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U. O. DOTTORATI

Dottorato in Scienze della Terra e del Mare
Dipartimento di Scienze della Terra e del Mare (DiSTeM)
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SEA SALT: INNOVATIVE TECHNOLOGIES FOR TRACEABILITY, SECURITY AND NUTRACEUTICS – INDUSTRIAL APPLICATIONS

IL DOTTORE
Eleonora Fioravanti

IL COORDINATORE
Prof. Marco Milazzo

IL TUTOR
Prof. Marco Milazzo

IL CO-TUTOR
Prof.ssa Concetta Maria Messina

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SUMMARY

Salt production is a sustainable process based on the use of renewable resources such as wind, sun and seawater. Through fractional precipitation, induced by wind and sun, almost pure sodium chloride precipitates in crystallising basins. This process involves the use of simple technologies, modified over millennia only by the introduction of mechanical means. Thus, sea salt production can be considered a technologically mature production process. However, it is still possible to intervene by adding value through innovation in areas related to the traceability of the product and the exploitation of the enormous potential arising from the unique biodiversity present in these environments. The innovation envisaged is based on the use of DNA purification technologies, next-generation sequencing and in vitro evaluation. Using advanced molecular biology techniques, it is in fact possible to characterise the population of Bacteria and Archaeobacteria included in the salt crystals during their formation process and to define their geographical origin. Furthermore, with extraction techniques, culture characterisation and *in vitro* evaluation on cells, it is possible to exploit bioactive compounds extracted from halophilic microorganisms for applications in nutraceutics, pharmaceutics and cosmeceutics. Thus, despite the technological maturity of the main production process, it is possible to intervene with a high degree of innovation to help increase its overall profitability.

The characterisation of the microbiome present in salt crystals is crucial for the traceability and safety of the resource. The testing of bioactive extracts, originating from saline waters, in cosmeceutical and pharmaceutical applications will enable products derived from these applications to benefit from the related traceability, safety and human health results, thus bringing significant benefits to their eco-sustainable image.

The aims of this research fall within the principles of the National Strategy for Intelligent Specialisation (SNSI) approved by the European Commission, which promotes and subsidises such scientific approaches and objectives. Already in the past, the European Community has granted the Protected Geographical Indication for

Trapani's sea salt, in the wake of which other European countries have applied for it. Sea salt is produced in coastal salt works, which have generally been transformed into protected environments. For this reason, the certificate of origin is associated with an incisive image of eco-sustainability, which derives from the place of production being represented by peculiar natural environments.

The close relations between the various scientific themes make the aforementioned PhD project innovative, applied, connected to human activities and consumption, consistent with national strategies and in line with the European objectives.

The purpose of the PhD thesis contributes to the application of current Blue Economy strategies in accordance with the "Blue Growth" and the Horizon programme, thus enabling the development of future prospects for improving the circular economy and marine production chain to obtain compounds that are useful for nutraceutical, cosmeceutical and pharmaceutical applications.

During the years of my PhD thesis, were been performed several experiments with the main goal of defining and validating techniques in traceability of produced salt and methodologies for the use of sea salt and halophilic organisms in nutraceuticals and cosmeceuticals.

For this purpose, the attention was focused on six commercial salt from different Mediterranean salt works (Aigues Mortes, Cervia, Margherita di Savoia, Nubia, S. Antioco, Sfax) to salt work community determination, in **Chapter 2**. The results showed a significant variability in the community of halophiles present in salt crystals among salt samples from different salt works.

In **Chapter 3** the pharmaceutical potential of brine from salt works was been validated by carrying out *in vitro* tests.

Chapter 4 is focused on validation of the pharmaceutical and cosmeceutical properties of bioactive compounds obtained from salt work, focusing on antioxidant properties tested *in vitro*. The intrinsic antioxidant capacity of salt work waters ensures increased antioxidant capacity at the cellular level, confirming the importance

of using *in vitro* cell systems and biomolecular markers for the research of bioactives for cosmeceutical applications.

CHAPTER 1

1. GENERAL INTRODUCTION

1.1 Sea salt

Sea salt, NaCl up to >98% pure, is produced all over the world using the same raw material: seawater, with the same production system: salt work. However, in spite of this remarkable homogeneity of raw material, extraction technique and composition, the quality of salt produced in different parts of the world can be very different. The difference is determined by the components defined as "impurities," namely trace elements, which make up a small percentage, ranging from 0.5% to 2.5% (Sedivy, 2009; Donadio et al., 2010; Silva et al., 2010a; 2010b; Oren, 2010, Galvis-Sanchez et al., 2011).

Sea salt is structurally composed of: Potassium mixed with halite (NaCl), Iodine, Magnesium, Sulfur, Aluminum, Calcium, Copper, Iron, Minerals (Sodium oxide, Calcium oxide, Potassium oxide, Magnesium oxide, Sulfur oxide, Aluminum oxide, Iron oxide, Silica oxide, Bromine oxide, Strontium oxide), (Yalcin and Mutlu, 2012). Among the trace elements in sea salt, the greatest effect on "quality" is determined by the organic component (Silva et al., 2015).

Salt (Sodium Chloride, NaCl) has always been a fundamental resource for human consumption and essential for cellular health, being involved in water transfer, muscle contraction, stomac pH control (Kurlansky, 2002). Salt is naturally present in fruits and vegetables, contributing 20% to daily intake, and is an ingredient used in food preparations. Indeed salt is added to food during processing (50% contribution) or directly during cooking meals (30%). Salt is consumed in excess of the recommended amount, 5 to 10 times more than the recommended 2g per day (Brown et al., 2009; He and MacGregor, 2003).

Salt's history dates back to antiquity, probably with the discovery of agriculture and early food preservation (Desrosier, 1977). The use of salt in the food and beverage industry has three main functions. First, it imparts a specific flavour to the product, enhancing and modifying the flavour of other ingredients and reducing the sensation of bitterness. Second, it aids the handling and processing of many products: it makes gluten in bread more stable and less extensible; it regulates the activity of starter cultures in cheese, increases water retention and improves the tenderness of meat. Thirdly, and most important, salt acts as a food preserving agent by reducing water content (Hutton, 2002).

Salt applications for food and feed account for 3% of European consumption, while industrial or chemical applications and road de-icing account for 66% and 29% respectively (The European Salt Producers' Association, 2004).

Salt can come from underground, known as rock salt, or from the evaporation of seawater in salt work, known as sea salt.

Solar salt works consist of a series of shallow ponds, normally less than 1 m deep, affording a large surface area for evaporation, with salinity increasing from seawater up to the saturation point of halite (NaCl). Different types of evaporite minerals precipitate on the bottom of the ponds according to the degree of evaporation. Sea salt from Mediterranean salt works is obtained from coastal evaporative basins, consisting of a complex system of functionally interconnected tanks (Isaji et al., 2019), where seawater is evaporated for the purpose of fractionating the solutes present to obtain, in crystallizing tanks, 90% pure NaCl (Jhala, 2000).

1.2 Salt work and production process

Solar Salt process is environmental friendly. Salt has been produced by solar evaporation from seawater since time immemorial, as mentioned above. This process also helps to preserve wetlands, which are becoming increasingly scarce as cities expand. Salting seawater consists of progressive brine evaporation in large ponds using solar heat and natural wind. As the brine evaporates, it becomes concentrated and the salts crystallise in a fixed order. The sodium chloride fraction is separated from the brine in a series of flat rectangular ponds over a defined concentration range and deposited as a uniform crust. A variety of methods, ranging from simple hand labour to mechanical harvesters, are used to harvest the salt crust. The chlorides and sulphates of magnesium, calcium and potassium, which are impurities in the salt, crystallise from the concentrated brine in addition to sodium chloride. The harvested salt, in the form of wet crystals, is washed with brine to remove the insoluble substances as well as the soluble impurities. (Jhala, 2006).

The Solar Salt process can be broken down into four parts:

1. Brine Management, 2. Crystallization, 3. Harvesting salt,4. Up-grading.

1. Brine Management

The initial specific gravity varies according to the location, but is normally between 1.02 and 1.025, i.e. around 3.0* Be (degree Baumé, density measurement of an aqueous solution). Solar evaporation concentrates it to 1.21, or just over 25* Be. Initially, the ponds are large, known as reservoirs. By gradual evaporation, when the density reaches 10 degrees Be, the volume of sea water is reduced to 37%. After that, the original volume is reduced to 20% and the liquid stays unsaturated up to 17 degrees Be. At 12 degrees Be, it is sometimes observed that some gypsum and calcium and magnesium carbonate separate. When the concentration reaches 17 deg Be, calcium sulphate ($CaSO_4$ 2H2O) begins to separate in the form of a thin layer, first floating and then settling. Most of the CaSO4 precipitates between 17 and 25 degrees Be. In the last stage, the separation of sodium chloride begins. It is separated

together with other salts as impurities of sodium chloride. At this stage, the rate of evaporation reduces the sweet water to less than 50%. As evaporation continues, 36.5 grams of sodium chloride precipitate for every 100 grams of water evaporated from the saturated solution.

2. Crystallizers

The crystallisers are the heart of the saltworks. It is therefore of paramount importance for a saltworks to get the best out of them. The crystallisers should give the maximum yield, the best quality of salt with the minimum consumption of brine. It has its own importance. For proper quality control it is necessary that in addition to checking the specific gravity/density, Ca and Mg should also be determined before charging the brine to the crystallisers. It is necessary to continue monitoring the density and Ca and Mg in the brine and in the precipitated salt after the brine has been fed to the crystallisers. Usually a specific density of 25.17 to 29 degrees should be maintained in the crystallizers. Maintaining a brine depth of about 20cm is also important. In crystallisers with a deep charge, the formation of crystals is of the cubic type, whereas in crystallisers with a shallow charge the formation of crystals is of the hollow type. Deep bottom allows the brine to reach high temperature, wave action promotes on evaporation in the tank, the cubic shape of the salt crystal allows easy removal of impurities by washing, the presence of microorganisms in the tank increases the crystallization phase.

3. Harvesting

The first crust of salt, approximately 30 cm thick, is used as a permanent bed, after which subsequent crusts, 10 to 30 cm thick, are harvested and sent to the washing plant.

4. Up grading

Salt is freshly harvested. It contains impurities from seawater. Washing with brine removes about 70% of these impurities. (Jhala, 2006).

1.3 Blue economy

The interest in the sustainability of the ocean economy is growing. The terms “Blue Economy” and “Blue Growth” were introduced at the Rio+20 United Nations Conference on Sustainable Development in 2012 (Mulazzani & Malorgio, 2017).

A general definition of "Blue Economy" was given by the United Nations (UN): the economy of the oceans that is aiming to “the improvement of human well-being and social equity, while significantly reducing environmental risks and ecological scarcities” (UNCTAD, 2014).

Sea salt is produced all over the world using the same raw material, seawater, in the same production system: the salt work. Sea salt production is a sustainable activity with important benefits for the economies of the regions that practice it and for environmental conservation. Most areas where salt pans are located now fall within Nature Reserves. This productive activity, therefore, is intrinsically associated with environmental protection and recently salt pans in Italy and Europe have become tourist attractions for naturalistic and traditional intrinsic value.

Solar Salt process is environmental friendly: solar evaporation and crystallisation processes are suitable due to the use of free solar energy that means a low energy cost, the low cost of equipment for installation and operation of the solar pond system, and human intervention results in the phase of moving the water between the various tanks and in the final collection (Schultheis et al., 2001; Jhala, 2000). The relatively low cost of constructing solar ponds makes the use of solar energy attractive in economic terms, especially in areas with high insolation. (Schultheis et al., 2001).

For example, the World Bank's most recent definition of the blue economy is “the sustainable use of ocean resources for economic growth, improved livelihoods and jobs while preserving the health of ocean ecosystem” (World Bank, 2017).

The World Bank's definition is a concept that encompasses many aspects of ocean sustainability, from sustainable fisheries to ecosystem health and pollution prevention. (Voyer *et al*., 2018).

Within the Blue Economy, the value of natural capital should be included in salt for its sustainable development in terms of food security, sustainable livelihoods and income.

The concept of the Blue Economy has been endorsed by the World Bank, the EU, the African Union, the OECD and the UN. Coastal states are assessing the economic opportunities that exist both within and beyond their maritime jurisdictions. (Voyer *et al*., 2018).

However, to date, there is no generally accepted definition of the Blue Economy, as the term has been used for different purposes (Voyer & van Leeuwen, 2019).

According to Silver (Silver et al., 2015), Voyer (Voyer et al., 2018) and Voyer & van Leeuwen (Voyer & van Leeuwen, 2019), four Blue Economy 'lenses' have been identified:

1. The 'oceans as natural capital' lens, in relation to ecotourism, marine protected areas (MPAs) and payment for ecosystem services; the aim of this lens is to quantify the conservation benefits and economic opportunities from increased protection of the oceans;

2. The 'oceans as livelihoods' lens, the main sectors of which are tourism and small-scale fisheries. These lenses suggest that Blue Economies can help address poverty and food security;

3. The 'oceans as good business' lens, which focuses on large multinational companies in the shipping and industrial fishing sectors, emphasises the scale of the economic contributions of ocean-based industries to global markets to put into play the importance of these sectors and their ability to provide increased growth;

4. The "oceans as a driver of innovation" lens, whose focus is on technical and technological innovations (ocean-based renewable energy, biotechnology and seabed mining), imagining the oceans as sources of new discoveries and wealth (Silver *et al.*, 2015; Voyer *et al.*, 2018; Voyer & van Leeuwen, 2019).

According to Blue Economy concepts, the implementation of sustainable practices allows benefits to be derived from the oceans and coastal regions while respecting their carrying capacity for human activities. For this reason, in order to maintain the potential of the oceans over time, human activities must be managed in a way that ensures the health of the oceans and the maintenance of economic productivity (The EU Blue Economy Report, 2021).

"Blue Growth, promoted by the 2014 FAO report, is defined as "a coherent approach to the environmentally sound, integrated and socio-economically sensitive management of aquatic resources, including marine, freshwater and brackish water environments". Blue Growth takes definition on very different meanings and approaches depending on the social contexts in which it is used, like the Blue Economy. (Eikeset *et al.*, 2018).

Internationally, the concepts of Blue Economy and Blue Growth are gaining visibility, given their prominent role in Rio+20. These concepts are part of Goal 2 ('End hunger, achieve food security and improved nutrition, and promote sustainable agriculture') and Goal 14 ('Conserve and sustainably use the oceans, seas and marine resources for sustainable development') of the Sustainable Development Goals (SDGs) of the UN's 2030 Agenda. (Ababouch & Carolu, 2015).

Therefore, sea salt and the biotechnological application of its bioactive extracts are a resource that fully falls under the concept of blue economy and blue growth, as it involves onshore plants based on renewable energy in its marine production (Fig. 1.1).

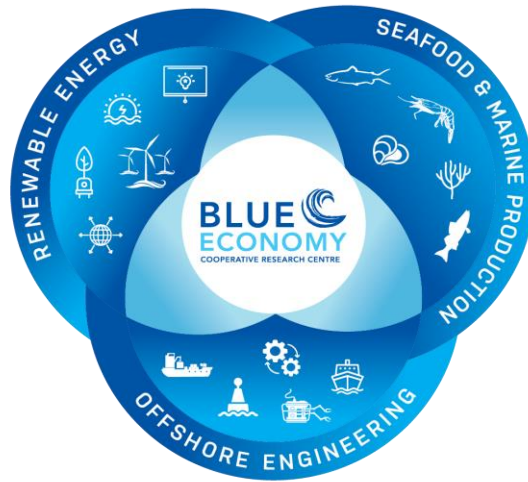


Figure 1.1 The opportunities to grow the blue economy are vast. Source: (Blue Economy CRC)

1.4 Innovation

Sea salt production can be considered a technologically mature production process. However, it is still possible to intervene by adding value through innovation in areas related to product traceability and the exploitation of the enormous potential arising from the unique biodiversity present in these environments. The planned innovation is based on advanced molecular biology techniques with which it is possible to characterize the population in Bacteria and Archaea included in salt crystals during their formation process and to define their geographical origin. In addition, with extraction techniques, culture characterization and *in vitro* evaluation on normal and cancer cells, it is possible to exploit bioactive compounds extracted from halophilic microorganisms for various applications. Thus, despite the technological maturity of the main production process, it is possible to intervene with a high degree of innovation to help increase its overall profitability.

Characterization of the components of sea salt, which are critical for traceability and safety of the resource, will include analysis of the nutritional value of the elements that make up the salt, characterization of the microbiome present in salt crystals, and identification of possible contaminants, including microplastics.

The extraction of bioactive compounds from extremophilic microorganisms present in salt water will allow their molecular characterization and the possibility of developing methodologies for producing the molecules *in vitro*.

The testing of bioactive extracts in nutraceutical and cosmeceutical applications will allow the products resulting from such applications to benefit from the related results, with regard to traceability, safety and human health, thus deriving significant benefits on their eco-sustainable image.

The aims of such research fall within the principles of the National Strategy for Intelligent Specialization (SNSI) approved by the European Commission, which promotes and subsidizes such scientific approaches and objectives. Already in the past, the European Community has granted Protected Geographical Indication for Trapani sea salt, in the wake of which other European countries have applied for it. Sea salt production takes place in coastal salt works, which, generally, have been transformed into protected environments. Therefore, the certificate of origin is associated with an incisive image of eco-sustainability, which comes from the place of production represented by distinctive natural environments.

