 Chemicals

All chemicals and reagents were of analytical grade. The enzymes Food Pro PNL, Corolase 8000, and Corolase 7089 were obtained from Nofima, Norway. EDTA, tris(hydroxymethyl) aminomethane, sodium dodecyl sulphate (SDS), β-mercaptoethanol (β-ME), glycerol, bovine serum albumin, and potassium persulfate ($K_2S_2O_8$) (dipotassium peroxdisulfate) were purchased from Sigma-Aldrich Corp. (USA). Sodium hydroxide, hydrochloric acid and sodium chloride were procured by Scharlo (Scharlo Co., Spain). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and ABTS [2,20-Azinobis (3-ethylben-zothiazoline-6-sulfonic acid) diammonium salt] were obtained from Santa-Cruz Biotechnology (CA, USA)

2.3 Preparation of protein hydrolysates

The frozen starfish were thawed under cold tap water. Then the samples were chopped into small pieces (0.5mm×0.5mm) before using for hydrolysis. A part of the sample was subjected to deproteinization by mixing the chopped sample with 0.1 M NaOH in 1:10 ratio (w/v) and subjecting it to homogenization at 4000 rpm for 2.5 min with the Silverson homogenizer. The homogenized sample was centrifuged at 2000 × g for 2 min at 4 °C, and the supernatant was discarded. The precipitate obtained was mixed with cold water in 1:10 ratio (w/v) and pH was adjusted to 7.4. Then, it was dewatered by centrifuging at 5000 × g for 5 min. The precipitate was then used for the preparation of protein hydrolysate. Demineralized starfish sample was prepared by using EDTA solution after deproteinization (mentioned earlier). During demineralization, the deproteinized sample was mixed with 0.5 M EDTA-2Na solution keeping the ratio of 1:15 (w/v sample to solution). The demineralization was carried out in cold room (4 °C) for 24 h by stirring with the fresh EDTA solution changed at 12 h. Residual EDTA was removed from demineralized samples by washing with cold water. Then, dewatering of the samples was done by centrifugation at 5,000 × g (5 min at 4 °C).

Hence, 3 samples including chopped whole starfish (SF), deproteinized starfish (DMSF), and demineralized starfish (DMSF) were used for the preparation of hydrolysates.

Three enzymes namely Food Pro PNL, Corolase 8000, and Corolase 7089 were used for the preparation of hydrolysates from starfish. 15 g of chopped starfish, deproteinized starfish, and demineralized starfish, were taken in 50 mL falcon tubes, separately, and mixed with 0.1 N phosphate buffer, pH 7.0 (1:1 w/v ratio). Then, the samples were preheated in a shaking incubator at 55 °C for 10 min. The preheated samples were added with enzymes at 2% (w/w), and the hydrolysis was continued for 2 h. After the hydrolysis the samples were centrifuged at 8500 rpm for 20 min to separate the undissolved matter. Then, the samples were heated at 90 °C for 10 mins to inactivate the enzymes. Later, the hydrolysates were lyophilized to get the dry powder.

2.4 Determination of protein degree of hydrolysis (DH)

DH was determined according to Nielsen, et al. (2001) with slight modifications. Briefly, 0.5 mL hydrolysate sample was mixed with 3.75 ml o-phthaldialdehyde (OPA) reagent, vortexed for 5 sec, followed by incubation for 2 min at room temperature. Prior to incubation, hydrolysate samples were diluted with pure distilled water to keep the absorbance reading below 1.0, and the absorbance was measured at 340 nm using a spectrophotometer (Cary 60 UV–vis, Agilent technologies, 117 USA).

2.5. Characterization of hydrolysates

2.5.1 Yield and protein content

The yield of hydrolysates was calculated using the following formula:

Yield (%) = (Weight of freeze-dried hydrolysates)/(Weight of initial wet raw material) × 100

Total nitrogen content was determined using a nitrogen analyzer (LECO), and then the crude protein content in sample was calculated using a nitrogen to protein conversion factor of 5.58 (Mariotti et al., 2008).

2.5.2 Amino acid composition of hydrolysates

Amino acid composition of the hydrolysates was analyzed based on the method of Ozcan and Senyuva (2006) with some modifications. Freeze-dried hydrolysates samples (10 mg) were mixed with 4 mL of 6 N HCl and hydrolyzed at 110 °C for 24 h. Hydrolyzed samples were diluted using 0.2 M acetic acid, and automatically injected to LC/MS (Agilent 1100 HPLC, Waldbron, Germany) in replicates, and compared against amino acid standards. Tryptophan and cysteine were not recovered with this method.

2.5.3 SDS-PAGE of hydrolysates

SDS-PAGE analysis was conducted according to the methodology established by Laemmli (1970). The hydrolysate samples, prepared in a solution containing 5% SDS, were combined with the sample buffer (Bio-Rad, USA) at a ratio of 1:2, while also incorporating 10% β-ME, thereby achieving a final protein concentration of 2 µg protein/µL. Subsequently, 10 µL of each sample, along with 5 µL of an ultra-low range molecular weight marker (1.06–26.6 kDa, Sigma, USA), were loaded onto a 16.5% Mini-protein Tris-Tricine Gel (Bio-Rad, USA). The proteins were then subjected to electrophoresis at a consistent current of 125 V, employing a Mini Protein II unit (Bio-Rad, USA). The gel was fixed in a solution of 5% glutaraldehyde that had been freshly prepared for a duration of 1 hour. Subsequently, the gel underwent a washing process for 5 minutes, which was repeated three times. Staining was done using 0.02 % (w/v) Coomassie Brilliant Blue R-250 in 10 % (v/v) acetic acid for 1 h, followed by destaining 10 % (v/v) acetic acid for 1 h to overnight with several changes of destaining solution. Finally, an image capturing the gel was obtained using the Bio GelDoc Go Imaging system (Bio-Rad, USA).