1.4.1 Traceability

Traceability systems providing information on the origin, processing, trade and final destination of food are needed at a pan-European level (Tatsadjieu et al., 2010). Such systems should increase consumer confidence in food and enable regulatory authorities to identify and withdraw from the market food that is unsafe or inedible (Schwägele, 2005).

Sea salt is composed of more than 98.5% NaCl, this characteristic would seem to make geographic typing based on composition very difficult, however, the production system and the environment in which it is produced significantly influence the composition of the remaining 1.5%, making its geographic characterization possible (Sedivy, 2009).

Halophilic microorganisms present in the water of the salt work basins, such as Archaea, Bacteria and unicellular Algae, permit to characterize the geographical origin of the salt and to obtain high value bioactive compounds that they produce as an intrinsic mechanism of resistance to the changing of environmental factors.

Identification of geographical origin is part of the requirement of the Food Production Traceability System that guarantees an increase of the added value to the food products. From a methodological point of view, the innovation of product traceability consists in analyzing the microbial composition of the community, which has been found to be peculiar for each salt work. During crystallization process, Archaea and Bacteria can be entrapped in salt crystals, where they can survive for very long period. It has been demonstrated that microbial community of basins is different in different saltwort (Fernandez et al., 2014).

Thus, characterization of microbial community could be used to identify the salt work were sea salt was produced (Figure 1.2).

By means of metagenomic analysis of 16S rRNA gene extracted from halophiles and Archaea salt crystals and Next Generation Sequencing (NGS), it is possible to characterize the microbial community, thus tracing the geographical origin of the salt (Dufossé et al., 2013; Henriet et al., 2014).

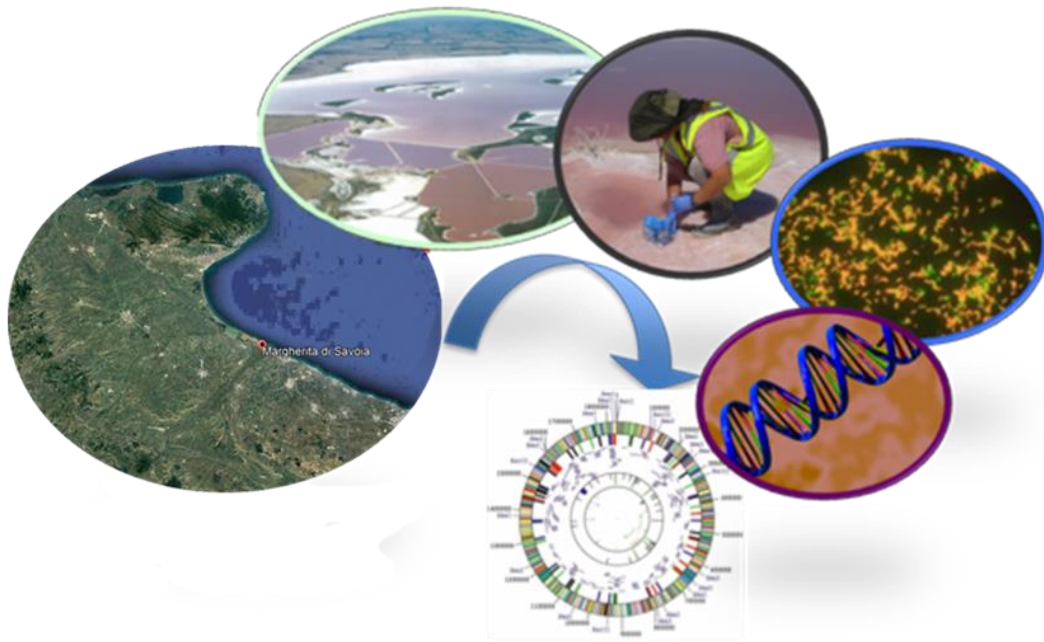


Figure 1.2 Study of environmental genomics to tracing the geographical origin. Source: (FAO)

1.4.2 Security

Sea salt is extracted from peculiar marine environments located along the coast, under particular conditions of high salinity water and in association with animal and plant species. Such peculiar environments result in extreme vulnerability, especially concerning anthropogenic pollution. High concentrations of pesticides, from crops, and industrial by products increase the level of vulnerability of coastal areas, causing deleterious impact on aquatic systems and the quality of the sea salt produced. Various authors have reported the presence of contaminants found in salt marsh waters, in *Artemia* crustaceans living in salt marshes or in sea salt after the crystallization process, such as pesticides (e.g. benzofuran and amylphenol derivatives, chlorophosphates) such as herbicides, insecticides and fungicides, urban and industrial pollutants (e.g., caffeine, toluene), polychlorinated biphenyl compounds (PCBs), flame retardants (PBDEs), halogenated, aliphatic and aromatic hydrocarbons, and ketones (Serrano et al., 2012). Also plasticizing substances and fragrances (e.g., galaxolide, a synthetic compound from musks used for fragrances), included within pharmaceuticals and personal wellness products (e.g., benzophenone and derivatives to filter UV), (Serrano et al., 2011).

Contaminants, frequently, are transported by rivers and plantation wastewater flows to salt works, estuaries and river deltas; this makes such areas vulnerable, and the sea salt produced may contain contaminants in the water, which remain included in it after concentration and crystallization processes. Consequently, there is a need for monitoring on the presence of organic contaminants in sea salt and knowledge of its quality, as salt is widely used in human and animal nutrition, as well as in aquaculture activities (Serrano et al., 2011).

As sea salts are harvested from seawater, they might contain contaminants present in the marine environment, but also the production and food processing methods may lead to increased concentrations of microplastics and trace elements.

Since 1960, plastic production has increased annually by 8.7%, involving the world's industry in its use, and in 2004 the term "microplastics" was introduced to describe the product, not disposed, of waste and degradation of this material (Smith et al., 2018). Microplastics, defined as plastic materials or fragments measuring less than 5 mm (Yang et al., 2015), are often subdivided by primary type, <5 mm, and secondary type, resulting from the breakdown of larger objects (Smith et al., 2018). Microplastics, are now considered an important class of emerging contaminants, and the most commonly treated types of plastics are Polyethylene and Polypropylene. In addition to the additive chemicals associated with plastic debris, persistent organic substances (POPs) that have accumulated on microplastics, such as biphenyl polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides such as DDT and HCB, flame retardants (PBDEs) have been found in the oceans (Smith et al., 2018). The widespread presence of microplastics and subsequent accumulation is found in marine, freshwater and terrestrial ecosystems as a consequence of improperly managed product disposal. The 80% of plastics are derived from terrestrial sources, with the remainder from marine operations. Waste plastic materials enter the marine environment as garbage, industrial waste or refuse through waterways, sewage discharges, and transported by wind and tides. Waste generation and dispersal are linked and proportionally associated with economic development, local infrastructure, and legislation (Smith et al., 2018).

The presence of microplastics in the seas is relatively well known, but little is known about their presence in salt for commercial use that is consumed massively by the world's population (Karami et al., 2017).

In various studies, to estimate the quantity and quality of microplastics present, they used extraction techniques by hydrogen peroxide treatment to extract the microplastics from the salt and dissolve the organic matter, filtration techniques for

separation, microscopic and chemical analyses for characterization of microplastics (Seth et al., 2018).
Microplastic (MP) occurrence in seawater is relatively well understood, but nothing is known about their presence in most commercial salts used by people worldwide. In the study of Karami et al. (2017), 72 particles were extracted and 41.6% were plastic polymers, 23.6% were pigments, 5.50% were amorphous carbon, and 29.1% remained unidentified. The particle size (mean ± SD) was 515 ± 171 μm. The most common plastic polymers were polypropylene (40.0%) and polyethylene (33.3%). Fragments were the main form of MPs (63.8%), followed by filaments (25.6%) and films (10.6%), (Karami et al., 2017).

To this end, some studies have turned to the study of salt from the world's largest producers of this resource China, the United States, and India, which is taken over Europe and which export salt for human and industrial consumption worldwide. In 2015, 78% of the salt produced in India was marine-derived, although the presence of microplastics in marine sediments, beaches and invertebrates was well documented. The level of microplastic contamination in Indian sea salt is unknown, just as there are no strategies to prevent contamination of the salt produced. These rather critical shortcomings do not prevent India's worldwide export of salt (Seth et al., 2018); it therefore turns out that traceability of salt is important, which can guarantee its origin, enhance the production system, track the components under European and Italian laws with a view to food control and environmental management.

1.4.3 Nutraceutic, pharmaceutic and cosmeceutic

Microbial communities provide a range of benefits to mankind, including pigment production by a number of microbes whose deserved importance has been highlighted in recent years, and other unexplored sources of many unknown pigment compounds. These compounds are also known to exhibit cytotoxic, antioxidant, antimicrobial, antimalarial, anticancer, antitumour and antifouling activities.The importance of microbial pigments has been highlighted in various applications such as cosmetics, food, pharmaceuticals and textiles. (Ramesh et al., 2019).

Pigments are molecules that absorb a specific wavelength of light and reflect the remaining visible spectrum (380-750nm). One of the charismatic features of microbes is the production of pigments. It seems that microbial pigments are not just colours. They are a mixture of different chemical components with a wide range of potential biological activities.

Over the past two decades, there has been a tremendous increase in the study of pigmented micro-organisms from terrestrial and marine ecosystems. Microbial pigmented molecules such as bacteriochlorophylls, carotenoids, flavins, indigoids, melanins, pheomelanin, monascins, phenazines, phenazostatin D, prodigiosin, quinone precursors, violacein, glaukothalin, pycocyanin, xanthomonadin, phenazine, canthaxanthin, astaxanthin, β carotene and others are produced as by-products by several microorganisms. (Velmurugan et al., 2020).

Many of these compounds and their derivatives have been reported to exhibit a wide range of cell-specific biological activities, which are expressed in terms of effective/inhibitory/lethal concentrations such as effective dose (ED), growth inhibitory concentration (GIC), minimum inhibitory concentration (MIC), half maximal effective concentration (EC_{50}), half maximal growth inhibition (GI_{50}), half maximal inhibitory concentration (IC_{50}), half maximal lethal concentration (LC_{50}),

half maximal lethal dose (LD_{50}), and 50% cancer growth inhibition (TGI_{50}), (Ramesh et al., 2019).

In recent years, bioactive molecules have been introduced as ingredients for cosmetic products. Bioactive molecules, or "actives", are characterized by their ability to actively modulate biological processes occurring in human skin, for example, by inducing beneficial properties or by interfering with pathways known to lead to skin damage. To reflect the pharmaceutical properties of their active ingredients, cosmetic products containing such active molecules have been termed cosmeceuticals. The increasing use of actives has already changed and will continue to change our perception of cosmetic products, because the more we know about the biological properties of these molecules, the better we can use them to develop more effective and targeted products.

Given the ongoing demographic changes in societies, the need for new actives to protect human skin from environmental threats, and in particular from extrinsic skin ageing, is constantly increasing and therefore studies into how these molecules work are of great interest. Typically, active ingredients can be either chemically produced or directly derived from natural sources (Grether-Beck et al., 2007).

In hypersaline environments, interest in living microbes, i.e. halophilic microorganisms, has increased with the recent discovery of new taxa useful for various biotechnological applications and processes, including biopolymers, biosurfactants, exopolysaccharides, compatible solutes and bioactive compounds (carotenoids, anti-tumour and antimicrobial substances, etc.), (Chattopadhyay et al., 2008; Fernández-García et al., 2012).

In the study of Oren and Trupen in 1990, Halophilic archaea with red carotenoid pigments improved light absorption in the brine and promoted evaporation by increasing temperature. Carotenoids have received increasing attention because they are the most abundant pigments in nature, and marine carotenoids differ structurally from those found in terrestrial environments. In halophilic archaea, bacterioruberin is

considered to be the main representative of C50 carotenoids (Abbes et al., 2013). Carotenoids, a group of lipid-soluble compounds responsible for the yellow and red colours of many plants, have been shown to be effective in the prevention of several chronic diseases, including skin cancer and cardiovascular disease (Lu et al., 1995). Carotenoids are also widely distributed in nature and have considerable potential as nutraceuticals and dietary antioxidants (Elliot, 2005). In addition, bacterioruberin is known to contain 13 pairs of conjugated double carbon bonds that endow biological tissues with effective hydroxyl radical scavenging power and singlet oxygen quenching activity. This pigment can protect halobacteria from lethal injury under intense light (Mandelli et al., 2012) and confers bacteria with resistance to oxidative DNA damage from radiography, UV-irradiation, and H_2O_2 exposure (Shahmohammad et al., 1998). Bacteriorubin also has other equally important roles in membrane fluidity, including acting as a water barrier and being responsible for the permeability of oxygen and other molecules, thereby enhancing bacterial survival in hypersaline and low temperature environments. (Fong et al., 2010). Considering the promising properties and attributes of carotenoids, recent attention has been paid to the discovery of novel natural carotenoids with practical functional applications in the prevention of human health diseases. (Ganesan et al., 2011). In this context, carotenoids and flavonoids have been reported to form complexes with metal ions that alter or inhibit metabolic pathways. Carotenoids from terrestrial origins, such as β-carotene and lycopene, have particularly been investigated as cancer preventive agents (Abbes et al., 2013).

1.5 Halophilic microorganisms

One of the most critical environmental factors is salinity, which determines habitability of aquatic environment. Salinity is one of the most critical environmental factors determining the habitability of aquatic environments. Although salinity is potentially a strong habitability limiting factor, hypersaline environments support a surprising diversity of microbial life, especially in shallow environments that support benthic microbial mats (Oren, 2002; Ley et al., 2006; Oren et al., 2009). It follows that several processes are active in the shallow hypersaline environment, strongly influencing the biogeochemical cycles and chemical properties of the system.
system. In addition, by altering various environmental factors (salinity, temperature, pH, light conditions, etc.), seawater evaporation induces transitions in the state and composition of the microbial community. These changes lead to changes in biological processes, which in turn have a strong impact on the environment. Therefore, not only the physical and chemical processes induced by evaporation, but also the biological processes within the system determine the chemical characteristics of the evaporating seawater (Isaji et al., 2017).

In the "red waters" of crystallizing tanks (Figure 1.3), halophilic archaebacteria can reach and exceed a concentration of 10^7 – 10^8 cells per ml (Oren, 2010). When sodium chloride begins to precipitate these microorganisms become trapped in the fluids included in the crystals (Castanier et al., 1999; Davies, 2009; Oren, 2010). Numerous organic compounds originate from these microorganisms, including hydrocarbons, alcohols, phenols, aldehydes, ketones, terpenoids, norisoprenoids, etc. (Donadio et al., 2010; Silva et al., 2010a; 2010b; Galvis-Sanchez et al., 2011).

Figure 1.3 Red water in the tanks from a salt work on Isola Lunga (Stagnone Lagoon, Trapani). Source: (Santulli, 2014)

Microorganisms trapped in sea salt crystals, generally halophilic bacteria and archaebacteria, which can account for up to 2% of the salt's wet weight (Lefond, 2012), retain motility for more than 6 months (Norton and Grant, 1988) and have been "awakened" and cultured for up to 5 years after salt crystals have formed (Mc Genity et al., 2000).

The different ecological conditions of the various orders of tanks in a salt marsh, determined by increasing salinity, result in the establishment of different microbiological communities. At moderate salinities (up to 15°Bè), moderately halophilic bacteria such as *Halomonas spp*. are established (Oren, 1994); at salinities above 20/25°Bè, red halophilic archaebacteria dominate, resulting in the characteristic red color of the water in the crystallizing tanks (Oren et al., 1992; Oren and Dubinsky, 1994). At lower salinities, the unicellular alga *Dunaliella* is present in the serving tanks, contributing to this coloration (Oren et al., 1992; Oren and Dubinsky, 1994; Oren, 2009). The red color of these microorganisms is due to the presence of carotenoids, archaebacteria lycopene, etc., which together with osmolytes and compatible solutes (Roberts, 2005) enable these organisms to survive in these extreme conditions and overcome stresses induced by salinity, intense solar irradiation and high temperature, desiccation, free radicals, etc. (Brown, 1976; Shahmohammadi et al., 1998).

Salt marshes are to be considered a giant bioreactor where biological processes, which occur upstream, significantly influence the formation of salt crystals in crystallizing pools (Javor, 2002).

In fact, the presence of halophilic bacteria in salt pond waters can have direct influences on salt production through:

- the increase in the size and number of NaCl crystals (Lopez-Cortes et al., 1994);
- the acceleration of crystal formation (Norton and Grant, 1988);
- the initiation of the crystallization process (Castanier et al., 1999).

During the annual cultivation cycle, the biological component is directly and indirectly exploited by using it to grow salt marsh.

The direct use of the organic component comes from the reuse of brines from the crop made in the previous year. The spent brines, drained from the crystallizing tanks before the salt is harvested, and stored during the winter in the service tanks, act as a yeast to trigger the new production. These waters contain, in fact, bacteria, unicellular algae and *Artemia salina* cysts, which, finding themselves in favorable environmental conditions again, begin to divide and reproduce, helping to establish the environmental conditions necessary for abundant production of high-quality salt.