2.5.4 Size Exclusion Chromatography (SEC)

The collagen peptides of starfish were submitted to size exclusion chromatography (SEC) to estimate their molecular mass distribution. Briefly, samples were solved with MilliQ water to a concentration of 10 mg/mL, centrifuged at 10,000 × g for 10 min, and then the resulting supernatant was filtered (0.45 µm, Fisher Scientific) and used for the SEC analysis. AdvanceBio SEC 150 Å Protein Standard (Agilent Technologies) was used for the analysis of molecular weights. For each sample, 20 µL were injected into two Agilent Bio SEC columns (150 Å and 100 Å) maintained at a constant temperature of 25 °C. The mobile phase consisted of 30% acetonitrile and 0.05% trifluoroacetic acid in Milli-Q water (v/v). The chromatographic runs were controlled from the Chromeleon software version 7.2 SR 4 (Thermo Schientific, Walham, MA, USA). From the chromatographic runs of both the standards and hydrolysates, a UV trace of 214 nm was monitored. The retention times of molecular mass standards were used to calculate the molecular mass distribution of the collagen peptides according to the retention time of the different peaks present in the chromatogram.

2.5.5 ABTS Radical Scavenging Activity Assay

The scavenging activity of the ABTS radical was determined according the method provided by Ahn et al. (2004), adapted for 96-well microplates. Briefly, an ABTS stock solution (7 mM ABTS, in water) was mixed with 2.45 mM $K_2S_2O_8$ (final concentration) and left in the dark for 16–18 h at room temperature, allowing ABTS radical cation generation. Then, it was diluted with water to acquire an ABTS working solution whose absorbance is 0.7 ± 0.05 at 734 nm. To 4 µL (50 mg/ml) of each sample were added 196 µL of $ABTS^{\bullet+}$ solution, mixed and kept in the dark at 25–27 °C for 7 min; the absorbance was read at 734 nm. The scavenging activity was expressed using Equation:

Scavenging activity (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100$

where $A_{control}$ is the absorbance of the $ABTS^{\bullet+}$ solution; A_{sample} is the absorbance of the sample mixed with ABTS. Trolox was used as standard, and the results were expressed in nmol of Trolox equivalent per mg of dry weight.

2.5.6 Determination of cell density by trypan blue dye assay

In a microtube, 1 part of 0.4 % trypan blue and 1 part of cell suspension were mixed. The mixture was loaded into a Bürker chamber and, within 5 min of mixing, the dead cells and the total number of cells were counted to evaluate the percentage of viable cells. (Strober, 2001; 2015).

2.5.7 *In vitro* Anti-proliferative Assay

Human cells were bought at ATCC. Human melanoma cell (A2058; ATCC® CRL-11147™) and human normal fibroblast (MRC-5; ATCC® CCL-171™) were cultured in DMEM high glucose supplemented with 10% Fetal Bovine Serum, 1% L-glutamine, 1% Pen-Strep solution in a humidified incubator at 37 °C and 5% CO_2. To estimate the in vitro anti-proliferative effects MRC-5 and A2058 cells were seeded in 96-well microtiter plate at density of $1x10^4$ cells/well and incubated at 37 °C to allow for cell adhesion in the plates. After 24 h, the medium was replaced with fresh medium containing increasing concentrations of the extracts (10 µg/mL, 50 µg/mL, and 100 µg/mL) dissolved in sterilized water (milliQ), and further incubated for 72 h. Each concentration was tested at least in triplicate. After 72 h of treatment, cell viability was assessed using the MTT test 3-(4,5-dimethyl-2-thizolyl)-2,5-diphenyl-2H-tetrazolium bromide; A2231,0001, Applichem Panreac Tischkalender, Darmstadt, GmbH) (Baudelet et al.,2013; Martinez et al., 2022). MTT/PBS solution (0.5 mg/ml) was then added to the wells and incubated

for 3 h at 37 °C in a humidified atmosphere. The reaction was stopped by removal of the supernatant and the formazan products was dissolved with 100µL of isopropanol. Absorbance was measured at OD = 570 nm using a microplate reader (Multiskan^{TM} FC Microplate Photometer, Thermo Fisher Scientific, Waltham, MA, United States). The assay was performed according to the manufacturer's instructions. Cell survival was expressed as a percentage of viable cells in the presence of the tested samples, with respect to untreated control cultures. Extracts with cell viabilities above 50% were not considered active. The percentage of cell viability was calculated as follows: mean (A570–A630) and compared to cells supplemented with media alone. Mean, standard deviation and statistical analysis were calculated on biological triplicates using GraphPad Prism8 software (GraphPad, San Diego, CA, USA).

2.6. Statistical analysis

The preparation of hydrolysates from all the samples were carried out at least twice. Analyses of the hydrolysates was then done in duplicates and average values from these analyses were subjected to analysis of variance (ANOVA) to determine significant differences between different hydrolysates. Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie 1980). Data was regarded as significantly different when $p<0.05$. Statistical analysis was performed using the Statistical Package for Social Science (IBM SPSS 28.0 for Windows, SPSS Inc., Chicago, IL, USA).

3. Result and Discussion

3.1 Yield and Protein content

The yields of protein hydrolysates obtained from the starfish *A. rubens* prepared using different enzymes are depicted in Figure 1a. The hydrolysates SF-FP, SF-C7, and DMSF-C8 showed the highest yield, on the contrary, all the DPSF hydrolysates (obtained by using all 3 different enzymes) showed the lowest yield. This indicated that the enzyme activity varies depending on the raw material and enzyme type. In particular, the enzymes Food Pro PNL and Corolase 7089 showed good activity on the whole starfish, while Corolase 8000 showed greater activity on demineralized starfish than other samples. Hence, the high yield of protein hydrolysates obtained from the whole starfish was mainly due to the presence of both collagenous and non-collagenous proteins, while the low yield obtained from the deproteinized one was due to the removal of non-collagenous proteins, and the presence of minerals along with collagenous proteins in the overall

starting raw material. Since the demineralized sample was almost pure collagen protein, it resulted in the higher yield compared to deproteinized sample. However, the difference in the yield starting from the same raw material could be mainly attributed to the activity of the enzymes.

The protein content in hydrolysates (Figure 1b) was highest in DPSF-FP and DMSF-C8 followed by DPSF-C7, on the contrary it was least in SF-C8 (Figure 1b). The hydrolysates prepared from whole starfish by using all three enzymes had the lowest protein content, indicating that they had other components than protein. This indicated that with the higher yield the protein purity was less in hydrolysates prepared from whole starfish. This could be due to the presence of minerals found mainly in the calcareous endoskeleton of starfish (Blowes et al., 2017). The protein content in hydrolysates prepared from both deproteinized and demineralized starfish majorly collagenous since non-collagenous proteins were removed by deproteinization. Hence the hydrolysates from deproteinized and demineralized starfish more likely contained collagen peptides.

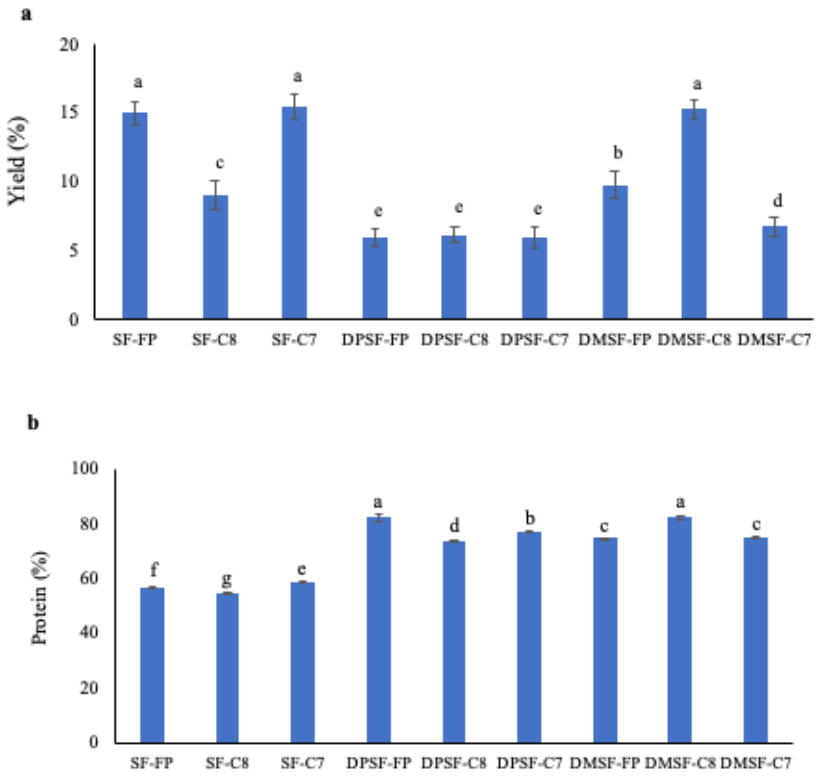
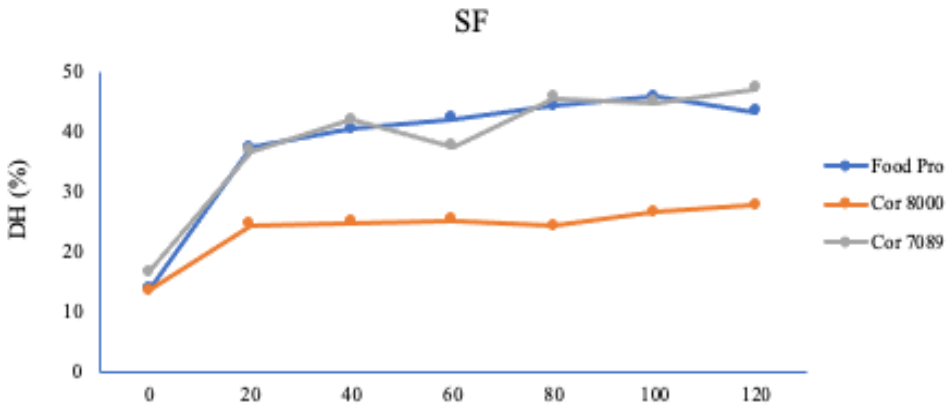


Figure 1. Yield (a) and protein content (b) of whole starfish (SF), deproteinized starfish (DPSF), demineralized starfish (DMSF) hydrolysates, obtained by Food Pro PNL (FP), Corolase 8000 (C8), or Corolase 7089 (C7). Data show mean values ±SD (n=3).