Each environment has specific microorganisms; within solar salt pans, halotolerant and halophilic microorganisms develop, which, through specific biological mechanisms, are able to control their osmotic pressure in environments with high salinity (Margesin et al., 2001) or high light intensity. The microbiology of crystallizing salt ponds has been reevaluated from a molecular perspective by applying targeted 16S rRNA analysis methods to identify their microbial composition. Archaea and Bacteria coexist in this extreme environment, and generally among the archaea the predominant species, with 50% of the total number of cells is *Haloquadratum walsbyi*; the second component of the biome, quantitatively relevant, and accounting for 20-25% of the total prokaryotic community is *Salinibacter ruber* (Elevi Bardavid et al., 2008). Another community domain is attributed to the primary producer, *Dunaliella*, a unicellular green alga.

Many other archaea and bacteria (*Halobacterium, Halococcus*) have been described and characterized as producers of pigments, such as carotenoids (salinixanthin, b-carotene, spirilloxanthin, bacterioruberine), (Dufossè et al., 2013). In other studies, such as that of Fernandez et al. in 2014, the metagenome is obtained by pyrosequencing prokaryotic DNA extracted from saline tank waters, usually with salinities at 19% and 37%, finding sequences never before described. In addition, a simplified metabolism for carbon and nitrogen cycling in extreme habitats has been characterized. Light is widely used as an energy source by bacteriorodopsins and other rhodopsins.

For the determination of geographical origin, one way to trace the source of the product is to analyze the bacterial communities present on food samples. In this regard, molecular techniques (such as PCR- DGGE, which also allows the detection of culturable and non-culturable bacteria (Leesing, 2005)) are used by employing 16S rDNA profiles to infer variations in salt bacterial community structures from various regions. In addition, it is important to be able to access or create new sets of primers for Archaea, designed for the 16S rRNA target, which would allow for not underestimating the insufficiently known Archaea community (Gantner et al., 2011). Following appropriate statistical analysis, different bacterial composition of the salt from different regions is shown and could be used as a "barcode" to certify the different origin of the salt. These profiles could serve as specific markers for different locations. Such a method is proposed as a new traceability tool that allows the origin of the place of salt production to be traced back to the store directly through a specific unique code (Dufossè et al., 2013). In addition, through the described analyses, traceability can also be extended to food products such as fish (Le Nguyen et al., 2008; Tatsadjieu et al., 2010) and fruit (El Sheikha et al., 2010 and 2011).

1.6 Bioactive compounds

Although the study of systematics is still at an exploratory stage, the study of possible applications of secondary metabolites produced by bacteria and archaea in extreme environments, in various fields such as nutraceutics and cosmeceutics, is in full swing. Halophilic bacteria have evolved certain biochemical strategies to resist the risks given by the oxidative stresses to which they are subjected, such as an efficient antioxidant system, represented by lycopene, bacterioruberins, bacteriorodopsins, and bacteriopsins, which have strong activity against radicals. The carotenoid Lycopene, which is mostly contained in tomatoes but is also produced in considerable amounts by extremophiles, has been identified as the most potent antioxidant (Peck et al., 2002). The antioxidant properties of lycopene can be widely used in both pharmaceutical and nutraceutical applications (Gharbi et al., 2017). Recently, attention has been focused on the antiproliferative effects of lycopene in pharmaceuticals (Rao et al., 1999).

For example, Zeaxanthin extracted from *Flavobacterium sp*. is a yellow pigment also known as 3,3'-di-hydroxy-b-carotene and can be used as a food additive in poultry feed to reinforce the yellow color of animals' skin or to accentuate the color of the yolk of their eggs (Alcantara et al., 1999). The compound is also suitable for use as a coloring agent, for example, in the cosmetics and food industries. Cultures of *Flavobacterium sp*. in a nutrient medium containing glucose or sucrose, sulfur-containing amino acids such as methionine, cysteine, pyridoxine and metal ions were able to produce 190 mg (zeaxanthin)/L, with a specific cell concentration of 16 mg/g of dried cell mass (Dufossè, 2006).

Cantaxanthin extracted from the photosynthetic bacterium, *Bradyrhizobium sp*. or from the extremophile *Halobacterium sp*. is a carotenoid pigment and has been used dissolved in water for many years in order to impart red color to the flesh of farmed salmonids (Hannibal et al., 2000). Interest in this keto-carotenoid is waning as a side effect of overdosing on this molecule is the deposition of tiny crystals in the consumer's eye, with debate still open (Baker, 2001).

Astaxanthin is present in 10 types of carotenoids. Because C40 carotenoids represent a group of pigments that has gained increasing commercial interest in recent years, numerous screenings have been conducted in order to characterize new biological sources of astaxanthin, and positive targets such as *Paracoccuscarotini faciens* (Tsubokura et al., 1999) and *Halobacterium salinarium* (Calo et al., 1995) have been isolated (Dufossè, 2006).

1.7 Aims of the thesis

My PhD research project consider the potential value of halophilic microorganisms present in the water of the salt work basins, such as Archaea, Bacteria and unicellular Algae, to characterize the geographical origin of the salt and to obtain high value bioactive compounds that they produce as an intrinsic mechanism of resistance to the changing of environmental factors. Identification of geographical origin is part of the requirement of the Food Production Traceability System that guarantees an increase of the added value to the food products fall within the principles of the National Strategy for Intelligent Specialisation (SNSI) approved by the European Commission, which promotes and subsidises such scientific approaches and objectives. Sea salt is produced in coastal salt works, which have generally been transformed into protected environments. For this reason, the certificate of origin is associated with an incisive image of eco-sustainability, which derives from the place of production being represented by peculiar natural environments.

From a methodological point of view, the innovation of product traceability consists in analyzing the microbial composition of the community, which has been found to be peculiar for each salt work, and it is crucial for the traceability and safety of the resource. The testing of bioactive extracts, originating from saline waters, in cosmeceutical and pharmaceutical applications will enable products derived from these applications to benefit from the related traceability, safety and human health results, thus bringing significant benefits to their eco-sustainable image.

Aim of this thesis was to define techniques in traceability of produced salt and methodologies for the use of sea salt and halophilic organisms in nutraceutic, pharmaceutic and cosmeceutic.

The close relations between the various scientific themes make the aforementioned PhD project innovative, applied, connected to human activities and consumption, consistent with national strategies and in line with the European objectives.

The purpose of the PhD thesis contributes to the application of current Blue Economy strategies in accordance with the "Blue Growth" and the Horizon programme, thus enabling the development of future prospects for improving the circular economy and marine production chain to obtain compounds that are useful for nutraceutical, cosmeceutical and pharmaceutical applications.

CHAPTER 2

2. BACTERIAL COMMUNITY

2.1 Introduction

The world of halophilic microorganisms is very diverse. Microbes adapted to live at high salt concentrations can be found in all three domains of life: Archaea, Bacteria and Eucarya. In some ecosystems, salt-loving microorganisms are so abundant that their presence can be detected without a microscope. The brines of salt crystallizer ponds around the world are coloured pink-red by archaea (*Haloquadratum* and other members of the *Halobacteriales*), bacteria (*Salinibacter*) and eucarya (*Dunaliella salina*). Hypersaline environments, such as salt pond brines and natural salt lakes, provide the ecologist with relatively simple ecosystems with low diversity and high community densities (Ma et al., 2010, Gagnon et al., 2023). Different types of halophiles have solved the problem of coping with salt stress (and often other forms of stress) in different ways, so studying microbial life at high salinity can answer many fundamental questions about how microorganisms adapt to their environment. Most known halophiles are relatively easy to grow, and genera such as *Halobacterium, Haloferax* and *Haloarcula* have become models for studies of the archaeal domain because they are much easier to handle than methanogenic and hyperthermophilic archaea. (Ma et al., 2010). Most habitats studied for the presence of halophiles are thalassohaline environments, formed by evaporation of seawater, reflecting the ionic composition of seawater and having a near neutral to slightly alkaline pH. (Ma et al., 2010). As brines dry and halite crystals form, small fluid inclusions remain trapped within the crystals. Microorganisms that inhabited the brine can become trapped in these inclusions. (Baati et al., 2008).

The question of the longevity of different types of halophiles in salt crystals has become a popular topic, relevant to disciplines such as geology, biogeography, evolution and even space exploration (McGenity et al., 2000), since the first

controlled studies showed that such microorganisms can retain their viability for long periods of time (Norton et al., 1988, Favreau et al., 2023).

Molecular biology techniques involving sequencing of the 16s ribosomal RNA gene have made it possible to demonstrate considerable variability in the microbial community even in salt works in the same country, such as in Australia (Oh et al., 2010) or in Tunisia where Baati et al. (2008), confirming the geographic effect on the microbial population, observe that the salt marshes of Sfax (Tunisia) exhibit distinctive characteristics not found in any other salt marshes in the world, ascribed to the climate and nutrient composition of the water feeding the salt works. In these salt marshes the bacterial clones are dominated exclusively by *Proteobacteria* and *Bacteroidetes*, while among the *Archaea* the dominant clones appear closely related to the order *Halobacteriales* (phylum *Euryarchaeota* class *Halobacteria*).

Tsiamis et al. (2008), in a study aimed at characterizing changes in the prokaryotic community profile during different stages of processing in the Messolonghi-Tourlis salt works (Greece), reported the largest prokaryotic community ever described for a salt work. Through an approach based on in vitro culture and techniques that do not involve this step, these authors isolated 132 strains of *Archaea*, essentially *Halobacteriacea*, and 219 strains of *Bacteria*, with a high diversity as evidenced by the 416 subfamilies identified. Significant variability in short-term community structure is evident from the results, dependent on the stage of salt work processing, an observation that confirms the close relationship between biotic component and salt production. Tsiamis et al. (2008) also confirm the significant effect that geographical location has on microbial community structure. In the Messolongi salt work, *Halovibrio, Salicola* and *Salinibacter* common in other salt works were not found, but a group of *Archaea* correlated with *Halobacterium salinarum* are present, as in the Maras salt marshes (Peruvian Andes), (Maturrano et al., 2006).

Dufossé et al. (2013) demonstrated, through the analysis of 16s RNA gene profiles obtained by the PCR-DGGE technique, that the bacterial communities present in salt crystals from some French salt pans is variable. These authors, therefore, suggest that

this technique can be a useful tool to identify the geographic origin of commercial salt.

2.2 Materials and methods

2.2.1 Study area and sample collection

The samples were represented by six commercial salt from different Mediterranean salt works: Aigues Mortes (France), Cervia (Ra), Margherita di Savoia (BAT), Nubia (Tp), S. Antioco (Ca), Sfax (Tunisia), (Figure 2.1). The unwashed crystals, collected in 2016, were stored in the dark at 4°C until use.

Figure 2.1 Salt samples location in Italy, France and Tunisia.

The sourcing locations of marine salt samples are mostly protected natural areas dedicated to sustainable industrial salt production and ecotourism. In Italy, despite 8300 km of coastline (ISPRA 2011), the only remaining industrial sea salt producers are in the salt work of S. Antioco (Sardinia), Trapani (Sicily), S.Margerita di Savoia (Apulia) and Cervia (Emilia Romagna).

2.2.2 Determination of salt work community

A metagenomic analysis of halophilic microorganisms was carried out to identify of the salt-associated bacterial community.

The methodological approach was the validation of a metagenomic characterization of microorganisms entrapped in salt crystals.

For each six salt samples, 100 ml of 25% solution was prepared from a sterile salt solution 12.5% in order to minimize osmotic stress on the bacterial cells present.

Considering the high densities of bacteria in the solutions, an initial 1:10 dilution was made for each sample using a sterile 25% NaCl solution filtered through Whatman GF/C glass fiber filters.

Each resulting solution was filtered on 0.45 μm pore size of cellulose acetate filters Millipore and centrifuged at 4000 rpm for 15 minutes. The obtained pellet was transferred into sterile 1.5 ml eppendorfs and stored at -20°C for subsequent extraction of the DNA present in the sample (Dufossé et al., 2013; Henriet et al., 2014).

DNA was extracted with Zymo Research's Kit, ZR Soil Microbe DNA MiniPrep™, specifically for DNA extraction from soil-isolated bacteria, which involves a mechanical lysis step using microbeads and EURx ltd's Kit: GeneMatrix Bacterial and Yeast Genomic DNA purification Kit (www.zymoresearch.com - ZR soil microbe DNA Miniprep).

The 16S rRNA genes were amplified using the bacteria-specific forward primer 008F or the archaeal-specific primer 21F in combination with the universal reverse primer 1390R (Baati et al., 2010).

The PCR thermal profile started as an initial denaturation at 94°C for 5 minutes, continued as primer annealing at 59°C for 1 minute and extension at 72°C for 1.5 minutes. The final elongation step was extended to 15 minutes.

The DNA was then visualized by electrophoresis on 1% agarose gel with ethidium bromide staining (Baati et al., 2010).

DNA quantities were estimated by running purified DNA samples by using spectrophotometer: the spectrophotometric method exploits the ability of nucleic acids to absorb UV light with an absorption maximum at a wavelength of 260 nm; the absorption spectrum ranges from 230 nm to 280 nm.
The drop of DNA eluate obtained earlier, was introduced into the machine directly (Multiscan-Sky Microplate Reader, Thermo-Scientific TM, USA). After that were read the absorbance (A) at 260 nm and the absorbance (A_{280}) at 280 nm and then was calculated the ratio of the readings at 260 nm and 280 nm. If this ratio is ≥ 1.7, it means that the DNA preparation is pure; otherwise, if the ratio is < 1.7, it means that contamination by protein is present.

The sequencing of the 16S rRNA gene was carried out by facilities outside the University of Palermo, and the sequences identified were attributed to the various possible taxonomic levels.

2.2.3 Statistical analysis

In order to visually assess the differences in the bacterial strains considered of the 6 salt samples used, the results obtained were processed by Cluster analysis, based on the Bray-Curtis similarity matrix, using the PRIMER version 6.0 statistical software. This statistical tool allows all data to be analyzed simultaneously, providing a graph as output.

Data, also, were analyzed by Multi-Dimensional Scaling (MDS) using the PRIMER version 6.0 statistical software. MDS was calculated with the similarity matrix between variables (sites) and using Euclidean distance.

The Kruskal-Wallis H test, performed with R 3.5.1 statistical software, is a rank-based non-parametric test that can be used to determine whether there are statistically significant differences between the six groups of an independent variable on a continuous or ordinal dependent variable. It is considered the non-parametric alternative to one-way ANOVA and is an extension of the Mann-Whitney U test to allow comparison of more than two independent groups (Laerd statistics).

Finally community indices (Dominance, Simpson index, Shannon index, Equitability) were calculated with R 3.5.1 statistical software.

2.3 Results and discussion

Cluster analysis (Figure 2.2) and Multidimensional Scaling (MDS) with goodness of fit index=0, i.e. perfect fit of the test to the data (Figure 2.3), supported by Kruskal-Wallis tests (Table 2.1), showed that there is significant variability in the community of halophiles present in the crystals among the salt samples from the Mediterranean salt works. In this last test, the p-value is < 0.05, so the null hypothesis was rejected and the sites highlighted with (*) are significantly different from each other.

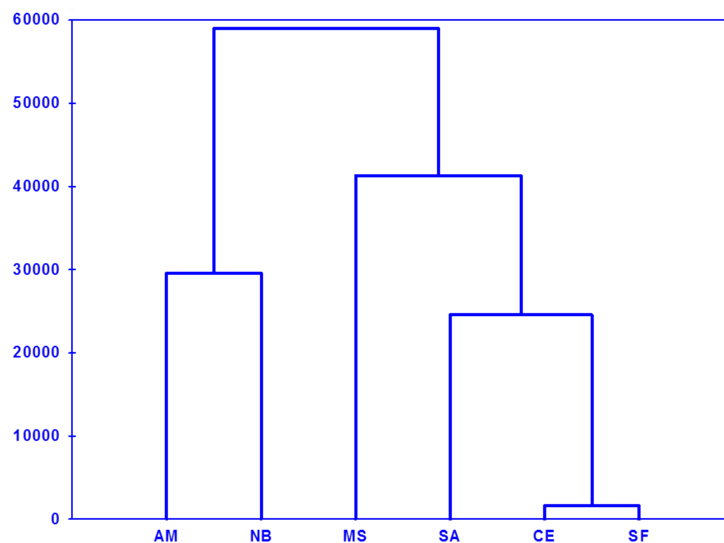


Figure 2.2 Cladogram by Cluster Analysis: Aigues Mortes (AM), Nubia (NB), Margherita di Savoia (MS), Sant'Antioco (SA), Cervia (CE), Sfax (SF).