3.2 Degree of hydrolysis

The degree of hydrolysis (DH) of different enzymes on whole starfish (SF), deproteinized starfish (DPSF), and demineralized starfish (DMSF) is depicted in Figure 2. The Food Pro PNL and Corolase 7089 showed higher DH for whole starfish and deproteinized starfish compared to DH for Corolase 8000. On the contray, Corolase 8000 showed the highest DH for the demineralized starfish. This was in accordance with the yield result (Figure 1a). Overall, the DH for the deproteinized sample was the lowest. This could be likely due to the removal of non-collagenous proteins, and the presence of minerals which affected the enzyme activity. Hence, deproteinized starfish samples also had the lowest yield. The whole starfish DH was higher than that obtained with the deproteinized samples, as enzyme acted on both collagenous and non-collagenous proteins. The Corolase 8000 enzyme showed a higher activity for demineralized samples than those obtained for whole and deproteinized starfish, indicating that it could be used for the preparation of collagen hydrolysates or collagen peptides. The lower activity of Corolase 8000 on the whole starfish and deproteinized starfish compared to the demineralized starfish indicated that its activity was affected by the presence of minerals. Overall, the results indicated that the enzymes Food Pro PNL and Corolase 7089 could be suitable for the preparation of hydrolysates from non-collagenous proteins, and Corolase 8000 enzyme for the preparation of hydrolysates starting from collagenous proteins.



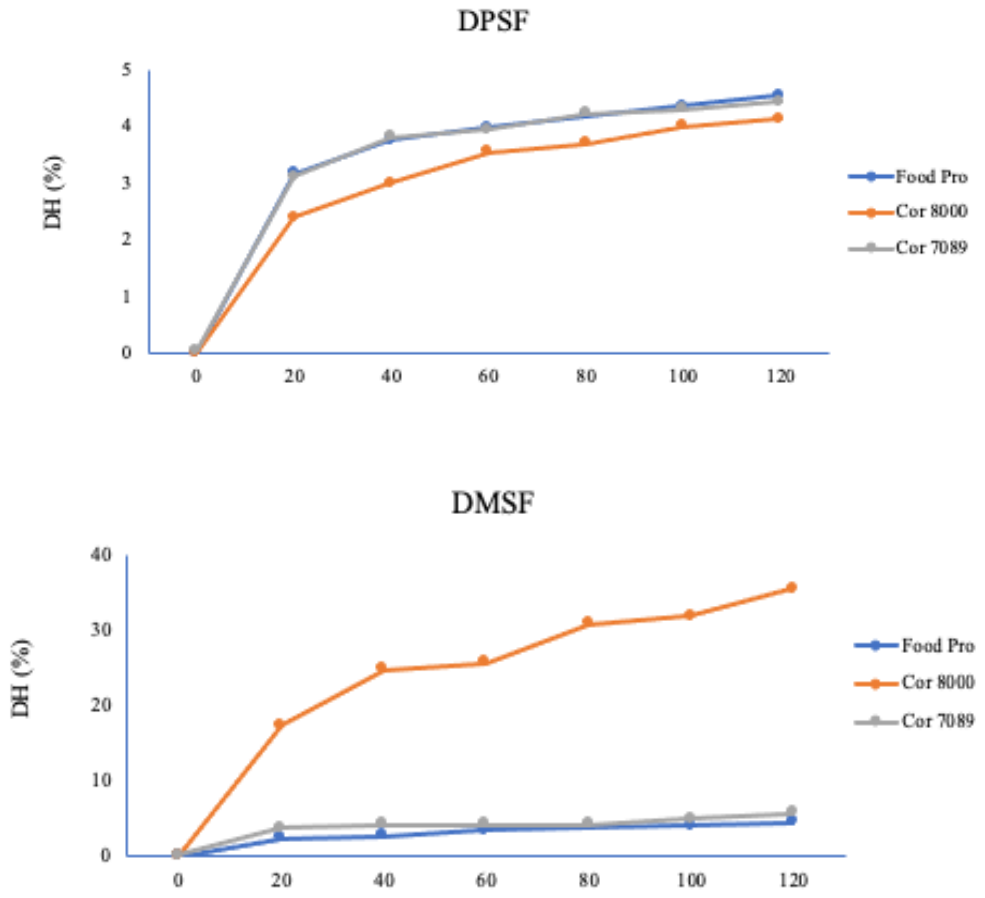


Figure 2. Degree of hydrolysis of different enzymes on whole starfish (SF), deproteinized starfish (DPSF), demineralized starfish (DMSF).