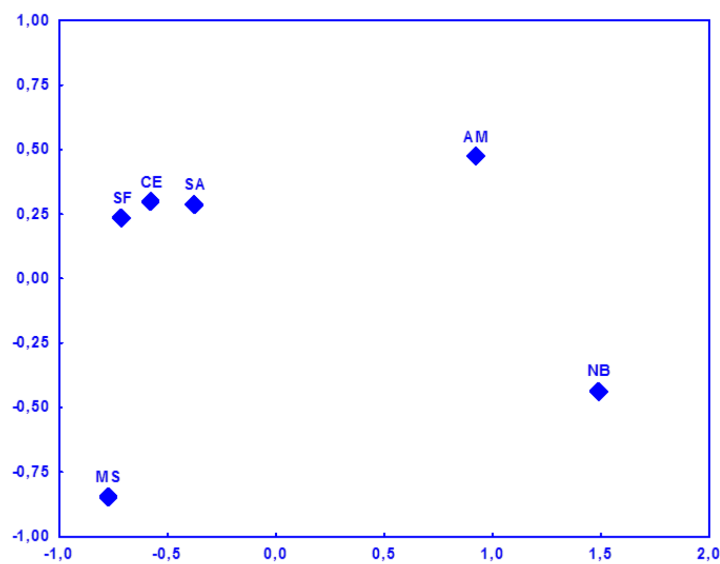


Figure 2.3 Multidimensional scaling, stress= 0, Aigues Mortes (AM), Nubia (NB), Margherita di Savoia (MS), Sant'Antioco (SA), Cervia (CE), Sfax (SF).

Table 2.1 Output of Kruskal- Wallis test: *p-value < 0.05.

SITES	Sfax	Cervia	S.Antioco	Margherita	Nubia	AiguesM.
Tunisia	0	0,2541	0,4024	0,06419	3,24E-09*	0,8951
Cervia		0	0,0524	0,004388*	3,34E-06*	0,2347
Palma			0	0,2517	2,03E-11*	0,5004
Margherita				0	1,04E-12*	0,1272
Culcasi					0	2,58E-08*
Camargue						0

Surveys with ecological community indices (Table 2.2), showed that phyla in the salt works tend not to be equally present; there are more phyla coexisting at Sfax and Cervia than at the other sites; at St. Antioco there is prevalence of a few phyla with many individuals, while at Sfax and Cervia there are communities with many phyla and a few individuals for each; at Sfax and Cervia the individuals are more evenly divided in the phyla present.

Table 2.2 Ecological community indices: Taxa (S), Individuals (number); Dominance (D), Simpson index (1-D), Shannon index (H), Evenness (J).

	Sfax	Cervia	S.Antioco	Margherita	Nubia	AiguesM.
Taxa_S	5	7	6	6	9	6
Individuals	17248	18250	37890	45642	147067	107945
Dominance_D	0,4296	0,4633	0,9027	0,8054	0,6968	0,7703
Simpson_1-D	0,5704	0,5367	0,0973	0,1946	0,3032	0,2297
Shannon_H	1,022	1,017	0,2557	0,418	0,6457	0,468
Evenness_J	0,6348	0,5225	0,1427	0,2333	0,2939	0,2612

Taxa corresponds to the phyla; Dominance (range 0-1) indicates the dominance of one phyla over others (Figure 2.4); Simpson index ($D=\sum [(ni/N)^2]$; range 0-1) shows community uniformity i.e. whether there are phyla that coexist without dominance, not to be confused with Shannon index; Shannon index ($H' = -\sum [ni/N * \log(ni/N)]$; range 0- high values) measures entropy. High values correspond to communities with many phyla and few individuals for each; Evenness (*J=H'/H'max*. If J = 1 well-distributed individuals) measures the uniformity with which individuals are divided into the phylum present.

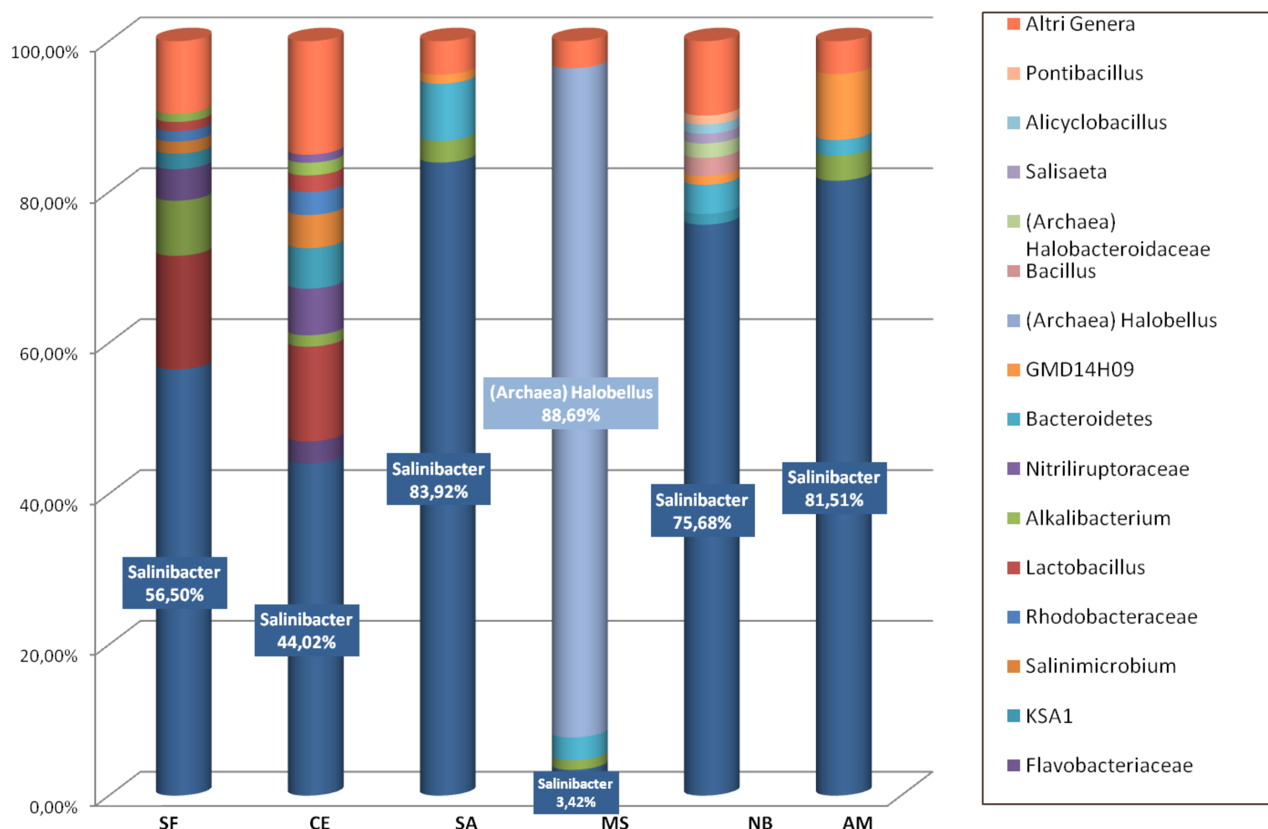


Figure 2.4 Graphic output of Dominance in the six salt work community: Aigues Mortes (AM), Nubia (NB), Margherita di Savoia (MS), Sant'Antioco (SA), Cervia (CE), Sfax (SF).

Archaea and *Bacteria* coexist in this extreme environment, and generally among the bacterii the predominant genus, with well over 50% of the total number of sequences at most sites, is *Salinibacter*; at the Margherita di Savoia site the predominant genus is *Halobellus*. In the first two sites a more heterogeneous community is noted, although there is dominant *Salinibacter*. All detected *Archaea* belong to the family *Halobacteriaceae*. Genera have been included in this graph where possible, otherwise the family, order or class where the taxonomic resolution of metagenomics did not yield more specific results; in some cases of genus homonymy the species has been specified. Populations included in the graph are those present with a minimum of 1% of the sequences analyzed.

A metagenomic analysis has been carried out on 6 salt samples produced by the solar evaporation of hypersaline waters. It has been reported that these extreme environments maintain high densities of halophilic archaea dominated by a limited

number of genera, mainly *Haloquadratum, Halorubrum* and *Haloarcula* (Bidle et al., 2005; Bowman et al., 2000; Oh et al., 2010; Pašić et al., 2005). In this study, haloarchaeal diversity in food-grade salts showed that *Halobellum*, an archaea, dominated in one salt (Margherita di Savoia), whereas *Halobacteroidacae*, not better identified, were present in some other salts. In the other five salt works, especially in Sant'Antioco, Aigues Mortes and Nubia, genera *Salinibacter*, an extremely halophilic member of the domain Bacteria with ecological relevance in crystallisation ponds, appeared to be a major actor of salt diversity, according to Elevi Bardavid et al. (2008). This is of interest because this genus has been reported on several occasions as being the most important organism in solar saline waters (Antòn et al., 2005). Analysis of a 16S rRNA gene clone library generated from saltern waters showed that 69% of the archaeal clones were closely related to *Hqr.walsbyi*, with the remaining related to *Halobacterium salinarum* (Henriet et al., 2014). However, the results identified two major genera in the six saltern samples (*Salinibacter* and *Halobellus*), but *Hqr.walsbyi* and *Halobacterium salinarum* were not detected.

2.4 Conclusion

Evaluation of the results obtained shows that there is significant variability in the community of halophiles present in salt crystals among salt samples from Aigues Mortes, Margherita di Savoia and Nubia salt works, according to Henriet et al. (2014) and Fontana et al. (2023). This variability is a useful prerequisite for tracking the geographic origin of the product.

Although the results of the cluster analyses and MDS show a significant dissimilarity in the composition of the micro-organism community between the 3 sites mentioned above, a high degree of similarity and an inability to identify the expected dissimilarities is evident for the other 3 salt works (Sant'Antioco, Cervia and Sfax). Therefore, these results do not allow the molecular approach to be considered sufficient for a standardised tracking approach to discern the geographical origin of sea salt, even though the molecular approach results useful to investigate on microorganism community composition (Gugliandolo et al., 2015) and to compare eachother (Fernandez et al., 2013 and 2014).

CHAPTER 3

3. PHARMACEUTICAL POTENTIAL OF BRINE

3.1 Introduction

In the progression of many inflammatory diseases, the production of reactive oxygen species (ROS) plays an important role. For the prevention and treatment of these pathologies, the search for antioxidants with the ability to scavenge free radicals from the body's cells and reduce oxidative damage is essential. Haloarchaea are extremely halophilic microorganisms. They live in hypersaline environments, such as salt flats, where they can tolerate high salinity and increased ultraviolet (UV) and infrared radiation. In order to cope with these extreme conditions, haloarchaea have developed unique mechanisms to maintain an osmotic equilibrium with the medium, and are endowed with unique compounds, not found in other species, with bioactive properties that have not yet been fully explored. (Avila-Roman et al., 2023).

Carotenoids

Carotenoids are important natural lipid-soluble pigments. They are produced by many microorganisms and plants. The structures of carotenoids are derived from five carbon isoprene units that are enzymatically polymerised to form regular, highly conjugated 40-carbon structures. One or both ends of the carbon skeleton can be cyclized to form ring β-ionone end groups, which can be further substituted by oxo, hydroxy or epoxy groups at various positions to form the various xanthophylls. In addition to their use as pigments, carotenes have recently become of commercial interest because of their beneficial effects on human health through their antioxidant properties, including their potential role in cancer prevention and enhancement of the immune response. Carotenoids have commercial applications in pharmaceuticals, neutraceuticals and animal feed additives. In addition to

carotenoids have biological functions as diverse as vitamin A precursor, immune system modulation and anti-tumour activity. β-Carotene as a vitamin A precursor can be enzymatically converted to vitamin A (retinol) and used in cosmetic products. A recent review describes the role of the carotenoids cantaxanthin and zeaxanthin as antioxidants and other commercial applications (Kim et al., 2008).

Carotenoids are liposoluble pigments responsible for the yellow-orange or orange-red colours of plants, algae, microorganisms and animals. Animals cannot produce carotenoids, but they do ingest them in their food. The carotenoids bacterioruberin, carotene, lycopene, canthaxanthin, 3-hydroxy-echinenone, lycopersene, phytoene, phytofluene and 2-isopentenyl-3,4-dehydrorhodopin have already been identified in halophilic archaea. Some of these carotenoids are found at low concentrations, suggesting that they may be precursors of other carotenoids. Usually, bacterioruberin is the major carotenoid from halophilic archaea. Halophilic archaea probably synthesise carotenoids via the mevalonate pathway. Bacterioruberin has important biological functions in halophilic archaea. It acts as a reinforcement of the cell membrane by increasing membrane rigidity and decreasing water permeability. It also protects the microorganism against DNA damaging agents such as ionising radiation, ultraviolet radiation and hydrogen peroxide, probably due to its antioxidant capacity (Bhong, 2023).

With the aim of enhancing the salt works environment with the application of marine biotechnology, it was conducted an experiment aimed at *in vitro* evaluation of the possible antioxidant and protective effects of salt works brine and their potential use in nutraceutical and cosmetic industries.

PPARs and LDL

Peroxisome proliferator-activated receptors (PPARs) are transcription factors that belong to the ligand-activated nuclear receptor superfamily. They have ubiquitous expression throughout the body. They initiate transcription of a number of genes involved in energy homeostasis when activated by endogenously secreted prostaglandins and fatty acids. To date, three major types have been identified: PPAR-α, PPAR-β/δ and PPAR-γ (Kota et al., 2005).

One of the most studied roles of PPARs is their involvement in inflammatory processes. Numerous studies have shown that agonists of PPARα and PPARγ exert anti-inflammatory effects both *in vitro* and *in vivo* (Pinna, 2023, Li et al., 2023).

PPAR-α is a receptor for structurally diverse compounds, including hypolipidaemic fibrates. PPAR-α is expressed in numerous tissues in rodents and humans, including liver, kidney, heart, skeletal muscle and brown fat, and is also expressed in a number of vascular cells such as endothelial cells, VSMCs and monocytes/macrophages (Kota et al., 2005). PPAR-α is also involved in skin homeostasis. PPAR-α controls keratinocyte proliferation/differentiation, contributes to wound healing and regulates skin inflammation. Activation of PPAR- α exerts anti-inflammatory effects in various skin conditions such as irritant and allergic contact dermatitis, atopic dermatitis and UV-induced erythema, and therefore investigations into the functions of PPAR- α are necessary to provide a better understanding for the treatment of many inflammatory skin diseases (Helder et al., 2023).

Despite extensive research on PPAR-α, the functional identity of PPAR-β remains unclear. PPAR-β is expressed in a wide range of tissues and cells, with relatively higher levels in brain, adipose tissue and skin (Kota et al., 2005).

Targeted activation of PPARbeta/delta may represent a promising therapeutic approach for the treatment of diseases in which inflammation is a central component of the pathophysiology (Wagner and Wagner, 2023).

Lipoprotein lipase (LPL) plays a central role in lipid metabolism by hydrolysing triglyceride-rich lipoproteins and releasing fatty acids (Yang et al., 2023).
The study by Ziouzenkova et al. (2003) suggests a novel anti-inflammatory role for LPL.

Further studies show specificity for PPAR activation by lipolysis in terms of lipoprotein substrate (VLDL ≫ LDL > HDL), PPAR isoform (PPARα ≫ PPARδ > PPARγ) and among fatty acid releasing lipases. These PPAR responses required intact LPL catalytic activity. *In vivo*, transgenic mice overexpressing LPL had increased peroxisome proliferation, but not in the genetic absence of PPARα. Although human plasma has minimal PPARα activation despite containing abundant free fatty acids, a marked PPARα activation is seen in human plasma after LPL addition *in vitr*o or systemic release *in vivo*. These data suggest a previously uncharacterised pathway by which the key lipolytic enzyme LPL can act on circulating lipoproteins to generate PPARα ligands, providing a potentially important link between lipoprotein metabolism and distal PPARα transcriptional effects (Ziouzenkova et al., 2003).

3.2 Materials and methods

3.2.1 Study area and sample collection

On August 3rd 2020, from the salt works of Trapani "Culcasi", water samples (4 bottles for each tank) were been taken from 8 tanks that differed in function within the salt work, and in the physical-chemical parameters (salinity, pH, conductivity, redox potential, sulphides and temperature) that characterized them.

Samples of water were sampled from the selected basins reported in tables 3.1, having different salinities, according to the production process of the salt. The water was put in sterile containers keep in cold.

On these samples, were been performed the separation and quantification of the organic and inorganic fraction and the extraction and spectrophotometric determination of carotenoids and chlorophyll-a.

For the cell treatments, the samples were filtered under 0.22 µm (MF-Millipore Membrane), aliquoted in sterile corning vials and maintained at 4°C. From all samples, a 4% stock solution in distilled water was prepared.

After, volumes of different saltwater were sampled and diluted in cell culture media, in order to have a final salinity equal to 0.2% (Dai et al., 2023).

Table 3.1 Different temperature and salinity in the 8 basins referred to the picture, where the water flow and direction are showed.

Tank	Temperature (°C)	Salinity%	Name
V0	30,8	3,8	mare
V1	31,6	5,8	fredda
V2	32,5	10	coltivo
V3	31,3	14,4	calda
V4	32,2	17	controsentina
V5	34,4	28	sentina 1
V6	37,2	32	sentina 2
V7	37	34	cristallizzante

3.2.2 Physical-chemical and organic parameters

On the field were recorded salinity (refractometer up to 25 ppt), pH and temperature with portable pH meter (Orion 290 A).

In the laboratory were recorded redox potential (mV), with redox electrodes and conductivity (mS/cm) with conductivity meter (Analytical control model 131 microcomputer).

The electrochemical determination of sulphides (Wildish et al., 1999, 2001) was carried out on surface sediments (0-2 cm depth) using an ion-selective Ag+/S2-9616BN electrode (Orion) connected to a portable pH meter (Orion 290 A), a membrane electrode in which the membrane potential is selective for one or more ions and which measures the activity of the free ion, while the species to which the ion is bound, especially those that are not ionised, are not estimated.

The Total Suspended Solids (g/l) were carried out using 0.45 µm Micropore filters.

The Total Organic Matter (TOM) was determined by ashing. 0.5 g of samples was ashed for 5 hours at 500°C. The difference between the initial and final weights were used to calculate the TOM.

Carotenoids and Chlorophyll-a were determined according to the protocol of Porra et al., 1989, using 1 mm glass filter digested with 80% acetone for extraction, and by spectrophotometer reading (Multiscan-Sky Microplate Reader, Thermo-Scientific TM, USA) at 663 nm (preferential chlorophyll-a absorption), 470 nm (preferential carotenoid absorption).

One of the project's goals was the extraction of carotenoids from the halophile microalga *Dunaliella salina* and the investigation of possible applications in nutraceutics.

For this purpose, a unicellular green alga was isolated from a water sample collected in a glass bottle at the Trapani Salt Works in August 2019, and a culture of the primary producer *Dunaliella salina* was introduced. The colonies were kept alive in both liquid and solid media. However, when the microalgae were subjected to stress

conditions of light and salinity, they did not produce visible levels of carotenoids. It is therefore likely that either the growth medium or the stress conditions to which the colonies were subjected did not result in the production of secondary metabolites such as carotenoids.

It was not possible evaluate the growth, the photosynthetic and antioxidant activities of *Dunaliella salina* under light and salinity stress conditions (Venkatachalam et al., 2019), and found the right nutrient and salinity concentrations to allow the production of secondary metabolites even in controlled laboratory environments (Abu-Rezq et al., 2010).

3.2.3 Cell treatments

Using saline water from four salt work basins, were conducted *in vitro* tests on cell cultures. Tests involved saline water from the following basins: basin 0 “mare”, basin 2 “coltivo”, basin 4 “controsentina, basin 7 “cristallizzante”.

In order to evaluate oxidative stress impact on skin cell lines and to see the possible protective effect of water was chosen one kind of cell 3T3 L1 pre-adipocyte cell line from mouse. Cells were grown as a monolayer in 75cm^2 plastic tissue culture flasks and incubated in a humidified atmosphere at 37°C, 5% CO_2 and 95% air in HEPA class 100 water jacketed CO_2 incubator (Thermo Fisher, US). The culture medium used was Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% calf serum, 2 mM glutamine, and 100 µg/ml penicillin–streptomycin.

To study the effects of saline water, the cells, after being detached with trypsin-EDTA (1X), were sown in equal numbers on 6-well plates (3x104 cells/1000 µl), in the presence of the culture medium, and subjected to UV treatments in order to assess the antioxidant effect against oxidative stress, induced experimentally through exposure to UV rays (lamp UV KW 254 nm).

The exposure protocol developed on the murine preadipocyte cell line 3T3 involved:

1. CO- flasks (cells maintained under standard conditions);
2. CO/UV flasks (cells exposed to UV, and not treated with saline water);
3. SWW flasks (cells treated with saline water for one hour);
4. SWW/UV flasks (cells first subjected to saline water treatment for one hour and then exposed to UVB radiation - 20 W, wavelength of 311 nm - for a period of 30 minutes).

Them the cells were treated with salt water (SWW) for a time of 1 hours in DMEM. After 1 hour, the medium was removed and replaced with phosphate buffered saline (PBS). The cells treated with salt waters were exposed, with the cells to which no salt water was added (CO/UV), to UVB radiation (20 W, wavelength of 311 nm) for a period of 30 minutes. The experiment also included cells as a negative control (CO-),

which were not exposed to either radiation or salt water. All conditions resume by Table 3.2.

Table 3.2 Conditions of cell treatments: In the 1st column there is negative control (CO-) where cells were not exposed to either radiation (UV) or salt water (SWW) but grew in standard medium. In the 2nd column cell (CO+) were exposed to UV only, while in the 3rd column cells (saltwork/UV) treated with salt waters were exposed to UVB radiation.

	CO-	**CO/UV**	**saltwork/UV**
St.Medium	+	-	-
SWW	-	-	+
UV	-	+	+

Preliminary viability and toxicity tests were conducted using the MTT colorimetric test in order to evaluate the protective ability of saline waters against the effects caused by exposure to UV radiation.

According to the technique of Mosmann, cell viability was assessed by a quantitative colourimetric assay employing the compound 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT). MTT is a tetrazolium salt which, after reduction by mitochondrial dehydrogenase, develops a blue-violet colour intensity which is directly proportional to the number of living cells and can be measured spectrophotometrically. MTT (final concentration 1 mg/ml) was added to cultured and treated cells in 96-well plates, which were then incubated for 2 hours at 37°C. After removal of the culture medium, a lysis buffer (0.1 ml of 50% SDS-dimetilformaldehyde 20%, pH 4.7) was added to lyse the cells and release the coloured product obtained by the reduction of MTT. After incubation at 37°C for a few minutes, the absorbance was measured spectrophotometrically using a microplate reader (Multiscan-Sky Microplate Reader, Thermo-Scientific TM, USA). Absorbance was measured at wavelengths of 570 nm and 690 nm. Results were expressed as the percentage of viable cells relative to the control. Each viability experiment was performed in triplicate.

After exposure the PBS was removed, cells were washed using PBS and 1 mL of PUREzol (Bio-Rad, USA) was added to the plates for each well. The PUREzol containing the RNA from cells was obtained and stored at -80 °C prior to analyses. Total cellular RNA was isolated from the samples in PUREzol using the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, USA) and the concentration was determined spectrophotometrically at 260 nm. The absorbance ratios A260/A280 and A260/A230 were evaluated as indicators of RNA purity. For each sample, 1 μg of RNA was reverse transcribed in a volume of 20 μL using the 5X iScript Reaction Mix Kit (Bio-Rad, USA) according to the manufacturer's instructions. Amplification was performed in a total volume of 20 μL containing 0.4 μmol.L-1 of each primer, cDNA diluted 1:10 of the final reaction volume, 1X IQ SYBR Green Supermix (Bio-Rad, USA) and nuclease-free water. Real-time PCR conditions were optimised in a gradient cycler (C1000 Touch Thermal Cycler, Bio-Rad, USA) using the following run protocol: an initial activation step at 95 °C for 3 min, followed by 39 cycles of 95 °C for 10 s and 60 °C for 30 s, with a single fluorescence measurement. The melting curve programme was achieved at 65-95 °C with a heating rate of 0.5 °C/cycle and continuous fluorescence measurement. All reactions were carried out in triplicate. For each PCR, the linear range of a standard curve of serial dilutions was checked. The relative quantification of PPARα, PPARβδ, LPL gene expression was evaluated after normalisation with the reference genes. All these genes being implied in anti-inflammatory activity.

3.3 Results and discussion

The results obtained from the analysis of organic and inorganic parameters show that salinity increases along the salt tanks in accordance with the increase in temperature and evaporation (Figure 3.1)

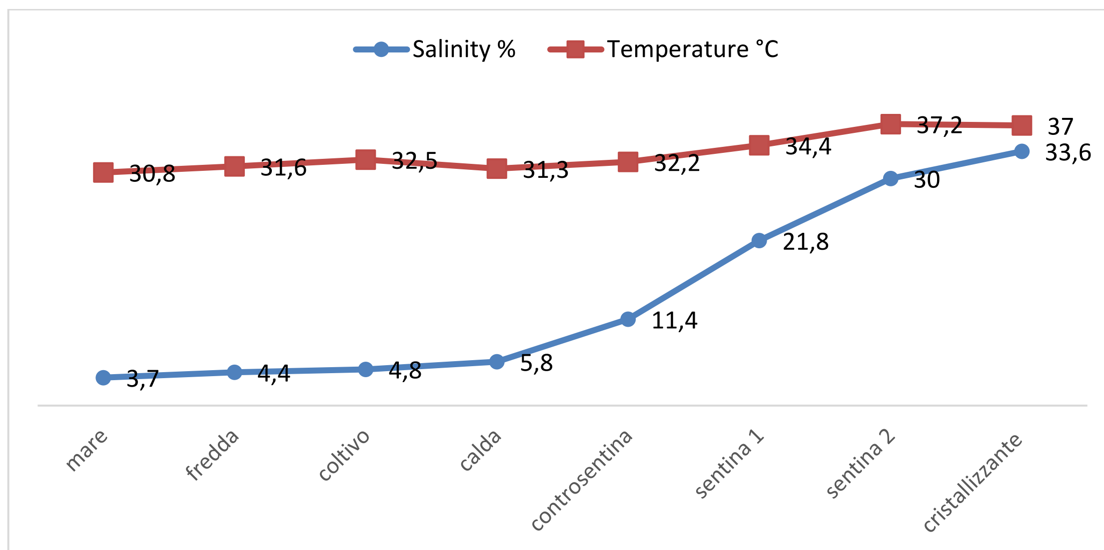


Figure 3.1 Salinity (%) and temperature (°C) values among salt tanks in Culcasi salt work. Values are represented by the mean with the relative standard deviation represented.

Each material has a specific conductivity value that affects the passage of heat through the body: the higher the value of k the greater the ease with which heat will pass through (Ko et al., 2023). Pure water has a very low conductivity (10 µS/cm), but substances dissolved in it increase this value (Light et al., 2004). In accordance with this physical evidence, conductivity increases in tanks from the sea, where it is lowest, to bilge tanks where NaCl concentration is highest. In the crystallizing tank, the now precipitated salt allows the conductivity value to decrease (Figure 3.2)

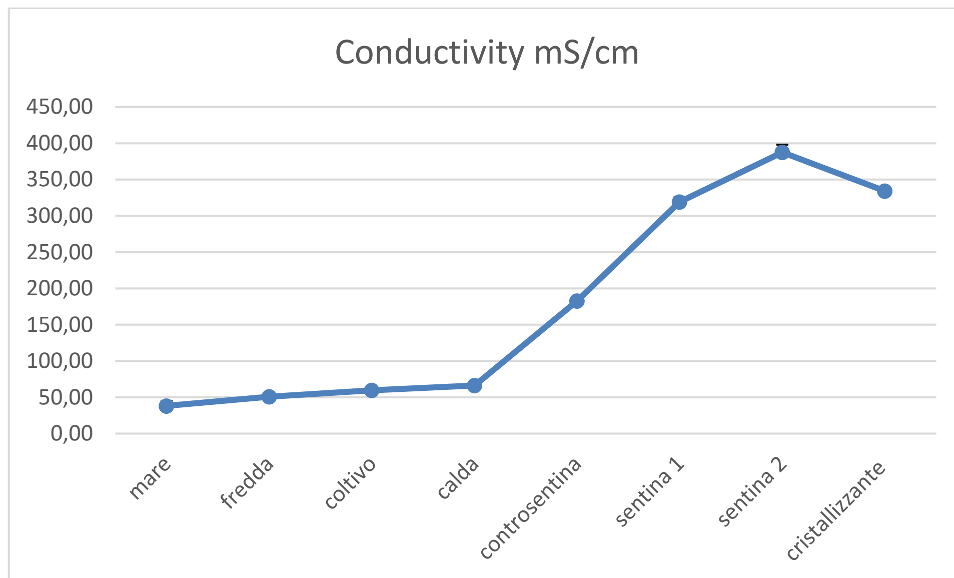


Figure 3.2 Conductivity among salt tanks in Culcasi salt work. Values are represented by the mean with the relative standard deviation represented.

Redox potential (Eh) is a measure of the tendency of a chemical species to acquire electrons, that is, to be reduced. The greater the reduction potential, the greater the tendency to acquire electrons i.e., the greater the reduction potential the greater the oxidizing character.

The measured potential, described by Nernst equation, refers to the amount of the measured ion in solution:

$E=E_0+S\ \log A$

Where

E = measured electrode potential

E_0= reference potential.

A = ion activity in solution.

S = electrode slope determined by standard calibration (Brown et al., 2011).

The redox potential depends on the activity of hydrogen ions, i.e., the pH value of the solution in which these reactions take place as well as, to a lesser extent, the temperature (Figure 3.3).

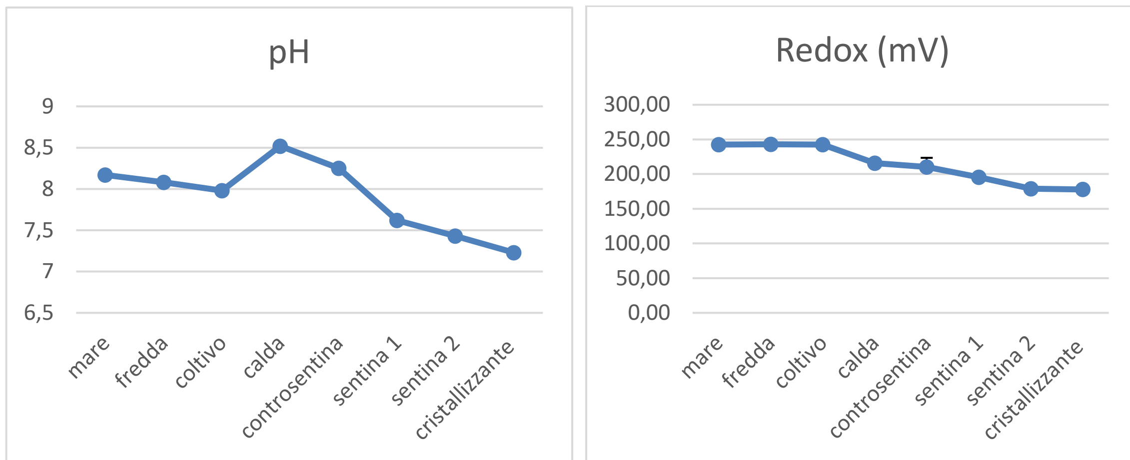


Figure 3.3 pH and redox trend among salt tanks in Culcasi salt work. Values are represented by the mean with the relative standard deviation represented.

Sulfides could not be detected because of values that were below the probe's detection threshold.

The graphs of total suspended solids and total organic matter are mirrored (Figure 3.4), as the values increase with increasing evaporation in the tanks, which implies a greater component of suspended solids and greater production of organic molecules by primary producers.

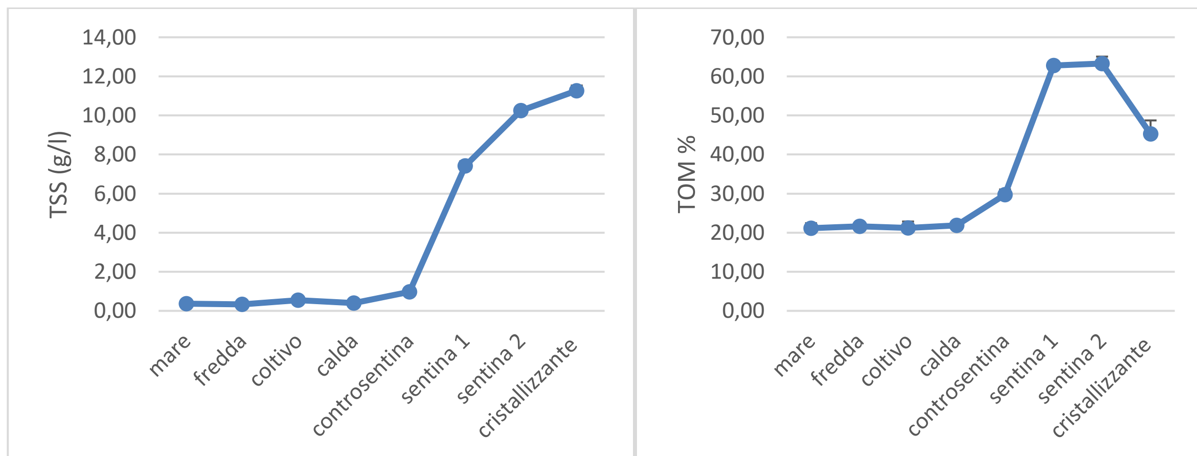


Figure 3.4 Total suspended solids and total organic matter trend among salt tanks in Culcasi salt work. Values are represented by the mean with the relative standard deviation represented.

Trend of carotenoids reflects the molecular response of the microorganisms in the salt ponds under stress conditions: it appears that the concentration of carotenoids increases from the sea to the crystallizing tank, where temperature, salinity and light radiation intensity gradually increase and oxygen is reduced. Carotenoids as a product of secondary metabolism provide microorganisms with protection.
Chlorophyll-a has an inverse trend: there is an increase in its concentration from the sea to the "controsentina" tank, in accordance with the increase in light exposure of the photosynthesizing microorganisms, then under increased stress conditions and with the progression of the crystallizing phase there is a decrease, due to the reduction in photosynthetic activity (Figure 3.5).