3.3 Amino acid content of hydrolysates

Amino acid content of the hydrolysates obtained from starfish using different enzymes is shown in Table 1. The amino acid glycine was found in the highest quantity in all the hydrolysates regardless of raw material and enzyme type. Hydrolysates prepared from whole starfish had lower glycine content compared to those prepared from deproteinized and demineralized starfish. This was mainly because the whole starfish contained both collagenous and non-collagenous proteins, while those prepared from deproteinized and demineralized starfish had mainly collagenous proteins. Among all samples the highest glycine content was observed in the hydrolysate prepared from deproteinized starfish using the enzyme Corolase 8000, followed by that prepared from demineralized starfish using the same enzyme. This was more likely due to the higher collagenous protein content in the deproteinized and demineralized samples. The amino acid sequence of

hydrolysates from deproteinized and demineralized samples was comparable with the distinctive amino acid sequence for collagen, in which glycine is found at every third amino acid residue (Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). The hydrolysate prepared from whole starfish using Corolase 8000 had the lowest glycine content. This indicated that the enzyme Corolase 8000 was suitable for the sample with collagenous proteins. The hydrolysates prepared from whole starfish had glutamine and asparagine as the second and third major amino acids, similar to the amino acid content of starfish *Acanthaster planci* (Luo et al., 2011). The hydrolysates prepared from deproteinized and demineralized starfish had glutamine and proline as the second and third major amino acids, suggesting that these were collagen peptides as amino acid profile of collagen subunits is a repeating chain of Glycine-X-Y, where X and Y are variables, although they are usually occupied by proline and hydroxyproline, respectively (Abdollahi et al., 2018). The amino acid sequence of hydrolysate samples from deproteinized and demineralized samples were comparable with that of collagen peptides from *A. pectinifera* which also had glycine as the main amino acid (Han et al., 2021). Glycine was also the major amino acid in collagen extracted from *A. planci* (Tan et al., 2013), seastar *A. amurensis* (Lee et al., 2009) and from purple sea urchin (*Anthocidaris crassispina*) (Nagai & Suzuki, 1999).

Table 1: Amino acid composition of whole starfish (SF), deproteinized starfish (DPSF), demineralized starfish (DMSF) hydrolysates, obtained by Food Pro PNL (FP), Corolase 8000 (C8), or Corolase 7089 (C7) (mg/g protein). Results are given as mean±S.D (n=3). Different superscripts in the same row indicate significant differences (p<0.05).

Aminoacid/ Sample	SF-FP	SF-C8	SF-C7	DPSF-FP	DPSF-C8	DPSF-C7	DMSF-FP	DMSF-C8	DMSF-C7
Lysine	49.48	44.43	53.59	23.28	46.94	35.98	24.03	47.15	19.73
Histidine	4.88	3.05	5.21	2.42	4.82	2.73	0.54	4.08	0.02
Arginine	21.68	13.53	23.49	54.05	75.61	57.23	37.99	76.02	23.57
Glycine	89.29	75.48	88.59	234.46	326.27	261.68	177.53	305.22	114.97
Alanine	31.80	22.31	31.93	75.69	112.93	88.22	59.47	108.71	42.48
Serine	6.56	5.40	6.69	13.14	17.76	14.47	10.70	17.14	8.02
Threonine	1.23	0.75	1.21	1.40	2.96	1.56	0.28	2.08	0.15
Glutamine	70.50	55.46	76.23	129.36	189.58	149.98	108.31	193.90	77.03
Asparagine	54.39	41.09	56.95	86.62	131.59	101.08	69.75	131.40	51.53
Proline	30.61	18.76	30.64	102.98	149.03	120.03	86.87	147.10	59.94

Valine	28.51	23.24	29.93	31.86	46.67	36.14	25.56	43.96	20.40
Methionine	9.61	5.87	10.85	15.78	27.54	19.99	10.59	25.06	5.31
Tyrosine	17.57	12.41	17.23	16.60	25.64	16.87	8.83	19.06	7.07
Isoleucine	23.97	19.74	24.88	25.62	37.67	29.71	21.75	35.91	17.37
Leucine	35.61	28.95	37.11	28.68	42.90	33.79	24.09	41.67	20.06
Phenylalanine	19.64	15.44	19.72	8.66	15.80	10.71	6.12	13.59	5.64

3.4 Protein Pattern of starfish hydrolysates

The protein pattern of hydrolysates from starfish prepared using different enzymes is shown in Figure 3. The protein pattern differed based on the sources of hydrolysates. Protein pattern of hydrolysates prepared from whole starfish using Food Pro PNL and Corolase 8000 enzymes had higher molecular weight peptides (> 26.6 kDa). The hydrolysates DPSF-FP and DPSF-C7 had lower molecular weight peptides showing bands at 3.39 kDa, 14.2 kDa, 17.0 kDa, and 26.6 kDa. The hydrolysates prepared from demineralized starfish using Food Pro PNL had higher molecular weight bands, above 26.6 kDa, and the one prepared using Corolase 7089 had bands ranging from 17.0 kDa to above 26.6 kDa. The hydrolysates prepared from a particular source had similar protein patterns irrespective of enzyme type, but the enzyme activity changed based on the type of raw material.

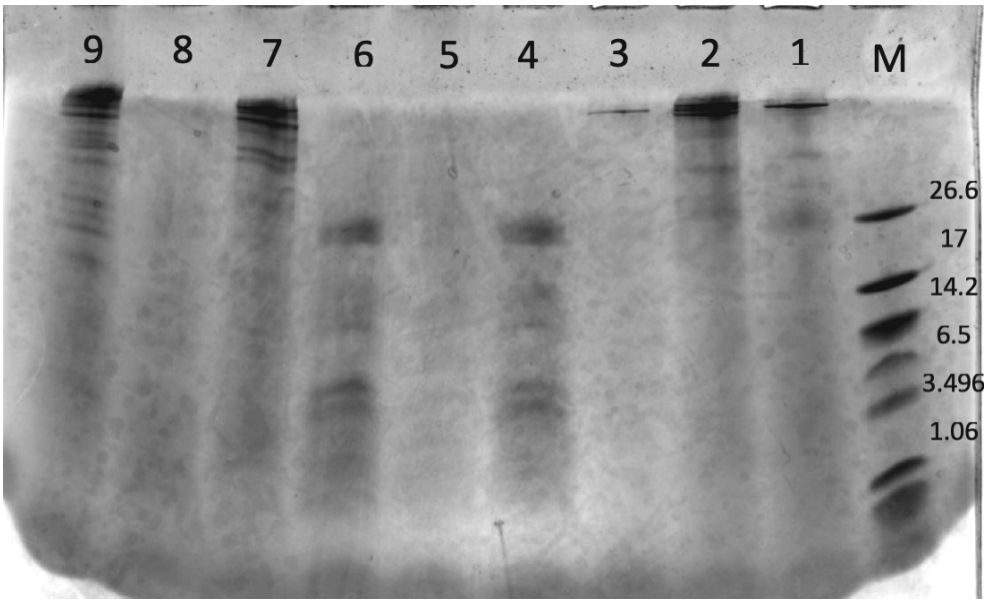


Figure 3. Polypeptide patterns of starfish protein hydrolysates prepared using different enzymes, M: Marker; 1: SF-FP; 2: SF-C8; 3: SF-C7; 4: DPSF-FP; 5: DPSF-C8; 6: DPSF-C7; 7: DMSF-FP; 8: DMSF-C8; 9: DMSF-C7.

3.5 Chromatogram

Chromatographic analysis of starfish hydrolysates obtained by using different enzymes is shown in Figure 4. Size exclusion chromatography of starfish hydrolysates showed that all the samples had highest peak at 45 kDa range. This was in accordance with the SDS-PAGE results as most of the hydrolysates had peptides with the size more than 26.6 kDa marker. The hydrolysates prepared form whole starfish using Food Pro PNL and Corolase 7089 showed small peaks at 17.0 kDa and 6.7 kDa.

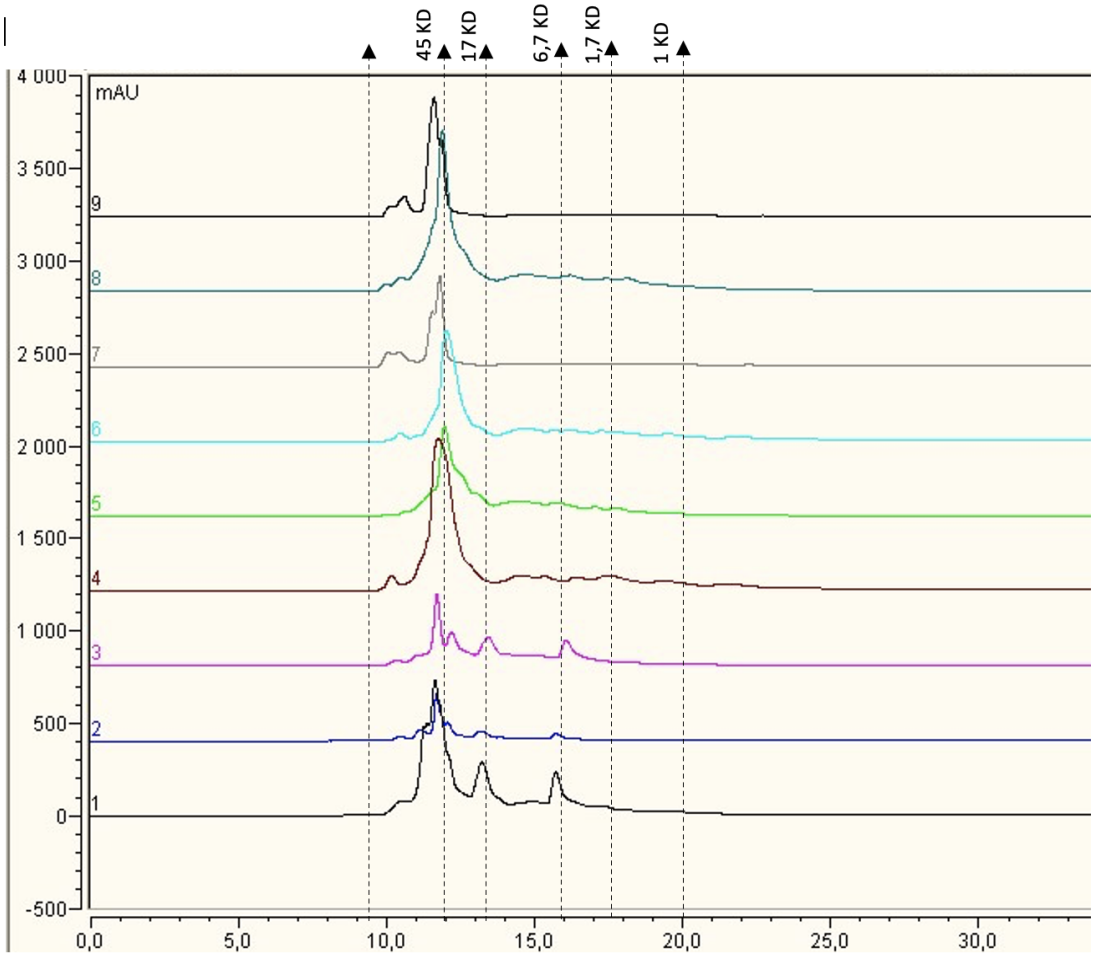


Figure 4. Chromatogram of starfish protein hydrolysates prepared using different enzymes, 1: SF-FP; 2: SF-C8; 3: SF-C7; 4: DPSF-FP; 5: DPSF-C8; 6: DPSF-C7; 7: DMSF-FP; 8: DMSF-C8; 9: DMSF-C7.