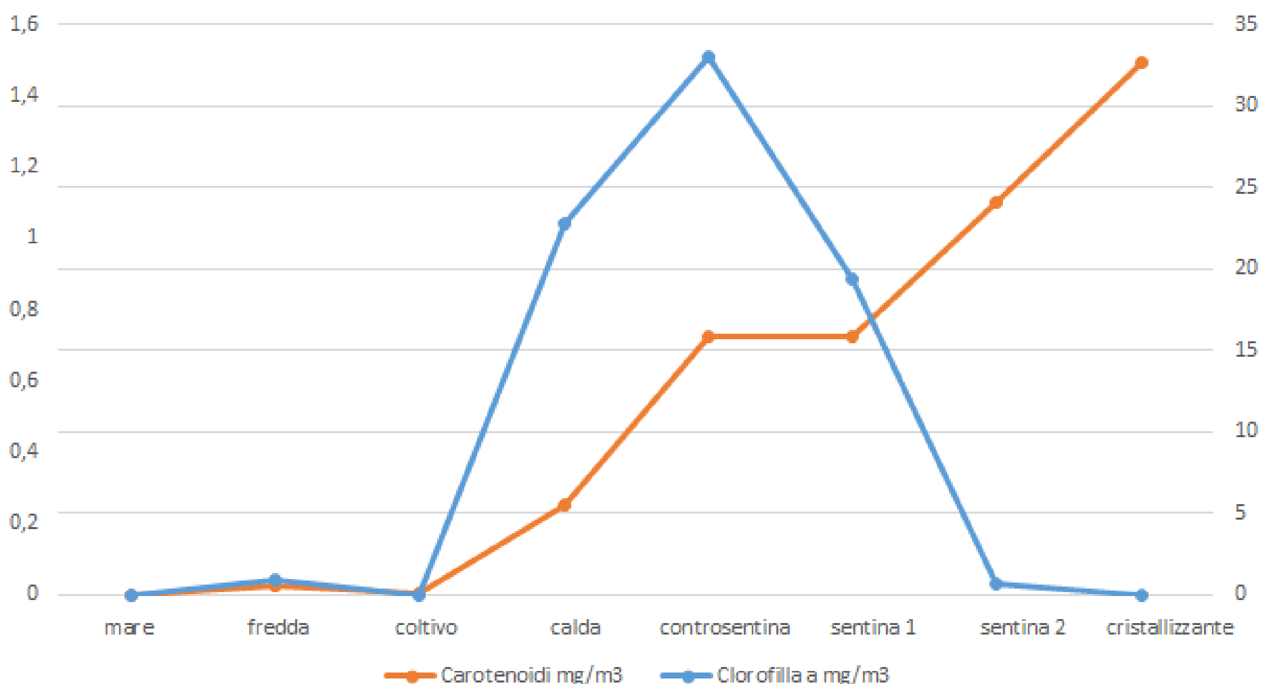
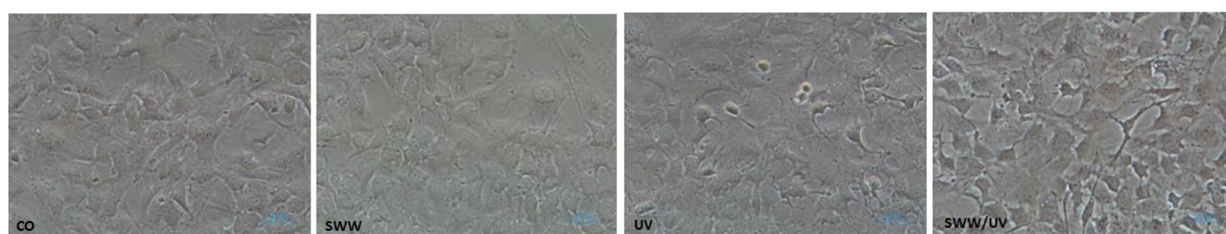


Figure 3.5 Determination of Chlorophyll-a and carotenoids among salt tanks in Culcasi salt work. Values are represented by the mean with the relative standard deviation represented.

From the cell viability test results obtained by the MTT assay, was been observed that cells pre-treated with saline water from crystallizer tank – V7 (SWW), showed no change on cell viability compared to untreated cells (CO). In addition, cells pre-treated with saline water – V7 for one hour and then exposed to UV radiation (SWW/UV) showed increased viability compared to untreated and UV-exposed (UV) cells. After exposition and treatment all cells were stored in incubator and were focal microscopic observation (Nikon Eclipse E200) so as to note cell viability after treatment but also to see if salt water can have protective effect against UV induced stress. Pictures was taken from the observation by microscope (Figure 3.6).
From the results of the viability test, it was observed that cells treated for one hour with saline water and then exposed to UV radiation had lower viability than the control, but higher viability than cells exposed directly to UV. This allows the assumption that the saline water has a protective effect on cells.



	CO	SWW	UV	SWW/UV
Cell viability (%)	100	100	75	87

Figure 3.6 Focal microscopic observation (20x) related to cell viability: untreated cells (CO) = viability 100%, cells pre-treated with saline water (SWW) = viability 100%, untreated and UV-exposed (UV) cells = viability 75%, cells pre-treated with saline water for one hour and then exposed to UV radiation (SWW/UV) = viability 87%

The relative expression of PPARα, PPARβδ, LPL genes in cells treated with brine water and then subjected to UV stress is high, compared with cells subjected directly to UV (Figures 3.7). This result suggests that saline water, which is rich in secondary metabolites produced as protection from stressful situations, shields cells from UV, and allows a better cellular reaction to stress, such that the expression of PPARα, PPARβδ, LPL genes known to be implied in anti-inflammatory activity is increased.

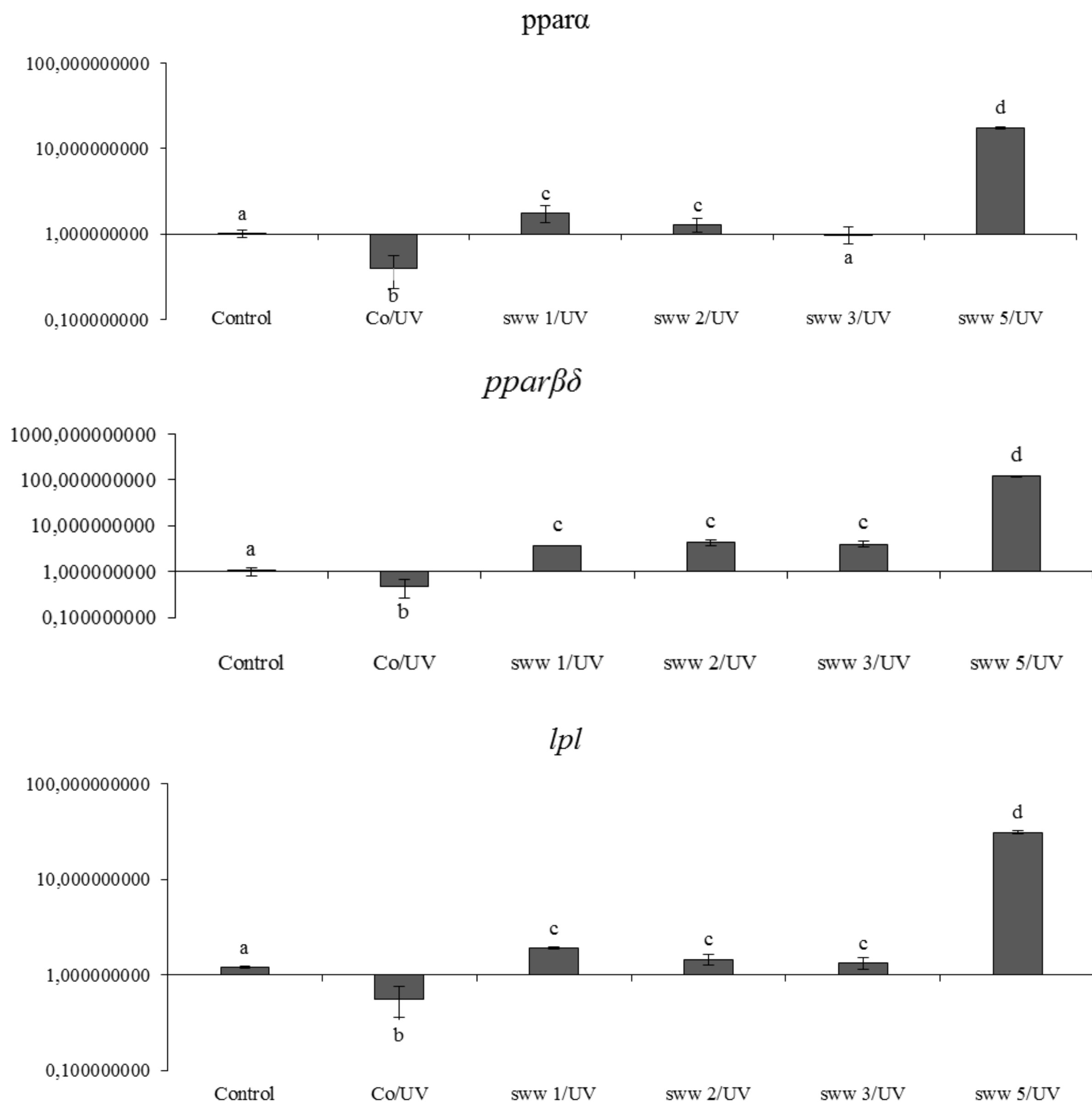


Figure 3.7 Relative PPARα, PPARβδ, LPL gene expression in the 3T3 cell line under standard condition (control), UV radiation (Co/UV), salt work water – basin 0 "mare" – treatment before UV radiation (sww1/UV), salt work water – basin 4 "controsentina" - treatment before UV radiation (sww2/UV), salt work water – basin 2 "coltivo" – treatment before UV radiation (sww3/UV) and salt work water – basin 7 "cristallizzante"- treatment before UV radiation (sww5/UV). Lowercase letters indicate significant differences between different extract ($p < 0.05$).

The cells treated with water deriving from the crystallizing basin and then subjected to UV stress showed greater expression of the 3 genes investigated than the other salt work water treatment derived from the other three basins. Cells treated with treatment sww5/UV showed an over expression of the PPARα, PPARβδ, LPL genes,

responsible for the anti-inflammatory effect. The water of the crystallizing basin, which based on the parameters determined by water analyses, appears to be richer in carotenoids produced by a greater presence of primary producers, based on the high concentration values of total suspended solids and total organic matter. These microorganisms that find themselves in stressful conditions in the crystallizing basin with extreme values of pH, redox, temperature and salinity produce secondary metabolites, which give the water protective properties on the cells.

The major limitation of the study is the use of saline water without the identification of several classes of compounds present in saline water, an investigation reported in some studies, as the one of Centini et al. (2015). The analyses were limited to some organic and inorganic parameters of saline water, which allowed the water to be characterized for the administration of its aliquots on murine cells. However in the experiment, it is provide evidence that saline water of crystallising basin from the salt work in Trapani "Culcasi" improve anti-inflammatory activity in 3T3 L1 pre-adipocyte cells from mouse. Results showed that saline water, with its unidentified compounds, induced the transcriptional expression of PPARα, PPARβδ, LPL genes which are involved in inflammatory processes, causing anti-inflammatory effects in UV-induced diseases (Helder et al., 2023).

3.4 Conclusion

Secondary metabolites, present in the inorganic fraction of saline waters can be considered to have a protective effect on the 3T3 cell line by inducing the expression of genes PPARα, PPARβδ, LPL. In particular cells treated with saline water arising from crystallizer basin showed a greater expression than the other treatment.

Salt work brines are emerging as a valuable lead series with great potential as anti-inflammatory agents and as promising candidates for further efficacy evaluation, as was found in studies of Dead-sea salts isolated from brine by Levi-Schaffer et al. (1996). Owing to the antioxidant and anti-inflammatory effects, evidenced by the increase of anti-inflammation related genes expression using salt work brine could have potential health benefits.

With regard to the experiment carried out on *Dunaliella salina*, the initial phase did not produce the desired results. In the future, it will be possible to evaluate the growth, photosynthetic and antioxidant activities of the halophilic microalgae species under light and salinity stress conditions (Venkatachalam et al., 2019), in order to find the right nutrient and salinity concentrations to allow the production of secondary metabolites even in controlled laboratory environments (Abu-Rezq et al., 2010).

CHAPTER 4

4. POTENTIAL USE OF SEA SALT PRODUCTS IN NUTRACEUTIC AND COSMECEUTIC

4.1 Introduction

Salt work water

Many chemical defences to adapt to the environment have been developed by marine organisms, producing a wide range of biologically important compounds, that can be exploited for biomedicine, food, nutraceutical and cosmeceutical purposes (Messina et al., 2021).

Halophiles present in brines can have a positive influence on the quality of the salt produced, due to the production of metabolites such as carotenoids. Secondary metabolites were made by halophiles to protect themselves from ultraviolet radiation (UV). It is well known from the literature that secondary metabolites are substances with antioxidant, anti-inflammatory, anti-tumour, antimicrobial, bactericidal and fungicidal properties (Gao et al., 2015; Quist et al., 2011; Abu-Jdayil et al., 2008).

Human skin is particularly sensitive to UV. Therefore, chronic or acute responses to UV cause a variety of biological cellular responses, including sunburn, pigmentation, DNA damage, connective tissue alteration, and even skin cancer (Lee et al., 2010). So the evaluation of some biomarkers related to these aspects were considered. It is well known that saline water have benefical effects against oxydative stress that can be referred to bioactive compounds produced by micro- organisms living in the salt work (Spilioti et al., 2017, Margesin et Shinner, 2001). Most of these properties are referred to anti- oxydant protection against UV, employing an experimental model system setup in the laboratory (Montenegro et al., 2019). It is possible to draw considerable conclusions about the anti- oxidant, in order to assess the protective effects monitoring the expression of some genes related to: stress, oxidative stress, cell damage, apoptosis and cancer.

Normal Human Epidermal Keratinocytes (NHEK)

During evolution in the seven layers of the epidermis, keratinocytes undergo numerous morphological, functional, and biochemical transformations due, for example, to hormonal activity in the basal cell layer or intense enzyme activity in the transition zone. In particular, it has been found (Wiegand et al., 2014; Vessey et al., 1995) that the differentiation of keratinocytes in culture is accompanied by an increase in Glutathione system (GSH) enzymes, in addition to the increase in total glutathione in differentiated cultures. This increase suggests that growing and differentiating keratinocytes acquire increased antioxidant defense capacity (Cassier-Chauvat et al., 2023, Tan et al., 2023).

The skin is a model used for studies of cellular aging. Two types of skin aging have been identified: intrinsic aging, generally due to genetic causes, and extrinsic, photo-aging, due to the resultant of environmental stresses, most notably UV. Some simplified in vitro study models based on cellular aging have shown the relationship between UV and aging. The long-term effects of subtoxic UV exposures of cultured NHEK showed a progressive arrest of proliferation and the attainment of a plateau of cells in a senescent state. This was followed by detachment of most cells; the few remaining in culture were partially transformed with tumor traits, associated with ROS accumulation (Debacq- Chainiaux et al., 2012). Lewis et al. (2008) showed that low doses of UVB radiation induced premature cellular aging in NHEK.

The experiment was conducted on the HEK (Human Epidermal Keratinocyte) cell line, human keratinocytes, the cell type most commonly found in the epidermis (>90%) and the skin's first line of defense against environmental stresses. In the epidermis, dividing stem cells in the basal layer give rise to the progeny of cells destined to migrate to the upper layers up to the superficial stratum corneum, where they will be lost near the superficial horny part of the epidermis. This process, in which undifferentiated, proliferative keratinocytes are converted into highly differentiated, nondividing cells, is termed cell differentiation (Eckert, 1989; Pastar et al., 2014; Guenin-Mace et al. 2023).

UV stress

Human skin is particularly sensitive to ultraviolet (UV) radiation because it is one of the few organs directly exposed to the sun. One of the main factor for the cellular over-production of radical species, such as ROS, is the excessive and prolonged exposure to ultra-violet radiation (UV) (Aseervatham,et al. 2013). Cellular systems are able to maintain a balance between the production of ROS and the cellular ability to detoxify the reactive intermediates under normal physiological conditions (Vasarri et al., 2021).

Therefore, chronic or acute responses to ultraviolet radiation (UVR) cause a variety of biological responses, including sunburn, pigmentation, DNA damage, connective tissue alteration, and even skin cancer. Repeated exposure to UVR (i.e., photo-aging) primarily causes premature aging of the skin, which is easily recognized by the formation of wrinkles, mottled pigmentation, and histological changes, including thicker epidermis and altered connective tissue structure. The integrity of skin connective tissue, which maintains skin strength and resilience, depends on dermal extracellular matrix (ECM) proteins, such as collagen types I and III. Matrix metalloproteases (MMPs) may be responsible for the degradation of collagen and other extracellular matrix proteins, which are the main targets for alleviating skin photo-aging. Alterations in elastin, fibronectin, and proteoglycans are also strongly associated with the aged appearance of skin (Lee et al., 2010).

Skin is a model of choice for the study of ageing. Indeed, skin ageing can be modulated by internal and external factors, reflecting its complexity. Two types of skin ageing have been identified: intrinsic, mainly genetic, and extrinsic, "photo-aging", resulting from the effects of environmental stress and, more specifically, UV radiation. Simplified *in vitro* models based on cellular senescence have been developed to study the relationship between UV and ageing. These models vary in terms of cell type (fibroblasts or keratinocytes, normal or immortalised) and the type of UV used (UVA or UVB) (Huang et al., 2023).