3.6 Antioxidant activity

In the present study, starfish hydrolysates were analyzed for their radical scavenging activity by the ABTS assay. It is a spectrophotometric method extensively used as a

screening assay of antioxidant natural products (Munteanu e Apetrei 2021). The antioxidant activity was reported as nmol of Trolox equivalents (TE) per g of dry weight, and shown in Figure 5.

Results highlight that the antioxidant activity of deproteinized starfish hydrolysates (DPSF-FP, DPSF-C7, DPSF-C8) is remarkably higher compared with whole starfish (SF-FP, SF-C7, SF-C8) or demineralized starfish (DMSF-FP, DMSF-C7, DMSF-C8) hydrolysates. Moreover, the sample containing deproteinized starfish hydrolysates, obtained by using the enzyme Corolase 8000 (DPSF-C8) showed the best antioxidant activity (174200 nmol TE/ g of dry weight). The demineralized samples show an antioxidant activity that is approximately half that of the deproteinized hydrolysates (DMSF-FP, DMSF-C7, DMSF-C8 showed values of 85300, 82000, and 84800 nmol TE/ g of dry weight, respectively). The minor antioxidant activity was reported for untreated starfish hydrolysates. The higher antioxidant activity of the deproteinized samples could be anticipated to the presence of lower molecular weight peptides as shown on SDS-PAGE (Figure 2).

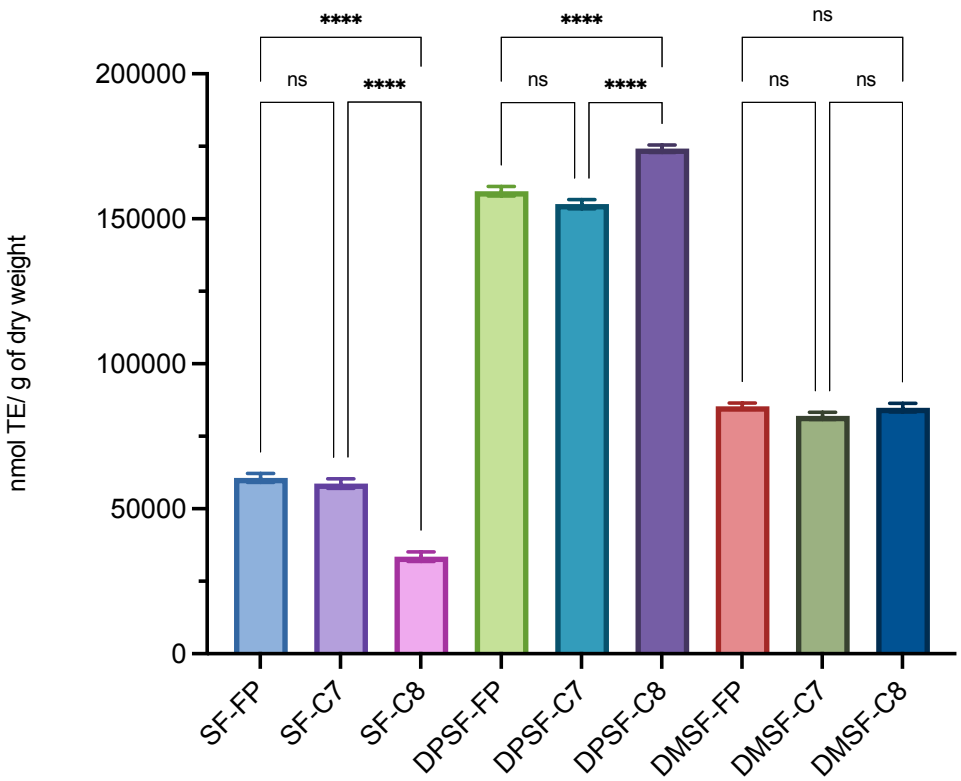


Figure 5. Antioxidant activity of whole starfish (SF), deproteinized starfish (DPSF), demineralized starfish (DMSF) hydrolysates, obtained by Food Pro PNL (FP), Corolase 8000 (C8), or Corolase 7089 (C7). Data are the mean values of three independent

experiments performed in three technical replicates. The antioxidant activity is expressed as nmol of TE per mg of protein ± standard deviation. ANOVA ordinary one-way Bonferroni's MULTIPLE COMPARISON 0.1234 (ns), <0,0001 (****).