Normal human epidermal keratinocytes (NHEK) are the major cell type of the epidermis and are the first defence of the skin against environmental stress. They proliferate in the basal layer before progressing to the suprabasal layers through a complex differentiation programme that culminates in fully differentiated dead cells in the keratinised superficial layer, maintaining a strong impermeable barrier. Anout the long-term effect of subcytotoxic UV exposure on NHEKs, show a progressive proliferation arrest and reach a senescence plateau after about 15-25 population doublings (depending on the donor) in culture. This plateau is followed by massive detachment of almost all cells, lasting only a few days to 2-3 weeks. A few remaining cells with partially transformed and tumorigenic properties will then spontaneously form senescent cultures, which is linked to the accumulation of reactive oxygen species during senescence. Senescent keratinocytes show morphological changes, including increased cytoplasmic and perinuclear organelle content (Debacq-Chainiaux et al., 2013). Lewis et al. demonstrated that low doses of UVB irradiation induce premature cellular senescence in NHEKs.

Biomarkers

Superoxide dismutase1 (SOD1)

Cells have evolved a sophisticated antioxidant system that allows the continuous processing of reactive oxygen species, particularly superoxide (O_2-), which is highly reactive but has a low half-life, and hydrogen peroxide (H_2O_2), which is less reactive but persists in cells long enough to reach their nucleus and is therefore much more damaging than other free radicals.

The mechanism of ROS removal involves the enzymes superoxide dismutase, catalase, thioredoxin, and glutathione; antioxidants are generally regenerated in their reduced active state by specific reductase enzymes (Tsang et al., 2014).

SODs are a class of highly conserved enzymes that catalyze the inactivation of superoxide to oxygen and hydrogen peroxide. In eukaryotic cells, there are three distinct superoxide dismutases: SOD1 is a soluble Cu/Zn enzyme that is found most in the cytosol, with a small percentage (3%) found in the intermembrane space of mitochondria (Switzer et al., 2023).

The Sod1 gene, like the others, encodes for the enzyme superoxide dismutase, which acts by catalyzing the reaction of superoxide to hydrogen peroxide (H2O2), reducing reactive oxygen species; cells deprived of this gene are subject to extensive oxidative damage and DNA damage (Tsang et al., 2014).

Sirtuin1 (SIRT1)

Sirtuins are a family of proteins found in all domains of life. The first discovered sirtuin, Sir2 (silent information regulator 2), from the yeast *Saccharomyces cerevisiae* regulates ribosomal DNA recombination, gene silencing, DNA repair, and chromosomal stability and longevity (Michan et al., 2007). The mammalian gene homologous to Sir2 is Sirtuin 1 (Sirt1), which belongs to the NAD-dependent histone deacetylase family and is implicated in aging, metabolism and stress resistance (Lee et al., 2010). Sirt1 controls the cell's response to oxidative stress by regulating the FOXO family of proteins (Brunet et al., 2004). The mammalian Forkhead class O

(FOXO) transcription factors are implicated in the regulation of various cellular processes, such as: cell cycle and longevity, apoptosis, DNA repair, stress resistance, and metabolism. Similar to the tumor suppressor p53, FOXO is activated by stressors that, through phosphorylation, translocation to the nucleus and acetylation/deacetylation, induce the expression of genes that contribute to cell cycle arrest (Furukawa- Hibi et al., 2005).

The results of the study by Brunet et al. (2004) show that Sirt1 and FOXO3 form a protein complex within the cell in response to oxidative stress, and Sirt1 deacetylates, both in vitro and in vivo, having a dual effect on FOXO3 function: sirtuin increases FOXO3's ability to induce cell cycle arrest and resistance to oxidative stress, but inhibits FOXO3 from inducing cell death.

Therefore Sirt1 may increase the longevity of the organism by delaying the FOXO3 response that would result in cell apoptosis and directing it toward stress resistance.

Aquaporin3 (AQP3)

Aquaporins (AQPs) are a family of water channel proteins expressed in various tissues. To date, ten members of the aquaporin family (AQP0- AQP9) have been cloned from mammals and divided into two subgroups based on amino acid sequence and molecular function (Sugiyama et al., 2001).

AQP3 is the most abundant aquaglyceroporin in the skin and is permeable to glycerol and urea in addition to water. AQP3 plays an important role in the hydration of the mammalian epidermis; moreover, recent studies have found that glycerol transport by AQP3 is involved in both lipid metabolism in the skin and the regulation of keratinocyte proliferation and differentiation (Boury-Jamot et al., 2009).

The epidermis, as the primary barrier between the body and the environment, is exposed to abrupt changes in gradients. In particular, between the stratum granulosum and stratum corneum there is a constantly altered water gradient as the epidermis has an increasingly low water content (Warner et al, 1988; Verdier-Sèvrain et al., 2007; Barel et al., 2014). Similarly, the pH value that on the skin

surface is around 5 goes up to 7 in the stratum corneum (Ohman et Vahlquist, 1994; Elias, 2005; Lambers et al., 2006). Hyperosmolarity stresses mammalian cells due to osmotic flux of water and various biological events such as activation of protein-kinase pathways and heat shock transcription factor 1, stimulation of cytokine production, and gene expression changes.

Furthermore, transcriptional regulation of AQP has been shown to occur due to alteration of external osmolarity. The barrier formed by the skin against excessive water loss is located more in the stratum corneum and is compromised by chemicals and UV radiation, leading to increased transepidermal water loss. Therefore, keratinocytes in the epidermis undergo marked dehydration, suggesting that FPAs play a protective role against possible water loss. Furthermore, the epidermis, which lacks capillary vessels, has an active water uptake mechanism from the dermis to maintain homeostasis, in which AQPs are involved to supply keratinocytes with water (Sugiyama et al., 2001).

AQP3 is constitutively expressed by epidermal keratinocytes. The sudden decrease in the expression of AQP3 in keratinocytes, below the stratum corneum, as well as the inhibition of its function as a water channel at acidic pH, are both in agreement with the water loss prevention function of the stratum corneum. Therefore, AQP3 forms a short-range water circuit between the base of the epidermis and the stratum corneum with the aim of maintaining a constant water content and preventing the formation of continuous water gradients between the two layers (Sougrat et al., 2002).

Prostaglandin-endoperoxide synthase-2 (COX-2)

The COX-2 protein, prostaglandin-endoperoxide synthase-2, is the product of an "immediate-early" gene that is rapidly inducible and tightly regulated. COX-2 expression is highly restricted, under basal conditions; however, COX-2 is upregulated dramatically during the process of inflammation. The mechanisms underlying the association between COX-2 overexpression and potentially cancer cells may include resistance to apoptosis or programmed cell death, thus pro-

apoptotic mechanisms. Overexpression of COX-2 undoubtedly plays a role in disease processes characterized by increased local prostaglandin production (Kyurkchiev et al., 2014; Crofford 1997).

The human epidermis is a tissue that undergoes the active metabolism of arachidonic acid into prostaglandins, regulated by the action of prostaglandin H synthase (also known as cyclooxygenase or COX2). Thus, prostaglandins (PGs) are products of cyclooxygenase (COX) synthesis from arachidonic acid. COX1 is constitutively expressed by almost all tissues, while COX2 is induced under inflammatory conditions and preferentially metabolizes prostaglandin E2, which acts as a messenger molecule in a paracrine and autocrine manner on surrounding cells (Kyurkchiev et al., 2014). UV radiation activity can trigger COX2 to induce prostaglandin formation (Pisarchik et al., 2004).

Recent work (Luo et al., 2011; Itoh et al., 1999; Gilroy et al., 1999) has shown that nuclear factor erythroid-2-related factor 2 (Nrf2) confers protection against oxidative stress. In addition, COX2-dependent electrophilic oxide-derived molecules (EFOX) have been shown to act as anti-inflammatory mediators through activation of the Nrf2-dependent antioxidant response element (ARE). These studies provided more information on COX2-mediated events and its active role in the oxidative stress response.

The function of all tissues, particularly epithelial and endothelial tissues, decreases with age, leading to the production of reactive oxygen species (ROS). COX2 expression increases with aging in most tissues, partly due to the accumulation of ROS and to chemical reactions. Luo et al. (2011) hypothesize that COX-2 levels increase during the aging process because the increase in ROS levels requires the involvement of COX-2-dependent EFOX as an anti-inflammatory and Nrf2/ARE signaling as an antioxidant.

Cosmeceuticals and nutraceuticals

Cosmeceuticals, which include anti-aging creams and moisturisers, are cosmetic products with drug-like benefits that enhance or protect the appearance or integrity of the human body. From the words "cosmetic and pharmaceutical" comes the name cosmeceutical. Cosmeceuticals can be applied to products such as creams, lotions and ointments and contain active ingredients such as vitamins, phytochemicals, enzymes, antioxidants and essential oils. Because of their beneficial effects on human health, cosmeceuticals have recently attracted increased attention. Marine organisms, an extremely heterogeneous group in the oceans, are an excellent reservoir for the identification and extraction of biologically active substances that have the potential to be used as pharmaceuticals, food supplements and cosmetics,
cosmeceuticals, enzymes and fine chemicals. Bioactive substances derived from marine organisms have a wide range of functional roles as secondary metabolites, and these properties can be exploited for the development of novel pharmaceuticals and cosmeceuticals. Extensive research on the general aspects of the chemical structures, physical and biochemical properties and biotechnological applications of bioactive compounds from marine organisms has been carried out in recent years. This makes these substances a potentially rich source for a wide range of chemical products with applications in functional foods, cosmetics, cosmeceuticals, pharmaceuticals and even the food industry.

The aim of this work was to present the products of salt microorganisms for possible use in cosmeceuticals, with marked antioxidant and anti-inflammatory properties, from the perspective of biotechnological applications of bioactive substances derived from marine organisms (Kim et al., 2008).

4.2 Materials and methods

4.2.1 Study area and sample collection

In support of nutraceutical and cosmeceutical applications, the bioactive properties of salt work were tested *in vitro* screening on Human Epidermal Keratinocyte (HEK) cell line, with the aim of testing the anti- oxidant effects of sea salt products.

Human Epidermal Keratinocyte (HEK) (Sigma-Aldrich, St. Louis, MO, USA) were grown as a monolayer in flasks, using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 µg/mL penicillin–streptomycin, incubated in a humidified atmosphere at 5% CO2, 95% air and 37 °C, under sterile conditions.

Cells at 80% of confluence were detached, utilizing a trypsin solution (0.05% of trypsin in PBS, pH 7.2–7.4) and pelleted by centrifugation (1000 rpm, 10 min, 25 °C). The cell suspension was dispensed in 96-well plate at a density of 8000 cells/well and incubated for 24 h before the exposure to salt work water from different salt tanks. In order HEK cells were exposed to NaCl solutions (0.9%), water tank from "mare", water tank from "calda", water tank from "cristallizzante" and consequently were analyzed biomarker RNA expressions.

HEK cells, in the presence of the culture medium, were subjected to UV treatments in order to assess the antioxidant effect of salt work waters against oxidative stress, induced experimentally through exposure to UV rays (lamp UV KW 254 nm) at dose rate of 105 erg/mm2/sec for 5 min.

Cells not treated with water salt work and not UV irradiated were cultivated as controls.

Through the RNA extraction protocol (Aurum Total RNA Fatty and Fibrous Tissue Kit) It was possible to extract RNA from HEKs and its subsequent quantification. Purity and concentration of RNA were analyzed by the spectrophotometric method (Multiscan-Sky Microplate Reader, Thermo-Scientific TM, USA), which employ the capacity of nucleic acids to adsorb the UV light at a specific wave-length of 260 nm. Absorbance at 260 nm cannot discriminate between RNA and DNA, however the

relative purity of the solution can be evaluated via the ratio between A260 and absorbance 280 nm. Impurities as proteins, which have a peak of absorption at 280, will reduce the ratio. The ratio between A260/A280 should be for RNA samples compered between 1.8-2.

In order to evaluate the gene expression, RNA samples must be converted into cDNA. This step is performed through an enzyme, the retrotrascriptase. Is important then to know exactly the right concentration of RNA samples, because the RT-PCR is essentially a quantitative method and then is fundamental to amplify in each sample the same amount of substrate. In RT-PCR (PCR sprint machine) was used Work solution (cDNA+ SYBR Green Supermix). Target cDNAs were amplified using gene-specific primers designed for the transcribed region of each gene based on the work of Spilioti et al., 2017.

4.2.2 Biomarker primers

Gene specific primers (Table 4.1) were used to amplify target cDNAs of SOD1, SIRT1, AQP3, COX2.

Glyceraldehyde diphosphate (GADPH) was the housekeeping gene, the control always expressed.

Each gene expression analysis were performed on three biological repeats.

Table 4.1. PCR primers for quantitative real-time PCR.

Gene	Forward	Reverse
GAPDH	5’-GAAGGTGAAGGTCGGAGTC-3’	5’-GAAGATGGTGATGGGATTTC-3’
SOD1	5’-ACTGGTGGTCCATGAAAAAGC-3’	5’-AACGACTTCCAGCGTTTCCT-3’
SIRT1	5′-TAGGCGGCTTGATGGTAATC-3′	5′-TGGCATGTCCCACTATCACT-3′
AQP3	5′-GGCTGAAGCAGGAGAATCAC-3′	5′-GCTGAGTCCCAGCTGTTTTT-3′
COX2	5'-GGCCTGTGTGTGGTCATGATCATC-3'	5'-CAGGATGTGGTGGTGACTGTC-3'

4.2.3 Statistical analysis

One-way analysis of variance (ANOVA) was performed and Tukey's post hoc test was used to compare means between samples (± standard deviation).

The degree of heterogeneity was assessed using the Cochran test. Significance was accepted at a probability of $p < 0.05$. ANOVA was performed using STATISTICA (version 8.0, Statsoft Inc., Tulsa, OK, USA).

4.3 Results and discussion

The analyses still in progress showed, among the evaluated genes, some peculiar aspects related to four key genes as Superoxyde dismutase 1, Sirtuin 1, Aquaporin 3 and Prostaglandin-endoperoxide synthase-2.

Superoxyde dismutase 1 (SOD1):

In eukaryotic cells encodes for the enzyme superoxide dismutase that catalyse the inactivation of superoxide to oxygen and hydrogen peroxide (H_2O_2), reducing reactive oxygen species; cells deprived of this gene are subject to extensive oxidative damage and DNA damage (Tsang et al., 2014).

The up-regulation of the Sod1 gene in cells treated with saline water, characterized by certain chemical-physical parameters, could indicate a saline water-induced antioxidant effect (Gao et al., 2015; Milani et al., 2013). The up-regulation of the SOD1 gene suggested that the saline water treatment was attributable to the antioxidant activity of the water itself. HEK cells treated with saline water and subjected to UVB radiation overexpressed the SOD1 gene more in cells treated with warm and crystallizer water than in the control (Figure 4.1b). Consequently HEK cells, aided by the antioxidant effects of saline water, implemented defence mechanisms to cope with the emerging oxidative damage (Shi et al., 2016; Milani et al., 2013).

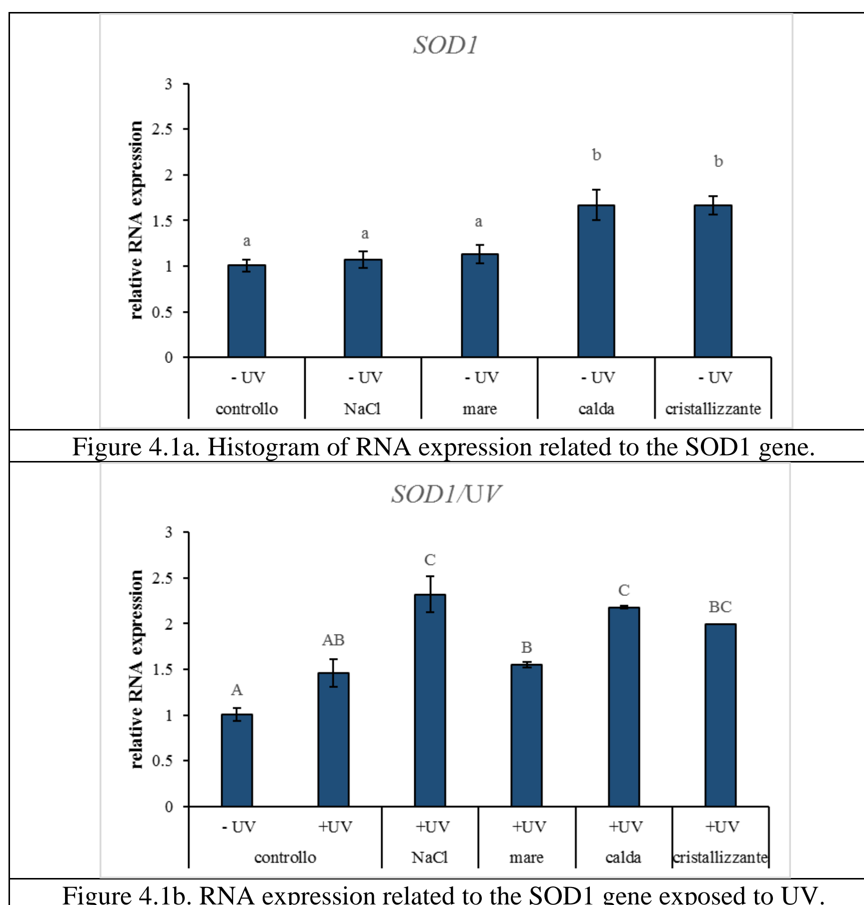


Figure 4.1a. Histogram of RNA expression related to the SOD1 gene.