3.7 *In vitro* anti-proliferative test

Starfish hydrolysates were tested for their possible anti-proliferative activity against human malignant melanoma cells A2058 and normal fibroblast MRC-5 by using the MTT test. The two cell lines were incubated in presence of three concentrations for each sample (10 µg/mL, 50 µg/mL, and 100 µg/mL) and in absence of them. After 72 h (Figure 6) of incubation at 37 °C with the samples, cell survival was measured by MTT test. As shown in Figure 6, at the end of the 72 h treatment, following a comparison with the results obtained under the same experimental conditions on MRC-5 control cells, the compound SF-FP showed an anti-proliferative activity against A2058 melanoma cells. In particular, SF-FP showed a reduction of cell proliferation A2058 to 33% at concentrations of 100 µg/ml, 78% at 50 µg/ml and 68% at 10 µg/ml. All the other compounds, even if decreased cell viability, did not show a significant reduction below 50%, such as the compound DPSF-FP which reduced cell viability to 56% in A2058 at 50 µg/ml and 80% at 100 µg/ml, and DPSF C8 which showed a reduction of viability to 52% at 50 µg/ml. Similarly, dichloromethane extract taken from Brittle star (*Ophiocoma erinaceuss*) had antiproliferative effect on B16F10 melanoma cells especially at the concentrations above 31 µg/ml (Mostafapour, Baharara, e Amini 2015).

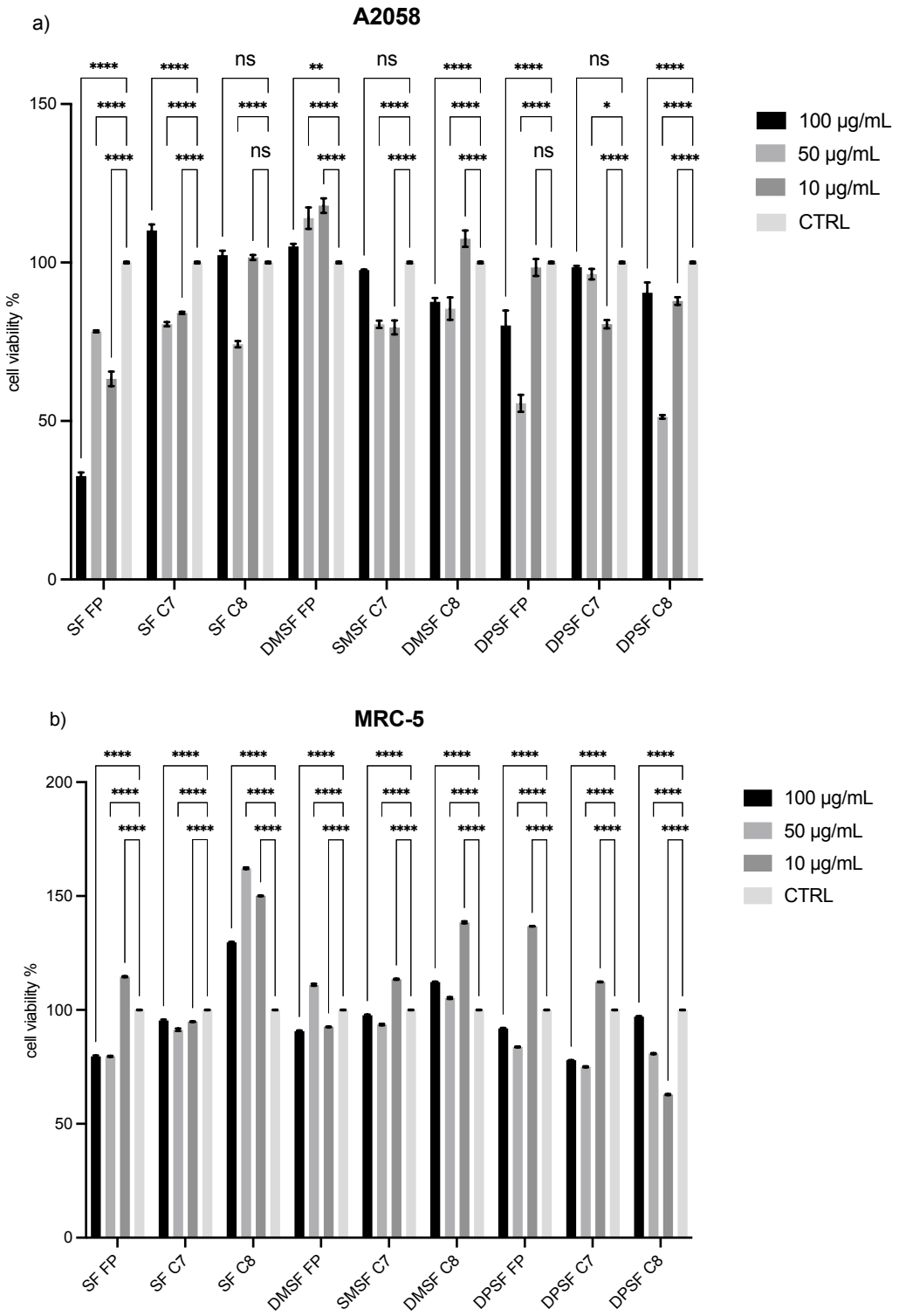


Figure 6. *In vitro* Anti-proliferative effect of whole starfish (SF), deproteinized starfish (DPSF), demineralized starfish (DMSF) hydrolysates, obtained by Food Pro PNL (FP), Corolase 8000 (C8), or Corolase 7089 (C7) on A2058 cell line (malignant melanoma) (a) and MRC-5 (control cell line) (b) and the respective controls only with the culture medium. Results are expressed as percentage of cell survival after 72h exposure (n = 3).

ANOVA ordinary one-way Dunnett''s MULTIPLE COMPARISON 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0,0001 (****)

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