Figure 4.1b. RNA expression related to the SOD1 gene exposed to UV.

Figure 4.1. (a) SOD1 RNA expression: cells without any treatment (controllo), NaCl treatment (NaCl), sea water treatment (mare), warm tank treatment (calda), crystallizer tank treatment (cristallizzante).

(b) SOD1 RNA expression stressed by UVB: cells without any treatment (controllo), NaCl treatment (NaCl), sea water treatment (mare), warm tank treatment (calda), crystallizer tank treatment (cristallizzante).

Lowercase/uppercase letters represent significant differences among different extract ($p < 0.05$).

Sirtuin 1 (SIRT1):

Sirt1, which belongs to the NAD-dependent histone deacetylase family and it is implicated in ageing, metabolism and stress resistance (Lee et al., 2010). Sirt1 controls the cell response to oxidative stress by regulating the FOXO family of proteins, which induce cell death, and may increase the longevity of the organism (Brunet et al., 2004). HEK cells treated with saline water increased the expression of Sirt1 in all treatment compared to the control (Figure 4.2a). In contrast, Sirt1 was under expressed when cells were exposed to UVB (Figure 4.2b).

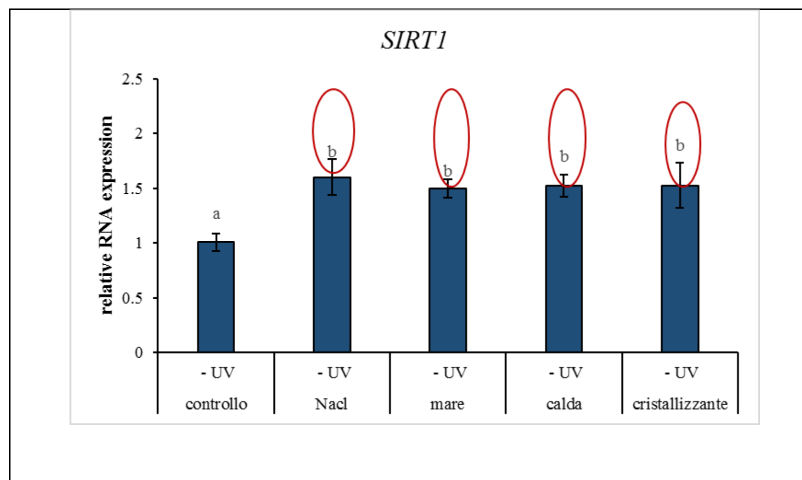


Figure 4.2a. Histogram of RNA expression related to the SIRT1 gene.

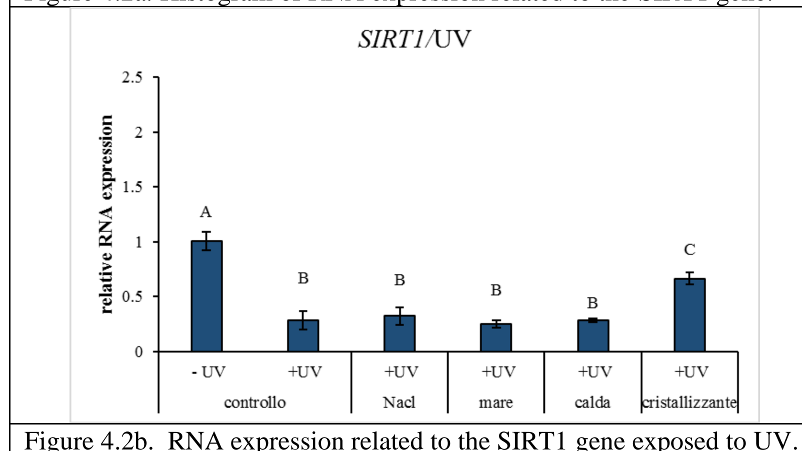


Figure 4.2b. RNA expression related to the SIRT1 gene exposed to UV.

Figure 4.2. (a) SIRT1 RNA expression: cells without any treatment (controllo), NaCl treatment (NaCl), sea water treatment (mare), warm tank treatment (calda), crystallizer tank treatment (cristallizzante).

(b) SIRT1 RNA expression stressed by UVB: cells without any treatment (controllo), NaCl treatment (NaCl), sea water treatment (mare), warm tank treatment (calda), crystallizer tank treatment (cristallizzante).

Lowercase/uppercase letters represent significant differences among different extract ($p < 0.05$).

Cells treated with saline water from the crystallizer tank and then exposed to UV showed an up-regulation of the gene, compared to the stressed control. According to Chou et al. (2013), Sirt1 was under expressed when the cell was subjected to strong and prolonged UVB radiation, as the cell could not cope with the damage caused by the radiation.

Aquaporin 3 (AQP3):

Subjecting HEK cells to saline water treatment shows that AQP3 is underexpressed compared with the control in both warm and crystallizing saline water-treated cells (Figure 4.3a) so, in accordance with the function of preventing water loss (Sougrat et al, 2002) caused by an external hypertonic environment consisting of saline water, cells decrease the expression of AQP3 by attempting to limit water loss as a result of the osmotic stress to which they are subjected and preventing dehydration. In this case, saline water appears to have no positive effect on cells.

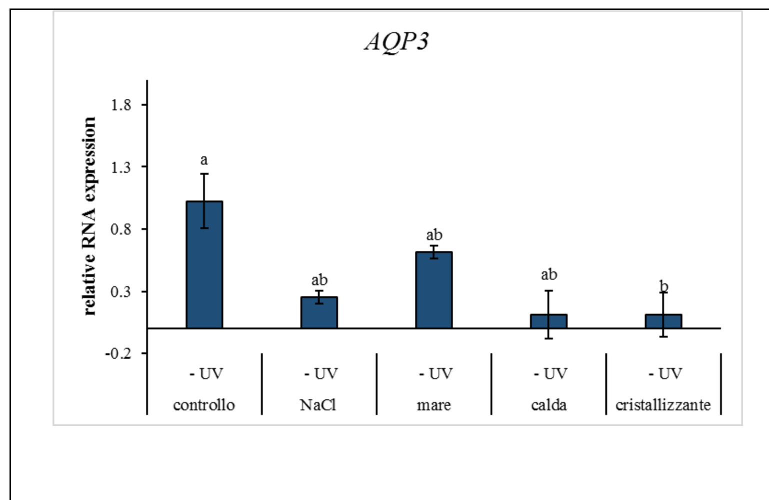


Figure 4.3a. Histogram of RNA expression related to the AQP3 gene.

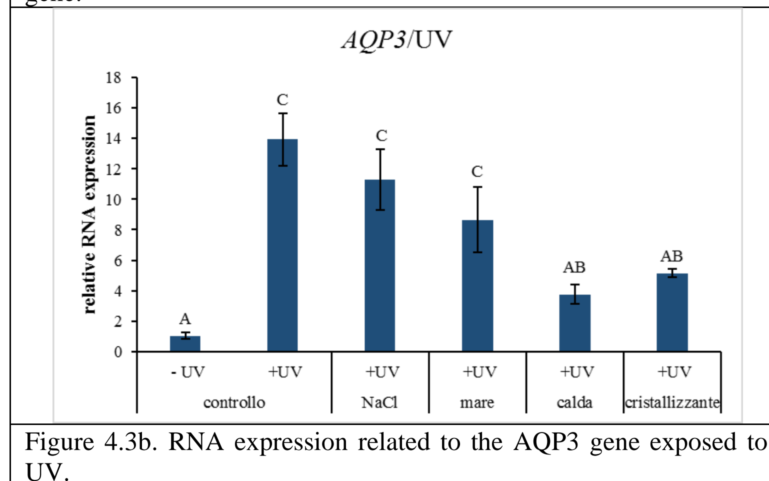


Figure 4.3b. RNA expression related to the AQP3 gene exposed to UV.

Figure 4.3. (a) AQP3 RNA expression: cells without any treatment (controllo), NaCl treatment (NaCl), sea water treatment (mare), warm tank treatment (calda), crystallizer tank treatment (cristallizzante).

(b) AQP3 RNA expression stressed by UVB: cells without any treatment (controllo), NaCl treatment (NaCl), sea water treatment (mare), warm tank treatment (calda), crystallizer tank treatment (cristallizzante).

Lowercase/uppercase letters represent significant differences among different extract ($p < 0.05$).

HEK cells treated with saline water and exposed to UVB exhibit down- regulation of the AQP3 gene compared with the stressed control (cells exposed to UVB but not treated with saline water), (Figure 4.3a).

This result is due to the combination of two factors: over-expression of AQP3 due to UVB exposure and under-expression of AQP3 due to exposure to hypertonic environment (Figure 4.3b); thus, AQP3 has a balanced expression to be able to cope with oxidative stress by acting as an anti aging, while still being able to maintain the protective function of water loss in hyperosmotic environment.

When HEKs are exposed to UVB light, the expression of AQP3 increases in accordance with the work on NHEK by Pisarchik et al. (2004) and the study on human skin fibroblasts (NHF) by Xie et al. (2013), highlighting the protective action against oxidative stress of AQP3 against the cell. Induction of the AQP3 gene may also be accompanied by increased immunoreactivity of the cell (Bellemere et al., 2007). When oxidative stress increases, high levels of AQP3 prevent the cell from going into apoptosis (Xie et al., 2013).

Prostaglandin-endoperoxide synthase-2 (COX2):

HEK cells treated with saline water increase COX2 expression compared with control, as if saline water treatment represented an inflammatory event (Figure 4.4a). HEK cells subjected to UVB and treated with saline water significantly increase COX2 expression compared with control (Figure 4.4b).

The increase in COX2 allows the cell to activate biochemical pathways for antioxidant protection (Luo et al., 2011) and to engage the pro-apoptotic pathway limiting damage (Healy et al., 2005).

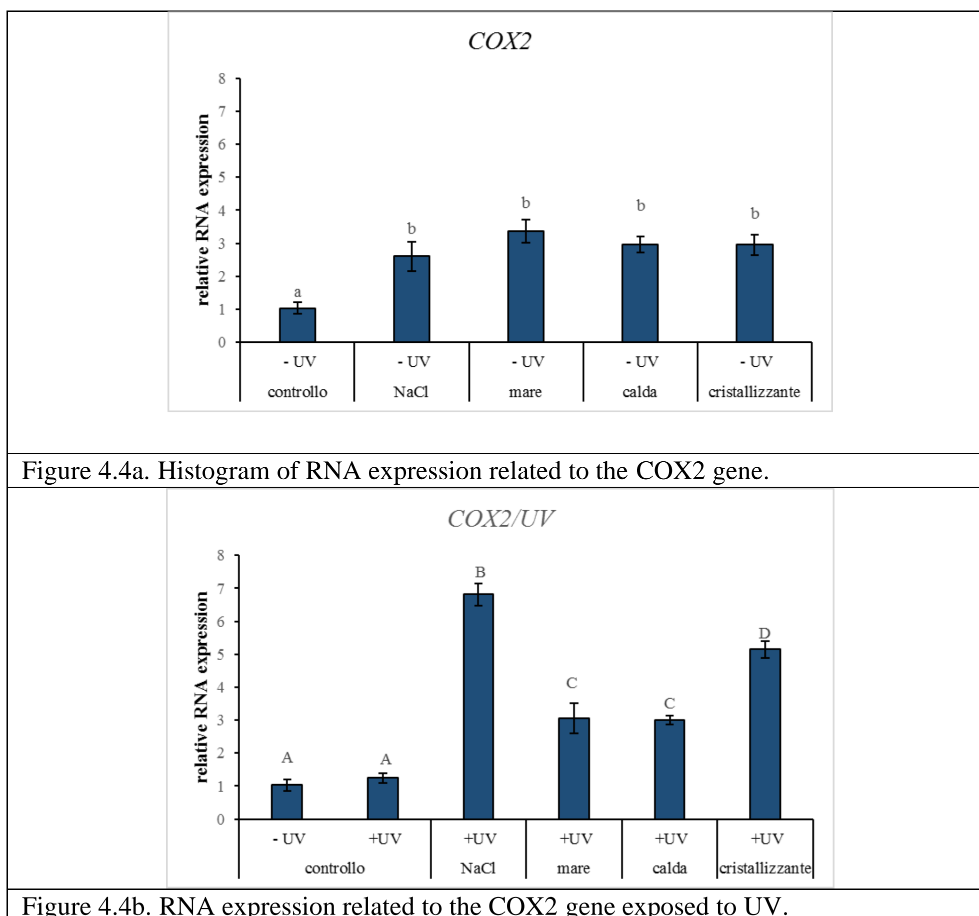


Figure 4.4a. Histogram of RNA expression related to the COX2 gene.

Figure 4.4b. RNA expression related to the COX2 gene exposed to UV.

Figure 4.4. (a) COX2 RNA expression: cells without any treatment (controllo), NaCl treatment (NaCl), sea water treatment (mare), warm tank treatment (calda), crystallizer tank treatment (cristallizzante).

(b) COX2 RNA expression stressed by UVB: cells without any treatment (controllo), NaCl treatment (NaCl), sea water treatment (mare), warm tank treatment (calda), crystallizer tank treatment (cristallizzante). Lowercase/uppercase letters represent significant differences among different extract ($p < 0.05$).

4.4 Conclusion

The up-regulation of SOD1, SIRT1, COX2 and the down -regulation of AQP3 in cells treated with saline water from salt work, in particular from the crystallizer tank, allowed us to hypothesise that saline water had a beneficial effect on cells, ensuring a state of integrity that allowed them to express genes to counteract both oxidative UVB damage and cell death by apoptosis, in according to Spilioti et al. (2017).

The results demonstrate the potential use of saline waters as antioxidant and photoprotective agents for skin protection. Human skin has been the main target of photo-oxidation as it is continuously exposed to UV radiation. At the cellular level, this exposure can induce a state of oxidative stress and damage. In order to obtain molecules with important therapeutic properties for human health, scientific research has focused on the discovery of new natural compounds of marine origin. Potential candidates for preventing the harmful effects of UV radiation on the skin are saltwork waters.

These results confirm the importance of using *in vitro* cell systems and biomolecular markers in the study of bioactives for cosmeceutical applications, and conclude that the intrinsic antioxidant capacity of seawater provides increased antioxidant capacity at the cellular level. In this way, reliable results can be obtained for the validation of the efficacy of bioactive molecules of marine origin.

This experiment contributes to the application of current Blue economy strategies in accordance with the "Blue Growth" and the Horizon programme, thus enabling the development of future prospects for improving the circular economy and marine production chain to obtain compounds that are useful for cosmeceutical and pharmaceutical applications.

5. CONCLUDING REMARKS

During the three years of my research activity, it was conducted an experiment based on molecular biology to investigate the microbiological profile of salt samples (through molecular techniques, 16s rDNA, NGS). Salterns are excellent models for studying the ecology and diversity of microorganisms. Comparing haloarchaeal communities from the six Mediterranean salt work, significative differences are showed in the microbiota and in the microbial community structure, however, it has not been possible to establish a standardised method of tracing or a readily applicable method, transferable to industry and the company, for characterising the geographical origin of sea salt.

In line with the "Blue Growth" objectives, cosmeceuticals represent a promising area for the valorisation and use of marine bioactive molecules.

Secondary metabolites, in recent studies (Messina et al. 2015, 2019, 2021) are interesting candidates for the prevention of adverse effects of UV radiation on human skin cells, showing to be anti-oxidant effective against photo-induced oxidative damage to the skin, such as skin ageing.

Many studies (Dudossè et al., 2005; Grether-Beck et al., 2007, Abu-Jdayil et al., 2008; Kim et al., 2008, 2010; Quist et al., 2011; Abbes et al., 2013; Centini et al. 2015; Gao et al., 2015; Spilioti et al., 2017) indicate that the extraction of bioactive compounds from marine sources, such as sea mud, brine, microorganisms and algae, and its uses in the nutraceutic, cosmeceutic and pharmaceutic fields, emphasises biological properties of marine bioactive molecules, bringing direct benefits on human health. Consequently, such evidence brings significant benefits to the eco-sustainable image of marine products.

In the two experiments conducted involving saline water treatment on the murine 3T3 cell line and the HEK cell line respectively, it was found that the molecular markers investigated were over-expressed by the cells *in vitro* following UV-B treatment. In conclusion, these studies demonstrated that cells pre-treated with saline water had

antioxidant activity under UV stress condition than cell without pre- treatment. The observations obtained from the investigated biomarkers were in agreement with the sources (Sugiyama et al., 2001; Pisarchik et al., 2004; Brunet et al., 2004; Yang et al., 2006; Bellemere et al., 2007; Spanier et al., 2009; Lee et al., 2010; Cheng et al., 2011; Schrader et al., 2012; Milani et al., 2013; Xie et al., 2013; Chou et al., 2013; Russo et al., 2013; Jeon et al., 2014; Shi et al. 2016) and allow the conclusion that treatment of cells with saline water has beneficial effects that can be used in the nutraceutical and cosmeceutical fields.

The limit of the experiments conducted were the impossibility of extracting and determining bioactive compounds from the saltwater (Kovac et al., 2013) and the impossibility of validating them on an industrial scale, caused by the lack of reproducibility of the individual experiments.

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